USE OF VIBRATOME SECTIONING AND PHOSPHOR
IMAGING TO CHARACTERIZE DISTRIBUTION OF $^{14}$C
IN BRAIN AFTER ADMINISTRATION OF RADIO-
LABELED ACETALDEHYDE AND ACETATE.

Master’s Thesis Defense

By

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1. BACKGROUND AND SIGNIFICANCE:

While a low concentration of alcohol has been associated with better health and longer life than is abstinence, the heavy consumption of alcohol, especially over a period of many years, can lead to serious health problems and even death. Alcoholism is considered to be a disease, and the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, published by American Psychiatric Association as well as the International Classification Diseases, published by World Health Organization include the following symptoms of alcohol abuse:

1) Craving: Strong need and urge to drink.
2) Loss of control: Not being able to stop drinking once drinking has begun.
3) Physical dependence: Withdrawal symptoms, such as nausea, sweating, shakiness, and anxiety after stopping drinking.
4) Tolerance: The need to drink greater amounts of alcohol to get desired effects.

Alcohol dependence is considered to be one of the most costly health care problems in the American society. The yearly dollar cost for alcoholism is projected to be more than $185 billion [1]. Violence is commonly associated with alcohol use, with an estimated 26% of offenders using alcohol at the time of their crime [2]. It has been estimated that at least 15% of men and around 10% of women in the US meet the criteria for alcohol dependence at some point in their lives. A 2002 national survey estimated previous year rates of abuse or dependence to be about 8% overall. It was18% for people of age 18-25 years [3].
The average person with alcoholism first demonstrates the clustering of major alcohol related life problems in mid 20’s to early 30’s and most people with alcoholism present for treatment are in their early 40’s [4]. If the alcohol consumption continues, the person is expected to die 15 years earlier than the average expected age for the general population, with leading causes of death being (with decreasing order of frequency) heart disease, cancer, accidents, and suicide [5].

1.1 MEDICAL COMPLICATIONS OF ALCOHOLISM

1.1.1 Gastrointestinal Tract and Pancreas:
Alcohol abuse may lead to inflammation of the gastrointestinal tract. It causes decrease in the peristaltic movement and also decreases the sphincter tone, which may lead to reflux esophagitis with pain and stricture formation [6]. Alcohol decreases gastric emptying and increases gastric secretion. This causes disruption of the mucosal barrier, allowing hydrogen ions to pass through mucosa releasing histamine and causing bleeding. Acute gastritis is characterized by vomiting, anorexia and epigastric pain. Alcohol consumption impairs absorption of folate, vitamin B12, thiamine and vitamin A as well as some amino acid and lipids. Intestinal enzyme activity is altered as well [7]. High amounts of alcohol also increase the risk of acute pancreatitis (4:1 ratio for individuals with consumption of alcohol of about 35 drinks/week to that of individuals with lower or no alcohol consumption) [8].
1.1.2 Liver:

Three types of lesions occur in the livers of the alcohol abusers. In 90% of cases fatty liver is observed (hepatic stenosis); 10-35% show alcoholic hepatitis, and around 10-20% show alcoholic cirrhosis. Hepatic stenosis is a reversible condition which may develop to cirrhosis in about 7% of cases [9]. Symptoms of alcoholic stenosis include nausea, vomiting, hepatomegaly, right upper quadrant pain, and tenderness. Symptoms of hepatitis include anorexia, nausea, vomiting, fever, chills, hepatomegaly, right upper quadrant tenderness and abdominal pain. Cirrhosis develops as a result of prolonged damage to hepatocytes which causes centrilobular inflammation and fibrosis. This condition leads further to development of portal hypertension and varices development. Cirrhosis also leads to hepatic encephalopathy, secondary malnutrition, ascites and clotting deficiencies [10].

1.1.3 Nutrition:

Alcoholics generally show deficiencies of thiamine, B vitamins, ascorbic acid and folate. These deficiencies may further lead to increased levels of homocysteine, and ultimately promotes atherosclerosis and thrombosis [11]. Alcohol may lead to increased protein turnover, inhibition of lipolysis and negative nitrogen balance [12]. Ethanol also suppresses appetite through its effects on the CNS. Malnutrition may result due to damage to stomach, intestine, liver or pancreas.
1.1.4 **Cardiovascular System:**

Left ventricular function declines in a dose dependent manner and alcohol is involved in at least 30% of all dilated cardiomyopathies [13]. The contractility of heart decreases through alcohol’s effect of increased calcium flow into muscle cells, decreased protein synthesis and mitochondrial disruption [14]. Alcohol increases blood pressure independently of age and body weight in men, but not in women when age and body mass index were controlled [15]. Heavy alcohol intake is also associated with increased risk of hemorrhagic stroke and ischemia [16]. It may also cause vasoconstriction of cerebral blood vessels. Although heavy alcohol consumption possesses risk for cardiovascular diseases, low-to-moderate alcohol drinking may be beneficial for the cardiovascular system. Light drinkers (less than 2 drinks/day) show a 20% decrease in risk of coronary artery diseases [17]. We may describe protective actions of alcohol as a U shaped curve. Non drinkers and heavy drinkers show increased risk of coronary artery disease compared to light drinkers.

1.1.5 **Nervous System:**

Ethanol damages the Central Nervous System (CNS) as well as the Peripheral Nervous System (PNS) by variety of mechanisms, which are discussed later on. Alcoholism may lead to Wernicke-Korsakoff syndrome, which involves thiamine deficiency, atrophy of brain and dementia. Hepatic encephalopathy is another major complication associated with hepatic cirrhosis. The most frequent complication of chronic alcohol consumption is toxic polyneuropathy due to malnutrition [18].
1.1.6 **Hematology:**

Anemia is commonly associated with chronic alcohol consumption and results from hemorrhage, hemolysis or bone marrow hypoplasia. Folate deficiency may lead to development of megaloblastic anemia. One may frequently observe occurrence of transient thrombocytopenia (decrease in platelets) after consumption of large amount of alcohol in binge drinker [19]. Leucopenia (decrease in white blood cells) may sometimes result. This is also due to malnutrition. Alcohol also delays the clotting as well as thrombotic functions due to deficiencies in Vitamin K dependent factors.

1.1.7 **Endocrine Function:**

Alcohol interferes with testicular, pituitary and hypothalamic functions. Chronic alcohol consumption leads to testicular atrophy, decreased libido and diminished sperm counts. Thyroid dysfunction is also commonly seen in alcoholics, with decreased thyroxine levels. Alcohol intoxication leads to activation of the hypothalamic pituitary axis and results in increased levels of glucocorticoids. With the development of tolerance due to chronic alcohol consumption, this effect may be reduced. However, chronic exposure to high levels of glucocorticoids may lead to premature aging.

1.1.8 **Fetal Alcohol Syndrome:**

Fetal Alcohol Syndrome (FAS) is an irreversible condition associated with excessive consumption of alcohol by pregnant women. Victims suffer from physical deformities and often mental deficiencies. They suffer from these problems for their entire lives. While most cases occur among children of alcoholic mothers who consume alcohol
heavily throughout their pregnancies, no one knows for certain what level of alcohol consumption is safe for a pregnant woman. The fetus is most vulnerable to effects of alcohol during the first trimester.

1.1.9 Other Complications:
Apart from the above mentioned diseased states alcohol consumption may lead to development of other conditions. The most prevalent among them is cancer. Alcohol is associated with cancer of the oral cavity, larynx and esophagus. Statistically significant risk are also found for cancers associated with stomach, colon, rectum, breast, ovaries and liver [20].

Skin diseases like psoriasis have been associated with alcohol abuse. Flushing, rapid muscle breakdown and eczema also may be found in alcoholics. Musculoskeletal diseases like rhabdomyolysis as well as osteoporosis are seen in alcoholics. Alcoholics also may have impaired immune system, placing them at higher risk of infections like Hepatitis C.

1.2 PHARMACOLOGY OF ETHANOL

The mechanisms of action of ethanol in brain are very complex and poorly understood. It is really difficult to identify the effects of ethanol and its metabolites on various neurochemicals in the brain. Changes in the neurochemical behavior of brain vary for acute administration and chronic administration of ethanol. Thus, ethanol does not follow the principle of “one drug one effect”. Various theories have been proposed for the effects of alcohol on brain. These are briefly discussed below.
In previous years, one theory of ethanol was based on its property of altering the properties of lipids in the neuronal membrane. A direct relationship has been observed between the anesthetic properties of alcohol and changes in the lipid solubility of these membranes resulting in increased fluidity of the membrane as well as increasing permeability. This general property may contribute to the biological effects of very high doses of ethanol, but it is likely that more specific mechanisms play more important roles.

The pleasurable effects experienced on ingestion of ethanol are probably due to increased dopamine signaling in the ventral tegmental area and nucleus accumbens. Ethanol has shown to increase levels of dopamine metabolites and the number of functional dopamine receptors [21, 22]. All drugs associated with reinforcement and reward appear to activate mid-brain dopamine circuits. However, the precise way that ethanol enhances dopaminergic neurotransmission is not fully understood.

Alcohol also affects serotonergic neurotransmission. Acute administration of ethanol increases the release of serotonin, while chronic exposure tends to decrease the release. Specific serotonin receptors including 2A, 2C and 1B are involved in ethanol’s actions [23, 24].

One of the most important mechanisms for alcohol’s action involves GABAergic transmission. It is the system involved in benzodiazepines’ actions. Acute doses of alcohol increases the transmission of this system with a mechanism which might be
different from that of benzodiazepines. Animals that self administer of alcohol have a GABA receptor system less sensitive to alcohol [25-27].

Other neurotransmitters are associated with alcohol’s effects. Glutamate receptors of the N-methyl D-aspartate (NMDA) subtype also appear to be important for ethanol’s action. Acute administration of ethanol decreases the sensitivity of NMDA receptors, while chronic use and alcohol withdrawal are associated to increased responsiveness of these receptors.

Apart from its effects on the neurochemistry of the brain, it may also possess some other interactions with the brain’s opioid receptor system. At least theoretically, the metabolite of alcohol, acetaldehyde can combine with neurochemicals, such as dopamine and serotonin to produce substances, the tetrahydroisoquinolines and the beta carbolines, that may have opioid like effects. Mu-opiod receptors have been shown to be involved in ethanol self administration in rodents [28].

Many other theories related to alcohol metabolism have been proposed which includes its effect on acetylcholine system, on cannabinoid receptors, adenosine receptors, potassium channels and on the elements of intracellular second messenger system.
1.3 METABOLISM OF ALCOHOL IN THE BODY AND THE BRAIN

Acetaldehyde, the initial metabolite of ethanol, may contribute to ethanol’s actions in the CNS (see Figure 1). However, several arguments have been advanced against this idea. The first is that the metabolism of acetaldehyde in the liver maintains its blood levels very low, which may not be high enough for a large amount of acetaldehyde to enter the brain. Secondly, acetaldehyde may not seem to penetrate the blood brain barrier (BBB), except when present at very high blood levels. A third reason to doubt the importance of acetaldehyde in the overall action of ethanol is that when methyl pyrazole (an inhibitor of alcohol dehydrogenase) is administered to animals, intoxication due to ethanol is still observed. This suggests that acetaldehyde may not be important for ethanol’s CNS actions. The possibility of conversion of alcohol to acetaldehyde in the brain eliminates all the above arguments. The same enzymes that are responsible for alcohol metabolism in liver, catalase, cytochrome P450 (Cyp2E1) and alcohol dehydrogenase, also mediate the conversion of ethanol to acetaldehyde in the brain [29].

**Figure 1:** Metabolism of ethanol in body
Furthermore, consumption of alcohol is associated with many centrally mediated effects including euphoria, relief of anxiety, sedation and others. Some of these may be viewed as desirable (relief of anxiety, sedation). Others such as loss of motor co-ordination, loss of self control and “hangover” are harmful and/or unpleasant. In fact, alcohol induces a constellation of actions that depend on dose, time after injection, intake of other substances, prior history of use of alcohol and prior and current use of other drugs. Acetaldehyde may play an important role in some of alcohol’s effect and/or modulate effects primarily due to alcohol itself.

1.4 CONSEQUENCES OF OXIDATION OF ETHANOL TO ACETALDEHYDE IN BRAIN

Aldehydes are chemically reactive compounds. Thus, acetaldehyde in the brain could form other compounds with potential pharmacological and toxicological actions. These may be divided to two main categories. One is direct binding of acetaldehyde to nucleic acids, proteins and phospholipids. Another potential mechanism involves indirect actions of acetaldehyde via inhibition of metabolism of other aldehydes. For example, aldehydes produced by action of monoamine oxidases on dopamine, nor epinephrine or epinephrine accumulate when acetaldehyde is present [30]. Thus, acetaldehyde could potentially influence the concentrations of endogenous compounds that affect the brain’s functioning.
One idea is that acetaldehyde condense with the same neurotransmitters to produce tetrahydroisoquinolines that could act at neurotransmitter receptors. Aldehydes may also condense with serotonin-like compounds to produce tetrahydro-beta carbolines which may similarly be active in the brain. Also recently, studies have proposed that acetaldehyde may compete with malondialdehyde or 4-hydroxynonenal, aldehyde products after the breakdown of lipids. These aldehydes may also decrease the activity of alcohol dehydrogenase and aldehyde dehydrogenase [31, 32]. This would increase the levels of acetaldehyde as well as other toxic aldehydes. These reactions may lead to development of behavioral changes.

1.5 INTRODUCTION TO CYCLONE STORAGE PHOSPHOR IMAGER

Figure 2: Storage phosphor imager working principle

Storage phosphor imaging (Figure 2) works on the principle of radiation induced emission of photostimulated luminescence and can be used for quantitative image analysis [33]. The phosphor screens used for the storage phosphor imager consist of photostimulable crystals of barium fluorohalide with small amounts of bivalent europium ions as an activator (BaFBr : Eu$^{2+}$). The phosphor crystals absorb and store energy from the decay of radioactive material when the radiolabeled sample is exposed to the
phosphor screen. The energy from radioactive decay oxidizes Eu$^{2+}$ ions to Eu$^{3+}$ ions and electrons are liberated. The electrons move towards the conduction band of the phosphor crystals, where they travel freely until they are trapped by ‘F centers’ (Farbzentrum center which is the German word for color) present in the halide crystals to form metastable BaFBr$^{-1}$ excited complexes. These electrons have energy slightly lower than the conduction band, so they cannot recombine with Eu$^{3+}$. These trapped electrons constitute a latent image and their number is proportional to incident radiation. The phosphor screen, which now contains a latent image, is then exposed to red laser light (633 nm), which excites the metastable electrons to the high energy conduction band where they recombine with Eu$^{3+}$ and return to low energy valence Eu$^{2+}$. This results in liberation of energy in the form of blue light (390 nm). The intensity of the emitted light is proportional to the radioactivity from the sample. The emitted blue light is detected by a photo-multiplier tube and the data is stored as a digital image. The intensity and location of radioactivity in the sample can be inferred from these two-dimensional digital images. The autoradiogram can be then analyzed using image analysis software, and if the standards are included along with the samples, the image can be quantified discretely. The phosphor screen can be reused by deleting any image simply by exposing the plate to white light.
2. SPECIFIC AIMS

2.1 OBJECTIVES

1) To evaluate the use of a vibrating microtome and phosphor imager to measure brain regional concentrations of radiotracers labeled with $^{14}$C.

2) To evaluate the optimum slice thickness necessary for clear image quality.

3) To compare the $^{14}$C distribution in mouse brain after administration of [1-$^{14}$C]acetaldehyde and [1-$^{14}$C]acetate.

2.2 HYPOTHESES

1) We will be able to obtain high resolution maps of $^{14}$C distribution that will allow measurement of $^{14}$C in small (sub mm) regions of mouse brain.

2) The slice thickness that optimizes the technical difficulties of sectioning and good quality images will be between 300 and 500 $\mu$M.

3) Distribution of [1-$^{14}$C]acetate and [1-$^{14}$C]acetaldehyde will be similar, but will show differences that might be due to the formation of acetaldehyde-biogenic amines adducts.
3. RESULTS

3.1 ANALYSIS OF DIFFERENT RADIOTRACERS IN DIFFERENT TISSUES IN MICE

Table 1: Percentage of injected radioactivity in different tissues for various time points.

<table>
<thead>
<tr>
<th>Radiotracer</th>
<th>Tissue</th>
<th>0.25 Minute</th>
<th>1 Minute</th>
<th>4 Minute</th>
<th>16 Minute</th>
<th>30 Minute</th>
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<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>[1,2-¹⁴C]acetate</td>
<td>BL</td>
<td>3.94</td>
<td>0.94</td>
<td>2.36</td>
<td>1.12</td>
<td>0.45</td>
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<tr>
<td></td>
<td>LV</td>
<td>1.66</td>
<td>0.87</td>
<td>3.13</td>
<td>1.50</td>
<td>2.61</td>
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<tr>
<td></td>
<td>KD</td>
<td>5.65</td>
<td>2.32</td>
<td>10.48</td>
<td>4.16</td>
<td>5.67</td>
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<tr>
<td></td>
<td>AD</td>
<td>17.67</td>
<td>6.83</td>
<td>26.62</td>
<td>23.15</td>
<td>9.77</td>
</tr>
<tr>
<td></td>
<td>HR</td>
<td>16.79</td>
<td>3.81</td>
<td>8.58</td>
<td>4.14</td>
<td>2.55</td>
</tr>
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(SD = Standard Deviation, BL = Blood, LV = Liver, KD = Kidney, AD = Adrenal Gland, HR = Heart)

Table 1 shows the data from some of the experimental work I have undertaken in Dr Gatley's laboratory. In this work, which was shared by myself and Somdutta Mitra, we measured levels of ¹⁴C in heart, liver, kidneys, adrenal glands and blood from mice.
injected intravenously with [1-^{14}C]ethanol, [2-^{14}C]ethanol, [1-^{14}C]acetate, [2-^{14}C]acetate or [1,2-^{14}C]acetaldehyde. These tissues were obtained from animals studied by Dr Gatley's former postdoc Lavina Faleiro, who is now an assistant professor at the Royal College of Physicians of Ireland. Dr. Faleiro had measured radioactivity in the brains of these animals and the other tissues dissected out of the animals had been kept at -80°C until they were analyzed by Ms. Mitra and myself. Since there were 5 tissues, 5 radiotracers, 5 time-points and 8 mice per time-point, we had about 1000 samples for work-up. Additional experiments were also done, including measurement of radioactivity in mice that were also given pharmacological as well as radiotracer doses of ethanol, and high performance liquid chromatography analysis of radioactivity in hearts given ^{14}C-acetate.

Different radiotracers (acetaldehyde, [1-^{14}C]acetate, [2-^{14}C]acetate, [1-^{14}C]ethanol and [2-^{14}C]ethanol) were injected in mice with tail vein injection (n=8 for all groups). Mice were sacrificed after different time points (0.25 minutes, 1 minutes, 4 minutes, 16 minutes and 30 minutes). Tissues (blood, liver, kidney, adrenal gland and heart) were collected from mice, weighed and frozen until use.

3.1.1. For blood, adrenal gland, kidney:

When required tissues were taken out and then dissolved in solvable (Perkin Elmer) (volume of solvable: blood -1 mL, adrenal gland-1 mL and kidney-2 mL) and allowed to dissolve. Then liquid scintillation fluid was added and radioactivity was counted with liquid scintillation counter.
3.1.2. For heart:

We planned to perform HPLC on heart tissues, so 1 molar perchloric acid (2 mL) was added to the tissues. Then it was homogenized, centrifuged and the supernatant was taken and neutralized with potassium bicarbonate. Then again it was centrifuged to remove any salt formed. Half of the volume of solution was taken and measured with liquid scintillation counter.

3.1.3. For liver:

Tissues were dissolved in 2 mL of propriety tissue solubilizer and bleached with 30% w/w hydrogen peroxide. 100 μL of this solution was taken and counted with liquid scintillation counter.

Although several features of the data in Table 1 warrant detailed discussion, most of these are beyond the scope of this thesis. They include:

- the generally higher tissue concentrations, at longer time-points, of radioactivity from [2-\(^{14}\)C]acetate or ethanol than from the [1-\(^{14}\)C] tracers;
- the high uptake of label from acetate by the heart;
- the broadly similar tissue concentrations of acetate and ethanol at later time-points;
- the higher uptake of ethanol than acetate or acetaldehyde by the kidneys, at later time-points.
A particularly prominent feature is the high concentration of label from acetaldehyde in adrenal glands (Figure 3). Although the data are noisy, reflecting the fact that the mouse adrenal gland is a tiny structure whose weight is difficult to measure because of variable amounts of extra-adrenal tissue in dissections, there seems no doubt concerning the greater adrenal uptake of [1,2-\textsuperscript{14}C]acetaldehyde than of the other radiotracers.

The notion that some components of the psychoactive effects of alcohol are due to the actions of compounds formed by reaction of amines with the ethanol metabolite acetaldehyde in the brain is intriguing. So, we aimed at looking at the distribution of metabolites of [1-\textsuperscript{14}C]acetate and [1-\textsuperscript{14}C]acetaldehyde in mouse brain slices cut with the vibratome and images obtained by autoradiography with cyclone phosphor imager.
3.2 EVALUATION OF STORAGE PHOSPHOR IMAGING FOR QUANTITATIVE ANALYSIS OF TLC PLATE AND BRAIN SLICE

Autoradiographic studies have made major contributions to neuroscience [34] in terms of mapping distributions of radioactive metabolic substrates and neurotransmitter receptor ligands. In the general technique, the brain is sliced into thin sections which are then apposed to photographic film which is sensitive to beta particles emitted by the radiotracer. But I have used the method of autoradiography with storage phosphor imager instead of film autoradiography. Phosphorimaging employs plates coated with radiosensitive crystals. The plates offer poorer spatial resolution than photographic film, but unlike film give a signal that increases linearly with the radioactivity concentration to which they are exposed. They also exhibit a large dynamic range compared to film. Another advantage is the avoidance of the extreme light-sensitivity and darkroom work associated with photographic emulsions. While working with Dr. Gatley and Dr. Duclos, I used a phosphorimager in thin-layer-chromatography experiments to measure radiolabeled substrate and product of an enzymatic reaction (diacylglycerol lipase). This led to the concept of using the phosphorimager for ex vivo autoradiography of mouse brain.

Despite the simplicity of the storage phosphor imager, there are a number of issues that need to be evaluated to ensure quantitative accuracy and good image quality. It was important to characterize the parameters such as optimum exposure length for our
settings, whether fade effects linearity following long exposure time, which plate to use and the resolution settings on the instrument.

We spotted $^{14}$C labeled 2-deoxy glucose on a TLC plastic sheet (silica gel 60 F$_{254}$, EMD Chemicals Inc., NJ). The radioactivity of the solution prepared was 50 μCi/mL. Spots of 0.5 μL, 1.0 μL, 2 μL, 4.0 μL, and 8 μL were plotted on a plate. Duplicate volumes of these solutions were also counted with the liquid scintillation counter (Multipurpose scintillation coulter, Beckman Coulter). The results obtained are given in Table 2.

**Table 2:** Liquid scintillation counter results.

<table>
<thead>
<tr>
<th>Volume(μl)</th>
<th>CPM</th>
<th>DPM = CPM/Efficiency* (x10$^3$)</th>
<th>Experimentally determined Radioactivity (μCi) = DPM/2.22x10$^6$</th>
<th>Calculated radioactivity (μCi)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.50</td>
<td>48278</td>
<td>54</td>
<td>0.024</td>
<td>0.025</td>
</tr>
<tr>
<td>1.00</td>
<td>92439</td>
<td>103</td>
<td>0.046</td>
<td>0.05</td>
</tr>
<tr>
<td>2.00</td>
<td>183338</td>
<td>204</td>
<td>0.092</td>
<td>0.10</td>
</tr>
<tr>
<td>4.00</td>
<td>385277</td>
<td>428</td>
<td>0.193</td>
<td>0.20</td>
</tr>
<tr>
<td>8.00</td>
<td>745260</td>
<td>828</td>
<td>0.373</td>
<td>0.40</td>
</tr>
</tbody>
</table>

*Efficiency for $^{14}$C is 90%
The graph obtained was linear.

The next thing was to check whether we obtained the same linearity of response with respect to radioactivity with cyclone phosphor imager. The TLC plate prepared was exposed for various time points (12 minutes, 30 minutes, 55 minutes, 20 hours and 45 minutes, and 116 hours) to Super resolution phosphor screens (Size: 12.5 x 25.2 cm, Perkin Elmer). Images were obtained using cyclone storage phosphor imager and OptiQuant software was used for image analysis.

Analysis was done by circling the spots obtained (T1) and then copying the regions onto the part of the plate where no radioactive material was exposed (T2). This gave the amount of background DLU (T2) in the defined area for that plate. This background digital light unit (DLU) (T2) was subtracted from DLU obtained for the radioactive material (T1). Thus, T1-T2 gave the actual value for radioactivity (shown in Table 3). Graphs were plotted for DLU obtained vs volume for various exposure times (Figure 5).
Also, we normalized these counts with respect to time and plotted DLU/minute vs volume of radioactive material to check the effect of time on signal response.

**Table 3:** DLU for various exposure times for serial dilution for various time points.

<table>
<thead>
<tr>
<th>Volume (μL)</th>
<th>DLU (x10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 Minutes</td>
<td>30 Minutes</td>
</tr>
<tr>
<td>0.50</td>
<td>3.5</td>
</tr>
<tr>
<td>1.00</td>
<td>6.0</td>
</tr>
<tr>
<td>2.00</td>
<td>10.6</td>
</tr>
<tr>
<td>4.00</td>
<td>32.4</td>
</tr>
<tr>
<td>8.00</td>
<td>62.1</td>
</tr>
</tbody>
</table>

Thus, we can see the increase in DLU with increase in exposure time until 21 hours, then we observe decrease in DLU obtained over time (for 48 hours and 116 hours). Maximum response is seen at 21 hour exposure.

**Figure 5:** DLU vs. Volume for different exposure time
Also, we wanted to check the signal sensitivity with respect to time. So, we normalized counts obtained from the exposure for various time points with respect to time and plotted a graph. Results are shown in Table 4 and were plotted as shown in Figure 6.

**Table 4:** DLU/minute vs. Exposure time for different amount of radioactive material

<table>
<thead>
<tr>
<th>Volume (ul)</th>
<th>DLU/Minute (x10^5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12 Minutes</td>
</tr>
<tr>
<td>0.50</td>
<td>2.87</td>
</tr>
<tr>
<td>1.00</td>
<td>4.97</td>
</tr>
<tr>
<td>2.00</td>
<td>8.83</td>
</tr>
<tr>
<td>4.00</td>
<td>26.99</td>
</tr>
<tr>
<td>8.00</td>
<td>51.77</td>
</tr>
</tbody>
</table>

**Figure 6:** DLU/minute vs. Exposure time (hours) for different volume of radioactive material

Here we can see fading of signal becomes very evident after 21 hours, for the 48 hours and for 116 hours time points.
So, from the results shown above, it was determined that 1 day (24 hours) was optimum exposure time for our analysis because it gave us enough signal to measure radioactivity in our sample and we were also able to avoid unnecessary signal fading with longer time of exposure.

**3.3 QUANTITATIVE TLC WITH THE HELP OF STORAGE PHOSPHOR IMAGER**

**3.3.1 Radiosynthesis Analysis Using Storage Phosphor Imager**

We prepared radiolabeled $2-O-[1^\prime,14^C]agranidonyl-1-O$-stearoyl-$sn$-glycerol (1,2-diacylglycerol, 1,2-DAG) that is a substrate for diacylglycerol lipase (DAGL). This DAGL enzyme converts 1,2-DAG to 2-arachidonoylglycerol (2-AG) that is a type of endogeneous cannabinoid (endocannabinoid).[35] We synthesized the radiolabeled 1,2-DAG with an objective of using it for the assay of h-DAGL enzyme activity as well as to check the effect of a series of novel enzyme inhibitors. $2-O-[1^\prime,14^C]arachidonoyl-1-O$-stearoyl-$sn$-glycerol was prepared from the commercially available substrate $2-O-[1^\prime,14^C]arachidonoyl-1-O$-stearoyl-$sn$-glycero-$3$-phosphocholine. The phosphocholine substrate was reacted with phospholipase C obtained from Bacillus cereus at a hexane and phosphate buffer interface. The reaction was performed on a 20 µCi scale and gave radiolabeled 1,2-DAG in a yield of 83% and radiochemical purity of 99% by TLC.

**3.3.1.1. Purity Of The Substrate:**

The substrate $2-O-[1^\prime,14^C]arachidonoyl-1-O$-stearoyl-$sn$-glycero-$3$-phosphocholine (GE Healthcare CFA655) had a specific activity of 57 mCi/mmol and was found to be >98% pure by TLC (60:30:5 chloroform/methanol/water system, Rf 0.33).
3.3.1.2. Reaction:
Phospholipase C (Sigma P9439 from Bacillus cereus) was reconstituted with water. Hexane was used as the upper phase for the reaction. Fresh enzyme solution was added every 20 min for 2 hours to catalyze the hydrolysis. The hexane phase was separated from aqueous phase. The addition of acetone to reach acetone/hexane to 30:70 was done. Then, complete removal of trace starting material was done by filtration through a short silica gel pad that was then washed with 30:70 acetone/hexane mixture.

Figure 7: TLC of 2-[1’-14C]arachidonoyl-1-sn-stearoyl-glycerol (Rf 0.95) from the phospholipase C hydrolysis of the phosphodiester 2-[1’-14C] arachidonoyl-1-stearoyl-sn-glycero-3-phosphocholine

3.3.1.3 TLC:
TLC was carried out for the filtration elution as well as for the column wash. All of the TLC plates used were silica gel 60 on glass of 250 µM thickness (E. Merck). The spots were made on the TLC plate, the plate was eluted with a 60:30:5 chloroform/methanol/water mixture, then
removed and allowed to dry thoroughly. The TLC plate was placed in contact with a multisensitive phosphor screen (Perkin-Elmer) for 24 hours. The digital image was produced on a Perkin-Elmer Cyclone Phosphor Imager and is shown in Figure 7. Lane 1 (left) is the filtration elution and lane 2 (right) is the column wash. The purity of both spots was found to be 99% (Rf value of 0.95). There was no evidence of starting material, which if was present, would have shown a 0.33 Rf value.

3.3.2 Diacylglycerol Lipase (DAGL) Activity Using Storage Phosphor Imager

We also used the same TLC technique to check the enzyme activity of murine diacylglycerol lipase subtype beta (mDAGL-β) and the effects of some inhibitors. The radioactive substrate prepared above, 2-O-[1'-'14C]arachidonoyl-1-O-stearoyl-sn-glycerol (radiolabeled 1,2-DAG), was hydrolyzed with mDAGL-β and various inhibitors were evaluated to assay their inhibition activity. Figure 8 shows the results of this experiment.

The TLC plate was exposed for 24 hours to a medium sensitivity plate. Then the plate was analyzed using the cyclone storage phosphor imager (Figure 8). The image obtained was analyzed using OptiQuant software. Regions with radioactivity were circled and amount of radioactivity was determined as DLU. T1 region (1-12) represents the substrate. T2 region (1-12) represents product produced due to the enzyme activity. T3 and T4 region are the copies of T1 and T2 respectively on the part of the plate where no radioactive substance was exposed to determine the background counts. Results are shown in table 5. The percent conversion was calculated as:

\[
\text{% Conversion} = \frac{[T2-T4/(T1-T3)+(T2-T4)]}{100}
\]
Lane 1 contained no enzyme and no inhibitor. No conversion was seen as this was the negative control with a minimum conversion response. Lanes 2-11 contained the mDAGL-β enzyme. Lane 2 had ten minute pre-treatment with vehicle (dimethysulfoxide solvent) before the radioactive substrate was added, and is the positive control with the maximum response signal for the assay. Lanes 3-11 had inhibitors present in the pretreatment solvents. Lanes 3 to 9 were novel DAGL inhibitors prepared in Center of Drug Discovery (NEU, MA). Lane 10 contained 10 µM tetrahydrolipstatin (THL) and Lane 11 contained 1.0 µM THL in the pretreatment. The IC₅₀ for THL is below 1 µM, while none of the tested compounds (Lanes 3-9) showed significant inhibition even at the 10 µM concentration. Lane 12 was another positive control with commercially available lipoprotein lipase, which has hydrolytic activity comparable to the diacylglycerol lipases, but can also hydrolyze the product 2-AG to glycerol and labeled arachidonic acid that is evidenced by the low Rf spot.

**Figure 8:** DAGL assay TLC plate 1
Table 5: Analysis of DAGL assay plate 1

<table>
<thead>
<tr>
<th>Lane</th>
<th>Gross DLU (x10^6)</th>
<th>DLU-BG (x10^6)</th>
<th>%Conversion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T1</td>
<td>T2</td>
<td>T3</td>
</tr>
<tr>
<td>1</td>
<td>90.2</td>
<td>0.2</td>
<td>0.6</td>
</tr>
<tr>
<td>2</td>
<td>69.5</td>
<td>3.5</td>
<td>0.5</td>
</tr>
<tr>
<td>3</td>
<td>68.5</td>
<td>3.4</td>
<td>0.7</td>
</tr>
<tr>
<td>4</td>
<td>61.1</td>
<td>3.5</td>
<td>0.6</td>
</tr>
<tr>
<td>5</td>
<td>66.8</td>
<td>2.6</td>
<td>0.6</td>
</tr>
<tr>
<td>6</td>
<td>69.0</td>
<td>3.8</td>
<td>0.8</td>
</tr>
<tr>
<td>7</td>
<td>62.1</td>
<td>3.1</td>
<td>0.8</td>
</tr>
<tr>
<td>8</td>
<td>56.7</td>
<td>3.3</td>
<td>0.7</td>
</tr>
<tr>
<td>9</td>
<td>60.4</td>
<td>3.0</td>
<td>0.6</td>
</tr>
<tr>
<td>10</td>
<td>72.9</td>
<td>0.5</td>
<td>0.8</td>
</tr>
<tr>
<td>11</td>
<td>69.1</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>12</td>
<td>3.2</td>
<td>38.1</td>
<td>0.4</td>
</tr>
</tbody>
</table>

The second set of compounds was screened in another experiment that was carried out to check their activity as DAGL inhibitors. The same negative control was used in Lane 1 and positive controls with mDAGL-β in Lane 2 and lipoprotein lipase in Lane 13. Lane 11 contained 10 µM tetrahydrolipstatin (THL) and Lane 12 contained 1.0 µM THL in the pretreatment. None of the novel compounds in Lanes 3-10 inhibited mDAGL-β at the 10 µM concentrations used. The results obtained are shown in Figure 9 and Table 6.
**Figure 9:** DAGL assay TLC plate 2

![DAGL assay TLC plate 2](image)

**Table 6:** Analysis of DAGL assay plate 2

<table>
<thead>
<tr>
<th>Lane</th>
<th>Gross DLU (x10⁶)</th>
<th>DLU-BG (x10⁶)</th>
<th>%Conversion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T1</td>
<td>T2</td>
<td>T3</td>
</tr>
<tr>
<td>1</td>
<td>41.6</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>2</td>
<td>37.0</td>
<td>0.9</td>
<td>0.2</td>
</tr>
<tr>
<td>3</td>
<td>33.3</td>
<td>0.9</td>
<td>0.2</td>
</tr>
<tr>
<td>4</td>
<td>35.3</td>
<td>1.0</td>
<td>0.2</td>
</tr>
<tr>
<td>5</td>
<td>33.7</td>
<td>1.0</td>
<td>0.2</td>
</tr>
<tr>
<td>6</td>
<td>33.3</td>
<td>1.0</td>
<td>0.2</td>
</tr>
<tr>
<td>7</td>
<td>32.3</td>
<td>1.3</td>
<td>0.3</td>
</tr>
<tr>
<td>8</td>
<td>32.5</td>
<td>1.1</td>
<td>0.3</td>
</tr>
<tr>
<td>9</td>
<td>33.0</td>
<td>1.4</td>
<td>0.3</td>
</tr>
<tr>
<td>10</td>
<td>33.0</td>
<td>1.3</td>
<td>0.3</td>
</tr>
<tr>
<td>11</td>
<td>34.0</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>12</td>
<td>34.6</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>13</td>
<td>1.6</td>
<td>19.7</td>
<td>0.2</td>
</tr>
</tbody>
</table>
3.4. VIBRATOME SECTIONING

3.4.1. ANIMALS

All animal procedures were conducted in accordance to the NIH Guide for the use of Laboratory Animals with a protocol approved by Institutional Animal Care and Use Committee. Male Swiss Webster mice were purchased from Charles River Laboratories (Cambridge, MA), maintained on a 12-hr light-dark cycle (7am-7pm) and given access to food and water ad libitum. Care was taken to minimize pain and discomfort for all procedures. All mice weighed from 25-30 gm.

3.4.2. SECTIONING

Sectioning was done with the help of vibrating microtome (World Precision Instrument – NVSL Manual Advance Vibroslice). Vibrating blade microtomes are extensively used in biological research for sectioning of non frozen tissues. Freezing or embedding tissue before sectioning is useful, especially when very thin slice like 1 µm are required. However, these treatments can create artifacts, alter morphology, destroy enzyme activity and produce other deleterious effects. Also, it is hard to preserve the sections produced in this manner. Another alternative to this is the use of slicing method involving vibratome, which enables us to section the fresh and unfrozen tissues by moving a vibrating blade through a specimen submerged in a liquid bath. This method preserves the ultrastructure which is essential for experiments utilizing microscopy, immunohistochemistry [36], cell tracing and living brain slicing techniques [37].

Brain was cut to form a flat base. Brain stem and cerebellum were removed. The flat end of the brain was glued (ethyl cyanoacrylate) to the plate in the center of the stage. The stage of the microtome was placed in the tissue bath. The cutting head was rotated
vertically and a razor blade was inserted into the blade holder; the blade was tightened in place by tightening the stainless steel screw. The cutting head was rotated ventrally until the lower surface of the blade rested against 20-25 angle of the tissue [38]. The tissue was then moved to the most extreme position in the front of the blade, by rotating the screw beneath the tissue bath. The bath was filled with enough of saline to cover the specimen. The circuit was turned on and the tissue was manually advanced toward the vibrating blade and a section was cut. The process was concluded by moving the tissue to the most extreme position in front of the blade. The section was removed from the bath with a paint brush and mounted on the slide. Then the blade was lowered vertically by 500 μm or 300 μm by rotating the knob and another section was cut using the same procedure described above. After all sectioning was completed, the circuit was turned off and stage of microtome was removed and tissue bath was emptied.

Vibratome frequency was set at its maximum amplitude. It is suggested in the literature that for soft tissue use the highest amplitude frequency and slow progression of tissue through the blade [38].

3.4.3. BRAIN SLICES AUTORADIOGRAPHY AFTER INJECTING [2-14C] DEOXYGLUCOSE

First we started off with cutting 500 μm slices of mouse brain after injecting 14C labeled 2-deoxyglucose. The mice were sacrificed after 30 minutes and after 1 hour of injections. 500 μm slices were taken and then autoradiography was performed. The images obtained are shown in Figure 10.
Figure 10: Autoradiographic images after injecting $^{14}$C labeled 2-deoxyglucose (500 μm).

Regions were marked within striatum, Cortex, hippocampus, thalamus and hypothalamus and DLU/mm$^2$ for each of the region were determined and averaged. Then the ratio of average of DLU/mm$^2$ of each region and that of striatum was taken to determine any significant difference between them. And following results were obtained.
Table 7: $^{14}$C labeled 2-deoxyglucose 500 μ slices

<table>
<thead>
<tr>
<th>Regions</th>
<th>30 Minutes</th>
<th>1 hour</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average DLU/mm$^2$</td>
<td>Ratio (DLU/mm$^2$ of region/DLU/mm$^2$ of striatum)</td>
</tr>
<tr>
<td>Striatum</td>
<td>124,177</td>
<td>1.00</td>
</tr>
<tr>
<td>Cortex</td>
<td>130,457</td>
<td>1.05</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>120,922</td>
<td>0.97</td>
</tr>
<tr>
<td>Thalamus</td>
<td>151,001</td>
<td>1.22</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>106,272</td>
<td>0.86</td>
</tr>
</tbody>
</table>

Slices of 300 μm were then taken after sacrificing mouse 30 minutes post injection of $^{14}$C labeled 2-deoxyglucose. Slices were exposed to super sensitive plates for 24 hours and the digital images were created using the cyclone storage phosphor imager.
Figure 11: Autoradiographic images of mouse brain slices obtained after administration of $^{14}$C labeled 2-deoxyglucose (300 μ)

Three slices from the above image are shown below to show how different regions can be distinguished from each other within a brain slice.

Figure 12: Individual brain slice specimens

1) Cortex
2) Periform Cortex
3) Anterior Commissural
4) Corpus Callosum
5) Olfactory Tubercle
6) Caudate Putamen
Regions were marked within striatum, Cortex, hippocampus, thalamus, hypothalamus and corpus callosum and DLU/mm² for each of the region were determined and averaged. Then the ratio of average of DLU/mm² of each region and that of striatum was taken to determine any significant difference between them. And following results were obtained.
Table 8: $^{14}$C labeled 2-deoxyglucose 300 μ slices

<table>
<thead>
<tr>
<th>Region</th>
<th>Average DLU/mm²</th>
<th>Ratio(DLU/mm² of region/DLU/mm² of striatum)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Striatum</td>
<td>659,203</td>
<td>1</td>
</tr>
<tr>
<td>Cortex</td>
<td>674,072</td>
<td>1.022556</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>614,954</td>
<td>0.932875</td>
</tr>
<tr>
<td>Thalamus</td>
<td>852,007</td>
<td>1.29248</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>640,269</td>
<td>0.971277</td>
</tr>
<tr>
<td>Corpus callosum</td>
<td>266,006</td>
<td>0.403527</td>
</tr>
</tbody>
</table>

Thus, there we can find higher amount of radioactivity in thalamus, while the least was present in corpus callosum among these regions.

After looking at these images we decided 300 μm slices to be optimum for our experiments.

3.4.4. BRAIN SLICES AUTORADIOGRAPHY AFTER INJECTING $[1^{-14}C]$ ACETATE

Three mice were taken for this group. They were injected with $[1^{-14}C]$acetate and were sacrificed after 30 minutes. The mice were then dissected to take out the brain and then the brains were spliced into slices of 300 μm with the vibrating microtome. Then the slices were allowed to dry for one day and then exposed to supersensitive plate for one day along with the standard prepared. The plate was then scanned with storage phosphor imager at 600 dpi. Images obtained are shown in Figure 13. Then analysis was done by
first of all subtracting the background DLU/mm² from that obtained with the image and then dividing those values by that obtained with standard. When values were obtained for all regions, they were divided with respect to striatum and then these ratios were averaged and standard deviation was calculated for each region. The results are shown in table 9.

**Figure 13:** Images after injecting [1-¹⁴C]acetate.

Mouse 1: [1-¹⁴C]acetate.
Mouse 2: [1-\textsuperscript{14}C]acetate

Mouse 3: [1-\textsuperscript{14}C]acetate
Table 9: Results for [1-^{14}\text{C}]acetate

<table>
<thead>
<tr>
<th>Regions</th>
<th>Mouse 1</th>
<th>Mouse 2</th>
<th>Mouse 3</th>
<th>Average</th>
<th>Std dev</th>
</tr>
</thead>
<tbody>
<tr>
<td>Striatum</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Cortex</td>
<td>1.99</td>
<td>1.95</td>
<td>2.54</td>
<td>2.16</td>
<td>0.33</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>2.98</td>
<td>2.86</td>
<td>2.95</td>
<td>2.93</td>
<td>0.06</td>
</tr>
<tr>
<td>Thalamus</td>
<td>2.26</td>
<td>2.68</td>
<td>2.53</td>
<td>2.49</td>
<td>0.21</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>2.47</td>
<td>2.45</td>
<td>2.77</td>
<td>2.56</td>
<td>0.18</td>
</tr>
</tbody>
</table>

3.4.5. BRAIN SLICES AUTORADIOGRAPHY AFTER INJECTING [1-^{14}\text{C}] ACETALDEHYDE

Similarly, four mice were taken for this group. They were injected with [1-^{14}\text{C}] acetaldehyde and were sacrificed after 30 minutes. The mice were then dissected to take out the brain and then the brains were spliced into slices of 300 μ with the vibrating microtome. Then the slices were allowed to dry for one day and then exposed to supersensitive plate for one day along with the standard prepared. The plate was then scanned with storage phosphor imager at 600 dpi. Images obtained are shown in Figure 14. Then analysis was done by first of all subtracting the background DLU/mm\(^2\) from that obtained with the image and then dividing those values by that obtained with standard. When values were obtained for all regions, they were divided with respect to striatum of all mice and then these ratios were averaged and standard deviation was calculated for each region. The results are shown in Table 10.
**Figure 14:** Images after injecting $[1^{-14}C]$acetaldehyde.

Mouse 1: $[1^{-14}C]$acetaldehyde

Mouse 2: $[1^{-14}C]$acetaldehyde
Mouse 3: [1-^{14}C]acetaldehyde

Mouse 4: [1-^{14}C]acetaldehyde
Table 10: Results for [1-14C]acetataldehyde

<table>
<thead>
<tr>
<th>Regions</th>
<th>Mouse 1</th>
<th>Mouse 2</th>
<th>Mouse 3</th>
<th>Mouse 4</th>
<th>Average</th>
<th>Std dev</th>
</tr>
</thead>
<tbody>
<tr>
<td>Striatum</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0.00</td>
</tr>
<tr>
<td>Cortex</td>
<td>1.98</td>
<td>1.94</td>
<td>2.06</td>
<td>1.79</td>
<td>1.94</td>
<td>0.11</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>2.09</td>
<td>2.17</td>
<td>1.66</td>
<td>1.48</td>
<td>1.85</td>
<td>0.33</td>
</tr>
<tr>
<td>Thalamus</td>
<td>2.19</td>
<td>2.26</td>
<td>2.08</td>
<td>2.20</td>
<td>2.18</td>
<td>0.07</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>2.36</td>
<td>2.34</td>
<td>2.15</td>
<td>2.22</td>
<td>2.27</td>
<td>0.10</td>
</tr>
</tbody>
</table>

The comparison for distribution of [1-14C]acetate and [1-14C]acetaldehyde in various regions is shown in Table 11.

Table 11: Comparison of distribution of label from 14C acetate and acetaldehyde

<table>
<thead>
<tr>
<th>Regions</th>
<th>[1-14C]acetate</th>
<th>[1-14C]acetaldehyde</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average</td>
<td>Std Deviation</td>
</tr>
<tr>
<td>Striatum</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Cortex</td>
<td>2.16</td>
<td>0.33</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>2.93</td>
<td>0.06</td>
</tr>
<tr>
<td>Thalamus</td>
<td>2.49</td>
<td>0.21</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>2.56</td>
<td>0.18</td>
</tr>
</tbody>
</table>
Figure 15: Comparison of distribution of label from $^{14}$C acetate and acetaldehyde.

Thus, a higher ratio of DLU/mm$^2$ was obtained for hippocampus with acetate compared to acetaldehyde. There were no significant difference for other regions between acetate and acetaldehyde.

Higher amounts of radioactivity were found in all of the brain regions for acetaldehyde compared to acetate except for hippocampus. This is shown in Figure 16.

(*: $p=0.003$)
3.5. MICRODISSECTION:

Phosphor imaging plate autoradiography of vibratome sections showed quite similar distribution patterns but indicated a greater relative concentration of label in hippocampus from acetate than from acetaldehyde. To further evaluate this finding, we conducted microdissection experiments 30 minutes after IV administration of 1 μCi of [1-\textsuperscript{14}C]acetate and [1-\textsuperscript{14}C]acetaldehyde to male swiss webster mice weighing 23-27 g. A significantly greater fraction of administered radioactivity was found in brain after acetaldehyde than after acetate (1.98 ± 0.25 versus 1.23 ± 0.12 percent injected radioactivity per gram (%IA/g), n=8, p<0.001).

Results are shown in Table 12.
TABLE 12: Microdissection results for [1-14C]acetate and acetaldehyde

<table>
<thead>
<tr>
<th>Regions</th>
<th>ACETALDEHYDE</th>
<th></th>
<th>ACETATE</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Standard Deviation</td>
<td>Mean</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>3.65</td>
<td>0.52</td>
<td>2.12</td>
<td>0.47</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>2.15</td>
<td>0.23</td>
<td>1.54</td>
<td>0.25</td>
</tr>
<tr>
<td>Striatum</td>
<td>1.81</td>
<td>0.20</td>
<td>1.15</td>
<td>0.15</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>2.12</td>
<td>0.21</td>
<td>1.21</td>
<td>0.35</td>
</tr>
<tr>
<td>Thalamus</td>
<td>2.14</td>
<td>0.22</td>
<td>1.37</td>
<td>0.11</td>
</tr>
<tr>
<td>Temporal Cortex</td>
<td>2.17</td>
<td>0.35</td>
<td>1.46</td>
<td>0.18</td>
</tr>
<tr>
<td>Frontal Cortex</td>
<td>1.90</td>
<td>0.45</td>
<td>1.57</td>
<td>0.24</td>
</tr>
<tr>
<td>Rest of brain</td>
<td>1.88</td>
<td>0.27</td>
<td>1.16</td>
<td>0.09</td>
</tr>
<tr>
<td>Whole brain</td>
<td>1.98</td>
<td>0.25</td>
<td>1.23</td>
<td>0.12</td>
</tr>
</tbody>
</table>

%IA/g = % Injected Radioactivity/gram

Figure 17: Microdissection results for [1-14C]acetate and acetaldehyde

For acetaldehyde, the brain regional concentration of label varied from 3.65 ± 0.52 to 1.81 ± 0.20 %IA/g and the descending order of radioactivity concentration was
hypothalamus, temporal cortex, hippocampus, thalamus, cerebellum and dorsal striatum. For acetate they varied from 2.12 ± 0.47 to 1.15 ± 0.15 %IA/g, and the order was hypothalamus, frontal cortex, hippocampus, temporal cortex, thalamus, cerebellum and dorsal striatum. When results were normalized to total brain radioactivity for each radiotracer, a significantly higher hippocampus to whole brain ratio was confirmed for acetate compared to acetaldehyde (1.25 ± 0.20 versus 1.09 ± 0.12 %IA/g, p<0.01), shown in Figure 17. A higher frontal cortex to whole brain ratio was also seen (1.28 ± 0.19 versus 0.96 ± 0.23 %IA/g, p<0.01). For all other regions there was no significant difference between acetate and acetaldehyde.
4. CONCLUSION:

We obtained distribution maps of good quality for $^{14}$C after injecting [1-$^{14}$C]acetate and [1-$^{14}$C]acetaldehyde illustrating the usefulness of the vibratome/phosphorimager technique. Quantitative analysis of the images showed significant differences in relative radioactivity concentration between acetaldehyde and acetate in the hippocampus, with acetate levels higher than acetaldehyde. To confirm the results, microdissection experiments of mice brains were conducted, and radioactivity quantified using liquid scintillation counting. When the results were normalized to total brain radioactivity for each radiotracer, a significantly higher hippocampus to whole brain ratio was confirmed for acetate compared to acetaldehyde. In contrast to the relative higher uptake of label in hippocampus after administration of acetate, the absolute brain uptake was higher for acetaldehyde in all regions. This suggests that there is not only brain uptake of acetate derived from acetaldehyde by oxidation in periphery, but that there is also formation of acetate within the CNS. It is also possible that there is formation of adducts of acetaldehyde with amine neurotransmitters in the brain. However, our data do not clearly support the formation of long-lived acetaldehyde-dopamine adducts, since levels of $^{14}$C from acetaldehyde were not higher in striatum, the brain region that contains the highest concentrations of dopamine. It is possible, however, that relatively unstable species are formed from acetaldehyde, but these do not make a large contribution to brain regional concentrations of radioactivity at 30 minutes post injection.
5. FUTURE DIRECTIONS:

Studies involving identification of the chemical forms of radioactivity in brain regions would be necessary to explain our findings of higher absolute levels of radioactivity after acetaldehyde, but lower relative uptake of acetaldehyde in the hippocampus. These might involve extraction of $^{14}$C from brain, followed by high performance liquid chromatography using detection of radioactivity. Studies with metabolic inhibitors, for example, 4-methylpyrazole (alcohol dehydrogenase) or cyanamide (aldehyde dehydrogenase) might also give useful information on the behavior of acetaldehyde within the brain.
6. REFERENCES

The preparation of 2-O-[1′-14C]arachidonoyl-1-O-stearoyl-sn-glycerol

Richard I. Duclos Jr, a* S. John Gatley, b Shachi R. Bhatt b and Meghan Johnston a

2-O-Arachidonoyl-1-O-stearoyl-sn-glycerol is the most abundant molecular species of the 1,2-diacyl-sn-glycerol signaling lipids in neural tissue. The facile preparation of 2-O-[1′,14C]arachidonoyl-1-O-stearoyl-sn-glycerol from 2-O-[1′,14C]arachidonoyl-1-O-stearoyl-sn-glycero-3-phosphocholine at a hexane and phosphate buffer interface with phospholipase C was demonstrated on a 20 μCi scale in 83% radiochemical yield. The specific activity of the product 2-O-[1′,14C]arachidonoyl-1-O-stearoyl-sn-glycerol was 57.0 mCi/mmol and the radiochemical purity was determined to be >99% by TLC. The hydrolysis of this lipid biosynthetic intermediate with lipoprotein lipase was shown to produce 2-O-[1′,14C]arachidonoylglycerol (2-AG). The 14C-radiolabeled monoacylglycerol 2-AG is an endogenous cannabinoid receptor-signaling molecule (endocannabinoid).

Keywords: 2-O-arachidonoyl-1-O-stearoyl-sn-glycerol; 14C-labeled; diacylglycerol; 2-arachidonoylglycerol; 2-AG

Introduction

1,2-Diacyl-sn-glycerols have important physico-chemical and juxtacrine signaling functions.1–4 Although diacylglycerols are found in extremely low concentration in cell membranes, 2-O-arachidonoyl-1-O-stearoyl-sn-glycerol is the most abundant molecular species in brain6 and peripheral nerve.7 The hydrolysis of this lipid by diacylglycerol lipase (DAGL) produces the endocannabinoid 2-O-arachidonoylglycerol (2-AG).7,8 The endocannabinoid system is the focus of our research and includes the two most abundant endocannabinoid lipids, 2-AG and N-arachidonoylthanolamine (AEA), each having distinct signaling functions.5,10 Radiolabeled 2-O-arachidonoyl-1-O-stearoyl-sn-glycerol (2) (see Scheme 1) was required for our studies of DAGL enzyme activity and the biosynthesis of 14C-labeled endocannabinoid 2-AG (3). 2-O-[1′,14C]Arachidonoyl-1-O-stearoyl-sn-glycerol (2) has recently been used to assay the activity of hDAGL-α preparations,11,12 including in the presence of inhibitors, but is no longer commercially available.

This report describes the convenient preparation of radiolabeled 2-O-[1′,14C]arachidonoyl-1-O-stearoyl-sn-glycerol (2) via a biomimetic route that uses the phosphocholine phosphodiester group for protection. Syntheses of labeled 1,2-diacyl-sn-glycerols have previously used the 3-phosphocholine group,13–18 3-phosphate group,19 or 3-phosphinositole group20 as protecting groups in chemo-enzymatic syntheses. Other classical protecting groups have also been used in purely chemical syntheses of unlabeled21–23 and labeled11,12-diacyl-sn-glycerols and labeled rac-1,2-diacylglycerols,24,25 although such routes involve the technical challenge of avoiding acyl group migration during a microscale radiosynthesis.

Our conversion of the commercially available labeled substrate 2-O-[′14C]arachidonoyl-1-O-stearoyl-sn-glycero-3-phosphocholine (1) utilized the regiospecificity of the enzymatic hydrolysis by phospholipase C.26 The hydrolysis of the phosphatidylcholine 1 with phospholipase C from Bacillus cereus was performed at neutral pH. The mild reaction conditions are critical for 1,2-diacyl-sn-glycerols, where variation of pH from 7 or increased temperature results in rearrangement of 1,2-diacyl-sn-glycerol to 1,3-diacyl-sn-glycerol.27–29 1,2-Diacylglycerols are reportedly more stable in hydrocarbon solution.29 The 1,2-diacylglycerol and 1,3-diacylglycerol isomers are readily separable by analytical TLC28,31,32 and HPLC.24,25 However, our 1,2-diacyl-sn-glycerol isolation following the enzymatic protecting group cleavage required only flash chromatographic filtration to remove trace amounts of unreacted starting material as well as the phosphocholine salt byproduct origin materials. We had previously used this methodology with unlabeled 2-O-arachidonoyl-1-O-stearoyl-sn-glycero-3-phosphocholine on scales of up to 10 mg to give unlabeled 2-O-arachidonoyl-1-O-stearoyl-sn-glycerol that was homogeneous by TLC (30:70 acetone/hexane, developing with phosphomolybdic acid reagent) and characterized by 1H NMR.

Results and discussion

The substrate 2-O-[1′,14C]arachidonoyl-1-O-stearoyl-sn-glycero-3-phosphocholine (1) (GE Healthcare CFA655) contained a
radiochemical purity) by TLC (Rf 0.00, 30:70 acetone/hexane; Rf 0.33, 60:30:5 chloroform/methanol/water). The tailing of the 1,2-diacyl-

\( \text{sn} \)

2-glycerol (\( \text{sn} \)) enzymatically at a hexane and phosphate buffer interface. Analytical TLC (86:14:0.75 chloroform/methanol/aqueous ammonium hydroxide) of the enzymatic conversion of 2-O-[\( ^{14}\text{C} \)]arachidonoyl-1-O-stearoyl-sn-glycerol (\( 2, \text{Rf } 0.92 \)) to 2-O-[\( ^{14}\text{C} \)]arachidonoylglycerol (3, 2-AG) was not further characterized as this radiolabeled 2-AG was prepared only on an analytical scale.

Thus, the facile preparation of the \( ^{14}\text{C} \)-radiolabeled 1,2-diacyl-sn-glycerol signaling lipid molecule 2 free of any 1,3-diacyl-sn-glycerol rearrangement byproduct was demonstrated on a 20 \( \mu \text{Ci} \) scale. This 2-O-[\( ^{14}\text{C} \)]arachidonoyl-1-O-stearoyl-sn-glycerol (2) activates protein kinase C and is the biosynthetic

\( ^{14}\text{C} \)-label in the carbonyl of the arachidonoyl group, and the [\( ^{1}
\text{C} \text{]-arachidonoyl group was assayed to be } 97.5\% \text{ in the sn-2 position. This substrate } 1 \text{ had a specific activity of } 57.0 \text{ mCi/mmol and was found to be nearly homogeneous (}>98\% \text{ radiochemical purity}) by TLC (Rf 0.00, 30:70 acetone/hexane; Rf 0.33, 60:30:5 chloroform/methanol/water). The phospholipase C (Sigma P9439 from \( \text{B. cereus} \)) was reconstituted with water to give a phosphate buffered solution that could be frozen, stored at \(-20^\circ \text{C} \), and reused. Generally, PLC hydrolyses have been run at an interface of diethyl ether and aqueous buffer,\(^{13,16,17,33,34}\) We found that the reaction proceeds when hexane is used, although the enzyme activity does fall off and the conversion must be driven to completion by adding fresh enzyme solution at 20 min intervals over 2 h. The phase separation of hexane from aqueous buffer was easier than when using diethyl ether. There is still some solubility of the oily phosphocholine substrate from aqueous buffer was easier than when using diethyl ether.

A small amount of 1-O-[\( ^{14}\text{C} \)]arachidonoyl-1-O-stearoyl-sn-glycerol (2) was 57.0 mCi/mmol and the radiochemical purity was determined to be >99\% by TLC (Rf 0.28, 4:96 acetone/chloroform; Rf 0.49, 30:70 acetone/hexane; Rf 0.95, 60:30:5 chloroform/methanol/water). The tailing of the 1,2-diacyl-sn-glycerol 2 spot was comparable with that seen using a related solvent system.\(^{29}\) A small amount of 1-O-[\( ^{14}\text{C} \)]arachidonoyl-2-O-stearoyl-sn-glycerol may be present from the starting phosphocholine. Rearrangement of 2-O-[\( ^{14}\text{C} \)]arachidonoyl-1-O-stearoyl-sn-glycerol (2) to 3-O-[\( ^{14}\text{C} \)]arachidonoyl-1-O-stearoyl-sn-glycerol would have given a slightly higher Rf spot for this byproduct in the TLC analysis with 30:70 acetone/hexane.\(^{22-23}\) The presence of the rearrangement impurity could affect the study of the DAGL enzyme where the 1,3-diacylglycerol byproduct could act as a substrate or inhibitor.

We used the 2-O-[\( ^{14}\text{C} \)]arachidonoyl-1-O-stearoyl-sn-glycerol (2) for our preliminary experiments with lipoprotein lipase (from \( \text{Pseudomonas sp.} \), Sigma), which has excellent 1,2-diacyl-sn-glycerol lipase activity with high selectivity for the sn-1 acyl group.\(^{35-37}\) The lipoprotein lipase readily hydrolyzed 2-O-[\( ^{14}\text{C} \)]arachidonoyl-1-O-stearoyl-sn-glycerol (2) enzymatically at a hexane and phosphate buffer interface. Analytical TLC (86:14:0.75 chloroform/methanol/aqueous ammonium hydroxide) of the enzymatic conversion of 2-O-[\( ^{14}\text{C} \)]arachidonoyl-1-O-stearoyl-sn-glycerol (2, Rf 0.92) to 2-O-[\( ^{14}\text{C} \)]arachidonoylglycerol (3, Rf 0.59) after 15 min with 58 units of lipoprotein lipase (from \( \text{Pseudomonas sp.} \)).
precursor of the endocannabinoid signaling molecule 2-AG 3. The regioselective hydrolysis of 14C-radiolabeled 1,2-diacyl-sn-glycerol 2 by an enzyme with DAGL activity to give 2-AG 3 was also demonstrated.

Experimental

General

All TLC used silica gel 60 on glass that was 250 μm thickness (E. Merck). After elutions and thorough air drying, latent images of the TLC plates were made on multisensitive phosphor screens (Perkin–Elmer) that were then quantified on a Perkin–Elmer Cyclone phosphoimaging system.

2-O-[11,14C]Arachidonoyl-1-O-stearyl-sn-glycerol (2)

In a 1.5 mL screw-top Wheaton vial was added 19.95 μCi of 2-O-[11,14C]arachidonoyl-1-O-stearyl-sn-glycerol-3-phosphocholine (1) (GE Healthcare CFA655, 57.0 mCi/mmol) in 1:1 toluene:ethanol in 200 μL portions that were evaporated to dryness in a gentle argon stream at ambient temperature. The phosphatidylcholine 1 residue was immediately partitioned between 100 μL of phospholipase C (Sigma P9439 from B. cereus, 0.2 unit/μL, phosphate buffer, pH 7.0) and 400 μL of hexane and stirred gently with a triangular magnet at ambient temperature under argon. Additional 50 μL portions of phospholipase C in buffer were delivered by pipette to the bottom (aqueous) phase at 20 min intervals for a total of 400 μL. A sample of the hexane phase was removed after 2 h and found to contain > 95% product 1,2-diacyl-sn-glycerol 2 by TLC (Rf 0.49, 30:70 acetone/hexane; Rf 0.95, 60:30:5 chloroform/methanol/water). The bottom (aqueous) phase was removed by pipette, found to contain only 3% of the radioactivity by scintillation counting, and was discarded. The top (hexane) phase was transferred to a clean screw-top vial and dried briefly over Na2SO4. The dry hexane solution was transferred to a new vial, and was transferred to a clean screw-top vial and dried briefly over hexane (Rf 0.49), however, there was no evidence of the elution. The tailing appeared more significant in 30:70 acetone/hexane, however, there was no evidence of the rearrangement byproduct 1,3-diacyl-sn-glycerol that would have had a slightly higher Rf than that for the 1,2-diacyl-sn-glycerol 2. The product 2-O-[11,14C]arachidonoyl-1-O-stearyl-sn-glycerol (2) was stored in the acetone/hexane solution under an argon atmosphere at −20 °C when not in use and was stable for months.

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