Following the same procedure but using foots which had been hydrolyzed to reduce their neutral oil content to less than 1%, one-pass processing esterified 91% to 92% of the fatty acids, with a second pass increasing the percentage of fatty acids esterified to 96% or 97%.

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The Fungicidal Activity of the Unsaturated Fatty Acids and Quaternary Salts Prepared from Fish Oils [1]

BORIS SOKOLOFF, MASAMICHI TOYOMIZU, WALTER TRAUNER, and GEORGE RENNINGER Southern Bio-Research Institute, Florida Southern College, Lakeland, Florida

POLLOCK (1) and Axelrod *et al.* (2) have reported that fatty acids with three double bonds in a molecule exert an inhibitory influence upon some micro-organisms. This observation was confirmed by Laser (3) and Wyss (4). As a rule, only gram-positive organisms are inhibited by the action of unsaturated fatty acids, according to Kodicek (5) and Dubos (6). These investigators have reported that oleic, linoleic, and linolenic acids inhibited *Lactobacillus helveticus* and other gram-positive bacteria. No such effect was evidenced in regard to *E. coli* and *Proteus vulgaris*. It appears that oxidation of unsaturated fatty acids increases their antibiotic activity. Tomiyasu *et al.* (7) found that unsaturated fatty acids or highly unsaturated fatty acid fractions from fish oils did not inhibit the growth of *Debaryomyces membranefaciens*, but by oxidation they manifested an activity against this micro-organism. Ferlin *et al.* (8) and Karbinos *et al.* (9) have suggested that this phenomenon depends on the formation of pelargonic acid, a by-product of the oxidation of fatty acids.

Apparently nonionic surface-active compounds have little, if any, effect on microbial metabolism and do not show any antibiotic activity. Baker *et al.* (10) and Hotchkiss (11) were unable to disclose any inhibitory effect of such compounds in spite of the fact that they were effective as depressants of surface tension. According to Baker *et al.* (12), the cationic surface-active compounds at a physiologic pH are more biologically active than the anionic compounds. Furthermore Baker *et al.* (13), Birkeland *et al.* (14), Ordal and Borg (15), and others have reported that cationic surface-active compounds exerted the same activity against both gram-positive and gram-negative organisms while the anionic surface-active compounds showed a rather selective activity.

Among the cationic surface-active compounds, quaternary ammonium compounds were found to be biologically effective, even in high dilutions. Numerous investigations have been conducted on the antibacterial and antifungal activities of these compounds. Valko and DuBois (16) found that some quaternary ammonium compounds were effective against *Staphylococcus aureus* and *E. typhosa* in concentrations from 1:10,000 down to 1:19,000. Heineman (17), Dunn (18), Howard and Keil (19), Lawrence (20) and others have reported that some of the quaternary compounds possess fungicidal and fungistatic action toward some of the pathogenic fungi in highly diluted concentrations.

In the present study the fungicidal activities of the unsaturated fatty acids prepared from fish oils and their quaternary salts were investigated.

Experimental

Method of Testing Antifungal Activity of Fatty Acids. Several methods of testing fatty acids for their antifungal activity were investigated in the present study. Both the partition method on agar-agar plates and the paper-disc method were used in some tests. However it was found that the method of two-fold dilution, with Sabouraud's peptone-dextrose medium at the pH 6.1, was the most satisfactory and reliable. The fatty acids and fatty acid fractions were emulsified according to the following formula: 0.6 ml. Tween 20; 0.3 ml. propylene glycol; and 9.1 ml. medium. Their activity was tested on three fungi: *Candida albicans, Aspergillus niger,* and *Rhyzopus nigricans.* One drop of the suspension of fungi culture was inoculated into 5 ml. of the mixture, containing various degrees of dilution of the fatty acids. After 48 hrs. the growth of the organism was determined by turbidity of the medium and microscopic examination. The antifungal activity of the material was expressed by the minimum amount of sample for a complete inhibition of growth of the organism.

Material

The following fatty acids and unsaturated fatty acid fractions from fish oil were tested on *Candida albicans:*

a) four saturated fatty acids: caproic, undecanoic, myristic, and palmitic;

b) four unsaturated fatty acids:[2]

[1] Aided by a grant from the Collett-Week Company, Ossining, N. Y.

TABLE I
The Minimum Concentrations of Fatty Acids Required for Complete Inhibition of Growth of *Candida Albicans*

	Fresh	Oxidized
	(%)	(%)
Caproic acid	0.16	.....
Undecanoic acid	0.02	.....
Myristic acid	1.28	.....
Palmitic acid	>1.28	.....
Oleic acid	>1.28	>1.28
Linoleic acid	>1.28	>1.28
Linolenic acid	>1.28	>1.28
10-hendecenoic acid	0.02	0.02
Methyl 10-hendecenate	1.28	1.28
10-hendecenyl alcohol	0.02	0.02
Ethyl undecylate	>1.28	.....
Unsaturated fatty acid fraction of pilchard oil, i.v. 287.2	>1.28	0.64
Unsaturated fatty acid fraction of menhaden oil, i.v. 244.7	>1.28	0.64
Ethyl ester of unsaturated fatty acid fraction of pilchard oil, i.v. 267.1	>1.28	0.64
Ethyl ester of unsaturated fatty acid fraction of menhaden oil, i.v. 253.1	>1.28	0.64
Ethyl ester of unsaturated fatty acid fraction of cod liver oil, i.v. 269.9	>1.28	0.64
Unsaturated fatty acid fraction of pilchard oil, i.v. 182.7	>1.28	>1.28
Unsaturated fatty acid fraction of pilchard oil, i.v. 85.3	>1.28	>1.28
Unsaturated fatty acid fraction of safflower oil, i.v. 169.7	>1.28	>1.28
Unsaturated fatty acid fraction of cottonseed oil, i.v. 110.2	>1.28	>1.28
Ethyl ester of unsaturated fatty acid fraction of safflower oil, i.v. 148.6	>1.28	>1.28

oleic acid, U.S.P., iodine value 89.9; acid value 196.1; saponification value 199; from coula oil; by hydrolysis;

10-henedecenoic acid, U.S.P., iodine value 133.0; acid value 301; saponification value 303; volatile matter 0.1%; from ricinoleic acid by pyrolysis;

linoleic acid; iodine value 179.5; acid value 197.3; from dehydrated castor oil;

linolenic acid, iodine value 270.9; acid value 198.6; from linseed oil;

c) methyl 10-hendecenate, 10-hendecenyl alcohol, ethyl undecylate;[3]

d) ten unsaturated fatty acid fractions and ethyl esters of unsaturated fatty fractions from fish and vegetable oils, listed in Table I.[3] The unsaturated fatty acid fractions were prepared by the urea complex procedure, and the ethyl esters of unsaturated fatty acids by the standard Hilditch method.

In order to determine the effect of oxidation on antifungal activity, unsaturated fatty acids were oxidized by bubbling oxygen into them in the presence of M/100 Cu-stearate at 100°C. for 1.5 hrs. (7). The results are summarized in Table I.

Analysis of the data presented in Table I indicates that undecanoic acid, 10-hendecenyl alcohol, and 10-hendecenoic acid possess the highest activity, inhibiting growth of *Candida albicans* in the concentration of 0.02%.

Similar but not identical results were obtained by testing the fatty acid compounds on *Aspergillus niger* and *Rhyzopus nigricans*. 10-Hendecenoic acid inhibited the growth of *Aspergillus niger* at 0.16%, and of *Rhyzopus nigricans* at 0.02%. Caproic acid arrested the growth of these fungi at 0.04%. Myristic acid was active against *Aspergillus niger* at 1.28% and against *Rhyzopus nigricans* at 0.16% concentration.

By oxidation the highly unsaturated fractions from pilchard and menhaden oils and the ethyl esters of unsaturated fatty acid fractions of menhaden, pilchard, and cod liver oils showed an increase in their antifungal activity.

Antifungal Activity of Unsaturated Quaternary Ammonium Derivatives Prepared from Fish Oil. In order to compare the activity of unsaturated quaternaries from fish oil with that of saturated quaternary compounds, six saturated quaternary compounds were prepared. These were: tetradecyltriethylammonium bromide, hexadecyltriethylammonium bromide, octadecyltriethylammonium bromide, tetradecylpyridinium bromide, hexadecylpyridinium bromide, and octadecylpyridinium bromide.

Methods of Preparation. Three triethylammonium bromides were prepared from three bromide fractions of calamary liver oil: C-1, C-2, and C-3. Seven pyridinium bromides were prepared from three bromide fractions from calamary liver oil: C-1, C-2, and C-3 and four bromide fractions from menhaden oil: M-1, M-2, M-3, and M-4.

Hexadecyltriethylammonium bromide was prepared by heating bromohexadecane with triethylamine in ethyl alcohol (21). Hexadecylpyridinium bromide was prepared by heating bromohexadecane with pyridine (22). By these methods two triethylammonium bromides and two pyridinium bromides were prepared from bromotetradecane and bromo-octadecane.

The unsaturated quaternaries were prepared from menhaden oil by forming, first, unsaturated alkyl alcohol; secondly, unsaturated alkyl bromide; and finally, unsaturated alkyl triethylammonium bromide or unsaturated pyridinium bromide.

Unsaturated alkyl alcohol was prepared from menhaden oil (iodine value 176.7, saponification value 194.7, acid value 0) by the alkaline method, with sodium and ethylene glycol monoethyl ester in xylene, as suggested by Blatt (23).

Unsaturated alkyl bromide was prepared by bromination of unsaturated alkyl alcohol with phosphorus tribromide (23).

After the bromination, unsaturated alkyl bromide was fractioned by vacuum into four fractions described in Table II.

Unsaturated alkyl pyridinium bromide was prepared from each of these fractions by following the method of Knight and Shaw (22).

The changes in the iodine value during the preparation procedure of quaternaries were investigated on calamary oil. In this case calamary oil was first saponified and esterified in methyl ester, and then fractionated. The iodine values and saponification values of the three fractions are given in Table III.

The decrease of iodine value was remarkable in the alcoholization process, but the degrees were almost the same in every fraction. Saponification value of the high boiling point fraction was lower than that of the low boiling point fraction, and iodine value of the high boiling-point fraction was higher than that of the low boiling-point fraction. Therefore quaternaries prepared from the high boiling point fraction were more unsaturated and longer in carbon chain length than those prepared from the low boiling-point fraction.

TABLE II
Fractionation of Unsaturated Alkyl Bromide Prepared from Menhaden Oil

	Boiling point °C./2 mm.	Iodine value
M-1	149–164	43.2
M-2	164–170	59.3
M-3	170–180	79.5
M-4	180–194	120.9

[2] Obtained from the Bios Laboratories, New York, N. Y.
[3] Generously supplied by the Collett-Week Company, Ossining, N. Y.

TABLE III

Changes in Iodine Value During the Preparation Procedure of Quaternaries from Calamary Oil

	Methyl ester			Alcohol		Alkyl bromide	
	Boil. pt. °C./mm.	S.V.[a]	I.V.	B.P.	I.V.	B.P.	I.V.
C-1	165–186/4	204.2	71.8	129–142/1.5	57.0	145–181/2.0	40.1
C-2	186–208/4	187.0	167.7	168–178/2.5	130.6	164–180/1.0	93.0
C-3	208–229/4	173.3	266.1	190–217/2.5	201.4	194–209/2.0	147.2

[a] Saponification value.

The quaternary ammonium salts prepared from fish oils were analyzed on their bromide and nitrogen contents. The results of this analysis are presented in Table IV. The values of C-1 and C-2 of both the bromides were nearly the same as the calculated values for hexadecyl and octadecyl, respectively. The average carbon lengths are C_{16} and C_{18}. The values for C-3 are very close to those of eicosyl-triethylammonium bromide and -pyridinium bromide, indicating the average carbon length of C_{20}. Similar values were obtained for the M- series.

TABLE IV

Analysis of Quaternary Ammonium Salts

	Percentage bromine[a]		Percentage nitrogen[b]	
	Calcd.	Found	Calcd.	Found
Triethylammonium bromide				
Tetradecyl	21.12	21.57	3.70	3.73
Hexadecyl	19.66	20.04	3.44	3.44
Octadecyl	18.39	18.14	3.22	3.12
C-1	.....	20.28	.....	3.43
C-2	.....	18.10	.....	3.26
C-3	.....	16.33	.....	3.03
Pyridinium bromide				
Tetradecyl	22.42	21.90	3.93	3.84
Hexadecyl	20.79	20.06	3.64	3.56
Octadecyl	19.37	18.97	3.40	3.34
M-1	.....	20.90	.....	3.76
M-2	.....	19.48	.....	3.48
M-3	.....	19.32	.....	3.41
M-4	.....	18.07	.....	3.29
C-1	.....	20.34	.....	3.57
C-2	.....	19.15	.....	3.43
C-3	.....	17.18	.....	3.25

[a] Estimated by micro-Carius method.
[b] Estimated by micro-Dumas method.

Results

The results of testing the six saturated quaternary compounds and 10 unsaturated quaternaries from fish oil on their activity against *Candida albicans* are shown in Table V, and against *Aspergillus niger* and *Rhyzopus nigricans* in Table VI.

The data presented in these tables indicate that both hexadecyltriethylammonium bromide and hexadecylpyridinium bromide exert similar activity against the three fungi. *Candida albicans* and *Rhyzopus nigricans* were inhibited at the concentration of 0.16 mg. % while *Aspergillus niger* at 1.28 mg. %. The unsaturated quaternaries from fish oils gave similar inhibition. Among saturated quaternary compounds the C_{16} compounds were more active than the C_{14} and C_{18} compounds. Unsaturated quaternaries prepared from the lowest boiling-point fractions, the C-1 and M-1, possessed a milder activity than the C-2 and C-3, or M-3 and M-4.

Taken as a group, the quaternaries possessed an antifungal activity much higher than the unsaturated fatty acids. The most active fatty acid, undecylic acid, inhibited the growth of *Candida albicans* at the concentration of 0.02% while the most active quaternaries arrested the growth of the same fungus at 0.32 mg. % or at a concentration six hundred times smaller.

Discussion

It has been demonstrated that unsaturation is an important factor in determining the activity of fatty acids against gram-positive bacteria. Moreover Solamides (24) and Hanel *et al.* (25) have proved that the highly unsaturated fatty acid fractions from cod liver oil inhibited bacterial growth. On the other hand McKee *et al.* (26) postulated that the length of the carbon chain was more important than unsaturation as far as the antibacterial activity of fatty acids was concerned. They stated that the C_8–C_{14} acids possessed optimal inhibitory activity. Onr findings (Table I) showed that undecylic acid and caproic acid inhibited the growth of *Candida albicans* but that unsaturated fatty acid fractions from fish oil, even highly unsaturated ones, were ineffective. It may be assumed that the length of the carbon chain is an essential factor in the antifungal activity of fatty acids.

By oxidation of unsaturated fatty acid fractions and ethyl ester fractions from fish oil, only highly unsaturated fractions showed a slight activity. Oxidation of oleic, linoleic, and linolenic acid did not improve their activity. On the other hand, it appears

TABLE V

Inhibiting Activity of Saturated and Unsaturated Alkyl Quaternaries on *Candida Albicans*

mg. %	0.64	0.32	0.16
Triethylammonium bromide			
Tetradecyl	—	++	++
Hexadecyl	—	—	±
Octadecyl	—	—	+
C-1	—	—	++
C-2	—	—	+
C-3	—	—	+
Pyridinium bromide			
Tetradecyl	—	++	++
Hexadecyl	—	—	±
Octadecyl	—	—	+
M-1	—	±	++
M-2	—	—	++
M-3	—	—	+
M-4	—	—	+
C-1	—	—	++
C-2	—	—	+
C-3	—	—	+

— = No growth; + = slight growth; ± = questionable growth; ++ = normal growth.

TABLE VI

Inhibiting Activity of Saturated and Unsaturated Alkyl Quaternaries on *Aspergillus Niger* and *Rhyzopus Nigricans*

	Aspergillus niger	*Rhyzopus nigricans*
	(mg.%)	*(mg.%)*
Triethylammonium bromide		
C-3	2.56	0.32
Hexadecyl	1.28	0.16
Pyridinium bromide		
C-3	2.56	0.32
M-4	2.56	0.32
Hexadecyl	1.28	0.16

that oxidation of the unsaturated fractions which have more than four double bonds rendered them active.

Discussing the activity of quaternaries, it should be pointed out that systematic studies of the structure of saturated quaternary compounds and their bactericidal functions have uniformly emphasized the importance of carbon chain length in determining their activity. As to attachment of the long-chain alkyl group which contained unsaturated linkage to quaternary compound, there are a few reports concerning 9-octadecenyl group only. Valko and DuBois (16) have stated that 9-octadecenyldimethylbenzylammonium chloride was about six times as active as octadecyl compound. Shelton *et al.* (27) prepared a series of quaternary ammonium compounds of the type represented by the formula $R(CH_3)_3NBr$, in which R stood for a straight-chain alkyl group. When R represented the 9-octadecenyl group, the germicidal activity was higher than when it represented an octadecyl group. They suggested that the substitution of the unsaturated alkyl group in place of the saturated group increased the activity of quaternary ammonium compounds. However the substitution of the highly unsaturated alkyl group has not been studied as yet. Regarding carbon chain length, it was found that the maximum activity was observed at the C_{16} in the general type of $RN^+(CH_3)_3$ (16, 27), but it was reached at the C_{14} in the series of higher aliphatic dimethylbenzylammonium chlorides (16). Replacement of the methyl groups in hexadecyltrimethylammonium bromide with ethyl groups had no adverse effect on the activity (27). As to alkyl pyridinium bromide, Kolloff *et al.* (28) and Shelton *et al.* (27) discovered that the C_{16} compound was the most active. For the above reasons, the authors prepared triethylammonium bromides and pyridinium bromides from fish oil.

On the basis of saponification values of the unsaturated methyl ester fractions derived from calamary oil (Table III), the average carbon chain lengths of unsaturated alkyl groups of C-1, C-2, and C-3 in methyl esters are almost C_{15}, C_{17}, and C_{19}; the carbon chain lengths of unsaturated alkyl groups in quaternaries prepared from C-1, C-2, and C-3 are C_{16}, C_{18}, and C_{20}, respectively (Table IV). If saturated, the quaternaries prepared from C-1 should be most active. But as shown in Tables V and VI, triethylammonium bromide and pyridinium bromide prepared from C-1 are the weakest. Pyridinium bromides prepared from menhaden oil show similar results.

Alkyl bromide fractions which are prepared from fish oil are unsaturated, and the pattern of unsaturation is as follows: C-3>C-2>C-1 and M-4>M-3>M-2>M-1.

All in all, the data presented in Tables V and VI suggest that two factors seem to determine the activity of quaternaries prepared from fish oil. Any lengthening of the carbon chain above C_{16} decreases the antifungal activity while an increase of unsaturation enhances it.

The antifungal activities of unsaturated groups in quaternary compounds and of unsaturated fatty acids apparently have somewhat different patterns of action. This might partially depend on the differences in their water solubility. The surface of micro-organisms is, as a rule, negatively charged. Since the unsaturated group in a quaternary compound in aqueous solution is in the portion which is positively charged, this might be a decisive factor in their highly inhibitory activity against fungi. On the other hand, the unsaturated group in a fatty acid is in the portion which is negatively charged, which explains their relatively lower antifungal activity. Further investigations along these lines, which are under way, will, it is hoped, clarify more exactly the nature of these differences in the patterns of action of these two groups of fatty acids.

Summary

Saturated and unsaturated fatty acids and 10 unsaturated fatty acid fractions and ethyl esters of unsaturated fatty acid fractions prepared from fish oils were tested on their inhibitory activity against *Candida albicans*.

Oxidation of highly unsaturated fractions from fish oil and ethyl esters of unsaturated fatty acid fractions of menhaden, pilchard, and cod liver oils increases their antifungal activity.

Saturated and unsaturated quaternaries were tested for their antifungal activity.

Hexadecyltriethylammonium bromide and hexadecylpyridinium bromide showed the highest activity against *Candida albicans, Aspergillus niger,* and *Rhyzopus nigricans.*

Any lengthening of the carbon chain more than C_{16} weakened the activity of both saturated triethylammonium bromide and pyridinium bromide. An increase of unsaturation enhanced it.

The antifungal activity of quaternaries prepared from fish oils was about 4,000 times stronger than that of oxidized highly unsaturated fatty acid fractions prepared from fish oils.

The decisive factor in the highly inhibitory activity of quaternaries against fungi might depend on their positively charged portion since the surface of microorganisms is, as a rule, negatively charged.

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