METHODS AND MATERIALS

a) Animals

Albino rats and guinea pigs of particular breed were used in all the experiments. Both young and 6 to 12 months' old animals were used in this study. Only male animals were generally employed. The older animals were fed on a standard diet ad libitum, however, younger animals during the weanling period were mostly reared up on mother's milk. Animals were maintained in a big airy room. The room was cooled with air conditioning facilities during summer and during winter, the room was heated with the help of hot air blower.

b) Mode of slaughter and dissection of tissue

The animals were killed by decapitation. The skin over the skull was cut and retracted and the brain exposed by cutting the skull with scissors along the dorsal side and removed whole. Tissue was freed of extraneous material by means of filter paper and forceps. The whole operation from killing of the animals to removal and cleaning of the tissues did not take more than two minutes and every effort was made to reduce the time.

c) Preparation of homogenate

Fresh tissue was used in all experiments. Before preparing the homogenates the tissue was rapidly minced with stainless steel scissors to get a homogeneous mass. Frozen material was never employed. The suspension medium was 0.32 M sucrose. Potter-Elvehjem homogenizer was used to homogenize brain tissue. 9 ml of

medium was used for 1 gm of tissue. The homogenizer was operated for two minutes interval with a gap of one minute. The cycle of upward and downward movement of the tube took place 20 to 25 times, the contents being held chilled in a bath of ice.

d) Method of subcellular fractionation of brain homogenate

Nuclear, crude mitochondrial and microsomal fractions were prepared by differential centrifugation. The crude mitochondrial fraction was purified as described by Whittaker (113) by centrifuging it into a discontinuous sucrose gradient. The whole process may be summarized as below.

(i) A 10% homogenate was made in 0.32 M sucrose (i.e. every gm of tissue was made upto a final volume of 10 ml).

(ii) The homogenate was centrifuged in PR-1 type of centrifuge at 1,000 g for 10 minutes.

(iii) The supernatant (S_1) was carefully tipped off into a container in ice. Pellet (P_1) was resuspended in cold 0.32 M sucrose. P_1 is the fraction containing mainly nuclei, large myelin fragments and large debris i.e. blood vessels and cells, with small amounts of synaptosomes, glia cells.

(iv) The supernatant (S_1) was then centrifuged at 17,000 g for 60 minutes in Spinco L 265B (Att 30 rotor) centrifuge.

(v) The supernatant was poured off called S_2 and placed in ice..

(vi) The pellet from step (iv) was P_2 and was resuspended in 0.32 M sucrose (approximately 2 to 3 ml for every gm of original tissue). This was the crude mitochondrial fraction. It contained small myelin fragments, synaptosomes and mitochondria.

(vii) This was then carefully layered over a density gradient (made up one hour before use) consisting of layers of 1.2 M and 0.8 M sucrose and spun for 2 hours at 53,000 g in SW 25 rotor in a Spinco Model L 265 B.

(viii) The material was separated into three layers: which were (from top to downward) P_2A (small myelin), P_2B (synaptosomes) and P_2C (mitochondria).

e) Preparation of calibration curve for ammonia

This curve was prepared by using same colorimeter and same reagents which were used in the determination of enzyme activity. One stock solution of ammonium sulphate was prepared which contain 100 μg equivalent of ammonia per ml of solution.

0.1, 0.2, 0.3, 0.4 and 0.5 ml of stock solution were taken in the test tubes. The volume was made upto 2 ml with distilled water and 2 ml of 2N NaOH and 1 ml Nessler's reagent were added

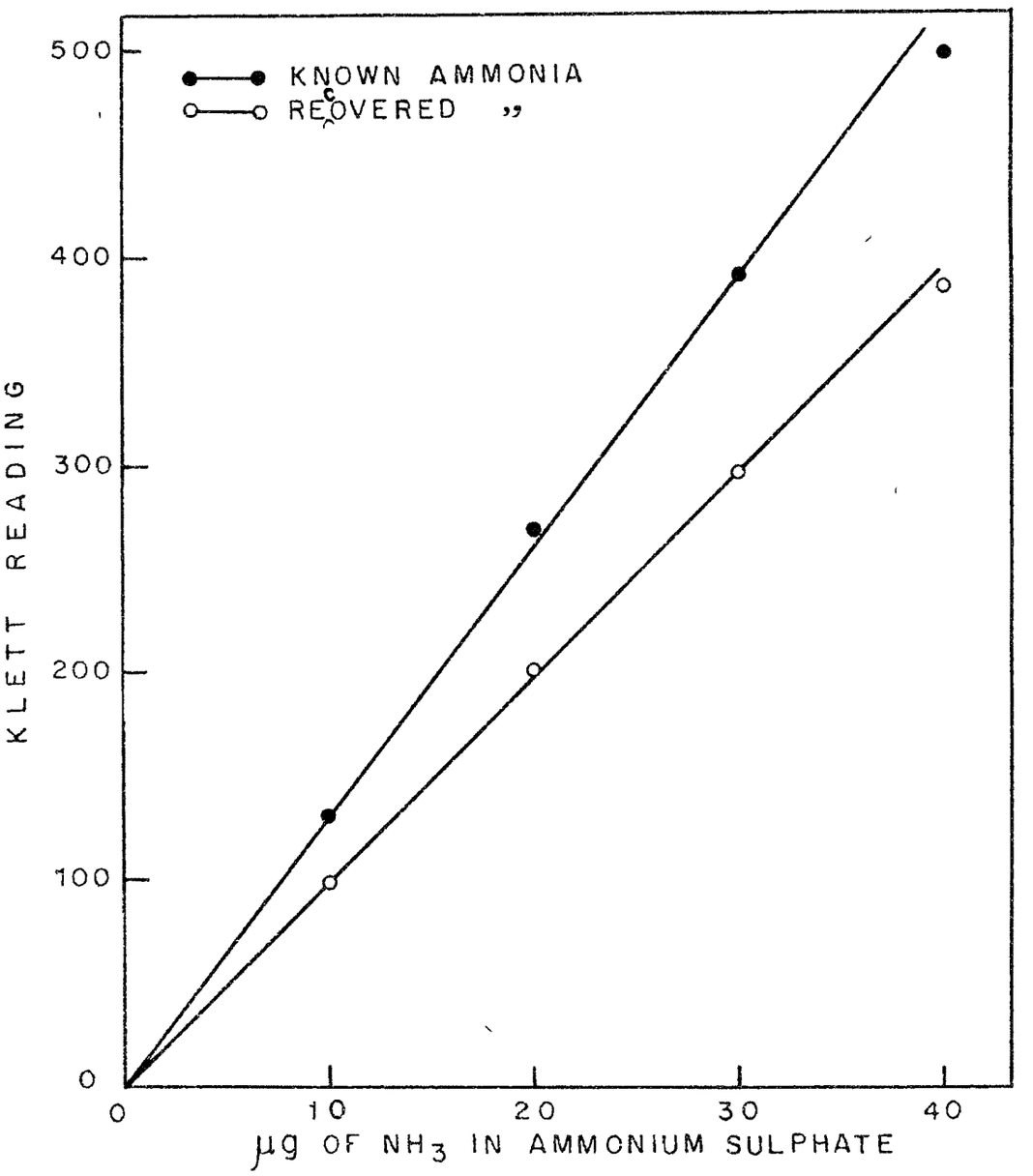

Fig. 1·1 Calibration curve for ammonia .

subsequently. The intensity of color was measured at the end of 30 minutes at 425 mμ (violet filter) in a Klett-Summerson colorimeter.

A calibration chart was made by plotting on a millimeter graph paper, the optical density on the ordinate scale and amount of ammonia in ammonium sulphate (in μg) on the abscissa (Fig. 1.1).

f) Recovery of ammonia by Conway's diffusion cell

Recovery of ammonia was measured by taking 1 ml of 1N sulphuric acid in central chamber and 0.1, 0.2, 0.3, 0.4 and 0.5 ml respectively of stock solution of ammonium sulphate in outer chamber of diffusion cells (114). Volume was made upto 3 ml with distilled water and 0.3 ml of saturated K_2CO_3 was added. The cells were incubated at 37°C for 60 minutes. After incubation material of the central chamber was transferred quantitatively by washing with distilled water to respective test tubes. The volume was made upto 2 ml with distilled water and 2 ml of 2N NaOH and 1 ml Nessler's reagent were added subsequently. The intensity of color was measured at the end of 30 minutes at 425 mμ (violet filter). A calibration chart was made by plotting on a millimeter graph paper, the optical density on the ordinate scale and amount of ammonia as ammonium sulphate (in μg) recovered in the inner chamber of diffusion cell, on the abscissa.

g) Determination of glutaminase activity

The determination of ammonia was done by the microdiffusion method of Conway and Byrne (114).

The activity of the enzyme was assayed following the method of Weil-Malherbe (27) using L-glutamine as substrate.

The central chamber of the Conway unit contained 1 ml of 1 N sulphuric acid solution. The outer chamber contained the appropriate quantity of the mitochondrial preparation in 0.32 M sucrose containing 1 mg of protein per incubation, 0.02 M tris-HCl buffer at pH 7.4 and phosphate (sodium salt, pH 7) to a final volume of 3 ml. The reaction was commenced by the addition of 0.3 ml of L-glutamine (final concentration 0.005 M), to the outer chamber. Other additions were made before the addition of glutamine. At the end of 60 minutes incubation at 37°C, 0.3 ml of saturated K_2CO_3 solution was added to the outer chamber to stop the reaction and to release the ammonia formed. In the control experiment substrate was added just before the addition of saturated K_2CO_3. The cells were again incubated at 37°C for 60 mins. Materials of central chamber was transferred quantitatively by washing with distilled water to a final volume of 2 ml. 2 ml of 2N NaOH and 1 ml of Nessler's reagent were added subsequently. The intensity of color was measured at the end of 30 minutes at 425 mμ (violet filter) in a Klett-Summerson colorimeter. Amount of ammonia formed was calculated from the calibration curve of recovered ammonia.

The protein was estimated by Biuret method (115).

Materials

L-glutamine, palmitic acid and myristic acid were obtained from British Drug House; Tris, Na-L-thyroxine were from Sigma Chemical Company; 2:4 DNP and digitonin of reagent grade were from E. Merck and Company, Germany; GSH and BSA were from Nutritional Biochemicals Corporation; oleic acid was from Reidel and Company, Germany. Other reagents were of reagent grade.

Solution of fatty acids were prepared by dissolving them in minimum quantity of alcohol and Tris-HCl buffer was added to make the solution neutral. Corresponding control experiments were carried out in presence of same concentration of alcohol and Tris-HCl buffer at same pH.

Digitonin of 2% stock solution was prepared by adding warm 0.32 M sucrose to the powdered digitonin mixing briskly and sonicating for 1-2 minutes, in an ultrasonic bath. The resulting water clear solution was kept at $0^{\circ}C$. All digitonin solution were prepared immediately before use.
