A Study of Phenoxyl Radicals from Bisphenol-A By Fast-flow ESR Spectroscopy

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INTRODUCTION

Certain molecules have the ability to mimic hormones, specifically estrogen, and are examples of harmful chemicals that can cause lowered fertility, harmed development, and even behavioral problems [1]. One such estrogen mimic, Bisphenol-A, was shown to cause proliferation of cultured breast cancer cells [2]. Its molecular structure was noted to be similar to estradiol (**Figure 1&2**).

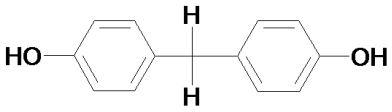

Figure 1: Bisphenol-A

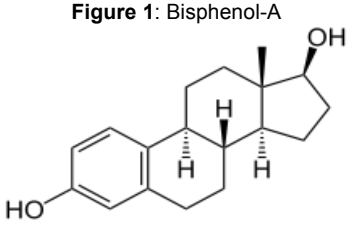

Figure 2: Estradiol [10]

Bisphenol-A is commonly known as BPA, and was commonly found in plastics and served as a plasticizer in food containers, toys, and metal food cans. Additionally, BPA found in plastic toys are a danger to children [3]. Fortunately, BPA taken orally at low doses is less harmful but does not mean Bisphenol-A does not have an effect on the body or that it is completely harmless. [4].

Radicals, molecules with unpaired electrons, can have serious biological consequences through the mechanism known as oxidative stress [5]. Since radicals can cause damage to an organism's DNA, proteins, and lipids, the body has defense systems aimed at countering actual destructive results of those radicals [6].

Through a series of reactions, diagrammed in **Figure 3**, phenoxyl radicals, of which Bisphenol-A can be precursor, is converted by the body's defensive mechanisms back into a non-radical phenolic groups. However, when the cell's antioxidant defenses suppress BPA phenoxyl radicals, they form secondary free radicals. Subsequently, the restored BPA phenol can react again: the phenol group can, in the presence of enzymes, revert back to a radical once again. Thus a small number of radicals can very easily and readily generate a much larger number of radicals [7]. As a result, the body's defensive

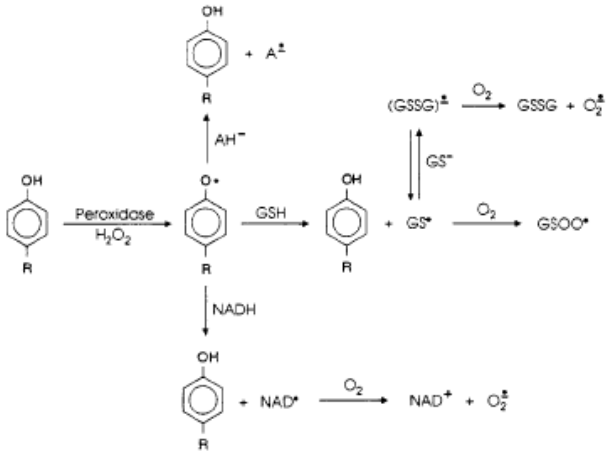

Figure 3: Futile metabolism of phenoxyl radical

mechanisms will be depleted as the reaction is repeated. Nicotinamide adenine dinucleotide (NADH), one of the antioxidants shown in Figure 3, reacts with phenoxyl radicals. Depletion of NADH is harmful to the cell because NADH is critical to the production of adenosine triphosphate (ATP), the energy source for cellular activity [8]. Also shown in Figure 3 are reactions with other cellular antioxidant protectors such as glutathione (GSH) and ascorbate (AH, Vitamin C) [9]. Except for ascorbate, which forms a relatively stable radical, these specific defenses themselves turn into radicals, and contribute to oxidative stress [11]. This may cause cell death and subsequent cell division that may result in DNA replication errors and, ultimately, cancer.

This report has its primary goal in demonstrating whether or not compounds related to BPA that are now being used as alternative plasticizers have the potential, in the presence of enzymes occurring *in vivo*, to become radicals. Observation of free radicals is carried out in three ways. The reaction between phenoxyl radicals and the body's defensive antioxidants consumes oxygen in order to produce radicals as shown in Figure 3. Therefore, one can simply monitor the activity of oxygen in solution in order to track the progress of the reactions of radicals [12]. The phenoxyl radical is unstable (hence of its potential for biological damage), and therefore direct spectroscopic observation can be difficult. One method to circumvent this problem is to attach the radical to another molecule, known a spin trap, which is stable and therefore allows the use of the spectroscopic observation technique known as

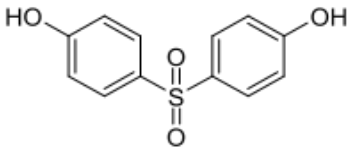

Figure 4: Bisphenol-S [17]

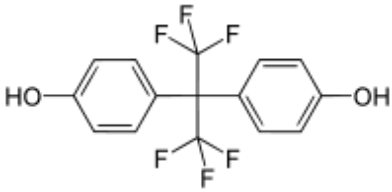

Figure 5: Bisphenol-AF [18]

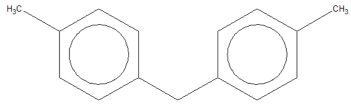

Figure 6: BHM Molecule

electron spin resonance spectroscopy (ESR). However, the results show merely the presence of radicals, and do not give information about the original, unstable radical itself [13]. Therefore, we will rely primarily on the third option this summer, a technique known as fast-flow ESR.

By using a specially designed sample mixing flat cell, two solutions are mixed as they enter the ESR cavity. The resulting radicals are observed and then pushed out through the top of the sample tube before they can decompose, and a fresh mixture replaces them in the flat cell [14]. The main disadvantage of this fast flow technique is cost, as the molecules being observed are constantly being discarded and replaced. This continual flow, combined with the fact that the enzymes used to generate radicals are typically very expensive, results in cost being a prohibitive factor. Fortunately Yamasaki discovered that relatively cheap hemoglobin molecules could act as enzymes to produce radicals in the same manner that the much more expensive molecules do, making fast-flow ESR a viable option even on limited budgets [15]. In addition to BPA itself, I plan to study three related compounds, Bisphenol-S (BPS) and Bisphenol-AF (BPAF), and 4,4'-Dihydroxydiphenyl methane (BHM), the structures of which are shown in **Figures 4, 5 and 6**, respectively. These compounds are under active investigation by the National Toxicology Program of the NIEHS (National Institute of Environmental Health Sciences) [16].

The results of these investigations, while completed *in vitro*, may provide insight into possible mechanisms of toxicity of BPA and BPA-related compounds by establishing if they have the same free radical chemistry and biochemistry as analogous phenolic compounds, of which no research has been done.

RESULTS

The primary goal of this research was to see if BPS, BPAF, and BHM would all produce radicals under the same conditions that BPA would produce radicals. Experimentation with BPS revealed that radicals were produced when reacting with the Ce(SO4)2 system and were highly visible Gaussian calculations were then produced for accuracy of the WinSim BPS HFCC's. The theoretical HFCC's and WinSim HFCC's are close enough to deem the WinSim HFCC's close to what the actual value may be (**Figure 7**). BPAF molecule was tested with the Ce(SO2)4 system and the ESR recorded a spectrum consisting of radical BPAF. WinSim simulations revealed that the six fluorides were affecting the HFCC's. The Gaussian DFT HFCC's were compared14, and seemed close enough to be relevant. BHM was the final molecule that was tested using the Ce(SO2)4 system, and spectrum of radical BHM15 was produced and recorded by the ESR.

Experiments were done to determine the best flow rate16 using BPA when trying to create free radicals. The equation that best represents how the rate of flow affects the intensity is a linear one, having a correlation of about .99193.

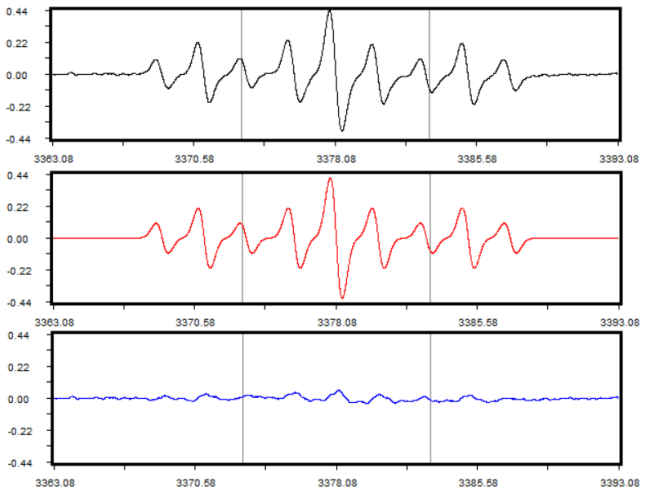

Figure 7: BPS Spectrum, Winsim Simulation, and Winsim Residual with a correlation value of .990316.

METHODS AND MATERIALS

BPA: 1.8263g of BPA (Sigma Aldrich Lot #0632PO) was dissolved in a 20% ETOH solution to create a 4L 2mM solution of BPA, and then bubbled for 5 minutes in N2 gas. This solution reacted with the 4L 1.8mM $Ce(SO_4)_2$ solution in the flat cell as the ESR recorded the spectrum.

BPA was also used in a study to determine the effects of flowrate. Solutions of the BPA/$Ce(SO_4)_2$ were made that were tested at different flowrates ranging from 30ml/min incrementing by 20ml/min until reaching 190mL/min.

Another solution included .9130g BPA dissolved into a dilute 2L 20% ETOH solution that contained 4.55mL of H_2O_2. This solution was bubbled for 5 minutes and then reacted with the Hb solution in the flat cell as the ESR recorded the spectrum.

BHM: 1.6018g of BHM (TCI Lot #GL01-FLPM) was dissolved in 20% ETOH solution to create a 4L 2mM solution of BHM, and then bubbled for 5 minutes in N2 gas. This solution reacted with the 4L 1.8mM $Ce(SO_4)_2$ solution in the flat cell as the ESR recorded the spectrum.

Another solution included .8009g BHM dissolved into a dilute 2L 20% ETOH solution that contained 4.55mL of H_2O_2. This solution was bubbled for 5 minutes and then reacted with the Hb solution in the flat cell as the ESR recorded the spectrum.

BPAF: 2.6898g of BPAF was dissolved in 20% ETOH solution to create a 4L 2mM solution of BPAF, and then bubbled for 5 minutes in N_2 gas. This solution reacted with the 4L 1.8mM $Ce(_SO_4)_2$ solution in the flat cell as the ESR recorded the spectrum.

Another solution included 1.3410g of BPAF dissolved into a dilute 2L 20% ETOH solution that contained 4.55mL of H_2O_2. This solution was bubbled for 5 minutes and then reacted with the Hb solution in the flat cell as the ESR recorded the spectrum.

BPS: 2.0021g of BPS was dissolved in 20% ETOH solution to create a 4L 2mM solution of BPS, and then bubbled for 5 minutes in N_2 gas. The solution reacted with the 4L 1.8mM $Ce(_SO_4)_2$ solution in the flat cell as the ESR recorded the spectrum.

BPS was also used in a study depicting the effects of the molarity of solutions in free radical reactions. The molarity of BPS starts at .5mM and each consecutive test doubled the molarity until reaching 4mM. The molarity of the $Ce(SO_4)_2$ was at a .9 ratio to the molarity of BPS in each test.

Another solution included 1.0079g BPS dissolved into a dilute 2L 20% ETOH solution that contained 4.55mL of H_2O_2. This solution was bubbled for 5 minutes and then reacted with the Hb solution in the flat cell as the ESR recorded the spectrum.

$Ce(SO_4)_2$:100mL of concentrated H2O2 was diluted to 4L. In this diluted solution, 2.3921g of $Ce(SO_4)_2$ (Fisher, Lot # not specified) was dissolved and then bubbled with N2 gas for 5 minutes.

Hb System: 200 mL of Phosphate Buffer was diluted to 2L and then 0.04 DTPA (Sigma Aldrich Lot #23617BB) was dissolved. Before the addition of 2g Hb, the solution was bubbled with N_2 gas for 5 minutes.

ESR: For reaction in the ESR each solution was poured into a separate reservoirs. The solutions were then reacted into the flat cell using a peristaltic pump at the desired mL/min. As the solutions would continue to flow, the ESR would be started in order to detect if radicals were formed by producing a spectrum of the reactions happening in the flat cell.

WinSim:The spectrum was then moved to the WinSim program for simulation. The simulation was then subtracted in the WinSim program to produce a residual, and a correlation between the simulation and spectrum from the ESR would be found. The simulation would also provide Hyper Fine Correlation Constants (HFCCs).

Gaussian: The simulated HFCCs would then be compared the theoretical Gaussian HFCC's that were found by running the Gaussian Program to verify the integrity of the WinSim HFCC's.

The above research was completed using a JEOL RE-1X Electron Spin Resonance Spectrometer, a Wilmad mixing flat cell, a MasterFlex microprocessor controlled peristaltic pump.

CONCLUSION

BPA has been proven by other research students to create radicals [15]. These new compounds, BPS, BHM, and BPAF have similar BPA backbone. These compounds have all also been shown to create radicals when oxidized with $Ce(SO_2)_4$; however, the primary concern is whether these compounds will create radicals *in vivo*. Tests were conducted using a Hemoglobin system, replacing the costly horse radish peroxidase; and they all showed except for BPS, due to a lack of time. Further tests should be done to reproduce these results especially *in vivo* where a matching of HFCCs will be enough to show the correlation.

Another point of interest that occurred during the research was the behavior of BPAF. Originally it was predicted that the fluorine would not show or affect the HFCCs. However, this was not true. All six fluorine were present and were verified by the DFT calculations to be an accurate representative.

Furthermore, since the structure and radicalization of these compounds were similar, it would be worthwhile to produce more experiments in which these compounds can be tested for human effects.

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