Methods for detection of reactive metabolites of oxygen and nitrogen: in vitro and in vivo considerations

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Tarpey, Margaret M., David A. Wink, and Matthew B. Grisham. Methods for detection of reactive metabolites of oxygen and nitrogen: in vitro and in vivo considerations. Am J Physiol Regul Integr Comp Physiol 286: R431–R444, 2004; 10.1152/ajpregu.00361.2003.—Facile detection of reactive oxygen and nitrogen species in biologic systems is often problematic. This is a result of the numerous cellular mechanisms, both enzymatic and nonenzymatic involved in their catabolism/decomposition, the complex and overlapping nature of their reactivities, as well as the often limited intracellular access of detector systems. This review describes approaches to the direct and indirect measurement of different reactive metabolites of oxygen and nitrogen. Particular attention to a method’s applicability for in vivo determinations will be addressed.

THE DISCOVERY by McCord and Fridovich (83, 84) that the erythrocyte-associated enzyme superoxide dismutase (SOD) decomposes the free radical superoxide launched a frenzy of research activity that remains unabated over the past 35 years. Indeed, the discovery of SOD defined an entirely new area of biomedical research that represents one of the fundamental advances in biology over the past 50 years. This discovery suggested that reactive free radicals derived from oxygen could be produced in vivo in significant quantities. Until this landmark observation, chemists and biochemists believed that free radicals of any type were much too reactive and therefore much too toxic to be produced in vivo. With investigators more receptive to the idea that small molecular weight reactive radical species play important roles in normal physiology as well as pathophysiology, it was not surprising when Ignarro, Furchgott, and Murad identified the nitrogen-based free radical nitric oxide (NO) as the elusive endothelium-derived relaxing factor (EDRF). In fact, NO has been shown to be important in pathophysiology as well as specific reactive oxygen and nitrogen species using methods and instrumentation commonly found in most research laboratories. Particular attention will be given to those approaches that use methods for the in vivo determinations of these reactive species.

However, measurement of the in situ production of reactive species is difficult for several reasons. For example, in many tissues, low intracellular steady-state concentrations of superoxide occur as a result of the balance between the basal rates of partial reduction of oxygen to superoxide and the diffusion-limited scavenging of superoxide by both cytoplasmic and mitochondrial SODs, resulting in intracellular superoxide concentrations estimated to rarely exceed 1 nM. (13) Extracellular release of small proportions of intracellularly formed superoxide may occur via diffusion through anion channels (78, 107). In addition, superoxide formed from plasma membrane-bound oxidases remains at relatively low levels due to serum and extracellular fluid components, including low molecular weight oxidant scavengers and the heparin-binding extracellular-SOD (1, 46, 81, 105, 119). Thus the relatively short half-life (seconds) of reactive species and the efficient and redundant systems that have evolved to scavenge them require that any detection technique must be sensitive enough to effectively compete with these intra- and extracellular antioxidant components for reaction with the substance in question. Additionally, methods for analysis of reactive species must have adequate intracellular access to faithfully reflect intracellular conditions. Finally, the often overlapping reactivities of reactive species with detection systems may hamper unequivocal identification and quantification of the responsible substance.

This review will focus on common approaches to detect oxidative stress as well as specific reactive oxygen and nitrogen species using methods and instrumentation commonly found in most research laboratories. Particular attention will be given to those approaches that use methods for the in vivo determinations of these reactive species.

METHODS TO DETECT OXIDATIVE STRESS

Reduction and Oxidation (Redox) Potential of the GSH/GSSG Couple: Indicator of Oxidative Stress in Vivo

Detoxification of reactive oxygen species is one of the prerequisites for all aerobic life forms, and multiple levels of
enzymatic and nonenzymatic defenses have evolved to form what has been termed the oxidant defense network. An imbalance between these defense mechanisms and pro-oxidative forces in favor of the latter creates what has been termed “oxidative stress.” Indeed, reduced glutathione (GSH) is recognized as one of the most important nonenzymatic oxidant defenses within the body. It exists in very large amounts (mM levels) within cells where it acts to detoxify peroxides as well as maintain other physiologically important antioxidants (e.g., α-tocopherol, ascorbic acid) in their reduced forms. Reduced glutathione is continuously regenerated from its oxidized form GSSG by the action of an NADPH-dependent reductase. Because the rates of synthesis of GSH, export of GSH and GSSG from the intracellular compartment to the extracellular space, and formation of protein-bound GSH mixed disulfides are slow relative to the rates of reduction of GSSG and oxidation of GSH, “the balance of GSH and GSSG provides a dynamic indicator of oxidative stress” in vivo (60). Indeed, alterations in tissue redox environment may reflect alterations in cell growth, differentiation, and apoptosis. Quantification of redox state of the GSH/GSSG pool in tissue and/or plasma that takes into account the correct stoichiometry of 2 GSH oxidized per GSSG formed may be determined by the redox potential (Eh), which is calculated according to the Nernst equation: Eo(mV) = Eo + 30 log([GSSG]/[GSH]2), where GSH and GSSG are molar concentrations and Eo is −264 mV for pH 7.4 (60). GSH and GSSG may be determined biochemically or by HPLC according to the method described by Jones (60). To avoid artifactual overestimation or underestimation of plasma GSH and GSSG, one must avoid hemolysis, limit oxidation, and inhibit the GSH degrading enzyme α-glutamyltranspeptidase. Because oxidation of thiols may occur rapidly on tissue disruption, it is essential that tissue be rapidly excised and freeze clamped or placed in liquid nitrogen prior to disruption (60).

Measurement of F2-Isoprostanes as Indicators of Lipid Peroxidation

Lipid peroxidation has been and remains one of the most widely used indicators of oxidant/free radical formation in vitro and in vivo. Unfortunately, many of the methods used to detect lipid peroxidation in urine, blood plasma, or tissue are nonspecific, relying on the detection of thiobarbituric acid (TBA)-reactive substances such as malondialdehyde (MDA) or other reactive aldehydes generated from the in vivo or ex vivo decomposition of lipid peroxidation products (102). In addition, it is well known that a variety of different bio-organic substances (e.g., bile acids, carbohydrates, nucleic acids, certain antibiotics, and amino acids) react with TBA to varying degrees, rendering this method sensitive but nonspecific (47). In 1990, Roberts and coworkers (88) discovered that nonenzymatic, free radical-induced lipid peroxidation produced F2-like prostaglandins of arachidonic acid. Because these oxidized lipids are isomers of cyclooxygenase-generated prostaglandin F2α, they were termed F2-isoprostanes (IsoP). It had been known for many years that potent oxidants such as hydroxyl radical, peroxyl radicals, nitrogen dioxide, peroxynitrite, and higher oxidation states of heme and hemoproteins (ferryl heme) are capable of initiating peroxidation of polyunsaturated fatty acids (43). Roberts and Morrow (106) found that these types of oxidizing agents induced IsoP formation in vitro and in vivo as depicted in Fig. 1. Subsequent studies by this same group demonstrated that IsoPs are formed initially as esterified fatty acids attached to phospholipids and then are released to their free form by the action of phospholipases (106). There are several reasons why measurement of IsoPs makes this approach one of the most reliable attractive markers for assessing oxidative stress in vivo. First, these compounds are stable and not produced by any known enzymatic pathways using arachidonate such as the cyclooxygenase or lipoxygenase pathways. In addition, IsoP levels can be quantified in extracellular fluids such as plasma and urine making this relatively noninvasive approach particularly useful for human and whole animal studies.

Another important consideration of IsoP detection is that these oxidized lipids increase in vivo in response to known free radical generators and oxidative stresses such as diquat or CCl4 administration (106). Furthermore, their enhanced production may be modulated by supplementation with different antioxidants including vitamin E and/or selenium (106). Finally, it has been confirmed that IsoP levels are not altered by lipid content in the diet. The major drawbacks of this approach relate to the detection systems required to quantify these oxidized lipids. Extensive analytic work has established that the most sensitive, reliable, and quantitative method for the detection of IsoPs is by mass spectrometry making the detection of IsoPs an expensive and time-consuming endeavor (106). Several commercially available ELISA kits have been developed for quantifying different IsoPs; however, there exists considerable interference with substances found in biological fluids and tissue extracts. It is highly recommended that samples be partially purified before analysis can be performed.

The above mentioned methods are used for the determination of generalized oxidative stress in vivo and in vitro. If one wishes to measure specific reactive oxygen species, there are additional methods, with varying degrees of applicability to the in vivo situation, available as described below.

SUPEROXIDE DETECTION

Cytochrome c Reduction

The reduction of ferricytochrome c to ferrocytochrome c has been used to measure rates of formation of superoxide by numerous enzymes, whole cells, and vascular tissue (3, 71, 82). At pH 8.5 and room temperature, the rate constant has been estimated at ∼1.5 × 105 M/s (4, 4, 70)

$$Fe^{3+} {\text{cyt c}} + O_2^{-} \rightarrow Fe^{2+} {\text{cyt c}} + O_2$$

The spectrophotometric reaction is followed at 550 nm; the extinction coefficient for ferricytochrome c is 0.89 × 10^4 M/cm, whereas that for ferrocytochrome c is 2.99 × 10^4 M/cm. Therefore, E_m 550nm = 2.1 × 10^4 M/cm (82).

There are several precautions when using this reaction to detect superoxide. Reduction of cytochrome c is not absolutely specific for superoxide. Cellular reductants such as ascorbate and glutathione are capable of cytochrome c reduction and may be present in high concentration within tissue extracts, as are cellular reductases that enzymatically mediate cytochrome c reduction. Additionally, enzymes such as xanthine oxidase are capable of reducing quinones or redox-active dyes that may
also be present and whose reduced forms are capable of directly reducing cytochrome c. It is important therefore to demonstrate specificity for superoxide of this reaction by the extent of inhibition of cytochrome c reduction by exogenous SOD (34). Reduced cytochrome c can be reoxidized by cytochrome oxidases, cellular peroxidases, and oxidants, including hydrogen peroxide (H$_2$O$_2$) and peroxynitrite (ONOO$^-$) (134). Because the apparent rate of cytochrome c reduction is decreased, these reoxidation reactions will underestimate the rate of superoxide formation. Enzyme inhibitors (10 μM CN for cytochrome oxidase) or scavengers of reactive species (100 U/ml catalase for H$_2$O$_2$, 10 mM urate for ONOO$^-$) can be added to the reaction mixture to avoid this drawback.

To enhance the specificity of the cytochrome c assay for superoxide, cytochrome c can be acetylated (93). Acetylation of lysine residues present within ferri-cytochrome c decreases direct electron transfer by mitochondrial and microsomal reductases to cytochrome c and from cytochrome c by cytochrome oxidase, while maintaining superoxide’s ability of to reduce cytochrome c (3). Succinoylation of cytochrome c is more effective in decreasing the reduction of cytochrome c by NADPH-cytochrome P-450 reductase or cytochrome b$_5$ and oxidation by cytochrome c oxidase. However, succinoylation of cytochrome c decreases the rate constant of the reaction with superoxide by ~90% compared with native cytochrome c, thus requiring the use of higher concentrations of the succinoylated cytochrome c (69).

Because of size and charge considerations, cytochrome c has restricted intracellular access. This can reduce the value of the reaction when attempting to measure rates of superoxide formation within intact cells, as opposed to cellular or tissue extracts. Although the use of the cytochrome c reaction has achieved “gold standard” status for the detection of superoxide, particularly with in vitro assays and activated leukocytes, its relative insensitivity to detect low rates of superoxide formation, as well as interference by a variety of endogenous reducers, limits its applicability for in vivo detection. It has, however, been used to detect superoxide formation in vascular tissue (71).

**Inhibition of Aconitase**

Alterations in aconitase activity have been used as a sensitive index of changes in steady-state levels of superoxide in vitro and in vivo (36, 38, 49, 115, 140). Aconitase catalyzes the interconversion of citrate to isocitrate and is present in both cytosolic and mitochondrial forms. Superoxide inactivates aconitase due to oxidation followed by reversible loss of Fe from its cubane [4Fe–4S] cluster, with a rate constant of $10^6–10^7$ M/s (19, 32, 48). Because of ongoing basal superoxide formation, a fraction of aconitase at any moment is inactive, but capable of reactivation; this ratio of inactive to active aconitase will increase as rates of superoxide production are augmented (36). Cell and tissue aconitase activity is thus determined by the relative rates of inactivation by superoxide or other oxidants, and the rate of reactivation by reduction and restoration of iron to the cluster (36, 49). Using this method, steady-state superoxide concentrations have been estimated at ~8–30 pM in A549 lung epithelial cells under normal conditions (86%
active aconitase) and 50–200 pM in cells when 50% of aconitase is in the inactive form (38). Aconitase activity has been used to determine relative rates of superoxide formation in a variety of cell types including cultured neuronal cells, macrophages, and fibroblasts, as well as liver, heart, lung, and brain tissues (37, 85, 96).

Aconitase activity in cell or tissue homogenates is measured spectrophotometrically by following the conversion of isocitrate 20 mM to cis-aconitate at A240nm. A coupled assay where citrate serves as substrate for aconitase and the isocitrate product is then converted to α-ketoglutarate by NADP+-dependent isocitrate dehydrogenase allows monitoring of NADPH formation at A340nm and can diminish background absorbance interference. Reactivation of aconitase activity can be accomplished by addition of the reducing agent dithiothreitol, ferrous ammonium sulfate, and Na2 S to cellular extracts and remeasuring aconitase activity (49).

Other oxidants, in addition to superoxide, can modify aconitase activity. Peroxynitrite will oxidize the [Fe4–4S] cluster of the enzyme with subsequent loss of activity, which is restored by the addition of thiol reagents and reduced iron (19, 48). The rate constant of this bimolecular reaction has been estimated at 105 M/s. (19) It has also been reported that NO itself can inhibit aconitase activity, but it is unclear whether, in vivo, NO is directly responsible for significant enzyme inactivation or it is the result of ensuing ONOO− formation. The effect of NOS inhibition on extents of aconitase inactivation may help to determine the role(s) of superoxide and NO-derived species in enzyme inactivation. Oxygen and H2O2 have also been noted to inhibit aconitase activity, albeit with rate constants four to five orders of magnitude lower than those noted for superoxide or ONOO− so that a direct contribution of oxygen and H2O2 to oxidant-mediated aconitase inactivation is minimal (32).

**Hydroethidine Oxidation by Superoxide**

Hydroethidine (dihydroethidium, HE) is a cell-permeant compound that can undergo a two-electron oxidation to form the DNA-binding fluorophore ethidium bromide or a structurally similar product (8, 143). The reaction is relatively specific for superoxide, with minimal oxidation induced by H2O2 or hypochlorous acid (HOCl) (8). The intracellular oxidation of HE to ethidium by superoxide has been analyzed with flow cytometry in cell suspensions (18), and visualization of cellular and anatomic regions displaying increased rates of oxidant production with digital imaging microscopy has been described in vivo (126) and in tissue slices and isolated blood vessels (9, 87). In contrast to other “intracellular” probes for superoxide, such as lucigenin, there is little capacity for artifactual formation of superoxide by HE due to redox cycling. However, there are critical reactions that may limit the use of HE conversion to ethidium as a quantitative marker for superoxide production (8). First, cytochrome c is also capable of oxidizing HE—this may be of importance 1) when mitochondria are the primary source of superoxide production or 2) when cytochrome c is released into the cytosol under conditions leading to apoptosis (42). Because oxidant stress has been suggested to initiate apoptotic processes and apoptosis itself has been proposed to increase mitochondrial superoxide production (17), it would be difficult to ascribe HE conversion to ethidium solely to superoxide; thus localization of cellular sites of superoxide generation would be suspect under these circumstances. Furthermore, use of high concentrations of HE can result in superoxide-independent fluorescence as a result of ethidium formation that exceeds the binding capacity of mitochondrial nucleic acids, allowing the ethidium cation to bind to nuclear DNA with marked enhancement of fluorescence (15). Quantitation of superoxide production using HE may also be inaccurate due to its capacity to enhance rates of superoxide dismutation to H2O2 (8). Thus, under these conditions, initial rates of superoxide formation will be underestimated. Finally, as with the use of any fluorophore for tissue localization of the compound of interest, the potential for autofluorescence must be recognized and taken into account (120). Because of these considerations, the use of HE for the determination of superoxide in vivo appears to have significant limitations.

**Chemiluminescence Reactions**

Chemiluminescent methods for superoxide detection have been frequently employed because of the potential for access to intracellular sites of superoxide generation, the alleged specificity of reaction of the chemiluminescent probe with superoxide, minimal cellular toxicity, and increased sensitivity when compared with chemical measurements. The most widely used chemiluminescent compound for superoxide detection is lucigenin. However, the potential for redox cycling of lucigenin and artifactual generation of superoxide have raised questions concerning its appropriate use for determining quantitative rates of superoxide formation (75, 130). Although it has been suggested that reducing the concentration of lucigenin to 5 μM will eliminate the potential for redox cycling (89, 118), other investigations have demonstrated that redox cycling with increased oxygen consumption and enhanced formation of superoxide occurs at this concentration (5, 58). Interestingly, the presence of different electron donors (NADH vs. NADPH) in the reaction mixture influences both lucigenin-dependent redox cycling and chemiluminescent intensities (58). Despite its limitations in precisely estimating rates of superoxide formation, the use of low concentrations of lucigenin can be useful in providing qualitative information concerning the involvement of superoxide in a given experimental condition.

In an effort to make use of the advantages of chemiluminescent techniques, other nonredox-cycling compounds have been studied for their utility as probes for superoxide. Coelenterazine [2-(4-hydroxybenzyl)-6-(4-hydroxyphenyl)-8-benzyl-3,7-dihydroimidazo [1,2-α] pyrazin-3-one] is a lipophilic luminescence originally isolated from the coelenterate Aequorea and is the light-producing chromoprotein in the aequorin complex (90, 91, 116, 132). Superoxide-stimulated chemiluminescence occurs after direct oxidation of coelenterazine (39, 132), thus eliminating the redox cycling-dependent artifactual generation of superoxide that generates controversy with lucigenin (75, 132, 137). Although the intensity of light emitted from the interaction of coelenterazine with superoxide is greater than lucigenin-dependent chemiluminescence, coelenterazine-dependent chemiluminescence is not entirely specific for superoxide, as ONOO− will also result in luminescence in the presence of coelenterazine (131). Selective use of NOS inhibitors, as well as ONOO− scavengers, may aid in discriminating between the contributions of superoxide and ONOO− to chemiluminescence under these circumstances. In addition to
in vitro chemical assays, coelenterazine-dependent chemiluminescence has been used in cultured cells (25), neutrophils (77), vascular tissues (51, 121, 131), and isolated mitochondria (104). Structural analogs, CLA [2-methyl-6-phenyl-3,7-dihydroimidazo[1,2-α] pyrazin-3-one] and MCLA [2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo[1,2-α] pyrazin-3-one] have also been used to assess superoxide formation in a variety of conditions, including neutrophil and macrophage activation (92, 125), cultured cells (57, 129), and vascular tissue (117). Of note, with the use of MCLA chemiluminescence, in vivo superoxide production has been reported from the surface of liver (135), intestine (114), heart (156), and lung (77, 91, 127).

Electron Spin Resonance and Spin Trapping

Electron spin resonance (ESR) spectroscopy, also known as electron paramagnetic resonance (EPR), is at present the only analytic approach that permits the direct detection of free radicals. This technique reports on the magnetic properties of unpaired electrons and their molecular environment. Unpaired electrons can exist in two orientations, either parallel or antiparallel with respect to an applied magnetic field. The energy differences of these states correspond to the microwave region of the electromagnetic spectrum. While the unpaired electrons of species such as NO, superoxide, and hydroxyl radical (·OH) are too low in concentration and short lived to be directly detected by ESR in biological systems, this dilemma can be avoided by ESR measurement of more stable secondary radical species. For superoxide this generally consists of converting short-lived oxygen centered adducts to longer-lived carbon adducts.

These more stable radical species are formed by adding exogenous “spin traps”—molecules that react with primary radical species to give more enduring radical adducts, with characteristic ESR “signatures.” These spin traps, frequently nitroxide and nitrore derivatives, can also be used to label biomolecules and probe basal and oxidative-induced molecular events in protein and lipid microenvironments (10, 139). With a limit of sensitivity of ~10⁻⁹ M, ESR spectroscopy is capable of detecting the more stable free radical-derived species produced during oxidative and inflammatory injury, including ascorbyl radical, tocopheroxyl radical, and heme-nitrosyl complexes (73). The use of spin traps such as phenyl-tert-butyl nitronate (PBN) and 5,5-dimethylpyrroline-N-oxide (DMPO) have had an illustrious history in detecting organic radical products. Cyclic hydroxylamines such as 1-hydroxy-3-carboxy-pyrrolidine are oxidized by superoxide or ONOO⁻ to form stable radical adducts that can be detected by ESR and these adducts are less reactive to cellular radicals such as ascorbate and glutathione (24), suggesting their potential utility in measuring reactive species production in intact cells. Reactive species have been successfully detected in vivo with ESR (35, 50, 103). Although probe instability, tissue metabolism, lack of spin trap specificity, and the cost of ESR spectrometers makes this technique less than ideal for the in vivo determination of free radicals, one may enhance the dependability of this technique by combining it with other strategies for detecting specific reactive species (see above and below).

DETECTION OF HYDROGEN PEROXIDE

Horseradish Peroxidase-Linked Assays

Numerous assays for the detection of H₂O₂ depend on the oxidation of a detector compound. In the presence of H₂O₂, hydrogen donors are oxidized by HRP (11)

\[ \text{HRP} + \text{H}_2\text{O}_2 \rightarrow \text{HRP-H}_2\text{O}_2 [\text{Compound I}] \]

\[ \text{HRP-H}_2\text{O}_2 + \text{AH}_2 \rightarrow \rightarrow \text{HRP} + 2\text{H}_2\text{O} + \text{A} \]

The amount of H₂O₂ present is estimated by following the decrease in fluorescence of initially fluorescent probes such as scopoletin (7-hydroxy-6-methoxy-coumarin) (12) or by monitoring the increase in fluorescence from previously nonfluorescent hydrogen donors such as diacetyldichlorofluorescin (52), p-hydroxyphenylacetate (54), homovanillic acid (3-methoxy-4-hydroxyphenylacetic acid) (113), or Amplex Red (N-acetyl-3,7-dihydroxyphenoxazine) (21, 144). Oxidation of tetr methylbenzidine (124) or phenol red (100) can be followed spectrophotometrically using the same principle. Several precautions are required for accurate interpretation of reporter molecule test systems for detection of H₂O₂ in biological systems.

Numerous biological compounds including thiols and ascorbate can serve as substrate for horseradish peroxidase (HRP) and thus compete with the detector molecule for oxidation, leading to underestimation of H₂O₂ formation.

Competition with HRP by endogenous catalase for H₂O₂ can also lead to underestimation of H₂O₂. For example, in the scopoletin-HP coupled assay, rates of H₂O₂ formation in isolated mitochondria or sub mitochondrial particles ranged from 2 to 76% of those detected by either oxygen consumption or by H₂O₂-induced cytochrome c peroxidase Compound I formation. Quenching of fluorescent signals by cell and tissue components can lead to overestimation of H₂O₂ in the case of scopoletin or underestimation, as with homovanillic acid.

When measuring cellular or subcellular H₂O₂ accumulation, accuracy of the assay can be improved by separation of the incubation media from cellular components after addition of the detection system, thus limiting confounding interactions of the detection system with cellular elements (124).

Spectrophotometric assays using HRP-coupled reactions also have limitations. Direct reduction of the oxidized detector molecule by electron transport components can limit the utility of tetr methylbenzidine in H₂O₂ determination, e.g., in mitochondria (124). Although spectrophotometric detection of H₂O₂ with phenol red has been used successfully in purified enzyme-substrate mixtures and to detect H₂O₂ release from activated leukocytes (100), the phenol red-HP assay is exquisitely pH dependent and less sensitive than fluorescent
methods, reducing its utility when attempting to detect H₂O₂ from cell types with low endogenous rates of oxidant formation. Taken together, the HRP-linked assays represent a particularly useful method for the quantification of H₂O₂ levels in cultured cells, organ cultures, and isolated buffer perfused tissue preparations. However, these methods are not suitable for determinations of H₂O₂ in plasma or serum because many reducing agents present in extracellular fluid (e.g., GSH, ascorbate, etc.) will interfere with the assay.

Dichlorofluorescein Fluorescence

The oxidation of 2′,7′-dichlorofluorescin (DCFH) to the fluorescent compound 2′,7′-dichlorofluorescein (DCF) was initially thought to be a relatively specific indicator of H₂O₂ formation (63) and has been used extensively in the detection of oxidants produced during the respiratory burst in inflammatory cells, either in cell lysates or in intact cells using flow cytometry (6, 97). The diacetate form of DCFH (DCFH-DA) is taken up by cells, where DCFH-DA is metabolized by intracellular esterases to DCFH, which has been suggested to remain “trapped” intracellularly. In the presence of H₂O₂, DCFH is oxidized to DCF; fluorescence is measured with excitation at 498 nm and emission at 522 nm. However, there are limitations to the interpretation of DCF fluorescence as a specific marker for quantitative intracellular H₂O₂ formation.

The H₂O₂-dependent oxidation of DCFH to DCF occurs slowly, if at all, in the absence of ferrous iron (110). Furthermore, DCF formation is greatly enhanced in the presence of heme-containing substances such as hematin, peroxidases, or cytochrome c or other redox-active metal ions (20, 53, 74, 110). Critically, peroxidases are capable of inducing DCFH oxidation in the absence of H₂O₂ (72, 74, 108). Thus alterations in cellular peroxidase or heme activity are likely to be equally if not more important than H₂O₂ in determining rates of DCF formation and cellular fluorescence (16, 94). Furthermore, a recent report emphasizes the critical role that intracellular iron trafficking plays in mediating H₂O₂-dependent DCF fluorescence (128).

Intracellularly formed DCFH does not necessarily remain within the confines of the intracellular space, but rather can reaccumulate in extracellular media where it would be available for reaction with extracellular oxidants (112).

The peroxidase-dependent formation of DCF from DCFH, both in the presence and absence of H₂O₂, is complex and generates both the DCF semiquinone free radical DCF⁺ (108), the DCF phenoxyl radical DCF⁻ (109), as well as superoxide. The dismutation of superoxide to H₂O₂ would then yield artifactual DCFH oxidation and amplification of DCF fluorescence.

In addition to peroxidase-dependent oxidation of DCFH, other substances are capable of directly inducing DCF formation in the absence of H₂O₂, including ONOO⁻ and HOCl (22, 68). Lipid peroxides are also capable of inducing DCF fluorescence in the presence of heme-containing compounds (20).

A structurally related analog of DCFH is dihydrorhodamine 123 (DHR), a cell-permeant, mitochondrial-avid compound that is oxidized to the fluorophore rhodamine 123. Although a direct reaction of DHR 123 with H₂O₂ does not occur, it can be catalyzed by heme-containing peroxidases such as HRP (74) or other heme compounds, including cytochrome c. (112) How-

ever, several other cell-derived oxidants such as ONOO⁻ and HOCl are also capable of directly oxidizing DHR 123 (22). Although careful application of SOD, catalase, and ONOO⁻ scavengers may provide more precise identification of the substance(s) responsible for DHR oxidation, in many instances, several of these compounds may be produced concomitantly, resulting in multiple pathways responsible for the oxidation of DHR 123 to rhodamine within the cellular milieu.

Because of the various biologic substances that can lead to DCF and rhodamine fluorescence and the inherent uncertainty relating to endogenous vs. artificial oxidant generation, these fluorescent assays are probably best applied as qualitative markers of cellular oxidant stress, rather than as precise indicators of rates of H₂O₂ formation for in vitro and in vivo systems.

Aminotriazole Inhibition of Catalase

A major pathway for clearance of H₂O₂ is through reaction with catalase. The intermediate complex, Compound I, that is formed in catalase-dependent metabolism of H₂O₂ is relatively stable. Aminotriazole, an irreversible inhibitor of catalase, reacts with the Compound I intermediate; thus catalase is inhibited by aminotriazole only in the presence of H₂O₂ (80). Intracellular rates of production of H₂O₂ can therefore be estimated by determining the half-time of aminotriazole-dependent inactivation of catalase; steady-state H₂O₂ concentrations can be approximated according to (65, 95)

\[ [\text{H}_2\text{O}_2] = \frac{k_w}{k_i} \left( \frac{0.5}{t_{1/2}} \right) = -k_{cat} \text{ and } k_i = 1.7 \times 10^7 \text{ M/s} \]

This method has in fact been used to assess rates of H₂O₂ formation in numerous isolated cell types as well as in vivo (45, 99, 142). It should be remembered, however, that under conditions where glutathione peroxidase-reductase significantly contributes to H₂O₂ metabolism, this method will underestimate actual H₂O₂ concentrations (65).

METHODS TO DETECT NO AND ITS DECOMPOSITION PRODUCTS IN VIVO

More than 20 years ago it was demonstrated that rodents and humans excrete much larger amounts of nitrate (NO₃⁻) than could be accounted for by ingestion of food. Subsequent studies revealed that germ-free rats also excreted significantly more NO₃⁻ than could be accounted for by dietary ingestion, suggesting that mammalian cells have the biochemical machinery necessary to synthesize oxidized metabolites of nitrogen. Subsequent studies from several disciplines converged to identify NO as the precursor of NO₃⁻ and an important regulatory molecule involved in regulation of the immune, cardiovascular, and nervous systems. It has been known for many years that NO is relatively unstable in the presence of molecular O₂ and will rapidly and spontaneously autoxidize to yield a variety of nitrogen oxides

\[ 2\text{NO} + \text{O}_2 \rightarrow 2\text{NO}_2 \]
\[ 2\text{NO}_2 + \text{H}_2\text{O} \rightarrow 4\text{NO}_3^- + 4\text{H}^+ \]

where NO₂, N₂O₃, and NO₃⁻ represent nitrogen dioxide, dinitrogen trioxide, and nitrite, respectively. It has been defi-
tively demonstrated that the only stable product formed by the spontaneous autoxidation of NO in oxygenated solutions is NO$_3^-$, However, when one analyzes urine or plasma, predominantly NO$_2^-$ is found in these extracellular fluids. Although the mechanisms by which NO is converted to NO$_3^-$ in vivo are not entirely clear, there are at least two possibilities. One mechanism, proposed by Ignarro et al. (56), suggests that the NO$_2^-$ derived from NO autoxidation is rapidly converted to NO$_3^-$ via its oxidation by certain oxyhemoproteins (P-Fe$^{2+}$O$_2$) such as oxyhemoglobin or oxymyoglobin.

$$2P-Fe^{2+}O_2 + 3NO_2^- + 2H^+ \rightarrow 2P-Fe^{3+} + 3NO_3^- + H_2O$$

or

$$4P-Fe^{2+}O_2 + 4NO_2^- + 4H^+ \rightarrow 4P-Fe^{3+} + 4NO_3^- + O_2 + 2H_2O$$

It should be noted, however, that these investigators used large concentrations of NO (300 μM), which will rapidly autoxidize to NO$_3^-$. Although the authors suggested that the NO$_3^-$ would in turn react with the hemoproteins, this reaction is quite slow, requiring 2–3 h. A second, possibly more reasonable explanation for the presence of predominantly NO$_3^-$ in vivo may have to do with the fact that the levels of NO produced by NO synthase (NOS) in vivo would be much smaller and thus the half-life of NO would be much longer. In this case, NO would react directly and very rapidly with oxyhemoproteins to yield NO$_3^-$ before it has an opportunity to autoxidize to NO$_3^-$. It should be noted that saliva does contain relatively high concentrations of NO$_3^-$, which many investigators suggest is synthesized by the oral bacteria.

Approximately 56% of the human body is fluid. Although most of the fluid is localized within the intracellular compartment (intracellular fluid), ~30% of the total body water is found in the spaces outside the cells and is termed extracellular fluid. Examples of some extracellular fluids include plasma, lymph, urine, cerebrospinal fluid (CSF), intraocular fluid, sweat, and tears, as well as a variety of gastrointestinal fluids, including salivary, gastric, intestinal, pancreatic, and biliary secretions. Extracellular fluids such as plasma, lymph, and CSF are continuously being transported via the circulation to all parts of the body. This rapid transport and subsequent mixing between the plasma and extracellular fluid via diffusion through the capillary endothelial cells allows all cells in the body to be exposed to essentially the same extracellular environment. Thus the presence of certain metabolites in extracellular fluid provides a good indicator for those metabolic processes that occur at the cellular and tissue level. Furthermore, because some extracellular fluids (e.g., urine, saliva, tears) are normally excreted, these fluids may be collected and metabolites analyzed in a noninvasive manner, thereby allowing the continuous determination of systemic levels of various metabolites. One metabolite that has been known to be present in plasma and urine is nitrate (NO$_3^-$). Because saliva and lymph also contain substantial amounts of nitrite (NO$_2^-$) and NO$_3^-$, one would predict that other fluids, such as CSF, bile, tears, sweat, and intraocular fluid also contain this low molecular weight metabolite.

The methods outlined below describe methods to quantify NO$_3^-$ and NO$_2^-$ in extracellular fluids such as plasma, urine, and/or lymph. The same methods may also be used for other extracellular fluids (e.g., saliva, tears, etc.), cell culture fluid, and/or organ culture supernatants.

**Preparation of Extracellular Fluids: Complications and Considerations**

Determination of urinary or plasma levels of NO$_3^-$ and NO$_2^-$ provides a useful method to quantify systemic NO production in vivo. Analysis of urine for the presence of nitrogen oxides remains one of the easiest methods to assess noninvasively systemic NO metabolism. It should be remembered that urinary (or plasma) NO$_3^-$ levels reflect not only endogenous NO production but also total NO$_3^-$ ingestion from the diet as well as the minor contribution made by bacteria found in the gut. Thus animals should either be fasted or allowed to ingest NO$_3^-$/NO$_2^-$-free diets before determinations. It has been our experience that a 24-h fast will reduce plasma NO$_3^-$ levels by 60–80%, demonstrating that the majority of the circulating nitrate in rats is contributed to their diet. Furthermore, it is important to include “antibiotics,” such as propanol or penicillin/streptomycin in collection tubes, to prevent bacterial growth during collection (41).

The collection and processing of plasma and lymph also require some precaution. We have found the heparinized plasma or lymph may form a precipitate on addition of the highly acidic Griess reagent, rendering these samples unusable for analysis (44). Interestingly, the precipitation does not occur in all samples and will occasionally occur even when protein is first removed by addition of ZnSO$_4$ or ultrafiltration. Because of these inconsistent results it has been difficult to determine the mechanism of this precipitation reaction. We determined that the presence of heparin may promote precipitation of the plasma or lymph samples when acidified by the Griess reagent. We propose that the inability to produce consistent precipitation in all samples may be the result of the small but significantly different amounts of heparin used for each sample, as the volume of heparin is never accurately measured when using this anticoagulant. We have found that the use of citrate as an anticoagulant or allowing plasma to clot to yield serum completely prevents this problem. We have also determined that when the need arises to assess plasma or lymph NO$_3^-$ and NO$_2^-$ levels in heparinized fluid, we can do so if the heparin is removed using protamine sulfate precipitation before the addition of the Griess reagent.

**Determination of Nitrate and Nitrite Using the Griess Reaction**

One method for the indirect determination of NO involves the spectrophotometric measurement of its stable decomposition products NO$_3^-$ and NO$_2^-$. This method requires that NO$_3^-$ first be reduced to NO$_2^-$ and then NO$_2^-$ determined by the Griess reaction as shown in Fig. 2. Briefly, the Griess reaction is a two-step diazotization reaction in which the NO-derived nitrosating agent (e.g., N$_2$O$_3$), generated from the acid-catalyzed formation of nitrous acid from nitrite (or the interaction of NO with oxygen), reacts with sulfanilic acid to produce a diazonium ion that is then coupled to N-(1-naphthyl)ethylenediamine to form a chromophoric azo product that absorbs strongly at 543 nm (44). For quantification of NO$_3^-$ and NO$_2^-$ in extracellular fluids, we have found enzymatic reduction of...
NO$\text{\textsuperscript{3-}}$ to NO$\text{\textsuperscript{2-}}$ using a commercially available preparation of nitrate reductase (NR) to be the most satisfactory method (44)

$$\text{NO}_3^- + \text{NADPH} \xrightarrow{\text{NR}} \text{NO}_2^- + \text{NADP}^+$$

Aspergillus NR (Boehringer Mannheim, Indianapolis, IN) is highly efficient at reducing very small amounts of NO$\text{\textsuperscript{3-}}$ to NO$\text{\textsuperscript{2-}}$. After the incubation, any unreacted NADPH is oxidized by addition of lactate dehydrogenase and pyruvic acid because reduced pyridine nucleotides (NADPH, NADH) strongly inhibit the Griess reaction. An alternative method for oxidizing any unreacted NADPH is to replace the LDH/pyruvate system with 1 mM potassium ferricyanide (44). A known volume of premixed Griess reagent is then added to each incubation mixture and incubated for 10 min, and the absorbance of each sample was determined at 543 nm.

As mentioned previously, we found that the presence of heparin in plasma or lymph sample may produce precipitation on addition of the Griess reagent. Therefore, heparin must be removed before the addition of the Griess reagent using addition of a small aliquot of protamine sulfate as previously described (44).

**Determination of NO Using Fluorescence Spectroscopy**

**Diaminonaphthalene assay.** Although the Griess reaction is a simple, rapid, and inexpensive assay for NO$\text{\textsuperscript{2-}}$ and NO$\text{\textsuperscript{3-}}$ in physiological fluids, it has a practical sensitivity limit only 2–3 μM. In attempts to enhance the sensitivity of measuring NO$\text{\textsuperscript{2-}}$ or NO generated under physiological conditions, different fluorimetric methods have been developed that exploit the ability of NO to produce N-nitrosating agents. One of these methods employed the use of the aromatic diamino compound 2,3-diaminonaphthalene (DAN) as an indicator of NO formation (86). The relatively nonfluorescent DAN reacts rapidly with the NO-derived N-nitrosating agent (N$_2$O$_3$) generated from the interaction of NO with oxygen or from the acid-catalyzed formation of nitrous acid from nitrite to yield the highly fluorescent product 2,3-naphthotriazole (NAT; Fig. 3). This assay offers the additional advantages of specificity, sensitivity, and versatility. This assay is capable of detecting as little as 10–30 nM (i.e., 10–30 pmol/ml) NAT and may be used to quantify NO generated under biologically relevant conditions (e.g., neutral pH) with minimal interference by nitrite decomposition (86). As with the Griess reaction, the DAN assay can be used to quantify NO production at under physiological conditions and/or stable decomposition products of NO in physiological fluids as well as tissue culture media and organ culture supernatants.

**Diaminofluorescein-2 assay.** In addition to the DAN assay, more recent studies demonstrated that diaminofluorescein-2 (DAF-2) may be used to determine the presence of NO in vitro and in situ (66, 67). Like DAN, nitrosation of the diamino...
group results in a nitrosamine that, through an internal rearrangement, forms the fluorescent triazole (Fig. 4). The advantages of this compound are that wavelength associated with fluorescenc can be used, making equipment currently used for other bioassays as well as cell and tissue imaging easily adapted to detect NO in vitro and in vivo. Although NO-derived \( \text{N}_2 \text{O}_3 \) is to be the primary mechanism by which cells and tissue generate the triazole derivative, recent investigations suggest that oxidative nitrosation may represent an alternative pathway for triazole generation (29, 31). This implies that, under biological conditions, the triazole can be formed from either nitrosative or oxidative chemistry.

In solution or media these two pathways can be differentiated. The use of nitrosative scavengers such as azide will prevent nitrosation but not oxidation. Conversely, urate will quench oxidative nitrosation but not nitrosative nitrosation. Using different scavengers, the chemistry of DAF-2 formation can be teased out in different experiments (29, 31). Although DAF-2 has been thought to be an indicator of NO, it recently has been shown that nitrosyl (HNO) reacts with DAF-2 giving even higher yields of triazole than NO (30). In light of the potential for HNO in biological systems, it could be interesting to entertain the possibility that some of the NO detected by DAF-2 is in fact HNO. One cautionary note is that using powerful light sources such as lasers can result in photochemistry that can lead to false positives. It should be noted that control tests need to be done to ensure that it is the chemistry from NO and not some artifact of the photochemistry.

**DETECTION OF S-NITROSOTHIOLS BY COLORIMETRIC AND FLUOROMETRIC METHODS**

The formation and biological properties of NO-derived S-nitrosothiols (RSNOs) play an important part of the biology of NO (2, 122, 123). It is well appreciated that autoxidation of NO in the presence of thiols (RSH) generates RSNOs via the following mechanism

\[
2\text{NO} + \text{O}_2 \rightarrow 2\text{NO}_2
\]

\[
2\text{NO} + 2\text{NO}_2 \rightarrow 2\text{N}_2\text{O}_3
\]

\[
2\text{N}_2\text{O}_3 + 2\text{RSH} \rightarrow 2\text{RSNO} + 2\text{NO}_2
\]

Different RSNOs are known to stimulate guanylate cyclase thereby promoting vasorelaxation. Although controversial, studies have also proposed that S-nitrosohemoglobin adducts are involved in homeostasis of vascular tone and oxygen delivery (40, 59). Furthermore, the S-nitrosation of proteins has been suggested as an important step in cell signaling as well as inhibition of a variety of enzymes. Here we describe methods for the colorimetric and fluorometric detection of RSNO (141).

The detection of RSNO has often employed the Saville reaction, which involves the displacement of the nitrosonium ion (NO\(^+\)) by mercury salts (Fig. 5). The resulting nitrite or NO generated from the spontaneous decomposition of NO\(^+\) is detected by methods such as chemiluminescence or HPLC. These methods involve equipment and expertise not always found in most laboratories. Other techniques for the detection of RSNO employ colorimetric methods such as the Griess reaction to measure the nitrite formed from the treatment of RSNO with mercuric chloride. However, samples that contain large amounts of nitrite can interfere with and limit the detection range of these methods under acidic conditions. To overcome these problems, two methods have been devised to detect RSNO-derived nitrosating species at neutral pH (141). The colorimetric method uses the components of the Griess reaction while the fluorimetric method uses the conversion of DAN to its fluorescent triazole derivative (141). These methods may be conducted at neutral rather than acidic pH, which eliminates the interference of contaminating nitrite and allows the detection of nitrosation mediated by the presence of NO.

The colorimetric reaction uses the same chemistry as described previously in which the nitrosating species nitrosonium (NO\(^+\)) generated from the interaction between RSNO and mercuric chloride interacts with sulfanilamide to form a diazonium ion (Fig. 5). The resulting diazonium salt then reacts with naphthylethylenediamine to form the colored azo complex (Fig. 4). The fluorometric assay is based on the reaction of DAN with NO\(^+\) liberated from RSNO after mercuric chloride addition to yield a primary nitrosamine, which is converted rapidly to a fluorescent triazole (Fig. 6). The colorimetric assay has a detection range of 0.5–100 \(\mu\)M, while the fluorometric assay is effective in the range of 50–1,000 nM RSNO (141). The combination of the two assays provides a detection range from 50 nM to 100 \(\mu\)M RSNO, required for most biological experiments. Variations of these methods have been used successfully to quantify high and low molecular weight RSNOs in human and rat plasma as well as the S-nitrosated derivatives of human and rat hemoglobin (61, 62).
QUANTIFICATION OF 3-NITROTYROSINE: SPECIFIC FOOTPRINT FOR PEROXYNITRITE?

NO may rapidly interact with the free radical $O_2^-$ to yield the potent oxidants peroxynitrite ($\text{ONOO}^-\text{H}_2\text{O}$) and its conjugate acid peroxynitrous acid ($\text{ONOOH}$) (7):

$$O_2^- + \text{NO} \rightarrow \text{ONOO}^- + H^+ \rightarrow \text{ONOOH}$$

Although a tremendous number of investigations have been performed that suggest that $\text{ONOO}^-$ may play an important role in several pathophysiological situations, the question of whether $\text{ONOO}^-$ is actually formed in vivo and exerts any physiological and/or pathophysiological activity remains the subject of vigorous debate. Because it has been reported that only $\text{ONOO}^-$ will nitrate tyrosine residues in proteins and peptides under physiological conditions, the presence of nitrotyrosine (NT) has been used as evidence for $\text{ONOO}^-$ production in vivo. Indeed, the vast majority of studies implicating $\text{ONOO}^-$ as an important cytotoxic specie have used immunohistochemical or HPLC determinations of NT as proof of $\text{ONOO}^-$ formation in vivo. In reality, NT may be generated by multiple pathways suggesting that the presence of NT is not a specific “footprint” for $\text{ONOO}^-$ formation in vivo (Fig. 7). For example, work by several laboratories demonstrated that tyrosine can be nitrated by peroxidase (or heme)-catalyzed, hydrogen peroxide-dependent oxidation of nitrite to form NO$_2$ (Fig. 6) (28, 133):

$$\text{H}_2\text{O}_2 + \text{peroxidase (heme)} \rightarrow \text{oxidant (porphyrin radical)}$$

$$\text{Oxidant} + \text{NO}_2^- \rightarrow \text{NO}_2$$

$$2 \text{NO}_2^- \rightarrow \text{NT}$$

It has been shown that MPO and other peroxidases will mediate this reaction. Colocalization of these peroxidases with NT strongly indicates this reaction is the primary source of tyrosine nitration in vivo (26). Although some studies have indicated the possibility of peroxidase-independent sources of NT formation (14), it should be remembered that virtually any hemoprotein (e.g., hemoglobin, myoglobin, cytochromes) pos-

![Fig. 5. Detection of S-nitrosothiols (RSNO) by the Saville reaction. Liberation of the nitrosating specie nitrosium ($\text{NO}^+$) by interaction of RSNO with mercury salts in the presence of the Griess reagents, results in the formation of the same diazo product as described in Fig. 2.](image)

![Fig. 6. Fluorometric detection of RSNO. Liberation of nitrosium ($\text{NO}^+$) by interaction of RSNO with mercury salts in the presence of the DAN results in the formation of the same fluorometric triazole derivative described in Fig. 3.](image)

![Fig. 7. Multiple pathways for the formation of 3-nitrotyrosine. 3-Nitrotyrosine may be generated by peroxynitrite ($\text{ONOO}^-$), autoxidation of NO, nitroxyl (HNO) in the presence of oxygen, heme, or hemoprotein catalyzed, $\text{H}_2\text{O}_2$-dependent oxidation of nitrite (NO$_2^-$), NO$_2^-$ interaction with HOCl, and acidified NO$_2^-$.](image)
sesses the potential to catalyze the $H_2O_2$-dependent oxidation of nitrite to yield NT (64). In addition to heme or hemoprotein-mediated NT formation, the MPO-derived oxidant hypochlorous acid (HOCl) will interact with nitrite to yield the potent nitrating agent nitryl chloride (Cl-NO$_2$), which, in and of itself, will nitrate tyrosine to form NT (27).

Another example of ONOO$^-$-independent generation of NT is the reaction of tyrosine with acidic nitrate

$$\text{HNO}_2 + H^+ \rightarrow H_2\text{NO}_2 + NO_2^-$$
$$\text{NO}_2^- \rightarrow \text{NO}_2 + NO$$

$$2 \text{NO}_2 + \text{Tyrosine} \rightarrow \text{NT} + \text{NO}_2^-$$

When the pH approaches 4–5, nitrite can slowly generate NT (101). Whether acidic nitrate can produce NT in vivo remains the subject of active investigation. It is possible that this reaction may be important in acidic microenvironments such as the lipid bilayer, phagolysosomes, etc., where NO accumulation and oxidation to nitrite would readily occur.

It has been found that the autoxidation of NO can also generate NT (98). Although possible in free solution, nitration via the autoxidation of NO (i.e., the NO/NO$_2$ reaction) occurs optimally only with supraphysiological fluxes of NO (i.e., 1 mM), suggesting that this mechanism most probably does not occur in vivo in cell cytosol or extracellular fluid (98). However, it should be remembered that NO (and O$_2$) are known to concentrate to very large amounts within the lipid bilayer, suggesting that the autoxidation of NO in this hydrophobic environment would produce NO$_2$ and N$_2$O$_3$ that would readily form NT (76).

Finally, interaction of tyrosine with nitroxyl (HNO) may represent a potential pathway for NT in vivo. It has been demonstrated that HNO combines with oxygen to form a peroxynitrite like species (87a, 87b). However, unlike peroxynitrite, this oxidant will not nitrate the amino acid tyrosine (94a). However, exposure to large concentrations of HNO donor can result in nitrated protein. The mechanistic differences are still being addressed and represent a possible HNO source, although probably only under specific conditions.

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