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by M. Kochi

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Studies on the occurrence of 3,11- and 5,11- Eicosadienoic acids in Sea-Urchin lipids*

p.83

By
Masayuki KOCHI

* Contribution from the Shimonoseki University of Fisheries No. 773, received July 8, 1976

Contents.

Introduction p.85

The strong and unique flavour of sea-urchin gonads is greatly appreciated, and they are used as a delicacy among sea-foods. With the rise of eating standard in recent years, the demand for raw sea-urchin and for shiokara (salted sea-urchin) has increased, and they have preogressed from the status of a regional specialty to that of an important luxury food[1,2].

There are many studies available of the composition and components of both raw sea-urchins and salted sea-urhins from the standpoints of dietetics and of food chemistry[3 - 12].

There have also been many studies of the influence of the lipid composition on the quality of the sea-urchins with regard to taste and on the changes of taste during storage[1 - 3, 5, 14 - 18, 22 -30].

The lipid content of the sea-urchin gonads depends on the species, the district, the age, the sex and the degree of ripeness[1 - 3, 5, 14 - 18, 22 - 25].

Muraskiuni (Anthocidaris crassispina)* and Akauni (Pseudocentrotus depressus) contain more lipids than Bafununi (Strongylocentrotus pulcherrimus) and Ezobafununi (Strongylocentrotus intermedius)[1]. Miwa[25] found that the lipid content of the gonads of S. intermedius varied with ripeness, gradually increasing during growth after spawning and reaching a maximum during the rips period. Matsuzaki et al[15] also found that the lipid content of A. crassispina along the coast of Kitaura in Yamaguchi Prefecture increased around March to June.

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* The text and the Tables mix Japanese common names and biological names. A list is therefore added for reference after the English language summary. Translator

There have also been a few studies of the fatty acid composition of sea-urchin lipids[25 - 30]. Toyama et al[26] investigated the fatty acids in the gonad lipids, using S. pulcherrimus as the main source, and reported about 50% of monoethylene unsaturated fatty acids, and about 25% each of saturated fatty acids and of highly unsaturated fatty acids. About 50% of the monoethylene unsaturated fatty acid was oleic acid.

Tsuyuki et al[27] state that 83% of the lipids in A. crassispina were lower unsaturated fatty acids.

Higashi et al[28] compared the fatty acid composition of the gonad lipids of various sea-urchins and found that there was more 14:0 acid in Mexican sea-urchins than in S. pulcherrimus or A. crassispina from Japan, but that there was little C_{20} polyenoic acid.

Miwa[25] also found that the fatty acid composition of S. intermedius was quite different from that of other marine animals, the proportion of 16:1 acid being quite high, with only a small amount of 16:2 present.

Hatakeyama et al[29] found that S. pulcherrimus from Sanriku had a total lipid content of 9.8%, the phospholipids in the lipids being 70%, with 12% of the triglycerides, and that the fatty acid composition was very peculiar with more than 50% of C_{20} or higher acids.

Fujino et al[30] investigated the changes during storage in the lipids in salted sea-urchin made from S S. intermedius and found that the 18:2, 18:4, 20:1, 20:2, acids and the 20:4 or 22:0 and the 22:1 acids could easily be separated during storage.

The results of the analyses of the fatty acids were obtained by using gas chromatography of the methyl esters or of their hydrogenation products to identify the constituent fatty acids and to determine the amounts.

As Miwa[25] and Hatakeyama[29] have found, the lipids in sea-urchins have unique fatty acid compositions which differ from those in other marine animals. Peaks are present in the gas chromatogram retention times which can be considered to be mixed peaks due to the superposition of two or more different types of fatty acids. Thus it is difficult to determine the detailed fatty acid composition solely from the results of gas chromatography of the methyl esters of the specimens. In order to detect the minor components and to obtain the true fatty acid composition, it is necessary to combine gas chromatography with other methods of separation.

The methods available for discrimination between the fatty acids include the low temperature crystallization method[31-34], the urea addition fractionation method[35-40], the lithium salt acetone method[36], the mercury adduct method[34, 51, 42], the counter-current distribution method[43-45], paper chromatography[46-49], column chromatography[50-57], thin layer chromatography[58-72], and collecting gas chromatography[73-77]. However, these methods are in general used in the separating and refining stages of studies of the fatty acid composition, and have only had a small amount of use in the preliminary stages of separation for gas chromatography when the objective has been to determine the fatty acid composition.

Shimma et al[78, 79] used the urea fractionation method to decide the number of double bonds in the component fatty acids in their fatty acid analyses of the back flesh of fish and shellfish. Wolfe et al[80] used the mercury fractionation method to obtain the detailed fatty acid composition of crawfish. Sano et al[40, 81, 84], used the urea fractionation method and silver nitrate impregnation thin layer chromatography for the detailed structure of fatty acids in whale oil, and observed a large number of minor constituents.

The urea fractionation method and the silver nitrate impregnation thin layer chromatography method were investigated for their suitability as the preliminary stages of the analysis by gas chromatography of the fatty acids in sea-urchin lipids. It was verified that silver nitrate impregnation thin layer chromatography was a usable step, and that it could be used in the identification of the fatty acid components. In the analyses it was found that the 11,14 eicosadienoic acid in the sea-urchin lipids is accompanied by a unique organization of a large quantity of eicosadienoic acids. p. 86

A large number of highly unsaturated fatty acids are present in the oil from marine animals, and it is known that these highly unsaturated fatty acids have common structural characteristics[85 - 87]. In those above C_{18} the relative positions of the double bonds are all of the divinylmethane type in which, counting from the end furthest from the carboxyl group, the double bonds are between the 9th, 6th or 3rd carbon atoms and their nearest neighbours on the carboxyl group side. Fatty acids having this structure are classified with respect to fat metabolism as belonging to the oleic acid series, the linoleic acid series or the linolenic acid series[88].

Fatty acids which do not belong to these series are also present in nature[89 - 90]. Fatty acids with special double bond position have particularly been observed in oils from land plants[32, 34, 39, 74, 91 - 99].

The author has extracted eicosadienoic acids with double bond positions not related to the oleic acid series, the linoleic acid series or the linolenic acid series from sea-urchin lipids. There are no other reports of the presence of large amounts of fatty acids with such peculiar structure in the lipids of marine animals and plants.

In addition, the eicosadienoic acid isomers having the 20:2 acid structure have been obtained from the sea-urchins and have been separated and identified by determining the positions of the double bonds. The result has been to establish the presence of two types of 20:2 acid isomers of similar structure in sea-urchin lipids. The first to be found in nature was the 3,11- 20:2 acid, and the second to be established was the 5,11- 20:2 acid, which was not known to be present in marine animals and plants in other than small amounts[100, 101].

Attention was next directed to the distribution of the 20:2 acid isomers in marine animals and plants, and to the proportions in which they are present in the lipids.

This report will discuss the experiments which were made on this subject, and the results which were obtained.

Chapter 1

The analysis of fatty acids in sea-urchin gonad lipids

The fatty acid composition of animal and plant lipids can be analyzed by means of gas chromatography (GLC), but in order to investigate the composition in detail other methods of separation are used in conjunction with GLC[40, 78 - 84].

In comparison with fish oils, the fatty acid composition of the sea-urchin gonad lipids is quite complicated, and it is difficult to obtain an exact composition from the results of GLC analyses alone.

In order, therefore, to investigate the exact fatty acid composition of the sea-urchin gonad lipids, the methyl esters of the specimen materials were first separated according to the number of double bonds, and the fractions so obtained were further resolved by GLC.

SECTION 1

The analysis of fatty acids by the joint use of urea fractionation and gas chromatography

Analysis of fatty acids by urea fractionation is an easy process with the advantage that a large number of samples can be simultaneously handled, and it is widely used as the means for the separation and determination of unsaturated fatty acids[35 - 39]. It is also used an an auxiliary stage in the analysis by GLC of the fatty acids in lipids of complicated composition[40, 78, 79].

The fatty acid composition of the gonad lipids of a number of species of sea-urchins was analysed by combining this method with GLC. p. 87

Experimental methods

The species of sea-urchin gonads used and their habitats are shown in Table 1. All of the sea-urchins were captured during the main fishing season (June to September).

Table 1. Specification of sea-urchin gonad used as raw materials.

Sample No.	Species	Source	Treatment
1	Bafun-uni (*Strongylocentrotus pulcherrimus*)	Kitaura	Shucked in fresh state
2	Murasaki-uni (*Anthocidaris crassispina*)	Kitaura	Shucked in fresh state
3	Kitamurasaki-uni (*S. nudus*)	Hokkaido	Shucked and the gonad was salted prior to storage at 0-3°C for two months
4	Murasaki-uni (*A. crassispina*)	Korea	Shucked and the gonad was salted prior to storage at 0-3°C for twelve months
5	Aka-uni (*Pseudocentrotus depressus*)	Korea	Shucked and the gonad was salted prior to storage at 0-3°C for twelve months

Table 2. Properties of the lipids from sea-urchin gonad.

Sample No.	Saponification value	Iodine value
1	202.8	134.9
2	186.3	103.1
3	188.6	164.2
4	194.0	125.5
5	203.3	150.4

Table 3. Fractionation of fatty acid methyl esters as urea inclusion compounds.

Fraction No.	Reference
1	Crystallized compounds in the incubation at 15-20°C for three hours after the addition of 15 g of urea.
2	Crystallized compounds in the incubation at 15°C for three hours after the addition of 15 g of urea to the fraction obtained from removing fraction 1.
3	Crystallized compounds in the incubation at 15°C for three hours after the addition of 10 g of urea to the fraction obtained from removing fraction 2.
4	Crystallized compounds in the incubation at 15°C for three hours after the addition of 10 g of urea to the fraction obtained from removing fraction 3.
5	Crystallized compounds in the overnight incubation of the filtrate from fraction 4 at 0-1°C.

Table 4. Conditions for gas-liquid chromatography.

Apparatus : SHIMADZU Gas Chromatograph Model GC-1B
Column dimentions : 300×0.4 cm i.d. stainless steel
Solid support : Shimalite W (60/80 mesh)
Stationary phase : Diethylene glycol succinate polyester (10 : 90)
Temperatures : Column, 190°C; Injection, 260°C; Detector, 205°C
Carrier gas : Nitrogen; Flow rate, 60 ml/min; Inlet pressure, 2.0 kg/cm^2; Outlet pressure, atmospheric
Detector : SHIMADZU Hydrogen Flame Ionization Detector Model HFD-1
Flow rate of hydrogen gas : 48 ml/min
Flow rate of air : 1.8 l/min

The gonads of specimens from Kitaura (the area along the Japan Sea coast in the neighbourhood of Shimonoseki) were used in the fresh condition. An appropriate quantity of salt was added to the gonads of sea-urchins from Hokkaido and Korea immediately after capture, and after a small amount of water had been removed they were placed in containers and frozen for two to twelve months before being used.

The extraction of the lipids

The soluble lipids were extracted from the sea-urchin gonads by acetone and ethyl ether, and were used as the lipid specimens. The saponification values and the iodine values of the lipids obtained are shown in Table 2.

Preparation of fatty acid methyl esters

The fatty acids were saponified, and after the unsaponified matter was removed the mixed fatty acids were obtained by hydrolysis. They were added to a 3% solution of hydrogen chloride in methanol and refluxed for one hour at about 75% to prepare the methyl esters of the fatty acids.

Methyl esters hydrogenation

1.0 g of methyl ester sample was dissolved in n-hexane and hydrogenated with platinum black as a catalyst[102].

Urea fractionation of the methyl esters

15.0 g of the specimen methyl esters were dissolved in 150 ml of anhydrous methanol, and fractionated with urea according to the method of Sano[48]. Five fractions were separated as shown in Table 3. p. 88

Fatty acid analysis

The conditions for GLC are shown in Table 4. In order to identify the fatty acids use was made of the straight line relation[103] between the number of carbon atoms and the logarithm of the retention times of standard fatty acids or their comparative or relative retention times (with the time for stearic acid as unity).

The standard acids used were the commercial methyl esters of the 12:0, 14:0, 16:0, 18:0, 20:0, 22:0, 18:1ω9, 18:2ω6. 18:3ω3, and 22:1ω9 acids, and also the methyl esters of the 18:4ω3, 20:3ω3, 20:4ω3, 20:5ω3, 22:5ω3, and 22:6ω3 acids present in marine fish oils. The quantitative determination of the fatty acids was made from the peak area (by the half-width method) and the percentage area expressed as the quantitative percentage. The number of double bonds in the individual fatty acids methyl esters was estimated from the gas chromatograms of the fractions obtained by urea fractionation, and the identifications and the quantitative amounts were obtained from the exact retention times.

Results of experiments

The gas chromatograms obtained from the methyl esters of fatty acids in sea-urchin lipids had characteristics quite different from those obtained from fish oils. All components with retention times up to 50 minutes were observed, but peaks corresponding to highly unsaturated C_{22} acids and to acids with 23 or more carbons were not observed.

A large number of peaks corresponding to medium fatty acids were observed, but these peaks were believed to be mixed peaks resulting from the combination of several fatty acids having almost identical retention times. It was therefore difficult to obtain a detailed identification or quantitative analysis of the specimen methyl esters from the gas chromatograms alone. The specimen methyl esters were therefore separated by urea fractionation and the fractions obtained were analyzed by GLC.

The chromatograms of the fractions obtained by urea fractionation of the methyl esters of fatty acids from the lipids of S. nudus are shown in Figure 1. Peak A

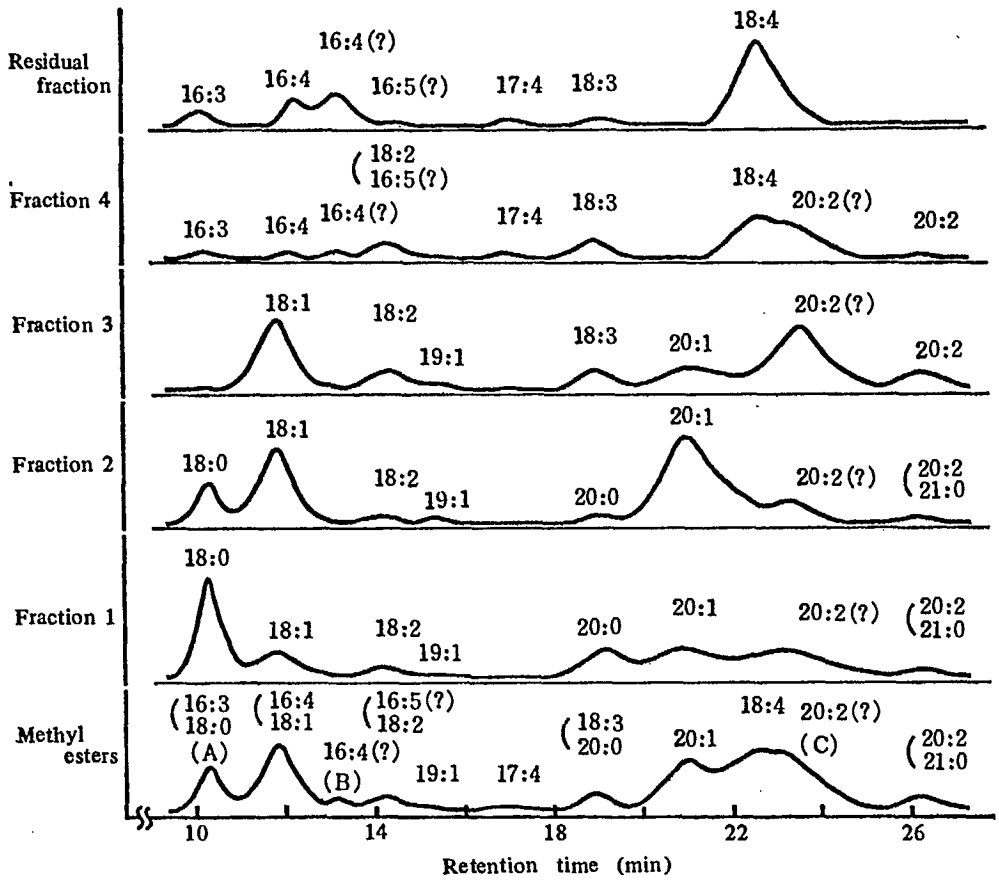

Fig. 1. Gas chromatograms of fatty acid methyl esters from *S. nudus* and of their fractions separated by urea fractionation.

is largest in fraction 1 and in the residual fraction, so that it is evidently a mixed peak consisting of two components of almost identical retention times which are identified as the 18:0 acid to be observed in fractions 1 and 2, and as the 16:3 acid in fraction 4 and the residual fraction. This procedure is used to identify the fatty acids by combining the observations of the variation of the other peaks in the chromatograms between the various fractions separated by urea fractionation.

Peak B could not be identified even by this process. The peak has exactly the retention time appropriate to the 19:0 acid, but in urea fractionation it does not behave as a saturated fatty acid and appears to be a trienoic or tetraenoic acid. However, no fatty acid is known to correspond to this peak. It was supposed to be any one of the isomers of 16:4 or of 17:3 or to be oxyacid of 16:4. According to the quantitative value of the hydrogenated compound it is not a C_{17} acid, so it is supposed to be a C_{16} acid. Iverson et al[37] and Schlenk[38] have shown that it is more difficult to form urea fractionation products from branched chain fatty acids or oxyacids than from straight chain acids, and in regard to urea fractionation the behaviour of this peak is similar to that of the 16:4 acid. It is therefore concluded that the peak B component is a double bond position isomer of the 16:4 acid[104 - 106]. This 16:4 peak was found only as a small peak in S. nudus. There was no distinct peak with P. depressus though a very small quantity was found in the urea fractionation chromatogram. It was not present in the other sea-urchins.

Similarly, an apparently distorted peak which p.89
seems to be a superposition of two components is observed between the 20:1 and the 20:2 peaks. This peak (C) appears

Table 5. Fatty acid composition of the lipid from sea-urchin gonad (% of total fatty acid methyl esters).

Fatty acid	Sample No.					Fatty acid	Sample No.				
	1	2	3	4	5		1	2	3	4	5
12 : 0	0.1	0.1	Trace	0.1	Trace	18 : 0	2.1	2.2	2.2	2.3	2.3
12 : 4	0.1	0.2	0.2	0.1	0.2	18 : 1	5.8	6.5	4.6	7.3	6.1
13 : 1	0.1	Trace	0.1	0.2	0.1	18 : 2	1.7	0.8	1.7	1.4	0.8
14 : 0	8.2	15.9	6.9	11.4	8.3	18 : 3	1.5	2.5	2.7	0.9	1.6
14 : 1	0.2	0.7	0.3	0.5	0.4	18 : 4	8.1	5.0	6.3	5.2	6.6
14 : 2	Trace	Trace	Trace	Trace	0.1	18 : 5(?)	1.6	0.4	1.3	0.7	0.8
14 : 3	0.1	Trace	Trace	0.1	0.2	19 : 1	0.2	Trace	Trace	0.2	0.1
14 : 4(?)	0.7	0.7	0.6	0.7	0.6	19 : 4	0.7	0.7	0.8	0.8	0.7
15 : 0	0.3	0.3	0.2	0.4	0.2	20 : 0	5.2	2.3	3.0	2.4	2.8
15 : 1	0.1	Trace	0.1	0.1	0.1	20 : 1	5.3	10.1	6.3	7.8	8.5
16 : 0	16.7	16.5	14.2	16.5	15.5	20 : 2(?)	5.5	8.8	6.7	8.0	5.3
16 : 1	3.0	4.1	4.9	4.6	4.5	20 : 2	0.2	0.9	0.3	0.8	0.8
16 : 2	0.2	0.1	0.2	0.2	0.2	20 : 3	7.4	5.8	8.1	7.9	10.3
16 : 3	0.8	0.7	0.4	0.6	0.7	20 : 4	2.7	2.0	2.1	1.7	1.7
16 : 4	4.2	0.9	0.5	0.8	0.2	20 : 5	9.5	5.4	16.2	8.6	11.8
16 : 4(?)	—	—	0.6	—	0.2	21 : 0	Trace	Trace	0.3	Trace	Trace
16 : 5(?)	0.7	0.6	0.4	0.4	0.8	21 : 1	1.0	1.2	1.2	1.3	1.0
17 : 0	0.1	0.1	0.1	0.2	0.1	22 : 0	1.2	0.6	0.9	1.3	1.1
17 : 1	0.1	0.1	0.2	0.1	0.2	22 : 1	3.1	2.2	2.5	2.7	2.7
17 : 4	0.2	0.3	0.3	0.4	0.3	22 : 2	1.3	1.3	2.6	1.3	2.1

Table 6. Proportion of fatty acid methyl esters from sea-urchin gonad (% of total fatty acid methyl esters).

Sample No.	Based on unsaturation			Based on chain length		
	Saturated acids	Monoenes	Polyenes	C_{12}-C_{14} acids	C_{15}-C_{18} acids	C_{20}-C_{22} acids
1	33.9	18.9	47.2	9.5	48.1	42.4
2	38.0	24.9	37.1	17.6	41.8	40.6
3	27.8	20.2	52.0	8.1	41.7	50.2
4	34.6	24.8	40.6	13.1	43.1	43.8
5	30.3	23.7	46.0	9.9	42.0	48.1

in urea fractionation fraction 1 and is largest in fraction 3, but there is no fatty acid peak known to correspond to it. From the amount of hydrogenation product it is thought to be a C_{20} acid, and its behaviour in urea fractionation is analogous to that of 20:2, so it is inferred to be a cis-trans or position isomer of the 20:2 acid.

The behaviour in urea fractionation was used with this process to determine or to infer the component fatty acids in the peaks of the methyl esters and to measure the amounts. The fatty acid compositions of the sea-urchin gonads so found are shown in Table 5. Some 30-40 fatty acids were found to be included in the make-up of the fatty acids in the sea-urchin gonads. The most important of them are the 14:0, 16:0, 18:1, 18:4, 20:1, 20:3, and 20:5 acids. Also a fatty acid estimated to be an isomer of acid 20:2 was one of the fatty acids with a main composition. Also observed is a large number of C_{14}, C_{16}, C_{18}, and C_{20} acids of other degrees of unsaturation or of other structure. There were no striking differences in the fatty acid components or the percentage composition which depended on the species or the habitat. However, there are small differences in the proportions of each fatty acid. The amounts of 16:4, 18:4, and 20:0 acids in S. pulcherrimus from Kitaura are greater than in other sea-urchins. The amounts of 14:0 and 20:1 acids were high and the content rate of 20:5 acid was low in A. crassispina from Kitaura. A. crassispina from Hokkaido has a high proportion of 20:5 acid and a low proportion of 14:0 acid.

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Table 6 shows for comparison the results of classifying the component fatty acids according to the degree of unsaturation or to the carbon chain length. Although C_{22} polyenoic acids are not found in the sea-urchin gonad lipids, there are large amounts of polyenoic acids with more than 20 carbon atoms. In particular in S. nudus the proportion of such fatty acids is more than 50%. The proportion of monoenes is about 20% in all cases, which is low when compared with the results of Toyama et al[26] and Tsuyuki et al[27]. The composition of A. crassispina from Korea

and Kitaura are similar, but the compositions of the other sea-urchins are slightly different.

Discussion p. 91

Miwa[25] found 17 types of fatty acids among the components of sea-urchin gonad lipids, and Hatakeyama et al[29] found 23, but by using urea fractionation jointly with GLC in the analysis, 39 or 40 fatty acids have been identified or inferred. It has thus been possible to separate the component fatty acids in mixed peaks and, by detecting the minor components, to obtain the fatty acid composition in more detail.

The principal fatty acids in the sea-urchin lipids are the 14:0, 16:0, 18:1, 18:4, 20:1, 20:3, and 20:5 acids, and in addition it has been inferred that an important component fatty acid is an isomer of the 20:2 acid. There has been little report of the presence of such an isomer in the lipids of marine animals or plants. Kikuchi[107] reports the presence of a small amount of what may be 20:2 acid in shako* from Tokyo Bay, but it is not known whether this is the same isomer.

Comparison of sea-urchin lipids with fish oils shows that there is a higher proportion of 14:0, 18:4, 20:1 and 20:3 acids, and a lower proportion of 18:0, 18:1, and 22:1 acids. Ueda[108] analysed the fatty acid composition of 33 types of marine fish oils and classified them as of Tuna type (Thunnus), Horse Mackerel type (Trachurus), and Mackerel type (Scomber), but the fatty acid composition of sea-urchin lipids does not fit into any of these types. It is also quite different from the pattern found by Shimma et al[79] to be typical of the component fatty acids of marine Teleostei.

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* Shako, see page 139

The fatty acid composition of the ovaries in marine fish is similar to that in the flesh lipids[109, 110]. The fatty acids of the same gonads in the sea-urchin is therefore quite different from that in fish eggs.

The fatty acid composition in seaweed lipids varies greatly according to the species[111, 112], but the fatty acid composition of the sea-urchin is quite different again. However, the algae and the sea-urchins agree in the absence from the lipids of C_{22} polyenoic acids and of fatty acids with more than 22 carbon atoms. The sea-urchin lipids are also remarkable for high proportions of 14:0, 16:0, 18:1, 18:4, 20:3, and 20:5 acids, whatever the species.

Sea-urchin lipids also differ considerably in fatty acid composition from shellfish lipids[78, 113], and from plankton lipids[114 - 118].

Sea-urchins tend to be omnivorous, but they are principally vegetarian, feeding on seaweeds[119, 120]. One may therefore suppose that the sea-urchin gonad lipids will be affected by the seaweed lipids, though the fatty acid composition is different in character from that of marine animal lipids or seaweed lipids.

Section 2

Analysis of fatty acids by the joint use of silver nitrate impregnation thin layer chromatography and gas chromatography

The previous section described the joint use of urea fractionation and GLC for analysis, and the fractions obtained were not all satisfactorily resolved. An alternative to the urea fractionation method for separating

fatty acids according to the degree of unsaturation is the use of silver nitrate impregnated thin layer chromatography (argentation TLC). This method is based on the formation of complexes between silver ions and unsaturated compounds, and it is widely used as a stage in the separation and identification of unsaturated fatty acids [59, 62, 67 - 69, 72].

This section describes an analysis of the fatty acids in sea-urchin lipids in which argentation TLC is used as a method of treatment preliminary to GLC.

Experimental methods

Extraction of lipids

An appropriate amount of salt was added to the gonads of A. crassispina, a small amount of water was removed and they were placed in barrels and kept in frozen storage for ten months. The acetone-soluble lipids were extracted from the gonads, three times the quantity of ethyl ether was added to extract the lipids three times at room temperature. The non-polar lipids so obtained were again extracted with five times the quantity of acetone, and then used as the specimen lipids.

Preparation of fatty acid methyl esters[121]

The specimen lipids were added to 10 times the amount of a 3% solution of hydrogen chloride in methanol and refluxed for four hours at 75°C in order to prepare the fatty acid methyl esters.

Identification of the fatty acid methyl esters

Silica gel TLC[58, 61] was used. A plate of silica gel G (made by Merck) 600 μ thick and 20 x 20 cm was prepared, and activated by heating to 110°C for 60 minutes. A strip of 80 $m\ell$ of the methyl esters was placed on the plate and developed with petroleum ether - ethyl ether - acetic acid (90 : 10 : 1 v/v). After air current drying

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it was sprayed with 2', 7' - dichlorfluorescein, the bands were located under ultraviolet light, and the methyl ester fractions were separated and used as the specimen methyl esters.

Fractionation by argentation TLC[40, 67]

A plate 600μ thick and 20 x 20 cm was prepared by adding 60ml of a 12,5% (w/v) solution of silver nitrate to 20g of silica gel G. It was activated by heating to 110°C for 90 minutes. A strip of 80 mℓ of the specimen methyl esters was placed on the plate and developed in a dark place with n-hexane - ether - acetic acid (70 : 30 : 1, v/v). After air current drying it was sprayed with dichlorfluorescein and the bands were located. It was divided into seven fractions.

Analysis of the fatty acids

The conditions of GLC and of identification and measurement of the fatty acids were the same as in Section 1. Additional standard fatty acids were used as the methyl esters made by Applied Science Lab, and were 20:2ω6, iso-14:0, iso-16:0, iso-18:0, iso-20:0, anteiso-15:0, anteiso-17:0, anteiso-19:0 and anteiso-21:0. In addition, attention was given to the end carbon chain (ECC or ω)[103] and the separation factor (SF)[122] in inferring the position of the double bonds in the unsaturated fatty acids.

Experimental results

Figure 2 shows a chromatogram obtained by argentation TLC from the fatty acid methyl esters of the non-polar lipids in sea-urchin gonads. The specimen methyl esters were separated into more than 10 bands, but they were divided into 7 fractions by joining neighbouring bands together. The fractions were numbered 1, 2, 3 .... in order according to the size of the R_f value, the band with

the smallest value of R_f being joined with the origin as fraction number 7.

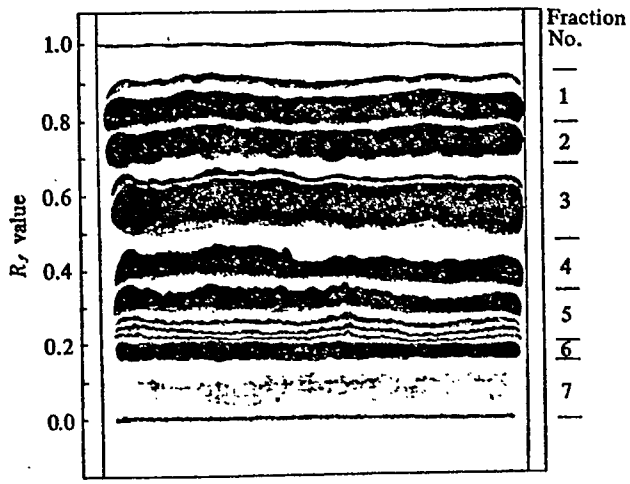

Fig. 2. Thin-layer chromatogram of fatty acid methyl esters on silica gel impregnated with silver nitrate. Developer: *n*-hexane–ethyl ether–acetic acid (70 : 30 : 1, v/v)

Figure 3 shows a gas chromatogram of each of the methyl ester fractions obtained by argentation TLC from the specimen methyl esters. 29 peaks were obtained in the specimen methyl ester chromatograms. Since peaks numbered 12, 15, 20, and 21 are to be discussed, the chromatograms are reproduced in the Figure up to peak 22.

Peak 12 is found in all the argentation TLC fractions except fraction 4. Since peak A is found in fractions 1, 2, and 3 with exactly the same retention time, it is taken as the peak for the same fatty acid in each fraction, and is identified from the relation between carbon chain length and retention time shown in Figure 4 to be a 17:1 acid. In fraction 1, this peak can be seen to have a small peak B on the trailing edge. This peak remains present in chromatograms of the hydrogenation

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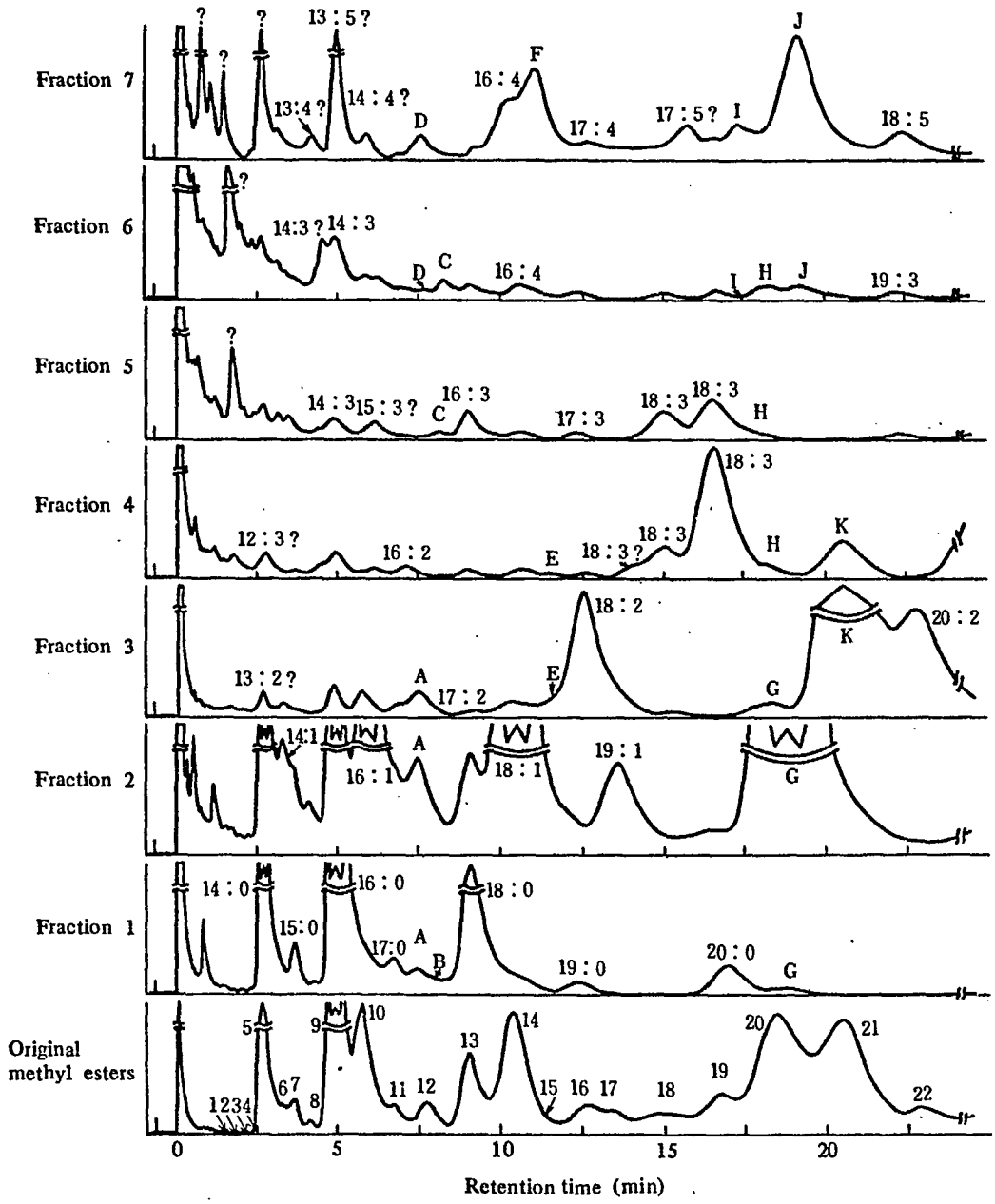

Fig. 3. Gas chromatograms of fatty acid methyl esters fractionated into homologues by thin-layer argentation chromatography.

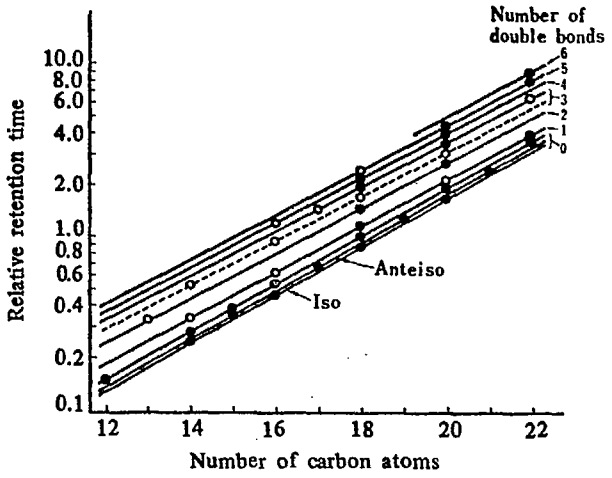

Fig. 4. Relative retention time plotted against the number of carbon atoms of fatty acids.
●: authentic methyl esters
○: methyl esters separated from samples

products of the specimen methyl esters, and since it has a relative retention time which agrees with that of iso-18:0 acid, peak B is identified as iso-18:0 acid. Peak C is observed in fractions 5 and 6, and is present in quantity in fraction 5 which principally contains trienoic acids. From the relative retention time it is inferred to be a 16:3 acid, but it does not lie on the line connecting the trienoic (ω3) acids and is located centrally between the lines for the dienoic (ω6) acids and the trienoic (ω3) acids. It is known that 16:3ω4 acid and 16:3ω6 acid are present in marine animals and plants[85]. From the relation between retention time and the ECC in the polyester column[122, 123], and from the SF relative to the 16:3ω4 acid, peak C is identified as 16:3ω6 acid. Several fatty acids other than peak 12 were found to be located centrally between the dienoic acid and the trienoic acid lines. When these fatty acids were joined, a straight line was obtained (the dashed line in Figure 4) which was exactly parallel to the

trienoic acid ($\omega 3$) line and contained the 16:3ω6 acid. Thus fatty acids lying on this straight line are identified as trienoic acids of the ($\omega 6$) series. In fraction 6, 16:3ω6 acid is already known to be present, and the fatty acid of peak D which appears distinctly in fraction 7 was inferred from its behaviour in argentation TLC to be a tetraenoic acid. However, in the relation between carbon number and retention time, it lies somewhat below the tetraenoic ($\omega 3$) acid line. For this reason peak D was inferred to be a double bond isomer of 15:4 acid. Thus peak 12 is a mixed peak containing four types of fatty acids, 17:1 acid, iso-18:0 acid, 16:3ω6 acid and a 15:4 acid isomer.

Peak 15 is found in argentation TLC fractions 3, 4, and 7. Of these, Peak E which appears in fractions 3 and 4 in a position before 18:2ω6 acid, is inferred to be a dienoic acid and from the SF with respect to 18:1ω9 and 18:2ω6 acids it was identified as 18:2ω9 acid. Peak F, which appears in fraction 7, could be a tetraenoic acid or a pentaenoic acid, but it is located halfway between the tetraenoic ($\omega 3$) line and the pentaenoic ($\omega 3$) line in the retention time-carbon number diagram. From the relation between ECC and retention time, and from the SF with respect to 16:4ω3 acid, peak F was identified as 16:4ω1. This fatty acid is known to be widely distributed in marine animals and plants[85]. Thus peak 15 was recognized to be a mixed peak containing two fatty acids, 18:2ω9 and 16:4ω1. p. 95

Peak 20 is found in all the argentation TLC fractions. Peak G occurs in fractions 1, 2 and 3, peak H in fractions 4, 5 and 6, and the two peaks I and J in fractions 6 and 7. Of these, peaks G and J are identified from their retention times as 20:1ω9 and 18:4ω3. Since

peak H appears in the fraction which contains most of the trienoic acids, it was inferred to be 19:3 acid, but its retention time did not agree with either the 19:3ω3 or the 19:3ω6 acid. Since no peak for a 19:0 branched chain fatty acid appeared in the chromatogram of the hydrogenation products of the specimen methyl esters, unsaturated C_{19} branched chain acids are not thought to be present. For this reason, it was inferred that peak H was an isomer of 19:3 acid. Because peak I appears in fractions 6 and 7 it was thought to be a tetraenoic acid, and was inferred from its retention time to be 18:4. However, from the SF with respect to 18:4ω3 and 18:3ω6 it is not thought to be 18:4ω6. Peak I was therefore inferred to be an isomer of 18:4 acid. Accordingly, peak 20 is considered to be a mixed peak containing four types of fatty acids, 20:1ω9, 18:4ω3, 19:3 isomer and 18:4 isomer.

Peak 21 corresponds to the peak which in the preceding section was inferred from its behaviour in urea fractionation to be a 20:2 acid isomer. It also appears as peak K in the argentation TLC fraction 3 which contains the largest proportion of the dienoic acids. Moreover, according to the relative proportion of the fatty acids, it reached 50% of the total fatty acids in fraction 3. This peak K is located centrally between the monoenoic (ω9) acid line and the dienoic (ω6) acid line in the retention time - carbon number diagram but from its SF with respect to 20:1ω9 and 20:2ω6 it is not thought to be 20:2ω9 acid. Moreover, since no peak for a 20:0 branched chain fatty acid was found in the chromatogram of the hydrogenation products of the specimen methyl esters, it is not thought to be a C_{20} unsaturated branched chain fatty acid. Thus peak 21 is inferred to be a double bond position isomer of 20:2 acid (20:2 acid isomer*).

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* In Chapter 4 of this paper it is shown to be a mixture of 3,11- and 5,11- eicosadienoic acids, whose structure was determined.

The fatty acid components of mixed peaks other than those discussed above were identified or inferred by consideration of the fractions into which they were separated by argentation TLC. The results are shown in Table 7. Since the peaks detected in the gas chromatograms of the specimen methyl esters were nearly always mixed peaks containing two or more types of fatty acid, a total of 76 types were detected among the fatty acids forming the sea-urchin gonad lipids.

The separation of the fatty acids into the argentation TLC fractions on the basis of the degree of unsaturation was obtained from Table 7 and is shown in Table 8. The monoenoic and dienoic acids were principally present in fractions 1, 2 and 3, and the trienoic acids in fractions 4 and 5. The pentaenoic acids were present in fraction 7 but since there was not much tetraenoic acid in the sea-urchin lipids no fraction was found to consist principally of tetraenoic acids. However, since the proportion of tetraenoic acid was greatest in fraction 6, they can be considered to be present in this fraction.

Next, the results of quantitative measurements of each fatty acid are shown in Table 9. In the mixed peaks, the exact retention times for the individual peaks of the component fatty acids obtained in the gas chromatograms of each of the fractions separated by argentation TLC and the proportion contained in each fraction were obtained. By forming a reference diagram of these proportions, the percentages of each individual fatty acid was determined. The main fatty acid components of the sea-urchin non-polar lipids were 14:0, 16:0, 18:1ω9, 20:1ω9, 20:2 acid isomer, 20:3ω3 and 20:5ω3. In addition a large number of branched chain fatty acids, of double bond position isomers of

Table 7. Gas chromatographic analyses of fatty acids from sea-urchin gonad fractionated into homologues by thin-layer argentation chromatography.

Peak No.	Fatty acid	Relative retention time	Fraction No. 1	2	3	4	5	6	7
1	12 : 0	0.15	0.2	0.1					
2	12 : 1	0.18	0.1	0.1					
3	13 : 0	0.21	0.1	Trace					
4	13 : 1	0.24	0.1	Trace					
5	14 : 0	0.28	18.2	5.3	0.7				
	12 : 3 ?	0.28				0.9	2.1	1.1	
	13 : 2 ?	0.33			0.3	0.3	2.4		
6	14 : 1ω7 Anteiso	0.34	0.1	0.8					
	15 : 0	0.35	0.8						
	12 : 4 ?	0.36						0.8	0.1
	13 : 3 ?	0.37					1.5	0.6	
7	15 : 0	0.38	2.1	0.5					
	14 : 2ω9	0.39			0.2	0.2			
	13 : 3 ?	0.41					Trace	0.4	
	13 : 4 ?	0.45						Trace	0.1
8	15 : 1	0.46		0.4					
	16 : 0 Iso	0.47	0.4						
	14 : 3 ?	0.49				0.6	0.7	1.3	
	14 : 3ω6	0.52				1.1	1.9	1.5	
9	16 : 0	0.53	52.0	9.5	1.1				
	13 : 5 ?	0.55							1.0
	14 : 4 ?	0.62						0.7	0.3
10	16 : 1ω9	0.63	0.7	12.9	1.4				
	17 : 0 Anteiso	0.66	1.0						
	15 : 3 ?	0.67				0.6	3.1	0.6	
	17 : 0	0.72	2.1	0.8					
11	16 : 2ω7	0.74			0.4	0.8			
	15 : 4 ?	0.75						0.4	0.2
	17 : 1	0.81	1.2	1.6	1.0				
12	15 : 4 ?	0.83						0.3	0.3
	18 : 0 Iso	0.88	0.5						
	16 : 3ω6	0.94					1.1	0.9	
	16 : 3ω4	0.99				0.6	6.4	0.5	
13	18 : 0	1.00	12.7	1.8					
	17 : 2ω8	1.01			0.2	Trace			
	16 : 4 ?	1.09						Trace	0.2
	18 : 1ω9	1.14	1.6	18.5	1.2				
14	17 : 3 ?	1.18				0.8	1.1	0.3	
	16 : 4ω3	1.21						0.7	0.7
	16 : 4ω1	1.26							1.0
15	18 : 2ω9	1.34			1.4	0.4			
	19 : 0	1.37	1.5	0.5					
	17 : 4ω6	1.42						Trace	0.1
16	17 : 3ω3	1.43					0.7	0.3	
	18 : 2ω6	1.44			12.0	0.4			
	19 : 1	1.52		2.7	1.1				
17	18 : 3 ?	1.60				1.2			
	18 : 3ω6	1.70				3.1	8.9	0.4	
18	19 : 2 ?	1.70			0.3	Trace			
	17 : 5 ?	1.72							0.4
	17 : 5 ?	1.85							0.2
19	20 : 0	1.87	3.7	0.7					
	18 : 3ω3	1.93				14.8	12.5	0.7	
	18 : 4 ?	2.00						Trace	0.5
20	20 : 1ω9	2.09	0.9	32.0	1.6				
	19 : 3 ?	2.10				1.4	1.9	1.2	
	18 : 4ω3	2.20						1.2	2.2
21	20 : 2 ?	2.34			48.6	5.0			
	18 : 5ω3	2.44							0.6
22	19 : 3ω3	2.60					Trace	0.4	
	20 : 2ω6	2.60			15.1				
	19 : 4 ?	2.72						Trace	0.1
23	20 : 3ω9	2.81				15.1	3.1		
	19 : 4ω3	2.89						0.5	
	21 : 1	2.91		4.5	Trace				
	20 : 3ω6	3.03				19.2	2.0		
24	21 : 2 ?	3.16			1.0	Trace			
	20 : 3ω3*	3.45				33.5	50.6	67.7	0.3
25	20 : 4 ?	3.70						Trace	0.3
	22 : 1ω9	3.88		7.3	2.6				
26	20 : 4ω3	3.90						16.6	0.5
	22 : 2ω9	4.33			9.8				
27	20 : 5ω3	4.40							87.8
28	21 : 4ω5	5.18						Trace	1.1
	21 : 5ω2	6.10							2.0
29	22 : 3ω3	6.26						0.9	

Figures in fatty acid composition (the fourth column) indicate the percentage of total fatty acid methyl esters in individual fractions.

*May include 20 : 4ω6 acid

Table 8. Percentage distribution of fatty acid methyl esters fractionated into homologues on the basis of the degree of unsaturation.

Degree of unsaturation	Fraction No.						
	1	2	3	4	5	6	7
0	95.3	19.2	1.8				
1	4.7	80.8	8.9				
2			89.3	7.1	2.4		
3				92.9	97.6	78.8	0.3
4						21.2	7.7
5							92.0

Table 9. Fatty acid composition of non-polar lipid from sea-urchin gonad (% of total fatty acid methyl esters).

Fatty acid	Hydrogenated	Unhydrogenated
12 : 0	0.1	0.1
12 : 1		Trace
12 : 3 ?		0.1
12 : 4 ?		Trace
13 : 0	0.1	Trace
13 : 1		Trace
13 : 2 ?		0.1
13 : 3 ?		0.1
13 : 3 ?		Trace
13 : 4 ?		Trace
13 : 5 ?		Trace
14 : 0	7.9	6.2
14 : 1ω7		0.3
14 : 2ω9		Trace
14 : 3 ?		Trace
14 : 3ω6		0.5
14 : 4 ?		0.1
Anteiso 15 : 0	0.4	0.4
15 : 0	0.8	0.6
15 : 1		Trace
15 : 3 ?		0.3
15 : 4 ?		Trace
15 : 4 ?		Trace
Iso 16 : 0	0.2	0.2
16 : 0	19.7	15.2
16 : 1ω9		3.6
16 : 2ω7		0.1
16 : 3ω6		0.2
16 : 3ω4		0.6
16 : 4 ?		Trace
16 : 4ω3		Trace
16 : 4ω1		Trace
Anteiso 17 : 0	0.2	0.2
17 : 0	0.9	0.6
17 : 1 ?		0.5
17 : 2ω8		Trace
17 : 3 ?		0.1
17 : 3ω3		Trace
17 : 4ω6		Trace
17 : 5 ?		Trace
17 : 5 ?		Trace
Iso 18 : 0	0.1	0.2
18 : 0	15.9	3.4
18 : 1ω9		5.7
18 : 2ω9		0.3
18 : 2ω6		2.0
18 : 3 ?		0.2
18 : 3ω6		1.3
18 : 3ω3		2.1
18 : 4 ?		Trace
18 : 4ω3		0.7
18 : 5ω3		Trace
19 : 0	1.4	0.4
19 : 1		0.7
19 : 2 ?		Trace
19 : 3 ?		0.3
19 : 3ω3		Trace
19 : 4 ?		Trace
19 : 4ω3		Trace
20 : 0	43.7	1.1
20 : 1ω9		9.9
20 : 2 ?		9.9
20 : 2ω6		2.9
20 : 3ω9		1.5
20 : 3ω6		1.0
20 : 3ω3*		9.5
20 : 4 ?		Trace
20 : 4ω3		1.4
20 : 5ω3		6.8
21 : 0	2.2	—
21 : 1		1.3
21 : 2 ?		0.1
21 : 4ω5		0.2
21 : 5ω2		0.6
22 : 0	6.4	—
22 : 1ω9		2.9
22 : 2ω9		3.5
22 : 3ω3		Trace

* May include 20 : 4 ω 6 acid

unsaturated fatty acids, and of highly unsaturated fatty acids with add numbers of carbon atoms was detected.

Branched chain fatty acids have been reported to occur in the muscle oil of the porbeagle (Lamna)[124], in White Spotted Dolphin oil (Stenella attenuate)[125], in cod liver oil[126] and in whale oil[40, 81, 82, 127]. In the present experiments the quantities in the argentation TLC fraction 1 or in the hydrogenation products of the specimen methyl esters were very small, but iso-16:0, iso-18:0, anteiso-15:0 and anteiso-17:0 acids were shown to be present.

It is also known that double bond position isomers of the unsaturated fatty acids occur in a large number of marine plants and animals. These include C_{16} and C_{18} from monoenoic to pentaenoic acids, C_{20} from monoenoic to pentaenoic acids, and C_{22} monoenoic acids and pentaenoic acids[85, 87, 106, 128]. The sea-urchin lipids were found to contain, in addition to these acids, small quantities or traces of isomers of C_{13}, C_{14}, C_{17}, C_{18} and C_{19} trienoic acids, C_{16}, C_{18}, C_{19}, and C_{20} tetraenoic acids and C_{17} pentaenoic acid isomers. It was also particularly noticeable that there was about 10% of 20:2 acid isomers. No other report has been seen of these isomers as important component fatty acids in marine animals or plants.

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Highly unsaturated fatty acids of odd carbon number already known to occur are 17:4, 19:4, 19:5, 21:4, 21:5 and 23:5 acids[40, 89, 126, 127, 129 - 132]. In the present experiments small quantities or traces of 17:4, 17:5, 19:4, 21:4 and 21:5 acids were detected. Of these, 21:5 acid, which is believed to be widely distributed in marine animals and plants, was present in the greatest amount, the proportion being 0.6%.

Discussion

The sea-urchin lipids were well separated according to the degree of unsaturation by argentation TLC. By means of gas chromatography of each of the fractions so obtained the component fatty acids in the mixed peaks present in the gas chromatograms of the specimen methyl esters were individually separated and identified.

This resulted in the identification of a total of 76 types of fatty acids including those present in small quantity or as traces, and those formerly unknown. Some of them including the iso-18:0, 16:3ω6 and 18:2ω9 acids, and the fatty acid isomers 15:4, 18:4, 19:3 and 20:2 acids, were hardly recognized as present in marine animals and plants. In particular it was noticed that a fairly large amount of 20:2 acid isomers was present.

It was also shown that TLC was superior to urea fractionation as a method of separating unsaturated fatty acids and small quantities or trace components could be well detected in mixed peaks. It was also concluded that argentation TLC was an effective method for use in the preliminary separation stage of the analysis by GLC of lipids containing complex components.

Summary

The joint use of urea fractionation or of argentation TLC with GLC was investigated as a means of analyzing the fatty acids in sea-urchin gonad lipids.

The urea fractionation method had the advantage p. 99 that a large number of specimens could be simultaneously processed, but it was by no means always satisfactory in separation according to the degree of unsaturation. It was therefore not to be considered as an effective separating stage in the analysis of fatty acids.

On the other hand, argentation TLC makes a good separation of fatty acid methyl esters according to degree of unsaturation, and had the advantage that very small quantities of specimens could be handled in a short time. It was therefore concluded to be the superior method to use as the preliminary separation stage of the analysis of fatty acids by GLC. By the use of this method, a total of 76 types of fatty acids were identified as components of the sea-urchin lipids, including those present in small quantities or as traces, and a number of types of fatty acids which were hardly known as components of marine animal or plant lipids. It was possible to obtain the exact fatty acid composition. It was particularly worthwhile to note that there was a considerable quantity of 20:2 isomers.

Chapter 2
The separation of the eicosadienoic acid isomers forming part of the sea-urchin lipids

In the previous chapter, the detailed fatty acid composition of the sea-urchin gonad lipids was investigated by means of the joint use of urea fractionation or argentation TLC with GLC. It was found that about 5% to 10% of all the sea-urchin lipids used as specimens consisted of fatty acids with retention times on the polyester column rather less than that of the 11, 14- 20:2 acid, which were taken to be 20:2 acid isomers.

Next, the composition of the lipids in the gonads and the viscera was investigated and also the fatty acid composition of each component lipid, in order to elucidate the distribution of these isomers among the lipid components.

Experimental methods

Specimens

Use was made of the gonads and of the viscera other than the gonads (to be called simply the viscera) of S. pulcherrimus from Hokkaido. The weights and the lipid contents of the gonads and viscera obtained are shown in Table 10.

Table 10. Description of the sample examined.

Number of individuals	Testa diameter (cm)	Total weight (g)	Gonad		Viscera*	
			Weight (g)	Lipid content (%)	Weight (g)	Lipid content (%)
116	2.2–3.7	1148	126.5	4.4	125.3	1.8

* Excluded gonad

The extration and separation of the lipids

The total quantities of the lipids (TL) were extracted separately from the gonads and the viscera by the method of Folch[133], 2 g of the TL obtained was weighed out, and injected into a 2 x 14 cm column made by suspending silicic acid (Mallinckrodt manufacture, 100 mesh, heat activated for two hours) in chloroform. The non-polar lipids (PL) were eluted with repeated use of 350 ml of methanol. Next the method of Dittmer et al[134] was used to separate the non-polar lipids into free fatty acids (FA) and neutral lipids by means of a sodium carbonate solution. One gram of the neutral lipids obtained was weighed out, and again injected into a silicic acid column. The method of Barron et al[135] as used for the separation into the six types of lipid components shown in Figure 5. Silica gel TLC (developer: paraffin ether - ethyl ether - acetic acid, 80 : 30 : 1,v/v) was used to identify the component lipids of each of the fractions obtained.[136]

Fatty acid analysis

The methyl esters of the fatty acids were prepared from the TL and from each of the lipid components by the hydrogen - chloride - methanol method, and analysed by GLC under the conditions shown in Table 11. The fatty acids were identified and the quantities measured by the methods described in Chapter 1, Section 2. To assist in the identification and quantitative measurement of the fatty acids, 20% of the fatty acids obtained by argentation TLC from the TL, the PL and the HG were hydrogenated, and the SE, the DG, and the visceral FA were also hydrogenated. p.100

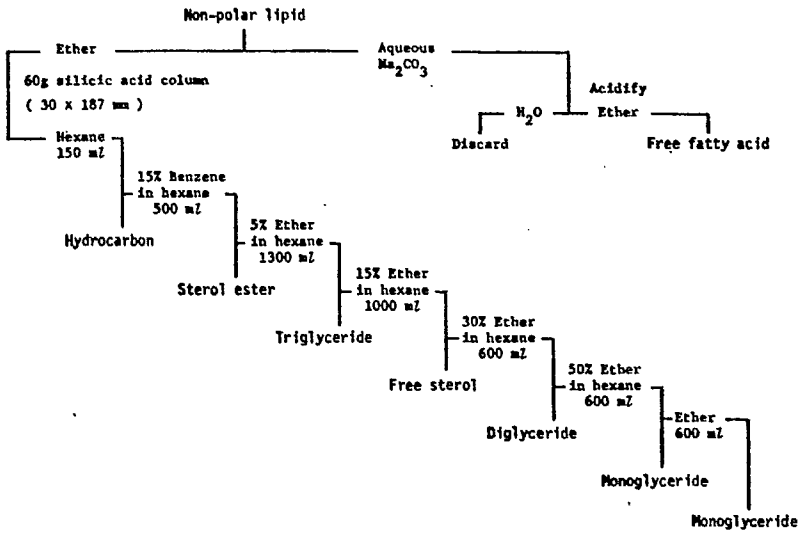

Fig. 5. Separation of non-polar lipid into their main constituent classes.

Table 11. Conditions for gas-liquid chromatography.

Apparatus :	SHIMADZU Gas Chromatograph Model GC-5A
Column dimensions :	300×0.3cm i.d. stainless steel
Solid support :	Shimalite W (60/80 mesh)
Stationary phase :	Diethylene glycol succinate polyester (10 : 90)
Temperatures :	Column 190°C, injection and detector 260°C
Carrier gas :	Nitrogen at 30 ml/min
Detector :	SHIMADZU Hydrogen Flame Ionization Detector Model FID-5
Sample size :	0.1 to 0.2μl
Analysis time :	Approximately 7.9 min to methyl octadecanoate

Experimental results

The lipid composition of the gonads and the viscera is shown in Table 12. In both the important constituent lipids were PL, TG, and S. However, there were differences between the gonads and the viscera in the proportions of the lipid components; the gonads having a high proportion of TG and the viscera having high proportions of PL and S. On comparing these results with those formerly reported for the composition of sea-urchin lipids[27, 29, 30] the PL content of the gonads and viscera is seen to be much greater in S. pulcherrimus than in A. crassispina[27] or in S. intermedius[30]. Hatakeyama[29] also reported the proportion of PL in S. pulcherrimus from Sanriku to be higher than the proportion of TG. p.101

Next, the fatty acid components of the TL of the gonads and the viscera, and of each of the component lipids, were analyzed, with the results shown in Tables 13 and 14. The important fatty acids common to all the component lipids were the 14:0, 16:0, 18:1ω9, 20:1ω9, 20:3ω3, 20:5ω3 and the isomers of 20:2 acids. The proportions of the 20:2 acid isomers were 5.0 - 7.5% in the lipid components of the gonads and 5.4 - 8.3% in the viscera. It is thus seen that these isomers are not confined to the gonads, but they are found in the viscera and in all the component lipids and that there is very little difference in the proportions in the gonad or viscera lipids or in each of the various component lipids.

The fatty acids in the lipids in the food are known to exert a considerable influence on the fatty acid composition of the body lipids of fish[115, 137, 138], and since the lipids in the body shift into the ovary during oogenesis, it has been suggested that the food will also affect the egg lipids[139, 140]. Since sea-urchins

Table 12. Lipid composition of gonad and viscera of sea urchin. (%)

Lipid class	Gonad	Viscera
Polar lipid (PL)	44.0	54.7
Free fatty acid (FA)	0.2	3.2
Hydrocarbon (HC)	0.03	0.3
Sterol ester (SE)	2.2	2.1
Triglyceride (TG)	43.5	26.1
Free sterol (S)	6.6	10.3
Diglyceride (DG)	2.7	1.6
Monoglyceride (MG)	0.7	1.7

Table 13. Fatty acid composition of lipid classes obtained from gonad of sea urchin. (%)

Fatty acid	Relative retention time	TL*	PL	FA	SE	TG	DG	MG
12 : 0	0.18	Trace	Trace	—	—	Trace	Trace	0.2
Anteiso 13 : 0	0.21	Trace	Trace	—	0.5	Trace	—	0.3
: 0	0.24	Trace	Trace	—	0.2	Trace	Trace	—
Anteiso 14 : 0	0.28	Trace	—	—	1.1	Trace	0.2	0.6
: 0	0.32	6.0	3.5	1.4	4.6	9.2	6.9	4.3
: 1ω7	0.38	0.3	0.2	Trace	1.3	0.4	0.2	0.7
Anteiso 15 : 0	0.37	0.3	0.2	—	—	0.3	0.6	—
: 0	0.43	1.0	0.5	1.4	1.4	0.9	1.2	1.5
: 2 ?	0.57	0.1	Trace	—	0.2	Trace	Trace	Trace
: 3ω3	0.79	0.1	—	—	—	0.4	—	—
Iso 16 : 0	0.47	0.3	0.2	1.0	10.4	0.2	0.3	0.6
: 0	0.56	17.0	12.2	3.2	14.9	21.3	17.6	14.5
: 1ω9	0.66	3.8	1.2	1.5	3.5	5.1	5.6	6.3
: 2ω7	0.80	0.2	0.2	—	0.2	0.2	0.4	1.0

Table 13. (Cont'd)

Fatty acid	Relative retention time	TL*	PL	FA	SE	TG	DG	MG
16 : 2 ?	0.84	1.0	0.2	0.8	0.5	1.0	1.0	0.7
: 3ω4	1.04	0.5	Trace	0.8	1.0	0.8	1.0	0.6
: 4 ?	1.05	—	—	—	—	0.3	—	—
: 4ω3	1.19	0.4	0.1	—	0.6	0.6	0.8	0.8
: 4ω1	1.27	0.3	0.2	—	—	0.3	—	—
17 : 0	0.75	0.5	0.4	2.4	1.7	0.3	0.7	0.7
: 1	0.85	0.1	Trace	—	—	Trace	—	—
: 2 ?	0.98	0.1	0.1	—	—	—	—	—
: 2ω8	1.06	0.1	0.1	—	—	0.1	—	—
Iso 18 : 0	0.83	0.5	0.8	0.4	8.8	0.3	0.2	0.7
: 0	1.00	2.6	3.8	3.1	3.9	1.2	2.0	3.0
: 1ω9	1.15	6.0	4.9	2.9	5.1	7.3	7.2	4.4
: 2ω9	1.33	0.2	0.2	—	—	0.4	—	—
: 2ω6	1.40	2.4	1.7	2.9	0.3	2.9	3.3	0.9
: 3ω6	1.64	0.3	0.1	—	2.1	0.2	1.4	12.1
: 3ω3	1.83	2.4	2.2	2.0	1.7	3.0	3.3	2.0
: 4ω3	2.12	1.5	1.2	8.2	1.3	1.6	1.5	1.1
19 : 0	1.32	0.4	0.5	—	0.5	Trace	0.2	0.8
: 1	1.48	0.2	—	—	0.8	—	0.5	0.8
: 2 ?	1.69	Trace	0.3	—	—	0.3	—	—
: 3ω6	2.14	0.6	0.5	2.0	—	0.6	Trace	—
: 5 ?	3.13	0.1	0.2	—	—	—	—	—
Iso 20 : 0	1.49	Trace	0.3	0.4	1.3	Trace	0.3	0.5
: 0	1.78	0.8	0.7	0.9	0.9	1.0	1.5	0.8
: 1ω9	1.99	7.2	8.7	2.0	4.4	5.8	6.7	3.8
: 2 Isomer	2.18	6.8	6.5	6.7	5.8	7.5	5.9	5.0
: 2ω6	2.46	1.9	1.6	1.4	1.2	2.1	2.1	1.2
: 3ω9	2.63	0.7	0.9	3.0	0.9	0.6	1.2	0.3
: 3ω6	2.82	1.0	0.3	1.9	1.3	1.2	1.4	1.0
: 3ω3	3.20	8.3	12.4	4.8	4.4	5.3	7.0	3.2
: 4ω3	3.66	Trace	Trace	0.4	0.7	0.6	0.5	1.0
: 5ω3	4.16	15.2	22.4	37.5	6.0	10.0	9.3	5.5
21 : 1	2.67	1.2	1.3	1.0	0.3	1.3	1.1	0.7
: 2 ?	2.97	0.1	0.3	—	—	Trace	—	—
: 3ω6	3.76	0.7	0.2	—	—	0.6	0.8	—
: 3ω3	4.18	0.2	0.4	0.3	0.3	0.4	—	—
22 : 1ω9	3.48	2.3	4.1	1.1	1.2	1.0	1.6	0.7
: 2ω9	3.96	1.0	0.7	—	1.0	1.1	1.1	—
: 2ω6	4.28	1.0	0.4	1.8	0.8	0.8	1.0	0.5
: 2 ?	4.56	0.9	1.1	1.9	1.2	0.6	0.8	17.2
: 3ω3	5.53	0.4	0.3	—	—	0.3	0.4	—
: 5ω3	7.18	Trace	0.2	—	—	Trace	—	—
: 6ω3	8.15	0.7	0.6	—	—	0.6	0.8	—
23 : 1	4.64	0.3	0.6	0.9	—	Trace	0.4	—
Iso 24 : 0	4.70	—	Trace	—	1.7	—	—	—
: 1	6.25	—	0.3	—	—	—	—	—

* TL, total lipid; other abbreviations are shown in Table 12.

Table 14. Fatty acid composition of lipid classes obtained from viscera of sea urchin. (%)

Fatty acid	Relative retention time	TL	PL	FA	SE	TG	DG	MG
12 : 0	0.18	0.1	Trace	Trace	Trace	0.1	Trace	0.2
: 1	0.22	Trace	Trace	Trace	—	Trace	0.3	0.5
Anteiso 13 : 0	0.21	0.1	—	—	0.3	—	0.2	—
: 0	0.24	0.1	Trace	Trace	0.2	Trace	0.4	—
: 4 ?	0.48	0.2	0.2	—	—	Trace	—	—
Anteiso 14 : 0	0.28	0.1	Trace	Trace	1.2	Trace	0.4	0.5
: 0	0.32	6.7	3.7	5.6	5.0	9.8	6.3	2.5
: 1ω7	0.38	0.4	0.2	0.3	0.3	0.7	0.4	0.2
: 2ω9	0.42	Trace	0.1	—	0.5	—	0.2	0.3
: 3ω6	0.54	0.4	0.4	—	—	—	—	—
: 5 ?	0.83	Trace	0.2	—	—	Trace	—	—
Anteiso 15 : 0	0.37	0.1	—	Trace	0.6	—	0.2	—
: 0	0.43	0.5	0.4	0.4	0.9	0.8	0.6	0.2
: 1	0.49	0.5	—	—	—	—	—	—
: 3ω6	0.71	0.3	0.3	—	1.3	—	—	—
: 4ω6	0.80	0.2	0.3	—	0.9	Trace	—	—
Iso 16 : 0	0.47	0.2	0.1	Trace	2.2	0.3	0.3	0.3
: 0	0.56	16.2	11.4	14.0	20.0	21.1	13.2	8.2
: 1ω9	0.66	3.5	1.1	3.2	2.0	4.9	4.0	2.9
: 2ω7	0.80	0.2	—	—	—	0.1	—	—
: 2 ?	0.84	0.3	0.4	0.4	—	0.4	—	0.6
: 3ω4	1.04	0.4	0.4	1.0	—	0.4	—	—
: 4ω3	1.19	0.3	0.2	—	—	0.3	—	—
: 4ω1	1.27	0.2	—	—	—	—	—	—
: 5 ?	1.39	0.2	0.2	—	—	Trace	—	—
Anteiso 17 : 0	0.67	Trace	0.3	0.5	—	—	0.2	—
: 0	0.75	0.2	0.1	0.8	2.9	0.7	0.7	Trace
: 1	0.85	0.2	0.4	—	—	Trace	—	—
17 : 2 ?	0.98	Trace	0.1	—	—	—	—	—
: 3ω3	1.37	0.1	0.2	—	—	—	—	—
: 5 ?	1.80	0.1	0.2	—	—	Trace	—	—
Iso 18 : 0	0.83	0.6	1.0	Trace	3.0	0.2	0.3	0.2
: 0	1.00	3.1	3.6	4.2	6.7	2.3	3.0	4.0
: 1ω9	1.15	6.0	3.7	7.0	5.7	7.9	5.5	5.4
: 2ω6	1.40	1.2	1.3	1.2	1.6	2.0	1.0	1.2
: 3ω6	1.64	0.4	0.3	0.6	2.2	0.7	0.8	7.3
: 3ω3	1.83	1.5	1.0	2.6	1.8	2.1	2.6	2.3
: 4ω3	2.12	1.4	1.4	1.9	0.8	1.6	1.8	1.0
: 5ω3	2.38	—	—	—	—	0.3	—	—
Anteiso 19 : 0	1.20	—	0.1	—	—	—	0.9	—
: 0	1.32	0.3	0.1	0.7	0.2	0.7	0.4	0.7
: 1	1.48	0.4	0.5	0.5	0.8	0.6	0.6	0.2
: 3ω6	2.14	0.3	0.4	0.4	1.0	0.2	—	—
: 4ω6	2.45	0.1	—	—	—	—	—	—
: 4ω3	2.79	0.2	0.2	—	—	0.2	—	—
: 5 ?	3.13	0.2	0.2	—	—	Trace	—	—
Iso 20 : 0	1.49	0.1	0.2	0.2	0.5	—	0.7	—

Table 14. (Cont'd)

Fatty acid	Relative retention time	TL	PL	FA	SE	TG	DG	MG
20 : 0	1.78	0.3	0.2	0.3	0.5	0.6	0.5	—
: 1ω9	1.99	8.0	8.7	10.9	9.5	9.0	6.7	6.1
: 2 Isomer	2.18	7.6	8.3	7.6	5.4	7.8	8.0	5.8
: 2ω6	2.46	1.4	1.3	1.8	0.6	1.4	1.2	1.1
: 3ω9	2.63	1.1	1.8	—	—	0.4	—	—
: 3ω6	2.82	0.8	0.6	1.0	1.6	0.8	0.9	0.7
: 3ω3	3.20	9.0	12.6	9.8	7.8	4.5	9.5	6.3
: 4ω3	3.66	1.9	1.7	1.0	1.0	0.9	1.0	1.5
: 5ω3	4.16	13.6	20.0	16.2	3.3	7.2	16.1	13.8
Anteiso 21 : 0	2.15	0.9	1.0	0.9	—	0.7	0.8	—
: 0	2.35	0.1	Trace	—	—	0.3	—	—
: 1	2.67	0.6	0.6	1.6	0.7	0.7	0.9	0.9
: 3ω6	3.76	0.2	0.3	—	—	0.2	—	—
: 3ω3	4.18	0.2	0.3	—	0.6	—	0.4	—
: 5ω2	5.51	0.1	Trace	—	—	Trace	—	—
Iso 22 : 0	2.65	Trace	0.3	—	0.6	0.2	0.2	—
: 1ω9	3.48	1.9	2.8	1.4	1.4	1.2	1.5	1.2
: 2ω6	4.28	1.3	1.9	0.4	1.4	0.8	1.2	0.3
: 2 ?	4.56	1.3	0.4	0.4	0.6	3.1	1.2	14.2
: 3ω3	5.53	0.3	0.3	0.3	—	0.3	0.5	0.8
: 4ω3	6.40	0.5	0.5	Trace	—	0.3	Trace	0.7
: 5ω3	7.18	0.3	0.2	Trace	—	0.3	0.3	0.4
: 6ω3	8.15	0.7	0.7	0.6	—	0.4	1.0	0.8
Anteiso 23 : 0	3.90	Trace	—	—	0.5	—	0.7	—
: 1	4.64	0.3	0.3	0.3	0.4	0.5	—	—
Iso 24 : 0	4.70	Trace	—	Trace	1.5	—	1.6	—
: 4 ?	11.17	Trace	0.3	—	—	Trace	0.8	6.7

Abbreviations are the same as those in Table 13.

Table 15. Distribution of isomeric eicosadienoic acid among the main lipid classes of sea urchin.

Lipid class	Gonad		Viscera	
	Amount of the isomer (mg/100g of whole visceral organs)	Distribution of the isomer (% in whole visceral organs)	Amount of the isomer (mg/100g of whole visceral organs)	Distribution of the isomer (% in whole visceral organs)
PL	80.6	27.0	51.9	17.4
FA	0.5	0.2	3.6	1.2
SE	2.1	0.7	0.8	0.3
TG	118.6	39.8	30.0	10.1
DG	5.6	1.9	1.8	0.6
MG	1.1	0.4	1.3	0.4
Total	208.5	70.0	89.4	30.0

Abbreviations are shown in Table 12.

do not possess subcutaneous fatty tissue for the storage of nutrients, lipids are accumulated not only in the viscera but also in the gonads[25, 141]. It is therefore to be supposed that the fatty acid composition of the visceral and gonad lipids will be influenced by the changes in the fatty acids of the food lipids. The extent of influence of the food lipids will depend on the lipid components. The relative contents may vary according to the influence received from the food, and therefore attention was given to the quantities of lipids and the amounts of 20:2 isomers present in each lipid class was computed and compared. From the proportion of 20:2 acid isomers in each of the lipid components and the yield of methyl esters, and from the weight, lipid quantity and lipid composition of the gonads and the viscera, the quantity of 20:2 acid isomers present in each of the lipid classes was obtained. The results are shown in Table 15. The largest amounts of the isomers are present in the gonad TG, followed in order by the gonad PL and the PL and the TG of the viscera. Moreover 70% of the total amount of 20:2 acid isomers present in the sea-urchin is in the gonads, and 30% is in the viscera. About 50% of the isomers is in the TG, about 44% in the PL and there are very small amounts in the other lipid classes. p.105

The distribution of the isomeric 20:2 acids in the PL and the TG was compared with the quantities of the main component fatty acids observed in each of the lipid classes in the sea-urchins. These acids are 16:0, 20:1ω9, and 20:5ω3. As shown in Figure 6, the TG contained 61% of 16:0 acid, which is known to figure principally as a storage lipid and the PL contained 65% of 20:5ω3 which is principally a tissue lipid. The distribution pattern

of the 20:1ω9 acid was more similar to that of the 16:0 acid than to the 20:5ω3 acid. However the isomeric 20:2 acid was more similar to the 20:5ω3 acid than to the 16:0 acid.

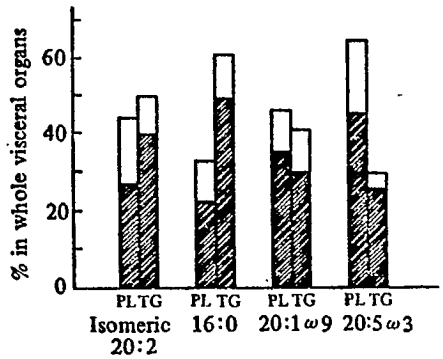

Fig. 6. Comparison of distribution of major component fatty acids in sea urchin.

Discussion

A number of 20:2 acids with double bonds in positions other than 8,11 and 11,14 are known to occur in nature[89, 100, 142]. However the proportion of these 20:2 acids is normally low. The sea-urchin lipids are unusual in having a large quantity of isomeric 20:2 acids.

The fact that the sea-urchin lipids show a peculiarly high content of isomeric 20:2 acids can be related to their physiology and their associations. Moreover these isomers are not found only in the gonads but also in the viscera, and form important components of all the lipid classes. More is found in the gonads than in the viscera, but in both cases they are mostly found in the TG and the PL. The distribution pattern between the TG and the PL ressembles that of the 16:0 acid rather than that of the 20:5ω3 acid.

Summary

An investigation was made of the distribution of the isomeric 20:2 acids in the gonads and the viscera of S. pulcherrimus. It was established that these isomers are distributed throughout all the lipid classes in the gonads and the viscera, being 5.0 to 7.5% of the gonad lipids and 5.4 to 8.3% of the viscera lipids. p.106

70% of the isomeric 20:2 acid in the sea-urchin is in the gonads and 30% in the viscera. About 50% of the isomer is in the TG and about 44% in the PL. The distribution pattern between the TG and the PL ressembles that of the 16:0 acid rather than that of the 20:5ω3 acid.

Chapter 3

The isolation and confirmation of the isomeric eicosadienoic acids

The isomeric 20:2 acids found in sea-urchin lipids differ from the oleic acid, linoleic acid and linolenic acid series of fatty acids which are widely distributed in marine plants and animals, and it is inferred that they have special positions of the double bonds.

In order to elucidate the structure of these isomers, they were first isolated and refined from the sea-urchin gonad lipids. It was then confirmed that the fractions obtained were isomeric 20:2 acids.

Section 1

The isolation of the isomeric eicosadienoic acids

There are many methods of isolating and confirming fatty acids or their methyl esters[31 - 77], and a combination of a number of methods is generally used.

The author used a combined seven-stage process for the isolation and confirmation of isomeric 20:2 acids from fatty acid methyl esters of the sea-urchin gonad lipids. The principal methods were fractionation according to degree of unsaturation (by silver-nitrate impregnated silicic acid column chromatography) and fractionation according to carbon atom number (n-undecane impregnated silica gel thin layer chromatography).

Experimental method

Specimens

The fatty acid methyl esters used were prepared from the gonads of A. crassispina in the way described in Chapter 2.

Fatty acid analysis

The same methods were used as in Chapter 2. The standard eicosadienoic acid used was 11,14-20:2 - methyl ester. (Applied Sciences Lab)

Wintering

After being left quietly overnight in a refrigerator at 5°C, the solid methyl esters and the liquid methyl esters were separated.

Fractional Vacuum distillation

The liquid methyl esters were distilled in a current of nitrogen gas at a pressure of 11mm Hg.

Urea fractionation

The method of Chapter 1, section 1 was used.

Silver nitrate impregnated silicic acid column chromatography

This was done by the method of Privett et al[52]. A mixed column (2.6 x 63.5 cm) was prepared by adding 50g of celite to 100g of silver nitrate impregnated silicic acid prepared by the method of de Vries[53,54] (Mallinckrodt manufacture, 100 mesh, impregnated with about 0.5g of silver nitrate to 1.0g of silicic acid, heat activated at 120°C for 16 hours). About 1g of the methyl esters was injected into the column and the ethyl ester - petroleum ether eluants were allowed to flow down.

n-Undecane impregnation silica gel TLC[65]

A 20 x 20 cm plate of silica gel G 500μ thick was activated by heating to 110°C for 120 minutes. After cooling it was at once stood up in a developing tank containing a solution of n-undecane in petroleum ether and impregnated with n-undecane by the ascent method. The impregnation was continued for twice the time required for the n-undecane solution to reach the end of the plate. After

the plate had been dried in a current of air for about five minutes at room temperature, about 20mg of the methyl esters were spotted in a belt and developed by the ascent method with acetone - acetonitryl (1:1 v/v). As the developing agent a mixture of 9 parts saturated with one part unsaturated n-undecane was used.

Silicic acid column chromatography

A column 1.2 x 10 cm was prepared with silicic acid activated by heating to 110°C for 180 minutes. The eluants used were 75 ml and 25 ml of hexane followed in order by 75 ml and 25 ml of ethyl ester.

Silica gel TLC

(1) To confirm the removal of the n-undecane, a 20 x 20cm plate 250μ thick of silica gel G activated by heating to 110°C for 60 minutes was used and developed in n-hexane - ethyl ether (94 : 6, v/v). p 107

(2) To remove impurities, a 20 x 20 cm plate of silica gel G 500μ thick activated by heating to 110°C for 180 minutes was used and developed in n-hexane - ethyl ester (90 : 10, v/v). After air drying, the positions of the bands were confirmed by spraying both edges with a 1% solution of iodine methanol and the isomeric 20:2 acids were scraped off.

Experimental results

The specimen methyl esters were separated by the processes shown in Figure 7. At each stage in the process, the amount of isomeric 20:2 acids in the fractions obtained was checked by GLC, and the fraction containing the largest amount was used in the next stage. The following results were obtained.

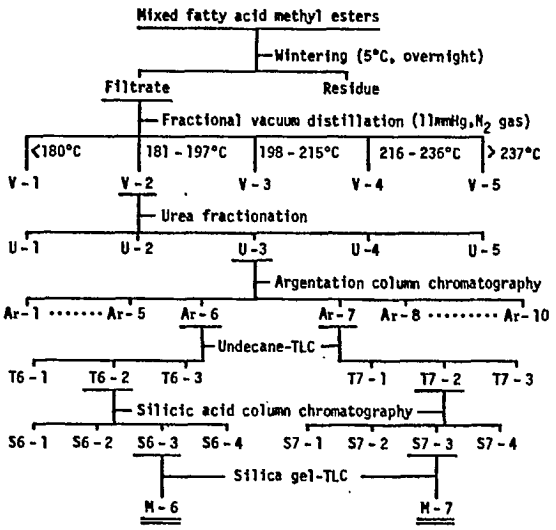

Fig. 7. Preparation of isomeric eicosadienoate from mixed fatty acid methyl esters of sea-urchin gonad.

Table 16. Content of isomeric eicosadienoate in the fractions obtained by wintering, fractional vacuum distillation and urea fractionation.

Sample analyzed	Content
Original methyl esters	8.0%
Filtrate of wintering	9.9
Fraction V-2 *	10.7
Fraction U-3 *	18.7

* Each fraction is obtained by fractional vacuum distillation and urea fractionation, respectively, as shown in Fig. 7.

Wintering, fractional vacuum distillation, and urea fractionation

The solid methyl esters were separated by wintering and filtered out, and the liquid methyl esters were separated into five fractions by fractional vacuum distillation. The fraction with the highest content of 20:2 isomeric acids was V-2 which came over at 181°- 197°C. This V-2 fraction was next urea fractionated and separated into five fractions according to the number of double bonds. The fraction containing the greatest amount of isomeric 20:2 acids was U-4, with 21.6%, but since a small quantity of 20:3 acid was present in this fraction, the U-3 fraction, which contained no trienoic acid, was used as the material for the next stage of separation. p 108

The proportions of isomeric 20:3 acids in each of the fractions obtained in these stages and in the specimen methyl esters are shown in Table 16. The proportion of isomeric 20:2 acids was increased by these stages from 8% to about 19%.

Silver nitrate impregnated silic acid chromatography

The U-3 fraction was again separated according to the number of double bonds. The resulting elution diagram is shown in Figure 8. Nine peaks, A to I, were detected in this chromatogram. The peaks with the highest amounts of isomeric 20:2 acids were peaks E and F, but since the leading half of peak E contained 11,14- 20:2 acid, Peak E was divided into two fractions, the leading half (Tube no. 77 to 87) and the trailing half (Tube no. 88 to 93). The content of isomeric 20:2 acid and of 11,14- 20:2 acid in each of the fractions is shown in Table 17. Fractions Ar-6 and Ar-7 had the greatest amounts of isomeric 20:2 acid. Both of these fractions contained small amounts of 18:2 and 22:2 acids, but the 11,14- 20:2 acid had been separated and removed.

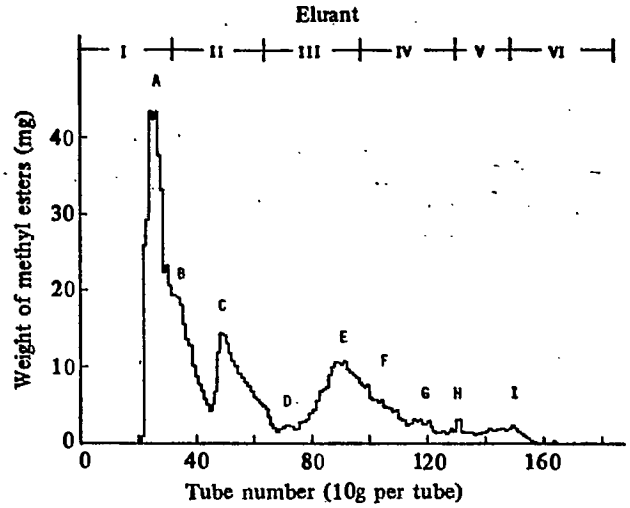

Fig. 8. Column chromatogram of fraction U-3 on silicic acid impregnated with silver nitrate.

Column: 2.6 × 63.5 cm

Eluant: (I) 3% ethyl ether in petroleum ether, 600 m*l*;
(II) 5% ethyl ether in petroleum ether, 600 m*l*;
(III) 6% ethyl ether in petroleum ether, 600 m*l*;
(IV) 8% ethyl ether in petroleum ether, 600 m*l*;
(V) 10% ethyl ether in petroleum ether, 300 m*l*;
(VI) ethyl ether alone, 600 m*l*

Flow rate: 2.0 m*l*/min

Recovery of methyl esters: 94.7%

Table 17. Content of isomeric eicosadienoate in the fractions obtained by argentation column chromatography.

Peak No.	Fraction No.	Yield	Eicosadienoate		Impurities
			[11, 14]	Isomer*	
		g	%	%	
A	Ar- 1	0.2778	0	0	18 : 1, 20 : 1, 14 : 0?
B	Ar- 2	0.2088	0	0	16 : 1, 18 : 1, 17 : 1, 20 : 1
C	Ar- 3	0.1717	4	5	16 : 1, 14 : 1, 18 : 1
D	Ar- 4	0.0147	8	18	22 : 2, 21 : 2, 18 : 2
E	Ar- 5	0.0568	28	39	22 : 2, 18 : 2
	Ar- 6	0.0614	0	87	18 : 2, 22 : 2
F	Ar- 7	0.1100	0	79	18 : 2, 22 : 2
G	Ar- 8	0.0346	0	69	18 : 2, 22 : 2
H	Ar- 9	0.0196	0	65	18 : 2, 22 : 2
I	Ar-10	0.0349	0	30	18 : 2, 22 : 2

*The positions of double bonds are not known at present.

n-Undecane impregnated silicic acid TLC

From results obtained with 20:2 isomers and with commercial fatty acid methyl esters it was thought that the 16:0; 18:1 and 22:3 acids would form the "critical pairs"[61, 143] with the 20:2 isomers. p 109

Consequently the 18:2 and 22:2 acids mixed into the Ar-6 and Ar-7 fractions were fractionated and removed. The results are shown in Table 18. Both Ar-6 and Ar-7 were individually fractionated into three fractions. Fractions T6-2 and T7-2 had the highest contents of isomeric 20:2 acids and it had been possible to remove most of the admixed fatty acids. However, there was a large amount of n-undecane in these fractions.

Silicic acid column chromatography

The n-undecane was removed from the fractions T6-2 and T7-2 by passage through a silicic acid column. They were then fractionated into the eight fractions S6-1 to S6-4 and S7-1 to S7-4. The residual n-undecane in these fractions (other than S6-1 and S7-1) was measured by silica gel TLC. The thin layer chromatograms obtained are shown in Figure 9. It can be seen from these chromatograms that residual n-undecane is present in fractions S6-2 and S7-2, but that it is completely absent from fractions S6-3 and S7-3. However, in addition to isomeric 20:2 acids, impurities which were thought to be other separate fatty acids were detected in fractions S6-3 and S7-3. p 110

Silica gel TLC

Fractionation of fractions S6-3 and S7-3 by silica gel TLC gave almost exactly the same chromatograms as in Figure 9. Fractions M6 and M7 were prepared to recover the 20:2 isomer fraction. The gas chromatograms of these fractions are shown in Figure 10. The fatty acid composition is shown in Table 19. In the chromatogram obtained

Table 18. Content of isomeric eicosadienoate in the fractions obtained by thin-layer chromatography on silica gel impregnated with *n*-undecane.

Fraction No.	R_f range	Isomeric eicosadienoate	Impurities
		%	
T6-1	0.74 - 0.66	3.7	16 : 0, 16 : 1, 17 : 2?, 18 : 2, 19 : 2
2	0.64 - 0.54	97.9	16 : 0, 17 : 2?, 18 : 2, 19 : 2
3	0.53 - 0.44	86.5	21 : 2, 22 : 2
T7-1	0.76 - 0.65	6.7	16 : 0, 16 : 1, 18 : 2
2	0.64 - 0.54	98.6	16 : 0, 17 : 2?, 18 : 2
3	0.54 - 0.44	90.3	16 : 0, 22 : 2

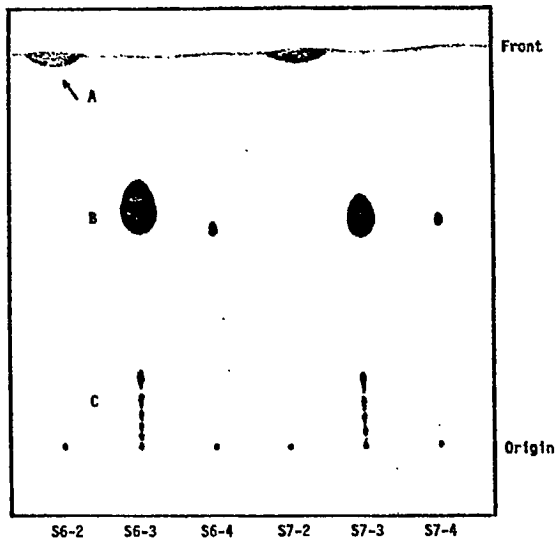

Fig. 9. Thin-layer chromatogram of the fractions obtained by silicic acid chromatography.
Peaks: A, *n*-undecane; B, eicosadienoate; C, impurities

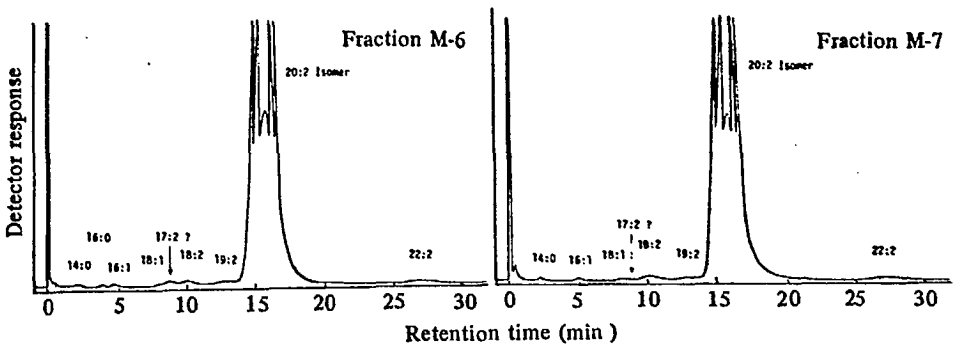

Fig. 10. Gas chromatograms of isomeric eicosadienoate fractions, M-6 and M-7.

Table 19. Fatty acid composition of the fractions M-6 and M-7 obtained by silica gel TLC.

Fatty acid	Relative retention time*	M-6	M-7
		%	%
14 : 0	0.31	0.1	0.1
16 : 0	0.57	0.1	—
16 : 1 ω9	0.69	0.1	Trace
18 : 1 ω9	1.18	0.1	0.1
18 : 2 ?	1.28	0.3	0.1
18 : 2 ω6	1.44	0.6	0.7
19 : 2 ω6	1.83	0.5	0.3
20 : 2 Isomer	2.24	97.8	98.4
22 : 2 ω9	3.97	0.4	0.3

*Relative to methyl octadecanoate

the 20:2 isomer peak and peaks many times smaller are observed. Moreover, the retention times of the 20:2 isomers were the same in M-6 and M-7. p 111

By the combined use of these seven stages of processing, 12.3 mg of fraction M-6 containing 97.8% of the 20:2 acid isomers and 23.3 mg of fraction M-7 containing 98.4% were prepared from 20.6 grams of the specimen methyl esters.

Discussion

The dienoic acid fraction was recovered by a process of separating the acids according to the number of double bonds, in which wintering of the specimen fatty acid methyl esters, fractional vacuum distillation, and urea fractionation were used as pre-processing for silver nitrate impregnated silica gel chromatography. The fraction obtained was fractionated by silver nitrate impregnated silver gel chromatography. As a result it was possible, by separating and removing most of the admixed fatty acids of other degrees of unsaturation, to obtain two fractions, Ar-6 and Ar-7, with high contents of the isomeric 20:2 acids. By this chromatography, the 11,14- 20:2 acid was separated and removed from the isomeric 20:2 acids but it was not possible to remove all of the 18:2 and 22:2 acids. This is thought to be because the proportions of these acids in fraction U-3 was considerably larger than the proportion of 11,14- 20:2 acid. The GLC retention times of the isomeric 20:2 acids in fractions Ar-6 and Ar-7 exactly agreed, but because there were differences in the amount of isomeric 20:2 acids and in the compositions of the admixed fatty acids, both fractions were further separated and refined.

Because the principal admixed fatty acids in fractions Ar-6 and Ar-7 were 18:2 and 22:2 acids, the next step was to separate the acids by n-undecane impregnated silica gel TLC according to the carbon number. In this TLC the fatty acid methyl esters were separated principally according to the carbon number but there was also some separation according to the number of double bonds. However, since the critical pairs for the isomeric 20:2 acids are 16:0, 18:1 and 22:3, it was possible to separate and remove most of the 18:2 and 22:2 acids.

In the next separation process the introduced and original impurities were removed.

In this way, by combining separations based on number of double bonds and on carbon number, and by other refining processes, it was possible to produce fraction M-6 containing 97.8% of the isomeric 20:2 acids and M-7 containing 98.4%.

Section 2

Confirmation of the eicosadienoic acid position isomers.

The identification of the fatty acids in this fatty acid analysis of sea-urchin lipids is based entirely on GLC. This does not guarantee that the principal fatty acids in the fractions described in the previous section are in fact isomeric 20:2 acids. p 112

The identity of these fatty acids as 20:2 isomers was therefore confirmed by carbon chain length analysis, by measurement of the number of double bonds, by relative retention times in GLC and by SF comparison.

Experimental methods

Specimens

Fraction M-7 as prepared in the previous section was used.

Hydrogenation

The method described in Chapter 1, section 1 was used*.

Measurement of hydrogen uptake

The Warping pressure gauge method was used[144]. About one mg of the methyl ester was weighed out, dissolved in 3 ml of n-hexane, introduced into the main chamber of the pressure gauge container, and 15 mg of platinum black was introduced into the measuring chamber. After the air in the equipment had been completely replaced by hydrogen gas, it was immersed in a constant temperature tank at 25°C and shaken (40 times per minute) until it had reached temperature equilibrium. After the platinum black had been transferred to the main chamber it was shaken vigorously (80 times per minute) and the hydrogen uptake was measured every 10 minutes.

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* Sic, but I cannot find such a description. Translator

Experimental results

Carbon chain length analysis

Fraction M-7 and its hydrogenation products were analysed by GLC, and the exact carbon chain length of the principal fatty acid in this fraction was measured. The results obtained are shown in Table 20. A large peak due to arachidic acid was observed in the gas chromatogram of the hydrogenation product. The percentage contained in this peak was 98.1%, agreeing with the proportion of 20:2 isomers in the M-7 fraction. It was therefore confirmed that the isomer was a C_{20} straight chain fatty acid.

Table 20. GLC analyses of isomeric eicosadienoate fraction, M-7 , before and after hydrogenation.

Fatty acid	Before hydrogenation		After hydrogenation	
	Relative retention time	% Area	Relative retention time	% Area
18 : 0	—	—	1.00	0.8
: 1	1.14	0.1	—	—
: 2	1.38	0.4	—	—
19 : 0	—	—	1.32	0.5
: 2	1.79	0.4	—	—
20 : 0	—	—	1.79	98.1
: 2 Isomer	2.19	98.4	—	—
22 : 0	—	—	3.20	0.6
: 2	4.01	0.8	—	—

Measurement of the number of unsaturated bonds

The hydrogen uptake of 0.932g of the fraction M-7 was measured. The measured uptake is shown in Figure 11. The uptake reached equilibrium after about 60 minutes, and the amount was 128.0 μl. This was 98.8% of the theoretical value for a 20:2 acid. If the principal fatty acid in the fraction were eicosamonoenoic acid (20:1 acid) the theoretical value would be the same. However, it is known that the ECL value of the 9-18:1 acid is just the same as that of the 18:3ω3 acid, and larger than that of the 11-18:1 acid[90]. Thus it is thought that the ECL value of the 20:1 acid would be rather larger than that of a 20:2 isomer acid and just the same as that of 20:3ω3. Moreover, in separating processes based on the number of double bonds, such as urea fractionation and silver nitrate impregnated column chromatography, this 20:2 isomer behaves in exactly the same way as other dienoic acids. It is thus confirmed that the principal fatty acids in fraction M-7 are 20:2 isomers.

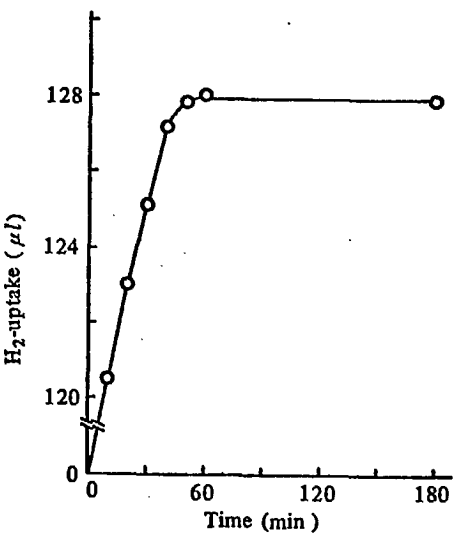

Fig. 11. Hydrogen-uptake of isomeric eicosadienoate fraction, M-7 (0.932 mg).

Comparison of relative retention times

The relative retention time of fraction M-7 in gas chromatography was measured, the SF relative to the 20:1ω9 and 20:2ω6 used as standards were calculated and compared with Ackman's values[145]. The results are shown in Table 21. There was a slight difference between the relative retention time of 20:2ω6 and Ackman's result. This difference is thought to be due to a difference in the aging of the column used[146]. However, the SF of the isomer relative to 20:1ω9 and to 20:2ω6 was not the same as the SF of 20:2ω9. It was therefore shown that the isomer is a 20:2 acid whose structure differs from that of 20:2ω9.

p 113

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Table 21. Comparison of relative retention time and separation factor of methyl eicosadienoates in present study with those in reference.

Fatty acid	Present study		Reference*	
	Relative** retention time	Separation factor	Relative** retention time	Separation factor
20 : 2 ω6	2.46		2.53	
20 : 1 ω9	1.99	1.24	2.01	1.26
20 : 2 ω6			2.53	
20 : 2 ω9			2.37	1.07
20 : 2 ω6	2.46			
20 : 2 Isomer	2.18	1.13		
20 : 2 ω9			2.37	
20 : 1 ω9			2.01	1.18
20 : 2 Isomer	2.18			
20 : 1 ω9	1.99	1.10		

* Calculated from ACKMAN'S data[145], DEGS column at 190°C

** Relative to methyl octadecanoate

Discussion

It was confirmed that the principal fatty acids in the M-6 and M-7 fractions prepared from sea-urchin gonad lipids were 20:2 isomer acids.

The production of arachidic acid from the principal fatty acids of fraction M-7 showed that it was a C_{20} straight chain acid, and the hydrogen uptake showed that it was a C_{20} dienoic acid. From the relative retention times in GLC and from the SF with respect to 20:1ω9 acid and 20:2ω6 acid it was shown to be a 20:2 acid whose structure was different from that of 8,11- 20:2 acid, and thus to be isomeric 20:2.

Fraction M-6 was prepared from fraction Ar-6, which was the trailing half of the peak E containing 11,14- 20:2 acid obtained, in the preceding section, by silver nitrate impregnated silicic acid column chromatography. Fraction M-7 was prepared from Fraction Ar-7. In neither of these fractions was the isolation complete. However, the retention times of the principal fatty acids of fractions M-6 and M-7 were identical. From these facts it was unmistakeable that the principal fatty acid in fraction M-6 was isomeric 20:2 acid, and that the principal fatty acid in fraction M-7 was either identically the same 20:2 acid or one of extremely similar structure.

The acids known to occur in nature other than the 8,11-, and 11,14- 20:2 acids are the 5,11-[91,92,100,101,147,148], the 5,13-[100, 101, 148], the 7,10-[100, 149], the 10,13-[149] and the 14,17- 20:2 acids[91]. In addition, the 6,11-[148] and the 8,13- 20:2[148] have been inferred to be present. The 20:2 isomer acids found in the sea-urchin lipids cannot be supposed to be the 10,13- or the 14,17- 20:2 acids because of the relation between the double bond

positions in fatty acids and the GLC retention times[105,106,122,123,150]. These isomers were therefore thought either to be the 5,11-, 5,13-, 6,11-, 7,10 or 8,13-20:2 acids or to be some other fatty acids of a different nature.

Summary

The isomeric 20:2 acid was isolated and refined from the methyl esters of the fatty acids in the sea-urchin gonad lipids by a combined process with seven stages of separation, and fractions containing 98% of the isomer were prepared.

It was confirmed by means of carbon chain length analysis, measurement of the number of unsaturated bonds, and comparison of the relative retention times and SF in GLC that the fatty acid in the fraction so obtained was isomeric 20:2 acid with a structure differing from the 8,11-, 11,14-, 10,13- or 14,17-20:2 acids.

Chapter 4

The structure of the eicosadienoic acid position isomers

In the previous chapter, it was confirmed that the 20:2 acid isomer isolated and refined from sea-urchin gonad lipids was a straight chain 20:2 acid. However, it was found to differ from the 8,11- and the 11,14- 20:2 acids which are widely distributed in nature, and was supposed to be a 20:2 acid with a peculiar double bond location. Such a 20:2 acid was not known to occur in marine animals and plants other than shell fish[100, 101].

The position of the double bonds in this isomer was therefore determined.

Section 1

Mass analysis

The mass spectra of fatty acids or their esters have been applied to the determination of double bond positions[151, 152]. Also, since the higher order fatty acids are found to produce large molecular ion peaks, they have also been applied to the determination of molecular weight[153 - 157].

The molecular weight of the isomer was therefore determined by measurement of the mass spectrum. p 115

Experimental methods

Materials

The M-7 fraction prepared in the preceding chapter was used.

Mass spectrum

Measurement was made by using a Nippon Denshi type JMS-01SG mass spectrometer, with an ionizing voltage of 75 eV and a specimen temperature of 32°C.

Experimental results

The mass spectrum of fraction M-7 is shown in Figure 12. The molecular ion of the principal fatty acid methyl ester in this fraction has an m/e of 322, in agreement with the molecular weight of a 20:2 acid. It is thus confirmed that this fatty acid is isomeric 20:2.

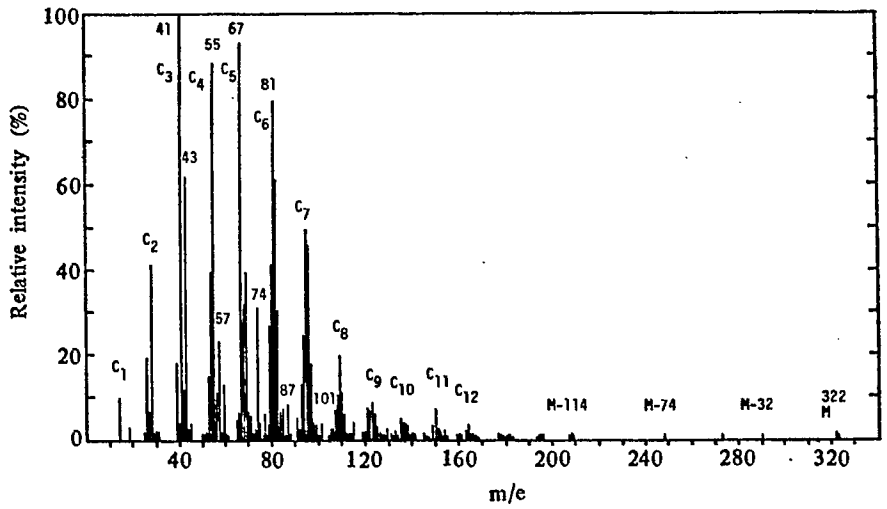

Fig. 12. Mass spectrum of isomeric eicosadienoate fraction, M-7, obtained from lipid of sea-urchin gonad by chromatographic procedures.

Normally, the methyl esters of high order fatty acids produce strong molecular ion peaks[153 - 157], but with this 20:2 isomer it was weak. This shows that the molecular ion of the isomer is easily fragmented. The peaks with m/e of 31 and 74 which are characteristic of straight chain fatty acids, and the methoxycarbonyl ion fragments at m/e 59, 87, 101, 115 and 129 were found. There is the normal hydrocarbon fragment peak at m/e 41, and a large number of C_1 to C_{12} hydrocarbon fragments. However, no fragments particularly related to the position of the double bonds were found.

Discussion

The 20:2 isomer present in the sea-urchin lipids was shown in the preceding chapter to be a straight chain 20:2 acid, and this was confirmed by mass spectroscopy. The molecular ion peak which is known to be usually strong in higher order fatty acid methyl esters was weak, and the normal peak at m/e 41 was small. No conclusion about the position of the double bonds could be directly obtained from the mass spectrum.

Section 2

Oxidative cleavage by means of sodium periodate and potassium permanganate

Oxidative cleavage of unsaturated fatty acids by oxidants cuts them at the unsaturated bond positions. Thus by investigating the composition of the cleavage products by means of GLC, the position of the unsaturated bonds can be determined[87]. p 116

The methods normally used for sectioning double bonds are the sodium periodate-potassium permanganate method[158 - 164] and the ozone cleavage method[163, 165, 166].

The former of these methods was used here to determine the positions of the double bonds in the 20:2 isomer acids.

Experimental methods

Materials

The fractions M-6 and M-7 as prepared in the previous chapter were used.

Oxidative cleavage

Following the method of Rudloff[159], 3 mg of fraction M-6 and M-7 were oxidatively sectioned in the double bond positions by a solution of sodium periodate and potassium permanganate in tert-butanol. One part of the liquid extract after being distilled with ethyl ester under reduced pressure was methyl esterified and the esters obtained were analysed by GLC. The residual extract was fractionated into mono- and dicarboxylic acids and methyl esterfied. The mono and dicarboxyl methyl esters obtained were separately analysed by GLC and used to identify the fatty acids.

Fatty acid analysis

The methods of Chapter 2 were used. However, for the analysis of the lower monocarboxylic acids and dicarboxylic acids the column temperatures were 120°C and 160°C. In these cases the standard acids used were methyl esters of C_6 - C_{10} monocarboxylic acids and of C_2 - C_{11} dicarboxylic acids.

Experimental results

Fractions M-6 and M-7 were separately oxidized. The gas chromatogram of the oxidative cleavage products of fraction M-7 is shown in Figure 13. Nine peaks, A to I, were found in this chromatogram. Peaks B, D, F and H were identified as nonanoic acid, glutaric acid, adipic acid and suberic acid and the retention time of peak A agrees with that of heptanoic acid. Peaks C, E, G and H* do not correspond to any known fatty acid. They are taken to be peaks produced by by-products of the oxidation cleavage process.

The mono- and dicarboxylic acid composition of the results of oxidative cleavage are shown in Table 22. In both fractions, the monocarboxylic acids were almost entirely nonanoic acid, and the dicarboxylic acids were glutaric acid, adipic acid and suberic acid. However, the proportions of the dicarboxylic acids produced by the two fractions were somewhat different, with more suberic acid from M-6 and more adipic acid and glutaric acid from M-7. Furthermore, there was only a trace of the malonic acid which should be produced by the oxidative cleavage of the double bond portion of a fatty acid having the divinylmethane structure. This can be split during oxidative cleavage[160], but probably it is because the divinylmethyl structure is absent.

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* Sic, though this disagrees with the last sentence Translator

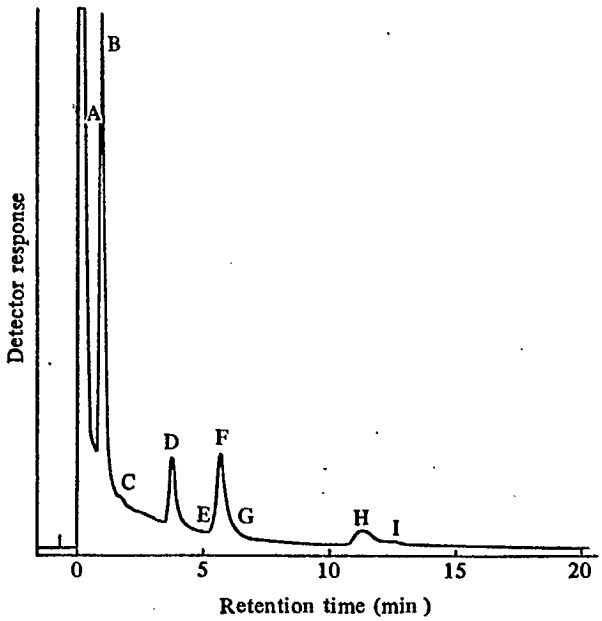

Fig. 13. Gas chromatogram of the methyl esters of the acids resulting from oxidative cleavage of isomeric eicosadienoate fraction, M-7.
Column temperature: 160°C

Table 22. Composition of oxidative cleavage products of isomeric eicosadienoate fractions, M-6 and M-7 , estimated as methyl esters of mono- and dicarboxylic acids (mole %).

Fatty acid	Fraction	
	M-6	M-7
Monocarboxylic		
C_4	Trace	—
C_8	Trace	Trace
C_9	100.0	100.0
Dicarboxylic		
C_3	Trace	—
C_4	Trace	—
C_5	22.3	34.6
C_6	27.7	50.0
C_7	Trace	Trace
C_8	50.0	15.4
C_9	Trace	—

M-6 and M-7 were fractionated from lipid of sea-urchin gonad by chromatographic procedures.

The 20:2 acid structures which can be inferred from the above oxidation cleavage products are shown in Figure 14.

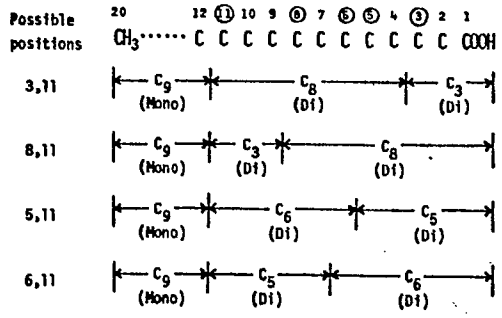

Fig. 14. Possible positions of double bonds in isomeric eicosadienoic acid deduced from C_9-monocarboxylic acid, and C_5-, C_6- and C_8-dicarboxylic acids obtained by periodate-permanganate oxidation.

In the GLC analysis of fractions M-6 and M-7 described in Chapter 3, section 1, it was found that the isomeric 20:2 peaks were identical, but from the results of the oxidative cleavage it is reasonable to conclude that there is a mixture of two to four fatty acids with different double bond positions, and that they are present in different proportions in fractions M-6 and M-7. Thus, it could be inferred from Figure 14 that the main constituents of M-6 are the 3,11- or the 8,11- 20:2 acids, and those of M-7 are the 5,11- or the 6,11- 20:2 acid.

Now the oxidative cleavage of 5,11- or of 6,11- 20:2 acid should theoretically produce equal mole quantities of glutaric and adipic acids. However, both M-6 and M-7 produced more glutaric acid than adipic adic. It could be thought that this was due to the admixture of compounds produced as by-products of the oxidative cleavage, with the

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same retention times in GLC as adipic acid methyl ester, such as malonic semi-aldehyde from the carboxyl side carbon chain of 3,11- 20:2 acid or malonic aldehyde from the carbon chain between the double bonds in 8,11- 20:2 acid. This was therefore tested by the oxidative cleavage of glutaronic acid and linoleic acid under the same conditions, but such by-product compounds were not found. Thus the differences in the amounts of glutaric and adipic acids could be ascribed to the fact found by Korn[167] that the great solubility of glutaric acid in water contributes to the difficulty of its recovery.

Discussion

The principal fatty acids in fractions M-6 and M-7 produced a single peak in packed column GLC and had the same retention times, so that it was inferred that there was only one 20:2 isomer acid in the sea-urchin lipids. However, it is inferred from the results of the present experiments that at least two and perhaps more isomers are present.

From the fact that GLC analysis of the oxidative cleavage products shows that the only monocarboxylic acid is nonanoic acid, one of the double bonds in the 20:2 isomers must be between the ninth and the tenth carbon atoms from the methyl radical end, that is to say between the eleventh and twelfth carbon atoms from the carboxyl radical end. From dicarboxyl acid composition it is inferred that the principal fatty acid in the M-6 fraction could be either 3,11- or 8,11- 20:2 acid. However, as mentioned in the previous chapter, the retention time of this fatty acid is considerably less than that of the 8,11- 20:2 acid. It is therefore inferred that the main fatty acid in fraction M-6 is 3,11- 20:2 acid.

However, the main fatty acid in fraction M-7 could be either 5,11- 20:2 acid or 6,11- 20:2 acid.

Section 3

Analysis by the ultra-violet absorption spectra before and after alkaline isomerization

Conjugated unsaturated fatty acids have a characteristic absorption in the ultraviolet region, and when the divinylmethane type of unsaturated fatty acid is heated with alkali, the unsaturated bond are conjugated and an ultraviolet region absorption is produced[168], but this isomerization is extremely difficult to produce in unsaturated fatty acids in which the separation of the double bonds is of the divinylethane type or greater[169].

The principal fatty acid in fraction M-6 was inferred to be either 3,11- or 8,11- 20:2 acid, and from the retention time it was supposed to be 3,11- 20:2 acid, but the exact structure was uncertain. p. 119

The ultraviolet spectra were therefore analyzed before and after isomerization, in order to ascertain whether the principal fatty acid in fraction M-6 was 3,11- or 8,11- 20:2 acid.

Experimental methods

Materials

The fraction M-6 produced in the previous chapter was used. It was used for the test after removal of oxidation products by the method of Shono et al[170].

Alkaline isomerization

The alkaline isomerization followed the standard method[171] using a 6% solution of potassium hydroxide in ethylene glycol.

Measurement of the conjugated unsaturated fatty acids

The specimen methyl esters were dissolved in n-hexane, the absorption was determined at wavelengths from 210 to 346 mμ by the standard method[171] and the quantity of conjugated unsaturated fatty acid was calculated.

Measurement of the unconjugated unsaturated fatty acids

After alkaline isomerization the reaction fluid was diluted with methanol, the ultraviolet absorption at wavelengths of 210 to 346 mμ was measured, and the quantity of conjugated unsaturated fatty acid was calculated as before. The quantity of divinyl type unconjugated unsaturated fatty acid was calculated from the difference in the quantities of conjugated unsaturated fatty acids before and after alkaline isomerization. For reference, the methyl ester of linoleic acid (made by Wako Pure Chemicals, high grade) was tested under the same conditions.

Experimental results

The ultraviolet absorption spectra of fraction M-6 and of methyl linoeleate before and after alkaline isomerization are shown in Figures 15 and 16. Before isomerization, the fraction M-6 and the methyl linoleate both showed very little ultraviolet absorption, but after isomerization both showed the conjugated diene absorption at the wavelength of 233 mμ. However, the absorption of the M-6 fraction was very much weaker than the absorption of the methyl

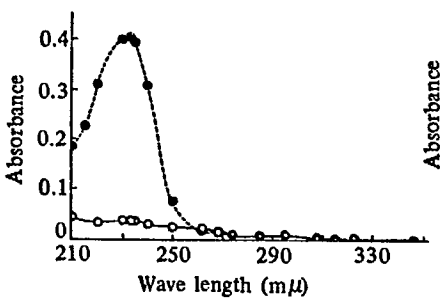


Fig. 15. Absorption spectra of isomeric eicosadienoate fraction, M-6, before and after alkaline isomerization.
Solid line, before alkaline isomerization (0.1080 g/l)
Dotted line, after alkaline isomerization (0.0600 g/l)

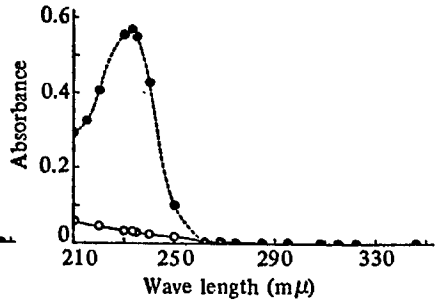


Fig. 16. Absorption spectra of methyl linoleate before and after alkaline isomerization.
Solid line, before alkaline isomerization (0.1080 g/l)
Dotted line, after alkaline isomerization (0.0064 g/l)

Table 23. Proportion of conjugated acids in isomeric eicosadienoate fraction, M-6 and control specimen, commercially available linoleate before and after alkaline isomerization.

Fatty acid	Fraction M-6		Methyl linoleate	
	Before isomerization	After isomerization	Before isomerization	After isomerization
	%	%	%	%
Conjugated dienes	0.30	6.02	0.20	94.72
〃 trienes	0	0.02	0.02	0.38
〃 tetraenes	0	0	0	0
〃 pentaenes	0	0	0	0
Others	99.70	93.96*1	99.78	4.90*2

*1 Almost exclusively isomeric eicosadienoate

*2 Almost exclusively oleate

Table 24. Composition of isomeric eicosadienoate fraction, M-6 and control specimen, commercially available linoleate, calculated from the data shown in Table 23, compared with their composition by GLC analysis.

Fatty acid	Fraction M-6		Methyl linoleate	
	Alkaline isomerization	GLC	Alkaline isomerization	GLC
	%	%	%	%
Conjugated dienes	0.30	0.3	0.20	—
〃 trienes	0	—	0.02	—
Methylene interrupted				
〃 dienes	5.72	1.5	94.52*3	94.1
〃 trienes	0.02	—	0.36*4	0.2
Others	93.96*1	98.2	4.90*2	5.7

*1 and *2, the same as those shown in Table 23

*3, linoleate ; *4, linolenate

linoleate which had been treated in the same way for comparison. The quantities of conjugated unsaturated fatty acids were calculated from the absorption before and after isomerization, with the results shown in Table 23. In the conditions used, the methyl lineolate was isomerized and the conjugated diene acid produced, but most of the 20:2 isomeric acid in the M-6 fraction was not isomerized. Table 24 compares the results of GLC with the quantities of fatty acids containing double bond arrangements of the divinyl methane type, as calculated from the results shown in Table 23. The results of both analyses are almost in agreement for the methyl lineolate, but in the M-6 fraction there are differences in the quantities of divinylmethane type diene acids and of other fatty acids. These differences are due to the mixture of a small quantity of 8,11- 20:2 acid with the 3,11- 20:2 acid in the fraction. In GLC, it was included in the large peak produced by the 3,11- 20:2 acid, and being unobserved was included in the measurement of the 3,11- 20:2 acid. This is clear from the smallness of the differences in detail between the results of the two types of analysis of the methyl lineolate. p 120

Discussion

Since practically no conjugated acid is produced by the alkaline isomerization of the principal fatty acids in the M-6 fraction, it is evidently a 20:2 acid in which the double bond arrangement is either of the divinylethane type or more widely separated. Thus this fatty acid which had already been inferred to be 3,11- 20:2 acid is confirmed as the 3,11- 20:2 acid. Comparison of the fatty acid compositions derived by GLC and by alkaline isomerization shows that a small quantity of the 8,11- 20:2 acid is present in the fraction.

Section 4

Capillary column gas chromatography

It was concluded in section 2 from the results of oxidative cleavage that there could be two to four 20:2 fatty acids of differing structures in fractions M-6 and M-7. It was shown in section 3 that the principal fatty acid in fraction M-6 was the 3,11- 20:2 acid and that there was also a small quantity of the 8,11- 20:2 acid present.

The number of types of 20:2 acids present in fractions M-6 and M-7 was therefore investigated by capillary column chromatography[126].

Experimental methods

Materials

Fractions M-6 and M-7 produced in the previous chapter were used.

GLC analysis

The conditions of use of capillary column GLC are shown in Table 25. As standard fatty acids, 16:0, 18:0 and 11,14- 20:2 acid methyl esters were used.

Table 25. Conditions for gas-liquid chromatography using capillary column.

Apparatus :	SHIMADZU Gas Chromatograph Model GC-5A
Column :	45 m×0.05 cm, stainless steel
Solid support :	Column wall
Stationary phase :	Butanediol succinate polyester (BDS)
Temperatures :	Column, 168°C; injection and detector, 280°C
Carrier gas :	Nitrogen at 2.2 m*l*/min
Detector :	SHIMADZU Hydrogen Flame Ionization Detector Model FID-5
Sensitivity :	1000 MΩ
Range :	0.08 V
Theoretical plates (*n*) :	Approximately 23,000
Analysis time :	Approximately 23 min to methyl palmitate

Experimental results

The gas chromatograms obtained by capillary column GLC of fractions M-6 and M-7 are shown in Figure 17. Fraction M-6 gave four peaks, A to D, and fraction M-7 gave two peaks, A and B. Peak D has the same retention

time as the standard 11,14- 20:2 acid, and was identified as 11,14- 20:2 acid. Peak C, with a relative retention time of 2.43 and a SF from 11,14- 20:2 acid of 1.07, was identified as 8,11- 20:2 acid[122].

The 20:2 isomeric acids which were observed as a single peak in packed column GLC were found to give two peaks, A and B from both fraction M-6 and M-7 with capillary column chromatography. Thus it was seen that both fractions contained, in addition to the 3,11- 20:2 acid, some other 20:2 acid which could be the 5,11- or the 6,11- 20:2 acid.

Next, a comparison was made between the relative amounts of 3,11- 20:2 acid and the 5,11- or 6,11- 20:2 acid as determined by capillary column GLC and by the oxidative cleavage described in section 2. This comparison is shown in Table 26. In both fractions, the proportion of peak A is almost the same as the proportion of 5,11 or 6,11- 20:2 acid, and that of peak B is the same as the proportion of 3,11- 20:2 acid. Thus it is clear that peak B consists of the 3,11- 20:2 acid.

The relative retention times and the SF from 11,14- 20:2 acid of these peaks are shown in Table 27. The retention p 122 times of these isomers are slightly less than that of the 8,11- 20:2 acid. However, the relative retention times of the two isomers differ very little, so that they will be difficult to isolate completely even in the capillary column.

Discussion

From the results of capillary column GLC it was p 123 shown that both fractions M-6 and M-7 contain two types of 20:2 acid isomers with special double bond arrangements. By comparison with the results of oxidative cleavage given in section 2, it was shown that the principal fatty acid in fraction M-6 was the 3,11- 20:2 acid, and that in M-7 it could be the 5,11- or the 6,11- 20:2 acid.

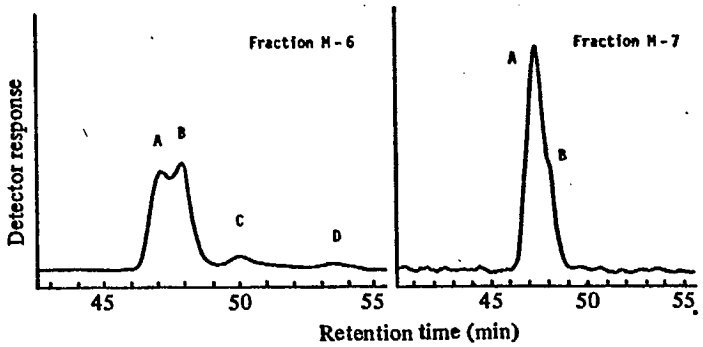


Fig. 17. Gas chromatograms of isomeric eicosadienoate fractions, M-6 and M-7 (capillary column, BDS, 45m).

Peaks: A, 5,11- or 6,11-eicosadienoate; B, 3,11-eicosadienoate; C, 8,11-eicosadienoate; D, 11,14-eicosadienoate

Table 26. Comparison between fatty acid composition based on capillary column analyses and oxidative cleavage.

Fraction	Composition based on			
	Capillary column analyses		Oxidative cleavage	
	Peak A	Peak B	3, 11-20 : 2	5, 11- or 6, 11-20 : 2
	%	%	%	%
M-6	31.4	68.6	70.6	29.4
M-7	70.8	29.2	30.3	69.7

Table 27. Relative retention times and separation factors of eicosadienoates separated by capillary column.

Peak No. position of double bond	A 5,11	B 3,11	C 8,11	D 11,14
r_{18}	2.29	2.33	2.43	2.61
SF	1.14	1.12	1.07	—

r_{18} : Retention times relative to methyl octadecanoate

SF: Separation factors between 11,14-eicosadienoate and others

There was also shown to be a small quantity of the 8,11- 20:2 acid present in fraction M-6, which was not found by packed column GLC. This agrees with the alkaline isomerization results of section 3.

Section 5

Hydrazine partial reduction and oxidative cleavage

In order to decide whether the principal fatty acid in fraction M-7 was 5,11 or 6,11- 20:2 acid, hydrazine partial reduction and oxidative cleavage was used. When a 20:2 isomer is partially reduced in a hydrazine-methanol solution, two types of 20:1 acid are produced by hydrogenation of one or other of the double bonds[72]. Oxidative cleavage will cut the 20:1 acids produced at the position of its double bond, and by investigating the composition of the cleavage products the position of the double bonds in the isomer is determined.

Experimental methods

Materials

A fraction M-7' was produced from new specimens by following the method by which the M-7 fraction was produced in Chapter 3, section 1.

Partial reduction

Following the method of Aylward[172] the M-7' fraction was added to a hydrazine-methanol solution and the double bonds were partially reduced by reacting for 4 hours at 50°C.

Argentation chromatography

A 20 x 20 cm plate was prepared by the method of Chapter 1, section 2 and activated by heating to 110°C for 120 minutes. After the methyl esters had been spotted in a band, it was developed with methanol - chloroform (0.5 : 99.5, v/v). After air current drying, both edges of the plate were sprayed with a 1% solution of iodine in methanol to locate the positions of the bands, and the monenoic acid fraction was collected.

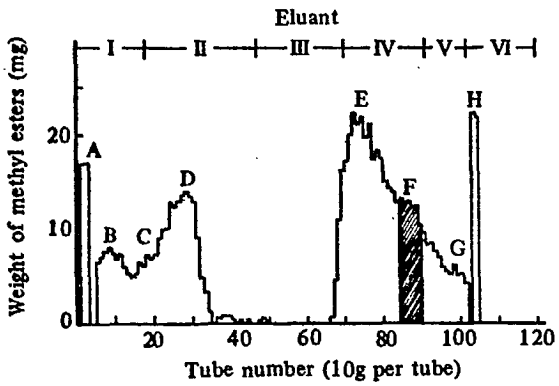


Fig. 18. Separation of isomeric eicosadienoate on silicic acid impregnated with silver nitrate.

Column: 2.6 × 62.5cm
Eluant: (I) 5% ethyl ether in petroleum ether, 500 m*l*;
(II) 6% ethyl ether in petroleum ether, 600 m*l*;
(III) 7% ethyl ether in petroleum ether, 600 m*l*;
(IV) 8% ethyl ether in petroleum ether, 400 m*l*;
(V) 9% ethyl ether in petroleum ether, 200 m*l*;
(VI) ethyl ether alone, 400 m*l*
Flow rate : 2.0 m*l*/min

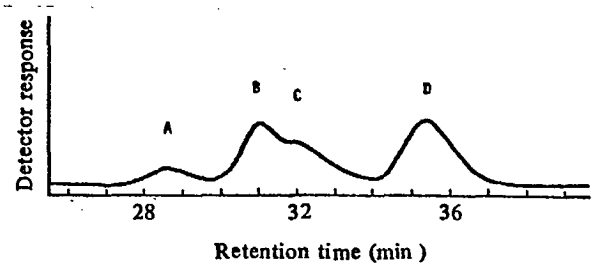


Fig. 19. Gas chromatogram of hydrazine reduced isomeric eicosadienoate fraction, M-7' (packed column, DEGS, 3m).

Peaks: A, arachidate; B, assumable other eicosenoate;
C, 11-eicosenoate; D, isomeric eicosadienoate

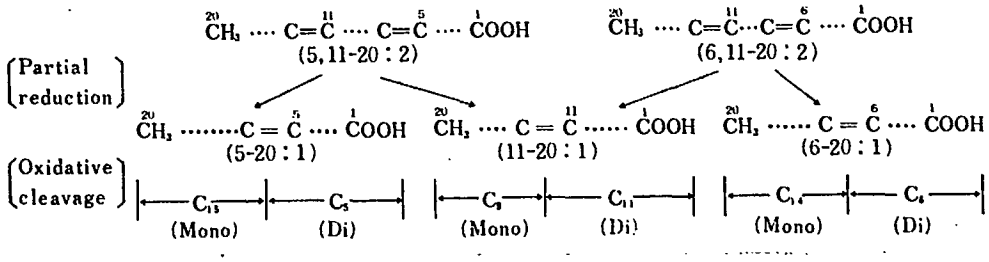
Oxidative cleavage and fatty acid analysis

This was the same as in section 2.

Experimental results

A fraction of M-7' containing a large amount of 5,11- or 6,11- 20:2 acid was prepared from A. crassispina gonads in the same way as the M-7 fraction. The silver nitrate impregnated silicic acid column chromatography elution diagram is shown in Figure 18. According to GLC analysis, the 20:2 isomeric acids are present in peaks E and F. According to the results obtained in section 2 by oxidative cleavage, and to the results of capillary column GLC in the preceding section, the 5,11- or 6,11- 20:2 acid will be concentrated in peak F (shown hatched in the Figure). Accordingly, this fraction was isolated and refined and a fraction M-7', containing 93.7% of the 20:2 isomer acids was obtained.

This M-7' fraction was investigated to find whether the 20:2 acid isomer in the sea-urchin lipids was 5,11- or 6,11- 20:2 acid. It is to be expected that hydrazine partial reduction and oxidative cleavage of the 5,11- or 6,11- 20:2 acids will have the following products.



Thus the structure of the 20:2 isomer acid can be determined from the composition of the mono and dicarboxylic acids produced. p 124

The gas chromatogram of the hydrazine partial reduction products is shown in Figure 19. In this

chromatogram there are two 20:1 acid peaks, B and C. Of these, C is identified as the 11-20:1 peak, and B is thought from its slightly less retention time to be the peak of the 5- or 6- 20:1 acid.

The partial reduction products were separated by argentation TLC and after the monoenoic acid fraction had been collected it was added to a solution of sodium periodate and potassium permanganate for oxidative cleavage. The composition of the mono- and dicarboxylic acids produced was investigated by GLC, with the results shown in Table 28. The moncarboxylic acids produced in quantity were nonanoic acid and pentadecylic acid, and the dicarboxylic acids were glutaric acid and undecanedioic acid.

Table 28. Composition of oxidative cleavage products of monoenoic acids formed by hydrazine partial reduction of isomeric eicosadienoate fraction, M-7', estimated as methyl esters of mono- and dicarboxylic acids.

Monocarboxylic acid		Dicarboxylic acid	
Component	mole %	Component	mole %
C_8	0.8	C_4	1.1
C_9	37.2	C_5	35.4
C_{10}	0.5	C_6	3.6
C_{12}	0.6	C_7	3.4
C_{13}	1.9	C_8	1.6
C_{14}	8.4	C_9	7.1
C_{15}	36.4	C_{10}	11.8
C_{16}	14.2	C_{11}	36.0
		C_{12}	Trace

Discussion

p 125

Two types of 20:2 acid were obtained by hydrazine partial reduction of fraction M-7', and one of them was identified as 11- 20:1 acid. Thus one of the double bonds in the main 20:2 acid in this fraction is between the eleventh and twelfth carbon atoms. This agrees with the results obtained in section 2.

The mono- and dicarboxylic acids obtained in large amounts by oxidative cleavage of the 20:1 fraction were nonanoic acid, pentadecylic acid, glutaric acid and undecanedioic acid. Of these, the nonanoic acid and the udecanedioic acid are the products of the 11- 20:1 acid. Because pentadecylic acid and glutaric acid were obtained in quantity, the other 20:1 acid must be the 5- 20:1 acid. Thus the structure of the main fatty acid in the M-7' fraction is shown to be 5,11- 20:2 acid. Myristic acid and adipic acid were also observed among the partial reduction products, but the proportions of these acids were low compared with the pentadecylic acid and the glutaric acid, and there are great differences in the amounts of the two acids. They are by-products of the oxidative cleavage of the fatty acids[161], and are not considered to show the presence of the 6,11- 20:2 acid.

Summary

The position of the double bonds in the 20:2 isomeric acids present in the sea-urchin lipids was determined by mass analysis, oxidation cleavage with sodium periodate and potassium permanganate, alkaline isomerization, capillary column GLC and hydrazine partial reduction.

It was thereby shown that two types of 20:2 acids with unusual double bond arrangements were present, and it was determined that their structures were those of the 3,11- 20 acid and the 5,11- 20:2 acid.

It is known that many of the unsaturated fatty acids found in nature have common structural characteristics, and that many of them are of the same type as oleic, linoleic, and linolenic acids and have the divinylmethane arrangement of double bonds[85 - 88]. However, fatty acids

with unusual structures other than these fatty acids have been found[89, 90]. Of the 20:2 acids, 8,11- and 11,14- 20:2 acids are widely distributed in nature[85, 89], but the ω3 series related 14,17- 20:2 acid is not much in evidence[91]. Other 20:2 acids known to be present include 5,11-[91, 92, 100, 101, 147, 148], 5,13-[100, 101, 148], 7,10-[100, 149], and 10,13- 20:2 acids[149], and in addition the presence of 6,11-[148] and 8,13- 20:2 acids[148] has been inferred.

The 3,11- 20:2 acid found in the sea-urchin lipids has a double bond at the third carbon atom, but it is a fatty acid of unusual structure with a hexamethylene interrupted arrangement of the two double bonds, and it is the first such fatty acid to be discovered in nature. Other fatty acids with a double bond on the third carbon atom known to be present in nature are 3- 16:1[93, 95], 3- 18:1[74, 94, 95], 3,6- 16:2[149], 3,9- 18:2[92], 3,9,12- 18:2[93-96], and 3,9,12,15- 18:4[97]. Fatty acids with the hexamethylene interrupted structure which have been reported to exist p 126 are 5,13- 20:2[100,101,148], 5,13- 22:2[32,34,74], and 7,.5- 22:2[101]. However, no fatty acid has been found with the structure characteristic of 3,11- 20:2 acid.

The 5,11- 20:2 acid is found in the gingko nut and in tea[91, 92] and in rat liver[147, 148], and has recently been discovered in marine shellfish[101, 100]. There is a dienoic 18:2 acid with the 5,11- position structure[75, 91, 92, 173], which is an important constituent fatty acid in slime moulds[75]. Apart from these fatty acids, a large number of fatty acids with a tetramethylene interrupted arrangement occur in nature[39, 91-98, 101, 147, 174], but apart from some vegetable nut oils[93, 95-97], the quantities are normally small. The sea-urchin is a remarkable marine animal in having a relatively large amount of the 5,11- 20:2 acid.

In general, fatty acids with unusual double bond arrangements are often found in the plant world in vegetable nut oils[32, 34, 39, 74, 91-99], but they are scarcely known to occur in animal lipids[101, 147-149]. The occurrence of the 3,11- and the 5,11- 20:2 acids in sea-urchins is of great interest for comparative biochemistry.

Chapter 5

The distribution of position isomers of eicosadienoic acid among marine plants and animals

There have been a large number of studies of the principal component fatty acids in marine animals and plants, and they have been summarized by Gruger[175]. However, there are very few studies of the detailed fatty acid composition, and there are not more than two or three reports of the presence of 20:2 acid isomers[100, 101, 142].

The 20:2 isomeric acids have almost exactly the same retention time on a polyester column used for GLC as the 18:4ω3 acid, and consequently they will not be detected unless there is some associated chemical preprocessing. Thus it may in the past have been measured as 18:4ω3 acid.

The distribution of these isomers in a number of marine plants and animals was therefore investigated by making a detailed analysis of their fatty acid composition by the joint use of argentation TLC and GLC.

Section 1

Mollusca The distribution in Bivalvia and Gastropods

There have been a number of studies of the fatty acid composition of shellfish lipids[78, 113, 176 - 179], but all of them have dealt with the fatty acid composition of the muscle lipids. Recently, Ackman et al[100] have found 20:2 isomers to occur in a species of periwinkle, and Watanabe et al[142] and Paradis et al[101] have found them in an oyster species.

For this reason, an investigation was made of the fatty acid composition of three species of gastropods which feed on marine seaweeds like sea-urchins and of two species of bivalves which feed on phytoplankton. In addition to establishing the distribution of 20:2 acid isomer, comparative measurements were made of the differences between bivalves and gastropods and between muscle and viscera.

Experimental methods

Materials

Two Bivalvia species Mategai, razor clam, Solen strictus, and Kagamigai, Venus shell, Phacosoma japonica and three Gastropod species, Kuroawabi, abalone, Nordotis discus, Sazae, Top shell, Batillus cornutus, and Ookoshitakagangara, Tegula, Omphalius pfeifferi carpenteri were used. They were caught offshore from Yoshimi in Shimonoseki. The season of capture, the number of individuals used, the shell length or shell diameter and the weight of the viscera used are shown in Table 29.

Extraction of lipids and fatty acid analysis

The same methods were used as in Chapter 2. p 127

Experimental results

The lipid content of the muscle and the viscera is shown in Table 30. The amounts are low, 1 - 2% in muscle and 2 - 3% in viscera, but about 5% in Tegula viscera.

Gas chromatograms of the muscle lipids of the razor clam before and after hydrogenation are shown in Figure 20. In the gas chromatogram of the hydrogenation products there are peaks between the 14:0 and 16:0 large peaks, between 16:0 and 18:0, between 18:0 and 20:0 and between 20:0 and 22:0. One of these peaks is produced by an unsaturated fatty acid with an odd number of carbon atoms in the chain, but the others are not produced by straight chain fatty acids. All of these peaks A to H are presumed to be due to unsaturated branched chain fatty acids. As p 128 shown in Table 31, all of these peaks, except for peaks D and H, have equivalent chain lengths (ECL)[123] which agree with the ECL values of standard iso or anteiso unsaturated branched chain fatty acids. Thus peak A is identified as iso 14:0, C as iso 16:0, E as iso 18:0, G as iso 20:0, B as anteiso 15:0 and F as anteiso 19:0. Peaks D and H

Table 29. Description of the sample examined.

Common name	Japanese name	Season	Number of individuals	Average size (cm)	Total weight (g)	Stripped shellfish			
						Muscle		Viscera	
						Weight (g)	Yield (%)	Weight (g)	Yield (%)
Razor clam	Mategai	Dec.	330	8.7*	1600	590	36.9	140	8.8
Venus shell	Kagamigai	Dec.	72	5.2*	2413	319	13.2	130	5.4
Abalone	Kuroawabi	Jan.	17	8.0*	966	382	39.5	166	17.2
Top shell	Sazae	Jan.	18	5.3**	1564	264	16.9	260	16.6
Tegula	Ookoshitakagangara	Nov.	135	3.4**	2400	197	8.2	201	8.4

* Shell length

** Shell diameter

Table 30. The lipid content of shellfishes.

Species	Lipid content (%)	
	Muscle	Viscera
Razor clam	1.3	2.5
Venus shell	1.2	2.3
Abalone	1.1	2.7
Top shell	1.7	3.4
Tegula	1.7	4.8

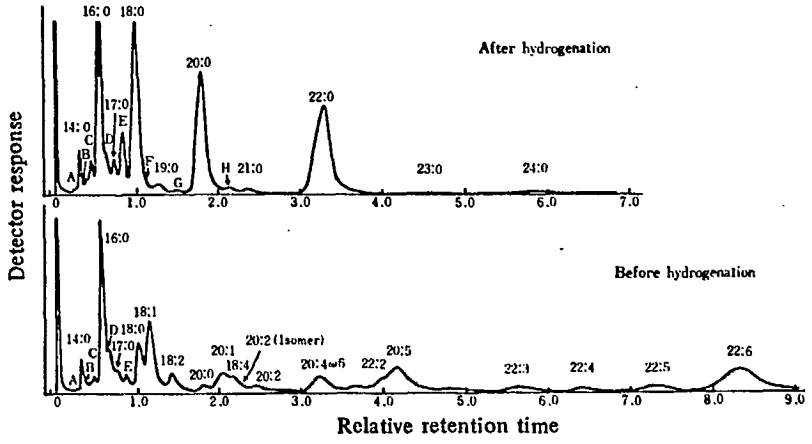


Fig. 20. Gas chromatograms of fatty acid methyl esters from muscular lipid of razor clam.

Table 31. Equivalent chain lengths (ECL) of authentic branched chain fatty acid methyl esters and the esters (A-H) appeared on chromatogram after hydrogenation (*cf.* Fig. 20).

Authentic esters			Examined esters	
		ECL	Symbol	ECL
Iso	14:0	13.48	A	13.48
Anteiso	15:0	14.58	B	14.55
Iso	16:0	15.47	C	15.43
—		—	D	16.47
Anteiso	17:0	16.66	—	—
Iso	18:0	17.55	E	17.51
Anteiso	19:0	18.74	F	18.70
Iso	20:0	19.57	G	19.53
—		—	H	20.52
Anteiso	21:0	20.74	—	—

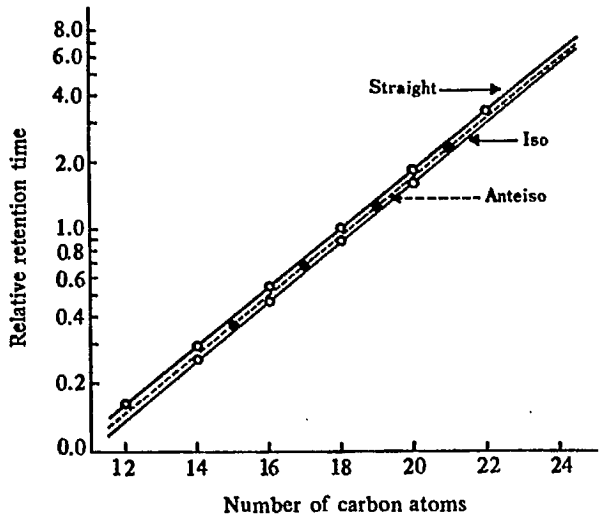


Fig. 21. Relative retention time plotted against the number of carbon atoms of fatty acids.

lie on the line in Figure 21 connecting the iso unsaturated branched chain fatty acids in the relative retention time - carbon atom number diagram, and Peak D is identified as iso 17:0 and Peak H as iso 21:0. The proportion of Peak E in the specimen methyl esters is 1.9%, but since there is 5.4% in the hydrogenation products, it is presumed that C_{18} iso unsaturated branch chain fatty acids are present as well as iso 18:0. In the chromatograms of the specimen methyl esters these unsaturated branch chain fatty acids appear in what seems to be a double peak containing 18:0 or 18:1 only. Wolfe et al[80] found 18:1 p 130 unsaturated branched chain fatty acids in the lipids of the fresh water crawfish and report the relative retention time as 1.01. Since the argentation TLC fractions containing the saturated acids and the monoenoic acids have large peaks for the 18:0 and 18:1 acids, it is not possible to verify a peak for 18:1. and there is no iso 18:2 peak in the dienoic fraction. For this reason the C_{18} iso unsaturated branched chain fatty acid in question is presumed to be iso 18:1. The 20:2 acid isomers appear in the chromatogram of the specimen methyl esters as a peak on the trailing shoulder of the 18:4 peak, and cannot be observed as a definite peak, but in the chromatogram of the argentation TLC dienoic fraction there is a clear peak before the 20:2ω6 peak, so their presence is confirmed.

The fatty acid composition of the razor shell viscera lipids and of the lipids in the muscle and viscera of the other shellfish were analyzed in the same way, with the results shown in Table 32. From 47 to 55 fatty acids were observed in the make-up of the shellfish lipids. The principal fatty acids in the razor shell muscle were 16:0, 18:1, 20:5 and 22:6, and in the viscera they were 16:0, 18:1, 18:2, 20:1, 20:5 and 22:6. In the venus shell muscle

Table 32. Fatty acid composition of the lipids from shellfishes. (%)

Fatty acid	Razor clam		Venus shell		Abalone		Top shell		Tegula	
	Muscle	Viscera	Muscle	Viscera	Muscle	Viscera	Muscle	Viscera	Muscle	Viscera
12 : 0	Trace	0.1	Trace	Trace	Trace	0.1	Trace	0.1	Trace	0.1
13 : 0	Trace	Trace	Trace	Trace	0.1	0.1	Trace	Trace	Trace	0.1
Iso 14 : 0	0.1	0.1	0.1	0.1	—	—	Trace	0.1	0.1	0.1
: 0	1.8	2.1	0.7	2.4	3.0	6.3	1.6	4.7	1.8	5.9
: 1	0.2	0.2	0.2	0.2	Trace	0.3	0.3	0.6	0.1	0.4
: 3 ω6 ?	Trace	Trace	Trace	Trace	Trace	0.8	0.2	0.1	Trace	Trace
Anteiso 15 : 0	0.3	0.6	0.1	0.4	—	—	Trace	Trace	0.3	0.4
: 0	0.7	0.6	0.4	0.5	1.0	1.1	2.6	1.9	1.1	1.0
Iso 16 : 0	1.7	1.1	1.4	0.8	1.8	1.2	0.5	0.3	1.6	1.1
: 0	16.3	12.3	18.2	16.1	21.9	23.1	23.3	18.9	20.0	23.8
: 1 ω7	1.2	2.1	0.5	2.9	0.9	0.9	1.2	1.3	1.8	4.2
: 2 ω4	0.2	0.1	Trace	Trace	Trace	0.2	0.2	0.8	Trace	Trace
: 3 ω4	0.2	0.1	Trace	Trace	Trace	Trace	Trace	0.8	Trace	Trace
: 4 ω3	Trace	1.3	0.2	0.1	0.1	0.1	Trace	Trace	Trace	Trace
Iso 17 : 0	2.7	1.8	2.6	2.2	—	—	Trace	Trace	2.2	1.1
: 0	2.7	2.2	2.5	1.9	1.3	1.4	2.2	1.6	3.6	2.1
: 1	0.1	0.2	Trace	Trace	Trace	Trace	Trace	0.4	Trace	0.4
Iso 18 : 0	1.9	1.7	2.7	1.4	2.0	2.3	3.4	2.3	5.0	3.8
: 0	3.5	2.9	0.3	2.5	4.5	3.1	1.8	1.8	1.3	3.4
Iso : 1	3.5	3.0	5.0	3.3	0.4	—	3.4	1.4	2.4	—
: 1 ω9	10.6	16.5	8.2	14.8	12.0	10.2	8.0	12.1	9.1	15.0
: 2 ω6	3.2	7.1	1.7	4.3	1.3	2.1	3.4	2.6	2.5	2.5
: 3 ω6	0.3	0.7	0.3	0.5	0.1	0.3	0.2	0.4	0.3	Trace
: 3 ω3	1.1	1.7	1.0	1.7	2.8	4.1	1.6	1.8	1.5	2.2
: 4 ω3	2.5	2.1	3.3	2.2	1.0	2.6	0.5	1.3	1.2	0.7
Anteiso 19 : 0	0.5	0.4	0.5	0.4	—	—	—	—	—	—
: 0	0.2	—	Trace	0.4	—	—	—	0.3	Trace	—
: 1	0.2	0.2	Trace	Trace	Trace	0.2	0.2	Trace	Trace	0.4
: 5 ?	0.3	0.4	0.6	0.1	0.7	0.6	0.6	0.6	1.2	0.5
Iso 20 : 0	0.3	0.2	0.6	0.4	0.4	0.6	0.5	0.5	0.5	0.4
: 0	0.1	0.3	Trace	0.3	0.5	0.8	0.3	0.4	0.3	0.7
: 1 ω9	4.4	5.4	3.1	4.7	3.7	4.7	1.7	4.2	2.6	4.0
: 2 Isomer	1.1	1.2	0.8	1.1	0.4	1.1	0.4	1.0	0.5	1.0
: 2 ω6	1.5	1.7	1.6	1.2	0.3	0.5	0.3	0.6	0.4	0.4
: 3 ω9	Trace	0.5	—	—	—	—	—	—	0.3	0.4
: 3 ω6	0.3	0.4	0.2	0.2	0.2	0.3	0.3	0.6	0.3	0.4
: 4 ω6*	4.0	3.4	3.7	2.7	8.7	7.6	11.3	12.5	9.0	4.9
: 4 ω3	0.6	0.2	0.5	0.7	1.3	0.8	1.2	0.7	0.2	0.7
: 5 ω3	7.0	5.3	10.7	9.8	8.8	9.3	5.6	7.3	7.5	5.8
Iso 21 : 0	0.3	0.6	0.7	0.6	0.4	0.4	0.3	0.3	0.5	0.5
: 0	Trace	—	—	—	Trace	—	Trace	—	—	—
: 1 ω9 ?	Trace	—	—	—	0.2	0.3	Trace	—	Trace	—
: 2 ω7 ?	0.2	0.3	—	—	0.4	0.3	Trace	0.5	0.4	0.3
: 3 ω6 ?	—	Trace	0.3	0.1	0.3	0.3	0.2	0.2	Trace	0.2

Table 32. (Cont'd)

Fatty acid	Razor clam		Venus shell		Abalone		Top shell		Tegula	
	Muscle	Viscera	Muscle	Viscera	Muscle	Viscera	Muscle	Viscera	Muscle	Viscera
21 : 5 ω2	0.7	0.4	0.7	0.5	—	—	0.8	0.6	0.3	0.3
Iso 22 : 0	—	—	—	—	—	—	Trace	0.2	—	—
: 0	Trace	—	0.7	0.3	—	0.2	0.2	Trace	—	—
: 1 ω9	0.3	1.0	0.3	0.5	0.3	0.4	1.0	0.4	0.7	0.2
: 2 ω6	4.1	3.9	4.2	4.3	4.9	4.2	3.9	3.3	5.2	4.1
: 3 ω6	0.6	0.8	0.3	0.3	0.3	0.4	0.5	0.6	0.5	0.3
: 3 ω3	0.9	0.5	1.0	0.4	2.3	1.2	4.1	2.7	1.0	0.3
: 4 ω3	1.0	0.9	1.0	0.6	0.4	Trace	0.7	0.5	1.1	0.5
: 5 ω3	2.1	1.5	2.3	1.5	9.3	5.0	8.9	4.2	7.1	3.3
: 6 ω3	14.0	8.3	15.1	9.8	0.4	Trace	0.9	0.7	2.3	1.4
Anteiso 23 : 0	—	0.7	0.6	Trace	—	—	—	—	0.6	—
: 0	Trace	0.3	—	—	—	—	0.5	0.7	0.6	—
: 2 ω6?	—	—	—	—	Trace	0.2	—	—	—	—
: 3 ω6?	—	—	0.5	0.5	—	Trace	—	—	—	—
Iso 24 : 0	—	—	—	—	0.4	—	0.4	—	—	0.3
: 0	0.5	0.5	0.6	0.3	0.6	—	0.4	0.6	0.8	0.4
: 1	—	—	—	—	—	0.3	—	—	—	—
Iso 25 : 0	—	—	—	—	—	—	0.4	Trace	—	—
: 0	—	—	—	—	—	—	—	0.5	—	—
26 : 0	—	—	—	—	0.6	—	—	—	—	—

*Includes a small amount of 20 : 3 ω3 acid

they were 16:0, iso 18:1, 18:1, 20:5 and 22:6, and in the viscera they were 16:0, 18:1, 20:5 and 22:6. There was no striking difference between the viscera and muscle lipid fatty acid composition of the razor clam and the venus shell.

In the three species of gastropods there was again great similarity between the muscle and the viscera lipid fatty acid compositions. The principal constituent fatty acids of the muscle were 16:0, 18:1. 20:4ω6, 20:5 and 22:5. Those of the viscera were 14:0, 16:0, 18:1, 20:4ω6 and 20:5. There was a definite difference between the muscle and the viscera in the amounts of 14:0 and 22:5, the proportion of 22:5 being greater in the muscle and that of 14:0 in the viscera.

Comparison of the fatty acid composition of the muscle lipids of the bivalves and the gastropods shows a definite difference in 20:4ω6, 22:5 and 22:6, the proportion of 22:6 being highest in the bivalves and that of 20:4ω6 and 22:5 in the gastropods. Shimma et al[78] found the same trends in shellfish muscle lipids. The visceral lipids showed the same tendencies, with no striking differences from the muscle lipids. p 131

The proportion of 20:2 isomeric acids in the shellfish specimens used was 0.8 - 1.2% in the bivalves and 0.4 - 1.1% in the gastropods. There was no great difference between the muscle and viscera lipids of the bivalves of the gastropods, though there was slightly less in the gastropod muscles.

Discussion

20:2 isomeric acids were shown to be present in all the lipids of the muscles and viscera of two species of bivalves and of three species of gastropods. The proportions

were 0.4 to 1.2%, just the same as in former reports[100]. There was a clear difference between bivalves and gastropods in the proportions of 20:4ω6, 22:5 and 22:6 acids, but there was no noticeable difference in the proportions of these isomers.

The gastropods are herbivorous like the sea-urchins and they live in the same area of the sea, but their lipids contain a lower proportion of 20:2 isomer acids, and when the quantity of lipids is also taken into account, the quantity of these isomers in the gastropods is extremely small in comparison with that in the sea-urchins. Thus it is not to be supposed that the 20:2 isomer acids are characteristic of herbivorous, seaweed eating marine animals.

Section 2

Distribution in Echinoderms

The distribution of 20:2 isomeric acids in shellfish has been discussed in section 1.

Sea-urchins are echinoderms, and in this section the distribution of 20:2 isomeric acids in two other echinoderms is discussed.

Experimental methods

Materials

Manamako (trepang, Stichopus japonicus) and Itomakihitode (sea star, Asterina pectinifera) caught along the north shore of Yamaguchi prefecture were used.

Extraction of lipids and fatty acid analysis

The same methods were used as in Chapter 2.

Table 33. Description of the sample examined.

Species	Season	Number of individuals	Body length (cm)	Boby weight (g)	Yield	
					Muscle (%)	Viscera (%)
Manamako	Nov.	5	10.5-18.0	44.0-75.0	92.3	7.7
Itomakihitode	Dec.	7	3.5-4.2*	21.5-34.2	—	—

*Disc length

Experimental results

The lipid contents of the viscera and muscle of Stichopus and of Asterina are shown in Table 34. The lipid contents of all echinoderms are small, the greatest being 0.5%.

Table 34. The lipid content of echinoderms.

	Manamako		Itomakihitode
	Muscle	Viscera	Whole
	%	%	%
Lipid content	0.4	0.4	0.2

The fatty acid composition of these lipids is shown in Table 35. The fatty acid compositions of the muscle and the viscera of Stichopus are very similar, those of Asterina are somewhat different. In all the lipids the principal component fatty acids are 16:0, 18:0, 18:1, 20:1, 20:3ω3, and 20:5, but after these the first important acid in the Stichopus muscle is 16:1 and in the viscera 22:6. p 132

The 20:2 isomeric acids were present in all the the lipids, with 1.0% in the muscle and 0.6% in the viscera of Stichopus and 3.4% in Asterina. p 133

Discussion

The 20:2 isomeric acids were shown to occur in the muscle and the viscera of trepang, Stichopus japonicus, and of the sea star Asterina pectinifera. However the amounts of the acids in Stichopus and in Asterina are quite different. The content of the carnivorous Asterina is high, but that of Stichopus, which eats detritus and diatoms, is small.

The proportion of 20:2 isomeric acids contained in Asterina is lower than that of the sea-urchins but higher than that in the shellfish. The proportion in the Stichopus

Table 35. Fatty acid composition of the lipids from echinoderms.

Fatty acid	Manamako		Itomaki-hitode
	Muscle	Viscera	
	%	%	%
12 : 0	0.6	1.9	0.1
1	0.3	1.1	0.1
Iso 13 : 0	0.5	Trace	—
0	Trace	0.5	0.1
1	0.4	—	0.3
Iso 14 : 0	—	Trace	0.4
Anteiso 14 : 0	0.6	Trace	—
0	1.4	1.0	1.0
1	0.2	0.1	0.1
2	—	—	Trace
3ω3	Trace	0.1	—
4 ?	—	—	Trace
Iso 15 : 0	—	—	1.1
Anteiso 15 : 0	2.4	1.1	—
0	0.9	1.0	0.7
1	Trace	0.3	0.1
2	—	—	0.1
3 ?	Trace	Trace	—
3ω3	Trace	Trace	Trace
5 ?	0.1	0.1	0.4
Iso 16 : 0	0.8	0.6	0.7
0	9.2	5.0	4.7
1ω9	7.6	3.1	1.7
2ω7	—	—	0.4
3ω6	0.2	0.3	—
3ω3	0.1	0.3	—
5 ?	Trace	Trace	Trace
Iso 17 : 0	1.1	0.5	0.6
0	2.0	0.8	0.9
1	0.3	0.3	0.2
3 ?	Trace	0.1	0.7
3ω3	Trace	0.2	Trace
4ω3	—	—	Trace
Iso 18 : 0	0.6	0.5	0.6
0	6.3	4.6	7.7
1ω9	5.7	6.5	8.2
2ω6	1.2	0.9	0.6
3ω6	—	—	0.8
3ω3	0.6	0.3	0.2
18 : 4ω3	Trace	—	Trace
5 ?	Trace	Trace	Trace
Anteiso 19 : 0	0.6	Trace	0.5
0	0.9	0.9	1.2
1	0.6	0.6	1.5
2	0.3	0.2	Trace
4ω3	Trace	Trace	0.1
Iso 20 : 0	Trace	Trace	0.4
0	1.1	1.3	0.6
1ω9	7.0	4.8	18.8
2 Isomer	1.0	0.6	3.4
2ω9	0.4	0.4	0.2
2ω6	0.6	1.9	2.0
3ω6	0.3	0.4	—
3ω3	13.5	16.9	13.4
4ω3	Trace	0.1	0.1
5ω3	10.0	15.9	13.1
Iso 21 : 0	—	—	1.1
Anteiso 21 : 0	Trace	0.6	—
0	1.0	1.2	0.4
1	1.4	1.0	1.1
2	—	—	0.2
3 ?	0.1	0.7	0.8
3ω3	0.3	0.9	—
5ω2	Trace	Trace	0.3
22 : 0	1.5	1.6	0.9
1ω9	1.8	1.6	1.0
2ω6	—	—	0.3
3ω6	Trace	0.3	0.3
3ω3	2.2	0.4	0.3
4ω3	1.6	2.0	0.3
5ω3	1.0	3.1	0.5
6ω3	3.8	6.0	1.8
Anteiso 23 : 0	—	Trace	Trace
0	1.3	0.9	0.4
1	2.2	2.2	0.2
24 : 0	0.4	0.4	0.6
1ω9	2.0	2.0	0.9
25 : 0	—	—	0.9

muscle and viscera was the same as in the shellfish. Thus one can hardly consider that the 20:2 isomeric acids are distinctive fatty acids of the sea-urchins.

Section 3

Sections 1 and 2 have discussed the distribution of the 20:2 isomeric acids in bivalves, gastropods and echinoderms. This section discusses the distribution of the 20:2 isomeric acids in several species of seaweeds which form the diet of sea-urchins.

Experimental Methods

Materials

The seaweeds used were obtained from the north shore of Yamaguchi Prefecture. They were Arame (Eisenia bicyclis) Kajime (Ecklonia cava), Wakame (Undaria pinnatifidia), Hijiki (Hizikia fusiforme), Umitoranoo (Sargassum thunbergii) Nokogirimoku (Sargassum serratifolium), Makusa (Gelidium amansii) and Anaaosa (Ulva pertusa).

Preparation of fatty acid methyl esters

These were prepared as in Chapter 1, section 1.

Fatty acid analysis

The same methods were used as in Chapter 2.

Experimental results

The lipid contents and the fatty acid compositions of the seaweeds are shown in Tables 36 and 37. The lipid contents of the seaweeds were in general small, being 0.1% to 0.6%.

Table 36. The lipid content of seaweeds.

Species	Lipid content
Brown algae	
Arame	0.2
Kajime	0.2
Wakame	0.1
Hijiki	0.3
Umitoranoo	0.6
Nokogirimoku	0.4
Red algae	
Makusa	0.2
Green algae	
Anaaosa	0.1

Table 37. Fatty acid composition of the lipids from seaweeds. (%)

Fatty acid	Arame	Kajime	Wakame	Hijiki	Umito-ranoo	Nokogi-rimoku	Makusa	Anaaosa
12 : 0	0.2	0.1	—	0.2	—	0.2	0.2	0.3
1	0.6	0.7	—	0.1	0.4	—	0.1	Trace
3	—	—	—	0.4	—	—	0.1	Trace
Anteiso 13 : 0	—	—	—	0.3	—	0.2	—	—
0	0.5	0.5	—	0.5	0.3	0.3	0.2	—
1	—	—	—	Trace	0.3	0.3	—	—
Iso 14 : 0	—	0.5	—	0.4	0.4	0.4	—	—
0	5.4	6.1	0.6	4.8	4.9	4.3	6.8	0.9
1	Trace	Trace	—	0.1	0.1	Trace	0.5	0.4
2	0.2	Trace	—	—	—	0.3	0.1	—
3 ?	—	—	—	—	—	—	Trace	0.3
4 ?	—	—	—	—	0.9	—	0.2	—
Anteiso 15 : 0	2.5	1.5	—	1.7	0.6	0.3	—	—
0	0.3	0.6	0.6	0.2	0.2	0.1	0.9	0.7
1	0.1	—	—	0.2	Trace	Trace	—	—
3 ω6	Trace	0.3	—	—	Trace	—	—	—
3 ω3	—	0.3	—	Trace	Trace	—	—	—
4 ?	2.0	1.5	—	2.0	—	Trace	Trace	Trace
4 ω3	Trace	Trace	—	—	0.3	—	—	—
Iso 16 : 0	0.4	0.3	—	0.4	0.5	0.3	—	—
0	19.2	19.3	30.3	31.4	28.3	27.4	70.0	31.8
1 ω7	9.3	9.4	3.2	7.0	7.5	5.3	2.8	6.9
2 ω6	0.6	0.7	Trace	Trace	2.2	0.5	0.6	1.8
2 ?	0.7	0.5	—	0.6	—	—	—	Trace
3 ω3	0.5	0.8	—	0.3	0.2	0.5	Trace	Trace
4 ω6	—	—	—	—	—	—	—	0.3
4 ω3	Trace	—	—	1.1	0.2	Trace	Trace	11.3
5 ?	—	—	—	0.6	0.2	Trace	0.3	—
Iso 17 : 0	—	—	—	—	1.5	—	—	—
0	0.2	0.7	—	1.0	—	0.3	—	—
1	0.3	0.3	—	—	0.6	0.1	0.6	1.8
3	0.5	—	—	—	Trace	Trace	0.4	0.2
5	—	—	0.7	—	—	—	—	—
Iso 18 : 0	0.6	—	—	—	—	0.3	—	—
0	0.6	0.5	1.6	0.6	1.5	0.9	3.0	0.6
1 ω9	10.3	14.0	11.6	11.0	6.8	10.8	5.0	10.6
2 ω6	7.5	6.7	11.7	4.2	4.9	5.1	0.3	8.6
3 ω6	2.1	2.0	—	0.6	0.5	0.4	0.6	1.5
3 ω3	5.0	5.5	6.4	6.7	13.0	9.9	0.3	10.6
4 ω3	5.5	5.8	7.9	1.8	0.8	6.0	1.8	2.9
5 ω3	—	—	—	—	—	—	0.3	0.3
Anteiso 19 : 0	1.1	—	—	—	1.0	—	—	—
0	—	—	—	0.4	—	Trace	Trace	—
2	0.6	—	—	—	—	—	—	—
3 ω6	—	—	—	0.5	0.7	0.4	—	—

Table 37. (Cont'd)

Fatty acid	Arame	Kajime	Wakame	Hijiki	Umito-ranoo	Nokogi-rimoku	Makusa	Anaaosa
19 : 4 ω3	—	—	—	—	—	—	Trace	1.1
5 ?	—	0.7	—	—	—	—	—	0.6
20 : 0	0.6	—	2.0	0.6	—	1.8	0.5	—
1 ω9	6.9	0.4	—	2.4	2.5	1.0	0.6	0.7
2 Isomer	0.2	Trace	Trace	Trace	0.7	Trace	0.2	0.1
2 ω6	0.2	0.6	Trace	Trace	0.3	0.6	0.4	0.4
3 ω6	Trace	0.6	—	—	Trace	0.4	Trace	1.1
3 ω3*	9.6	13.2	14.0	10.3	8.7	13.8	3.2	1.1
3 ?	—	—	—	—	—	—	—	0.3
4 ω3	0.9	1.3	Trace	1.6	1.8	0.8	0.8	0.3
5 ω3	4.3	4.6	3.0	2.6	5.4	4.8	1.3	0.3
21 : 0	—	—	—	—	—	—	—	1.1
3 ω3	—	—	1.0	—	—	—	Trace	0.4
5 ω3	—	—	—	—	—	—	—	0.3
Iso 22 : 0	—	—	—	—	—	—	—	2.5
0	—	—	—	0.3	—	0.7	—	1.0
1 ω9	—	—	—	2.5	1.3	1.0	—	0.6
3 ω3	—	—	—	—	—	—	—	0.5
24 : 1	—	—	—	—	—	—	—	Trace

*May include 20 : 4 ω6 acid

The principal fatty acids in the seaweeds were p 135
14:0, 16:0, 16:1, 18:1, 18:2, 18:3ω3, 18:4ω3 and 20:3ω3. However there were considerable differences between the amounts depending on the species.

The 20:2 isomeric acids were present in all the seaweed specimens, but the contents were low. Thus, the highest was 0.7% of the lipids of S.thunbergii (Umitoranoo), there was 0.1 to 0.2% in the lipids of Eisenia bycyclis (Arame) Gelidium amansii (Makusa) and Ulva pertusa (Anaaosa), and in the other seaweeds only traces were observed.

Discussion

There have been several studies of the fatty acid composition of the lipids in seaweeds[111, 112, 180, 181], but there have been no reports of the presence of 20:2 isomeric acids. Six species of brown algae, one species of red algae and one species of green algae were investigated with the result that the fatty acid composition of all these seaweeds was found to include the 20:2 isomeric acids. The contents were extremely small, and apart from Sargassum thunbergii (Umitoranoo) the greatest amount was 0.2%

Sea-urchins are known to have a preference for seaweed species such as Umitoranoo, Kajime and Nokogirimoku[119,120]. The content of the 20:2 isomeric acids was higher in Umitoranoo than in the other seaweeds, but considering the quantity of lipids in Umitoranoo the content of these isomers was very much less than in the sea-urchins. It would therefore be difficult to suppose that 20:2 isomeric acids present in sea-urchins originate directly in the seaweed diet.

Section 4

Distribution in fish

The 8,11- and 11,14- 20:2 acids are widely distributed in fish[85, 89], but the content of these 20:2 acids is normally low. The presence of 20:2 isomeric acids has not been reported. This section discusses an investigation of the distribution of 20:2 isomeric acids in three species of fish from the same or nearby areas of sea as the sea-urchins. p 136

Experimental methods

Materials

The materials used were the muscle, the liver, the gonads and the viscera (other than the liver, the gonads and the gills) of male and female Sasanohabera (a wrasse, Pseudolabrus japonicus)and Kurodai (a porgy, Acanthopagrus schlegelii) caught off the north coast of Yamaguchi prefecture in October, and of Tsukushitobiuo (a flying fish, Cypselurus heterurus doerderleini)caught off Tsushima, Nagasaki prefecture, towards the end of June.

Lipid extraction and fatty acid analysis

The same methods were used as in Chapter 2.

Experimental results

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The lipid contents of the Sasanohabera, Kurodai and Tsukushitobiuo specimens are shown in Table 38. There are considerable differences in content between the species, with high content in Sasanohabera and Kurodai, and low content in Tsukushitobiuo.

The quantities of the eicosadienoic acids contained in these fish lipids are shown separately for 20:2 isomers, 20:2ω9 and 20:2ω6 are shown in Table 39. The 20:2ω6 acid was widely distributed in the fish lipids. The 20:2ω9 acid was found in the Pseudolabrus and the Acanthopagrus lipids but not in the Cypselurus lipids.

Table 38. The lipid content of marine fishes.

Species	Male				Female			
	Muscle	Liver	Gonad	Viscera*	Muscle	Liver	Gonad	Viscera*
	%	%	%	%	%	%	%	%
Sasanohabera	1.3	7.0	2.3	12.0	1.7	8.2	5.1	17.3
Kurodai	2.7	13.2	2.3	15.5	2.7	3.4	11.1	8.1
Tsukushitobiuo	0.7	4.1	2.4	4.7	0.9	3.6	2.6	3.1

*Excluded liver and gonad

Table 39. Distribution of eicosadienoic acids in the lipids of marine fishes.

			Isomeric 20 : 2	20 : 2ω9	20 : 2ω6
			%	%	%
Sasanohabera	Male	Muscle	0	Trace	0.5
		Liver	0.2	0.2	0.5
		Gonad	Trace	Trace	0.5
		Viscera	0.3	0.2	0.8
	Female	Muscle	0	Trace	Trace
		Liver	0.2	0.1	0.4
		Gonad	Trace	0.1	0.4
		Viscera	0.1	0.1	0.4
Kurodai	Male	Muscle	0	0.8	0.8
		Liver	0	0.4	0.5
		Gonad	0.3	0.3	0.4
		Viscera	0.3	0.4	0.3
	Female	Muscle	0.5	0.4	0.2
		Liver	0.3	0.4	0.1
		Gonad	0.4	0.3	0.2
		Viscera	0	0.8	0.5
Tsukushitobiuo	Male	Muscle	0	—	0.3
		Liver	0	—	0.4
		Gonad	0	—	0.1
		Viscera	Trace	—	0.3
	Female	Muscle	0	—	0.2
		Liver	Trace	—	0.4
		Gonad	0	—	0
		Viscera	0	—	0

The 20:2 isomeric acids were found in the Pseudolabrus lipids other than the muscle, and in the lipids of the male gonads and viscera and of the female muscle, viscera and gonads of Acanthopagrus, but they were not detected in Cypselurus. The content was always low, not more than 0.5%.

Investigations were also made of the presence of 20:2 isomeric acids in the muscle of Wanieso (a lizard fish, Saurida tumbil) and Kinguchi (Yellow croaker, Pseudosciaena manchurica) and in the ovaries of Katsuo (Bonito, Katsuwonus pelamis), and Kihada (yellow fin tuna, Neothunnus macropterus) but they were not found in any of these species.

Discussion

There are no known reports of the presence of 20:2 isomeric acids in fish. However the present studies have shown that they are present in the male and female liver, gonads and viscera of the wrasse Pseudolabrus japonicus, in the male gonads and viscera and the female muscle, liver and gonads of theporgy Acanthopagrus schlegelii and in the male viscera and the female liver of the flying fish Cypselurus heterurus doerderleini.

The 20:2 isomeric acids were found in all the lipids of the sea-urchins, echinoderms, shellfish and seaweeds which were used as specimens, but they were present in less than half of the lipids of the fish. They were found in Pseudolabrus and Acanthopagrus which live in the same region of the sea as the sea-urchins, but were practically absent from Cypselurus which lives nearby. The amounts of the isomers in the fish lipids were less than in the lipids from the other marine plants and animals, being no more than 0.5%

Traces or very small quantities were present in the ovaries and the seminal glands of Pseudolabrus and Acanthopagrus, but there was none in the ovaries or seminal glands of Cypselurus or in the ovaries of the bonito or tuna. These isomers can therefore not be considered to be distinctive fatty acids of the gonads of marine animals.

Summary

An investigation was made of the distribution in marine plants and animals of the 20:2 isomeric acids which the author had found, for the first time in marine animals, in sea-urchin lipids. It was found that the 20:2 isomeric acids were not confined to sea-urchins but were widely distributed among marine plants and animals. They were present in shellfish (razor shell Solen strictus, Venus shell Phacosoma japonica, Abalone Nordotis discus, Top shell Batillus cornutus and Tegula Omphalius pfeifferi carpenteri), in echinoderms (trepang Stichopus japonicus, sea star Asterina pectinifera), in seaweeds (Eisenia bicyclis, Ecklonia cava Undaria pinnatifidia, Hizikia fusiforme, Saragassum serratifolium, Geliduim amansii and Ulva pertusa) and in fish (wrasse, Pseudolabrus japonicus, porgy Acanthopagrus schlegelii and flying fish Gypselurus heterurus doerderleini). However, with the exception of Asterina, the proportion in the lipids was small, being about 1% or less.

It is clear from this that the 20:2 isomer acids are not solely to be found in sea-urchin lipids. Nevertheless, compared with the sea-urchins, the amounts in these marine plants and animals were small.

Chapter 6

The relative amounts of 3,11- and 5,11- eicosadienoic acid in the lipids of various marine plants and animals and in sea-urchins

It was shown in the preceding chapter that the 20:2 isomeric acids were not confined to sea-urchins but were present in various species of marine plants and animals. In Chapter 4, the 20:2 isomeric acids present in sea-urchin gonad lipids were identified as the 3,11 and the 5,11- 20:2 acid isomers, and the difficulty of separating these ismoers by GLC in a packed column was discussed.

The relative amounts of 3,11- and 5,11- 20:2 acids present in the sea-urchins and in the species of marine animal and plant lipids in which the presence of the 20:2 isomers had been verified were therefore investigated by capillary column GLC.

Section 1

The relative amounts present in sea-urchin lipids

It was shown in Chapter 4 that the 20:2 isomer acids present in sea-urchin lipids were the 3,11- and 5,11- 20:2 acids. However the relative proportions of these isomers in the sea-urchin were not found.

The relative proportion of 3,11- and 5,11- 20:2 acids in the sea-urchin gonads and viscera were therefore investigated.

Experimental methods

Materials

The fatty acid mehyl esters of the TL, PL and non-polar lipids (NL) of the sea-urchin gonads and viscera prepared in Chapter 2 were fractionated by argentation TLC and the dienoic acid fraction was used.

Fatty acid analysis

The same methods were used as in Chapter 4, section 4.

Table 40. Relative amounts of eicosadienoic acids in the lipids from gonad and viscera of sea urchin.

Lipid class		Position of double bond			
		[3, 11]	[5, 11]	[8, 11]	[11, 14]
		%	%	%	%
Gonad	TL	30.6	44.6	4.2	20.6
	PL	46.0	37.7	2.4	13.9
	NL	29.8	44.8	3.8	21.6
Viscera	TL	34.8	48.8	4.5	11.9
	PL	45.3	45.9	3.1	5.7
	NL	32.9	47.1	4.9	15.1

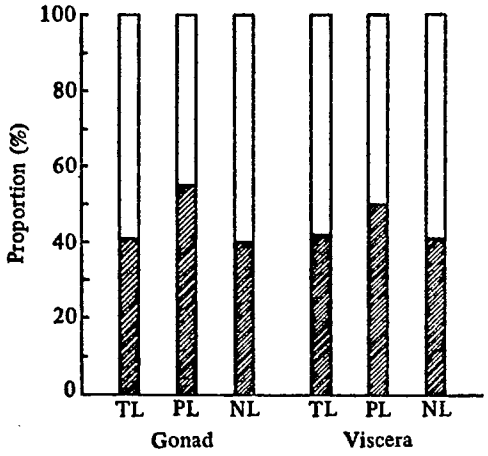

Fig. 22. Proportion of 3,11- and 5, 11-eicosadienoic acids in the lipids from sea urchin.

▨ 3,11-20:2 □ 5,11-20:2

Experimental results

Four 20:2 acid peaks were detected in the gas chromatograms of each of the TL, PL and the NL of the sea-urchin gonads and viscera. From their retention times and from the SF relative to the standard 11,14- 20:2 acid they were identified as the 3,11-, 5,11-, 8,11- and 11-14- 20:2 acids. The relative proportions of these 20:2 acids are shown in Table 40, and those of 3,11- 20:2 and 5,11- 20:2 are shown in Figure 22. In all the lipids of the gonads or viscera, the proportions of 3,11- and 5,11- 20:2 acids were high, and that of 8,11- 20:2 was lowest.

In both the TL and the NL of the gonads and the viscera, the proportion of 5,11- 20:2 acid was larger than that of 3,11- 20:2 acid, reaching about 60% of the 20:2 isomers. Conversely the proportion of 3,11- 20:2 acid was slightly the greater in the PL, being about 55% of the 20:2 isomers in the gonad PL and about 50% in the visceral PL. p 139

Summary

It has been found that sea-urchins contain considerably more 20:2 isomeric acids than other marine plants and animals, and that the 3,11- 20:2 acid and 5,11- 20:2 acids are present in the gonads and the viscera.

There is rather more 5,11- 20:2 acid than 3,11- 20:2 acid in the TL and NL. There are just equal amounts of each in the PL of the viscera, and in the gonad PL there is rather more 3,11- 20:2 acid than 5,11- 20:2 acid. From the fact that there is a high proportion of 3,11- 20:2 acid in the PL, especially the gonad PL, it is inferred that the 3,11-20:2 has some physiological role in sea-urchin reproduction.

Section 2

The relative proportions in marine plant and animal lipids

In the previous section the relative proportions of the 3,11- and the 5,11- 20:2 acids in the sea-urchin gonad and visceral lipids were discussed.

This section discusses the relative proportions of these isomers in the various marine plant and animal lipids which have been shown to contain the 20:2 isomeric acids.

Experimental methods

Materials

Use was made of the dienoic acid fractions obtained in the previous chapter by argentation TLC from marine plant and animal lipids.

Fatty acid analysis

The same methods were used as in Chapter 4, Section 4. p 141

Experimental results

Six peaks produced by 20:2 acids were observed in the gas chromatogram of the Top Shell viscera lipids. Four of these peaks were identified from their retention times and their SF relative to 11,14- 20:2 acid as agreeing with the 3,11-, 5,11-, 8,11-, and 11,14- 20:2 acids found in sea-urchin lipids. The remaining two peaks had retention times of 2.39 and 2.51 and their component fatty acid structure was not determined. In the specimens other than Top Shell viscera, 4 to 6 peaks were observed. The 3,11- and 5,11- 20:2 acids were not present in the Hijiki lipids, the 3,11- 20:2 acid was not present in the Sea star and razor clam muscle and viscera lipid, and the 5,11- 20:2 acid was not present in the Arame, Makusa and Anaaosa lipids. Both the 3,11- and the 5,11- 20:2 isomers were present in all the other lipids.

Table 41. Relative amounts of 3,11- and 5,11-eicosadienoic acids in various marine animals and seaweeds.

Materials			(3, 11)	(5, 11)
Shellfish			%	%
Mategai		Muscle	0	100
		Viscera	0	100
Kagamigai		Muscle	84	16
		Viscera	54	46
Kuroawabi		Muscle	63	37
		Viscera	35	65
Sazae		Muscle	55	45
		Viscera	57	43
Ookoshitakagangara		Muscle	74	26
		Viscera	100	0
Echinoderm				
Manamako		Muscle	49	51
		Viscera	48	52
Itomakihitode		Whole	0	100
Seaweed				
Arame		Whole	100	0
Kajime		Whole	5	95
Wakame		Whole	24	76
Hijiki		Whole	0	0
Umitoranoo		Whole	5	95
Nokogirimoku		Whole	14	86
Makusa		Whole	100	0
Anaaosa		Whole	100	0
Fish				
Sasanohabera	(Male)	Liver	21	79
		Gonad	34	66
		Viscera	43	57
	(Female)	Liver	33	67
		Gonad	79	21
		Viscera	43	57
Kurodai	(Male)	Gonad	56	44
		Viscera	56	44
	(Female)	Muscle	70	30
		Liver	63	37
		Gonad	68	32
Tsukushitobiuo	(Male)	Viscera	47	53
	(Female)	Liver	60	40

The relative amounts of the 3,11- and the 5,11- 20:2 acids in these marine plant and animal lipids are shown in Table 41. The relative amount of the 3,11- 20:2 acid was the greater in the Venus shell muscle, the Abalone muscle, the Tegula muscle and viscera, in Arame, Makusa and Anaaosa, in the female Wrasse gonads, the female Porgy gonads, muscle and viscera, and in the female Flying fish liver. The relative amount of the 5,11- 20:2 acid was the greater in Razor clam muscle and viscera, Abalone viscera, in Sea star, Kajime, Wakame, Umitoranoo, and Nokogirimoku, in male Wrasse liver and gonads and female liver. In the other specimens there was no great difference between the amounts of the two isomers.

Discussion

Except for Hijiki, all the marine animals and plants contained one or both of the 3,11- and 5,11- 20:2 acids.

In general there was more 3,11- 20:2 acid than 5,11- 20:2 acid in the shellfish, though there was more of the 5,11- 20:2 acid in the Abalone viscera, and there was only the 5,11- 20:2 acid in the viscera and muscle of the razor clam.

In the echinoderms, there were about equal amounts of both isomers in the trepang muscle and viscera, but there was only the 5,11- 20:2 acid in the sea star.

In the seaweeds, there was 3,11- 20:2 acid but no 5,11- 20:2 acid in the lipids of Arame, Makusa and Anaaosa, but there was considerably more of the 5,11- 20:2 acid in the other lipids.

In the fish, the lipids of the wrasse other than the female gonads were high in 5,11- 20:2 acid, but in the porgy all the lipids were high in 3,11- 20:2 acid. In the male flying fish viscera lipids the amounts of both isomers were about equal, and there was slightly more of the 3,11- 20:2 acid in the female liver.

These facts show that there was no definite trend for the relative proportions of the 3,11- and the 5,11- 20:2 acids to depend on the species, the diet, the sex or on the muscle, the liver, the gonads or the viscera.

In 1975, Paradis et al[101] reported that the lipids of the American oyster contained the 5,11- and 5,13- 20:2 acids and inferred the presence of 7,10- 20:2 acid, but they did not mention the presence of 3,11- 20:2 acid. The American oyster may thus be considered the same as the razor clam in the absence of 3,11- 20:2 acid. In the present study it was ascertained that apart from the sea-urchins not only the shellfish but many marine animals and plants contained two types of 20:2 acids of unknown structure. That with a relative retention time of 2.51 appears between the 8,11- 20:2 acid and the 11,14- 20:2 acid and may perhaps be inferred to be the 10,13- 20:2 acid. The fatty acid with the relative retention time 2.39 appears between the 3,11- 20:2 acid and the 8,11- 20:2 acid, and is present in a high relative proportion in abalone, top shell, tegula and hijiki and in the liver lipids of the female porgy. Since the relative retention times and SF relative to 11,14- 20:2 acid of the 5,13- and the 7,10- 20:2 acid are not given in the report of Paradis et al, it is not known whether the fatty acids are in fact these two.

Thus the 3,11- and 5,11- 20:2 acids are not solely to be found in sea-urchins but are also widely distributed among marine plants and animals, though the proportions are quite small compared with those in sea-urchins.

After the uniquely large amounts of the isomers in sea-urchins had been recognized, consideration was given to their origin from the points of view of the food chain and of lipid metabolism. Sea-urchins tend to be omnivorous,

but their principal diet is seaweed. The quantity of the isomers in the equally herbivorous gastropods was small, and there was only a small amount in the seaweeds. There was up to 15 times as much of the 5,11- 20:2 acid in the gastropods, and in the sea-urchins 20 times as much in the viscera and 50 times in the gonads. The amount of 3,11- 20:2 acid in the gastropods was about 10 to 100 times as much as in Arame, and in the sea-urchins the amounts reached about 150 times in the viscera and about 300 times in the gonads. p 142

Watanabe et al[142] have shown that the oyster rapidly converts the fatty acids in the lipids of its seaweed diet into its characteristic fatty acid composition. Since the 16:1ω7 acid which forms a large proportion of the seaweed lipids is absent from the sea-urchin lipids, it seems that the sea-urchin also rapidly converts the fatty acids in the lipids of its diet.

It would be appropriate, from this to suppose that the 3,11- and 5,11- 20:2 acids in the sea-urchin originate both directly from the seaweed lipids and from conversion of their fatty acids.

Kayama has studied the lipid metabolism of fish[88,114,115]. There are also a number of studies of fatty acids with unusual positions or with unusual arrangements of double bonds[147,148,173,182]. Fulco et al[182] have shown that Bacillus mageterium differs from normal animals in producing unsaturation, and that it produces 5- 16:1 acid from 16:0, and 5- 18:1 from 18:0. Davidoff et al[173] have shown that slime moulds produce 5,9- 16:2 from 9- 16:2, and 5,11- 18:2 by way of 11- 18:1, and that they also form 5,9- 18:2 from 18:0 by way of 9- 18:2. This desaturation occurs first in position 9 and then in position 5. Ullman et al[147] report the formation of 5,11,14- 20:3 acid from 11,14- 20:2 acid and of 5,11- 20:2 acid from 11- 20:1 acid in the rat. In the same way Egwim et al[148] infer that

the 5,11- or6,11- 20:2 acid present in the rat is formed by carbon chain extension of 9- 18:1 acid and removal of hydrogen from the resulting 11- 20:2 acid. Morris et al[93] inferred that the3-16:1, 3- 18:1, 3,9- 18:2 and 3,9,12- 18:3 acids in Aster alpinus seed oil were formed from 16:0, 18:0, 9- 18:1 and 9,12- 18:2 acids.

In the sea-urchin lipids, the proportions of the 16:0 and the 18:1ω9 acids are lower than in the seaweed lipids, and the proportions of 20:1ω9 acid and of the 20:2 isomers is considerably higher. There is also no great difference between the amounts of the 20:0 isomers*. Thus we may suppose that the fatty acids in the diet lipids are converted in the sea-urchin along the following path, in which the 3,11- and 5,11- 20:2 acids are formed by dehydrogenation of the 11- 20:1 acid.

16:0 ⟶ 18:0 ⟶ 9- 18:1 ⟶ 11- 20:1 ⟶ 3,11- 20:2
16:0 ⟶ 18:0 ⟶ 9- 18:1 ⟶ 11- 20:1 ⟶ 5,11- 20:2

However the reason for the introduction of the second double bond in a position other than in the normal divinylmethane arrangement is not known.

The sea-urchin contains unique sulpholipids which have only been found in plants, and contains them in proportions much greater than the plants. These sulpholipids are concentrated in the gonad membranes, where they are to be supposed to have some function[183]. Sea-urchins also contain alkaloids and trigonelline which have not been found in the animal kingdom but only in plants[184]. The author[9] has reported the presence in sea-urchins of guanylic acid, cytydilic acid and uridylic acid but not of inosinic acid, and also the presence of large quantities of not only 5'- nucleotides but 3' nucleotides.

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* Sic, but presumably 20:2. Translator.

These facts show that the sea-urchin has a peculiar nucleotide and fatty acid composition which differs from both marine plants and animals, and contains a number of compounds present only in plants. Taking into consideration the presence of the 5,11- 20:2 acid in the seed oils of land plants, the presence of large amounts of both 3,11- and 5,11- 20:2 acids in sea-urchin lipids is of great interest for comparative biochemistry.

Summary

The relative proportions of the 3,11- and 5,11- 20:2 acids in sea-urchin and various marine plant and animal lipids were measured.

The 3,11- and 5,11- 20:2 acids were present in both the gonads and the viscera of the sea-urchin, and in general the proportion of 5,11- 20:2 acid was slightly higher. However the proportion of 3,11- 20:2 acid was slightly higher in the gonad PL.

One or both of the 3,11- and 5,11- 20:2 acids were present in the lipids from all the marine plants and animals other than Hijiki. The proportion of 3,11- 20:2 acid was generally greater in the shellfish, though there was no 3,11-20:2 acid in the razor clam. In the echinoderms, the amounts of the two acids were about equal in the trepang lipids, but there was no 5,11- 20:2 acid in the sea star lipids. The proportion of the 5,11- 20:2 acid was the higher in many of the seaweeds, but it was not present in the lipids from Arame, Hakusa and Anaaosa. in the fish, the proportion of 5,11- 20:2 acid was higher in the wrasse, and of 3,11- 20:2 acid in the porgy. The proportions of the two isomers were about equal in the male flying fish viscera lipids, but proportion of 3,11- 20:2 acid was slightly greater in the female liver lipids.

It was evident from this that the relative proportions of the two isomers shows no definite trend according to species, diet or sex.

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By comparing the amounts of 3,11- and 5,11- 20:2 acids in sea-urchins and seaweed, it appears that the 3,11- and 5,11- 20:2 acids in the sea-urchin arise not only from accumulation of those originally in the seaweed lipids, but also by conversion of the seaweed lipid fatty acids in the sea-urchin body. From the relative composition of the sea-urchin lipids and the seaweed lipids the following conversion path was inferred

$$16{:}0 \longrightarrow 18{:}0 \longrightarrow 9\text{-}\ 18{:}1 \longrightarrow 11\text{-}\ 20{:}1 \begin{cases} \nearrow 3,11\text{-}\ 20{:}2 \\ \searrow 5,11\text{-}\ 20{:}2 \end{cases}$$

It is also inferred, from the high relative proportion of the 3,11- 20:2 acid in the PL, especially the gonad PL, that it has some physiological function in sea-urchin reproduction.

Chapter 7
Recapitulation

The component fatty acid composition of a number of marine plants and animals was found by the application of GLC fatty acid analysis. In order to obtain an exact fatty acid composition it is necessary to use two or more differently directed methods of identification and to combine GLC with other chemical means of separation. This combination is particularly suitable for the detailed fatty acid analysis of trace components and previously unknown components.

The fatty acid composition of the sea-urchin gonad lipids is more complicated than that of fish oils, and it is difficult to obtain an exact composition from the results of GLC alone. The usefulness of the joint application of urea fractionation or argentation TLC with GLC was investigated.

By the joint use of urea fractionation trace components could be detected and a fairly detailed fatty acid composition could be obtained, but separation by this method on the basis of the degree of unsaturation was by no means satisfactory.

Argentation TLC however produced good separation according to the degree of unsaturation. By this means it was possible to obtain an exact composition, and to identify 76 fatty acids including those present in small quantities or as traces and a number of fatty acids which were scarcely known to occur in marine plant and animal lipids.

By the use of the joint methods of analysis it was found that a number of the sea-urchin lipids contained considerable quantities of 20:2 isomeric acids not known to be present in marine animals and plants.

The distribution of the 20:2 isomeric acids among the sea-urchin lipids was investigated. The isomers were distributed among all classes of lipids from the sea-urchin

gonads and viscera, and formed 5.0 to 7.5% of the gonad lipids and 5.4 to 8.3% of the viscera lipids. 70% of the total 20:2 isomer acids present in the sea-urchin was in the gonads, and 30% in the viscera, and in both there were large amounts in the TG and the PL.

Since it was presumed from the retention times of these 20:2 isomer acids and from their SF that they had unusual double bond positions, they were isolated and refined and it was established that they were 20:2 acids.

The process used involved wintering, reduced pressure distillation, urea fractionation, silver nitrate impregnated silicic acid column chromatography, n-undecane impregnated silica gel TLC, silicic acid column chromatography and silica gel TLC. The 20:2 isomeric acids were isolated and refined from the methyl esters of the fatty acids in the gonads of Murasakiuni, Anthocidaris crassispina, and fractions containing about 98% of the isomers were prepared. Using these fractions, it was next established p 144 by carbon chain length analysis, measurement of the number of unsaturated bonds, and the relative retention times and SF in GLC, that these 20:2 isomer acids differed from the 8,11-, 11,14-, 10,13- or 14,17- 20:2 acids.

The positions of the double bonds in the 20:2 isomers were then determined by mass spectrography, by oxidative cleavage with sodium periodate and potassium permanganate, by alkaline isomerization, by capillary column GLC and by the oxidative cleavage of the hydrazine partial reduction products.

It was found that there were two 20:2 acids with unusual double bond arrangements, and their structure was determined to be 3,11- 20:2 and 5,11- 20:2. This was the first time that the 3,11- 20:2 acid had been found in nature. The 5,11- 20:2 fatty acid had not been found in marine plants and animals except in small quantities in shellfish.

The distribution of 20:2 isomeric acids in marine plants and animals was therefore investigated. The 20:2 isomeric acids were found to be present in shellfish, echinoderms, seaweeds and fish, and to be widely distributed in marine plants and animals.

It was thus evident that the 20:2 isomers were not distinctive components of the sea-urchins. However, the quantities found were very much less in the marine plants and animals than in the sea-urchins.

Consideration was next given to investigation the relative proportions of the 3,11- and the 5,11- 20:2 isomers in the sea-urchins and in the marine plants and animals in which the 20:2 isomers had been found.

Both the 3,11- and the 5,11 20:2 acids were present in the sea-urchin gonads and viscera. In the TL and the NL theproportion of 5,11- 20:2 acid was slightly greater, but in the gonad PL the proportion of 3,11- 20:2 was slightly greater.

Either one or both of the 3,11- and the 5,11- 20:2 acids was present in all the marine plants and animals except Hijiki. In the shellfish the proportion of 3,11- 20:2 acid was higher, though there was no 3,11- 20:2 acid in the razor clam lipids. In the echinoderms, the proportions of the two isomers were about equal in the trepang, but only the 5,11- 20:2 acid was present in the sea star. The proportion of 5,11- 20:2 acid was higher in the seaweeds, though there was no 5,11- 20:2 in Arame, Makusa or Anaaosa lipids. Among the fish, the proportion of 5,11- 20:2 was higher in the wrasse, but that of 3,11- 20:2 was higher in the porgy. In the male viscera of the flying fish the proportions of the two isomers were about equal, but there was rather more 3,11- 20:2 acid in the female liver lipids.

Thus no tendency of the relative proportions of the isomers to depend on species, diet or sex could be established.

From comparisons of the fatty acid composition of the sea-urchin lipids and of the amounts of 3,11- 20:2 and 5,11- 20:2 acids, it was concluded that these isomers in the sea-urchins originated directly from the seaweed lipids, and that in addition to being accumulated by the sea-urchin they were formed in the sea-urchin body by conversion of the seaweed lipid fatty acids along a path which was presumed to be the following.

$$16{:}0 \longrightarrow 18{:}0 \longrightarrow 9\text{-}\ 18{:}1 \longrightarrow 11\text{-}\ 20{:}1 \begin{cases} \nearrow 3,11\text{-}\ 20{:}2 \\ \searrow 5,11\text{-}\ 20{:}2 \end{cases}$$

Since these isomers are present in uniquely large amounts in the sea-urchins, it is presumed that they are associated with the sea-urchin physiology. From the fact that the relative amount of the 3,11- 20:2 acid is higher in the PL, particularly the gonad PL, it is inferred that it is associated with the physiology of reproduction of the sea-urchin.

Fatty acids with unusual bond arrangements occur in quantity in land plant oils, but do not occur in quantity in animal lipids. The fact that large quantities of the 3,11- and 5,11- 20:2 acids occur in sea-urchin lipids is of great interest for comparative biochemistry.

Acknowledgements

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Summary

The component fatty acids of a vast number of marine organisms have been clarified since the gas-liquid chromatographic technique came to be applied to fatty acid analysis in biochemical research. However, most of these investigations have dealt with the composition of major fatty acids.

The component fatty acids of the gonad lipid from sea urchin are very complex as compared with those of other marine animals, and show severe overlapping of many peaks in gas-liquid chromatography (GLC) on DEGS column. The elucidation of the fatty acid composition of the gonad lipid will be of great value not only from the viewpoint of lipid research of marine organisms, but also from the standpoint of food chemistry.

Urea-fractionation technique or argentation thin-layer chromatography was, therefore, adapted to preliminary fractionation of the mixed fatty acid methyl esters according to the degree of unsaturation for the purpose of detailed studies of component fatty acids of the gonad lipid, including the detection of minor components and unusual fatty acids. And then the fractions obtained were applied to GLC.

By means of GLC combined with urea-fractionation technique, it was found out that fatty acid composition of the gonad lipids from sea urchins, *Strongylocentrotus pulcherrimus, Anthocidaris crassispina, Strongylocentrotus nudus* and *Pseudocentrotus depressus,* differed from that of marine fish oils in comparatively high percentages of 14:0, 18:4, 20:1 and 20:3 acids, and in a high proportion of long-chain fatty acids in spite of the fact that C_{22} polyunsaturated fatty acids and longer chain ones than C_{24} fatty acid were not detected. The differences in the fatty acid composition among the different species and sources of sea urchin were not distinct. Through urea-fractionation technique, however, the total esters could not be resolved efficiently enough for quantification of the components.

The combination of GLC and argentation TLC showed satisfactory separation, giving detailed and accurate fatty acid composition of the gonad lipid from a sea urchin, *A. crassispina.* A total of 76 fatty acids, including a number of branched-chain fatty acids, polyunsaturated odd-numbered ones and positional isomers of unsaturated fatty acids, were tentatively identified on the basis of chromatographic behavior. The major components were 14:0, 16:0, 18:1ω9, 20:1ω9, 20:3ω3 and 20:5ω3 acids.

In these experiments isomeric 20:2 acid which had not been previously reported in marine source, was detected in fairly large quantities in the gonad lipids of all sea-urchin species examined.

This finding led the author to the clarification of the distribution of the isomer in the lipid classes of a sea-urchin species, *S. pulcherrimus.* The lipids from the gonads and the other viscera were analysed for their fatty acid components by GLC, after being fractionated into the main lipid classes by silicic acid column chromatography. The isomer is widely distributed in all the lipid classes from both the gonads and the other viscera. Its percentages in the gonads and the other viscera ranged from 5.0 to 7.5%, and 5.4 to 8.3%, respectively. Furthermore, much of the isomer was present in only two classes, i.e., polar lipids and triglycerides, in the amounts of about 95% of the total weight of the isomer in the whole visceral organs. Its quantity in the gonads amounted to about 70% of the total.

The isomeric 20:2 acid was separated in considerably high purity from the gonad lipid of a sea-urchin species, *A. crassispina,* by using a variety of analytical techniques. The mixed fatty acid methyl esters were subjected to wintering, fractional vacuum distillation and urea fractionation. A fraction rich in the isomer was thus obtained. The fraction was purified further by argentation column chromatography, TLC on silica gel impregnated with *n*-undecane, silicic acid column chromatography and silica gel TLC. Two fractions of comparatively high purity of the isomer were obtained after the final process, the purities, expressed as percentages according to GLC peak areas, being 97.8% and 98.4% respectively.

In addition to the results of GLC analysis, both chain length analysis and hydrogen uptake measurement were carried out with one of the final products. Quantitative hydrogenation of an aliquot of the isomer produced methyl arachidate, its hydrogen uptake being 98.8% of the theoretical value for 20:2 acid. Accordingly, the isomer was confirmed to be a dienoic fatty acid with straight chain of 20 carbons. Relative retention time and separation factors of the isomer were not same as those of 8,11-20:2 acid. These observations gave evidence that the acid is isomeric 20:2 acid itself.

The double bond positions in the isomeric 20:2 acid were determined by means of mass spectometry, periodate-permanganate oxidation, alkaline isomerization, GLC analysis on capillary column and hydrazine partial reduction followed by periodate-permanganate oxidation.

The isomeric 20:2 acid of the lipid from sea urchin was proved to consist of two positional isomers on the basis of GLC analyses of periodate-permanganate oxidation products of the final two products. One of the isomers yielded mainly nonanoic and suberic acids on oxidative cleavage, and gave no characteristic absorption of conjugated dienes in the UV region after alkaline isomerization. These findings indicated that the isomer was a hitherto unknown acid, i.e., 3,11-20:2 acid. The other isomer was identified as 5,11-20:2 acid which is rarely demonstrated in marine organisms. It produced nonanoic, glutaric and adipic acids on direct oxidative cleavage and nonanoic, pentadecanoic, glutaric and undecanedioic acids on oxidative cleavage of its partial reduction products.

It would be interesting to find out if such isomers are distributed in the lipids of marine organisms. For that purpose, the distribution of isomeric 20:2 acids in various marine organisms was investigated. The isomers were proved to be present not alone in the lipids of sea urchin but in other lipids from many marine animals and seaweeds.

In the shellfishes examined, the isomers were widely distributed in both the muscular and visceral lipids from all the species with a range from 0.4 to 1.0%. No distinct difference in the percentages was appreciable between the following two classes, i.e., two bivalves, *Solen strictus* and *Phacosoma japonica,* and three gastropods, *Nordotis discus, Batillus cornutus* and *Omphalius pfeifferi carpenteri.*

Of the echinoderms, trepang, *Stichopus japonicus,* contained the isomers in a level similar to those of shellfishes in the muscular and visceral lipids, but the lipid of sea star, *Asterina pectinifera,* showed a high percentage of the isomers second to sea urchin among the marine organisms examined.

The lipids from the seaweeds, six of the brown algae, *Eisenia bicyclis, Ecklonia cava, Undaria pinnatifida, Hizikia fusiforme, Sargassum thunbergii* and *Sargassum serratifolium,* one of the red algae, *Gelidium amansii,* and one of the green algae, *Ulva pertusa,* contained

the isomers in traces or in very low percentages.

In the marine fishes, the distribution of the isomers in the muscular, liver, gonad and visceral lipids of both the male and female varied widely with the species. Labridae, *Pseudolabrus japonicus,* and sparidae, *Acanthopagrus schlegelii,* contained the isomers in traces or very low percentages in the lipids from several different tissues. Flying fish, *Cypselurus heterurus döderleini,* however, contained the isomers in traces in the lipids from only two different tissues.

Their quantity was much smaller in these marine organisms than in sea urchin. These findings disclosed that the isomeric 20:2 acids were no characteristic constituent of sea urchin.

The proportion of 3,11- and 5,11-20:2 acids in the lipids of sea urchin, *S. pulcherrimus,* and of marine organisms in which the isomeric 20:2 acids were detected, was estimated. All the lipids of sea-urchin gonads and viscera were proved to contain both 3,11- and 5,11-20:2 acids. In the total lipids and non-polar lipids, 5,11-20:2 acid was present in a slightly higher proportion than 3,11-20:2 acid, but in the polar lipid of gonads the relative proportion was reversed.

Shellfish lipids showed a high proportion of 3,11-20:2 acid and the lipids of tegula, *O. p. carpenteri,* contained only the acid. However, the acid was present neither in the muscular lipid nor in the visceral lipid from razor clam, *S. strictus.*

Of the echinoderms, trepang, *S. japonicus,* showed about equal proportion of the two acids in the muscular and visceral lipids, but the lipid of sea star, *A. pectinifera,* contained only 5,11-20:2 acid.

Seaweed lipids showed a high proportion of 5,11-20:2 acid, but the acid was not detected in the lipids of *E. bicyclis, G. amansii* and *U. pertusa.*

Of the marine fishes, *P. japonicus* and *A. schlegelii,* showed a high proportion of 5,11-20:2 acid and 3,11-20:2 acid in the lipids, respectively. Flying fish, *C. h. döderleini,* showed about equal proportion of the two acids in the visceral lipid of males and a slightly high proportion of 3,11-20:2 acid in the liver lipid of females.

The 3,11- and/or 5,11-20:2 acids were found not only in sea-urchin lipids but also in various lipids of marine animals and seaweeds examined, excepting *H. fusiforme.* There was no regular pattern relating to any of the taxonomical groups, tissues, feeding habits or sexes in the proportion of the acids. However, sea urchin was distinct from other marine organisms with large amounts of the structurally unusual fatty acids, 3,11- and 5,11-20:2 acids.

The origin of the acids in sea urchin, therefore, had to be considered. Sea urchin feeds mainly on seaweed, although it has a omnivorous tendency. Essential difference was found in the amounts of 3,11- and 5,11-20:2 acids between sea urchin and seaweeds. Thus, the amounts of 3,11- and 5,11-20:2 acids in the gonads were respectively about 300 and 50 times as much as those in seaweeds which contained each acid in largest amounts among their species, and these amounts in viscera about 150 and 20 times of those in the seaweeds, respectively.

This difference between herbivorous gastropods and seaweeds was not so distinct as that between sea urchin and seaweeds. In view of these differences, it is almost unlikely that the acids derived directly from diets are accumulated remarkably only in sea urchin. Accordingly, biochemical conversion of the dietary fatty acids in sea urchin may be

responsible for the accumulation of greater part of the acids.

The lipid from sea urchin showed lower percentages of 16:0 and 18:1ω9 acids, and higher percentages of 20:1ω9 and isomeric 20:2 acids as compared with the lipids of seaweeds, but the percentage of 20:0 acid was not so distinctly different in both the lipids.

The possibility of biosynthesis of fatty acids with double bonds at the 3,4- or 5,6-position has been presumed or proved in *Aster alpinus*, slime mold and rat.

The following probable conversion pathway may be analogized from the basis of above-mentioned findings for 3,11- and 5,11-20:2 acids in sea urchin.

$$16{:}0 \longrightarrow 18{:}0 \longrightarrow 9\text{-}18{:}1 \longrightarrow 11\text{-}20{:}1 \begin{cases} \nearrow\ 3,11\text{-}20{:}2 \\ \searrow\ 5,11\text{-}20{:}2 \end{cases}$$

Fatty acids containing non-methylene interrupted arrangement of double bonds have been previously demonstrated in terrestrial plant oils, and were recently found in some marine shellfish species.

It is noteworthy, however, that the sea urchin contains fatty acids with hexamethylene- and tetramethylene-interrupted arrangement of double bonds, 3,11- and 5,11-20:2 acids, in larger amounts than any other marine organism. It is possible to consider that 3,11-20:2 acid may play a physiological role associated with reproduction of sea urchin.

List of animals and plants

Sea-urchins	
Murasakiuni (Purple sea-urchin)	Anthocidaris crassispina
Akauni (Red sea-urchin)	Pseudocentrotus depressus
Bafununi (Horsedung sea-urchin)	Strongylocentrotus pulcherrimus
Kitamurasakiuni (Northern purple sea-urchin)	Strongylocentrotus nudus
Bivalves	
Sazae (Top shell)	Batillus cornutus
Kuroawabi (Abalone)	Nordotis discus
Ookoshitakagangara (Tegula)	Omphalius pfeifferi carpenteri
Gastropods	
Mategai (Razor clam)	Solen strictus
Kagamigai (Venus shell)	Phacosoma japonica
Echinoderms	
Manamako (Trepang)	Stichopus japonicus
Itomakihitode (Sea star)	Asterina pectinifera
Seaweeds	
Arame	Eisenia bicyclis
Kajime	Ecklonia cava
Wakame	Undaria pinnatifidia
Hijiki	Hizikia fusiforme
Umitoranoo	Sargassum thunbergii
Nokogirimoku	Sargassum serratifolium
Makusa	Gelidium amansii
Anaaosa	Ulva pertusa

Fish

Sasanohabera (Wrasse)	Pseudolabrus japonicus
Kurodai (Porgy)	Acanthopagrus schlegelii
Tsukushitobiuo (Flying fish)	Cypselurus heterurus
Wanieso (Lizard fish)	Saurida tumbil
Kinguchi (Yellow croaker)	Pseudosciaena manchuria
Katsuo (Bonito)	Katsuwonus pelamis
Kihada (Yellow fin tuna)	Neothunnus macropterus

Shako

This may refer either to the giant clam Tridachna gigas or to the mantis shrimp Squilla oratorio. I have not been able to consult the original reference. Translator.