APPENDICES

APPENDIX A

Preparation of Tissue Sections

PREPARATION OF TISSUE SECTIONS

Procedures:

1. The brains of the animals were perfused transcardially with 4% paraformaldehyde in 0.1M phosphate buffer pH 7.4.
2. Following the perfusion, the brain were removed and postfixed with 4% paraformaldehyde in 0.1M phosphate buffer overnight at 4 °C.
3. Tissues were rinse with phosphate buffer and infiltrated with 30% sucrose solution in order to provide cryoprotection.
4. The specimens were frozen rapidly with dried ice.
5. After freezing, 30 μm thick of specimens were cut on dried ice sectioning machine.
6. Sections were stored in phosphate buffer and they were picked up on slides coat with 0.5 g aluminiumsulphate and 0.1 g gelatin.

Reference

Kril JJ, Halliday GM, Svoboda MD, Cartwright H. The cerebral cortex is damaged in chronic alcoholic. **Neuroscience** 1997; 79(7): 983-8.

APPENDIX B

Preparation of Phosphate Buffer Solution

PREPARATION OF PHOSPHATE BUFFER SOLUTION

0.01 M phosphate buffer saline

Reagents:

1. KCL	0.8 g
2. KH_2PO_4	0.8 g
3. NaCl	32 g
4. $NaHPO_4$	4.6 g

Procedures:

1. Add KCL, KH_2PO_4 , NaCl and $NaHPO_4$ in 3800 ml of distilled water.
2. Dissolve these chemicals on magnetic stirrer.
3. Adjust to pH 7.4 with NaOH.
4. Make up a final volume to 4000 ml.

Reference:

Merante F, Raha S, Reod JK, Proteau G. The Simultaneous Isolation of RNA and DNA from Tissues and Cultured Cells. In: Harwood AJ, editor. **Methods in Molecular Biology: Basic DNA and RNA Protocols,** London: Humana Press; 1996. p. 3-9.

APPENDIX C

Cresyl Violet Staining for Nissl Substance

CRESYL VIOLET STANING FOR NISSL SUBSTANCE

Cresyl violet can be used to demonstrate Nissl substance. The rationale of the technique is a simple acid-base reaction, where the cationic dyes bond with the anionic RNA of the Nissl substance, plus the DNA and RNA of cell nuclei.

Staining solution:

0.5% g/ml aqueous solution of cresyl violet	100 ml
10% acetic acid	7 ml

Add 10% acetic acid 7 ml in 0.5% g/ml aqueous cresyl fast violet solution 100 ml and adjust pH to 3.5-3.8. Stand the solution at room temperature for 24-48 hours. The solution should be heated gentle and filtered before use.

Procedures:

1. Immerse slides into xylene solution for 2 times, approximate 2-3 minutes each.
2. Hydrate the sections in serial concentration of alcohol; absolute, 95% and 70% alcohol approximate 3 minutes per each process.
3. Wash the sections in distilled water.
4. Stain the sections in cresyl violet solution for 3-5 minutes. Nissl body should be violet.
5. Immerse the sections in a serial concentration of alcohol; 70%, 95% and absolute alcohol for 1 minute or longer per each process until the background is relatively clear.
6. Clear the sections in xylene solution for 2-3 minutes.
7. Mount the slides and coverslipped with DePeX permount.

Results: Nissl body: violet

Reference:

Paxinos G, Chorles W, Cresyl Violet. In: Paxinos G, Chorles W, editors. **The rat brain in stereotaxic coordinates**. London: Academic Press; 1981. p. 9-17.

APPENDIX D

Immunohistochemical Study of Tyrosine Hydroxylase (TH) Enzyme

IMMUNOHISTOCHEMICAL STUDY OF TYROSINE HYDROXYLASE (TH) ENZYME

Reagents:

1. KPBS-BT (Kreb phosphate buffer saline containing bovine serum albumin and triton x-100).
2. 0.05 M Tris-HCL buffer pH 7.6.
3. 0.5% H_2O_2 in methanol.
4. Primary antibody against tyrosine hydroxylase (TH) dilute 1:1000.
5. DAKO Strept ABC Complex/HRP duet kit. This kit consist of reagent A: Streptavidin, B: biotinylated horseradish. Reagent C: biotinylated goat antibody to mouse immunoglobulin.

Working solution of biotinylated goat antibody to mouse immunoglobulin

Add regent C 10 µl in 1 ml of KPBS-BT.

Working solution of Strept AB Complex/HRP

Add 10 µl of reagent A and B into 1 ml of KPBS-BT.

6. 0.4% H_2O_2 and diaminobenzidine in 0.05 M Tris-HCL.
7. 5% normal horse serum in KPBS-BT.

Procedures:

1. Inhibit endogenous peroxidase activity by incubating in 0.5% H_2O_2 in methanol for 30 minutes.
2. Wash slides in running tap water for 1 minute than was slides again in distilled water for 1 minute?
3. Wash slides in KPBS and KPBS-BT for 5 minutes per each process.
4. Remove excess buffer, then apply the 5% normal goat serum in KPBS-BT to the sections and incubate in moist chamber for 30 minutes in order to minimize background staining.
5. Drain off excess normal goat serum.

6. Incubate sections in mouse primary antibody against TH dilute 1:1000 in KPBS-BT at room temperature for 2 hours and then incubate at 4°C for 48 hours (This step is omitted in control slide).
7. Wash off excess antiserum and wash slides in KPBS-BT for two 7 minutes changes.
8. Drain off excess buffer and incubate slides with 100 µl of working solution of biotinylated goat antibody to mouse for 4 hours at room temperature.
9. Wash slides in KPBS-BT for two 7 minutes changes.
10. Drain off excess buffer and incubate slides with 100 µl of working solution of Strept AB Complex/HRP for 4 hours at room temperature.
11. Wash slides in KPBS-BT for 1 minute and then was slides again with KPBS for 10 minutes two times.
12. React for peroxidase activity in KPBS-BT containing 0.025% diaminobenzidine (DAB) and 0.01% H_2O_2 for 24 hours at room temperature.
13. Wash in running tap water, let dry and mount section in DePeX permount.

Reference:

Wood GS, Warnke R. Suppression of endogenous avidin-binding activity in tissues and its relevance to biodin-avidin detection systems. **J Histochem Cytochem** 1981; 29(10): 1196-1204.

APPENDIX E

Preparation of Tissue Homogenate

PREPARATION OF TISSUE HOMOGENATE

After the last injection of substances, all animals were anesthetized with intraperitoneal injection of pentobarbital sodium (Nembutal ®) at dose of 50 mg/kg BW. Brains were isolated and kept cool in ice buckets. Then these tissues were homogenized in 4 volume of 1.15% KCL with a glass Potter-Elvehjem homogenizer.

Reference:

Marzel P. General principle and procedure for drug metabolism in vitro. In: La Du BN, Mandel HG, Way EL, editors. **Fundamental of drug metabolism and drug deposition.** New York: Krieger Publishing Company; 1979. p. 527-52.

APPENDIX F

Protein Determination

PROTEIN DETERMINATION

Reagents

1. Solution A

   0.1 g of solution tartate ($Na_2C_4H_4O_6 \cdot 2H_2O$), 10 g of sodium carbonate (Na_2CO_3) and 1.2 g of sodium hydroxide (NaOH). Dissolve the chemicals in distilled water to make 500 ml.

2. Solution B

   Dissolve 0.5 g of copper sulphate ($CuSO_4 \cdot 5\ H_2O$) with distilled water 100 ml.

3. Solution C

   Freshly mix 50 ml of solution A and 1 ml of solution B.

4. Solution D

   Dilute commercial 2.0 N Folin phenol with distilled water 1:1 and use immediately.

5. Standard protein

   Dissolve bovine serum albumin 60 mg to 100 ml with distilled water.

Procedure	Volume		
	Blank	Standard	Unknown
DW	0.2	0.1	0.1
Standard BSA	-	0.1	-
Sample (1:200)	-	-	0.1
Solution C	5.0	5.0	5.0
Mix and let stand at RT for 10 min			
Solution D	0.5	0.5	0.5
Mix and let stand at RT for 1 h			
Read OD at 650 nm by spectronic 20 against the reagent blank			

Calculation

Total protein (g/100ml) = $\frac{\text{OD unknown}}{\text{OD standard}} \times \frac{\text{Concentration of Std.}}{1000} \times \text{dilution}$

100 = Conversion factor from mg% to gm%

Reference

Lowry OH, Roseburgh NJ, Farr AL, Randall RJ. Protein measurement with Folin phenol reagent. **J Biol Chem** 1951; 193(1): 265-75.

APPENDIX G

Estimation of Malondialdehyde

DETERMINATION OF MALONDIALDEHYDE

Reagents

1. 8.1% SDS (sodium dodecyl sulfate)
2. 20% acetic acid solution adjust to pH 3.5 with NaOH
3. 0.85% TBA (thiobabituric acid)
4. TMP (1,1,3,3-tetramethoxy propane), and the level of peroxide was expressed as nmol of MDA (malondialdehyde)

Procedure

1. Add the following substances in the table into the series of glass tube with screw capped.

	Blank (ml)	Standard (ml)	Unknown (ml)
*Sample	-	-	0.1
8.1% SDS	0.1	0.1	0.1
20% Acetic acid (pH 3.5)	0.75	0.75	0.75
0.8% TBA	0.75	0.75	0.75
TMP Stock standard	-	0.1	-
Distilled water	0.4	0.3	0.3

2. Heated the tubes in the water bath at 95°C for 60 min.
3. After cooling with tap water, 1.0 ml of distilled water and 5.0 ml of the mixture of n-butanol : pyridine (15:1 v/v) 2.5 ml are added and shaken vigorously.
4. After centrifugation at 4000 rpm for 10 minutes, the organic layer is taken and its absorbance at 532 nm is measured.
5. The content of lipid peroxide is expressed in terms of nmol MDA/100 mg protein.

Calibration Curve

1. Prepare a series of tubes containing TMP stock standard in water in the following concentration: 2.0 nmol/0.2 ml, 4.0 nmol/0.2 ml, 6.0 nmol/0.2 ml, 8.0 nmol/0.2 ml, 10.0 nmol/0.2 ml.
2. Perform the procedure as in step2.
3. Determine the absorbance at 532 nm. The OD was plotted against concentration of MDA which expressed as nmol MDA/100 mg protein.

Reference

Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxide in animal tissues by thiobabituric acid reaction. **Anal Biochem** 1979; 95(2): 351-358.

APPENDIX H

Enzymatic Assay of Superoxide Dismutase

ENZYMATIC ASSAY OF SUPEROXIDE DISMUATSE

Principle

Superoxide dismutase (SOD) catalyzes the reaction:

$$2O^{\bullet}_2 + 2H^+ \xrightarrow{SOD} O_2 + H_2O_2$$

Superoxide dismutase activity is measured as the inhibition of the reduction of cytochrome c by the superoxide radical, observed at 550 nm:

$$\text{Cytochrom } c \text{ (oxidized)} + 2O^{\bullet}_2 \longrightarrow \text{Cytochrome } c \text{ (reduced)} + O_2$$

The superoxide radical is produced enzymatically by the reaction:

$$\text{Xanthine} + O_2 + H_2O \xrightarrow{XOD} \text{Uric acid} + 2O^{\bullet}_2 + H^+$$

Abbreviation used:

SOD = Superoxide dismuatse

XOD = Xanthine oxidase

Condition: T = 25º C, pH = 7.8, A550nm, Light path = 1 cm

Method: Continuous Spectrophotometric Rate Determination

Reagents:

1. Reagent A: 216 mM Potassium phosphate buffer, pH 7.8 at 25º C (Prepare 50 ml in deionized water using potassium phosphate monobasic anhydruos, Sigma Prod. No. P-5379, adjust to pH 7.8 at 25º C with 1 M KOH.)

2. Reagent B: 10.7 mM Ethylenediaminetetraacetic acid solution (EDTA) (Prepare 50 ml in deionized water using ethylenediaminetetraacetate acid disodium salt dihydrate, Sigma Stock. No. ED2SS.)

3. Reagent C: 1.1 mM Cytochrome *c* solution (Cyt *c*) (Prepare 2 ml in deionized water using cytochrome c, from horse heart, Sigma Prod. No. C-7752.)

4. Reagent D: 0.108 mM Xanthine solution (Xanthine)

(Prepare 500 ml in deionized water using xanthine, Sigma Prod. No. X-0626. Titrate into solution with 1 M KOH.)

5. Reagent E: Xanthine oxidase enzyme solution (XOD)

(Immediately before use, prepare a solution containing 0.05 units/ml of xanthine oxidase, Sigma Prod. No. X-1875, in cold deionized water.)

6. Reagent F: Superoxide dismutase enzymatic solution

(Immediately before use, prepare a solution containing 10 units/ml of superoxide dismutase in cold deionized water.)

Procedure:

Prepare a reaction cocktail by pipetting (in milliliters) the following reagents into a suitable container:

Deionized Water	23.0
Reagent A (Buffer)	25.0
Reagent B (EDTA)	1.0
Reagent C (Cyt c)	1.0
Reagent D (Xanthine)	50.0

Mix and adjust to pH 7.8 at 25º C, if necessary, with 1 M HCL or 1 M KOH. Pipette (in milliliters) the following reagents into suitable cuvettes:

	Test 1	Test 2	
	Uninhibited	Inhibited	Blank
Reaction Cocktail	2.80	2.80	2.80

Equilibrate to 25º C. Monitor the absorbance, A550 nm, until constant, using a suitably thermostatted spectrophotometer. Then add:

Deionized Water	0.10	-----	0.20
Reagent E (XOD)	0.10	0.10	-----
Reagent F (Enzyme solution)	-----	0.10	-----

Immediately mix by inversion and recorded the increase in A550 nm for approximately 5 minutes. Obtain the ΔA 550 nm/minute by using the maximum linear rate for the both the Uninhibited (Test 1), Inhibited (Test 2) and Blank.

Calculation:

$$\% \text{ Inhibition 2} = \frac{\Delta A550 \text{ nm/min Uninhibited} - DA550 \text{ nm/min Inhibited}}{\Delta A550 \text{ nm/min Uninhibited} - DA550 \text{ nm/min Inhibited}} \times 100$$

$$\text{Units/ml enzyme} = \frac{\% \text{ Inhibition (df)}}{(50\%)\ (0.1)}$$

df = Dilution factor

0.1 = Volume (in milliliters) of enzyme used

50% = Inhibition of the rate of cytochrome c reduction as per the Unit

Definition

$$\text{Units/ml solid} = \frac{\text{units/ml enzyme}}{\text{mg solid/ml enzyme}}$$

$$\text{Units/ml protein} = \frac{\text{units/ml enzyme}}{\text{mg protein/ml enzyme}}$$

Unit definitions:

One unit will inhibit the rate of reduction of cytochrome *c* by 50% in a coupled system, using xanthine and xanthine oxidase at pH 7.8 at 25º C in a 3.0 ml reaction volume. The xanthine oxidase concentration should produce an initial (uninhibited) ΔA550 nm of 0.025 " 0.005 per minute.

Final assay concentration:

In a 3.00 ml reaction mix, the final concentrations are 50 mM potassium phosphate, 0.1 mM ethylenediaminetetraacetate acid, 0.01 mM cytochrome *c*, 0.05 mM xanthine, 0.005 unit xanthine oxidase and 1 unit superoxide dismutase.

Reference:

McCord JM, Fridovich I. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). **J Biol Chem** 1996; 244(22): 6049-6055, 1969.

APPENDIX I

Enzymatic Assay of Glutathione Peroxidase

ENZYMATIC ASSAY OF GLUTATHIONE PEROXIDASE

Principle:

$$2\ GSH + H_2O_2 \xrightarrow{\text{Glutathione peroxidase}} GSSG + 2\ H_2O$$

$$GSSG + \beta\text{-NADPH} \xrightarrow{\text{Glutathione reductase}} \beta\text{-NADP} + 2\ GSH$$

Abbreviations used:

GSH = Glutathione, reduced form

GSSG = Glutathione, oxidized form

β-NADPH = β-Nicotinamide Adenine Dinucleotide Phosphate, reduced form

β-NADP = β-Nicotinamind Adenosine Dinucleotide Phosphate, oxidized form

Condition: T = 25º C, pH = 7.01, A340nm, Light path = 1 cm

Method: Continuous Spectrophotometric Rate Determination

Reagents:

1. Reagent A: 50 mM sodium phosphate buffer with 0.40 mM ethylenediaminetetraacetate acid (EDTA), pH 7.01 at 25º C (Prepare 50 ml in deionized water using sodium phosphate monobasic anhydrous, Sigma Prod. No. S-0751 and ethylenediaminetetraacetate acid tetrasodium salt, Sigma Stock. No. ED4SS. Adjust to pH 7.01 at 25º C with 1 M NaOH.)

2. Reagent B: 1.0 sodium azide solution (Buffer w/azide)
(Prepare 50 ml in reagent A using sodium azide, Sigma Prod. No. S-20002.2)

3. Reagent C: β-Nicotinamide Adenine Dinucleotide Phosphate, reduced form (β-NADPH) (Use 1.0 mg vial of Nicotinamide Adenine Dinucleotide Phosphate, reduced form tetrasodium salt, Sigma stock No. 201-201.)

4. Reagent D: Glutathione reductase enzyme solution (GR)
(Immediately prepare before use, prepare a solution containing 100 units/ml of glutathione reductase, Sigma Prod. No. G-3664, in cold deionized water.)

5. Reagent E: Glutathione, reduced (GSH) (Prepare 5 ml in deionized water using glutathione, free acid, reduced form, Sigma Prod. No. G-4251.)

6. Reagent F: 10.0 mM sodium phosphate buffer with 1.0 mM dithiothreitol, pH 7.0 at 25º C (Buffer w/DTT) (Prepare 100 ml in deionized water using sodium phosphate monobasic anhydrous, Sigma Prod. No. S-0751 and DL-Dithiothreitol Sigma Prod. No. D-0632. Adjust to pH 7.0 at 25º C with 1 M NaOH.)

7. Reagent G: Glutathione peroxidase enzyme solution
(Immediately before use, prepare a solution containing 1.5-3.0 units/ml of glutathione peroxidase in cold reagent F.)

8. Reagent H: 0.042% (w/w) hydrogen peroxide (H_2O_2) (Prepare 5 ml in deionized water using hydrogen peroxide, 30% (w/w) solution, Sigma Prod. No H-10093. Prepare fresh)

Procedure:

Prepare a reaction cocktail by pipetting (in milliliters) the following reagents into reagent C (β-NADPH via):

Reagent B (Buffer w/Azide)	9.20
Reagent D (GR)	0.10
Reagent E (GSH)	0.05

Mix by inversion and adjust to pH 7.0 at 25º C with 1 M HCl or 1 M NaOH, if necessary. Pipette (in milliliters) the following reagents into suitable cuvettes:

	Test	**Blank**
Reaction cocktail	3.00	3.00
Reagent F (Buffer w/DTT)	-----	0.05
Reagent G (Glutathione peroxidase)	0.05	-----

Mix by inversion and equilibrate to 25º C. Monitor the A340nm until constant, using a suitably thermostatted spectrophotometer. Then add:

	Test	**Blank**
Reagent H (H_2O_2)	0.05	0.05

Immediately mix by inversion and record the decrease in A340nm for approximately 5 minutes. Obtain the ‘ A340nm/minute using the maximum linear rate for both the test and blank.

Calculation:

$$\text{Units/ml enzyme} = \frac{(\text{‘ A340nm/min Test- ‘A340nm/min Blank})(2)(3.1)(df)}{(6.22)(0.05)}$$

2 = 2 umole of GSH produced per umole of β-NADPH oxidized

3.1 = Total volume (in milliliters) of assay

df = Dilution factor

6.22 = Millimolar extinction coefficient of β-NADPH at 340 nm

0.05 = Volume (in milliliters) of enzyme used

$$\text{Units/ml solid} = \frac{\text{units/ml enzyme}}{\text{mg solid/ml enzyme}}$$

$$\text{Units/ml protein} = \frac{\text{units/ml enzyme}}{\text{mg protein/ml enzyme}}$$

Unit definition:

One unit will catalyze the oxidation by H_2O_2 of 1.0 umole of reduced glutathione to oxidized glutathione per minute at pH 7.0 at 25º C.

Final assay concentration:

In a 3.05 ml reaction mix, final concentrations are 48 mM sodium phosphate, 0.38 nM ethylenediaminetetraacetate acid, 0.12 mM β-nicotinamind adenosine dinucleotide phosphate, reduced form, 0.95 mM sodium azide, 3.2 units of glutathione reductase, 1 mM glutathione, 0.02 mM DL-dithiothreitol, 0.0007% (w/w) hydrogen peroxide and 0.075-0.15 unit of glutathione peroxidase.

Reference:

Wendel A. Glutathione Peroxidase. In: Jakoby WB, editor. **Enzymatic basis of detoxification.** New York: Academic Press; 1980. p. 333-53.

APPENDIX J

Enzymatic Assay of Catalase

ENZYMATIC ASSAY OF CATALASE

Principle

$$2\ H_2O_2 \xrightarrow{\text{Catalase}} 2\ H_2O + O_2$$

Conditions: T = 25º C, pH = 7.0, A240nm, Light path = 1 cm

Method: Continuous spectrophotometric rate determination

Reagents:

1. Reagent A: 50 mM potassium phosphate buffer pH 7.0 at 25º C (Prepare 200 ml in deionized water using potassium phosphate monobasic anhydrous, Sigma Prod. No. P-5379. Adjust to pH 7.0 at 25º C using 1 M KOH.)
2. Reagent B: 0.036% Hydrogen peroxide solution (H_2O_2) (Substrate solution) (Prepare in reagent a using hydrogen peroxide, 30% (w/w) Sigma Prod. No. H-1009. Determine the A240nm of this solution using reagent A as a blank. The A240nm should be between 0.550 and 0.520 absorbance units. Add hydrogen peroxide to increase the absorbance and reagent A to decrease the absorbance.)
3. Reagent C: Catalase solution (Immediately before use prepare a solution containing 50-100 units per ml cold reagent A.)

Procedure:

Pipette (in milliliters) the following reagents into suitable cuvettes.

	Test	Blank
Reagent A	-----	3.0
Reagent B (Substrate solution)	2.9	-----

Equilibrate to 25º C. Monitor the A240nm until constant and spectrophotometer. Then add:

	Test	Blank
Reagent C (Catalase solution)	0.1	-----

Immediately mix by inversion and record the time required for the A240nm to decrease from 0.45 to 0.40 absorbance units.

Calculation:

$$\text{Units/ml enzyme} = \frac{(3.45)\,(df)}{(min)\,(0.1)}$$

3.45 = Corresponds to the decomposition of 3.45 micromole of hydrogen peroxide in a 3.0 ml reaction mixture producing a decrease in the A240nm from 0.45 to 0.40 absorbance units

df = Dilution factor

min = time in minutes required for the A240nm to decrease from 0.45 to 0.40 absorbance units

0.1 = Volume (in milliliter) of enzyme used

$$\text{Units/ml solid} = \frac{\text{units/ml enzyme}}{\text{mg solid/ml enzyme}}$$

$$\text{Units/ml protein} = \frac{\text{units/ml enzyme}}{\text{mg protein/ml enzyme}}$$

Unit definition:

One unit will decompose 1.0 umole of H_2O_2 per minute at pH 7.0 at 25º C, while the H_2O_2 concentration falls from 10.3 mM to 9.2 mM. The rate of disappearance of H_2O_2 is followed by observing the rate of decrease in the absorbance at 240 nm.

Final assay concentration:

In a 3.00 ml reaction mix, the final concentrations are 50 mM potassium phosphate, 0.035% (w/w) hydrogen peroxide, and 5-10 units catalase.

Reference:

Beer RF Jr, Sizer IW. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. **J Biol Chem** 1952; 195: 133-40

Stern KG. On the absorption spectrum of catalase. **J Biol Chem** 1937; 121: 561-72.

APPENDIX K

Enzymatic Assay of Acetylcholinesterase

ENZYMATIC ASSAY OF ACETYLCHOLINESTERASE

Solutions:

1. 0.1 M phosphate buffer (PB), pH 8.0
2. 0.075 M acetylthiocholine iodide (ATCId)
3. 0.01 M dithiobisnitrobenzoate (DTNB)

Prepare brain homogenates:

A. Weight a sample (about 30 mg) and add 1 ml of PB/30 mg tissue (30 mg/ml). Homogenize this solution until the brain is uniformly dispersed in the buffer. Place the tube on ice.

B. Assay

   1. Turn on the spectrophotometer and set at 412 nm. Let it warm up for at least 15 min prior to reading.
   2. Label the assay tubes-four tubes (3 for the assay and one for a control).
   3. Pipette 3 ml PB into each assay tube.
   4. Using a pipette add 200 µl of sample homogenate to each of the four labeled assay tubes. Vortex a tube by setting the needle to 0 transmittance.
   5. Zero the spectrophotometer.20 without a tube by setting the needle to 0 transmittance.
   6. Add 100 µl DTNB to the first cortex tube, vortex, and place it in a test tube rack for 5 min. This allows the solution to reach room temperature.
   7. Vortex and quickly wipe the outside of the tube with kimwipe. Place the tube in the spectrophotometer.20 and zero the spectrometer to 0 absorbance. This will be a baseline reading before measuring product formation.
   8. Take the tube out of the spectrophotometer, quickly add 20 µl ATCId and vortex.
   9. Immediately return the tube to the spec.20. Note the time and take a zero reading of absorbance. Take reading at 30 sec, 60 sec, 2 min and 3 min and record the data in a table.

10. Repair the procedure (step 6-10) for the other sample homogenates. Run the control through the same procedure except do not add substrate (ATChI) but add 20 µl PB instead.

C. Calculate of the rate of the reaction:

1. Graph the data for the different brain region-change in absorbance/mim.against time. Are the graphs linear?

2. Calculate the rate of color change per minute for each reading and average the rates within each three minute run. Then average the arts between each run for each brain region, calculate the rate of the reaction according to the following equation:

$R = \Delta A/(1.36*10^4)x1/(200/3320)Co = 1.22(10^{-3})\ A/Co$

R = rate, in moles substrate hydrolyzed/ min. g. tissue

ΔA = change in absorbance/min

Co = original concentration of tissue (mg/ml) 200/3320 are volume correction

$1.36\ (10^4)$ = the extinction coefficient of the yellow product

3. Make a bar graph to show the enzyme activity of each brain region.

Reference:

Thomson RF. **The Brain: A Neuroscience Primer**. New York: W.H. Freeman and Company; 1993.

Robertson RT, Holunann CF, Bruce JL, Coyle JT. Neonatal enucleation reduces specificity activity of acetylcholinesterse and developing rat visual cortex. **Devel Brain Res**., 1988; 39: 298-302.