Anal. Chem. **1995**, *67*, 4261–4268

Use of 2,3-Naphthalenedicarboxaldehyde Derivatization for Single-Cell Analysis of Glutathione by Capillary Electrophoresis and Histochemical Localization by Fluorescence Microscopy

Owe Orwar,[†] Harvey A. Fishman,[†] Noam E. Ziv,[‡] Richard H. Scheller,[‡] and Richard N. Zare*,[†]

Departments of Chemistry and Molecular and Cellular Physiology, Howard Hughes Medical Institute, Stanford University, Stanford, California 94305

We report that 2,3-naphthalenedicarboxaldehyde reacts rapidly with glutathione and its precursor, γ-glutamylcysteine, to form highly fluorescent derivatives under physiological conditions. In contrast to previous accounts of 2,3-naphthalenedicarboxaldehyde labeling of primary amines, no additional CN^- ion or any other additional nucleophile is required. The fluorescence spectral properties of the chromophores ($\lambda_{exc\ max}$ = 472 nm, $\lambda_{em\ max}$ = 528 nm) make these derivatives amenable to excitation and detection by optical instrumentation that is optimized for fluorescein wavelengths. This selective labeling chemistry enabled quantitative determination and histochemical localization of glutathione in neurobiological samples. Intracellular glutathione was labeled by incubating cultured cells or cell suspensions in a 2,3-naphthalenedicarboxaldehyde-supplemented, DMSO-containing physiological buffer (pH = 7.4) for 2–10 min. Applications include imaging of cultured NG 108-15 cells (mouse neuroblastoma × rat glioma) and primary glial and neuronal cell cocultures (rat hippocampus) using epiluminescent and confocal fluorescence microscopy. Quantitative determination of glutathione in single NG 108-15 cells was accomplished using laser-induced fluorescence detection and capillary electrophoresis.

Glutathione (γ-Glu-Cys-Gly, GSH) is the most abundant low-molecular-weight thiol-containing species in mammalian tissues.[1,2] It is often involved in protective and detoxifying functions of the cell such as maintaining the proper thiol–disulfide status of proteins and quenching reactive free radical species. GSH also participates in other important biochemical reactions, including DNA[3] and protein synthesis and amino acid uptake.[1,2] Also, GSH protects against excitotoxin-induced brain damage[4] and may be involved in HIV expression;[5] pro-GSH drugs have been proposed for anti-HIV therapy.[6,7] Some cases of drug and radiation resistance in cancer therapy have been attributed to abnormally high concentrations of GSH in tumor cells.[8] In an attempt to counteract this high GSH concentration in tumor cells, one clinical trial is testing the use of L-buthionine-(*S*,*R*)-sulfoximine (BSO), an inhibitor of GSH synthesis, as an adjunct in cancer therapy.[8]

Because of the biological and clinical significance of GSH, numerous assays have been developed to determine its concentration. In separation techniques, electrochemical detection is often used because the thiol moiety can be readily detected at modestly positive potentials on Hg/Au amalgam electrodes.[9] Several histochemical methods have been developed for GSH localization, including fluorescence labeling with Prussian blue,[10] mercury orange,[11] *o*-phthalaldehyde (OPA),[12,13] and monochlorobimane (MCB).[14] Immunohistochemical methods have also been developed that are based on antisera raised against bovine serum albumin-coupled GSH.[15]

Derivatizations of GSH with MCB[14,16,17] and with OPA[18–21] represent two of the most selective schemes for fluorescence microscopy, flow cytometry, and separation-based analyses. The reaction of GSH with MCB is catalyzed by GSH S-transferases,[14,16,17] which makes it highly specific for GSH. GSH S-transferases are heterogeneously distributed in different tissues

† Department of Chemistry.
‡ Department of Molecular and Cellular Physiology.

(1) Meister, A.; Tate, S. S. *Annu. Rev. Biochem.* **1976**, *45*, 559.
(2) Meister, A.; Anderson, M. *Annu. Rev. Biochem.* **1983**, *52*, 711–760.
(3) Reichard, P.; Thelander, L. *Annu. Rev. Biochem.* **1979**, *48*, 133–158.
(4) Bridges, R. J.; Koh, J.-Y.; Hatalski, C. G.; Cotman, C. W. *Eur. J. Pharmacol.* **1991**, *192*, 199–200.
(5) Staal, F. J. T.; Roederer, M.; Herzenberg, L. A.; Herzenberg, L. A. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 9943–9947.
(6) Kalebic, T.; Kinter, A.; Poli, G.; Anderson, M. E.; Meister, A.; Fauci, A. S. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 986–990.
(7) Roederer, M.; Ela, S. W.; Staal, F. J. T.; Herzenberg, L. A.; Herzenberg, L. A. *AIDS Res. Hum. Retroviruses* **1992**, *8*, 209–217.
(8) Meister, A. *Cancer Res.* **1994**, *54*, 1969S–1975S.
(9) Allison, L. A.; Shoup, R. E. *Anal. Chem.* **1983**, *55*, 8–12.
(10) Smith, M. T.; Loveridge, N.; Wills, E.; Chayen, J. *Biochem. J.* **1979**, *182*, 103–108.
(11) Asghar, K.; Reddy, B. G.; Krishna, G. *J. Histochem. Cytochem.* **1974**, *23*, 774–779.
(12) Murray, G. I.; Burke, M. D.; Ewen, S. W. B. *Histochem. J.* **1986**, *18*, 434–440.
(13) Treumer, J.; Valet, G. *Exp. Cell Res.* **1986**, *163*, 518–524.
(14) Bellomo, G.; Vairetti, M.; Stivala, L.; Mirabelli, F.; Richelmi, P.; Orrenius, S. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 4412–4416.
(15) Hjelle, O. P.; Chaudry, F. A.; Ottersen, O. P. *Eur. J. Neurosci.* **1994**, *6*, 793–804.
(16) Rice, G. C.; Bump, E. A.; Shrieve, D. C.; Lee, W.; Kovacs, M. *Cancer Res.* **1986**, *46*, 6105–6110.
(17) Fernandez-Checa, J. C.; Kaplowitz, N. *Anal. Biochem.* **1990**, *190*, 212–219.
(18) Cohn, V. H.; Lyle, J. *Anal. Biochem.* **1966**, *14*, 434.
(19) Morineau, G.; Azoulay, M.; Frappier, F. *J. Chromatogr.* **1989**, *467*, 209.
(20) Neuschwander-Tetri, B. A.; Roll, F. J. *Anal. Biochem.* **1989**, *179*, 236.
(21) Orwar, O.; Sandberg, M.; Jacobson, I.; Sundahl, M.; Folestad, S. *Anal. Chem.* **1994**, *66*, 4471–4482.

0003-2700/95/0367-4261$9.00/0

and within different intracellular compartments. Some cell types have very low GSH S-transferase activities, and some GSH S-transferase isoenzymes do not catalyze the conjugation reaction between MCB and GSH, thus severely limiting use of this labeling method.[22-24] Moreover, nonspecific staining using MCB has also been shown.[22,23] OPA is another highly selective fluorogenic reagent for GSH which also reacts with GSH's precursor, γ-glutamylcysteine (γ-GC). These two peptides undergo cyclization reactions with OPA that involve both the thiol and the primary amine of the peptides.[19-21] It is notable that cysteine, the other prominent low-molecular-weight thiol-containing amino compound besides GSH, does not form a fluorescent derivative when reacted with OPA.[25] Further, because the concentration of GSH is 3 orders of magnitude higher than that of γ-GC, at least in rat brain,[26] interferences from this dipeptide are expected to be minimal. These favorable properties have made OPA amenable for histochemical evaluation of the GSH content using fluorescence microscopy and flow cytometry.[12,13] OPA-labeling chemistry has several drawbacks, however, including prominent side reactions with imidazole-containing compounds such as histamine,[27,28] histidine, and histidine-containing peptides.[29,30] For histochemical localization of the OPA-GSH adduct using fluorescence microscopy, it is thus necessary to treat the tissue with buffered formalin, which complexes imidazolyl compounds.[12,30] Very long incubation times of 30 to 60 min have also been used with these microscopy-based methods.[12,31] Other drawbacks to using OPA are the excitation wavelengths of the OPA-GSH and OPA-γ-GC chromophores in the UV and moderate fluorescence quantum yields.

We describe a method that overcomes these problems based on selective fluorogenic derivatization of GSH and γ-GC using 2,3-naphthalenedicarboxaldehyde (NDA). Fluorophore formation with GSH and γ-GC was rapid under physiological conditions (the apparent first-order rate constant for NDA-GSH formation was 3.4×10^{-2} s^{-1}). NDA diffused rapidly into cells, and maximal fluorescence from condensation of intracellular GSH was achieved in <2 min. Fluorescence microscopes and flow cytometers that operate at fluorescein wavelengths can be used for excitation and detection of the chromophores. The selectivity for GSH is >1000-fold higher than that for histidine and histamine and ~100-fold higher than that for arginine. Problems with spontaneous oxidation of the peptides to their corresponding disulfide dimers are largely avoided because the dye is added to living cells.

EXPERIMENTAL SECTION

Apparatus. Capillary electrophoresis (CE) experiments in 800-mm × 27-μm-i.d. fused silica capillaries (Polymicro, Phoenix, AZ) were performed using a high-voltage power supply (Glassman, Whitehouse Station, NJ) operated at ~25 kV.

Laser-induced fluorescence (LIF) detection in CE experiments was performed using the 457.9-nm line from an Ar^+ ion laser (Spectra Physics 2017, Mountain View, CA). A notch interference filter centered at 457 nm with a 10.5-nm fwhm (Omega Optical, Brattleboro, VT) was used to reject plasma discharge emission and unwanted laser light. The laser beam was focused onto the fused silica capillary using a 50-mm-focal length lens. The beam waist at the focal point ($1/e^2$ intensity) was calculated to be 11 μm. A linear-graded, neutral density filter (Melles Griot, Irvine, CA) was used to select optimal excitation laser powers. The detection zone of the capillary (produced by stripping off the polyimide layer on a 5-mm length by heating) was housed in a parabolic mirror. The fluorescence emission was collected at a right angle with respect to the incident laser beam and focused by a 150-mm-focal length plano-convex lens onto the photomultiplier tube (PMT; Hamamatsu Model R4632, San Jose, CA). The PMT was biased at 600 V. The fluorescence emission was filtered to reject Raman and Rayleigh scattered light by a 532-nm notch interference filter (~30 nm fwhm) and a 480-nm long-pass filter (~92% transmission between 500 and 580 nm), both from Omega Optical. The PMT current was amplified by a picoammeter, converted to voltage and digitized using an analog-to-digital board (Chrom-1, Omega Engineering, Stamford, CT), and displayed using commercial software (Galactic Industries, Salem, NH) run on a personal computer.

Confocal images were obtained using a modified Bio-Rad MRC 500 laser scanning unit coupled to a Zeiss IM-35 inverted microscope as described by Ryan et al.[32] Excitation was performed using the 488-nm line of an air-cooled Ar^+ ion laser with ~20 μW of power delivered to the back aperture of the objective. Fluorescence and differential interference contrast (DIC) images were aquired simultaneously.

Fluorescence microscopy was performed using a Nikon Diaphot-TMD-EF inverted microscope (Tokyo, Japan) with an oil-immersion lens, 100× (0.8 NA). The cells were illuminated with 470–490-nm light (Nikon filter block B-1A), and fluorescence (>520 nm) was imaged onto an R928 PMT with a Microflex PFX photomicrographic attachment (Nikon). The current from the PMT was converted to a voltage and amplified with an operational amplifier. High-frequency noise was removed by a low-pass filter with a RC time constant of 1 s. The voltage signal was digitized by a DAS 8 analog-to-digital board (Metrabyte) and displayed with customized software written in QuickBasic (Microsoft) on a personal computer. Images were photographed using a Nikon N8008s camera.

Fluorescence and UV–vis spectra were recorded on a spectrofluorometer (Model 8000, SLM Inc., Urbana-Champaign, IL) and a diode array spectrophotometer (Model 8450A, Hewlett Packard, Palo Alto, CA), respectively.

Fluorescence quantum yields (Φ_f) were recorded and calculated according to the procedures described by Parker and Rees.[33] Fluorescein in 0.1 N sodium hydroxide was used as a reference compound ($\Phi_f = 0.85$).[33]

Reagents and Chemicals. GSH, γ-GC, *S*-methyl glutathione, glutathione (oxidized form), amino acid standards, L-buthionine-

(22) Hedley, D. W.; Chow, S. *Cytometry* **1994**, *15*, 349–358.
(23) Van der Ven, A. J. A. M.; Mier, P.; Peters, W. H. M.; Dolstra, H.; Van Erp, P. E. J.; Koopmans, P. P.; Van der Meer, J. W. M. *Anal. Biochem.* **1994**, *217*, 41–47.
(24) Cook, J. A.; Iype, S. N.; Mitchell, J. B. *Cancer Res.* **1991**, *51*, 1606–1612.
(25) Hissin, P. J.; Hilf, R. *Anal. Biochem.* **1976**, *74*, 214–226.
(26) Sandberg, M.; Li, X.; Folestad, S.; Weber, S. G.; Orwar, O. *Anal. Biochem.* **1994**, *217*, 48–61.
(27) Rönnberg, A. L.; Hansson, C.; Håkanson, R. *Anal. Biochem.* **1984**, *139*, 338–344.
(28) Allenmark, S.; Bergström, S.; Enerbäck, L. *Anal. Biochem.* **1985**, *144*, 98–103.
(29) Håkanson, R.; Rönnberg, A. L.; Sjölund, K. *Anal. Biochem.* **1974**, *59*, 98–109.
(30) Mokrasch, L. C.; Teschke, E. J. *Anal. Biochem.* **1984**, *140*, 506–509.
(31) Philbert, M. A.; Beiswanger, C. M.; Waters, D. K.; Reuhl, K. R.; Lowndes, H. E. *Toxicol. Appl. Pharmacol.* **1991**, *107*, 215–227.
(32) Ryan, T. A.; Reuter, H.; Wendland, B.; Schweizer, F. E.; Tsien, R. W.; Smith, S. J. *Neuron* **1993**, *11*, 713–724.
(33) Parker, C. A.; Rees, W. T. *Analyst* **1960**, *85*, 587–600.

(*S,R*)-sulfoximine, and sodium tetraborate were obtained from Sigma Chemicals (St. Louis, MO). Dimethyl sulfoxide (DMSO) was obtained from Aldrich Chemicals (Milwaukee, WI). Peptide and amino acid standards were prepared in distilled deionized water immediately prior to derivatization to avoid spontaneous oxidation of thiol-containing amino acids and peptides. The NDA reagent (1−100 mM) for derivatization of standards was prepared in acetonitrile. The CE buffer was ~11 mM borate buffer (pH = 9.1). Deionized water was prepared using a Millipore Milli-Q system (Millipore, Bedford, MA).

Cell Culture, Dye Loading, and Derivatization. NG 108-15 cells (passages 5−10) were grown in Dulbecco's modified eagle medium (Gibco BRL/Life Technologies, Grand Island, NY) supplemented with 10% (v/v) fetal calf serum, 5% (v/v) penicillin−streptomycin (Gibco), 5% (v/v) 200 mM L-glutamine, and 5% (v/v) solution consisting of 10 μM sodium hypoxanthine, 40 μM aminopterin, and 1.6 mM thymidine (Gibco). Cells from the stock cultures were plated onto No. 1 coverslips several hours before fluorescence measurements. Glial and pyramidal cell cocultures, obtained from freshly dissociated coronal sections of rat hippocampal slices, were used on the eighth day of culturing. The NDA reagent used for cell loading was prepared by dissolving ~500 μg of NDA (Aldrich Chemicals) in 50 μL of DMSO (Aldrich Chemicals) and 5 μL of pluoronic (Molecular Probes, OR). A 5−40-μL aliquot of this solution was diluted in DMSO to 50 μL and added to 500 μL of a HEPES buffered saline solution (HBS) containing 135 mM NaCl, 5 mM KCl, 10 mM glucose, 2 mM $MgCl_2$, 2 mM $CaCl_2$, and 10 mM HEPES (pH = 7.4). The cells were loaded for 2−10 min at room temperature. The dye loading was terminated by washing the cells several times with HBS to remove unreacted reagent. The cells were maintained in HBS during microscopy.

For single-cell analyses using CE-LIF detection, NG 108-15 cell suspensions were derivatized using the same dye solution recipe as used for the fluorescence microscopy experiments. Derivatization and cell-injection procedures were performed essentially as described by Hogan and Yeung.[34] Briefly, the polyimide layer on the inlet end of the CE capillary was stripped on a 1-cm length by heating and fixed on a coverslip by a piece of adhesive tape. The capillary mount was placed on the stage of a microscope (Zeiss Telaval 31). Approximately 10 μL each of cell suspension and dye solution were placed over the inlet end of the capillary. After a 10-min reaction time, single cells (or small clusters) were gently sucked into the capillary by a gas-tight syringe connected to the outlet end of the capillary. While this procedure was being performed, the capillary was observed in the microscope. After a cell entered the capillary, the capillary was reinserted into a buffer vial, and the separation voltage was applied immediately.

Extracts and standards were derivatized by adding 100 μL of 11 mM borate buffer (pH ~ 9.1) to 100 μL of extract or standard solution and then 100 μL of the NDA reagent solution. After a 10-min reaction time, samples were injected into the separation capillary.

RESULTS AND DISCUSSION

Reaction Kinetics and Fluorescence Properties of the NDA-GSH and NDA-γ-GC Derivatives. NDA reacted rapidly with GSH and γ-GC to form fluorescent adducts. For the reaction of GSH using 100-fold molar excess of NDA, the apparent first-order rate constants, k_{app}, were 2.5×10^{-1} and 3.4×10^{-2} s^{-1} in 50 mM borate buffer (pH = 9.1) and HBS (pH = 7.4), respectively (Figure 1). The rate constant obtained using a 100-fold molar excess of GSH over reagent concentration (borate buffer, pH = 9.1) was not significantly different, $k_{app} = 2.5 \times 10^{-1}$ s^{-1}. Considering that the reaction with the peptides and NDA is so rapid, particularly in basic media, these products may also be formed in derivatization schemes employed for amino acids analyses using NDA/CN^- labeling with biological samples.

In analogy with the reaction of GSH and γ-GC with OPA,[19-21] we propose that the predominant products formed in the reaction with the respective peptides and NDA are the tetracyclic derivatives shown in Figure 2. That the thiol moiety of the peptides participate in the reaction is supported by the finding that S-methylated glutathione did not react with NDA (see below). Formation of these derivatives probably follows the reaction

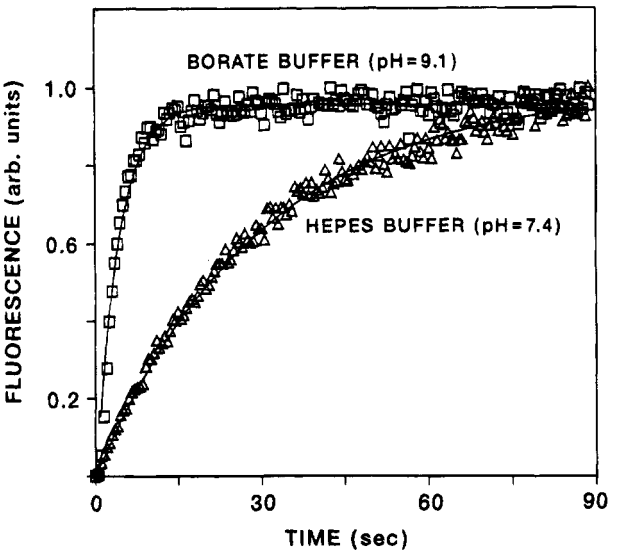
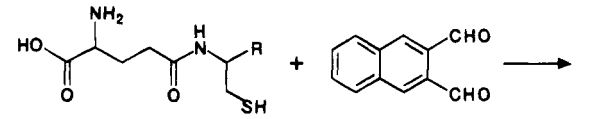


Figure 1. Formation of the NDA-GSH derivative as a function of time in two different aqueous buffers. Fluorescence measurements (λ_{exc} = 460 nm, λ_{em} = 528 nm) were performed on continuously stirred aerated solutions. □: Normalized data obtained with 8.3 mM NDA and 83 μM GSH in 50 mM borate buffer (pH = 9.1) that contained 33% acetonitrile by volume. The best-fit curve to this data set yielded an apparent first-order reaction rate constant of 2.5×10^{-1} s^{-1}. △: Normalized data obtained with 8.3 mM NDA and 83 μM GSH in a HEPES buffer (pH = 7.4) that contained 33% acetonitrile by volume. Here, an apparent first-order reaction rate constant of 3.4×10^{-2} s^{-1} was obtained.



γ-Glutamylcysteine: R=COOH
Glutathione: R=$CONHCH_2COOH$

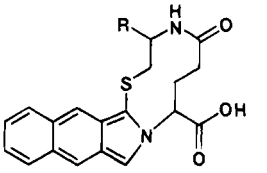


Figure 2. Reaction scheme showing the structure of the proposed predominant reaction product between 2,3-naphthalenedicarboxaldehyde and GSH and 2,3-naphthalenedicarboxaldehyde and γ-GC, respectively.

(34) Hogan, B. L.; Yeung, E. S. *Anal. Chem.* **1992**, *64*, 2841−2845.

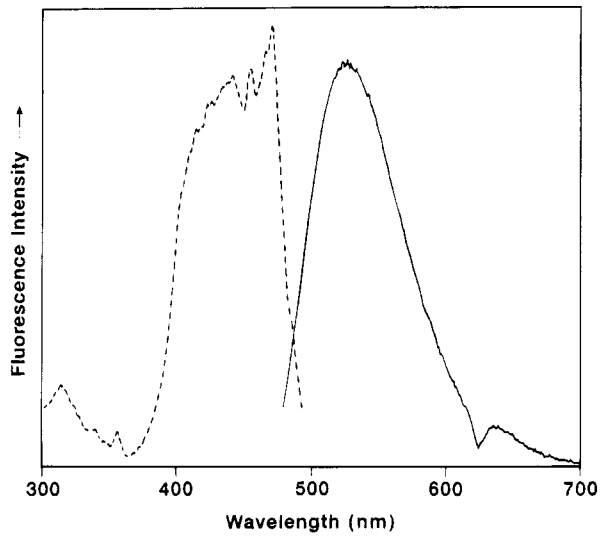


Figure 3. Excitation (- - -) and emission (—) fluorescence spectra (λ_{exc} = 460 nm, λ_{em} = 528 nm) of the NDA-GSH product obtained in 50 mM borate buffer (pH = 9.1) that contained 33% acetonitrile by volume.

scheme proposed by Sternson et al.[35] for the formation of 1-(alkylthio)-2-alkylisoindoles from OPA, a primary amine, and a thiol. Thermal degradation rates (293 K) of the NDA-GSH and NDA-γ-GC derivatives were slow because almost no decomposition was observed after 24-h storage of the derivatized solutions (50 mM borate buffer (pH = 9.1)/acetonitrile, 4:1 v/v) (data not shown).

The molar absorptivities of the chromophores in aqueous alkaline solution—NDA-GSH (ϵ = 5095 M^{-1} cm^{-1}, λ_{abs} = 460 nm) and NDA-γ-GC (ϵ = 5008 M^{-1} cm^{-1}, λ_{abs} = 460 nm)—are moderate but similar to those observed for structurally related cyanobenz-[*f*]isoindoles.[36] Increased molar absorptivities of the NDA-GSH and NDA-γ-GC chromophores were observed at shorter wavelengths (data not shown). Figure 3 shows the fluorescence spectra of the NDA-GSH derivative ($\lambda_{exc\ max}$ = 472 nm, $\lambda_{em\ max}$ = 528 nm). Qualitatively, the fluorescence spectra of the NDA-γ-GC derivative were similar (data not shown). The broad excitation band makes excitation with several different laser lines feasible (for example, the 442-nm line of a HeCd laser and the 457.9- and 488-nm lines of Ar^+ lasers). An advantage is that the NDA-GSH and NDA-γ-GC chromophores can be used with microscopy and flow cytometric equipment that are optimized for fluorescein measurements. The fluorescence quantum yields of the chromophores in 0.1 N sodium hydroxide—NDA-GSH (Φ_f = 0.58) and NDA-γ-GC (Φ_f = 0.55)—were improved over the corresponding OPA derivatives[21]—OPA-GSH (Φ_f = 0.42) and OPA-γ-GC (Φ_f = 0.40).

NDA was originally used as a selective reagent for arginine under conditions for which reaction with thiol-containing compounds was blocked by the addition of *N*-ethylmaleimide.[37] Later, NDA was employed with CN^- as a coreagent to label primary amine-containing species.[38] NDA/CN^- has been succesfully adopted for amino acid analyses in single cells using open-tubular liquid chromatography and CE with LIF detection.[39,40] It is an advantage that CN^- ion is not required for fluorophore formation between NDA and the peptides in the present study because CN^- can induce condensation reactions of aromatic aldehydes, forming fluorescent products that supposedly interfere with the chromatographic analysis of NDA/CN^--labeled amino acids.[41]

The NDA-Arg adduct has a fluorescence spectrum similar to those of NDA-GSH and NDA-γ-GC derivatives and is thus a potential source of interference in our analysis. Moreover, both histidine and histamine react with OPA, and we assume that those two imidazolyl compounds could also react with NDA. In addition to these amines, several other amino acids and peptides were tested for interference using fluorescence spectroscopy. The amines (100 μL, 1 mM) were derivatized with NDA (200 μL, 10 mM) for 10 min in 400 μL of HBS (pH = 7.4) and diluted three times with HBS. The fluorescence of the products was measured at 530 nm (λ_{exc} = 460 nm). The following values were obtained (expressed as percent relative fluorescence intensity): γ-GC and GSH, 100%; Arg, ~1%; Homocys, Cys, His, histamine, Ala, Val, Phe, Trp, Tyr, Asp, Glu, Met, Asn, Ser, Glu, Gln, *S*-methyl glutathione, and GSSG (oxidized form of glutathione), all <0.1%. The interference expected from Arg in biological samples is small because, in addition to a 100-fold lower fluorescence intensity of the NDA-Arg derivative relative to the NDA-GSH derivative, the concentration of Arg in the mammalian brain is low, e.g., ~0.1 mM in rat, monkey, and human brain[42] compared to a GSH concentration of about 2.2 mM.[43] OPA readily forms highly fluorescent derivatives with histamine,[27,28] His, and histidine-containing peptides.[29,30] Mokrasch and Teschke[30] obtained the following relative fluorescence intensities of GSH, His, and various His-containing peptides in phosphate-buffered solution (pH = 8) after derivatization with OPA: GSH, 100%; homocarnosine (γ-amino-*n*-butyrylhistidine), 44.2%; carnosine (β-alanylhistidine), 4.4%; His, 13.8%; His-Ser, 18.6%; and His-Gly, 12.6%. Thus, despite the structural similarities of the NDA and OPA molecules, differences exist either in their reactivity with imidazolyl compounds or in the photophysical and photochemical properties of the formed derivatives. The side reactions with imidazolyl compounds observed for OPA are problematic because imidazolyl compounds are, generally, present in high concentrations in mammalian cells: homocarnosine, ~0.33–0.61 mM in brain tissue;[44–46] free His, ~0.09–0.15 mM in human brain.[46] To minimize interferences from endogenous imidazolyl compounds when measuring the GSH content by OPA derivatization, formalin treatment of the tissue is required prior to incubation with OPA.[12,30]

CE-LIF Detection of the NDA-GSH Derivative in Single Cells and Cell Extracts. A CE separation of the NDA-GSH and NDA-γ-GC derivatives is shown in Figure 4a. Both derivatives

(35) Sternson, L. A.; Stobaugh, J. F.; Repta, A. J. *Anal. Biochem.* **1985**, *144*, 233–246.
(36) Matuszewski, B. K.; Givens, R. S.; Srinivasachar, K.; Carlson, R. G.; Higuchi, T. *Anal. Chem.* **1987**, *59*, 1102–1105.
(37) Miura, T.; Kashiwamura, M.; Kimura, M. *Anal. Biochem.* **1984**, *139*, 432–437.
(38) Srinivasachar, K.; Carlson, R. G.; Givens, R. S.; Matuszewski, B. K. *J. Org. Chem.* **1986**, *51*, 3978.
(39) Kennedy, R. T.; Oates, M. D.; Cooper, B. R.; Nickerson, B.; Jorgenson, J. W. *Science* **1989**, *246*, 57–63.
(40) Gilman, S. D.; Ewing, A. G. *Anal. Chem.* **1995**, 67, 58–64.
(41) Kvakman, P. J. M.; Koelewijn, H.; Kool, I.; Brinkman, U. A. Th.; De Jong, G. J. *J. Chromatogr.* **1990**, *511*, 155–166.
(42) Clarke, D. D.; Lajtha, A. L.; Maker, H. S. *Basic Neurochemistry*, 4th ed.; Raven Press: New York, 1989; Chapter 28.
(43) Folbergova, J.; Rehncrona, S.; Siesjö, B. K. *J. Neurochem.* **1979**, *32*, 1621.
(44) Crush, K. G. *Comp. Biochem. Physiol.* **1970**, *34*, 3–30.
(45) Perry, T. L.; Hansen, S.; Berry, K.; Mok, C.; Lesk, D. *J. Neurochem.* **1971**, *18*, 521–528.
(46) Nakamura, H.; Zimmerman, C. L.; Pisano, J. J. *Anal. Biochem.* **1979**, *93*, 423–429.

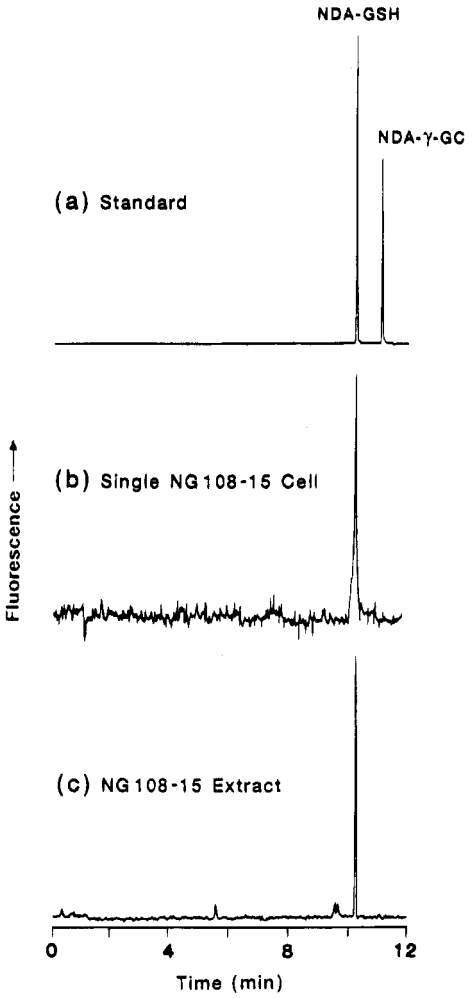


Figure 4. (a) Separation of a NDA-GSH and NDA-γ-GC standard (100 μM of each derivative injected). (b) Electropherogram of a single NDA-derivatized NG 108-15 cell. (c) Electropherogram of a NDA-derivatized NG 108-15 cell extract. See Experimental Section for details.

Table 1. Precision, Linearity, and Detection Limits of 2,3-Naphthalenedicarboxaldehyde-Labeled Glutathione and γ-Glutamylcysteine

derivative	corr coeff (r)[a]	CLOD/nM[b]	MLOD/amol[b]	RSD/%[c]
NDA-GSH	0.9976	250	126	4.4
NDA-γ-GC	0.9960	376	188	7.0

[a] Product−moment correlation coefficients obtained in the range 1.67−167 μM (n = 9). [b] Concentration and mass limits of detection (CLOD and MLOD, respectively) obtained using a 27-μm-i.d. capillary and a 5-s injection period (injection volume estimated to be 200 pL, see ref 53). [c] Relative standard deviation of peak heights (n = 6).

are negatively charged in the HBS buffer (pH = 7.4) used for the CE separations. Table 1 lists detection limits, linearity, and precision. Electropherograms of NDA-derivatized single cells showed the NDA-GSH peak as the main feature (Figure 4b). From ruler-calibrated confocal DIC images, NG 108-15 cells were determined to have cell radii of 7.8 ± 1.4 μm (mean ± SD, n = 39) and cell volumes (spherical cell geometry approximation) of 2.2 ± 1.1 pL (mean ± SD, n = 39). It is important to point out that what appears to be a narrow distribution of radii is really a large variation in cell volumes. Using CE-LIF detection, we found the intracellular content of GSH in single NG 108-15 cells to be 1.28 ± 1.15 fmol (mean ± SD, n = 10, six single-cell injections, one injection of two cells, two injections of three cells, and one injection of four cells). In these measurements, cell-to-cell variations in size are not taken into account. This result, however, gives a mean intracellular GSH concentration of 1.2 mM (assuming that the nucleus occupies half the cell volume and that only cytoplasmic GSH is labeled). The large variations in the amount of GSH contained in each cell may result from variations in cell size but may also be a function of intracellular GSH heterogeneity, similar to that reported for astrocytes in primary cultures.[47] Interestingly, the same variability in relative fluorescence intensity of NDA-labeled cells was observed in the experiments designed for evaluating the effect of BSO (see below). The mean concentration of GSH in NG 108-15 cell lysates has been determined previously as 50 nmol/mg protein or ~5 mM (wet weight),[48] a value somewhat higher than our estimate. When single and small numbers of cells were injected, the peak shape and retention time showed some variability, probably reflecting cell lysis at different locations within the capillary and nonuniform disintegration of cell clusters. Injections of NDA-derivatized NG 108-15 extracts (n = 5, single extraction using methanol/distilled water, 75:25 v/v, containing 1 mM Na_2EDTA) produced electropherograms with only one prominent feature (Figure 4c). This peak was identified as the NDA-GSH derivative by comparison of migration times of NDA-GSH standards (Figure 4a). The recovery (peak height measurements, ~1.5 μM each of the injected standard and extract) of NDA-GSH standards added to NG 108-15 cell extracts was 86 ± 32% (n = 6). In one run, the peak centered at ~5.5 min (Figure 4c) was of equal height to that of the NDA-GSH derivative.

Hogan and Yeung demonstrated the feasibility of using monobromobimane (MBB) as a reagent for labeling intracellular GSH in single erythrocytes,[34] and similar analysis using MBB-labeling has been performed on hemolysates using liquid chromatography.[49] The electropherograms and chromatograms obtained in these respective studies, however, are more complex than ours because MBB is inherently fluorescent and reacts with thiols other than glutathione. This is, of course, not a problem when using MBB in conjunction with separations, but it makes the reagent less suited for histochemical assays that probe the GSH content.

Fluorescence Microscopy. Because NDA-labeled single NG 108-15 cells and lysed cell suspensions produced only one prominent feature in the electropherograms (see above), we tested the use of NDA as a reagent for histochemical evaluation of the GSH content using fluorescence microscopy. When cultured NG 108-15 cells were incubated with NDA and excited by 470−490-nm light, green fluorescence was localized mainly to the cytoplasm (Figure 5A). Cells developed strong fluorescence even when incubated with NDA for short periods of time (<30 s). It is interesting to note that very long incubation times (30−60 min) have been used for labeling of intracellular GSH with OPA.[12,31] One reason for such long incubation times could be that OPA, because it is more hydrophilic, does not penetrate biological membranes as easily as NDA. Control cultures incubated with the same media without NDA showed no detectable fluorescence (data not shown). Experiments were performed to test whether

(47) Devesa, A.; O'Connor, J. E.; Garcia, C.; Puertes, I. R.; Vina, J. *Brain Res.* **1993**, *618*, 181−189.
(48) Patel, N.; Kumagai, Y.; Unger, S. E.; Fukuto, J. M.; Cho, A. K. *Chem. Res. Toxicol.* **1991**, *4*, 421−426.
(49) Nardi, G.; Cippolaro, M.; Loguerico, C. *J. Chromatogr.* **1990**, *530*, 122−128.

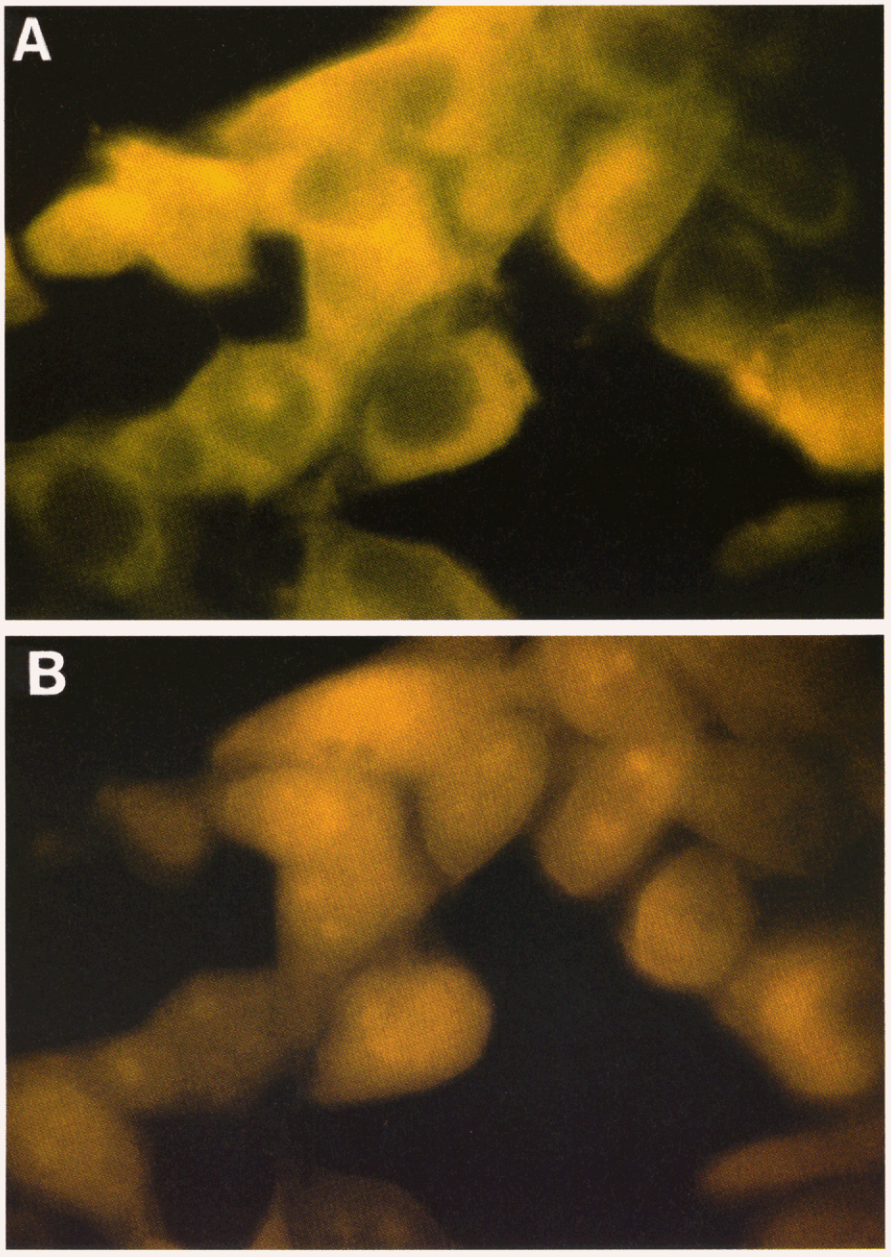


Figure 5. Fluorescence photomicrographic images obtained after ~15 s and ~5 min of illumination for NG 108-15 cells with NDA present in the cell-bathing media. (A) Fluorescence images obtained after ~15 s of illumination with 470−490-nm light. After NDA is washed from the incubating media, no further fluorescence is observed from the cells; they subsequently undergo photobleaching. Note that fluorescence is localized mainly to the cytoplasm. (B) Photomicrographic fluorescence images of the same field as in Figure 5A obtained after ~5 min of irradiation. Two major changes are apparent: the fluorescence color has changed from green to yellow, and the fluorescence is present throughout the cells, including the nuclei.

the chemical identity of the formed fluorophore is indeed that of the NDA-GSH derivative. Because γ-GC is present in brain cells at concentrations 3 orders of magnitude lower than GSH,[26] we do not expect to detect that species in single cells. As already shown, analyses of single NDA-derivatized cells as well as lysed cell suspensions using CE-LIF showed the NDA-GSH adduct as the main component. Furthermore, NG 108-15 cells incubated with BSO (2 mM, 18 h), an inhibitor of γ-GC synthetase activity, showed ~50% reduced relative fluorescence intensity ($p < 0.001$, Student's t-test), 652.4 ± 289.6 (mean ± SD, $n = 40$, four dishes) compared with control cells (BSO-free media), 1278.2 ± 853.2 (mean ± SD, $n = 36$, four dishes). Here, the fluorescence intensity was measured after a 2-min incubation with NDA by adjusting the aperture size to the PMT (biased at 650 V) so that the fluorescence from a single cell was collected. The BSO treatment resulted in a high number of necrotic cells that did not fluoresce after incubation with NDA; these cells were not included in the statistical evaluation. Treatment with 2 mM BSO of NG 108-15 cells for 24 h has been suggested to deplete intracellular GSH stores by ~97%.[48] The reason that this reduction is higher than our value is probably that it is an average over both viable and necrotic cells. We cannot, however, completely rule out the possibility that other structures such as proteins may account for some of the staining observed using NDA.

In conclusion, NDA diffuses through cell membranes and reacts rapidly with intracellular analytes to form fluorescent products. The predominant chromophore observed upon exciting the cells with 470−490-nm light and monitoring the fluorescence at 530 nm appears to be that of the NDA-GSH adduct. In experiments in which NDA remained in the media, the fluores-

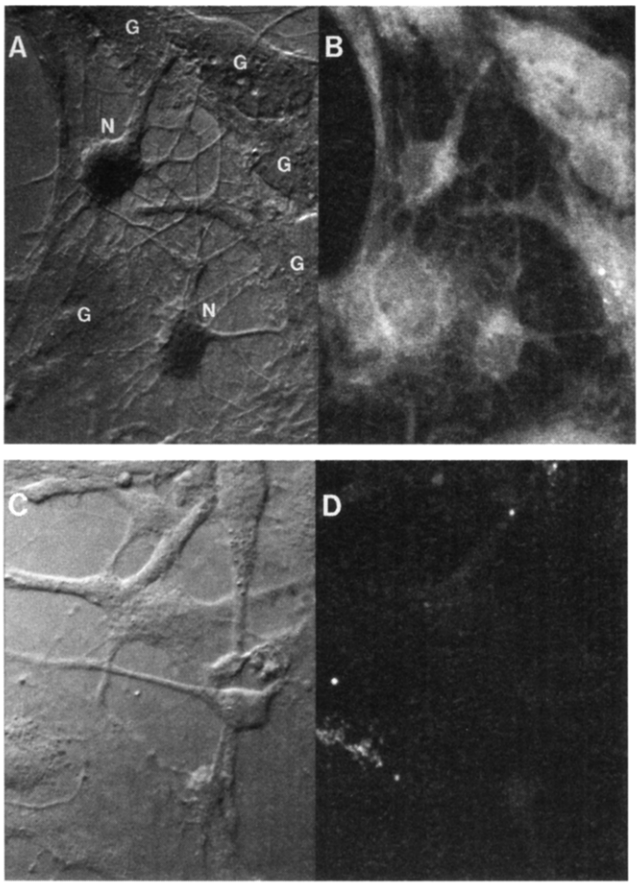


Figure 6. Fluorescence and DIC confocal images of glial and neuronal cell hippocampal cocultures. (A) DIC image of NDA-labeled glial and neuronal cells with the different cell types depicted. Key: N, neuronal cell; G, glial cell. (B) Confocal fluorescence image of the same field as in Figure 6A. (C) DIC image of unlabeled glial and neuronal cells. (D) Confocal fluorescence image of the same field as in Figure 6C.

cence intensity increased and the observed color from the emitted fluorescence changed from green to yellow (Figure 5B). We believe this increased fluorescence intensity and color change are caused mainly by the NDA-GSH (and NDA-γ-GC) chromophores that undergo a photochemical reaction to form highly photostabile derivatives. Prominent staining was observed in the nuclei after 5 min of continuous illumination, consistent with reports on GSH localization in that compartment.[14,15] Another study, however, showed that the MCB-GSH conjugate formed intracellularly, as well as other water-soluble fluorescent markers, diffused into the nucleus and accumulated there.[50] Further experiments are needed (1) to identify the chemical nature of the photoproducts formed from the NDA-GSH (and NDA-γ-GC) derivatives in biological environments upon excitation with 470−490-nm light and (2) to investigate whether or not the NDA-GSH derivative spontaneously relocalizes from the cytoplasmic compartment to the nucleus.

Confocal Fluorescence Microscopy of Glial and Neuronal Cell Cocultures. A DIC image of a NDA-labeled glial and neuronal cell hippocampal coculture is shown in Figure 6A. Figure 6B shows a confocal fluorescence image of the same field.

Fluorescence images were obtained in 12 experiments (three fluorescence images acquired from four different culture dishes) and showed similar staining patterns. In the neuronal cells, fluorescent staining was confined to a small portion of the cytoplasm. In contrast, glial cells were stained evenly throughout the entire cytoplasm. Cellular processes were also stained following incubation with NDA (Figure 6B), which is in agreement with immunocytochemical methods showing GSH localization in cellular processes.[15] Even if intensity analysis of the fluorescence was not performed, the image in Figure 6B suggests a greater localization of GSH in glial cells than in pyramidal neurons. These data are consistent with previous reports that have demonstrated a higher concentration of GSH in glial cells than in pyramidal neurons.[51,52] Control experiments with media lacking NDA showed some autofluorescence, but the staining pattern did not resemble that of NDA-loaded cells (Figure 6D). When glial and neuronal cell cocultures were incubated with BSO (0.5 mM) for 24 h (four dishes compared to four control dishes), several neuronal cells were unstained, but glial cells still showed some fluorescence (data not shown). Cultures containing only neuronal cells have been shown previously to be GSH deficient, whereas cultures with only glial cells contain high levels of GSH.[51] These observations have been explained by the difference that neurons require cysteine for GSH synthesis, whereas glial cells can utilize cystine.[51] Cysteine, however, does not persist in culture media because it is readily autoxidized to cystine; thus, under typical cell culture conditions, the precursor needs for GSH synthesis are satisfied for glial cells but not for neurons.

CONCLUSION

We show that NDA is a fluorogenic label that has high selectivity for GSH under physiological conditions. By using CE-LIF detection and fluorescence microscopy, both quantitative and qualitative analysis of intracellular GSH can be performed. This approach represents an advantage over immunohistochemical methods because of speed of analysis and ease of quantitation.

NDA should be more generally applicable for GSH labeling than MCB, which is dependent on catalysis by GSH S-transferases. NDA appears to be more selective for GSH than MBB, which reacts with several other thiols and is fluorescent itself. Further, NDA labeling of GSH represents several advantages over OPA labeling: (1) the chromophores have higher fluorescence quantum yields, (2) formalin treatment of the cells is not needed because no side reactions occur with histidine or histamine to result in interfering fluorescent products, (3) shorter times are required for loading the cells with dye, and (4) the chromophores are excited in the visible range (440−490 nm) and detected at ~530 nm, thereby allowing for the use of instrumentation set for fluorescein wavelengths. However, dye-loading conditions, side reactions with proteins, and the photochemistry of these chromophores in biological environments require further investigation before full utilization of the presented microscopy-based histochemical methods can be realized.

(50) Briviba, K.; Fraser, G.; Sies, H.; Ketterer, B. *Biochem. J.* **1993**, *294*, 631−633.
(51) Sagara, J.; Miura, K.; Bannai, S. *J. Neurochem.* **1993**, *61*, 1672−1676.
(52) Raps, S. P.; Lai, J. C. K.; Hertz, L.; Cooper, A. J. L. *Brain Res.* **1989**, *293*, 398−401.
(53) Fishman, H. A.; Amudi, N. M.; Lee, T. T.; Scheller, R. H.; Zare, R. N. *Anal. Chem.* **1994**, *66*, 2318.

ACKNOWLEDGMENT

O.O. is supported by the Swedish Natural Science Research Council (No. K-PD 10481-301). H.A.F. is a W. R. Grace fellow. The authors gratefully acknowledge help from Dr. M. Sandberg, Dr. N. Allbritton, Dr. J. Ames, Dr. S. J. Smith, and H. Dris. This work was supported by grants from the National Institute of Mental Health (MH45423-03 and MH45324-05) and Beckman Instruments, Inc.

Received for review May 23, 1995. Accepted August 30, 1995.[⊗]

AC950494W

[⊗] Abstract published in *Advance ACS Abstracts,* October 1, 1995.