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Conversion of Cholesterol to Coprostanol and Cholestanol in the Estuary Sediment

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Abstract

This paper deals with the conversion of cholesterol-4-^{14}C to radioactive coprostanol, cholestanol, and cholestenone in the estuary sediment from Kagoshima Bay, Kagoshima, Japan.

The incubation of cholesterol-4-^{14}C with the sediment and sea water gave radioactive coprostanol, cholestanol, and cholestenone. The identification of these radioactive compounds was performed by thin-layer chromatography and preparative gas-liquid chromatography followed by radioactive measurements. However, the incubation of cholesterol-4-^{14}C with sterilized sediment and with sea water (or sterilized sea water) produced extremely small amounts of the above mentioned conversion products. These results indicated that the conversion of cholesterol to coprostanol, cholestanol, and cholestenone is effected by the action of microorganisms in the sediment.

Several workers have attempted to use coprostanol (5β-cholestane-3β-ol) as an indicator of fecal pollution in water[1~7]. Since the feces of human and mammals are regarded as the only source of coprostanol in natural environments, the occurrence of coprostanol has been conceived to be indicative of water pollution in the cause of human life.

As to the marine environments, the authors have pointed out the presence of coprostanol in both sea water and the sediments in Ariake Sea, indicating that the concentration of coprostanol was markedly high in the sediments as compared with that of sea water[7]. Later, we have found that the estuary sediment from Kagoshima Bay contained large amounts of 5β-stanols such as coprostanol, 24-methylcoprostanol (24-methyl-5β-cholestane-3β-ol), and 24-ethylcoprostanol (24-ethyl-5β-cholestane-3β-ol) and 5α-stanols such as cholestanol (5α-cholestane-3β-ol), 24-methylcholestanol (24-methyl-5α-cholestane-3β-ol), and 24-ethylcholestanol (24-ethyl-5α-cholestane-3β-ol) besides common unsaturated sterols[8]. Generally, living organisms contain only small amounts of saturated sterols in their bodies[9], except for some species of sponges[10]. Therefore, it is open to question that the saturated sterols including coprostanol in the estuary sediments are formed by microbiological or chemical hydrogenation from corresponding unsaturated sterols such as cholesterol. In order to resolve

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this question, we intend to examine the conversion of cholesterol in the estuary sediment by using tracer techniques. The present paper deals with these results and discussion.

Materials and Methods

Incubation of cholesterol-4-^{14}C with the sediment and sea water Cholesterol-4-^{14}C (specific activity, 143 μCi/mg; Radiochemical Centre, Amersham, England) was purified by thin-layer chromatography (TLC) on Kieselgel G with chloroform[8] and on 10 % (w/w) $AgNO_3$-Kieselgel G with ethanol-free chloroform[11]. Sea water (surface water) and the sediment (0-5 cm depth) were aseptically collected near the estuary of River-Koutsuki, July 10, 1977. Cholesterol-4-^{14}C was incubated in a 100 ml-Erlenmyer flask containing sea water (10 ml) and the estuary sediment (10 g in wet weight) without shake at 20°C as shown in Table 1.

Table 1. Incubation of cholesterol-4-^{14}C with the sediment and sea water

Incubation No. *1	Incubation medium	Cholesterol-4-^{14}C added (μCi)*2	Incubation period (days)
1	Sterilized sediment (10 g) + Sterilized sea water (10 ml)	2	10
2	Sterilized sediment (10 g) + Sea water (10 ml)	2	10
3	Sediment (10 g) + Sea water (10 ml)	2	10
4	Sediment (10 g) + Sea water (10 ml)	2	30
5	Sediment (10 g) + Sea water (10 ml) + Yeast extract (5 mg) + Polypeptone (10 mg)	2	10

*1 Incubation was carried out without shaking at 20°C.
*2 Cholesterol-4-^{14}C was dissolved in 0.15 ml of 5 % Tween 80 and added to each flask.

Extraction and identification of conversion products After incubation, lipids were extracted twice with chloroform-methanol-water (2: 2: 1) according to the method of BLIGH and DYER[12] from the incubation mixture. Lipids were saponified with 10 % KOH in ethanol at 80°C for 3 hr, and the unsaponifiable matters were isolated from the saponification mixture by extraction with ether. The unsaponifiable matters were separated into '5β-stanol fr.' and 'sterol fr.' by thin-layer chromatography (TLC) on Kieselgel G with chloroform[8]. '5β-Stanol fr.' contained coprostanol and cholestenone (cholest-4-en-3-one), whereas 'sterol fr.' involved cholestanol and other unsaturated sterols. 'Sterol fr.' was acetylated with dry pyridine-acetic anhydride (1: 1) and then subjected to 10 % (w/w) $AgNO_3$-Kieselgel G with ethanol-free chloroform[11] to separate Δ^0-, Δ^7-, and Δ^5-sterol fractions. Finally, the incorporation of radioactivity into each sterol was ascertained by preparative gas-liquid chromatography (GLC) on 1.5 % OV-17 (column temperature, 250°C) followed by radioactive measurements[13]. Radioactivity was determined on a Beckman liquid scintillation counter LS-230 using a solution of 0.6 % PPO as a scintillator. The efficiency of counting of

radioactive steroids was about 90 per cent. In addition, the presence of radioactive compounds was checked by means of autoradiography after TLC.

Results

Cholesterol-4-^{14}C was incubated with the sediments and sea water as shown in Table 1. The results are given in Tables 2 and 3. The recoveries of radioactivity in lipids were low in incubations No. 3, No. 4, and No. 5 which contained non-sterilized both sediment and sea water. GASKELL and EGLINTON[14] have pointed out the difficulty in quantitative extraction of sterols from the sediment. However, the low recoveries of radioactivity in incubations No. 3, No. 4, and No. 5 were attributable to loss of ^{14}C-activity, probably as an expired carbon dioxide during the incubation period, because of the high recoveries of

Table 2. Incorporation of ^{14}C activity into lipid fractions

Incubation No.	Radioactivity (dpm × 10^3) and incorporation (%)				
	Lipids	Unsap. matters*1	'5β-Stanol fr.'	'Sterol fr.'*2	Unknown polar comp.*3
1	4,150 (94.3%)	4,100 (93.2%)	4.1 (0.09%)	3,900 (88.7%)	197 (4.5%)
2	4,220 (96.0%)	4,200 (96.0%)	4.2 (0.09%)	4,020 (91.3%)	211 (4.8%)
3	3,560 (80.9%)	3,310 (75.2%)	76.1 (1.7 %)	3,080 (69.9%)	156 (3.5%)
4	2,700 (61.4%)	2,260 (51.3%)	108 (2.5 %)	2,030 (46.1%)	122 (2.8%)
5	3,400 (77.2%)	3,020 (68.6%)	117 (2.7 %)	2,770 (62.9%)	130 (3.0%)

% Incorporation was expressed as percentage of cholesterol-4-^{14}C (2 μCi) added to each incubation flask. *1 Unsaponifiable matters. *2 Sterols except 5β-stanols. *3 TLC on Kieselgel G of the unsaponifiable matters showed the presence of radioactive substances (Rf 0.00, 0.05) besides two radioactive bands corresponding to 5β-stanols and other sterols. These radioactive compounds are referred as 'unknown polar compounds'.

Table 3. Distribution (%) of radioactivity in '5β-stanol fr.' and 'sterol fr.' and incorporation (%) of radioactivity into coprostanol, cholestenone, cholesterol, cholestanol, and cholest-7-enol

Incubation No.	'5β-Stanol fr.'				'Sterol fr.'			
	Corpostanol		Cholestenone		Cholestanol		Cholesterol	Cholest-7-enol
	% Distr.*1	% Incorp.*2	% Distr.	% Incorp.	% Distr.	% Incorp.	% Distr.	% Distr.
1	49	0.04	51	0.05	3.7	3.3	96.3	—
2	56	0.05	44	0.04	3.2	2.9	96.8	—
3	48	0.82	52	0.88	15.8	11.0	84.2	—
4	42	1.05	58	1.45	27.4	12.6	72.6	trace*3
5	50	1.35	50	1.35	12.2	7.7	87.8	trace

*1 % Distribution of radioactivity in '5β-stanol fr.' or 'sterol fr.' determined by using TLC.
*2 % Incorporation expressed as percentage of cholesterol-4-^{14}C added to each incubation flask.
*3 Less than 0.1 %.

radioactivity in lipids from incubations No. 1 and No. 2 which contained either sterilized or non-sterilized sea water in addition to sterilized sediment. Also, the results of the present study suggests that cholesterol is undergone degradation to carbon dioxide within a short period in the estuary sediment; namely, about 40 % of cholesterol-4-^{14}C was suspected to be converted to carbon dioxide in incubation No. 4 (30-day incubation).

Aliquots of the radioactive unsaponifiable matters were subjected to TLC on Kieselgel G and autoradiographed. The autoradiograms of the unsaponifiable matters from incubations No. 3, No. 4, and No. 5 showed the presence of four

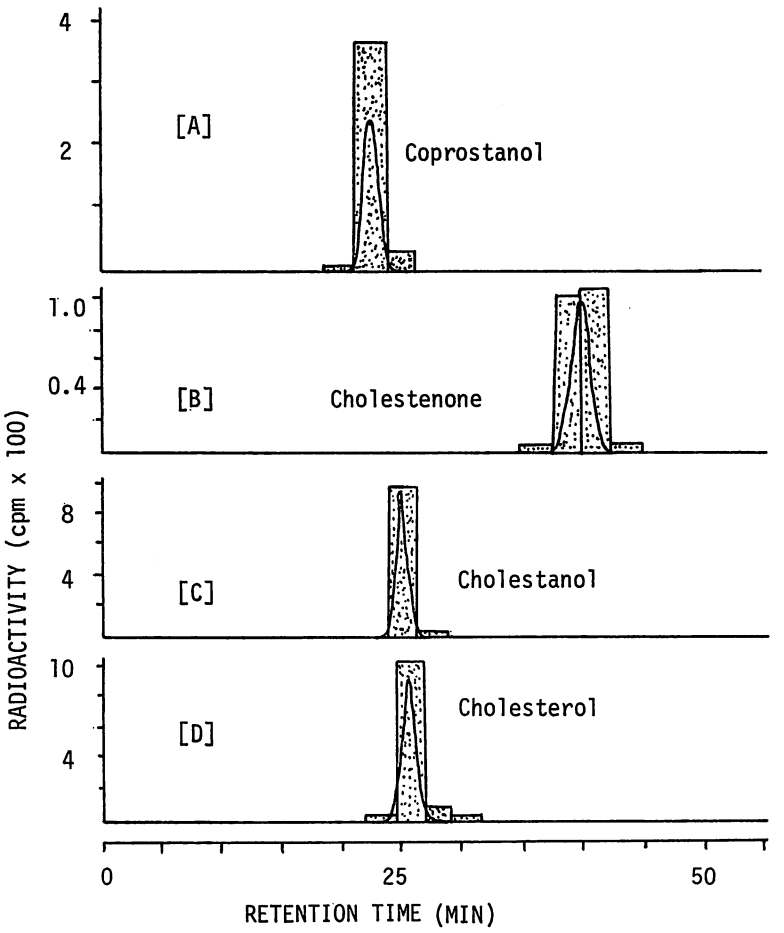

Fig. 1. Preparative GLC on 1.5% OV-17 of the fractions corresponding to coprostanol [A], cholestenone [B], Δ^0-sterols [C], and Δ^5-sterols [D].

To each fraction obtained by TLC, coprostanol, cholestenone, cholestanol, and cholesterol were added as carriers and subjected to GLC. Trapped samples were then subjected to determination of radioactivity with a liquid scintillation counter.

radioactive bands with the identical Rf values to coprostanol (Rf 0.45; referred as '5β-stanol fr.'), cholesterol (Rf 0.40; referred as 'sterol fr.'), and unknown polar compounds (Rf 0.05 and 0.00). Since the unknown polar compounds were also detected in incubations No. 1 and No. 2, they were assumed to be formed by inorganic process.

The '5β-stanol fr.' was acetylated with dry pyridine-acetic anhydride and then subjected to TLC on Kieselgel G with chloroform. The autoradiograms after TLC gave two radioactive bands corresponding to coprostanyl acetate (Rf 0.80) and cholestenone (Rf 0.45) in incubations No. 3, No. 4, and No. 5. In incubations No. 1 and No. 2, radioactive bands were not observed definitely in the autoradiograms, however the zone corresponding to coprostanyl acetate and cholestenone gave radioactivity slightly by means of liquid scintillation counting. Since the percentage incorporation of cholesterol-4-^{14}C into coprostanol and cholestenone was higher in incubations No. 3, No. 4, and No. 5 than in incubations No. 1 and No. 2, the conversion of cholesterol to both the steroids is suggested to be effected by the microorganism in sediment rather than by inorganic process. To confirm the identity of radioactive coprostanol and cholestenone, the zones corresponding to both compounds were scraped from TLC plates and steroids were eluted with ether for preparative GLC analysis followed by radioactive measurements. The preparative GLC analysis showed that coprostanol and cholestenone were the sole radioactive compounds of their respective fractions (Fig. 1).

The 'sterol fr.' was acetylated and then separated into $\triangle^0$-, $\triangle^7$-, and $\triangle^5$-steryl acetates by using TLC on 10 % $AgNO_3$-Kieselgel G. The autoradiograms after TLC showed radioactive bands corresponding to cholestany (Rf 0.65) and cholesteryl (Rf 0.57) acetates in all incubations. However, the percentage incorporation of cholesterol to cholestanol was higher in incubations No. 3, No. 4, and No. 5 than in incubations No. 1 and No. 2. The results indicated that the hydrogenation of cholesterol to cholestanol was also operated mainly by microbial transformation as well as the formation of coprostanol and cholestenone. The preparative GLC analysis of $\triangle^0$- and $\triangle^5$-steryl acetates from TLC on $AgNO_3$-Kieselgel G revealed that radioactivity was associated with cholestanyl and cholesteryl acetates but not with C_{28}- and C_{29}-steryl acetates (Fig. 1).

On the basis of the above results, we concluded that cholesterol is converted to coprostanol, cholestenone, and cholestanol in the estuary sediment mainly by the action of microorganisms.

Discussion

Steroids have been regarded as a usuful indicator of biological origin of organic matters in recent[15,16] and ancient sediments[17~21] and petroleum[22]. Apart from these studies, coprostanol is also regarded to reflect fecal pollution in water[1~7]. Our previous study[8] has shown that the estuary sediment collected

from the same station as the present study contained coprostanol, cholesterol, and cholestanol in the weight ratio about 2.6: 1.0: 0.7, with suggestion that a part of coprostanol may be formed from cholesterol in the sediment. The present study showed that cholesterol is transformed to coprostanol besides cholestanol and cholestenone. Recently, GASKELL and EGLINTON have also demonstrated by using tracer techniques that cholesterol is hydrogenated to cholestanol and coprostanol in the sediment of Rostherne Mere (Cheshire, England), a highly productive lake, and in the model environment of anaerobic sewage sludge[14]. They have reported that the rate of hydrogenation of cholesterol is approximately 0.5% in 3 months. In the present study, the approximate % conversion rates of cholesterol to coprostanol and cholestanol were 1% and 13%, respectively, in 30 day-incubation (incubation No. 4, see Table 3). These values on % conversion may not always agree with those on % conversion of cholesterol to coprostanol and cholestanol in the estuary sediment under natural environments. However, the results of the present study suggest that the amounts of coprostanol deriving from cholesterol in the sediments should be taken into consideration in evaluation of fecal pollution using coprostanol concentration of sediments. Since both cholestanol and coprostanol are formed from cholesterol in the sediments, it may give available informations on the origin of coprostanol to determine the concentrations of cholestanol and cholesterol and the weight ratio of coprostanol to cholesterol and/or cholestanol.

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