MATERIAL
AND
METHODS
2. MATERIAL AND METHODS

2.1. Experimental Animals:

One hundred male guinea pigs, weighing 250 - 500 g and thirty-six male albino rats, weighing 150 - 250 obtained from Central Animals House, Jawaharlal Nehru Medical College, Aligarh Muslim University, Aligarh were used in this study. Animals were housed in individual cages and fed commercial laboratory diet (Hindustan Lever Laboratory Feeds, India) and drinking water ad libitum. The pellet composition corresponded to nutritional standards recommended by the U.S. National Research Council’s Publication No. 990, entitled, "Nutritional Requirements of Laboratory Animals". The experimental and control groups consisted of equal number of animals. Each group, used for a given experiment, invariably comprised of six guinea pigs/rats. Illumination in the colony room was maintained on 12 hour light, 12 hour dark schedule.

2.2. Animal Exposures:

2.2.1. Design, Fabrication and Operation of Exposure Chamber for Testing Atmospheric Pollutants by Inhalation;

2.2.2. Design:

Exposure Chamber for toxicological investigation of the
effects of various air contaminants on animals has been especially designed and constructed in this laboratory (Baider et al., 1981) taking into account the following basic requirements for such an "exposure chamber":

(i) Exposure chamber should be cubical in geometrical configuration to minimize the surface area, since the quantity of surface exposed per unit volume is influenced by shape and size.

(ii) A large size exposure chamber be constructed so that the contaminated atmosphere of "exposure chamber" may not be altered even on the presence of a number of animals.

(iii) Exposure chamber should be air tight to eliminate leakage.

(iv) Wall surface is an important factor since the adsorption of the contaminant by the wall of the exposure chamber poses problem. The material selected for the construction of the exposure chamber was made inert to various contaminants.

(v) The sides of the exposure chamber were necessarily made of transparent glass so as to observe the physical condition of animals during the course of experiments.

(vi) An essential feature of the exposure chamber was the provision of baffle for dispersion of gas and outlet for the contaminant on the opposite side of the exposure chamber.

(vii) Three most important objectives were achieved in this exposure chamber:

(a) Preparation of a known dynamic concentration of a contaminant gas.

(b) The contaminant gas was constantly and easily mixed with a measured air-flow rate.

(c) The flow of the contaminated atmosphere was regulated by a regulatory valve at the inlet.

(viii) Plastic made air-intake and discharge pipes were used to prevent the possible chemical reaction of contaminant gases.

(ix) An air filter (Whatman paper) was used for efficient collection of a wide range of particle sizes, by high-speed air flow. This filter holder was inserted proximal to the gas injector for purified air supply in the exposure chamber.
(x) Another essential requirement of the exposure chamber was the provision of an appropriate meter for measuring the volume of the contaminant gas accurately. This was reliably done by displacing small amounts of the gas from a flask by a liquid from a source at a known head. The displacement of the gas was measured accurately by the controlled rate liquid transference into the flask.

(xi) For the promotion of the turbulent flow and ensuring proper mixing of the contaminated air in the exposure chamber, the contaminant was injected by a thin bent tube, of uniform bore, so as to provide a high velocity flow subsequently, the contaminated air was also passed through a deflector.

(xii) It was necessary to measure temperature, pressure, and humidity inside the exposure chamber. For this purpose, a mercury thermometer, a pressure gauge and a hygrometer were provided.

(xiii) For the entry of animals into the exposure chamber, a sliding door was provided, keeping in mind that no dilution of the contaminated air was to be allowed.

(xiv) Contaminant gas was collected in an absorber containing aqueous solution for the accurate analysis of contaminant by titration.

2.2.3. Description of the Exposure Chamber:

Exposure chamber, cubical in shape, having a volume of one cubic metre was designed to obtain controlled concentration of atmospheric pollutants. The assembly of various parts of the exposure chamber has been shown in Figs. 6 and 7. The large size of the exposure chamber was preferred to maintain a uniform atmosphere of the exposure chamber that would not be affected by the presence of the animals. The walls of the exposure chamber were made of 18 mm thick plywood board and other four sides comprised of transparent glass (thickness, 6 mm). The inner surface of the plywood side was painted with a synthetic enamel adhesive paint which was impervious to the
EXPOSURE CHAMBER FOR TESTING ATMOSPHERIC POLLUTANTS BY INHALATION
chemicals. The exposure chamber can easily be cleaned from inside by removing the glass sides which were fixed by screws through rubber packings. The atmosphere inside the exposure chamber was generated by mixing a known quantity of ambient air with a contaminant gas. The ambient air was drawn into the exposure chamber through a pipe with the help of a blower having a capacity of 947 liter/hour (No. 21, Fig. 7). A valve (No. 1, Fig. 7) was provided on the inlet side of the exposure chamber to regulate the ambient air supply. The inlet pipe (No. 2, Fig. 7) was in two parts. A galvanized iron pipe was connected to the Orifice meter, thereafter a plastic pipe (No. 11, Fig. 7) was used to eliminate possibility of any chemical reaction with the contaminant gas which was injected into the air stream through this part. A British Standard Orifice Meter (Linford, 1951) (No. 3, Fig. 7), 5 cm in diameter, was installed in the inlet pipe to measure the quantity of ambient air entering the exposure chamber. A 1/8" tube water manometer (No. 4, Fig. 7) was used to measure the pressure difference across the Orifice Meter. The plastic portion of the inlet pipe was also provided with a piece of perforated gauze (No. 12, Fig. 7) for thorough mixing of the ambient air and the contaminant gas. A whatman filter paper (No. 13, Fig. 7), 0.1 mm in diameter, was kept inside the inlet pipe to filter the ambient air from dust particles prior to mixing with the known contaminant gas. The deflector (No. 10, Fig. 7) inside the exposure chamber ensures a uniform distribution of contaminant gas.
2.2.4 Measurements:

(i) Measurement of Temperature:
A mercury in glass thermometer (No. 17, Fig. 7) was used to measure the temperature inside the exposure chamber.

(ii) Measurement of Pressure:
A pressure gauge (No. 15, Fig. 7) was fixed inside the exposure chamber to record the pressure of the contaminated air.

(iii) Measurement of Humidity:
The humidity inside the exposure chamber was monitored with the help of a hair hygrometer (No. 16, Fig. 7).

(iv) Measurement of the Volume (ml) of the Contaminant Gas Mixed with the Ambient Air:
An air tight flask (No. 19, Fig. 7) was filled with the saturated solution of a contaminant gas, then the gas generator (No. 5 or No. 6, Fig. 2) was connected through a capillary tube to the flask and the gas was produced. The solution in the flask was displaced fully by the gas and collected from the other capillary tube into a trough (No. 19, Fig. 7). A known quantity of the same liquid was
transferred at a controlled rate from a graduated aspirator (No. 17, Fig. 7), placed at a height of 2.2 m. The displaced gas was then ejected at a constant rate into an air stream by means of a bent tube (No. 10, Fig. 7), 0.8 cm in diameter. The volume of the contaminant gas forced into the exposure chamber per hour was measured by the quantity of the liquid transferred from the graduated aspirator into the flask during the course of the experiment.

2.2.5. Calculations;

(1) Calculation of the quantity of ambient air and a contaminant gas entering into the chamber. The calculations were based upon the following formula (Linford, 1951):

\[ Q = 35.9 \text{ cm}^2 \sqrt{h/\rho} \]

where,

\( Q \) = quantity of air discharge in cubic ft/hr.
\( C \) = coefficient of water = 0.604.
\( B \) = value of approximate factor = 1.0328.
\( h \) = water level difference between two arms of 'U' tube manometer = 0.9 cm.
\( \rho \) = density of the water = 0.073.
\( h = 0.9/2.54 = 0.35 \text{ ft of } H_2O \).

Now substituting the values in the formula, we get:
\[ q = 358.9 \text{ cm}^2 \sqrt{\frac{h}{\rho}} \]
\[ \text{or } q = 358.9 \times 0.604 \times 1.0328 \times 4 \sqrt{\frac{0.354}{0.075}} \]
\[ \text{or } q = 1945.12 \text{ cubic ft/hr} \]
\[ \text{or } q = 1945.12/60 \text{ cubic ft/min.} \]
\[ \text{or } q = 32.41 \text{ cubic ft/min.} \]
\[ \text{or } q = 32.41/35.345 = 0.917 \text{ m}^3/\text{min} \times 10^6 \text{ for ml.} \]
\[ \text{or } q = 91700 \text{ ml/min.} \]
\[ q = 917 \text{ liter/min.} \]

(11) Calculation of the concentration of hydrogen sulfide \( (H_2S) \) inside the Exposure Chamber:

(a) 1100 c.c. of \( H_2S \) was injected per hour.

quantity of \( H_2S \) injected into the stream of air per minute = 18.33 c.c./min.

(b) Area of exposure chamber is one cubic meter.

Total amount of \( H_2S \) present in the exposure chamber

\[ \frac{18.33}{100 \times 100 \times 100} = 0.001833 \text{ m}^3/\text{min} \]

9,1700 ml of air contains 18.33 ml of \( H_2S \)

\[ 10^6 \text{ (1 million) air contains } 18.33/0.91700 \times 10^{-6} \]

Total concentration of \( H_2S \) inside the exposure chamber is 19.99/1000000 ppm.
(iii) (a) Calculation of sulphur dioxide ($SO_2$) in ppm inside the exposure chamber:

(1) 562.5 ml of SO$_2$ was injected per hour.

Quantity of $SO_2$ injected into the air stream per minute

\[ \frac{562.5}{60} = 9.4 \text{ ml/min.} \]

(b) Total amount of $SO_2$ present in the exposure chamber:

\[ \frac{9.4}{100 \times 100 \times 100} = 0.00094 \text{ ml/min.} \]

9.17,000 ml of ambient air contains 9.4 ml of H$_2$O.

\[ \frac{9.4}{10^6 \text{ ml of ambient air}} = \frac{9.4}{0.91700 \times 10^{-6}} \]

The contamination of SO$_2$ inside the exposure chamber is 10.2/1000000 ppm.

(iv) Calculation of Exposure levels in SI Units:

1 litre = 1000 cm$^3$
1 cm$^3 = 10^{-6}$ m$^3$

Air was supplied at the rate of 917 litre/hour.

Air = $9.17 \times 10^{-3}$ m$^3 = 9.17 \times 1000 \times 10^{-6}$ m$^3$
Air = $9.17 \times 10^{-3}$ m$^3$/min = $1.522 \times 10^{-5}$ m$^3$/Sec.

(a) H$_2$O was injected at the rate of 19.33 ml/min.

19.33 cm$^3 = 19.33 \times 10^{-6}$ m$^3$/min = $3.055 \times 10^{-7}$ m$^3$/Sec.
(b) $SO_2$ was injected at the rate of $9.7 \text{ ml/min}$

$$9.4 \text{ cm}^3 = 9.4 \times 10^{-6} \text{ m}^3/\text{min} = 1.500 \times 10^{-7} \text{ m}^3/\text{sec}.$$ 

2.3. Exposure of Animals to Air Pollutant $SO_2$ (10 ppm):

Thirty six male adult guinea pigs, weighing 250 - 500 g were divided into two equal groups. They were fed a pellet diet of Hindustan Lever Ltd., India and tap water ad libitum. Control animals were kept inside the exposure chamber under "normal environmental conditions", while the experimental animals were exposed to $SO_2$, a concentration similar to N.A.C. levels for one hour daily for 21 days. Contaminated air, a mixture of ambient air (917 litre/min) and $SO_2$ (9.4 ml/min), was supplied into the exposure chamber by a blower. The contaminated air was purified from dust by a filter. Saturated solution of $SO_2$ was filled into the flask before Woulf's bottle was connected through a capillary tube to this flask and $SO_2$ was produced. The solution in the flask was completely displaced by $SO_2$.

A known volume (562.5 ml/hr) of the same liquid was transferred from an aspirator at a height of 2.2 m to the flask at a controlled rate. The displaced $SO_2$ was then ejected into the air stream through a beat tube at a constant rate. The quantity of $SO_2$ forced into the chamber was measured by the volume of liquid transferred from the aspirator into the flask during a given hour. The exposure chamber temperature was $29 \pm 2^\circ C$, pressure recorded 750 mm Hg and relative humidity varied up to $65 \pm 15\%$. The contaminated air samples were analyzed by the method of Jacobs (1960).
In another experiment twenty four male albino rats, weighing 150 - 200 g were divided into two equal groups. Experimental rats were exposed to the contaminated atmosphere of SO₂ (10 ppm) for 1 hour daily for 30 days according to the procedure described earlier in section 2.3. During this investigation, temperature was kept at 25 ± 2°C, pressure remained 760 mm Hg and relative humidity was 60 ± 10%.

2.4 Exposure of Animals to Air Pollutant H₂S;

Twelve male adult guinea pigs, weighing 350 - 500 g were divided into two equal groups. They were fed a pellet diet (Hindustan Lever Ltd., India) and water ad libitum. The animals were exposed 1 hour daily for 11 days to H₂S - concentration of 20 ppm equivalent to Maximum Allowable Concentration (M.A.C.) levels. Control group of the animals were kept in the same exposure chamber under "normal environmental conditions". Ambient air at the rate of 917 litre/min (1.528 x 10⁻³ m³/sec) and H₂S, at the rate of 19.33 ml/min (1.566 x 10⁻⁷ m³/sec.) were drawn into the exposure chamber by means of a blower. Prior to its entry into the exposure chamber, the contaminated air was passed through a filter to collect dust particles. The flask was filled with a saturated solution of H₂S and Hpp's apparatus was connected through a capillary tube to this flask before H₂S was generated. The solution in the flask was displaced fully by H₂S and collected from other capillary tube into a trough. A measured quantity (100 ml/h) of the same liquid was transferred from the graduated aspirator at a height of 2.2 metres to the
flask at a controlled rate. The displaced $H_2F$ was thereafter ejected into the air stream through a bent tube at a constant rate. The quantity of $H_2F$ forced into the chamber was the same as the liquid transferred from the aspirator into the flask of $H_2F$. During this experiment the ranges of temperature, pressure and relative humidity recorded were $25 \pm 5^\circ C$, 760 mm Hg and $40 \pm 20\%$ respectively. The contaminated air samples were analyzed by the procedure of Jacobs et al. (1957).

2.5. Exposure of Animals to an Atmosphere Contaminated by $H_2F$ and $SO_2$: Fifty two male guinea pigs, weighing 250 - 400 g were divided into two equal groups. Control animals were kept under "normal environmental conditions", whereas experimental group was exposed to the contaminated atmospheres of $H_2F$ (20 ppm) and $SO_2$ (10 ppm) in the exposure chamber alternately for one hour daily for 30 days. The exposure technique was the same as described in Sections 2.4. and 2.5. During this experiment the temperature was $25 \pm 5^\circ C$, pressure remained 760 mm Hg and humidity was $50 \pm 15\%$. The contaminated air samples were analyzed by the methods of Jacobs et al. (1957) and Jacobs (1950) respectively.

2.6. Analysis of Contaminated Air for $SO_2$ (Jacobs, 1950): This method is applicable to the determination of $SO_2$ in ambient air in the concentration range from about 0.01 to 10.0 ppm.
Reagents:

1) Absorbing solution, hydrogen peroxide, 0.03 N, pH 5. 3.4 ml of 30% \( \text{H}_2\text{O}_2 \) was diluted to 2 litres with DDW and titrated with 0.002 N HCl to determine the alkalinity of the solution.

II) Mixed indicator, 0.1%. 0.05 gm of bromocresol green and 0.04 gm methyl red were dissolved in 100 ml of methanol and stored in an amber bottle at room temperature.

III) Standard sulfuric acid solution, 0.002 N. This solution was prepared by appropriate dilution of concentrated \( \text{H}_2\text{SO}_4 \).

IV) Standard sodium hydroxide solution, 0.002 N. It was prepared by diluting 1N sodium hydroxide with DDW.

Analytical Procedure:

The samples were absorbed in 0.03N \( \text{H}_2\text{O}_2 \) reagent and transferred to stoppered glass container, then three drops of mixed indicator were added and titrated with standard 0.002N NaOH until the color changed from red to green. A reagent blank was titrated in the same manner, and this result was subtracted from the sample titer. Thus the net titer of 0.002N NaOH (in ml) multiplied by 20.47 gave the volume of \( \text{SO}_2 \) in millilitres.
Analysis of Contaminated Air Samples for H₂S (Jacobs et al., 1957):

Reagents:

1) *Ammonium nitric acid solution*: 50 ml of H₂SO₄ was added to 30 ml of double distilled water and cooled, then 12.0 g of Ni(NH₂)₆-dimethyl-p-phenylene diamine was added to the solution and 25 ml of this solution was diluted to one liter with H₂SO₄ and DDW (1:1).

2) *Ferric chloride solution*: 100 g of FeCl₃·6 H₂O was dissolved in DDW and volume was made up to 100 ml.

3) *Absorption mixture*: 0.3 g of cadmium sulfate (CdSO₄·8 H₂O) was dissolved in double distilled water and was mixed with a solution of 0.3 g NaOH in double distilled water and diluted to one liter. This mixture was stirred well before being used.

4) *Hydrogen sulfide solution*: 0.71 g of sodium sulfide (Na₂S·9 H₂O) was dissolved in 1 liter of double distilled water.

Analytical Procedure:

50 ml of the absorption mixture was added to a standard impinger and contaminated air, at a measured rate, was passed for 30 minutes. To this was added 0.5 ml of amine-sulfuric acid test solution and 1 drop of ferric chloride solution and shaken
after each addition. This mixture was transferred to a 50 ml volumetric flask, volume was made up to the mark and allowed to stand for 30 minutes. A reference reagent blank was prepared by adding the above amounts of test reagents and ferric chloride solution to 45 ml of absorption mixture in a 50 ml volumetric flask, made up to volume, and allowed the mixture to stand for 30 minutes.

The spectrophotometer was set to zero optical absorbance with 25 ml of the reference blank at 670 nm and the absorbance of 25 ml of the sample was then determined. By reference to a standard calibration curve, the concentration of $\text{H}_2\text{S}$ in the sample was measured.

Calculation:

The results on the air sample may be calculated in parts per thousand million (billion), by volume as follows:

$$\text{H}_2\text{S (ppb)} = \frac{\mu g \text{H}_2\text{S} \times 2 \times 719}{\text{volume of air sample in liters at } 25^\circ\text{C and } 760 \text{ mm}}$$

2.3. Biological Investigations:

2.3.1. Removal of Brain and Spinal Cord:

Overnight fasted guinea pigs & rats were sacrificed by decapitation. The brains and cervical spinal cord were removed rapidly
and placed in deep freeze at -20°C after removing the adherent blood clots from the surface.

2.9.2. Dissection of Various Parts of the Brain:

The brains were dissected out on an ice plate into cerebral hemisphere, cerebellum and spinal cord as shown in Fig. 8 and 9.

The superficial gray matter was carefully dissected out from the underlying white matter of the cerebrum. Thereafter, the white matter was removed and the caudate nucleus, lentiform nucleus and amygdaloid nucleus together with the lateral part of the thalamus were removed. These nuclei constitute the so-called basal ganglia. They were weighed to the nearest milligram on a single pan electrical balance.

2.9.3. Extraction of Lipids from Discrete Brain Areas:

Different parts of the brain, weighing 30 - 300 mg, were homogenised in a glass homogenizer to a final volume of 6 ml chloroform-methanol (2:1, v/v) according to the method of Folch et al. (1951). This method was partly modified in our laboratory (Islam et al., 1980) for isolation of lipids from discrete areas of the brain. Each homogenate was shaken periodically for an hour and filtered under vacuum through a sintered glass funnel (0.45) (Fig. 10). The residue of each test tube was again homogenised with 2 ml chloroform-methanol and filtered. The test tubes were rinsed with fresh chloroform-methanol (2:1) and again
Fig. 9: Diagram of guinea pig brain, sagittal section, showing landmark used and region obtained in dissection procedures.
Fig. 9: Diagram of guinea pig brain, sagittal section, showing landmark used and region obtained in dissection procedures.
EXPLANATION OF THE FIGURE

Fig. 10: Filter adapter and sintered glass funnel for the quick filtration of brain lipid homogenate directly in the 18 x 150 mm test tube. Filter adapter is connected to vacuum through the side arm tube.
filtered. The final volume of each extract was made up to 10 ml with fresh chloroform-methanol mixture. Thereafter, 2.5 ml of saline solution was added to the extract in each test tube (4:1, v/v). For the other parts, weighing 200 - 500 mg, the final volume of each extract was 15.0 ml and 3.75 ml saline solution was added to it. This was shaken vigorously on a test tube mixer for thorough mixing and placed at -20°C in a deep freeze overnight for complete separation of the two layers. The junction of the layers of each test tube was marked, the upper layer was used for the estimation of gangliosides and desired amount of the lower layer of each test tube was collected in stoppered tubes with the help of a syringe (Fig. 11) and stored at -20°C for 24 hours. The test tubes, in which the two layers were separated, were dried and the volume of the lower layer of each test tube was measured. The extract was used for the estimation of total lipids, phospholipids, cholesterol, free fatty acids and esterified fatty acids.

2.9. Estimation of Total Lipids:

Total lipids were estimated according to the method of Woodman and Price (1972) as follows:

Reagents:

1) A standard solution of 0.5 mg brain/ml of chloroform-methanol (2:1) was prepared by diluting 1.0 ml of refrigerated stock solution (50 mg brain/10 ml, chloroform-methanol 2:1) in a
Fig. 11: A simple device of microtipped bulb pipette using 10 ml syringe. Length of the needle 14 cm.
10.0 ml standard flask and volume was made up to 10.0 ml with chloroform-methanol mixture.

ii) Concentrated Sulfuric Acid A.R.

iii) Coloring Reagent: 6.0 g Potassium dihydrogen orthophosphate and 0.39 vanilin were dissolved by heating in a 100.0 ml volumetric flask and volume was made up to 100.0 ml with DDW.

iv) Brain Lipids: Lipids were extracted from 3 guinea pig brains in 50.0 ml chloroform-methanol mixture. The homogenate was centrifuged at 5000 rpm for 15 min. The supernatant was taken in a separating funnel and washed thrice with an equal volume of physiological saline and the lower layer was collected after 15 min. of each washing. After final washing the lower layer was kept in a refrigerator overnight for the complete separation of the two layers. The lower layer was collected in a 15 cm diameter petridish and the solvent was evaporated at room temperature by continuous shaking of the petridish. The dried lipids were again dissolved in chloroform-methanol mixture and centrifuged to remove undissolved lipids. The extract was once again evaporated in a petridish and dried lipids were stored at -20°C.

v) Procedure: Duplicate brain samples, containing 0.05 ml of the aliquots, were taken in 16 x 150 mm corning test tubes. Concentrated H₂SO₄ (2.5 ml) was added to each test tube and heated on a boiling water bath for 20 minutes. After cooling, 5.0 ml of coloring reagent was added to it and absorption was read at 530 nm, exactly after 10 minutes against a reagent
A calibration curve was prepared by taking different concentrations (50 to 500 μg) of standard brain lipids by adopting the same procedure as described above. The values of the standard curve were plotted by least square method. The concentration of total lipids in brain samples were calculated by the formula given below:

### Calculation:

\[
\text{Total lipids (μg/g fresh weight)} = \frac{C \times V}{V_t \times w_t}
\]

- **C** = Concentration of lipids in μg in 0.1 ml brain extract (volume taken into estimation).
- **V** = Total volume of the lower layer.
- **V_t** = Volume taken for the estimation.
- **w_t** = Fresh weight of the tissue in mg.

The above formula was used for calculating the concentration of total lipids, phosphate, cholesterol and free fatty acids.

2.10. **Estimation of Phosphate:**

Phosphate was estimated by the well known method of Fiske and Subbarow as described by Marinetti (1952) by utilizing the following procedure:

**Reagents:**

1) A standard of 0.01 mg/inorganic phosphate/ml was prepared by
the dilution of 5.0 ml of refrigerated stock solution (0.43 g \( \text{NH}_2\text{PO}_4 \)/500.0 ml DDW) in a 100.0 ml volumetric flask and volume was made up to 100.0 ml with DDW.

ii) Ammonium Molybdate solution 2.5% was prepared by heating in a boiling water bath.

iii) Perchloric acid 70% A.R.

iv) Reducing reagent: Sodium bisulfite 3.0 g (Sigma Chemical Co., U.S.A.), sodium sulfite 0.1 g (May & Baker, U.K.) and recrystallized 1-amino-2-naphthol-4-sulfonic Acid (ANSA) 0.05 g were dissolved in 20.0 ml DDW. A slight yellow solution thus obtained was stored in amber colored bottle. The color is stable for a week at the room temperature.

v) Recrystallization of ANSA: Sodium metabisulfite 15.0 g, sodium sulfite (anhydrous) 1.0 g and crude ANSA 1.5 g were dissolved in 100.0 ml of boiling DDW. Hot solution was filtered through the filter paper. One ml concentrated Hydrochloric Acid was added to the filtrate and stirred. The precipitate was filtered through the suction pump and washed with 30.0 ml double distilled water and finally with alcohol till washing is colorless. This purified ANSA is dried in oven at 100°C for 1 hour with least possible exposure to light and transferred to an amber colored bottle.

Procedure:

Draw aliquots 0.1 ml in duplicate were pipetted in
10 x 150 mm corning test tubes and all the solvent was removed by heating on a boiling water bath. One ml of analytical grade 70% perchloric acid was added to the samples and heated on a digester for 30 minutes or until the sample became clear. After complete digestion, they were cooled at the room temperature. Thereafter, 1.5 ml ammonium molybdate, 0.2 ml reducing agent (MBA), and 7.0 ml double distilled water were added to it. The contents were mixed after each addition. The tubes were heated on a boiling water bath for 7 minutes. The color intensity was read at 700 nm after 30 minutes. A calibration curve was drawn by taking 1/μg to 5/μg of phosphorus and the blank was prepared with 1.0 ml perchloric acid alone. Similar procedure was adopted as described above and the values of the calibration curve were plotted by the least square method. The absorption is a linear function of the phosphorus content and the amount in unknown samples can be calculated by direct proportion with the absorbance obtained for the standard. The amount of phospholipid was calculated by multiplying the values with a factor 25.

2.11. Estimation of Cholesterol:

Cholesterol was estimated by Liebermann-Burchard reaction as described by Moor et al. (1922).

Reagents:

1) Standard solution of cholesterol 1.0 mg/ml in chloroform was
prepared by the dilution of 1.0 ml refrigerated stock solution of recrystallized cholesterol (100 mg/ml) in a 10.0 ml standard flask and volume was made upto 10 ml with analytical grade chloroform.

11) **Color Reagent**: Analytical grade acetic anhydride, 50.0 ml, was kept in deep freeze for one hour and 5.0 ml concentrated analytical grade sulfuric acid was added dropwise with stirring. The reaction mixture was allowed to stand for additional 10 minute at room temperature before use. This reagent was prepared fresh each time it was used.

**Procedure**;

Aliquots of brain extracts, 0.5 ml were taken in 15 x 125 mm screw capped culture tubes. To each tube, 4.5 ml analytical grade chloroform and 1.0 ml of coloring reagent were added and mixed. The tubes were kept in dark at 25°C for 30 minutes. The color intensity was read at 660 nm against a reagent blank.

A calibration curve with different concentrations of cholesterol (100 - 800 μg) was drawn according to the same method as described above. The values were plotted by the least square method and concentration of the cholesterol was calculated as in the case of total lipids.

2.12. **Estimation of Free Fatty Acids**;

Free Fatty Acids were assayed by the procedure as described by Heding (1955).
Reagents:

1) Standard solution of 0.25 mg palmitic acid/ml heptane was prepared by diluting 1.0 ml of refrigerated stock solution (25 mg recrystallized palmitic acid/10.0 ml in heptane) to the final volume of 10.0 ml with heptane.

2) Barbitol Phenol Red Reagent: The stock buffer indicator consisted 1.0% phenol red (Sigma Chemical Co., U.S.A.) in 0.12M sodium barbitol (0.25 g/10.0 ml). This solution was diluted before use. Nitrogen gas was passed in a mixture containing 50.0 ml absolute ethanol and 100.0 ml heptane. To this mixture 0.5 ml of buffer was added and mixed gently.

3) Extraction Mixture: Isopropanol-heptane-5-Sulfuric acid (80:10:1, v/v/v).

Procedure:

In stoppered test tubes, 0.5 ml brain aliquots, 5.0 ml of extraction mixture, 3.0 ml of heptane and 2.0 ml of double distilled water were added and shaken vigorously for 3 minutes and allowed to stand for 30 minutes at room temperature for the complete separation of the two phases. Two ml of the heptane phase (upper layer) was pipetted into the 15 x 125 mm screw capped culture tubes and was inerted by nitrogen gas, otherwise dissolved carbon dioxide gives high values of free fatty acids. Thereafter, 3.0 ml of barbitol phenol red reagent was added. To avoid evaporation and interference with atmospheric
carbon dioxide, the tubes were capped after addition of the reagent. The optical density values, which reach equilibrium quickly, were read at 560 nm against pure heptane-ethanol. A calibration curve with various concentrations of palmitic acid (50 - 300 μg) was drawn by the same procedure. To get a linear curve the absorbance of the unknown samples was subtracted from the absorbance of the blank. By knowing the absorbance of the unknown sample, the concentration of the FFA can be calculated from the calibration curve.

2.13. Estimation of Esterified Fatty Acids:

The analysis of esterified fatty acids was performed by using the method of Stern and Shapiro (1953).

1) Standard Solution: A standard solution of 4.0 μeq of tricolein in alcohol-ether (3:1) was prepared by the dilution of 1.0 μeq stock solution (59 mg tricolein in 5 ml alcohol-ether, 3:1) to the final volume of 10.0 ml with alcohol-ether mixture.

2) 2.0 N Hydroxylamine Hydrochloride (Solution I): 13.9 g of hydroxylamine hydrochloride was dissolved in double distilled water by dilution to 100 ml.

3) 3.5 N Sodium Hydroxide (Solution II): 14.0 g of sodium hydroxide was dissolved in 100 ml double distilled water.

4) Hydroxylamine-Sodium Hydroxide: It was made before using by mixing equal volume of Solution I and II.
v) 0.37 M Ferric Chloride in 0.1 M HCl: 0.0 g FeCl₃ was dissolved in 100 ml of 0.1 M HCl.

vi) 4.0 M Hydrochloric Acid Solution (Approximately): The solution was prepared by adding 1 part of analytical grade concentrated HCl to 2 parts of double distilled water.

vii) Alcohol-Ether (3:1) Mixture: 3 parts of 95% ethanol were added to 1 part of ethyl ether (Peroxide free).

viii) Peroxide Free Ether: A little of hydroxylamine hydrochloride was added to the stock ether bottle prior to distillation of ether. The ether used in the procedure was freshly distilled.

ix) Procedure: Aliquots of brain extracts, 0.2 ml were pipetted in 16 x 150 mm screw capped culture tubes and dried at room temperature. Then 3.0 ml aliquot of alcohol and ether (3:1) was pipetted to each tube and 1.0 ml of hydroxylamine hydrochloride solution was added, the contents were mixed and allowed to stand for 20 minute at room temperature. Thereafter, 0.6 ml of 4 N HCl and 0.5 ml of ferric chloride solution were added to it and mixed after each addition. A dark brown color developed which was measured at 520 nm against a reagent blank.

A calibration curve was drawn by using 0.1 to 0.6 ml of the standard solution (4.0 µg/ml) and the volume was made up to 3.0 ml with alcohol-ether mixture and the procedure was carried out as described above.
2.14 Estimation of Gangliosides:

Gangliosides were estimated according to the method of Follet et al. (1978) essentially as follows:

Reagents:

1) Standard solution: A standard solution of 100 μg N-acetyl neuraminic acid (Sigma Chemical Co., U.S.A.) per ml double distilled water was made by diluting 1.0 ml of the refrigerated stock solution (10.0 mg N-acetyl neuraminic acid/10 ml double distilled water) to the final volume of 10 ml with double distilled water.

ii) Resorcinol Reagent: This reagent was prepared by mixing 10.0 ml of 3% resorcinol solution in double distilled water, 80 ml of concentrated HCl, 0.25 ml of 0.1 M copper sulfate and water up to 100.0 ml.

iii) Procedure: To 2.0 ml of the upper layer of the lipid extracts was added 2.0 ml of resorcinol reagent. The test tubes were heated in boiling water bath for 30 minute. After cooling, 5.0 ml of a mixture of butyl acetate-n-butanol (85:15, v/v) was added to each tube. The tubes were shaken thoroughly and kept for 15 minute to separate the organic phase. About 3 to
4 ml of the organic phase was taken and absorbance was measured at 580 nm against reagent blank. A standard curve with different concentrations of N-acetyl-neuraminic acid (5 - 30 μg) having 2.0 ml final volume of water was prepared by treating similarly.

iv) Calculation:

\[
\text{Gangliosides (mg/g fresh weight)} = \frac{C \times V}{Vt \times Wt}
\]

- **C** = Concentration in μg in 2.0 ml extract
- **V** = Total volume of the upper layer
- **Vt** = Volume taken for the estimation
- **Wt** = Fresh weight of the tissue in mg

2.15. Determination of Lipid Peroxidation:

The amount of malondialdehyde formed/30 minute during lipid peroxidation was estimated according to the method of Utaly et al. (1967) as described below:

i) **0.15M - Potassium Chloride:** 2.2368 g of KCl was dissolved in 200 ml double distilled water.

ii) **10% (v/v) Trichloroacetic Acid:** 10.0 g TCA was dissolved in 100 ml double distilled water.

iii) **0.67% 2-Thiobarbituric Acid (TBA):** This was prepared by dissolving 0.67 g of TBA in 25 - 50 ml double distilled water.
by adding two pellets of NaOH and volume was made up to 100 ml with double distilled water. The pH of the solution was adjusted to 7.2 with the 1N HCl.

iv) Procedure: Different parts of the brain were homogenized (10% w/v) in chilled 0.15M potassium chloride. One ml of each homogenate was taken in 25 ml conical flask and incubated at 37°C ± 1°C in a metabolic shaker (120 strokes/min; amplitude 1 cm) for 3 hours. After incubation, 1.0 ml of 10% TCA was added to it for the precipitation of protein. Thereafter, 1.0 ml of each reaction mixture was pipetted in centrifuge tubes and centrifuged at 3000 rpm for 10 minutes. One ml of the clear supernatant was mixed with 1.0 ml 0.67% TBA and 1.0 ml double distilled water. The test tubes were placed in a boiling water bath for 10 minutes, cooled and the absorbance of the coloring solution was read at 535 nm. The rate of lipid peroxidation is expressed as nanomoles of malonaldehyde formed per 30 minute using extinction coefficient 1.56 x 10^5 as described by Utley et al. (1967). Lipid peroxidation was calculated using the following formula:

\[ X = \frac{0.0D \times 30 \times 1000 \times 1000 \times 1000 \times 3 \times 2}{1.56 \times 100000 \times 1000 \times 150} \]

Where, \( X \) = nanomoles of malonaldehyde formed/30 minute

\[ X = \frac{0.0D \times 10}{1.56} \]
2.16. Determination of Lipase Activity:

The activity of lipase was measured by the method of Tiets and Fierro (1986). This procedure was partly modified in our laboratory (Haider et al., 1981) for the determination of lipase activity in various regions of the brain as described below:

Reagents:

1) **Lipase Substrate** (Sigma Chemical Co., U.S.A.): It contained 50% olive oil (v/v) and Sodium Azide 0.1% as preservative. It was kept in a refrigerator at 5°C and shaken thoroughly before use.

2) **Trisma Buffer**: Reagent grade tris 0.2M (hydroxymethyl) aminomethane (Sigma Chemical Co., U.S.A.) dissolved in double distilled water, pH 8.0 at 25°C and sodium Azide (0.1%) as preservative. It was stored at room temperature.

3) **Thymolphthalein Indicator Solution**: 0.9% thymolphthalein (v/v) in Ethanol.

4) Ethyl alcohol, 95%

5) 0.05M, Sodium hydroxide.

6) **Procedure**: Various parts of the brain were homogenized (10% w/v) in chilled 0.15M Potassium chloride. The homogenates were centrifuged at 5000 rpm for 20 minute. Thereafter,
one ml of the supernate of each part of the brain was pipetted in a 50 ml conical flask, marked 'BRAIN' and was placed at 0°C for 3 hours. Then two sets of the test tubes were taken, one set was marked 'TEST' and other as 'BLANK'. One ml aliquot of each part of the brain was taken only in the set of test tubes marked 'TEST', thereafter, into both sets of test tubes (TEST and BLANK) were pipetted 3.0 ml lipase substrate 1.0 ml trisna buffer and 2.5 ml of double distilled water. Sets of both test tubes were placed in a constant water bath at 37°C for incubation for 3 hours.

After the incubation, contents of the test tube (TEST) were poured into 50 ml flask and marked 'TEST'. The contents of the test tube (BLANK) were poured into the 'BLANK' flask which was kept at 0°C. Sets of both test tubes were rinsed with 3.0 ml alcohol and poured into their respective conical flasks. To both sets of the flasks, 6 drops (0.25 ml) of thymolphthalein indicator were added and titrated with 0.05N NaOH to a light blue color.

vii) Calculation:

\[
\text{Volume (ml) of NaOH used for TEST - Volume (ml) of NaOH used for BLANK} = \delta
\]

Brain lipase activity in International Unit per gram (IU/g) = \delta \times 10 \times 0.25

Brain lipase (IU/g) = \delta \times 2.5

where, 0.25 a multiple factor.
Results were given as mean ± S.E.M. Statistical analysis for significance between the means of the experimental and control groups was checked by Student's *t* test, and the 'F' values calculated. 'F' values less than 0.05 were considered be significant.

2.18. Analysis of Variance (ANOVA):

ANOVA was calculated. The object of ANOVA was to break up the total variation into components due to each of the two factors:

(i) Variation within regions

(ii) Variation due to CO and H2S exposure.

They were compared by 'F' test.