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STUDIES ON THE STEROL METABOLISM IN MARINE CRUSTACEANS*

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INTRODUCTION

Sterols are present in all aminals, plants, and microorganisms except bacteria as a constituent of lipids. In higher animals, cholesterol is found from their tissues as

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a main sterol. In marine invertebrates, belonging to the more primitive animals, however, there is a great diversity in the occurrence and distribution of sterols as compared with higher animals. For example, (1) a number of sterols differing in the number and position of double bonds in rings or side-chain, or the attachment to the side-chain of substituents such as alkyl groups at C-24 occur in marine invertebrates; (2) a certain family or class of marine invertebrates contains one or more sterols in addition to cholesterol in their tissues; (3) some marine invertebrates contain other sterols in stead of cholesterol as a main sterol. The above facts are interesting in the viewpoint of comparative biochemistry. BERGMANN (1952, 1962) has discussed the relationship between the species of animals and the types of sterols. However, the relationship is not fully established on account of the difficulty in separating completely the sterol mixtures occurring in marine animals, especially minor components.

On the other hand, no report has been presented on the metabolism of sterols in marine invertebrates except a few mollusks, although it has been suggested that the sterol metabolism in marine invertebrates is not necessarily similar to that of higher animals. For this reason, the followings are conceivable: (1) The pure sterols are not obtainable from commercial sources except for a few sterols; (2) the separation of sterols with a closely related structure is not always easy; (3) it is relatively difficult to cultivate marine crustaceans by the diet composed of chemically known substances; (4) the suitable radioactive sterols can not be prepared so easily by chemical methods.

Recently, the culture of marine crustaceans not to speak of the prawn, *Penaeus japonicus*, has been practiced in the various districts of Japan. However, the nutritional requirements of these crustaceans have not been well establisehd. In the insects, belonging to the same arthropods as marine crustaceans, it is generally recognized that most insects lack sterol-synthesizing ability from acetate and/or mevalonate and require sterols as a diet for their normal growth in contrast with higher animals. Hence, the author has attempted to clarify the metabolism of sterols in marine crustaceans.

On the sterol composition of marine crustaceans, there are only a few reports although this class embraces a great number of species. An early investigation has demonstrated that cholesterol is regarded as a typical sterol for marine crustaceans. In exceptional case, the barnacle, *Balanus glandula*, was found to contain a relatively large amount of 24-dehydrocholesterol(35%) in addition to cholesterol (65%) (FAGERLUND & IDLER, 1957). However, the sterol compositions reported in an earlier report may be somewhat corrected, because the identification of sterols was not performed by sensitive method such as gas-liquid chromatography (GLC). In the present study, therefore, the sterol composition of the several marine crustaceans was first clarified by GLC prior to the investigation of sterol metabolism in them. Next, the sterol-synthesizing ability was investigated on the lobster, *Panulirus japonica*, the prawn, *P. japonicus*, the crab, *Portunus trituberculatus*, and *Artemia salina*. From the above experiments, it was assumed that most marine crustaceans lack sterol-synthesizing ability or the sterol synthesis proceeds extremely slowly in these animals. In the case of the

prawn, *P. japonicus,* it was also demonstrated by the nutritional experiment that this crustacean requires sterols as a diet. The above results suggested that the sterols found in marine crustaceans originate from exogenous sources and some endogenous sterols are derived from dietary C_{28}- and C_{29}-sterols. Hence, the utilization and bioconversion of dietary sterols were investigated by the feeding trials and tracer techniques. In addition, for the purpose of clarifying some function of sterols in marine crustaceans, the distribution and fate of exogenous cholesterol in the tissues and the bioconversion of cholesterol to steroid hormones were examined by use of the lobster, *P. japonica.* Furthermore, the *in vitro* bioconversion of progesterone by the slices of the ovaries and testes of the crab, *P. trituberculatus,* was investigated in order to clarify the steroidgenesis in marine crustaceans.

The present paper deals with the results of the above experiments.

The trivial names and the corresponding systematic names used in this paper are as follows: Cholesterol, cholest-5-ene-3β-ol; 22-dehydrocholesterol, cholest-5, 22-diene-3β-ol; desmostrol, cholest-5, 24-diene-3β-ol; brassicasterol, 24(S)-24-methyl-cholest-5, 22-diene-3β-ol; campesterol, 24 (R) -24-methylcholest-5-ene-3β-ol; 24-methylenecholesterol, ergost-5, 24(28)-diene-3β-ol; ergosterol, ergost-5, 7, 22-triene-3β-ol; stigmasterol, stigmast-5, 22-diene-3β-ol; β-sitosterol, stigmast-5-ene-3β-ol; progesterone, pregn-4-ene-3, 20-dione; 17α-hydroxyprogesterone, 17α-hydroxy-pregn-4-ene-3, 20-dione; androstenedione, androst-4-ene-3, 17-dione; testosterone, androst-4-ene-17β-ol-3-one; deoxycorticosterone, 21-hydroxy-pregn-4-ene-3, 20-dione; corticosterone, 11β, 21-dihydroxy-pregn-4-ene-3, 20-dione; cortisone, 17α, 21-dihydroxy-pregn-4-ene-3, 11, 20-trione; cortisol, 11β, 17α, 21-trihydroxy-pregn-4-ene-3, 20-dione; 11-deoxycortisol, 17α, 21-dihydroxy-pregn-4-ene-3, 20-dione; 11β-hydroxyandrostenedione, 11β-hydroxy-androst-4-ene-3, 17-dione; 11-ketotestosterone, 17β-hydroxy-androst-4-ene-3, 11-dione; 1α-hydroxycorticosterone, 1α, 11β, 21-trihydroxy-pregn-4-ene-3, 20-dione.

Part–1. Sterol Composition of Marine Crustaceans

It is a well-known fact that some marine invertebrates contain a variety of sterols in their tissues. The occurrence and distribution of the sterols in animals were extensively reviewed by BERGMANN (1952, 1962) and by AUSTIN (1970). The diversity of sterols in marine invertebrates is of interest in the viewpoint of both chemotaxonomy and comparative biochemistry. In the early studies, however, the separation of closely related sterols is not always complete for lack of a suitable isolation method. Recently, KRITCHEVSKY *et al.* (1967) have shown by using gas-liquid chromatography (GLC) that a certain crab contains 22-dehydrocholesterol (3.9%), cholesterol (57.4%), brassicasterol (36.7%), and 24-methylenecholesterol (2.0%) as a sterol. Furthermore, IDLER and WISEMAN (1968) have demonstrated by GLC that the Alaskan king crab, *Paralithodes camtschatica,* and the North Atlantic queen crab, *Chionoecetes opilio,* contain six and three sterols in adidtion to cholesterol, respectively, and they have pointed out that these crabs contain a relatively large amount of desmosterol which was found only in the chick embryo (STOKES *et al.*, 1956) and the barnacle, *Balanus*

glandula, (FAGERLUND & IDLER, 1957). Considering these results, the sterol composition of marine crustaceans reported in early papers should be reinvestigated by a more sensitive method, especially on the minor sterols.

Therefore, the author attempted to clarify the composition of naturally occurring sterols in marine crustaceans prior to the investigation of sterol metabolism in them. This part deals with the sterol composition of the several marine crustaceans, the sterols of which have not been shown so distinctly.

Materials and Methods

Crustaceans. The lobster, *Panulirus japonica*, prawn, *Penaeus japonicus*, and crab, *Portunus triberculatus*, were obtained from the commerical sources, in July, 1969. The Amphipoda, *Caprella* sp., and Mysidaces, *Neomysis intermedia*, were collected at the Hanase-point and Shibushi-bay in Kagoshima, respectively, in June, 1969. The three species of mantis crabs, *Gonodactylus chiragra*, *Gonodactylus falcatus*, and *Odontodactylus scyllarus*, were caught on the seashore of Zamami Island in Okinawa during the August in 1969, and they were pooled in acetone until use.

Isolation of sterols. The lobster, prawn, crab, and Mysidacea were minced with a meat mincer, and the lipids were extracted with chloroform-methanol according to the method of BLIGH and DYER (1959). The Amphipoda was extracted with ether in a Soxhlet apparatus for 48 hours after being bried below 70°C. In the case of the mantis crabs, the samples pooled in acetone were filtered through a glass filter, and then the lipids were extracted with ether from the residues by using a Soxhlet apparatus. The filtrate and ether extract were combined and concentrated under reduced pressure with a rotary evaporator. The lipids so obtained from the eight species of marine crustaceans were saponified with 10% solution of potassium hydroxide in 90% aqueous ethanol, and then non-saponifiable materials were extracted with ether in the usual manner. The sterols were isolated from the non-saponifiable materials by the digitonin-precipitation method (IDLER & BAUMANN, 1952) or by the column chromatography on alumina (grade II, Merck) (VAN DEN OORD, 1964). In the column chromatography, the elution was carried out with hexane, hexane-benzene (1: 1), benzene, benzene-ethyl acetate (9: 1), benzene-ethyl acetate (85: 15), and ethyl acetate-methanol (96: 4), successively. The presence of sterols in the eluates was checked by thin-layer chromatography on Kiesel gel G (Merck) with benzene-ethyl acetate (4: 1). The sterols on the plate were detected by spraying conc. sulfuric acid -ethanol (1: 1, v/v) followed by heating 5 minutes at 100–105°C.

GLC of sterols. The sterols so isolated were purified by recrystallization from methanol and subjected to GLC. During the present study, a Shimadzu gas-chromatographic unit, model GC-3AF, with dual hydrogen flame inonization detectors was used. The identification of sterols was performed by using at least two or more different columns. In the use of the column 1.0% NGS–1.0% XE-60 (1: 1), the sterols were trimethylsilylated (LUUKKAINEN *et al.*, 1961) and then subjected to GLC. The operating condition in GLC is shown in Table 1. Stigmasterol and desmosterol

were purchased from Gas-chrokogyo Co., Ltd. Ergosterol and cholesterol were obtained from Nakarai Chemicals Co. and purified by recrystallization from methanol. β-Sitosterol obtained from Tokyo Chemicals Co. Ltd. was determined to be the mixture of β-sitosterol and campesterol (1: 1) by GLC on 1.5% SE-30 and mass spectral analysis. 22-Dehydrocholesterol, brassicasterol, and 24-methylenecholesterol were isolated in this laboratory from the red alga, *Porphyridium cruentum*, the diatom, *Cyclotella nana*, and the clam, *Tapes philippinarum*, respectively.

Table 1. The operating condition in GLC.

Column packing	Column length	Column temp. (°C)	Hydrogen (kg/cm²)
1.5% SE-30 on 60-80 mesh Chromosorb W	2.0 m×4 mm I. D.	225	1.0
1.5% OV-17 on 80-100 mesh Shimalite W	3.0 m×4 mm I. D.	233	1.0
1.0% XE-60 on 60-80 mesh Chromosorb W	2.0 m×4 mm I. D.	205	1.0
1.0% NGS-1.0% XE-60 (1: 1, v/v) on 60-80 mesh Chromosorb W	2.5 m×4 mm I. D.	215	0.75

* Nitrogen: 2.0 kg/cm²

Infrared and mass spectral analyses. Infrared absorption spectrum was obtained with a Nippon Bunko DS-301 spectrometer in chloroform. Mass spectrum was measured on a Hitachi RMU-6D instrument (chamber volatage, 70 eV).

Results and Discussion

Recently, for the purpose of clarifying the sterol composition of marine invertebrates the several workers have attempted to separate the sterol mixtures by GLC. Consequently, the problem of separating the sterol mixtures occurring in these animals appears to be completely resolved by the use of GLC. IDLER *et al.* have reported that a variety of sterols in the red algae (IDLER *et al.*, 1968) and the crabs (IDLER & WISEMAN, 1968) are separable by the GLC using a combined system of 3% NGS-3% XE-60 (1: 1) and 14 ft column. In spite of good separation, however, there is only the inconvenience that the GLC adopted by them was considerably time-consuming. In the present study, their method was slightly modified as follows: Sterols were subjected to GLC as trimethylsilyl derivative instead of methyl ether and analyzed by using 1.0% NGS-1.0% XE-60 (1: 1) and 2.5 m of column. As shown in Table 2, the sterol mixture containing brassicasterol and desmosterol which have not been separated by the GLC using a usual column was separated in considerably short times.

The content and composition of the sterols isolated from the eight species of the marine crustaceans examined in the present study are given in Tables 3 and 4. All crustaceans examined were found to contain mainly cholesterol as a sterol, although considerable differences were perceived in the content and composition of sterols among these species. In the case of the Amphipoda, prawn, Mysidacea, however, other sterols such as 22-dehydrocholesterol, brassicasterol, desmosterol, and 24-methylenecholesterol were detected as a minor component. Fig. 1 shows the results of GLC of the sterols isolated from the Amphipoda. 22-Dehydrocholesterol was contained

Table 2. Relative retention time of sterols in GLC.

Sterol	Relative retention time			
	Column			
	SE-30	XE-60	OV-17	NGS-XE-60*
Cholestane	1.00 (8.80)	1.00 (4.20)	1.00 (8.50)	—
22-Dehydrocholesterol	1.67	4.26	1.69	0.90
Cholesterol	1.83	4.29	1.79	1.00 (18.4)
Brassicasterol	2.04	5.02	—	1.07
Desmosterol	2.04	5.03	2.00	1.20
24-Methylenecholesterol	2.32	—	—	1.28
Ergosterol	2.20	5.98	2.19	1.43
Campesterol	2.34	6.61	2.29	1.31
Stigmasterol	2.59	6.00	2.51	1.40
β-Sitosterol	2.93	8.01	2.84	1.63

* The trimethylsilyl derivative of sterols was subjected to GLC.
( ): Retention time (min.)

Table 3. Content of sterols in the marine crustaceans.

Animal	Total fresh weight (g)	Lipids		Non-sap. materials		Sterols	
		g	(%)	g	(%)	g	(%)
Lobster	200.0	6.12	(3.1)	2.03	(1.02)	0.455	(0.22)
Prawn	30.0	0.74	(2.5)	0.20	(0.65)	0.019	(0.064)
Crab	143.0	3.50	(2.4)	0.21	(0.15)	0.024	(0.017)
Amphipoda	7.50*	0.43	(5.8)	0.16	(2.10)	0.014	(0.019)
Mysidacea	1000	16.3	(1.6)	2.10	(0.21)	0.69	(0.069)
G. chiragra	700	14.6	(2.1)	3.67	(0.52)	0.15	(0.021)
G. falcatus	46.6	0.56	(1.2)	0.32	(0.69)	0.003	(0.07)
O. scyllarus	31.2	0.71	(2.3)	0.15	(0.49)	0.001	(0.03)

* dry weight

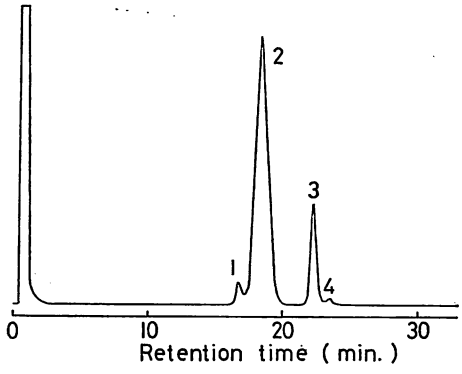


Fig. 1. GLC on 1.0% NGS-1.0% XE-60 (1: 1) of the sterols from the Amphipoda.
1, 22-dehydrocholesterol; 2, cholesterol;
3, desmosterol; 4, 24-methylenecholesterol

Table 4. Percentage composition of the sterols isolated from the eight species of crustaceans determined by GLC.

Sterol	% Composition							
	A	B	C	D	E	F	G	H
22-Dehydrocholesterol	—	3	—	4	3	—	—	—
Cholesterol	100	90	100	73	78	100	100	100
Brassicasterol	—	—	—	—	9	—	—	—
Desmosterol	—	—	—	22	—	—	—	—
24-Methylenecholesterol	—	7	—	1	10	—	—	—
Stigmasterol	—	trace	—	—	—	—	—	—
β-Sitosterol	—	trace	—	—	trace	—	—	—

A; lobster, *P. japonica*
B; prawn, *P. japonicus*
C; crab, *P. trituberculatus*
D; Amphipoda, *Caprella* sp.
E; Mysidacea, *N. intermedia*
F; mantis crab, *G. chiragra*
G; mantis crab, *G. falcatus*
H; mantis crab, *G. scyllarus*

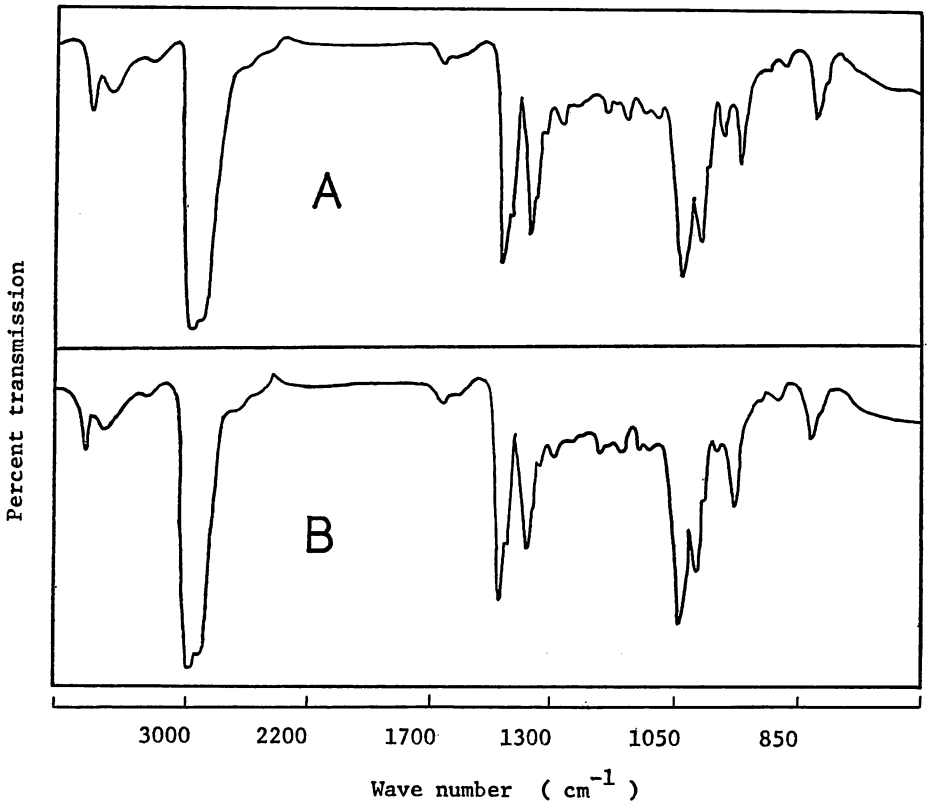


Fig. 2. Infrared spectra of the sterol (A) isolated from the mantis crab, *G. chiragra*, and authentic cholesterol (B).

in the Amphipoda (4%), the prawn (3%), and the Mysidacea (3%). 24-Methylenecholesterol was found in the above three species of crustaceans. Desmosterol and brassicasterol were present in the Amphipoda (22%) and the Mysidacea (9%), respectively. In the case of the crab, lobster, and three species of mantis crabs, the isolated sterol was composed of only cholesterol. On the sterol isolated from the mantis crab, *G. chiragra*, the sterol was further analyzed by infrared absorption and mass spectrometries in order to confirm the identification The results are shown in Figs. 2 and 3. The infrared spectrum of the sterol from the mantis crab was identical with that of authentic cholesterol. Also, the mass spectrum of this sterol showed one molecular ion peak at m/e 386 corresponding to cholesterol.

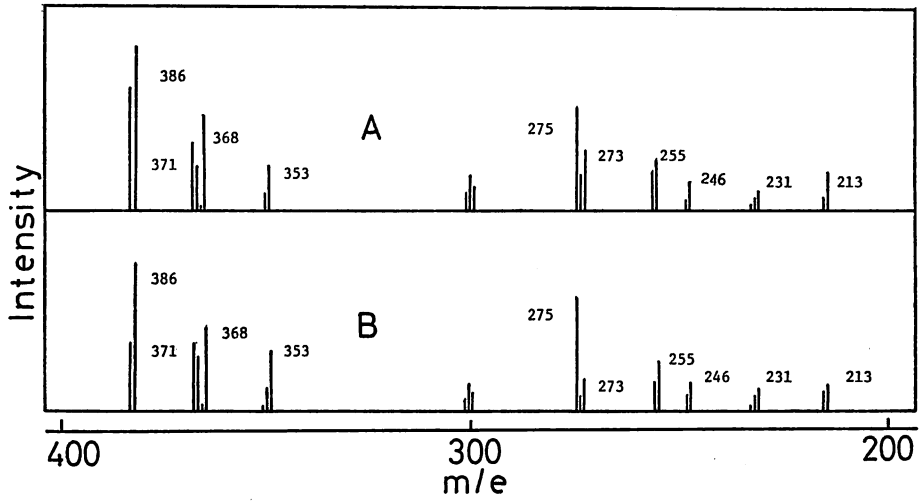


Fig. 3. Mass spectra of the sterol (A) isolated from the mantis crab, *G. chiragra*, and authentic cholesterol (B).

The other peaks were interpreted as follows: m/e 371 (M^+-CH_3), 368 (M^+-HOH), 353[$M^+-(CH_3+HOH)$], 275 [$M^+-(HOH+C_7H_9)$], 273 (M^+-R, R=alkyl side chain), 255 [$M^+-(R+HOH)$], 246 [$M^+-(R+27)$], 231 [$M^+-(R+42)$], and 213 [$M^+-(R+42+HOH)$] (KNIGHTS, 1967). These results indicated that the sterol of this crab is identical with cholesterol.

As stated above, it was demonstrated that some crustaceans contain other sterols besides cholesterol. In the present study, however, it was obscure whether these sterols originate from exogenous or endogenous sources. On the other hand, it is a well known fact that the minor sterols contained in the marine crustaceans are also found in some algae and marine animals as a major sterol. Accordingly, it may be assumed that some sterols occurring in crustaceans originate from exogenous sources such as diets.

Summary

In this part, the composition of naturally occurring sterols in the lobster, *Panulirus*

japonica, prawn, *Penaeus japonicus*, crab, *Portunus trituberculatus*, Amphipoda, *Caprella* sp., Mysidacea, *Neomysis intermedia*, and three species of mantis crabs, *Gonodactylus chiragra*, *Gonodactylus falcatus*, and *Odontodactylus scyllarus*, was investigated mainly by using GLC. The results obtained are as follows:

1) The sterols isolated from the eight species of crustaceans were mainly composed of cholesterol (73–100%).

2) In the case of the prawn, Amphipoda, and Mysidacea, several sterols besides cholesterol were contained as minor constituents. The prawn was found to contain 22-dehydrocholesterol (3%), 24-methylenecholesterol (7%), stigmasterol (trace), and β-sitosterol (trace) in addition to cholesterol (90%). The Amphipoda contained 22-dehydrocholesterol (4%), desmosterol (22%), and 24-methylenecholesterol (1%) besides cholesterol (73%). The Mysidacea contained 22-dehydrocholesterol (3%), brassicasterol (9%), 24-methylenecholesterol (10%), β-sitosterol (trace), and cholesterol (78%).

3) On the other hand, the lobster, crab, and three species of mantis crabs were found to contain only cholesterol as a sterol.

Part-2. Sterol Biosynthesis in Marine Crustaceans

It is commonly recognized that animals are capable of synthesizing cholesterol from acetate via squalene. As an exceptional case, it was shown that the insects possess no ability for sterol-synthesis and require cholesterol or other sterols as a diet for their normal growth (BLOCH *et al.*, 1956; CLARK & BLOCH, 1959a; KODICEK & LEVINSON, 1960; ROBBINS *et al.*, 1960; KAPLANIS *et al.*, 1961; SEDEE, 1961). The utilization of sterols by insects was extensively reviewed by CLAYTON (1964). Recently, it has been found that several marine invertebrates are incapable of synthesizing cholesterol from acetate or mevalonate as also occurs in insects. The crab, *Cancer pagurus* (VAN DEN OORD, 1964), the crayfish, *Astacus astacus* (ZANDEE, 1922, 1964a, 1966a, b; GOSSELIN, 1965), and the lobster, *Homarus gammarus* (ZANDEE, 1964b, 1967a), were shown to be incapable of incorporating acetate-1-^{14}C and/or mevalonate-2-^{14}C into squalene and cholesterol. In other marine invertebrates, it has also been reported that the sea urchin, *Paracentrotus lividus* (SALAQUE *et al.*, 1966), the whelk, *Buccinum undatum* (VOOGT, 1967b), the oyster, *Ostrea gryphea* (SALAQUE *et al.*, 1966), the cuttle fish, *Sepia offcinalis* (ZANDEE, 1967b), and the Japanese holothurian, *Stichopus japonicus* (NOMURA *et al.*, 1969) lack ability for sterol synthesis. However, the mussel, *Mytilis californianus* (FAGERLUND & IDLER, 1960), the clam, *Saxidomus giganteus* (FAGERLUND & IDLER, 1960), the snails, *Helix pomatia* (ADDINK & VERVERGAERT, 1963) and *Arion rufus* (VOOGT, 1967a), and some marine Annelida (WOOTON & WRIGHT, 1962) have been demonstrated to synthesize their sterols from acetate and mevalonate.

On the other hand, it was demonstrated that some marine invertebrates contain a variety of sterols in addition to cholesterol. For example, the Alaskan king crab,

Paralithodes camtschatica, was shown to contain 22-dehydrocholesterol (3.1%), cholesterol (61.3%), brassicasterol (1.7%), desmosterol (31.1%), 24-methylenecholesterol (< 0.1%), β-sitosterol (0.8%), fucosterol (<0.1%) and unknown components (1.0%) (IDLER & WISEMAN, 1968). The source of these sterols was obscure although it has been assumed that some originate from the diets. In investigating the sterol metabolism of marine invertebrates, therefore, sterol-synthesizing ability should be first clarified because it appears to be different according to the species of marine animals.

In this part, the sterol-synthesizing ability of the carnivorous crustaceans, the lobster, prawn, and crab, and the herbivorous crustacean, *Artemia salina*, was investigated by using acetate-1-^{14}C as a tracer.

A. Sterol-synthesizing ability in the carnivorous crustaceans, the lobster, *Panulirus japonica*, the prawn, *Penaeus japonicus*, and the crab, *Portunus trituberculatus*.

Materials and Methods

Animals and injection of acetate-1-^{14}C. Three species of marine crustaceans, lobster, prawn, and crab, were obtained from the Subtropical Marine Biological Laboratory, Kagoshima, in August 1969. During the experimental period, these animals were kept in a circulatory trough at 20–22°C, and no food was given to them. One lobster, 200 g body weight, was injected three times with acetate-1-^{14}C at intervals of 24 hours. Each injection consisted of 30μCi of sodium acetate-1-^{14}C (specific activity, 50.0 mCi/mM) in 0.1 ml of distilled water. By a similar method, two prawns, 30 g total body weight, and one crab, 143 g body weight, were injected twice with 12μCi and 10 μCi of acetate-1-^{14}C, respectively.

Isolation of lipids. At 2 days after the last injection, the experimental animals were killed by freezing at −20°C. All tissues of the animals were chopped up with a meat mincer and homogenized for 2 minutes in a Waring Blender. The lipids were extracted from the homogenates with chloroform-methanol (BLIGH & DYER, 1959), and saponified with 2N potassium hydroxide in 80% methanol for 3 hours. The saponification mixtures were filtered, and the non-saponifiable materials and fatty acids were separated by extraction with ether in the usual manner. In the case of the lobster, the non-saponifiable materials were separated into the various fractions by column chromatography on alumina (grade II). The elution was carried out with hexane, hexane-benzene, benzene-ethyl acetate and ethyl acetate-methanol successively (VAN DEN OORD, 1964). The crude sterol fraction, eluted with benzene-ethyl acetate (85: 15), was purified by thin-layer chromatography (TLC) on Kiesel gel G using benzene-ethyl acetate (4: 1) as a solvent, and then recrystallized several times from methanol. The hydrocarbon fraction, eluted with hexane, was subjected to TLC using heptane-hexane (3: 2) and the crude squalene fraction was isolated. The zone corresponding to squalene detected with iodine vapour was scraped off from the

plate, and then squalene was extracted with petroleum ether and subjected to the measurement of radioactivity. The presence of squalene in the eluate was investigated by gas-liquid chromatography (GLC) on 1.5% SE–30. In the case of the prawn and crab, the sterols were isolated from the non-saponifiable materials by the digitonin-precipitation method (IDLER & BAUMANN, 1952). After decomposing by pyridine, the sterols so obtained were purified by recrystallization from methanol. The composition of the purified sterols was investigated by GLC using two different columns, 1.5% SE–30 and 1.0% NGS–1.0% XE–60 (1 : 1). The details of the operating conditions in GLC have been described in part-1.

Measurement of radioactivity. The radioactivity of the materials was measured with a Beckman liquid scintillation counter, model LS–150. The toluene solution of 2,5-diphenyloxazole (PPO) (0.6%) and 2,2′-*p*-phenylene-bis-5-phenyoxazole (POPOP) (0.02%) was used as a scintillator. The efficiency of counting of radioactive materials was approximately 65 per cent.

Results

After injection of acetate-1-^{14}C, the radioactivity of the lipid fractions isolated from the lobster, prawn, and crab was measured. The results are shown in Tables 5, 7, and 9. In GLC, the sterols isolated from these crustaceans were composed of the following components: Lobster (cholesterol, 100%), prawn (cholesterol, 90%; 22-dehydrocholesterol, 3%; 24-methylenecholesterol, 7%) and crab (cholesterol, 100%). In all crustaceans, a relatively high radioactivity was recovered in the fatty acids and non-saponifiable materials. These results indicated that the animals utilized acetate for the synthesis of fatty acids and some non-saponifiable materials. In all animals, however, the sterols were labelled to a strikingly low degree in comparison with the other lipid fractions, and the radioactivities of the sterols were rapidly lost according to the consecutive crystallizations as shown in Talbes 6, 8, and 10. Finally, no significant radioactivity was found in the sterols from the three species of crustaceans.

In the case of the lobster, the hydrocarbon fraction was chromatographed by TLC and the crude squalene fraction was obtained. In GLC, however, no squalene was

Table 5. Radioactivity of the lipid fractions isolated from the lobster, *P. japonica*, after injection of acetate-1-^{14}C.

Lipid fraction	Weight (mg)	Radioactivity	
		Total activity (dpm)	Specific activity (dpm/mg)
Total fresh weight	200,000	—	—
Non-saponifiable materials	2,034	2,560,000	1,260
Fatty acids	1,028	732,000	712
Hydrocarbon fraction	698	2,530,000	3,370
Crude squalene fraction*	42.0	280,000	6,970
Crude sterols	12.9	3,920	304

* Obtained from the hydrocarbon fraction by TLC

Table 6. Recrystallization of the sterols isolated from the lobster, *P. japonica*

Crystallization	Specific activity (dpm/mg)
1 st	112
2 nd	32.3
3 rd	0

Table 7. Radioactivity of the lipid fractions isolated from the prawn, *P. japonicus*, after injection of acetate-1-^{14}C.

Lipid fraction	Weight (mg)	Radioactivity	
		Total activity (dpm)	Specific activity (dpm/mg)
Total fresh weight	30,000	—	—
Total lipids	741	980,000	1,320
Non-saponifiable materials	195	501,000	2,570
Fatty acids	170	136,000	797
Crude 3β-sterols*	19.2	2,230	116

Table 8. Recrystallization of the 3β-sterols isolated from the prawn, *P. japonicus*.

Crystallization	Specific activity (dpm/mg)
1 st	90.5
2 nd	22.2
3 rd	0

Table 9. Radioactivity of the lipid fractions isolated from the crab, *P. trituberculatus*, after injection of acetate-1-^{14}C.

Lipid fraction	Weight (mg)	Radioactivity	
		Total activity (dpm)	Specific activity (dpm/mg)
Total fresh weight	143,000	—	—
Total lipids	3,501	1,690,000	483
Non-saponifiable materials	2,112	785,000	372
Fatty acids	342	50,400	148
Crude 3 β-sterols*	4.8	390	81.3

* Obtained by the digitonin -precipitation method

Table 10. Recrystallization of the 3β-sterols isolated from the crab, *P. trituberculatus*.

Crystallization	Specific activity (dpm/mg)
1 st	70.2
2 nd	12.3
3 rd	0

detected in this fraction which revealed a high radioactivity. Accordingly, no further investigation was carried out on the hydrocarbon fraction. From the results mentioned above, it may be suggested that the lobster, *P. japonica*, the prawn, *P. japonicus*, and the crab, *P. trituberculatus*, lack sterol-synthesizing ability or that the biosynthesis of sterols proceeds extremely slowly in these crustaceans.

B. Sterol-synthesizing ability in the hervivorous crustacean, *Artemia salina*.

Materials and Methods

Artemia salina was obtained from the Brine Shrimp Sale Co., Inc. (Hayward, California). The hatched *Artemia* was cultivated in a trough ($30 \times 30 \times 60$ cm.) The cultivation was carried out in circulating natural sea water of which the specific gravity had been adjusted previously to 1.040 with sodium chloride (SHIMAYA *et al.*, 1967). The *Artemia* was cultivated with "Wakamoto" (Wakamoto Pharmaceutical Co., Ltd. Japan) until this crustacean grew into 5–6 mm in body length, spooned up with the net (0.5 mm I.D.), and subjected to the incubation with acetate-1-^{14}C (57.0 mCi/mM). In group 1, *Artemia* (about 50 g in fresh matter) was transferred into 3 liter Erlenmeyer flask containing 2 liters of the natural sea water filtered through a glass filter (No. 5). To this, 50 μCi of acetate-1-^{14}C, antibiotics (10^6 units of penicillin G and 25 mg of streptomycin), and 5 g of the sterilized "Wakamoto" were added, and the flask was kept with aeration at 25–26°C for 30 hours. In group 2, *Artemia* was incubated with acetate-1-^{14}C and non-sterilized "Wakamoto" without addition of antibiotics under the same conditions as in group 1. After incubation, the *Artemia* was collected with the net, and washed thoroughly with a tap water, and the lipids were extracted with chloroform-methanol and fractionated into the non-saponifiable materials, fatty acids, and crude sterols by the same method as mentioned in part-2-A.

Results

In both incubation groups, a high radioactivity was recovered in the non-saponifiable materials and fatty acids as shown in Table 11. On the other hand, the crude sterols obtained by column chromatography on alumina revealed a low radioactivity in comparison with both the above fractions. To ascertain the incorporation of acetate-1-^{14}C into sterols, the sterols were recrystallized several times from the different solvent systems. During the consecutive crystallizations, the radioactivity of the sterols decreased extremely. Finally, no detectable radioactivity was perceived in the crystal of sterols. In the present study, a significant difference on the incorporation of acetate-1-^{14}C was not perceived between two incubation groups. The above results suggest that *Artemia salina*, herbivorous crustacean, lacks sterol-synthesizing ability from acetate or that the biosynthesis of sterols proceeds extremely slowly in *Artemia* as well as the carnivorous crustaceans.

Table 11. Incorporation of acetate-1-^{14}C into the lipid fractions of *Artemia*.

Lipid fraction	Incubation					
	Without antibiotics			With antibiotics		
	Weight (mg)	Activity (dpm)	Specific activity (dpm/mg)	Weight (mg)	Activity (dpm)	Specific activity (dpm/mg)
Total lipids	169.4	2,130,000	12,500	124.3	1,570,000	12,700
Non-sap. materials	55.2	575,000	10,400	38.5	364,000	9,460
Fatty acids	72.2	1,250,000	17,300	40.5	946,000	23,400
Crude sterols*	4.1	11,500	2,810	2.3	5,750	2,500

* The crude sterols were obtained from the non-saponifiable materials by column chromatography on alumina (grade II) with hexane-benzene.

Table 12. Recrystallization of the sterols isolated from the *Artemia* incubated with acetate-1-^{14}C.

Crystallization	Solvent system	Specific activity (dpm/mg)*	
		Incubation	
		Without antibiotics	With antibiotics
1 st	Methanol	75.0	16.5
2 nd	Methanol	25.1	5.6
3 rd	Acetone-water	0	0

* To the crude sterols isolated from the *Artemia*, about 10 mg of non-radioactive cholesterol was added and recrystallized.

Discussion

In the present study, it was shown that both the herbivorous and carnivorous crustaceans examined may lack sterol-synthesizing ability or that the biosynthesis of sterols proceeds extremely slowly in them. In the rat, LONGDON and BLOCH (1953) have reported that the cholesterol-synthesizing ability was reduced by addition of a large amount of cholesterol to the diet. In the present experimental conditions, however, it was not conceivable that the crustaceans revealed no sterol-synthesizing ability due to such a reason.

These findings have also been reported for several marine crustaceans. ZANDEE (1962, 1964a, 1966a, b) has shown that the crayfish, *A. astacus*, lacks the ability to synthesize cholesterol from acetate and mevalonate. Furthermore, he has demonstrated no sterol-synthesizing ability in the lobster, *H. gammarus* (ZANDEE, 1964b, 1967a). VAN DEN OORD (1964) has reported that the crab, *C. pagurus*, did not incorporate acetate and mevalonate into sterols. The results obtained in the present study agree with the above results for other crustaceans. In addition, the absence of sterol-synthesizing ability has been also perceived in other groups of Arthropoda, the spider, *Avicularia avicularia*, and the millepede, *Graphidostreptus tumuliporus* (ZANDEE, 1967a). From these facts, it may be suggested that a number of marine crustaceans are probably incapable of synthesizing their sterols from acetate. Therefore, it may be also assumed that these animals require cholesterol or other sterols as

a nutrient and that the herbivorous ones such as the brine shrimp probably modify phyto-sterols, which are found in algae, protozoa, and phyto-planktons, to cholesterol.

Summary

1) In this part, the sterol-synthesizing ability of the lobster, *Panulirus japonica*, the prawn, *Penaeus japonicus*, the crab, *Portunus triberculatus*, and *Artemia salina* was investigated by using acetate-1-^{14}C.

2) It was shown that these crustaceans incorporated acetate-1-^{14}C into the fatty acids and some non-saponifiable materials but not into sterols.

3) From the results, it was suggested that the lobster, prawn, crab, and *Artemia* lack sterol-synthesizing ability or that the biosynthesis of sterols proceeds extremely slowly in these crustaceans.

Part-3. Sterol Requirements of Marine Crustaceans: Sterol Requirements of the Prawn, *Penaeus japonicus*

It is generally accepted that insects possess no ability for sterol-synthesis in contrast with higher animals. In part-2, the author showed that the several marine crustaceans are incapable of incorporating acetate into sterols, and suggested that marine crustaceans may lack sterol-synthesizing ability and require sterols as a diet. However, it has not been demonstrated up to the present whether marine crustaceans require sterols or not, probably on account of the difficulty in cultivating them only with the artificial diet composed of chemically defined materials. Recently, KANAZAWA *et al.* (1970) have succeeded in preparing the artificial diet sufficient for the feeding trials as to the nutritional requirements of the prawn, *Penaeus japonicus*.

Hence, in this part, the requirements of the prawn for sterols were investigated by using the artificial diet.

Materials and Methods

Prawn. The prawn, *P. japonicus*, 0.5–1.0 g body weight, was kindly supplied from the Subtropical Marine Biological Laboratory (Kagoshima) and the Fisheries Experimental Stations of Kumamoto and Kagoshima. After transportation to the laboratory, the prawn was precultivated with a clam, *Tapes philippinarum*, for 1 week.

Sterols. Cholesterol, ergosterol, and stigmasterol were obtained from Nakarai Chemicals Co., Ltd. (Japan). β-Sitosterol was purchased from Nutritional Biochemicals Co. (U.S.A.). The purity of these sterols was checked by gas-liquid chromatography on 1.5% SE–30 and the results obtained are as follows: Cholesterol, 99.9%; ergosterol, 99.8%; stigmasterol, 99.9%; β-sitosterol, 94.4% β-sitosterol and 5.6% campesterol. In the present study, the commercial β-sitosterol was used without further purification on account of the difficulty in separating campesterol from β-sitosterol.

Test diets. In the present experiment, the artificial diet for nutritional requirements of the prawn, *P. japonicus*, devised by KANAZAWA *et al.* (1970) was used as a basal diet (sterol-free). The composition of the basal diet is given in Table 13. The soybean oil obtained from Nakarai Chemicals Co., Ltd. was found to contain 0.2% of sterols (β-sitosterol and stigmasterol). However, the content may be regarded as negligible in comparison with the amount of sterols added to the test diets. The test diets were prepared as follows: To the basal diet, the sterols dissolved in a small amount of ethanol were added, mixed thoroughly, and then the diets were sealed up in a Kurehalon tube, heated at 100°C for 30 minutes, and stored in a refrigerator until use.

Table 13. Composition of the basal diet.

Ingredient	Composition (%)	Ingredient	Composition (%)
Glucose	5.5	Glycine	0.1
Sucrose	10.0	Citric acid	0.3
Starch	4.0	Succinic acid	0.3
Chitin	4.0	Soybean oil	8.0
Glucosamine-HCl	1.5	Salt mixture*	7.7
Cellulose powder	4.0	Vitamin mixture**	2.6
Soybean casein	50.0	Morin	0.1
Methionine	1.0	Total	100.0
Tryptophan	0.2	Agar	5.0
Glutamic acid	0.2	Distilled water	100.0

* Salt mixture (g/100 g of dry diet): K_2HPO_4, 2.313; KCl, 0.723; $MgSO_4$, 1.149; $FeSO_4 \cdot 7H_2O$, 0.107; $Ca_3(PO_4)_2$, 2.109; $MnSO_4 \cdot 7H_2O$, 0.015; $CaCO_3$, 1.293

** Vitamin mixture (mg/100 g of dry diet): p-Aminobenzoic acid, 5; biotin, 0.2; inositol, 200; niacin, 20; Ca-pantothenate, 30; pyridoxine-HCl, 6; riboflavin, 4; thiamine-HCl, 2; menadione, 2; β-carotene, 4.8; α-tocopherol, 10; cyanocobalamin, 0.04; calciferol, 0.6; ascorbic acid, 2000; folic acid, 0.4; choline-HCl, 300

Feeding method. The prawn was cultivated with the several test diets in a circulatory trough ($30 \times 30 \times 60$ cm) at 25°C for 30 or 40 days. The feeding method was essentially the same as reported by KANAZAWA *et al.* (1970).

Results and Discussion

Cholesterol is present in the tissues of the prawn, *P. japonicus*, as a predominant sterol (see part-1.). Accordingly, the requirement for cholesterol was first investigated. The results of the feeding trials of the prawns maintained on the sterol-free and cholesterol-containing diets are shown in Table 14.

In the experiments 1, 2, and 3, the prawn fed on the cholesterol-containing diet grew normally to outward appearances, and the growth rate and survival rate of the prawn were 56–98% and 86–95%, respectively. On the other hand, the growth rate of the prawn fed on the sterol-free diet was inferior to that of the prawn fed on the cholesterol-containing diet, although a definite difference was not perceived between both the experimental groups on the survival rate. Also, a detectable abnormality on the length of antennae, the color of body, and the furtive movement, creeping

Table 14. Results of the feeding experiments on requirement for cholesterol.

Experiment No.	Experimental period (days)	Cholesterol added (%)	Number of prawn at start	Survival rate (%)	Average body weight (g) Initial	Final	Growth rate* (%)
1	30	0	20	45	1.08	1.70	64
		0.5	20	95	1.57	2.28	72
2	40	0	23	78	0.57	0.88	28
		0.5	23	86	0.51	1.59	98
3	30	0	24	63	1.49	1.73	22
		0.5	24	88	1.26	1.83	56

Date of experiment were as follows:
Experiment 1; Jan. 27, 1969–Feb. 26, 1969
Experiment 2; Oct. 25, 1969–Dec. 4, 1969
Experiment 3; July 20, 1970–Aug. 19, 1970

* Growth rate $(\%)=\frac{A}{B}\times 100$

A: Body weight gain (g) of the prawn fed on the test diets
B: Body weight gain (g) of the prawn fed on the clam, *T. philippinarum*

Table 15. Effect of dietary cholesterol levels on the growth and survival rates of the prawn.

Cholesterol added (g/100 g of diet)	Experimental period (days)	Number of prawn at start	Survival rate (%)	Growth rate* (%)
0.05	30	24	82	45
0.1	30	24	88	57
0.5	30	24	88	84
1.0	30	24	92	84
5.0	30	24	83	42

* Growth rate: See Table 14

into the sands, etc., was not perceived in the prawn fed on the sterol-free diet throughout the experimental period. From the results, it was conceivable that the prawn requires to some extent cholesterol or other sterols as a nutrient, although the metabolic role of sterols in this crustacean was obscure.

Hence, to estimate the quantitative requirement of the prawn for cholesterol, the prawns were divided into five groups and fed on the test diets containing the different levels of cholesterol (0.05, 0.1, 0.5, 1.0, and 5.0 g/100 g diet). The results are shown in Table 15. On the survival rate, a definite difference was not observed among the five experimental groups. However, the high growth rate was found to attain when the diet contained 0.5–1.0 g of cholesterol per 100 g of diet. From the results, the quantitative requirement of the prawn, *P. japonicus*, for cholesterol was estimated to be near 0.5 g per 100 g of diet under the experimental conditions adopted. The value obtained in this experiment generally agreed with the requirement of the insects for cholesterol (ITO, 1961; ISHI, 1964; CLAYTON, 1964).

A phytoplankton or yeast has been generally used as a diet for young prawn. Hence, the nutritional effect of other sterols such as mycosterols and phytosterols on the prawn was investigated. In the present study, ergosterol, stigmasterol, and β-sitosterol

were each added to the basal diet and supplied to the prawns. The results are shown in Table 16. The survival rate of the prawns fed on the test diets containing ergosterol, stigmasterol, and β-sitosterol (83–96%) was similar to that of the prawn fed on the cholesterol-containing diet. On the growth rate, ergosterol, stigmasterol, and β-sitosterol were seen to be effective, but inferior to cholesterol. These results suggest that the prawns is capable of utilizing these C_{28}- and C_{29}-sterols to some extent for normal growth. However, it is obscure whether these sterols are digested and accumulated as an endogenous sterol in the prawn.

Table 16. Effect of cholesterol and other sterols on the growth and survival rates of the prawn.

Sterol added (0.5 g/100 g of diet)	Survival rate (%)			Growth rate (%)*		
	Experiment**			Experiment**		
	1	2	3	1	2	3
Cholesterol	95	86	86	72	98	56
Ergosterol	94	87	92	51	79	48
Stigmasterol	96	83	88	62	67	56
β-Sitosterol	89	83	92	56	29	50

* Growth rate: See table 14.

** Experimental period: Experiment, 1, for 40 days; experiment 2 and 3 for 30 days.

In parts-1 and 2, it was shown that the naturally occurring sterols of the prawn are composed of cholesterol (90%), 22-dehydrocholesterol (3%), and 24-methylenecholesterol (7%), and that the prawn did not incorporate acetate-1-^{14}C into sterol fraction. Considering these facts, it is conceivable that the prawn, *P. japonicus*, probably lacks sterol-synthesizing ability and requires cholesterol or other sterols for normal growth, and that this crustacean may modify some C_{28}- and C_{29}-sterols to endogenous sterols somewhat.

Summary

In this part, the requirements of the prawn, *Penaeus japonicus*, for sterols were investigated by the feeding trials using the artificial diet devised by KANAZAWA *et al.* (1970). The results obtained are as follows:

1) The prawn fed on the cholesterol-containing diet grew normally, and the survival and growth rates were 86–95% and 56–98%, respectively. However, the growth rate of the prawn fed on the sterol-free diet was poor (22–64%).

2) The quantitative requirement of the prawn for cholesterol was estimated to be approximately 0.5 g per 100 g of diet under the experimental conditions adopted.

3) The survival rate of the prawns fed on the test diets containing ergosterol, stigmasterol, and β-sitosterol was similar to that of the prawn fed on the cholesterol-containing diet. However, the growth rate of the prawns fed on these sterols was inferior to that of them fed on cholesterol.

From the above results, it was suggested that the prawn, *P. japonicus*, requires sterols for normal growth.

Part-4. Utilization of Dietary Sterols by Marine Crustaceans

In part-2, it has been assumed that most marine crustaceans probably lack ability for sterol-synthesis as also occurs in insects. In the case of the prawn, *Penaeus japonicus*, it has been manifested by the nutritional experiment that this crustacean requires sterols as a diet for normal growth (see part-3). In this part, the author intended to obtain the knowledge on the utilization of sterols other than cholesterol by marine crustaceans. In this approach, the prawn, *P. japonicus*, and *Artemia salina* were used as experimental animals.

A. Utilization of the dietary sterols by *Artemia*.

Materials and Methods

Sterol composition of *Cryptococcus albidus* and "Wakamoto". The sterol composition of the diets used for *Artemia*, marine-occurring yeast, *Cryptococcus albidus*, and "Wakamoto" (Wakamoto Pharmaceutical Co., Japan), was first investigated. *C. albidus* was incubated on a reciprocal shaker at 25°C for 48 hours according to the same method as reported elsewhere (TESHIMA & KANAZAWA, 1971), and then the cells were collected by the centrifugation method (3000 rpm, 15 min.) and subjected to isolation of sterols. The incubation medium used for *C. albidus* was composed of 8 g of molasses, 1 g of polypeptone, 0.5 g of yeast extract, and 1000 ml of the natural sea water filtered through a glass filter No. 5. The cells of *C. albidus* and "Wakamoto" were saponified with alcoholic potassium hydroxide, and then the sterols were isolated from the non-saponifiable materials by column chromatography on alumina (grade II). The composition of sterols was analyzed by gas-liquid chromatography (GLC). The identification of sterols was carried out by using at least two or more columns (1.5% SE-30, 1.5% OV-17, 1.0% XE-60, and 1.0% NGS-1.0% XE-60). The details of the procedures for isolation of sterols and for GLC were mentioned in part-1.

Feeding experiment using marine-occurring yeast and "Wakamoto".

The hatched *Artemia* was cultivated with the two types of diets, marine-occurring yeast, *C. albidus*, and "Wakamoto" The cultivation was carried out at 25–27°C for 8 days by the similar manner to that described in part-2-B. After cultivation, the sterols were isolated from each *Artemia* fed on the above diets, and then the sterol composition was analyzed by GLC.

Results

The sterol composition of *C. albidus* and "Wakamoto" is shown in Table 17. The GLC analysis showed that *C. albidus* contained ergosterol (72%) and campesterol (28%), and also that "Wakamoto" contained ergosterol (58%), stigmasterol (1%), β-sitosterol (28%), and an unknown sterol (13%). No cholesterol was detected in

Table 17. Composition of the sterols isolated from *C. albidus* and "Wakamoto".

Sterol	RRT in SE-30*	% Composition	
		C. albidus	"Wakamoto"
Unknown	0.53	—	13
Cholesterol	1.00	—	—
Ergosterol	1.23	72	58
Campesterol	1.29	28	—
Stigmasterol	1.42	—	1
β-Sitosterol	1.61	—	28

* Relative to cholesterol (16.1 min.)

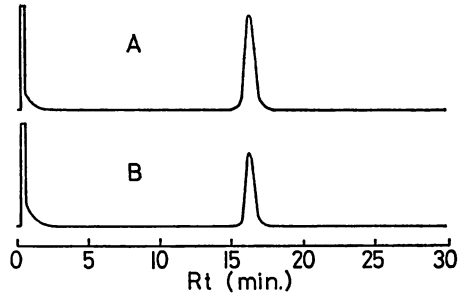


Fig. 4. GLC of the isolated sterol from the *Artemia* fed on *C. albidus* and "Wakamoto".
A, *C. albidus*; B, "Wakamoto"
Column 1.5% SE-30; column temp. 225°C.

both the diets for *Artemia*. However, *Artemia* grew normally and began to copulate at 8 days after hatch. Moreover, Fig. 4 showed that the *Artemia* contained only cholesterol in spite of being cultivated with the diets containing C_{28}- and C_{29}-sterols and no cholesterol. In part-2-B, *Artemia* was found to be incapable of synthesizing sterols from acetate. Considering these results, it is suggested that *Artemia* modifies ergosterol and β-sitosterol to cholesterol.

B. Utilization of the dietary sterols by the prawn, *P. japonicus*.

Materials and Methods

Analysis of the sterols of clam. The clam, *Tapes philippinarum*, was obtained from the commercial source in May, 1970. From the edible parts of clams, the sterols were isolated by the same manner as described in part-1, and then the composition of sterols was investigated by gas-liquid chromatography (GLC) on 1.5% SE-30 and 1.0% NGS-1.0% XE-60.

Feeding experiment using the artificial diet and the clam. The prawn, *P. japonicus*, 1-2 g body weight, was cultivated with the clam and the artificial diets with cholesterol, ergosterol, stigmasterol, or β-sitosterol or without sterols for 30 days at 20-22°C. In this experiment, each 0.5 g of sterols was added to 100 g of the basal

diet (sterol-free) devised by KANAZAWA *et al.* (1970). The details of the methods for preparation of diets and for feeding were essentially the same as reported by KANAZAWA *et al.* (1970). After cultivation, the sterols were isolated from the whole tissues of the prawns fed on the six different diets, and then the content and composition of sterols were determined by the digitonin method (IDLER & BAUMANN, 1952) and by GLC on 1.5% SE–30, respectively.

Results

The sterol content of the prawns fed on the clam and artificial diet is shown in Table 18. After 30 days, the sterol content of tissues slightly decreased in the prawn fed on the sterol-free diet. In the prawns fed on the clam and the sterol-containing

Table 18. The sterol content of the prawns fed on the clam and artificial diets.

Diet supplied	Sterols isolated from the prawns (%)*
Sterol-free diet	0.11
Cholesterol-diet	0.18
Ergosterol-diet	0.17
Stigmasterol-diet	0.15
β-Sitosterol-diet	0.16
Clam	0.18
Prawn before cultivation	0.17

* Per fresh matter

diets, however, the sterol content was approximately equal to that of the prawn before cultivation. These results suggest that the prawn digested cholesterol and other sterols and accumulated as endogenous sterols. The sterol composition of the prawns fed on the artificial diets is given in Table 19. The sterols isolated from the prawn before cultivation were composed of cholesterol (90%), 22-dehydrocholesterol (3%), 24-methylenecholesterol (7%), and β-sitosterol (trace). After 30 days, the sterol composition of the prawns fed on the artificial diets changed considerably. In the prawns fed on the diets containing only ergosterol, stigmasterol, and β-sitosterol, no detectable

Table 19. Sterol composition of the prawns fed on the artificial diets.

Sterol identified	% Composition of the sterols in the prawns*					
	Before cultivation	After cultivation with the artificial diets**				
		1	2	3	4	5
22-Dehydrocholesterol	3	—	trace	—	—	trace
Cholesterol	90	98	99	99	99	96
Desmosterol	—	1	—	1	—	3
24-Methylenecholesterol	7	1	1	trace	1	1
Stigmasterol	—	—	—	—	—	—
Ergosterol	—	—	—	—	—	—
β-Sitosterol	trace	trace	trace	trace	trace	trace

* Determined by GLC on 1.5% SE–30

** 1, sterol-free; 2, cholesterol-containing; 3, ergosterol-containing; 4, stigmasterol-containing; 5, β-sitosterol-containing

increase of these sterols was perceived. Moreover, the percentages of cholesterol were found to be high as compared with that of the prawn before cultivation. These results suggest that the prawn probably converts these C_{28}- and C_{29}-sterols to cholesterol.

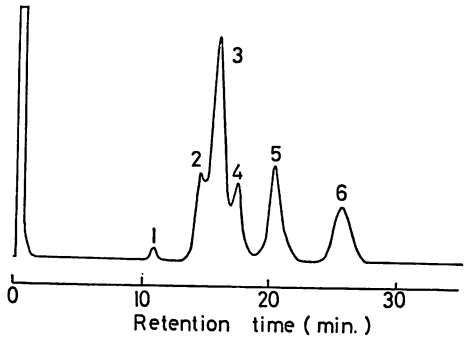


Fig. 5. GLC of the sterols isolated from the clam, *T. philippinarum*. Column 1.5% SE-30; column temp. 225°C
1, unknown sterol; 2, 22-dehydrocholesterol; 3, cholesterol 4, desmosterol; 5, 24-methylenecholesterol; 6, β-sitosterol

On the other hand, the sterols of the clam were composed of 22-dehydrocholesterol (4%), cholesterol (39%), desmosterol (17%), 24-methylenecholesterol (24%), β-sitosterol (15%), and an unknown sterol (1%) as shown in Fig. 5 and Table 20. From the prawn fed on the clam containing the above sterols, large amounts of desmosterol and 24-methylenecholesterol were detected as compared with that of the prawn fed on the sterol-free diet. The results may lead the following assumptions: (1) Some desmosterol and 24-methylenecholesterol found in the tissues of wild prawn may originate from dietary sources such as clams. (2) The turnover of desmosterol and 24-methylenecholesterol may proceeds slowly in comparison with that of ergosterol, stigmasterol, and β-sitosterol if the conversion of the two sterols to cholesterol takes place in the prawn.

Table 20. Sterol composition of the clam and the prawn fed on the clam.

Identified sterol	% Composition of sterols	
	Clam	Prawn fed on the clam
Unknown sterol	1	trace
22-Dehydrocholesterol	4	trace
Cholesterol	39	88
Desmosterol	17	9
24-Methylenecholesterol	24	3
β-Sitosterol	15	trace

Discussion

In part-2 and part-3, it has been shown that the prawn, *P. japonicus*, probably lacks

sterol-synthesizing ability from acetate and requires sterols as a diet. In marine crustaceans, however, there is no report on the utilization of sterols other than cholesterol. In the insects, it has been shown that cholesterol is generally effective for growth of the insects, although ergosterol, stigmasterol, and β-sitosterol are also utilized considerably as a substituent for cholesterol (CLAYTON, 1964). Furthermore, it has been pointed out that the ability for utilization of sterols may be related to the feeding habits of insects: That is, most herbivorous and omnivorous insects seem to be capable of utilizing phyto- and fungal sterols (C_{28}- and C_{29}-sterols) in addition to zoosterols (C_{27}-sterols), whereas carnivorous ones are incapable of utilizing C_{28}- and C_{29}-sterols (FRAENKEL *et al.*, 1941: LEVINSON, 1960). In the present study, it was demonstrated that *Artemia,* herbivorous crustacean, is capable of utilizing ergosterol and/or campersterol for normal growth, and also that the prawn may digest ergosterol, stigmasterol, and β-sitosterol and convert these sterols to cholesterol. Considering these results, it may be assumed that marine crustaceans possess ability for modification of some phyto- and fungal sterols. However, this assumption should be confirmed by a more suitable experiment.

Summary

In this part, the utilization of sterols in marine crustaceans was investigated by using *Artemia salina* and the prawn, *Penaeus japonicus*. The results obtained are as follows:

1) In spite of being supplied with the diets containing ergosterol, stigmasterol, campesterol, and β-sitosterol, *Artemia* was found to contain only cholesterol as a sterol. in the tissues

2) In the prawns fed on the artificial diets containing each stigmasterol, ergosterol, and β-sitosterol, the percentages of cholesterol increased in comparison with that of the prawn before cultivation, although the sterol content was approximately equal to that of the prawn before cultivation.

3) From the prawn fed on the clam, the sterols of which were composed of 22-dehydrocholesterol (4%), cholesterol (39%), desmosterol (17%), 24-methylenecholesterol (24%), β-sitosterol (15%) and an unknown sterol (1%), large amounts of desmosterol and 24-methylenecholesterol were detected as compared with that of the prawn fed on the sterol-free diet.

4) From the above results, it was assumed that *Artemia* is capable of modifying ergosterol and/or campesterol for normal growth, and also that the prawn, *P. japonicus*, may digest ergosterol, stigmasterol, and β-sitosterol and convert them to cholesterol. However, the turnover of desmosterol and 24-methylenecholesterol was conceivable to proceed relatively slowly in the prawn.

Part–5. Bioconversion of Sterol Molecules in Marine Crustaceans

Sterol biosynthesis in marine crustaceans was first investigated using tracer technique

by ZANDEE (1962), and he demonstrated that the crayfish, *Astacus astacus*, is incapable of synthesizing cholesterol from acetate. In other marine crustaceans, it was also found that the crab, *Cancer pagurus* (VAN DEN OORD, 1964), and the lobster, *Homarus gammarus* (ZANDEE, 1964, 1967), lack sterol-synthesizing ability from acetate or mevalonate. In addition, the author showed in part-2 that the lobster, *Panulirus japonica*, the prawn, *Penaeus japonicus*, and the crab, *Portunus trituberculatus*, probably lack sterol-synthesizing ability, though acetate-1-^{14}C was utilized to yield both radioactive fatty acids and non-saponifiable materilas. These findings suggest that most marine crustaceans probably lack sterol-synthesizing ability from acetate and mevalonate. Therefore, some sterols found in their tissues may originate from dietary sources. Especially, in the herbivorous ones, the phyto- and fungal sterols such as β-sitosterol, stigmasterol, and ergosterol may be transformed metabolically to C_{27}-sterols such as cholesterol.

However, the metabolic fate of dietary sterols in marine crustaceans has not been shown so distinctly probably due to the following reasons: (1) The pure sterols cannot be obtainable from commercial sources except a few sterols; (2) the separation of sterol mixture with a closely related structure is not always easy in a usual chromatographic method; (3) the suitalbe radioactive sterols for investigation of sterol metabolism are not obtainable from commercial sources, and it is very difficult to prepare radioactive sterols by chemical methods.

In the present study, the author intended to labell sterols with ^{14}C by the biological method. As a result, the author succeeded in labelling the several sterols with ^{14}C by incubating the protozoan, *Euglena gracilis*, marine-occurring yeast, *Cryptococcus albidus*, and diatom, *Cyclotella nana*, with acetate-1-^{14}C. The present part deals with the bioconversions of ergosterol, β-sitosterol, 24-methylcholesterol,and brassicasterol to cholesterol in the several marine custaceans.

A. Bioconversion of ergosterol to cholesterol in *Artemia salina*.

Materials and Methods

Chemicals. Authentic cholesterol and ergosterol were obtained from Nakarai Chemicals Co., Ltd. (Japan) and purified by recrystallization from methanol. Acetate-1-^{14}C (50.0 mCi/mM) was purchased from Daiichi Chemicals Co., Ltd. (Japan).

Incubation of *Euglena*. Two loops of the cells of *Euglena* were incubated in 5 ml of HUTNER's neutral medium (HUTNER *et al.*, 1949) at 30°C for 6 days. The large-scale incubation was carried out in 1000 ml Erlenmeyer flask containg 500 ml of the same medium. During the present study, the incubation of *Euglena* was carried out in the dark.

Biosynthesis of ergosterol-^{14}C in *Euglena*. The cells of *Euglena* were collected by centrifugation (1500 rpm, 5 min.) and 1 g of cells was incubated in 10 ml of phosphate buffer (pH 7.4) with 100 μCi of acetate-1-^{14}C at 30°C for 6 hours. After incubation, the cells were harvested and washed three times with phosphate buffer by using

the centrifugation method. The wet cells were saponified with alcoholic potassium hydroxide solution (95% ethanol-50% aqueous potassium hydroxide (10: 4)) at 80°C for 3 hours (TESHIMA & KANAZAWA, 1971). The saponification mixture was filtered through a glass filter and then non-saponifiable materials were extracted with ether in the usual manner. The crude sterols were isolated from the non-saponifiable materials by the digitonin-precipitation method (IDLER & BAUMANN, 1952). After decomposing the digitonide by dry pyridine, the sterols so obtained were recrystallized several times from methanol and the radioactivity of the crystals was measured. The composition of sterols was investigated by gas-liquid chromatography (GLC). In addition, as the diet of *Artemia,* 6 g of the cells of *Euglena* was incubated with acetate-1-^{14}C (46 μCi × 6) by the same method.

Bioconversion of ergosterol-^{14}C in *Artemia*. The hatched *Artemia* was cultivated in a trough (30 × 30 × 60 cm) at 25°C. The cultivation was carried out in circulating natural sea water of which the specific gravity had been adjusted previously to 1.040 with sodium chloride (SHIMAYA *et al.*, 1967). The *Artemia* was given "Wakamoto" (Wakomoto Pharmaceutical Co.) as the diet. When *Artemia* began to copulate, 6.0 g of the ^{14}C-labelled *Euglena* was supplied to *Artemia* over 3 days. From the *Artemia* fed on ^{14}C-labelled *Euglena,* the lipids were extracted with chloroform-methanol (BLIGH & DYER, 1959) and saponified with 2N potassium hydroxide in 80% methanol. The crude sterols were obtained from the non-saponifiable materials by the same method as used for *Euglena.* The crude sterols were recrystallized several times from methanol or acetone-water. The radioactivity and composition of the sterols were investigated by a liquid scintillation counter and by GLC, respectively.

GLC and measurement of radioactivity. In GLC, a Shimadzu model GC–3AF gas-chromatographic unit with hydrogen flame ionization detectors and glass column (200 cm × 4 mm I. D.) was used. The column was 1.5% SE–30 on 60–80 mesh Chromosorb W (see part–1). The radioactivity was measured with a Beckman liquid scintillation counter, model LS–150. The toluene solution of PPO (0.6%) and POPOP (0.02%) was used as a scintillator. The efficiency of counting of radioactive sterols was approximately 65 per cent.

Results and Discussion

For investigation of the ergosterol-^{14}C biosynthesis from acetate in *Euglena,* 1 g of the resting cells of *Euglena* was incubated with 100 μCi of acetate-1-^{14}C. After incubation, the sterols were isolated and analyzed by GLC on 1.5% SE–30. In GLC, the *Euglena* was found to contain only ergosterol as shown in Fig. 6. The presence of ergosterol in *Euglena gracilis* was already reported by STERN *et al.* (1960). To the sterol (0.308 mg) isolated from the *Euglena,* about 8.0 mg of authentic ergosterol was added, recrystallized and then the radioactivity of the crystals was measured. The results are shown in Table 21. The results indicate that acetate-1-^{14}C was incorporated into the sterol fraction of *Euglena.* From these facts, *E. gracilis* was conceivably capable of synthesizing ergosterol from acetate.

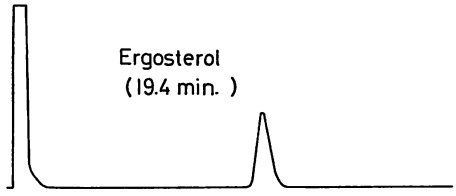


Fig. 6. Gas-chromatogram of the sterol isolated from the *Euglena* after incubation with acetate-1-^{14}C.

Table 21. Recrystallization of the sterol isolated from the *Euglena* incubated with acetate-1-^{14}C.

Crystallization	Solvent system	Specific activity* (dpm/mg)
1 st	Methanol	2340
2 nd	Methanol	2110
3 rd	Methanol	2160

* To the sterol isolated from the *Euglena*, about 8.0 mg of authentic ergosterol was added and recrystallized.

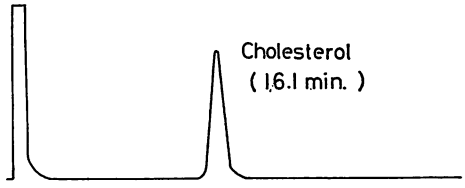


Fig. 7. Gas-chromatogram of the sterol isolated from the *Artemia* fed on ^{14}C-labelled *Euglena*.

Table 22. Recrystallization of the sterol isolated from the *Artemia* fed on ^{14}C-labelled *Euglena*.

Crystallization	Solvent system	Specific activity* (dpm/mg)
1 st	Methanol	3010
2 nd	Acetone-water	2920

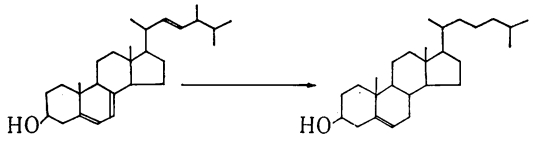
* To the sterol isolated from the *Artemia*, about 10 mg of authentic cholesterol was added and recrystallized.

From the *Artemia* fed on the *Euglena* labelled with ^{14}C, the sterols (3.4 mg) were isolated and subjected to GLC. As shown in Fig. 7, the gas-chromatogram of the sterols showed that the *Artemia* contained only cholesterol in spite of being supplied with the *Euglena* containing only ergosterol as a sterol. To the sterol isolated from the *Artemia*, about 10 mg of authentic cholesterol was added, recrystallized and the radioacitvity of the crystals was measured. As shown in Table 22, a relatively high radioactivity was recovered from the crystals. On the basis of the above data, it was indicated that *Artemia* converts the dietary ergosterol to cholesterol metabolically.

On the bioconversion of the sterol molecules in marine invertebrates, relatively little

has been known until now. FAGERLUND & IDLER (1960) have reported that the starfish, *Pisaster ochraceus,* converts injected cholesterol-4-^{14}C to 7-cholestenol, and they assumed that such bioconversion of Δ^5-sterols to Δ^7-sterols may occur in other marine invertebrates. Furthermore, they have demonstrated that cholesterol-26-^{14}C injected into the clam, *Saxidomus giganteus,* can undergo unsaturation in at least two positions, viz. at C–22 and C–25 (FAGERLUND & IDLER, 1961a), and also that cholesterol could be converted to 24-methylenecholesterol *in vivo* by this clam, with the suggestion of the possible pathway in the biosynthesis of C_{28}-sterols in lamellibranchs (FAGERLUND & IDLER, 1961b). In addition, a few nutritional studies have been carried out on the sterol metabolism in marine invertebrates. TAMURA *et al.* (1964a) have reported that β-sitosterol added to the diet was digested and accumulated as endogenous sterols in the small oyster, *Grassostrea virginica.* In part–3 and part–4, the author suggested that the prawn, *Penaeus japonicus,* may convert dietary ergosterol and β-sitosterol to cholesterol. However, no report is to be found on the bioconversion of ergosterol to cholesterol in marine invertebrates.

In insects, it has been demonstrated that the cockroach, *Blattela germanica,* slightly transformed a part of ergosterol to $\Delta^{5,22}$-cholestadienol (CLARK & BLOCH, 1959b; CLAYTON, 1960), although no enzyme systems for the reduction of the Δ^{22}-bond was found in this species (NOLAND, 1954). As far as the author knows, the present study is the first one showing evidence of the biological conversion of ergosterol to cholesterol.



Ergosterol Cholesterol

B. Bioconversion of ergosterol to cholesterol in the crab, *Portunus trituberculatus*

Materials and Methods

Chemicals. Authentic cholesterol and ergosterol were obtained from Nakarai Chemicals Co., Ltd. (Japan) and purified by recrystallization from methanol. Cholesteryl acetate was prepared by the acetylation of cholesterol with acetic anhydride-dry pyridine (1 : 1). The purity of these steroids was ascertained by gas-liquid chromatography (GLC) using at least two columns (1.5% SE–30 and 1.5% OV–17). Acetate-1-^{14}C (57.0 mCi/mM) was purchased from the Radiochemical Centre, Amersham (England).

Preparation of the ergosterol-^{14}C-containing diet. In order to labell ergosterol with ^{14}C, 11.5 g of the resting cells of *Euglena gracilis* was incubated with 120 μCi of acetate-1-^{14}C in 50 ml of phosphate buffer (pH 7.0) at 30°C for 6 hours according to the same method as described in part–5–A. As a result, *Euglena*-^{14}C containig only

ergosterol as a radioactive sterol was obtained. To 14.0 g of the artificial diet (sterol-free) for the prawn, *P. japonicus,* devised by KANAZAWA *et al.* (1970), 10.0 g of the *Euglena*-^{14}C containing ergosterol-^{14}C (25,100 dpm) was added, mixed thoroughly, heated on a water-bath at 80°C for 30 minutes, and then cooled in a refrigerator until use.

Administration of the ergosterol-^{14}C-containing diet to the crab. The crab, *Portunus trituberculatus,* 10–17 g body weight, was caught at the Kinko-bay, Kagoshima, in August, 1970. During the experimental period, the crab was kept in a plastic trough equipped with circulating systems by filtration through a sand bed at 25°C as shown in part–3. The diet was cut into about 5 mm cube and supplied to eight crabs over 10 days.

Analysis of the sterols. From the crab fed on the diet containing ergosterol-^{14}C, the lipids were extracted with chloroform-methanol (BLIGH & DYER, 1959), saponified with alcoholic potassium hydroxide, and then the sterols were isolated from the non-saponifiable materials by column chromatography on alumina (grade II). The details of the procedure for isolation of sterols were described in part–1. The sterols obtained by column chromatography were recrystallized several times from methanol and the radioactivity of the crystals was measured with a liquid scintillation counter, Beckman LS–150. The composition of the sterols so obtained was investigated by GLC using three different columns (1.5% SE–30, 1.5% OV–17, and 1.0% NGS–1.0% XE–60). For the purpose of separating sterols, the radioactive sterols were acetylated and subjected to column chromatography on a mixture of silver nitrate-silicic acid (Mallinckrodt Chemical Works, U. S. A.) (1: 4, w/w) (VROMAN & COHEN, 1967). The column was eluted step-wise with hexane-benzene, and the fractions were monitored by GLC on 1.5% SE–30.

Results and Discussion

From the crab fed on the ergosterol-^{14}C-containing diet, 60.9 mg of the sterols (13,200 dpm) was isolated. The GLC analysis showed that the sterol was composed of only one component corresponding to cholesterol as shown in Fig. 8. The results

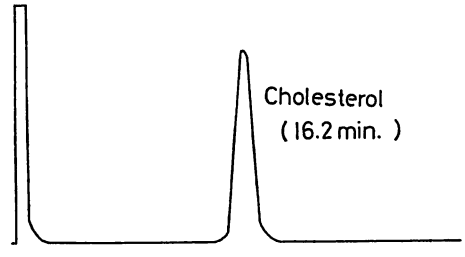


Fig. 8. Gas-chromatogram of the sterol isolated from the crab fed on ^{14}C-labelled *Euglena.*
Column 2.0 m × 4 mm I.D., 1.5% SE–30
Column temp. 225°C
Nitrogen 2.0 kg/cm^2

sugested that the crab, *P. trituberculatus,* converts the dietary ergosterol to cholesterol, because it was already shown in part–2 that this crab probably lacks sterol-synthesizing ability from acetate.

To confirm the bioconversion of ergosterol to cholesterol, the radioactive sterol was acetylated and chromatographed on a silver nitrate-impregnated silicic acid. As shown in Fig. 9, the radioactive steryl acetate gave one peak corresponding to cholesteryl acetate in this chromatography. The radioactive cholesteryl acetate-^{14}C

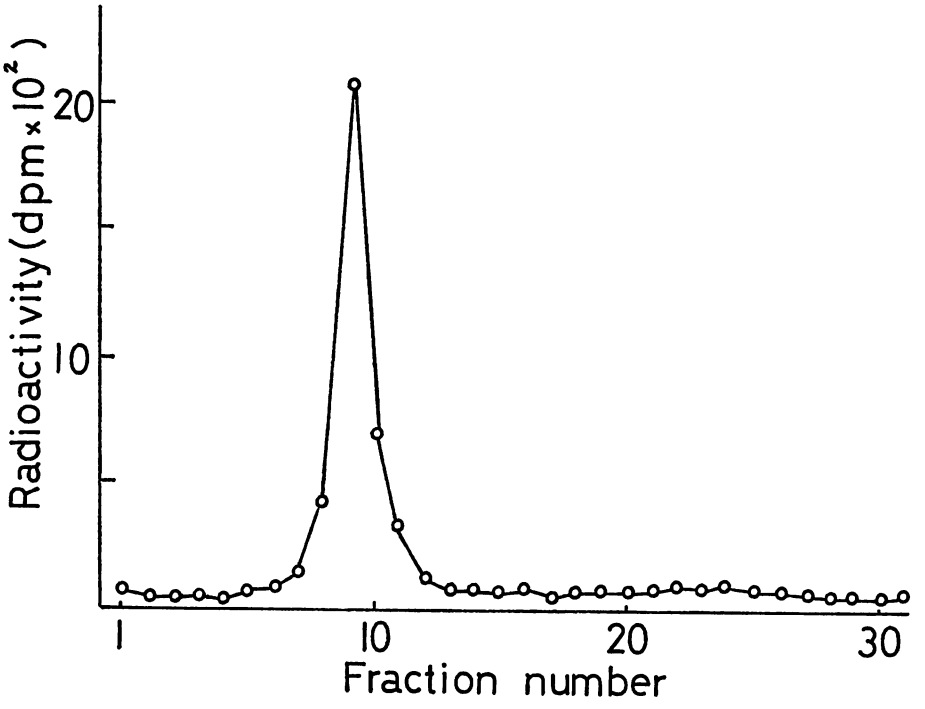


Fig. 9. Column chromatography of the sterol (as acetate) isolated from the crab, *P. trituberculatus.*

fraction was recrystallized several times from chloroform-metahnol. Finally, to the presumed cholesteryl acetate-^{14}C, about 5 mg of authentic cholesteryl acetate was added, recrystallized, and then the radioactivity of the crystals was measured. As shown in Table 23, the specific activity of the crystals was constant in the last three crystallizations.

On the basis of the above data, it was concluded that the crab, *Portunus trituberculatus,* is capable of converting ergosterol to cholesterol. In part–5–A, the author showed by using tracer technique that *Artemia salina* converts the dietary ergosterol to cholesterol. The present study further demonstrated that the above conversion of ergosterol to cholesterol takes place in the crab, *P. trituberculatus,* carnivorous crustacean, as well as in *Artemia,* herbivorous crustacean. From these facts, it is assumed that other marine crustaceans may possess the ability for conversion of ergosterol to cholesterol.

Table 23. Recrystallization of cholesteryl acetate-^{14}C isolated from the crab, *P. trituberculatus*, fed on the diet containing *Euglena*-^{14}C.

Crystallization	Solvent system	Specific activity* (dpm/mg)
1 st	Methanol	132
2 nd	Methanol	127
3 rd	Acetone-water	135

* To the cholesteryl acetate fraction (30 mg) obtained by column chromatography, about 5 mg of non-radioactive cholesteryl acetate was added and recrystallized.

C. Bioconversion of 24-Methylcholesterol to cholesterol in *Artemia salina*.

Materilas and Methods

Chemicals. Cholesterol and ergosterol were obtained from Nakarai Chemicals Co., Ltd. (Japan) and purified by recrystallization from methanol. 24-Methylcholesterol (probably campesterol) was isolated from the marine-occurring yeast, *Cryptococcus albidus* (Teshima & Kanazawa, 1971). The purity of these sterols was checked by gas-liquid chromatography (GLC) on 1.5% SE-30 and 1.5% OV-17. Acetate-1-^{14}C (57 mCi/mM) was purchased from the Radiochemical Centre, Amersham (England).

Analytical methods. GLC was performed as described in part-1. Column chromatography on a silver nitrate-impregnated silicic acid and thin-layer chromatography (TLC) on a paraffin-impregnated Kieselguhr were carried out according to the method of Vroman and Cohen (1967) and that of de Souza and Nes (1969), respectively. Radioactivity was measured with a Beckman liquid scintillation counter by using PPO-POPOP as a scintillator. Mass spectrum was obtained with a Hitachi RMU-6D instrument (chamber voltage, 70 eV).

Marine-occurring yeast. One loop of the cells of *C. albidus* was transferred into the sterile medium containing 0.15 g of molasses, 0.15 g of polypeptone, 0.075 g of yeast extract, and 150 ml of the natural sea water filtered through a glass filter (No. 5). The culture was incubated on a reciprocal shaker at 26°C. At the log-phase of growth, the cells of *C. albidus* were collected by centrifugation (4000 rpm, 15 min.).

Incubation of *C. albidus* with acetate-1-^{14}C. The resting cells of *C. albidus* (10 g) were incubated in 150 ml of phosphate buffer (pH 7.0) with 150 μCi of acetate-1-^{14}C for 4 hours. After incubation, the cells were collected and washed three times with phosphate buffer by the centrifugation method. The cells were saponified with alcoholic potassium hydroxide at 80°C for 3 hours as described in part-5-A, and then the sterols were isolated from the non-saponifiable materials by column chromatography on alumina (grade II). The identification of sterols was carried out by GLC and by mass spectrometry (Knights, 1967).

Administration of ^{14}C-labelled *C. albidus* to *Artemia*. The hatched *Artemia* was cultivated with "Wakamoto" until it began to copulate, and then the adult *Artemia* was fed on the *C. albidus* labelled with ^{14}C over 3 days. After cultivation, the sterols were isolated from the cells of *Artemia* (30 g), and then the radioactivity and composition of sterols were investigated. The details of the procedures for cultivation of *Artemia* and for isolation of sterols were described in part-5-A.

Results and Discussion

From the cells of *C. albidus* incubated with acetate-1-^{14}C, the sterols were isolated and purified by recrystallization from methanol. The purified sterols (24.0 mg) showed a relatively high radioactivity (34,400 dpm). In GLC on 1.5% SE-30, the sterols were found to contain two components corresponding to ergosterol (73%) and campesterol (27%) as shown in Fig. 10. The mass spectrum of the sterols showed

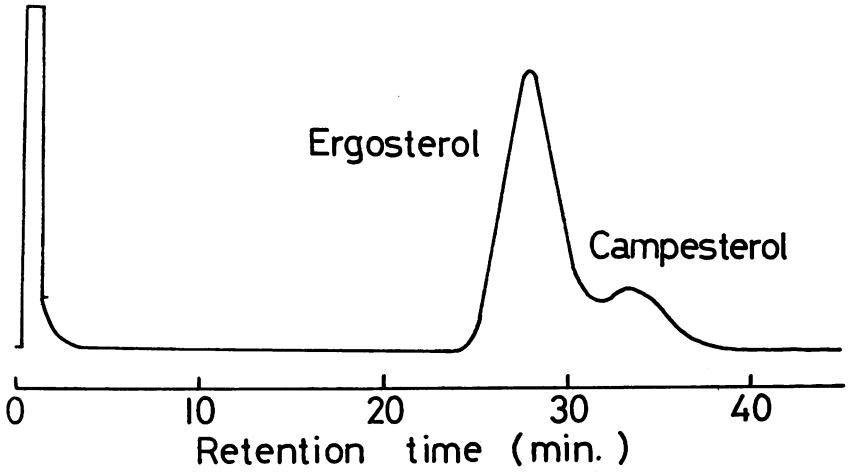


Fig. 10. Gas-chromatogram of the sterols isolated from the *C. albidus*. Column 1.5% SE-30; column temp. 215°C

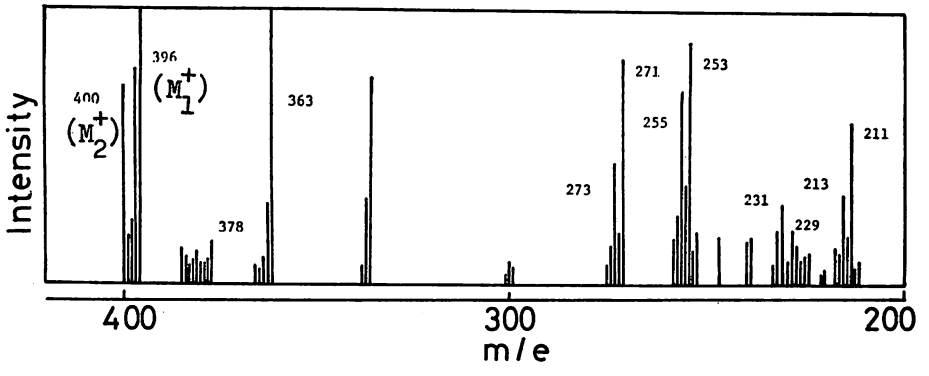


Fig. 11. Mass spectrum of the sterols isolated from the *C. albidus*.

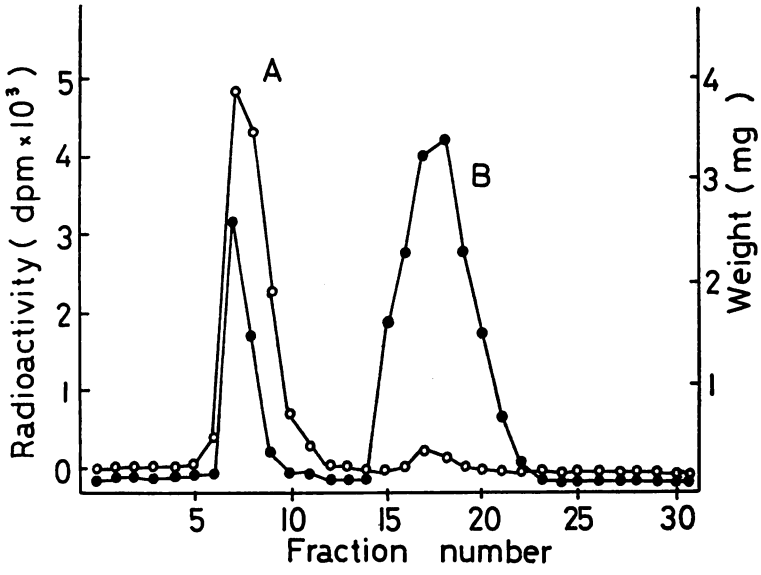


Fig. 12. Column chromatography of the sterols isolated from the *C. albidus* incubated with acetate-1-^{14}C.

The sterols were acetylated with acetic anhydride-dry pyridine (1:1) and then subjected to column chromatography on a silver nitrate-impregnated silicic acid. •——•, weight of steryl acetate; ○——○, radioactivity; A, corresponding to campesterol; B, corresponding to ergosterol

the presence of two molecular ion peaks at m/e 400 (M^{+}_{2}; corresponding to campesterol and 396 ($M;^{\dagger}_{1}$ corresponding to ergosterol) and other prominent peaks at m/e 385 ($M^{\ddagger}_{2}-CH_3$), 381 ($M^{\ddagger}_{2}-CH_3$), 382 ($M^{\ddagger}_{2}-HOH$), 378 ($M^{\dagger}_{1}-HOH$), 367 [$M^{\ddagger}_{2}-(CH_3+HOH)$], 363 [$M^{\dagger}_{1}-(CH_3+HOH)$], 273 ($M^{\ddagger}_{2}-R_2$, R_2=alkyl side chain of campesterol), 271 ($M^{\dagger}_{1}-R_1$, R_1=alkyl side chain of ergosterol), 255 [$M^{\ddagger}_{2}-(R_2+HOH)$], 253 [$M^{\dagger}_{1}-(R_1+HOH)$], 231 [$M^{\ddagger}_{2}-(R_2+42)$], 229 [$M^{\dagger}_{1}-(R_1+42)$], 213 [$M^{\ddagger}_{2}-(R_2+42+HOH)$], and 211 [$M^{\dagger}_{1}-(R_1+42+HOH)$] as shown in Fig. 11. The results of mass spectrometry also supported that the sterols isolated from *C. albidus* are composed of ergosterol and campesterol, although the configuration of ethyl and methyl groups at C-24 was left undetermined.

The radioactive sterols were acetylated and chromatographed on a silver nitrate-impregnated silicic acid with hexane-benzene (Vroman & Cohen, 1967). In this chromatography, the steryl acetate mixture was separated into two substances corresponding to campesteryl and ergosteryl acetates as shown in Fig. 12. The radioactive peaks were also identical with the above two steryl acetates in positions. From the above results, it was clarified that *C. albidus* is capable of synthesizing campesterol and ergosterol from acetate. Under the present experimental conditions adopted, however, acetate-1-^{14}C was mainly incorporated into campesterol fraction (90% of radioactivity).

From the *Artemia* fed on the *C. albidus*, containing radioactive campesterol and ergosterol, the sterols (6.8 mg) were isolated. The sterols gave a relatively high radio-

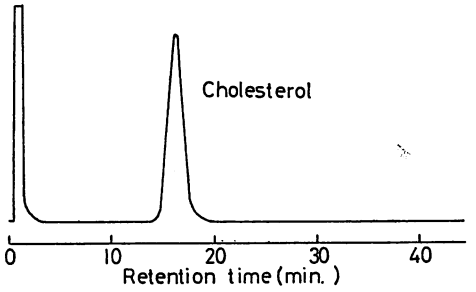


Fig. 13. Gas-chromatogram of the sterol isolated from the *Artemia* fed on the *C. albidus* incubated with acetate-1-^{14}C.
Column 1.5% SE-30; column temp. 225°C

activity (11,900 dpm). The GLC analysis indicated that the sterol was composed of only cholesterol as shown in Fig. 13. These results suggest that *Artemia* may convert both campesterol and ergosterol to cholesterol. In part-5-A, it was already demonstrated that *Artemia* is capable of converting the dietary ergosterol to cholesterol. In this part, considering the high radioactivity recovered in the sterol of *Artemia*, campesterol as well as ergosterol was conceivable to be also converted to cholesterol. To confirm the identification, an aliquot of the radioactive sterol isolated from the *Artemia* was subjected to thin-layer chromatography on a paraffin-impregnated Kieselguhr (DE SOUZA & NES, 1969) and radioautographed. As shown in Fig. 14, the radioautogram gave one spot showing the same mobility as cholesterol. Finally,

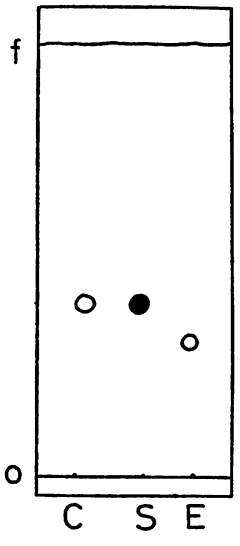


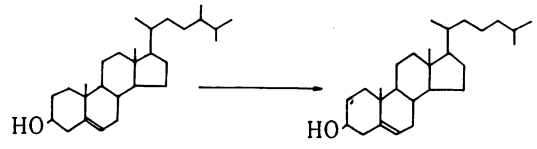
Fig. 14. Radioautogram of the sterol isolated from the *Artemia* fed on the *C. albidus*-^{14}C
The samples were applied to TLC on a paraffin-impregnated Kieselguhr with the system, paraffin oil/acetone-water (4: 1). S indicates the radioactive sample. The reference sterols (E, ergosterol; C, cholesterol) were detected by spraying conc. sulfuric acid-ethanol (1: 1) on the plate.

Table 24. Recrystallization of the radioactive cholesterol isolated from the *Artemia* fed on the *C. albidus*-^{14}C.

Crystallization	Solvent system	Specific activity (dpm/mg)
1 st	Methanol	243
2 nd	Methanol	162
3 rd	Ethanol	151
4 th	Acetone-water	158

to the presumed cholesterol-^{14}C, about 10 mg of non-radioactive cholesterol was added, recrystallized, and then the radioactivity of the crystals was measured. As shown in Table 24, the specific activity (dpm/mg) of the crystals was constant in the last three crystallizations.

On the basis of the above data, it was concluded that *Artemia salina* is capable of converting 24-methylcholesterol (probably campesterol) to cholesterol. The result suggested that *Artemia* may possess the enzyme systems for the demethylation at C-24 of 24-methylcholesterol. In insects, a few literatures contain the several references to the bioconversion of C_{28}- and C_{29}-sterols to C_{27}-sterols (CLAYTON, 1964). However, no report has been presented on the bioconversion of 24-methylcholesterol to cholesterol. The author believes that this study is the first one showing the biological conversion of 24-methylcholesterol to cholesterol in aminals.



24-Methylcholesterol (Campesterol) Cholesterol

D. Bioconversion of brassicasterol to cholesterol in *Artemia salina*.

Materials and Methods

Incubation of *Cyclotella nana* with acetate-1-^{14}C. *C. nana* was incubated in 1000 ml Erlenmeyer flask containing 600 ml of the sterile artificial sea water devised by TAKANO (TAKANO, 1964) under the white fluorescent lamp (40 W) at 21-22°C for 10 days. The cells were harvested by the centrifugation method (4000 rpm, 10 min.). The resting cells were incubated in 10 ml of phosphate buffer (pH 7.4) with 15 μCi of acetate-1-^{14}C (57.0 mCi/mM) at 25°C for 6 hours. In total, 16 g of the cells was used for incubation. After incubation, the cells were collected and washed three times with 20 ml of phosphate buffer by the centrifugation method. The cells were saponified with alcoholic potassium hydroxide at 80°C for 3 hours, and then the sterols were isolated from the non-saponifiable materials by column chromatography on alumina (grade II) according to the same method as described in part-2. The

composition of the sterols so obtained was determined by gas-liquid chromatography (GLC) on 1.5% SE-30. Furthermore, the identification was further confirmed by infrared absorption and mass spectral analyses. In order to clarify the incorporation of acetate-1-^{14}C into sterols, the radioactivity of the sterols was measured with a liquid scintillation counter. Furthermore, an aliquot of the sterols was chromatographed on Kiesel gel G with benzene-ethyl acetate (4: 1) and radioautographed by exposing the chromatogram for 2 weeks.

Administration of *Cyclotella*-^{14}C to *Artemia*. In addition, as the diet of *Artemia*, 10 g of the cells of *Cyclotella* was incubated with acetate-1-^{14}C (15 μCi$\times$10) by the same method. *Artemia* was cultivated with "Wakamoto" in a circulatory trough according to the same method as described in part-4-A. When *Artemia* grew into 4-5 mm body length, about 9.4 g of the ^{14}C-labelled *Cyclotella* was given to them as a diet and they were cultivated for 4 days successively. After cultivation, the sterols were isolated from the *Artemia* and then the composition and radioactivity were investigated by GLC on 1.5% SE-30 and column chromatography on a silver nitrate-impregnated silicic acid and with a liquid scintillation counter, respectively. The details of the procedures for measurement of radioactivity and for chromatography have been described in part-1, part-2, and part-5-B.

Results and Discussion

From the cells (16.0 g) of the *Cyclotella* incubated with acetate-1-^{14}C, the sterols (4.7 mg) were isolated by column chromatography on alumina and further purified by TLC on Kiesel gel G with heptane-ethyl acetate (8: 2). The purified sterol (46,300 dpm) was found to be composed of only brassicasterol by GLC on 1.5% SE-30 as shown in Fig. 15. To confirm the identification, the sterol was further subjected to infrared absorption and mass spectral analyses. The mass spectrum of this sterol showed the molecular ion at m/e 398 (M^+; corresponding to brassicasterol) and other prominent peaks at m/e 383 (M^+-CH_3), 380 (M^+-HOH), 365 [$M^+-(CH_3+HOH)$], 355 [M^+-43 ($C_{25}-C_{27}$)], 337 [$M^+-(43+HOH)$], 300 [$M^+-(97$ ($C_{22}-C_{28}$)$+1H)$], 273 (M^+-R, R=alkyl side chain of brassicasterol), 271 [$M^+-(R+2H)$], 255 [$M^+-(R+HOH)$], 253 [$M^+-(R+2H+$

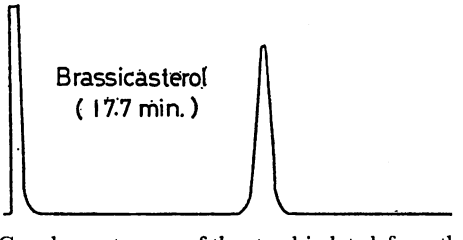


Fig. 15. Gas-chromatogram of the sterol isolated from the *Cyclotella*.
Column: 1.5% SE-30
Column temp.: 225°C

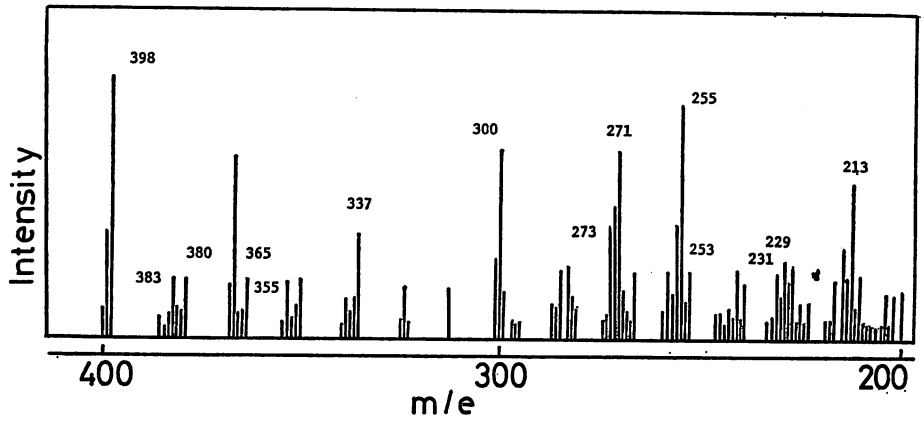


Fig. 16. Mass spectrum of the sterol isolated from the *Cyclotella*.

HOH)], 231 [$M^+-(R+42)$], 229 [$M^+-(R+27+HOH)$], and 213 [$M^+-(R+42+HOH)$] as shown in Fig. 16. The peak at m/e 300 [$M^+-(97+1H)$] was indicative of the presence of a $\Delta^{5,22}$-sterol (WYLLIE & DJERASSI, 1968).

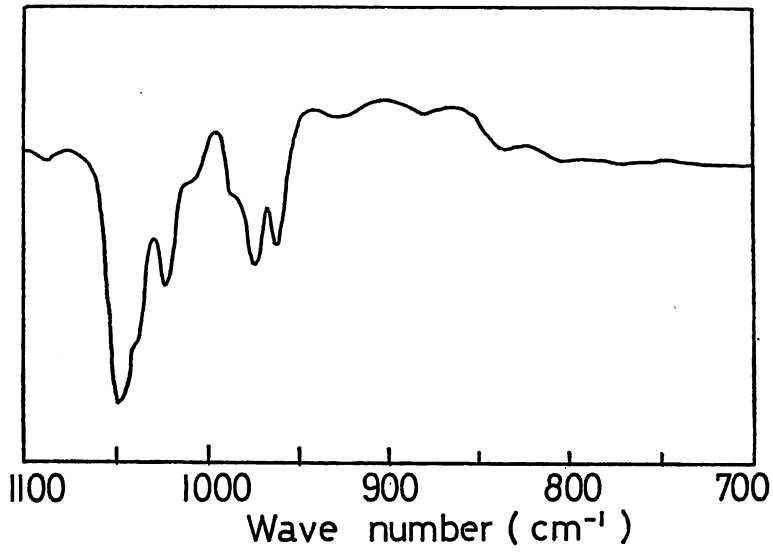


Fig. 17. Infrared absorption spectrum of the sterol isolated from the *C. nana*.

The infrared absorption spectrum of this sterol in Fig. 17 showed a significant absorption at 962 and 973 cm^{-1} showing the presence of a *trans* double bond at C-22 of steroid-side chain (JONES, 1950; COLE, 1956; TAMURA *et al.*, 1964b).

The above data indicated that the sterol isolated from the *Cyclotella* was composed of only brassicasterol. Hence, in order to check the radiochemical purity, the sterol was subjected to TLC on Kiesel gel G with benzene-ethyl acetate (4: 1) and

radioautographed. As shown in Fig. 18, the radioautogram showed the presence of one radioactive substance corresponding to brassicasterol.

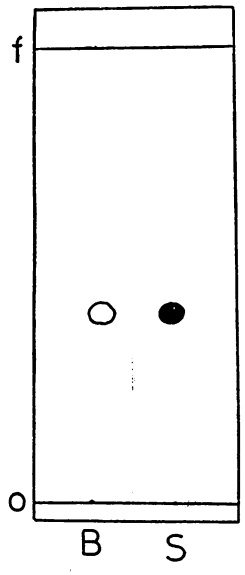


Fig. 18. Radioautography of the sterol isolated from the *Cyclotella* incubated with acetate-1-^{14}C.
After TLC, the plate was covered with X-ray film and exposed for 2 weeks. B and S indicate authentic brassicasterol and the radioactive sample, respectively.

Finally, to the presumed brassicasterol-^{14}C, about 2 mg of authentic brassicasterol was added and recrystallized from chloroform-methanol. As shown in Table 25, the crystals of the sterol showed constant specific activity during the crystallizations. From the above data, it was demonstrated that *Cyclotella nana* is capable of synthesizing brassicasterol from acetate.

From the *Artemia* (42 g) fed on the *Cyclotella*-^{14}C, containing brassicasterol-^{14}C, 6.3 g of the radioactive sterols (56,000 dpm) was isolated. The GLC of the sterol

Table 25. Recrystallization of brassicasterol-^{14}C isolated from the *Cyclotella* incubated with acetate-1-^{14}C.

Crystallization	Solvent system	Specific activity (dpm/mg)
1 st	Chloroform-methnol	11,500
2 nd	Chloroform-methanol	11,100
3 rd	Chloroform-methanol	11,500

showed the presence of one component corresponding to cholesterol as shown in Fig. 19. Hence, in order to confirm the identification, about 10 mg of non-radioactive cholesterol was added to the presumed cholesterol-^{14}C, acetylated with acetic anhydride-dry pyridine (1: 1), and then the steryl acetate was chromatographed on a mixture of silicic acid-silver nitrate (4: 1, w/w) with hexane-benzene. As shown in Fig. 20, the steryl acetate gave one radioactive peak corresponding to cholesteryl acetate.

Finally, the cholesteryl acetate-^{14}C fraction obtained by the column chromatography was recrystallized several times from chloroform-methanol. As shown in Table 26, the crystals of the steryl acetate showed constant specific activity in the last three crystallizations.

On the basis of the above data, it was concluded that *Artemia* is capable of converting the dietary brassicasterol to cholesterol.

The author has demonstrated in part–5–A and part–5–B that the bioconversion of ergosterol to cholesterol takes place in *Artemia* and the crab, *P. trituberculatus*. The

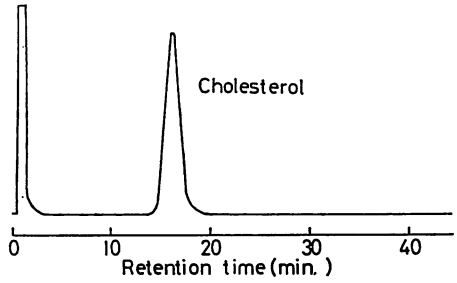


Fig. 19. Gas-chromatogram of the sterol isolated from the *Artemia* fed on the *Cyclotella*-^{14}C.
Column 1.5% SE-30; column temp. 225°C.

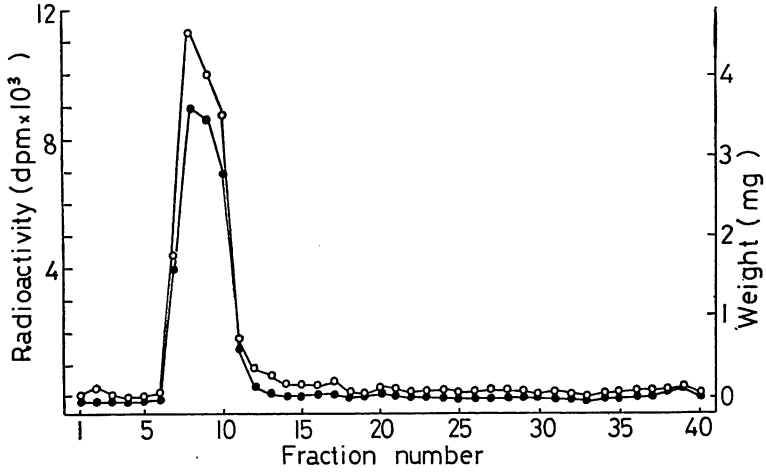


Fig. 20. Silver nitrate-impregnated silicic acid column chromatography of the sterol (as acetate) isolated from the *Artemia* fed on the *Cyclotella*-^{14}C.
o——o, radioactivity; •——•, weight of steryl acetate

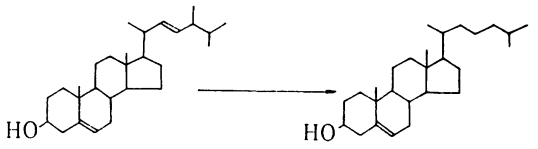
Table 26. Recrystallization of cholesteryl acetate-^{14}C isolated from the *Artemia*.

Crystallization	Solvent system	Specific activity (dpm/mg)
1 st	Chloroform-methanol	1240
2 nd	Chloroform-methanol	1230
3 rd	Chloroform-methanol	1250

above conversions of ergosterol to cholesterol were conceivable to necessitate at least the removal of methyl group at C-24 and the reduction of double bonds at C-7 and C-22 from the molecule of ergosterol. Accordingly, it seems reasonable to assume that cholesterol is formed from ergosterol at least via two intermediates such as brassicasterol and 24-methylcholesterol. However, no intermediate has been detected in the experiments performed in part-5-A and part-5-B. In the present part, it

was evidently proved that *Artemia* is capable of converting brassicasterol to cholesterol, and suggested that this crustacean possesses the enzyme systems for the demethylation at C–24 and for the reduction of double bond at C–22 of brassicasterol. Furthermore, it has been clarified in part–5–C that *Artemia* converts the dietary 24–mehylcholesterol to cholesterol.

Considering these facts, it may be postulated that brassicasterol and/or 24-methylcholesterol are one of the possible intermediates in the bioconversion of ergosterol to cholesterol in *Artemia*.



Brassicasterol Cholesterol

E. Bioconversion of β-sitosterol to cholesterol in the prawn, *Penaeus japonicus*

Materials and Methods

Chemicals. β-Sitosterol-4-^{14}C (61 mCi/mM) was purchased from the Radiochemical Centre, Amersham (England). Cholesterol and β-sitosterol were obtained from Nakarai Chemicals Co., Ltd. (Japan). The purity of these sterols was ascertained by thin-layer chromatography (TLC) and by gas-liquid chromatography (GLC).

Injection of β-sitosterol-4-^{14}C. The prawn, *Penaeus japonicus,* about 3 g body weight, was kindly supplied from the Fisheries Experimental Station, Kagoshima Prefecture, in June, 1970. Four prawns were each injected with 0.2 μCi of β-sitosterol-4-^{14}C in 0.01 m*l* of ethanol and kept in a circulating sea water at 20–24°C. During the experimental period, no food was given to them. After 48 hours, the prawns were killed by freezing at −20°C.

Isolation of sterols. From the whole tissues of the prawns injected with β-sitosterol-4-^{14}C, the lipids were extracted with chloroform-methanol (BLIGH & DYER, 1959), saponified with 10% alcoholic potassium hydroxide at 80°C for 2 hours, and then the sterols were isolated from the non-saponifiable materials by the digitonin-precipitation method (IDLER & BAUMANN, 1952).

Chromatography. The composition of sterols was determined by GLC using at least two or more columns such as 1.5% SE–30, 1.5% OV–17, 1.0% XE–60, and 1.0% NGS −1.0% XE–60. In TLC, three types of adsorbents were used. For the preliminary purification of sterols, sterols were subjected to TLC on Kiesel gel G with benzene-ethyl acetate (4: 1). For the separation of sterols, free sterols and steryl acetates were subjected to chromatography on a paraffin-impreganated Kieselguhr

with the system (paraffin oil/ acetone-water (4: 1)) devised by DE SOUZA and NES (1969) and on a silver nitrate-impreganated Kiesel gel with benzene-haxane (3: 2) (BAISTED, 1969), respectively.

Measurement of radioactivity. Radioactivity was determined with a Beckman liquid scintillation counter model LS-150 using a solution of PPO (0.6%) and POPOP (0.02%) as a scintillator. In radioautography, a thin-layer plate was covered with a Sakura X-ray film (Konishiroku Photo Ind. Co., Ltd. Japan) and exposed for 2 weeks.

Results and Discussion

From the four prawns injected with β-sitosterol-4-^{14}C, the sterols (13.9 mg) were isolated. In GLC on 1.5% SE-30, the sterol was found to be composed of only cholesterol (see Fig. 21). In GLC on 1.0% XE-60, 1.5% OV-17, and 1.0% NGS —1.0% XE-60, the sterol also gave one peak corresponding to cholesterol. However, the radioautography of the sterols showed the presence of two radioactive substances

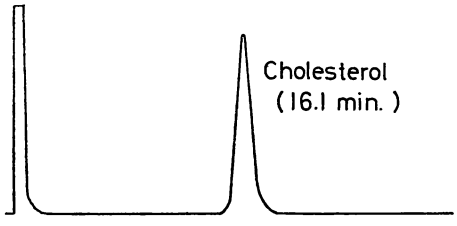


Fig. 21. Gas-chromatogram of the sterol isolated from the prawn injected with β-sitosterol-4-^{14}C.
Column 1.5%SE-30; column temp. 225°C

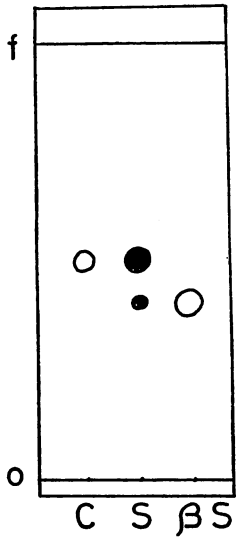


Fig. 22. Radioautography of the sterols isolated from the prawn injected with β-sitosterol-4-^{14}C.

The samples were applied to TLC on a paraffin-impregnated Kieselguhr. The reference sterols were detected by spraying conc. sulfuric acid-ethanol (1:1). C, S, and β-S indicate cholesterol, radioactive sterols isolated from the prawn, and β-sitosterol, respectively.

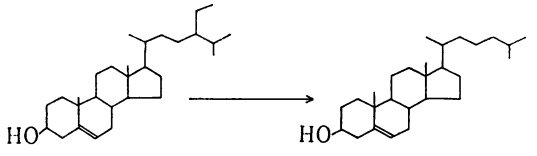
which revealed the same mobilities as cholesterol (Rf, 0.51) and β-sitosterol (Rf, 0.40) as shown in Fig. 22. An aliquot of the radioactive sterols was acetylated with acetic anhydride-dry pyridine (1: 1) and chromatographed on a silver nitrate-impregnated Kiesel gel. In this type of TLC, the radioactive sterols showed one spot corresponding to cholesteryl and β-sitosteryl acetates. Accordingly, the zone corresponding to cholesterol in the former TLC was scraped off from the plate and the sterol was eluted with dichloromethane-methanol (9: 1) (IDLER *et al.*, 1966). To the presumed cholesterol-^{14}C, about 5 mg of non-radioactive cholesterol was added and crystallized repeatedly from the several solvent systems. In the last three crystallizations, the crystals of sterol gave constant specific activity (dpm/mg) as shown in Table 27.

Table 27. Recrystallization of the radioactive cholesterol isolated from the four prawns injected with β-sitosterol-4-^{14}C.

Crystallization	Solvent system	Specific activity (dpm/mg)
1 st	Methanol	339
2 nd	Ethanol	349
3 rd	Acetone-water	344

On the basis of the above data, it was concluded that the prawn, *P. japonicus*, is capable of converting β-stiosterol to cholesterol. In part-3, the author demonstrated by the nutritional experiment that the requirement of the prawn for cholesterol was considerably replaced by β-sitosterol. Furthermore, it was suggested by the feeding trials in part-4 that this prawn may convert β-sitosterol to cholesterol. However, there was no direct evidence that the dealkylation at C–24 of β-sitosterol takes place in marine invertebrates. In insects, a few reports have been presented on the removal of ethyl radical at C–24 of β-sitosterol (CLAYTON, 1964). For example, ROBBINS *et al.* (1962) have shown that the cockroach, *Blattela germanica*, converted the dietary β-sitosterol to cholesterol. On the other hand, KAPLANIS *et al.* (1963) have reported that the housefly, *Musca domestica*, was incapable of converting the dietary β-sitosterol -^{3}H to cholesterol.

From the viewpoint of comparative biochemistry, it is of great interest that the prawn, *P. japonicus*, belonging to the same arthropods as insects, possesses the enzyme systems for the dealkylation at C–24 of β-sitosterol.



β-Sitosterol Cholesterol

Summary

The present study was designed to clarify the *in vivo* bioconversion of dietary C_{28}-

and C_{29}-sterols in marine crustaceans. After the administration of ^{14}C-labelled sterols to the several crustaceans, the sterols were isolated from the tissues of them, and then the bioconversion products were investigated. The identification of sterols was performed by thin-layer chromatography, gas-liquid chromatography, column chromatography, infrared absorption spectroscopy and mass spectrometry. The criterion of identification of bioconversion products was carried out according to constant specific activity (dpm/mg) of the crystals in the serial crystallizations. The results obtained are as follows:

1) It was evidently demonstrated that *Artemia salina* is capable of converting the dietary ergosterol, 24-methylcholesterol, and brassicasterol to cholesterol.

2) It was shown that the crab, *Portunus trituberculatus,* carnivorous crustacean, as well as *Artemia salina,* herbivorous crustacean, also converts ergosterol to cholesterol.

3) The prawn, *Penaeus japonicus,* was found to possess the ability for converting the injected β-sitosterol to cholesterol.

4) From the above results, it is suggested that most marine crustaceans may convert some C_{28}- and C_{29}-sterols to cholesterol.

Part-6. Distribution and Fate of Cholesterol in the Lobster, *Panulirus japonica*

In the previous parts, it has been shown that the several marine crustaceans may probably lack sterol-synthesizing ability (see part-2) and require a dietary source of cholesterol or other sterols (see part-3), and also that some C_{28}- and C_{29}-sterols are converted to cholesterol in marine crustaceans (see part-5). However, the distribution of sterols in the tissues and the sites of sterol metabolism in marine crustaceans are still obscure. In this part, therefore, the fate of injected cholesterol-4-^{14}C was investigated by using the lobster, *Panulirus japonica.*

Materials and Methods

Injection of cholesterol-4-^{14}C to the lobster. The lobster, *P. japonica,* was obtained from the commercial source in August, 1970. During the experimental period, the lobster was kept in a circulatory trough at 20–22°C, and the clam, *Tapes philippinarum,* was given to the lobster as a diet. Three lobsters, 300 g total body weight, were each injected with 1 μCi of cholesterol-4-^{14}C (33.5 mCi/mM, Radiochemical Centre) in 0.01 ml of ethanol into the abdomen.

Determination of the radioactive compounds in the tissues. At 2, 10, and 20 days after injection, the lobsters were taken out from the trough, and then the gonads, heart, intestines, muscle, gills, stomach, hepatopancreas, and shell were dissected out. The tissues were immediately weighed and then stored in a refrigerator until use. From the each tissue, the lipids were extracted with chloroform-methanol according to the method of Bligh and Dyer (1959) and the radioactivity of lipids was measured with a liquid scintillation counter by the same method as mentioned in

part-2.

Fractionation of the radioactive lipids into the free sterols, steryl esters, and polar compounds. An aliquot of the lipids from the tissues of lobsters kept for 10 and 20 days after injection was subjected to thin-layer chromatography (TLC) on Kiesel gel G with cyclohexane-ethyl acetate (7: 3). In this TLC, the lipids were fractionated into the steryl ester (Rf, 0.64–0.69), free sterol (Rf, 0.39–0.45), and polar compound (Rf, 0.00–0.26) fractions. The radioactivity of the three fractions was also measured.

Results

The distribution of radioactive compounds in the lobster tissues after injection of cholesterol-4-^{14}C is shown in Table 28. At 2 days after injection, 32.9% of the administrated radioactivity was recovered in the tissues of lobster, and ^{14}C-activity was detected in the every tissue examined. The radioactivity was very high in the gonads, hepatopancreas, heart, and intestines, but slightly low in the gills and stomach and extremely low in the muscle and shell. The highest radioactivity was detected in the gonads.

With the lapse of day, the total radioactivity in the lobster decreased as shown in Table 28. In the gonads, heart, intestines, hepatopancreas, and gills, the radioactivity decreased within 10 to 20 days after injection, whereas that in the muscle and shell stayed relatively unchanged. In the stomach, the radioactivity appeared to decrease slightly.

The lipids isolated from the tissues of the lobsters kept for 10 and 20 days were fractionated into the steryl ester, free sterol, and polar compound fractions. As shown in Table 29, it was demonstrated that the radioactive compounds from the lobster kept for 10 days were mainly present as free sterols in every tissue, although ^{14}C-activity was also detected in the steryl ester and polar compound fractions. However, a considerable difference was perceived among the tissues on the percentage of the above three fractions. In the gonads, intestines, muscle, gills, and shell, the

Table 28. Distribution of radioactive compounds in the tissues of the lobster, *P. japonica*, injected with cholesterol-4-^{14}C.

Tissue	Radioactivity (dpm/g of wet tissue)		
	Keeping period after injection		
	2 days	10 days	20 days
Gonads	33,600	14,500	6,190
Heart	24,600	11,500	5,290
Intestines	21,800	21,100	14,600
Muscle	3,810	4,760	3,010
Gills	10,300	6,190	2,370
Stomach	11,300	5,290	7,060
Hepatopancreas	26,500	14,600	6,501
Shell	3,320	2,880	1,560
Total	135,000	80,800	46,600

Table 29. Distribution of radioactive free sterols, steryl esters, and polar compounds in the tissues of the lobster kept for 10 and 20 days after injection of cholesterol-4-^{14}C.

Tissue	Day after injection	Relative percentage of ^{14}C-compounds		
		Fraction		
		Free sterol	Steryl ester	Polar compound
Gonads	10	82.8	11.7	5.5
	20	24.7	11.8	63.5
Heart	10	60.6	18.4	21.0
	20	63.4	26.0	10.6
Intestines	10	80.5	8.0	11.5
	20	61.0	29.5	9.5
Muscle	10	90.6	3.1	6.3
	20	79.3	12.4	8.3
Gills	10	70.9	11.7	17.4
	20	33.3	19.3	47.4
Stomach	10	52.0	5.2	42.8
	20	69.2	5.5	25.3
Hepatopancreas	10	60.9	25.9	13.2
	20	51.8	24.1	24.1
Shell	10	86.1	5.1	8.8
	20	75.4	9.0	15.6

radioactivity of free sterol fraction amounted to 70.9–90.6% of the total radioactivity of the three fractions, and the steryl ester and polar compound fractions gave only low radioactivity. In the heart, stomach, and hepatopancreas, the radioactivity of free sterol fraction was low as compared with the above four tissues, and a considerable high radioactivity was recovered in the steryl ester and/or polar compound fractions. In the stomach, nearly half of radioactive compounds was present as polar compounds.

After 20 days, the percentages of radioactive three fractions of lipids varied remarkably in the several tissues. The percentage of free sterol fraction decreased in every tissue except the stomach and heart, in which the free sterol fraction seemed to decrease slightly or to be almost constant. In the gonads, the free sterol fraction decreased remarkably as compared with the other tissues. On the other hand, the percentage of steryl ester fraction appeared to increase slightly or to stay unchanged in all tissues except the intestines in which a considerable increase of steryl esters was perceived. The polar compound fraction increased remarkably in the gonads and decreased in the stomach and heart. However, a notable change was not seen in the other tissues on the polar compound fraction although the polar compounds appeared to increase slightly during the experimental period.

Discussion

Sterols are assumed to be an essential substance for normal growth of marine crustaceans as well as insects. However, no information has been obtained so far on the turnover of sterols and the sites of sterol metabolism in marine crustaceans. In the insects, belonging to the same arthropods as crustaceans, a few reports have been

presented on the fate of exogenous cholesterol in the tissues. CASIDA *et al.* (1957) have studied the distribution of cholesterol-4-^{14}C in the tissues of the American cockroach, *Periplaneta americana,* kept for 16 hours after injection, and shown that the radioactive compounds were present in all tissues examined, with the highest concentration appearing in the gut tissues. Also, it has been clarified that the adult house fly (KAPLANIS *et al.,* 1960) and the German cockroach (ROBBINS *et al.,* 1961) retained administrated sterols for an extended period. Furthermore, ISHII *et al.* (1963) have investigated the fate of cholesterol-4-^{14}C in the different tissues of the American cockroach, *P. americana,* at intervals of 1, 10, and 20 days after injection, and shown that the concentration of radioactive compounds was high in the fat body, foregut, and mid gut, but low in the muscle and testes. They have also indicated that more than 80% of the administrated radioactivity was recovered in the cockroach even at 20 days after injection, with the suggestion that this insect has a strict sterol economy (ISHII *et al.,* 1963).

In the present study, cholesterol-4-^{14}C was injected into the lobster, *P. japonica,* and the distribution and fate of radioactive cholesterol were investigated at intervals of 2, 10, and 20 days after injection. At 2 days after injection, 32.9% of the administrated radioactivity was found to be retained in the lobster tissues. The result indicated that the lobster excreted exogenous cholesterol and/or its metabolites within short times as compared with the insects. In the lobster, it was shown that the concentration of radioactive compounds was high in the gonads, hepatopancreas, heart, and intestines, but low in the other tissues at 2 days after injection, and that the radioactive compounds decreased in all tissues except the muscle and shell with the lapse of day. On the other hand, ISHII *et al.* (1963) have perceived in the American cockroach, *P. americana,* that the radioactive compounds considerably increased in the testes and muscle and decreased in the foregut, fat body, and mid-gut at 10 days after injection of cholesterol-4-^{14}C. Comparing the above data obtained in the lobster and the insects, it may be postulated that the sites of sterol metabolism and the mode of sterol metabolism in marine crustaceans are not always similar to those of insects.

In all tissues of the lobster kept for 10 days, the radioactive steryl esters and some polar compounds were also present in addition to free sterols. In the American cockroach, it has been reported that the percentage of steryl esters increased considerably in the fat body, wings, and alumentary tract during 20 days of experimental period (ISHII *et al.,* 1963). In the case of the lobster, however the percentage of steryl esters was not so markedly increased in all tissues except the intestines. In contrast with the insects, the most notable change in the relative composition of radioactive compounds during the experimental period was perceived in the free sterol and polar compound fractions rather than the steryl esters.

Summary

The distribution and fate of cholesterol-4-^{14}C were studied in the tissues of the

lobster, *Panulirus japonica,* kept for 2, 10, and 20 days after injection. The results obtained are as follows:

1) At 2 days after injection, the concentration of ^{14}C-compounds was high in the gonads, hepatopancreas, heart, and intestines, but low in the other tissues. With the lapse of day, the radioactivity decreased markedly in the gonads, heart, hepatopancreas, and gills, whereas that in the stomach, muscle, and shell decreased slightly or stayed unchanged.

2) Throughout the experimental period, free sterols were predominant in all tissues examined except the gonads. In the gonads of the lobster kept for 20 days after injection of cholesterol-4-^{14}C, the percentage of polar compounds (63.5%) was higher than that of free sterols (24.7%). With the lapse of day, the percentage of free sterols and polar compounds changed considerably, whereas that of steryl esters was relatively constant.

3) The most notable change in the concentration and composition of radioactive compounds was found in the gonads.

Part-7. Bioconversion of Cholesterol to Steroid Hormones in the Lobster, *Panulirus japonica*

In the insects, a few hypotheses have been proposed on the function of sterols: (1) The dietary sterols may be utilized as an element of cellular membrane structure because the concentration of sterols in the insect tissues was relatively constant throughout the developmental stages (Lasser *et al.,* 1963; Levinson & Silverman, 1954); (2) Cholesterol or its metabolites may be concerned with the control of reproduction (Chauvin, 1949); (3) Cholesterol itself plays a significant role as a brain hormone responsible for stimulating the production of ecdysone, insect-molting hormone, by the prothroracic gland (Kobayashi *et al.,* 1962; Kirimura *et al.,* 1962). Furthermore, it has been generally assumed that sterols may be converted to some insect-molting hormones (Karlson & Hoffmeister, 1963; Hikino *et al.,* 1969). vitamin D, and steroid hormones. However, the decisive function of sterols in insects has not been demonstrated.

On the other hand, a great interest attaches to the function of sterols in marine crustaceans from the viewpoint of comparative biochemistry as they molt at the growing stages and probably lack sterol-synthesizing ability from acetate as occurs in insects. However, no report has been presented on the function of sterols in them. Hence, the author intended to obtain some knowledges on the function of sterols in marine crustaceans. In this approach, the bioconversion of cholesterol to steroid hormones in the lobster, *Panulirus japonica,* was investigated by using cholesterol-4-^{14}C.

Materials and Methods

Chemicals. Cholesterol-4-^{14}C (33.5 mCi/mM) was purchased from the Daiichi

Pure Chemicals Co., Ltd. (Japan). The purity of this substance was checked by thin-layer chromatography (TLC) and by gas-liquid chromatography (GLC). Non-radioactive steroids were obtained from Sigma Chemicals Co. (U.S.A.) and Nakarai Chemicals Co., Ltd. (Japan).

Injection of cholesterol-4-^{14}C. The lobster, *P. japonica,* about 150 g body weight, was injected into the muscle with 10 μCi of cholesterol-4-^{14}C in 0.1 ml of ethanol and kept in a circulating sea water at 22°C. During the experimental period, no food was given to the lobster. After 48 hours, the lobster was taken out from the trough and dissected, and then the hepatopancreas, ovaries, and blood (from the heart) were taken out and chilled until use.

Separation of bioconversion products. From the ovaries, hepatopancreas, and blood, the metabolites were extracted with 7 volumes of dichloromethane, and then the solvent was removed below 40°C under reduced pressure. To the residue, about 10 μg each of non-radioactive cortisol, cortisone, corticosterone, deoxycorticosterone, testosterone, 17α-hydroxyprogesterone, androstenedione, and progesterone was added as a carrier for radioactive steroids produced. The dichloromethane extract was fractionated twice in one dimension by TLC on Kiesel gel G-Kiesel gel GF_{254} (5: 1) using the first solvent, ethyl acetate-cyclohexane-toluene (10: 10: 1), and the second system, chloroform-methanol-water (90: 10: 1). The carrier steroids on the plate were located under the ultraviolet-light (wave length, 253 mμ), eluted with dichloromethane-methanol (9: 1) (IDLER *et al.*, 1966), and subjected to TLC and paper-chromatography (PPC) in order to identify the steroids produced from cholesterol-4-^{14}C. The solvent systems used in TLC were: S1, chloroform-methanol-water (188: 12: 1); S2, benzene-acetone (75: 25); S3, chloroform-methanol (97: 3); S4, ethyl acetate-cyclohexane-toluene (10: 10: 1); S5, chloroform-methanol-water (90: 10: 1); S6, ethyl acetate-chloroform-water (90: 10: 1); S7, ethyl ether; S8, benzene-acetone (80: 20); S9, benzene-methanol (90: 10); S10, heptane-ethyl acetate (80: 10) (HARA & TAKEUCHI, 1963; IDLER & MACNAB, 1967; ARAI, 1967a; IDLER *et al.*, 1969). PPC was carried out by the descending method using BUSH-type solvent systems (BUSH, 1961): TM–70, toluene-70% methanol; HBM–70, heptane benzene (1: 1)—70% methanol; HM–80, heptane–80% methanol.

Measurement of radioactivity. Radioactivity was determined with a Beckman liquid scintillation counter model LS–150 using a solution of 0.6% PPO and 0.02% POPOP as a scintillator.

Chemical derivation of metabolites. In order to confirm the identification of metabolites, a part of chromatographically purified metabolites was acetylated with acetic anhydride-dry pyridine (1: 1) and also oxidized with 0.5% chromium trioxide in 90% acetic acid. In acetylation, a radioactive metabolite was dissolved in 0.5 ml of dry pyridine, to which 0.5 ml of acetic anhydride was added and mixed thoroughly, and then the mixture was allowed to stand for 24 hours at 37°C. The reagents were eliminated by a stream of nitrogen. For oxidation, a radioactive material was dissolved in 1 ml of 0.5% chromium trioxide in 90% acetic acid and

kept for 10 minutes at room temperatures. Then, to the reaction mixture, 3 ml of distilled water was added and the materials were extracted 3 times with 5 ml of dichloromethane. The dichloromethane layer was washed twice with 2 ml of distilled water, dried over anhydrous sodium sulfate, and concentrated under reduced pressure.

Results

Metabolites of cholesterol-4-^{14}C from the hepatopancreas. The dichloromethane extract from the hepatopancreas was initially chromatographed by TLC and fractionated into nine fractions as shown in Table 30.

Table 30. Initial TLC of the dichloromethane extract from the hepatopancreas.

Fraction	Rf	UV-absorption	Radioactivity	
			dpm	%
1	0.00–0.20	−	9,690	0.36
2	0.22	+	6,020	0.23
3	0.33	+	8,900	0.33
4	0.35–0.50	−	11,700	0.44
5	0.51	+	13,000	0.49
6	0.56	+	50,200	1.88
7	0.60	+	267,000	10.0
8	0.78	+	612,000	22.9
9*	0.80–0.90	−	1,690,000	62.3

* Fraction 9; Cholesterol-4-^{14}C (precursor)

The materials 2, 3, 5, 6, 7, and 8 which revealed both high radioactivity and UV-absorption were investigated. The materials 2 and 3 corresponding to carrier cortisol, cortisone, and corticosterone in the initial TLC were successively rechromatographed by TLC using systems S1, S6, and S5 and by PPC using system TM–70. The carrier cortisol and cortisone have lost the radioactivity during these purification steps. On the other hand, the carrier corticosterone maintained its radioactivity throughout the purification steps.

The materials 5 corresponding to carrier deoxycorticosterone and testosterone was rechromatographed by TLC using system S3 and separated into the two radioactive materials corresponding to deoxycorticosterone (5–1) and testosterone (5–2). The material 5–1 was rechromatographed by TLC using systems S2, S1, S3, and S4, successively. In these TLC, this material gave the same mobility as standard deoxycorticosterone and revealed high radioactivity. Acetylation of this substance gave a radioactive material corresponding to deoxycorticosterone 21-monoacetate in TLC using system S2. On the other hand, the material 5–2 was rechromatographed by TLC using systems S2, S1, and S3, successively. In these chromatographic purification steps, this material gave the same mobility as authentic testosterone and showed high radioactivity. Acetylation of this substance gave a radioactive material

corresponding to testosterone 17 β-monoacetate in TLC using system S2. When this substance was oxidized with 0.5% chromium trioxide, a radioactive compound which exhibited the same mobility as androstenedione in TLC using system S2 was obtained. Finally, to the presumed testosterone-^{14}C, about 10 mg of non-radioactive testosterone was added and recrystallized several times from the different solvent systems. As shown in Table 31, the specific activity of the crystals was constant in the last three crystallizations.

Table 31. Recrystallization of testosterone-^{14}C isolated from the hepatopancreas of the lobster injected with cholesterol-4-^{14}C.

Crystallization	Solvent system	Specific activity (dpm/mg)
1 st	Dichloromethane-heptane	119
2 nd	Hexane-acetone	115
3 rd	Hexane-acetone	108

When the material 6 corresponding to 17α-hydroxyprogesterone was chromatographed successively by TLC using systems S2, S1, S3, S4, and S2, this material gave the same mobility as authentic 17α-hydroxyprogesterone. Oxidation of this substance gave radioactive androstenedione.

The material 7 corresponding to androstenedione was rechromatographed by TLC using systems S2, S7, and S8, and PPC using system HBM–70, and TLC using system S2, successively. Throughout these chromatographic purification steps, this material revealed a significant radioactivity and the same mobility as standard androstenedione.

The material 8 corresponding to progesterone was rechromatographed by TLC using systems S2, S7, S7, and S8, successively. In these TLC, this material showed the same mobility as progesterone. Finally, to the presumed progesterone-^{14}C, about 10 mg of non-radioactive progesterone was added and then recrystallized several times. As shown in Table 32, the specific activity of the crystals was constant in the last three crystallizations. Table 33 summarizes the chromatographic purification steps for radioactive metabolites and the identified metabolites from the extract of the hepatopancreas.

Table 32. Recrystallization of progesterone-^{14}C isolated from the hepatopancreas of the lobster injected with cholesterol-4-^{14}C.

Crystallization	Solvent system	Specific activity (dpm/mg)
1 st	Methanol-water	273
2 nd	Acetone-water	268
3 rd	Methanol-water	275

Table 33. Purification step and identification of the metabolites of cholesterol-4-^{14}C from the hepatopancreas.

Fraction	Chromatographic purification step	Identified steroid
3	TLC: Sl, PPC: TM-70, TLC: S6, S5	Corticosterone
5-1	TLC: S2, S1, S3, S4 Acetylation (TLC: S2) TLC: S2, S1, S3	Deoxycorticosterone
5-2	Acetylation (TLC: S2) Oxidation (TLC: S2)	Testosterone
6	TLC: S2, S1, S3, S4, S2 Oxidation (TLC: S2)	17α-Hydroxyprogesterone
7	TLC: S2, S7, S8, PPC: HBM-70 TLC: S2	Androstenedione
8	TLC: S2, S7, S7, S8	Progesterone

Metabolites of cholesterol-4-^{14}C from the ovaries. The dichloromethane extract from the ovaries was initially chromatographed and fractionated into eight fractions as shown in Table 34.

Table 34. Initial TLC of the dichloromethane extract from the ovaries.

Fraction	Rf	UV-absorption	Radioactivity	
			dpm	%
1	0.00-0.10	−	441	0.11
2	0.12	+	566	0.14
3	0.25	+	1,700	0.43
4	0.27-0.45	−	1,980	0.50
5	0.47	+	4,040	1.01
6	0.56	+	3,210	0.80
7	0.62	+	327,000	82.0
8*	0.64-0.81	−	60,000	15.0

* Fraction 8: Cholesterol-4-^{14}C (precursor)

The materials 5, 6, and 7 which revealed relatively high radioactivity and UV-absorption were subjected to the investigation of metabolites. In the present study, however, the materials 1, 2, 3, 4, and 8 were left un-analyzed.

The materials 5, 6, and 7 corresponding to carrier testosterone, 17α-hydroxyprogesterone, and progesterone showed respectively the same mobility as authentic tes-to

Table 35. Identification of the metabolites of cholesterol-4-^{14}C from the ovaries.

Fraction	Chromatographic purification step	Identified steroid
	TLC: S7, S8, S8	
5	Acetylation (TLC: S8) Oxidation (TLC: S8)	Testosterone
6	TLC: S7, S8, S9	17α-Hydroxyprogesterone
7	TLC: S7, S9, S8	Progesterone

sterone, 17α-hydroxyprogesterone, and progesterone throughout the TLC purification steps, and maintained significant radioactivity. Furthermore, acetylation and oxidation of the chromatographically purified testosterone-^{14}C gave radioactive testosterone 17β-monoacetate and androstenedione, respectively. Table 35 summarizes the identified metabolites from the extract of ovaries.

Metabolites of cholesterol-4-^{14}C from the blood. The dichloromethane extract from the blood was initially chromatographed by TLC and separated into eight fractions as shown in Table 36. The materials 6 and 7 which revealed relatively high radioactivity and UV-absorption were subjected to the investigation of metabolites. The other materials which showed low radioactivity or non-UV absorption were left un-analyzed in the present study.

Table 36. Initial TLC of the dichloromethane extract from the blood.

Fraction	Rf	UV-absorption	Radioactivity	
			dpm	%
1	0.00–0.30	–	512	0.27
2	0.32	+	280	0.15
3	0.44	+	306	0.16
4	0.45–0.63	–	340	0.18
5	0.65	+	956	0.50
6	0.75	+	2,020	1.06
7	0.88	+	179,000	93.6
8*	0.90–1.00	–	7,740	4.05

* Fraction 8: Cholesterol-4-^{14}C (precursor)

The materials 6 and 7 were rechromatographed by TLC using systems S2, S4, S7, and S10, and by PPC using system HM–80, successively. In these TLC and PPC, the materials 6 and 7 gave the same mobility as authentic testosterone and progesterone, respectively. Acetylation and oxidation of the chromatographically purified testosterone-^{14}C gave radioactive testosterone 17β-monoacetate and androstenedione, respectively. Table 37 summarizes the identified metabolites from the extract of blood.

Table 37. Identification of the metabolites of cholesterol-4-^{14}C from the blood.

Fraction	Chromatographic purification step	Identified steroid
6	TLC: S2, S7 Acetylation (TLC: S2) Oxidation (TLC: S1)	Testosterone
7	TLC: S2, S7, S10, S4, PPC: HM–80	Progesterone

Discussion

In vertebrates, it has been generally recognized that cholesterol is a precursor of steroid hormones such as sexual and adrenocortical hormones. However, little in-

formation has been obtained about invertebrates. GOTTFRIED *et al.* (1967) have reported that pregnenolone was isolated from the albumen gland of the giant land slug, *Ariolimax californicus,* by incubation without addition of exogenous precursor, and they have suggested the presence of a cholesterol side-chain cleaving enzyme in this species. Moreover, GOTTFRIED and DORFMAN (1970) have shown that the incubation of the male phase ovotestes of the same animal with cholesterol-1,2-^{3}H yielded redioactive pregnenolone. On the other hand, IDLER *et al.* (1969) have failed to convert radioactive cholesterol to pregnenolone by the gonads from the scallop, *Placopecten magellanicus.*

In the present study, the several steroid hormones were identified from the hepatopancreas, ovaries, and blood of the lobster injected with cholesterol-4-^{14}C as summarized in Table 38. From the hepatopancreas, ovaries, and blood, progesterone and testosterone were obtained as metabolites of cholesterol-4-^{14}C. In the case of the hepatopancreas, the above two metabolites were conclusively identified by recrystallization to constant specific activity. In addition, from the hepatopancreas, 17α-hydroxyprogesterone, androstenedione, deoxycorticosterone, and corticosterone were obtained as a metabolite. 17α-Hydroxyprogesterone was also detected in the ovaries. From the above results, it was conceivable that the lobster, *Panulirus japonica,* possesses ability for conversion of cholesterol to steroid hormones.

Table 38. Metabolites of cholesterol-4-^{14}C isolated from the hepatopancreas, ovaries, and blood of the lobster after injection of cholesterol-4-^{14}C.

Steroid hormone	Hepatopancreas	Ovaries	Blood
Progesterone	+	+	+
17α-Hydroxyprogesterone	+	+	−
Androstenedione	+	−	−
Testosterone	+	+	+
Deoxycorticosterone	+	−	−
Corticosterone	+	−	−

Summary

In this part, the *in vivo* bioconversion of cholesterol-4-^{14}C to steroid hormones was investigated by using the lobster, *Panulirus japonica.* The results obtained are summarized as follows:

1) After injection of cholesterol-4-^{14}C into the lobster, the hepatopancreas, ovaries, and blood were taken out, and then the metabolites of cholesterol were investigated.

2) The identification of metabolites was carried out mainly by TLC and PPC and by derivative formations.

3) From the hepatopancreas, progesterone, 17α-hydroxyprogesterone, androstenedione, testosterone, deoxycorticosterone, and corticosterone were obtained as metabolites. Progesterone and testosterone were also detected in the ovaries and blood. In the case of the ovaries, 17α-hydroxyprogesterone was detected in addition to the above two metabolites.

4) These results indicated that the lobster, *P. japonica,* is capable of converting cholesterol to some steroid hormones although the sites of bioconversion are obscure.

Part-8 Metabolism of Steroid Hormones in Marine Crustaceans: Biosynthesis of Steroid Hormones in the Crab, *Portunus trituberculatus*

From the viewpoint of comparative endocrinology, a number of workers have studied on the steroidgenesis in lower vertebrates (GOTTFRIED, 1964; ARAI & EGAMI, 1968). As far as the present author knows, however, the knowledge on the steroidgenesis in marine invertebrates is relatively little. In the early studies, the occurrence of steroid-hormone-like materials in marine invertebrates has been suggested by the several workers (DONAHUE, 1940, 1948, 1952; HAGERMAN *et al.*, 1957; SCHWERDTFEGER, 1932; STEIDLE, 1930). Recently, the chemical identification of these effects has been carried out with the eggs of the lobsters (DONAHUE, 1957; LISK, 1961; BRODZICKI, 1963) and the ovaries of the starfish and sea urchin (BOTTICELLI *et al.*, 1960, 1961). Furthermore, it was shown by the histochemical methods that the tissues of the oyster, *Grassostrea gigas,* contain Δ^5-3β-hydroxysteroid dehydrogenase (MORI *et al.*, 1964) and 17β-hydroxysteroid dehydrogenase (MORI *et al.*, 1965). On the other hand, it has been demonstrated by the incubation of tissues with radioactive precursors that the several marine invertebrates contain the enzyme systems for steroidgenesis. For example, HATHAWAY (1965) has reported that the gonads of the sea urchin and the oyster are capable of converting estradiol-17β to estrone and testosterone to androstendeione. GILGAN and IDLER (1967) have shown the conversion of androstenedione to testosterone by some tissues of the lobster, *Homarus americanus.* Moreover, IDLER *et al.* (1969) have demonstrated that the gonadal tissues of the scallop, *Placopecten magellanicus,* transformed 17α-hydroxyprogesterone to androstenedione. However, their attempts to convert normal precursors to cortisol and cortisone with the gonadal tissues and the hepatopancreas of this mollusks were unsuccessful. In marine invertebrates, no report has been presented on the corticoidgenesis except for the bioconversion of progesterone to deoxycorticosterone by the androgenic gland of the blue crab, *Callinectes sapidus* (TCHOLAKIAN & EIK-NES, 1969).

In part–7, the author demonstrated that the lobster, *Panulirus japonica,* converts cholesterol-4-^{14}C to steroid hormones. In this part, the *in vitro* bioconversion of progesterone was examined in order to clarify the biosynthetic pathways of steroid hormones in marine crustaceans. This part deals with the results of the bioconversion of non-radioactive and radioactive progesterone to steroid hormones by the slices of ovaries and testes of the crab, *Portunus trituberculatus.*

A. Bioconversion of non-radioactive progesterone by the slices of ovaries.

Materials and Methods

Animals and incubation. The crab, *P. trituberculatus,* was obtained from the

commercial source, in April, 1969. The crab, 130 g average body weight, was carefully dissected, the ovaries being taken out. The ovaries were cut into small pieces, weighed, transferred into the incubation flask (50 ml Erlenmeyer flask) containing the incubation medium, gassed with pure oxygen for 2 minutes, and then subjected to incubation. The incubation medium contains the following salts (g/1000 ml): NaCl, 23.0; KCl, 0.964; $MgCl_2 \cdot 6H_2O$, 5.0; $MgSO_4 \cdot 7H_2O$, 7.27; Tris, 3.0; $CaCl_2$, 1.119; $NaH_2PO_4 \cdot H_2O$, 0.138; pH 7.4. The sliced ovaries (1000 mg) were preincubated in a mixture of 8 ml of the incubation medium with progesterone (1.0 mg), dissolved in 0.1 ml of ethanol, antibiotics (9000 units of penicillin G and 240 μg of aureomycin) and cofactors (2.5 mg of glucose-6-phosphate, 1 unit of glucose-6-phosphate dehydrogenase, and 1.0 mg of nicotinamide-adenine dinucleotide phosphate) (IDLER *et al.*, 1969; IDLER & FREEMAN, 1966). The incubation was carried out with shaking at 20°C. After 1 hour, the medium was changed with the fresh one containing antibiotics and cofactors. To this, 1.0 mg of progesterone was added and incubated for 4 hours successively. The medium, cofactors, and antibiotics were refreshed every 120 minutes and gassed with pure oxygen. After incubation, the tissues and two changes of medium were combined and extracted with 7 volumes of dichloromethane, and then the solvent was removed under nitrogen by use of a rotary evaporator below 40°C. In total, 8.0 g of the ovaries was used for incubation.

For the purpose of investigating the steroids produced from endogenous precursors, 8.0 g of the ovaries was incubated without addition of progesterone under the same condition. In addition, the analysis of steroid hormones in the fresh ovaries was carried out as control.

Chromatographic analysis. In the present study, thin-layer chromatography (TLC) and gas-liquid chromatography (GLC) were used in order to isolate and identify steroid hormones. In TLC, a mixture of Kiesel gel G-Keisel gel GF_{254} (5: 1) was spreaded in a layer, 0.25 or 0.5 mm in thickness, and the plate was heated

Table 39. Solvent system in TLC.

Solvent system	Composition
S1	Chloroform-methanol (97: 3)
S2	Benzene-acetone (8: 2)
S3	Benzene-chloroform-methanol-ether (2: 2: 1: 1)
S4	Benzene-acetone (3: 1)
S5	Benzene-methanol (9: 1
S6	Heptane-ethyl acetate (8: 2)

at 105°C for 60 minutes. The solvent systems (HARA & TAKEUCHI, 1963; ARAI, 1967a) used in TLC are shown in Table 39. The dichloromethane extract was initially chromatographed on a plate (20×20 cm, 0.5 mm in thickness) with chloroform-methanol (97: 3) as a solvent. The steroids on the plate were detected by UV-light (at 253 mμ) and by blue-tetrazolium reagent (GOSZTONYI *et al.*, 1963). The UV-absorbing substance was eluted from the plate with dichloromethane-methanol (9: 1) (IDLER *et al.*, 1966), and then rechromagotraphed on a thin-layer using three

or more solvent systems, successively. In these purification steps, the chromatographic homogenity and mobility of UV-absorbing substance were investigated by TLC along with authentic steroid hormones. Finally, the final eluate from TLC was analyzed by GLC on 1.0% QF-1 and on 1.5% OV-17.

The outlines of purification and analysis of each UV-absorbing substance are shown in Figs. 23 and 24.

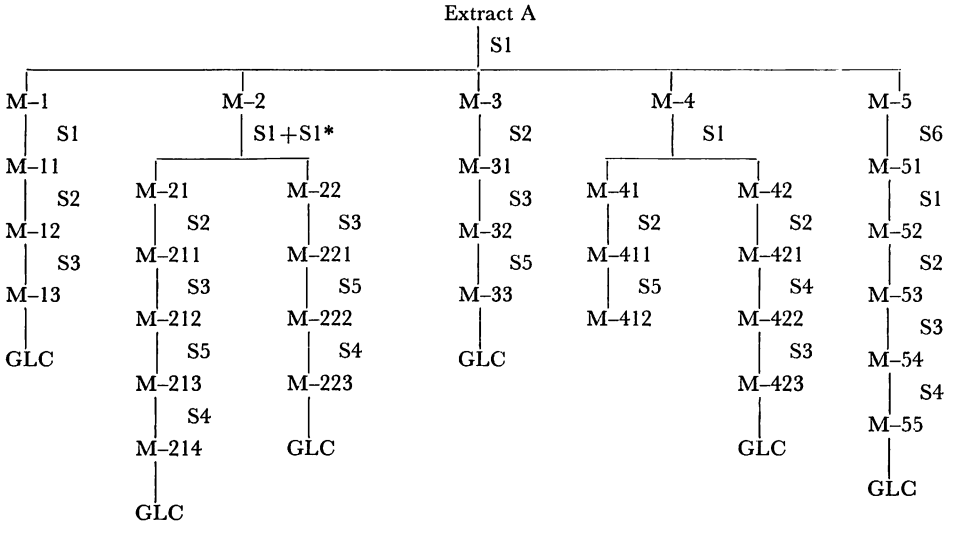


Fig. 23. Purification step and analysis of steroids.
Extract A, ovaries incubated with progesterone; S, solvent systems used in TLC; M, the steroids eluted from thin-layer plate
*Developed repeatedly by use of system S1

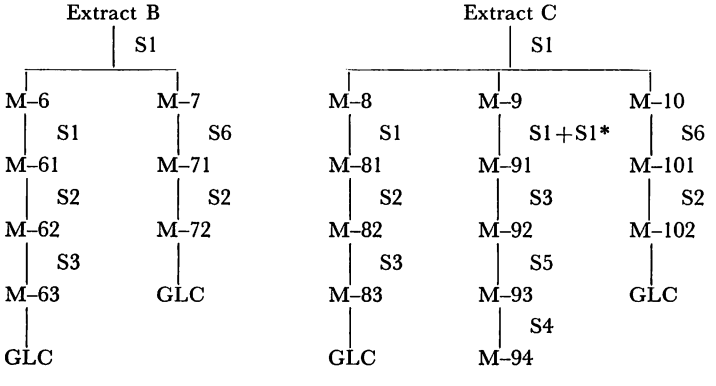


Fig. 24. Purification step and analysis of steroids.
Extract B, non-incubated ovaries; Extract C, ovaries incubated without addition of progesterone
*Developed repeatedly by use of system S1

Results

In fish, progesterone, androgen, and corticoids have been demonstrated to be synthesized mainly through the pathways as shown in Fig. 25 (Arai & Egami, 1968). Accordingly, the production of Δ^4-3-ketosteroids from progesterone by the slices of

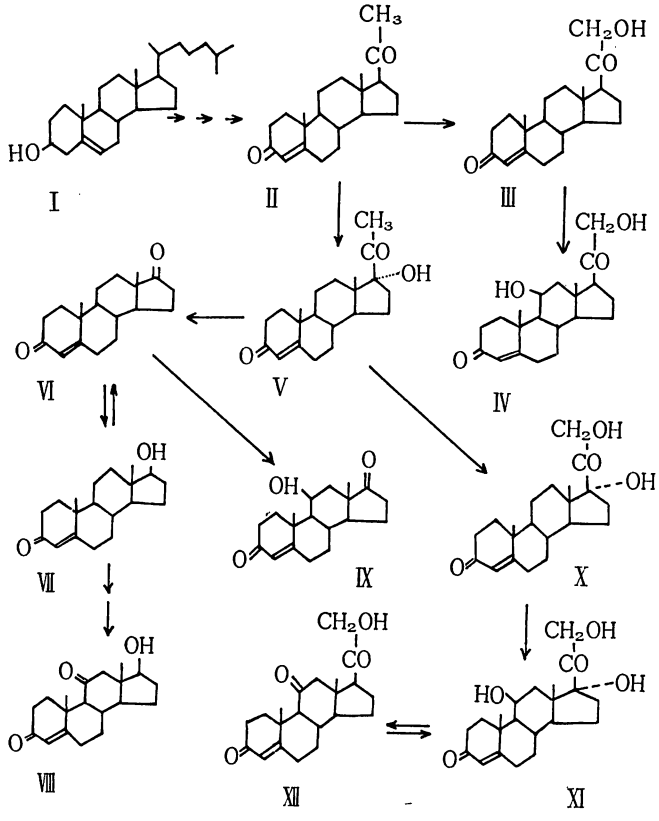


Fig. 25. Possible biosynthetic pathways of steroid hormones in fish.
I, cholesterol; II, progesterone; III, deoxycorticosterone; IV, corticorsterone; V, 17α-hydroxyprogesterone; VI, androstenedione; VII, testosterone; VIII, 11-ketotestosterone; IX, 11β-hydroxyandrostenedione; X, 11-deoxycortisol; XI, cortisol; XII, cortisone

the ovaries of crab, *P. trituberculatus,* was investigated in the present study. The results are shown in Figs. 26 and 27 and Table 40.

From the ovaries incubated with progesterone, 17α-hydroxyprogesterone, testosterone, 11-ketotestosterone, and 11β-hydroxyandrostenedione were tentatively identified by both TLC and GLC. Under the experimental conditions adopted, 17α-hydroxyprogesterone was present in high concentration in comparison with the other steroids. In addtion, two UV-absorbing substances were detected in the initial TLC of the extract from the ovaries incubated with progesterone. One (M-4: Rf,

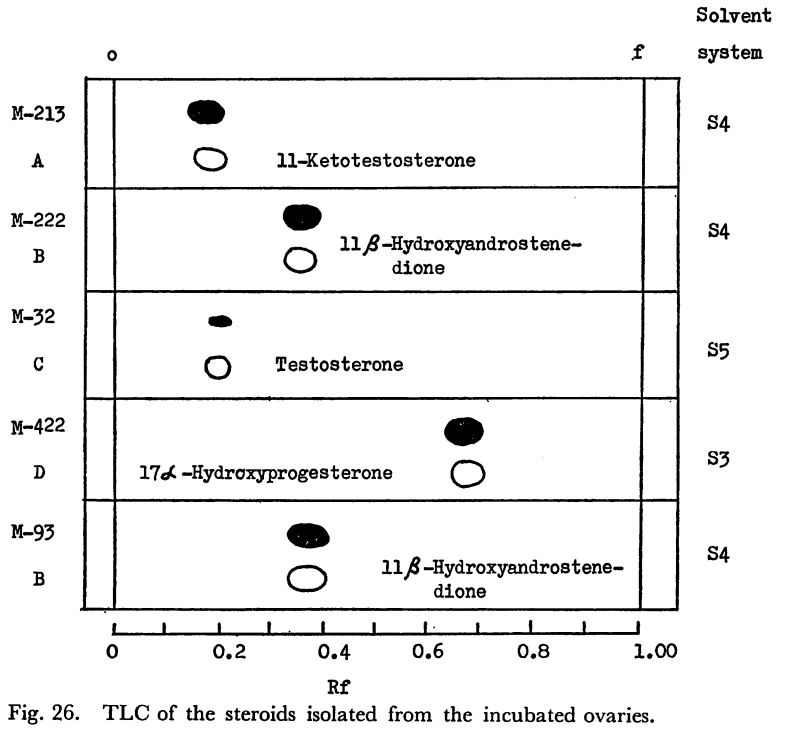


Fig. 26. TLC of the steroids isolated from the incubated ovaries.
A, B, C, and D: Authentic steroids

Table 40. Rf value in TLC and relative retention time in GLC of the isolated steroids from the ovaries incubated with progesterone.

Steroid identified	Rf in TLC (Solvent system)					Relative Rt in GLC* Column **	
	S1	S2	S3	S4	S5	QF-1	OV-17
11-Ketotestosterone	0.21	0.17	0.50	0.17	0.12	7.86	0.72
11β-Hydroxyandrostenedione	0.38	0.29	0.60	0.35	0.17	11.47	0.86
Testosterone	0.45	0.30	0.56	—	0.20	3.88	—
17α-Hydroxyprogesterone	0.55	0.35	0.67	0.44	—	17.65	1.20
Progesterone (precursor)	0.67	0.53	0.73	0.53	0.50	8.59	0.93

* Relative to cholestane

** QF-1 : Column 2.0 m×4 mm I.D., 1.0% QF-1 on 60–80 mesh Chromosorb W; Column temp. 210°C; N_2 1.0 kg/cm²

OV-17: Column 3.0 m×4 mm I.D., 1.5% OV-17 on 80–100 mesh Shimalite W; Column temp. 210°C; N_2 1.0 kg/cm²

0.60), blue tetrazolium-positive, showed the same mobility as authentic deoxycorticosterone in TLC using systems S1, S2, and S5, however it was left un-analyzed by GLC. The other (M-1: Rf, 0.15), blue tetrazolium-negative, was found to be more polar than both cortisol and 11-ketotestosterone in TLC using systems S1, S2, and S3. Also, in GLC on 1.0% QF-1 and 1.5%OV-17, the retention times of this substance was

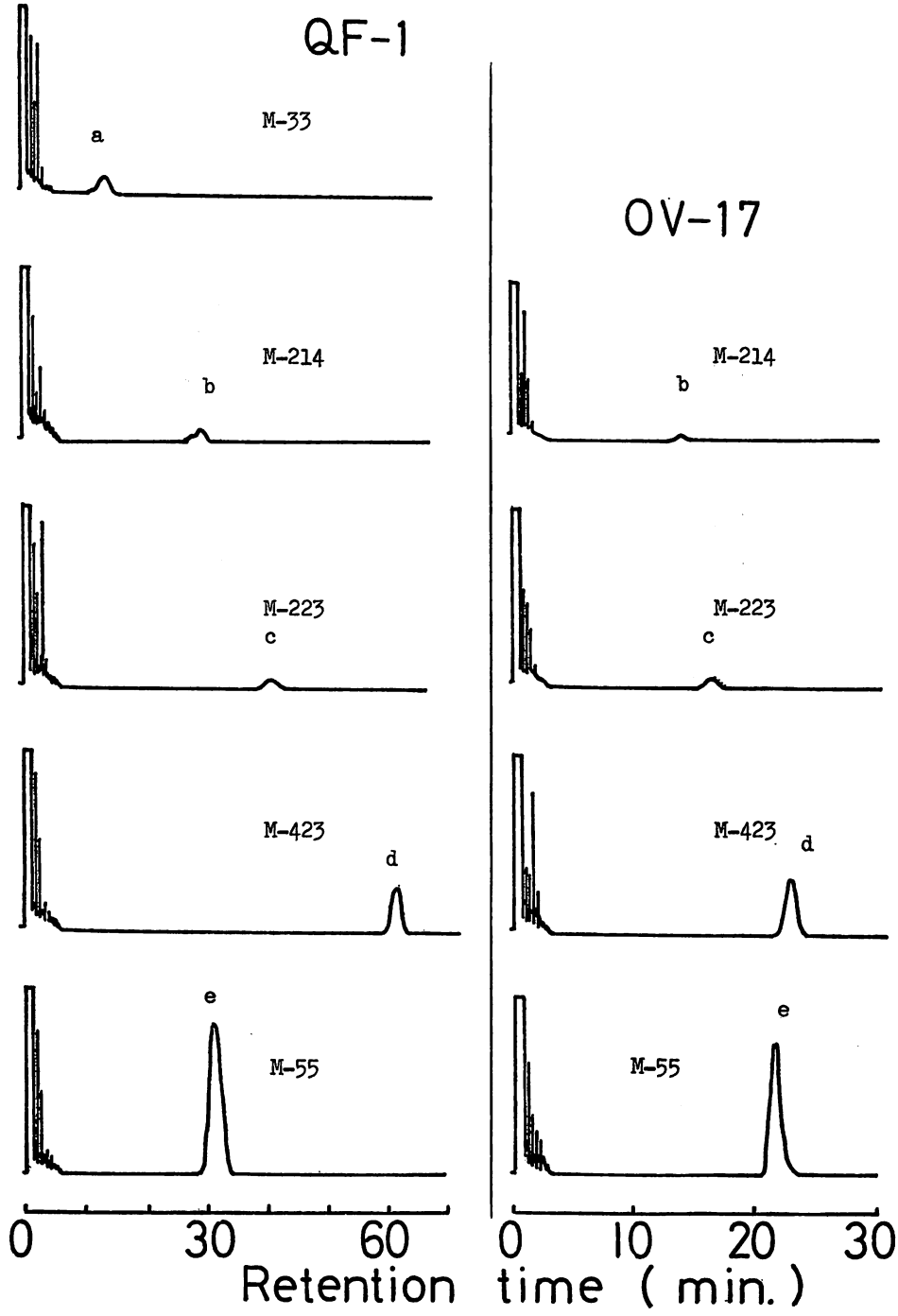


Fig. 27. GLC of the steroids isolated from the ovaries incubated with progesterone. a, testosterone; b, 11-ketotestosterone; c, 11β-hydroxyandrostenedione; d, 17α-hydroxyprogesterone; e, progesterone

not identical with those of the steroid hormones usually found in the ovaries of animals.

From the ovaries incubated without addition of progesterone, the three UV-absorbing substances were detected in the initial TLC, although these substances were in extremely minute quantities. One of them (M–9; Rf, 0.38) was identical with authentic 11β-hydroxyandrostenedione in TLC using systems S1+S1, S3, S5, and S4. However, no further examination to identify this substance could be done due to insufficient amount. The other substances (M–8: Rf, 0.15 and M–10: Rf, 0.75) were conceivable to be different from steroid hormones from the mobilities in successive TLC using systems S1, S2, S4, and S6, and from the retention times in GLC on 1.5% OV–17 (relative retention times to cholestane, 0.10 and 0.12).

From the fresh ovaries, the two UV-absorbing substances were detected in the initial TLC. From the results of sequential TLC and GLC, these substances were assumed to be different from steroid hormones. Moreover, the insufficient materials did not allow to carried out further investigation.

Table 41 summarizes the identified steroids tentatively from the ovaries incubated and non-incubated.

Table 41. Steroid hormone identified from the incubated and non-incubated ovaries of the carb, *P. trituberculatus*.

Steroid identified	Non-incubated ovaries	Incubated ovaries	
		Without progesterone	With progesterone
11-Ketotestosterone	−	−	+
11β-Hydroxyandrostenedione	−	±	+
Testosterone	−	−	+
17α-Hydroxyprogesterone	−	−	++
Progesterone (precursor)	−	−	+++

−, ±, and + indicate the following mean:
−: no presence of detectable amount of steroids under the present experimental conditions adopted
±: detectable but not enough to identify it conclusively by using both TLC and GLC
+: identified by using both TLC and GLC

Discussion

In the present study, 11-ketotestosterone, 11β-hydroxyandrostenedione, testosterone, and 17α-hydroxyprogesterone were detected in the ovaries incubated with progesterone. On the other hand, from the fresh ovaries and the ovaries incubated without addition of progesterone, the above steroids were not isolated enough to identify them by TLC or GLC. From the results, it may be suggested that the crab, *Portunus trituberculatus*, possesses the enzyme systems at least for synthesis of 11-ketotestosterone, 11β-hydroxyandrostenedione, testosterone, and 17α-hydroxyprogesterone.

11-Ketotestosterone first isolated from the blood of sockeye salmon (IDLER *et al.*, 1960) has also been obtained from the blood of the Atlantic salmon (SCHMIDT & IDLER, 1962) and the common mullet (EYLATH & ECKSTEIN, 1969), and the steroid

was found to reveal strongly androgenic activity for the sockeye salmon (IDLER *et al.*, 1961) and for the medaka, *Oryzias latipes* (ARAI, 1967b). Also, the *in vitro* production of this compound has been demonstrated by the Atlantic salmon tissue (IDLER & MACNAB, 1967), the testes of rainbow trout (ARAI & TAMAOKI, 1967c, d) and the ovaries of mollusk (GOTTFRIED & LUSIS, 1966). However, it has not yet been isolated from marine invertebrates such the crab. This study is the first one showing the production of 11-ketotestosterone in marine invertebrates.

B. Bioconversion of progesterone-4-^{14}C by the slices of ovaries.

Materials and Methods

Substrate and other chemicals. Progesterone-4-^{14}C (29.3 mCi/mM) was obtained from Daiichi Pure Chemicals Co., Ltd. (Japan). The purity of this substance was ascertained by thin-layer chromatography (TLC). Non-radioactive steroids were obtained from Sigma Chemicals Co., Ltd. (U.S.A.). Nicotinamide-adenine dinucleotide phosphate (NADP), glucose-6-phosphate (G-6-P), glucose-6-phosphate dehydrogenase (G-6-P DH) were obtained from Nutritional Biochemicals Co., Ltd. (U.S.A.).

Incubation. From the crab, *P. trituberculatus,* 160 g body weight, the ovaries were excised and subjected to incubation with progesterone-4-^{14}C. The incubation method was essentially the same as described in part-8-A. Five hundred mg of the slices of ovaries was preincubated in 8 ml of the incubation medium containing cofactors (1.0 mg of NADP, 2.5 mg of G-6-P, and 1 unit of G-6-P DH) and antibiotics (9000 units of penicillin G and 240 μg of aureomycin) at 20°C for 30 minutes. At the end of half an hour, the medium was changed with the fresh one containing cofactors and antibiotics. To this, 1 μCi of progesterone-4-^{14}C (dissolved in 0.1 ml of propylene glycol-ethanol (1: 1)) was added and incubated for 6 hours successively. The incubation medium, cofactors, and antibiotics were refreshed every 120 minutes and gassed with pure oxygen. The composition of the incubation medium was described in part-8-A. After incubation, the medium was decanted, combined with the two changes of medium, and then extracted twice with 7 volumes of dichloromethane.

Separation of bioconversion products. To the dichloromethane extract, about 50 μg each of 17α-hydroxyprogesterone, androstenedione, testosterone, 11β-hydroxyandrostenedione, 11-ketotestosterone, deoxycorticosterone, corticosterone, cortisone, cortisol, and 11-deoxycortisol was added as a carrier for radioactive steroids produced. The dichloromethane extract of metabolites was initially chromatographed by TLC using chloroform-methanol (97: 3), and then the steroids were located under UV-light at 253 mμ. The UV-absorbing and non-UV-absorbing zones were scraped off from the plate contiguously, and the metabolites were eluted with dichloromethane-methanol (9: 1). The each eluate was subjected to TLC and paper-chromatography (PPC) in order to purify the radioactive metabolite as shown in

Fig. 29. The solvent systems used in TLC were: S1, chloroform-methanol (97: 3); S2, benzene-acetone (8: 2); S3, benzene-chloroform-methanol-ether (2: 2: 1: 1); S3, benzene-acetone (3: 1); S7, chloroform-methanol-water (188: 12: 1); S8, ethyl acetate-cyclohexane-toluene (10: 10: 1). PPC was carried out by the descending method using BUSH-type solvent system (BUSH, 1961): HBM-70, heptane-benzene (1: 1) —70% methanol. The elution of steroids on paper chromagotram was conducted with methanol by using descending PPC. Furthermore, the conclusive identification of radioactive metabolites was based on constant specific activity (dpm/mg) of the crystals. Radioactivity was measured with a Beckman liquid scintillation counter LS-150. Radioactive sample was transferred into a counting vial (Beckman Co., Ltd.). After evaporation of solvent, 20 ml of a toluene solution of PPO (0.6%) and POPOP (0.02%) was added to each vial as a scintillator. The efficiency of counting of radioactive materials was approximately 65 per cent.

Chemical derivation of metabolites. For the purpose of confirming the identification, an aliquot of chromatographically purified metaboiltes was acetylated with acetic anhydride-dry pyridine (1: 1) and also oxidized with 0.5% chromium trioxide in 90% acetic acid.

Results

The dichloromethane extract of metabolites was initially chromatographed by TLC and fractionated contiguously into twelve fractions as shown in Fig. 28. In the present study, the materials (E–8, 9, and 10) which revealed both radioactivity and UV-absorption were investigated. The outlines of isolation and identification of metabolites

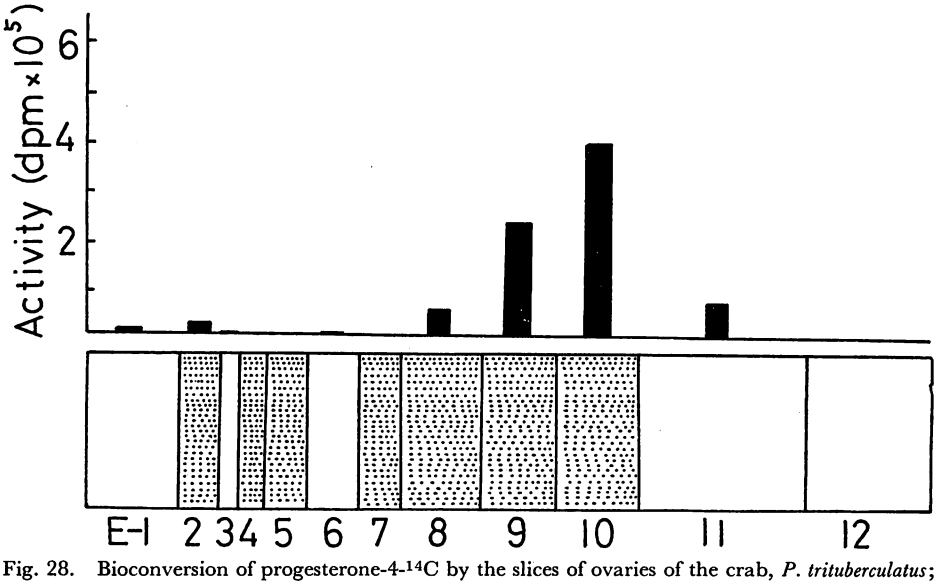


Fig. 28. Bioconversion of progesterone-4-^{14}C by the slices of ovaries of the crab, *P. trituberculatus*; radioactivities and UV-absorbing zones in the initial TLC.

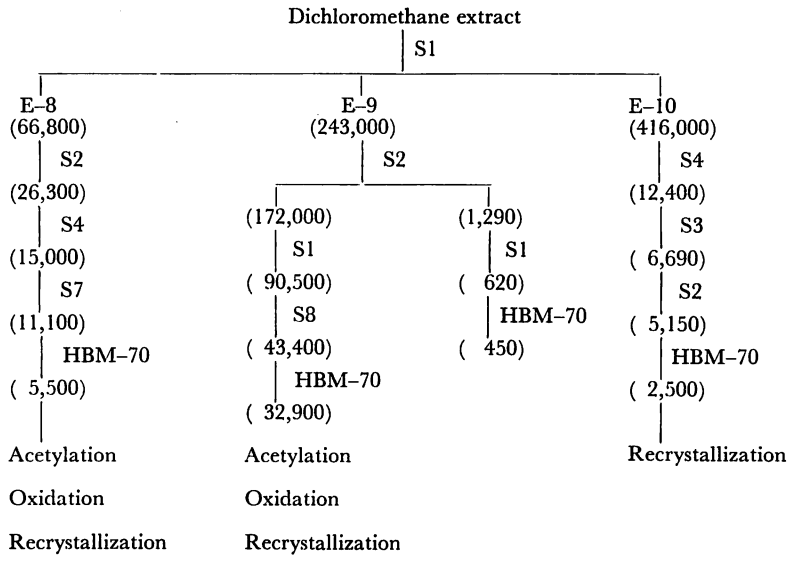


Fig. 29. Isolation and identification of the metabolites of progesterone-4-^{14}C produced by the ovaries of crab, *P. trituberculatus*.
( ): radioactivity of the metabolites purified by chromatography
S1, S2, S3, S7, and S8: solvent systems in TLC.
HBM-70: solvent system in PPC

are shown in Fig. 29. The materials which gave extremely low radioatcivity in the initial TLC were left un-analyzed. As a results, testosterone, 17α-hydroxyprogesterone, and deoxycorticosterone were identified as a main metabolite of progesterone produced by the slices of ovaries.

Identification of testosterone. The UV-absorbing material (E-8) corresponding to testosterone in the initial TLC was eluted and rechromatographed successively by TLC using systems S2, S4, and S7, and by PPC using system HBM-70. In these TLC and PPC, the substance gave the same mobility as authentic testosterone and relatively high radioactivity. Acetylation of this substance gave a radioactive material corresponding to testosterone 17β-monoacetate in TLC using system S2. When the substance was oxidized with 0.5% chromium trioxide, a radioactive compound was obtained which exhibited the same mobility as authentic androstenedione in TLC using system S2. Finally, to the presumed testosterone-^{14}C, about 10 mg of non-radioactive testosterone was added and its radiochemical homogenity was checked

Table 42. Recrystallization of testosterone-^{14}C isolated from incubation of progesterone-4-^{14}C with the ovaries of the crab, *P. trituberculatus*.

Crystallization	Solvent system	Specific activity (dpm/mg)
1 st	Dichloromethane-heptane	117
2 nd	Hexane-acetone	130
3 rd	Hexane-acetone	123

by the serial crystallizations followed by specific activity determination of each crystal. As shown in Table 42, the specific activity of the resultant crystals was constant in the last three crystallizations.

Identification of 17α-hydroxyprogesterone. When the material (E–9) on the initial TLC was rechromatographed by TLC using systems S2, two UV-absorbing zones, which showed the same mobilities as authentic androstenedione and 17α-hydroxyprogesterone, were detected. The material corresponding to androstenedione was further chromatographed sequentially by TLC using system S1 and by PPC using system HBM–70. However, the material lost its ^{14}C-activity progressively during the chromatographic purification steps. Accordingly, it was unsuccessful to obtain significant specific activity by crystallization. On the other hand, the material corresponding to 17α-hydroxyprogesterone showed the same mobility as authentic 17α-hydroxyprogesterone and high radioactivity throughout the chromatographic purification steps by TLC using systems S1 and S8 and by PPC using HBM–70. Oxidation of this material gave a radioactive substance with the same mobility as androstenedione in TLC using systems S1 and S2. Finally, to the presumed 17α-hydroxyprogesterone-^{14}C, about 20 mg of non-radioactive 17α-hydroxyprogesterone was added and re-

Table 43. Recrystallization of 17α-hydroxyprogesterone-^{14}C isolated from incubation of progesterone-4-^{14}C with the ovaries.

Crystallization	Solvent system	Specific activity (dpm/mg)
1 st	Hexane-acetone	812
2 nd	Dichloromethane-heptane	800
3 rd	Acetone-water	819

crystallized from the several solvent systems. As shown in Table 43, the specific activity of the crystals was constant in the last three crystallizations.

Identification of deoxycorticosterone. The material (E–10) obtained in the initial TLC was found to contain carrier deoxycorticosterone and progesterone (precursor) by TLC using system S4. In the sequential purification steps by TLC using systems S3 and S2 and by PPC using system HBM-70, the material corresponding to deoxycorticosterone showed the same mobility as authentic deoxycorticosterone and significant radioactivity. Finally, to the chromatographically purified deoxycorticosterone-^{14}C, about 5 mg of non-radioactive deoxycorticosterone was added and recrystallized from the different solvent systems. As shown in Table 44, the crystals gave the constant specific activity in the last three crystallizations.

Table 44. Recrystallization of deoxycorticosterone-^{14}C isolated from incubation of progesterone-4-^{14}C with the ovaries.

Crystallization	Solvent system	Specific activity (dpm/mg)
1 st	Hexane-acetone	105
2 nd	Hexane-acetone	100
3 rd	Acetone-heptane	101

Discussion

In fish, many reports have been presented on the occurrence and biosynthesis of steroid hormones (ARAI & EGAMI, 1968). Considering the informations obtained up to the present, the metabolic pathways of steroid hormones in fish appear to be similar to those of higher animals. However, it has been reported that 11-ketotestosterone, which had not been found in mammals, was isolated from the blood of sockeye salmon (IDLER *et al.*, 1960) and the Atlantic salmon (SCHMIDT *et al.*, 1962). Moreover, it was shown that 1α-hydroxycorticosterone was isolated as a new adrenal steroid from the blood of elasmobranchinii (IDLER & TRUSCOTT, 1966) and it was present widely in these fishes (TRUSCOTT & IDLER, 1968). The above findings may suggest that the steroidgenesis in lower marine animals is not always similar to that of higher animals.

In part-8-A, the author demonstrated tentatively by using TLC and GLC that the crab, *P. trituberculatus*, may possess the enzyme systems for biosynthesis of some steroid hormones. In the present part, the incubation of the sliced ovaries with progesterone-4-^{14}C gave 17α-hydroxyprogesterone, testosterone, and deoxycorticosterone as a metabolite. The result showed evidently that the ovaries of the crab, *P. trituberculatus*, contain the enzyme systems for 17α-hydroxylation, side-chain cleavage of progesterone, and 21-hydroxylation. As one of the possible biosynthetic pathways of these metabolites, it is conceivable that 17α-hydroxyprogesterone and deoxycorticosterone were directly formed from progesterone by 17α-hydroxylation and 21-hydroxylation, respectively. In marine invertebrates, 21-hydroxylation of progesterone has been demonstrated in the androgenic gland, posterio vas deferens, and hepatopancreas of the blue crab, *Callinectes sapidus* (TCHOLAKIAN & EIK-NES, 1969). On the other hand, the biosynthetic pathways of testosterone are not proposed so distinctly as the above two metabolites, although this metabolite is conceivable to be formed probably from 17α-hydroxyprogesterone via androstenedione. Under the experimental conditions adopted, radioactive androstendione enough for conclusive identification was not detected as a metabolite. This result may lead the following assumptions: (1) Testosterone might be produced via a pathway not involving androstenedione as a intermediate, or (2) androstenedione might be converted rapidly to testosterone under the present experimental conditions. GILGAN and IDLER (1967) have reported the bioconversion of androstenedione to testosterone by some lobster tissue. Furthermore, IDLER *et al.* (1969) have indicated the bioconversion of 17α-hydroxyprogesterone to androstenedione by the gonads and hepatopancreas of the scallop, *Palcopecten magellanicus*. The presence of 17β-hydroxysteroid dehydrogenase activity was also shown by the histochemical method in the several tissues of the oyster, *Grassostrea gigas* (MORI *et al.*, 1965). In addition, considering the wide distribution of 17β-hydroxysteroid dehydrogenase in animals, the above findings in the marine invertebrates may place a slight reliance in the latter hypothesis.

C. Bioconversion of progesterone-4-^{14}C by the slices of testes.

Materials and Methods

Incubation and extraction. The crab, *P. trituberculatus*, 140 g body weight, was used in this experiment. The details of the procedures for incubation of testes and for extraction of metabolites were essentially the same as described in part-8-B. After preincubation without precursor at 20°C for 30 minutes, the slices of testes (500 mg) were incubated with 1 μCi of progesterone-4-^{14}C (29.3 mCi/mM) in 8 ml of the medium containing cofactors (1.0 mg of nicotinamide-adenine dinucleotide phosphate, 2.5 mg of glucose-6-phosphate, and 1 unit of glucose-6-phosphate dehydrogenase) and antibiotics (12,000 units of penicillin G and 240 μg of aureomycin) for 6 hours successively. After incubation, the metabolites were extracted twice with 7 volumes of dichloromethane, and washed with distilled water, and then the solvent was removed under reduced pressure.

Analysis of bioconversion products. To the dichloromethane extract, about 50 μg of non-radioactive 17α-hydroxyprogesterone, androstenedione, testosterone, 11-ketotestosterone, deoxycorticosterone, corticosterone, cortisol, 11-deoxycortisol, and cortisone was added as a carrier for radioactive metabolites produced.

The isolation and tentative identification of metabolites were carried out by TLC and by PPC. The solvent systems used in TLC were as follows: S1, chloroform-methanol (97: 3); S2, benzene-acetone (8: 2); S8, ethyl acetate-cyclohexane-toluene (10: 10: 1); S9, chloroform-95% ethanol (95: 5); S10, dichloromethane-methanol (9: 1). PPC was conducted with the BUSH-type solvent system: HBM-70. Moreover, a part of the radioactive metabolites was subjected to acetylation with acetic anhydride-dry pyridine (1: 1) and also to oxidation with 0.5% chromium trioxide in 90% acetic acid, and then the chromatographic behavior of derivatives was compared with that of authentic steroids. Finally, about each 10 mg of non-radioactive steroids was added to radioactive metabolites, recrystallized from the various solvent systems, and then the specific activity (dpm/mg) of the crystals was investigated. The weight and radioactivity of crystals were measured with a Shimadzu electric balance PMB-1 and with a Beckman liquid scintillation couter LS-150, respectively. The criterion of identification of metabolites was carried out according to the constant specific activity of the crystals in the last three crystallizations.

Results

The dichloromethane extract of metabolites was initially chromatographed by TLC using system S1, and fractionated into fifteen fractions as shown in Fig. 30. The materials (E-8, 9, 10, and 12) which revealed relatively high radioactivity were eluted from the plate and subjected to the investigation of metabolites. The materials (E-1, 2, 3, 4, 5, 6, 7, 11, 13, 14, and 15) which gave extremely low radioactivity were left un-analyzed in the present study. The material (E-8) corresponding to carrier testosterone in the initial TLC was subjected to TLC using systems S1, S2, S10, and S1+

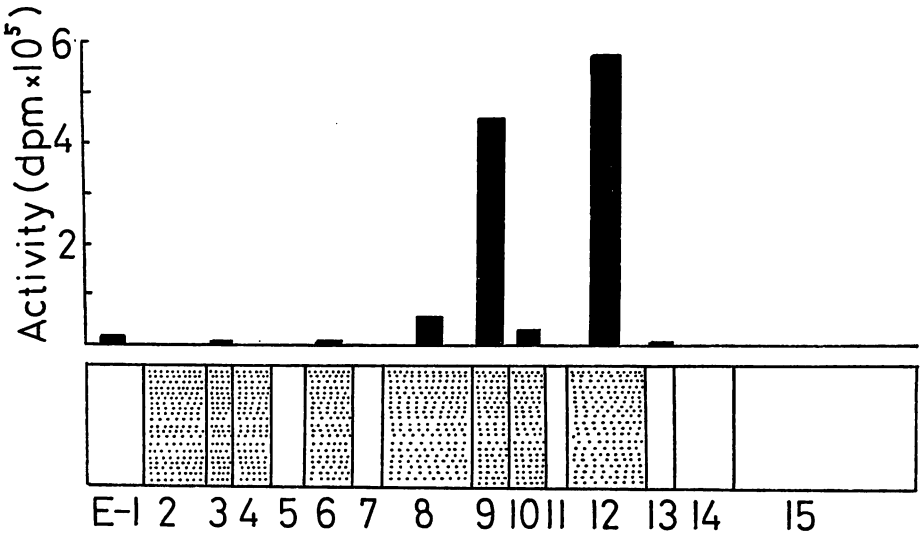


Fig. 30. Bioconversion of progesterone-4-^{14}C by the testes of crab; radioactivities and UV-absorbing zones in the initial TLC.

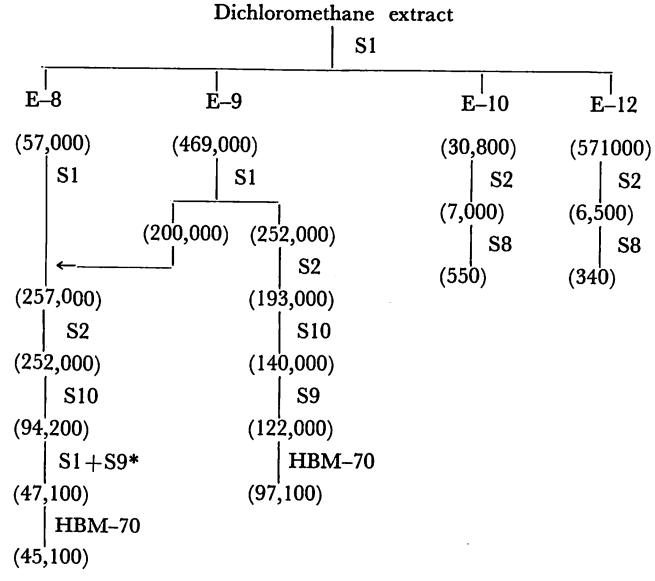


Fig. 31. Isolation and identification of the metabolites of progesterone-4-^{14}C produced by the testes of crab, *P. trituberculatus*.

( ): radioactivity of the metabolites purified by chromatography.

S1, S2, S8, S9, S10: solvent systems in TLC

HBM-70: solvent system in PPC

* Developed repeatedly by use of systems S1 (first) and S9 (second)

S9, and to PPC using system HBM–70, as shown in Fig. 31. In these chromatographic purification steps, the biosynthetic testosterone showed the same mobility as authentic testosterone and gave relatively high radioactivity. Acetylation of this substance gave a radioactive material corresponding to testosterone 17β-monoacetate. When the substance was oxidized with 0.5% chromium trioxide in 90% acetic acid, a radioactive compound was obtained which exhibited the same mobility as authentic androstenedione in TLC using system S2.

The material (E–9) corresponding to 17α-hydroxyprogesterone was rechromatographed by TLC using systems S1, S2, S10 and S9, and by PPC using system HBM–70, successively. Throughout these purification steps, the material showed the same mobility as authentic 17α-hydroxyprogesterone and significant radioactivity. Acetylation and oxidation of the chromatographically purified material gave a radioactive compound corresponding to 17α-hydroxyprogesterone and androstenedione, respectively.

Finally, to the presumed testosterone-^{14}C and 17α-hydroxyprogesterone-^{14}C, about 10 mg of authentic testosterone and 17α-hydroxyprogesterone were each added and recrystallized. As shown Tables 45 and 46, the specific activity of the crystals from both metabolites was constant in the last three crystallizations, respectively.

Table 45. Recrystallization of testosterone-^{14}C isolated from incubation of progesterone--^{14}C with the testes of crab.

Crystallization	Solvent system	Specific activity (dpm/mg)
1 st	Hexane-acetone	6300
2 nd	Dichloromethane-heptane	6200
3 rd	Hexane-acetone	6150

Table 46. Recrystallization of 17α-hydroxyprogesterone-^{14}C isolated from incubation of progesterone-4-^{14}C with the testes of crab.

Crystallization	Solvent system	Specific activity (dpm/mg)
1 st	Acetone-water	3280
2 nd	Hexane-acetone	3650
3 rd	Hexane-acetone	3310

On the other hand, the materials (E–10 and 12) corresponding to androstenedione and deoxycorticosterone in the initial TLC lost the radioactivity during the purification steps by TLC using systems S2 and S8. Accordingly, further analysis was not carried out on these metabolites.

Discussion

In fish, androgen, progesterone, and estrogen have been isolated from the testes of the several species of the teleosts and elasmobranches: the dogfish, *Scyliorhinus stellaris* (CHIEFFI & LUPO, 1961), rainbow trout, *Salmo irideus* (GALZIGNA & D'ANCONA,

1961), carp, *Cyprinus carpio* (GALZIGNA & D'ANCONA, 1961), marine teleost, *Morone labrax* (LUPO & CHIEFFI, 1963), and sockeye salmon, *Oncorhynchus nerka* (GRAJCER & IDLER, 1963). Moreover, the *in vitro* bioconversion of steroid hormones by the testes has been demonstrated in the dogfish, *Squalus acanthias* (SIMPSON *et al.*, 1964a, b), Japanese dace, *Tribolodon hakonensis* (ARAI *et al.*, 1964), rainbow trout, *Salmo gairdneri* (ARAI & TAMAOKI, 1967c, d), and the Atlantic salmon, *Salmo salar* (IDLER & MACNAB, 1967). In marine invertebrates, however, only a few reports have been presented on the occurrence and metabolism of steroid hormones. GILGAN and IDLER (1967) have reported that the androgenic gland of the American lobster, *Homarus americanus*, converted androstenedione to testosterone. TCHOLAKIAN and EIK-NES (1969) have shown the bioconversion of progesterone to deoxycorticosterone by the androgenic gland of the blue crab, *Callinectes sapidus*.

In the present study, it was shown that progesterone was converted to 17α-hydroxyprogesterone and testosterone by the slices of testes of the crab, *P. trituberculatus*. The results indicate that the testes of this crab contain the enzyme systems for 17α-hydroxylation and side-chain cleavage of progesterone. The bioconversion of progesterone to the above two metabolites has also been demonstrated in the ovaries of this crab (see part-8-B). In the case of the testes, however, the bioconversion of progesterone to deoxycorticosterone was not demonstrated in contrast with the ovaries. In the blue crab, *C. sapidus*, it was shown that steroid 21-hydroxylase activity was high in the androgenic gland as compared with that in the postrio vas deferens and hepatopancreas (TCHOLAKIAN & EIK-NES, 1969). In addition, steroid 21-hydroxylase activity has been demonstrated in the testes of many animals (DOMINGUEZ *et al.*, 1960; SIMPSON *et al.*, 1963; GOTTFRIED, 1964; SIMPSON *et al.*, 1964b). Therefore, the failure of conversion of progesterone to deoxycorticosterone in the present study may not necessarily imply the lack of steroid 21-hydroxylase in the testes of the crab, *P. trituberculatus*.

In part-7 and this part, it was shown that cholesterol is metabolized to several steroid hormones in the lobster, *P. japonica*, and also that the ovaries and testes of the crab, *P. trituberculatus*, possess at least the enzyme systems related for some androgen metabolism. These results suggest that cholesterol plays a role of physiological importance as precursor of steroid hormones in marine crustaceans.

Some steroids are found to produce certain biological effects in higher animals. For example, progesterone is concerned, in the latter half of the menstrual cycle, mainly with preparing the endometrium for nidation of the fertilized ovum if conception has occurred. During gestation, progesterone, in association with estrogen, appears to be related to maintenance of a state of quiescence of the uterine muscle. Testosterone is found to induce the appearance of male secondary sexual characteristics such as the growth of male genital organs. Corticoids, mainly being produced at adrenal cortex, are concerned with controlling electrolyte, water, carbohydrate, and fat metabolism.

Considering that marine crustaceans contain relatively large amounts of sterols in their tissues and require cholesterol or other sterols and are lacking in sterol-synthesiz-

ing ability, it seems reasonable to presume that some steroids derived from cholesterol possess certain physiological function as a hormone in crustaceans as well as in mammals.

Summary

In this part, the *in vitro* bioconversion of progesterone by the slices of the ovaries and testes of the crab, *Portunus trituberculatus*, was investigated. The results obtained are as follows:

1) When the ovaries were incubated with non-radioactive progesterone, 11-ketotestosterone, 11β-hydroxyandrostenedione, testosterone, and 17α-hydroxyprogesterone were tentatively identified as a bioconversion product by using TLC and GLC.

2) The incubation of the ovaries with progesterone-4-^{14}C gave 17α-hydroxyprogesterone, testosterone, and deoxycorticosterone as a metabolite. The identification of these metabolites was carried out by chromatography, derivative formation, and by constant specific activity in consecutive crystallizations. The results indicated that the ovaries of this crab contain steroid 17α-hydroxylase, steroid C_{17}–C_{20} lyase, and steroid 21-hydroxylase.

3) The incubation of the testes with progesterone-4-^{14}C gave 17α-hydroxyprogesterone and testosterone as a metabolite. The results indicated that the testes contain steroid 17α-hydroxylase and steroid C_{17}-C_{20} layse.

4) From the above data, it was suggested that the crab, *P. trituberculatus*, possesses at least the enzyme systems for the biosynthesis of some androgen and deoxycorticosterone.

Part–9 ABSTRACT

Sterols occur in all animals, plants, and microorganisms as a constituent of lipids. It is well known that in the animal kingdom the more primitive species such as marine invertebrates contain a great variety types of sterols while evolutionally higher animals often contain only cholesterol, the most widely distributed sterol in animals. In the early studies on the sterols of marine invertebrates, however, the great majority of works have dealt with the elucidation of chemical structure of naturally occurring sterols, and there were few reports on the metabolism of sterols. Hence, the author intended to clarify the sterol metabolism in marine crustaceans which are important animals in the field of fisheries culture. The present study is concerned with an understanding of the composition of naturally occurring sterols, sterol-synthesizing ability, nutritional requirements for sterols, and *in vivo* transformation of sterol molecules in marine crustaceans. Furthermore, for the purpose of obtaining some knowledge on the function of sterols in crustaceans, the distribution and fate of exogenous cholesterol in the tissues, and the biosynthesis of steroid hormones from cholesterol and progesterone were investigated by using the lobster, *Panulirus japonica*, or the crab, *Portunus trituberculatus*. This paper deals with the results and discussion on these problems.

The occurrence and distribution of sterols in marine invertebrates have been extensively reviewed by BERGMANN (1962) for publications up to about 1960. Most recently, AUSTIN (1970) has attempted to reclassify marine invertebrates and plants on the basis of their sterol compositions. On the sterols of crustaceans, there are only a few reports although this class embraces a great number of species. The fragmentary investigations have shown that several crabs, lobsters, prawns, and shrimps contained mainly cholesterol in thier tissues. As an exceptional case, it has been found that the barnacle, *Balanus glandula* (FAGERLUND & IDLER, 1957) contained desmosterol for 34% of the total sterols. In the present study, it has been clarified that all crustaceans examined (eight species) contained cholesterol (73–100%) as a principal sterol. In the case of the prawn, *Penaeus japonicus*, Amphipoda, *Caprella* sp., and Mysidacea, *Neomysis intermedia*, other sterols such as 22-dehydrocholesterol, brassicasterol, desmosterol, 24-methylenecholesterol, stigmasterol, and β-sitosterol were detected as a minor component in addition to large amounts of cholesterol. This result pointed out that a sensitive method such as gas-liquid chromatography (GLC) should be used to obtain reliable data on sterol composition of marine crustaceans and to evaluate the relationship between taxonomy and sterols. Also, the author demonstrated that the sterol composition of marine crustaceans was simple as compared with that of other marine invertebrates, Molluscs, Echinoderms, Porifera and Coelenterates which were reviewed by AUSTIN (1970).

It is generally recognized that animals except insects are capable of synthesizing cholesterol from acetate via mevalonic acid, squalene, lanosterol, etc., and also that plants synthesize mainly a variety of C_{28}- and C_{29}-sterols from lower units probably via desmosterol (C_{27}-sterol). In marine crustaceans, however, there are only little informations on the biosynthesis of sterols. On the other hand, some C_{28}- and C_{29}-sterols and other minor sterols found in the tissues of crustaceans are often contained in algae and planktons as a main sterol. Accordingly, the question was raised whether cholesterol and other sterols occurring in crustaceans are formed endogenously or are derived from exogenous sources such as diets. The present study showed that the lobster, *P. japonica*, prawn, *P. japonicus*, crab, *P. trituberculatus*, and *Artemia salina* are incapable of incorporating acetate-1-^{14}C into sterol fraction, although acetate is utilized for the synthesis of fatty acids and some non-saponifiable materials. This result pointed out the deletion or incompleteness of enzyme systems for sterol biosynthesis in the marine crustaceans, and also substantially agreed with that obtained in other crustaceans and other groups of Arthropoda; the crayfish, *Astacus astacus* (Zandee, 1962, 1964a, 1966a, b; GOSSELIN, 1965), the crab, *Cancer pagurus* (Oord, 1964), the lobster, *Homarus gammarus* (ZANDEE, 1964b, 1967a), the spider, *Avicularia avicularia* (ZANDEE, 1967a), the millepede, *Graphidostreptus tumuliporus* (ZANDEE, 1967a), and the insects (CLAYTON, 1964). As to marine crustaceans, there is no report showing the evidence that sterols are synthesized from acetate and mevalonate. Therefore, further study such as nutritional experiment should be performed to confirm the absence of sterol-synthesizing ability in crustaceans, in addition to the tracer experiment using possible

precursor in cholesterol biosynthesis.

In the case of the prawn, *P. japonicus*, the absence of sterol-synthesizing ability was also supported by the feeding trial using the artificial diet composed of chemically known substances: That is, it was found that the growth of the prawn fed on the sterol-free diet was inferior to that of the prawn fed on the sterol-containing diet. This indicated that the prawn requires sterols as a diet for normal growth. Under the experimental conditions adopted in this study, the requirement of the prawn for cholesterol was estimated to be approximately 0.5 g per 100 g of the diet. The value for sterol requirement was nearly similar to that of the insects belonging to the same Arthropoda as marine crustaceans (ITO, 1961; ISHII, 1964; CLAYTON, 1964).

Generally, phytoplanktons and yeasts are good diet for young crustaceans. Hence, one may interest in the nutritional effect of sterols other than cholesterol for the prawn and other crustaceans. In this approach, the author intended to clarify the utilization of dietary C_{28}- and C_{29}-sterols by the feeding trials using the artificial diet and the natural diets (the clam, *Tapes philippinarum*, the marine yeast, *Cryptococcus albidus*, and "Wakamoto"). The survival rates of the prawns fed on the three artificial diets containing each ergosterol, stigmasterol, and β-sitosterol as a sole source of sterol were similar to that of the prawn fed on the cholesterol-containing diet, although the growth rates appeared to be slightly poor. However, the GLC analysis of the sterols isolated from the prawns supplied with the above three sterols showed no detectable increase of these sterols in the tissues. Furthermore, it was shown that the sterol composition of the prawn fed on the clam containing 22-dehydrocholesterol, cholesterol, desmosterol, 24-methylenecholesterol and β-sitosterol was slightly similar to that of the clam. In the case of the *Artemia*, it was shown that they became an adult in spite of being cultivated with the diets (the marine yeast and "Wakamoto") containing ergosterol, 24-methylcholesterol, stigmasterol, and an unknown sterol but not cholesterol, and that the sterol isolated from the *Artemia* was composed of only cholesterol. These results in the feeding trials using the prawn and *Artemia* suggested that some C_{28}- and C_{29}-sterols such as ergosterol, stigmasterol, 24-methylcholesterol (probably campesterol), and β-sitosterol are utilized to some extent for their growth as a substituent for cholesterol. Furthermore, it was assumed that the bioconversion of some C_{28}- and C_{29}-sterols to cholesterol may take place in marine crustaceans.

Considering the above facts, it seems reasonable to assume that most marine crustaceans as also occurs in the insects lack sterol-synthesizing ability and require cholesterol or other sterol as a diet, and also that the sterols occurring in the tissues of marine crustaceans originate from dietary sources and/or metabolites of dietary sterols. Compared with the nutritional requirements of higher animals, it is unique that crustaceans lack ability for sterol synthesis and may possess the ability for modification of C_{28}- and C_{29}-sterols to cholesterol. From the viewpoint of comparative biochemistry, therefore, it is very interesting to know the metabolic fate of exogenous sterols in crustaceans.

Hence, to obtain the evidence for bioconversion of C_{29}- and C_{29}-sterols to endogenous

sterols such as cholesterol, the several sterols were labelled with ^{14}C by using the biological methods and supplied to the crustaceans as a diet. The radioactive ergosterol, 24-methylcholesterol and brassicasterol were obtained by the incubation of the protozoan, *Euglena gracilis*, the marine yeast, *C. albidus*, and the diatom, *Cyclotella nana*, with acetate-1-^{14}C, respectively. From the crustaceans supplied with the above sterols-^{14}C, the sterols were isolated and the bioconversion of sterols was investigated. As a result, it has been evidently demonstrated that the *Artemia* and the crab, *P. trituberculatus*, are capable of converting ergosterol to cholesterol. For the above bioconversion of ergosterol to cholesterol, it was conceivable to necessitate at least the removal of the methyl group at C–24 and the reduction of the double bonds at C–7 and C–22 from the molecule of ergosterol. Accordingly, it seemed reasonable to assume that cholesterol is formed from ergosterol via at least two intermediates. In this study, it was further clarified that *Artemia* is capable of converting both 24-methylcholesterol and brassicasterol to cholesterol. From these facts, it may be postulated that brassicasterol and/or 24-methylcholesterol are one of the possible intermediates in the bioconversion of ergosterol to cholesterol in *Artemia*. In addition, it was also shown that the prawn, *P. japonicus*, is capable of converting the injected β-sitosterol-4-^{14}C to cholesterol, with the suggestion that this crustacean may possess the enzyme systems for dealkylation at C–24 of β-sitosterol. These findings suggested that most marine crustaceans probably possess the ability for modification of some C_{28}- and C_{29}-sterols to cholesterol. In the insects, there are a few evidences that several insects possess ability for dealkylation at C–24 of β-sitosterol (Robbins *et al.*, 1962) and for reduction of the double bond at C–22 of ergosterol (Clark & Bloch, 1959b; Clayton, 1960). In this respect, the sterol metabolism of crustaceans appeared to be essentially similar to that of insects. However, no biological conversion of ergosterol, 24-methylcholesterol, and brassicasterol to cholesterol has been demonstrated in animals yet. The author believes that this study is the first one showing evidently the bioconversion of the above three sterols to cholesterol.

As stated above, cholesterol or other sterols were conceivable to be essential for normal growth of marine crustaceans. However, the function and metabolic fate of cholesterol in crustaceans are still obscure. Hence, for the purpose of clarifying some function of cholesterol, the distribution of cholesterol-4-^{14}C injected into the tissues and the *in vivo* bioconversion of cholesterol to steroid hormones were investigated. The cholesterol-4-^{14}C injected into the lobster, *P. japonica*, was found to be distributed throughout the every tissue examined, with high concentration in the gonads and hepatopancreas. The chromatographic analysis of labelled substances recovered from the tissues showed the presence of radioactive free sterols, esterified sterols, and polar substances in every tissue. With the lapse of day, the relative percentage composition of the above three substances changed; the most notable change was perceived on the gonads. These results suggested that the hepatopancreas and gonads may be one of the significant sites related to the metabolism of exogenous cholesterol in crustaceans.

In mammals and/or insects, it has been generally recognized that cholesterol is metabolized to bile acid, steroid hormones, vitamin D, and insect molting hormones, although the function of cholesterol has not been fully elucidated yet. In the present study, after injection of cholesterol-4-^{14}C into the lobster, *P. japonica*, the attempt to identify radioactive steroid hormones from the hepatopancreas, ovaries, and blood was carried out. As a result, it was found that cholesterol is converted to progesterone, 17α-hydroxyprogesterone, testosterone, and androstenedione. In invertebrates, the question is still left unresolved whether cholesterol is utilized as a precursor of steroid hormones or not. GOTTFRIED *et al.* (1967) and GOTTFRIED and DORFMAN (1970) have suggested the presence of enzyme systems for formation of pregnenolone from cholesterol in the slug, *Ariolimax californicus*. On the other hand, IDLER *et al.* (1969) have failed to convert cholesterol to steroid hormones by using the gonads of the scallop, *Placopecten magellanicus*. In the case of the lobster, however, it was undoubted that cholesterol is utilized as a precursor of steroid hormones.

The results obtained by the studies on the metabolism of cholesterol in the lobster suggested that other crustaceans probably possess the enzyme systems for bioconversion of cholesterol to steroid hormones. However, the studies on the steroidgenesis in marine invertebrates involving crustaceans are only an open issue. Accordingly, it was still obscure whether the whole biosynthetic pathways of steroid hormones established in higher animals are present or not. Therefore, the *in vitro* production of steroid hormones from progesterone was investigated by using the sliced tissues of the crab, *P. trituberculatus*, in order to obtain the knowledge on the biosynthetic pathways. The *in vitro* incubation of non-radioactive progesterone with the ovaries gave 11-ketotestosterone, 11β-hydroxyandrostenedione, testosterone, and 17α-hydroxyprogesterone as a metabolite. In addition, the incubation of progesterone-4-^{14}C with the ovaries gave radioactive 17α-hydroxyprogesterone, testosterone, and 11-deoxycorticosterone, with the suggestion that the ovaries of this crab contain steroid 17α-hydroxylase, steroid C_{17}–C_{20} lyase, and steroid 21-hydroxylase. The incubation of progesterone-4-^{14}C with the testes gave radioactive 17α-hydroxyprogesterone and testosterone as a metabolite. These results indicated that the gonadal tissues of the crab, *P. trituberculatus*, are significantly related to the metabolism of steroid hormones.

It is a well known fact that in mammals some steroids demonstrate a biological activity as sex hormones. For example, progesterone is known to be concerned, in the latter half of menstrual cycle, mainly with preparing the endomentrium for nidation of fertilized ovum if conception has occurred. During gestation, progesterone, in association with estrogen, appears to be related to maintenance of a state of quiescence of the uterine muscle. Testosterone is found to introduce the appearance of male secondary sexual characteristics such as the growth of male genital organs. Also, corticoids are concerned in controlling electrolyte, water, carbohydrate, and fat metabolism.

Considering that marine crustaceans contain relatively high amounts of sterols and require cholesterol or other sterols and are lacking in sterol-synthesizing ability,

the presence of enzyme systems for biosynthesis of steroid hormones in the crab, *P. trituberculatus*, strongly suggest that some steroids formed from cholesterol probably possess certain physiological activity as hormones in marine crustaceans as well as in mammals.

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