Plant Physiol. (1996) 110: 1177–1186

N^4-Hexanoylspermidine, a New Polyamine-Related Compound That Accumulates during Ovary and Petal Senescence in Pea[1]

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A previously unknown polyamine conjugate that accumulates in senescing ovaries of pea (*Pisum sativum* L.) was shown by mass spectrometry, nuclear magnetic resonance, and chemical synthesis to be N^4-hexanoylspermidine (hexanoyl-spd). This structure was indicated by analysis of the dansylated polyamine using fast atom bombardment mass spectrometry, following purification by high-performance liquid chromatography. Furthermore, acid hydrolysis of the compound yielded spermidine and hexanoic acid. ^{1}H-nuclear magnetic resonance suggested that spermidine was substituted at N^4 in the conjugate. Hexanoyl-spd was synthesized, and its didansyl derivative was shown to have an identical mass spectrum and high-performance liquid chromatography retention time as the derivatized natural compound. Further confirmation of its structure was obtained by comparison of the synthetic and natural polyamines as trifluoroacetyl derivatives using gas chromatography-mass spectrometry. This new polyamine conjugate is present in pea ovaries at low levels at anthesis and its concentration remains low in developing seeded fruit or in parthenocarpic fruit that have been induced by application of growth regulators to emasculated flowers or by topping the plant. Conjugate levels are also low in parthenocarpic fruit induced naturally in the slender (*la cry*^{s}) mutant. However, levels of hexanoyl-spd increase progressively in senescing petals and ovaries, beginning at anthesis or 2 d later, respectively.

The major polyamines of interest in plant physiology, put, spd, and spm, occur naturally as free bases or bound to phenolic acids, other low-molecular-weight compounds, or macromolecules (Galston and Kaur-Sawhney, 1990). Polyamines are found in relatively high concentrations in young, actively growing tissues, but their levels often decline with age in various organisms, including plants, in which the decline may begin with the onset of senescence (Kaur-Sawhney and Galston, 1991). Application of polyamines to excised leaves or leaf segments of monocots and dicots retards dark-induced leaf senescence, suggesting a role for polyamines in the control of senescence (Kaur-Sawhney and Galston, 1991). On the other hand, there are examples in which a decline in polyamine levels is not correlated with senescence and in which exogenous polyamines do not delay this process. For example, in the G_2 line of peas (*Pisum sativum* L.), senescence of the apical buds in long-day photoperiods begins before polyamine concentrations decline (Smith et al., 1983). Moreover, application of spm accelerates senescence in the presence of light in oat leaves (Kaur-Sawhney and Galston, 1979). As Galston and Kaur-Sawhney (1990) suggested, retardation of dark-induced senescence by exogenous polyamines might be an indirect, nonspecific effect due to interference with the formation of enzymes involved in ethylene biosynthesis.

In unpollinated pea ovaries, the onset of senescence is preceded by a decrease in put and spd contents and by an increase in that of spm. Furthermore, spm levels decrease when fruit growth is induced by application of plant growth regulators, such as auxin and GA, or by pollination (Carbonell and Navarro, 1989). Analysis of PCA-soluble polyamines in pea ovaries revealed two major components, spd and spm, and two minor components, one of which was put, but the second was unidentified. In the present work, we show that the unidentified polyamine is identical with hexanoyl-spd and that its level increases substantially during ovary and petal senescence.

[1] This work was supported by grant PB92–0018-C02–01 and M.A.P.-A. received a fellowship from Dirección General de Investigación Científica y Técnica of Spain. IACR receives grant-aided support from the Biotechnology and Biological Sciences Research Council of the United Kingdom.

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Abbreviations: amu, atomic mass unit; BOC, butoxycarbonyl; FAB, fast atom bombardment; GC-ECD, GLC-electron capture detection; hexanoyl-spd, N^4-hexanoylspermidine; HD, 1,6-hexanediamine; LC-MS, liquid chromatography-MS; pa, post anthesis; PCA, perchloric acid; put, putrescine; spd, spermidine; spm, spermine.

MATERIALS AND METHODS

Plant Material

Plants of *Pisum sativum* (L.) cv Alaska were grown in a greenhouse as previously described (Carbonell and García-Martínez, 1985). Only the first and/or the second flowers were used for experiments. Unpollinated ovaries were obtained by emasculation of the flowers (petals and stamens removed) 2 d before anthesis. Parthenocarpic fruits were induced by application of 20 μL of 0.33 mM GA_3 (Fluka), 0.44 mM BA (Fluka), or 0.045 mM 2,4-D (Fluka). All solutions contained 0.1% Tween 80 as a wetting agent. All treatments were carried out on the day of anthesis (d 0). Samples of ovaries, fruits, and petals were collected at different stages of development. Sepals and anthers were collected from flowers at the day of anthesis or 2 d before, respectively. Leaves, stems, and roots were collected from flowering adult plants. All samples were weighed, frozen in liquid N_2, and stored at −80°C.

Extraction and Dansylation of Polyamines

Extraction and quantification of polyamines were carried out as previously described (Carbonell and Navarro, 1989). Samples were homogenized in a chilled mortar with 5 to 9 volumes of cold 0.2 N PCA and 1 μmol g^{-1} fresh weight HD as an internal standard. Homogenates were clarified by centrifugation at 20,000*g* for 15 min at 4°C. One hundred microliters of supernatants were mixed with 200 μL of saturated Na_2CO_3 and 400 μL of dansyl-chloride (10 mg mL^{-1} acetone) and incubated in the dark for 1 h at 60°C in tapered reaction vials. One hundred microliters of L-Pro (100 mg mL^{-1}) were added and, after a further 30-min incubation, vials were opened and dansyl-polyamines were extracted twice with 500 μL of toluene. The organic phase was dried under a stream of N_2 at 40°C, and residues were dissolved in 200 μL of acetonitrile (HPLC grade) and ultrafiltered through HV-4 filters (Millipore). Standards of put, spd, and spm, as well as synthetic hexanoyl-spd, were made in 0.2 N PCA (100 μg mL^{-1} each) and analyzed as described above.

For HPLC analysis, aliquots (20–50 μL) were injected into a Hypersil-ODS reverse-phase column (200 × 4.6 mm, 5 μm; Hewlett-Packard) equilibrated in acetonitrile:water (60:40, v/v). Elution was done by increasing the percentage of acetonitrile to 90 over 30 min in a constant flow of 1.5 mL min^{-1} at 35°C. Dansyl-polyamines were detected using a fluorescence spectrophotometer with 365 and 425 nm excitation and emission wavelength filters, respectively. The solvent gradient was generated using two Waters 510 pumps controlled with the Millennium 2010 software package (Waters).

Purification of Dansyl-Hexanoyl-Spd and Dansyl-Spd from Senescent Ovaries

For the purification of the didansyl derivative of natural hexanoyl-spd, 1 to 8 g of senescent ovaries between d 4 and 5 pa were homogenized with a pestle in 4 volumes of cold 0.2 N PCA in a chilled mortar, and homogenates were centrifuged at 20,000*g* for 15 min at 4°C. Dansylation reactions were as described above, except that 500-μL aliquots of supernatants were used. Final dry samples were dissolved in acetonitrile, water was added to 40%, and the solution was ultrafiltered before being injected into the HPLC column described above. Fractions eluting between 11.0 and 12.5 min (hexanoyl-spd) and at 16 to 17 min (spd) were recovered and dried under vacuum at 50°C, and the residues were dissolved in 2 mL of acetonitrile. For quantification, a 50-μL aliquot of the purified HPLC fraction was mixed with a known amount of HD (approximately 1 μg) and dansylated as described above.

Analysis of Dansyl-Hexanoyl-Spd by FAB

Positive-ion FAB was carried out on a Kratos MS80RFA mass spectrometer (Manchester, UK) using an Ion Tech Saddle Field Gun (Teddington, UK) at 8 kV. Xenon atoms were used to bombard the sample in a thioglycerol matrix on a copper probe tip. Calibration was performed using Fomblin M (Fluorochem, Old Glossop, UK), and data were acquired at a scan speed of 10 s $decade^{-1}$ from *m/z* 1,000 to 80.

Combined LC-MS

LC-MS was carried out as described elsewhere (Moritz and Monteiro, 1994) using a JEOL double-focusing SX102A mass spectrometer fitted with a frit FAB LC interface. The mobile phase for HPLC was isocratic 80% methanol containing 1% acetic acid and 1% glycerol. Positive ions were obtained at a rate of 5 s per scan over a mass range of 20 to 1500 amu. For mass determination the conditions were the same, except that 0.02% PEG 600 was added to the mobile phase and the mass range was scanned from 550 to 800 amu at a resolution of 5,000 to 10,000, analyzed by acceleration voltage scanning.

Synthesis of Hexanoyl-Spd and Dansyl-Hexanoyl-Spd

N^1,N^8-bis-BOC-Spd (Fig. 1, 1)

N^1,N^8-bis-BOC-spd was a gift from Professor P.M. Cullis (University of Leicester, UK) and was synthesized as described by Cohen et al. (1992).

N^4-Hexanoyl-N^1,N^8-bis-BOC-Spd (Fig. 1, 2)

N^1,N^8-bis-BOC-spd (27 mg) in CH_2Cl_2 (5 mL) was treated with 100 μL of triethylamine and 50 μL of hexanoyl chloride. After 2 h at room temperature, the solvent was evaporated and the residue partitioned between ethyl acetate and saturated aqueous $NaHCO_3$. After the sample was washed with water, the ethyl acetate was evaporated to give a gum, which was purified by preparative TLC on silica gel using ethyl acetate:hexane (3:2, v/v). The band at R_F 0.5 gave 33 mg of N^4-hexanoyl-N^1,N^8-bis-BOC-spd (Fig. 1, 2). Electron impact MS *m/z* (relative intensity) 443 ($[M]^+$, 1%), 344(8), 270(12), 232(17), 214(20), 70(37), 59(100) and 57(73). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.43(1H, *br*, NH), 4.63(1H, *br*, NH), 3.39(2H, *t*, J = 6.5 Hz, H_2-3), 3.23(2H, *t*, J

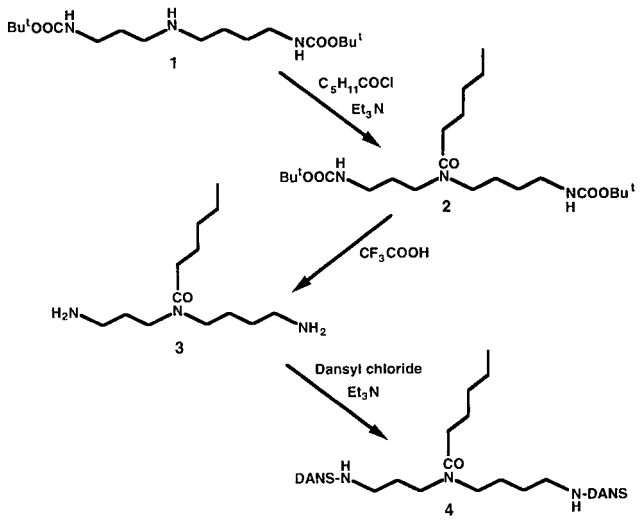

Figure 1. Scheme for synthesis of hexanoyl-spd (3) and N^1,N^8-bis-dansyl-hexanoyl-spd (4). 1, N^1,N^8-bis-BOC-spd; 2, N^4-hexanoyl-N^1,N^8-bis-BOC-spd.

= 8 Hz, H_2-5), 3.14(2H, *t*, *J* = 6.5 Hz, H_2-1), 3.04(2H, *dt*, *J* = 6.5, 6.0, H_2-8), 2.29(2H, *t*, *J* = 7.5 Hz, H_2-2'), 1.64 and 1.46(8H, two multiplets, H_2-2, 6, 7, and 3'), 1.43(18H, *s*, 2x^{t}Bu), 1.32(4H, *m*, H_2-4' and 5') and 0.90(3H, *t*, *J* = 7 Hz, H_3-6'). ^{13}C-NMR (100 MHz, $CDCl_3$) C: δ 173.63 (2'-CO), 156.22 and 156.06 (^{t}BuOCO), 78.87 and 79.40 (^{t}BuO); CH_2: δ 47.44, 45.41, 42.36, 40.00, 37.22, 33.11, 31.72, 27.68, 26.19, 25.32, 22.56; CH_3: δ 28.43 and 28.46 (^{t}Bu), 14.0 (6').

Hexanoyl-Spd Trifluoroacetate Salt (Fig. 1, 3)

N^4-hexanoyl-N^1,N^8-bis-BOC-spd (Fig. 1, 2) (30 mg) was dissolved in 2 mL of TFA. After 20 min of dissolution, the solvent was evaporated in a stream of N_2 and the residue dissolved in methanol and evaporated again. After the sample was dried in vacuo, the solid product was washed with hexane. The insoluble material (28 mg) was identified as N^4-hexanoyl-spd trifluoroacetate salt (Fig. 1, 3). Electron impact MS *m/z* (relative intensity) 471 ($[M]^+$, absent), 243(M-2xCF_3COOH, 8%), 213(26), 200(23), 156(27), 144(19), 127(25), 115(34), 101(33), 98(41), 84(49), 69(80), 44(100). ^{1}H-NMR (400 MHz, CD_3OD): δ 3.48 (2H, *t*, *J* = 6.5 Hz, H_2-3), 3.39 (2H, *m*, H_2-5), 2.98 (2H, *m*, H_2-1), 2.90 (2H, *t*, *J* = 6.5 Hz, H_2-8), 2.41 (2H, *t*, *J* = 7.5 Hz, H_2-2'), 1.91 (2H, *m*, H_2-2), 1.68 and 1.62 (6H, two multiplets, H_2-6, 7, and 3'), 1.33 (4H, *m*, H_2-4' and 5'), 0.91 (3H, *t*, *J* = 7 Hz, H_3-6'). ^{13}C-NMR (100 MHz, CD_3OD) C: δ 176.80 (2'-CO), 163.00 (*q*, J_{CCF} = 43 Hz, CF_3COO-), 118.50 (*q*, J_{CF} = 286 Hz, CF_3COO-); CH_2: δ 48.81, 46.26, 43.46, 40.56, 38.67, 33.92, 32.80, 26.97, 26.44, 25.98, 23.6; CH_3: δ 14.38 (6').

N^1,N^8-bis-Dansyl-Hexanoyl-Spd (Fig. 1, 4)

N^4-Hexanoyl-spd-bis-CF_3COO salt (Fig. 1, 3) (25 mg) was dissolved in 3 mL of acetonitrile and treated with 100 μL of triethylamine and 50 mg of dansyl chloride. After 1 h, water and ethyl acetate were added. The organic layer was evaporated and the residue purified by preparative TLC on silica gel with hexane:ethyl acetate (1:1, v/v). The fluorescent band at R_F 0.35 was collected to give N^1,N^8-bis-dansyl-hexanoyl-spd (Fig. 1, 4) (56 mg). Positive-ion FAB: *m/z* 710 $[M+H]^+$, 612 ($[M+H]^+$ -$C_6H_{10}O$), 477 ($[M+H]^+$-dansyl), 170 (dimethylaminonaphthyl). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.6–8.2 (6H, complex multiplet, dansyl hydrogens), 7.5 (4H, *m*, dansyl), 7.15 (2H, *d*, *J* = 7.5 Hz, dansyl), 3.18 (2H, *t*, H_2-3), 3.05 (2H, *m*, H_2-5), 2.84 (12H, *s*, 2xNMe_2), 2.84 and 2.80 (4H, 2 *m*, H_2-1 and 8), 2.10 (2H, *t*, H_2-2'), 1.45 (4H, *m*, H_2-2 and 3'), 1.36 (4H, *m*, H_2-6 and 7), 1.20 (4H, *m*, H_2-4' and 5'), 0.87 (3H, *t*, H_3-6'). ^{13}C-NMR (100 MHz, $CDCl_3$) C: δ 173.81 (2'-CO); dansyl C and CH: δ 152.05, 151.70, 135.57, 134.67, 130.52, 130.03, 129.85, 129.80, 129.61, 129.53(2), 129.04, 128.47, 128.21, 123.19, 123.13, 119.31, 118.56, 115.23, 115.14; CH_2: 47.14, 42.72, 41.91, 39.88, 32.84, 31.53, 27.67, 26.83, 25.73, 25.09, 22.46; CH_3: δ 45.41 (NMe_2), 13.95 (6').

Acid Hydrolysis of Natural and Synthetic Dansyl-Hexanoyl-Spd

Approximately 100 μg of natural or synthetic dansyl-hexanoyl-spd (corresponding to approximately 35 μg of hexanoyl-spd) were dissolved in 500 μL of 6 N HCl and transferred to flame-sealable glass vials. Vials were closed, and acid hydrolysis was carried out for 24 to 48 h at 110°C. Before the vials were opened, these were cooled to room temperature. For short-chain fatty acid analysis, reaction mixtures were extracted twice in 50 μL of $CHCl_3$ and the organic phase was used for esterification. The aqueous phase was used for polyamine analysis and quantification. The reaction mixtures were centrifuged at 20,000*g* for 30 min at room temperature and dried under a stream of N_2 at 60°C, and residues were dissolved in 1 volume of 0.2 N PCA. One-hundred-microliter aliquots were processed for qualitative HPLC analysis. For quantification, 100-μL aliquots were mixed with a known amount of HD (approximately 1 μg) and dansylated as described above.

Analysis by GC-ECD of Fatty Acids as Their 2,2,2-Trichloroethyl Esters

Esterification of fatty acids from acid hydrolysis of natural or synthetic dansyl-hexanoyl-spd was carried out essentially as described by Alley et al. (1976). Hexanoic acid (caproic acid, C_6) and *n*-hexadecanoic acid (palmitic acid, C_{16}) were dissolved in $CHCl_3$ at 10 μg mL^{-1} and 250 mg mL^{-1}, respectively. Hexanoic acid from acid hydrolysis was extracted twice in 50 μL of $CHCl_3$. Samples or standards of hexanoic acid (50 μL) were mixed with 50 μL of 2,2,2-trichloroethanol (1:99, v/v, in $CHCl_3$) and 150 μL of heptafluorobutyric acid and allowed to react for 30 min at room temperature. Subsequently, 50 μL of *n*-hexadecanoic acid (250 mg mL^{-1}) and 50 μL of heptafluorobutyric acid were added, and the vials were incubated for a further 15 min. Chloroform (100 μL) and 100 μL of 0.1 N HCl were then added and the organic phases were transferred to a new vial, to which 100 μL of 0.1 N NaOH were added, and the resulting aqueous phase was removed. A small amount of anhydrous sodium carbonate was added to dry the

organic phase, which was then transferred to a new vial, dried under a stream of N_2, and redissolved in 100 μL of diethyl ether for GC-ECD using a Hewlett-Packard 5890 series II gas chromatograph. Aliquots (1 μL) of the esterification reaction mixtures were injected into an HP-5 column (5% phenyl methyl siloxane, cross-linked, 50 m × 0.2 mm, 0.33 mm; Hewlett-Packard). The column temperature was programmed from 140 to 270°C at 5°C min^{-1}. Helium was used as the carrier gas at a flow rate of 1 mL min^{-1}.

Formation of Trifluoroacetyl Derivatives

Acylation of polyamines was carried out as described by Laleye et al. (1987). Five hundred milligrams of senescent ovaries (at d 5 pa) were homogenized with a pestle in 3 mL of cold 0.2 N PCA in a chilled mortar and centrifuged at 20,000*g* for 15 min at 4°C. The supernatant (3 mL) was eluted through a C_{18} reverse-phase cartridge (SepPak, Millipore) as follows. The cartridge was equilibrated with 20 mL of methanol and 20 mL of water at pH 2.0. The sample was loaded and the retained compounds were eluted with 5 mL of methanol. Aliquots (50 μL) of the initial homogenate, as well as eluted fractions, were analyzed by HPLC after dansylation, as described above. Hexanoyl-spd was retained on the cartridge and eluted with methanol, which was removed under a stream of N_2 at 40°C. The residue was dissolved in 3 mL of 50 mM pyridine in benzene and 1.5 mL of trifluoroacetic anhydride and heated at 60°C for 30 min. After the samples were cooled to room temperature, 3 mL of benzene were added to the vials, then the solvents were evaporated under a stream of N_2 at 40°C, and the residues were dissolved in 0.5 mL of acetonitrile and 1.2 mL of water (final percentage 30:70 [v/v] acetonitrile:water). The pH was increased to 5.0 with NaOH and the solutions were ultrafiltered through HV-4 filters. Aliquots (300 μL) were injected into a reverse-phase Hypersil ODC column (Hewlett-Packard) equilibrated in acetonitrile:water (30:70, v/v). Trifluoroacetyl derivatives were eluted in a gradient of 30 to 90% (v/v) acetonitrile:water over 30 min that was generated using a 626 pump 600S pump controller, and Millennium 2010 software package (Waters). Detection was performed with a 996 Photodiode Array Detector (Waters) with a wavelength range from 190 to 250 nm. Standards were prepared in 1 N HCl (100 μg mL^{-1} each), from which aliquots of 200 μL were dried under a stream of N_2 at 60°C, dissolved in 200 μL of 50 mM pyridine in benzene and 150 μL of trifluoroacetic anhydride and reacted as described above. HPLC fractions containing trifluoroacetyl derivatives, either from pea ovary extracts or standards, were recovered in glass vials, dried under a stream of N_2 at 50°C, and analyzed by GC-MS.

GC-MS of Trifluoroacetyl Polyamines

GC-MS was carried out using the Kratos MS80 system. The GC conditions were: on-column injection at 70°C onto a 25-m × 0.32-mm × 0.5-μm film thickness BP1 column (Scientific Glass Engineering, Melbourne, Australia) and after 1 min, programming at 8°C min^{-1} to 340°C. MS conditions were: interface and source temperatures, 260 and 220°C, respectively; electron energy, 70 electron volts; and scanning from 600 to 50 amu at 1 s mass $decade^{-1}$.

RESULTS

We reported previously that spd and spm were the major polyamines present in unpollinated pea ovaries and young developing fruits, whereas the level of put was much lower (Carbonell and Navarro, 1989). HPLC chromatograms of dansylated extracts from pea ovaries contained an unidentified minor peak at 11.8 min. In a new analysis using a larger set of dansyl derivatives of free and bound polyamine standards, the compound eluting at 11.8 min did not correspond to any of the dansylated standards available:

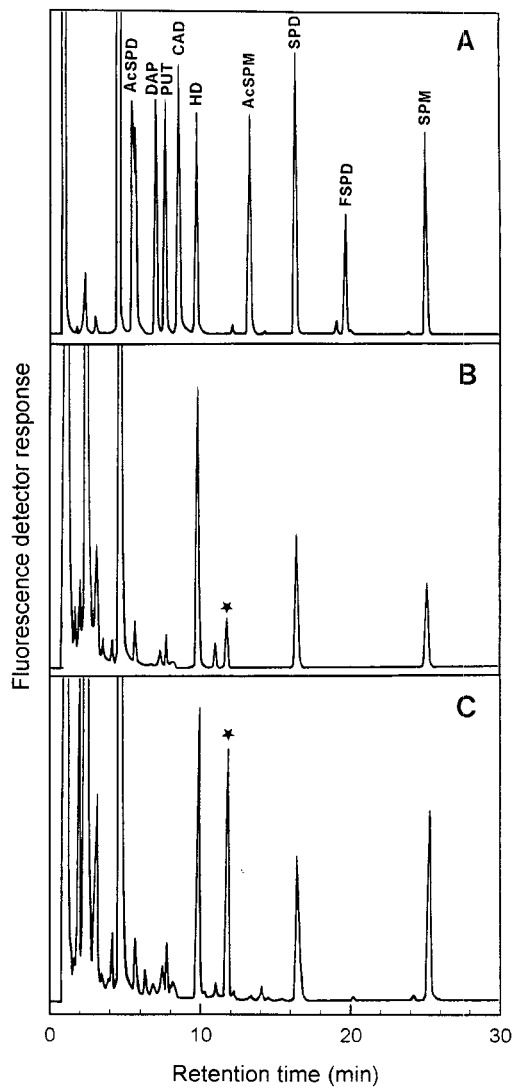

Figure 2. HPLC chromatograms of the dansyl derivatives of polyamines. A, Polyamine standards: AcSPD, N^1- and N^8-acetyl-spd; DAP, 1,3-diaminepropane; CAD, cadaverine; AcSPM, N^1-acetyl-spm; and FSPD, N^1-feruloyl-spd. B, Dansylated extract from parthenocarpic fruits, at d 5 pa, induced by treatment of unpollinated flowers with GA_3. C, Dansylated extracts from senescent ovaries at d 5 pa. The star indicates the unknown compound at 11.8 min.

N^1-acetyl-spd and N^8-acetyl-spd, 5.6 min; 1,4-diaminopropane, 7.1 min; put, 7.7 min; cadaverine, 8.5 min; HD, 9.8 min; N^1-acetyl-spm, 13.2 min; spd, 16.4 min; N^1-feruloyl-spd, 20.3 min; and spm, 25.3 min (Fig. 2A). The polyamine with the closest retention time was N^1-acetyl-spm. The height and area of the peak at 11.8 min remained constant during fruit growth but increased markedly compared with the internal standard when the ovary began to senesce at approximately d 2 or d 3 pa (Fig. 2, B and C).

Analysis by FAB

As a first approach to identifying the compound(s) associated with the peak at 11.8 min, we isolated the HPLC peaks corresponding to put, spd, spm, and the unknown compound(s) from dansylated extracts of senescent ovaries and analyzed the contents by positive-ion FAB. The mass spectra for the HPLC fractions corresponding to put, spd, and spm were identical with those for dansylated authentic standards (data not shown). The FAB mass spectra of the dansylated unknown compound and $(dansyl)_3$-spd are shown in Figure 3. The former shows a $[M+H]^+$ ion at *m/z* 710 (Fig. 3A). The ion at *m/z* 612, corresponding to a fragment resulting from loss of 99 amu from the molecular ion, is also present in the spectrum of $(dansyl)_3$-spd (Fig. 3B). A third ion, at *m/z* 477, would be produced by a loss of a dansyl group from the molecular ion. If we assume that the compound is derivatized with two dansyl groups, it would have an M_r of 243 in the underivatized form, corresponding to spd conjugated with a moiety of M_r 99. This could correspond to a hexanoyl group ($C_5H_{11}CO$), bound to a primary or secondary amine of spd.

Analysis of the contents of the HPLC peak by ^{1}H-NMR suggested that the dansyl groups were coupled to the primary N atoms of the unknown polyamine. This was evident from comparison of the aromatic hydrogen chemical shifts of the unknown dansyl polyamine with those in the spectra of $(dansyl)_2$-put, $(dansyl)_3$-spd, and $(dansyl)_4$-spm. In particular, an isolated doublet at 8.0 is

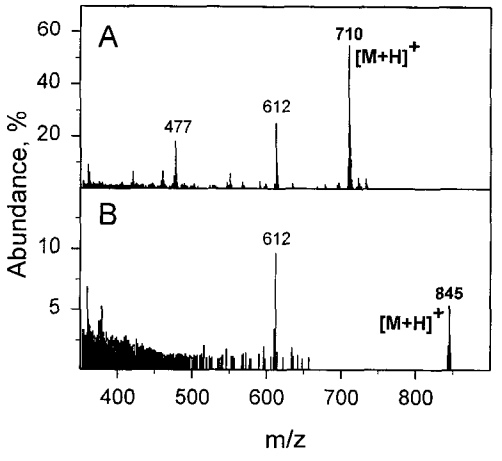

Figure 3. FAB mass spectra of dansyl derivative of unknown compound (A) and dansyl-spd (B) purified from dansylated extracts of senescent ovaries by HPLC. Ions with relative abundance <1% were omitted.

indicative of the tertiary dansyl group(s) in $(dansyl)_3$-spd and $(dansyl)_4$-spm. This signal is not seen in the spectrum of $(dansyl)_2$-put or that of the unknown compound. Thus, we concluded that the two dansyl groups in the unknown compound were terminal, and the putative hexanoyl moiety was on the internal amino group. Apart from the presence of the terminal ethyl group of the aliphatic chain (3H triplet at the 0.9), little further information could be obtained from the NMR spectrum, since it contained a number of extra peaks, indicating that the compound was not pure. The sample was, thus, examined further by LC-MS, using an instrument with a frit FAB interface (Moritz and Monteiro, 1994). However, the mass spectrum obtained was essentially identical with that observed by direct FAB, and no other components in the HPLC peak were apparent.

Synthesis and Analysis of Synthetic Hexanoyl-Spd

The mass spectral and NMR data suggested that the unidentified polyamine could be spd with a hexanoyl moiety linked as an amide to the central N atom. Consequently, hexanoyl-spd and its $(dansyl)_2$ derivative N^1,N^8-didansyl-hexanoyl-spd were synthesized as shown in Figure 1. Selective protection of spd with BOC groups at the terminal primary amino groups was described by Cohen et al. (1992). N^1,N^8-bis-BOC-spd (Fig. 1, 1) was acylated at N^4 with hexanoyl-chloride to give compound 2 in Figure 1. Removal of the BOC groups was accomplished by brief treatment with TFA, which yielded the required hexanoyl-spd as the trifluoroacetate salt (Fig. 1, 3), as shown by the presence of quartets in the ^{13}C-NMR spectrum at the 163.0 (J = 43 Hz) and 118.5 (J = 286 Hz). Dansylation of compound 3 in Figure 1 yielded N^1,N^8-didansyl-hexanoyl-spd (Fig. 1, 4), which had an identical FAB mass spectrum with that of the dansylated natural product. Similarly, signals for dansyl-hexanoyl-spd (Figs. 1 and 4) were all present in the ^{1}H-NMR of the dansylated natural product, although the sample contained other dansylated impurities. The HPLC retention time of synthetic dansyl-hexanoyl-spd was identical with that of the derivatized natural compound and, when they were co-injected, they eluted as a single peak at 11.8 min, even under isocratic eluting conditions (data not shown).

Acid Hydrolysis of Natural and Synthetic Hexanoyl-Spd

The products from acid hydrolysis of the natural polyamine were compared with those from the synthetic compound. *n*-Hexanoic acid, hexanoyl-spd, and spd were detected in the hydrolysates either from synthetic dansyl-hexanoyl-spd after 24 and 48 h of hydrolysis or from the dansylated natural compound after 24 h of hydrolysis (Table I). The efficiency of the reaction was approximately 45% after 24 h and increased to only approximately 65% after 48 h. No fatty acids were detected by GC analysis of methyl esters using flame ionization detection, and it was necessary to increase the sensitivity of the assay using trichloroethyl esters and ECD. Figure 4 shows GC-ECD chromatograms of hexanoic and *n*-hexadecanoic acid stan-

Table I. *Analysis of products from acid hydrolysis of the dansyl derivatives of synthetic and natural hexanoyl-spd*

Synthetic, 36 μg of synthetic hexanoyl-spd hydrolyzed (108 μg of its dansyl derivative). Natural, 33 μg of natural hexanoyl-spd hydrolyzed (100 μg of its dansyl derivative).

Type	Hexanoyl-spd	Spd[a]	Hexanoic Acid	Recovery	Hydrolysis
Synthetic					
24 h	17.0	8.6 (14.6)	++[b]	31.6 (93%)	46%
48 h	9.7	10.3 (17.4)	++	27.1 (80%)	64%
Natural					
24 h	14.6	6.5 (11.0)	++	25.6 (77%)	43%

[a] Equivalent amount of hexanoyl-spd detected as spd (μg spd × 1.69). [b] Significant amount of hexanoic acid detected by GC-ECD.

dards (Fig. 4A), chromatograms of ether extracts of the acid hydrolysates of the dansyl derivative of the natural compound (Fig. 4B), and chromatograms of synthetic didansyl-hexanoyl-spd (Fig. 4C). The trichloroethyl ester of hexanoic acid was detected at 13.8 ± 0.1 min, indicating the release of this short-chain fatty acid from the polyamine.

GC-MS of Natural Hexanoyl-Spd and Endogenous Polyamines

To obtain definitive evidence for the identity of the endogenous compound, full-scan GC-MS was carried out on trifluoroacetylated extracts. The natural unknown compound was purified from extracts of senescent ovaries on reverse-phase C_{18} cartridges. Whereas endogenous put, spd, and spm were not retained by the cartridge, the unknown polyamine bound and could be eluted with methanol. Partially purified extracts from senescent ovaries were converted to trifluoroacetyl derivatives, after which the derivatized polyamines were purified by HPLC and then analyzed by GC-MS. The HPLC retention times for trifluoroacetyl derivatives of polyamine standards were: put, 5.5 min; HD, 9.3 min; spd, 11.8 min; hexanoyl-spd, 14.5 min; and spm, 17.5 min. The elution profile of trifluoroacetyl polyamines was similar to that of dansyl-polyamines, except that hexanoyl-spd eluted between spd and spm as the trifluoroacetyl derivative, whereas the dansyl derivative eluted between put and spd. The trifluoroacetyl derivatives of synthetic hexanoyl-spd and the natural compound eluted with the same retention times and their co-injection gave a single peak. On GC-MS, synthetic and natural hexanoyl-spd gave identical mass spectra (Fig. 5) and, furthermore, they eluted as a single peak when co-injected. We conclude that the natural compound is identical with hexanoyl-spd.

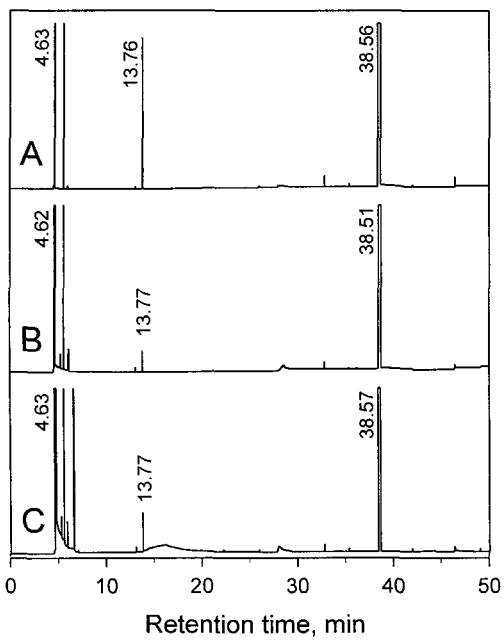

Figure 4. GC-ECD chromatograms of 2,2,2-trichloroethanol derivatives of hexanoic (13.8 min) and hexadecanoic (38.6 min) acids. Standards (A) and ether extracts of the acid hydrolysate of dansyl derivatives of the natural compound (B) or synthetic hexanoyl-spd (C) are shown.

Time Course of Hexanoyl-Spd Levels during Ovary Senescence

Synthetic hexanoyl-spd was used to obtain a calibration curve for quantification of the natural compound, using HD as the internal standard. Different relative amounts of synthetic hexanoyl-spd and HD were dansylated and their peak areas determined after HPLC (Carbonell and Navarro, 1989). A linear correlation between relative areas and relative amounts ranging between 0 and 10 mg hexanoyl-spd mg^{-1} HD ($y = 0.2799x + 0.0488$, $r = 0.9970$) was obtained, with a response factor of 3.5723.

Levels of hexanoyl-spd increased during the senescence of unpollinated pea ovaries (Fig. 6). The concentration of hexanoyl-spd remained constant between d 0 and d 2 pa, when ovaries continued to develop, but increased when the ovary entered the senescence phase (d 2–d 4 pa). At d 4 pa, levels of hexanoyl-spd were the highest when compared with levels of put, spd, and spm (data not shown). By contrast, when senescence was prevented, either by treating the ovaries with plant growth substances that induced parthenocarpic fruit development (GA_3, 2,4-D, or BA) or by topping the plant, hexanoyl-spd levels remained constant (Fig. 6, inset). Constant hexanoyl-spd levels were also observed during fruit development induced by natural pollination of cv Alaska (data not shown) or in the slender (*la cry*^{s}) pea mutant, in which development of parthenocarpic fruits is induced spontaneously (Fig. 7). In this mutant, the same levels of hexanoyl-spd were observed in fruits from emasculated and pollinated flowers.

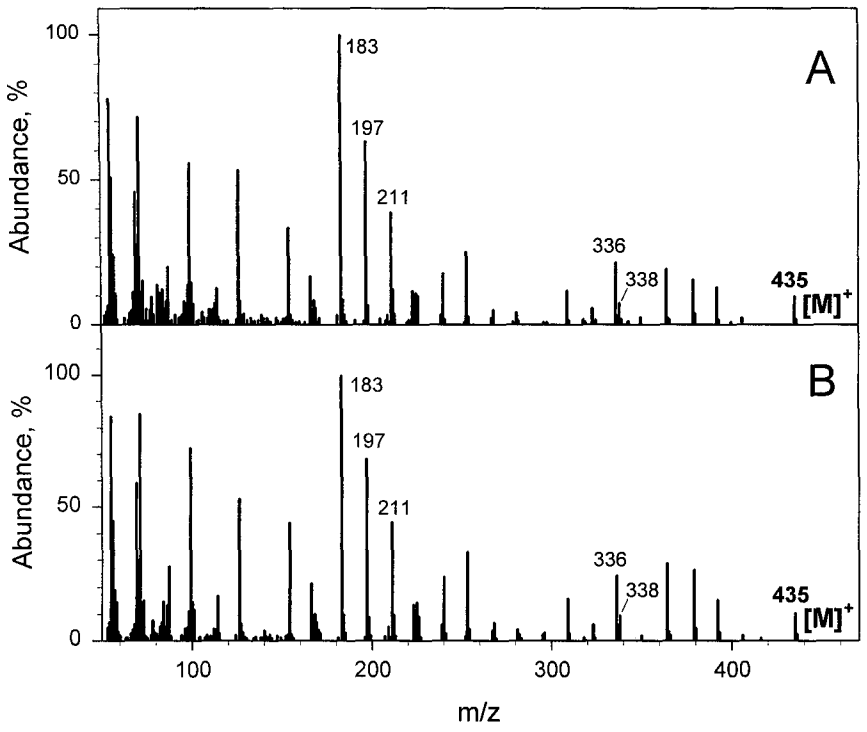

Figure 5. Electron impact mass spectra of the TFA derivatives of synthetic hexanoyl-spd (A) and natural hexanoyl-spd (B) after GC-MS. Ions with relative abundance <1% are omitted.

Organ Specificity of Hexanoyl-Spd Accumulation

To check for the presence of hexanoyl-spd in different tissues, extracts from the outer whorls of the mature flower, as well as from shoot apex, leaves, stems, and roots, were analyzed by HPLC as described for ovaries and fruits (Table II). Hexanoyl-spd was present in all of the organs tested; the highest levels were in young leaves and shoot apex, which is composed mainly of the youngest leaves. By contrast, the compound is barely detectable in roots. In flowers, hexanoyl-spd was most abundant in sepals but was present at very low concentrations in anthers. It was also observed that the content of the polyamine decreased during leaf development. These data indicate that hexanoyl-spd is present mainly in green tissues and at very low levels in nongreen tissues, such as roots, mature anthers, and petals at anthesis. The concentrations of spd are also included in Table II. As for hexanoyl-spd, spd was present at highest levels in young leaves and shoot apex and decreased in leaves during development. In contrast to hexanoyl-spd, spd was present at a very high concentration in mature anthers. Levels of both polyamines were similar in stems.

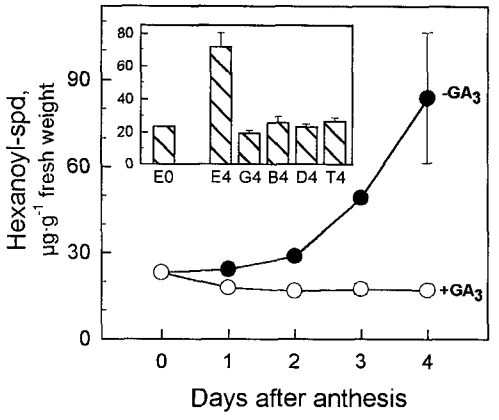

Figure 6. Time course of hexanoyl-spd levels during ovary development and senescence (●) or fruit development (○) induced by treatment of emasculated flowers with GA_3. Inset, Hexanoyl-spd levels in ovaries at the day of anthesis (E0) or at d 4 pa (E4) and in fruits at d 4 pa induced by treatment of emasculated flowers with GA_3 (G4), BA (B4), and 2,4-D (D4) or by topping (T4).

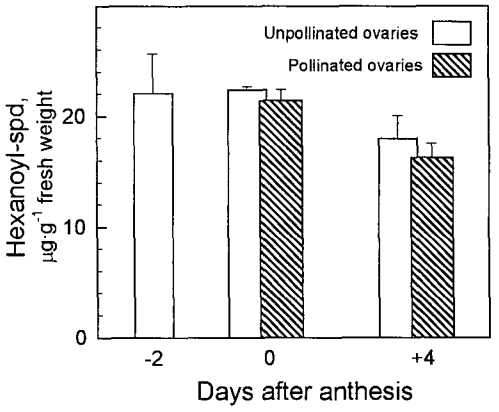

Figure 7. Levels of hexanoyl-spd in fruit of the slender (*la cry*^{s}) pea mutant after self-pollination of intact flowers or self-induced parthenocarpic fruits after emasculation of flowers 2 d before anthesis.

Table II. *Concentrations of hexanoyl-spd and spd in different organs of the adult pea plant*

Organ	Hexanoyl-spd	spd
Flower	$\mu g\ g^{-1}$	$\mu g\ g^{-1}$
Ovary[a]	30.36 ± 1.75[c]	94.71 ± 13.93
Petal[a]	14.20 ± 0.74	33.97 ± 1.24
Sepal[a]	54.49 ± 1.96	22.96 ± 0.89
Anther[b]	2.52 ± 0.12	148.57 ± 3.38
Shoot apex	63.81 ± 1.02	187.82 ± 5.57
Young leaf	78.18 ± 3.36	222.81 ± 10.22
Adult leaf	29.10 ± 1.01	29.38 ± 1.19
Stem	18.53 ± 0.34	14.91 ± 0.43
Root	0.87 ± 0.09	13.34 ± 0.28

[a] Samples were collected at the stage of anthesis. [b] Anthers were collected from flowers 2 d before anthesis. [c] Mean ± SE, $n = 3$.

Time Course of Hexanoyl-Spd Levels and Content during Petal Senescence

The concentration of hexanoyl-spd in petals was measured throughout the growth and senescence of the corolla (Fig. 8). Fresh weight increased during corolla growth (d −2 to d 0), with a maximum at anthesis, and decreased during its senescence (d 0 to d 2 pa) (Fig. 8A). During the growth phase, hexanoyl-spd levels were almost constant, but, as in ovaries, when the organ entered the senescence process, levels increased. At the end of senescence (d 2 pa), hexanoyl-spd was the most abundant polyamine in the corolla, being 3 times more concentrated than at anthesis. Since corolla fresh weight decreased to about one-third during senescence, the increase in hexanoyl-spd concentration is due primarily to loss of water rather than an increase in hexanoyl-spd content. The accumulation of hexanoyl-spd in corolla and ovaries during the growth and senescence of these tissues follows very similar patterns (Fig. 8B) and indicates that the production of this polyamine occurs during the growth and in the first hours of senescence of both tissues.

Figure 9 shows time courses of concentrations (Fig. 9A) and amounts per organ (Fig. 9B) of put, spd, and spm in petals during growth and senescence. The concentrations of these polyamines showed different patterns. Put levels decreased during growth and remained constant at a minimum level during senescence. The pattern is similar for spd, but spm levels decreased during petal growth and increased during senescence. In addition, the amounts of put, spd, and spm showed a different profile from that of hexanoyl-spd. Put content remained almost constant, whereas spd and spm content followed the same time course as petal fresh weight, with maxima at anthesis.

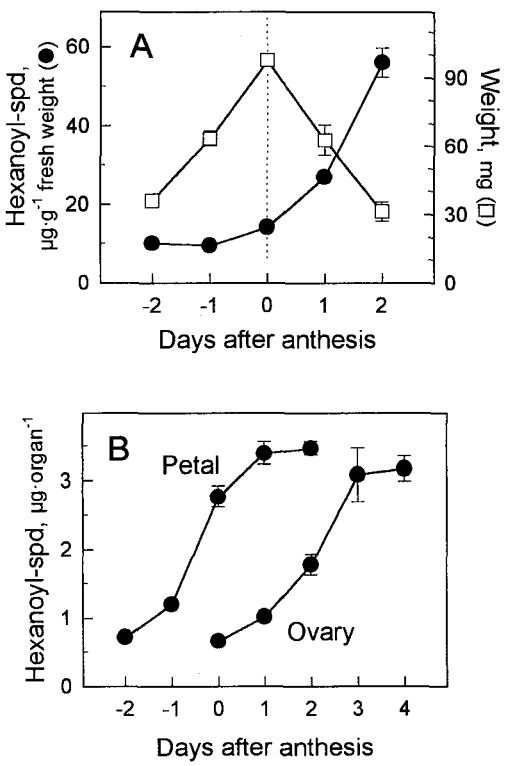

Figure 8. Time course of hexanoyl-spd levels and content during petal growth and senescence. A, Level of hexanoyl-spd (●) and fresh weight (□) of the corolla. Dashed line indicates the stage of flower anthesis. Results are means ± SE, $n = 3$. B, Content of hexanoyl-spd in petal and ovary during their growth and senescence. Results are means ± SE, $n = 3$.

DISCUSSION

In this paper, we provide evidence to support the identification of a new, natural polyamine conjugate from pea ovaries as hexanoyl-spd (Fig. 1, 4). This compound accumulates during ovary and petal senescence and becomes the most abundant polyamine in these organs. A first indication of its chemical composition was obtained by purification of its dansyl derivative by HPLC followed by FAB analysis. Its mass spectrum was consistent with it

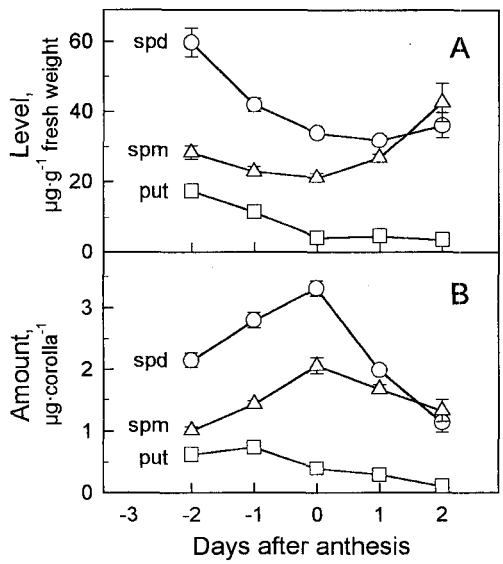

Figure 9. Time course of levels (A) and contents (B) of put, spd, and spm during growth and senescence of the corolla. Results are means ± SE, $n = 3$.

being spd conjugated with a moiety of M_r 99, which corresponds to a hexanoyl group. This was supported by the release of spd and hexanoic acid when the polyamine was subjected to acid hydrolysis. Final characterization was established by synthesis of the proposed structure and comparison of the electron impact mass spectrum of the trifluoroacetyl derivatives of the natural and synthetic compounds after GC-MS.

Polyamine Conjugates and Their Function in Plants

There are no previous reports of polyamine conjugates with short-chain fatty acids in plants. However, solapalmitine and solapalmitenine, conjugates of the longer fatty acids hexadecanoic acid and hexadec-2-enoic acid, respectively, and the triamine solamine are present in *Solanum tripartitum* and have significant inhibitory activity against cells derived from a type of human carcinoma (Kupchan et al., 1969). Also, a derivative of the monoamine *iso*butylamine with icosa-2,4-8-trienoic acid, a long-chain fatty acid, was isolated from *Piper officinarum* (Gupta et al., 1977). Other polyamine conjugates are quite complex (Smith et al., 1983). Most of these are amides of hydroxycinnamic acids (caffeic, ferulic, and *p*-coumaric acid) and common polyamines (Leubner-Metzger and Amrhein, 1993). These compounds are associated with several physiological processes (flowering and fruit/seed development) and with the hypersensitive response to viral or fungal infection (Flores and Martín-Tanguy, 1991). Other related compounds, acetyl-polyamines, have been found only in animals and a few bacterial strains (Seiler, 1987). Acetyl-polyamines, in which the primary amine groups are acetylated, are considered intermediate metabolites in the catabolism of spd and spm and in the polyamine interconversion reactions. Acetylation results in a decrease in the net positive charge of polyamines and thus reduces the affinity of polyamines for anionic binding sites. In addition, the acetyl derivatives are more easily removed from cells by transport and catabolism.

Biological Significance of Hexanoyl-Spd

At anthesis, hexanoyl-spd levels in ovaries are low, similar to those of put and much less than those of spd and spm (Carbonell and Navarro, 1989). The levels remain constant if fruit-set is induced, either by natural pollination or, in unpollinated ovaries, by application of plant growth regulators, topping the plant, or using the slender pea mutant, which displays natural parthenocarpic fruit-set. However, once senescence is initiated, there is a marked increase in hexanoyl-spd levels in untreated unpollinated ovaries, such that hexanoyl-spd becomes the most abundant polyamine. This accumulation is coincident with a decrease in the levels of put and spd. This decrease in put was shown to be associated with very low Arg decarboxylase activity and increased put oxidase activity in senescent ovaries (Pérez-Amador and Carbonell, 1995). A similar increase in hexanoyl-spd levels was observed during the senescence of the corolla. It is likely that spd is converted to hexanoyl-spd, presumably via a reaction similar to the acetylation of polyamines and for which hexanoyl-CoA and spd would be substrates. The suggestion that spd is the immediate precursor of hexanoyl-spd is supported by the observation that the spd concentration decreased as hexanoyl-spd levels increased, both in petals (Figs. 8A and 9) and ovaries (Carbonell and Navarro, 1989). In addition, levels of total spd conjugates are very low during ovary growth, whereas significant amounts could be measured only in senescent ovaries (data not shown), perhaps because of the synthesis of hexanoyl-spd from spd.

Hexanoyl-spd concentrations are high in green tissues and low in nongreen tissues, such as roots, mature anthers, and petals at anthesis. By contrast, in anthers the level of free spd is very high. These data are different from those reported for tomato, in which levels of spd are high in gynoecium, medium in stamen, and low in sepal and petal (180, 55, 25, and 25 $\mu g\ g^{-1}$ fresh weight, respectively) (Rastogi and Sawhney, 1990). An earlier work by Palavan and Galston (1982) with flowers of *Phaseolus vulgaris* indicated that spd levels were lower than in tomato, but with a similar pattern, i.e. high in pistil, medium in stamen, and low in petal and sepal (40, 13, 7, and 5 $\mu g\ g^{-1}$ fresh weight, respectively). As in pea, spd levels decreased during leaf development in *Heliotropium* (Birecka et al., 1984).

There is not a clear understanding of the functions of individual polyamines. In general, a decrease in polyamine levels is associated with senescence and aging in leaves, and addition of polyamines inhibits processes associated with senescence of excised plant organs (reviewed by Evans and Malmberg, 1989). The decrease in the levels of put and spd in unpollinated ovaries is accompanied by an increase in spm level in such tissues, reaching a maximum d 3 pa, when senescence is initiated (Carbonell and Navarro, 1989). However, the level of hexanoyl-spd is correlated more closely with the course of ovary senescence than is that of spm. The difference between concentration and amount per organ of hexanoyl-spd and those for the other polyamines suggests that this new conjugated polyamine would remain in the senescent organ, not being mobilized to the rest of the plant, and also that it is not catabolized.

Conjugation of spd with hexanoic acid, like acetylation, should reduce its positive charge and thereby affect its interactions with anionic groups present in nucleic acids and membrane phospholipids (Seiler, 1987). If the latter interaction stabilizes membranes, then polyamine conjugation may lead to membrane destabilization and thereby contribute to senescence. In pea ovaries, senescence results in a dramatic alteration in membrane structure (Vercher et al., 1987; Vercher and Carbonell, 1991). It may, in fact, be difficult to establish whether hexanoyl-spd formation contributes to pea ovary and petal senescence or is a consequence of it. However, this new polyamine may prove to be a good marker for pea ovary and petal senescence. Further work on the biochemistry of hexanoyl-spd biosynthesis and degradation in pea should be informative.

ACKNOWLEDGMENTS

The authors wish to thank Mrs. A. Ahuir, A. Blasco, and M.A. Argomániz for technical support and Mr. R. Martínez-Pardo and A. Villar for their work in the greenhouse. We are also grateful to Professor P. Cullis (University of Leicester, UK) for the gift of N^1,N^8-bis-BOC-spd.

Received November 6, 1995; accepted January 6, 1996.
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