Base- and acid-catalyzed interconversions of O-acyl- and N-acyl-ethanolamines: a cautionary note for lipid analyses

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Abstract **The isolation and quantification of ethanolamine containing lipids from animal tissues may expose neutral lipid extracts to acidic or basic conditions during chromatographic separations or derivatization chemistry. While investigating the acid- and base-catalyzed production of anandamide in chromatographic fractions of rat brain extracts not containing anandamide, we observed that O,N-acyl migrations are facile. O,N-acyl migrations are well documented in synthetic organic chemistry literature, but are not well described or recognized with regard to methods in lipid isolation or lipid enzyme studies. We report here the synthesis and characterization of O- and N-acyl (palmitoyl- or arachidonoyl-)ethanolamines. Their rearrangements in base and acid are quantified by liquid chromatography–electrospray ionization mass spectrometry. The rearrangements proceed through a cyclic intermediate that is also formed during chemical reactions commonly used for derivatization of acylethanolamines for gas chromatography–mass spectrometry. The isolation and characterization of N- or O-acylethanolamines and their enzymatic formation requires awareness and consideration of the proclivity of these compounds to chemically rearrange.**—Markey, S. P., T. Dudding, and T-C. L. Wang. **Base- and acid-catalyzed interconversions of O-acyl- and N-acyl-ethanolamines: a cautionary note for lipid analyses.** ***J. Lipid Res.*** **2000.** 41: **657–662.**

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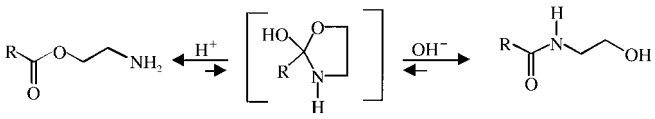
During a study on the GC/MS quantification of anandamide (N-arachidonoylethanolamine, NAE) in extracts of rat tissues, we observed acid- and base-catalyzed production of NAE from chromatographic fractions that did not contain NAE (1). We noted that identical analytical separations and mass spectrometric analyses resulted whether O-arachidonoylethanolamine or N-arachidonoylethanolamine was used as the starting material. In investigating this observation, we found that O,N acyl migrations in ethanolamines have been well documented in organic synthesis since the observations of Wolfheim in 1914 (2), later used by several authors in preparative syntheses (3–7). Mechanistic studies described the probability of a cyclic intermediate and defined the reaction conditions for aqueous O-to-N acyl migration (8, 9). A pH greater than 7.5 was reported to be sufficient to effect instantaneous rearrangement of aminoethyl ester hydrochlorides to their corresponding ethanolamides. The rearrangement has been used most frequently in synthetic applications involving migrations of aromatic acyl groups, but there are previous reports of aliphatic acyl group migrations (10, 11). During the characterization of N-acylphosphatidylethanolamine in pea seeds, Dawson, Clarke, and Quarles (12) observed that the N-acylethanolamine generated by acid hydrolysis invariably exhibited two components on thin-layer chromatography. Both components were isolated and individually identified as N-acylethanolamine and O-acylethanolamine. Dawson et al. (12) then demonstrated that the O-acylethanolamine readily reverted to the N-acyl product upon re-chromatography. The authors proposed N-to-O acyl interchange as a consequence of exposure of these compounds to acid and base.



Studies of the endogenous brain levels of anandamide and its biosynthesis have led several investigators to use conditions for biosynthetic studies (incubations at pH 9, reaction quenching with strong base) and analysis (ammonia-containing thin-layer chromatographic solvents). These conditions could result in O-to-N acyl migration if O-arachidonoylethanolamine were present, either in tissue or as a result of O-acyl esterification or post-mortem metabolism (13–15). We report here that basic treatment of O-arachidonoylethanolamine or O-palmitoylethanolamine causes migration to their respective N-amides, because very high levels of N-arachidonoylethanolamine have been reported following purposeful exposure of tissue extracts to base (conditions milder than those required for

Abbreviations: NAE, N-arachidonoylethanolamine; OAE, O-arachidonoylethanolamine; NPE, N-palmitoylethanolamine; OPE, O-palmitoylethanolamine; TMS, trimethylsilyl.

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hydrolysis of N-arachidonoylphosphatidylethanolamine). Conversely, acid treatment of N-arachidonoylethanolamine causes rearrangement to O-arachidonoylethanolamine. These acyl group migrations have not been described in biochemical studies of anandamide. Furthermore, chemical derivatives from N- and O-acylethanolamines obtained by using acidic reagents generate singular and identical products, probably formed as a result of cyclic intermediates.

MATERIALS AND METHODS

Arachidonic acid was purchased from Biomol (Plymouth Meeting, PA); ethanolamine, palmitic acid, di-tert-butyldicarbonate, 4-dimethylaminopyridine, 1,3-dicyclohexylcarbodiimide from Aldrich Chemical Company (St. Louis, MO). N-Palmitoylethanolamine and N-arachidonoylethanoamine were prepared as described in the literature (16).

N-(tert-butoxycarbonyl)ethanolamine [1]

To a stirred solution of methanol (25 mL) containing (3 mL) of triethylamine was added ethanolamine (1 g, 16 mmol), followed by di-tert-butyl dicarbonate (6.98 g, 32 mmol) and the resulting mixture was heated to 50°C for 30 min. The solution was then allowed to cool to room temperature and the solvent was removed by reduced pressure rotary evaporation. The resulting residue was briefly stirred with dilute ice-cold HCl (pH = 2.15, 10 mL) followed immediately by repeated extraction using ethyl acetate (5 × 10 mL), the organic layer was dried over $MgSO_4$, filtered and concentrated, and dried under vacuum affording (1) 2.52 g (98%).

O-palmitoylethanolamine [2]

To an anhydrous solution of palmitic acid ($C_{16}H_{32}O_2$, 4.01 g, 15.7 mmol) in CH_2Cl_2 (30 mL) was added N,N′-dicyclohexylcarbodimide (3.56 g, 17.3 mmol) and 4-dimethylaminopyridine (0.38 g, 3.1 mmol). The solution was stirred 10 min and a (5 mL) tetrahydrofuran solution of (1) (2.52 g, 15.7 mmol) was added dropwise. The resulting mixture was heated to reflux and stirred for 5 h, cooled, filtered through celite, and concentrated affording a clear oil which was purified by flash column chromatography (hexane–ethyl acetate, 9:1) yielding (2) 5.32 g (85%).

O-palmitoylethanolamine-N-trifluoroacetate salt [3]

A solution of (2) (200 mg; 0.5 mmol) in CH_2Cl_2 (2 mL) under an inert atmosphere was cooled to 0°C and trifluoroacetic acid (2 mL) was added. The reaction was allowed to warm to room temperature and stirred for 2 h; solvent removal under vacuum afforded the desired product (3) 200 mg (97%) yield. In a similar procedure, arachidonic acid (0.1 g, 0.33 mmol) was converted to O-arachidonoylethanolamine-N-trifluoroacetate salt, 0.13 g (85%).

Base-catalyzed interchange of O-to-N-acylethanolamine

Aliquots of 20 μL ethanol containing 10 μg/μL of either O-arachidonoyl or O-palmitoylethanolamine were added to 180 μL of $NaHCO_3/Na_2CO_3$ buffer at pH 9.2 or 10.2 and incubated at 37°C. At various time points, 5-μL aliquots were removed and mixed with 1 mL of acetonitrile–water (65:35), and 5 μL of that mixture was immediately analyzed by LC/MS.

Acid catalyzed interchange of N-to-O-arachidonoylethanolamine

A 20 μL aliquot containing 10 μg/μL N-arachidonoylethanolamine was added to 80 μL 3 N HCl solution, sonicated (Kontes Micro KT40 Ultrasonic Cell Disruptor, Vineland, NJ) at 50% power output for times varying from 30 sec to 4 min. After 15 min, 100 μL ethyl acetate was added and the solution was vortex mixed. A 5-μL aliquot of the ethyl acetate layer was added to 1 mL of acetonitrile–water (65:35), and 5 μL of that mixture was immediately analyzed by LC/MS.

Mass spectrometry

Electron ionization GC/MS experiments were performed on a Finnigan TSQ-70 (Sunnyvale, CA) equipped with a Varian 3400 (Walnut Creek, CA) gas chromatograph. A DB-5MS (15 m × 0.25 mm i.d., 0.25 μm film thickness, J & W Scientific, Folsom, CA) column was used for the analysis of derivatized acylethanolamines with an injector temperature of 220°C. Derivatizations were performed with N,O-(bis-trimethylsilyl)-trifluoroacetamide (Pierce, Rockford IL) or pentafluorobenzoylchloride (Aldrich) using published conditions (1, 17).

LC/MS experiments were performed using a Finnigan LCQ equipped with an electrospray ion source. A Hewlett-Packard 1050 (Palo Alto, CA) liquid chromatograph was used to deliver 0.3 mL/min acetonitrile–water (65:35) containing 0.05% trifluoroacetic acid. A pre-column split of 10:1 was accomplished with an LC Packings (San Francisco, CA) ACURATE splitter with a flow calibrator for 1.0 mm i.d. column. A C18 column (Zorbax, 15 cm × 1.0 mm i.d., 5 μ, Micro-Tech Scientific, Sunnyvale, CA) was connected after the splitter for chromatographic separations. A measured 35 μL/min flow was sprayed at a voltage of 4.8 kV, a capillary temperature of 200°C (capillary voltage 30 V), and tube lens voltage 25 V into the ion trap. A scan range of *m/z* 50 to 400 was used for palmitoylethanolamines (MH^+ 300, base peak); O-palmitoylethanolamine eluted at 7.6 min, N-palmitoylethanolamine at 10.4 min. A scan range of *m/z* 150–450 was used for arachidonoylethanolamines (MH^+ 348, base peak); O-arachidonoylethanolamine eluted at 6.1 min, N-arachidonoylethanolamine at 7.6 min.

RESULTS

Gas chromatographic retention times for underivatized and trimethylsilylated (TMS) N- and O-acylethanolamines reveal that the compounds are readily separated chromatographically. O-acylethanolamines elute consistently before N-acylethanolamines whether as native compounds or TMS derivatives. Dehydrated, cyclized products (2-oxazolines) are formed thermally, more readily from O-acyl than N-acyl ethanolamines, eluting prior to their respective acyclic precursor as previously reported (18, 19). The O-acylethanolamines are more prone to dehydrate to the 2-oxazolines than the N-acyl compounds; they are also able to thermally rearrange to N-acylethanolamines. Ion chromatograms of O-acylethanolamines evidence the presence of three species: the dehydrated 2-oxazoline (minor), the acyclic O-acylethanolamine (predominant), and the N-acylethanolamine (minor). The electron ionization mass spectrum of the 2-oxazoline from O-arachidonoylethanolamine is similar to that published (18, 19), except that the $M^{+\cdot}$ ion at *m/z* 329 is more prominent than the MH^+ that was reported using an ion trap. The mass spectra of acyclic N- and O-arachidonoylethanolamine are identical to each other, exhibiting ions at *m/z* 79, 91, 103 (base peak), 175, 222, 236, 276, and 347 ($M^{+\cdot}$). The mass spectra of acyclic N- and O-palmitoylethanolamine are also identical to each other with ions *m/z* 30, 43, 103 (base peak), 116, 239, and 299 ($M^{+\cdot}$).

Electron ionization mass spectrometric fragmentation patterns for TMS derivatives of N- and O-acylethanolamines indicate that no rearrangement accompanies derivatization with neutral silylation reagents. Using conditions described, the N-TMS derivative of O-palmitoylethanolamine elutes at 7 min 10 sec, approximately 20 sec before the O-TMS derivative of N-palmitoylethanolamine. The N-TMS derivative of O-arachidonoylethanolamine elutes at 8 min 5 sec, approximately 25 sec before the O-TMS derivative of N-arachidonoylethanolamine. The spectra of both N-TMS derivatives of O-acylethanolamines exhibit base peaks at *m/z* 102, an ion characteristic of trimethylsilylated primary amines ($CH_2 = N^+H{-}TMS$)(20). The spectra of both O-TMS derivatives of N-acylethanolamines match those published previously (21).

In contrast to trimethylsilylation with neutral reagents, pentafluorobenzoylation with pentafluorobenzoylchloride does promote cyclization. Electron ionization mass spectra of the pentafluorobenzoyl (PFBz) derivatives of N- and O-acylethanolamines indicate that the both N- and O-acylethanolamines yield a single product, most likely a cyclic derivative. N- and O-palmitoylethanolamine-PFBz eluted at 8 min 50 sec, and exhibit identical mass spectra with major fragments at *m/z* 167, 195, 209, 238, 256, 297, and 493 ($M^{+\cdot}$); N- and O-arachidonoylethanolamine-PFBz eluted at 9 min 50 sec, and exhibit identical mass spectra with major fragments at *m/z* 167, 195, 209, 238, 256, and 541 ($M^{+\cdot}$).

Electrospray ionization mass spectra of N- and O-arachidonoylethanolamine are shown in **Fig. 1**. Protonated molecular ions at *m/z* 348 dominate both spectra. N-arachidonoylethanolamine (NAE) exhibits a consistent *m/z* 287 fragment for the loss of 61 daltons, whereas O-arachidonoylethanolamine (OAE) consistently loses 17 daltons with a resulting fragment at *m/z* 331. The electrospray ionization mass spectra of the pamitoylethanolamines are similarly dominated by protonated molecular ions. The chromatographic separation of N- and O-arachidonoylethanolamines is shown in the lower panel of Fig. 1, obtained by monitoring *m/z* 348 as the compounds elute from a reverse phase column.

Figure 2 shows the rate of O-to-N- acyl interchange for arachidonoyl- and palmitoyl-ethanolamines at pH 9.2 and 10.2 determined by measuring product area ratios from electrospray data. The interchange rates are dependent upon pH and acyl group, with palmitoyl interchange proceeding at a significantly faster rate than arachidonoyl. The acid catalyzed reverse N- to O-acyl exchange is shown in **Fig. 3** for N-arachidonoylethanolamine. No appreciable acid catalyzed interchange was observed in the absence of sonication.

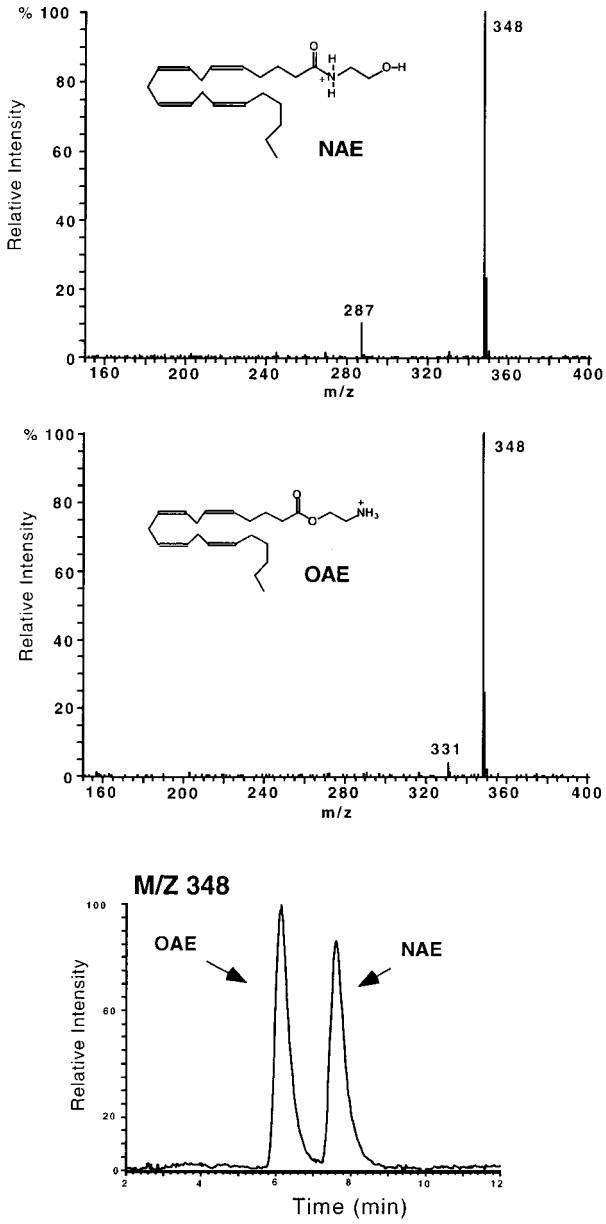

Fig. 1. (Top) Electrospray mass spectrum of N-arachidonoylethanolamine (NAE); (middle) electrospray mass spectrum of O-arachidonoylethanolamine (OAE); (lower) mass chromatogram of *m/z* 348 recorded after the injection of equal quantities of NAE and OAE.

DISCUSSION

The use of gas chromatography–mass spectrometry to characterize and quantify N-acylethanolamines has been reported by several authors (18, 19, 21). The dehydrated cyclized 2-oxazoline rather than the acyclic native molecule has been reported as the major component when underivatized N-acylethanolamines are analyzed (18, 19). However, we observe that the 2-oxazolines are minor components detectable in the analyses of either the O- or N-acylethanolamines. We recorded mass spectra from each of the synthesized materials and concluded that the O- or the N-acylethanolamines can withstand temperatures required for gas phase chromatography without cyclization and thermal dehydration. Thermal dehydration is highly dependent upon injection port characteristics and surface activity. The similarity of the electron ionization mass spectra of underivatized N- and O-acylethanolamines suggest that cyclization is very likely upon ionization. The trimethylsilyl derivatives of the N-acylethanolamines exhibit mass spectra identical to those published (21) with

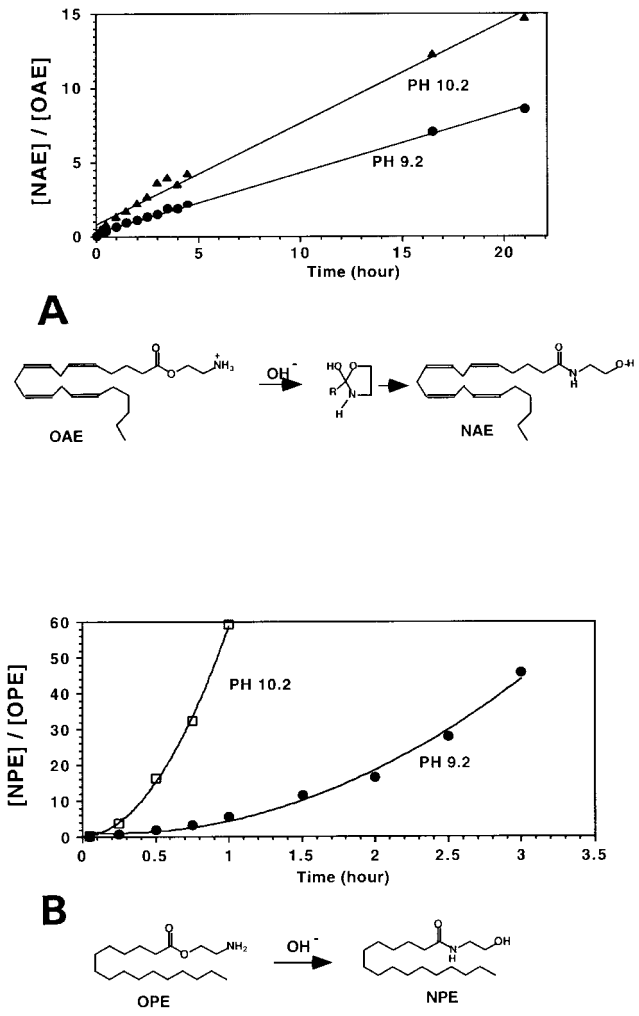

Fig. 2. Base catalyzed O-to-N-acyl interchange. A: Rate of arachidonoyl interchange at pH 9.2 or 10.2 in aqueous sodium bicarbonate/carbonate buffer at 37°C. Aliquots were diluted and analyzed by LC/MS (electrospray) using selected ion detection at *m/z* 348 as shown in Fig. 1. Peak area ratios were measured and plotted. B: Rate of palmitoyl interchange using the similar conditions and analyses as for arachidonoyl interchange.

major characteristic fragments indicating the presence of a primary alcohol. The O-acylethanolamine TMS derivatives exhibit prominent ions characteristic of primary amines and provide additional evidence that the TMS derivatives retain the acyclic structure of their precursors when formed using neutral reaction conditions and N, O-(bis-trimethylsilyl)-trifluoroacetamide. In contrast, the pentafluorobenzoyl (PFBz) derivatives of N- and O-acylethanolamines indicated that both N- and O-acylethanolamines react to yield a single and identical chromatographic product, most likely through acid catalysis and formation of a cyclic derivative in pentafluorobenzoylchloride or in the gas chromatograph injector. These observations are consistent with the fact that in anhydrous acids, N-acylethanolamines undergo facile cyclization and equilibration with O-acylethanolamines (8, 9).

Although O-to-N-acylethanolamine rearrangement has been observed and reported previously, the facile nature of this reaction has not been considered and discussed with reference to the isolation and characterization of anandamide and related compounds. Very mild treatment with aqueous base (pH 9.2) is sufficient to produce O-to-N acyl migration with N-acylethanolamide production in preference to hydrolysis to ethanolamine and free fatty acid. The rate of migration is highly dependent upon the chemical nature of the acyl group (Fig. 2). At 30 min at 37°C, about 2% of the O-palmitoylethanolamine rearranges to N-palmitoylethanolamine, but at pH 10, there is 16% rearrangement. Rates are appreciably slower for the arachidonoyl interchange, with 0.5% interchange of O-to-N-arachidonoylethanolamine at 30 min at pH 9.2.

While these chemical rearrangement rates may not at first seem likely to have influenced prior reports on enzymatic studies, there are conditions used in such studies that require consideration of this non-enzymatic reaction. Most characterization of amidase activities have utilized radioactive substrates (either radiolabeled ethanolamine or fatty acid), and only a few percent of the substrate is converted to measured product during these assays (13–15). The use of crude enzymatic preparations makes it likely that there are acyl transferase and esterase as well as amidase activities present, with the result that the production of O-acyl ethanolamines at basic pH provides substrates for non-enzymatic formation of N-acylethanolamines. The pH maximum for the amidase enzyme reaction is between 9 and 9.5 (14). Investigators studying amidase reactions chose sample workup and characterization methods based upon the stability of the product and N-acylethanolamines are known to be stable in base. Consequently, they halted the enzyme reaction with concentrated base to denature proteins (14), with the likely result of inadvertently accelerating chemical rearrangements of any O-to-N-acylethanolamines. Other investigators used ammonia-containing thin-layer chromatographic solvents for the separation of anandamide and related lipids (13, 22, 23), which introduced an opportunity for base catalyzed rearrangement of O-acyl ethanolamines.

The reverse rearrangement of N-to-O-acylethanolamines requires either anhydrous acid or significant heat or sonication in order to effect the amide-to-amine conversion (Fig. 3). We chose to demonstrate this process using sonication in this study because it is commonly used for cell and tissue disruption, and the chemical consequences of this treatment are usually assumed to be negligible. Once anandamide and related amides are formed, these compounds are unlikely to rearrange and lead to the chemical formation of O-acylethanolamines unless forcing conditions such as strong anhydrous acid or sonication and heat are used. However, several investigators have reported unknown compounds detected on thin-layer chromatography with properties corresponding closely to those noted by Dawson for O-acylethanolamine in the characterization of N-acylphosphatidylethanolamine (13, 15, 22). Thus, O-arachidonoylethanolamine may be the prominent unidentified metabolite of radiolabeled anandamide

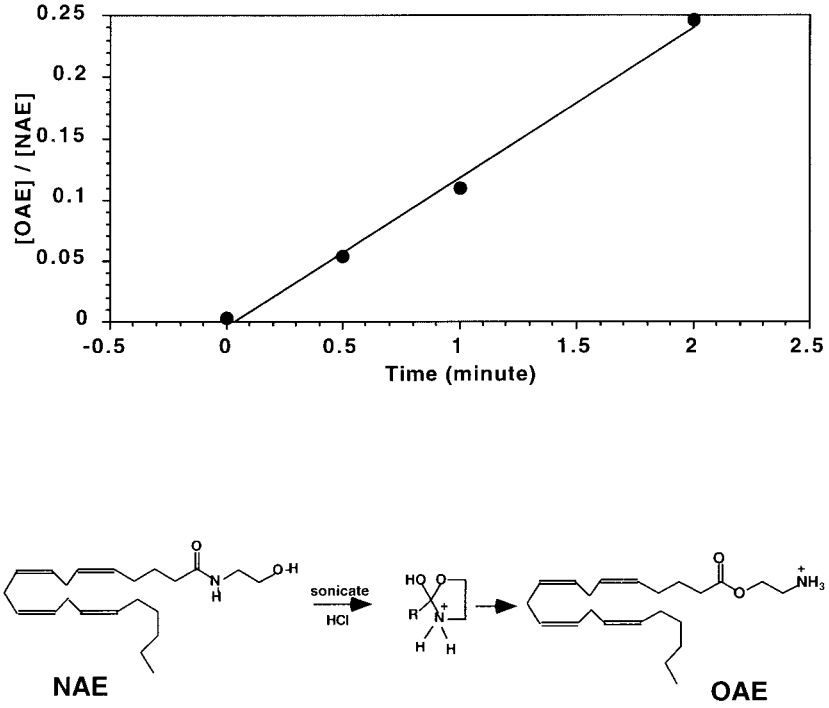

Fig. 3. The extent of acid catalyzed N-to-O acyl interchange is proportional to the duration of sonication for the interconversion of NAE to OAE as measured by LC/MS (electrospray) using conditions as described for Figs. 1 and 2. Note that unlike the base catalyzed rearrangement that proceeds spontaneously over the time shown in Fig. 2, acid catalyzed interchange does not proceed without sonication.

observed after incubation of [^{3}H]anandamide with neuroblastoma and glioma cells (13). O-arachidonoylethanolamine is also consistent with the properties of the prominent unknown in thin-layer chromatograms reported by DiMarzo et al., (22) isolated from extracts of macrophages preincubated with [^{3}H]arachidonic acid. These unidentified components raise the question whether O-acylethanolamines arise chemically or enzymically by transacylase catalyzed rearrangement of N-acylethanolamines.

We conclude that the characterization and determination of biological formation of either N- or O-acylethanolamines requires awareness and consideration of the chemistry of this class of molecules and their proclivity to undergo N-to-O and O-to-N acyl rearrangements.

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