Characterization of free endogenous C_{14} and C_{16} sphingoid bases from *Drosophila melanogaster*

Henrik Fyrst,* Deron R. Herr,† Greg L. Harris,† and Julie D. Saba[1,*]

Children's Hospital,* Oakland Research Institute, 5700 Martin Luther King Jr. Way, Oakland, CA 94609; and Department of Biology and Molecular Biology Institute,† San Diego State University, San Diego, CA 92007

Abstract **Sphingolipid metabolites function as signaling molecules in mammalian cells, influencing cell proliferation, migration, and death. Recently, sphingolipid signaling has been implicated in the regulation of developmental processes in *Drosophila melanogaster*. However, biochemical analysis of endogenous *Drosophila* sphingoid bases has not been reported. In this study, a rapid HPLC-based method was developed for the analysis of free sphingoid bases endogenous to *Drosophila*. Four molecular species of endogenous free sphingoid bases were observed in adult flies and identified as C_{14} and C_{16} sphingosine (Sph) and C_{14} and C_{16} dihydrosphingosine (DHS). The C_{14} molecular species were the most prevalent, accounting for ~94% of the total free sphingoid bases in adult wild-type flies. An Sph kinase (SK) mutant demonstrated significant accumulation of all four sphingoid bases, whereas a serine palmitoyltransferase mutant demonstrated low but detectable levels. When endogenous sphingoid bases were evaluated at different stages of development, the observed ratio of Sph to DHS increased significantly from early embryo to adulthood. Throughout development, this ratio was significantly lower in the SK mutant as compared with the wild-type. This is the first report describing analysis of free C_{14} and C_{16} sphingoid bases from *Drosophila*. The biochemical characterization of these lipids from mutant models of sphingolipid metabolism should greatly facilitate the analysis of the biological significance of these signaling molecules.**—Fyrst, H., D. R. Herr, G. L. Harris, and J. D. Saba. **Characterization of free endogenous C_{14} and C_{16} sphingoid bases from *Drosophila melanogaster*. *J. Lipid Res.* 2004.** 45: **54–62.**

Supplementary key words high performance liquid chromatography • sphingolipid • long chain sphingoid base • sphingosine • dihydrosphingosine • signaling

The biochemical pathways of sphingolipid metabolism have been well characterized in the budding yeast, *Saccharomyces cerevisiae*, and in mammalian cells (**Fig. 1**). The free long-chain sphingoid bases (LCBs) and their phosphorylated (LCBP) and acylated (ceramide) derivatives formed in these pathways are potent signaling molecules that have been implicated in signaling pathways that regulate cell death, survival, differentiation, migration, and lipid homeostasis (1–6). Accordingly, methods have been developed for the analysis of these compounds in different cell types. Sphingolipids have been most thoroughly characterized in mammalian cells, in which the predominant molecular species of free LCBs are C_{18} and C_{20} sphingosine (Sph) and C_{18} and C_{20} dihydrosphingosine (DHS), and in *Saccharomyces cerevisiae*, in which the predominant molecular species are C_{18} and C_{20} phytosphingosine and C_{18} and C_{20} DHS (7–11). Sphingolipid molecular structures have also been determined for numerous other species (12–19). However, in most of the latter, the sphingoid backbone structures have been determined through degradative analysis of higher order sphingolipids, whereas the structural characterization and quantitation of LCB signaling molecules have not been reported. Despite this caveat, it appears that significant diversity exists among LCBs of different species with regard to carbon chain length, hydroxylation and methylation state, and saturation.

Recently, sphingolipid intermediates have been implicated in the regulation of development and physiology (20–26). In *Drosophila*, we have found that sphingolipid intermediates are involved in maintaining reproductive function, viability, and muscle integrity (26). *Drosophila* provides a powerful genetic model system for dissecting signaling pathways and determining their roles in animal development. Although only a few *Drosophila* enzymes of sphingolipid metabolism have been characterized (25–28) (Fig. 1), the identification of candidate genes and the ease of their genetic manipulation make this an ideal system for elucidating developmental and physiological roles of sphingolipid signal transduction at the level of the whole organism. In this study, we describe the identifica-

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Abbreviations: DHS, dihydrosphingosine; LCB, free long-chain sphingoid base; LCBP, phosphorylated free long-chain sphingoid base; OPA, *ortho*-phthalaldehyde; SK, sphingosine kinase; Sph, sphingosine; SPT, serine palmitoyltransferase.

[1] To whom correspondence should be addressed.
e-mail: jsaba@chori.org

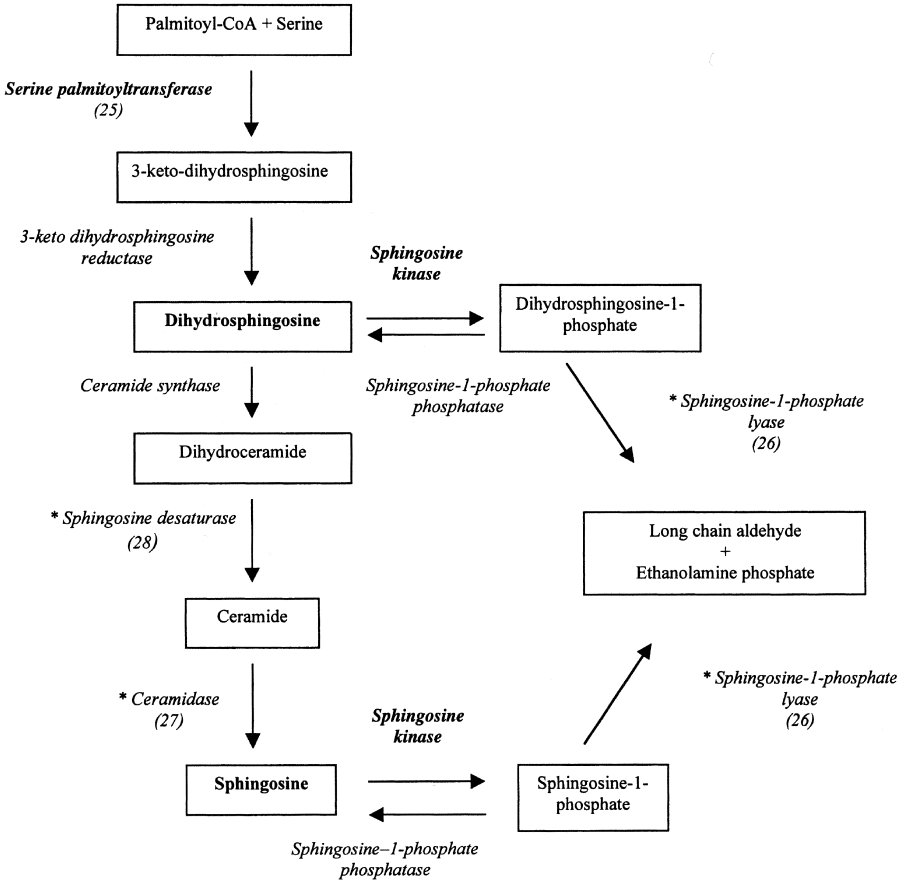

Fig. 1. Mammalian sphingolipid metabolic pathway. *Drosophila* endogenous sphingolipid molecules and enzymes corresponding to mutant models examined in this study are shown in bold. Asterisks indicate other enzymes of sphingolipid metabolism that have been characterized in *Drosophila*.

tion and quantification of C_{14} and C_{16} LCBs endogenous to *Drosophila* and evaluate their presence throughout fly development. Knowledge of *Drosophila* endogenous LCB structure and metabolism should afford the most effective use of this system in moving toward a better understanding of the biological significance of these enigmatic lipids.

EXPERIMENTAL PROCEDURES

Drosophila melanogaster lines

The *lace* gene encodes one subunit of a *Drosophila* serine palmitoyltransferase (SPT). Inheritance of two $lace^{k05305}$ null alleles is reported to be uniformly lethal, whereas the heterozygous allelic combination used in these experiments, $lace^{k05305}/lace^{2}$, leads to severe developmental phenotypes and a low percentage of viable progeny (25). A *Drosophila* line homozygous for a null allele (unpublished observations) of one of two putative Sph kinase (SK) genes was also utilized in these experiments. This mutant ($Sk2^{KG05894}$) was created by the insertion of a P-element into the 5′ UTR of CG2159, as previously described (29). The product of this gene demonstrates SK activity against a wide range of LCB substrates and functionally complements a yeast SK mutant (unpublished observations). Wild-type Canton-S (BL-1), $lace^{2}$ (BL-3156), $lace^{k05305}$ (BL-12176), and $Sk2^{KG05894}$ (BL-14133) lines were obtained from the Bloomington *Drosophila* Stock Center (Indiana University, Bloomington, IN). Flies were reared on standard fly media at room temperature. In all cases, control and mutant flies were reared in parallel under identical conditions. For developmental analysis, adult flies were allowed to deposit embryos on grape juice agar plates. After the collection period, plates were removed from the collection chamber, covered, and aged at room temperature to obtain appropriately staged embryos. For example, to collect 6–12 h embryos, adults were exposed to plates for 6 h, and plates were removed and aged for an additional 6 h before embryos were collected. Embryos were removed from the plates by washing with 0.7% sodium chloride-0.03% Triton X-100, rinsed extensively with water, and frozen at −70°C for storage.

Preparation of *Drosophila* lipid extracts

Samples containing 25 mg of frozen intact fly material were placed in a 7 ml Potter Elvehjem homogenizer. Twenty microliters of a mixture of internal LCB standards (Matreya Inc., Pleasant Gap, PA) containing 250 to 500 pmol of each LCB were then added. Flies were homogenized in 2 ml of chloroform-methanol (1:1; v/v) with a loose pestle, followed by a tight pestle until it moved smoothly. Extracts were further homogenized with a tip sonicator (3 × 20 s) while on ice, then transferred to a glass tube and centrifuged at 1,500 *g* for 10 min. Supernatants were recovered and dried down in a speed vac. Extracts were resuspended in 200 μl of methanol containing 0.1 M potassium hydroxide, followed by vortexing, bath sonication, and incubation at 37°C for 2 h to allow hydrolysis of esterified acyl chains. Following hy-

drolysis, the samples were cooled to room temperature, dried down in a speed vac, and resuspended in 2 ml of chloroform-methanol (2:1; v/v). Five hundred microliters of water was then added, and samples were vortexed, followed by centrifugation at 1,500 *g* for 5 min to obtain a clear separation of the two phases. The organic phase was recovered and washed three times with water. The washed organic phase was dried down in a speed vac and resuspended in 500 μl of methanol-water (1:1; v/v) containing 0.1% glacial acetic acid (solvent A).

Solid-phase extraction on a Strata C18-E column

The Strata C18-E solid-phase extraction column (50 mg/ml) (Phenomenex, Torrance, CA) was initially wetted with 200 μl of methanol, followed by equilibration with 1 ml of solvent A. Fly extracts or LCB standards in solvent A were applied to the equilibrated Strata C18-E column, followed by a wash with 1 ml of solvent A. A second wash of the column was performed by the addition of 500 μl of methanol. LCBs were eluted from the column with either 500 μl methanol-20 mM ammonium acetate (9:1; v/v) or 500 μl chloroform-methanol-20 mM ammonium acetate (4.5:4.5:1; v/v/v) and dried down in a speed vac. The recovery of LCBs from the Strata C18-E column was determined by comparing the HPLC peak area obtained for each sphingoid base before and after Strata C18-E extraction.

HPLC analysis

LCBs were derivatized with *ortho*-phthalaldehyde (OPA) (Sigma, St. Louis, MO) prior to HPLC analysis, as previously described (30). Samples were clarified by spinning at 14,000 *g* for 2 min, and the OPA-derivatized LCBs were separated on a reverse-phase column (Luna RP-18, 3 μ, 4.6 × 75 mm) (Phenomenex) with the mobile-phase methanol-10 mM ammonium acetate (pH 5.2) (82:18; v/v). Flow rate was 1 ml/min. The HPLC system used was a Beckman System Gold with a 125 solvent module. The fluorescent LCBs were detected and quantified using a Spectra-Physics fluorescence detector (SP 8410).

Mass spectrometry analysis of *Drosophila* LCBs

A Strata C18-E column-purified lipid extract from adult $Sk2^{KG05894}$ flies or a C_{14} Sph standard were analyzed on a Micromass Quattro LCZ instrument following direct injection of 10 μl of sample. The mobile phase was 80% methanol containing 0.1% formic acid. Flow rate was 0.2 ml/min. Structural confirmation of LCBs was obtained by positive electrospray ionization (ESI+) mass spectrometry. LCBs were detected by precursor ion scans of structurally distinct ion fragments as described (31). Applying 3.5 kV to the capillary started the spray, and the collision-induced decomposition spectra, at a cone voltage of 20 V, were recorded at a collision energy of 15 eV with argon as collision gas.

RESULTS

HPLC separation of sphingoid bases

A limited number of reports describing the structural analysis of higher order sphingolipids from dipteran insects indicate that the sphingoid backbone found in these complex molecules is most often a C_{14} Sph and, to a lesser extent, a C_{16} Sph (17–19). On the basis of these reports, an HPLC method was developed for the separation of LCBs with a carbon number of 14 to 18. **Figure 2A** illustrates the HPLC separation of five different LCB standards with 14 to 18 carbon atoms. (C_{14} DHS is not commercially available at the present time and is, therefore, not included as a standard in this experiment).

Solid-phase extraction of sphingoid bases

HPLC analysis of LCBs from crude chloroform-methanol lipid extracts from adult flies was hampered by the presence of a high content of contaminating fluorescent material (results not shown). Consequently, a solid-phase extraction step using a Strata C18-E column prior to HPLC analysis was introduced. **Table 1** shows the recovery of LCB standards from the Strata C18-E column using different elution solvents. Surprisingly, when methanol was employed as the eluting solvent, recovery of all the LCB standards was less than 2%. The inadequate recovery of the LCB standards from the Strata C18-E column was vastly improved by addition of 10% by volume of a 20 mM ammonium acetate solution to the methanol elution solvent. By employing this elution system, recovery in the range of 50% to 95% was obtained for the C_{14} and C_{16} LCB standards. However, inadequate recoveries were still obtained for the C_{18} LCB standards. In an attempt to improve the recovery of the C_{18} LCBs from the column, the elution solvent chloroform-methanol (1:1; v/v) was introduced. By employing this more hydrophobic elution solvent, a very moderate recovery was found for all the LCB standards. In agreement with the results obtained with methanol, the addition of ammonium acetate vastly improved the recovery of all the LCB standards from the column. Recovery of the C_{14} to C_{18} LCB standards with the elution solvent chloroform-methanol-20 mM ammonium acetate (4.5:4.5:1; v/v/v) was in the range of 60% to 80%. Losses associated with the two-phase extraction and subsequent washes were demonstrated to be consistently $<16\%$ for all LCB standards (data not shown).

HPLC analysis of LCBs from *Drosophila*

Elution of adult fly lipids from the Strata C18-E column with chloroform-methanol-20 mM ammonium acetate (4.5:4.5:1; v/v/v), resulted in an HPLC spectrum with significant unwanted background fluorescence (data not shown). Therefore, on the basis of the recovery of the LCB standards from the Strata C18-E column under various solvent conditions (Table 1), a methanol wash was introduced prior to elution with chloroform-methanol-20 mM ammonium acetate (4.5:4.5:1; v/v/v) (see Experimental Procedures for details). Figure 2 demonstrates an HPLC run of Strata C18-E column-purified LCBs using this method. Figure 2A shows the HPLC separation of LCB standards. All the C_{14} to C_{18} LCB standards evaluated were well separated on the HPLC in the 40 min run. Lipid extracts from three different lines of adult *Drosophila* flies were analyzed. The lipid profile of wild-type flies (Fig. 2B) was compared with that of an SK (*Sk2*) mutant (Fig. 2C) and an SPT (*lace*) mutant (See Experimental Procedures) (Fig. 2D). The *Sk2* mutants would be predicted to manifest a reduced capacity to phosphorylate LCBs and, as a consequence, should demonstrate increased levels of

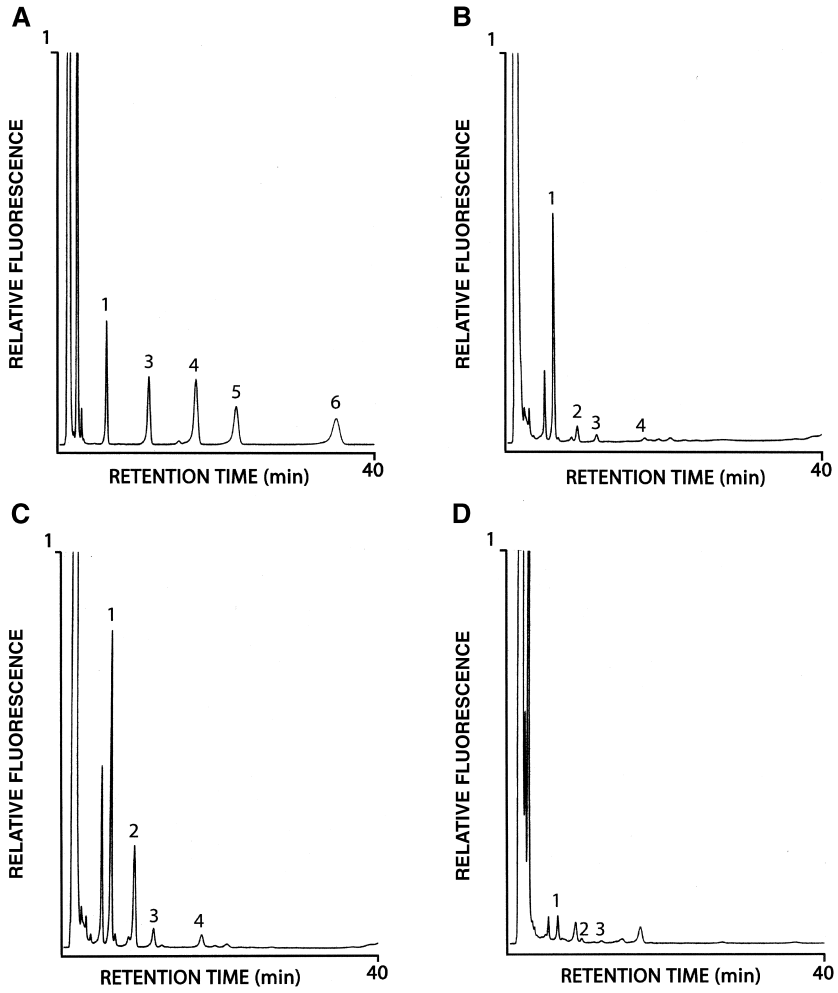

Fig. 2. HPLC analysis of *ortho*-phthalaldehyde (OPA)-derivatized free long-chain sphingoid base (LCB) standards and *Drosophila* lipid extracts following Strata C18-E column purification. Samples were separated on a C18 Luna column (4.6 × 75 mm) using isocratic elution with the solvent system methanol-10 mM ammonium acetate (pH 5.2), (82:18; v/v). Flow rate was 1 ml/min. HPLC analysis was performed on LCB standards containing 150–200 pmol of each standard (A) and lipid extracts obtained from 25 mg of adult wild-type (B), sphingosine kinase 2 (*Sk2*) mutant (C), and *lace* mutant (D). Peaks were identified as C_{14} sphingosine (Sph) (peak 1), C_{14} dihydrosphingosine (DHS) (peak 2), C_{16} Sph (peak 3), C_{16} DHS (peak 4), C_{18} Sph (peak 5), and C_{18} DHS (peak 6).

LCBs (Fig. 1). In contrast, the hypomorphic *lace* mutants are defective in the first step of sphingolipid de novo biosynthesis and would be predicted to exhibit diminished levels or complete absence of LCBs. The HPLC runs demonstrated five peaks that were increased in the *Sk2* mutant and decreased in the *lace* mutant. Three peaks eluting with the same retention times as the C_{14} Sph, C_{16} Sph, and C_{16} DHS standards were identified in the fly extracts (peaks 1, 3, 4). In addition, a peak with a retention time between that of C_{14} Sph and C_{16} Sph (peak 2) and a peak with a retention time shorter than that of C_{14} Sph were identified. No peaks that eluted with retention times corresponding to the C_{18} LCB standards were observed.

Following isocratic elution from a C18 reverse-phase HPLC column, a plot of the carbon length of a derivatized sphingoid base standard against the log of the retention time shows a linear correlation between sphingoid bases belonging to the same molecular class (11). This can be useful for the identification of an unknown sphingoid base. **Figure 3** demonstrates a plot of OPA-derivatized LCB standards and the unknown peak 2. As shown in the figure, a linear correlation exists between the retention time of the unknown peak 2 and the two DHS standards in this plot. This finding strongly suggests that peak 2 is C_{14} DHS. The identity of the peak that elutes ahead of C_{14} Sph remains unknown. It is likely to represent a sphingolipid, and its retention time could suggest a C_{12} DHS. However, mass spectrometry analysis gave no indication of endogenous C_{12} LCBs in the flies (see below).

Mass spectrometry analysis of LCBs from *Drosophila*

LCBs can be identified through their patterns of collision-induced dissociation and precursor ion scans using ESI+ (31). Based on their unique molecular structures,

TABLE 1. Recovery of sphingoid base standards following solid-phase extraction on a Strata C18-E column

Eluting Solvent	Sphingoid Base				
	C_{14} Sph	C_{16} Sph	C_{16} DHS	C_{18} Sph	C_{18} DHS
			% Recovery		
Methanol	1.7 ± 0.9	1.4 ± 1.1	nd	nd	nd
Methanol-20 mM ammonium acetate (9:1; v/v)	96.0 ± 2.1	80.4 ± 7.9	53.5 ± 5.6	29.8 ± 6.2	15.4 ± 0.8
Chloroform-methanol (1:1; v/v)	45.2 ± 3.3	38.8 ± 4.5	24.5 ± 5.1	31.4 ± 5.3	25.0 ± 0.3
Chloroform-methanol-20 mM ammonium acetate (4.5:4.5:1; v/v/v)	77.6 ± 1.0	78.3 ± 1.5	72.7 ± 2.3	69.8 ± 1.3	63.7 ± 0.7

DHS, dihydrosphingosine; nd, no detectable recovery. Values are shown as mean ± SD for at least three independent measurements. Percent recovery was found by comparing the HPLC peak area obtained for each sphingoid base before and after Strata C18-E extraction.

typical decomposition products arise from the loss of two water molecules. **Figure 4A** shows a precursor ion scan of a C_{14} Sph standard. The precursor ion spectrum of *m/z* 208 (C_{14} Sph minus two water molecules) shows parents as *m/z* 244 (C_{14} Sph) and *m/z* 226 (C_{14} Sph minus one water molecule). To verify the existence of C_{14} DHS in *Drosophila*, we analyzed a Strata C18-E column-purified lipid extract by ESI+. A lipid extract from the *Sk2* mutant was chosen for the analysis, because it demonstrated elevated levels of LCBs (Fig. 2C). Initially, we sought the presence of endogenous C_{14} Sph. Figure 4B shows a precursor ion spectrum of *m/z* 208 identifying C_{14} Sph (*m/z* 244) in the extract. Subsequently, we sought the presence of C_{14} DHS. Figure 4C shows a precursor ion spectrum of *m/z* 210 identifying endogenous C_{14} DHS (*m/z* 246). In addition, precursor ion scans of *m/z* 236 and *m/z* 238 identified endogenous C_{16} Sph and C_{16} DHS in the fly extract (results not shown). Precursor ion scans of *m/z* 264 and *m/z* 266 failed to identify C_{18} LCBs in the fly extract, supporting the results obtained from the HPLC analysis (Fig. 2). In addition, precursor ion scans of *m/z* 180 and *m/z* 182 failed to identify C_{12} LCBs in the fly extract.

C_{14} and C_{16} sphingoid bases in *Drosophila* models of sphingolipid metabolism

Endogenous *Drosophila* LCBs were quantified by performing HPLC separation of Strata C18-E column-purified extracts either with or without the addition of a defined amount of C_{14} Sph, C_{16} Sph, and C_{16} DHS standard. Separation was followed by comparison of the integrated area obtained for each fluorescent LCB peak (**Table 2**). To quantify C_{14} DHS, an estimated value for its percent recovery through the Strata C18-E column was found based on the percent recovery of the C_{16} DHS standard and the difference in the percent recovery of the C_{14} and C_{16} Sph standard (see Table 2 legend). Interestingly, *lace* and *Sk2* mutant flies differed appreciably from wild-type flies in both the total amount and composition of LCBs, as determined by analysis of lipid extracts from each line. The total amount of LCBs in the wild-type was ~1.3 nmol/100 mg of whole flies. The *Sk2* mutants exhibited a 1.8-fold increase, and the *lace* mutants an 8.4-fold decrease in the total amount of LCBs in comparison to wild-type flies. Sph accounted for ~90% of the total amount of LCBs in the wild-type flies, whereas DHS accounted for ~10%. Therefore, the molar ratio of Sph to DHS was ~9:1. In the *Sk2* mutant, the corresponding values were 66% Sph and 34% DHS, resulting in a molar ratio of ~2:1, whereas in the *lace* mutant the corresponding values were 84% Sph and 16% DHS, resulting in a molar ratio of ~5:1.

Sphingoid bases in *Drosophila* development

Genetic studies have implicated a role for sphingolipid intermediates in the process of development (20–26). However, quantification of these molecules throughout development has not been performed. To investigate whether a biochemical basis for the potential role of sphingolipid intermediates exists, we evaluated the endogenous LCBs at different stages of *Drosophila* development. **Table 3** lists the results obtained for the wild-type fly. The total amount of LCBs remained fairly constant throughout

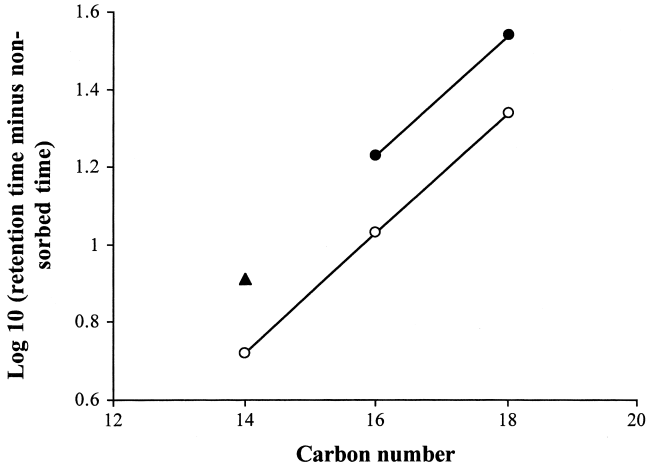

Fig. 3. Plot of log 10 (retention time minus nonsorbed time; 0.6 min) versus carbon number of OPA-derivatized sphingoid bases. Sph standards (open circles), DHS standards (closed circles), and peak 2 from Fig. 3 (closed triangle). Values for retention time are shown as the mean of three independent measurements, because the calculated standard deviation was less than 2% for all retention times evaluated.

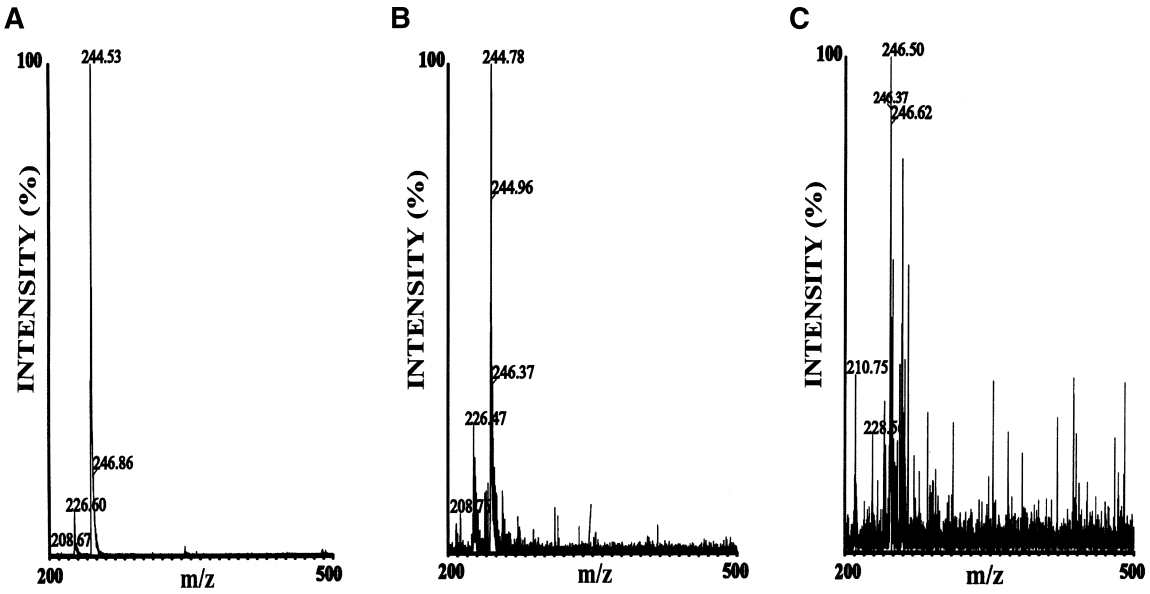

Fig. 4. Precursor ion spectra of sphingoid bases. Precursor ion spectra of *m/z* 208 were obtained for a C_{14} Sph standard (A) and a Strata C18-E column-purified *Sk2* mutant extract (B). Precursor ion spectrum of *m/z* 210 was obtained for a Strata C18-E column-purified *Sk2* mutant fly extract (C).

development, ranging from 1.25 to 2.10 nmol LCBs/100 mg of fly material. The C_{14} LCBs accounted for 92% to 96% of the total LCBs, except at the larval stage, where the C_{14} LCBs only accounted for 72%. Developmental progress resulted in a significant increase in the molar ratio of Sph to DHS. Development from early embryo to adulthood resulted in a 10-fold increase, from 0.89 to 9.12, in the Sph:DHS ratio (Tables 2, 3).

To further investigate the role of LCBs in development, we performed an analysis of the *Sk2* mutant (**Table 4**). Several differences were observed when the *Sk2* mutant was compared with the wild type. The first was the presence of a significant increase in the level of total LCBs at all stages of development in the *Sk2* mutant. Throughout development from early embryo to pupa, the level of LCBs was increased ~4- to 7-fold, and in the adult fly, the level was increased ~2-fold (Tables 2, 4). Second, the ratio of Sph to DHS was significantly lower in the *Sk2* mutant at all stages analyzed, and the development from early embryo to adulthood resulted in a 6-fold increase, from 0.35 to 1.98, in the ratio of Sph to DHS (Tables 2, 4). Third, the *Sk2* mutant displayed a much larger variation in the total amount of LCBs throughout development, ranging from 1.98 to 13.72 nmol/100 mg of fly material (Tables 2, 4). Fourth, the amount of C_{16} LCBs in the *Sk2* mutant at the larval and pupal stage was 9- and 13-fold higher, respectively, than the amount in the wild type. More than half of the LCBs found in the *Sk2* larvae were accounted for by C_{16} molecular species.

In summary, these results suggest that under normal conditions, the total amount of LCBs remains fairly constant throughout development. In contrast, the ratio of Sph to DHS increases, and when the fly reaches adulthood, the ratio of Sph to DHS is ~9:1. Lack of Sk2 activity clearly affects these parameters and leads to an increase in total LCBs as well as a lower Sph:DHS ratio.

TABLE 2. HPLC analysis of endogenous free long-chain sphingoid bases in various adult *Drosophila* lines

	Wild-Type	*Sk2*	*lace*
C_{14} Sph (nmol/100 mg of flies)	1.122 ± 0.066	1.472 ± 0.133 (131.2)	0.120 ± 0.010 (10.7)
C_{14} DHS[a] (nmol/100 mg of flies)	0.108 ± 0.014	0.691 ± 0.159 (639.8)	0.025 ± 0.008 (23.1)
C_{16} Sph (nmol/100 mg of flies)	0.063 ± 0.012	0.138 ± 0.016 (219.0)	0.011 ± 0.007 (17.5)
C_{16} DHS (nmol/100 mg of flies)	0.022 ± 0.010	0.122 ± 0.037 (554.5)	nd
Total LCBs (nmol/100 mg of flies)	1.315 ± 0.112	2.424 ± 0.345[b] (184.3)	0.156 ± 0.025[c] (11.9)
Sph:DHS (mol:mol)	9.12	1.98	5.24

LCB, free long chain sphingoid base; SK, sphingosine kinase; Sph, sphingosine. Values are shown as mean ± SD for three independent measurements. Numbers in parentheses represent percent of wild type.

[a] An estimated value for the recovery of C_{14} DHS from the Strata C18-E column was determined using the following formula: % recovery of C_{14} DHS = % recovery of C_{16} DHS × (% recovery of C_{14} Sph/% recovery of C_{16} Sph).

[b] Significantly different from wild-type; $P < 0.01$.

[c] Significantly different from wild-type; $P < 0.0001$.

TABLE 3. HPLC analysis of endogenous LCBs in different stages of wild-type *Drosophila* development

	Embryo (0–6 h)	Embryo (6–12 h)	Embryo (12–18 h)	Embryo (18–24 h)	Larva	Pupa
C_{14} Sph (nmol/100 mg of material)	0.601 ± 0.121	0.967 ± 0.217	0.977 ± 0.065	0.918 ± 0.225	0.739 ± 0.175	1.651 ± 0.201
C_{14} DHS (nmol/100 mg of material	0.784 ± 0.099	0.455 ± 0.087	0.435 ± 0.058	0.236 ± 0.013	0.479 ± 0.079	0.363 ± 0.024
C_{16} Sph (nmol/100 mg of material)	0.108 ± 0.006	0.052 ± 0.013	0.056 ± 0.021	0.068 ± 0.029	0.161 ± 0.052	0.063 ± 0.016
C_{16} DHS (nmol/100 mg of material)	0.011 ± 0.005	0.013 ± 0.004	0.044 ± 0.001	0.024 ± 0.013	0.306 ± 0.081	0.026 ± 0.010
Total LCBs (nmol/100 mg of material)	1.504 ± 0.231	1.487 ± 0.321	1.512 ± 0.145	1.246 ± 0.280	1.685 ± 0.387	2.103 ± 0.251
Sph:DHS (mol:mol)	0.89	2.18	2.16	3.97	1.15	4.41

Values are shown as mean ± SD for three independent measurements.

DISCUSSION

This work describes the identification and quantification of LCBs endogenous to *Drosophila*. By developing a simple method based on solid-phase extraction and HPLC separation, we found that the predominant *Drosophila* LCBs were C_{14} and C_{16} molecules. Whereas the predominant endogenous sphingoid bases of mammalian cells and the yeast *Saccharomyces cerevisiae* are C_{18} and C_{20} structures, neither HPLC nor mass spectrometry analysis provided any indication that C_{18} sphingoid bases exist in *Drosophila* under the conditions examined. This does not preclude the possibility that negligible amounts of C_{18} sphingoid bases could exist at baseline and could potentially increase under certain conditions.

A substantial recovery of the C_{14} to C_{18} LCBs was obtained from the Strata C18-E column following elution with the solvent system chloroform-methanol-20 mM ammonium acetate (4.5:4.5:1; v/v/v). However, the total amount of lipid eluted in this solvent system was several-fold higher than the lipid eluted in methanol-20 mM ammonium acetate (9:1; v/v) (results not shown). Therefore, when only shorter chain LCBs such as C_{14} and C_{16} are to be quantified or recovered, it may be advantageous to elute with methanol-20 mM ammonium acetate (9:1; v/v) to attain maximum purity of the sample. In contrast, to obtain a sufficient recovery of longer chain LCBs, or when the LCB chain length of a sample is unknown, a more hydrophobic solvent such as chloroform-methanol-20 mM ammonium acetate (4.5:4.5:1; v/v/v) may be employed.

Our finding of C_{14} and C_{16} LCBs in *Drosophila* has several important ramifications for sphingolipid biochemistry and metabolism in *Drosophila*. The first is that these LCBs are likely to exhibit significant differences in biophysical properties, subcellular localization, effects on membranes, and mechanisms of transport, in comparison with the longer chain sphingoid bases of mammalian cells and yeast. LCBs with a C_{14} backbone (as compared with a C_{18} backbone) are considerably less hydrophobic and, as a consequence, will exchange much more rapidly with a hydrophilic environment. This increased rate of exchange of the C_{14} compound could have important implications in the process of translocation and potentially in signal transduction in the fly. Second, these findings indicate that *Drosophila* enzymes of sphingolipid metabolism are likely to differ in their substrate specificities from those of mammalian cells and yeast. For example, palmitoyl-CoA is the preferred fatty acyl substrate for the yeast and mammalian SPT, which catalyzes the first step in sphingolipid biosynthesis through the condensation of palmitic acid and serine (32–34). This enzymatic reaction results in the formation of a C_{18} sphingoid base. On the basis of our findings, it seems probable that a C_{12} fatty acyl-CoA is the preferred substrate of *Drosophila* SPT (or, more accurately, serine acyltransferase) for LCB formation. It has been reported that *Drosophila* fatty acid synthetase activity produces a substantial amount of C_{12} and C_{14} fatty acids (35). Hence, a ready pool of C_{12} and C_{14} acyl-CoA esters for LCB formation is likely to exist.

Sphingoid bases can be derived either from de novo synthesis or from catabolism of higher order sphingolipids, as described for the sphingolipid metabolic pathway in mammalian cells (Fig. 1). In mammalian cells, the enzymatic conversion of DHS to Sph during de novo synthesis occurs subsequent to the acylation of DHS (36). The ratio of Sph to DHS can, therefore, serve as an indicator for the relative rate of de novo sphingolipid biosynthesis compared with sphingolipid degradation. We observed a substantial increase in the ratio of Sph to DHS during development in wild-type flies, although the total amount of LCBs remained fairly constant (Tables 2, 3). This indicates an increase in the breakdown of sphingolipids through the metabolic pathway (Fig. 1), and thereby suggests that particular LCB species may be important during the process of development. We observed a less-predominant increase in the ratio of Sph to DHS during development in the *Sk2* mutant. Moreover, the Sph:DHS ratio was significantly lower at every stage of development analyzed, and large variations were observed in the total LCB content. Consistent with the notion that a tightly regulated LCB content and composition may be important in development is the finding of reproductive and flight defects in homozygote *Sk2* mutant flies (unpublished observations).

That a sufficient level of Sph is needed for proper development of the fly has been suggested in studies of the hypomorphic *lace* mutant. Homozygotes of the *lace* null allele die during the first instar larval stage, and hypomorphic alleles used in this study result in pronounced morphological defects and reduced viability. These phenotypes can be overcome by feeding with Sph (25). The altered Sph:DHS ratio suggests a significant perturbation of sphingolipid metabolism in both the *lace* mutant and in the *Sk2* mutant. The precise biochemical mechanism responsible for this effect is unknown. However, in view of the diminished biosynthesis of sphingolipids in the *lace* mutant, it is feasible that a certain amount of DHS is con-

TABLE 4. HPLC analysis of endogenous LCBs in different stages of *Sk2* mutant *Drosophila* development

	Embryo (0–6 h)	Embryo (6–12 h)	Embryo (12–18 h)	Embryo (18–24 h)	Larva	Pupa
C_{14} Sph (nmol/100 mg of material)	1.980 ± 0.251	3.153 ± 0.348	2.662 ± 0.213	2.529 ± 0.567	1.161 ± 0.429	6.200 ± 0.991
C_{14} DHS (nmol/100 mg of material	5.856 ± 0.997	7.375 ± 1.106	4.614 ± 1.033	1.900 ± 0.066	2.467 ± 0.345	6.315 ± 0.758
C_{16} Sph (nmol/100 mg of material)	0.119 ± 0.033	0.188 ± 0.051	0.172 ± 0.014	0.166 ± 0.020	1.408 ± 0.268	0.666 ± 0.060
C_{16} DHS (nmol/100 mg of material)	0.220 ± 0.016	0.317 ± 0.060	0.242 ± 0.029	0.168 ± 0.047	2.688 ± 0.645	0.539 ± 0.086
Total LCBs (nmol/100 mg of material)	8.175 ± 1.297[a] (543.6)	11.033 ± 1.565[b] (742.0)	7.690 ± 1.289[c] (508.6)	4.763 ± 0.709[c] (382.3)	7.724 ± 1.687[c] (458.4)	13.720 ± 1.895[b] (652.4)
Sph:DHS (mol:mol)	0.35	0.43	0.58	1.3	0.49	1.03

Values are shown as mean ± SD for three independent measurements. Numbers in parentheses represent percent of wild type.
[a]Significantly different from wild-type; $P < 0.001$.
[b]Significantly different from wild-type; $P < 0.0005$.
[c]Significantly different from wild-type; $P < 0.005$.

served as needed for proper cellular function. Impaired incorporation of LCBs into sphingolipid biosynthesis might also be responsible for the accumulation of DHS in the *Sk2* mutant. In support of this notion, it has been suggested that proper sphingolipid biosynthesis in yeast is dependent on a phosphorylation/dephosphorylation cycle mediated by SK and LCBP phosphatase activity (37).

Biochemical analysis of the sphingolipid profiles of mutant *Drosophila* models in comparison to wild-type flies can provide important information complementary to genetic studies performed in this organism. In the case illustrated by the *lace* mutants analyzed in our study, the finding of residual LCBs indicates that some residual SPT activity exists in these mutants, thus accounting for the occasional survivors and reinforcing the vital function of sphingolipids in all metazoan and eukaryotic models studied thus far. Although it is conceivable that an activity exists in flies that converts C_{18} and C_{20} yeast sphingoid bases present in fly medium to C_{14} and C_{16} LCBs, no such activity has been described in any organism and thus we consider this highly unlikely. In the case of the *Sk2* mutant, measurement of endogenous LCBs throughout development in comparison to wild-type flies indicates that significant perturbation of LCB metabolism is present in this mutant, even though a second SK (*Sk1*) gene is present and presumed to be functional. Analysis of LCBs in single and combination SK mutant models should clarify the contribution of each SK gene to LCB metabolism, uncover unique substrate specificities of each, and demonstrate the physiological consequences of LCB accumulation and LCBP depletion in the fly.

In summary, we have developed an HPLC-based method for measurement of LCBs in *Drosophila* that is easily adopted for use in other organisms. We identified four free sphingoid bases of *Drosophila* as C_{14} and C_{16} Sph and DHS. This method was employed to characterize the LCB profile of adult wild-type flies as well as two interesting *Drosophila* mutants of sphingolipid metabolism. Furthermore, the LCB profile was characterized during fly development in a wild-type line and an *Sk2* mutant line. The further identification and characterization of *Drosophila* genes of sphingolipid metabolism and the analysis of sphingolipids in corresponding mutant models should help define aspects of sphingolipid biochemistry unique to this powerful genetic system and facilitate the analysis of the biological significance of these signaling molecules.

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