The Sterol Components of *Undaria pinnatifida* and the Incorporation of ^{14}C-1-acetate into Them

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미역의 스테롤 組成과 ^{14}C-1-식초산염의 스테롤 轉換에 關하여

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미역의 스테롤 組成을 가스크로마토그라피로 調査하였고, 또한 미역줄기에 ^{14}C-1-acetate를 注射하여, 120時間後 各脂質區分 및 스테롤의 比放射能을 調査하여 ^{14}C-1-acetate의 스테롤에로의 轉換에 對하여 고찰하였다.

實驗에 使用한 미역成分組成은 炭化水素 1.6%, 색소 및 스테롤에스텔 2.5%, 글리세리드 3.3%, 유리지방산 2.2%, 스테롤 3.8%, 클로로필系色素 18.8% 및 極性脂質이 67.3%였다.

미역의 스테롤組成을 보면 콜레스테롤 3.5%, 24-메칠렌콜레스테롤 11.2% 및 프코스테롤이 85.3%였다. 미역줄기에 ^{14}C-1-아세트산염을 注射한 後, 120時間 經過한 各脂質 區分의 比放射能을 보면, 總脂質에 4,648 dpm/*mg*, 極性脂質에 2,754 dpm/*mg*, 클로로필 系色素에 373 dpm/*mg*, 스테롤에 22,481 dpm/*mg*, 유리지방산에 6,520 dpm/*mg*, 스테롤에스텔에 786 dpm/*mg*, 炭化水素에 358 dpm/*mg*로서 스테롤 區分에 가장 높았고, 各 스테롤의 比放射能을 보면, 콜레스테롤에 115 dpm/*mg*, 24-메틸렌 콜레스테롤에 147,821 dpm/*mg*, 프코스테롤에 20,887 dpm/*mg* 였다.

Introduction

Most Japanese species of Rhodophyta [5] contain primarily cholesterol, though some species contain large amounts of desmosterol [1] or 22-dehydrocholesterol [2], while Rhodophyta [3] from British waters contain sitosterol. These and many other questions concerning the occurrence and identity of sterols in algae have been resolved in recent years by a reexamination of the species using recently developed methods of analysis such as gas and thinlayer chromatography and mass spectroscopy.

Carter *et al.* [4] reported the dominant sterol in the brown algae was fucosterol. According to Patterson [5], most Phaeophyta contain traces of cholesterol and biosynthetic precursors of fucosterol, and he [6] also reported that the sterols from two species of *Laminaria* collected from the coast of Maine, U.S.A., were composed of fucosterol, 24-methylene cholesterol, cholesterol, saringosterol and demosterol.

By Teshima *at al.* [7] ^{14}C-labelled 22-dehydrocholesterol and cholesterol were proved to be biosynthesized in the culture media contained ^{14}C-1-acetate of *Porphyridium cruentum*, and Katayama[8] reported that ^{14}C-carotenoid could be obtained by injection of ^{14}C-2-mevalonate in the stems of *Codium intricatum*, a green alga.

The present study is undertaken to know whether ^{14}C-1-acetate can be incorporated into the lipid fractions and sterols of *U. pinnatifida*, popularly eaten in Korea and Japan, and to identify the companions of fucosterol and to note whether or not their structures suggest that they are biosynthetically related to fucosterol as the companions of cholesterol in higher animals were biosynthetically related to it.

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Materials and Metheds

^{14}C-1-acetate administration: *U. pinnatifida* were collected off Matsushima Bay, Miyagi Prefecture, Japan, on January 25, 1975. The brown alga brought to the isotope laboratory, where all volatile and water-soluble ^{14}C-compounds metabolites from this alga were filtered, had been cultured in three plastic baths (dimension, 45×25×20 *cm*) for 7 days, in which sea water was being aerated and circulating. After 120 hours from the injection of ^{14}C-1-acetate (50 μci) into the stems of the brown alga (100 *g*, on wet base), the total lipids were extracted by the method of Bligh and Dyer[9]. The cold alga was kept on culturing in other baths on the same conditions.

Lipid classification by column chromatography [10]: Total lipids (81 *mg*) adsorbed on Silica Gel (100*g*, Mallinckrodt, 80-100 mesh) activated by various eluants, were eluted in different fractions with solutions of petroleum ether(p. e.), 3% ether-p. e., 7% ether-p. e., 15% ether-p. e., 25% ether-p. e., ether, chloroform and 25% chloroform-methanol.

Radioactivity detection and counting of each lipid fraction: Small quantities of the hot lipids applied to TLC coated with Wakogel B-5 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) were developed in a solution of p. e.: ether: acetic acid (80:20:1), and then the radioactivity of each band on TLC, was checked with a TLC scanner equipped with a radioactivity counter. The specific radioactivity of each lipid class scraped off TLC bands on a level of 10*mg*, was counted by a Packard's automatic scintillation counter.

The sterol isolation: The lipids were saponified with a solution of 10% KOH in 90% ethanol for 30 minutes. Upon cooling, the solution was diluted with distilled water, the unsaponifiable matters were extracted with anhydrous ether, and the ether phase was washed several times with distilled water. The sterol fraction was recrystallized with cold methanol sereval times, and further purified by column chromatography, if contaminated with impurities.

Gas liquid chromatographic analysis of the sterol acetates: The pure sterol fraction obtained from cold sample was acetylated [11] with a solution of anhydrous acetic acid and pyridine (1:1) in a refrigerator overnight. GLC apparatus used in this study was a dual column Hitachi Model GC-2C with a flame ionization detector. The coiled stainless(3 *mm*×2 *m*, i. d.) column packed with 1.5% OV-17 and 1.5% SE-30 on Chromosorb W was used, and the column operating temperature was 250°C. The sterol identification was conducted by comparing the relative retention time to cholesterol to those of the authentic sterols and, in some cases, confirmed by IR, Mass spectra and NMR spectra.

The separation of the hot sterol acetates on TLC [12,13]: The hot sterol acetates on TLC impregnated with 10% silver nitrate in the solvent system, n-hexane : benzene 5:3, and the bands were detected by spray of 0.1% Rhodamine 6G-acetone, followed by irradiation of long wave ultraviolet (365 μm). Small quantities of sterol carriers were added, if necessary. The radioactivity counting was done as the same above-mentioned, and the specific radioactivities of the sterols were carried out as follows; the sterol bands encircled on TLC were collected to some extent, respectively. Each sterol filtered from Silica Gel and 15 *ml* of scintillator* were added in a vial. The vials were kept in a dark place overnight. Counting was conducted by a Packard's automatic scintillation counter, and the radioactivity was calculated on the channel ratio method.

Spectra analysis: Infrared absorption spectra were conducted on KBr films, and mass spectra were measured on Hitachi RMU-7, its operating conditions were as follows: chamber voltage 80 eV, total emission 80μA, ion chamber temperature 160℃, vacuum 5×10^{-7} *mm*Hg. NMR spectra

* Solution of 4 *g* PPO (2,5-diphenyloxazole) and 0.1 *g* POPO {1,4-bis-2-(4-methyl-5- phenyloxazolyl)-benzene} dissolved in 1 liter of toluene.

were taken on 60Mc/s JNM-MH-60-11, $CDCl_3$ and tetramethylsilane (TMS) were used as a solvent and a standard shielding reagent, respectivelv.

Results

The lipid classes were composed of polar lipids, 67.3%; chlorophyll, 18.8%; sterol, 3.8%; triglyceride, 3.3%; unknown pigment and sterol ester, 2.5% and hydrocarbon, 1.6%; respectively. The lipid composition of *U. pinnatifida* examined is given in Table 1.

Table 1. Fractionation of lipid of *U. pinnatifida* incorporated with ^{14}C-1-acetate

Eluant	Volume (*ml*)	Fraction	Wt (*mg*)	%*
100% Petroleum ether (p. e)	200	Hydrocarbon	1.2	1.6
3% Ether:p e	200	Pigment & sterolester	1.8	2.5
7% Ether:p. e	300	Triglyceride	2.4	3.3
15% Ether:p. e	300	Fatty acid	1.6	2.2
25% Ether:p. e	300	Sterol	2.8	3.8
Ether	500	Chlorophyll	13.8	18.8
Chloroform	200			
Chloroform:methanol (1:4)	800	Polar lipids	49.4	67.3

* % to total recovery weight.

Radioactivities of various lipid classes: The radioactivities on the bands of polar lipids, sterol, unknown (probably fatty alcohol), free fatty acid, triglyceride, and hydrocarbon were recorded on radiogram, but so didn't on chlorophyll band. The radiogram of each lipid class separated on TLC is presented in Fig. 1.

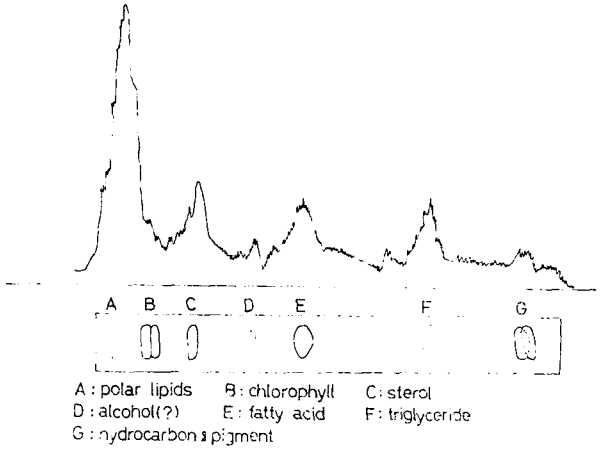

Fig. 1. A radiogram of the lipid classes from *U. pinnatifida* incorporated with ^{14}C-1-acetate.

In specific radioactivities of various lipid classes, the highest specific radioactivity was counted in the sterol fraction, 22,481 dpm/*mg*, while the lowest in the chlorophyll fraction, 373 dpm/*mg* as summarized in Table 2.

Table 2. Radioactivity distribution of various lipid classes fractionated from *U. pinnatifida*

Classes	dpm/*mg*
Total lipids	4,648
Polar lipids	2,754
Chlorophyll	373
Sterol	22,481
Fatty acid	6,520
Sterol ester	786
Hydrocarbon	358

GLC of the sterol acetates: In GLC on 1.5 % OV-17, the sterol mixture isolated from *U. pinnatifida* was found to be composed of three components as shown in Fig. 2. The peaks 1, 2 and 3 were identified by the relative retention times to cholesterol as cholesterol, 24-methylene cholesterol and fucosterol respectively. Their composition is listed in Table 3.

Table 3. The sterol composition of *U. pinnatifida*

Rt (*min.*)	Rrt	Sterol	% (as methyl ester)
24.5	1.00	Cholesterol	3.5
32.8	1.34	24-Methylenecholesterol	11.2
42.1	1.72	Fucosterol	85.3

GLC condition: Column temperature 250℃ 1.5% OV-17.

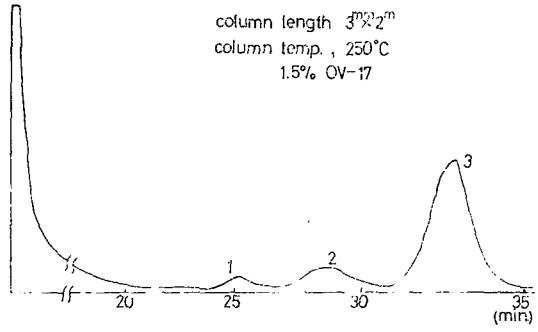

Fig. 2. The GLC chromatogram of the sterols from *U. pinnatifida.*

The sterol acetates from *U. pinnatifida* were separated into distinct three bands fluoresced in long wave violet light. Each sterol acetate band impregnated with 15% silver nitrate significantly shows radioactivity. The scanning radiogram of TLC is shown in Fig. 3, and the specific radioactivities of cholesterol, 24-methylenecholesterol and fucosterol were 115 dpm/*mg*, 147,821 dpm/*mg*, and 20,887 dpm/*mg*, respectively (Table 4).

Table 4. Radioactivity distribution of the sterols from *U. pinnatifida*

Sterol	dpm/*mg*
Cholesterol	115
24-Methylenecholesterol	147,821
Fucosterol	20,887

Mass and IR spectra of the sterol acetates identification of cholesterol : The mass spectra of the peak 1 in Fig. 4—a, showed no molecularion peak corresponding to cholesterol acetate (428*m*/e), but the other peaks are interpreted as follows; 368 (M^+-CH_3COOH), 353[$M^+-(CH_3COOH+CH_3)$], 325[$M^+-(CH_3COOH+43)$], 255

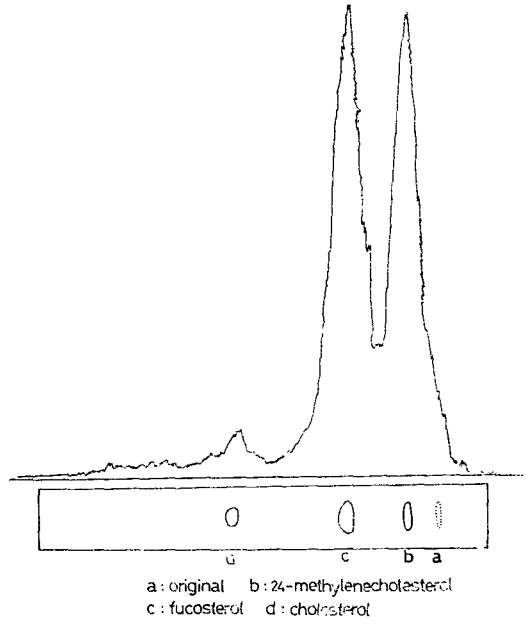

Fig. 3. Radiogram of the hot sterols from *U. pinnatifida.*

[$M^+-(CH_3COOH+R, R$;side chain)], 253[$M^+-(CH_3COOH+R+2H)$], as shown in Fig. 5—a, the IR absorption spectra of the peak 1 is identical to those of standard cholesterol acetate.

Identification of 24-methylenecholesterol : In case of the peak 2 on GLC, the mass spectra gave prominent peaks at m/e 380, 365, 296, 255 and 253. The peak at m/e 440 corresponding to that of molecular ion(M^+) of the sterol acetate was not observed. As shown in Fig. 4—b, however, a relatively high peak is seen at m/e 380 corresponding to one for the loss of acetic acid [14] from the molecular ion. The other peaks are interpreted as [15] follows; m/e 365[$M^+-(CH_3COOH+CH_3)$], 296[$M^+-(CH_3COOH+CH_3+C_6H_{12})$],

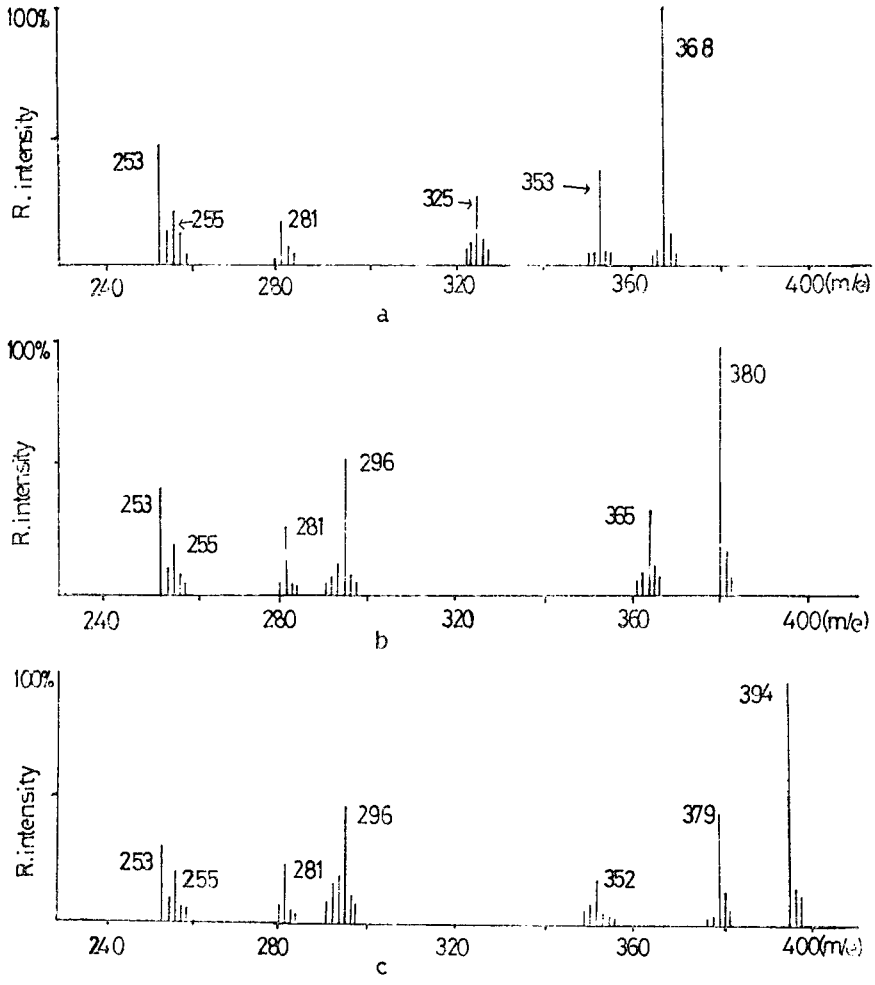

Fig. 4. The Mass spectra of the steroid acetates from *U. pinnatifida*.
a: Peak 1 (Cholesterol acetate)
b: Peak 2 (24-methylenecholesterol acetate)
c: Peak 3 (Fucosterol acetate)

255[M^+—(CH_3COOH+R, R; side chain)], 253[M^+—(CH_3COOH+R+2H)], the ion peak 296 is characteristic in all the sterols included the side chains with a double bond at C24—C28. On the IR spectra, as shown in Fig. 5—b, the absorption peaks at 885cm^{-1} and 1640 cm^{-1}, suggest the presence of vinylidene ($CH_2=C\langle^{R}_{R}$) in the side chain of the sterol.

Identification of fucosterol: The mass spectra of the peak 3 gave strong peaks at m/e 394, 379, 352, 296, 281, 255 and 253 (Fig. 4—c). The molecular ion peak being not seen, the parent peak shown at m/e 394 corresponds to that for the loss of acetic acid from the molecular ion. The other peaks are interpreted as follows; 379 [M^+—(CH_3COOH+CH_3)], 352[M^+—(CH_3COOH +CH_3CH_2)], 296[M^+—(CH_3COOH+C_7H_{14})], 281 [M^+—(CH_3COOH+CH_3+C_7H_{14})], 255[M^+— (CH_3 COOH+R)], 253[M^+— (CH_3COOH+R+2H)]. The IR spectra of the peak 3 resemble those of fucosterol with peaks at 840$cm^{-1}$and 800cm^{-1} for the 4^5 bond[16] (Fig. 5—c), and have another peaks at 825cm^{-1} attributed to the out-of-plane bending frequency of the hydrogen at C28.

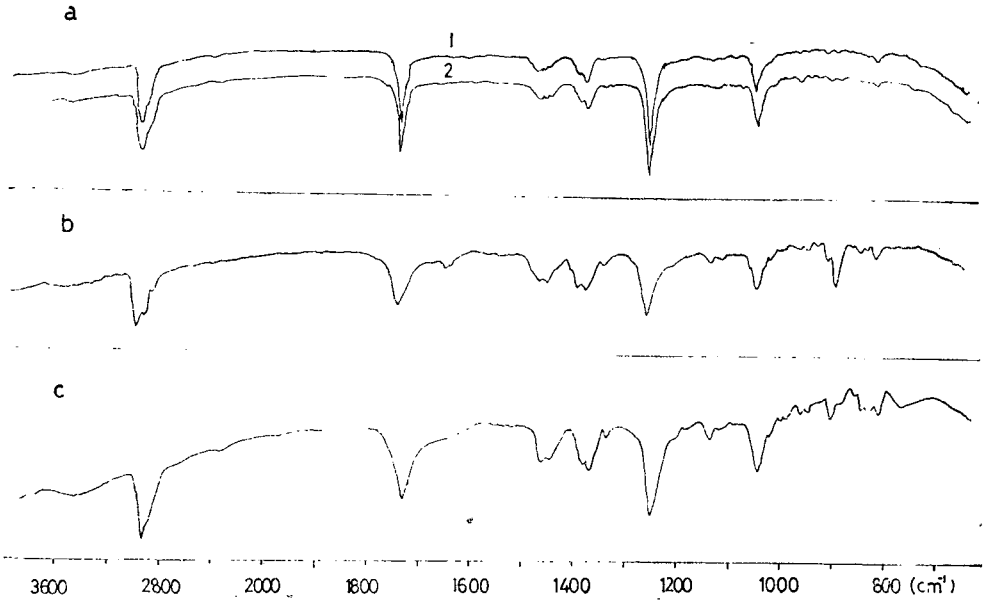

Fig. 5. IR spectra of the sterol acetates from *U. pinnatifida*.
a-1: Cholesterol acetate, 2: Peak 1 on GLC, b: Peak 2 on GLC, c: Peak 3 on GLC.

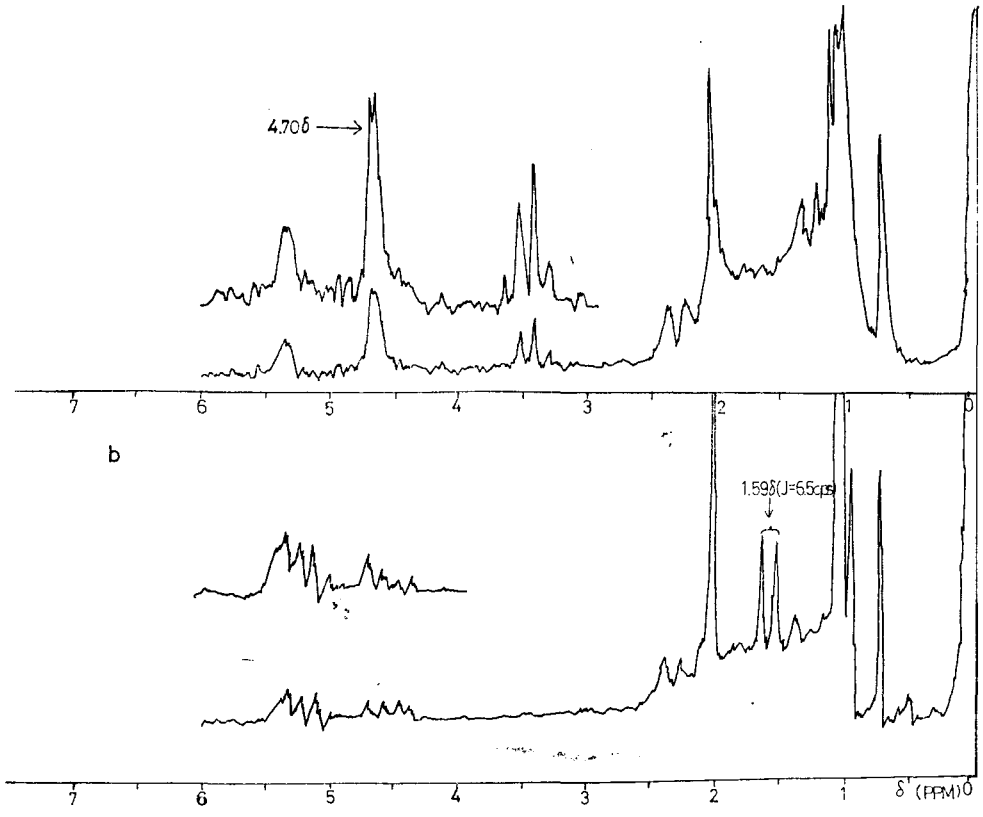

Fig. 6. NMR spectra of the sterol acetates from *U. pinnatifida*. a: 24-methylenecholesterol acetate, b: fucosterol acetate.

NMR spectra of the sterol acetates: NMR spectra of the sterol peak 2 on GLC gave a singlet at 4.70δ for the proton of methylene radical at C24 (Fig. 6—a). The chemical shift 1.59δ (doublet, J=6.5 cps) of the sterol peak 3 on GLC indicated the ethylidene (=C—CH_3) (Fig. 6—b).

The peaks 1, 2 and 3 on GLC are identified as cholesterol, 24—methylenecholesterol and fucosterol, respectively.

Discussion

Vroman and Cohen[13] separated the acetates of cholesterol and desmosterol on Silica Gel H impregnated with silver nitrate, developing with n-hexane: benzene 5:3, and Idler *et al.*[12] completely separated the sterol acetate mixture from scallop on Silica Gel-HF254 and 366 impregnated with silver nitrate. According to them, the sterol mixture unseparable on a usual method could be individually separated with the double bond numbers and the structure of sterol on 10% $AgNO_3$ -HF356 TLC by slightly changing the polarity of developing solvent and repeating developments.

In this study, simple sterol components of *U. pinnatifida* were completely separated on TLC impregnated with silver nitrate with higher Rfs.

It is of great interest that the sterol fraction showed the highest specific radioactivity among all the lipid classes, while that of chlorophyll was the lowest (373 dpm/*mg*). But the specific radioactivity of chlorophyll rapidly increased by the time passing after ^{14}C-1-acetate injection (no data presented).

Since the earliest work of Carter *et al.*,[4] it has been recognized that fucosterol is the predominant sterol of brown algae *Laminaria*[6] apparently contains fucosterol, 24-methylenecholesterol, saringosterol and desmosterol. According to Ikegawa *et al.*,[19] nine Phaeophyta apparently contain fucosterol as its primary sterol with small amounts of cholesterol, 24-methylenecholesterol and saringosterol. But they did not detect desmosterol from nine Phaeophyta.

Patterson[6] suggested that cholesterol in *Laminaria* could be produced by a reduction of desmosterol and by some modification of saringosterol. But these two sterols were not detected in *U. pinnatifida*.

Though cholesterol can be apparently biosynthesized in *U. pinnatifida*, the biosynthesis mechanism of cholesterol can not be completely explained from the results of this study.

It is well known in plant that 24-methylenecholesterol is the precursor[20),21)] of fucosterol and methyl radical attached to C24 site of C29 sterol is donated from methionine. In this study, the specific radioactivity of fucosterol is lower than that of 24-methylenecolesterole. This fact is considered to be ascribed to slow methylation at C28 site of 24-methylenecholesterol..

Summary

The present study was carried out to know the sterol components of *U. pinnatifida* and their incorporation abilities of ^{14}C-1-acetate injected into it.

The results obtained are as follows;

1. The total lipids are classified as hydrocarbon 1.6%, pigment and sterol ester 2.5%, triglyceride 3.3%, free fatty acid 2.2%, free sterol 3.8%, chlorophyll 18.8%, and polar lipids 67.3%.
2. The sterol mixture from *U. pinnatifida* are composed of cholesterol 3.5%, 24-methylenecholesterol 11.2%, fucosterol 85.3%.
3. The radioactivities of the lipids classes from *U. pinnatifida* injected with ^{14}C-1-acetate are distributed 4,648 dpm/*mg* in total lipid, 2,754 dpm/*mg* in polar lipids, 373 dpm/*mg* in chlorophyll, 22,481 dpm/*mg* in free sterol, 6,520 dpm/*mg* in free fatty acid, 789 dpm/*mg* in sterol ester and 358 dpm/*mg* in hydrocarbon respectively.
4. The specific radioactivities of the sterols are 115 dpm/*mg* in cholesterol, 147,821 dpm/

mg in 24-methylenecholesterol, 20, 887 dpm/*mg* in fucosterol.

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