Technical

# Lipids and Fatty Acids of the Gastropod Mollusc *Cerethidea cingulata*

**Ashis K. Dutta[a], Prasanta K. Pal[a], Amitabha Ghosh*[,a], Suniti Misra[b], S. Nandi[b] and A. Choudhury[b]**
[a]Department of Chemistry, Bose Institute, Calcutta, India, and [b]Department of Marine Science, University of Calcutta, Calcutta, India

**Fatty acids of total lipid, neutral lipid, triglyceride, sterol ester and phosphatidylcholine of *Cerethidea cingulata* (Gmelin) have been determined. Levels of 16:1 (14%), 20:5ω3 (15%) and nonsaponifiables (40%) were found.**

The molluscs constitute one of the more important invertebrate groups in the animal kingdom and are divided taxonomically into seven classes, of which Gastropoda, Bivalvia and Cephalopoda comprise major marine fishery resources and are commercially important worldwide (1). In addition to their commercial value, current interest in the role of dietary polyunsaturated fatty acids in human health, particularly that of eicosapentaenoic (20:5ω3) acid in amelioration of certain cardiovascular diseases (2-4), also focuses attention on the molluscs, as well as other marine fishery products, which are excellent sources of polyunsaturates.

Sunderbans mangrove environment constitutes a complex ecosystem at the confluence of the deltaic estuarine complex of the river Hooghly and the Bay of Bengal. The enormous salt marshes and mud flats of this mangrove system support a luxuriant benthic macrofauna, of which molluscs, perhaps, constitute the major population. Fatty acid distributions in various lipid classes of the gastropod *Cerethidea cingulata,* one of the dominant gastropod animals of this mangrove area, have been determined. They were not investigated previously.

## EXPERIMENTAL

*Sample collection and extraction of lipids.* Samples were collected from the southwest coast of Sagar island, between the latitude 21° and 21.53°N and longitude 88.02° and 88.15°E. Flesh of 100 animals was taken out of the shells, and lipids were extracted according to the method of Bligh and Dyer (5). Lipid was weighed and stored in n-hexane at -18 C. Analytical data for the lipid was acquired by the official methods of AOCS (6).

*Saponification of total lipid, separation of nonsaponifiables and fatty acids.* The total lipid was saponified in an atmosphere of nitrogen gas, according to Hilditch (7), using methanolic KOH. Nonsaponifiables were extracted with diethyl ether, pooled, dried and weighed. Fatty acids were liberated by acidifying the aqueous layer with 4N $H_2SO_4$ in an atmosphere of nitrogen and extracted by diethyl ether. Pooled ethereal extract of fatty acid was dried and methylated using diazomethane (8).

*Fractionation of mixed methyl esters of total lipids by argentation column chromatography.* The mixed methyl esters of the total lipid were fractionated by argentation column chromatography (9). Altogether seven fractions, from saturated to hexaenoate esters, were obtained as described previously (10).

*Fractionation of total lipid into various classes.* The total lipid was fractionated into neutral lipid and phospholipid by column chromatography on silicic acid (11). The neutral lipid was fractionated into hydrocarbons, sterol esters, triglycerides, free fatty acids and sterols by preparative TLC (12), using a solvent system of light petroleum ether (40-60 C), diethyl ether and acetic acid (80:20:1, v/v/v). With this solvent system hydrocarbon and sterol ester bands were overlapped; these were resolved by rechromatography using n-hexane as developing solvent. Phospholipids were resolved into components on preparative TLC plates coated with alkali treated adsorbent (13).

*Extraction and methylation of fatty acids of neutral lipid, triglyceride and phosphatidylcholine.* The fatty acids of neutral lipid, triglyceride and phosphatidylcholine were obtained by alkaline hydrolysis (7) and were methylated by diazomethane (8).

*Lipolysis of sterol ester on TLC plates and separation of the products.* Sterol ester was lipolyzed on a TLC plate (14), using lipid-free (15) porcine pancreatic lipase. Sterols and fatty acids were separated on the same TLC plate and were recovered. Fatty acids were methylated using diazomethane.

*Separation of the components of nonsaponifiables.* Sterols, hydrocarbons and alkoxy lipids were separated and isolated from nonsaponifiables by preparative TLC (16) using light petroleum ether (40-60 C) and diethyl either (1:1, v/v). The alkoxy lipids were recovered from the origin, which gave yellow spots with 2,4-dinitrophenylhydrazine reagent (17).

*Gas liquid chromatography.* The instrument used was a Pye Unicam model 104 gas chromatograph equipped with dual glass column (1.8 m x 3 mm) and dual flame ionization detector. Fatty acid methyl esters were analyzed on 10% DEGS liquid phase supported on 80-100 mesh Diatomite C (AW, DMCS treated). Sterols were analyzed as acetates and also as trimethylsilyl ether derivatives on 3% SE-30 and 3% OV-17 columns (14), respectively. The hydrocarbons were analyzed directly on 3% OV-17 column (17). For preparative GLC, a 15% DEGS column (2.1 m x 8 mm) was used (28).

*Catalytic reduction.* Aliquots of all the fatty acid methyl ester samples were reduced catalytically, using platinum oxide catalyst (20).

*Peak identification.* Fatty acid methyl ester peaks were identified using (i) cod liver oil fatty acid methyl esters as secondary standard according to the method of Ackman and Burgher (18); (ii) semilogarithmic plot (19) of relative retention times (RRT) against carbon chainlengths of the fatty acids of cod liver oil and fitting the logarithm of RRT of the fatty acids under investigation into these plots; (iii) comparison of the equivalent chainlength (ECL) values with those published (18,27); and (iv) comparison of the chromatograms of the hydrogenated and nonhydrogenated samples.

Hydrocarbon peaks were identified by comparison of the retention times with those of authentic standards and by semilogarithmic plots of RRT against carbon chainlengths.

*Mass, NMR, UV and IR spectroscopy.* The mass spectra of 20:3ω9, 20:4ω6 and 20:5ω3 acids were taken on a low resolution mass spectrometer, model MS-30 by AEI, UK. The NMR spectra of these three fatty acid methyl esters were recorded on a 90 MHz spectrometer by Varian Associates, California. The UV and IR spectra were recorded on instruments by Hilger-Watts, UK.

*Determination of double bond positions of 20:3ω9, 20:4ω6 and 20:5ω3.* The double bond positions of the three fatty acids were determined by (i) partially reducing them with hydrazine hydrate (21) to produce maximum amounts of monoenoic acids; (ii) recovery of monoenes by TLC on silver nitrate-treated plates (22); (iii) permanganate-periodate oxidation (23) of the monoenoic acids; (iv) separation of mono- and dicarboxylic

*To whom correspondence should be addressed at Bose Institute, 93/1, A.P.C. Road, Calcutta 700 009, India.

acids on TLC plates (24), and (v) final identification of the mono-and dicarboxylic acid esters by GLC.

## RESULTS AND DISCUSSION

The total lipid was 10% of the tissue on a wet weight basis, with an iodine value (Wijs') of 70. Nonsaponifiable content was 40% of the total lipid, which was quite high but comparable to that of *B. boddaerti* (25), another detritus-feeding benthic animal of this ecosystem.

The total lipid was fractionated into neutral lipid and phospholipid by column chromatography. The neutral lipid fraction was further fractionated into various classes, which have been presented in Table 1. Phosphatidylcholine was over 93% of the total phospholipid and was also the major component among all the lipid classes. Other minor constituent phospholipids were phosphatidylserine, phosphatidylethanolamine and lysophosphatidylcholine. The abnormally high level of nonsaponifiables was due to substantially high levels of sterol and sterol ester in the total lipid. GLC analysis of the derivatized sterols from the nonsaponifiables revealed the presence of a component (80%) with retention time identical to that of cholesterol and three minor components with higher retention times. Although the major sterol in the present study had a retention time identical to that of cholesterol, the structure may be different, particularly with respect to the side chain, which is common in marine sterols (26). GLC analysis of the hydrocarbon fraction indicated the presence of saturated components with 20- to 38-carbon chainlengths, of which 21-, 31-and 33-carbon chainlengths were absent. Major components were C-28 (30.5%), C-29 (12%), C-30 (8%), C-32 (6%) and C-36 (6%). Among the branched chain hydrocarbons of the iso-series, C-23 (2%) and C-29 (4.5%) were present.

The mixed methyl esters of total lipid were fractionated by argentation column chromatography into seven fractions containing 0-6 double bonds. The three biologically important fatty acids, viz 20:3ω9, 20:4ω6 and 20:5ω3, were isolated by preparative GLC of the fractions containing tri-, tetra- and pentaenoic fatty acids, respectively. The mixed methyl esters of neutral lipid, triglyceride, sterol ester and phosphatidylcholine were analyzed directly by GLC. The peaks were identified on the basis of RRT, ECL and hydrogenation data. The fatty acid methyl ester samples did not show the presence of any con-

TABLE 1

**Composition of Various Lipid Components of *C. cingulata***

| Components | % w/w of total lipid |
|---|---|
| Hydrocarbon | 1.7 |
| Sterol ester | 12.0 |
| Triglyceride | 9.3 |
| Free fatty acid | 2.1 |
| Sterol | 31.1 |
| Phosphatidylcholine | 39.2 |
| Other phospholipids | 2.6 |
| Alkyl glycerol ethers | 2.0 |

TABLE 2

**Fatty Acid Compositions (% w/w) of Various Lipids of *C. cingulata***

| Fatty acids[a] | Total lipid | Neutral lipid | Triglyceride | Sterol ester | Phosphatidyl choline | ECL values[b] |
|---|---|---|---|---|---|---|
| 12:0 | 0.8 | 0.5 | 0.5 | 1.5 | 1.0 | 12.0 |
| 14:0 | 0.7 | 2.0 | 2.0 | 5.5 | 1.5 | 14.0 |
| 16:0 | 12.0 | 13.5 | 14.0 | 18.0 | 13.0 | 16.0 |
| 18:0 | 4.0 | 7.0 | 7.5 | 12.0 | 3.0 | 18.0 |
| 22:0 | 0.3 | 2.5 | 4.5 | 3.0 | 0.5 | 22.0 |
| 12:1 | 0.2 | 0.5 | 0.5 | 0.5 | 0.3 | 12.4 |
| 14:1 | 6.0 | 6.0 | 6.5 | 6.5 | 5.5 | 14.5 |
| 16:1 | 14.0 | 13.0 | 13.5 | 11.5 | 13.5 | 16.6 |
| 17:1 | 1.5 | 0.5 | 0.5 | 0.5 | 1.0 | 17.4 |
| 18:1 | 9.5 | 11.5 | 12.0 | 15.5 | 9.5 | 18.5 |
| 20:1 | 1.0 | 1.0 | 1.5 | 1.0 | 1.0 | 20.4 |
| 21:1 | 0.8 | 0.5 | 0.5 | 0.2 | 0.5 | 21.3 |
| 22:1 | 0.5 | 0.5 | 0.5 | 0.2 | 0.5 | 22.7 |
| 18:2ω6 | 4.5 | 6.0 | 6.0 | 6.0 | 4.0 | 19.4 |
| 20:2ω9 | 0.8 | 1.0 | 1.5 | 1.0 | 0.8 | 20.85 |
| 22:2ω9 | 0.5 | 1.0 | 1.0 | 0.5 | 0.5 | 22.75 |
| 18:3ω3 | 8.0 | 7.5 | 7.5 | 6.0 | 10.0 | 20.4 |
| 20:3ω9 | 3.5 | 2.5 | 2.0 | 1.0 | 3.5 | 21.6 |
| 18:4ω3 | 1.0 | 0.5 | 0.5 | 0.5 | 0.5 | 21.3 |
| 20:4ω6 | 3.0 | 2.5 | 2.0 | 1.0 | 3.5 | 22.8 |
| 20:5ω3 | 15.5 | 12.8 | 10.5 | 6.0 | 15.0 | 24.0 |
| 21:5ω3 | 4.5 | 1.5 | 0.3 | 0.3 | 3.5 | 24.95 |
| 22:5ω6 | 1.5 | 1.5 | 1.5 | 0.5 | 2.0 | 25.5 |
| 22:5ω3 | 1.9 | 1.0 | 1.2 | 0.5 | 2.4 | 26.0 |
| 22:6ω3 | 4.0 | 3.2 | 2.0 | 0.8 | 3.5 | 26.25 |

[a]Isomeric monoenoic esters could not be distinguished.
[b]Equivalent chainlength (ECL) values.

jugated unsaturation or unusual groups, on examination by UV or IR spectroscopy. The fatty acid compositions of various samples and the ECL values of the various fatty acids have been presented in Table 2.

For the structural confirmation of 20:3ω9, 20:4ω6 and 20:5ω3 acids, they were isolated in the pure state by preparative GLC. Oxidation of the monoenes obtained by the partial reduction of these acids produced mono- and dicarboxylic acids, carbon chainlengths of which were determined by GLC; double bond positions were ascertained (25). Catalytic reduction of the three acids produced arachidic acid (20:0). Molecular weights of the methyl esters of 20:3ω9, 20:4ω6 and 20:5ω3 acids were 320, 318 and 316, respectively, as determined from the mass spectra. NMR spectra of 20:3ω9, 20:4ω6 and 20:5ω3 were identical to those of the synthetic 20:3ω9 acid (28), authentic 20:4ω6 obtained from Nu-Chek-Prep. Inc., Elysian, Minnesota, and previously isolated 20:5ω3 acid (25), respectively.

The fatty acid compositions of total lipid, neutral lipid, triglyceride, sterol ester and phosphatidylcholine have been presented in Table 2. Qualitatively, all the lipid components have identical fatty acid compositions with quantitative variations. In all the lipids, proportions of unsaturated fatty acids to saturated ones were higher. Highest proportion (82.2%) of unsaturated fatty acids was in total lipid, whereas the lowest (60.0%) was in sterol ester. The proportion of unsaturated fatty acids in phosphatidylcholine was also comparable (81.9%) to that in the total lipid. Among the unsaturated fatty acids, the proportions of monoenoic fatty acids were highest in all the lipids and were always over 30% of the total fatty acids. Among the polyunsaturated fatty acids, proportions of total pentaenoic fatty acids were relatively high in the total lipid (23.4%) and phosphatidylcholine (22.9%). Of the pentaenoic fatty acids, levels of 20:5ω3 were as high as 15.5% in the total lipid, comparable to that in *B. boddaerti* (25). The 20:5ω3 fatty acid is important because it can form biologically active prostaglandins (29,30). The highest level of 20:5ω3 hitherto reported ranges up to 18% in pacific pilchard oil (31). The other prostaglandin precursor acid, viz 20:4ω6 (32), was present in low levels. The other biologically important fatty acid, viz 20:3ω9, which is produced in animals with essential fatty acid deficiency (33), was present in all the lipids, though in low levels. The overall fatty acid profile of *C. cingulata* lipid was comparable to that of *B. boddaerti* (25), another detritus-feeding benthic animal of this ecosystem.

## ACKNOWLEDGMENTS

This research has been carried out with financial assistance from the Department of Science and Technology, Government of India, Project No. 18(6)/77-SERC. A.K. Barua, acting director, and D.P. Chakraborty, chairman, provided laboratory facilities.

## REFERENCES

1. Joseph, J.D., *Prog. Lipid Res. 21*:109 (1982).
2. Dyerberg, J., H.O. Bang and N. Njorne, *Am. J. Clin. Nutr. 28*:958 (1975).
3. Dyerberg, J., H.O. Bang, E. Stofferson, S. Moncada and J.R. Vane, *Lancet 2*:117 (1978).
4. Dyerberg, J., and H.O. Bang, *Ibid. 2*:433 (1979).
5. Bligh, E.G., and W.J. Dyer, *Can. J. Biochem. Physiol. 37*:911 (1959).
6. *Official and Tentative Methods of the American Oil Chemists' Society, Vols. I and II,* Third edition, AOCS, Champaign, IL, 1964 (revised to 1974).
7. Hilditch, T.P., and P.N. Williams, in *The Chemical Constitution of Natural Fats,* Chapman and Hall, London, Fourth edition, 1964, p. 679.
8. Schlenk, H., and J.L. Gallerman, *Anal. Chem. 32*:1412 (1960).
9. Ghosh, A., M. Hoque and J. Dutta, *J. Chromatogr. 69*:207 (1972).
10. Hoque, M., A. Ghosh and J. Dutta, *JAOCS 50*:29 (1973).
11. Rouser, G., G. Kritchevski and A. Yamamoto, in *Lipid Chromatographic Analysis,* Vol. I, edited by G.V. Marinetti, Marcel Dekker, New York, NY, 1967, pp. 118-119.
12. Mangold, H.K., in *Thin Layer Chromatography,* edited by E. Stahl, Springer-Verlag, New York, NY, 1969, pp. 374-379.
13. Renkonen, O., and P. Varo, in *Lipid Chromatographic Analysis,* Vol. I, edited by G.V. Marinetti, Marcel Dekker, New York, NY, 1967, pp. 54-57.
14. Misra, S., A. Choudhury, A.K. Dutta and A. Ghosh, *Phytochem. 23*:2823 (1984).
15. Misra, S., A. Choudhury, A.K. Dutta, J. Dutta and A. Ghosh, *Lipids 19*:302 (1984).
16. Misra, S., A. Ghosh and J. Dutta, *J. Sci. Food Agric. 35*:59 (1984).
17. Mangold, H.K., and W.J. Bauman, in *Lipid Chromatographic Analysis,* Vol. I, edited by G.V. Marinetti, Marcel Dekker, New York, NY, 1967, pp. 343-345.
18. Ackman, R.G., and R.D. Burgher, *JAOCS 42*:38 (1965).
19. Ackman, R.G., and R.D. Burgher, *Can. J. Biochem. Physiol. 41*:1627 (1963).
20. Ghosh, A., and J. Dutta, *Trans. Bose Res. Inst. 35*:13 (1972).
21. Aylward, F., and C.V.N. Rao, *J. Appl. Chem. 6*:248 (1956).
22. Morris, L.J., M.O. Marshall and E.W. Hammond, *Lipids 3*:91 (1968).
23. Chang, T.C.L., and C.C. Sweeley, *J. Lipid Res. 3*:170 (1962).
24. Wu, J., A. Ghosh and J.L. Beal, *Lloydia 43*:360 (1980).
25. Misra, S., A.K. Dutta, T. Dhar, A. Ghosh, A. Choudhury and J. Dutta, *J. Sci. Food Agric. 34*:1413 (1983).
26. Djerassi, C., N. Theobold, W.C.M.C. Kokee, C.S. Pak and R.M.K. Carlson, *Pure Appl. Chem. 51*:1815 (1979).
27. Ackman, R.G., *JAOCS 40*:558 (1963).
28. Ghosh, A., M. Koley and J. Dutta, *Lipids 17*:314 (1982).
29. Whitaker, M.O., A. Wyche, F. Fitzpatric, H. Sprecher and P. Needleman, *Proc. Natl. Acad. Sci. U.S.A. 76*:5919 (1979).
30. Needleman, P., A. Raz, M.S. Minkes, A.J. Ferrendelli and H. Sprecher, *Ibid. 76*:944 (1979).
31. Ackman, R.G., and J.C. Sipos, *J. Fish Res. Bd. Can. 21*:841 (1964).
32. Lands, W.E.M., M.E. Hemler and C.G. Crawford, in *Polyunsaturated Fatty Acids,* edited by W.H. Kunau and R.T. Holman, American Oil Chemists' Society, Champaign, Illinois, 1977, p. 193.

[Received April 23, 1985]