ether linkage, were not measured by the modified procedure (see Table V). Reaction conditions of the alumina DNP-hydrazine column are not rigorous enough to effect hydrolysis of the bound carbonyls.

Reproducibility of the method is satisfactory and no difficulties were encountered in analyzing various fats and oils, with the exception of marine oils. Attempts to obtain reproducible values on oxidized marine oils failed. The main problem preventing accurate analysis was due to the formation of an interfering red color when obtaining blank readings on oil samples in benzene-ethanolic KOH solutions.

In previous work Lillard and Day (1) found a high correlation between the concentration of volatile alk-2-enals and the oxidized flavor intensity of milk fat. Since the modified Pool and Klose procedure appears to measure the volatile carbonyls, it is believed that values obtained for the alk-2-enals will show a comparable correlation with oxidized flavor intensity of milk fat.

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On the Structure of Highly Unsaturated Fatty Acids of Fish Oils by High Resolution Nuclear Magnetic Resonance Spectral Analysis

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Abstract

Methyl esters of highly unsaturated fatty acid concentrates were prepared from fish oils by the urea-adduct method. The nuclear magnetic resonance spectra of the mixed esters and some related pattern compounds were analyzed. As a result, it was concluded that the structure of highly unsaturated fatty acids has divinylmethane arrangement of the ethylenic bonds and no divinylethane arrangement, and that one methylene group is present between the terminal methyl group and the double bond located at the remotest position from a carboxyl group in the acids.

Introduction

FISH OILS, especially marine fish oils, generally contain great portions of highly unsaturated fatty acids having more than three double bonds. A great number of works have been made regarding the structure of the acids. Many contributions recently worked out suggest that the structure of highly unsaturated fatty acids are classified into two types: The first (structure I) is the structure comprising solely divinylmethane arrangement of the ethylenic bonds

$$(CH{=}CH{-}CH_2{-}CH{=}CH{-}).^2$$

The arrangement is identical to that in linoleic and linolenic acids. The second (structure II) is the structure having solely divinylethane arrangement

$$(-CH{=}CH{-}CH_2{-}CH_2{-}CH{=}CH-)$$

or having both divinylethane and divinylmethane arrangements.[3] However, since no authors have reported that highly unsaturated fatty acids of structure I and II occur together in a fish oil, it suggests that the acids occurring in the same sample will have one of the two types of structure.

It is the purpose of this work to clarify which type of structure would be correct, by analyzing the results obtained by a non-destructive analytical method or the nuclear magnetic resonance technique using methyl esters of highly unsaturated fatty acid concentrates.

The structures of the above two types have hitherto been determined by chemical method whereby isolation of highly unsaturated fatty acid in a pure state was indispensable. Because of readiness in autoxidation and isomerization of highly unsaturated fatty acids, the isolation from fish oil, including various kinds of highly unsaturated fatty acids, is complicated and difficult. Hence, in this work the samples containing various kinds of highly unsaturated fatty acids have been concentrated from fish oils without isolating individual acids, to determine the type of structure by utilizing the nuclear magnetic resonance spectral analysis.

The resonance frequency of the hydrogen atom in the nuclear magnetic resonance spectrum depends

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[2] An excellent review of the previous works has been published by O. Notevarp in "Fish As Food," edited by G. Borgstrom, Academic Press, Inc., New York, N.Y., 1961, Vol. 1, pp. 260–263.

[3] Excellent reviews of the previous works have been published by Tsuchiya T. in "Fish As Food," edited by G. Borgstrom, Academic Press, Inc., New York, N.Y., 1961, Vol. 1, pp. 215–218 and also by T. P. Hilditch, J. Chem. Soc., 243 (1948).

upon its chemical environment. Hence, if a sample would be the mixture of various highly unsaturated fatty acid esters, the chemically equivalent atoms in each ester should have the signal at the same magnetic field. Considering from the above conception, it is expected that the spectrum of the mixture of various highly unsaturated fatty acid esters have the signals raised from the following functional groups in each ester: olefinic group; carbomethoxy group; methylene group adjacent to a carbonyl group; methylene group adjacent to a single double-bonded carbon; ordinary methylene group; terminal methyl group; a methylene group adjacent to two double-bonded carbons in the structure having the divinylmethane arrangement; and dimethylene groups between two double bonds in the structure having the divinylethane arrangement. Hence, when special attention would be concentrated against the signals of olefinic groups, a methylene group adjacent to two double-bonded carbons, and dimethylene groups between two double bonds, it is considered possible to presume the type of structure of highly unsaturated fatty acid.

In this work, the samples composed of highly unsaturated fatty acid esters were prepared from mixed fatty acid esters of fish oils by using the urea-adduct method which allows the following advantages: It avoids isomerization and facilitates preparation of highly unsaturated fatty acid esters containing no oxidized products since the oxidized products do not form urea adducts (1).

Nuclear magnetic resonance spectra of the samples from fish oils and some related pattern compounds were measured. The arrangement of the ethylenic bonds in the highly unsaturated fatty acid chain was presumed from the basis of the spectra and chemical characteristics of the samples. Furthermore, the position of a double bond situated at the nearest position from terminal methyl group was discussed from the pattern of terminal methyl group in the spectra of highly unsaturated fatty acid esters as compared with that of the group in the spectra of methyl esters of stearic, oleic, linoleic, and linolenic acids and of pentene-1 and hexene-1.

Experimental

Measurement of n.m.r. Spectra. The measurements were made with a Varian DP 60 Spectrometer operating at 60 MC. The spectra of all samples were obtained at 25–28C in 5 mm i.d. sample tubes, which were spun. The spectra of the fatty acid methyl esters were given in CCl_4 solutions containing approximately 25% of the esters, and the spectra of 1,5-hexadiene, pentene-1, and hexene-1 were obtained on pure liquid samples. Benzene was used as the internal reference except in the case of pentene-1, and for convenience, the chemical shifts (see Fig. 5) are given on the τ-value (τ-value of the proton resonance in benzene: 2.73 ppm). The amplitudes of oscillating magnetic field were 60–73 db and sweep rates 4.84–4.73 c.p.s./sec. Area intensity of the signals was measured by a Varian NMR integrator.

Cuttlefish Ester Sample (No. 1). The oil of cuttlefish (*Ommastrephus pacificus,* A). from Hokkaido neighboring waters has the following characteristics: N_D^{25} 1.4812; acid number 16.6; saponification number 182.2; iodine number [4] (Wijs) 200.9. The oil was shaken with 2-N alcoholic KOH solution at room temp for 5 hr in a dark-brown glass bottle filled with nitrogen gas.[5] The soap solution was then diluted with water and extracted with diethyl ether to remove unsaponifiable matters. Fatty acids were obtained from the soap by adding diluted hydrochloric acid. Fifty g of the mixed methyl esters prepared from the acids were dissolved into 500 ml of methanol containing 80 g of urea. The solution was then allowed to stand for about 5 hr at 18–20C. The crystal deposited was filtered off on a Buchner funnel. Forty g of urea was added to the mother liquor and dissolved by gentle heating. Then the solution was allowed to stand for about 5 hr at 18–20C. The treatment with urea was repeated five times at 18–20C, once at 7–8C, and once at about 0C. The final urea adduct deposited at 0C was treated with diluted hydrochloric acid and methyl ester liberated was then extracted with diethyl ether. The sample extracted was composed of highly unsaturated fatty acid esters with the following chemical characteristics and its yield was 4.8 g: saponification number 171.6; and iodine number 409.1. The ultraviolet absorption spectrum of the sample showed the trace of conjugated diene.[6]

Cuttlefish Ester Sample (No. 2). Fifty g of the mixed methyl esters of cuttlefish fatty acids, mentioned above, were treated by the same urea-adduct method as described in the case of the cuttle fish ester sample (No. 1) five times at 18–19C and finally once at about 0C. Nine g of the sample with the following chemical characteristics were obtained from the final urea adduct: saponification number 172.9; iodine number 405.9.

Cuttlefish Ester Sample (No. 3). Seven g of the cuttlefish ester sample (No. 2) were submitted to distillation under a reduced pressure of 2 mm Hg and 1.5 g of fraction distilled at 205C with the following chemical characteristics obtained: saponification number 168.1; iodine number 414.3.

Mackerel Ester Sample. Mixed methyl esters were prepared from the internal organ oil of mackerel (*Scomber japonicus,* H.) captured near Chiba Prefecture (characteristics of the oil: N_D^{25} 1.4756; acid number 4.6; saponification number 186.7; iodine number 161.2). Fifty g of the esters were treated by the same urea-adduct method as that in the case of the cuttlefish ester sample (No. 1) and 3.5 g of the sample with the following characteristics were obtained: saponification number 172.0; iodine number 372.5.

Sardine Ester Sample (A). Mixed methyl esters prepared from oil of sardine (*Amblygaster melanostictum,* T. & S.) captured near Hokkaido (characteristics of the oil: N_D^{40} 1.4713; acid number 22.1; saponification number 192.3; iodine number 169.9) were treated by the same urea-adduct method as that in the case of the cuttlefish ester sample (No. 1) and 3.6 g of the ester of highly unsaturated fatty acid concentrates were obtained. The ester was subjected to absorption chromatography on alumina adsorption column (alumina: aluminium oxydatum standard made by E. Merck AG.), using redistilled petroleum ether, boiling range 40–65C as eluant; and 2.5 g of refined ester with the following characteristics were obtained: saponification number 178.4; iodine number 390.5.

Sardine Ester Sample (B). The mixed methyl esters were prepared from the oil of sardine (*Amblygaster melanostictum,* T. & S.) captured near Chiba Prefecture (characteristics of the oil: N_D^{40} 1.4732; acid

[4] Iodine numbers of all samples were measured by Wijs' Method.

[5] In order to prevent the oxidation of the samples, the following operations were carried out under nitrogen gas, when possible.

[6] All esters prepared in this work have trace or none of conjugated diene, and the amount of conjugated diene was negligible to discuss.

number 12.3; saponification number 182.3; iodine number 176.6) by the same procedure as that in the case of the sardine ester sample (A). Chemical characteristics and yield of the sample obtained were as follows: yield 3.3 g.; saponification number 168.3; and iodine number 409.0.

Methyl Stearate. The ester was prepared from commercial product by solvent crystallization using 95% ethyl alcohol. Characteristics of the ester were as follows: mp 39.0C; saponification number 188.5 (theoretical 188.1) and iodine number 0.0 (theoretical 0).

Methyl Oleate. Lithium salts of the liquid fatty acids separated from Tsubaki (*Camellia Japonica,* L.) oil fatty acids by Twitchell's lead salt-alcohol method were repeatedly crystallized from 50% ethyl alcohol solution. Methyl ester prepared from the finally crystallized salt were refined by vacuum distillation. Characteristics of the refined ester were as follows: saponification number 190.0 (theoretical 189.2) and iodine number 85.1 (theoretical 85.6).

Methyl Linoleate. A vacuum distilled fraction obtained from safflower seed oil methyl ester was refined by low temp solvent crystallization and by urea-adduct method. Characteristics of the refined ester were as follows: saponification number 190.2 (theoretical 190.7) and iodine number 172.1 (theoretical 172.5).

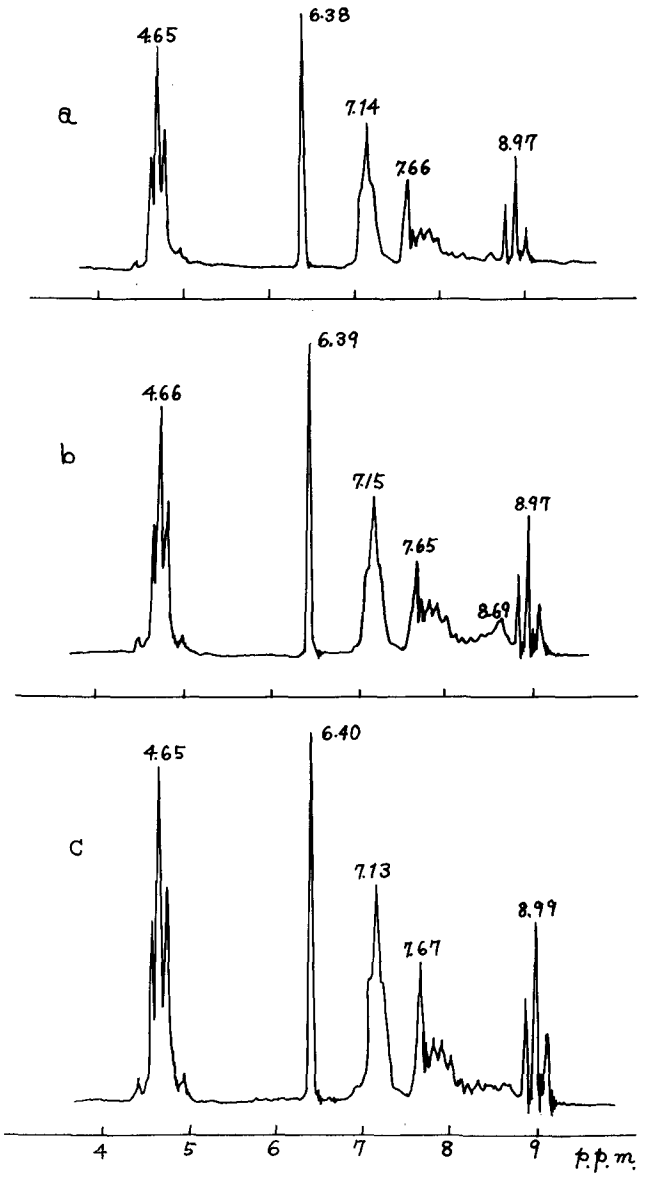

FIG. 1. n.m.r. spectra of fish oil ester samples: (a) cuttlefish (No. 2); (b) mackerel; (c) sardine (A).

Methyl Linolenate. The ether insoluble bromide prepared from linseed oil fatty acids was debrominated by conventional method. Methyl ester thus obtained was refined by vacuum distillation. Characteristics of the refined ester were as follows: saponification number 191.5 (theoretical 192.0) and iodine number 259.0 (theoretical 260.6).

1,5-Hexadiene, Pentene-1, and Hexene-1. These materials were commercial products (Tokyo Kasei Kogyo Co., Ltd.). The gas chromatograms showed no impurities.

Results and Discussion

The nuclear magnetic resonance (n.m.r.) spectra of the samples prepared from fish oils are shown in Figure 1. Since the curves of three cuttlefish ester samples are practically identical and similar in the curves of two sardine ester samples, the spectra of cuttlefish ester samples (No. 1 and 3) and sardine ester sample (B) were omitted. Moreover, the n.m.r. spectra of methyl esters of linolenic, linoleic, oleic, and stearic acids were identical with those shown by Storey (2). Hence, the spectra of esters of oleic and stearic acids were omitted, but the spectra of esters of linolenic and linoleic acids are shown in Figure 2 for comparison with Figure 1.

Since the samples are mainly composed of highly unsaturated fatty acid esters as are shown from the chemical characteristics, the signals in the n.m.r. spectra of the samples (Fig. 1) are to be attributed to the various proton resonance in highly unsaturated fatty acid esters.

Considering from basic patterns of n.m.r. spectra of methyl esters of linolenic, linoleic, oleic, and stearic acids (Fig. 2) (2), the assignment of signals in the n.m.r. spectra of the samples from fish oils is attributed to the following groups: the signal at 4.7 ppm is due to the olefinic groups; at 6.4 ppm to the carbomethoxy group; 7.2 ppm to the methylene group adjacent to two double-bonded carbons; at 7.5–8.2 ppm to α-methylene groups, or in other words, methylene groups adjacent to a carbonyl group or to a single double-bonded carbon; at 8.7 ppm to ordinary methylene groups; and the triplet at the highest magnetic field to the terminal methyl group.

The presence of the signal at 7.2 ppm shows clearly that the highly unsaturated fatty acid esters in the samples prepared in this work have the divinylmethane arrangement of the ethylenic bonds, while it is impossible to show that the divinylethane is present or not in the samples only from the information mentioned above.

The n.m.r. spectrum of 1,5-hexadiene is shown in Figure 3. In the spectrum the signal at 7.8–7.9 ppm is assigned to the dimethylene groups between the two double bonds and this assignment agrees with the result reported by Tiers (3). Hence, if the highly unsaturated fatty acid esters in the samples would have the divinylethane arrangement, the signal of the dimethylene groups should be isolated from that of the methylene group of divinylmethane arrangement, and may overlap with that of the α-methylene groups.

The area intensity of the signals of $=\overset{|}{C}-CH_2-\overset{|}{C}=$ and $-CH=CH-$ in the samples from fish oils was obtained by a Varian Integrator. Intensity ratio of the two signals was compared against the proton number ratios of $=\overset{|}{C}-CH_2-\overset{|}{C}=$ to $-CH=CH-$ in the proposed structures, calculated as follows: a) the mean numbers of double bonds in each sample were calcu-

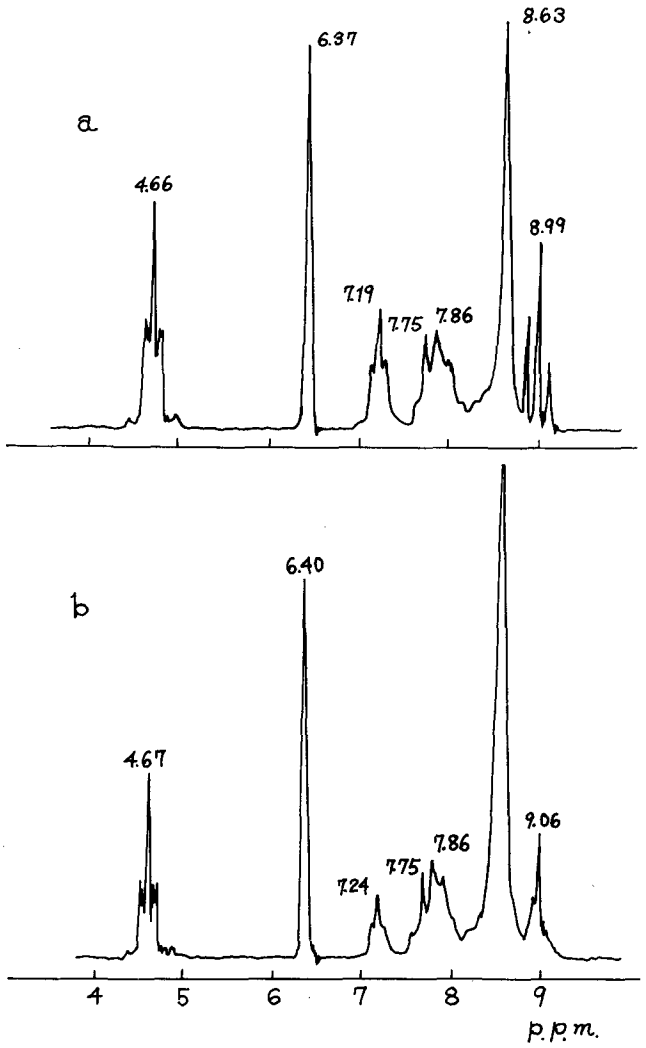

FIG. 2. n.m.r. spectra of methyl esters: (a) linolenate; (b) linoleate.

lated from the mean mol wt, obtained from the saponification numbers, and from the iodine numbers which give the mean proton numbers of the ethylenic group; b) the mean proton numbers of $=\overset{|}{C}-CH_2-\overset{|}{C}=$

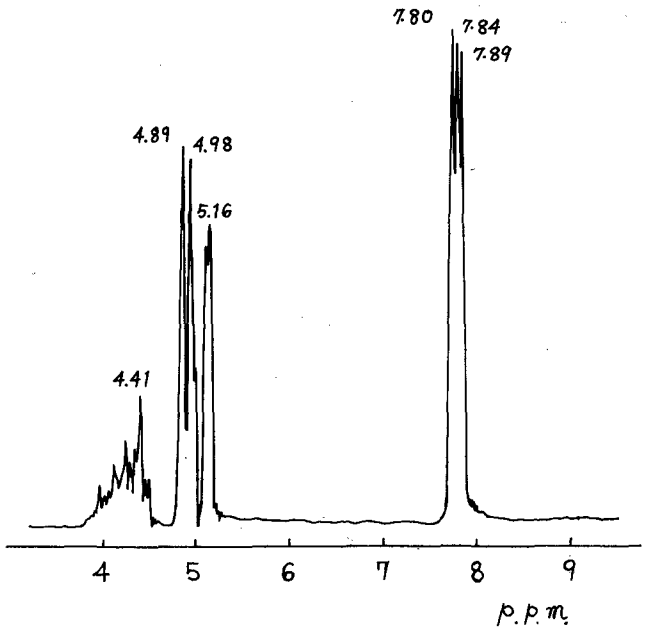

FIG. 3. n.m.r. spectrum of 1,5-hexadiene.

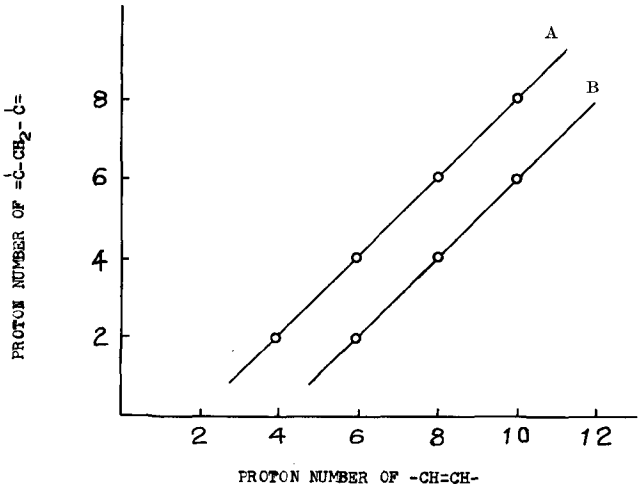

FIG. 4. Plots of proton numbers of $=\overset{|}{C}-CH_2-\overset{|}{C}=$ vs. proton numbers of $-CH=CH-$: A—the structure having solely divinylmethane arrangement; B—the structure having one divinylethane and divinylmethanes.

in each sample can be estimated from Figure 4, in which it was plotted against the mean proton number of the ethylenic group (the circles in the figure were obtained by simple arithmetical calculation). In Figure 4, "A" is the case in which mixture of all highly unsaturated fatty acid esters contained in each sample prepared from fish oils have solely divinylmethane arrangement, and "B" is the case in which the arrangements of the ethylenic bonds in all mixed acid esters have one divinylethane and the remainders are the divinylmethanes.

Ratio of mean proton numbers of $=\overset{|}{C}-CH_2-\overset{|}{C}=$, obtained graphically from Figure 4, to those of $-CH=CH-$ (ratio "A" and "B") and the area intensity ratio of the n.m.r. signals (ratio "n.m.r.") are given in Table I.

Analysis from Table I clearly shows that the ratio "n.m.r." agrees with the ratio "A" but does not with the ratio "B" in all cases. And if there exist two or more divinylethanes in all esters, the disagreement between the ratio "n.m.r." and the calculated ratio will become more notable. The samples prepared from fish oils contain, of course, various kinds of highly unsaturated fatty acid esters, but from the results mentioned above it is presumed that all highly unsaturated fatty acid esters in each sample have, from a statistical view, solely divinylmethane arrangement.

There is another evidence to support the above mentioned conclusion. If the highly unsaturated fatty acids in the samples would have the divinylethane arrangement, the signal of the dimethylene groups between the two double bonds should, as mentioned previously, overlap with that of the α-methylene

TABLE I

Area Intensity Ratio of $=\overset{|}{C}-CH_2-\overset{|}{C}=$ Signal to $-CH=CH-$ Signal and Proton Number Ratio Obtained by Calculation

Ratio of $=\overset{\|}{C}-CH_2-\overset{\|}{C}=$ to $-CH=CH-$	Cuttlefish			Mackerel	Sardine	
	No. 1	No. 2	No. 3		A	B
Ratio n.m.r.	0.77	0.78	0.81	0.79	0.87	0.80
Ratio A	0.81	0.81	0.82	0.80	0.80	0.81
Ratio B	0.63	0.62	0.64	0.60	0.59	0.62

Ratio n.m.r.: The area intensity ratio of signals in the n.m.r. spectra.
Ratio A: The proton number ratio of the mixed acid esters having solely divinylmethane structure.
Ratio B: The proton number of the mixed acid esters having one divinylethane and divinylmethanes.

TABLE II

Area Intensity Ratio of the Signal at 7.5–8.2 ppm to Olefinic Group Signal and Proton Number Ratio Obtained by Calculation

Ratio of α-methylene group to olefinic group	Cuttlefish			Mackerel	Sardine	
	No. 1	No. 2	No. 3		A	B
Ratio n.m.r.	0.50	0.54	0.56	0.66	0.61	0.60
Ratio A	0.57	0.57	0.55	0.63	0.62	0.58
Ratio B	0.95	0.96	0.92	1.04	1.03	0.96

Ratio n.m.r.: The area intensity ratio of signals in the n.m.r. spectra.
Ratio A: The proton number ratio of the mixed acid esters having solely divinylmethane structure.
Ratio B: The proton number ratio of the mixed acid esters having one divinylethane and divinylmethanes.

groups which must appear at 7.5–8.2 ppm and thereby the n.m.r. area intensity ratio of the signal at 7.5–8.2 ppm to that of olefinic groups should be larger than the proton number ratio of α-methylene groups to olefinic groups.

Table II shows the n.m.r. data and the proton number ratios of the two proposed structures, the calculation of which is as follows: In the Table II, ratio "A" indicates the proton number ratio of the structure having solely divinylmethane arrangement, i.e., the ratio of the proton numbers in α-methylene groups (6 protons) to the numbers in olefinic groups calculated from the chemical characteristics of the samples. Ratio "B" indicates the proton number ratio of the structure having one divinylethane and divinylmethanes, i.e., the ratio of the proton numbers in α-methylene groups and two methylene groups between the two double bonds (10 protons) to the numbers in olefinic groups.

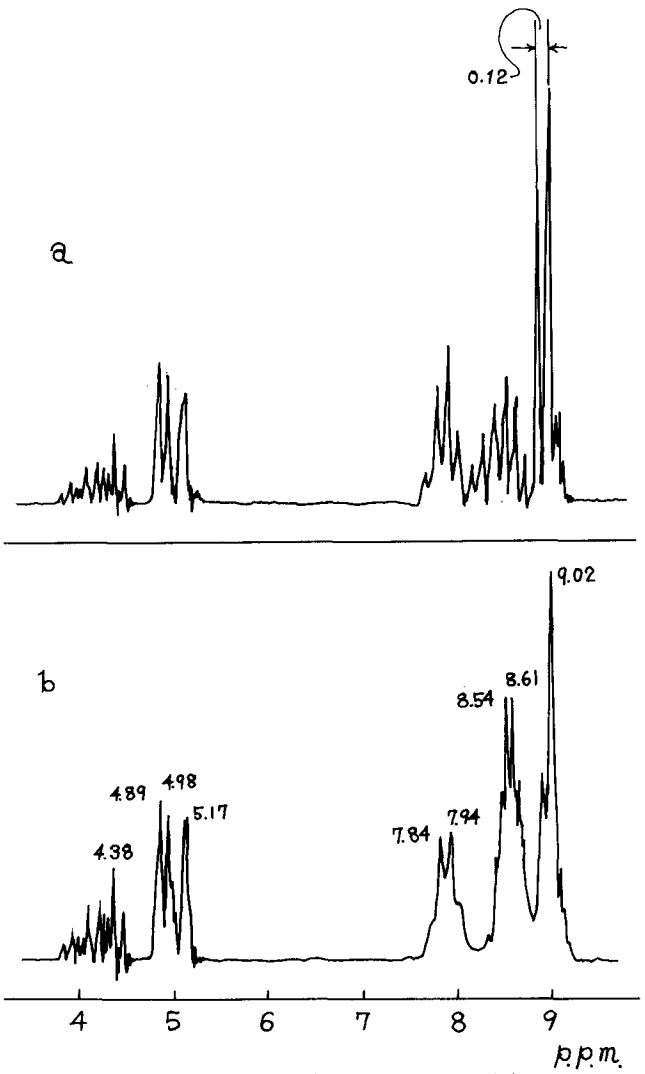

FIG. 5. n.m.r. spectra of (a) pentene-1 and (b) hexene-1.

The ratio of the n.m.r. data shown in Table II agrees with the proton number ratio of the structure having solely divinylmethane arrangement, but not with the structure having one divinylethane and divinylmethanes in all cases. And if there exist two or more divinylethanes in all esters, the disagreement between the ratio "n.m.r." and the calculated ratio will become more notable.

From Tables I and II, it is conclusively presumed that the structure of highly unsaturated fatty acids occurring in cuttlefish, internal organs of mackerel, and sardine oils used in this work has solely divinylmethane arrangement of the ethylenic bonds.

The pattern at the highest magnetic field in the n.m.r. spectra of the samples of fish oils shown in Figure 1, being assigned to the terminal methyl group, shows a clear triplet and its shape, τ-value and coupling constant are similar to those in methyl linolenate (Fig. 2a). On the other hand, all of the patterns raised from the terminal methyl group in the spectra of methyl linoleate, methyl oleate, and methyl stearate show no such clear triplet. The chemical shifts of the main peak, appearing in the patterns of linoleate, oleate, and stearate, are somewhat different from those in linolenic and highly unsaturated fatty acid esters. These results show that the shape, τ-value, and coupling constant of pattern of the terminal methyl group have, presumably, a relationship to the number of methylene groups between the terminal methyl group and the double bond located at the remostest position from a carboxyl group.

In connection with the result, the n.m.r. spectrum of hexene-1, having three methylene groups between the terminal methyl group and the double bond, and that of pentene-1, having two methylene groups, are shown in Figure 5. The pattern assigned to the terminal methyl group in the spectrum of hexene-1 shows a strong peak with two weak peaks on both sides and, in appearance, resembles roughly that of methyl linoleate. The pattern assigned to the group in the spectrum of pentene-1 has two sharp and strong peaks and a weak one with splittings. Namely, when one methylene group is present between the terminal methyl group and the double bond, the pattern of the terminal methyl group shows a clear triplet as in a case of methyl linolenate, and when two or more than two methylene groups are present, the pattern shows no clear triplet.

Considering from the similarity of the pattern raised from the terminal methyl group in the n.m.r. spectra of the samples of fish oils contrasted to that of methyl linolenate, it is therefore suggested that one methylene group is present between the terminal methyl group and the double bond located at the remotest position from a carboxyl group in the highly unsaturated fatty acid esters.

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