Biological Effects of Low Level Exposures

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PART 1. The Role of ROS in Health And Disease
PART 2. Proposing a Definition of Hormesis

INTRODUCTION

This issue of the BELLE Newsletter is devoted to two significant topics - that of (1) assessing the role of reactive oxygen species (ROS) in health and disease and (2) proposing a definition of hormesis. In the case of the ROS section, I invited a number of recognized experts to independently address the following questions in 2,500 words or less.

a. Does the concept of ROS being metabolic messengers affect the dose response function regarding defined cellular reactions to changes in cellular ROS concentrations?

b. Do various levels of cellular ROS concentrations from different sources affect the paradigm of chemical and/or radiation carcinogenesis?

c. May concentration dependent responses to ROS in mammalian cells affect the practice of quantitative risk assessment from exposure to ROS-delivering reactions such as by way of ionizing radiation, as currently practiced by most regulatory agencies worldwide?

The experts were informed that their contribution would be summarized by Dr. Ludwig Feinendegen, formerly of the NIH.

The defining hormesis paper was written by Linda Baldwin and myself. It was sent to a broad spectrum of scientists from academia, government and industry. Their unedited commentary is presented. We then offered a response to their commentary. Finally, we encourage the readership to submit letters to the editor in response to these papers.

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PART 1: THE ROLE OF ROS IN HEALTH AND DISEASE

OXIDANTS AND ANTIOXIDATIVE DEFENSE

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The role of oxygen free radicals and other oxidants in several diseases has been well established over the past decade. Whereas it was long known that high doses of oxidants may damage or kill cells the effect of low doses or long time exposure to small flux rates of oxidants are in the focus of the free radical research until now. This includes effects of oxidants on signal transduction pathways and gene expression as well as the role of oxidants in carcinogenesis, fibrosis or other.

For long time the effect of free radicals and other oxidants on cells or tissues was described as bad and as to be prevented by antioxidants. At that time the antioxidative defense was mostly seen as what today is called the primary antioxidative defense. This primary antioxidative defense includes the well known and described catalases, superoxide dismutases, the glutathione system and an increasing number of antioxidants, including several vitamins. The impact of secondary antioxidative defense systems, like repair mechanisms and damage removal systems, was largely unknown. Consequently intake of antioxidants was thought to be the most effective influence on the antioxidant system. This opinion was supported additionally by numerous companies and the resulting support of the research into this direction. Today we gathered an increasing knowledge of the action and interaction within the antioxidant network. Therefore, one has to ask the question whether it is really healthy to disturb the antioxidant balance and redox chains by supplementation with single compounds? Since we know that the number of naturally occurring antioxidants amounts to several hundred and probably more compounds, mostly with unknown bioavailability and metabolism, it is difficult to foresee the effects of an unnatural high intake both of a single or several compounds.

As mentioned above it is known today that the primary antioxidative defense systems are only one part of the complex cellular strategies to deal with oxidative stress. Repair mechanisms, like DNA repair, are long time known. Especially under the highlight of apoptosis research the DNA/chromatin repair seems to be one key event where the cellular fate is decided: apoptosis or survival. The activation and action or the cleavage and inactivation of the poly(ADP)-ribose polymerase (PARP) is one of the decision points on the way to the apoptotic cell death. Other repair mechanisms of cellular systems are only in the research interests within the last time, including protein repair and removal [1] and membrane repair [2]. But secondary antioxidative defense seems to be more than the removal or repair of damaged compounds. One of the key events is the adaptive response of most cell types and organisms to oxidative stress. Although, the adaptability seems to be important for survival on cellular and organism level for the single organisms and during evolution, relatively little is known about the mechanisms involved. It is known that stress situations, including those with oxidative components, are followed by adaptation in humans [3, 4]. The adaptation strategies are complex and include several organ systems, e.g. the cardial system and regulation of the blood flow, the immune system etc., and on the other hand the regulation of gene expression and protein synthesis on a single cell level.

Attempts were made to search for the mechanism of the oxidant induced gene regulation in mammalian cells. Until now no conclusive model could be demonstrated. Most probably oxidants can act via changes of the redox equilibrium of the cell and on the other side it is possible that several "sensor sites" of proteins can be oxidized by specific compounds. It is most likely that these "sensor sites" are acting as an intracellular second messenger and pass the activation signal to several transcription factors, like NF-kB, AP-1 or CREB. It was demonstrated that at least 40 various genes and perhaps more can be activated by hydrogen peroxide in mammalian cells [5]. Several of these genes are known, like enzymes of primary or secondary antioxidative defense, but there is also a number of genes with unknown function. Therefore, mammalian cells posses a wide spectrum of responses to oxidative stress and ROS depending on the oxidizing compound, the doses and flux rates of this compound, the environment and many more.

A summary of the effect of oxygen free radicals on mammalian cells was given by Davies [6]. Here the dose dependency of oxygen free radicals on dividing mammalian cells was reviewed. Small doses of oxidants (in most studies hydrogen peroxide was used) cause an increase of proliferation of these cells. Rising doses cause a temporary growth arrest. The time of the growth arrest is in the order of several hours and usually ends with "adopted" cells. That means if these cells are treated repeatedly with oxidants they are more resistant. Since this adaptation is transient after longer time periods, the cells are again naive. (This is different to several selec-

tion strategies, which one may use to get clones with a permanent higher resistance towards oxidation induced damage.) If one increases the doses furthermore cells can come into a state of permanent growth arrest. These cells do not divide any more, but they fulfil normal metabolic functions. Often this stage was misinterpreted as dead cells, especially in assays which are relying on cell division parameters. Several authors take these cells in permanent growth arrest as a model system for senescent cells. If one treats cells with even higher concentrations of oxidants the cells begin to die via the apoptotic or via the necrotic way.

Therefore, this variety of cellular responses to oxidative stress beginning from mitogenic responses at low doses and ending with death at high doses, rises questions about the effects of chronic exposures, the effect of various oxidant species, and the effect of the site of oxidant generation. Unfortunately most of these questions are unsolved until today and give a wide field of research within the following years. The relevance of these questions seems to be very important in regard to human health problems. On the other hand it is questionable whether antioxidant therapies interfere with attempts to treat cancer patients. It seems to be likely, that the antioxidative defense of normal tissue may be as well improved as that of cancer cells, at least in certain forms of cancer. On the other hand it seems to be interesting to undertake further attempts to interfere with the secondary antioxidative defense strategies of cells and with adaptive mechanisms. Under these aspects the exposure of cancer cells to non-lethal doses of irradiation seems to be dangerous, taking the adaptation and/or selection of more resistant cancer cell clones into consideration. This danger is not only real for irradiation based therapies of cancer cells, but also for the chemical interventions.

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OXIDANT SIGNALING IN CARCINOGENESIS: A COMMENTARY

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The accumulation of reactive oxygen species (ROS) as byproducts of normal energy metabolism and in response to inflammatory conditions or ROS generating environmental exposures has long been associated with the pathogenesis of cancer in rodents and humans(1). In this context ROS are considered toxic, and disrupt cellular homeostasis through damage of macromolecules such as lipids, proteins, and nucleic acids.

However, the concept that ROS serve a signaling role in carcinogenesis is relatively new. Investigations in this field suggest that ROS acting as metabolic messengers influence mainly the promotion and progression stages of experimental carcinogenesis. Certain cell-signaling molecules involved in these events are targets for redox modification and functional alterations that mediate oxidant –induced cellular responses.

The review article by Hsu et al.(2) extensively discusses the role(s) of oxidant signaling in transformation. Clonal variants of mouse epidermal JB6 cells identified for their differential susceptibility to tumor promoters also show differential reduction –oxidation (redox) responses. Oxidative events are known to regulate transactivation of transcriptional factors such as AP-1 and NFkB. The authors demonstrated that AP-1 and NFkB showed differential protein levels or activation in response to tumor promoters in JB6 cells. Furthermore, AP-1 and NFkB are both required for maintaining the transformed phenotypes.

Protein Kinase C (PKC) is another important target of ROS that has relevance in tumor progression. Activation of PKC is a critical event in carcinogenesis. By activating PKC, phorbol esters and related tumor promoters appear to bypass the normal cellular mechanisms for regulating cell proliferation. Gopalakrishna and Jaken(3) have reviewed the role of oxidant signaling and PKC in tumor promotion and progression stages of carcinogenesis. Oxidants selectively react with the regulatory domain of PKC, stimulate cellular PKC activity, and signal for tumor promotion.

ROS also act as mediators of mitogenic signaling in

fibroblasts transformed by oncogenic ras, a pathway that requires the activation of a NAD(P)H oxidase regulated by the small GTPase rac1(4). Along these same lines, Gupta et al.(5) conducted studies in mouse keratinocyte cell line that carries an activated ras gene. Constitutive Erk-1/2 and p38 MAPK activities were identified as important components of ROS-mediated mitogenic signaling in these radiation- and MNNG-progressed tumor cell lines.

Oxidant signaling can also modulate gene expression that may have important function(s) in carcinogenesis. A recent article by Deng et al.(6) identified the mechanism by which hepatocarcinogen 2-acetylaminofluorene (2-AAF) efficiently activates rat mdr1 expression. 2-AAF treatment led to generation of intracellular ROS, which causes activation of IKK kinase, degradation of IkappaB beta and subsequent activation of NFkB that upregulates mdr1b.

In contrast to experimental carcinogenesis, humans are generally exposed to mixtures of agents that can simultaneously act at different stages of the carcinogenesis process. Thus in humans, promotional events which frequently increase cellular proliferation or decrease apoptosis may at least theoretically influence subsequent initiation events. Thus oxidant signaling by influencing promotional events may influence initiation events in human carcinogenesis.

In summary, aided by recent studies demonstrating a role for ROS as metabolic second messengers in tumor cells, the state of knowledge in the field of carcinogenesis is rapidly expanding. Most studies suggest that the signaling function of ROS in the promotion and progression phases of experimental carcinogenesis supports existing paradigms for oxidants in tumor development. It is likely that such studies will identify novel molecular targets that will spur the development of more effective chemopreventive and chemotherapeutic agents.

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EXPOSURE TO LOW LEVEL CHEMICALS AND IONIZING RADIATION: REACTIVE OXYGEN SPECIES AND CELLULAR PATHWAYS

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BACKGROUND

The very essence of life is the exploitation of thermodynamically favorable reactions that provide the bases for metabolism, self-replication, healing, adaptation to the environment, and the ability to modify the environment. Key to such reactions under homeostatic conditions are extra- and intracellular energy landscapes consisting of reactive molecules and local ion concentrations, *inter alia*, which cells both control and respond to in ways that decisively figure into the regulation of protein-protein and protein-nucleic acid interactions and their functional outcomes. Contributors to the energy landscapes in the micromilieu in and around our cells are reactive oxygen species (ROS), including superoxide anions, hydrogen peroxide, hydroxyl radicals and oxides of nitrogen of metabolic origin or that occur as a consequence of reactions secondary to metabolism. Reaction of these with other biomolecules, such as lipids, proteins and DNA, can lead to the production of yet other reactive molecules. Balancing the modifying and potentially detrimental effects of ROS and their reactive products on cellular molecules are yet other sets of molecules that serve to limit the duration of availability of ROS, either via enzymatic conversion of the species or by performing as free radical scavengers. Additional biochemical mechanisms maintain the reducing potentials in the various cellular subcompartments, while still others cope with oxidatively damaged biomolecules. The latter includes the enzymatic repair of DNA, which

can undergo oxidative modifications even under normal basal metabolic conditions, or, as in the case of proteins with oxidized residues, accelerations in their rates of degradation, all again being driven by local energy landscapes.

When appropriately orchestrated, the inherent protective strategies diminish the likelihood that endogenously produced ROS will cause untoward effects such as cell killing, DNA/gene mutations and carcinogenesis, while preserving the roles of ROS as mediators in many crucial, finely tuned cellular pathways. At the level of the whole cell, this condition may best be defined as a state of cellular redox balance, with recognition of the existence of localized macro- and nanoscale differences in oxidative and reduction potentials in different cellular subcompartments.

The abrupt imposition of extraordinarily high levels of ROS due to acute exposures to ionizing radiation (IR) and chemicals that either directly produce or stimulate the metabolic production of ROS would reasonably be expected to alter the overall redox status of cells, however temporarily until defensive countermeasures are put into place. As a consequence of an excessive preponderance of ROS, energy landscapes that drive protein-protein and protein-nucleic acid assemblies and dissembles correspondingly would be expected to be disturbed by their actions, with attendant alterations in cell functions in addition to inducing molecular damage *per se*. Predicting outcomes from such changes, especially when ROS are only modestly increased above basal levels, which can result from some low dose exposure scenarios, remains elusive. Progress toward doing so, however, likely will be made as our understanding of relationships among local extra- and intracellular energy landscapes, reactive oxygen species, and the regulation and functions of proteins and their complexes are further elucidated.

The concept of a "toxic threshold" for ROS, which can vary in level of occurrence as a function of ROS load and ROS type in one cell phenotype to another and be altered by a cell's prior history of exposure to ROS-generating stimuli as well, has become essentially dogma in the scientific literature. Accordingly, the toxic threshold is surpassed and pathogenic responses occur when elevated ROS concentrations overcome antioxidant defenses and are excessively abundant beyond levels required for normal physiological regulations. However, it is unclear that even the normal basal fluctuations and levels of ROS in our cells are always below the so called toxic threshold as defined above, given, for example, the occurrence of DNA oxidative adducts in our cells. In what follows, responses to the questions mainly focus on sublethal levels of ROS that are induced by low dose IR and low doses of chemicals that modestly, yet significantly increase ROS either directly or via activation of ROS-generating metabolic pathways.

1. Does the concept of ROS being metabolic messengers affect the dose response function regarding defined cellular reactions to changes in cellular ROS concentrations?

There is no doubt that ROS generated by a variety of enzymatic reactions play important signaling roles in the regulation of numerous cellular activities, e.g., proliferation, smooth muscle cell tone, antimicrobial defense, and gene expression, to name a few (e.g., Rao and Berk, 1992; Burdon et al., 1994). Clearly, exposures to high doses of ionizing radiation (IR) and some chemicals that cause the generation of high cellular levels of ROS are frankly harmful and result in biological effects ranging from cell cycle delays to senescence to apoptosis. Unfortunately, there is only limited information about how the imposition of supra-basal, yet sublethal levels of cellular ROS by environmental stresses like low-level IR modifies normal biochemical reactions in cells that involve basal levels of and metabolically controlled fluctuations in ROS. Further, where information is available, interpretations of how supra-basal levels of ROS affect the dose-response characteristics of ROS-mediated reactions are mainly inferential in terms of "cause-and-effect". Nevertheless, mounting evidence suggests that environmentally-induced, low level increases in ROS can substantially impact on ROS-related mechanisms in a manner that activates pathways, which otherwise may be transiently silent in the absence of normal stimuli or cell state. As a specific example, exposure of human cells to a low 1 cGy dose of α-particles can result in persisting increases in intracellular superoxide anions and hydrogen peroxide via activation of NADPH/NADH oxidase (Narayanan et al., 1997). The transfer of supernatants from the irradiated cells causes the same level of induction of ROS in unirradiated cells as a so called "bystander effect" (Narayanan et al., 1997). While IR and cellular ROS are often considered in the context of radiolytic mechanisms at play during exposure, it is now evident that low level IR and its interactions with biomolecules can result in more persisting increases in ROS that are of metabolic origin. After manifesting a cell cycle delay as of 1 day after exposure, which was attributable to increases in the tumor suppressor protein TP53, the directly irradiated cells subsequently showed increases in their growth rates relative to control cells. Cells that received the supernatants from irradiated cells showed no evidence of a cell cycle delay, and, in fact, grew even faster. The supra-basal increases in intracellular ROS and enhanced cell growth rates have sequentially been attributed to an initial IR-induced generation of extracellular ROS and ROS-associated prompt increases in transforming growth factor-β1 in irradiated cell supernatants, which, in turn, activate cellular NAD(P)H oxidase (Lehnert and Iyer, 1999; Iyer and Lehnert, 2000). Numerous lines of evidence indicate that low level increases in intracellular ROS occurs in proliferating cells and that cell proliferation can be enhanced by treating cells with low level, but not excessive ROS (Burdon, 1995).

We have also observed that the transcription factor NFkB is translocated to the nuclei of cells experiencing the supra-basal increases in ROS, which accounted for

increases in the subsequent transcription and cellular production of the chemokine interleukin-8 (Narayanan et al., 1999). Thus, ROS-sensitive pathways involved in the regulation of gene expression and cell proliferation, can be activated under some conditions by ROS-inducing environmental stresses. Such effects likely are due to direct actions of ROS on specific proteins/protein complexes involved in signal transducing pathways that mediate the effects, as well as the redox status of transcription factors. On the other hand, decreases in species like glutathione conceivably could affect the redox status of signal transducing proteins and proteins involved in cell cycle regulation (Burdon et al., 1994) as another side of oxidant-antioxidant balance. Regardless, the induction of metabolically produced ROS by low dose IR minimally appears to "mimic" endogenous conditions that stimulate cell growth. Presumably this is accomplished by ROS serving as second messengers in the same cellular pathways as they do in response to growth factors and cytokines (e.g., Sundaresan et al., 1995; Thannickal et al., 1993; Umans and Levi, 1995). The "central dogma" of pre-modern radiation biology that DNA damage and repair determine the biological effects of IR observed at the cellular level obviously requires reconsideration.

Do ROS-generating environmental stimuli affect cellular responses involving ROS and related pathways upon subsequent exposure to ROS-inducing stresses in a manner that affects cellular dose-response profiles? The literature regarding adaptive responses induced by ROS-generating stimuli suggests the answer here is generally "yes", at least in the context of adaptive-type responses. However, investigations of adaptive responses have yet to provide generalized insight into this matter in terms of harm or benefit from all possible biological outcomes. Decreased radiosensitivity, for example, points to a fundamental alteration in the IR dose-response characteristics as one potentially positive effect that may have ROS underpinnings. Yet, reports that cells experiencing an IR/ROS-associated adaptive response can manifest more complex types of DNA damage when subsequently exposed to another bout of IR than they would otherwise (Ueno et al., 1996) and even become more susceptible to malignant transformation (Sasaki, 1996) are especially troublesome from a human health perspective.

2. Do various levels of cellular ROS concentrations from different sources affect the paradigm of chemical and/or radiation carcinogenesis?

Most models of chemical and radiation-induced carcinogenesis include genomic mutations and cell proliferation. In addition to stand breaks, a large number of different types of DNA base modifications caused by ROS have been detected. Coupled with the fact that oxidative DNA adducts like 8-hydroxyguanine are particularly prone to yield GC to TA base-pair transversions, cell division contributes to the mutagenic effects by converting DNA lesions to point mutations, deletions, or translocations. The idea that a persistent state of oxidative stress plays many roles in cancer, ranging from activation of transcription factors like NFkB, the induction of proto-oncogenes, e.g., c-*fos*, c-*jun*, c-*myc*, and the induction of modified DNA base products and strand breaks, which presumably can result in progressive accumulations of the necessary array of mutations for cell transformation and metastasis, is gaining experimental support in the inflammation and cancer literature (e.g., Christen et al., 1999; Wiseman and Halliwell, 1996). With inflammation, the source of ROS can usually be attributed to their metabolic production by inflammatory leukocytes stimulated by innate and/or adaptive immune mechanisms. That ROS from these sources can be mutagenic and malignantly transform other targets cells was demonstrated years ago (Weitzman and Stossel, 1981; Weitzman et al., 1985). Such findings indicate that extracellular sources of ROS like those produced by phagocytes undergoing respiratory bursts capably gain access to and damage the DNA of yet other cells. How this is accomplished in target cells in terms of the ultimate mediator(s) of DNA damage in a background of cellular antioxidant defense mechanisms is unclear. Nevertheless, a lesson here seems to be that external sources of ROS generated by diverse environmental stimuli including low dose IR (Iyer and Lehnert, 2000) and materials such as asbestos (Kamp et al., 1995) may have important DNA-damaging consequences, while additionally favoring enhancements in cell growth. ROS in this regard hypothetically may induce a transient condition akin to a mutator phenotype, perhaps more persistently under chronic conditions of ROS exposure. Perhaps a key to understanding ROS, mutations, and cell growth as they pertain to cancer may be gained from experiments in which cells are exposed chronically to near ambient, yet supra-basal cellular levels of ROS as opposed to the acute exposures to much higher concentrations of ROS that are often used in many investigations of the role of ROS in carcinogenesis and mechanisms of related phenomena like genomic instability.

Intracellular, metabolically-derived sources of ROS are numerous. These include the diffusion of hydrogen peroxide produced by activation of cell membrane associated NADH oxidase and superoxide anions generated from activated cell membrane-associated NADPH oxidase, which gain intracellular access via anion channels, ROS from mitochondria, and ROS from peroxisomes, to cite only some. While ROS from all of these sources have been variously implicated in carcinogenesis, details about their endogenous levels of ROS production and how the imposition of exogenous sources of low yet supra-basal levels of ROS by chemicals and IR affects the initiation and promotion of cancer are lacking. Even so, if ROS-associated adaptive responses, for example, indeed provide protection against subsequent exposures to further bouts of oxidative stress via up-regulations in DNA repair mechanisms and antioxidant defenses, then

the possibility exists that the mutational and cell proliferative components of conventional carcinogenesis paradigms may become less active in cells so affected under some circumstances. Associations between conditions of chronic inflammation and cancer, however, seem to directionally argue against this possibility. Again, keys to this matter may be the levels of ROS that are produced relative to basal levels, the types of ROS that may be involved, and responding cell phenotypes.

3. May concentration dependent responses to ROS in mammalian cells affect the practice of quantitative risk assessment from exposure to ROS-delivering reactions such as by way of ionizing radiation, as currently practiced by most regulatory agencies world-wide?

Risk assessors are continuously striving to decrease the uncertainty in risk assessment by refining epidemiological studies and by incorporating biological mechanisms into their analyses. Yet, models of risk to a disease such as cancer relative to exposure to ROS-delivering or inducing stimuli, particularly low dose exposures, remains a daunting challenge. The wide range of environmental stimuli that can lead to the production of ROS in humans in even relatively pristine settings and how these "background" and likely fluctuating levels of ROS in our tissues may affect subsequent biological responses to other ROS challenges like low dose IR in an antagonistic, additive, or synergistic manner must be understood. Other matters such as the heterogeneity in antioxidant defenses and DNA repair capabilities that can occur in one person to the next need to be understood as well if the future promise of individualized risk assessment to low dose IR or chemicals is to ever be realized.

Biologists obviously have an important role toward these ends, but our tasks, as with the risk assessors, are likewise challenging. While experiments may be straightforward, interpretations of their results that may be subsequently incorporated in risk models are not necessarily so. Based mainly on cell survival data, most investigators, for example, believed that the untoward effects of high LET a particles required nuclear "hits" by the a particles. Numerous microdosimetric models, including the Human Respiratory Tract Model for Radiological Protection (1994), have been developed for assessing a-radiation dose to sensitive airway cells in the lower respiratory tract and for estimating cancer risk due to the inhalation of radon/radon progeny (James, 1988; Hui et al., 1990; Fisher et al., 1991; Hofman et al., 1991). An underlying assumption shared by these models is that traversals of a particles through the nuclei of target cells, e.g., basal and secretory cells, along the conducting airways alone are of primary concern in terms of posing a cancer threat. In this regard, prior expensive and meticulous efforts had been directed toward morphometrically characterizing distances to the cell nuclei for use in calculating the probability density in specific energy delivered to the cell nuclei by a+particles from the decay of inhaled radon/radon progeny. We now know that many effects associated with a particle-induced carcinogenesis can occur in the absence of nuclear traversals and even whole cell hits, and that some of the processes are associated with the extracellular and intracellular generation of ROS.

Some mention should be made about how ROS-related mechanisms may affect the very nature of dose-response relationships for ROS-generating stimuli like IR in the context of cancer. While carcinogenic effects of high doses of IR can not be denied, a widely held belief remains that exposure to IR is detrimental to human health, regardless of dose. Even among radiation biologists and regulatory scientists, this latter view, which presupposes existence of a proportionality between high-level exposures and the health consequences of low-level IR, continues to dominate in scientific and regulatory circles. Such notions have led to another "radiation paradigm" (summarized in Loken and Feinendegen, 1993) upon which is based potentially overly restrictive exposure standards and expensive remediation efforts at environmental sites that have even minor radioactive contamination. According to this paradigm: 1) radiation exposure is harmful at all doses, i.e., "linear-no threshold theory", and 2) there are no effects at low doses that cannot be predicted from effects observed at high doses. Thus, the paradigm does not allow for the possibility that the detrimental effects of ionizing radiation (IR) may only occur above a threshold dose and/or that low dose IR may induce responses that may be adaptively beneficial and not pathogenic. After all, living organisms have been exposed to natural background IR since life began on earth.

We have obtained evidence that another bystander response to low dose IR that occurs along with the generation of supra-basal levels of ROS is a decrease in basal protein levels of TP53 and $p21^{Waf\text{-}1,\ Cip1}$ and increases in the proteins PCNA and CDC2, which are all accompanied by the condition of enhanced cell growth (Iyer and Lehnert, 2000) discussed earlier. Unlike normal cells that show an induction of TP53 after irradiation with as little as 1 cGy of α-particles or γ-rays, cells experiencing these effects fail to mount a normal TP53 response when likewise irradiated. More recently, we have found that irradiation of cells experiencing these effects show diminished radiosensitivity and increases in the DNA repair and redox protein AP-endonuclease, i.e., a bystander-mediated adaptive-type response (submitted for publication). Such findings: 1) provide further evidence that IR-induced effects can occur in unirradiated cells as a further complexity that must ultimately figure into risk assessment models, and 2) demonstrate that the very nature of "normal" IR dose-response profiles can be altered in pre-existing conditions in which basal levels of ROS are elevated. A remaining challenge is to determine if the modified dose-response profiles in bystander cells and directly

irradiated cells showing adaptive responses are harmful or beneficial and under what conditions they may be either or both. We must conclude that quantitative, realistic risk assessment models must accommodate these new and related types of results, preferably from a predictive perspective based on new knowledge about how ROS affect proteins and the functions of their complexes, our cells' nanomachines.

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REACTIVE OXYGEN SPECIES AS DOUBLE-EDGED SWORDS IN CELLULAR PROCESSES: LOW DOSE CELL SIGNALING VERSUS HIGH DOSE TOXICITY

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INTRODUCTION

Over the past several decades, studies have clearly shown the involvement of reactive oxygen species (ROS) in the elaboration of multiple pathologies such as radiation injury, carcinogenesis, aging, ischemia-reperfusion, and atherosclerosis [1, 2]. ROS damage cells by interactions with critical macromolecules including DNA, proteins, and lipids leading to cell death, mutation, and other toxicities. The recognition of ROS-associated mutagenesis and carcinogenesis stimulated discussion and speculation regarding the shape of the dose-response curve for exogenous agents. Is the curve linear at low doses or are there cellular antioxidant defenses that protect cells from low doses of oxidative damage? Whether the dose response curve for ROS-mediated damage is linear, nonlinear, or exhibits a threshold is not known. High doses are clearly toxic, but the effects of low doses are less clear. In addition to the toxic effects, low levels of ROS may modulate protein structure and function in a physiologically relevant manner resulting in activation of signal transduction pathways leading to modulation of gene expression. The recent discovery of involvement of ROS production in a multitude of normal biological processes defines a new role of ROS in normal cell homeostasis rather than just toxic damage to cellular macromolecules [3, 4]. This may result in a reduced or enhanced effect of the toxic actions of ROS.

In addition to understanding the impact of ROS, it is important to understand the beneficial (and harmful) effects of antioxidants. Antioxidants can protect against toxic effects of ROS but also inhibit the normal physiologic role of ROS. Furthermore, during the cancer process, preneoplastic cells acquire a pro-oxidant state that elevates the rate of cell death of the cancer cell. Antioxidants may protect cancer cells from oxidative stress-induced suicide and thereby accelerate cancer progression. Thus, the role of ROS in maintaining redox balance in the cell may influence cell balance in favor of clonal expansion instead of increased cell death. This apparent paradoxical relationship between ROS production, cellular redox status, and cell function may qualitatively alter cellular responses at different levels of oxidative stress and complicates consideration of risk assessment in the context of ROS production and inhibition.

SOURCES AND ACTIONS OF ROS

Sources. **The potential contribution of ROS to cellular processes is apparent when considering the availability and diversity of ROS from numerous sources. Normal oxidative metabolism is a key endogenous generator of ROS with the mitochondria serving as a major site of superoxide production. Other endogenous sources of ROS include NADPH cytochrome P450 reductase, xanthine oxidase, NADPH oxidase, lipoxygenase, and cyclooxygenase [2]. Immune responses by phagocytic cells also produce a spectrum of ROS including superoxide, hydrogen peroxide, and hypochlorous acid [5]. Major exogenous sources of ROS-mediated pathogenesis are tobacco smoke, fatty acids in foods, transition metals, and ethanol which produce lipid peroxides, organic radicals and hydroxyl radicals [5]. Physical agents inducing ROS include gamma ray, x ray, and ultraviolet irradiation. Other widely accepted producers of ROS that alter cellular responses include redox cycling quinones, thiol alkylating agents, aldehydes, and heavy metals. Clearly, numerous and diverse sources of ROS exist that may disrupt cellular homeostasis or damage cells.**

Mutagenesis. **Increased oxidative stress and excessive ROS production damage DNA. Both endogenous and exogenous sources can cause base modifications and DNA strand alterations. Oxygen radicals produce more than thirty different DNA adducts with each potentially mutagenic and contributing to the etiology of cancer [6]. Moreover, the release of ROS from endogenous oxidative metabolism alone is estimated to cause ~10^4 mutations per day [7]. Although the majority of damage is**

removed by specific and non-specific repair processes, a small number of lesions escape repair and possibly accumulate with age.

High levels of unrepairable damage to DNA lead to cell loss by one of two forms of cell death that are distinct morphologically and biochemically. Severe and sudden injury by excessive ROS can lead to necrosis that is characterized by extensive plasma membrane damage and loss of cellular homeostasis. Alternately, cells may die by apoptosis which is an active process of cellular self-destruction that removes genetically damaged, pre-neoplastic, or senescent cells without eliciting an immune response. Apoptosis can also be induced by cytotoxic insults including ROS [8].

Effect of antioxidants on mutagenesis. Endogenous and exogenous antioxidants prevent mutagenic damage to DNA by quenching ROS production [9, 10]. In addition, numerous endogenous enzymatic and non-enzymatic antioxidants attenuate ROS-mediated damage. Enzymatic antioxidants, which include glutathione peroxidase, catalase, and superoxide dismutase, inactivate organic peroxides, hydrogen peroxide, and superoxide. Non-enzymatic antioxidants, which include glutathione, thioredoxin, vitamins C and E, uric acid, and selenium, also function as ROS sinks [11]. The interplay between ROS production and antioxidant defenses establishes a balance that protects cells against toxicity. However, the paradoxical need for ROS in cell signaling suggests that antioxidants may inhibit both cell proliferation and cell death, which may inhibit or enhance mutagenesis.

Redox status. Cells maintain a reduced intracellular environment compared to the oxidizing extracellular milieu. As a result, ROS concentrations are relatively controlled and compartmentalized [12]. Cellular signaling systems are under close redox regulation. Moreover, as signaling pathways proceed, the redox state of the cell is regulated through feedback establishing a close interdependent linkage of signal induction and propagation. Alterations in intracellular redox state occur primarily through oxidation of protein sulhydryl groups and can modulate cell signaling, DNA synthesis, enzyme activation, selective gene expression, regulation of cell cycle, and cell survival [13]. High doses of ROS may inactivate signaling pathways because sulfhydryl-containing proteins are highly susceptible to oxidation. Thus, the fate and function of a cell may be determined by the level of ROS, which ultimately participates as an intermediate in cross talk between cellular signaling systems and the cellular redox state [2]. This is critical because lower redox status in the cell may influence the balance in favor of proliferation over apoptosis via changes in signaling pathways.

ROS stimulation of cell signaling pathways. ROS are known to stimulate signaling of numerous cellular pathways. Hydrogen peroxide treatment of cells causes activation of NF-kB/Rel, AP-1, and mitogen activated kinases [13]. Oxidants can also simulate receptor tyrosine kinases even in the absence of ligand as well as the downstream effectors in signal transduction pathways including ras, protein kinase C, phospholipase C gamma, mitogen activated kinase, and c-jun-N-terminal kinase [2, 14, 15]. Thus, it is apparent that ROS can stimulate numerous, diverse pathways, suggesting a common role in cellular responses.

ROS production of growth factors and cytokines. ROS are also produced at various steps within signaling cascades activated by receptors. Many growth factors or cytokines bind their cognate receptors and generate rapid increases in ROS. In addition, the results of in vitro studies demonstrate that ligands such as platelet derived growth factor, epidermal growth factor, angiotensin II, and numerous cytokines clearly trigger rapid, intracellular production of ROS [1]. Moreover, the proto-oncogenic G protein ras also produces elevated ROS in fibroblasts upon stimulation [16]. The widespread overlap of ROS production and signal stimulation suggests the potential for cross talk among pathways and potential feedback regulation.

Effect of antioxidants on signaling. Application of antioxidants can interrupt cell signaling by quenching free radical production. In fact, addition of catalase, a potent hydrogen peroxide quencher, can inhibit the ability of platelet derived growth factor to stimulate tyrosine phosphorylation of numerous proteins [17]. The observations that transgenic animals overexpressing antioxidants exhibit abnormalities in function also suggest a critical role for antioxidants perhaps in maintaining a redox balance [18].

CANCER

Apoptosis versus proliferation. The discussion thus far has centered on the roles of ROS and the potential of interaction of antioxidants with these functions in normal cells. Clearly, cellular redox imbalance is under the control of an heirarchy of antioxidants and other redox-active proteins and is extremely complex [18]. But within the context of low level ROS production and redox status, how does this affect cancer? Cancer is a collection of diseases based on disruption of a delicate cell number balance controlled by cellular proliferation and cell death (apoptosis). The involvement of ROS in apoptosis is clear. ROS can also enhance mitogenesis in numerous cell types, including normal cells, initiated cells, and cancer cells. Low levels of ROS stimulate cell division and promote tumor growth, presumably through regulation of proliferative genes [5, 19]. Superoxide and hydrogen peroxide are known to stimulate growth in vitro in epidermal cells, fibroblasts, osteoblast, leukemia cells, and smooth muscle cells [20]. ROS also stimulate growth of bacteria and yeast [19]. The specific contributions and mechanisms of low level ROS to mitogenic pathways are unknown. It is likely that there is a delicate balance between cell growth and death that must be maintained.

Paradoxical effects of antioxidants on cancer. The involvement of ROS in diverse biological processes is evident. With such complex interactions, it is difficult to extrapolate how antioxidants may affect this balance. Although

supplemental antioxidants may attenuate proliferation, the concomitant suppression of apoptosis may permit clonal expansion of pre-neoplastic cells based on the redox state of the cell at the time of insult.

Numerous epidemiological studies support a protective role of dietary antioxidants in protection against carcinogenesis. However, a growing body of literature suggests that dietary antioxidants may also exhibit carcinogenic effects and enhance malignant progression. In two large intervention trials, high risk subjects, i.e., smokers, supplemented with carotenoids displayed significant, elevated incidences of lung cancer and mortality [21]. Studies in transgenic mice show that animals fed antioxidant replete diets displayed larger brain tumors than animals fed antioxidant-deficient diets [22]. In a study of breast cancer, the larger more aggressive and invasive cancers correlated with elevated plasma vitamin E levels and lower concentrations of free radical byproducts [23]. Moreover, stromal cells facilitate rapid transport and accumulation of vitamin C in leukemia cells, prostate tumors, and breast cancer cells when compared to normal cells [24]. Thus, dietary and supplemental antioxidants may inhibit carcinogenesis; however, compelling data also demonstrate that antioxidants can exacerbate progression of the cancer process.

DOSE RESPONSE CURVE

The outcome of a cellular response is clearly dependent on the level of ROS production. Excessive ROS production results in cell death and likely will not cause mutations relevant to pathogenesis, i.e., cancer, because cells are removed by necrosis and/or apoptosis. However, subtoxic ROS production may affect cellular responses via alterations in cell signaling. It is well accepted that relatively low levels of ROS promote cellular proliferation rather that cause cell degeneration or death [1]. Neoplastic cells are reported to experience persistent oxidative stress compared to non-neoplastic cells, ensuring activation of transcription factors, expression of proto-oncogenes, induction of DNA damage, and activation of antioxidant responses in neoplastic cells [25]. Furthermore, ROS may modulate not only the expression of transcription factors but also their transcriptional activity via redox regulation, thereby enhancing proliferative potential particularly of neoplastic cells. Moreover, there appears to be a dose-related increase in oxidative stress in premalignant cells with tumor progression. Given the evolution of precancer cells through the stages of initiation, promotion, and progression, there may be distinct dose responses to exogenous ROS agents at different stages of neoplastic development.

ROS induces and eliminates mutated cells by apoptosis depending on ROS dose. The activation by ROS of receptor-associated cascades and cell proliferation further confounds the ability to predict a dose response relationship for exogenous oxidative stressors. The capacity for ROS to affect multiple cellular processes suggests that no one answer exists regarding the shape of the dose response curve.

CONCLUSION

ROS are diverse and abundant in biological systems. While excessive ROS production clearly damages DNA, low levels of ROS affect cell signaling particularly at the level of redox modulation. Moreover, the specific contributions of ROS to apoptosis and mitogenesis in maintenance of cell number homeostasis remains to be elucidated. ROS dose is a critical parameter in determining the ultimate cellular response; however the shape of the dose response curve is unpredictable.

When cells are stimulated with ROS, cell signaling cascades are activated. It appears that the cellular redox potential is an important determinant of cell function and interruption of redox balance may adversely affect cell function. As a result, compounds such as antioxidants may intercept critical ROS signaling molecules and both protect cells and foster pathogenesis. As a result, further study is needed to unravel the role of ROS in redox regulation and the potential outcome of antioxidant administration on cellular responses.

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REDOX-MODULATED XENOBIOTIC ACTION AND ROS FORMATION: A MIRROR OR A WINDOW

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An extensive body of evidence has established that a number of xenobiotics exert their toxicity *via* a sequence of redox mechanisms[1-3]. In turn, these mechanisms both involve the formation of reactive oxygen species (ROS), and lead to the formation of reactive intermediates of the native molecule. Thus, a combination of effects may occur, partly due (1) to ROS activity (e.g. by hydroxyl adduct formation)[4-5], and partly (2) by the specific action of the xenobiotic's end-product(s) (e.g. cross-linking with biopolymers)[6-7]. To what extent which of these two events may be prevailing is an open question, firstly related to the specific mechanistic features of any class of agents and, in a given class, depending on the individual structure analogues being considered[8]. By making the question further more complex, it should be noted that the action mechanisms of a given xenobiotic may be modulated by the extra- and intracellular environment, including e.g. ambient oxygen levels, the endogenous oxidative activities, pH and other ionic balance, and the co-presence of other bioactive agents (hormones or other xenobiotics)[9-12].

A closely related field of investigation deals with the numerous physiological roles of ROS (see below), and of reactive nitrogen species (RNS) in modulating or signalling a number of key-events, e.g. in cell division, differentiation, embryogenesis, and antimicrobial defense[13-21]. Oxidative and nitrosative activities have been shown to trigger a number of basic mechanisms in cellular and organismal life stages, from ovocyte fertilisation to ageing and death.

A frequent attitude among present-day scientists consists of optimising their technical skills and knowledge in their specialist disciplines, yet often disregarding

developments in other areas. With regard to the roles for ROS in physiology and in pharmacology/toxicology, respectively, the scientific community has a wealth of information in each of these two related fields, yet with a general lack of studies suitable to interface these two disciplines.

Two examples drawn from previous studies will now be discussed in order to suggest interdisciplinary approaches that may clarify the biological significance of ROS.

FANCONI'S ANAEMIA DEFINITION VS. MITOMYCIN C AND DIEPOXYBUTANE TOXICITY MECHANISMS

Fanconi's Anaemia (FA) is inherited as an autosomal recessive disorder, and is characterised by progressive bone marrow failure, chromosomal instability, cancer proneness and excess cellular sensitivity to mitomycin C (MMC) and diepoxybutane (DEB)[22-24]. A growing body of evidence points to the occurrence of an inborn "prooxidant state" in FA, that may provide a realistic link to bone marrow failure and to cancer proneness[25-30].

The diagnostic test relies on the excess sensitivity of FA cells to DEB or MMC, causing a dramatic increase in chromosome breaks and characteristic translocations[22]. The sensitivity to DEB and MMC also relates to a current attribution of FA phenotype, currently referred to as «crosslinker sensitivity»[31]. This term is due to the recognized formation of DNA crosslinks as an ultimate outcome of DEB and MMC toxicity[8;23]. Hence, this sensitivity has engendered the widespread belief, and consequent definition, of FA as a DNA repair disorder[22-23]. However, both MMC- and DEB-associated toxicities involve redox mechanisms that play a major role in their effects[3;5;32-34]. In the case of MMC, a consistent body of literature points to a redox-cycling mechanism in MMC toxicity[3;5;8;32]. A number of studies demonstrated that MMC must be activated, like other quinone drugs, by a series of redox reactions leading to quinone reduction coupled with ROS formation. This well-established mechanistic information provided consistent explanation for MMC-associated toxicity, that was dependent on O_2 levels[5], and was decreased or removed by a number of antioxidant agents or conditions (as hypoxia or thioredoxin overexpression)[9;32;35].

Through different pathways, also DEB toxicity is associated with redox mechanisms[32-34]. Its epoxide structure implies the redox-mediated catalysis in the rearrangement of oxygen bonds[36]. Similar to MMC, DEB-induced cytogenetic damage is decreased by overexpression of thioredoxin gene[35]. A major role in modulating DEB toxicity has been reported for GSH[37-38]. Thus, DEB-induced testicular toxicity in mice was enhanced by preliminary GSH depletion and an increase in DEB-induced micronuclei was observed in *GSTT1*-null mutant cells (defective in the glutathione S-transferase T1 gene)[38]. A recent study from our laboratory has shown that DEB-induced toxicity is significantly correlated to oxygen levels[32]. The effects of MMC on the expression of catalase and MnSOD, and of DEB on glutathione levels (as a GSH depleter) showed different mechanisms underlying either DEB- or MMC-associated toxicity[32]. Thus, the available information is consistent with the occurrence of redox-dependent mechanisms for both MMC and DEB. It could be postulated that FA cell sensitivity to MMC and DEB should reflect some deficiency(ies) in coping with oxidative stress, as compared to non-FA cells. Two recent papers provided evidence for a direct association of FA complementation group C (FANCC) protein with glutathione S-transferase and a NADPH cytochrome P450 reductase[31;39]. It should be noted that the two activities are directly related to DEB- and MMC-associated toxicity mechanisms, respectively.

The redox deficiencies in FA may thus be regarded as an example of a direct relationship of redox-dependent modulation of toxicity mechanisms. Other oxidative stress-related disorders might provide scope for future research and further evidence in this crucial field[40].

OXIDATIVE ACTIVITIES IN CELL FUNCTIONS

A number of basic cellular mechanisms depend on a defined balance in oxidative activities, including e.g. cell division and early life stages (fertilisation and embryogenesis)[13-19]. A decrease in ROS formation or in oxidative activities results in damage to embryogenesis or cell differentiation, as developing embryos or differentiating cells are affected by hypoxia or antioxidants[18;41-42]. Moreover, the adverse effects of antioxidants were shown to depend on precise timing of administration during early development, the earliest phases being the most sensitive to antioxidant effects[43]. The physiological significance of ROS formation in cell differentiation and in embryogenesis has been reported previously, as summarised in Table 1. Fertilisation-associated "respiratory" burst has been related to the earliest events in embryogenesis, including fertilisation envelope formation[16], and a messenger role for hydroxy fatty acids[13] in modulating membrane structure and cell cycling. The demand for a prooxidant state in cell differentiation was related to ROS-induced triggers of calcium fluxes which, in turn, would modulate basic mechanisms in cell division and differentiation[14;43]. Most of those papers referred to ROS production as a "harmful" event to cells; thus the antioxidant store in oocytes[44] was viewed as a response or a "shield" to oxidative damage. Few reports, however, pointed to the essential role(s) for ROS *per se* in promoting cell differentiation[18] and cell proliferation[45]. We reported that DNA hydroxylation (measured as 8-hydroxy-2'-deoxyguanosine, 8-OHdG) increases, along with a decrease in luminol-dependent chemiluminescence during sea urchin embryogenesis[43]. The current term of oxidative "damage"[4;46] appears to be more appropriate when referring to the changes induced by ROS to differentiated or adult cells, whereas a balanced

Table 1. Major published evidence pointing to the roles of oxidants/antioxidants in early development and in cell proliferation and differentiation.

Events	Testing objects	Observations	Interpretation	References
Fertilisation	Sea Urchin or Medaka Eggs	↑ Oxygen Consumption ↑ Ionic Fluxes ↑↓ LDCL, ↑ H_2O_2 ↑ Lipid Peroxidation	Start for Embryogenesis Preventing Polyspermy Early Mitotic Signalling	13;16;19;43;53
Cleavage	Mouse Oocytes	Mitotic Arrest (2-cell stage)	↑ ROS Formation Due to: a) O_2 in Culture or b) Phenytoin Derivatives	12;17;19;54
Cell Proliferation & Differentiation	Several Cell Models and Organisms	↑↓ Cu,ZnSOD, MnSOD, Glutathione and Peroxides ↑ ROS Formation as a Mitotic or Differentiation Signal	Coping with Oxidative Stress Viewed as Damage to Embryogenesis ROS as a Necessary Factor in Cell Proliferation and Differentiation	16;17;39;43
Embryogenesis	Several Cell Models and Organisms	Antioxidant-induced Anomalies and/or Arrest	Attributed to Ionic (Ca^{2+}) Imbalance	42;43

ROS formation (and DNA oxidation) may represent a *sine qua non* **in cell differentiation[41;43].**

A role for ROS in modulating prokaryotic gene expression has been reported by Storz *et al.*[47]**. A body of evidence points to DNA methylation in eukaryotes in modulating cell differentiation and gene expression[48-49]. An association was reported between oxidant-induced alterations in specific DNA sequences and changes in cytosine methylation, thus suggesting that DNA hyper- or hypomethylation as well as hydroxylation may play complementary roles in DNA signaling, gene expression and cell differentiation[50-52]. These interconnections between DNA oxidation and methylation require further investigations that might unveil poorly understood, yet crucial phenomena.**

Altogether, the available evidence points to substantially different assets in dividing *vs.* **resting cells, as well as in early life stages** *vs.* **adult stage, in terms of spontaneous and** *necessary* **oxidative activities. This background information cannot be without consequences when evaluating toxicity mechanisms, and** *ad hoc* **studies are expected to provide relevant information.**

CONCLUSION

Beyond the Mirror, Alice is trespassing from the current world towards a land of unexpected findings (Fig. 1). This may represent the challenge of moving from the present set of information on oxidative "stress" and xenobiotic-associated mechanisms towards a more integrative physiological and toxicological perspective.

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Fig. 1. Alice's mirror, or a window instead?

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WHY HAVE CELLS SELECTED REACTIVE OXYGEN SPECIES TO REGULATE CELL SIGNALING EVENTS?

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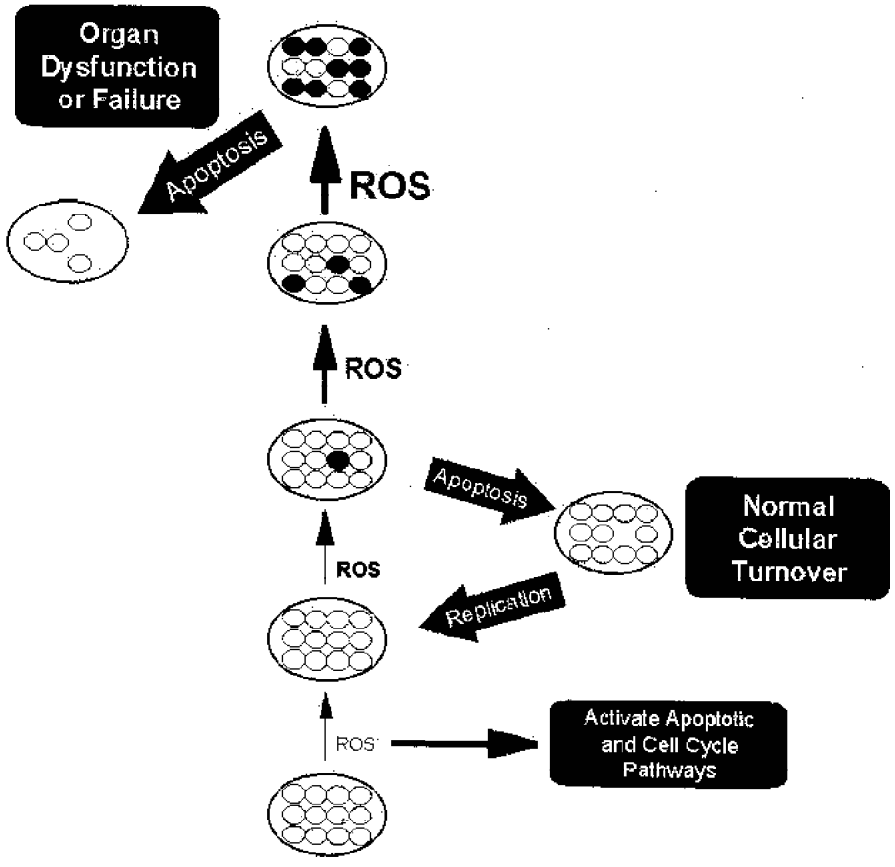
There is a growing body of evidence demonstrating that exposure of cells to reactive oxygen species (ROS) leads to oxidative modification of nucleic acids, proteins, and lipids, and that such modifications can contribute to the development of a number of diseases and aging. This raises the question: If ROS are so damaging to cells, why have cells selected ROS to trigger activation of so many cell signaling pathways? It makes good sense that elevation of ROS levels leads to activation of pathways leading to an increase in the rate of synthesis of several antioxidant enzymes needed to prevent formation of toxic levels of ROS. But why are ROS, especially H_2O_2, used also to trigger pathways leading to cell proliferation on the one hand and cell death (apoptosis) on the other?

We suggest that the use of H_2O_2 for the latter purpose is linked to the fact that when certain organs reach maturity, cell division essentially stops. It follows that, if cells in a given tissue become damaged and nonfunctional, the only way the tissue can be repaired is to get rid of the damaged cell to make room for its replacement with a good cell. Perhaps this is why ROS are implicated in the activation of both cell replication and apoptosis. As illustrated in Fig. 1, we propose that under normal conditions the steady state level of H_2O_2 is maintained at a low value determined by the rate of H_2O_2 formation and its removal by antioxidants. However, under conditions of oxidative stress, the level of H_2O_2 starts to increase above the normal steady state level. But, after a modest increase in the level of H_2O_2 occurs, the cell signaling systems are alerted (activated) to prepare for the eventuality that further increases in H_2O_2 could lead to serious cellular damage. Then, if the level of H_2O_2 continues to rise and damage to cells does occur, the pathway for apoptosis is primed to kill and remove the defective cell, and the cell proliferation pathway is activated to replace the bad cell with a good cell. Thus, H_2O_2 coordinately regulates apoptosis and cell division to keep constant the organ cell numbers.

Although such speculation offers a reasonable explanation for the fact that H_2O_2 activates pathways for both cell replication and cell death, evidence for such an explanation is still lacking.

FIG. 1



REACTIVE OXYGEN SPECIES (ROS) IN CELL RESPONSES TO TOXIC AGENTS

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INTRODUCTION

This journal issue brings six contributions that arose from questions to the authors who graciously complied with their answers. The questions addressed the potential modulating effects of cellular ROS in the initiation of cellular responses to different concentrations of potentially toxic agents:

1) Does the concept of ROS being metabolic messengers affect the dose response function regarding defined cellular reactions to changes in cellular ROS concentrations?

2) Do various levels of cellular ROS concentrations from different sources affect the paradigm of chemical and/or radiation carcinogenesis?

3) May concentration dependent responses to ROS in mammalian cells affect the practice of quantitative risk assessment from exposure to ROS-delivering reactions such as by way of ionizing radiation, as currently practiced by most regulatory agencies worldwide?

The treatment of these pointed questions in the preceding six contributions attests to the complexity of the task and the difficulty in defining the role of ROS in the initiation of cellular responses to toxic agents that directly or indirectly involve ROS related reactions. Yet, these questions need be tackled (2). No doubt, the current knowledge of the role of cellular ROS underpins their essential function far beyond toxic effects. These were long held to be the main consequences of ROS from different sources including ionizing radiation. Metabolically created ROS are major contributors to aging and cancer (1; 10; 15; 16). Increasingly, research focuses on better defining the delicate balance in the physiological role of ROS signaling in various species and cell types (4; 5; 9; 17; 21; 22).

ROS are constantly produced in cells that metabolize oxygen. The main site of production is in the mitochondria. From here, between 0.1 and 4.5 % of ROS have been described to escape into the cytoplasm (2). A total of 10^9 ROS may arise in the cytoplasm per cell per day by this route alone. A network of antioxidant defenses keeps the ROS concentrations for given cell types at physiological levels. Moreover, bursts of ROS are physiologically produced as mini-bursts in the course of normal cellular metabolism, for instance by the binding of growth factors to appropriate cellular receptors. ROS are, on the one hand, signals for essential cell functions including that for adaptive responses, cell proliferation as well as cell suicide, apoptosis. On the other hand, ROS may cause damage to cellular substrates with long lasting consequences especially if the target is the DNA. The balance of these effects appears largely to depend on the intracellular ROS concentrations. Thus, relatively low and moderately high concentrations of ROS may cause lasting molecular damage, such as via oxidative DNA adducts, yet they also have signaling functions that initiate adaptive responses and can decide cellular survival or death. When ROS concentrations reach high levels, damage is the overriding result and at very high levels they even initiate cell necrosis. Collateral cellular functions may modulate the types of ROS generated, their production rate, compartmental flux, and their effects.

The intracellular levels of ROS concentration are subject to various external interferences. Exposure to hydrogen peroxide in the experimental setting is being used to study the effect of changes in ROS concentration. Small and large bursts of ROS may arise at any time accidentally from external sources. Such bursts come, for instance, by radiolysis of water brought about by the absorption of ionizing radiation at random in the exposed tissue. Energy deposition events from single or a few electron tracks caused by the absorption of low-LET type radiation, such as x- or gamma rays, instantaneously create from less than hundred to thousands of ROS along a particle track. High-LET radiation, such as α-particles, may produce bursts of tens of thousands ROS per track. Such bursts temporarily change cellular homeostasis, change cellular signaling and cause damage in the hit cells and also in the neighborhood of particle hits. Whether change in physiological signaling or damage prevails, depends on the size and site of these bursts (2; 4; 5; 6; 7; 8).

Whereas physiological mini-bursts of ROS are likely primarily confined to specific cellular compartments, radiation-induced ROS bursts are nearly always distributed stochastically and unpredictably anywhere in the exposed tissue and cells. Depending on their size and location ROS bursts can initiate biochemical feedback controls that temporarily change cellular sensitivity to higher ROS concentrations; the underlying biochemical

reactions are summarily termed adaptive responses. These have been subject of discussion in a previous set of publications in this journal and have led to the present discourse. This presentation attempts to summarize the six statements on ROS function in this journal and relate them to biological consequences of low doses of ionizing radiation with ROS bursts being created widely spaced in time and tissue mass. The hypothesis emerges that depending on cell types and tissue background radiation physiologically bears upon the homeostasis of tissue by initiating removal of predamaged cells (apoptosis), and by protecting normal cells and promoting their proliferation.

EPITOME OF REPORTS IN THIS JOURNAL

Martin and Barret (14) in their paper draw attention to the role of ROS being a double-edged sword in cellular functions. The authors pitch the potential benefits versus potential detriment to the human body from antioxidant administration. The arguments are based on a careful analysis of the action of various antioxidants and on the sources of ROS. The interplay between antioxidants and ROS affects mutagenesis and cellular signaling pathways with the possible net consequence of oncogenesis. Here, especially high- ROS-induced apoptosis is held against the low-ROS induction of enhanced cell proliferation. Since apoptosis caused by elevated ROS concentrations eliminates a damaged cell, low ROS concentrations during antioxidant administration may prevent apoptosis in favor of cell survival and proliferation also in neoplastic cells and thus rather promote cancer than protect against it. Cancer cells are usually at a higher oxidative stress than normal cells, causing different kinds of stimulating and damaging effects including activation of antioxidant defenses with ensuing enhancement of cell proliferation, probably depending on the stage of neoplastic development. The principal dual effect of low ROS concentrations in the cell on protection and fostering damage has been observed experimentally and by epidemiology and needs to be seen in the context of numerous and diverse signaling pathways with "the potential for cross talk among pathways and potential feedback regulation." The authors, therefore, argue for the unpredictability of the shape of the dose response curve because of the variability of cellular responses.

The paper by Lehnert and Iyer (13) answers the three above listed questions separately. They again emphasize the crucial importance of the cellular redox balance in signaling for metabolic functions and point to the variation of a toxic threshold of ROS in the cell depending on its previous history of ROS exposure. Adaptive responses may be linked to intra- and extra-cellular ROS exposure at various concentrations. Regarding the first question, the phenomenon of adaptive responses is discussed on the basis of own work and that cited in the literature. It involves ROS-triggered activation of metabolic cascades, for instance the transport of transcription factor (NFκB) to the cell nucleus with subsequent production of interleukin-8, and other corresponding changes in a number of different metabolic pathways including the antioxidant defense systems of the cell. The responses may both temporarily protect against renewed elevation of ROS and with it damaging consequences to cellular constituents, especially the DNA, but also may lead to an increase in complex types of DNA damage and even in genetic instability when later exposed to a higher concentration of ROS. Indeed, the cellular as well as extracellular irradiation causes endogenous ROS bursts. The time intervals between consecutive exposures appear crucial in that the development, full expression, and disappearance of the adaptive responses are to be considered.

With respect to the second question, Lehnert and Iyer again cite many data on ROS generation and triggering of cell responses in terms of signaling cascades and damage to the DNA. The need for much more work especially in the realm of cancer promotion is obvious. The effects of exogenously caused supra-basal yet not toxic levels of ROS on top of the endogenous level escape a detailed understanding in their various reaction circuits. An example lists, on the one hand, the undisputed role of ROS associated adaptive responses with protection against the accumulation of damage in tissues from renewed exposure to ROS bursts, when, on the other hand, chronic infection with its increased levels of ROS may cause increased cancer incidence in the affected tissue. This contradiction is unresolved.

In dealing with the third question, Lehnert and Iyer point to the presently appearing futility of various models that link risk of cancer to the exposure to any ROS delivering agent such as ionizing radiation. A case in point is the effect of α-particles in multi-cellular tissue. So-called bystander effects induce a variety of responses in neighboring non-irradiated cells. Such responses include both damage to the DNA and also protective mechanisms such as induction of apoptosis, and activation of different gene expressions that also elicit adaptive responses in these non-irradiated cells. The corresponding intercellular signaling substances include ROS. It is unknown whether the various responses in bystander cells cause a net benefit or damage to the exposed tissue. A common denominator in these studies is the temporary induction of supra-basal levels of ROS. The conclusion asks for the need of including all these new experimental findings in various cell systems into models that eventually allow a realistic quantitative risk assessment of low-dose irradiation. This plea also demands more interdisciplinary work between specialists in the field of ROS, in cell biology, and radiation biology.

In their comment on oxidant signaling in carcinogenesis, Deshpande and Irani (3) summarize the role of ROS in tumor development from various experimental approaches. These include analyses of defined signaling pathways and their modulation of gene expression through stimulation of transcription factors. The authors reemphasize the role of ROS as metabolic second messengers as supportive for the theory of causation of cancer by

oxidants. Both tumor initiation and promotion may be enhanced by ROS at supra-basal levels, as these appear to be also effective in maintaining transformed phenotypes. The usual multiple exposure mode of humans to ROS-delivering agents lets one expect a simultaneous influence at different stages of carcinogenesis in the exposed tissue, for example, involving both an enhanced cell proliferation and a decreased rate of apoptosis both favoring carcinogenesis.

The paper by Grune (11) deals preferentially with oxidants in the context of antioxidant defenses. These include primary and secondary levels of action. Whereas the glutathione system, superoxide dismutase and catalase are grouped into the first category, the author prefers to refer to DNA repair mechanisms and damage removal systems as belonging to a secondary level of defense against oxidative damage in tissue. Among the latter, poly-(ADP)-ribose polymerase (PARP) is one key for the induction of DNA repair or apoptosis. All anti-oxidative defense mechanisms likely interact within a complex metabolic network. Because of this, the question as to the benefit from antioxidant treatments arises. In case of such a treatment, it should encompass more than one component so that the function of the network remains coherent and effective. Adaptive responses following exposure to supra-basal yet not toxic levels of ROS are temporary and appear universal. They involve successively complex levels of biological organization. Thus, a changed equilibrium in the cellular redox state, for example in human on physical exercise, may lead to a wide range of responses. These involve enhanced anti-oxidative defenses, alterations in gene expressions and protein synthesis, with DNA repair and damage removal, as well as structural changes in heart and blood vessels and increased immune competence, all leading to an improved tolerance to stress. The role of ROS appears crucial here. H_2O_2 is reported to activate more than 40 genes many of which have yet unknown functions. Confounding factors, moreover, are various types of ROS, their concentration levels, flux rates, compartmentalization and their cellular environment; many of these factors remain elusive. One may, in general, ascribe a gradient of responses to increasing levels of ROS concentrations: At low level increases, cells appear to experience a temporarily increased rate of proliferation; at higher levels, a temporary growth arrest leading to adapted cells may be prominent; at still higher levels, permanent growth arrest results with remaining metabolic functions; even higher levels of ROS appear to initiate apoptosis and eventually necrosis. Various cells in different species are expected to have different response patterns following exposure to increased ROS concentrations. Important questions remain unsolved, such as the difference between responses to pulsed versus chronic ROS exposures, specific reactions to different ROS species, and finally the influence on the response by the site of ROS generation in the cell. Regarding cancer therapy, the author discourages the application of non-lethal doses of ionizing radiation, since sublethal irradiation may eventually initiate some cellular resistance against further treatment be it chemo- or radiotherapy.

Besides endogenous oxygen metabolism and irradiation, a large range of toxic chemical species asserts their effect through action on the cellular redox state, as Pagano in his paper (18) points out. These interferences encompass direct ROS activity and reactive molecular intermediates that oxidize cellular constituents, such as biopolymers. The essential role of ROS in cellular signaling is reemphasized in adult as well as in embryonic mammalian cells beginning from fertilization of the egg cell by the sperm, in cell differentiation, maturation, and defense against infections. Many extensive studies are largely confined to specialized laboratories with little interaction with other fields in cell biology. The author pleads for more interdisciplinary work and cites two examples: Fanconi's anemia and specific oxidative activities in cell function. Fanconi's anemia may be defined as a disease based on a deficiency of redox mechanisms, more precise, on ineffective oxidative defenses so that the patients cannot adequately cope with oxidative stress. Various biochemical mechanisms of cellular responses to mitomycin-c and diepoxybutane both acting in association with redox systems, are listed in support for this claim. Regarding oxidative activities in cell function as second example, the author first focuses on the essential role of ROS in embryogenesis. Depressed levels of ROS in the embryo cause disturbances in cell proliferation and differentiation. ROS may trigger essential cellular Ca fluxes. These citations contradict the general appraisal of ROS being harmful. Even DNA oxidation may have a physiological role in essential DNA methylations in adult cells. Thus, oxidative adducts in specific DNA sequences appear to be associated with cytosine methylation implying complimentary mechanisms in DNA hydroxylation and –methylation in DNA signaling, gene expression and cell differentiation. The emphasis on necessary oxidations in cells, as in previous papers, warns against simplification in assessing the consequences of changing physiological levels of ROS concentrations in cells.

Stadtman and Levine (23) prefer to briefly summarize their position regarding the function of ROS in regulating cell signaling events. First they emphasize the undisputed involvement of ROS in causing cellular damage in terms of oxidative modification of all cellular constituents. On the other hand, cells have acquired to use ROS in triggering many cell signaling pathways. Thus, increased ROS levels stimulate antioxidant defenses and also may trigger cell proliferation as well as apoptosis. Removal of damaged cells needs be linked to the replacement with healthy cells in order to maintain tissue homoeostasis. The authors propose that ROS, here in terms of H_2O_2, are maintained at steady state low levels of concentrations through the action of antioxidants. Upon oxidative stress, a moderate elevation of the ROS level above normal steady state initiates adaptive responses in order to protect in case of a repeat exposure to higher ROS concentrations

and to prevent damage. Still higher concentrations of ROS will trigger apoptosis and at the same time signal for cell replacement by initiating proliferation of good cells. In this way, cell numbers in tissue are kept constant. The authors relate their hypothesis to experimental observations on H_2O_2 effects on cells; yet, evidence for their explanation regarding tissue effects needs further research.

AN ATTEMPT AT SYNOPSIS REGARDING EXPOSURE TO IONIZING RADIATION

When one assesses in a synoptic way the various contributions in this journal issue, one should attempt to link the wealth of experimental data on the role of ROS at various concentrations in mammalian cells also to the environmental natural background exposure to ROS inducing agents (2). Among these, ionizing radiation is a major contributor. With this view, the degree, time and duration of exposure to a given ROS concentration in a cell is paramount for understanding the relationships between the various effects of ROS. They indeed project a double-edged sword, as Martin and Barret said in their paper (14). In a homeostatic steady state, the ROS concentration in cells is kept within a physiological range of values with physiological mini-bursts occurring probably at rather frequent and changing intervals. About 10^9 ROS can be assumed to escape mitochondria into the cytoplasm per average cell per day (2; 19; 20). This would generate about 11600 ROS per second per average cell via mitochondrial escape, and change with the demand for metabolic oxygen supply.

Regarding the contribution to endogenous ROS from natural background of ionizing radiation, one needs to acknowledge that on average each cell, or more accurately, each nanogram of tissue being the average mass of a mammalian cell, is being directly affected once to twice per year (7; 8). This occurs from charged particles, which are created in the human body randomly by absorption of background radiation. Such single energy deposition events, or hits, cause a sudden burst of ROS in the hit cells as well as in the intercellular matrix and may affect non-hit neighboring cells through intercellular signal substances.

About 30 eV is used per creation of a single ROS by radiolysis of water and 1 mGy per nanogram expresses the absorption of 6.24 keV in this mass (12). Thus, particle hits of some 0.4 to 1 mGy per nanogram, for instance, from the exposure to the low-LET ^{137}Cs-γ rays and 250 kVp x-rays respectively, will generate bursts of a total of less than a hundred to several hundred ROS per average hit per nanogram within less than a microsecond. The average number of ROS per burst from absorption of densely ionizing α-particles per nanogram reaches much higher values, ranging up to some 70000 for a 4 meV α-particle, again within less than a microsecond.

In other words, instant ROS bursts of different sizes, which mainly fall in the moderately high to high level range of concentrations, occur naturally several times a year per nanogram tissue directly and indirectly on top of the endogenously created steady state level of ROS concentration with physiological, relatively frequent min-bursts. Both the quantitative relationships between endogenous steady state ROS levels and exogenously caused ROS bursts are likely different in various cell types and species, as are the qualitative consequences of the endogenous and radiation caused ROS. The latter appear principally different to some degree because of their specific chemical nature and the site and spacing of their generation. Nevertheless, low-dose induced adaptive responses have been unequivocally shown in mammalian cells to involve ROS (6).

When one wants to evaluate the consequences of radiation-induced supra-basal ROS bursts, both the signaling effect and structural damage must be considered. The latter is over a wide range proportional to the density of energy deposited along a particle track with the concomitant clustering of ROS. A 6 to 7 keV electron track passing at random through a mammalian cell may thus produce about two hundred ROS but on average only two damages to DNA, most of which are rapidly repaired. Whereas the damage here is hardly measurable, the ROS effects are readily observed (5; 6; 7). It follows that the ratio of signaling to local structural damage per energy deposition event per nanogram is expected to be high for low-LET radiation and low for high-LET radiation. The spectrum of radiation qualities from background exposure indicates orders of magnitude more energy deposition events of the low-LET type than of the high-LET type.

These considerations force an integrated view of actions of ROS from the various sources, i.e., from endogenous metabolism and natural or induced exposure to ionizing radiation (5; 6; 19; 20). The wealth of data discussed in the papers in this issue leads one to entertain the hypothesis that a low level of steady state ROS and thus an optimal antioxidant life style is principally beneficial in that damage from oxidative modification of all cellular constituents is kept low (1,10). This benefit, however, apparently requires a trade-of in that ROS bursts are essential, as they occur endogenously and inevitably naturally several times a year from exogenous sources such as from background exposure to ionizing radiation, as described above. These bursts are considered to temporarily facilitate essential signals that induce both damage removal by apoptosis of heavier damaged cells including those with oncogenic transformation and replacement of lost cells by proliferation of good cells (23), perhaps also by way of bystander effects (13). Other adaptive responses elicited by such ROS bursts include delayed appearing and temporarily lasting stimulation of antioxidant defenses and probably also DNA repair seen after low-dose irradiation. Immune responses in various mammals were improved following low-level irradiation and are likely linked to low-LET radiation-induced ROS bursts in the respective precursor cells.

Moreover, the question arises as to whether the above mentioned relatively high ratio of signaling to damaging effects of low-dose, low-LET irradiation with the corresponding ROS bursts may be beneficial if properly spaced in time on top of the relatively low level of endogenously generated ROS (7; 8; 19; 20). The time interval may be crucial for signaling effects with adaptive responses to operate optimally. The experimental data so far largely encompass cell system responses and much more work is needed to understand the delicate net of biochemical reactions that cause these responses. However, from what has become known it appears indeed unlikely that the relationship between cancer risk and low doses of ionizing radiation can be expressed by a linear function. Indeed, hormetic responses need to be expected within the low dose range of low-LET irradiation of eukaryotes and complex tissues in contrast to simple biological entities where linearity of the dose-effect response is unquestioned. The need for more interdisciplinary work is obvious.

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PART 2: PROPOSING A DEFINITION OF HORMESIS

DEFINING HORMESIS

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ABSTRACT

Much confusion surrounds the concept of hormesis and what its biological meaning represents. This paper provides a definition of hormesis that addresses its historical foundations, quantitative features, and underlying evolutionary- and toxicologically-based mechanistic strategies. Hormesis should be considered an adaptive response characterized by biphasic dose responses of generally similar quantitative features with respect to amplitude and range of the stimulatory response that are either directly induced or the result of compensatory biological processes following an initial disruption in homeostasis. Given the limited magnitude of the stimulatory response (i.e., usually 30-60% greater than controls at maximum), heightened study design and replication requirements are often necessary to ensure reliable judgments on causality. Even though hormesis is considered an adaptive response, the issue of beneficial/harmful effects should not be part of the definition of hormesis, but reserved to a subsequent evaluation of the biological and ecological context of the response.

INTRODUCTION

The phenomenon of hormesis is becoming more broadly discussed in the biomedical literature, especially in toxicology and radiation biology/health physics as well as in the general scientific and lay literature. What characterizes much of this literature is the lack of a generally agreed upon definition of hormesis with respect to conceptual understanding, quantitative features, mechanistic framework, and biological significance. A plethora of terms has been applied to similar descriptive dose-response phenomena such as beneficial effects of low doses, intermediate disturbance hypothesis, subsidy-stress gradient, U-shaped, J-shaped, biphasic, stimulatory-inhibitory, facilitation-inhibition, reverse, bi-directional, dual, bell-shaped, compensatory and paradoxical dose responses as well as a string of biological "laws" including Hebb's (1955), Yerkes-Dodson (Broadhurst, 1957; Teigen, 1994) and Arndt-Schulz (Calabrese and Baldwin, 1999). Such terminological diversity for similar appearing descriptive dose-response phenomena reflects, at least in part, significant professional/academic isolation and lack of conceptual integration across scientific disciplines. This lack of consistency impedes progress to design and test hypotheses related to this phenomenon and to differentiate and generalize complexities of biological responses to low dose exposures.

The current paper offers a definition of hormesis that is based on a comprehensive assessment of the historical literature relevant to the concept of hormesis in the chemical and radiation domains from the late 19th to the middle of the 20th century (Calabrese and Baldwin, 1999, 1999a, 2000, 2000a, 2000b) and an assessment of several thousand articles with evidence of hormetic effects based on quantitative evaluation criteria (Calabrese and Baldwin, 1997, 1997a, 2001).

DECOUPLING BENEFICIAL EFFECTS FROM THE DEFINITION OF HORMESIS

The concept of a beneficial effect within the context of a dose-response study is difficult to determine due to considerable biological complexity and the fact that beneficial effects are often seen with reference to a specific and relative setting. What may be beneficial for the individual due to low-dose exposures may be harmful for a population. Longevity may be enhanced at low doses but at the expense of fecundity or the reverse. What may be beneficial may be different when assessing the effects of the treatment on the host or the attacking organism. A cancer chemotherapeutic drug may be effective at high doses due to inhibitory effects on cell proliferation, but harmful to the patient at lower doses where it may stimulate cell proliferation and therefore tumor growth. In this case the low dose may be assumed to be harmful to the patient while enhancing the tumor (Foeken et al., 1992). In a similar situation, a high dose of antibiotic may be bactericidal, thereby permitting the patient to survive (Calabrese and Baldwin, 1999). However, at lower doses the treatment may enhance the survival of the bacteria to the detriment of the patient. Low doses of certain cardiac glycosides may enhance cell proliferation of prostatic smooth muscle at low doses while having an inhibitory effect at higher doses. Yet, low-dose exposure may enhance the likelihood of functional impeding of urine flow in males (Chueh et al., 2001). In this case the hormetic-stimulatory response at low dose would not be beneficial for the patient. These examples illustrate that the definitional characterization of hormetic dose responses as a beneficial effect at low doses is often complex, situation specific, sometimes overly simplistic, encouraging of ideologically-based support or criticism of the hormesis concept and there-

fore not generally useful. This does not mean that beneficial or harmful characterizations should not be made. Such judgments need to made, but at a subsequent and more advanced stage of analysis.

UNRAVELLING HISTORICAL CONFUSIONS: Is the hormetic stimulation the result of a direct stimulation or an overcompensation response?

In the early-to-mid-decades of the 20th century, a significant issue in the area of radiation-induced biological effects emerged as to whether reported stimulatory responses due to low doses of radiation were the result of a direct stimulation (i.e., often referred to as a biopositive effect) or an overcompensatory response following injury. The Arndt-Schulz Law, which was based on the research of Hugo Schulz in the 1880s, assumed that a direct stimulatory response accounts for the low-dose stimulatory phase of the dose response. This was viewed by leading experts in radiation biology/health such as Shields Warren during the 1940s-1960s as incompatible with substantial experimental data indicating that stimulation caused by low-dose radiation exposure occurred only as a result of an overcompensation reparative response to an initial disruption in homeostasis (see Calabrese and Baldwin, 2001). Thus, the Arndt-Schulz Law was essentially discounted by mainstream radiation health researchers. The lack of resolution of this issue has continued to the present time and provides a principal basis for current confusion over the concept of hormesis.

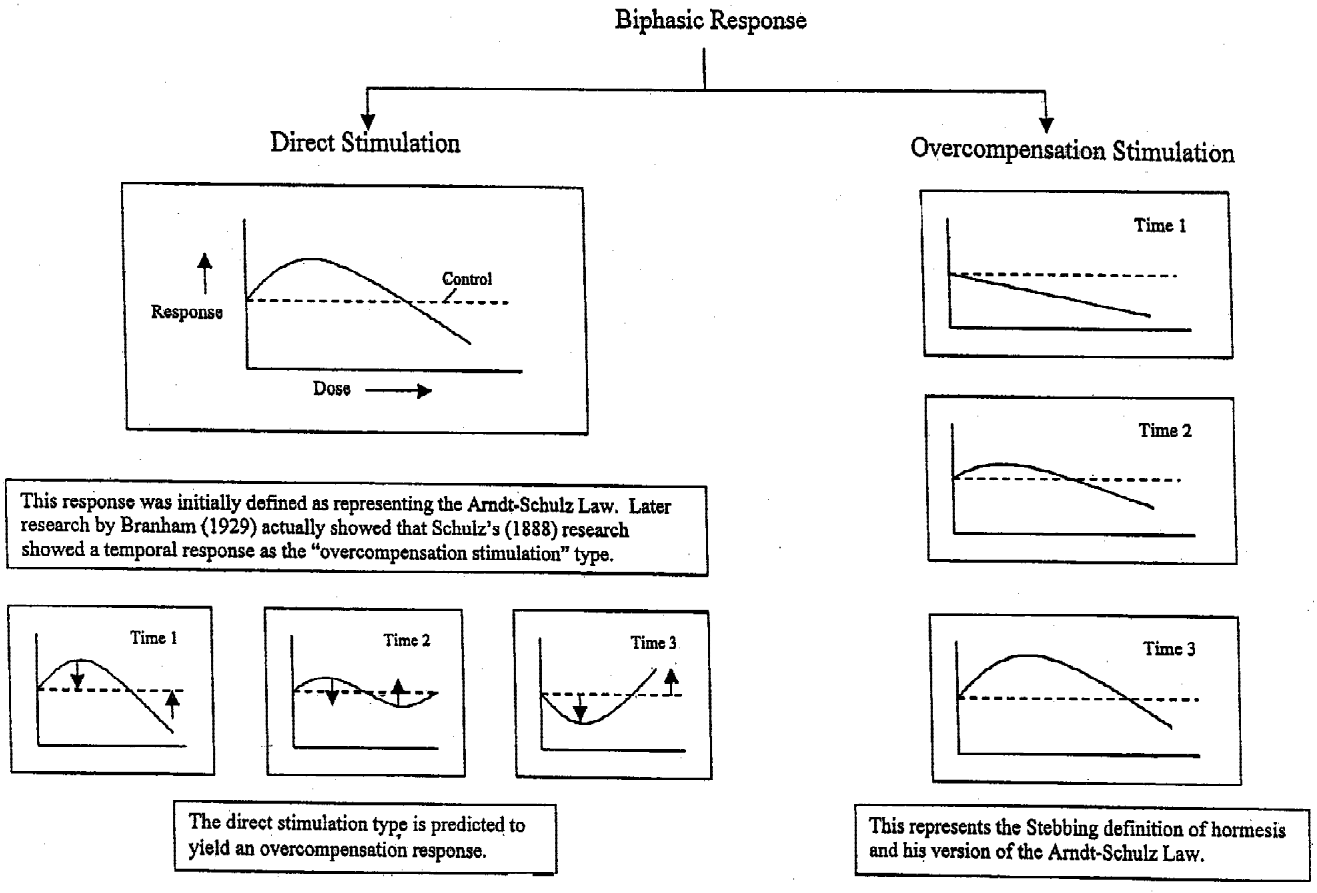
DEFINING HORMESIS

Hormesis is an adaptive response characterized by biphasic dose responses of generally similar quantitative features with respect to amplitude and range of the stimulatory response that are either directly induced [i.e., direct stimulation hormesis (DSH)] or the result of compensatory biological processes following an initial disruption in homeostasis [i.e., overcompensation stimulation hormesis (OCSH)].

Overcompensation Stimulation Hormesis

Overcompensation hormesis is an adaptive response to low levels of stress or damage resulting in enhanced fitness for some physiological systems for finite periods and, under specific defined circumstances such as colony growth, indefinitely. It results from a modest overcompensation to a disruption in homeostasis. The key conceptual features of OCSH are the disruption of homeostasis, the modest overcompensation, the reestab-

Figure 1. Comparison of direct stimulation and overcompensation stimulation hormesis (modified from Calabrese and Baldwin, 2001a.)



lishment of homeostasis and the adaptive nature of the process. Figure 1 depicts the general form of the OCSH dose response relationship including the temporal sequence of the dose response (Calabrese and Baldwin, 2001a).

The "disruption of homeostasis" phrase establishes the toxicological nature of hormesis distinguishing it from the concept of essentiality of nutrients and DSH (Figure 1). Disruption of homeostasis, within the context of hormesis, is not restricted to gross toxicological damage whereby macromolecular changes predominate but should be more broadly seen as comprising a continuum from a general stress response, as evidenced by alterations in glucocorticoid levels, to those changes that include limited macromolecular damage. The "modest overcompensation" feature of the process leading to the expression of hormesis is essential because it functionally links hormetic responses to homeostasis, a universal biological concept, providing the theoretical foundation for the broad generalizability of hormetic phenomena.

This modest overcompensation response suggests a highly regulated, optimization process providing additional adaptive equity as a type of biological insurance policy that remains after the costs of tissue repair have been satisfied. This concept implies a continuous responding to compensatory regulatory messages until the homeostatic condition is reestablished. Efficiency in reestablishing homeostasis demands that resources be appropriately allocated. Compensation responses should be quantitatively linked to the extent of damage incurred; that is, the repair response would correspond to the extent of the damage, with sufficient, but not excessive, biological resources allocated to ensure that the repair function is completed.

Hormesis represents the advantage gained by the individual from resources initially and principally allocated for repair activities but modestly in excess of that needed to repair the immediate damage. This process could also preadapt the organism against damage from a subsequent and more massive exposure within a limited time period. Therefore, the limited overcompensation response may satisfy two functions: the assurance that the repair was adequately accomplished in a timely fashion and protection against subsequent and possibly more massive insults. The value of this latter function is commonly assessed in chemical and radiation toxicological studies of the adaptive response. In this case a low dose (e.g., X-rays, many heavy metals, organic solvents such as carbon tetrachloride, endogenous compounds such as β-amyloid peptide) administered prior to a higher and more threatening dose of the same agent, often reduces the toxic potential of the subsequent massive exposure. Furthermore, if no subsequent toxic exposure occurs, the overestimated application of resources to the initial damage (i.e., the overcompensation response) may be employed for other useful functions (e.g., reducing background stressor damage, providing additional vegetative growth, etc.). This is, in fact, what is typically measured in studies assessing hormesis.

The modest extra resources to assure reestablishment of the homeostatic condition have been broadly adopted by many species. Despite this common adaptive strategy, various biological systems may have evolved different specific approaches to achieve the compensatory response, depending on the significance of the function needing restoration, the availability of resources, as well as the extent to which biological redundancy occurs in the affected systems. This is analogous to the case with other adaptive strategies such as enzyme-mediated xenobiotic detoxification/excretion processes where probably all species follow the general norm of converting lipophilic substances to more hydrophilic metabolites but may use different specific chemical substrate strategies (e.g., glucose vs. sulfate, glycine vs. glutamate) to achieve this hydrophilic metabolite detoxification/excretory goal. Thus, the process of natural selection of hormetic strategies within the diverse range of biological species is likely to follow a generally similar broad goal with specific strategies tailored to the unique ecological niche features of the species. Within an evolutionary paradigm of diversity linked to a common framework, the nature of the hormetic dose-response curve across species is quantitatively consistent suggesting a high degree of genetically based conservation.

Direct Stimulation Hormesis

Examples of hormetic dose responses exist in which detailed temporal features were included without the observation of an overcompensating response. Such findings indicate that hormetic responses can occur via direct (biopositive) mechanisms. However, as suggested above, lack of temporal features in most studies precludes such differentiation.

DSH displays similar quantitative features as OCSH with respect to amplitude and dose range of the stimulatory response. This suggests that it is also tightly regulated with major resource constraints. The endpoints that are assessed represent functions that maintain normal multi-system responsiveness and homeostasis. The physiological systems and endpoints measured with DSH are often those reported in experiments in which OCSH is observed. This suggests that OCSH and DSH may be mediated via similar regulatory systems and therefore are bounded by similar resource and system plasticity contraints, accounting for their common quantitative features. However, the initial action that generates the DSH is not a response to a disruption in homeostasis but an adaptive response that operates within normal maintenance functions that allow for metabolic excursions within the 2-fold range of background. It would use fewer resources as compared to OCSH since there is no obvious damage to repair and disruption to overcome. Nonetheless, it represents a type of steady-state adaptive response that reflects normal, modulatory physiological dynamics.

QUALITATIVE/QUANTITATIVE FEATURES OF HORMESIS

Qualitative Features

Hormetic responses are characterized as biphasic dose-response relationships exhibiting a low-dose stimulation and a high-dose inhibition. That is, both the stimulatory and inhibitory dimensions of the hormetic phenomenon must be present to satisfy the qualitative definition of hormesis. This is necessary in order to establish the hormetic response within the traditional toxicological dose-response continuum. Dose-response relationships exhibiting stimulation at low doses but where the inhibitory response is not demonstrated either because the response at higher doses does not diminish below control values or because the upper end of the dose-response spectrum was not assessed do not satisfy this definition. Whether the hormetic response displays a U- or an inverted U-shaped dose response is a function of the endpoint measured. For example, an inverted U-shaped dose response would be observed when the endpoints were longevity or growth; a U-shaped dose response would be seen when the endpoints were disease incidence such as cancer or heart disease. Consequently, hormesis is a general term for biphasic dose-response relationships of a U- or inverted U-shaped nature.

Quantitative Features

Further confusing the understanding of the term hormesis is that the historical use of this term did not define nor imply specific quantitative and temporal features of the dose-response relationship. Based on an investigation of several thousand published studies offering qualitative consistency with the hormetic dose-response relationship, Calabrese and Baldwin (1997, 1999) noted that such effects could be quantitatively characterized by a maximum stimulatory response that generally did not exceed 2-fold of the control with most maximum responses only 30 – 60% greater than controls. The width of the stimulatory response was typically (i.e., 90% of 2609 examples) in the 5- to 100-fold dosage range, immediately below the toxicity threshold; reliable exceptions to the 5- to 100-fold stimulatory dose range exist in which ranges $\geq 10^3$-fold of dose have been reported.

The fact that the stimulatory zone can be so broad suggests that multiple mechanisms are involved. Furthermore, while evidence exists that overcompensation to a disruption in homeostasis may extend over a 100-(Garcea et al., 1985) to at least a 300-fold (Smith, 1935) dose range, the direct stimulatory response may have the capacity to affect stimulatory responses over a range considerably larger than observed with the overcompensation-based phenomenon based on preliminary assessments of selected pharmacological dose-response systems (Calabrese and Baldwin, 2001). While this remains essentially an unexplored area, further sub-classification according to the range of stimulation may be necessary but, as of yet, there is insufficient biological understanding to guide on how to proceed.

Limited insight exists concerning why the stimulatory range of hormetic dose-response relationships can vary widely based on research in pharmacology and experimental psychology. In the field of pharmacology, the administration of parathyroid hormone to pancreatic islets cells *in vitro* affects a highly reproducible hormetic dose-response relationship concerning the release of insulin. However, if the level of calcium in the medium is changed it alters the nature of this hormetic dose response by changing the stimulatory range from approximately 8- to 100-fold but not the amplitude of the response (Fadda et al., 1990). To our knowledge this represents the first pharmacological/toxicological example of an experimental modulation of the stimulatory range of the hormetic dose-response relationship.

The range of the stimulatory response has been readily assessed in the field of experimental psychology where more complicated study designs have been routinely used. For example, it is common that the effects of different levels of stress on various types of performance are evaluated. However, experimenters often incorporate a second variable, tasks of different complexity, to be solved. In these experiments the hormetic dose response is typically seen to have a similar amplitude across the different levels of stress but the range of the stimulatory response is much more restricted under conditions of greater complexity. These types of hormetic-like dose-response relationships have been referred to as Hebb's Law (i.e., when there is a single level of complexity) and the Yerkes-Dodson Law (i.e., when there are multiple levels of complexity).

These two models (i.e., release of insulin from pancreatic islet cells and those representing examples of the Yerkes-Dodson Law), by which the range of the stimulatory response may be modified, have significant implications not only for the design of toxicological investigations but also for understanding the underlying mechanisms that account for the range of stimulatory responses in the low-dose zone.

NOMENCLATURE

The term hormesis was selected to represent the biphasic dose-response phenomenon described here because of its widespread use in the fields of radiation biology/health physics and ecological and human toxicology. In addition to numerous articles, two books have been published written on the topic (Luckey, 1980, 1991). Even though there has been a lack of precision/agreement over the meaning of hormesis it has been reasonably focused on and consistent with the currently proposed interpretation and quantitative characteristics. Other terms like U-shaped, J-shaped, biphasic, stimulatory-inhibitory, dual, and bidirectional are valuable but too general. The terms intermediate disturbance hypothesis and subsidy-stress gradient are more specific, and probably are examples of OCSH but need to be better demonstrated and assessed. While we believe that

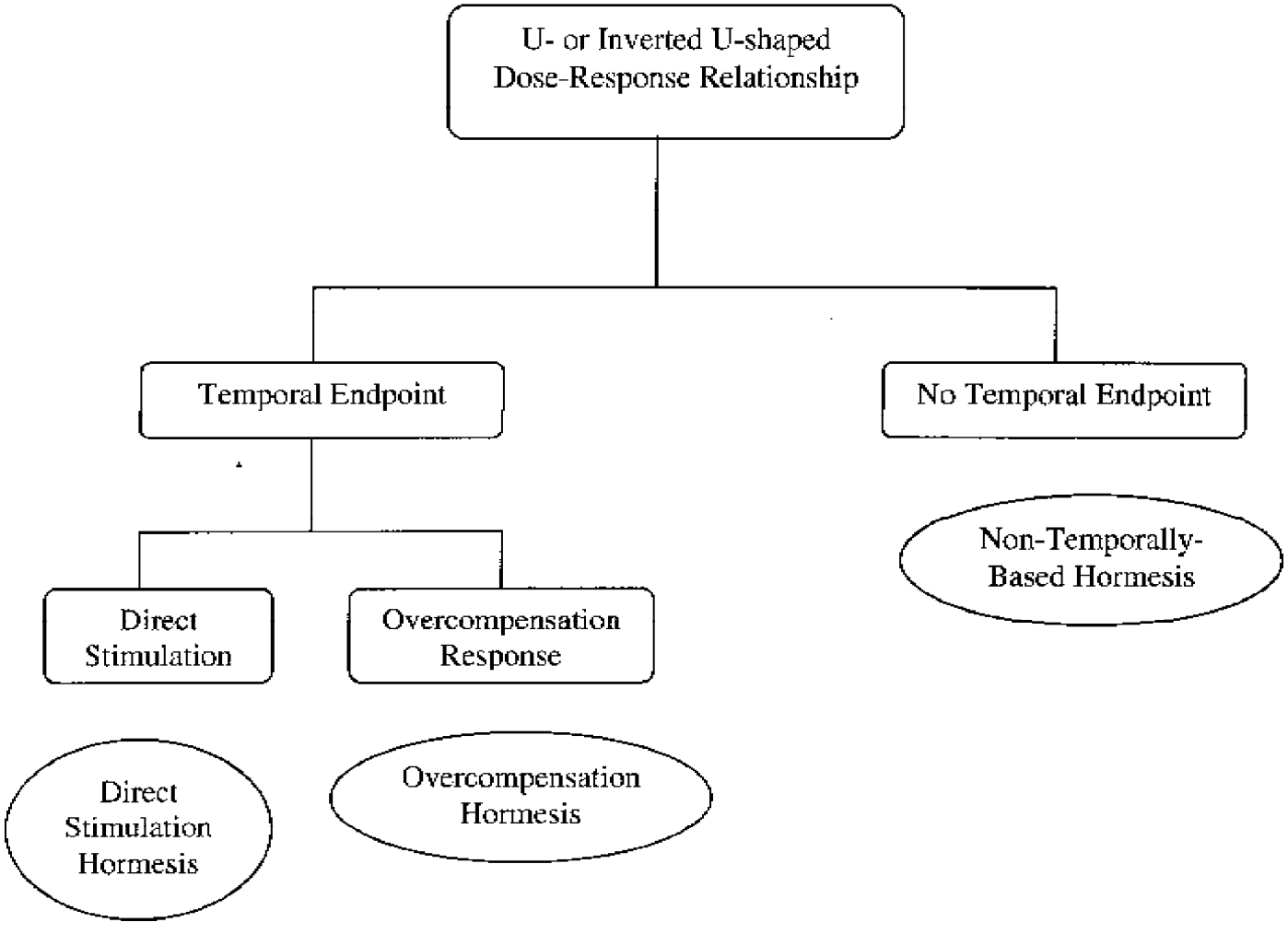
hormesis is a highly predictable process, the characterization of it as a "Law" is excessive and unnecessary. Thus, we believe that the term hormesis warrants the primary focus for common use in this area.

In Figure 2, a hormetic nomenclature is proposed that both recognizes the descriptive similarity of hormetic-like biphasic dose-response relationships, as well as general features of differentiation based on quantitative aspects of dose responses and temporal responsiveness. The three basic features involve hormetic-like responses in the presence or absence of temporal data. If appropriate temporal data are available then it may be possible to differentiate between directly stimulatory or overcompensation stimulatory hormetic responses. A further common level of differentiation may be applied to the three general dose-response classifications based on quantitative features of the dose response concerning the magnitude and range of the stimulatory response. Under the assumption that hormesis represents a modest overcompensation to a disruption in homeostasis, there is a biologically based expectation that any overcompensation response would be limited; such responsiveness would assure that homeostasis would be efficiently re-established (Calabrese and Baldwin, 2001). Based on such observations it is judged that maximum stimulatory responses greater than 3- to 4-fold are likely to represent different phenomena than hormesis.

DIFFERENTIATING THE DEFINITION OF HORMESIS FROM THE PROOF OF HORMESIS

The principal problem with the above definition of hormesis is that of determining if the definition has been satisfied, especially the low-dose stimulation. The recognition that hormetic effects in the stimulatory range are likely not to exceed 30-60% of the control places heightened experimental requirements on claims that the dose response was a real stimulation not accounted for by normal variation. While there is no absolute guidance to be offered in this area, demands to derive a causality conclusion require consideration of the strength and appropriateness of the study design, adequacy of statistical power and reproducibility of findings. The demands on factors impacting decisions on proof become even more difficult when the temporal parameter is included because of the multi-dose, multi-

Fig. 2. Schematic of hormetic nomenclature.



time period study design considerations. This is a significant contributory factor leading to the more limited number of studies that adequately document both the dose and temporal features of the hormetic phenomenon.

The challenge of proof also requires the use of a biological model and endpoint selection that can be assessed within the context of a hormetic dose-response relationship. That is, the endpoint must have the potential to display a biphasic dose-response relationship and temporal responsiveness. Animal models with disease incidence essentially negligible would be unable to assess the occurrence of possible biphasic responses. This is a serious experimental issue since some commonly used cancer and teratogenicity bioassay models have been selected in part because of a low background disease incidence. Likewise, if the initial comparison data were normalized to 100% and these values could not be increased it would not be possible to estimate stimulatory responses, but only decreased responses. Thus, while it is essential to have a clear definition of hormesis it is also important that investigators interested in studying this phenomenon be properly guided with respect to model and endpoint selection, temporal considerations and study design/statistical power and replication concerns.

COMMON STRATEGY, BUT NO SINGLE HORMETIC MECHANISM

The common features of hormetic dose-response relationships that are extremely widespread across the biological and toxicological sciences suggest a common regulatory strategy for biological resource allocation as well as plasticity of regulatory processes within the context of an evolutionary framework. Thus, even though the definition of hormesis is of a descriptive nature, its generalizability indicates the occurrence of basic biological regulatory processes and strategies.

The issue of whether there is a hormetic mechanism may be evaluated within the above framework. Current evidence suggests that the key feature of hormetic dose responses is that resource allocation must be carefully controlled and regulated via physiological set points linked to molecular switching mechanisms. This framework provides the basis by which direct stimulatory or overcompensation stimulatory effects are regulated and display similar hormetic-like biphasic quantitative dose-response relationships. Within this context, there is no expectation that a single hormetic mechanism would have evolved and be broadly applicable. While hormetic responses would be expected to occur in most tissues, precisely how such biological responses occur would be biologically framed within the unique endogenous and exogenous environments of each biological sub-system. While it is clear that hormetic dose-response relationships display limited amplitude variation, the range of the stimulatory response may be very broad. Such recognition is critical to understand for hazard assessment, risk assessment and therapeutic purposes.

CONCLUSION

This paper argues that hormesis is an adaptive response with distinguishing dose-response characteristics that is induced by either direct acting or overcompensation-induced stimulatory processes at low doses. In biological terms hormesis represents an organismal strategy for optimal resource allocation that ensures homeostasis is maintained. This strategy dictates the quantitative features of the dose-response relationship that typifies hormesis including the modest amplitude of the stimulatory response, the range of the stimulatory response and the relationship of peak stimulatory zone to the onset of toxicity regardless of mechanism by which the low dose stimulation originated (i.e., direct acting stimulation versus overcompensation stimulation following initial toxicity). Since numerous mechanisms have evolved to achieve this resource allocation regulatory goal, no single hormetic mechanism is expected, but a common evolutionary-based homeostasis maintenance regulatory strategy is evident.

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DEFINING HORMESIS – COMMENTS ON CALABRESE AND BALDWIN

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INTRODUCTION

Calabrese and Baldwin's (2002) paper defining hormesis is an important step forward towards understanding the range of responses encompassed by hormesis. There are several points made in the paper that merit further examination, some in terms of positive steps forward, others in terms of clarifications, and one in terms of other options.

A SCIENTIFIC DEFINITION

The definition of hormesis proposed by Calabrese and Baldwin (2002) is, appropriately, purely scientific. Removing beneficial/harmful effects from the definition of hormesis is a major, key step forward, which will change the debate regarding hormesis significantly. Previously hormesis has been generally defined in terms of potential beneficial effects. Such definitions confused the phenomenon of hormesis with its possible outcomes. Beneficial effects at the individual organism level may not apply to communities of organisms and may adversely affect other organisms or other aspects of an individual's life-cycle or metabolic processes (Chapman, 2002).

Terms such as "beneficial" or "positive", previously part of the definition of hormesis, were typically not defined. Thus it was not clear whether beneficial or positive responses applied to ecosystems (e.g., helped insure their continuance) or increased the value of the ecosystem for humans. In many cases, previously, hormesis was defined in terms of stakeholder values, i.e., non-scientifically.

However, there should now no longer be confusion between scientific issues (hormesis itself) and manage-

ment issues (the significance of hormesis). It is now clear that the possible outcomes of hormesis, whether or not hormesis has beneficial (or harmful) effects to the individual, other individuals, or to populations and communities, comprise a separate issue. To put this in terms of risk assessment and risk management, determining whether or not hormesis occurs is a risk assessment issue; determining the significance of hormesis is a risk management issue (the "more advanced stage of the analysis" noted by Calabrese and Baldwin, 2002).

HORMESIS AND RISK MANAGEMENT

Risk management involving hormesis can now focus on risk:risk comparisons. For instance, how do the risks of eutrophication and species composition changes resulting from nutrient additions to nutrient-poor lakes compare with the risks of not adding those nutrients? Risk:risk comparisons are surprisingly rare in risk management based on ecological risk assessment, compared to life-cycle assessment (LCA) which is usually applied to products, not biotic phenomena. However, evaluating the significance of hormetic responses in a risk management framework may well require adaptation of LCA procedures given the complexity of the real environment.

For instance, Stuijfzand et al. (2000) found that growth of the midge *Chironomus riparius* was less inhibited by toxicants in polluted rivers than predicted from laboratory tests. In fact, a hormetic stimulation was observed. The stimulation was due to overcompensation based on the quality (not the quantity) of the food in the river water as opposed to that provided in the laboratory. The stimulation was on average >30%, which fits the hormetic model. These results could be interpreted as meaning that pollution of the river waters is not a problem so long as there is sufficient quality and quantity of food to compensate. However, a fully informed decision would require that the life-cycle of the pollutants be examined in all environmental compartments where they are likely to interact, hence the suggestion for applying an LCA model to such situations.

HORMESIS AND EVOLUTION

The concept of preadaptation resulting from "modest overcompensation" implies that at least some hormetic responses may be genetic not solely somatic. If so, then the benefits of such responses can be passed onto subsequent generations. This is more a Lamarkian view of evolutionary change than a Darwinian view. Testing whether and which hormetic responses can be adaptive should be done, focusing on whether such responses are inheritable. The results of such studies should be published in journals read by scientists studying evolutionary processes; such cross-fertilization would appear essential for fully determining the evolutionary basis for and significance of hormesis.

IS AROUSAL HORMETIC?

It was not completely clear from Calabrese and Baldwin (2002) which of the "plethora of terms" are actually included in their definition of hormesis. It seems certain that the definition encompasses the laboratory experimental literature involving organisms other than humans (e.g., U-shaped, J-shaped, bell-shaped). It is less certain that the definition encompasses terms from ecology which have recently been recognized as potentially hormetic (e.g., intermediate disturbance hypothesis, subsidy-stress gradient – Gentile, 2000; Chapman, 2001a,b, 2002). Specificity would be useful, in the form of a summary of what phenomena and "laws" are or are not included in this new definition of hormesis, and a justification or explanation, possibly in tabular format.

For instance, it is not clear whether the Yerkes-Dodson "law" would be included as a form of hormesis, given Figure 1 of Calabrese and Baldwin (2002), even though that "law" is referred to as an example of hormesis. In Figure 1 the basal levels prior to the hormetic response are always higher than the levels following the hormetic response. However, arousal, as originally defined by Yerkes and Dodson (1908) and now a well-established phenomenon in psychology (Corcoran, 1965; Revelle and Loftus, 1992) is characterized by a return to the basal level following removal of stimuli such as caffeine or stress.

Arousal simply means to be wide awake, alert, and full of energy; the reverse conditions would be sleepy, sluggish, tired or relaxed. The baseline for arousal is sleep, the high point would be extreme effort or intense excitement, and if the high point is extended (e.g., hyper-activity or excessive stress), it can be detrimental to the individual (and to others). The baseline before and after arousal remains the same; arousal is a pure U-shaped or bell-shaped response, and one that is physiological, though it is subject to a very wide variety of different mechanisms (Revelle and Loftus, 1992). Given that "both stimulatory and inhibitory dimensions of the hormetic phenomenon must be present to satisfy the qualitative definition of hormesis", arousal does not seem to fit the definition. The definition as exampled and explained in Calabrese and Baldwin (2002) clearly requires clarification in the case of the Yerkes-Dodson "law", and probably also for other "laws" and phenomena noted in their paper.

IS "HORMESIS" THE CORRECT TERM?

I am not convinced that the term "hormesis" is a suitable umbrella term for low dose exposure-responses (Chapman, 2002), and neither are others (Menzie, 2001; Suter, 2001; Giesy, 2001). The term hormesis is generally associated with single-species adaptive responses; higher-level hormetic effects may not be mediated by the same mechanisms as lower-level effects. Ecosystem responses to stress are best characterized as complex and non-linear. And lower-level effects (i.e., at the individual

level) probably encompass a variety of different metabolic and other strategies, as noted by Calabrese and Baldwin (2002). Further, the existence of a "common evolutionary-based homeostasis maintenance regulatory strategy" is not as clearly evident as claimed.

In addition, the term "hormesis" has certain negative, historical connotations (Calabrese and Baldwin, 2000) that probably preclude it being a satisfactory umbrella term. While I like the general approach taken by Calabrese and Baldwin (2002), I favour a new term that will not have any "baggage". Hormesis could be one of the sub-terms, as could more ecological terms such as subsidy stress. As noted by Calabrese and Baldwin (2002), the differentiations they propose may not be the only ones possible, or reasonable.

CONCLUSIONS

The paper by Calabrese and Baldwin (2002) is a major step forward in many respects. However, some clarifications are required as noted above to improve the clarity of the definition, and the utility of the descriptions. The use of "hormesis" as an overall umbrella term remains arguable.

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DEFINING HORMESIS: THE NECESSARY TOOL TO CLARIFY EXPERIMENTALLY THE LOW DOSE-RESPONSE RELATIONSHIP

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ABSTRACT

The authors comment on Calabrese and Baldwin's paper "Defining Hormesis" which, to date, is the first attempt to provide a definition of hormesis that goes beyond the different interpretations of this phenomenon reported in the literature.

While appreciating the effort made in this study to place hormesis in a general and at the same time specific context, the authors believe some clarifications are needed as regards the quantitative features of this phenomenon. In this connection, they speculate on whether Calabrese and Baldwin think it appropriate to include hormesis assessment criteria in the document, referring in particular to those reported in a previous paper. The authors share Calabrese and Baldwin's conclusion that future experimental models designed to study hormetic phenomena must necessarily include the time factor which not only guarantees this phenomenon will be detected, but is also able to detect it's the specific type of hormesis.

Keywords: Hormesis, NOAEL, Low doses, Exposure

The paper presented by Calabrese and Baldwin makes a significant contribution to defining hormesis. In this work they set out to define and generalize this phenomenon in order to allocate an appropriate place to hormesis in a number of different disciplines such as biology, medicine and toxicology.[1,2] The Authors' analysis of the concept of hormesis makes it comprehensible to the reader since it goes beyond the heterogeneous definition attributed to it in the literature. In fact early attempts to define hormesis were characterized by lack of uniformity.[3-6]

In this paper Calabrese and Baldwin define hormesis as

> **"…an adaptative response characterized by biphasic dose responses of generally similar quantitative features with respect to amplitude and range of stimulatory response…"**

This definition makes it possible to establish the design for experimental studies in this field whose aim will be to demonstrate the widespread nature of the phenomenon. In fact, although the biomedical literature examined by the Authors in Calabrese et al.[7] and another study[8] mentioned in this paper is very extensive, there are relatively few articles that have provided evidence of the hormetic mechanism. The Authors are therefore to be praised for their efforts in defining hormesis as the first necessary step to correctly designing any experiment on this matter.

Up to now, lack of uniformity in the definition of hormesis has led to a disparity of opinion that has impeded, if not prevented, the formulation and testing of hypotheses connected with this phenomenon. This has probably hampered, to some extent, a wider recognition of hormesis on the part of the scientific community.

Calabrese and Baldwin also deserve merit for clarifying the concept of low dose exposure. The concept of low doses, and more generally that of hormesis, has been quite often linked to beneficial effects. The Authors use some examples to show that dose-response relationships can result in both beneficial and harmful effects at low levels, and that even if there is a beneficial effect on a single subject, the possibility of harmful results in the general population cannot be excluded, e.g. the case of longevity.[9] We should however remember that in the case of stimulation by chemical or physical agents, any possible beneficial or harmful effects might lead to a misunderstanding of relevant scientific findings, a misinterpretation of occupational exposure limits or even cause political mismanagement of results. This might occur in the occupational sector where the phenomenon of hormesis could be exploited by employers' and trade union representatives for their own purposes.

Calabrese and Baldwin demonstrate that hormesis can be either overcompensation stimulation (OCSC) or direct stimulation (DHS). This wider definition, which encompasses two types of hormesis, facilitates the understanding phenomena linked either to overcompensation (that occurs in order to restore homeostasis

following a disruption) or direct stimulation.

In our opinion, the Authors provide a good definition of the qualitative nature of hormesis "as biphasic dose-response relationships exhibiting a low-dose stimulation and a high-dose inhibition".

However, the definition of hormesis from a quantitative point of view could be improved by clarifying what is meant by "modest overcompensation", even if the Authors have reported that in most cases maximum responses are to be found in the 30 - 60% range compared to the control value. Furthermore, it is not clear whether, when the maximum stimulation response exceeds a given threshold, the phenomenon observed can still be considered hormetic.

In their previous study, Calabrese and Baldwin[8] refer to No Observed Adverse Effect Level (NOAEL), pointing out that, under some circumstances, it is still difficult to obtain an accurate measurement of this parameter. As regards the NOAEL, the Authors could explain why they have eliminated all reference to this in their current paper, and if, should their document be reconsidered, they intend to reintroduce their comments on NOAEL and "control value".

Clarification should be provided for the statistical criteria used both in the definition of NOAEL (in the absence of an experimental measurement) and of inhibition. For example, are the criteria of 90% (for U-shaped curves) and 110% threshold values (for inverted U-shape curves) congruous with the variability of biological experiments and the biological significance of 10% variations? Similar questions could be put forward for Alternative Quantitative criteria proposed for assessing hormesis. Taken globally, what is the quantitative impact that the choice of different (even slightly different) statistical criteria would have on the final number of hormetic-type dose-response relationships?

However, even though we deem the statistical criteria used by Calabrese and Baldwin for confirming hormesis to be valid, different types of statistical evaluations should not be overlooked.[10,11]

It would be appreciable to know if the Authors have identified (even in general terms), the common strategy that leads to the appropriate allocation of biological resources and the flexibility in regulatory processes referred to in their paper.

In our opinion, the concept of hormesis needs further investigation that could indicate the direction to be followed when defining endpoints, taking into consideration, of course, the biological diversity of the living organisms or substructures under examination.

It is our belief that Calabrese and Baldwin's paper represents not only the first comprehensive approach to defining hormesis in relation to current scientific knowledge, but it also provides a framework in which the qualitative and quantitative aspects of hormesis can be viewed from a historical angle without ambiguity.

In conclusion, we agree that future studies on hormesis should be carefully designed in relation to their endpoints, to the doses used, the NOAEL value (if the Authors still intend to take this parameter into consideration in the definition of hormesis) and the possibility of measuring response variations to an increase in exposure levels. Finally, we agree with the Authors that the inclusion of the time parameter in any experimental model will be a decisive factor in understanding and interpreting hormetic phenomena. The statistical approaches should make it possible to distinguish a genuine hormetic occurrence from a mere increase in response.

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DEFINING, EXPLAINING AND UNDERSTANDING HORMESIS

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I wish to make three points in this invited commentary:

1. Good points of the definition.

The proposed definition of hormesis should do fine in clearing up some misunderstanding and misperceptions. The thought that hormesis is an evolutionary-based adaptive response based on optimal resource allocation is certainly an interesting thought. I do not know how such a possibility would be experimentally tested or argued either for or against.

2. What the "definition" of hormesis lacks.

There is no basis in a "superior" science such as physics, chemistry, biochemistry, endocrinology or pharmacology that explains what hormesis is at the level of atoms, molecules and/or cellular macromolecules. Let us use as a comparative example the drug-receptor theory of dose-response commonly used in endocrinology and pharmacology.

The word drug could be replaced by the word hormone or ligand depending on the chemical of interest. Well-known sources of information on drug-receptor theory include commonly available books such as Goodman and Gilman's The Pharmacological Basis of Therapeutics and Casarett & Doull's Toxicology The Basic Science of Poisons. A better, more detailed look at drug-receptor interactions is in the Textbook of Receptor Pharmacology (Foreman and Johansen, 1996). This book (Foreman and Johansen, 1996) defines the association and then disassociation between a drug A with its receptor R:

A + R ——k+1———> AR
<——k-1———

In simple drug-receptor theories, the biological effect is proportional to the concentration of the chemical species AR (bound drug or occupied receptors). Plotted on a linear scale, a rectangular hyperbola is the mathematical form of the dose-response relationship. Plotted on a logarithmic x axis of drug concentration, a S shaped sigmoidal curve is the mathematical form of the dose-response relationship for drug-receptor mediated interactions.

It is this type of firm foundation in the chemical law of mass action that hormesis is lacking. From such a simple theory of drug-receptor binding and complex formation, many useful scientific concepts have been developed, many types of testable experiments can be performed and several versatile mathematical equations can be derived (e.g. saturation binding experiments, theories of full and partial agonists, antagonism and the Hill-Langmuir equation). Nothing similar comes from the definition of hormesis. This limitation or omission in the definition or understanding of hormesis is recognized by Calabrese and Baldwin when they write:

" ... the definition of hormesis is of a descriptive nature, ..."

3. A problem in "understanding and explaining" hormesis.

It is hard for an advocate of hormesis to "prove" only one mechanisms is operating throughout all the dose-response range and this single mechanism is "beneficial" in the low dose range and "toxic" in a higher dose range.

Hormesis may consist of a initial beneficial dose region where several mechanisms are operating (just for the sake of argument let us say 3 mechanisms) and the overall sum of these 3 mechanisms is "beneficial" to the organism. At higher, toxic, doses, many more mechanisms are operating (just for the sake of argument let us say 8 mechanisms) and the sum of all these 8 mechanisms puts the organism in the "toxic" part of the biphasic dose-response curve.

Unlike an advocate of hormesis, an advocate of the drug-receptor theory could give very convincing evidence that binding of a drug to its receptor forms a new chemical complex containing bound to the receptor drug and that the biological response is proportional to bound drug throughout the entire dose-response relationship.

A problem that hormesis has in being more scientifically accepted is (1) proving that only one mechanism accounts for both the "beneficial" and "toxic" parts of the biphasic dose-response curve and (2) giving substantial evidence against the interpretation that "hormesis" is the sum of many different mechanisms which add up to either "beneficial" or "toxic" in two different parts of the dose-response curve.

Disclaimer:

This manuscript has been reviewed in accordance with the policy of the National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

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INVITED RESPONSE TO DEFINITION OF HORMESIS (CALABRESE EJ AND BALDWIN LA)

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DEFINITION OF HORMESIS

According to Calabrese and Baldwin [1,2], hormesis is an adaptive biphasic response. Both responses have similar quantitative amplitudes and ranges resulting from compensation to disturbance(s) of homeostasis. The benefit or harm of compensatory response(s) can be defined by considering both the biological and ecological context of the response. Thus, hormesis can have a low-level adaptive response, no response at an intermediate level, and a second response at a high level of deviation from homeostasis, ie a U- or J-shaped response [1].

EVALUATION OF THE DEFINITION

This definition allows moderate to low-level acute stress to alert multi-cellular organisms, trigger a slight overcompensation on a whole-organism basis and return to homeostasis. The requirement for the response to be biphasic either ignores the conventional dose response or argues for a different mechanism for the response[1]. To see how well the definition works, we chose to apply it to pulmonary oxidant injury, to radiation injury to white blood cells, and to selenium concentration's relationship to cancer.

PULMONARY INJURY

Air pollutant oxidant stresses to lung tissue (eg ozone < 0.1 ppm, nitrogen dioxide < 5 ppm) is expected to destroy type I pneumocytes. If examined from a whole

body perspective, at low doses healing or type II alveolar pneumocyte hyperplasia occurs. At higher doses, healing by secondary intent or emphysema and death occur. This whole body response is at least biphasic. In our opinion, different responses, encompassing different mechanisms occur at different levels of the pulmonary-injury dose response. As Calabrese and Baldwin have indicated, duration of the 2 responses is similar. For example, type I pneumocyte death is naturally healed by type II cell hyperplasia and differentiation into type I alveolar pneumocytes. If type I cell death is excessive, fibroblasts are no longer prevented from growing and being chemically attracted, and healing is by secondary intent. If fibroplasia is not possible, tissue loss and pulmonary emphysema occur [3-6]. Because of the different mechanisms, amplitude of the 2 phases is not routinely compared.

RADIATION INJURY TO WHITE BLOOD CELLS

In the 2nd example, external radiation kills white blood cells, and at a higher dose no leukocyte response occurs and the irradiated animal dies [7]. In the low dose response, the bone marrow is stimulated and new white blood cells are made. At higher radiation doses the more resistant precursor cells are killed as well. The second phase of adaptation, radiation kills the bone marrow responsible for making replacement cells so that presumably damaged cells will not be made. From the whole body standpoint, this appears to be a biphasic response [7]. The duration of each phase of response is similar. However, because of the different mechanisms the amplitude has not, to our knowledge, been meaningfully compared.

SELENIUM AS AN ESSENTIAL ELEMENT, REDUCER OF CARCINOGENESIS AND A POTENTIAL TOXIN

In the 3rd example, selenium produces a different biphasic response [8]. At low levels, selenium has been shown an essential nutrient for rats. Selenium is incorporated to form selenoamino acids, cofactors to essential enzymes. In contrast, at higher levels selenium is effective in preventing the initiation of cancer. Although the mechanism is uncertain, it most likely relates to the generation of catalytic selenium metabolites—selenols and selenides. This proposed mechanism differs from the way selenium serves as an essential nutrient. Finally, at higher selenium doses, the formation of selenoamino acids in keratin and other cutaneous proteins leads to the first stages of selenium toxicity. From a whole body standpoint, this response is at least biphasic, but relies on at least 2 separate mechanims[1, 2, 8]. The duration of each phase is approximately similar. However, because of the difference in mechanisms, the amplitude has not, to our knowledge, been meaningfully compared.

From a whole body standpoint, each of these 3 examples—radiation or chemical injury to lung, radiation's effect on white blood cells, or selenium's biological interactions to reduce cancer—has more than 1 phase as the toxicant dose increases. Each phase has approximately the same temporal distribution, but different biological mechanisms. Because of these differences in biological mechanisms, the response amplitudes have not been compared.

GENERALIZATION OF HORMESIS TO A THEORY OR EXPLANATION OF A NATURAL PHENOMENON

To explain a natural phenomenon and promulgate a theory, one must gather data, evaluate that data, give a sense of that which is not known, formulate a list of working hypotheses, and attempt to prove and disprove the most likely hypothesis and any conflicting hypotheses. This process allows refining of the most likely hypothesis or hypotheses by continuing to search that which is not known and stating the current limitations of it [9].

Gathering the data requires use of a good design to collect a meaningful amount of data. The data that Calabrese and Baldwin [1, 2] reviewed was published and extensive; thus, one has few doubts about it being of good scientific design. Moreover, when there were limitations to the scientific design, they stated them [1, 2].

Secondly, data should be logically consistent with the explainers' experiences. Since Calabrese and Baldwin try to combine diverse sets of data within the framework of hormesis, we can define their process as having logical consistency. Their data appear to be sufficiently cohesive. They describe low level responses not previously identified. Attempts to explain or disprove these new low-level responses are a major part of molecular biological investigations today, and the discoverers of these responses are to be congratulated. From the whole-body standpoint, the reactions appear biphasic and related to different mechanisms.

Thirdly, explainers-theorists must examine all the data in a disinterested way that does not bias their data or its interpretation. Inevitably such analyses will reveal limitations to the explanation, such as the fact that different mechanisms operate at different levels. Then the hypothesis that the explainers believe to be central and any competing hypotheses must be examined. The limitations must be clearly stated and used as a basis to prove and disprove this and all competing hypotheses. This step is quite important, because its judicial use puts the explainers in the best possible position to use critiques from peers to strengthen the most likely of their hypotheses. The explainers should strengthen this phase of their proof by stating the competing hypotheses and the reasoning process by which their central hypothesis reached maximum likelihood.

If the explanation is to be the "standard" against which all others are judged, its acceptance must be critiqued by vigorous questioning. Calabrese and

Baldwin are to be commended for seeking comments from peers and using such comments as a basis of further refining the hypothesis. Experimental limitations may place such proof or disproof in abeyance, but they do not negate the necessity of examining each hypothesis in turn, stating the limitations of each, and refining the strongest hypothesis in this process of proof/disproof. It is not unlikely that the strongest hypothesis will be repeatedly refined during this process. If one examines Calabrese and Baldwin's earlier work [2], it appears that their hormesis framework has undergone considerable refinement.

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COMMENTS ON THE ARTICLE "DEFINING HORMESIS," BY E.J. CALABRESE AND L.A. BALDWIN

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In view of the diversity of biological responses and the extent to which they remain poorly elucidated in many instances, there is merit in the suggestion by Calabrese and Baldwin that the term "hormesis" should be applicable to those adaptive responses which are characterized by biphasic dose-response relationships, without reference to any associated beneficial or harmful effects, since the latter can be evaluated properly only in the context of each response and should not enter into the definition of "hormesis" itself.

According to the above definition, many adaptive responses to ionizing radiation can be regarded as "hormetic" in nature, including the capacity for enhanced repair of damage to DNA and chromosomes (UNSCEAR, 1994; UNSCEAR, 2000). It remains to be determined, however, whether the dose-response relationships for mutations and chromosome aberrations are similarly biphasic in nature; i.e., whether the frequencies of such lesions, and of the cancers to which they may give rise, can be reduced below control levels by appropriately small doses of radiation. Given the potential importance of such a possibility and the fact that it is not excluded by the existing data (NCRP, 2001), the issue clearly warrants further research.

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RESPONSE TO EXPERT COMMENTATORS

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The reviewers all shared the view that the time had come for a more clarifying and specific definition of hormesis. They therefore welcomed the attempt that was provided. While generally supportive of the broad features of the definition, each reviewer had ideas, issues and concerns that the definition either did not adequately address, stress, or otherwise clarify. In the case of the commentary provided by Kitchin there was the recommendation that hormesis seek a firm biochemical foundation and a variety of examples were used as illustrations. We agree with the basic thrust of these comments and indicate that we have identified a large number of studies within the pharmacological literature that define reliable mechanisms of hormetic-like biphasic dose-response relationships within the context of drug-receptor binding and complex formation (Calabrese and Baldwin, 2001). Consequently, we believe that the understanding and, indeed, the demonstration of how hormetic-like biphasic dose responses can occur has been clarified in a large number of systems and in detail. In all such cases the explanation has been within the context of mainstream pharmacological principles. There are still many examples of hormetic-like biphasic dose responses for which mechanisms are not yet known. However, this is no reason to question the concept of hormesis and to establish two camps such as "advocate of hormesis" or "advocate of drug receptor theory". In fact, these scientists are one and the same. That is, it is typical for investigators to observe an hormetic dose response and then spend the next few years trying to understand its underlying mechanism.

The comments of Upton were generally supportive of the concept and definition of hormesis. However, he raises the issue of whether hormesis could be applied to radiation induced mutation and cancer. We would like to note that this is beyond the scope of the article "Defining Hormesis" but would point the readership to two articles by us specifically addressing the issue of

hormesis and chemical- (Calabrese and Baldwin, 1998) and radiation- (Calabrese and Baldwin, 2002) induced cancer. In general, we believe that there is a sufficient core of well-designed and conducted experiments in the cancer domain that demonstrate the occurrence of hormesis. We also believe that the evidence supports the broad generalizability of this concept such that it should extend to all endpoints, models and chemical/physical agents.

We appreciate the emphasis of Chapman on the differentiation of hormetic effects from its application in risk management activities. We strongly agree with the perception of Chapman that the removing of beneficial/harmful effects from the definition of hormesis will significantly alter the debate over the role of hormesis in the field of toxicology and risk assessment. In addition, Chapman correctly questions whether some of the diverse biological phenomena displaying biphasic dose responses (e.g., Yerkes-Dodson Law) are examples of hormesis. In general, these various and similarly appearing phenomena have never been assessed in a comparative manner. This is an important area of research and should be actively pursued. The issue of terminology is raised by Chapman and this is one we have struggled with as well. However, we have come to the conclusion that the most significant problem with the term hormesis has been its lack of definition and the entanglement of the science of hormesis with its application to risk management. With these issues both addressed and hopefully resolved in the Defining Hormesis paper we believe that terminological issues will not dominate future discussion.

The comments of Carelli and Iavicoli were generally supportive of the proposed definition of hormesis, but raised the question about the role of the NOAEL in the definition of hormesis. They asked why the term NOAEL was not used in the entire article. We appreciate this insightful perspective since in the actual practice of assessing hormetic effects the identification of the NOAEL is essential. While the term was not needed for this definitional paper we refer the readership to our recently published paper of how to assess hormetic dose responses where the NOAEL information is of critical importance (Calabrese and Baldwin, 2001).

Finally, we agree with the general perspective of Pickrell and Oehme that our understanding and concept of hormesis has evolved and been refined as a result of the peer-review process. With respect to the technical point of their paper, we agree that hormetic-like biphasic responses have tended to be ignored because multiple mechanisms likely contributed to the formation of the biphasic dose response. However, as in the response to Kitchin, we do not believe this is a limitation of the hormesis definition, but rather a potential explanation.

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