Why antioxidant therapies have failed in clinical trials

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Abstract

In spite of considerable research, and many clinical trials involving thousands of patients, there is a conspicuous lack of antioxidant therapies available. In this paper we present results for the interaction and neutralization of a free radical species. We adopt two modeling techniques, one based upon Gillespies Stochastic Simulation Algorithm and one based upon a discrete Markov chain. An advantage of these models is that they incorporate values for the number of molecules present per unit volume, and with the Markov chain model, the relative dimensions of these molecules in contrast to ordinary differential equations. By means of thiese models we question the basis of antioxidant therapies based trapping or scavenging of reactive species. We demonstrate the extraordinary capacity of the enzymatic antioxidant defenses relative to non-enzymatic defenses. We conclude that, if the concentration of an non-enzymatic antioxidant is too low there is little chance of collision and interaction with a free radical species. Furthermore, if the rate of reaction between the free radical and the non-enzymatic antioxidant is below a necessary threshold then the effect of an antioxidant will be dwarfed by the free radical defense systems naturally present. As such we suggest that failure of most antioxidant therapies in clinical trials is to be expected.

Keywords: Antioxidant, Free Radical, ROS, Gillespie, Markov Model

1. Introduction

The free radical theory of ageing, Harman (2016), put forward the hypothesis that free radicals, usually reactive oxygen species, generated as a by product of respiration are a causal factor in the process of ageing. This is based on the supposition that free radicals are highly reactive and are a cause of continual and cumulative damage to cellular protein, lipid, and DNA (the

soma). This damage, unless repaired, will lead to a progressive deterioration of cellular function. Cumulative damage by free radicals has been associated with many diseases, notably age related neurodegenerative conditions such as Alzheimer's. This is in part because the brain has a high metabolic demand and a subsequent high oxygen demand, while being essentially post-mitotic, Cobley et al. (2018). While it is well established that antioxidants like ascorbic acid can neutralize many free radical species, the use of antioxidants in clinical trials has shown them to be predominately ineffective, Steinhubl (2008). The chemistry of the free radical trapping proporties of vitamin c (ascorbic acid) are well documented, however, just because the chemistry is plausible does not necessitate that it occurs *in vivo* or or that it is effective. Ascorbic acid can be taken orally but but there is negligable absorbtion at doses greater than 500mg (about 5 oranges worth) at which point it will increase the plasma concentration to a steady state of 80 micromolar, Padayatty (2003). The cells and tissues of a typical and well nourished person will already contain ascorbic acid at 1-4 millimolar. One would assume that if ascorbic acid is already present at a high concentration and provided an effective defense against free radicals, but would be even more effective at a slightly higher concentration, that this would have occured.

To address the question of the effectiveness of antioxidants, the process of oxidative damage and the mechanisms to avoid it can be reduced to a number of basic processes. Namely, the generation of free radicals and their subsequent neutralization by specific enzymatic means, neutralization by non-enzymatic antioxidants, or damage to the soma. This is shown schematically in Fig 1.

In this paper we adopt two modeling techniques, namely Gillespie's Stochastic Simulation Algorithm (using Kinetiscope) and a discrete Markov chain. The two models yield comparable results.

2. Model of Free Radical Interactions

In this section we develop a model of free radical interactions by imagining a highly simplified organelle, it functions much like a mitochondrion. The parameters for the number and size of molecules in the cell were taken from the *By the numbers* website[1] and associated publications, Milo and Phillips
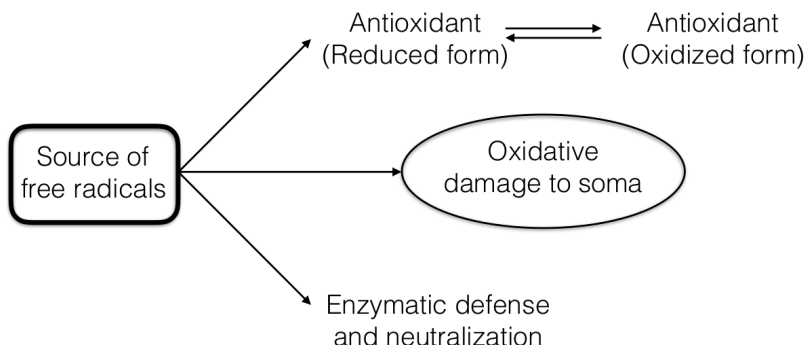
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[1]http://bionumbers.hms.harvard.edu/

Figure 1: Generalized scheme for the interactions of free radicals. After their generation free radical species can be neutralized by scavenging antioxidants, enzymatic defenses, or interact with and damage components of the cell.

(2015). A typical human cell such as a fibroblast has a volume of $2,000\mu m^3$, and the mass of mitochondria is 20-25% of the total cell volume. As such, we defined the volume of the imaginary organelle as $400\mu m^3$ and assigned arbitrary values to the cell contents, that is, 1×10^9 protein molecules, 1×10^{10} molecules of water, and 1×10^8 molecules of a non-enzymatic antioxidant, Bergsten et al. (1989). Of the protein present 3×10^7 molecules (3%) are an enzymatic antioxidant. For interaction with the free radical the initial rate constant assumed for the non-enzymatic antioxidant was $3 \times 10^5 M^{-1}s^{-1}$, and for the enzymatic antioxidant it was $1 \times 10^9 M^{-1}s^{-1}$. The high value for the enzymatic antioxidant is due to the fact that superoxide dismutase is an unusual enzyme and operates far faster than most enzymes, and may be as fast as it is theoretically possible.

The modeling software used was Kinetiscope from Columbia Hill Technical Consulting[2]. The algorithm is based upon Gillespie's Stochastic Simulation Algorithm, Gillespie (2016). The algorithm calculates the probabilities of all reactions in the system. For each iteration, reactions are selected at random, weighted by their probabilities which are derived from their respective rate constants.

The values used were loosely based on the rate constants for the reactions of superoxide with glutatione, Winterbourn (2016), with ascorbic acid,

Buettner and Jurkiewicz (1996), and superoxide dismutase, Milgrom (2016). The rate for damage to the soma was set at $300 M^{-1} s^{-1}$ based of the rate of superoxide interaction with methionine protein residues, Davies (2016). An arbitrary rate of free radical generation of $1 \times 10^{-6} s^{-1}$ was selected to give a measurable rate of protein oxidation in a reasonable time span. The actual rate of generation of free radicals, specifically superoxide, by mitochondria has not been accurately determined, Murphy (2009). This is, in part, due to the high catalytic rate of enzymes such as superoxide dismutase and catalase which maintain a low steady state level of superoxide and hydrogen peroxide.

In the absence of enzymatic antioxidants or non-enzymatic antioxidants the rate of damage to the protein was, as would be expected, proportional to the rate of generation of free radicals (not shown). Enzymes involved in the defence against free radical damage such as superoxide dismutase and catalase have extremely high catalytic rates, and are some of the most abundant proteins present in the cell ($\sim 1-3\%$ from PaxDb: Protein Abundance Database[3]). If the concentration of the enzymatic antioxidant is set to 3% of the total protein and the k_{cat}/K_M is varied, it can be seen (see Fig 2) that in order to reduce the rate of damage to the soma (protein in this model) to a negligible level a k_{cat}/K_M of $3 \times 10^8 M^{-1} s^{-1}$ to $1 \times 10^9 M^{-1} s^{-1}$ is required. Coincidentally this is close to the observed k_{cat}/K_M of enzymes such as catalase and superoxide dismutase, Milgrom (2016). Conversely if we define the k_{cat}/K_M of the enzymatic antioxidant as $1 \times 10^9 M^{-1} s^{-1}$ and vary the concentration of the enzymatic antioxidant, the concentration at which there is negligible oxidative damage to the soma is at 0.3-1% of the total protein. This is reasonably close to the concentration of enzymes such as catalase and superoxide dismutase at 1-3% (Fig 3).

In order to be effective as an antioxidant, it is necessary to be present at a relatively high concentration and to have a high forward rate constant (k_{cat}/K_M). If a non-enzymatic antioxidant is added to the system at a high concentration (1×10^8 molecules per cell) with a moderate $k_{cat}/K_M \approx 3 \times 10^5 M^{-1} s^{-1}$, in the absence of any enzymatic antioxidant, it can be seen that it can function as an antioxidant for a short while (see Fig 4). However, in the absence of a mechanism for the efficient regeneration of the reduced form from the oxidized form, the antioxidant is rapidly depleted.

These results indicate that, in comparson to endogenous antioxidants,
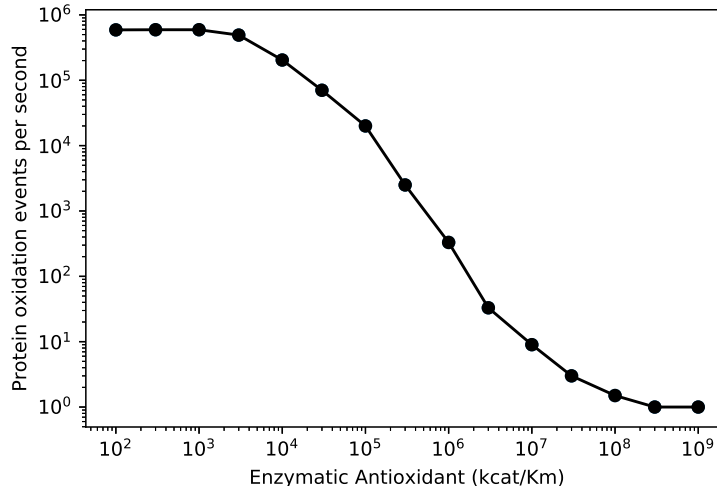
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[3]https://pax-db.org

Figure 2: Effect of variation in the amount of enzymatic antioxidant. To inhibit the rate of oxidation of cellular protein the enzymatic antioxidant has to be present at 10^8 molecules per cell or greater.

non-enzymatic antioxidant are relatively ineffective at ameliorating oxidative damage from free radicals. This is contrary to the conventional wisdom that exogenous antioxidants can reduce free radical damage, as such, we desired confirmation of this result. Thus, we developed a second model based upon a discrete time Markov chain which we describe in Section 3 below.

3. Markov Chain

In this section we describe a discrete time Markov chain to model the trajectory of a free radical within a cell. The cell in this model is comprised of water molecules, protein molecules, enzymatic antioxidants, and non-enzymatic antioxidants as previously defined. We further assume that there is a steady rate of free radical production. During each time interval a free radical encounters either a water molecule, a protein, an enzymatic antioxidant, or a non-enzymatic antioxidant. We consider the trajectory of a free radical within a cell and its likelihood of an encounter with each of the above components. These likelihoods are governed by the relative abundance of each component in the cell and their relative size. Unlike the previous
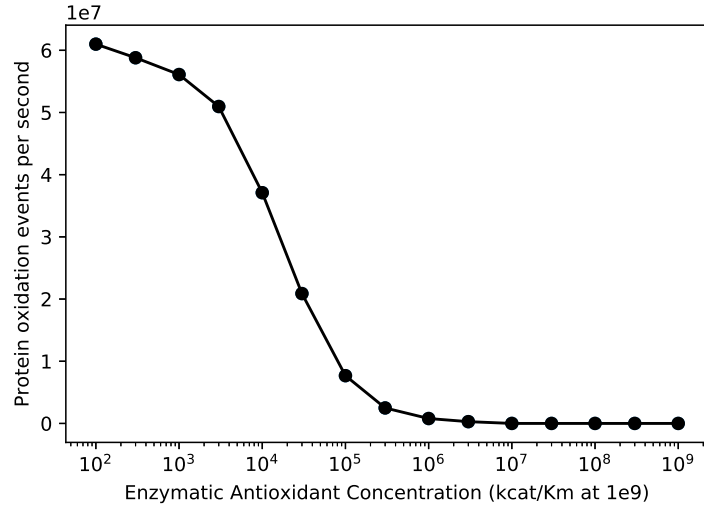
Figure 3: The assigned rate constant for the reaction of enzymatic antioxidant with the free radical species can be varied. If the number of molecules per organelle is defined as 10^8, then a k_{cat}/K_M greater than $10^7 M^{-1} s^{-1}$ is necessary. For reference the k_{cat}/K_M of superoxide dismutase is $10^9 M^{-1} s^{-1}$
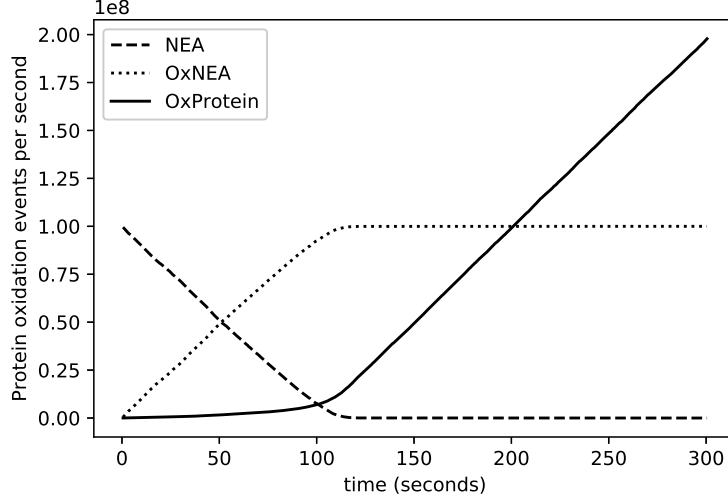
Figure 4: Rapid depletion of a non-regenerating non-enzymatic antioxidant, with accumulation of the oxidized form. When the store of the non-enzymatic antioxidant is exhausted there is rapid oxidation of the cellular proteins.

model our Markov model takes into account the relative volume occupied (size and number) of the molecules involved. Water, for example, is the most abundant molecule but is considerably smaller than a protein molecule.

An encounter with a water molecule (which we call state w) has no effect on the free radical which continues on its trajectory into the next time interval (and, consequently, the next state). The probability of an encounter with a water molecule $p(w)$ is derived from the ratio of water and the total amount of matter in the cell, thus:

$$p(w) = \frac{m_w v_w}{M_{total}} \tag{1}$$

We compute the total matter M_{total} in the cell as:

$$M_{total} = m_w v_w + m_{prot} v_{prot} + m_{ea} v_{ea} + m_{nea} v_{nea} \tag{2}$$

where m_w, m_{prot}, m_{ea} and m_{nea} are the number of molecules of water, protein, enzymatic antioxidant, and non-enzymatic antioxidant, respectively. Likewise, v_w, v_{prot}, v_{ea} and v_{nea} are the respective volumes for water, protein, enzymatic antioxidant, and non-enzymatic antioxidant.

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The probability of an encounter with a protein is derived from the ratio of the amount of protein and the total amount of matter in the cell (M_{total}). However, the effect of an encounter with a protein molecule can yield two different results. Either the encounter is productive and the free radical reacts with the protein of the encounter is *futile* resulting in no reaction. A productive encounter results in a neutralisation of the free radical and its trajectory is terminated. A productive encounter is an *absorbing* state. We should point out that a *productive* encounter of a free radical with protein results in cell damage and equates to oxidative damage. If the encounter is futile the state is *non-absorbing* and the free radical continues on its trajectory into the next time interval. Consequently, we have transition probabilities for each of the productive and futile states, namely, $p(prot_a)$ and $p(prot_{na})$, respectively. The fraction of futile encounters for the protein (φ_{prot}) is derived from Bar-Evan et al. (1996):

$$\varphi_{prot} = 1 - k_{prot}/\tilde{k}_1 \tag{3}$$

where k_{prot} is the k_{cat}/K_M value for protein and $\tilde{k}_1$ is the diffusion limit rate constant. According to Bar-Evan et al. (1996) $\tilde{k}_1$ is between 10^9 and $10^{10} M^{-1}s^{-1}$. The purpose of this paper we use $\tilde{k}_1 = 10^{10}$. From Table 1, $k_{cat}/K_M = 300 M^{-1}s^{-1}$.

The probability $p(prot_a)$ is given by:

$$p(prot_a) = \frac{(1 - \varphi_{prot})m_{prot}v_{prot}}{M_{total}} \tag{4}$$

The probability $p(prot_{na})$ is given by:

$$p(prot_{na}) = \frac{\varphi_{prot}m_{prot}v_{prot}}{M_{total}} \tag{5}$$

As with protein molecules, we model an encounter with enzymatic antioxidant as productive and futile encounter states to reflect the effect on the free radical, that is, the encounter either results in a reaction or no reaction with the enzymatic antioxidant, respectively. With the enzymatic antioxidant (as well as the non-enzymatic antioxidant) a *productive* encounter with a free radical neutralizes it. We use ea_a to represent the productive encounter state and ea_{na} to represent the futile encounter state. State ea_a is absorbing because the trajectory of the free radical is terminated during the current time interval. Conversely, the ea_{na} state is non-absorbing as the free radical

does not react with the enzymatic antioxidant and its trajectory continues on into the next time interval. The fraction of futile encounters for the enzymatic antioxidant is computed, thus:

$$\varphi_{ea} = 1 - k_{ea}/\tilde{k}_1 \qquad (6)$$

The term $k_{ea} = 10^9$ is the k_{cat}/K_M value for the enzymatic antioxidant (see Table 1). The probability $p(ea_a)$ is given by:

$$p(ea_a) = \frac{(1 - \varphi_{ea})m_{ea}v_{ea}}{M_{total}} \qquad (7)$$

The probability $p(ea_{na})$ is given by:

$$p(ea_{na}) = \frac{\varphi eam_{ea_{na}}v_{ea_{na}}}{M_{total}} \qquad (8)$$

Like the enzymatic antioxidant, a non-enzymatic antioxidant has a productive encounter state (nea_a) and a futile encounter state nea_{na} which are absorbing and non-absorbing, respectively. Functionally, there is no difference between an enzymatic antioxidant or non-enzymatic antioxidant with rapid regeneration, the main factor is the processing time, which can be viewed as the equivalent of the forward rate constant. The fraction of futile encounters with the non-enzymatic antioxidant is given by:

$$\varphi_{nea} = 1 - k_{nea}/\tilde{k}_1 \qquad (9)$$

where $k_{ea} = 10^5$ is the k_{cat}/K_M value for the non-enzymatic antioxidant (Table 1). The probability $p(nea_a)$ is given by:

$$p(nea_a) = \frac{\varphi_{nea}m_{nea_a}v_{nea_a}}{M_{total}} \qquad (10)$$

The probability $p(nea_{na})$ is given by:

$$p(nea_{na}) = \frac{(1 - \varphi_{nea})m_{nea_{na}}v_{nea_{na}}}{M_{total}} \qquad (11)$$

Table 1 shows the values used to compute quantities of material in the cell along with their corresponding k_{cat}/K_M values (where applicable). These values are used to derive transition probabilities for the transition matrix $\mathbf{T}$ of the Markov model:

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	No. molecules	Vol. molecules nm^3	k_{cat}/K_M $M^{-1}s^{-1}$
Water	1.3×10^{13}	0.01	NA
Protein	1.0×10^9	65.40	300
Enzymatic antioxidant	3.0×10^7	65.40	1×10^9
Non-enzymatic antioxidant	1.0×10^8	1.00	1×10^5

Table 1: Assigned values for the abundance, dimensions, and rate constants in the model

$$\mathbf{T} = \begin{bmatrix} p(w) & p(prot_a) & p(prot_{na}) & p(ea_a) & p(ea_{na}) & p(nea_a) & p(nea_{na}) \\ 0 & 1 & 0 & 0 & 0 & 0 & 0 \\ p(w) & p(prot_a) & p(prot_{na}) & p(ea_a) & p(ea_{na}) & p(nea_a) & p(nea_{na}) \\ 0 & 0 & 0 & 1 & 0 & 0 & 0 \\ p(w) & p(prot_a) & p(prot_{na}) & p(ea_a) & p(ea_{na}) & p(nea_a) & p(nea_{na}) \\ 0 & 0 & 0 & 0 & 0 & 1 & 0 \\ p(w) & p(prot_a) & p(prot_{na}) & p(ea_a) & p(ea_{na}) & p(nea_a) & p(nea_{na}) \end{bmatrix} \tag{12}$$

The probability of the system being in state i at time k is π_i^k:

$$\pi = \pi\mathbf{T} \tag{13}$$

where $\lim_{k \to \infty} \pi = \pi^{(k)}$. Without any loss of generality, we assume that the initial state of system is an encounter with water, thus, the initial state is:

$$\pi^0 = \begin{bmatrix} 1 & 0 & 0 & 0 & 0 & 0 & 0 \end{bmatrix} \tag{14}$$

We ran the model for varying levels of non-enzymatic antioxidant concentrations, namely, $10^3 \geq m_{nea} \geq 10^{17}$ and observed the effect on protein reaction rates. The rates of protein reaction (that is, damage to the cell) for various m_{nea} are shown in Fig 5. Under these conditions it can be seen that the non-enzymatic antioxidant has a negligible effect for any realistic value of m_{nea}. While mechanistically different, the rate of neutralization of free radical species by enzymatic antioxidants is far greater than the rate of neutralization by non-enzymatic antioxidants, which in turn occurs at a much faster rate than damage to proteins by the free radical species, that is,
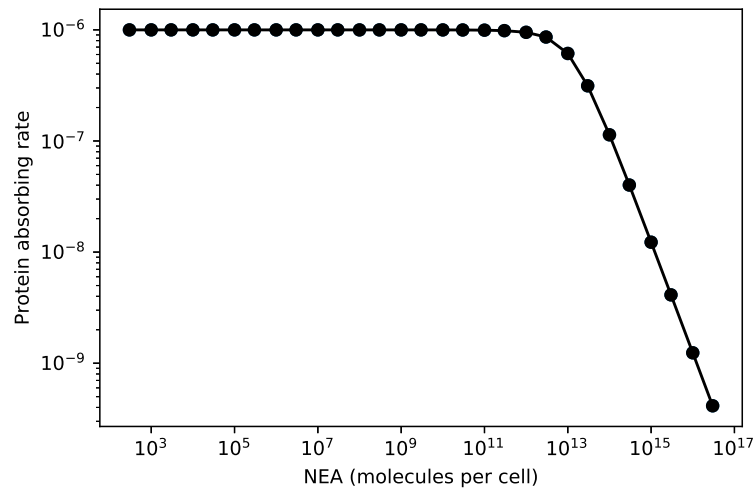
Figure 5: Protein reaction. It is apparent that, under the conditions specified in the model, that in order to reduce the rate of damage to protein by free radicals the non-enzymatic antioxidant would need to be present at physiologically unrealistic concentrations. A reduction in the rate of protein oxidation does not occur until the non-enzymatic antoxidant is present at greater than 10^{13} molecules per cell.

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$k_{ea} \gg k_{nea} \gg k_{prot}$. Furthermore, these rates differ by several orders of magnitude. Molecules with a size of the order of molecules such as glutathione and ascorbic acid are relatively small compared to cell components such as protein. To be effective physical interaction between the free radical and the putative antioxidant is necessary, this interaction is limited by physical size and numerical presence.

4. Conclusions

In this paper we present two models of free radical interactions in a cell. The first employs Gillespies Stochastic Simulation Algorithm from the stochastic modeling tool, Kinetiscope. The second model is based upon a discrete Markov chain. These models are, purposely, highly abstracted and reductive because they are not intended to mimic or replicate an actual biological system. The purpose was to explore the parameters that most influence the potential interaction and termination of a free radical species. The models used different approaches but their results are in qualitative agreement.

They illustrate that enzymes involved in defense against oxidative damage, such as superoxide dismutase and catalase are already extremely effective. At the concentrations seen *in vivo* enzymatic antioxidants such as superoxide dismutase or catalase will essentially eliminate oxidative damage. This is not surprising, to achieve the catalytic rates observed empirically, enzymes such as superoxide dismutase and catalase must have been subject to strong selective pressure and as such are probably optimal in terms of catalytic rate and concentration. Non-enzymatic antioxidants such as glutathione or ascorbic acid have relatively modest rates of reaction with superoxide, in spite of the presumably identical selective pressures that acted on the enzymatic antioxidants. Based on these results we would infer that defense against free radicals is not a major role for these compounds. The results also permit a number of conclusions to be made concerning the role of therapeutic non-enzymatic antioxidants in the prevention of oxidative damage to the soma:

1. To be effective, a non-enzymatic antioxidant that operates by trapping or scavenging free radicals, needs to be present at a sufficiently high concentration to give a reasonable chance of interaction between the antioxidant, rather than with other components of the cell. These high concentrations likely to be physiologically unrealistic.

2. A potential free radical scavenging agent would also need to have a sufficiently high rate constant to effectively compete with the enzymatic defense system. Unless the rate of reaction is of the order of $1 \times 10^7 M^{-1}s^{-1}$ or greater, they will have little effect on the rate of protein oxidation.
3. There also needs to be an effective and efficient means of regenerating the reduced form of the antioxidant from the oxidized form. Without a mechanism to regenerate the reduced form of an antioxidant from the oxidized form an exogenous antioxidant would be rapidly depleted. For a synthetic or non-endogenous compound it is unlikely a mechanism for this will be present.

The models presented in this paper are limited in the number of parameters considered and assumes that the reactions take place in an aqueous environment. Non-enzymatic antioxidants may have utility in a lipid membrane environment. In lipids oxidative damage can propagate by means of a chain reaction. Nevertheless, within the scope of these models we suggest that therapeutic potential for non-enzymatic antioxidants that function by trapping or scavenging free radical species, is limited. In order to reduce the rate of damage to protein by free radicals the non-enzymatic antioxidant would need to be present at physiologically unrealistic concentrations. A reduction in the rate of protein oxidation does not occur until the non-enzymatic antoxidant is present at greater than 10^{13} molecules per cell. Therefore, the failure of many putative antioxidant compounds to demonstrate a therapeutic effect is to be expected.

5. References

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