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A CRITICAL ANALYSIS OF CLAIMS OF RADIOSYNTHESIS BY FUNGI

A Chapter Style Thesis Submitted in Partial Fulfillment of the Requirements for the  
Degree of Master of Science in Biology

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# A CRITICAL ANALYSIS OF CLAIMS OF RADIOSYNTHESIS BY

## FUNGI

By Eric D. Walberg

We recommend acceptance of this thesis in partial fulfillment of the candidate's requirements for the degree of Master of Science in Biology.

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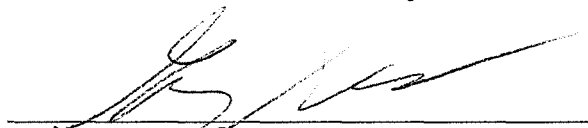


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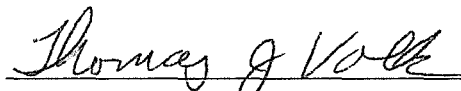


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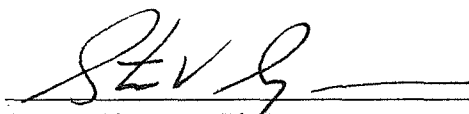
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## ABSTRACT

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'Radiosynthesis' had been described as the capture and metabolic use of energy from ionizing radiation. Past literature (Dadachova, E., Bryan, R. A., Huang, X., Moadel, T., Schweitzer, A. D., Aisen, P., Nosanchuk, J. D., Casadevall, A. (2007). *PloS one*, 2(5): e457, and papers and patent following) has purportedly demonstrated this in fungi, wherein increased fungal growth was seen upon treatment with low-dose ionizing radiation. Melanin was proposed as the agent of energy capture and transduction. Examination of the previous work reveals a number of issues that call into question both radiosynthesis and a mechanism involving melanin. Notably, the energy deposited in their irradiated systems was too low to show the amount of growth seen, and the carbon sources provided in the media were sufficient to support the observed growth and proliferation. Further, the results often fail to show what they are claimed to show, or are misinterpreted. Finally, indirect evidence provided does not support either radiosynthesis or that the mechanism for the observed growth requires melanin. This thesis re-evaluates past work, and concludes that radiosynthesis has not been demonstrated. This thesis also proposes an alternate hypothesis: misinterpretation of the effects of radiation as though from a different stimulus, resulting in an expressional shift appropriate for that stimulus or a more generalized response. Finally, this thesis proposes that a micro-scale stochastic view may be useful for future research in examining the responses of fungi and single-celled organisms in low-dose ionizing radiation.

## ACKNOWLEDGEMENTS

I would be eternally remiss if he did not acknowledge Dr. Tom Volk. I have frustrated him immensely, hoarded space in his lab unduly, and pestered his other grad students maddeningly. And in his lab, this work was subject to numerous fits and starts. Nevertheless he has stuck by me, something nobody should be expected to do under the circumstances.

I am also grateful to many. To Dr. Heather Schenck for teaching me to write in other than poorly translated Russian, for checking my assumptions before I made a fool of myself more publicly, and for coaching me on diplomacy – though whether I employ it is another matter. To Dr. Gregory Sandland with whom I spent many hours; despite that experiments are not presented in this thesis, Dr. Sandland was immensely helpful in statistical analysis and data advice. And to Dr. Shelly Leshner for setting a high standard and ensuring that numerous dubious and unhelpful things are not interred herein.

I am both very grateful and feel very guilty toward Dr. Ruth Bryan, one of the coauthors of some of the papers which are dealt with herein. Proliferation in ionizing radiation was never successfully demonstrated in this thesis, but this was by no means due to any lack of help from Dr. Bryan. Kind, open-minded, and collaborative, Dr. Bryan helped me immensely, always answering questions and providing resources, even though I had come to disagree with the idea being invalidated by this thesis.

Finally, I apologize to Buttons, Bones McCat, and Malessezia Furfur for abandoning them, if only temporarily. They deserve better. I'm sorry kids. I'll be home soon. I miss you, and I love you.

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## CHAPTER I

### INTRODUCTION

The phenomenon of ‘radiosynthesis,’ the capture and metabolic use of energy from ionizing radiation, has purportedly been demonstrated in fungi (Dadachova *et al.* 2007, Dadachova *et al.* 2014, Biello 2007, Balter 2007, Norris 2007, Dadachova and Casadevall 2011). Melanin was proposed as the primary agent of energy capture and transduction. The idea has its origins in an increasing body of literature showing resistance of melanized fungi to the effects of ionizing radiation and in peculiar patterns of growth often specific to strains isolated from high-radiation sites. These observations culminated in experiments that reported increased fungal growth on exposure to ionizing radiation. Melanized species and strains reportedly showed higher growth than non-melanized ones (Dadachova *et al.* 2007).

Many fungi have been isolated from radioactively contaminated or irradiated areas. For example, fungi have been isolated from Chernobyl nuclear reactor's containment building (Mironenko *et al.* 2000, Vember and Zhdanova 2001, Zhdanova *et al.* 2000), from radioactively contaminated soils such as those around the Chernobyl reactor (Zhdanova *et al.* 1994), and from chronically externally irradiated soils in the ‘irradiated forest’ at Brookhaven National Laboratory (Gochenaur and Woodwell 1974). Very high radiation environments tend to select for simple communities dominated primarily by melanized species. Presumably, therefore, melanization affords protection against the effects of ionizing radiation (Zhdanova *et al.* 1994, Gochenaur and

Woodwell 1974).

A number of traits for fungal survival in high-radiation environments have been identified. These include, but are not limited to, increased expression of antioxidants or protective enzymes (Tugay 2007, Tugay *et al.* 2011), hyphal aggregation (Gessler *et al.* 2007), and increased melanin production (Tugay 2007, Tugay *et al.* 2011). Most of the observed traits are adaptations for increased tolerance to oxidative stress (Tugay 2007, Tugay *et al.* 2011, Ivanova *et al.* 2005), or repair of DNA damage and cell cycle regulation (Bennett *et al.* 2001, Dighton *et al.* 2008). This makes sense, as the main biological effects of ionizing radiation are due to production of reactive species – often oxidizing radicals – in bulk aqueous solutions, and to DNA damage.

Peculiarities have become apparent, however. For example, there is purported growth toward, and degradation of, ‘hot particles’ (bits of graphite from the Chernobyl reactor containment vessel) (Zhdanova *et al.* 1991). This observation suggested a test of whether the fungi were reacting to the carbon or to the radiation. Zhdanova *et al.* (2004) purportedly demonstrated attraction by radiation itself, so termed ‘radiotropism,’ by measuring mean growth angles relative to a radiation source. Thereafter, Tugay *et al.* (2006) demonstrated that some isolates from the Chernobyl contamination showed stimulation of germination and growth of emergent hyphae under ionizing radiation. Though some isolates from Chernobyl showed no effect or a decrease in germination, isolates from un-contaminated areas unanimously showed unchanged or decreased germination. Karpenko *et al.* (2006) showed a high correlation between strains demonstrating radiotropism and strains demonstrating phototropism. This correlation hints at potential common mechanisms, though they attribute this to light generated by

radioluminescence – an unlikely scenario (Tarasov *et al.* 2007). In all studies, radiotropism was highly strain-dependent, although some showed correlations with dose. The likelihood that a strain would show radiotropism peaked in isolates from areas of ‘low’ contamination; isolates from un-contaminated areas were less likely to show radiotropism, while isolates from regions of high doses (~1 Gray or more, unit of time not specified in source) almost universally showed no radiotropism (Zhdanova *et al.* 2004, Dighton *et al.* 2008).

Zhdanova *et al.* (2004) believed radiotropism to be an attraction to the radiation itself, and concluded by proposing three alternate hypothetical mechanisms: 1. Response to lower energy emissions due to effects of radiation on the medium, 2. Response to a temperature gradient where the radiation intersected the medium, and 3. Metabolic use of the energy from the ionizing radiation. In their analysis, Zhdanova *et al.* (2004) discarded only the second hypothesis as likely insignificant. This could perhaps be the origin of the idea which was later termed ‘radiosynthesis’ for fungi.

Sparked by a number of the observations above, as well as several lines of ‘indirect evidence,’ Dadachova *et al.* (2007) proposed to test the third hypothesis of Zhdanova *et al.* (2004). Dadachova *et al.* (2007) purportedly demonstrated that some melanized fungi showed higher cell counts and faster nutrient uptake when grown in low doses ( $5 \times 10^{-5}$  Gy / hr) of ionizing radiation. Therein it was also claimed and purportedly demonstrated that melanized fungi showed a greater effect than non-melanized controls. Three different fungi were tested (*Cryptococcus neoformans*, *Cladosporium sphaerospermum*, and *Wangiella dermatitidis*); each fungus was melanized or de-melanized by a different mechanism and compared to closely-related strains or within

strains. This was to rule out confounding factors from each method of de-melanization. Dadachova *et al.* (2007) further explored how the properties of melanin changed after irradiation, including melanin's redox transfer properties. The results of Dadachova *et al.* (2007) have been described in a number of popular science news casts, wherein statements were made by the author affirming the idea of radiosynthesis (e.g. Biello 2007, Balter 2007, Norris 2007). Furthermore, in a patent application submitted that same year, the idea was clearly stated and the term 'radiosynthesis' was applied for the first time to fungi (Dadachova *et al.* 2014). This patent was granted in 2014. Such ideas have since been repeated elsewhere (e.g. Dadachova and Casadevall 2011).

The increased growth and proliferation seen in Dadachova *et al.* (2007) has been repeated (Robertson *et al.* 2012, Shuryak *et al.* 2014). Furthermore, similar stimulatory effects have been seen sporadically in the literature in other organisms. These include but are not limited to the single-celled eukaryotes *Paramecium tetraurelia* (Croute *et al.* 1982a) and *Tetrahymena pyriformi* (Luckey 1986), and the cyanobacterium *Synechococcus lividus* (Conter *et al.* 1984). In the latter species, the effect was demonstrated to depend on several factors including growth phase (Conter *et al.* 1987b). Along similar lines, a decrease in growth has been demonstrated by shielding *P. tetraurelia* (Planel *et al.* 1976, Kawaishi *et al.* 2012), *T. pyriformi* (Luckey 1986), *S. lividus* (Conter *et al.* 1983), and mouse cells (Takizawa *et al.* 1992, Kawanishi *et al.* 2012) from background radiation. If correct, then the effects seen in low-dose irradiation experiments are exaggerations of a more ubiquitous effect present to a lesser degree at background radiation levels.

Were 'radiosynthesis' in fungi – or any organism – indeed occurring, the

implications could be profound. Many have assumed this interpretation to be correct, with a number of works citing Dadachova *et al.* (2007) in the affirmative, if sometimes hesitantly. Unfortunately, this conclusion is incorrect or unsupportable for several reasons.

- Energy calculations can show that the dose was far too low to show the observed growth if used metabolically.
- The carbon sources available to the fungi in the experiments that showed increased cell counts are estimated to be sufficient to support the observed growth, including the increase seen under ionizing radiation.
- The results of the experiments are open to alternate interpretations or occasionally misinterpreted.
- Indirect evidence used to justify either radiosynthesis or the importance of melanin therein does not, under scrutiny, support the hypothesis or the conclusions.

This thesis addresses these issues in the literature supporting radiosynthesis.

This thesis also proposes an alternate hypothesis: the fungi tested may be ‘misinterpreting’ the effects of ionizing radiation as if from a different stimulus, and undergoing a metabolic shift usually reserved for this alternate stimulus. This may occur due to tethering of components of condition-specific responses to a generic DNA damage or oxidative stress response (Goldman *et al.* 2002). Finally, this thesis proposes that a micro-scale stochastic view may be useful in future research, especially with single celled organisms in suspension. This view is used to examine possible targets in low-dose irradiated systems. This view has potential implications for future irradiation procedures

within this field. Despite the pitfall of the idea of radiosynthesis, the responses of single-celled organisms to low-dose ionizing radiation should continue to be studied; the real cause and progression of the irradiated organisms' responses seems yet to be determined.

## **CHAPTER II**

### **CALCULATIONS ARGUING AGAINST RADIOSYNTHESIS IN FUNGI**

The dose is defined as the amount of energy added to an amount of mass by radiation. In this thesis, a commonly used unit will be the Gray (Gy), equivalent to 1 Joule of energy deposited per kg of mass.

Three recent papers have demonstrated increased growth and proliferation of fungi in low-dose ionizing radiation: Dadachova *et al.* (2007), Robertson *et al.* (2012), and Shuryak *et al.* (2014). In the two former reports,  $\gamma$ -rays sources were used, while in the latter X-ray sources were used. The high-energy photons from each source are prone to ignoring matter, though they will occasionally interact. At the photon energies in these studies, the primary interactions with matter are Compton scatter at higher energies, and the photoelectric effect at lower energies. In Compton scatter, the photons strike electrons, ejecting the electrons from their respective atoms with a portion of the photon's energy. In the photoelectric effect, the photon is absorbed by an electron, thus the electron is ejected from the atom with all of the photon's energy, minus the electron's binding energy.

Both Compton scatter and the photoelectric effect result in electrons traveling through the material. In the case of the three studies referred to above, the material is the medium or cells. In the case of the experiments dealt with in this thesis, the medium and occasionally the cells. These electrons lose energy as they travel through the medium by interacting with the electrons around the molecules. The high-energy electrons excite and



ionize atomic and molecular electrons in a process termed linear energy transfer (LET). The amount of energy transferred is the dose. Under normal circumstances, most of this dose is not in a biologically useful form. A portion of the energy is transferred to non-electronic orbital excitations; in water, about 70% of the energy lost by the electron becomes waste heat (Bushberg *et al.* 2002, Cherry *et al.* 2003, Lide 2008). In water-based media or in organisms, water is the primary component; excited and ionized molecules of water will break apart into reactive species. For example, excited water may break into hydroxyl radicals (OH<sup>•</sup>) and hydrogen radicals (H<sup>•</sup>), while free electrons resulting from ionization events become stabilized in solution as solvated electrons (e<sup>-</sup><sub>(aq)</sub>). Such products subsequently react with each other or with other local molecules, often with diffusion-limited kinetics. Therein further energy is lost (Ward 1988).

Dadachova *et al.* (2014) proposes an antenna-like mechanism wherein high-energy electrons are slowed by melanin. Were this mechanism viable, the loss of energy due to LET in the solution might be mitigated. Pi-bonded or conjugated systems are said to take a greater share of the energy of an electron due to the addition of molecular excitation bands (Swallow 1966, Swallow and Inokuti 1988). Melanins are made of stacks of well-conjugated oligomers (Cheng *et al.* 1994b, Stark *et al.* 2003b, Kaxiras *et al.* 2006, Meredith and Sarna 2006, Meng and Kaxiras 2008a), thus without reason to think otherwise it seems reasonable that melanins might demonstrate high LET – this could be tested.

Here shall be presented a series of calculations demonstrating that the radiation dose was too low to show growth and proliferation by metabolic use, and that the carbon sources were sufficient to show the observed growth and proliferation. The discrepancy

is irrespective of any proposed mechanism of energy capture. These calculations do not dispute the possibility of metabolic use of ionizing radiation. Dadachova *et al.* (2007) and Dadachova and Casadevall (2008) rightly cite the example of bacteria making use of hydrogen and sulfate arising from radiolysis in chemosynthesis (Lin 2006). These calculations merely dispute that experiments have demonstrated radiosynthesis in fungi. Though many different calculations could be used, these few were selected for their ease and because they address particular claims or experiments in Dadachova *et al.* (2007). None of these calculations pertain directly to melanin; it is the sheer quantitative discrepancy, regardless of the proposed mechanism involving melanin, that is the foundation of this argument. Table 1 contains a list of values and conversions that were used in these back of the envelope (BOTE) calculations.

Table 1. Values and conversions useful for BOTE calculations in this section.

---

Energy conversions		
1 Joule (J)	= 6.242×10 <sup>18</sup> electron volts (eV)	
1 kilowatt hour (kWh)	= 3.6×10 <sup>6</sup> J	= 2.25×10 <sup>25</sup> eV
1 Gray (Gy)	= 1 J/kg	= 6.242×10 <sup>15</sup> eV/g
Doses reported in Dadachova <i>et al.</i> (2007)		
5×10 <sup>-5</sup> Gy/hr	= 3.121×10 <sup>14</sup> eV/(kg×hr)	= 3.121×10 <sup>11</sup> eV/(mL×hr)
1.5×10 <sup>-3</sup> Gy/30 hr	= 9.363×10 <sup>15</sup> eV/(kg×30 hr)	= 9.363×10 <sup>12</sup> eV/(mL×30 hr)
1.8×10 <sup>-2</sup> Gy/15 days	= 1.124×10 <sup>17</sup> eV/(kg×15 days)	= 1.124×10 <sup>14</sup> eV/(mL×15 days)
Food energy (energy available from food through respiration)		
Carbohydrates ~ Protein	= 17 kJ/g	= 1.06×10 <sup>23</sup> eV/g
Organic Acids (Acetate)	= 10 kJ/g	= 6.24×10 <sup>22</sup> eV/g
Average solar insolation at Lat: 42°50'N, Lon: 32°E (Point on Black Sea (Overmann <i>et al.</i> 1992))		
4.15 kWh/(m <sup>2</sup> ×day)	= 0.173 kWh/(m <sup>2</sup> ×hr)	= 3.89×10 <sup>24</sup> eV/(m <sup>2</sup> ×hr)
30% average solar insolation at point above (photosynthetically available light)		
1.25 kWh/(m <sup>2</sup> ×day)	= 0.052 kWh/(m <sup>2</sup> ×hr)	= 1.17×10 <sup>20</sup> eV/(cm <sup>2</sup> ×hr)
Energy absorbed from 30% solar insolation assuming 1% photosynthetic efficiency		
0.0125 kWh/(m <sup>2</sup> ×day)	= 5.2×10 <sup>-4</sup> kWh/(m <sup>2</sup> ×day)	= 1.17×10 <sup>18</sup> eV/(cm <sup>2</sup> ×hr)
Redox potentials		
NAD <sup>+</sup> + H <sup>+</sup> + 2e <sup>-</sup> → NADH		ΔE' <sup>o</sup> = -0.32 V
½O <sub>2</sub> + 2H <sup>+</sup> + 2e <sup>-</sup> → H <sub>2</sub> O		ΔE' <sup>o</sup> = +0.82 V
NAD <sup>+</sup> + H <sub>2</sub> O → ½O <sub>2</sub> + NADH + H <sup>+</sup>		ΔE' <sup>o</sup> = -1.14 V

---

### Example of Precedent For These Calculations

The work analyzed in this thesis is not the first claim pertaining to ‘radiosynthesis’, nor the coining of the term ‘radiosynthesis’. The idea was broached at least once before, in Kriss and Rukina (1953), to explain the observed carbon fixation deep in the Black Sea, in the hydrogen sulfide zone. As a precedent for the following calculations, Kuznetsov (1956) addressed these claims with some basic energetics calculations. The results were compared to actual data, showing that energy entering the system from forms of ionizing radiation (cosmic rays, radioactivity in natural waters) was far too little to explain the observed activity, and so was unlikely to be the cause of the observed synthesis.

The primary argument in Kuznetsov (1956) was briefly as follows:

1. The energy of formation of  $C_6H_{12}O_6$  (and  $6O_2$ ) from  $6H_2O$  and  $6CO_2$  is:

$$708 \text{ kCal/mole}$$

(our known values have been honed since, but it was not horribly far off).

2. 1 Ci (1 Curie =  $2.22 \times 10^{12}$  decays/minute) of radium produces heat of:

$$\sim 2.3 \text{ calories/minute}$$

3. The observed synthesis of carbon products in Black Sea waters was:

$$1 \text{ } \mu\text{g}/(\text{L} \times \text{day}) = 0.0039 \text{ calories}/(\text{L} \times \text{day})$$

which could be supplied by:

$$3.75 \times 10^9 \text{ decays}/(\text{L} \times \text{day}) = 2.6 \times 10^3 \text{ decays}/(\text{mL} \times \text{minute}) \sim 10^{-3} \mu \text{ Ci/mL}$$

4. The measured activity of natural bodies of water varied, but only between:

$$\sim 10^{-9} \text{ to } 10^{-7} \mu \text{ Ci/mL}$$

The activity of deep ocean silts was:

$$\sim 10^{-5} \text{ } \mu\text{Ci/mL.}$$

Kuznetsov (1956) concluded that ‘radiosynthesis’ was probably not the energy source powering the observed carbon fixation seen deep in the Black Sea.

Kuznetsov (1956) is not perfect, containing a few unit-based typographical errors and many assumptions for simplicity. However, this reference demonstrated that there was a vast discrepancy between what basic calculations say would be necessary for radiosynthesis and what was observed. The calculations presented here are of similar ilk, though hopefully devoid of errors. The discrepancy in the amount of energy added to the system by radiation in Dadachova *et al.* (2007) is similarly large. Therefore these calculations can make assumptions for simplicity without loss of strength. One can see similar examples of calculations of energy deposited by a given dose in Hall and Giaccia (2006).

### **Calculations Demonstrating That Radiosynthesis Has Not Been Shown in Fungi Comparison to Food In The System**

The experiments in Dadachova *et al.* (2007) were done:

*to show the ability of melanized fungi to utilize ionizing radiation as an energy source in conditions of limited nutrient availability* (Dadachova *et al.* 2014).

To demonstrate that the amount of energy added to the system via ionizing radiation is insignificant, it shall be compared to the ‘food energy’ of carbon sources provided to the fungi in each growth experiment.

From Table 1, the usual values for food energy for carbohydrates is  $\sim 10^{23}$  eV/g, and for simple organic acids is  $\sim 6.2 \times 10^{22}$  eV/g. This is a bomb-calorimetric approximation of the amount of energy an organism can maximally derive from a food

source when metabolized via respiration.

The amount of energy reported to be added to the system in Dadachova *et al.* (2007) by gamma radiation was  $1.5 \times 10^{-3}$  Gy/30hr, which is  $\sim 10^{16}$  eV/(L $\times$ 30hr). This amount of energy is equivalent to the ‘food energy’ of:

- $10^{-7}$  g/L of carbohydrates
- $1.5 \times 10^{-7}$  g/L of acetate

This is about six orders of magnitude less than the actual concentrations of carbon sources available to the fungi in any of the growth experiments in Dadachova *et al.* (2007). These amounts were as follows:

- *Cladosporium sphaerospermum* was tested with and without 0.1 g/L sucrose
- *Wangiella dermatitidis* was tested in ‘essential salts’ with 0.12 g/L sucrose
- *Cryptococcus neoformans* was tested in ‘essential salts’ with 1 mM sodium acetate, i.e. 0.060 g/L acetate

These values can be converted to food energies. The food energy from carbon sources in the growth experiments of Dadachova *et al.* (2007) were as follows:

- $10^{22}$  eV/L sucrose for *Cl. sphaerospermum*
- $1.2 \times 10^{22}$  eV/L sucrose for *W. dermatitidis*
- $3.7 \times 10^{21}$  eV/L acetate for *Cr. neoformans*

**Addendum 1: Food energy and whole cells.** With a few assumptions, the question can be asked: what would be the energy of a whole cell? This calculation shall assume the reverse: approximately how much energy could you get out of a cell if, say, one were to mineralize it? Such a question can demonstrate the dose discrepancy.

Assume 5  $\mu$ m diameter (small) *Cr. neoformans* cells, excluding mass expanding

beyond that range (such as the capsule), with an approximate dry weight of 1/20<sup>th</sup> its wet weight (a common estimate for fungi), and a dry weight density (compacted) of ~1 g/cm<sup>3</sup>. For simplicity, assume the cell is composed of primarily carbohydrates and proteins, thus having a ‘food energy’ content of ~1.06×10<sup>23</sup> eV/g.

For this hypothetical cell, the ‘food energy’ of its mass would be:

$$4/3\pi(2.5 \times 10^{-4} \text{ cm})^3 \times (1/20 \text{ g/cm}^3) \times (1.06 \times 10^{23} \text{ eV/g}) = 6.94 \times 10^{11} \text{ eV/cell}$$

From this can be calculated an approximate number of cells that could be formed from the energy of the dose in Dadachova *et al.* (2007):

$$\frac{9.363 \times 10^{12} \text{ eV}/(\text{mL} \times 30\text{hr})}{6.94 \times 10^{11} \text{ eV/cell}} = 13.5 \text{ cell}/(\text{mL} \times 30\text{hr})$$

Therefore, with the assumptions mentioned, the total dose would be the equivalent of the approximate bond energy of 13.5 cells. Though imprecise and containing assumptions for simplicity, this calculation gives an idea of the order of the discrepancy; in Dadachova *et al.* (2007), *Cr. neoformans* cell growth experiments began with 10<sup>5</sup> cells. The *difference* in growth between irradiated and unirradiated treatments was on the order of ~10<sup>5</sup> - 10<sup>6</sup> cells. This is a discrepancy of five orders of magnitude.

**Addendum 2: Food in the system to cells grown.** Joining the two previous calculations, how many cells could one make with the food in-system? Is it sufficient to show the growth observed? *Cr. neoformans* was grown in 1 mM sodium acetate (60 µg/mL acetate). If each cell is about 6.94×10<sup>11</sup> eV/cell, and assuming the above value of ~6.24×10<sup>23</sup> eV/g ‘food energy’ for organic acids, the acetate in 1 mL of the system is the ‘food energy’ equivalent of approximately:

$$\frac{(6 \times 10^{-5} \text{ g}) \times (6.24 \times 10^{22} \text{ eV/g})}{\sim 6.94 \times 10^{11} \text{ eV/cell}} = \sim 5.4 \times 10^6 \text{ cells}$$

The *Cr. neoformans* experiments in Dadachova *et al.* (2007) ended with  $\sim 10^6$  cells/mL. If acetate is the primary carbon source, as it should be in the system in question, this order of magnitude difference means that there may be an excess of food. If a metabolic shift were to cause more of this acetate to be used structurally rather than energetically, there is, by this calculation, enough of an excess to account for the observed extra growth in irradiated samples.

One telling fact lies in the comparison of Dadachova *et al.* (2007) and Shuryak *et al.* (2014) *Cr. neoformans* growth assays: both experiments end around  $10^6$  cells. However, Dadachova *et al.* (2007) began with  $10^5$  cells and the experiment ran for 30 hours, while Shuryak *et al.* (2014) began with  $10^3$  cells and the experiment ran for 48 hours (or were left to grow to 72 hours). Cells at 30 hours in Dadachova *et al.* (2007) were showing obvious slowing of growth around  $\sim 10^6$  CFUs, and Shuryak *et al.* (2014) observed the same at 72 hours around  $\sim 10^6$  CFUs. While stimulation may lead to a greater number of cell counts and greater acetate uptake, it is likely that it is acetate itself that is limiting. The difference in growth and proliferation is likely supported by a difference in acetate uptake and partitioning. Furthermore, the back-of-the-envelope estimate of the maximum number of ‘small cells’ producible from the carbon sources in-system is on the order of the observed maximum number of cells in either experiment. This fact demonstrates the approximate validity of the estimates herein.

### **NADH/NADPH reduction, Fatty Acid Synthesis, and Membrane Produced**

Dadachova *et al.* (2014) states:

*One can speculate by analogy with photosynthesis that in melanized cells the energy of ionizing radiation is harvested by an antenna complex and funneled to*

*the reaction center where this energy is used to split water molecules. Splitting of water molecules starts a flow of electrons, which are used to reduce NAD<sup>+</sup> to NADH (“portable electrons”) and convert ADP by addition of phosphate into ATP (“portable energy”) in a process termed herein as “radiosynthesis.”*

This analogy points to a model system by which one could examine the claim in terms of its final energetics: fatty acid synthesis in the *Cr. neoformans* experiments. In Dadachova *et al.* (2007), *Cr. neoformans* was grown in 60 mg/L acetate. Acetate can be converted to acetyl-CoA by acetyl-CoA ligase, which *Cr. neoformans* is reported as having (KEGG IDs: CNA07740, CNBA7580). Acetyl-CoA is converted to malonyl-CoA by acetyl-CoA carboxylase, without which *Cr. neoformans* would be unlikely to survive, given the necessity of fatty acid synthesis. Therefore these estimates need not assume any carbon fixation, as the cells could use the acetate in-system.

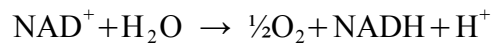
Fatty acid synthesis proceeds by enzymatic addition of 2-carbon units, from 3-carbon malonyl-CoA, to an initial acetyl-CoA. Each malonyl-CoA unit requires 2 NADPH to add to the growing chain; thus biosynthesis of a 16-carbon chain (the fatty acid palmitate) requires 14 NADPH (Stryer 1995). Carboxylation of the acetyl-CoA to malonyl-CoA requires 1 ATP as well, for a net use of 7 ATP. An approximation of ATP formed per NADH is around 2.5 ideally (Stryer 1995), so the synthesis of one Palmitate uses about 17 NADH. The Gibbs energy of formation of NADH and NADPH can be used to examine this system.

The source of energy used to reduce NADH is presumed by the quote above to be melanin: DOPA melanin (eumelanin, approximately) for *Cr. neoformans*, and DHN melanin for *W. dermatitidis* and *Cl. sphaerospermum* (though these were tested only for



growth). Melanins have been recalcitrant in giving up their secrets, including their basic redox potentials. If, however, melanin is assumed to act catalytically, providing only the mechanism and energy for driving the reaction, it can be discounted for simplicity. The beginning and end points, electron donor and acceptor, can be dealt with alone. To address the claim quoted above, assume NAD(P)H to be the electron acceptor and H<sub>2</sub>O to be the electron donor, as in photosynthesis.

The redox reaction for reduction of NADH and oxidation of H<sub>2</sub>O presented in the statement above would be:



$$\Delta E'^0 = -0.32 \text{ V} + -0.82 \text{ V} = -1.14 \text{ V}$$

from which can be solved the equilibrium constant, K':

$$K' = \exp\left[\frac{-1.14 \times 2 \times F}{R \times T}\right] = \exp\left[\frac{-1.14 \times 2 \times 9.65 \times 10^4}{8.314 \times 293}\right] = 5.95 \times 10^{-40}$$

The equilibrium concentrations for the coupling in the system are heavily therefore against the reduction of NAD<sup>+</sup> to NADH and the oxidation of H<sub>2</sub>O to O<sub>2</sub>, because:

$$\frac{[\text{O}_2]^{1/2} \times [\text{NADH}] \times [\text{H}^+]}{[\text{NAD}^+] \times [\text{H}_2\text{O}]} = 5.95 \times 10^{-40}$$

Now a more 'real' value can be derived from common known values. The concentration of O<sub>2</sub> at saturation in water at room temperature is ~9 mM. According to Nichols and Ferguson (2002), common ratios for NAD<sup>+</sup>:NADH and NADP<sup>+</sup>:NADPH, are 10:1 and 1:100 respectively. Experiments were often done at or around pH 6, while ΔE'<sup>o</sup> is defined at pH 7. Finally, the molarity of pure water is 55.5, so assume 55M water. These numbers give values of -0.08 V and +0.01 for NAD<sup>+</sup>/NADH and NADP<sup>+</sup>/NADPH respectively. The calculation for the non-steady state redox potential is:

$$\Delta E = \Delta E^{\circ} + \frac{RT}{(nF)} \ln \left( \frac{[\text{Prod1}]^{p1} [\text{Prod2}]^{p2} [\text{Prod3}]^{p3}}{[\text{React1}]^{r1} [\text{React2}]^{r2}} \right)$$

thus

$$\Delta E_{\text{NADH}} = -1.14 \text{ V} - 0.08 \text{ V} = -1.06 \text{ V}$$

$$\Delta E_{\text{NADPH}} = -1.14 \text{ V} + 0.1 \text{ V} = -1.15 \text{ V}$$

Every order of magnitude difference between the [Prod]ucts and [React]ants will change the  $\Delta E$  by about 0.03 V, or 0.015 V for  $\text{O}_2$ . It is, for practical reasons, unlikely that a cell would be capable of greatly decreasing the final energy value, thus BOTE calculations can proceed with an approximation of 1 V to give the benefit of the doubt. Furthermore, the mechanism stated in Dadachova *et al.* (2007) requires melanin, which in *Cr. neoformans* is extracellular (Eisenman *et al.* 2005). Accordingly, it would be difficult for a cell to maintain the gradient of NAD(P)<sup>+</sup> and NAD(P)H across the membrane necessary to greatly increase the favor of this reaction.

To use this calculation for comparison, an associated energy must be calculated.

The redox potential and the Gibbs energy are convertible:

$$\Delta G = -nF\Delta E$$

$$\Delta G = -(-1 \text{ J/C}) \times (9.65 \times 10^4 \text{ C/mole}) \times (2)$$

$$= 1.95 \times 10^5 \text{ J/mol} = 1.205 \times 10^{24} \text{ eV/mol}$$

This is approximately the input it would take to oxidize 1 mol of NADH from NAD<sup>+</sup> using  $\text{H}_2\text{O}$  as the electron source. Assuming perfect capture:

$$\frac{9.363 \times 10^{12} \text{ eV} / (\text{mL} \times 30 \text{ hr})}{1.205 \times 10^{24} \text{ eV/mol}} = 7.77 \times 10^{-12} \text{ mol} / (\text{mL} \times 30 \text{ hr})$$

Finally, synthesis of palmitate from acetate would take approximately 17 NADH; the amount of palmitate that can be formed with the above of NADH is:

$$\frac{7.77 \times 10^{-12} \text{ mol NADH} / (\text{mL} \times 30 \text{ hr})}{17 \text{ mol NADH/mol Palmitate}} = 4.57 \times 10^{-13} \frac{\text{mol palmitate}}{\text{mL} \times 30 \text{ hr}}.$$

For biological perspective, palmitate in a cell membrane has a surface area per molecule of  $\sim 20 \text{ \AA}^2$  (Jain 1988), thus

$$\frac{(4.57 \times 10^{-13} \text{ mol palmitate}) \times (20 \text{ \AA}^2 / \text{molecule}) \times (6.02 \times 10^{23} \text{ molecules/mole})}{2 \text{ layer}}$$

$$= 2.75 \times 10^{12} \text{ \AA}^2 = 2.75 \times 10^{-8} \text{ m}^2$$

To compare, the surface area of a smallish spherical  $5 \text{ \mu m}$  diameter *Cr. neoformans* cell (*Cr. neoformans* ranges from 2-10  $\text{\mu m}$  diameter) is:

$$4\pi r^2 = 4\pi (2.5 \times 10^{-6} \text{ m})^2 = 7.85 \times 10^{-11} \text{ m}^2$$

thus the calculated palmitate would produce enough outer membrane for

$$\frac{2.75 \times 10^{-8} \text{ m}^2}{7.85 \times 10^{-11} \text{ m}^2 / \text{cell}} = 3.5 \times 10^2 \text{ cells}$$

To summarize: under the reported dose of  $5 \times 10^{-5} \text{ Gy/hr}$  for 30 hours into 1 mL and assuming perfect capture, it would be possible to synthesize enough outer membrane for  $\sim 350$  spherical cells of  $5 \text{ \mu m}$  diameter.

To give the benefit of the doubt, this number is a low estimate; membranes are about half protein. Also, this calculation assumed that the source of electrons was water. If, instead, the source of electrons was closer in redox potential to NADH (though what this would be remains unknown), the amount of NADH formed per unit energy input could be higher.

Unfortunately the energy addition was assumed to be the total ‘dose’ in usable form. Much of the actual energy added to the system would be lost as described earlier. This is especially true if melanin is the transducer, due to its tendency to dissipate excitation energy as heat (Meredith and Sarna 2006, Delevoye *et al.* 2011, Olsen *et al.*

2007, Meng and Kaxiras 2008b). The actual value would likely be much lower than this. Furthermore, this number accounts for the outer membrane; a value for internal membranes would be orders of magnitude less.

### **Comparison to Photosynthesis: Regular Sunlight**

The quote above invokes analogy to photosynthesis. What, then, is the energetic comparison between typical photosynthesis and the experiments in Dadachova *et al.* (2007)? From table 1, near 42.5°N the solar insolation in the visible spectrum averages  $1.17 \times 10^{20}$  eV/(cm<sup>2</sup>×hr). At 1% efficiency, a common value for photosynthesis, the energy captured would be  $1.17 \times 10^{18}$  eV/(cm<sup>2</sup>×hr). For brief comparison,

$$5 \times 10^{-5} \text{ Gy/hr} = 3.121 \times 10^{11} \text{ eV/(mL} \times \text{hr)}$$

between 6 and 7 orders of magnitude lower than the energy captured for photosynthesis. As usual, the actual discrepancy is probably much larger, as that difference assumes maximum efficiency of capture of ionizing radiation.

To avoid dismissing an effective amount of energy, a comparison is in order. Overmann *et al.* (1992) found a strain of the green sulfur bacterium *Ch. phaeobacteroides* in the Black Sea growing in about 0.0005% the surface light. This would be a light intensity of:

$$(3.89 \times 10^{20} \text{ eV/(cm}^2 \times \text{hr})) \times (5 \times 10^{-6}) = 1.945 \times 10^{15} \text{ eV/(cm}^2 \times \text{hr)}$$

From the data provided, one should hesitate to assume a photosynthetic efficiency, considering the special low-light adaptations of *Ch. phaeobacteroides*. Even assuming a low 1% efficiency, however, the amount of energy taken up by the *Ch. phaeobacteroides* would be  $1.945 \times 10^{13}$  eV/cm<sup>2</sup>/hr; this rate of energy uptake is nearly two orders of magnitude greater than  $3.12 \times 10^{11}$  eV/(mL×hr), the rate of total energy addition to 1 mL

at a radiation dose in Dadachova *et al.* (2007).

Similarly, Dadachova *et al.*'s (2007) refers to Beatty *et al.* (2005), which reports on photosynthetic bacterium found photosynthesizing at a hydrothermal vent. Beatty *et al.* (2005) states:

*The photon flux at 750 +/- 50 nm (corresponding to the long wavelength absorption peak of light-harvesting BChl c in GSB1) at the orifice of a 370°C black smoker was  $\sim 10^8$  photons/cm<sup>2</sup>/s/sr.*

Beatty *et al.* (2005) helpfully gives this number a comparison:

*Within 1-2 cm of 332°C flange pools on black smoker chimneys, the total photon flux ( $\approx 10^{11}$  photons  $\times$  cm<sup>-2</sup>  $\times$  s<sup>-1</sup>  $\times$  sr<sup>-1</sup>) over the 600- to 1,000-nm range was estimated to be of the same order of magnitude as the solar photon availability for a green sulfur bacterium living at 80 m depth in the Black Sea”*

The mentioned bacterium was used in the comparison above. Beatty *et al.* (2005) also states that the geothermal vent bacterium

*may be thought of as eking out an existence by infrequent harvesting of rare geothermal photons.*

Finally, the Black Sea bacterium was calculated as having a doubling time of approximately 2.8 years.

This doubling time, and the statements above for *Ch. phaeobacteroides*, should put into perspective the vast energetic discrepancy in the claims of Dadachova *et al.* (2007), with observed doubling times of less than a day for cells of much greater mass.

## Chapter II's Conclusions

The above calculations establish that the energy added to each system by radiation in growth experiments of Dadachova *et al.* (2007) was low. This energy could not have supported the observed increase in the growth of irradiated samples compared to unirradiated samples. The energy addition from radiation was low even by standards of deep-lake or deep-ocean photosynthetic bacteria, whose dividing times are said to be on the order of years. Instead, the amount of energy from carbon sources provided to the fungi was sufficient to support the observed growth. To show viability of the hypothesis of radiosynthesis in fungi one would need to explain away a minimum of five to six orders of magnitude discrepancy in energy. It is therefore unlikely that 'radiosynthesis' was demonstrated in these experiments purported to support it. Some other effects are likely the cause of the increased carbon uptake and reproduction seen when select fungi are grown under low doses of ionizing radiation.

### **CHAPTER III**

#### **ADDRESSING INDIRECT EVIDENCE GIVEN IN SUPPORT OF RADIOSYNTHESIS**

In the work supporting radiosynthesis, and in particular the use of melanins therein, a number of examples of indirect evidence have been presented. This includes evidence in the introduction and conclusions of Dadachova *et al.* (2007) and in Dadachova *et al.* (2014). Additionally, the review article, Dadachova and Casadevall (2008), offers “*The indirect evidence of radiosynthesis*” (Dadachova, Personal Communication, June 27 2010). The following section will examine some of the claims outlined in these previous works.

The following is a list of some statements and indirect evidence addressed in this section, with a brief supporting quote for each:

##### **A. Photosynthesis Without Light:**

*There are recent reports that certain life forms can utilize non-conventional forms of energy - microbes in geothermal vents at the bottom of the ocean can harvest thermal radiation as an energy source –*  
(Dadachova *et al.* 2007)

*the phenomenon of “photosynthesis without light” has been recently reported to take place in the geothermally illuminated environment on the bottom of the ocean, thus establishing the precedent that certain life forms can utilize unusual forms of energy –* (Dadachova *et al.* 2014).

B. Microorganisms Living in Mines:

*some microorganisms living in mines exploit energy from radiolysis of water. – (Dadachova et al. 2007).*

C. Species from Evolution Canyon in  $^{60}\text{Co}$  Radiation:

*Interestingly, when species of Alternaria, Aspergillus, Humicola, Oidiodendron, and Staphylotrichum from both slopes were subjected to high doses (up to 4000 Gy) of  $^{60}\text{Co}$  radiation, the isolates from the south slope grew at greater rates than the isolates from the north slope. – (Dadachova and Casadevall 2008).*

D. Melanized Fungi in Radioactively Contaminated Areas, and Radiotropism:

*Both in the laboratory and in the field several other species of melanized fungi grew towards soil particles contaminated with different radionuclides, gradually engulfing and destroying those particles – (Dadachova et al. 2007 (quoted), Dadachova and Casadevall 2008, Dadachova and Casadevall 2011, Dadachova et al. 2014).*

E. Paleobiological Importance of Melanins:

*Many fungal fossils appear to be melanized. Melanized fungal spores are common in the sediment layers of the early Cretaceous period when many species of animals and plants died out which coincides with the Earth's crossing the "magnetic zero" resulting in the loss of its "shield" against cosmic radiation. Additionally, radiation from a putative passing star called Nemesis has been suggested as a cause of extinction events. – (Dadachova et al. 2007, Dadachova and Casadevall 2011)*



F. Constitutive Melanin Synthesis, Despite Lack of Need for a Radioprotector:

*Since the overwhelming majority of fungi, melanized or not, can withstand doses up to  $1.76 \times 10^4$  Gy, there is no apparent requirement for melanin as a radiation protector: – (Dadachova et al. 2007)*

G. High Elevation Dose:

*in high elevation regions inhabited by melanotic fungi the background radiation levels are approximately 500–1,000 higher than at sea level – (Dadachova et al. 2007)*

For each of the claims above, alternate interpretations will be offered to argue that each claim is incorrect or unsupportive of radiosynthesis.

**A. Photosynthesis Without Light**

The bacterium described in Beatty *et al.* (2005) was a green sulfur bacterium, a group of photosynthetic bacteria known for growing at low light intensities and at high temperatures. This bacterium was found to be photosynthesizing using infrared from geothermal vents. This bacterium had evolved to use primarily bacteriochlorophyll c. This pigment has an absorbance peak spanning from 700 to 800+ nm – far-red to infrared wavelengths, in intact chlorosomes in aqueous solutions (Beatty et al. 2005, Grimm *et al.* 2006). Hydrothermal vents and associated flange pools emit black body radiation in this range, as the water may reach temperatures of 300+ °C (Van Dover *et al.* 1996). The photon flux was deemed sufficient for growth of this bacterium; a few centimeters from the flange pool surface, the flux was comparable to 80 meters depth in the Black Sea. From such depths, a similarly efficient green sulfur bacterium was previously isolated (Beatty *et al.* 2005, Overmann *et al.* 1992).

This is not “*Photosynthesis without light*”; this bacterium was undergoing slow, efficient, but otherwise normal photosynthesis. This does not “*establish the precedent that certain life forms can utilize unusual forms of energy*” as no novel form of energy use is described, nor is any novel capture mechanism posed.

### **B. Microorganisms Living in Mines**

Bacteria were found to be living in a stable ecosystem, whose only energy input was from radiation (Lin *et al.* 2006). To summarize, waters were tested from rocks from 4 km below the earth’s surface. This water was estimated to have been secluded from all non-geothermal influence for millions of years. The organisms found were presumed to be related to *Desulfotomaculum*, based on previous observations of *Desulfotomaculum*-related bacteria both in other deep environments and in water from a nearby borehole. The main energy source was determined to be oxidation of sulfide to sulfates and formation of Hydrogen, both from products of water radiolysis. This was determined by isotopic and concentration observations of sulfate and sulfide, combined with energetics calculations. The bacteria were sulfate reducers, which reduce the sulfate with the hydrogen to extract energy. Other mechanisms for energy and carbon acquisition have been proposed, such as production of acetate from CO<sub>2</sub> and H<sub>2</sub>; H<sub>2</sub> remains the commonly-proposed, abundant, chemically stable reductant in most proposed mechanisms. The radiation dose in the waters within the rock strata in question, the Ventersdorp Supergroup metabasalt, was calculated to be  $\sim 6.4 \times 10^{-7}$  Gy/hr (Lin *et al.* 2005).

Lin *et al.* (2006) presents an actual example of bacterial radiosynthesis: a biome almost entirely energetically sustained by products of radiolysis of water. However, there

is an energetic discrepancy of many orders of magnitude between the bacteria presented in Lin *et al.* (2006) and the yeast grown in Dadachova *et al.* (2007).

The dose in the water in question was two orders of magnitude lower than the dose in the system used in Dadachova *et al.* (2007). However, Lin *et al.* (2006) states that the observed sulfate reduction rates would be the equivalent of bacterial cell turnover rates on the order of tens to hundreds of years, with a population of  $4 \times 10^7$  bacterial cells/L. The proliferation in *Cryptococcus neoformans* seen in Dadachova *et al.* (2007) is an order of magnitude overall within 30 hours or less, with a starting population of  $10^8$  yeast cells/L and an ending population of  $\sim 10^9$  yeast cells/L. Yeast cells are greater in volume and mass than these bacterial cells by around three order of magnitude. Since the yeast cells in Dadachova *et al.* (2007) were greater in concentration and larger in size than the bacterial cells in question, the total dose per cell to yeast cells is lower while the needs per cell are higher compared to bacterial cells in Lin *et al.* (2006). Furthermore, Lin *et al.* (2007) point out how extremely efficient or recalcitrant the bacterial cells must be to maintain their population on such a low energy input.

To compare these two reports, the dose in Dadachova *et al.* (2007) was two orders of magnitude greater, but the authors reported four to five orders of magnitude lower dividing times in cells. This was done with cells one or more orders of magnitude larger than the bacteria in Lin *et al.* (2006), from a starting population double that in Lin *et al.* (2006). In Dadachova *et al.* (2007), the increase in growth of irradiated samples over unirradiated ones alone was about an order of magnitude greater than the steady state population in Lin *et al.* (2006). Tallying up the minimum discrepancies for each, this is an approximate minimum total discrepancy of  $4 + 3 + 1 - 2 = 6$  orders of magnitude.

### **C. Species from Evolution Canyon in $^{60}\text{Co}$ Radiation**

In Volz. *et al.* (1997), fungal isolates were collected from the north-facing slope (NFS) and south-facing slopes (SFS) of the site dubbed Evolution Canyon. These were subject to high doses of  $^{60}\text{Co}$  radiation, and response was compared between isolates from opposite slopes. Dadachova and Casadevall (2008), states:

*Interestingly, when species of Alternaria, Aspergillus, Humicola, Oidiodendron, and Staphylotrichum from both slopes were subjected to high doses (up to 4000 Gy) of  $^{60}\text{Co}$  radiation, the isolates from the south slope grew at greater rates than the isolates from the north slope.*

This is not correct. Volz *et al.* (1997) does not state that the five species mentioned grew faster when subjected to the  $^{60}\text{Co}$  radiation. Rather, the fungi listed in Dadachova and Casadevall (2008) was the list of species that grew faster from the SFS versus the NFS *before* irradiation. Dadachova and Casadevall (2008) neglects to mention three genera that grew faster when isolated from the NFS than from the SFS, and two genera that showed no difference, within this unirradiated group.

Unfortunately, Volz *et al.* (1997) wrote their results out, while a table would likely have alleviated all misinterpretation. Table 2 contains a helpful compilation of the growth comparisons for all doses and both slopes from Volz *et al.* (1997).

### **D. Melanized Fungi in Radioactively Contaminated Areas, and Radiotropism**

Ionizing radiation selects for communities rich in melanized fungi, but this has been demonstrated only for very high doses. Gochenaur and Woodwell (1974), for example, reports such a shift only at doses around or above 5 Gy/day. 20 Gy/day was the maximum dose in the study. Below this dose, no particular selection was seen on

Table 2. Comparison of isolates by the growth at different doses of  $^{60}\text{Co}$  radiation, compared between slopes in Volz *et al.* (1997). Listing is by isolates that showed faster growth than the isolate of the same species from the opposite slope at a given dose. Note that the list of species listed by Dadachova and Casadevall (2008) as growing faster at 4,000 Gy (400,000 rads) when isolated from the SFS is instead the list when unirradiated.

Dose (Gy)	North-facing Slops	South-facing Slope	Showed No Difference
0	<i>Sordaria fimicola</i> <i>Fusarium solani</i> <i>Stachybotrys chartarum</i> <i>Fusarium sambucinum</i>	<i>Staphylotrichum coccosporum</i> <i>Humicola grisea</i> <i>Alternaria alternata</i> <i>Oidiodendron cerealis</i> <i>Aspergillus niger</i>	<i>Mucor hiemalis</i> <i>Ulocladium consortiale</i>
$4 \times 10^2$	<i>Fusarium solani</i> <i>Stachybotrys chartarum</i> <i>Alternaria alternata</i> <i>Fusarium sambucinum</i>	<i>Staphylotrichum coccosporum</i> <i>Oidiodendron cerealis</i> <i>Humicola grisea</i>	<i>Mucor hiemalis</i> <i>Ulocladium consortiale</i> <i>Aspergillus niger</i> <i>Sordaria fimicola</i>
$4 \times 10^3$	<i>Staphylotrichum coccosporum</i> <i>Humicola grisea</i>	<i>Fusarium solani</i> <i>Oidiodendron cerealis</i> <i>Stachybotrys chartarum</i> <i>Alternaria alternata</i> <i>Fusarium sambucinum</i>	<i>Aspergillus niger</i> <i>Sordaria fimicola</i> <i>Ulocladium consortiale</i>
$1 \times 10^4$	<i>Fusarium sambucinum</i> <i>Fusarium solani</i> <i>Sordaria fimicola</i> <i>Stachybotrys chartarum</i>	<i>Alternaria alternata</i> <i>Humicola grisea</i> <i>Mucor hiemalis</i> <i>Oidiodendron cerealis</i>	<i>Aspergillus niger</i> <i>Staphylotrichum coccosporum</i> <i>Ulocladium consortiale</i>
$2 \times 10^4^*$		<i>Alternaria alternata</i> <i>Aspergillus niger</i> <i>Staphylotrichum coccosporum</i>	<i>Aspergillus niger</i>

\* Only these four isolates survived.

melanized forms overall. Similarly, Zhdanova *et al.* (1994) analyzed soil fungal communities in contaminated soils after the Chernobyl explosion. They found that in only the most highly contaminated soils did melanized fungi dominate, namely in soils whose activities were above the order of  $10^4$  Bq/kg. This activity would impart a dose of  $\sim 10^4$  Gy/year, or between  $10^1$ - $10^2$  Gy/day (Tugay *et al.* 2007). This is the same order of magnitude at which the shift toward melanized fungi was seen in Gochenaur and Woodwell (1974). Zhdanova *et al.* (1994) also notes that this balance of species is in

contrast to the population before the Chernobyl disaster, in which non-melanized species dominated.

Zhdanova *et al.* (2000) also reported a shift toward melanized species in the inner regions of the containment structure of Chernobyl, but their reporting of dose is ambiguous. Their conclusions state that the calculated dose is hundreds of Gy/year, while in the text it often refers to a maximum dose of 220 mR/hour (milliRoentgen: a measure of dose). This is  $\sim 2 \times 10^{-3}$  Gy/hour or  $\sim 5 \times 10^{-2}$  Gy/day. The authors noted a shift toward melanized species, but this shift was less apparent at the lowest doses (whatever they may be). Furthermore, they found that seasonality was a stronger determinant than dose in influencing the ratio of melanized vs unmelanized species. The trend from spring to fall in high dose regions was a shift from 30% to 100% melanized species, while in the lowest dose regions the shift from spring to fall was from 17% to 63%. From these studies, it is apparent that shifts towards melanized species occur only at very high doses, therefore protection is likely to be important.

Radiotropism, the growth toward a source of ionizing radiation, has also been cited as evidence supporting some benefit to organisms growing in ionizing radiation. In their own examination of this issue, Dadachova and Casadevall (2008) point out that, because of ambiguity and incongruity between works on this topic, there are limitations to the interpretation.

Zhdanova *et al.* (2004) demonstrated radiotropism in the emergent hyphae of germinating spores. Similarly, Tugay *et al.* (2006) demonstrated stimulation of germination and increased emergent hyphal growth from spores in response to radiation exposure. A third study, Tugay *et al.* (2007) could only be found in Russian so is not

examined here.

Zhdanova *et al.* (2004) contains a discussion about melanin. Too few species were tested to see an effect due to melanization specifically, and both melanized and non-melanized species showed radiotropism. Instead, they discuss the possibility that melanins and other natural quinines may take part in regulation of developmental pathways. They posed this as an explanation for the increase in melanized species in ‘long-term low levels of ionizing radiation,’ citing Zhdanova *et al.* (1994). As discussed above, however, that Zhdanova *et al.* (1994) claimed a large preponderance of melanized species only at higher, not low, doses. The authors proposed that this was because of higher resistance to the effects of radionuclides in melanized species.

Though the description and pictures of the setup in Zhdanova *et al.* (2004) are ambiguous, the growth seems to be presented as not toward the radiation itself, but toward an impingement point of that radiation on the media. It states that samples were placed on top of the lead box surrounding the source, while some were taped to the side, each over a 1 mm diameter hole. The paper does not bring up any distinction between these positions, thus the samples taped on the side are also likely flat against the side. As such, the impingement of the beam is perpendicular to the surface of the medium, effecting a 1mm diameter cylinder, toward which the fungi grew. In the case of the  $^{109}\text{Cd}$  gamma source, which emits 88 keV photons, both the Compton electrons and photoelectrons resulting from interaction with the medium have ranges of less than 0.1 mm (Berger *et al.* 2005), so the effects would be highly localized to that cylinder.

The experiment in Tugay *et al.* (2006) was similar in setup, though they used different radioisotopes, and utilized only the flat, top hole of the lead container.

Nevertheless, an effect is seen under doses from both radioisotopes. Compton electrons and photoelectrons from the lower energy emission of the two, 27 keV from  $^{121}\text{Sn}$ , would again have ranges less than 0.1mm. The direct effects, therefore, are again likely localized to the 1mm cylinder of media, and the observed changes elsewhere likely due to diffusive elements produced. The doses reported for the medium are on the order of a few hundred Gy, which would produce biologically relevant quantities of the products of radiolysis. Furthermore, as with the previous, there was no melanin dependence on the spore germination or hyphal elongation in this study.

Given the localization of the radiation's effects on the media in both papers, the fungi are more likely to be reacting to diffusive species produced in the medium.  $\text{H}_2\text{O}_2$ , for example, is purported to lead to stimulated growth in some fungi (Ivanova *et al.* 2005, Aslanidi *et al.* 2007). If a gradient were formed by the diffusive species, as could be expected, this gradient could result in the directional growth up this gradient. Germination of spores has been shown to be dependent on the production of ROS, tying the germination experiments to this model (Aguirre *et al.* 2005, Gessler 2007). This is a possible explanation for the observed effects, and the concentrations produced at the reported dose would likely be sufficient to show it.

Karpenko *et al.* (2006) examined the phototropic and radiostimulatory response in radiotropic and non-radiotropic strains. The results support three important points. First, radiotropism or radiostimulation seems to have no melanin-dependence. This paper tested a number of melanized and non-melanized species, and found radiotropism in both groups with similar frequencies. The tendency to show an effect seems instead to be species dependent, and within that there is consistency of effect by strain. This and the



previous works also all demonstrated a distinct dependence on radioactivity of the site from whence they were isolated. Second, phototropism was highly correlated with radiotropism, pointing possibly to a common mechanism. Third, two isolates showed an effect only either in germination, or in reproductive structure formation. These observations point to a signaling effect that could potentially tie in with a hypothesis involving ROS stimulation or signaling at high doses.

To summarize, melanin's role in high radiation environments is likely protective. Lower doses show no preferential selection on melanization. Radiotropism, furthermore, is not dependent on melanization, and the effect may have an overlapping mechanism with a phototropic response. The available information can be fit, admittedly *ad hoc*, to a hypothesis involving stimulation by ROS produced in media by ionizing radiation, but only at doses capable of producing biologically relevant amounts.

### **E. Paleobiological Importance of Melanins**

The following quote is given describing the paleobiological importance for melanins:

*The role of melanin in microorganisms living in high electromagnetic radiation fluxes is even more intriguing when the pigment is considered from a paleobiological perspective. Many fungal fossils appear to be melanized. Melanized fungal spores are common in the sediment layers of the early Cretaceous period when many species of animals and plants died out which coincides with the Earth's crossing the "magnetic zero" resulting in the loss of its "shield" against cosmic radiation. Additionally, radiation from a putative passing star called Nemesis has been suggested as a cause of extinction events. The*

*proliferation of melanotic fungi may even have contributed to the mass extinctions at the end of Cretaceous period.” – (Dadachova et al. 2007).*

In support of the above statements, Dadachova *et al.* (2007) cites Taylor *et al.* (2005), Jansonius and Kalgutkar (2000), Hulot and Gallet (2003), Davis *et al.* (1985), and Casadevall (2005).

The paleobiological importance attributed to any of the work does not support radiosynthesis or a mechanism therein involving melanin. Instead, the conclusion they are said to support is:

*Melanins are ancient pigments that have probably been selected because they enhance the survival of melanized fungi in diverse environments and, perhaps incidentally, in various hosts” – (Dadachova et al. 2007)*

These statements and this conclusion are repeated throughout the body of literature, presented in each of Dadachova *et al.* (2007), Dadachova and Casadevall (2008), Dadachova *et al.* (2008), Dadachova and Casadevall (2011), and Dadachova *et al.* (2014). This thesis takes no issue with the conclusion in the second quotation above. The statement in the first quote, however, are arguably unsupported themselves. The primary issues are a lack of support provided for the claims by any of the cited literature, incorrect interpretations within the claims’ respective fields, or irrelevance of the statement in a biological context.

**“Many fungal fossils appear to be melanized.”**

Melanins are recalcitrant molecular structures; melanins’ chemical stability, aggregation, and insolubility make degradation of melanins difficult. Melanins are degraded by powerful oxidants (Bell and Wheeler 1986, Wang and Casadevall 1996,

Butler and Day 1998, Gallas *et al.* 2000, Kayatz *et al.* 2001). Because melanins are recalcitrant, they are said to contribute to the humic fractions of soils (Henson *et al.* 1999). So tough are melanins that the melanosomes of dinosaurs were purportedly found to be preserved in some dinosaur fossils, while all other organic matter had long since disappeared (Zhang *et al.* 2010) (Earlier work has concluded that similar granules were bacterial impressions, but Zhang *et al.* (2010) claims to have controlled for this in considering the granules).

Melanized spores, or at least their melanin component, would be more rigid, more chemically recalcitrant, and more likely to be preserved in the fossil record than non-melanized spores. In sedimentation and in laboratory treatment, spores show disintegration that varies by species (Wolf 1968, Wolf 1969). Often some wall layers are removed entirely, but the melanin layer tends to remain (Weber and Hess 1976). Furthermore, apparent dominance of melanized fungal spores in the literature cited could exist because large, multicellular mitospores tend to be melanized. Such distinct spores may be more easily noticed and recognized than hyaline, round, single-celled spores, leading to sampling bias. These are some of the many basic null hypotheses that would need to be addressed to validate the claim that the fossil record reflects a preponderance of melanized fungal spores.

On the other hand, there are many reasons for spores or hyphae to be melanized, and many modern species are. Only constancy is needed to explain why the use of melanin would be conserved. Furthermore, the idea that melanins are ‘ancient pigments’ depends on within-group interpretation. The use of different melanins has also been re-derived several times in several major groups, from several different monomers,

including in the process of detoxifying exogenous precursors (Butler and Day 1998).

The fact that many fungal fossils are melanized could be important if this presented a preponderance of melanized spores. Such a fact could tie this statement to those that follow it. However, neither Taylor *et al.* (2005) nor Jansonius and Kalgutkar (2000), mention such a preponderance of melanized spores. Taylor *et al.* (2005) describes evidence for a perithecial plant pathogen from plant fossils within the Rhynie Chert. The structures and spores of the fungus described are melanized. However, the single fungus scope of the paper, and the lack of comparative spore abundance for other species, represent insufficient evidence toward any claimed abundance of species with melanized spores. Jansonius and Kalgutkar (2000) redescribed a number of fossilized fungal spores previously described and illustrated in four other publications. The spores they dealt with are primarily melanized, but they are also primarily large dictyosporae. As per the potential null hypothesis, these are more likely to be seen and recognized, or to have enough detail to warrant a second glance in the literature. Jansonius and Kalgutkar (2000) chose to re-examine specimens from four prior papers, with “imperfect illustrations.” They mentioned that there existed many other imperfect presentations they could have re-examined, but did not examine them for reasons of practicality. The goal of Jansonius and Kalgutkar (2000) was not an examination of the breadth of abundance of fossil fungal spores, and should not be interpreted as representative of fossilized spore diversity or abundance.

**"Melanized fungal spores are common in the sediment layers of the early Cretaceous period when many species of animals and plants died out..." and "The proliferation of melanotic fungi may even have contributed to the mass extinctions at the end of Cretaceous period."**

The only cited paper that speaks of a preponderance of spores at a time in the fossil record is Casadevall (2005). This work cites Vajda and McLoughlin (2004) speaking of a sudden 'spike' in fungal spore mass at the Cretaceous-Tertiary boundary. This boundary is famous for the extinction of most dinosaurs ~65 MYA. Vajda and McLoughlin (2004) make no mention of the spores being melanized. They propose that the observed populations are likely saprophytes growing on the abundant dead matter at the time. Casadevall (2005) does not contradict this idea, citing Vajda *et al.* (2001) as evidence of major deforestation and abundance of substrate for saprophytes. Casadevall (2005) deals with melanization as one of several known virulence factors for growing in animal hosts. It does not, however, claim that a preponderance of the spores at the K-T boundary are melanized.

Similar fungal population spikes to those mentioned for the K-T boundary have also been described for the Permian-Triassic (P-T) boundary (the 'End-Permian Extinction' ~250MYA). Visscher and Brugman (1986) demonstrates a spike in fungal remains in aquatic sediments from this time period. The authors associate this observation with a large influx of land-derived plant matter due to devastation of land plants. The spike disappears shortly thereafter during re-establishment of land ecosystems. Similarly, at the P-T boundary, Eshet *et al.* (1995) shows the fungal spike occurring with a spike in terrestrial plant debris in sediments of shallow marine origin,

and Steiner *et al.* (2003) describes a spike in fungal remains and woody plant debris in freshwater river deposits. Both interpret the spike as saprophytes flourishing on abundant plant debris. In the fungal spikes at the K-T and P-T boundaries, the fungi come to absolutely dominate in an apparently high-nutrient landscape: from low abundance before and after, to often 90% or more of the fossil remains in the spike.

These spores are not said to be specifically melanized. Furthermore, these claims are questionable in importance to melanized fungi in “*high electromagnetic radiation fluxes*” (Dadachova *et al.* 2007); Vajda and McLoughlin (2004) attributes decreased sunlight, thus likely lower need for melanin, and high humidity as likely contributing to the transient dominance of saprophytes. Furthermore, high radiation has not generally been posed to have caused the K-T extinction. Finally, the timing stated in the first quote is amiss; the cited literature pertain only to the end-cretaceous, not to the early cretaceous. This will be discussed more later.

**“...which coincides with the Earth’s crossing the “magnetic zero” resulting in the loss of its “shield” against cosmic radiation.”**

The paper cited in support of this quote, Hulot and Gallet (2003), deals with ‘Superchrons’: long periods of time (tens of millions of years) in which the earth’s magnetic field remains in one polar orientation. Specifically the paper deals with analyzing patterns during more normal times, when the pole reverses several times per million years on average (Merrill 2010). It hones in on times just before and after the Cretaceous Normal Superchron which began at ~120MYA and ended ~83MYA.

Evidence for a link between superchrons and extinctions may exist, but no such link is directly related to the loss of the magnetic field. Courtillot and Olson (2007)

proposed that the conditions that generate the instabilities that end the superchrons may produce deep mantle plumes that have been linked to several extinction events occurring 10 – 20 MY post superchron. When a reversal does occur, models predict that the magnetic field does not disappear, instead becoming chaotic for a time (Glatzmaier and Roberts 1995, Roberts and Glatzmaier 2000). Similar variations in intensity – down to about 10% of the current field value – are seen in more normal times (Guyodo and Valet 1999, Glassmeier and Vogt 2010). Even if the magnetic field did disappear, a majority of impinging cosmic particles would be attenuated by the atmosphere (Rossi 1964, Wilson 1976, Glassmeier and Vogt 2010), and the number of particles would only double (Raisbeck *et al.* 1985).

There is also an issue in timing; Dadachova *et al.* (2007) cites extinctions in both the Early and Late Cretaceous. However the only notable extinction in the early cretaceous was primarily seen in benthic bivalves (molluscs) in what is now Europe and western Asia (Raup and Sepkoski 1982, Hallam 1986, Hallam and Wignall 1997). This event has been interpreted as being due to the regression of shallow epicontinental seas.

There have been a number of claims of possible mechanisms by which radiation from space might cause extinctions on Earth. Dartnell (2011) outlines some of these hypothesized events, including nearby supernovae, solar superflares, gamma ray bursts and/or associated cosmic ray bursts, and others. Notably, the events are calculated to be very rare, potentially affecting Earth at frequencies on the order of once in tens of millions of years or longer. As such, these events would pose only very infrequent, intermittent selective influences on life. Even if melanized organisms were better able to survive the event, such events would be unlikely to maintain selection favoring

melanization in more moderate times. Recall from previous discussion that radiation was found to select for melanized fungi only at doses which would be quickly deadly to multicellular organisms (Gochenaour and Woodwell 1974, Zhdanova *et al.* 1994, Tugay *et al.* 2007). If the events were capable of selecting for melanized fungi, they would be of such significance as to devastate macroscopic and multicellular organisms.

### **"Radiation from a putative passing star called Nemesis."**

The idea that the sun had a companion was but one of several explanations that were proposed for the supposed periodicity of extinctions, with an approximately 26 MY cycle (Ray 1985). This periodicity may or may not be a statistical artifact (Stanley 1987, Erwin 1993, Taylor 2004, Erwin 2006). The proposal is that the sun is not actually a lone star but a wide binary, with its much smaller partner orbiting with a ~26 MY orbit.

Radiation from this hypothesized solar companion was not proposed to be the cause of extinctions. Instead, the hypothesized solar companion was said to gravitationally disturb objects in the Oort Cloud in passing as it reached and returned from its perihelion in its eccentric orbit (Davis *et al.* 1985, Clube and Napier 1985, Norton 2001). This influence would throw some of these objects into new orbits, with trajectories that would bring them into the inner and outer solar system. Bombardment by these objects was the proposed mechanism for the observed extinctions. To date, however, no such solar companion has been found.

### **Summary of Paleobiological Indirect Evidence**

The paleontological claims addressed in this section are not supported by any of the cited literature. This includes the abundance of melanized fossils, the timing of extinction events, the causes of said extinctions, or claims dealing with solar companion



theory.

#### **F. Constitutive Melanin Synthesis Despite Lack of Necessity for Radioprotection**

Dadachova *et al.* (2007) proposed the idea that melanin may be used for energy capture at least in part because many species produce melanins constitutively despite a lack of apparent need for radioprotection. This idea was posed because environmental radiation levels are far below fungitoxic levels even in the highest radiation levels seen in natural environments on Earth. Constitutive melanin synthesis despite a lack of need for radioprotection does not specifically imply a possible use in energy capture because there are a number of other reasons to synthesize melanins constitutively.

#### **The Thneed That Everyone Needs: Other Uses of Melanin**

Melanins have a number of uses in an organism other than as protection from ionizing radiation. Since environments with high ionizing radiation are rare on earth, melanization is likely used to protect from other, more common, stressors such as UV and visible light (Dadachova *et al.* 2007, Durrell and Shields 1960). Peculiarly, Dadachova *et al.* (2007) invokes a novel use for melanin because of its lack of requirement for radioprotection, but also directly states that it protects against UV.

The UV protection afforded by melanins is important. A relatively long list of studies have shown sensitivity of melanin-lacking fungi to UV when compared to melanized (often wild type) strains. These include *Monilinia fructicola* conidia (Rehnstrom and Free 1996), *Verticillium dahliae* microsclerotia (Hawke and Lazarovits 1994), *Alternaria alternata* (Kawamura *et al.* 1999), *Aspergillus carbonarius* conidia (Curtis 1970), *Ustilago nuda* teliospores (Will *et al.* 1987), *Metarhizium anisopliae* conidia (Braga *et al.* 2006), *Cr. neoformans* melanized with L-dopa (Wang and

Casadevall 1994), and many more. Often near-complete killing of non-pigmented varieties occurs at doses resulting in only 50% inactivation of pigmented varieties.

Melanins also provide cell wall integrity. Melanin granules have been shown to adhere and possibly cross-link to components in the cell wall (Zhong *et al.* 2008).

Melanins' placement has been shown to be required for the proper organization of cell wall components and structural rigidity (Pihet *et al.* 2009, Carzaniga *et al.* 2002). If synthesized extracellularly in pre-placed vesicles in the cell wall, the granules can grow and join into a coherent structure, as in *Cr. neoformans* (Wang *et al.* 2006, Eisenman *et al.* 2005).

Melanins have also been implicated as providing tolerance to water stress. Three main mechanisms have been proposed for this effect. The first and most popular explanation is that a melanin layer in the cell wall acts to decrease pore size and limit permeability to compatible solutes such as glycerol. This mechanism was proposed for desiccation and osmotic stress tolerance seen in *Cenococcum geophilum* (Fernandez and Koide 2013), *Hortaea werneckii* in (Kogej *et al.* 2007, Plemenitaš *et al.* 2008).

Alternatively, and more simply, water loss may be inhibited by the reduced pore size and hydrophobic layer, or simply the properly-arranged cell wall (Fernandez and Koide 2013)

The second common explanation is that melanins' contribution to cell wall rigidity may also help maintain cell-wall integrity upon rehydration. Sudden rehydration before compatible solutes can be metabolized or sequestered could result in excess turgor pressure which would put strain on the cell wall (Fernandez and Koide 2013). Finally, melanins may increase survival simply by scavenging ROS often formed during osmotic stress or desiccation (Fernandez and Koide 2013, Kranner *et al.* 2008, Kranner and Birtic

2005).

Making use of both cell wall rigidity and the potential to act as a size selective membrane, a melanin layer is often integral to appressoria or similar structures in many plant pathogens (Howard *et al.* 1991, Galhano and Talbot 2011, de Jong *et al.* 1997, Talbot 2003). This combination of rigidity and size selection allows appressoria to build up high turgor pressure by producing high concentrations of glycerol.

Melanins may also protect against ROS and similar species from external sources, though not against intracellular sources (Jacobson *et al.* 1993). Extracellular sources of ROS include one's own or one's host's defenses, or attack by competitors or parasites (Wang *et al.* 1995, Bolwell 1997, Aver'yanov 1997, Langfelder *et al.* 1998, Schnitzler 1999, Da Silva *et al.* 2006, Plonka and Grabacka 2006, Gessler *et al.* 2007). Similarly, melanins have been shown to protect against exogenous redox agents such as hypochlorite and permanganate, though results are mixed on melanin's effect on H<sub>2</sub>O<sub>2</sub> (Jacobson and Tinnell 1993, Jacobson *et al.* 1995). Furthermore, melanins sequester redox-active metals and reduce the damage done by them. For example, Fe(II) bound to melanin was less able to undergo Fenton reactions, and Fe(III) was more difficult to reduce. Thus the bound Fe was less likely to redox cycle, and OH<sup>•</sup> from Fenton reactions at the melanins' surface was often scavenged by the melanin due solely to proximity (Rózanowska 2011).

Melanins have been implicated in protecting against some small molecules by exclusion or by surface binding. Well-studied examples in the literature often focus on antifungal agents of human interest. The binding is often specific to the molecule or molecular group, the type of melanin, or the species in question. The protection may be

broad, mixed or specific (Ikeda *et al.* 2003, Nosanchuk and Casadevall 2006, Polak and Dixon 1989, da Silva *et al.* 2006).

Finally, a melanin layer affords protection against enzymatic degradation such as occurs in soil communities, where predation and competition are regularly encountered. Melanized fungi are more resistant to enzymatic lysis when compared to nonmelanized varieties. A melanin layer may block enzymes from the cell wall, or bind to and sequester enzymes (Old and Robertson 1970, Kuo and Alexander 1967, Rosas and Casadevall 2001, Bull 1970, Doering *et al.* 1999, Rehnstrom and Free 1996).

### **Why Not Wait Until Later?**

Many fungi likely synthesize melanin constitutively because of the number of assaults melanins can help an organism tolerate. Other assaults are constant in the environment, such as UV in exposed habitats, lysis in soils, desiccation, or need for cell wall integrity; melanins are likely constantly beneficial. Many of the assaults are transient or sudden, such as sudden desiccation or rehydration, predation or infection, heating, sudden UV exposure, heat, cold, or sudden chemical or enzymatic attack.

Melanins are generally produced slowly, hyphae in culture darkening with time. This is partly because melanins are not normally something that can be just synthesized in the cytoplasm like carotenoids. The production of melanins involves many steps, and these steps are compartmentalized in most organisms because they also involve harmful products and diffusion-based reactions.

Melanins are synthesized from hydroquinones or similar molecules. Examples include dihydroxynaphthalene in ascomycetes, or DOPA melanins in animals. Oxidation of these molecules results in quinones, which have disrupted aromaticity and are

therefore relatively reactive. These react with local molecules, with each other, or with growing oligomers of each other, until around 4 to 8 monomers or more have bonded in an entirely conjugated series (Cheng *et al.* 1994a, Cheng *et al.* 1994b, Stark *et al.* 2003a, Stark *et al.* 2003b, Kaxiras *et al.* 2006, Meredith and Sarna 2006, Meng and Kaxiras 2008a). The resulting oligomers  $\pi$ -stack with each other, aggregating to form the granules and filaments that we recognize as the melanin (Clancy and Simon 2001, Watt *et al.* 2009). This view of melanins as made of many different possible oligomers is termed the chemical disorder model of melanins' structure (Meredith *et al.* 2006). This model explains many of melanins' properties, and seems to be the currently accepted structural model.

Almost all higher organisms that produce melanins do so in specialized structures – sometimes intracellular, sometimes extracellular. This accommodation is likely needed to avoid cytoplasmic harm from reactive intermediates, and/or to sequester monomers to ensure that they react only with each other (Delevoye *et al.* 2011). In many organisms these structures are produced in a series of steps. These steps sometimes involve production of preliminary vesicles, laying down of a protein scaffold to ensure that the melanin deposits uniformly rather than just at the membrane, and addition of monomers and a wide array of proteins for proper melanin production and vesicle maintenance. In many cases, mature structures must then be transported outside the cell and incorporated into the cell wall (Franzen *et al.* 2008, Walker *et al.* 2010).

Not all microorganisms undergo all of these steps, nor do the structures they produce have all the complexity of, say, animal melanosomes. *Candida albicans* lacks a scaffold, thus produces hollow shells (Walker *et al.* 2010). *Cr. neoformans* produces

vesicles containing laccase that are placed in the cell wall to await potential exogenous monomers (Eisenman *et al.* 2005, Eisenman *et al.* 2009). However, *Agaricus bisporus* and *Fonsecaea pedrosoi* were observed to make complex structures that have been likened to those of animals (Hegnauer *et al.* 1985, Franzen *et al.* 2008).

Processes that involve de novo synthesis of special compartments and their subsequent maturation and transport take time. Also, melanization is itself a slow process by necessity, possibly due to the reactivity of intermediates. To demonstrate, a small pile of L-dopa can be placed on a petri plate of fully-grown *Cr. neoformans*. The nearest *Cr. neoformans* to the pile fail to melanize, while those farther away melanize as normal. It is therefore unlikely that melanins can be produced quickly to provide protection on-the-fly. Instead, to receive the benefits melanins provide when dealing with sudden unexpected stressors, the melanin would need to have been synthesized preemptively – that is, constitutively. Additionally, constitutive synthesis may simply be due to melanins' use in proper cell wall formation.

To address the particular claim pertaining to high elevations, such locales tend to be drier, colder habitats. There is often little in the way of protection from UV, and the soil is often thin. In these ways, high elevations are similar in character to arctic habitats. Melanized species in such a habitat would presumably be more tolerant of these stressors, potentially accounting for any preponderance of melanized fungi in these habitats. Melanized forms have been said to predominate in arctic/antarctic habitats for these reasons (Ruisi *et al.* 2007).

### **G. High Elevation Dose**

Briefly, Dadachova *et al.* (2007) states:

*in high elevation regions inhabited by melanotic fungi the background radiation levels are approximately 500–1,000 higher than at sea level*

The dose at some of the highest solid elevations, the high Himalayas, are said to receive a dose from cosmic rays of  $\sim 1 \times 10^{-6}$  Gy/hr. This is only 33 times higher than at sea level, which is about  $3 \times 10^{-8}$  Gy/hr. Furthermore, at such extremes even fungi are not growing, due to constraints such as temperature. As such, the dose to fungi growing at the extremes of livable height is somewhat lower. Such values as even 100 times sea level are not seen until above the top of Mt. Everest (Ford 2004).

## **CHAPTER IV**

### **REINTERPRETATION OF RESULTS CLAIMED TO SUPPORT RADIOSYNTHESIS OR THE USE OF MELANIN THEREIN**

A number of experiments in Dadachova *et al.* (2007) are cited as providing evidence either of radiosynthesis or of the mechanism therein involving melanin. Some of the results, notably the increased growth under ionizing radiation, have been repeated in other works such as Robertson *et al.* (2012) and Shuryak *et al.* (2014). However, the results and interpretations of some experiments in Dadachova *et al.* (2007) contain issues. These issues limit or negate the experimental support of radiosynthesis or of a mechanism therein involving melanin. Some of these issues will be addressed herein.

#### **Growth Assays**

Some of the most important evidence given in support of radiosynthesis in fungi is the demonstrated increased fungal growth in ionizing radiation. For each assay, there is no doubt as to the increased cell counts in irradiated samples. Reinterpretation of the data and conclusions, or repetition in the literature, however, call into question whether melanin was involved.

#### ***Cryptococcus neoformans* Growth and Acetate Uptake Assay (Dadachova *et al.* 2007, Shuryak *et al.* 2014)**

*Cr. neoformans* has been documented several times to show increased proliferation under ionizing radiation, purportedly when melanized. This behavior was first shown in Dadachova *et al.* (2007). Therein, WT cells (laccase competent, capable of



producing melanin when exogenous substrates are supplied) were melanized, then treated or untreated with ionizing radiation. Lac(-) cells, a strain lacking in the laccase needed for production of melanin from exogenous substrates, were grown and tested similarly. Each sample was plated for CFU counts (Colony Forming Units), to demonstrate growth. In a similar experiment,  $^{14}\text{C}$ -acetate uptake was measured as an indicator of growth. In this experiment,  $^{14}\text{C}$ -acetate uptake was found to closely parallel the results of the CFU count experiments. Finally, dry weight was used as a third measure of growth.

In Dadachova *et al.* (2007), melanized irradiated samples produced more CFUs than melanized unirradiated samples, unirradiated lac(-) mutants produced more CFUs than unirradiated melanized WT cells, and there was a slight increase in CFUs in irradiated lac- samples compared to unirradiated lac- samples. The authors interpret these results as follows:

*The crucial difference between the wild type H99 and Lac(-) cells is that the exposure to ionizing radiation produced approximately 2.5 times more CFUs in irradiated melanized cells than in unirradiated melanized controls, while irradiation of Lac(-) cells resulted only in a 1.1-fold increase in CFUs.*

Irradiated WT and lac(-) cell growth were not significantly different. The interpretation above focuses on the vertical difference between irradiated and unirradiated treatments. Dadachova *et al.* (2007) dealt with the possibility of detriments due to melanization by proposing potentially reduced porosity. The authors proposed that lower porosity might limit nutrient diffusion. Alternately they proposed such inhibition could be due to cytotoxicity of oxidized melanin precursors. The latter suggestion might seem dubious, because the cells were irradiated in media devoid of L-Dopa, the exogenous

precursor to melanin used therein. However it is conceivable that stress due to this cytotoxicity might lead to a longer lag phase when inoculated into fresh media. The former hypothesis will be addressed further below, but is additionally questionable because this constraint would be present regardless of irradiation. Nutrient restriction by lowered porosity would likely affect the  $^{14}\text{C}$ -acetate assay results for all melanized samples. Furthermore, the mere '1.1 fold' change may be an artifact of the high variability as depicted by the error bars in Dadachova *et al.* (2007). The mean values in the figures therein for unirradiated and irradiated lac- samples lie closer to  $0.8 \times 10^6$  and  $1.15 \times 10^6$  respectively. This difference is 1.4 to 1.5 fold greater CFUs, a greater change than reported.

Until experiments determine the cause of the decreased growth in the absence of radiation, the converse to deriving benefit from radiation must be considered. That is, radiation may provide release from some inhibition caused by melanization. One can impose this logic on the  $^{14}\text{C}$ -acetate uptake assay, since the data between the CFUs and  $^{14}\text{C}$ -acetate uptake assays show identical trends. For some reason, melanized, unirradiated cells take up less carbon than all other treatments and controls, and this correlates closely to observed growth as measured by CFUs.

This interpretation of hindrance of the unirradiated rather than benefit to the irradiated samples may also affect the results of Shuryak *et al.* (2014). Therein, cell concentration was measured as a number of descendants from initial cells:

$$Q_e = \frac{(X_r(i) - X_c)}{X_0}$$

where  $X_c$  is the CFU counts of the unirradiated control and  $X_r(i)$  is the CFU counts of the irradiated samples. This study demonstrated an effect due to radiation in all treatments,

and an effect due to melanin in some. If, however,  $X_c$  was low because of the hindrance of melanized, unirradiated samples as observed in Dadachova *et al.* (2007), the  $Q_e$  values may be artificially high in melanized, unirradiated samples. This would unduly lead to the same attribution to benefit by melanin in irradiated samples.

The second major issue is that cells were irradiated (or unirradiated) in media lacking L-Dopa. New buds formed in the absence of L-Dopa would lack melanin (Nosanchuk and Casadevall 2003). The total number of melanized cells would remain constant in solution regardless of growth:  $10^5$  cells/ml in treatments in Dadachova *et al.* (2007), and  $10^3$  cells/mL in treatments in Shuryak *et al.* (2014). This poses a conundrum: why do samples continue to show effects due to melanization, such as decreased growth, long after constraints due to cellular melanization are lifted? In Dadachova *et al.* (2007), between 18 and 30 hours, only  $1/4^{\text{th}}$  or less of the cells are melanized in the unirradiated samples (and an even lower proportion in irradiated samples), and each new cell budded is released from the effects of melanization. Similarly, in Shuryak *et al.* (2014) the initial  $10^3$  cells are melanized, but by 48 hours  $10^5$  to  $10^6$  cells exist, diluting the melanized cells  $10^2$  to  $10^3$  times. Comparing growth of a fungus that is not intrinsically melanized, like *Cr. neoformans*, is a good idea at lower growth times. Later, however, any effect due to melanin would be more a function of spheres of melanin present in solution, rather than the melanin's position around particular cells. Since all experiments have tested growth through time, hindrance of initial budding could also contribute to lower cell counts at later times.

A study that incorporates non-melanized cells and dead melanized cells, or which begins with a lower number of melanized cells in the same system (as in Shuryak *et al.*

2014) might be illuminating. Fortunately, the former was performed in Dadachova *et al.* (2008), while the latter was performed in Shuryak *et al.* (2014). In the former, the presence of *Cr. neoformans* melanin ghosts was found to impart some radiation protection against cytotoxic radiation doses. However, this is not necessarily relevant to the effects of radiation doses many orders of magnitude lower. A similar setup to Dadachova *et al.* (2008) but under conditions of Dadachova *et al.* (2007) and Shuryak *et al.* (2014) would test for effects due merely to the presence of melanin in solution in growth experiments.

#### ***Wangiella dermatitidis* Growth Assay (Dadachova *et al.* 2007, Robertson *et al.* 2012)**

*W. dermatitidis* is one of the three fungi studied in-depth, although the results have varied between reports. In Dadachova *et al.* (2007), the irradiated and unirradiated melanin-deficient mutant and the unirradiated wild type showed nearly no difference from each other. The irradiated wild type samples and complemented melanin-deficient mutants (made capable of melanin production) showed higher growth than each of these. This showed an effect that could be attributed to the combination of melanization and low-dose irradiation. Robertson *et al.* (2012), however, showed differences between irradiated and unirradiated cells regardless of melanization; the changes were due almost solely to irradiation. Thus, increased growth under low-dose radiation has been reproduced, but the necessity for melanin has not.

It may also be worth mentioning that there is an interesting relationship in the dose-growth relationship in Robertson *et al.* (2012). In a growth by dose assay, growth stimulation at 24 hours peaks at  $5 \times 10^{-4}$  Gy/hr, shows possible beginnings of inhibition at  $5 \times 10^{-3}$  Gy/hr, and shows apparently distinct inhibition at  $5 \times 10^{-2}$  Gy/hr. Further

experiments showed distinct growth enhancement at 48 hours with little to no sign of inhibition at doses of  $2 \times 10^{-3}$  Gy/hr and  $2 \times 10^{-2}$  Gy/hr. Due to the difference in measurement times (24 vs 48 hours) and specific dose increments, these 24 and 48 hour time points are not necessarily comparable. However these results give an interesting picture of potentially initial inhibition at high doses and low times that is overcome at longer times, leading irradiated cells to catch up.

#### ***Cladosporium sphaerospermum* Growth Assay (Dadachova *et al.* 2007)**

In Dadachova *et al.* (2007), *Cl. sphaerospermum* was tested for increased growth in both melanized and non-melanized forms. The non-melanized samples were produced by inhibition of DHN melanin synthesis by tricyclazole. In two experiments, *Cl. sphaerospermum* was grown with and without sucrose as a carbon source. Within each experiment, samples were irradiated or unirradiated. Irradiation showed little to no effect on samples lacking sucrose, but resulted in increased growth in samples containing sucrose. This effect demonstrates that carbon fixation was not likely occurring to any great degree. Like the  $^{14}\text{C}$ -acetate uptake experiment with *Cr. neoformans*, this report also showed potentially increased substrate use due to irradiation. Growth was measured by colony diameter. The possibility that any differences may be due to morphologic changes such as hyphal aggregation is properly addressed in Dadachova *et al.* (2007). This experiment does not support radiosynthesis or an effect due to melanin because of issues with statistics, data presentation, and interpretation.

The statistics used are not addressed in the article. The error bars are thus likely to be the standard error, though only top bars are shown. If a bottom bar is considered, very few time points in the sucrose-lacking treatment showed significant difference from

each other in terms of colony size. Exceptions include on the second and, barely, third day for the melanized samples, and the final day on the non-melanized samples.

Questionable data are also presented for radial growth rates and volumes. Some of the data are missing or unreported, or the methods are poorly stated. The radial growth rate is reported as having been calculated by:

$$K = \frac{(R_t - R_0)}{(t - t_0)} \quad (\text{Dadachova } et al. 2007)$$

The first data points are presented at T = 4 days; radial growth rates begin at what looks like 96 hours, the same time. At this time, all radial growth rates are depicted as positive, while all 'volumes' are zero. Presumably this means that all volumes are calculated relative to day 4 while all radial growth rates are relative to the 0-hour time point. The article is silent on this point, but if this is the case, the two methods should be stated and the subtraction presented accordingly.

This interpretation of the presentation is supported by two pieces of data. First, at the T=12 day time point the radial growth rate in the melanized unirradiated sucrose-lacking treatment is depicted as matching, not exceeding, the growth rate of the melanized irradiated treatment. In the same time point, the volumes of the unirradiated and irradiated treatments have yet to become equal. Unirradiated colonies would require a period of radial growth greater than the unirradiated colonies to overtake them in volume (therefore radius), which apparently occurs at T=15 days. This also shows that data for the T=15 day time point is inexplicably omitted from the radial growth rate data.

Second, in the sucrose-containing treatment, at T=4 days, the radial growth rates of both non-melanized treatments are greater than the melanized unirradiated treatment. At T=8 on the volume graph, however, the volumes are lower. Therefore, the first point

of the radial growth rate does indeed begin at  $T=4$  minus  $t = 0$ , while the volume is normalized to  $T=4$ . Were it otherwise (were the 96 hour point on the radial graph numbers for  $T = 8$  minus  $T = 4$ ), the value would be above that of the non-melanized samples.

There are three implications to this chosen presentation of data. First, the presentation of the data is overcomplicated. Total radial growth rather than volume would likely have sufficed. Such a presentation would have made the radial growth rate depiction redundant and rendered normalization unnecessary. Second, the actual data processing is not stated, and requires effort to parse. These first two issues may seem aesthetic, but they make the data very difficult to interpret, which could obfuscate. Third, the final time point, the 15-day time point, is inexplicably left out of the radial growth rate data. As a final, minor observation, the publication states that colonies were counted and measured daily, yet only 4-day increments are shown. This inconsistency may be a simple clerical error.

With respect to the sucrose-containing treatment, Dadachova *et al.* (2007) states:

*Exposure of melanized cells to radiation was associated with increased growth of colonies in all conditions*

meaning sucrose-containing and sucrose-lacking conditions. The article also states:

*On agar with sucrose, the irradiated melanized colonies of Cl. sphaerospermum grew significantly more in regard to their volume and faster as shown by their radial growth rate than control melanized or control melanin-deficient ones.*

*Irradiated melanized colonies grew more than irradiated melanin deficient cells.*

Finally, it notes:

*The same trend was observed for cells grown without sucrose - the largest and fastest growing colonies were observed for irradiated melanized cells in comparison with the other 3 controls.*

This observation is interpreted in Dadachova *et al.* (2007) in the following manner:

*the irradiated melanized cells experienced increased growth even in the conditions of starvation.*

Tricyclazole had a distinct inhibitory effect on growth in addition to inhibiting melanin synthesis. Because of this, melanized samples grew better than the non-melanized samples. The quoted statements address only comparison of the melanized irradiated treatment to all others. Comparison *within* non-melanized treatments, however, returns the same trend seen when comparing *within* melanized treatments: irradiated colonies grew more than unirradiated colonies. The experiments therefore, while certainly showing some effect due to radiation, do not support the idea that melanization can be implicated in the effect; the stimulatory effect of radiation is seen, and is seemingly equal in magnitude between irradiated and unirradiated non-melanized samples as between irradiated and unirradiated melanized samples.

Finally, the interpretation that, in sucrose-lacking experiments, irradiated, melanized *Cl. sphaerospermum* grew faster than unirradiated, melanized *Cl. sphaerospermum* is barely borne out in the data presented in Dadachova *et al.* (2007). In the 'volume' data, significance beyond the standard error is seen only at the day 8 and possibly day 4 time points in the melanized samples, and at the day 15 time point in the de-melanized samples. Furthermore, the samples in sucrose-lacking media grew despite the purported lack of carbon sources, and the lack of agarase induction by radiation.



However, media truly lacking a carbon source would not have shown growth in sucrose-lacking treatments on the same order as the sucrose-containing samples. Yet the melanized sucrose-lacking treatments grew very similarly to the unirradiated melanized sucrose-containing treatment. This alludes either to a mechanism by which agar can be degraded that is not specifically induced by radiation, or to the presence of some carbon source that is unaccounted for. Therefore the purported increased growth seen in all samples, ambiguous though it is in sucrose-lacking samples, could be due to the induction of carbon source transport mechanisms by low-dose radiation. The transport mechanism so induced may simply be tailored to sucrose, hence the more efficient growth in the presence of sucrose. Similarly, the mechanism induced by radiation may include sucrose metabolism as seen in *W. dermatitidis* in Robertson *et al.* (2012), along with induction of transport of glucose and/or fructose. This would explain why uptake of the likely unaccounted for carbon sources in the media lead to a much lower increase in growth due to low-dose ionizing radiation.

### **Summary of Reinterpretation of Growth Assays**

Increased growth is one of the most important demonstrations in support of radiosynthesis. However, when the data reported in Dadachova *et al.* (2007) are re-examined or the experiments are reproduced by other workers, the putative beneficial effect due to the combination of irradiation and melanization becomes less certain. Irradiation alone, regardless of melanization, seems to be able to induce the observed increase in growth. The data in the *W. dermatitidis* growth study in Dadachova *et al.* (2007) do, however, seem to show a specific need for melanin to show the stimulatory effect. Why this was the case is unknown herein. Apparently later tests such as in

Robertson *et al.* (2012) saw stimulation regardless of melanization.

### ***Cr. neoformans* XTT and MTT Assays (Dadachova *et al.* 2007)**

The XTT and MTT assays are usually fluorometric tests for metabolic activity. The compounds are tetrazolium salts that change color when reduced. XTT (sodium 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium) is colorless when oxidized but turns orange when reduced. MTT (3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) is yellow when oxidized but turns purple when reduced. Both are often used for cell proliferation assays, though each can be used in other ways.

XTT is said to remain extracellular due to a net negative charge. This dye is generally reduced by electron transport at the plasma membrane, though this reaction is very slow (Berridge *et al.* 2005). To bring the speed up to usability within an assay, an intermediate redox cycling compound is used. Menadione was used in Dadachova *et al.* (2007). These intermediate compounds are reduced, and then reduce XTT. MTT is similar in concept, but simpler: MTT is able to enter the cell, and is reduced therein by various reduced substrates, notably NADH (Berridge *et al.* 2005).

In Dadachova *et al.* (2007), the XTT assay supposedly showed that the site of increased electron transfer was extracellular, where the *Cr. neoformans* melanin layer lies. It was claimed therein that this assay showed an increase in metabolic activity in melanized *Cr. neoformans* cells. However there is a discrepancy with the controls which may have led to a misinterpretation of the data. Furthermore, the assay may not be valid necessarily in the system used, in context of known issues (though these issues were partially addressed by Dadachova *et al.* (2007)). The results are nonetheless very

interesting. Properly interpreted, the results of this assay allude to an interesting interaction between melanization and live cells that may merit further study.

### **Discrepancy In Controls**

The discrepancy with the controls involves the XTT assay, and seems to have led to misinterpretation of the results. The XTT assay was done on two treatment regimes: irradiated vs. unirradiated cells, and cells grown at 22 °C vs. cells grown at 30 °C. Importantly, all radiation experiment treatments were done at 22 °C. This means that all of the controls between the irradiation and temperature experiments were the same control: unirradiated and at 22 °C. Unfortunately, the controls show a distinct lack of consistency within experiments when compared to between experiments. The ratios of the melanized (mel) treatment controls vary relative to each other and to other controls between experiments, while the non-melanized (non-mel) controls do not.

To illustrate this discrepancy, Figure 1 shows the data from Dadachova *et al.* (2007) for both XTT experiments. Several different conclusions can be drawn depending on which of the controls in the irradiation experiment are considered to be consistent with those of the temperature experiment. Each combination must be considered, because the between-experiment ratios need not stay the same necessarily. Instead, it is the within-experiment ratios between experiments that is important.

1. Assume non-mel treatments and controls are all consistent between experiments.

Accordingly, within irradiated experiment: mel dead shows an effect due to radiation, and mel live shown no effect due to radiation. Mel live control is anomalously low and mel dead control is anomalously high.

2. Assume mel live control is consistent between experiments. Accordingly, within

irradiated experiment: there is some effect of radiation on mel live and mel dead.

Mel dead control and all controls and treatments in non-mel live and non-mel dead are anomalously high.

3. Assume mel dead control is consistent between experiments. Accordingly, within irradiated experiment: there is some positive effect of radiation on mel dead but some negative effect of radiation on mel live. Mel live control and all non-mel treatments and controls are anomalously low.

Specific absorbance values are set aside, since, again, numbers can vary between treatments so long as the ratios within treatments remain the same. Regardless, of which control one considers to be consistent between experiments, at least some of the controls – which, again, are technically the same treatment between experiments – vary relative to each other, thus showing some discrepancy. Therefore, if one is to conclude, for example, that there is a change due to irradiation in live melanized samples by comparison to the melanized live control, one must acknowledge a peculiar rise in the

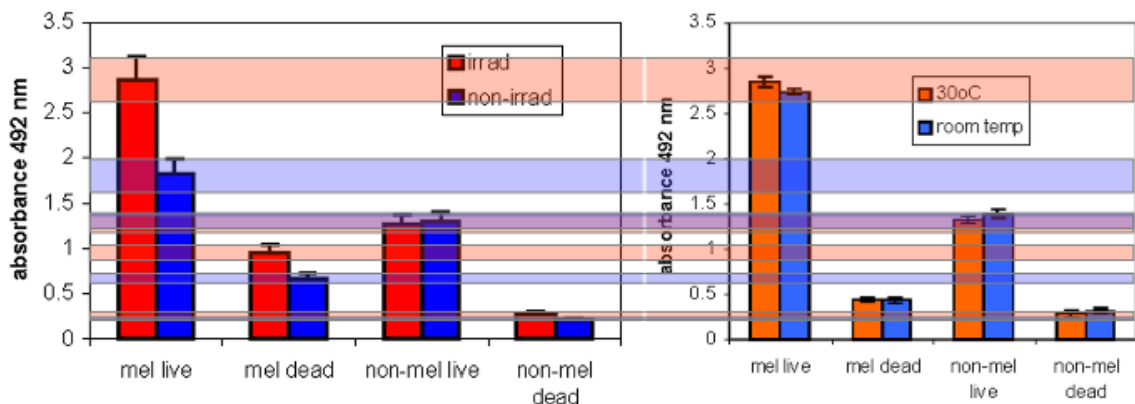


Figure 1. XTT assays from Dadachova *et al.* (2007), reproduced and modified under PlosONE's Creative Commons Attribution license (<http://journals.plos.org/plosone/s/content-license>, May 30 2015). Size is modified to match axes between graphs. Shaded boxes are set to match the high and approximate low error bars surrounding the mean values of the irradiation experiment. This allows for visual comparison between the results of the irradiation experiment and those of the temperature experiment.

non-melanized samples and the melanized dead control.

### **Reinterpretation of Data**

With the above in mind, the presented data can be reanalyzed. To begin, the abstract of Dadachova *et al.* (2007) states:

*XTT/MTT assays showed increased metabolic activity of melanized Cr. neoformans cells relative to non-melanized cells.*

In the description of the XTT/MTT assay, the paper states:

*The XTT assay showed significant increase in electron-transfer events in the irradiated melanized cells in comparison with unirradiated melanized or irradiated non-melanized cells*  
*melanin can reduce XTT reagent by itself*

and

*Irradiation of dead cells caused significant increase in the XTT reduction, thus confirming our hypothesis that radiation enhances electron-transfer properties of melanin.*

In their conclusions, Dadachova *et al.* (2007) states

*comparative analysis of MTT/XTT reduction assays revealed that radiation-induced effects on the electron transfer properties of melanin were localized to the extracellular space thus establishing a spatial relationship between the site for electron-transfer events and the location of the melanin pigment.*

Some of these interpretations were addressed above in the analysis of the discrepancy between the identical controls. The remaining interpretations will be examined in the following reanalysis of the data, with help from the literature.

For simplicity, and because of the above, ignore the melanized controls from the irradiation experiment. Without those controls, there is little difference between the results due to the slight difference in temperature, or due to irradiation. Next, consider each of the factors examined – live and melanized – as if one were performing an analysis of variance. Each of these factors contributes its own effect to the total reduction of XTT. If the total reduction when factors are combined is greater than the sum of their relative reductions apart, then there is some effect due to a combination of factors that is not explained by either of the factors separately.

Tables 3 and 4 contain the data approximated by lines drawn across the graphs of Dadachova *et al.* (2007) for the MTT and XTT assays respectively. Lines A, B, and C therein represent the respective contributions due to the presence of cell material (dead non-melanized), the effect of being alive (live - dead), and the effect due to the presence of melanin (dead melanized – dead non-melanized). In the MTT assay, there may be a slight effect due to being both live and melanized, which is not accounted for by live cells or the presence of melanin alone. In the XTT assay, however, this difference is much more distinct.

### **Alternate Explanation**

The XTT assay data do support an interesting interpretation: there is some effect due to the combined factors of being live and melanized that is not merely the sum of live cells and the presence of melanin. However, the interpretation that this effect is due to greater total metabolic activity as stated in the abstract of Dadachova *et al.* (2007) should be re-examined. It is interesting that melanin itself seems to be able to result in reduction of MTT (alone) and XTT (via menadione).



One important fact to note is the sensitivity of the MTT and XTT assays to superoxide ( $O_2^{\cdot-}$ ).  $O_2^{\cdot-}$  affects the MTT assay by reducing MTT directly.  $O_2^{\cdot-}$  may have some effect on the XTT assay, though purportedly through the intermediate electron transfer agent (menadione).  $O_2^{\cdot-}$  shows very little direct reduction of XTT, but may reduce the intermediate which subsequently reduces XTT (Berridge *et al.* 2005). Menadione is known to reduce  $O_2$  to  $O_2^{\cdot-}$ , but whether the reverse reaction would be of significance seems to be unknown. This basic  $O_2^{\cdot-}$  mechanism is unlikely to explain the results of the XTT assay because the mechanism would be controlled for by live, non-melanized cells.

However, melanin was shown to contribute to reduction of XTT (possibly but not necessarily via menadione) and MTT on its own, and has been shown to catalyze redox reactions (Gan *et al.* 1976, Dadachova *et al.* 2007). If some mechanism existed for reduction of melanin by live cells, this could explain why live, melanized cells show such high rates of XTT reduction. Melanin of dead cells would have no source of reducing power, while live, non-melanized cells would lack melanin to act as a redox catalyst. Therefore, only in live, melanized cells would this increased reductive capacity be apparent. Such a mechanism has been shown in *Cr. neoformans* by Jacobson and Hong (1997). The mechanism therein involved the reduction of melanin by Fe(II) generated by the cell. The phosphate-buffered saline medium used in Dadachova *et al.* (2007) in the XTT and MTT assays would not contain sufficient iron to maintain reduction of melanin. If, however, the cells used other, endogenously-produced compounds or stored Fe(II) for reduction of melanin, maintenance of reduced melanin could explain the results. Indeed, *Cr. neoformans* was found to, in iron-starved media, secrete extracellular reductants to



reduce Fe(III) to Fe(II) for uptake (Nyhus *et al.* 1997). The main constraint would be that the reductant only slowly reduce XTT, MTT, or menadione. Melanin would therefore increase the reduction rate by catalyzing the redox reaction between the reductant and XTT, MTT, or menadione. Alternatively, some compound might be produced and secreted by *Cr. neoformans* only when melanized for the reduction of its melanin; this, too, would explain the XTT results.

Returning to  $O_2^{\cdot-}$ , melanins have been shown to both reduce and oxidize  $O_2^{\cdot-}$ , with a possible preference for oxidation of  $O_2^{\cdot-}$  to  $O_2$  (Korytowski *et al.* 1985, Korytowski *et al.* 1986). In a solution containing both  $O_2^{\cdot-}$  and an oxidizer, this preference would be exacerbated because the melanin would be maintained in a more oxidized state. To summarize, melanins have been shown to oxidize  $O_2^{\cdot-}$  and to reduce XTT and MTT. Menadione can be reduced by  $O_2^{\cdot-}$  (Winterbourn *et al.* 1978), but also produces  $O_2^{\cdot-}$  by reducing  $O_2$ . Therefore, the mechanism by which live, melanized cells show increased reduction of XTT and to a lesser extent MTT may involve simply melanin acting as an electron transfer agent between  $O_2^{\cdot-}$  and XTT, MTT, and/or menadione. This idea is very similar to the previous, but would require only a common ROS capable of reducing melanin. Live cells would be required to produce  $O_2^{\cdot-}$ , while melanin would be required to increase the rate of transfer of electrons between  $O_2^{\cdot-}$  and XTT, MTT, or menadione. Therefore this might be a mechanism by which live, melanized cells would show greater reduction of XTT and to a lesser extent MTT than can be accounted for by melanin alone or live cells alone. Unfortunately,  $O_2^{\cdot-}$  diffuses only poorly across membranes (Rosen and Freeman 1984), and melanins are only particularly useful for protecting against extracellular ROS (Jacobson and Tinnell 1993, Jacobson *et al.* 1995). If, however,

mechanisms exist for creating or allowing for the export of  $O_2^{\cdot-}$ , the melanin could be reduced. Though this is similar to the first idea, it is presented separately because it would be easy to test. If  $O_2^{\cdot-}$  is involved in the mechanism by which melanized, live cells show greater reduction of XTT and to a lesser extent MTT, addition of superoxide dismutase (SOD) during the test could mitigate the effect as in Berridge *et al.* (2005).

To give one final alternate hypothesis,  $O_2$  could compete for electrons with (or from) menadione or MTT (Berridge *et al.* 2005). A melanized cell wall could perhaps physically limit  $O_2$  diffusion, while cells use up internal and local  $O_2$ . If XTT/menadione and MTT are in vast excess compared to the available reducing power, diffusion for these compounds would be a non-issue. The removal of  $O_2$  as a competing electron scavenger near the cell surface, while the remaining  $O_2$  is used up by the live cell, would increase the reduction of XTT/menadione or MTT by the cell.

### **Validity**

This experiment provides evidence of a mechanism in which being melanized increases the reduction of the XTT or MTT dyes by live cells beyond what is explainable by the presence of melanin alone or of live cells alone. However, too many confounding factors exist to conclude that this result is due to increased metabolic activity, or to enhancement of electron transfer properties of the melanin due to low-level irradiation. This latter conclusion is unsupported by the data when controls are accounted for.

The 'control' wherein cells were heat-killed at 65 °C for an hour may not be a proper control for testing the effects of melanin, as heat treating is a potential compounding variable. The comparison is not just live vs. dead, but live vs. dead, heat-treated cells. The heat-treating is not trivial: the NADH/ferricyanide coupling experiment

in Dadachova *et al.* (2007) (see below) showed a 4-fold increase in the electron transfer catalysis rate due to heating at 75 °C relative to unheated controls. This result was observed despite that the cells had been long-since cooled before being placed in the experimental solution.

There was an increase in XTT reduction in the 30 °C treatment, although the interpretation of this can be re-written. Dadachova *et al.* (2007) concludes

*Overall, these experiments showed the increase in electron transfer properties of melanin in melanized cells post exposure to ionizing radiation and to less extent - to heat.*

It was only the melanized live cells at 30°C that showed a (very small) significant increase in XTT reduction, not the melanized dead cells. Therefore, the effect was likely attributable not to the electron transfer properties of melanin, but to some combination of being melanized and live at 30 °C in this system vs. to being melanized and live at 22 °C. In any case, the difference between each other is ever so slight, if real, but neither was significantly different from the irradiated, melanized, live cell treatment.

#### **ESR Signal Post-irradiation (Dadachova *et al.* 2007)**

In support of melanins being involved in energy capture, Dadachova *et al.* (2007) states

*The electronic complexity of melanins allows them to scatter/trap photons and electrons, which was evidenced in this study by the following observations: 1. changes in the electronic structure of melanin post radiation exposure as measured by amplitude changes in the ESR signal.*

The paper also notes

*One important indication of melanin interaction with ionizing radiation was a large change in ESR signal of Cr. neoformans dry melanin “ghosts” after they were subjected to 0.3 kGy irradiation and subsequently suspended in water.*

The data and claims provided, however, show unexplained results. In melanins, the ESR signal is due primarily to the concentration of semiquinone radicals present in the melanin. Semiquinone radicals are the intermediate redox state between the quinone (oxidized) and hydroquinone (reduced) moieties within the DOPA melanin. Some equilibrium generally exists between the reduced and oxidized moieties and their respective half-reduced radical. Schweitzer *et al.* (2009) demonstrated, in various DOPA-based melanins, ‘spins per molecule’ ranging from ~1 in 4000 to 1 in 500.

Changes in the number of radicals are read as changes in the amplitude of an ESR signal. A number of factors have been shown to shift this equilibrium, resulting in differing concentrations of semiquinone radicals. These factors include temperature (Chio *et al.* 1980, Chio *et al.* 1982, Blois *et al.* 1964), light absorption (Sarna and Sealy 1984, Collins *et al.* 1995), monomer protonation i.e. pH (Chio *et al.* 1982), the presence of other constituents in solution that interact with spin states (Blois *et al.* 1964), redox state (Chauffe *et al.* 1975), and hydration (Blois *et al.* 1964, Chauffe *et al.* 1975, Mostert *et al.* 2012). Instead of ‘trapping’ of electrons, for example, the change could perhaps be due to the residual excitations from Compton electrons. If produced akin to those from high intensity light, this population would persist for at least several hours (Collins *et al.* 1995). Worth noting is the fact that the dose that showed the purported change was 300 Gy, 6-7 orders of magnitude above the dose in growth experiments (described above) in the same work. The effects on the melanin by radiation are therefore not necessarily

comparable between these experiments.

The ESR spectrum of *Cr. neoformans* melanin post-irradiation shows a quality not usually characteristic of ESR spectra: negative absorbance. ESR data are routinely presented as the first derivative of the electron absorbance curve, as was done in Dadachova *et al.* (2007). However, in the post-irradiation *Cr. neoformans* melanin, the initial drop followed by a tall spike represent an absorbance that is at least in part distinctly negative. A negative absorbance – perhaps an emission – would be a peculiar and potentially novel quality if real. Negative absorbance does not seem to have been demonstrated elsewhere. Other ESR signals in the same work and in many others show the same characteristic peaks that change only in height, rarely if ever shape, and do not show negative absorbance. Dadachova *et al.* (2007) interpret the amplitude of the signal as important. Indeed it is, but the ESR data should have been stacked in their depiction, because ESR data have arbitrary units and are only useful by comparison to each other. Instead, the shape is what is truly odd. Other issues can account for these data, and this list is not comprehensive:

1. The ESR data for irradiated *Cr. neoformans* melanin are presented backwards in the paper
2. Some novel property is causing negative electron absorbance in ESR, possibly Induced emission from a trapped spin state
3. The data are merely poor – a failed measurement, Schmutz

#### **NADH Oxidation by Ferricyanide (Dadachova *et al.* 2007)**

Gan *et al.* (1976) demonstrated that eumelanin can act as a catalyst in coupling redox reactions, using several couplings of oxidizers and reducers. Several important

factors were demonstrated therein.

First, melanin could be oxidized or reduced. For example, in a solution containing ferricyanide and melanin, the authors report that ferricyanide was reduced to a point, at which the reaction halted. Re-oxidation by adding NADH facilitated further reaction, but only until the NADH was used up. Only NADH and melanin together was shown to have different kinetics, continuing at a constant rate, but that observation was attributed to the presence of molecular oxygen in the setup.

Second, in the system containing NADH, ferricyanide, and melanin, the rate of reduction of ferricyanide and oxidation of NADH was greatly accelerated compared to only ferricyanide and NADH together. The extent of the reaction, however, was determined by the ferricyanide and NADH concentrations. The melanin was therefore acting catalytically. The ability to behave as a catalyst has been attributed to the spontaneous oxidation of melanin by ferricyanide, and oxidation of NADH by oxidized melanin.

Third, the reactions were shown to be somewhat specific, with only select oxidizer-reducer couplings being efficiently catalyzed.

Fourth, the rates of reaction were subject to inhibition by end products of the reaction (Ferrocyanide or NAD<sup>+</sup>). Inhibition implies a need for contact with an ‘active site’ of sorts.

Dadachova *et al.* (2007) showed that the ability of melanin to reduce ferricyanide and oxidize NADH increases when the melanin is irradiated. The rate of reaction is presented as the initial velocity of increase as measured at 2.5 minutes. The study was thorough, testing for effects from other treatments, namely heat and light. All trials

showed an approximately 4x increase in the velocity of reaction of the ferricyanide/NADH couple. Interestingly, there was also an increase in the rate of ferricyanide reduced in the absence of NADH, though comparable data are not presented for this experiment under treatments of light and heat. It would be interesting to know if the total amount of ferricyanide reduced was variable. In Gan *et al.* (1976), the reduction of ferricyanide proceeded quickly at first and then slowly for 20 minutes before coming slowly to a halt.

It was thorough that Dadachova *et al.* (2007) tested a number of energy input treatments. However, the similarity between the results points to a common mechanism. Each test for initial rate involved treating the melanin (e.g. with light, heat, or radiation), placing the melanin on dry ice, and placing the melanin in the experimental solution. The lag-time between treatment and measurement implies that the reason for the increased rate of catalysis involves some change in the melanin itself. The similarity of the results for different treatments implies that the change is something melanin can undergo with as little as heating at 75°C.

### **Tentative Hypothesis**

Though it is beyond the scope of this thesis to study this mechanism, a hypothesis – or at least an educated guess – is offered herein. The extrinsic radical population (the population produced by external treatment) may be responsible for the increased rate of catalysis. If this hypothesis is correct, any treatment that increases the radical population could increase the reaction rate. In Seagle *et al.* (2005), the reactive site that scavenged  $O_2^{\cdot -}$  was one of two melanin semiquinone radicals. Seagle *et al.* (2005) stated that this particular radical was destroyed in the process, but that its population was replenished by

conversion of the second radical type. Collins *et al.* (1995) demonstrated the generation of a radical population that survived long after treatment ended. The final radical population remained, after treatment, at a around 1.4x - 1.5x the untreated level (though the area under the curve is more indicative of the actual population). If this stable population is more prone to reacting with oxidants or reductants, it could explain the observed increase in the initial rate of redox catalysis. The consistent 4x maximum increase in the initial rate of reaction catalysis regardless of the treatment of the melanin could be due to a saturation- or equilibrium-level radical population, as likely seen in Collins *et al.* (1995).

A study demonstrating time-dependence of reaction rate on treatment, with calculation of the total energy added, would help show whether the 4x maximum increase is a coincidence, or a limit for the melanin studied. Dadachova *et al.* (2007) tested dependence on treatment time for only ionizing radiation, but the dose at 14Gy/min is near the order of the light flux from the 250 Watt bulb at 1 m distance. Such a dose rate of visible light might show similar time or dose dependence as that observed in ionizing radiation, although the exact times are likely to vary due to differences in mechanisms of excitation. Change over time could be tested with variable flux. Rates could also be measured while under treatment directly. This might be telling, as a larger radical population has been shown to exist during illumination, much of which decays quickly when treatment ends (Collins *et al.* 1995). Constant formation of radicals beyond the stable saturation point, and use of transient radicals as they are formed, may show a further increased rate of reaction catalysis as seen in Seagle *et al.* (2005).

The quinone moieties within melanins can exist in several states:



- Indolequinone methide (IQM) where the hydrogen is on the indole group
- Quinone-imine (QI) where the hydrogen is on the oxygen nearer this group
- Quinone-methide (QM) where the hydrogen is on the oxygen farther from the amino group.

Conversion between these groups has been studied as one of the mechanisms of energy dissipation in melanin monomers and by extension in melanins (Pezzella *et al.* 2006).

The ground state of the IQM is lower than the ground state of QI, thus ground-state monomers tend to prefer the IQM ground state. However, the first excited state of IQM is higher than the first excited state of QI. Some amount of energy can be dissipated from excited IQM by conversion to excited QI (Olsen *et al.* 2007). This mechanism of energy dissipation is termed proton transfer.

Some of the long-lived stable radical population produced by illumination may be quinone-imine-based radical. One rationale for this hypothesis is that the gap between the quinone-methide and quinone-imine states is a mere 0.14 eV, but the barrier energy of conversion between these states is ~0.3 eV (Olsen *et al.* 2007, Meng and Kaxiras 2008b) and involves several bond rearrangements. Quinone-imines may be formed by conversion of a quinone-methide, or due to a shift in equilibrium. Formation of a quinone-imine may be followed by dissipation of excess energy by other means, leaving the quinone-imine in the ground state. This could 'trap' semiquinones in the quinone-imine state. The respective semiquinones of each of the above quinone monomers may demonstrate tendencies similar to the quinone counterparts.

## **Validity**

One important factor not demonstrated by this experiment would support the ability of melanin to be an energy transduction agent, e.g. in analogy to photosynthesis. Melanins have not been shown to run a reaction 'uphill'. The most basic demonstration, because of its prevalence in the literature, would be the ability to reduce NADH. Reduction of NADH to any notable extent is unlikely. Though melanins' reduction potentials are difficult to study, values have been estimated. Recent estimates for the reduction potential of eumelanin have hovered around -0.15 to -0.2 V. While energy input could allow this to run uphill, melanins dissipate a majority of the energy they absorb non-radiatively as heat (Meredith and Sarna 2006). For example, estimates for the efficiency of conversion of visible light to heat range around 99.8-99.9 %. Therefore not only would eumelanin require very specific conditions to reduce NADH, but the quantum yield of energy would likely be low. On the other hand, since the efficiency of photosynthesis is commonly at most a few percent, the usually low quantum yield does not invalidate the idea of energy transduction by melanins.

## **Summary of Reinterpretation of Previous Results**

The experiments and results presented in Dadachova *et al.* (2007) were invoked in Dadachova and Casadevall (2008), Dadachova and Casadevall (2011), Dadachova *et al.* (2014), and others, as supporting evidence for radiosynthesis, or supporting the use of melanin therein. Some of these results are examined in this section. For each result addressed, the data can be re-analyzed to yield different hypotheses, or fail to support the claims made based on them. Re-interpretation or reproduction of growth experiments elsewhere have shown that melanin is unnecessary to show increased growth in ionizing

radiation. The results from XTT and MTT assays were said to show that radiation had an effect due to low-dose radiation; these data are very unlikely to support that claim on scrutiny. The ESR data for *Cr. neoformans* melanins are difficult to explain and likely represent either a bad run, a clerical error, or a physically unlikely scenario. The NADH/Ferricyanide experiment reproduces results in the literature that show that melanins can catalyze redox reactions. The result does not demonstrate the ability to run any reactions backwards, such as reduction of NADH. The catalysis rate may already have explicable demonstrations in the literature due to other mechanisms.

That said, the experiments and their results are sometimes inspiring, bringing up large questions in radiation biology or pointing to important properties of melanins. Misinterpretation of the results, however, may limit their utility. For example, a link between the electron transfer catalysis rate and the radical population could be probed. A combination of the experimental setup of Seagle *et al.* (2005) and the NADH/ferricyanide coupling rate increase with different energy excitations of Dadachova *et al.* (2007) might shed light on the results of Collins *et al.* (1995). The results of the XTT/MTT assay in Dadachova *et al.* (2007) may be due to, and may demonstrate a mechanism for, maintenance of melanin's reduced state akin to that seen in Jacobson and Hong (1997), though possibly by other means. Growth experiments as in Dadachova *et al.* (2007) and Shuryak *et al.* (2014), incorporating gene expression studies as in Robertson *et al.* (2012), could point to the mechanism and cause of the stimulation of cells in low-dose radiation – especially with alternate hypotheses in mind (next section). It is the hopes of the author of this thesis that the treatment herein inspires future researchers. In this section, re-interpreting results or putting results in a proper context brings up many potentially

important lines of research.

## CHAPTER V

### ALTERNATE HYPOTHESIS: MISINTERPRETATION

Much of this thesis has focused on specifically addressing lines of evidence used to support the idea of radiosynthesis or the use of melanin therein, and showing why these pieces of evidence fail to do so. However, removing one idea leaves only a gap with no answers. Hereafter this thesis shall focus on presenting constructive ideas to galvanize further research and possibly to fill in the gap. To begin, this thesis will present an alternate hypothesis to explain previous observations.

The hypothesis posed herein to explain the observations made in previous research is that the fungi (and conceivably other organisms) are misinterpreting the effects of ionizing radiation. The primary cellular effects of ionizing radiation are the production of reactive species and DNA damage. Doses of radiation at which growth and proliferation were seen are low, but are nonetheless rare in nature. However, other sources of ROS and causes of DNA damage are notably common, and include:

- Normal aerobic respiration (Feinendegen *et al.* 2004, Murphy 2009, Tang *et al.* 2014)
- Backups in the electron transport chain (Murphy 2009, Hamanaka and Chandel 2010, Liochev 2011)
- Internal signaling for numerous processes where superoxide ( $O_2^{\cdot-}$ ) is generated commonly by NADPH oxidase (NOX) enzymes (Nivière and Fontecave 1995, Aguirre *et al.* 2005, Stone and Yang 2006, Gessler *et al.* 2007, Finkel 2011). This

includes, somewhat ironically, the DNA damage response (Rowe *et al.* 2008)

- Light, often by photosensitization, where an absorbing molecule transfers energy to oxygen generating singlet oxygen (DeRosa and Crutchley 2002, Krieger-Linzkay 2005)
- UV, which causes direct DNA damage (Nilsson 1996)
- $\beta$ -oxidation of long chain fatty acids (Nivière and Fontecave 1995)
- CYP450 reductases and other metabolic enzymes (Nivière and Fontecave 1995)
- Heat, by disrupting protein complexes notably in the mitochondria (Davidson *et al.* 1996)
- Melanin production (Borovansky and Riley 2011)
- Redox-active metals such as chromium or iron, and redox-cycling compounds such as menadione or paraquat (Greenberg and Demple 1989, Brennan and Schiestl 1996, Angelova *et al.* 2005, Criddle 2006)
- Host signaling or attack (Bolwell and Wojtaszek 1997, Gessler 2007, Nanda *et al.* 2010)
- Secretions by competitors in the environment, e.g. aflatoxin (Lin *et al.* 2014)

and many others.

Instead of some specific response to ionizing radiation, the effects of radiation are more likely misinterpreted by the cells as indicators of some condition commonly experienced within the cells' normal ecological niche. The fungi would mount a response to a condition or conditions that commonly lead to the production of ROS or to DNA damage, despite that such conditions are not the present in this case.

The most basic response to ROS or DNA damage involves protection of the cell: a

DNA repair response and an oxidative stress / antioxidant response were seen in Robertson *et al.* (2012). These responses are likely to be present under any dose capable of eliciting a response. These responses are also likely linked to other responses, such as those resulting in growth, proliferation, and/or metabolic shifts. A metabolic shift should explain previous observations such as increased uptake of nutrients, increased budding, some of the metabolic shifts in Robertson *et al.* (2012), and others. A few candidate responses are discussed herein that may be tied to a DNA damage or oxidative stress response:

1. Response as though conditions are more aerobic
2. Response as though conditions are hypoxic
3. Response as though nutrients are bountiful, or conditions more reducing
4. Response as though in the presence of light
5. Secondary response to other cellular conditions

This list may seem to cover some contingencies from end to end. Perhaps it does, but ROS and DNA damage signals are complex. A given species might react to ROS as if one or more of these conditions are the case, based on the species' normal ecology.

To the author of this thesis, an erroneous response to environmental conditions seems an obvious mechanism for explaining many of the observations made in the literature. This mechanism invokes nothing entirely novel, and assumes no energy capture. Nevertheless, the idea of radiosynthesis in the literature may have led others from this conclusion. For example, Robertson *et al.* (2012) pose the following explanation for the up-regulation of photolyases and carotenoid biosynthesis in their expression analysis:

*However, our experiments were conducted in the absence of light and UV.*

*Therefore, up-regulation of those genes in response to low dose ionizing radiation might suggest that those gene products are not only able to safeguard DNA but also able to potentially utilize ionizing radiation as energy for DNA repair.*

The suggestion posed in the quote requires the assumption that the up-regulation of light-active proteins is indeed proper for ionizing radiation – an unlikely case. The suggestion also invokes a hitherto unknown mechanisms of activity for enzymes of well-known function. The hypothesis of mistaken signaling deals with the up-regulation of photolyases and of carotenoid biosynthesis thusly: light and UV can produce ROS, can cause DNA damage, and are common. Therefore, some portion of a response to light and/or UV – at least some of the protective mechanisms – may be tethered to the otherwise generic DNA damage response or antioxidant response. By this mechanism, low doses of radiation might also induce some extent of light response.

To give a similar example, Chen *et al.* (2014) cited Robertson *et al.* (2012) in stating the following:

*A previous study demonstrated that ionizing radiation also significantly upregulated gene expression of cryptochrome CryA, a well-known photoreceptor.*

*This suggests that this photosensory protein may also sense radiation.*

However, this comment skips a step in the signaling process where many other proteins may exert effect, implicating CryA itself unduly. Up-regulation of CryA does not imply that CryA was what sensed the radiation or its effects, unless CryA was the only agent resulting in its own up-regulation. Mere transcription of CryA, not activation, was seen in Robertson *et al.* (2012); the cryptochrome would likely be impotent without light. The



mistaken signaling hypothesis can again be easily applied: CryA expression is more likely tied in with a response tailored to other, more common stimuli – probably light or UV. CryA may, for example, be up-regulated because the fungus sensed ROS or DNA damage, and expects to soon or currently be challenged by light and/or UV.

Tethering of multiple responses could conceivably be a common phenomenon; instead of sensing every nuance of the environment independently, more general or redundant responses can be mounted to very common stimuli. This linkage would lead some specific stimuli to up-regulate, in a lab setting, genes that might not seem useful in that specific setting – hence the misinterpretations above. To give an example, in *N. crassa*, 300+ genes are regulated by light, ~6% of the genome. Of those genes, about 22 % are for carbon source metabolism. An additional 17 % are for biosynthesis of various molecules such as pigments, vitamins, cofactors, and secondary metabolites (Chen *et al.* 2009, Belozerskaya *et al.* 2011). A particular environmental signal can have many consequences for an organism, and fungi may tether responses accordingly.

It is the hope of the author of this thesis that the analysis herein will inspire future researchers. However, this author also urges that when information is given in this thesis, it should be double checked and confirmed in the literature.

### **Respective Responses to Radiation: What is Without Dispute (Herein)**

Previous sections of this thesis questioned many lines of evidence presented in the recent previous literature. However a number of important observations in the literature are undisputed herein. The following list contains some of those observations that have been repeated elsewhere and/or are not questioned herein. These observations may require some explanation or further study, or provide information useful for analyzing to

what incorrect stimulus cells may be responding. Some of these will be examined further.

1.  $C^{14}$ acetate uptake in *Cryptococcus neoformans* under ionizing radiation in Dadachova *et al.* (2007).
2. Rate of NADH/Ferricyanide redox catalysis by *Cr. neoformans* melanin. In particular, the rate increase upon irradiation with very high-dose ionizing radiation, but also under heat, light, ect., in Dadachova *et al.* (2007).
3. Increased growth or proliferation of a number of both fungal and non-fungal organisms under low-dose ionizing radiation (or peroxide), or detriment by shielding therefrom:
  1. *Cladosporium sphaerospermum* (Dadachova *et al.* 2007)
  2. *Cr. neoformans* (Dadachova *et al.* 2007, Shuryak *et al.* 2014)
  3. *Wangiella dermatitidis* (Dadachova *et al.* 2007, Robertson *et al.* 2012)
  4. *Synechococcus lividus* (Conter *et al.* 1983, Conter *et al.* 1984, Dupouy *et al.* 1985, Conter *et al.* 1986, Conter *et al.* 1987a, Conter *et al.* 1987b)
  5. *Paramecium tetraurelia* (Planel *et al.* 1976, Croute *et al.* 1982, Kawanishi *et al.* 2012)
4. Mitigation of ‘benefit’ of radiation by shaking (compared to static conditions) or ‘rich media’ (as compared to in ‘minimal media’) seen in Robertson *et al.* (2012). Shaking or rich media brought the proliferation of controls up to par with irradiated samples.
5. Gene expression profiles and their implications in irradiated vs. unirradiated, and melanized vs. non-melanized, cells in Robertson *et al.* (2012). Up-regulated by

ionizing radiation:

- Translesion synthesis (DNA replication despite damage)
- DNA repair including excision repair and photolyases
- Fatty acid synthesis (particularly unsaturated and structural fatty acids)
- Aquaporins and similar (water and other transport)
- Antioxidant genes (including mitochondrial SOD)
- Carotenogenesis
- Sugar transport (especially in the melanized WT)
- Protein maintenance
- Complex sugar breakdown (e.g. starch, sucrose)

Down-regulated by ionizing radiation:

- Cell cycle and cytoskeleton, including DNA damage checkpoint proteins and DNA replication
- Fatty acid breakdown
- Ribosomal biogenesis
- Amino acid degradation
- Nucleotide synthesis
- Carbohydrate metabolism
- Energy metabolism (e.g. oxidative phosphorylation)

Note: the author of this thesis cannot speculate why the albino *W. dermatitidis* would up-regulate ribosomal biosynthesis in response to ionizing radiation but the WT would not.

6. Increased survival in long-term starvation by *W. dermatitidis* in Robertson *et al.* (2012).

### **Proposing Ideas of Possible Mistaken Responses**

According the proposed hypothesis, the cellular effects of ionizing radiation are likely misinterpreted as though arising from other conditions. The following are some ideas for conditions that the effects of ionizing radiation might mimic. What these hypotheses propose is essentially a number of responses that might reasonably be tethered to an oxidative stress or DNA damage response.

This treatment is by no means exhaustive. The list also contains much speculation and educated guessing in the production of a number of sub hypotheses (some of which are mutually exclusive). These speculations should therefore never be quoted as providing actual information or proven reinterpretations. These and other potential mistaken responses are not always mutually exclusive. The final metabolic profile, such as that observed in Robertson *et al.* (2012), may be a combination of responses tethered to the same signal, or in response to changing conditions due to previous signals. This discussion focuses on the expression profiles of *W. dermatitidis* from Robertson *et al.* (2012). The discussion includes attempts to interpret the observed metabolic profile within the context of each hypothetical mistaken response. However, the expression profile from Robertson *et al.* (2012) compares cells only after 48 hours of low-dose irradiation, in which the cells had budded more in irradiated treatments than controls. For much of the expression profile, it is not known whether expression is due to radiation, due secondarily to new conditions within cells following response ramp-up, or due merely to the differing needs of many smaller cells.

The expression profiles of any two species are likely to be different. For example, light was shown to regulate transcription of ~6% of the genomes of both *Neurospora crassa* and *Trichoderma atroviridis*. There was, however, only ~10 % overlap between the genes so regulated in each of these species. Moreover, some of those common genes were light-specific, such as photolyases and light signaling pathway proteins (Rodriguez-Romero *et al.* 2010). While this example is specific to light, it illustrates how different the expression for a given stimulus can be between species. Important for the research in this field, the expression profiles of *Cr. neoformans* and *W. dermatitidis* will likely differ greatly. Speculations relying on Robertson *et al.* (2012) should not be necessarily applied to *Cr. neoformans* in other work, despite that ideas may be generalized herein.

Expression profiles of *Cr. neoformans* in ionizing radiation will need to be built independently.

### **Oxidative Stress, Metabolic Consequences, and Cell Cycle Proteins**

Regardless of the signal that may be mimicked, there is an expected oxidative stress and DNA damage response. This response could be due to detection of reactive species (including ROS) in the cytoplasm and nucleus, and/or more likely to DNA damage. Both the DNA repair and antioxidant paths will likely be up-regulated in all tests that show any proliferation or metabolic shift, simply by the nature of the trigger: reactive species and/or DNA damage, which go hand-in-hand (Rowe *et al.* 2008). This fact likely explains why DNA repair, DNA damage bypass, and antioxidant/scavenging pathways were up-regulated in *W. dermatitidis* following irradiation (Robertson *et al.* 2012). Oxidative stress and DNA repair responses need not be repeatedly described when examining potential mistaken responses. These responses can be assumed, as they

are likely the primary response; it is likely to these responses that other responses may be tethered.

Some portions of an oxidative stress response have inherent metabolic consequences. For example, in Angelova *et al.* (2005), cyanide-resistant respiration was up-regulated in all twelve fungal species examined under oxidative stress in both  $\text{H}_2\text{O}_2$  and  $\text{O}_2^\cdot$ . Commonly, cyanide-resistant respiration involves alternative oxidase, an enzyme that results in the non-ATP-generating ‘waste’ of reducing power by shunting electrons directly onto oxygen (Vanlerberghe and McIntosh 1997, Akhter *et al.* 2003, Vanlerberghe 2013). This process serves to decrease the pool of reduced cofactors in the electron transport chain so as to reduce autooxidation by  $\text{O}_2$ . This process also locally depletes  $\text{O}_2$  in a relatively ROS-free manner, and to regenerates  $\text{NAD}^+$  or similar molecules for other metabolic processes. Consequently, a reaction to external ROS sources by up-regulation of alternate oxidase serves to validate the idea of misinterpreted signals. The usual sources of ROS would be internal and often due to the electron transport chain for one reason or another. Whether low-dose ionizing radiation up-regulated this path seems not to have been reported, but Robertson *et al.* (2012) did report a decrease in expression of genes for energy-generating pathways. Enzymes associated with production of reducing power can also sometimes be up-regulated in oxidative stress. One example is glucose-6-P-dehydrogenase (G6PD), one of the first steps in glucose metabolism (Angelova *et al.* 2005). These responses serve to provide antioxidant enzyme systems such as glutathione reductase with the reducing power they need to handle ROS efficiently.

Robertson *et al.* (2012) already provided sufficient analysis of why cell-cycle

proteins and DNA damage checkpoints were down-regulated: a large proportion of cells had budded and the young, budded cells were arrested in G1 (growth, non-reproduction) at the time of examination. DNA damage checkpoint proteins might also be conjectured to be low if the cell was set on passing through the cell cycle despite possible damage. Cells may want to reproduce regardless of possible damage due perhaps to what is incorrectly perceived as a time of plenty or other conditions.

### **1: As Though O<sub>2</sub> is Higher**

The most straightforward mistaken response to conceptualize is to the presence of oxygen. ROS (reactive oxygen species), in particular O<sub>2</sub><sup>•</sup> and H<sub>2</sub>O<sub>2</sub>, are produced at relatively consistent rates during normal aerobic respiration. ~0.1 – 3% of electrons sent to the electron transport chain ‘leak out’ due to autoxidation of cofactors by O<sub>2</sub>, forming O<sub>2</sub><sup>•</sup>. The production of reactive species or DNA damage by radiation could be misinterpreted by a cell as an indicator that O<sub>2</sub> is abundant, when O<sub>2</sub> may actually be less so. The probability of premature oxidation in the electron transport chain, or autoxidation of other components by O<sub>2</sub>, would likely rise as the O<sub>2</sub> concentration rises. Unfortunately for this mistaken signal idea, however, such effects are often saturated at low O<sub>2</sub> levels.

Energy is likely less limiting while aerobic than while anaerobic, and more of a cell’s carbon source can be used structurally. For example, *Saccharomyces cerevisiae* rarely divide when anaerobic, as energy is limited to what they can obtain by fermenting carbon sources. When aerobic, however, *S. cerevisiae* maintains growth rates typical of fast-growing yeast. This is notable because *S. cerevisiae* has been shown to express fermentative genes even in aerobic conditions. Metabolic shifts are therefore possibly less important in determining growth; the presence of oxygen itself is mediating the

growth in *S. cerevisiae*. However, in other species, the awareness that oxygen is higher or lower may have metabolic consequences. As oxygen is depleted, *Cr. neoformans* up-regulates hexose uptake and ethanol production genes, while *Candida albicans* up-regulates glycolytic genes (Ernst and Tielker 2009).

In terms of *Cr. neoformans*, or *W. dermatitidis*, response as though O<sub>2</sub> is higher in concentration could account for the increased proliferation and structural use of acetate. The cell may be misinterpreting reactive species or DNA damage as if conditions may be aerobic or may remain aerobic longer. The cell might assume energy should be in abundance, down-regulate functions that produce ATP, and up-regulate functions that use or store energy or carbon sources.

Robertson *et al.* (2012) briefly noted that both richer media and shaking negated the ‘benefit’ provided by ionizing radiation in terms of cell proliferation. Shaking might oxygenate the medium, while negating the O<sub>2</sub>-poor and carbon-poor microhabitat that would likely develop around a cell in static conditions. Such changes would be highly relevant at the bottom of a tube, where cells were observed to settle rapidly in experiments done for this thesis.

## **2: As Though Hypoxic**

A metabolic shift could be due to a response to lower oxygen. In conversion to anaerobic conditions, an ‘ROS burst’ is associated with a shift in many cell functions. This anaerobic response results in a metabolic shift in an organism’s carbon assimilation profiles that is dependent on the organism in question. For example, *Cr. neoformans* up-regulates hexose uptake and ethanol production genes. *Candida albicans* up-regulates glycolytic genes. *S. cerevisiae* remains unique in expressing fermentative genes



regardless of oxygen levels (Ernst and Tielker 2009). *Cr. neoformans* also up-regulates genes for aerobic metabolism, presumably in an attempt to funnel O<sub>2</sub> down energy-producing paths as O<sub>2</sub> becomes limiting.

However, for a response to hypoxia or anoxia to be invoked, the ROS burst would need to be of great concentration, sufficient to activate and inactivate proteins on a mass scale in the cytoplasm (Aguirre *et al.* 2005, Gessler 2007). Furthermore, this should result in a decrease in cell cycle progression. Therefore a response as though in anoxia is unlikely to be induced by low-dose radiation.

If cells sensed oxygen were running out, an expression shift appropriate for hypoxia would be apparent. This shift often includes up-regulation of oxygen-requiring genes in crucial pathways such as heme or ergosterol synthesis (Kwast *et al.* 1998, Zhang and Hatch 1999, Hughes *et al.* 2005, Chang *et al.* 2007, Ernst and Tielker 2009).

### **3: As Though in Bountiful Nutrients, or to General Buildup in Reducing Power**

Production of ROS in aerobic conditions requires two factors: oxygen and a source of reducing power. If higher-nutrient conditions prevail, the production of reducing power could be expected to increase, leading to a more reducing state within the cell. A more reducing state would result in increased ROS in the cell, as oxygen is autoxidized by an abundance of reduced molecules and enzyme cofactors (Murphy 2009, Hamanaka and Chandel 2010). The ROS or DNA damage from ionizing radiation could be misinterpreted as being due to a build-up of reducing power. This hypothetical mistaken signal has potentially good explanatory value for the metabolic shifts observed in *W. dermatitidis* in Robertson *et al.* (2012). Consequently, the level of carbon sources in solution may merely be below a threshold of sensation, yet sufficient for the observed

growth once the metabolic shift occurs.

In Robertson *et al.* (2012), sugar transporters were up-regulated in *W. dermatitidis*, especially in the melanized wild type. Melanin layers are said to potentially limit the permeability of the cell wall (Howard *et al.* 1991, Eisenman *et al.* 2005, Kogej *et al.* 2007, Plemenitaš *et al.* 2008, Fernandez and Koide 2013). If such decrease in permeability were manifest, but the cell were to incorrectly sense that the external carbohydrate concentration were higher, the cell might up-regulate transport. Unfortunately, the converse might instead be expected to be true: up-regulation of transport might occur in starvation to help acquire dwindling nutrients. For this mechanism to be of any ecological benefit, a fungus would have to have some sense that the amount of sugar may be higher or lower – and likely higher, as producing transporters is expensive.

If a build-up of reducing power is commonly associated with excess sugars or other carbon sources, a cell would alleviate the reducing power by shifting away from metabolic processes that reduce cofactors. This could explain why irradiated *W. dermatitidis* decreased glycolysis, fatty acid metabolism, amino acid metabolism, and energy metabolism. The cell could further alleviate the build-up by up-regulating pathways that use up reduced cofactors, such as the observed increase in fatty acid synthesis. Robertson *et al.* (2012) briefly noted that both richer media and shaking negated the ‘benefit’ provided by ionizing radiation in terms of cell proliferation, by causing controls to proliferate equally well compared to irradiated samples. Richer media would be an actual increase in the available carbon sources. Accordingly, shaking might negate the low-carbon microhabitat that would likely develop around a cell in

static conditions. If the carbon source is present but merely below the threshold of detection, increasing transporters, albeit due to some improper signal, might allow for increased carbon source uptake and growth. However, richer media might instead cause cells to enter exponential-like phase, which might change the response to radiation (Conter *et al.* 1984, Dupouy *et al.* 1985, Conter *et al.* 1986, Conter *et al.* 1987a, Conter *et al.* 1987b)

Other changes beyond an increase in available carbon can lead to a build-up of reducing power. Buildup of ATP, for example, would lead to a decrease in oxidative phosphorylation, a back-up of reduced cofactors in the ETC, and buildup of reducing power in the cell. These states could occur despite a paucity of carbon if ATP is in low demand by the cell. The observed shifts in carbon and energy metabolism would alleviate the reducing status of the cell due to such a buildup of ATP, again by merely decreasing the production, and increasing the use, of reducing molecules. Furthermore, such an increase in ATP would normally be indicative of production greater than need, which implies that conditions are favorable for growth and storage rather than subsistence. Thus, again, the ROS or DNA damage from ionizing radiation might be misinterpreted as evidence of abundance, or at least as evidence of greater than current need.

#### **4: As Though in Light**

One of the nonspecific signals for the presence of light can be the detection of ROS (Yoshida and Hasunuma 2004). A quantity of singlet oxygen ( $^1\text{O}_2$ ) is produced by sensitization. Singlet oxygen converts to  $\text{O}_2^-$  by taking an electron from surrounding molecules, or can undergo a number of sometimes exotic electrophilic attacks (DeRosa

and Crutchley 2002).  $O_2^{\cdot -}$  is in turn converted to  $H_2O_2$  by superoxide dismutase (SOD). Moreover, UV can cause DNA damage by base modification, including by producing base dimers. Thus, light can be associated with either an ROS response, or a DNA damage response.

It is noteworthy that cyclobutane pyrimidine dimer photolyase, cryptochrome/6-4 photolyase, and carotenoid biosynthesis were up-regulated by *W. dermatitidis* in response to ionizing radiation in Robertson *et al.* (2012). While other mechanisms such as excision repair (which was also up-regulated) are capable of handling many lesions, photolyases are usually useful only for repairing nucleotide dimers due to UV damage. Photolyases also require blue light for activity, thus are only useful in light. The up-regulation of these light-dependent enzymes for repair of UV-dependent damage is evidence that at least some portion of a light response may be tethered to either the oxidative stress or DNA damage response. Carotenogenesis can be similarly considered evidence of such a tethering; light has been shown to induce carotenoid synthesis (Geis and Szaniszlo 1984, Robertson *et al.* 2012); more on that later.

In *Cr. neoformans*, light is known to regulate sexual development and UV protection via a white collar complex (WCC) homolog (Idnurm and Heitman 2005). This protein complex directly senses light via a bound FADH cofactor. UV protection likely would not involve up-regulation of photolyases, as *Cr. neoformans* is believed to lack these repair enzymes (Lucas-Lledó and Lynch 2009). Studies on the effects of light on *Cr. neoformans* beyond sexual development have not been reported. The lack of photolyases implies that lesions such as nucleotide dimers would be repaired by more general DNA repair mechanisms such as excision repair. It would therefore be all the

more viable, evolutionarily, to tether responses that may be mediated also by light to the typical DNA damage response.

## **5. Not All Things Need To Be Tethered Directly**

Instead of a direct response, some changes may be secondary to others. For example, as mentioned above, some of the metabolic changes observed in irradiated samples in Robertson *et al.* (2012) may be due to different needs of many small, newly-budded cells, rather than to radiation. This idea invokes the important question of timing, akin to the chicken and the egg. Do radiation-induced metabolic shifts result in cellular excesses, and do these excesses lead to growth and proliferation? Or does induction of proliferation result in cells with needs different from their predecessors, which accordingly undergo a metabolic shift?

To give examples, the metabolic shifts up-regulate pathways that use acetate, such as fatty acid synthesis. If metabolic shifts are what are signaled for, rather than proliferation, the proliferation may then be a function of the resulting excesses of such molecules as fatty acids, which make up much of a cell by mass. Similarly, as presented above, some of the observed responses are likely merely a function of the average cells' growth stage: arrest in G1. A lack of need for new nucleotides could result in down-regulation of nucleotide synthesis in non-reproducing cells.

The opposite may also be true. If, for example, proliferation were stimulated directly, many of the additional effects might be only secondary to budding. The up-regulation of transporters, aquaporins, fatty acid synthesis, complex sugar metabolism, and ribosomal synthesis (in albino mutants) might be merely to meet the needs of many young cells. Similarly, the down-regulation of many catabolic pathways such as amino

acid metabolism, simple sugar metabolism, and oxidative phosphorylation might simply be to reserve resources for structural use in these new cells.

Worth noting is an observation from Robertson *et al.* (2012): in irradiated cells, the internal ROS concentration was lower than in unirradiated cells. This afforded protection in long-term starvation, presumably because the excessive antioxidant response helped buffer against greater stress. The zealous protective response mounted to low-dose radiation led to lower internal ROS loads than normal, despite otherwise normal conditions most of the time. Cells may react to changes in their own internal oxidative status with a metabolic shift. For example, cells maintain a certain ambient equilibrium between production and destruction of ROS. Some portions of that equilibrium include mechanisms for production of reducing power – some of the enzymes for which were down-regulated in Robertson *et al.* (2012).

Two interesting cases involves growth of the single-celled eukaryote *Paramecium tetraurelia* and the cyanobacterium *Synechococcus lividus* in Croute *et al.* (1982), Dupouy *et al.* (1985), and Conter *et al.* (1987b). Growth seen in low-dose ionizing radiation and in H<sub>2</sub>O<sub>2</sub> was interpreted as due to up-regulation of G6PD and nucleic acid synthesis, and production of NADP<sup>+</sup> by use of glutathione reductase. NADP<sup>+</sup> is interpreted as a possible limiting reagent in both G6PD activity and the later steps of the same path. Glutathione reductase uses NADPH to reduce glutathione disulfide (oxidized, dimerized glutathione) to glutathione so that glutathione can scavenge H<sub>2</sub>O<sub>2</sub>. Up-regulation of G6PD increases reducing power for antioxidant systems such as glutathione reductase that protect against oxidative stress. However, up-regulation of nucleic acid synthesis and other effects was interpreted as a shunt pathway for the product of G6PD.

In this way, G6PD perhaps only accidentally provided more resources for other systems, using up stored carbon in the process. If this theory is correct, the antioxidant system accidentally provided a ‘boost’ to other systems or functions, which resulted in growth and proliferation. While this mechanism could perhaps work for H<sub>2</sub>O<sub>2</sub> treatment, this specific mechanism cannot be correct for ionizing radiation. The doses in that research were on the order of 10<sup>-6</sup> Gy/hr, and the amount of any products of radiolysis would be insufficient to power such a system by NADP<sup>+</sup> formation. That said, this proposed mechanism could indeed be valid under radiation if NADP<sup>+</sup> is not limiting. Indeed, NADP<sup>+</sup> is unlikely to be limiting in the previous work in this field. To illustrate, up-regulation of fatty acid synthesis in Robertson *et al.* (2012) would produce NADP<sup>+</sup>.

### **Carotenogenesis: Some Tethering and Cross Signaling**

The hypothesis of this thesis hinges on an improper response, as if to other environmental conditions. An example of response tethering and species-specific differences therein is carotenoid biosynthesis. Carotenoid biosynthesis is often controlled by light and/or ROS. The following are examples of studies where ROS, light, and low-dose radiation were shown to, or sometimes shown not to, elicit carotenoid biosynthesis. Sometimes the results are contradictory or specific to the fungus in question, such as whether light is or is not required to up-regulate carotenoid biosynthesis in *Neurospora crassa*. Carotenoid biosynthesis in particular makes a useful example because carotenoids are often used as antioxidants in addition to light/UV blockers and excited state quenchers, thus can ecologically serve a purpose both in light and in oxidative stress independently.

In Schroeder and Johnson (1995), the yeast *Phaffia rhodozyma* was shown to

accumulate carotenoids in response to light-generated  $^1\text{O}_2$ . Exposure to peroxides merely shifted concentrations of specific carotenoids but not overall concentrations, presumably in the dark. This shows that up-regulation of carotenogenesis requires light-signaling in this organism.

In Igusa *et al.* (2005), the presence of  $\text{H}_2\text{O}_2$  or  $\text{O}_2$  was shown to enhance carotenoid production in *N. crassa*, but only during exposure to light. In the dark, carotenoid production was enhanced neither by oxygen nor by 10 mM  $\text{H}_2\text{O}_2$ . Thus, while ROS might be endogenous or exogenous, this system still required light. Presumably light activated the White Collar Complex (WCC), so any ROS would simply be produced as part of the downstream signal. Though light was required for the effect,  $\text{O}_2$  and  $\text{H}_2\text{O}_2$  mimicked each others effects, pointing to the necessary presence of  $\text{O}_2$  to produce  $\text{H}_2\text{O}_2$ , such as by NADPH oxidase (NOX).

In Michán *et al.* (2002), *N. crassa* mutants deficient in CAT-3 showed increased carotenoid synthesis over the wild type even in dark conditions. CAT-3 is the cytosolic oxidative stress response catalase of *N. crassa* (Yamashita *et al.* 2007). Light was not necessary for this effect, but exacerbated it; the CAT-3-deficient mutant still produced far more carotenoids compared to the wild type similarly illuminated. The CAT-3-deficient mutant also showed increased hyphal aggregation, sporulation, height and abundance of aerial hyphae, and protein oxidation (a marker of oxidative stress). Oxidative stress due to normal metabolic ROS above the normal levels (due to a lack of control by CAT-3) was associated with dramatic morphological changes. Thus environmental increase in ROS could signal for changes in carotenogenesis. In this case, ROS were capable of eliciting a distinct response that included carotenogenesis, for which light was not



necessary but was nevertheless enhancing.

In Yoshida and Hasunama (2004), *N. crassa* SOD-1 knockout mutants were shown to hyper-accumulate carotenoids, though this observation required activation by light. Before/without light, SOD-1 deficient mutants did not differ from wild type in the dark. Furthermore, WC-1 and WC-2 (the two components of the WCC) knockout mutants showed almost no carotenogenesis. Dual knockout WC-1/SOD-1 and WC-2/SOD-1 mutants both failed to up-regulate carotenogenesis regardless of light conditions. De-novo synthesis of WC-1 or VVD (VIVID, a regulatory component of the WCC) was not necessary. From this it can be gleaned that ROS affected carotenogenesis specifically by affecting WCC signaling, but only post-translationally and only once the WCC was initially activated by light. As a side note, Yoshida and Hasunama (2004) also found that SOD-1 deficient mutants showed disturbed perithecial polarity (the direction of the opening or ‘beak’ of the perithecium). The perithecia did not lose polarity altogether unless WC-1 or WC-2 were knocked out. In the latter case, SOD-1 deficiency showed no additional effect, pointing to the necessity of the WCC for polarity. Since NOX-1 is needed for proper sexual development in *N. crassa* (Cano-Domínguez *et al.* 2008), the loss of polarity is due to the loss of control of ROS. In other words, proper perithecial production required both the purposeful production of ROS, and the ability to bring the resulting concentration back under control.

Carotenogenesis was induced in *Fusarium aquaeductuum* by wavelengths of light to which *F. aquaeductuum* is usually blind (reds) by addition of photosensitizing dyes (Lang-Feulner and Rau 1975, Yoshida *et al.* 2004, Avalos and Limón 2014). Presumably neither the red light nor the dyes would activate WCC homologs. *F. aquaeductuum* may

thus respond to ROS without light and up-regulate carotenoid synthesis.

Importantly, Robertson *et al.* (2012) showed that ionizing radiation up-regulated carotenoid biosynthesis in *W. dermatitidis*. This behavior was independent of light. Interestingly, while carotenoid synthesis was up-regulated, DHN melanin synthesis was not; melanin synthesis was constitutive in these conditions. This example is particularly important for this thesis, because it brings a function usually thought of as being triggered by light or ROS under the purview of ionizing radiation. Recently, Chen *et al.* (2014) compared *W. dermatitidis* responses to pH stress and low-dose radiation from Robertson *et al.* (2012). Carotenoid biosynthesis genes were, in general, differently expressed between these two stressors. Ionizing radiation led to up-regulation, with pH generally showing less change or down-regulation. This indicates that the response to radiation was not merely a generic stress response, and that the responses to pH and ionizing radiation are different.

The above examples should illustrate the complexity of light signaling and the interplay of ROS and potentially DNA damage. In some organisms, expression of protective mechanisms is tied with another effect. For example, in *Phaffia rhodozyma*, carotenoid production seems to be tethered to light signaling. In *F. aquaeductuum* carotenoid production is inducible by oxidative stress alone. *W. dermatitidis* showed up-regulated carotenogenesis under ionizing radiation and required no light. The latter response is likely tethered to a DNA damage response or to an oxidative stress response like in *F. aquaeductuum*. Finally, *N. crassa* showed light-dependent, or light-independent but light-modified, signaling for carotenogenesis.

### **Side Note About Carotenoids**

Robertson *et al.* (2012) observed a rise in carotenoids in *W. dermatitidis*, likely part in parcel with the up-regulation of other stress protection mechanisms. The carotenoid biosynthesis in *Cr. neoformans* proceeds via the mevalonate pathway, which uses Acetyl-CoA as a building block. This might lead one to speculate that carotenoid accumulation could contribute to the uptake of C<sup>14</sup>acetate uptake observed in Dadachova *et al.* (2007). However, the carotenoid content of carotenoid-containing yeasts, including *Cryptococcus*, range in concentration from ~50-400 ug/g dry weight (Libkind and Broock 2006, Moliné *et al.* 2009). As long as this holds approximately true in Dadachova *et al.* (2007), there are not enough cells in the initial or final solutions therein to use an appreciable amount of the acetate for the production of carotenoids. Other metabolic uses such as fatty acid synthesis, as described in chapter II, are much more likely to have been where the C<sup>14</sup>acetate was used.

### **A Call for More Expression Comparisons**

Among the most useful works for the above discussion was Robertson *et al.* (2012). Expanding on that work, Chen *et al.* (2014) tested cells under pH stress, and found that the expression profiles were distinct. Expansion of these expression profiles may be illuminating, particularly if radiation-based cell activation strategies can be tailored (discussed in the Chapter VI). Expanding expression not only to different stimuli but to different species (notably *Cr. neoformans*) would allow response similarities to be mapped. If incorrect signaling is occurring, the signal or portion of a signal to which a false response is being made could be identified.

The following is a non-comprehensive list of conditions under which fungi might

be tested to determine what misinterpretation is occurring under irradiation. The changes that affect the response to radiation could be assessed thereby.

- Cellular oxidative stress of several types (e.g. H<sub>2</sub>O<sub>2</sub>, Superoxide)
  - Constant, low-level sources of ROS, to mimic the effects of low dose radiation on the medium, as was attempted in Crouete *et al.* (1982)
  - Sudden and transient oxidative stress, perhaps by sudden photosensitization or by an ROS bolus or temporary treatment, to mimic some of the internal effects of low dose radiation on cells (discussed in Chapter VI)
- DNA damage
  - Constant, low-level DNA damage, such as by low-level doses of toxins, as in Povirk (1996)
  - Sudden and transient, low-level DNA damage followed by periods without, so as to mimic the internal effects low-dose radiation (discussed in Chapter VI)
- Visible Light, as per Karpenko *et al.* (2006), the expressional observation in Robertson *et al.* (2012) and observations herein (below)
- UV, as a specific form of DNA damage – preferably in the range beyond FAD absorbance to avoid light response activation
- Shaking or general oxygenation in comparison to static or oxygen-lacking conditions, per observation in Robertson *et al.* (2012)
- Media gradient from starvation to rich, per observation in Robertson *et al.* (2012)
  - Different carbon sources in otherwise poor media, per observations in *Cladosporium sphaerospermum* in Dadachova *et al.* (2007) and per expression changes in Robertson *et al.* (2012).

- Presence of antioxidants, especially intracellularly, which should decrease the ROS concentration of normal conditions, thus mimicking the cell's lower oxidative state as observed in irradiated cells Robertson *et al.* (2012).
- Growth phase and response differences between growth phases, per observations in Conter *et al.* (1984), Dupouy *et al.* (1985), Conter *et al.* (1986), Conter *et al.* (1987a), Conter *et al.* (1987b)

Growth over time should also be assessed for each treatment when not confounded by necessary growth conditions for a given treatment. Proliferation is the current gold standard, as it was reproduced between Dadachova *et al.* (2007), Robertson *et al.* (2012), and Shuryak *et al.* (2014). For any given treatment, the combined treatment with radiation might also be illuminating, such as if any treatments mitigate the effects of radiation either by increasing proliferation or by negating proliferation due to radiation. For example, a lack of sucrose inhibited *Cl. sphaerospermum* from showing stimulation in Dadachova *et al.* (2007), pointing to a mechanism requiring specific external carbon sources rather than internal reserves.

Though results of experiments are otherwise unentered in this thesis, the author poses an observation. Sparked by discussion with Dr. Ekaterina Dadachova, the effect of light on *Cr. neoformans* proliferation was tested as part of a pilot experiment alongside several other treatments including radiation. Of all the treatments (including radiation), only light elicited any proliferative response. This response was regardless of all other independent variables including *Cr. neoformans* strain and melanization. This response was correlated with amount of light: 3 minutes of direct bright light showed the lowest response, 30 minutes an intermediate response, and 24 hours the highest response (data

not shown). This response may be in line with that observed in Karpenko *et al.* (2006), which showed distinct overlap between filamentous fungi showing radiotropism and photostimulation, hinting at an underlying mechanism in common. The stimulation therein was also seen regardless of melanization, being instead strain-dependent. However they attribute the effect to radioluminescence, a suggestion that is unlikely to be applicable at low radiation doses as in Dadachova *et al.* (2007) or Robertson *et al.* (2012) (Tarasov *et al.* 2007). Recall that in Robertson *et al.* (2012), photolyases were up-regulated in *W. dermatitidis* which are generally useful only in light for repairing damage from UV. *W. dermatitidis* therefore may have tethered at least some of a light response to a DNA damage response. This linkage makes sense because UV causes DNA damage. *Cr. neoformans* lacks photolyases (Lucas-Lledó and Lynch 2009). Accordingly, *Cr. neoformans* may be even more likely to tether a light response to a normal DNA damage response, as UV damage would be repaired by more general DNA repair mechanisms such as excision repair. Based on these observations, the author of this thesis encourages future researchers applying expression analysis to consider light high on the list of priorities.

## **CHAPTER VI**

### **MICRO-SCALE, STOCHASTIC VIEW OF LOW-DOSE RADIATION ON FUNGI AND OTHERS**

Much of this thesis focused on dispelling a particular idea, radiosynthesis, and pointing out some of the flaws in the evidence claimed to support it. Thereafter, an alternate hypothesis was posed that may explain observations in the past literature in this field while invoking no separately novel ideas. To finish, this thesis shall point out a potentially important way of thinking of how the doses of  $\gamma$  radiation in the research dealt with herein interact with diffuse cells in solution: each cell experiences only very occasional, somewhat dramatic events when rare high-energy electrons pass through. To this end, a simple, unrefined, but seemingly predictive approximation of the probability of a cell or nucleus being affected by an electron will be briefly presented. The resulting estimates of probabilities imply that the responses of single cells in solution, or of nuclei within filamentous fungi, must occur following singular or few electron events. This way of thinking may have implications for irradiation strategies used in future research on the responses of single-celled organisms or filamentous fungi to low doses of ionizing radiation.

In the study of responses of single-celled organisms or filamentous fungi to low doses of radiation, there is a tendency to treat the system as if it is affected by a given average dose. For example, Shuryak *et al.* (2014) speaks of a binary “on/off” switch, but states that the threshold for this occurs at very low dose rates,  $<0.002$  mGy/hr. This dose

is only just over an order or magnitude above background radiation. Views that use the average radiation dose are sufficient at high radiation doses, around doses of  $\sim 10^{-1}$  Gy/hr or above, because cells and nuclei are repeatedly affected in short order by high-energy electrons. However, the view from the cell's perspective is very different at low radiation doses. When using radiation doses of  $\sim 10^{-2}$  Gy/hr or less, researchers studying the responses of organisms should shrink their view down to the level of the cells and the individual electron tracks. At that scale, and at doses of  $\sim 10^{-2}$  Gy/hr or less, the tracks of electrons are small and locally infrequent.

Such micro-scale, stochastic views are not novel to this thesis. This view has historically been applied to animal tissues subject to very low radiation doses, often near background levels of  $\sim 10^{-7}$  Gy/hr. At such low doses, the cells in an animal may experience single hits on the order of about once a year (Feinendegen *et al.* 2004). However there are two important distinctions between much of the literature applying stochastic models which focuses primarily on mammalian cells, and the studies in question in this thesis.

First, single-celled organisms or filamentous fungi are likely to show very different responses from mammalian cells, and to take different amounts of DNA damage from a given dose. Much of the discussion of stochastic effects in the literature focuses on probabilities of oncogenesis and apoptosis versus adaptive response (e.g. Nygaard *et al.* 2013). Apoptosis is unlikely to be of great importance for fungi at low doses ( $< 10^{-2}$  Gy/hr), though apoptosis is nearly unstudied in fungi within these radiation dose regions. Similarly, discussion based on mammalian cells often involves bystander effects, where unaffected cells mount an adaptive response to a treatment due to signals from affected



cells. While such effects might be discovered in cytoplasmically-linked filamentous fungi if studied, only extracellular signaling would be likely to affect single-celled organisms. Extracellular bystander signaling was shown to be unimportant in *Schizosaccharomyces pombe* at nonlethal doses (DeVeaux *et al.* 2006). However, there may be epigenetic effects that may be passed down to an affected cell's progeny. For example, genomic instability, often seen in animal cells post-irradiation, has been identified in *Saccharomyces cerevisiae*. This effect was shown to last several generations post irradiation (Brennan *et al.* 2001). Furthermore, buds might maintain stress responses mounted in a parent cell before budding. Finally, fungal genomes are around two orders of magnitude smaller than mammalian genomes. The amount of DNA damage dealt by high-energy electrons is calculated to be about linear with genome size (Ward 1988, Westmoreland *et al.* 2009, Stewart *et al.* 2011, Robertson *et al.* 2012). Therefore an impinging electron may deal a similarly-scaled two orders of magnitude lower damage to DNA in a fungal nucleus.

Second, the radiation doses in the research dealt with herein lie in a region transitional between ways of thinking about radiation dose. As mentioned, at doses around or above  $10^{-1}$  Gy/hr, the average dose is a fair way to conceptualize the dose even in single cells. At very low doses, the literature primarily focuses on rare events in primarily mammalian cells, with moderate increases above background such as would be due to diagnostic x-rays or environmental contaminants. The radiation doses in the works dealt with herein, such as Croute *et al.* (1982), Dadachova *et al.* (2007), Robertson *et al.* (2012), and several works by Conter *et al.*, lie within a region between. The radiation doses in these reports range from two to five orders of magnitude above typical

background doses. Doses of such magnitude are estimated herein to result in single electron events spaced apart on the order of days to weeks at lower doses, and on the order of minutes to hours at higher doses. It seems as though because the dose seems so much higher than background, researchers have been treating these systems as though the average dose is applicable. However, it will hopefully become apparent in the following discussion that these radiation doses lie within the region where occasional stochastic events, so called micro-dose hits (Feinendegen *et al.* 2004), are still far more important than the average dose. Furthermore, due to the probability of a cell being affected by a given number of electrons at low radiation doses, it is unlikely that all cells are affected equally.

### **The Average Dose and The Medium**

The average dose would be appropriate if the medium, all the fluid in the extracellular space, were the ‘target,’ that with which radiation interacts with in a way that affects cells. To compare radiation and normal biological sources of ROS, one needs to use G-values to calculate the reactive species produced by a dose of radiation. A G-value is the amount of a particular product of radiolysis produced per given dose. Table 5 shows common G-values produced by electrons in water. While some additives to

Table 5. Common G-values for products of radiolysis of pure water by electrons, in order of abundance (Choppin *et al.* 2002)

Product	G-value (Number / 100 eV)
OH <sup>•</sup>	2.7
e <sup>-</sup> <sub>(aq)</sub>	2.7
H <sub>2</sub> O <sub>2</sub>	0.7
H <sup>•</sup>	0.6
H <sub>2</sub>	0.45
O <sub>2</sub> <sup>•-</sup>	0.026

systems can change these outcomes, often by scavenging particular products, these numbers are a good approximation for the initial products of radiolysis in biological systems. The overall total of reactive species sum to 6.73 molecules / 100 eV. A dose of  $5 \times 10^{-5}$  Gy/hr equates to  $3.12 \times 10^{14}$  eV/(L×hr). At this dose, and assuming the values from table 5, the following rates of production apply for pure water:

$$\text{H}_2\text{O}_2 = 3.63 \times 10^{-12} \text{ M/hr}$$

$$\text{O}_2^{\bullet -} = 1.35 \times 10^{-13} \text{ M/hr}$$

$$\text{OH}^{\bullet} = 1.40 \times 10^{-11} \text{ M/hr}$$

$$\text{e}_{(\text{aq})}^{-} + \text{H}^{\bullet} = 1.76 \times 10^{-11} \text{ M/hr}$$

$$\text{Overall} = 3.49 \times 10^{-11} \text{ M/hr}$$

Common values of steady state ROS concentrations in non-pathological conditions are cited as  $10^{-9}$  to  $10^{-7}$  M  $\text{H}_2\text{O}_2$ , and  $10^{-12}$  to  $10^{-11}$   $\text{O}_2^{\bullet -}$  (Nivière and Fontecave 1995). The above-calculated values are low compared to these steady state values.

From the above values, the surrounding medium can essentially be excluded as the ‘target’; it is unlikely that effects of low-dose radiation on the extracellular medium lead to cellular activation. Reactive species generated in the medium would quickly diffuse outward and thus would be poorly localized. Therefore, the average dose is applicable to irradiation of the medium, but the above calculations show that the amount of reactive species produced would be unlikely to be biologically relevant. While  $\text{e}_{(\text{aq})}^{-}$  and  $\text{OH}^{\bullet}$  may be abundant, these are so reactive that they are unlikely to travel more than a few nanometers. As such, these moieties are even less likely to pass all the way through a *Cryptococcus neoformans* capsule and membrane. The value of  $\text{H}_2\text{O}_2$  generated would be unlikely to be biologically relevant when added externally.

Intracellular  $\text{H}_2\text{O}_2$ -degrading enzymes such as catalase and peroxiredoxins maintain a gradient of  $\text{H}_2\text{O}_2$  across the plasma membrane on the order of from  $\sim 1:10$  to up to around  $\sim 1:10^3$  (Antunes and Cadenas 2000, Huang and Sikes 2014). Studies that have shown stimulatory effects by extracellular doses of  $\text{H}_2\text{O}_2$  on various organisms tend to see stimulation around  $10^{-7}$  to  $10^{-5}$  M, which is far above what is produced by radiolysis at low doses.

$\text{O}_2^{\cdot-}$  might conceivably be able to build up to biologically relevant concentrations. In oxygenated solutions, many  $e^-_{(\text{aq})}$  could be scavenged by oxygen producing  $\text{O}_2^{\cdot-}$ , thus increasing the rate of production by as much as 2 orders of magnitude over that predicted by radiolysis of pure water. However,  $\text{O}_2^{\cdot-}$  is still likely to be subject to extracellular scavenging (albeit at rates less than  $e^-_{(\text{aq})}$  and  $\text{OH}^{\cdot}$ ) and is therefore unlikely to build up.  $\text{O}_2^{\cdot-}$  is also likely to experience a gradient across the plasma membrane due to the proverbial minefield of superoxide dismutase (SOD) and scavengers within the cell.

It is possible that this assessment unduly dismisses the medium as the target. Conter (1987) showed that pre-irradiation of media affected *Synechococcus lividus* cells, inducing proliferation. In that work, media were pre-incubated in shielding (thus receiving less than background radiation) or at  $2.1 \times 10^{-2}$  Gy/year, which is  $2.3 \times 10^{-6}$  Gy/hr, for 21 days. By the G-values above, this would produce peroxide of  $\sim 1.67 \times 10^{-13}$   $\text{MH}_2\text{O}_2/\text{hr}$ , or  $8.41 \times 10^{-11}$  M total over 21 days. Irradiated media stimulated cells in transition phase but inhibited cells in exponential phase relative to shielded media. The addition of active catalase at the time of inoculation negated the stimulation of transition-phase cells (though not the inhibition of exponential-phase cells). Inactivated catalase failed to negate stimulation. Why this would show stimulation when mathematical

treatment is very much against the idea is beyond the knowledge of the author of this thesis. One might pose that a different component were at fault, such as  $O_2^{\cdot-}$  which might be produced in oxygenated media at a greater rate than by the G-value above due to scavenging of  $e^-_{(aq)}$  by  $O_2$ . However why catalase – and only active catalase – would be protective against the effects of  $O_2^{\cdot-}$  is unknown. Despite the results of Conter (1987), the thesis author believes that effects on the media are not what lead to the responses observed in other literature.

### **The Basic Micro-Scale Stochastic Model: A Bud's Eye View**

Above was given reason why the medium is an unlikely target. Henceforth, cells and their nuclei shall be dealt with as probable targets. These would be unlikely to see the effects of ionizing radiation distributed evenly throughout the medium. On the scale of cells and sub-cellular components, impinging electrons are infrequent, noteworthy events. The electron tracks may be substantial, but are transient. Excluding the medium (above) implies that only those cells across whose cytoplasms or nuclei the electron travels will experience any effects from the electron, as in Figure 2.

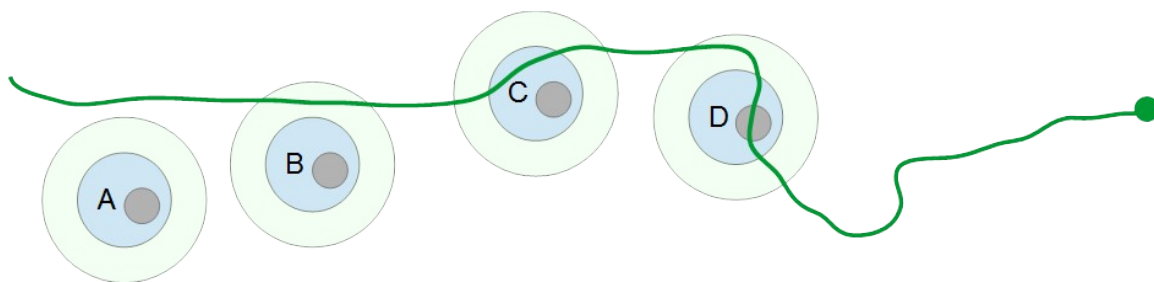


Figure 2. *Cryptococcus neoformans* cells near the main track of an electron. Assuming extracellular effects do not affect a cell, A = unaffected, B = unaffected (electron traverses the capsule), C = affected in the cytoplasm, D = affected in the cytoplasm and nucleus.

Consider, for the following estimates, a  $7.5\ \mu\text{m}$  diameter *Cr. neoformans* cell – a medium-sized cell. This cell would contain a volume of  $2.21 \times 10^{-13}$  L. Nuclear size has been found to consistently scale with cell size with little respect even for changing

genome size. *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* nuclear size rarely vary from ~7-9% of the cell volume (Jorgensen *et al.* 2007, Neumann and Nurse 2007, Webster *et al.* 2009). Based on 8% cell volume, then, this 7.5  $\mu\text{m}$  cell should then have a nucleus of 3.23  $\mu\text{m}$  diameter and contain a volume of  $1.76 \times 10^{-14}$  L. This diameter is a good approximation of *Cr. neoformans* nuclei (Yamaguchi *et al.* 2009).

Also consider hereafter  $10^4$  eV and  $10^5$  eV electrons. These energies are generally representative of lower- and higher-energy electrons that would be produced by Compton scatter from 172 keV photons, such as would be produced by a  $^{188}\text{W}/^{188}\text{Re}$  generator.  $10^4$  eV electrons have tracks 2.5  $\mu\text{m}$  long in water, while  $10^5$  eV electrons have tracks 143  $\mu\text{m}$  long in water (Berger *et al.* 2005). Both would exhibit higher linear energy transfer (LET) as they slow, and would therefore distribute energy faster near their ends. However, for simplicity, assume that they distribute their energy evenly, and assume the G-values of pure water as in Table 5.  $10^4$  eV electrons would, then, produce ~673 reactive species total or 269 reactive species/ $\mu\text{m}$ , and  $10^5$  eV electrons would produce 6730 reactive species total or 47 reactive species/ $\mu\text{m}$ . Finally, since a Gray is a unit of energy per kg, and the density of 1 L of water or media is usually ~1 kg/L, let us assume for ease of calculation that the cells are present in 1 L of uniformly irradiated water.

One final important fact is needed to build a model of the system on the micro-scale with the above numbers. The existence of a cell in a given place at any given time and the passage of an electron through a given region of space are independent events. This fact allows for a stochastic model of the cells' experience to be built, based on the probability of a cell existing within a region of space, and the probability of an electron passing through or otherwise affecting that region.

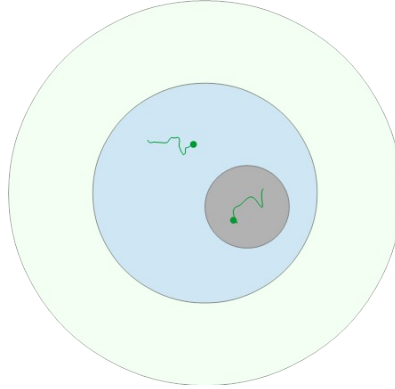


Figure 3. 7.5  $\mu\text{m}$  diameter *Cr. neoformans* cell with a 3.23  $\mu\text{m}$  nucleus and a surrounding capsule, and  $10^4$  eV electron tracks in its cytoplasm and nucleus. The track is shorter than the diameter of the cell or nucleus, and can be estimated as contained therein.

From the ranges above, it should be apparent that a  $10^4$  eV electron is capable of dissipating all of its energy inside of a cell, or less likely but possibly, a nucleus, as in Figure 3. To affect a cell, then, such an electron must either be generated inside or very near to a cell. This allows the probability of this track coexisting with a single specific cell to be estimated by the volume of the cell:

$$\frac{V_{\text{cell}}}{V_{\text{total}}} = \frac{2.21 \times 10^{-13} \text{ L}}{1 \text{ L}} = P_{1e^-} = 2.21 \times 10^{-13}$$

This number is useful because it allows the opposite to be calculated: the probability of being *unaffected* by an electron. Or, more appropriate for a situation involving irradiation, the probability of being *unaffected* by  $N_e$  electrons:

$$P_{0_{1e^-}} = (1 - P_{1e^-})^{N_e}$$

A dose of  $5 \times 10^{-5}$  Gy/hr implies  $3.12 \times 10^{14}$  J/(L $\times$ hr). Distributed among  $10^4$  eV electrons, this dose would result in  $3.12 \times 10^{10}$  electrons/(L $\times$ hr). The probability of a specific cell being unaffected by any one electron in this analysis, then, is:

$$P_{0_{1e^-}} = (1 - 2.21 \times 10^{-13})^{3.12 \times 10^{10}} = 0.993$$

What this means is, according to the assumptions above, at a dose of  $5 \times 10^{-5}$  Gy/hr, 99.3%

of the cells in solution should be *unaffected* by at least one electron in an hour. State as the reverse, 0.7 % of cells should be *affected* by at least 1 electron in an hour. This number rises to 15.2 % affected by 24 hours. For a 3.23  $\mu\text{m}$  nucleus, the above calculations yield values of 0.05% affected at 1 hr, and 1.3 % affected after 24 hours.

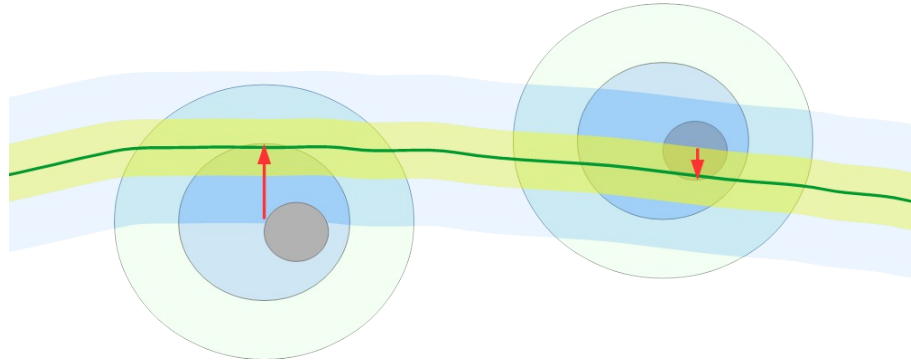


Figure 4. Cylinders around the track of an electron (line) defining the region within which a cell's center (left, thick region around line, blue) or nucleus (right, moderate region around line, yellow) must exist to be affected by that electron, per assumptions.

Similar estimates can be done for  $10^5$  eV electrons. However, the tracks of these electrons cannot be contained within a cell. It is more appropriate to use a different estimate of the region they affect: assume a cylinder around the electron's (admittedly tortuous) path, as in Figure 4. This cylinder is the region within which a cell must exist to be affected by an electron. The radius of such a cylinder would be defined by the farthest distance it could pass from the center of the cell or nucleus and still pass through the cell or nucleus.

For a 7.5  $\mu\text{m}$  diameter cell with a 3.23  $\mu\text{m}$  diameter nucleus, and the 143  $\mu\text{m}$  long track, the volume enclosed by the cylinders around the track is  $6.32 \times 10^{-12}$  L for cells and  $1.17 \times 10^{-13}$  L for nuclei. A dose of  $5 \times 10^{-5}$  Gy/hr dose would be distributed among  $3.12 \times 10^9$  electrons/(L $\times$ hr). These values give the chance of a cell being affected by at least one  $10^5$  eV electron as 2 % by 1 hour and 33.7 % by 24 hours. Nuclei so considered



Table 6. Percent probabilities of a 7.5  $\mu\text{m}$  diameter cell or its 3.12  $\mu\text{m}$  diameter nucleus being affected at a given time by at least 1, 2, 10, or 100 high-energy electrons at a given dose rate after 1 and 24 hours. These values are divided assuming all electrons are either  $10^4$  or  $10^5$  eV, per the estimates and assumptions above.

Electron Energy (KeV)	Dose Rate (Gy) / hr	Compartment	Time							
			1 hr				24 hr			
			1 e <sup>-</sup>	2 e <sup>-</sup>	10 e <sup>-</sup>	100 e <sup>-</sup>	1 e <sup>-</sup>	2 e <sup>-</sup>	10 e <sup>-</sup>	100 e <sup>-</sup>
$10^4$	$5 \times 10^{-5}$	cytoplasm	0.7	0.0	0.0	0.0	15.2	2.3	0.0	0.0
		nucleus	0.1	0.0	0.0	0.0	1.3	0.0	0.0	0.0
	$5 \times 10^{-4}$	cytoplasm	6.7	0.4	0.0	0.0	80.9	65.4	12.0	0.0
		nucleus	0.5	0.0	0.0	0.0	12.4	1.5	0.0	0.0
	$5 \times 10^{-3}$	cytoplasm	49.8	24.8	0.1	0.0	100.0	100.0	100.0	100.0
		nucleus	5.4	0.3	0.0	0.0	73.3	53.8	4.5	0.0
	$5 \times 10^{-2}$	cytoplasm	99.9	99.8	99.0	90.3	100.0	100.0	100.0	100.0
		nucleus	42.3	17.9	0.0	0.0	100.0	100.0	100.0	100.0
$10^5$	$5 \times 10^{-5}$	cytoplasm	2.0	0.0	0.0	0.0	37.7	14.2	0.0	0.0
		nucleus	0.4	0.0	0.0	0.0	8.4	0.7	0.0	0.0
	$5 \times 10^{-4}$	cytoplasm	17.9	3.2	0.0	0.0	99.1	98.2	91.5	41.2
		nucleus	3.6	0.1	0.0	0.0	58.4	34.1	0.5	0.0
	$5 \times 10^{-3}$	cytoplasm	86.1	74.1	22.3	0.0	100.0	100.0	100.0	100.0
		nucleus	30.6	9.4	0.0	0.0	100.0	100.0	99.8	98.5
	$5 \times 10^{-2}$	cytoplasm	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
		nucleus	97.4	94.9	77.0	7.3	100.0	100.0	100.0	100.0

would be 0.4 % likely to be affected by at least one  $10^5$  eV electron after 1 hr and 8.4 % by 24 hr.

From the above, the probability of a cell being affected by at least two electrons, at least three electrons, and so forth is easily calculable. Table 6 contains results from the above estimates based on the dose of  $5 \times 10^{-5}$  Gy/hr at 1 hour and at 24 hours as in the growth experiment in Robertson *et al.* (2012). Despite these being approximate values, this model is believed to have some predictive power. For example, by the approximations therein, one can see a possible reason why the maximum proliferation in Robertson *et al.* (2012) was seen around a dose of  $5 \times 10^{-4}$ : the number of cells potentially

affected by at least one electron is reaching a maximum, but the number and timing of electrons has yet to reach a stressful level. The effective radii of the cylinders for *Wangiella dermatitidis* therein would of course differ, therefore these values would differ.

The above estimate could be improved in a number of practical ways that are beyond the scope of this thesis. These include more realistic effective radii for calculated cylinders to account for tracks and spurs (possibly resulting in effective cones or other shapes), accounting for the actual Compton electron and photoelectron energy distributions for a given radiation source (Feinendegen *et al.* 2004), application of an appropriate hit-size weighting function (Bond and Varma 1983), proper geometry and an account of size variance in the cell population, and others.

### **Constraints and the Likely Target**

The micro-scale stochastic view for effects of low-dose radiation places constraints on any mechanism for cellular detection and response. Assuming the medium is not the target, per examination above, the mechanism must respond to events that are:

1. Infrequent, possibly singular
2. Sudden, on the order of microseconds
3. Transient, as reactive species will quickly react with cell components or be mopped up
4. Moderate to low impact
5. Highly localized, along the track of an electron or its blobs and spurs

The mechanism of sensation and cellular response may begin at a DNA damage response. The nucleus may usually be a relative sanctuary (Malinouski *et al.* 2011). A sudden track of damage – especially clustered damage along an electron's path that

prevents simple repair (Ward 1988, Semenenko and Stewart 2006) – may be sufficient to elicit a significant DNA damage response as if it were a double-strand break. Cells are particularly sensitive to double-strand breaks: one double-strand break is sufficient, if sensed, to lead to cell cycle arrest (Frankenberg-Schwager and Frankenberg 1990). Around 20-30% of double-strand breaks due to radiation would have additional damage in close proximity, making the damage more complex and difficult if not impossible to repair (Goodhead 1994, Nikjoo *et al.* 2001). A similar clustered damage profile would exist for other, lesser lesions, likely causing them to be treated by a cell with the fervor of a double strand break (Ward 1988). In experiments showing decreased reproduction in mouse cells shielded from background radiation, a line of mouse cells deficient in double-strand break repair via mutations in XRCC4 was reported as showing no decrease in growth in low-radiation environments, implying a link between proliferation or growth and DNA damage response (Kawanishi *et al.* 2012). In general, DNA damage is often invoked to explain the regulatory effects of low-dose ionizing radiation (Feinendegen *et al.* 2004). Once the response activates transcription, translation will lag behind, and the resulting protein products will persist for potentially days to weeks. Thus, a sudden, transient event is said to have long-term implications for the cell so affected.

Unfortunately, however, it is questionable whether the amount of DNA damage dealt should be sufficient to enable detection and response. Several numbers exist in the literature for estimating the number of DNA lesions per unit time due to radiation. Ward (1988) gives values for mammalian cells: 1 Gy produced 1000 single-strand breaks, 1150+ base modifications, and 40 double-strand breaks. Damage profile values tend to scale with genome size, and mammalian cells generally contain ~6 Gb of DNA. Values

above therefore imply more general damage rates of  $\sim 170$  single strand breaks/(Gb $\times$ Gy),  $\sim 200+$  base modifications/(Gb $\times$ Gy), and  $\sim 7$  double-strand breaks/(Gb $\times$ Gy).

Westmoreland *et al.* (2009) gave a double-strand break value of 8.3 double strand breaks/(Gb $\times$ Gy) from *Saccharomyces cerevisiae*. Stewart *et al.* (2011) state values of 650 damaged bases/(Gy $\times$ Gb) and 217 single strand breaks/(Gy $\times$ Gb). These values are all on about the same order, so comparison should be consistent. The genome size of *Cr. neoformans* is  $\sim 19$  Mb, and that of *W. dermatitidis* is  $\sim 26$  Mb.

Robertson *et al.* (2012) helpfully point out that the average dose is unlikely to deal significant damage: they calculated that a dose of  $3 \times 10^{-4}$  Gy/hr over 48 hr would lead to  $\sim 0.003$  double strand breaks per *W. dermatitidis* nucleus. By similar calculation using values above, the total base damage rate is  $\sim 7 - 17$  lesions/(Gy $\times$ cell) in *Cr. neoformans* and  $10 - 23$  lesions/(Gy $\times$ cell) in *W. dermatitidis*.  $3 \times 10^{-4}$  Gy/hr over 48 hr, then, would lead to  $\sim 0.10 - 0.24$  lesions/cell in *Cr. neoformans* and, and  $\sim 0.14 - 0.33$  lesions/cell in *W. dermatitidis*. This number of total lesions is small compared to the likely number of lesions produced per day under normal conditions. Assuming the probability of damage scales with genome size, a fungal genome is likely to experience  $10^3 - 10^4$  base modifications and single-strand breaks per genome per day due to steady-state ROS (Feinendegen *et al.* 2004).

This number is an average-dose view. The problem improves somewhat if one zooms down closer. If a  $10^4$  eV electron deposits all of its energy inside a cell, the effective instantaneous dose to the  $7.5 \mu\text{m}$  diameter cell is  $7.25 \times 10^{-3}$  Gy within the cell. More appropriately, if this electron were to deposit all of this energy in a nucleus of  $3.12 \mu\text{m}$  diameter, the dose immediately therein would be  $9.1 \times 10^{-2}$  Gy within the nucleus.

Notably, this single-hit value would not change with the average dose; only the probability of such a hit would change. Therefore average doses would more dramatically underestimate what a cell would experience – albeit experience less frequently or with lower probability – at lower doses. This instantaneous dose would predict, on average, ~0.65 – 1.51 lesions in a cell so hit in *Cr. neoformans*, and 0.89 – 2.07 lesions in a cell so hit in *W. dermatitidis*. These numbers are still unnervingly low; however, at least now the probability of multiple lesions has been increased. Notably, 30-40 % of DNA damage is dealt directly by an electron's interaction with DNA; the remainder is due to products of radiolysis (Feinendegen *et al.* 2004 citing Hall 2000).

Some argument could be made for the cytoplasm as the target that, when struck, might lead to a cellular response. As shown in Table 6, the cytoplasm is a much larger target, which greatly increases the probability of electron impingement at a given low dose. The instantaneous concentration of reactive species produced by, for example, a  $10^4$  eV electron – around ~673 assuming pure water – would be on the order of  $5 \times 10^{-9}$  M. The protein damage by  $\text{OH}^\bullet$  and  $\text{e}^-_{(\text{aq})}$  at these levels might be sufficient to elicit a response if mechanisms exist to detect it. Similarly, if some significant portion of  $\text{e}^-_{(\text{aq})}$  are scavenged by  $\text{O}_2$  producing  $\text{O}_2^{\bullet-}$ , the instantaneous  $\text{O}_2^{\bullet-}$  concentration could be up to 2 orders of magnitude higher than the estimated normal physiological steady state estimates ( $10^{-11}$  –  $10^{-12}$  M from Nivière and Fontecave (1995)). The difference between physiological steady states and instantaneous reactive species concentrations could be exacerbated if a cell is in a state where endogenous ROS production is low, such as starvation or near stasis.

Unfortunately, however, most of the reactive species produced would be

scavenged only by small molecule antioxidants or would react non-specifically with local proteins with diffusion-limited kinetics. The cytoplasm experiences ROS leakage from mitochondria, peroxisomes, and the endoplasmic reticulum, with a predicted  $10^9$  ROS produced internally per day in mammalian cells (Feinendegen *et al.* 2004). During times of signaling, the  $H_2O_2$  concentrations is said to rise as high as  $10^{-4} - 10^{-5}$  M (Woo *et al.* 2010); since this  $H_2O_2$  is produced by dismutation of NOX-generated  $O_2^{\cdot-}$  by SOD, presumably the  $O_2^{\cdot-}$  concentration would have risen quite high as well. The cytoplasm contains numerous enzymes for handling both ROS and their effects, and small molecules to scavenge highly reactive species, even under non-oxidizing conditions. As such, it may yet be difficult for the number of reactive species produced by a high-energy electron to reach a level, or to cause an amount of damage, that the components of the cytoplasm could not immediately handle or would even notice.

### **Suggestions and Implications for Research**

The simple model presented above predicts an approximate probability of a cell being affected by some number of electrons. Furthermore, it shows that the observed proliferative effects need to be attributable to as few as a single electron. If this analysis is correct, there are a number of potentially useful implications for research in this field.

First, the model would need to be validated experimentally. Unfortunately, the author of this thesis was unable to demonstrate any increased proliferation under ionizing radiation, despite several attempts and mimicking the irradiated systems of previous groups. Nevertheless it is hoped that the ideas presented herein will serve in some way to support future investigations.

To validate the model, the ‘binary,’ switch-like nature of response activation will

need to be tested. One of the most straightforward ways to do this would be to irradiate with one or more short doses calculated to ‘hit’ a proportion of the cells in solution. For example, instead of irradiating with a dose of  $5 \times 10^{-4}$  Gy/hr over 12 hours, cells could be subject to a dose of  $6 \times 10^{-3}$  Gy within an hour or less. Hypothetically this moderate, shorter dose should activate a similar number of cells as the lower, longer dose. The exact doses and timing of doses would be determined by the researcher. Of course a sudden moderate dose can be unlike a longer low dose if the probability of multiple hits is high: it is different if a cell is affected by multiple electrons in short order compared to single electrons hours apart. Irradiation schemes may consider accounting for repair time, often  $\sim 10$  minutes for simple DNA lesions (Feinendegen *et al.* 2004).

If the model proves to be valid, the assumption that radiation’s effect on the medium is not that which elicits a response will need to be tested, especially given the results of Conter (1987). This could be done numerous ways. Methods that irradiate solutions but do not do so for cells could be devised. *Cr. neoformans* cells were observed to be prone to settling in experiments for this thesis, and this trait could be used to easily exclude them from being irradiated. Or a small, concentrated aliquot of cells with catalase, SOD, and extracellular scavengers could be given a defined radiation dose and immediately diluted into much greater volumes (though lacking catalase, SOD, and extracellular scavengers). This variation would both inhibit formation of products in the medium during irradiation, and dilute any possible products formed, while still potentially activating cells directly.

If the model is found predictive, and effects of radiation on the media are found to not be the target, then experiments could be done to find better parameters and

adjustments. These include the true effective radius for a cylinder or cone after spurs, blobs, and other factors are taken into account, hit weighting functions, spectra of responses, and so on. Blobs and spurs are unlikely to change the effective radius much, as they have ranges of a mere few nanometers. Nevertheless, these clusterings of damage along the path account for ~95+ % of the energy deposited by an electron (Hall 2000). An assay could be developed to view the response activation in single cells. While it is presumed herein that the nucleus and DNA therein is the target which, when struck, results in a response, this hypothesis needs to be confirmed in a way that excludes the cytoplasm.

If the micro-scale stochastic model herein is found to be true, there are a number of implications for research in this field. First, as described above, cell responses could be activated with more sudden, moderate, unsustained doses. This would improve study of cellular responses to low-dose ionizing radiation. For example, one flaw in Robertson *et al.* (2012) was that the population was examined for expressional analysis at only 48 hours, and the irradiated treatment had budded more than the control. The irradiated population was higher in new small cells, which implies different needs and expressional profiles. How much of the response to radiation therein was lost to time and development? How many artifacts have cropped up? If the model proposed herein is correct, the problem would be intractable while long, low radiation doses are used. The population would at any time be a mix of unactivated cells, newly activated cells, and cells that had received their activation long in the past. To mitigate the issue one or more sudden, unsustained, moderate doses could activate a significant portion of the population, allowing expression to be followed from short times through to long times.



Similarly, other notable factors that might affect a cell's response could be examined. For example, Conter *et al.* (1984), Dupouy *et al.* (1985), Conter *et al.* (1987a), and Conter *et al.* (1987b) showed that in *Synechococcus lividus*, response to H<sub>2</sub>O<sub>2</sub> and to low-dose ionizing radiation both depended on growth phase and metabolic conditions. Therein cells were merely grown until the desired phase, then placed in the usual long, low radiation dose conditions. Instead, however, cells could be grown to a desired phase, and very suddenly – and simultaneously – irradiated. This would allow for pinpointing what, if any, cellular conditions must prevail in order for radiation to show a stimulatory effect.

## SUMMARY

The idea of radiosynthesis as described in Dadachova *et al.* (2007 patent) based on research in Dadachova *et al.* (2007), and as presented in later reports, is unfounded according to arguments presented in this thesis. The idea fails basic mathematical scrutiny, the data and their interpretations are often flawed, and the indirect evidence is unsupportive or incorrect. This invalidates by default the idea that melanin has been shown to be involved in capture and metabolic use of ionizing radiation. Moreover, experiments in Robertson *et al.* (2012) and Shuryak *et al.* (2014), as well in works including (but not limited to) Planel *et al.* (1976), Croute *et al.* (1982), Conter *et al.* (1983, 1984, 1986, 1987b,c), and Takizawa *et al.* (1992), have shown proliferative effects of low-dose ionizing radiation on several very different organisms, in each case independent of the presence of melanin.

With the idea of radiosynthesis set aside, the systems can be examined more accurately for the cause of this proliferation. The hypothesis of a somewhat accidental effect was proposed, wherein organisms respond as though to a more common source or cause of ROS or DNA damage. Similar ideas exist in the aforementioned articles from the 1970's and 1980's. Therein the effect was interpreted as likely due to up-regulation of a mechanism of an oxidative stress response that incidentally provided resources for other metabolic pathways. The main pathway, the pentose phosphate pathway, is important for growth, and use of this pathway as a shunt would provide building blocks for growth. However, these early works provided a picture limited by technology, and

possessed some flaws in assumptions as to what led to stimulation. Modern repetition and examination will likely elucidate (or confirm) the mechanism(s) by which various organisms are led to grow or proliferate under low-dose ionizing radiation.

To this end, a simple probabilistic model is proposed that may be useful to researchers when dealing with responses of diffuse cells under low doses of radiation. This model assumes that at low doses of  $\gamma$  radiation, cells are directly affected in a way that leads to a response only when a Compton electron or photoelectron passes through the cell's cytoplasm or nucleus. With this assumption, the probability of a cell or nucleus being affected can be estimated using volumes and electron ranges in the medium. If the assumption is correct, this model has implications for irradiation strategies that may increase the versatility of studies seeking to examine the effects of low-dose radiation on cells.

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