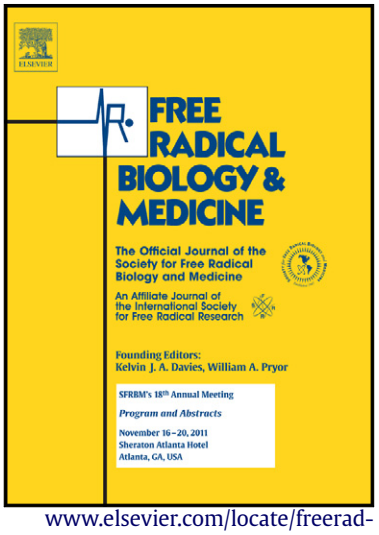

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Even free radicals should follow some rules: A Suggested Guide to Free Radical Research Terminology and Methodology

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EVEN FREE RADICALS SHOULD FOLLOW SOME RULES: A Suggested Guide to Free Radical Research Terminology and Methodology

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Highlights

- Dye oxidation alone is insufficient evidence for detection of reactive species in biology.
- Non-enzymatic lipid peroxidation assessment requires product analysis by MS.
- Antioxidant claims require quantitative dose-response lowering of oxidative stress.
- ROS and RNS should be used only when defined and the actual species are unknown.

Abstract

Free radicals and oxidants are now implicated in physiological responses and in several diseases. Given the wide range of expertise of free radical researchers, application of the greater understanding of chemistry has not been uniformly applied to biological studies. We suggest that some widely used methodologies and terminologies hamper progress and need to be addressed. We make the case for abandonment and judicious use of several methods and terms, and suggest practical and viable alternatives. These changes are suggested in four areas: use of fluorescent dyes to identify and quantify reactive species, methods for measurement of lipid peroxidation in complex biological systems, claims of antioxidants as radical scavengers, and use of the terms for reactive species.

Introduction - The Problem and the Need for Some Rules:

The Free Radical field has undergone massive expansion in recent years. Emerging data indicate that the biological generation and reactivity of oxidants is harnessed to regulate numerous redox-dependent physiological processes. In turn, uncontrolled production and dysregulation of redox signaling is implicated in the initiation and propagation of several pathological conditions. Considering the vastly different backgrounds and training of free radical 'practitioners,' perhaps it is not surprising that agreement on common practices, including terminology, can be difficult.

While we have no intent to impose restrictions on freedom of expression, we do suggest that some widely used practices are detrimental to progress in our field and need to be addressed. The goal of this paper is to identify such practices, make the case for their abandonment, and suggest practical and viable alternatives.

We have selected four areas that frequently cause concern and contention: 1) The use of fluorescent dyes to identify and quantify reactive species, 2) The Thiobarbituric Acid Reactive Substances (TBARS) assay for lipid peroxidation in complex biological systems, 3) Antioxidants as radical scavengers, and 4) Recommended terminology.

Use of Fluorescent Dyes to Measure Reactive Species.

Although there are published methods that use the oxidation of fluorescent dyes to detect specific reactive species, misinterpretation of the data obtained from such dyes is a major problem in the free radical field. Recently, the editors of *Free Radical Biology & Medicine* addressed this issue [1] and made the following main recommendations:

(1) The reaction of reactive species with reporter dyes results in the generation of both specific, often less abundant, oxidized products and more abundant non-specific

products. Therefore the detection of a specific reactive species requires identification, separation and quantification of the specific oxidation products. Furthermore, wise utilization of fluorescence dyes requires performing a series of controls in conjunction with molecular or pharmacological inhibitors for the identification of the reactive species involved.

(2) The most commonly used dye, dichlorodihydrofluorescein diacetate (DCFH-DA), is cell-permeable and undergoes intracellular hydrolysis to form the DCFH carboxylate anion, which is retained in the cell. Two-electron oxidation of DCFH results in the formation of the fluorescent product, dichlorofluorescein (DCF). However, DCFH does not react with superoxide, hydrogen peroxide or nitric oxide. Instead, DCF fluorescence results from oxidation by potent oxidants, such those produced from metal ion- and peroxidase-catalyzed reactions and from proton- and carbon dioxide-catalyzed decomposition of peroxynitrite. Moreover, DCF-dependent fluorescence can be self-amplified by redox-cycling of the one-electron oxidized dye [2]. Possibly DCFH oxidation largely reflects the relocation of lysosomal iron to the cytosol [3] and peroxidase-catalyzed oxidation [4].

(3) Recommended sources for the measurement of reactive species include papers by, Kalyanaraman *et al.* [1], Rhee *et al.* [5], Markvicheva *et al.* [6], and Van de Bittner *et al.* [7].

We suggest that the time has come for rigorous and precise use of these methods. We must stop accepting claims that reactive species are involved in a biological process based solely on the use of dye oxidation. It is essential that verification by separation of products or a more specific methodology be employed as suggested in the references cited above [1, 5-7]. As such we recommend that scientific journals should not accept manuscripts containing improper interpretation of dye oxidation, and request revisions. Provided that appropriate methodologies have been employed authors should be able to address the issue by appropriate revision of the text.

Thiobarbituric Acid Reactive Substances (**TBARS):**

In a test-tube experiment involving the oxidation of pure unsaturated fatty acids, thiobarbituric acid may be used to detect the formation of the lipid peroxidation product malonyldialdehyde (or malondialdehyde, or MDA) by production of a pink colored product. In simple or highly purified systems, the TBA test for MDA gives an entirely appropriate estimate of lipid peroxidation.

Unfortunately, in more complex biological systems, many compounds (including simple and complex carbohydrates, protein oxidation products, and nucleic acid oxidation products) react with thiobarbituric acid to produce colored adducts. Thus, one cannot directly equate the measurement of TBARS with MDA or lipid peroxidation when measured in a complex biological system.

Therefore the use of TBARS as a sole indicator of lipid peroxidation in a complex biological system is not appropriate. In contrast however, separation and mass spectroscopic analysis of thiobarbituric acid products, particularly MDA, has value in

accessing the role of lipid peroxidation in oxidative stress as demonstrated by the multi-laboratory investigation referred to as "BOSS II" [8].

We further suggest however, that the best general indicator of non-enzymatic lipid peroxidation under normoxic conditions, currently available, is the measurement of F2-isoprostanes. These should be measured by mass spectrometry because the antibodies used in ELISA are not specific for F2-isoprotanes [9].

Antioxidants as Scavengers of Radicals and Hydroperoxides:
There are thousands of compounds that exhibit antioxidant chemistry *in vitro* and appear to have some 'antioxidant effect' *in vivo*. Reactions of small organic compounds with most radicals are actually a competition in which the rate constants for the reactions are usually very close to one other. Thus, for a molecule to be effective as a scavenger, it would need to outcompete all other potentially reactive molecules present in the system. Only in cases where an unusually high, localized concentration of an 'antioxidant' molecule can be reached is this possible. An exception may be α-tocopherol. Based on consideration of its specific uptake and relatively rapid kinetics of reaction with lipid hydroperoxyl radicals (LOO•) compared with the propagation reaction (LOO• + lipid), α-tocopherol may be an effective chain breaker in lipid peroxidation [10].

Scavenging of superoxide, hydrogen peroxide and other hydroperoxides, all of which react relatively slowly with small organic molecules, is carried out efficiently by enzyme-catalyzed reactions that have rate constants that are 100,000 times faster than for their non-enzymatic counterparts. Thus, with the possible exception of α-tocopherol (and positively charged ubiquinone analogs that accumulate in mitochondria), physiologically meaningful scavenging by non-enzymatic reactions is essentially insignificant. Instead, many of the compounds referred to 'antioxidants' are most probably acting through their effects on signaling pathways, rather than reacting as true antioxidant scavengers. Please see Forman et al. [10] for a more complete discussion.

<u>*Hydroxyl radical scavenging:*</u> There are numerous misconceptions about so-called 'hydroxyl radical scavenging' in biological systems, by small molecules including polyphenols, which have unfortunately resulted in many erroneous statements appearing in published papers. All organic compounds react with hydroxyl radicals with rate constants approaching the diffusion limitation. Thus, in solution, no compound really has any more significant hydroxyl radical scavenging activity than hundreds or thousands of other compounds (proteins, lipids, nucleic acids, amino acids, numerous metabolites, *etc.*) already present in any biological system. Therefore, for any compound to be even 50% effective in solution it would have to be present at equal or greater concentrations than all of those other compounds together. Thus, there are no antioxidants for •OH.

Spin traps are often used as scavengers of hydroxyl radicals based on the formation of characteristic EPR-detectable hydroxyl spin adducts. However, spin traps only need to react with a minute fraction of the total hydroxyl radicals generated in any given

situation in order to yield a measurable EPR-sensitive radical adduct. Thus, although spin traps (e.g., DMPO) may not quantitatively inhibit hydroxyl radicals, they can still be used to detect hydroxyl radicals because of the high sensitivity and specificity of the EPR technique.

Thus, unless a molecule can convincingly be demonstrated to act *in vivo* as a direct scavenger of reactive species (as has been shown for α-tocopherol) we suggest that claiming such activity is no longer acceptable. This is not the same as showing that the molecule in question decreases the (damaging) effects of a reactive species, which may be through a different mechanism than direct scavenging. Additionally, limited intestinal absorption often significantly diminishes the effective concentration of an agent that can actually be achieved *in vivo*. We strongly encourage our colleagues to intensively investigate the mechanism(s) by which their potentially effective 'antioxidants' actually work *in vivo*, rather than just assuming that a test-tube antioxidant must also act as a scavenging antioxidant *in vivo*.

Demonstrating that an agent acts a scavenger when a reactive species is added to cells in culture alone, is also not sufficient to identify a new physiological antioxidant scavenger, since the molecule in question may simply scavenge the reactive species outside the cells.

Although we feel that appropriately controlled studies of antioxidants in animals and humans are to be strongly encouraged, merely phenomenological accounts that do not rigorously investigate mechanism(s) of action, are strongly discouraged. Furthermore, if a study involves treatment of patients or animals with an antioxidant, it must follow the same rules as apply to studies of other potential pharmaceutical agents. In this regard, it must be shown that the dose of "antioxidant" tested actually lowered oxidative stress using quantitative methodology.

Recommended Terminology: ROS, RNS and other Non-standard Abbreviations:

We recommend that the abbreviations ROS and RNS not be used without definition. Winterbourn has discussed this issue [11] and concluded that, "The term ROS is generally taken to encompass the initial species generated by oxygen reduction (superoxide or hydrogen peroxide) as well as their secondary reactive products. Reactive nitrogen species (RNS) is also in common usage to describe reactive species derived from nitric oxide. There is clearly overlap and crosstalk between the production, function and decomposition of the two groupings, especially because of the highly favored reaction between superoxide and nitric oxide to give peroxynitrite."

Therefore we suggest that the preferred practice should be to use the name of the identified species. Of course we understand using the terms ROS or RNS when the species is unknown, or when it might be one of several molecules that have been implicated without certainty. But, it is then essential that this be clearly stated. The use

of ROS and RNS in the text should be discouraged, particularly when the actual species are known or can be reasonably surmised. As an example, NOX (if defined as NADPH oxidase) may be used but its products $O_2^{\cdot -}$ and/or H_2O_2, along with downstream products including hypochlorous and other hypohalous acids, should not be referred to as ROS. We suggest that ROS and RNS, as well as other non-standard terms and abbreviations, should not be used in manuscript titles, or in table or figure legends. The use of ROS or RNS in the text should be as stated above; i.e., only when it is clearly stated that the species is unknown, or one of several implicated molecules without certainty.

Our goal here is not to reproach or reprimand anyone, but instead to contend that, at this point in the evolution of free radical biology and medicine, adherence to appropriate and accurate terminology and methodology are really needed to advance the field.

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