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Ascorbic Acid Assays of Individual Neurons and Neuronal Tissues Using Capillary Electrophoresis with Laser-Induced Fluorescence Detection

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Ascorbic acid is an important cellular metabolite involved in many biochemical pathways. A method to quantitate ascorbic acid and dehydroascorbic acid in individual neurons and neuronal tissues is described with detection limits of 320 pM (430 zmol). The method uses microvial sampling, derivatization with 4,5-dimethyl-1,2-phenylenediamine, capillary electrophoresis separation, and laser-induced fluorescence detection and quantifies the ascorbic acid and dehydroascorbic acid levels with less than a 15-min total analysis time including sample preparation and derivatization. Ascorbic acid and dehydroascorbic acid levels are measured using functionally characterized and identified neurons of *Aplysia californica*, *Pleurobranchaea californica*, and *Lymnaea stagnalis* − three well-recognized models in cellular and system neuroscience. Multiple assays of a particular identified neuron (e.g., metacerebral cells from *Aplysia*) show a high level of reproducibility, while endogenous intracellular concentrations of ascorbate are neuron-specific. Ascorbic acid concentrations in the neurons studied range from 0.19 to 6.2 mM for *Aplysia* and 0.12 to 0.22 mM for *Lymnaea*. In contrast, concentrations of ascorbic acid observed in heterogeneous tissues such as ganglia (with connective tissues, glia, blood vessels, neuropile, and areas with intercellular spaces), 4−190 μM, are significantly lower than the single-cell values.

Ascorbic acid (AA), commonly referred to as ascorbate or vitamin C, is an important micronutrient with many physiological roles.[1−3] AA is an essential ingredient in the diet of a small number of vertebrate species that lack the enzyme L-gulono-1,4-lactone oxidase[2] such as guinea pigs, some fruit-eating bats, several birds, and most primates including humans.[3] In both plants and animals, the primary function of AA is proposed to be that of a reducing agent: neutralizing toxic peroxides, stabilizing free radicals, and playing a role in normal photosynthesis.[1] AA is a cytosolic antioxidant operating in cooperation with lipid-soluble membrane antioxidants such as tocopherol or carotene and may increase the capability of the cells to cope with reactive oxygen metabolites generated by the activated phagocytic apparatus of the cells.[4,5] Neuronal tissues have one of the highest level of ascorbate. However, the role of ascorbate in neuronal function is unclear. Apart from a well-recognized role of ascorbate as an antioxidant protecting neurons against ischemic and excitotoxic insults,[6,7] there is growing evidence suggesting additional functions of this widespread molecule. For example, AA can alter the redox state of neurotransmitter receptors, stimulate release of various neurotransmitters, and act as a critical cofactor in norepinephrine synthesis.[8] The importance of AA as a modulatory molecule is further emphasized by the heterogeneity of its distribution.[9] Regional variations in brain AA concentration in rat and human have been measured, suggesting its association with different neuronal subtypes.[10,11] Unfortunately, it has not been measured directly at the single-cell level. Such measurements are important due to the expected heterogeneity of AA concentrations in neurons located in close proximity to each other, even in a well-defined brain region.

In the presence of an oxidizing species, AA is degraded to dehydroascorbic acid (DHA) as shown in Figure 1. In a healthy organism, the level of DHA is kept low due to high endogenous concentrations of glutathione.[12] An elevation in DHA levels above the normal threshold (∼ 5% of the corresponding AA level) is observed with plant stress and as a result of damage and metabolic disturbances such as diabetes and Alzheimer's disease.[1,12] However, DHA is the form of AA delivered to the cells,[5,13] indicating the importance of maintaining an appropriate balance of DHA and AA in a biological system.[1] Thus, simultaneous measurement of

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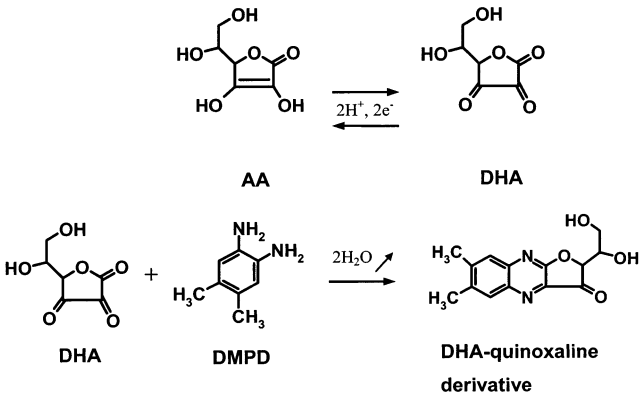

Figure 1. Redox structures of ascorbic acid and the derivatization of dehydroascorbic acid with 4,5-dimethyl-1,2-phenylenediamine to form a highly fluorescent quinoxaline derivative.

the DHA and AA is required to monitor the redox state of the cellular metabolism. Typical AA levels can be as high as 0.5 mM in the extracellular space of mammalian brain tissue, and its intracellular concentrations may reach 2−10 mM (an extrapolation from multicellular sample analyses).[3,15] Here we developed an assay for AA, compatible with picoliter to microliter volumes of single cells and small tissue samples.

Accurate measurement of AA in biological systems in vitro is difficult because of its rapid oxidation at physiological pH, temperature, and oxygen saturation. Traditional methods include titration, spectrophotometry, electrochemistry,[9] absorbance, enzymatic activity, and chemiluminescence[14] measurements. The majority of these methods suffer from one or more of the following: long analysis times, which promote the degradation of AA, overestimations due to the presence of other oxidizable species, inability to independently measure both AA and DHA, electrode contamination, interference by overlapping UV absorbance profiles with other matrix biomolecules, large sample size requirements, and poor sensitivity. These problems have been partially addressed by combining chromatographic separation via HPLC with fluorometric detection.[16−19] A high-resolution separation and species-specific fluorometric derivatization chemistry imparts a two-part selectivity enhancement while the sensitivity is also increased through selection of an appropriate fluorophore.

Capillary electrophoresis (CE) is our method of choice [20−22] and is well-suited for the analysis of individual cells, primarily because of the high separation efficiencies, ultra-small-volume sample requirements, and sensitive detection methods associated with the technique.[23,24] There have been several prior assays for AA and DHA using CE with a variety of detection modes.[25−28] While most have used UV absorbance or electrochemical method for detection, laser-induced fluorescence (LIF) detection was chosen in this study. LIF allows for the determination of intracellular components either directly or through derivatization. Limits of detection (LODs) are unparalleled, with the most impressive examples ranging from zeptomole[29] levels to single enzyme molecules.[30] For AA and DHA measurements, we isolate the cell or tissue of interest and derivatize the DHA (or first oxidize the AA and then derivatize) with 4,5-dimethyl-1,2-phenylenediamine (DMPD: see also Figure 1). Here, we validate the ability to work with biological samples using well-characterized neurons of three different model organisms. Both neuronal tissues and identified neurons from *Aplysia californica*, *Pleurobranchaea californica*, and *Lymnaea stagnalis* are used as the model organisms because of the wealth of biochemical, physiological, and functional information available,[31−33] as well as a long history of identified neurons from these animals being used for validating new single-cell analytical approaches.[21,23,34−36]

EXPERIMENTAL SECTION

Reagents. All reagents were of the highest available purity and were obtained from Sigma (St. Louis, MO) unless otherwise noted. CE buffer, 30 mM sodium borate, contained 0.03 g of boric acid (H_3BO_3) and 0.97 g of sodium borate decahydrate ($Na_2B_4O_7 \cdot 10H_2O$) in 100 mL of ultrapure water (Millipore, Bedford, MA), adjusted to pH 9.85 ± 0.05 with 3 M NaOH (Fisher Scientific, Fair Lawn, NJ). Sample buffer, 50 mM sodium acetate at pH 3.7 ± 0.1, was prepared by combining 0.074 g of anhydrous sodium acetate ($NaC_2H_3O_2$, EM Science, Gibbstown, NJ) and 0.23 mL of glacial acetic acid ($C_2H_4O_2$) in 100 mL of ultrapure water; EDTA (1 mM final concentration) was added to chelate trace metals. The sample buffer was purged with a stream of nitrogen gas for at least 5 h to eliminate molecular oxygen. DHA and AA standards were prepared by dissolving a measured quantity of high-purity solid in this sample buffer. The desired concentrations were achieved using the method of serial dilutions. Standard calibration curves of at least four points were generated each day. All buffers and reagents were also freshly prepared each day.

Animals. *A. californica* (100−150 g) and *P. californica* (200−500 g) were obtained from the *Aplysia* Research Facility (Miami,

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FL) and Sea-Life Supply (Sand City, CA), respectively. Upon arrival, all animals were stored in artificial seawater at 12−14 °C until use. Specimens of *L. stagnalis*, a freshwater pond snail, were obtained from stock raised at the aquaculture facilities at the Vrije Univesiteit, Amsterdam, and maintained in aerated freshwater aquaria under constant light. The animals were fed lettuce twice weekly. *Lymnaea* selected for further experimentation were adults at least 25 mm in shell length.

Cell Isolation and Sample Preparation. Individual ganglia and single neurons were isolated from the central nervous systems (CNSs) of *Aplysia*, *Pleurobranchaea*, and *Lymnaea* using maps published elsewhere.[36−39] Before dissection, animals were maintained for ∼1 h at 4 °C for anesthesia. All microdissection steps were performed in the appropriate physiological solutions: for *Lymnaea* see Moroz et al.[39] and for *Aplysia* and *Pleurobranchaea* see Cruz et al.[36] After the connective tissues were softened with proteases (1% trypsin, 60 min for *Aplysia*; 1% protease−dispase 15 min for *Pleurobranchaea*; 0.25% protease E, 10 min for *Lymnaea*), individual neurons were isolated with sharpened tungsten needles (2−5 μm tip diameter) and polished plastic micropipets. Isolated neurons were placed in sterile 30-mm Petri dishes, their diameters were measured, and within 1−2 min neurons were transferred into a 0.75-mL polypropylene tube (Fisher Scientific) with 2 μL of N_2-purged sodium acetate buffer. The total volume of physiological solution and neurons of interest was 0.5 μL. Immediately, after the transfer, the acetate buffer containing the cell was flash-frozen on dry ice to disrupt the cell membrane. An additional 4 μL of acetate buffer was added to dilute the cell and increase the sample volume. Next, samples were sonicated for up to 1 min. Centrifugation over ice was performed for ∼30 s to reduce the likelihood of capillary contamination by cellular debris. The resulting supernatant was divided into two 2.5-μL aliquots for DHA and total DHA plus AA analyses.

Ganglia were also isolated from animals under cold anesthesia. After the proteolytic treatment, connective tissues were removed from each ganglion. The ganglia were blotted and placed in a polypropylene vial for homogenization with a Teflon rod. The homogenized material was sonicated (∼30 s) and briefly centrifuged (2−5 min at 14000*g*) to remove cell membranes and any remaining sheath material. Care must be taken not to heat the sample during this process. The supernatant was carefully removed and diluted with sample buffer if needed.

Derivatization. Derivatization of single neuron and neuronal tissue samples was carried out by adding 1.5 μL of DMPD solution (∼2 mg of DMPD in 2 mL of sample buffer) to the sample aliquot. The reaction took place in 4 min at room temperature. Oxidation of AA to DHA was accomplished by adding 0.1 μL of a diluted Br_2 solution (10 μL of saturated bromine water (Fisher Scientific), in 500 mL of ultrapure H_2O), allowing oxidation to take place for 30 s, and then removing the excess Br_2 under a stream of flowing N_2 gas. Initial and final sample plus vial weights were measured to ensure that neither net addition nor net loss of fluid occurred during the oxidation procedure as this would effect analyte quantitation.

CE-LIF Analysis. A home-built instrument was employed for these experiments. CE separations took place in an 80-cm-long, 50-μm-i.d., 365-μm-o.d. fused-silica capillary (Polymicro Technologies, Phoenix, AZ). Injections took place at 8 kV (12 μA) for 8 s, corresponding to a 7.6-nL injection volume, controlled by a Bertan power supply (Bertan High Voltage, series 230, model 30R, Hicksville, NY). Separations were performed at 20 kV (40 μA).

Detection took place on-capillary, 40 cm beyond the point of injection. The 350−356-nm excitation from an Ar/Kr mixed-gas laser (Coherent, Innova 70 Spectrum, Palo Alto, CA) was conditioned by passing through an interference filter (Omega, 330WB80, Brattleboro, VT). The resulting fluorescence was collected at 90° by an all-reflective microscope objective (Ealing Electrooptical, Holliston, MA) and filtered spatially by a machined 3-mm pinhole and spectrally by a 80-nm fwhm, 500-nm interference filter (03FIB004, Melles Griot, Irvine, CA) and a high-pass filter (400EFLP, Janos Technology, Townshend, VT). The signal was then measured by a photomultiplier tube (PMT) (Hamamatsu, HC125−03, Bridgewater, NJ) operating in photon counting mode. The PMT signal, consisting of a series of TTL pulses, was counted by a data acquisition card (National Instruments, 6024E, Austin, TX). All counting, instrumentation, and voltages were controlled by a custom-tailored program written in Labview (National Instruments, version 5.0.1).

DHA was measured from very fresh cellular samples within minutes after isolation. AA concentration was later determined by oxidizing all AA in the sample to DHA with the Br_2 solution, therefore quantitating the total DHA plus AA, and then subtracting the DHA measurement from the total concentration.

RESULTS AND DISCUSSION

Stability of Ascorbic Acid. To assay AA and DHA accurately, oxidation of AA and degradation of DHA should be minimized during sample preparation and separation. The best way to avoid sample degradation is to measure each sample rapidly after isolation. To confirm the integrity of DHA and AA standards, aliquots of each were probed by infrared (IR) spectrometry to investigate the possibility of preoxidation. Results (not shown) demonstrate the purity of the AA standard used (99.9 ± 0.1%, $n = 3$).

In general, oxidation of AA in aqueous solutions depends on a number of factors such as pH, temperature, light, and the presence of molecular oxygen or trace metals.[1,40,41] When AA was dissolved in phosphate buffer (pH 7.4) or CE run buffer (borate buffer, pH 9.85) and kept at room temperature, more than 40% of AA was oxidized to DHA within 30 min. Several authors recommend acidic buffer for AA storage.[40,42,43] Acetate buffer was tested as an alternative; however, significant oxidation was also observed within minutes due to molecular oxygen and trace metal contamination. Therefore N_2-purged acetate buffer (final pH 3.7 ± 0.1) containing EDTA as a metal scavenger was chosen as a sample buffer. As a consequence, oxidative degradation can be minimized to less than 5% of the total AA.

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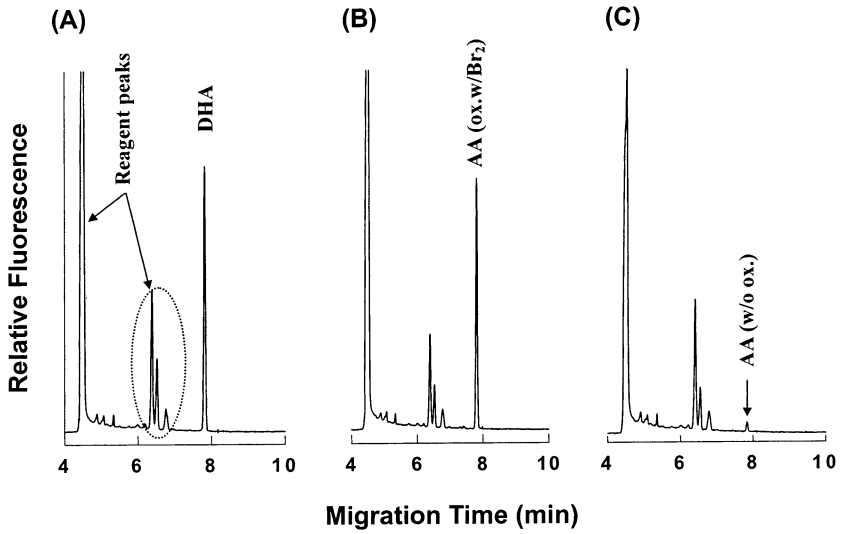

Figure 2. Electropherograms of standard ascorbic acid and dehydroascorbic acid: (A) 0.31 μM dehydroascorbic acid; (B) 0.31 μM ascorbic acid after oxidation process with Br_2 solution; (C) 0.31 μM ascorbic acid without Br_2 oxidation. Conditions: CE run buffer, 30 mM borate at pH 9.85; capillary dimensions, 80 cm ×b0 50-μm i.d. (365-μm o.d.) with 40-cm effective length; electrokinetic injection, 8 s at 8 kV; electrophoresis voltage, 20 kV; excitation, 350−356 nm Ar/Kr mixed-gas laser.

Selection of the Derivatization Reagent. Previously reported fluorometric reagents for AA analysis include *o*-phenylenediamine[17,44] and several of its derivatives.[16,19] One of these reacts with DHA to form a highly fluorescent quinoxaline derivative (Figure 1). In this work, three derivatization reagents were evaluated for selectivity and signal intensity: 1,2-phenylenediamine (PD), 4-methoxy-1,2-phenylenediamine (MPD), and 4,5-dimethyl-1,2-phenylenediamine (DMPD). While the MPD derivatization product has an absorption maximum that overlaps the laser lines in use, DMPD gives a stronger fluorescence yield with maximum excitation occurring at a slightly longer wavelength.[45] PD, although having good wavelength overlap, is reported to have the poorest fluorescence yield.[45]

Optimum derivatization conditions, including time, temperature, pH, and buffer composition, were found for each reagent. PD was found to react best at 50 °C for 2 min in a 50 mM acetate buffer at pH 4. MPD required 5 min of reaction time under these conditions, but produced increased signal intensity. DMPD reacted in 2 min using the same buffer, but had to be incubated at 55 °C during the reaction time for optimum yield.

The resulting derivatized products were directly compared to select the optimum reagent. Results show that CE-LIF sensitivity increased in the order PD < MPD < DMPD, while baseline simplicity (absence of extraneous peaks or shifts in baseline) increased in the order MPD < PD = DMPD. The fluorescent products displayed migration times in the order MPD = PD > DMPD, so that DMPD had the fastest assay time. Hence, DMPD was selected as the reagent of choice for AA quantitation experiments using CE-LIF with 350-nm excitation. However, reaction conditions may effect accurate quantitation because AA oxidizes much faster at higher temperatures, producing 2,3-diketogulonate by a cleavage of a lactone ring in as little as 1 h. DHA is also unstable. Therefore, an excess of reagent was used for derivatization instead of higher reaction temperatures. Results were similar for a ~4-min reaction at room temperature and a ~2-min reaction in an elevated temperature water bath.

Figure 2 demonstrates typical electropherograms for DHA and AA standards using DMPD. As DMPD derivatizes only DHA and not AA, methods to efficiently oxidize AA to DHA were investigated. AA was effectively oxidized to DHA after treatment with a Br_2 solution, producing a peak with the same signal intensity as a DHA solution of the same concentration. Without intentional oxidation of AA using Br_2, the AA signal was almost undetected as shown in Figure 2C. The correlation coefficients of standard calibration curves were consistently ≥0.99 during this work, and the LOD for DHA, at a S/N ratio of 3, was 320 pM (430 zmol). The tested dynamic range for this assay extended over more than three decades, and the highest concentration tested, 3.0 mM, was within the linear range.

AA Levels in Ganglia from Three Model Species. To confirm the ability to measure AA in heterogeneous neuronal tissues, a variety of neuronal samples were assayed for DHA and AA. AA levels were first measured in eight ganglia (each with between 300 and 3000 neurons) taken from freshwater and marine species significantly different in the ionic composition of the hemolymph. Figure 3 shows typical electropherograms of representative ganglia for DHA and DHA plus AA assays; the data are summarized in Table 1. The range of AA was found to be from 1.9 to 21 pmol. By measuring the size of the ganglion, its total volume can be approximated and the concentrations of AA and DHA determined. The AA concentrations ranged from ~4 to ~190 μM. Overall, AA concentration varied by ganglion type and species and may reflect different cell types and species-specific differences.

AA and DHA Levels in Individual Metacerebral Cells (MCCs). The MCC serotonergic neurons are the best recognized pair of feeding interneurons involved in feeding arousal.[21,38] There are only two neurons of this type in the whole nervous system, and MCC homologues were found in all opistobranch and pulmonate molluscs investigated to date. AA and DHA levels were

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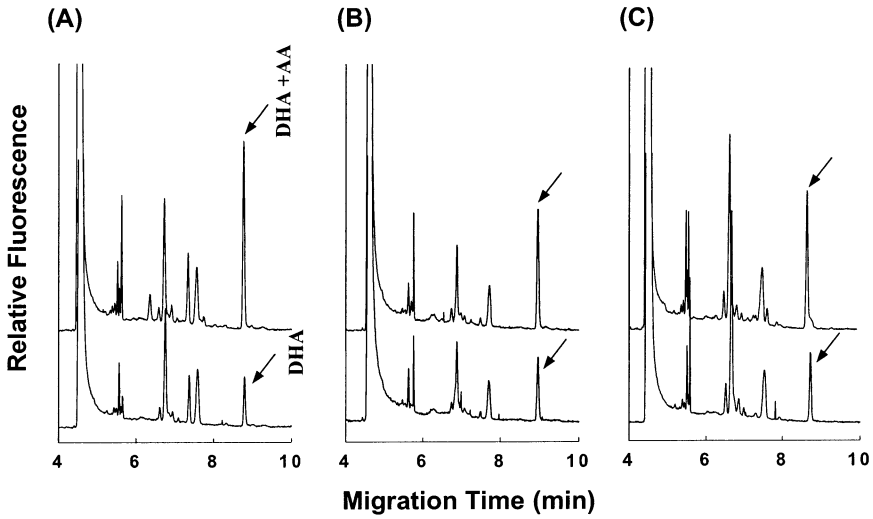

Figure 3. Electropherograms from three different ganglia before (dehydroascorbic acid only) and after (dehydroascorbic acid + ascorbic acid) oxidation by bromine: (A) left pleural ganglion from *Aplysia*; (B) pedal ganglion from *Pleurobranchaea*; (C) visceral ganglion from *Lymnaea*.

Table 1. Summary of Ascorbic Acid Amounts and Concentrations Calculated from Representative *Aplysia*, *Pleurobranchaea*, and *Lymnaea* Ganglia

species	ganglion	diameter (μm)	volume[a] (μL)	concn (mM)	mass (pmol)
Aplysia	left pleural	1200 × 975 × 975	0.60	0.018	11
Aplysia	right pleural	1050 × 900 × 900	0.45	0.0042	1.9
Pleurobranchaea	visceral	375 × 300 × 300	0.018	0.19	3.4
Pleurobranchaea	pedal	1050 × 450 × 450	0.11	0.039	4.3
Pleurobranchaea	buccal	1200 × 450 × 450	0.13	0.010	1.3
Pleurobranchaea	cerebral	1425 × 1125 × 675	0.57	0.037	21
Lymnaea	visceral	480 × 375 × 300	0.028	0.18	5.0

[a] Cell volumes were calculated using a modification of the spherical volume equation ($V = 4/3\pi r_x r_y r_z$).

analyzed in 11 MCC neurons taken from six *A. californica*, the cellular dissections having been performed by three individuals, to test the reproducibility of single-neuron measurements. These cells were assayed for DHA within several minutes of isolation to ensure accurate assessment of the metabolite levels in the living animal. AA concentration was calculated based on subtraction of the DHA contribution from the total ascorbic acid measurement. Figure 4 shows typical electropherograms of MCC neurons for DHA and DHA plus AA assays. Table 2 provides the actual data for all 11 cells studied, along with the calculated average and standard error of the mean (SEM) for each metabolite. AA levels dominate the total ascorbic acid measurement, and the average concentration of DHA is ~19% of the measured AA levels (the ratio of AA/DHA is 5.4). Several precautions were taken during sample preparation to prevent oxidation of the AA within these samples before the DHA assay, including transferring the cells on ice and performing fast analyses so that the measured values would be close to those found within a living cell. The ascorbic acid levels of ~1.5 mM for these cells fall at the low end of the 2−10 mM/cell range indirectly estimated for mammalian neurons, using multicellular sample populations.[3,15]

Total Ascorbic Acid Levels in Selected Neuron Populations. Different classes of identified neurons have been assayed from two distantly related species − *Aplysia* and *Lymnaea*. The sample preparation procedures were almost identical to those employed for MCC neuron analysis with the exception of excluding the initial DHA measurement. Because several of the *Lymnaea* neurons are significantly smaller, only a single total DHA plus AA measurement was made; a 2-μL volume of acetate buffer containing the cell was directly oxidized with bromine solution,

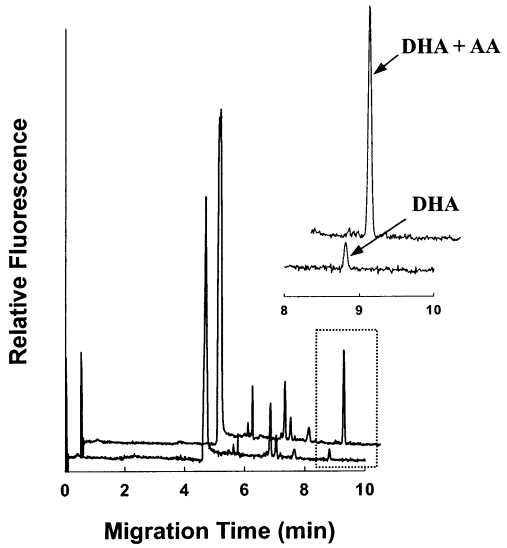

Figure 4. Electropherograms generated from metacerebral cells before (dehydroascorbic acid) and after (dehydroascorbic acid + ascorbic acid) oxidation by bromine. The inset expands the DHA and DHA + AA peaks.

Table 2. Summary of Ascorbic Acid and Dehydroascorbic Acid Amounts and Concentrations Calculated from 11 *Aplysia* Metacerebral Cell Assays

	diameter (μm)	volume[a] (nL)	ascorbic acid concn (mM)	ascorbic acid mass (pmol)	dehydroascorbic acid concn (mM)	dehydroascorbic acid mass (pmol)
MCC 1	330	19	0.86	16	0.098	1.9
MCC 2	300	14	1.6	22	0.32	4.5
MCC 3	440	45	0.29	13	0.042	1.9
MCC 4	220	5.6	1.3	7.3	0.25	1.4
MCC 5	210	4.9	2.6	13	0.66	3.2
MCC 6	210	4.9	1.8	8.7	0.45	2.2
MCC 7	230	6.4	0.58	3.7	0.16	1.0
MCC 8	230	6.4	0.89	5.7	0.34	2.1
MCC 9	240	7.2	1.4	9.7	0.079	0.56
MCC 10	225	6.0	3.0	18	0.52	3.1
MCC 11	240	7.2	2.2	16	0.15	1.1
mean ±SEM			1.5 ±0.2	12 ±2	0.28 ±0.06	2.1 ±0.3

[a] cell volumes were calculated assuming spherical geometry.

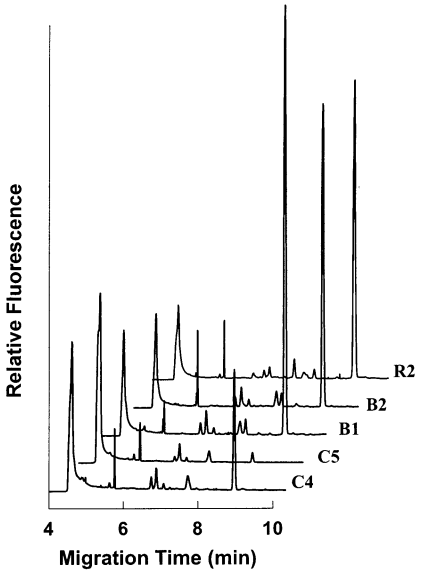

Figure 5. Representative electropherograms from five individual identified neurons from *A. califonica*.

derivatized with 1.5 μL of DMPD, and injected into the CE column without further dilution.

As previously mentioned, one goal is to determine the heterogeneity of AA levels in the CNS. Figure 5 compares electropherograms of five individual, identified neurons (B1, B2, C4, C5, R2) from *A. californica*. As this figure shows, different neurons in a single species contain ascorbic acid concentrations that vary by more than 40-fold. In the case of *Lymnaea*, there are two adjacent peaks, highlighting the need for a separation even with such a selective derivatization method. Spiking a cellular sample with a DHA standard facilitated identification of the AA in the presence of unknown compounds. The data calculated from *Aplysia* and *Lymnaea* single-neuron assays are summarized in Table 3.

In Figure 6, electropherograms of homologous MCC neurons from *Aplysia* and *Lymnaea* are compared; each species contains reproducible but very different concentrations of total ascorbic

Table 3. Summary of Total Ascorbic Acid[a] Amounts and Concentrations Calculated from Representative *Aplysia* and *Lymnaea* Single-Neuron Assays

species	cell	diameter (μm)	volume (nL)	concn (mM)	mass (pmol)
Aplysia	B1	195	3.9	3.7	14
Aplysia	B2	195	3.9	2.7	10
Aplysia	C4	105	0.61	6.0	3.7
Aplysia	C5	135	1.3	0.19	0.25
Aplysia	R2	525 × 375 × 375	39	6.2	240
Lymnaea	B1	75	0.22	0.22	0.048
Lymnaea	B2	75, 75	0.44	0.20	0.088
Lymnaea	B4	75	0.22	0.12	0.026
Lymnaea	LPeD1	105, 105	1.2	0.17	0.20
Lymnaea	MCC	90	0.38	0.13	0.049

[a] Ascorbic acid + dehydroascorbic acid.

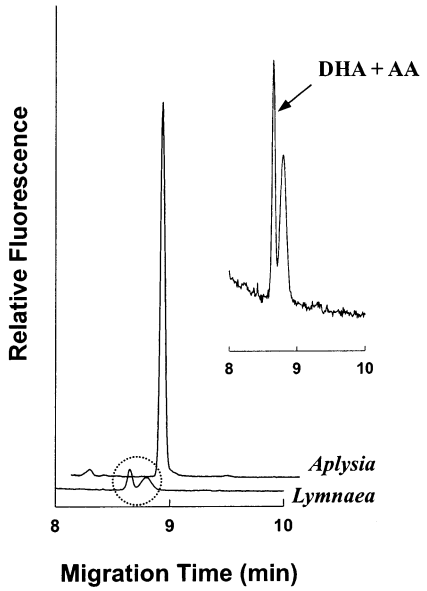

Figure 6. Comparison of total AA levels in MCCs from two different species. Cell size and volume measured in *Aplysia* were 250 μm and 8.7 nL while in *Lymnaea* these measurements were 105 μm and 1.2 nL, respectively.

acid. The concentrations of AA in *Aplysia* MCC neurons were higher than in *Lymnaea* MCC neurons.

Ascorbate Levels in the Molluscan Circulatory System. In mammals, the intracellular concentrations of AA are up to 40-fold higher than plasma concentrations.[46] Moreover, a considerable amount of intracellular AA is derived from rapid conversion of DHA.[13,47] Thus, low values of AA were expected in hemolymph, the equivalent of the blood in molluscs. In *Aplysia*, while the average concentration of AA in six identified neurons including the MCC was 3.4 mM, the average hemolymph level from three different animals (data not shown) was 2.9 μM. In *Lymnaea*, the average concentration of AA in five neurons and in hemolymph measured from four different animals was 170 and 3.5 μM, respectively. The ratio of AA/DHA in *Aplysia* hemolymph and in *Lymnaea* hemolymph was 2.2 and 2.1, respectively. These ratios

(46) Bánhegyi G.; Marcolongo, P.; Puskás, F.; Fulceri R.; Mandl, J.; Benedetti, A. *J. Biol. Chem.* **1998**, *273*, 2758−2762.
(47) Rumsey, S. C.; Kwon, O.; Xu, G. W.; Burant, C. F.; Simpson, I.; Levine, M. *J. Biol. Chem.* **1997**, *272*, 18982−18989.

are ∼2.4 times smaller than that of MCC (5.4) in *Aplysia*. Most remarkable is that the AA levels in the larger neurons chosen for study are 1 order of magnitude higher than in the ganglia and nearly 3 orders of magnitude higher than in hemolymph, demonstrating the effective ascorbate uptake system in these particular neurons and an asymmetric AA distribution.

CONCLUSIONS

A versatile and selective CE-LIF method has been developed and characterized for AA and DHA measurements with sufficient sensitivity and selectivity to detect this compound in single cells. Ascorbic acid levels in selected neurons and neuronal tissues of three invertebrate model organisms *A. californica, P. californica,* and *L. stagnalis* have been measured. Given the concentrations of AA measured and the assay LODs, we estimate that AA can be reliably detected in cells down to ∼20 μm, within the size range of the larger mammalian neurons. Interestingly, many of the identified cells studied showed amounts of AA and DHA that were species- and neuron-dependent.

While the AA and DHA concentrations in different cell types are likely related to differences in the biochemical state of the cell, further study is required to understand the consequences of this variability. Recent results have shown that the free radical NO was generated from nitrite in a pH-dependent manner, with larger amounts seen after the addition of AA.[16] Particularly intriguing is the fact that several cells with high levels of AA also have been reported to have high nitrite levels,[32,48] suggesting the possibility that nonenzymatic NO production in specific neurons should be further investigated.

(48) Boudko, D. Y.; Cooper, B. Y.; Harvey, W. R.; Moroz, L. L. *J. Chromatogr., B,* in press.

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