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Nitric Oxide and Peroxynitrite. The Ugly, the Uglier and the Not So Good*

A Personal View of Recent Controversies

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Nitric oxide, a gaseous free radical, is poorly reactive with most biomolecules but highly reactive with other free radicals. Its ability to scavenge peroxyl and other damaging radicals may make it an important antioxidant *in vivo*, particularly in the cardiovascular system, although this ability has been somewhat eclipsed in the literature by a focus on the toxicity of peroxynitrite, generated by reaction of $O_2^{\bullet-}$ with $NO^{\bullet}$ (or of NO^- with O_2). On balance, experimental and theoretical data support the view that $ONOO^-$ can lead to hydroxyl radical ($OH^{\bullet}$) generation at pH 7.4, but it seems unlikely that $OH^{\bullet}$ contributes much to the cytotoxicity of $ONOO^-$. The cytotoxicity of $ONOO^-$ may have been over-emphasized: its formation and rapid reaction with antioxidants may provide a mechanism of using $NO^{\bullet}$ to dispose of excess $O_2^{\bullet-}$, or even of using $O_2^{\bullet-}$ to dispose of excess $NO^{\bullet}$, in order to maintain the correct balance between these radicals *in vivo*. Injection or instillation of "bolus" $ONOO^-$ into animals has produced tissue injury, however, although more experiments generating $ONOO^-$ at steady rates *in vivo* are required. The presence of 3-nitrotyrosine in tissues is still frequently taken as evidence of $ONOO^-$ generation *in vivo*, but abundant evidence now exists to support the view that it is a biomarker of several "reactive nitrogen species". Another under-addressed problem is the reliability of assays used to detect and measure 3-nitrotyrosine in tissues and body fluids: immunostaining results vary between laboratories and simple HPLC methods are susceptible to artefacts. Exposure of biological material to low pH (e.g. during acidic hydrolysis to liberate nitrotyrosine from proteins) or to H_2O_2 might cause artefactual generation of nitrotyrosine from NO_2^- in the samples. This may be the origin of some of the very large values for tissue nitrotyrosine levels quoted in the literature. Nitrous acid causes not only tyrosine nitration but also DNA base deamination at low pH: these events are relevant to the human stomach since saliva and many foods are rich in nitrite. Several plant phenolics inhibit nitration and deamination *in vitro*, an effect that could conceivably contribute to their protective effects against gastric cancer development.

Keywords: Nitric oxide, peroxynitrite, deamination, 3-nitrotyrosine, xanthine, hypoxanthine, reactive nitrogen species, gastric cancer, lipid peroxidation, superoxide, hydroxyl radical, nitrous acid, nitrite, catechins

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INTRODUCTION: THE BASIC CHEMISTRY OF NITRIC OXIDE

Nitric oxide (official chemical name *nitrogen monoxide*) is a colourless gas, moderately soluble in water (up to 2 mM at 20°C and about 1.6 mM at 37°C) and rather more soluble in organic solvents[1] (hence NO may, like O_2 tend to concentrate within membranes *in vivo*).[2] Since it has an unpaired electron, nitric oxide is by definition a free radical and so it is written as $NO^\bullet$ from now on in this article. If the electron is lost, nitrosonium cation results (a non-radical)

$$NO^\bullet \rightarrow e^- + NO^+$$

If an electron is gained, nitroxyl anion (another non-radical) is the product

$$NO^\bullet + e^- \rightarrow NO^-$$

and this species can react with oxygen to give peroxynitrite

$$NO^- + O_2 \rightarrow ONOO^-$$

For example, such reactions happen when $NO^\bullet$ interacts with ferrous cytochrome c.[3] On exposure to O_2, $NO^\bullet$ reacts to form the brown gas nitrogen dioxide $NO_2^\bullet$, also a free radical and much more reactive than is $NO^\bullet$. The overall reaction is

$$2NO^\bullet + O_2 \rightarrow 2NO_2^\bullet$$

although it proceeds as the sum of several stages. The final oxidation product of $NO^\bullet$ in aqueous solution is mostly nitrite,[4] according to the overall equation

$$4NO^\bullet + O_2 + 2H_2O \rightarrow 4H^+ + 4NO_2^-$$

Extensive reviews have been written on the basic chemistry of $NO^\bullet$, its biosynthesis and its multitude of biological roles,[1,5–8] so we will not dwell on these issues here. The focus of this review is on the research on $NO^\bullet$ and its derivatives that has taken place in our laboratories.

NITRIC OXIDE IS A GOOD FREE RADICAL SCAVENGER

Nitric oxide is fairly low down on the "reactivity-scale" of free radicals. Unlike such indiscriminately-reactive species as $OH^\bullet$, $NO^\bullet$ reacts slowly, if at all, with most biological molecules. However, most biological molecules are not free radicals. By contrast, $NO^\bullet$ is astoundingly reactive with other free radicals: some representative rate constants are listed in Table I.[9–13] Thus $NO^\bullet$ can scavenge $OH^\bullet$

$$NO^\bullet + OH^\bullet \rightarrow HNO_2 \quad k > 10^{10}\,M^{-1}\,s^{-1}$$

peroxyl radicals[12]

$$RO_2^\bullet + NO^\bullet \rightarrow ROONO \quad k > 10^9\,M^{-1}\,s^{-1}$$

and tyrosyl radicals, the latter probably by a reversible addition reaction.[14] The literature on the free radical reactions of $NO^\bullet$ has largely focussed on its fast reaction with superoxide to give peroxynitrite[11]

$$O_2^{\bullet-} + NO^\bullet \rightarrow ONOO^- \quad k > 10^9\,M^{-1}\,s^{-1}$$

TABLE I Some rate constants for reaction of nitric oxide with other free radicals[9–13]

Radical	Rate constant ($M^{-1}\,s^{-1}$)
Superoxide/$HO_2^\bullet$	$>10^9$
Peroxyl	$>10^9$
Tyrosyl	$>10^9$
Tryptophanyl	$>10^9$
Ethanol	$>10^9$
Hydroxyl ($OH^\bullet$)	$>10^{10}$
e^- (aq)	$>10^{10}$
$H^\bullet$	$>10^{10}$

but reactions of $NO^{\bullet}$ with other free radicals are equally fast (Table I). Another potentially-deleterious effect of $NO^{\bullet}$ is its ability to reversibly inhibit ribonucleotide reductase by reaction with a tyrosyl radical essential for catalytic function.[14] However, we would argue that the free radical reactivity of $NO^{\bullet}$ is, overall, beneficial *in vivo*. Thus the ability to scavenge $RO_2^{\bullet}$ radicals makes $NO^{\bullet}$ a powerful inhibitor of lipid peroxidation.[15,16] Evidence suggests that $NO^{\bullet}$ may have an anti-atherosclerotic effect,[5,6] perhaps by inhibiting lipid peroxidation. Nitric oxide also antagonizes the pro-oxidant properties of haem proteins in the presence of peroxides, quenching both haem ferryl species and amino acid radicals on the protein.[17,18]

NITRIC OXIDE, SUPEROXIDE AND PEROXYNITRITE

The bioactivity of $NO^{\bullet}$ (e.g. its EDRF activity) in many biological systems is enhanced by addition of SOD, suggesting that interactions of $O_2^{\bullet-}$ and $NO^{\bullet}$ may be a frequent occurrence. Indeed, it was suggested in 1989[19] that the vascular endothelium may generate $O_2^{\bullet-}$ as a means of regulating the bioactivity of $NO^{\bullet}$. There is now considerable evidence that vascular endothelial cells are *capable* of generating $O_2^{\bullet-}$ using NAD(P)H oxidases or xanthine oxidase, and that excess $O_2^{\bullet-}$ generation can contribute to the pathology of hypertension and atherosclerosis and to the development of nitrate tolerance.[20–26] However, evidence for release of $O_2^{\bullet-}$ under normal physiological conditions in the vascular endothelium is, to date, less convincing (reviewed in Ref. [27]) In any case, the seminal paper of Beckman *et al.*,[28] published in 1990, diverted attention away from the possibility of physiological $O_2^{\bullet-}/NO^{\bullet}$ antagonism to the likelihood of damage by the reaction product, $ONOO^-$. Addition of $ONOO^-$ to biomolecules, cells and tissues at pH 7.4 was soon shown to lead to nitrosylation, nitration and oxidation of biomolecules, cytotoxicity, cell death and tissue injury (reviewed in Ref. [1,29–32]). Some of the damage caused by $ONOO^-$ addition to biological systems was initially suggested to be due to formation of $OH^{\bullet}$, or a species resembling $OH^{\bullet}$

$$ONOO^- + H^+ \rightarrow NO_2^{\bullet} + OH^{\bullet}$$

and experiments involving aromatic hydroxylation, deoxyribose degradation, spin-trapping and other techniques gave some evidence consistent with generation of $OH^{\bullet}$ or a species closely resembling it.[28,33–37] However, other evidence did not support this view.[38–40] The data seemed equally matched, but unfortunately the negative studies tended to be given more weight because of a powerfully-presented thermodynamic argument that homolysis of ONOOH is a very unlikely reaction pathway.[41] However, equally-powerful arguments by expert chemists support the opposite view.[42,43] Not being convinced of the applicability of classical thermodynamics to living systems,[44] we opted to continue experimentation.[45] Detailed aromatic hydroxylation studies supported $OH^{\bullet}$ generation, although were not totally conclusive of its production.[45]. Our current view is that the balance of evidence does favour some $OH^{\bullet}$ generation from $ONOO^-$ at pH 7.4, although it is only a minor reaction product[36,45–47] and may be, at best, a small contributor to the damage caused when $ONOO^-$ is generated *in vivo*. Peroxynitrite generated in extracellular fluids may preferentially react with the HCO_3^-/CO_2 system (simply because of the high concentration of HCO_3^- present in such fluids *in vivo*),[47] whereas $ONOO^-$ produced in the intracellular environment might be predicted to react with GSH (present at millimolar levels) in addition.[32,48] Reactions with urate and ascorbate are also feasible in some cases.[48] The end-products[49–51] of such reactions include $NO^{\bullet}$. Is it possible, therefore, that $ONOO^-$ is not very toxic *in vivo*, and its high reactivity with a wide range of intracellular and extracellular antioxidants[1,32,48,52] means that it is quickly disposed of? Have we been wrong in focusing

on the cytotoxicity of $ONOO^-$? Reactions of $ONOO^-$/ONOOH with several biomolecules generate some $NO^\bullet$. Do we then have mechanisms (additional to SOD) for using $NO^\bullet$ to dispose of unwanted $O_2^{\bullet-}$ *in vivo*?

$$O_2^{\bullet-} + NO^\bullet \rightarrow ONOO^- \ (+\text{antioxidants}) \rightarrow \text{some } NO^\bullet \text{ regenerated}$$

This mechanism could also use $O_2^{\bullet-}$ to dispose of excess $NO^\bullet$ in situations where $NO^\bullet$ or its autoxidation products are causing damage.[53,54] Reaction of $ONOO^-$ with some antioxidants (e.g. urate[48]) can lead to formation of secondary damaging species but these may be in turn disposed of by reaction with other antioxidants, such as ascorbate.[55]

PEROXYNITRITE CYTOTOXICITY *IN VIVO*

It is well-established by studies with isolated biomolecules and whole cells that $ONOO^-$ can be cytotoxic at pH 7.4 and its addition can lead to cell death, often by apoptosis.[56–63] Of course, bolus addition of $ONOO^-$ may not be a good model for the events that occur when $O_2^{\bullet-}$ and $NO^\bullet$ are co-generated *in vivo*,[64–67] but in a few studies evidence has been presented that $ONOO^-$ is being generated *in vivo* to cause damage.[57,67]

It is important therefore to examine directly the effects of $ONOO^-$ *in vivo*. For example, it is pro-inflammatory when introduced into the colon[68] and induces airway hyper-responsiveness when instilled into guinea pig airways.[69] Ridger *et al.*[70] showed that intradermal or intraplantar injection of $ONOO^-$ into rats increased plasma extravasation and microvascular blood flow. The effects of intradermal injection were not decreased[70] by SOD, catalase, indomethacin or antagonists of histamine, 5-HT or tachykinin NK1. More experiments of this type are needed, especially (if possible) using "$ONOO^-$ donors" so that $ONOO^-$ is generated at a steady rate rather than being added as a (often highly-alkaline) bolus. The suitability of SIN-1 as a generator of $ONOO^-$ *in vivo* has recently been questioned.[71]

TYROSINE NITRATION IS NOT EVIDENCE FOR PEROXYNITRITE FORMATION *IN VIVO*

Despite some cautions in the literature,[65,72] the presence of 3-nitrotyrosine (3-NT) *in vivo* is still frequently taken as *prima facie* evidence of $ONOO^-$ generation. In some cases, the evidence is strengthened by showing that SOD (or SOD mimics) and NOS inhibitors can each block 3-NT formation, consistent with $O_2^{\bullet-}$ and $NO^\bullet$ being both involved in the formation of the tyrosine-nitrating species.[58,67] However, the cases where this has been shown are only a minor fraction of the published literature (Table II).

How else could tyrosine nitration occur?

(1) $NO^\bullet$ reacts fast with tyr-$O^\bullet$ radicals (Table I). The addition product(s) may under certain circumstances be converted to nitrotyrosine[73] by an oxidation process. How often this might happen *in vivo* remains to be determined.

(2) H_2O_2 plus NO_2^- can nitrate tyrosine at acidic pH, although $ONOO^-$ is probably involved here.[74] Indeed, addition of H_2O_2 to acidified NO_2^- is a standard laboratory method for preparation of $ONOO^-$

$$HNO_2 + H_2O_2 \rightarrow HOONO + H_2O$$
$$HOONO \underset{NaOH}{\rightarrow} ONOO^-$$

(3) Cigarette smoke can nitrate tyrosine,[75] although it is again possible that $ONOO^-$ could be involved.[76]

(4) Nitrogen dioxide, also a free radical ($NO_2^\bullet$), can nitrate tyrosine *in vitro*,[77] although high levels of $NO_2^\bullet$ are required: the levels present in polluted air seem insufficient.[72,78]

(5) Myeloperoxidase can oxidize tyrosine to tyrosyl radicals, and nitrite anion to a nitrating species, probably $NO_2^\bullet$ (although

TABLE II Nitrotyrosine in pathology: a literature summary

Disease	Journal/Author
CANCER	1. Ambs, S., Merriam, W.G., Bennett, W.P., FelleyBosco, E., Ogunfusika, M.O., Oser, S.M., Klein, S., Shields, P.G., Billiar, T.R. and Harris, C.C. Frequent nitric oxide synthase-2 expression in human colon adenomas: implication for tumor angiogenesis and colon cancer progression. *Cancer Research* **58**: 334–341, 1998. 2. Goto, T., Haruma, K., Yoshihara, M., Kitadai, Y., Ito, M., Mihara, M., Kamada, T., Tanaka, S., Sumii, K. and Kajiyama, G. Inducible nitric oxide synthase and nitrotyrosine in gastric mucosa of gastric cancer patients. *Gastroenterology* **114**: G2473, 1998. 3. Gal, A., Tamir, S., Kennedy, L.J., Tannenbaum, S.R. and Wogan, G.N. Nitrotyrosine formation, apoptosis, and oxidative damage: relationships to nitric oxide production in SJL mice bearing the RCsX tumor. *Cancer Research* **57**: 1823–1828, 1997.
LUNG DISEASES	1. Ischiropoulos, H., Almehdi, A.B. and Fisher, A.B. Reactive species in ischemic rat lung injury – contribution of peroxynitrite. *American Journal of Physiology – Lung Cellular and Molecular Physiology* **13**: L158–L164, 1995. 2. Kooy, N.W., Royall, J.A., Ye, Z.Z., Kelly, D.R. and Beckman, J.S. Evidence for *in vivo* peroxynitrite production in human acute lung injury. *American Journal of Respiratory and Critical Care Medicine* **151**: 1250–1254, 1995. 3. Haddad, I.Y., Pataki, C., G. Hu, P., Galliani, C., Beckman, J.S. and Matalon, S. Quantitation of nitrotyrosine levels in lung sections of patients and animals with acute lung injury. *Journal of Clinical Investigation* **94**: 2407–2413, 1994. 4. Wizemann, T.M., Gardner, C.R., Laskin, J.D., Quinones, S., Durham, S. K., Goller, N.L., Ohnishi, S.T. and Laskin, D.L. Production of nitric-oxide and peroxynitrite in the lung during acute endotoxemia. *Journal of Leukocyte Biology* **56**: 759–768, 1994. 5. Sadeghi-Hashjin, G., Folkerts, G., Henricks, P.A.J., Muijsers, R.B.R. and Nijkamp, F.P. Peroxynitrite in airway diseases. *Clinical and Experimental Allergy* **28**: 1464–1473, 1998.
Bronchopulmonary dysplasia	1. Banks, B.A., Ischiropoulos, H., McClelland, M., Ballard, P.L. and Ballard, R.A. Plasma 3-nitrotyrosine is elevated in premature infants who develop bronchopulmonary dysplasia. *Pediatrics* **101**: 870–874, 1998. 2. Banks, B.A., McClelland, M.M., Ischiropoulos, H., Seri, I., Planer, B.C., Ballard, R.A. and Ballard, P.L. Plasma nitrotyrosine as an indicator of nitric-oxide derived oxidant stress in infants with bronchopulmonary dysplasia. *Pediatric Research* **39**: 1935, 1996.
Respiratory failure	1. Hallman, M., Bry, K., Turbow, R., Waffarn, F. and Lappalainen, U. Pulmonary toxicity associated with nitric oxide in term infants with severe respiratory failure. *Journal of Pediatrics* **132**: 827–829, 1998.
Obliterative bronchiolitis	1. Mason, N.A., Springall, D.R., Pomerance, A., Evans, T.J., Yacoub, M.H. and Polak, J.M. Expression of inducible nitric oxide synthase and formation of peroxynitrite in posttransplant obliterative bronchiolitis. *Journal of Heart and Lung Transplantation* **17**: 710–714, 1998. 2. McDermott, C.D., Gavita, S.M., Shennib, H. and Giaid, A. Immunohistochemical localization of nitric oxide synthase and the oxidant peroxynitrite in lung transplant recipients with obliterative bronchiolitis. *Transplantation* **64**: 270–274, 1997.
Emphysema	1. Meng, Q.H., Bishop A.E., RodriguezChacon, M. and Polak, J.M. High levels of inducible nitric oxide synthase (iNOS) and nitrotyrosine in alveolar type II cells in emphysema. *Journal of Pathology* **184**: A42, 1998.
Asbestos inhalation	1. Tanaka, S., Choe, N., Hemenway, D.R., Zhu, S., Matalon, S. and Kagan, E. Asbestos inhalation induces reactive nitrogen species and nitrotyrosine formation in the lungs and pleura of the rat. *Journal of Clinical Investigation* **102**: 445–454, 1998. 2. Choe, N., Tanaka, S. and Kagan, E. Asbestos fibres and interleukin-1 upregulate the formation of reactive nitrogen species in rat pleural mesothelial cells. *American Journal of Respiratory and Cell Molecular Biology* **19**: 226–236 (1998).
Pulmonary fibrosis	1. Saleh, D., Barnes, P.J. and Giaid, A. Increased production of the potent oxidant peroxynitrite in the lungs of patients with idiopathic pulmonary fibrosis. *American Journal of Respiratory and Critical Care Medicine* **155**: 1763–1769, 1997.

TABLE II (*Continued*)

Disease	Journal/Author
Pulmonary granulomatous inflammation	1. Setoguchi, K., Takeya, M., Akaike, T., Suga, M., Hattori, R., Maeda, H., Ando, M. and Takahashi, K. Expression of inducible nitric-oxide synthase and its involvement in pulmonary granulomatous inflammation in rats. *American Journal of Pathology* **149**: 2005–2022, 1996.
Adult respiratory distress syndrome	1. Haddad, I.Y., Hu, P., Ye, Y., Galliani, C.A., Beckman, J.S. and Matalon, S. Detection of nitrotyrosine in patients with the adult respiratory distress syndrome (ARDS). *FASEB Journal* **8**: A896, 1994 (also see papers listed at the beginning of this section).
Nasal allergy	1. Sato, M., Fukuyama, N., Sakai, M. and Nakazawa, H. Increased nitric oxide in nasal lavage fluid and nitrotyrosine formation in nasal mucosa – indices for severe perennial nasal allergy. *Clinical and Experimental Allergy* **28**: 597–605, 1998.
Cigarette smoking	1. Petruzzelli, S., Puntoni, R., Mimotti, P., Pulera, N., Baliva, F., Fornai, E. and Giuntini, C. Plasma 3-nitrotyrosine in cigarette smokers. *American Journal of Respiratory and Critical Care Medicine* **156**: 1902–1907, 1997. 2. Chambers, D.C., Tunnicliffe, W.S. and Ayres J.G. Acute inhalation of cigarette smoke increases lower respiratory tract nitric oxide concentrations. *Thorax* **53**: 677–679, 1998.
Influenza infection/ Lung infection	1. Akaike, T., Noguchi, Y., Ijiri, S., Setoguchi, K., Suga, M., Zheng, Y.M., Dietzschold, B. and Maeda, H. Pathogenesis of influenza virus-induced pneumonia – involvement of both nitric-oxide and oxygen radicals. *Proceedings of the National Academy of Sciences of the USA* **93**: 2448–2453, 1996. 2. Yamazaki, C., Hoshimo, J., Sekiguchi, T., Hori, Y., Mizuno, S. and Horie, K. Production of superoxide and nitric oxide by alveolar macrophages in the bleomycin-induced interstitial pneumonia mice model. *Japanese Journal of Pharmacology* **78**: 69–73, 1998.
Asthma	1. Saleh, D., Ernst, P., Lim, S., Barnes, P.J. and Giaid, A. Increased formation of the potent oxidant peroxynitrite in the airways of asthmatic patients is associated with induction of nitric oxide synthase: effect of inhaled glucocorticoid. *FASEB Journal* **12**: 929–937, 1998.
NEURODEGENERATIVE DISEASES	
ALS	1. Bruijn, L.I., Beal, M.F., Becher, M.W., Schulz, J.B., Wong, P.C., Price, D.L and Cleveland, D.W. Elevated free nitrotyrosine levels, but not protein-bound nitrotyrosine or hydroxyl radicals, throughout amyotrophic lateral sclerosis (ALS)-like disease implicate tyrosine nitration as an aberrant *in vivo* property of one familial ALS-linked superoxide dismutase 1 mutant. *Proceedings of the National Academy of Sciences of the United States of America* **95**: 7606–7611, 1997. 2. Strong, M.J., Sopper, M.M., Crow, J.P., Strong, W.L. and Beckman, J.S. Nitration of the low molecular weight neurofilament is equivalent in sporadic amyotrophic lateral sclerosis and control cervical spinal cord. *Biochemical and Biophysical Research Communications* **248**: 157–164, 1998. 3. Abe, K., Pan, L.H., Watanabe, M., Konno, H., Kato, T. and Itoyama, Y. Upregulation of protein-tyrosine nitration in the anterior horn cells of amyotrophic lateral sclerosis. *Neurological Research* **19**: 124–128, 1997. 4. Beal, M.F., Ferrante, R.J., Browne, S.E., Matthews, R.T. Kowall, N.W. and Brown, R.H. Increased 3-nitrotyrosine in both sporadic and familial amyotrophic lateral sclerosis. *Annals of Neurology* **42**: 644–654, 1997. 5. Delanty, N., Przedborski, S., Bandele, A.N., Lynch, T. and Trifiletti, R.R. Elevated protein nitrotyrosine immunoreactivity in patients with amyotrophic lateral sclerosis. *Annals of Neurology* **42**: T199, 1997. 6. Abe, K., Pan, L.H., Watanabe, M., Kato, T. and Itoyama, Y. Induction of nitrotyrosine-like immunoreactivity in the lower motor neuron of amyotrophic-lateral-sclerosis. *Neuroscience Letters* **199**: 152–154, 1995. 7. Chou, S.M., Wang, H.S. and Komai, K. Immunoreactivities of nitric-oxide synthase and nitrotyrosine in neurofilamentous spheroids and conglomerates of amyotrophic lateral sclerosis. *Annals of Neurology* **38**: 293– 294, 1995.

Stroke	1. Forster, C., Clark, H.B., Ross, M.E. and Iadecola, C. Inducible nitric oxide synthase expression in human cerebral infarcts. *Acta Neuropathologica* **97**: 215–220, 1999 (also see ischaemia-reperfusion).
Parkinson's disease	1. Good, P.F., Hsu, A., Werner, P., Perl, D.P. and Olanow, C.W. Protein nitration in Parkinson's disease. *Journal of Neuropathology and Experimental Neurology* **57**: 338–342, 1998. 2. Przedborski, S., Bandele, A.N., Jackson Lewis, V., Kostic, V. and Trifiletti, R.R. Nitrotyrosine and Parkinson's disease (PD). *Neurology* **48**: 22 001, 1997. 3. Ferrante, R.J., Hantraye, P., Brouillet, E. and Beal, M.F. Increased nitrotyrosine immunoreactivity in substantia nigra neurones in MPTP-treated baboons is blocked by inhibition of neuronal nitric oxide synthase. *Brain Research* **823**: 177–182, 1999.
AIDS dementia	1. Boven, L.A., Gomes, L., Hery, C., Gray, F., Verhoef, J., Portegies, P., Tardieu, M. and Nottet, H.S.L.M. Increased peroxynitrite activity in AIDS dementia complex: implications for the neuropathogenesis of HIV-1 infection. *Journal of Immunology* **162**: 4319–4327, 1999.
Multiple sclerosis	1. Cross, A.H., Manning, P.T., Keeling, R.M., Schmidt, R.E. and Misko, T.P. Peroxynitrite formation within the central nervous system in active multiple sclerosis. *Journal of Neuroimmunology* **88**: 45–56, 1998. 2. Oleszak, E.L., Zaczynska, E., Bhattacharjee, M., Butunoi, C., Legido, A. and Katsetos, C.D. Inducible nitric oxide synthase and nitrotyrosine are found in monocytes/macrophages and/or astrocytes in acute, but not in chronic, multiple sclerosis. *Clinical and Diagnostic Laboratory Immunology* **5**: 438–445, 1998. 3. Zabaleta, M.E., Bianco, N.E. and DeSanctis, J. Serum nitrotyrosine levels in patients with multiple sclerosis: relationship with clinical activity. *Medical Science Research* **26**: 407–408, 1998. 4. Bagasra, O., Michaels, F.H., Zheng, Y.M., Bobroski, L.E., Spitsin, S.V., Fu, Z.F., Tawadros, R. and Koprowski, H. Activation of the inducible form of nitric oxide synthase in the brains of patients with multiple sclerosis. *Proceedings of the National Academy of Sciences USA* **92**: 12 041–12 045, 1995. 5. Giovanni, G., Miller, R.F., Heales, S.J.R., Land, J.M., Harrison, M.J.G. and Thompson, E.J. Cerebrospinal fluid and serum nitric oxide metabolites in patients with multiple sclerosis. *Multiple Sclerosis* **4**: 27–30, 1998.
Alzheimer's disease	1. Paris, D., Parker, T.A., Town, T., Suo, Z.M., Fang, C.H., Humphrey, J., Crawford, F. and Mullan, M. Role of peroxynitrite in the vasoactive and cytotoxic effects of Alzheimer's beta-amyloid (1–40) peptide. *Experimental Neurology* **152**: 116–122, 1998. 2. Smith, M.A., Harris, P.L.R., Sayre, L.M., Beckman, J.S. and Perry, G. Widespread peroxynitrite-mediated damage in Alzheimer's disease. *Journal of Neuroscience* **17**: 2653–2657, 1997. 3. Su, J.H., Deng, G.M. and Cotman, C.W. Neuronal DNA damage precedes tangle formation and is associated with upregulation of nitrotyrosine in Alzheimer's disease brain. *Brain Research* **774**: 193–199, 1997. 4. Good, P.F., Werner, P., Hsu, A., Olanow, C.W. and Perl, D.P. Evidence for neuronal oxidative damage in Alzheimer's disease. *American Journal of Pathology* **149**: 21–28, 1996. 5. Hensley, K., Maidt, M.L., Yu, Z.Q., Sang, H., Markesbery, W.Y. and Floyd, R.A. Electrochemical analysis of protein nitrotyrosine and dityrosine in the Alzheimer brain indicates region-specific accumulation. *Journal of Neuroscience* **18**: 8126–8132, 1998.
Huntington's disease	1. Galpern, W.R., Matthews, R.T., Beal, M.F. and Isacson, O. NGF attenuates 3-nitrotyrosine formation in a 3-NP model of Huntington's disease. *Neuroreport* **7**: 2639–2642, 1996.
Progressive supranuclear palsy	1. Komori, T., Shibata, N., Kobayashi, M., Sasaki, S. and Iwata, M. Inducible nitric oxide synthase-like immunoreactivity in argyrophilic, tau-positive astrocytes in progressive supranuclear palsy. *Acta Neuropathologica* **95**: 338–344, 1998.
CNS demyelination	1. Cross, A.H., Manning, P.T., Stern, M.K. and Misko, T.P. Evidence for the production of peroxynitrite in inflammatory CNS demyelination. *Journal of Neuroimmunology* **80**: 121–130, 1997 (also see section on multiple sclerosis).

TABLE II (*Continued*)

Disease	Journal/Author
Encephalomyelitis	1. Okuda, Y., Sakoda, S., Fujimura, H. and Yanagihara, T. Nitric oxide via an inducible isoform of nitric oxide synthase is a possible factor to eliminate inflammatory cells from the central nervous system of mice with experimental allergic encephalomyelitis. *Journal of Neuroimmunology* **73**: 107–116, 1997 (also see section on multiple sclecosis). 2. Cross, A.H., Misko, T.P. and Manning, P.T. Detection of nitrotyrosine immunoreactivity in experimental autoimmune encephalomyelitis-affected CNS tissues – evidence for the presence of peroxynitrite in inflammatory CNS demyelination. *Annals of Neurology* **40**: M116, 1996.
CNS inflammation	1. van der Veen, R.C., Hinton, D.R., Incardonna, F. and Hofman, F.M. Extensive peroxynitrite activity during progressive stages of central nervous system inflammation. *Journal of Neuroimmunology* **77**: 1–7, 1997.
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NO_2^+ is conceivable if a two-electron oxidation takes place).[79] The tyrosyl radical and $NO_2^\bullet$ can then combine locally to give nitrotyrosine. Breakdown of $NO^\bullet$ in aqueous solution gives NO_2^- as the major end-product and micromolar levels of NO_2^- have been reported in human body fluids (reviewed in Ref. [72]) Since NO_2^- is a preferred substrate for the enzyme, these reactions are feasible *in vivo*.[80,81]

(6) Nitrite and hypochlorous acid (HOCl) can interact to form a product that can nitrate tyrosine and other aromatic compounds[80,82,83] and oxidize lipids in low-density lipoproteins.[84] The product is probably[83] nitryl chloride, NO_2Cl. Hypochlorous acid is readily scavenged by such antioxidants as GSH[85] and ascorbate[86] which will compete *in vivo* with NO_2^- for any HOCl generated.[81] However, these agents are also good inhibitors of $ONOO^-$-dependent tyrosine nitration,[48,87] i.e. peroxynitrous acid and HOCl have comparable degrees of "indiscriminate reactivity". Hence phagocytes may promote tyrosine nitration *in vivo* by multiple mechanisms in which myeloperoxidase plays an especially important role, both directly and as a source of HOCl. To make the situation even more complex, HOCl can destroy nitrotyrosine, both free and in proteins.[123]

NITROUS ACID AND TYROSINE NITRATION

Another mechanism of tyrosine nitration is HNO_2 dependent: exposure of tyrosine to nitrite at low pH causes nitrotyrosine formation.[29,77,88,89] High concentrations of NO_2^- are found in saliva ($> 50\,\mu M$) and in many foods,[90,91] so the generation of HNO_2 in the human stomach is a likely event. It might occur also to some extent in ischaemic tissues.[124] The gastric production of HNO_2 and oxides of nitrogen may have antibacterial actions.[90,91] HNO_2, or species derived from it, can also deaminate DNA bases, converting guanine to xanthine and adenine to hypoxanthine.[89,92] No oxidized DNA bases are produced (Figure 1). Several phenolic compounds are inhibitors of tyrosine nitration and DNA base deamination and their presence in foods of plant origin may serve to modulate any potentially-deleterious consequences of excess generation of HNO_2 in the stomach.[89,92] Several plant phenolics can also inhibit $ONOO^-$-dependent tyrosine nitration *in vivo*.[93]

3-NITROTYROSINE: A BIOMARKER OR A TOXIN?

3-Nitrotyrosine formation seems to be a biomarker of the attack of "reactive nitrogen species" on tyrosine, either free or within proteins, rather than a specific marker of $ONOO^-$-dependent damage.[72] Is it only a biomarker, or does it have metabolic effects in its own right? Nitration of tyrosine should affect signal transduction pathways, but this has not been demonstrated *in vivo* (a difficult task). Like many other nitro-compounds, free nitrotyrosine can be reduced and undergo redox cycling to produce $O_2^{\bullet -}$, although much higher levels than those measured *in vivo* are needed for substantial $O_2^{\bullet -}$ generation and so the physiological significance is as yet unclear.[94] Major urinary metabolites produced from nitrotyrosine administered to rats were identified as 3-nitro-4-hydroxyphenylacetic acid (44%) and 3-nitro-4-hydroxyphenyllactic acid (5%): the former metabolite has been detected in human urine.[95] Some eukaryotic cell lines can incorporate nitrotyrosine into tubulin, causing cytoskeletal distortion and eventual apoptosis.[96] Proteins containing nitrotyrosine may turn over at an accelerated rate *in vivo*,[95,97] and/or there may be mechanisms for "de-nitrating" nitrotyrosine within proteins without degrading them.[98] These pathways could conceivably explain why elevated levels of free, but not protein-bound,

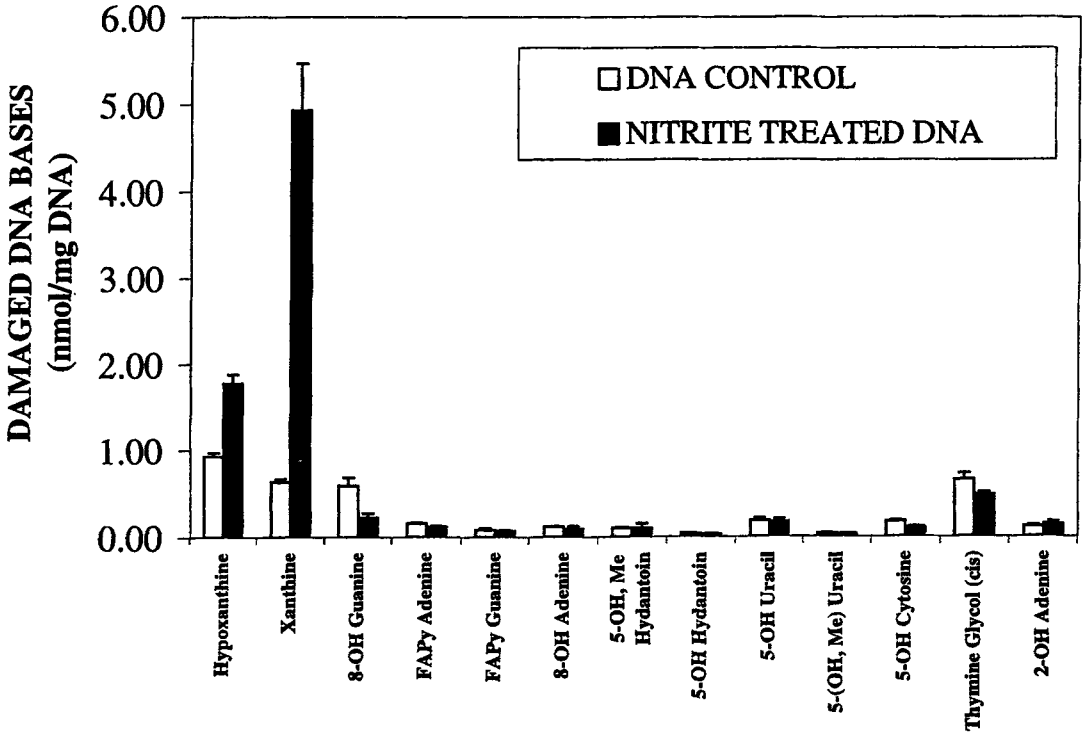

FIGURE 1 Levels of oxidized and deaminated DNA bases in control (untreated), and nitrite-treated calf thymus DNA at pH 3.0 as revealed by GC/MS. DNA (0.5 mg/ml) was incubated at 37°C for 15 min and NO_2^- added, the samples mixed and incubated for a further 60 min. After this time the samples were dialysed against water for 25 h, freeze-dried and hydrolysed with 60% (v/v) formic acid for 45 min at 150°C. Oxidized and deaminated DNA base products were then analysed by GC/MS. Results are expressed as mean ± s.d. of 3 or more separate determinations. Nitrite does not affect DNA when incubated with it at pH 7.4.

nitrotyrosine have been detected in patients with amyotrophic lateral sclerosis,[99] and why approximately 0.5 μM levels of free nitrotyrosine were detected in plasma and synovial fluid from some patients with rheumatoid arthritis.[100].

HOW TO ASSAY NITROTYROSINE: A CONTROVERSIAL AREA

Nitrotyrosine is most commonly identified in tissues by immunostaining[30] although variable results are obtained in different laboratories.[101–103] For example, we have been unable to detect 3-nitrotyrosine in human atherosclerotic tissue by immunostaining or HPLC analysis,[104] an observation in agreement with the studies of Dean *et al.*[105] However, several other groups have detected it in similar material by immunostaining.[106–110] It is rare to see immunostaining reports confirmed by direct chemical analysis for nitrotyrosine: in at least one case the two do not match.[111] Hypochlorous acid can destroy nitrotyrosine: hence the extent of formation of HOCl, which has been reported in some lesions on the basis of measurements of 3-chlorotyrosine, could be an important variable.[123]

HPLC procedures for analysis of nitrotyrosine (both free, and released from proteins after acidic hydrolysis) have been developed in many laboratories.[30,100,112–118] However, artefactual peaks that are dithionite-reducible (a criterion often used to identify nitrotyrosine, which is reduced to aminotyrosine by dithionite) and co-elute with 3-nitrotyrosine have been described in human

brain tissue.[115] Simple absorbance detection of nitrotyrosine, or electrochemical detection at a single voltage, is not in our view a reliable method to assay nitrotyrosine:[115] at the very least, diode array[115] or examination of electrochemical behaviour at several voltages (e.g. using coulometric array detectors) should be carried out.[113,117,118] An alternative approach, which promises more rigorous identification, is the use of mass spectrometric techniques.[119–121] An ELISA method to measure nitrated proteins in human body fluids has been described.[122]

THE NITRATION ARTEFACT

Analytical techniques to measure nitrotyrosine are frequently applied to biological material after acidic hydrolysis to liberate nitrotyrosine from proteins. However, there is considerable potential for artefact if NO_2^- is present. Traces of NO_2^- are present in many tissues and body fluids, and levels tend to be higher in disease states, since $NO^\bullet$ production is increased. Exposure of NO_2^- to acid can cause artefactual nitration of tyrosine via HNO_2 generation (see above). It is possible that this could have happened in the studies reported in Ref. [110], an apparent chemical validation of the presence of nitrotyrosine in human atherosclerotic lesions. An MS-based assay for nitrotyrosine that avoids acidic derivatization conditions has been described.[119] Similarly, the exposure of tissue sections to acidic fixatives, or to H_2O_2, could conceivably generate artefacts in immunohistochemical studies if NO_2^- is present. For example, tissue myeloperoxidase could use H_2O_2 to oxidize NO_2^- as described above,[79] and acid will generate HNO_2 from NO_2^-.

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