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De novo biosynthesis of linoleic acid in two non-insect invertebrates: the land slug and the garden snail[1]

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Abstract. The de novo biosynthesis of linoleic acid [18:2(n-6)], a fatty acid considered to be essential for most animals, was demonstrated in the land snail, *Bulimulis alternatus mariae*, and the garden slug, *Arion circumscriptus*. Radiolabeled acetate injected into the animals was incorporated into both 18:2 and 20:2, as demonstrated by radio-high performance liquid chromatography (radio-HPLC) and radio-gas chromatography (radio-GC). GC-mass spectrometry (GC-MS) of the methoxy derivatives of the 18:2 and 20:2 isolated from the snail showed that major isomers had the double bonds in the n-6,9 positions. Radio-gas-liquid chromatography (radio-GLC) of the ozonolysis products from the labeled dienoic fatty acid methyl esters showed that both ends of the molecules were labeled, confirming de novo synthesis. The production of linoleic acid by these animals suggests the capability to produce linoleic acid may be widespread in invertebrates.
Key words. Linoleic acid biosynthesis; essential fatty acid; Δ^{12} desaturase; *Bulimulis alternatus mariae*; *Arion circumscriptus*.

Until recently, linoleic acid [18:2(n-6)] had been considered to be an essential fatty acid for all animals[3]. The requirement for this fatty acid in the animal diet is due to the absence of a Δ^{12} desaturase, the enzyme responsible for the insertion of the second double bond in oleic acid. Plants[4], some fungi[5] and protozoa[6] possess a Δ^{12} desaturase and thus readily produce linoleic acid. Studies in the 1980s demonstrated that many, although by no means all, insect species also possess the ability to produce linoleic acid[7–10], rendering them free from a dietary requirement of fatty acid. The Δ^{12} desaturase was subsequently characterized in the house cricket[11] and the American cockroach[12]. The observation that many insect species could produce linoleic acid suggested the possibility that other invertebrates might also have this capability. The results reported herein demonstrate that the land snail and the garden slug can biosynthesize linoleic acid and suggest that this biosynthetic capability is much more widespread among animals than previously thought.

Materials and methods

Land snails, *Bulimulis alternatus mariae*, were obtained from Carolina Biological Supply Company, Burlington, NC. Garden slugs, *Arion circumscriptus*, were collected from gardens and marshy areas in Reno, NV. The animals were injected with 1 μCi each sodium [1-^{14}C]acetate (50 μCi/mmol), and, after 24 h at room temperature, were killed by freezing at −20 °C. After thawing, lipid was extracted by homogenization in chloroform:methanol:water (1:2:1), and lipids were extracted by the method of Bligh and Dyer[13]. Lipid classes were separated by thin layer chromatography (TLC)[7], and fatty acids from polar lipids and triacylglycerols were methylated as described[14]. Labeled fatty acid methyl esters were analyzed by radio-HPLC on a Supelco LC-8 reverse-phase column with acetonitrile:water (75:25) as eluant. Radiolabeled fatty acid methyl esters were detected with a Radiomatic Instruments Flo-one/Beta flow through liquid scintillation detector. Dienoic fatty acid methyl esters were separated on silver nitrate (10%) impregnated TLC plates developed in hexane:diethyl ether (80:20). The dienes were analyzed by radio-GC[7] using a Radiomatic Beta/Flo-one combustion flow-through proportional counter. A portion of the dienoic fatty acid methyl esters was subjected to ozonolysis as described by Beroza and Bierl[15], and the products analyzed by radio-GC[7].

The dienoic fatty acid methyl esters obtained from snails not injected with [1-^{14}C]acetate were converted to their monomethoxy derivatives[16]. They were subjected to GC-mass spectrometric analyses on a Hewlett-Packard GC equipped with a Hewlett-Packard 5970B mass selective detector, which was interfaced to a Hewlett-Packard Chemstation computer.

Results

Radio-HPLC of fatty acid methyl esters. Radiolabel was recovered predominantly in the polar lipids and triacylglycerols from both snails and slugs injected with [1-^{14}C]acetate (data not shown). Radio-HPLC analyses of the methyl ester derivatives of these fractions (fig. 1A, B) demonstrated the incorporation of radioactivity into fatty acids that co-eluted with 18:2 and 20:2 standards. The labeled dienoic fatty acid methyl esters from both the snail and slug were isolated by silver

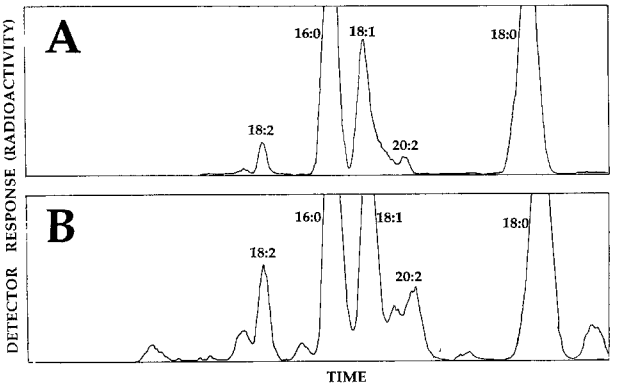

Figure 1. Radio-HPLC analysis of the fatty acid methyl esters from triacylglycerols obtained from the land snail (A) and slug (B) after incubation with [1-^{14}C]acetate. The incubations were performed and lipid extracted, isolated, methylated and analyzed as described in 'Materials and methods'.

nitrate TLC and analyzed by radio-HPLC, and the results showed that both 18:2 and 20:2 were labeled (data not shown).

Positions of the double bonds in 18:2 and 20:2. The positions of the double bonds in the 18:2 and 20:2 fatty acids from snails were determined by GC-MS analyses of their monomethoxy derivatives (fig. 2). The two main components in the dienoic fraction co-eluted with 18:2 and 20:2 when analyzed by GC (fig. 2A). The mass spectrum of the monomethoxy derivative of the 18:2 (fig. 2B) gave fragments that showed that the double bonds were in the $\Delta^{9,12}$(n-6,9) positions. If the 18:2 was the n-6 isomer, then methoxy derivatives should be formed on positions 9, 10, 12 and 13. Cleavage in the mass spectrometer on either side of the methoxy derivative with the charge remaining on the fragment that contained the methoxy group showed that methoxy groups were on position 9, 10, 12 and 13, indicating that the major component was the $\Delta^{9,12}$ isomer. Similarly, the mass spectrum (fig. 2C) of the monomethoxy derivatives of 20:2 gave fragments that showed that the methoxy groups were on positions 11, 12, 14 and 15, demonstrating that the double bonds were in the $\Delta^{11,14}$(n-6,9) positions.

Ozonolysis of dienes. The radiolabeled dienoic fatty acid methyl esters from the snail were isolated by silver nitrate-TLC. Radio-gas-liquid chromatography (radio-GLC) analyses of this fraction showed two major peaks which had the same retention times as did 18:2 and 20:2 standards (fig. 3A). The ozonolysis products from this fraction gave three peaks that corresponded to C6, C9 and C11 fragments, which were interpreted as arising from the methyl end of both the 18:2 and 20:2 (C6 fragment) and the carboxyl end of the 18:2 (C9 fragment) and 20:2 (C11 fragment). Thus, the data show that both ends of the molecule were labeled, which indicates de novo synthesis of the dienoic fatty acids.

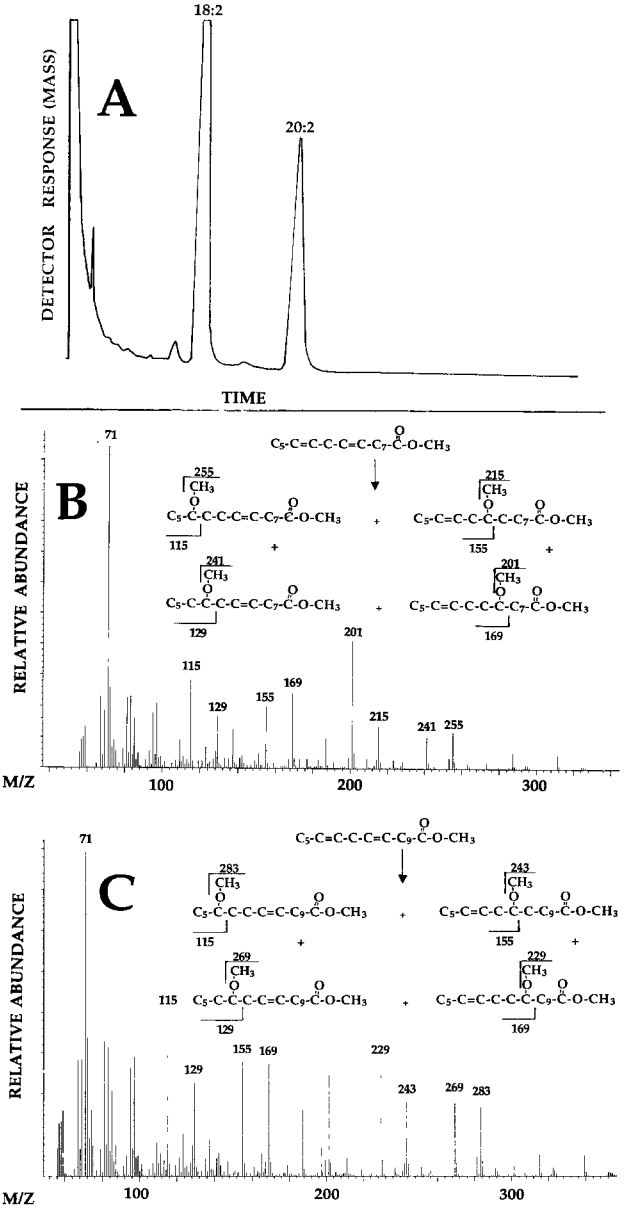

Figure 2. GC trace of the dienoic fatty acid methyl esters (A) and mass spectra of the monomethoxy derivatives of 18:2 (B) and 20:2 (C) from the land snail. The dienoic acids were extracted, isolated, derivatized and analyzed as described in 'Materials and methods'.

Discussion

The discovery that many insect species can biosynthesize linoleic acid raised the question of just how widespread the ability to produce linoleic acid is among animals. Since the report in 1930[17] that vertebrates require linoleic acid in their diets, the dogma evolved that all animals are unable to synthesize this 'essential' fatty acid and therefore require it in their diet. This dogma was almost universally accepted[18] despite occasional reports to the contrary that indicated that certain organisms either didn't require linoleic acid in the diet or that they could biosynthesize it[19]. However, in the

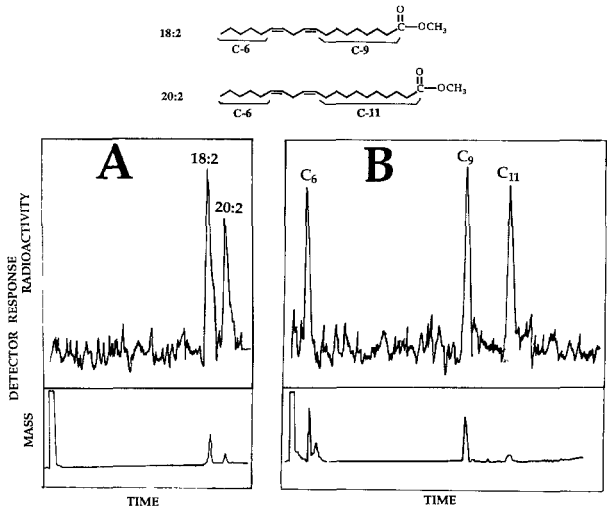

Figure 3. Radio-GLC of the dienoic methyl esters (A) and their ozonolysis products (B) from the land snail. The labeled fatty acid methyl esters were obtained and analyzed as described in 'Materials and methods'.

1980s incontrovertible evidence was obtained that showed that many insect species could produce linoleic acid[7–10], and the Δ^{12} desaturase was characterized in two insect species[11,12].

Van der Horst et al.[20] reported a number of years ago that the pulmonate land snail, *Cepaea nemoralis*, was able to incorporate [1-^{14}C]acetate into a fatty acid that co-eluted on GC with linoleate. However, without further characterization of the 18:2 and evidence showing that the entire molecule was labeled, this report did not receive wide attention. Indeed, textbooks on invertebrates[21] contain statements to the effect that, although lipid requirements have not been as widely studied in invertebrates as in vertebrates, linoleic acid is assumed to be an essential fatty acid for invertebrates.

The data presented herein demonstrate that the land snail and the garden slug can synthesize linoleic acid [18:2(n-6)] from radiolabeled precursors, and furthermore, show that both ends of the newly-synthesized molecule are labeled. This rules out the possibility that the snail simply chain shortens dietary 18:2 and then adds a labeled acetate unit to form 18:2. Extensive and very involved studies were performed in insects to demonstrate that insect tissue and not microorganisms contained the Δ^{12} desaturase[22,23]. Similar studies have not yet been performed in the snail or the slug, but considering the relatively high amounts of linoleic acid formed, it appears unlikely that microorganisms contribute markedly to linoleic acid synthesis.

The ability to produce linoleic acid by those invertebrates that possess a Δ^{12} desaturase leads to the questions of just how widespread this enzyme is and whether possession of this enzyme was a primitive state, subsequently lost by many higher animals. Alternatively, the Δ^{12} desaturase found in some insect species and mollusks may reflect an example of convergent evolution. The results presented here showing that linoleic acid is synthesized by a snail and a slug, along with the work indicating linoleic acid synthesis is widespread in insects, form the basis for continuing studies on the evolution of this intriguing enzyme.

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