Free Radicals and Antioxidants in the Year 2000

A Historical Look to the Future

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ABSTRACT: **In the late 1950's free radicals and antioxidants were almost unheard of in the clinical and biological sciences but chemists had known about them for years in the context of radiation, polymer and combustion technology. Daniel Gilbert, Rebeca Gerschman and their colleagues related the toxic effects of elevated oxygen levels on aerobes to those of ionizing radiation, and proposed that oxygen toxicity is due to free radical formation, in a pioneering paper in 1956. Biochemistry owes much of its early expansion to the development and application of chromatographic and electrophoretic techniques, especially as applied to the study of proteins. Thus, superoxide dismutase (SOD) enzymes (MnSOD, CuZnSOD, FeSOD) were quickly identified. By the 1980's Molecular Biology had evolved from within biochemistry and microbiology to become a dominant new discipline, with DNA sequencing, recombinant DNA technology, cloning, and the development of PCR representing milestones in its advance. As a biological tool to explore reaction mechanisms, SOD was a unique and valuable asset. Its ability to inhibit radical reactions leading to oxidative damage *in vitro* often turned out to be due to its ability to prevent reduction of iron ions by superoxide. Nitric oxide (NO·) provided the next clue as to how SOD might be playing a critical biological role. Although NO· is sluggish in its reactions with most biomolecules it is astoundingly reactive with free radicals, including superoxide. Overall, this high reactivity of NO· with radicals may be beneficial *in vivo*, e.g. by scavenging peroxyl radicals and inhibiting lipid peroxidation. If reactive oxygen species are intimately involved with the redox regulation of cell functions, as seems likely from current evidence, it may be easier to understand why attempts to change antioxidant balance in aging experiments have failed. The cell will adapt to maintain its redox balance. Indeed, transgenic animals over-expressing antioxidants show some abnormalities of function. There must therefore be a highly complex interrelationship between dietary, constitutive, and inducible antioxidants within the body, under genetic control. The challenge for the new century is to be able to understand these relationships, and how to manipulate them to our advantage to prevent and treat disease.**

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FROM TIES TO JEANS

The working environment in government-funded hospital laboratories during the late 1950's and early 1960's was unbelievably different from that which we know today. This was an era without computers, automated laboratory equipment, fax machines, or micro chips. Laboratory life was often formal, and a bachelors degree in science was often all that was needed to head a department. Whilst working in a hospital laboratory in the UK at this time, John Gutteridge well remembers there being different canteens for graduates and non-graduates, and an incident when a junior colleague was sent home for not wearing a neck tie. As a mild challenge to the system John came to work wearing a bow tie, although it never looked so elegant as Dan's bow ties do on him. The University environment was also much more structured than it is now and the Heads of Department reigned supreme. In his early days as a lecturer in Biochemistry, Barry Halliwell was forbidden to set foot in the Department of Chemistry for having the insolence to use a piece of equipment in that department without asking the Professor, even though the Chemistry lecturer to whom it belonged was enthusiastic about collaboration.

The formality which pervaded all aspects of society (at least in the UK) at this time was challenged and irreversibly broken by youth culture, being expressed through fashion and pop music, with the iconoclastic views of John Lennon being particularly influential. Thus, Jeans (rather wide at the ankle) entered laboratories as the preferred mode of dress, reflecting the dramatic change in attitudes to formality. Perhaps this may have gone too far—or are we being old-fashioned? We have both attended weddings and funerals where many people show up in jeans and T-shirts, and even the best restaurants in the world have minimized their dress code requirements almost to vanishing point. There are still outposts however: Barry Halliwell rejoiced when at a recent scientific meeting in a Caribbean country, over 80% of the scientists were deemed inadequately dressed to eat in the hotel restaurants (including all the American speakers). Had he been there, Dan would have surely qualified to eat with the select few in elegant splendor.

FROM JEANS TO GENES

In the late 1950's free radicals and antioxidants were almost unheard of in the clinical and biological sciences but chemists had known about them for years in the context of radiation, polymer and combustion technology. In a pioneering paper in 1954, Dan, Rebecca Gerschman and their colleagues related the toxic effects of elevated oxygen levels on aerobes to those of ionizing radiation, and proposed that oxygen toxicity is due to free radical formation.[1] This concept did not capture the imagination of most life scientists, however, until the discovery of the superoxide dismutase enzymes by McCord and Fridovich in 1968.[2] These authors had previously observed the ability of several proteins to inhibit the reduction of cytochrome c by xanthine oxidase, and in a flash of inspiration realized that the inhibitory effects were due to a novel protein contaminating the other proteins tested. They purified this contaminant and thus identified the first enzyme (copper and zinc-containing superoxide dismutase, CuZnSOD) known to act on a free radical substrate.[3,4] Barry Halliwell

had at the same time observed that illuminated chloroplasts reduce cytochrome c and this was inhibited by catalase, but could not understand how removing H_2O_2 could prevent a reduction reaction. After reading the seminal papers of McCord and Fridovich, he learned the answer: the commercial catalase used was contaminated by SOD.[5] Thus Dan's pioneering "free radical theory of oxygen toxicity" became the "superoxide theory of oxygen toxicity."

Biochemistry owes much of its early expansion, after its separation from Chemistry, to the development and application of chromatographic and electrophoretic techniques, especially as applied to the study of proteins. Thus new SOD enzymes (MnSOD, FeSOD) were quickly identified. By the 1980's Molecular Biology had evolved from within biochemistry and microbiology to become a dominant new discipline, with DNA sequencing, recombinant DNA technology, cloning, and the development of PCR representing milestones in its advance.

KNOCKABOUTS AND KNOCKOUTS

The highly stable CuZnSOD enzymes are easy to purify and could be sold at high profit by suppliers. Hence they were readily available to researchers by the late 1970's, resulting in an explosion of interest in superoxide and other reduction intermediates of oxygen in biological systems. A protein antioxidant catalyst which had as its substrate a small inorganic free radical was something novel to biochemistry at that time. Now we readily accept the important biological roles of small inorganic molecules, especially the free radical nitric oxide (NO·) and increasingly, carbon monoxide (if one can call CO inorganic). One of the authors recently proposed that SO_2 might be a biological signalling system and, in investigating this, rediscovered sulfite toxicity.[6] Indeed, investigation of sulfite oxidation mechanisms was the first example of the use of SOD as a "probe" for $O_2^{-\cdot}$.[3] Things tend to come full circle in this field, as we shall see again when considering Fenton chemistry.

Not all were prepared to accept that SOD was exclusively a biological enzyme and preferred to view it more as a copper transport protein. The "copper controversy"—catalyst or non-catalyst—unnecessarily dominated several of the early conferences dedicated to this new research field. People love stand-up controversy at meetings, but it usually achieves little. Since all those involved were good scientists and generated valid data, then there must be ways of accommodating all their observations and exactly this has happened with time. CuZnSOD is an important contributor to the "pool" of total intracellular copper, and other metalloproteins can sometimes replace SOD in removing $O_2^{-\cdot}$.[7]

Nevertheless, the superoxide theory of oxygen toxicity has held its ground, although there is no general agreement on why elevated levels of $O_2^{-\cdot}$ can be toxic. Is it direct selective damage by $O_2^{-\cdot}/HO_2\cdot$, or the $O_2^{-\cdot}$-dependent formation of peroxynitrite or of the infamous hydroxyl radical? Is SOD really important *in vivo*? To answer the question directly, simply remove SOD and see what happens, as Touati *et al.* first did with *E. coli.*[8] *E. coli* mutants lacking FeSOD and MnSOD were viable, but sick (we now know they still had some SOD, as CuZnSOD in the periplasmic space). Mice lacking mitochondrial SOD (MnSOD) are also very sick: this enzyme is essential for healthy aerobic life.[9,10] But what a surprise with CuZnSOD and

glutathione peroxidase! Mice can survive without them, although in the latter case they are more sensitive to certain toxins and in the former subtle defects (e.g. in reproductive potential) are gradually being discovered.[11,12]

Thus mice can adapt to live without these enzymes (but remember that mice are not men). How they do so remains to be discovered, but information is coming thick and fast. When Barry Halliwell was doing his Ph.D. in a plant biochemistry laboratory in the 1970's, everyone knew about the key role of thioredoxin in redox regulation and antioxidant defence in plants.[13] Only now is its importance in animals being fully appreciated,[14] and thioredoxin-linked peroxidases (peroxidoredoxins) are the new "flavor of the month."

"If free radicals play important roles in disease pathologies, then SOD might have remarkable properties as a drug for human medicine," was the next obvious step forward proffered by many researchers. With hindsight, we now know that cellular redox balance, under the control of a hierarchy of antioxidants and other redox-active proteins, is extremely complex and minimally influenced by extra "spoonfuls" of constitutive antioxidants. For example, oxidizing conditions can activate NFκB in some cell types, but the oxidized protein will not bind to DNA, i.e., its activity is redox-regulated. SOD, as a pharmaceutical, has made little or no impact on human medicine.

IRON TOOLS

As a biological tool to explore reaction mechanisms, SOD was a unique and valuable asset.[3] Its ability to inhibit radical reactions leading to oxidative damage *in vitro* often turned out to be due to its ability to prevent reduction of iron ions or iron chelates by superoxide. Thus SOD would inhibit superoxide-driven Fenton chemistry (reaction **A**), since removal of $O_2^{\cdot-}$ prevented the formation of ferrous ions

$$\begin{aligned} O_2^{\cdot-} + \text{Fe(III)} &\rightarrow Fe^{2+} + O_2 \\ 2\,O_2^{\cdot-} + 2H^+ &\rightarrow H_2O_2 + O_2 \\ H_2O_2 + Fe^{2+} &\rightarrow OH^- + {\cdot}OH + \text{Fe(III)} \end{aligned} \qquad \textbf{(A)}$$

However, SOD would not inhibit Fenton chemistry resulting from the mixing of Fe^{2+} with H_2O_2, or from the autoxidation of ferrous salts at physiological pH values.

$$\begin{aligned} Fe^{2+} + O_2 &\rightleftharpoons \text{Fe(III)} + O_2^{\cdot-} \\ 2\,O_2^{\cdot-} + 2H^+ &\rightarrow H_2O_2 + O_2 \\ Fe^{2+} + H_2O_2 &\rightarrow OH^- + {\cdot}OH + \text{Fe(III)} \end{aligned} \qquad \textbf{(B)}$$

For many years the prevention of iron reduction and consequent OH· generation seemed a major explanation for the protective role of SOD. Some groups embraced it enthusiastically and took our 1984 article proposing the importance of iron *in vivo*[15] as a bible, citing it as evidence that OH· is responsible for most or all of the oxidative damage occurring *in vivo.* Indeed, we are amused to note that this 1984 paper has been often cited by groups who appear not to have read it. For example, it

has been quoted as saying that OH· plays a key role as an initiator of biological iron-dependent lipid peroxidation when in fact we said the opposite.[15,16] Others vehemently opposed the concept of iron as a promoter of oxidative damage, stating that there is no iron "catalytic" for free radical reactions *in vivo* and that $O_2^{-\cdot}$ couldn't reduce it even if there was, since cells are full of other reducing agents. Another controversy started, although at least the participants were polite to each other. Time has again shown us that all the data were correct: organisms carefully restrict the availability of "catalytic" iron ions *in vivo* to minimize oxidative damage.[17] Yet cells have a transit pool of low molecular mass iron (recently demonstrated directly[18]) and oxidative stress can liberate further iron catalytic for free radical reactions, as was shown over a decade ago for ferritin and heme proteins.[19,20] Superoxide can also liberate iron from certain iron-sulfur clusters[21] and the concept of oxidative stress leading to iron ion release has been repeatedly re-presented as if it were novel. Tissue injury also liberates catalytic metal ions and cells "sense" their iron levels using iron-responsive elements and regulate the turnover of mRNAs encoding iron sequestration proteins accordingly.[22] Mice lacking transferrin have a large "pool" of catalytic iron and soon die: thus iron sequestration in non-catalytic forms is almost as important as MnSOD to their survival.[23]

MALIGNANT SPIRITS

Another source of pointless controversy is the "iron wheel," the regularly resurrected circular argument about whether or not OH· *is* the key species in Fenton chemistry. In 1932 Bray and Gorin proposed that a ferrous salt plus hydrogen peroxide produced a ferryl species (FeO^{2+}), and not the hydroxyl radical (·OH), and that ferryl rather than OH· was responsible for most of the damage seen. This concept has been picked up and re-presented as novel multiple times in the past 25 years. Much of the evidence against OH· was based on the idiosyncratic behavior of a variety of "OH· scavengers" added to Fenton systems damaging biological molecules. To many of us working with iron chelators, it has been obvious from the beginning that iron-binding, both weak and strong, dictates site-specific reactions that often overrule established second-order rate constants for attack of OH· upon molecules added to Fenton reactions (for a detailed discussion see refs. 24 and 25). Other arguments have been based on the fact that the end-products expected when biomolecules were added to Fenton systems were not "typical of OH·." Walling[25] showed decades ago that iron salts influence the fate of the primary radicals generated by attack of OH· on molecules, so that the end-products will not be the same as for radiation-generated OH·. Ferryl species could conceivably be intermediates in OH· generation, e.g.

$$Fe^{2+} + H_2O_2 \rightarrow \boxed{\begin{array}{c}\text{oxo-iron}\\ \text{species}\end{array}} \rightarrow Fe(III) + OH\cdot + OH^-$$

but their formation in "simple" biological Fenton chemistry has never been clearly demonstrated by classical chemical methods (although *heme* ferryl species are well-established). Walling[25] has summarized it beautifully: "The formulation of complexes to explain reactions and kinetic data is a great temptation but, particularly when they cannot be independently demonstrated, has little more intellectual justification

than invoking malignant spirits. However, since suitable complexes can fit almost any data, the temptation is real..."

NITRIC OXIDE AND PEROXYNITRITE: FROM DOGMA TO DOUBT

Nitric oxide provided the next clue as to how SOD might be playing a critical biological role. Although NO· is sluggish in its reactions with most biomolecules it is astoundingly reactive with free radicals, including superoxide. Overall, this high reactivity of NO· with radicals may be beneficial *in vivo*, e.g. by scavenging peroxyl radicals and inhibiting lipid peroxidation.[26,27] However, the product of reaction of $O_2^{-\cdot}$ with NO·, peroxynitrite ($ONOO^-$), is widely thought to be a "baddie," although this view is not universally shared. *In vitro* at pH 7.4, addition of $ONOO^-$ is cytotoxic and damages most biomolecules.[26] If its concentrations are great enough, nitric oxide can out-compete SOD for reaction with $O_2^{-\cdot}$. Normally, however, SOD might "control" the levels of $O_2^{-\cdot}$, balancing the action of NO· as a vasodilator.[28] Why has NO· research, after a relatively short time span, been awarded a Nobel Prize, whereas $O_2^{-\cdot}$ research has not? The answer probably reflects the clinical, pharmacological and medical impact of NO· knowledge and application, which has not yet materialized for $O_2^{-\cdot}$.

Addition of peroxynitrite at physiological pH will oxidize, nitrate or nitrosylate a wide variety of biological molecules. Many researchers also observed hydroxylation of aromatic molecules to occur, which led to the suggestion that ONOOH could dissociate to yield OH· radicals. This provoked an outcry from certain chemists, who like to predict results based upon thermodynamic and other theoretical considerations. The irrelevance of these calculations to biomedical science is illustrated by the fact that merely changing one of the assumed parameters in the calculation changes the reaction

$$ONOOH \rightarrow NO_2\cdot + OH\cdot$$

from "thermodynamically impossible" to "the preferred reaction pathway."[29] One can never know the real values of thermodynamic parameters *in vivo* and thus can never make meaningful calculations. Experimental data show that $ONOO^-$ at pH 7.4 may make some OH·, but not much[30,31] and data should always be preferred over theory. One should never be dogmatic in science—look at what happened to the "central dogma"! The god-like hospital consultants of the early days of the National Health Service in the UK always "knew better" than their patients or their GPs. It has been said that "they were often in error, but never in doubt."

FROM TBA TO DIODE ARRAY

When we came to write the first edition of our textbook *Free Radicals in Biology and Medicine* in 1985,[17] the only chapter dealing with the measurement of biomolecular damage by free radicals was that on lipid peroxidation. This was mainly because there were few other simple methods available for application to clinical and biological material, and partly because lipid peroxidation, as a radical chain

reaction, has intrigued scientists since its elucidation in the 1940's, incidentally, not by life scientists but by polymer scientists at the British Rubber Producers Association.[17] The key role of lipid peroxidation in causing rancidity in foods became increasingly apparent with the trend towards more pre-packaged "long life" food materials, kindling intense interest in the quantification of food oxidative deterioration and the development of protective antioxidants (of which the simplest is to pack food *in vacuo* or under nitrogen). The first toxin shown to act by a free radical mechanism, CCl_4, causes damage by stimulating lipid peroxidation, easily detected in animals, cells and microsomal fractions.[32]

Another reason perhaps why we all stayed with lipid peroxidation for so long, without looking for damage to other key biological molecules, was that almost all the methods used to detect it were so simple, such as measuring peroxides with iodide, thiobarbituric acid reactivity (TBAR) and UV spectra (the dreaded diene conjugates, which have generated almost as many artifacts as TBAR). TBAR came originally from the food industry where it was used to detect rancidity in foods containing polyunsaturated fatty acids. Just heat some food, biological tissue or fluid with 2-thiobarbituric acid under acid conditions and you will get a beautiful pink color—anybody can do it! And so they did. Free radicals could now be implicated in every disease process known to man (TBAR probably goes up in all of them). Understanding of what this method really measures took decades to penetrate the free radical community and penetration is not yet complete. To many (but not all) editors and reviewers, during the 1990's TBA has become "that bloody assay." The TBA test does have value in examining the oxidation of defined lipid systems (foods, microsomes, etc.) but cannot be used to compare peroxidation of different systems with different PUFA compositions (different PUFAs generate different levels of TBAR) and, used alone, is an unreliable index of levels of lipid peroxidation in cells, tissues or body fluids.[17]

The appeal of the microsome and its abnormal propensity to undergo lipid peroxidation *in vitro* (especially if stored frozen for long periods) eventually began to fade. During the late 1980's and early 1990's interest in free radical damage in biological systems started to move away from lipids and consider proteins and nucleic acids, especially DNA. Ironically, many basic chemical studies of DNA and protein oxidation had already been done (reviewed in refs. 33–35). The knowledge just needed application to biological material. The days of the HPLC and mass spectrometer had arrived: techniques that were also applied to get much more accurate estimates of lipid peroxidation in biological materials. This gave us for the first time an overall picture of the oxidative damage that occurs in cells and tissues.[17,34,35] We learned that (unlike CCl_4) most toxins acting by free radical mechanisms do not primarily act via lipid peroxidation, and that oxidative damage to DNA and/or proteins is often more important as a primary cytotoxic mechanism than is lipid peroxidation. This change in emphasis is reflected in the third edition of our textbook: lipid peroxidation no longer merits a chapter to itself.[17]

CAUSE OR CONSEQUENCE

TBAR-material (and, more recently, better-authenticated products of lipid peroxidation) are found in body fluids and tissues in increased amounts in almost any disease state and in animals given virtually any toxin. During the 1970's–1980's this led to the assumption that free radicals were the cause of many diseases and lipid peroxidation the primary mechanism of action of many toxins. If the former were true, the hope was, particularly for SOD, that antioxidants inhibiting peroxidation would cure many diseases. It was soon clear to many researchers that free radicals did not cause a plethora of diseases, neither were "spoonfuls" of SOD or vitamin E going to modify them, let alone cure them. In 1984, we pointed out[36] that increased free radical formation was probably an inevitable consequence of most diseases in which tissue damage occurred. Although considered by many to be heresy at the time, it is now a widely accepted (although still periodically "rediscovered") explanation for increased free radical activity. For example, reactive oxygen species are generated in increased amounts in cells undergoing apoptosis triggered by a range of mechanisms, but they are not essential constituents of the apoptotic pathway.[37] The same "consequence but not cause" concept is probably true also for many other alleged mediators of tissue injury in human disease, including NO· and cytokines. Levels of these, as well as of reactive oxygen species, all go up as part of the response to injury.[38] In some diseases, excessive production of NO· will turn out to be important (e.g. septic shock perhaps), in others, one or more of the cytokines (e.g. TNFα in rheumatoid arthritis perhaps). The same is true of the reactive oxygen species. There is good evidence for their role in atherosclerosis, suggestive (but not conclusive) evidence for their importance in the major neurodegenerative and chronic inflammatory diseases, and little evidence for an important role in the majority of human diseases.[17,38]

By the 1990's it was clear that antioxidants are not a panacea for aging and disease, and only fringe medicine still peddles this notion. What has become clearer however, is the importance of certain dietary antioxidants in *preventing* life-threatening diseases, such as heart disease and certain types of cancer (reviewed in refs. 39 and 40). Even here, however, simplistic assumptions about the biomolecules responsible (mistaking correlation for causation) may have given us a biased view of which constituents of fruits, grains, vegetables are the most protective (discussed in ref. 41). Thus diets rich in β-carotene protect against cancer development, but β-carotene itself does not (at least, not by an antioxidant mechanism). Twenty years of nutrition research have told us that for "advanced" countries the way to a healthy lifestyle is to eat more plants, a concept familiar to Hippocrates. What it has not told us is exactly why. Nevertheless, the evidence for vitamin E as an agent maintaining the health of the cardiovascular system is growing daily.[39] Research using accurate measures of oxidative damage to proteins, lipids and DNA ("biomarkers") should help us to learn more about the other protective antioxidants in a plant-rich diet.[41]

LET'S TALK

The general lack of success of antioxidant therapy (at least using SOD or chain-breaking antioxidants) may have been a major contributor to the shift away from limitation of the damage caused by reactive oxygen species to one of interest in their physiological roles, e.g. in biological signaling. Thus, sub-toxic levels of reactive oxygen species have been shown to play important roles as signal, trigger, and messenger molecules in cell culture systems.[42–45] Nitric oxide is well-known for playing such roles *in vivo*, but as yet evidence that reactive oxygen species do the same is sparse. One must be wary: cells in culture are frequently hyperoxic and usually lacking many of the antioxidants that would surround them *in vivo*. In particular, ascorbate is rarely added to culture media. Cells adapt to growth in culture. It is not impossible that, with tumor cell lines in particular, adaptations to favor growth may occur by using oxygen radicals to trigger pathways that are triggered by other means *in vivo*.[46]

An early clue that such roles for ROS and RNS might be biologically important came from comparing the different patterns of antioxidant protection used by cells, membranes, and extracellular fluids. Briefly, cells use enzymes and other antioxidants to control ROS levels so that intracellular iron can signal within cells for synthesis of iron-containing proteins to achieve iron homeostasis.[24] In extracellular fluids, however, we see the reverse situation whereby proteins remove or control iron so that reactive species persist for a short while, perhaps to act as signal molecules.[47] This is why introduction of excess iron or copper ions into the extracellular environment has the potential to cause oxidative damage.[47] *In vivo*, normal cells function in a reducing environment, and as this is changed to a more oxidizing (or less reducing) environment cell functions change. Cell proliferation followed by apoptosis, and eventually necrosis are observed *in vitro* when cells in culture are subjected to ever increasing conditions of oxidation.[44,48] Even the caspases of apoptosis are redox-regulated: oxidation by high levels of reactive oxygen species inactivates them[48] and it has been proposed that tumor cells have evolved an abnormally high "oxidation state" to do exactly this.[49]

If reactive oxygen species are intimately involved with the redox regulation of cell functions, which seems likely based on current evidence, then it is perhaps easier to understand why attempts to change antioxidant balance in aging experiments have failed. The cell will adapt to maintain its redox balance. Indeed, transgenic animals over-expressing antioxidants (e.g. CuZnSOD and glutathione peroxidase) show some abnormalities of function.[50,51] There must therefore be a highly complex interrelationship between dietary, constitutive, and inducible antioxidants within the body, under genetic control. The challenge for the next century is to be able to understand these relationships, and how to manipulate them to our advantage to prevent and treat disease.

CONCLUSION

Dan and his colleagues make a fundamental contribution to biology in their seminal paper.[1] Look how far it has taken us, and how far we have yet to go. We look forward to the future with confidence, and await the surprises that will surely come.

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