The amt of 9,15-type diene (Diene Fraction E) and its corresponding fraction from nickel 1, which are larger than expected by chance, may indicate that the 12,13 double bond in the triene is reduced more rapidly than the 9,10 or 15,16 double bonds. However, this larger amt of 9,15 diene is also caused by the slower reduction of 9,15 dienes (isolinoleate type) compared with 9,12 linoleate. Previous workers have reported such differences in reduction rates for 9,15 and 9,12 linoleates (2,13) and it is reasonable to assume that similar differences occur with the *trans*-containing isomers which are also present in the hydrogenating mixture. In the monoene from a nickel catalyzed linolenate, there are less 12 monoenes than 9 or 15. From the platinum monoenes, however, one would predict that the double bonds farthest from the carboxyl are reduced more rapidly, as believed by Inoue and Suzuki (6). The difference in monoene composition may be caused by differences in the relative rates of hydrogenation of isolinoleic acid-type dienes and other dienes.

The reaction pathways by which this complex mixture of diene and monoene isomers is formed are still not clear. Certainly the isomerized trienes and dienes that are present in small amt participate in the reaction, and some of them—especially conjugated esters—may be quite reactive intermediates. Interpretation of data, therefore, must be tentative, and more information on the identity and relative reaction rates of the intermediates is needed before the reduction is well understood.

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[Received August 7, 1963—Accepted May 18, 1964]

Phospholipids of the South African Pilchard (*Sardina ocellata Jenyns*)

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Abstract

Chromatographic resolution of phospholipids from the flesh of the South African pilchard (*Sardina ocellata Jenyns*) revealed the presence of cardiolipins, cephalins, inositol phosphatides, cerebrosides, sphingomyelins, lecithins, lysolecithins and plasmalogens.

Column and paper chromatographic techniques were used to identify ethanolamine, serine, choline, sphinogsine and inositol in pilchard phospholipid hydrolysates.

The fatty acids of pilchard phospholipids comprised large amt of C_{22} hexaenoic, C_{20} pentaenoic and C_{16} saturated acids, with smaller amt of C_{18} dienoic, C_{18} monoenoic and C_{18} saturated acids.

Introduction

THE PILCHARD (*Sardina ocellata Jenyns*) is abundant in South African waters, and is of considerable economic importance in the Republic of South Africa.

The triglyceride composition of pilchard oil has been investigated in considerable detail (30,33), but the phospholipids have not been examined previously.

The phospholipids of cod and haddock have been described in a series of papers by Lovern and Olley (19,20), Olley and Lovern (25,26), Garcia et al. (8) and Lovern et al. (21), while tuna phospholipids have been examined by Igarashi et al. (10,11,12), Zama and Igarashi (13,15) and Katada (14).

These investigations, together with reports concerning the phospholipid composition of other fish, have been reviewed by Lovern (22).

Pilchard phospholipids were isolated according to a modified procedure which was based on the method of Lovern et al. (21) for codfish.

Experimental

Isolation of Pilchard Phospholipids

Method: Operations were carried out under an atmosphere of carbon dioxide produced by adding dry ice to all extracts and containers. Phosphorus contents were determined according to the method of Bartlett (2).

1) *Solvent Extraction of Mixed Triglycerides and Phospholipids.* Fresh pilchards, caught during the previous 24 hr, were received in the laboratory packed in ice at 0C. The fish were decapitated, degutted, deboned and descaled. After coarse mincing of the flesh with adhering skin, a weighed portion (750 g) was extracted by thorough stirring with 375 ml acetone. The slurry was filtered on a Buchner funnel and the tissue was further drained by subjecting it to a pressure of 2000 lb/sq. in. in the canvas bag of a laboratory Carver press. The extraction process was repeated with a further 375 ml acetone and both acetone extracts were discarded.

The presscake from the second acetone extraction was macerated for 10 min in a Waring blender with approx 700 ml $CHCl_3$:MeOH (2:1, v/v). The suspension was filtered on a Buchner funnel and the disintegrated tissue again blended with 700 ml $CHCl_3$:MeOH (2:1, v/v). After filtering, the residue was suspended in a further 700 ml $CHCl_3$:MeOH (2:1, v/v) and allowed to stand overnight at −20C before removal of the solvent.

The combined $CHCl_3$:MeOH extracts were dis-

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tributed between several containers and submerged in a large volume of tap water at 1C to remove the MeOH and water soluble impurities by diffusion according to the method of Folch et al. (7). The washing process was repeated after adding MeOH to the residual $CHCl_3$, retaining the fluffy layer which separated at the interface. All $CHCl_3$ layers were then combined with the addition of ethanol to dissolve the fluffy material and rapidly filtered. Evaporation of the $CHCl_3$ at reduced temp and pressure with a leak of oxygen-free nitrogen (24) gave 19.5 g dark yellow oil consisting of a mixture of triglycerides and phospholipids. This oil contained 1.3% P.

2) *Separation of Pilchard Triglycerides from the Phospholipids by Chromatography on Activated Silica Gel.* Silica gel (120 g, 50–100 mesh chromatographic, L. Light & Co., Colnbrook, Bucks., England) was activated at 120C for 4 hr, slurried with petroleum ether (bp 40–60C) and packed to a height of 30 cm in a column 3.3 cm in diam.

The mixture of triglycerides and phospholipids (19.15 g) was dissolved in 20 ml petroleum ether (bp 40–60C) and carefully run into the top of the column. The material was washed in with 2 x 12.5 ml portions of petroleum ether and the chromatogram developed with 300 ml of the same solvent. A yellow band was found to move with the solvent front leaving a ± 2 cm band of pale yellow material adsorbed at the top of the column.

The eluate was collected in a CO_2 atmosphere and evaporated at reduced temp and pressure with a leak of oxygen-free nitrogen to yield 5.47 g concn pilchard phospholipids as a solid resinous yellow-brown material containing 3.3% P and 1.7% N.

(The yellow material near the top of the column was desorbed by washing with purified diethyl ether until the eluate became clear. Evaporation of the ether at reduced temp and pressure gave 13.43 g clear yellow triglyceride oil containing 0.17% P).

3) *Separation of Pilchard Phospholipids into Component Fractions by Chromatography on Activated Silicic Acid.* Silicic acid (220 g Mallinkrodt A.R.) was activated at 120C for 16 hr, slurried with 250 ml $CHCl_3$:MeOH (7:1, v/v) and packed to a height of 52 cm in a column 3.3 cm in diam. The chloroform contained 2% ethanol as a preservative. The slurry was de-gassed before packing and the column was washed with 100 ml $CHCl_3$:MeOH (7:1, v/v) before use.

The phospholipid mixture (5.41 g) was placed on the column in 15 ml $CHCl_3$:MeOH (7:1, v/v) and washed with 2 x 7.5 ml portions of the same solvent. The column was pressurized with nitrogen at 10 cm Hg and the eluate was collected in 14.5 ml fractions at a flow rate of 1.5 ml/min.

Development was carried out with 690 ml $CHCl_3$:MeOH (7:1, v/v) followed by 190 ml $CHCl_3$:MeOH (4:1, v/v). After a final wash with 55 ml $CHCl_3$:MeOH (2:1, v/v) the eluted fractions were combined on the basis of their phosphorus content to represent a large initial peak (tubes 5–14, Fraction I) and four other incompletely resolved components (tubes 16–25, 26–30, 32–37 and 40–49 representing Fraction II–V respectively).

The washed column was extruded and cut into sections corresponding to the positions of three yellow bands which were 5 cm, 3 cm and 1 cm in width and adsorbed with their upper edges at respective distances of 11, 5 and 0 cm from the top. These bands, representing Fractions VII, VIII and IX, were each desorbed with 150 ml absolute MeOH which was filtered before evaporation at reduced temp and pressure. The section of the column below the visibly adsorbed bands was similarly extracted to yield material containing the remainder of the incompletely eluted fraction in tubes 40–49 (Fraction VI).

All fractions were stored under CO_2 at −20C.

4) *Re-Chromatography of Phospholipid Material Strongly Adsorbed During Preliminary Fractionation.* The yellow material desorbed from the top of the preliminary chromatogram (Fraction IX) was refractionated on silicic acid (40 g Mallinkrodt A.R., activated 16 hr at 120C), and packed as a slurry in 50 ml $CHCl_3$:MeOH (4:1, v/v) to a height of 25 cm in a column of 1.9 cm diam.

The mixture (250 mg) was placed on the column in 2 ml $CHCl_3$:MeOH (4:1, v/v) and was washed in with 2 x 1 ml portions of the same solvent. The column was pressurized with nitrogen at 10 cm Hg and the eluate was collected in 13 ml fractions at a flow rate of 15 ml/hr.

Development was carried out with successive 100 ml portions of $CHCl_3$:MeOH mixtures (4:1, 2:1, 1:1, 1:2 and 1:4, v/v) followed by 150 ml absolute MeOH, 50 ml MeOH:H_2O (24:1, v/v) and 100 ml MeOH:H_2O (17:3, v/v). Fractions were combined on the basis of their phosphorus content to represent three well-defined peaks (tubes 1–4 (Fraction IXa), 5–6 (Fraction IXb), 10–12 (Fraction IXc) and three incompletely resolved peaks (tubes 18–20 (Fraction IXd), 23–24 (Fraction IXe), 27–30 (Fraction IXf).

The washed column was extruded and cut into sections corresponding to the positions of a 1-cm yellow band at the top (Fraction IXj) and another 5.5 cm yellow band adsorbed just beneath it (Fraction IXi) The clear portion of the column below the yellow bands was arbitrarily divided into two 9 cm lengths (Fraction IXg and IXh). Each extruded section was washed with absolute MeOH, MeOH:H_2O (1:1, v/v), H_2O and again with absolute MeOH to give an extract which was rapidly filtered before evaporation at reduced temp and pressure.

All phospholipid fractions were stored under CO_2 at −20C.

Identification of Pilchard Phospholipid Components

The phospholipids were characterized both by direct chromatography on silicic acid impregnated paper and by paper chromatographic analysis of their hydrolysis products.

Paper Chromatography of the Total Pilchard Phospholipid Mixture

Chromatography on silicic acid impregnated paper was carried out according to Marinetti et al. (23) with a solvent system of di-isobutyl ketone, acetic acid, water (8:5:1, v/v) for five hr (5). Marker compounds, D.L. α-cephalin, D.L. α-lecithin and sphingomyelin (obtained from L. Light & Co., Colnbrook, Bucks., England) were run with each chromatogram. The spots were detected by staining with Rhodamine 6G. The ninhydrin and phosphomolybdic acid reagents of Lea et al. (16) were used to distinguish between spots containing phospholipids with a primary amino group and others with choline moieties. Plasmalogens were identified as brown spots by immersing the developed

TABLE I

The Yield, Phosphorus Content and Qualitative Analysis of Fractions Obtained by Chromatography of the Phospholipids from Pilchard Flesh

Fraction	% of total phospholipids	Phosphorus content %	Serine R_F = 0.22	Ethanolamine R_F = 0.37	Choline R_F = 0.39	Sphingosine	Inositol
Total phospholipids + triglycerides	100	1.3	..	..	..	..	..
Total triglycerides	.	0.2	..	..	..	..	..
Total phospholipids	100	3.3				..	
I	9.7	2.9	0	1+	0	0	0
II	1.4	2.7	0	1+	0	3+	0
III	0.6	2.5	0	1+	0	0	0
IV	1.5	3.0	3+	1+	0	0	2+
V	5.6	3.5	2+	4+	0	0	0
VI	17.6	2.3	0	4+	0	0	1+
VII	16.2	3.1	1+	3+	4+	0	5+
VIII	18.6	3.4	0	2+	4+	0	4+
IX	8.3	3.5	2+	2+	3+	3+	4+

papers in a 0.15% solution of 2:4-dinitrophenylhydrazine in 3N HCl (23).

Hydrolysis of Pilchard Phospholipid Components

1) *Hydrolysis of Lecithins, Cephalins and Inositol Phosphatides.* A portion (4–40 mg) of each chromatographic fraction was dissolved in $CHCl_3$:MeOH and the solution transferred to an ampoule with a constricted neck. The solvent was evaporated and 5 ml 6N HCl was added to each ampoule before sealing. The tubes were opened after heating 48 hr at 115C and the HCl poured into a filter. The residual tar was thoroughly leached with hot H_2O and poured into the same filter. The aqueous extract was evaporated twice to near dryness on a water bath and the residue taken up in 2 ml H_2O. This solution was retained for paper chromatography after centrifuging to remove a small quantity of insoluble tar.

2) *Hydrolysis of Sphingomyelins and Cerebrosides.* For the identification of sphingomyelins and cerebrosides, 10–40 mg of each chromatographic fraction was hydrolysed with 2N methanolic-HCl and the liberated sphingosine purified by small-scale silicic acid chromatography according to the method of Sweeley and Moscatelli (31). The purified base was dissolved in MeOH and retained for identification by paper chromatography.

Chromatography of Pilchard Phospholipid Hydrolysis Products

Choline. This hydrolysis product was identified as its phosphomolybdate after ascending chromatography for 16 hr on Whatman No. 1 paper with a solvent system of butanol:diethylene glycol:water (4:1:1, v/v) according to the method of Levine and Chargaff (17).

A second spot with R_F = 0.52 was observed in all cases where choline (R_F 0.39) was identified. Levine and Chargaff (18) and Phillips (27) also reported the presence of an unknown compound with an R_F greater than that of choline, but were unable to establish its identity. The substance has now been identified as (2-chloroethyl) trimethylammonium chloride which is produced as an artifact during hydrolysis with HCl (6).

Ethanolamine and Serine. Ethanolamine and serine were identified as ninhydrin reacting spots with respective R_F values of 0.37 and 0.22 on Whatman No. 2 paper chromatograms developed for 43 hr with a descending solvent system of butanol:diethylene glycol:water (4:1:1, v/v).

Inositol. This substance was identified by chromatography on Whatman No. 1 papers developed for 3.5 hr with acetone:H_2O (4:1, v/v). The solvent was allowed to drip from the lower edge of the paper in order to remove contaminating reducing substances such as glycerol.

The air-dried chromatograms were dipped in 50 ml aqueous acetone containing 2.5 ml saturated aqueous $AgNO_3$, and dried again for 5 min at 60C following the method of Anet and Reynolds (1). The papers were then dipped in 1% NaOH in acetone: H_2O (1:1, v/v) and the spots allowed to develop for approx 3 min in air. The chromatograms were fixed in 10% aqueous $Na_2S_2O_3$ and washed for 2 hr in running water before drying.

The acetone used for development and spraying was previously purified by distillation over $AgNO_3$, according to Werner (32).

Sphingosine. All fractions hydrolysed according to the method of Sweeley and Moscatelli (31) were examined for the presence of sphingosine by ascending chromatography for 4 hr on Whatman No. 1 paper with a solvent system representing the upper phase of a butanol:acetic acid:water (4:1:5, v/v) mixture. The chromatograms were dried 0.5 hr in air, dipped in 0.25% sodium fluorescein according to Saito (28) and washed one hr in running water to reveal the sphingosine as an orange-pink spot with R_F 0.87.

The Fatty Acid Composition of Total Pilchard Phospholipids

Total pilchard phospholipids were transmethylated in dry methanol saturated with HCl and the liberated fatty acid esters saponified according to the method of Böttcher et al. (4). After removal of the nonsaponifiable material, the unsaturated acids were remethylated and submitted to analysis by gas-chromatography over a polyethylene glycol glutarate stationary phase at 190C with Argon as carrier gas.

The average eqivalent wt of the fatty acid mixture was determined by titration with 0.01N-NaOH after quantitative hydrogenation over a 2.5% palladium-$BaSO_4$ catalyst.

Results

The Yield, Phosphorus Content and Qualitative Analysis of Pilchard Phospholipid Fractions

Table I shows the yield, phosphorus content and qualitative analysis of nine fractions obtained by chromatography of 5.41 g total phospholipids from pilchard flesh. The most strongly adsorbed Fraction IX was re-chromatographed to yield ten sub-fractions with the properties shown in Table II.

In Tables I and II the relative intensities of the spots corresponding to each marker compound are indicated by the number of positive integers assigned on the basis of visual inspection. The Tables may thus be regarded as semiquantitative for each individual component but not with respect to the relative proportions of different components.

Chromatographic Analysis of Total Pilchard Phospholipids

Chromatograms of the total pilchard phospholipids on silicic acid impregnated paper showed five different spots when stained with Rhodamine 6G. Marker compounds together with the ninhydrin and phospho-

TABLE II

The Yield, Phosphorus Content and Qualitative Analysis of Pilchard Phospholipid Fractions Obtained by Rechromatography of Fraction IX, Table I

Fraction	% of total phospholipids	Serine R_F = 0.22	Ethanolamine R_F = 0.37	Choline R_F = 0.39	Sphingosine	Inositol
IX a	11.7	2+	0	0	..	0
IX b	2.7	2+	0	0	..	0
IX c	6.4	2+	1+	0	..	4+
IX d	19.8	2+	0	3+	0	0
IX e	7.6	2+	0	4+	4+	0
IX f	11.8	2+	0	3+	3+	0
IX g	5.1	1+	1+	0	..	0
IX h	11.6	Trace	0	0	..	0
IX i	4.3	2+	0	Trace	..	0
IX j	4.8	2+	0	0	..	0

molybdic acid reagents of Lea et al. (16) were used to identify four of these components as lysolecithin (R_F 0.32), sphingomyelin (R_F 0.39), lecithin (R_F 0.49) and cephalin (R_F 0.62). The remaining spot with R_F 0.97 probably represented material of the cardiolipin type. Inositol phosphatides were not detected separately since these were resolved with lysolecithin in the solvent system used (23). Plasmalogens were detected as brown spots in the lecithin, cephalin and cardiolipin positions by immersing the chromatograms in acidified 2:4-dinitrophenylhydrazine solution. The presence of plasmalogens was confirmed by a positive Schiff reaction (23) on the total phospholipids prior to chromatography.

Fatty Acid Composition of Total Pilchard Phospholipids

Gas-chromatographic analysis of the fatty acid methyl esters derived from pilchard phospholipids showed the presence of the following constituents:

C_{16} saturated (36.6%); C_{16} monoenoic (2.1%);
C_{18} saturated (5.7%); C_{18} monoenoic (6.9%);
C_{18} dienoic (0.9%); C_{20} pentaenoic (13.0%);
C_{22} hexaenoic (31.8%).

Several unidentified minor constituents amounting to 3% of the total fatty acid esters, were also detected.

Gas-chromatography of the same fatty acid esters following complete hydrogenation over 2.5% palladium-$BaSO_4$ catalyst, showed the chain length distribution to be C_{16} (35.2%); C_{18} (14.5%); C_{20} (12.2%) and C_{22} (36.5%). Minor peaks representing 0.7% of the total saturated esters were also observed.

A 225.7 mg sample of the mixed fatty acids isolated from the total pilchard phospholipids absorbed 50.9 cc hydrogen at NTP and the hydrogenated derivatives showed an average equivalent wt of 318 ± 8 by titration with 0.01N-NaOH. These values correspond to an average of 3.2 double bonds/molecule compared with 2.4 double bonds/molecule for pilchard depot fatty acids which have an average equivalent wt of 282 (29).

Discussion

The results show that pilchard phospholipids comprise lecithins, lysolecithins, cephalins, inositol phosphatides, sphingomyelins and plasmalogens. The presence of cerebrosides may be inferred from the observation that Fraction II (Table I) liberated sphingosine on hydrolysis but not choline which would also have been detected if Fraction II had contained sphingomyelins instead of cerebrosides. The first peak eluted from the chromatogram of total pilchard phospholipids (Fraction I) showed only traces of ninhydrin reacting substances and was very probably of the cardiolipin type (3,9).

Preliminary acetone extraction of the minced flesh was carried out to remove most of the triglyceride oil which was present in large quantity. Subsequent treatment with $CHCl_3$:MeOH (2:1, v/v) undoubtedly extracted the bulk of the total phospholipids occurring in pilchard flesh, but it is likely that a proportion of these were removed together with the triglycerides during the initial acetone extraction. Thus the yields reported can only be regarded as semi-quantitative.

The crude extract of 19.5 g lipid from 750 g coarsely minced flesh corresponds to 2.6% by wt or 0.9% by wt of phospholipids based on the phosphorus analysis (22). However, the actual yield of 5.5 g purified phospholipids obtained after removal of triglyceride from the crude extract corresponds to 0.7% of the wet wt of flesh used—a figure which is in keeping with the values quoted by Lovern for the herring (22).

Precise fractionation of the water soluble phospholipid hydrolysis products was not achieved so that the data regarding the proportions of these components shown in Tables I and II are again only semi-quantitative.

Pilchard phospholipid fatty acids comprised large amounts of C_{22} hexaenoic, C_{20} pentaenoic and C_{16} saturated acids, with smaller amt of C_{18} dienoic, C_{18} monoenoic, C_{16} monoenoic and C_{18} saturated acids. The phospholipid fatty acids showed a greater average chain length and a higher degree of unsaturation than the acids of pilchard triglyceride oil.

Pending the isolation and characterization of individual components, it would seem that the qualitative composition of pilchard phospholipids is very similar to that of other fish (22) and animal organisms. Pilchard phospholipids may be distinguished to a certain extent by the fact that they contain very highly unsaturated fatty acids as part of their structure.

ACKNOWLEDGMENTS

Gas-chromatographic analysis of the phospholipid fatty acids by L. H. Krut.

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[Received December 9, 1963—Accepted May 18, 1964]