ON THE STEROLS OF TWENTY FIVE SPECIES OF MARINE INVERTEBRATES IN JAPANESE WATERS

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In 1955 Idler and Fagerlund[1] separated 24-methylenecholesterol from some bivalves and established its structure by the formation of formaldehyde and 24-ketocholesterol by means of ozonization and the analysis of the infrared spectra. Bergmann's reinvestigation[2] on the chalinasterol obtained from a sea anemone proved that the structure of the chalinasterol was not 24 a-methylcholesta-5, 22-dien-3 β-ol but 24-methylenecholesterol. Ostreasterol which had been assumed to be impure chalinasterol was also identical with 24-methylenecholesterol. So this sterol occurs not only in such marine invertebrates like pelecypods but also in sea anemones and sea sponges.

The Japanese investigators in this field, however, seem to have been unable to separate it from the sterol fraction of any marine invertebrate, from the literature available so far. In our former studies the animals were usually dried either in an electric oven at a temperature below 80°C or in the open air before the lipids were extracted. This can be the reason why the 24-methylenecholesterol was not isolated from their sterol fractions, for the end methylene group is seemingly unstable to the heat or the air. If the invertebrates in this side of the Pacific Ocean contain different types of sterols, it would be much interesting.

Considering these points, in this study the lipids were extracted directly from the fresh animals without drying. Of twenty five samples, two were dried as before. The scientific names and their classification were given in Table 1. Among these samples, most of them except the following nine species, *Cynthia karasboja*, *Pentacta doliolum*, *Mitella mitella*, *Nerita japonica*, *Tegula argyrostoma basilirata*, *Tegula pfeifferi*, *Calliostoma unicum*, *Barbatia obtusoides* and *Onithochiton hirasei* were already investigated by Toyama and his collaborators, but this time they were taken up once again as the raw materials because the way of the lipid extraction, the time and the district of sample collection were different.

Being mostly small in amount, the samples could not be fully examined. The lipid, nonsaponifiable matter and sterol were determined. The melting point, optical rotation and provitamin D content were measured and infrared spectrum was taken. Among the naturally occurring steroids, it is not only 24-methylenecholesterol but also vitamin D_2, $\Delta^{5,7,22,25}$-cholestatetraenol[3] and 24(28)-dehydroergosterol[4] that have an end methylene group. Besides these, 20-methylenesterols are also suspected to occur in nature, though they have not been isolated yet. Owing to $RR' = CH_2$ in them, all these compounds should show the absorption maximum at both 890 cm^{-1} and 1640 cm^{-1}.[5] In this study, however, the samples

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TABLE 1. List of Marine Invertebrates Examined

Scientific name	Number	Weight (g)	Lipid (g)	Nonsaponif. matter (%)	Sterol			
					%	M.p. (°C)	$[\alpha]_D^\circ$	$\Delta^{5,7}$-Sterol (provitamin D) (%)
PROTOCHORDATA								
Urochordata:								
Cynthia karasboja	10	530	1.167	26.21	51.93	119–122	−46.3	4.9
ECHINODERMA								
Echinoidea:								
Heliocidaris crassispina	48	1260	8.384	15.73	72.70	142–143	−41.5	0
Pseudocentrotus depressus	3	102	1.502	8.60	69.97	140–141	−40.7	3.6
Holothurioidea:								
Pentacta doliolum	16	72	3.230	17.65	3.35	—	—	—
Crinoidea:								
Comanthus japonica	1	17	0.271	26.76	31.54	128–129	—	0
Ophiuroidea:								
Ophioplocus japonica	40	122	—	—	9.45	129–131	—	0.6
ARTHROPODA								
Crustacea:								
Mitella mitella	66	300	0.595	23.43	38.19	140–142	−51.9	0.2
MOLLUSCA								
Gastropoda:								
Onchidium verruculatum	83	67	1.234	11.78	44.53	104–109	+ 3.9	1.8
Chicoreus asianus	7	220	0.817	30.02	60.33	115–122	−28.7	10.0
Cellana toreuma	26	117	1.121	17.38	52.33	137–140	−55.5	0.7
Nerita japonica	1170	520	1.494	29.33	55.76	140–142	−29.4	5.3
Monodonta labio	184	440	1.908	26.02	53.00	141–143	−44.8	1.8
Lunella coronata coreensis	49	305	2.403	13.37	55.66	143–145	−39.2	1.6
Turbo cornutus	16	300	1.264	23.34	67.25	142–143	−42.9	0.8
Tegula argyrostoma basilirata	215	790	6.815	10.06	62.35	142	−41.9	1.8
Tegula pfeifferi	54	470	3.139	12.15	58.80	140–142	−37.4	2.1
Thais bronni	55	390	3.320	18.26	35.69	132–135	−49.3	14.1
Thais clavigera	341	1120	8.118	16.32	52.33	138–140	−44.3	17.3
Calliostoma unicum	18	50	0.320	29.88	35.50	138–141	—	3.9
Pelecypoda:								
Barbatia obtusoides	148	920	5.822	13.15	60.27	132–134	−47.1	9.8
*Pecten yessoensis**	—	—	—	—	64.64	132–134	−41.2	—
Loricata:								
Liolophura japonica	58	190	1.928	14.19	27.36	115–118	0	3.5
Onithochiton hirasei	114	214	2.333	13.74	60.57	114–116	0	4.7
Cryptoplax japonica	14	41	0.481	22.45	29.44	110–120	0	12.5
ANNELIDA								
Polychaeta:								
*Nereis japonica**	—	11490	135	21.48	40.66	140–141	−23.5	3.5

Notes: The yields of nonsaponifiable matter and sterol were expressed on the basis of lipid and nonsaponifiable matter, respectively. $\Delta^{5,7}$-Sterol was determined for the sterol fraction obtained by recrystallization of nonsaponifiable matter from methanol. The content of $\Delta^{5,7}$-sterol in the total sterol was estimated on the assumption that the sterol fraction remaining in methanol has the same content of $\Delta^{5,7}$-sterol as the sterol fraction crystallized out from methanol.

* For these samples, nonsaponifiable matter was separated from the acetone-soluble fat fraction.

which showed the absorption maximum at the both regions were assumed that they contained 24-methylenecholesterol. On the basis of this assumption, the bivalves, *Pecten yessoensis* and *Barbatia obtusoides* showed distinctly the presence of 24-methylenecholesterol. Besides, *Pseudocentrotus depressus*, *Comanthus japonica*, *Ophioplocus japonicus*, *Mitella mitella*, *Onchidium verruculatum*, *Cellana toreuma*, *Monodonta labio*, *Tegula pfeifferi* and *Calliostoma unicum* seemed to have the weak bands in these regions. On the contrary, the molluscs which belong to Loricata were found to contain no 24-methylenecholesterol at all. *Cynthia karasboja*, *Heriocidaris crassispina*, *Nerita japonica*, *Lunella coronata coreensis*, *Turbo cornutus*, *Tegula argyrostoma basilirata*, *Thais bronni*, *Thais clavigera*, and *Nereis japonica* seemed to give the negative results, though some of them were very ambiguous. In molluscs, 24-methylenecholesterol has been found exclusively in pelecypods, but among the samples examined in this study there are only two bivalves. Further studies must be done on more kinds of bivalves.

It has been noticed in a previous study[6] that the sterols from chitons (Loricata) contain considerable amount of Δ^7-sterol, while their $\Delta^{5,7}$-sterol content is practically negligible. The present investigation, however, revealed that their $\Delta^{5,7}$-sterol content was much higher than was expected. It was found to constitute as much as 12.5% of the crude sterol of *Cryptoplax japonica*, 4.7% of *Onithochiton hirasei* and 3.5% of *Liolophura japonica*. These results do not accord with the former observation. It may be too early to give any conclusion here but the discrepancy may be attributed to the fact that the condition of the lipids extraction is quite different.

It is already known that the crude sterols of *Sunetta menstrualis* and *Cellana toreuma* show the absorption maximum at 255 mμ of ultraviolet region.[7] This time too *Cynthia karasboja*, *Heliocidaris crassispina*, *Lunella coronata coreensis* and *Tegula argyrostoma basilirata* had a particular absorption at the same wavelength. As Toyama and Tanaka[8] have noticed, the sterols obtained from chitons have a low melting point which comes from the fact that their principal sterol is Δ^7-sterol, but the sterol of *Onchidium verruculatum* has even lower melting point as 104°–109°C. This is noteworthy because the animal is classified in the particular family Oncidiidae.

In a previous paper, Toyama and Takagi[9] described that *Cucumaria chronhjelmi* and *Stichopus japonicus* of Holothurioidea contained extremely small quantity of sterol in their nonsaponifiable matter and they were able to isolate batyl alcohol from them. In the present study, the same result was obtained for *Pentacta doliolum* which belongs to Holothurioidea.

The chromatography of azoylester of crude sterol obtained from *Pecten yessoensis* afforded crude 24-methylenecholesterol fraction (m.p. 139°–149°C, m.p. of the acetate 135°C) and clionasterol fraction (m.p. 136°–137°C). The melted benzoate of the latter sterol developed green color upon cooling. From the most strongly adsorbed part of the column, methyl azoylate was isolated, indicating that the methanol used as a solvent was not completely removed and remained in the crude sterol. It may be added here that pectsterol was separated from *Pecten yessoensis* by Kuwata and Yoshiki.[10] The sample which was used for the separation of 24-methylenecholesterol by Idler and Fagerlund was the commercial frozen scallop and the scientific name was *Pecten caurinus*. It may also belong

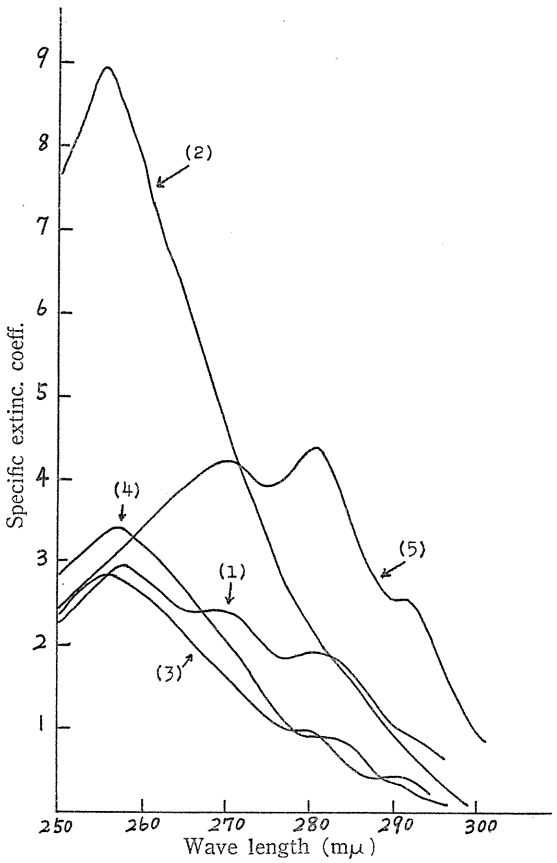
to the same family as *Pecten yessoensis*, though the identification is not settled yet at present.

As to Annelida, Bock and Wetter[11] studied on the sterol of the earthworm, *Lumbricus terrestris* and observed that the sterol fraction contained ergosterol in an amount of more than 20% together with cholesterol. The sterols of marine Annelida seem not to be adequately investigated before. The present study on the sterol of *Nereis japonica* revealed that it contained 3.5% of $\Delta^{5,7}$-sterol. Upon bromination it afforded dibromide of m.p. 116.5°C in good yield. The debrominated sterol and its derivatives were found to be identical with those of cholesterol.

Experimental

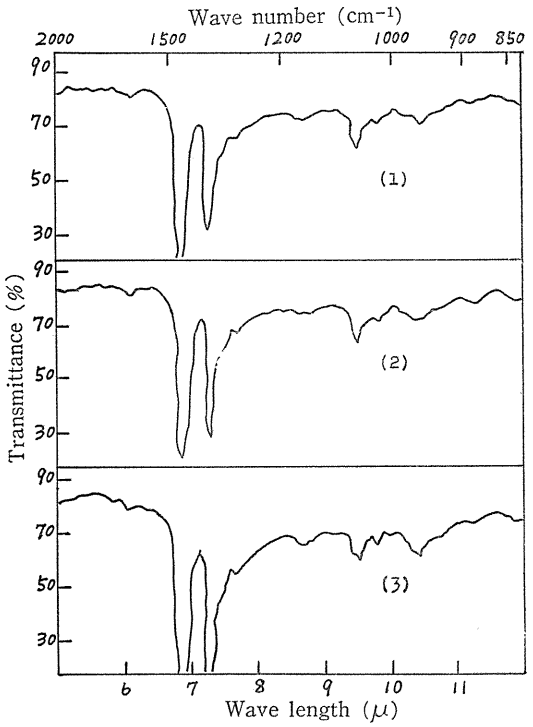
1. Samples

All animals except *Pecten yessoensis* and *Nereis japonica* were collected at the



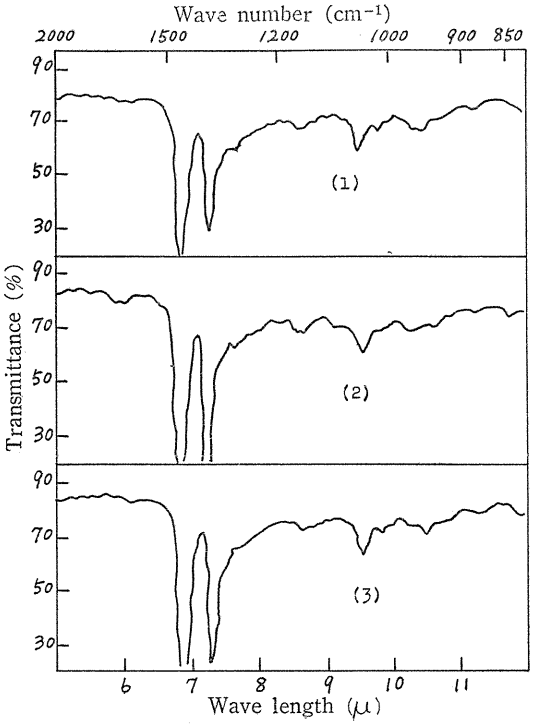
(1) *Cynthia karasboja*
(2) *Heliocidaris crassispina*
(3) *Lunella coronata coreensis*
(4) *Tegula argyrostoma basilirata*
(5) *Cryptoplax japonica*

FIG. 1. Ultraviolet absorption curves for sterols.



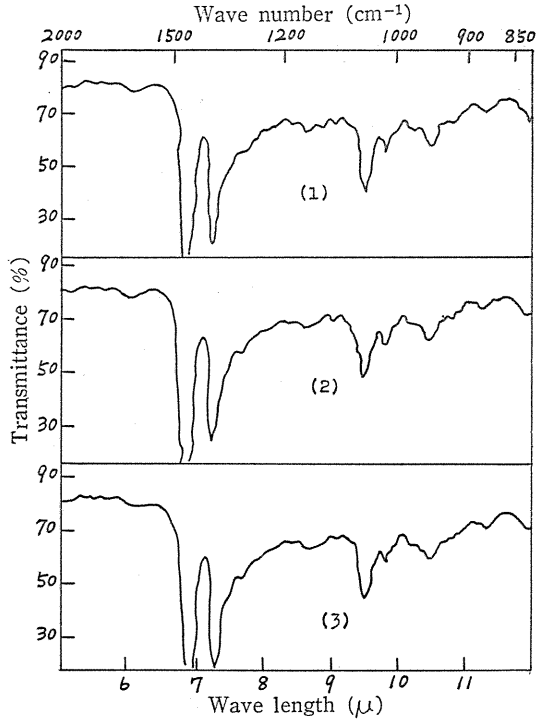
(1) *Pseudocentrotus depressus*
(2) *Comanthus japonica*
(3) *Ophioplocus japonicus*

FIG. 2. Infrared spectra for sterols.



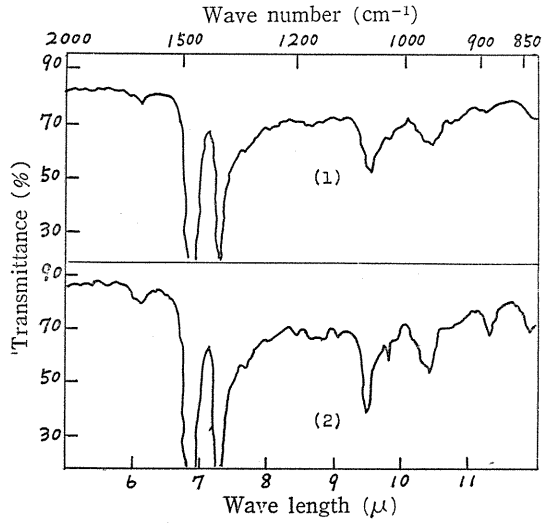
(1) *Mitella mitella*
(2) *Onchidium verruculatum*
(3) *Cellana toreuma*

FIG. 3. Infrared spectra for sterols.



(1) *Monodonta labio*
(2) *Tegula pfeifferi*
(3) *Calliostoma unicum*

FIG. 4. Infrared spectra for sterols.



(1) *Barbatia obtusoides*
(2) *Pecten yessoensis*

FIG. 5. Infrared spectra for sterols.

coast of Sugashima, Mie-ken in the middle of June 1958. The fresh animals were extracted with ether, after they were treated with ethanol two times. The both solutions were combined, filtered and washed with water and the solvents were distilled. Benzene was added to the residue and the water was removed by the codistillation with benzene. The lipid obtained here was saponified and the nonsaponifiable matter was extracted with ether. Recrystallization of the nonsaponifiable matter from methanol afforded the crude sterol, and the sterol remaining in the methanol was determined with digitonin. The ultraviolet absorption of the crude sterol was measured in the region ranging from 250 mμ to 300 mμ. The $\Delta^{5,7}$-sterol content was calculated from the extinction coefficient at the wavelengths of 277 mμ, 282 mμ, and 290 mμ.[7] All these results were summarized in Table 1. The infrared spectra were measured with Perkin Elmer, Type 21, using the Nujol mull method. The ultraviolet spectra of sterols which have the absorption maximum at the wavelength of 255 mμ, and that of *Cryptoplax japonica* were shown in Fig. 1 and the infrared spectra having the absorption band corresponding to the end methylene group were shown in Fig. 2 to Fig. 5.

2. Nonsaponifiable matters and sterols of some samples

(i) ***Pentacta doliolum.*** The lipid of *Pentacta doliolum* was a viscous liquid of red brown color and the nonsaponifiable matter was a crystalline solid. Upon treatment with methanol the nonsaponifiable matter afforded white powder of m.p. 66°–67°C. From 3.25 g of lipid, 88.8 mg of this substance was obtained. The melting point was 66°–69°C after several recrystallizations from methanol. An excess of 1% solution of digitonin in ethanol was added to the solution of nonsaponifiable matter in ethanol and the sterol was removed by filtration of the digitonide. The melting point of the crystalline substance obtained from the filtrate was 67°C. The phenylurethane and bis-*p*-nitrobenzoate melted at 99°–101°C and 66°–68°C, respectively.

(ii) ***Pecten yessoensis.*** The scallop was supplied by Dr. Yamada of the Faculty of Fisheries, Hokkaido University in December, 1954. Dried material (3.15 kg) was obtained from 14.6 kg of stripped shellfish, which afforded 641 g of lipid with ether extraction. The lipid was treated with about ten times its weight of acetone and 584 g of acetone-soluble fat was obtained. The fat was dark brown viscous liquid and its characteristics were as follows: acid value 72.9, saponification value 190.3, iodine value (Wijs method) 117.5 and nonsaponifiable matter 5.28%. The nonsaponifiable matter was yellow solid. Five hundred and fifteen g of the fat afforded 27.2 g of it which upon treatment with hot methanol gave 9.4 g of the crude sterol. Sterol was found to constitute 64.64% of the nonsaponifiable matter according to the digitonin method. The crude sterol showed m.p. 132°–134°C and $[\alpha]_D^{20} = -41.2$. To 1.204 g of the crude sterol were added 1 g of *p*-phenylazobenzoylchloride and 30 cc of dry pyridine, and the mixture was heated for two hours on a water bath. After the reaction mixture was poured into ice water, the precipitate was taken in ether and the ethereal solution was washed with water, dilute hydrochloric acid, sodium bicarbonate solution and water. After drying, the ether was distilled off. The yield of *p*-phenylazobenzoyl ester was 1.673 g. Ten cc of the solution of 0.83 g of the ester in benzene and hexane (1 : 3) was added to the column packed with 120 g of Hyflo

Super-Cel* and 240 g of silicic acid** of 60 mm diameter and 750 mm height employing the same technique as was described by Idler and Baumann.[12] After 25 hours run four bands were observed. The variegated column was pushed out of the open end and cut into four portions. The eluted fractions were named I, II, III and IV from the bottom. The yield was I 0.296 g, II 0.310 g, III 0.174 g and IV 0.024 g, respectively. The intensity of the color was strongest in the upper zone IV which afforded orange-red needles of m.p. 126°–127°C upon recrystallization from benzene and ethanol. This ester had the absorption maximum at 323 mμ. Anal. Calcd. for $C_{14}H_{12}O_2N_2$, N 11.66%; Found 11.65%. The melting point of the ester obtained from the zone III was 195°C which afforded fluffy needles of m.p. 197°C upon recrystallization from benzene and ethanol. After saponification and acetylation, the acetate obtained was refluxed for sixteen hours with a small amount of maleic anhydride in xylene. Saponification of the product followed by extraction with ether and acetylation gave the acetate of m.p. 129°C which reached a constant m.p. 135°C on recrystallization from acetone. The sterol regenerated from the acetate had m.p. 139°–140°C and its infrared spectrum was given in Fig. 6. Zones I and II of the chromatography showed m.p. 187°C and 192°C, respectively. They were combined, saponified and acetylated. The yield of the acetate was 0.36 g and its melting point was 131°–132°C. This was taken up in ether and brominated by the usual manner. Small amount of white powder (m.p. 145°–146°C) was formed which removed by filtration. When the filtrate was concentrated and ethanol was added after the excess bromine had been washed, there was obtained solid bromide which was debrominated with zinc dust and glacial acetic acid. The debrominated acetate had m.p. 136°–138°C and $[\alpha]_D^{25} = -46.8°$. The free sterol melted at 136°–137°C and the benzoate had m.p. 138°–139°C and $[\alpha]_D^{25} = -22.4°$, the melt of which developed brilliant green color upon cooling.

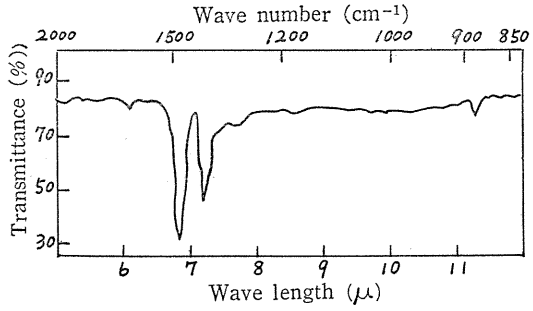


FIG. 6. Infrared spectra for a crude 24-methylenecholesterol fraction from *Pecten yessoensis*.

(iii) ***Nereis japonica.*** *Nereis japonica* was caught, off Haneda, Tokyo, in January 1958. The fresh animal weighed 11.49 kg which was dried in a vacuum oven at a temperature below 80°C to give 1,220 g of powdered dry material.

* Wako Pure Chemicals.

** Mallinckrodt Chemical Works, 100 mesh for chromatography.

Ether extraction gave 135 g of lipid which was treated with about ten times its weight of acetone. The acetone-soluble fat was 67.5 g. This was dark brown liquid at room temperature and had the following characteristics: d_4^{20} 0.9438, n_D^{20} 1.4854, acid value 34.5, saponification value 167.2 iodine value (Wijs method) 129.1 and nonsaponifiable matter 21.48%. Sixty one g of the fat was saponified and the nonsaponifiable matter was extracted with ether. The yield was 14 g. The sterol was found to constitute 40.66% of the nonsaponifiable matter according to the digitonide determination. The crude sterol obtained by recrystallization of the nonsaponifiable matter from methanol showed m.p. 140°–141°C and $[\alpha]_D^{20} = -23.5°$. $\Delta^{5,7}$-Sterol content was found to be 3.5%. The acetate of the crude sterol had m.p. 113.5°–114°C and iodine value (pyridine sulphate dibromide method) 71.2. In 7 cc of ether 1.09 g of the crude sterol was dissolved by gentle warming and 5 cc of a solution of bromine and sodium acetate in glacial acetic acid was added.[13] The yield of the dibromide was 1.1 g which melted at 116.5°C. The debromination with zinc dust, glacial acetic acid and ether afforded plates of m.p. 148°C and $[\alpha]_D^{20} = -37.9°$. The acetate and benzoate prepared from the sterol showed m.p. 114°C and m.p. 146°C (turbid), 178°C (clear), respectively. The melting point of the crude steryl acetate (m.p. 113.5°–114°C) rised to 122°–125°C by several recrystallizations from acetone. From 5.6 g of the acetate, 0.48 g of the high melting fraction was obtained. This was dissolved in hexane and passed through a column packed with alumina and two fractions of m.p. 100°–101°C (0.16 g) and m.p. 122°–123°C (0.30 g) were obtained. The latter gave a small amount of crystals of m.p. 134°–135°C after repeated recrystallizations from ethanol. The sterol obtained by saponification melted at 146°C and its $\Delta^{5,7}$-sterol content was found to be 0.15%.

Summary

The lipids were extracted mostly from the fresh animals, without being dried, of Protochordate, Echinoderm, Crustacean, Mollusca and Annelid. The nonsaponifiable matter and sterol were determined. Both U. V. and I. R. spectra were taken to calculate the provitamin D content and to detect the end methylene group. The sterols of *Pecten yessoensis* and *Barbatia obtusoides* showed distinctively the presence of the end methylene group, on the other hand the mollusca which belong to the Loricata gave the negative results. The sterols of *Cynthia karasboja, Heriocidaris crassispina, Lunella coronata coreensis* and *Tegula argyrostoma basilirata* had an absorption maximum at the wavelength of 255 mμ. Batyl alcohol was isolated from the nonsaponifiable matter of *Pentacta doliolum.* Clionasterol and 24-methylenecholesterol fractions were separated from the sterol mixture of *Pecten yessoensis* by the chromatography of its azoylester. Cholesterol was found to be predominant in the sterol fraction of *Nereis japonica.*

We are indebted to Dr. M. Yamada of the Faculty of Fisheries, Hokkaido University for the sample of *Pecten yessoensis* and to Dr. T. Kaneda of the Tokai Regional Fisheries Research Institute for the sample of *Nereis japonica.* Also we wish to thank Dr. M. Momotani of the Momotani Junten Kan for the generous use of infrared spectrophotometer and Mr. K. Naito of the Osaka Industrial Research Institute for interpretation of the infrared spectra.

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