IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Patent of:	SAMPALIS, Fotini	Confirmation No.:	1897
Control No.:	95/001,774	Group Art Unit:	3991
Filed:	October 19, 2011	Examiner:	CAMPELL, Bruce R.

FOR: **INTER PARTES REEXAM OF U.S. PATENT 8,030,348: NATURAL MARINE SOURCE PHOSPHOLIPIDS COMPRISING POLYUNSATURATED FATTY ACIDS AND THEIR APPLICATIONS**

Mail Stop Declaration
Commissioner for Patents
P.O. Box 1450
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DECLARATION OF JACEK JACZYNSKI, PH.D. UNDER 37 C.F.R. § 1.132

I, Jacek Jaczynski, declare as follows:

1. I am a tenured Associate Professor of Food Science and Technology at West Virginia University; Davis College of Agriculture, Natural Resources, and Design; Division of Animal and Nutritional Sciences. My appointment is 50% research and 50% teaching. I have been a professor at West Virginia University since 2002.

2. I earned a Ph.D. in Food Science and Technology in 2002 from Oregon State University, Seafood Research and Education Center. Immediately following my doctoral work, I joined West Virginia University as a faculty member. For the past 14 years I have been actively pursuing scientific research specializing in aquatic foods, with an emphasis on krill. I have published 15 book chapters and over 50 peer-reviewed articles on food science and technology, many in high impact journals as indexed by Journal Citation Reports.® For example, one of my peer-reviewed publications directly concerns solvent extraction of krill

oil.[1] In addition, I am the sole inventor of an issued patent (U.S. 7,763,717) and the inventor of two other patent applications currently under examination. One focus of my patent and patent applications is a method for isolating lipids from krill.

3. I serve on the Editorial Board for the *Journal of Aquatic Food Product Technology* and as a peer-reviewer for several food science journals, such as *Food Chemistry* and the *Journal of Agricultural and Food Chemistry*. I am a professional member of the Institute of Food Technologists ("IFT"), the American Chemical Society, the World Aquaculture Society, and Gamma Sigma Delta, an honorary society of agricultural scientists. I served as a Chair of the Division of Aquatic Food Products of the IFT for the 2010-2011 term. For the past 10 years I have also taught food science-related courses at West Virginia University, many of which enroll over 300 students annually. My curriculum vitae is attached as **Appendix A**.

4. In December of 2011, I was engaged by counsel for Neptune Technologies and Bioressources, Inc. ("Neptune") to review U.S. Patent 8,030,348 ("the '348 patent") and its substantive prosecution history, the Corrected Request for Reexamination filed by Aker Biomarine ("Aker"), listed as U.S.S.N. 95/001,714, including the Declaration of Mr. Bjorn Ole Haugsgjerd and the Declaration of Dr. Thomas Gundersen, and supporting materials, and to provide my expert scientific opinion regarding whether Gundersen and Haugsgjerd accurately followed the process disclosed in patent publication WO 00/23546 ("Beaudoin I") and CA 2,251,265 ("Beaudoin II") and therefore whether the data presented by Aker accurately characterized the krill extract obtained by Beaudoin. Also, I was asked to express my opinion on why intact phospholipids bearing omega 3 fatty acids, such as those found in krill oil extracts, are superior to other forms of omega 3 fatty acids, such as the triglyceride-bound forms seen in fish and algal oils, as well as free fatty acids.

5. I have had no prior direct involvement with either Neptune or Aker. I am being compensated at my customary hourly rate for my time spent on developing, forming, and expressing the facts and opinions in this declaration. I have no personal interest in the ultimate outcome of the reexamination proceedings involving the '348 patent.

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[1] *See* Gigliotti *et al.* "Extraction and Characterisation of Lipids from Antarctic Krill (*Euphausia superba*)" *Food Chemistry* 125(3): 1028-1036 (April, 2011), **Appendix B**.

6. I have carefully read the information provided and also conducted my own search of relevant, peer-reviewed scientific literature. Below I provide my expert scientific opinion.

Gundersen and Haugsgjerd Did Not Accurately Replicate Beaudoin I or Beaudoin II.

7. In my opinion, Gundersen and Haugsgjerd did not accurately reproduce the methodology for total lipid extraction from krill that is disclosed in Beaudoin I or II. Specifically, Gundersen did not sufficiently heat the krill oil samples in a manner that was appropriate to replicate Beaudoin I or II, and Haugsgjerd did not accurately replicate the extraction method of Beaudoin I or II because he added a significant step to the Beaudoin protocol. For at least these reasons, it is my opinion that Haugsgjerd and Gundersen failed to opine on the specific process of Beaudoin I or II and therefore failed to characterize the krill extract actually produced by Beaudoin I or II.

Gundersen Did Not Appropriately Heat the Samples.

8. Gundersen conducted the last step of the krill oil extraction procedure (which was partially conducted by Haugsgjerd). In doing so, Gundersen applied heat in a manner inconsistent with Beaudoin I or II to the krill oil extracted by Haugsgjerd. Specifically, Gundersen alleges that he conducted a heat treatment at 125°C for 15 minutes or at 70°C for 5 minutes, in an attempt to reproduce Beaudoin I and II (*see* Gundersen Declaration, Exhibit 2, Analytical Report second of two pages numbered 1, between page 5 and page 7).[2] However, in his attempt to heat the oil, Gundersen placed a heat block inside the oven of a gas chromatograph set to either 70°C or 125°C for at least one hour (*see* Gundersen Declaration, Analytical Report second page numbered 1, between page 5 and page 7). A vial of krill oil extract was then heated using the heat block for 15 minutes at 125°C or 5 minutes at 70°C (*see* Gundersen Declaration, Exhibit 2, Analytical Report second of two pages numbered 1, between page 5 and page 7). After Gundersen heated the vials, they were allowed to cool on a laboratory bench to room temperature (*see* Gundersen Declaration, Exhibit 2, Analytical Report second of two pages numbered 1, between page 5 and page 7).

[2] I respectfully note that the confusion regarding page numbers in the Gundersen declaration stems from the declaration apparently being submitted either out of order or with incorrect pagination.

9. In my opinion, this heat treatment did not allow the oil to be heated to the temperature disclosed by Beaudoin I or II for the time specified by Beaudoin I or II due to slow heat transfer to the oil from the heat block. Gundersen's heating method was mediated primarily by air-liquid convection and not conduction. It is a well-established fact that conduction results in much quicker heat transfer than convection.[3] In simple terms, heated air contains relatively fewer molecules that can transfer heat from one object to another, as compared to heated liquids, such as oils as in a heated oil bath. Therefore, the transfer of heat via convection is much slower than conduction; thus, the samples heated as described by Gundersen were not maintained at the temperature of 125°C for 15 minutes or 70°C for 5 minutes.

10. A simple analogy allows illustration of this complex phenomenon. Consider placing one's hand in a standard kitchen oven set at a moderate temperature, say 400°F (which is about 200°C). One could easily hold one's hand in this oven for a period of time before experiencing physical discomfort or injury. If one were to place one's hand in a pot of boiling water (*i.e.*, around 100°C), however, one would immediately experience a burning sensation. This common scenario is explained by the difference between heat transfer by a slower method, convection (*i.e.*, the stove in the analogy), versus a faster method, conduction (*i.e.*, the boiling pot of water in the analogy).

11. Accordingly, when Dr. Gundersen placed the extracted krill oil in a heat block, he relied on heat transfer by convection to allegedly heat the oil to 125°C (or 70°C). Like the hand in the oven described above, the oil samples themselves did not reach and maintain a temperature of 125°C for 15 minutes. In contrast, during the prosecution of U.S. Patent 8,030,348, the applicant submitted data obtained after heating for 15 minutes at 125°C by placing the extracted oil in an oil bath, which, in my opinion, accurately re-created Beaudoin I.[4] Using this appropriate heat transfer method, mediated by conduction, the oil reached 125°C and therefore experienced a full 15 minute exposure to this temperature.

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[3] *See, e.g.*, Singh and Heldman, *Introduction to Food Engineering* (3rd ed.), New York, NY: Academic Press, 2008 (pp. 222-27), **Appendix C**; Heldman and Lund, *Handbook of Food Engineering*, New York, NY: Marcel Dekker, 1992 (pp. 247-59), **Appendix D,** both of which are fundamental food engineering textbooks.

[4] As noted in ¶4 above, I reviewed the office Action response filed on May 31, 2011 in the prosecution of the U.S. Patent 8,030,348.

12. I also note that the proper use of a heat block to heat an oil extract effectively has been described in the literature. For example, in Herman and Groves,[5] the authors conduct an experiment in which they thermally stress lipid emulsions containing phospholipids and observe hydrolysis of the fatty acids off of the phospholipids from this heating. Specifically, they describe, at page 775:

> "**Thermal stress was applied by filling heating block chambers** (Dry Baths, Fisher Scientific, Itasca, IL; 60 chambers per block, each 12 mm diameter and 50 mm deep) **with oil and immersing the 2-mL ampoules** containing the emulsion at the desired temperature, covering the blocks with aluminum foil to minimize thermal fluctuation" (emphasis added).

Such a protocol would allow effective heat transfer to the samples because it relies on conduction through hot oil, as was performed in obtaining the data presented in the prosecution of U.S. 8,030,348. Gundersen did not follow this known protocol.

13. In my expert opinion, the ineffective heating applied by Gundersen had a significant effect on the extent of hydrolysis of the ester bonds connecting fatty acids (*e.g.* DHA and EPA) to the glycerol backbone of the phospholipids. Accordingly, Gundersen only allegedly observed a residual mass spectrometry signal of phospholipids bearing DHA and EPA (or EPA/EPA or DHA/DHA).

14. Further, I also note that Gundersen provides an unclear trend as to the effect of heating. Comparing the HPLC-MS data presented in Appendix A, Gundersen appears to detect the same intensity peaks for non-heated, heated to 60°C or 70°C, and heated to 125°C (*see, e.g.*, chromatograms labeled P308-1, P308-2, and P308-3). This further underscores the ineffective heating approach used by Gundersen.

Haugsgjerd and Gundersen Added an Experimental Step Not Disclosed in Beaudoin I or Beaudoin II.

15. Further, it is my opinion as an expert on krill oil extraction that not only did Haugsgjerd and Gundersen fail to establish that the oils were sufficiently heated to replicate Beaudoin I or II,

[5] Herman and Groves, "The Influence of Free Fatty Acid Formation on the pH of Phospholipid-stabilized Triglyceride Emulsions," *Pharmaceutical Research*, 10(5): 774-76 (1993), **Appendix E.**

Haugsgjerd also performed an *additional* experimental step in his extraction procedure that *deviated* from Beaudoin I and II. Specifically, Haugsgjerd reports the following steps to generate Fractions IIa and IIb (Haugsgjerd Declaration, ¶ 3, Gundersen Declaration, Exhibit 2, Analytical Report second of two pages numbered 1, between page 5 and page 7):

- extracting with either ethanol or ethyl acetate;
- filtering solvent and evaporating under reduced pressure;
- "flush[ing] samples with nitrogen gas" and "stor[ing] at -20C until further analysis;" and
- sending samples from Haugsgjerd to Gundersen and having Gundersen unsuccessfully attempt to heat to 125°C for 15 minutes.

I note that the Beaudoin I or II protocol, as successfully replicated to generate the data presented in the prosecution of U.S. 8,030,348, did not involve flushing oil fractions with nitrogen gas and freezing at -20°C before heating. On the contrary, in both the disclosed Beaudoin process and the experiments conducted to generate the data presented in the prosecution of U.S. 8,030,348, following evaporation to partially remove solvent, the fractions were immediately heated (*see* Response to Office Action of April 29, 2010 in U.S. 10/485,094, Appendix I; Beaudoin I, pages 7 and 10). There is no mention in Beaudoin I or II of either storing samples at -20°C or under nitrogen gas.

16. In my opinion, Haugsgjerd's deviation from the process disclosed in Beaudoin I or II is very significant. By freezing the sample before heating, Haugsgjerd *further suppressed* any hydrolysis of ester bonds found on the phospholipids being analyzed. It is my opinion that after Beaudoin completed the penultimate step of removing the solvent by rotary evaporation, he heated to remove the residual "volatile matter and humidity" from fraction I and fraction II (*see* Beaudoin I, page 10). Note that in Table 13 of Beaudoin I "volatile matter and moisture levels" of 10% and 6.8%, respectively, for fractions I and II are reported. These levels of solvent and humidity would have rendered those oils crude for Beaudoin's purposes of determining total lipids generated by his procedure, and therefore prompted him

to "get rid of traces of solvents [by] briefly heat[ing] (to about 125°C, for about 15 min.) the oil under nitrogen" (*see* Beaudoin I, pages 7 and 10). This heating inevitably led to the hydrolysis of ester bonds. As a result, the phospholipids were degraded and consequently released free fatty acids such as EPA and DHA. It is my opinion that Haugsgjerd suppressed this inevitable hydrolysis by storing his samples at -20°C. This is a step not taught by Beaudoin I or II, which causes one to question why Haugsgjerd did this. It is also my opinion that had Haugsgjerd heated the samples in an oil bath or with a heating jacket after removing the solvents by rotary evaporation and then stored the final product under nitrogen at -20°C for subsequent evaluation by Gundersen, Gundersen would not have allegedly observed even residual amounts of phospholipids carrying two of DHA and/or EPA. *I note, as evidence for this opinion, the paper cited in ¶ 12 above in which the authors thermally stressed phospholipid emulsions in heating blocks containing oil and described hydrolysis of ester bonds in phospholipids. As an inevitable consequence of this thermal hydrolysis, free fatty acids were released from the phospholipids.*

17. In summary, it is my opinion that, due to inadequate sample heating and the addition of an experimental step to the extraction protocol, Haugsgjerd and Gundersen did not accurately reproduce the methodology of oil extraction from krill as disclosed in Beaudoin I or II, and therefore the mass spectrometric data presented by Gundersen fails to accurately characterize the krill oil actually produced by Beaudoin I or II.

Intact Phospholipids Containing Omega 3 Polyunsaturated Fatty Acids Possess Desirable Properties Not Observed in Other Forms of Lipids Such as Triglycerides Containing Omega 3 Polyunsaturated Fatty Acids.

18. I have been asked to express my opinion, based on the peer reviewed literature, including my laboratory's published research, on the superior properties of omega 3 fatty acids in phospholipid form as compared to other forms (or lipid classes), such as triglycerides.

Possible Forms of Omega 3 Fatty Acids and Their Presence in Various Extracts.

19. The '348 patent teaches extracts, including krill extracts, and more specifically, intact phospholipids bearing omega 3 polyunsaturated fatty acids, such as EPA and DHA. These compositions are distinguishable from fish oil and algal oil, as fish and algal oil extracts feature EPA and DHA bound to triglycerides. My laboratory recently conducted a comparative study among krill oil, fish oil, and algal oil.[6] Thin-layer-chromatography (TLC) analysis clearly demonstrated that krill oil contained significant amounts of phospholipids, while the amount of detected phospholipids in fish oil and algal oil was negligible. Further, TLC experiments showed that krill oil contained negligible amounts of triglycerides, while fish oil and algal oil contained significant amounts.

20. Further, the Kassis *et al.* study showed that there are high amounts of EPA and DHA in krill oil (47% of total fatty acids) and these omega 3 polyunsaturated fatty acids are primarily esterified in phospholipids. On the other hand, EPA and DHA in fish oil and algal oil are not significantly esterified in phospholipids, but are largely esterified in triglycerides.

21. Therefore, krill oil, unlike fish oil and algal oil contains significant amounts of omega 3 fatty acids esterified in phospholipids.

Phospholipids Have Superior Physiological Absorption Profiles.

22. Based on the recently developing research in this field, it appears that the chemical "carrier" of omega 3 polyunsaturated fatty acids in krill extracts, *i.e.*, phospholipids, provides superior physiological absorption profiles, especially as compared to triglyceride "carriers."

23. Phospholipids are *amphiphilic* and triglycerides are not. The chemical structure of an amphiphilic compound contains functional groups that allow *simultaneous* water- and lipid-solubility due to the presence of hydrophilic and hydrophobic moieties. The hydrophilic moiety in krill phospholipids is present at the *sn*-3 position, where the nitrogen-containing moiety (*e.g.*, the choline in phosphatidylcholine) provides positive charges and the phosphate bridge provides negative charges. These positive and negative charges interact with charges on the water dipoles, thereby, resulting in phospholipid solubility in water. The hydrophobic

[6] *See* Kassis *et al.*, "Characterization of Lipids and Antioxidant Capacity of Novel Nutraceutical Egg Products Developed with Omega-3-Rich Oils" *J Sci Food Agr* 92(1): 66-73 (2012), **Appendix F.**

moiety in krill phospholipids is present at the *sn*-1 and *sn*-2 positions in the form of the long hydrocarbon chains of omega 3 polyunsaturated fatty acids. In contrast, the triglycerides of fish oil and algal oil are not amphiphilic, as their *sn*-1, *sn*-2, and *sn*-3 positions are occupied with hydrophobic fatty acids that have long hydrocarbon chains. Accordingly, triglycerides are lipid-soluble but not water-soluble. By way of analogy, phospholipids (such as those of the '348 patent) are like ambidextrous people, having the ability to write with both the right and left hand, while triglycerides (such as those of fish oil and algal oil) are like right- or left-handed people, having the ability to write with only a single hand.

24. This duality of phospholipids has implications for absorption in the body. When lipids (*i.e.*, typical oils and fats composed of triglycerides, such as fish oil and algal oil) are ingested, because of their water insolubility and lower density than water, they float on top of and form a layer that is separate from the digestive juices in the stomach and small intestine. These digestive juices contain lipolytic enzymes that digest lipids. Therefore, before water-insoluble lipids (*i.e.*, triglycerides) can be digested and absorbed, they first have to be emulsified by bile. However, amphiphilic compounds such as phospholipids (like those in the '348 patent) skip the emulsification process because they are water soluble. In fact, phospholipids enhance emulsification by bile and, thereby, aid in digestion of water-insoluble oils.[7]

25. Phospholipids and triglycerides are also digested by different enzymes, and many authors have attributed this difference to the enhanced digestion and absorption of phospholipids. Lingual, gastric, and pancreatic lipases initiate digestion of triglycerides by cleaving ester bonds preferentially at external positions, *sn*-1 and *sn*-3, yielding a 2-monoglyceride and two free fatty acids; while phospholipases, responsible for digestion of phospholipids, cleave at the center position, *sn*-2, and yield a 1-lysophospholipid and a free fatty acid.[8] Carnielli *et al.* (1998) suggests that cleavage at the center position results in enhanced digestion.[9]

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[7] *See* O'Doherty *et al.*, "Role of Luminal Lecithin in Intestinal Fat Absorption" *Lipids* 8: 249-55 (1973), **Appendix G**.

[8] *See* Mattson *et al.* "The Digestion and Absorption of Triglycerides" *J Biol Chem* 239: 2772-7(1964), **Appendix H**; Tso *et al.*, "Evidence for Separate Pathways of Chylomicron and Very Low-Density Lipoprotein Assembly and Transport by Rat Small Intestine" *Am J Physiol* 247: G599-G610 (1984), **Appendix I**.

[9] Carnielli *et al.* "Intestinal absorption of long-chain polyunsaturated fatty acids in preterm infants fed breast milk or formula" *Am J Clin Nutr* 67: 97-103 (1998), **Appendix J**.

Additionally, triglycerides in fish oil containing omega 3 fatty acids are relatively resistant to digestion by human lipase.[10] Further, Carnielli *et al.* and Morgan *et al.*[11] have shown that phospholipids containing omega 3 polyunsaturated fatty acids are digested and absorbed better than triglycerides.

26. Digested and absorbed lipids have to be delivered, via the blood stream, to destination organs. However, because the digested lipids (except phospholipids) are water insoluble, they are packaged in micelles for absorption and subsequently in chylomicrons before they enter the blood stream and reach the destination organs. Chylomicrons are specialized spherical, delivery vehicles for digested lipids which are distributed in the blood stream. It is important to emphasize that the surface of chylomicrons is coated with phospholipids that provide water solubility in the blood, while triglycerides are buried in the interior.

27. Amate *et al.* (2001) suggests that lipids following their digestion and absorption are re-esterified to the same chemical form (*i.e.*, either triglycerides or phospholipids) that they were in prior to digestion and absorption (*i.e.*, the same chemical form as they were in the diet).[12] Chylomicrons in the blood stream exchange their components with high-density-lipoproteins (HDLs, also referred to as "good cholesterol").[13]

28. If the lipids in the diet are in the phospholipid form (such as in krill oil) and since phospholipids coat the exterior of chylomicrons, they have a higher likelihood of affecting the blood lipid panel (*i.e.*, total blood cholesterol, total blood triglycerides, HDLs, and low-density-lipoproteins or LDLs commonly referred to as "bad cholesterol") and thus, affecting cardiovascular disease, than triglycerides. Therefore, the transport of phospholipids to

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[10] Bottino *et al.*, "Resistance of Certain Longchain Polyunsaturated Fatty Acids of Marine Oils to Pancreatic Lipase Hydrolysis" *Lipids* 2, 489-93 (1967), **Appendix K**; Hernell *et al.*, "Does the Bile Salt-Stimulated Lipase of Human Milk Have a Role in the Use of the Milk Long-Chain Polyunsaturated Fatty Acids?" *J Pediatr Gastroenterol Nutr* 16: 426-31(1993), **Appendix L**.

[11] Morgan *et al.* ("Fatty Acid Balance Studies In Term Infants Fed Formula Milk Containing Long-Chain Polyunsaturated Fatty Acids" *Acta Paediatr* 87: 136-42 (1998), **Appendix M**.

[12] Amate *et al.* "Feeding Infant Piglets Formula with Long-Chain Polyunsaturated Fatty Acids as Triacylglycerols or Phospholipids Influences the Distribution of these Fatty Acids in Plasma Lipoprotein Fractions" *J Nutr* 131: 1250-55 (2001), **Appendix N**.

[13] *See* Mattson *et al.* "The Digestion and Absorption of Triglycerides" *J Biol Chem* 239(9): 2772-77 (1964), **Appendix H**.

destination organs is also more easily facilitated as compared to triglycerides which are buried in the interior of chylomicrons and are water-insoluble (*i.e.*, insoluble in blood). Amate *et al.* (2001) confirmed this concept in piglets that were fed egg phospholipids containing DHA. The piglets had higher concentrations of DHA in high density lipoproteins.

29. One could use a simple analogy to illustrate this complex concept. Imagine driving a car with two passengers that must reach the National Mall in Washington D.C. in rush hour. The delivery of the two passengers to the National Mall will be significantly delayed if the driver takes the typical, always congested, highways of the D.C. area. However, if the driver takes the HOV-lane, the two passengers will be able to reach the National Mall more efficiently. The two passengers are analogous to the two essential omega 3 polyunsaturated fatty acids, EPA and DHA, that have to reach a destination organ (the National Mall). The slow highways in the D.C. area are like the triglyceride absorption mechanism and the HOV-lane is like the phospholipid absorption mechanism. It is clear that the HOV-lane (*i.e.*, phospholipids) will allow more efficient delivery of the two passengers (*i.e.*, EPA and DHA) to the National Mall (*i.e.*, destination organs) when compared to the typical, slow highways (*i.e.*, triglycerides) in the DC area.

Omega 3 Polyunsaturated Fatty Acids in Phospholipid Form Have Superior Medical Effects.

30. A number of specific health benefits in humans are associated with EPA and DHA. Although these health benefits do not intrinsically depend on whether EPA and DHA are provided in the diet as phospholipids or triglycerides, as described above, the delivery of these two essential omega-3 polyunsaturated fatty acids to the destination organs is more efficient via phospholipids. Therefore, the *extent* of the health benefits has been shown to be greater when EPA and DHA are bound in phospholipids instead of triglycerides.

31. Two major classes of health benefits associated with dietary intake of EPA and DHA are anti-inflammatory and cardiovascular benefits. These health benefits are based on how EPA and DHA are metabolized in the human body. As EPA is metabolized, it competitively inhibits two enzymes (cyclooxygenase and 5-lipoxygenase). If these enzymes are not inhibited, they catalyze production of eicosanoids that initiate inflammation. However, adequate supply of EPA results in reduced production of eicosanoids, thereby having an anti-

inflammatory effect, which ultimately reduces platelet aggregation and adherence as well as vasoconstriction. As a final result, the vasodilation is stimulated.[14] It is apparent that EPA reduces the development of atherosclerotic plaques, and as such, improves cardiovascular health. There is another set of anti-inflammatory compounds (D-series resolvins, docosatrienes, and neuroprotectins) associated with DHA metabolism. These compounds have direct anti-inflammatory properties unlike the indirect effect of EPA via competitive enzyme inhibition. These DHA-derived compounds clear inflammation sites of cellular debris, suppress pro-inflammatory interleukins, and also have neuroprotective properties.[15] Similar to EPA, it is apparent that adequate supply of DHA improves cardiovascular health.

32. As mentioned above, the *extent* of the health benefits of omega 3 polyunsaturated fatty acids is greater when these molecules are esterified as intact phospholipids, as compared to being esterified to triglycerides. My krill research group recently published a review article describing, in part, the health benefits associated with consumption of krill oil, which contains intact phospholipids bearing omega 3 polyunsaturated fatty acids, EPA and DHA.[16] This article was featured as a Special Article on the cover of the February 2007 issue of *Nutrition Reviews*. As described therein, the effect of krill oil and fish oil on hyperlipidemia was investigated using human subjects diagnosed with mild to high blood cholesterol and total triglycerides.[17] Krill oil (2-3 g/day) reduced total blood triglycerides by 27-28%, while fish oil had no effect. Krill oil and fish oil both reduced blood cholesterol, but krill oil resulted in higher reduction (up to 18%) than fish oil. Krill oil reduced LDLs by up to 39%, whereas fish oil had no effect. In addition, krill oil increased HDLs by up to 60%, while fish oil had no effect. In infants, DHA was absorbed better from egg phospholipids than algal

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[14] Simopoulos, "Omega-3 Fatty Acids in Inflammation and Autoimmune Diseases" *J Am Coll Nutr* 21(6): 495-505 (2002), **Appendix O**.

[15] Hong *et al.*, "Novel Docosatrienes and 17S-resolvins Generated from Docosahexaenoic Acid in Murine Brain, Human Blood, and Glial Cells. Autacoids in Anti-Inflammation" *J Biol Chem* 278(17): 14677-87 (2003), **Appendix P**.

[16] *See* Tou *et al.*, "Krill for Human Consumption: Nutritional Value and Potential Health Benefits" *Nutr Rev* 65(2): 63-77 (2007), **Appendix Q**.

[17] *See* Tou *et al.* (citing Bunea *et al.*, "Evaluation of the Effects of Neptune Krill Oil on the Clinical Course of Hyperlipidemia" *Altern Med Rev* 9: 420-28 (2004), **Appendix R**.

triglycerides despite a 2.5 times higher dose of DHA in algal triglycerides.[18] In rats, krill protein concentrate (KPC) containing DHA in phospholipids resulted in better accretion of DHA in the brain and liver compared to triglycerides.[19] This is important because DHA is indispensable and critical for proper brain development and the liver is the main organ that processes fatty acids including DHA after absorption and digestion. Consumption of EPA and DHA from krill oil resulted in an increased HDLs/triglycerides ratio compared to fish oil even though the DHA concentration in fish oil was more than 2 times higher than in the krill oil administered.[20] Further, krill oil resulted in reduction of several symptoms commonly associated with premenstrual syndrome and dysmenorrhea to a greater degree than fish oil.[21] This was attributed to more efficient delivery of EPA/DHA esterified in phospholipids in krill oil as opposed to triglycerides in fish oil.

The Extract of the '348 Patent, But Not Beaudoin's Oil, Would Likely Possess the Above Effects.

33. The '348 patent teaches intact phospholipids containing EPA and DHA (*see* Tables 9 and 10 of the '348 patent). These phospholipids are <u>not</u> heat damaged (*see* Column 18 of the '348 patent). Therefore, in my opinion, the extract of the '348 patent would posses the superior phospholipid-associated physiological effects observed in the articles discussed above. In contrast, the Beaudoin oil, containing far fewer intact phospholipids and being heat damaged, would not possess the extent of these beneficial characteristics. This is bolstered by the fact that Beaudoin discloses that the fractions I and II, *before heating*, have free fatty acid amounts of 23.7 ± 1.1% and 20.3 ± 0.3%, respectively (*see* Beaudoin I, Table 14). That is to say, almost one quarter of the Beaudoin oil is species that are *not attached* to the phospholipid carrier *before heating*. After exposing the oil to 125°C for 15 minutes, the amount of phospholipids that are intact is less, as seen in the experiments conducted in the prosecution of the '348 patent and supported by the literature.

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[18] Carnielli *et al.*, "Intestinal Absorption of Long-Chain Polyunsaturated Fatty Acids in Preterm Infants Fed Breast Milk or Formula" *Am J Clin Nutr* 67: 97-103 (1998), **Appendix J.**

[19] Bridges *et al.*, "Determination of Digestibility, Tissue Deposition, and Metabolism of the Omega-3 Fatty Acid Content of Krill Protein Concentrate in Growing Rats" *J Agric Food Chem* 58: 2830-7 (2010), **Appendix S.**

[20] Ulven *et al.*, "Metabolic Effects of Krill Oil are Essentially Similar to Those of Fish Oil But at Lower Dose of EPA and DHA, in Health Volunteers" *Lipids* 46: 37-46 (2011), **Appendix T**

[21] Sampalis *et al.*, "Evaluation of the Effects of Neptune Krill Oil™ on the Management of Premenstrual Syndrome and Dysmenorrheal" *Altern Med Rev* 8: 171-9 (2003), **Appendix U.**

34. Specifically, heat-induced hydrolysis will occur upon this heating. The extracted krill oils produced by Beaudoin contained 10% moisture and volatiles. This moisture would be sufficient to allow a heat-induced hydrolytic reaction in phospholipids. The literature is replete with examples of such; for instance, in Herman and Groves, referenced above, hydrolysis of the fatty acids from phospholipids occurred upon thermal stress during sterilization at 121°C in a soy oil-based emulsion.

35. Accordingly, it is my opinion that the intact, non-heat damaged phospholipids disclosed and claimed in the '348 patent would provide superior physiological and medical effects as compared to the heat damaged phospholipids of Beaudoin for at least the reasons discussed above.

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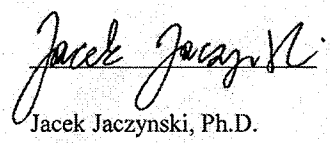
36. I further declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true, and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of U.S. Patent 8,030,348.

By: 

Jacek Jaczynski, Ph.D.

Dated: March 16, 2012

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Appendix A

Curriculum Vitae of Dr. Jacek Jaczynski

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Education:

- Oregon State University, Food Science and Technology, Ph.D., 2002, dissertation: "Surimi seafood under thermal and e-beam processing"
- Oregon State University, Food Science and Technology, M.S., 1998, thesis: "Antimicrobial activity of nisin and hen lysozyme acting in tandem against *Listeria monocytogenes* on a model surface"
- Agricultural University of Poznan (Poland), Biotechnology, M.S., 1995, thesis: "Optimization of a high-cell density propionic acid fermentation in a continuous bio-reactor system with microcarriers"

Work and Teaching Experience:

- Associate Professor (teaching – 50%, research – 50%), West Virginia University, 2008 to present
- Assistant Professor (teaching – 50%, research – 50%), West Virginia University, 2002-2008
- Graduate Research Assistant, Oregon State University Seafood Laboratory, 1998-2002
- Graduate Research Assistant, Oregon State University, 1996-1998

Annual Teaching:

- HN&F 126 – Society and Food (HN&F – Human Nutrition and Food), 3 CR, fall and spring semesters, annual enrollment ~220, approved for General Education Curriculum (GEC). I significantly modified this course and have enjoyed a steady increase in student enrollment from approximately 55 to 110 students per semester since I started teaching. The enrollment limitation is 110. I find this course very rewarding, because it provides me with a considerable challenge as a college educator to teach a large group of diverse majors at their early college career (mainly freshmen). At WVU, as at most major universities, teaching is assessed by the Student Evaluation of Instruction (SEI). The average for SEI's for all of the semesters I have taught HN&F 126 is 4.72 (scale 0.00-5.00) ranging from 4.07 to 4.99.
- FDST 200 – Food Science and Technology, 3 CR, fall semesters, annual enrollment ~65, approved for General Education Curriculum (GEC). I developed this course at WVU. As I have continually upgraded this course, the student enrollment grew to approximately 65. The enrollment limitation is 66. I genuinely enjoy teaching FSDT 200 – a course that I developed from the ground up and always look forward to the start of each fall semester to meet my new FDST 200 class. The average for SEI's for all of the semesters I have taught FDST 200 is 4.86 ranging from 4.80 to 4.95.
- FDST 445/545 – Food Microbiology, 3 CR, spring semesters, annual enrollment ~25. I developed this senior/graduate level course at WVU. Since the course approval, FDST 445/545 has experienced a steady increase in student enrollment and currently attracts approximately 25 senior/graduate level students from our college (various majors) as well as from the Medical School, Biology, and Chemistry majors. FDST 445/545 is a senior/graduate level course in my area of research specialization; and therefore, I have an enthusiastic approach to teaching this course. The average for SEI's for all of the semesters I have taught FDST 445/545 is 4.86 ranging from 4.71 to 4.95.

Professional Affiliations:

- Institute of Food Technologists
- American Chemical Society
- World Aquaculture Society
- Gamma Sigma Delta

Patents (* – denotes primary inventor):

- Jaczynski J. 2010. Continuous protein and lipid recovery from food animal processing byproducts. U.S. Patent and Trademark Office. Patent number 7,763,717.
- Matak KE, Jaczynski* J. 2011. Conversion of dark chicken meat processing by-products to white chicken meat food products with nutraceutical properties. U.S. Patent and Trademark Office. Application number 61/574,263.
- Matak KE, Jaczynski* J. 2011. The YumEGGa™ stick – Nutraceutical, ready-to-eat egg stick. U.S. Patent and Trademark Office. Application number 61/574,262.

Book Chapters (* – denotes corresponding author):

2004

- Jaczynski* J, Park JW. 2004. Temperature, Color and Texture Prediction Models for Surimi Seafood Pasteurization. In: Shahidi F, Spanier A, Ho CT, Braggins T, editors. *Quality of Fresh and Processed Foods*. New York: Kluwer Academics/Plenum Publishing. p 121-34.
- Jaczynski* J, Park JW. 2004. Application of Electron Beam to Surimi Seafood. In: Komolprasert V, Morehouse K, editors. *Irradiation of Food and Packaging: Recent Developments*. Washington, DC: American Chemical Society. p 165-79.

2005

- Su* YC, Daeschel MA, Frazier J, Jaczynski J. 2005. Microbiology and Pasteurization of Surimi Seafood. In: Park JW, editor. *Surimi and Surimi Seafood*, 2nd ed. Boca Raton: CRC Press. p 583-648.
- Jaczynski* J, Hunt A, Park JW. 2005. Safety and Quality of Frozen Fish, Shellfish and Related Products. In: Sun DW, editor. *Handbook of Frozen Food Processing and Packaging*. Boca Raton: CRC Press. p 341-377.

2007

- Torres* JA, Chen YC, Rodrigo-García J, Jaczynski J. 2007. Recovery of By-products from Seafood Processing Streams. In: Shahidi F, editor. *Maximizing the Value of Marine By-products*. Boca Raton: CRC Press. p 65-90.

2008

- Jaczynski* J. 2008. Protein and Lipid Recovery from Food Processing By-products Using Isoelectric Solubilization / Precipitation. In: Papadopoulos KN, editor. *Food Chemistry Research Developments*. Hauppauge: Nova Science Publishers. p 167-198.

2009

- Jaczynski* J. 2009. Application of Electron Beam to Food Processing with Emphasis on Seafood Products: Inactivation of Food-borne Pathogens and Effects on Food Quality. In: Bellinghouse VC, editor. *Food Processing: Methods, Techniques, and Trends*. Hauppauge: Nova Science Publishers. p 211-247.
- Jaczynski* J, Chen YC, Velazquez G, Torres JA. 2009. Procesamiento con haz de Electrones. In: Legarreta IG, Rosmini MA, Armenta R, editors. *Tecnologia de Productos de Origen Acuatico Pescado*. Mexico City: Editorial Limusa, S.A. de C.V. p 405-426.
- Jaczynski* J, Chen YC, Velazquez G, Torres JA. 2009. Recuperacion de Proteinas y Lipidos. In: Legarreta IG, Rosmini MA, Armenta R, editors. *Tecnologia de Productos de Origen Acuatico Pescado*. Mexico City: Editorial Limusa, S.A. de C.V. p 461-474.
- Matak KE, Jaczynski* J. 2009. Food Preservation with Electron Beam. In: Hulsen I, Ohnesorge E, editors. *Food Science Research and Technology*. Hauppauge: Nova Science Publishers. p 229-246.

2010

- Rodrigo-García J, Jaczynski J, Torres* JA. 2010. Recovery and Utilization of Proteins from Surimi Processing Water. In: Bechtel PJ, Smiley S, editors. *A Sustainable Future: Fish Processing Byproducts*. Fairbanks: Alaska Sea Grant, University of Alaska. p 147-160.

- Gehring CK, Gigliotti JC, Tou JC, Moritz JS, Jaczynski* J. 2010. The Biochemistry of Isoelectric Processing and Nutritional Quality of Proteins and Lipids Recovered with This Technique. In: Haugen S, Meijer S, editors. *Handbook of Nutritional Biochemistry: Genomics, Metabolomics and Food Supply.* Hauppauge: Nova Science Publishers. p 255-288.

2011

- Tahergorabi R, Hosseini SV, Jaczynski* J. 2011. Seafood Proteins. In: Phillips GO, Williams PA, editors. *Handbook of Food Proteins*. Cambridge: Woodhead Publishing Ltd. p 116-149.

2012

- Jaczynski* J, Tahergorabi R, Hunt A, Park JW. 2012. Safety and Quality of Frozen Aquatic Food Products. In: Sun DW, editor. *Handbook of Frozen Food Processing and Packaging*, 2nd ed. Boca Raton: CRC Press. p 343-385.
- Su* YC, Daeschel MA, Frazier J, Jaczynski J. 2012. Microbiology and Pasteurization of Surimi Seafood. In: Park JW, editor. *Surimi and Surimi Seafood*, 3rd ed. Boca Raton: CRC Press. In press.

Peer-Reviewed Publications (* – denotes corresponding author; impact factors and journal rankings based on 2010 Journal Citation Reports):

2002

- Jaczynski J, Park* JW. 2002. Temperature prediction during thermal processing of surimi seafood. *Journal of Food Science* 67(8):3053-3057 (impact: 1.733, rank: 35/128).

2003

- Jaczynski J, Park* JW. 2003. Predictive models for microbial inactivation and texture degradation in surimi seafood during thermal processing. *Journal of Food Science* 68(3):1025-1030 (impact: 1.733, rank: 35/128).
- Jaczynski J, Park* JW. 2003. Physicochemical properties of surimi seafood as affected by electron beam and heat. *Journal of Food Science* 68(5):1626-1630 (impact: 1.733, rank: 35/128).
- Jaczynski J, Park* JW. 2003. Microbial inactivation and electron penetration in surimi seafood during electron beam processing. *Journal of Food Science* 68(5):1788-1792 (impact: 1.733, rank: 35/128).

2004

- Jaczynski J, Park* JW. 2004. Physicochemical changes in Alaska pollock surimi and surimi gel as affected by electron beam. *Journal of Food Science* 69(1):53-57 (impact: 1.733, rank: 35/128).

2005

- Moritz* JS, Parsons AS, Buchanan NP, Baker NJ, Jaczynski J, Gekara OJ, Bryan WB. 2005. Synthetic methionine and feed restriction effects on performance and meat quality of organically reared broiler chickens. *Journal of Applied Poultry Research* 14:521-535 (impact: 0.745, rank: 26/56).

2006

- Black JL, Jaczynski* J. 2006. Temperature effect on inactivation kinetics of *Escherichia coli* O157:H7 by electron beam in ground beef, chicken breast meat, and trout fillets. *Journal of Food Science* 71(6):M221-227 (impact: 1.733, rank: 35/128).
- Chen YC, Nguyen J, Semmens K, Beamer S, Jaczynski* J. 2006. Enhancement of omega-3 fatty acid content in rainbow trout (*Oncorhynchus mykiss*) fillets. *Journal of Food Science* 71(7):C383-389 (impact: 1.733, rank: 35/128).

2007

- Chen YC, Nguyen J, Semmens K, Beamer S, Jaczynski* J. 2007. Physicochemical changes in omega-3-enhanced farmed rainbow trout (*Oncorhynchus mykiss*) muscle during refrigerated storage. *Food Chemistry* 104(3):1143-1152 (impact: 3.458, rank: 5/128).
- Chen YC, Jaczynski* J. 2007. Gelation of protein recovered from whole Antarctic krill (*Euphausia superba*) by isoelectric solubilization / precipitation as affected by functional additives. *Journal of Agricultural and Food Chemistry* 55(5):1814-1822 (impact: 2.816, rank: 10/128).

- Tou* JC, Jaczynski J, Chen YC. 2007. Krill for human consumption: nutritional value and potential health benefits. *Nutrition Reviews* 65(2):63-77 (impact: 4.077, rank: 9/70).
- Chen YC, Jaczynski* J. 2007. Protein recovery from rainbow trout (*Oncorhynchus mykiss*) processing by-products via isoelectric solubilization / precipitation and its gelation properties as affected by functional additives. *Journal of Agricultural and Food Chemistry* 55(22):9079-9088 (impact: 2.816, rank: 10/128).
- Chen YC, Tou JC, Jaczynski* J. 2007. Amino acid, fatty acid, and mineral profiles of materials recovered from rainbow trout (*Oncorhynchus mykiss*) processing by-products using isoelectric solubilization / precipitation. *Journal of Food Science* 72(9):C527-535 (impact: 1.733, rank: 35/128).
- Black JL, Jaczynski* J. 2007. Effect of ionic strength on inactivation kinetics of *Escherichia coli* O157:H7 by electron beam in ground beef, chicken breast meat, and trout fillets. *International Journal of Food Science and Technology* 42(7):894-902 (impact: 1.223, rank: 54/128).
- Chalise PR, Hotta E, Matak KE, Jaczynski* J. 2007. Inactivation kinetics of *Escherichia coli* by pulsed electron beam. *Journal of Food Science* 72(7):M280-285 (impact: 1.733, rank: 35/128).

2008

- Black JL, Jaczynski* J. 2008. Effect of water activity on inactivation kinetics of *Escherichia coli* O157:H7 by electron beam in ground beef, chicken breast meat, and trout fillets. *International Journal of Food Science and Technology* 43(4):579-586 (impact: 1.223, rank: 54/128).
- Sommers* CH, Rajkowski KT, Jaczynski J, Matak KE. 2008. Letter to the editor: Radiation sensitivity of *Escherichia coli* JM109 and DH5α. *Journal of Food Science* 73(1):vii-viii (impact: 1.733, rank: 35/128).
- Levanduski L, Jaczynski* J. 2008. Increased resistance of *Escherichia coli* O157:H7 to electron beam following repetitive irradiation at sub-lethal doses. *International Journal of Food Microbiology* 121(3):328-334 (impact: 3.143, rank: 7/128).
- Chen YC, Nguyen J, Semmens K, Beamer S, Jaczynski* J. 2008. Effects of dietary alpha-tocopheryl acetate on lipid oxidation and alpha-tocopherol content of novel omega-3-enhanced farmed rainbow trout (*Oncorhynchus mykiss*) fillets. *LWT – Food Science and Technology* 41(2):244-253 (impact: 2.292, rank: 22/128).
- Chen YC, Nguyen J, Semmens K, Beamer S, Jaczynski* J. 2008. Chemical changes in omega-3-enhanced farmed rainbow trout (*Oncorhynchus mykiss*) fillets during abusive-temperature storage. *Food Control* 19(6):599-608 (impact: 2.812, rank: 11/128).
- Gigliotti JC, Jaczynski J, Tou* JC. 2008. Determination of the nutritional value, protein quality and safety of krill protein concentrate isolated using an isoelectric solubilization / precipitation technique. *Food Chemistry* 111(1):209-214 (impact: 3.458, rank: 5/128).

2009

- Chen YC, Tou JC, Jaczynski* J. 2009. Amino acid and mineral composition of protein and other components and their recovery yields from whole Antarctic krill (*Euphausia superba*) using isoelectric solubilization / precipitation. *Journal of Food Science* 74(2):H31-39 (impact: 1.733, rank: 35/128).
- Taskaya L, Chen YC, Beamer S, Tou JC, Jaczynski* J. 2009. Compositional characteristics of materials recovered from whole gutted silver carp (*Hypophthalmichthys molitrix*) using isoelectric solubilization / precipitation. *Journal of Agricultural and Food Chemistry* 57(10):4259-4266 (impact: 2.816, rank: 10/128).
- Taskaya L, Jaczynski J. 2009*. Flocculation-enhanced protein recovery from fish processing by-products by isoelectric solubilization / precipitation. *LWT – Food Science and Technology* 42(2):570-575 (impact: 2.292, rank: 22/128).
- Taskaya L, Chen YC, Jaczynski* J. 2009. Functional properties of proteins recovered from whole gutted silver carp (*Hypophthalmichthys molitrix*) by isoelectric solubilization / precipitation. *LWT – Food Science and Technology* 42(6):1082-1089 (impact: 2.292, rank: 22/128).
- Taskaya L, Chen YC, Beamer S, Jaczynski* J. 2009. Texture and colour properties of proteins recovered from whole gutted silver carp (*Hypophthalmichthys molitrix*) using isoelectric solubilisation / precipitation. *Journal of the Science of Food and Agriculture* 89(2):349-358 (impact: 1.360, rank: 48/128).

- Gehring CK, Davenport MP, Jaczynski* J. 2009. Functional and nutritional quality of protein and lipid recovered from fish processing by-products and underutilized aquatic species using isoelectric solubilization / precipitation. *Current Nutrition and Food Science* 5(1):17-39 (impact: NA, rank: NA).
- Gehring CK, Jaczynski J, Moritz* JS. 2009. Improvement of pellet quality with proteins recovered from whole fish using isoelectric solubilization-precipitation. *Journal of Applied Poultry Research* 18:418-431 (impact: 0.745, rank: 26/56).
- Lansdowne L, Beamer S, Jaczynski J, Matak* KE. 2009. Survival of *Listeria innocua* after isoelectric solubilization and precipitation of fish protein. *Journal of Food Science* 74(4):M201-205 (impact: 1.733, rank: 35/128).
- Lansdowne L, Beamer S, Jaczynski J, Matak* KE. 2009. Survival of *Escherichia coli* after isoelectric solubilization / precipitation of fish. *Journal of Food Protection* 72(7):1398-1403 (impact: 1.720, rank: 37/128).

2010

- James DL, Jaczynski J, Matak* KE. 2010. Electron beam irradiation on nalidixic acid-resistant *Salmonella* Montevideo in cooked tomato puree of various pH values. *Journal of Food Safety* 30(3):515-525 (impact: 0.702, rank: 77/128).
- Hvizdzak AL, Beamer S, Jaczynski J, Matak* KE. 2010. Use of electron beam irradiation for the reduction of *Salmonella enterica* serovars Typhimurium and Tennessee in peanut butter. *Journal of Food Protection* 73(2):353-357 (impact: 1.720, rank: 37/128).
- Matak* KE, Hvizdzak AL, Beamer SK, Jaczynski J. 2010. Recovery of *Salmonella enterica* serovars Typhimurium and Tennessee in peanut butter after electron beam exposure. *Journal of Food Science* 75(7):M462-467 (impact: 1.733, rank: 35/128).
- Taskaya L, Chen YC, Jaczynski* J. 2010. Color improvement by titanium dioxide and its effect on gelation and texture of proteins recovered from whole fish using isoelectric solubilization / precipitation. *LWT – Food Science and Technology* 43(3):401-408 (impact: 2.292, rank: 22/128).
- Kassis N, Drake SR, Beamer SK, Matak KE, Jaczynski* J. 2010. Development of nutraceutical egg products with omega-3-rich oils. *LWT – Food Science and Technology* 43(5):777-783 (impact: 2.292, rank: 22/128).
- Kassis N, Beamer SK, Matak KE, Tou JC, Jaczynski* J. 2010. Nutritional composition of novel nutraceutical egg products developed with omega-3 rich oils. *LWT – Food Science and Technology* 43(8):1204-1212 (impact: 2.292, rank: 22/128).
- Bridges KM, Gigliotti JC, Altman S, Jaczynski J, Tou* JC. 2010. Determination of digestibility, tissue deposition, and metabolism of omega-3 fatty acid content of krill protein concentrate in growing rats. *Journal of Agricultural and Food Chemistry* 58(5):2830-2837 (impact: 2.816, rank: 10/128).

2011

- Gigliotti JC, Smith AL, Jaczynski J, Tou* JC. 2011. Consumption of krill protein concentrate prevents early renal injury and nephrocalcinosis in female Sprague-Dawley rats. *Urological Research* 39(1):59-67 (impact: 1.172, rank: 51/69).
- Gigliotti JC, Davenport MP, Beamer SK, Tou JC, Jaczynski* J. 2011. Extraction and characterization of lipids from Antarctic krill (*Euphausia superba*). *Food Chemistry* 125(3):1028-1036 (impact: 3.458, rank: 5/128).
- Gehring CK, Gigliotti JC, Moritz JS, Tou JC, Jaczynski* J. 2011. Functional and nutritional characteristics of proteins and lipids recovered by isoelectric processing of fish by-products and low-value fish: A review. *Food Chemistry* 124(2):422-431 (impact: 3.458, rank: 5/128).
- Pietrowski BN, Tahergorabi R, Matak KE, Tou JC, Jaczynski* J. 2011. Chemical properties of surimi seafood nutrified with ω-3 rich oils. *Food Chemistry* 129(3):912-919 (impact: 3.458, rank: 5/128).
- Simmons CA, Turk P, Beamer S, Jaczynski J, Semmens K, Matak* KE. 2011. The effect of a flaxseed oil-enhanced diet on the product quality of farmed brook trout *(Salvelinus fontinalis)* fillets. *Journal of Food Science* 76(3):S192-197 (impact: 1.733, rank: 35/128).

- Otto RA, Paker I, Bane L, Beamer S, Jaczynski J, Matak* KE. 2011. Survival of *Listeria innocua* after isoelectric solubilization/precipitation with acetic and citric acids. *Journal of Food Protection* 74(8):1348-1352 (impact: 1.720, rank: 37/128).
- Otto RA, Beamer S, Jaczynski J, Matak* KE. 2011. The effect of using citric or acetic acid on survival of *Listeria monocytogenes* during fish protein recovery by isoelectric solubilization and precipitation process. *Journal of Food Science* 76(8):M579-583 (impact: 1.733, rank: 35/128).
- Tesfai AT, Beamer SK, Matak KE, Jaczynski* J. 2011. Microbial radio-resistance of *Salmonella* Typhimurium in egg increases due to repetitive irradiation with electron beam. *Radiation Physics and Chemistry* 80(4):591-596 (impact: 1.132, rank: 31/33).
- Tesfai AT, Beamer SK, Matak KE, Jaczynski* J. 2011. Radioresistance development of DNA repair deficient *Escherichia coli* DH5α in ground beef subjected to electron beam at sub-lethal doses. *International Journal of Radiation Biology* 87(6):571-578 (impact: 1.861, rank: 37/113).
- Tahergorabi R, Beamer SK, Matak KE, Jaczynski* J. 2011. Effect of isoelectric solubilization/precipitation and titanium dioxide on whitening and texture of proteins recovered from dark chicken-meat processing by-products. *LWT – Food Science and Technology* 44(4):896-903 (impact: 2.292, rank: 22/128).

2012

- Tahergorabi R, Sivanandan L, Jaczynski* J. 2012. Dynamic rheology and endothermic transitions of proteins recovered from chicken-meat processing by-products using isoelectric solubilization/precipitation and addition of TiO_2. *LWT – Food Science and Technology* 46(1):148-155 (impact: 2.292, rank: 22/128).
- Pietrowski BN, Tahergorabi R, Jaczynski* J. 2012. Dynamic rheology and thermal transitions of surimi seafood enhanced with ω-3-rich oils. *Food Hydrocolloids* 27(2):384-389 (impact: 2.659, rank: 13/128).
- Kassis N, Gigliotti JC, Beamer SK, Tou JC, Jaczynski* J. 2012. Characterization of lipids and antioxidant capacity of novel nutraceutical egg products developed with omega-3-rich oils. *Journal of the Science of Food and Agriculture* 92(1):66-73 (impact: 1.360, rank: 48/128).
- Tahergorabi R, Jaczynski* J. 2012. Physicochemical changes in surimi with salt substitute. *Food Chemistry*. In press (impact: 3.458, rank: 5/128).
- Tahergorabi R, Matak KE, Jaczynski* J. 2012. Application of electron beam to inactivate *Salmonella* in food: Recent developments. *Food Research International*. In press (impact: 2.416, rank: 20/128).
- El-Rawass AL, Hvizdzak A, Davenport MP, Beamer SK, Jaczynski J, Matak* KE. 2012. Electron beam irradiation on quality indicators of peanut butter over a storage period. *Food Chemistry*. In press (impact: 3.458, rank: 5/128).
- Sedoski HD, Beamer SK, Jaczynski J, Partington S, Matak* KE. 2012. Sensory evaluation and quality indicators of nutritionally-enhanced egg product with omega-3 rich oils. *LWT – Food Science and Technology*. Under review.
- Tahergorabi R, Sivanandan L, Beamer SK, Matak KE, Jaczynski* J. 2012. A three-prong strategy to develop functional food using proteins from chicken processing by-products recovered with isoelectric solubilization/precipitation. *Journal of the Science of Food and Agriculture*. Under review.
- Tahergorabi R, Beamer SK, Matak KE, Jaczynski* J. 2012. Physicochemical properties of surimi gels with salt substitute. *LWT – Food Science and Technology*. Under review.
- Tahergorabi R, Beamer SK, Matak KE, Jaczynski* J. 2012. Functional food products made from fish protein isolate recovered with isoelectric solubilization/precipitation. *LWT – Food Science and Technology*. Under review.
- Tahergorabi R, Beamer SK, Matak KE, Jaczynski* J. 2012. Isoelectric solubilization/precipitation as a means to recover protein isolate from striped bass (*Morone saxatilis)* and its physicochemical properties in a nutraceutical seafood product. *Journal of Agricultural and Food Chemistry*. Under review.

Invited Non-Peer-Reviewed Professional Publications:

- Jaczynski J. 2005. Protein, lipid recovery from fish-processing by-products. *Global Aquaculture Advocate* 8(2):34-6.
- Jaczynski J. 2005. E-beam treatments could improve seafood safety, shelf life. *Global Aquaculture Advocate* 8(3):34-6.
- Jaczynski J. 2005. Pilot system recovers protein, lipids from fish by-products. *Global Aquaculture Advocate* 8(6):30-1.
- Jaczynski J. 2008. Recovering proteins and fats from fish by-products. *Fish Tales* 6(2):3-4.
- Jaczynski J. 2012. A three-prong strategy to develop nutraceutical seafood products using protein isolates recovered with isoelectric solubilization/precipitation from fish processing by-products. *Global Aquaculture Advocate*. In press.

Invited Lectures:

- Jaczynski J, Le Formal G, Park JW. 2003. Application of electron beam to surimi seafood and salmon. National Fisheries Institute Annual Meeting, Long Beach, CA. Oct. 9-11, 2003.
- Jaczynski J, Park JW. 2003. Application of electron beam to surimi seafood and smoked salmon. National Fisheries Institute Seafood Technology Innovations Conference, Orlando, FL. Feb. 4-7, 2003.
- Jaczynski J, Chen YC, Beamer S, Taskaya L. 2006. Continuous protein and lipid recovery system from fish processing by-products. The 31st Catfish Processors Conference, Mississippi State University (Starkville, MS). May 16-17, 2006.
- Chalise PR, Hotta E, Matak KE, Jaczynski J. 2007. Low-energy pulsed electron beam technique for microbial inactivation. The 34th IEEE International Conference on Plasma Science and the 16th IEEE International Pulsed Power Conference, Albuquerque, NM. June 17-22, 2007.
- Jaczynski J. 2008. Protein recovery from food processing by-products: From proof of concept through pilot scale to industrial application. Annual BIT Peptide and Protein Technology Conference: From Concept to Market, Shenzhen, China. April 22-24, 2008.
- Tou J, Gigliotti J, Jaczynski J. 2008. Krill as a source of protein and n-3 long-chain PUFAs. Canadian Institute of Food Science and Technology/Agriculture and Agri-Food Canada Conference, Charlottetown (Prince Edward Island), Canada. May 25-27, 2008.
- Jaczynski J. 2008. Conversion of dark chicken meat processing by-products to value-added white chicken meat food products: From proof of concept through pilot scale to industrial process. Seminar for PGG Quality Animal Ingredients SL, Ribera d'Ondara – Lleida, Spain. June 2-6, 2008.
- Jaczynski J, Matak KE. 2011. Application of e-beam to control foodborne pathogens in seafood. Seafood Products Association Pathogen Intervention Strategies, Seattle, WA. April 27, 2011.

Conference Abstracts:

2002

- Jaczynski J, Park JW, Zinn CA. 2002. Non-thermal electron beam affects gelation properties of fish proteins. Abstract #44-2. Institute of Food Technologists, Anaheim, CA.
- Jaczynski J, Park JW, Zinn CA. 2002. Electron beam penetration in seafood. Abstract #76E-1. Institute of Food Technologists, Anaheim, CA

2003

- Kamireddy N, Kenny PB, Jaczynski J, Slider SD, Kiser RA. 2003. Time and temperature affect storage stability of rainbow trout fillets. Abstract #76A-25. Institute of Food Technologists, Chicago, IL.

2004

- Kamireddy N, Kenney PB, Jittinandana S, Slider SD, Kiser RA, Mazik PM, Hankins JA, Jaczynski J. 2004. Effect of vitamin E supplementation on storage stability of rainbow trout (*Oncorhynchus mykiss*) fillets. Abstract #49B-1. Institute of Food Technologists, Las Vegas, NV.
- Jaczynski J, Beamer SK. 2004. Protein and lipid recovery from rainbow trout (*Oncorhynchus mykiss*). Abstract #83A-20. Institute of Food Technologists, Las Vegas, NV.

2005

- Jaczynski J, Black J. 2005. Assurance of food safety/security and shelf-life extension of aquatic foods using non-thermal electron beam. Abstract #190. World Aquaculture Society, New Orleans, LA.
- Jaczynski J, Beamer S. 2005. Development of value-added human foods from aquatic foods processing by-products. Abstract #191. World Aquaculture Society, New Orleans, LA.
- Chen YC, Jaczynski J. 2005. Gelation of proteins recovered from krill. Abstract #89B-20. Institute of Food Technologists, New Orleans, LA.
- Chen YC, Nguyen J, Semmens K, Beamer SK, Jaczynski J. 2005. Storage stability of omega-3-enhanced rainbow trout (*Oncorhynchus mykiss*): 1. Refrigerated storage temperature. Abstract #89A-38. Institute of Food Technologists, New Orleans, LA.
- Chen YC, Nguyen J, Semmens K, Beamer SK, Jaczynski J. 2005. Storage stability of omega-3-enhanced rainbow trout (*Oncorhynchus mykiss*): 2. Abusive storage temperature. Abstract #89A-24. Institute of Food Technologists, New Orleans, LA.
- Chen YC, Nguyen J, Semmens K, Beamer SK, Jaczynski J. 2005. Omega-3 fatty acid and total fat contents in omega-3-enhanced farmed rainbow trout (*Oncorhynchus mykiss*). Abstract #89A-20. Institute of Food Technologists, New Orleans, LA.
- Chen YC, Nguyen J, Semmens K, Beamer SK, Jaczynski J. 2005. Effects of dietary alpha-tocopheryl acetate on lipid oxidation of omega-3-enhanced farmed rainbow trout (*Oncorhynchus mykiss*). Abstract #89A-23. Institute of Food Technologists, New Orleans, LA.
- Chen YC, Jaczynski J. 2005. Gelation of proteins recovered from rainbow trout (*Oncorhynchus mykiss*) processing by-product. Abstract #89B-19. Institute of Food Technologists, New Orleans, LA.
- Taskaya L, Jaczynski J. 2005. Flocculation-enhanced protein recovery from trout processing by-products by isoelectric solubilization/precipitation. 7th Joint Meeting 50th Annual Atlantic Fisheries Technology Conference and 29th Annual Seafood Science and Technology Society of the Americas, Norfolk, VA.

2006

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- Development of value-added food based on proteins and lipids recovered from trout processing by-products; Agency: USDA/CSREES; Amount funded (year): $51,566 (2002); Role in project: co-PI.
- Development of pH-driven protein recovery for trout processing waste; Agency: WVU Senate; Amount funded (year): $12,076 (2002); Role in project: PI.
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- The effects of omega-3 fatty acid fortified fish on C-reactive protein levels; Agency: WVU Bridge Grants & Research Development Grants; Amount funded (year): $11,741 (2003); Role in project: co-PI.
- Development of value-added food based on proteins and lipids recovered from fish processing waste; Agency: WVU Research Corporation; Amount funded (year): $27,895 (2003); Role in project: PI.
- Protein and lipid recovery from fish processing by-products: Process scale-up from laboratory-batch to continuous semi-industrial application – part 1; Agency: USDA/CSREES; Amount funded (year): $115,579 (2004); Role in project: co-PI.
- Protein and lipid recovery from fish processing by-products: Process scale-up from laboratory-batch to continuous semi-industrial application – part 2; Agency: USDA/CSREES; Amount funded (year): $144,798 (2005); Role in project: co-PI.
- Production of brook trout and assessment of attributes for niche markets among distributors and restaurants; Agency: USDA/CSREES; Amount funded (year): $16,902 (2006); Role in project: co-PI.
- Processing strategies for the production of long-chain fatty acids by algae; Agency: USDA NRI; Amount approved (year): $100,000 (2006); Role in project: co-PI.
- Inactivation of *Escherichia coli* O157:H7 on ready-to-eat fresh vegetables with electron beam; Agency: WVU Research Corporation; Amount funded (year): $26,860 (2007); Role in project: PI.
- A high-speed centrifuge for the Division of Animal and Nutritional Sciences at West Virginia University; Agency: USDA NRI; Amount approved (year): $54,700 (2007); Role in project: co-PI.
- Enhanced lipid removal during isoelectric protein recovery from rainbow trout (*Oncorhynchus mykiss*) processing by-products; Agency: USDA/CSREES; Amount funded (year): $43,699 (2007); Role in project: co-PI.
- Application of dissolved-air-floatation (DAF) to protein recovery with isoelectric solublization/precipitation; Agency: USDA/CSREES; Amount funded (year): $40,213 (2008); Role in project: co-PI.
- Investigating different structural forms and ratios of eicosapentaenoic and docosahexaenoic acid on tissue deposition and lipid responses; Agency: USDA NRI; Amount approved (year): $112,000 (2008); Role in project: co-PI.
- Protein recovery from food processing by-products invited lecture, Shenzhen, China; Agency: Davis College Faculty Development Awards; Amount approved (year): $3,571 (2008); Role in project: PI.
- Down-stream processing – development of nutraceutical fish sticks from trout processing by-products: A niche market opportunity for WV aquaculture; Agency: USDA/NIFA; Amount funded (year): $44,147 (2010); Role in project: co-PI.

Appendix B

Gigliotti et al. "Extraction and Characterisation of Lipids from Antarctic Krill (Euphausia superba)" Food Chemistry 125(3): 1028-1036 (April, 2011)

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Extraction and characterisation of lipids from Antarctic krill (*Euphausia superba*)

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ABSTRACT

There is significant commercial interest in oil extraction from krill because it is rich in omega-3 polyunsaturated fatty acids (*n*-3 PUFA) such as eicosapentaenoic (EPA, 20:5*n*3) and docosahexaenoic (DHA, 22:6*n*3) acids. The objectives were to determine oil extraction efficiency using different solvent systems and the composition of extracted oil and spent krill following extraction. Extraction efficiency was the highest ($P < 0.05$) for one-step extraction using freeze-dried krill with 1:12 or 1:30 krill:solvent ratio (w:v) compared to Folch, Soxhlet, and conventional two-step extraction. Extracted oils contained predominantly phospholipids (20–33%), polar non-phospholipids (64–77%), and minor triglycerides (1–3%). Triglycerides contained much less ($P < 0.05$) total *n*-3 (4.0%), DHA (1.1%), and EPA (2.3%), but more ($P < 0.05$) saturated FA (38.7%) than phospholipids (total *n*-3-47.4%, DHA-18.0%, EPA-28.2%, saturated FA-23.5%). Antioxidant capacity of krill oil extracted by one-step extraction (9.4–14.2 μmol Trolox Equivalents/ml oil) was generally similar to antioxidant capacity of krill oil extracted by ethanol (22.9), but greater ($P < 0.05$) than antioxidant capacity of krill oil extracted by acetone (1.2) and Folch method (1.5). The spent krill following oil extraction contained protein (72.9–75.8%, dry basis). Based on the extraction efficiency and composition of the extracted oil, the one-step extraction using 1:12 krill:solvent ratio is recommended.

1. Introduction

Antarctic krill (*Euphausia superba*) are small, shrimp-like crustaceans. Commercial capture is simple because krill form high-density surface swarms. Despite their small size, krill likely has the largest biomass of any multi-cellular animal species on earth (Nicol, James, & Pitcher, 1987). Although it is difficult to accurately determine the sustainable biomass for krill harvest, this significant resource may be comparable to the biomass of all other aquatic species currently harvested. The total annual capture from all fisheries has been approximately 130 million tons (MT) since 2000 (FAO, 2007). By comparison, krill biomass has been estimated at 400–1550 MT with a sustainable harvest at 70–200 MT (Suzuki & Shibata, 1990). However, newer estimates suggest that the krill biomass may be lower (Priddle, Boyd, Whitehouse, Murphy, & Croxall, 1998; Smetacek & Nicol, 2005). Nicol and Foster (2003) estimated the annual krill capture to be 0.1 MT, making krill an underutilized species. However, due to the role that krill play in marine ecology, an internationally monitored and governed ecosystem approach is a necessity for a long-term sustainability of this fishery (Everson, 2000; Hureau, 1985; Laws, 1985).

Grantham (1977) reported that krill contains 77.9–83.1% moisture, 0.4–3.6% lipids, 11.9–15.4% protein, and ~2% chitin and glucides. Saether, Ellingsen, and Mohr (1986) determined that due to seasonality lipid content ranges widely from 12–50% (dry basis). Lipid content and its composition in krill also depend on species, age, and the time between capture and freezing (Kolakowska, 1991). Kolakowska, Kolakowski, and Szczygielski (1994) reported that the *n*-3 PUFA, EPA and DHA are particularly abundant, which was attributed to krill consuming single-cell marine micro-algae. However, shellfish are often perceived as high in cholesterol; and therefore, reduce its acceptance as food by consumers. Cholesterol level in krill is higher than fish, but lower than shrimp (Tou, Jaczynski, & Chen, 2007). Also, it is important to emphasise that two-thirds of the sterols in shellfish are non-cholesterol sterols, which interfere with absorption of dietary cholesterol (Feeley, Criner, & Watt, 1972; Vahouny, Connor, Roy, Lin, & Gallo, 1981).

Despite its potential as a high quality lipid and protein source (Bridges, Gigliotti, Altman, Jaczynski, & Tou, 2010; Chen, Tou, & Jaczynski, 2009; Gigliotti, Jaczynski, & Tou, 2008; Gigliotti, Smith, Jaczynski, & Tou, 2010; Tou et al., 2007), the use of krill as human food has been limited (Suzuki & Shibata, 1990). Krill is mainly used by reduction fisheries for manufacture of fish feeds due to its high astaxanthin content. In addition, encapsulated krill oil is used as a dietary supplement with various potential health benefits including protection against cardiovascular disease (CVD) (Bunea, El

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Farrah, & Deutsch, 2004; Sampalis, 2007; Sampalis et al., 2003). Bunea et al. (2004) attributed some of these benefits to the *n*-3 PUFA in krill being mainly associated with phospholipids (PL); unlike in fish where the *n*-3 PUFA are associated with triglycerides (TG). The oxidative stability of krill oil has been attributed to its antioxidants content, in particular astaxanthin (Suzuki & Shibata, 1990). Krill oil may be valuable in the development of nutraceutical food products (Kassis, Beamer, Matak, Tou, and Jaczynski, 2010; Kassis, Drake, Beamer, Matak, & Jaczynski, 2010; Kassis, Gigliotti, Beamer, Tou, and Jaczynski, submitted for publication).

A major hindrance to commercial processing of krill and development of new krill-based food products may be due to high activity of krill lipases and proteases (Anheller, Hellgren, Karlstam, & Vincent, 1989). These enzymes are released immediately upon the demise of krill, resulting in autolysis, which leads to a rapid spoilage. The enzymes combined with its small size makes krill processing for human food a significant challenge. Another concern is high fluoride content in the exoskeleton. However, centrifugation removes fluoride (Christians & Leinemann, 1983; Karl et al., 1986).

Krill oil is currently extracted by two-step solvent extraction using acetone and ethanol in the first and second step, respectively (Beaudoin & Martin, 2004; Sampalis, 2007). However, this extraction requires two separate extraction steps and takes a relatively long time. In addition, the two-step extraction does not mention water removal from krill prior to oil extraction. Water interferes with solvent extraction and water removal prior to oil extraction results in greatly improved extraction efficiency and less water in the extracted oil (Dunford, Temelli, & LeBlanc, 1997; Nilsson, 1996). Another process to extract krill oil takes advantage of superctitical-CO_2 entrained with up to 20% ethanol (Bruheim et al., 2008). However, this process requires thermal inactivation of lipases at over 50 °C prior to oil extraction. Although heat likely inactivates lipases resulting in reduced hydrolysis of ester bonds and consequently fewer free FA, it simultaneously denatures heat-labile krill muscle proteins (Carvajal, Lanier, & Macdonald, 2005).

Due to structural changes, the recovery of denatured proteins would be difficult and even if krill proteins were recovered, the proteins would exhibit reduced functionalities (i.e., gel-forming ability, extractability, water-holding-capacity, etc.). Bruheim et al.'s (2008) process is similar to the two-step solvent extraction (Beaudoin & Martin, 2004; Sampalis, 2007), but does not require water removal prior to processing. However, freeze-drying of krill prior to oil extraction with superctitical-CO_2 has been shown to increase extraction efficiency approximately three times (Yamaguchi et al., 1986). The protein remaining in the residual spent krill following oil extraction can be recovered with techniques such as isoelectric solubilisation/precipitation and if protein functionalities are retained, this protein could be used in human food products contributing to the fuller use of this tremendous resource (Chen & Jaczynski, 2007a,b; Chen et al., 2009; Gehring, Gigliotti, Moritz, Tou, & Jaczynski, 2010; Jaczynski, 2010; Torres, Chen, Rodrigo-Garcia, & Jaczynski, 2007).

It is hypothesised that one-step extraction with acetone:ethanol mixture for 2 h from whole krill will result in high extraction efficiency. The objectives were to determine oil extraction efficiency from whole krill using different solvent systems and characterise the composition of extracted lipids and residual spent krill following oil extraction.

2. Materials and methods

2.1. Sample preparation and oil extraction

Whole frozen Antarctic krill (*Euphausia superba*) was obtained from Krill Canada (Langley, BC, Canada). The krill blocks were transported overnight to the West Virginia University food science laboratory in heavily insulated industrial strength boxes filled with dry ice. Upon arrival the boxes were immediately stored at −80 °C until use. Whole frozen krill was freeze-dried without thawing (VirTis Genesis 35SQ Super XL freeze-dryer, Virtis, Gardiner, NY, USA), vacuum-packed and stored at −80 °C until processed. A flow diagram of oil extraction from krill is shown is Fig. 1.

In the one-step extraction, oil was extracted from freeze-dried krill using 1:1 acetone:ethanol (v:v) solvent mixture (ACS grade acetone, Fisher Scientific, Fairlawn, NJ, USA; ACS grade 95% ethanol, Pharmco, Brookfield, CT, USA). The following krill:solvent ratios were tested 1:6, 1:9, 1:12, and 1:30 (w:v). The weight of the initial freeze-dried krill was recorded in order to determine extraction efficiency (see below). The 1:30 ratio was used in the present study to mimic the 1:6 ratio of whole fresh krill as described by Beaudoin and Martin (2004) as well as Sampalis (2007). Fresh whole krill is currently used in commercial oil extraction (Beaudoin & Martin, 2004; Bruheim et al., 2008; Sampalis, 2007). However, in the present study freeze-dried krill was used (Dunford et al., 1997; Yamaguchi et al., 1986). Therefore, 1:30 ratio (freeze-dried krill) used in the present study approximately corresponded to 1:6 ratio (fresh whole krill) during commercial krill oil extraction based on the lipid content in relation to the solvent volume. Freeze-dried krill was dispersed in the solvent mixture (acetone:ethanol) by homogenisation for 30 s using a laboratory blender (model 51BL31, Waring Commercial, Torrington, CT, USA). Oil extraction was conducted for 2 h at 4 °C using a continuous shaker (model Excella E25R, New Brunswick Scientific, Edison, NJ, USA) followed by centrifugation at 10,000*g* and 4 °C for 20 min (model Sorvall RC-5B Refrigerated Superspeed, Kendro Laboratory Products, Newtown, CT, USA). The supernatant (i.e., extracted krill oil) was decanted and air dried at atmospheric pressure. The sediment (residual spent krill including protein, shell, etc.) was also dried under air at atmospheric pressure and analysed for crude protein (Kjeldahl), total fat (Soxhlet), and ash content (see below).

In the two-step extraction oil was extracted from freeze-dried krill using two separate extractions. The weight of the initial freeze-dried krill was recorded in order to determine extraction efficiency (see below). The freeze-dried krill was first mixed with acetone at a 1:6 ratio (krill:acetone, w:v), centrifuged, and then the sediment was mixed with ethanol at a 1:6 ratio (krill:ethanol, w:v), followed by final centrifugation. Therefore, two separate extracts were obtained. Acetone extract was obtained in step 1 and ethanol extract in step 2. In step 1, freeze-dried krill was dispersed in acetone by homogenisation for 30 s using the laboratory blender. Oil extraction in step 1 was conducted for 2 h at 4 °C using the continuous shaker followed by centrifugation at 10,000*g* and 4 °C for 20 min. The supernatant (i.e. acetone extract) was decanted and air dried at atmospheric pressure; while the sediment was subjected to step 2 of the extraction. Step 2 was conducted in the same manner as step 1 except ethanol was used instead of acetone. Therefore, total extraction time (i.e., step 1 and 2) was 4 h. Following step 2, final centrifugation at 10,000*g* and 4 °C for 20 min was applied. The supernatant (i.e., ethanol extract) was decanted and air dried at atmospheric pressure. The sediment (residual spent krill including protein, shell, etc.) was analysed for crude protein (Kjeldahl), total fat (Soxhlet), and ash content (see below).

Dry krill oils (i.e., krill oil from one-step extraction, acetone extract from two-step extraction, and ethanol extract from two-step extraction) were clarified in 2:1 chloroform:methanol (v:v) mixture (ACS grade chloroform, Fisher Scientific, Fairlawn, NJ, USA; HPLC grade methanol, Fisher Scientific, Fairlawn, NJ, USA) with 20 ml of 10% NaCl in water added to a separation funnel (Folch, Lees, & Sloane, 1957). This clarification removed any residual water in the krill oil samples. Sufficient volume of the chloroform:methanol mixture was added until there was no visible separate layers. Following clar-

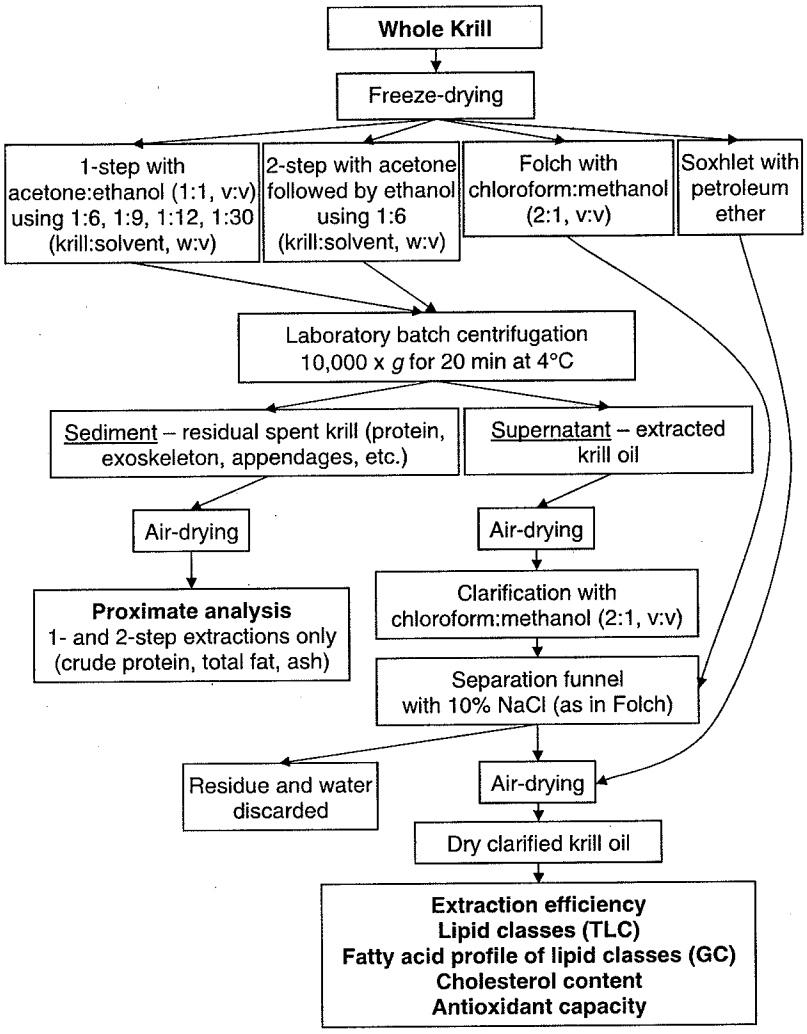

Fig. 1. A flow diagram for oil extraction from krill and subsequent analyses of the extracted oil and residual spent krill.

ification, the krill oil samples were air re-dried at atmospheric pressure. Dry clarified krill oils were weighed and compared to the initial weight of the freeze-dried krill subjected to extraction in order to determine extraction efficiency (see below).

For comparison, oil was also extracted from freeze-dried krill using the Folch method (Folch et al., 1957) and Soxhlet extraction (AOAC, 1995). In Folch method, 3 g of freeze-dried krill were dispersed in 60 ml of the 2:1 chloroform:methanol mixture by homogenisation for 30 s using the laboratory blender, followed by filtration through Whatmann #40 paper (Whatman International, Maidstone, UK) in the separation funnel. A 20 ml aliquot of 10% NaCl in water was added to the separation funnel and the mixture was manually shaken and allowed to stand until the phases had completely separated. The bottom organic solvent phase was removed and air dried at atmospheric pressure. The drying resulted in solvent evaporation yielding krill oil. The weight of krill oil was recorded in order to determine extraction efficiency. In Soxhlet extraction, 3 g of freeze-dried krill were dispersed in petroleum ether (ACS grade petroleum ether, VWR International, Bridgeport, NJ, USA) (AOAC, 1995). The extraction was carried out in a standard Soxhlet apparatus for 18 h (Chen, Nguyen, Semmens, Beamer, & Jaczynski, 2006, 2007), followed by air drying at atmospheric pressure. The drying resulted in evaporation of petroleum ether yielding krill oil. The weight of krill oil was recorded in order to determine extraction efficiency.

In preliminary experiments, the above solvent systems were applied to extract oil from fresh krill and freeze-dried krill. The extraction efficiency was significantly lower ($P < 0.05$) for all solvent systems using fresh krill when compared to freeze-dried krill. These preliminary experiments confirmed earlier reports by Yamaguchi et al. (1986) and Dunford et al. (1997). Therefore, only freeze-dried krill was used in the present study. All oil extractions were performed in triplicate ($n = 3$) using the same batch of freeze-dried krill.

2.2. Determination of extraction efficiency

The weight of the initial freeze-dried krill prior to the extraction with different solvent systems was recorded. Following extraction, the weight of extracted oil was also recorded. The weight of acetone extract and ethanol extract were combined in order to determine an overall extraction efficiency of the two-step extraction. The extraction efficiency was determined according to the following equation:

$$\text{Extraction efficiency} = \frac{\text{weight of extracted krill oil(g)}}{\text{weight of freeze-dried krill subjected to extraction(g)}} \times 100 \quad (1)$$

Extraction efficiency is expressed as g of extracted oil per 100 g of freeze-dried krill subjected to the extraction.

2.3. Determination of lipid classes in extracted krill oil

Thin layer chromatography (TLC) was applied to resolve lipid classes of oils extracted by one-step, two-step, Folch, or Soxhlet. For krill oil extracted by the two-step extraction, TLC and subsequent densitometry analysis were applied to acetone and ethanol extracts. A 10 ml aliquot of extracted krill oil was dissolved in 1:1 chloroform:methanol (v:v) and loaded onto TLC plates (Whatman K6F silica plates with 60 A pore sizes, P.J. Cobert Associates, St. Louis, MO). The TLC plates were developed using a 80:20:1.5 hexane:ether:acetic acid solution (v:v:v) as a mobile phase. Once developed, plates were air dried for 5 min.

Plate images were captured using a digital camera interfaced with a PC and spot densitometer (Fluorchem 8000, Alpha Innotech Corp., San Leandro, CA) using transluminating white light (Alpha Innotech Corp., San Leandro, CA). The images were analysed using the Fluorchem software (version 1.0, Alpha Innotech Corp., San Leandro, CA). Phospholipids (PL) and triglycerides (TG) were identified using R_F values obtained from triolein (Sigma–Aldrich, St. Louis, MO) and soybean lecithin (Fisher Scientific, Fairlawn, NJ) standards. Once identified, the bands corresponding to PL, TG, and polar non-PL class were scraped from the TLC plates and suspended in 1:1 chloroform:methanol (v:v) for determination of fatty acid profile (FAP) by gas chromatography (GC). The densitometry data are reported as mean values (±standard deviation) of at least three replicates and the mean values are expressed as percent of lipid class in total krill oil.

2.4. Fatty acid profile (FAP) of extracted krill oil

The PL and TG were separately scraped from the TLC plates. The FAP of the PL and TG scrapes was determined (Chen, Nguyen, Semmens, Beamer, & Jaczynski, 2007, 2008a, 2008b; Folch et al., 1957; Taskaya, Chen, Beamer, Tou, & Jaczynski, 2009). FA were transmethylated by the addition of 4 ml of 4 g/100 ml H_2SO_4 in anhydrous methanol and heated in a water bath set at 90 °C for 60 min. The mixture was saponified by transferring through a Na_2SO_4-filled glass Pasteur pipette and subsequently dried under N_2 in a water bath set at 60 °C for 60 min. The FA methyl esters (FAME) were re-suspended in filtered iso-octane (HPLC grade iso-octane, Fisher Scientific, Fairlawn, NJ, USA). The FAME were analysed by a gas chromatograph (GC) (model CP-3800, Varian Analytical Instruments, Walnut Creek, CA, USA) using flame ionisation detector fitted with a WCOT-fused silica capillary column (50 m length, 0.25 mm inside diameter; Varian Analytical Instruments, Walnut Creek, CA, USA). Injection and detection temperatures were maintained at 220 °C and column temperature was 190 °C. The stationary phase was CP-Silica 88 (Varian Analytical Instruments, Walnut Creek, CA, USA). Nitrogen was the carrier gas and a split ratio of 10 to 1 was used. The FA were identified by comparing their retention times with those of known standards and references (Ackman, 1980). Peak area and the amount of each FA were computed by an integrator using the Star GC workstation (version 6, Varian Analytical Instruments, Walnut Creek, CA, USA). The data are reported as mean values (±standard deviation) of at least three replicates and the mean values are expressed as percent of a fatty acid in total fatty acids.

2.5. Determination of cholesterol content in extracted krill oil

Due to the prevalence of polar non-PL, the TLC and densitometry analysis did not allow determination of cholesterol content. For krill oil extracted with the two-step extraction, cholesterol content was separately determined for acetone and ethanol extracts. Cholesterol content of oil extracted from krill by one-step, two-step, Folch, and Soxhlet procedure was determined using a colorimetric assay (EMD Chemicals Inc., Darmstadt, Germany). This cholesterol assay relies on the oxidation of cholesterol by added cholesterol oxidase and yielding H_2O_2. The resulting H_2O_2 interacts with a cholesterol probe to produce resorufin that is detected spectrophotometrically.

Krill oil samples were prepared in a cholesterol reaction buffer. In a 96 well microplate, 50 µl of the buffered krill oil samples were mixed with 50 µl of reaction mix (containing cholesterol oxidase and cholesterol probe). Samples were covered and incubated at 37 °C for 1 h. Absorbance was read at 570 nm using a Spectramax Plus microplate reader (Molecular Devices, Sunnyvale, CA). In order to determine cholesterol content in krill oil, standard curve was constructed using cholesterol standards mixed with 50 µl of the reaction mix. The cholesterol content is reported as mean values (± standard deviation) of at least three replicates and the mean values are expressed as grams of cholesterol per 100 grams of krill oil.

2.6. Antioxidant capacity of extracted krill oil

It has been reported that krill oil is rich in antioxidants, in particular astaxanthin (Kolakowska, 1991; Kolakowska et al., 1994; Suzuki & Shibata, 1990; Tou et al., 2007). These antioxidants were likely included in the polar non-PL class. Therefore, total antioxidant capacity for the oil extracted from krill by the different extraction methods was determined according to a colorimetric antioxidant assay (Cayman Chemical Company, Ann Arbor, MI). For krill oil extracted with the two-step extraction, total antioxidant capacity was separately determined for acetone and ethanol extracts. This antioxidant assay relies on the ability of endogenous antioxidants in krill oil to inhibit the metmyoglobin-mediated oxidation of ABTS (2,2′-Azino-di-[3-ethylbenzthiazoline sulphonate]) to $ABTS^+$. ABTS and metmyoglobin were added to krill oil samples as reagents and the amount of $ABTS^+$ was determined spectrophotometrically. The capacity of the endogenous antioxidants in krill oil to prevent ABTS oxidation was compared with that of a water-soluble tocopherol analogue, trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid).

In a 96 well microplate, 10 µl of krill oil samples were mixed with 10 µl of metmyoglobin and 150 µl of ABTS. To initiate the reaction, 40 µl of H_2O_2 was added to each well. Samples were covered and incubated at room temperature on a shaker for 5 min. Absorbance was read at 750 nm using a Spectramax Plus microplate reader (Molecular Devices, Sunnyvale, CA). In order to determine trolox

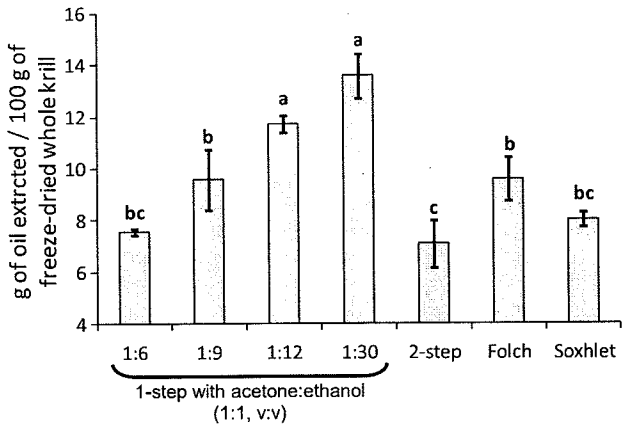

Fig. 2. Extraction efficiency* of different solvent systems for extraction of oil from freeze-dried whole krill. *Different letters on the top of data bars indicate significant differences (Tukey's test, $P < 0.05$) between mean values (±SD, $n = 3$).

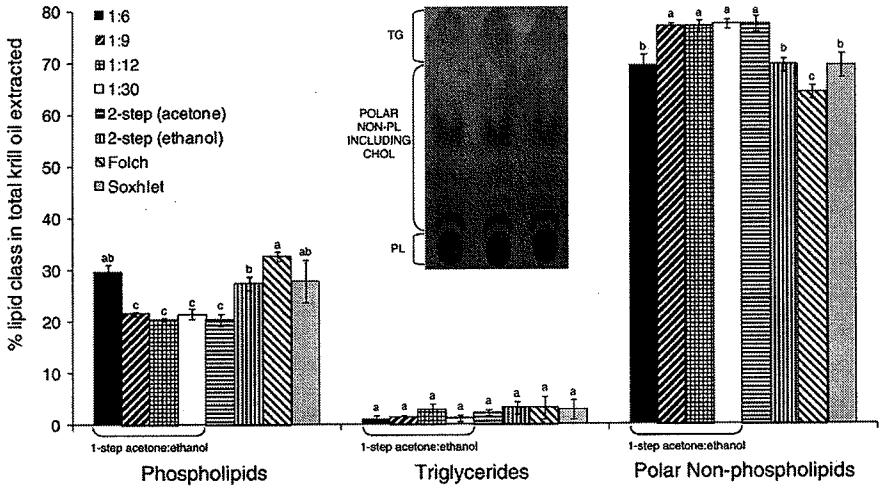

Fig. 3. Densitometry analysis of thin layer chromatography (TLC) plates. For two-step extraction, TLC plates with acetone and ethanol extracts were analysed separately. Insert: An example TLC plate showing lipid classes of krill oil extracted with one-step extraction using 1:12 krill:solvent ratio (w:v) (triplicate). TG – triglycerides, PL – phospholipids, CHOL – cholesterol. Different letters on the top of data bars indicate significant differences (Tukey's test, $P < 0.05$) between mean values (±SD, $n = 3$) within a lipid class.

equivalent (TE) for krill oil, standard curve was constructed using trolox standards mixed with 10 μl of metmyoglobin and 150 μl of ABTS. The antioxidant capacity is reported as mean values (±standard deviation) of at least three replicates and the mean values are expressed as μmols of TE per ml of krill oil.

2.7. Proximate analysis of the residual spent krill following oil extraction

To determine proximate composition (i.e., crude protein, total fat, and ash content) on dry basis, the residual spent krill was analysed for moisture content. A sample (2 g) was placed on an aluminium dish (Fisher Scientific, Fairlawn, NJ, USA), spread evenly across the dish and oven dried (105 °C for 24 h) (AOAC, 1995). The crude protein, ash content, and total fat were determined in the residual spent krill following oil extraction with one- and two-step extraction only (i.e., sediment in Fig. 1). Crude protein was determined by Kjeldahl assay (AOAC, 1995). Ash content was determined by incinerating samples in a muffle furnace at 550 °C for 24 h (AOAC, 1995). Residual fat content was determined according to the Soxhlet extraction method (AOAC, 1995). The proximate data are reported as mean values (±standard deviation) of at least three replicates and the mean values are expressed as grams per 100 g of the residual spent krill following oil extraction (dry weight basis).

2.8. Statistical analysis

The oil extraction experiments were performed in triplicate ($n = 3$). For each triplicate, at least three measurements were performed. One-way independent measures analyses of variance (ANOVA) were used to determine individual differences between treatments except for the differences of FA content between TG and PL where Student's *t*-test was used. Post-hoc analysis was conducted using Tukey's test with a significance level of ($P < 0.05$). ANOVA statistical comparisons were conducted using KYPlot (KyensLab, Tokyo, Japan).

3. Results and discussion

3.1. Extraction efficiency

Extraction of krill oil using the two-step procedure resulted in similar ($P > 0.05$) efficiency as the one-step procedure using a krill:solvent ratio of 1:6 (Fig. 2). Extraction efficiency for the one-step procedure increased ($P < 0.05$) as the krill:solvent ratio increased, with 1:12 and 1:30 ratios having the greatest ($P < 0.05$) efficiencies. There was no ($P > 0.05$) significant difference in extraction efficiency between the one-step procedure using 1:12 and 1:30 ratio. The krill:solvent ratio is a critical parameter for extraction efficiency using the one-step procedure. Likely, if the krill:solvent ratio were increased in the two-step extraction, the extraction efficiency would similarly increase. However, one-step extraction is simpler than the two-step procedure; and therefore, one-step extraction with acetone:ethanol (1:1, v:v) using 1:12 krill:solvent ratio is recommended.

3.2. Lipid classes in extracted krill oil

Major lipid classes of the extracted krill oils were separated using TLC (Fig. 3 insert), quantified by densitometry and presented as % of lipid class in total krill oil extracted (Fig. 3). The extraction procedure did not ($P > 0.05$) affect TG content in the extracted oils, which ranged 1.0–3.2% (Fig. 3). However, extraction procedure had an effect ($P < 0.05$) on PL content, with Folch, Soxhlet, and 1-step (1:6 krill:solvent ratio) extractions having the highest ($P < 0.05$) content of PL. The PL content of all extracted oils ranged from 20.4–32.7% (Fig. 3). A high PL content in krill oil has been described previously, with total PL accounting for approximately 40% of krill oil (Bottino, 1975). The high PL content makes krill oil unique as compared to other dietary lipids. The TG content in fish oils is approximately 60% (Tou et al., 2007).

The major lipid classes in all of the oils extracted from krill were the polar non-PL classes (>60%). These classes consist of cholesterol, mono- and di-glycerides, and the red pigment primarily astaxanthin. The association of the red pigment with the polar non-PL classes made quantifying each class individually with TLC difficult. Thus, these classes were combined and accounted for as the polar non-PL class. The one-step extraction with krill:solvent ratios greater than 1:6 and the acetone fraction from the two-step extraction had the highest ($P < 0.05$) polar non-PL class content. The differences in the polar non-PL class content should be of commercial interest because an extraction procedure that yields the oil with lower cholesterol content, but higher antioxidant capacity would provide the healthiest oil. Therefore, cholesterol content and total antioxidant capacity were determined (see Section 3.4 and 3.5, respectively).

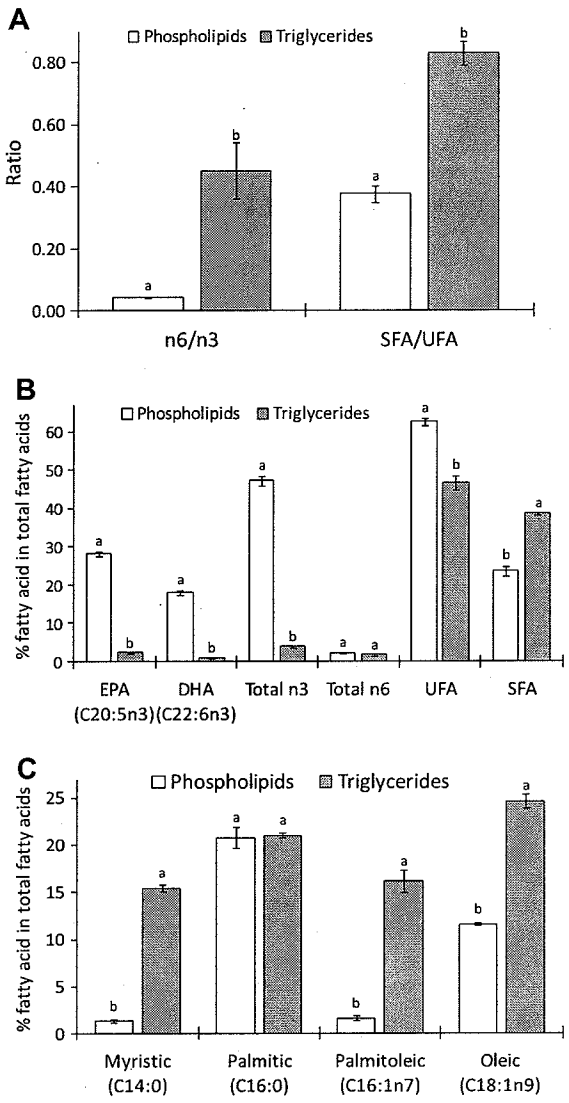

Fig. 4. Major FA* associated with phospholipids (PL) and triglycerides (TG) in oil extracted from krill by one-step extraction using 1:12 krill:solvent ratio (w:v). The PL, TG, and polar non-PL class were resolved with TLC (Fig. 3). The polar non-PL showed no detectable FA. This class likely contained antioxidants including astaxanthin. *n*3 – ω-3 FA, *n*6 – ω-6 FA, UFA – total unsaturated FA, SFA – total saturated FA, EPA – eicosapentaenoic acid (20:5n3), DHA – docosahexaenoic acid (22:6*n*3). *Different letters on the top of data bars indicate significant differences (Student's *t*-test, $P < 0.05$) between mean values (±SD, $n = 3$) within the FA.

3.3. Fatty acid profile (FAP) of extracted krill oil

The PL was the major lipid class containing FA (PL – 20–33% vs. TG – 1–3%; Fig. 3). Only PL and TG had detectable levels of FA. Preliminary experiments were conducted to determine if the different extraction methods had an effect ($P < 0.05$) on the FAP. The FAP was not ($P > 0.05$) different regardless of the extraction method (data not shown). Therefore, extraction methods tested in the present study did not ($P > 0.05$) affect FAP of the extracted oils. In addition, Fig. 2 indicates that one-step extraction resulted in the highest ($P < 0.05$) extraction efficiency for 1:12 and 1:30 krill:solvent ratio (w:v); while the extraction efficiency at 1:12 and 1:30 was similar ($P > 0.05$). Therefore, only FAP for PL and TG extracted with one-step extraction using 1:12 krill:solvent ratio (w:v) is reported (Fig. 4).

Following TLC analysis, TG and PL spots were scraped from the TLC plates and methylated for FAP by GC (Fig. 4). The ratios of *n*6/*n*3 and saturated FA/unsaturated FA were lower ($P < 0.05$) in PL than TG (Fig. 4A). Oil with a lower *n*-6/*n*-3 and saturated FA/unsaturated FA is considered healthier. EPA and DHA were predominant ($P < 0.05$) in PL, contributing to much higher ($P < 0.05$) content of total *n*-3 FA and unsaturated FA in PL than in TG (Fig. 4B). EPA and DHA are the bioactive *n*-3 PUFA associated with health benefits such as reduced risk of CVD (cardiovascular disease). The TG had a higher ($P < 0.05$) content of myristic, palmitoleic, and oleic acid than PL (Fig. 4C).

The PL and TG require different digestive enzymes and therefore, there may be differences in bioavailability and tissue accretion of *n*-3 PUFA. In turn, this may result in different physiological and health effects (Arnate, Gil, & Ramirez, 2001, 2002; Matthews et al., 2002). Therefore, having EPA and DHA esterified as PL in krill oil may have significant implications for human health. The preferential esterification of EPA and DHA into PL is intriguing and has been noted previously (Saether et al., 1986). However, available data comparing the benefits of consuming ω-3 PUFA as krill oil compared to other sources is scarce. Therefore, further studies are needed to compare and understand the nutritional value and health effects of krill oil versus other sources of *n*-3 PUFA.

3.4. Cholesterol content in extracted krill oil

The one-step extraction procedures yielding oil with the highest ($P < 0.05$) content of polar non-PL (one-step extractions in Fig. 3) did not ($P < 0.05$) result in the highest cholesterol content (Fig. 5A). The one-step procedures with krill:solvent ratios ⩾1:9 generally had the lowest cholesterol content. Cholesterol content of the two-step, Folch, and Soxhlet procedure was similar to the content of the polar non-PL (Fig. 3), suggesting that the cholesterol content contributed more to the content of polar non-PL in these procedures than the one-step procedures.

In general, cholesterol content of krill is lower than shrimp, and slightly higher than fish (Tou et al., 2007). Krill oil resulted in 18% reduction in total serum cholesterol compared to 6% with fish oil in hyperlipidemic patients (Bunea et al., 2004). The hypolipidemic effects of seafood oils are not fully understood, but are significantly influenced by *n*-3 PUFA. Since krill oil is rich in *n*-3 PUFA bound in PL and is low in cholesterol, the hypolipidemic effects of krill oil could be additive.

3.5. Antioxidant capacity of extracted krill oil

The red colour of krill oil is due to the carotenoid astaxanthin, a potent antioxidant. Frozen krill contains 3–4 mg of carotenoids/100 g and astaxanthin is >80% of the total carotenoids (Yamaguchi et al., 1983). By measuring the total antioxidant capacity of the krill oil extracted in the present study, the antioxidative effect of astaxanthin was accounted for. *In vitro* studies have shown that astaxanthin decreases membrane oxidative injury to a greater degree than α-tocopherol, an antioxidant commonly used in food products (Kurashige, Okimasu, Inoue, & Utsumi, 1990).

Oils extracted with the one-step procedure using 1:9 and 1:30 krill:solvent ratios as well as the ethanol extract of the two-step procedure had the greatest ($P < 0.05$) antioxidant capacity (Fig. 5B). The antioxidant capacities of oils extracted with one-step procedures was similar to their respective polar non-PL content (Fig. 3), suggesting that astaxanthin was the primary component of their polar non-PL. Despite having the highest ($P < 0.05$) content of polar non-PL (Fig.3), the acetone extract of the two-step procedure had the lowest ($P < 0.05$) antioxidant capacity. Therefore, the polar non-PL of the acetone extract may be predominately cholesterol. The Folch extraction also yielded oil with the lowest ($P < 0.05$) antioxidant capacity.

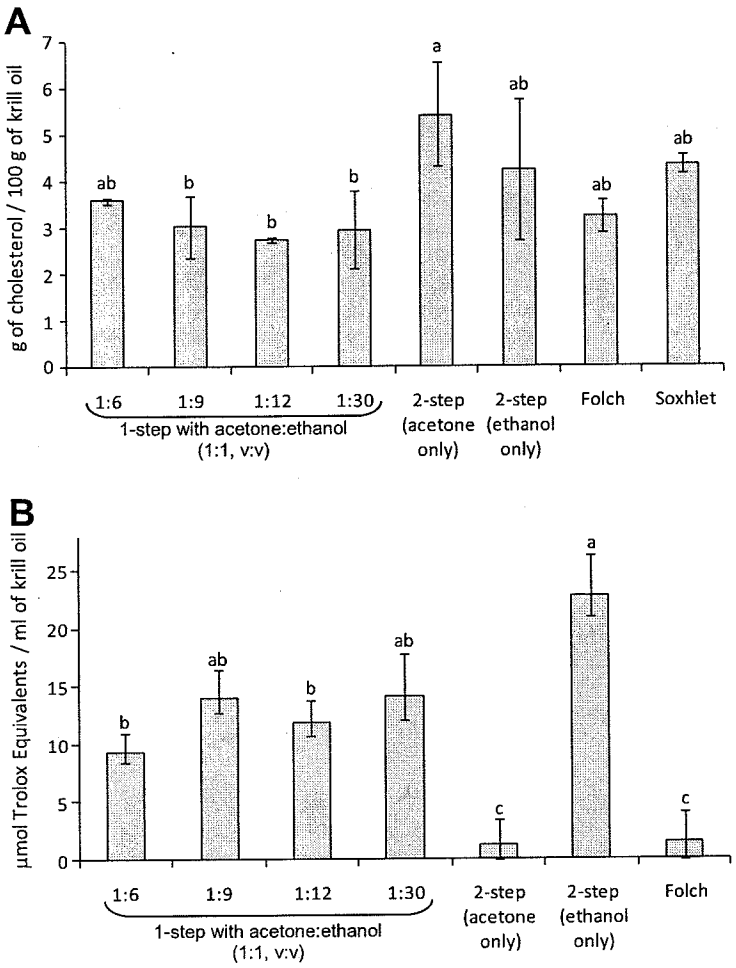

Fig. 5. Cholesterol content (A) and antioxidant capacity (B) of oil extracted from freeze-dried whole krill using different solvent systems. *Different letters on the top of data bars indicate significant differences (Tukey's test, $P < 0.05$) between mean values (±SD, $n = 3$).

Table 1
Crude protein*, total fat*, and ash content* of the residual spent krill following oil extraction with one- and two-step extraction.

	Extraction method				
	1-Step extraction with acetone:ethanol (1:1, v:v) at different krill:solvent ratio (w:v)				2-Step extraction
	1:6	1:9	1:12	1:30	
Crude protein (g/100 g, dry basis)	72.9 ± 1.0 a	73.8 ± 0.8 a	74.8 ± 0.8 a	75.8 ± 1.0 a	75.3 ± 4.0 a
Ash content (g/100 g, dry basis)	17.7 ± 0.7 a	17.3 ± 0.5 a	17.9 ± 0.5 a	16.1 ± 0.6 b	15.6 ± 0.4 b
Total fat (g/100 g, dry basis)	6.4 ± 1.0 a	6.5 ± 1.2 a	4.4 ± 1.0 b	3.7 ± 0.7 b	3.4 ± 1.1 b

* Different letters indicate significant differences (Tukey's test, $P < 0.05$) between mean values (±SD, $n = 3$) within the same row.

3.6. Proximate composition of krill following oil extraction

Following oil extraction, the residual spent krill was removed from the solvent/oil mixture by centrifugation. The spent krill was collected in the sediment (Fig. 1). Table 1 shows that the sediment was primarily composed of protein (~74 g/100 g, dry weight). The amount of protein in the residual spent krill was not ($P > 0.05$) affected by oil extraction procedure. The lipid content decreased ($P < 0.05$) as the krill:solvent ratio increased from 1:9 to 1:30 in the one-step extraction. The two-step extraction and one-step extraction using 1:12 and 1:30 krill:solvent ratios resulted in the lowest ($P < 0.05$) lipid content in the residual spent krill. The ash content in the residual spent krill was comparable to the ash content in whole krill prior to extraction (ash 17.4 g/100 g, dry basis, Chen et al., 2009). Therefore, the fluoride (F) content was likely high and would need to be reduced if the protein in the residual spent krill were to be recovered and used for human/animal consumption.

The proximate data clearly shows that there is high protein content in the residual spent krill; though ash (and likely F) is also high. The protein could be recovered with the isoelectric solubilisation/precipitation (ISP). It has been shown that the proteins recovered from whole krill and other aquatic resources using ISP yield good quality heat-set gels (Chen & Jaczynski, 2007a; Taskaya, Chen, Beamer, and Jaczynski, 2009; Taskaya, Chen, & Jaczynski, 2009). Application of ISP to whole krill also significantly reduces ash content (and likely F) in the recovered proteins (Chen, Tou, & Jaczynski, 2007; Chen et al., 2009). Therefore, a fuller utilisation

of krill as a human food would be possible by extracting oil as shown in the present study, followed by protein recovery by ISP for subsequent inclusion in human food products.

4. Conclusions

Based on the present study, subjecting freeze-dried krill to one-step extraction (acetone:ethanol, 1:1, v:v) using 1:12 krill:solvent ratio (w:v) resulted in the highest oil extraction efficiency. Phospholipids (PL) were the main lipid class in the extracted oil containing omega-3 fatty acids (*n*-3 FA). The PL contained considerably more total *n*-3 FA, unsaturated FA, eicosapentaenoic (EPA, 20:5n3) and docosahexaenoic (DHA, 22:6*n*3) acids, but less saturated FA and similar content of total *n*-6 FA compared to triglycerides (TG). The oil extracted from freeze-dried krill with one-step procedure using 1:12 krill:solvent ratio also had low cholesterol content and high antioxidant capacity compared to other treatments. The protein left over in the residual spent krill following oil extraction could potentially be recovered with isoelectric solubilisation/precipitation (ISP). The ISP also results in reduction of ash, and thus, fluoride content in the ISP-recovered krill protein.

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Appendix C

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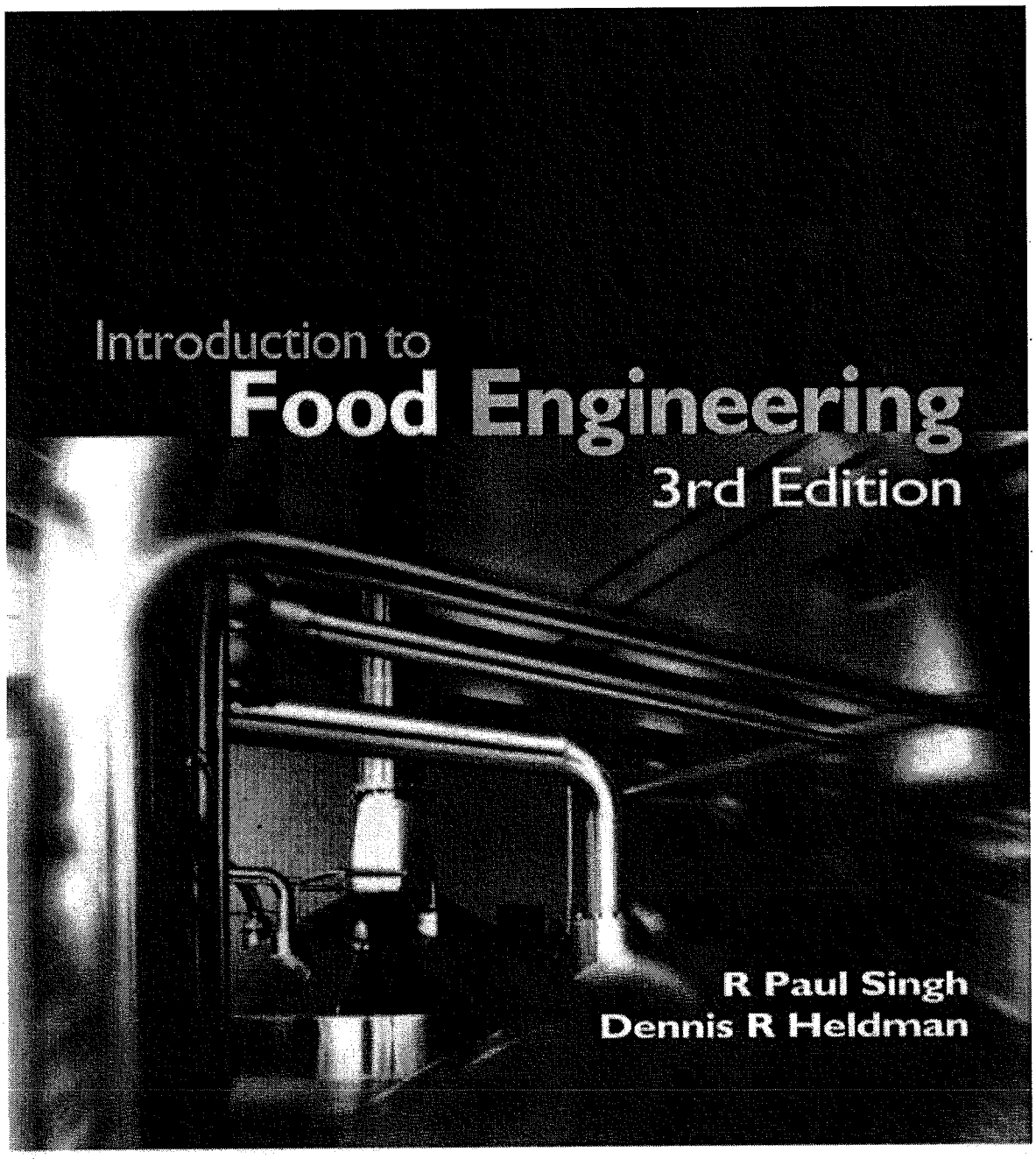

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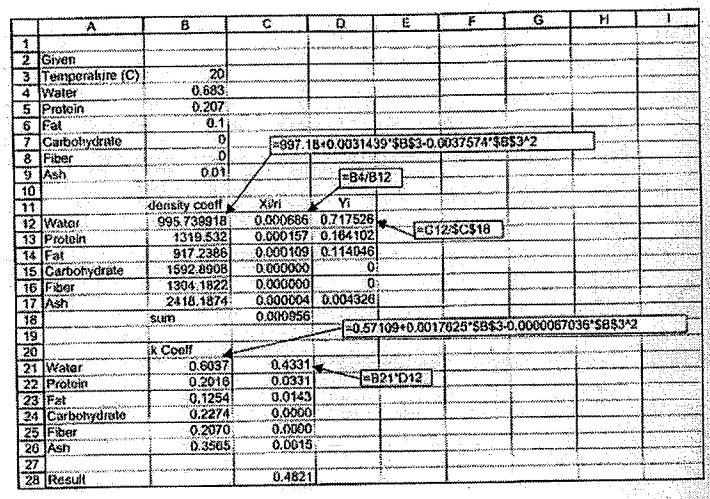
Figure E4.2 Spreadsheet for data given in Example 4.2.



	A	B	C	D	E	F	G	H	I
1									
2	Given								
3	Temperature (C)	20							
4	Water	0.683							
5	Protein	0.207							
6	Fat	0.1							
7	Carbohydrate	0	=997.18+0.0031439*B3-0.0037574*B3^2						
8	Fiber	0							
9	Ash	0.01		=B4/B12					
10									
11		density coeff	Xiri	Yi					
12	Water	995.739918	0.000686	0.717526					
13	Protein	1319.532	0.000157	0.164102	=C12/C18				
14	Fat	917.2386	0.000109	0.114046					
15	Carbohydrate	1592.8908	0.000000	0					
16	Fiber	1304.1822	0.000000	0					
17	Ash	2418.1874	0.000004	0.004326					
18		sum	0.000956						
19					=0.57109+0.0017625*B3-0.0000067036*B3^2				
20		k Coeff							
21	Water	0.6037	0.4331						
22	Protein	0.2016	0.0331	=B21*D12					
23	Fat	0.1254	0.0143						
24	Carbohydrate	0.2274	0.0000						
25	Fiber	0.2070	0.0000						
26	Ash	0.3565	0.0015						
27									
28	Result		0.4821						

Solution

(1) Using Eq. (4.8)

$$k = 0.08 + (0.52 \times 0.683)$$
$$= 0.435 \text{ W/(m °C)}$$

(2) Next we will program a spreadsheet as shown in Fig. E4.2 using the composition of hamburger beef from Table A.2.8 and coefficients of Eqs (4.10) and (4.11) given in Table A.2.9. We will use a temperature of 20°C.

(3) The thermal conductivity predicted by Eq. (4.8) is 0.435 W/(m °C), whereas using Eq. (4.10) it is 0.4821 W/(m °C). While Eq. (4.8) is easier to use, it does not include the influence of temperature.

4.3 Modes of Heat Transfer

In Chapter 1, we reviewed various forms of energy, such as thermal, potential, mechanical, kinetic, electrical, and nuclear. Our focus in this chapter will be on thermal energy, commonly referred to as heat energy or heat content. As noted in Section 1.19, heat energy is simply the sensible and latent forms of internal energy. Recall that the heat content of an object such as a tomato is determined by its mass, specific heat, and temperature. The equation for calculating heat content is

$$Q = mc_p\Delta T \tag{4.14}$$

where m is mass (kg), c_p is specific heat at constant pressure (kJ/[kg K]), and ΔT is the temperature difference between the object and a reference temperature (°C). Heat content is always expressed relative to some other temperature (called a datum or reference temperature).

Although determining heat content is an important calculation, the knowledge of how heat may *transfer* from one object to another or within an object is of even greater practical value. For example, to thermally sterilize tomato juice, we raise its heat content by transferring heat from some heating medium such as steam into the juice. In order to design the sterilization equipment, we need to know how much heat is necessary to raise the temperature of tomato juice from the initial to the final sterilization temperature using Eq. (4.14). Furthermore, we need to know the rate at which heat will transfer from steam into the juice first passing through the walls of the sterilizer. Therefore, our concerns in heating calculations are twofold: the quantity of heat transferred, Q, expressed in the units of joule (J), and the rate of heat transfer, q expressed as joule/s (J/s) or watt (W).

We will first review some highlights of the three common modes of heat transfer – conduction, convection, and radiation – and then examine selected topics of rates of heat transfer important in the design and analysis of food processes.

4.3.1 Conductive Heat Transfer

Conduction is the mode of heat transfer in which the transfer of energy takes place at a molecular level. There are two commonly accepted theories that describe conductive heat transfer. According to one theory, as molecules of a solid material attain additional thermal energy, they become more energetic and vibrate with increased amplitude of vibration while confined in their lattice. These vibrations are transmitted from one molecule to another without actual translatory motion of the molecules. Heat is thus conducted from regions of higher temperature to those at lower temperature. The second theory states that conduction occurs at a molecular level due to the drift of free electrons. These free electrons are prevalent in metals, and they carry thermal and electrical energy. For this reason, good conductors of electricity such as silver and copper are also good conductors of thermal energy.

Note that in conductive mode, there is no physical movement of the object undergoing heat transfer. Conduction is the common mode of heat transfer in heating/cooling of opaque solid materials.

From everyday experience, we know that on a hot day, heat

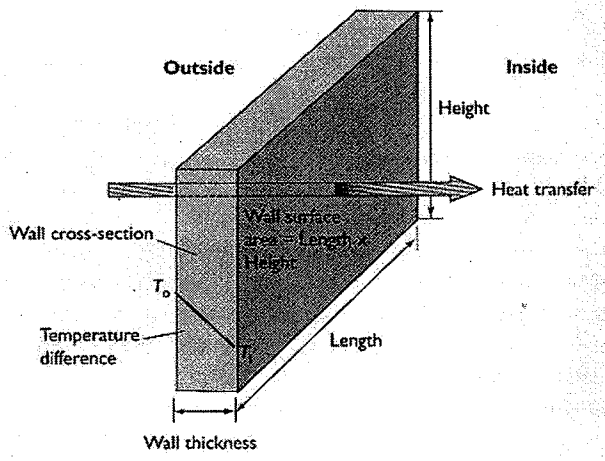


Figure 4.11 Conductive heat flow in a wall.

transfer from the outside to the inside through the wall of a room (Fig. 4.11) depends on the surface area of the wall (a wall with larger surface area will conduct more heat), the thermal properties of construction materials (steel will conduct more heat than brick), wall thickness (more heat transfer through a thin wall than thick), and temperature difference (more heat transfer will occur when the outside temperature is much hotter than the inside room temperature). In other words, the rate of heat transfer through the wall may be expressed as

$$q \propto \frac{\text{(wall surface area)(temperature difference)}}{\text{(wall thickness)}} \tag{4.15}$$

or

$$q_x \propto \frac{A\,\mathrm{d}T}{\mathrm{d}x} \tag{4.16}$$

or, by inserting a constant of proportionality,

$$q_x = -kA\frac{\mathrm{d}T}{\mathrm{d}x} \tag{4.17}$$

where q_x is the rate of heat flow in the direction of heat transfer by conduction (W); k is thermal conductivity (W/[m °C]); A is area (normal to the direction of heat transfer) through which heat flows (m^2); T is temperature (°C); and x is length (m), a variable.

Equation (4.17) is also called the Fourier's law for heat conduction, after Joseph Fourier, a French mathematical physicist. According to the second law of thermodynamics, heat will always conduct from higher temperature to lower temperature. As shown in Fig. 4.12, the gradient $\mathrm{d}T/\mathrm{d}x$ is negative, because temperature

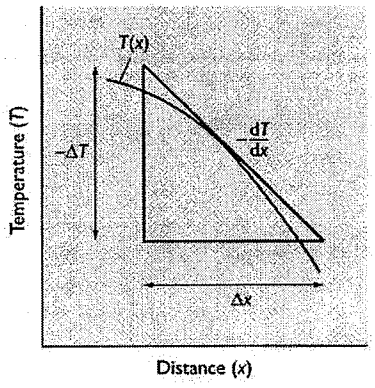


Figure 4.12 Sign convention for conductive heat flow.

decreases with increasing values of x. Therefore, in Eq. (4.17), a negative sign is used to obtain a positive value for heat flow in the direction of decreasing temperature.

Example 4.3

One face of a stainless-steel plate 1 cm thick is maintained at 110°C, while the other face is at 90°C (Fig. E4.3). Assuming steady-state conditions, calculate the rate of heat transfer per unit area through the plate. The thermal conductivity of stainless steel is 17 W/(m °C).

Given

Thickness of plate = 1 cm = 0.01 m
Temperature of one face = 110°C
Temperature of other face = 90°C
Thermal conductivity of stainless steel = 17 W/(m °C)

Approach

For steady-state heat transfer in rectangular coordinates we will use Eq. (4.17) to compute rate of heat transfer.

Solution

(1) From Eq. (4.17)

$$q = -\frac{17[\mathrm{W/(m\ ^\circ C)}] \times 1[\mathrm{m^2}] \times (110 - 90)[^\circ\mathrm{C}]}{(0 - 0.01)[\mathrm{m}]}$$

$$= 34{,}000\ \mathrm{W}$$

(2) Rate of heat transfer per unit area is calculated to be 34,000 W. A positive sign is obtained for the heat transfer, indicating that heat always flows "downhill" from 110°C to 90°C.

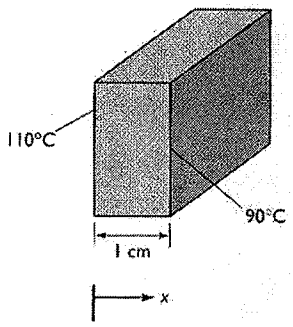


Figure E4.3 Heat flow in a plate.

4.3.2 Convective Heat Transfer

When a fluid (liquid or gas) comes into contact with a solid body such as the surface of a wall, heat exchange will occur between the solid and the fluid whenever there is a temperature difference between the two. During heating and cooling of gases and liquids the fluid streams exchange heat with solid surfaces by convection.

The magnitude of the fluid motion plays an important role in convective heat transfer. For example, if air is flowing at a high velocity past a hot baked potato, the latter will cool down much

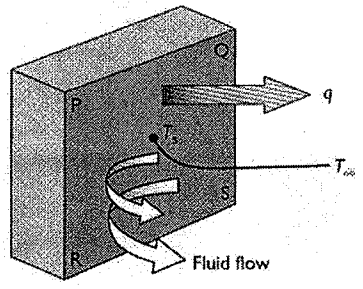


Figure 4.13 Convective heat flow from the surface of a flat plate.

faster than if the air velocity was much lower. The complex behavior of fluid flow next to a solid surface, as seen in velocity profiles for laminar and turbulent flow conditions in Chapter 2, make the determination of convective heat transfer a complicated topic.

Depending on whether the flow of the fluid is artificially induced or natural, there are two types of convective heat transfer: **forced** convection and **free** (also called **natural**) convection. Forced convection involves the use of some mechanical means, such as a pump or a fan, to induce movement of the fluid. In contrast, free convection occurs due to density differences caused by temperature gradients within the system. Both of these mechanisms may result in either laminar or turbulent flow of the fluid, although turbulence occurs more often in forced convection heat transfer.

Consider heat transfer from a heated flat plate, PQRS, exposed to a flowing fluid, as shown in Fig. 4.13. The surface temperature of the plate is T_s, and the temperature of the fluid far away from the plate surface is T_∞. Because of the viscous properties of the fluid, a velocity profile is set up within the flowing fluid, with the fluid velocity decreasing to zero at the solid surface. Overall, we see that the rate of heat transfer from the solid surface to the flowing fluid is proportional to the surface area of solid, A, in contact with the fluid, and the difference between the temperatures T_s and T_∞. Or,

$$q \propto A(T_s - T_\infty) \tag{4.18}$$

Or,

$$q = hA(T_s - T_\infty) \tag{4.19}$$

The area is A (m^2), and h is the convective heat-transfer coefficient (sometimes called surface heat-transfer coefficient), expressed as $W/(m^2\ ^\circ C)$. This equation is also called Newton's law of cooling.

Table 4.1 Some Approximate Values of Convective Heat-Transfer Coefficient

Fluid	Convective heat-transfer coefficient (W/[m^2 K])
Air	
Free convection	5–25
Forced convection	10–200
Water	
Free convection	20–100
Forced convection	50–10,000
Boiling water	3000–100,000
Condensing water vapor	5000–100,000

Note that the convective heat transfer coefficient, h, is not a property of the solid material. This coefficient, however, depends on a number of properties of fluid (density, specific heat, viscosity, thermal conductivity), the velocity of fluid, geometry, and roughness of the surface of the solid object in contact with the fluid. Table 4.1 gives some approximate values of h. A high value of h reflects a high rate of heat transfer. Forced convection offers a higher value of h than free convection. For example, you feel cooler sitting in a room with a fan blowing air than in a room with stagnant air.

Example 4.4

The rate of heat transfer per unit area from a metal plate is 1000 W/m^2. The surface temperature of the plate is 120°C, and ambient temperature is 20°C. (See Fig. E4.4.) Estimate the convective heat transfer coefficient.

Given

Plate surface temperature = 120°C
Ambient temperature = 20°C
Rate of heat transfer per unit area = 1000 W/m^2

Approach

Since the rate of heat transfer per unit area is known, we will estimate the convective heat transfer coefficient directly from Newton's law of cooling, Eq. (4.19).

Solution

(1) From Eq. (4.19)

$$h = \frac{1000[\text{W/m}^2]}{(120 - 20)[^\circ\text{C}]}$$
$$= 10\ \text{W/(m}^2\ ^\circ\text{C)}$$

(2) The convective heat transfer coefficient is found to be 10 W/(m^2 °C).

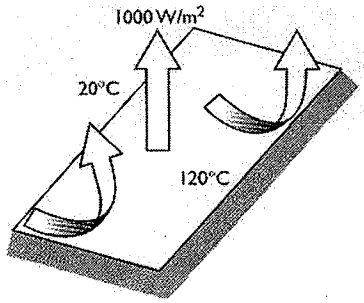


Figure E4.4 Convective heat transfer from a plate.

4.3.3 Radiation Heat Transfer

Radiation heat transfer occurs between two surfaces by the emission and later absorption of electromagnetic waves (or photons). In contrast to conduction and convection, radiation requires no physical medium for its propagation – it can even occur

Appendix D

Heldman and Lund, Handbook of Food Engineering, New York, NY: Marcel Dekker, 1992 (pp. 247-59)

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5

Heating and Cooling Processes for Foods

R. Paul Singh

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1. INTRODUCTION

Heating and cooling processes are an important part of food processing operations. Many desirable changes, as well as undesirable reactions, occur in foods when they are heated or cooled. The rate and extent of these reactions can be controlled by controlling the rate of heat transfer. Thus, heating and cooling characteristics of foods must be well understood to bring about intended changes in foods during processing.

As a food material is heated or cooled, there is an initial period of unsteady state when the temperature at a given location in the material is changing with time. After a certain time has elapsed, the rate of heat transfer reaches a steady state where the temperature may vary from one location to another, but at any given location, there is no change in temperature with time.

In this chapter a mathematical description of both steady-state and unsteady-state heat transfer in foods is presented. A description of important thermal properties of foods is given with a particular emphasis on mathematical models. Knowledge of these thermal properties is essential to the general study of heat transfer.

The topic of heat transfer covers extensive material. Several excellent textbooks give detailed coverage of this area. This chapter provides a summary of different modes of heat transfer relevant to food processing. Several mathematical expressions will be given without derivations. For additional background material, the reader is referred to books by Holman (1986), Chapman (1974), and Kreith and Black (1980).

2. THERMAL PROPERTIES OF FOODS

2.1. Thermal Conductivity

Thermal conductivity k is the rate of heat transfer q through a unit cross-sectional area A when a unit temperature difference $(T_1 - T_2)$ is maintained over a unit distance L.

$$k = \frac{qL}{A(T_1 - T_2)} \tag{1}$$

The definition above, which implies steady-state heat transfer conditions, has been used to design experiments for measuring thermal conductivity of foods. In addition, transient techniques are also used for more rapid determination of thermal conductivity. These experimental methods have been reviewed by Choi and Okos (1986) and Reidy and Rippen (1971). The steady-state methods include the guarded hot-plate method, the concentric cylinder method, and the concentric sphere method. The transient methods include the Fitch method, the line heat source or probe method, and the plate heat source method. Experimental data on thermal conductivities measured for various food groups have been expressed by mathematical relationships. These models are useful in estimating thermal conductivity of food materials. Some of the commonly used models are presented in the following.

Riedel (1949) presented the following model to predict thermal conductivity of fruit juices, sugar solutions, and milk:

$$k = (326.58 + 1.0412T - 0.00337T^2) \times (0.46 + 0.54X_w) \times 1.73 \times 10^{-3} \tag{2}$$

It was estimated that between 0° and 180°C there was an error of 1% when this model was used.

Sweat (1974) suggested the following equation, obtained with regression analysis of data on thermal conductivities of several fruits and vegetables:

$$k = 0.148 + 0.00493 \times (\%\ \text{water}) \tag{3}$$

Equation (3) should predict thermal conductivity within ±15% of experimental values for fruits and vegetables with moisture contents greater than 60%. This model is unsuitable for low-density products, or foods with void spaces, such as apples. Experimental values of thermal conductivity of selected foods are given in Table 1.

Choi and Okos (1986) have suggested the following model for liquid foods based on the food composition:

$$k = \sum_i k_i X_i^v \tag{4}$$

where the estimated volume fraction, $X_i^v = (X_i^w/\rho_i) / \Sigma(X_i^w/\rho_i)$.

The values of thermal conductivities of pure components of liquid foods are given in Table 2. For porous foods, a review of thermal conductivity values and mathematical models has been given by Wallapapan and Sweat (1986).

Thermal conductivities of anisotropic materials vary with the direction of heat transfer. For example, for meats the thermal conductivity along the meat fibers is different

Table 1 Thermal Conductivity of Selected Food Products

Product	Moisture content (%)	Temperature (°C)	Thermal conductivity (W/m·K)
Apple	85.6	2 to 36	0.393
Applesauce	78.8	2 to 36	0.516
Beef, freeze dried			
1000 mmHg pressure	—	0	0.065
0.001 mmHg pressure	—	0	0.037
Beef, lean			
Perpendicular to fibers	78.9	7	0.476
	78.9	62	0.485
Parallel to fibers	78.7	8	0.431
	78.7	61	0.447
Beef fat	—	24 to 38	0.19
Butter	15	46	0.197
Cod	83	2.8	0.544
Corn, yellow dent	0.91	8 to 52	0.141
	30.2	8 to 52	0.172
Egg, frozen whole	—	−10 to −6	0.97
Egg, white	—	36	0.577
Egg, yolk	—	33	0.338
Fish muscle	—	0 to 10	0.557
Grapefruit, whole	—	30	0.45
Honey	12.6	2	0.502
	80	2	0.344
	14.8	69	0.623
	80	69	0.415
Juice, apple	87.4	20	0.559
	87.4	80	0.632
	36.0	20	0.389
	36.0	80	0.436
Lamb			
Perpendicular to fiber	71.8	5	0.45
		61	0.478
Parallel to fiber	71.0	5	0.415
		61	0.422
Milk	—	37	0.530
Milk, condensed	90	24	0.571
	—	78	0.641
	50	26	0.329
	—	78	0.364
Milk, skimmed	—	1.5	0.538
	—	80	0.635
Milk, nonfat dry	4.2	39	0.419
Olive oil	—	15	0.189
	—	100	0.163
Oranges, combined	—	30	0.431
Peas, black-eyed	—	3 to 17	0.312
Pork			
Perpendicular to fibers	75.1	6	0.488
		60	0.54
Parallel to fibers	75.9	4	0.443
		61	0.489

Table 1 Continued

Product	Moisture content (%)	Temperature (°C)	Thermal conductivity (W/m·K)
Pork fat	—	25	0.152
Potato, raw flesh	81.5	1 to 32	0.554
Potato, starch gel	—	1 to 67	0.04
Poultry, broiler muscle	69.1 to 74.9	4 to 27	0.412
Salmon			
Perpendicular to fibers	73	4	0.502
Salt	—	87	0.247
Sausage mixture	64.72	24	0.407
Soybean oil meal	13.2	7 to 10	0.069
Strawberries	—	−14 to 25	0.675
Sugars	—	29 to 62	0.087 to 0.22
Turkey, breast			
Perpendicular to fibers	74	3	0.502
Parallel to fibers	74	3	0.523
Veal			
Perpendicular to fibers	75	6	0.476
		62	0.489
Parallel to fibers	75	5	0.441
		60	0.452
Vegetable and animal oils	—	4 to 187	0.169
Wheat flour	8.8	43	0.45
		65.5	0.689
		1.7	0.542
Whey		80	0.641

Product	Water (%)	Specific heat, experimental (kJ/kg·K)
Beef (hamburger)	68.3	3.52
Butter	15.5	2.051–2.135
Milk, whole pasteurized	87.0	3.852
Skim milk	90.5	3.977–4.019
Egg yolk	49.0	2.810
Fish, fresh	76.0	3.600
Beef, lean	71.7	3.433
Potato	79.8	3.517
Apple, raw	84.4	3.726–4.019
Bacon	49.9	2.01
Cucumber	96.1	4.103
Potato	75.0	3.517
Veal	68.0	3.223
Fish	80.0	3.60
Cheese, cottage	65.0	3.265
Shrimp	66.2	3.014
Sardines	57.4	3.014
Beef, roast	60.0	3.056
Carrot, fresh	88.2	3.81–3.935

Source: Reidy (1968).

Table 2 Thermal Property Models

a. Major food components

Thermal property	Major component	Group model temperature function	Standard error	Standard % error
k(W/m·°C)	Protein	$k = 1.788\,1 \times 10^{-1} + 1.195\,8 \times 10^{-3}T - 2.717\,8 \times 10^{-6}T^2$	0.012	5.91
	Fat	$k = 1.807\,1 \times 10^{-1} - 2.760\,4 \times 10^{-3}T - 1.774\,9 \times 10^{-7}T^2$	0.0032	1.95
	Carbohydrate	$k = 2.014\,1 \times 10^{-1} + 1.387\,4 \times 10^{-3}T - 4.331\,2 \times 10^{-6}T^2$	0.0134	5.42
	Fiber	$k = 1.833\,1 \times 10^{-1} + 1.249\,7 \times 10^{-3}T - 3.168\,3 \times 10^{-6}T^2$	0.0127	5.55
	Ash	$k = 3.296\,2 \times 10^{-1} + 1.401\,1 \times 10^{-3}T - 2.906\,9 \times 10^{-6}T^2$	0.0083	2.15
α (m^2/s)	Protein	$\alpha = 6.871\,4 \times 10^{-2} + 4.757\,8 \times 10^{-4}T - 1.464\,6 \times 10^{-6}T^2$	0.0038	4.50
	Fat	$\alpha = 9.877\,7 \times 10^{-2} - 1.256\,9 \times 10^{-4}T - 3.828\,6 \times 10^{-8}T^2$	0.0020	2.15
	Carbohydrate	$\alpha = 8.084\,2 \times 10^{-2} + 5.305\,2 \times 10^{-4}T - 2.321\,8 \times 10^{-6}T^2$	0.0058	5.84
	Fiber	$\alpha = 7.397\,6 \times 10^{-2} + 5.190\,2 \times 10^{-4}T - 2.220\,2 \times 10^{-6}T^2$	0.0026	3.14
	Ash	$\alpha = 1.246\,1 \times 10^{-1} + 3.732\,1 \times 10^{-4}T - 1.224\,4 \times 10^{-6}T^2$	0.0022	1.61
ρ (kg/m^3)	Protein	$\rho = 1.329\,9 \times 10^{3} - 5.184\,0 \times 10^{-1}T$	39.9501	3.07
	Fat	$\rho = 9.255\,9 \times 10^{2} - 4.175\,7 \times 10^{-1}T$	4.2554	0.47
	Carbohydrate	$\rho = 1.599\,1 \times 10^{3} - 3.104\,6 \times 10^{-1}T$	93.1249	5.98
	Fiber	$\rho = 1.311\,5 \times 10^{3} - 3.658\,9 \times 10^{-1}T$	8.2687	0.64
	Ash	$\rho = 2.423\,8 \times 10^{3} - 2.806\,3 \times 10^{-1}T$	2.2315	0.09
C_p (kJ/kg·°C)	Protein	$C_p = 2.008\,2 + 1.208\,9 \times 10^{-3}T - 1.312\,9 \times 10^{-6}T^2$	0.1147	5.57
	Fat	$C_p = 1.984\,2 + 1.473\,3 \times 10^{-3}T - 4.800\,8 \times 10^{-6}T^2$	0.0236	1.16
	Carbohydrate	$C_p = 1.548\,8 + 1.962\,5 \times 10^{-3}T - 5.939\,9 \times 10^{-6}T^2$	0.0986	5.96
	Fiber	$C_p = 1.845\,9 + 1.830\,6 \times 10^{-3}T - 4.650\,9 \times 10^{-6}T^2$	0.0293	1.66
	Ash	$C_p = 1.092\,6 + 1.889\,6 \times 10^{-3}T - 3.681\,7 \times 10^{-6}T^2$	0.0296	2.47

b. Water and ice as a function of temperature

	Temperature functions[a]	Standard error	Standard % error
Water	$k_w = 5.710\,9 \times 10^{-1} + 1.762\,5 \times 10^{-3}T - 6.703\,6 \times 10^{-6}T^2$	0.0028	0.45
	$\alpha_w = 1.316\,8 \times 10^{-1} + 6.247\,7 \times 10^{-4}T - 2.402\,2 \times 10^{-6}T^2$	0.0022×10^{-6}	1.44
	$\rho_w = 9.971\,8 \times 10^{2} + 3.143\,9 \times 10^{-3}T - 3.757\,4 \times 10^{-3}T^2$	2.1044	0.22
	$C_{pw1} = 4.081\,7 - 5.306\,2 \times 10^{-3}T + 9.951\,6 \times 10^{-4}T^2$	0.0988	2.15
	$C_{pw2} = 4.176\,2 - 9.086\,4 \times 10^{-5}T + 5.473\,1 \times 10^{-6}T^2$	0.0159	0.38
Ice	$k_i = 2.219\,6 - 6.248\,9 \times 10^{-3}T + 1.015\,4 \times 10^{-4}T^2$	0.0079	0.79
	$\alpha_i = 1.175\,6 - 6.083\,3 \times 10^{-3}T + 9.503\,7 \times 10^{-5}T^2$	0.0044×10^{-6}	0.33
	$\rho_i = 9.168\,9 \times 10^{2} - 1.307\,1 \times 10^{-1}T$	0.5382	0.06
	$C_{pi} = 2.062\,3 + 6.076\,9 \times 10^{-3}T$	0.0014	0.07

[a] C_{pw1} = for the temperature range −40 to 0°C.
C_{pw2} = for the temperature range 0 to 150°C.
Source: Choi and Okos (1986).

than it is across the fibers. These differences were considered by Kopelman (1966). His models for thermal conductivity are presented by Heldman and Singh (1981).

2.2. Density

Density of a food material is the mass of the sample divided by its volume. Experimental determination of density can be done using a pycnometer, an air comparison pycnometer, and a platform scale method (Choi and Okos, 1986). Mathematical models of density of foods have been developed for prediction purposes. For fruit juices, Riedel (1949) suggested measuring the index of refraction of the juice, s, and using the following relationship:

$$\rho = \frac{s^2 - 1}{s^2 + 2} \times \frac{62.4}{0.206} \times 16.0185 \tag{5}$$

Other researchers have reported specific models for milk, cream, and tomato juice (Short, 1955; Phipps, 1969; Choi and Okos, 1983).

Using the compositional information on liquid foods, Choi and Okos (1966) have suggested the following model:

$$\rho = \frac{1}{\sum(X_i^w/\rho_i)} \tag{6}$$

The density values of pure components are given in Table 2.

2.3. Specific Heat

Specific heat of a food material is a measure of the amount of energy required by a unit mass to raise its temperature by a unit degree. Specific heat, or the mass heat capacity of food materials, has been determined experimentally by several methods, including the method of mixtures, method of guard plate, and using a differential scanning calorimeter (Choi and Okos, 1986).

For high-moisture foods, above the freezing point, Siebel (1982) developed the following equation:

$$c_p = 0.837 + 3.349X_w \tag{7}$$

A similar equation was suggested by Dickerson (1968) for high-moisture foods.

Charm (1971) suggested the following model:

$$c_p = 2.093X_F + 1.256X_s + 4.187X_w \tag{8}$$

Using a similar approach, Choi and Okos (1986) have suggested the following model for the specific heat for liquid foods:

$$c_p = \sum c_{pi}X_i^w \tag{9}$$

where the specific heat of pure components is given in Table 2.

A review of specific heat values for porous foods is given by Wallapapan and Sweat (1986).

2.4. Thermal Diffusivity

Thermal diffusivity, α, can be expressed in terms of thermal conductivity, density, and specific heat as

$$\alpha = \frac{k}{\rho c_p} \tag{10}$$

If the values of properties on the right-hand side of equation are known, thermal diffusivity can be calculated. Most researchers use this procedure to determine thermal diffusivity. There are a few direct methods for experimental determination, such as the use of a cylinderical object and time–temperature data, use of a spherical object and time–temperature data, and use of a thermal conductivity probe (Choi and Okos, 1986).

Thermal diffusivity is strongly influenced by the water content as shown by the following models developed by Dickerson (1969) and Martens (1980), respectively:

$$\alpha = 0.088 \times 10^{-6} + (\alpha_w - 0.088 \times 10^{-6})X_w \tag{11}$$

$$\alpha = 0.057363X_w + 0.00028(T + 273) \times 10^{-6} \tag{12}$$

Based on the composition of liquid foods, Choi and Okos (1986) suggest the following model:

$$\alpha = \sum \alpha_i X_i^v \tag{13}$$

where the values of thermal diffusivity for pure components are given in Table 2.

Information on thermal properties of porous foods is presented in a review paper by Wallapapan et al. (1983). Thermal diffusivity values of selected foods are given in Table 3.

EXAMPLE A new engineered food has the following composition: water 79.4%, protein 2%, fat 0.1%, carbohydrate 17.6%, and ash 0.9%. Estimate thermal conductivity, density, specific heat, and thermal diffusivity at 20°C.

Solution The thermal properties will be determined using the models suggested by Choi and Okos (1986). The models shown in Table 2 may be programmed in a spreadsheet to yield the following results:

Thermal conductivity: $k = 0.54$ W/m·°C
Density: $\rho = 1082$ kg/m^3
Specific heat: $c_p = 3.65$ kJ/kg·°C
Thermal diffusivity: $\alpha = 0.137 \times 10^{-6}$ m^2/s

3. STEADY-STATE HEATING AND COOLING OF FOODS

3.1. Conduction Heat Transfer

The conduction mode of heat transfer involves energy transfer from regions of higher to lower temperatures. This energy transfer occurs mainly by the contact of matter at a given location with adjacent matter. There is no physical movement of the mass from one location to another. The rate of heat transfer due to conduction was described by Fourier using the following equation, also called *Fourier's law*:

Table 3 Thermal Diffusivity of Some Foodstuffs

Product	Water content (wt %)	Temperature[a] (°C)	Thermal diffusivity ($\times 10^{-5}$ m^2/s)
Fruits, vegetables, and by-products			
Apple, whole, Red Delicious	85	0–30	1.37
Applesauce	37	5	1.05
	37	65	1.12
	80	5	1.22
	80	65	1.40
	—	26–129	1.67
Avocado, flesh	—	24,0	1.24
Seed	—	24,0	1.29
Whole	—	41,0	1.54
Banana, flesh	76	5	1.18
	76	65	1.42
Beans, baked	—	4–122	1.68
Cherries, tart, flesh	—	30,0	1.32
Grapefruit, Marsh, flesh	88.8	—	1.27
Grapefruit, Marsh, albedo	72.2	—	1.09
Lemon, whole	—	40,0	1.07
Lima bean, pureed	—	26–122	1.80
Pea, pureed	—	26–128	1.82
Peach, whole	—	27,4	1.39
Potato, flesh	—	25	1.70
Potato, mashed, cooked	78	5	1.23
	78	65	1.45
Rutabaga	—	48,0	1.34
Squash, whole	—	47,0	1.71
Strawberry, flesh	92	5	1.27
Sugarbeet	—	14,60	1.26
Sweet potato, whole	—	35	1.06
	—	55	1.39
	—	70	1.91
Tomato, pulp	—	4.26	1.48
Fish and meat products			
Codfish	81	5	1.22
	81	65	1.42
Corned beef	65	5	1.32
	65	65	1.18
Beef, chuck[b]	66	40–65	1.23
Beef, round	71	40–65	1.33
Beef, tongue	68	40–65	1.32
Halibut	76	40–65	1.47
Ham, smoked	64	5	1.18
Ham, smoked	64	40–65	1.38
Water	—	30	1.48
	—	65	1.60
Ice	—	0	11.82

Source: Singh (1982).

[a]Where two temperatures separated by a comma are given, the first is the initial temperature of the sample, and the second is that of the surroundings.

[b]Data are applicable only where juices that exuded during heating remain in the food samples.

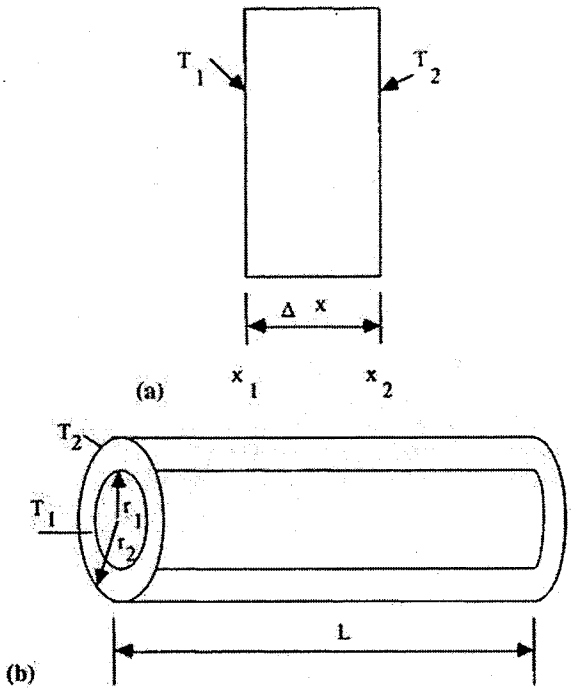

Figure 1 Schematic of (a) a plane wall, (b) a cylinder, (c) a composite wall, and (d) a composite cylinder.

$$\frac{q}{A} = -k\frac{dT}{dx} \tag{14}$$

From Eq. (14) it is evident that the rate of heat transfer per unit area is proportional to the temperature gradient along the x-axis. The negative sign indicates that heat flow occurs from a hotter region to a colder region. The thermal conductivity is a unique property of the material. Thermal conductivity of food materials may exhibit a strong dependence on temperature and location. A further description of this property is given in Section 2.1.

Fourier's law may be solved for a rectangular, cylindrical, or spherical coordinate system, depending on the geometrical shape of the object being studied. Some commonly used solutions of Fourier's law are presented below.

1. Conduction heat transfer in a flat plate (Fig. 1a):

$$q_x = -kA\frac{\Delta T}{\Delta x} = -kA\frac{T_2 - T_1}{x_2 - x_1} \tag{15}$$

2. Conductive heat transfer in a hollow pipe (Fig. 1b):

$$q = \frac{2\pi Lk(T_2 - T_1)}{\ln(r_2/r_1)} \tag{16}$$

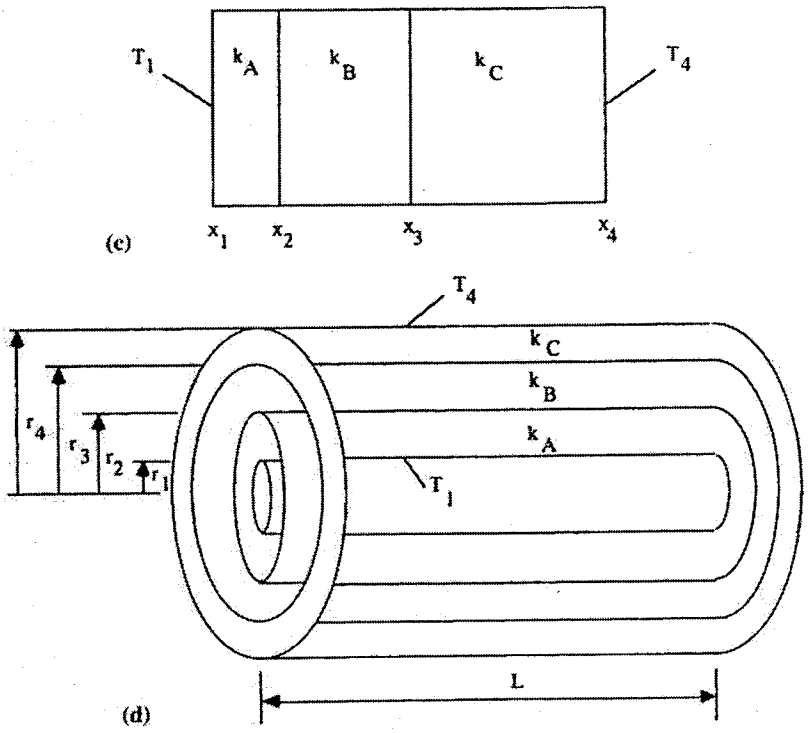

Figure 1 Continued

3. Conductive heat transfer in a three-layered wall where thermal conductivities of the three layers are k_A, k_B, and k_C; and thickness of each layer is $x_2 - x_1$, $x_3 - x_2$, and $x_4 - x_3$, respectively (Fig. 1c).

$$q = \frac{A(T_4 - T_1)}{(x_2 - x_1)/k_A + (x_3 - x_2)/k_B + (x_4 - x_3)/k_C} \tag{17}$$

4. Conductive heat transfer in a three-layered composite cylindrical tube (Fig. 1d):

$$q_r = \frac{2\pi L(T_4 - T_1)}{\ln(r_2/r_1)/k_A + \ln(r_3/r_2)/k_B + \ln(r_4/r_3)/k_C} \tag{18}$$

5. Conductive heat transfer in a hollow sphere:

$$q_r = -4\pi k r_1 r_2 \frac{T_2 - T_1}{r_2 - r_1} \tag{19}$$

6. Steady-state heat transfer with heat generation:

Certain foods, especially fruits, vegetables, and cereal grains respire during storage. The heat of respiration must be taken into account when determining steady-state heat

transfer for these products. A steady-state solution for temperature for a one-dimensional case is as follows:

$$T_o = \frac{qL^2}{2k} + T_w \tag{20}$$

This solution suggests a parabolic temperature distribution for heat conduction involving heat generation.

3.2. Convection Heat Transfer

Convection heat transfer is the major mode of heat transfer between the surface of a solid material and the surrounding fluid. The rate of convective heat transfer depends on the properties of the fluid and the fluid flow characteristics. Originally suggested by Prandtl, the resistance to heat transfer may be considered to be localized in a boundary layer within the fluid present at the surface of the solid material. Although this concept is for ideal situations, it has been widely used in studying convective heat transfer.

Using the boundary layer concept, the rate of convective heat transfer may be written as

$$q = \frac{k}{\delta} A(T_s - T_\infty) \tag{21}$$

Since δ, the thickness of the boundary layer, cannot be measured, the quantity k/δ is expressed by h, the convective heat transfer coefficient. Then

$$q = hA(T_s - T_\infty) \tag{22}$$

The convective heat transfer coefficient, h, has been measured by numerous researchers for a variety of different conditions. This coefficient is dependent on fluid properties, such as k, ρ, c_p, and μ, velocity of flow, and the geometrical shape of the object undergoing heating or cooling. Using dimensional analysis, correlations have been developed to determine the convective heat transfer coefficient. Some of these relationships that are important to food processing are presented in the following section.

There are two modes of convective heat transfer depending on the fluid flow characteristics. The first mode of heat transfer is called forced convection. The fluid flow is artificially induced, such as blowing air with a fan or a blower, pumping liquid on a heating (or cooling) surface. On the other hand, if the fluid flow is due primarily to changes in fluid density that are caused by differences in temperature, heat transfer occurs by free (or natural) convection.

3.2.1. *Forced Convection in Newtonian Fluids*

The convective heat transfer coefficient, under forced convection conditions, has been measured experimentally for a variety of different conditions. The experimental results are typically presented by correlations developed using dimensional analysis. Some of these correlations are presented in the following.

Laminar flow inside circular pipe (horizontal or vertical):

$$N_{Nu} = 1.86\,(N_{Gz})^{1/3} \left(\frac{\mu}{\mu_w}\right)^{0.14} \qquad \text{for } N_{Re} < 2100 \tag{23}$$

Transitional flow inside circular pipe:

$$N_{Nu} = 0.116[(N_{Re})^{0.667} - 125](N_{Pr})^{1/3}\left[1 + \left(\frac{d}{L}\right)^{0.667}\right]\left(\frac{\mu}{\mu_w}\right)^{0.14} \quad (24)$$

for $2100 < N_{Re} < 10{,}000$

Turbulent flow inside a circular pipe:

$$N_{Nu} = 0.023\,(N_{Re})^{0.8}(N_{Pr})^{0.667}\left(\frac{\mu_b}{\mu_w}\right)^{0.14} \quad (25)$$

Flow of liquid normal to a single cylinder:

$$N_{Nu} = [0.35 + 0.56(N_{Re})^{0.52}](N_{Pr})^{0.3} \qquad \text{for } N_{Re} = 0.1\text{–}300 \quad (26)$$

Flow of gases past a sphere:

$$N_{Nu} = 2 + 0.6\,(N_{Re})^{0.5}(N_{Pr})^{0.33} \qquad \text{for } N_{Re} < 325 \quad (27)$$

and

$$N_{Nu} = 0.4(N_{Re})^{0.6}(N_{Pr})^{0.33} \qquad \text{for } 325 < N_{Re} < 70{,}000 \quad (28)$$

Flow of gases past a sphere:

$$N_{Nu} = [0.97 + 0.68\,N_{Re}^{0.52}]\,(N_{Pr})^{0.3} \quad (29)$$

3.2.2. *Free Convection in Newtonian Fluids*

In the free-convection mode of heat transfer, the temperature of the fluid affects its density, which causes buoyant forces to develop. The following functional relationship is used to determine the convective heat transfer coefficient:

$$N_{Nu} = f(N_{Gr}, N_{Pr}) \quad (30)$$

Experimentally obtained results have been expressed using the following equation:

$$N_{Nu} = a(N_{Gr}N_{Pr})^b \quad (31)$$

where a and b are evaluated from Table 4 for respective conditions.

3.2.3. *Convective Heat Transfer in Non-Newtonian Fluids*

Piston Flow For a Graetz number larger than 500, the following expression presented by Metzner et al. (1959) is useful:

$$N_{Nu} = \frac{8}{\pi} + \frac{4}{\pi}(N_{Gz})^{0.5} \quad (32)$$

Fully Developed Velocity Profile For a power law fluid, the following equation may be used:

$$N_{Nu} = 1.75\left(\frac{3n + 1}{4n}\right)^{1/3}(N_{Gz})^{1/3} \quad (33)$$

Table 4 Constants a and b for Eq. (31)

Configuration	$N_{Gr}N_{Pr}$	a	b
Vertical plates and cylinders			
Length > 1 m			
Laminar	$<10^4$	1.36	1/5
Laminar	$10^4 < N_{Gr}N_{Pr} < 10^9$	0.55	1/4
Turbulent	$>10^9$	0.13	1/3
Spheres and horizontal cylinders			
Diameter < 0.2 m			
Laminar	$10^3 < N_{Gr}N_{Pr} < 10^9$	0.53	1/4
Turbulent	$>10^9$	0.13	1/3
Horizontal plates			
Heated plate facing up (or cooled plate facing down)			
Laminar	$10^5 < N_{Gr}N_{Pr} < 2 \times 10^7$	0.54	1/4
Turbulent	$2 \times 10^7 < N_{Gr}N_{Pr} < 3 \times 10^{10}$	0.14	1/3
Heated plate facing down (or cooled plate facing up)			
Laminar	$3 \times 10^5 < N_{Gr}N_{Pr} < 3 \times 10^{10}$	0.27	1/4

Another expression that is useful for a fully developed velocity profile was proposed by Charm and Merrill (1959):

$$N_{Nu} = 2(N_{Gz})^{1/3}\left[\frac{m_b}{m_s}\frac{3n+1}{2(3n-1)}\right]^{0.14} \tag{34}$$

3.3. Radiation Heat Transfer

The study of heat transfer by radiation includes three important properties of food materials: emissivity, ϵ; absorptivity, α; and transmittance, τ.

The energy emitted from a surface can be described using the Stefan–Boltzmann law:

$$q = \sigma A \epsilon T_A^4 \tag{35}$$

where $\sigma = 5.67 \times 10^{-8}$ W/m$^2 \cdot$ K^4 and T_A = absolute temperature (K).

Equation (35) can be used to determine radiative energy exchange between a surface A of a body and the surroundings at temperature T_2 that envelope the body. An example is baking bread inside an oven.

$$q_{1-2} = \sigma A_1(\epsilon_1 T_{A_1}^4 - \phi_{1-2}T_{A_2}^4) \tag{36}$$

According to Kirchhoff's law, the emissivity of a body is equal to its absorptivity for the same wavelength.

An example of radiative heat exchange that is commonly encountered in food processing is radiation between two parallel gray surfaces:

Appendix E

Herman and Groves, "The Influence of Free Fatty Acid Formation on the pH of Phospholipid-stabilized Triglyceride Emulsions," Pharmaceutical Research, 10(5): 774-76 (1993)

The Influence of Free Fatty Acid Formation on the pH of Phospholipid-Stabilized Triglyceride Emulsions

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KEY WORDS: phospholipids; thermal degradation; triglyceride emulsions; pH; free fatty acids.

INTRODUCTION

The intravenous administration of triglyceride emulsions stabilized with phospholipid emulsifiers has been employed for parenteral nutrition for over 30 years (1). Terminally heat sterilized, these systems are required to be physically and chemically stable in order to avoid harming the patient (2). Nevertheless, slow hydrolysis of the phospholipids is known to occur after the initial sterilization-induced degradation (3).

It is implicitly assumed that the subsequent fall of product pH is due to the formation of free fatty acids (4,5). We have recently demonstrated that the initial hydrolysis of the phospholipids during the heat sterilization process paradoxically promotes physical stabilization of the emulsion system, most probably because of the formation of liquid crystalline structures at the oil/water interface (3,6). The principal degradation process is due to the hydrolysis of the diacylphosphatidylcholines and diacylphosphatidylethanolamines to their corresponding monoacyl (lyso-) derivatives and free fatty acid (FFA) moieties. In turn, the lyso derivatives can degrade to the corresponding glycerophosphoryl compounds, with the formation of additional FFA. FFA can also be formed by the hydrolysis of emulsified triglycerides to the corresponding mono- and diglycerides, although this reaction is believed to be relatively slow compared to the breakdown of the diacylphosphatidyl derivatives (3).

The emulsion systems are unbuffered and the formation of FFA will inevitably lower the pH from the initial value of 8.0 (1) over a period of time poststerilization. Håkansson (2) demonstrated that the degradation rate decreases until pH 6.5 is reached, after which there is again an acceleration of the degradation process. This effect has been confirmed by Grit *et al.* (7). Stabilization may, therefore, be improved by the addition of extraneous FFA, a suggestion made by Washington and Davis (4), who evaluated the effect induced by the addition of oleic acid to their emulsion systems. However, the value of this suggestion is unclear since, during the phospholipid hydrolysis process, lyso compounds are produced in addition to FFA and these materials also contribute to the emulsion stabilization process (3,6).

Measurement of pH could, therefore, provide an indirect method of determining the FFA content of phospholipid-stabilized emulsions. This concept was evaluated measuring the FFA content directly by potassium hydroxide titration of the degrading emulsion at the same time as taking the pH of the system with a glass electrode.

MATERIALS AND METHODS

Materials

Purified egg phospholipid, Asahi Injectable grade (lot 900-80201), was received as a gift from Austin Chemical, Chicago, IL. Dipalmitoyl phosphatidylethanolamine (Lot 1 60PE-45) (DPPE) and hydrogenated egg phosphatidylcholine (Lot HEPC −44) (HEPC) were purchased from Avanti Polar Lipids, Birmingham, AL. Pharmaceutical-grade and "superrefined"-grade soybean oils USP were received as gifts from Croda Inc., Edison, NJ. Glycerol, sodium hydroxide, potassium hydroxide, and potassium hydrogen phthalate were all used as received from Fisher Scientific, Itasca, IL. A Milli-Q ion-exchange water system was used.

Model Emulsion

The model emulsion was made to the following formula: soybean oil, 20 g; egg phospholipid, 1.2 g; glycerol, 2.25 g; and water to 100 mL.

The egg phospholipid was dispersed in the glycerol and about 95% of the water at 70°C, the oil mixed in, and the coarse emulsion passed through a Microfluidics Model 110T homogenizer at a pressure of 10,000 psig for a total of 10 times to ensure minimal particle size (8). The system was washed through the homogenizer and made up to volume with water, and the pH adjusted to 8.0 with 0.01 *N* sodium hydroxide and packed and sealed in 2-mL volumes in 2-mL glass ampoules. Gas sparging with nitrogen or oxygen was carried out for 15 min when required. Hydrogenated phospholipid-stabilized emulsions were prepared by the same method, using 0.24 g DPPE and 0.96 g HEPC instead of the 1.2 g egg phospholipids.

Samples were stressed and analyzed in replicate; $n = 4$.

pH Measurement

An Orion Model 811 glass electrode pH meter was used. Potassium chloride was added to counteract the ion adsorbing effects of charged droplets (9), a process also used in the USP XX11 for the measurement of the pH of Dextrose solutions. After experimentation to determine conditions required to give reproducible results, measurements were made following the addition of 50 μL of a saturated aqueous potassium chloride solution to 2 mL of emulsion sample.

Free Fatty Acid Measurement

FFA measurement of a solution of the emulsion sample

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was by direct titration to neutrality with 0.01 *N* potassium hydroxide solution standardized using potassium hydrogen phthalate, with phenolphthalein as indicator (USP XXII). The IUPAC method (10) specifies ethanol:diethyl ether (95:5) as the solvent of choice. However, for safety reasons, the solvent was changed to ethanol:chloroform (2:1), each titration being preceded by bringing the solvent to neutrality with 0.01 *N* potassium hydroxide solution.

Thermal Stress

Thermal stress was applied by filling heating block chambers (Dry Baths, Fisher Scientific, Itasca, IL; 60 chambers per block, each 12 mm diameter and 50 mm deep) with oil and immersing the 2-mL ampoules containing the emulsion at the desired temperature, covering the blocks with aluminum foil to minimize thermal fluctuation. Temperatures were determined with calibrated mercury-in-glass thermometers placed at random in the block chambers.

In general, all ampouled emulsion samples were initially sterilized at 121°C (F_o = 18) using a Getinge BioF$_o$OE autoclave. Unautoclaved controls were stored at 5°C prior to evaluation.

RESULTS AND DISCUSSION

The rate of pH change and the rate of FFA formation are compared in Fig. 1 for a model emulsion prepared with the pharmaceutical grade of soy oil. Results obtained using the "superrefined" grade of oil were similar. It is evident that the rate of formation of FFA is slower than the rate at which the pH drops, suggesting that some other factors are involved. As shown in Fig. 2, emulsions prepared with unsaturated or saturated acyl groups on the phospholipid moieties and sparged with nitrogen prior to sterilization and storage changed pH at almost identical rates. However, it should be noted that these two emulsions had markedly different hydrolysis rates, (3), which suggested that the lowering of pH was due to some other factor not necessarily associated with phospholipid hydrolysis. As noted earlier, free fatty acids can also arise by hydrolysis of triglycerides but the total (titratable) FFA in the system, irrespective of its source, is clearly unable to account for the relatively rapid lowering of pH.

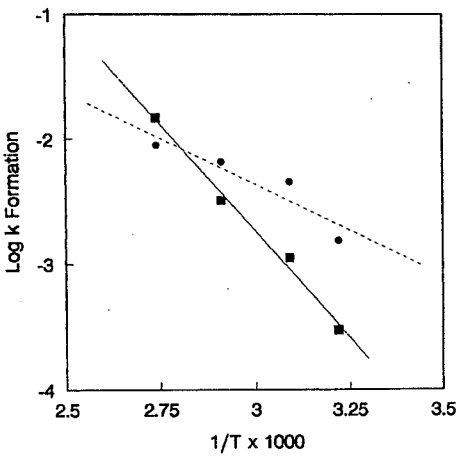

Fig. 1. The rate of FFA formation (by KOH titration) and the fall of pH (glass electrode) in a 20% soy oil emulsion stabilized with egg lecithin after sterilization and storage over the range 25–90°C. pH (slope = −3.42), ——■——; FFA (slope = −1.47), ---●---. *K*, rate of formation (slope of change against time); T, absolute temperature (kelvins).

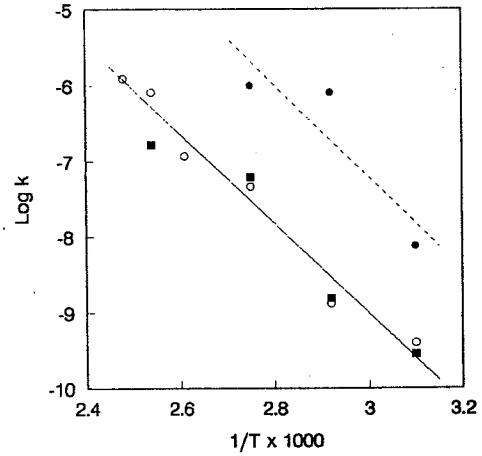

Fig. 2. The rate of pH change in a 20% soy oil emulsion stabilized with egg lecithin or saturated phospholipids and sparged with nitrogen or oxygen before sterilization and storage over the range 25–90°C. Control: egg phospholipids, nitrogen sparged (estimated slope = −5.93), ——○——; saturated phospholipids, nitrogen sparged (estimated slope = −5.30), ■; egg phospholipids, oxygen sparged (estimated slope = −6.11), ----●----.

When sparged with oxygen, the pH change in the control emulsion was considerably increased (Fig. 2), and this observation suggests that the effect of oxygen on the emulsion pH was more pronounced than that produced by the heat-induced hydrolytic degradation reaction.

The solubility of gases in liquids is described by Henry's law (11):

$$P_A = X_A K_a$$

where P_A is the vapor pressure of a solution containing solute A, X_A is the mole fraction of A, and K_a is Henry's law constant. Thus, intuitively, since the K_a for gases in nonaqueous solvents is generally higher than the corresponding value in water, it would appear that the oxygen is likely to preferentially dissolve in the oil phase of the emulsion. Atkins (11) discussed this issue in relation to benzene at standard temperature and pressure and observed that, in all cases, the gas was more soluble in the benzene than in the water.

Based on this consideration, it seems feasible to suggest that residual oxygen may remain dissolved in the triglyceride oil phase after preparation and manipulation of the emulsion. Some of the triglycerides contain unsaturated acyl centers, which could, therefore, become partially oxidized, although not necessarily to the point where they would be titratable with alkali and estimated as FFA. These moieties would affect the pH of the unbuffered system, in addition to the effects produced by the FFA resulting from hydrolysis of phospholipid and triglyceride entities. The evident complexity of the physical and chemical structure of phospholipid-

stabilized emulsions makes this suggestion difficult to confirm at present.

ACKNOWLEDGMENTS

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Appendix F

Kassis et al., "Characterization of Lipids and Antioxidant Capacity of Novel Nutraceutical Egg Products Developed with Omega-3-Rich Oils" J Sci Food Agr 92(1): 66-73 (2012)

Research Article

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Characterization of lipids and antioxidant capacity of novel nutraceutical egg products developed with omega-3-rich oils

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Abstract

BACKGROUND: Cardiovascular disease has had an unquestioned status of the number one cause of death in the US since 1921. Omega-3 polyunsaturated fatty acids (ω-3 PUFAs) have cardio-protective benefits. However, egg is typically a poor source of ω-3 PUFAs and, in general, the American diet is low in these cardio-protective fatty acids. Novel, nutritionally enhanced egg products were developed by substituting yolk with ω-3 PUFA-rich flaxseed, menhaden, algae, or krill oil. Experimental egg products matched composition of hen egg (whole egg). The experimental egg products, mixed whole egg, and a liquid egg product (Egg Beaters™) were microwave-cooked and compared.

RESULTS: Although fat, protein, and moisture contents of experimental egg products matched ($P > 0.05$) mixed whole egg, experimental egg products had more ($P < 0.05$) ω-3 PUFAs, lower ($P < 0.05$) ω-6/ω-3 ratio, and depending on oil added, a higher ($P < 0.05$) unsaturated/saturated fatty acids ratio compared to mixed whole egg. Triglycerides were the main lipid class in all experimental egg products except those developed with krill oil, which had even more phospholipids than mixed whole egg. Analysis of thiobarbituric acid reactive substances showed that lipid oxidation of experimental egg products was lower ($P < 0.05$) or similar ($P > 0.05$) to mixed whole egg, except for experimental egg products with krill oil. However, peroxide value showed that all egg samples had minimal oxidation. Experimental egg products developed with menhaden or flaxseed oil had the highest ($P < 0.05$) concentration of the antioxidant, ethyoxquin compared to all other egg samples. However, experimental egg products with krill oil likely contained a natural antioxidant, astaxanthin.

CONCLUSION: This study demonstrated an alternative approach to developing novel, nutraceutical egg products. Instead of dietary modification of chicken feed, yolk substitution with ω-3 PUFAs oils resulted in enhancement of ω-3 PUFAs beyond levels possible to achieve by modifying chicken feed.

Keywords: egg products; nutraceutical food products; functional food products; egg nutritional composition; egg fatty acid profile; egg lipid classes and oxidation; flaxseed oil; fish oil; algae oil; krill oil; food product development

INTRODUCTION

For the last decade, egg consumption in the US has exceeded 6 billion dozen eggs per year, rendering eggs a staple food.[1] The egg is one of the best and least expensive sources of high quality dietary protein. However, approximately half of the total fat in egg is saturated fat.[2] Nutrients in egg are not equally distributed. Fat, including saturated fat is only in yolk; while egg white does not contain fat. The saturated fat intake increases serum total and low-density lipoprotein (LDL)-cholesterol concentrations, which are established risk factors for cardiovascular disease (CVD).[2] According to the American Heart Association, CVD has had an unquestioned status of the number one cause of death in the US since 1921.[3]

Omega-3 polyunsaturated fatty acids (ω-3 PUFAs) have been demonstrated to have cardio-protective benefits. The ω-3 PUFA intake decreases inflammatory markers, blood pressure and serum triglycerides, which are established risk factors for CVD.[4] The ω-3 PUFAs also improve endothelial functions; reduce platelet aggregation, vasoconstriction and the risk of sudden cardiac death.[4] Anderson and Ma[5] provided a comprehensive review of health benefits associated with specific ω-3 PUFAs. Aquatic foods are the major sources of ω-3 PUFAs, eicosapentaenoic (EPA, 20 : 5ω-3) and docosahexaenoic acids (DHA, 22 : 6ω-3). Sources of plant-derived ω-3 PUFAs include flaxseed, walnuts, canola, soybean and algae. However, plant-derived oils mainly contain α-linolenic acid (ALA, 18 : 3ω-3) with the exception of algae such as *Crypthecodinium cohnii* and *Schizochytrium* sp. that bio-synthesize DHA. Populations with total fat intake greater than 30% of total energy predominantly from fish and plant oils maintain low mortality from CVD.[6] The American diet is low in ω-3 PUFAs despite their health benefits.[7]

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Nutraceutical or functional foods are food products that contain added, technologically developed ingredients with specific health benefits.[8] The ω-3 PUFA-fortified food products provide a means to achieve desired biochemical effects of these nutrients without the ingestion of dietary supplements, medications or a major change in dietary habits. A potential consequence of addition of ω-3 PUFAs is higher susceptibility to oxidation, which leads to the formation of peroxides, off-flavors, changes of taste, texture and color, and loss of nutrients.

Eggs are not naturally rich in ω-3 PUFAs. Dietary manipulation of ω-3 PUFAs in poultry feed yields eggs with more ω-3 PUFAs.[9–11] However, the increase obtained this way is limited likely due to chicken physiology. Even a three-fold increase of ω-3 PUFAs in egg obtained by increased dietary intake of ω-3 PUFAs by chickens should be considered relatively small, particularly when compared with the ω-3 PUFAs daily intakes for humans recommended by the Canadian, Scandinavian and British governments (up to 2000 mg day^{-1}). Consumers responded positively to ω-3-fortified eggs in acceptability study.[12] According to the International Food Information Council,[13] 89% of Americans recognize that certain foods have benefits beyond basic nutrition and reduce the risk of diseases.

There are liquid egg and egg substitute products on the market, such as Egg Beaters™. Their base ingredient is egg white and since they do not contain yolk, they are devoid of saturated fat. At the same time, they do not contain ω-3 PUFAs and, therefore, lack the potential health/nutraceutical and marketing benefits.

The objective of this study was to characterize proximate composition, fatty acid profile, lipid classes, fat oxidation, and antioxidant capacity of nutritionally enhanced cooked egg products as compared to mixed whole egg and Egg Beaters™. The nutritionally enhanced egg products were developed by replacing yolk with ω-3 PUFA-rich oils from flaxseed, menhaden, algae or krill.

MATERIALS AND METHODS

Development of experimental egg products

Fresh, quality eggs were purchased from a local chain grocery store. National brand liquid egg product (hereafter called 'Egg Beaters™') was also compared. The eggs and Egg Beaters™ were stored under refrigeration. The storage time did not exceed 3 days. The development of experimental egg products has been reported previously.[14,15] The experimental egg products consisted of fresh egg whites, alternative oil, freeze-dried egg whites, non-iodized salt (NaCl), and annatto. The egg whites were manually separated from whole eggs. The yolks were not used in the experiments. Care was taken to remove chalazae membranes from egg whites. Annatto (cheese coloring CM500A) was obtained from Grape and Granary (Akron, OH, USA). Annatto is a plant-derived yellow pigment with amphiphilic properties allowing for simultaneous water and lipid solubility. Therefore, annatto was used to obtain a color of the experimental egg products that would resemble the color of cooked mixed whole egg (i.e. egg yolk and white mixed together). The following alternative oils were used in the formulation of experimental egg products: flaxseed oil was obtained from Jedwards International, Inc. (Quincy, MA, USA); menhaden oil (Omega Pure 8042TE) was obtained from Omega Pure (Reedsville, VA, USA); two types of algae oil (DHAS and DHASCO) were obtained from Martek Biosciences (Columbia, MD, USA); and krill oil (4225F) was obtained from Enzymotec USA, Inc. (Springfield, NJ, USA).

The DHAS oil is a cheaper, but less concentrated source of algal DHA than DHASCO. In addition, the DHA oil contains soy lecithin and DHASCO does not. This is why both oils were used in the present study. The objective for the formulation of experimental egg products was to achieve moisture, crude protein, and total fat that would be similar ($P > 0.05$) to the proximate composition of mixed whole egg. An optimization spreadsheet was set up and preliminary experiments (data not shown) were conducted to meet this objective. The optimized composition of the experimental egg products containing all of the above alternative oils except the DHASCO algae oil was as follows:

- 430 mL of fresh egg whites
- 50 mL of alternative oil (four alternative oils listed above)
- 15 g freeze-dried egg whites
- 5 g non-iodized salt (NaCl)
- 750 µL annatto

The batter composition that included the DHASCO algae oil included 420 mL of fresh egg whites, 40 mL of DHASCO oil, 20 g of soybean lecithin (catalog number 03 376-250; Fisher Scientific, Fairlawn, NJ, USA), 15 g of freeze-dried egg whites, 5 g of non-iodized salt (NaCl), and 750 µL of annatto. It was found in the preliminary experiments (data not shown) that 20 g of soybean lecithin prevented phase separation and following cooking, the resultant gels were uniform. The DHAS oil contained soybean lecithin. The same fresh egg whites were used for freeze drying (VirTis Genesis 35SQ Super XL freeze-dryer; Virtis, Gardiner, NY, USA) as the fresh egg whites used in the formulation of experimental egg products. The freeze-dried egg whites were added in order to increase crude protein content in the experimental egg products so that it would be similar ($P > 0.05$) to mixed whole egg (i.e. egg yolk and white mixed together). Final volume was approximately 500 mL.

Mixing and cooking of experimental egg products, mixed whole egg, and Egg Beaters™

The 500 mL of experimental egg products, mixed whole egg (i.e. egg yolk and white), or Egg Beaters™ were mixed in a 1 L beaker. However, approximately 18 h prior to addition of the other ingredients, the 15 g of freeze-dried egg whites were added to the 430 mL of fresh egg whites (or 420 mL when the DHASCO algae oil was used) and held under refrigeration. Mixing was not used during these 18 h. It was determined in the preliminary experiments (data not shown) that this procedure allowed hydration of freeze-dried egg whites and following cooking the resultant gels were uniform. After 18 h of hydration, all other ingredients were added and the experimental egg products were mixed for 2 h at room temperature, while mixed whole egg and Egg Beaters™ for 1 h also at room temperature. The 1 L beaker with a standard 3-inch magnetic stir bar was placed on a mixing plate (Thermix Strirring Hot Plate Model 310T; Fisher Scientific). The mixing plate was set at speed 5 for experimental egg products, while speed 3 was used for mixed whole egg and Egg Beaters™. It was determined in the preliminary experiments (data not shown) that these mixing conditions prevented phase separation and following cooking, the resultant gels were uniform.

Following mixing, the experimental egg products, mixed whole egg, or Egg Beaters™ were poured into polycarbonate tubes (length, 18 cm; internal diameter, 1.90 cm; wall thickness, 0.635 cm) and cooked using a microwave oven. The tubes had polycarbonate screw caps at both ends that were sealed with standard o-rings. A standard 1100 W household microwave oven

(Model JES1139WL; GE Appliances, Louisville, KY, USA) was set at 50% power and the experimental egg products, mixed whole egg, or Egg Beaters™ were cooked for 60 s. Preliminary experiments (data not shown) showed that these settings were optimal for gelation of the experimental egg products, mixed whole egg, and Egg Beaters™, resulting in uniformly cooked gels without over- or under-cooking.

Proximate composition analysis of cooked gels

The total fat, crude protein, ash content, and moisture content were determined for cooked gel samples (i.e. experimental egg products, mixed whole egg, and Egg Beaters™) according to standard AOAC methods.[16] All proximate analyses are reported as mean values (± standard deviation) of at least three replicates and the mean values are expressed as g per 100 g of a cooked gel sample (dry and wet weight basis).

Determination of fatty acid profile with gas–liquid chromatography

Fatty acid profile was determined for cooked gel samples (i.e. experimental egg products and mixed whole egg) according to AOAC methods 965.49 and 996.06.[17] Fatty acid profile was not determined for Egg Beaters™ because they contained much less ($P < 0.05$) total fat (2.6 g 100 g^{-1}, dry basis) than all other samples (>40 g 100 g^{-1}, dry basis) (Table 1). Lipids were extracted by acid hydrolysis into ether and methylated to fatty acid methyl esters, which were measured quantitatively by using a capillary gas–liquid chromatograph (GLC) (Model 7890A equipped with a 7683B series injector; Agilent Technologies, Santa Clara, CA, USA) against an internal standard (triundecanoin, C11 : 0). Helium was used as the carrier gas with a 0.75 mL min^{-1} flow rate and a 200 : 1 split ratio. The initial temperature of 100 °C was held for 4 min and then increased to the final temperature of 240 °C at a heating ramp of 3 °C/min. The final temperature was held for 15 min. The injector and detector temperatures were 225 and 285 °C, respectively. The data are reported as mean values (± standard deviation) of at least three replicates and the mean values are expressed as % of a fatty acid in total fatty acids.

Determination of lipid classes with thin layer chromatography

Lipid classes of cooked gel samples (i.e. experimental egg products and mixed whole egg) were resolved with thin layer chromatography (TLC). Lipid classes were not identified for Egg Beaters™ because they contained much less total fat than all other samples (Table 1). Lipids were extracted from the cooked gel samples according to Bligh and Dyer.[18] The extracted lipids were dissolved in chloroform/methanol (1 : 1; v/v) and loaded onto TLC plates (Whatman K6F silica plates with 60 A pore sizes; P.J. Cobert Associates, St Louis, MO, USA). The TLC plates were developed using a hexane/ether/acetic acid solution (80 : 20:1.5, v/v/v) as a mobile phase. Plate images were captured using a PC-interfaced digital camera. Phospholipids (PLs) and triglycerides (TGs) were identified using triolein (Sigma-Aldrich, St Louis, MO, USA) and soybean lecithin standards (Fisher Scientific). Other lipid classes such as sterols (cholesterol), mono- and di-glycerides were not identified because the experimental egg products were developed using commercial oils. These oils contained various lipid derivatives such as antioxidants (e.g. astaxanthin in krill oil) as well as other additives (e.g. emulsifiers) and, therefore, were not of interest and would be difficult to properly identify with TLC. This is why lipid classes other than TGs and PLs were cumulatively named 'polar non-phospholipid class'. At least three replicate TLC plates were run per sample and a representative image of the three replicates is shown in Fig. 1.

Measurement of thiobarbituric acid reactive substances

Oxidative rancidity of cooked gel samples (i.e. experimental egg products and mixed whole egg) was measured by a 2-thiobarbituric acid reactive substances (TBARS) assay of malondialdehyde as previously described.[19–21] TBARS were not determined for Egg Beaters™ because they contained much less total fat than all other samples (Table 1). The absorbance was measured at 535 nm using a UV–visible spectrophotometer (model DU530; Beckman Instruments, Fullerton, CA, USA). The TBARS values were calculated based on molar absorptivity of malondialdehyde (156 000 M^{-1} cm^{-1}) at 535 nm. The TBARS values are reported as mean values (± standard deviation) of at least three replicates and the mean values are expressed as mg of malondialdehyde per kg of a cooked gel sample.

Table 1. Proximate analysis* (g 100 g^{-1} on a dry and a wet weight basis) of cooked gels

Constituent	Whole egg	Egg Beaters™	Flaxseed	Menhaden	DHAS	DHASCO	Krill
Total fat							
Dry basis	42.3 ± 2.6^a	2.6 ± 0.9^b	41.7 ± 2.5^a	40.7 ± 2.8^a	40.7 ± 2.7^a	41.5 ± 2.0^a	40.3 ± 8.7^a
Wet basis	11.3 ± 0.6^a	0.3 ± 0.2^b	11.2 ± 0.6^a	10.5 ± 0.7^a	10.9 ± 0.7^a	10.8 ± 0.5^a	10.7 ± 2.2^a
Crude protein							
Dry basis	51.5 ± 0.8^b	78.8 ± 0.7^a	51.9 ± 1.9^b	52.1 ± 1.3^b	52.2 ± 0.8^b	51.8 ± 0.3^b	51.8 ± 0.3^b
Wet basis	13.8 ± 0.2^a	9.9 ± 0.2^b	13.9 ± 0.5^a	13.5 ± 0.3^a	14.0 ± 0.2^a	13.5 ± 0.1^a	13.8 ± 0.1^a
Ash content							
Dry basis	3.7 ± 0.0^b	6.8 ± 0.3^a	6.6 ± 0.3^a	6.9 ± 0.3^a	6.8 ± 0.2^a	6.7 ± 0.3^a	6.8 ± 0.2^a
Wet basis	1.0 ± 0.0^b	0.9 ± 0.1^b	1.8 ± 0.1^a	1.8 ± 0.1^a	1.8 ± 0.1^a	1.7 ± 0.1^a	1.8 ± 0.1^a
Moisture content	73.3 ± 1.0^b	87.5 ± 0.7^a	73.2 ± 2.1^b	74.1 ± 1.2^b	73.2 ± 1.1^b	73.9 ± 1.2^b	73.4 ± 1.9^b

Proximate composition of cooked experimental egg products developed with addition of flaxseed, menhaden, algae (DHAS and DHASCO), and krill oils was compared to cooked mixed whole egg and Egg Beaters™.
* Data are given as mean values ± standard deviation ($n = 3$). Different letters within the same row indicate significant differences (Fisher's least significant difference, $P < 0.05$) between mean values.

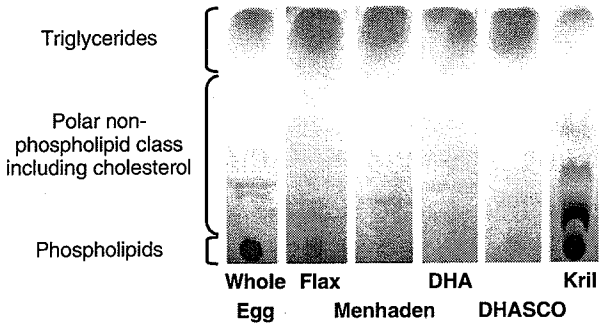

Figure 1. Lipid classes present in cooked gels. The lipids were extracted from cooked mixed whole egg as well as cooked experimental egg products developed with addition of flaxseed, menhaden, algae (DHAS and DHASCO), and krill oils. Lipid classes present in the extracted lipids were resolved with thin layer chromatography.

Measurement of peroxide value

The oxidative rancidity of cooked gel samples (i.e. experimental egg products and mixed whole egg) was also analyzed by determining peroxide value according to the AOAC method 965.33.[17] Peroxide value was not determined for Egg Beaters™ because they contained much less total fat than all other samples (Table 1). Lipids were extracted from the cooked gel samples and dissolved in acetic acid/chloroform solution (3 : 2, v/v). Titration was performed with sodium thiosulfate and a starch solution was used as an indicator. A blank titration was conducted without the sample. The peroxide value values are reported as mean values (± standard deviation) of at least three replicates and the mean values are expressed as milliequivalents of peroxide per kg of oil in a cooked gel sample.

Assessment of antioxidant capacity by determining ethoxyquin concentration with liquid chromatography

The concentration of ethoxyquin in cooked gel samples (i.e. experimental egg products and mixed whole egg) was determined according to AOAC method 996.13.[17] The ethoxyquin concentration was not determined for Egg Beaters™ because they contained much less total fat than all other samples (Table 1). The ethoxyquin concentration was used as an indicator of antioxidant capacity in the cooked gel samples. Ethoxyquin was extracted from gel samples with acetonitrile. The ethoxyquin concentration was determined by isocratic liquid chromatography (LC) equipped with fluorescence detector and LC column (250 × 4.6 mm i.d., C_{18} octadecylsilane, 5 μm spherical, and 100 μm pore size). The LC was calibrated and its linearity was established with ethoxyquin standard solutions. The LC was operating at 35 °C and a flow rate of 1.3 mL min^{-1}. The excitation and emission of the fluorescence detector were set at 360 and 432 nm, respectively. Ethoxyquin concentrations are reported as mean values (± standard deviation) of at least three replicates and the mean values are expressed as μg of ethoxyquin per gram of a cooked gel sample (dry weight basis).

Statistics

The experiments were performed in triplicate ($n = 3$). In each triplicate, at least three measurements were performed. Data were subjected to one-way analysis of variance (ANOVA). A significant difference was used at 0.05 probability level and differences between treatments were tested using the Fisher's least significant difference test.[22] The statistical analysis was performed using R version 2.0.9 software.[23] The data are reported as mean values ± standard deviation (SD).

RESULTS AND DISCUSSION

Proximate composition analysis of cooked egg gels

The proximate composition of cooked experimental egg products was compared to cooked mixed whole egg and cooked Egg Beaters™ (Table 1). The moisture content, crude protein, and total fat of experimental egg products matched ($P > 0.05$) mixed whole egg. This result was expected since experimental egg products were formulated to match the proximate composition of mixed whole egg.

Fat and protein are essential dietary macronutrients and mixed whole egg contains nearly equal amounts of both (Table 1). One whole egg contains approximately 6 g of protein, over half of which is in the white; and 5 g of fat, all contained within the yolk. However, egg white is approximately two thirds of the whole egg volume in addition to having much higher moisture content than the yolk. Therefore, removing the yolk from experimental egg products in the present study not only eliminated the egg total fat including saturated fat, but also reduced the protein content. Thus, freeze-dried egg whites were added to experimental egg products to match the crude protein content of mixed whole egg. The fat of experimental egg products was solely derived from ω-3-rich oils and it was comparable ($P > 0.05$) to the fat content of mixed whole egg (Table 1). Although mixed whole egg does contain some ω-3 PUFAs, it also contains considerable amount of saturated fatty acids (Fig. 2B, Table 2), which are an established dietary risk factor known to increase CVD when consumed in excess. Thus, during the development of experimental egg products the fat in whole egg was replaced with heart-healthy ω-3 PUFAs oils from alternative sources (flaxseed, menhaden, algae and krill).

The USDA reported proximate composition values of whole egg as 75.8, 47.4, 41.0 and 3.7 g 100 g^{-1} for moisture, crude protein, total lipid, and ash, respectively.[24] The proximate composition of experimental egg products developed in this study and the mixed whole egg used as a control were similar to the composition data reported by the USDA.[24] Egg Beaters™ had higher ($P < 0.05$) crude protein (dry basis) and moisture content as well as considerably lower ($P < 0.05$) total fat (dry basis) than experimental egg products and mixed whole egg. However, the crude protein on wet basis (often referred to as 'as-is' basis) for Egg Beaters™ was 9.9 g 100 g^{-1} (Table 1). On a wet basis, therefore, Egg Beaters™ contained less ($P < 0.05$) crude protein than experimental egg products and mixed whole egg. This is because the base ingredient for Egg Beaters™ are egg whites that have considerably higher moisture content when compared to mixed whole egg. The ash content (dry basis) of experimental egg products was similar ($P > 0.05$) to Egg Beaters™ and higher ($P < 0.05$) than in mixed whole egg. A possible explanation is that the addition of the non-iodized salt and freeze-dried egg whites may have contributed to slightly higher ash content in experimental egg products.

Fat characterization: Fatty acid profile and lipid classes

Although the proximate analysis proved that the total fat content of experimental egg products matched ($P > 0.05$) mixed whole egg (Table 1), the fatty acid profile and thin layer chromatography (TLC) revealed considerable differences in fatty acid composition and lipid classes, respectively (Fig. 1, Fig. 2, Table 2).

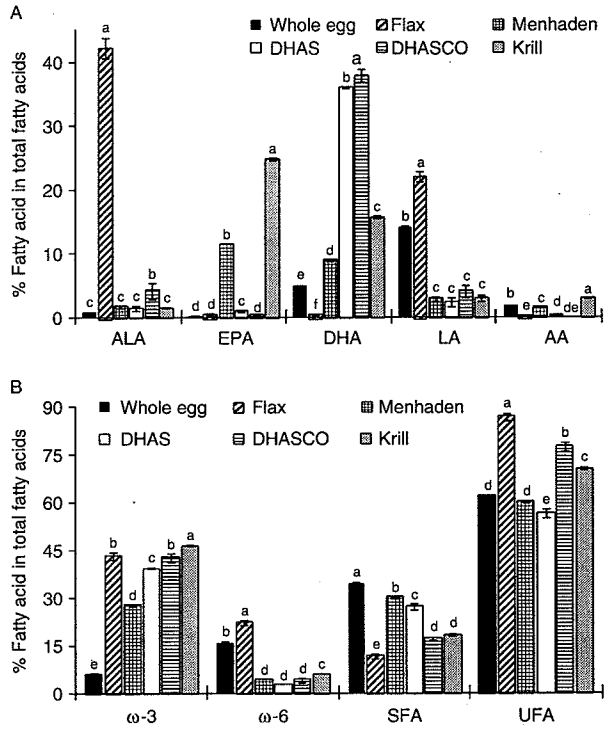

Figure 2. Major fatty acids (% fatty acid in total fatty acid) of cooked gels. The fatty acid composition of cooked experimental egg products developed with addition of flaxseed, menhaden, algae (DHAS and DHASCO), and krill oils was compared to cooked mixed whole egg. Data are given as mean values ± SD ($n = 3$). Different letters on data bars indicate significant differences (Fisher's least significant difference, $P < 0.05$) between mean values within a fatty acid. ALA, linolenic (18 : 3ω3); EPA, eicosapentaenoic (20 : 5ω3); DHA, docosahexaenoic (22 : 6ω3); LA, linoleic (18 : 2ω6); and AA, arachidonic (AA, 20 : 4ω6); ω3, total ω-3 fatty acids; ω6, total ω-6 fatty acids; SFA, total saturated fatty acids; UFA, total unsaturated fatty acids.

The α-linolenic (ALA, 18 : 3ω-3), eicosapentaenoic (EPA, 20 : 5ω-3), and docosahexaenoic acids (DHA, 22 : 6ω-3) were the main ω-3 PUFAs, whereas linoleic acid (18 : 2ω-6) and arachidonic acid (20 : 4ω-6) were the main ω-6 PUFAs detected in cooked experimental egg products and mixed whole egg (Fig. 2A). A current status of clinically proven health benefits associated with specific ω-3 PUFAs has been recently reviewed.[5] Experimental egg products contained much more ($P < 0.05$) ω-3 PUFAs (27.6–46.5% of total fatty acids) and less ($P < 0.05$) ω-6 PUFAs (2.7–5.8% of total fatty acids with the exception of experimental eggs with flaxseed oil that had 22.1%) than mixed whole egg (ω-3 PUFAs, 5.9%; ω-6 PUFAs, 15.6%) (Fig. 2B). Furthermore, mixed whole egg contained higher ($P < 0.05$) levels of saturated fatty acids than experimental egg products (whole egg, 34.4% vs. experimental eggs, 11.5–29.9%).

ALA is the major fatty acid in flaxseed oil.[25] Thus, experimental egg products developed with flaxseed oil contained the highest ($P < 0.05$) amount of ALA (42.2% of total fatty acids) compared to the other experimental egg products (DHAS, 1.4%; krill, 1.4%; menhaden, 1.6%; DHASCO, 4.1%) as well as mixed whole egg (0.8%) (Fig. 2A). It has been shown that incorporation of flaxseed into hen's feed increases ALA content up to 11% of total fatty acids, which is almost four times less than in the present study.[9,11] Experimental egg products developed with menhaden or krill oil had the greatest ($P < 0.05$) level of EPA, at 11.3% and 24.7%, respectively; whereas all other eggs had EPA at below 1%. This is consistent with the fact that seafood-derived oils are abundant in EPA and DHA.[26] The DHA was detected at the highest ($P < 0.05$) levels in experimental egg products developed with both types of algae oil, DHAS and DHASCO at 35.9% and 37.8% of total fatty acids, respectively; and were much greater ($P < 0.05$) than mixed whole egg and other experimental egg products developed with ω-3-rich oils (krill, 15.6% DHA of total fatty acids; menhaden, 8.8%; whole egg, 4.8%; and flaxseed, 0.3%). Single-cell marine micro-algae such as *Crypthecodinium cohnii* bio-synthesize lipids very efficiently. This particular alga accumulates DHA at 40% of algal biomass (dry basis) and DHA is the main ω-3 PUFA bio-synthesized.[27] Both oils used in the present study, DHAS and DHASCO, were extracted from *C. cohnii*. This is why experimental egg products developed with both types of algae oil had the highest DHA content. Krill feed on single-cell marine micro-algae and, therefore, are a rich source of DHA.[28] This is why experimental egg products developed with krill oil also contained high level of DHA.

Experimental egg products developed with flaxseed had the highest ($P < 0.05$) content of linoleic acid followed by whole egg. As a result, these samples had the highest ($P < 0.05$) content of ω-6 PUFAs. Experimental egg products developed with krill oil had the highest ($P < 0.05$) content of arachidonic acid; however, the arachidonic acid content in all of the samples was relatively low. Arachidonic acid is a major constituent of the cell membrane phospholipids and krill oil contains high levels of phospholipids (Fig. 1); values as high as 54–58% have been reported.[29] As a consequence of the differences between fatty acid composition of fat in mixed whole egg and experimental egg products, the ratio of ω-6/ω-3 fatty acids was much higher ($P < 0.05$) in mixed whole egg (2.64) than experimental egg products (0.07–0.51) (Table 2). However, it is important to note that besides the differences in ω-6/ω-3 fatty acids, the ratio of unsaturated fatty acids/saturated fatty acids in mixed whole egg (1.81) was similar ($P > 0.05$) to experimental egg products developed with menhaden (2.01) and DHAS (2.09) oils, but lower ($P < 0.05$) than with krill (3.86) and flaxseed (7.62) oils. Milinsk *et al.*[10] demonstrated that fatty

Table 2. The ratios* of omega-6/omega-3 fatty acids (ω-6/ω-3) and unsaturated/saturated fatty acids (UFAs/SFAs) determined for cooked gels

Fatty acids	Whole egg	Flaxseed	Menhaden	DHAS	DHASCO	Krill
ω-6/ω-3	2.64 ± 0.226^{a}	0.51 ± 0.005^{b}	0.14 ± 0.001^{c}	0.07 ± 0.002^{c}	0.09 ± 0.016^{c}	0.12 ± 0.01^{c}
UFAs/SFAs	1.81 ± 0.024^{c}	7.62 ± 0.667^{a}	2.01 ± 0.014^{c}	2.09 ± 0.118^{c}	4.56 ± 0.210^{b}	3.86 ± 0.052^{b}

The ω-6/ω-3 and UFAs/SFAs ratios of cooked experimental egg products developed with addition of flaxseed, menhaden, algae (DHAS and DHASCO), and krill oils were compared to cooked mixed whole egg.
* Data are given as mean values ± standard deviation ($n = 3$). Different letters within the same row indicate significant differences (Fisher's least significant difference, $P < 0.05$) between mean values.

acid composition of whole egg can be modified by altering the lipid source of hen's diet; however, it was possible only to a limited degree. The modification of the egg fatty acid composition is constrained by the animal physiology unlike the approach presented in this study.

Figure 1 shows lipid classes present in the cooked mixed whole egg and experimental egg products as resolved by TLC. Although all of the egg samples contained TGs and PLs, experimental egg products developed with krill oil and mixed whole egg had the highest content of PLs followed by experimental egg products with flaxseed; while experimental egg products with krill oil had the least TGs. TGs were most abundant in experimental egg products developed with flaxseed, algae, and menhaden oils; while mixed whole egg had slightly fewer TGs.

Egg yolk is naturally rich in PLs (lecithin); and therefore, yolk or whole egg has excellent emulsification properties. Krill oil also contains large amounts of PLs, phosphatidylcholine and phosphatidylethanolamine. As evidenced by the darker PL stain for experimental egg products with krill oil, krill oil contained slightly more PLs than mixed whole egg. Therefore, krill oil likely has very good emulsification properties. Krill oil is used as a high-value dietary supplement with potential nutraceutical benefits such as protection against CVD and management of premenstrual syndrome.[30] Some of these benefits have been attributed to the ω-3 PUFAs in krill oil being associated with PLs; unlike in fish oil such as menhaden where ω-3 PUFAs are associated with TGs. The stability of krill oil has been attributed to its antioxidants, in particular astaxanthin.[26] The TLC image for mixed whole egg shows a dark band in the 'polar non-phospholipid' class, which likely corresponds to cholesterol content. This band is absent in experimental egg products. One egg contains about 200 mg of cholesterol and so far it has not been shown to be possible to reduce cholesterol content in egg by a dietary modification of hen's feed.[10,31]

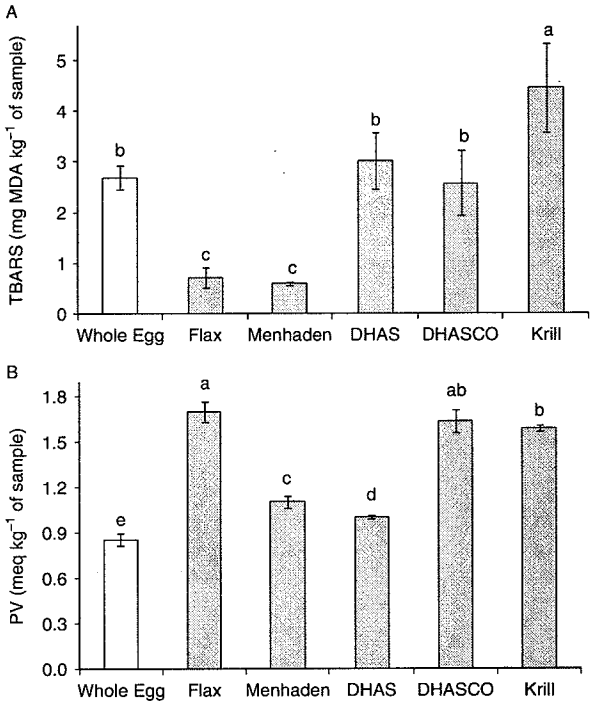

Figure 3. Lipid oxidation of cooked experimental egg products developed with addition of flaxseed, menhaden, algae (DHAS and DHASCO), and krill oils was compared to cooked mixed whole egg. Data are given as mean values ± SD (n = 3). Different letters on data bars indicate significant differences (Fisher's least significant difference, $P < 0.05$) between mean values. (A) Lipid oxidation assessed with 2-thiobarbituric acid reactive substances (TBARS). (B) Lipid oxidation assessed with peroxide value.

Fat oxidation: TBARS, peroxide value and antioxidant capacity

The primary products of lipid oxidation, peroxides are estimated by determining peroxide value. Peroxides are generated at relatively initial stages of lipid oxidation in foods as opposed to malonaldehyde, which is a group of secondary lipid oxidation products measured with TBARS assay.[32] Thus, the best understanding of lipid oxidation in food is likely accomplished to employing these two analyses.

Figure 3A shows the results of TBARS analysis of the cooked experimental egg products and cooked mixed whole egg. Experimental egg products developed with krill oil were the most ($P < 0.05$) susceptible to oxidation, which can be explained by their fatty acid profile (Fig. 2). These eggs products contained the highest ($P < 0.05$) level of ω-3 PUFAs as EPA and DHA (Fig. 2), making them more prone to oxidation than other experimental egg products. Experimental egg products with both types of algae oils had TBARS values comparable ($P > 0.05$) to mixed whole egg, but higher ($P < 0.05$) than experimental egg products with flaxseed and menhaden oils. Both types of algae oils contained added antioxidants (mixed tocopherols, rosemary extract, etc.). This is probably why, despite higher content of ω-3 PUFAs in experimental eggs developed with algae oils, they had similar ($P > 0.05$) TBARS to whole eggs. Experimental egg products with algae oils had slightly lower content of ω-3 PUFAs than those with krill oil (Fig. 2B), which may partially account for their lower ($P < 0.05$) TBARS values. Experimental egg products with flaxseed and menhaden oils had the lowest ($P < 0.05$) TBARS values from all of the samples. Experimental egg products with flaxseed oil had generally comparable levels of ω-3 PUFAs to those with algae oils (Fig. 2B), but were composed of ALA instead of DHA (Fig. 2A). ALA has a lower unsaturation level than DHA, which makes ALA less susceptible to oxidation. Experimental egg products with menhaden had the lowest content of ω-3 PUFAs compared to all experimental egg products, which may explain their lower TBARS value. Experimental egg products had peroxide values higher ($P < 0.05$) than mixed whole egg (0.9 meq kg^{-1}) (Fig. 3B). The peroxide values for experimental egg products were between 1.0 and 1.7 meq kg^{-1}. Peroxide values of 1–5 meq kg^{-1} indicate low levels of lipid oxidation.[32]

Four of the five oils used in the present study were of aquatic origin (menhaden, two types of algae, and krill). All of these oils are commercially available sources of ω-3 PUFAs and therefore, likely to contain some antioxidants. Various antioxidants are often added to commercially available ω-3-fortified food products to inhibit lipid oxidation. Ethoxyquin is a common primary antioxidant used to protect unsaturated hydrocarbon systems. This antioxidant is commonly found in seafood-derived oils and fishmeal, but also other oils, fats and meals.[33] In the present study ethoxyquin concentration was determined as an indicator of antioxidant capacity. Experimental egg products developed with menhaden oil had the highest ($P < 0.05$) concentration of ethoxyquin (0.74 μg g^{-1}) followed by those with flaxseed oil (0.41 μg g^{-1}) (Fig. 4). This data correlates well with the lowest ($P < 0.05$) oxidation of experimental egg products developed with these two oils as assessed by TBARS assay (Fig. 3A). Experimental egg products with DHAS and DHASCO oils had minimal levels of

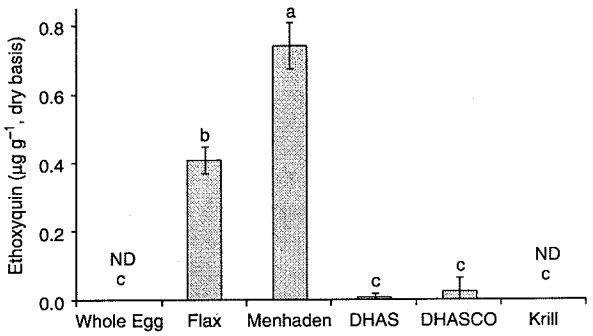

Figure 4. Antioxidant capacity was assessed by determining ethoxyquin concentration. The ethoxyquin concentration of cooked experimental egg products developed with addition of flaxseed, menhaden, algae (DHAS and DHASCO), and krill oils was compared to cooked mixed whole egg. Data are given as mean values ± SD ($n = 3$). Different letters on data bars indicate significant differences (Fisher's least significant difference, $P < 0.05$) between mean values. ND, not detected.

ethoxyquin. This antioxidant was not detected in mixed whole eggs or experimental egg products developed with krill oil. However, krill oil naturally contains an antioxidant, astaxanthin, that extracts with lipids and inhibits oil oxidation.[26] In the present study, the presence of astaxanthin was indicated by a halo stain in the TLC image of experimental egg products with krill oil above the PL stain in the 'polar non-phospholipid class' (Fig. 1).

CONCLUSIONS

Substitution of the egg yolk with ω-3 PUFA-rich oils (flaxseed, menhaden, algae or krill oil) resulted in the development of nutritionally enhanced cooked egg products. Although ω-3 PUFA-enhanced eggs are typically developed through a dietary modification of chicken feed, the approach presented in the present study offers development of nutritionally enhanced cooked egg product via processing. This approach allowed achieving contents of health beneficial ω-3 PUFAs in the egg products beyond levels that have been demonstrated possible so far by dietary modification of chicken feed. The experimental egg products had similar composition to mixed whole egg in terms of total fat, crude protein, and moisture content. However, depending on the ω-3 PUFA-rich oil used, the experimental egg products had much higher content of ω-3 PUFAs than mixed whole egg, in particular health beneficial ALA, EPA, and DHA.

Based on the present study, a ready-to-eat, microwave-cooked and vacuum-packed egg stick similar to cheese stick can be developed. However, egg proteins are regarded as a reference protein and have higher nutritional value compared to milk protein, casein. Not only are the egg proteins of higher nutritional value, but the proposed egg stick has a healthier fat profile (more total ω-3, unsaturated fatty acid, ALA, EPA and DHA; while less saturated fatty acid, cholesterol, and lower ω-6/ω-3) than cheese stick. Therefore, the proposed egg stick would have nutraceutical/marketing advantage. Although the results of this study point towards the potential for a novel food product, sensory tests and storage stability study are recommended.

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Appendix G

O'Doherty et al., "Role of Luminal Lecithin in Intestinal Fat Absorption" Lipids 8: 249-55 (1973)

Role of Luminal Lecithin in Intestinal Fat Absorption

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ABSTRACT

The effects of biliary lecithin on fat absorption were studied in 1 day bile fistula rats fed micellar solutions of bile salt, monoglyceride and radioactive free fatty acids. By electron microscopy and measurement of uptake of radioactivity into liver and adipose tissue, it was shown that in the absence of bile lecithin there was significant impairment of fat release from mucosa. Fat clearance was effected by the feeding of phosphatidyl choline or choline, but not phosphatidyl ethanolamine, inositol or cholesterol. In the absence of luminal choline there was a decrease in incorporation of radioactive leucine into mucosal protein. It is concluded that biliary and dietary lecithin or choline play an important role in triglyceride transport out of intestinal mucosa, by providing surfactant lecithin for the chylomicron envelope and by supporting mucosal protein biosynthesis.

INTRODUCTION

Previous studies from our laboratory have suggested that the synthesis and release of chylomicrons from isolated mucosal cells and everted sacs (1) is dependent on both protein and phospholipid biosynthesis. In the present experiments we have tested the role of lecithin synthesis in the formation and release of chylomicrons in vivo by interfering with the supply of the precursors of phosphatidyl choline that reach the intestinal mucosa from the lumen. For this purpose we compared uptake and clearance of fatty meals in normal and bile fistula rats supplemented with various phospholipids, choline and inositol. The results show that deprivation of dietary or biliary lecithin or choline leads to significant impairment of fat absorption. Since protein biosynthesis is also decreased under these conditions, it is not possible at the present time to dissociate the role of lecithin and protein biosynthesis in chylomicron formation and release. A preliminary account of this work has been presented (2).

MATERIALS AND METHODS

Materials

9,10[^{3}H]-Palmitic acid (specific activity 200/mc/mM) and 1[^{14}C]-leucine (specific activity 20 mc/mM) were obtained from New England Nuclear (Boston, Mass.). Monoolein, taurocholic acid and choline were purchased from Eastman Organic Chemicals (Rochester, N.Y.). Oleic and palmitic acids were obtained from Nutritional Biochemicals Corp. (Cleveland, Ohio). Cholesterol was purchased from the British Drug Houses (Toronto); and inositol from the Sigma Chemical Co., (St. Louis, Mo.). Phosphatidyl choline (3) and phosphatidyl ethanolamine (4) were prepared from egg yolk.

Animal Experimentation

Male Wistar rats (300 g), which had been maintained on laboratory chow, were fasted 24 hr prior to experimentation. Bile fistula animals were prepared as follows. The animals were opened under ether anaesthesia and the bile duct was cannulated by inserting a thin polyethylene tube of suitable diameter. After passing the distal end of the cannula through a wound in the back of the animal, the abdomen was closed by suturing. Sham-operated animals were used as controls. After 1 day, the animals were fed by stomach tube, a micellar solution containing 800 mg of monoolein and free fatty acids (equimolar palmitic and oleic), in a 1:2 molar ratio. The monoolein and free fatty acids were prepared as a micellar solution by sonication with 1% taurocholic acid. For the radioactive experiments, 10 μc 9,10[^{3}H]-palmitate was added.

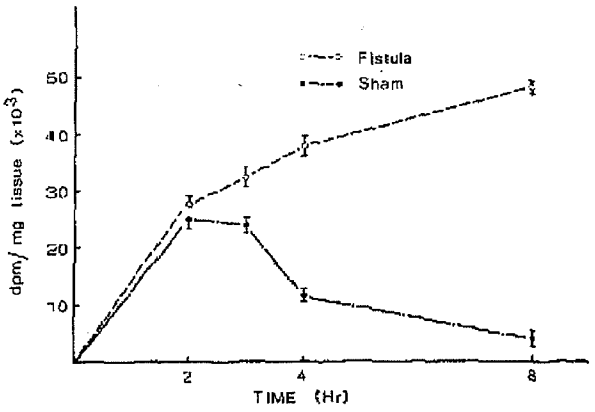

FIG. 1. Time course for rate of uptake and clearance of labeled lipid by rat jejuna. A suitable length of upper jejunum (6 in.) was taken for determination of radioactivity. Each point represents mean of three rats.

In Vitro Incubations

Everted sacs were prepared (5) from the jejuna of both fasted and fat-fed sham-operated and bile-fistula rats. The animals were sacrificed under ether anaesthesia, the jejuna excised, flushed with ice cold Krebs bicarbonate buffer, pH 7.4, everted and the mucosal side washed free of the luminal contents. Suitable lengths (5-10 cm) of the jejuna were allowed to fill with buffer, the ends tied and the sacs submitted to incubations as described in Table IV.

Assay of Protein Biosynthesis

Protein biosynthesis was assessed by incubating the everted sacs with radioactive leucine under the conditions described in Table IV. The reactions were stopped by the addition of 5 ml 10% trichloracetic acid containing 10 mg unlabeled L-leucine per milligram, and the suspensions homogenized. The protein precipitates were washed, extracted, and the radioactivity counted (6). The protein concentration was determined by the method of Hübscher et al. (7).

Lipid Analysis

Total lipid extracts were prepared according to the method of Folch et al. (8). The lipid extracts were resolved into fractions corresponding to triglycerides, free fatty acids, diglycerides, monoglycerides and phospholipids by thin layer chromatography on Silica Gel H (Merck & Co.) using heptane-isopropyl ether-acetic acid 60:40:3 v/v/v. The bands were detected by spraying with 0.05% 2,7-dichlorofluorescein in 50% methanol, and were visualized under UV light. Appropriate standards were used in all chromatographic separations. Lipid samples were extracted from gel scrapings with chloroform-methanol 2:1 v/v by partitioning with water at one-fifth the volume, including that of the sample. For measurement of radioactivity, fluorescein was removed by adding 4M NH_4OH as one-tenth the water volume. The chloroform layer was filtered through anhydrous Na_2SO_4 and a cotton plug into a scintillation vial, and dried at 40 C under nitrogen. To the dried residue was added 10 ml scintillation fluid, 0.5% 2,5-diphenyloxazole and 0.3% 1,4-bis(2-[5-phenyloxazole]) benzene (Amersham-Searle) in toluene. Radioactivity was measured in a Nuclear Chicago liquid-scintillation counter. Correction for quenching was made by the channels ratio method (9). The counting efficiency was 40%.

Electron Microscopy

Electron microscopy was performed as described previously (10).

RESULTS

Uptake and Clearance of Micellar Lipid in Normal and Fistula Rats

Figure 1 shows the rate of uptake and clearance of the fed radioactive lipid mixture by the jejuna of sham-operated and bile fistula rats. Almost all of the mucosal radioactivity was in the neutral fraction, less than 5% being found as free fatty acid. In the sham-operated animals a maximal concentration of label in the gut wall was reached ca. 2-3 hr after introducing the meal by stomach tube, after which it decreased and approached prefeeding levels by the end of a 6-8 hr period. This course of events corresponds to that noted by Gallagher et al. (11), who monitored the appearance of radioactivity in the lymph of normal rats after feeding a micellar solution of radioactive triolein. They found that the major part of the fat appeared in the lymph in the first 4 hr. In the bile fistula rats the initial uptake of radioactivity followed the course noted for the sham-operated controls. Instead of subsiding after the 3 hr period, the radioactivity in these animals continued to rise, although at a slower rate, and at the end of the 8 hr experimental period had reached twice the peak level of the intact rats. These results are also consistent with those of Gallagher et al. (11), who observed an abnormally low and delayed recovery of fat in the thoracic duct lymph of bile fistula rats. Clear-cut differences in fat absorption between control and bile fistula animals were also found recently by Rampone (12), who noted that the fistula group retained more of the label in the gut wall after 8 hr than the normal group and that there was less lipid recovered in the lymph.

Table I shows the distribution of radioactivity among the neutral lipid, free fatty acid and phospholipid fractions recovered from the gut wall during the course of the experiments. At no time did the free fatty acid level exceed 5% in the normal animals, which suggested that esterification of the monoglyceride was not impaired. In the fistual animals, the proportion of the free fatty acid was much higher. The highest amount of radioactivity in the free fatty acid fraction occurred in the bile fistula rats sacrificed at the 8 hr time interval. There was, however, no comparable increase in the level of monoglyceride at 8 hr. Dietschy (13) has claimed that mucosal triglyceride synthesis is normal in bile fistula rats, but this claim is based on rates of incorporation of fatty acid by tissue slices and may not reflect the present circumstances.

The impairment of clearance of mucosal fat by the bile fistula rat is further documented in

TABLE I

Per Cent Distribution of Radioactivity in Mucosal Lipids[a]

	Triglycerides		Diglycerides		Monoglycerides		Free Fatty Acids		Phospholipids	
Time, hr	Sham	Fistula	Sham	Fistula	Sham	Fistula	Sham	Fistula	Sham	Fistula
2	72 ± 4	69 ± 3	8 ± 2	11 ± 2	2 ± 0.5	3 ± 1	5 ± 1	11 ± 3	13 ± 2	6 ± 3
4	75 ± 3	72 ± 2	10 ± 1	9 ± 2	1 ± 0.5	2 ± 0.5	4 ± 2	13 ± 5	10 ± 2	4 ± 2
8	71 ± 4	63 ± 1	11 ± 3	14 ± 3	2 ± 1	1 ± 0.5	4 ± 2	18 ± 6	12 ± 3	4 ± 2

[a]Each figure represents the mean ±SEM of three animals. In each case a uniform portion of the jejunum (6 in.) was taken for determination.

Table II, which compares the uptake of radioactivity by adipose tissue and liver in the control and bile fistula animals. Although the radioactivities measured per gram of tissue do not allow the calculation of the actual amounts of fat absorbed by the two types of animal preparations, there remains little doubt that the bile fistula animal has suffered a severe impairment in its ability to produce or release chylomicrons, which could then be cleared by the liver and adipose tissue in the normal manner.

The above observations are supported by electron micrographic evidence. Micrographs prepared from the intestinal mucosa of a normal and bile fistula rat 2 hr after administration of the experimental meal, showed that both preparations contained large numbers of lipid particles, and there was little difference in the character of these particles between the two preparations. Clearly absent from the fistula preparations, however, were the extensive accretions of chylomicrons seen in the intercellular spaces of the mucosa of the control animal. Electron micrographs of the normal and the fistula rat 5 hr after the experimental meal showed that the normal animal had cleared most of the fat out of the mucosal cells by this time, as indicated by the absence of particles from the cell and chylomicrons from the intercellular spaces. There was little change in the appearance of the electron micrographs between the 2nd and 5th hr following the meal in the bile fistula animals, as would be expected from the analytical data presented above.

Effect of Phospholipids on Clearance of Mucosal Fat

In order to restore the mucosal formation and release of chylomicrons in the bile fistula rat, the experimental meals were supplemented with various bile lipid components or their precursors in amounts comparable to those supplied by normal bile flow over a 24 hr period. Since the experimental meals already included bile salts, which had no effect on the chylomicron release at least, this aspect of the bile composition was not further examined. Table III shows the effect of addition of egg yolk lecithin (50 mg) or choline (5 mg) on the amount of dietary fat retained by the mucosa of the bile fistula rat. For this comparison, total lipid extracts were prepared from the upper 6 in. of the jejunum 5 hr after the meal. In both instances the obtained clearance approached that noted for the sham-operated animals. Electron micrographs of the intestinal mucosa of the bile fistula rats that received lecithin or choline along with their fat meals, and were prepared 5 hr after the feeding, showed that there was an essentially complete disappearance of the fat particles from the mucosal cells.

TABLE II

Uptake of Radioactivity in Extrajejunal Tissues by Sham and Bile Fistula Rats[a]

	←dpm/g Liver→		←dpm/g Adipose tissue→	
Time, hr	Sham	Fistula	Sham	Fistula
2	12,200 ±1,900	960 ±400	1,300 ± 180	170 ±50
4	15,300 ±2,200	1,280 ±340	2,260 ± 340	190 ±40
8	16,100 ±2,300	1,310 ±400	3,180 ± 310	210 ±60

[a]Values are means ± SEM from six rats.

TABLE III

Fat in Jejunum after 5 Hours[a]

Preparation	Fat, mg
Sham	91 ± 18
Fistula	396 ± 32
Fistula + PC	82 ± 17
Fistula + choline	87 ± 22

[a]Each figure represents the mean ±SEM of six rats. A uniform portion (6 in.) of the upper jejunum was taken for determinations.

Figure 2 shows that inclusion of either lecithin or choline in the experimental meal led to a rapid appearance of radioactive palmitate in the adipose tissue and liver of the fistula rats, which approached the rates of uptake noted for the sham-operated animals. It is noteworthy that supplementation of the test meal with lecithin or free choline also led to a slightly increased uptake of radioactivity by the liver and adipose tissue of the sham-operated animals. This suggests that fat absorption may not normally proceed at maximum rates, unless dietary choline or lecithin is present. These observations suggest that lecithin, or at least some compound of choline, is intimately involved in chylomicron formation and release, presumably as a component of the lipoprotein envelope. This conclusion is in agreement with previous studies, which have established that much of both dietary and biliary lecithin is recovered in the chylomicron membrane during fat absorption.

The inclusion of egg yolk phosphatidyl ethanolamine (50 mg), cholesterol (1%) or inositol (5 mg) in the test meal had no effect on the clearance of fat from the intestine of the bile fistula rat (Fig. 3). This is effectively illustrated by the negligible amounts of radioactivity detected in the liver and adipose tissue of the fistula animals over the 8 hr examination period. This impression was supported by electron micrographs, which revealed fat laden cells in the intestinal mucosa of all the fistula animals 5 hr after feeding the above experimental meals. The addition of phosphatidyl ethanolamine, cholesterol and inositol to the meals of the sham-operated animals also failed to show any enhancement of the uptake of radioactivity by the liver or adipose tissue. The lack of promotion of mucosal clearance by phosphatidyl ethanolamine would be expected, if the effect is specific for lecithin, since it has been already reported (14) that the intestinal mucosa lacks the ability to convert phosphatidyl ethanolamine into phosphatidyl choline via methylation. The lack of effect of inositol on the mucosal clearance of lipid is in contrast to its effect in liver, where inositol is known to act as a lipotropic agent (15). It is possible that the amounts of cholesterol required for chylomicron formation and secretion are probably already available to the intestine, since it has been reported that cholesterogenesis increases eight-fold in biliary diversion (13). In any event, the amounts of phosphatidyl ethanolamine, phosphatidyl inositol and cholesterol found in the chylomicron membrane are rather small in comparison to the content of lecithin.

Effect of Phospholipids on Biosynthesis of Mucosal Protein

Table IV gives the results of the incorporation of radioactive leucine into the mucosal proteins of everted sacs from fasted and fat-fed normal and bile fistula animals. It is seen that the specific activities of the mucosal protein are ca. 30% lower in the fasted bile fistula rats, and that this difference increases to ca. 44%, when comparisons are made in the fed animals. It is possible that the synthesis of the secretory lipoproteins is inhibited even more completely than the analysis of the specific activities of the total protein would indicate. This could not be

TABLE IV

Effect of Phospholipids on Biosynthesis of Mucosal Proteins[a]

Preparation	Fasted animal	Fed animal
Sham	5,180 ± 340	14,870 ± 990
Sham + PC	6,330 ± 780	18,680 ± 1,240
Sham + Choline	6,690 ± 810	18,190 ± 1,380
Fistula	2,670 ± 310	9,260 ± 640
Fistula + PC	6,750 ± 840	17,230 ± 1,760
Fistula + Choline	7,190 ± 680	18,470 ± 1,590

[a]Everted sacs were prepared from jejuna as described in Methods. Incubation flasks contained everted sacs in Krebs-bicarbonate buffer, pH 7.4 plus ^{14}C-leucine (2×10^6 dpm). Reactions were carried out for 1 hr at 37 C in a Dubnoff metabolic shaker. Lecithin (50 mg) and choline (5 mg) were fed to animals as before and animals then sacrificed after 3 hr. Results represent mean of three experiments.

immediately ascertained, because the chylomicron proteins cannot be readily isolated in the absence of significant chylomicron release. The above results suggest that synthesis of protein in the normal fed animals is increased over that seen in the normal fasted animal and that the bile fistula preparation is unable to supply the increased needs for protein during fat absorption, when either choline or lecithin is lacking in the diet or in the bile. Addition of lecithin or choline resulted in a restoration of the leucine incorporation to the levels noted in the sham-operated animals. Furthermore, the effect was more pronounced in the fistula animals that had received the experimental fat meal than in the fasting animals.

The latter experiments suggest that the effect of lecithin or choline upon chylomicron formation and release is at least partially dependent upon an effective synthesis of secretory or membrane proteins, which accompany the fat globules as they leave the cell. This interpretation of the results is in agreement with the findings of Mookerjea (16), who claimed that the triglyceride accumulation in the livers of choline-deficient rats is due to an impairment in the biosynthesis of the lipoproteins responsible for the transport of triglycerides out of the liver. Choline supplementation brought about an immediate release of the block in protein synthesis and secretion.

DISCUSSION

From the results obtained, by both electron microscopy and measurement of uptake of radioactivity by extrajejunal tissues, it is obvious that the absence of both dietary and biliary lecithin results in significant impairment of fat clearance from rat intestinal mucosa. Addition of either lecithin or choline caused a release of fat at a rate comparable to that of the control animals. To our knowledge, this is the first report that demonstrates a role for biliary lecithin in fat absorption.

A function for lecithin in fat absorption was proposed in earlier studies. Sinclair (17) first suggested, in 1929, that a phospholipid was an obligatory intermediate in the absorption of fat. Frazer (18,19) claimed that, in the absence of dietary choline, rats fed triglyceride accumulated fat in the intestinal mucosa, and that addition of dietary choline markedly enhanced fat clearance. Later, Augur et al. (20) reported that lecithin increased the digestibility and absorption of fat in man, while Tidwell (21) concluded that by counting chylomicra he could demonstrate a small increase in the rate of absorption when free choline is added to a

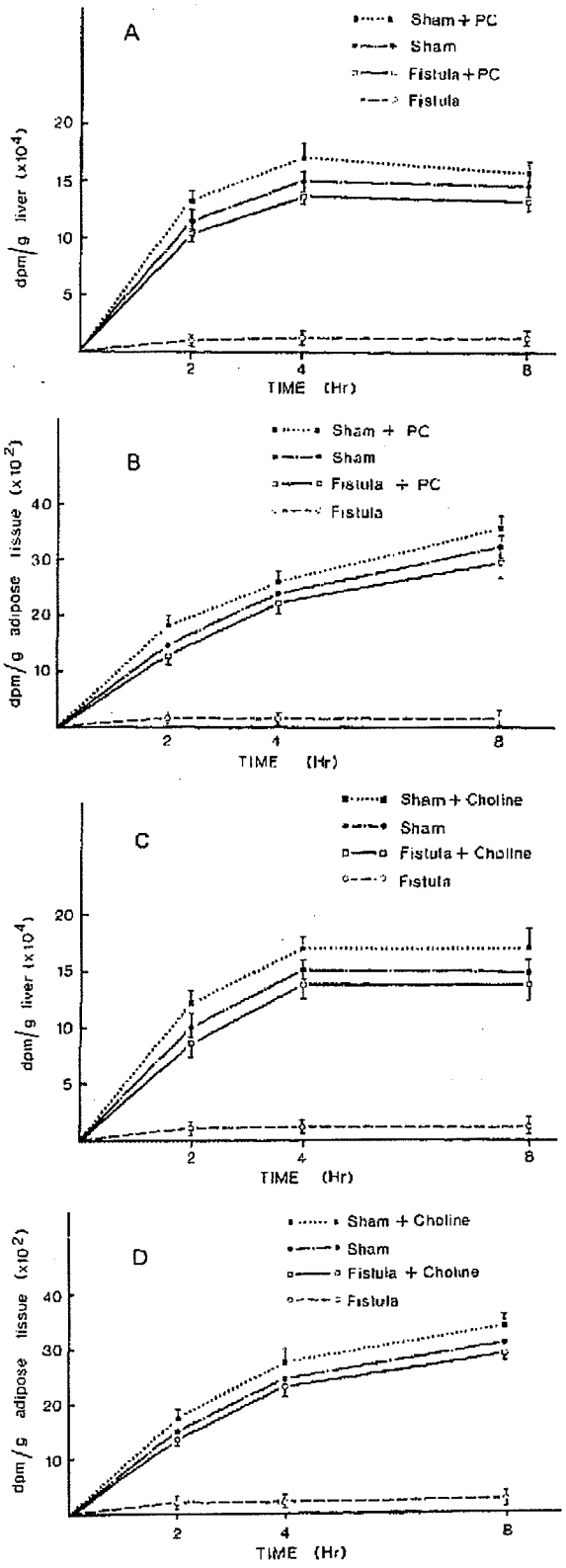

FIG. 2. Effect of lecithin (50 mg) and choline (5 mg) on uptake of labeled lipid by liver and adipose tissue. A and B show effect of lecithin on uptake by liver and adipose tissue, respectively. C and D show effect of choline. Each point represents mean of six rats. Vertical bars are standard error of mean.

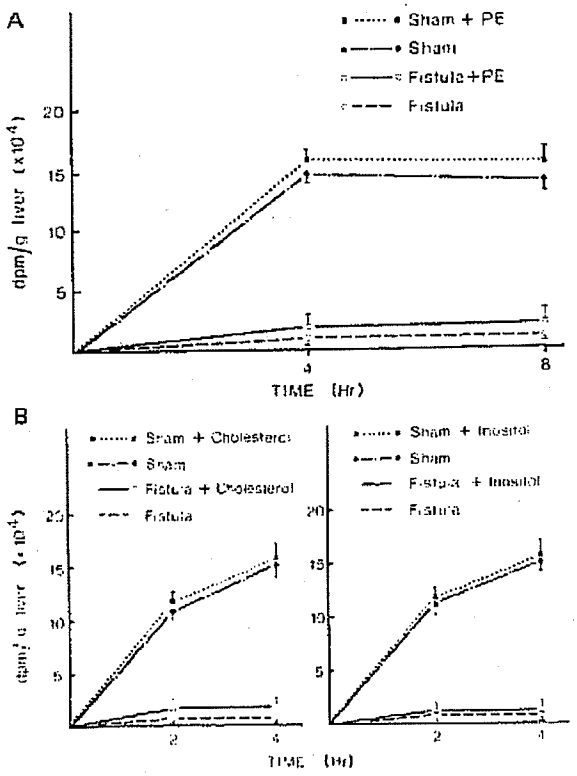

FIG. 3. Effect of phosphatidyl ethanolamine (50 mg), cholesterol (1%) and inositol (5 mg) on uptake of labeled fat by liver. A shows effect of phosphatidyl ethanolamine on uptake by liver. B shows effect of cholesterol and inositol. Each point represents mean of six rats. Vertical bars are standard error of mean. Uptake of labeled fat by adipose tissue showed a similar pattern.

fat meal. Tasker and Hartroft (22), using morphological techniques, were unable to confirm the findings of either Frazer or Tidwell. In addition, Zilversmit et al. (23), using [^{32}P], found that the turnover of mucosal phospholipids was low, which presumably ruled out their participation as intermediates in fat absorption. It could not rule out a limited synthesis of lecithin necessary for chylomicron formation, much of which could proceed normally via the lysolecithin pathway.

Of the phospholipids in rat chylomicrons, 70-80% is lecithin (24). The fatty acid composition of chylomicron lecithin has been shown to be rather constant and largely independent of the composition of the fatty meal given (25). In addition, Scow et al. (26) have concluded that a maximum of 40% of chylomicron lecithin was derived from dietary sources, even when the concentration of lecithin in the diet was two to three times that in the chylomicrons. Of the 60% or more of the chylomicron lecithin which therefore came from endogenous sources, the bile would probably be a major contributor. If lecithin is important in the total absorption process, one would expect an increase in its synthesis during absorption. Indeed Hübscher et al. (27) found that, of all the phospholipids of the intestinal mucosa, only lecithin showed a five-fold increase in specific activity during fat absorption, as compared with carbohydrate-fed controls.

It is possible that the various components of the bile have a distinct function in the different stages of fat absorption. The bile acids appear to aid absorption of monoglycerides and free fatty acids into the intestinal mucosa, while the lecithin moiety may be involved in secretion out of the intestine. This can be deduced from the present work along with the earlier studies of Morgan and Börgstrom (28) who showed a marked impairment in absorption into the intestine, if micellar solutions of dietary glycerides were made with phospholipids and not bile salts. Rampone (12) has recently found, both in experiments where bile fistula rats were perfused with a micellar solution of bile salt, monoglyceride and free fatty acid, and in experiments using everted sacs from small intestine of bile fistula rats, that absorbed lipid accumulated in the intestine. He concluded that bile contained one or more components that played a role in the phase of absorption involved with transport out of the mucosa. From the present studies, one such component would appear to be lecithin. Morgan (29) had previously claimed little difference in the lymphatic output of esterified fat between normal and bile fistula rats. In view of the present data, the lack of effect by biliary diversion observed by Morgan must have been due to lecithin which was present at the level of 1.2% in the commercial emulsion.

The importance of phospholipid in fat clearance can also be inferred from the work of Friedman and Cardell (30), who have reported the effects of puromycin on the structure of the intestinal mucosa during fat absorption. Puromycin treatment caused the absorptive cells to accumulate increased quantities of lipid that were devoid of membrane during fat absorption, and, in addition, puromycin-treated cells contained much less rough endoplasmic reticulum and Golgi membranes. When such membranes were absent, the cell's ability to discharge chylomicra was impaired and lipid accumulated. The findings of Friedman and Cardell are in agreement with some biochemical evidence for inhibition of phospholipid synthesis by puromycin (1). In addition, Van den Berg and Hulsmann (31) have reported that even lack of the appropriate diglyceride lowered lecithin synthesis in the gut and that this

impaired chylomicron synthesis and release. Since phospholipid is an integral part of membranes, its biosynthesis must therefore be essential for membrane formation.

It is also obvious that the presence of lecithin or choline supports or stimulates mucosal protein synthesis. This role of lecithin or choline in promoting protein synthesis in the gut is similar to the effect of phosphoryl choline in promoting protein synthesis in livers of normal and choline-deficient rats (32-33). The exact mechanisms by which biliary lecithin is involved in the formation and release of chylomicrons and the nature of its involvement in the synthesis of chylomicron protein must await further investigation.

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Appendix H

Mattson et al. "The Digestion and Absorption of Triglycerides" J Biol Chem 239: 2772-7(1964)

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The Digestion and Absorption of Triglycerides

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Studies both *in vitro* and *in vivo* have established that the hydrolysis of triglycerides in the intestinal tract can yield diglycerides, monoglycerides, free fatty acids, and free glycerol. Thus far it has not been possible to establish which of these materials are absorbed as such into the intestinal wall. That a solution to this problem has not been forthcoming was undoubtedly attributable to the lack of satisfactory techniques for testing the various hypotheses that have been proposed. Thus, information has been gained by the use of unusual compounds, such as dimethyl stearic acid (1) or conjugated linoleic acid (2), but there is the possibility that the results from such experiments are not applicable to triglycerides containing the usual fatty acids. Even when normal fatty acids are used, the results are not necessarily conclusive. Particular examples of this are the recent studies of Savary, Constantin, and Desnuelle (3) and Mattson and Volpenhein (4), in which the digestion and absorption of triglycerides containing both saturated and unsaturated fatty acids were followed. These experiments demonstrated that only a portion of the fatty acids that are esterified with the β position of the dietary triglycerides undergo hydrolysis during the processes of digestion and absorption. As we pointed out at that time, the absorption of palmitic acid is poor when the acid is fed in the free form, improved when the acid occupies the α position on a triglyceride, and greatest when the acid is esterified at the β position of the triglyceride. Thus, no final conclusions could be drawn from these experiments as to the nature of the materials that are absorbed. The shortcomings of our earlier study have been overcome by preparing a series of glycerides containing labeled oleic acid as the sole fatty acid and labeled glycerol. The hydrolytic and synthetic products of the processes of digestion and absorption were determined when glycerol and oleic acid were fed either in the free form or as mono-, di-, or triolein; in the last instance, the fatty acids at the α and β positions were studied separately. From these results, together with the studies reported by others, it is possible to elucidate the composition of the lipids that are absorbed, the way they arise by enzymatic hydrolysis, and how they are subsequently resynthesized into the triglycerides that are found in the lymph.

EXPERIMENTAL PROCEDURE

Carboxyl-labeled oleic acid and α-^{14}C-glycerol were obtained from the Volk Radiochemical Company, New York. The oleic acid was purified by countercurrent distribution, 400 transfers, with the use of the two-phase solvent system resulting from a mixture of heptane, dimethyl formamide, and glacial acetic acid, 3:2:1 (v/v). Both compounds were analyzed by paper, column, and thin layer chromatography and found to be of better than 99% radiochemical purity. Gas-liquid chromatography of the unlabeled oleic acid showed it to be 99% pure. A series of glycerides was synthesized from these materials.

α-Labeled Mono-olein (*G*O*--*)[1]—This compound was prepared from labeled oleic acid and labeled glycerol by the method of Hartman (5).

α,α'-Labeled Diolein (*G*O*-O**)—Labeled glycerol and labeled oleoyl chloride in a molar ratio of 1:2 were refluxed in pyridine-chloroform. The α,α'-diolein was separated from the resulting mixture of glycerides by silica gel chromatography and crystallization (6).

β-Labeled Triolein (*G*OO*O*)—β-Mono-olein-1-^{14}C was prepared from labeled oleic acid and labeled glycerol by the method of Martin (7). This was acylated with unlabeled oleoyl chloride (8).

α,α'-Labeled Triolein (*G*O*OO**)—α-Mono-olein-1-^{14}C was prepared from labeled oleic acid and labeled glycerol by the method of Hartman (5). This was acylated with unlabeled oleoyl chloride (8).

Other Labeled Trioleins—Labeled glycerol was acylated (9) with 1-^{14}C-oleoyl chloride to obtain triolein in which all the constituents were labeled (G*O*O*O*). The same method but with appropriate radioactive or inactive glycerol or oleoyl chloride was used for the preparation of triolein in which the glycerol was labeled but the fatty acids were not (G*OOO) and of triolein in which the fatty acids were labeled but the glycerol was not (GO*O*O*).

The purity of these preparations was confirmed by thin-layer chromatography. The distribution of labeled acid in these glycerides was determined by the method of Mattson and Volpenhein (8). None of the glycerides was found to contain more than 1% of compounds of other structures.

These experimental fats were administered as emulsions (4) to rats whose thoracic ducts had been cannulated. The test meals, each containing 1.5 g of fat, were fed at 0 and 24 hours. Lymph was collected for the 48 hours after the first feeding. Triglycerides were isolated from the lymph lipids by silicic acid column chromatography, and the location of the labeled fatty acid in these triglycerides was determined by hydrolysis with pancreatic lipase (8). Measurements of radioactivity were made on the free glycerol and the free fatty acids that were obtained from the

[1] Since the interpretation of these experiments depends on the location of the labeled fatty acid on the glycerol moiety, we have selected a graphic designation of the structure of the triglycerides, giving the order of the fatty acid esters on the glycerol backbone and using an asterisk (*) to indicate the labeled component. For example, triolein in which the fatty acids esterified at the α and α' positions and the glycerol are labeled is abbreviated G*O*OO*. For the partial glycerides the location of the free hydroxyl groups is indicated by a hyphen. For example, β-mono-olein in which the glycerol and fatty acid are labeled is abbreviated G*-O*-.

glycerides by saponification, acidification, and separation into ether-soluble and water-soluble components. Counting was done on a Packard TriCarb liquid scintillation counter (17).

RESULTS AND DISCUSSION

The specific activity of the fatty acid moiety of the lymph triglycerides relative to that of the diet triglycerides is given in Tables I and III. The relative specific activity of the lymph fatty acids was approximately constant, regardless of the form in which the oleic acid was fed. Thus, the oleic acid was equally well absorbed whether it was fed as the free acid or as any of several ester forms. The differences in absorbability encountered in our earlier study (4) have thus been avoided by the use of only oleic acid.

The average relative specific activity of the lymph fatty acids based on all of the experiments was 88%. This decrease in relative specific activity is due to the presence of fatty acids of endogenous origin in the thoracic duct lymph. In a number of animals fed a fat-free diet, we have found that this endogenous lipid amounts to approximately 150 mg per day, a quantity sufficient to account for the 12% decrease in specific activity.

In contrast to the fatty acids, the relative specific activity of the lymph glycerol was markedly dependent on the form in which it was fed. When one considers only those experiments in which the labeled glycerol was fed as part of a triglyceride (Experiments 1, 2, and 3), the average specific activity of the lymph glycerol relative to that of the dietary glycerol was 69%. This decrease in relative activity could be due not only to dilution by endogenous glycerides, as in the case of the oleic acid, but also to the complete hydrolysis of a portion of the triglyceride and replacement of the resultant free glycerol by endogenous glycerol for the resynthesis of triglycerides. The decrease in glycerol specific activity due to endogenous lipid can be corrected for by adjusting the glycerol activity in proportion to the dilution of oleic acid activity by endogenous fatty acids. The values obtained after this adjustment are given in the last column of Table I; they averaged 78%, when the glycerol was fed as part of a triglyceride. Thus, there was a 22% decrease in specific activity of the glycerol as the result of the complete hydrolysis of a portion of the dietary triglyceride and the lack of utilization of part or all of the resulting free glycerol in the resynthesis of triglycerides.

When free glycerol was fed (Experiments 4 and 5), only about 5% of it appeared in the lymph as a constituent of triglycerides. These results are in agreement with those reported by others (11–13), the highest average value from any one laboratory being 14% (14). Thus, little of the free glycerol, which is formed by the complete hydrolysis of triglycerides in the lumen of the intestine, is used in the resynthesis of triglycerides in the intestinal wall. The relative insolubility of glycerol in the lipids that are being absorbed affords a ready explanation of this phenomenon.

The distribution of the labeled fatty acid in the triglyceride molecules of the lymph is given in Table II. In Experiments 6 and 7, a small amount of labeled free oleic acid or methyl oleate was fed along with a large amount of unlabeled triolein. In both experiments approximately 14% of the labeled acid was found in the β position of the lymph triglycerides. The amount of labeled acid fed was quite small compared to the free fatty acids that would result from the hydrolysis of the triglycerides. The labeled acid then can be used to follow the fate of the free fatty acids resulting from glyceride digestion. The results show that about 14% of the free fatty acids resulting from the hydrolysis

TABLE I

Specific activity of glycerol and fatty acid moieties in lymph relative to that in diet

Experiment	Material fed	Relative activity[a]		Glycerol-to-fatty acid recovery ratio in lymph
		Glycerol	Fatty acid	
		%		%
1	G*O*O*O*[b]	65.2	87.8	74.2
		58.6	81.1	72.3
		71.5	86.7	82.5
		70.4	90.2	78.0
		74.5	90.2	82.7
		73.7	91.6	80.5
2	G*O*OO*	64.2	88.3	72.7
		67.7	88.3	75.5
		64.4	88.3	72.8
3	G*OO*O	73.4	90.4	81.3
		72.8	90.4	80.6
		69.8	88.9	78.7
		68.8[c]		77.6[c]
4	10% G* + 90% MeO*	6.3	85.7	7.4
		6.1	83.8	7.3
		5.6	88.8	6.3
5	1% G* + 99% GO*O*O*	3.5	92.8	3.8
		3.2	89.8	3.6
		3.5	94.1	3.7
			87.7[c]	

[a] Specific activity of lymph fatty acid or glycerol as percent of specific activity of the corresponding moiety of the compound or compounds fed.

[b] See Footnote 1 in the text.

[c] Average.

of triolein were re-esterified to the β position and 86% to the α and α' positions. Thus, there is considerably more hydrolysis and re-esterification taking place at the α and α' positions than at the β position.

In Experiment 2, the labeled acids were in the α and α' positions of the dietary triolein. The lymph triglycerides of these animals were found to contain 12.5% of the labeled acid in the β position and 87.5% in the α and α' positions. All of the labeled oleic acid that was fed in Experiment 3 was in the β position of the triolein; in the lymph triglycerides, 77.6% of the labeled acid was in the β position and 22.4% in the α and α' positions. These results are in terms of percentage distribution, but they are not directly comparable because the number of labeled positions in the two experiments was not the same. Thus, 100 moles of triglycerides in which the α positions were labeled contained 200 moles of labeled acid and 100 moles of unlabeled acid, whereas 100 moles of triglyceride in which the β position was labeled consisted of 100 moles of labeled acid and 200 moles of unlabeled acid. The data must be transferred from a percentage to an equivalance basis before comparisons can be made. When they are expressed on such an equivalance basis, there were 25.0 labeled acids that moved from the α positions

TABLE II

Proportion of labeled fatty acid in β position of lymph triglycerides after ingestion of various lipids

Experiment	Material ingested	Proportion of label in β position
2	G*O*OO*[a]	13.6 11.8 12.1 12.5[b]
3	G*OO*O	80.0 74.2 78.6 77.6[b]
4	10% G* + 90% MeO*	33.1 25.5
12	O*	29.1 31.9 29.8 29.2[b]
6	1% O* + 99% GOOO	13.8 13.7
7	0.2% MeO* + 99.8% GOOO	16.2 14.8 12.1 14.1[b]
9	5% G*O*-- + 95% O	28 30 29[b]
10	5% G*O*-- + 95% GOOO	16.0 15.0 13.8 14.9[b]
13	5% G*O*-O* + 95% O	29.5 32.7 35.7 32.6[b]
11	5% G*O*-O* + 95% GOOO	14.4 13.0 13.7[b]

[a] See Footnote 1 in the text.

[b] Average.

to the β position in Experiment 2 and 22.4 labeled acids that moved from the β position to the α positions in Experiment 3. These two numbers should be the same. That they are not could be due either to experimental error or to the more rapid absorption of the free fatty acids (15, 16) resulting from the hydrolysis of triglycerides. In either event, this difference would be compensated for by averaging the values obtained in Experiments 2 and 3. Thus, when the fatty acids esterified at the α and α′ positions of the dietary triglyceride were labeled, the best value for the movement of the acids to the β position was 23.8.

The hydrolysis of triglycerides in the intestinal tract proceeds by a series of directed stepwise reactions from triglycerides to α,β-diglyceride to β-monoglyceride (17). Although a portion of the monoglyceride is hydrolyzed, it is likely that this hydrolysis occurs only after isomerization to α-monoglyceride. Since the exchange of fatty acids in the lumen of the intestine is limited almost exclusively to the α and α′ positions (18), the only way for a fatty acid that was originally in the α position to enter the β position is by synthesis *de novo* of a triglyceride. When the labeled acids are in the α positions of the dietary triglyceride, the amount of labeled fatty acid in the β position of the resulting lymph triglyceride will be proportional to the amount of triglyceride that was completely hydrolyzed. The level of activity in the β position of the lymph triglycerides will depend on the activity of the free fatty acid pool from which the acids are drawn for the synthesis *de novo*. The activity of this free fatty acid pool will be a function of the proportion of diglyceride, monoglyceride, and free glycerol resulting from the hydrolysis of triglyceride. Thus, the following relationship exists among the possible products of digestion and the movement of labeled fatty acids from the α positions to the β position:

$$G_S \times \frac{DG + 2\,MG + 2\,G_H}{DG + 2\,MG + 3\,G_H} = 23.8$$

where G_S is the glycerol used for synthesis *de novo* and the hydrolysis products are DG, diglyceride, MG, monoglyceride, G_H, glycerol, all in mole per cent and $G_H + MG + DG = 100\%$. G_S and G_H will have the same value because the amount of synthesis *de novo* will be equal to the amount of triglyceride that is completely hydrolyzed. The denominator represents the total free fatty acids, and the numerator represents the labeled free fatty acids resulting from the partial or complete hydrolysis of this triglyceride. In order to solve the equation, values for DG from the point at which it constituted none to the point at which it was the sole product of hydrolysis, *i.e.* from 0 to 76.2%, were inserted into the equation. The values obtained for G_S and G_H ranged only from 27, if no monoglyceride was present, to 29, if no diglyceride was present. Other combinations of mono- and diglyceride give values for G_S and G_H lying between 27 and 29. Thus, approximately 28% of the dietary triglyceride is completely hydrolyzed and there is a corresponding amount of synthesis *de novo*.

In Experiments 6 and 7, it was found that the esterifications taking place at the β position constitute 14% of the total that are occurring. In the preceding paragraph it was shown that during the digestion and absorption of 100 molecules of dietary triglycerides there will be 28 molecules of triglyceride synthesized *de novo* and hence 28 esterifications taking place at the β position. Therefore, per 100 molecules of triglyceride absorbed, approximately 28/0.14 or 200 total esterifications are taking place and at least 86%, (200 − 28)/2, of the fatty acids in the α positions were hydrolyzed and esterified. Since the same source of error—a more rapid absorption of free fatty acid—as was discussed in connection with Experiments 2 and 3 would be operative in

Experiments 6 and 7, this value of 14% is probably slightly high, and hence the value for total esterifications is in excess of 200. These results together with observations reported by others, which will be discussed later, suggest that all of the primary esters of the dietary triglycerides are hydrolyzed.

The values for the recovery of labeled glycerol and fatty acid in the lymph when partial glycerides were fed are given in Table III. In the second column of this table, the activity of the lymph fatty acids relative to that of the dietary fatty acids was calculated by the same method used in obtaining the values in Table I. However, the same method could not be used in obtaining the glycerol values because, relative to a triglyceride, the dietary fats in Experiments 10 and 11 contained an excess of glycerol and those in Experiments 9 and 13 had a deficit of glycerol. To determine the recovery of glycerol relative to that of oleic acid, the total amount of glycerol and fatty acid activity recovered was used. Although the recovery of neither of these was complete, and although it varied between experiments, the relative recovery of the two in any one experiment would be the same. Since it is the ratio of recovery that is sought, the values obtained by this method are meaningful.

As shown in Table III, when glycerol- and fatty acid-labeled α-mono-olein (Experiment 9) or α,α'-diolein (Experiment 13) was fed together with free oleic acid, the ratio of glycerol to fatty acid recovered was approximately the same as when glycerol- and fatty acid-labeled triolein (Table I, Experiments 2 and 3) was fed. On the other hand, when these partial glycerides were fed with triolein, only 40% of the dietary monoglyceride-glycerol (Experiment 10) and 61% of the dietary diglyceride-glycerol (Experiment 11) were recovered. It is interesting that the differences between 100 and these last two values approximate the deficiency in fatty acid needed by these partial glycerides in order to make triglycerides. In all four experiments some of the glycerol of the partial glycerides was used for the synthesis of the triglycerides found in the lymph. The extent of this utilization depended on the availability of an adequate amount of fatty acids to make triglycerides.

The distribution of the fatty acids of the dietary partial glycerides in the lymph triglycerides is shown in Table II. When the partial glycerides were fed together with free oleic acid (Experiments 9 and 13), the labeled acid was distributed almost equally among all three positions in the triglyceride molecule; the distribution was similar to that obtained when methyl oleate or free oleic acid was fed (Experiments 4 and 12). Such results indicate the complete hydrolysis of the partial glycerides. If the partial glycerides were incompletely hydrolyzed, the proportion of the labeled acid in the β position of the lymph triglycerides would be reduced to the same extent. Thus, if only 50% of the partial glycerides were hydrolyzed, 16% of the labeled acids in the lymph triglyceride would be in the β position. When the mixture fed was partial glyceride and triglyceride (Experiments 10 and 11), approximately 14% of the labeled acid was found in the β position of the lymph triglyceride; this value is quite similar to that found when labeled free oleic acid or methyl oleate was fed together with triolein (Experiments 6 and 7). Here, too, if all of the mono- and diglycerides were not hydrolyzed, the proportion of labeled acid in the β position would be reduced. In this instance, hydrolysis of only 50% of the partial glycerides would result in triglycerides in the lymph in which 7% of the labeled acids were in the β position. Thus, in all four of these experiments in which partial glycerides were fed,

TABLE III

Recovery of labeled glycerol and labeled oleic acid in lymph after ingestion of partial glycerides

Experiment	Material ingested	Relative activity of lymph fatty acids[a]	Labeled moiety recovered[b]		Total glycerol-to-fatty acid recovery ratio in lymph
			Glycerol	Fatty acid	
		%	%		%
9	5% G*O*--[c] + 95% O	91.2	72.7	85.0	85.6
		88.2	65.0	76.2	85.4
					85.5[d]
10	5% G*O*-- + 95% GOOO	84.6	36.5	83.2	43.8
		84.6	32.3	81.7	39.5
		90.3	32.3	89.5	36.1
					39.8[d]
13	5% G*O*-O* + 95% O	87.8	64.3	85.7	75.2
		84.1	73.7	83.3	88.4
		88.8	69.6	83.3	83.7
					82.4[d]
11	5% G*O*-O* + 95% GOOO	81.7	46.5	72.8	63.8
		87.2	47.2	80.5	58.7
		86.8			61.2[d]

[a] Specific activity of lymph fatty acid as percentage of specific activity of dietary fatty acids.

[b] Recovery of total activity in lymph fatty acid or glycerol as percentage of the total activity of the corresponding moiety of the compounds fed.

[c] See Footnote 1 in the text.

[d] Average.

the distribution pattern of the labeled acids in the lymph triglycerides resembled that obtained when a labeled free fatty acid was fed, and thus complete hydrolysis of the partial glyceride is indicated. As will be shown in the next paragraph, these results are in seeming contradiction to those obtained when the glycerol of the partial glyceride was the labeled material.

The recovery in the lymph of dietary free glycerol as reported here (Table I, Experiments 4 and 5) and by others (11–14) show that little, if any, of the free glycerol that is present in the lumen of the intestinal tract is used for the resynthesis of triglycerides in the intestinal wall. The fatty acid distribution data obtained with the labeled α-mono-olein and α,α'-diolein point to complete hydrolysis of these partial glycerides. However, the recovery of labeled glycerol (Table III) in these same experiments shows reutilization of the glycerol comparable to that of dietary triglyceride-glycerol, when account is taken of the fatty acids and glycerol available for triglyceride synthesis. The recovery of glycerol thus indicates that these partial glycerides are not completely hydrolyzed. A reasonable solution to this dilemma is that the dietary monoglyceride is absorbed as such and hydrolyzed in the intestinal wall, where the resulting free glycerol is then used for the resynthesis of triglycerides. This agrees with an earlier suggestion of Reiser and Williams (19). The fate of the diglycerides is the same, although it is likely that they are

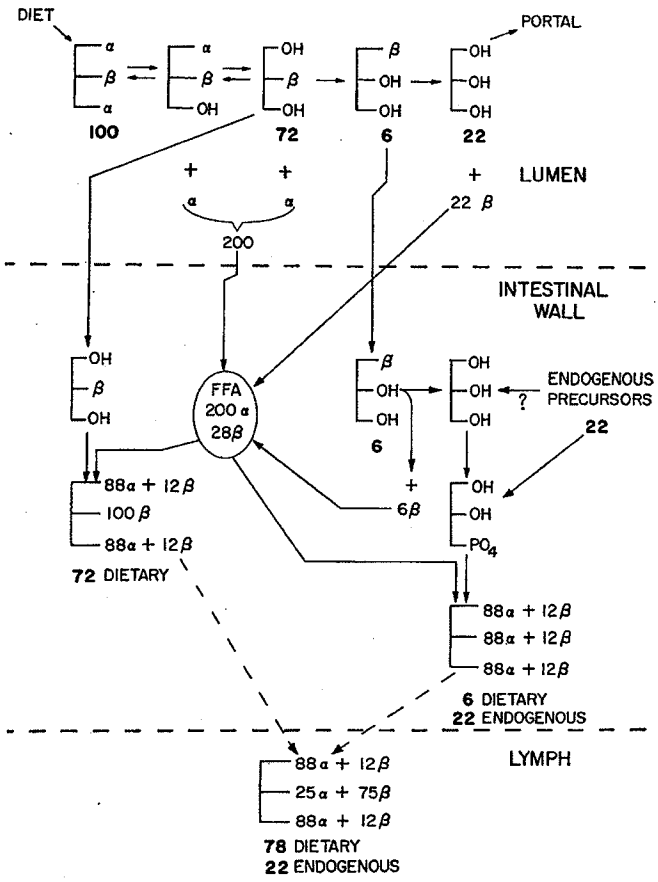

FIG. 1. The digestion, absorption, and resynthesis of glycerides. The proportions of the various products of digestion and their resynthesis into triglycerides, which appear in the lymph, are shown. The *bold-faced numbers beneath the formulas* are the values for the glycerol moiety, and the *light-faced numbers* are the values for the fatty acid moiety. The *Greek letters* indicate fatty acids that occupied the α, β, or α' position in the original triglyceride.

split to α-monoglycerides in the lumen before absorption. The data obtained by Skipski, Morehouse, and Deuel (20) led them to the conclusion that dietary α-monoglyceride was absorbed without hydrolysis. However, from their experiments, the further fate of the compound could not be determined.

It should be emphasized that it is the α-monoglyceride and not the β-monoglyceride that is involved in this proposed scheme of hydrolysis after absorption. It is likely that the acylase of the intestinal wall can esterify only β- and not α-monoglycerides.

The conditions that exist in the intestinal tract are particularly well suited for the generation of monoglycerides as the chief end product of triglyceride digestion. In the first place, there is an abundance of lipolytic enzyme present. We[2] have found that the rat excretes into the intestinal tract about 25 ml of pancraetic juice per day. Under conditions of enzyme assay (8), 1 ml of juice is capable of hydrolyzing 7 g of fatty acid ester per hour. Because of the specificity of the enzyme for the primary ester groups (21), the chief end product of the hydrolysis is β-monoglyceride, the formation of free glycerol being limited primarily by the rate at which the β-monoglyceride is isomerized to α-monoglyceride.

[2] Unpublished observations.

The physical nature of the various lipids in the intestinal tract also explains why monoglyceride is the end product of digestion. Hofmann and Borgström (22, 23) separated the material recovered from the lumen of the intestine into an oil phase and a water phase by centrifugation. The di- and triglycerides were in the oil phase, which contained little or no monoglyceride and free fatty acid. The monoglycerides were in the water phase, probably as extended micelles consisting of monoglycerides, free fatty acids, and bile salts. Since Sarda and Desnuelle (24) have shown that pancreatic lipase acts only at an oil-water interface and cannot hydrolyze water-soluble substrates, the di- and triglycerides in the lumen of the intestine will be hydrolyzed readily, whereas the monoglycerides, being in a water-soluble form, cannot be hydrolyzed. Thus, the conditions in the lumen of the intestinal tract are ideal for the rapid hydrolysis of triglycerides and diglycerides. However, the hydrolysis of the resulting β-monoglycerides depends on their isomerization and subsequent cleavage while they are still in the oil phase.

The above data, together with the reports of others, make it possible to propose an integrated system for the digestion and absorption of triglycerides. This system is shown in Fig. 1. Values are given for the proportion of the various digestion products and the distribution of the dietary fatty acids in the lymph triglycerides. Digestion commences in the intestinal tract with the hydrolysis of triglycerides by a series of directed stepwise reactions from triglyceride to α,β-diglyceride to β-monoglyceride (17). This hydrolysis takes place at an oil-water interface. About 28% of the β-monoglyceride isomerizes to α-monoglyceride, and in turn, about 75% of the α-monoglyceride is further hydrolyzed to free glycerol. This free glycerol enters the intestinal wall independently of the lipids and is not further used in the process of lipid absorption. The β-monoglycerides are not hydrolyzed because of their transfer to a water-soluble phase (22, 23) and because of enzyme specificity (21, 22). The digestion of 100 moles of triglycerides yields, for absorption, 72 moles of β-monoglyceride, 6 moles of α-monoglyceride, 200 moles of fatty acids from the α and α' positions, and 22 moles of fatty acids from the β position. These values are similar to those proposed earlier by Reiser *et al.* (2). Borgström, Tryding, and Westoo (1) concluded from their studies that 60% of the glyceride ester bonds are hydrolyzed and 40% of the triglyceride is completely hydrolyzed.

In the intestinal wall the α-monoglyceride is hydrolyzed. The occurrence of this reaction has been demonstrated in isolated systems (25–27). Both the free fatty acid and glycerol are available for the resynthesis of triglycerides. It is likely that the free glycerol resulting from the hydrolysis of the α-monoglyceride is first converted to α-glycerol phosphate (28). A system for the conversion of glycerol to triglyceride by this path has been described by Clark and Hübscher (29). The remaining glycerol comes from endogenous precursors such as fructose 1,6-diphosphate (30) or glucose (31). On the other hand, the β-monoglyceride is not hydrolyzed. It can be acylated directly to triglyceride without passing through a phosphorylated intermediate (32–34).

According to this scheme, the triglycerides of the lymph would contain 78% of the dietary glycerol. Of the fatty acids that were in the β position of the dietary triglycerides, 25% would be found in the α and α' positions of the lymph triglycerides, and there would be a comparable movement of fatty acids from the α and α' positions of the dietary triglyceride to the β position of

the lymph triglycerides. Thus, this scheme is compatible with the experimental results reported here.

SUMMARY

Rats were fed triolein, diolein, and mono-olein in which the fatty acids occupying specific positions of the glyceride molecule and the glycerol were labeled. The recovery of labeled glycerol and oleic acid and the location of the labeled acid in the triglyceride molecules of the lymph were determined.

From these data and the results obtained by others, the following scheme is proposed for the digestion and absorption of oleoyl glycerides. In the lumen of the intestine, triglycerides are hydrolyzed by way of α,β-diglycerides to 72 parts β-monoglyceride, 6 parts α-monoglyceride, and 22 parts free glycerol. This hydrolysis results in the cleavage of all of the fatty acids esterified at the α and α' positions and 22% of those esterified at the β position of the dietary triglyceride. Thus, approximately 75% of the glycerol of dietary triglyceride is absorbed as monoglyceride, and 75% of the fatty acids of dietary triglyceride are absorbed as free acids. The free glycerol is absorbed independently of the lipids, and little of it is used in glyceride synthesis. In the intestinal wall the β-monoglycerides are re-esterified to triglycerides. The α-monoglycerides are hydrolyzed in the intestinal wall, but the resulting free glycerol, as well as the fatty acid, is used in the synthesis of triglycerides.

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Appendix I

Tso *et al.*, "Evidence for Separate Pathways of Chylomicron and Very Low-Density Lipoprotein Assembly and Transport by Rat Small Intestine" *Am J Physiol* 247: G599-G610 (1984)

Evidence for separate pathways of chylomicron and very low-density lipoprotein assembly and transport by rat small intestine

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TSO, PATRICK, DEBORAH S. DRAKE, DENNIS D. BLACK, AND SEYMOUR M. SABESIN. *Evidence for separate pathways of chylomicron and very low-density lipoprotein assembly and transport by rat small intestine.* Am. J. Physiol. 247 (Gastrointest. Liver Physiol. 10): G599–G610, 1984.—Previously, we demonstrated that the hydrophobic surfactant Pluronic L-81 (L-81) inhibits the intestinal formation and transport of chylomicrons (CM) but not of very low-density lipoprotein-sized (VLDL) particles. The present study was undertaken to determine whether infusion of egg lecithin results mainly in secretion of VLDL by the small intestine and whether L-81 has any effect on their formation and secretion. Intestinal fistula rats were infused intraduodenally at a rate of 3 ml/h with a lipid emulsion containing 20 mM egg lecithin and 19 mM sodium taurocholate for 8 h. This was then followed by another 8 h of infusion of a similar lipid emulsion but with 0.5 mg/h of L-81 added. Lymphatic lipid output was measured, and lymph lipoproteins were sized by use of electron microscopy. Whether L-81 was present or not, no significant difference was detected in the lymphatic triglyceride, phospholipid, or cholesterol outputs. Based on agarose gel electrophoresis, sizing of intestinal lymph lipoproteins, and also the determination of lipid in the intestinal lymph CM and VLDL as separated by ultracentrifugation, VLDL were the major lipoproteins present in lymph during the infusion of egg lecithin. Thus, intraduodenal infusion of egg lecithin in the rat results mainly in the transport of VLDL and is not affected by the administration of L-81. The results suggest that CM and VLDL are assembled separately by the enterocytes and indicate the usefulness of L-81 in further investigating the pathways and regulation of intestinal lipoprotein synthesis, assembly, and secretion.

hydrophobic surfactant; lipid transport; lipid metabolism

DESPITE THE WEALTH OF INFORMATION gathered in the last 20 yr about intestinal lipid absorption, relatively little is known about the factors that regulate the intracellular assembly, modification, and secretion of lipoproteins from the small intestinal epithelial cells (enterocytes). This is partly due to the complexity of the processes involved and to the lack of good experimental models for studying the various steps. During the past few years, we have studied the effect of a hydrophobic surfactant, Pluronic L-81, on the formation and transport of intestinal lipoproteins (29–31). Intraduodenal administration of 0.5 mg/h of Pluronic L-81 (L-81) did not inhibit the uptake of lipolytic products or reesterification of monoglyceride and fatty acid to form triglyceride (29, 30). Nevertheless, this small dose of L-81 markedly reduced the secretion of chylomicrons (CM) by the enterocytes into the mesenteric lymph (29, 30). Consequently, the transport of both triglyceride and cholesterol into lymph was greatly impaired. Preliminary electron microscopic data suggested that L-81 causes an accumulation of triglyceride in vesiculated smooth endoplasmic reticulum and prevents the movement of prechylomicrons into the Golgi complex (29). In contrast to the striking inhibition of CM transport, the formation and transport of very low-density lipoprotein (VLDL)-sized particles were not impaired. The inhibitory action of L-81 is extremely rapid ($t_{1/2}$ of 69 min for 0.5 mg/h dose and 35 min for 1 mg/h), and the inhibition of CM transport can be reversed quickly through the cessation of L-81 infusion.

Recently, it has been reported that the intraduodenal infusion of lecithin (phosphatidylcholine, PC) in humans stimulates VLDL production (1). This could result from an abundance of intracellular PC, which then promotes the formation of small triglyceride-rich particles, i.e., VLDL. Currently, the separation of intestinal triglyceride-rich lipoproteins into CM and VLDL is based mainly on operational criteria. Lipoproteins whose Svedberg flotation (S_f) rate exceeds 400 are classified as CM, and those with S_f rates of 20–400 are defined as VLDL (17). The intestinal VLDL particles have also been called "small chylomicrons" by some investigators (10). Although it has not been proven conclusively, it has been suggested that CM and VLDL represent two distinct populations of lipoproteins. The first evidence came from the study of Ockner, Hughes, and Isselbacher (23), who showed that the infusion of palmitate caused a marked increase in VLDL transport, whereas with oleate and linoleate VLDL output remains unchanged. In contrast, CM output was markedly increased when oleate and linoleate were infused. Furthermore, the fatty acid composition of the triglyceride in VLDL was different from the triglycerides of CM, therefore suggesting a different pathway for the assembly of VLDL and CM particles. The ultrastructural and biochemical study conducted by Mahley and co-workers (18), showing that intestinal

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Golgi vesicles contained either CM or VLDL particles with little mixing of particle sizes, also provided evidence for separate biosynthetic pathways for these lipoproteins. The present study was conducted to address two hypotheses: *1*) are VLDL the major lipoproteins transporting dietary lipid into lymph when PC is infused and *2*) are there two pathways for the intestinal transport of CM and VLDL and does L-81 only affect the secretion of CM. Thus, if VLDL is the major lipoprotein secreted during egg PC infusion, intestinal lipid transported should be unaffected by L-81 infusion.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (250–300 g) were used for all experiments. The animals were fasted overnight before surgery. Under ether anesthesia, the intestinal lymph duct was cannulated with clear vinyl tubing (0.8 mm OD), according to the method described by Bollman et al. (2). The only modification to the original surgical procedure was that the cannula was secured through the application of a drop of Eastman Kodak 910 glue, instead of suture. A silicone tube (1.6 mm OD), tipped inside with a clear vinyl tubing (1 mm OD), was introduced about 2 cm down the duodenum through the fundus of the stomach. The tube was secured in the duodenum through a transmural suture, and the fundal incision was closed by a purse-string suture. Postoperatively, the animals were infused with 3 ml/h of a saline solution (145 mM NaCl) containing KCl (4 mM) and glucose (0.28 M). The animals were allowed to recover for at least 36 h in restraining cages kept in a warm chamber (approximately 30°C) before lipid infusion. Morgan (20) demonstrated that bile fistula rats absorbed lipid significantly better at 48 h than at 24 h after the operation.

Experimental plan. Our preliminary data indicated that the order in which PC or PC plus L-81 was infused had no bearing on lymphatic lipid outputs. Consequently, we have chosen the following experimental protocol. On the day of the experiment, the lipid emulsion containing 60 μmol egg PC and 57 μmol sodium taurocholate in 3 ml of phosphate-buffered saline (pH 6.4) was infused at 3 ml/h for 8 h. This infusion was then replaced for another 8 h by the same lipid emulsion containing 0.17 mg/ml of Pluronic L-81. Our control animals were infused intraduodenally with the lipid emulsion described above, except no L-81 was added, for 16 h.

Preparation of lipid emulsion. The appropriate volume of stock solution containing 10 mg/ml of egg yolk PC was dispensed into a beaker. For the L-81-containing infusate, 2 ml of an L-81 solution (2.5 mg/ml) were added. The chloroform was evaporated under nitrogen, and 30 ml of phosphate-buffered saline (6.75 mM Na_2HP_4, 16.5 mM NaH_2PO_4, 115 mM NaCl, and 5 mM KCl, pH 6.4) containing 19 mM sodium taurocholate were added. This was then sonicated using a Fisher sonic dismembrator model 300, and the final infusate was a stable liquid crystalline solution. The infusate remained stable during the entire infusion period. Aliquots were analyzed for glyceride ester and lipid phosphorus at the beginning and end of infusion and agreed within ±5%.

Collection of lymph. Lymph was collected into precooled tubes for 2 h before the lipid infusion was begun. This lymph sample was analyzed as the fasting lymphatic output before lipid infusion. Further lymph samples were collected every 2 h for the entire lipid infusion period (16 h).

Analysis. Part of the lymph collected was extracted for lipid by the method of Folch et al. (7). Aliquots were taken for esterified fatty acid (28), cholesterol (35), and phospholipid determination (25).

Negative staining of lymph lipoproteins. The size distribution of lymph lipoproteins was studied by negative-stain electron microscopy. A Formvar-coated grid was floated on a drop of the lymph and the excess removed by a tissue. The grid was then floated on a drop of a 2% sodium phosphotungstate solution (pH 5.9) for about 40 s and the grid dried by a tissue. The negatively stained sample was examined with a Zeiss EM-10 electron microscope (Carl Zeiss, Thornwood, NY). To measure the distribution of particles with different diameters, 600–800 particles on the enlarged prints of the electron micrographs were measured as the mean of the two estimates perpendicular to each other. The mean diameter was calculated from the above measurements.

Agarose gel electrophoresis. Small aliquots of intestinal lymph were used for the separation of lipoproteins by agarose gel electrophoresis and stained with fat red 7B using the apparatus and method supplied by Corning-ACI (Palo Alto, CA).

Isolation of CM and VLDL. After the lymph was collected, 1/25th volume of a preservative solution containing 25 mM EDTA, 2.5 mg/ml gentamycin sulfate, 1.25 mg/ml chloramphenicol, 5 mg/ml sodium azide, and 1.5 mM phenylmethylsulfonyl fluoride was added. The concentrations of the various preservative agents should prevent the hydroperoxidation, bacterial degradation, and proteolysis of the apolipoprotein of the lymph lipoproteins. Lipoproteins were separated by ultracentrifugation in a Beckman L8-70 ultracentrifuge using a swinging-bucket 41 rotor (Beckman Instruments, Spinco Division, Palo Alto, CA). CM were isolated by layering 2 ml of lymph with 11 ml of saline at $d = 1.006$, followed by ultracentrifugation at 17°C for 1.5×10^6 *g* average minute. The upper 2.5 ml of CM were removed by tube slicing and washed once by ultracentrifugation under identical conditions prior to lipid and apolipoprotein analysis. More $d = 1.006$ salt solution was then added to the remaining lymph sample, and the VLDL was then isolated by ultracentrifugation in the same rotor at 1.8×10^8 *g* average minute. The VLDL fraction was washed once prior to analysis. The lipid and the apolipoprotein composition of the isolated CM and the VLDL fractions was then determined.

Analysis of apolipoprotein composition. Lipoprotein samples were dialyzed against 0.01% disodium EDTA (pH 7.4). The dialyzing solution also contained preservatives at the same concentration as described above. The protein concentration of dialyzed lipoproteins was determined by the procedure of Markwell et al. (19). The apolipoprotein composition of the CM and the VLDL

was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Ten percent of the hourly output of CM and VLDL protein were separated by gradient (3.5–27%) SDS-PAGE. The procedure for SDS-PAGE was modified from the SDS-PAGE system of Laemmli (15). A vertical slab-gel apparatus (LKB Scientific Instruments, Bromma, Sweden) was used. Separating gels were composed of a 3.5–27% gradient, and the sample buffer was twice the concentration of the Laemmli buffer. Gels were stained with 0.05% Coomassie brilliant blue R-250 and destained in the solutions of Weber and Osborn (32). The apolipoprotein bands were identified on the basis of molecular weights: *1*) as determined by reference to purified protein standards and *2*) by comparison to published SDS-gel electrophoretograms of rat apolipoproteins (3, 4, 9, 34).

The relative changes in the hourly output of each individual apolipoprotein were compared among periods of fasting and infusion of PC with or without added L-81. Although we cannot measure the absolute amount of each of the apolipoproteins secreted because of differences in chromogenicity of individual apolipoproteins, comparison of changes in concentration of one particular apolipoprotein due to different treatments remains valid.

Materials. Egg yolk PC was purchased from Sigma Chemical, St. Louis, MO. Sodium taurocholate was supplied by Calbiochem-Behring, La Jolla, CA, and analyzed by thin-layer chromatography using a propionic acid: isoamyl acetate:water:*n*-propanol system (15:20:5:10, vol/vol). Pluronic surfactant was kindly donated by Dr. Schmolke of BASF Wyandotte, Wyandotte, MI. All reagents and solvents used were of analytical grade.

RESULTS

Three control rats infused intraduodenally with egg PC (60 μmol/h) were studied for the entire 16 h. Additionally studied were seven experimental rats infused for the first 8 h with egg lecithin, followed by another 8 h of infusion of egg lecithin plus L-81. As shown in Table 1, both the triglyceride and phospholipid outputs increased after lipid infusion. After reaching an almost steady lipid output at the end of 4 h of lipid infusion, the lipid output remained relatively unchanged for the duration of the infusion period. The triglyceride and phospholipid outputs observed in the control rats were nearly superimposable over those observed in the experimental rats, which will be described in the following. Since the central theme of the study is directed toward observations on the experimental rats, the remainder of the paper is devoted to a discussion of this particular group of rats.

Lymph flow rate. Seven rats were used for this study. The fasting lymph flow rate was 2.59 ± 0.13 ml/h (mean ± SE). As a result of PC infusion, the lymph flow increased considerably to reach a steady output of 3.87 ± 0.29 ml/h during the 6–8 h of PC infusion (Fig. 1). The lymph flow then decreased steadily following PC plus L-81 infusion, and the flow rate was 2.78 ± 0.25 ml/h during the 14–16 h of infusion. The change in lymph flow rate between the infusion of PC and PC plus L-81 will be discussed later.

TABLE 1. *Triglyceride and phospholipid output in control animals*

Fast	0–2	2–4	4–6	6–8	8–10	10–12	12–14	14–16
Triglyceride output, μmol/h								
4.6 ±1.1	9.9 ±0.9	21.4 ±6.7	22.1 ±4.4	20.9 ±5.8	21.1 ±3.2	21.0 ±2.7	20.0 ±2.8	22.5 ±3.2
Phospholipid output, μmol/h								
1.3 ±0.2	2.6 ±0.3	4.1 ±0.3	3.9 ±0.9	3.8 ±0.8	3.9 ±0.8	3.7 ±0.7	4.1 ±0.6	4.0 ±0.7

Values are means ± SD; n = 3 rats. The animals were infused intraduodenally with egg lecithin (60 μmol/h) for 16 h. The analytical procedures involved are described in MATERIALS AND METHODS.

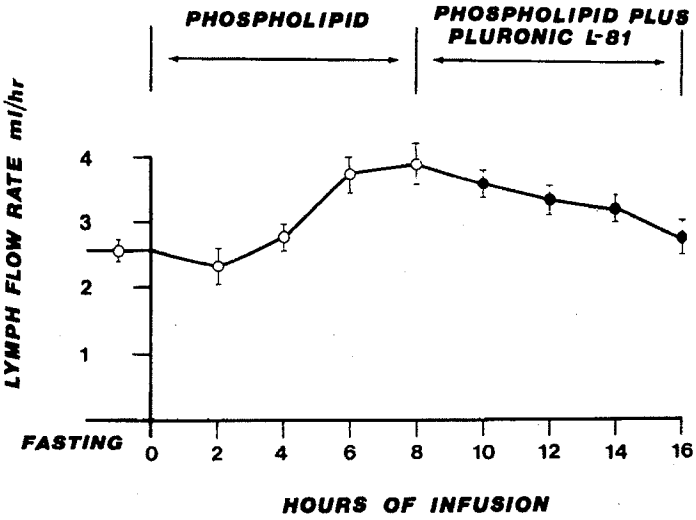

FIG. 1. Intestinal lymph flow. Intestinal lymph flow was measured in milliliters per hour before lipid infusion (fasting) and also every 2 h during 16 h of infusion of 60 μmol/h of egg lecithin without (first 8 h) and with 0.5 mg/h of Pluronic L-81 added (between 8 and 16 h). Seven animals were used for this study, and values are expressed as means ± SE.

Lymphatic triglyceride output. Figure 2 shows the lymphatic triglyceride output. The fasting triglyceride output was 4.23 ± 0.68 μmol/h in agreement with our previous studies (30). The triglyceride output increased drastically during PC infusion and reached a relatively steady output at the end of the 4th h of lipid infusion. The lymphatic triglyceride output then remained relatively constant (22–25 μmol/h) for the rest of the infusion period. The addition of L-81 did not affect the lymphatic triglyceride output. One difference observed in this study was that the triglyceride output observed was slightly lower (22–25 μmol/h) compared with our previous studies (30–32 μmol/h) (30). This is probably related to the metabolism of the products of PC lipolysis, fatty acid and lysolecithin, by the enterocytes. This point will be discussed later.

Phospholipid output. The fasting lymphatic phospholipid output was 1.34 ± 0.15 μmol/h, which is in agreement with out previous studies (Fig. 3). Phospholipid output increased concomitantly with triglyceride output during egg PC infusion. At the end of 4 h of lipid infusion, the phospholipid output into lymph was reaching a pla-

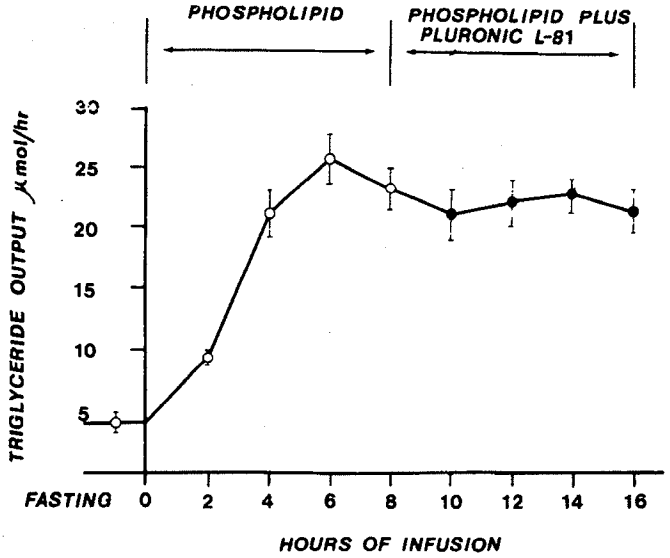

FIG. 2. Lymphatic triglyceride (TG) output. Lymph TG output was measured chemically during fasting (before lipid infusion) and also every 2 h during 16 h of infusion of 60 μmol/h of egg lecithin without (first 8 h) and with 0.5 mg/h of Pluronic L-81 added (between 8 and 16 h). Seven animals were used for this study, and values are expressed as means ± SE.

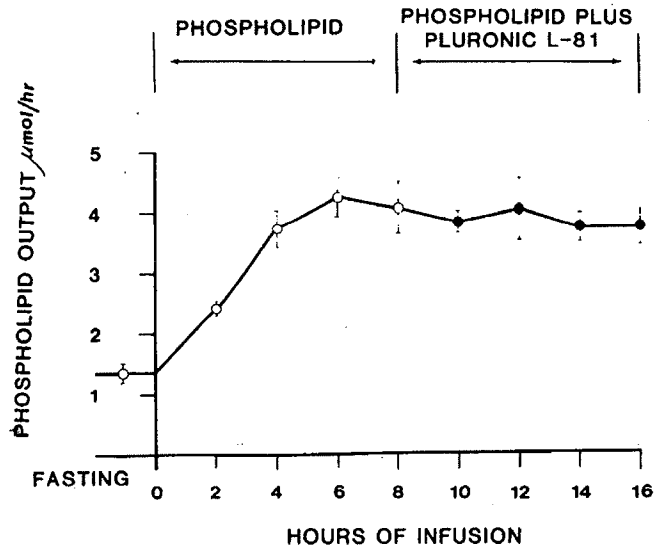

FIG. 3. Lymphatic phospholipid (PL) output. Lymph PL output was measured chemically during fasting (before lipid infusion) and also every 2 h during 16 h of infusion of 60 μmol/h of egg lecithin without (first 8 h) and with 0.5 mg/h of Pluronic L-81 added (between 8 and 16 h). Seven animals were used for this study, and values are expressed as means ± SE.

teau. The phospholipid output into lymph then remained relatively unchanged (3.8–4.1 μmol/h) for the rest of the lipid infusion period. The lack of suppression of lymphatic phospholipid output by L-81 paralleled its effect on triglyceride output. Although the esterified fatty acid infused in this study was egg PC, the phospholipid output observed in this study was comparable with that observed in our previous studies when the esterified fatty acid infused was mainly in the form of triolein.

Cholesterol output. The fasting cholesterol output was 1.69 ± 0.21 μmol/h. The lymphatic cholesterol output

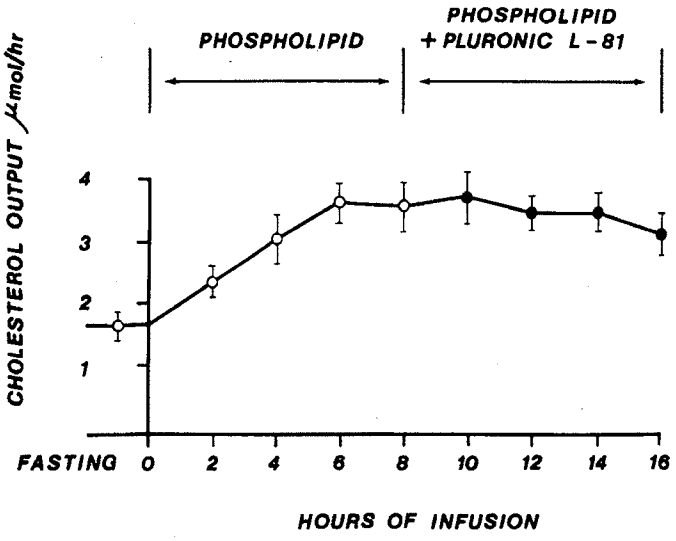

FIG. 4. Lymphatic cholesterol output. Lymph cholesterol output was measured chemically during fasting (before infusion of lipid) and also every 2 h during 16 h of infusion of 60 μmol/h of egg lecithin without (first 8 h) and with 0.5 mg/h of L-81 added (between 8 and 16 h). Seven animals were used for this study, and values are given as means ± SE.

closely paralleled that of triglyceride and phospholipid output (Fig. 4). The lymphatic cholesterol output increased following the feeding of egg PC and reached a steady output of 3.10–3.50 μmol/h. The cholesterol output in the presence of L-81 was significantly higher (3.1–3.4 μmol/h) than that observed in our previous studies (1.2–1.3 μmol/h).

Agarose gel electrophoresis of lymph lipoproteins. Figure 5*A* shows the electrophoretogram of intestinal lymph lipoproteins obtained from a rat actively absorbing lipid after administration of 0.5 ml olive oil via a gastric tube. The lipoproteins present were predominantly CM and pre-β migrating VLDL. Figure 5*B* shows the intestinal lipoproteins of lymph during fasting and following 0–2, 2–4, 4–6, and 6–8 h, respectively, of egg PC infusion without L-81. In contrast to the lipoprotein pattern shown in Fig. 5*A*, both fasting lymph and the intestinal lymph of rats fed with egg PC contained predominantly the VLDL particles and with trace amount of CM at the origin. Figure 5*C* shows the electrophoretograms of intestinal lymph collected during the infusion of egg PC plus L-81, i.e., the 8–10, 10–12, 12–14, and 14–16th h, respectively. The major lipoproteins present were the pre-β migrating VLDL. In contrast to the lymph electrophoretogram observed during PC infusion, VLDL secreted during PC plus L-81 infusion were noted to migrate faster and formed a wider zone. A trace of CM was present at the origin.

Particle size distribution of intestinal lymph lipoproteins. Lymph lipoproteins from four different groups were negatively stained, and their diameters were measured as the mean of two estimates perpendicular to each other. Six to eight hundred particles were counted in each group. Since we measured only between 200 and 300 intestinal lymph particles from each rat, there were at least three rats observed in each group. The four

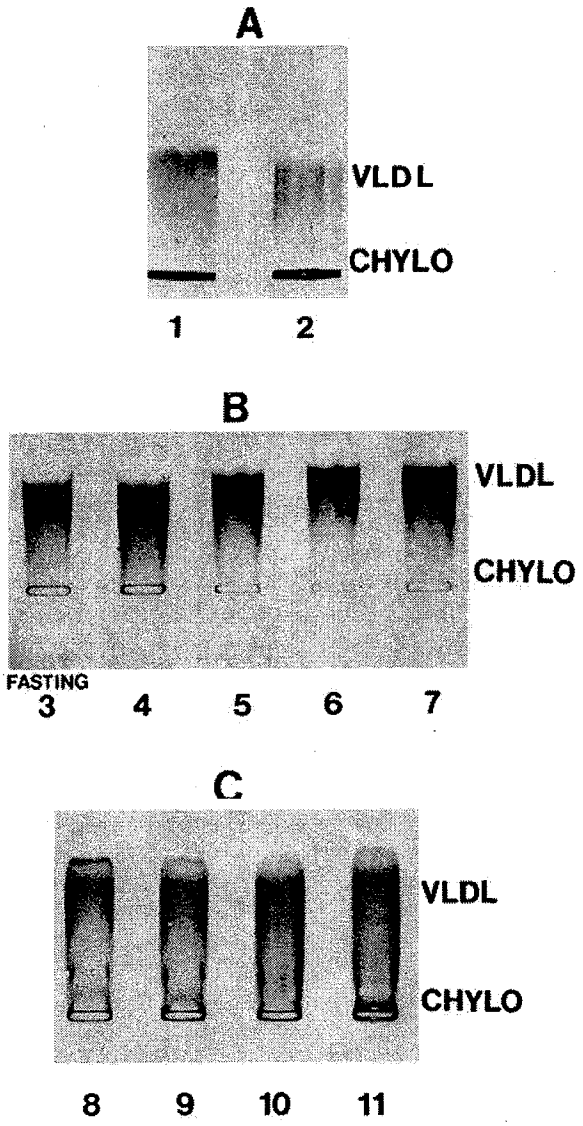

FIG. 5. Agarose gel electrophoresis of lymph lipoproteins. Small aliquots of intestinal lymph were used for separation of lipoproteins by agarose gel electrophoresis and stained with fat red 7B using apparatus and method supplied by Corning-ACI. *Lanes 1* and *2* correspond to intestinal lymph of a rat actively absorbing triolein; *lane 3*, fasting intestinal lymph; *lanes 4–7*, intestinal lymph collected during 0–2, 2–4, 4–6, and 6–8 h of infusion of egg lecithin; and *lanes 8–11*, intestinal lymph collected during 8–10, 10–12, 12–14, and 14–16 h of infusion of egg lecithin plus L-81.

groups were *1*) fasting intestinal lymph, *2*) intestinal lymph from a rat actively absorbing lipid after triolein infusion, *3*) intestinal lymph from a rat during the 7th and 8th h of egg PC infusion, and *4*) intestinal lymph from a rat during the 7th and 8th h of infusion of egg PC plus 0.5 mg/h of L-81. In *group 1* (Fig. 6*A*), the lymph contained predominantly VLDL-sized particles ranging in diameter from 200 to 900 Å, with a mean diameter of 465 Å. After infusing a lipid emulsion containing triolein, the size of the lipoproteins transported in intestinal lymph increased dramatically (Fig. 6*B*). Although some VLDL-sized lipoproteins were still present, the majority of the lipoproteins transported in lymph were of CM size with diameters ranging between 1,000 and 2,800 Å. Of the CM transported in lymph, the majority of the particles had diameters ranging from 1,200 to 1,500 Å. In contrast, the lipoproteins produced by the rat small intestine after infusion of egg PC were predominantly VLDL-sized particles (Fig. 7*A*), with a mean diameter of 584 Å. As shown in Fig. 7*B*, the infusion of L-81 with PC did not alter the size distribution of lipoproteins transported by the rat small intestine as compared with the rats infused with egg PC alone (cf. Fig. 7, *A* and *B*). Primarily VLDL-sized lipoproteins were observed, with a mean diameter of 523 Å. To summarize the morphological data, VLDL-sized particles were the major lipoproteins produced and transported by the rat small intestine during fasting and also during the infusion of egg PC, with or without the concomitant infusion of L-81. In contrast, CM were the predominant lipoproteins secreted in lymph when the rats were fed intraduodenally with triolein.

Lipid output in CM and VLDL. The CM and VLDL were isolated separately by ultracentrifugation from the fasting lymph, 6- to 8-h lymph (i.e., during egg PC infusion), and lymph during subsequent hours of infusion of egg PC plus L-81 (i.e., 8–16 h). Lipid from the lymph lipoprotein fraction was extracted by the method of Folch et al. (7), and the phospholipid, cholesterol, and triglyceride outputs were determined chemically (Table 2).

Phospholipid output. During fasting, most of the phospholipid transported in intestinal lymph was carried by VLDL (approximately 70% total lymph phospholipid) (Table 2). However, CM accounted for 15–20% of the fasting intestinal lymph phospholipid. After the infusion of egg PC, phospholipid output into both lymph CM and VLDL fractions increased significantly. There was twice as much phospholipid transported in the VLDL fraction (1.89 ± 0.09/μmol/h, mean ± SE) as in the CM fraction (0.90 ± 0.07 μmol/h). Although one-third of the lymphatic lipid was carried by the CM particles ($S_f > 400$), the majority of these lipoprotein particles possessed a diameter less than 1,000 Å (see the histogram in Fig. 7*A*). As demonstrated by Fraser (8), there could be a considerable overlap in size between different subfractions of CM of different S_f values. Thus, what we have isolated as the CM fraction by ultracentrifugation contains predominantly the "small CM," which could be called "large VLDL." The difference in intestinal CM and VLDL particles is based on a difference in their flotation characteristics. However, CM and VLDL particles overlap in physical characteristics and chemical compositions. The phospholipid output into both intestinal lymph CM and VLDL fractions remained unchanged during the subsequent 8 h of infusion of egg PC plus L-81. The 14- to 16-h phospholipid output was 0.83 ± 0.04 μmol/h for the CM and 2.07 ± 0.09 μmol/h for the VLDL.

Triglyceride output. The triglyceride output into lymph CM and VLDL closely followed that of phospholipid. During fasting, the triglyceride output into CM and VLDL was 0.81 ± 0.43 and 2.42 ± 0.71 μmol/h, respectively (Table 2). After egg PC infusion, the triglyceride output into CM and VLDL increased significantly and reached a steady output of 8.78 ± 1.18 μmol/h for the

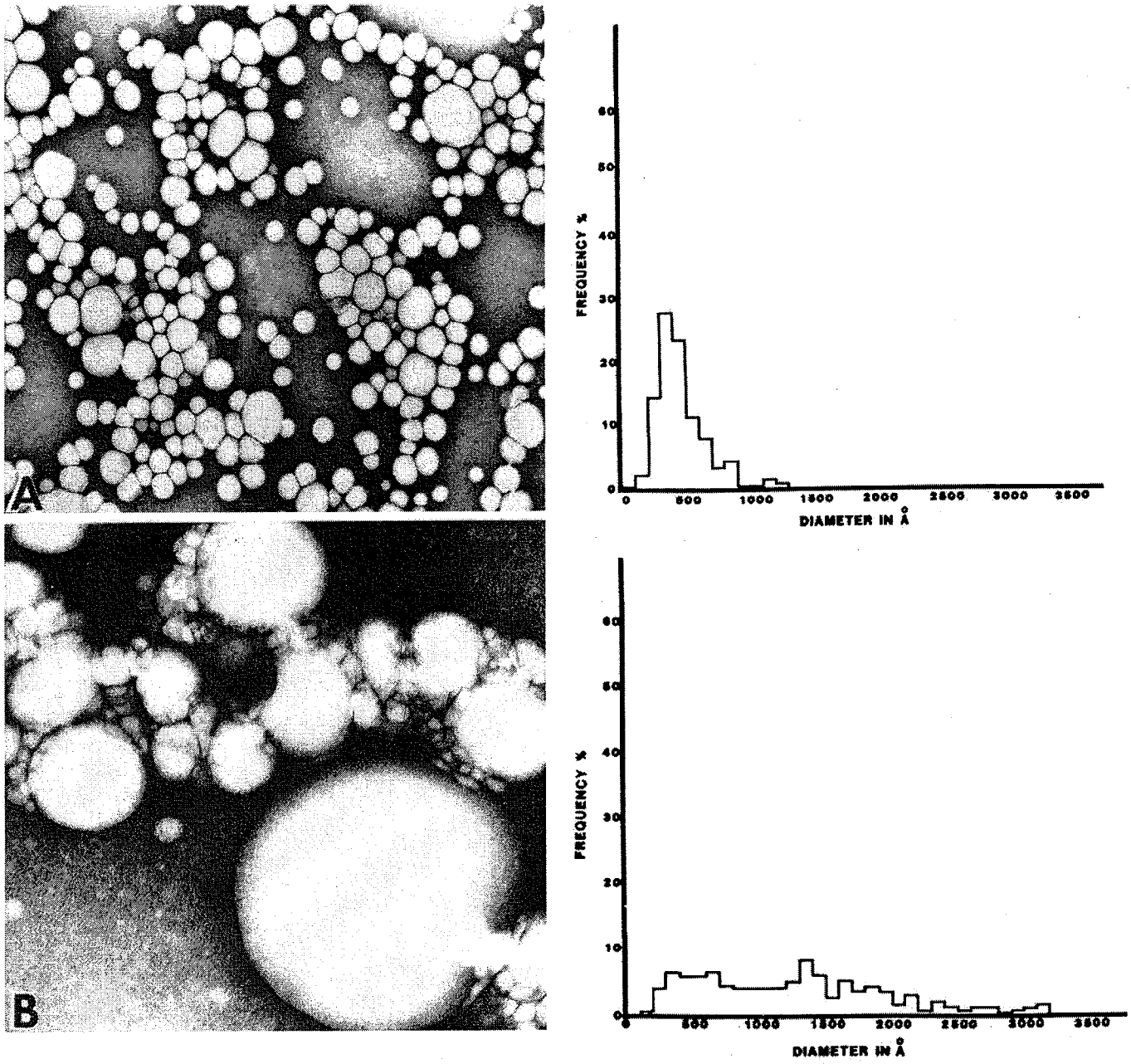

FIG. 6. Distribution of diameter of lymph lipoproteins. Electron micrograph and histogram of distribution of intestinal lymph lipoproteins negatively stained with 2% phosphotungstate (pH 5.9) and examined with a Zeiss 10 electron microscope. Magnification of micrographs is ×75,000. Histogram on right represents distribution of mean of diameter of 2 measurements perpendicular to each other of 600–800 particles. *A*: fasting intestinal lymph. *B*: intestinal lymph from a rat actively absorbing triolein.

CM fraction and 12.92 ± 3.21 μmol/h for the VLDL. The triglyceride output data confirmed that there were significantly more VLDL than CM carrying the lipid secreted by the small intestine when phospholipid, instead of triglyceride, was infused. As pointed out in the last section, there could be considerable overlap in the physical characteristics and chemical compositions between the CM and VLDL, separated by their flotation characteristics. Thus, what we have isolated as the CM fraction by ultracentrifugation probably contains mainly large VLDL particles. The triglyceride output into CM and VLDL for the 14- to 16-h lymph was 7.59 ± 2.31 and 12.57 ± 2.10 μmol/h, respectively.

Cholesterol output. The fasting CM and VLDL cholesterol outputs were 0.46 ± 0.14 and 0.70 ± 0.20 μmol/h, respectively (Table 2). The CM and VLDL cholesterol outputs increased as a result of egg PC infusion and reached a maximum of 0.82 ± 0.35 μmol/h for the CM and 1.49 ± 0.54 μmol/h for the VLDL during the 6–8 h of lipid infusion. Again, the presence of L-81 did not affect the cholesterol output into lymph CM and VLDL by the small intestine. Thus, the cholesterol output into CM and VLDL remained relatively constant during the infusion of egg PC plus L-81.

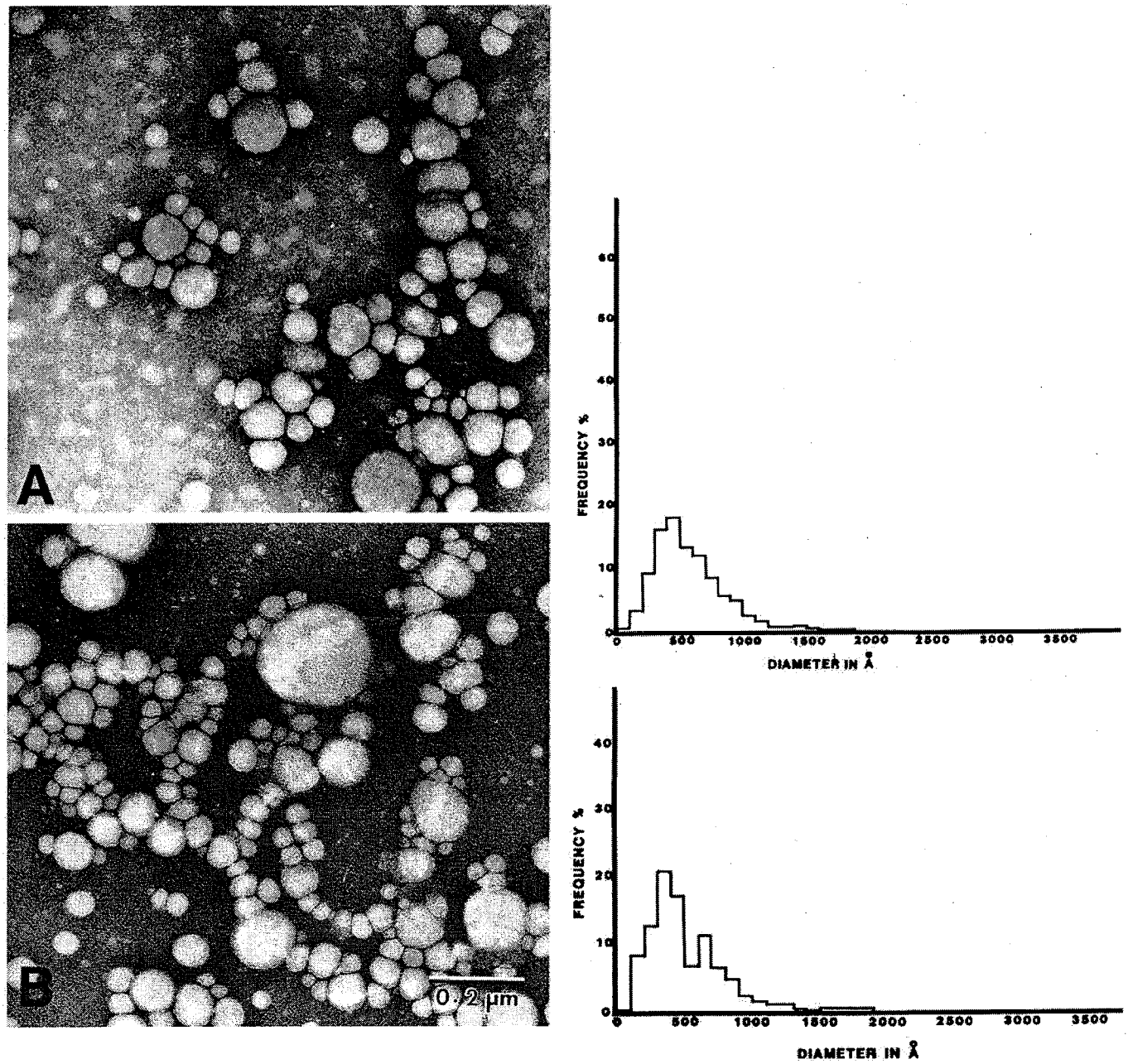

FIG. 7. Electron micrograph and histogram of distribution of intestinal lymph lipoproteins negatively stained with 2% phosphotungstate (pH 5.9) and examined with a Zeiss 10 electron microscope. Magnification of micrographs is ×75,000. Histogram on right represents distribution of mean of diameter of 2 measurements perpendicular to each other of 600–800 particles. *A*: intestinal lymph during infusion of egg lecithin (6–8 h). *B*: intestinal lymph harvested during infusion of egg lecithin plus L-81 (14–16 h).

Apolipoprotein composition of CM and VLDL. Figure 8, *A* and *B*, shows the apolipoprotein composition of the CM and VLDL as analyzed by SDS-PAGE, respectively. Ten percent of the hourly output of the CM protein isolated from the intestinal lymph during fasting, during the infusion of egg PC (6–8 h), and also during the infusion of egg PC plus L-81 (8–10, 10–12, 12–14, and 14–16 h) were applied onto the gradient (3.5–27%) polyacrylamide gel prior to electrophoresis. In fasting lymph only a small amount of apoC, apoA-I, and apoB was present and apoE and apoA-IV were absent. After infusion of egg PC, the output of apoA-I and apoC increased significantly, as indicated by the increased intensity of staining of these bands and apoA-IV appeared. Other than an increase in apoB output, the CM apolipoprotein pattern remained relatively unchanged during the subsequent 8 h of infusion of egg PC plus L-81. Figure 8*B* shows the analyses performed on the VLDL protein. As shown in the SDS-PAGE gel, there was significantly more protein in the VLDL during fasting compared to the CM fraction. Also, in contrast to the fasting CM, traces of apoA-IV and apoE were present in the fasting lymph VLDL. After beginning egg PC infusion, the output of all the major apolipoproteins increased signifi-

TABLE 2. *Phospholipid, triglyceride, and cholesterol output in experimental animals*

	Fast	Hour of Lipid Infusion 6–8	8–10	10–12	12–14	14–16
		Phospholipid output, μmol/h				
CM	0.34 ±0.06	0.90 ±0.07	0.77 ±0.05	0.90 ±0.06	1.01 ±0.08	0.83 ±0.04
VLDL	0.92 ±0.05	1.89 ±0.09	1.78 ±0.11	1.82 ±0.13	1.99 ±0.10	2.07 ±0.09
		Triglyceride output, μmol/h				
CM	0.81 ±0.16	8.78 ±0.45	6.90 ±0.75	6.71 ±0.91	7.00 ±0.59	7.59 ±0.87
VLDL	2.43 ±0.27	12.92 ±1.21	10.60 ±0.93	11.65 ±0.41	11.53 ±0.53	12.57 ±0.79
		Cholesterol output, μmol/h				
CM	0.46 ±0.14	0.82 ±0.35	0.95 ±0.30	0.87 ±0.19	0.85 ±0.18	0.93 ±0.32
VLDL	0.70 ±0.20	1.49 ±0.54	1.40 ±0.43	1.34 ±0.48	1.40 ±0.47	1.49 ±0.57

Values are means ± SE; $n = 7$ rats. The animals were infused intraduodenally with egg lecithin (60 μmol/h) for the first 8 h, followed by another 8 h of infusion of egg lecithin plus 0.5 mg/h of L-81. The chylomicrons (CM) and very low-density lipoproteins (VLDL) from the different lymph samples were isolated by ultracentrifugation, and lipid was extracted prior to chemical determination.

cantly in the VLDL. The apolipoprotein composition of the intestinal lymph VLDL was little affected by the presence of L-81, except that output of apoA-IV continued to increase even after infusion of egg PC plus L-81.

DISCUSSION

This study was conducted to address two hypotheses: *1*) that infusion of esterified fatty acid in the form of egg PC, instead of triglyceride, results in the formation and secretion of VLDL particles as the major vehicle for the transport of absorbed lipid, and *2*) that there are separate pathways of CM and VLDL assembly and transport by the enterocytes. We have demonstrated previously that L-81 interferes only with CM secretion (29); therefore, L-81 should not inhibit intestinal lipid transport with the infusion of egg PC since VLDL are the major intestinal lipoproteins synthesized under these experimental conditions. To compare the results of this investigation with the previous studies that we have conducted on the effect of L-81 on the absorption and transport of triolein, we infused egg PC at a rate of 120 μmol of esterified fatty acid per hour. This is similar to our previous studies, in which we infused 40 μmol/h of triolein (120 μmol/h of esterified fatty acid).

The lymph flow rate increased significantly after infusion of egg PC (3.87 ± 0.29 ml/h for the 6–8 h), and it was comparable with that observed when rats were infused with triolein (30). The lymph flow decreased as the result of infusion of egg PC plus 0.5 mg/h L-81 (2.78 ± 0.25 ml/h during the 14–16 h). The decrease in lymph flow rate could be explained by negative fluid balance resulting from the first 8 h of infusion of egg PC only, but might also be related to the addition of L-81 after 8 h.

The presence of L-81 had little effect on the intestinal lymphatic output of triglyceride, phospholipid, and cholesterol. It is important, however, to note that the triglyceride output observed in this study (22–25 μmol/h during the 6–8 h) was lower than that observed by us when the same amount of esterified fatty acid was infused as triolein (30–32 μmol/h). The difference in the lymphatic triglyceride output we have observed between the two studies could be partly attributed to differences in the availability of the fatty acids for triglyceride biosynthesis after triglyceride or PC infusion. Lecithin in the intestinal lumen is broken down by phospholipase A_2 to form lysolecithin and fatty acid, which are then absorbed without further degradation (21, 27). In the intestinal mucosa, the absorbed 1-lysolecithin can enter either one of two metabolic pathways. It can be converted back to PC after reacylation with acyl-CoA (16, 21), or it can be hydrolyzed to glycerophosphorylcholine and fatty acid by lysolecithinase present in the intestinal mucosa (5, 24). When triolein is infused, the lipolytic products 2-monoglyceride and oleic acid are absorbed and then rapidly resynthesized to triglyceride and assembled into CM (26). In contrast, fatty acids derived from PC are resynthesized into triglyceride via the glycerol-3-phosphate pathway. The glycerol-3-phosphate pathway is also involved in the de novo synthesis of PC via the provision of diglyceride for the Kennedy pathway (11, 12). Consequently, less fatty acids are available for triglyceride biosynthesis when esterified fatty acids are infused intraduodenally in the form of PC rather than triglyceride.

The failure of L-81 to inhibit lymphatic lipid transport when egg PC instead of triolein is infused is important and prompted us to determine specifically the class of lipoproteins synthesized by the intestinal epithelial cells when egg PC is infused. Agarose gel electrophoresis showed that CM were the major class of lipoproteins in intestinal lymph after intraluminal triolein infusion. In contrast, the pre-β migrating VLDL were the major lipoproteins in intestinal lymph after egg PC infusion. This observation was further supported by the electron microscopic quantitation of lipoprotein size. VLDL-sized particles were the main lipoproteins in intestinal lymph during fasting, confirming the earlier observation of Ockner and co-workers (22); however, after fat feeding, lipoprotein size changed according to the type of lipid fed. With triolein feeding, the major lipoproteins in intestinal lymph were CM, ranging in diameter mostly between 1,200 and 1,500 Å. In contrast, VLDL-sized particles were the major lipoproteins secreted by the enterocytes after feeding egg PC. The mean diameter of these VLDL particles was 523 Å, not too different from 465 Å in fasting VLDL. The infusion of egg PC with L-81 had no effect on the lipoprotein particle size transported in intestinal lymph.

To further validate that VLDL were the major lipoproteins secreted into intestinal lymph when egg PC was fed, CM and the VLDL were isolated from the lymph by ultracentrifugation and lipid outputs determined. The CM output-to-VLDL output ratio were 0.48, 0.65, and 0.55 for phospholipids, triglycerides, and cholesterol, respectively, during the 6–8 h of egg PC infusion. There-

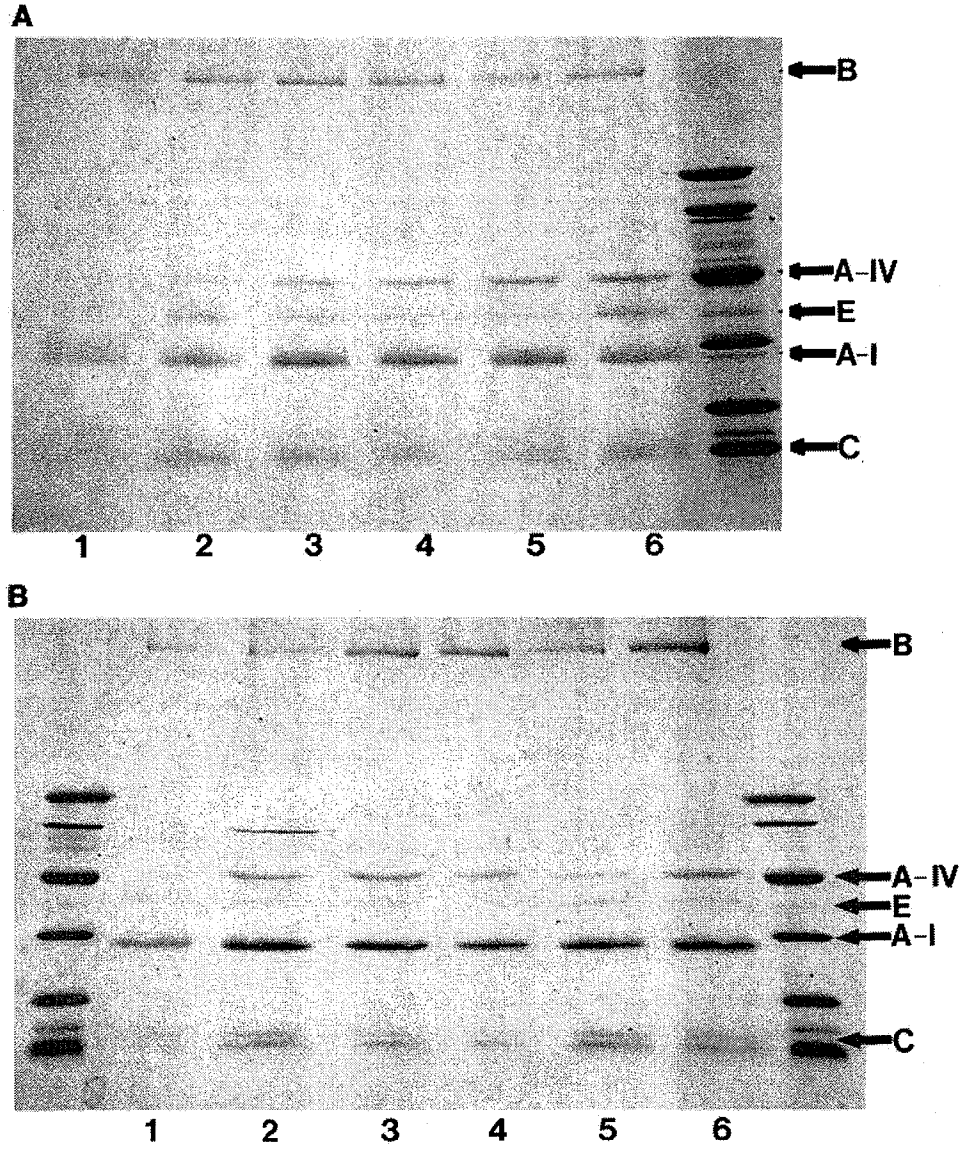

FIG. 8. Apolipoprotein composition of intestinal chylomicrons (CM) and very low-density lipoproteins (VLDL). *A*: apolipoprotein composition of CM as analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Ten percent of hourly output of CM protein isolated from intestinal lymph during fasting (*lane 1*), during infusion of egg lecithin (6–8 h, *lane 2*), and also during infusion of egg lecithin plus L-81 (8–10, 10–12, 12–14, and 14–16 h corresponding to *lanes 3–6*, respectively) were applied onto gradient (3.5–27%) polyacrylamide gel prior to electrophoresis. Staining and destaining of gel and also identification of various apolipoproteins are described in MATERIALS AND METHODS. Protein standards at both sides of CM samples consist of phosphorylase B, 92,500; bovine serum albumin, 66,200; ovalbumin, 45,000; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; and lysozyme, 14,400. *B*: gradient gel of corresponding VLDL samples. Ten percent of hourly output of VLDL protein was applied in each lane.

fore, again these data confirmed that there were considerably more VLDL than CM being secreted by the small intestine with egg PC feeding. As discussed in RESULTS, there is considerable overlap in the physical properties and chemical composition of CM and VLDL separated by ultracentrifugation. Thus, the majority of the particles in the CM fraction isolated by ultracentrifugation in our study are probably large VLDL particles. This is supported by the fact that almost all of the lymph lipoproteins are less than 1,000 Å. Although our observation supports that of Beil and Grundy (1) that VLDL are the major lipoproteins produced by the small intestine when soybean PC is fed in humans, it contradicts their observation in the rat. They observed that the rat intestine transported mainly CM when the rat was fed comparable doses of soybean PC (1). The discrepancy observed between our observations and that of Beil and Grundy (1) could be explained by the type of PC used. The egg PC used in our study is rich in palmitate (33%) and poor in linoleate (17%), whereas soybean PC is poor in palmitate (13%) but extremely rich in linoleate (67%). Ockner et al. (23) demonstrated that feeding of saturated fatty acid (palmitic acid) stimulated the production of VLDL, and yet feeding of unsaturated FA (linoleic acid) resulted in the production of mainly CM particles. Thus, the difference between our observations and those of Beil and Grundy (1) may be due to a difference in the fatty acid composition of the PC fed. Furthermore, there are other differences in experimental design that may be responsible for the discrepancy in RESULTS. We allowed our rats to recover for 36 h prior to fat feeding, whereas Beil and Grundy (1) only allowed 6–8 h. We allowed a long recovery period in our experiments because Morgan (20) demonstrated that rats absorbed lipid significantly better at 48 h than at 24 h after operation. This may explain the big difference in the total fatty acids recovered in lymph in the two rats they studied (49% in one and 94% in the other) (1).

The apolipoprotein composition of the intestinal lymph VLDL and the CM was quite similar and agreed with the observation of other investigators. The presence of L-81 did not affect the apoB content, as analyzed by

SDS-PAGE, of the intestinal CM and VLDL. The only modification in apolipoprotein composition of CM and VLDL caused by L-81 was in apoA-IV. The amount of apoA-IV relative to the other apolipoproteins in the VLDL fraction increased significantly during the 8–16 h of lipid infusion (i.e., phospholipid plus L-81). The increase in apoA-IV was probably caused by the phospholipid infusion rather than the infusion of L-81. Krause et al. (13) reported a similar increase in the secretion of apoA-IV by the rat small intestine during the 24 h of continuous intraduodenal administration of a lipid emulsion.

In this study, we have demonstrated that the infusion of egg PC stimulated the small intestine to transport mainly VLDL-size lipoproteins, which have also been called "small CM" by some investigators (10). We have also shown that L-81 has no effect on lipid transport into lymph when infused with egg PC, supporting our previous observations suggesting that L-81 only affects the assembly of CM particles (31). There are two different mechanisms that can explain the observation made in this study. First, there could be two separate pathways for the packaging and secretion of intestinal CM and VLDL. While L-81 inhibits the pathway for the production of CM, the packaging and secretion of VLDL are unaffected. Alternatively, one can propose that L-81 inhibits the formation of large CM, which are derived directly from small CM, suggesting one process for the formation of CM and VLDL. However, our previous observations (29) would argue against the latter explanation as we failed to observe an increase in the transport of VLDL-sized particles in the lymph of rats infused intraduodenally in the triolein plus L-81 group. Rather, the lymphatic triglyceride output was markedly reduced as compared with rats given the triolein only.

The concept that there are two separate pathways for the packaging of CM and VLDL in the small intestine is supported by observations reported by different investigators. Ockner, Hughes, and Isselbacher (23) showed that the infusion of palmitate resulted in a marked increase in VLDL transport, whereas with the infusion of oleate and linoleate VLDL output in lymph remain unchanged. Furthermore, the fatty acid composition of the triglyceride from VLDL was different from that of CM, therefore suggesting different pathways of assembly for the VLDL and the CM particles. Mahley and co-workers (18) showed that rat enterocyte Golgi vesicles contained either CM or VLDL particles, with little mixing of particle sizes. Recent studies by Feldman et al. (6) demonstrated that cholesterol and triglyceride differentially affect the particle size of intestinal lymph lipoproteins. With increasing cholesterol absorption, more lipid was carried by VLDL in contrast to the preferential increase in CM when more triglyceride was absorbed. The present studies provide the first observations showing that L-81 may be a unique tool for studying the two separate pathways for the intestinal formation and transport of CM and VLDL. Despite the evidence for two pathways for CM and VLDL production, there is undoubtedly some overlap in the size and the chemical composition of the lipoproteins produced by the intestine. This overlap probably accounts for the observation that the intestinal lymph of fat-fed rats contains a continuous spectrum of triglyceride-rich lipoproteins ranging in size from 300 to 4,500 Å (33, 36).

We propose the following hypothesis depicting the intracellular packaging of intestinal CM and VLDL. When triglyceride and PC are infused separately, the fatty acids derived from triglyceride and PC are handled differently by the enterocytes (Fig. 9). The fatty acids from the triglyceride are used mainly for the production of CM, whereas those from PC stimulate VLDL formation predominantly. The pathways for the formation of CM and VLDL are different, and the former is sensitive to the effect of L-81, whereas the latter is not. The pre-

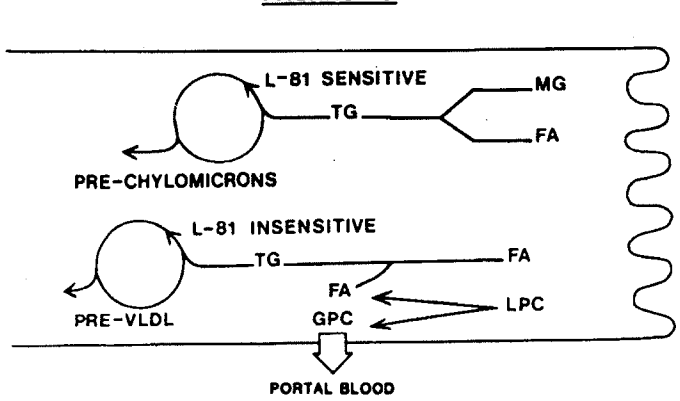

FIG. 9. Formation of intestinal pre-chylomicron (CM) and pre-very low-density lipoprotein (VLDL) particles. This diagram depicts packaging of pre-CM and pre-VLDL particles by enterocytes of small intestine. Absorbed monoglyceride (MG) and fatty acid (FA), major digestion products of triglyceride (TG), are reconstituted in cell to form TG by monoglyceride pathway, which is then subsequently packaged into predominantly pre-CM particles. This pathway is inhibited by presence of L-81. On contrary, absorbed FA and also FA derived from hydrolysis of absorbed lysolecithin are used to form TG via the α-glycerophosphate pathway, which is then packaged into pre-VLDL particles. Unlike formation of pre-CM, this pathway is not sensitive to L-81.

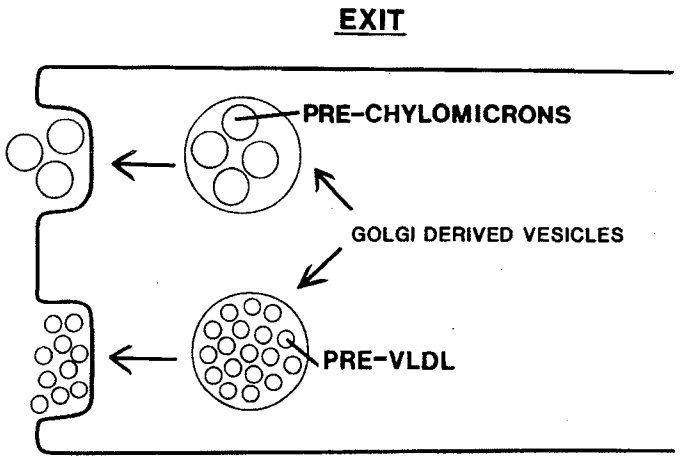

FIG. 10. Packaging and secretion of intestinal chylomicrons (CM) and very low-density lipoproteins (VLDL). This diagram depicts Golgi-derived vesicles containing either pre-CM or pre-VLDL particles. There is little admixing of pre-CM and pre-VLDL particles in those vesicles. Basis for this diagram is derived from our present data and data of Mahley et al. (18).

CM and pre-VLDL particles are then packaged in the Golgi complex into either pre-CM- or pre-VLDL-containing vesicles (Fig. 10). However, there are questions that we are currently unable to explain by our hypothesis, e.g., why should L-81 preferentially inhibit CM secretion if the final common pathway for secretion of CM and VLDL is packaging in the Golgi apparatus. Preliminary morphological study showed that, when L-81 alone was infused intraduodenally to a fasting rat, numerous VLDL-sized particles were present in the Golgi cisternae of the cells from the villus tip of the upper jejunum. In contrast, when the cells from a similar region of the small intestine were examined by electron microscopy after infusion of triolein plus L-81, numerous large lipid droplets accumulated in dilated cisternae of the endoplasmic reticulum. Some osmiophilic lipid droplets were as large as 15,000 Å and the Golgi cisternae were devoid of CM-sized particles. These preliminary morphological observations suggest a difference in the packaging of intestinal CM and VLDL particles, although both lipoprotein particles are processed in the Golgi apparatus. In our laboratory, ultrastructural studies are currently underway to examine further the formation and secretion of CM and VLDL during various conditions of lipid infusion and the effects of L-81 on these pathways.

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Appendix J

Carnielli et al. "Intestinal absorption of long-chain polyunsaturated fatty acids in preterm infants fed breast milk or formula" Am J Clin Nutr 67: 97-103 (1998)

Intestinal absorption of long-chain polyunsaturated fatty acids in preterm infants fed breast milk or formula[1–3]

Virgilio P Carnielli, Giovanna Verlato, Fabio Pederzini, Ingrid Luijendijk, Anneke Boerlage, Dino Pedrotti, and Pieter JJ Sauer

ABSTRACT The importance of long-chain polyunsaturated fatty acids (LCPs) in the development of preterm infants is now well accepted but the source of dietary LCPs to be added to infant formulas remains controversial. We measured dietary intakes, fecal output, and percentages of intestinal absorption of n−6 and n−3 LCPs in healthy preterm infants fed exclusively preterm breast milk (PBM; $n = 20$), formula without LCPs added (NLCPs; $n = 19$), formula with LCPs derived from phospholipids (PL-LCPs; $n = 19$), or formula with LCPs from triacylglycerols (TG-LCPs; $n = 19$). Intestinal absorption of arachidonic acid was not different in the four groups but docosahexaenoic acid was better absorbed from PL-LCPs than from PBM (88.3 ± 1.8% compared with 78.4 ± 4.0%, $P < 0.05$) Total absorption of n−6 LCPs was not different between groups but total n−3 LCPs were better absorbed from PL-LCPs than from PBM or TG-LCPs (88.7 ± 1.9%, 79.2 ± 4.4%, and 80.4 ± 2.2%, respectively). In conclusion, docosahexaenoic acid and arachidonic acid were absorbed as efficiently from TG-LCPs formula as from breast milk fat. Absorption of docosahexaenoic acid and n−3 LCPs was greater from PL-LCPs formula than from PBM or TG-LCPs formula. *Am J Clin Nutr* 1998;67:97–103.

KEY WORDS Polyunsaturated fatty acids, feeding study, preterm infants, infant formula, breast milk, fat absorption, premature infants, arachidonic acid, docosahexaenoic acid

INTRODUCTION

Arachidonic acid (AA, 20:4n−6) and docosahexaenoic acid (DHA, 22:6n−3) are the predominant long-chain polyunsaturated fatty acids (LCPs) derived by chain elongation and desaturation of the parent essential fatty acids linoleic acid (18:2n−6) and linolenic acid (18:3n−3), respectively. LCPs may be conditionally essential for premature infants (1–3). AA was associated with growth of fetuses (4) and infants (5). Although it was shown that both preterm (6) and full-term infants (7) are capable of synthesizing AA and DHA from linoleic acid and linolenic acid, infants who consumed formulas containing DHA had better early visual acuity than control infants (1–3, 8). The finding of higher concentrations of LCPs in the brains of infants who died from sudden infant death and were breast-fed than in the brains of bottle-fed control infants also supports the current view of the importance of dietary LCPs during infant development (9, 10).

Very little information is available on the amount and ideal source of LCPs to be added to formulas consumed by infants. It is still unclear whether the fat blend in infant formulas should mimic that in breast milk as much as possible or whether dietary intake of LCPs should produce plasma concentrations and biological effects similar to those in the fetus in utero or in breast-fed infants.

Breast milk fat consists of 98% triacylglycerols, 1% phospholipids, and 0.5% cholesterol and cholesterol esters (11). Although this composition remains rather constant throughout lactation, fatty acid composition of these lipid classes is affected by diet. DHA, for instance, is found in amounts ranging from 0.05% to 1.4% of total fatty acids (12). LCPs in breast milk are mainly in triacylglycerols, in which they are primarily esterified at the *sn*-2 and *sn*-3 positions (13, 14), but they also occur in phospholipids (15).

In formulas for infants, LCPs are added to the fat blend by using relatively highly unsaturated lipids, such as fish oil (mainly triacylglycerols), designer oils (mainly triacylglycerols) derived from unicellular organisms, or egg phospholipids. These products differ from breast milk lipids and differences in metabolism might be expected. The differences could result from a different fatty acid composition, from different proportions of triacylglycerols and phospholipids in the fat blend, and from differences in molecular structure.

Little is known about the intestinal handling of highly unsaturated molecular species reported to be relatively resistant to the action of pancreatic lipase (16). Hernell at al (17) found relative resistance to hydrolysis of triacylglycerols containing AA and eicosapentaenoic acid (20:5n−3) when triacylglycerols from rat chylomicrons were incubated in vitro with human pancreatic lipase and colipase. Few data are currently available (18) on intestinal absorption of LCPs in preterm infants, who often have impaired fat absorption during the first weeks of life (19–22). We studied dietary intakes, fecal excretion, and intestinal absorption of LCPs in preterm infants fed exclusively preterm breast milk

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(PBM), preterm formula without LCPs added (NLCPs), or preterm formulas supplemented with LCPs derived from egg phospholipids (PL-LCPs) or LCPs from triacylglycerols derived from unicellular organisms (TG-LCPs).

SUBJECTS AND METHODS

Subjects and clinical design

Four groups of preterm infants were fed either PBM (n = 20), NLCPs (n = 19), PL-LCPs (n = 19), or TG-LCPs (n = 19) formula. Composition of feedings is shown in **Table 1**. All infants were fed exclusively with either PBM or one of the study formulas until they were ≥5-wk old. Infants were free of major diseases and were growing at the time of the study. Clinical characteristics of the infants are shown in **Table 2**. Participation in the study was voluntary and written informed consent was obtained from the infants' parents. The project was approved by the Ethics Committee of Sophia Children's Hospital/Erasmus University Rotterdam, Rotterdam, Netherlands, which agrees with the principles in the Declaration of Helsinki. Subject assignment was done randomly on the basis of unavailability of breast milk or parental decision not to breast-feed. Analyses were conducted by researchers who were blinded to the dietary assignment.

The study consisted of 72-h metabolic balance assessments and gas-chromatographic determinations of dietary and fecal fatty acids. The balance evaluations were performed in infants 28-d old. Fat and fatty acid composition of PBM was obtained from direct determinations in milk samples representative of the milk fed to each infant during the study. Values for the protein and carbohydrate contents of PBM were obtained from the study by Anderson et al (23). Total lipids were extracted from milk by using the Röse-Gottlieb method (24) and amounts were determined gravimetrically. Plasma concentrations of fatty acids in infants fed PBM were reported recently (25). The NLCPs and TG-LCPs formulas were produced exclusively for this study (Nutricia Research, Zoetermeer, Netherlands) and had similar compositions, including similar fatty acid profiles, except for the addition of LCPs. The PL-LCPs formula used is currently on the market (Aptamil with Milupan; Milupa, Friedrichsdorf, Germany).

TABLE 1
Composition of the study feedings[1]

	PBM (day 28) (n = 20)	NLCPs (n = 10)	PL-LCPs (n = 6)	TG-LCPs (n = 10)
Energy				
(kJ/L)	2923[2]	3328	3328	3328
(kcal/L)	720[2]	800	800	800
Protein (g/L)	18.1[2]	22	22.8	22
Carbohydrates (g/L)	69.5[2]	80	88.0	80
Lactose (g/L)	—	40	57.1	40
Dextrine maltose (g/L)	—	40	30.8	40
Fat (g/L)	39 ± 4	44	40.0	44
Fatty acid (% by wt)				
8:0	0.29	2.29	1.23	2.20
10:0	1.69	1.92	1.41	1.88
12:0	7.85	14.08	5.27	14.34
14:0	9.04	6.72	5.40	6.47
16:0	22.24	9.33	26.59	8.65
18:0	6.4	4.35	7.35	4.48
20:0	0.21	0.27	0.24	0.29
16:1n−7	2.22	0.21	1.04	0.17
18:1n−9	33.12	43.15	32.50	42.49
18:1n−7	1.99	0.63	2.05	0.63
18:2n−6	10.29	14.14	11.92	14.05
20:4n−6	0.48	0	0.35	0.84
18:3n−3	0.47	1.23	0.98	1.21
20:5n−3	0.07	0	0.03	0
22:6n−3	0.26	0	0.24	0.64
Total n−6 LCPs	1.34	0	0.55	0.97
Total n−3 LCPs	0.53	0	0.45	0.64

[1] PBM, preterm breast milk; LCPs, long-chain polyunsaturated fatty acids; NLCPs, no added LCPs; PL-LCPs, LCPs from phospholipids; TG-LCPs, LCPs from triacylglycerols.

[2] Values from Anderson et al (23).

Balance studies

Intakes of formulas or PBM were determined by weighing bottles or, in the case of tube feeding, by recording the volume in syringes. Balance studies with separate collections of urine (24 h) and feces (72 h) were begun when infants were 28-d old. Fecal collections were carried out by bracketing with carmine red. Fifty milligrams of carmine red was dissolved in 3 mL distilled water and given either through a nasogastric tube or orally just before the 1200 feeding. Feces were then collected, including the first red stool and excluding the "second" first red stool about 3 d later. Collection was done by using plastic sheets placed inside diapers. Corrections were made for accidental losses of feces in diapers (double weighing of the diaper) and for feces sticking to the buttocks of the infants (double weighing of the cleaning swabs).

The total amount of feces collected during the 3-d balance period was weighed and homogenized and a small sample of the homogenate was freeze-dried. Fat excretion was determined by using a modification of the method of Jeejeebhoy et al (26), with twice as much hydrochloric acid added. Excretion amounts were calculated by multiplying the volume of feces produced by the concentration of the compound of interest. Intestinal absorption was calculated by dividing the apparent amount absorbed (intake minus excretion) by the intake and multiplying by 100.

Determination of fatty acids in formulas and feces

Contents of individual fatty acids in the feedings and feces were determined by high-resolution capillary-gas chromatography. Analyses were done in triplicate. Fresh fecal samples (5–10 mg each) were subjected to *trans*-esterification by hydrochloric acid methanol after the addition of nonanoic and heptadecanoic acids as internal standards. Fatty acid methyl esters were separated and identified with a gas chromatograph (5890 II; Hewlett Packard, Amstelveen, Netherlands) equipped with a fused silica column (Supelcowax 10; 60 m × 0.20 mm internal diameter; 0.20-μm film thickness; Supelco, Zwijndrecht, Netherlands), a flame ionization detector (280 °C), and a split-splitless injector used in splitless mode (280 °C).

The gas chromatograph was operated with the following temperature program: 60 °C initially for 5 min, raising of the oven temperature by 20 °C/min until 205 °C was reached, and holding at this temperature for 15 min. The temperature was then increased by 0.20 °C/min until 222 °C was reached. Helium was used as a carrier gas (2 mL/min) and peak areas were calculated with HP-Chem station software (Hewlett Packard) by using nonanoic and

TABLE 2
Clinical characteristics of the infants studied[1]

Characteristic	PBM (n = 20)	NLCPs (n = 19)	PL-LCPs (n = 19)	TG-LCPs (n = 19)
Sex (M/F)	9/11	8/11	8/11	10/9
Birth weight (kg)	1.10 ± 0.31[2]	1.09 ± 0.22	1.21 ± 0.20	1.04 ± 0.25
Gestational age (wk)	29.8 ± 2.6	29.1 ± 2.2	30.4 ± 2.2	29.3 ± 2.0
Weight at balance study (kg)	1.42 ± 0.41	1.48 ± 0.29	1.46 ± 0.19	1.51 ± 0.2

[1] n = 77; PBM, preterm breast milk; LCPs, long-chain polyunsaturated fatty acids; NLCPs, no added LCPs; PL-LCPs, LCPs from phospholipids; TG-LCPs, LCPs from triacylglycerols.
[2] $\bar{x}$ ± SD; there were no significant differences between groups.

heptadecanoic as internal standards. Fatty acids were identified by comparing retention times with known standards (NuChek Prep, Elysian, MN). All reagents were analytic grade.

Total n−6 LCPs were calculated as the sum of the following fatty acids: 20:2n−6, 20:3n−6, 20:4n−6, 22:4n−6, and 22:5n−6. Total n−3 LCPs resulted from the sum of 20:3n−3, 20:4n−3, 20:5n−3, 22:5n−3, and 22:6n−3. For the purposes of this study, fecal hydroxy fatty acids were assumed to be represented by the gas-chromatographic peaks that changed retention time after acetylation of fatty acid methyl esters. Acetylation of hydroxy fatty acid methyl esters was done in selected fecal samples with acetyl chloride for 1 h at 100°C (Merck, Milan, Italy). Under the gas-chromatographic conditions used, no coelution occurred between fecal hydroxy fatty acids and any of the LCPs. Selected fatty acids were identified with use of a quadrupole mass spectrometer (MD 800 GC-MS; Fisons, Milan, Italy). No attempt was made to identify *trans* fatty acids.

Statistical analysis

Data are presented as group means ± SEMs unless otherwise stated. Group comparisons were done with analysis of variance; differences among groups were tested with Tukey posttests. All calculations were done with the SYSTAT statistical package (version 5.2; Systat Inc, Evanston, IL).

RESULTS

Clinical characteristics of the infants studied were not different between groups (Table 2). Dietary intakes of PBM or formula, fecal output, and fat-balance data are given in **Table 3**. Infants fed PBM had a higher fluid intake, which is often recommended with breast milk, which has a lower energy and protein content than preterm formulas. Fluid intake was also higher in infants fed PL-LCPs formula than in those fed the other two formulas. This made fat intakes comparable among the study groups.

The infants fed PBM had the lowest fecal fat content (226.0 ± 69.9 mg/g); the amount was not significantly different from that in the PL-LCPs group (226.4 ± 62.7 mg/g). Infants in the other two formula groups had significantly higher values (305.3 ± 92.6 and 273.7 ± 59.9 mg/g, respectively, in the NLCPs and TG-LCPs groups; P = 0.002). These differences were also found for percentages of total fat absorption because fecal output was not different among the four groups.

Intakes and output of individual fatty acids are given in **Tables 4** and **5**. Palmitic acid intake in the PBM and PL-LCPs groups was not different but the intake values were significantly higher that those in the NLCPs and TG-LCPs groups. In the PBM group, AA intake was 32.0 ± 2.1 mg·kg^{-1}·d^{-1} but intake of total n−6 LCPs was three times higher (92.9 ± 5.6 mg·kg^{-1}·d^{-1}). The same difference applied to intakes of DHA and total n−3 LCPs, which were 18.0 ± 1.3 and 37.7 ± 2.9 mg·kg^{-1}·d^{-1}, respectively, indicating the presence of other n−3 and n−6 polyunsaturated fatty acids in addition to AA and DHA. Lower amounts of these other fatty acids were found in the PL-LCPs formula but there were virtually none in the TG-LCPs formula.

Metabolizable intakes of individual fatty acids in the 77 infants are shown in **Table 6**. Intestinal absorption of each individual fatty acid for which dietary intake could be calculated is shown in **Table 7**. Palmitic acid was better absorbed from PBM than from formulas. Linoleic acid was also better absorbed from PBM (88.1 ± 3.0%) and PL-LCPs formula (91.3 ± 1.7%) than from the NLCPs (69.7 ± 3.4%) and TG-LCPs (68.9 ± 4.0%) formulas. AA was absorbed in similar percentages from PBM, PL-LCPs, and TG-LCPs. Percentages of DHA absorption were similar in the PBM and TG-LCPs

TABLE 3
Dietary intakes, fecal output, and fat-balance data in preterm infants fed preterm breast milk or formula[1]

Variable	PBM (n = 20)	NLCPs (n = 19)	PL-LCPs (n = 19)	TG-LCPs (n = 19)	P value[2]
Dietary intake (mL·kg^{-1}·d^{-1})	173 ± 14^{a}	154 ± 5^{b}	160 ± 5^{a}	153 ± 11^{b}	0.000
Fecal output (g·kg^{-1}·d^{-1})	5.3 ± 2.9	7.0 ± 2.1	6.8 ± 1.8	7.0 ± 1.7	0.080
Fecal fat (mg/g)	226.0 ± 69.9^{a}	305.3 ± 92.6^{b}	226.4 ± 62.7^{a}	273.7 ± 59.9^{a,b}	0.002
Fat intake (g·kg^{-1}·d^{-1})	7.5 ± 2.1a	6.6 ± 0.4^{a,b}	6.1 ± 0.2^{b}	6.6 ± 0.5^{b}	0.002
Fat output (g·kg^{-1}·d^{-1})	1.3 ± 0.9^{a}	2.1 ± 0.6^{b}	1.5 ± 0.4^{a,b}	1.9 ± 0.7^{b}	0.001
Fat absorption (%)	82.3 ± 13.8^{a}	68.6 ± 9.2^{b}	75.6 ± 6.9^{a,b}	69.9 ± 10.9^{b}	0.000

[1] $\bar{x}$ ± SD; n = 77. PBM, preterm breast milk; LCPs, long-chain polyunsaturated fatty acids; NLCPs, no added LCPs; PL-LCPs, LCPs from phospholipids; TG-LCPs, LCPs from triacylglycerols. Values with different superscript letters are significantly different, $P \leq 0.05$ (Tukey test).
[2] By ANOVA.

groups but lower than that in the PL-LCPs group.

DISCUSSION

This study showed that in small preterm infants fed PBM, LCPs are not absorbed completely, as has been believed, and that LCPs bound to phospholipids are better absorbed than triacylglycerol LCPs from either formula or breast milk. The study provided further evidence that intestinal absorption of total fat and palmitic acid is better from breast milk than from formula.

Reliable calculation of intestinal absorption of individual polyunsaturated fatty acids requires processing of feces immediately after excretion to avoid bacterial and oxidative degradation of the most unsaturated fatty acids. Use of high-resolution capillary-gas chromatography is also necessary to minimize coelution of several unknown peaks (mostly hydroxy fatty acids) that may interfere with determination of the more common dietary fatty acids. We paid attention to these conditions and found that ≈80% of AA and DHA from breast milk was absorbed. In PBM-fed infants, this value approximated the amount of total fat absorption and was similar to the palmitic acid absorption (78.4 ± 4.0%) but lower than that of oleic acid absorption (85.8 ± 3.2%). Absorption of LCPs from breast milk was thus not better than absorption of saturated fat.

In breast milk, palmitic acid is preferentially esterified to the center *sn*-2 position and LCPs are more represented at the *sn*-2 and *sn*-3 positions (14, 15, 27). It is thus conceivable that LCPs at the external position may have a disadvantage compared with fatty acids attached to the center position, which does not have to be cleaved for absorption. Lingual, gastric, and pancreatic lipases preferentially hydrolyze the *sn*-1 and *sn*-3 ester bonds of triacylglycerols but their activity is greater toward short- and medium-chain fatty acids than toward long-chain fatty acids, especially LCPs (16, 28, 29).

Intestinal absorption of AA and DHA from TG-LCPs formula was similar to that from PBM. This is an important finding because this formula contained highly unsaturated triacylglycerol molecular species, with up to 40% DHA and up to 30% AA. Unlike in breast milk triacylglycerols, in which DHA has a clear positional specificity (14), DHA in algal oils does not have a strong positional specificity; there are similar proportions at the *sn*-1, *sn*-2, and *sn*-3 positions (data not shown). On the basis of work by Bottino et al (16), who described resistance of certain LCPs of fish oils to hydrolysis by pancreatic lipase, and data of Hernell et al (17), relatively low absorption might also have been expected with the highly unsaturated triacylglycerols from algae. However, our data indicated that LCPs from these triacylglycerols were absorbed as efficiently as those from breast milk, even without the lipolytic enzymes that are present in breast milk but not in formulas (17). Differences in the molecular structure of these algal triacylglycerols and fish oils (with which most studies have been performed) could be responsible for the different digestibility and absorption of these two products.

DHA from PL-LCPs was absorbed better than DHA from PBM. This finding clearly indicates that intestinal absorption of phospholipids in preterm infants is efficient. Data on intestinal absorption of phospholipids in human infants and adults is lacking and information on this issue is derived mainly from studies in animals (30). It is known, however, that under normal dietary conditions in human adults, dietary phospholipids are a minor portion of the phospholipids presented to the gut for intestinal absorption; the majority are phospholipids of biliary origin (31). Whether this also applies in newborn infants is not known. However, to ensure intake of LCPs from a phospholipid source in the range of intake of breast milk, dietary intake of phospholipids must far exceed the phospholipid content of breast milk (18).

Proportions of intestinal absorption representing the sum of n−6 and n−3 LCPs were similar to those of AA and DHA,

TABLE 4
Fatty acid intake in preterm infants fed exclusively preterm breast milk or formula at ≈4 wk of age[1]

Fatty acid	PBM (n = 20)	NLCPs (n = 19)	PL-LCPs (n = 19)	TG-LCPs (n = 19)	P value[2]
			$mg \cdot kg^{-1} \cdot d^{-1}$		
8:0	18.6 ± 1.2[a]	136.2 ± 1.7[b]	68.0 ± 0.5[c]	130.0 ± 2.5[b]	0.000
10:0	109.0 ± 8.5[a]	114.2 ± 1.4[a]	77.6 ± 0.6[b]	111.4 ± 2.1[a]	0.000
12:0	519.7 ± 51.5[a]	837.2 ± 10.4[b]	290.7 ± 2.3[c]	847.8 ± 16.4[b]	0.000
14:0	601.7 ± 55.6[a]	399.4 ± 5.0[b]	298.2 ± 2.4[b]	382.2 ± 7.2[b]	0.000
16:0	1506.1 ± 122.0[a]	554.9 ± 6.9[b]	1467.0 ± 11.7[a]	511.2 ± 9.7[b]	0.000
18:0	431.1 ± 34.1[a]	258.8 ± 3.2[b]	405.4 ± 3.2[a]	264.9 ± 5.0[b]	0.000
20:0	14.0 ± 1.0[a]	16.1 ± 0.2[b]	13.5 ± 0.1[a]	17.0 ± 0.3[b]	0.000
16:1n−7	151.4 ± 16.7[a]	12.4 ± 0.2[b]	57.5 ± 0.5[c]	10.2 ± 0.2[b]	0.000
18:1n−9	2209 ± 154.9[a]	2565.8 ± 31.9[b]	1793.2 ± 14.2[c]	2511.6 ± 47.5[a,b]	0.000
18:1n−7	133.4 ± 10.1[a]	37.7 ± 0.5[b]	113.2 ± 0.9[c]	37.4 ± 0.7[b]	0.000
20:1n−9	30.3 ± 2.1[a]	10.2 ± 0.1[b]	9.1 ± 0.1[b]	10.7 ± 0.2[b]	0.000
18:2n−6	687.4 ± 64.7[a]	840.4 ± 10.4[b]	657.8 ± 5.2[a]	828.7 ± 16.1[b]	0.000
20:4n−6	32.0 ± 2.1[a]	0	19.4 ± 0.2[b]	49.7 ± 1.0[c]	0.000
18:3n−3	38.8 ± 4.1[a]	72.9 ± 0.9[b]	54.1 ± 0.4[c]	71.5 ± 1.3[b]	0.000
20:5n−3	5.0 ± 0.9[a]	0	2.1 ± 0.4[b]	0	0.003
22:6n−3	18.0 ± 1.3[a]	0	13.6 ± 0.1[b]	37.8 ± 0.7[c]	0.000
Total n−6 LCPs	92.9 ± 5.6[a]	0	29.9 ± 0.2[b]	57.2 ± 1.1[c]	0.000
Total n−3 LCPs	37.7 ± 2.9[a]	0	25.9 ± 0.5[b]	37.9 ± 0.7[a]	0.000

[1] $\bar{x}$ ± SEM; n = 77. PBM, preterm breast milk; LCPs, long-chain polyunsaturated fatty acids; NLCPs, no added LCPs; PL-LCPs, LCPs from phospholipids; TG-LCPs, LCPs from triacylglycerols. Values with different superscript letters are significantly different, $P < 0.05$ (Tukey test).

[2] By ANOVA.

TABLE 5
Fecal output of fatty acids in preterm infants fed exclusively preterm breast milk or formula at ≈4 wk of age[1]

Fatty acid	PBM (n = 20)	NLCPs (n = 19)	PL-LCPs (n = 19)	TG-LCPs (n = 19)	P value[2]
			$mg \cdot kg^{-1} \cdot d^{-1}$		
8:0	1.2 ± 0.2	1.6 ± 0.4	1.9 ± 0.2	1.8 ± 0.2	0.201
10:0	1.9 ± 0.7	3.2 ± 1.4	1.4 ± 0.2	2.2 ± 1.4	0.632
12:0	27.3 ± 8.7^{a}	55.4 ± 7.2^{b}	16.0 ± 1.0^{a}	39.9 ± 5.9^{a,b}	0.000
14:0	62.9 ± 15.5	74.6 ± 6.0	47.8 ± 3.2	59.2 ± 6.8	0.254
16:0	305.5 ± 49.0^{a}	268.8 ± 16.6^{a}	604.8 ± 30.7^{b}	255.2 ± 19.4^{a}	0.000
18:0	161.1 ± 19.8^{a}	190.5 ± 10.5^{a,b}	242.6 ± 14.0^{b}	197.6 ± 14.0^{a,b}	0.003
20:0	5.4 ± 0.7^{a}	19.8 ± 1.3^{b}	11.5 ± 0.7^{c}	22.7 ± 1.5^{b}	0.000
16:1n−7	13.7 ± 3.6^{a}	1.3 ± 0.2^{b,c}	8.3 ± 0.8^{ac}	0.7 ± 0.2^{b}	0.000
18:1n−9	307.1 ± 64.1^{a}	641.0 ± 54.5^{b}	209.9 ± 31.4^{a}	550.0 ± 64.3^{b}	0.000
18:1n−7	26.3 ± 4.2^{a}	18.6 ± 2.6^{a,b}	24.6 ± 2.4^{a,b}	15.2 ± 2.1^{b}	0.034
20:1n−9	8.1 ± 1.2^{a}	13.7 ± 1.2^{b}	3.3 ± 0.3^{c}	15.2 ± 1.3^{b}	0.000
18:2n−6	78.4 ± 22.5^{a}	255.2 ± 28.9^{b}	57.2 ± 10.8 ^{a}	255.2 ± 31.9^{b}	0.000
20:4n−6	6.0 ± 1.2^{a}	1.2 ± 0.1^{b}	3.0 ± 0.3^{a,b}	9.8 ± 1.2^{c}	0.000
18:3n−3	3.6 ± 1.0^{a}	8.4 ± 1.1^{b}	3.0 ± 0.6^{a}	6.9 ± 1.2^{a,b}	0.000
20:5n−3	2.3 ± 0.4^{a}	1.7 ± 0.4^{a,b}	0.7 ± 0.2^{b}	2.2 ± 0.4^{a}	0.011
22:6n−3	3.9 ± 0.7^{a}	0.3 ± 0.1^{b}	1.6 ± 0.2^{b}	7.4 ± 0.9^{c}	0.000
Total n−6 LCPs	19.8 ± 3.4^{a}	4.6 ± 1.2^{b}	8.3 ± 0.7^{b,c}	13.3 ± 1.3^{c}	0.000
Total n−3 LCPs	7.2 ± 1.3^{a,b}	4.2 ± 1.9^{b}	2.9 ± 0.5^{b}	9.8 ± 1.1^{a}	0.002

[1] $\bar{x}$ ± SEM; n = 77. PBM, preterm breast milk; LCPs, long-chain polyunsaturated fatty acids; NLCPs, no added LCPs; PL-LCPs, LCPs from phospholipids; TG-LCPs, LCPs from triacylglycerols. Values with different superscript letters are significantly different, $P \leq 0.05$ (Tukey test).
[2] By ANOVA.

respectively. This indicated that other minor LCPs behave in a similar way with respect to absorption. In the PBM group, intakes of n−6 and n−3 LCPs were more than double than those of AA and DHA, respectively. This resulted from the presence of several intermediates of the n−6 and n−3 families in breast milk. Because these fatty acids have biological importance and may be converted into other LCPs, their dietary intakes should be taken into account, especially when breast milk is compared with supplemented formulas. Infants fed PL-LCPs formula had total n−6 and n−3 LCPs intakes that were much higher that those of AA and DHA alone. Eicosapentaenoic acid contributed to 13.5% of total n−3 LCPs in PBM ((5 mg $\cdot kg^{-1} \cdot d^{-1}$) and 8.1% in PL-LCPs (2.1 mg $\cdot kg^{-1} \cdot d^{-1}$). This difference may deserve attention in future studies. With the TG-LCPs formula, AA and

TABLE 6
Metabolizable intake of fatty acids in preterm infants fed exclusively preterm breast milk or formula at ≈4 wk of age[1]

Fatty acid	PBM (n = 19)	NLCPs (n = 19)	PL-LCPs (n = 19)	TG-LCPs (n = 19)	P value[2]
			$mg \cdot kg^{-1} \cdot d^{-1}$		
8:0	17.4 ± 1.2^{a}	134.6 ± 1.8^{b}	66.0 ± 0.6^{c}	128.5 ± 2.5^{b}	0.000
10:0	107.1 ± 8.4^{a}	111.0 ± 2.5^{a}	76.2 ± 0.7^{b}	109.2 ± 2.7^{a,b}	0.005
12:0	492.3 ± 51.1^{a}	781.8 ± 12.5^{b}	274.6 ± 2.8^{c}	808.0 ± 16.9^{b}	0.000
14:0	538.8 ± 56.3^{a}	324.9 ± 7.5^{b}	250.3 ± 4.3^{b}	323.2 ± 10.4^{b}	0.000
16:0	1200.7 ± 124.2^{a}	286.1 ± 17.7^{b}	862.3 ± 33.5^{c}	256.0 ± 24.8^{b}	0.000
18:0	269.9 ± 34.0^{a}	68.3 ± 11.2^{b}	162.7 ± 14.0^{c}	67.4 ± 17.0^{b}	0.000
20.0	8.6 ± 1.1^{a}	−3.8 ± 1.2^{b}	2.0 ± 0.7^{c}	−5.7 ± 1.7^{b}	0.000
16:1n−7	137.8 ± 16.1^{a}	11.0 ± 0.2^{b}	49.2 ± 1.0^{c}	9.5 ± 0.3^{b}	0.000
18:1n−9	1902.8 ± 150.2^{a,b}	1924.7 ± 60.6^{a,b}	1583.4 ± 37.0^{a}	1961.6 ± 83.1^{b}	0.023
18:1n−7	107.2 ± 10.8^{a}	19.0 ± 2.6^{b}	88.5 ± 2.3^{a}	22.3 ± 2.2^{b}	0.000
20:1n−9	22.2 ± 2.0^{a}	−3.5 ± 1.2^{b}	5.7 ± 0.3^{c}	−4.4 ± 1.4^{b}	0.000
18:2n−6	608.9 ± 60.7	585.2 ± 28.8	600.7 ± 13.0	575.4 ± 38.0	0.933
20:4n−6	26.0 ± 2.1^{a}	NA	16.4 ± 0.4^{a}	40.0 ± 1.4^{b}	0.000
18:3n−3	35.2 ± 3.8^{a}	64.5 ± 1.2^{b}	51.0 ± 0.8^{c}	64.7 ± 1.8^{b}	0.000
20:5n−3	2.7 ± 1.0	NA	1.4 ± 0.4	NA	0.242
22:6n−3	14.1 ± 1.2^{a}	NA	12.0 ± 0.3^{a}	30.6 ± 1.1^{b}	0.000
Total n−6 LCPs	73.0 ± 6.0^{a}	NA	21.6 ± 0.7^{c}	43.8 ± 1.5^{b}	0.000
Total n−3 LCPs	30.5 ± 3.0^{a}	NA	23.0 ± 0.7^{b}	30.4 ± 1.1^{a}	0.011

[1] $\bar{x}$ ± SEM; n = 77. PBM, preterm breast milk; LCPs, long-chain polyunsaturated fatty acids; NCPs, no added LPCs; PL-LCPs, LCPs from phospholipids, TG-LCPs, LCPs from triacylglycerols. Values with different superscript letters are significantly different, $P \leq 0.05$ (Tukey test).
[2] By ANOVA.

TABLE 7
Intestinal absorption percentages of fatty acids in preterm infants fed exclusively preterm breast milk or formula at ≈4 wk of age[1]

Fatty acid	PBM (n = 20)	NLCPs (n = 19)	PL-LCPs (n = 19)	TG-LCPs (n = 19)	P value[2]
			%		
8:0	93.5 ± 1.0[a]	98.8 ± 0.3[b]	97.2 ± 0.3[a]	98.6 ± 0.2[b]	0.000
10:0	98.4 ± 0.6	97.0 ± 1.4	98.2 ± 0.2	98.1 ± 1.5	0.761
12:0	94.8 ± 1.6	93.4 ± 0.9	94.5 ± 0.4	95.3 ± 0.7	0.596
14:0	88.9 ± 2.7[a]	81.3 ± 1.5[b]	84.0 ± 1.1[a,b]	84.4 ± 1.7[a,b]	0.039
16:0	78.4 ± 4.0[a]	51.4 ± 3.0[b]	58.7 ± 2.2[b]	49.3 ± 4.2[b]	0.000
18:0	60.2 ± 5.5[a]	26.2 ± 4.2[b]	40.2 ± 3.5[b]	24.0 ± 6.2[b]	0.000
20:0	59.5 ± 6.1[a]	−23.4 ± 7.9[b]	14.8 ± 5.1[c]	−35.8 ± 10.2[b]	0.000
16:1n−7	90.6 ± 2.5[a,b]	89.6 ± 1.8[a,b]	85.5 ± 1.5[b]	93.3 ± 2.2[a]	0.035
18:1n−9	85.8 ± 3.2[a,c]	75.0 ± 2.1[b]	88.3 ± 1.8[c]	77.9 ± 2.5[ab]	0.001
18:1n−7	78.4 ± 4.0[a]	50.2 ± 6.8[b]	78.3 ± 2.0[a]	59.4 ± 5.6[b]	0.000
18:2n−6	88.1 ± 3.0[a]	69.7 ± 3.4[b]	91.3 ± 1.7[a]	68.9 ± 4.0[b]	0.000
20:4n−6	81.1 ± 3.6	0	84.7 ± 1.7	80.4 ± 2.3	0.481
18:3n−3	90.3 ± 2.6	88.5 ± 1.5	94.4 ± 1.1	90.5 ± 1.6	0.146
20:5n−3	57.0 ± 6.9[a]	0	64.3 ± 9.5	0	0.547
22:6n−3	78.4 ± 4.0[a]	0	88.3 ± 1.8[b]	80.6 ± 2.1[a,b]	0.044
Total n−6 LCPs	78.0 ± 4.0	0	74.0 ± 2.8	76.8 ± 2.2	0.381
Total n−3 LCPs	79.2 ± 4.4	0	88.7 ± 1.9	80.4 ± 2.2[b]	0.064

[1] $\bar{x}$ ± SEM; n = 77. PBM, preterm breast milk; LCPs, long-chain polyunsaturated fatty acids; NLCPs, no added LCPs; PL-LCPs, LCPs from phospholipids; TG-LCPs, LCPs from triacylglycerols. Values with different superscript letters are significantly different, $P \leq 0.05$ (Tukey test).

DHA intakes were >85% of total n−6 and n−3 LCPs, respectively, and eicosapentaenoic acid was absent. Mean intake of total n−3 LCPs was lower in the PL-LCPs group than in the PBM and TG-LCPs groups (25.9, 37.7, and 37.9 mg·kg^{-1}·d^{-1}, respectively, in the three groups).

Intakes of LCPs in PBM-fed preterm infants were slightly higher than those reported by Boehm et al (32), who compared a group fed breast milk with groups fed an unsupplemented formula and a supplemented formula similar to the PL-LCPs formula in our study. This difference in intake of LCPs in our study and that in the study by Boehm et al may have resulted from different fluid intakes or from the fact that the infants in our study were fed a PL-LCPs formula with a higher energy concentration than the infants in the study by Boehm et al (332.8 compared with 292.9 kJ/d).

Fecal output data indicated that losses of LCPs in preterm infants are not negligible. Infants fed NLCPs formula lost ≈4.6 ± 1.2 mg n−6 LCPs·kg^{-1}·d^{-1} and 4.2 ± 1.9 mg n−3 LCPs·kg^{-1}·d^{-1}. A portion of these fecal LCPs may derive from endogenous sources—-bile lipids, enterocytes shed into the gut lumen, and even bacterial walls. It was shown that bile in human adults contains LCPs (33), but no information is available on children or premature infants nor do data exist on the amount of bile produced. It is likely, however, that a large portion of the LCPs of bile lipids is absorbed along the intestinal tract. Although it is theoretically possible that LCPs in feces are not a reflection of gut absorption, circumstantial evidence suggests that this is not the case. First, much higher amounts are found in stools of infants consuming LCPs in their diets. Second, when LCP content of PBM rises, so does the amount excreted in the stool.

We also calculated metabolizable intakes (Table 6) because we believe that these values provide better estimates of the amounts of LCPs entering the body than do values derived from diet alone. Furthermore, we believe that fatty acid content, especially LCP content, in infant formulas is better specified in absolute amounts than as a percentage of total fat because fat content of infant formulas may differ markedly. A difference in total fat of 1.2 g/d (for example, an increase from 3.2 to 4.4 g/d) could lead to a difference in estimated LCP intake of 28%. It is also worth emphasizing that in our subjects ≈20–25% of dietary LCPs were lost in the feces.

In conclusion, our study showed that there are substantial amounts of LCPs in the feces of PBM- and formula-fed preterm infants. We also found that DHA from egg phospholipids was absorbed better than DHA from PBM. DHA and AA from single-cell oils were absorbed as efficiently as DHA and AA from breast milk fat.

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Appendix K

Bottino et al., "Resistance of Certain Longchain Polyunsaturated Fatty Acids of Marine Oils to Pancreatic Lipase Hydrolysis" Lipids 2, 489-93 (1967)

synthesis cannot be specified.

Many organs had significant ^{14}C activity in one or more fatty acids having retention times equal to those for 20:5, 22:2, 22:3, 22:4, 22:5 and 22:6. In the case of fatty acids from lungs and from brain, two of these were shown to be 22:2 and 22:4. In brain tissue about 40% of the ^{14}C activity was in fatty acids other than 20:4 and of this about half was in a fraction tentatively identified as 18:2. Though the chemical identity of this compound was not established further, it may be $\Delta^{8,12}$ octadecadienoic acid, synthesized from Δ^{9} 16:1, or $\Delta^{6,9}$ octadecadienoic acid. The presence of the latter isomer in pig brain tissue has been reported by Kishimoto and Radin (15). Formation of $\Delta^{6,9}$ octadecadienoic acid from ^{14}C-oleic acid by rat liver microsomes was shown by Holloway et al. (16). An 18:2 fatty acid (presumably the same isomer) was shown to be synthesized from 1-^{14}C-acetyl CoA by subcellular particles of rat liver by Harlan and Wakil (17). We have previously also observed biosynthesis of this isomer by rat liver microsomes incubated with ^{14}C-acetyl CoA (18).

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Resistance of Certain Long-Chain Polyunsaturated Fatty Acids of Marine Oils to Pancreatic Lipase Hydrolysis

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ABSTRACT

When whale oil triglycerides were subjected to pancreatic lipase hydrolysis, eicosapentaenoic and docosahexaenoic acids were found mainly in the di- and triglyceride products, suggesting that they are in the 1,3-positions but resistant to the action of the lipase. Their presence in the 1,3-positions was confirmed. Their resistance to pancreatic lipase hydrolysis was demonstrated by analysis of the products of the enzyme action on: (a) a concentrate of highly unsaturated whale oil triglycerides; (b) the latter after randomization; and (c) synthetic 1,2-di-octadecenoyl-3-eicosapentaenoyl glycerol.

Docosapentaenoic acid was also shown to be present in the 1,3-position of whale oil triglycerides but was not lipase resistant. It is postulated that the presence of a double bond near the carboxyl group exercises an inhibitory effect, or that the location of the double bonds in the resistant acids places their terminal methyl groups close to the carboxyl, producing a steric hindrance effect.

INTRODUCTION

IN A STUDY OF THE STRUCTURE of marine mammal oils by the use of pancreatic lipase, the distribution of fatty acids in the hydrolytic products of whale oil suggested that eicosapentaenoic (20:5) and docosahexaenoic (22:6) acids, but not docosapentaenoic (22:5) acid, are resistant to the action of that hydrolytic enzyme. The results of the present study confirm the resistance of those acids to pancreatic lipase action, even though the acids are located in the 1,3-positions of whale oil triglycerides. A preliminary report of this work has been presented (1).

EXPERIMENTAL

The location of the 20:5, 22:5, and 22:6 acids in the whale glyceride molecules and the resistance of these acids to the activity of pancreatic lipase were determined by analyses of the products of the enzyme action on: (a) unmodified whale oil; (b) a concentrate of highly unsaturated whale oil triglycerides; (c) the latter after randomization; and (d) synthetic 1,2-di-octadecenoyl-3-eicosapentaenoyl glycerol.

Methods

The triglycerides of two samples of whale oil[1] were purified by preparative thin-layer chromatography (TLC). A highly unsaturated fraction was prepared from one of them by crystallization at –60C (2). Menhaden oil was provided by the Department of Oceanography, Texas A&M University. Lipase (EC 3.1.1.3) from hog pancreas, PL-III, was purchased from Worthington Biochemical Corporation, Freehold, N. J. Lipase hydrolyses were performed in vitro by the procedure of Luddy et al. (3), including the determination of the fatty acid composition of the free fatty acids and of the mono-, di-, and triglyceride products.

Randomization of the highly unsaturated concentrate of whale oil was achieved by treatment with 0.1 M lithium secondary butylate in dimethyl formamide (4). The reaction mixture was kept under nitrogen at room temperature for 3 days. The rearranged triglycerides were purified by preparative TLC.

Purification of triglycerides by TLC was achieved on 0.25-mm thick layers of silica gel (Adsorbosil-1, Applied Science Laboratories, State College, Pa.) on 20 x 20 cm glass plates. The developing solvent system was a mixture of petroleum ether (30-60C bp)-ethyl ether-acetic acid (60:40:1.6, v/v/v).

Gas-liquid chromatography (GLC) was performed in a Research Specialties Model 600 gas chromatograph (Warner-Chilcott Laboratories Division, Richmond, Calif.). The chromatograph was equipped with an argon ionization detector and a 6 ft x 1/4 in. column packed with 15% diethylene glycol succinate on 60-80 mesh Chromosorb W. The column was operated isothermally at 195C. The identities of the quantitatively more important peaks were ascertained by comparing their relative retention times with those of known standards.

[1] One of the samples of whale oil was from the Arista Company, New York. The other was obtained through the courtesy of H. S. Olcott.

Infrared spectra were obtained in a IR8 Beckman infrared spectrophotometer between sodium chloride pellets.

1,2-Di-octadecenoyl-3-eicosapentaenoyl glycerol was synthesized from 1,2-diolein and eicosapentaenoyl chloride and purified by TLC. A manuscript describing this synthesis is in preparation. Eicosapentaenoic acid, 91% pure, isolated from menhaden oil, was purchased from the Hormel Institute, Austin, Minn.

RESULTS AND DISCUSSION

Evidences of Resistance

After 50% pancreatic lipase hydrolysis of the whale oil triglycerides, the concentrations of the 20:5 and 22:6 acids were lower in both the fatty acid and the monoglyceride fractions, but higher in the diglyceride and triglyceride fractions of the resultant mixture than in the original oil (Table I). This suggests that these two polyunsaturated fatty acids are in the 1- and 3- positions but are resistant to the action of the lipase. That the 20:5, 22:5, and 22:6 acids of the whale oil are in the 1,3-positions has been reported by Brockerhoff and Hoyle (5). The accumulation of long-chain polyunsaturated fatty acids in the diglycerides after lipase hydrolysis of marine oils has also been reported by others (4, 6, 7).

Not all the polyunsaturated acids of whale oil behave as the 20:5 and 22:6 acids. The 22:5 acid was present in the free fatty acids and was not enriched in the di- and triglycerides, although like the 20:5 and 22:6 acids it was in low concentration in the monoglyceride products of hydrolysis (Table I). Therefore, the 22:5 acid must be considered as also present in the 1,3-positions; but, in contrast to the 20:5 and 22:6 acids, susceptible to the action of pancreatic lipase.

TABLE I

Major Fatty Acid Components of Whale Oil Triglycerides and Its Lipase Hydrolysis Products

Acid[a]	Sample	Whale oils: Original TG	Products of hydrolysis[b]: FA	MG	DG	TG
		percentage.				
14:0	I[c]	8.7	8.1	9.3	9.2	7.1
	II	4.6	3.4	7.7	3.9	2.4
16:0	I	14.8	20.1	5.5	9.9	12.5
	II	14.9	21.1	10.0	7.9	7.3
16:1	I	16.7	11.3	28.9	17.8	12.0
	II	14.4	9.2	24.1	16.6	8.6
18:1	I	32.2	37.5	40.9	29.4	23.7
	II	33.6	38.7	45.0	30.1	19.0
20:1	I	2.6	5.3	0.3	1.1	1.9
	II	2.1	2.3	1.1	2.1	2.8
20:5	I	6.6	2.0	2.1	11.8	13.8
	II	8.1	3.0	2.4	17.7	26.0
22:5	I	3.9	2.9	0.5	2.2	2.9
	II	5.2	5.9	tr	3.9	6.8
22:6	I	4.9	2.6	0.8	5.3	10.1
	II	5.8	3.9	tr	8.1	17.3

[a] Chain length: number of double bonds.

[b] FA = Fatty Acids; M = Monoglycerides; DG = Diglycerides; TG = Triglycerides.

[c] Average of duplicate analyses.

Since the concentration of some of the polyunsaturated acids were low in the original whale oil, a highly unsaturated concentrate was obtained by removal of the more saturated glycerides by crystallization from acetone at −60C (2). The concentrate was then subjected to pancreatic lipase hydrolysis. The results are presented in Table II-A. It can be seen that, as compared to a level of about 22% in the concentrate, there were only 7% and 8% of the 20:5 acid in the free fatty acid and monoglyceride fractions, respectively. There were 26% in the diglycerides and 30% in the triglycerides. The results from the concentrate thus reinforce previous indications of resistance. The distribution of the 22:6 acid in the hydrolysis products also indicates resistance but to a somewhat lesser degree. The 22:5 acid was hydrolzed normally as shown by its relatively high level in the fatty acid fraction.

TABLE II

The Effect of Randomization on the Products of Pancreatic Lipase Action on a Highly Unsaturated Fraction from Whale Oil Triglycerides (major fatty acids only)

Acid[b]	(A) Whale oil highly unsaturated TG[a]: Concentrate (original TG)	Products of hydrolysis: FA	MG	DG	TG	(B) Randomized whale oil highly unsaturated TG: Randomized TG	Products of hydrolysis: FA	MG	DG	TG	(C) Recalculation of (B) omitting 20:5 and 22:6: Randomized TG	Products of hydrolysis: FA	MG	DG	TG
	percentage					percentage					percentage				
14:0	4.9	4.1	6.3	4.1	4.7	4.6	8.5	7.3	4.6	4.3	6.9	9.0	9.5	7.7	7.6
16:0	2.4	4.6	1.1	1.3	2.4	2.3	5.1	3.0	2.0	2.0	3.5	5.4	3.9	3.4	3.5
16:1	15.6	16.9	34.5	20.3	12.1	14.1	24.0	18.6	14.6	12.9	21.3	25.5	24.2	24.5	22.8
18:1	25.5	38.0	27.0	20.0	19.4	25.3	35.1	26.8	17.1	17.8	38.2	37.3	34.9	28.7	31.5
20.1	3.5	2.3	3.2	3.6	3.1	3.1	1.5	2.7	4.5	3.9	4.7	1.6	3.5	7.6	6.9
20:5	22.3	7.0	8.2	26.4	30.1	22.4	3.2	16.4	29.7	29.6					
22:5	4.6	8.0	0.9	3.1	4.3	4.8	3.6	4.5	5.2	5.1	7.2	3.8	5.9	8.7	9.0
22:6	12.6	8.5	2.0	11.4	15.9	11.3	2.8	6.8	10.8	13.9					

[a] FA = Fatty Acids; MG = Monoglycerides; DG = Diglycerides; TG = Triglycerides.

[b] Chain length: number of double bonds.

TABLE III

Major Fatty Acid Components of Menhaden Oil Triglycerides and Its Lipase Hydrolysis Products

Acid[a]	Original TG	Products of hydrolysis[b]: FA	MG	DG	TG
	percentage				
14:0	11.1	11.0	14.2	7.7	6.3
16:0	19.4	27.2	24.9	14.7	17.4
16:1	16.1	17.6	13.6	9.1	8.1
18:0	5.6	7.6	3.2	3.4	3.7
18:1	16.2	20.1	5.7	4.7	5.0
20:1	3.8	1.6	2.8	6.6	6.0
20:5	10.5	2.0	11.4	22.8	25.5
22.5	1.4	0.6	2.5	2.2	tr
22:6	7.3	1.5	15.1	15.0	16.0

[a] Chain length: number of double bonds.

[b] FA = Fatty Acids; MG = Monoglycerides; DG = Diglycerides; TG = Triglycerides.

In order to rule out position in the triglyceride molecule as the determining factor in the low degree of hydrolysis of the 20:5 and 22:6 acids, an aliquot of the highly unsaturated concentrate was randomized by chemical treatment. Whale oil offers unusual resistance to rearrangement by the use of standard procedures. Several combinations of catalysts, solvents and different times of treatment were tested before satisfactory results could be obtained. Sodium methoxide in methanol solution produced methyl esters difficult to separate from the randomized triglycerides. A xylene suspension of the same catalyst (8) was only partially effective. Lithium secondary butylate in dimethyl formamide solution (4) was found to be effective when the reaction period was prolonged for 3 days at room temperature. This procedure was therefore used. The randomized triglyceride products, purified by TLC, were analyzed by GLC and subjected to pancreatic lipase hydrolysis. The results are presented in Table IIB. Since the fatty acid compositions of the four products of hydrolysis are not similar, one might conclude that the randomization is incomplete. However, this criterion would only be valid if all the acids were equally susceptible to the lipase, a condition which is not met due to the presence of the resistant 20:5 and 22:6 acids. If the data are recalculated omitting the 20:5 and 22:6 acids or, in other words, making the nonresistant acids equal to 100%, the figures shown in Table IIC are obtained. The quite similar concentrations of the six major acids in all four fractions indicates effective randomization.

The presence of significant amounts of 20:5, 22:5, and 22:6 acids in the monoglycerides after, but not before randomization (Table IIB), indicates that they were not originally located in the 2-position in the whale oil triglycerides. Finally, the very low levels of 20:5 and 22:6 acids in the free fatty acid fraction of the pancreatic lipase hydrolysis products of the randomized oil indicate that the reduced degree of hydrolysis of those acids is not due to the positional specificity of the enzyme, but is due to a characteristic of the fatty acid molecule itself.

In order to compare the behavior of the 20:5 and 22:6 acids in the pancreatic lipase hydrolysis of whale oil with their behavior when located mainly in the 2-position as in fish oils, menhaden oil triglycerides were subjected to pancreatic lipase hydrolysis (Table III). The distribution of the 20:5 and 22:6 acids in the hydrolysis products of menhaden oil is different from that in whale oil products (Table I), although their concentrations in the two oils are quite similar. This is further evidence that the distribution of these acids in the two oils is different and that in whale oil hydrolysis their resistance to pancreatic lipase is independent of their position.

It required about 2 min to attain 50% hydrolysis of the untreated whale oils under the conditions used. An extended reaction time should increase the general degree of hydrolysis but leave higher concentrations of the resistant 20:5 and 22:6 acids in the unhydrolyzed di- or triglycerides. This was found to be true only for 20:5, whose concentrations after 2, 3, and 5 min of hydrolysis were 11.8, 14.4 and 18.2% respectively in diglycerides and 13.8, 13.3, and 19.7% respectively in triglycerides. The concentration of 22:6 after 2, 3, and 5 min of hydrolysis was 5.9, 5.3, and 5.4% respectively in diglycerides and 11.0, 7.2, and 6.7% respectively in triglycerides. The lack of increase in percentage of 22:6 in the di- and triglycerides with time might be due to its having approached maximum levels at the 2-min period.

It was also found that the rate of hydrolysis decreased appreciably after half the triglyceride acids were released. This is a logical consequence of distribution in the 1,3-position of the 20:5 and 22:6 acids, their resistance to hydrolysis, and the reported presence of the

C_{20} and C_{22} acids in only 50% of whale oil triglycerides (2).

Proof of Resistance

Proof of the resistance of the 20:5 acid (and by inference of the 22:6 acid) was obtained by study of the action of pancreatic lipase on synthetic 1,2-di-octadecenoyl-3-eicosapentaenoyl glycerol. The results are presented in Table IV. The fatty acid compositions of the triglycerides before lipase hydrolysis and of the monoglyceride and triglyceride products of hydrolysis show that the substance synthesized is, in fact, 1,2-di-octadecenoyl-3-eicosapentaenoyl glycerol, with some contamination due to impurities in the starting materials.

The experimental values for the composition of the fatty acid and diglyceride fractions are closer to the values calculated on the assumption of resistance than on the assumption of nonresistance. The small amount of monoglycerides produced is another indication of resistance. The presence of 17% 20:5 acid in the fatty acid fraction indicates that some hydrolysis of that acid took place. This could be due to the resistance to the enzyme not being absolute, to the presence of a hydrolyzable isomer of the 20:5 acid in the starting material, or to an alteration in the structure of the all *cis* 20:5 acid during the chemical synthesis of the triglyceride. Analyses of the starting material showed that there were 9% impurities as ascertained by GLC and that only 75% of the theoretical amount of glutaric acid was produced by $KMnO_4$ oxidation in acetic acid medium (9). Examination of the original 20:5 acid and the 1,2-di-octadecenoyl-3-eicosapentaenoyl glycerol by infrared spectrometry showed that only traces of *trans* isomerization occurred during the synthesis.

TABLE IV

Products of the Action of Pancreatic Lipase on 1,2-Di-octadecenoyl-3-eicosapentaenoyl Glycerol

Acid[b]	Original TG	Products of hydrolysis[a] FA	MG	DG	TG
		Mole percent[c]			
		Theoretical (nonresistance)			
18:1 + impur.[d]	69.7	54.6	100	77.7	69.3
20:5	30.3	45.4	0	22.3	30.3
		Theoretical (absolute resistance)			
18:1 + impur.	69.7	100	[e]	54.5	69.7
20:5	30:3	0	[e]	45.5	30.3
		Experimental			
18:1 + impur.	71.5	83.0	99.1[f]	60.8	73.0
20:5	28.5	17.0	0.9[f]	39.2	27.0

[a] FA = Fatty Acids; MG = Monoglycerides; DG = Diglycerides; TG = Triglycerides.

[b] Chain length: double bond.

[c] The detector response to the 20:5 acid was found to be 0.88 times that of the 18.1. However, no correction was applied since it would have had no significant effect on the conclusions.

[d] The preparation of 20:5 acid used had 8.9% impurities of other fatty acids. Since they are not expected to be lipase resistant, their percentages are added to that of oleic acid.

[e] No MG should be obtained.

[f] Very small amount of MG obtained.

Mechanism of Resistance

It is evident that in spite of being located at the 1,3-positions of the whale oil triglycerides, the 20:5 and 22:6 acids resist pancreatic lipase hydrolysis while the 22:5 acid is hydrolyzed without difficulty. The explanation for this phenomenon may lie in differences in their molecular structures:

20:5 $CH_3CH_2(CH{=}CHCH_2)_5 - CH_2CH_2COOH$

22:5 $CH_3CH_2(CH{=}CHCH_2)_5 - CH_2CH_2CH_2CH_2COOH$

22:6 $CH_3CH_2(CH{=}CHCH_2)_5 - CH{=}CHCH_2CH_2CO^3OH$

In view of the evidence presented by others (10) the ω3 structure is assumed for these three acids. Since their terminal 17 carbon chains are identical, any differences in behavior must be assumed to be caused by differences in their structure at the carboxyl end of the chain. The responsible factor could be the proximity of the double bond to the carboxyl group, since the first double bond of the resistant 20:5 and 22:6 acids lies closer to the carboxyl group than does that of the nonresistant 22:5 acid. This view is strengthened by the demonstration by Kleiman et al. (11) that the *trans*-3-enoic acids of *Grindelia oxylepis* seed oil are also resistant to lipase hydrolysis. The presence of methyl groups in a position close to the carboxyl end has also been shown to hinder hydrolysis by the lipase (12).

Another difference in structure between the resistant and the susceptible polyunsaturated acids lies in the space relations of their terminal methyl to their carboxyl groups. As shown in the photographs of the molecular models (Figure 1) the terminal methyl groups of the resistant acids lie close to their carboxyl groups. This proximity may cause a steric hindrance effect on the hydrolysis by the lipase.

Metabolic Implications

The resistance of some of the polyunsaturated fatty acids of whale oil to pancreatic lipase hydrolysis provides an explanation for the finding by Garton et al. (13) that whale

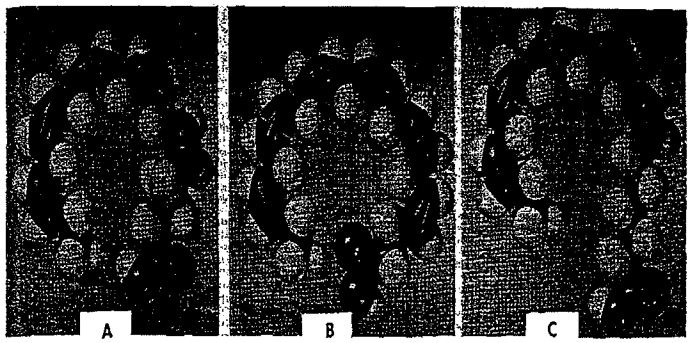

FIG. 1. Molecular models of the 20:5 (A), 22:6 (B), and 22:5 (C) acids of marine oils.

oil can be crystallized almost unchanged from the depot tissues of pigs fed high doses of the oil for a prolonged period of time. In preliminary experiments in this laboratory, however, neither the triglycerides, nor the phospholipids of thoracic duct lymph of rats administered by stomach tube one dose of the highly unsaturated concentrate of whale oil, contained the marine long-chain polyunsaturated acids. The presence of whale glycerides in the tissues of Garton's pigs may have been the product of a low degree of intestinal absorption over a long period of ingestion.

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Appendix L

Hernell et al., "Does the Bile Salt-Stimulated Lipase of Human Milk Have a Role in the Use of the Milk Long-Chain Polyunsaturated Fatty Acids?" J Pediatr Gastroenterol Nutr 16: 426-31(1993)

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Does the Bile Salt-Stimulated Lipase of Human Milk Have a Role in the Use of the Milk Long-Chain Polyunsaturated Fatty Acids?

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Summary: Long-chain polyunsaturated (LCP) fatty acids derived from linoleic (18:2 n-6) and α-linolenic (18:3 n-3) acids are considered essential nutrients in preterm infants. The efficiency by which such fatty acids are released as absorbable products from triacylglycerol was explored in vitro using rat chylomicron triacylglycerol as substrate. When incubated with purified human pancreatic colipase-dependent lipase and colipase, arachidonic acid (20:4 n-6) was released less efficiently than linoleic acid from such triacylglycerol. This difference was not seen when purified human milk bile salt-stimulated lipase (BSSL) was incubated with the triacylglycerol substrate, and it was almost abolished when colipase-dependent lipase (with colipase) and BSSL acted simultaneously, as they do in breast-fed infants. There was no difference in arachidonic acid and eicosapentaenoic acid (20:5 n-3) release rates with either colipase-dependent lipase or BSSL, albeit the release was more rapid with the milk enzyme than with colipase-dependent lipase. Again, the most efficient release as absorbable free fatty acids was achieved when the two lipases operated together. The relative resistance to hydrolysis of arachidonic acid and eicosapentaenoic acid by colipase-dependent lipase was best explained by the localization of the first double bond to the δ-5 position of the respective fatty acid. The results obtained suggest that BSSL is of importance for the efficient use of human milk LCP fatty acids. **Key Words:** Bile salt-stimulated lipase—Colipase-dependent lipase—Long-chain polyunsaturated fatty acids—Fat digestion—Breast-fed infant.

In recent years there has been an increasing awareness that long-chain polyunsaturated (LCP) fatty acids may be an essential nutrient for preterm infants. The reason is that preterm infants have a relatively low capacity to synthesize these derivatives from the precursor fatty acids, that is, linoleic acid (18:2 n-6) of the n-6 series and α-linolenic acid (18:3 n-3) of the n-3 series (1,2).

Some LCP fatty acids, e.g., dihomo-γ-linolenic acid (20:3 n-6) and arachidonic acid (20:4 n-6), both derived from linoleic acid, and eicosapentaenoic acid (20:5 n-3), derived from α-linolenic acid, are precursors of the biologically active eicosanoids. LCP fatty acids, particularly arachidonic acid and docosahexaenoic acid (22:6 n-3), are also qualitatively important constituents of membrane phospholipids (3), the proportion being particularly high in the gray matter of the brain and in the photoreceptor cells of the retina (4–6). Hence, the requirement for these fatty acids is particularly high during the period of rapid development of the central nervous system, that is, the last trimester of pregnancy through early infancy. Calculations have shown that in fully breast-fed preterm infants the content of these LCP fatty acids in human milk suffice to meet the requirements for tissue accretion (1,7). Recently it has been recommended to supplement infant formulas intended for preterm infants with LCP fatty acids approximating the amount and composition of human milk (8,9). Supplementation with only n-3 LCP fatty acids causes depletion of n-6

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LCP fatty acids, that is, arachidonic acid (8,10, 11).

There is still limited information about the pathways by which these fatty acids are used when occurring in human milk, or in infant formulas supplemented with lipids containing such fatty acids. In human milk, LCP fatty acids are enriched in the phospholipid fraction. Because triacylglycerol constitutes >98% of the milk lipids, most of the milk LCP fatty acids are carried by this lipid fraction (12). In the present article we have used chylomicron triacylglycerol containing labeled linoleic and arachidonic acids, or labeled arachidonic and eicosapentaenoic acids, as a model substrate for incubations in vitro with purified human pancreatic colipase-dependent lipase and bile salt-stimulated milk lipase, the two lipases involved in intestinal fat digestion in breast-fed infants (13,14). We have thus compared the relative rates of release of these fatty acids as absorbable products when incubated with either lipase alone or with the two enzymes in combination.

MATERIALS AND METHODS

Preparation of Enzymes and Radioactive Chylomicrons

Human pancreatic colipase-dependent lipase (15) and colipase (16) were purified according to Sternby and Borgström and bile salt-stimulated lipase (BSSL) was purified from human milk as described by Bläckberg and Hernell (17). [1-^{14}C]-labeled 18:2 n-6 (52.6 mCi/mmol), [5,6,8,9,11,12,14,15-^{3}H]-labeled 20:4 n-6 (83.8 Ci/mmol), and [5,6,8,9,11, 12,14,15,17,18-^{3}H]-labeled 20:5 n-3 (79.0 Ci/mmol) were purchased from New England Nuclear.

In the first series of experiments, chylomicrons were obtained by feeding a mesenteric lymph duct cannulated rat (18) 50 μCi [^{14}C]18:2 n-6 and 50 μCi [^{3}H]20:4 n-6 dispersed in 1 ml 20% Intralipid (Kabi Vitrum AB, Stockholm, Sweden) through a gastric fistula 24 h after cannulation as described (19). In a second set of experiments, 50 μCi [^{14}C]20:4 n-6 and 50 μCi [^{3}H]20:5 n-3 were mixed with 1 ml of a chloroform solution containing 1 mg egg phosphatidylcholine/ml. The solvent was evaporated under a stream of N_2 and immediately dispersed in 0.9% NaCl by buzzing. The dispersion was then mixed with 10% (vol/vol) fish oil triacylglycerol (MAX-EPA, Naturprodukter, Örebro, Sweden) emulsion prepared by ultrasonication of 1% gum arabic in 0.9% NaCl for 2 min. The fish oil contained as major n-3 fatty acids 20:5 n-3 (16.5%) and 22:6 n-3 (12.3%). The fish oil emulsion thus prepared (2 ml) was fed via a gastric fistula to another lymph duct cannulated rat. After feeding the radioactive compounds, chyle was collected on ice. Na_2 EDTA was added to a final concentration of 2 m*M*, and the chyle was then stored at 4°C. After the chyle had been diluted with 1.1% NaCl containing 2 m*M* EDTA, chylomicrons were isolated by ultracentrifugation at 25,000 rpm for 2 h at 4°C using a Beckman SW 41 swinging bucket rotor (19).

Incubation Conditions and Lipid Analysis

The incubation mixture was composed of 10 m*M* Hepes buffer (pH 7.4), 2.5 m*M* $CaCl_2$, and 0.12 *M* NaCl containing 2.0 m*M* sodium taurodeoxycholate (NaTDC) and 1.5 m*M* sodium taurocholate (NaTC). Incubations were performed at 37°C in a total volume of 3 ml with purified human colipase-dependent lipase and colipase, with purified BSSL, or with the two lipases in combination. The amount of each enzyme added is given in Figs. 1 and 2. After preincubating the medium with the enzyme(s) for 5 min, the labeled chylomicrons were added. During the following 60 min of incubation, aliquots were withdrawn at various time intervals as indicated in Figs. 1 and 2. The lipids were immediately extracted with 7–8 vol of chloroform/methanol (1:1, vol/vol) containing 0.005% butylated hydroxytoluene. The lipid fractions were separated by thin-layer chromatography, the spots visualized by staining with iodine, the different lipid fractions (triacylglycerol, diacylglycerol, monoacylglycerol, and free fatty acid) transferred into counting vials, and the radioactivity determined as described (20). The triacylglycerol content of the chylomicron substrates was determined by a colorometric enzymatic kit method (Boehringer-Mannheim GmBH).

Data are expressed as percentage of total radioactivity (^{14}C and ^{3}H, respectively) present in each lipid class. All values are means of duplicate samples. Although radioactivity also was present in polar lipids, this fraction remained stable during the incubations (data not shown).

RESULTS

Hydrolysis of [^{3}H]arachidonic and [^{14}C]linoleic Acid-Labeled Chylomicrons

When colipase-dependent lipase (together with colipase) was incubated with [^{14}C]18:2 n-6- and

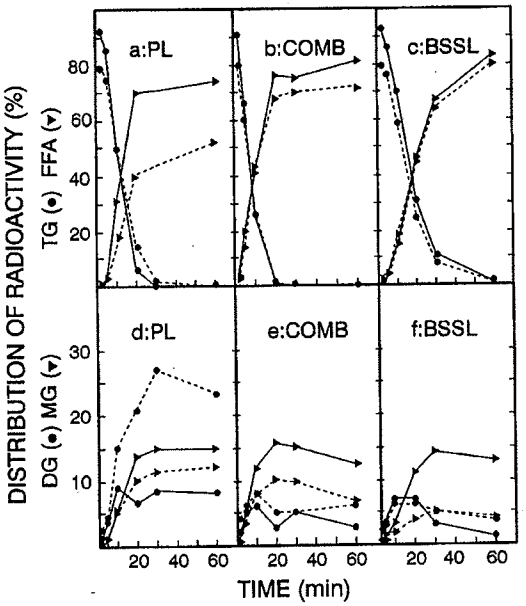

FIG. 1. Chylomicrons with triacylglycerol containing [^{14}C]-linoleic acid and [^{3}H]arachidonic acid were prepared and used as substrate under the conditions described in Materials and Methods. To the chylomicrons, pancreatic colipase–dependent lipase (0.25 μg/ml) and colipase (0.25 μg/ml) (**a** and **d**) (PL), BSSL (14 μg/ml) (**c** and **f**), or a combination of the two (**b** and **e**) (COMB) were added. At the times indicated, samples were withdrawn, the reaction stopped, and the mol% of the different fatty acids (linoleic acid, ——; arachidonic acid, - - -) in each lipid class determined. **a–c:** Triacylglycerol (●) and free fatty acids (▲). **d–f:** Diacylglycerol (●) and monoacylglycerol (▲). The diacylglycerol values represent the sum of 1,2(2,3)- and 1,3-diacylglycerol. To each incubation, 100 μl of chylomicrons containing 500 μg of triacylglycerol was added, the total radioactivity of chylomicrons being 1.7×10^5 dpm of ^{3}H and 2.3×10^5 dpm of ^{14}C.

[^{3}H]20:4 n-6-labeled chylomicrons as the triacylglycerol substrate, the rate of release as free fatty acid was much faster for 18:2 n-6 than for 20:4 n-6. This was evident after 10 min of incubation (Fig. 1a). After 20 min of incubation, the amount of 20:4 released as free fatty acid was only 57% that of 18:2, and after 60 min it was no more than 70%. When colipase-dependent lipase and BSSL were allowed to operate together, this difference was much less pronounced: the amount released as 20:4 was about 90% that of 18:2 after 20 min as well as after 60 min (Fig. 1b). The explanation was that BSSL, in contrast to colipase-dependent lipase, did not discriminate between the two fatty acids. This is illustrated in Fig. 1c, where the milk lipase alone was incubated with the chylomicron substrate.

The reason behind the slower release of 20:4 by colipase-dependent lipase was that this fatty acid, but not 18:2, accumulated in the diacylglycerol fraction; >25% as compared with <10% after 30 min of incubation and still a more than twofold higher concentration after 60 min (Fig. 1d). No obvious difference in accumulation in the diacylglycerol fraction was observed when the two lipases operated together (Fig. 1e), or when BSSL acted alone (Fig. 1f). More 18:2 than 20:4 accumulated in the monoacylglycerol fractions in all incubations, the difference being most pronounced for BSSL alone (Fig. 1d–f).

Hydrolysis of [^{14}C]arachidonic and [^{3}H]eicosapentaenoic Acid-Labeled Chylomicrons

When identical incubations were conducted with chylomicrons labeled with [^{14}C]20:4 n-6 and

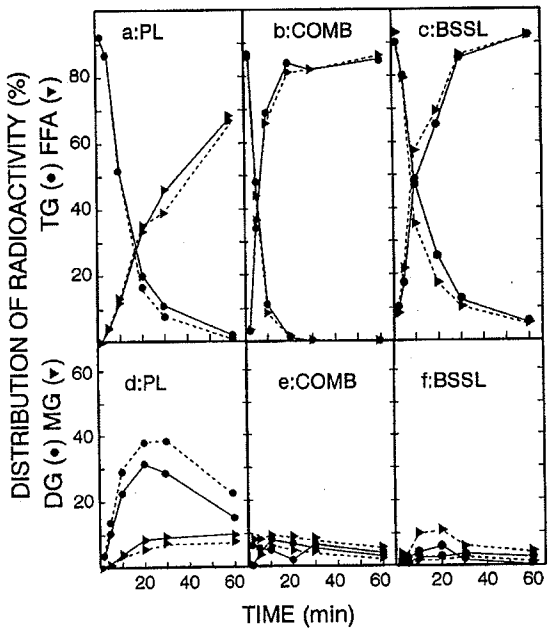

FIG. 2. Chylomicrons with triacylglycerol containing [^{14}C]arachidonic and [^{3}H]eicosapentaenoic acid were prepared and used as substrate under the conditions described in Materials and Methods. To the chylomicrons, pancreatic colipase–dependent lipase (0.25 μg/ml) and colipase (0.25 μg/ml) (**a** and **d**) (PL), BSSL (14 μg/ml) (**c** and **f**), or a combination of the two (**b** and **e**) (COMB) were added. At the times indicated, samples were withdrawn, the reaction stopped, and the mol% of the different fatty acids (arachidonic acid, ——); eicosapentaenoic acid, - - -) in each lipid class determined. **a–c:** Triacylglycerol (●)and free fatty acids (▲). **d–f:** Diacylglycerol (●) and monoacylglycerol (▲). The diacylglycerol values represent the sum of 1,2(2,3)- and 1,3-diacylglycerol. To each incubation, 100 μl of chylomicrons containing 480 μg of triacylglycerol was added, the total radioactivity of chylomicrons being 1.5×10^5 dpm of ^{3}H and 1.8×10^5 dpm of ^{14}C.

[^{3}H]20:5 n-3, there was no apparent difference in the rates by which the two fatty acids were released as free fatty acid during incubation with colipase-dependent lipase (Fig. 2a), with BSSL alone (Fig. 2b), or with the two lipases in combination (Fig. 2c). However, when hydrolysis by colipase-dependent lipase on the one hand and BSSL on the other were compared, both fatty acids were released at a slower rate by the former lipase (compare Fig. 2a and c). After 10 min of incubation, when ~50% of triacylglycerol had been hydrolyzed, <15 mol% had been released as free fatty acids by colipase-dependent lipase (Fig. 2a), whereas the corresponding value for BSSL alone was >40 mol% (Fig. 2c). After 60 min of incubation, when all triacylglycerol had been hydrolyzed, the corresponding values were 70 and 90 mol%, respectively. The promoting effect of BSSL is clearly illustrated when the lipases are operating together (compare Fig. 2a and b). Together the two lipases had released >60 mol% of the fatty acids as free fatty acids after 10 min of incubation (Fig. 2b). Again, a much larger proportion of the fatty acids were accumulating in the diacylglycerol fraction during hydrolysis by colipase-dependent lipase alone (Fig. 2d) than when incubated by BSSL alone (Fig. 2f) or by the two lipases in combination (Fig. 2e).

DISCUSSION

It has recently been recommended that infant formulas intended for preterm infants should contain certain concentrations not only of linoleic and α-linolenic acids, but also various LCP fatty acid derivatives of the n-6 and n-3 series (8,9), yet there are almost no studies concerning the pathways and efficiency by which these fatty acids are used when fed as formula or in human milk lipids (21). Some previous studies suggest that they may behave differently from fatty acids containing 16–18 carbon atoms, including the respective n-6 and n-3 precursors.

During hydrolysis of whale oil triacylglycerols by pig pancreatic lipase in vitro, eicosapentaenoic acid and docosahexaenoic acid were found to be relatively resistant to hydrolysis (22). From studies of hydrolysis by the same lipase of a set of synthetic triacylglycerols, each containing one positional isomer of cis-18:1, the double-bond position varying from the δ-2 to the δ-16 positions, the conclusion was drawn that the double-bond position was of decisive importance for hydrolysis of the ester bond (23). Isomers containing the double bond in the δ-2 to δ-7 positions, δ-1 being the carboxyl carbon, were relatively resistant to hydrolysis, the most hindered being the δ-5 position; no discrimination was seen for positions beyond δ-7. Interestingly, the δ-5 position coincides with the first double bond in arachidonic acid and eicosapentaenoic acid, whereas it is δ-9 for linoleic acid and α-linolenic acid.

Our observation that 20:4 n-6 as compared with 18:2 n-6 was released more slowly by colipase-dependent lipase confirms these results and is compatible with the view that the double-bond position is the cause of resistance to hydrolysis. A likely alternative explanation could have been that 20:4 n-6 to a larger extent than 18:2 n-6 was esterified to the *sn*-2 position of the triacylglycerol, which is resistant to hydrolysis by colipase-dependent lipase (24). If that had been the case, 20:4 n-6 should have accumulated in the monoacylglycerol fraction during hydrolysis. However, no such accumulation was observed. Rather, accumulation occurred in the diacylglycerol fraction, which supports the view that the fatty acid structure rather than the position in the triacylglycerol molecule caused the hindrance.

Because 20:4 n-6 and 20:5 n-3, both having their first double bond in position δ-5, were released at the same rate by colipase-dependent lipase, these results also are supportive of an effect of the double-bond position on hydrolysis. However, it should be noted that the efficiency in use is not dependent on whether the acid belongs to the n-3 or n-6 series per se.

The BSSL is inactive in milk as secreted, but becomes activated when mixed with primary bile salts in duodenal contents (25). It has a broad substrate specificity, and thus hydrolyses a variety of lipid substrates independent of their chemical structure and physical state (26). In contrast to colipase-dependent lipase, it does not possess positional specificity, but also hydrolyses the fatty acid esterified in the *sn*-2 position of a triacylglycerol (24). In the present study this lipase, in contrast to colipase-dependent lipase, did not discriminate between the fatty acids with respect to their double-bond position; 18:2 n-6 and 20:4 n-6 were released as free fatty acids at the same rate as were 20:4 n-6 and 20:5 n-3. With this lipase, a larger proportion of 20:5 n-3 than of 20:4 n-6 was found in the diacylglycerol fraction after 20 min of incubation. However, this difference was seen in the 1,3-diacylglycerol frac-

tion rather than in the 1,2-diacylglycerol fraction (data not shown). After 60 min of incubation, <5% of the labelled 20:5 n-3 remained as diacylglycerol. As for 20:4 n-6, ~90% had been released as free fatty acid (Fig. 2c and f).

Recently Chen et al. (19) in similar systems found that human pancreatic carboxyl ester lipase (carboxylic ester hydrolase) hydrolyzed rat chylomicrons containing [^{3}H]20:4 n-6 and [^{14}C]18:2 n-6 at a slow rate compared with colipase-dependent pancreatic lipase. With the latter lipase, increasing the bile salt concentration in the incubation increased the amount of 20:4 n-6 accumulating in the 1,2-diacylglycerol fraction. The suggestion was made that diacylglycerol containing LCP fatty acids formed during hydrolysis by colipase-dependent lipase could serve as a substrate for carboxyl ester lipase. When the two lipases were combined, efficient hydrolysis was obtained. The same investigators also studied the hydrolysis of chylomicrons containing labelled 20:4 n-6 and 20:5 n-3. The results were similar to those found in the present study, that is, both fatty acids accumulated in the diacylglycerol fraction during hydrolysis by colipase-dependent lipase alone, but not when the two lipases were combined or when these chylomicrons were incubated with human duodenal contents containing both lipases (27). They concluded that both lipases participate in the hydrolysis of 20:4 and 20:5 ester bonds of dietary triacylglycerol.

Because human milk BSSL and human pancreatic carboxylic ester hydrolase are very similar with respect to their properties, we have suggested that the two enzymes may actually be coded for by a common gene (28). Recently we cloned and sequenced the milk lipase and the greater part of the pancreatic counterpart and found the two proteins to be identical (29); these data were confirmed by Reue et al (30). Hence, the concert in results obtained with respect to hydrolysis of LCP fatty acid esters can be explained at the molecular level.

In the newborn infant, particularly the preterm infant, exocrine pancreatic function is not fully developed, and the postprandial activities in duodenal contents of pancreatic lipases is much lower than in adults (31). In the breast-fed infant, the major part of the combined BSSL and carboxylic ester hydrolase activity recorded in duodenal contents after a human milk meal originates in the milk (32). Therefore, in regard to the results presented here, it appears that breast-fed infants have a greater capacity to use LCP fatty acids from dietary triacylglycerol than infants fed formulas supplemented with such a triacylglycerol. This is likely for two reasons. The BSSL contributes to an overall efficient use of the milk triacylglycerol (13,14), which explains why the coefficient of fat absorption generally is higher from raw human milk than from formulas or heat-treated milk (33,34). The results in the present study, in analogy to previous observations, show that LCP fatty acids are released at a slower rate than the precursor fatty acid (e.g., linoleic acid) by colipase-dependent lipase. No such discrimination was seen with BSSL, so the discrimination was almost abolished when the two lipases acted simultaneously, as they do in the breast-fed infant. Although it is difficult to draw conclusions as to the in vivo situation from these data, they are compatible with the view that BSSL is of importance for an efficient use of the milk LCP fatty acids.

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Appendix M

Morgan et al. ("Fatty Acid Balance Studies In Term Infants Fed Formula Milk Containing Long-Chain Polyunsaturated Fatty Acids" Acta Paediatr 87: 136-42 (1998)

Acta Pædiatr 87: 136–42. 1998

Fatty acid balance studies in term infants fed formula milk containing long-chain polyunsaturated fatty acids

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Long-chain polyunsaturated fatty acids (LCP) are thought to be required for optimal nervous system development in the newborn. A commercial milk formula containing LCP (Aptamil-LCP) with a fatty acid profile closely resembling breast milk, has recently been introduced for term infants. The absorption of fatty acids in term infants was examined in a double-blind randomized controlled trial comparing Aptamil-LCP ($n = 20$) and standard Aptamil ($n = 20$). Formula-fed newborn infants were studied from birth for 14 d. Fat balances (3 d) were performed from d 10. A 3-d stool collection was performed from d 10 in a parallel breastfed group ($n = 21$). Plasma samples were taken on d 6. Median fat excretion (mg kg^{-1}) was 897.1, 615.0 and 355.2 with Aptamil, Aptamil-LCP and breastfeeding, respectively. The median total fat absorption coefficient in Aptamil-LCP-fed infants was higher than in those fed standard Aptamil ($p < 0.01$). These findings were accounted for by differences in the excretion and absorption of long-chain saturated fatty acids (C14:0, C16:0 and C18:0). Higher fat excretion was associated with bulkier and firmer stools. Only trace amounts of LCP were detected in the stools of all groups. This accounted for less than 4% of dietary intake in Aptamil-LCP-fed infants. No differences in the utilization of LCP from Aptamil-LCP and breast milk feeding were apparent. Plasma phospholipid fatty acid composition data reflected differences in dietary LCP intake. Thus, PL LCP levels were highest in the breastfed infants and lowest in the Aptamil-fed infants, with values for the Aptamil-LCP-fed group falling in between. □ *Fat balance, polyunsaturated fatty acids, term infant formula*

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Long-chain polyunsaturated fatty acids (LCP) have recently been added to a commercial infant formula. This represents the first significant change to term infant formula in more than a decade. LCP are derived from the essential fatty acids. Linoleic acid (LA; C18:2n-6) and α-linolenic acid (LNA; C18:3n-3) are the precursors to the n-6 LCP and n-3 LCP, respectively (1). LCP are important components of all cell membranes but particularly in the retina and nervous system (2). Evidence suggests that newborn infants are unable to meet the LCP requirements of the developing nervous system without preformed LCP in the diet (2, 3). Breast milk contains preformed LCP but, until recently, artificial formula milks did not. Differences have been demonstrated between breastfed and formula-fed preterm infants when visual acuity and retinal function were assessed (4). More recent studies have reproduced these findings in term infants (5). These neurological differences are abolished by supplementing the formula with n-3 LCP (6). However, unbalanced n-3 LCP supplementation without adding n-6 LCP appears to be detrimental to growth (7).

A formula containing balanced quantities of n-3 and n-6 LCP, similar to those in human milk, has been recommended (8). Aptamil-LCP is a term infant formula containing a fat blend that meets these recommendations. This fat blend uses egg phospholipid as a source of n-6 and n-3 LCP. However, there is little information on how well LCP may be utilized from such a formula or, indeed, from breast milk. Moreover, it is important to establish whether the alternative lipid source used in Aptamil-LCP alters the overall fatty acid absorption profile compared to a standard fat blend.

These issues were examined using 3-d fat balance studies in infants randomized to receive Aptamil-LCP and a similar standard formula, Aptamil (containing no LCP). A 3-d stool collection was also performed in a parallel breastfed group.

Subjects and methods

This study received the approval of the local ethical committee. All term infants, of gestational age 37–42 weeks and birth weight 2.5–4.5 kg, were eligible for study. Recruitment took place antenatally and, occasionally, immediately following delivery. Parental consent was obtained for eligible infants before the first feed. At this time, infants were randomized to receive either Aptamil-LCP or Aptamil using a time-balanced, computer-generated randomization table (IDV Rancode). The groups were

further divided into male and female subgroups to ensure balanced numbers of each gender in the main groups. A parallel breastfed group was also recruited over the same period. Any infant failing to complete the study was withdrawn. Formula-fed infants were replaced (in order) according to their randomization number, once the last number in that group had been reached. All withdrawals were carefully documented.

Infants were fed trial formula in ready-to-feed bottles for the first 14 d of life. Neither the parents nor the nurse were aware which formula the infant was receiving. Breastfed and formula-fed infants was supervised at home by the clinical research nurse. Anthropometric data were recorded at birth and on d 14 in all infants. Additional weight measurements were performed at the start and finish of the fat balance. Weight was measured to the nearest 5 g using top-loading electronic scales. Length was measured to the nearest 1 mm using a Holtain length board. Head and mid-upper arm circumferences were measured to the nearest 1 mm with a non-extendible tape measure. Triceps and subscapular skinfold thicknesses were measured to the nearest 0.1 mm using a Holtain skin calliper.

Fat balances and stool collections (72 h) were performed from d 10 and closely supervised by the clinical research nurse. Specimens were collected on two or three visits per day during the fat balance. After each feed, the bottle and any remaining milk were stored in a sealed container for collection. Formula milk intake was calculated by collecting and weighing bottles before and after all feeds. Weights were recorded to the nearest 0.01 g using electronic scales. Vomiting was quantitatively assessed by weighing preweighed bibs after feeds and correcting the intake accordingly.

Each fat balance began with a feed containing carmine marker (100 mg per 100 ml milk). Infants wore nappies with a rayon nappy liner in place for the duration of the fat balance. All stools were collected following the appearance of the first carmine marker. Any stool adhering to the skin was wiped on to the nappy liner. Nappies were stored in an ice-packed cool box. Liners and stool were transferred to collection pots by the supervising nurse. Specimens were then immediately stored at −40°C. After 72 h the second carmine marker feed was given. Stool collection continued until the second carmine marker appeared.

Stool collections (72 h) were performed from d 10 in breastfed infants, as described above. No quantitative assessment of the volume of breast milk taken could be made. However, a 2 ml sample of breast milk was taken following one feed during the stool collection (random breast, mid-afternoon sample). This was stored at −40°C. The number of stools passed by each infant was documented. The consistency of each stool was recorded as watery, seedy, soft, formed or hard. This was based on a comparison with standard photographs as described in an earlier study (9).

Blood samples were obtained at the same time as the Guthrie test (d 6). Cells and plasma were separated by centrifuge. The plasma was retained and stored at −40°C.

Analytical methods

Samples were analysed blind at the Department of Child Health, University Hospital (Nottingham, UK) as described previously (9, 10). In brief, faecal samples were combined, weighed and homogenized. Lipids were then extracted using the method described by Folch et al. (11). The following internal standards were added to the filtrate: 100 μg heptadecanoic acid, 104.8 μg ml^{-1} triheptadecanoin and 141.2 μg ml^{-1} L-α-phosphatidylcholine diheptadecanoyl. The filtrate was separated into free fatty acid (FFA), triacylglycerol (TG) and phospholipids (PL) using thin-layer chromatography. The resulting samples were transmethylated using the method of Glass (12). Plasma samples were similarly treated.

The final extracts were applied to a Perkin Elmer gas chromatograph model 8140, fitted with a flame ionization detector, using the method described previously (10). In brief, a fused silica column, 30 m in length with an internal diameter of 0.32 mm coated with SP2330 (bicyanopropylphenyl polysiloxane) at a film thickness of 0.2 μm, was run isothermically at 185°C. LCP separation (as indicated by time between peaks) was efficient and occurred over 23 min. Comparison with the retention times and response factors for known fatty acids allowed identification. Response factors were measured for different peaks of interest and correction factors were made if factors were different from 1. This method's limit of detection (10) allowed measurement of FFA ($5 \times 10-4$ μmol 500 ml^{-1} sample) and measurement of TG and PL (1×10^{-3} μmol ml^{-1} sample).

Milk samples were analysed in a similar way, except that the lipid fractions were not separated using thin-layer chromatography. During the study, three batches of each formula was used. In order to avoid effects of variation between batches, 20 samples from each batch were analysed and fatty acid concentration was recorded separately for each batch.

Statistics

All results are presented as medians with 95% confidence intervals (CI) for the median in parentheses. The Kruskal–Wallis test [non-parametric one-way analysis of variance (ANOVA)] was used to compare data from the three groups. The p-value for the test statistic H, resulting from the ANOVA, was tabulated. Values of $p < 0.05$ were taken to be significant These data were subsequently analysed using two-sample Mann–Whitney tests. The subsequent p-values were corrected for multiple comparisons using the Bonferroni method. Zero values appear in the tables where the fatty acid in question was undetectable (see limit of detection above).

Results

During the study, four formula-fed infants (two in each group) were withdrawn, all for violations of the protocol. They were replaced as described previously. Breastfed

Table 1. Anthropometric data during 14-d study period.

Variable	Group			ANOVA
	Aptamil ($n = 20$)	Aptamil-LCP ($n = 20$)	Human milk ($n = 21$)	
Birthweight (g)	3770 (3460–4070)	3430 (3050–3980)	3290 (3050–3570)	0.03[a]
Weight gain (g)	250 (185–295)	200 (140–265)	220 (95–330)	0.53
Weight gain (g kg^{-1} d^{-1})	4.6 (3.3–6.2)	4.1 (2.6–5.6)	5.0 (2.0–7.6)	0.61
Length (mm)	519 (496–528)	520 (498–526)	511 (498–526)	0.81
Gain (mm)	14 (11–20)	13 (10–15)	9 (6–15)	0.053
OFC (mm)	350 (344–360)	347 (334–351)	345 (336–355)	0.38
Gain (mm)	12 (11–15)	10 (8–14)	12 (6–14)	0.58
MUAC (mm)	113 (108–119)	108 (96–117)	108 (101–109)	0.06
Gain (mm)	3 (2–4)	3 (2–4)	2 (1–3)	0.028[a]
TSFT (mm)	49 (41–51)	47 (39–50)	42 (40–49)	0.23
Gain (mm)	2 (2–3)	2 (1–2)	3 (2–4)	0.16
SSFT (mm)	48 (42–52)	45 (40–51)	42 (39–45)	0.18
Gain (mm)	3 (1–3)	1 (0–2)	3 (2–4)	0.11

OFC, Occipital frontal head circumference; MUAC, mid-upper arm circumference; TSFT, triceps skinfold thickness; SSFT, subscapular skinfold thickness.

Values are medians with 95% CI for the median in parentheses; p values are tabulated for the test statistic H, resulting from the ANOVA. Where $p < 0.05$, the following paired comparisons (Mann–Whitney test) are made: Aptamil with breastfeeding (a), Aptamil-LCP with breastfeeding (b), and Aptamil with Aptamil-LCP feeding (c).

[a] $p < 0.05$.

infants had a lower median birth weight than formula-fed infants, although only the differences between Aptamil and breastfeeding were statistically significant. No other differences in anthropometric data were evident (Table 1). In particular, weight gain during the fat balance was very similar in all three groups.

The median fat content (all batches, $n = 60$) was 3.38 and 3.29 g dl^{-1} for Aptamil and Aptamil-LCP, respectively. The fatty acid profiles (all batches $n = 60$) of the two formulae were similar, apart from LCP content (Table 2). LCP were present in Aptamil, but only in trace amounts. In Aptamil-LCP, levels of LCP were at the low end of the range for those obtained by other workers from breastfeeding mothers on a typical Western diet. The median fat content ($n = 21$) was 2.72 g dl^{-1} (range 2.07–4.09) for breast milk. The fatty acid profiles (apart from LCP) of the two formulae were similar to that of breast milk (Table 2). The LCP content (wt.%) of breast milk was higher than that of

Table 2. Milk fatty acid composition.

Fatty acid	Aptamil	Aptamil-LCP	Human milk
C10:0	0.92 (0.39–1.40)	0.49 (0.58–1.43)	0.42 (0.29–0.61)
C12:0	4.81 (3.51–5.84)	4.79 (4.35–5.51)	4.38 (3.62–4.84)
C14:0	5.81 (5.50–6.11)	5.42 (5.04–5.67)	6.32 (4.69–8.07)
C14:1n-7	0.01 (0.00–0.12)	0 (0.00–0.13)	0.18 (0.00–0.29)
C16:0	28.38 (28.12–28.57)	27.73 (26.52–28.39)	24.80 (23.40–25.50)
C16:1n-7	0.48 (0.34–0.66)	0.86 (0.71–0.98)	2.34 (1.81–2.42)
C18:0	9.01 (8.44–9.36)	7.00 (6.52–8.62)	7.20 (6.24–8.74)
C18:1n-9	37.94 (36.45–39.59)	37.12 (35.91–37.81)	38.00 (34.90–40.40)
C18:2n-6 (LA)	11.25 (11.13–11.34)	13.61 (13.31–13.98)	12.70 (8.78–17.30)
C18:3n-3 (LNA)	0.29 (0.25–0.33)	0.64 (0.48–0.75)	0.49 (0.23–0.89)
C18:3n-6/C20:0	0.85 (0.77–0.90)	0.58 (0.42–0.91)	0.26 (0.18–0.37)
C20:2n-6	0.09 (0.00–0.12)	0.14 (0.06–0.25)	0.62 (0.49–0.74)
C20:3n-6	0.01 (0.00–0.12)	0.05 (0.02–0.13)	0.41 (0.29–0.63)
C20:4n-6 (AA)	0.07 (0.02–0.12)	0.30 (0.29–0.32)	1.30 (0.77–1.82)
C22:4n-6	0.01 (0.00–0.15)	0.05 (0.00–0.14)	0.07 (0.00–0.23)
Total n-6 LCP	0.18 (0.02–0.41)	0.54 (0.42–0.84)	2.38 (1.98–3.20)
C20:5n-3	0.02 (0.00–0.04)	0.07 (0.03–0.12)	0.07 (0.00–0.15)
C22:5n-3	0 (0.00–0.10)	0.05 (0.02–0.08)	0.00 (0.00–0.12)
C22:6n-3 (DHA)	0.05 (0.02–0.06)	0.27 (0.13–0.63)	0.58 (0.24–0.71)
Total n-3 LCP	0.07 (0.02–0.20)	0.39 (0.23–0.83)	0.76 (0.47–1.22)
Others	0.23 (0.04–0.60)	0.33 (0.13–0.63)	0.19 (0.00–0.43)

Values are medians (wt%) with 95% CI for the median in parentheses.

Table 3. Milk fatty acid intake during 3-d fat balance.

Fatty acid	Aptamil ($n = 20$)	Aptamil-LCP ($n = 20$)
Main formula milk (g kg^{-1})		
Total fat	15.4 (12.7–16.6)	14.57 (13.4–15.7)
C14:0	0.896 (0.737–0.961)	0.797 (0.730–0.856)
C16:0	4.37 (3.92–4.69)	4.08 (3.79–4.38)
C18:0	1.37 (1.23–1.47)	1.00 (0.934–1.08)
C18:1n-9	5.87 (5.26–6.29)	5.32 (4.95–5.72)
C18:2n-6 (LA)	1.73 (1.55–1.86)	1.90 (1.77–2.05)
C18:3n-3 (LNA)	0.045 (0.040–0.048)	0.099 (0.092–0.106)
LCP (mg kg^{-1})		
C20:4n-6 (AA)	7.7 (6.9–8.3)	45.2 (42.0–48.5)
Total n-6 LCP	37.0 (33.2–39.7)	90.3 (83.9–97.0)
C22:6n-3 (DHA)	7.6 (6.8–8.1)	39.3 (36.5–42.3)
Total n-3 LCP	20.1 (18.0–21.5)	52.5 (48.7–56.3)

Values are medians with 95% CI for the median in parentheses.

Aptamil-LCP, particularly for n-6 LCP. The median AA concentration (mg 100 ml^{-1} milk) was 2.4 (0.7–4.1), 9.9 (9.5–10.5) and 26.6 (20.3–45.3) for Aptamil, Aptamil-LCP and breast milk, respectively. The median DHA concentration (mg 100 ml^{-1} milk) was 1.7 (0.7–2.0), 8.9 (4.2–20.7) and 12.2 (7.8–28.3) for Aptamil, Aptamil-LCP and breast milk, respectively. This suggests that LCP intake (especially n-6 LCP) may be considerably higher in breastfed infants than in Aptamil-LCP-fed infants. However, as the volumes of breast milk consumed are unknown, this comparison must be guarded.

Total fat and fatty acid intake (Table 3) were calculated for each infant using the specific data from the batch of milk fed to that infant. The median volumes of milk (ml kg^{-1} d^{-1}) were 153.7 (138.4–165.6) and 143.7 (133.5–154.3) for Aptamil and Aptamil-LCP feeding, respectively (not statistically significant).

Faecal fat analysis revealed that the most fat was excreted as FFA in all groups (Table 4). However, TG accounted for a greater proportion of the fat in breastfed stool. At first sight, these results suggest poorer TG hydrolysis and higher TG excretion in breastfed infants. However, fat excretion is much lower in breastfed infants (described below) and so absolute levels of stool TG are similar in formula-fed and breastfed infants. Therefore, it can be concluded that the extra fat excreted by formula-fed infants is as FFA. Subsequent results combine values from all three sources.

Median total fat excretion was 897 mg with Aptamil feeding, 615 mg with Aptamil-LCP feeding and 355.2 mg with breastfeeding (Table 5). All two-sample comparisons were statistically significant. Most fat excreted was as long-chain saturated fatty acids, with more than half accounted for by C16:0 in both groups (Table 5). The excretion pattern for each of the long-chain saturated fatty acids (C14:0, C16:0 and C18:0) mirrored that for total fat excretion and thus accounted for the main trend between the three groups. Small amounts of α-linolenic acid were detectable in breastfed infants' stools but none in formula-fed infants' stools.

LCP were detected in tiny amounts in the stools of term infants. Comparisons between the three groups revealed no statistical differences. However, the breastfed infants demonstrated a wide variation in LCP excretion, with some individuals excreting relatively high amounts. This may be a reflection of the higher intake. The fact that excreted LCP levels obtained with Aptamil and Aptamil-LCP feeding were similar suggests that some excreted LCP may be endogenously derived.

The median absorption coefficients (Table 6) for total fat

Table 4. Stool composition over 3-d fat balance.

Fatty acid	Aptamil ($n = 20$)	Aptamil-LCP ($n = 20$)	Human milk ($n = 21$)	ANOVA
Stool frequency	6.5 (5–12)	5 (4–6)	12 (10–15)	$< 0.001^{ab*}$
Stool weight (g kg^{-1})	16.9 (11.5–20.1)	13.0 (10.8–16.1)	10. 5(6.8–11.2)	$< 0.01^{c}$
Score (consistency)	206	221	309	
Total fat (mg kg^{-1})	897.1(711.2–1246)	615.0(524.4–810.8)	355.2 (234.4–609.2)	$< 0.01^{ab}$
FFA (wt%)	92.7 (87.3–97.3)	89.7 (86.6–98.7)	87.0 (75.0–92.0)	0.031^{a}
TG (wt%)	6.6 (2.6–12.5)	7.8 (0.8–13.0)	11.0 (8.9–24.9)	0.019^{a}
PL (wt%)	0 (0.0–4.9)	0 (0.0–4.4)	0 (0.0–0.1)	

FFA, free fatty acids; TG, triacylglycerol; PL, phospholipid.

Values are median with 95% CI for the median in parentheses.

p values are tabulated for the test statistic H, resulting from the ANOVA. Where $p < 0.05$, the following paired comparisons (Mann–Whitney testing) are made: Aptamil with breastfeeding (a), Aptamil-LCP with breastfeeding (b), and Aptamil with Aptamil-LCP feeding (c).

$^{ac}p < 0.05$, $^{ab}p < 0.01$, $*p < 0.001$.

Table 5. Fatty acid excretion in stools over 3 d.

Fatty acid	Aptamil ($n = 20$)	Aptamil-LCP ($n = 20$)	Human milk ($n = 21$)	ANOVA
Total fat	897.1 (711.2–1246)	615.0 (524.4–810.8)	355.2 (234.4–609.2)	$< 0.01^{a}$
C14:0	29.7 (18.1–47.5)	18.4 (10.2–31.8)	9.5 (2.8–19.8)	$< 0.01^{a}$
C14:1n-7	3.4 (2.9–5.0)	2.6 (1.9–3.9)	2.3 (0.6–6.0)	0.15
C16:0	536.8 (410.4–763.1)	374.3 (363.4–462.9)	126.6 (79.3–194.2)	$< 0.001^{ab}$
C16:1n-7	1.8 (0.8–1.9)	0.6 (0.2–1.1)	2.0 (0.5–10.6)	0.015^{bc}
C18:0	198.0 (164.4–273.7)	128.7 (98.9–171.2)	73.7 (36.3–127.0)	$< 0.001^{ac}$
C18:1n-9	74.0 (46.7–128.2)	42.8 (27.1–75.8)	84.9 (26.4–162.8)	0.16
C18:2n-6 (LA)	7.4 (1.0–17.4)	4.5 (3.2–10.7)	15.2 (5.6–31.6)	0.080
C18:3n-3 (LNA)	0	0	1.3 (0.0–4.5)	$< 0.01^{ab}$
C18:3n-6 & C20:0	18.4 (13.7–23.9)	3.9 (2.7–5.3)	4.4 (2.8–9.4)	$< 0.001^{ac}$
C20:2n-6	0.95 (0.00–1.38)	0.42 (0.00–0.77)	2.78 (0.60–5.43)	$< 0.01^{b}$
C20:3n-6	0.06 (0.00–0.79)	0.55 (0.00–1.20)	1.60 (0.03–2.28)	0.15
C20:4n-6 (AA)	0.91 (0.00–2.41)	1.10 (0.00–3.16)	3.25 (1.02–9.87)	0.16
C22:4n-6	0	0	0 (0.00–1.38)	0.12
Total n-6 LCP	2.89 (1.84–4.17)	2.98 (1.45–6.43)	10.80 (4.82–19.48)	0.056
C20:5n-3	0	0	0	
C22:5n-3	0	0	0	
C22:6n-3 (DHA)	0 (0.00–1.57)	0 (0.00–1.77)	0 (0.00–3.68)	0.44
Total n-3 LCP	0 (0.00–1.83)	2.27 (0.00–3.32)	1.82 (0.00–3.68)	0.12
Others	3.0 (2.1–4.5)	2.2 (0.5–5.9)	11.0 (2.3–27.0)	0.060

Values are medians (mg kg^{-1} body weight) with 95% CI for the median in parentheses.
p-Values are tabulated for the test statistic H, resulting from the ANOVA. Where $p < 0.05$, the following paired comparisons (Mann–Whitney testing) are made: Aptamil with breastfeeding (a), Aptamil-LCP with breastfeeding (b), and Aptamil with Aptamil-LCP feeding (c).
$^{aa}p < 0.05$, $^{ab}p < 0.01$, $^{*}p < 0.001$.

were 94.3% (91.8–95.3%) for Aptamil and 95.6% (94.6–96.5%) for Aptamil-LCP. The narrow CI suggest a high level of reproducibility. The difference between the formulae was small but statistically significant ($p < 0.01$). This resulted mainly from differences in individual saturated fatty acid absorption, with C14:0, C16:0 and C18:0 all showing statistically significant differences between the groups ($p < 0.05$). Absorption declined with increasing saturated fatty acid chain length in both groups, as previously reported. This effect was more marked in the Aptamil group. Median absorption coefficients for the 18-carbon unsaturated fatty acids all exceeded 99%.

For LCP we describe LCP disappearance rather than absorption coefficients. This reflects the limited interpretation of such data where dietary content of specific fatty acids is low, but also the importance of relating fatty acid excretion data to dietary intake. Median AA disappearance values (%) were 88.9 (67.6–100) and 98.3 (96.1–100) for Aptamil and Aptamil-LCP, respectively. Median total n-6 LCP values (%) were 92.7 (83.5–94.1) and 96.4 (94.4–98.3) for Aptamil and Aptamil-LCP feeding, respectively. Most infants did not excrete n-3 LCP in detectable quantities, equating to 100% disappearance.

Clear differences were apparent in stool frequency (Table 4). All two-sample comparisons showed statistically significant differences. In contrast, median stool weight was lower in breastfed than in formula-fed infants. Therefore, in general, breastfed infants had frequent, low-weight stools compared with the formula-fed infants. These differences were also reflected in the stool composition data (Table 4). This is given as a score for each group. Breastfed infants tended to have softer stools (reflected in the total score), with 77.5% described as seedy and none as formed or hard. In the Aptamil-LCP group, soft stools

Table 6. Fatty acid absorption.

Fatty acid	Aptamil ($n = 20$)	Aptamil-LCP ($n = 20$)	p value
Total fat	94.3 (91.8–95.3)	95.6 (94.6–96.5)	$< 0.01^{*}$
C14:0	96.2 (94.6–97.8)	97.8 (96.5–98.6)	0.037^{*}
C14:1n-7	11.9 (–26.1–29.7)	6.0 (–27.1–43.3)	0.96
C16:0	87.0 (84.7–90.2)	90.2 (88.4–92.0)	0.030^{*}
C16:1n-7	98.4 (97.5–98.7)	99.5 (98.9–99.8)	$< 0.001^{*}$
C18:0	84.7 (77.7–86.3)	87.6 (84.8–89.6)	0.023^{*}
C18:1n-9	98.8 (97.2–99.0)	99.2 (98.7–99.5)	0.062
C18:2n-6 (LA)	99.6 (99.0–99.9)	99.8 (99.5–99.8)	0.15
C18:3n-3 (LNA)	100.0	100.0	

Values are medians (%) with 95% CI for the median in parentheses.
p values result from Mann–Whitney testing; $^{*}p < 0.05$.

predominated (69.6%), with only 4% as formed or hard. The Aptamil group had the firmest stools, with 25.7% described as formed or hard. These findings suggest that reduced fat absorption is associated with bulkier, firmer stools.

Plasma phospholipid (PL) fatty acid compositions were also measured, although these were not the main part of the study. Median PL total n-6 LCP (wt.%) were 16.20 (14.28–17.75), 20.53 (16.64–22.32) and 25.52 (21.94–28.29) for Aptamil, Aptamil-LCP and human milk feeding, respectively ($p < 0.001$). Median PL total n-3 LCP (wt.%) were 4.19 (1.03–6.42), 4.38 (2.40–5.91) and 4.47 (2.62–5.72) for Aptamil, Aptamil-LCP and human milk feeding, respectively (not statistically significant).

Discussion

The n-6 LCP levels in the present breast milk samples are higher than those generally reported for mothers on typical Western diets (13). However, this represents data from mature human milk whereas our samples are from d 10–11. Transitional term human milk has a higher LCP content than mature term human milk (14). There is no obvious explanation in the maternal diet, although this was not formally recorded. Analysis of human milk was performed simultaneously with formula milk. In contrast, levels recorded in Aptamil-LCP were lower than those anticipated (by Milupa) from formula milk composition data, based on the contribution and composition of the different lipid sources. Nevertheless, subsequent separate analysis of study Aptamil-LCP by Milupa's own laboratories closely agreed with our data.

Lipid is absorbed well from both formulae. Indeed, the absorption coefficients are higher than those previously reported for term infants (15, 16). However, no recent fat balance studies have been conducted on healthy term infants using current commercial formulae for comparison. Falsely high absorption coefficients would result from either overestimating the milk intake or underestimating stool output. However, this study specifically used ready-to-feed formula to reduce inaccuracies in feed preparation that may have been present in previous studies that used formula reconstituted from powder. It is possible that underestimating losses through vomiting could have led to an overestimation of intake. However, all bibs were preweighed and retained for quantitative adjustment of intake. Underestimating stool output is, of course, difficult to exclude in a study performed at home, even under close supervision. However, lost stool specimens would have had to have been similar in all patients to achieve the relatively narrow CI for fat excretion and absorption. This seems an unlikely scenario.

The decline in absorption coefficient with increasing saturated fatty acid chain length has most recently been shown in preterm infants (9). Likewise, the highly efficient absorption of 18-carbon unsaturated fatty acids is well described (9, 15). The superior fat absorption with Aptamil-LCP feeding, mainly apparent in the long-chain saturated fatty acids, was unexpected. This emphasizes the importance of fully assessing the results of changes in lipid sources for formula milks.

Egg yolk is the source of 17.5% of the lipid in the Aptamil-LCP fat blend. This is the main new constituent and is the primary source of LCP (17). It has been shown that the long-chain saturated fatty acids esterified to the *sn*-2 position of the TG molecules are better absorbed in newborn infants (18). If egg yolk TG demonstrate this more favourable structure then this may explain the improved long-chain saturated fatty acid absorption. However, egg yolk is also rich in PL. Addition of these polar lipids to artificial formula milk may improve the stability of the emulsion (19). This may optimize TG exposure to intestinal lipase activity and so enhance lipid absorption. It is possible that dietary LCP themselves may contribute to gut function. Changing the fatty acid composition of cell membranes can alter the membrane structure and function of cells, including enterocytes. Indeed, animal studies have demonstrated improved water, electrolyte and lipid absorption in the presence of polyunsaturated fatty acid-enriched diets (20).

The fact that breastfed infants absorb fat better than formula-fed infants has been known for some time (15). This is implicit in our fat excretion data, although no formal fat balance was performed in our breastfed infants. TG structure, the nature of the human milk fat emulsion and the presence of a human milk lipase are probably among the contributing factors.

Little is known about LCP utilization in the neonatal period. However, Bottino et al. (21) found that EPA and DHA in whale oil TG were relatively resistant to hydrolysis by pancreatic lipase. Heimermann and Holman (22) later showed this was due to the proximity of the first double bond to the carboxyl terminus. This resistance to hydrolysis does not hold for the other lipases. Thus, the importance of gastric lipases in neonatal fat digestion would appear to favour LCP utilization (23). This is compounded in breastfed infants by the presence of bile-salt-stimulated lipase (24). Interestingly, n-6 LCP excretion was highest in the breastfed infants, although this may reflect the higher n-6 LCP milk concentrations.

In human milk, more than 90% of LCP are present as esters of TG. In Aptamil-LCP approximately 70% of LCP are present as esters of PL. This may be expected to result in impaired utilization of LCP. Gastric lipases have no activity against PL ester (23). Furthermore, it would be expected than phospholipase A_2 concentration in the neonatal intestinal lumen would be low, as with other pancreatic enzymes. However, Aptamil-LCP-fed infants showed no evidence of excess PL or LCP excretion.

It is likely that in humans any difficulty in utilizing dietary LCP occurs at the stage of hydrolysis. This is supported by evidence, in humans, that EPA and DHA are only 68% and 57% absorbed from fish oil TG, respectively, but completely absorbed from fish oil FFA (25).

It is possible that the gut flora may alter undigested fatty acids, including LCP. There is evidence that saturation of

unsaturated fatty acids may occur under the anaerobic conditions of the gut and that desaturation may occur to LA in the aerobic conditions near the anus (26). However, experiments on germ-free and conventional rats (27) indicate greater lipid excretion (C16:0 and C18:0) in the latter, although it was clear that this did not result from hydrogenation of unsaturated fatty acids. Further evidence to support unaltered excretion of long-chain unsaturated fatty acids comes from studies with erucic acid (28). Shedding of gut enterocytes into the intestinal lumen may also contribute to excreted LCP. However, as this represents an endogenous source, this can be considered a legitimate part of the fatty acid balance.

We would accept that, given the relatively low dietary LCP intake and the fact that it is not possible to exclude an effect of gut flora on excreted LCP, certain limitations are placed on the interpretation of the LCP excretion data. However, if these findings are considered along with preterm infant data (29), which show a similar pattern but with much higher levels of LCP excretion, there is circumstantial evidence that excreted LCP reflect LCP utilization. Moreover, to establish conclusively the fate of dietary LCP, it is likely that isotope labelling of dietary LCP would be required. It would be essential to have the LCP excretion data that we describe, before embarking on such a study.

The plasma data provide some supportive evidence for the fat balance data. However, there is evidence that PL n-3 LCP composition differences are not apparent before d 7 (30). This is consistent with the present findings. It is not clear whether the failure of formulae containing LCP to attain levels seen in breastfed infants are a reflection of differences in LCP absorption characteristics or milk LCP composition.

This study indicates that LCP are excreted in tiny amounts (less than 4% intake) in term infants fed Aptamil-LCP. There is no evidence of poorer utilization of dietary LCP from the lipid source used in the artificial formula compared to breast milk. In addition, the altered fat blend of Aptamil-LCP showed a small but statistically significant improvement in the absorption coefficients of long-chain saturated fatty acids.

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Appendix N

Amate et al. "Feeding Infant Piglets Formula with Long-Chain Polyunsaturated Fatty Acids as Triacylglycerols or Phospholipids Influences the Distribution of these Fatty Acids in Plasma Lipoprotein Fractions" J Nutr 131: 1250-55 (2001)

Feeding Infant Piglets Formula with Long-Chain Polyunsaturated Fatty Acids as Triacylglycerols or Phospholipids Influences the Distribution of These Fatty Acids in Plasma Lipoprotein Fractions[1]

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ABSTRACT Several sources of long-chain polyunsaturated fatty acids (LCP) are currently available for infant formula supplementation. These oils differ in their fatty acid composition, the chemical form of the fatty acid esters [triacylglycerols (TG) or phospholipids (PL)] and presence of other lipid components. These differences may affect LCP absorption, distribution and metabolic fate after ingestion. The purpose of the present study was to evaluate the influence of different chemical forms of dietary LCP on the composition of plasma, plasma lipoproteins, liver and jejunum in infant piglets. Thirty pigs (5 d old) were bottle-fed different diets for 4 wk: a control diet (C), a diet containing LCP as TG from tuna and fungal oils (TF-TG) or a diet containing LCP as PL from egg yolk (E-PL). We measured lipid and fatty acid composition of plasma and lipoproteins, as well as lipid composition of liver and intestinal mucosa. The arachidonic and docosahexaenoic acids in HDL-PL were significantly higher in piglets fed the E-PL diet than in those fed the TF-TG diet. Opposite results were found in the LDL-PL diet. No significant differences were found between groups in TG or cholesterol concentrations of plasma or lipoproteins. Arachidonic acid in plasma PL and cholesteryl esters was significantly higher in the E-PL group than in the TF-TG group. The chemical form in which LCP esters are present in different dietary sources influences their distribution in plasma lipoproteins. This may be important for infant nutrition and suggests that not all LCP sources may be biologically equivalent. J. Nutr. 131: 1250–1255, 2001.

KEY WORDS: • *lipoproteins* • *long-chain polyunsaturated fatty acids* • *phospholipids* • *piglets* • *triacylglycerols*

Human milk contains high levels of long chain polyunsaturated fatty acids (LCP)[3] (Jensen et al. 1995). LCP with 20 and 22 carbon atoms, mainly arachidonic acid (AA) and docosahexaenoic acid (DHA), are critical components of cellular membranes and are especially enriched in the developing retina and gray matter of the brain (Bazan et al. 1986, Clandinin et al. 1980). Some reports indicate that LCP status is improved in infants fed LCP-containing formulas (Clandinin et al. 1992, Koletzko et al. 1989). In addition, other studies show that such improvement could be correlated with improved visual acuity and cognitive development (Birch et al. 1992a, 1992b and 1998, Carlson et al. 1993 and 1996, Makrides et al. 1995, Uauy et al. 1990). Based on these observations, international committees have recommended that infant formula, especially for preterm infants, be supplemented with AA and DHA at levels normally found in human milk (British Foundation 1992, ESPGAN 1991, FAO/WHO 1994, ISSFAL 1994). On the other hand, the need to supplement formulas for term infants with preformed LCP continues to be a matter of debate (Auestad et al. 1997, Heird et al. 1997).

Lipid components of infant formulas are obtained from vegetable oils, which contain fatty acids of up to 18 carbon atoms in length. For this reason, LCP supplementation to the diet requires other lipid sources, generally of animal origin. There are several LCP-enriched sources available, such as fish oils, oils from unicellular organisms and egg oil fractions. Fish and unicellular oils are mainly composed of triacylglycerols (TG), and commercial egg oil fractions are rich in phospholipids (PL). Furthermore, these lipid sources also differ in fatty acid composition, presence of other lipid components and the molecular structure of their TG and PL. Because lipid digestion is a complex process involving enzymes, it is possible that these differences between lipid sources affect LCP absorption, distribution and metabolic fate.

Triacylglycerols provide >90% of the total energy of dietary fat; therefore, TG digestion and absorption have been extensively studied. On the other hand, little information is available about dietary PL. Intestinal hydrolysis of TG and PL yields different products: 2-monoacylglycerols and free fatty acids in the case of TG and 1-lysophospholipids and free fatty acids in the case of PL (Pufal et al. 1995, Thomson et al. 1988). Those products are taken up by enterocytes, reesterified and secreted into lymph chylomicrons.

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[3] Abbreviations used: AA, arachidonic acid; DHA, docosahexaenoic acid; LCP, long-chain polyunsaturated fatty acids; PL, phospholipids; TG, triacylglycerols.

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A number of studies have focused on TG absorption and metabolism, mainly on the importance of the *sn*-2 position (Christensen et al. 1995, Jensen et al. 1994, Pufal et al. 1985). Less attention has been paid to dietary PL, but there is evidence of impaired TG absorption when the supply of exogenous PL is insufficient for micelle formation during fat digestion and absorption (Levy and Roy 1989). Moreover, clinical trials with premature infants found that DHA from egg PL was better absorbed than DHA from breast milk and DHA TG from single-cell oils (Carnielli et al. 1995). Other studies have also shown a positive effect of PL-LCP supplementation on fat absorption (Morgan et al. 1998). PL are also essential for intestinal lipoprotein formation and for fat distribution outside the enterocytes. Data from animal studies suggest that the intraduodenal infusion of triolein results in the formation of chylomicrons, whereas the infusion of egg phosphatidylcholine favors the formation of intestinal VLDL-size particles (Tso et al. 1984).

Based on previous work, we hypothesized that the chemical structure and composition of LCP sources used to supplement infant formula may influence the distribution of these important fatty acids and that therefore LCP sources may not be biologically equivalent. For this purpose, we evaluated the effects of dietary LCP as TG or PL on plasma and plasma lipoproteins in infant piglets. Because small intestine and liver are key organs in the metabolism of lipoproteins, we also determined the lipid composition of jejunum and liver.

MATERIALS AND METHODS

Animals and diets. Thirty 5-d-old Yorkshire piglets at term gestation (obtained from Ntra. Sra. de las Mercedes Farm, La Guardia, Jaen, Spain) were randomly assigned to each of three dietary groups. Each group of piglets was housed together and freely fed by bottle three times a day for 4 wk. Heating was provided with spot heat lamps attached above each cage. The study was approved by the Animal Care Committee at the University of Granada and conforms to the European Union Regulation of Animal Care for the care and use of animals for research.

Three powdered formulas, identical in all ingredients except for fat composition, were designed to meet the nutrient requirement of growing piglets (Miller and Ullrey 1987). The formulas were dissolved in warm water at a concentration of 188 g/L. The general composition of the control formula has been previously reported (Lopez-Pedrosa et al. 1998). Control fat was composed of a blend of olive, soy and coconut oils and milk fat. LCP were added by supplementation with either tuna and fungal oil (TF-TG diet) or egg yolk PL (E-PL diet). The final fatty acid composition of the diets is given in **Table 1**. Tuna oil with a low 20:5(n-3)/22:6(n-3) ratio was supplied by Mochida (Tokyo, Japan), and fungal oil was supplied by Suntory (Tokyo, Japan). Part of the vegetable fat blend in the control formula (2.7 g/100 g) was replaced by tuna and fungal oils to reach 0.6 and 0.3 g of AA and DHA/100 g, respectively. E-PL (Ovothin 160) was supplied by Lucas Meyer (Hamburg, Germany). For this diet, 13.2 g/100 g of the fat blend in the control formula was replaced with this source of PL to reach the same concentration of AA and DHA as in the TF-TG diet.

Because the E-PL source contained cholesterol, the control and TF-TG diets were supplemented with cholesterol to reach a final concentration of 3.3 g/100 g diet. In this fashion, dietary cholesterol effects on lipoprotein metabolism were avoided, and it was ensured that any differences between the LCP supplemented groups were due to the particular forms of LCP in the diets.

Analytical procedures. After a 4-wk feeding period, piglets were deprived of food overnight and bled to death via jugular vein puncture while under anesthesia. Blood was collected with tripotassium EDTA (2.7 mmol/L) as anticoagulant and centrifuged at 3000 × *g* for 10 min at 4°C. An aliquot of plasma (10 mL) was collected for lipoprotein isolation; the remaining plasma was frozen in liquid nitrogen and stored at −80°C until analyzed. Livers were excised, washed in cool saline solution and dried with filter paper to remove excess water. Proximal jejunum (60 cm) was removed, rinsed thoroughly with cold saline solution, opened lengthwise and blotted dry. The mucosa was removed by scraping the entire luminal surface with a glass coverslip over an ice-cold Petri dish. The livers and jejunum mucosa were also frozen in liquid nitrogen and stored at −80°C.

TABLE 1

Fatty acid composition of adapted milk formula (control) and the same diet supplemented with (n-6) and (n-3) long-chain polyunsaturated fatty acids from fungal and tuna oils (TF-TG) or from egg yolk phospholipids (E-PL)

Fatty acid	Control	TF-TG	E-PL
	g/100 g total fatty acid		
8:0	2.3	1.9	1.7
10:0	2.8	2.5	2.2
12:0	1.6	1.7	1.8
14:0	5.6	6.2	6.2
16:0	22.3	23.4	24.9
18:0	8.6	8.4	9.9
16:1(n-7)	0.6	0.7	0.8
18:1(n-9)	36.7	35.8	35.2
18:2(n-6)	13.6	12.2	11.6
18:3(n-3)	1.2	1.1	1.2
20:4(n-6)	0.1	0.6	0.6
22:6(n-3)	—	0.3	0.3

Plasma density was adjusted to 1300 kg/L with KBr and overlaid with NaCl (0.15 mol/L). Lipoproteins were separated through discontinuous density gradient ultracentrifugation with a Beckman L8-70M ultracentrifuge equipped with a vertical vTi50 rotor (Beckman Instruments, Palo Alto, CA) at 240,000 × *g* for 2 h at 10°C. After centrifugation, bands corresponding to VLDL, LDL and HDL were collected and frozen at −80°C until analysis. To determine the different lipid classes of plasma and lipoproteins, these were extracted using hexane/isopropanol (3:2) according to Kolarovic and Fournier (1986) and dried under nitrogen. Lipid fractions from plasma and lipoprotein extracts were separated by thin-layer chromatography on Silica Gel 60 plates (0.5 mm; Merck, Darmstadt, Germany) using hexane/isopropyl ether/acetic acid (75:25:1.5) according to the method previously described by Skipski and Barclay (1969).

To analyze the lipid composition, 0.2 g of liver and intestinal mucosa was homogenized in distilled water and extracted with hexane/isopropanol (3:2). Lipid extracts were dissolved in chloroform, aliquots were taken in duplicate for each measurement and the solvent removed under nitrogen. Isopropanol (100 μL) was added to facilitate mixing with enzymatic reagents, and total and free cholesterol and triacylglycerol concentrations were determined through spectrophotometry with commercial kits (Roche Diagnostic GmbH, Mannheim, Germany). PL concentrations were determined as inorganic phosphorous after sample mineralization (Ziversmit et al. 1950).

Cholesterol and triglycerides in plasma were measured directly with the commercial kits mentioned and according to the supplier's instructions. In lipoproteins, those lipids were measured by the same method but adapted for a microplate assay. PL in plasma and lipoproteins were also measured in lipid extracts (Ziversmit et al. 1950).

Lipid from different plasma fractions and lipoproteins were converted to fatty acid methyl esters through transmethylation as previously described by Lepage and Roy (1986). Fatty acid methyl esters were separated and quantified by gas-liquid chromatography with a Hewlett Packard model 5890 gas chromatograph (Palo Alto, CA) equipped with a flame ionization detector and a 60 m × 0.32 mm internal diameter SP-2330 capillary column (Supelco, Bellefonte, PA) as previously described (Amate et al. 1999).

Statistical analysis. Diet group differences were investigated by one-way ANOVA with the 7D program of BMDP Statistical Software PC 90 version (Los Angeles, CA) (Dixon et al. 1990). The

homogeneity of variances was analyzed by Levene's test. If variances were not homogeneous, Welch's test was used to study group differences. When a significance difference was found ($P < 0.05$), Bonferroni's test was used to examine individual comparisons. Values in the text are means ± SEM.

RESULTS

Growth and formula intake. The overall mean body weight at the beginning of the study was 2321 ± 46.9 g. Body weight increased exponentially over time. No significant differences were found between groups, with the overall body weight at the end of the study 5848 ± 195.4 g. The daily energy intake was 1239 ± 23 kJ/kg body.

Fatty acid composition of plasma and plasma fractions. Proportions of AA and DHA in plasma total lipids and its fractions generally were higher in both LCP-supplemented groups than in the control group (**Table 2**). Oleic acid in plasma and plasma PL and CE were higher in the control group than in both LCP-supplemented groups, except in PL of piglets fed TF-TG. Proportions of 18:2(n-6) in plasma PL of control piglets were also higher than those of the experimental groups.

The proportion of AA in PL and CE was significantly higher in the E-PL group than in the TF-TG group. No significant differences were found in the fatty acid composition of plasma TG among the three groups.

Fatty acid composition of lipoproteins. As in plasma lipids, proportions of DHA were significantly greater in both LCP-supplemented groups compared with the control group in all lipoprotein fractions (**Table 3**). AA was also greater in HDL lipids of LCP-supplemented groups and in VLDL and LDL lipids of the E-PL group. On the other hand, the VLDL and HDL 18:1(n-9) level was lower in the E-PL and TF-TG groups than in the control group. A lower proportion of 18:2(n-6) was present in HDL from both LCP-supplemented groups compared with the control group. Proportions of AA and DHA in HDL-PL were significantly higher in piglets fed the E-PL diet than in the control and TF-TG piglets (**Fig. 1**). In LDL-PL, the TF-TG group had higher proportions of both AA and DHA than the E-PL and control groups.

Lipid composition of plasma, lipoproteins, liver and jejunum. No significant differences were found between the control and LCP-supplemented groups or between the LCP-supplemented groups in the concentrations of TG, PL and total, free and esterified cholesterol of plasma and lipoproteins (data not shown). Total cholesterol concentrations of liver was significantly lower in both LCP-supplemented groups than in the control group (data not shown). Concentrations of cholesterol, TG and PL in liver did not differ between the E-PL and TF-TG groups. Lipid composition of jejunal mucosa did not differ between the study groups (data not shown).

DISCUSSION

There have been many studies dealing with the influence of LCP-supplemented diets during the postnatal period in experimental animals (Arburckle et al. 1991, Foote et al. 1990) and in infants (Birch et al. 1992a, 1992b and 1998, Carlson et al. 1993 and 1996, Clandinin et al. 1992, Koletzko et al. 1989, Makrides et al. 1995, Uauy et al. 1990). However, to our knowledge, only two of these studies compared diets containing LCP from different sources. In one study, a comparison of LCP absorption in preterm infants fed either breast milk, infant formula (without LCP), formula with LCP derived from TG or formula with LCP from PL was reported (Carnielli et al. 1995); the other study reported the fatty acid composition of brain cortical areas and non-neural tissues in piglets fed either sow's milk, a control formula, formulas enriched with (n-3) fatty acids or formulas enriched with (n- 3) and (n-6) fatty acids from either egg yolk or pig brain PL (Goustard-Langelier et al. 1999). None of them studied lipoprotein composition.

In the present study, we report the influence of dietary

TABLE 2

Selected fatty acid composition of plasma lipids and its fractions in infant piglets fed the control diet or diets supplemented with long-chain polyunsaturated fatty acids (LCP) as triacylglycerols (TG) or phospholipids (PL)[1]

	16:0	18:0	16:1(n-7)	18:1(n-9)	18:2(n-6)	AA[2] 20:4(n-6)	22:5(n-6)	18:3(n-3)	20:5(n-3)	DHA 22:6(n-3)
	g/100 g total fatty acids									
Total lipids										
Control	18.1 ± 0.5	11.9 ± 0.2	0.7 ± 0.0	27.2 ± 0.8[a]	25.9 ± 0.5	5.5 ± 0.3[b]	0.26 ± 0.0[b]	0.9 ± 0.0	0.19 ± 0.0[b]	1.3 ± 0.1[b]
TF-TG	19.9 ± 0.4	11.9 ± 0.4	0.5 ± 0.0	23.8 ± 0.3[b]	24.4 ± 0.3	7.1 ± 0.3[a]	0.19 ± 0.0[b]	0.8 ± 0.0	0.29 ± 0.0[a]	2.8 ± 0.2[a]
E-PL	19.2 ± 0.3	12.8 ± 0.3	0.6 ± 0.0	21.7 ± 0.6[b]	24.8 ± 0.7	8.1 ± 0.4[a]	0.46 ± 0.0[a]	0.7 ± 0.0	0.17 ± 0.0[b]	2.6 ± 0.2[a]
Phospholipids										
Control	24.9 ± 0.7	29.3 ± 1.0	0.5 ± 0.1	14.8 ± 1.1[a]	12.8 ± 0.6[a]	3.9 ± 0.4[b]	0.4 ± 0.0	—	—	0.9 ± 0.1[b]
TF-TG	26.9 ± 0.9	29.7 ± 0.8	0.5 ± 0.1	13.5 ± 1.1[a]	10.5 ± 0.7[b]	4.3 ± 0.5[b]	0.5 ± 0.2	—	—	1.4 ± 0.3[a]
E-PL	26.5 ± 1.3	32.3 ± 1.0	0.4 ± 0.0	9.9 ± 0.8[b]	9.9 ± 0.8[b]	6.4 ± 0.8[a]	0.5 ± 0.1	—	—	2.1 ± 0.4[a]
Triacylglycerols										
Control	27.3 ± 0.5	9.9 ± 0.4	0.8 ± 0.1	38.7 ± 0.7	10.5 ± 0.8	0.3 ± 0.1	—	—	—	—
TF-TG	29.2 ± 0.3	9.6 ± 0.5	0.9 ± 0.1	37.9 ± 0.6	8.8 ± 0.8	0.8 ± 0.2	—	—	—	0.2 ± 0.1
E-PL	29.5 ± 1.0	10.2 ± 0.6	0.8 ± 0.1	33.9 ± 0.8	10.7 ± 1.3	1.6 ± 0.4	—	—	—	0.5 ± 0.1
Cholesteryl ester										
Control	16.2 ± 0.9	4.2 ± 0.4	1.2 ± 0.1	41.9 ± 1.2[a]	25.7 ± 1.5	1.0 ± 0.2[b]	—	0.6 ± 0.1	—	0.2 ± 0.0
TF-TG	19.9 ± 0.6	3.6 ± 0.3	1.1 ± 0.1	36.9 ± 1.0[b]	25.6 ± 1.3	1.5 ± 0.3[b]	—	0.5 ± 0.1	—	0.3 ± 0.0
E-PL	17.9 ± 0.9	3.4 ± 0.4	1.0 ± 0.1	33.0 ± 2.5[b]	31.4 ± 2.6	2.7 ± 0.6[a]	—	0.7 ± 0.1	—	0.3 ± 0.0

[1] Values are means ± SEM, $n = 10$. Means with a different superscript letter differ, $P < 0.05$.

[2] AA, arachidonic acid; DHA, docosahexaenoic acid; Control, group fed adapted milk formula for piglets; TF-TG, group fed adapted milk formula for piglets supplemented with LCP from fungal and tuna oils; E-PL, group fed adapted milk formula for piglets supplemented with LCP from egg yolk phospholipids; —, not detected.

TABLE 3

Selected fatty acid composition of plasma lipoproteins in infant piglets fed the control diet or diets supplemented with long-chain polyunsaturated fatty acids (LCP) as triacylglycerols (TG) or phospholipids (PL)[1]

	16:0	18:0	16:1(n-7)	18:1(n-9)	18:2(n-6)	AA[2] 20:4(n-6)	18:3(n-3)	DHA 22:6(n-3)
	g/100 g of total fatty acids							
VLDL								
Control	20.9 ± 0.3	9.43 ± 0.3	0.7 ± 0.1	36.0 ± 0.8[a]	18.8 ± 0.4	2.7 ± 0.4[b]	0.5 ± 0.1	0.7 ± 0.1[b]
TF-TG	22.4 ± 0.4	9.6 ± 0.3	0.8 ± 0.1	31.9 ± 0.6[b]	17.7 ± 0.4	3.9 ± 0.3[b]	1.2 ± 0.1	2.2 ± 0.2[a]
E-PL	22.6 ± 0.7	10.4 ± 0.3	0.9 ± 0.1	29.2 ± 1.2[b]	17.3 ± 0.8	4.5 ± 0.5[a]	1.1 ± 0.1	1.9 ± 0.2[a]
LDL								
Control	14.9 ± 0.5	9.7 ± 0.3	0.7 ± 0.0	29.9 ± 1.2	28.2 ± 0.9	5.1 ± 0.4[b]	—	1.2 ± 0.2[b]
TF-TG	16.2 ± 0.3	9.5 ± 0.4	0.6 ± 0.0	28.2 ± 1.1	27.8 ± 0.5	6.3 ± 0.3[b]	—	2.0 ± 0.1[a]
E-PL	15.7 ± 0.2	9.2 ± 0.3	0.7 ± 0.0	26.0 ± 1.2	29.7 ± 0.9	6.8 ± 0.4[a]	—	1.7 ± 0.2[a]
HDL								
Control	18.6 ± 0.4	14.7 ± 0.1	0.4 ± 0.0	21.0 ± 0.4[a]	25.1 ± 0.4[a]	7.6 ± 0.3[b]	—	2.1 ± 0.1[b]
TF-TG	19.9 ± 0.3	14.7 ± 0.3	0.6 ± 0.2	19.2 ± 0.4[b]	21.4 ± 0.6[b]	9.8 ± 0.5[a]	—	3.8 ± 0.4[a]
E-PL	18.7 ± 0.2	15.7 ± 0.3	0.3 ± 0.0	17.8 ± 0.3[c]	22.8 ± 0.5[b]	10.5 ± 0.3[a]	—	3.9 ± 0.2[a]

[1] Values are means ± SEM, $n = 10$. Means with a different superscript letter differ, $P < 0.05$.

[2] AA, arachidonic acid; DHA, docosahexaenoic acid; Control, group fed adapted milk formula for piglets; TF-TG, group fed adapted milk formula for piglets supplemented with LCPs from fungal and tuna oils; E-PL, group fed adapted milk formula from piglets supplemented with LCPs from egg yolk phospholipids; —, not detected.

forms of LCP (either PL or TG) on the lipid and fatty acid composition of plasma and plasma lipoproteins and on the lipid composition of liver and jejunal mucosa in infant piglets.

The direct effect of fatty acids ingested with the diet on plasma and tissue fatty acid composition has been largely proved (Dougherty et al. 1987, Hrboticky et al. 1990). LCP-supplemented groups had lower proportions of 18:1(n-9) or 18:2(n-6), or both, in some of the plasma fractions (PL and CE) and lipoproteins (VLDL and HDL) compared with the control group. This effect was probably caused by the slightly lower proportion of these fatty acids in the LCP diets (Table 1) or by the replacement of these fatty acids to maintain the unsaturation index. As expected, LCP supplementation resulted in higher proportions of AA and DHA in plasma and all lipoproteins of piglets fed TF-TG and E-PL diet compared with the control piglets.

The most important difference found between the piglets fed LCP-PL and those fed LCP-TG was the distribution of AA and DHA in HDL-PL and LDL-PL. In fact, although there were no differences in the fatty acid composition of whole lipoproteins, HDL-PL contained a higher proportion of LCP when those fatty acids were fed as PL. Opposite results were found in LDL-PL, which contained a higher proportion of LCP when those fatty acids were fed as TG. Fatty acid composition of plasma fractions partly reflected this difference; AA was also higher in CE and PL, the main components of HDL, of piglets fed the PL diet than of those fed the TG diet. No effect was found on plasma TG, mainly representative of VLDL TG.

This different distribution of LCP in lipoprotein PL may be explained if after digestion and absorption, LCP are reesterified to the same chemical structure in which they were added to the diet (as PL or TG), being assembled mainly as PL on the chylomicron surface in the case of the E-PL group and mainly as TG in chylomicron core in the case of the TF-TG group. Plasma chylomicrons exchange some of their components with HDL during intravascular catabolism (Posner 1986); therefore, HDL from piglets fed an E-PL diet would contain a higher proportion of AA and DHA in HDL-PL, which is consistent with our observation.

The metabolic fate of LCP from our experimental diets can be determined taking into account, on one hand, the digestion and absorption process, and on the other hand, the positional distribution of fatty acids in the lipid sources used to supplement the experimental diets. We previously reported that AA and DHA were mainly esterified to the *sn*-2 position of E-PL. For example, 50% of DHA acid was present in the *sn*-2 position of tuna oil, whereas nearly 80% of AA was esterified to the outer positions of the TG molecule in fungal oil (Amate et al. 1999). Therefore, after digestion, LCP would be released as free fatty acids in case of the E-PL diet, and DHA mainly as 2-monoacylglycerols and AA mainly as free fatty acid in the case of the TF-TG diet. 2-Monoacylglycerols are reestified to newly form TG, which would support in part our hypothesis that dietary LCP-TG may contribute to chylomicron TG. However, little information is available concerning lipids released as free fatty acids in the intestinal lumen (Pufal et al. 1985, Thomson et al. 1988). The metabolic fate of LCP from different dietary sources in lymph chylomicrons deserves further research.

LCP supplementation to the diet as both PL or TG lowered the hepatic cholesterol concentration. This result was in agreement with Ikeda et al. (1994), who also found less cholesterol in the livers of rats fed a DHA-containing diet. This effect may be due to a reduction in hepatic cholesterol synthesis, because (n-3) LCP inhibits HMG-CoA reductase activity (Choi et al. 1989). On the other hand, (n-3) LCP have been shown to lower plasma and liver TG (Harris 1996, Rustan et al. 1988). We did not find a lowering effect of (n-3) LCP on liver TG. However, it should be taken into account that the TG-lowering effect of (n-3) LCP has been shown in humans and experimental animals in studies of the prevention or treatment of cardiovascular disease using high doses of (n-3) LCP, mainly 20:5(n-3) (Rustan et al. 1988). Both the E-PL and TF-TG diets contained 0.3 g/100 g total fatty acids as DHA with no 20:5(n-3) (E-PL) or <0.05 g/100 g 20:5(n-3) (TF-TG). It is not clear whether DHA has the same effects as 20:5(n-3) on plasma and tissue lipids (Berge et al. 1999). Moreover, the presence of AA in our experimental diets may modulate the TG-lowering effects of (n-3) LCP; in fact, it has been reported that AA increases circulating levels of TG (Whelan et al. 1995).

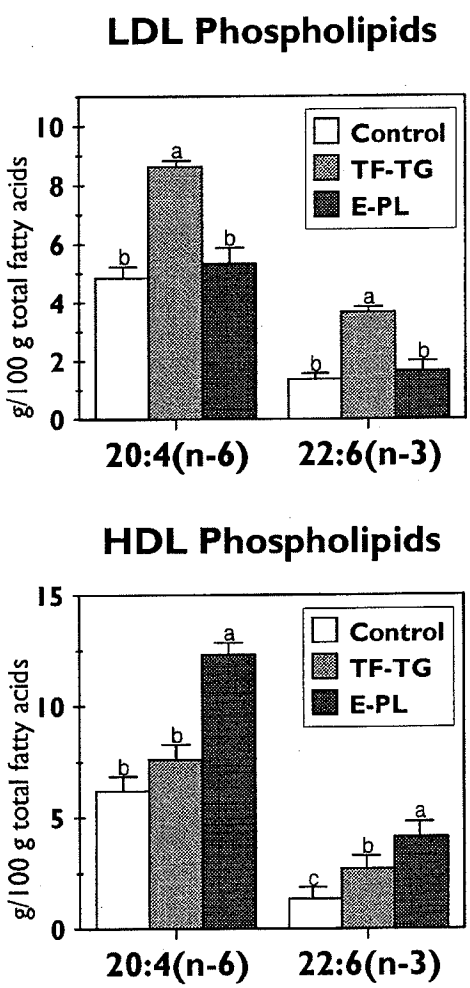

FIGURE 1 Arachidonic [20:4(n-6)] and docosahexaenoic [22:6(n-3)] acid proportions in HDL and LDL phospholipids of piglets fed the control diet or diets supplemented with long-chain polyunsaturated fatty acids (LCP) as triacylglycerols or phospholipids. Values are means ± SEM, $n = 10$; means without a common superscript letter differ, $P < 0.05$. Control, group fed adapted milk formula for piglets; TF-TG, group fed adapted milk formula for piglets supplemented with LCP from fungal and tuna oils; E-PL, group fed adapted milk formula for piglets supplemented with LCP from egg yolk phospholipids.

Regarding comparisons between the group fed LCP as PL and the group fed LCP as TG, there have been two previous studies that showed beneficial effects of LCP-PL: one by Carlson et al. (1998) and one by our research group (Lopez-Pedrosa et al. 1995 and 1999). Carlson et al. (1998) reported a lower incidence of necrotizing enterocolitis in preterm infants fed a formula with E-PL. The authors justified their finding with one or more components of E-PL, such as AA and choline. López-Pedrosa et al. (1995 and 1999) found that malnutrition in piglets affects the composition of jejunal mucosa and liver and plasma lipoproteins. They also reported that dietary LCP-PL from pig brain improved the recovery of damaged intestine and normalized the lipid and fatty acid composition of liver and plasma lipoproteins to the levels of healthy animals of the same age. As in the study of Carlson et al. (1998), those results were explained not only by dietary LCP themselves but also by the form in which they were esterified or the presence of other components within the PL source.

The studies by Carlson et al. (1998) and López-Pedrosa et al. (1995 and 1999) pointed out a positive effect of dietary LCP-PL on some kind of intestinal or hepatic alterations. However, the results of our study did not show an effect of LCP-PL on the lipid composition of liver and jejunum in healthy animals. Some of the effects found in those reports may be explained by the presence of other components in the LCP-PL sources, such as cholesterol (Carlson et al. 1998, Lopez-Pedrosa et al. 1995 and 1999). In the present study, cholesterol was added to the control and TF-TG diets to match the composition of the E-PL diet to overcome potential effects of this component.

We found a different distribution of LCP in the PL fraction of HDL and LDL when piglets were fed a diet containing LCP from E-PL or TF-TG. The physiological importance of these results remains to be determined, but on one hand, LCP added as TF-TG or as E-PL are transported in different lipoprotein fractions, which may affect tissue uptake. On the other hand, differences on the composition of HDL and LDL PL may affect particle fluidity of the lipoproteins involved in the transport and removal of cholesterol from tissues, which means that LCP in the form of PL or TG may influence cholesterol metabolism.

In summary, this study showed that the chemical form in which LCP are present in different dietary sources influences their distribution in plasma lipoproteins. This fact should be taken into consideration when LCP sources are selected for infant formula supplementation.

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Appendix O

Simopoulos, "Omega-3 Fatty Acids in Inflammation and Autoimmune Diseases" J Am Coll Nutr 21(6): 495-505 (2002)

Review

Omega-3 Fatty Acids in Inflammation and Autoimmune Diseases

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Key words: inflammation, cardiovascular disease and major depression, autoimmune diseases, IL-1, IL-6, TNF, background diet, omega-6/omega-3 ratio

Among the fatty acids, it is the omega-3 polyunsaturated fatty acids (PUFA) which possess the most potent immunomodulatory activities, and among the omega-3 PUFA, those from fish oil—eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)—are more biologically potent than α-linolenic acid (ALA). Some of the effects of omega-3 PUFA are brought about by modulation of the amount and types of eicosanoids made, and other effects are elicited by eicosanoid-independent mechanisms, including actions upon intracellular signaling pathways, transcription factor activity and gene expression. Animal experiments and clinical intervention studies indicate that omega-3 fatty acids have anti-inflammatory properties and, therefore, might be useful in the management of inflammatory and autoimmune diseases. Coronary heart disease, major depression, aging and cancer are characterized by an increased level of interleukin 1 (IL-1), a proinflammatory cytokine. Similarly, arthritis, Crohn's disease, ulcerative colitis and lupus erythematosis are autoimmune diseases characterized by a high level of IL-1 and the proinflammatory leukotriene LTB_4 produced by omega-6 fatty acids. There have been a number of clinical trials assessing the benefits of dietary supplementation with fish oils in several inflammatory and autoimmune diseases in humans, including rheumatoid arthritis, Crohn's disease, ulcerative colitis, psoriasis, lupus erythematosus, multiple sclerosis and migraine headaches. Many of the placebo-controlled trials of fish oil in chronic inflammatory diseases reveal significant benefit, including decreased disease activity and a lowered use of anti-inflammatory drugs.

Key teaching points:

- In Western diets, omega-6 fatty acids are the predominant polyunsaturated fats. The omega-6 and omega-3 fatty acids are metabolically distinct and have opposing physiologic functions.
- Eicosapentaenoic acid (EPA) is released to compete with arachidonic acid (AA) for enzymatic metabolism inducing the production of less inflammatory and chemotactic derivatives.
- Animal and human studies support the hypothesis that omega-3 PUFA suppress cell mediated immune responses.
- In experimental animals and humans, serum PUFA levels predict the response of proinflammatory cytokines to psychologic stress. Imbalance in the omega-6/omega-3 PUFA ratio in major depression may be related to the increased production of proinflammatory cytokines and eicosanoids in that illness.
- The increased omega-6/omega-3 ratio in Western diets most likely contributes to an increased incidence of cardiovascular disease and inflammatory disorders.
- Patients with autoimmune diseases, such as rheumatoid arthritis, inflammatory bowel disease and asthma, usually respond to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) supplementation by decreasing the elevated levels of cytokines.

Introduction

The first evidence of the important role of dietary intake of omega-3 polyunsaturated fatty acids (PUFAs) in inflammation was derived from epidemiological observations of the low incidence of autoimmune and inflammatory disorders, such as psoriasis, asthma and type-1 diabetes, as well as the complete absence of multiple sclerosis, in a population of Greenland

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Eskimos compared with gender- and age-matched groups living in Denmark [1]. Most of these diseases are characterized by inappropriate activation of T cells resulting on and ultimately destruction of host tissues.

In the 1980's several independent lines of evidence suggested that changes in the natural history of hypertensive, atherosclerotic and chronic inflammatory disorders may be achieved by altering availability of eicosanoid precursors. Native Greenland Eskimos [2] and Japanese [3] have a high dietary intake of long chain omega-3 PUFA from seafood and a low incidence of myocardial infarction and chronic inflammatory or autoimmune disorders, even when compared to their Westernized ethnic counterparts. Diets containing omega-3 PUFA have also been found to reduce the severity of experimental cerebral [4] and myocardial [5] infarction, to retard autoimmune nephritis and prolong survival of NZB × NZW F_1 mice [6,7] and reduce the incidence of breast tumors in rats [8].

The 1980s were a period of expansion in our knowledge about PUFAs in general and omega-3 fatty acids in particular. Today we know that omega-3 fatty acids are essential for normal growth and development and may play an important role in the prevention and treatment of coronary artery disease, hypertension, arthritis, other inflammatory and autoimmune disorders and cancer [9]. Research has been carried out in animal models, tissue cultures and human beings. The original observational studies have given way to controlled clinical trials.

In this paper, I review the anti-inflammatory aspects of omega-3 fatty acids relative to prostaglandins and cytokines and their clinical effects in inflammatory and autoimmune diseases, such as cardiovascular disease, major depression, arthritis, inflammatory bowel disease, asthma and psoriasis.

Omega-6 and Omega-3 Fatty Acids and Prostaglandin Metabolism

Omega-6 fatty acids account for the majority of polyunsaturated fatty acids (PUFA) in the food supply. They are the predominant PUFA in all diets, especially Western diets. When diets are supplemented with omega-3 fatty acids, the latter partially replace the omega-6 fatty acids in the membranes of practically all cells (i.e., erythrocytes, platelets, endothelial cells, monocytes, lymphocytes, granulocytes, neuronal cells, fibroblasts, retinal cells, hepatic cells and neuroblastoma cells).

Competition between the omega-6 and omega-3 fatty acids occurs in prostaglandin formation. Eicosapentaenoic acid (EPA), an omega-3 fatty acid, competes with arachidonic acid (AA), an omega-6 fatty acid, for prostaglandin and leukotriene synthesis at the cyclooxygenase and lipoxygenase level (Fig. 1). When humans ingest fish or fish oil, the EPA and docosahexaenoic acid (DHA) from fish or fish oil lead to (1) a decreased production of prostaglandin E_2 (PGE_2) metabolites, (2) a decrease in thromboxane A_2, a potent platelet aggregator and vasoconstrictor (3) a decrease in leukotriene B_4 formation,

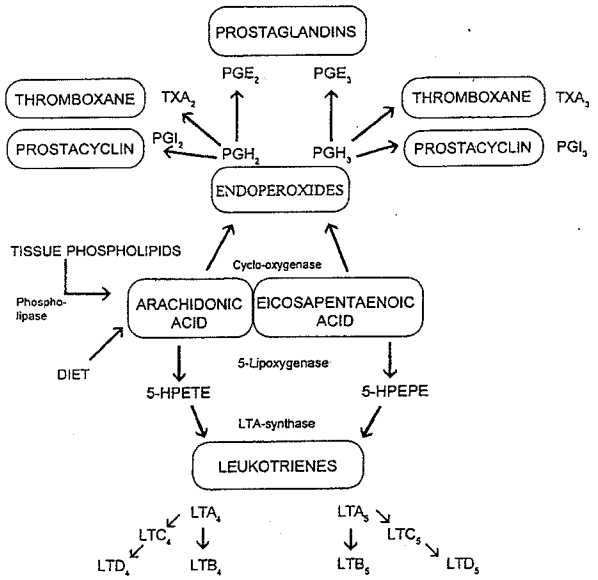

Fig. 1. Oxidative metabolism of arachidonic acid and eicosapentaenoic acid by the cyclooxygenase and 5-lipoxygenase pathways. 5-HPETE denotes 5-hydroperoxyeicosatetranoic acid and 5-HPEPE denotes 5-hydroxyeicosapentaenoic acid.

an inducer of inflammation and a powerful inducer of leukocyte chemotaxis and adherence, (4) an increase in thromboxane A_3, a weak platelet aggregator and a weak vasoconstrictor, (5) an increase in prostacyclin PGI_3, leading to an overall increase in total prostacyclin by increasing PGI_3 without a decrease in PGI_2 (both PGI_2 and PGI_3 are active vasodilators and inhibitors of platelet aggregation) and (6) an increase in leukotriene B_5, a weak inducer of inflammation and a weak chemotactic agent [10,11]. Omega-3 fatty acids modulate prostaglandin metabolism and decrease triglycerides and, in high doses, lower cholesterol and have antithrombotic and anti-inflammatory properties. These studies were extensively reviewed and reported [12–17].

Many factors contribute to the complex course of inflammatory reactions. Microbiological, immunological and toxic agents can initiate the inflammatory response by activating a variety of humoral and cellular mediators. In the early phase of inflammation, excessive amounts of interleukins and lipid mediators are released and play a crucial role. Pro-inflammatory eicosanoids of AA metabolism are released from membrane phospholipids in the course of inflammatory activation. EPA is released to compete with AA for enzymatic metabolism inducing the production of less inflammatory and chemotactic derivatives.

A variety of substances that inhibit the COX pathway have been investigated, including non-steroidal anti-inflammatory drugs (NSAIDs) used for the treatment of inflammation, pain and fever. Although NSAIDs inhibit COX and are efficacious anti-inflammatory agents, serious adverse effects limit their use. Two forms of COX have been identified, a constitutively

expressed COX-1 and a cytokine inducible COX-2. It has been suggested that NSAID toxicity is due to inhibition of COX-1, whereas therapeutic properties are derived from COX-2 inhibition at the site of inflammation [18,19]. In addition, there is evidence that COX-2 inhibition can suppress the growth of colorectal cancer [20].

A new arena for omega-3 fatty acids has emerged as adjuvants to drug treatment leading to synergism (potentiating the effects of drugs) or to decreasing their toxicity (Table 1) [21–32].

Similarly, increasing the intake of omega-3 fatty acids while decreasing the omega-6 fatty acids in the diet has led to improvements and a decrease of non-steroidal anti-inflammatory agents in patients with rheumatoid arthritis [33,34] and asthma [35].

Dietary fish oils, rich in omega-3 PUFA, are rapidly incorporated into the membrane phospholipids of circulating human (monocyte) cells, suggesting that they are likely to have an effect on several aspects of cell function. Moderate dietary supplementation with omega-3 PUFA significantly increases their level in monocytes within two weeks [36]. The levels of EPA reached a maximum accumulation after six weeks' supplementation and DHA reached a peak at 18 weeks [37]. EPA returned rapidly to pretreatment levels in monocytes (although plasma levels remained significantly elevated from baseline after 24 weeks of washout) whereas DHA levels declined more slowly [37].

Omega-3 Fatty Acids, Interleukin-1 (IL-1) and Tumor Necrosis Factor (TNF)

The interactions between immune and inflammatory cells are mediated in large part by proteins, termed interleukins (IL), that are able to promote cell growth, differentiation and functional activation. TNF-α and IL-1 and IL-6 are the most important cytokines produced by monocytes and macrophages. Production of appropriate amounts of TNF, IL-1 and IL-6 is beneficial in response to infection, but in inappropriate amounts or overproduction can be dangerous and these cytokines, especially TNF, are implicated in causing some of the pathological responses that occur in inflammatory conditions. They induce fever and the synthesis of acute phase proteins by the liver, activate T and B lymphocytes and endothelial cells and are involved in many other aspects of the acute phase response.

In addition to their anti-inflammatory effects by suppressing

Table 1. Conditions in which Omega-3 Fatty Acids Have Been Shown to Have Synergistic Effects with Drugs

Human Studies	Reference	Animal Studies	Reference
Hypertension	[21]	Autoimmune Disorders	[27]
Arthritis	[22,23]		
Psoriasis	[24]		
Ulcerative Colitis	[25]		
Restenosis	[26]		

LTB_4, omega-3 supplementation to healthy volunteers suppresses the capacity of monocytes to synthesize interleukin-1 (IL-1) and tumor necrosis factor (TNF) (Table 2) [38]. Omega-3 fatty acids suppress IL-1 mRNA [40,41]. These observations led to studies in patients with inflammatory and autoimmune diseases. The suppression of cytokine synthesis could also be achieved by dietary alteration without fish oil supplementation [34]. The cytokine suppression is probably achieved at the level of transcription, since IL-1 mRNA was decreased. This effect may account for the beneficial effects of omega-3 fatty acids in models of chronic inflammatory disease. IL-1 and TNF influence a wide array of biological functions [42]. Many of the biological functions of IL-1 are shared by TNF [43]. IL-1 potentiates procoagulent activity, increases production of plasminogen activator inhibitor and endothelin and the formation of eicosanoids. Furthermore, it increases leukocyte adhesion by inducing the expression of adhesion molecules and it promotes endothelial protein permeability.

Pharmacologic agents known to reduce the synthesis of IL-1 and TNF are corticosteroids and cyclosporin. Since IL-1 and TNF are principal mediators of inflammation, reduced production of these cytokines contributes to the amelioration of inflammatory symptoms in patients taking omega-3 fatty acid supplements. Studies in normal volunteers indicate that omega-3 fatty acid supplementation reduced the ability of monocytes to produce IL-1β upon stimulation with endotoxin. The effect was most pronounced 10 weeks after stopping the supplementation and suggests prolonged incorporation of omega-3 fatty acids into a pool of circulating monocytes [44]. The capacity of the monocytes from these donors to synthesize IL-1β returned to the pre-supplement level 20 weeks after ending supplementation. Similar results were observed for IL-1α and TNF. These findings have led to trials with omega-3 fatty acids since the above effects (suppression of such magnitude) have been observed and can only be achieved pharmacologically by administration of glucocorticoids or cyclosporin A, which have well-known adverse side effects, particularly during long-term administration. In a one-year intervention trial with dietary fish oil, 66 patients, after renal transplantation and on cyclosporin, randomized, double-blind study, 6 gm of fish oil daily (3 gm of omega-3 fatty acids), had a beneficial effect on renal hemodynamics and on blood pressure. Furthermore, the fish-oil group had significantly fewer rejection episodes than the control group, and there was a trend to increased graft survival [45]. In patients with IgA nephropathy, treatment with fish oil for two years retards the rate at which renal function is lost [46]. The omega-3 fatty acids in fish oil affect eicosanoid metabolism and cytokine production, two important classes of inflammatory modulators, and therefore have the potential to alter renal hemodynamics and inflammation. IgA nephropathy is the most common glomerular disease in the world. Omega-3 fatty acids lower plasma triglycerides and improve red cell flexibility in patients with lupus nephritis [47,48].

Caughey *et al.* [49] demonstrated that a diet enriched with

Table 2. Effects of Omega-3 Fatty Acids on Factors Involved in the Pathophysiology of Inflammation

Factor	Function	Effect of ω3 Fatty Acid
Arachidonic acid	Eicosanoid precursor, aggregates platelets, stimulates white blood cells	↓
Thromboxane	Platelet aggregation, vasoconstriction, increase of intracellular Ca^{++}	↓
Prostacyclin ($PGI_{2/3}$)	Prevent platelet aggregation, vasodilation, increase cAMP	↑
Leukotriene (LTB_4)	Neutrophil chemoattractant, increase of intracellular Ca^{++}	↓
Fibrinogen	A member of the acute phase response and a blood clotting factor	↓
Tissue plasminogen activator	Increase endogenous fibrinolysis	↑
Platelet activating factor (PAF)	Activates platelets and white blood cells	↓
Platelet-derived growth factor (PDGF)	Chemoattractant and mitogen for smooth muscles and macrophages	↓
Oxygen free radicals	Cellular damage, enhance LDL uptake via scavenger pathway, stimulate arachidonic acid metabolism	↓
Lipid hydroperoxides	Stimulate eicosanoid formation	↓
Interleukin 1 and tumor necrosis factor	Stimulate neutrophil O_2 free radical formation, stimulate lymphocyte proliferation, stimulate PAF, express intercellular adhesion molecule-1 on endothelial cells, inhibit plasminogen activator, thus, procoagulants	↓
Interleukin-6 [39]	Stimulates the synthesis of all acute phase proteins involved in the inflammatory response: C-reative protein, serum amyloid A, fibrinogen, α_1-chymotrypsin and haptoglobin	↓

Adapted and modified from [38].

flaxseed oil can inhibit the *ex vivo* production of these cytokines by 30% in four weeks, whereas nine grams of fish oil for another four weeks inhibited IL-1β by 80% and TNFα by 74%. Flaxseed increased EPA but not DHA levels in monocytes. Thromboxane A_2 is a facilitator of cytokine synthesis in human monocytes [49]. Results of animal and human studies support the hypothesis that omega-3 PUFA suppress cell mediated immune responses, in part at least by inhibiting antigen presenting-cell function, increase membrane fluidity and alter the expression of membrane proteins, possibly by influencing the vertical displacement of the proteins within the membrane. Most of the human studies have shown that omega-3 fatty acids inhibit proinflammatory cytokines TNF and IL-1. Several studies performed in mice show that omega-3 fatty acids have a stimulatory effect on TNF and IL-1 [50–54]. This species-specific effect may be due to differences in the cell population affected by the PUFAs between the various species [55].

Omega-3 fatty acids suppress platelet activating factor (PAF). PAF is a potent platelet aggregator and leukocyte activator, and it strongly promotes AA metabolism (Table 2). It has been proposed that PAF, a phospholipase A_2 (PLA_2) dependent phospholipid, plays a crucial role in the pathogenesis of rheumatoid arthritis, asthma, endotoxin shock and acute renal transplant rejection.

Other Inflammatory Markers, Interleukin-6 (IL-6) and Cardiovascular Disease

Atherosclerosis and inflammation share similar basic mechanisms involving the adhesion of leukocytes to vascular endothelium in their early phases. There is a strong association between systemic inflammation and coronary artery disease. This association is thought to be causal, i.e. inflammation increases the risk of the disease, rather than simply marking the presence of atherosclerosis, which is an inflammatory process [56,57]. The relationship between infection and cardiovascular disease is likely to have several mediators (including possibly an autoimmune response against protein on the arterial endothelial cell wall) [58]. Cigarette smoking is a well established cardiovascular disease risk factor [59–64], as is high body mass index (BMI) [60], both providing a link between increased inflammation and increased risk. Although regular exercise reduces the risk of cardiovascular events, severe exercise has been shown to be associated with a systemic inflammatory response [65] and increased risk of myocardial infarction [66,67]. In contrast to the risks of severe exercise, moderate exercise and physical fitness are associated with lower baseline levels of inflammatory mediators [62,65,68–70].

Inflammatory markers such as C-reactive protein and fibrinogen are raised in affected people in both chronic coronary artery disease [60,62,63] and peripheral vascular disease compared with unaffected people [71]. The degree of inflammation correlates with disease severity [71–73].

Interleukin-6 (IL-6) is produced and released into the systemic circulation from subcutaneous adipose tissue as well as from cells of the immune system [74] (Table 2). The levels correlate with BMI and percent body fat. A recent theory is that increased IL-6 may be the link between obesity and insulin resistance [75]. Adipose tissue secretes IL-6 whose levels and those of C-reactive protein also correlate with obesity and insulin resistance. There is strong evidence supporting the central role of IL-6 in the inflammatory response. IL-6 is a 26 kDa cytokine, produced by many different cells in the body, including lymphocytes, monocytes, fibroblasts and endothelial cells. Various cytokines are involved in acute phase protein synthesis, including TNFα and IL-1β. However, IL-6 is the only cytokine that can stimulate the synthesis of all the acute

phase proteins involved in the inflammatory response: C-reactive protein, serum amyloid A, fibrinogen, α_1-chymotrypsin and haptoglobin [76]. There is evidence that phospholipase A_2 and cyclooxygenase pathways of AA metabolism are involved in the action of IL-6 in platelets (aggregation). Khalfoun *et al.* [77] examined the effects of PUFA on the production of IL-6 by human unstimulated endothelial cells and stimulated endothelial cells with TNFα, IL-4, LPS (lipopolysaccharide) or PBL (allogeneic peripheral blood lymphocytes). The addition of EPA and DHA significantly reduced the production of IL-6 whereas AA was ineffective even at highest concentrations. EPA was more potent than DHA.

Interleukin-6 occupies a central place in the inflammatory response. Woods *et al.* [39] suggest a link between IL-6 and cardiovascular disease and the pathways involved (Fig. 2). The discovery of genetic polymorphisms involving a change of a single base, from guanine to cytosine, at position—174 in the 5′ flanking region of the interleukin-6 gene is of great importance because the G allele is associated with higher IL-6 production than the C allele. It is quite possible that genetic variation could account for the different responses to omega-3 fatty acids, both in terms of suppression of IL-6 and the inflammatory response [78]. *In vivo* studies found basal IL-6 levels to be twice as high in volunteers with the GG allele than in those with the CC allele. Therefore, the understanding of the genetic mechanisms controlling the IL-6 levels as well as knowing the frequency of GG alleles in the population would provide further evidence that the higher levels of inflammation seen in patients with cardiovascular disease are primary rather than secondary in the development of cardiovascular disease.

Fatty Acids, Cytokines, and Major Depression

Psychologic stress in humans induces the production of proinflammatory cytokines such as interferon gamma (IFNγ), TNFα, IL-6 and IL-10. An imbalance of omega-6 and omega-3 PUFA in the peripheral blood causes an overproduction of proinflammatory cytokines. There is evidence that changes in fatty acid composition are involved in the pathophysiology of major depression. Changes in serotonin (5-HT) receptor number and function caused by changes in PUFA provide the theoretical rationale connecting fatty acids with the current receptor and neurotransmitter theories of depression [79–81].

The involvement of changes in fatty acid composition in the pathophysiology of major depression also revolves around its role in immune function and production of cytokines. There is now evidence that major depression is accompanied by an acute phase response, increased secretion of eicosanoids, such as prostaglandins; cytokines, i.e. the monocyte cytokines, IL-1β and IL-6, as well as the Th-1-like cytokines, IL-2 and IFNγ. IL-1, IL-2 and TNFα activate the hypothalamic adrenal (HPA) axis where proinflammatory cytokines can induce resistance to the effects of glucocorticoid hormones by influencing glucocorticoid receptor expression. In experimental animals and humans (students facing an academic examination), external stressors increase the production of inflammatory cytokines, such as IL-6, TNFα and IFNγ [82], and serum PUFA levels predict the response of proinflammatory cytokines to psychologic stress [83]. The increased C20:4ω6/C20:5ω3 ratio and the imbalance in the omega-6/omega-3 PUFA ratio in major depression may be related to the increased production of

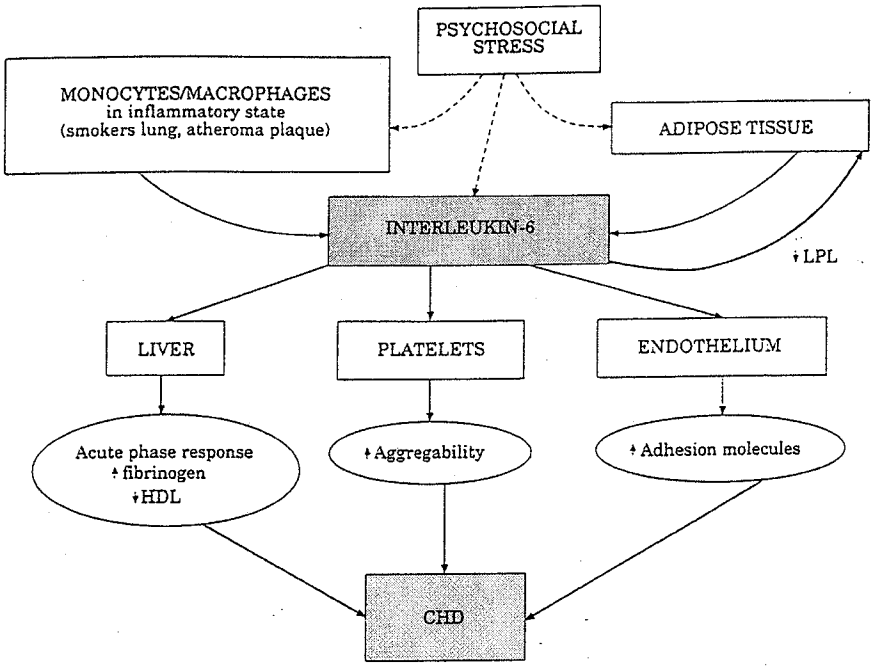

Fig. 2. The central role of interleukin-6 and its effect on the development of coronary artery disease [39].

proinflammatory cytokines and eicosanoids in that illness [79]. The increased omega-6/omega-3 ratio in Western diets most likely contributes to an increased incidence of cardiovascular disease and inflammatory disorders. Lower serum HDL cholesterol and an increased C20:4ω6/C20:5ω3 ratio are related both to depression and to a higher risk of cardiovascular disease, which shows a strong comorbidity with depression [79,80]. There are a number of studies evaluating the therapeutic effect of EPA and DHA in major depression. Stoll and colleagues have shown that EPA and DHA prolong remission, that is, reduce the risk of relapse in patients with bipolar disorder [84,85].

Rheumatoid Arthritis

Interest in the use of omega-3 fatty acids EPA and DHA in rheumatoid arthritis began in the mid-eighties, following the demonstration in several autoimmune strains of mice [NZB (NZB × NZW) F_1, MRL/1pr, and BxSB/Mpj] that omega-3 fatty acids reduced the severity of diffuse proliferative glomerulonephritis. Dose-response studies demonstrated that DHA is more effective than EPA, and that diets with combinations of these two omega-3 fatty acids are synergistic [6,86–88]. Kremer in 1985 [89] carried out a pilot study in 17 patients with rheumatoid arthritis who consumed 1.8 g EPA and 0.9 g DHA. This was a double-blind, controlled, randomized trial of 12 weeks' duration with a follow up evaluation one to two months after the diets and supplements were discontinued. Standard clinical measures of arthritis activity were performed at baseline and after 4, 8 and 12 weeks and at follow up. The results showed a significant difference in morning stiffness between the two groups at the time of the 12-week evaluation, which represented a worsening in the control group, while the fish oil supplemented group remained unchanged. In a subsequent trial, Kremer [90] measured neutrophil LTB_4 production, which was decreased in the patients receiving fish oil. The prolonged suppression of LTB_4 beyond the period of supplementation with fish oils most likely accounted for the continued clinical benefits observed after the period of discontinuation of fish oil. Prolonged effects on the immune system were subsequently reported in normal volunteers ingesting fish oil. Therefore, a crossover format is not appropriate to study the clinical or immune effects of fish oil in patients with inflammatory disease [44]. In a study [91] which examined potential mechanisms of EPA + DHA supplementation in patients with rheumatoid arthritis, 12 patients with active disease consumed 3.6 g EPA and 2.4 g DHA daily for a period of six weeks. After six weeks of fish oil ingestion, LTB_4 was decreased by 33%, and there was a 37% decrease in the quantity of platelet activation factor (PAF). Analyses of fatty acid composition of neutrophil membranes after six weeks' ingestion of fish oils revealed a decline of 33% in AA with a simultaneous twentyfold rise in EPA content from the pre-diet period. DHA was not detected. In the study by Endres [44], IL-1β, IL-1α and TNF were suppressed by 42% at six weeks, but a further decrease was observed 10 weeks after discontinuation. It is therefore possible that fish-oil induced suppression of IL-1 contributes to the amelioration of clinical signs and symptoms of disease activity in patients with rheumatoid arthritis to a greater extent than does inhibition of leukotriene metabolism.

There are at least 13 randomized controlled clinical trials that show benefit from fish oil supplements in patients with rheumatoid arthritis [92]. A common feature of the studies has been a reduction in symptoms and in the number of tender joints. There was a reduction in the dose of analgesic anti-inflammatory drugs. In a subsequent meta-analysis, morning stiffness was decreased, as well as the number of tender joints [93]. Cleland and James have attempted to develop a standard laboratory index of omega-3 nutritional status. They have explored the feasibility of using an assay to guide prevention and therapeutic treatments with omega-3 fatty acids. They have established that there is little diurnal variation in levels of plasma phospholipid EPA, no relationship with meals and a close correlation with cellular EPA levels. Plasma phospholipid EPA correlated very closely with peripheral blood mononuclear cell EPA levels ($r = 0.97$). Thus, they measure non-fasting EPA level, mononuclear cell EPA level and the degree of inhibition in the synthesis of the inflammatory cytokines IL-1β and TNF *ex vivo* in human volunteers given diets fortified with omega-3 fatty acids [49]. They noted substantial inhibition of IL-1β and TNF when the mononuclear cell level of EPA was equal to or greater than 1.5% of total cell phospholipid fatty acids and correlated with a plasma phospholipid EPA level equal to or greater than 3.2%. In their clinic, patients achieving the target EPA level tended to have higher discontinuation rates of NSAIDs [92]. This non-fasting plasma phospholipid EPA may prove to be a useful assay to support the use of dietary omega-3 fatty acids in the treatment of autoimmune diseases and possibly in their prevention. Relative to prevention of rheumatoid arthritis, changes in the diet are recommended in patients with family history who are at special risk for the disease because they carry the HLA-DRβ susceptibility alleles. Population studies suggest that omega-3 fatty acids may have a preventive effect in rheumatoid arthritis. Therefore, persons at a higher risk because of genetic susceptibility are good subjects to carry out preventive measures through dietary change by decreasing the omega-6 fatty acid and increasing the omega-3 fatty acid intake.

Inflammatory Bowel Disease

Crohn's disease and ulcerative colitis, collectively known as inflammatory bowel disease, are related but distinct complex disorders with immunologic, environmental and genetic components [94]. The recent approach to the management of ulcerative colitis has centered on soluble mediators of inflammation. The mediators that have been studied most extensively are

the AA metabolites, prostaglandins, leukotrienes and cytokines. Many others probably remain to be identified, as do all of the cells of origin. Ongoing studies are attempting to define the effects of inflammatory mediators on the functional capabilities of various relevant cell types. The exact point in the pathway of inflammation at which the available therapeutic agents have their greatest effect has not yet been defined. Patients with ulcerative colitis have increased amounts of LTB_4, the end product of AA via the 5-lipoxygenase pathway (Fig. 1), and IL-1β (Table 2). Stenson *et al.* [25] in a double-blind crossover comparison with placebo showed that fish oil supplementation, which increases production of B_5 and decreases production of B_4, did indeed reduce the contents of rectal dialysates of leukotrienes. The clinical improvement was modest. However, the steroid dose could be reduced, and histologic improvement was impressive, even if the gross sigmoidoscopic improvement was not. Encouraging results have been reported by others [95,96]. Belluzzi *et al.* [97] showed a reduced rate of relapse in patients with Crohn's disease in remission by supplementation of 2.7 g of omega-3 enteric-coated fish oil preparation. Endres *et al.* [98] reviewed the evidence of the therapeutic effect of omega-3 fatty acids in patients with inflammatory bowel disease and concluded that some studies have shown a significant improvement in clinical activity and a steroid-sparing effect while others have shown only a trend towards improvement. This variation in response may be due to the heterogeneity of inflammatory bowel disease.

Asthma

Asthma is a mediator driven inflammatory process in the lungs and the most common chronic condition in childhood. The leukotrienes and prostaglandins are implicated in the inflammatory cascade that occurs in asthmatic airways. There is evidence of airway inflammation even in newly diagnosed asthma patients within two to twelve months after their first symptoms [99]. Among the cells involved in asthma are mast cells, macrophages, eosinophils and lymphocytes. The inflammatory mediators include cytokines and growth factors (peptide mediators) as well as the eicosanoids, which are the products of AA metabolism, which are important mediators in the underlying inflammatory mechanisms of asthma (Fig. 1, Table 2). Leukotrienes and prostaglandins appear to have the greatest relevance to the pathogenesis of asthma. The leukotrienes are potent inducers of bronchospasm, airway edema, mucus secretion and inflammatory cell migration, all of which are important to the asthmatic symptomatology. Broughton *et al.* [35] studied the effect of omega-3 fatty acids at a ratio of omega-6:omega-3 of 10:1 to 5:1 in an asthmatic population in ameliorating methacholine-induced respiratory distress. With low omega-3 ingestion, methacholine-induced respiratory distress increased. With high omega-3 fatty acid ingestion, alterations in urinary 5-series leukotriene excretion predicted treatment efficacy and a dose change in >40% of the test subjects (responders) whereas the non-responders had a further loss in respiratory capacity. A urinary ratio of 4-series to 5-series of <1 induced by omega-3 fatty acid ingestion may predict respiratory benefit.

Psoriasis

The recognition that AA metabolism is altered in psoriasis prompted attempts to inhibit the generation of proinflammatory lipoxygenase products, LTB_4 and 12-hydroxyeicosatetraenoic acid (12-HETE), which are markedly elevated in the psoriatic lesions [100]. The addition of MaxEPA® to the standard treatment produced further improvement and a decrease in LTB_4 and 12-HETE (Fig. 1) [11]. In other studies fish oil was successfully used in combination with etretinate to reduce the hyperlipidemia caused by that drug. In patients treated with ultraviolet B (UVB), omega-3 fatty acids prolong the beneficial effects of a course of phototherapy [24]. Fish oil in combination with cyclosporin reduces nephrotoxicity, which is the major side effect of that drug [24].

Conclusions

The anti-inflammatory properties of ω3 fatty acids, especially EPA, are due to competition with arachidonic acid (AA) as a substrate for cyclooxygenases and 5-lipoxygenase. The eicosanoids from the ω6 and ω3 fatty acids have opposing properties. The eicosanoids are considered a link between PUFA, inflammation and immunity. In addition to their effects on prostaglandins, thromboxanes and leukotrienes, ω3 fatty acids suppress the production on interleukin 1 (IL-1β) by suppressing the IL-1β mRNA, as well as the expression of Cox2 (cytooxygenase) mRNA that is induced by IL-1β. Cox2 is overexpressed in colon cancer cells. Both ALA, and EPA and DHA are involved in immune function. The precise effect of ALA depends on the level of linoleic acid (LA) and total PUFA content of the diet. A high dose of ALA (about 15 g/day) will suppress human IL-1 and TNF (tumor necrosis factor). It is unclear whether ALA itself exerts these effects or whether they are the result of its conversion to EPA. Excessive intake of ω6 fatty acids characteristic of Western diets produces an imbalance of ω6 to ω3 PUFAs which leads to an overproduction of the proinflammatory prostaglandins of the ω6 series and cytokines. Supplements of LA rich vegetable oils increase IL-1 and TNFα. Humans given ω3-rich flax seed oil or fish oil supplements have sharply reduced stimulated production of IL-1, IL-2 and TNFα, as well as suppressed mononuclear cell proliferation and expression of IL-2 receptors. Thus, in humans, LA increases proinflammatory cytokine secretion, whereas fish oil reduces proinflammatory cytokine secretion.

Experimental studies have provided evidence that incorporation of omega-3 fatty acids modifies inflammatory and immune reactions, making omega-3 fatty acids potential therapeutic agents for inflammatory and autoimmune diseases. Their effects are brought about by modulation of the type and amount

of eicosanoids and cytokines and by altering gene expression. A number of studies have been carried out in patients with coronary heart disease, cancer, obesity, arthritis, inflammatory bowel disease, psoriasis, asthma, lupus erythematosus, multiple sclerosis, major depression and bipolar depression. Clinical studies indicate that omega-3 fatty acids improve the clinical condition and biochemical factors of patients with arthritis, but the clinical intervention studies in other autoimmune conditions have given conflicting results, most likely due to lack of an adequate number of subjects in some and not taking into consideration the background diet or genetic variation. There is a clear need for more carefully designed and controlled clinical trials in the therapeutic application of omega-3 fatty acids to human autoimmune and inflammatory conditions. Nutritional supplementation with omega-3 fatty acids either as an alternative or adjunct therapy is potentially important, especially since current therapies with drugs have many side effects and the diseases are heterogeneous. In designing clinical interventions, genetic variation should be taken into consideration, since the level of cytokines is to a great extent genetically determined and the dose or amount of omega-3 fatty acids to suppress the proinflammatory state may vary.

The importance of omega-3 essential fatty acids in the diet is now evident, as well as the need to return to a more physiologic omega-6/omega-3 ratio of about 1-4/1 rather than the ratio of 20-16/1 provided by current Western diets. In order to improve the ratio of omega-6/omega-3 essential fatty acids, it will be necessary to decrease the intake of omega-6 fatty acids from vegetable oils and to increase the intake of omega-3 fatty acids by using oils rich in omega-3 fatty acids and increase the intake of fish to two to three times per week or take supplements. Omega-3 fatty acids have been part of our diet since the beginning of time. It is only for the past 150 years that omega-3 fatty acids have been decreased in Western diets due to agribusiness and food processing. The need to return the omega-3 fatty acids into the food supply has been recognized by industry, which is already producing omega-3 enriched products.

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Appendix P

Hong et al., "Novel Docosatrienes and 17S-resolvins Generated from Docosahexaenoic Acid in Murine Brain, Human Blood, and Glial Cells. Autacoids in Anti-Inflammation" J Biol Chem 278(17): 14677-87 (2003)

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Novel Docosatrienes and 17S-Resolvins Generated from Docosahexaenoic Acid in Murine Brain, Human Blood, and Glial Cells

AUTACOIDS IN ANTI-INFLAMMATION*

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Docosahexaenoic acid (DHA, C22:6) is highly enriched in brain, synapses, and retina and is a major ω-3 fatty acid. Deficiencies in this essential fatty acid are reportedly associated with neuronal function, cancer, and inflammation. Here, using new lipidomic analyses employing high performance liquid chromatography coupled with a photodiode-array detector and a tandem mass spectrometer, a novel series of endogenous mediators was identified in blood, leukocytes, brain, and glial cells as 17S-hydroxy-containing docosanoids denoted as docosatrienes (the main bioactive member of the series was 10,17S-docosatriene) and 17S series resolvins. These novel mediators were biosynthesized via epoxide-containing intermediates and proved potent (pico- to nanomolar range) regulators of both leukocytes reducing infiltration *in vivo* and glial cells blocking their cytokine production. These results indicate that DHA is the precursor to potent protective mediators generated via enzymatic oxygenations to novel docosatrienes and 17S series resolvins that each regulate events of interest in inflammation and resolution.

Chemical mediators and autacoids such as local acting lipid mediators derived from arachidonic acid are well established regulators of key events of interest in host defense, coagulation, inflammation, and cancer (1). The tight control of the enzymes that regulate conversion of unesterified arachidonic acid to key classes of mediators, including prostaglandins, thromboxane, leukotrienes, and lipoxins highlights the importance of arachidonic acid as an essential fatty acid and precursor of these potent bioactive eicosanoids (2). The potential for dysregulation of each of the individual classes of eicosanoids has been suspect as important molecular events associated with several human diseases, including, inflammatory diseases, atherosclerosis, cardiovascular disorders, Alzheimer's disease, and cancer. The control in physiologic systems of these lipid mediators is an ongoing area of intense investigation, because early results indicated that deficiency disease can be initiated by exclusion of fat from the diet (3), and arachidonic acid was also uncovered as the precursor to prostanoids that play key roles in the regulation of parturition and renal function (4, 5).

Results from recent studies indicate that arachidonic acid is not the only fatty acid precursor that is transformed to potent bioactive mediators in inflammation and resolution (6, 7). Both DHA[1] and eicosapentaenoic acid (EPA), the well-known ω-3 fatty acids present in fish oils, appear to be effective as dietary supplements in the treatment of a wide range of human disorders (8–11). For example, ω-3 fatty acid supplementation is reported to have a beneficial impact in treating asthma, atherosclerosis, cancer, and cardiovascular disorders (for a recent review, see Ref. 12). Of interest, the lack of ω-3 fatty acid consumption has also been shown to correlate with mental depression (13). Several clinical trials aimed at testing the therapeutic value of ω-3 supplementation have convincingly established that ω-3 fatty acids can display beneficial actions reducing the incidence and the severity of disease (14). In many of these, aspirin therapy was also used in conjunction, although apparently unintentionally, along with the ω-3 supplementation (*i.e.* see Refs. 14 and 15). Recent results evaluating the impact of aspirin in the transformation of ω-3 in inflammatory exudates *in vivo*, namely acute inflammation and spontaneous resolution, demonstrate that DHA and EPA are each converted via independent pathways to potent bioactive local mediators. These new di- and tri-hydroxy-containing compounds derived from ω-3 fatty acids were termed "resolvins," because they are (*a*) formed within the resolution phase of acute inflammatory response, at least in part, as cell-cell interactions products, (*b*) "stop" neutrophil entry to sites of inflammation, and (*c*) reduce exudates (7). These findings, together with earlier results (16–21), suggest that ω-3 fatty acids, in addition to arachidonic acid, an n-6 fatty acid (2, 4), can serve as precursors for potent

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[1] The abbreviations used are: DHA, C22:6, docosahexaenoic acid; 4S-HDHA, 4S-hydroxydocosa-5E,7Z,10Z,13Z,16Z,19Z-hexaenoic acid; 4S,17R/S-diHDHA, 4S,17R/S-dihydroxydocosa-5E,7Z,10Z,13Z,15E,19Z-hexaenoic acid; 7S,17R/S-diHDHA, 7S,17R/S-dihydroxydocosa-4Z,8E,10Z,13Z,15E,19Z-hexaenoic acid; 10,17R-docosatriene, 10,17R-dihydroxydocosahexaenoic acid; 10,17S-docosatriene, 10,17S-dihydroxydocosahexaenoic acid; 10R/S-OCH_3-17S-HDHA, 10R/S-methoxy-17S-hydroxydocosahexaenoic acid; 17R/S-H(p)DHA, 17R/S-hydro(peroxy)docosa-4Z,7Z,10Z,13Z,15E,19Z-hexaenoic acid; COX-2, cyclooxygenase 2; LC-PDA-MS-MS, liquid chromatography-photodiode array detector-tandem mass spectrometry; LO, lipoxygenase; PMN, polymorphonuclear leukocytes; HPLC, high performance liquid chromatography; EPA, eicosapentaenoic acid; PBS, phosphate-buffered saline; TNF, tumor necrosis factor; IL, interleukin.

bioactive molecules with distinct functions (22). Hence, it is likely that the resolvins and related compounds identified might represent the active products responsible, at least in part, for the many reported beneficial responses obtained in clinical studies with patients given high doses of ω-3 supplementation (see Refs. 6 and 7 and references within).

Another aspect for the many years of sustained interest in DHA lies in the fact that the brain is lipid-rich and that DHA is highly enriched in the membranes of brain synapses and in the retina (16). DHA declines in brain neurons with age and may result in loss in mental function (20). Also, it is now clear that DHA is required for fetal brain development and is held to be critical in the newborn for appropriate development and intelligence (23). Hence, from these and many other recent studies (24), it is now apparent that, in humans, DHA serves a critical role in both physiologic and pathophysiologic responses. Our recent finding that aspirin therapy can lead to the biosynthesis of unique series of 17*R* resolvins generated from DHA led us to question whether the significant roles reported for DHA in the many biological systems noted above were related to DHA conversion to potent local bioactive mediators. The reason for this line of thinking is that in most experimental systems, where the actions of either DHA or EPA were assessed either clinically or in animal models, the concentrations required to evoke beneficial actions are usually in the high microgram to high milligram range. In this context, it is difficult to envision direct and specific molecular actions responsible for the many beneficial outcomes from *in vivo* studies. Thus, with the coordinates (physical and biologic properties) in hand for the new aspirin-triggered 17*R* series resolvins obtained using lipidomics and bioassays (see Ref. 7), we determined in the present experiments whether DHA is converted to bioactive mediators *de novo*. The present results indicate that (*a*) DHA is a precursor to a potent family of bioactive docosanoids that include novel docosatrienes as well as the 17*S* epimer resolvin series generated in human blood cells, mouse brain, and by human glial cells; (*b*) these compounds display potent actions on leukocyte trafficking as well as on glial cell functions down-regulating cytokines expression. Hence, our present results indicate that DHA is a precursor in novel biosynthetic pathways to previously unrecognized potent molecules.

EXPERIMENTAL PROCEDURES

Materials—Zymosan A and soybean lipoxygenase (fraction IV) were purchased from Sigma Co. (St. Louis, MO). Docosahexaenoic acid (C22:6, DHA) was from Cayman Chemical Co. (Ann Arbor, MI); other synthetic standards, hydroxy fatty acids, and intermediates used for MS identification and fragment ion references were purchased from Cascade Biochem Ltd. (Reading, UK). Authentic docosanoids of known hydroxy-containing products from DHA used to characterize physical properties, *i.e.* LC-MS-MS diagnostic ions and MS-MS spectra, were 4*S*-hydroxy-5*E*,7*Z*,10*Z*,13*Z*,16*Z*,19*Z*-docosahexaenoic acid (4*S*-HDHA), and the racemate 17*R/S*-hydroxy-4*Z*,7*Z*,10*Z*,13*Z*,15*E*,19*Z*-docosahexaenoic acid (denoted 17*R/S*-HDHA) purchased from Penn Bio-Organics (Bellefonte, PA). For each, NMR analyses were used to establish stereochemistry (*i.e.* double-bond configurations). Using recombinant enzymes (*i.e.* COX-2, 5-LO, and others) and additional reactions, we prepared and isolated 10,17*S*-docosatriene, 10,17*R*-docosatriene, 7*S*,17*S*-diHDHA, 4*S*,17*S*-diHDHA, 4*S*,5,17*S*-triHDHA, 4*S*,11,17*S*-triHDHA, 7*S*,8,17*S*-triHDHA, and 7*S*,16,17*S*-triHDHA, similar to (6, 25). Additional materials used in LC-MS-MS analyses were from vendors reported in (6, 25).

Biogenic Synthesis of 17S Series Compounds—The reference materials were prepared by incubation of DHA and soybean lipoxygenase (Type IV) and/or 5-LO from potato using different ratios of substrate to enzyme that gave specific product profiles (see below). Each reaction condition used was optimized according to UV spectrometric monitoring and high performance liquid chromatography coupled with a photodiode-array detector and a tandem mass spectrometer (LC-PDA-MS-MS, ThermoFinnigan, San Jose, CA). In brief, soybean lipoxygenase (260 kilounits, 14 mg of protein/ml, 360 kilounits/mg of protein, Sigma, St. Louis, MO) was incubated with DHA (100 μg) in 10 ml of Dulbecco's PBS (phosphate-buffered saline with Ca^{+2}/Mg^{+2}, pH 8.0) at 4 °C. To obtain 17*S*-hydro(peroxy)-DHA (H(p)DHA), aliquots (2 ml) were removed at the initial 15-min interval. To obtain both di- and tri-HDHA, lipoxygenase (260 kilounits), and/or potato 5-LO was added to the remaining 17*S*-H(p)DHA incubations in one round-bottomed flask held at 2–4 °C. At 15-min intervals, product profiles were assessed using LC-PDA-MS-MS following $NaBH_4$ reduction. The 17*R* series resolvins were prepared as in a previous study (7).

Hemoglobin-catalyzed Conversion of 17-H(p)DHA—17*S*-H(p)DHA was prepared and incubated with hemoglobin (Sigma H7379A) to identify non-enzymatic transformation products. Incubations were carried out according to those used for hemoglobin-catalyzed transformation of 15*S*-hydro(peroxy)eicosatetraenoic acid (H(p)ETE) (26). In brief, 17*S*-H(p)DHA (30 μg/ml) was incubated with human hemoglobin (300 μg/ml) in PBS (pH 7.4, 37 °C) for 15 min. Incubations were terminated by acidification to pH 3.2 and taken for immediate solid-phase extraction (SepPak). Mono-, di-, and trihydroxy docosanoid reaction products were each separated by combined column chromatography (CC7) followed by solid-phase extraction. Products were identified and quantitated by LC-PDA-MS-MS lipidomic analysis.

Human Whole Blood, PMN, and Murine Brain Incubations—Human whole (venous) blood (10 ml) was collected with or without heparin from healthy volunteers (that declined taking medication for ~2 weeks before donation; Brigham and Women's Hospital protocol 88-02642). Following collection, whole blood was immediately incubated with DHA (10μg) in a covered water bath for 40 min (37 °C). Human PMN were freshly isolated from the whole blood by Ficoll gradient and enumerated as in a previous study (7). PMN ($30–50 \times 10^6$ cells/ml) were exposed to zymosan A (100μg/ml, Sigma) followed by addition of either 17*S*-hydroxy-DHA (3 μg/ml) or DHA (3 μg/ml). Cell suspensions were incubated for (37 °C, 40 min) in a covered water bath. For identification of epoxide-containing intermediates, isolated human PMN ($30–50 \times 10^6$ cells/incubation) were incubated with 17*S*-hydro(peroxy)-DHA and zymosan A (100 μg/ml) in Hanks' buffer containing 1.8 mM Ca^{2+} and 10 mM HEPES (pH 7.4, at 37 °C for 15 min). The incubations were immediately poured into 10 volumes of cold acidified (apparent pH 3.1) methanol and kept (30 min, 4 °C) before analysis. Lipidomic and lipid mediator profile analyses were performed as in previous studies (7, 27, 28).

DHA Uptake and Agonist-induced Release of Novel Docosanoids—Human glial cells (DBTRG-05MG, ATCC) were cultured as in a previous study (7). Media were removed from semiconfluent monolayers (~75%) and replaced with Hanks' buffer (37 °C, 10 ml) containing HEPES, 10 mM, pH 7.4, and [1-^{14}C]DHA (55 mCi/mmol, American Radiolabeled Chemicals, Inc., 10 μM), or unlabeled C22:6 (~10 μM, Oxford Biomedical Research) was added in EtOH (5–10 μl). Cells were placed in an incubator with an atmosphere of 5% CO_2 at 37 °C for 90 min, and aliquots (100 μl) were removed at 30-min intervals and analyzed by scintillation counting to determine cellular [1-^{14}C]DHA. At 90 min, buffer was removed and cells were washed with Hanks' buffer (10 ml, 37 °C). Hanks' buffer (10 ml, 37 °C) containing HEPES (10 mM, pH 7.4) and $CaCl_2$ (1.6 mM) was added to the flasks, and cells were exposed to either zymosan A (100 ng/ml), calcium ionophore (A23187, 5 μM), or buffer (with vehicle, 0.01% EtOH) alone. Cells were placed in an incubator with an atmosphere of 5% CO_2 (37 °C for 30 min), and aliquots were removed at initiation and 30 min post agonist addition to assess the cellular release of [^{14}C]DHA and ^{14}C-labeled oxygenated products. After 30 min buffer was removed and immediately added to 2 volumes of MeOH (4 °C) and kept at −80 °C for >30 min. Cells were detached, lysed with 5 N NaOH and neutralized with 1 M Tris (pH 8.0) to quantitate cell-associated 1-^{14}C-labeled DHA and novel docosanoids carrying label. For the experiments aimed at determining the sites for DHA incorporation into major classes of phospholipids, triglycerides, and neutral lipids, incubations (37 °C, 30 min) were stopped with $CHCl_3$/MeOH (2:5, v/v) and cells were detached. Lipids were extracted using the method of Bligh and Dyer (29), materials from the organic phase were suspended in chloroform, and the identity of lipid classes was established with authentic standards and standard procedures (29). Phosphorimaging analysis was used for quantitation of the ^{14}C content and demonstrated that 80–86% of the total cell associated DHA was in phospholipid and ~11–18% in neutral lipid pools (range from two separate experiments). DHA was predominantly acylated into phosphatidylethanolamine (39–41%) and phosphatidylcholine (29–36%), findings that are consistent with studies in human platelets (30), neutrophils (31), and a rat glioma cell line (32). Selected samples were taken for additional analysis and extracted, and methyl formate-eluted products were analyzed by PDA-MS-MS for identification as in Ref. 7.

Reversed-phase HPLC radioprofiles were carried out using a 1100 series liquid chromatography system (Agilent Technologies) as done previously (7, 27). Radioactivity in 60-s fractions was quantified with an LS6500 scintillation counter (Beckman).

Substrate Competition with Human rCOX-2—Human recombinant COX-2 was overexpressed in *Sf*9 insect cells (ATCC). The microsomal fractions (~8 μl) were suspended in Tris (100 mM, pH 8.0) as done previously (33). [1-^{14}C]Docosahexaenoic acid (55 mCi/mmol, American Radiolabeled Chemicals, Inc., 10 μM) was added to rCOX-2 microsomal membrane suspensions followed immediately by arachidonic acid (C20:4, Cayman Chemical) at increasing concentrations or vehicle alone (0.5% EtOH). Suspensions were incubated at 37 °C for 30 min and stopped with a solution (4 °C) containing ether:MeOH:citrate (30:4:1, v/v). The organic phase was collected and analyzed by thin layer chromatography at 4 °C using as a mobile phase the organic fraction of ethyl acetate:2,2,4-trimethylpentane:acetic acid:water (110:50:20:100, v/v) as in a previous study (34). Total and product-specific radioactivity were each quantitated using a PhosphorImager. Human rCOX-2 conversion of DHA (10 μM) to specific products was 95 ± 0.5% (n = 6) and was used as the 100% value to determine the impact of arachidonic acid on conversion of DHA.

Acute Inflammatory Exudates: Murine Dorsal Air Pouch and Peritonitis—In the 6- to 8-week-old male FVB mice (fed laboratory rodent diet 5001 (Lab Diet, Purina Mills) containing 1.49% eicosapentaenoic acid, 1.86% DHA, and <0.25% arachidonic acid, inflammatory exudates were initiated by intra-pouch injection of recombinant mouse TNFα (100 ng/pouch, R&D Systems). Four hours later mice were sacrificed, in accordance with the Harvard Medical Area Standing Committee on Animals protocol 02570, air pouch lavages were collected, and cells were enumerated. Inhibition of TNFα (100 ng/pouch) stimulated PMN infiltration, with intravenous tail injection of *S* series resolvins and docosatrienes (as prepared with biogenic synthesis; see below) was determined with pouch lavages taken at 4 h. To assess the impact of novel compounds in peritonitis, mice (FVB) were anesthetized with isoflurane and docosatrienes, and *S* series and *R* series of resolvins (suspended in 120 μl of saline) were administered intravenously and followed (~1–1.5 min) by an intraperitoneal injection of zymosan A (1 mg) in 1 ml of sterile saline. Two hours after the intraperitoneal injections, mice were euthanized and peritoneal lavages were rapidly collected for enumeration. Cytosensor® analyses were evaluated using a Cytosensor® microphysiometer (Molecular Devices) and computer workstation as in previous work (35, 36). Also, reverse transcription-PCR analyses of TNFα-induced IL1β was carried out according to a previous study (7).

RESULTS

Novel Docosanoids via Lipidomics: Formation of 17S Series Resolvins and Docosatrienes by Human Whole Blood and Murine Brain—Because brain and blood share many biochemical and signaling pathways in common (37, 38), we compared the profiles of bioactive docosanoids produced by murine brain, human blood, and leukocytes to those generated by glial cells. To this end, we first determined whether human blood generates bioactive docosanoids such as the recently described resolvins (7) by examining the transformation of DHA in human whole blood and endogenous products produced by murine brain on addition of agonists and utilizing LC-PDA-MS-MS-based lipidomic analysis. Fig. 1*A* shows representative LC-MS selective ion monitoring for [M-1] at *m/z* 375 for trihydroxy-containing docosanoids and *m/z* 359 for dihydroxy- and *m/z* 343 for monohydroxy-containing docosanoids, respectively. These results demonstrate for the first time that, in addition to docosanoids with physical properties similar to those of the aspirin-triggered 17*R* series resolvins generated in resolving murine inflammatory exudates (7), shown in Fig. 1, human blood converts DHA to 17*S* series resolvins as well as novel dihydroxy-containing docosanoids. LC-MS plots of selective ion chromatograms and mass spectra also showed the presence of novel dihydroxy-containing docosanoids. For example, the MS-MS spectrum of a dihydroxy-containing DHA is reported in Fig. 1*B* with prominent fragment ions consistent with the structure shown in the insert, namely 10,17*S*-dihydroxydocosahexaenoate and ions at *m/z* 359 (M-H), 341 (M-H-H_2O), 323 (M-H-2H_2O), 315 (M-H-CO_2), 297 (M-H-H_2O-CO_2), and 277 (M-H-H_2O-CO_2-2H). Additional diagnostic ions consistent with the carbon 10 and carbon 17 alcohol-containing positions were observed at *m/z* 153, 163, 181, 189, 205, 217 (216-CO_2+H), 243 (261-H_2O+H), 261, and 289. The UV spectrum of this docosanoid gave a maximum absorbance wavelength (λ_{max}) of 269 nm with shoulders at 260 and 279 nm indicating the presence of a conjugated triene chromophore (*i.e.* three conjugated dou-

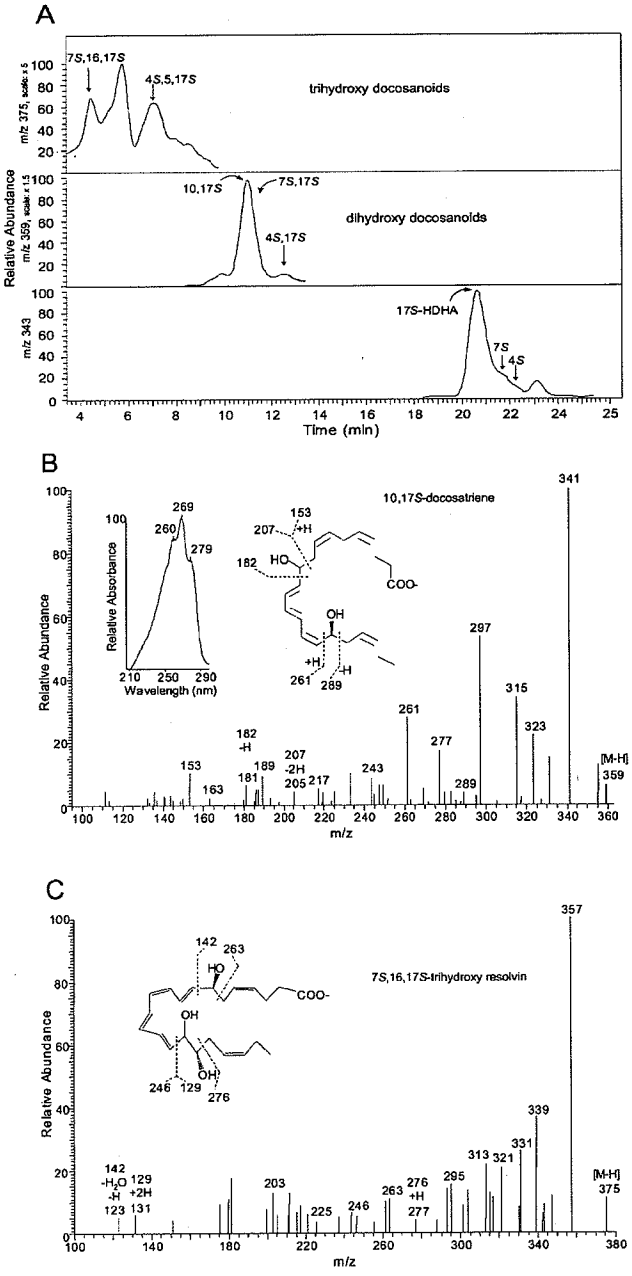

FIG. 1. **Formation of novel docosatrienes and 17*S* series resolvins.** Lipidomic analysis was carried out with heparinized blood incubated (10 ml, 37 °C, 30 min) with DHA and products identified by LC-PDA-MS-MS (see "Experimental Procedures" and text, n = 3). *A*, selected ion chromatograms of trihydroxy-carrying (*m/z* 375, *upper panel*), dihydroxy-carrying (*m/z* 359, *middle panel*), and monohydroxy-carrying (*m/z* 343, *lower panel*) products from DHA. Identification of the ions diagnostic for specific hydroxy group positions present in the trihydroxy, dihydroxy, and monohydroxy products generated are given in the *insets* (see text). *B*, MS-MS and UV (*inset*) spectra of 10,17*S*-docosatriene (depicted with tentative stereochemistry assignments). *C*, MS-MS spectrum of the 17*S* series trihydroxy resolvin: 7*S*,16,17*S*-triHDHA.

ble bonds) in the new structure. The configuration of the alcohol at position 17 was confirmed as being in predominately the *S* configuration using materials prepared with isolated lipoxygenases and DHA as substrate with co-elution experiments (see "Experimental Procedures"). The predominant products, also observed from endogenous sources of DHA in isolated murine brain incubated with ionophore, were 10,17*S*-docosatriene and 4*S*,17*S*-diHDHA ($n = 3$, not shown). Taken together with the chromatographic behavior, these physical properties were consistent with the formation of 10,17*S*-diHDHA (docosatriene), a novel carbon 17 position-oxygenated product formed from DHA.

We questioned whether these 17-hydroxy-carrying docosanoids from human whole blood and murine brain could be formed via 15-lipoxygenation, which is an enzymatic activity in human leukocytes and is induced during the resolution phase of acute inflammation (39) and whether these new compounds were biosynthetically interrelated. Along these lines, earlier results with isolated soybean 15-lipoxygenase showed that this enzyme can rapidly transform DHA to 17*S*-HDHA *in vitro* (34) that were confirmed during the course of the present experiments with isolated enzyme and LC-PDA-MS-MS analyses ($n > 12$, not shown). Results shown in Fig. 1 demonstrate that human whole blood generated 17*S*-hydroxydocosahexaenoic acid (λ_{max} of 237 nm and diagnostic MS-MS ion *m/z* 245) from DHA. This human product of whole blood was matched in physical properties to those of authentic 17*S*-hydroxydocosahexenoate produced via biogenic synthesis (see "Experimental Procedures"). In addition, a novel product 7*S*,16,17*S*-trihydroxydocosahexaenoic acid was uncovered that exhibited an MS-MS spectrum (Fig. 1*C*) with diagnostic ions at *m/z* 123 (142-H_2O-H), 131(129 + 2H), 203 (246-CO_2-H), 225 (263–2H_2O-2H), 246, 263, 277 (276+H), 295 (M-H-2H_2O-CO_2), 313 (M-H-H_2O-CO_2), 321 (M-H-^3H_2O), 331 (M-H-CO_2), 339 (M-H-2H_2O), 357 (M-H-H_2O-CO_2), and 375 (M-H). The presence of this 7*S*,16,17*S*-trihydroxy-containing docosanoid suggested a pathway for its formation (see below) similar to the generation of the anti-inflammatory eicosanoid lipoxin B_4, a 5-lipoxygenase interaction product generated from arachidonic acid (*e.g.* 5*S*,14,15*S*-trihydroxyeicosatetraenoic acid), and the newly described 17*R* epimer termed 7*S*,16,17*R*-trihydroxydocosahexaenoic acid-resolvin (as in 7). Of interest, the 17*R* series and 17*S* series of di- and tri-hydroxy products appear to give different chromatographic retention times (see "Experimental Procedures").

Lipidomic-based analysis employing LC-PDA-MS-MS also identified the presence of distinct docosanoids that were consistent with the presence of and conversion of DHA by both lipoxygenase and cyclooxygenase-like enzymatic activities that act on DHA present in whole blood (Fig. 1*A*). In these analyses, we also identified 13-HDHA (UV λ_{max} 237 nm and diagnostic ion *m/z* 221), a potential cyclooxygenase-2-derived product from DHA (Ref. 7 and data not shown). Along with these compounds shown in Fig. 1*A*, we also observed LC-MS and MS-MS spectra consistent with the production of both 11-HDHA and 14-HDHA the known 12-lipoxygenase products reported earlier (17, 40), as well as the 4*S*- and 7*S*-hydroxy-containing products of DHA that can be generated by a 5-lipoxygenase-like reaction as exemplified by the potato 5-lipoxygenase enzyme (41, 42). It is noteworthy that both clotted whole blood (non-heparinized) as well as heparinized blood generated the identified monohydroxydocosanoid profile (Fig. 1). In contrast, the dihydroxy products from DHA, including the novel docosatriene (10,17*S*-diHDHA), were not observed with clotted whole blood (data not shown). In addition, incubation of 17*S*-hydro(peroxy)-DHA with hemoglobin as used with 15-H(p)ETE conversion (26) produced several new non-enzymatic epoxy alcohols and trihydroxydocosanoids (*i.e.* 16,17*S*-epoxy-15-hydroxydocosapentaenoic acid (16,17*S*-epoxy-15-HDPA), 13,14-epoxy-17*S*-hydroxydocosapentaenoic acid (13,14-epoxy-17*S*-HDPA), 16,17*S*-epoxy-13-hydroxydocosapentaenoic acid (16,17*S*-epoxy-13-HDPA), 15,16-epoxy-17*S*-hydroxydocosapentaenoic acid (15,16-epoxy-17*S*-HDPA), 13,16,17*S*-tri-hydroxydocosapentaenoic acid (13,16,17*S*-HDPA), 15,16,17*S*-tri-hydroxydocosapentaenoic acid (15,16,17*S*-tri-HDPA), 13,14,17*S*-tri-hydroxydocosapentaenoic acid (13,14,17*S*-tri-HDPA); see "Experimental Procedures"). These compounds were not formed in substantial quantities in whole blood (see below) but may be relevant in other pathophysiologic scenarios. It is likely that they were not formed in substantial amounts in these incubations, because the 17*S*-H(p)DHA, once formed, remained intracellular, limiting its ability to interact with heme proteins in a non-selective fashion. The bioactions of these will be reported elsewhere. Together these results indicated that human blood and murine brain converts DHA to several previously unappreciated novel di- and tri-hydroxy-containing docosanoids. Some of these new docosanoids resembled the physical properties of the recently identified 17*R* series resolvins (7), namely those denoted here as their 17*S* carbon position, *e.g.* hydroxy epimers or the 17*S* series resolvins.

A Role for Cell-Cell Interactions in the Formation of 17S Series Resolvins and Docosatrienes—Human PMN were exposed to 17*S*-H(p)DHA to determine if the likely intermediates in the formation of 17*S* series resolvins and docosatrienes were transformed by leukocyte-associated lipoxygenase-like activity (*i.e.* 5-LO, 15-LO) to the novel dihydroxy- and trihydroxy-containing docosanoids identified in blood and murine brain (see Fig. 1). Indeed, PMN freshly isolated from peripheral blood, transformed 17*S*-hydro(peroxy)docosahexaenoic acid to two distinct 5-lipoxygenase products 7*S*,17*S*-diHDHA and the less prominent product 4*S*,17*S*-diHDHA as indicated in the selective ion chromatogram plotted at *m/z* 359 (Fig. 2*A*). The 4*S*,17*S*-diHDHA was identified based on the MS-MS spectrum that displayed diagnostic ions at *m/z* 101, 257, 239 (257-H_2O), 261, 267, 287, 245 (290-CO_2-H), 359 (M-H), 341 (M-H-H_2O), 315 (M-H-CO_2), 297 (M-H-CO_2-H_2O), and 277 (M-H-CO_2-2H_2O-2H) and on its UV spectrum that gave a λ_{max} 234 nm revealing its conjugated diene structure (Fig. 2*B*, *inset*). In addition to these dihydroxydocosanoids, the MS-MS and UV spectral analysis also confirmed the presence and formation of the novel docosatriene 10,17*S*-diHDHA (see Fig. 2*A*), results that are consistent with spectra obtained from murine brain and human whole blood lipidomic analysis (Fig. 1). The apparent ratio for 10,17*S*-docosatriene to the other main dihydroxy product 7,17*S*-diHDHA generated by human PMN was ~1.12 (calculated based on the analysis of UV chromatographic peak areas at their respective λ_{max} 269 and 242 nm using recorded on line PDA spectra). Further MS-MS and UV spectral analysis revealed the formation of ω-22-hydroxy-10,17*S*-docosatriene (Fig. 2*C*), a likely ω-oxidation product of the 10,17*S*-docosatriene in human leukocytes. Human leukocytes and several other tissues possess a prominent ω-oxidation apparatus that can rapidly inactivate local bioactive lipid mediators (25, 43). The structure of ω-22,10,17*S*-docosatriene was determined by analysis of the MS-MS spectrum (Fig. 2*C*). This compound gave ions of diagnostic value at *m/z* 375 (M-H), 357 (M-H-H_2O), 343 (344-H), 339 (M-H-H_2O), 331 (M-H-CO_2), 324 (344-H_2O-2H), 309 (344–2H_2O+H), 300 (344-CO_2), 295 (M-H-2H_2O-CO_2), 273 (290-H_2O+H), 265 (344–2H_2O-CO_2+H), 261 (260+H), 217 (260-CO_2+H), 193, 181, and 163 (181-H_2O). Taken together these findings suggest transformation of 17H(p)DHA via two prominent and distinct metabolic pathways to generate 5-LO-

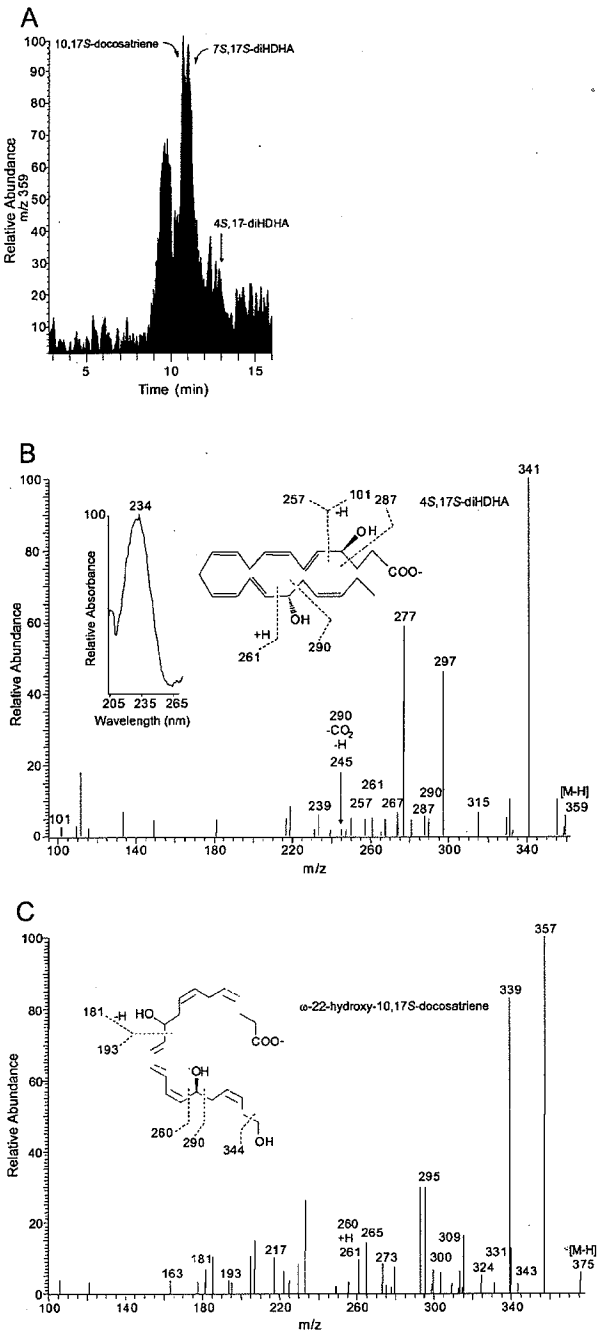

FIG. 2. **Human PMN produce both 17*S* series resolvins and docosatrienes.** Human neutrophils (30–50 × 10^6 cells/incubation) were exposed to zymosan A and 17*S*-H(p)DHA, and products were analyzed using LC-PDA-MS-MS (see "Experimental Procedures," n = 5). *A*, selected ion chromatogram (*m/z* 359) for dihydroxy-DHA products. *B*, MS-MS and UV (*inset*) spectra of the 17*S* series resolvin 4*S*,17*S*-diHDHA. *C*, MS-MS of ω-22-hydroxy-10,17*S*-docosatriene. Identification of 17*S* series diHDHAs and 10,17*S*-docosatriene are indicated with tentative stereochemistry assignments.

like interaction products (*i.e.* the double dioxygenase products 4*S*,17*S*-diHDHA and 7*S*,17*S*-diHDHA) and the novel 10,17*S*-docosatriene.

Human Glial Cells Generate and Release Novel Docosanoids: Docosatrienes—Next, we determined whether the *S* series resolvins and docosatrienes were formed from cellular stores of DHA and if they could also be produced by a single cell type. To this end, we labeled human glial cells with [1-^{14}C]DHA. As anticipated, glial cells rapidly incorporated labeled DHA as indicated by a statistically significant increase in total radiolabel associated with cellular stores ($p > 0.05$, $n = 3$). Radioactivity associated with cellular stores increased from 35 ± 8% at $t = 30$ min to 51 ± 3% at $t = 60$ min and 61 ± 4% at $t = 90$ min post addition of [1-^{14}C]DHA ($n = 3$) indicating that cellular uptake of labeled DHA was time-dependent. Because glial cells are involved in host defense and inflammation in neural tissues, we exposed them to the microbial product, zymosan A, as well as a non-selective stimulus for cellular calcium (calcium ionophore, A23187) to test whether they were agonists for the formation of novel docosanoids from cellular DHA stores. Activation of glial cells induced a statistically significant release ($p > 0.05$, $n = 4$) of cell-associated label as well as formation of the novel docosanoids. Results in Fig. 3*A* indicated release of ~4.1 ± 1.1% of total cellular DHA following exposure to zymosan A and ~5.6 ± 2.4% with calcium ionophore when directly compared with glial cells that were exposed to vehicle alone (1.6 ± 0.6%). Reversed-phase-HPLC radioprofiles demonstrated that zymosan A challenge of glial cells released several distinct 1-^{14}C-labeled compounds from cellular DHA stores (see *inset* of Fig. 3*A*). In addition to distinct docosanoids that carried radioactivity in the profile there was also label associated with material that corresponded to the retention time of native DHA that accounted for 74 ± 6% ($n = 3$) of the total released radioactivity. Exposure of human glial cell to either zymosan A or calcium ionophore gave agonist-dependent and selective loss of [1-^{14}C]DHA from phosphatidylethanolamine that ranged from 13 to 28% and from 3 to 22%, respectively (see Fig. 3*A* and "Experimental Procedures").

To identify the endogenous docosanoids formed from cellular stores of DHA in human glial cells, we analyzed the released DHA-derived products by LC-PDA-MS-MS based lipidomics (as in Fig. 1). Again, activation of glial cells with either calcium ionophore (Fig. 3*B*) or zymosan A stimulated the formation (not shown) of novel 17-lipoxygenation product 10,17*S*-diHDHA as well as its ω-oxidation product 10,17*S*,22-triHDHA (note that both were also produced in blood; see Fig. 1). The structure of the ω-22 product was determined by analysis (at *m/z* 375) of the MS-MS spectrum (Fig. 3*C*). The material beneath the peaks corresponded to 10,17*S*-docosatriene and 17*S*-HDHA (not shown) and gave MS-MS and UV spectra essentially identical to the 17*S*-oxygenation products of DHA that were detected with human whole blood (see 10,17*S*-docosatriene in Fig. 1*B*). Of the theoretically possible multiple isomers, only one major peak eluted 10,17*S*-docosatriene produced by glial cells (see "Experimental Procedures"). The ω-22-hydroxy-4,17-diHDHA was also formed as shown in the LC-MS selective ion chromatogram at *m/z* 375 (Fig. 3*C*). This structure was consistent with the MS-MS (at *m/z* 375) and prominent ions of diagnostic value at *m/z* 216 (260-CO_2-H), 236 (273–2H_2O-H), 247 (303-^3H_2O-2H), 272 (290-H_2O), 273, 293 (M-H-2H_2O-CO_2-2H), 300 (344-CO_2), 303, 313 (M-H-H_2O-CO_2), 337 (M-H-2H_2O-2H), 345 (344+H), 357 (M-H-H_2O), and 375 (M-H) together with the UV spectrum (λ_{max} 238 nm, Fig. 3*C*, *inset*). The formation of ω-22-hydroxy-4,17-diHDHA suggests conversion of 17*S*-H(p)DHA by a 5-lipoxygenase-like mechanism that can oxygenate DHA at the carbon 4 position as shown for DHA to give 7*S*- or 4*S*-HDHA (41, 42). Hence, with 17*S*-H(p)DHA as substrate, this LO-like reaction can generate 4*S*,17*S*-diHDHA that is subsequently ω-oxidized at carbon 22.

To assess the potential pathways involved in the formation of these new compounds, human glial cells were exposed to exogenous DHA in the presence of calcium ionophore. Lipidomic-based analysis using LC-PDA-MS-MS as shown in Fig. 4 yielded products consistent with the presence of 15-LO, 12-LO,

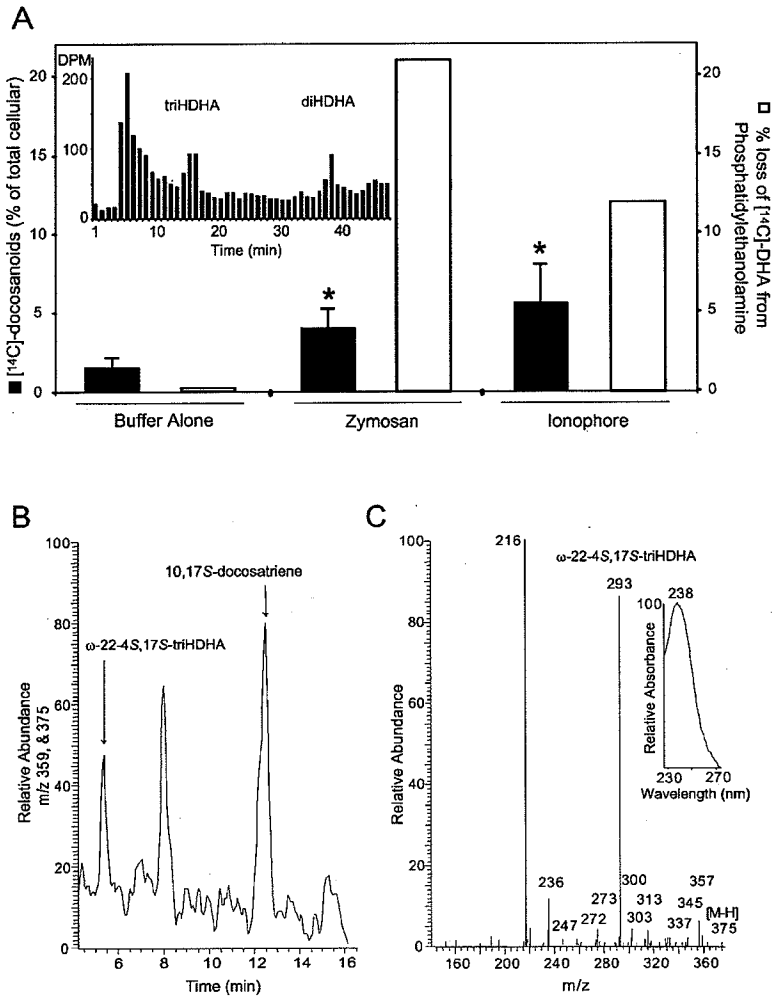

FIG. 3. **Human glial cells release and transform DHA.** *A*, human glial cells were labeled with [1-^{14}C]DHA and exposed to either PBS alone, zymosan A, or calcium ionophore (see "Experimental Procedures"). Released [1-^{14}C]docosanoids were quantified (n = 4). The *asterisk* denotes significant differences from control (PBS alone), $p < 0.05$ analysis of variance, Newman-Keuls test. The *inset* shows a representative HPLC radiochromatogram of released [1-^{14}C]docosanoids (see "Experimental Procedures") from glial cells exposed to zymosan A (n = 3). Phospholipids from glial cells were identified, and the ^{14}C content in phosphatidylethanolamine (*PE*) was determined. The percent loss in PE ^{14}C content from cells exposed to agonists was determined (n = 2). *B*, lipidomic profile of human glial cells exposed to Ca^{2+} ionophore: LC-MS total ion chromatogram of released products. *C*, LC-MS selective ion chromatogram of released DHA-derived products from glial cells exposed to calcium ionophore. MS-MS and UV spectra of ω-22-hydroxy-4*S*,17*S*-diHDHA.

5-LO, and COX-2 activity in glial cells. This was further supported by the formation of stereospecific oxidation products that were also identified in murine brain and blood (see text and Fig. 1). Formation of double oxygenation products 7*S*,17*S*-diHDHA and 4*S*,17*S*-diHDHA was confirmed by MS-MS and UV spectra that proved to be identical to those obtained with human whole blood (Fig. 1). The formation of ω-22,16,17*S*-docosatriene was confirmed by MS/MS (at m/z 375) spectrum (Fig. 4*B*). Its spectrum showed ions at m/z 309 (344–$2H_2O$+H), 345 (344+H), 217 (260-CO_2+H), 243 (260-H_2O+H), 259 (260-H), 271 (290-H_2O-H), 111 (145–$2H_2O$+2H), 143 (145–2H), 231, 277 (M-H-^3H_2O- CO_2), 295 (M-H-$2H_2O$-CO_2), 311 (M-H-H_2O-CO_2-2H), 331 (M-H -CO_2), 339 (M-H-$2H_2O$), 357 (M-H-H_2O), and 375 (M-H). These results suggested formation of a 16,17*S*-epoxide-containing intermediate that opens to form the diol 16,17*S*-diHDHA (see below), which is subsequently transformed by ω-oxidation to give the trihydroxy-containing product.

Substrate Competition with Human rCOX-2—Human COX-2 generates stereospecific oxygenated fatty acids from multiple substrates such as eicosatetraenoic acid, eicosapentaenoic acid (6), and docosahexaenoic acid (7). These lipid mediators as potential COX-2 products are of interest, because COX-2 may regulate homeostasis in the brain, kidney, and digestive tract as well as being potentially linked to inflammatory diseases, fetal development, and carcinogenesis (44). 13-HDHA, a COX-2 product, was identified using LC-MS-MS-based lipidomic analyses in both blood and human glial cell incubations. We investigated the impact of arachidonic acid, the COX-2 substrate for prostaglandins and 15-epi-lipoxin formation (45), on docosahexaenoic conversion by human recombinant enzyme. COX-2 rapidly transformed docosahexaenoic acid to specific docosanoids that were determined using both thin layer chromatography and LC-PDA-MS-MS. The percentage of docosahexaenoic acid transformation at 10 μM substrate by rCOX-2 to specific products mainly 13-hydroxydocosanoid was 94.6 ± 0.5% (n = 3, d = 2 in each). This conversion of DHA was inhibited in a concentration-dependent fashion when exposed to increasing concentrations of arachidonic acid (Fig. 5), which reached 70 ± 15% inhibition at 1 mM arachidonic acid (n = 3). These results indicate that DHA is an effective substrate for oxygenation and can compete for the enzyme's active site *in vitro*. Hence, these findings suggest that within cells or tissues the rate-limiting step for DHA oxygenation is substrate availability. They do not, however, preclude the involvement *per se* of as yet to be defined enzymes or ones that can specifically act on DHA to produce bioactive local mediators (see below).

Anti-inflammatory Properties of 17S Series Resolvins and Docosatriene: Inhibition of PMN Recruitment and Cytokine Gene Expression—To determine whether the 17*S* series compounds in the 17*S* series carry biologic properties, we tested their topical and systemic actions in established *in vivo* models of acute inflammation (Fig. 6*A*). The novel docosanoids proved to be potent inhibitors of TNFα-induced leukocyte trafficking to the air pouch both via topical and systemic application. The 17*S* series resolvins reduced PMN within the exudates by

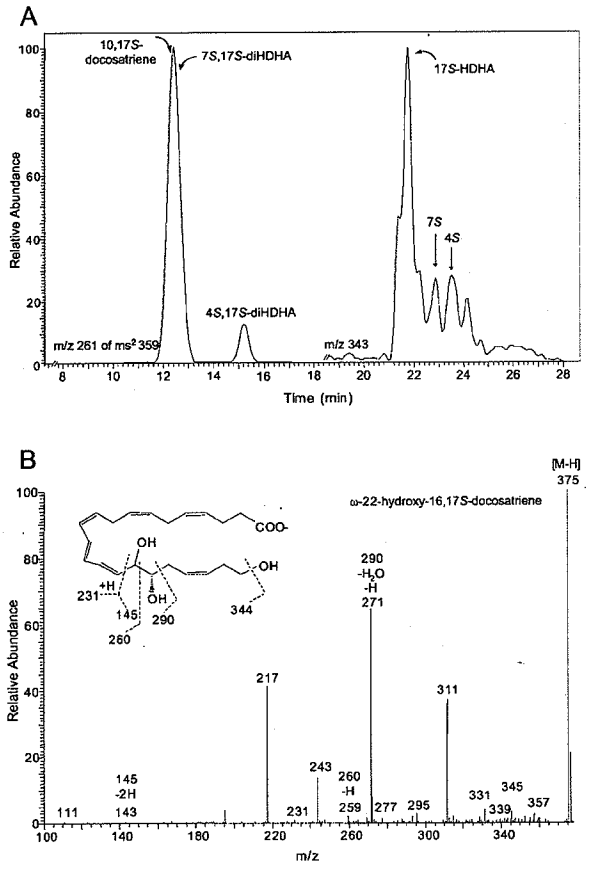

FIG. 4. **Glial cells generate 17*S* series resolvins and 10,17*S*-docosatriene.** Human glial cells were exposed to DHA and calcium ionophore (37 °C, 30 min). The docosanoids were identified by LC-PDA-MS-MS analysis. *A*, LC-MS-selective ion chromatograms showing generation of specific mono-HDHAs (*right*) and di-HDHAs (*left*). *B*, MS-MS of ω-22-hydroxy-16,17*S*-docosatriene.

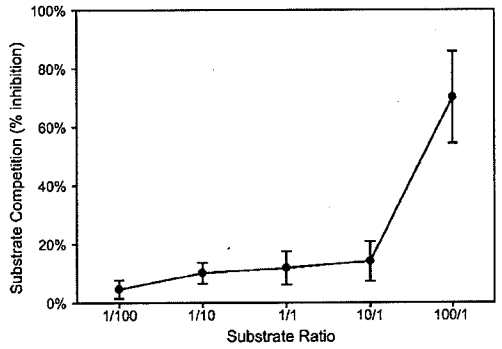

FIG. 5. **Substrate competition at human recombinant COX-2.** Human rCOX-2 microsomal membranes from Sf9 cells were incubated with [1-^{14}C]DHA (C22:6, 10 μM) in the presence of increasing concentrations of unlabeled arachidonic acid (C20:4). COX-2 conversion of [1-^{14}C]DHA and specific product profiles were quantitated (n = 3). rCOX-2 activity is expressed as the inhibition of [^{14}C]C22:6 product formation (95 ± 0.5%; conversion of DHA was set to 100%).

82.2 ± 5.6% given topically and by 49.6 ± 8.2% when given intravenously (n = 4). Systemic treatment with the 17*S* series resolvins also inhibited zymosan A-induced PMN recruitment to the peritoneum by 45.1 ± 0.8% (n = 4), a value that was equipotent to the recently described 17*R* series resolvins (7). The direct side-by-side comparison showed that the 17*R* series resolvins inhibited PMN recruitment by 42.6 ± 8.0% (Fig. 6*A*). Treatment with the isolated novel docosatriene (10,17*S*-diHDHA), a member of the 17*S* series (see Fig. 8), proved to be a potent systemic inhibitor of PMN recruitment as evidenced by its ability to abate PMN in the peritoneal exudates by ~42%. In this regard, it proved to be as potent as indomethacin, a well-characterized non-steroidal anti-inflammatory drug (46), which reduced PMN peritoneal in exudates by ~40% (Fig. 6*A*).

Properties with Glial Cells—The 17*S* series DHA compounds were also evaluated as potential regulators of cytokine-induced gene expression in the human glioma cells (Fig. 6*B*). Semi-qualitative reverse transcription-PCR analyses demonstrated that both the 17*S*-HDHA and 10,17*S*-docosatriene inhibited TNFα-induced IL-1β transcript levels and gave a dose-dependent inhibition with an IC_{50} of ~0.5 nM (Fig. 6*B*). Analysis using microphysiometry can give a rapid real-time indication of receptor ligand-operated cellular events (47). To determine if the 17*S* series docosanoids interact with recognition sites on human glial cells, we evaluated whether these novel lipid mediators could evoke ligand-operated extracellular acidification using a four-channel Cytosensor®. Results shown in Fig. 6*C* demonstrate that the novel 10,17*S*-docosatriene and a mixture of both 17*S* series docosanoids each added to the cells separately evoked rapid ligand-specific extracellular acidification ($p < 0.03$). In sharp contrast, the precursor DHA did not evoke extracellular acidification. These findings are consistent with the ability of both 17*S*-HDHA and 10,17*S*-docosatriene to regulate gene expression in human glial cells (Fig. 6*B*) and suggest the presence of recognition sites for 17*S* series docosanoids in human glial cells.

Trapping of Epoxide Intermediates—To evaluate the formation of potential epoxide-containing intermediates during the biosynthesis of 17*S* series resolvins and docosatrienes, we exposed PMN to excess acidic alcohol and analyzed the extracted materials for the presence of methoxy-trapping products as in previous work (28, 48, 49) that could be formed as direct marker of epoxide-containing biosynthetic intermediates using LC-PDA-MS-MS. Results shown in Fig. 7 demonstrate formation of two prominent 16-methoxy-17*S*-hydroxydocosahexaenoate isomers, denoted *Ia* and *Ib*, as well as two 10-methoxy-17*S*-hydroxydocosahexaenoate isomers, denoted *IIa* and *IIb* in Fig. 7*A*. The 16-methoxy-containing trapping products were identified based on the UV and MS-MS (at *m/z* 373) spectral analysis (Fig. 7*B*) of the corresponding LC peaks in Fig. 7*A*. The presence of a chromophore with a triple band of absorbance at λ_{max} 270 nm with shoulders at 261 and 281 nm (see Fig. 7*B*, *inset*) and diagnostic MS-MS ions of *m/z* 355 (M-H-H_2O) and 329 (M-H-CO_2), 230, 143, 275 (274+H), 241 (274-MeOH-H), 304, 271 (304-MeOH), and 210 (304-H_2O-MeOH-CO_2) were consistent with the racemic alcohol-trapping products at position 16 denoted a and b isomers, namely, 16*R/S*-methoxy-17*S*-hydroxy-docosahexenoic acid. The 10-methoxy-containing trapping products were also identified by the presence of chromophores that gave a λ_{max} 270 nm with shoulders at 260 and 281 nm (see *inset* in Fig. 7), together with diagnostic ions present in their MS/MS at *m/z* 355 (M-H-H_2O), 341 (M-H-MeOH), 329 (M-H-CO_2), 323 (M-H-H_2O-MeOH), 311 (M-H-H_2O-CO_2), 297 (M-H-MeOH-CO_2), 279 (M-H-H_2O-MeOH-CO_2), 152, 177, 203 (221-H_2O), and 119 (196-H-MeOH-CO_2), 304, 275 (274+H), and 227 (304-H-MeOH-CO_2). The results of LC-PDA-MS-MS analysis also uncovered the presence of low levels of 4*S*-OH-, 11-methoxy-, 17*S*-OH-DHA (data not shown). The identification of these major methoxy-trapping products indicated that both a 16,17-epoxide-containing intermediate and 4,5-epoxide intermediate were generated from 17*S*-H(p)DHA by human PMN. The nucleophilic addition of excess MeOH in acidic conditions to form two main groups of methoxy- derivatives at carbon position 10 and/or 16 position is consistent with open 16,17-epoxy and 4,5-epoxy rings and is in

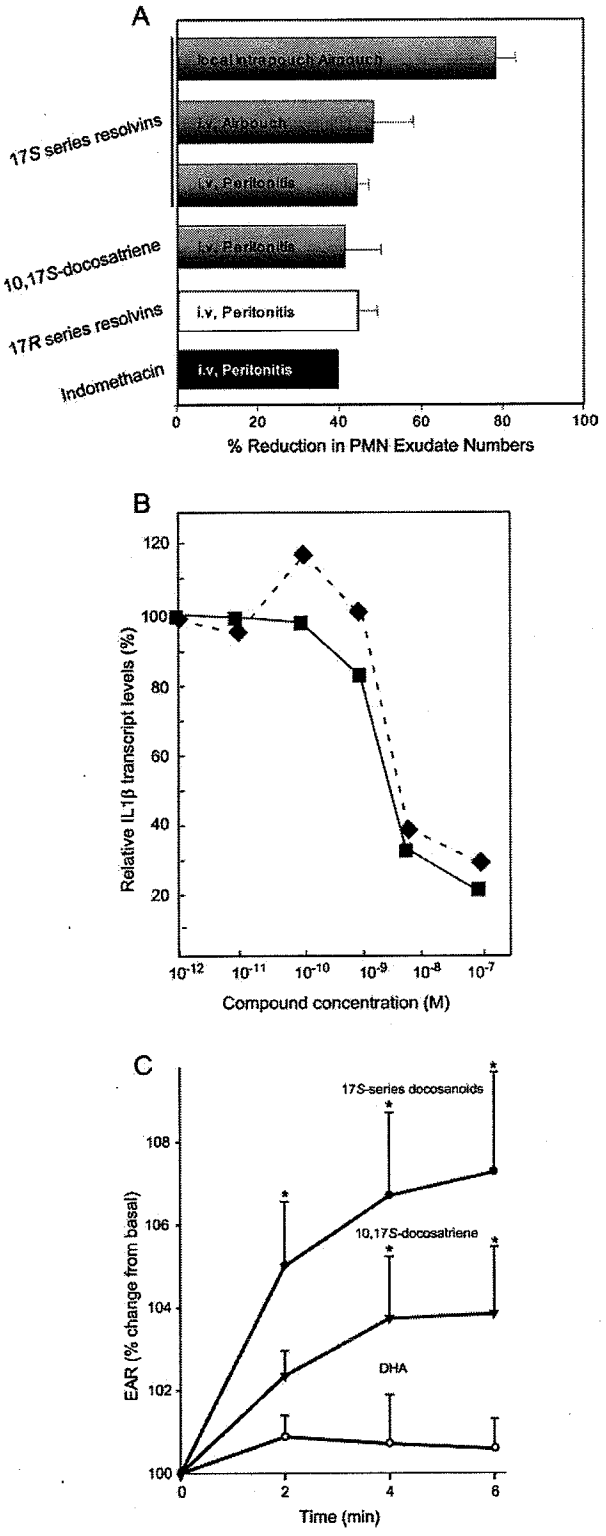

FIG. 6. **Anti-inflammatory properties of 17*S* series resolvins and 10,17*S*-docosatriene.** *A*, inhibition of PMN infiltration in murine peritonitis and dorsal skin pouch following systemic and topical application. Peritonitis: 17*S* series docosanoids (100 ng) or indomethacin (100 ng) was injected intravenously into mouse tails followed by zymosan A into the peritoneum. Mice were sacrificed, and peritoneal lavages were collected (2 h) and cells enumerated ($n = 4$). Air pouch, 17*S* series compounds (100 ng) were injected intrapouch or intravenously followed by intrapouch injection of TNFα. For direct comparison, mice were treated with 17*R* series resolvins via intrapouch injection followed by TNFα. At 4 h, air pouch lavages were collected and enumerated. Values represent mean ± S.E. from three to four different mice; all gave $p <$ 0.05 when infiltrated PMN were compared with vehicle control. *B*, inhibition of TNFα-stimulated IL1β transcripts in human glial cells. Human glial cells were stimulated for 16 h with TNFα in the presence of specified concentrations of 17*S*-HDHA (*solid line*) or 10,17*S*-docosatriene (*dotted line*), and expression of IL-1β transcripts was analyzed. Data are representative of $n = 2$. *C*, glial cells: 17*S* series docosanoids evoke ligand-operated extracellular acidification. Changes in extracellular acidification rates (EAR) were analyzed using Cytosensor® microphysiometry. Cells were superfused (100 μl/60 s) with DHA, 17*S* series docosanoids ($n = 3$), or 10,17*S*-docosatriene ($n = 4$). Values are expressed as EAR (μV/s) normalized to baseline (100%), and ligands were added at 2 min.

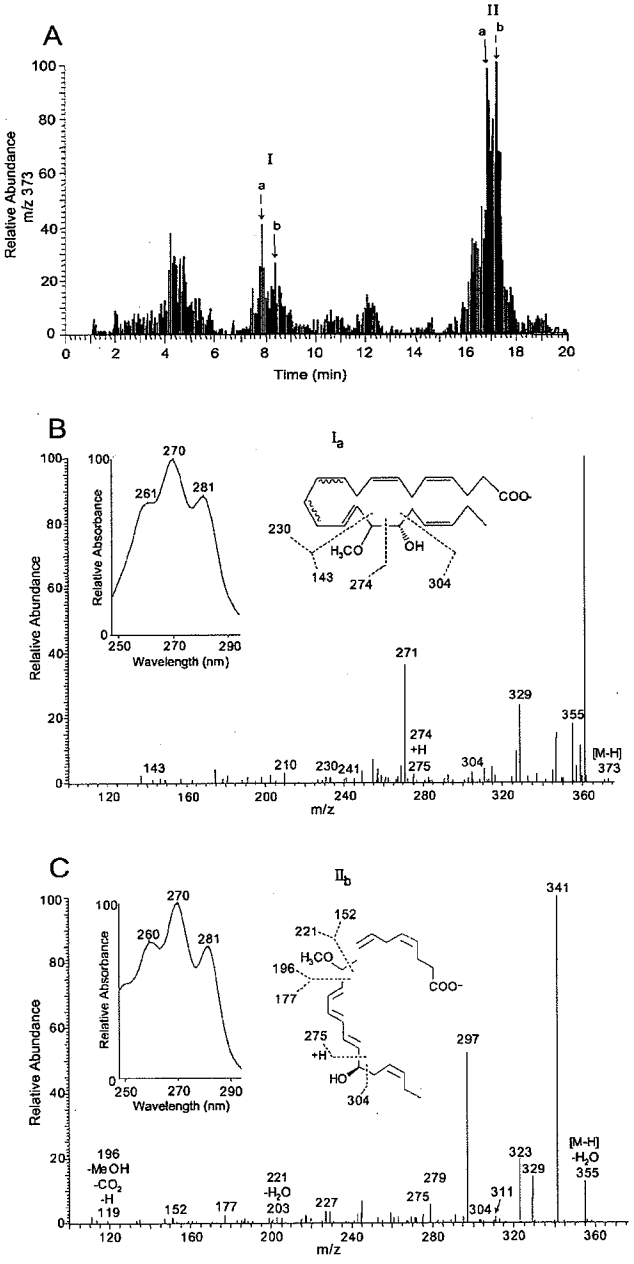

FIG. 7. **Epoxide-containing intermediates in the biosynthesis of docosatrienes and 17*S* series resolvins.** PMN (30–50 × 10^6 cells/incubation) were incubated with 17*S*-hydroperoxy-DHA and zymosan A and stopped, and alcohol-trapping products were extracted. *A*, LC-MS chromatogram obtained from selective ion monitoring for methoxy-trapping products at *m/z* 373 shows two 16-OCH_3 (*Ia* and *Ib*) and two 10-OCH_3 (*IIa* and *IIb*) isomers. *B*, MS-MS and UV (*inset*) spectra of 16-OCH_3 product I; *C*, MS-MS and UV (*inset*) spectra of 10-OCH_3 product II.

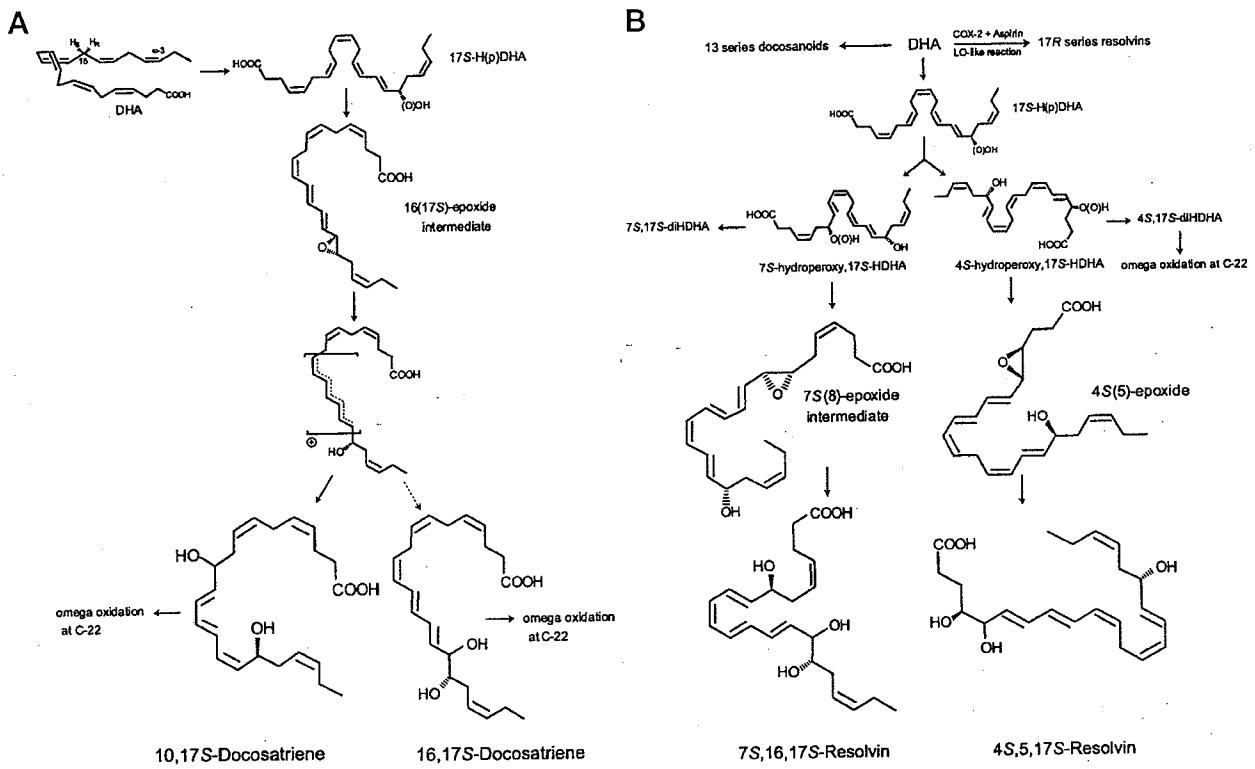

FIG. 8. **Biosynthetic scheme proposed for 10,17*S*-docosatrienes and 17*S* series resolvins.** These transformations may involve novel enzymes in addition to those that serve in the biosynthesis of eicosanoids (see text for further detail). The complete stereochemistry for the docosatrienes (*A*) and 17*S* series resolvins (*B*) remain to be established and are depicted in their tentative configurations based on biogenic total synthesis, lipidomic analyses, and alcohol trapping profiles. The carbonium cation intermediate shown in *A* is also likely to be the enzymatic basis of the epoxide conversion in *B*.

agreement with the involvement of specific epoxy intermediates as those involved in leukotriene and lipoxin generation from arachidonic acid (28, 48, 49).

DISCUSSION

The present results indicate that DHA is a precursor to bioactive products, the basic structures of which were established, and the presence of previously undisclosed pathways in mammalian cells to convert DHA to potent endogenous compounds that display anti-inflammatory properties. Results of alcohol trapping experiments provide evidence for a 16(17)-epoxide intermediate generated from the 17*S*-hydroperoxy precursor in both human glial cells and leukocytes. Taken together with the results in Figs. 1–7, they support the scheme proposed in Fig. 8. In single cell-type systems, as for example, in glial cells, agonists stimulate the release of unesterified DHA predominantly from phosphatidylethanolamine (Fig. 3), which is converted via a lipoxygenase-like reaction to 17*S*-hydroperoxy derivative, which is further transformed to the *trans*-16(17)-epoxide-containing intermediate (Fig. 8*A*).

This *trans* epoxide intermediate can undergo aqueous hydrolysis via a carbonium cation intermediate to produce 10,17*S*-docosatriene carrying a conjugated triene and the 17 position in the *S* configuration retained from 17*S* or the vicinol diol triene (Fig. 8*A*). Once the epoxide intermediate at the 16(17) position is produced via a second hydrogen abstraction mechanism, which appears to be similar to that established for human lipoxygenases and related enzymes such as leukotriene A_4 hydrolase (see Refs. 43, 50–52), it gives a carbonium cation intermediate that can then be converted to the dihydroxy docosatrienes (Fig. 8*A*). Once produced, these dihydroxy-containing docosatrienes exert their actions, in the present studies preventing leukocyte infiltration and down-regulating the production of inflammatory cytokines.

The lipoxygenase product 17*S*-H(p)DHA is converted to 16(17)-epoxide, which, if generated enzymatically, is likely to be a *trans* epoxide by analogy with the formation of leukotriene A_4 (48, 51, 52). If 10,17-docosatriene is generated via non-enzymatic aqueous hydrolysis of the 16(17)-epoxide, the products would be likely to yield the position 10 racemates (10*R/S*) with all-*trans* double-bond configurations for the resulting conjugations. We have observed, in human cells and whole blood, production of only one dominant 10,17-dihydroxy product, indicating the biosynthesis and most bioactive product is likely to be enzymatically formed *in situ*. This enzymatic hydrolysis could proceed via a mechanism analogous to the leukotriene A_4 hydrolase. Its double-bond geometry would likely be 10*R*-hydroxy-Δ11*E*,13*E*,15Z as depicted in Fig. 8 (48, 51, 52). Although 10,17*S*-docosatriene is generated endogenously and carries potent bioactivity, the chirality of the 10 position alcohol remains to be established. Nevertheless, once these dihydroxy products are formed and impart their bioactions, they can undergo ω-oxidation at sites of inflammation and within exudates. They can then undergo ω-oxidation at the carbon 22 position (see Fig. 8 and "Results"). This type of ω-oxidation route is usually associated with rapid inactivation of bioactive eicosanoid mediators (for example, see Refs. 43 and 53). These are also novel trihydroxy docosanoids and implicate that the DHAs carry bioinformation. This was further substantiated with the results obtained from *in vivo* experiments indicating that 10,17*S*-docosatriene is a potent regulator of peritonitis-activated leukocyte recruitment.

The major bioactive product generated from endogenous

DHA released by glial cells focused our attention on the formation and actions of 10,17*S*-docosatriene (see Figs. 3, 4, and 6 for bioactions). Our results obtained with glial cells, murine brain, whole blood, and human leukocytes indicate that the biosynthesis of the docosatrienes can occur in a single cell type and appears to involve a 15-lipoxygenase-like activity and/or related enzymes. Whether the newly uncovered isoforms of human 15-lipoxygenase A or B (see Ref. 54 for a recent perspective) or possibly other, yet to be discovered, novel enzymes are involved in this pathway remains to be established. The initial step to form monohydro(peroxy)-containing intermediates is not likely to occur via a classic p450 enzyme as the predominant pathway (55), because we found the production of the 16(17)-epoxide intermediate as direct evidence for formation of alcohol-trapping products, *i.e.* the markers of epoxides that arise from allylic epoxides (Fig. 8). From our present results, it appears that the major enzymatic route to formation in glial cells involves tightly controlled enzymatic transformations. These reactions appear to be akin to those used for eicosanoid biosynthesis and related enzymes but instead handle DHA. These novel pathways may also involve very specialized enzyme systems to produce in brain, neural systems, and cells of hematopoietic origin the docosanoids established in the present study, namely the docosatrienes and 17*S* series resolvins.

The results of the alcohol trapping and labeling experiments also support the scheme in Fig. 8*B* for the formation of 17*S* series resolvins. Once produced, 17-hydroperoxy-DHA can via transcellular biosynthesis be converted by human neutrophils (see Fig. 8*B*) to two main intermediates following the insertion of the hydroperoxy at either the 4 or 7 position in their precursor. This is rapidly transformed to both 7,8-epoxide and 4,5-epoxide intermediates as also observed with the recently elucidated 17*R* series resolvins produced when aspirin treatment is administered. These two pathways in leukocytes give rise to the dihydroxy- and trihydroxy-containing products termed 17*S* series resolvins (Fig. 8*B*). These are the main products derived from human leukocytes as determined here and are observed both in human whole blood and in inflammatory exudates (see Ref. 7). The 17*S* series compounds appear to be equally potent as the 17*R* series compounds *in vivo* as shown in Fig. 6. This is analogous to the flexibility in stereochemistry noted earlier for the carbon 15 position of arachidonic acid as *R* or *S* observed in lipoxin and aspirin-triggered 15-epi-lipoxin formation and action where the chirality of the other two alcohol positions and double bond geometries are highly critical to maintain bioactions (7). Also, there appear to be one or more specific ligand receptor systems responsible for activating glial cell receptors as indicated by the results with microphysiometry analyses (see Fig. 6*C*), a finding that is in line with other lipid mediators derived from arachidonic acid, namely that there are specific receptors that transduce their wide range of actions (see Refs. 43 and 56). The receptor system(s) and cell types identified in the present experiments with DHA and glial cells as well as formation and action of 10,17-docosatriene provide opportunities to uncover novel receptors signaling used by the docosatrienes and resolvins to evoke their actions *in vivo*.

Results from earlier studies demonstrated that DHA is released from membrane-rich sources in retina and synaptic terminals (57, 58) and underscores an important role for this essential fatty acid in neuronal development (20) and function (59). Importantly, the molecular mechanism(s) for these reported DHA actions remained of interest to explain precise actions in many organ systems. Our present results establish that glial cells can release and transform DHA to novel docosatrienes. In addition, during multicellular events and cell-cell interactions, for example, leukocytes can take up 17-HDHA and convert it to resolvins. These results underscore the importance of cell-cell interaction in generating products that neither cell type can generate alone. This theme in multicellular responses appears to be a key mechanism in the generation of a diverse array of bioactive oxygenated lipid mediators. Because fish are rich in DHA and are able to produce essentially equal amounts of 4 and 5 series eicosanoids (49) from arachidonic acid and EPA, it is possible that DHA transformation to both docosatrienes and 17*S* series resolvins is a conserved primordial signaling pathway in certain human tissues. Hence, results from the present experiments establish the enzymatic transformation pathways for the production of docosatrienes and 17*S* series resolvins and document their actions in models *in vivo*. These new pathways and structures could represent the active components responsible for some of the beneficial actions reported with dietary supplementation of DHA. Moreover, these results indicate that DHA is a precursor for two novel families of bioactive mediators, namely docosatrienes and 17*S* series resolvins.

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Appendix Q

Tou et al., "Krill for Human Consumption: Nutritional Value and Potential Health Benefits" Nutr Rev 65(2): 63-77 (2007)

Special Article

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Krill for Human Consumption: Nutritional Value and Potential Health Benefits

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The marine crustacean krill (order Euphausiacea) has not been a traditional food in the human diet. Public acceptance of krill for human consumption will depend partly on its nutritive value. The aim of this article is to assess the nutritive value and potential health benefits of krill, an abundant food source with high nutritional value and a variety of compounds relevant to human health. Krill is a rich source of high-quality protein, with the advantage over other animal proteins of being low in fat and a rich source of omega-3 fatty acids. Antioxidant levels in krill are higher than in fish, suggesting benefits against oxidative damage. Finally, the waste generated by the processing of krill into edible products can be developed into value-added products.

Key words: human health, krill, nutrition

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INTRODUCTION

Human consumption of fish-derived food products has been increasing steadily, however, the global capture of fish has remained fairly stable for the past 20 years and has been forecasted as unlikely to increase in the future.[1] Reports of health benefits has contributed to the rise in seafood consumption. For example, the American Heart Association recommends eating fish at least twice a week as part of their guidelines for reducing heart disease.[2] One way to minimize the gap between the steadily increasing consumption and dwindling resources of fish is to identify new sources that may be utilized for human consumption. Krill is one such resource.

The Norwegian word "krill" translates into "young fry of fish" and has been adopted as the term used to describe marine crustaceans belonging to the order Euphausiacea. Krill is widely known as whale food, but is also a source of food for seals, squid, fish, seabirds, and, to a much lesser degree, humans. In appearance, krill resembles shrimp (Figure 1).[3] Similar to other crustaceans, krill possess a chitinous exoskeleton but are distinguishable from other crustaceans by the presence of visible external gills, luminous organs, and a cephalothorax content consisting of extremely active proteolytic enzymes. During harvest, these proteolytic enzymes are released, resulting in krill meat being rapidly liquefied.[4] A more detailed discussion of the biology of krill is available elsewhere.[5]

Krill range in size from 0.01 to 2 g wet weight and from 8 mm to 6 cm length.[6] Despite their small size, krill are capable of forming large surface swarms that may reach densities of over 1 million animals per cubic meter of seawater,[7] making them an attractive species for harvesting. In addition, krill are found in oceans worldwide, making them among the most populous animal species. Despite this abundance, the commercial harvest of krill has mainly focused on its use as feed in aquariums, aquaculture, and sport fishing.[8] Of the different species of krill, only Antarctic krill (*Euphausia superba*) and Pacific krill (*Euphausia pacifica*) have been harvested to any significant degree for human consumption. The underutilization and abundance of krill make it an untapped food source for humans that, when coupled with a conscientious ecosystem approach to managing krill stocks, should result in its long-term sustainability.

Commercial krill products currently available for human consumption consist mainly of frozen raw krill, frozen boiled krill, and peeled krill meat.[9] However, interest in krill as a food source for human consumption is expected to increase with the emergence of technological advances and new product development. Still, widespread acceptance of krill as part of the human diet will

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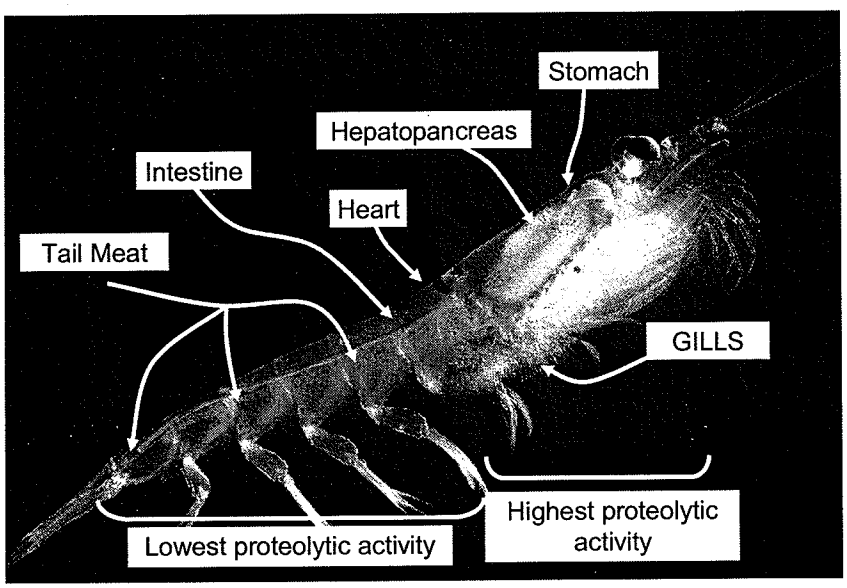

Figure 1. Photograph showing krill body structure. (Adapted with permission from Torres et al.[3])

depend on the consumer's perception of it as a nutritious as well as "healthy" food. Therefore, the aims of this article are to evaluate the nutritional value of krill as a food for human consumption and to review the scientific evidence regarding its health benefits.

NUTRIENT COMPOSITION OF KRILL

Seafood is low in calories compared with other animal foods. For example, a 100-g serving of shrimp provides approximately 106 kcal, whereas the same amount of fish provides 110 to 150 kcal, lean beef 250 kcal, and roasted chicken 200 kcal.[10] Proximate analysis of whole krill shows a range of 77.9% to 83.1% for moisture, 0.5% to 3.6% for total lipids, 11.9% to 15.4% for crude protein, 3% for ash, and 2% for chitin and glucides.[11] To assess the food value of krill, the nutrient composition of krill meat was compared with other seafoods in the human diet. Shrimp was selected for comparison because it is a crustacean familiar to the human diet; fish was also selected because it is widely regarded to be a "healthy" food.

As shown in Figure 2, the nutrient composition of krill closely resembles that of shrimp. Total protein and ash content of krill are comparable to fish, but its total lipid content is lower than fatty and lean fish species. Overall, krill resembles other seafood in being low in fat and a good source of protein. Based on proximate analysis, krill offers an attractive food addition to the human diet. However, proximate analysis does not provide information regarding the type of fat or the quality of the protein provided by krill. The next sections of the article assess individual components of krill for nutritive value and potential health benefits.

KRILL OIL

Current dietary recommendations suggest reducing fat consumption because high-fat diets have been implicated in weight gain and in increased risk of various diseases, most notably cardiovascular disease (CVD). In addition to the amount of fat, the type of fat also has an important impact on health. Foods high in saturated fatty acids (SFAs) have been linked to increased risk of CVD, whereas the omega-3 polyunsaturated fatty acids (ω-3 PUFAs), particularly eicosapentanoic acids (EPA, 20:5ω-3) and decosahexanoic acid (DHA, 22:6ω-3), have been linked to reduced risk of CVD.[12] Thus, the nutritive value of krill oil was evaluated due to the consumer's desire for foods that are low in fat and SFAs and high in ω-3 PUFAs.

Nutritional Value

Saether et al.[13] analyzed the lipid content of three species of krill and reported values ranging from 12% to 50% on a dry-weight basis. The wide range in lipid content was attributed to the sampling occurring during different seasons. A reduction in lipid content occurred in the spring, when food was scarce, whereas it increased in the autumn and early winter, when food was abundant. Kolakowska[14] reported that the lack of reproductive activity in the winter raised the lipid content of female krill to over 8% of their wet weight. Thus, the lipid

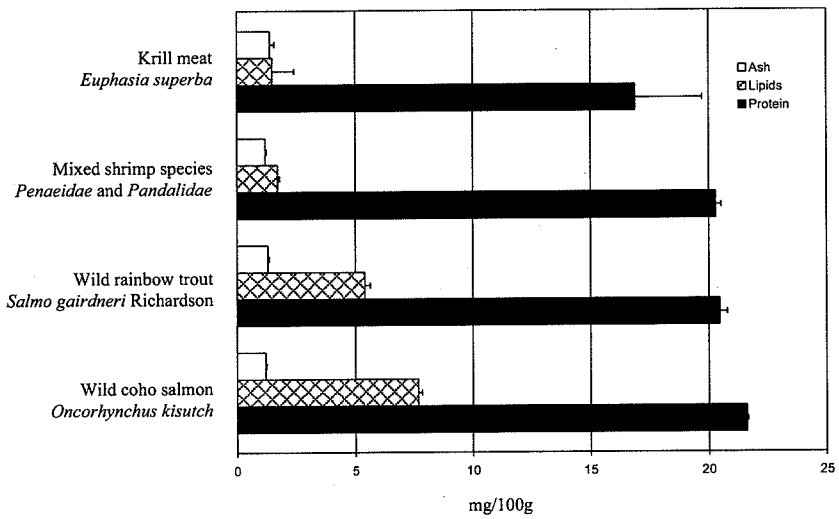

Figure 2. Proximate analysis of krill, shrimp, and lean (trout) and fatty (salmon) fish. Values for krill are based on Suzuki and Shibata[9]; values for shrimp, trout, and salmon are based on based on USDA.[10]

content and profile of krill may vary considerably depending on factors such as season, species, age, and the lag time between capture and freezing. These factors must be taken into consideration to ensure the consistency of krill oil. Regardless of this variability, krill is similar to other seafood in being low in fat compared with other animal foods. The lipid content of krill meat is 1.5%,[9] compared with approximately 26% for lean beef, 3.6% for chicken, 5.9% for fatty fish, and 3.5% for lean fish on a wet-weight basis.[10]

The lipid content in krill was analyzed for fatty acid composition. Table 1 shows that krill provides both of the essential fatty acids: α-linolenic acid (ALA, 18:3 ω-3) and linoleic acid (LA, 18:2 ω-6). In addition, krill is low (26.1%) in both SFAs and (24.2%) monounsaturated (MUFAs) but high (48.5%) in PUFAs. Palmitic acid (16:0) is the predominant SFA, oleic acid (18:1ω-9) is the predominant MUFA, and the PUFAs consist mainly of ω-3 fatty acids. Kolakowska et al.[15] reported that ω-3 PUFAs accounted for approximately 19% of total fatty

Table 1. Lipid Content and Fatty Acid Composition of Krill, Shrimp, and Lean (Trout) and Fatty (Salmon) Fish*

Lipid	Krill	Shrimp	Trout	Salmon
Total lipids (g/100 g)	1.50	1.73	3.46	5.93
SFA (%)	26.1	19.0	20.9	21.1
14:0	4.9	1.2	2.3	4.5
16:0	18.8	10.6	12.2	12.7
18:0	1.0	6.0	4.0	3.5
MUFA (%)	24.2	14.6	32.6	36.0
16:1 (ω-7)	4.9	4.8	5.9	8.5
18:1 (ω-9)	16.4	8.5	17.7	20.3
PUFA (%)	48.5	38.7	35.8	33.6
18:2 (ω-6)	3.3	1.6	6.9	6.5
18:3 (ω-3)	1.1	0.8	3.4	2.6
20:4 (ω-6)	0.5	5.0	3.2	2.2
20:5 (ω-3) EPA	17.4	14.9	4.8	7.2
22:6 (ω-3) DHA	12.4	12.8	12.1	11.1
Cholesterol (mg/100 g)	66.1	152	59	45

* Whole krill (*Euphausia superba*), mixed shrimp species (*Penaeidae* and *Pandalidae*), wild rainbow trout (*Salmo gairdneri* Richardson), and wild Coho salmon (*Oncorhynchus kisutch*). Values for krill are based on Suzuki and Shibata[9]; values for the other species are based on the USDA.[10]

DHA, decosahexanoic acid; EPA, eicosapentanoic acid; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

acids in Antarctic krill caught during the winter. Of the ω-3 PUFAs, EPA and DHA were particularly abundant. This was not surprising given that krill feed on marine phytoplankton such as single-cell microalgae, which synthesize large amounts of EPA and DHA.

The fatty acid composition of krill meat was compared with that of shrimp, rainbow trout (a lean fish), and coho salmon (a fatty fish). As shown in Table 1, the DHA content of krill is similar to that of shrimp and fish, but its EPA content is higher than either lean or fatty fish. Overall, the fatty acid profile of krill resembles that of shrimp and fish. However, most of the fatty acids in fish are incorporated into triglycerides, whereas 65% of the fatty acids in crustaceans are incorporated into phospholipids.[16] Arai et al.[17] reported that phospholipids comprise 29.9% of the lipid content of krill, whereas Bottino[18] reported even higher levels of 54% to 58%. The variations in krill phospholipid content in these studies may be due to differences in krill species, age, season, or harvest time.

Another lipid class of interest is cholesterol. Consumer perception of krill as a food high in cholesterol may reduce its acceptance in the human diet. The cholesterol level in krill is higher than that of fish but lower than that of shrimp (Table 1), and ranges from 62.1 to 71.6 mg/100 g in tissue and from 17 to 76.3 mg/g in krill oil.[19] However, it should be noted that two-thirds of the sterols in shellfish are non-cholesterol sterols.[20] The non-cholesterol sterols in shellfish have been reported to interfere with cholesterol absorption.[21] Rats fed krill lipids for 3 weeks[22] or 2 months[17] failed to show an increase in liver or blood cholesterol. Any hypocholesterolemic effects associated with the cholesterol content of krill may be negated by its non-cholesterol sterols, low fat, low SFA, and high ω-3 PUFA content. More studies on the effects of non-cholesterol sterols and ω-3 PUFA-rich phospholipids derived from krill on CVD risk factors are needed before any definitive statements can be made regarding the impact of krill on heart disease. Consumer interest in reducing heart disease through dietary modification makes addressing the role of krill on CVD risk an important issue in its acceptance for human consumption.

Cardiovascular Health Benefits

Although krill oil is being advertised as a supplement with protective effects against heart disease, few published studies exist. Shagaeva et al.[23] reported that feeding krill meat to patients with type 1 diabetes reduced their incidence of atherosclerosis. However, we were unable to critically review this article because it is written in Russian. In a recent study, the effect of krill oil on hyperlipidemia was investigated.[24] The study design was a double-blind trial comprised of 120 male and female subjects (mean age of 51 ± 9.5 years) diagnosed with mild to high blood cholesterol and triglycerides. Subjects were randomly assigned to one of the following treatment groups: 1) low-dose krill oil: 1 g/d if BMI was under 30 kg/m^2 and 1.5 g/d if BMI was over 30 kg/m^2; 2) high-dose krill oil: 2 g/d if BMI was under 30 kg/m^2 and 3 g/d if BMI was over 30 kg/m^2; 3) 3 g/d of fish oil containing 180 mg EPA and 120 mg DHA; or 4) placebo containing microcrystalline cellulose. Assigned treatments were given daily for 12 weeks. The primary end points measured were total cholesterol, triglycerides, low-density lipoproteins (LDLs), and high-density lipoproteins (HDLs) at baseline and at 90 d.

The results on blood lipids indicated that placebo, fish oil, and low-dose (1.0–1.5 g/d) krill oil had no significant effect on triglycerides, whereas high-dose (2–3 g/d) krill oil significantly ($P < 0.05$) reduced triglycerides by 27% to 28% (Figure 3A). While the results of this study showed the absence of a significant effect of fish oil on triglycerides, others have reported that a similar dose of 3 g/d fish oil lowered triglycerides by 30%.[25] Total cholesterol was elevated in subjects taking a placebo, while both fish oil and krill oil treatment reduced total cholesterol ($P < 0.05$; Figure 3A). Krill oil induced higher reductions in cholesterol than fish oil, with the low-dose krill oil decreasing total cholesterol by of 13% to 14% and the high-dose krill oil by 18%.

The results on circulating lipoproteins indicated increased ($P < 0.05$) LDLs in subjects taking a placebo. Fish oil had no significant effect on LDLs, whereas krill oil significantly reduced LDLs. Low-dose krill oil reduced LDLs by 32% to 36%, and high-dose krill oil by 37 to 39% (Figure 3B). In addition, krill oil significantly increased HDLs compared with fish oil. Low-dose krill oil increased HDLs by 43% to 44%, and high-dose krill oil by 55% to 60% (Figure 3B). Based on these findings, Bunea et al.[24] concluded that krill oil was more effective at improving blood lipids and lipoproteins than fish oil. Smith and Sahyoun[26] reviewed the evidence regarding fish oil consumption on CVD and concluded that fish oil produced only modest changes on lipoproteins. In some cases, high doses (3 g/d) of fish oil have been observed to raise LDLs.[27] Bunea et al.[24] attributed the greater lipogenic action of krill oil to the ω-3 PUFAs in krill being associated with phospholipids; the ω-PUFAs in fish are mainly associated with triglycerides. Future research should compare the lipogenic effects of providing ω-3 PUFAs as phospholipids or triglycerides.

Limitations of the Bunea et al.[24] study include the small subject size and the absence of adjustments for

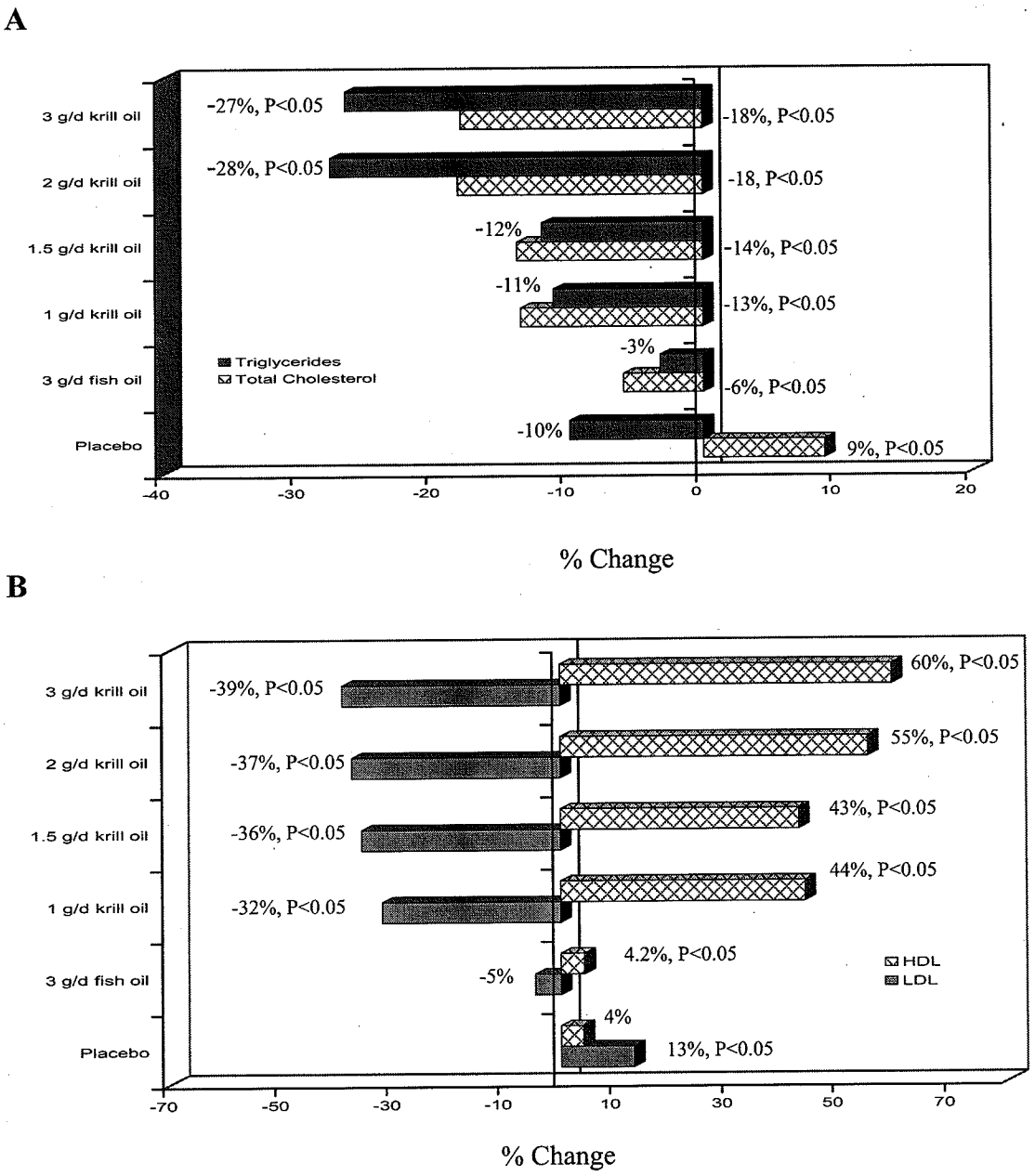

Figure 3. The effect of krill oil, fish oil, or placebo on blood lipids and lipoproteins. Adapted with permission from Bunea et al.[24] $P < 0.05$ indicates a significant change from baseline to day 90 of treatment.

other factors such as diet, smoking, gender, and genetics, all of which influence blood lipids and lipoproteins. Furthermore, the anti-atherosclerotic effects of EPA and DHA in fish oil have been attributed to mechanisms other than lipogenic actions. Fish oil has been reported to reduce CVD risk through diverse mechanisms of reducing blood pressure, inflammation, arrhythmia, and atherosclerotic plaque growth, as well as by promotion of endothelial function, anti-thrombosis, and the improvement of insulin sensitivity.[28] Whether ω-3 PUFAs provided from food sources other than fish oil exert similar effects remains to be determined.

Other Possible Health Benefits

The major focus on ω-3 PUFAs has been their effects on CVD. However, ω-3 PUFA research has expanded into other health issues such as neurological

function, retinal and brain development, cancer, arthritis, immunological conditions, diabetes, kidney disease, and skin disorders.[29] Thus, there are tremendous opportunities for research regarding the potential health effects of krill. Recently, a role of krill oil in the management of premenstrual syndrome was investigated.[30] The study design was a double-blind trial comprised of 70 women of reproductive age who met the diagnostic criteria for PMS. Subjects were randomly assigned to take two gel capsules containing 1 g of krill oil or 1 g of fish oil (18% EPA and 12% DHA) daily at mealtime for a duration of 3 months. At 45 and 90 d of treatment, the subjects completed a self-assessment questionnaire on symptoms associated with PMS and also recorded all analgesics consumed to alleviate menstrual-related pain.

The results showed that women taking krill oil consumed fewer pain relievers and reported fewer PMS symptoms of breast tenderness, joint pain, swelling, and bloating compared with women receiving fish oil. The decrease in physical symptoms of PMS was attributed to the ω-3 PUFAs in krill oil reducing the production of pro-inflammatory 2-series prostaglandins and increasing the production of anti-inflammatory 3-series prostaglandins.[30] Women taking krill oil also reported fewer emotional symptoms associated with PMS, such as feelings of being overwhelmed, irritability, stress, and depression. Alleviation of emotional symptoms of PMS by krill oil has been attributed to the influence of DHA on brain function. Based on these findings, Sampalis et al.[30] concluded that krill oil was more effective than fish oil at improving both the physical and emotional symptoms of PMS. Several limitations of the Sampalis et al.[30] study are the small subject size, no measurements of blood 2- and 3-series eicosanoid concentrations, and problems of validity and reliability inherent to self-assessment questionnaires. Thus, more studies are needed before any definitive statement regarding the effect of krill oil on PMS can be made.

Although the consumption of foods high in ω-3 PUFAs are recommended for health benefits, there are safety issues to consider. Foods rich in PUFAs are highly susceptible to lipid peroxidation, which results in oxidative products that cause deterioration of food quality and upon ingestion may cause cellular damage. Increased oxidation has been associated with increased risk of CVD, cancer, and cataracts, and also accelerates aging. Despite its high PUFA content, krill oil is considered relatively resistant to oxidation. The stability of krill oil has been attributed to its antioxidant content.[9] The next section of the article assesses the antioxidant and vitamin content of krill.

KRILL VITAMIN CONTENT

Antioxidants neutralize free radicals and protect against cellular damage. Table 2[32] shows the level of selected antioxidant vitamins in krill, shrimp, and fish, along with the Recommended Dietary Allowances (RDAs) of these vitamins for adults. The level of vitamin A in krill, shrimp, and fish is below the RDA for adults. However, the vitamin A content in krill is greater than that in shrimp, trout, or salmon. Kinumaki[31] reported a wide range of 197 to 1446 IU/100 g for the vitamin A content in raw or boiled krill. Krill also contains provitamin A. The principal carotenoid in krill is astaxanthin. The reported amount of 1.5 to 2.0 mg astaxanthin in 100 g of krill tissue indicates that krill is a rich source of astaxanthin.[8]

Astaxanthin has been used as a colorant added to fish feed to give farmed salmon their pink color. The discovery of astaxanthin's powerful antioxidant properties has led to research on its potential health benefits. In

Table 2. Levels of Selected Fat-Soluble and Water-Soluble Vitamins in Krill, Shrimp, and Lean (Trout) and Fatty (Salmon) Fish*, Along With the RDA for Adults†

Vitamin	Krill	Shrimp	Trout	Salmon	RDA
Fat-Soluble Antioxidants					
Vitamin A (IU/100 g)	380	180	62	100	3000 IU/d
Vitamin E (mg/100 g)	15	1.10	ND	0.65	15 mg/d
Water-Soluble B-Complex					
Thiamin (mg/100 g)	ND	0.028	0.123	0.113	1.2–1.2 mg/d
Riboflavin (mg/100 g)	0.002	0.034	0.105	0.140	1.2–1.3 mg/d
Niacin (mg/100 g)	0.07	2.552	5.384	7.23	14–16 mg/d
Vitamin B_6 (mg/100 g)	0.001	0.140	0.406	0.549	1.3–1.7 mg/d
Folate (µg/100 g)	60	3	12	9	400 µg/d
Vitamin B_{12} (µg/100 g)	16	1.16	4.45	4.17	2.4 µg/d

* Whole krill (*Euphausia superba*), mixed shrimp species (*Penaeidae* and *Pandalidae*), wild rainbow trout (*Salmo gairdneri* Richardson), and wild Coho salmon (*Oncorhynchus kisutch*). Values for krill are based on Suzuki and Shibata[9]; values for the other species are based on the USDA.[10]
† RDA values based on the Dietary Reference Intakes for Individuals.[32]
ND, Not determined.

a recent review article,[33] health benefits attributed to astaxanthin included reduced risk of cataracts, diabetes, heart disease, neural deterioration, and certain cancers; however, most of these studies were conducted using in vitro or animal models. In a human trial, young adult male volunteers provided astaxanthin in a daily beverage for 14 d showed protection against premature LDL oxidation in the absence of any other dietary changes.[34] Despite the promising results for astaxanthin, research is needed regarding the health benefits of astaxanthin derived from krill.

One of the most important antioxidants is vitamin E, which functions specifically to protect against lipid peroxidation in biological membranes. Due to methodological difficulties, vitamin E has not been determined extensively in foods. According to Table 2, krill meets the RDA for vitamin E and contains higher amounts of vitamin E than either shrimp or fish. Thus, the stability of krill may be attributed to its high levels of vitamin E. However, other components may also protect krill from oxidation. Dunlap et al.[35] identified a marine-derived tocopherol with enhanced antioxidant effects on cellular lipids. Venkatraman et al.[36] reported that mice fed a diet containing 10% krill oil had higher liver expression of endogenous antioxidant enzymes (i.e., catalase, glutathione peroxidase, and superoxide dismutase) and lower peroxide and thiobarbituric acid values compared with mice fed a 10% corn oil diet. The high content of vitamin E and the presence of other antioxidant components suggest that krill may have beneficial effects against oxidative damage.

The water-soluble vitamins play an important role in maintaining health by acting as coenzymes in energy metabolism. Table 2 shows the levels of selected water-soluble vitamins in krill, shrimp, and fish, along with the RDA for these vitamins in adults. The levels of thiamin, riboflavin, and niacin are higher in fish compared with shellfish (i.e., krill and shrimp), but are below the RDA for adults, indicating that in general seafood is a poor source of these vitamins. Table 2 also shows the levels of the B-complex vitamins B_{12}, B_6, and folate in krill, shrimp, and fish. These vitamins play a role in the metabolism of homocysteine, so deficiencies can lead to high blood homocysteine. This has important implications because a high blood level of homocysteine is an independent predictor of heart disease and stroke.[37] The vitamin B_6 content of most seafood is below the RDA, and levels in krill are below that of either shrimp or fish. Although krill is higher in folate than shrimp and fish, levels are still below the RDA. On the other hand, vitamin B_{12} in krill (16 μg/100 g) is substantially higher than in shrimp (1.16 μg/100g) or fish (4.17 to 4.45 μg/100g) and exceeds the RDA of 2.4 μg/d for adults. The assessment of the nutritive value of krill based on vitamin content indicates that it has considerable appeal for human consumption because it provides a good source of vitamin B_{12}, vitamin E, and astaxanthin, as well as other potential antioxidant compounds.

KRILL PROTEIN QUALITY

Whole Krill

Krill is a high-protein food, having a protein content estimated in the range of 60% to 65% dry weight.[8] Similar to other animal foods, the protein derived from krill is a complete protein, as indicated by the presence of all nine of the indispensable amino acids required by adults. As shown in Table 3,[38] the level of all of the indispensable amino acids in whole krill met the FAO/WHO/UNU amino acid requirement for adults[39] (with the exception of histidine). In contrast, several indispensable amino acids in fish were below the amino acid requirement for adults, most notably methionine and cysteine. However, it should be noted that the indispensable amino acid levels in whole krill were below the reference protein, egg. Tamura[40] reported an amino acid score in the range of 0.85 to 1.00, indicating that the amount of indispensable amino acid provided by krill protein met the protein requirement of adults. Although considered a high-quality protein, krill is of lower quality than whole egg, which has an amino acid score of 1.21.

In practice, protein quality is determined not only by the amino acid composition but also by its digestibility. Iwantani et al.[41] fed rats a diet containing either egg protein or defatted, freeze-dried whole krill for 4 weeks, and then determined the weight gain of the animals to calculate the protein efficiency ratio. Biological value and net protein utilization were also measured to assess protein availability and digestibility. Rats fed whole krill gained less weight, had a reduced protein efficiency ratio, biological value, and net protein utilization compared with animals fed egg protein. The decreased digestibility of krill protein may have been due to the presence of the exoskeleton. Obatake[22] determined the nutritive value of the protein in krill meat after removal of the exoskeleton, and found that it was still inferior to that of egg protein. Findings in the animal studies were not always indicative of the findings in humans. In a human trial, Tamura[42] fed boiled krill or whole egg to adult men for 21 days and reported a net protein utilization of 55% for krill and 61% for whole egg, with no differences in their digestibility. Based on the evidence, krill appears to be a good source of high-quality protein. The large biomass and high-quality protein offered by krill provides an economical replacement for commer-

Table 3. Amino Acid Composition of Krill and Lean (Trout) and Fatty (Salmon) Fish* Compared With Egg, Along With the Amino Acid Requirement for Adults†

Amino Acid	Krill	Trout	Salmon	Egg	Requirement
	mg/g				*mg/g protein*
Isoleucine	25	9.4	10.0	63	13
Leucine	40	16.6	17.6	88	19
Lysine	44	18.8	19.9	70	16
Methionine + cysteine	24	8.3	8.7	56	17
Phenylalanine + tyrosine	50	14.9	15.7	98	19
Threonine	22	9	9.5	49	9
Tryptophan	7	2.3	2.4	16	5
Valine	26	10.5	11.1	72	13
Histidine	11	6	6.4	24	16
Arginine	38	12.3	12.9	28.4	ND
Alanine	29	12.4	13.1	26.4	ND
Aspartic acid	53	21	22.1	47.6	ND
Glutamine	67	30.6	32.3	61.9	ND
Glycine	34	9.8	10.4	15.9	ND
Proline	23	7.2	7.6	18.9	ND
Serine	19	8.4	8.8	35.2	ND

* Whole krill (*Euphausia superba*), wild rainbow trout (*Salmo gairdneri* Richardson), and wild Coho salmon (*Oncorhynchus kisutch*). Values for krill are based on Torres et al.[3]; values for trout, salmon, and egg are based on the USDA[10]; values for egg are also based on Young and Pellett[38]; requirement for adults based on FAO/WHO/UNU.[39]
ND, Not determined.

cially available protein sources. In addition, commercial protein sources available for human consumption such as casein, whey, soy protein, lactoalbumin, and wheat gluten have various limitations. For example, casein is low in sulfur amino acids, particularly cysteine, which must be added to the diet. Products in which krill protein may substitute for other proteins are nutritional supplements, sports drinks, infant formulas, and milk replacers. For these products, protein concentrates rather than whole krill or krill meat are used.

Krill Protein Concentrate

Protein concentrates are produced by technologies that concentrate the proteins in food so that levels are higher than those in the original food, making them an inexpensive source of available protein. Preparation of protein concentrate from fish is accomplished by extracting the lipids, removing the bones, and drying, so that the resultant product is 85% to 95% higher in protein than the original fish. Lack of proper technology for protein recovery from krill has hindered progress in the commercial development of krill protein concentrates. Some research has been carried out that made use of krill's high content of proteolytic enzymes to produce a product with protein recovery as high as 80%.[43] There is technology available for isolating muscle protein in a continuous mode from whole krill to produce krill protein concentrate. The continuous protein recovery technology is based on a principle of isoelectric solubilization followed by isolelectric precipitation of the protein. This basic biochemical principle has long been used in the dairy industry to manufacture cottage cheese, and also in other food processing industries such as those for soy protein isolates and concentrates. More recently, the principle of isoelectric point has been applied to fish muscle[44] and other animal muscle.[45] The protein recovery (dry basis) yield from krill using isoelectric solubilization/precipitation is approximately 90% or greater in a continuous mode. A more in-depth discussion of the isoelectrical solubilization recovery of krill is available elsewhere.[3]

Table 4[46] shows the amino acid composition of krill protein concentrate compared with that of other commercially available protein concentrates such as casein, whey, and soy protein. The indispensable amino acid profiles of the protein concentrates were compared with the indispensable amino acid requirements for adults and infants because protein concentrates are frequently used in infant formulas. As shown in Table 4, krill protein concentrate contains all indispensable amino acids in amounts that exceed the requirements for healthy adults. Krill protein concentrate also contains all of the indispensable amino acids in amounts that meet the requirements for infants (although leucine, tryptophan, and histidine levels were slightly below). Milk-

Table 4. Amino Acid Composition of Krill (*Euphausia superba*) Protein Concentrate, Whey Protein Concentrate, Sodium Caseinate, and Soy Protein Concentrate Compared with the Amino Acid Requirements for Infants and Adults*

Amino Acid	Krill	Whey	Sodium Caseinate	Soy	Infant Requirement	Adult Requirement
	mg/g				*mg/g protein*	
Indispensible Amino Acids						
Isoleucine	56	49.7	45.9	29.4	46	13
Leucine	88	106.6	88.9	49.2	93	19
Lysine	84	88.1	77.5	39.3	66	16
Methionine + cysteine	44	79.7	32	17	42	17
Phenylalanine + tyrosine	96	58.2	101.4	55.8	72	19
Threonine	46	68.7	40.5	23	43	9
Tryptophan	16	17.3	10.4	8.3	17	5
Valine	56	18.4	56.4	30.5	55	13
Histidine	25	7.8	25	15.8	26	16
Dispensible Amino Acids						
Arginine	54	27.1	33.5	46.4	ND	ND
Alanine	62	55.5	27.5	26.8	ND	ND
Aspartic acid	53	91.8	75.7	72.5	ND	ND
Glutamine	67	158.4	218.4	120.1	ND	ND
Glycine	34	53.2	17.3	26.9	ND	ND
Proline	23	66.6	93.3	33	ND	ND
Serine	19	53	55.4	33.7	ND	ND

* Values for krill are based on Torres et al.[3]; values for whey and soy protein concentrate are based on USDA[10]; values for sodium caseinate are based on Sindayikengera and Xia[46]; requirements for infants and adults are based on FAO/WHO/UNU[39].
ND, Not determined.

derived whey protein concentrate, sodium caseinate, and soy protein concentrates are more deficient in the indispensable amino acids for infants than are krill protein concentrates.

The amount of indispensable amino acids in krill protein concentrate is higher than that in soy protein concentrate and similar to that of the milk protein casein. Sidhu et al.[29] reported that rats fed krill protein concentrate had similar weight gain and protein efficiency ratio as rats fed casein. Although higher than casein, krill protein concentrate is low in threonine, leucine, lysine, and the sulfur amino acids (methionine and cysteine) compared with the whey protein concentrate (Table 4). This has important health implications because sulfur amino acids are involved in DNA transcription and RNA translation and may play a role in reducing the risk of CVD, dementia, cirrhosis, and immunomodulation.[47] The health benefits and safety issues regarding the use of alternative protein sources need to be investigated in more detail.

The Health Benefits and Safety of Krill Protein

Adequate protein intakes are necessary for synthesis of structural components of the muscle and of enzymes, hormones, hemoglobin, and other body tissues. Sidhu et al.[29] reported that rats fed krill protein concentrate showed no difference in organ weights and hemoglobin counts than rats fed casein, indicating that krill protein is capable of supporting protein synthesis. Too much rather than too little protein is typical of the Western diet. Consuming protein beyond recommended amounts is common among athletes interested in increasing their muscle mass. The general population has also recently developed an interest in increasing their protein intakes due to suggestions that high-protein diets support weight loss, and there is some evidence that short-term consumption of high-protein diets increases satiety and thermogenesis and reduces energy intake.[48] However, despite growing interest in proteins, the long-term effects of high-protein diets on weight loss are unknown and there may be harmful effects associated with such diets. For example, diets high in protein have been suggested to increase the risk for cardiac, renal, bone, and liver disease.[49]

High-protein diets have also been suggested to increase the risk of CVD by inducing negative effects on blood lipid profiles. However, the negative effects on blood lipids are more likely due to the high SFA intakes associated with most high-protein diets. Krill, unlike

other animal sources of proteins, is low in SFAs. Obatake[22] reported no difference in serum cholesterol levels for rats fed boiled whole krill or krill meat compared with animals fed casein.

Another area of concern is the use of alternate protein sources in infant formulas. Soy protein has been reported to accelerate puberty in female rats compared with whey and casein.[50] Other concerns regarding alternative protein sources are their ability to support growth and their long-term health effects.[50] In addition, all food proteins have the potential to be allergenic to some people. Approximately 1% to 2% of adults and up to 5% to 7% of children experience food allergies with symptoms ranging from a mild rash to life-threatening anaphylaxis.[51] The Food and Agricultural Organization of the United Nations includes crustaceans on its list of the eight most significant food allergens.[52] According to Wild and Lehrer,[53] shellfish is the number-one cause of food allergies in adult Americans. To our knowledge, no studies have examined whether krill protein or krill protein concentrate causes food allergies. Determining the protein allergens present in krill and the relative allergenic activity of krill compared with other major protein substitutes is important. For example, soy protein used to manage cow's milk allergies in infants has been reported to have less allergenic reactivity compared with milk proteins; however, concerns have been raised regarding the isoflavone content in soy infant formulas affecting neurobehavioral development, immune, endocrine, and thyroid function.[54] Krill protein concentrate, which has an indispensable amino acid profile superior to that of soy protein concentrate, may be a better protein substitute in infant formulas if its allergenic reactivity is found to be lower than that of other protein sources.

Finally, the high protein levels achievable with protein concentrates and isolates have been suggested to increase the risk of kidney damage, particularly in the immature kidneys of infants. Rats fed semi-purified diets containing soy protein or casein have been reported to have an increased incidence of nephrocalcinosis.[55] Nephrocalcinosis may be caused by a number of conditions, including excretion of excess calcium by the kidneys. Increased calcium excretion (calciuria) by the kidneys due to high-protein diets has been attributed to the sulfur amino acids increasing the acid load in the body, followed by the release of calcium from the tissues to restore acid-base balance.[56] Calcium released from the bone to restore acid-base balance has led to suggestions that high-protein diets may contribute to bone loss. In addition, rats fed semi-purified diets containing soy protein have been reported to have reduced mineral absorption.[55] The calciuria and reduced mineral absorption associated with high-protein diets may contribute to negative mineral balance, leading to bone loss and increasing the future risk of osteoporosis. Furthermore, minerals may be lost during the processing of whole foods into protein concentrates. The next section assesses the mineral content of whole krill and krill following processing into edible products.

KRILL MINERAL CONTENT

The mineral content of shellfish is to a large extent located in the exoskeleton, which is removed to produce krill meat and krill protein concentrate. Figure 4A compares the level of selected minerals in whole krill, krill meat, and krill protein concentrate, along with the RDA of these minerals for adults. In whole krill, the content of major minerals involved in bone health (calcium, phosphorus, and magnesium) met the RDA. Following removal of the exoskeleton to produce krill meat, calcium was reduced by 84.4%, phosphorus by 47.3%, and there were minute effects on magnesium. As a result, krill meat was below the RDA for calcium and phosphorus (Figure 4A). Processing of whole krill into krill protein concentrate reduced calcium by 78.6%, phosphorus by 14.4%, and magnesium by 83.5%. The phosphorus content in krill protein concentrate met the RDA, but calcium and magnesium were both below the RDA (Figure 4A).

Another important dietary mineral is iron. Identifying good food sources of iron is important because iron deficiency is the most common nutritional disorder worldwide.[57] Anemia due to iron deficiency has been associated with significantly lower scores in cognitive and educational achievement tests in school-age children[57] and lower work productivity in adults.[58] Removal of the exoskeleton to obtain krill meat and processing to krill protein concentrate have minute effects on the level of iron in krill (Figure 4B). Still, the iron content of krill does not meet the RDA for adult women (18 mg/d) or the lower RDA for adult men (8 mg/d). King et al.[59] determined the mineral content of shellfish commonly marketed in the northwestern United States, and found that shellfish in general is low in iron, with the exceptions of oysters, mussels, and clams, which have levels comparable to red meat.

Most foods are low in fluoride, but krill is an exception. Fluoride is important for the mineralization of bone and teeth and in the prevention of dental caries. Furthermore, the use of high-dose fluoride is being investigated for the prevention of osteoporosis.[60] The fluoride content of krill is concentrated in the exoskeleton, where it may reach concentrations of 350 mg/100g dry weight.[61] At death, the fluoride in krill is capable of migrating from the exoskeleton into the soft tissues.

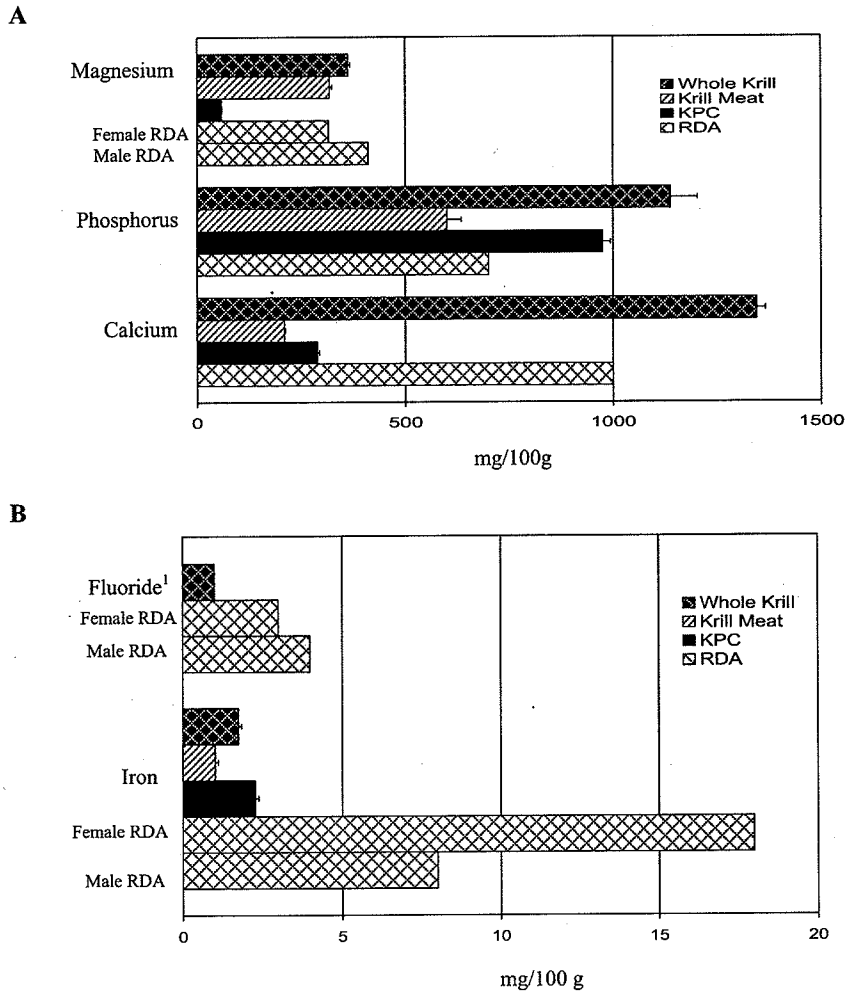

Figure 4. Mineral content of whole krill and krill following processing into meat and protein concentrate along with the recommended dietary allowance of these minerals for adults. (Fluoride value in krill meat is based on Virtue et al.[61])

Budzinski et al.[62] reported fluoride levels as high as 9 mg/100 g wet weight in krill meat. The fluoride derived from krill is also highly bioavailable. Tenuta-Filho and Alvarenga[63] reported that weanling male rats fed krill exoskeleton had 80% absorption of fluoride. The high fluoride content of krill, together with the high bioavailability of this fluoride, has led to concerns about the potential for toxicity. Careful removal of the exoskeleton before consuming krill and immediate removal of the shell upon harvest to prevent migration of fluoride into the muscle can minimize potential toxicity since over 99% of the fluoride content of krill is associated with the exoskeleton.[64]

Overall, the evaluation of the mineral content of krill indicated that it is a poor source of iron. Whole krill meets the RDA for minerals important for bone health; however, depending on the technology used to process krill into edible products, mineral losses may occur that result in mineral levels below the RDA. On the other hand, processing typically removes potentially toxic levels of fluoride. In the processing of shellfish, by-products represent approximately 50% to 70% of the weight of the raw material, much of which is the exoskeleton.[65] Whether the by-products generated by processing of krill into food may be developed into value-added products is another important consideration. In the next section, potential value-added products generated from krill are assessed.

VALUE-ADDED PRODUCTS

Chitin

Chitin is a polysaccharide comprised of units of N-acetyl-2 amino-2-deoxy-D-glucopyraose ($C_8H_{15}O_6N$)

bound by $\beta(1, 4)$ glycosidic links found in the exoskeleton of crustaceans, insects, and fungi.[66] Chitin and its derivative chitosan have a wide range of applications in the food, pharmaceutical, textile, agriculture, and cosmetic industries.[67] Crustacean by-products are the major source of commercially available chitin.[68] Figure 5 compares the amount of chitin in the exoskeleton of various crustaceans. Krill has a higher percentage of chitin than other more highly exploited shellfish such as crab and shrimp. The chitin composition of whole krill has been reported to be between 2.4% and 2.7% of their dry weight, making it an abundant source of chitin.[69]

Chitin is the raw material used for the production of chitosan, which has been promoted as a supplement for reducing body weight, hypercholesterolemia, and hypertension.[70] Chitosan differs from other weight-loss products because it does not claim to increase energy expenditures or induce satiety. Instead, orally administered chitosan binds to dietary fat and prevents it from being absorbed.[71] Increased fat excretion during chitosan administration has been demonstrated in animal studies.[72] However, the dosages of chitosan used in animal studies are 15 to 22 times higher than the recommended dose for humans. In a review by Mhurchu et al.,[73] the effectiveness of chitosan as a weight loss treatment in humans was examined. Evidence indicated that chitosan was more effective in short-term studies than placebo for promoting weight loss in overweight and obese subjects. However, the authors cautioned that many of the studies were of poor quality and recommended that more research be performed before any definitive conclusion regarding the role of chitosan as a weight loss agent be made.

Chitosan has also been suggested to play a role in CVD by binding negatively charged substances such as fatty acids and bile acid. This in turn reduces fat absorption and increases fecal sterol excretion, which lowers serum cholesterol. Chitosan has been demonstrated to produce significant hypocholesterolemic activity in different experimental animals,[67] but only a few studies have been conducted using human subjects. In a review by Ylitalo et al.,[74] dietary chitosan was found to have only modest effects on total cholesterol and LDL. Other health benefits attributed to chitosan are reduced systolic and diastolic blood pressure and anti-ulcer, anti-arthritic, and anti-uricemic properties.[67] Potential adverse effects of chitosan include gastrointestinal symptoms such as constipation, flatulence, and the risk of fat-soluble vitamin deficiency due to reduced fat absorption. Along with krill enzymes, chitin is one of the potentially lucrative by-products generated by the production of krill into edible products.

Krill Enzymes

The digestive glands of krill produce hydrolytic enzymes including proteases, carbohydrases, nucleases, and phospholipases.[75] These enzymes are released upon the demise of krill, resulting in rapid autolysis. Autolysis leads to spoilage and is a major hindrance to the harvest of krill. However, there is growing interest in the medical applications of krill enzymes, which have been studied for use in treating ulcers and promoting wound healing due to their effective debridement of necrotic tissue.[76-77] Melrose et al.[78] indicated that krill enzymes may be used as a chemonucleolytic agent to reduce the height of

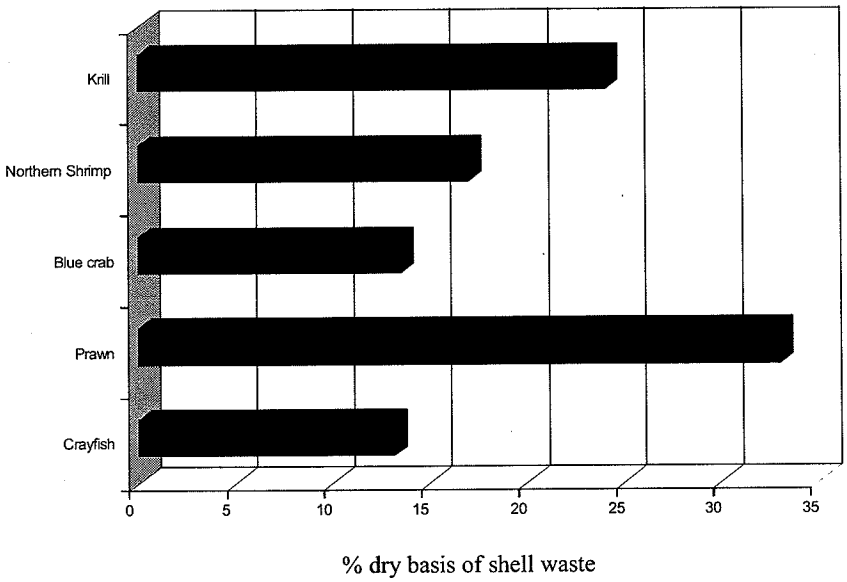

Figure 5. Chitin content in the exoskeletons of various shellfish. (Adapted from Synowiecki and Al-Khateeb.[68])

vertebral disks, which diminishes disk pressure on inflamed nerve roots, thereby alleviating sciatic pain. Studies have also reported that krill enzymes release microbes from plaque in vitro and remove plaque on dentures, indicating potential benefits in oral health.[79] Thus, krill products for human consumption and medical applications for krill by-products may be developed in parallel.

CONCLUSIONS

Based on its nutrient composition, krill appears to be a suitable food for human consumption. It has the benefits of being high in ω-3 PUFAs and a rich source of high-quality proteins. However, further research is required to determine whether components such ω-3 PUFAs and protein derived from krill exert the same health benefits as when provided by different sources. The development of krill into edible foods must also take into consideration that processing may affect its nutritive value; for example, the mineral losses associated with removal of the exoskeleton. Other areas requiring further research include the development of value-added products from the by-products generated by the processing of krill into food. Finally, it is critical to be cognizant that despite the seemingly limitless biomass provided by krill, a careful approach to krill stock management needs to be in place to ensure its long-term sustainability as a food source for humans.

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Appendix R

Bunea et al., "Evaluation of the Effects of Neptune Krill Oil on the Clinical Course of Hyperlipidemia" Altern Med Rev 9: 420-28 (2004)

Evaluation of the Effects of Neptune Krill Oil on the Clinical Course of Hyperlipidemia

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Abstract

OBJECTIVE: To assess the effects of krill oil on blood lipids, specifically total cholesterol, triglycerides, low-density lipoprotein (LDL), and high-density lipoprotein (HDL). METHODS: A multi-center, three-month, prospective, randomized study followed by a three-month, controlled follow-up of patients treated with 1 g and 1.5 g krill oil daily. Patients with hyperlipidemia able to maintain a healthy diet and with blood cholesterol levels between 194 and 348 mg/dL were eligible for enrollment in the trial. A sample size of 120 patients (30 patients/group) was randomly assigned to one of four groups. Group A received krill oil at a body mass index (BMI)-dependent daily dosage of 2-3 g daily. Patients in Group B were given 1-1.5 g krill oil daily, and Group C was given fish oil containing 180 mg eicosapentaenoic acid (EPA) and 120 mg docosahexaenoic acid (DHA) per gram of oil at a dose of 3 g daily. Group D was given a placebo containing microcrystalline cellulose. The krill oil used in this study was Neptune Krill Oil (NKO), provided by Neptune Technologies & Bioresources, Laval, Quebec, Canada. OUTCOME MEASURES: Primary parameters tested (baseline and 90-day visit) were total blood cholesterol, triglycerides, LDL, HDL, and glucose. RESULTS: Krill oil 1-3 g/day (BMI-dependent) was found to be effective for the reduction of glucose, total cholesterol, triglycerides, LDL, and HDL, compared to both fish oil and placebo. CONCLUSIONS: The results of the present study demonstrate within high levels of confidence that krill oil is effective for the management of hyperlipidemia by significantly reducing total cholesterol, LDL, and triglycerides, and increasing HDL levels. At lower and equal doses, krill oil was significantly more effective than fish oil for the reduction of glucose, triglycerides, and LDL levels.

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Introduction

The balance of polyunsaturated essential fatty acids (PUFAs) in the body is critical for the maintenance of healthy cell membranes and hormone regulation. During the last few decades the fatty acid content of the U.S. diet has shifted so it now contains much higher levels of omega-6 and less omega-3 fatty acids. When long-chain omega-6 fatty acids predominate in the phospholipids of cell membranes, the production of pro-inflammatory type-2 prostaglandins (PGs) and type-4 leukotrienes (LTs) are encouraged; whereas, the presence of omega-3 fatty acids promotes the production of anti-inflammatory PGs and LTs.[1,2]

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Omega-6 fatty acids, mainly arachidonic acid, have been shown to initiate an inflammatory process by triggering a flux of inflammatory PGs and LTs.[3,4] Omega-3 fatty acids, mainly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), compete with the omega-6 species for the enzyme prostaglandin synthetase. Omega-3 fatty acids trigger secretion of less potent 5-series LTs and anti-inflammatory PGs of the 3 series (PE_3, PI_3 and thromboxanes-A_3).[4-9] Consequently, supplementation with EPA and DHA promotes the production of less potent PGs and LTs, resulting in a decrease in the formation of inflammatory mediators.[10-13]

The exact mechanism of action by which omega-3 fatty acids favorably modify cardiovascular disease and associated disorders is not yet fully confirmed. Evidence suggests an increased intake of EPA and DHA results in an increase of EPA and DHA in tissue, cellular lipids, and circulatory lipids.[14] In parallel, they result in a simultaneous reduction of omega-6 fatty acids in the body.[14] This fatty acid shift is predominantly marked in cell membrane-bound phospholipids and results in alteration of the physicochemical properties of cell membranes. This favorably modifies cellular functions, including cell signaling, gene expression, biosynthetic processes, and eicosanoid formation.[15]

Human studies have revealed the ability of EPA and DHA to significantly reduce circulating levels of blood triglyceride and very low-density lipoprotein (VLDL), which have been associated with increased risk of cardiovascular disease.[16,17]

Krill oil is extracted from Antarctic krill, *Euphausia superba*, a zooplankton crustacean rich in phospholipids carrying long-chain omega-3 PUFAs, mainly EPA and DHA. Krill oil also contains various potent antioxidants, including vitamins A and E, astaxanthin, and a novel flavonoid similar to 6,8-di-c-glucosylluteolin, but with two or more glucose molecules and one aglycone.

Krill oil has a unique biomolecular profile of phospholipids naturally rich in omega-3 fatty acids and diverse antioxidants significantly different from the usual profile of fish oils. The association between phospholipids and long-chain omega-3 fatty acids highly facilitates the passage of fatty acid molecules through the intestinal wall, increasing bioavailability and ultimately improving the omega-3:omega-6 fatty acid ratio.[18,19]

Materials and Methods

A 12-week, double-blind, randomized trial was conducted comparing krill oil to high EPA and DHA (3:2 ratio) fish oil and placebo. Eligible patients were 18-85 years and had at least a six-month diagnosis of mildly high to very high blood cholesterol (193.9-347.9 mg/dL) and triglyceride levels (203.8-354.4 mg/dL). Patients with familial hypercholesterolemia, severely high cholesterol (>349 mg/dL), pregnancy, known or suspected allergy to fish or seafood, known alcohol or drug abuse within the previous year, known coagulopathy or receiving anticoagulant therapy, or co-morbidity that would interfere with study results were excluded from the study.

Enrolled patients were randomly assigned to one of four groups:

▲ Group A: Krill oil (2-3 g once daily)
Body Mass Index (BMI) < 30 – 2 g/day
BMI > 30 – 3 g/day

▲ Group B: Krill oil (1-1.5 g once daily)
BMI < 30 – 1 g/day
BMI > 30 – 1.5 g/day
Follow-up 500 mg/day for 90 days

▲ Group C: Fish oil (3:2) containing 180 mg EPA and 120 mg DHA per gram (3 g once daily)

▲ Group D: placebo (3 g once daily)

Patients were allowed to continue lipid-lowering medications at the usual daily dose and asked to report any change in dosage. Natural health products were discontinued for a two-week washout period prior to study initiation and thereafter for the study duration. Patients were asked to record concomitant medications taken daily.

Table 1. Results of Krill Oil (1.0 g/day) on Lipids

1.0 g Krill Oil	Time (d)/mg/dL		% Change	p-value
	0.00	90.00		
Total Cholesterol	235.83	204.12	-13.44%	0.000
LDL	167.78	114.05	-32.03%	0.000
HDL	57.22	82.35	43.92%	0.000
Triglycerides	120.50	107.21	-11.03%	0.114

Table 2. Results of Krill Oil (1.5 g/day) on Lipids

1.5 g Krill Oil	Time (d)/mg/dL		% Change	p-value
	0.00	90.00		
Total Cholesterol	231.19	199.49	-13.71%	0.000
LDL	164.74	105.93	-35.70%	0.000
HDL	58.76	83.89	42.76%	0.000
Triglycerides	126.70	111.64	-11.89%	0.113

The primary parameters tested were blood glucose, cholesterol, triglycerides, low-density lipoprotein (LDL), and high-density lipoprotein (HDL). Fasting blood lipids and glucose were analyzed at baseline as well as 30 and 90 days after study initiation for all groups, and at 180 days for the 30 patients in Group B.

Statistical Rationale and Analysis

A sample size of 120 patients (30 patients/group) provided 90-percent power to detect a 15-percent change in total cholesterol from baseline to three months.

Within-group differences reflecting changes over time for the same patient were assessed for statistical significance with the Paired Student's t-test. Between-group differences were assessed with planned comparisons of one-way analysis of variance.

Results

One-hundred-twenty patients with a mean age of 51 years (standard deviation 9.46) and ranging between 25 and 75 years were enrolled in the trial. BMI, a tool indicating weight status in adults, was calculated according to the metric formula ([weight in kilograms/(height in centimeters) x (height in centimeters)] x 10,000).[20,21] Of the 120 patients enrolled, 30 (25%) had moderate-to-severe obesity, with a BMI higher than 30. Sixty-four (53%) subjects were overweight, and 26 (22%) were normal weight, with a BMI between 25 and 30 and lower than 25, respectively. Women had a higher

mean BMI (28.2±5.1) compared to men (25.4±3.9) ($p<0.001$).

Among the 60 patients in the two groups receiving krill oil, 42 (70%) had a BMI of 30 or less. In Group A, 19 patients received 2 g krill oil daily and the remaining 11 received 3 g daily. In Group B, 23 patients were treated with a daily dose of 1 g krill oil and 7 with 1.5 g. All patients in Group B continued for an additional 90 days with a maintenance dose of 500 mg krill oil daily.

Baseline analysis of demographic criteria, laboratory data including total cholesterol and triglyceride levels, comorbidity, and concomitant medication at baseline showed no significant differences among the four groups (p=0.102-0.850).

After 12 weeks of treatment, patients receiving 1 or 1.5 g krill oil daily had a 13.4-percent and 13.7-percent decrease in mean total cholesterol, from 236 mg/dL and 231 mg/dL to 204 mg/dL (p=0.000) and 199 mg/dL (p=0.000), respectively (Tables 1 and 2). The group of patients treated with 2 or 3 g krill oil showed a significant respective reduction in mean total cholesterol of 18.1 and 18 percent. Levels were reduced from a baseline of 247 mg/dL and 251 mg/dL to 203 mg/dL (p=0.000) and 206 mg/dL (p=0.000), correspondingly (Tables 3 and 4). In comparison, people receiving 3 g fish oil had a mean reduction in total cholesterol of 5.9 percent, from a baseline 231 mg/dL to 218 mg/dL (p=0.000) (Table 5). Those enrolled in the placebo group showed a 9.1-percent increase in mean total cholesterol, from 222 mg/dL to 242 mg/dL (p=0.000) (Table 6).

Table 3. Results of Krill Oil (2.0 g/day) on Lipids

2 g Krill Oil	Time (d)/mg/dL		% Change	p-value
	0.00	90.00		
Total Cholesterol	247.42	202.58	-18.13%	0.000
LDL	182.86	114.43	-37.42%	0.000
HDL	51.03	79.25	55.30%	0.000
Triglycerides	160.37	116.07	-27.62%	0.025

Table 4. Results of Krill Oil (3.0 g/day) on Lipids

3 g Krill Oil	Time (d)/mg/dL		% Change	p-value
	0.00	90.00		
Total Cholesterol	250.52	205.67	-17.90%	0.000
LDL	172.81	105.16	-39.15%	0.000
HDL	64.18	102.45	59.64%	0.000
Triglycerides	152.77	112.27	-26.51%	0.028

Table 5. Results of Fish Oil (3.0 g/day) on Lipids

3 g Fish Oil	Time (d)/mg/dL		% Change	p-value
	0.00	90.00		
Total Cholesterol	231.15	217.55	-5.88%	0
LDL	121.67	117.83	-4.56%	0.141
HDL	56.64	59.03	4.22%	0.002
Triglycerides	140.87	136.44	-3.15%	0.239

Table 6. Results of Placebo on Lipids

Placebo	Time (d)/mg/dL		% Change	p-value
	0.00	90.00		
Total Cholesterol	221.91	242.01	9.06%	0.000
LDL	136.47	154.25	13.03%	0.000
HDL	56.83	56.70	4.00%	0.850
Triglycerides	143.53	129.36	-9.88%	0.215

An analogous effect on LDL levels was observed in all groups. Krill oil at a daily dose of 1 g, 1.5 g, 2 g, or 3 g achieved significant reductions of LDL of 32, 36, 37, and 39 percent, respectively (p=0.000). Baseline levels were decreased in the krill oil 1-g/day group from 168 mg/dL to 114 mg/dL, in the 1.5-g/day group from 165 mg/dL to 106 mg/dL, and in the 2- and 3-g/day groups from 183 mg/dL and 173 mg/dL to 114 mg/dL and 105 mg/dL, respectively. The laboratory results of patients treated daily with 3 g fish oil did not achieve a significant reduction in LDL (4.6%), with blood levels decreased from 122 mg/dL at baseline to 118 mg/dL (p=0.141) after 12 weeks. Patients receiving placebo showed a negative effect, with a 13-percent increase in LDL levels, from 137 mg/dL to 154 mg/dL (p=0.000).

HDL was significantly increased in all patients receiving krill oil (p=0.000) or fish oil (p=0.002). HDL levels increased from 57.2 mg/dL to 82.4 mg/dL (44% change) at krill oil 1 g/day; 58.8 mg/dL to 83.9 mg/dL (43% increase) for krill oil 1.5 g/day; 51 mg/dL to 79.3 mg/dL (55% increase) at krill oil 2 g/day; and from 64.2 mg/dL to 102.5 mg/dL (59% increase) at a daily krill oil dose of 3 g. Fish oil taken at 3 g/day increased HDL from 56.6 mg/dL to 59.03 mg/dL (4.2% increase). No significant decrease of HDL (p=0.850) was observed within the placebo group, with levels of HDL remaining almost stable, 56.8 mg/dL to 56.7 mg/dL.

Krill oil taken 1 g/day reduced blood triglycerides by a non-significant 11 percent, from 120.5 mg/dL to 107.2 mg/dL (p=0.114). A daily dose of 1.5 g krill oil resulted in a non-significant

11.9-percent reduction of triglycerides, from 122.7 mg/dL to 112 mg/dL (p=0.113). Subjects achieved a significant reduction of triglycerides at daily doses of 2 g and 3 g daily krill oil – 28 percent (p=0.025) and 27 percent (p=0.0228) – decreasing from baseline levels of 160.4 mg/dL and 152.8 mg/dL to 116.1 mg/dL and 112.3 mg/dL, respectively. Fish oil at 3 g/day did not achieve a significant reduction of triglycerides (3.2%), decreasing from 140.9 mg/dL to 136.4 mg/dL (p=0.239). Interestingly patients in the placebo group experienced a 9.8-percent decrease in triglycerides (p=0.215).

Blood glucose levels were reduced by 6.3 percent, from 105 mg/dL to 98 mg/dL (p=0.025), in patients receiving 1 g and 1.5 g krill oil daily, and 5.6 percent, from 92 mg/dL to 88 mg/dL (p=0.011), in those receiving 2 g and 3 g krill oil daily. A daily dose of 3 g fish oil reduced blood glucose by 3.3 percent, from 90 mg/dL to 87 mg/dL (p=0.275). Placebo treatment resulted in a non-significant blood glucose increase of 0.1 percent, from 92 mg/dL to 93 mg/dL (p=0.750).

The between-group comparison showed 1 g and 1.5 g krill oil daily was significantly more effective than 3 g fish oil in reducing glucose and LDL, whereas 2 g and 3 g krill oil demonstrated a significantly greater reduction of glucose, triglycerides, and LDL compared to 3 g fish oil. Both fish oil and krill oil performed significantly better than placebo for the regulation of glucose, triglycerides, total cholesterol, and HDL.

As mentioned previously, patients receiving 1 g and 1.5 g daily krill oil continued for another 12 weeks with a lower maintenance dose of 0.5 g krill oil daily (Table 7). These patients maintained a mean total cholesterol level of 192.5 mg/dL, a reduction of 19 percent (p=0.000) from baseline. LDL was further reduced from baseline by 44 percent, a reduction from 233 mg/dL to 107.5 mg/dL (p=0.000). A moderate decrease in HDL was seen, from 36 percent increase at 90 days to 33 percent after 180 days of treatment, which was still a significant increase from baseline (p=0.000). Triglycerides were slightly decreased further to a reduction of 25 percent from baseline (p=0.000), compared to the 12-percent reduction observed after 90 days of treatment. Blood glucose decreased by 6.6 percent from baseline (p=0.20), versus the 6.3-percent decrease at 90 days.

Table 7. Effect of a Lower Maintenance Dose of Krill Oil on Lipids

0.5 g Krill Oil	Time (d)/mg/dL		% Change	p-value
	0.00	180.00		
Total Cholesterol	235.83	192.53	18.90%	0.000
LDL	167.78	107.47	44.40%	0.000
HDL	57.22	77.71	33.40%	0.000
Triglycerides	120.50	89.89	25.40%	0.025

Discussion

Arteriosclerosis is the generic term for a number of diseases in which arterial walls become thickened and lose elasticity, with atherosclerosis being considered the most important. With its effects on the brain, heart, kidneys, and other vital organs and extremities, and despite medical advancements, atherosclerotic heart disease and stroke combined remain the number one cause of morbidity and mortality in the United States, Canada, and most Western countries.[22]

In the United States, cardiovascular disease has a mortality rate of 39.9 percent for males and 43.7 percent for females, a 15-21 percent difference from malignant disease, which ranks second.[22] It is estimated that 59.7 million Americans have one or more forms of cardiovascular disease.[22] Of the population with self-reported heart disease, 56-64 percent report restricted activity, 23-37 percent require one or more disability days per week, and 28-34 percent are unemployed because of disability or illness.[22] The primary lesion of atherosclerosis is the fatty streak, which eventually evolves into a fibrous plaque. Numerous randomized trials have proven that lowering serum cholesterol slows or reverses progression of coronary artery disease (CAD) and reduces coronary events.[22-29]

A daily intake of 1-3 g EPA and DHA or 3-9 g fish oil is currently recommended to reduce the risk of cardiovascular diseases.[22,23] Nevertheless, epidemiological studies evaluating the effects of fish oil on coronary heart disease are contradictory, ranging from reverse associations to virtually no effect to a beneficial effect.[30-33] One issue in the efficacy of EPA/DHA may be the bioavailability of these fatty acids.

A recent study demonstrated *in vivo* PUFA bioavailability depends on several factors, such as the type of lipids in which they are esterified, their physical state; i.e., lipid solution or colloidal particle systems, and the presence of co-ingested lipids.[18] *In vivo* PUFA absorption was evaluated by fatty acid analysis of thoracic lymph of duct-cannulated rats after intragastric feeding of dietary fats.[19] Evidence demonstrates oral essential fatty acid supplementation in the form of phospholipids is more effective than triglycerides in increasing concentrations of long-chain PUFAs in liver and brain.[18,19] DHA is better absorbed when delivered by liposomes than by fish oil (relative lymphatic absorption equal to 91 percent and 65 percent after liposome and fish oil administration, respectively). The best bioavailability of DHA delivered by liposomes is revealed by an increase in DHA proportions in both lymphatic triacylglycerols and phospholipids, compared to a fish oil diet.[18,19]

Krill oil is a complex combination of multiple active ingredients with synergistic bioactivity. The exact mechanism of action for krill oil's lipid-lowering effects is not yet entirely clear. However, krill oil's unique biomolecular profile of omega-3 (EPA/DHA) fatty acids already incorporated into phospholipids has exhibited a lipid-lowering effect on the level of the small intestine, which distinguishes krill oil from other known lipid-lowering principals.[18,19] Werner et al demonstrated essential fatty acids in the form of phospholipids were superior to essential fatty acids as triglycerides in significantly decreasing the saturated fatty acid ratios of liver triglycerides and phospholipids (each $p < 0.05$), while significantly increasing the phospholipid concentrations of the long-chain PUFAs ($p < 0.05$).[19]

LDL oxidation is believed to increase atherosclerosis through high serum LDL levels inducing LDL particles to migrate into subendothelial space. The process by which LDL particles are oxidized begins with lipid peroxidation, followed by fragmentation to short-chain aldehydes. At the same time, lecithin is converted to lysolecithin, a selective chemotactic agent for monocytes, which become macrophages that ingest oxidized LDL. The new macrophage becomes engorged with oxidized LDL cholesteryl esters and becomes a foam cell. Groups of foam cells form a fatty streak, the earliest indication of atherosclerosis.[34,35]

The unique molecular composition of krill oil, with its abundance of phospholipids and antioxidants, may explain the significant effect of krill oil for blood lipid regulation. In comparison to fish oil, krill oil significantly lowered blood lipids at lower doses.

The effect of fish oil on cardiovascular disease is tempered by the presence of methylmercury in many fish.[33] In fact, the U.S. Food and Drug Administration has advised pregnant women and women who may become pregnant not to eat swordfish, king mackerel, tilefish, shark, or fish from locally contaminated areas.[36] Therefore, it may be prudent to obtain these essential fatty acids via supplementation. Krill oil, and most fish oil concentrates, are molecularly distilled to remove heavy metals.

Conclusion

Atherosclerotic cardiovascular disease is a major health problem in the Western world, with CAD being the leading cause of mortality in the United States. Extensive observational epidemiologic data strongly associate high CAD risk to elevated total and LDL cholesterol and low levels of HDL cholesterol. Extensive clinical trial evidence has established that favorably altering dyslipidemias produces clear improvements in CAD end points.[15-17]

The results of this clinical trial demonstrate that daily doses of 1-3 g krill oil are significantly more effective than 3 g EPA/DHA fish oil in the management of hyperlipidemia. Furthermore, a maintenance dose of 500 mg krill oil is significantly effective for long-term regulation of blood lipids. The unique molecular composition of krill oil, which is rich in phospholipids, omega-3 fatty acids, and diverse antioxidants, surpasses the profile of fish oils and offers a superior approach toward the reduction of risk for cardiovascular disease.

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Appendix S

Bridges et al., "Determination of Digestibility, Tissue Deposition, and Metabolism of the Omega-3 Fatty Acid Content of Krill Protein Concentrate in Growing Rats" J Agric Food Chem 58: 2830-7 (2010)

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Determination of Digestibility, Tissue Deposition, and Metabolism of the Omega-3 Fatty Acid Content of Krill Protein Concentrate in Growing Rats

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Krill protein concentrate (KPC) consists of high-quality protein (77.7% dry basis) and lipids (8.1% dry basis) that are rich (27% of total fatty acids) in omega-3 polyunsaturated fatty acids (ω-3 PUFAs). The objective of the study was to determine digestibility, tissue deposition, metabolism, and tissue oxidative stability of the ω-3 PUFAs provided by KPC. Young female Sprague−Dawley rats (n = 10/group) were fed ad libitum isocaloric diets for 4 weeks with either 10% freeze-dried KPC or 10% casein. The casein diet contained 5.3% added corn oil (CO), whereas the KPC contained 5.3% total lipids from 0.9% krill oil (KO) provided by KPC and 4.4% added corn oil (KO + CO). Fatty acid compositions of various tissues were analyzed by gas chromatography. Lipid peroxidation was determined by thiobarbituric acid reactive substances (TBARS). Total antioxidant capacity and urinary eicosanoid metabolites were determined by enzyme immunoassay. The ω-3 PUFAs provided in KO from KPC increased (P = 0.003) docosahexaenoic acid (DHA) concentration in the brain. DHA and eicosapentaenoic acid (EPA) content in fat pads and liver were increased (P < 0.01), whereas the ω-6 PUFA, arachidonic acid (AA), was decreased (P < 0.01) in rats fed the KPC diet containing the KO + CO mixture compared to rats fed the casein diet containing pure CO. Feeding the KPC diet decreased pro-inflammatory 2-series prostaglandin and thromboxane metabolites. There was no significant difference in TBARS or total antioxidant capacity in the tissues of rats fed the different diets. On the basis of the study results, the low amount of ω-3 PUFAs provided by the KO content of KPC provides beneficial effects of increasing tissue EPA and DHA deposition and reduced AA-derived 2-series eicosanoid metabolites without increasing lipid peroxidation. Therefore, consumption of KPC has the potential to provide a healthy and sustainable source of ω-3 PUFAs.

KEYWORDS: Krill protein concentrate; ω-3 PUFAs; docosahexaenoic acid; eicosapentaenoic acid; arachidonic acid

INTRODUCTION

Krill is a marine crustacean with the most abundant animal biomass on Earth (*1*). On the basis of body size, krill has the highest content of protein (> 65% dry weight) among all organisms (*2*). Lipid analysis showed that ~19% of the fatty acids in Antarctic krill (*Euphausia superba*) consisted of omega-3 polyunsaturated fatty acids (ω-3 PUFAs), with eicosapentaenoic acid (EPA, 20:5 ω-3) and docosahexaenoic acid (DHA, 22:6 ω-3) being particularly abundant (*2*). Furthermore, mercury concentrations in Antarctic krill are reportedly low (*3*). Yet, krill has remained virtually untapped as a food source for human consumption.

Commercial development of krill as a food has been hindered by the lack of an effective technology for meat recovery from krill. Using isoelectric solubilization/precipitation, krill protein concentrate (KPC) was recovered (*4*). KPC consisted of 77.7% crude protein (dry basis) with protein quality determined to be similar to the milk protein, casein (*5*). Jacques et al. (*6*) reported reduced circulating plasma cholesterol concentration in laboratory animals fed protein derived from seafood compared to the milk protein, casein. The dietary combination of seafood protein and lipid has been suggested to be synergistic (*7*). KPC also contains a small amount of lipid (8.1% dry weight). Analysis of the lipids associated with KPC showed that 27% of the fatty acids were ω-3 PUFAs. Of the ω-3 PUFAs, EPA accounted for 12.7% and DHA for accounted for 12.3% (*5*).

The ω-3 PUFAs in krill have been reported to be in both phospholipid (PL) and triacylglycerol (TAG) form (*8*). The structural form of fatty acids may affect bioavailability because PLs are digested differently from TAGs. Rats fed DHA as egg PLs showed better apparent absorption indicated by lower fecal excretion of DHA and higher feed efficiency ratios than rats fed DHA in TAG form (*9*). In turn, this may affect ω-3 PUFA incorporation into tissues. Valenzuela et al. (*10*) reported that feeding female rats DHA supplemented in the form of egg PLs resulted in higher accretion of DHA in the liver compared to

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supplementation in TAG form. In contrast, Song and Miyazawa (*11*) found that rats fed DHA in PL form resulted in lower incorporation in the liver than rats fed DHA in TAG form. Therefore, it is important to determine the structural form of fatty acids provided by KPC and their bioavailability.

Tissue incorporation of ω-3 PUFAs from KPC is important because EPA and DHA exert various health benefits. The ω-3 PUFA, EPA, competes with arachidonic acid (AA) for enzymes involved in the synthesis of eicosanoids. The long-chain omega-6 polyunsaturated fatty acid (ω-6 PUFA) AA (20:4 ω-6) is the precursor of the 2-series eicosanoids (i.e., prostanoids and thromboxanes). The 2-series prostanoids are pro-inflammatory, and 2-series thromboxanes facilitate platelet aggregation and vasoconstriction. At high concentrations, EPA competitively inhibits the 2-series eicosanoids, resulting in reduced inflammation, platelet aggregation, and vasodilation (*12*). On the other hand, at high concentrations the ω-3 PUFAs, EPA and DHA, are highly susceptible to lipid peroxidation due to their high degree of unsaturation (*13*). Song and Miyazawa (*11*) demonstrated that increased levels of tissue DHA increased thiobarbituric acid reactive substances (TBARS), but not when provided in PL form. Therefore, the objective of this study was to determine digestibility, tissue deposition, metabolism, and oxidative stability of the ω-3 PUFAs provided by KPC.

MATERIALS AND METHODS

Diets. Whole frozen Antarctic krill (*Euphausia superba*) was purchased from Krill Canada (Langley, BC, Canada). KPC was recovered from whole krill using an isoelectric solubilization/precipitation method (*4*). The proximate composition of recovered KPC after freeze-drying was 77.7% crude protein and 8.1% total lipid on a dry basis (*5*). Diets were based on a standard purified AIN-93G diet (*14*). **Table 1** shows the diet ingredients and modifications of the AIN-93G diet. Dietary modifications consisted of providing 10% protein as KPC or casein and replacing soybean oil with corn oil. Diets were adjusted to be isocaloric and isonitrogenous with both diets containing 5.3% total lipid. The casein diet contained 5.3% corn oil (CO), whereas the KPC contained 5.3% total lipids from the 0.9% KO provided by KPC and 4.4% added corn oil (KO + CO). Diets containing KPC were prepared weekly and stored at 4 °C.

Animal Feeding Study. All animal procedures were approved by the Animal Care and Use Committee at West Virginia University and were conducted in accordance with the guidelines set forth by the Institute of Laboratory Animal for the Care and Use of Laboratory Animals (*15*). Young (28 day) growing female Sprague–Dawley rats were purchased from Taconic Farms (Rockville, MD). Upon arrival at the animal care facility, rats were individually housed in metabolic cages throughout the study duration (4 weeks) to determine food intake and to collect urine and feces. Rats were kept caged in rooms maintained at 21 °C with a 12 h light/dark cycle. During a 14 day acclimation period, animals were given ad libitum access to deionized distilled water (ddH_2O) and AIN-93G diet (Harklan Teklad, Indianapolis, IN). Following the 14 day acclimation period, rats (n = 10/group) were randomly assigned to be fed ad libitum either 10% casein with 5.3% CO or KPC with 0.9% KO and 4.4% added CO (KO + CO). Food intake was measured biweekly. At the end of the 4 week study, rats were euthanized by CO_2 inhalation. Brain, liver, and retroperitoneal and gonadal fat pads were dissected and weighed.

Digestibility. The apparent digestibility of dietary lipid was determined using fecal samples collected during the final week of the 4 week study. Fecal samples were freeze-dried for 48 h. Total fecal lipid content was determined by Soxhlet extraction. Apparent digestibility (%) was determined according to the method of Deuchi et al. (*16*) using the formula [(lipid intake − fecal lipid)/(lipid intake)] × 100. Similarly, apparent digestibility (%) of individual fatty acids was determined using the formula [(fatty acid intake − fecal fatty acids)/(fatty acid intake)] × 100.

Fatty Acid Analysis of Oils and Lipid Classes. Lipids were extracted from the diet, various tissues, and feces according to the method by Bligh and Dyer (*17*). All samples were conducted in duplicates. Samples were weighed, and 48 μL of heptadecenoic acid (17:1) was added as an internal standard. Weighed samples were added to Tris/EDTA buffer, pH 7.4. A chloroform/methanol/acetic acid (2:1:0.15 v/v/v) solution was added to the samples. Samples were centrifuged at 900*g* for 10 min at 10 °C, and the bottom chloroform layer was collected. The collected chloroform was then filtered through one-phase separation filters to remove any remaining water and precipitated material. The remaining layer was then mixed with chloroform/methanol (4:1 v/v) and centrifuged at 900*g* for 10 min at 10 °C. The chloroform layer was collected and filtered. The extracted lipid was dried under nitrogen gas.

Table 1. Composition of Diets

ingredient	casein, corn oil[a] (g/kg of diet)	KPC, krill oil + corn oil[a] (g/kg of diet)
casein	115	0
KPC[b]	0	128
DL-methionine	1.5	0
sucrose	531.8	609.8
corn starch	200	229.4
corn oil	53.5	49.9
cellulose	52	59.6
vitamin mix[c]	10	11.5
ethoxyquin	0.01	0.01
mineral mix[c]	13.4	15.3
calcium phosphate	20.2	20.9
calcium carbonate	2.6	3.6
gross energy (kcal/g)	4.3	4.3

[a] The KPC diet contains a mixture of 0.9% krill oil and 4.4% corn oil. The casein diet contains 5.3% corn oil. [b] KPC, krill protein concentrate. Diet formulated for 872g of diet + 128g addition of KPC. [c] Based on the AIN-93G diet vitamin and mineral mixes Reeves et al. (*14*).

The extracted lipid samples were transmethylated following the procedure described by Fritsche and Johnston (*18*). Briefly, extracted fatty acids were methylated by adding 4% H_2SO_4 in anhydrous methanol to the dried lipid samples followed by incubation in a 90 °C water bath for 60 min. Samples were cooled to room temperature, and 3 mL of ddH_2O was added to stop the reaction. Chloroform was added to the methylated sample. Samples were dried under nitrogen gas, and iso-octane (3 mL) was used as a diluent.

The methylated lipid samples were analyzed by gas chromatography (CP-3800, Varian Inc., Walnut Creek, CA) using an initial temperature of 140 °C held for 5 min and then increased at 1 °C/min to a final temperature of 220 °C. A wall-coated open tubular fused silica capillary column (Varian Inc.) was used to separate fatty acid methyl esters with CP-Sil 88 as the stationary phase. Nitrogen was used as the carrier gas, and total separation time was 110 min. Quantitative 37 component fatty acid methyl ester Sigma Mix (Supelco, Bellefonte, PA) was used to identify fatty acid composition. Fatty acids were identified using retention time and peak area counts.

Thin-layer chromatography (TLC) was performed to separate the lipid classes. The oil sample (10 mg) was dissolved in chloroform/methanol (1:1 v/v) and spotted onto Whatman K6F silica plates with 60 Å pore size (PJ Cobert Associates, St. Louis, MO). Plates were developed using a hexane/ether/acetic acid solution (80:20:1.5 v/v/v) as the mobile phase. Plates were air-dried for 5 min and observed by Fluorchem 8000 densitometer (Alpha Innotech Corp., San Leandro, CA) using transluminating white light. Plate images were captured using a camera interfaced to a PC, and images were analyzed using the spot densitometer tab in the Fluorchem (version 1.0) computer program. PL and TAG were identified using R_f values obtained from triolein (Sigma-Aldrich, St. Louis, MO) and soybean lecithin (Fisher Scientific, Pittsburgh, PA) standards. PL and TAG bands were scraped from the plates and suspended in chloroform/methanol (1:1 v/v). To determine the fatty acid composition of the TAG and PL, samples were methylated and analyzed by gas chromatography as described above for dietary oil fatty acid composition.

Thiobarbituric Acid Reactive Substances (TBARS). Tissue TBARS were determined in homogenates of brain, liver, and retroperitoneal and gonadal fat pads. To determine urinary TBARS, rats were individually housed in metabolic cages to allow for urine collection. Urine samples were collected during the final week of the 4 week feeding study.

Table 2. Fatty Acid Composition of Oil and Lipid Classes of Diets Fed to Young Female Rats[a]

	KPC with 0.9% KO + 4.4% CO			casein with 5.3% CO	
fatty acid	FA content (mg/mL)	FA-TAG[b] (%)	FA-PL[b] (%)	FA content (mg/mL)	FA-TAG[c] (%)
ω-6 PUFAs					
18:2 (ω-6), LA	2.92 ± 0.24	0.31 ± 0.24	47.4 ± 0.24	4.5 ± 0.20	56.1 ± 0.86
20:4 (ω-6), AA	0.016 ± 0.002	0.23 ± 0.24	0.06 ± 0.24	ND	ND
ω-3 PUFAs					
18:3 (ω-3), ALA	0.40 ± 0.03	0.85 ± 0.24	0.81 ± 0.24	0.09 ± 0.04	0.90 ± 0.02
20:5 (ω-3), EPA	0.40 ± 0.03	ND	0.27 ± 0.24	ND	ND
22:6 (ω-3), DHA	0.27 ± 0.02	12.7 ± 0.24	0.03 ± 0.24	ND	ND

[a] Values are given as mean ± SEM of triplicate samples. Abbreviations: ND, nondetectable; KO, krill oil; CO, corn oil; PUFAs, polyunsaturated fatty acids; FA, fatty acid; TAG, triacylglycerol; PL, phospholipid; LA, linoleic acid; AA, arachidonic acid; ALA, α-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. [b] 0.9% KO + 4.4% CO consists of 22 ±0.2% phospholipids and 61 ± 1.2% triacylglycerol. [c] 5.3% CO consists of 67.1 ±1.9% triacylglycerol and non-detectable phospholipids.

Collected urine samples were centrifuged at 1500*g* for 10 min at 4 °C to remove any debris. Urine samples were aliquoted into fresh tubes and stored at −80 °C until assayed for lipid peroxidation. TBARS were measured using a commercially available enzyme immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI). Briefly, tissue homogenate or urine samples were mixed with sodium dodecyl sulfate solution and a color reagent containing thiobarbituric acid, acetic acid, and sodium hydroxide. Samples were incubated for 60 min in a 90 °C water bath and then incubated in an ice bath for 10 min to stop the reaction. Following centrifugation for 10 min at 1600*g* at 4 °C, absorbance was read at 540 nm using a Spectramax Plus microplate reader (Molecular Devices, Sunnyvale, CA). Values were expressed as micromolar malondialdehyde (μM MDA).

Total Antioxidant Capacity. Urine samples were collected during the final week of the 4 week feeding study. Collected urine samples were centrifuged at 1500*g* for 10 min at 4 °C. Urine samples were aliquoted into fresh tubes and stored at −80 °C until assayed for total antioxidant capacity. Total antioxidant capacity in urine samples was measured according to a commercially available Antioxidant Assay EIA kit (Cayman Chemical). Briefly, urine samples were diluted 1:20 v/v with 5 mM potassium phosphate buffer containing 0.9% sodium chloride and 0.1% glucose at pH 7.4. In a 96-well microplate, duplicate diluted samples were mixed with the water-soluble tocopherol analogue, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), and metmyoglobin and 2,2′-azinobis(3-ethylbenzthiazoline sulfonate). To initiate the reaction, hydrogen peroxide was added to each well. Samples were then incubated at room temperature on a shaker for 5 min. Absorbance was read at 750 nm using a Spectramax Plus microplate reader. Values were expressed as micromolar Trolox.

Eicosanoid Metabolite Measurements. The unstable eicosanoid PGE_2 is converted to the stable metabolite 13,14-dihydro-15-keto PGA_2, which can be easily quantified by EIA. Similarly, unstable TXA_2 is converted to the stable metabolite 11-dehydro TXB_2, which can be easily quantified by EIA. These prostaglandin and thromboxane metabolites were determined by commercially available EIA kits (Cayman Chemical). Briefly, urine samples were diluted 1:2 and in a 96-well microplate mixed with acetylcholinesterase conjugate tracer (50 μL) and rabbit antiserum (50 μL) specific to the metabolite being measured. The microplate was incubated on an orbital shaker for 18 h for the PGE_2 metabolite and for 2 h for the 11-dehydro TXB_2 at room temperature. Following incubation, the wells were washed (five times). Ellman's reagent (200 μL) was added to each well and the microplate covered and allowed to develop in the dark for 90 min. Absorbance was read at 412 nm using a Spectramax Plus microplate reader.

Statistical Analysis. Student's *t* test was used to compare differences between diet groups. The Mann−Whitney rank sum test was performed on data not normally distributed. Differences were considered to be significant at $P < 0.05$. Results are expressed as mean ± standard error of the mean (SEM). Results were analyzed using SigmaStat 3.1 statistical software program (Systat Software Inc., San Jose, CA).

RESULTS

Lipid Composition of Diets. Diets were based on a modification of the standard purified AIN-93G diet (*14*). Modification of diet ingredients consisted of reducing protein content to 10% and replacing casein with KPC as the crude protein source. Another dietary modification was the replacement of soybean oil in the AIN-93G diet with corn oil. Diet ingredients were adjusted so that the diets were isocaloric and isonitrogenous and contained 5.3% total lipid (**Table 1**). The casein diet contained 5.3% corn oil (CO), whereas the KPC contained a mixture of 0.9% krill oil (KO) provided by KPC with 4.4% added corn oil (KO + CO). Shown in **Table 2**, pure CO is rich in the essential ω-6 fatty acid and precursor of long-chain ω-6 PUFAs linoleic acid (LA, 18:2 ω-6). CO also contains the essential ω-3 fatty acid and precursor of long-chain ω-3 PUFAs α-linolenic acid (ALA, 18:3 ω-3). The contents of the long-chain ω-6 PUFA AA and long-chain ω-3 PUFAs EPA and DHA were negligible in CO. However, adding 0.9% KO from KPC to 4.4% CO resulted in measurable amounts of AA, EPA, and DHA.

Separation of the lipid classes by TLC showed that CO was composed of 67.1 ± 1.9% TAGs and no detectable PLs. The KO + CO mixture was composed of 22 ± 0.2% PLs and 61 ± 1.2% TAGs. **Table 2** shows the fatty acids of these different lipid classes. LA was associated with TAG in the casein diet with CO. In contrast, LA was predominantly in PL in the KPC diet containing KO + CO. However, the long-chain ω-6 PUFA AA was predominantly in TAG in the KO + CO mixture. ALA was associated with TAG in the casein diet containing pure CO and equally distributed between PL and TAG in the KPC diet containing KO + CO. The long-chain ω-3 PUFA EPA was associated with PL, whereas DHA was predominantly in TAG in the KPC diet.

Digestibility of Dietary Fatty Acids. There was no significant difference in food intake for rats fed the KPC diet containing the KO + CO mixture (16.1 ± 0.4 g/day) compared to rats fed the casein diet containing pure CO (16.5 ± 0.4 g/day). There were no significant differences in total lipid intake, total lipid content of feces, or apparent digestibility of total dietary lipids between the diet groups. However, there were differences in digestibility of the individual fatty acids. In **Table 3**, apparent digestibility of the ω-6 fatty acid LA was significantly decreased ($P = 0.02$) in rats fed the KPC diet containing the KO + CO mixture compared to rats fed the casein diet containing pure CO. Rats fed the KPC diet had a lower ($P = 0.001$) intake of LA, but no difference in fecal excretion of LA compared to rats fed casein containing CO. In rats fed the casein diet containing CO only, the content of the long-chain ω-6 PUFA AA was negligible. Rats fed the KPC diet showed low (22.75 ± 6.45%) apparent digestibility of AA.

Of the ω-3 fatty acids, the apparent digestibility of ALA was not statistically different between the diet groups despite rats fed the KPC diet containing the KO + CO mixture having higher ($P < 0.05$) intake of ALA than rats fed casein containing pure CO. Fecal excretion of ALA by rats fed the KPC diet containing the KO + CO mixture was higher, although not statistically, compared

Table 3. Apparent Digestibility of Lipids and Fatty Acids in Young Female Rats Fed Casein Diet Consisting of Corn Oil or Krill Protein Concentrate Diet Consisting of Krill Oil and Corn Oil Mix[a]

	intake (mg)		fecal excretion (mg)		apparent digestibility (%)	
fatty acid	KPC, KO + CO	casein, CO	KPC, KO + CO	casein, CO	KPC, KO + CO	casein, CO
ω-6 PUFAs						
LA (18:2 ω-6)	303.13 ± 6.17*	346.50 ± 13.79	17.34 ± 3.66	10.68 ± 2.54	93.76 ± 1.06*	96.93 ± 1.06
AA (20:4 ω-6)	1.27 ± 0.03	ND	0.97 ± 0.20	ND	22.75 ± 6.45	ND
ω-3 PUFAs						
ALA (18:3 ω-3)	6.32 ± 0.13*	5.49 ± 0.22	2.88 ± 0.65	2.07 ± 0.41	49.02 ± 10.58	62.86 ± 6.26
EPA (20:5 ω-3)	13.28 ± 0.27	ND	3.60 ± 0.57	ND	70.12 ± 3.52	ND
DHA (22:6 ω-3)	12.89 ± 0.29	ND	0.77 ± 0.21	ND	93.42 ± 1.64	ND
total lipids (g)	6.16 ± 0.13	5.91 ± 0.24	0.78 ± 0.11	0.76 ± 0.10	86.10 ± 1.36	87.17 ± 1.36

[a] Values are given as mean ± SEM of $n = 10$ rats/group. * indicates significant difference at $P < 0.05$ by Student's t test. Abbreviations: ND, nondetectable; KPC, krill protein concentrate; KO, krill oil; CO, corn oil; PUFAs, polyunsaturated fatty acids; LA, linoleic acid; ALA, α-linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

Table 4. Fatty Acid Analysis of Tissues of Young Female Rats Fed Casein Diet Consisting of Corn Oil or Krill Protein Concentrate Diet Consisting of Krill Oil and Corn Oil Mix[a]

	brain (mg/g of tissue)		liver (mg/g of tissue)		retroperitoneal fat pad (mg/g of tissue)		gonadal fat pad (mg/g of tissue)	
fatty acid	KPC, KO + CO	casein, CO	KPC, KO + CO	casein, CO	KPC, KO + CO	casein, CO	KPC, KO + CO	casein, CO
ω-6 PUFAs								
LA (18:2 ω-6)	0.20 ± 0.06	0.18 ± 0.041	3.38 ± 0.29	3.24 ± 0.25	38.25 ± 3.25	43.51 ± 4.12	58.06 ± 6.25	60.79 ± 3.74
AA (20:4 ω-6)	1.99 ± 0.16	2.09 ± 0.28	1.94 ± 0.10*	3.94 ± 0.15	0.32 ± 0.12*	0.90 ± 0.09	0.69 ± 0.11	1.04 ± 0.12
ω-3 PUFAs								
ALA (18:3 ω-3)	0.15 ± 0.06	0.21 ± 0.07	ND	ND	1.38 ± 0.12	0.99 ± 0.15	1.54 ± 0.15	1.67 ± 0.13
EPA (20:5 ω-3)	ND	ND	0.76 ± 0.04*	ND	0.74 ± 0.19*	ND	0.87 ± 0.26*	ND
DHA (22:6 ω-3)	2.85 ± 0.24*	2.46 ± 0.37	1.67 ± 0.10*	0.72 ± 0.026	0.78 ± 0.19*	ND	0.92 ± 0.36*	ND

[a] Values are given as mean ± SEM of $n = 10$ rats/group. * indicates significant difference at $P < 0.05$ by Student's t test. Abbreviations: ND, nondetectable; KPC, krill protein concentrate; KO, krill oil; CO, corn oil; PUFAs, polyunsaturated fatty acids; LA, linoleic acid; AA, arachidonic acid; ALA, α-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

to rats fed the casein diet containing pure CO. In rats fed the casein diet containing CO, the content of the long-chain ω-3 PUFAs EPA and DHA was negligible. Rats fed the KPC diet showed moderate (70.12 ± 3.52%) apparent digestibility of EPA and high (93.42 ± 1.64%) apparent digestibility of DHA.

Body Weight, Organ Weights, and Fatty Acid Analysis of Tissues. At the end of the 4 week study, there was no significant difference in the final body weight of rats fed the KPC diet (242 ± 3 g) compared to rats fed the casein diet containing CO (235 ± 6 g). There was no significant difference in brain weight between rats fed the KPC diet (1.63 ± 0.03 g) compared to rats fed the casein containing CO (1.69 ± 0.03 g). There was no significant difference in liver weight between rats fed the KPC diet (10.1 ± 0.2 g) and rats fed the casein diet containing CO (9.3 ± 0.3 g). Rats fed the KPC diet had higher ($P = 0.02$) retroperitoneal fat pad weight (3.33 ± 0.26 g) than rats fed the casein diet containing CO (2.54 ± 0.16 g). Rats fed the KPC diet had higher ($P = 0.03$) gonadal fat pad weight (6.28 ± 0.24 g) than rats fed the casein diet containing CO (5.34 ± 0.32 g).

According to **Table 4**, there were no significant differences in brain, liver, or fat pad LA content among the diets despite different fatty acid compositions of the dietary oils. Although AA content was higher in the KO + CO provided by the KPC diet, AA deposition was decreased in the liver ($P < 0.001$) and retroperitoneal fat pads ($P = 0.002$), and there was a tendency ($P = 0.056$) for lower AA deposition in the gonadal fat pads compared to rats fed the casein diet containing CO. No difference in AA brain deposition was found between the diet groups.

Of the ω-3 PUFAs, ALA showed no significant differences in brain, liver, or fat pad content among the diet groups. Rats fed the KPC diet providing 0.9% KO containing EPA resulted in increased tissue deposition of EPA in liver ($P < 0.001$) and retroperitoneal and gonadal fat pads ($P = 0.004$) compared to rats fed the casein diet with CO containing no EPA. EPA was not present in detectable amounts in brain tissue of either diet group. Rats fed the KPC diet with 0.9% KO containing DHA increased DHA in brain ($P = 0.003$), liver ($P < 0.001$), and retroperitoneal ($P = 0.003$) and gonadal ($P = 0.021$) fat pads compared to rats fed the casein diet with CO containing no DHA.

Oxidative Stability. **In Table 5**, rats fed the KPC diet containing the KO + CO mixture had decreased ($P = 0.03$) TBARS in the gonadal fat pads compared to rats fed the casein diet containing pure CO. There were no significant differences in retroperitoneal fat pads, liver, or brain TBARS between the diet groups. There were no significant differences in urinary TBARS or total antioxidant capacity concentrations between the diet groups. Correcting for creatinine, to limit variability due to changes in renal excretory function, resulted in no significant differences in urinary TBARS or total antioxidant capacity among the diet groups.

Eicosanoid Measurements. Efficacy in reducing the AA products, prostaglandin E_2 (PGE_2) and thromboxane A_2 (TXA_2), was determined. Unstable PGE_2 was converted to the stable metabolite 13,14-dihydro-15-keto PGA_2, which was measured in urine samples. There was no statistically significant difference in urinary 13,14-dihydro-15-keto PGA_2 concentration in rats fed the KPC diet containing the KO + CO mixture compared to rats fed the casein diet containing pure CO (**Figure 1A**). Correcting for creatinine resulted in reduced ($P = 0.009$) 13,14-dihydro-15-keto PGA_2 concentration in rats fed the KPC diet compared to rats fed the casein diet containing CO (**Figure 1B**).

Unstable TXA_2 was converted to the stable metabolite 11-dehydro TXB_2, which was measured in urine samples. There was no statistically significant difference in urinary 11-dehydro TXB_2

Table 5. TBARS and Total Antioxidant Capacity in Young Female Rats Fed Casein Diet Consisting of Corn Oil or Krill Protein Concentrate Diet Consisting of Krill Oil and Corn Oil Mix[a]

	KPC, KO + CO	casein, CO
TBARS		
gonadal fat pad TBARS (μM MDA/g of tissue)	1.14 ± 0.25*	2.00 ± 0.15
retroperitoneal fat pad TBARS (μM MDA/g of tissue)	2.30 ± 0.21	2.20 ± 0.23
liver TBARS (μM MDA/g of tissue)	13.40 ± 0.48	13.07 ± 0.76
brain TBARS (μM MDA/g of tissue)	5.57 ± 0.28	5.93 ± 0.38
urinary TBARS (μM MDA/day)	53.5 ± 11.8	56.7 ± 11
urine TBARS/creatinine (μM MDA/mg of creatinine)	11.7 ± 2.0	6.6 ± 1.5
total antioxidant capacity		
urinary total antioxidant capacity (μM Trolox/day)	3.5 ± 0.8	7.8 ± 2.3
urinary total antioxidants/creatinine (μM Trolox/mg of creatinine)	42.3 ± 11.9	30.2 ± 16.1

[a] Values are given as mean ± SEM of $n = 6-10$ samples. * indicates significant difference at $P < 0.05$ by Student's t test. Abbreviations: KPC, krill protein concentrate; CO, corn oil; KO, krill oil; TBARS, thiobarbituric acid reactive substances; MDA, malondialdehyde; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid.

concentration in rats fed the KPC diet containing the KO + CO mixture compared to rats fed the casein diet containing pure CO (**Figure 2A**). Correcting for creatinine resulted in reduced ($P = 0.009$) urinary 11-dehydro TXB_2 concentration in rats fed the KPC diet compared to rats fed the casein diet containing CO (**Figure 2B**).

DISCUSSION

Fatty acid bioavailability influences tissue incorporation and conversion to other fatty acids. The KPC diet provided EPA in PL form, which showed moderate (70.12 ± 3.52%) digestibility. DHA was predominantly in TAG form, which showed high (93.42 ± 1.64%) digestibility. This difference in digestibility may be due to intestinal hydrolysis of TAGs yielding 2-monoacylglycerols using enzymes other than PLs, which yields 1-lysophospholipids (*19*). Feeding growing rats KPC containing the KO + CO mixture resulted in higher ($P < 0.05$) DHA content in the brain compared to rats fed casein containing pure CO. Innis et al. (*20*) reported that feeding rats a diet composed of a 10% fish oil and 2% safflower oil blend (15.1% EPA, 7.3% DHA, 0.9% AA) for 4 weeks increased brain EPA and DHA and decreased AA content compared to rats fed 12% pure safflower oil. In our study, rats fed the KPC diet containing 0.9% KO + 4.4% added CO (12.5% EPA, 12.7% DHA, 1.5% AA) for 4 weeks did not lead to detectable EPA content in the brain. EPA is typically found in low to nondetectable amounts in brain tissue (*21*). However, rats fed the KPC diet had increased DHA without decreasing AA content in the brain. This is important because DHA in conjunction with AA is considered necessary to support proper brain development (*20*, *22*). In our study, DHA and AA were also detectable in the brains of rats fed the CO, despite the negligible content in the oil source. According to Rapoport et al. (*23*), DHA content in the brain can be maintained in the absence of dietary DHA by liver conversion of ALA to circulating DHA. In the present study, the fatty acid composition of the liver was also determined because it is the main site of fatty acid synthesis and conversion (*21*).

The body can synthesize EPA and DHA provided sufficient ALA is consumed in the diet. In our study, detectable DHA in the liver tissue of rats fed the casein diet with DHA-deficient CO as the oil source suggests conversion from ALA. In rats fed KPC, liver ALA content was negligible despite higher ALA content in the KPC diet containing KO + CO mixture compared to the casein diet containing CO only. ALA digestibility was 49.02 ± 10.58% in rats fed the KPC diet compared to 62.86 ± 6.26% digestibility in rats fed casein diet with pure CO. Lower digestibility in the KPC diet may be due to some ALA being distributed in PL form. Also, decreased ALA may be due to conversion to EPA and DHA. Werner et al. (*24*) found that feeding ALA in the PL form compared to the TAG form increased DHA content in the liver. In our study, DHA and EPA contents were increased in the liver of rats fed KPC. Therefore, reduced liver ALA in rats fed the KPC diet may be due to the poor digestibility of ALA in PL form as well as efficient conversion of ALA in PL form to the long-chain ω-3 PUFAs EPA and DHA.

The casein diet containing CO was rich in LA but negligible in AA. Detectable AA in the liver tissue of rats fed the casein diet with AA-deficient CO as the oil source suggests conversion from LA. In rats fed KPC diet, liver AA concentration was lower ($P < 0.05$) despite higher AA content in the KO + CO mixture compared to the casein diet containing pure CO. The AA provided by the KPC diet was predominantly distributed in TAGs and had low (22.75 ± 6.45%) digestibility. However, the body can synthesize AA provided sufficient LA is consumed in the diet. Rats fed the KPC diet had lower ($P = 0.001$) intake and reduced ($P = 0.02$) digestibility of LA compared to the rats fed the casein diet containing CO. In the KPC diet, LA was predominantly in PL form, whereas LA was in TAG form in the casein diet containing CO. Additionally, tissue AA content is affected by EPA and DHA due to competition for the sn-2 position of PLs in cell membranes (*25*). Liver EPA and DHA contents were increased in rats fed the KPC diet. Therefore, reduced liver AA in rats fed the KPC diet may be due to the poor digestibility of LA and AA and possibly competitive inhibition associated with higher EPA and DHA tissue incorporation.

Froyland et al. (*26*) found that feeding male rats a diet containing 94% of total fatty acids as pure EPA in the form of ethyl esters increased liver EPA 17-fold compared to CO. The authors also found that feeding 91% of total fatty acids as pure DHA in the form of ethyl esters increased liver DHA 3-fold compared to CO. In the current study, the EPA and DHA from 0.9% KO individually contributed to < 0.01% of the total fatty acids. Despite this low amount, the long-chain ω-3 PUFAs provided from KPC were capable of increasing EPA and DHA and lowering liver AA concentrations in the liver. Lower AA content in the liver, but not the brain, may have resulted from the liver being the major site for fatty acid synthesis, whereas fatty acid uptake occurs in peripheral tissues. Studies have reported that biosynthesis of fatty acids resulted in preferential accretion in some tissues (i.e., brain, retina, and skeletal muscles) and efflux from tissues known to process and/or store fatty acids (*27*).

In the present study, the adipose tissue was also analyzed because dietary fatty acids not incorporated into membranes or metabolized are stored in adipose tissue as TAG. Therefore, adipose tissue reflects the dietary intake of fatty acids. In human adipose tissue, LA is the most abundant ω-6 PUFA at ~12−16% of fatty acids and ALA is the most abundant ω-3 PUFA at

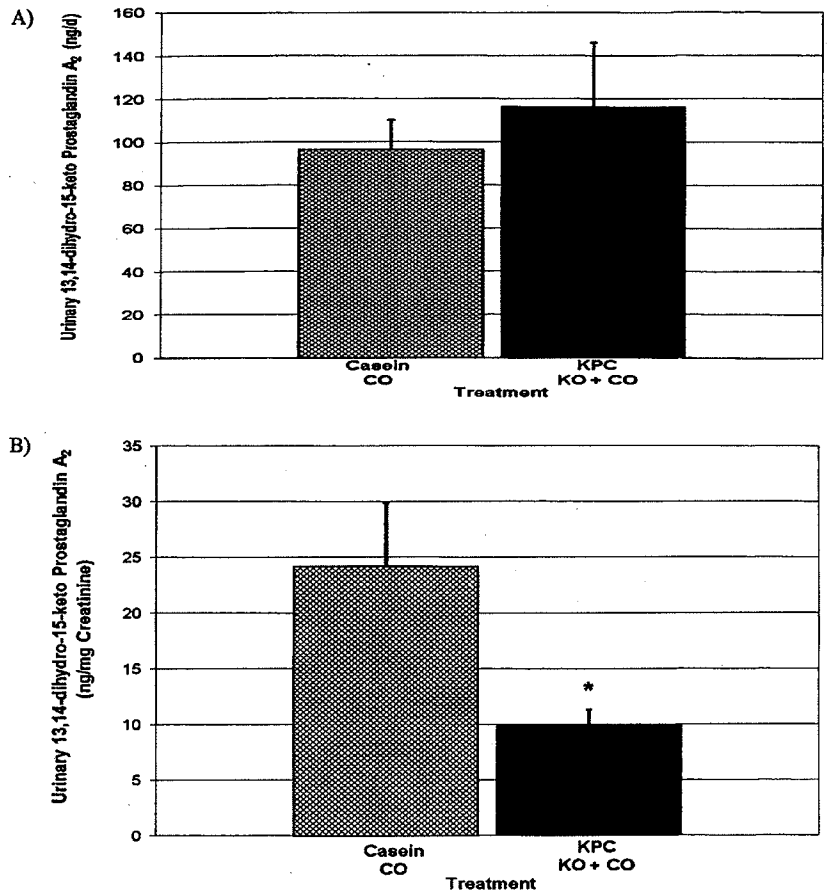

Figure 1. Effect of feeding young female rats diets consisting of casein containing 5.3% corn oil (CO) or krill protein concentrate (KPC) containing 0.9% krill oil and 4.4% added corn oil (KO + CO) on PGE_2 metabolite: (A) urinary 13,14-dihydro-15-keto PGA_2; (B) urinary 13,14-dihydro-15-keto PGA_2 corrected for creatinine to limit variability due to changes in renal excretory function. Values are given as mean ± SEM of $n = 6$ rats/group. * indicates significant difference at $P < 0.05$ by Student's t test.

~1% (*21*). In our animal study, LA and ALA contents of fat pads were similar in rats fed both diets. EPA and DHA were increased in the fat pads of rats fed the KPC diet containing the KO + CO mixture compared to rats fed the casein diet containing pure CO. Detectable AA content in the fat pads of rats fed AA-deficient CO provided by the casein diet indicated conversion from LA. Lower AA incorporation in the fat pads of rats fed the KPC diet likely resulted from the poor digestibility of AA. Froyland et al. (*26*) found that feeding male rats a diet with 94% of total fatty acids as EPA ethyl esters increased epididymal fat pad EPA content 41-fold compared to CO. The authors also found that feeding 91% of total fatty acids as DHA ethyl esters increased epididymal fat pad DHA 11-fold compared to CO. In our study, the long-chain ω-3 PUFAs provided by KPC were capable of increasing EPA and DHA in the adipose tissue. The results suggested that even the small lipid content provided by KPC improves ω-3 PUFA status. This is important because large doses of pure oil or isolated fatty acids are often used in studies to increase EPA and DHA tissue incorporation (*28*). However, small doses of EPA and DHA and oil mixes rather than pure oils are more representative of the human diet.

The present study also determined whether the increased tissue ω-3 PUFA content observed with KPC consumption altered lipid metabolism. At high concentrations, ω-3 PUFAs competitively inhibit AA, the precursor of 2-series eicosanoids such as prostaglandin E_2 (PGE_2) and thromboxane A_2 (TXA_2) (*12*). The 2-series PGE_2 is pro-inflammatory, and TXA_2 promotes platelet aggregation and vasodilation. Therefore, reduction in tissue content of the long-chain ω-6 PUFA AA has been suggested for health benefits. On the basis of the current study, KPC providing EPA or DHA at the level of 0.01% of the total fatty acids in the diet decreased PGE_2 and TXA_2 metabolites

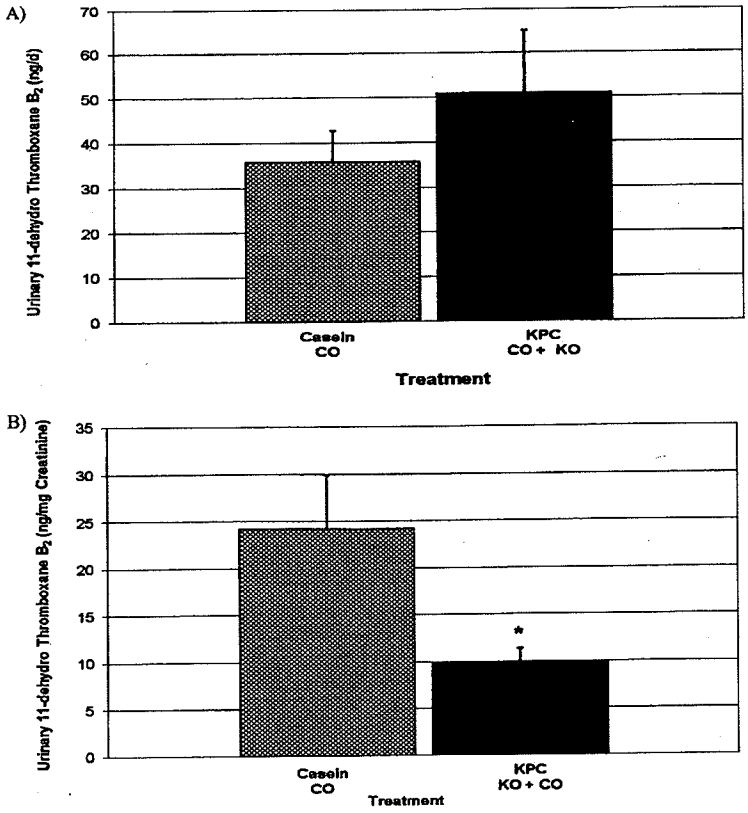

Figure 2. Effect of feeding young female rats diets consisting of casein containing 5.3% corn oil (CO) or krill protein concentrate (KPC) containing 0.9% krill oil and 4.4% added corn oil (KO + CO) on TXA_2 metabolite: (A) urinary 11-dehydro TXB_2; (B) urinary 11-dehydro TXB_2 corrected for creatinine to limit variability due to changes in renal excretory function. Values are given as mean ± SEM of $n = 6$ rats/group. * indicates significant difference at $P < 0.05$ by Student's t test.

compared to the casein diet with CO. This is consistent with reports that supplementation with either EPA and/or DHA decreases pro-inflammatory eicosanoid production (*25*).

Providing health benefits at low doses is important to consider because EPA and DHA are highly susceptible to lipid peroxidation due to their high degree of unsaturation. Saito and Kubo (*29*) found that feeding rats 8.4% total energy as purified DHA for 30 days significantly increased lipid peroxidation indicated by increased TBARS. In our study, tissue incorporation of EPA and DHA in rats fed the KPC diet containing the KO + CO mixture did not increase tissue and urinary TBARS or decrease total antioxidant capacity compared to rats fed the casein diet containing pure CO. Song and Miyazawa (*11*) observed that increased levels of DHA in membrane PLs did not increase lipid peroxidation when DHA was provided in the PL form. In our study, DHA was predominantly in the TAG form. Regardless of the structural form, the ω-3 PUFA content of KPC did not contribute significantly to oxidative stress.

In summary, KPC containing 0.9% KO provides some fatty acids in PL form. Generally, fatty acid digestibility was greater in TAG than PL form. The EPA and DHA associated with the KO provided by KPC increased tissue concentrations despite individually contributing to < 0.01% of the total fatty acids in the diet. The lipid content (KO) provided by KPC increased long-chain ω-3 fatty acids and decreased AA tissue accretion and pro-inflammatory eicosanoid metabolites without changing oxidative stability. A source of ω-3 PUFAs that provides high tissue accretion with the least lipid oxidation favors maximal health benefits. Therefore, the study results indicated that consumption of KPC provides a healthy source of ω-3 PUFAs with the advantage of offering a sustainable source of ω-3 PUFAs due to its large biomass and underutilization as a food source for humans.

ABBREVIATIONS USED

ω-3 PUFAs, omega-3 polyunsaturated fatty acids; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ALA, α-linolenic acid; ω-6 PUFAs, omega-6 polyunsaturated fatty acids; LA, linoleic acid; AA, arachidonic acid; KPC, krill protein concentrate; KO, krill oil; CO, corn oil; PL, phospholipid; TAG, triacylglycerol; TBARS, thiobarbituric acid reactive substances; MDA, malondialdehyde; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; TXA_2, thromboxane A_2;

TXB_2, 11-dehydrothromboxane B_2; PGE_2, prostaglandin E_2; PGA_2, 13,14-dihydro-15-keto prostaglandin A_2.

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RECENT PESTICIDE RESEARCH IN CHINA

Appendix T

Ulven et al., "Metabolic Effects of Krill Oil are Essentially Similar to Those of Fish Oil But at Lower Dose of EPA and DHA, in Health Volunteers" Lipids 46: 37-46 (2011)

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ORIGINAL ARTICLE

Metabolic Effects of Krill Oil are Essentially Similar to Those of Fish Oil but at Lower Dose of EPA and DHA, in Healthy Volunteers

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Abstract The purpose of the present study is to investigate the effects of krill oil and fish oil on serum lipids and markers of oxidative stress and inflammation and to evaluate if different molecular forms, triacylglycerol and phospholipids, of omega-3 polyunsaturated fatty acids (PUFAs) influence the plasma level of EPA and DHA differently. One hundred thirteen subjects with normal or slightly elevated total blood cholesterol and/or triglyceride levels were randomized into three groups and given either six capsules of krill oil ($N = 36$; 3.0 g/day, EPA + DHA = 543 mg) or three capsules of fish oil ($N = 40$; 1.8 g/day, EPA + DHA = 864 mg) daily for 7 weeks. A third group did not receive any supplementation and served as controls ($N = 37$). A significant increase in plasma EPA, DHA, and DPA was observed in the subjects supplemented with n-3 PUFAs as compared with the controls, but there were no significant differences in the changes in any of the n-3 PUFAs between the fish oil and the krill oil groups. No statistically significant differences in changes in any of the serum lipids or the markers of oxidative stress and inflammation between the study groups were observed. Krill oil and fish oil thus represent comparable dietary sources of n-3 PUFAs, even if the EPA + DHA dose in the krill oil was 62.8% of that in the fish oil.

Keywords Plasma lipoproteins · Plasma lipids · Dietary fat · Nutrition, n-3 fatty acids · Lipid absorption · Phospholipids

Abbreviations

EPA	Eicosapentaenoic acid
DHA	Docosahexaenoic acid
FA	Fatty acid
PL	Phospholipids
PUFA	Polyunsaturated fatty acid
TG	Triglycerides

Introduction

An association between consumption of fish and seafood and beneficial effects on a variety of health outcomes has been reported in epidemiologic studies and clinical trials [1–5]. These effects are mainly attributed to the omega-3

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long-chain polyunsaturated fatty acids (n-3 PUFAs) abundant in fish and seafood, and in particular to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The effects of marine n-3 PUFAs on various risk factors of cardiovascular disease (CVD) are in particular well documented. In large systematic reviews of the available literature, consistent reductions in triglyceride (TG) levels following consumption of n-3 PUFAs have been demonstrated as well as increases in levels of high-density lipoprotein (HDL) cholesterol [6, 7]. The net beneficial effects of these changes have been disputed, although several large intervention studies indicate that n-3 PUFAs reduce mortality in patients with high risk of developing coronary heart disease (CHD) [8]. Moreover, guidelines published by the American Heart Association for reducing CVD risk recommend fish consumption and fish oil supplementation based on the acknowledgement that EPA and DHA may decrease the risk of CHD, decrease sudden deaths, decrease arrhythmias, and slightly lower blood pressure [9].

Reports on health benefits have led to increased demand for products containing marine n-3 PUFAs. Since fish is a restricted resource, there is growing interest in exploiting alternative sources of marine n-3 PUFAs. Antarctic krill (*Euphausia superba*) is a rich source of n-3 PUFAs. Krill is by far the most dominant member of the Antarctic zooplankton community in terms of biomass, which is estimated to be between 125 and 750 million metric tons (according to the Food and Agriculture Organization of the United Nations; http://www.fao.org/fishery/species/3393/en), and thus attractive for commercial harvest. The DHA content of krill oil is similar to that of oily fish, but the EPA content is higher [10]. The overall fatty acid composition resembles that of fish. In fish, the fatty acids are mainly stored as TG, whereas in krill 30–65% of the fatty acids are incorporated into phospholipids (PL) [10]. Whether being esterified in TG or in PL impacts on the absorption efficiency of FAs into the blood and on effects on serum lipid levels are issues for discussion. In a study by Maki et al. [11] comparing the absorption efficacy of n-3 PUFAs from different sources it was shown that EPA and DHA from krill oil were absorbed at least as efficiently as EPA and DHA from menhaden oil (TG) [11], and studies in newborn infants have indicated that fatty acids in dietary PL may be better absorbed than those from TG [12–14]. Studies addressing the compartmental metabolism of dietary DHA have indicated that the metabolic fate of DHA differs substantially when ingested as TG compared with phosphatidylcholine, in terms of both bioavailability of DHA in plasma and accumulation in target tissues [15]. Only a limited number of studies addressing health outcomes following ingestion of krill oil as compared with fish oil are currently available, but some of these have shown promising effects of krill oil on serum lipids and on markers of inflammation and oxidative stress (reviewed in [10]).

The aim of this study is to investigate the plasma levels of EPA and DHA, and the effects on serum lipids and on some biomarkers of inflammation, oxidative stress, and hemostasis, after krill oil and fish oil administration in healthy subjects after a 7-week intervention period. Safety was evaluated based on assessment of hematology and biochemistry parameters, and registration of adverse events.

Experimental Procedures

Study Subjects

The 129 subjects included in the study were healthy volunteers of both genders with normal or slightly elevated total blood cholesterol (<7.5 mmol/L) and normal or slightly elevated blood triglyceride level (<4.0 mmol/L). Subjects with body mass index (BMI) >30 kg/m^2, hyperlipidemia, hypertension, coronary, peripheral or cerebral vascular disease were excluded from participating in the study. No concomitant medication intended to influence serum lipid level was permitted. All study subjects were informed verbally and in writing, and all subjects signed an informed consent form before entering the study. The study was approved by the Regional Ethics Committee.

Study Design

The study was an open single-center, randomized, parallel group designed study. Screening of subjects ($N = 149$) was performed at the first visit to include subjects that satisfied the eligibility criteria ($N = 129$). These were randomized into three study groups. Seven participants were lost before the baseline visit. The remaining 122 participants were given either 3 g krill oil daily ($N = 41$), 1.8 g fish oil daily ($N = 40$) or no supplementation ($N = 41$) for a period of 7 weeks. A total of 115 participants finished the study. The disposition of the subjects is illustrated in Fig. 1. None of the subjects regularly ate fatty fish more than once a week prior to inclusion or during the 7-week intervention period. None were using cod liver oil or other marine n-3 supplements during the study or at least 2 months prior to inclusion. All the participants were instructed by a nutritionist to keep their regular food habits during the study.

Study Products

The krill oil capsules contained processed krill oil extracted from Antarctic krill (*Euphausia superba*). The product was

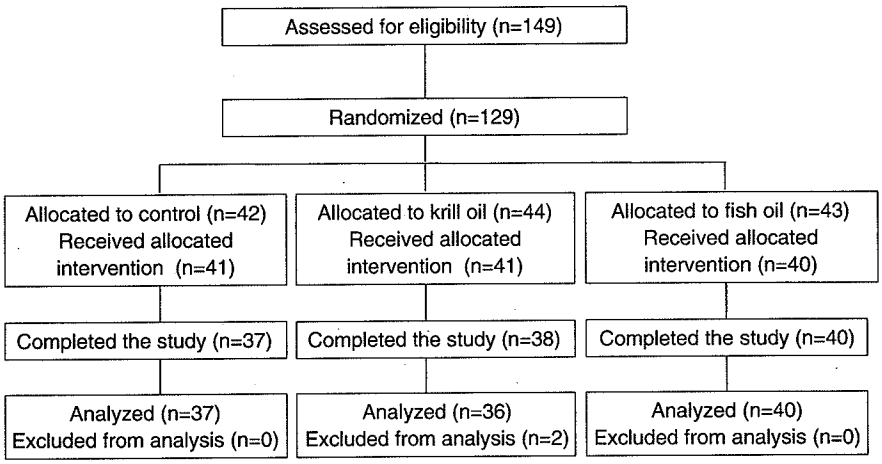

Fig. 1 Disposition of subjects

manufactured by Aker BioMarine ASA. Each capsule contained 500 mg oil that provided 90.5 mg EPA and DHA, and a total of 103.5 mg n-3 PUFAs. The capsules were made of gelatin softened with glycerol. The daily study dosage was six capsules (each of 500 mg oil). The comparator omega-3 fish oil product was manufactured by Peter Möller AS, Oslo, Norway. The daily study dosage was three capsules each containing 600 mg fish oil that provided 288 mg EPA and DHA, and a total of 330 mg n-3 PUFAs. The capsules were made of gelatin softened with glycerol. The fatty acid profile of the study products is presented in Table 1. The daily dose of EPA, DHA, and total n-3 PUFAs in the krill oil and fish oil groups is presented in Table 2. The daily EPA + DHA dose in the krill oil group was 62.8% of the dosage given in the fish oil group. DL-α-tocopheryl acetate (vitamin E), retinyl palmitate (vitamin A), and cholecalciferol (vitamin D) were added to the product.

Table 1 Relative content of fatty acids in the study products

Fatty acid	Fish oil (area %)	Krill oil (area %)
14:0	3.2	7.4
16:0	7.8	21.8
18:0	2.6	1.3
20:0	0.6	<0.1
22:0	0.4	0.2
16:1n-7	3.9	5.4
18:1n-9, -7, -5	6.1	18.3
20:1n-9, -7	2.0	1.2
22:1n-11, -9, -7	2.5	0.8
24:1n-9	<0.2	0.2
16:2n-4	0.7	0.5
18:2n-6	0.8	1.8
18:3n-6	<0.2	0.2
20:2n-6	0.3	<0.1
20:3n-6	0.2	<0.1
20:4n-6	1.5	0.5
22:4n-6	0.5	<0.1
18:3n-3	0.5	1.0
18:4n-3	1.9	1.6
20:3n-3	<0.2	<0.1
20:4n-3	<0.2	0.7
20:5n-3	27.0	19.0
21:5n-3	1.5	0.5
22:5n-3	4.8	0.5
22:6n-3	24.0	10.9
Other FA	7.2	6.4
Saturated FA	16.0	30.7
Monounsaturated FA	18.0	25.9
n-3	59.0	34.1
n-6	2.9	2.5

Clinical Assessment

Demographic characteristics (gender, age, height, and weight), concomitant medication, and medical history were recorded at the screening visit. In addition, all subjects went through a physical examination to confirm satisfaction of the eligibility criteria.

Changes in concomitant medication from screening, smoking and alcohol habits, and clinical symptoms before intake of the study products were also registered.

Serum Lipids and Blood Safety Parameters

Blood from venipuncture was collected after an overnight fast (≥12 h) at baseline and at final visit. The subjects were instructed to refrain from alcohol consumption and from vigorous physical activity the day before the blood sampling. Serum was obtained from silica gel tubes (BD Vacutainer), kept at room temperature for at least 30 min until centrifugation at 1,300 × *g* for 12 min at room temperature. Serum analysis of total, low-density lipoprotein

Table 2 n-3 fatty acid contents of the study products

Study product	Daily study dose	Daily dose EPA	Daily dose DHA	Daily dose EPA + DHA	Daily dose n-3 PUFAs
Fish oil	3 capsules (1.8 g oil)	450 mg	414 mg	864 mg	990 mg
Krill oil	6 capsules (3.0 g oil)	348 mg	195 mg	543 mg	621 mg

(LDL), and HDL-cholesterol, TG, apolipoprotein A1, and apolipoprotein B was performed at the routine laboratory at Department of Medical Biochemistry at the National Hospital, Norway using standard methods. Blood samples for analysis of hematology and serum biochemistry parameters including hemoglobin, leukocytes, erythrocytes, thrombocytes, hematocrit, glucose, calcium, sodium, potassium, urea, creatinine, alkaline phosphatase, alanine aminotransferase, bilirubin, albumin, and total protein were collected and analyzed at the routine laboratory at Department of Medical Biochemistry at the National Hospital, Norway using standard methods.

Plasma Fatty Acid Composition

Plasma was obtained from ethylenediamine tetraacetic acid (EDTA) tubes (BD Vacutainer) kept on ice immediately and within 12 min centrifuged at 1,300 × *g* for 10 min at 10°C. Plasma samples were kept frozen at −80°C until analysis. Plasma fatty acid composition was analyzed by Jurilab Ltd., Finland using a slight modification of the method of Nyyssonen et al. [16]. Plasma (250 μL), fatty acids, and 25 μL internal standard (eicosane 1 mg/mL in isopropanol) were extracted with 6 mL methanol–chloroform (1:2), and 1.5 mL water was added. The two phases were separated by centrifugation, and the upper phase was discarded. To the chloroform phase, 1 mL methanol–water (1:1) was added, and this extraction was repeated twice. The chloroform phase was evaporated under nitrogen. For methylation, the remainder was treated with 1.5 mL sulfuric acid–methanol (1:50) at 85°C for 2 h. The mixture was diluted with 1.5 mL water and extracted with light petroleum ether. The fatty acids from the ether phase were determined using a gas chromatograph (Agilent Technologies 6890)/mass spectrometer (Agilent Technologies 5973) with electron impact ionization and a HP-5ms capillary column (Hewlett Packard). For retention time and quantitative standardization, fatty acids purchased from Nu-Chek-Prep (Elysian, MN, USA) were used. All work was carried out under a certified ISO 9001/2000 quality system.

Plasma α-Tocopherol

Human plasma (100 μL) was diluted with 300 μL 2-propanol containing the internal standard tocol and butylated hydroxytoluene (BHT) as an antioxidant. After thorough mixing (15 min) and centrifugation (10 min, 4,000 × *g* at 10°C), an aliquot of 1 μL was injected from the supernatant into the high-performance liquid chromatography (HPLC) system. HPLC was performed with a HP 1100 liquid chromatograph (Agilent Technologies, Palo Alta, CA, USA) with a HP 1100 fluorescence detector (emission 295 nm, excitation 330 nm). Tocopherol isomers were separated on a 2.1 × 250 mm reversed-phase column. The column temperature was 40°C. A two-point calibration curve was made from analysis of a 3% albumin solution enriched with known concentration of tocopherols. Recovery is >95%, the method is linear from 1 to 200 μM at least, and the limit of detection is 0.01 μM. Relative standard deviation (RSD) is 2.8% (17.0 μM) and 4.6% (25.1 μM).

Urinary F2 Isoprostanes

Fasting urine samples were analyzed for 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) by a highly specific and validated radioimmunoassay as described by Basu [17]. Urinary levels of 8-iso-$PGF_{2\alpha}$ were adjusted by dividing the 8-iso-$PGF_{2\alpha}$ concentration by that of creatinine.

Markers of Inflammation and Hemostasis

Plasma interleukin-6 (IL-6), tumour necrosis factor-alpha (TNFα), monocyte chemotactic protein-1 (MCP-1), thromboxane B_2 (TxB_2), interferon-gamma (INFγ), soluble E-selectin and P-selectin, soluble intracellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) were determined by Fluorokine® MAP kits (R&D Systems, Inc., Minneapolis, MN, USA). Plasma leukotriene B_4 (LTB_4) and thromboxane B_2 (TxB_2) were assessed as described by Elvevoll et al. [18]. High-sensitivity C-reactive protein (hsCRP) evaluation was performed at the routine laboratory at Department of Medical Biochemistry at the National Hospital, Norway using standard method.

Statistics

All continuous variables were summarized by product group and visit number and described using standard statistical measures, i.e., number of observations, mean,

standard deviation (SD), median, minimum, and maximum. Absolute and percentage change from baseline to the week-7 visit are presented as summary statistics. All categorical (discrete, including ordinal) variables are presented in contingency tables showing counts and percentages for each treatment group at all time points. Continuously distributed efficacy laboratory parameters (lipids, EPA, DHA, and docosapentaenoic acid (DPA)) were analyzed by analysis of covariance (ANCOVA) using the following model: change in parameter value = baseline value + treatment + gender + age + error.

Change from baseline to week 7 was used as a dependent variable in the model. A linear model using SAS GLM with gender as fixed effect, subject as random effect, and baseline value and age as covariates was applied. A reduced ANCOVA model with baseline value and treatment was used for the secondary efficacy parameters. Significant treatment effects were analyzed by pairwise tests. Changes from baseline to end of intervention were tested by paired *t*-test.

Results

Subject Characteristics at Baseline

Figure 1 shows the disposition of all subjects. One hundred fifteen of 129 randomized subjects completed the study. Withdrawal rates were similar in all three groups (three withdrawals in the fish oil group, six withdrawals in the krill oil group, and five withdrawals in the control group). Three subjects discontinued the study due to clinical symptoms (all in the krill group), three subjects violated the exclusion criteria (two in the fish oil group and one in the krill oil group), and one subject in each group was lost to follow-up. Two subjects in the control group were withdrawn due to concomitant treatments, and three subjects withdrew their consent (one subject in the krill oil group and two subjects in the control group). Clinical symptoms included symptoms of common cold or gastrointestinal symptoms. During database clean-up, it was detected that two subjects (in the krill oil group) had been allowed to enter the study although they violated the entry criteria. They where therefore excluded from the per protocol subjects. The statistical analyses of efficacy were performed on the data collected from 113 per protocol subjects (Fig. 1).

The study groups were comparable in terms of weight, height, BMI, gender, and age at baseline (Table 3). Vital signs including systolic and diastolic blood pressure and heart rate were within normal ranges. More females than males were included in all study groups.

Table 3 Demographic information and body measurements

Parameter, mean (SD)	Study groups		
	Fish oil ($N = 43$)	Krill oil ($N = 44$)	Control ($N = 42$)
Age (years)	38.7 (11.1)	40.3 (14.8)	40.5 (12.1)
Height (cm)	171.2 (7.8)	171.3 (8.6)	172.2 (9.4)
Weight (kg)	71.7 (12.0)	69.8 (13.7)	71.7 (12.0)
BMI (kg/m^2)	24.4 (3.0)	23.6 (3.3)	23.9 (3.0)
Gender			
Female (*n*)	34 (79.1%)	31 (70.5%)	28 (66.7%)
Male (*n*)	9 (20.9%)	13 (29.5%)	14 (33.3%)

Fatty Acid Composition in Plasma

Plasma levels of EPA, DHA, and DPA increased significantly from baseline to the end of the intervention phase in the groups receiving fish oil and krill oil, but not in the control group. The changes in EPA, DHA, and DPA differed significantly between the subjects supplemented with n-3 PUFAs and the subjects in the control group, but there was no significant difference in the change in any of the n-3 PUFAs between the fish oil and the krill oil groups (Table 4).

There were significant within-group changes in individual FAs from start to end of intervention, but no clear trends in changes in the plasma FA composition were apparent in any of the study groups (Table 4).

The level of arachidonic acid (C20:4n-6) increased from baseline in the krill group, whereas a decrease was observed in the fish oil group. The changes in arachidonic acid between the fish oil and the krill oil groups, and the control group differed significantly ($p = 0.001$). Pairwise comparisons showed that the mean increase in arachidonic acid in the krill oil group was significantly different from the mean decreases in the fish oil and control groups, but there was no significant difference between the mean changes in arachidonic acid level between the fish oil and control groups.

Serum Lipids

Small changes in the levels of HDL-cholesterol, LDL-cholesterol, and TG were observed in all study groups from start to end of the intervention phase, but only the within-group increase in LDL-cholesterol seen in the fish oil group ($p = 0.039$) was statistically significant. The tests comparing the differences between the study groups gave no statistically significant results (Table 5). The HDL-cholesterol/TG ratio and the change from start to end of the intervention were calculated for all study groups. No significant changes in the HDL-cholesterol/TG ratio from start to end of the interventions were detected in the fish oil or

Table 4 Fatty acid composition in plasma

Parameter (μmol/L)	Treatment	*N*	Baseline	End of study	Change	*p*-Value[a] for change	*p*-Value[b] between groups
C14:0 myristic acid	Fish oil	40	67.4 ± 57.07	69.4 ± 62.32	2.0 ± 44.05	0.77	
	Krill oil	36	55.5 ± 32.43	57.8 ± 26.15	2.3 ± 30.93	0.65	0.71
	Control	37	60.5 ± 29.25	58.1 ± 38.01	−2.4 ± 35.46	0.69	
C15:0 pentadecanoic acid	Fish oil	40	18.2 ± 14.42	16.2 ± 8.22	−2.1 ± 12.01	0.28	
	Krill oil	36	14.7 ± 5.03	15.5 ± 3.69	0.8 ± 4.44	0.30	0.27
	Control	37	15.0 ± 5.02	15.0 ± 5.26	0.1 ± 4.32	0.94	
C16:0 palmitic acid	Fish oil	40	1,661.0 ± 496.5	1,522. ± 339.3	−139.0 ± 409.5	0.038	
	Krill oil	36	1,548.9 ± 477.9	1,547. ± 260.0	−2.3 ± 414.9	0.97	0.35
	Control	37	1,652.7 ± 374.2	1,578. ± 315.6	−74.6 ± 327.7	0.17	
C16:1n-7 palmitoleic acid	Fish oil	40	67.7 ± 35.41	63.0 ± 33.54	−4.7 ± 27.91	0.29	
	Krill oil	36	66.1 ± 49.18	61.8 ± 26.56	−4.4 ± 35.91	0.47	0.62
	Control	37	68.7 ± 32.98	63.9 ± 35.07	−4.8 ± 28.62	0.31	
C17:0 margaric acid	Fish oil	40	24.2 ± 8.38	23.9 ± 8.44	−0.3 ± 6.09	0.76	
	Krill oil	36	22.6 ± 5.85	24.0 ± 5.84	1.4 ± 5.49	0.14	0.089
	Control	37	23.3 ± 5.46	21.7 ± 5.84	−1.6 ± 4.85	0.048	
C18:0 stearic acid	Fish oil	40	580.6 ± 136.6	578.5 ± 130.2	−2.2 ± 133.2	0.92	
	Krill oil	36	548.5 ± 116.7	568.9 ± 112.6	20.4 ± 91.7	0.19	0.17
	Control	37	594.8 ± 103.5	562.4 ± 147.2	−32.4 ± 125.4	0.12	
C18:1n-9 oleic acid	Fish oil	40	558.9 ± 166.1	516.2 ± 146.8	−42.7 ± 154.0	0.087	
	Krill oil	36	532.8 ± 198.5	521.8 ± 109.1	−11.0 ± 191.0	0.73	0.71
	Control	37	570.2 ± 146.8	547.2 ± 156.1	−23.0 ± 151.9	0.36	
C18:2n-6 linoleic acid	Fish oil	40	829.3 ± 349.8	779.8 ± 254.5	−49.6 ± 251.3	0.22	
	Krill oil	36	742.0 ± 214.0	744.2 ± 187.9	2.2 ± 215.8	0.95	0.44
	Control	37	812.0 ± 219.6	735.7 ± 212.4	−76.3 ± 167.5	0.0088	
C18:3n-3 alpha-linoleic acid	Fish oil	40	61.7 ± 17.89	61.9 ± 20.22	0.2 ± 20.95	0.95	
	Krill oil	36	67.3 ± 25.24	68.4 ± 21.88	1.1 ± 20.08	0.73	0.15
	Control	37	68.3 ± 20.40	62.8 ± 22.07	−5.5 ± 19.55	0.094	
C20:3n-3 eicosatrienoic acid	Fish oil	40	39.6 ± 18.08	33.5 ± 17.97	−6.0 ± 10.00	0.0005	
	Krill oil	36	39.3 ± 19.20	34.9 ± 13.20	−4.4 ± 13.23	0.054	0.22
	Control	37	39.8 ± 18.24	37.7 ± 18.28	−2.1 ± 8.65	0.16	
C20:4n-6 arachidonic acid	Fish oil	40	192.6 ± 50.0	178.5 ± 45.3	−14.1 ± 29.6	0.0046	
	Krill oil	36	180.1 ± 52.4	192.1 ± 40.2	12.0 ± 32.8	0.035	0.0010
	Control	37	189.8 ± 44.2	182.8 ± 38.0	−7.0 ± 32.3	0.20	
C22:0 behenic acid	Fish oil	40	21.5 ± 6.05	21.8 ± 6.36	0.4 ± 3.30	0.49	
	Krill oil	36	20.2 ± 5.29	22.0 ± 6.25	1.9 ± 3.51	0.003	0.040
	Control	37	18.9 ± 6.02	18.3 ± 4.83	−0.6 ± 4.07	0.36	
C24:0 lignoceric acid	Fish oil	40	10.0 ± 4.07	10.3 ± 4.19	0.3 ± 1.71	0.22	
	Krill oil	36	9.7 ± 2.86	10.4 ± 3.15	0.7 ± 2.04	0.040	0.26
	Control	37	8.9 ± 3.37	8.5 ± 3.05	−0.4 ± 2.57	0.40	
C24:1n-9 neuronic acid	Fish oil	40	18.2 ± 6.88	18.9 ± 6.15	0.6 ± 3.75	0.29	
	Krill oil	36	16.7 ± 5.99	17.3 ± 5.87	0.6 ± 5.00	0.48	0.17
	Control	37	15.7 ± 5.39	16.7 ± 5.70	1.0 ± 5.67	0.30	
C20:5n-3 EPA	Fish oil	40	31.2 ± 23.11	76.3 ± 36.02	45.2 ± 29.65	<0.0001	
	Krill oil	36	30.4 ± 21.57	74.9 ± 38.66	44.5 ± 35.21	<0.0001	<0.0001
	Control	37	43.9 ± 40.74	37.2 ± 28.64	−6.6 ± 28.58	0.17	

Table 4 continued

Parameter (μmol/L)	Treatment	*N*	Baseline	End of study	Change	*p*-Value[a] for change	*p*-Value[b] between groups
C22:6n-3 DHA	Fish oil	40	47.0 ± 22.08	70.4 ± 25.70	23.4 ± 16.55	<0.0001	
	Krill oil	36	44.8 ± 21.36	64.2 ± 26.15	19.4 ± 23.75	<0.0001	<0.0001
	Control	37	57.4 ± 30.94	51.3 ± 23.70	−6.1 ± 21.25	0.088	
C22:5n-3 DPA	Fish oil	40	8.8 ± 3.98	12.7 ± 5.06	3.9 ± 3.24	<0.0001	
	Krill oil	36	8.2 ± 3.33	11.9 ± 3.56	3.6 ± 3.68	<0.0001	<0.0001
	Control	37	9.6 ± 5.09	8.6 ± 3.84	−1.0 ± 3.56	0.090	
Total fatty acids	Fish oil	40	4,250.7 ± 1,148.1	4,064.6 ± 890.8	−186.0 ± 921.8	0.21	
	Krill oil	36	3,958.1 ± 983.7	4,045.5 ± 662.5	87.4 ± 810.7	0.52	0.15
	Control	37	4,261.3 ± 804.2	4,016.8 ± 774.4	−244.5 ± 685.5	0.037	

[a] Test of within-group changes

[b] Test comparing change from start to end of intervention between the fish oil, krill oil, and control groups

control groups. In the krill oil group, however, there was a significant increase in the HDL-cholesterol/TG ratio. The test for differences between the study groups gave no significant results (Table 5).

Although the interventions did not significantly change TG levels a reduction was seen in those subjects in the krill oil group having the highest baseline values (Fig. 2).

The changes in levels of Apo B-100 from baseline to end of study were minor in all study groups. Moreover, the test for differences between the study groups in changes in Apo A1 was not significant. However, the within-group changes of Apo A1 levels from start to end of the interventions were statistically significant in the krill oil group.

Table 5 Serum lipids and lipoproteins

Parameter	Treatment	*N*	Baseline	End of study	Change	*p*-Value[a] for change	*p*-Value[b] between groups
HDL-cholesterol (mmol/L)	Fish oil	40	1.56 ± 0.384	1.61 ± 0.396	0.05 ± 0.157	0.063	
	Krill oil	36	1.50 ± 0.368	1.63 ± 0.517	0.13 ± 0.404	0.061	0.50
	Control	37	1.59 ± 0.354	1.63 ± 0.395	0.04 ± 0.228	0.29	
LDL-cholesterol (mmol/L)	Fish oil	40	2.96 ± 0.747	3.09 ± 0.827	0.13 ± 0.377	0.039	
	Krill oil	36	3.07 ± 0.724	3.16 ± 0.796	0.09 ± 0.390	0.18	0.45
	Control	37	2.98 ± 0.824	3.03 ± 0.802	0.05 ± 0.361	0.44	
Triglycerides (mmol/L)	Fish oil	40	0.95 ± 0.541	0.94 ± 0.542	−0.01 ± 0.462	0.84	
	Krill oil	36	1.10 ± 0.638	1.01 ± 0.649	−0.09 ± 0.417	0.21	0.65
	Control	37	0.92 ± 0.414	0.93 ± 0.523	0.02 ± 0.429	0.82	
HDL/triglycerides (%)	Fish oil	40	225.8 ± 151.08	216.8 ± 119.33	109.5 ± 44.62	0.19	
	Krill oil	36	196.9 ± 134.24	228.3 ± 146.62	129.2 ± 68.99	0.016	0.41
	Control	37	217.7 ± 138.65	234.4 ± 148.20	113.0 ± 49.28	0.12	
Total-cholesterol (mmol/L)	Fish oil	40	4.93 ± 0.778	5.13 ± 0.809	0.20 ± 0.424	0.0049	
	Krill oil	36	4.99 ± 0.815	5.20 ± 0.917	0.21 ± 0.496	0.014	0.78
	Control	37	4.95 ± 0.925	5.07 ± 0.861	0.12 ± 0.524	0.18	
Apo A1 (mmol/L)	Fish oil	40	1.64 ± 0.269	1.68 ± 0.250	0.04 ± 0.130	0.058	
	Krill oil	36	1.64 ± 0.241	1.73 ± 0.376	0.09 ± 0.267	0.047	0.70
	Control	37	1.68 ± 0.272	1.75 ± 0.272	0.07 ± 0.173	0.023	
Apo B-100 (mmol/L)	Fish oil	40	0.81 ± 0.184	0.80 ± 0.199	−0.01 ± 0.100	0.64	
	Krill oil	36	0.83 ± 0.208	0.81 ± 0.226	−0.02 ± 0.126	0.35	0.80
	Control	37	0.79 ± 0.197	0.78 ± 0.198	−0.01 ± 0.098	0.41	

[a] Test of within-group changes

[b] Test comparing change from start to end of intervention between the fish oil, krill oil, and control groups

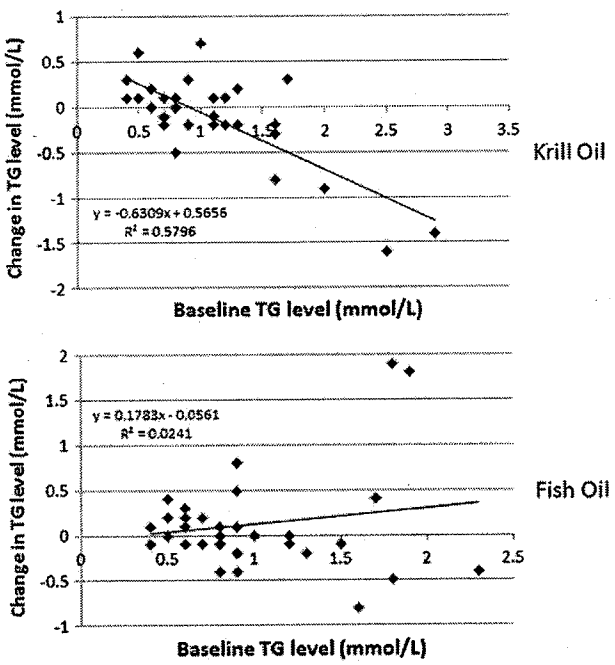

Fig. 2 Correlation between baseline TG levels and change in TG levels after 7 weeks of intervention with krill oil or fish oil

Oxidative Stress, Markers of Inflammation, and Hemostasis

α-Tocopherol is considered an antioxidant, and an increase in PUFAs may lead to increased oxidative stress. Although α-tocopherol was added to both supplements, no significant change in levels of α-tocopherol was detected (Supplementary Table). A tendency towards a reduced level of α-tocopherol was observed in all study groups. F2-isoprostanes, formed from free-radical-induced peroxidation of membrane-bound arachidonic acid, are considered a reliable biomarker of oxidative stress. However no differences were observed in urine F2-isoprostane, suggesting that there was not an increase in oxidative stress. No significant changes were observed in levels of hsCRP, markers of inflammation or hemostasis (Supplementary Table).

Discussion

The primary finding of the present study was that plasma concentrations of EPA, DPA, and DHA increased significantly in both the krill oil and fish oil groups compared with the control group following daily supplementation for 7 weeks. There was no statistically significant difference between these two groups in the levels of the increases in EPA and DHA. Since the subjects in the krill oil group received 62.8% of the total amount of n-3 PUFAs received by the subjects in the fish oil group, these findings indicate that the bioavailability of n-3 PUFAs from krill oil (mainly PL) is as, or possibly more, efficient as n-3 PUFA from fish oil (TG). This supports the results of a previous study with krill oil and menhaden oil in humans [11]. In the study performed by Maki et al., plasma EPA increased 90% and DHA increased 51% from baseline levels. In the current study EPA increased 146% and DHA increased 43% from baseline levels. The small discrepancy between these two studies might be related to different levels of EPA and DHA in the oils used, different treatment time (7 versus 4 weeks), and different dose used (3 g oil versus 2 g). It has been hypothesized that PL improve the bioavailability of lipids, which may facilitate absorption of EPA and DHA from marine PL compared with TG, but the extent to which this contributes to the efficient absorption observed in the krill oil group is unknown.

AHA dietary guidelines for long-chain n-3 PUFAs and fish for primary prevention of coronary diseases are two servings of fatty fish per week [9]. This recommendation will provide the order of 250–500 mg EPA + DHA per day [19]. In the present study we have shown that daily intake of 3 g krill oil containing 543 mg EPA + DHA increases the plasma level of EPA and DHA to the same extent as intake of fish oil containing 864 mg EPA + DHA. A food-based approach for achieving adequate intake of n-3 PUFAs is recommended [20]. However, for some individuals nutritional supplements may be needed, such as those who do not like fish or for other reasons choose not to include fish in their diet. This study demonstrates that supplementation with krill oil will be a good source of EPA and DHA in their daily diet.

Serum TG and HDL-cholesterol have been observed to be inversely related [21]. Although the metabolic relation that exists between HDL-cholesterol and TG is not fully understood, the ratio between TG and HDL-cholesterol has been shown to be a powerful risk predictor for CHD [22, 23]. In the present study, no statistically significant differences in HDL-cholesterol, TG or HDL-cholesterol/TG ratio were observed between the study groups. However, the change in the HDL-cholesterol/TG ratio in the krill oil group was statistically significant (Table 5). This observation supports the impression of a more pronounced effect of krill oil supplementation on HDL-cholesterol and TG compared with other n-3 PUFA supplements. However, to verify these effects of krill oil, they should be studied in a population with elevated blood TG levels and lowered HDL-cholesterol, i.e., in a population with markers of metabolic syndrome. The increase in HDL-cholesterol was slightly higher in the krill oil group than in the fish oil group (8.7% versus 3.2%), although not significantly so ($p = 0.061$). Compared with fish oil, krill oil contains a high amount of astaxanthin, which has been indicated to

increase HDL-cholesterol as well as decrease TG in humans [24]. Moreover, intake of PL may increase HDL-cholesterol [25]. The small increase in LDL-cholesterol, but no effect on HDL-cholesterol, in the fish oil group is in accordance with previous findings [7].

The analysis of the changes in the plasma fatty acid composition following 7 weeks of intervention with n-3 PUFAs showed that the levels of arachidonic acid and behenic acid significantly increased from baseline in the krill oil group as compared with the fish oil and control groups. Moreover, arachidonic acid was significantly decreased in the fish oil group. Intake of n-3 PUFAs from fish oil can be incorporated in cell PL in a time- and dose-dependent manner at the expense of arachidonic acid [26]. The explanation and importance of this finding are not clear. However, one possible explanation might be that arachidonic acid is mobilized from the cell membranes to the blood by EPA and DHA linked to the PL in the krill oil. However, the changes in plasma arachidonic acid were small compared with the changes in EPA and DHA, and there was no significant difference in the increase in EPA/arachidonic acid ratio between the two intervention groups.

The CRP level did not change during the study in any of the study groups, and no significant changes were observed in the other markers of inflammation and hemostasis (data not shown). This is in accordance with others who have examined the effect of fish oil among apparently healthy individuals [27–31]. Moreover, no statistically significant differences were found in α-tocopherol levels and F2-isoprostanes in urine, suggesting that no oxidative stress occurred.

The safety analysis revealed no clear patterns in the changes in any of the hematological or serum biochemical variables, vital signs or weight that might indicate a relation with administration of any of the studied products. Clinical symptoms registered during the study included mainly symptoms of common cold or gastrointestinal symptoms. However, one subject in the fish oil group experienced moderate bruises, and one subject in the krill oil group withdrew from the study because of an outbreak of rash that was possibly related to intake of the study products. Safety laboratory parameters and other safety observations such as occurrence of adverse events indicate that krill oil is well tolerated. There were no apparent differences in the rate of adverse events or blood safety parameters between the krill oil, fish oil or control groups.

In conclusion, the present study shows that n-3 PUFAs from krill oil in the form of PL are readily and effectively absorbed after ingestion and subsequently distributed in the blood. The krill oil supplement is safe and well tolerated. Krill oil thus represents a valuable source of n-3 PUFAs.

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Appendix U

Sampalis et al., "Evaluation of the Effects of Neptune Krill Oil™ on the Management of Premenstrual Syndrome and Dysmenorrheal" Altern Med Rev 8: 171-9 (2003)

Evaluation of the Effects of Neptune Krill Oil™ on the Management of Premenstrual Syndrome and Dysmenorrhea

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Abstract

PRIMARY OBJECTIVE: To evaluate the effectiveness of Neptune Krill Oil™ (NKO™) for the management of premenstrual syndrome and dysmenorrhea. SECONDARY OBJECTIVE: To compare the effectiveness of NKO for the management of premenstrual syndrome and dysmenorrhea with that of omega-3 fish oil. METHODS/ DESIGN: Double-blind, randomized clinical trial. SETTING: Outpatient clinic. PARTICIPANTS: Seventy patients of reproductive age diagnosed with premenstrual syndrome according to the Diagnostic and Statistical Manual of Mental Disorders, Third Edition, Revised (DSM-III-R). INTERVENTIONS: Treatment period of three months with either NKO or omega-3 fish oil. OUTCOME MEASURES: Self-Assessment Questionnaire based on the American College of Obstetricians & Gynecologists (ACOG) diagnostic criteria for premenstrual syndrome and dysmenorrhea and number of analgesics used for dysmenorrhea. RESULTS: In 70 patients with complete data, a statistically significant improvement was demonstrated among baseline, interim, and final evaluations in the self assessment questionnaire ($p<0.001$) within the NKO group as well as between-group comparison to fish oil, after three cycles or 45 and 90 days of treatment. Data analysis showed a significant reduction of the number of analgesics used for dysmenorrhea within the NKO group (comparing baseline vs. 45- vs. 90-day visit). The between-groups analysis illustrated that women taking NKO consumed significantly fewer analgesics during the 10-day treatment period than women receiving omega-3 fish oil ($p <0.03$). CONCLUSION: Neptune Krill Oil can significantly reduce dysmenorrhea and the emotional symptoms of premenstrual syndrome and is shown to be significantly more effective for the complete management of premenstrual symptoms compared to omega-3 fish oil.

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Table 1. Diagnostic Criteria for PMS

A. In most menstrual cycles during the past year, symptoms occurred during the last week of the luteal phase and remitted within a few days after the onset of the follicular phase. In menstruating females, these phases correspond to the week before, and a few days after, the onset of menses. (In non-menstruating females who have had a hysterectomy, the timing of luteal and follicular phase may require measurement of circulating reproductive hormones.)

B. At least five of the following symptoms have been present for most of the time during each symptomatic late luteal phase, at least one of the symptoms being either (1), (2), (3), or (4):
   1. marked affective lability, e.g., feeling suddenly sad, tearful, irritable, or angry;
   2. persistent and marked anger or irritability;
   3. marked anxiety, tension, feelings of being "keyed up" or "on edge;"
   4. decreased interest in usual activities, e.g., work, friends, hobbies;
   5. easy fatigability or marked lack of energy;
   6. subjective sense of difficulty in concentrating;
   7. marked change in appetite, overeating, or specific food cravings;
   8. hypersomnia or insomnia;
   9. other physical symptoms, such as breast tenderness or swelling, headaches, joint or muscle pain, a sensation of "bloating," weight gain.

C. The disturbance seriously interferes with work or with usual social activities or relationships with others.

D. The disturbance is not merely an exacerbation of the symptoms of another disorder, such as major depression, panic disorder, dysthymia, or a personality disorder.

Criteria A, B, C, and D are confirmed by prospective daily self-rating during at least two symptomatic cycles.

From: Spitzer RL et al. Late luteal phase dysphoric disorder and DSM-III-R. *Am J Psychiatry* 1989; 146: 892-897.

Introduction

Premenstrual syndrome (PMS) and dysmenorrhea are characterized by a combination of cyclic symptoms that occur during the luteal phase of the menstrual cycle. Symptoms are practically absent in the follicular phase of the menstrual cycle, begin at varying points after ovulation, peak in the late luteal phase, and subside with the onset of menses or shortly thereafter. The diagnostic criteria based on the Diagnostic and Statistical Manual of Mental Disorders (DSM III-R), Third Edition, Revised are outlined in Table 1.[1]

Multiple causes have been attributed to the complex interplay of neuroendocrine, chronobiological, and psychosocial interactions that comprise the symptomatology of PMS.[2-8] A review reveals more than 200 symptoms that have been reported to occur premenstrually.[2,7] Depending on the symptom, the causative factors include hormonal imbalance, psychological abnormalities, nutritional deficiency or excess, increased inflammatory prostaglandin synthesis, and neurotransmitter imbalance.[2,8] Nevertheless, laboratory findings vary from being completely normal in some women to an excess or deficiency of certain hormones, neurotransmitters, or nutrients in others. The most probable cause of the physical symptoms of PMS seems to be the combined interaction of hormones and essential nutrients leading to an increased inflammatory response. On the other hand, the emotional symptoms of PMS seem to be propagated by an exaggerated response of neurotransmitters to psychosocial stresses. These imbalances have been shown to differ extensively from person to person, as well as from cycle to cycle within the same person.

Evidence suggests that alpha-tocopherol can significantly reduce symptoms of PMS.[2,9-16] Tocopherol reduces release of arachidonic acid from phospholipids, resulting in a decrease in formation of prostaglandin E_2 (PGE_2) (inflammatory prostaglandins).[16] Alpha-tocopherol, which crosses the blood-brain barrier, also has modulating effects on neurotransmitters.[16] Several studies suggest a beneficial effect of vitamin A for the treatment of PMS, particularly for premenstrual headaches.[17,18]

The balance of polyunsaturated (essential) fatty acids in the body is critical for the maintenance of healthy cell membranes and hormone regulation. During the last several decades, the average Western diet has shifted to much higher levels of omega-6 and less omega-3 fatty acid intake. Long-chain omega-6 fatty acids, such as arachidonic acid, predominating in the phospholipids of cell membranes can encourage the production of pro-inflammatory type-2 prostaglandins (PGE_2), while omega-3 fatty acids promote the production of anti-inflammatory prostaglandins.[19-20]

Omega-6 fatty acids, mainly arachidonic acid, are released following the reduction of progesterone prior to menstruation,[21,22] initiating an inflammatory process by triggering a flux of inflammatory prostaglandins and leukotrienes in the uterus.[21,22] At the same time, cyclooxygenase metabolites of arachidonic acid, prostaglandins E_2 and F_2, cause vasoconstriction and myometrial contractions that lead to ischemia and eventually the pain of menstrual cramps.[21,22]

Research has uncovered abnormal fatty acid metabolism in women with PMS. Brush and colleagues examined plasma fatty acid levels in 42 women with premenstrual syndrome. They found that although levels of linoleic acid, the body's main dietary source of omega-6 fatty acids, were significantly above normal in all the women, levels of its anti-inflammatory metabolites, including gamma-linolenic acid, were all deficient,[23] reinforcing the theory that one of the main causes of PMS is inflammation.

The role of long-chain fatty acids for the management of PMS has been evaluated in several studies.[2,19-27] Omega-3 fatty acids, mainly eicosapentanoic acid (EPA) and docosahexanoic acid (DHA), compete with the omega-6 species for the enzyme prostaglandin synthetase. The omega-3 fatty acids trigger secretion of less potent leukotrienes and anti-inflammatory prostaglandins of the 3 series (PE_3, PI_3,and thromboxane A_3). [22,28-32] The result is a decrease of myometrial contractions and uterine vasoconstriction, relieving ischemia and reducing pain. This effect may be due to an increase in sensitivity of the uterus to the relaxing effects of beta-adrenergic catecholamines.[22,33-37]

While prostaglandin E_1 (PGE_1) inhibits glucose-induced insulin secretion, a deficiency – occurring either as a result of an inadequate intake of essential fatty acids in the form of cis-linoleic acid or problems with conversion to gamma-linolenic acid (GLA) – could result in symptoms of hypoglycemia, cravings for sweets, and increase in appetite reported by many PMS patients.[20,37] Long-chain omega-3 fatty acids in fish oil have been shown to be effective for the control of cravings occurring prior to menstruation.[2,22-32,34-36]

Neptune Krill Oil™ (NKO™) is a natural health product extracted from Antarctic krill also known as *Euphausia superba*. *Euphausia superba*, a zooplankton crustacean, is rich in phospholipids and triglycerides carrying long-chain omega-3 polyunsaturated fatty acids, mainly EPA and DHA, and in various potent antioxidants including vitamins A and E, astaxanthin, and a novel flavonoid (similar to the 6,8-Di-C-glucosyllutcolin, but with two or more glucose molecules and one aglycone).

Patients, Materials, and Methods

Women of reproductive age who fulfilled the DSM-III-R diagnostic criteria for PMS were eligible for the study. Women who were pregnant or breast feeding, on concomitant anticoagulant therapy, receiving psychotherapy, experiencing alcohol or drug dependence, on sex hormones except for oral contraceptives, with a known seafood allergy, or diagnosed with dementia, pituitary disease, coagulopathy, or a serious medical condition were not eligible for enrollment. Patients taking other dietary supplements or vitamins were asked to stop supplementation for two weeks at which time they were randomized in the trial.

The study was described to potentially eligible patients by the treating physician. Once the patient agreed to participate, an "eligibility form" was completed by the physician according to the patient's answers. If the patient (referred as "she") was eligible, she signed a written Patient Informed Consent Form and was enrolled for randomization. Patients were randomly allocated according to a list of random numbers. Once the patient was randomized to either the NKO or an omega-3 fish oil group, she underwent a physical examination for screening purposes. If the physical examination revealed no reason for the patient to be excluded from the study, she was asked to complete an initial self-assessment questionnaire, as well as to record her usual daily consumption of analgesics (including type and frequency) for menstrual pain.

Each patient periodically answered three identical self-assessment questionnaires, at baseline and one at each of the two follow-up visits. A questionnaire was given to a patient at each visit and she was asked to complete it on her own (no interviewer allowed) in the waiting room, prior to seeing the physician.

Each patient was asked to take two 1-gram soft gels of either NKO or omega-3 18:12 fish oil (fish oil containing 18% EPA and 12% DHA) once daily with meals during the first month of the trial. During the following two months, patients continued on a cyclic dose of two 1-gram soft gels eight days prior to and two days during menstruation. Study medication was given for three months, at which time patients were asked to record all analgesics consumed for menstrual pain. All patients were asked to follow a normal healthy diet consisting of 20-percent fat (less than 10-percent animal fat), 40-percent protein, and 40-percent carbohydrates. Patients were re-evaluated 45 and 90 days after recruitment.

The treating physician, the patient, and the epidemiologist performing the analysis were blinded until the completion of data analysis. The study was performed according to current International Conference on Harmonization guidelines on Good Clinical Practice. Reasons for withdrawal and adverse events were reported immediately. No crossover was allowed.

The primary objective of the study was to evaluate the effectiveness of Neptune Krill Oil for the management of premenstrual syndrome and dysmenorrhea. The secondary objective was to compare the effectiveness of NKO for the management of premenstrual syndrome with that of omega-3 fish oil. The study hypotheses were that Neptune Krill Oil can significantly reduce the physical and emotional symptoms of premenstrual syndrome and be significantly more effective for the management of PMS symptoms than fish oil.

Primary outcome measures were based on the scores of a self-assessment questionnaire for PMS based on the American College of Obstetricians & Gynecologists diagnostic criteria for premenstrual syndrome ranging from 0 (no symptoms) to 10 (unbearable),[37] and the difference in quantity of analgesic consumption for menstrual pain at baseline, 45-day, and 90-day visits. Secondary outcome measures were based on the overall treatment assessment, adverse event monitoring, and compliance checks.

Table 2. Summary of Scores on the Premenstrual Syndrome Self-assessment Questionnaire at each Visit

Symptom	Krill Oil						Control					
	Baseline*	45 days		90 days		Mean diff.**	Baseline*	45 days		90 days		Mean diff.**
		Score (SD)	P-value	Score (SD)	P-value			Score (SD)	P-value	Score (SD)	P-value	
Breast Tenderness	6.9	5.7(2)	<0.001	4.0(2)	<0.001	0.3	5.9	4.9(2)	=0.38	5.0(2)	=0.38	0.2
Overwhelmed	6.7	5.2(2)	<0.001	3.9 (2)	<0.001	0.3	7.0	5.9(1)	=0.06	6.7(2)	=0.40	0.1
Stress	7.2	5.7(2)	<0.001	4.5(3)	<0.001	0.2	6.9	5.4(2)	=0.07	6.1(2)	=0.07	0.1
Irritable	6.0	5.1(2)	<0.001	3.2(2)	<0.001	0.3	5.8	4.9(2)	=0.13	5.2(2)	=0.13	0.1
Depression	6.9	5.4(2)	<0.001	4.2(2)	<0.001	0.2	7.2	5.4(3)	=0.27	6.3(2)	=0.27	0.1
Joint Pain	5.8	4.7(2)	<0.001	2.1(2)	<0.001	0.5	5.2	3.7(3)	=0.18	4.0(2)	=0.18	0.2
Weight Gain	7.5	5.8(2)	<0.001	5.3(3)	<0.001	0.2	8.0	4.7(3)	=0.04	6.8(1)	<0.01	0.2
Abdominal Pain	7.4	5.6(2)	<0.001	4.9(2)	<0.001	0.2	7.0	4.9(4)	=0.04	5.6(3)	<0.001	0.2
Swelling	7.6	5.6(2)	<0.001	4.8(2)	<0.001	0.2	6.9	4.7(3)	=0.07	5.2(2)	<0.001	0.2
Bloating	7.6	6.1(2)	<0.001	6(2)	<0.001	0.1	7.1	6.0(3)	=0.08	6.4(2)	=0.08	0.1

Range:0-10
* Higher scores indicate more severe symptoms.
**Mean difference refers to the mean difference observed between baseline and 90 days of treatment.

Changes over time within the same group (intragroup differences) in the efficacy measures were assessed using the paired Student's t-test. Differences between the two groups, NKO versus fish oil (intergroup variance), with respect to the change in the efficacy measure were assessed with analysis of variance (ANOVA). The study was designed as a prospective, randomized, controlled, double-blind trial, with a 20-percent difference in the change in physical and emotional scores accepted as clinically significant, 90-percent power, and 5-percent significance. Seventy patients were enrolled in the study, randomly assigned to either the active (NKO) or the control (fish oil) group.

Results

Of the 70 patients enrolled, all patients completed the three-month study period. Of those, 36 were randomized to the active group and 34 to the control group. The mean (SD) age of the active group patients was 33(±5) and that of the control group was 32(±7).

As illustrated in Tables 2 and 3, the scores of the self-assessment questionnaire demonstrated a statistically significant difference ($p<0.001$) within the NKO group after intervals of both 45 days (first menstrual cycle) and 90 days (second and third cycle) in both emotional and physical symptoms related to PMS. By contrast, within the fish oil group a statistically significant difference was attained after 45 days only with symptoms of weight gain and abdominal pain ($p<0.04$). Other related physical

symptoms (breast tenderness, joint pain, swelling, and bloating) as well as emotional symptoms (feeling overwhelmed, stress, irritability, and depression) revealed no significant difference between baseline and 45-day follow-up visits. At the 90-day interval or third study visit of the fish oil group, significant differences were observed only with weight gain ($p<0.01$), abdominal pain ($p<0.001$), and swelling ($p<0.001$). All other physical and emotional symptoms revealed no significant difference between baseline and 90-day follow-up visit of patients in the control group.

The analysis of data showed the types of analgesics consumed most frequently by women in both groups were ibuprofen (68%), acetaminophen (28%), and acetylsalicylic acid (4%). Analysis of variance showed no significant difference between the two groups for daily analgesic consumption during PMS as well as during menstruation, prior to initiation of study medications. The reported mean consumption prior to initiation of the trial was the same for both study groups – 1.2 g ibuprofen or 2.5 g acetaminophen. The number of pain relievers used for menstrual pain by women taking NKO was significantly reduced to a mean daily consumption of 0.9 g ibuprofen and 1.5 g acetaminophen, a decrease of 33 percent and 40 percent, respectively, during the first treatment cycle (recorded at the 45-day visit). The mean reported analgesic consumption during the following treatment cycle(s) in the NKO group at the 90-day visit was 0.6 g ibuprofen (total decrease of 50%) or 1.0 g acetaminophen (total decrease of 50%) per day. Student's t-test analysis showed a significant intra-group reduction ($p<0.01$) for daily analgesic usage comparing baseline vs. 45 ($p<0.02$) vs. 90-day visit ($p=0.005$). Women in the fish oil group reported a similar decrease of 0.9 g ibuprofen and 1.65 g acetaminophen, a decrease of 33 and 34 percent, respectively, during the first treatment cycle. The analgesic use remained essentially constant during the second cycle, with reported daily quantities of 0.8 g ibuprofen and 1.48 g acetaminophen, a decrease of 33 and 41 percent, respectively. Student's t-test analysis showed an intra-group reduction for the fish oil group ($p<0.02$) for daily analgesic usage comparing baseline to 45-day ($p<0.02$) and 90-day ($p<0.02$) visits. At the end of the entire study, the comparative analysis between groups illustrated that women taking NKO consumed significantly fewer pain relievers during the 10 days of treatment than women receiving fish oil ($p<0.03$).

Table 3. Statistical Significance of Within and Between Group Variation

GROUP P-VALUE				
Symptom	NKO™		OMEGA-3 FISH OIL	
	45 days	90 days	45 days	90 days
Breast Tenderness	**<0.001**	**<0.001**	=0.38	=0.38
Overwhelmed	**<0.001**	**<0.001**	=0.06	=0.40
Stress	**<0.001**	**<0.001**	=0.07	=0.07
Irritable	**<0.001**	**<0.001**	=0.13	=0.13
Depression	**<0.001**	**<0.001**	=0.27	=0.27
Joint Pain	**<0.001**	**<0.001**	=0.18	=0.18
Weight Gain	**<0.001**	**<0.001**	**=0.04**	**<0.01**
Abdominal Pain	**<0.001**	**<0.001**	**=0.04**	**<0.001**
Swelling	**<0.001**	**<0.001**	=0.07	**<0.001**
Bloating	**<0.001**	**<0.001**	=0.08	=0.08

Analysis of variance showed that NKO was statistically more effective ($p<0.01$) than fish oil for the management of emotional symptoms (feeling overwhelmed, stress, irritability, and depression) ($p<0.01$); breast tenderness ($p<0.01$); and joint pain ($p<0.04$). There was no significant difference observed between NKO and fish oil for the management of weight gain, abdominal pain, and swelling ($p<0.5$).

No serious adverse events were reported during the duration of the trial. Three of the 36 women in the NKO group reported a reduction of the duration of the menstrual cycle by 3-7 days during the first month of treatment. This was no longer observed after the dose was reduced to 2 gel caps per day for 10 days per month. NKO group patients exhibited minor oiliness of the facial skin. Patients taking NKO did not experience any gastrointestinal difficulties such as regurgitation, while 64 percent of the women in the fish oil group complained of unpleasant reflux. On the contrary, the NKO subjects reported an increase of alertness, energy, and well-being.

Discussion

Premenstrual syndrome is a complex psychoneuroendocrine disorder characterized by a combination of physiological, psychological, and social symptoms. It is estimated that 85-97 percent of women of reproductive age experience some symptoms in the premenstrual phase of the cycle and 30-40 percent of these women will seek medical advice.[38] For 3-5 percent of women, symptoms are severe enough to significantly disrupt their everyday life.

Given the complexity in the cause and symptomatology of premenstrual syndrome, a vast array of treatments have been suggested. A meta-analysis performed by Wyatt et al[39] on the annual rates of diagnoses and prescribing patterns in premenstrual syndrome during the years 1993-1998 showed a yearly decrease in the number of prescriptions linked to PMS diagnoses. Simultaneously, the meta-analysis revealed a recent increase in popularity of alternative or natural remedies.[39] A survey of medical herbalists in 1998 showed PMS to be the second-most common condition treated with natural health products.[40]

Numerous studies have shown a beneficial effect of omega-3 fatty acids on menstrual pain.[41-43] This is consistent with the fact that menstrual pain and cramps are caused by inflammation mediated by omega-6 fatty acid-derived eicosanoids.

Following ovulation, there is shift of fatty acid balance in the phospholipids of the cell membranes.[43] Prior to menstruation, excessive amounts of arachidonic acid are released, and an increase in prostaglandins and leukotrienes (LTs) is triggered in the uterus. The inflammatory response initiated by the PGs and LTs results in vasoconstriction, myometrial contractions, and ischemia that cause pain; gastrointestinal symptoms such as nausea, vomiting, and bloating; and headaches.[43] Supplementation with omega-3 fatty acids mediates the production of less potent PGs and LTs, resulting in a reduction in the severity of myometrial contractions and uterine vasoconstriction, a decrease in the formation of inflammatory mediators, and subsequently reduced ischemia and improved blood flow.[41-43]

The results of the present study indicate that Neptune Krill Oil has statistically significant and clinically marked benefits against the inflammatory dysmenorrhea symptom complex as well as on the emotional symptomatology that characterizes premenstrual syndrome (intra-group difference $p<0.001$, CI 95%). When compared to omega-3 fish oil, the effect of NKO was comparable with respect to weight gain, abdominal pain, swelling, and bloating (inter-group variance ($p<0.5$, CI 95%). NKO was shown to be significantly more effective than fish oil for the management of all emotional symptoms of PMS ($p<0.01$, CI 95%), breast tenderness ($p<0.01$, CI 95%), and joint pain ($p<0.04$, CI 95%).

Evidence has shown that phospholipids of the brain have an especially high content of the long-chain omega-3 fatty acid DHA, and that these phospholipid species are centrally involved in brain function.[44-46] The effectiveness of NKO on emotional menstrual symptoms may thus be based on potential modulating effects on neurotransmitters that affect emotional and psychological symptoms. The synergistic effects of omega-3 fatty acids and phospholipids are specific to NKO since

the solvent-based cold extraction process used to produce this oil maintains the integrity of the phospholipids. Processes for fish oil extraction can involve conditions that irrevocably damage certain components like phospholipids.

Conclusion

The final results of the present study suggest within a high level of confidence that Neptune Krill Oil can significantly reduce the physical and emotional symptoms related to premenstrual syndrome, and is significantly more effective for the management of dysmenorrhea and emotional premenstrual symptoms than fish oil. NKO has a unique biomolecular profile of phospholipids, omega-3 fatty acids, and diverse antioxidants that surpasses the usual fish oil profile. The association between phospholipids and long-chain omega-3 fatty acids highly facilitates the passage of fatty acid molecules through the intestinal wall, increasing their bioavailability, and ultimately improving the omega-3:omega-6 ratio. Furthermore, phospholipid molecules play a major role in membrane fluidity, which may in turn play an active role in the management of emotional symptoms. Findings from this trial raise the possibility that Neptune Krill Oil has a positive benefit to risk profile for PMS.[47]

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