THE NEUROEXCITANT EFFECTS OF ASPARTAME: AN INVESTIGATION USING KINDLING

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The Neuroexcitant Effects of Aspartame: An Investigation Using Kindling.

BY

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A thesis submitted to the School of Graduate Studies in partial fulfillment of the requirements for the degree of Masters of Science

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Abstract

A great deal of controversial evidence has arisen regarding the artificial sweetener Aspartame (NutraSweet). This dipeptide is composed of two amino acids that have been shown to have detrimental effects on brain/behavior function (Olney and Ho, 1970; Nyhan, 1984; Wurtman, 1983B). Aspartame ingestion causes a significant increase in brain phenylalanine and tyrosine, and a decrease in brain tryptophan (Wurtman, 1983), which may change brain excitability (Racine and Coscina, 1979; Cadell, Harlow and Waisman, 1962). The purpose of the present study was to determine if aspartame consumption for a one week period changed brain excitability by reducing after-discharge thresholds as measured by the kindling technique. Animals were stereotactically implanted with one bipolar electrode in Area CA3 of the Hippocampus. After-discharges were obtained for all animals (Day 0). The groups were: Group 0, animals that consumed water, Group 200, animals that consumed 200mg/kg of aspartame in water, and Group 800, animals that consumed 800mg/kg of aspartame in water. After-discharge thresholds were obtained on Days 1 and 7 of liquid consumption. The mass kindling procedure began on the day following the end of the period of aspartame consumption. All animals were stimulated two or three times a day with the inter-stimulus interval being at least four hours. This was continued until three Stage-5 seizures were obtained. A generalized seizure triggering threshold (GST) was then obtained. The results show that there were no significant differences between food consumption, liquid consumption, weight gain or after-discharge thresholds between the groups. After-discharge thresholds were significantly different over days. A Newman-Kuels analysis comparing after-discharge thresholds for days showed Day0 > Day 1 > Day 7, and the ADT for Day 7 > GST. This was an expected finding since after-discharge thresholds do decrease with repeated stimulations (Racine, 1972A). However, brain excitability is not altered by aspartame consumption using the procedure employed in this study, nor was there any difference found in brain amino acid levels. Future studies should include a more detailed analysis on long-term aspartame consumption.

Key Words: Aspartame (APM), Kindling, Epilepsy, Phenylalanine (PHE), Aspartic Acid (ASP), Tyrosine (TYR), Tryptophan (TRP), Serotonin (5-HT), After-discharge Threshold (ADT), After-discharge (AD), Generalized Triggering Seizure Threshold (GST), Glutamate (GLU), Seizure.

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Introduction

Low calorie artificial sweetening agents appear to serve a useful social function. The availability of acceptable sweeteners has important therapeutic and psychological implications. A reduction in the intake of simple sugars is advocated for the prevention of dental cavities, prevention and treatment of obesity, and treatment of diabetes. Artificial sweetening agents provide a decrease in cavities, a decrease in carbohydrates for obese persons and a decrease in sugar levels in the blood for persons who are diabetic.

One of the most common artificial sweetening agents on the market today is Aspartame (NutraSweet). Aspartame (APM) is a dipeptide (L-aspartyl-L-phenylalanyl- methyl ester) with a sweetening power of 180 to 200 times that of sucrose in aqueous solution (Stegink, Filer and Baker, 1977) (see Figure 1). In the intestinal mucosa, APM is hydrolyzed into the two amino acids 40% aspartic acid (ASP), 50% phenylalanine (PHE), and methanol (Ranney, Oppermann, Muldoon and McMahon, 1976). The two amino acids are metabolized similarly to the amino acids arising from dietary protein. The sweet flavor in humans is a result of the aspartate molecule interacting with taste receptors on the tongue (Iwamura, 1980). However, in rats there is little evidence that APM is sweet or palatable (like sucrose) since no difference was found when APM or water consumption was measured (Sclafani and Abrams, 1986).

Questions regarding the safety of aspartame have arisen because of concern about the potential harmful effects of elevated levels of the amino acids, ASP and PHE, and the metabolite methanol and its by-product diketopiperazine.

The introduction will be sub-divided into the possible sub-sections that are affected or altered by the consumption of APM or the amino acids that compose APM.

```
                |                |
          O     |          O     |
  +       ||    |          ||    |
NH - CH - C -|- NH - CH - C -|- OCH
  3  |         |      |         |    3
     CH        |      CH        |
     | 2       |    //  2\\     |
     C. = O    |   HC     CH    |
     |-        |    |     |     |
     O         |   HC     CH    |
               |    \\    /     |
               |      CH        |
               |                |
  ASP          |     PHE        |    MEOH
```

Figure 1: A chemical configuration of the aspartame molecule, showing the position of the aspartic acid (ASP) and the methyl ester (MEOH) of phenylalanine (PHE).

Blood-Brain Barrier Effects.

Qne important source of potential harm is that ingestion of APM may alter brain levels of amino acids. The brain requires amino acids for the synthesis of peptides, proteins, and neurotransmitters, such as serotonin, dopamine, and norepinephrine. Amino acids that are not synthesized within the brain must be taken from the blood across the blood-brain barrier (BBB). In mammals the BBB is located at the endothelial cells of cerebral capillaries. The endothelial cells are joined by tight junctions and substrates must pass through two capillary membranes in order to reach brain extracellular fluid. Hence, transport through the BBB depends on lipid solubility or mediation of specific carriers in the capillary cell membranes (Pardridge, 1983). The transport mechanism is dependent upon the characteristics of the particular amino acid to be transported. There are three distinct carriers; one for neutral amino acids, one for basic amino acids, and one for dicarboxylic amino acids. Most of the amino acids enter the brain from blood by systems which show stereospecificity, competitive inhibition and saturation, that follow Michaelis-Menton saturation kinetics (Pardridge and Oldendorf, 1975). Neutral amino acid uptake into the brain is via a single facilitated system and is uniquely sensitive to competitive inhibition. Competition between many neutral amino acids for a common carrier system is an important aspect of the regulation of the amino acids that are available in the brain. All large neutral amino acids are assumed to compete for the same active carrier-mediated transport system located at the BBB (Fernstrom, 1981). Thus, if large imbalances in the plasma concentration of one or several neutral amino acids occur large changes in transport across the BBB and brain concentrations of all competing amino acids change. For example, in the disease Phenylketonuria (PKU). PHE cannot be converted to tyrosine (TYR) (a large neutral amino acid) in the liver, leaving an excessive concentration of PHE in the blood supply. When this large neutral amino acid reaches the BBB its concentration in the blood is

greater and therefore it has more molecules available to compete for carrier sites, hence causing an elevated PHE brain level. The metabolites that accumulate in PKU individuals (phenylactic acid and phenylacetic acid) inhibit 5-hydroxytryptophan decarboxylase (Nyhan, 1984) causing a decrease in 5-HT. Hence, in PKU children the levels of other amino acids (not including PHE) and neurotransmitters are decreased (Heuther, Schott, Sprotte, Thoemke and Neuhoff, 1984). The concentration in the brain of some amino acids are directly dependent on plasma concentrations while others are not. Fernstrom and Wurtman (1972) showed that brain TYR uptake and brain 5-HT levels were directly proportional to the ratio of the plasma concentration of TYR to the sum of the large neutral amino acids that compete with TYR for transport across the BBB. Wurtman, Larin, Mostafapour and Fernstrom (1974) have also shown that brain catecholamine formation is directly dependent on brain TYR concentration. The concentration of TYR in brain is itself directly proportional to the ratio of TYR to large neutral amino acids in plasma. The dependence of PHE and TYR levels in brain on their levels in blood has been shown many times. It was confused by Peng, Gubin, Harper, Vavich and Kemmerer (1973) who showed that with a high PHE diet the levels of leucine, isoleucine, methionine and histidine in brain fell while in blood they did not change. This was in agreement with Kennett, Curzon, Hunt and Patal (1986) who have shown that the amino acid levels in the blood and the amino acid levels in the brain appear to be partly independent. They showed that some amino acid levels in the blood can decrease while the amino acid levels in the brain remain stable or even increase. This demonstrates that some amino acids may very well be independent in their concentrations in blood versus brain, whereas others are not. Although the relationship of plasma/brain concentrations are not simple, the literature on amino acids show a consistent elevation in brain if plasma PHE is elevated. PHE has a powerful effect.

In a non-PKU individual, consumption of dietary PHE in the usual way, through protein ingestion, does not elevate plasma or brain levels of PHE any

more than other amino acids (Yokogoshi, Roberts, Caballero, and Wurtman, 1984). Amino acids are abundant in the plasma, and only the branched-chain amino acids, unlike PHE, are largely unmetabolized when they exit through the portal circulation. However, when PHE is consumed in the form of APM (34mg/kg of body weight) plasma PHE rises threefold above the normal rise in the other large neutral amino acids (Stegink, Filer, Baker, and McDonnell, 1980). This elevation in plasma PHE causes a proportional rise in brain PHE and decrease in the other large neutral amino acids in brain. Maher and Wurtman (1987) stated that APM is probably the only PHE-containing food that elevates the ratio of the concentration of PHE to the summed concentrations of the other large neutral amino acids.

Brain amino acid imbalances caused by competition at the BBB are important when studying the pathogenesis of genetic hyperphenylalaninemia or PKU (Choi and Pardridge, 1986). These problems (hyperphenylalaninemia or PKU) gain even more significance with the global use of APM. The postprandial hyperphenylalaninemia that is associated with APM ingestion causes a selective saturation of the neutral amino acid transport system at the BBB with PHE (Pardridge, 1986).

An exhaustive investigation of APM's effects on brain amino acids and neurotransmitter functions is warranted.

Serotonin Modulation Effects.

Brain neurotransmitters change as a result of food consumption. Two examples of these are catecholamines and serotonin. The rates at which the rate-limiting enzymes tyrosine hydroxylase and tryptophan hydroxylase convert TYR to l-dopa and tryptophan (TRP) to serotonin, respectively, can similarly be modulated by treatments that change brain levels of TYR and TRP. Ingestion of either pure TYR, APM or carbohydrates can increase brain TYR levels (Wurtman, 1983b).

The synthesis of serotonin (5-HT) begins in neurons by the hydroxylation of the essential amino acid TRP. Substances that can raise or lower the level of TRP in brain can rapidly change the rate at which TRP is hydroxylated and converted to 5-HT. Despite the fact that tryptophan hydroxylase is the rate-limiting step for 5-HT synthesis, 5-HT changes upon TRP availability. One example of this is the consumption of a high-carbohydrate, protein-poor meal. This meal elevates brain TRP levels and accelerates 5-HT synthesis (Fernstrom and Wurtman, 1971). When 3g/kg of glucose and 200mg/kg of APM was intubated into rats, the APM-carbohydrate combination doubled the PHE concentration in brain, increased the TYR level and suppressed by half the physiological increase in brain TRP that would normally accompany a carbohydrate-rich meal. APM also blocked the normal increase in brain 5-HT and 5-hydroxyindoleacetic acid that is produced by a carbohydrate meal (Wurtman, 1983B). Wurtman suggested that the carbohydrates alone caused an insulin-mediated decrease in plasma concentrations of the amino acids that compete with PHE and TYR for transport across the BBB (Wurtman, 1983B). If this decrease in the levels of the other plasma large neutral amino acids occurs, uptake of TRP into the brain is facilitated and brain 5-HT is elevated. TRP, TYR, PHE and other large neutral amino acids compete with one another for transport across the blood-brain barrier. However, if APM is the main carbohydrate substitute then two things should be expected to happen; brain TRP should decrease, and circulating TYR and PHE should be elevated, due to the high concentration of PHE in APM. When the rat consumes one test load of APM in water (200mg/kg), TYR and PHE are significantly increased (the means are 139.7 $\pm$ 8.4nmol/g and 75.5 $\pm$ 2.3nmol/g, respectively), as compared to water intake (54.4 $\pm$ 2.7nmol/g and 39.3 $\pm$ 0.9nmol/g respectively), and TRP and 5-HT (20.3 $\pm$ 0.6nmol/g and 484 $\pm$ 15nmol/g, respectively) remain unaltered in the rat brain (Wurtman, 1983b). Fernstrom, Fernstrom and Gillis (1983) showed that brain levels of TRP significantly decreased and brain levels of TYR and PHE significantly increased 30min and 60min after intubation of 200mg/kg of APM administration. However, the TRP changes were less reliable than the changes in the levels of PHE and TYR.

The rate of 5-HT synthesis in the mammalian brain is extremely sensitive to TRP supply. A chronic lowering of brain TRP causes a lowering of 5-HT hence there appears to be no long term compensatory mechanism for this neurotransmitter (Fernstrom, 1986). Therefore, any change in brain TRP levels that may result from chronic APM usage will cause a chronic change in 5-HT availability. De la Torre, Kawanaga and Mullon (1970) suggested that the brain 5-HT system plays an important role in epileptiform seizure activity. Hence an alteration in this system may actually cause a change in seizure activity. Racine and Coscina (1979) demonstrated that a decrease in 5-HT can increase seizure susceptibility. They found that the after-discharge thresholds were decreased in the amygdala but not the neocortex after raphe lesioning. These rats also required fewer amygdaloid stimulations to develop generalized convulsions. Hence, there appears to be an inhibitory role for 5-HT in kindling. Therefore, a decrease in 5-HT as a result of APM consumption may make brain tissue more excitable and this excitability would be even more pronounced in children or young rats as a result of their relatively immature BBB.

In agreement with the effects of 5-HT modulation, Yuwiler and Louttit (1961) showed that if hooded rats received a daily supplement of 5g/kg (of body weight) of l-PHE the mean serotonin content in plasma decreased significantly as compared to non-supplemented rats. However, it was noted that the animals that were fed the l-PHE diet ate less food than the non-supplemented animals. A second study by Yuwiler and Louttit (1961), in which the animals were supplemented with 2.8g/kg (of body weight)of l-PHE, showed no significant decrease in serotonin concentration or in the amount of food ingested. Truscott (1975) demonstrated that PHE and 5-HT show an inverse (almost linear) relationship regarding seizure severity in mice. He devised a 3 x 3 design where he used three levels of 5-hydroxytryptophan (a 5-HT precursor) (0mg, 0.75mg and 1.50mg) and three levels of l-PHE (0%, 1% and 2% of dietary PHE supplement). It was found that as the levels of l-PHE increased so did the severity of the audiogenic seizures. When the highest doses of both chemicals were used the

severity of the seizure was minimal. The high dose of 5-hydroxytryptophan paired with the low l-PHE dose also showed a minimal seizure. However, when the animal received the lowest 5-HT dose and the highest l-PHE dose the seizure reached a maximum on the severity scale. This demonstrates that high doses of 5-hydroxytryptophan appear to offer protection against audiogenic seizures. The seizure severity appears to be inversely related to the 5-hydroxytryptophan dosage, and as l-PHE increases and 5-hydroxytryptophan decreases the seizure becomes more severe.

Phenylalanine Modulation Effects.

PHE can also have a dramatic effect on plasma and brain levels of amino acids and neurotransmitters. When 200mg/kg of APM was administered to rats by a stomach tube, a two-fold increase in brain PHE levels occurred (Wurtman, 1983b). This effect was doubled if the rats received both APM (20mg/kg) and glucose (3g/kg). The APM alone completely blocked the normal rise in brain 5-HT produced by carbohydrate consumption and this reduction was greatly facilitated by glucose.

On the other hand, Stegink, Filer, Baker and McDonnell (1979) found that the maximum plasma and erythrocyte PHE levels following ingestion of 34mg/kg of APM in orange juice by adults and adult PKU heterozygotes were slightly above values noted postprandially in the infant and adult. Koch, Schaeffer and Shaw (1976) showed that if 34mg/kg (of body weight) of APM or the equivalent l- PHE was given to PKU homozygotes and normal children in one test day the serum PHE level was significantly increased for those with PKU. The adult PKU patient appears to be able to metabolize the dose of APM given here with no apparent problem over a day period. Caballero, Mahon, Rohr, Levy and Wurtman (1986) showed that humans ingesting relatively low levels of APM (10mg/kg) showed a significant increase in plasma PHE 1hr after ingestion. This pattern was also seen in PKU carriers. They also found that the ratio of PHE/large neutral amino acids

was significantly increased in these two groups. This elevation in plasma PHE/LNAA ratio potentiates the effects of APM on brain PHE levels. Thus large imbalances in the plasma concentration of PHE will lead to marked changes in transport and brain concentrations of all competing amino acids. The problem with the above studies is that they only used one test load which may not be generalizable since alterations in plasma amino acid levels may have been an immediate reaction instead of what one expects to see in the long-term. An individual would not consume this dosage in one sitting in the real world, nor would any equilibrium mechanisms have come in to play to return conditions to normal with the time frame between ingestion and measurement in these one test load studies. The possibility of a cumulative effect of APM after repeated, habitual, exposure has been explored by Matalon, Michals, Sullivan and Levy (1987). They found that normal adults ingesting APM (50mg/kg or 100mg/kg) for 12 weeks showed significantly higher urinary and blood concentration of PHE metabolites as compared to their baseline levels. The PKU heterozygotes also had significantly higher blood and urine concentration of the PHE metabolites as compared to their own baseline level as well as to the levels obtained from the normals upon repeated exposure to APM. Hence habitual use of APM does cause an elevation in PHE that may cause a prolonged decrease in brain 5-HT.

At an unknown level the PHE concentration in the brain can become toxic, as happens with PKU (Wurtman, 1983b). The toxic level in serum is taken to be 20mg/100ml. Cadell, Harlow and Waisman, (1962) raised monkeys from birth on a l-PHE rich diet and since the animals exhibited the same symptoms as a PKU child, they were called PKU monkeys. Hence, even though the animals apparently had the liver enzyme at birth the high level of PHE appeared to either inactivate the enzyme or make it less efficient so that PKU was induced. Forty percent of the PKU animals exhibited grand mal convulsions and this suggests that constant exposure to PHE may induce epileptic seizures. Untreated PKU is also correlated with intelligence (Fishler, Azen, Henderson and Friedman, 1987). They compared 33 PKU children who from birth had been maintained on a restricted PHE diet

for ten years with 72 PKU children who discontinued their restricted PHE diet after six years. These groups were compared on standardized tests of intelligence, language abilities, school achievement and perceptual skills. They found that the intelligence, spelling and reading scores were higher for children maintained on a PHE-restricted diet. This provides evidence to caution against the use of APM in specific populations such as PKU children and PKU heterozygote pregnant women. However, many pregnant women do not know if they are PKU heterozygotes, therefore all pregnant women should shy away from foods that are documented to possess an excess of PHE. The chances of having a baby with induced PKU may be increased since the fetus may be exposed to abnormally high levels of PHE.

The above evidence illustrates that APM intake affects plasma and erythrocyte levels of amino acids and neurotransmitter levels. These effects on neurotransmitter levels could cause an alteration in the excitability of neurons in the CNS.

Aspartic Acid

Olney (1978) hypothesized that the excitatory (depolarization) mechanism underlies the neurotoxicity of amino acid excitants, and that the toxic action is mediated through Glutamate (GLU) or ASP excitatory receptors on the dendrosomal surfaces of central neurons. These amino acids enter the brain rather slowly and even though they are metabolized very rapidly, competition and self-inhibition can still be demonstrated by sensitive techniques. GLU or ASP are effective in destroying the circumventricular organs (CVO) in either infant mice or rats if a dose as low as 0.5mg/kg is ingested. For example, Perez and Olney (1972) found that GLU or ASP administered systemically to 4-day old mice resulted in a significant influx of amino acids into specific brain regions, particularly the CVO. The CVO, even in an adult, are areas in which the BBB is relatively more permeable than in other areas, however, they are even more

permeable in the infant. The CVO include the organum vasculosum of the lamina terminalis, the median eminence, the subfornical organ, and the area postrema. It is suggested that the BBB in the immature rat brain is very permeable and allows some chemicals to pass through it. As the BBB matures these chemicals are excluded. Apparently, these chemicals only circulate in the CVO. However, GLU and ASP may attain hyperosmolar concentrations in the blood, thereby damaging the BBB. If this occurs toxic levels of GLU and ASP may enter additional brain regions. Several excitatory amino acids, including L-glutamate, N-methyl-D-aspartate, and L-ASP, produce convulsions in mammals, providing that the blood-brain barrier is relatively inefficient, as in immature animals (Cheema, Malathi, Padamanaban, and Sarma, 1969), or by-passed by the route of administration (Crawford, 1963). Johnston (1973) showed that the above excitant amino acids produced convulsions in 10-day old rats, when injected intraperitoneally. These convulsions were characterized by repeated tonic seizures, involving all limbs, head and tail. Convulsions lasted several minutes. The time of onset of seizures was dose dependent and was used to compare the relative potencies of the excitatory amino acids. This study suggested that the convulsions in 10-day-old rats were directly related to the neuroexcitatory properties of these amino acids. The view taken by Johnston (1973) was that the convulsant and excitant activities of these acidic amino acids have the same basic mechanism, i.e. the direct depolarization of central neurons. Weitz, Merlob, Amir and Reisner (1981) suggested that increased brain aspartate levels may have an epileptogenic effect in newborn humans. They found elevated ASP levels in cerebral spinal fluid in an infant who had intractable seizures.

Although young animals are more vulnerable to ASP-induced brain damage, this damage also occurs in adult animals (Olney, 1976). Price, Pusateri, Crow, Buchsbaum, Olney and Lowry (1984) treated adult mice intraperitoneally with ASP at one of several doses (0.47 to 3.75mmol/kg) and 30min later gave the same dose of ASP subcutaneously. They found that, in the CVO regions of the brain the ASP levels were 1.5 and 3.0 times above the levels in untreated animals,

following the lowest and highest doses, respectively. The tissue ASP levels remained unchanged in non-CVO brain regions.

Millan, Meldrum and Faingold (1986) found that a source of ASP (N-methyl-D-aspartate (NMDA)), when injected into the inferior colliculus, causes an increase in audiogenic seizures as measured by the animals response to high intensity acoustic stimulation. This study showed that high levels of ASP (in the form of NMDA) were responsible for the increased susceptibility of an animal to have a seizure. NMDA is also a potent convulsant when injected into the rat hippocampus, amygdala, cortex and cerebral ventricles (Turski, Meldrum and Collins, 1985; Zaczek and Coyle, 1982). However, when NMDA is injected into the ventral mid-striatum it prevents amygdala kindled seizures in rats. Since APM is a source of ASP it is important to determine if APM has any neural excitotoxic effects. It has been argued that GLU and ASP are safe food additives because humans have been exposed to these compounds in one form or another for many years without sustaining apparent harm. However, GLU and ASP-induced brain damage is a silent phenomenon and neuronal degeneration may be quite extensive before behavioral patterns are disrupted (Olney, 1978).

Moreover, approval of unlimited APM use in children may not be justified. Olney (1974) found that when children ingested GLU and APM they had a five to six fold increase in plasma levels of these two chemicals. Stegink, Shepherd, Brummel and Murray (1974) have estimated that a six to ten fold elevation of plasma GLU + APM is required to destroy neurons in the infant mouse hypothalamus. High doses of both ASP or GLU (1mg/gm of body weight) are capable of inducing retinal and hypothalamic damage in fetal and neonatal mice (Olney and Ho, 1970). GLU and APM appear to have an additive effect so that only half the dose (0.5mg/gm ASP + 0.5mg/gm GLU of body weight) produces the same hypothalamic and retinal damage. In agreement with the above study Daabees, Finkelstein, Stegink and Applebaum (1985) found that mice receiving 250mg/kg (of body weight) of both ASP or GLU developed neuronal necrosis and

as the doses of both chemicals increased separately so did the severity of the necrosis.

The administration of large amounts of ASP and/or GLU to neonatal rodents has unequivocally been shown to produce elevated plasma ASP and GLU concentrations and hypothalamic neuronal necrosis (as seen above). However, the ability of these amino acids to produce neuronal necrosis in the nonhuman primate is controversial. Olney and Sharp (1969) and Olney and Feigin (1972) reported neuronal necrosis in infant nonhuman primates given large doses of GLU. Contrary to this, Reynolds, Stegink, Filer and Renn (1980) found that infant monkeys receiving 2gm/kg (of body weight) of APM or 2gm/kg (of body weight) of APM plus 1gm/kg (of body weight) of MSG (monosodium glutamate - a glutamate source) developed no hypothalamic damage. Reynolds, Lemkey-Johnston, Filer and Pitkin (1971), Stegink, Reynolds, Filer, Pitkin, Boaz and Brummel (1975) and Heyward and Worden (1979) did not find neuronal necrosis even when plasma ASP and GLU concentrations may have been greatly elevated. These findings suggest that there is a definite species difference. Hypothalamic damage is not predictable in nonhuman primates as it is in rodents. Given these results the development of neuronal necrosis depends on the animal model being utilized.

Aspartame and Seizures.

As shown above, elevations of PHE and ASP (components of APM) and lowering 5-HT increases the probability of seizures. Wurtman (1985) showed that diminished brain levels of catecholamines were associated with lower seizure thresholds in the genetically epilepsy-prone rats used by Jobe, Ko and Dailey (1984). This suggests that exposure to large amounts of APM may ultimately decrease brain levels of norepinephrine and adversely affect some people predisposed to seizures. Convulsions were observed in a study in which monkeys were fed 3.6g/kg of APM for 30 weeks (Health and Welfare Canada, 1981).

However, one criticism of this study was that animals were not given free access to water. Therefore, it may not have been the APM that induced the seizure but plasma hyperosmolarity, produced by dehydration, that may have caused the seizures and alterations in brain electrical activity. In a similar study by Reynolds, Bauman, Stegink, Filer and Naidu (1984), monkeys received levels of APM up to 4g/kg for 270 days. Water was continuously available. No convulsions were observed in this study. Reynolds et al (1984) concluded that convulsions observed in the monkeys in the previous study were due to hyperosmolarity and/or hyperaminoacidemia induced by forced feeding.

Pinto and Maher (1986) have found that doses of 250, 500, and 750mg/kg of APM administered orally 1hr before pentylenetetrazole (PTZ) was subcutaneously administered to mice caused no change in the PTZ CD50 (concentration able to induce seizures 50% of the time). However, at 1000, 1500 and 2000mg/kg of APM the PTZ CD50 was significantly lowered. Seizures occurred in 75, 81 and 100%, respectively. This study demonstrates that APM does alter brain excitability. The effects of a high concentration of APM, in plasma, on mice are unknown. In rats the dosage required to produce equivalent plasma concentrations to humans (a dosage of 34mg/kg as defined as the 99 percentile daily human dose (Hattan, Henry, Montgomery, Bleiberg, Rulis and Bolger, 1983)) is 200mg/kg of APM (Stegnik, Koch, Blaskovics, Filer, Baker and McDonnell, 1981). More recent literature (Wurtman and Maher, 1988) indicated that 500mg/kg (of body weight) of APM in rats may be required to produce the same effect in plasma as 34mg/kg (of body weight) of APM does in humans. All APM doses that have been tested in humans raise plasma PHE relative to TYR. The PHE/TYR ratio in plasma of rodents is different to that of humans, following PHE ingestion, because hepatic PHE hydroxylase is more active in rodents. Thus there is no simple comparison for dosages of PHE between rodents and humans because equivalent PHE levels in plasma yield unequal PHE/TYR ratios. Hence, it may be more reasonable to determine APM doses that raise plasma PHE in humans to that equivalent dose that raises the plasma in rats. Therefore, the dose used by Pinto and Maher

(1986) may or may not have been reasonable and a more comprehensive analysis of plasma levels between species needs to be completed. A more sensitive form of seizure threshold determination may also be required. Nevins, Arnolde and Haigler (1987) found that APM at doses of 50 or 500mg/kg administered by gavage 0.5, 1, or 2hrs before PTZ-induced seizures or ECS in mice produced no effect on threshold. The threshold was measured by the presence or absence of clonus (PTZ) or tonus (ECS) that was recorded and the percent of mice having seizures in each group was calculated. The difference between the two studies indicates a possible dosage difference and if Wurtman and Mahers (1988) argument is correct than the dosage used in the Nevins, Arnolde and Haigler (1987) study was definitely too small.

High APM doses were found to potentiate seizures in rodents, whether the seizures were evoked by drugs, electroshock, or inhalation of fluorothyl (Maher and Wurtman, 1987). They gave animals doses of APM varying from 250mg/kg to 2000mg/kg one hour before animals were given the CD50 dose of PTZ. They found that as the dose of APM increased the number of animals that had seizures increased (50% to 100%, respectively for the above doses). They then gave another group of animals 1000mg/kg of APM and changed the dose of PTZ. They found that a lower dose of PTZ was required to produce seizures in APM animals. This enhanced susceptibility to PTZ-induced seizures was also seen among mice pretreated with an equimolar dose of PHE before receiving PTZ. When animals were given APM and valine (large neutral amino acid that competes with PHE at the BBB) the animals appeared to be protected from seizures. Therefore, we can conclude that PHE from the APM appears to have a seizure inducing ability when consumed in large amounts.

There have also been a number of APM-suggested medical complaints from the consumer. In 1984 the Center for Disease Control presented a paper that represented over 200 people. The complaints were all very subjective and diverse. They were: 67% reported neurological and behavioral symptoms including

dizziness, headaches and mood alterations, 24% reported gastrointestinal symptoms, 15% reported allergic type and/or dermatological symptoms, 6% reported changes in menstrual patterns, and 9% reported other symptoms. The Center for Disease Control concluded that these symptoms were not related to APM consumption. Bradstock, Serdula, Marks, Barnard, Crane, Remington and Trowbridge (1986) also found that people reported a number of complaints with regard to APM usage. They found that out of 517 reports, 67% had neurological/behavioral problems, 26% had gastrointestinal problems, 17% reported allergic reactions, and 6% reported menstrual problems. They also reported that in general these symptoms were unrelated to APM consumption but they do state that some specific subjects' symptoms appear to be related to APM consumption in commonly consumed amounts. Walton (1986) looked at a case study of a 54 year old woman who had no previous history of seizures. This woman had a seizure and manic episodes upon consumption of large amounts of APM. When the APM was eliminated from her diet so were the manic episodes and no more seizures were triggered. A child developed unexplained seizures and it was found that the mother had weaned her child from milk to diet Kool-Aid (Wurtman, 1987). Even though these cases are subjective and may be implicating the wrong product it is suggestive evidence.

In summary, the evidence indicates that the metabolic products of APM can alter CNS excitability and may induce convulsions. These data raise two very important issues that have not yet been approached in the literature on APM. The first is what are the effects of APM on brain excitability. The second is what are the effects of APM on persons who are predisposed to epilepsy. The first question will be dealt with in this study.

Kindling.

Kindling is a model of the development of epilepsy (Goddard, McIntyre and Leech, 1969). This is a procedure where low intensity stimulations, repeated daily,

to certain brain structures causes a progression of the epileptiform after-discharge (AD). The initial stimulation discharge, if it exceeds the after-discharge threshold (ADT), continues for a duration of 10s (Racine and Burnham, 1984), and the behavioral characteristic that accompanies this is behavioral arrest. With repeated stimulation, the AD increases in amplitude. As stimulations continue the amplitude of the wave increases, the duration of the AD increases three to ten fold and the wave form increases in complexity. Kindling progresses behaviorally, as well as physiologically. Physiologically, excitability can only be altered by inducing current intense enough to evoke an AD. It is the production of AD's that makes the process of kindling possible. Animals undergoing kindling proceed through five behavioral stages: Stage-1, mouth movement (chewing and smacking) began to appear, Stage-2, mouth movements and head clonus, Stage-3, mouth movement, head clonus and forelimb clonus, Stage-4, mouth movement, head and forelimb clonus, and rearing, and Stage-5, total loss of postural control (Racine, 1972B). These progressive stages of behaviors are comparable to complex-partial epilepsy (chewing, head-nodding) to those seen in temporal (limbic) lobe epilepsy (overt tonic-clonic seizures) (Stage-5 seizure as defined by Racine (1972A)). These changes are permanent (Goddard, McIntyre and Leech, 1969).

It is not the stimulation itself that causes kindling but the production of AD's. ADT's can be lowered in the hippocampus and amygdala by the daily stimulation that is delivered to these areas to produce kindling (Racine, 1972A). The area of interest in this study is area CA3 of the hippocampus and is one of the areas in the limbic system where temporal lobe epilepsy originates (Girgis, 1982).

Kindling itself alters brain tissue excitability. Susceptibility to kindling is a reflection of tissue excitability. Therefore, if brain tissue excitability is increased, kindling should be enhanced. ADT may be used to assess local convulsive excitability, the effects of drugs on this excitability may be measured either with respect to acute drug effects or with respect to rate of change of ADT with repeated stimulation. Thus kindling is a useful procedure for studying brain excitability.

Present Study.

The amount of water each animal consumes daily was previously determined to be approximately 45-50ml, hence animals will be given 55ml. Animals in the present study will have free access to their drinking bottle with "sweetened" water (55ml) or plain water (55ml) in it. This method of free access to a liquid was chosen because it provides the better analogy to a human consuming APM at their leisure. We want to mimic the effects of APM usage in humans to rats. Humans have access to a liquid the same as the rodents in this study do.

Most of the research on the APM issue uses the method of gavage. This is a good method if one wants to determine the immediate effects of a high dose of a chemical. However, when studying a daily consumed chemical that virtually has replaced carbohydrates in many households it is more beneficial and advantageous to have free access. This method eliminates the stress, both physical and mental, associated with gavage, and it mimics the constant alteration of blood amino acid levels that accompany the many APM ingestion sessions during the day for a human. Furthermore, the effect of a dose of relatively high concentration at one time in the day may be different from the same concentration ingested during the normal drinking schedule.

There are also many different protocols that could have been implemented for the present study. Of the many two will be discussed. In the first one ADT is assessed on day 0, day 1 (first day of APM ingestion) and day 7 (last day of APM ingestion). Starting on day 8 the animals are stimulated two to three times a day until they are kindled. The second design would be identical with the exception that APM would have been continued until the animal was kindled. These two designs are very different in terms of the issues they address. Design 1 addresses the issue of a permanent or long-term alteration induced by APM after APM had been discontinued. Hence, are there any permanent consequences as a result of

previous APM ingestion? Design 2 answers the question about effects during usage of APM. The hypothesis being tested in the present study is does APM ingestion cause a permanent effect in brain excitability? hence the method of choice here was design 1. If one wanted to determine if APM causes a transient change in excitability by using design 2 you would still have to use design 1 to determine if this excitability alteration is permanent or temporary.

The kindling method is a sensitive indicator of brain excitability and is the preferred method implemented when determining seizure thresholds.

The present study used three groups of animals; 0 group (animals received water), 200 group (animals received 200mg/kg (of body weight) of APM in water) and 800 group (animals received 800mg/kg (of body weight) of APM in water). All animals received the specified liquid for seven days. The dose of 200mg/kg (of body weight) of APM was chosen because if it is the PHE and TYR sum that is important then this dose is sufficient to produce an equivalent PHE elevation in plasma in rats if humans consume 34mg/kg (of body weight) of APM. However, if it is the ratio of PHE to the large neutral amino acids that is important in both humans and rodents then a dose of 500mg/kg (of body weight) of APM or more is required to increase PHE substantially more than TYR in rat plasma. Hence, 800mg/kg (of body weight) of APM was chosen. If there was a permanent change in brain excitability at 800mg/kg (of body weight) of APM (as determined by ADT assessments and rate of kindling) but not at the 200mg/kg (of body weight) of APM level then the critical dose could be determined. If there was no effect at either dose, as compared to the water group, then one can say that even though rodent plasma "equilibrates" humans in terms of the amino acids important here, APM does not change ADT nor does it cause a faster rate of kindling.

Purpose.

Sufficient evidence indicates that APM alters plasma and/or brain levels of

particular amino acids (PHE, TRY, ASP and TYR). All of these amino acids are directly or indirectly related to seizures as a result of their abundance and/or reduction due to APM ingestion.

The purpose of the present study was two-fold: 1. Are there any permanent effects of APM that cause animals to kindle faster even though APM consumption has ceased? and, 2. Do APM animals have immediate, or sustained lower ADT's? In other words, does APM alter local epileptic excitability and does prior ingestion of APM affect the subsequent development of a seizure disorder.

It was hypothesized that ADT would decrease as a result of APM consumption and that the animals receiving the higher dose of APM would kindle at a faster rate.

Method

Subjects. Fifty-eight male Long-Evans Hooded rats (250-260g) were obtained from Charles River Canada and were housed in the Animal Care Unit at Memorial University of Newfoundland. All animals were housed in groups of three per cage until two days prior to surgery when they were separated and placed in individual cages. Their room was maintained on a shifted 12-hr light, 12-hr dark schedule (i.e. dark period at 2:00 P.M. to 2:00 A.M.). All rats were implanted with one bipolar stimulating/recording electrode in area CA3 of the hippocampus. The rats were randomly assigned into three groups on Day 0 prior to ADT determination. The groups were: 0 Group (animals received only water to drink), 200 Group (animals received 200mg/kg (of body weight) in water to drink) and 800 Group (animals received 800mg/kg of APM in water). Dosages were determined by making up 200 or 800 mg/kg (of body weight) of APM in 55ml of water individually for the animals. Each animal was well adapted to the testing environment before starting the experimental manipulation. During this adaptation procedure the amount of water that any rat consumed was recorded. This was done to determine the general amount of liquid to give the animals. All animals were given free access to food and a liquid throughout the experiment. The amount of food and liquid consumed during the experimental procedure was recorded daily to determine if there were any consumption differences.

There was a large attrition factor in this experiment. During the surgery one animal died. Seven animals died due to a gastro-intestinal illness that was totally unrelated to the experiment (as determined by an autopsy), eleven animals were sacrificed due to displaced caps (as a result of the waiting period after the gastro-intestinal illness to ensure no sickness was introduced into the experiment and alter any variables), one animal was kindled in the 800mg/kg Group although there was no ADT found for Day 1. Finally, five animals could not be kindled. Four of these animals had incorrect placements and one had the correct placement but did not kindle.

Apparatus. There was one bipolar stimulating/recording electrode for each animal. The stimulation administered and the recordings obtained were also bipolar. The two tips of the bipolar electrode were separated at a distance of 0.25mm. All electrodes were teflon coated except for the tip. The electrode assembly and the anchor screws to keep the electrode in place were held by dental acrylic applied to the screws and the skull.

The animal's electrode was attached to a Lafayette 60 Hz sine wave stimulator (model no: 22408) which was connected to a 12V power supply that was used to provide power to the switching box. Electrodes were disconnected from a Beckman R411 Dynograph during stimulation by the switching box. Electrodes were reconnected by a changing of the internal switch of the switching box to enable recording of the electroencephalograph. The stimulus was delivered by a footpad that was also connected to the switching box and the stimulator. The switching box was in turn connected to a Beckman R411 Dynograph (ink-writing polygraph) where a permanent copy of brain activity was recorded for later analysis (see Figure 2). The filter setting of the dynograph was $\geq$ 30DC, the amplifier was set at 1mV/cm and the pre-amplifier was adjusted for animals.

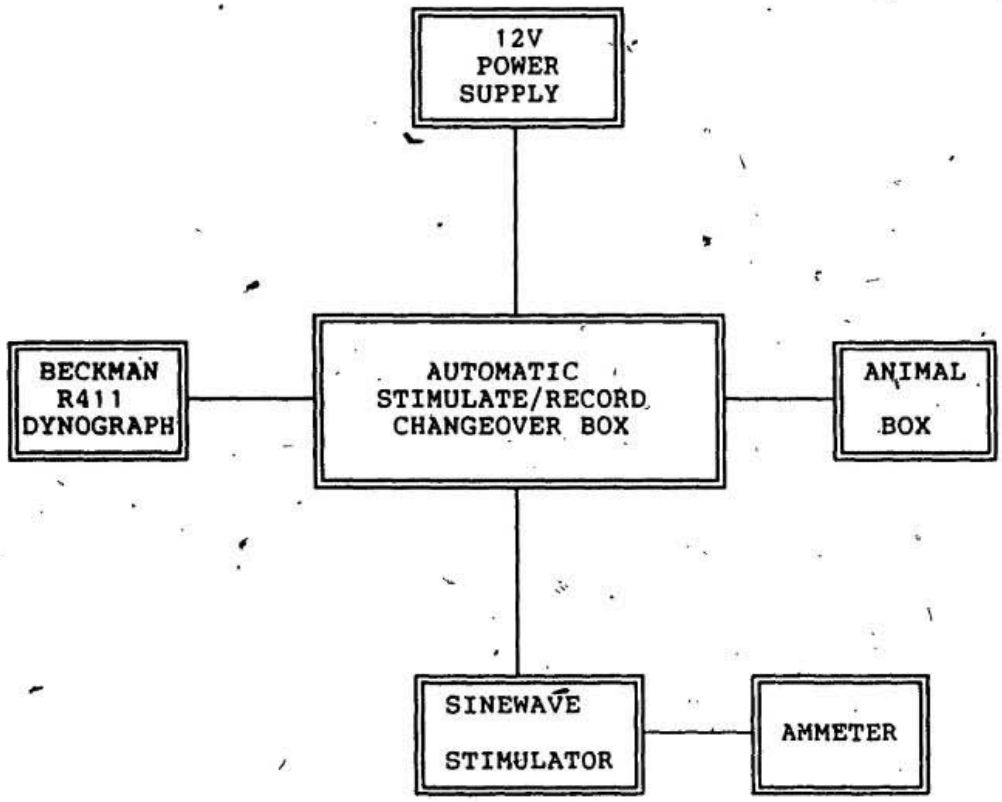


FIGURE 2: A block diagram of the apparatus used in this experiment.

Procedure.

Stereotactic Brain Surgery. The 58 animals were anesthetized i.p. by Avertin (1ml/100g). The bipolar electrode was stereotactically implanted in each animal (under aseptic conditions). The electrodes were aimed at the CA3 area of the hippocampus with coordinates at 3.8mm D-V (from skull), 2.8mm posterior (from bregma), and 2.8mm lateral (from midline) (Kairiss, Racine and Smith, 1984). Three skull screws were necessary to anchor the acrylic head assembly.

Ingestion Procedure. During the rats' recuperation from surgery the amount of water each animal drank each day was recorded for five days. This was done to determine the amount of liquid that had to be made-up. The rats drank an average of 45-50ml per day. Therefore, each rat was given 55ml of liquid per day so that any effect on seizure threshold could not be attributed to dehydration. All rats drank through a vertical drinking tube with ball-bearings at the end of the spigot to minimize spillage. All rats were randomly assigned to groups on Day 0 and were given free access to a liquid through-out the manipulation: 8 animals received water , 13 animals received 200mg/kg of APM in water, and 12 animals received 800mg/kg of APM in water. Animals received a fresh liquid at 11:00 A.M. every morning for seven days. The APM dose was determined individually for each animal by obtaining that particular animal's weight and multiplying it by the concentration of APM used for that animal's group. The dose of 200mg/kg (of body weight) of APM was chosen because it produces the equivalent plasma amino acid ratio in rats (Hatten, Henry, Montgomery, Bleiberg, Rulis and Bolger, 1983) as does 34mg/kg (of body weight) of APM in humans (Stegnik, Koch, Blaskovics, Filer, Baker and McDonnell, 1981). A very high dose of APM (800mg/kg (of body weight)) was used to determine if there was any effect on seizure thresholds. If none are obtained, then it is unlikely that APM would affect convulsive excitability at the levels consumed by the general population

Box Adaptation. All rats were handled daily after the stereotactic surgery and on the tenth and eleventh post-operative days they were each given 15min in the shielded test box. The rat was free to move as much as possible and no recording leads were attached. On the twelfth and thirteenth post-operative days the rats were again placed in the same box for 15min, however at these times the recording leads were attached to the electrode.

Threshold Determination. One day prior to the ingestion procedure (the fourteenth post-operative day-Day 0) the after-discharge threshold (ADT) for each rat was determined. The threshold was determined by using a stimulus train of 60Hz sine waves of 1.1sec duration. Threshold was determined to the nearest 5μA. There was an inter-stimulus interval of 5min between stimulus trains when determining the threshold. Threshold was defined as the lowest intensity of stimulation required to evoke an AD. The threshold was again determined after the animals had ingested APM or water for one day (Day 1) and again on the last day (seven days) of APM consumption (Day 7). This was done to determine if APM affects brain excitability immediately after ingestion or if the effect may occur after longer ingestion, if it occurs at all. Group 0 served as a control. Finally, a generalized seizure triggering threshold (GST) (Wada, Osawa and Mizoguchi, 1976) was determined one day after the third stage-5 seizure. This was obtained by reducing the kindling stimulation intensity by 5μA for every stimulation session to the point at which the stimulation was no longer sufficient to induce a stage-5 seizure. The interval of stimulations was the same as that used for the kindling procedure. The GST would be the lowest intensity required to evoke a Stage-5 seizure in a kindled animal and it was determined to the nearest 5μA.

Kindling Procedure. One day after the ingestion procedure (the day after

APM had been consumed for one week) the mass kindling procedure (Racine, 1972A) began for all animals. The kindling stimulations were the same as those used to determine threshold. The intensity each animal received was the lowest current required to evoke an AD on Day 7. The animal was stimulated two to three times every day, with an inter-stimulus interval of at least four hours, until the animal exhibited three stage-5 convulsions. Stage-5 convulsions consist of loss of postural control and head and bilateral clonus. The GST was determined on the day subsequent to the last stage-5 seizure.

Perfusion and Histology. All animals were sacrificed one week after being kindled. The animals were perfused transcardially with 0.9% buffered saline followed by a 10% formalin solution. The brains were removed and stored in 10% formalin for later sectioning. The tissue was frozen in isopropyl alcohol that was cooled in liquid Nitrogen. Tissue was sectioned at 38 microns and every third section was retained and stained using a metachromatic cresyl violet procedure.

Results

Only subjects with electrode placements in area CA3 of the hippocampus were reported in the analysis (see Figures 3 and 4).

Five animals were eliminated from the study because their ADT'S were above the maximum intensity used for ADT determination (400μA). The animals that did exhibit an ADT initially produced a wave of high frequency discharges superimposed over a normal stimulus-response wave form. The amplitude of the discharges decreased gradually and the AD's disappeared 10-20 sec after termination of the stimulus. When this pattern was obtained it was taken as the AD on Day 0. On Day 1 the AD was more defined and longer in latency. With the progression of more stimuli over days the AD became more characteristic of a seizure state with initial AD followed by a progression of the behavioral characteristics through the sequence of stages (Stage-1 through to Stage-5) (see Figures 5).

With regard to weight differences, there were no significant differences in weight gain between the three groups over the seven days of water/APM consumption, $\overline{X}_0 = 17.25$gm, $\overline{X}_{200} = 25.77$gm, and $\overline{X}_{800} = 21.75$gm; $\underline{F}(2,30) = 0.76$, $p > 0.05$. This referred to the weight gain between Days 1 and 7. Hence APM animals did not gain any more or less weight than the water animals. Therefore, any differences found in the dependent variable were not mediated by weight differences (see Table 1).

There was also no significant difference in food consumption between the three groups over the experimental manipulation ($\overline{X}_0 = 199.38$gm, $\overline{X}_{200} = 197.15$gm, and $\overline{X}_{800} = 189.0$gm; $\underline{F}(2,30) = 0.34$, $p > 0.05$). Hence, drinking APM in water does not decrease or increase food intake in rats (see Table 2).

Figure 3:

Distribution of of electrode tips in the hippocampus. Blocked area in CA3 indicates the concentration of the tips.

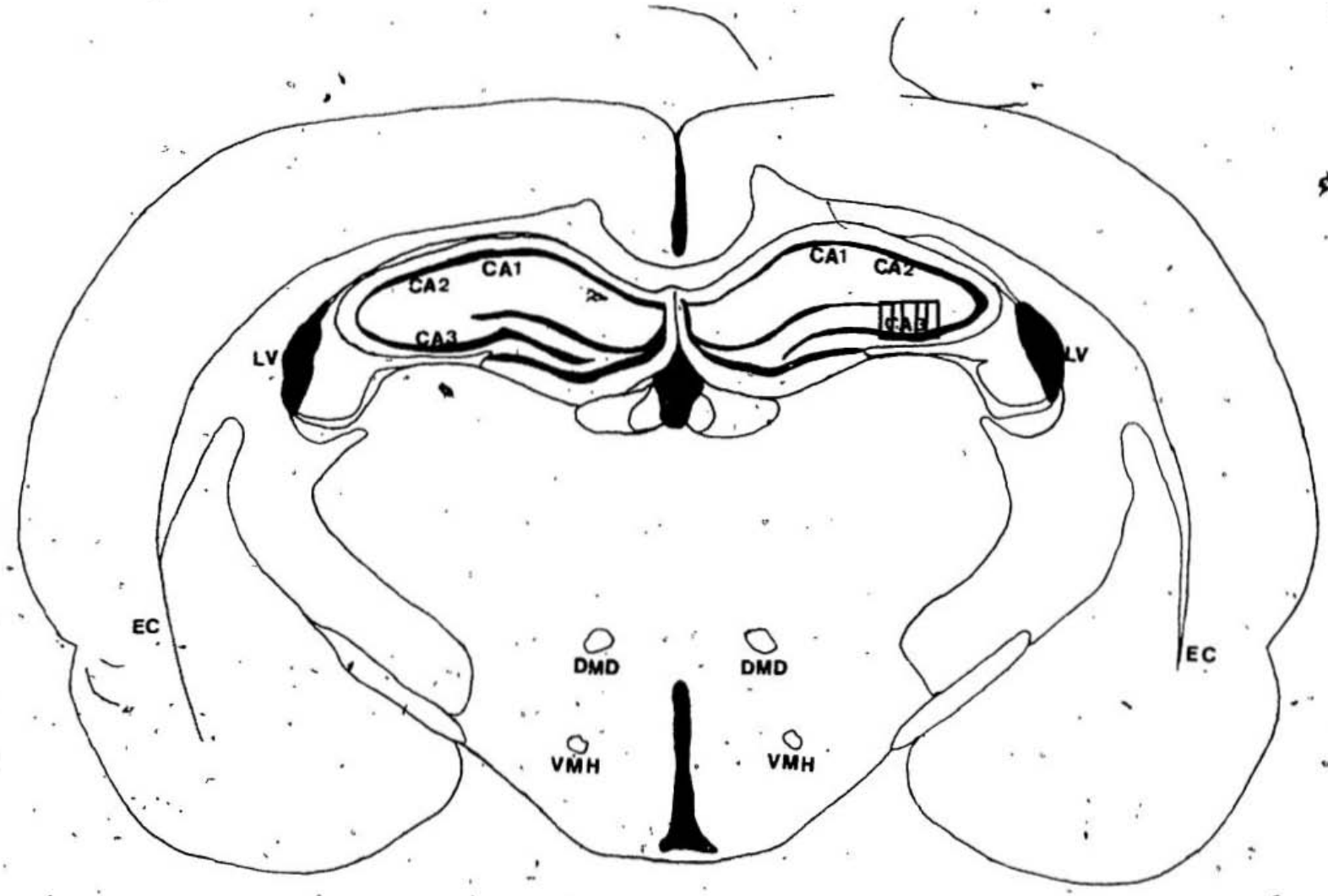

CA1
CA2
CA3
CA1
CA2
CA3
LV
LV
EC
EC
DMD
DMD
VMH
VMH

Figure 4:

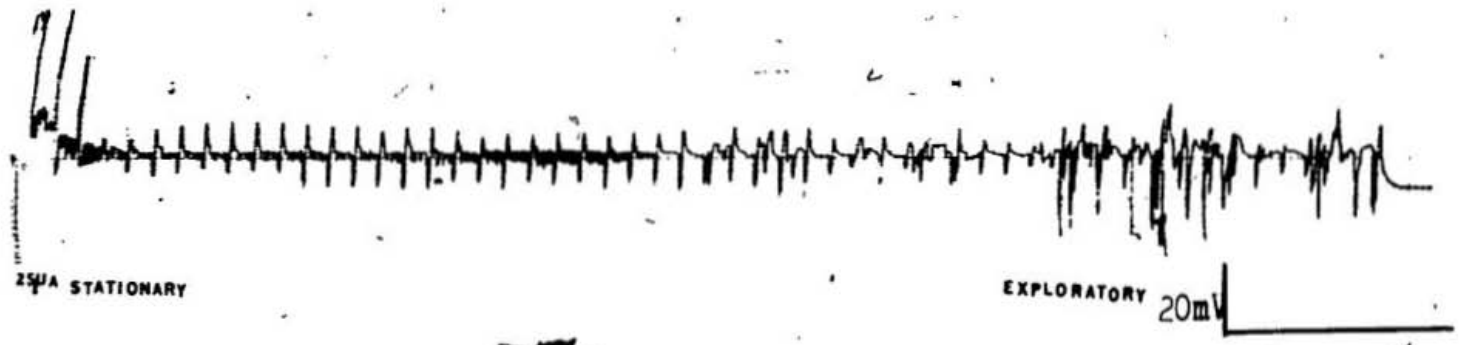
Photograph of a brain slice showing the electrode tip within Area CA3 of the hippocampus.

Figure 5: Two examples of the progressive increase in the duration of the after-discharge that occur as an animal comes closer to being kindled.

A: This shows the after-discharge exhibited by Animal 41.

B: This shows the after-discharge of Animal 19.

A


25μA STATIONARY
EXPLORATORY
20mV
1s

B

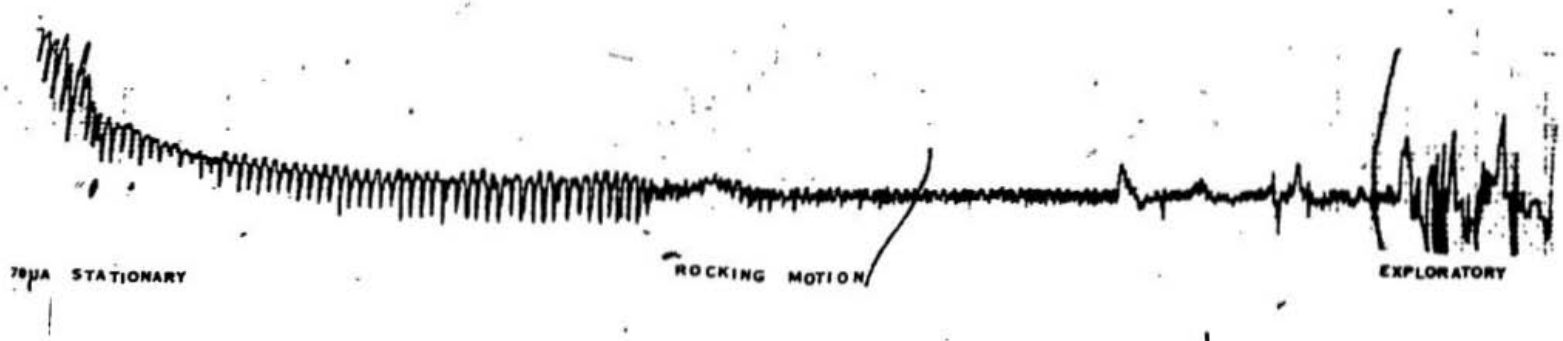

70μA STATIONARY
ROCKING MOTION
EXPLORATORY
5mV
1s

Table 1: One-Way Analysis of Variance to compare weight gain in subjects over 7 days of APM ingestion.

Source	df	SS	MS	F
G(GROUPS)	2	364.0	182.00	0.76
ERROR	30	7168.0	238.93	

Table 2: One-Way Analysis of Variance to compare food intake in subjects over 7 days of APM ingestion.

Source	df	SS	MS	F
G(GROUPS)	2	642.98	321.49	0.34
ERROR	30	28327.60	944.25	

There were also no significant differences in daily liquid consumption between the three groups ($\overline{X}_0 = 48.04$ml, $\overline{X}_{200} = 46.31$ml, and $\overline{X}_{800} = 42.95$ml; $F(2,30) = 1.03$, $p > 0.05$.). The total amount that an animal could consume per day was 55ml. There was no significant difference between groups over the seven days. Hence, all groups drank the same amount of liquid per day (see Table 3).

With regard to kindling, there were no significant differences in the number of stimulations required to evoke three Stage-5 seizures, $\overline{X}_0 = 45.1$, $\overline{X}_{200} = 43.1$, and $\overline{X}_{800} = 45.4$; $F(2,30) = 0.14$, $p > 0.05$. Hence, APM animals did not require fewer stimulations to produce seizures (see Table 4).

Figure 6 shows the change in ADT over days. It can be seen that groups 0 and 800 were initially the same at the start of the experiment with group 200 being slightly, but not significantly, above. It shows that all groups decreased over days and that group 200 decreased in ADT more than the other groups. It can also be seen that there was a crossover between day 7 and GST, but this was not significant. A detailed look at the analysis follows.

An overall Analysis of Variance was performed to determine whether there were any differences in ADT between groups over days and to determine whether there was an interaction between the groups over days. If there was a general APM effect it would be revealed in the interaction. There were no significant differences between the groups, $F(2,30) = 0.1913$, $p > 0.05$, nor was there an interaction between groups over days, $F(6,90) = 1.14$, $p > 0.05$. Hence, APM did not cause a difference in brain excitability. There was, however, a significant difference in ADT over all days, $F(3,90) = 37.6381$, $p \leq 0.001$ (see Table 5). The ADT between Day 0, Day 1, Day 7 and GST was not the same for any of the groups (see Table 6 for the means). A Newman-Kuels test comparing each day with every other showed that the ADT declined significantly for each day of testing (Day 0 > Day 1 > Day 7 > GST).

Figure 6: The change in ADT over days for the three groups.

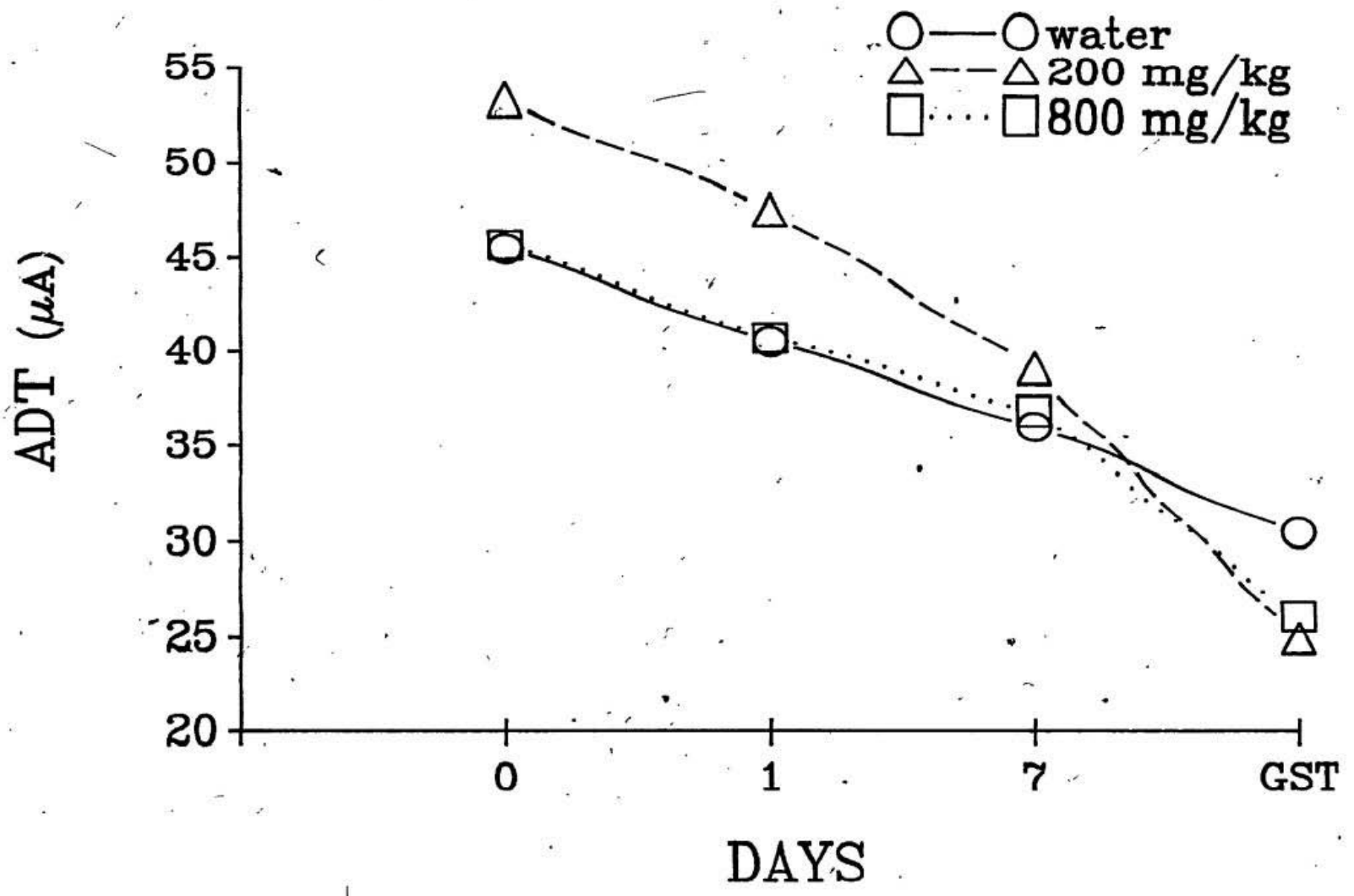

water
200 mg/kg
800 mg/kg
ADT (μA)
55
50
45
40
35
30
25
20
0
1
7
GST
DAYS

Table 3: Two-Way Analysis of Variance to compare liquid consumption per day of animals over the seven days of experimental manipulation.

Source	df	SS	MS	F
G(GROUPS)	2	964.62	482.31	1.03
S(SUBJECTS)	30	14062.60	468.75	
D(DAYS)	6	290.95	48.41	1.78
GxD	12	527.39	43.95	1.62
DxS	180	4886.24	27.15	

Table 4: One-Way Analysis of Variance to compare the number of stimulations required to kindle the animals (i.e. to reach 3 stage-5 seizures.

Source	df	SS	MS	F
G(GROUPS)	2	39	19.5	0.14
ERROR	30	4083	136.0	

Table 5: Two-Way Analysis of Variance for a comparison of the ADT for the three groups, for day 0, day 1, day 7 and GST.

SOURCE	df	SS	MS	F
G (GROUPS)	2	405.24	202.62	0.19
S (SUBJECTS)	30	31767.50	1058.92	
D (DAYS)	3	8770.27	2923.42	37.64 **
GxD	6	658.04	109.67	1.14
DxS	90	6990.47	77.67	

** $p < 0.01$

Table 6: The average ADT in μA for the three groups for Day 0, Day 1, Day 7 and GST.

	GROUPS			
DAYS	0mg/kg APM	200mg/kg APM	800mg/kg APM	overall
0	45.63	53.46	45.83	48.79
1	40.63	47.69	40.83	43.48
7	36.25	39.23	37.08	37.73
GST	30.63	25.00	26.25	26.82
overall	45.63	53.46	45.83	

To ensure that group differences were not present at the outset, a One-Way Analysis of Variance was completed for Day 0. It was found that there were no significant differences in the initial ADT for the groups on Day 0, before any APM was introduced into the experiment. Therefore, any difference seen cannot be attributed to differences with respect to baseline ADT (see Tables 6 and 7).

The short-term effects of APM ingestion were analyzed by performing an Analysis of Variance on ADT on Day 0 and Day 1. There was a days effect only on ADT, $F(2,30) = 9.97$, $p \leq 0.01$ (see Tables 6 and 8).

The long-term effects of seven days of APM ingestion were also examined by a 3(groups) x 2(days) analysis of variance. The two days of importance here were Day 7 and the day at which GST was measured. There was a significant difference in the threshold over the two days, $F(1,30) = 30.95$, $p \leq 0.01$ (see Tables 6 and 9). But there was no effect of APM ingestion on groups x days interaction.

Table 7: One-Way Analysis of Variance to compare the three groups for ADT on Day 0.

Source	df	SS	MS	F
G(GROUPS)	2	468.74	234.37	0.52
ERROR	30	13482.80	449.43	

Table 8: Two-Way Analysis of Variance for a comparison of the AD'T for the three groups, for day 0 and day 1.

SOURCE	df	SS	MS	F
G(GROUPS)	2	846.34	423.17	0.58
S(SUBJECTS)	30	21830.90	727.69	
D(DAYS)	1	464.02	464.02	9.97 **
GxD	2	2.33	1.17	0.03
DxS	30	1396.16	46.54	

** $p < 0.01$

Table 9: Two-Way Analysis of Variance for a comparison of the ADT for the three groups, for day 7 and GST.

SOURCE	df	SS	MS	F
G(GROUPS)	2	31.17	15.58	0.03
S(SUBJECTS)	30	13627.90	454.26	
D(DAYS)	1	1963.64	1963.64	30.96 **
GxD	2	183.44	91.72	1.45
DxS	30	1902.92	63.43	

** $p < 0.01$

Discussion

The hypothesis was not confirmed: APM did not produce changes in ADT during the ingestion procedure, nor did it decrease the rate of kindling after APM consumption was discontinued. It was expected that if APM altered brain excitability, even in the short-term, then ADT would change between day 0 and day 1. This was not the case here.

The determination of ADT for the subjects was observed from the behavioral correlate, although the physiological correlate was sometimes rather large when compared to the literature. This discrepancy may exist for several possible reasons; other literature studied ventral CA3 as compared to dorsal CA3, electrode impedance differences or filter setting differences.

In this experiment two variables could have changed brain tissue excitability: APM and kindling. Three possible outcomes could have occurred. The first, and expected, was that both of these variables would work together to produce a synergistic effect, hence excitability in APM animals would have been increased as a result of these two variables, as determined by a groups x days interaction in the analysis. The second possible outcome could have been an APM but no kindling effect. This would have been determined by a groups effect in the analysis, where groups would have been significantly different. If this had occurred it would have opposed previous literature since kindling itself affects ADT. Hence, if this occurred APM would act as protection against altered brain excitability. Therefore, if APM had an effect it was independent of kindling. The third possible outcome could have been a kindling but no APM effect. This would have been demonstrated by a days effect in the analysis. The change in ADT over days would have been significant.

Only a kindling effect was found. The ADT decreased over days as a result of induced excitability changes caused by the stimulations (as has been reported by

many researchers, for example Racine (1972A). The ADT for all groups decreased significantly for each testing day and there were no differences between water and APM groups. Hence the magnitude of change was unaltered and there was no interaction. Thus I conclude that under these conditions APM does not change brain excitability. This evidence is not in agreement with the findings of Maher and Wurtman (1987) and what was initially hypothesized in this study. They found that APM promotes seizures in animals who were already at risk (that is, animals treated with PTZ, fluorothyl or electroshock). These animals that were given APM before the seizure inducing treatment (PTZ, fluorothyl or electroshock) had more convulsions than animals that were not given any APM but just the seizure inducing treatment. The present study showed that APM had no effect on brain excitability even with the sensitive kindling technique. This study investigated here may be contrary to Maher and Wurtman (1987) because they gave their seizure inducing treatment one hour after APM ingestion. Hence, the plasma and brain levels of amino acids may have been altered, thus the results revealing an immediate brain excitability effect. The study performed here was more interested in the longer effects of APM and if any long term effects of brain excitability remained even after APM consumption ceased. Although the short term effects were studied here (by measuring ADT on days 0, 1 and 7) and it was found that APM did not have an immediate effect on brain excitability when studied by the kindling model. APM does not cause the rate of kindling to become faster nor does it decrease the intensity of stimulation needed to evoke an AD.

The fact that there were no differences among groups in the amount of liquid consumed was in agreement with Sclafani and Abrams (1986). Rats did not prefer APM over water for the animals receiving 200mg/kg or 800mg/kg of APM in their drinking water for seven days. APM also did not have an effect on food consumption or weight gain between the groups over the experimental manipulation. Therefore, it did not appear to upset their normal functions. The groups' data were all the same, regardless of APM consumption. If one of these features had changed along with ADT or number of stimulations to kindle

changed then it would have been difficult to say what was specifically causing the effect. However, this was not the case here.

The absence of APM effects on ADT and kindling raises two questions. First, did APM ingestion increase plasma PHE, ASP, TRP and TYR? Secondly, did elevated plasma levels of these amino acids change brain concentrations of 5-HT, TYR, PHE and TRP? There may still have been no effect, as was shown by kindling and ADT measurements, but instead an alteration in plasma or brain or plasma and brain concentration of amino acids. The long-term effect, if there is one, was not detected in brain excitability in Area CA3 of the Hippocampus, however, it is not to say that all amino acids are normal in the long-term, or even after three weeks. The next logical step is to determine plasma and brain amino acid levels after animals had consumed APM for one week. This study will tell one if the body compensates for constant alteration of the amino acid levels or does there exist a altered amino acid plasma/brain ratio. The subsequent study outlines such an experiment where plasma and brain amino acid levels were analyzed.

Amino Acid Study

Plasma and Brain Amino Acid Analysis

The previous study has shown that there were no significant differences between APM animals and water animals in the number of stimulations required to kindle Area CA3, nor was there any difference in the ADT between the groups. It is possible that there were no effects because although APM ingestion had raised brain tissue levels of various amino acids this had not altered kindling or ADT. Or it may be that the levels of APM ingestion had not altered amino acid levels in brain tissue. Although the levels used in the experiment were chosen on the basis of other work (Stegink, Koch, Blaskovics, Filer, Baker and McDonnell, 1981) the second possibility remains open.

In order to distinguish between these two explanations for the lack of effect a supplementary experiment was performed to determine if there actually was a difference in plasma and brain amino acid levels among the groups. If there was a difference in plasma and not brain one can say that the brain acts as a buffer for plasma amino acids (refer to BBB section of the introduction) hence not permitting entrance. If this is the case then intraventricular administration of the metabolites of APM may be a better route to determine the effects of APM on brain sensitivity. However, since we are interested in an analogue to the human situation then this route of administration is not acceptable. If there was a difference in brain levels among the groups one could say that APM does affect brain amino acid levels with the possible concomitant change in ratios of neurotransmitters (Fernstrom and Wurtman, 1971). In that case the tests of kindling and ADT used in the major experiment of this thesis were not sufficiently sensitive to detect an effect or there was no effect.

Method

Subjects.

Forty-five Long-Evans rats were maintained on APM for seven days as outlined in the previous study (15 animals on 0mg/Kg of APM; 15 animals on 200mg/Kg of APM; 15 animals on 800mg/Kg of APM). The animal's weight was recorded on day 1 and again on day 7. Animals also had free access to food at all times. The average weight of the animal for each group at the start of the experiment was 466.1g, 465.1g and 465.8g for the 0APM group, the 200APM group and the 800APM group, respectively. The animals were given free access to a liquid and the quantity drank was recorded daily. Plasma and brains were obtained from each animal. All animals were given a sufficient amount of Somnotol (65mg/Kg) for anesthesia. The skull bones were opened and the brains were removed before the plasma was extracted.

Procedure

This was a blind experiment. The person who extracted blood and brain coded the samples and then gave the samples to another person that performed the amino acid analysis procedure. Hence, biases were eliminated.

Plasma Extraction

Blood (2ml) was collected from the descending aorta in heparinized syringes. The blood was then transferred to centrifuge tubes and centrifuged for 10min at 4000RPM so that the plasma could be obtained. The plasma (supernatant) was mixed with an equal amount of Lithium Hydroxide (pH 2.2) and then injected into the HPLC amino acid analyzer (Beckman Bulletin 121M-TB-013) in the Biochemistry Department. The amino acids of interest have previously been discussed in the introduction and method of this thesis.

Brain Extraction

Each animal's brain was removed and sliced at the point where the spinal cord and cerebellum meet. The brain was immediately placed between pre-cooled (in liquid nitrogen) aluminium clamps for immediate freezing. The brain was then pulverized to a fine powder, with frequent additions of liquid nitrogen, homogenized with 40% Perchloric acid (4ml/gm of brain) and allowed to stand for 10min. on cold ice so that the protein precipitated. The brain was then centrifuged so that a 1ml aliquot of the supernatant could be obtained, which was adjusted to pH 2.2 by addition of sodium hydroxide. The HPLC analysis was performed in the Amino Acid Analysis facility of the Biochemistry Department at Memorial University of Newfoundland. One brain from each group was eliminated from the experiment as a result of breakage of the centrifuge tubes during centrifugation.

Results

One animal's data had to be removed from the statistical analysis. This animal had levels that were at least 3 standard deviations above the average in three of the plasma amino acids measured. Hence, this outlier was removed from the 800APM group.

A series of independent t-tests were performed between the three groups to determine if there were any significant differences among the groups. Plasma analysis revealed a significant difference between ASP levels in plasma between animals that drank 200mg/Kg of APM and 800mg/Kg of APM ($t(27) = -1.81$, $p < 0.05$), as well as, a significant difference between the 0mg/Kg of APM animals and 800mg/Kg of APM animals for ASP ($(t(27)) = -2.13$, $p < 0.05$). There was also a significant difference in tyrosine concentration for the 0mg/Kg of APM animals and the 800mg/Kg of APM animals ($(t(27)) = -2.66$, $p < 0.05$). There were no other differences found in the other amino acids analyzed. Table 10 shows the means for the amino acids analyzed in the three groups.

Another series of independent t-tests were also performed between the three groups to determine if there was any significant difference between the groups. Brain amino acid analysis revealed no significant difference in any amino acid studied here in brain between the three groups. Table 11 depicts the means for the amino acids analyzed in the three groups.

There were no differences in liquid consumption among the three groups over the seven days of APM/water ingestion. The average amount of liquid consumed per day for the 0mg/Kg APM animals was $\overline{X}_0 = 50.13$ml, the 200mg/Kg animals $\overline{X}_{200} = 52.75$ml, and the 800 mg/Kg APM animals $\overline{X}_{800} = 61.1$ml.

There were no differences in weight gain over the seven day ingestion period for the 0mg/Kg APM animals, the 200mg/Kg APM animals and the 800mg/Kg APM animals. The weight gains were 19.8g, 19.3g and 18.5g, respectively.

Discussion

Overall, there was a difference in plasma ASP between 200-800APM animals and 0-800APM animals. There was also a difference between plasma tyrosine concentration in the 0-800APM animals. However, there were no differences found between any amino acids tested in brain among the groups. This difference in plasma is not affecting brain levels at the concentrations of APM used in this experiment. It is also of interest to note that we have not found the PHE increment in plasma that has been found in previous research (Wurtman, 1983B). This was of importance because most previous research extracted brain/plasma immediately after APM consumption or every half hour up to two hours after APM ingestion. The other studies that found amino acid level differences also used one test load. Contrary to this, plasma and blood were extracted up to 6hr after APM/water consumption and these animals were maintained on the liquid for seven days. Therefore, insignificance in PHE may be due to a longer duration for the liver to metabolize the metabolites of APM or it may be that there is a compensatory mechanism that monitors and corrects high levels of circulating PHE when PHE is in the plasma at high concentrations for a prolonged period of time.

The lack of effect in brain levels of amino acids could not be attributed to methodology problems. The preparation of the liquids were done with standard procedure as that of high liquid chromatography. Hence, the results found in this experiment are probably not due to chance or experimental error.

Recent research by Wurtman and Maher (1988) suggests that the concentration of 200mg/Kg of APM in rats is not comparable to the 34mg/Kg of APM in humans. They suggest that due to the fast rate of metabolism in rats as compared to humans the concentration should be 500mg/Kg of APM. If this idea was correct than the concentration used in this experiment (800mg/Kg APM) should have been sufficient to produce an effect. However, this was not the case.

It is unlikely that humans would consume APM in higher concentrations than used here. Hence, there appears not to be any detrimental effect of APM on the brain with regard to ADT changes or possibly any other behavioral or neurological construct.

Table 10: The average amino acid concentration (nm/ml) in plasma for the three groups.

\Chemical Analyzed Group\	ASP	Glutamic Acid	Glutamine	TYR	PHE	TRY
0 APM	162.90	88.55	219.56	23.25	24.27	14.08
200 APM	168.41 *!	94.00	214.33	25.53	25.60	12.49
800 APM	239.29 *	91.79	261.48	28.25 *	26.69	13.43

$p < 0.05$, * refers to a significant difference between the 0APM and 800APM groups.

! refers to a significant difference between the 200APM and 800APM groups.

Table 11: The average amino acid concentration (nm/ml) in brain for the three groups.

\Chemical \Analyzed Group\	ASP	Glutamic Acid	Glutamine	TYR	PHE	TRY
0 APM	999.21	2652.69	1151.66	15.31	14.89	2.46
200 APM	1032.10	2611.70	1205.71	15.35	14.71	2.81
800 APM	1035.42	2777.48	1200.91	16.61	15.05	2.46

General Discussion

Overall, APM did not affect the rate of kindling in Area CA3 of the Hippocampus nor were there any differences in brain concentrations of the amino acids under study when animals ingested 0, 200, or 800 mg/Kg (of body weight) of APM. If the dosage equivalence between humans and rats is correct then from these two studies we can say that APM would not have any Central Nervous System effect on 'normal' subjects. However, there may be certain subjects that are sensitive to certain amino acid fluctuations. Also it may be that the effects of APM are additive and that over time the amino acids may become even higher in plasma and have more molecules available to compete for transfer across the BBB and then affect neurological functioning.

There are a number of other studies that can be done to determine the effects of APM on brain excitability. Some ideas are given in this paper. One way to determine the effects of APM on the development of seizures would be to obtain EEG's from animals for a baseline period and then give them APM to ingest for a three month period while obtaining daily EEG's. The animals can then be kindled to determine any difference in the rate of kindling between groups and the change in EEG'S during ingestion. Another study is to kindle animals first and then let them consume APM in water as was carried out here. This would continue for a reasonable amount of time with animals always being videotaped to detect if any spontaneous seizures were occurring. In this experiment you would be testing whether epileptics had more seizures during APM consumption or if there were any spontaneous seizures. This study should also test ADT at various times to determine if the ADT for APM epileptics was lower than the ADT for control epileptics. An alternate way to examine the question would be to take the same animals and only determine their GST to see if it has significantly decreased for APM animals. Yet another would be to replicate the study completed in this thesis with one exception, continue APM consumption from Day 1 until animals

exhibit three Stage-5 seizures. This study would test the transient effects of APM on ADT and rate of kindling.

The question of the effects of APM on brain excitability remains open. The method used in this thesis rely on demonstrating an average effect. It does not deal with the qeustion of effects on "susceptible" individuals. Given the present findings, research using methods appropriate to screening for sensitive individuals would be a useful area to examine.

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