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Insect tissues, not microorganisms, produce linoleic acid in the house cricket and the American cockroach[1]

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Summary. Biosynthesis of linoleic acid, 18:2(n−6), was unambiguously demonstrated to occur in the cockroach, *Periplaneta americana*, and the cricket, *Acheta domesticus*. Axenic tissue from both of these insect species was demonstrated by radio-gas-liquid chromatography (radio-GLC) and radio-high-performance liquid chromatography (radio-HPLC) to incorporate [1-^{14}C]acetate and [1-^{14}C]oleate into this essential fatty acid.
Key words. Linoleic acid biosynthesis; essential fatty acid; *Periplaneta americana*; *Acheta domesticus*.

The biosynthesis of linoleic acid, 18:2(n−6), ((Z,Z)-6,9-octacosadienoic acid), generally has been considered to occur only in plants[3], fungi[4], and protozoa[5]. The tenet that animals are unable to synthesize linoleic acid has been accepted despite occasional findings to the contrary, particularly among several insect species[6–8]. These early reports of linoleic acid synthesis in insects were discounted due to criticisms regarding inadequate analytical techniques and the possibility that microorganisms could contribute to linoleate production[9]. However, more recently, it has been shown by radio-GLC and radio-HPLC techniques that 12 of the 38 insect species investigated can synthesize 18:2 de novo from acetate[10–13]. In these studies, rigorous characterization of the newly-synthesized labeled 18:2 by gas chromatography-mass spectrometry (GC-MS), nuclear magnetic resonance (NMR), and radiotracer studies using analyses by radio-GLC, radio-HLPC, and ozonolysis followed by radio-GLC confirmed that 18:2(n−6) was formed.

The questions regarding the potential role of microorganisms have not been adequately addressed. Many insects have microorganisms in the gut tract, in specialized cells called mycetocytes or bacteriocytes, and on the cuticle. It is possible that these microorganisms are responsible for de novo synthesis of 18:2 from acetate. To determine whether insect tissue or associated microorganisms are involved in linoleate synthesis in insects, epidermal, fat body and testes tissue from *A. domesticus*, and epidermal and fat body tissue from *P. americana*, were examined under axenic conditions for their capability to produce linoleic acid. The results of these experiments reported here demonstrate unequivocally that it is insect tissue that produces linoleic acid.

Methods and materials

Cockroaches, *P. americana*, were reared in metal garbage cans and fed Purina dog chow and water ad libitum. Crickets, *A. domesticus*, were obtained from Fluker's Cricket Farm, Baton Rouge, Louisiana. Adult male cockroaches and penultimate instar male crickets were anesthetized with CO_2 and ligated at the neck with cotton thread. The insects were surface sterilized by immersion in 70% ethanol with 2 drops Tween 80/l for 5 min followed by immersion in Zephiran-Cl for 5 min, and rinsed in sterile distilled water for 5 min. Under sterile conditions, fat body, testes and epidermal-enriched tissues were dissected from the crickets, and fat body and epidermal-enriched tissues from the cockroach. Tissues were placed in 0.1 ml incubation medium in 96 well plates and incubated at 30 °C for 24 h. The incubation medium, L15B[14] contained penicillin (100 U/ml), streptomycin (100 µg/ml) and gentamycin (50 µg/ml), and either [1-^{14}C]acetate (0.75 µCi/0.1 ml) or [1-^{14}C]oleate (0.33 µCi/0.1 ml). The [1-^{14}C]oleate was suspended first in 100 µl ethanol, then added to 10 ml of the medium. The medium was then warmed (37 °C) and filtered. Following incubation, the wells were checked by microscopy for contamination and any showing evidence of either fungi or bacteria were discarded.

Tissues were obtained from six wells and pooled in Eppendorf tubes for a total of 3–4 replicates of each. Total

lipids were extracted as described by Bligh and Dyer[15]. Solvent was removed under nitrogen and the samples were frozen until analyzed. Lipids were resuspended in 1 ml chloroform:methanol (2:1), and a 10-µl volume was removed, dried and assayed for radioactivity in a Beckman liquid scintillation counter at about 90% efficiency. Lipid classes were separated by thin layer chromatography and methyl ester derivatives of fractions of interest were obtained as described[10-13]. Methyl esters were separated by HPLC on a Supelco C_8 reversed phase column with acetonitrile:water (80:20 for samples from oleate incubations and 75:25 for samples from acetate incubations) as solvents and radioactivity was monitored by a Radiomatic Flo-One flow-through detector with Scinti Vers LC as the scintillation cocktail.

The methyl ester derivatives of diunsaturated fatty acids were isolated on TLC plates impregnated with 10% (w/w) silver nitrate. The isolated 18:2 was then subjected to ozonolysis as described by Beroza and Bierl[16] and the products analyzed by radio-GLC as described[10].

Results

Incorporation of radioactivity into lipid classes. Following axenic incubations of cricket and cockroach tissues with [1-^{14}C]acetate, most of the radioactivity recovered in lipid extracts was in the triacylglycerol (53–77%) and polar lipid (14–28%) fractions (table 1). Similarly, as shown in table 2, after incubations with [1-^{14}C]oleate, the triacylglycerol fractions contained much of the radioactivity (33–58%). However, less oleate was incorporated into the polar lipids (7–23%), while more was recovered in the free fatty acid fraction (34–46%) (data not shown).

HPLC of lipids extracted from insect tissues. Figure 1 shows typical radio-HPLC chromatograms of the methyl ester derivatives of polar lipids from the cricket fat body (panel A) and the cockroach fat body (panel B) after incubation with [1-^{14}C]acetate. In both chromatograms, a peak eluting at the same retention time as an authentic 18:2(n−6) methyl ester standard is clearly visible. Similarly, after incubation with [1-^{14}C]oleate, 18:2(n−6) is found in polar lipids from both the cricket and cockroach fat body tissue (fig. 2, A and B).

The results of the HPLC analyses of the polar lipids and triacylglycerol fractions from tissues incubated with [1-^{14}C]acetate are presented in table 1, and from tissues incubated with [1-^{14}C]oleate in table 2. In incubations

Table 1. Synthesis of linoleic acid from [1-^{14}C]acetate in isolated tissue in axenic culture

	Distribution of radioactivity, % of total recovered in lipid extracts	18:2 Synthesized	
		% 18:2[a]	nmol[b]
Cricket			
Fat body			
Polar lipids	14	9.3 ± 10.7	10.0
Triacylglycerols	77	7.4 ± 04.4	39.3
Epidermis			
Polar lipids	15	2.8 ± 0.7	1.3
Triacylglycerols	75	3.2 ± 1.8	8.0
Testes			
Polar lipids	28	1.7 ± 1.0	0.5
Triacylglycerols	61	6.4 ± 1.9	8.3
Cockroach			
Fat body			
Polar lipids	13	7.0 ± 6.5	1.5
Triacylglycerols	77	3.6 ± 0.9	6.9
Epidermis			
Polar lipids	27	nd[c]	nd
Triacylglycerols	53	3.8 ± 1.9	0.6

[a]% of [1-^{14}C] recovered as 18:2; [b]nmol of 18:2 synthesized per pool (tissue from 6 insects); [c]not detected. n = 3.

Table 2. Synthesis of linoleic acid from [1-^{14}C]oleate in isolated tissue in axenic culture

	Distribution of radioactivity, % of total recovered in lipid extracts	18:2 Synthesized	
		% 18:2[a]	nmol[b]
Cricket			
Fat body			
Polar lipids	8	nd[c]	nd
Triacylglycerols	46	8.6 ± 1.4	9.1
Epidermis			
Polar lipids	9	1.7[d]	0.9
Triacylglycerols	40	1.2 ± 0.02	1.1
Testes			
Polar lipids	23	1.2 ± 0.3	0.6
Triacylglycerols	33	10.8 ± 5.4	8.0
Cockroach			
Fat body			
Polar lipids	14	3.0 ± 0.7	1.0
Triacylglycerols	54	8.5 ± 0.7	9.4
Epidermis			
Polar lipids	7	5.0[d]	0.7
Triacylglycerols	58	0.4 ± 0.2	0.4

[a]% of [1-^{14}C] recovered as 18:2; [b]nmol of 18:2 synthesized per pool (tissue from 6 insects); [c]not detected; [d]n = 1. n = 3.

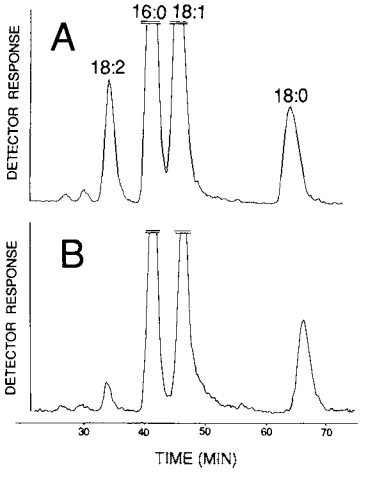

Figure 1. Radio-HPLC chromatograms of triacylglycerol fractions from *A. domesticus* (*A*) and *P. americana* (*B*) fat body tissues incubated with [1-^{14}C]acetate.

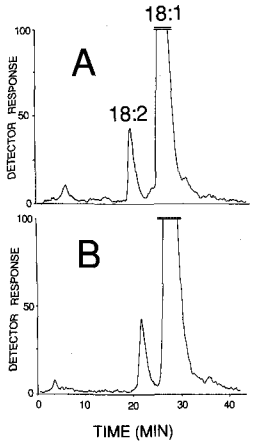

Figure 2. Radio-HPLC chromatograms of triacylglycerol fractions from *A. domesticus* (*A*) and *P. americana* (*B*) fat body tissues incubated with [1-^{14}C]oleate.

with both substrates and in both insects, most of the newly-synthesized 18:2 was recovered in the triacylglycerol fraction in fat body tissue. Incorporation of radiolabel was low in the diacylglycerol fractions (and the free fatty acid fractions in the case of acetate), thus radio-HPLC of these fractions showed few peaks above background noise and the data are not included. In tissues incubated with [1-^{14}C]oleate, 34–46% of the radiolabel was recovered in the free fatty acid fraction. None of this labeled material co-eluted with 18:2 standards.

Ozonolysis of the newly-synthesized 18:2. Samples shown by HPLC to contain 18:2 were separated by silver nitrate TLC and the diene fractions recovered. This isolated 18:2 was subjected to ozonolysis and the resultant fragments identified by radio-GLC. As shown in figure 3, fragments of 18:2 obtained from cricket and cockroach tissue incubated with [1-^{14}C]acetate showed radioactivity

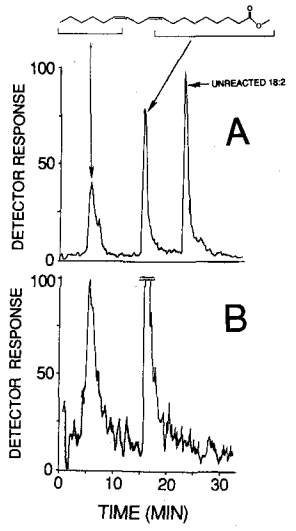

Figure 3. Radio-HPLC chromatograms of ozonolysis fragments of 18:2 isolated from *A. domesticus* (*A*) and *P. americana* (*B*) tissues incubated with [1-^{14}C]acetate.

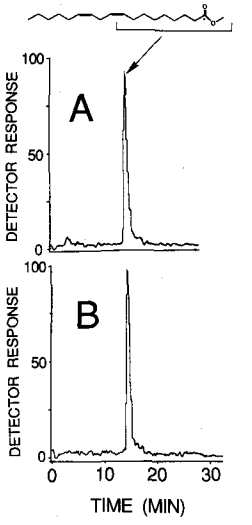

Figure 4. Radio-HPLC chromatograms of ozonolysis fragments of 18:2 isolated from *A. domesticus* (*A*) and *P. americana* (*B*) tissues incubated with [1-^{14}C]oleate.

in both hexanal and 9-oxo-methylnonanoate (panel A). Fragments from 18:2 obtained from incubations of both cricket and cockroach tissues with [1-^{14}C]oleate showed radioactivity only in the 9-oxo-methylnonanoate fragment (panel B).

Discussion

The role of microorganisms in linoleate synthesis in insects has been previously considered. Dwyer and Blomquist[17] presented evidence that desaturation of oleate (18:1) occurred in both fat body and epidermal tissue of the American cockroach, but not in isolated gut tract, with its resident microorganisms, indicating that these microorganisms did not contribute to the synthesis of linoleate (18:2(n−6)). Similarly, in termites whose gut tracts had been removed, synthesis of 18:2 was shown to continue[10,18]. Furthermore, in the American cockroach it is unlikely that the symbionts housed in mycetocytes or bacteriocytes were responsible for 18:1 desaturation, as these symbionts appear to be bacteria or bacteroids[19], and no reports have been confirmed that any bacteria can synthesize linoleic acid de novo[20]. Finally, in aphids treated with antibiotics, more than 50% of the mycetocytes were destroyed, and yet, desaturation of 18:1 to 18:2 continued at the same rate as in controls[12].

These results, taken together, have provided evidence that the synthesis of 18:2 in these insect species occurred in insect tissue, but in each case were not absolute. In most cases, contamination by surface microorganisms was not ruled out, and, although the case against them is strong, the role of bacteriocytes in 18:2 synthesis remained in question. However, not all insect species house endosymbionts in mycetocytes or bacteriocytes; in general, those species with either poor or very restricted diets

have been shown to rely on endosymbionts. *A. domesticus* has been shown not to contain intracellular symbionts[21]. Thus, the data presented here demonstrating 18:2(n−6) synthesis in axenic fat body tissues from *A. domesticus* clearly indicate that insect tissue produces 18:2. This conclusion for *A. domesticus* does not rule out the possible contribution of bacteriocytes in *P. americana*, but together with the inability of bacteria to synthesize 18:2 and the lack of any contaminating microorganisms in the incubations, the case is also strong for 18:2 synthesis by insect tissue in *P. americana*.

Extensive studies in plant tissue have shown that the enzyme responsible for the conversion of oleic acid to linoleic acid, the Δ^{12} desaturase, requires oleic acid esterified to phosphatidylcholine as a substrate[1]. In contrast, studies in the house cricket[22] and American cockroach (Borgeson, unpublished results) using microsomal preparations show that the substrate for the Δ^{12} desaturase is oleoyl-CoA. The results from the study reported here show that it is insect tissue that contains the novel Δ^{12} desaturase that converts oleoyl-CoA to linoleate.

These data show for the first time unambiguously that certain insect species can synthesize linoleic acid de novo from acetate, indicating that desaturation of oleic acid to linoleic acid occurs in these insects. This single step, not possible in any vertebrate, enables the insect to switch fatty acids from one family, (n−9), to another family, (n−6), thus forming de novo the precursor for the physiologically important eicosanoid, arachidonic acid (20:4(n−6)), which is in turn precursor to prostaglandins. Both the American cockroach and house cricket can form arachidonic acid de novo[23,24]. This step releases those insects with this capability from a nutritional dependency on plant-derived fatty acids.

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Changes in the protein kinase C activity or rat sternomastoid muscle during development and after denervation

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Summary. The relationship between the activity of protein kinase C (PKC) and muscle innervation was explored in the rat sternomastoid muscle (SM) from day 18 of gestation (E18) to adult age. Between E18 and birth, PKC activity rose 5-fold, and during the day after birth, diminished to a level characteristic of the mature muscle. The rise chiefly occurred in the neural part of the muscle, in both the membrane and the cytosol fractions. Between E18 and day 5 after birth, the ratios of membrane to cytosol PKC activity rose from 0.5 to 10 and 3 respectively in the neural and aneural parts of the muscle. Denervation of adult SM reduced PKC activity by half in the membrane fraction of the neural part but did not significantly change it in the membrane or cytosol fractions of the aneural parts. These results suggest that innervation plays an important part in determining the level of PKC activity in muscle.

Key words. Protein kinase C; sternomastoid muscle; development; denervation.