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N-Acylethanolamines in Seeds. Quantification of Molecular Species and Their Degradation upon Imbibition[1]

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***N*-Acylethanolamines (NAEs) were quantified in seeds of several plant species and several cultivated varieties of a single species (cotton [*Gossypium hirstutum*]) by gas chromatography-mass spectroscopy. The total NAE content of dry seeds ranged from 490 ± 89 ng g^{-1} fresh weight in pea (*Pisum sativum* cv early Alaska) to 1,608 ± 309 ng g^{-1} fresh weight in cotton (cv Stoneville 7A glandless). Molecular species of NAEs in all seeds contained predominantly 16C and 18C fatty acids, with *N*-linoleoylethanolamine (NAE18:2) being the most abundant (approaching 1,000 ng g^{-1} fresh weight in cottonseeds). Total NAE levels dropped drastically following 4 h of imbibition in seeds of pea, cotton, and peanut (*Arachis hypogea* cv Virginia), and this decline was most pronounced for NAE18:2. A novel enzyme activity was identified in cytosolic fractions of imbibed cottonseeds that hydrolyzed NAE18:2 in vitro. NAE degradation was optimal at 35°C in 50 mM MES buffer, pH 6.5, and was inhibited by phenylmethylsulfonyl fluoride and 5,5′-dithio-bis(2-nitrobenzoic acid), which is typical of other amide hydrolases. Amidohydrolase activity in cytosolic fractions exhibited saturation kinetics toward the NAE18:2 substrate, with an apparent K_m of 65 μM and a V_{max} of 83 nmol min^{-1} mg^{-1} protein. Total NAE amidohydrolase activity increased during seed imbibition, with the highest levels (about four times that in dry seeds) measured 2 h after commencing hydration. NAEs belong to the family of "endocannabinoids," which have been identified as potent lipid mediators in other types of eukaryotic cells. This raises the possibility that their imbibition-induced metabolism in plants is involved in the regulation of seed germination.**

NAPE is a membrane phospholipid of plant and animal cells with at least two proposed functional roles (Schmid et al., 1996): (a) to support the structural integrity of biomembranes, and (b) to act as a precursor for the production of lipid mediators. The existence of NAPE in higher plants was the subject of much controversy until a combination of biochemical and biophysical experimental evidence established unequivocally its natural occurrence in a wide range of plant tissues (Chapman and Moore, 1993). NAPE is not abundant under normal physiological conditions. For example, in cotton (*Gossypium hirsutum*) plants, NAPE content varies between 1.9 and 3.2 mol % of the total phospholipid, depending upon the tissue source and developmental stage (Sandoval et al., 1995; Chapman and Sprinkle, 1996). In mammals, NAPE accumulates only in membranes of damaged cells during tissue injury to about 10 mol % of the total phospholipid (Schmid et al., 1990). Biophysical studies indicate that NAPE is a bilayer-stabilizing lipid (LaFrance et al., 1997), and this has prompted some to speculate that it may be synthesized under stress conditions to help maintain membrane integrity and minimize cellular injury. In plants, NAPE is synthesized from two potential bilayer-destabilizing lipids—free fatty acids and PE (McAndrew and Chapman, 1998)—and so under certain conditions, NAPE biosynthesis may have a protective role in plant membranes.

NAPE is an *N*-acylated derivative of the common membrane phospholipid PE and is metabolized by a phosphodiesterase (of the PLD type) to yield phosphatidic acid and NAE (Chapman et al., 1995; Schmid et al., 1996; Chapman, 1998). NAPE is believed to be the precursor in vivo for the entire family of bioactive NAEs. In mammalian neurons, anandamide (*N*-arachidonylethanolamine) is an endogenous ligand for the cannabinoid receptor (for review, see DiMarzo, 1998) and is produced from *N*-arachidonyl PE by a Ca^{2+}-stimulated, PLD-type activity (Cadas et al., 1997). We recently identified a signal-mediated release of NAE from NAPE in elicitor-treated tobacco cell suspensions (Chapman et al., 1998) and leaves (S. Tripathy, B. Venables, and K.D. Chapman, unpublished results). These NAEs were identified by GC-MS as *N*-lauroyl- and *N*-myristoylethanolamine, and an enzyme activity was identified in tobacco microsomes that catalyzed the formation of NAE from NAPE in vitro. Also, homogenates of tobacco cell suspensions hydrolyzed NAE to form free fatty acids and ethanolamine, providing evidence for an intracellular amidohydrolase activity capable of metabolizing NAEs.

In recent years we have accumulated considerable information on the biosynthesis of NAPE during cottonseed development, germination, and seedling growth (Sandoval et al., 1995; Chapman and Sprinkle, 1996). NAPE biosynthesis is increased upon imbibition in cotyledons of cotton,

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Abbreviations: NAE, *N*-acylethanolamine; NAPE, *N*-acylphosphatidylethanolamine; PE, phosphatidylethanolamine; PLD, phospholipase D; TMS, trimethylsilyl.

and we were interested in the occurrence and fate of NAE (a presumed NAPE metabolite) in seeds as well. We report the levels of individual NAE molecular species (quantified by GC-MS) among seeds of several plant species and of several cultivars of cotton. NAE levels diminished rapidly in seeds during imbibition, and we identified and partially characterized a novel enzyme in imbibed cottonseeds that hydrolyzed NAE18:2 in vitro. Our results indicate a rapid metabolism of these potentially bioactive lipids during seed imbibition, and may suggest a role for these compounds in germination.

MATERIALS AND METHODS

Plant Material

Tomato (*Lycopersicon esculentum* Mill. cv Long Keeper), pea (*Pisum sativum* cv Early Alaska), castor (*Ricinus communis* cv Zanzabarensis), and peanut (*Arachis hypogea* cv Virginia) seeds were from Gurney's Seed and Nursery (Yankton, SD). Okra (*Abelmoschus esculentus* Moench cv Mammoth Pod) seeds were from plants propagated locally in our greenhouse (in the summer of 1996). Soybean (*Glycine max* cv Dare) seeds were a gift from Dr. Richard Wilson (North Carolina State University, Raleigh). Corn (*Zea mays*) seeds were purchased from Modern Biology (West Lafayette, IN). Cotton (*Gossypium hirsutum*) seeds were from Dr. Rick Turley (U.S. Department of Agriculture-Agricultural Research Service, Stoneville, MS), Dr. John Gannaway (Texas A&M University, Agricultural Experiment Station, Lubbock, TX), or Dr. John Burke (U.S. Department of Agriculture-Agricultural Research Service, Lubbock, TX), and the varieties are listed in Figure 4. All seeds were greater than 90% viable. For imbibition experiments, seeds were surface-sterilized in 10% commercial bleach and soaked in distilled water (in the dark) for 4 h at 30°C with aeration.

NAE Quantification

NAEs were isolated from crude lipid extracts by HPLC and these NAE-enriched fractions were identified and quantified as TMS-ether derivatives by GC-MS (Tripathy et al., 1999). The method is similar to that used by Piomelli and co-workers (Stella et al., 1997) for the analysis of anandamide in mammalian brain extracts, but with some modifications for quantification of unknown plant NAEs in lipid-rich seed extracts. One-gram portions of seeds were powdered in liquid N_2 in a mortar and added to hot 2-propanol (to inactivate any endogenous phospholipases) (Chapman et al., 1998). Lipids were extracted into chloroform, filtered, and subjected to normal-phase HPLC (4.6- × 250-mm Partisil 5 column, Whatman; model 712 HPLC system, Gilson, Middleton, WI). The lipids were suspended in chloroform (200 μL total volume) and separated with a linear gradient of 2-propanol in hexane (up to 40% 2-propanol over 20 min), followed by 5 min at 50% 2-propanol, and then 5 min at 100% hexane. Under these conditions NAEs eluted between 11 and 15 min (at about 30% 2-propanol in hexane), depending on the species, well away from most other lipids (see Fig. 1 for representative traces). A synthetic standard NAE20:4 with substantial UV absorbance at 214 nm was used to monitor NAE retention times and column performance on a daily basis.

The NAE-enriched HPLC fractions were collected, evaporated to dryness under N_2 gas, and derivatized in bis(trimethylsilyl)trifluoroacetamide at 50°C for 30 min. TMS-ether derivatives were suspended in hexane and analyzed by GC-MS. See Figure 2 for representative electron impact mass spectra of NAE18:2 identified in pea extracts and in the NAE18:2 synthetic standard. The GC (model 5890 series II, Hewlett-Packard) was equipped with a capillary column (30-m × 0.25-mm i.d. with a 0.25-μm film thickness; model DB-5.625, J&W Scientific, Folsom, CA). The injector temperature was 260°C and the oven temperature was programmed from 40°C to 280°C at 10°C/min.

The GC was coupled to a mass spectrometer (model HP5970, Hewlett-Packard) equipped with an electron impact source (70 eV) and operated for ultimate sensitivity in the selective ion monitoring mode. The $[M]^+$or $[M\text{-}15]^+$ ions, as well as two additional confirming masses, were monitored for each NAE species. Standard curves and mass spectra were prepared using injected masses of 0.1 to 30 ng of synthetic NAE from each species (NAE12:0, NAE14:0, NAE16:0, NAE18:0, NAE18:1$^{cis\Delta9}$, NAE18:2$^{cis\Delta9,12}$, NAE18:3$^{cis\Delta9,12,15}$, NAE18:3$^{cis\Delta6,9,12}$, and

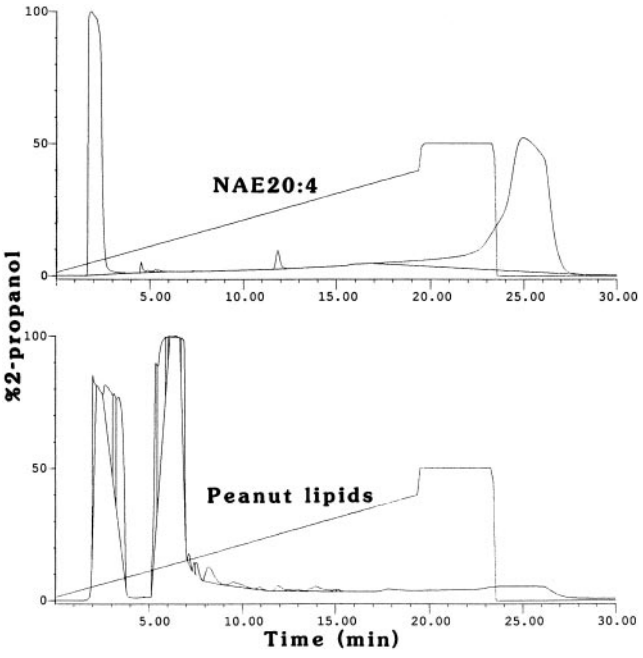

Figure 1. Representative A_{214} profiles (585 mV full scale) of synthetic NAE20:4 (upper trace) and crude peanut lipids (lower trace) fractionated in a 2-propanol gradient (0%–50% in hexane) by HPLC. The peak at approximately 12 min in the upper trace is NAE20:4 (confirmed by GC-MS), the standard that marks the relative retention time of NAEs in these separations. A fraction from 11 to 15 min was collected from HPLC separations of crude seed lipids (see lower trace for example); NAEs are enriched in the 11- to 15-min fraction and the majority of contaminating lipids (mostly triacylglycerols) were removed by 8 min. This represents a major "clean-up" step since, in comparison, peanut seeds contain about 45% oil by weight.

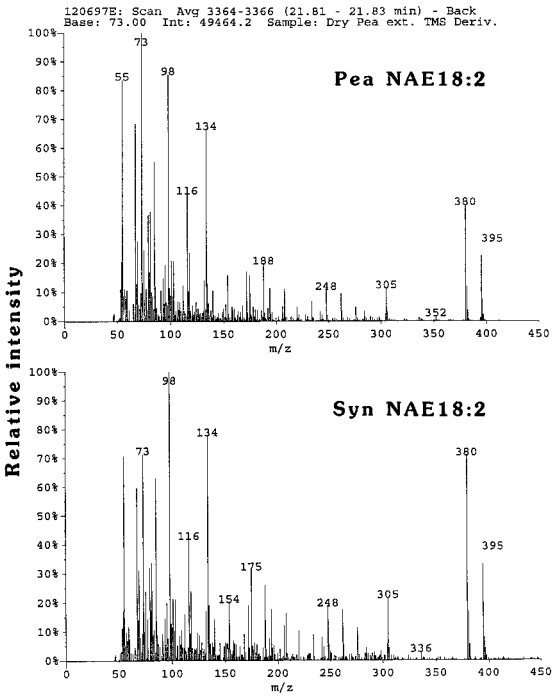

Figure 2. Representative electron impact mass spectra for TMS-ether derivatives of NAE18:2 isolated from pea seeds (upper) and, for comparison, our synthetic NAE18:2 quantitative standard (lower). These compounds have identical retention times on GC (21.82 min; not shown), and their electron impact mass spectra are virtually indistinguishable. Identifiable ions used for quantification purposes include the molecular ion M^+ at *m/z* 395, fragmentation ions $[M\text{-}15]^+$ at *m/z* 380, and $[M\text{-}90]^+$ at *m/z* 305. For all NAEs in seed extracts that were identified and quantified by GC-MS, identical GC retention times and electron impact mass spectra were obtained authentic synthetic standards (not shown), similar to the above example.

NAE20:4$^{cis\Delta5,8,11,14}$) in the presence of 10 ng of internal standard (decachlorobiphenyl). Final quantification of NAE species was calculated from the ratio of analyte (NAE) response to that of the internal standard. Method efficiency was evaluated by the recovery of NAE17:0 "surrogate" added to the preparation at the time of lipid extraction, and replicate values were adjusted for NAE17:0 recovery.

Synthetic NAE species were prepared from acyl chlorides in ethanolamine essentially as described previously (Devane et al., 1992). Purity and yield were confirmed by GC-MS.

Preparation of Cell Fractions

Imbibed cotton (cv Stoneville 7A glandless) seeds were homogenized in 1:1 (w/v) ratio of fresh weight to medium and fractionated by differential centrifugation as previously described (Chapman and Sriparameswaran, 1997). Fractions were characterized by distribution of marker enzyme activities (Chapman and Sriparameswaran, 1997). The supernatant from the 60-min spin at 150,000*g* was nearly devoid of any membrane markers and was designated the cytosolic fraction.

NAE Degradation in Vitro

An endpoint assay that follows the consumption of exogenously supplied NAE was developed to detect and measure NAE amidohydrolase activity in cottonseed extracts. It is based on the analysis of TMS-ether derivatives of NAE quantified by GC-flame ionization detection following incubation of synthetic NAE with cell fractions. Derivitization and GC conditions were exactly as described above, except the column had a 30-m × 0.32-mm i.d. External standard NAE 17:0 (200 ng) was added after the enzyme reaction was stopped, and the ratio of NAE17:0 standard to substrate was used to calculate the precise amount of NAE substrate remaining. Results from replicate samples were reproducible with this approach, and the accuracy of GC-flame ionization detection was verified with GC-MS. Assays were initiated by the addition of enzyme to NAE substrate, and were suspended by sonica-

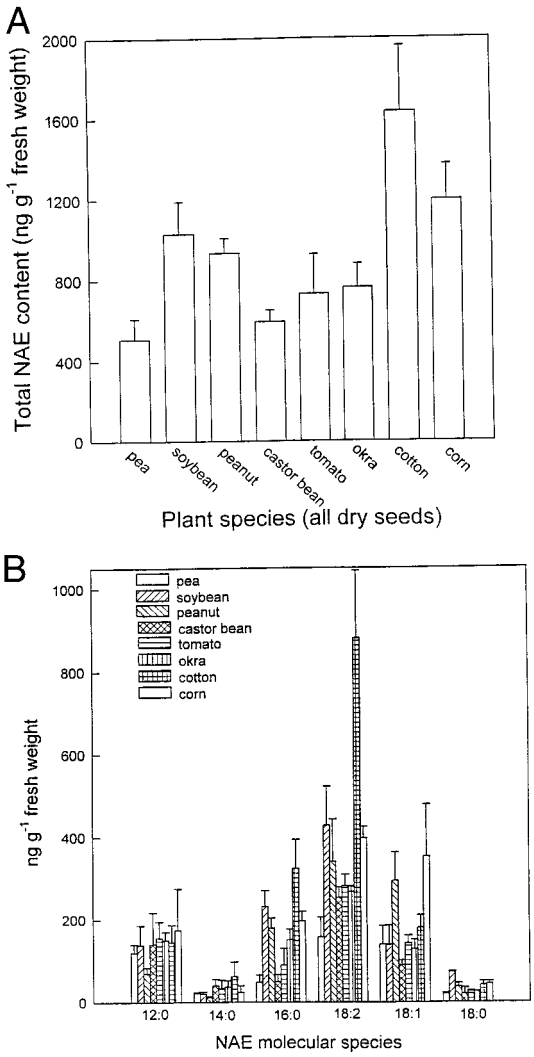

Figure 3. Quantification of NAE in dry seeds of pea, soybean, peanut, castor bean, tomato, okra, cotton, and corn. A, Total NAE content summed from individual molecular species profiles (B). Bars represent the means ± SD of three to six independent extractions and fractionations.

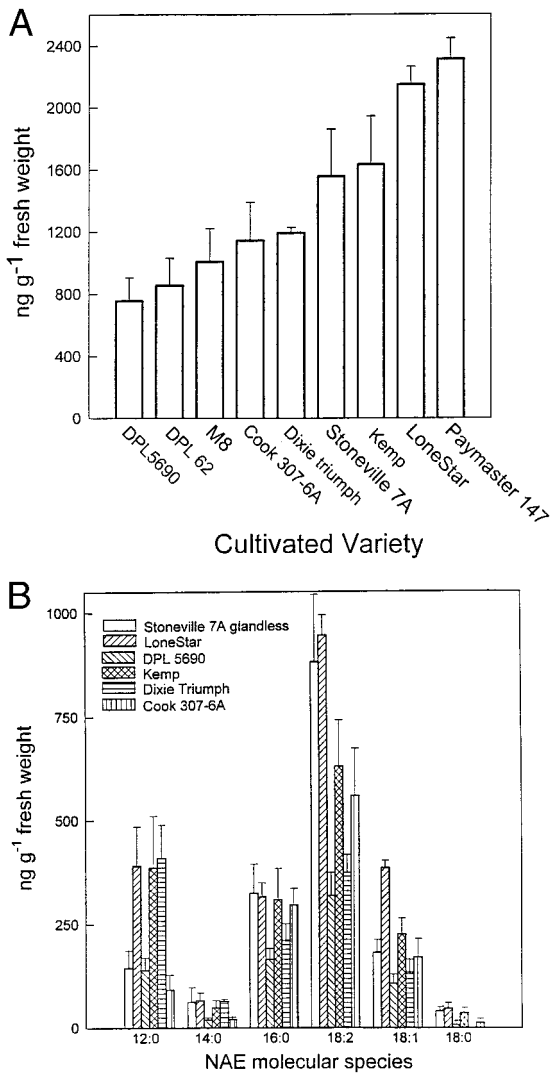

Figure 4. Quantification of NAE in dry seeds of several diverse, cultivated varieties of upland cotton (cvs DPL5690, DPL62, M8, Cook 307–6A, Dixie Triumph, Stoneville 7A glandless, Kemp, LoneStar, and Paymaster 147). A, Total NAE content summed from individual molecular species profiles (B). Bars represent the means ± SD of three to six independent extractions and fractionations.

tion in buffer in a final volume of 0.75 mL. Experiments were conducted in a shaking (60 rpm) bath to determine the optimal time, temperature, protein content, pH, and substrate concentration for amidohydrolase rate measurements. Activity was tested with radiolabeled NAE to confirm conversion of NAE to ethanolamine and free fatty acids (Chapman et al., 1998), and no NAE degradation was detected in the absence of enzyme or with enzyme that had been preincubated for 15 min at 100°C.

RESULTS

NAEs were quantified in lipid extracts from dry seeds of several plant species (Fig. 3). All seeds contained NAEs, but total NAE content varied about 3-fold among the species examined (Fig. 3A). Total NAE content was greatest in cotton (1,608 ± 309 ng g^{-1} fresh weight) and lowest in pea (490 ± 89 ng g^{-1} fresh weight). The individual molecular species of NAE were also analyzed (Fig. 3B). While NAE profiles in all seeds were similar qualitatively, there was considerable quantitative variability (particularly for NAE16:0, NAE18:2, and NAE18:1) among the species, which was not entirely accounted for by differences in total NAE content. NAE18:2 was the most abundant species in seeds, and NAE14:0 and NAE18:0 were the least abundant. This represents a marked difference from profiles of NAEs in leaves, where medium-chain, saturated NAE species were predominant (Chapman et al., 1998; Tripathy et al., 1999; K.D. Chapman and B. Venables, unpublished results). This may suggest distinctly different roles for NAE species in different plant tissues.

Similar to results among seeds of different plant species, we found about a 3-fold difference in total NAE content among several cultivars of cotton (Fig. 4). Total NAE levels ranged from just under 800 ng g^{-1} fresh weight for cv DPL5690 to nearly 2,400 ng g^{-1} fresh weight for cv Paymaster 147. As with seeds of different plant species, NAE18:2 was the most abundant species in the different cotton varieties. Variability in NAE content and acyl composition among cultivars of a single species was nearly as great as the variability among seeds of different species. These data would suggest that the variability is not necessarily physiologically significant and that there are likely cultivars of pea, for example, that have higher NAE levels than some cotton varieties. Perhaps the absolute amount of NAE (above a certain minimum level) in quiescent seeds is less important than its imbibition-induced metabolism (see below).

Because NAPE is the presumed precursor for NAEs, the *N*-acyl compositions of NAPE in dry seeds of peanut, pea,

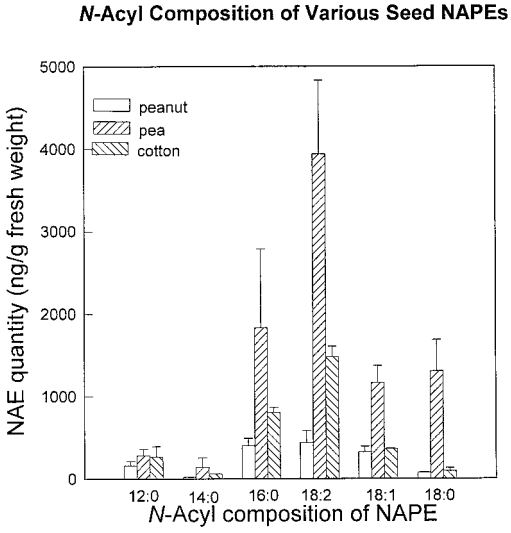

Figure 5. Comparison of the relative abundance of NAE moieties of dry seed NAPE generated enzymatically (see "Materials and Methods"). NAPE was purified by TLC from dry seeds of peanut, pea, and the cotton cv Stoneville 7A glandless, as described previously (Chapman and Moore, 1993). Bars represent the means ± SD of three independent extractions and fractionations.

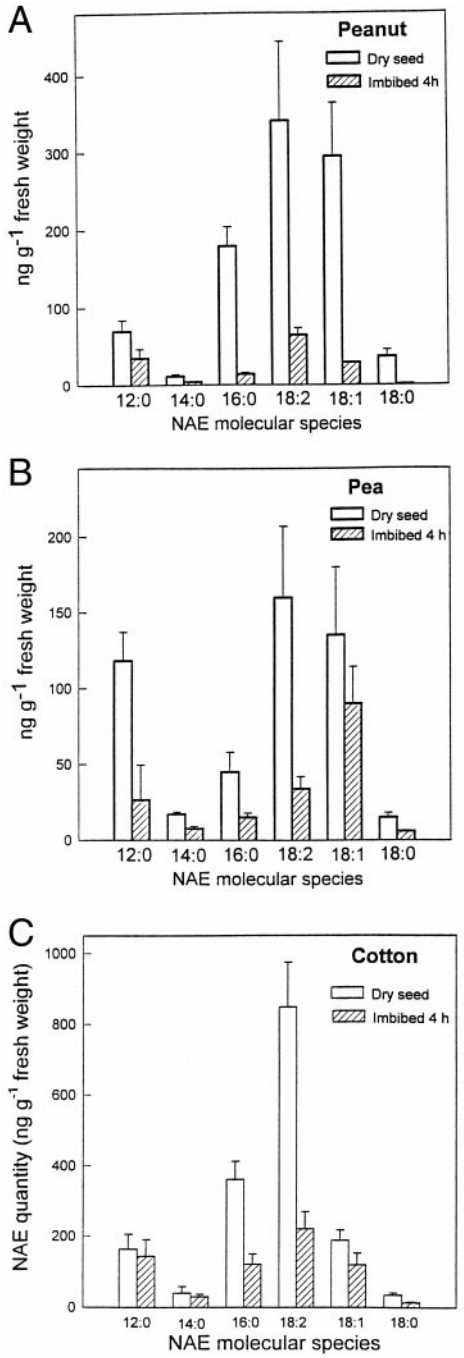

Figure 6. Quantification of NAE molecular species in dry (white bars) and 4-h-imbibed (hatched bars) seeds of peanut (A), pea (B), and the cotton cv Stoneville 7A glandless (C). Bars represent the means ± SD of three to six independent extractions and fractionations.

and cotton were compared (Fig. 5). The *N*-acyl portion of purified NAPE was generated by enzymatic digestion with a *Streptomyces chromofuscus* phosphodiesterase (Chapman and Moore, 1993), and the resulting NAE species were quantified by GC-MS. The same fatty acids that were constituents of seed NAEs (Fig. 3) were present in NAPE (Fig. 5), although their relative abundance was not identical. As expected, NAE18:2 and NAE16:0 were the most abundant

Table I. *Distribution of imbibed cottonseed NAE18:2 amidohydrolase activity in crude cell fractions*

Fractions were prepared in 100 mM potassium-phosphate (pH 7.2), 10 mM KCl, 1 mM EDTA, 1 mM EGTA, and 400 mM Suc, and assayed for amidohydrolase activity in the same buffer for 10 min at 35°C at a substrate concentration of 60 μM. Clarified homogenate (640*g*, 10 min supernatant) represents total activity. The 10,000*g* pellet is enriched in plastids, mitochondria, and glyoxysomes, while the 150,000*g* pellet is enriched in microsomes membranes derived from ER, Golgi, and plasma membranes (Chapman and Sriparameswaran, 1997). The 150,000*g* supernatant is enriched in cytosolic proteins. Values represent averages of duplicate assays from a single experiment. Similar results were obtained in replicate experiments.

Sample	Total Activity	Specific Activity
	nmol min^{-1}	*nmol min*$^{-1}$ *mg*$^{-1}$ *protein*
Clarified homogenate	307.8	5.1
10,000*g* (30 min) Pellet	17.3	1.2
150,000*g* (60 min) Pellet of a 10,000*g* supernatant	28.2	9.4
150,000*g* (60 min) Supernatant of a 10,000*g* supernatant	223.2	18.1

in NAPE isolated from all three species. For the most part, quantities of NAPE were in excess of the levels of NAEs in these seeds, which is consistent with the notion that NAEs are derived from NAPE. Interestingly, pea seeds contained the most NAPE (nearly 10 μg g^{-1} fresh weight), while they contained the least NAE of the seeds examined. While experimental conditions and analytical procedures were different, our results are consistent with previous findings that NAPE levels are substantial in dry pea seeds (Dawson et al., 1969).

NAE molecular species were quantified in imbibed (4 h) seeds of pea, cotton, and peanut (Fig. 6). Compared with dry seed levels, there was a dramatic reduction in most NAE species in all of these seeds, even considering the

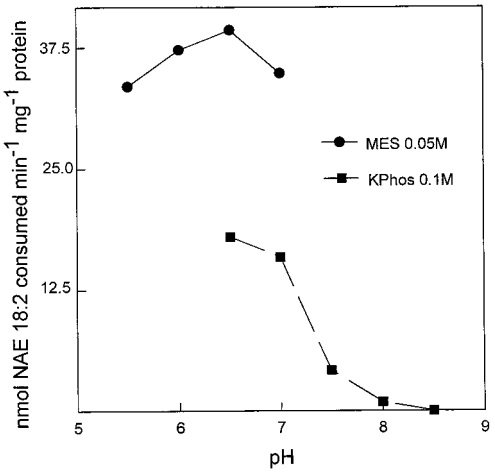

Figure 7. Degradation of NAE18:2 in cytosolic fractions of imbibed cottonseeds at varying pH. Assays were conducted for 10 min at 35°C, and were initiated by the addition of enzyme. Substrate (60 μM NAE18:2) was solubilized in buffer with sonication, and the final assay volume was 0.75 mL. Data points are averages of duplicate assays and are representative of replicate experiments.

Table II. *Influence of divalent cations and Ser and Cys group modifiers on NAE 18:2 degradation in vitro*

Assays were initiated by the addition of enzyme in 50 mM MES buffer, pH 6.5, at 100 μM NAE 18:2. Values are averages of duplicate assays (less than 10% variability) of a single cytosolic preparation. Similar results were obtained in replicate experiments with independently prepared cytosolic fractions.

Sample	NAE 18:2
	nmol consumed min^{-1} mg^{-1} protein
Cytosol[a] only	47.4
Cytosol +1 mM $MgCl_2$	47.7
Cytosol + 1 mM $MgCl_2$ +1 mM EDTA	49.7
Cytosol + 1 mM $MnCl_2$	48.2
Cytosol + 1 mM $MnCl_2$ + 1 mM EDTA	49.3
Cytosol + 1 mM $CaCl_2$	48.1
Cytosol + 1 mM $CaCl_2$ + 1 mM EGTA	47.5
Cytosol + 10 mM PMSF	25.8
Cytosol + 10 mM DTNB	10.8

[a] Cytosol was a 150,000*g*, 60 min supernatant (from a 10,000*g*, 30-min supernatant).

increase in fresh weight contributed by water (e.g. an increase of about 30% fresh weight in 4-h-imbibing cottonseeds). The drop in NAE levels was particularly notable for NAE18:2 and NAE16:0 in pea and cotton, while almost all NAEs were diminished in peanut seeds. Because of the short time period (4 h), and because a decrease in NAEs occurred in both oilseeds and non-oilseeds, it is unlikely that the metabolism of NAEs upon imbibition is associated with general lipid mobilization. Rather, we postulate that the metabolism of NAE is related specifically to the initiation of seed germination.

Exogenously supplied NAE18:2 was hydrolyzed in vitro in cell fractions of imbibed cottonseeds (Table I). NAE hydrolysis was highest in cytosolic fractions (83% of total activity was recovered in the 60-min 150,000*g* supernatant).

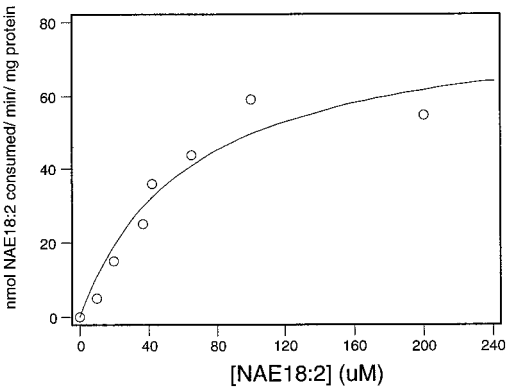

Figure 8. Plot of NAE18:2 degradation versus the concentration of NAE18:2. Assays were for 10 min in 50 mM MES buffer, pH 6.5, and 0.05 mg of cytosolic protein in a final volume of 0.75 mL. Substrate was solubilized in buffer with sonication, and the reaction was initiated by the addition of enzyme. Data points are averages of duplicate assays and are representative of replicate experiments. The solid line represents the data fit to the Michaelis-Menten equation (MacCurveFit software, produced by Kevin Reiner).

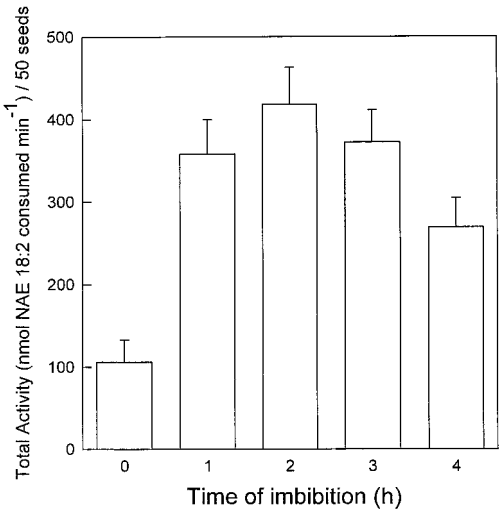

Figure 9. Time course of NAE 18:2 amidohydrolase activity measured in vitro in cell fractions (10,000*g*, 30-min supernatant) from dry or imbibed (for 1–4 h) cottonseeds. Results were calculated as total activity in extracts from 50 seeds at each time point, and values are the average ± SE from three separate experiments. Assay conditions were as described in the legend for Figure 8, except a final substrate concentration of 100 μM was used and the enzyme content was varied between 20 and 100 μg.

The rate of hydrolysis in cytosolic fractions was linear for 30 min with up to 120 μg of protein and optimal at 35°C (not shown). The reaction was pH dependent, with an optimum of 6.5, and the exchange of MES buffer (50 mM) for potassium-phosphate buffer doubled the rate of NAE degradation (Fig. 7). NAE hydrolysis in vitro apparently was not dependent on Mg^{2+}, Mn^{2+}, or Ca^{2+}, but was inhibited by both PMSF and 5,5′-dithio-bis(2-nitrobenzoic acid) (Table II), which is similar to amidohydrolases that degrade NAEs in mammalian systems (Schmid, 1996).

Hydrolysis of NAE18:2 in vitro was dependent upon substrate concentration, exhibiting typical saturation kinetics (Fig. 8). Fitting the data in Figure 8 to the Michaelis-Menten equation (solid line, $r^2 = 0.94$) gave an apparent K_m and V_{max} of 65 μM and 83 nmol min^{-1} mg^{-1} protein, respectively. The cytosolic fraction exhibited little activity toward NAE14:0 (not shown).

NAE amidohydrolase activity was detected in extracts of dry cottonseeds, and the relative activity increased about four times in just 2 h of imbibition (Fig. 9), suggesting a rapid activation of enzyme activity. A more complete characterization of this NAE amidohydrolase activity will not be not possible until it is purified; however, it is clear that imbibed seeds contain an enzyme(s) capable of hydrolyzing NAE18:2 that is likely responsible for imbibition-induced degradation of NAEs in vivo.

DISCUSSION

Until recently, NAPE was believed by many to be a misidentified artifact of lipid extraction, not an endogenous constituent of plant cells (for discussion, see Chapman and Moore, 1993). However, fast-atom bombardment-

MS/MS approaches helped to establish unequivocally the natural occurrence of NAPE in plants and to identify several molecular species of NAPE in cottonseeds (Chapman and Moore, 1993a; Sandoval et al., 1995). Radiolabeling experiments in vivo with [1,2-^{14}C]ethanolamine demonstrated the capacity of several plant species to synthesize NAPE de novo during postgerminative growth (Chapman and Moore, 1993a). The biosynthesis and turnover (Chapman et al., 1995) of NAPE to form NAE was reconstituted in vitro in microsomal membranes of cotton cotyledons, indicating that the cellular machinery for the metabolism of NAPE was present in germinated seeds. A developmental profile of NAPE biosynthesis indicated that NAPE synthesis was increased in cottonseeds during imbibition and germination (Chapman and Sprinkle, 1996). Perhaps NAPE biosynthesis is increased during seed imbibition/germination to replenish the cellular reserve of NAE precursor.

The *N*-acylation-phosphodiesterase pathway (Schmid et al., 1996) is apparently responsible for the generation of bioactive NAEs in animals (for review, see DiMarzo, 1998). This pathway involves the biosynthesis of a NAPE precursor that is cleaved in a signal-mediated fashion by a PLD-type enzyme to yield NAE. The specific NAE produced likely depends on a number of regulatory factors such as PLD specificity and the *N*-acyl composition of the precursor pool. Recent evidence indicates that NAPE/NAE metabolism is activated in elicitor-treated plant cells (Chapman et al., 1998; Tripathy et al., 1999) for the release of medium-chain, saturated NAEs (e.g. NAE14:0 levels increased from about 6–240 ng g^{-1} fresh weight in cryptogein-treated tobacco leaves). By comparison, NAE levels were considerably higher in quiescent seeds (Figs. 3 and 4), and the molecular species in seeds consist mainly of C16 and C18 fatty acids, with the most prevalent NAE species being the di-unsaturated NAE18:2. It is possible that different NAEs are accumulated at different developmental stages or in different tissues of plants for different purposes. Future work to identify the physiological role of NAPE/NAE metabolism in plants will require accurate quantification of these metabolites under differing physiological conditions, and the results presented here provide the basis for such future studies in seeds.

Most of the NAEs diminished rapidly upon seed imbibition (Fig. 6). Moreover, an active amidohydrolase activity was identified and partially characterized in the cytosolic fractions of imbibed cottonseeds (Tables I and II; Figs. 7 and 8) that hydrolyzed NAE18:2. This amidohydrolase activity was increased during seed imbibition (Fig. 9). The degradation of NAE by an amidohydrolase(s) is the mechanism by which the NAE neurotransmitter anandamide is inactivated following its selective uptake in mammalian neuronal cells (Cravatt et al., 1996; Beltramo et al., 1997). NAE formation and inactivation is emerging as a central signaling pathway in a variety of eukaryotic cell types (Schmid et al., 1996; Chapman, 1998). Therefore, the rapid changes in seed NAE levels raise the possiblity that their metabolism is involved in cell signaling during seed germination. While it is speculation, it is possible that NAE acts as an endogenous inhibitor that must be removed before germination can proceed.

Another possibility is that NAE metabolism is initiated as part of a protective mechanism to minimize imbibition-induced cellular damage. We previously noted that NAPE biosynthesis was increased in imbibing seeds, and proposed that the synthesis of this membrane-stabilizing lipid may be part of an effort to maintain cellular compartmentation during seed rehydration (for discussion, see Sandoval et al., 1995). NAPE is synthesized by a membrane-bound enzyme (designated NAPE synthase) from free fatty acids and PE (McAndrew and Chapman, 1998). Perhaps the NAE amidohydrolase activity provides free fatty acids for the NAPE synthase to allow cells of imbibing seeds to rapidly adjust their NAPE content. In any case, direct evidence of whether NAE is a lipid mediator or if its metabolism serves a protective role will await the ability to manipulate NAE levels in vivo.

NAE18:2 exhibits cannabimimetic properties when administered to animals (for review, see DiMarzo, 1998). However, these effects likely are indirect, because NAE18:2 was shown to competitively inhibit anandamide degradation by the amidohydrolase enzyme in mammalian cells (diTomaso et al., 1996; Maccarrone et al., 1998). Thus, the presence of NAE18:2 (also identified as a lipid constituent of brain) can potentiate the endogenous activity of anandamide. In the present study, NAE18:2 was the most abundant species of NAE in all dry seeds examined, with levels approaching 1 μg g^{-1} fresh weight in cottonseeds (Fig. 4B). The transient metabolic changes in cellular NAE levels has been shown to influence many physiological processes in vertebrates, including sleep, memory, pain, and immunity (Schmid et al., 1996; DiMarzo et al., 1998). Consequently, seeds may represent a natural source of new cannabimimetic compounds. In fact, identification of NAEs in processed cocoa powder prompted Piomelli and coworkers to propose that these compounds formed the molecular basis for chocolate cravings (diTomaso et al., 1996). The results reported in this manuscript accurately identify and quantify various NAE species in seeds of higher plants and, for the first time to our knowledge, place their metabolism in the physiological context of seed imbibition/germination.

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