

Effect of an Aspartame-Ethanol Mixture on *Daphnia magna* Cardiac Activity

Stephanie Schleidt¹, Danielle Indelicato², Ashley Feigenbutz¹, Cierra Lewis¹, and Rebecca Kohn²

¹Neuroscience Department, Ursinus College, Collegeville, Pennsylvania, 19426, ²Biology Department, Ursinus College, Collegeville, PA, 19426

Aspartame in conjunction with alcohol has been shown to increase the blood alcohol level in humans faster than alcohol and sucrose (Wu et al., 2006). To determine the potential effects of various mixtures of ethanol and aspartame on the nervous system, the heart rate of *Daphnia magna* (*D. magna*, water flea) was measured in deionized water (control), ethanol, aspartame, and five different mixtures of ethanol and aspartame. The heart rate was chosen as a representative measure since it is controlled by the nervous system and the heart rate of *D. magna* can easily be measured. The results were statistically evaluated by student's t-test. A significant increase in heart rate was observed for all mixed assays compared to both control and ethanol, but not to aspartame. The data suggests that the aspartame and alcohol mixture have a greater effect on *D. magna* heart rate than water or ethanol, but not aspartame alone. We propose that alcohol in combination with aspartame has potentially detrimental consequences for the nervous system.

Keywords: *Daphnia magna*; nervous system; aspartame; alcohol; metabolism; neuropathology; blood alcohol level

Introduction

Stimulants and depressants, specifically aspartame and alcohol, have been the focal point in many scientific studies both in conjunction with each other and as separate entities. The combination of aspartame and alcohol has resulted in a marked increase in blood alcohol levels when compared to alcohol and sugar (Wu et al., 2006). Anecdotal reports suggest that alcohol and aspartame, a component of diet sodas, are popular, particularly among women, to minimize the caloric intake of mixed alcoholic beverages (Henkel, 1999).

Aspartame

Aspartame, an artificial sweetener, was approved by the U.S. Food and Drug Administration in 1981 and has been used as a tabletop sweetener and in beverages, breakfast cereals, desserts, and chewing gum since then (Henkel, 1999). Aspartame consumption has

been shown to cause neurological and behavioral symptoms in certain individuals ranging from headaches and mood alterations to dizziness and insomnia (Bradsiock et al., 1986). These symptoms are thought to result from the role each component of aspartame plays once entering the body (Butchko et al., 2002).

Fifty percent of aspartame is comprised of phenylalanine, which is either converted in the liver to tyrosine (a non-essential amino acid) or remains unchanged. In order for either tyrosine or phenylalanine to cross the blood brain barrier (BBB), binding to a large neutral amino acid transporter (NAAT) must occur (Humphries et al., 2008). Since NAAT is the only way in which phenylalanine, tyrosine, and other amino acids can cross the BBB, a great deal of competition exists for the limited binding sites (Humphries et al., 2008). Increased aspartame intake increases the number of NAAT

sites occupied by phenylalanine, therefore inhibiting the transportation of other essential neutral amino acids into the brain. (Yokogoshi et al., 1984; Coulombe and Sharma, 1986). The increase of phenylalanine in the brain has been seen to cause a phenylketonuria effect, which is a hereditary disorder characterized by the accumulation of phenylalanine (Mehl-Madrona, 2005). This results in a decrease in the dopamine and serotonin production and since serotonin plays a critical role in behavior, control of sleep, appetite, and neuroendocrine function, a decrease in serotonin levels will have effects on these behaviors (Humphries et al., 2008).

Aspartic acid, also known as aspartate, is an excitatory neurotransmitter in the central nervous system (CNS) and comprises 40% of aspartame. Aspartic acid increases depolarization at the postsynaptic membrane. The increased depolarization can induce neuroendocrine disturbances and the rapid firing of neurons ultimately results in the failure of enzymes to function optimally (Humphries et al., 2008). Unlike other amino acids in the brain, aspartic acid is not one of the essential amino acids. As a result of the excitatory effects of aspartic acid, the concentration in the brain must be controlled to prevent excess stimulation of the nerve cells (Magnuson, 2007).

Methanol, constituting 10% of aspartame, is poisonous at temperatures above 86°F and therefore becomes toxic once aspartame is ingested. Methanol is found naturally in fruits and vegetables, but in that case it is bound to pectin. When it is bound to pectin, the body is unable to break down the molecule and therefore the methanol is never released into the bloodstream (Thomas, 2005). In aspartame, methanol is not bound to pectin and is therefore considered to be in its “free” form. In this case, the methanol is released to a greater degree and at an increased rate (Magnuson, 2007). The methanol present from the breakdown of aspartame is converted in the liver to formaldehyde, which is a known neurotoxin and carcinogen (Humphries et al., 2008). Formaldehyde is further broken down into formic acid, which accumulates in the brain, kidneys, spinal fluid, and other organs and can lead to excess acid in body fluids, which is

known as acidosis (Humphries et al., 2008). Methanol poisoning has been shown to result in dizziness, headaches, and behavioral alterations, which are all effects that have been reported from aspartame consumption (Thomas, 2005).

Several studies have shown the detrimental effects of aspartame metabolites on the nervous system. One specific study on the acetylcholinesterase of the frontal cortex of suckling rats concluded that the increased concentration of metabolites in the bloodstream resulted in seizures, memory loss, headaches, and cholinergic defects (Simintzi et al., 2007). This led researchers to conclude that high dosages (toxic levels) of aspartame are detrimental to the nervous systems of the rats, even though the consumption of normal levels of aspartame showed little effect on their frontal cortex. Interestingly, aspartame itself had little effect, but the metabolites were responsible for the decreased enzymatic activity (Simintzi et al., 2007). Not only has aspartame been shown to have an effect on enzyme activity, but it has been suggested to disrupt the metabolism of amino acids, protein structure, neuronal function, and catecholamine concentrations in the brain (Humphries et al., 2008).

Alcohol

Heavy alcohol consumption has been shown to cause depressive episodes, severe anxiety, insomnia, temporary cognitive defects, and peripheral neuropathy (Schuckit, 2009). Once alcohol is consumed, about 98% enters the bloodstream through the walls of the small intestine (Schuckit, 2009).

Alcohol, regardless of the dose, enhances the activity in the inhibitory γ -aminobutyric acid (GABA) systems throughout the brain (Schuckit, 2009). GABA is an inhibitory neurotransmitter that binds to specific transmembrane receptors in the plasma membrane of the pre-synaptic and post-synaptic neurons. Upon binding, ion channels open resulting in the influx of chloride ions or the efflux of potassium ions. When alcohol is introduced into the system, the inhibitory action of GABA is augmented. Since GABA is not present in one specific location in the brain, but rather in several locations, the presence of alcohol inhibits several activities in the brain

resulting in behavioral changes including muscle relaxation and somnolence (Schuckit, 2009).

Alcohol consumption also stimulates the neurons of the serotonergic system, which is located in the raphe nucleus at the base of the brain (Schuckit, 2009). This area influences brain functions that are related to attention, emotions, and motivation (Lovinger, 1991). Studies have shown that individuals who consume large amounts of alcohol have differences in brain serotonin levels in comparison to non-alcoholics (Lovinger, 1991). Lovinger (1991) also showed that both short and long term exposure of alcohol has an effect on the serotonin receptors that convert the signal produced by serotonin into functional changes in the signal-receiving cell. Even a single exposure to alcohol has an effect on the synaptic functions of serotonin. Upon alcohol ingestion, levels of serotonin metabolites in the urine and blood increase, indicating an increase in serotonin release from the nervous system. This has been suggested to be a result of enhanced signal transmission at serotonergic synapses (Lovinger, 1991). Research investigating the effects of ethanol on the nervous system has shown that alcohol has the ability to disrupt an active fragment of the activity-dependent neuro-protective protein (ADNP) called NAPVSIPQ (NAP) (Chen and Charness, 2008). NAP is an octapeptide that is necessary in signaling Fyn Kinase (a member of the Src Family Kinase), resulting in normal axonal outgrowth in cerebral granule cells, found in the brain (Chen and Charness, 2008). NAP has been shown to protect the nervous system against a wide variety of insults, including alcohol exposure. Therefore, disrupting the activity of NAP and the presence of excess ethanol due to social drinking in humans have resulted in neuronal migratory malfunctions, improper axonal-dendritic connections, as well as apoptosis of glial and neuronal progenitor cells (Chen and Charness, 2008).

Alcohol and Aspartame

In recent years, “sugar-free” alcoholic beverages have become more popular, but consuming alcoholic beverages that contain aspartame in place of sucrose may result in a greater degree of adverse effects (Wu et al,

2006). Participants in a study who consumed “diet-alcoholic” drinks containing aspartame were found to have higher blood-alcohol levels when compared to those consuming alcoholic drinks containing sucrose (Wu et al, 2006). In addition, alcohol, like aspartame, has been found to affect heart rate. Alcohol has a depressive effect on the nervous system by first depressing the highest cerebral centers of the brain (prefrontal cortex) then the motor and sensory centers, the cerebellum, spinal cord and finally the medulla (Karpas, 1916). The medulla is responsible for controlling breathing, heart rate and other basic involuntary functions in the body. By depressing this part of the brain, alcohol can also reduce heart rate.

Since the heart rate is modulated by different parts of the brain, the heart rate can be used in order to determine the effect of aspartame and alcohol, both combined and as separate entities, on the nervous system (Guyenet, 1990).

The small fresh water crustacean, *D. magna*, was used in this experiment because of their transparent carapace, which allows for increased visibility of the internal organs and makes monitoring the heart rate of the individual easier (Environmental Inquiry, 2006). It was hypothesized that treatment with aspartame (a stimulant) alone would increase the heart rate while treatment with alcohol (a depressant) would depress the heart rate. Since alcohol and aspartame have opposite effects on the nervous system, it was thought that the combination would result in a heart rate comparable to the base heart rate of *D. magna*. *D. magna* were treated with alcohol and aspartame alone and in different combinations to determine the effect on the nervous system.

Materials and Methods

Daphnia magna

All *D. magna* used in this experiment were purchased through Ward’s Natural Science© and housed in containers containing spring water. *D. magna* utilized were of different sizes and states of maturity.

Ethanol Solutions

Preliminary tests showed that a 2.0% alcohol stock solution, manufactured by Ward’s

Natural Science© (Rochester, New York), was fatal for *D. magna*, but 0.2% elicited a decrease in heart rate without fatalities. Four successive ethanol dilutions in deionized water were then prepared: 34.32 mM (0.2%), 17.16 mM (0.1%), 8.58 mM (0.05%), and 4.29 mM (0.025%). The amount of alcohol ingested could not be determined since the alcohol level in the fleas could not be measured. Forty *D. magna* were used in the ethanol assays, n=10 for each concentration.

Aspartame Solutions

Equal® was used as the source of aspartame. Three solutions with the following concentrations; 10.0 mM, 1.0 mM, and 0.1 mM in deionized water were prepared. It has been discussed that, in humans, aspartame only has negative effects at levels much higher than the recommended 40 – 50 mg/kg body weight/ day (Magnuson, 2007). Thus, a high concentration of aspartame was chosen for the initial assay. The solubility of aspartame is about 30 g/L at room temperature. Considering a molecular weight of 294.31 g/mole for aspartame, a starting solution of 10.0 mM was close to the maximum concentration at room temperature. It is difficult to judge how much of the compound was ingested by *D. magna*, but based on an estimated average body weight of 30.0 mg, the organisms were exposed to 49.05 g/kg body weight (10.0 mM), 4.905 g/kg body weight (1.0 mM), and 0.49 g/kg body weight (0.1 mM). Thirty *D. magna* were used in the ethanol assays, n=10 for each concentration.

Mixed Assays

Five mixed assays were made from initial concentrations of 34.32 mM (0.2%) ethanol and 10.0 mM aspartame. The concentration of ethanol in each successive mixture decreased while the aspartame concentration increased (Table 1). This type of varying solution was chosen to parallel alcoholic drinks with diet soda, since the proportion of alcohol in those drinks varies inversely with the amount of aspartame. Similarly, in this experiment the volume of liquid the organism was exposed to was limited. One-hundred and

twenty *D. magna* were used in the mixed assays. For Assay # 1 n=21, for Assay # 2 n=21, for Assay # 3 n=22, for Assay 4# n=29, and for Assay # 5 n=27.

Table 1. Mixed assay concentrations for ethanol and aspartame in 50 µL.

| Assay # | Ethanol (mM) | Aspartame (mM) |
|---------|--------------|----------------|
| 1 | 30.89 | 1.0 |
| 2 | 24.02 | 3.0 |
| 3 | 17.16 | 5.0 |
| 4 | 10.30 | 7.0 |
| 5 | 3.43 | 9.0 |

Heart Rate Acquisition

Each *D. magna* was chosen randomly and removed from a jar of fresh water with a plastic pipette and transferred individually onto a concave microscope slide. Any water remaining on the slide was entirely absorbed with a paper towel. Then *D. magna* were submerged in 50.0 µL of one of the following: deionized water for the base heart rate, alcohol solutions, aspartame solutions, or mixed assays. Subsequently, *D. magna* were allowed to acclimatize to the environment for two minutes, and then the heart rate was counted for one minute. Heart rate was recorded with the use of a hand held, manual counter. A different *D. magna* was used for each assay.

The base heart rate was acquired by transferring each *D. magna* into deionized water, allowing it to acclimatize for two minutes, and recording heart rates for one minute. Statistical analysis was performed similarly in the other parts of the experiment. Fifty *D. magna* were used for the base heart rate assay.

Statistical Analysis

The average heart rate and standard error of the mean (SEM) were calculated for each concentration of each part of the experiment (ethanol, aspartame, and mixed assays). Statistical significance at $\alpha = 0.05$ and $\alpha = 0.01$ was evaluated using the student's t-test. All calculations were performed on the statistical software SPSS.

Results

Base Heart Rate

To compare the effects of aspartame and alcohol on the cardiac activity of *D. magna*, a base heart rate (BR) of 204 +/- 7 beats per minute (bpm) was established (n=50).

Heart Rate in Ethanol

In order to evaluate changes in *D. magna* heart rate in ethanol the fleas were exposed to increasing ethanol concentrations (4.29, 8.58, 17.16, and 34.32 mM). The alcohol showed an overall depressive trend on heart rate. However, only ethanol concentrations 17.16 and 34.32 mM showed a significant decrease with

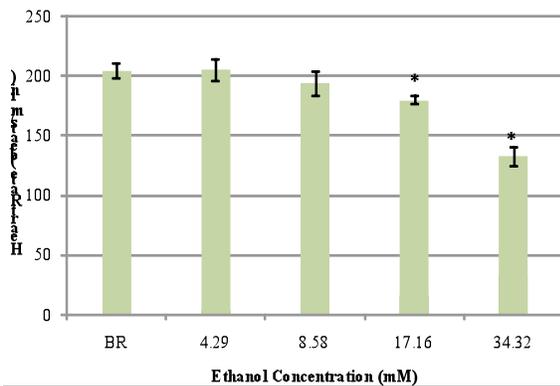


Figure 1. *Daphnia magna* heart rate change in ethanol. Heart rate in increasing ethanol concentrations was compared to base rate. Generally, a depressant trend was seen, but only concentrations 17.16 and 34.43 mM decreased heart rate significantly. 34.32 mM was used for subsequent testing. Error is SEM, n = 10 for each concentration, n = 50 for BR; * = $p < .01$.

total heart rates of 180 +/- 4 bpm (t (54.7) = 3.24, $p < .01$, n = 10) and 133 +/- 8 bpm (t (23.32) = 6.95, $p < .01$, n = 10) respectively (Fig. 1). For further investigation, an ethanol concentration of 34.32 mM was utilized to capitalize on the most pronounced depressive effect of this concentration.

Heart Rate in Aspartame

To assess changes of heart rate in aspartame three different concentrations of aspartame were examined (10.0 mM, 1.0 mM,

and 0.1 mM). When *D. magna* was submerged in aspartame, the fleas tended to exhibit higher

heart rates with increasing concentrations, compared to base rate (Fig. 2). When compared to the base rate the highest increase in heart rate was observed at a concentration of 10.0 mM with a heart rate of 243 +/- 9 bpm (t (30.6) = -4.09, $p < .01$, n=10) (Fig. 2).

Heart Rate in Mixed Assay

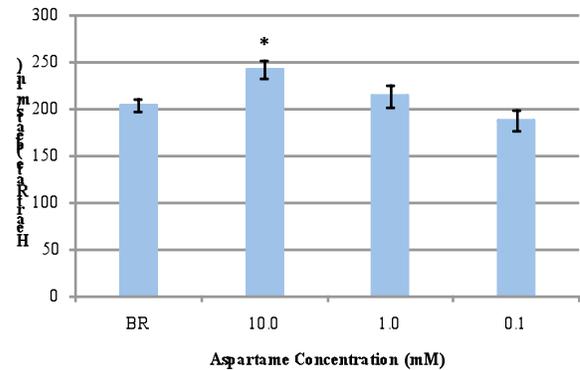


Figure 2. *D. magna* heart rate change in aspartame. *D. magna* heart rate was significantly increased from base rate in 10.0 mM aspartame but not in any of the two lower concentrations. Thus, in subsequent testing 10.0 mM of aspartame concentration was used. Bars represent SEM; n = 10 for 10.0 mM and 0.1 mM; n = 11 for concentration 1.0 mM; * = $p < .01$

To evaluate possible influences of ethanol in combination with aspartame on heart rate, a series of mixed assays were administered (Table 1). The base solutions for the mixed assays were 34.32 mM ethanol and 10.0 mM aspartame, which were chosen because each caused the greatest depression or stimulation of heart rate respectively.

Both ethanol and aspartame were observed to be different from base rate, as mentioned before. When compared to base rate, Assays # 1 through # 5 also showed significant differences with Assay #1 at 250 +/- 3 bpm (t (66.12) = -6.26, $p < .01$, n = 21), Assay # 2 at 248 +/- 4 bpm (t (68.82) = -5.50, $p < .01$, n = 21), Assay # 3 at 233 +/- 4 bpm (t (69.97) = -3.63, $p < .01$, n = 22), Assay # 4 at 231 +/- 3 bpm (t (63.68) = -3.67, $p < .01$, n = 29), and Assay # 5 at 234 +/- 5 bpm (t (74.98) = -3.63, $p < .01$, n = 27) (Fig. 3, significance denoted by *).

Increases in heart rate were found for all combination assays, when compared to heart rate in ethanol alone (Assay # 1 with t (29) =

16.38, $p < .01$; # 2 with $t(29) = 13.68$, $p < .01$; Assay # 3 with $t(30) = 11.93$, $p < .01$; Assay # 4 with $t(37) = 15.07$, $p < .01$, and Assay # 5 with $t(35) = 10.838$, $p < .01$ (Fig. 3, depicted by ^o).

When comparing each assay with its successive assay (Assay # 1 with # 2, Assay # 2 with # 3, Assay # 3 with # 4, and Assay # 4 with # 5), only Assay # 3 was observed to be significantly different from its preceding Assay # 2 ($t(41) = 2.39$, $p < .05$) (Fig. 3, denoted by [^]). Heart rate in aspartame was not different from any of Assays # 1-5 (Fig. 3).

Discussion

In the first experiments conducted (ethanol assay), a decrease in heart rate was observed with increasing ethanol concentrations demonstrating that ethanol has a depressive effect on heart rate. Heart rate was considered representative of the actions on the nervous system for the purpose of this experiment because the heart rate is regulated by the nervous system in *D. Magna*, and therefore changes in heart rate were used to assess changes in nervous system activity (Guyenet, 1990).

The second part of the experiment, in which *D. magna* were introduced to various concentrations of aspartame, resulted in an increase in heart rate as the concentration of aspartame solutions increased, confirming the stimulant effect of aspartame.

In the final part of the experiment, mixed assays of aspartame and ethanol were used to determine the combined effects of a stimulant and a depressant. The results of the mixed assay (Fig. 3) showed a marked increase of *D. magna* heart rate with even a minimal addition of aspartame to ethanol (Assay # 1). These findings suggest that the ingestion of aspartame has a greater impact on heart rate than alcohol. There was no systematic difference among Assays #1 – 5 or when comparing the assays with aspartame alone. This may indicate that any amount of aspartame in combination with alcohol has a significant influence on *D. magna* metabolism and heart rate. The difference between Assays # 2 and # 3 could

potentially be eliminated with further testing. The results obtained could be due to a novel metabolic pathway involved in the combined processing of ethanol and aspartame or due to the aspartame pathway that could partially override the depressive effect of alcohol.

The nervous system activity of *D. magna*, gauged by cardiac output, was greatly affected by the introduction of a stimulant, a depressant, and a combination of both. *D. magna*, has become an established model organism because they are easily cultured and have short reproductive cycles. While the main areas of focus on *D. magna* as a model organism lie in ecology and evolution, it has been shown that the crustacean exhibits about a 55% genetic homology with humans, suggesting it to be eligible as a model for some for human processes (Gilbert, 2007).

The experiments conducted were to understand the side effects and the mechanisms that underlie the process of how stimulants and depressants affect nervous system activity. Previous research on stimulants has found that they increase CNS or sympathetic nervous system activity with a spectrum of effects such as cardiovascular stimulation and increased energy levels (Rothman and Baumann, 2003). In the presence of stimulants, synaptic levels of monoamine neurotransmitters are elevated in neurons, specifically dopamine, serotonin, and norepinepherin (Sofuoglu and Sewell, 2008). Neurotransmitter concentrations increase as a result of blocking transporter-mediated reuptake inhibitors from the synapse (Rothman and Baumann, 2003). Excess levels of serotonin have been associated with cardiac and pulmonary disease, and stimulants such as amphetamine analogs act as substrates for the serotonin transporters, which release serotonin from platelets, in turn elevating plasma blood levels of the neurotransmitter (Zolkowska et al., 2006). Plasma serotonin stimulates mitogenic activity from cardiovascular cells (Zolkowska et al., 2006).

Depressants also affect CNS activity through different mechanisms. Ethanol, a depressant, enhances GABAergic synaptic inhibition by means of allosteric potentiation of postsynaptic GABA receptors (Ariwodola and Weiner, 2004). This mechanism plays a vital

role in the effects of ethanol, most notably on cognition. GABA receptors are responsible for mediating fast synaptic inhibition. Hippocampal GABA activity is potentiated by ethanol when there is a blockade of GABA B receptors (Ariwodola and Weiner, 2004). Ethanol was also found to increase GABAergic transmission into the ventral-tegmental area dopaminergic neurons, in which GABA inhibitory post synaptic currents are enhanced (Theile et al., 2008). The increase of overall GABA in the CNS produces a decrease in cardiovascular activity (Segura and Haywood, 1991).

Considering the large increase in *D. magna* heart rate in all five mixed assays, further investigation appears to be warranted aimed at identifying similar effects in larger organisms like mice, and eventually in humans. Examination of larger animals would allow controlling the amount of ingested alcohol and aspartame. Memory or behavior tasks could then be used to evaluate other effects of the mixture on nervous system functions. Furthermore, neurotransmitter levels in the brain of these animals could be measured. The results could shed light on the pathways involved in the metabolism of alcohol in conjunction with aspartame. We also suggest investigating the effect of aspartame doses that come closer to the maximum recommended amount of 40 – 50 mg/kg body weight/day.

Further research on the effects of aspartame and alcohol, similar to our experiment, should be particularly directed towards individuals who consume beverages containing aspartame and/ or alcohol. In one survey, two out of every five students were found to binge drink (Wechsler *et al.*, 1998) and research studies surveying the dietary habits of female college students (43%), found that artificial sweeteners (specifically aspartame) were frequently used to reduce weight gain in combination with other dietary methods (Malinauskas et al., 2006). Since both aspartame and alcohol are consumed on college campuses, the target population for future research could encompass college students and long-term or short-term effects of alcohol/aspartame consumption on the nervous system

Though our research was not conducted on humans, *D. magna* nervous system research

provides a basis for understanding the possible side effects of excessive consumption of aspartame in conjunction with alcohol. Human studies have shown that aspartame can cause migraines, seizures, depression and anxiety (Bradstock et al., 1986). Alcohol has been linked to depression, anxiety and cognitive deficits (Schuckit, 2009). Thus temporary or long-term nervous system alterations by alcohol and aspartame may cause serious health risks that have not yet been identified but could become manifest with increasing consumption of aspartame in the future.

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Corresponding Author

Cierra Jené Lewis
Ursinus College
rkohn@ursinus.edu
601 E Main Street
Collegeville, PA 19426

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