STUDIES ON THE ENZYMES OF CEREBRAL CELLULAR GLUTAMATE METABOLISM IN HYPERAMMONEMIA

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This is to certify that I, G.Y.C.V. Subbalakshmi, have carried out the research work embodied in the present thesis under the guidance of Dr. R.K. Murthy, for the full period prescribed under Ph.D. ordinances of the University

I declare to the best of my knowledge that no part of this thesis was earlier submitted for the award of research degree of any University.

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To
my parents

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C O N T E N T S

ABBREVIATIONS

Aspartate Aminotransferase

Adenosine 5-Diphosphate

Alanine Aminotransferase

Adenosine 5'-Monophosphate

Adenosine 5'-Triphosphate

Deoxyribonucleic acid

Dithio Nitro Benzoic Acid

Ethylene Diamine Tetra Acetic acid

Electroencephalograph

Y-aminobutyric Acid

Glutamate Dehydrogenase

Guanine 5'-diphosphate

Glutaminase

Glutamine Synthetase

Guanine 5'-Triphosphate

Hepatic Encephalopathy

Inosine 5'-Monophosphate

Lethal Dose at which 50% animals of the group die

Mg++ dependent Adenosine Triphosphosphatase

L-Methionine DL-Sulfoximine

Nicotinamide Adenine Dinucleotide

Nicotinamide Adenine Dinucleotide reduced

Ammonia

$NH4+$	Ammonium ion
Na+K+ATPase	$Na^{+}K^{+}$ activated, Mg++ dependent adenosine
$Na^{+}K+$ -ATPase	Na+,K+ activated Mg
Na+,K+-ATPase	triphosphatase
RNA	Ribonucleic acid
TCA	Trichloroacetic acid
TCA cycle	Tricarboxylic acid cycle

CHAPTER I

General Introduction and Scope of the Present Investigation

Of all the metabolic pathways in a wide variety of animals, the pathway concerned with the nitrogen metabolism has an unique position. Nitrogen metabolism is interesting in two aspects viz. (1) very few metabolic reactions are involved in the incorporation of inorganic nitrogen (in the form of ammonia) into organic nitrogen and (2) there are multitude of reactions which can liberate organic nitrogen in the form of ammonia. Much attention was focussed in the past decades to study the nitrogen metabolism in a wide variety of animals under variety of experimental conditions as accumulation of the ammonia either in the blood or in tissues would be lethal to the organism. A wide variety of biochemical, physiological and environmental adaptations are seen in animal phyla to circumvent the ammonia toxicity which are well documented.

In man,all the known hyperammonemic states are of metabolic origin, though, there are very few cases of direct exposure to ammonia. Brain is the most profoundly affected organ during hyperammonemic states and a majority of the clinical symptoms observed are related to the neurological disturbances. Voluminous work has been done to elucidate the basic pathophysiological mechanisms underlying the neurological disturbances induced by the ammonium ion. However, as yet, no conclusive mechanism has been proposed.

In the present investigation an effort was made to identify the cellular site of action of ammonia in the brain and to correlate the result with the observed neurological changes. Before pronouncing the scope of the present investigation, a brief introduction encompassing the available information on the cerebral ammonia metabolism both in normal and pathological states and the etiology and pathophysiology of the neurological disorders in hyperammonemic states would be provided.

Cerebral ammonia levels:

The ammonia content of the brain depends on the functional state at the time of study. Under normal physiological conditions the cerebral ammonia levels were reported to be between 0.15 and 0.35 μ moles/gm wet weight of the tissue. These values increased during heightened neuronal activity and declined following a decline in neuronal activity. Post-mortem changes were also found to have a profound influence on the determination of cerebral ammonia levels (107, 123, 134, 281, 282, 288, 311). Information on the regional heterogenity in the cerebral distribution of ammonia both in normal and pathological states is completely lacking. Such a study would be of interest since, the ammonia levels are used as an 'index of neuronal activity' in the normal brain (93). Under the pathological conditions, it would

help in understanding the vulnerability of different regions of brain.

Conditions influencing cerebral ammonia levels:

As mentioned earlier, cerebral ammonia levels fluctuate in parallel to the functional state. A reduction in functional activity was found to be associated with a decrease in cerebral ammonia content. Richter and Dawson reported an 80% reduction in brain ammonia content due to prolonged anaesthesia (226). A similar fall was noticed during sleep (300). However, an elevation of cerebral ammonia levels was found to be always associated with increased cerebral activity such as convulsions (93, 285, 287). Besides this, several convulsants were also found to increase brain ammonia content. These include MSI (93, 266), fluoroacetate, thiosemicarbazide, pentamethylene tetrazole (281) camphor (301), picrotoxin (226, 120), bicuculline (57) and insectisides such as telodrin (119) lindane, dieldrin, heptachlor and DDT (209) and bemigride (322). Such studies lead to a conclusion that the toxicity of these agents might be due to the liberation of ammonia as it was observed that ammonium salts by themselves are capable of producing convulsions. Not only the convulsant drugs, but also the pathological states associated with convulsions such as anoxia (226) hyperbaric oxygen (87), hypercapnia (92), hypoglycemia (2), ischemia(282),

audiogenic seizures (in sujceptible animals) (169) enhance cerebral ammonia levels. Deprivation of sleep (121) and painful shocks to extremities (286, 288) also elevated brain ammonia content.

Increased ammonia levels in brain and CSF were also noticed since long time in several pathological states such as hepatic encephalopathy (241, 328),after portocaval anastamoses (137, 323) and in the congenital disorders in the metabolism of amino acids and of urea cycle enzymes (69, 139, 241).

Sources and production of ammonia in brain:

Steady state level of ammonia in brain is governed by the relative rates of its uptake from blood and extra-cellular fluids, release into blood and extracellular fluids, its rate of formation from both endogenous and exogenous sources and finally by ammonia utilizing processes in brain. However, in the ensuing description, the role of extra-cellular space and extracellular fluids would be ignored as the contribution by this compartment to total cerebral volume was shown to be insignificant (accounting to 2-5/. of total volume (177, 250). The process involved in maintaining ammonia levels in brain might then be represented as follows:

Blood ammonia / Production from endogenous precursors.

BRAIN ANMONIA

Blood precursors / Utilization of both endogenous and exogenous ammonia.

Though it is well known that blood ammonia levels are strictly guarded and maintained well below the tissue levels (blood ammonia < 0.1 mM), its contribution to tissue pools cannot be ignored. This process assumes a greater importance especially in hyperammonemic states, when the blood ammonia levels would be higher than usual. In blood, it was shown that,the level of ammonia in red blood corpuscles was higher than plasma (50, 133). Several lines of evidences indicated that either the blood or extracellular ammonia rapidly equilibrates with tissue pools for eg., systemic administration of ammonium salts increased the brain ammonia levels in a very short time (123). Of the mechanisms proposed for ammonia transport across the neuronal plasma membranes, the pH gradient theory has been well accepted (191, 264).

Ammonia can exist in two forms - the unprotonated NH_3 form and the protonated NH4+ form. These two forms are in a state of dynamic equilibrium in solution and their interconversion was found to be rapid and solely dependent on pH of the medium (123). At alkaline pH, NH4+ dissociates

into NH_3 and H^+ and at acidic pH the NH3 form would be converted to NH4 form by accepting a proton. However, at physiological pH (7.4) at least 98/. of ammonia is in NH_4 form. In this state the molecule has a limited permeability across the plasma membrane while the NH_3 form penetrates the plasma membrane with ease as it is more lipid soluble.

Though, brain ammonia concentrations were found to be lower than blood, most of the blood ammonia enter the brain. It was observed that under physiological conditions, the pH of brain was lower than the blood pH (134). The NH_3 form of ammonia from blood would then rapidly enter the brain as it is more freely permeable. This perturbs the equilibrium between protonated and unprotonated forms and favours the dissociation of additional NH4+ to NH_3 in blood. Inside the cell, due to acidic pH, NH_3 accepts a proton and is converted to the impermeable NH4+ form. This process continues as long as the blood pH is higher than brain pH. Increased intra-cellular NH_3 levels would deplete protons and shift the intracellular pH towards alkaline range unless homeostatic mechanisms buffer the pH change. If they fail, not only further transport of ammonia would be inhibited but also other metabolic processes would be interfered. Evidences for the pH dependent transport of ammonia were provided by the following observations. (a) The tissue (brain and muscle) ammonia concentrations increased by 2-3 fold during metabolic

and respiratory alkalosis (264). (b) Variations in the LD_{50} dose of various ammonium salts tested were directly related to their ability to induce changes in the blood pH (310). Alkaline ammonium salts (NH_4OH, $(NH_4)_2CO_3$) were found to be more lethal even at very low doses than acidic salts of ammonia (NH_4Cl). (c) Acidosis induced by injection of hydrochloric acid had a protective effect on the mice injected with ammonium chloride (4).

In addition to the above said mechanism, carrier mediated or active uptake of ammonium ion (NH_4^+) by the cells was also proposed which was based on the following observations. (a) NH_4^+ substituted K^+ for the enzyme Na^+, K^+-ATPase due to the similarities in the radii of the hydrated ions of K^+ and NH_4 (4) (b) K^+ was replaced by NH_{4+} in the ouabain sensitive Na+ extrusion against concentration gradient in toad skeletal muscle and red blood cells (15, 219). However, this effect was observed only when NH_4^+ concentration was 3-7 times more than K+ . (c) Under in <u>vitro</u> conditions, the K^+ concentration in cortical slices decreased far in excess of the internal NH_{4+} when they were incubated with 10 mM NH_4Cl(17) (d) Administration of NH_4Cl or MSI (which increased brain ammonia levels) or addition of NH_4Cl to brain homogenates increased the Nat K^+-ATPase activity (235, 265, 266). (e) In infant brain an equal exchange of K+ with NH_4^+ was demonstrated. Such observations favour the hypothesis that NH_4^+ might be transported into the cell at the expence of ATP by the membrane bound Na+, K^+-ATPase (265).

In tissues like liver and muscle, amino acids from blood are avidly taken up and utilized not only for protein synthesis but also for energy generation, the latter process leads to ammonia production (167). However, in brain the operation of such a process appears to be unlikely because of the peculiarities of brain metabolism. It is well known that the blood-brain barrier is not freely permeable to all the amino acids (14). They are transported across the barrier by specific carrier proteins (14). As more than one amino acid share the same carrier protein for transport, the relative concentration but not the concentration of individual amino acid in the bathing medium, determine the rate of transport, which severely restricts their abundancy for utilization in energy generating reactions. Amino acids that have a relatively free permeability are glutamine and alanine (14). Generation of ammonia from glutamine might be severely restricted due to high endogenous glutamate concentrations in brain which inhibit glutaminase activity (147, 247) while the very low activity of alanine aminotransferase limits the utilization of alanine for ammonia production (8, 235, 268, 269). Further, the sole energy source for brain under physiological conditions is glucose and but for glutamate no other amino acid can support brain energy demands (14). However, exogenous (blood) glutamate may not be available for this purpose as the blood-brain barrier is impermeable to this amino acid. Hence, ammonia

reaction in brain from precursors supplied by blood might generation in brain physioological be of limited importance at least under physiological conditions

The inherent ability of brain to produce ammonia from its endogenous precursors was conclusively demonstrated during the in vitro studies with cortical slices. When slices were aerobically incubated in the absence of substrates supporting cerebral metabolism, about 14-16 moles of ammonia was generated per µgm. wet weight of tissue. Addition of substrate, such as glucose, suppressed ammonia formation to a large extent (>70%.). Metabolic inhibitors (iodoacetate), uncouplers of oxidative phosphorylation (2,4-Dinitro-phenol), electron transport chain inhibitors such as cyanide inhibit ammonia production in the absence of glucose and enhance in the presence of glucose. Anoxia diminished ammonia production both in the presence and absence of glucose under these conditions. These results suggested that ammonia production in brain could be an aerobic process (20).

Such a high rate of endogenous ammonia production in brain elicited interest in the search for a precursor to this process. In summary, the three major precursors proposed to serve as substrates were proteins, amino acids and nucleic acids.

Proteins: Proteins as a source for endogenous ammonia was suggested long ago (301, 318). It has been shown that of the total ammonia formed, during in vitro incubation of brain tissue in the absence of a substrate, at least 25-35/. was accounted by the deamidation of protein bound amide groups (301). It was estimated that 16% of the protein bound glutaminyl (but not asparginyl bonds) were deamidated in situ (321). This process was independent of the presence of glucose. Besides deamidation of protein bound amide groups, ammonia was also shown to originate from amino acids generated during proteolysis. The occurrence of latter process was evidenced by a rise in the content of essential amino acids during in vitro incubations (20, 317).

Amino acids: The free amino acid pool of brain differs from that of other tissues by the presence of large quanti-ties of glutamate family of amino acids (glutamate, glutamine, aspartate, alanine and Y-aminobuutyric acid). The glutamate concentration in the brains of various mammals was found to be approximately 10 µmoles/gm. wet weight while that of glutamine was 4-5 µmoles. As these two amino acids occur in such large concentrations, much attention was focussed on the changes in their content under a variety of metabolic states associated with ammonia production. Early studies in this direction indicated that at least 50% of the ammonia formed was accounted by the fall in glutamate content during

in vitro incubation of cortical slices (277). However, in these studies the corresponding increase in aspartate formed from endogenous glutamate by transamination, was not taken into account. Later studies indicated that the contribution made by glutamate to cerebral ammonia would be little higher than 30/. (19, 20) largely localized in the neurons and ammonia liberation occurs majorly from this site (19). Of exogenous glutamate taken up by brain, 49/. would be converted to aspartate and 37/. to glutamine (both the processes occur in glia) while only 14/. would be completely oxidized to ammonia and α-ketoglutarate, majorly in neurons (19). The glial cells as per these authors, do not produce ammonia. However, recent results suggest that glial cells are capable of oxidizing glutamate completely to ammonia and α-ketoglutarate (326).

During in vitro incubations of cortical slices, ammonia production was observed to be associated with a fall in the content of glutamine besides glutamate (20). 30/. of the ammonia formed could be accounted by the fall in the glutamine content. This value appears to be high as later studies indicated that ammonia production from exogenous glutamine proceeds at a lower rate, which was ascribed to the inhibition of the initial deamidation process by endogenous glutamate and ammonia (16). Further, the precise contributions made by α-amino and amido groups or spontaneous hydrolysis

of glutamine to cerebral ammonia pool is not known. Besides these two amino acids, other amino acids such as GABA were also proposed to act as precursors for ammonia production in the brain. However, the contribution made by amino acids other than glutamate and glutamine appears to be negligible.

Purine nucleotides: Depolarization of cortical slices *in vitro* was shown to be accompanied with the release of IMP, inosine and hypoxanthine (272). It was postulated quite early that in excitable tissues (such as brain and muscle) less phosphorylated nucleotides would be liberated as a result of depolarization (222). These results lead to the postulation that the purine nucleotides, especially AMP and adenosine might serve as precursors for ammonia production by a process of deamination in the purine nucleotide cycle (175, 251).

Enzymes involved in ammonia production: As the major precursors in cerebral ammonia production were shown to be proteins, glutamate and

and purine nucleotide cycle, a brief account of the enzymes involved in this process are given below.

Though, the contribution made by protein bound amide group is significant, it is surprising to note that no enzymatic mechanisms have been so far described in the

literature. The only study that gives a clue to the enzymatic mechanism involved was the inhibition of this process by MSI (321).

The enzyme glutamate dehydrogenase (GDH) oxidatively deaminates glutamate to ammonia and α-ketoglutarate and requires either NAD^+ or $NADP^+$ as the coenzyme. The same enzyme is also responsible for the reverse reaction (α-ketoglutarate+ammonia glutamate) in the presence of NAD(P)H. This enzyme is localized in the mitochondrial matrix and serves as a connecting link between amino acid and carbohydrate metabolisms. Though, Chee et al., (58) purified this enzyme from brain only recently, it was not thoroughly characterized as liver enzyme. The molecular weight of the purified enzyme from other sources ranges from 300,000 to 2,000,000 with a total number of polypeptide chains varying from 10-40 (31, 273). This enzyme exhibits association-dissociation phenomenon which appears to be under the control of various factors including its own concentration. At low concentrations, the enzyme dissociates into catalytically active subunits with a different substrate specificity (94,283). However, in liver mitochondria the concentration of this enzyme was found to be sufficiently high to exist in associated form (274). The association-dissociation kinetics of this enzyme are also influenced by physiological compounds such as nucleotides, inorganic ions and even NAD^+.

The activators promote the association of the subunits while the inhibitors dissociate the enzyme (284). The enzyme also exhibits sigmoid kinetics with allosteric properties. The allosteric activation by ADP, GDP and leucine and the allosteric inhibition by ATP and GTP are of physiological significance. The oxidative deamination of glutamate by this enzyme is also subjected by noncompetitive feed back inhibition by ammonia, if the concentration is high (20 mM) (43, 86). Further, the ratio of NAD(P)/NAD(P)H also influences the direction of the GDH reaction (18, 19, 317). In the presence of a substrate, ADP stimulates reductive amination of α-ketoglutarate by increasing affinity of GDH for ammonia (168).

Normally, the primary step in the metabolism of most of the amino acids is the transamination wherein the α-amino group is transferred to an α-ketoacid to form a corresponding amino acid. Though both oxaloacetate and pyruvate have the ability to participate in these reactions to form aspartate and alanine, in most of the transamination reactions α-keto-glutarate is the preferred substrate resulting in the forma-tion of glutamate which is later oxidized to produce ammonia by GDH reaction. This pathway is known as transdeamidation pathway. Of all the aminotransferases present in brain, the activity of aspartate aminotransferase (AAT) was found to be very high (7, 77, 144, 156) followed by alanine

aminotransferase (ALAT). Both the enzymes were found to have a dual localization in the cells, i.e., both in mitochondria and cytosol. Because of the high activity of AAT, it was shown that at least 80% of glutamate oxidation occurs through the transamination pathway (7). In addition to its role as a connecting link for amino acid and carbohydrate metabolisms, this enzyme also plays a major role in Borst cycle (4b) for the transfer of reducing equivalents from cytoplasm across the mitochondrial membranes. For this purpose, the enzyme acts in concert with malate dehydrogenase. The operation of this cycle in brain mitochondria is now well established (39, 77). This enzyme is also known to form stable macromolecular complexes with GDH in pig brain, though the significance of this process is yet to be understood (63).

Hydrolykic cleavage of glutamine would also generate ammonia from glutamine and the enzyme responsible for this process, glutaminase (GLNase) was found to be present in brain. Two major isozymes of GLNase have been identified in mammals (143). The phosphate independent GLNase is not susceptible either to ammonia or glutamate and is present mostly in kidney and liver. This enzyme is supposed to be absent in brain (140, 152, 153, 314-316) or even if present it is less than 10% of the total GLNase activity (201, 279).

The other form of GLNase, phosphate activated GLNase was found to be activated by low concentrations of phosphate. The enzyme is localized on both the sides of the mitochondrial inner membrane (153). This enzyme is inhibited by both the products, glutamate and ammonia under in vivo conditions. Further, excess substrate would also be inhibitory to this enzyme which was observed to be not due to the substrate per se but by the large amounts of products produced. This form of GLNase was demonstrated to be present in kidney, liver and brain. In the brain 90% of the GLNase activity was found to be due to phosphate activated form. Recent studies indicated a complex pattern of regulation of this enzyme in brain which depends on its cellular localization (154). The enzyme localized in the nerve ending particles was found to be inhibited by both glutamate and ammonia while the astroglial enzyme was found to be inhibited by glutamate alone. The enzyme from both the sources is activated by calcium ions (16,154). Besides generating ammonia, this enzyme is supposed to play a major role in generating the neurotransmitters such as glutamate and GABA from glutamine. However, under physiological conditions, it is believed that the activity of this enzyme is not fully expressed due to high glutamate levels which are inhibitory (18).

Ammonia production from purine neuleotides:

The operation of purine nucleotide cycle in brain was postulated by Lowenstein and his associates. Ammonia is generated in this pathway by the deamination of AMP by the enzyme AMP-deaminase with the concomitant production of IMP. IMP is converted to AMP and the necessary amino group is provided by aspartate. The net result of operation of this pathway is the oxidation of aspartic acid. The enzyme, AMP-deaminase is a cytosolic enzyme and exhibits allosteric properties. ATP acts as an allosteric activator and GTP as an allosteric inhibitor. It is believed that under physiological conditions, the inhibitory effect by GTP is far more than the ATP activation, hence, the enzyme exhibits low activity. The enzyme activity was also found to be stimulated by K+ and this inorganic ion could be substituted by Rb^{+} and NH4 + (256, 257).

In addition to AMP, adenosine was also shown to be deaminated in the brain by the enzyme adenosine deaminase. Hence, AMP might also be converted to adenosine by the enzyme 5'-nucleotidase and later deaminated. The presence of this latter enzyme was also demonstrated in brain and it was reported to be localized exclusively on the astroglial plasma membrane(332}

Liberation of inosine and hypoxanthine both in vivo and in vitro (by slices) into the incubation medium lends support to the operation of this pathway (222).

Minor reactions: Besides the above three processes, ammonia is also generated in small quantities from other sources such as guanine and guanosine deaminases, NAD-deaminase, glucosamine-6-phosphate deaminase, monoamine oxidase, serine dehydratase, histidase, etc.

As said earlier, the steady state levels of ammonia depends not only on the rate of transport and its endogenous synthesis, but also on the rate of its disposal or utilization. It is obvious that if the rate of removal of ammonia is low, then the tissue levels would be high due to ammonia accumulation. The latter condition may be seen in some pathological states especially where extracerebral tissues are involved.

Disposal of ammonia: Unlike the multitude of ammonia generating processes, only three pathways exist in animal systems to dispose of ammonia. They are (a) carbomyl phosphate biosynthesis (b) glutamate and (c) glutamine biosynthesis. As the carbomyl phosphate synthesis is negligible in brain (237), the latter two processes would be discussed.

Glutamate biosynthesis: The enzyme involved, in fixing inorganic ammonia into organic linkage of glutamate, is GDH, the description of which was given earlier.

$$\text{Ammonia} + \alpha\text{-ketoglutarate} \xrightarrow[\text{NADH}\ \ \text{NAD}^+]{} \text{glutamate}$$

Under physiological conditions, the equilibrium of this enzyme catalyzed reaction is poised in favour of glutamate biosynthesis. However, the contribution of this pathway to the total pool of cerebral glutamate ------- appears to be quantitatively lower than the transamination pathway. Glucose carbon would be rapidly converted to glutamate and aspartate by transamination pathway under physiological conditions when the cerebral ammonia levels are low (10).

Glutamine synthesis: In extrahepatic tissues, which are devoid of complete compliment of urea cycle enzymes,such as brain and muscle (237), the major pathway of ammonia detoxification is supposed to be glutamine biosynthesis (313). Glutamine acts as a temporary store house of ammonia and exerts less toxic effects when compared to ammonia. Further, it also serves as a nitrogen donor in several biosynthetic pathways leading to the formation of aminosugars, nicotinamide coenzymes, purine nucleotides, pyrimidines etc. In brain, glutamine has additional role as a precursor for neurotransmitter amino acids such as glutamate and GABA.

$$\text{Glutamate} + NH_3 \xrightarrow[\text{ATP}\ \ \text{ADP+Pi}]{} \text{glutamine}$$

This reaction is mediated by the enzyme glutamine synthetase (GS). Due to the endergonic nature, the reaction is virtually irreversible. The product ADP is inhibitory to the enzyme. The enzyme glutamine synthetase is ubiquitously distributed in ^^^^^^^^^^ all animal cells. In brain it is believed that this enzyme is exclusively localized in the astroglial cells (183, 206). Though much work is done on the regulation of bacterial glutamine synthetase, not much information is available on the brain enzyme and the available evidences indicate that this enzyme from animal tissues is not subjected to regulation by various ligands as the bacterial enzyme (167).

Metabolic compartmentation and ammonia in brain; Following infusion of ^{15}N-amrnonium acetate, it was observed that there was a significant increase in cerebral glutamine content without a corresponding drop in glutamate (29). Simultaneous determination of the fate of ^{15}N-label revealed heavy labelling of the amido nitrogen of glutamine than the -amino nitrogen of either glutamine or its precursor, glutamate. This anamoly in the precursor-product relationship was explained by assuming the existence of two metabolic pools of glutamate, of which one was larger and the other was smaller. It was postulated that the large pool of glutamate contains most of the tissue glutamate pool and has a slow turnover rate while small pool of glutamate contains less

glutamate and has a rapid turnover rate than the former. These two pools do not mix with each other. Further, it was shown that the rate of synthesis of glutamine was less in the large pool than the small pool and glutamate in the large pool would be metabolized by oxidative deamination while in the small pool it would be majorly involved in glutamine synthesis. The precursor for glutamate in the large pool was shown to be glucose while in the small pool besides glucose, β-hydroxybutyrate, acetate, carbon dioxide, butyrate, propionate, citrate, leucine, etc., would serve as precursors (9, 65, 193, 292). Further studies revealed that the large pool of glutamate is localized in the neurons and the small pool in the glia (18, 65, 100, 293). This suggested that the metabolism of glutamate would be different in the neurons and glia. The neurons metabolize glutamate primarily by oxidative deamination while in glial cells major portion of glutamate would be converted to glutamine and rest is transaminated to α-ketoglutarate prior to the oxidation through citric acid cycle ------ (19). Higher activities of GDH (231) in neurons than in the glia and the presence of high affinity uptake systems for glutamate in glia. (124, 127, 319) supported this concept. The glutamine so formed in the glia would be transported to nerve endings where it would serve as a precursor for releasable pool of glutamate and GABA. This concept received support from the observation that exogenous glutamine would serve as a better precursor

for releasable pool of both glutamate and GABA in synaptosomes (41) and in slices (112, 113) and the activity of GLNase was high *in* synaptosomes than *in* the astrocytes (320). Further, histochemical and biochemical localization of the enzyme GS exclusively in astrocytes favoured this concept (183, 206).

Thus according to the theory of metabolic compartmentation, ammonia would be generated by neurons and nerve endings from both glutamate and glutamine and liberated along with glutamate and GABA during functional states. The exogenous ammonia, glutamate and GABA would be transported into the astrocytes where ammonia is converted to glutamine. The glutamine in the astroglial cells would be transported into nerve endings where it is reconverted to glutamate and related neurotransmitters.

Cerebral effects of ammonia: Though ammonia is generated, released and utilized by brain during functional states, it is less well tolerated and the endogenous concentrations are very closely guarded. At elevated levels, ammonia exerts deleterious effects on cerebral functioning. Ammonium ion was shown to have a biphasic effect on cerebral activity, at low concentrations it is known to act as an excitant and at higher concentrations as a depressant(286). Elevated ammonia levels, associated with several pathological states, result

in either convulsions or coma. Though several hypotheses were proposed, the underlying mechanism in ammonia toxicity is yet an enigma. Walker and Schenker (306) have summarized the literature on the possible sites of action of ammonia. All the suggested mechanisms ultimately postulate that NH_{4+} would deplete cerebral ATP stores either directly or indirectly. Such a depletion of ATP in vital areas of brain has functional significance as it is believed that ATP is a prerequisite for the maintenance of cerebral electrical activity. Of the 8 sites suggested extensive literature is available only on few sites and the evidences for the effects on other sites are inconclusive.

Effects on ionic fluxes: Exogenous ammonia would encounter the plasma membrane before entering the cell and hence it might be assumed that at least some of the membrane functions would be influenced by the ammonium ion. The primary function of neuronal plasma membrane is the maintenance of ionic gradients essential for the generation of action potentials and other vital electrical activities. It was shown by several investigators that NH4+ exerts an influence on this process. Benjamin et al., reported that NH4+ suppressed spontaneous electrical activity in brain slices and promotes an efflux of K^+ with a concomitant increase in [Na+]0 (17). Administration of ammonium salts was shown to elevate plasma K^+ without any change in Na^+ (123). Loss

of K^+ from body fluids (hypokalemia) was also noticed in prolonged hyperammonia (218). These studies would then suggest that extracellular NH_4^+ affects the ionic fluxes across the neuronal plasma membrane. Besides the ionic channels (of several types), the membrane bound enzyme, Na^+, K^+-ATPase was shown to play a major role in the maintenance of ionic gradients (189), as this enzyme utilizes at least a third of total cerebral ATP stores (5). Na^+, K^+-ATPase was shown to transport 2 K^+ into the neuron and 3 Na^+ out of the cell at the expense of one ATP molecule (189). As mentioned earlier, NH_4^+ would compete with K^+ for the sites on this enzyme as the ionic radii of NH_4 and hydrated K^+ were found to be similar (262). It was shown that the activity of this enzyme in brain was elevated in hyperammonemic states (23b, 236, 26b). Stimulation of Na^+, K^+-ATPase would not only affect cellular ATP levels but also shifts the resting membrane potentials close to firing threshold (5, 16, 123). In addition, the release of neurotransmitters would also be affected as this enzyme was shown to be involved in this process (190, 299).

Besides these two ions, NH_4^+ was also shown to influence the permeability to chloride ions (Cl^-). NH_4^+ were shown to block the operation of outwardly directed chloride pump resulting in the disinhibition of postsynaptic inhibition of spinal neurons (178). Similar results were obtained by

other investigators (171, 179, 223, 224). An inward movement of Cl~ into rat brain cortical slices was shown to be dependent on the extracellular NH4+ concentration (17). At low concentration of NH4+ the inflow of Cl- was not associated with any fluid influx and at higher concentrations it was associated with a large influx of fluids. These authors concluded that the Cl- uptake at low [NH4+]0 was largely into neurons and the second phase of influx would be into glial cells. These permeability changes were believed to be due to local pH changes created by ammonium ion induced metabolic changes.

Depletion of α-ketoglutarate: Bessman and Bessman proposed this hypothesis to explain the mechanism of ammonia toxicity (33). The major contention of their proposal was that α-ketoglutarate (a KG) would be drained out of citric acid cycle in the detoxification process by way of glutamate formation (α-KG+NH_3——> glutamate) catalyzed by GDH. Depletion o+ αKG would interfere with the normal operation of TCA cycle and subsequently energy production. Though this hypothesis was theoretically attractive, the experimental results were highly contradictory. The early reports of reduced α-ketoglutarate levels in ammonia intoxication (32, 64, 259) did not receive support from later studies wherein the levels were reported to be either increased or remained unaltered (83, 123, 132). In spite of these contradictory

evidences this theory was still given credence due to the fact that these studies were carried out either with extracts of whole brain or with larger areas and this would mask any localized change in the levels of α-ketoglutarate. Further, no importance was given to the metabolic compartments. It is well known that about 80% of brain α-ketoglutarate would be present in the large compartment of glutamate and only 20% would be localized in the small compartment (99). No satisfactory method is at hand till today to study the localized changes in these compartments. However, it is interesting to note that Garfinkel and Hess (101), based on the data of Berl et al., (29), predicted a fall in the α-ketoglutarate levels in the small pool of glutamate, supposed to be localized in the astrocytes and such a change was considered to be possible (186).

Another implication of Bessman's hypothesis would be an increase in the glutamate content and its rate of formation due to enhanced reductive amination of α-ketoglutarate in hyperammonemic states. Evidence was provided to this much later by the elevated activity levels of cerebral GDH in hyperammonemic states (84, 202, 235, 236). It was observed that the magnitude of change was greater in the brain stem than in other regions which supported the earlier concept of greater vulnerability of this region (235, 236). However, on the contrary cerebral glutamate levels under

these conditions were reported to be either unaltered or decreased (29, 84, 90, 97, 123, 136, 180, 194, 289). This decrease was, however, explained to be due to the increased rate of glutamate utilization for glutamine biosynthesis, a process stimulated by enhanced cerebral ammonia levels.

Glutamine formation: As mentioned earlier, disposal of ammonia by way of glutamine biosynthesis would be a major pathway in brain during hyperammonemic states (313). As glutamine formation involves ATP utilization, it has been suggested that this would be another major energy depleting mechanism in hyperammonemia (198). In compliance with this hypothesis, an increase in cerebral glutamine levels was observed in hyperammonemic states of varied etiologies (133, 135, 137, 235, 297, 323, 328) and in cortical slices in the presence of ammonium salts (16, 19).Another line of evidence for this hypothesis was provided by the studies with glutamine synthetase inhibitor, methionine sulfoximine. Administration of this compound resulted in an elevation of cerebral ammonia levels with concomitant fall in glutamine levels and the animals succumbed to convulsions (311). The increase in cerebral glutamine levels in hyperammonemic conditions was shown to be partly due to decreased glutamine breakdown (inhibition of cerebral glutaminase by ammonia) (293) and also due to increased glutamine synthesis (26). This observation was supported by the unaltered activity of glutamine

synthetase in homogenates under hyperammonemic conditions
15
(68, 235). Metabolic studies with N ammonium salts indicated that the synthesis occurred exclusively in glial cells (29), an observation supported by immunohistochemical studies on the localization of this enzyme (206). The glial involvement in ammonia detoxification was further evinced by the cytological changes observed in glial cells in hyperammonemic states resulting in the formation of Alzheimer type II glia (53, 162, 203-205). These studies, in conclusion, supported the postulated role of glial cells in ammonia detoxification and energy depletion in these cells in hyperammonemic states.

Effects on cellular NADH/NAD ratio: Enhanced glutamate dehydrogenase in hyperammonemic states would affect the levels of not only α-ketoglutarate but also of NADH. As NADH is oxidized to NAD in this reaction, it was proposed that under hyperammonemic states, NADH availability for electron transport chain would be severely restricted thereby retarding ATP production (325). Hindfelt and Siesjo (136) confirmed this hypothesis by demonstrating a fall in the ratio of NADH to NAD in the mitochondria. The cytoplasmic ratio of NADH to NAD under these conditions was found to be elevated and enhanced lactate production was attributed to this. A similar profile reported later confirmed the above finding (260).

Other effects of increased cerebral ammonia levels in brain were supposed to be stimulation of phosphofructokinase (promotion of glycolysis) (136, 242), increased carbondioxide fixation (26, 29, 30, 304), and decreased ACh production (278, 308).

In conclusion, majority of the hypotheses on the mechanism of ammonia toxicity proposed an interference in ATP production, and depletion of TCA cycle intermediates. Though several efforts were made in the past to demonstrate such changes, the results obtained were inconclusive. Schenker and his associates have demonstrated a fall in ATP levels at least in brain stem and its associated structures (186, 259). But these results were contradicted by several other investigators (32, 123, 137, 235). The discrepancy might be due to various procedures adopted to arrest postmortem changes and the selection of different regions for ATP determination. Further, as the studies were made in homogenates, it would be difficult to detect the local changes in a small population of cells. Finally, it may be said that ammonium ion has a multipronged effect on brain metabolism and it would be difficult to predict which particular change triggers either convulsions or coma.

Pathological states associated with hyperammonemia; As mentioned earlier, in hyperammonemic states there would be

an elevation in both blood and tissue levels of ammonia resulting in a metabolic and functional derangement. Such hyperammonemic states could be due to defects either in the synthesis, transport or disposal of ammonia. The known hyperammonemic states might be categorized into congenital or acquired disorders. A brief description of the known states of hyperammonemia would ensue below, but detailed descriptions are available in several reviews (4, 74, 89, 241, 328).

Congenital disorders: In congenital hyperammonemic states defects were found in the enzymes directly related to ammonia metabolism on one hand and on the other hand several defects in the enzymes involved in the metabolism of amino acids not related to ammonia were noticed.

The only known congenital disorder with defective glutamate synthesis was reported to be Reye's syndrome. The defect in this syndrome is not in the enzymes of glutamate biosynthesis, but in the fragility of mitochondrial membranes which would be subjected to an acute insult (49, 214). Consequently, the integrity of these membranes would be lost leading to the liberation of all the mitochondrial enzymes inclusive of GDH and carbamyl phosphate synthetase which aggrevates the clinical condition (44, 228). Recently, it was shown that an endogenous inhibitor was bound to GDH

which would lower the residual activity of GDH (138). Due to the loss of both GDH and carbamyl phosphate synthetase, ammonia accumulates in the blood and tissues.

No reports are available on congenital defects in glutamine biosynthesis which might be because of the premature death of the embryos as glutamine has several other roles to play besides serving as an ammonia trap.

Several congenital disorders of urea cycle enzymes have been reported. Clinical symptoms and magnitude of hyperammonemia varies from mild to severe. The range of defects also vary. Total deficiency of carbamyl phosphate synthetase, though rarely occurs, was reported to be lethal, while in majority of cases, the deficiency was partial with various degrees of residual activity and in such cases other causes for hyperammonemia played a decisive role. The severity of ornithine transcarbamylase deficiency was reported to be fatal in males and benign in the females. The range of defectsvaried from total absence of the enzyme to altered affinity for ornithine. These patients had high blood glutamine levels besides ammonia, indicating increased glutamine synthesis as an alternate protective mechanism. Further, elevated blood levels of orotic acid and uracil were believed to be due to increased availability of carbomyl phosphate for their synthesis. Spongy cortical degeneration and mental retardation were reported in these patients (139).

In the congenital disorder of argininosuccinic acid synthetase, both citrulline and ammonia levels were elevated in the blood and the affected patients had an abnormal EEG and cerebral atrophy. The clinical outcome of this disorder was shown to be fatal. Massive accumulation of argininosuccinic acid was reported in argininosuccinase deficiency. The blood ammonia levels and clinical picture were highly variable and could be modified by restricting the protein intake. The survivors usually suffered with mental retardation (139).

Hyperammonemia was also observed in several other inherited disorders of amino acid metabolism. In ornithinemias due to a defective ornithine aminotransferase;ornithine accumulates in the blood and influences the operation of urea cycle leading to a hyperammonemic state. Due to the competitive inhibition exerted by lysine on arginase activity, hyperlysinemia was found to be associated with hyperammonemia. Disorders of branched chain amino acids, such as maple syrup disease, hypervalinemia, etc., were found to be accompanied with elevated blood ammonia levels and the relationship between these two is yet to be understood (139).

Acquired hyperammonemic states: A direct relationship between hepatic functioning and hyperammonemic states has been established. In several of the reported acquired hyperammonemic

states a severe deficiency of hepatic functioning was observed. The spectrum of neurological symptoms due to hepatic inadequacy was found to be highly variable and the term 'hepatic encephalopathy' (HEC) was coined to describe the changes. In the early stages of hepatic encephalopathy mental, personality and emotional changes were found to be very subtle. With the progress of encephalopathy,hypothermia, hyperventillation, confusion, drowsiness ensue which gradually lead to comatose condition. Neuromuscular changes like asterixis, hyper-reflexia, unsustained clonus were also noticed in early stages of HEC which disappear along with pupillary and corneal reflexes with the progress of the disease. Convulsions, decerebrate rigidity and decortical posture were found occasionally (241). HEC was found to be of two types. (i) Exogenous hepatic coma or chronic liver disease due to decreased liver mass and gradual shunting of blood around liver (liver bypass). These conditions are seen usually in cirrhosis and portal systemic shunting and the onset of neurological and other clinical symptoms are less violent and precipitated by exogenous factors such as high protein diet or by drugs. (ii) In acute liver disease (endogenous hepatic coma) the decrease in functional liver mass is due to acute viral infections, drugs and other toxins and the onset of clinical symptoms are very rapid and sudden.

Cerebral pathology varies with the duration and type of hepatic coma. In acute HEC cerebral edema is a prominent feature (309), while in chronic HEC,hypertrophy and hyperplasia of protoplasmic astrocytes are of common observation. These changes are seen principally in cerebral cortex and in other regions of brain (305). Neuronal cells do not show many of changes in the acute HEC while in chronic conditions degeneration and demyelination are exhibited (330). However, the major change is usually seen in the protoplasmic astrocytes. These cells, in chronic HEC, proliferate, enlarge and finally undergo hydropic degeneration. The changes induced would result in the formation of Alzheimer type II astroglial cells (1). The Alzheimer type II cells were also found in experimental HEC due to portocaval anastamosis in rats (150) and in induced hyperammonemias (53, 106, 162, 203-205). The development of Alzheimer cells were reported to be associated with the loss of glial acidic fibrillary protein in the grey matter (263). The Alzheimer changes in the glial cells under these conditions were believed to be due to abnormal biochemical events leading to altered electrolyte balance and other transport phenomena (52, 327).

Ammonia was inculcated since long time in the pathogenesis of HEC. Increased levels of ammonia in blood and CSF is a prominent laboratory finding in patients with HEC. The major site of origin of ammonia in these patients was

established to be intestine (210, 241, 328). The intestinal bacteria deaminate not only the dietary aminoacids but also urea to generate ammonia. In the absence of functional liver, this ammonia enters the systemic circulation. Tissue ammonia levels rapidly equilibrate with blood ammonia levels and as a result an overall hyperammonemic condition sets in. Increased ammonia levels are supposed to deplete TCA cycle intermediates and energy levels (due to glutamine formation) as described earlier which would ultimately result in the broad spectrum of neurological changes (241). Glial cells, as the seats of glutamine synthesis, were supposed to be involved in the detoxification process, and loose their energy resources which would be reflected in their morphology (203, 204). However, no direct relationship between the blood ammonia level and extent of coma was possible to establish. Instead a direct relationship with a good correlation exist between CSF glutamine levels and clinical state of coma. To overcome this anamoly various alternate hypotheses were proposed for the pathogenesis of hepatic coma, brief account of which are given below.

(a) False Neurotransmitters:

This theory was proposed by Fisher and Baldessarini (89, 142). Decreased levels of neutral amino acids (leucine isoleucine, and valine) with a concomitant rise in the levels of aromatic amino acids (phenylalanine, tyrosine and tryptophan)

would result in the preferential transport of the latter group of amino acids into brain. As their concentration builds up, the rate limiting initial hydroxylation reaction (in catecholamine and serotonin biosynthesis) would be bypassed and these are decarboxylated to form β-phenylethylamine, octopamine, synephrine ana tryptamine. These amines would displace the biogenic monoamines from the nerve endings and receptor sites resulting in an altered catecholaminergic and serotonergic transmission which might precipitate coma. The theory is wholly based on the partial disruption of blood brain barrier and a specific alteration in the amino acid transport.

( ) Short chain fatty acids:

Plasma and CSF levels of short chain fatty acids such as butyrate, valerate and octonate were reported to be elevated in conditions of HEC (331). These fatty acids either alone or synergestically with ammonia were shown to produce coma in experimental animals (307, 331) which was proposed to be one of the pathogenic mechanisms of HEC.

(c) Mercaptans:

Indoles and mercaptans were shown to be degradation products of methionine by intestinal bacteria liberated into the portal blood and detoxified by liver (59). In HEC due to hepatic inadequacy, these compounds enter the systemic

circulation attributing a characteristic musty odour to these patients (59). It was proposed that these compounds might be inculcated into the pathogenic mechanisms of HEC (329).

In addition to above hypotheses, the role of GABA produced by intestinal bacteria and affecting cerebral GABA-ergic functions due to a partial disruption of blood-brain barrier was recently proposed (184, 240). Another mechanism proposed was increased cerebral sensitivity to exogenous substances (241).

SCOPE OF THE PRESENT INVESTIGATION

Despite of an assumption that astroglial cells are highly vulnerable to ammonia toxicity, much of the earlier work on the cerebral effects of ammonia and the changes in cerebral metabolism in hyperammonemic conditions were carried out with extracts or subcellular fractions or slices prepared from either whole brain or from selected regions. It is well known that brain is a heterogenous organ having at least three different cell populations (neurons, astrocytes and oligodendrocytes) which are structurally, functionally and metabolically different from each other. No consious efforts were made to study the metabolic changes in cells other than astrocytes. This was partly because of the firm belief that

the astrocytes, which serve as the locale of the small compartment of glutamate hence, involved in the glutamine formation, were the seats of ammonia detoxification while the neurons encompassing the large compartment were not involved in this process. The major limiting factor to undertake such a study in the past was the difficulty to obtain a relatively pure preparation of the different cell populations. Though, this problem was answered recently by the use of cultured cells, in which the purity of the fraction might approach theoretical limits, certain limitations would restrict their use. One problem with cell culture is their considerable deviation in several biochemical and physiological parameters from the cells grown _in situ. However, the short life span of the brain cells under cultured conditions would also restrict their use. Moreover, the cells grown in the culture are totally devoid of the developmental influences of other cells which is quite normal in situ development.

In the present study an attempt was made to separate the different cell fractions from normal animals and from animals rendered hyperammonemiC. Efforts were also made to characterise the cells both physically and biochemically under these conditions. In addition, an attempt was also made to study the enzymes involved in the metabolism of amino acids of glutamate family (aspartate, alanine, glutamate and glutamine) in the cell fractions isolated from the normal

and hyperammonemic animals. The reason for selecting the amino acids of glutamate family is quite obvious as they are not only involved in the synthesis and the removal of ammonia, but they also have metabolic and functional roles to play in the cerebral tissues. The reason for selecting astrocytes to study the metabolic changes in hyperammonemia is based on the earlier observations that the astroglial cells are highly vulnerable to ammonia toxicity as they are seats of glutamine formation. Since, the metabolism of glutamate in the astrocytes is closely related and highly influenced by its metabolism in nerve endings, in the present study the metabolic changes in the nerve ending particles during hyperammonemic conditions were also studied. Such a study would throw light on the changes in glutamate metabolism in different metabolic compartments during pathological states. In the original theory of metabolic compartmentation though much was said about the involvement of neurons in the glutamate metabolism, the word 'neuron' was not clearly defined as the neurons have different structural elements such as perikarya, dendrites, axons and nerve endings. Of all these elements, only nerve endings are capable of releasing the neurotransmitters and thereby participating in the impulse transmission. Hence, it may be assumed that the word 'neuron' of the original theory of metabolic compartmentation might refer to the nerve ending particles. In other words, this theory ignored the contributions made

by neuronal perikarya to cerebral metabolism. Hence, it was felt necessary to study the metabolic contributions made by neuronal perikarya in normal and in hyperanmonemic states. Such a study would then yield results which would throw light not only on the cellular site of action of ammonia, but also on the neuro-glial interactions during the pathological states.

CHAPTER II

Materials and Methods

1. Animals;

Adult albino rats from an inbred colony of Wistar strain were chosen as experimental animals. The animals were of either sex and of same age having a body weight of 150-200 gms. The animals were maintained in an air conditioned room in groups of six in cages under natural light and dark periods. The animals had free access to food (balanced pellet diet from Hindustan Lever Limited) and water ad libitum.

2. Drug treatment;

2.1. Ammonium acetate treatment;

Ammonium acetate (25 mmoles/kg wt.) was administered intraperitonially using saline as a carrier, for both comatose and convulsive conditions.

2.2 MSI treatment;

L-Methionine-DL-Sulfoximine was administered intraperitonially using saline as a carrier. For acute treatment a dose of 300 mg/kg body weight was administered and the animals were sacrificed at the end of 3.5 hrs. For subacute treatment, the dose was reduced to 150 mg/kg body wt. and the animals were decapitated between 17-18 hrs.

3. Cell Isolation Procedure:

Both astrocytes and neurons were isolated by the method of Farooq and Norton (88) modified and described earlier (269, 270a). The basic cell isolation medium consisted of 8%, (w/v) glucose, 5% (w/v) fructose and 2% (w/v) Ficoll in 10 mM KH_2P0_4-NaOH buffer pH 6.0. Additional Ficoll was added to this basic medium to prepare solutions of increasing densities. Thus 7% Ficoll solution contained a total of 9% (w/v) Ficoll and so on. Brains devoid of cerebellum and brain stem were isolated from rats soon after decapitation. Cerebral cortex was quickly scooped out and 10-14 free-hand slices were prepared. Cortical slices were incubated in 5 ml of basic medium with acetylated trypsin to a final concentration of 0.1% at 37 C for 90 min. in a shaking water bath. After incubation, the medium was decanted, an equal amount of basic medium containing 0.1% soybean trypsin inhibitor was added and the mixture was cooled. The medium was discarded and the slices were washed 3-4 times with ice cold medium.

Disaggregation of the tissue was achieved by successive aspiration steps. A nozzle with a diameter of 2.4 mm at the tip and length of 3.4 cm was attached to the side arm of 100 ml suction flask. The washed tissue was aspirated through the nozzle into the medium under slight negative pressure. The coarse suspension was passed through

monofilament nylon screen with a mesh size of 300 . The residue on the screen was aspirated and filtered twice. Finally the undisrupted tissue on the screen was aspirated from the underside of the screen and all the filterates were combined. After mixing gently with a vortex mixer, the filterate was passed twice successively through nylon meshes of pore diameter 80 μ and 55 μ to remove debris and broken processes.

The coarse cell suspension was centrifuged at 720 g for 15 min. at 2°C. The cell rich pellet (P1) was suspended in 1% Ficoll solution and centrifuged at 720 g for 10 min. to separate a neuronal cell enriched pellet (P2). The supernatant was diluted to 1:1.125 with the basic medium and centrifuged at 1,120 g for 20 min. to get a pellet enriched with astrocytes (P_3). Instead of layering on density gradients as in the original procedure, P_2 was suspended in 30% Ficoll solution and P3 in 15% Ficoll solution and centrifuged at 8,500 g for 10 min. P30F contained relatively pure fraction of neurons and P_{15F} astrocytes. The pellets were washed once with the cell isolation medium.

The cells from experimental animals were isolated in the similar procedure but with slight modifications. The time for incubation with acetylated trypsin was reduced to 60 min. and the crude cell suspension after disaggregation

step was passed through 300 μ and 80 μ nylon meshes. The time for each differential centrifugation was increased by 5 min. P_2 was suspended in 26% Ficoll solution (instead of 30% and P_3 in 10% Ficoll solution (instead of 15%) for the final centrifugation step. (Flow diagram of the cell isolation procedure is enclosed at the end of the chapter).

The final cell pellets were suspended in 0.32 M sucrose and an aliquot was stained with 1% methylene blue. The cells were counted under a light microscope in a hemocytometer. The cell viability was assessed by dye exclusion method using 1% trypan blue. For enzyme assays and biochemical determinations cells were suspended in double distilled water and subjected to a single cycle of freezing and thawing.

Isolation of synaptosomes;

Synaptosomes were isolated by the procedure of Cotman (72). Cerebral cortex was homogenized in 5 ml of ice-cold 0.32 M sucrose (pH 7.0) in a Potter-Elvehjem type homogenizer with a teflon pestle. The homogenate was first centrifuged at 1000 g for 5 min. and the supernatant further at 15,000 g for 12 min. The resultant pellet (crude mitochondrial fraction) was suspended in 5 ml of 0.32 M sucrose and applied to density gradient consisting 5 ml of 4, 6 and

and 13% Ficoll in 0.32 M sucrose. After centrifugation at 63,500 g for 45 min. (MSE superspeed 75) the synaptosomal fraction from the 6/13% Ficoll interface was diluted with 4 volumes of 0.32 M sucrose (pH 7.0) and centrifuged at 50,000 g for 20 min. The pellet was suspended in distilled water and the protein concentration was adjusted to 1 mg/ml. (Flow diagram of the method is enclosed at the end of the chapter).

Microscopy of the cells:

Small drops of cell suspensions were taken on a glass slide and cover glass was placed. Phase contrast photo-micrographs were taken with Zeiss or Leitz photomicroscope.

Scanning electron microscopy of the cells was done according to the method of Hamberger et al., (114). Small drops of cell suspensions were placed on a cover glass and left for 5-10 min. Glutaraldehyde (2.5% in 0.1 M Na-phosphate buffer pH 7.4) was carefully added to the drops with a micro-pipette. The cells were left in glutaraldehyde for 90 min. in cold. The cover glass was then washed with decreasing concentrations of phosphate buffer and then with distilled water, 5O% ethanol, 96% ethanol and absolute ethanol. The specimens were coated with gold in a vacuum evaporator and examined with Jeol scanning electron microscope.

Biochemical determinations;

(i) Wet weight: Approximately 0.5x10 cells were suspended in Krebs Ringer-phosphate-glucose medium (NaCl 128 mM KC1 5 mM, $MgS0_4$ 1.3 mM, $CaCl_2$ 1.0 mM, Na-phosphate buffer 10 mM (pH 7.4), and glucose 10 mM) and filtered through Whatman GF/A filters or millipore membrane filters (0.45 µ) under negative pressure. The difference in the weights of filters with and without cells was taken as the weight of cells and average wet weight of a single cell was calculated and expressed as ng/cell.

(ii) Dry weight: The filters with and without cells were dried at 60°C to a constant weight. The difference between these two was taken as the dry weight of the cells and average dry weight of a single cell was calculated and expressed as ng/cell.

(iii) Water content: The difference between wet weight and dry weight was used for calculating water content, and was expressed as nl/cell.

(iv) Protein determination: Protein was estimated according to the method of Lowry et al., (176). An aliquot of the 0.32 M sucrose homogenate was precipitated with TCA (10% final concentration) and kept at 0°C for 30 min. and centrifuged to obtain a pellet. The pellet was washed with 10%

(w/v) TCA and suspended in 1 ml of 0.1 N NaOH. The protein content was estimated with Folin-Ciocalteau reagent (IN) using bovine serum albumin (100 µg/ml) as standard. For cell preparations the protein content was expressed as pg/cell and for synaptosomes as mg/gm weight of cerebral cortex.

(v) Nucleic acid determination; Nucleic acids (RNA, DNA) were estimated by the procedure of Schmidt and Thanhausser (24b). Nucleic acids present in 1 ml of cell suspension (in 0.32 M sucrose) was precipitated at 10% TCA concentration. The pellet was washed once with 10% TCA and delipidated by washing-twice with ethanol, ethanol:ether (3:1) and ether. The final pellet was dried overnight at room temperature and digested in 1 ml of 1 N KOH at 37°C for 2.5 hrs both DNA and protein were precipated 0.2 ml of 6N HC1 and 0.6 ml of *5%* (w/v) TCA. The supernatant contained RNA. The pellet was hydrolysed with 5% (w/v) TCA at 80°C for 30 min. and centrifuged after cooling to room temperature. The supernatant contained DNA and the pellet, protein. The absorbance of the nucleic acids was measured at 260 nm (Gilford spectrophotometer), using Calf thymus DNA and Yeast RNA as standards. Nucleic acid content per cell was calculated and expressed as pg/cell.

(vi) Ammonia determination; As it was not possible to estimate in the cellular and subcellular fractions, ammonia levels were determined in the whole brain homogenates. Rats were decapitated and the heads were allowed to fall into liquid nitrogen. The frozen heads were taken out after the fixation of the tissue for 15 min. and the brains were quickly scooped out and powdered under liquid nitrogen in a stainless steel mortar chilled with liquid nitrogen. The powder was transferred to tubes containing 5 ml of 5% (w/v) ice-cold PCA. Blood was collected from the Caracas soon after decapitation and the serum was separated. 0.5 ml of serum was precipitated with 5% (w/v) ice-cold PCA. The tubes were kept for 30 min. in an ice bath and centrifuged for 10 min. at 5000 rpm. The supernatants were neutralized with 2 N $KHC0_3$ to pH 7.0 and centrifuged. Ammonia was determined by phenol-hypochlorite method (141). To one ml of supernatant 1.5 ml of solution A (phenol 50 gm. and sodium nitroprusside 250 mg/3.75 1 water) and 2 ml solution B (8.4 gms NaOH, 89.2 gm Na_2HP0_4 and 10 ml of 5% NaOCl/lit) were added. After 20 min. the blue colour was read at 630 nm. Ammonium chloride (0.1-1 m mole) was taken as standard. Cerebral ammonia levels were expressed as μ moles ammonia per gm wet wt. of brain and the blood levels were expressed as μ moles ammonia per ml of serum.

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Marker Enzymes:

Carbonic anhydrase (E.C.2.1.1.1): Carbonic anhydrase was assayed by the method of Krebs and Roughton (148) using $KH^{14}CO_3$ and trapping liberated $^{14}CO_2$ with hyamine hydroxide.

$$H^{14}CO_3^- + H^+ \text{-----} \gg H_2O + {}^{14}CO_2$$

Assay mixture (250 μl) contained 100 µ moles of sodium phosphate buffer of pH 8.0, 50 µ moles of sodium bicarbonate with 10 µl ^{14}C-$KHCO_3$- and 15 µg of enzyme protein. The reaction tion was carried out at 4 C with shaking for 15 min. 14CO2 was trapped with 0.2 ml of IN hyamine hydroxide and radioactivity was determined after mixing with 10 ml of Bray's scintillation fluid (0.4/. diphenyl oxazole and 0.02/. 1,4-bis-(4-methyl-5-phenyloxazole-2-yl) benzene in toluene). Activity was expressed as μ moles of $^{14}CO_2$ liberated/mg protein/hr. or as n moles of 14Co2 liberated/cell/hr.

Lactate dehydrogenase (E.C.l.1.1.27); Lactate dehydrogenase was assayed by following the oxidation of NADH by the method of Bergmeyer and Bernt (25).

$$\text{Pyruvate} + \text{NADH} + H^+ \text{----} > \text{Lactate} + NAD^+$$

The reaction mixture (250 µl) contained 12 µ moles potassi urn phosphate buffer (pH 7.5), 15 µ moles of sodium pyruvate, 0.045 µ moles NADH and 2 µg of enzyme protein. After a

preincubation period of 5 min., pyruvate was added and NADH oxidation at 37 C was recorded for 5 min. at 340 nm. Enzyme activity was expressed as µ moles of NADH oxidized/mg protein/hr or nmoles of NADH oxidized/cell/hour.

Acetyl cholinesterase (E.C.3.1.1.7) and pseudocholinesterase; (E.C.3.1.1.8): The cholinesterases were assayed by the method of Ellmann et al. (85).

Acetylthiocholine iodide —-> Acetylthiol + choline iodide.

Butryl thiocholine iodide —> Butyrylthiol + choline iodide.

Free thiol group + DTNB —-» thio DTNB derivative.

Assay mixture for acetyl cholinesterase (250 µ1) contained 40 µ moles of sodium phosphate buffer (pH 7.9), 0.15 µmoles of acetylthiocholine iodide, 0.1 μmol of DTNB and 10 μq of enzyme protein.

Assay mixture for pseudocholinesterase (250 μl) contained 40 µ-mol of sodium phosphate buffer (pH 7.9), 0.75 µmoles of butrylthiocholine iodide, 0.1 µmoles of DTNB, 0.001 µmol of BW284 C51 (acetylcholinesterase inhibitor) and 10 µg of enzyme protein.

The reactions were carried out at 37 C and the increase in absorbancy was recorded at 412 nm. Enzyme activities were calculated by using the molar extinction

coefficient of DTNB thiol complex (1.36xl04) and expressed as µmoles of acetylthiocholine or butrylthiocholine hydrolyzed/mg protein/hr. or nmoles of acetylthiocholine or butrylthiocholine hydrolyzed/cell/hr.

Choline acetyltransferase (E.C.2.3.1.6); Choline acetyltransferase was assayed by the method of Fonnum (96). The incubation mixture contained 15 µmoles of NaCl, 2.5 µmoles of sodium phosphate buffer (pH 7.4), 0.4 µmoles of choline bromide, 1.25 µmoles of EDTA (pH 7.4), 10 nmoles of acetyl CoA (1- 14C acetyl CoA 4680 DPM/nmole) in a final volume of 50 µ1. Eserine sulfate in the original assay was replaced with BW284 C51 dibromide (0.1 µmole). Following the addition of 30 µg of enzyme protein incubation was carried out at 37°C for 15 min. In the controls 10 units of acetyl cholinesterase was added and BW284 C51 was omitted. At the end of incubation, 2 ml of acetonitrile containing 10 mg of tetraphenyl boron and 10 ml of toluene scintillation mixture (0.05/. diphenyl oxazole and 0.02% 1,4-bis-(4-methyl-5-phenyloxazole-2-yl)benzene in toluene) were added. The vials were shaken gently for a minute, to extract acetylcholine into toluene phase leaving behind acetyl CoA in the aqueous phase. The two layers were allowed to separate for 10 min. and the vials were counted for radioactivity in Beckman liquid scintillation counter (P-3133).

Glutamate dehydrogenase (GDH)(E.C.1.4.1.2): Glutamate dehydrogenase was assayed by the method of Chee et al., (58).

α-ketoglutarate + NH_3 + NAD+ → glutamate + NADH

0.25 µmoles of NADH,
The assay mixture (250 µl) contained/195 µmoles of sodium arsenate-EDTA (0.025 µmoles) buffer (pH 7.8), 12.5 µmoles of α-ketoglutarate and 10 µg of enzyme protein. Assay mixture without α-ketoglutarate was incubated for 5 min. and the fall in NADH absorbancy at 340 nm was recorded for every 15 sec. after the addition α-ketoglutarate.

The enzyme activity was expressed as µmoles of NADH oxidized/mg protein/hr. or n moles of NADH oxidized/cell/hr.

Glutamine Synthetase (GS)(6.3.1.2): Glutamine synthetase was assayed by the method described by Rowe et al. (233). The assay mixture (2 ml) contained 100 µmoles of imidazole-HC1 buffer (pH 7.2), 40 µmoles $MgCl_2$, 50 µmoles 2-mercapto-ethanol, 100 µmoles L-glutamate, 20 µmoles ATP, 200 µmoles of hydroxylamine hydrochloride and 200 µg of enzyme protein. In the blanks both ATP and L-glutamate were omitted. At the end of 15 min. incubation 3 ml ferric chloride reagent

per 100 ml

(6 gm $FeCl_3$, 3.35 gm TCA and 6.6 ml conc HCl) was added and centrifuged. The absorbancy of the supernatant was measured at 535 nm. Using γ-glutamyl monohydroxamate as standard,

the enzyme activity was calculated and expressed as µmoles of Y-glutamyl hydroxamate formed/mg protein/hr. or n moles of Y-glutamyl' hydroxamate formed/cell/hr.

Glutaminase (GLNase)(E.C.3.5.1.2); Glutaminase activity was estimated by the method as described by Nimmo and Tipton (201).

$$\text{L-Glutamine} \xrightarrow{(1)} \text{L-glutamate + ammonia}$$

$$\text{ammonia + } \alpha\text{-ketoglutarate + } {}^{+}\text{NADH+} \xrightarrow{(2)} \text{Glutamate + } \text{NAD}^{+} \text{ + H+}$$

(1) Glutaminase

(2) Glutamate dehydrogenase

The assay mixture (250 μl) contained 25 µmoles of tri-ethanolamine -HC1 buffer (pH 7.4) 0.025 µmoles of NADH, 1.125 (µmoles of L-glutamine, 0.75 µmoles of α-ketoglutarate, 10 units of GDH and 10 µg of enzyme protein. The reaction was carried out at 37°C and the NADH oxidation was measured at 340 nm. The enzyme activity was expressed as µ moles NADH oxidized/mg protein/hr. or as µ moles NADH oxidized/ cell/hr.

Aspartate aminotransferase (AAT)(E.C.2.6.1.1) and alanine aminotransferase (A1AT) (EX.2,6.1.2): Both the amino-transferases were assayed by the method of Bergmeyer and

Bernt (24). AAT assay was coupled to malate dehydrogenase (MDH) and A1AT assay was coupled to lactate dehydrogenase (LDH).

(1) L-Aspartate + α-ketoglutarate $\xrightarrow{AAT}$ L-glutamate + oxaloacetate

Oxaloacetate + NADH + H^+ $\xrightarrow{MDH}$ Malate + NAD^+ + H_2O

(2) L-Alanine + α-ketoglutarate $\xrightarrow{A1AT}$ L-glutamate + pyruvate

Pyruvate + NADH + H+ $\xrightarrow{LDH^+}$ Lactate + NAD+ + H_2O

Assay mixture for AAT (250 µl) contained 10 p,moles of potassium phosphate buffer (pH 7.4), 25 µmoles of L-aspartate, 0.065 µmoles of NADH, 4.5 µmoles of α-keto-glutarate 2.5 *µg* of MDH (in 50% glycerol) and 2 µg of enzyme protein. The change in NADH absorbance was recorded for 5 min.after adding α-ketoglutarate.

Assay mixture for A1AT (250 *µl)* contained 10 µmoles of potassium phosphate buffer (pH 7.4), 100 µmoles of L-alanine, 0.065 µmoles of NADH, 6.6 µmoles of α-ketoglu-tarate, 2-5 *µg* of LDH (in 50% glycerol) and 5 mg of enzyme protein. Preincubation was done for 10 min. and NADH oxidation was recorded for 5 min. following the addition of α-ketoglutarate.

Enzyme activities were calculated from the extinction coefficient of NADH (E mM6.2) and expressed as µmoles of NADH oxidized/mg protein/hr. or nmoles of NADH oxidized/cell/hr.

Assay for Na+, K+ -activated Mg++ dependent adenosine triphosphatase (E.C.3.6.1.3): Na+,K^+-activated Mg^{++} dependent adenosine triphosphatase was assayed by the method of Schousboe et al., (247) and the inorganic phosphate was estimated by the method of Lindberg and Ernster (170). The assay mixture (600 μl) for total ATPase contained 18 µmoles of histidine-HCl buffer (pH 7.4) 72 µmoles of NaCl, 12 µmoles of KC1, 0.6 µmoles of $MgCl_2$, 0.6 µmoles of ATP and 50 μg of enzyme protein. The assay mixture for Mg -ATPase contained 0.6 µmoles of ouabain along with the above components. After 20 min. of incubation at 37°C the reaction was terminated with 0.5 ml of 1.8 N-PCA and centrifuged. To the controls ATP was added at the end of incubation. The inorganic phosphate in a 0.5 ml aliquot of the supernatant was determined following the addition of 0.5 ml of 5N H_2S0_4, 0.5 ml of 2.5% (w/v) ammonium molybdate and 50 μl of reducing mixture (25 mg/ml solution of sodium sulfite: sodium bisulfite: i —- aminqhaphthol-4-sulfonic acid 1.28: 1.25:02 by weight). The solution was made upto 5 ml with distilled water and the colour was measured at 660 nm. after 20 min.

The activity of Na+ K+,-ATPase was calculated as the difference of total and ouabain insensitive ATPase and expressed as µmoles P. liberated/mg protein/hr. or µmoles p. liberated/cell/hr.

Validity of the enzyme assays: Initial rate kinetics of all the enzyme assays were given at the end of the chapter.

Statistical analysis: Statistical analysis of the data was done according to Ganong (98). P values were calculated by Students 't' test.

Materials: Nylon screens were purchased from Nybolt Co., Zurich. 2- C-acetyl CoA was purchased from Amersham Radiochemical Centre, $KH^{14}CO_3$ was supplied by Bhabha Atomic Research Centre, Bombay. Fico 11-400 was obtained from Pharmacia Chemicals.

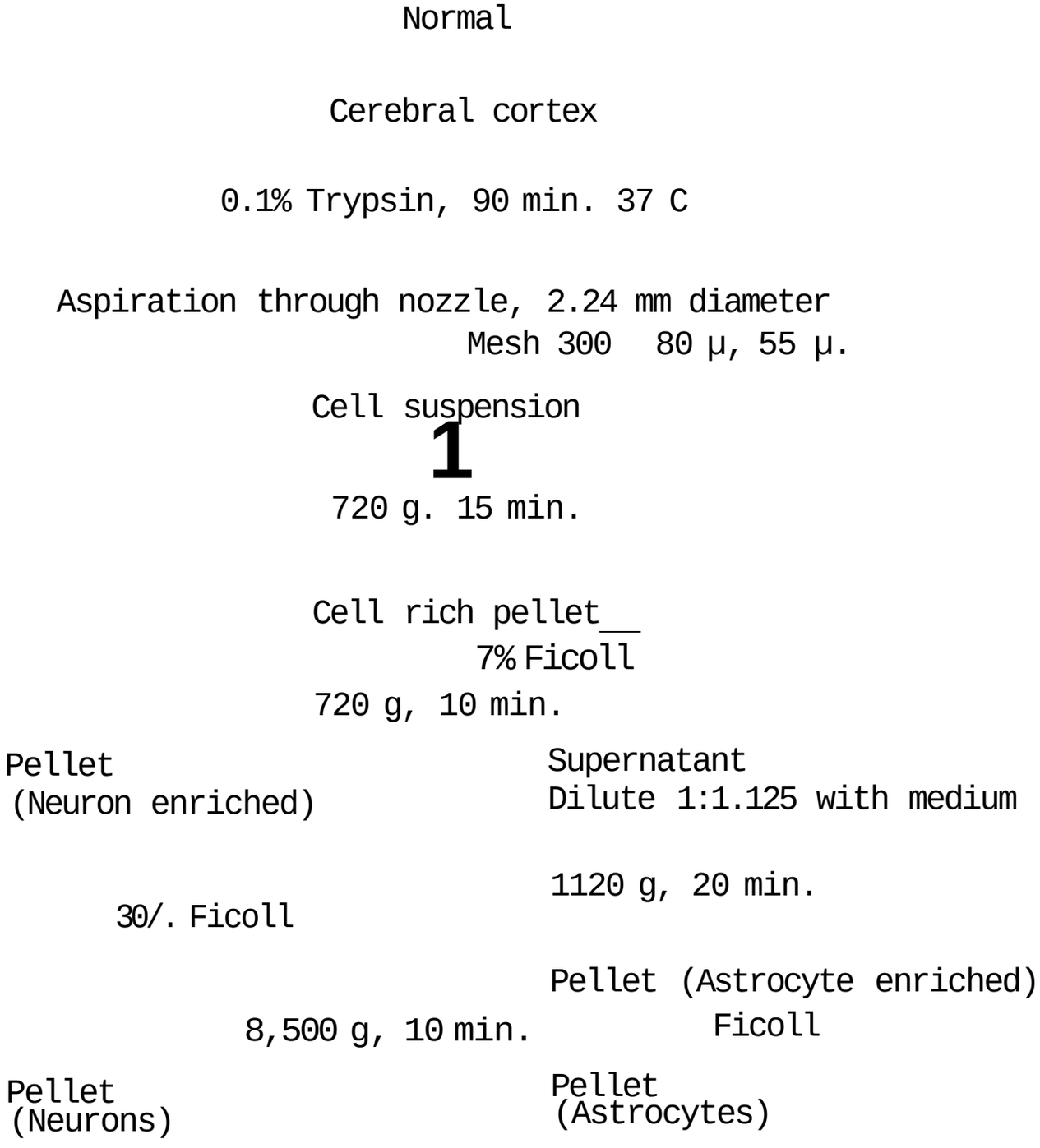
Acetylated trypsin, soybean trypsin inhibitor, α-ketoglutarate, β-NADH, L-aspartate, L-alanine, malate dehydrogenase, lactate dehydrogenase, glutamate dehydrogenase, sodium pyruvate, L-glutamine, ATP, EDTA, choline bromide, DTNB, acetylthiocholine iodide, butrylthiocholine iodide, diphenyl oxazole, 1,4-(bis-4-methyl-5-phenyl oxazole-2-yl benzene) and ouabain were from Sigma Chemical Co., St. Louis (USA).

BW284 C51 dibromide was a gift from Wellcome Ltd., Kent (UK).

All the other chemicals of AnalaR grade were obtained locally.

Spectrophotometric measurements were made with Gilford Spectrophotometer equipped with thermoprogrammer. Radioactivity measurements were made with Beckman Liquid Scintillation Spectrometer-LS3133P. Microscopic observations and photomicrographs were made with Zeiss Photomicroscope or Leitz Photomicroscope and Jeol scanning electron microscope. For centrifugations Mistral CL with high speed attachment or Remi-C 24 and MSE Superspeed 75 were used.

Flow diagram of the procedure for isolating neuronal and astrocyte enriched fractions from rat cerebral cortex.



Flow diagram of the procedure for isolating neuronal and astrocyte enriched fractions from rat cerebral cortex.

Experimental

Cerebral Cortex

↓

0.1% Trypsin, 60 min. 37 C

Aspiration through nozzle 2.4 mm diameter

I mesh 300 p., 80

Cell suspension

720 g, 20 min.

↓

Cell rich pellet

7% Fie oil

720 g, 15 min.

Pellet
(Neuron enriched)

Supernatant
Dilute 1:1.125 with medium

1120 g, 25 min

26% Ficoll

Pellet (Astrocyte enriched)

8,500 g, 15 min

10% Ficoll

Pellet (Neurons)

Pellet (Astrocytes)

Flow diagram of the procedure for isolating synaptosomes from cerebral cortex of normal and experimental rats.

Cerebral Cortex

10% homogenate in 0.32 M sucrose pH 7.0

1000 g, 5 min.

Pellet
(discard)

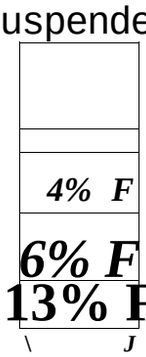
Supernatant

15000 g, 12 min

Pellet

Supernatant
(discard)

suspended in 5 ml 0.32 M sucrose



63, 580 g
45 min.

4% F
6% F
13%

dilute with
4 vol. 0.32 M
sucrose

50,000 g
20 min

Pellet
(Synaptosomes)

Supernatant
(discard)

w u o h eo ff r e q u e n c y

wu 099 of *Frequency*

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6.0

4

3.2 2.8 2.4 2.8

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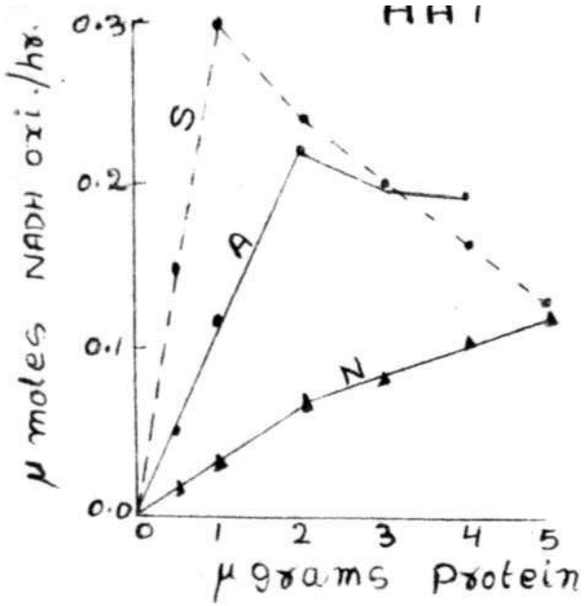
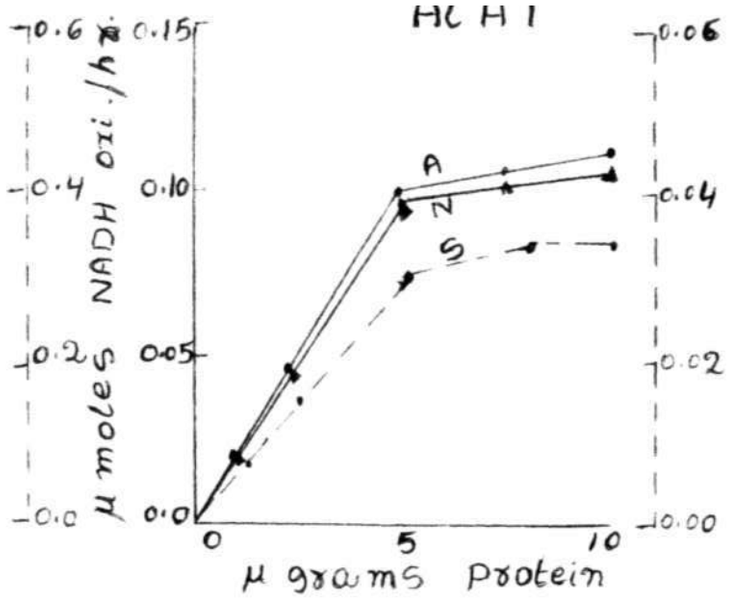
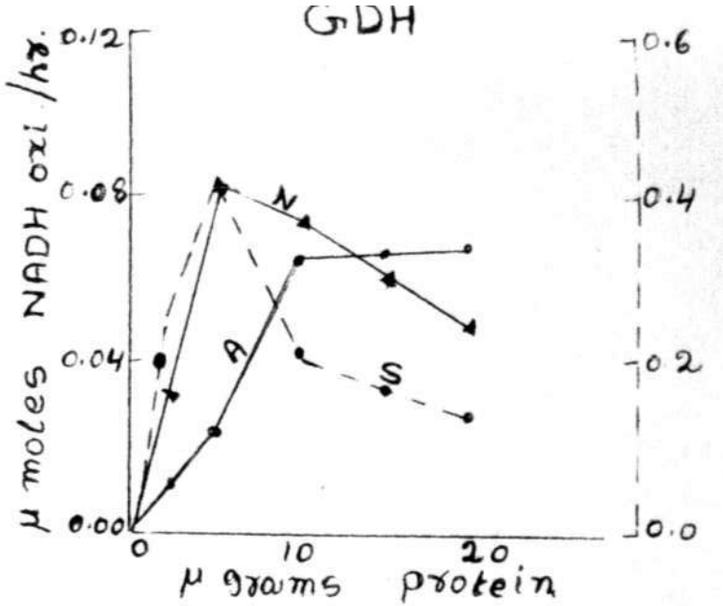
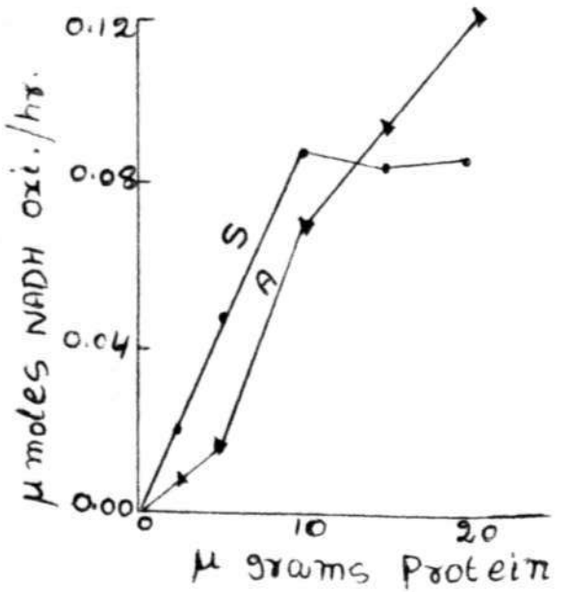
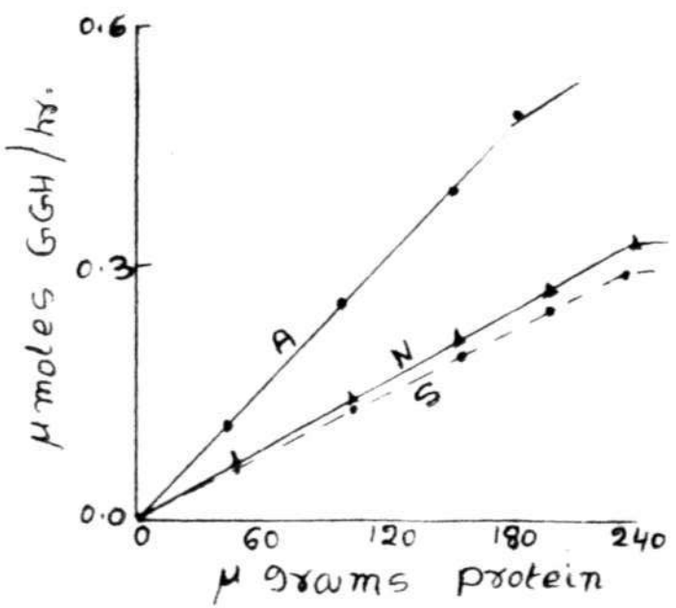
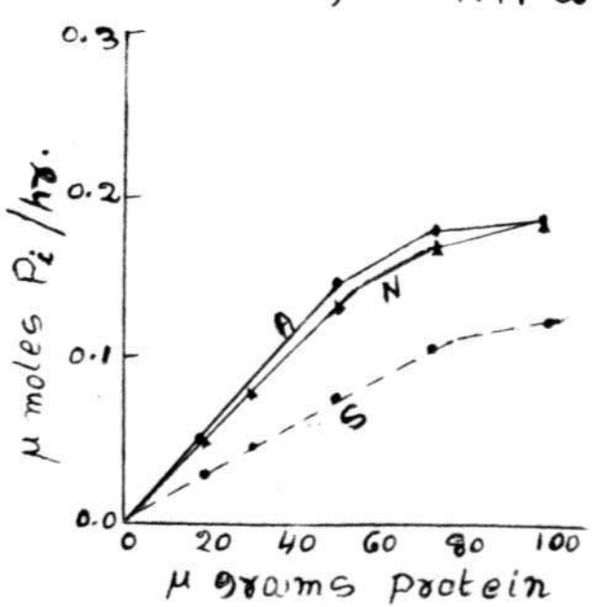

μ moles NADH oxi./hr.
0.3
0.2
0.1
0.0
S
A
N
0 1 2 3 4 5
μ grams Protein


μ moles NADH oxi./hr.
0.6
0.4
0.2
0.0
0.15
0.10
0.05
0.0
0.06
0.04
0.02
0.00
A
N
S
0 5 10
μ grams Protein


μ moles NADH oxi./hr.
0.12
0.08
0.04
0.00
0.6
0.4
0.2
0.0
N
A
S
0 10 20
μ grams protein


μ moles NADH oxi./hr.
0.12
0.08
0.04
0.00
S
A
0 10 20
μ grams Protein


μ moles GGH/hr.
0.6
0.3
0.0
A
N
S
0 60 120 180 240
μ grams protein


μ moles Pi/hr.
0.3
0.2
0.1
0.0
A
N
S
0 20 40 60 80 100
μ grams protein

CHAPTER III

Cell morphology, cytochemical parameters, marker enzymes and distributory profile of enzymes of glutamate metabolism in normal cells.

Following the early work of Rose (230) on the separation and partial characterization of the cell fractions from rat brain, several investigators have reported techniques to isolate these cell fractions with varying degrees of purity (88, 125, 196, 197, 253, 290). However, complete characterization of the cell fractions with respect to morphology, cytochemistry and marker enzymes was not done. In addition, information about the profiles of the enzymes of glutamate metabolism in these cell fractions is totally lacking in spite of voluminous work on metabolic compartmentation and cerebral glutamate metabolism. An attempt was made in the present study in these lines.

Results

Cell morphology and cell number: The physical appearance of the isolated cell fractions were shown in Plate I and II. Astroglia (Plate I: A, B, C, and D) had numerous processes and a well defined cell body. Its appearance resembled to that of a protoplasmic astrocyte. As the astrocytic processes were protoplasmic in nature and fold at random under the coverslip, it was difficult to obtain a phase contrast photomicrograph with lucid details. The perikarya (Plate II: A, B, C, and D) were usually devoid of processes such as dendrites and axons. The nucleus, being larger than that of the astrocyte, was very conspicuous. The number of cells were determined after staining a small aliquot

PLATE I

A and B : Phase contrast photomicrographs of bulk isolated astrocytes.

A x 800

B x 2,560 (arrow indicates cell body)

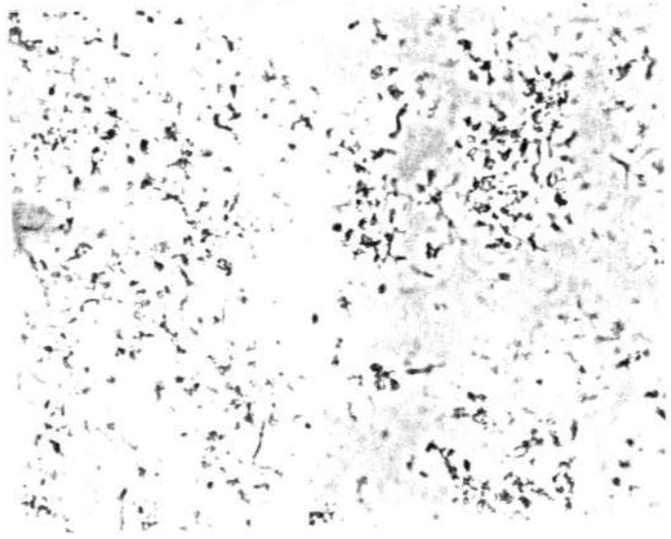
C and D : Scanning electron micrographs of bulk isolated astrocytes.

C x 602

D x 300

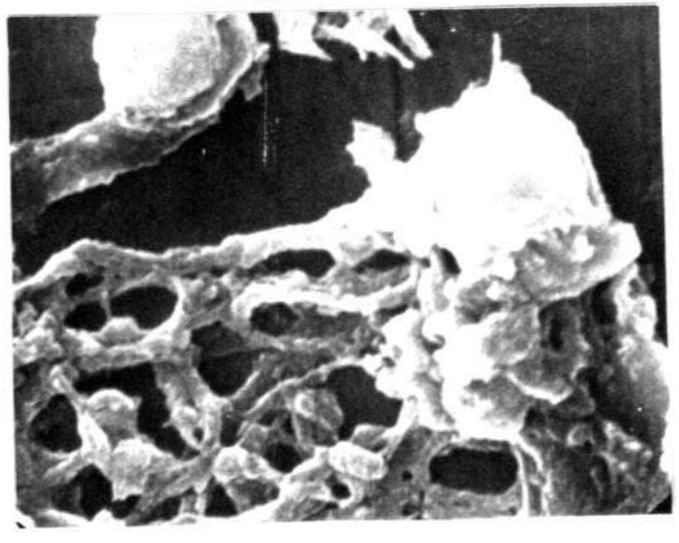
PLATE I

A



B

C



D

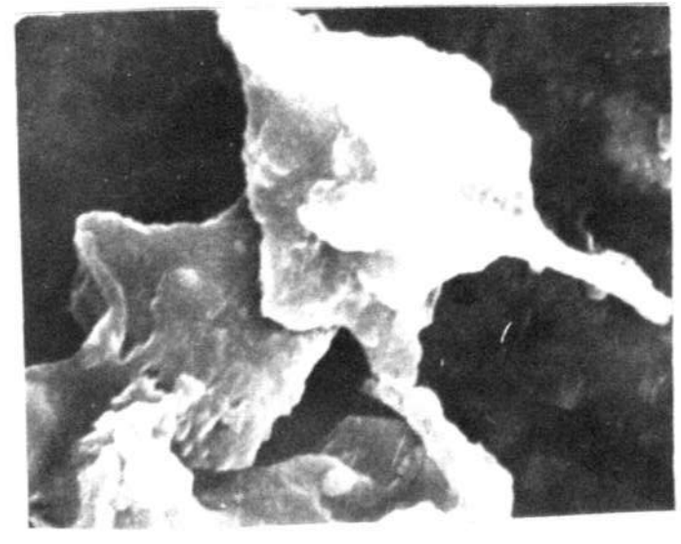


PLATE II

A and B : Phase contrast photomicrographs of bulk prepared neuronal perikarya

A x 400

B x 3,200

C and D : Scanning electron micrographs of bulk isolated neuronal perikarya

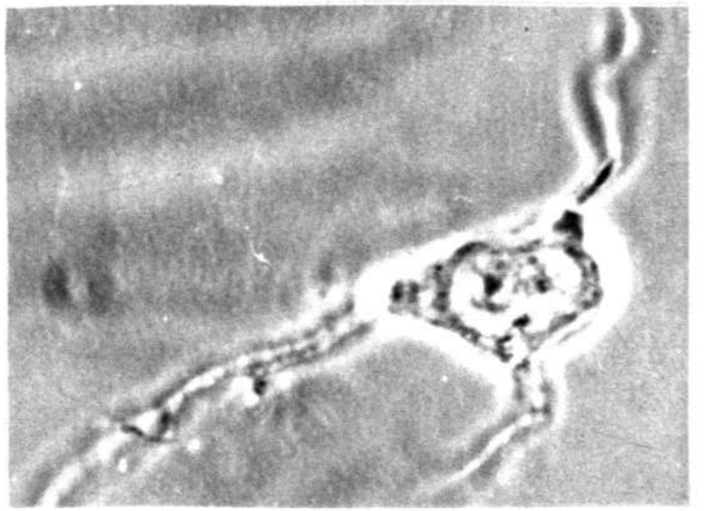
C x 602

D x 262

PLATE II

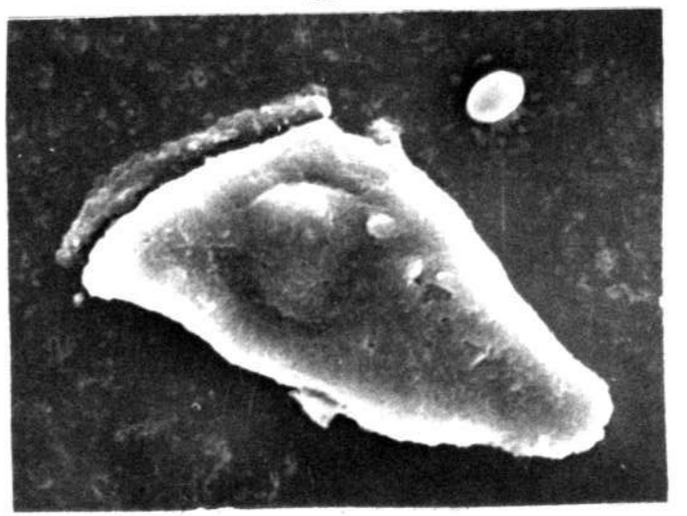
A

B



C

D



(10-20 µl) of the enriched cell preparations with methylene blue, using a hemocytometer. The number of the cells were counted at random from which the cell number was calculated using the formula

$$\text{cell number} = \frac{\text{number of cells per square} \times 1000 \times \text{volume of cell suspension (in ml).}}{0.00625}$$

Though this method could be applied successfully to count neurons and astrocytes it could not be used for determing the number of synaptosomes, because of their smaller size which was beyond the resolution of the microscope used. Though, it is believed that the ~~~~~~~~~~ *a*strocytes out-number the neurons in a ratio of 1:10 (230, 231), in the present investigation the number of neurons obtained was greater than the astrocytes (Fig.l). This might be due to a greater fragility of astrocytes compared to neurons. The number of astrocytes or neurons isolated from gm. wt. of cortex remained more or less constant in different experi-ments indicating the reproducibility of the method and the modifications made thereof. The number or astrocytes iso-lated by the present method was more or less equal to the number of cells isolated by Farooq and Norton (88).

Wet weight, dry weight and water contents: Various micro-scopic methods are available to determine the weight of individual cells (213). In the present investigation a

simple and highly reproducible method was deviced to determine the average weight of a single cell, which was described earlier (c.f. materials and methods).

In spite of a large number of processes, the wet weight of the astrocyte was less than that of a neuron which was devoid of processes (Fig.2). The magnitude of the difference between these two cells was about 4.7 fold. Similarly the dry weight of neuron was higher than the dry weight of astrocyte (Fig.2). The magnitude of difference was only 2.2 fold. This suggested that a greater portion of the neuronal wet weight was contributed by cell water rather than the particulate material. The observed water content of the cell preparations also supported this concept (Fig.2). Compared to astrocytes, neuronal water content was 6 fold higher. About 7b'/. of the weight of the neuron would then be contributed by cell water while in astrocyte it would be about 59/. of the wet weight. The observed difference in water contents might be because of large number of protoplasmic process with their inclusions in astrocytes. However, in neurons as the cytoplasm is compressed to a thin rim by the nucleus, which (nucleus) occupies 3/4 of the cell volume, the number of cytoplasmic organelles contributing to the dry weight would be comparatively less. The water content, wet weight and dry weight have greater bearing in assessing the pathological changes in the cells (to be discussed later).

Fig. 1 Cell number of astrocytes and neurons

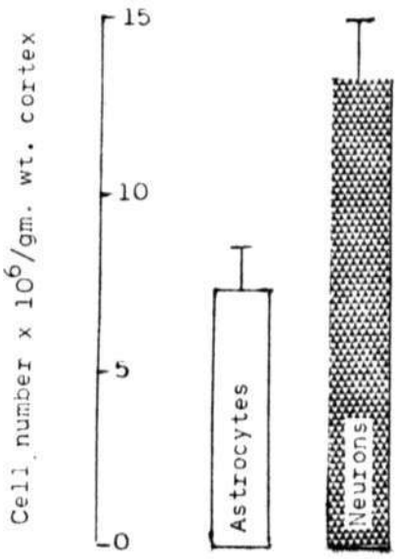
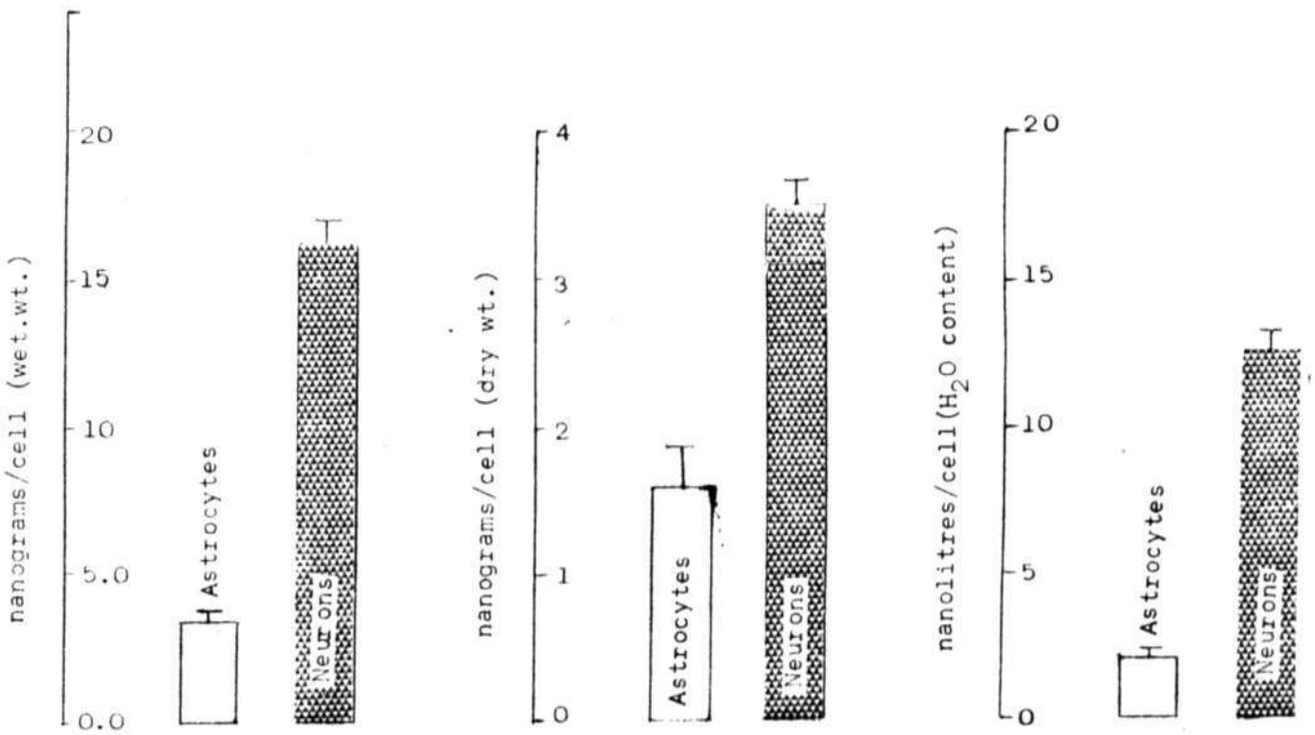


Fig. 2 Weights and intracellular water contents of astrocytes and neurons



DNA, RNA and protein:

The DNA content of the astrocyte was about 7 pgms/cell (Fig.4) and was comparable to the value reported by Farooq and Norton (88). It was also in concurrence to the reported DNA content of the normal diploid cell of rat (42). The DNA content of the neurons (30 pgms/cell) (Fig.3) was, however, higher than that of astrocyte and of the diploid cells of the rat or the values reported by other investigators (95, 207). The high DNA content of the neurons could be due to the polyploidy of these cells. Several reports were made in the past about the polyploidy of the neurons and this topic is highly controversial. Tetraploid DNA content was reported in the mature Purkinje cells, Betz cells, hippocampal pyramidal neurons and ventral horn cells (128, 129, 163, 208). Polyploidy to an order of 75,000-200,000 was observed in the neurons of Aplysia (66, 164) while a polyploidy of 16 was observed in insect neurons (276). However, the size of the nucleus or that of the cell was noticed to be unrelated to the state of ploidy (129). The earlier cytophotometric determinations of the DNA content made by these investigators was doubted and the validity of these reports has been questioned by recent investigators. No incorporation of 3H-thymidine into DNA was observed at the time of ploidy by Mares et al., in the Purkinje cells (181, 182) Cohen et al., failed to observe the polyploid DNA

content in the Purkinje cells isolated from the cerebellum (67). It was suggested that in the early cytospectrophotometric observations the nonspecific loss of incidental light passing through the cytoplasm was not accounted resulting in an erraneous estimation of DNA content. Though, the high neuronal DNA content obtained in the present study would support the concept of polyploidy in cerebral neurons, at present it is not possible to assess the true state of polyploidy in cerebral neurons.

The RNA content of astrocyte was more or less equal to its DNA content (Fig.3). This value corresponded with the values reported by Farooq and Norton (88) and was less than those reported by Norton and Poduslo (207). The neuronal RNA content was 4 fold higher than the astrocytic RNA content (Fig.3). The greater RNA content of neurons would indicate its protein synthetic capacity. Moreover, neurons are known to be enriched with the Nissl substance (ribonucleoprotein complex). Ultrastructural studies demonstrated the exclusive distribution of the Nissl substance in the perikaryon of the neuron (212), which might explain the high RNA content of the neuron. Such a greater protein synthetic capacity of the perikaryon would be required not only for its maintenance, but also for the higher functions. The high RNA content of the neurons would confirm basophilic staining properties to be perikaryon (212). However, in

the present study no efforts were made to characterize the relative contributions of the three different RNA species (m RNA, t RNA and r RNA) to the total RNA content.

The protein content of neurons and astrocytes did not differ much (225 and 219 pgms/cell) (Fig.4). These values were within the range of the values reported by Farooq and Norton (88). The discrepancy in the values with other investigators (125, 196, 197, 253) might be due to differences in the isolation procedures, mode of treatment with proteolytic enzymes, duration of the isolation procedure and the extent of contamination.

In spite of greater DNA and RNA contents, the neuron had more or less same amount of protein as the astrocytes. The presence of large number of protoplasmic processes in the astrocytes and the lack of processes such as axons, dendrites and nerve endings in neurons might account for such an observation. Further, the presence of a large nucleus with very little cytoplasm in the neurons compared to the astrocytes might be another reason for the low protein content in the former. It is also possible that the neuronal perikaryon might be synthesizing greater amounts of protein for export to the other sites than for its own use. Loss of these processes during isolation procedure would lessen the protein content in this cell fraction. No information

Fig. 3 Nucleic acid contents of astrocytes and neurons

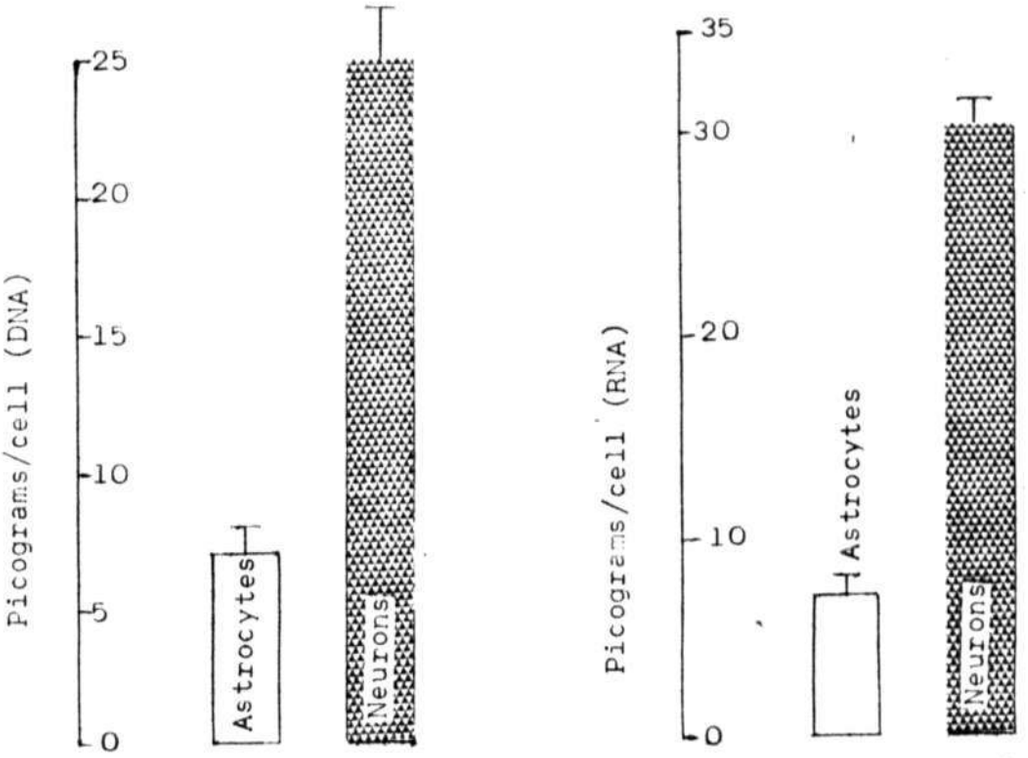
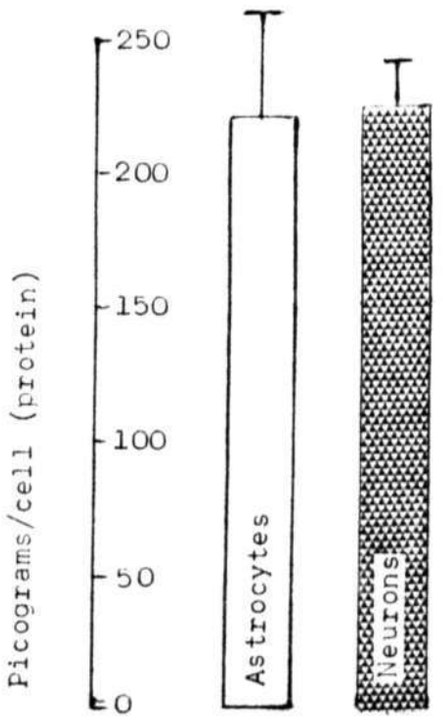


Fig. 4 protein content of astrocytes and neurons



is available on protein turnover rates of the individual brain cells.

Marker enzymes;

The purity of cell preparations may be assessed by two methods, viz., particle count method and by marker enzymes, the cellular localization of which have been well documented. In the particle count method, each preparation would be observed under the microscope for contamination by other cells. Though this method has an obvious advantage in the sense that the contamination would visualized directly, it would fail to detect the lysed cells and if contamination is membranous and submicroscopic in nature. Such adifficulty mi with an electron microscope. However, the time taken to prepare the material for electron microccopic observation would prohibit further studies with the cell preparations. In the present investigation it was observed that the contamination of astrocytes with neurons and vice versa was very minimal. With particle count method the maximum contamination level recorded was 5-7/. and 8-10% respectively. However, with this method it was not possible to detect the contamination of glial cells with synaptosomes and the synaptosomes with gliosomes. The gliosomes are known to be formed by the vesiculization of the glial processes. Both the gliosomes and synaptosomes are submicroscopic and require electron microscope for visualization.

The problems encountered in the particle count method were resolved to certain extent by determining the activity levels of marker enzymes in different cell fractions. While using marker enzymes for the characterization of either cellular or subcellular preparations following assumptions were made: the enzyme would be localized exclusively at a particular site in the cell or in a particular cell in the organ. Evidence for such an assumption was obtained majorly from histochemical studies. Precautions should be taken while interpreting the histochemical data especially about the sensitivity and specificity of the assay method. Usually histochemical methods measure the colour deposits either by visual means or by cytospectrophotometric methods. In the earlier method, the conclusions might be biased and in the later, nonspecific absorption of the light by the tissue preparation would be a major problem. The colorimetric methods employed in histochemistry are usually less sensitive than the isotopic or spectrophotometric methods employed in the biochemical methods. The problems in histochemical methods might be compounded by the permeability of the substrates and cofactors.

In brain, carbonic anhydrase (104, 105) pseudocholinesterase (13) and glutamine synthetase (183) are supposed to be localized in the glial cells, while choline acetyl transferase is known to be a marker for synaptosomes (187). No

specific marker enzymes were described in the literature for the neuronal perikarya. In addition to these, certain proteins such as S-100, glial fibillary acidic protein are known to be present exclusively in the glia (296) and 14-3-2 (neuronal specific enolase) is supposed to be a marker for neurons (217, 243, 244). In the present investigation the activities of carbonic anhydrase, pseudo cholinesterase, acetyl cholinesterase, choline acetyl transferase and glutamine synthetase were estimated as the marker enzymes in order to assess the purity of the cell preparations.

The activity of carbonic anhydrase was highest in the glial cells when compared to neurons and synaptosomes (Fig.5). The neurons had about 18% of the activity expressed by the glial cells and the synaptosomes about 14% indicating that the maximum contamination of the astrocytes by neurons and synaptosomes was 18 and 14/. respectively. The activity levels of acetylcholinesterase were surprisingly high in astrocytes than the neurons or synaptosomes (Fig.7). When compared to the synaptosomes, it was about 5 fold higher in astrocytes and about 2 fold in neurons. A similar profile was obtained for pseudocholinesterase but with marked differences in the activity of this enzyme between various preparations (Fig.6). Compared to synaptosomes, the activity of pseudo cholinesterase in astrocytes was about 30 fold higher. Based on pseudo cholinesterase

Fig. 5 Activity of carbonic anhydrase in different cell fractions

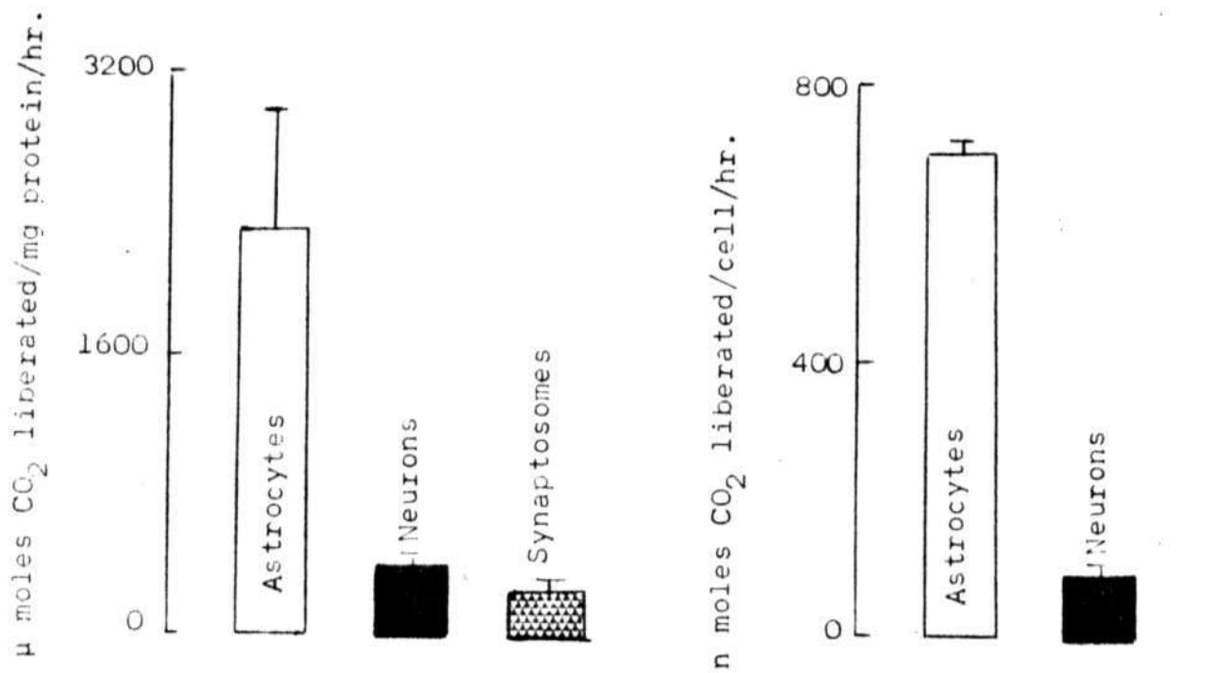
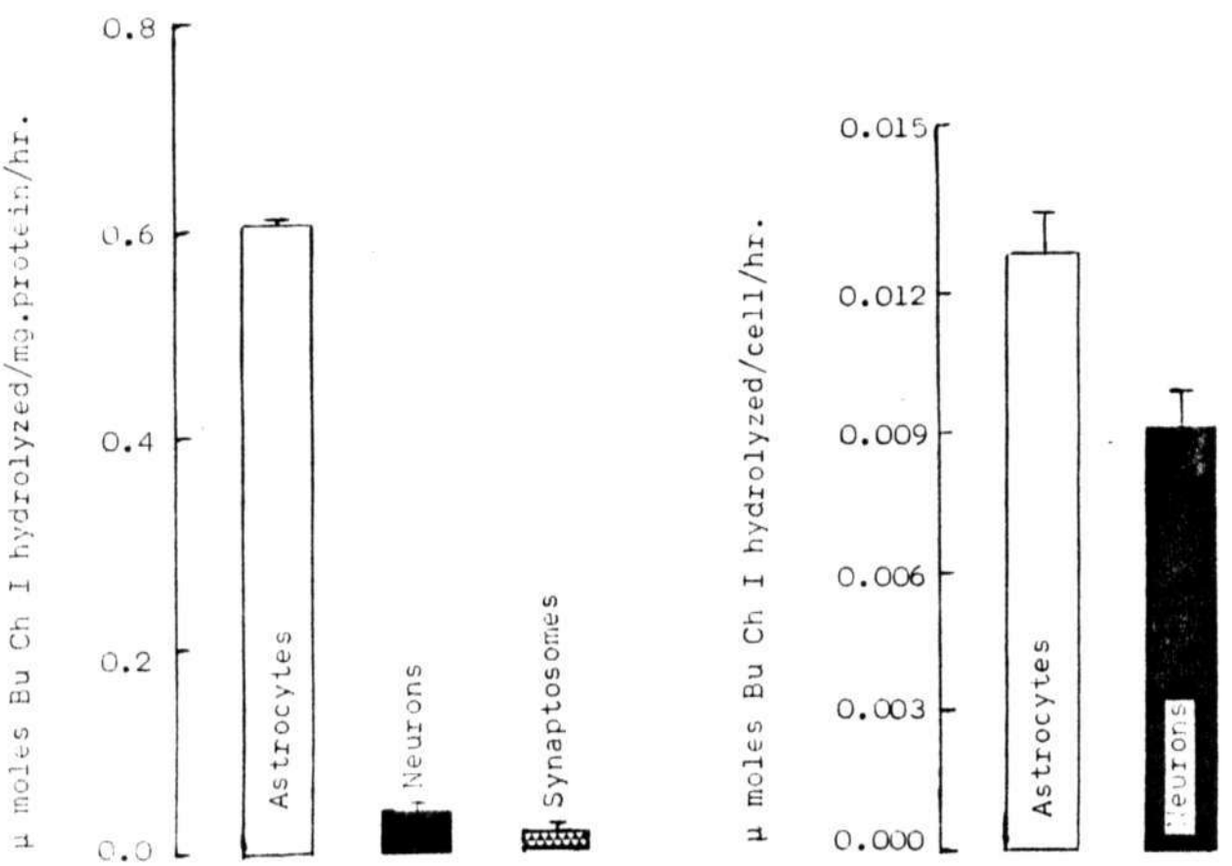


Fig. 6 Activity of pseudocholinesterase in different cell fractions



activity, the extent of contamination of astrocytes with the synaptosomes would be about 3% and about 7/. by neurons. The ratio of pseudocholinesterase to acetylcholinesterase activity was observed to be high in the astrocytes than in the synaptosomes and the contamination as assessed by this ratio was about 19/.. The distributory profile of glutamine synthetase, another marker for astrocytes, would be discussed later. All these observations indicated that the purity of astrocyte preparation by the present method was about 85-93% and the contamination never exceeded 10-12/.

The activity of choline acetyltransferase in synaptosomes was 10 fold higher than that of the astrocytes or neurons in which the activity levels were almost equal (Fig.8) Though, neuronal perikarya were established to be the sites of synthesis for this enzyme (187), lower activity observed in this fraction suggested that the enzyme was synthesized exclusively for transport to the nerve ending particles. Contamination of astrocytes or neurons with pinched off nerve endings (synaptosomes), as adjudged from the activity of this enzyme, was found to be not more than 9/. Though lactate dehydrogenase is considered to be a marker enzyme for synaptosomes its use is restricted for assessing the contamination of cell fractions with synaptosomes, because of the ubiquitous presence of this enzyme in all the cells.

Fig. 7 Activity of acetylcholinesterase in different cell fractions

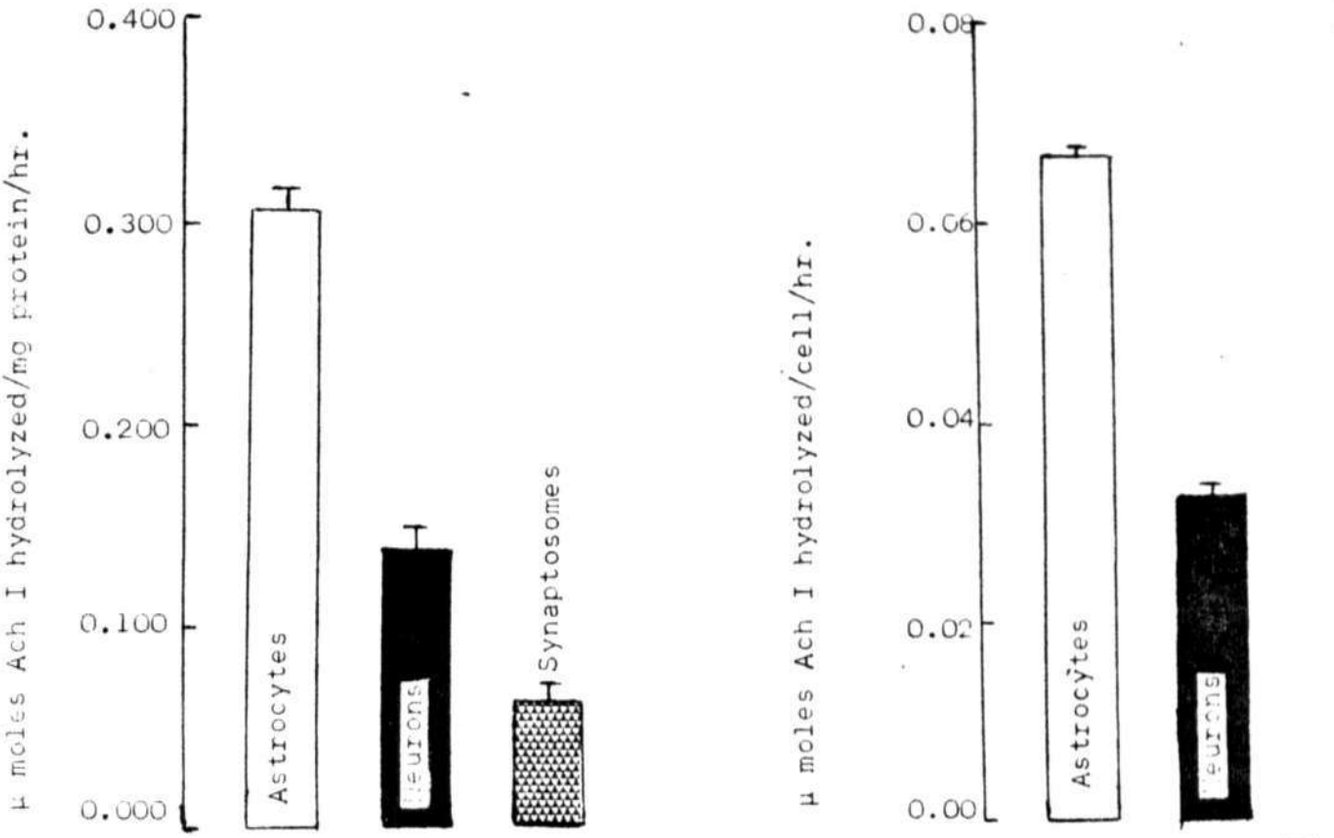
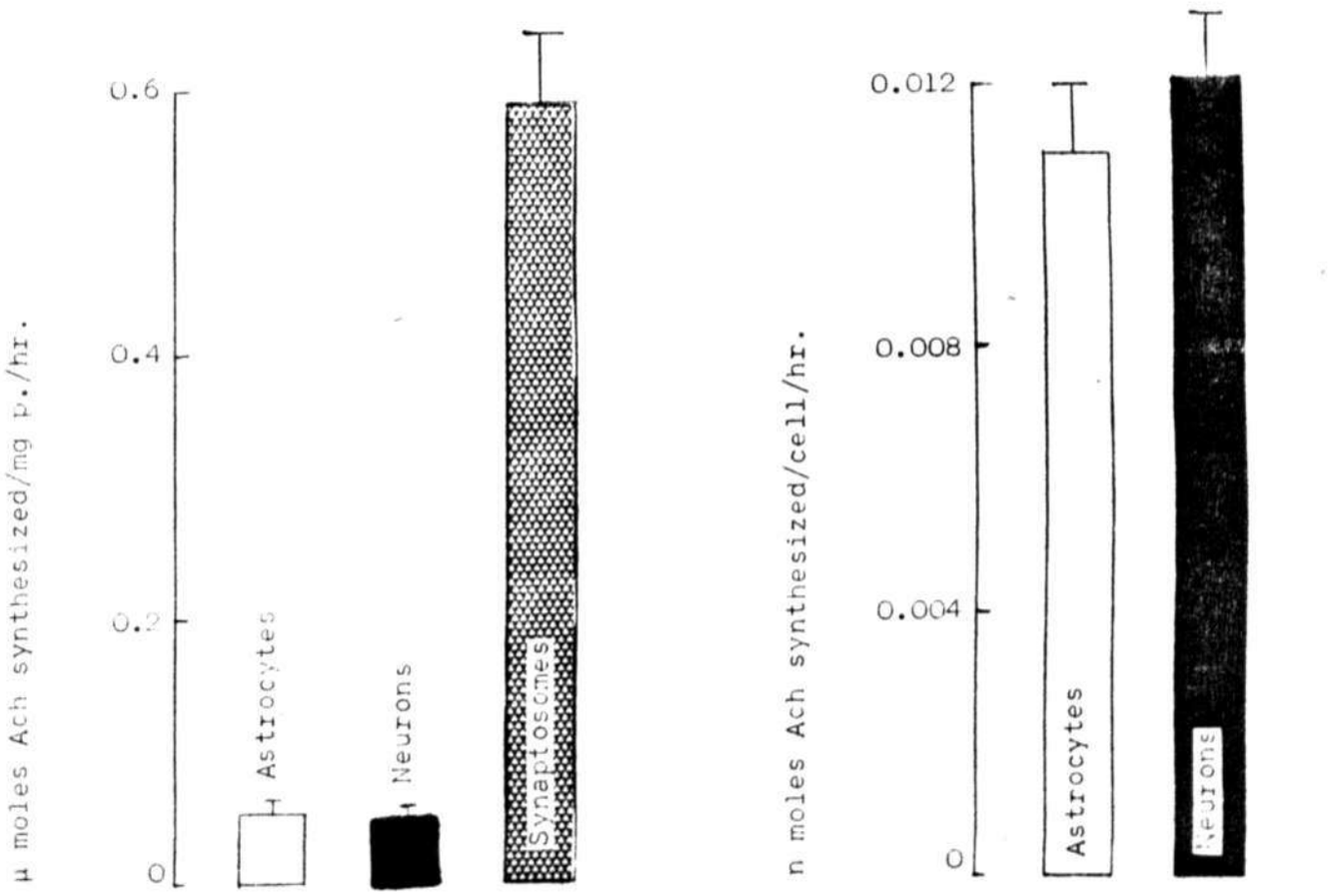


Fig. 8 Activity of choline acetyl transferase in different cell fractions



Distribution of enzymes of glutamate metabolism in cellular fractions: In the original model proposed by Berl et al., (28) , the amino acids glutamate and glutamine were supposed to exist in two compartments or pools. The large pool of glutamate, supposed to be localized in neurons would have its origin from glucose and pyruvate, and its rate of turnover was thought to be very low. The small pool of glutamate with a faster turnover rate than the large pool was presumed to be localized in the glial cells. The precursors for this pool were found to be acetate, propionate, butyrate, leucine, phenylalaine and CO2 besides glucose and pyruvate. The large and small pools of glutamate would not mix with each other as they are spatially separated and the fate of glutamate in these two pools was found to be totally different. In the large pool the glutamate was predicted to be converted to aspartate, GABA and α-ketoglutaric acid. Later studies of Quastel and his group (18-20) indicated that the glutamate released by neurons (exogenous) would be taken up by the glial cells and converted to glutamine. The loss of glutamate from the large compartment (eventually the dicarboxylic acids such as α-ketoglutarate and oxalocacetate) would be compensated by the transport of glutamine from the glial cells into the neurons where it would be reconverted to glutamate and its products. Further studies by Van den Berg (293) resulted in the identification of at least eight subcompartments (metabolic spaces) within the small compartment.

This theory of metabolic compartmentation was supported by incorporation of radioactive precursors such as glucose, glutamate, acetate, etc., into giutamate and glutamine (9, 65, 193, 292), by the pharmacological dissections (18) in cerebral slices using various drugs, known to affect the metabolism of a particular cell population in brain (tetra-dotoxin, fluoroacetate, fluorocitrate,malonate, etc.). Similarly, confirmative data for the theory of metabolic compartmentation was provided by using anaesthetics such as halothane (61, 62). Further evidence was provided by the immunohistochemical studies on the distribution of the enzymes of glutamate metabolism particularly of glutamine synthetase (183). Such a study indicated the presence of this enzyme exclusively in the astroglial cells, which contribute the small compartment of glutamate involved in the synthesis of glutamine. However, recent studies with cultured cells (154, 326) and studies with isolated cell fractions (269, 270) and excitotoxins (such as kianic acid) (149,200) yielded data contradicting the original theory of metabolic compartmentation.

One of the major drawbacks in the theory of meta-bolic compartmentation is the disregard for the functional and structural dissimilarities between the neuronal peri-karya and nerve ending particles. In fact, the neuronal compartment in the original theory does not distinguish the

neuronal perikarya from the nerve ending particles. It is well known that the releasable pool of neurotransmitters are exclusively present in the nerve terminals but not in the perikarya. It is also known that the major function of the perikarya is to receive the information from the dendrijc arborizations and to convey the same to the nerve endings via the axon. Such a structural and functional dissimilarity between the perikarya and nerve endings would always be associated with the dissimilarities in the metabolism, especially of the neurotransmitters. Hence, it is not known that the neuronal compartment (large pool) of the original hypothesis refers to the perikarya or to the nerve ending particle. However, based on the studies of Benjamin and Quastel (18) it might be assumed that this neuronal compartment refers to that of nerve ending particle.

No information is available with regard to the distribution of enzymes involved in the glutamate metabolism in the above said compartments. In the present study, an attempt was made to study the distribution of the enzymes in cell fractions ~~~~~~~~~ encompassing different metabolic compartments. In the process, a need was felt to identify neuronal perikarya as metabolically different entities from the nerve endings (synaptosomes).

Two different parameters viz. specific activity (activity/mg. protein/hr) and cellular activity (activity)

cell/hr) were used to express the enzyme activity. This was justified by the fact that the true comparision of an enzyme activity in two different cell populations would be valid only when their protein contents are similar but not when the variation is too high. Further, any changes in the protein content due to drug treatments would naturally mask the real changes in the enzyme activity expressed in relation to the total protein content which includes both enzymic and nonenzymic proteins. With the available material and the techniques, it would be highly impossible to determine the precise amount of enzyme protein of any given cell population. However, for synaptosomes the activity was expressed only per mg. protein and not per single synaptosomal particle, as it was not feasible to count the number of synaptosomal particles present in the assay mixture.

The specific activity of AAT was very high in synaptosomes than in other cell fractions (Fig.9). The activity of this enzyme in neuronal perikarya was found to be lowest among the three. When the activity was expressed per cell, it was observed that the astrocytes had a 2.4 fold higher activity than the neurons. A1AT was found to express lower activity than AAT which was on par with the results obtained with homogenates (6, 77, 234, 235). Distribution of this enzyme in the three fractions was observed to be more or less same (Fig.10). Similar results were obtained when the

Fig. 9 Activity of aspartate aminotransferase in different cell fractions

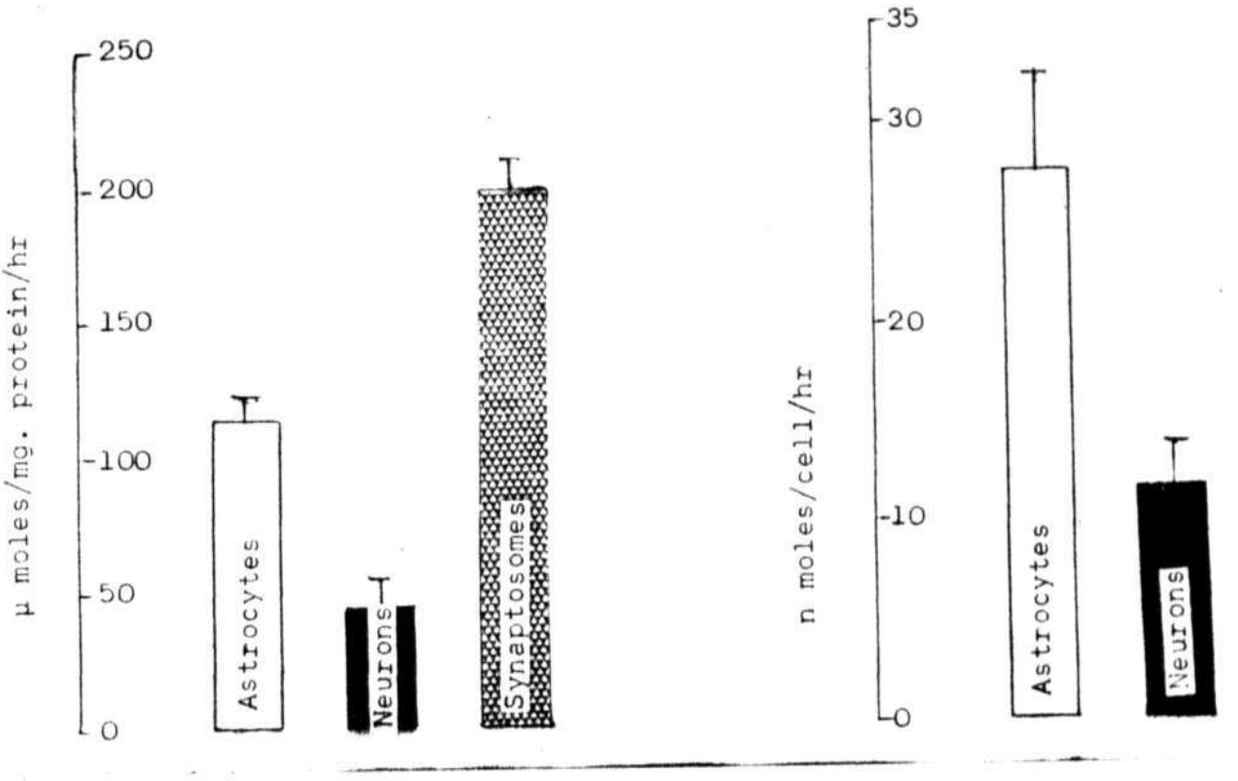
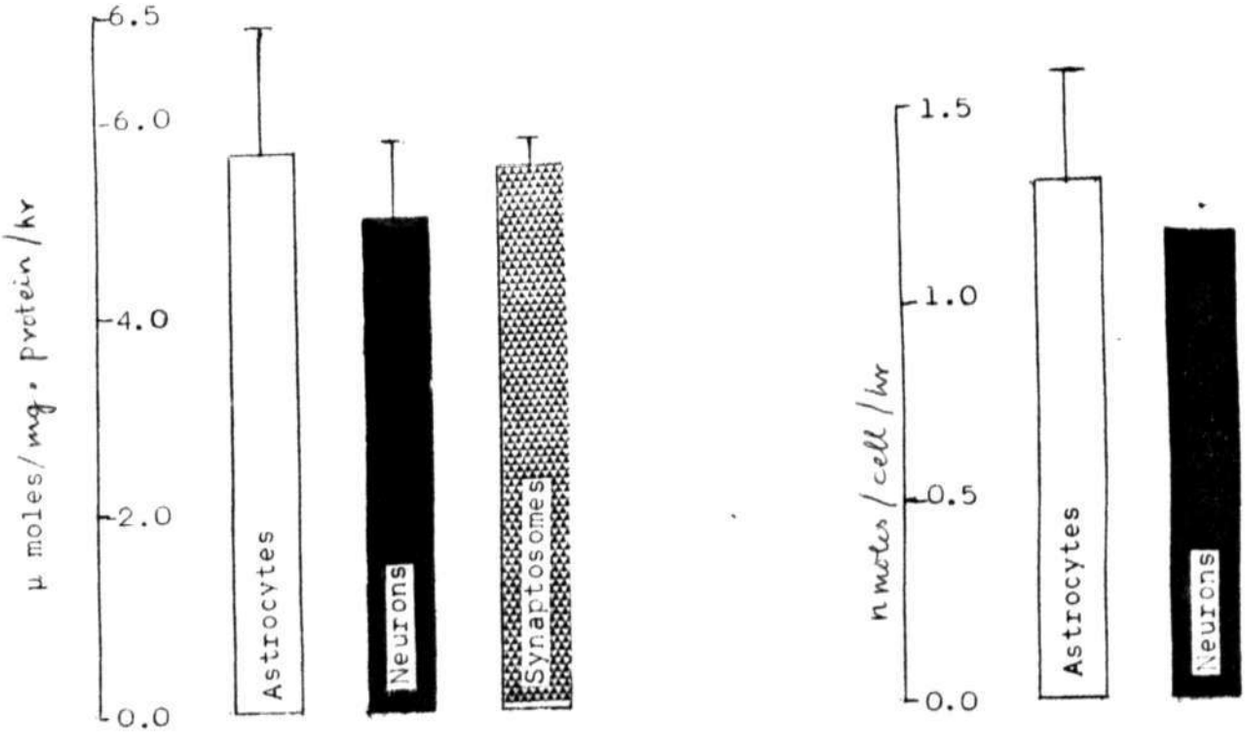


Fig. 10 Activity of alanine aminotransferase in different cell fractions



activity was expressed per cell. The specific activity of GDH was found to be equal in the neurons and glia, which was about 13-14 fold less than the activity found in synaptosomes. The cellular activity of GDH was found to be more in the astroglial fraction than in the neurons (Fig.11). GS activity was more or less equal in the neuronal perikarya and the synaptosomes which was lesser than the activity of the enzyme in astrocytes (Fig.12). Weiler et. al., reported the presence of glutamine synthetase in the glial cells as well as in neuronal perikarya and synaptosomes (320). However, the activity levels in all these fractions were lower than the values reported in the present study, though assay method was same. Further, they have observed that the activity in synaptosomes was far greater than the glia and neuronal perikarya which was reasoned to be due to the loss of the enzyme from astroglial cells and its subsequent uptake by other cell fractions. Hence, they have normalized the activity of GS with reference to lactate dehydrogenase. Discrepancy of their report with the present investigation might be due to methodological difficulties in the preparation of cell fractions, especially the neurons and glial cells, which would be reflected in the purity of the preparations. As the authors themselves have stated that the astroglial preparation was contaminated with unidentified membranes (38), the lowered specific activity in astrocytes might be due to the contaminating membranes contributing to the total protein

Fig. 11 Activity of glutamate dehydrogenase in different cell fractions

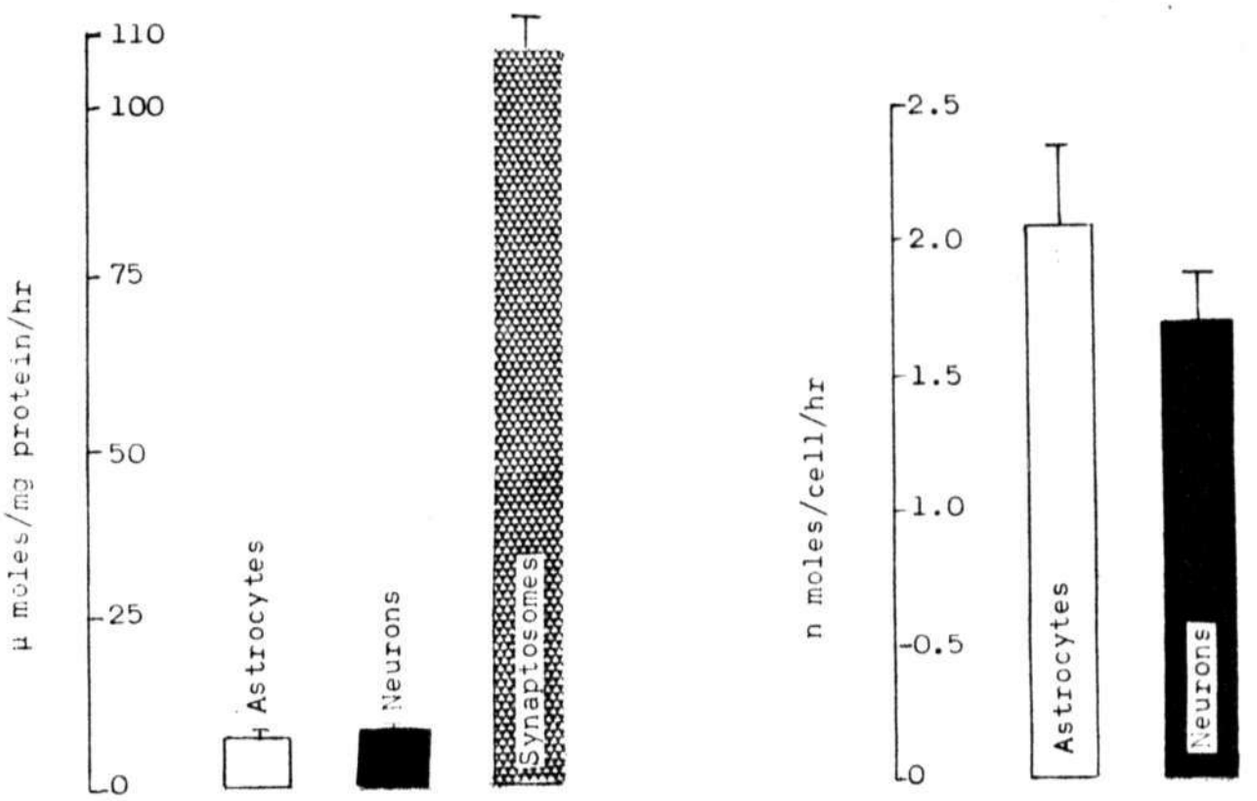
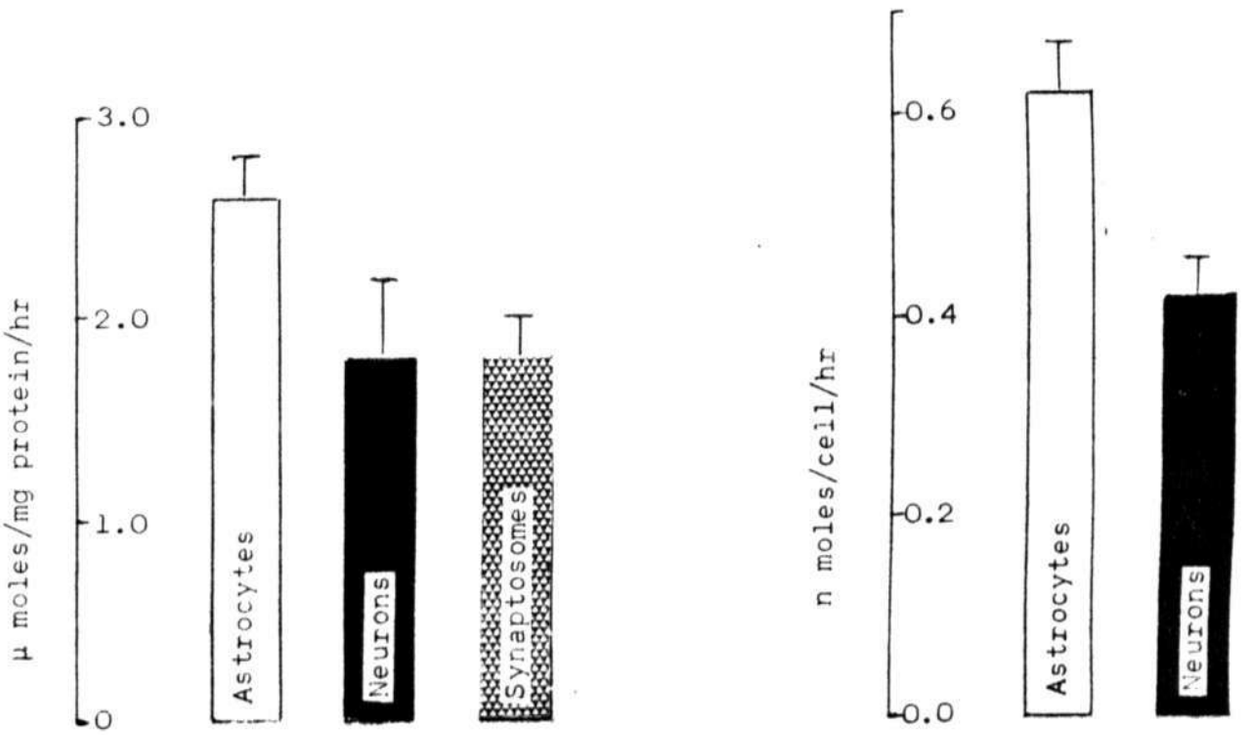


Fig. 12 Activity of glutamine synthetase in different cell fractions

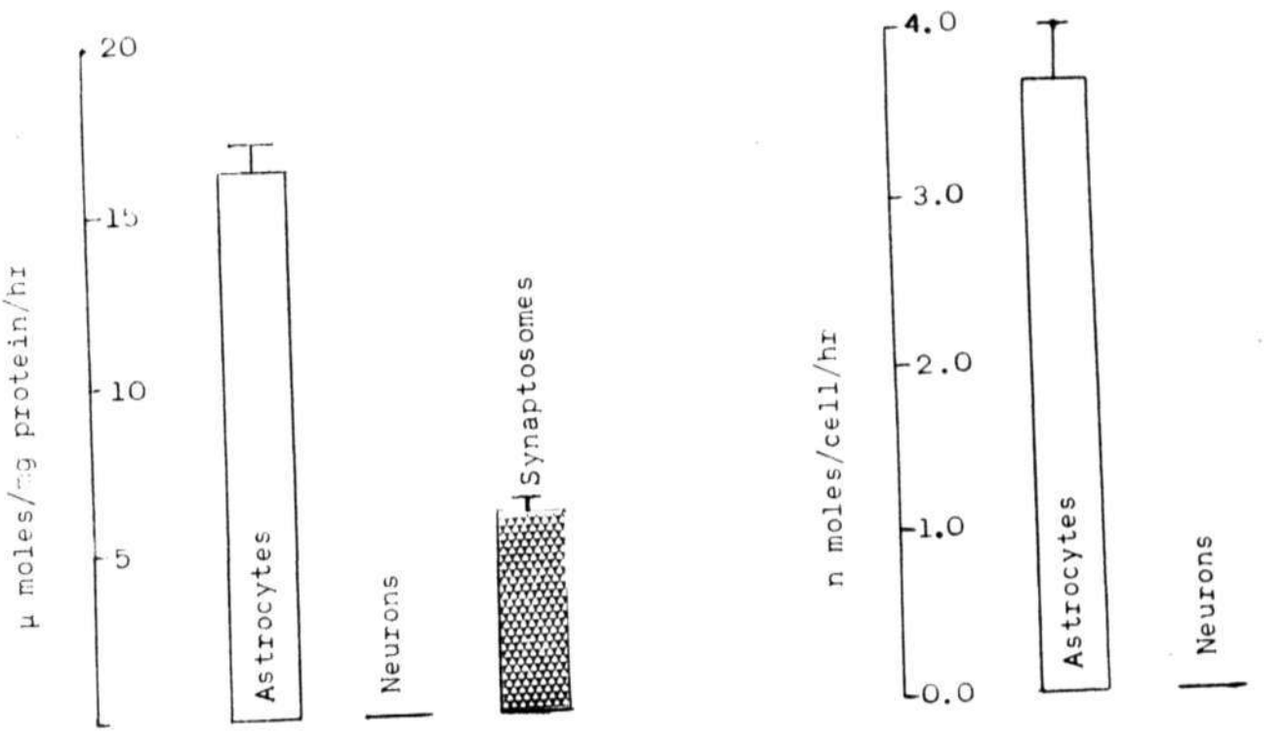


content of the preparation but not to enzyme activity. Further, normalization of GS activity with reference to lactate dehydrogenase may not be reflecting a true picture as lactate dehydrogenase is an ubiquitous enzyme present in all the cells and the activity levels of this enzyme in the respective cell preparations before isolation is difficult to obtain. The leakage of lactate dehydrogenase during isolation from the cellular cytoplasm was ignored by the authors. GS is known to be bound to the membranes of endoplasmic reticulum (254) so, its leakage during isolation appears to be improbable. Hence, a true comparision could not be made with the earlier data, presence of GS in neurons and'glial cells was also reported by Patel et al. (215). Cellular activity of this enzyme was found to be more in astrocytes than in the neuronal perikarya.

When expressed per mg protein the activity of GLNase in the astroglial fraction was about 2.5 fold higher than the synaptosomal faction. With the method employed presently the activity of this enzyme was not detected in neuronal perikarya (Fig.13). Based on the theory of metabolic compartmentation, it was predicted that the activity of GLNase would be higher in synaptosomes than in astrocytes and various authors have shown that at least 30-40% tissue GLNase might be present in the synaptosomes. Further, Weiler et al. (320) reported a three fold higher activity

Fig. 13

Activity of glutaminase in different cell fractions



of this enzyme in the synaptosomes than in the glia. A similar report was also made by Patel et al., (215) in the astrocytes and in granule cells and Purkinje cells isolated from rat cerebellum as well as in astrocytes cultured from the rat cerebellum. These results were once again not on par with the results reported in the present investigation. Such a contradictory result might be due to the membraneous contamination as described above (c.f. GS). The purity of the cell preparation reported by Patel et al., (215) was low (50-70*/.) compared to the present method. Further, the assay methods used in both the cases were also totally different. In the assay method used by these two investigators, incubations were made for a period of 15 min. It is well known that GLNase is inhibited both by NH_4^+ and glutamate (150,314). Prolonged incubations would then result in the product inhibition of enzyme activity. In the method used presently, it was observed that the rate of enzyme reaction was linear for only a period of two minutes, beyond which the linearity was not maintained. Hence, in the method used by the above authors, the incubation time (15 min.) would include a non-linear kinetic zone which might influence the calculation of the enzyme activity. Recent results of Kvamme et al. , (151) indicated that the cultured astrocytes contained very high activity of GLNase although, it was lower than that of synaptosomes. The activity of this enzyme was independent of the concentration of glutamine in the culture medium

which ruled out the possibility of induction of this enzyme by extracellular glutamine (151). The lower activity reported by these authors might be due to the differences in the homogenization media used.

In spite of the fact that no literature is available on the distribution of the enzymes of glutamate metabolism, a prediction was made in this regard based on the original hypothesis by subsequent investigations. As it was proposed that the precursors for the glutamate in large compartment were majorly glucose and pyruvate (18), it might be assumed that the enzymes AAT and to a certain extent GDH would express a higher activity in this compartment (i.e. in neuronal cells). As the release of neurotransmitters glutamate, aspartate and GABA would also occur from this compartment and as it was demonstrated repeatedly that this pool is derived from glutamine (41)258 , it might be inferred that the same compartment should also have a higher GLNase activity. In other words the neuronal compartment is supposed to be enriched with AAT, GDH and GLNase. The small compartment involved in the synthesis of glutamine from small pool of glutamate should then be enriched with GS. It was also shown that exogenous glutamate and the related neurotransmitter amino acids along with ammonia would be taken up into this compartment and a major portion of these amino acids are initially transaminated (49%) or converted to glutamine (37/.) and a

small portion is oxidized to CO_2 (19). Hence, it may be presumed that this compartment should be enriched with high GS and aminotransferases with poor activity levels of GDH.

The enzyme profiles obtained in the present investigation in astrocyte accord with the hypothesis. The high activity of AAT in synaptosomes would be responsible for the synthesis of glutamate from glucose. Such a proposal was strengthened by the observation that 80/. of the glucose carbon rapidly equilibrates with glutamate and related amino acids (10). Similarly a high activity of this enzyme in the astrocytes would be responsible for the conversion of a major portion of exogenous glutamate to aspartate as was observed by Benjamin and Quastel (19). Similarly, the high activity of GDH in synaptosomal fraction would be responsible for the reductive amination of α-ketoglutarate (derived from glucose or pyruvate) to glutamate. This observation was in accord with the results of Lai and Clark (155) that the synaptosomal mitochondria have 70% higher GDH activity than the nonsynaptic mitochondria.

The low activity of GDH in the astrocytes compared to that of synaptosomes would indicate that the glutamate formation by the reductive amination of α-ketoglutarate would be lower in these cells when compared to synaptosomes.

Though, Berl et al., (29, 30)~~~~ predicted that much of the GDH activity would be present in the small compartment, later evidences indicated that it might not be the case. Berl (26) himself and later by Cooper et al., (70) have shown that the specific radioactivity of α-amino group of glutamate or of glutamine was lower than that of amide nitrogen of glutamine when injected with ^{15}N or ^{13}N ammonia. This indicates that the glutamate (responsible for the glutamine synthesis) formation (by GDH activity) might not be taking place in the small compartment. Further studies of Nicklas et al., (200) and Krespan et al., (149) in which the kianic acid was used to destroy the large compartment, indicated that the GDH activity decreased in spite of glial proliferation and similarly synthesis of both glutamate and glutamine was lowered Results of present investigation along with the above said evidences would clearly suggest that ammonia fixation by GDH reaction would not be a major property of the small compartment. This would be explained later with more evidences in the following chapter.

Based on the studies with several precursors into glutamine the original proposals of metabolic compartmentation suggested GS would be localized in the small compartment. This concept was further strengthened by Benjamin and Quastel (19). The exclusive localization of GS in astrocytes was demonstrated by Martinez-Hernandez et al., by immunohistochemical method (183). Such a localization helped in

explaining the astroglial proliferation and Alzheimer type II astrocytosis in hyperammonemia conditions especially associated with hepatic failure. The results obtained in the present investigation were not in accord with the above mentioned observations. The activity of this enzyme in synaptosomes and in neuronal perikarya was at least 70-80/. of the astroglial activity. The presence of this enzyme in neuronal perikarya and synaptosomes, demonstrated in the present investigation, might not be due to the contamination of the cellular and subcellular fractions. The extent of the contamination of synaptosomes with gliosomes was not more than 15% (as assessed by carbonic anhydrase and cholinesterases ratios). The activity that could be expressed due to contamination should not be more than 15/. Failure to detect this enzyme immunohistochemically in the neuronal fraction might be due to redistribution of this enzyme during the tissue processing or by poor penetration of antibody into synaptosomes or neuronal perikarya. Similar conclusion was made by Van den Berg (293). In the present investigation the redistribution of this enzyme during the preparation of synaptosomes or the neuronal perikarya might also be ruled out as the enzyme in glial cells is bound to the endoplasmic reticulum. If the redistribution of the enzyme had occurred cholinesterase (microsomal enzyme) and carbonic anhydrase (cytoplasmic enzyme) should also have occurred resulting in a higher level of these enzymes in the synaptosomes as well as in neuronal perikarya.

The presence of GS in the synaptosomes might be due to the axonal transport from neuronal perikarya. As the neuronal perikarya are the sites of major biosynthetic reactions, the presence of this enzyme in the neuronal perikarya might be very essential. Whatever the reason for the presence of this enzyme in synaptosomes, it suggests the possibility of glutamine synthesis in this fraction which is the site of large compartment. Glutamine synthesis by synaptosomes was also implicated by the results of Nicklas et al., (200) and Krespan et al., (149). As mentioned earlier, these authors demonstrated that glutamine synthesis from ^{3}H-acetate and ^{14}C-GABA (precursors of small pool of glutamate) was inhibited by kianic acid while the effect was very moderate on the synthesis of glutamine from U-^{14}C-glucose, a large pool precursor. It is worth mentioning at this point that a caution must be exerted in interpreting the results with synaptosomes, as the synaptosomal population isolated from cerebral cortex is a heterogenous preparation with regards to the neurotransmitter specificity. With the methodology available presently, it is not possible to obtain a pure glutamitergic nerve ending fraction. Hence, the possibility of the absence of GS in pure glutamitergic nerve endings and its presence in the non-glutamitergic synaptosomes cannot be ruled out. A similar suggestion with regards to the presence of citrate lyase in cholinergic and noncholiner-gic nerve endings was made by Benjamin and Quastel (21).

Another contradictory observation made in the present investigation was the distribution of GLNase in the cellular and subcellular fractions. As mentioned earlier, higher levels of glutaminase activity in the synaptosomes than in the glial cells was reported (40, 41, 77, 82) and the possible reasons were also explained. It may be worthwhile to recall the observation of Kvamme et al., (151) on the presence of a high GLNase activity in the cultured astrocytes. It was also demonstrated by McGeer and McGeer (188) that the GLNase activity was present only in GABAnergic but not in glutamitergic nerve endings. The presence of low activity of this enzyme in the synaptosomes would question the hypothesis that the releasable pool of glutamate would be formed from glutamine in the nerve endings by the action of GLNase (40, 258). Further, the presence of high activity of this enzyme in astrocytes would doubt the role of astrocytes in glutamine formation as the whole glutamate-glutamine cycle becomes futile. However, it was observed that the glial enzyme was susceptible to inhibition by its product, ammonia (314). As the GDH activity inside the astrocytic mitochondria was very low, it might be expected that the astrocytic concentration of ammonia would be sufficiently high to inhibit the activity of this enzyme under physiological conditions. Glutamine transport into the astrocytic mitochondria would also play a major role in regulating the activity of this enzyme, as it was observed that at low concentrations

glutamine would be transported by a saturable carrier mediated mechanism while at higher concentrations the transport would be by diffusion (192). As the rate of efflux of glutamine from the astrocytes was reported to be very high (248) the glutamine formed in the astrocytic cytoplasm might be released into extracellular space before an adequate concentration responsible for diffusion would be achieved. The difference in the Vmax values of the efflux of glutamine from the astro-
Vmax
cytic cytoplasm (10 nmoles/min/mg protein) (130) was shown to be 10 times higher than the Vmax of mitochondrial transport (1 nmole/min./mg protein).

The theory of metabolic compartmentation implicated both glucose and glutamine as the precursors for glutamate in the large compartment (synaptosomes). The carbon skeleton for glutamate would be supplied by the α-ketoglutarate (generated in citric acid cycle during glucose oxidation) and localization of the enzymes of citric acid cycle exclusively in the mitochondria (except malate dehydrogenase) has been confirmed. Similarly, the mitochondrial localization of GDH was demonstrated conclusively (199, 239). Further, GLNase responsible for the formation of glutamate from glutamine, was also shown to be localized in the mitochondria (75, 153, 199, 239). In addition, glutamate would also be generated both by AAT and MAT, for which the mitochondrial localization has been established. In other words, glutamate

would be generated inside the mitochondria at least by three different reactions of which only the glutamate generated from glutamine acts as a neurotransmitter pool which would be liberated on depolarization of nerve endings. Under such conditions, it is paradoxical as to how these pools of glutamate generated in the same mitochondria do not mix with each other. Though this problem was recognized recently (40), no efforts were made to provide suitable answer.

To explain the incorporation of various precursors Van den Berg (292.) proposed the heterogenity of mitochondria might be responsible for the segregation of different pools of glutamate. Heterogenity in brain mitochondrial population was demonstrated repeatedly by several authors. Van den Berg *et al*/(293) demonstrated at least 9 different populations of mitochondria in brain while Lai et al., (157) demonstrated at least three populations of mitochondria. Neidle et al., (199) reported two different mitochondrial populations. The distribution of GLNase and GDH in these mitochondrial populations was shown to be different. The mitochondria with higher GLNase activity had lesser buoyant density than the mitochondria enriched with GDH activity. Based on these observations, it was proposed by these authors that the metabolism of glutamate and glutamine would be different in different mitochondrial populations. However, cellular localization of these different mitochondrial

populations is not known and it is difficult to envisage that the presence of different mitochondrial populations in a single nerve terminal and most probably nerve endings with different neurotransmitter specificities might harboar different mitochondrial populations. Further, the variations in the activities of enzymes of glutamate metabolism (GDH and GLNase) was not more than 15-20% between different mitochondrial populations isolated (157). As the information available was inadequate, a meaningful answer especially on mitochondrial heterogenity, would have to wait till further experimentation.

La Noue (246) proposed microcompartmentation in the kidney mitochondria to explain the acidosis induced changes in the glutamate metabolism. It was demonstrated that the glutamate originating by transamination and by deamination of glutamine do not mix in the mitochondria and exist as two separate pools, however, GDH has access to both these pools of glutamate. Hence, the compartmentation proposed in this case indicated the presence of two pools of glutamate within the kidney mitochondria and these two pools do not mix with each other. A similar possibility might be envisaged for brain mitochondria. It may be assumed that glutamate generated by GLNase would come out of mitochondria by rapid efflux through glutamate - (0H-) exchange system (77) and the ammonia liberated by this reaction would be utilized by GDH to

generate glutamate while in other microcompartment glutamate would be generated from aspartate by transmination and this pool of glutamate might be having a slow efflux rate possibly through glutamate-aspartate carrier. Most likely this microcompartment of glutamate would be involved in the maintenance of mitochondrial redox state and in the transport of reducing equivalents across the mitochondrial membranes through malate-aspartate shuttle (Borst cycle) (48).

The other alternative for the sequestration of the glutamate pools generated in the mitochondria might be due to the differences in the flux of metabolites through the enzyme systems and the competition for the common substrates between these two enzyme systems within the mitochondria. a-ketoglutarate is shared majorly by four different enzymes viz. α-ketoglutarate dehydrogenase, GDH, AAT and A1AT. Similarly oxaloacetate is shared by four different enzymes AAT, citrate synthase, malate dehydrogenase and pyruvate carboxylase. Competition for glutamate is seen at least between AAT and GDH. The availability of other precursors and cofactors such as ammonia, NAD , NADH, etc. would also determine the in <u>vivo</u> activity of these enzymes. The roles played by permeability, diffusion, rates of influx and efflux of the substrates would add to this complexity. Though activity of AAT (glutamate) is higher in the mitochondria than the other enzymes, it would be limited by

the availability of α-ketoglutarate. In the reverse direction oxaloacetate would be a limiting factor. It was reported earlier that the increased pyruvate oxidation would inhibit glutamate transamination due to the increased utilization of oxaloacetate for acetyl CoA condensation (6). As the available quantity of aspartate within the mitochondria would be very limited due to the impermeability of aspartate across the mitochondrial membranes (110) and is transported by glutamate-aspartate exchange (48) it may be assumed that the glutamate formation from aspartate would also be limited. In addition to aspartate, availability of α-ketoglutarate would also limit the glutamate formation by this enzyme. In the competition for α-ketoglutarate between GDH and α-ketoglutar

dehydrogenase it was shown that α-ketoglutarate is preferentially oxidized by α-ketoglutarate dehydrogenase (12) as the in <u>vivo</u> concentration of ammonia is extremely low (0.36 µmoles/gm. wt. cortex) and Km of GDH for ammonia is quite high (10-20 mmoles) (58) it may be assumed that the reductive amination of α-ketoglutarate would be severely limited due to the lack of adequate quantities of ammonia. Hence, the amount of glutamate generated by AAT as well as GDH may be inadequate to dilute the pool of glutamate generated from glutamine. As synaptosomal GLNase is known to be inhibited by both glutamate and ammonia (151), it would be worthwhile to include this parameter. The possible explanation may be the rapid removal of glutamate from the site of GLNase

by rapid efflux, as was suggested by Schoolwerth and La Noue (246) for kidney mitochondria. The ammonium ion generated in this reaction would lose a proton in mitochondrial matrix due to high alkalinity of the matrix. The unprotonated ammonia, thus formed, would freely diffuse across the mitochondrial membranes and after entering into cytoplasm, it is reconverted to ammonium ion. Thus ammonium ion may be converted to glutamine within the nerve terminal or in the astrocyte following its release into extracellular space (synaptic cleft).

Fig. 14 Activity of Mg^{2+} ATPase in different cell fractions

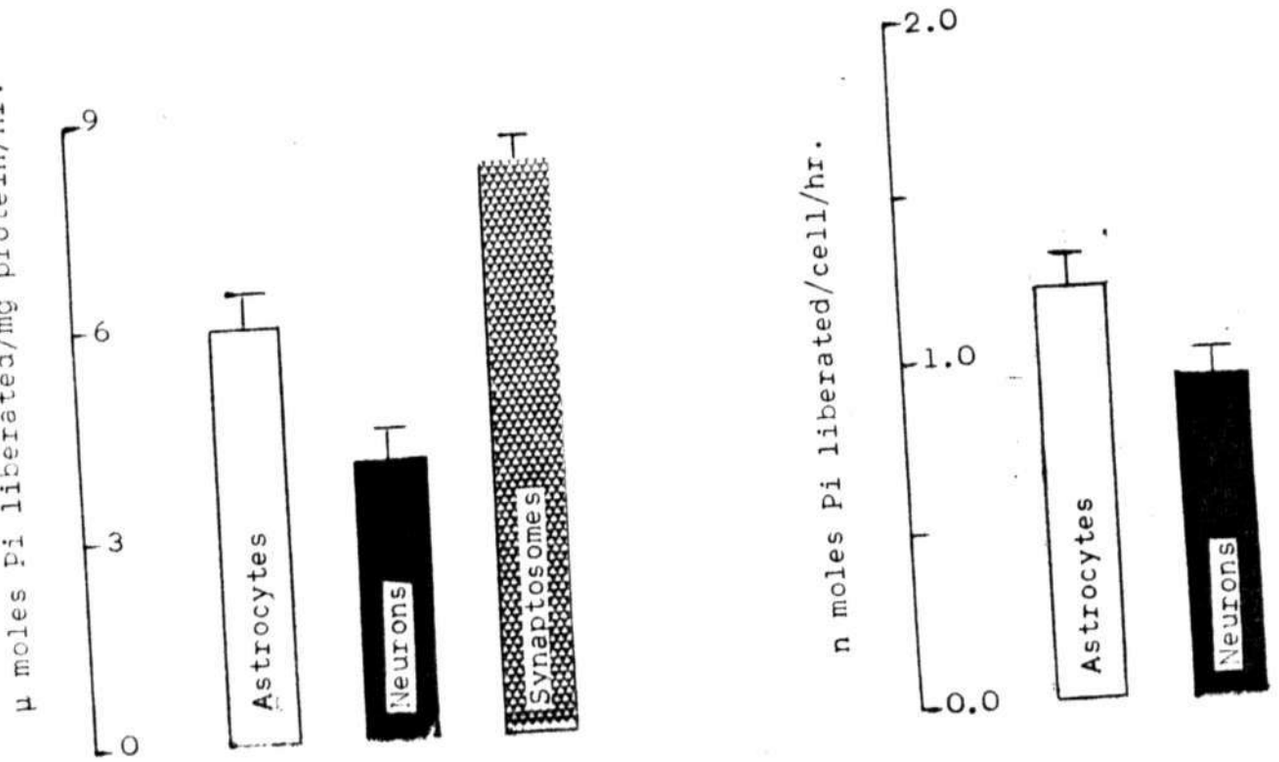
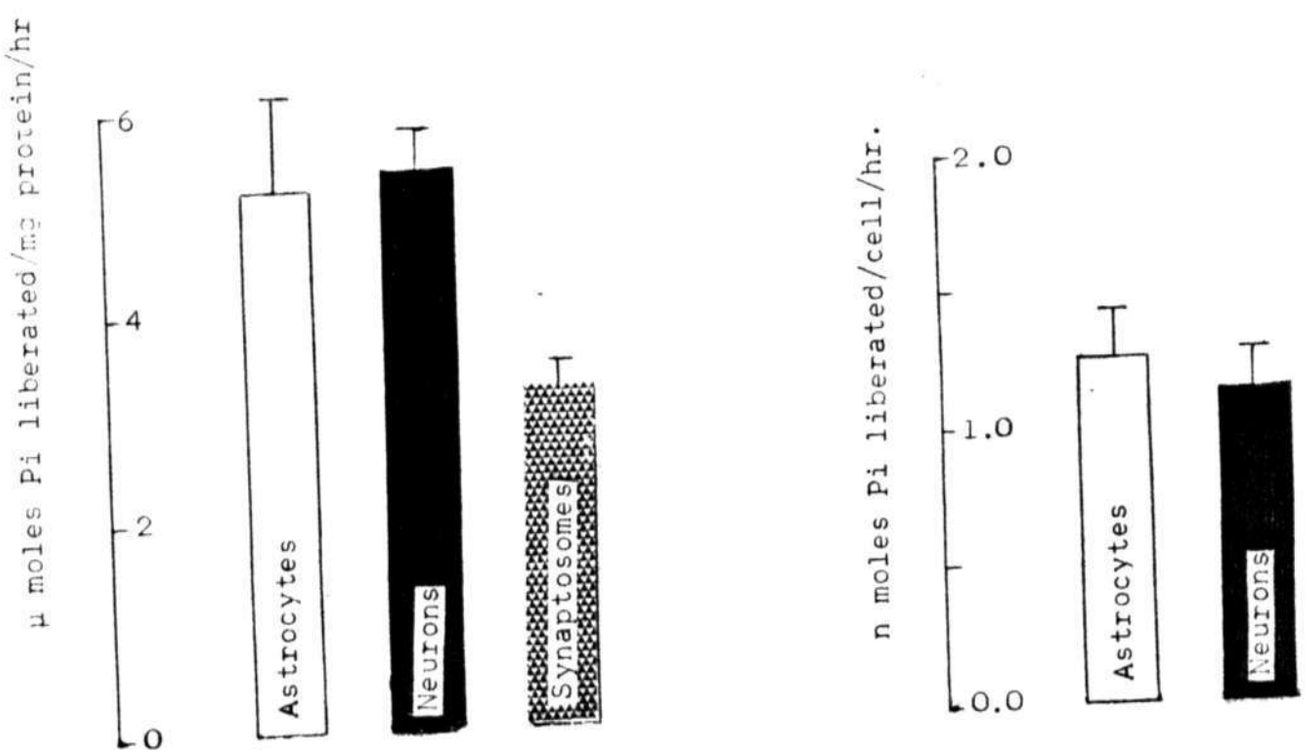


Fig. 15 Activity of Na^+, K^+-ATPase in different cell fractions



CHAPTER IV

Ammonia Toxicity

A great deal of work has been done in the past four decades to elucidate the mechanism of ammonia toxicity. Though ammonia is involved in the synthesis of amino acids, *an* increase in the levels of either blood or tissue ammonia (associated with several physiological disorders) was shown to be lethal (241). Several neurological and psychological symptoms were observed in hyperammonemic states (83a, 241, *4*). Though a great deal of work has been done on the neurological dysfunction due to elevated ammonia levels, no unified mechanism was proposed so far, which is not surprising as the effects of ammonia are highly complex and multipronged.

it was proposed that

As discussed earlier (c.f. introduction),cerebral energy reserves would be depleted in course of ammonia detoxification (83a, 241, 312). With the advent of the theory of metabolic compartmentation, major emphasis was laid on the role of glial cells in the ammonia toxicity, as they were supposed to be the seat of glutamine synthesis (166, 203, 204), which was supported by immunohistochemical studies on the localization of glutamine synthase (183, 206). It was demonstrated repeatedly that the glial cells undergo proliferation and enter into a reactive phase resulting in the formation of Alzheimer type II astroglial cells in hyperammonemic states (203, 204, 327). However,

no information is available on the biochemical changes or in the profiles of the enzymes involved in ammonia detoxification either in these reactive astrocytes or in neurons.

In the present investigation, as the cell preparations were relatively pure (85-90/), an attempt was made to study the physical, chemical and biochemical changes in these preparations in hyperanmonemic states. Two assumptions were made in this study (1) the artifacts (if any) induced in the course of cell isolation procedures would be the same in the cell preparations from the normal and experimental animals; (2) the changes, that have taken place in these cells prior to isolation (i.e., in the *in vivo* state), due to the drug treatment would be retained and survive the cell isolation procedure. No attempt was made to verify these facts due to the paucity of the methods, but the results obtained indicated that the assumptions made were true. One parameter not verified presently was the integrity of the plasma membrane of the isolated cells. This may not be having any influence on the present study in which the cells were lysed by osmotic shock to extract the enzymes and their activities were measured in these extracts. However, the plasma membrane was observed to be physically intact as seen in the photomicrographs of high magnification and also by estimating the enzyme activities in the homogenates prepared in

observed that the activity of the enzyme Na+, K^+-ATPase was very low when the homogenates were prepared in 0.32 M sucrose indicating that the extraction of the enzyme was incomplete when isotonic sucrose was used, which indirectly suggested the integrity of plasma membrane (results not reported). Another problem encountered in using such cell preparations would be the viability of the isolated cells. This aspect was verified routinely by the dye exclusion method using trypan blue. In addition, the $^{14}CO_2$ production from U- 14 C-glucose by the cells isolated from normal animals was also measured (astrocytes-1.08 nmoles 14 Co2 released/cell/hour; Neurons .- 7.5 $14 C0_2$ released/cell/hour). However, as the cells were lysed soon after preparation to extract the enzymes, the prolonged viability of the cells would not be a prerequisite for such a study. The problem of redistribution of enzymes during isolation was already discussed (page no.78).

Necessity for the modification or" method to isolate cells from drug treated animals;

Early attempts to isolate the cells from the drug treated (both ammonium acetate and MSI) using the method adopted to isolate the cells from normal animals yielded less number of cells (approximately 1-2x10/gm. wt. cortex). The reason for such a low yield might be due to either cell

necrosis induced by the administered drug or due to the loss of cells during the isolation procedure. The earlier possibility was ruled out based on the evidences presented in literature, indicating proliferation at least glial cells in hyperammonemic conditions (53, 162, 203-205, *241,* 327). Hence, assuming that the cells were being lost during isolation procedure, efforts were made to modify the procedure, so that the cell yield could be increased. When the cell number was counted at various stages of isolation, it was observed that the maximum loss was at the final stage of centrifugation. By reducing the Ficoll concentration from 15 to 10% for glial cells (final concentration of Ficoll 17% to 12%) and 30 to 26% for neurons (final concentration of Ficoll 32 to 28%) the yield was improved considerably. Further reduction of Ficoll concentration resulted in the sedimentation of debris or other contaminants, the nature of which was not determined. It was also observed that reducing the time of initial incubation of the coitical slices with trypsin and an increase in the time for all the differential centrifugation by 5 minutes resulted in a better yield. Hence, these two modifications were introduced in the isolation of glial cells and neurons from the animals treated with ammonium acetate and MSI. However, for synaptosomes no modifications were made to the method described already, as it was observed that the yield (mg_0 synaptosomal protein/gm. wt. of cortex) was not altered significantly in the experimental animals when compared to the normal ones.

The purity of the cell preparations were checked microscopically and biochemically to assess the contamination due to the modifications made in the procedure. As the activity of these marker enzymes might also be susceptible to the administered drug, a comparison of either the specific activity or per cell activity would result in erroneus conclusions. Hence, only the ratios of the enzyme activities were compared. The ratio of carbonic anhydrase and cholinesterases in the neurons and astroglial preparations were not altered significantly indicating that the cell purity was unaltered due to the modifications made (Fig.17). Since the procedure for the isolation of synaptosomes from drug treated animals was not modified, their purity was not assessed.

Necessity to use high concentrations of ammonium salts;

In the present investigation a dose of 2.5 mmoles/ 100 gm. body weight of ammonium acetate was administered to induce changes similar to those observed by other investigators. However, the dose of the administered ammonium salt was higher than that used by other workers (34). With the dosage suggested by these investigators (0.8 mmoles/ 100 gm. body wt.), no behavioural changes were noticed in our animals. This might be due to the differences in the strains of the rat or because of the age differences. No information is available as to the LD_{50} values for ammonium

Fig. 16 Effect of ammonium acetate on levels of ammonia in brain and serum

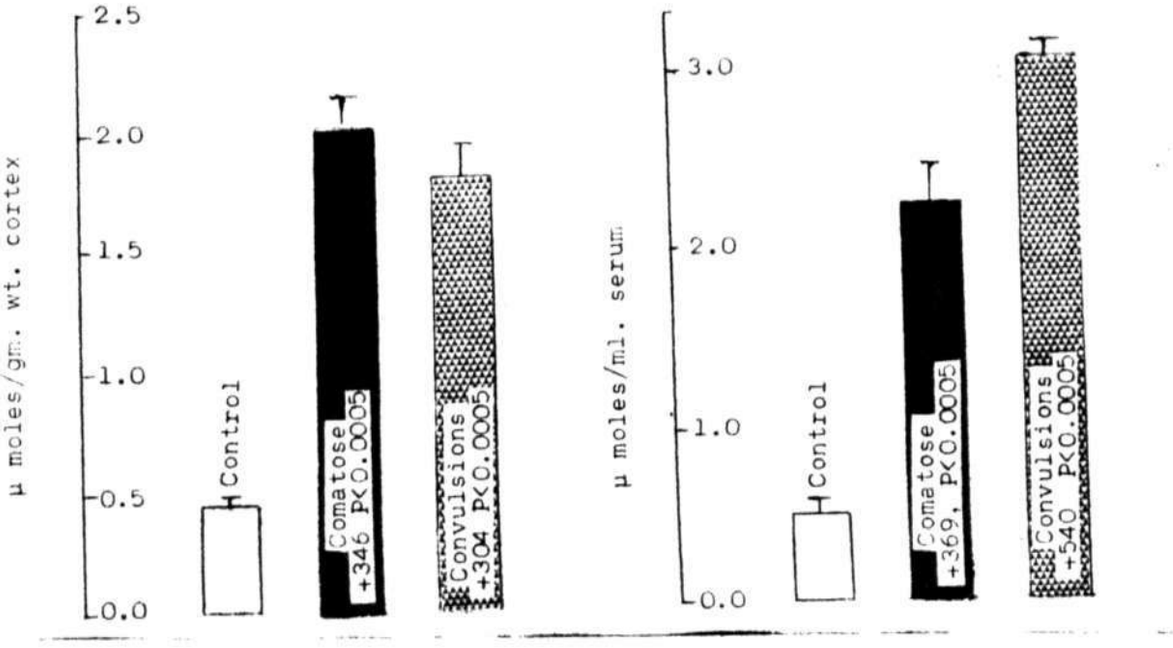
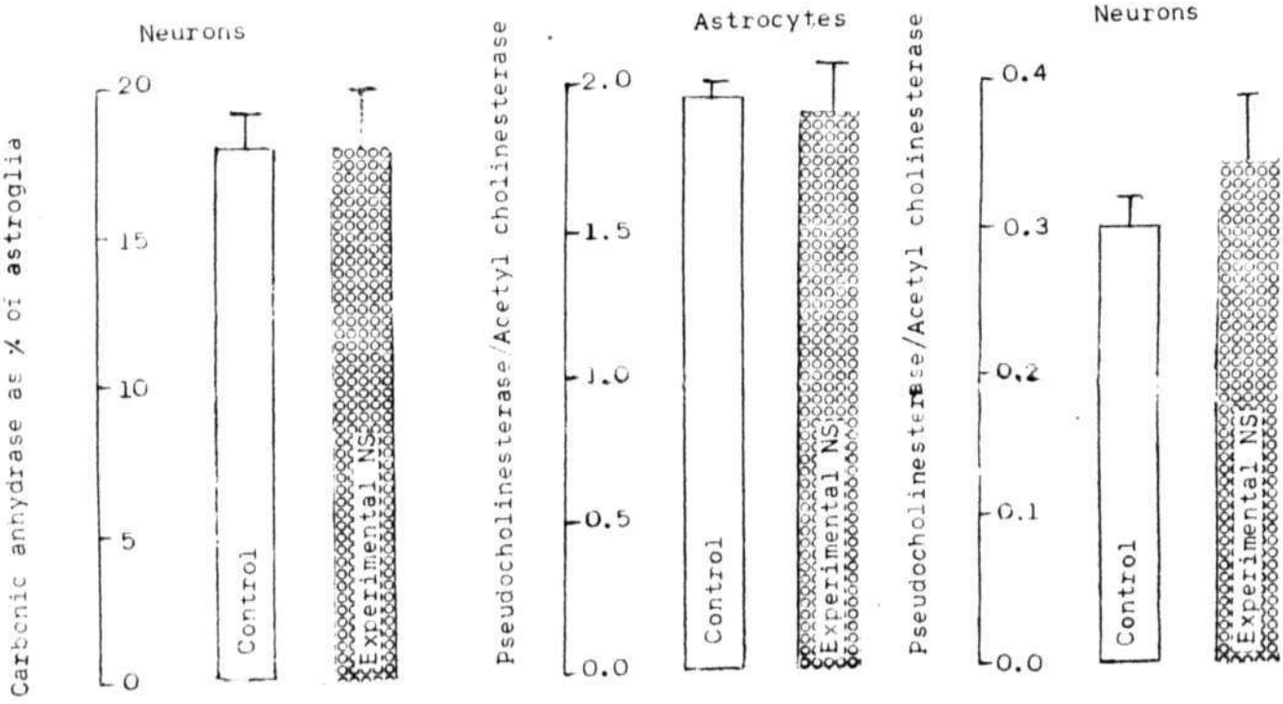


Fig. 17 Levels of marker enzymes in differerent cell fractions from experimental animals (modified procedure)



salts with reference to either strain or age. However, it is known that precipitation of coma or production of any cerebral disturbances is possible in rats only when massive doses of ammonium salts were administered (327). A dose dependent curve was prepared with the strain of rats used in this investigation. It was observed that with dosage used presently, rats entered into comatose state between 15-20 minutes, followed by convulsions between 25-30 minutes. When doses higher than 2.5 mmoles/lOO gm. body wt. were administered, the animals succumbed to death within 5 minutes and heavy bleeding was observed through rectum. Hence, in the present investigation the dose of 2.5 mmoles/100 gm. body wt.was used to induce both coma and convulsions.

Behavioural Changes:

All the behavioural observations made were only visual. No efforts were made to substantiate these observations by electrophysiological data. Before the onset of coma, behaviour of the animal was not significantly different from the normal animal. However, with the onset of coma between 15-20 minutes the animals exhibited an impairment in the alertness and were drousy. Motor responses of the animal to noxious cutaneous stimulation was inadequate. They also exhibited a delayed positive orientation (rightening reflex). The animals stopped feeding at this stage. If the experiment was not terminated at this stage, the comatose condition

observed in these animals led to convulsions. In the convulsive phase both tonic and clonic convulsions were observed. The duration of tonic phase was usually less than clonic phase. The animals were usually sacrificed at this phase. No distinction was made as to the stage of convulsions. Death was imminent in these animals 5-10 minutes after the convulsions.

Physical and Biochemical Changes in the Cell Fractions;

Cell number: Following the administration of ammonium acetate, there was a 55/. decrease in the number of neurons that could be isolated from gm. wt. of cortex, whereas the number of astrocytes isolated were more or less similar to that of controls (Figs.18 and 19). Though several investigators reported that the astrocytes would proliferate under prolonged hyperammonemic conditions (53, 162, 203 -205, 241, 327), more number of cells could not be isolated because of the brief duration of hyperammonemic state which might be inadequate for the cell proliferation. The reduction in the number of neurons might be due to neuronal death or loss of neurons during isolation. It was noticed, during microscopic observations, the neurons having smaller nuclei decreased considerably in number in experimental animals. Such a decrease in neuronal cell number was not reported hitherto in acute hyperammonic states. As mentioned earlier, the number of synaptosomes were not counted.

Fig. 18 Effect of ammonium acetate on astroglial cell number

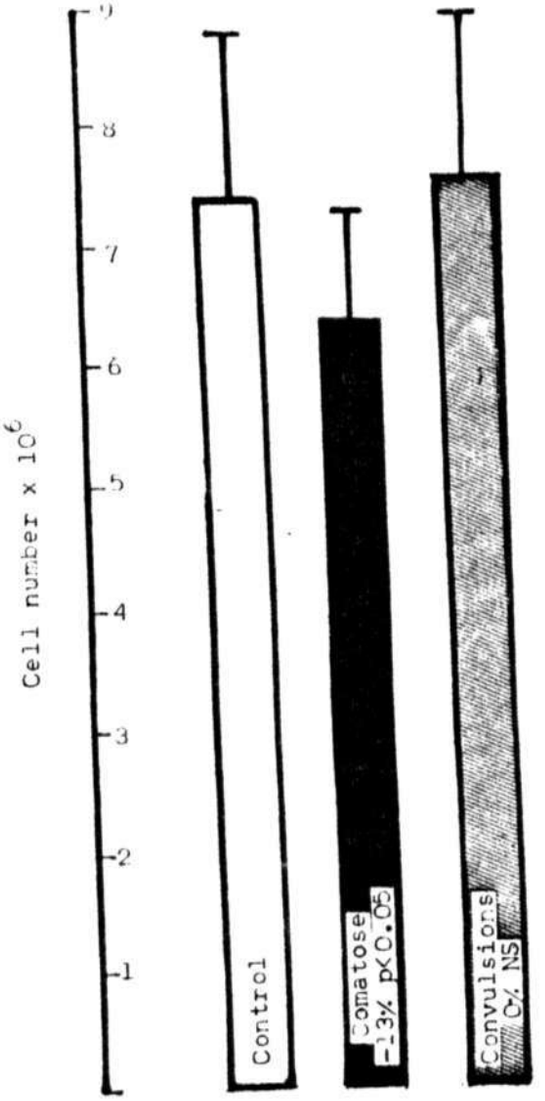
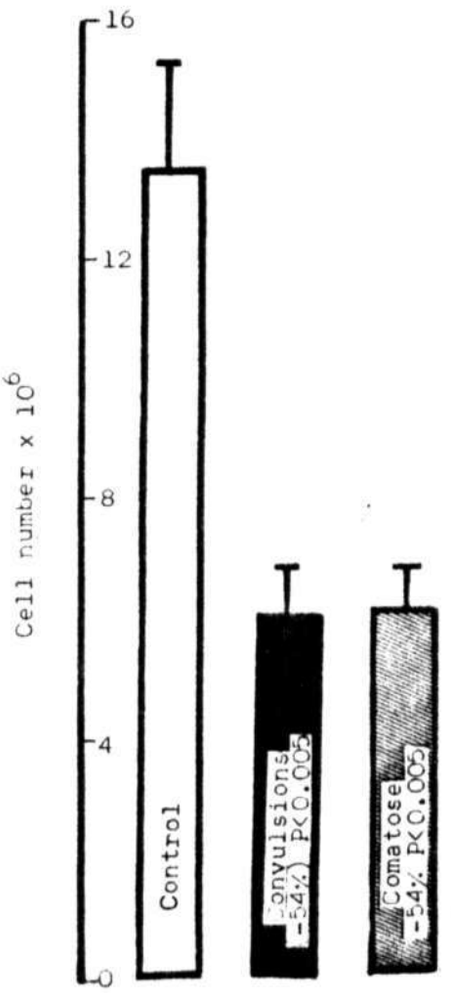


Fig. 19 Effect of ammonium acetate on neuronal cell number

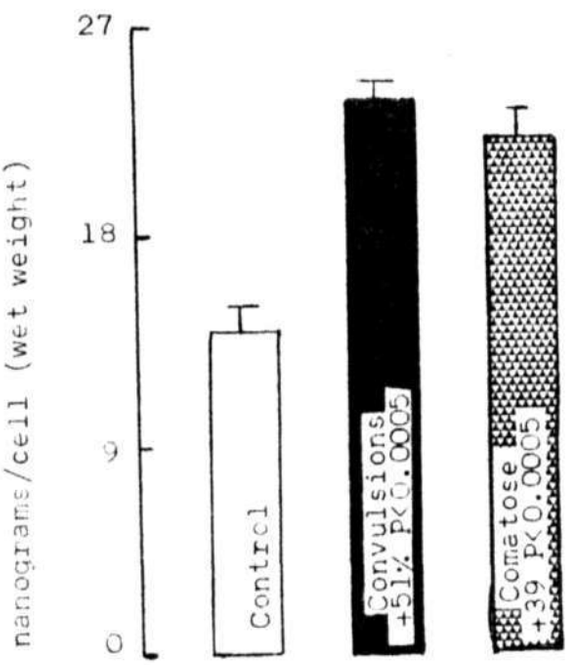


Wet weight, Dry weight and Water content;

Numerous reports have indicated the swelling of astroglia in hyperammonemic states but no efforts were made to determine the magnitude of swelling. Based on the assumptions that permeability properties of cells were retained even after isolation and rapid equilibrium of osmotic constituents would occur if the cells were suspended in an isoosmotic medium, an attempt was made to measure the water content of the cells suspended in Krebs-Ringer phosphate (containing glucose). While determining the water content, no distinction was made between bound and free water and it was assumed that the water content so determined would be an index of cell volume. The determination of wet weight and dry weight would also give additional information about the particulate material, if any, accumulated in cell as a result of drug treatment.

Acute administration of ammonium acetate resulted in an increase in both wet and dry weights as well as the water content of the neurons and astrocytes (Fig.20-23). The magnitude of this increase was at least 3 to 5 fold more in the astrocytes (Fig.21) than in neurons (Fig.20). In contrast to astrocytes, the wet weight decreased in the neurons isolated from animals undergoing convulsions compared to those in comatose state. The changes in dry weight showed

Fig. 20 Effect of ammonium acetate on neuronal weight



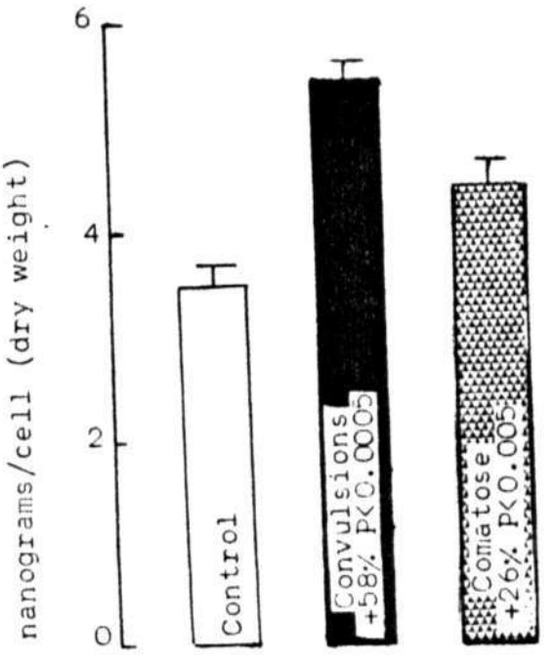
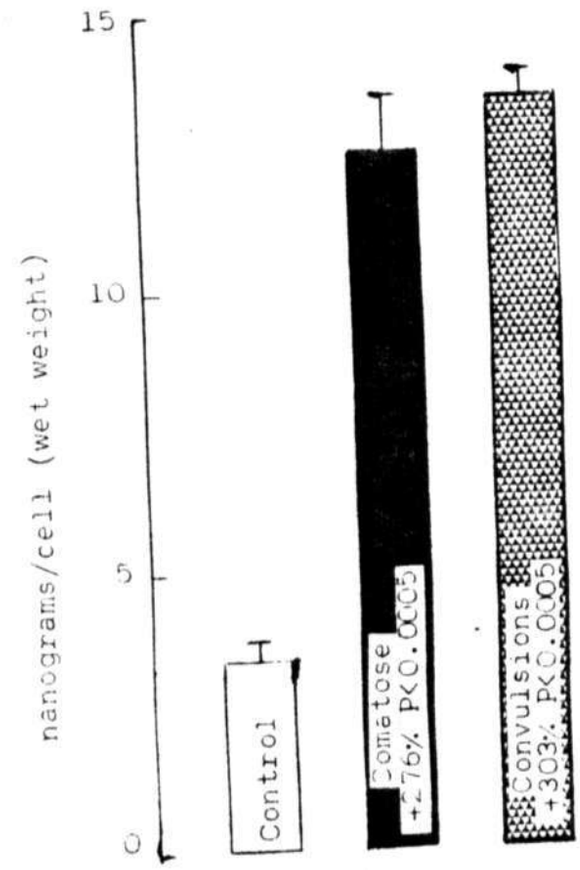
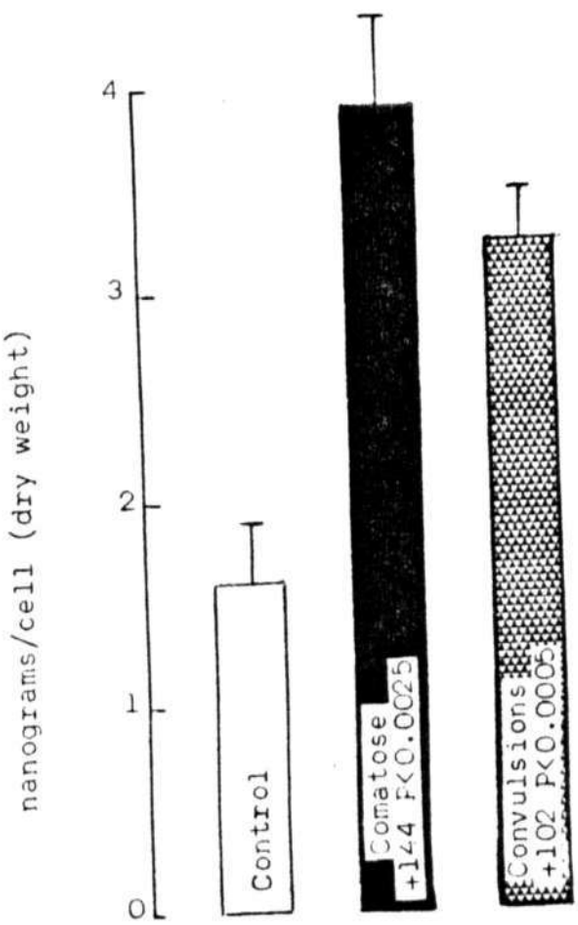


Fig. 21 Effect of ammonium acetate on glial cell weight





a similar pattern (decreased in the convulsive state) in both the cells. The water content (the cell volume) of the neurons remained more or less constant in comatose and convulsive states while in astrocytes this value increased in the convulsive state (Fig.22 and 23).

Greater vulnerability of the astrocytes to ammonia toxicity was suggested repeatedly (53, 162, 183, 205, 206, 327). It was noticed that under prolonged hyperammonemic conditions such as hepatic encephalopathy (204), protocaval anastomosis (53) urease treated animals (106, 165) and after carbon tetrachloride poisoning (3, 81), there was a marked proliferation of astrocytes. However, no reports are available in animals treated with an acute dose of ammonium salts (with or without normal liver function). Failure to observe any increase in the number of astrocytes in the experimental animals might be due to a very short time span between the administration of the drug and decapitation. The decrease in the neuronal cell number was surprising since in the earlier investigations neither loss nor any change in neuronal morphology was reported in animals even in prolonged hyperammonemic states such as protacoval shuntings (195, 204, 298). Similar observations were also reported by Gibson et al., in mice treated with urease (106). The only neuronal change observed under these conditions was the degeneration of myelinated fibres or loss of Purkinje

Fig. 22 Effect of ammonium acetate on intracellular water content of neurons

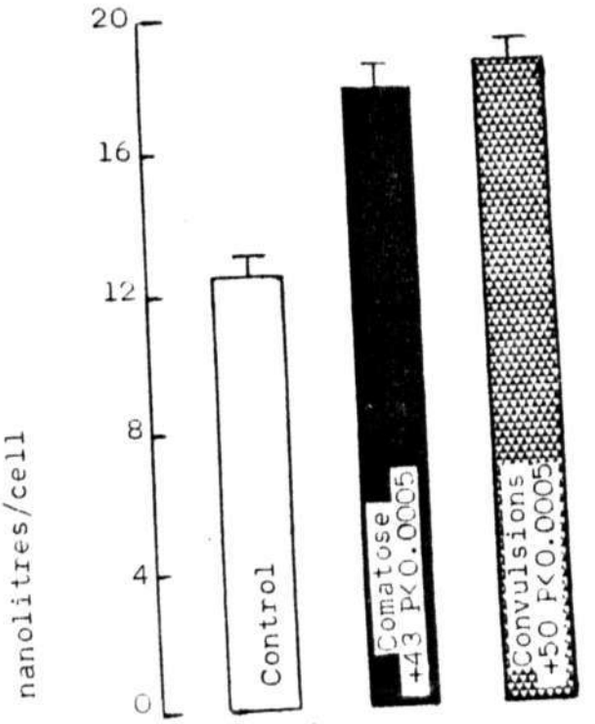
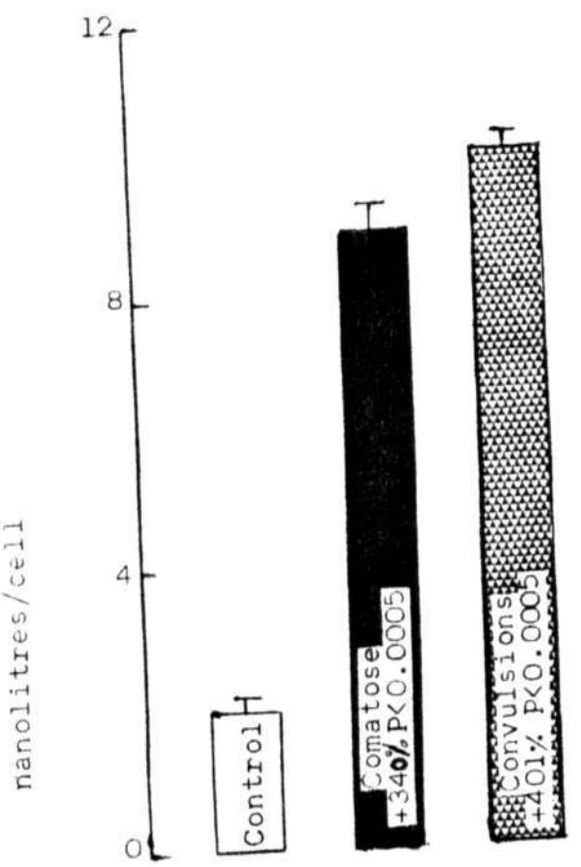


Fig. 23 Effect of ammonium acetate on on intracellular water content of astroglia



cells only in the cerebellum. No changes were observed in cerebral cortex. Though, the animals were hyperammonemic, the mode of induction was different from that of present investigation and as such no histological information is available regarding the neuronal changes in rats treated with an acute dose of ammonium salts. The loss of neurons observed in the present investigation might be real or due to the isolation procedures. As the neurons were swollen they might have become more vulnerable to the rapid changes in the tonicities of the medium (isotonic ---> hypertonic --> isotonic) which could induce cell lysis. The neuronal fraction isolated from normal animals consisted of 2 types of cells some with a large nucleus and others with a small nucleus. The neurons isolated from experimental animals were predominantly of only one type consisting of a large nucleus which suggested that the neurons with the smaller nucleus might have undergone some reactive changes which rendered them highly susceptible to the conditions employed in the isolation procedure or they might have degenerated within the animal due to drug treatment. The latter explanation seems to be more plausible as the tonicities employed in the cell isolation method for experimental animals were, infact, lesser than those employed for the controls and the supernatant obtained after pelleting the neurons was relatively free from debris or unsedimented neurons. It is not known whether this neuronal cell loss has any association with the induction of behavioural changes.

The increased water content of both neurons and astrocytes indicated their reactivity in the presence of ammonium ions. A higher magnitude of increase in the water content of the astrocytes in comparison to the neuronal perikarya concurred with the observations made by earlier investigators using histochemical techniques. It may be presumed that such an abnormal increase in water content would result in swelling of the cells. Astroglial swelling was observed not only in prolonged hyperammonemia but also in a number of conditions such as, thermal lesions (36, 37), allergic encephalomyelitis (161), brain anoxia (185), edema (174), and due to the administration of ouabain (71) and colchicin (116). Hyperammonemia induced by other methods such as urease treatment and CC14. treatment also resulted in astroglial swelling (106, 165). The astrocyte swelling might be due to a sudden increase in the extracellular fluids in the brain which flood the astrocyte. It might also be due to an increase in the osmotically active but impermeable components in astroglial cells without any change in their concentration in the extracellular compartment resulting in the influx of water from extracellular compartment. Several reports indicated the disruption of the normal blood brain barrier in hyperammonemic conditions (51, 128, 180) which might result in a large influx of water into extracellular compartment of the brain. The latter possibility i.e. an increase of osmotically active but impermeable components

in the astrocyte was also apparent from the dry weight measurements. Since proteins and nucleic acids are the major constituents of dry matter, an increase in these components as observed in the present investigation would eventually increase the osmotic pressure inside the cell and the water moves down the gradient resulting in the swelling of the cells. Besides the above mentioned possibilities, a fall in the energy charge (146) representing the available energy of the astrocyte due to the detoxification of ammonia by way of glutamine formation might also be responsible for the swelling of the cells. Benjamin et al.., (17) demonstrated that NHt in large concentration (beyond 5 mM) induced a large influx of Cl-" associated with fluid uptake into the glial cells. It was also interesting to note that the cells accumulated more water when they were isolated from convulsive animals than the cells from comatose animals which might be due to their prolonged exposure to ammonia. The increase in water content also indicated an altered membrane permeability under these conditions.

DNA, RNA and protein:

Administration of an acute dose of ammonium salts resulted in an elevation of DNA, RNA (Fig.24 and 25) and protein (Fig.26 and 27) levels both in neurons and astrocytes. The magnitude of increase in DNA content was more in the neurons than in the astrocytes. Both the cell fractions

Fig. 24 Effect of ammonium acetate on DNA, RNA (neuronal perikarya)

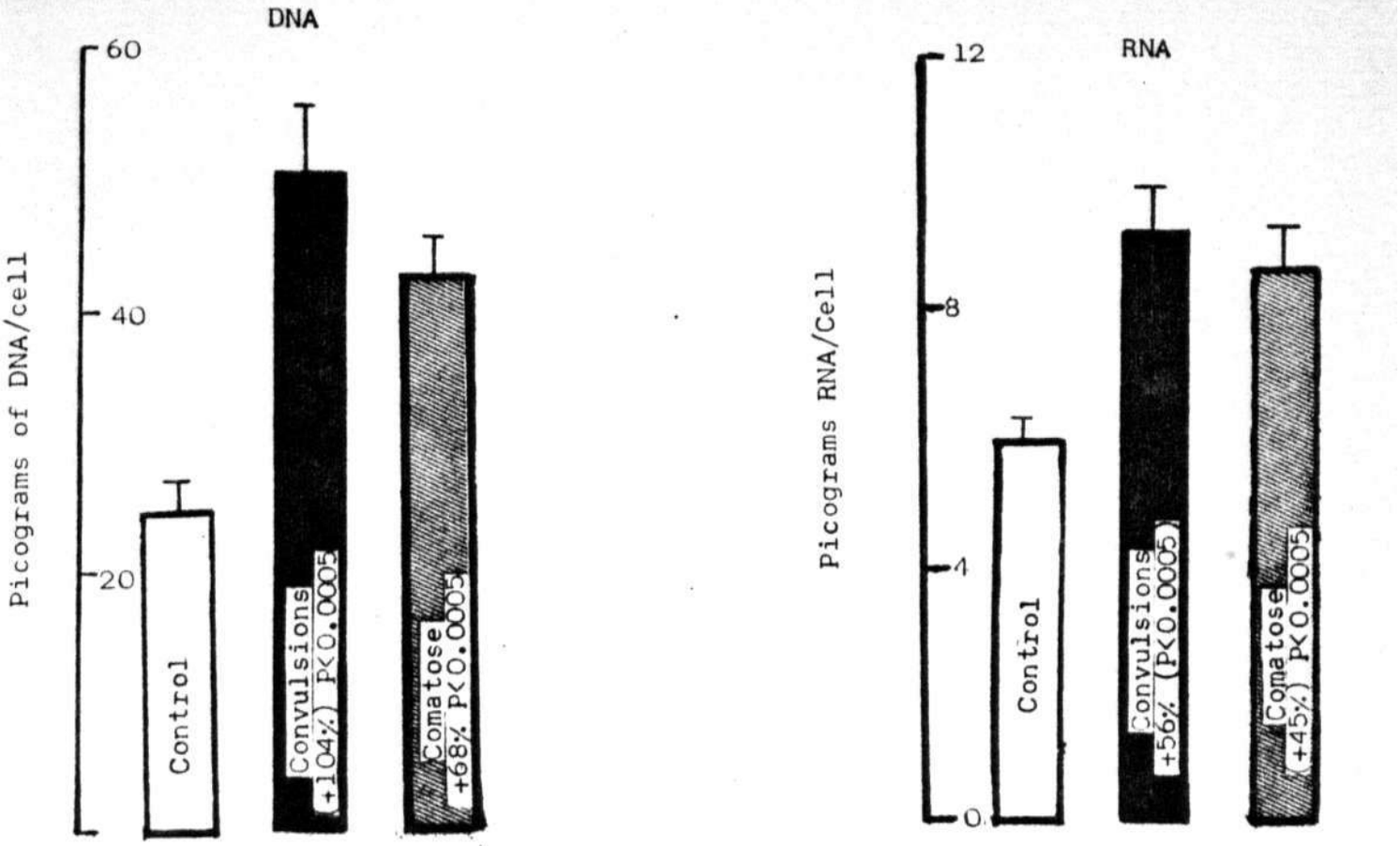


Fig. 25 Effect of ammonium acetate on glial DNA and RNA content

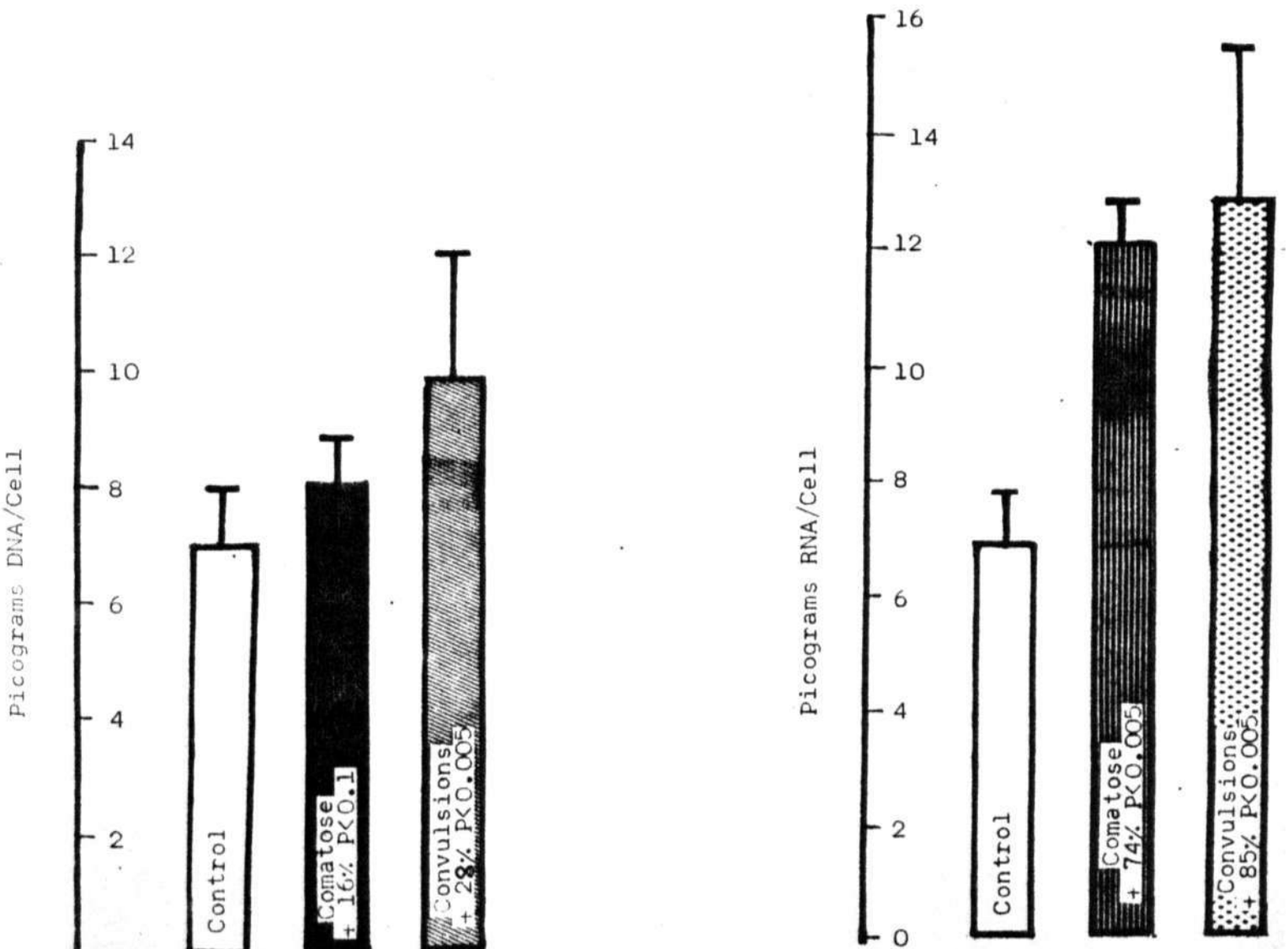


Fig. 26 Effect of ammonium acetate on neuronal protein content

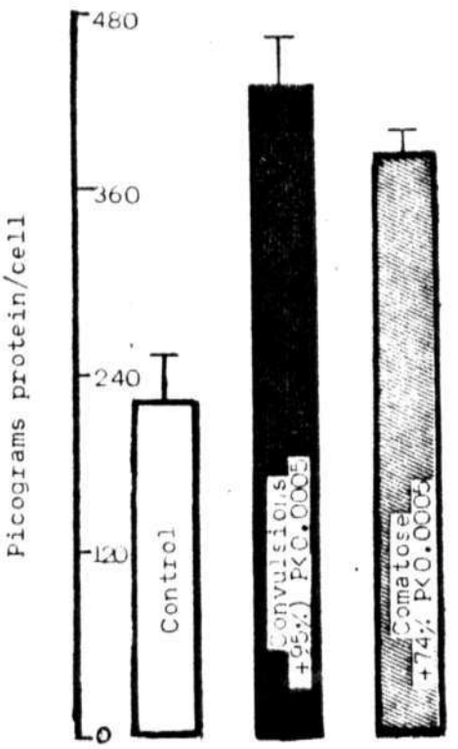
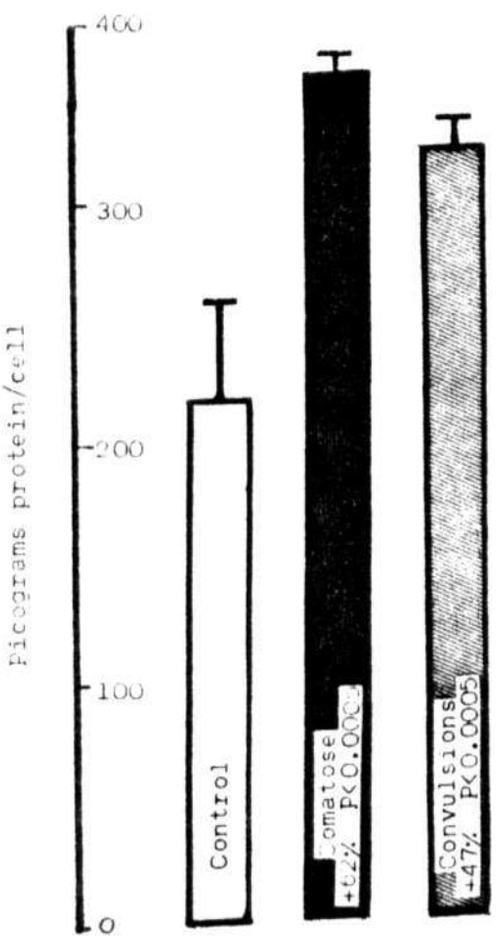


Fig. 27 Effect of ammonium acetate on protein content of astrocytes



isolated from convulsive animals had a higher DNA content than the cells isolated from comatose animals. In contrast to DNA, increase in RNA content in astrocytes was greater in magnitude when compared to that of neurons. Keeping in line with the increase in nucleic acid content, an increase in the protein content was observed following the administration of ammonium acetate. In neurons the increase in the protein content during convulsive phase was higher than that of the comatose phase, while in the astrocytes a reversed profile was observed.

The increased DNA content of astrocytes following the administration of ammonium ion correlated with the expected behaviour of the cell under these conditions. It has been demonstrated repeatedly that in the initial stages of hyperammonemia the primary astrocyte reaction would be cell proliferation. However, no mitotic spindles were observed at this stage suggesting a process akin to endomitosis (53, 162, 2X53-205, 241, 327). For the cell proliferation DNA synthesis is a prerequisite. An increased DNA content of astrocytes observed presently would support the concept of astrocyte reactivity to hyperammonemia. However, no information is available on the net rates of synthesis or the mechanisms involved in the stimulation of DNA synthesis under these conditions.

The increase observed presently in the neuronal DNA content was surprising as the neurons were reported to be relatively resistant to the hyperanimonemic conditions. Further, the decrease in cell number under these conditions makes it more difficult to understand this phenomenon. In addition, the well established fact that the neuronal DNA content is relatively stable after the maturation of the brain in mammals (23) adds to the complexity of the problem. The increase in the neuronal DNA content following the administration of ammonium acetate might be due to an increased rate of synthesis or a decreased rate of degradation. It is known that NH_4^+ stimulates the DNA transcription by increasing the transcribable portion of DNA (54-56). Though, the replicative synthesis of DNA is not known to occur in the adult neurons of rat, it was established that there would be repair synthesis of DNA (23). This process is mediated by the enzyme DNA polymerase-p (23), and virtually no information is available on the neuronal β-polymerase. Both acid and alkaline DNAses are known to be involved in the DNA turnover of mammalian brain (271) for which the regulation phenomenon is yet to be worked out. In the absence of such information it is difficult to predict the mechanism of accumulation of DNA under these conditions. However, the cells which survived hyperanimonemic insult and the isolation procedure might be assumed to be in a reactive phase. The increase in the DNA content, if considered along with the increased RNA and protein content would explain the increase observed in the activities of virtually all the

enzymes of glutamate metabolism observed in the present investigation.

The increase in RNA and protein contents in astro-
fee
glial cells would an index of increased translational activity under the experimental conditions. Incorporation of ^{3}H-uridine into cerebral RNA demonstrated during experimental hepatic-encephalopathy would be in support of such an observation (3). Proliferation of astroglia and its cellular organelles such as mitochondria, endoplasmic reticulum etc., (observed by earlier investigators) in hyperammonemic conditions (203, 204) would require the synthesis of new proteins. The increase translational activity observed in this investigation supports such a contention. The possibility of transfer of proteins from blood into the astroglial cells may also be worth considering as the blood brain barrier is disrupted under these conditions. It is interesting to note that no studies were undertaken to measure the cerebral protein turnover rates either in hyperammonemia or in hepatic encephalopathy. The only report on the cerebral protein synthesis in hyperammonemia so far available is that of Bessman and Pal (35).
14
They reported an inhibition of ^{14}C-valine incorporation into cerebral proteins in rats injected with ammonium acetate which was proportional to the blood $NH4^{+}$ levels, cerebral ATP and GTP levels and the state of consiousness.

Enzymes of glutamate metabolism:

Following the administration of ammonium acetate, cells and synaptosomes were isolated from the brain, and the enzyme activities were estimated in order to study the changes in glutamate metabolism. For the reasons mentioned earlier emphasis was laid on cellular activity than on the specific activity in the case of neurons and glia whereas for synaptosomes the results were expressed per mg. protein.

The activities of all the enzymes of glutamate metabolism excepting GLNase were increased Doth in comatose and convulsive conditions in the neuronal fraction of the rat Drain due to acute hyperammonemia. Of all the enzymes studied, the magnitude of increase in the activity of GDH was greater than that of any other enzyme (Fig.28). Based on the percent increase of the activities, the enzymes may be arranged in the following order GDH>GS>AAT>A1AT under comatose condition. The magnitude of increase in GDH activity was more or less same in both comatose and convulsive conditions, while that of the other three enzymes (AAT, A1AT and GS) was more during convulsions than in comatose state (Fig.28-31).

When the activities of the enzymes were represented as specific activities, the magnitude of increase was more or less same in all the enzymes except A1AT. The specific

activity of MAT remained unchanged under comatose condition while in convulsions it was elevated (Fig.28-31).

In contrast to neurons, the cellular activity of GDH in astrocytes was decreased by about 36% due to the administration of ammonium acetate (Fig.32). The activity of GS was elevated to the same magnitude (Fig.33). No changes were observed in glial AAT activity under these conditions (Fig.34). The activity of A1AT was, however, normal in comatose condition and increased by about 60% in convulsions (Fig.35). The change in the astroglial GLNase activity was significant only in comatose condition (Fig.36).

When the activities were expressed per mg. protein but for a significant decrease in the activities of GLNase (40%) and GDH, no changes were observed in the GS, AAT and A1AT (Fig.32-36).

The decline in the activity of synaptosomal GDH was highly significant (Fig.37) and the magnitude of decrease was far greater when compared to that of glial GDH. Under comatose condition this inhibition amounted to 93% while in convulsions it was relieved to a small extent (-72/). The activity of GS was observed to increase to the same extent as found in glial cells (+31%) in comatose condition. However, during convulsions this activity was reverted to

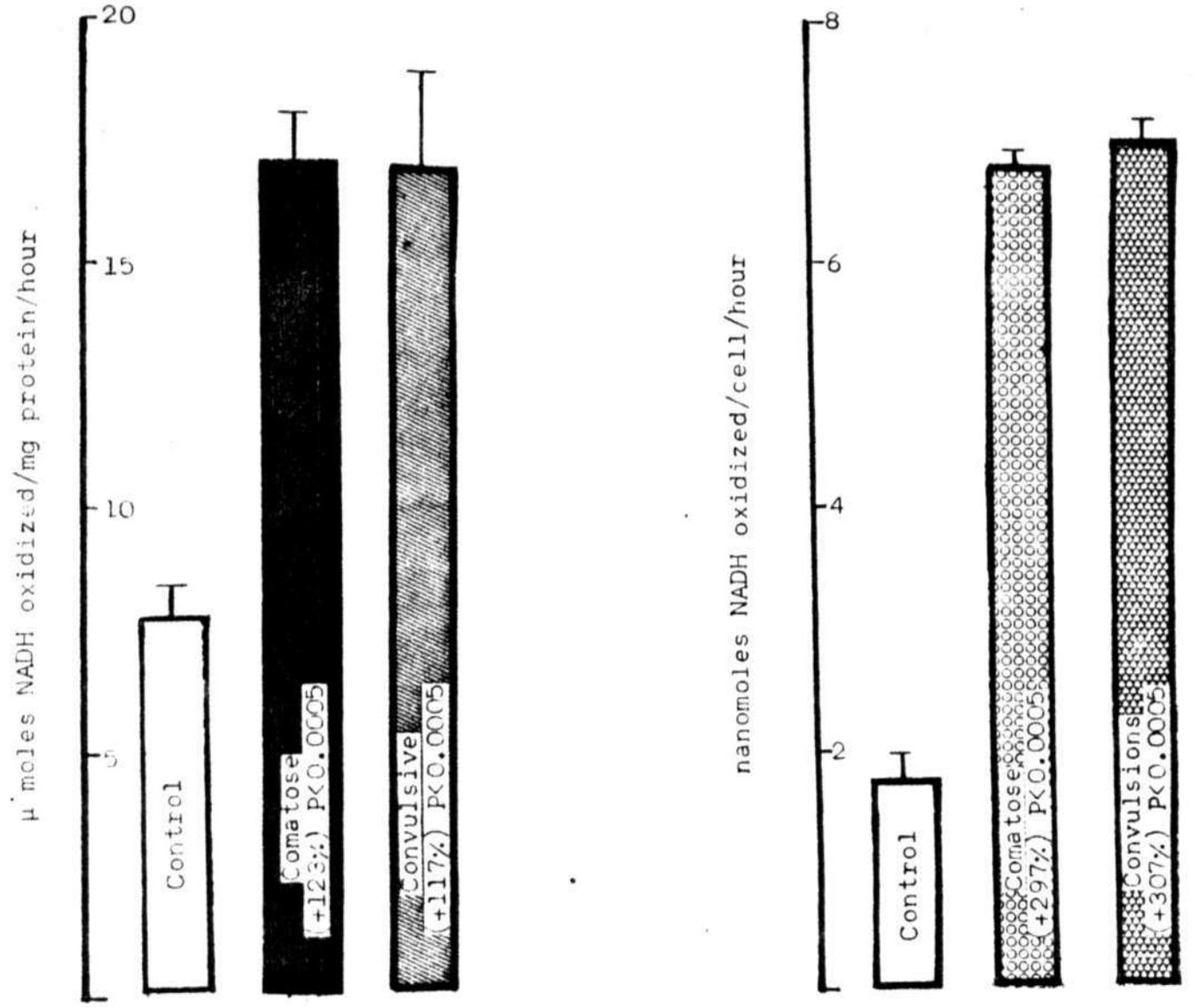


Fig. 29 Effect of ammonium acetate on neuronal glutamine synthetase

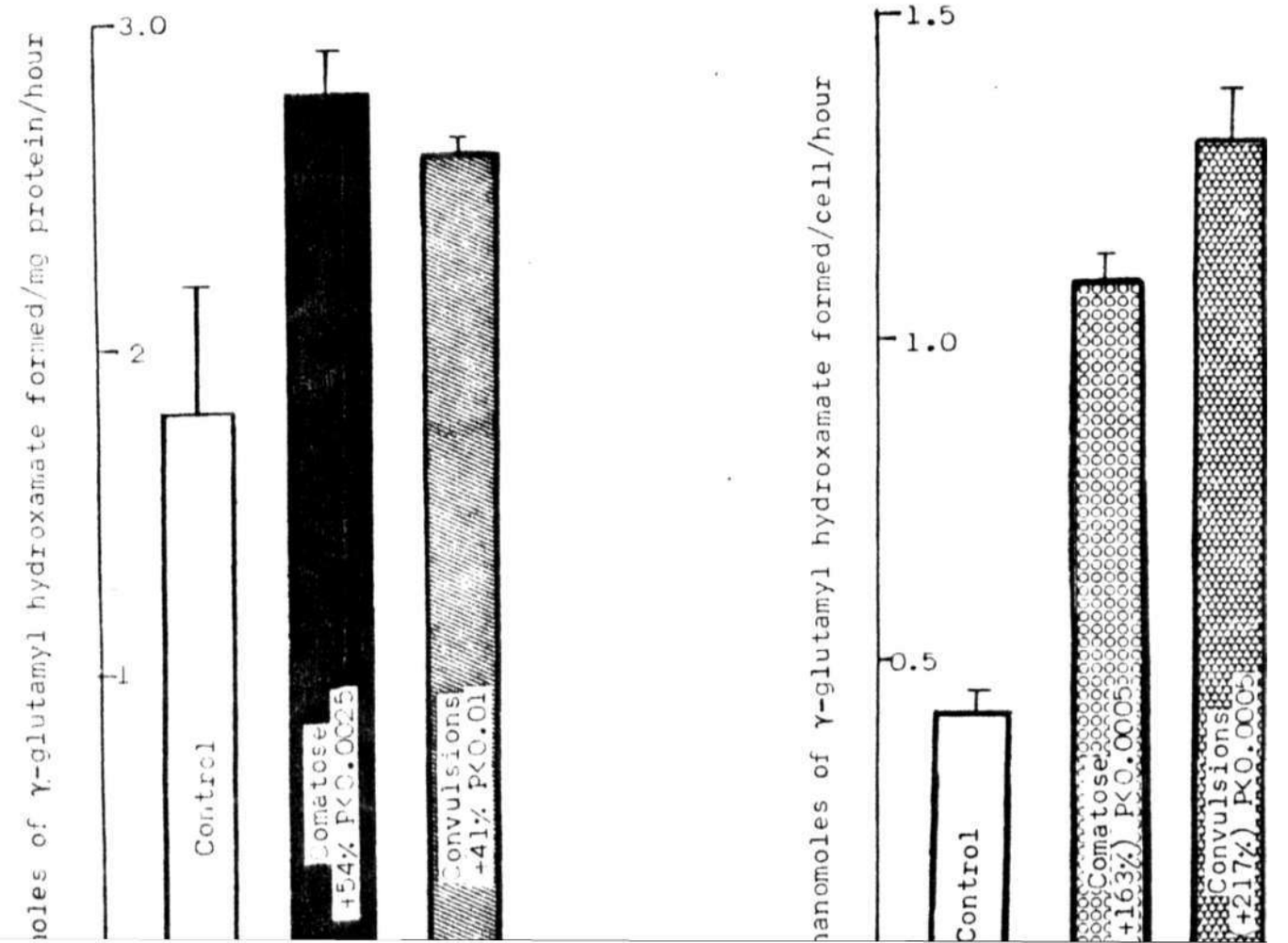


Fig. 30 Effect of ammonium acetate on neuronal aspartate aminotransferase

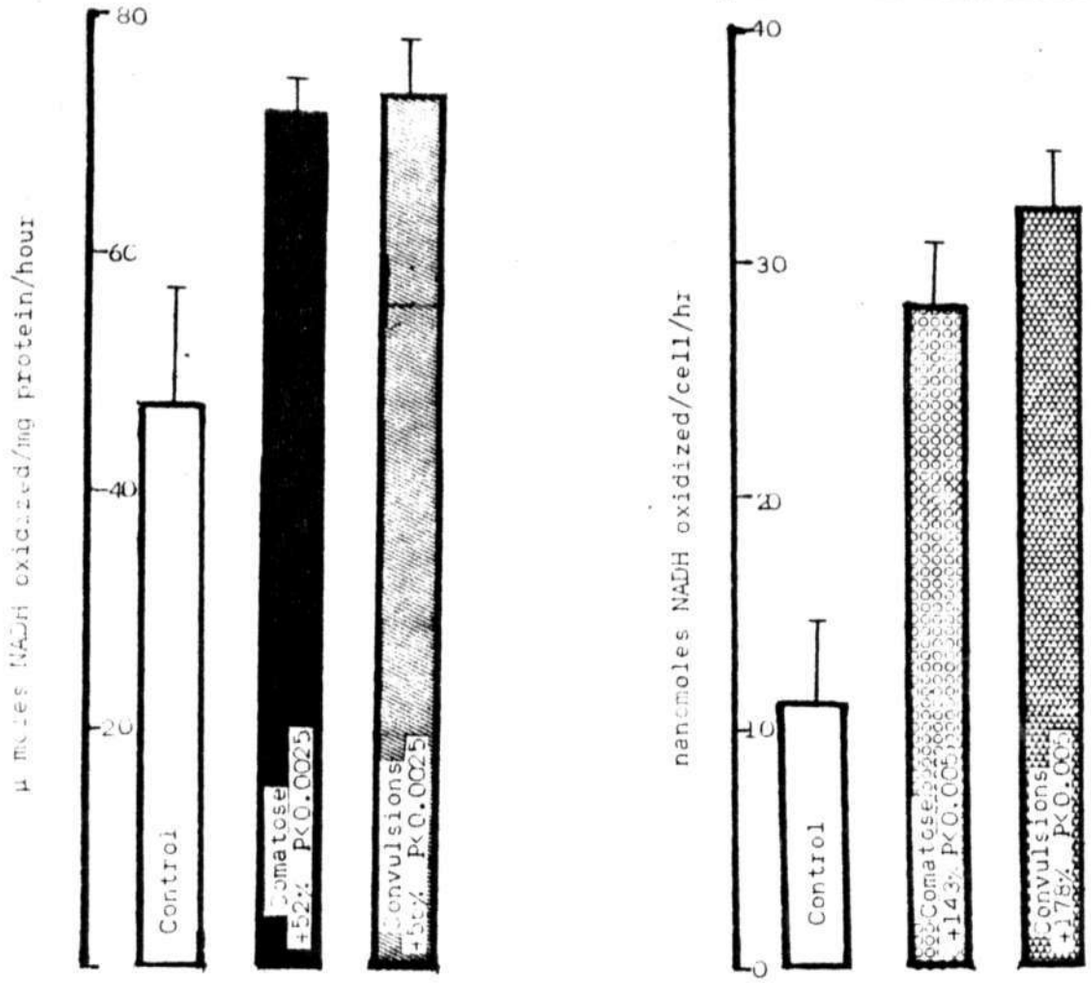


Fig. 31 Effect of ammonium acetate on neuronal alanine aminotransferase

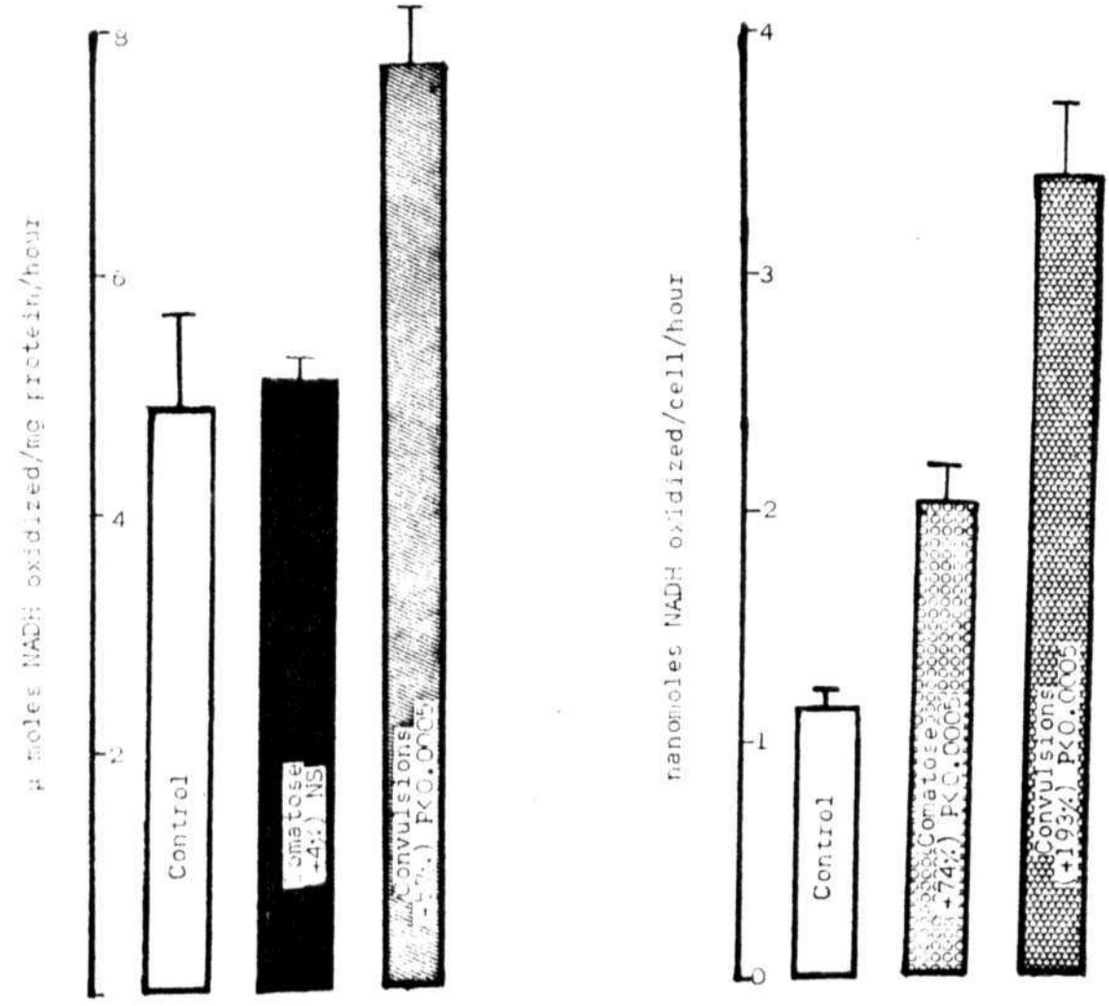


Fig. 32 Effect of ammonium acetate glial glutamate dehydrogenase

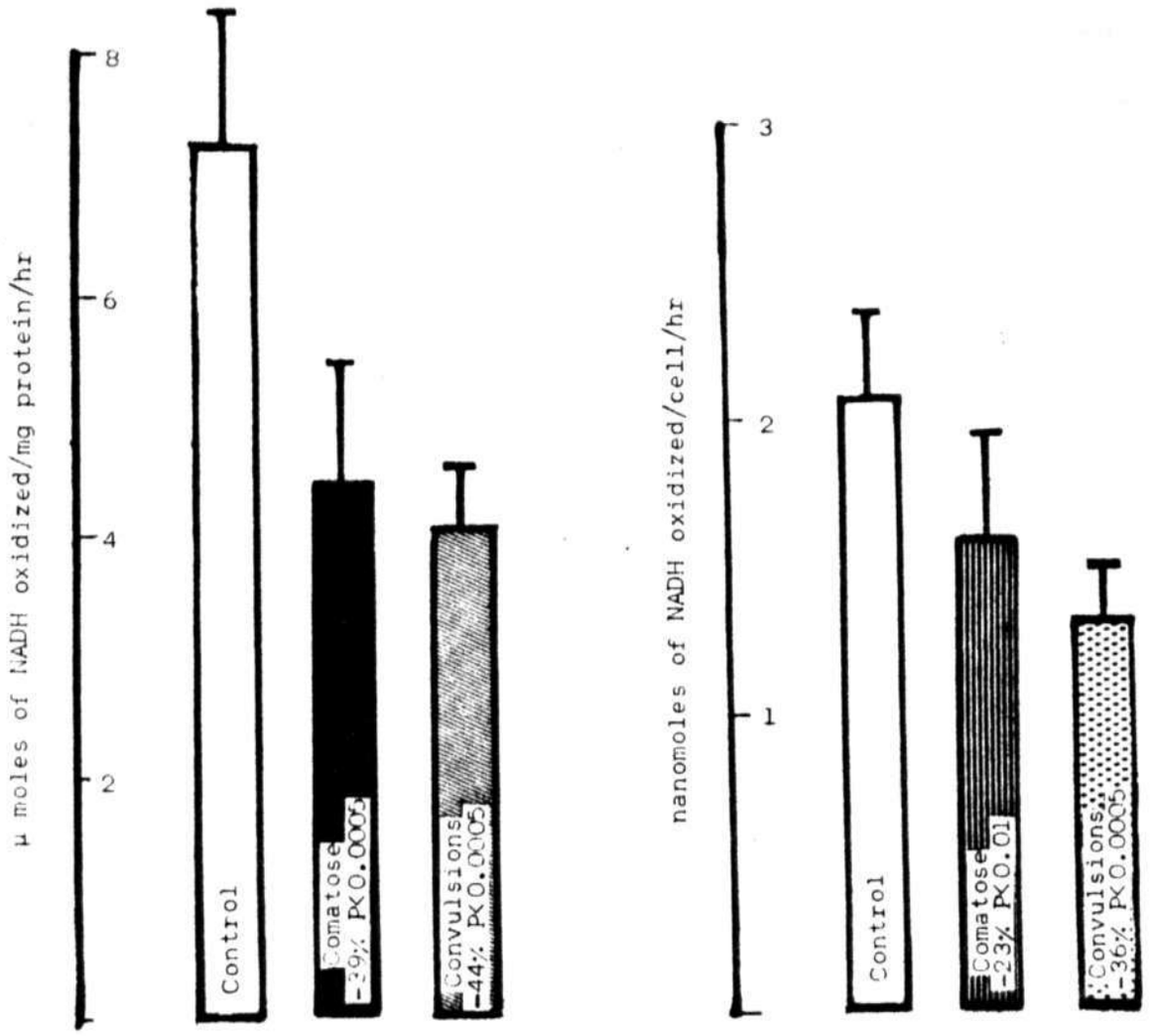


Fig. 33 Effect of ammonium acetate on astroglial glutamine synthetase

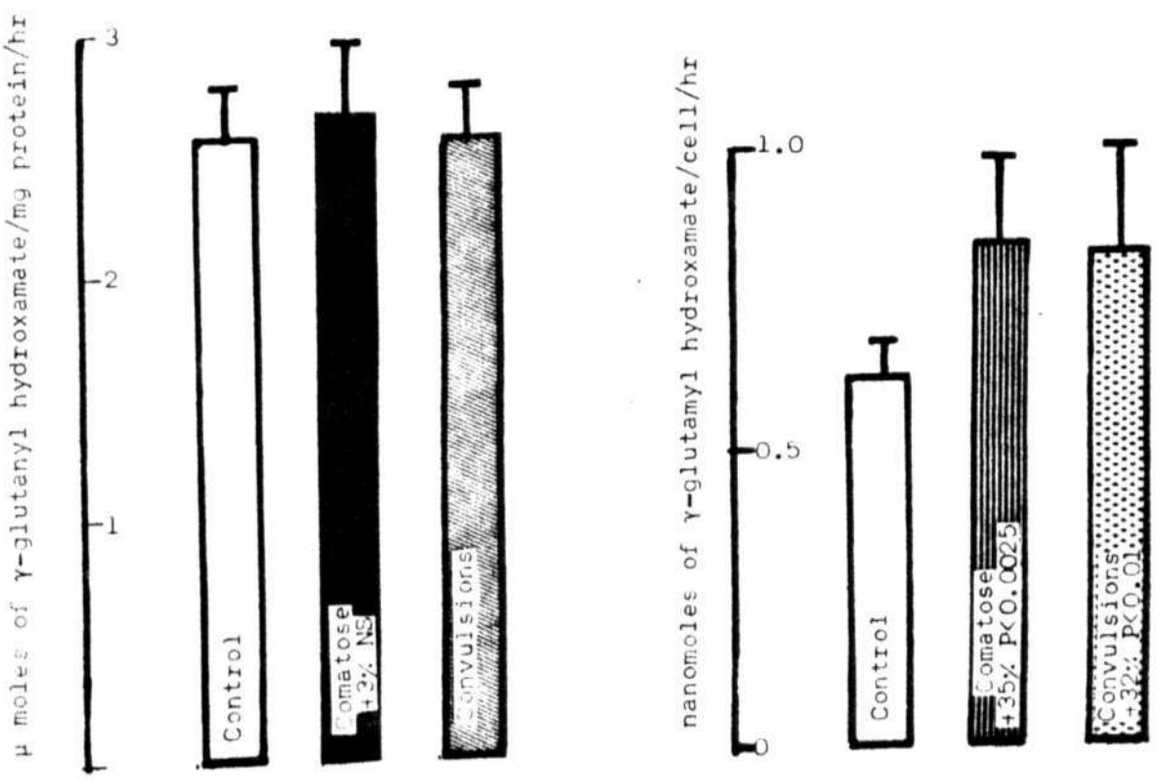


Fig. 34 Effect of ammonium acetate on astroglial aspartate aminotransferase

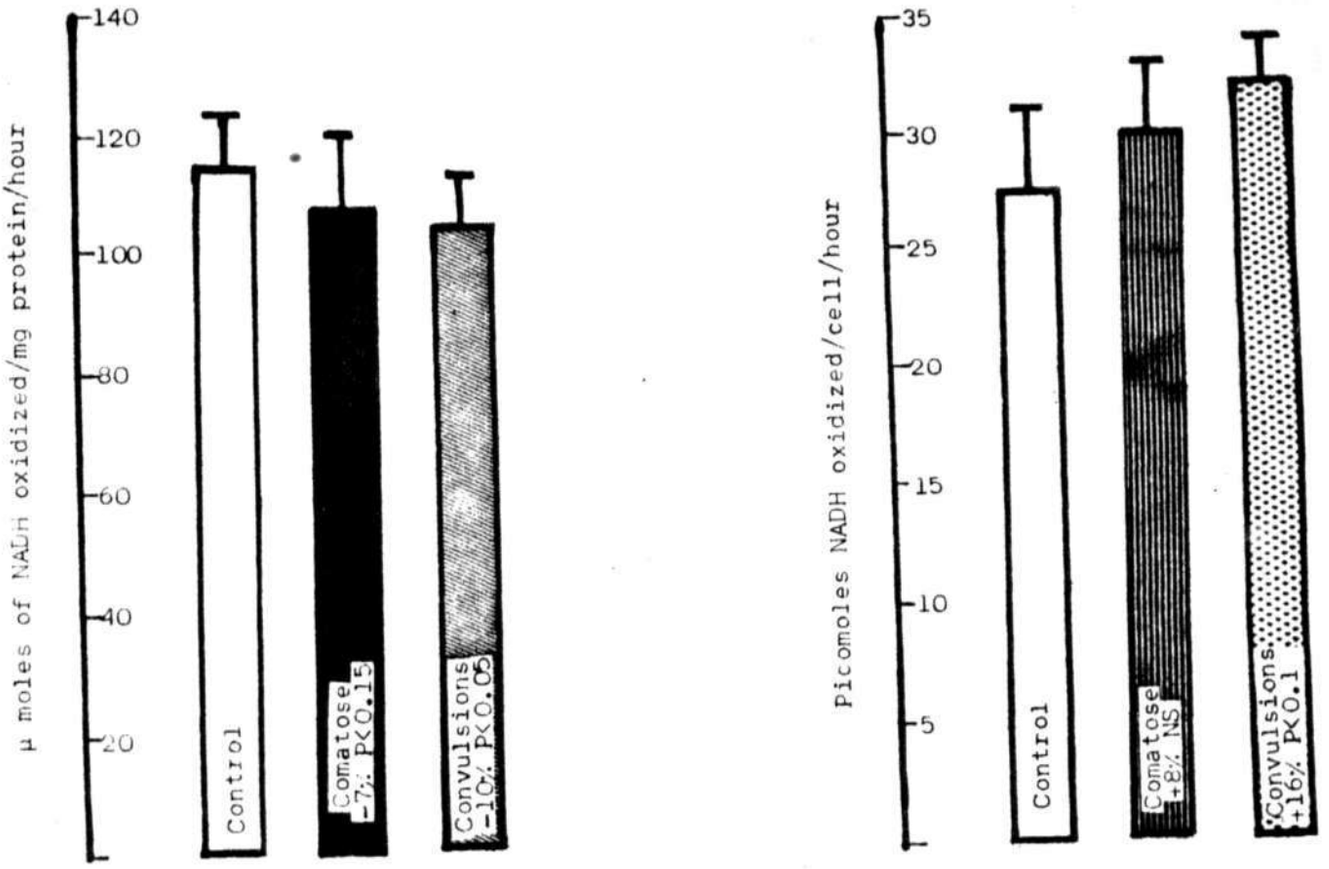


Fig. 35 Effect of ammonium acetate on glial alanine aminotransferase

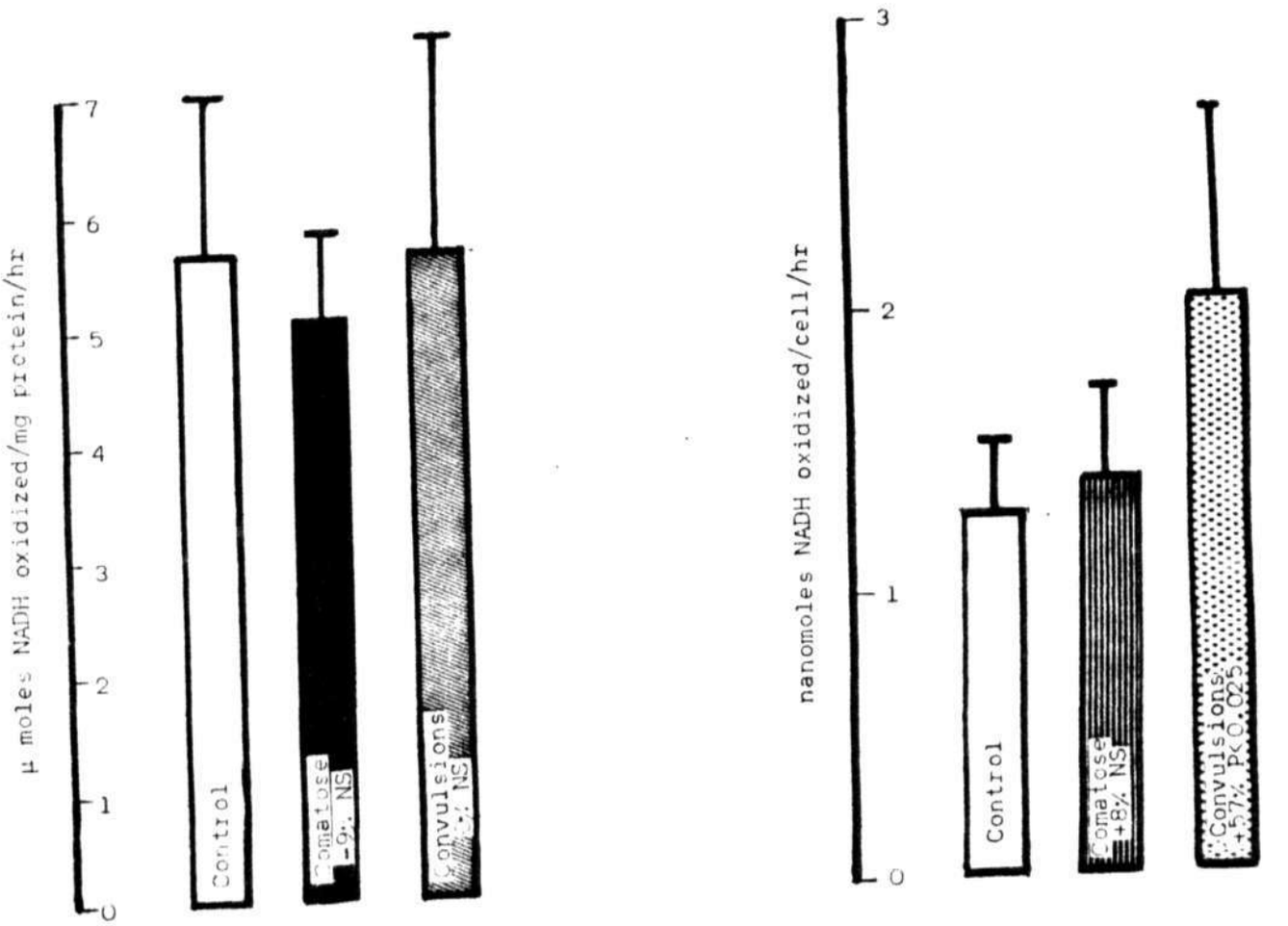


Fig. 36 Effect of ammonium acetate on astroglial glutaminase activity

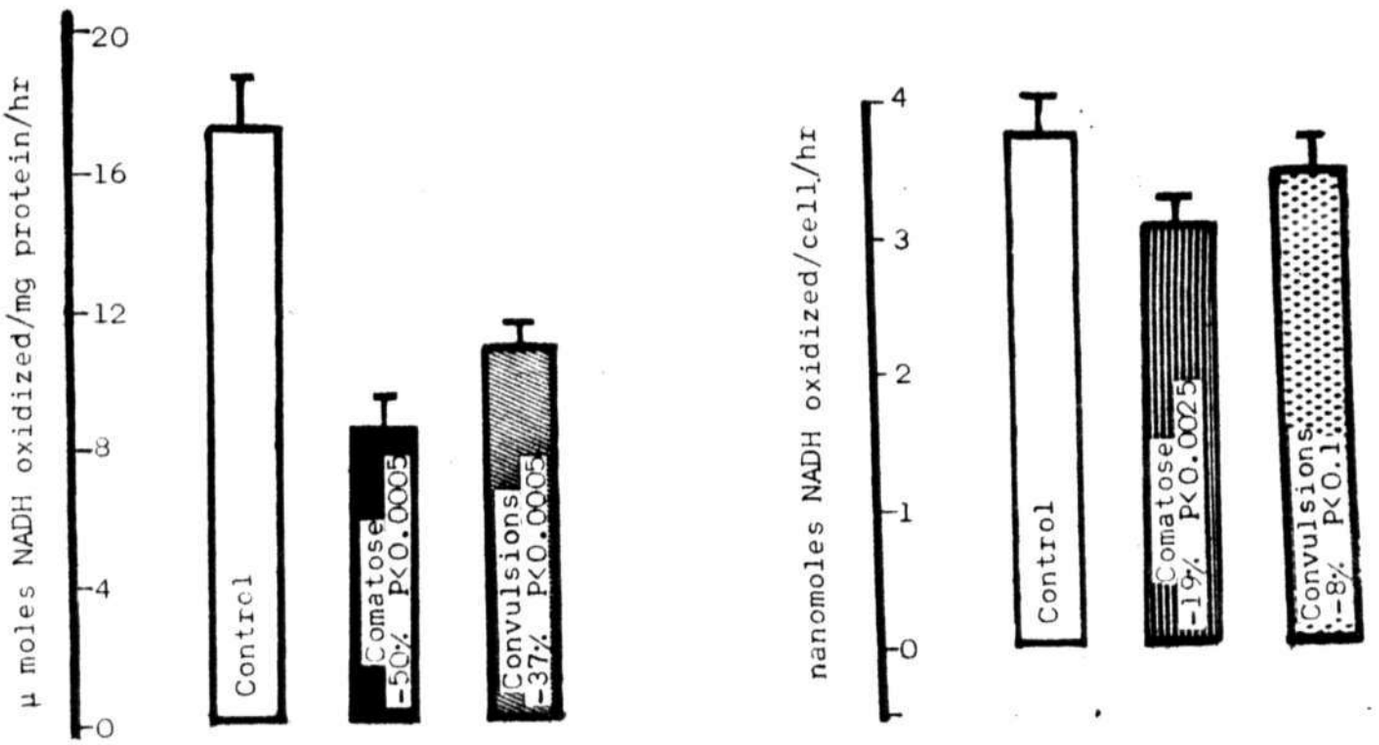
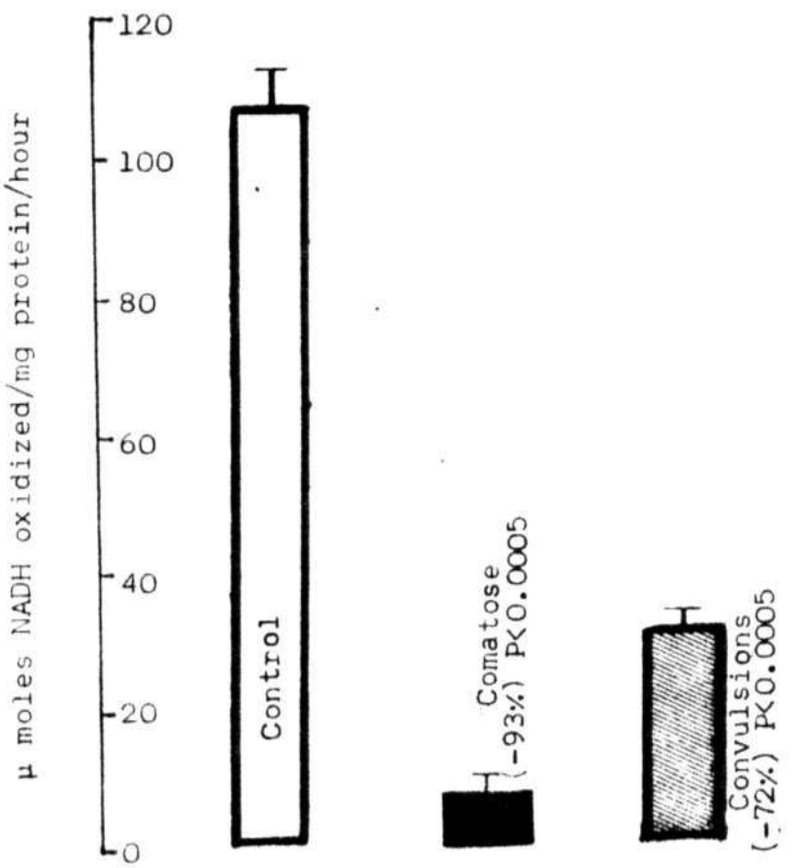


Fig. 37 Effect of Ammonia on synaptosomal GDH



normal levels (Fig.38). The suppression of AAT in both the conditions was more or less same (Fig.39). Unlike in the glial cells, the activity of MAT was elevated to a small extent in comatose condition. Similarly, the decrease in the activity of this enzyme in convulsions was not statistically significant (Fig.40). In contrast to glia, synaptosomal GLNase activity was elevated during comatose and was suppressed during convulsions (Fig.41).

Glutamate and glutamine formation is supposed to be the primary detoxification mechanisms of ammonia in extrahepatic tissues where the urea cycle is incomplete [237] and the enzymes GDH and GS are the enzymes involved in this process (313). It was suggested earlier that the operation of this detoxification pathway would deplete both α-ketoglutarate and ATP, resulting in the neurological dysfunction (33). Subsequent studies have shown that the site of detoxification was localized in the astrocytes (27). Much of the evidences for this concept was based on the differential labelling of glutamate and glutamine from various precursors in the presence of ammonium salts (27). No efforts were made by these investigators to study the changes in the enzyme activities under these conditions.

The results obtained in the present investigation were, however, not on par with the well established hypothesis of

Fig. 38 Effect of ammonium acetate on synaptosomal glutamine synthetase

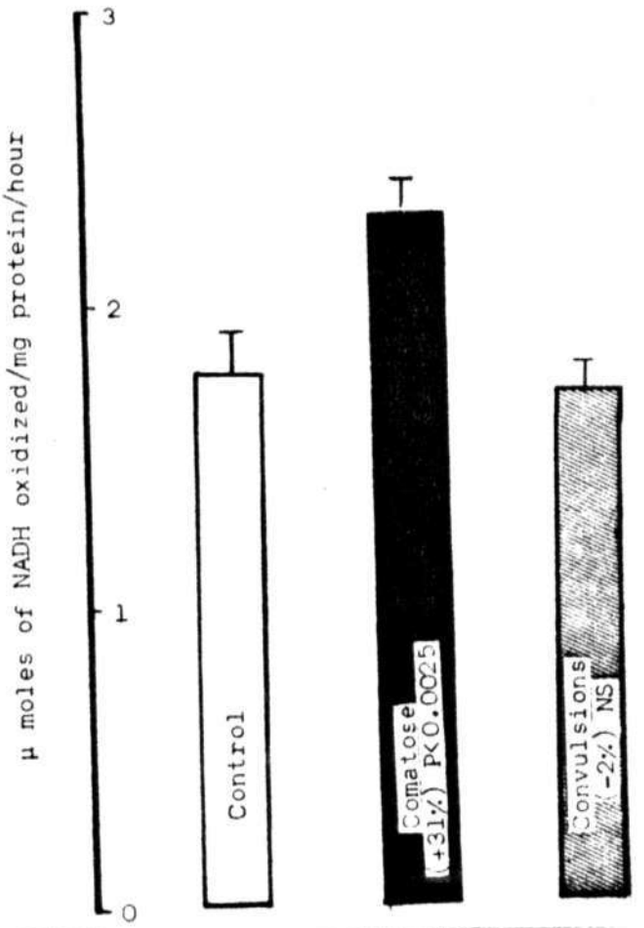


Fig. 39 Effect of ammonium acetate on synaptosomal aspartate aminotransferase

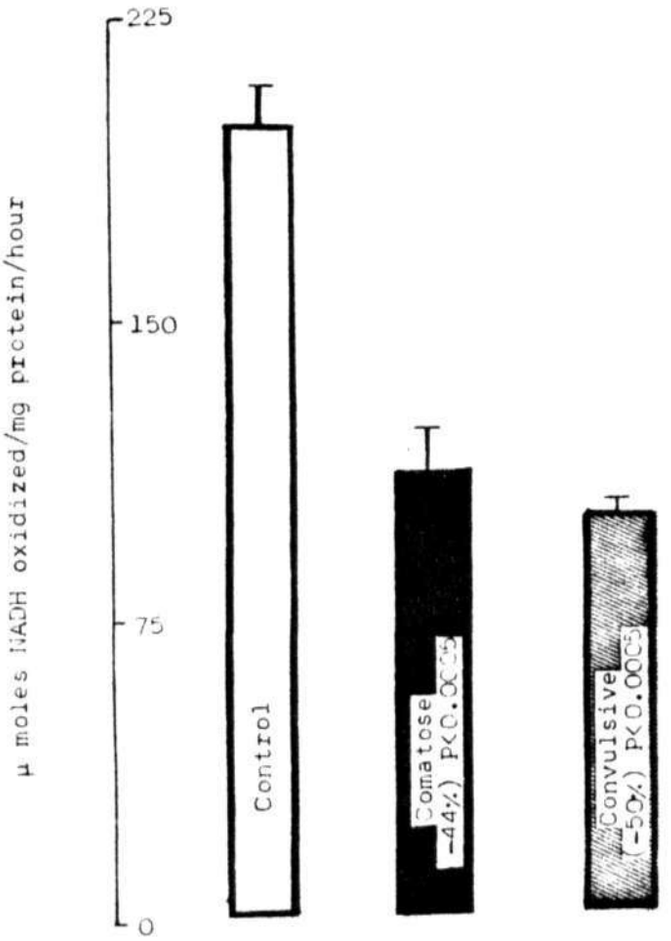


Fig. 40 Effect of Ammonium acetate on synaptosomal alanine aminotransierase

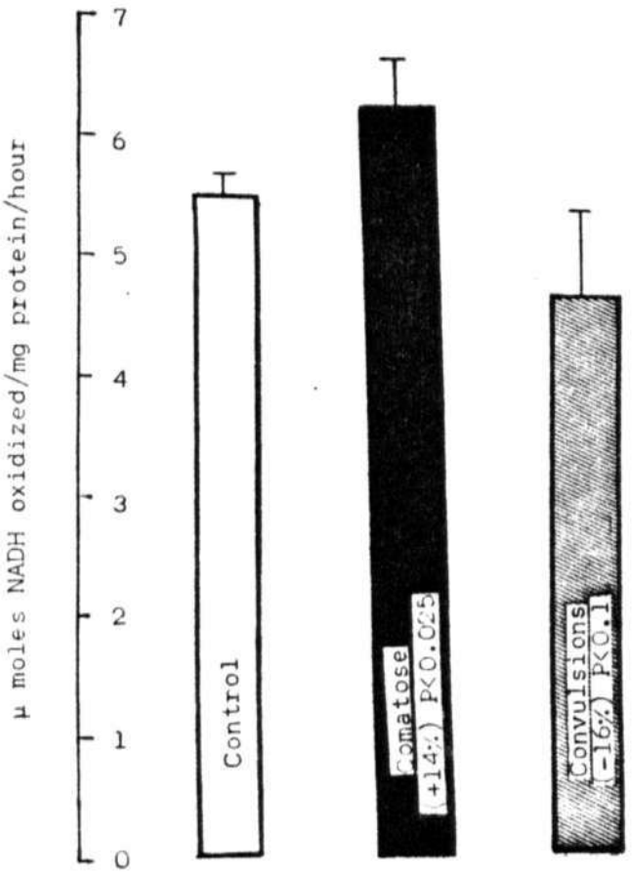
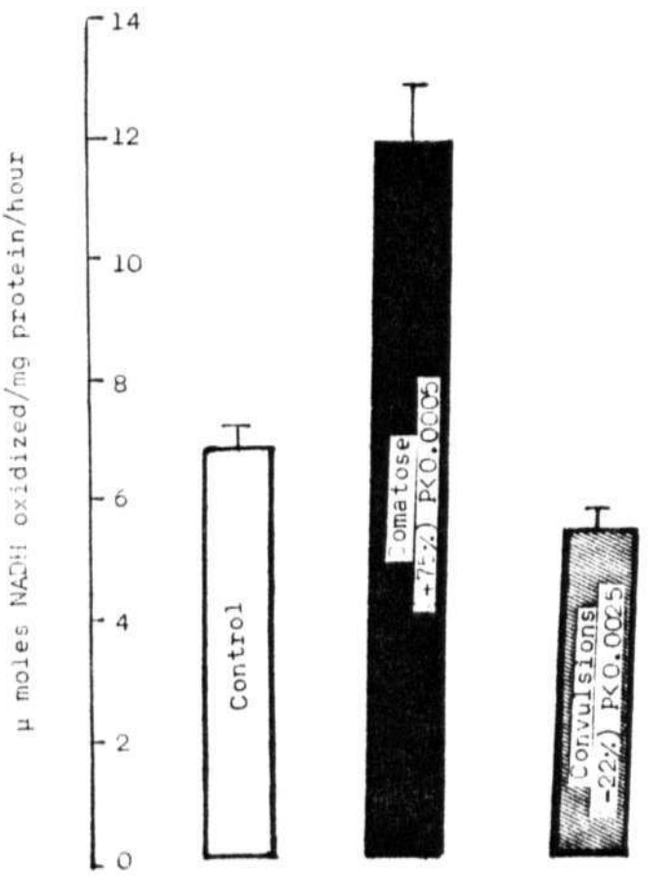


Fig. 41 Effect of Ammonia on synaptosomal glutaminase



the astrocyte vulnerability or its involvement in the process of ammonia detoxification. It was observed that the major changes in the activities of the enzymes of glutamate metabolism occurred in the neuronal perikarya rather in the astrocytes. The increase in the GDH activity in the neuronal perikarya would result in the detoxification of ammonia resulting in the formation of glutamate, depleting α-ketoglutarate from TCA cycle. The increased GS activity in this cell fraction would convert glutamate to glutamine in the presence of ammonia and ATP would be consumed in this process. These two processes would eventually deplete the citric acid cycle intermediates and ATP in this cell fraction. Though the reaction of AAT is reversible, under the present conditions due to enhanced glutamate levels and reduced α-ketoglutarate (due to an increase in GDH), it might be assumed that a fraction of glutamate formed in the detoxification process would be converted to aspartate. In this process oxaloacetate would be consumed and α-ketoglutarate regenerated might be utilized subsequently by GDH. The increased activity of this enzyme might also be of help in the transport of reducing equivalents across the mitochondrial membrane required for the GDH reaction, production of which would have been lowered due to an interferance in the operationof the later half of citric acid cycle (due to a-ketoglutarate depletion). Similarly it might be predicted that the A1AT would be operating in the direction of alanine formation

rather than in the direction of glutamate formation. Though this process would consume pyruvate, α-ketoglutarate is regenerated bypassing the earlier half of the citric acid cycle. The loss in the pyruvate would be made good by the increased glucose oxidation as it was shown that NH_4^+ would stimulate cerebral aerobic glycolysis (17, 22, 286). Carbon-dioxide fixation is another process in which the dicarboxylic acids are anaplerotically replenished. It is unfortunate that no information is available on the cellular localization of the enzymes involved, though earlier evidences indicated the localization of this process to be in the astrocytes (26, 60). It is interesting to note that all the above said processes were enhanced during convulsions when compared to comatose condition. Though the involvement of neuronal perikarya was not demonstrated, hitherto, indications were made in the literature (81). Based on the incorporation of ^{14}C-glucose into glutamine Cremer *et* al., suggested the involvement of large compartment in the glutamine formation (73). Similar suggestion was also made by Krespan et al., (149). Another interesting study in this direction was that of morni et al., (194). These authors demonstrated that acute administration of ammonium acetate increased the incorporation of ^{13}C-glucose into glutamate by *65%* under in vivo conditions. Since glucose incorporation to glutamate and glutamine is a property of large compartment, it may be appropriate to assume that these changes are taking

place in the neuron. If the astrocytes are the only cells responding to ammonia, then it would be highly difficult to explain either coma or convulsions during hyperammonia. The present results would then explain the observed neurological dysfunction.

As mentioned earlier, the results obtained with astrocytes were not in concurrence with the established hypothesis of involvement of small compartment of glutamate in the process of ammonia detoxification. Acute administration of ammonium salts resulted an inhibition of GDH in these cells rather than activation. Under such conditions, at least, a major portion of α-ketoglutarate would be available for the normal operation of citric acid cycle. The magnitude of increase in GS in this cell fraction was about 5-7 fold less compared to neuronal perikarya. However, the increase observed in the activity of this enzyme would suggest that a fraction of ammonia would be detoxified in these cells by way of glutamine formation. Inspite of a lesser magnitude of increase in GS the activity in astrocytes than neurons, the quantity of glutamine formed by these cells might be greater than neurons as the astrocytes outnumber the neurons by 1:10 (230, 231). Interestingly a reciprocal relationship was observed between GDH and GS in this cellular compartment which would help in preserving cellular energy pools. The present study also suggested that glutamate required for

glutamine formation in astrocytes may not be generated in the same cellular compartment (due to inhibition in GDH activity). In other words, the small rapidly turned over pool of glutamate which was supposed to be the precursor for glutamine synthesis was at least temporarily suspended in acute hyperammonemia. Under such conditions, it would be ~~~~~~~~~~ appropriate to assume that the glutamate required for astroglial glutamine formation would be generated elsewhere. This could be from the large compartment. Similar suggestion was already made by Cooper et al .,(70).

The lack of change in the AAT activity in the glial cells was on par with the aforesaid changes in GDH activity. As GDH was inhibited the requirement for the regeneration of α-ketoglutarate and the replenishment of reduced equivalents would also be minimal. A similar argument may be applicable to the lack of changes in A1AT activity at least in comatose condition. The increase in the activity of this enzyme under convulsions may spare the pyruvate for carbon dioxide fixation which is supposed to occur in this cellular compartment (27). The inhibition of GLNase activity observed in acute hyperammonemia, though not significant when expressed per cell, might be due to the inhibition of this enzyme by ammonium ion. The activity of this enzyme under these conditions when expressed per mg. protein, showed a very significant inhibition. The discrepancy might be due to

increased protein content in these cells under the present circumstances.

All the above observations suggested lack of active involvement of astrocytes in ammonia detoxification at least during acute hyperammonemic conditions. However, increased water content or swelling observed under these conditions would contradict the above suggestion. The results of Benjamin et al., (17) would help in resolving this problem. A biphasic influx of chloride ions into cortical slices was observed in the presence of ammonium chloride by these authors. At low concentration of NH_4 influx of chloride was not followed by fluid uptake, while at higher concentration this influx was associated with large amounts of fluid uptake. It was suggested that the initial phase of Cl~ influx was into the neurons and the later phase associated with fluid intake was supposed to be into glial cells. This would then suggest the swelling observed in the present investigation might be due to be large influx of Cl- into these cells. In conclusion, the swelling appears to be more or lessypassive process.

While reporting the inhibition of GDH in astrocytes, we suggested that the glutamate for glutamine biosynthesis might be originating in the synaptosomes (269). This conclusion was based on the reports of Hamberger .et al., (115) that only the evoked release but not the spontaneous release of

glutamate from hippocampal slices was inhibited by ammonium ions. Further, the inclusion of synaptosomes into small compartment by Hertz (130) provided additional support in such a concept. Keeping this in view it was assumed that the activity of GDH and other enzymes of glutamate metabolism would be stimulated in the synaptosomes in acute hyperammonia. However, when the activities were measured under similar conditions, an inhibition of all the enzymes of glutamate metabolism except GS was observed in synaptosomes. The magnitude of inhibition in GDH activity was far greater than that of astrocytes indicating that the detoxification process was greatly suppressed which would result in the accumulation of large quantities of ammonia in synaptosomes during hyperammonemia conditions. As in the case of astrocytes, the stimulation of GS in the synaptosomes would promote glutamine formation as an effort to alleviate the toxicity of accumulated ammonia. In this process some amount of ATP would be utilized. The inhibition of AAT in conjunction with GDH, would spare α-ketoglutarate and oxaloacetate for the operation of citric acid, probably at an enhanced rate, resulting in the generation of large amounts of ATP required in the maintenance of ionic gradients and also for glutamine formation. The behaviour of GLNase under these conditions was difficult to understand. The stimulation of the activity of this enzyme under comatose condition contradicted the reports of Van den Berg et al., (294) who demonstrated that in the initial phase of ammonia toxicity the increase in

glutamine concentration was not due to the enhanced synthesis of glutamine but due to a suppression in the conversion of glutamine to glutamate. This concept was supported by several observations wherein an inhibition of GLNase by both ammonia and glutamate was demonstrated (16, 150).

However, Svenneby (275) reported the presence of ammonia stimulated GLNase activity in the pig brain which is yet to be confirmed. Though the increased GLNase activity in comatose condition would be explained by the above said report, the physiological significance of such a process would be difficult to envisage. Such an increase would inevitably result in the breakdown of glutamine and generate additional ammonia in the synaptosomal mitochondria. Further glutamate-glutamine cycle would then become a futile cycle leading to a wasteful expenditure of energy. However, if the observations of McGeer and McGeer (188) are taken into consideration that GLNase is present only in GABAnergic nerve endings, under these conditions probably glutamine is converted to glutamate which would form a precursor pool in the synthesis of GABA resulting in an enhanced GABA formation. Since GABA is an inhibitory neurotransmitter, enhanced synthesis and release would depress the neuronal activity resulting in the comatose condition. Inhibition of the activity of GLNase during convulsions would depress the synthesis of glutamate serving as GABA precursor in the

GABA nergic nerve endings resulting in the disinhibition of the nervous system. Once the neurons are disinhibited , ammonia would stimulate the onset of convulsions by lowering the resting membrane potential (2). Though the involvement of GABA in ammonia toxicity was proposed repeatedly (11, 108, 240) conclusive evidences are lacking. It is worth mentioning at this juncture about the observations made by Polli (218) on a small but significant increase in the GABA content of cerebrophinal fluid in ammonia toxicity.

In conclusion, at least in acute ammonia toxicity it appears that the large compartment encompassing the neuronal perikarya would enter into a reactive phase and participates very actively in the ammonia detoxification while the small compartment represented by glia (and synaptosomes ? (Hertz, 1979) would play a passive role. The present observations also suggested at least a transitory deviation in the ammonia-glutamate-glutamine metabolism from the classical concept of metabolic compartmentation.

ATPases:

The perturbance in the ionic gradients by ammonium ion was repeatedly stressed (2, 17) as one of the mechanisms involved in its toxicity. The maintenance of ionic gradients in the nervous system is a complex phenomenon mediated not only by the ionic channels but also by ion pumps. Na+, K^+-ATPase

which is supposed to act as sodium pump, is an enzyme actively involved in the maintenance of ionic gradients (262) across neuronal membranes. As mentioned earlier this enzyme which exchanges Na^+ and K^+ in nonstoichiometric proportions, is a major consumer of cerebral energy reserves (1/3 of the energy reserve) (5). Hence, any change in the activity of this enzyme would eventually be reflected in the energy reserves. Further, the activity of this enzyme especially in nerve terminals is believed to regulate the release of neurotransmitters (190, 299). Because of its involvement in these three processes (ionic gradients, energy reserves and neurotransmitter release) much attention was devoted in the past in this laboratory on the changes in the activity of this enzyme during hyperammonemic conditions. It was observed that the activity of this enzyme in whole brain homogenates increased quite significantly in both acute and chronic hyperammonemia (235, 236) and MSI induced hyperammonemia (265-267). In fact these observations prompted us to propose that this enzyme would be responsible for the transport of NH4+ into the cells at least during hyperammonemic conditions. However, the use of brain homogenates yielded no clue as to the cellular localization of this change in the activity of Nat K^+-ATPase. In the present study an attempt was made in this direction. Since the estimation of Mg^{++}-ATPase is obligatory in the determination of Na+, K^+-ATPase, the changes in the activity of this enzyme

were also reported. As the Mg^{++} ions are not directly involved in the maintenance or generation of electrical activity of the neurons, the role of this enzyme is yet to be understood. Probably Mg++ -ATPase is a nonspecific enzyme, as many ATP dependent reactions require the presence of Mg^{++} ions (265). Hence, in the ensuing discussion, the changes in the activity of this enzyme would be considered in the light of such an assumption.

The activity of Mg++ -ATPase was stimulated both in comatose and convulsive states in the cellular and subcellular fractions of rat brain following the administration of ammonium acetate (Fig.42-44). This change was highly significant in the neurons and glia but not in the synaptosomes. Further, the magnitude of stimulation also followed the same pattern as the level of significance. The elevation was maximal in neurons when compared to glial cells and minimal in synaptosomes. Unlike Mg++ ATPase, which showed a uniform pattern of change in acute hyperammonemia, the changes in the activity of Nat K^{+}-ATPase varied from cell to cell and also with the neurological state. It was elevated in the neurons both in comatose and convulsive states. However, in glial cells the activity of this enzyme increased in convulsive state and was suppressed in comatose condition. In synaptosomes, this profile was reversed i.e., it increased in comatose and decreased during convulsions.

Fig. 42 Effect of ammonium acetate on neuronal Mg^{++} ATPase

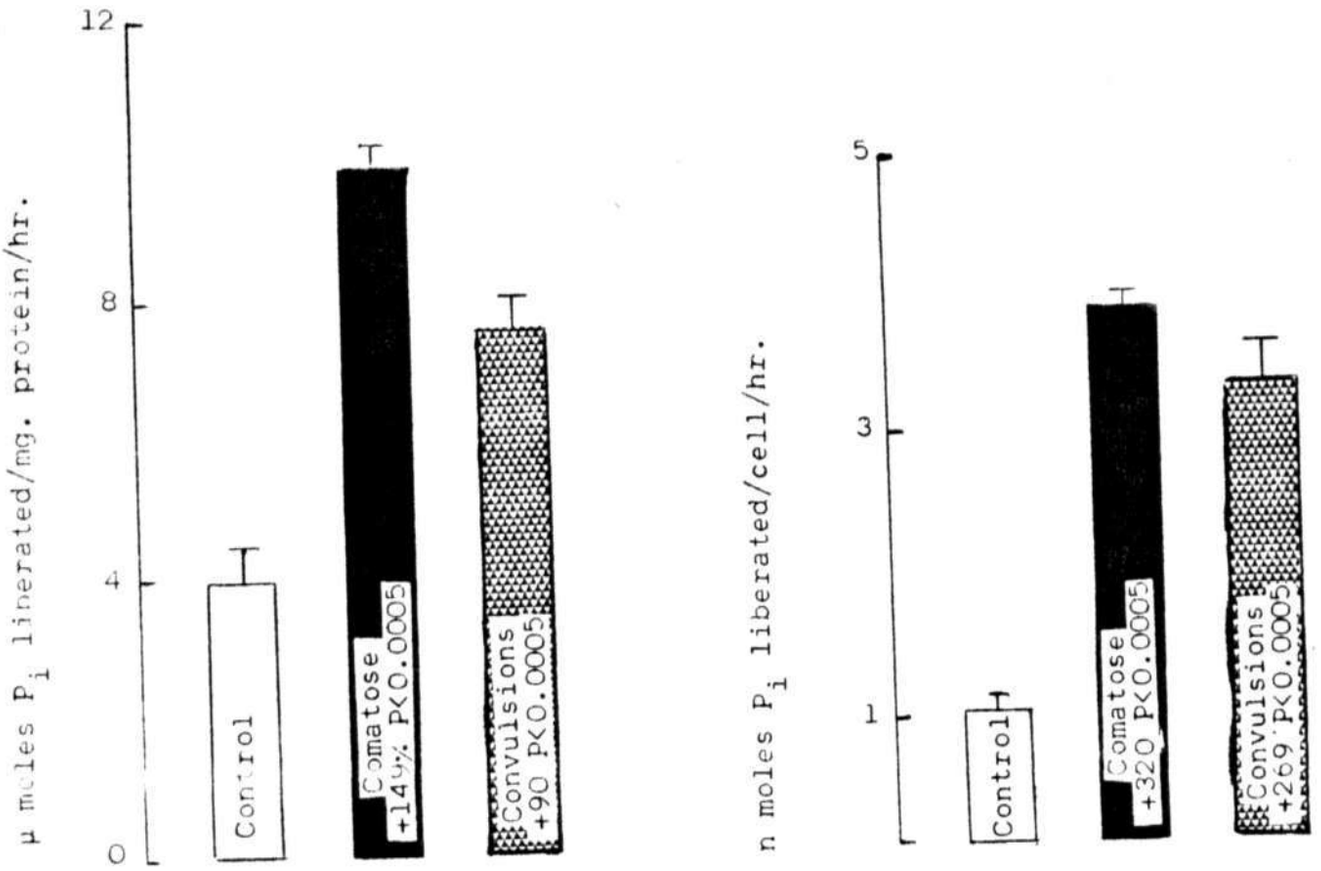
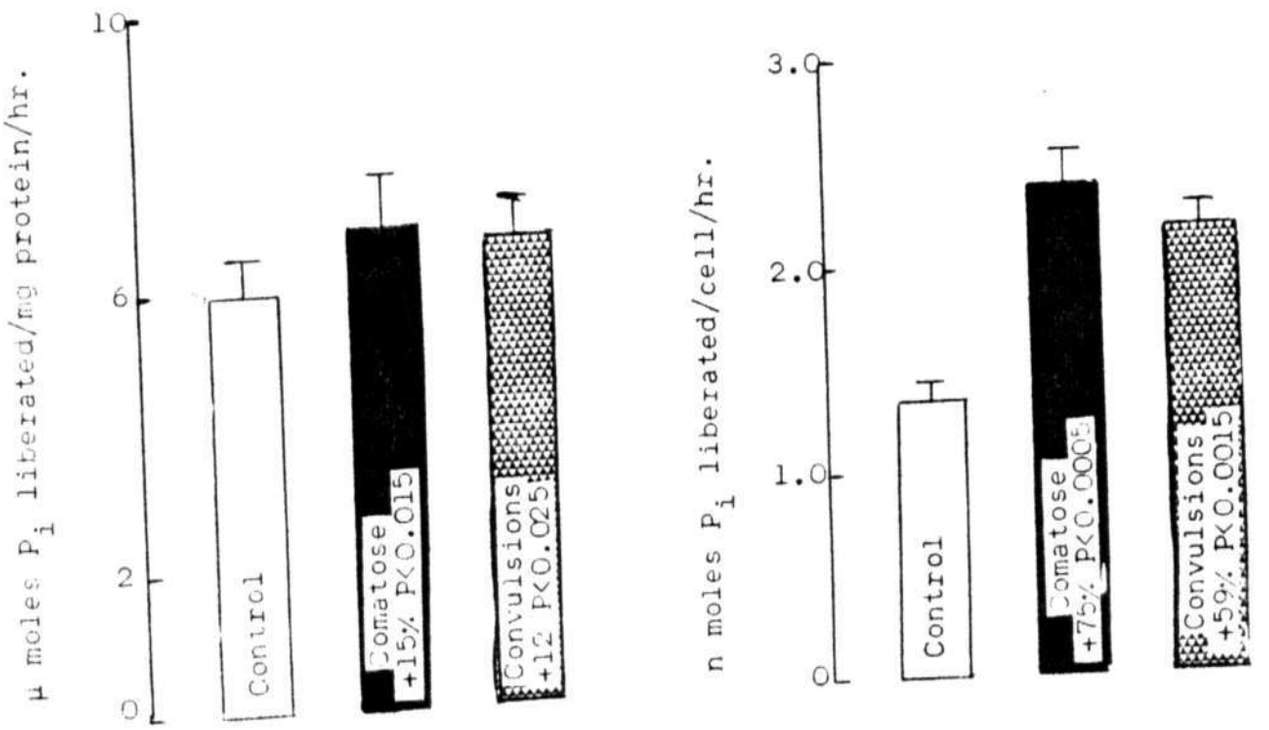


Fig. 43 Effect of ammonium acetate on astroglial Mg^{++} ATPase



A generalized increase in Mg^{++}-ATPase activity in acute hyperammonemia suggested an increase in the nonspecific ATP consuming reactions. Most likely these could be the initial reactions of glycolysis and the GS reaction since aerobic glycolysis and glutamine formation are known to be stimulated by ammonium ion (22, 286).

The increase in the activity of Nat K^{+}-ATPase in the neurons (including synaptosomes) under comatose conditions (Fig.45 and 47) would enhance the transport of NH4+ into these cells due to the similarities between the sizes of hydrated K^{+} ion and the NH4+ ions. The incoming NHt would be exchanged for the K+ resulting in an eflux of the latter. Such an exchange of NH4+ and K+ was reported to be in a ratio of 1:1 for rat brain cortical slices (287). Since NH4+ resemble K^{+} ions it may be assumed that large accumulation of these ions inside the neuron results in hyperpolarization. The stimulation of aerobic glycolysis by these ions leading to increased lactate formation, would decrease the pH and enhance the permeability of the membrane to chloride ions resulting in a large influx as suggested by Benjamin et al. (17). The enhanced concentrations of both NH4+ and Cl- inside the neuron would then synergestically act to hyperpolarize the neurons which might be responsible for the comatose state. The suppressed activity of this enzyme in the glial cell in this state (Fig.46) might enhance the availability

Fig. 44 Effect of ammonium acetate on synaptosomal Mg^{++} ATPase

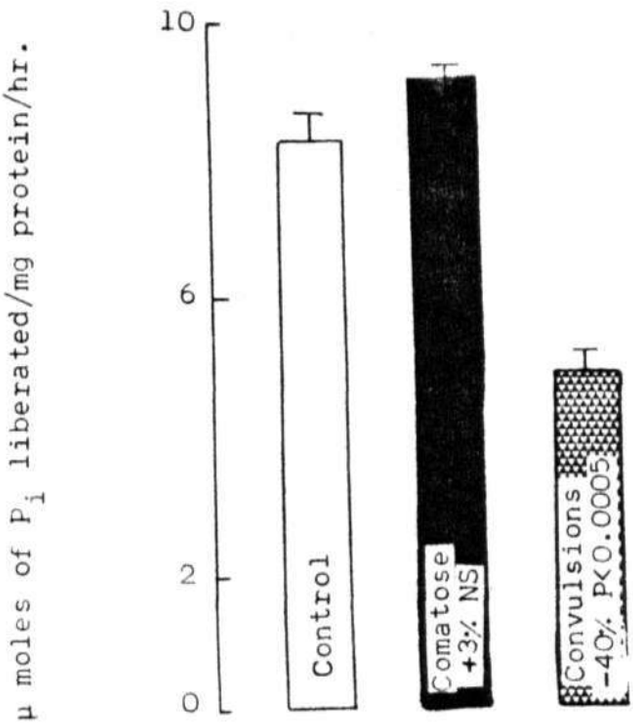
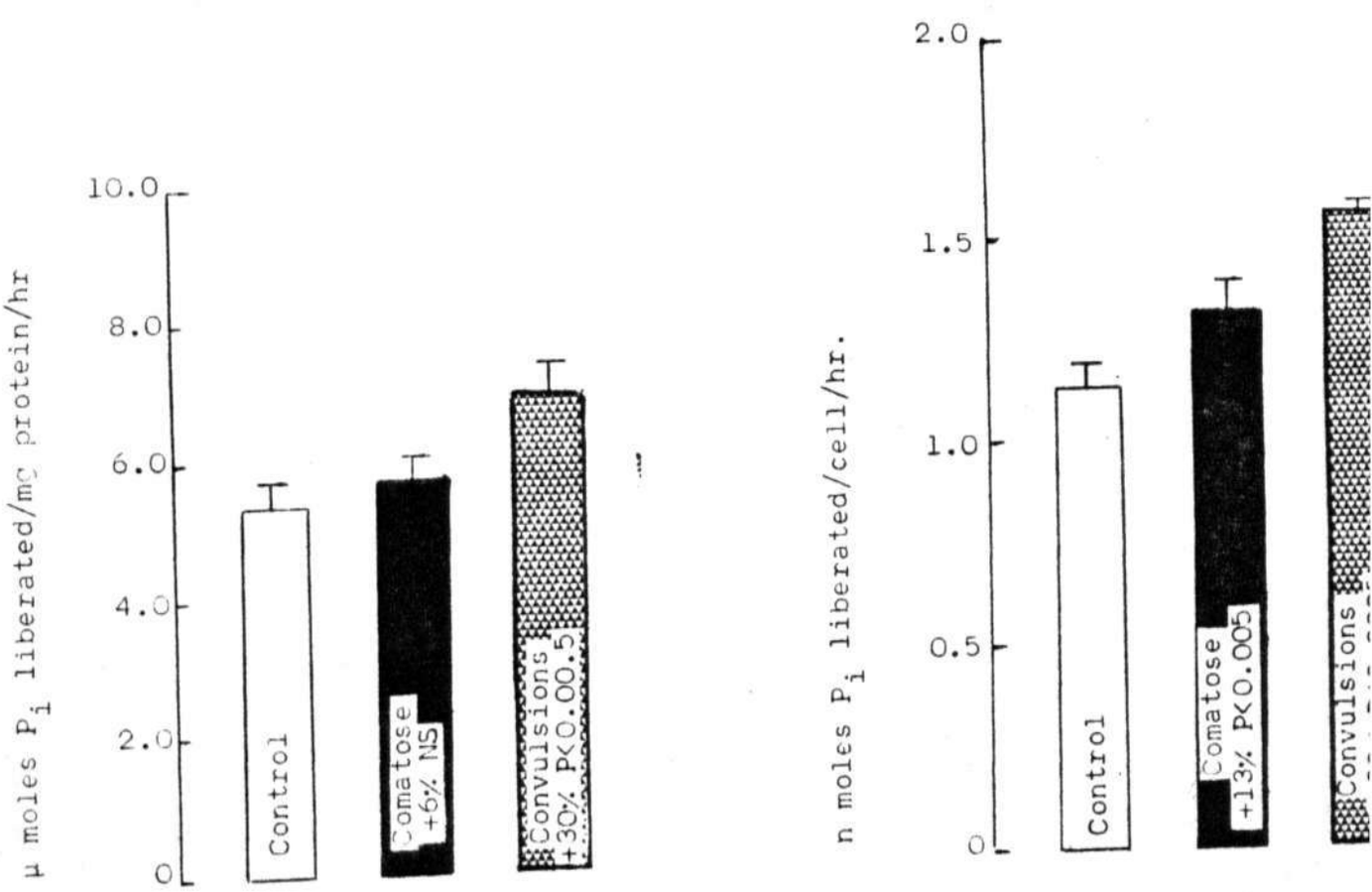


Fig. 45 Effect of ammonium acetate on neuronal Na^+, K^+-ATPase



of NH_4^+ for the neurons. The state of hyperpolarization may further be aggrevated by the extraneuronal accumulation of K^+ ions. Though extracellular K^+ is known to depolarize the neuron, prolonged depolarization would eventually lead to hyperpolarization. The glial, Na+, K^+-ATPase, which is known to be stimulated by K^+ ions would be ineffective in removing the extracellular K^+, because of the suppressed activity.

During convulsions, the activity of this enzyme was restored to the normal level in synaptosomes and was elevated in the glial cells (Fig.46 and 47). Under the same condition it is interesting to observe that there was a 50% fall in brain ammonia levels when compared to comatose state(Fig.l6). The increased activity of this enzyme in the glial cells during convulsions would not only decrease the accumulated extracellular K^+ but also allows NH_4^+ to be transported into these cells. At this stage probably, the second phase of Cl^- influx into cerebral tissues might be assumed to occur. As the second phase of Cl^- influx was supposed to be into glial cells along with the fluid uptake, the glial cells would continue to swell under these conditions, which was evident from the present studies on the water content of these cells (Fig. 23). This influx of Cl^- would deplete the extraneuronal chloride ions. Such a depletion might be associated with the reduction in accumulated K^+ ions and

Fig. 46 Effect of ammonium acetate on astroglial Na^+, K^+-ATPase

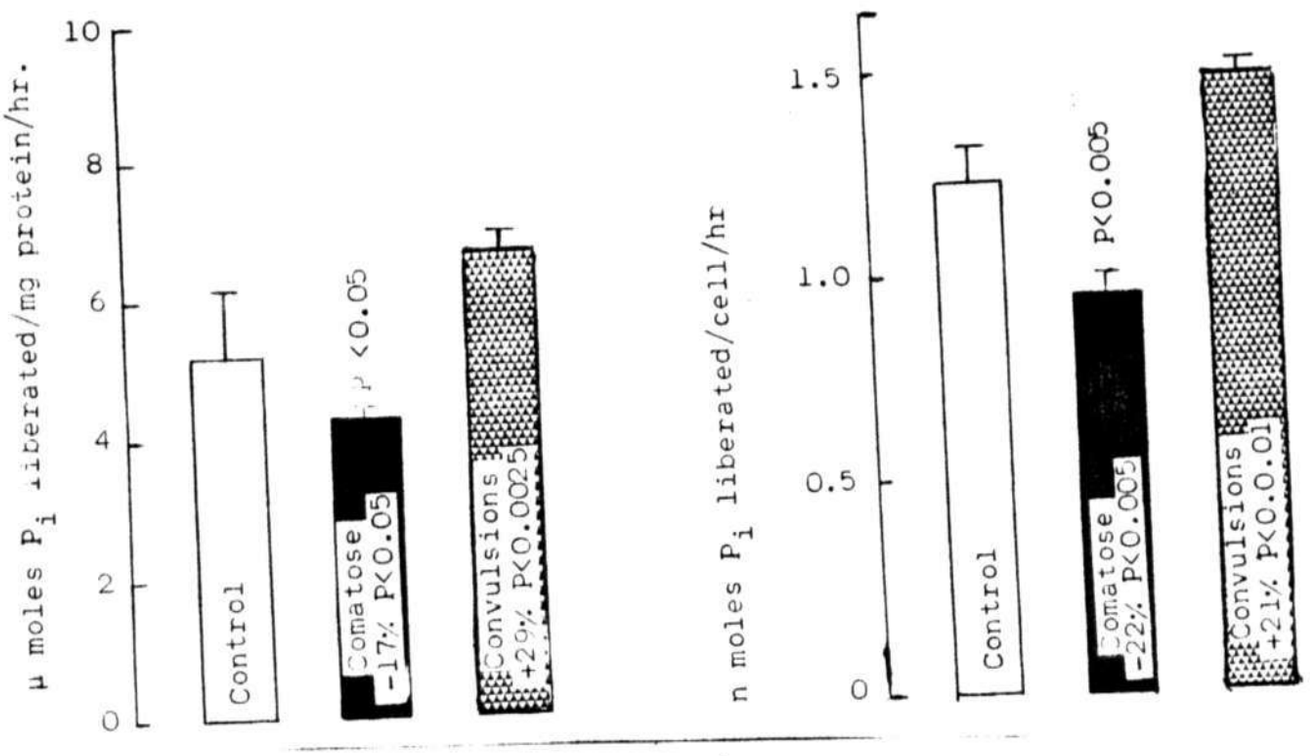
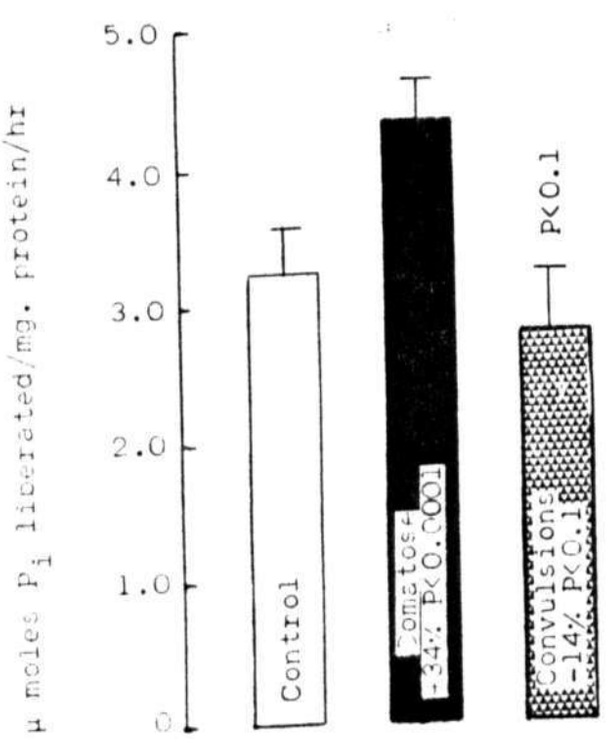


Fig. 47 Effect of ammonium acetate in synaptosomal Na^+, K^+-ATPase.



convert the hyperpolarized state to a depolarized state. This condition was clearly demonstrated by Benjamin et al., (17). Such a sudden release in the hyperpolarization may probably result in the convulsive activity. In other words, in the initial stages of ammonia toxicity NH_4^+ would effect the nerve endings and the glial cells at a later stage. This could be clearly seen in the changes observed in the activities of the enzymes of glutamate metabolism. In synaptosomes, the changes in the activity of AAT, GDH, GS and GLNase as well as Mg^{++}-ATPase were minimal during convulsions when compared to comatose state whereas in the glial cells these changes were either aggrevated or unaltered when compared to the comatose condition.

In conclusion, the comatose condition might be due to the enhanced GABA synthesis and due to changes in the ionic gradients as discussed above. The convulsions observed in the terminal stages of acute ammonia toxicity might be due to disinhibition of the nervous system by affecting the synthesis of GABA (through the reduction in the glutamate pools originating from glutamine) in conjunction with the reduction in the extraneuronal chloride ions and increased glial ATPase activity. Finally, it may be emphasized that the neuronal perikarya representing the large compartment are involved to a major extent in ammonia detoxification than was predicted earlier and are also involved in the supply of glutamate for the synthesis of glutamine in the glial cells

CHAPTER V

MSI Toxicity

Introduction:

Historical background:

It was observed that dogs underwent canine hysteria or running fits after the ingestion of wheat flour treated with agene ($NC1_3$) (261). Gastaunt et al., later observed that feeding dogs with agenized proteins resulted in epilepsy and suspected that some toxic principle might be responsible for the convulsions (102). Proler and Kellaway identified the toxic principle of the agenized protein diet as methionine sulfoximine (MSI) (220). Because of similarity of the convulsions to those of human epilepsy, this agent was the object of numerous physiological and neurochemical studies. The same authors found that MSI produces a complex syndrome with episodic behavioural changes consisting of turning of head, sniffing and crouching movements in cats.

Hathcote and Pace observed that MSI either abolishes or retards the growth of the microbial culture, Leuconostoc mesenteroides, and only glutamine could alleviate this effect (118). This observation led to the conclusion that MSI may block the biosynthesis of glutamine. Pace and McDermot extrapolated these studies to animal tissues (211). They studied the effect of MSI on some enzyme systems of glutamine metabolism and found that GS was the enzyme inhibited

by MSI. Peter and Tower observed a marked inhibition of GS in brain slices of MSI treated cats and a relationship was postulated between the amidation of glutamate in brain and seizures produced either with MSI or acoustic epileptogenic stimulation (216).

Sellinger and Weiler reported that MSI competetively inhibited GS in vitro (255). They have studied the nature of inhibition of GS in cat cerebral cortex with MSI and postulated that MSI causes depletion of ATP in a intracellular compartment within which the synthesis of glutamine occurs. Lamar and Sellinger reported that in in vivo this drug inhibited GS irreversibly (159, 160).

Molecular mechanism of MSI toxicity:

The studies of Pace and McDermot suggested that MSI inhibits glutamine formation by virtue of its interaction with GS (211). It was postulated that the inhibition of GS by MSI results in an elevation of brain ammonia levels, a condition known to be toxic to brain (216). Warren and Schenker studied the effect of MSI on the toxicity of administered ammonium salts (311). They noted that a four hour premedication with MSI prevented deaths in mice by a lethal dose of ammonium chloride given intraperitonially. These studies indicated that ammonia intoxication might not be

dependent upon the mere presence of high cerebral ammonia levels per se, but might be due to the mechanism of detoxification.

Folbergrova et al., observed an increase in free glutamate levels in the rat brain after MSI administration (93). They found that neurotoxic effects were apparent after subthreshold doses of MSI or under conditions when MSI was administered simultaneously with paraxysmal MSI doses thus preventing the onset of paraxysmal state. It was concluded that although MSI markedly affects the synthesis of free glutamine in the brain, impairment of this metabolic link might not be responsible for the onset of seizures. Furthermore, views on the mechanism of MSI toxicity, especially the production of seizures have been inconclusive (45, 103, 109).

Later Ronzio *et* al., studied the mechanism of inhibition of GS by MSI (229). They found that the inhibition by MSI required ATP and Mg^{++} and was associated with the cleavage of ATP to Pi and ADP. The inhibited enzyme was separated by gel filteration and was shown to contain MSI-$P0_4$ which was tightly bound to the enzyme (22*). The irreversible inhibition of GS is associated with the phosphorylation of MSI and tight binding of MSI-$P0_4$ to the enzyme (158).

Rowe and melster studied the effects of all the isomers of MSI and found that only L-Methionine-DL-sulfoximine was

the convulsant isomer (232). They have also achieved the chemical synthesis of MSI-$P0_4$ and found that L-MSI-$P0_4$ inhibited the enzyme much more effectively in the presence of Mg^{++} and ADP (46).

Behavioural changes:

When dogs were treated with white bread bleached with agene, they entered into a state called canine hysteria or running fits (261). Proler and Meyer observed in cats a complex syndrome consisting of episodic behavioural changes like head turning, sniffing and crouching movements, fear and running fits at the onset of which the animal might appear hystrical and enter into generalized tonic-clonic convulsions (221).

EEG studies of animals subjected to MSI intoxication revealed that crouching and head turning episodes were accompanied by a diffused pattern of EEG, with a high frequency during running attacks (221). Generalized tonic-clonic convulsions were almost identical with those seen in human beings (221). The tonic phase was characterized by high frequency, high voltage discharges in all regions, while clonic phase was characterized by bilaterally symmetrical bursts of spike and polyspike discharges which occurred synchronously with clonic jerks.

In their studies on ultrastructural changes in the rat brain following MSI treatment (15 mg/lOO gm. body weight), Gutierrez and Norenberg recorded the behavioural changes (111). A progressive decrease in physical activity observed and rats developed a substantially wobbly gait and splayed leggedness two hours after MSI administration. After 6 hours they lost the equilibrium and a series of involuntary movements consisting of extension and tortion of the head and choreiform movements were noticed occasionally. This stupurous but arousable state was followed by generalized tonic and clonic convulsions. Seizures were preceded by hyperactivity, facial scraching and twitching and clonic movements of the head to upper extremities. Wada et al., have observed that MSI causes transient reduction of audiogenic susceptibility in genetically sensitive rats (302, 303).

Ultrastructural studies:

Several anatomical studies in brain following the administration of MSI were performed to determine the site of action of MSI. De Robertis and Sellinger made both ultrastructural and neurochemical studies in the nerve endings of MSI convulsant rats (79). Accumulation of MSI in nerve ending fraction associated with striking ultrastructural changes were observed. Swelling of the nerve endings and loss of synaptic vesicles was noticed. The changes were more wide spread in non-cholinergic population

of nerve endings and it was postulated that MSI acts on nerve endings related to the glutamate-glutamine-GABA metabolism.

Later, Lodin et al., analyzed the effect of MSI on cell dimensions and cell dry mass in the nervous system (172, 173). An important ultrastructural study of Rizzuto and Gonatus demonstrated liquifactive necrosis of cerebral cortex, degenerative changes in neuron, swelling of glia and nerve endings and a spongy state of neuropile (227). Gutierrez and Norenberg performed a histological study of the brain after MSI administration, prior to the onset of seizures (111). The fundamental structural alteration in brain was the development of Alzheimer type II astrocytosis. Light microscopy revealed that astrocytic perikarya and nuclei were slightly enlarged in the MSI treated animals. The ultrastructural investigation showed that the cytoplasm was enlarged in *10%* of the astrocytes. On an average these enlarged cells were twice the size of normal cells. The astroglial processes were broadened and the perivascular end-feet were enlarged.

There was a three fold increase in the number of mitochondria which also became more oblong and pleomorphic. Rough endoplasmic reticulum proliferated with a seven fold increase in the number of oblong profiles. A pronounced accumulation of glycogen was observed in the smooth endoplasmic

reticulum. Increase in the number of mitochondria was attributed to increased intracellular ammonia levels which stimulated the detoxification process involving the mitochondrial GDH, the activity of which increased under these conditions. Alzheimer type II astrocytosis as mentioned earlier, was also observed in portocaval shunting, toxic liver damage and in other hyperammonemic conditions and this change seems to implicate ammonia directly in the etiology of ammonia toxicity (53, 162, 203-205, 241, 327).

Stimulation of the enzyme fructose-1,6-biphosphatase was observed in the cerebral cortex of rats submitted to MSI and this stimulation was shown to be due to increased synthesis of the enzyme (131). It was demonstrated that MSI modifie t-RNA pools of rat brain (78,238). N^2-methyl and N^2-dimethyl guanine t-RNA Methyl transferase activity was shown to be stimulated (238). It was also shown that MSI modified the $t\text{-}RNA^{Lys}$ and $t\text{-}RNA^{Phe}$ pools of developing rat brain (252).

In spite of all these studies, not much attention was focussed on the biochemical changes in MSI toxicity with particular reference to the enzymes of glutamate metabolism. Since the behavioural changes were observed to be close to human epilepsy and the glial changes (Alzheimer type II astrocytosis) was akin to that observed in hyperammonemic states (particularly hepatic encephalopathy), it would serve as a best experimental model for hyperammonemia. In the present investigation an attempt was made to fill the lacunae in the available knowledge on the effect of MSI on glutamate metaboli

The modifications and its validity was described earlier (C.f. ammonia toxicity page No. 89).

Cell number-: Following the administration of MSI, the neuronal yield was reduced by about 40/, while that of astrocytes increased to a small but significant extent (Fig. 48 and 49). It is interesting to note that the fall in the yield of neurons was very close to the decrease observed in acute ammonia toxicity (-50/). It appears that hyperarrmonemic conditions would inevitably result in the loss of at least some neurons. As mentioned earlier in detail, this loss could be during the isolation procedure. Since, many of the earlier reports indicated no change either in neuronal morphology or in its ultrastructure during hyperammonemic states (117) the other possibility could be the artifacts induced during the tissue preparation for histological studies (fixation, dehydration, etc.) which might have masked the changes especially if their magnitude were to be small.

The increase in the number of astrocytes was on par with the earlier reports on the effects of MSI (111). The major glial reaction in response to MSI is the proliferation of astrocytes. Gutteirrez and Norenberg reported a 43% increase in the astrocytic nuclei with a lesser dose of MSI at the end of 7 1/2 hrs (111). However, in the present investigation such an enormous increase in the yield of

Fig. 48 Effect of methionine sulfoximine on the neuronal cell number

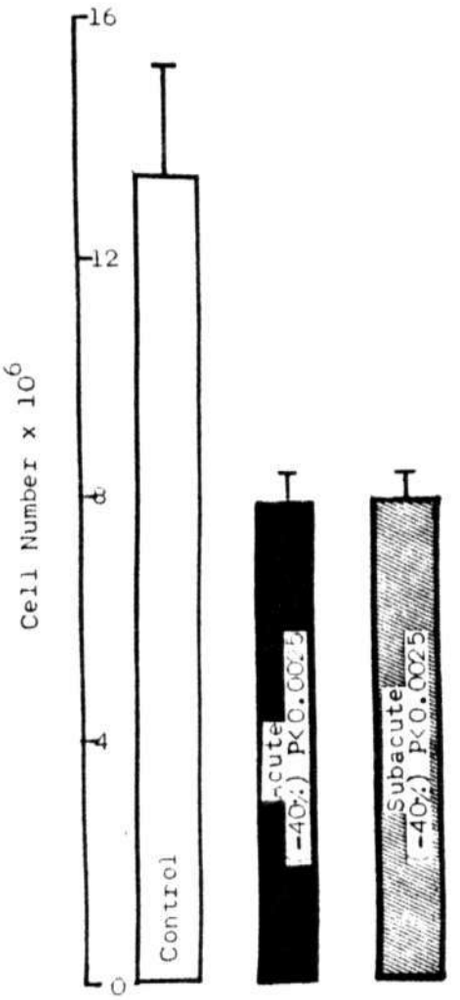
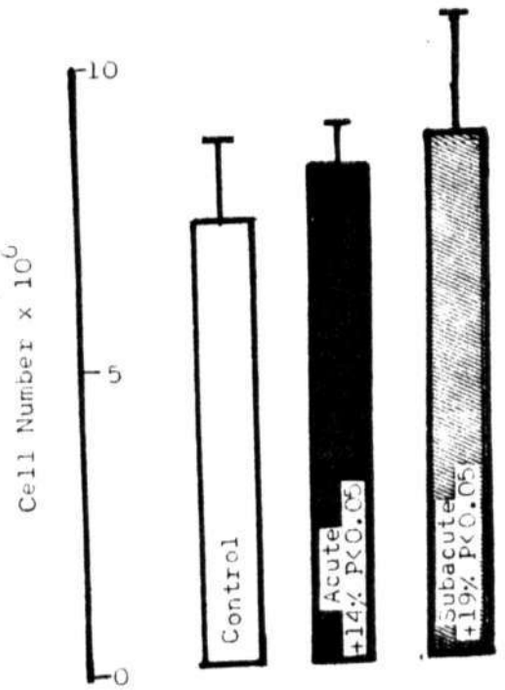


Fig. 49 Effect of methionine sulfoximine on glial cell number



astrocytes was not observed both in acute and subacute conditions. Probably, some of the astrocytes which were in degenerative phase failed to survive the isolation procedure.

Cell weights and water content;

The pattern of chahges in the wet weight of neurons and glial cells was observed to vary with the dosage of the drug administered. The wet weight of the neurons increased above the control value in acute and subacute conditions (Fig.50). The magnitude of change was at least twice as high in acute state than in the subacute state. The changes observed in glial cells were greater in magnitude than that of the neurons. In acute conditions, the astrocytic change was about 7 fold higher than the neuronal change and in subacute condition it increased to 30 fold. Further, the astrocytic change in subacute state was found to be at least 1.5 times higher than the acute stage (Fig.52).

The changes in the dry weights of both the neurons and glial cells were totally different from the changes in the wet weight (Fig.50 and 51). The increase in the dry weight of neurons was more or less same in both acute and subacute states. The increase in the water content of astrocytes in subacute stage was far greater than the acute stage. The difference between neurons and astrocytes in this parameter was about 3 to 4 fold in acute state and was

Fig. 50 Effect of methionine sulfoximine on neuronal weight

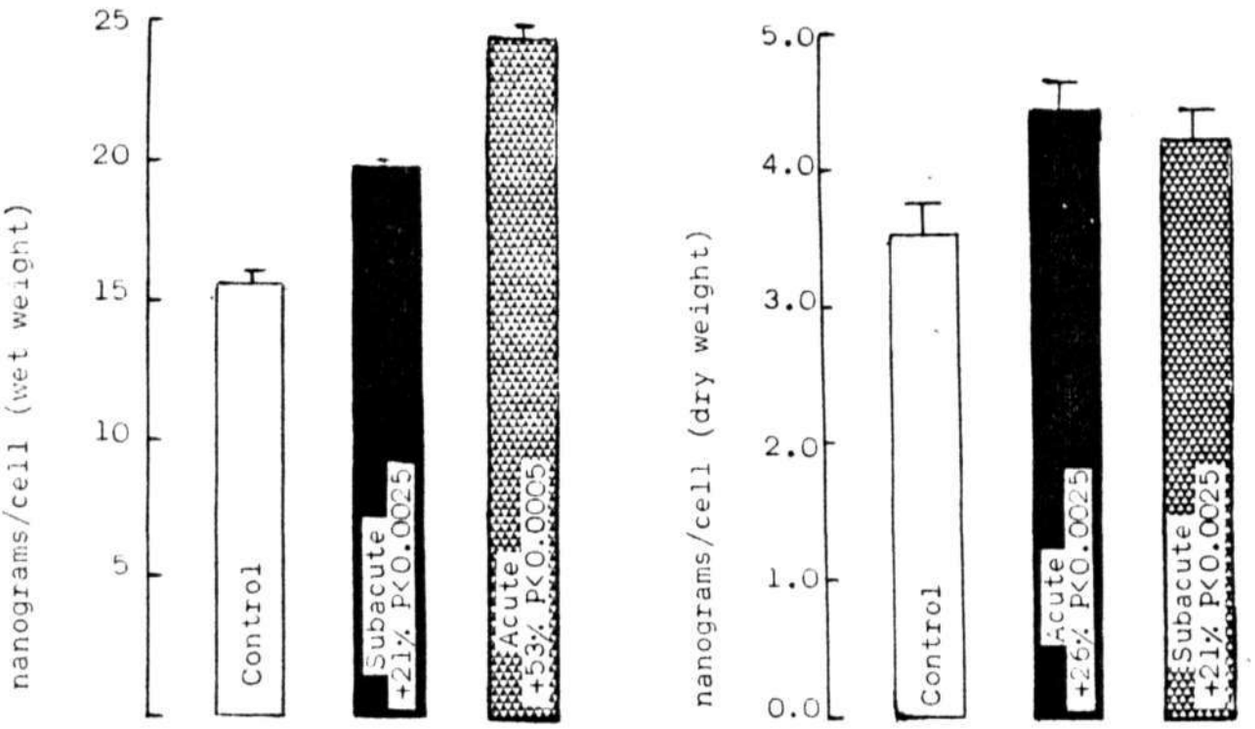
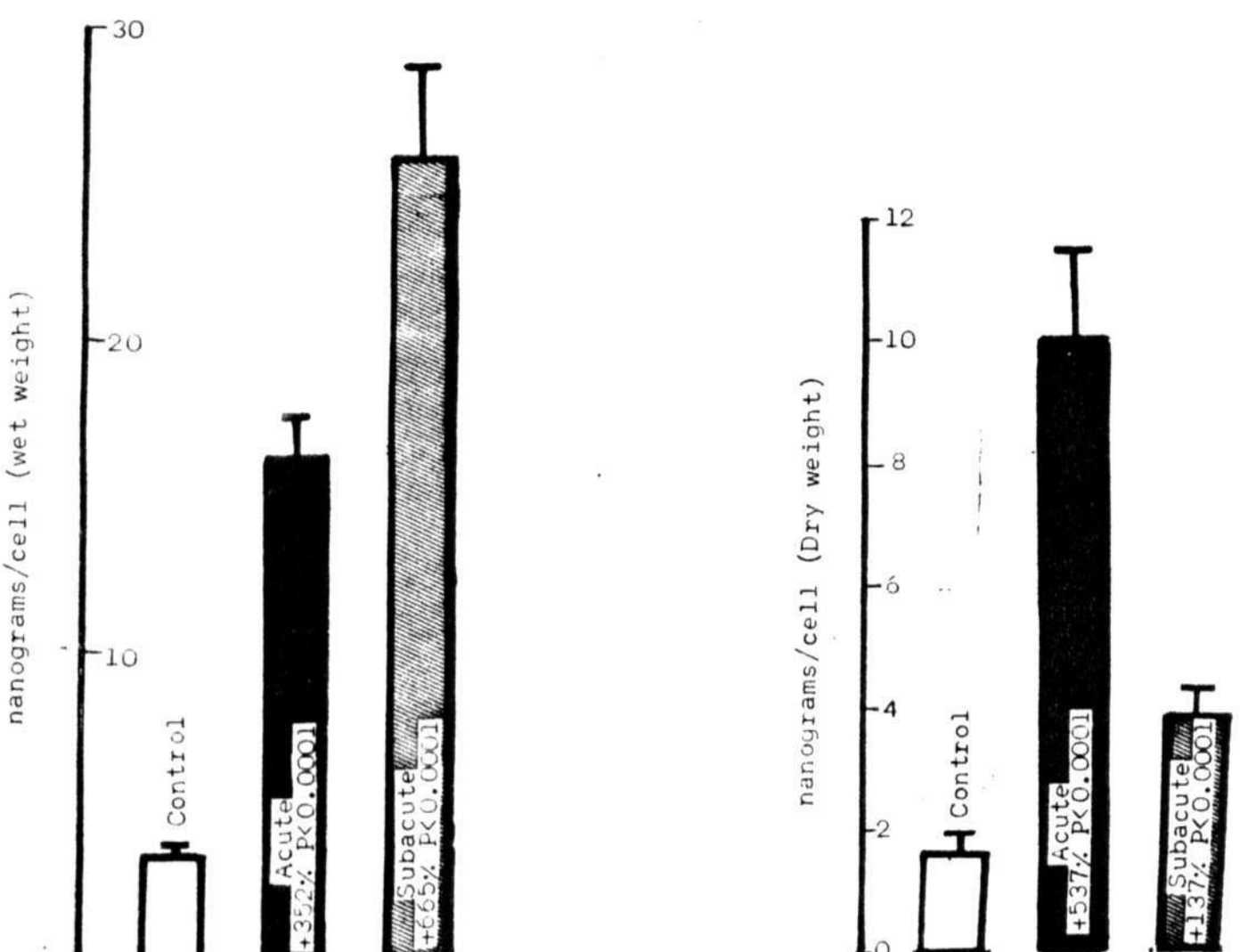


Fig. 51 Effect of methionine sulfoximine on glial cell weight



about 50 fold in the subacute state. In conclusion, the astrocytic response in these parameters was far greater than that of neurons after the administration MSI.

The increase in wet weight as discussed earlier would suggest an increase in both the particulate and soluble material, while a change in dry weight would reflect the changes in the particulate material. The increase in dry weight in the astrocytes under these conditions would represent the synthesis and accumulation of particulate material. This observation concurred with the existing reports of the increase in the mitochondrial profiles and smooth and rough endoplasmic reticulums and an increase in the macromolecules such as glycogen in astrocytes following MSI administration In addition to glycogen, other macromolecules such as protein and nucleic acids might also be expected to increase under these conditions as they are directly or indirectly involved in the process of cell proliferation. The changes in the neurons in this parameter though small, yet indicated the reactivity of these cells either to the drug or to the drug induced metabolic changes.

The increase in the water content was found to be (Fig.53) remarkable in the astrocytes especially in subacute (Fig.53). This observation once again was in accord with

the already reported changes in the astrocytes due to **MSI.** Harris (117) observed a dialation in glial cytoplasm in cm electron microscopic study in cerebral cortex following MSI administration. A similar change was also reported during hepatic encephalopathy (205). In the astrocyte the relative contribution of particulate and soluble materials to the wet weight in acute and subacute stages showed an interesting trend. In the acute stage as much of 2/3 of the wet weight was due to an increase in the particulate material, while in the subacute state it was less than 13% In other words, quite high during acute condition which declined in subacute state, most likely due to an augmentation of degradative processes and degenerative changes in the cell(equivalent to liquidative necrosis). In neurons the contribution made by dry matter was more or less constant in both the states. The changes in the wet weight were solely due to the increase in cell water content. Though the neurons accumulated more water during acute state, they lost this water in subacute state (Fig.52). The changes in water content would reflect in the changes of the cell size due to swelling. Accumulation of water both in neurons and glia would alter the buoyant density, which might be the reason for the differences in the sedimentation properties of these cells during isolation. The changes in the dry matter and the suggestions made therein would further be substantiated by subsequent results on macromolecular composition of the cells.

Fig. 52 Effect of methionine sulfoximine on intracellular water content of neuronal perikaryon

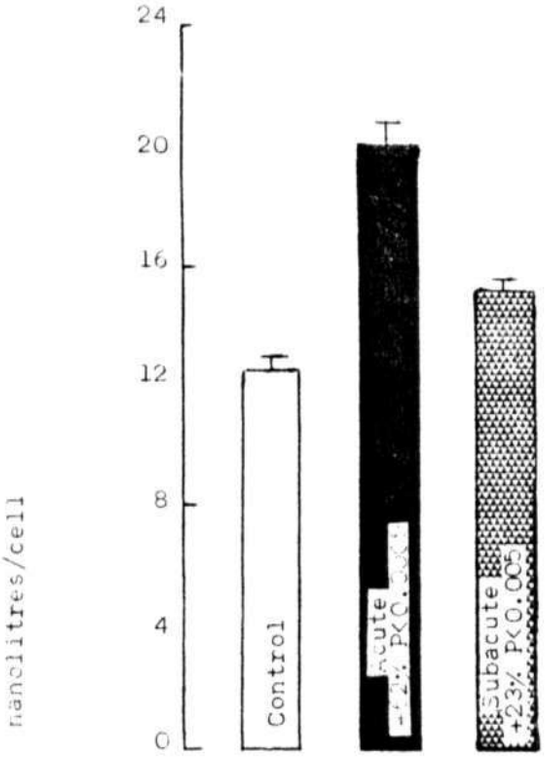
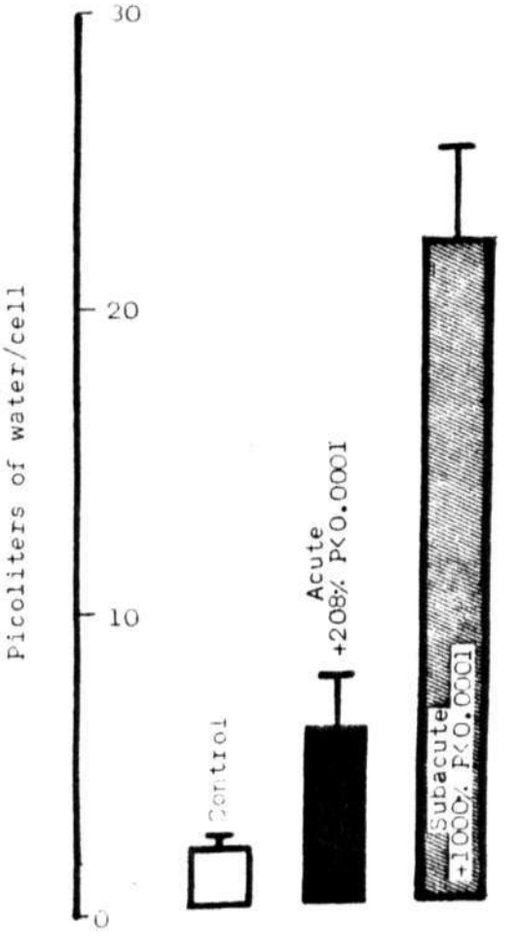


Fig. 53 Effect of methionine sulfoximine on intra cellular water content of astroglia



DNA, RNA and protein:

DNA: Following the administration of MSI, significant changes were observed in the macromolecular composition of the cells. The DNA content increased to a small extent in the neuronal perikarya both in acute and subacute stages (Fig.54). However, the increase in the astrocytes was far greater in magnitude than the neurons, the difference being 3-8 fold. The astrocytic DNA content was four fold greater in the subacute state than in the acute condition (Fig.55).

The increase in the DNA content in a given tissue woula be an index of cell proliferation. Though it is well known that MSI induces cell proliferation in the brain (111), surprisingly, no efforts were made to study the mechanism of this process. The interesting feature of MSI induced glial proliferation is the absence of mitotic spindles suggesting that the process might be endomitotic in nature (162). Since the values represented in the present investigation were per cell values, the increased DNA content would then indicate either increase in the synthesis or a decrease in degradation. Most probably in the neurons the accumulated DNA which was very small might be due to repair synthesis while in the astrocytes where the magnitude of accumulation was very high, it could be a replicative synthesis. This increase in the DNA content, if considered in conjunction with the absence of mitotic spindles, would then render

the cells polyploid at least under the present condition. At present it is not known whether this synthesis of **DNA** is a replicative synthesis or repair synthesis. Irrespective of mechanism of stimulation of DNA synthesis in the astrocytes, it suggested the reactive nature of these cells to the presence of the drug, MSI.

The changes in the cellular RNA content were more or less similar to the profile of DNA, excepting that the difference in the magnitude of change between neurons and glial cells was only about 3-4 fold. The increase in the RNA content in the neuronal perikarya was observed to be more or less same in both the conditions (Fig.54). In contrast, the increase in glial cells was found to be greater in subacute state than in the acute state (Fig.55). As in the case of DNA, this increase in the RNA content would be due to increased synthesis or decreased degredation or increased stabilization of RNA. Post-transcriptional modification of at least one of the RNA species (tRNA) was reported (76, 78, 238) which might play a significant role in stabilization of newly formed RNA molecules. In the present investigation no attempts were made to resolve the relative contributions of the three species of RNA. If the pattern is to be same in all the cells, then it might be assumed that the contribution of rRNA to total cellular RNA would be greater than that of tRNA or mRNA. This would concur with the electron microscopic observations made by several investigators on the ultrastructure of astrocyte

Fig. 54 Effect of methionine suifoximine on **neuronal nucleic acid content**

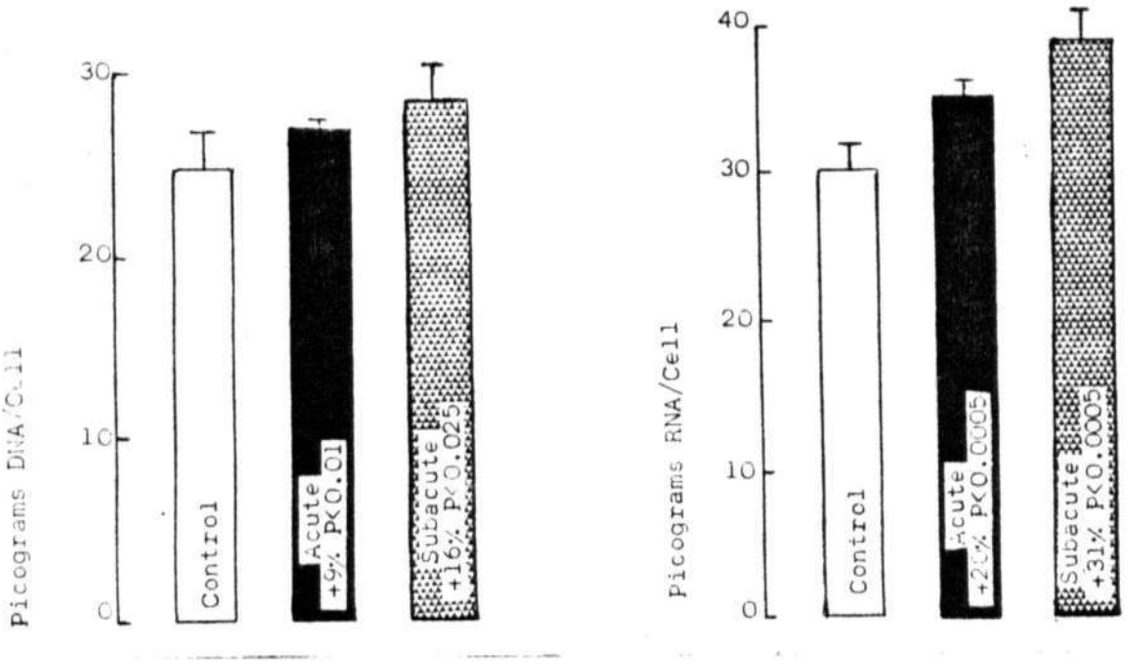
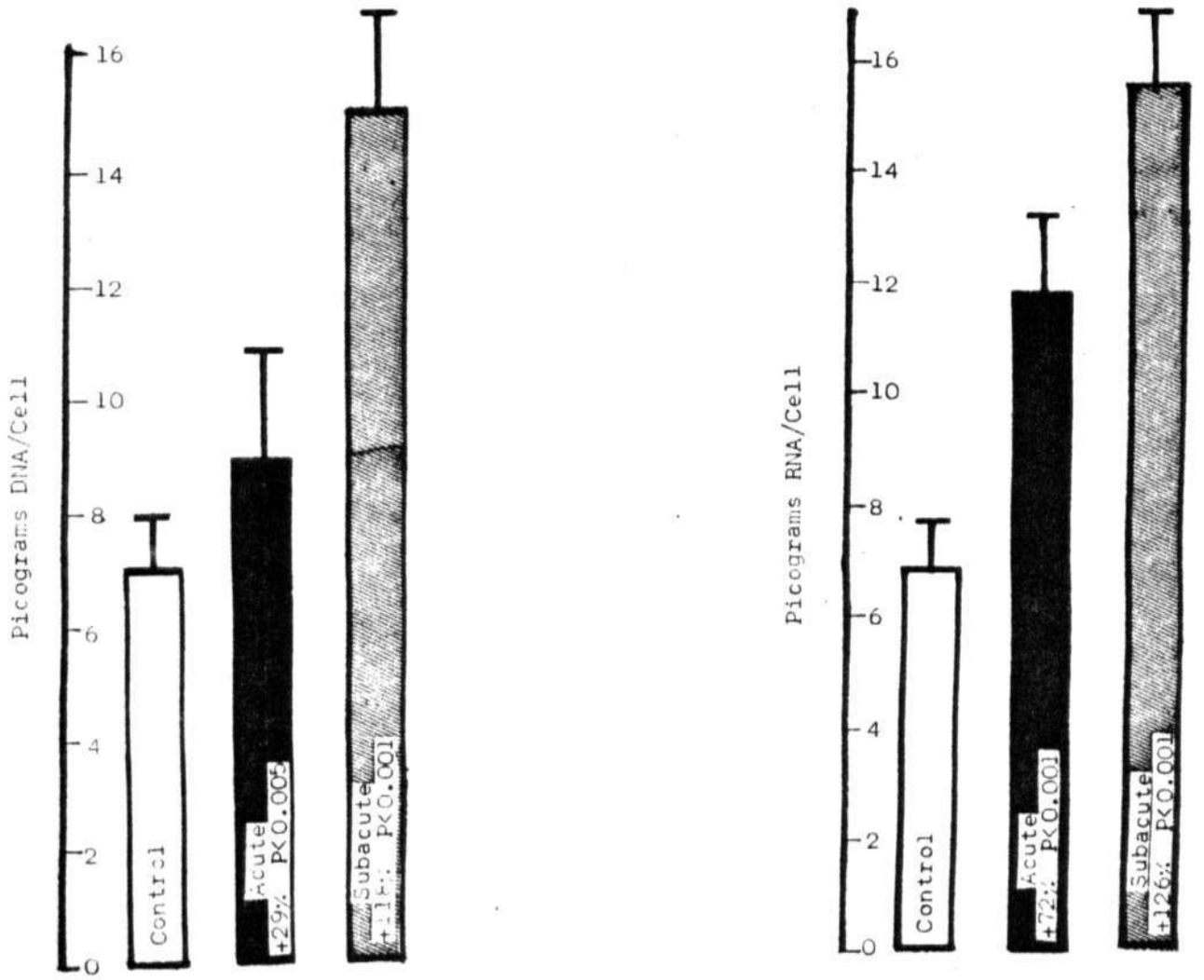


Fig. 55 Effect of methionine sulfoximine on astroglial nucleic acid content



in hyperammonemic state (106, 203-205, 327). The Alzheimer type II astrocytosis was associated with the proliferation of both smooth and rough endoplasmic reticula (111). As the ribosomes are constituents of rough endoplasmic reticulum,the observed increase in the RNA might be due to the changes in rRNA content. Other RNA species such as mRNA and tRNA might also be increased under these conditions. For the proliferation of the cells and organelles, protein synthesis is known to be a prerequisite. An increased incorporation of labelled uridine into RNA in hepatic encephal pathy was demonstrated, suggesting an increase in the synthesis of RNA in astroglial proliferation (3).

Parallel to the changes in DNA and RNA, protein content was found to increase both in neurons and astrocytes following administration of MSI (Fig.56 and 57). Unlike the changes in the other macromolecular components, wherein the magnitude of increase was greater in the subacute state than the acute state, the protein content both in astrocytes and neurons in the subacute state was less than that of acute condition. In the astrocytes isolated from the animals treated with acute dose of MSI, the increase in the protein content was almost twice to that of the neurons. However, in subacute state the percent increase was more or less same both in neurons and astrocytes. This suggested that the fall in the protein content in the subacute state was far greater in the astrocytes than the neurons.

Fig. 56 Effect of methionine suifoximine on neuronal protein content

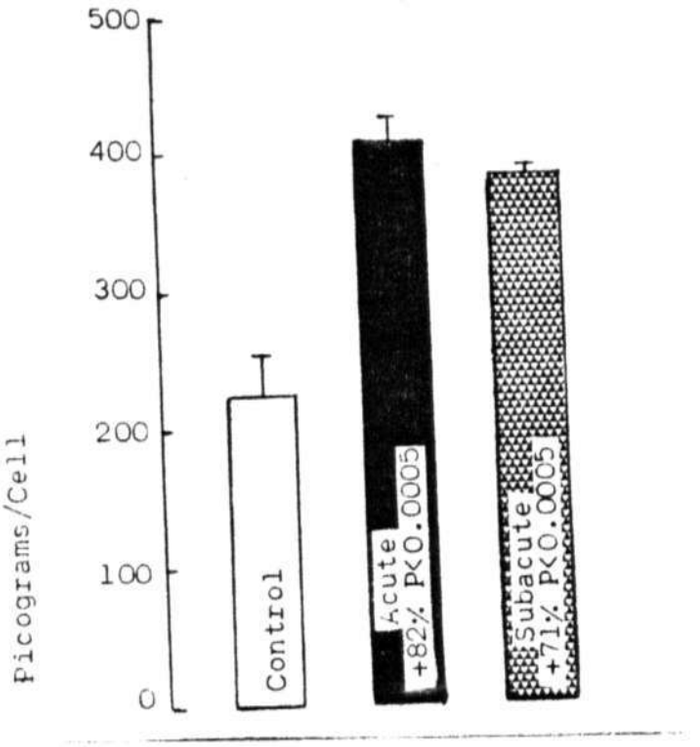
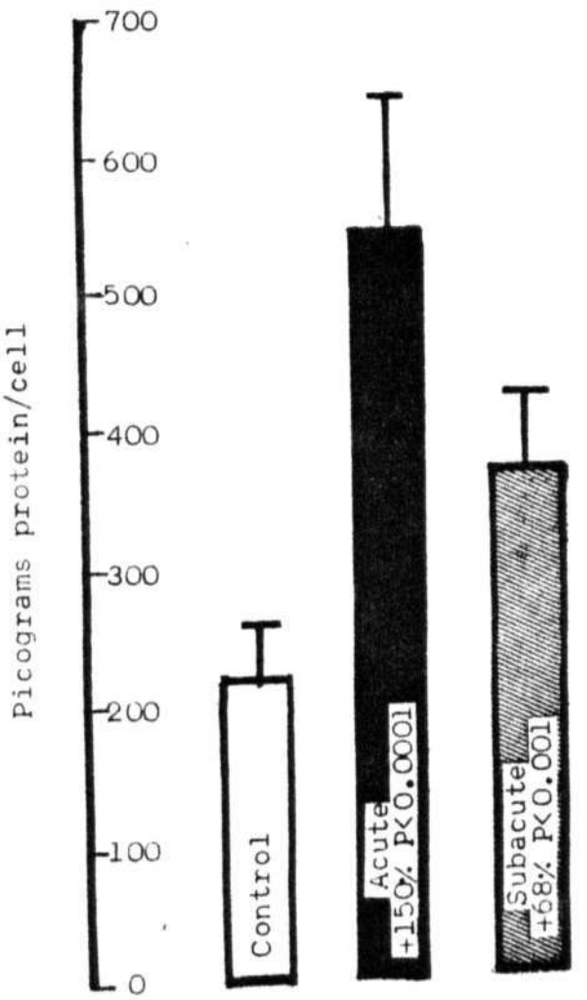


Fig. 57 Effect of methionine sulfoximine on glial protein content



As mentioned earlier, both for ultrastructural and biochemical changes observed in the reactive astrocytes, the protein synthesis is a prerequisite. The change in the protein content of the astrocytes observed in the present investigation would support the earlier observations on the reactive astrocytes under these conditions. It is interesting to note that the neuronal protein content increased despite of a decrease in cell number and a marginal elevation in the contents of DNA and RNA. Though, no ultrastructural changes were observed to indicate the reactive nature of neurons in MSI toxicity, the observed increase in the DNA, RNA and protein contents in the neuronal perikarya would indicate the occurrence of such a process at least to a small extent. Another interesting aspect of the changes of protein content observed in the present study was a lesser magnitude of accumulated protein in subacute state when compared to the cells in acute condition. In fact the increase DNA and RNA contents were far greater under these conditions and it is further believed that the changes responsible for the formation of Alzheimer type II astrocyte (proliferation of mitochondria, endoplasmic reticulum, etc.) would be maximal at this stage. In such conditions, a further increase in protein content(when compared to acute state) should have been observed but not a decline. Probably the machinery responsible for the degeneration of astrocytes was already in operation at this stage resulting an increased

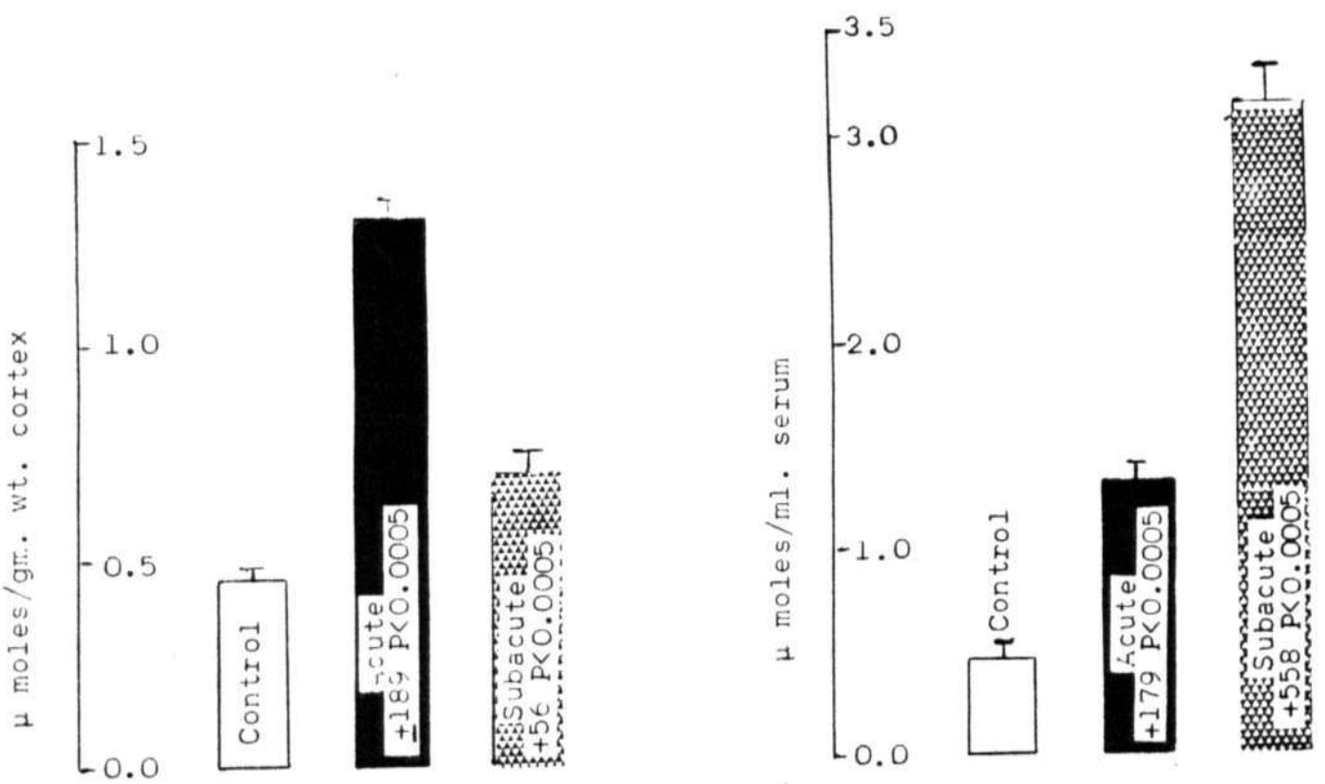
degradation and turnover of cellular proteins. However, unless further studies are made in this direction especially with reference to protein turnover rates under these conditions, it would be difficult to make an appropriate suggestion.

The changes observed in the macromolecular components of neurons and glia were in accord with the changes observed in the dry matter of the cells in acute and subacute states. The decrease in the dry matter (dry weight of the cell) was far greater in the astrocytes than the neurons in subacute state, a change similar to that observed in the protein profile of the astrocytes under these conditions. It is apparent from these studies that the proteins contribute a greater portion to the dry matter of the cell.

Ammonia levels:

Ammonia levels were determined in the serum and whole brain homogenates following the administration of both acute and subacute doses of MSI (Fig.58). Ammonia levels increased sharply in the serum in both the states. Though the quantity of the drug administered in subacute state was less than the acute dose, the magnitude of increase in serum ammonia levels was far greater than the acute condition. In contrast to the serum concentration, the increase of ammonia level in the brain under acute condition was far greater than the subacute state. As the cerebral ammonia levels were determined

Effect of methionine sulfoximine on levels of ammonia **in brain** and **serum**



in whole brain homogenates, it would be difficult to predict the regional heterogenity in this parameter. The rise in cerebral ammonia levels due to MSI administration observed in the present investigation concurred with the expected action of the drug and with the reports in literature (145, 280, 311). However, the observed rise in the serum values contradicted the observations made by certain earlier investigators (145, 280) but concurred with that of Warren and Schenker (311). Such an increase in the serum ammonia level might be due to the inhibition of GS in peripheral tissues such as liver, kidney and muscle. Probably, the contribution made by muscle to serum ammonia levels would be high because of its greater mass (80%) within the Caracas than the other tissues. It was interesting to note that the magnitude of increase in the ammonia levels both in the serum and brain in acute state was more or less same which suggested a rapid equilibration of ammonia between these two tissues. Increase in the cerebral ammonia content due to MSI administration was shown to be due to the inhibition of GS, an enzyme primarily involved in the ammonia detoxification. The lesser magnitude of increase in cerebral ammonia content in the subacute state might be due to the synthesis of GS which may help in lowering the ammonia detoxification or due to a restricted permeability of this ion into the brain. The greater increase in serum ammonia levels in this condition would suggest the later possibility. Further,

evidence for such a possibility would be shown in the latter part of the results, wherein total inhibition of GS was observed even in the subacute state, thus ruling out the earlier possibility.

Enzymes of qlutamate metabolism:

Following the administration of MSI, GS activity was totally inhibited in the glia and neurons in both the states (Fig.62 and 63). The synaptosomal enzyme was, however, partially inhibited in the acute state leaving about 15% of residual activity. The inhibition of this enzyme in the synaptosomes approached totality in the subacute state with only 5% of residual activity (Fig.64). Cellular activity of GDH increased both in neurons and glia in acute and subacute states (Fig.59 and 60). When the activity was expressed per mg. protein, a similar result was noticed except that the change in the activity in glia under acute condition was not statistically significant. In the former mode of expression of enzyme activity, it was noticed that the changes in the GDH activity was more in neuronal peri-karya in comparison to the glial cells. In the latter mode of expression (specific activity) the changes in the neuronal GDH activity were less than that of the astrocytes excepting in acute state. The synaptosomal GDH was inhibited both in acute and subacute states, the percent inhibition being greater in the acute condition (Fig.61).

F i g . 5 9 Effect of methionine caroximine on neuronal glutamate dehydrogenase

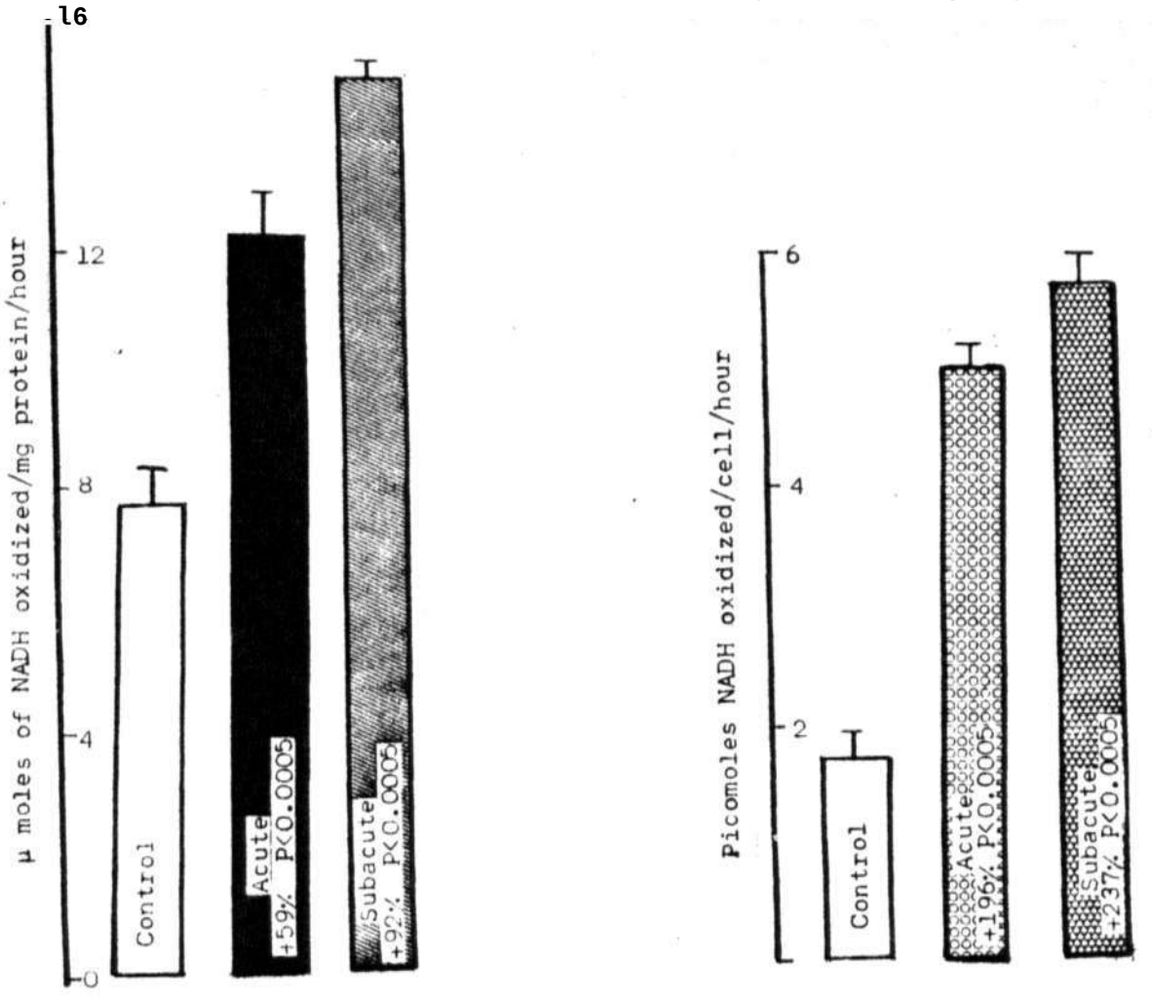


Fig. 60 Effect of methionine sulfoximine on astroglial glutamate dehydrogenase

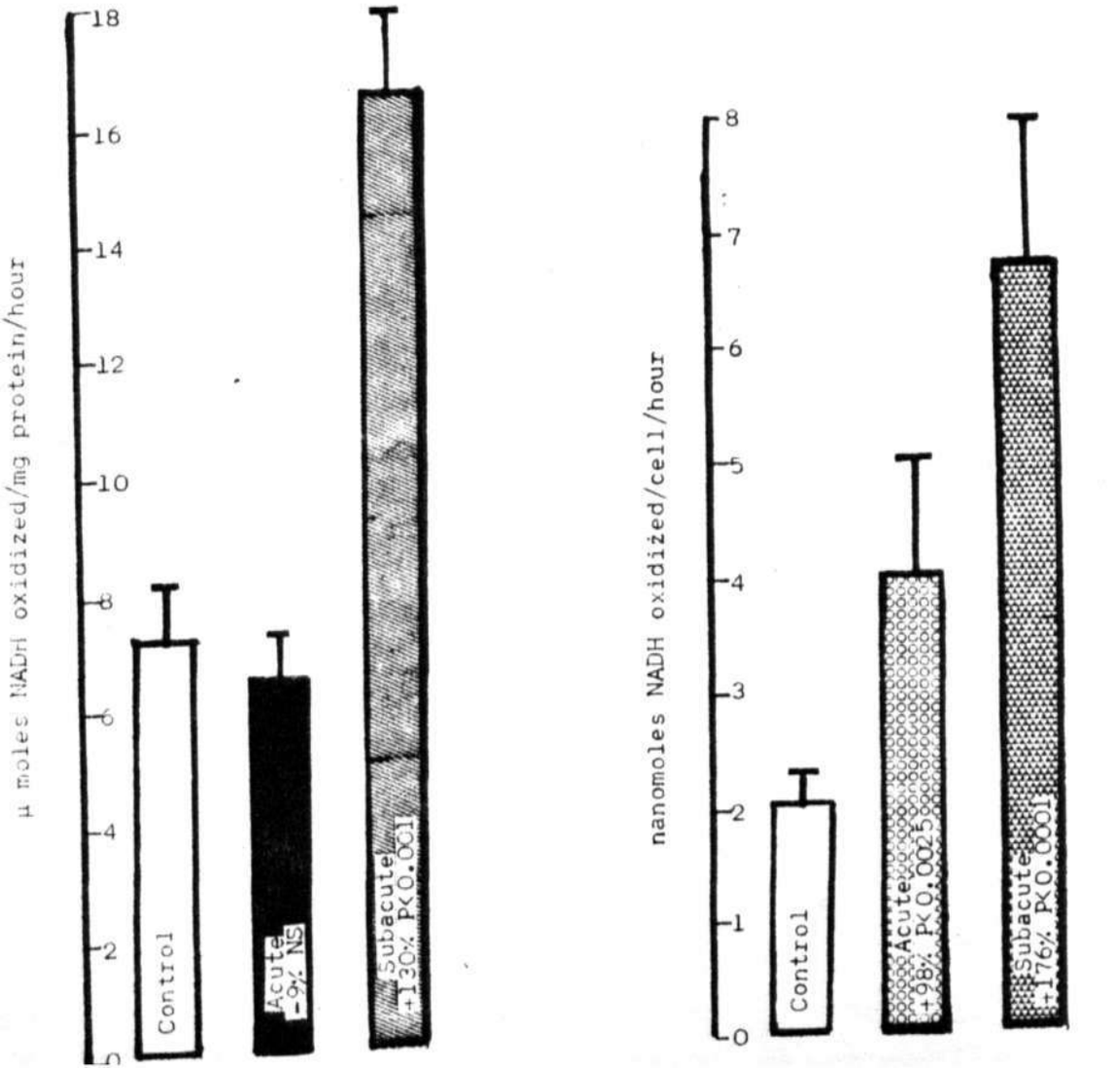


Fig. 61 Effect of methionine sulfoximine on synaptosomal GDH

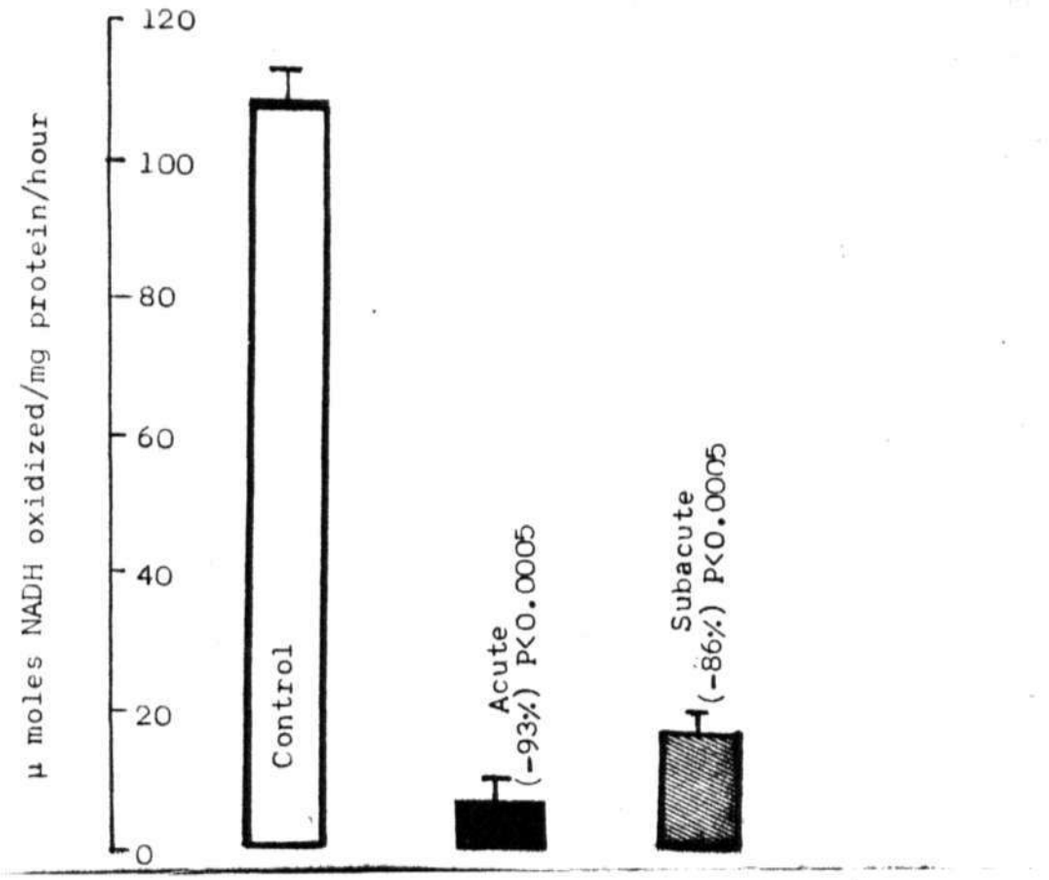


Fig. 62 Effect of methionine sulfoximine on neuronal glutamine synthetase

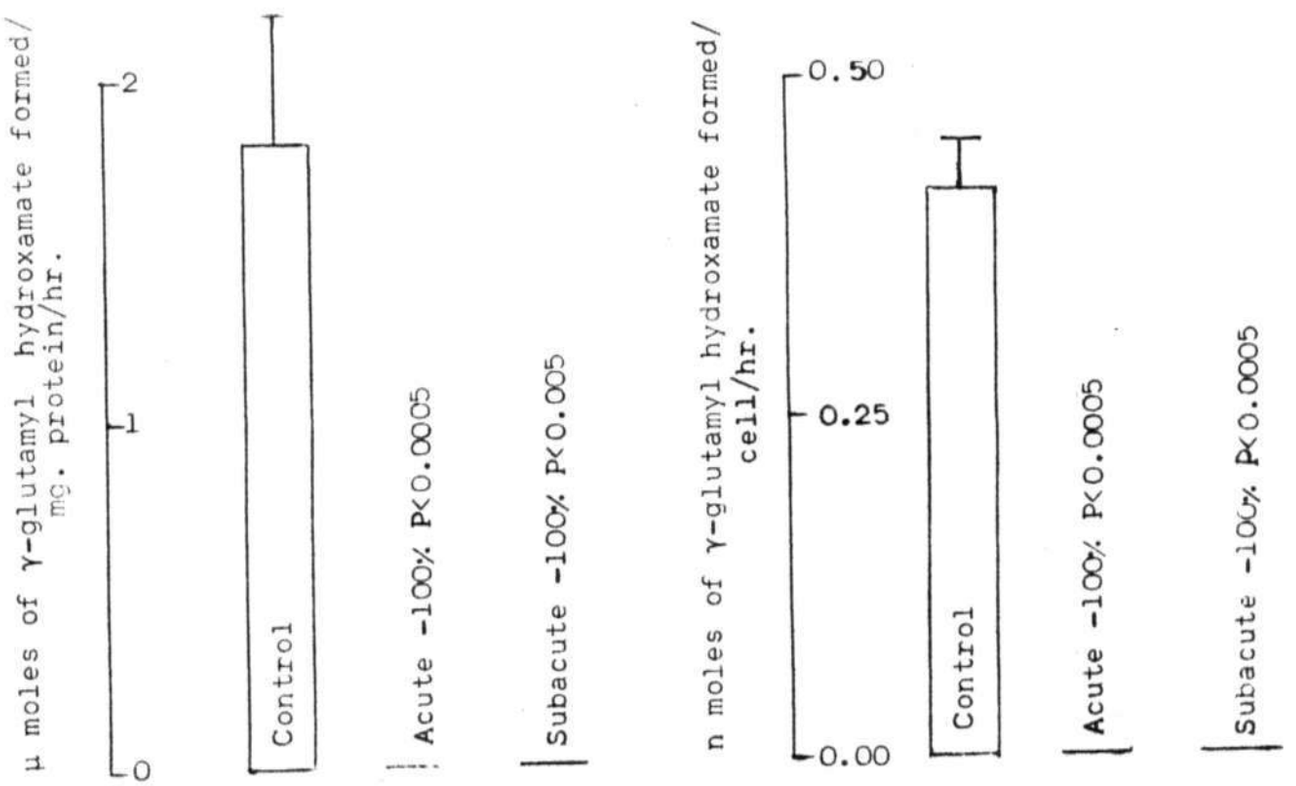


Fig. 63 Effect of methionine sulfoximine **on astroglial glutamine synthetase**

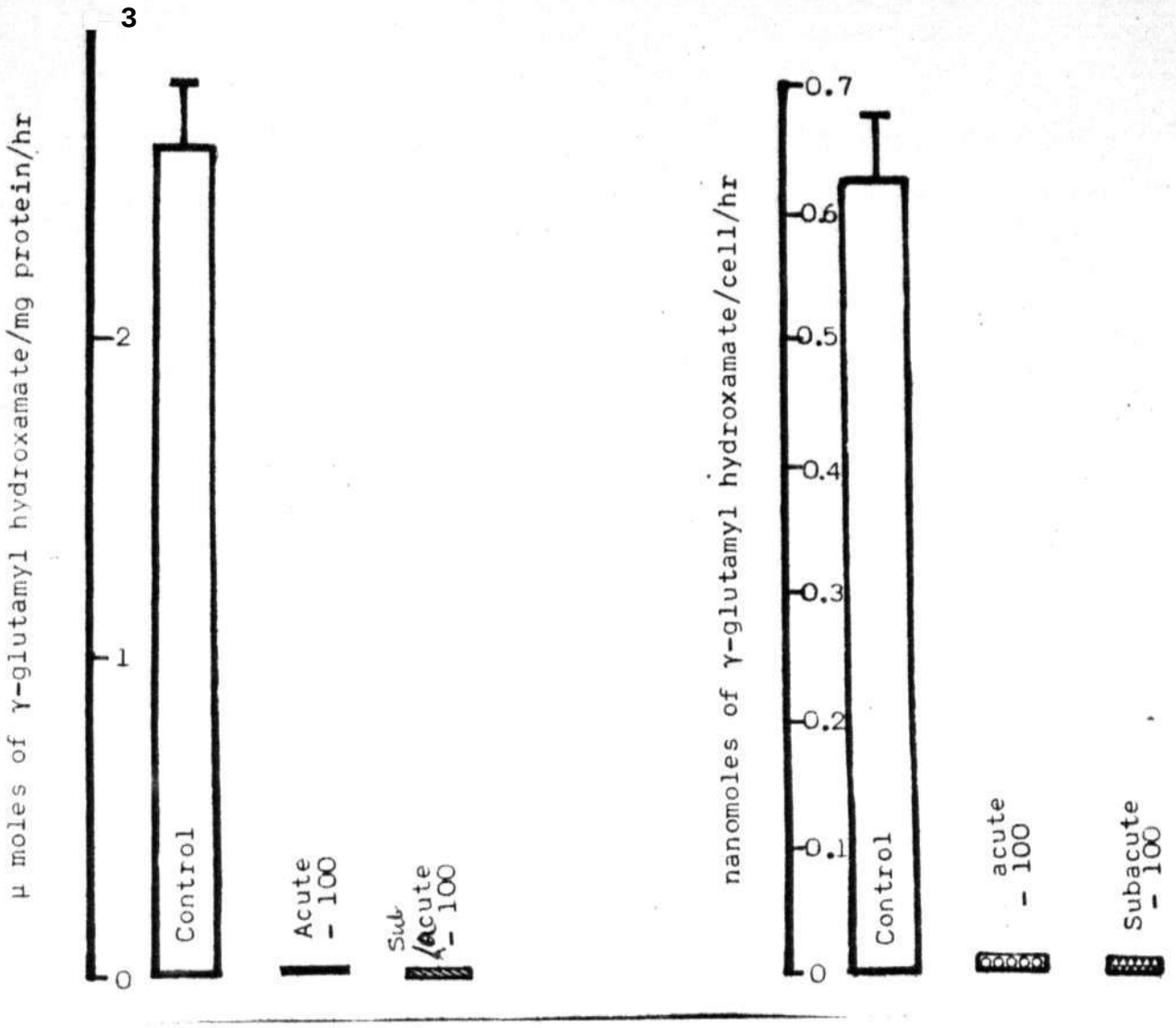
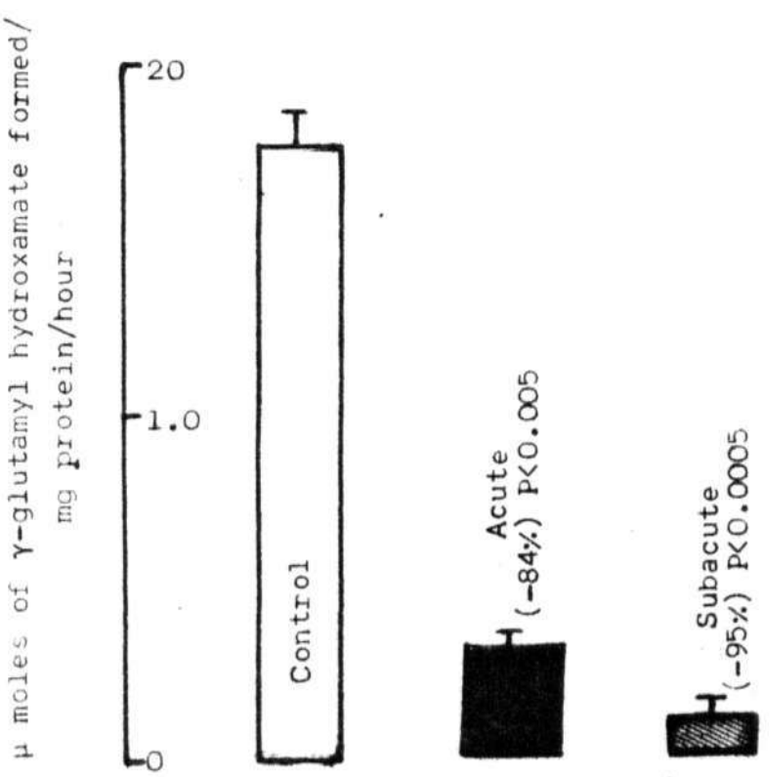


Fig. 64 Effect of methionine sulfoximine on synaptosomal glutamine synthetase



Statistically significant elevation in the cellular activity of AAT was observed only in the astrocytes in both the states (Fig.66). The elevation in the activity of this enzyme ~~~~~ observed in astrocytes in acute group was greater than that of subacute group. When the activity was expressed as specific activity, the magnitude of elevation observed in acute state was less. In the neuronal perikarya about 30% inhibition was observed in the specific activity of this enzyme in both the states (Fig.65). Synaptosomal activity of AAT was inhibited in acute and subacute states, the magnitude of inhibition being greater in acute condition (Fig.67). Irrespective of the mode of expression, A1AT activity was found to be depressed following the administration of MSI. However, the percent depression in the activity of this enzyme was less in acute condition. The activity of this enzyme was elevated in the acute state and was depressed in the subacute state (Fig.68-70). Astroglial glutaminase was inhibited to a similar extent both in acute and subacutes states when expressed specific activity. Marginal stimulation (18% was observed in acute state when the activity was expressed per cell (Fig.71). In the synaptosomes the activity of this enzyme was unaltered in acute state and was increased in the subacute condition (Fig.72).

Inhibition of GS observed in the present investigation concurred with the proposed mechanism of the action of

Fig. 65 Effect of methionine sulfoximine on neuronal aspartate aminotransferase

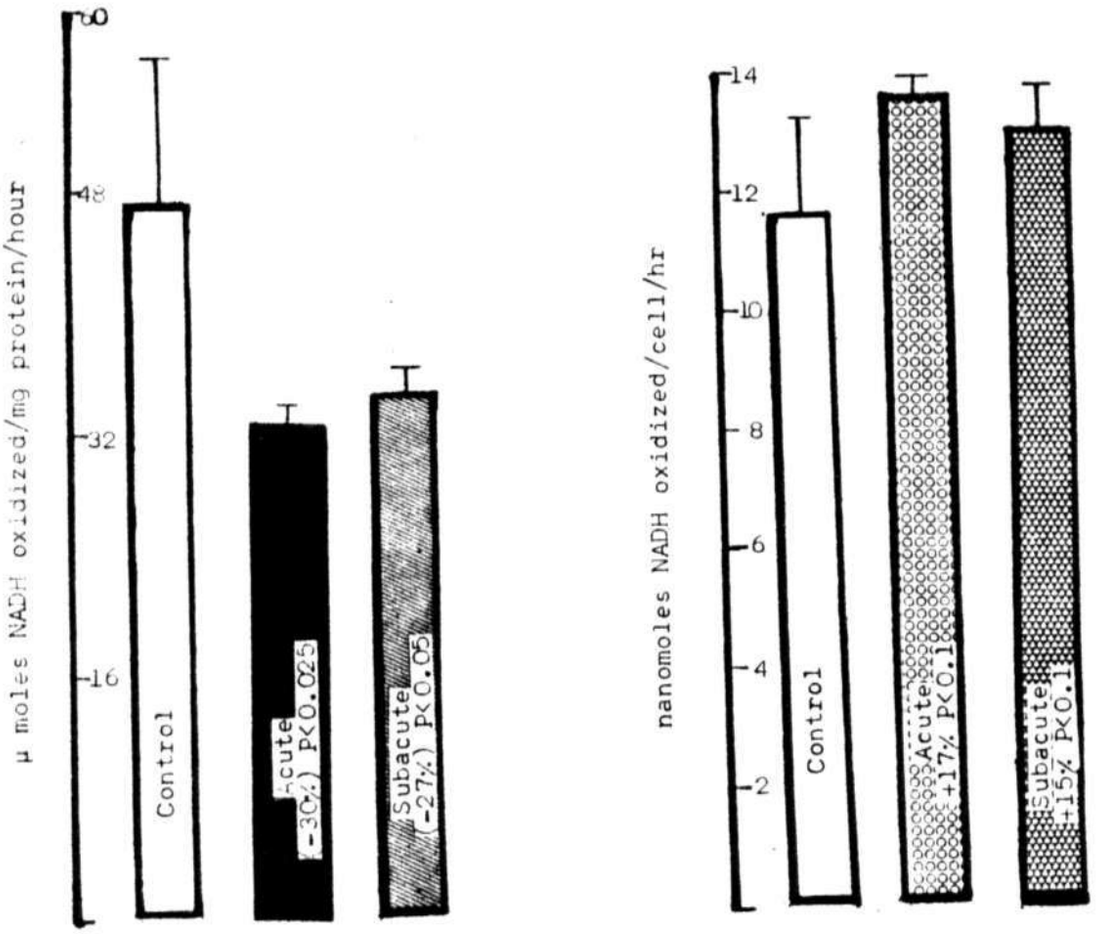


Fig. 66 Effect of methionine sulfoximine on glial aspartate aminotransferase

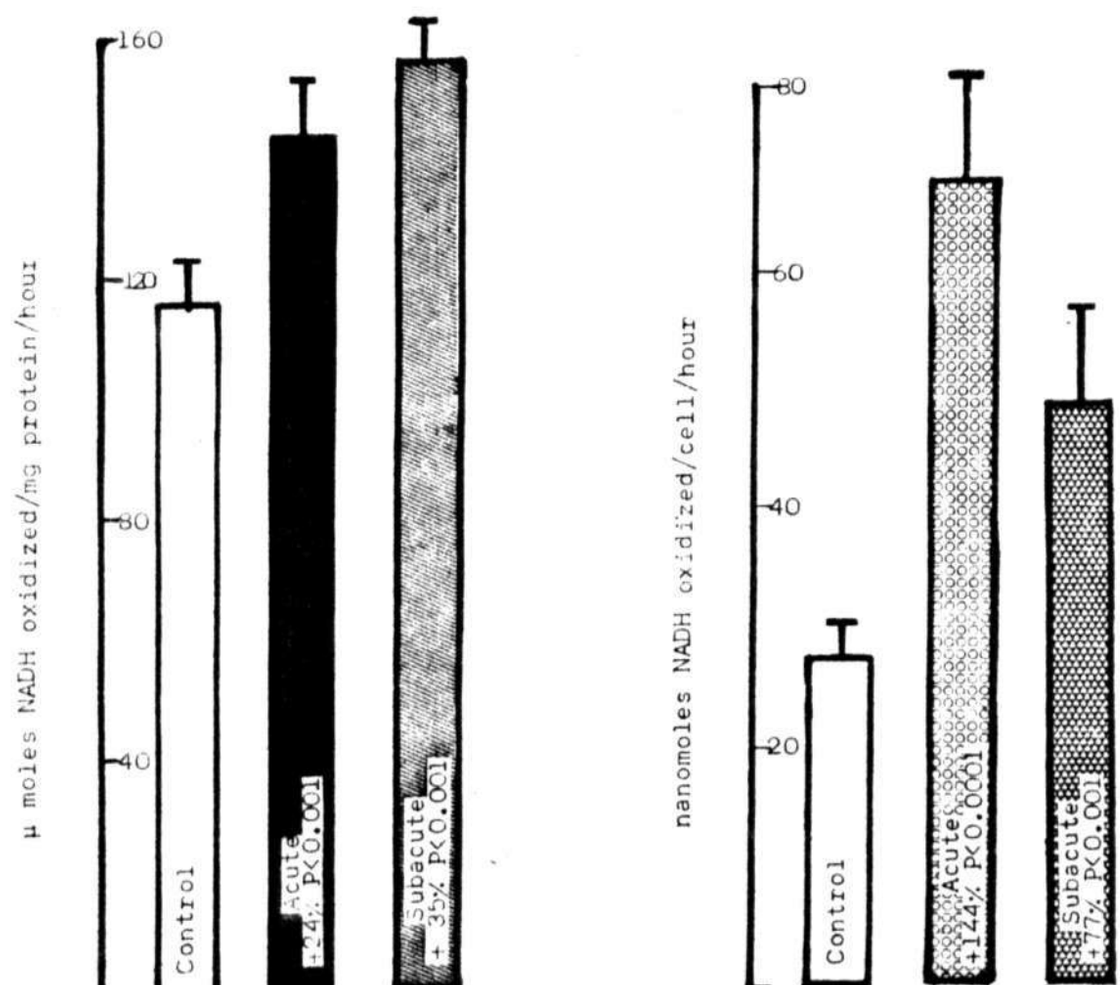


Fig. 67 Effect of methionine sulfoximine on synaptosomal aspartate **aminotransferase**

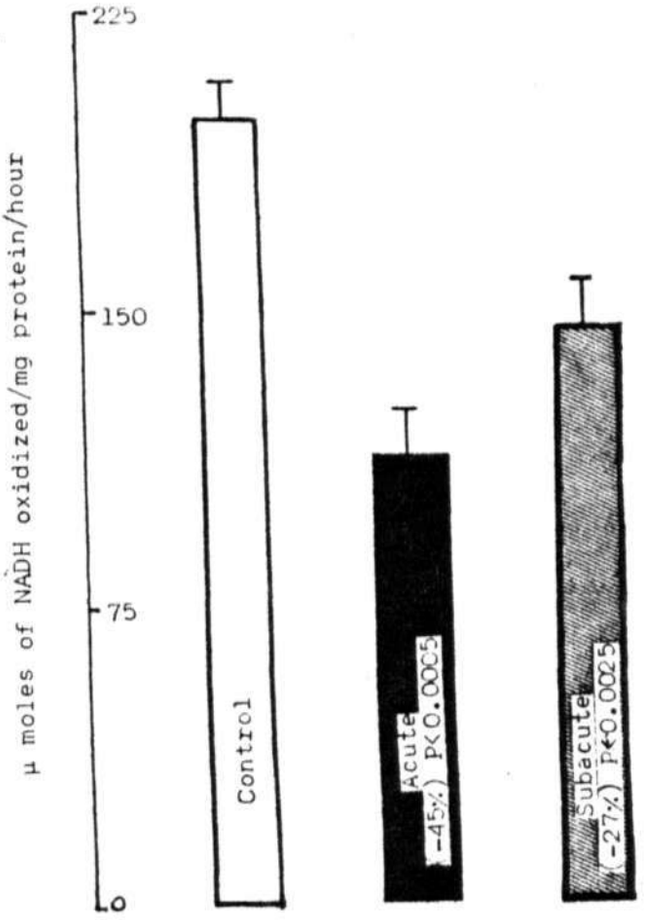


Fig. 68 Effect of methionine sulfoximine on neuronal alanine aminotransferase

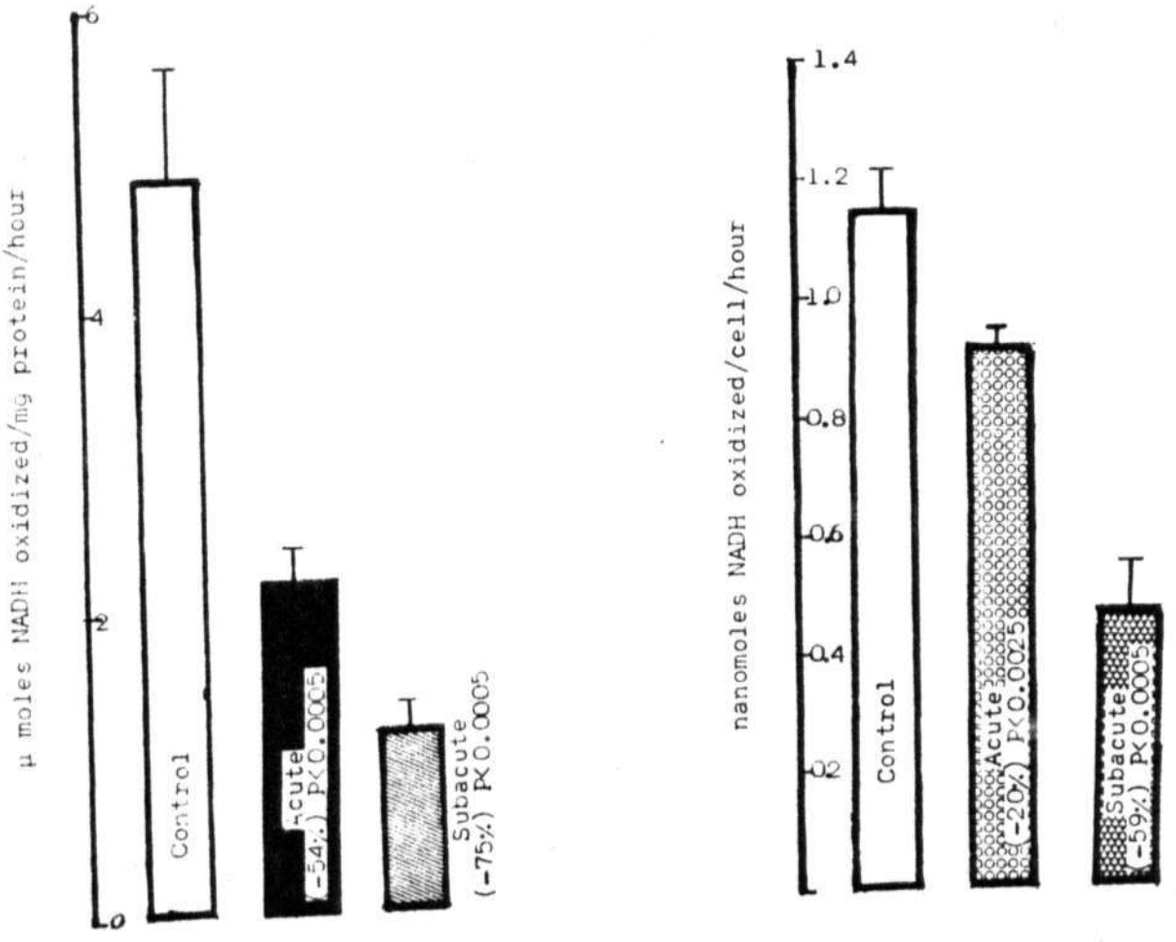


Fig. 69 Effect of methionine suifoximine on astroglial alanine aminotransferase

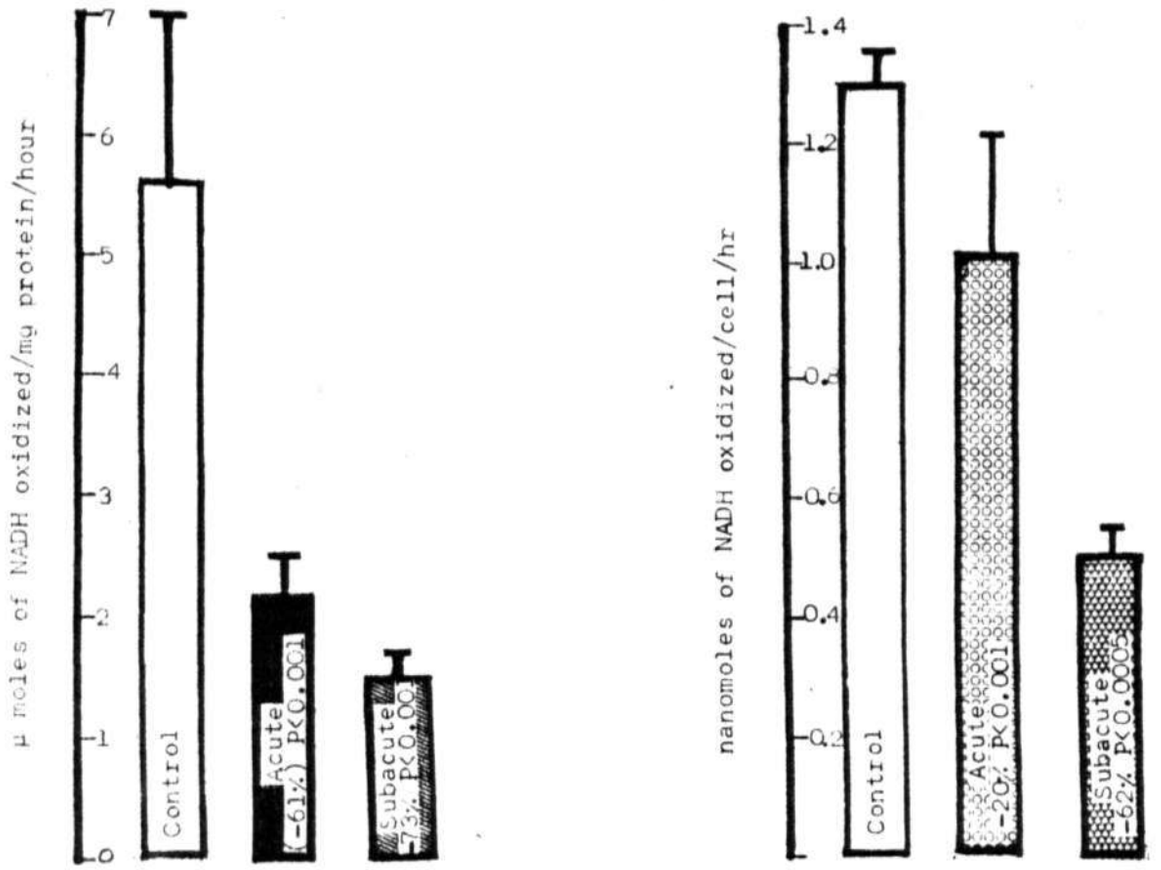


Fig. 70 Effect of methionine sulfoximine on synaptosomal alanine aminotransferase

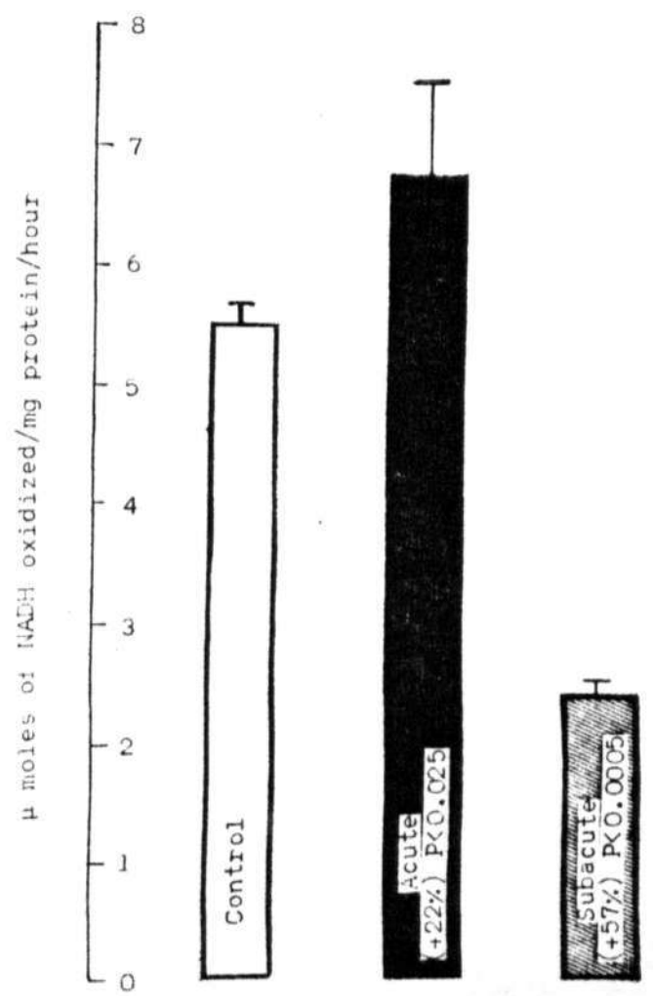


Fig. 71 Effect of methionine sulfoximine on astroglial glutaminase

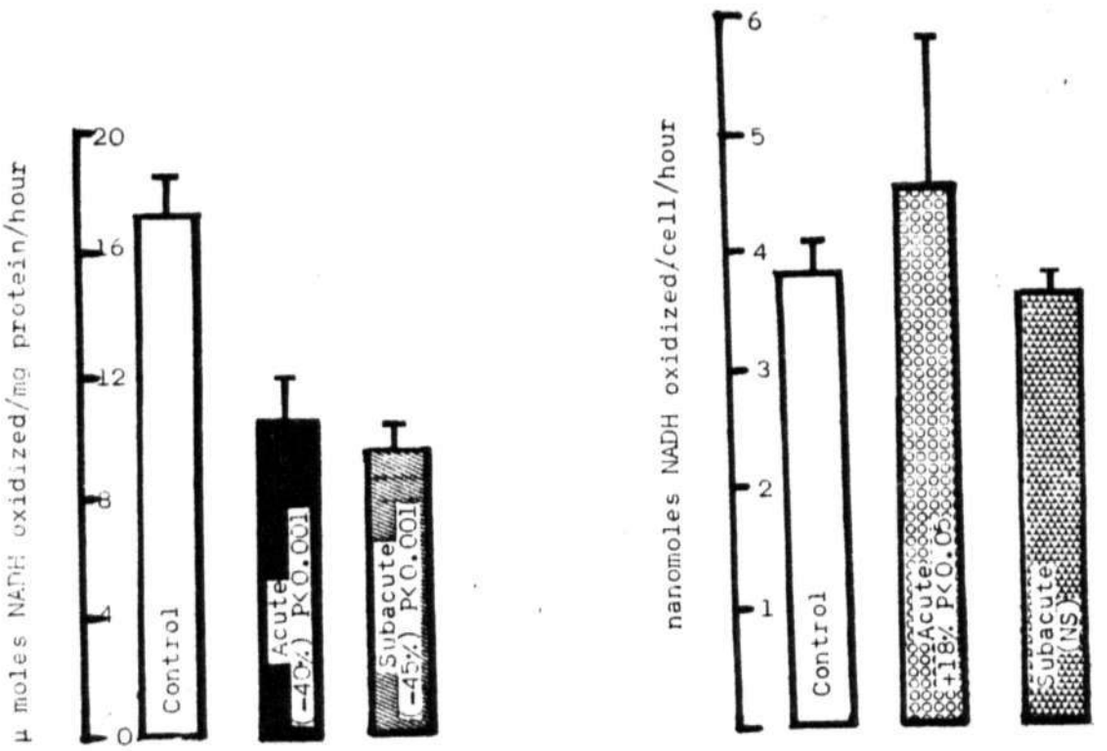
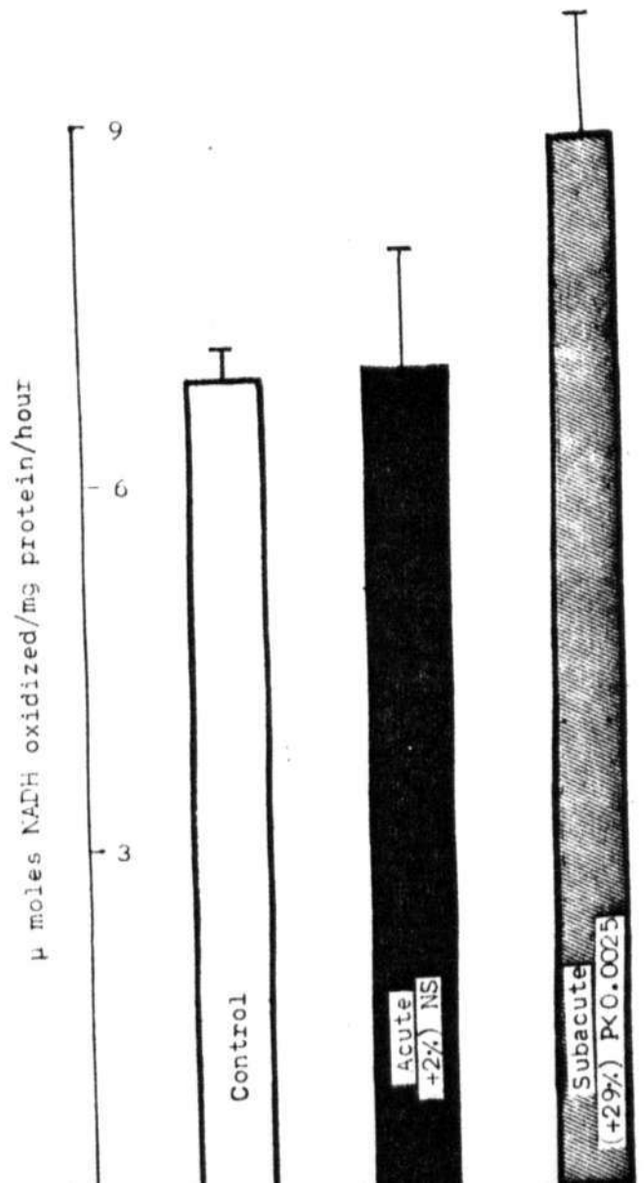


Fig. 72 Effect of methionine sulfoximine on synaptosomal glutaminase



MSI (159). The total inhibition of the enzyme in neurons and astrocytes at the end of 3.5 hrs. was also on par with the work of Lamar (158), wherein near maximal inhibition of the brain enzyme was demonstrated at the end of 4 hrs. with slightly lesser dose of MSI (200 mg/kg body wt.). It was interesting to note that the synaptosomal GS was not inhibited totally unlike the neuronal or glial enzyme under the same conditions. Though the exact mechanism behind the discrepancy is yet to be understood, this observation was akin to the reports of De Robertis & Sellinger (79) wherein 66% inhibition was observed in the activity of synaptosomal GS. The inhibition of AlAT due to the administration of MSI, also correlated with the reports of De Robertis & sellinger (79) and Lamar (158).

The elevation of GDH activity observed in the present investigation both in neurons and glia would help in detoxifying ammonia by way of glutamate formation in these two cell fractions. In this respect the action of MSI on glial GDH was different from that of ammonia wherein an inhibition rather than stimulation was observed in the glial cells. Such an effect indicated an inverse relationship in the activities of GDH and GS in this cellular compartment. In ammonia toxicity, GS was elevated and GDH was inhibited. In MSI toxicity where GS was inhibited GDH was elevated. Such an inverse relationship between these two enzymes would probably

help in the conservation of ATP. Under physiological conditions where the ammonia levels would be inadequate to saturate GDH, glutamine formation would be a major mechanism of ammonia disposal. When there is a surge of ammonia as in acute ammonia toxicity, if glutamine synthesis is not inhi-

continue to

bited, this process would/serve as major route of detoxification in the glial cells. Under these conditions, glutamate formation in glial cells would be limited and the required glutamate would be supplied, probably, by neurons. This inhibition of GDH would help in an uninterrupted flow of intermediates through TCA cycle generating the ATP required for glutamine formation. However, when GS is inhibited, as in the case of MSI toxicity, GDH would assume an important role in the detoxification of ammonia. This would, no doubt, interfere with the normal operation of TCA cycle and energy production. Probably the ATP meant for the glutamine formation would be utilized by the other energy requiring mechanisms The increase in glial GDH observed in the present investigation was similar to the results of Norenberg (202). This elevation in GDH might be due to increased synthesis of the enzyme as the number of mitochondria were found to increase under these conditions (205). However, the situation in neurons in ammonia toxicity appeared to be totally different wherein both GDH and GS appeared to play an equally important role in ammonia detoxification. Such a notion is supported by the elevation in the activity of GDH,observed in both the

conditions tested. The energy deficit due to ammonia detoxification in this cellular compartment probably replenished by an increase in glucose oxidation, as these cells are known to utilize considerably large amounts of glucose (Neurons - 7.5 pmoles $14C0_2$ released/cell/hr; astrocytes-1.08 pmoles 14CO2 released/cell/hr). Such an equal importance to these reactions in neuronal perikarya would naturally interfere with other equally important energy utilizing mechanisms such as maintenance of ionic gradients. The neurological dysfunction observed in both acute ammonia toxicity and MSI toxicity might be due to such an interference. The behaviour of synaptosomal compartment under these conditions was altogether different from neuronal perikarya and glia, as the activity of GDH was found to be inhibited without any relationship to the GS. Such an inhibition would eventually result in an accumulation of ammonium ions in this compartment which might interfere with the other vital processes such as state of the release of neurotransmitters leading to neurological dysfunction. The decrease in synaptosomal GDH activity observed in the present investigation contradicted the report of De Robertis and *SoXh*£ftY* (79).

The response of AAT both in neuronal perikarya and glia was different from that observed in acute ammonia toxicity (Fig.30,34). In MSI toxicity the activity of this enzyme was elevated in glial cells and was unaltered in

neuronal perikarya while in ammonia toxicity it was elevated in neuronal perikarya and was unaltered in glial cells. Though the exact reason for such a differential behaviour of the same enzyme in these two conditions is difficult to explain, an attempt would be made by considering the inhibition observed i the activity of GDH. In MSI toxicity glial GDH was elevated leading to the depletion of α-ketoglutarate from the citric acid cycle. As the segment of TCA cycle between succinate and α-ketoglutarate is irreversible, replenishment of α-keto-glutarate by the reversal of TCA cycle would be improbable under these conditions. Due to elevation in the GDH, gluta-mate would accumulate in the glial cells. This glutamate reacts with oxaloacetate, regenerating α-ketoglutarate with the formation of aspartate (AAT reaction). Under such conditions, the depletion of oxaloacetate would be inevitable and this could be made good by enhanced CCu fixation which was supposed to be localized in this cellular compartment. Besides regenerating α-ketoglutarate, the increased AAT would also help in the transport of reducing equivalents across the mitochondrial membrane required for glutamate formation especially when the operation of TCA cycle was disturbed. The unaltered activity of AAT in acute ammonia toxicity might be due to the fact that α-ketoglutarate depletion due to GDH activity was not induced.

A situation similar to astrocytes, might be envisaged in neuronal perikarya especially with respect to the utilization of α-ketoglutarate (due to an elevation of GDH activity). However, due to lack of change in AAT activity, α-ketoglutarate utilized for glutamate formation might not be regenerated in adequate amounts. Probably all the α-ketoglutarate required for GDH activity might have its origin exclusively from glucose in this cellular compartment (it is well known that neurons consume more glucose than astrocytes and glutamate in this compartment originates majorely from glucose). The pathway for the regeneration of α-ketoglutarate from oxaloacetate, as mentioned above, might not be operative in this compartment as it does not harbour the enzymes of CCU fixation. Hence, if α-ketoglutarate is drained out by GDH reaction, it would be lost permanently because of lack of regenerative mechanism and this would impose further strain on the operation of citric acid cycle. However, in ammonia toxicity, AAT activity was elevated in the neuronal perikarya, which would not fall in line with the above argument. It must be remembered that the hyperammonemic state in MSI toxicity would be totally different from that of acute ammonia toxicity, especially in the operation of GS. Though, superficially the enzymes AAT and *GS* appear to have no relationship to influence each other, subcellular localization of these enzymes would yield a clue in their relationships. GDH is

known to be localized in mitochondria. Hence, glutamate formed by this enzyme during hyperammonemic state would be in the mitochondria. The enzyme GS is known to be localized in microsomes and require glutamate as a substrate, which has to be transported across the mitochondrial membrane as it is not freely permeable. Hence, mechanisms must exist in the cell for the transport of glutamate across mitochondrial membrane, especially to support the GS activity. Two such mechanisms were proposed earlier. The glutamate -OH carrier was shown to transport glutamate from cytoplasm into mitochondria *{77)*. The reversal of this carrier action, i.e. from mitochondria to cytoplasm might not be in operation due to an unfavourable H^+/OH~ gradients (the mitochondrial matrix has more OH- and less H^+ than the medium) (167). The other suggested mode of exchange was aspartate-glutamate exchange (48). Glutamate from mitochondrial matrix would exchange with aspartate in cytoplasm in this mode of transport which serves as a part of malate-aspartate shuttle. In this process the enzyme AAT plays a major role in connecting the GDH, localized in mitochondria, with the GS localized in endoplasmic reticulum. For increased glutamine production as in acute ammonia toxicity, more glutamate has to be transported across mitochondrial membrane, hence, an elevation in AAT activity. In MSI toxicity, where GS was inhibited, there might not be a need for the increased transport of glutamate, hence, the activity of this enzyme was unaltered.

The situation in glia was slightly different in this aspect as glutamate required for glutamine formation would be derived from several precursors and the contribution by GDH would be small in comparison with normal conditions (when GS is not inhibited). This might be a reason for not observing any change in AAT in the glia in acute ammonia toxicity. This concept was further supported by the inhibition of AAT in synaptosomes both in acute ammonia toxicity and also in MSI toxicity, wherein GDH activity was inhibited more or less to a similar extent.

Though the mechanism behind the inhibition of A1AT in MSI toxicity is not understood, it might help in channelling pyruvate into TCA cycle or for $C0_2$ fixation.

Though a marginal stimulation in GLNase activity was observed in acute MSI toxicity, in subacute state, it was reverted to normal levels. As there would be no substrate for this enzyme, the changes expressed in the activity of this enzyme might not be of any significance. However, in synaptosomes some amount of glutamine might still be available due to the residual activity of GS and this glutamine could serve as a precursor for the releasable pool of glutamate and GABA.

Very few observations were made on the levels of amino acids in MSI toxicity and these reports were far from complete. Most of the studies were made in whole brain homogenates in the animals other than rat. In addition, at least in few reports where dogs were used as experimental animals(280), they were subjected to extensive surgery and were treated with several anaesthetics. Further, the results obtained in in vivo experiments were contradictory to the results obtained from in vitro experiments. The in, vivo experiments indicated a decrease in the levels of glutamate and aspartate (280, 295) which would contradict with the observations made in the present study where the increased GDH would necessitate an increased level of glutamate. Since most of the experiments were in whole brain homogenates it would be difficult to assess the changes in the cortex especially if there is a regional heterogenity in the drug susceptibility (265). In one of the in vitro studies (20) marginal increase in glutamate level was observed while in another study (29b) decrease was noticed in the slices. In the slice preparations the numbers of neuronal perikarya would be less compared to number of nerve endings. If the assumption that each neuron would bare approximately 40,000 nerve endings is considered (212), under such conditions one might expect a decrease in glutamate formation in the slice preparations as GDH was inhibited by MSI in synaptosomes.

and Van den Velden

The observations made by Van den Berg (295) were interesting and would support the observations made in present investigation, especially with reference to the changes with the glial GDH and AAT activities. These authors noticed an increase in the incorporation of ^{14}C label into glutamate and aspartate from ^{14}C-acetate, a small compartment precursor and a decrease in the incorporation of labelled carbon into these amino acids from 2-^{14}C-glucose, a large compartment precursor. The latter effect may be explained by assuming that the bulk of glucose utilization would be be in synaptosomes as they outnumber the neuronal perikarya.

In the present discussion about MSI toxicity not much stress was given on the metabolic compartmentation of glutamate because, both the cells have become reactive and expressed more or less similar changes (excepting AAT), a situation totally different from that of ammonia toxicity where the major changes were observed only in neuronal perikarya.

ATPases;

The activity of Mg^{++}-ATPase was observed to be elevated both in neuronal perikarya and glia in both the conditions of MSI toxicity (Fig.73 and 74). The percent elevation in the activity of this enzyme was more or less same in both acute and subacute states in neurons whereas

Fig. 73Effect of methionine sulfoximine on neuronal Mg++ ATPase

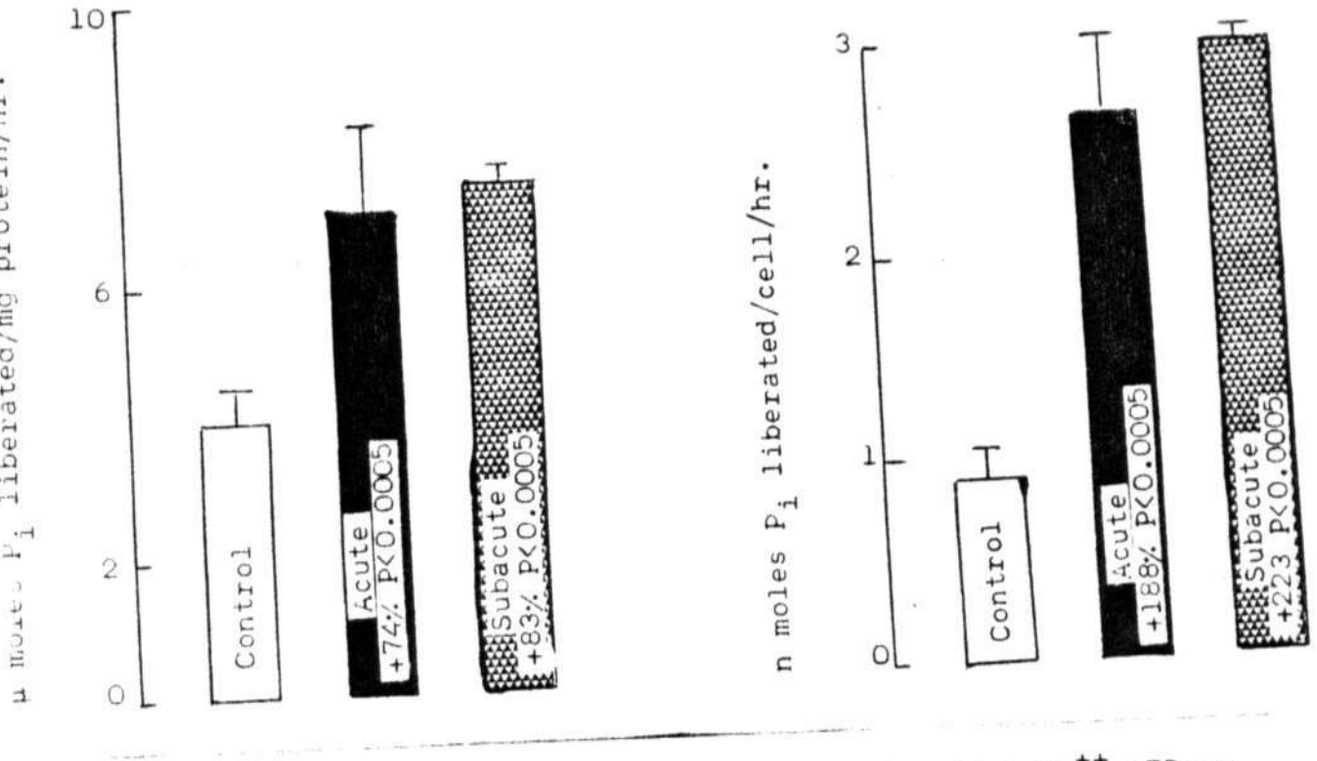
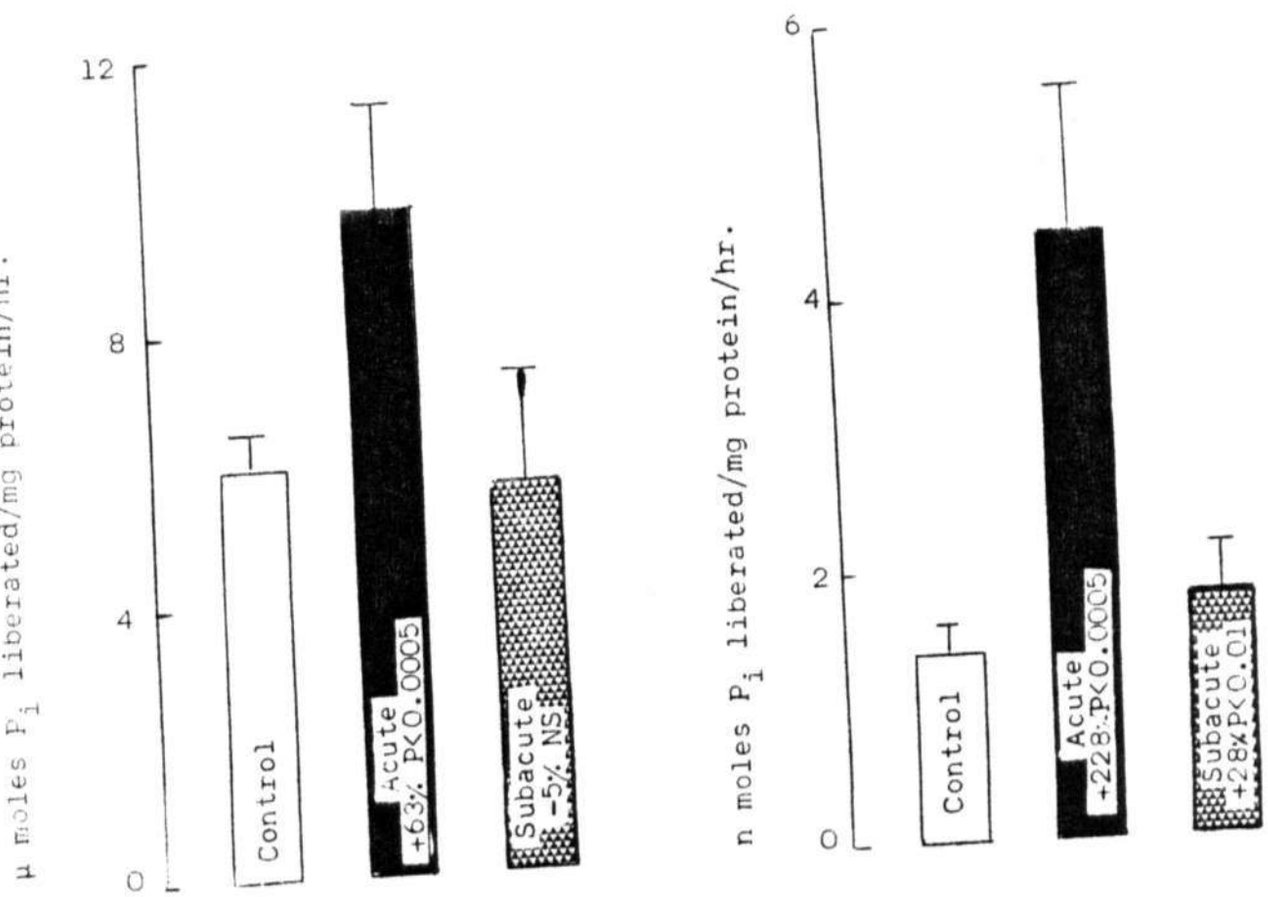


Fig. 74 Effect of methionine sulfoximine on astroglial Mg^{++} ATPase



it was less in subacute state in glial cells. No significant changes were observed in the activity of this enzyme in the nerve ending particles (Fig.75). The changes observed in the activity of Mg^{++}-ATPase in neuronal perikarya and in synaptosomes were akin to the changes observed in ammonia toxicity. As mentioned earlier, the activity of this enzyme(s) could be a representative of several ATP consuming reactions (219) especially those involved in glucose phosphorylation. The increased activity of this enzyme(s) would then represent an increase in the phosphorylation of glucose and larger influx of glucose carbon into TCA cycle, thereby ensuring an uninterrupted supply of α-ketoglutarate for glutamate formation. Lack of changes in the activity of this enzyme(s) in the synaptosomes would be in accord with this suggestion, as the glutamate formation was totally suppressed in MSI toxicity. The increased activity of this enzyme(s) in the glial cells in acute phase would also represent a similar situation. The decline in the activity in subacute phase might be due to the degenerative changes of the reactive glial cells, restricting α-ketoglutarate availability for the glutamate formation.

The increased activity of Na+, K^{+}-ATPase observed in MSI toxicity in all the three fractions (Fig.76-78) might be responsible for the earlier reported elevation in the activity of this enzyme in brain homogenates under similar

Fig. 75 Effect of methionine **sulfoximine** on synaptosomal **Mg^{++} ATPase**

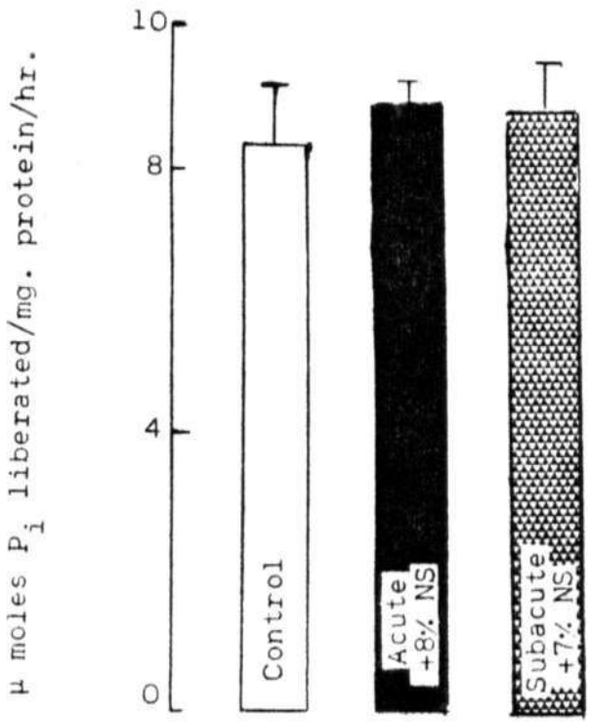
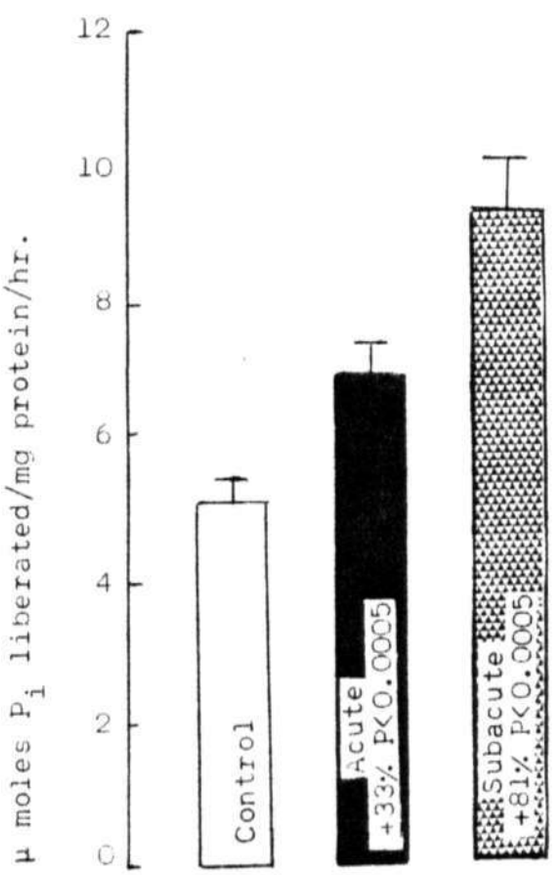


Fig. 76 Effect of methionine sulfoximine on neuronal Na^+, K^+-ATPase



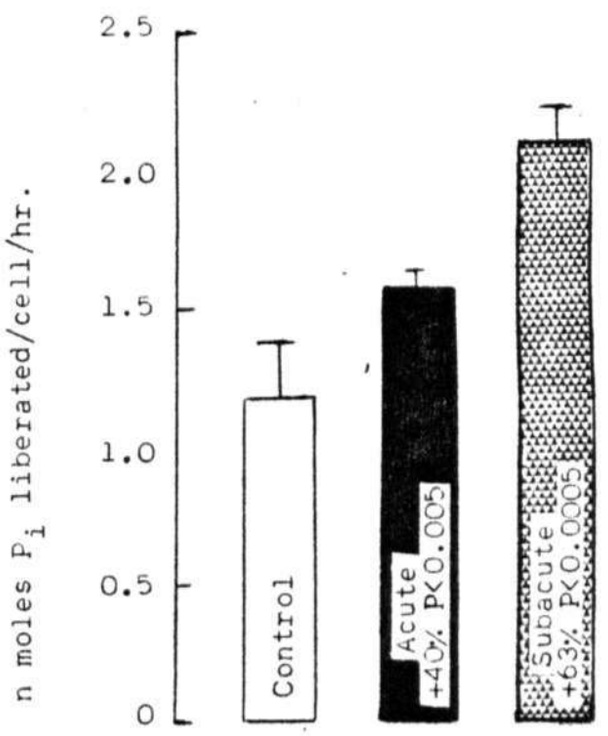


Fig. 77 Effect of methionine suifoximine on astroglial Na^+,K+ -ATPase

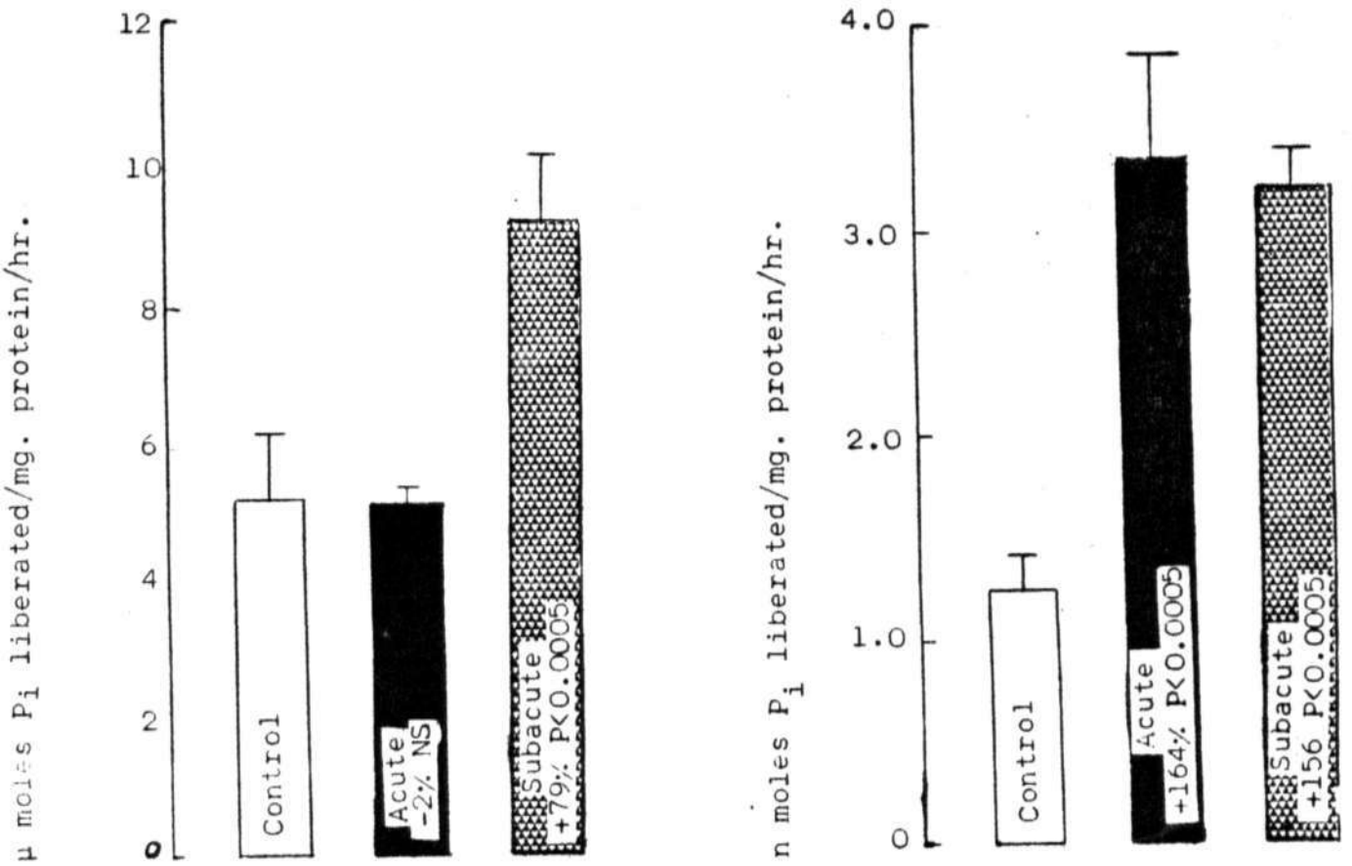
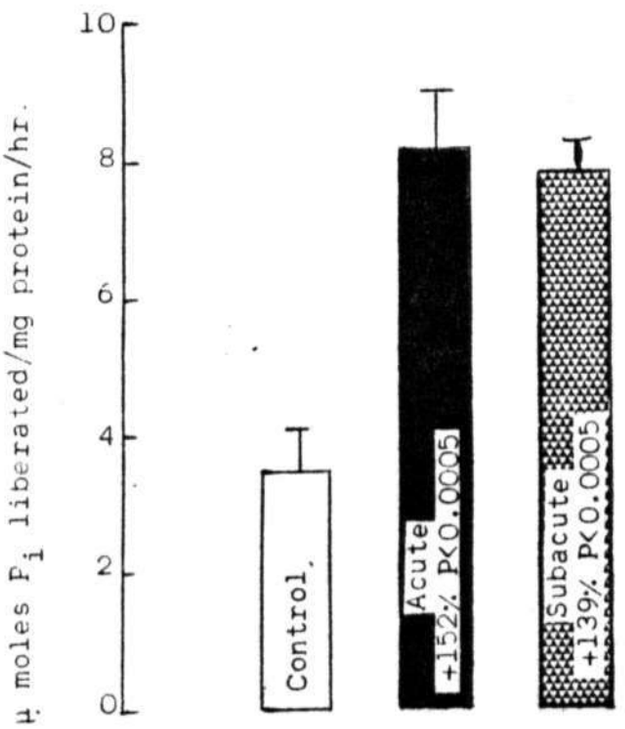


Fig. 78 Effect of methionine sulfoximine on synaptosomal Na^+, K^+-ATPase



conditions (265). The magnitude of changes in the activity Of Na+, K+ -ATPase in MSI toxicity was different from that of ammonia toxicity. The changes in the glial cells and synaptosomes were more or less equal and were greater than those of changes in neuronal perikarya. The increased activity of this enzyme might be responsible for the transport of larger quantity of NH_{4+} into glial cells and synaptosomes than into neuronal perikarya, due to reasons mentioned earlier, Increased transport of NH_{4+} due to the elevation of this enzyme activity into the synaptosomes, especially in the absence of normal detoxification mechanisms, as observed presently, would result in the accumulation of NH4+ inside and the resting membrane potential would be shifted more towards the threshold value (123). This might be responsible for the susceptibility of the normal mice to audiogenic stimuli following the treatment of MSI (302, 303) and also for the convulsions observed in the present investigation.

Finally, an attempt would be made to correlate the changes observed in cell morphology, cellular metabolism and the behaviour of the animals during MSI toxicity. The increased number of glial cells, as was discussed, might be an index of the proliferation of these cells in MSI toxicity. In contrast, a state of neuronal degeneration (decrease in cell number) was observed. The glial cells would enter into reactive phase by synthesizing and accumulating large

amounts of DNA and protein in addition to the glycogen. Accumulation of these components along with the metabolic changes might lead to the changes in the osmotic activity inside the cell resulting in large influx of water and the swelling of glial cells. The lack of detoxification process in the nerve endings would result in an accumulation of NH_4^+, which would affect the membrane potentials (and probably the state of excitation and release of neurotransmitters) without imposing any metabolic strain. In conclusion, the neurological symptoms observed might be due to multipronged action of this drug. From the present investigation, it would appear that the loss of at least few neuronal perikarya, depletion of α-ketoglutarate and probable changes in membrane potentials in the nerve endings might be responsible for the neurological symptoms elicited by the drug, MSI.

Before any cause-effect relationship is established in hyperammonemia similar studies should be conducted in several other regions of the brain, especially in cerebellum (responsible for the motor activity) and in brain stem (responsible for maintenance of consciousness through reticular activating system) and in hyperanmonemic syndromes of varied etiologies. Studies should also be made on the turnover rates of not only macromolecules but also of several metabolities and the rates of operation of various metabolic pathways in these cells isolated from different regions.

This study was a preliminary effort in such a direction and by no means is totally complete. However, it would help in understanding not only the cerebral mechanisms involved in the pathological states but also provides the much sought information on neuroglial interactions.

SUMMARY

SUMMARY

1. A modified method was described for the isolation of cells from rat brain both from normal animals and from animals injected with ammonium acetate and methionine sulfoximine (MSI). Synaptosomes were also isolated from these animals.

2. In addition to microscopic identification, marker enzymes were assayed to determine the purity of the isolated cell fractions. Activities of carbonic anhydrase, butyryl and acetyl cholinesterases and choline acetyltransferase indicated that the contamination was not more than 10-15/. of various cell fractions.

3. Neuronal perikarya had a greater wet weight than the astroglia. The water content of the neuronal perikarya (78/.) was greater than that of the astrocytes (59/.) while the contribution made by the particulate material (dry matter) to the cell weight was greater in astrocytes (47%) than in neuronal perikarya (21%).

4. The DNA content of the neurons was greater than that of astrocytes or that of a diploid cell of rat which might be due to polyploidy in the neurons. The RNA content was also higher in neuronal perikarya than the astrocytes, an observation in concurrence with the somatotrophic role

played by the neuronal perikarya. Protein content of the neurons and astrocytes was more or less same, in spite of a higher nucleic acid content of the neuronal perikarya, the reasons for which were discussed.

5. Distribution of the enzymes of glutamate metabolism was determined in the isolated cell fractions and synaptosomes from normal animals.

6. Glutamate dehydrogenase (GDH) activity was greater in the synaptosomes than in astrocytes and neurons in which the activity levels were similar. The distributory profile of aspartate aminotransferase (AAT) was: synaptosomes > astrocytes > neuronal perikarya, while that of alanine aminotransferase (A1AT) was equal in all the three fractions.

7. Glutamine synthetase (GS) activity was present not only in the astroglia but also in the neuronal perikarya and synaptosomes which could not be attributed to the contamination of these preparations with glial cells. It was suggested that this enzyme in neuronal perikarya might supply glutamine for various other vital biosynthetic reactions, in addition to its contribution to the neurotransmitter pool. The presence of this enzyme in synaptosomes might be due to axonal transport from the neuronal perikarya.

8. The activity of glutaminase (GLNase) was found to be greater in the astrocytes than in the synaptosomes and it was not detected in the neuronal perikarya.

9. Based on the distribution of the enzymes, it was suggested that the nerve terminals would derive a major portion of their glutamate from glucose and a part of this glutamate might be converted to glutamine in the nerve terminals. The glutamate for the biosynthesis of glutamine in the astrocytes might have its origin from the glutamate synthesized in synaptosomes and a very small amount of glucose carbon would enter into the glutamate in the astrocyte. A similar condition was also perceived for the neuronal perikarya. The suggestions made in the present investigation would differ from the classical theory of metabolic compartmentation with respect to the synthesis of glutamine both in neuronal perikarya and in the nerve endings and also with respect to the ability of astrocytes to synthesize glutamate from glucose. AAT was supposed to play a major role not only in the synthesis of glutamate and aspartate (utilizing the dicarboxylic acids from TCA cycle)but also in the transport of glutamate from the mitochondria for the synthesis of glutamine in the cytoplasm and in the transport of reducing equivalents across mitochondrial membranes.

10. Lesser number of neurons were isolated from gram weight of cortex of the animals injected with ammonium acetate, than the controls while the number of astrocytes isolated did not vary to a significant extent. The loss of neurons under these conditions was not due to the vagaries of the isolation procedure. Microscopic examination of neuronal preparation from experimental animals revealed that a population of neurons which are small in size (and have a small nucleus) were few in number than in the preparations from control animals. The neurons which were large in size (and have a large nucleus) were unaffected. The swelling of astroglia and neurons was microscopically evident. It was suggested that at least a population of neurons (possibly Golgi type II neurons or granule cells) were vulnerable to hyperammonemic states.

11. Both wet weight and dry weight of neurons and astrocytes increased following the administration of ammonium acetate. The magnitude of increase was greater in the astrocytes than in the neurons. A similar profile was obtained in the water content of the cells where, the increase in astrocytes was at least 8 fold higher than that of neuronal perikarya. The increase in the water content suggested swelling of the cells due to the administration of ammonium acetate.

12. The increase in the DNA content was greater in the neurons when compared to astrocytes, while a reverse profile was observed for the changes in RNA content following the administration of an acute dose of ammonium acetate. Parallel changes were also observed in the protein content of these cells. The magnitude of increase in protein content was greater in neurons than astrocytes. Though the reasons for the increase in DNA content could not be explained, the changes in the RNA and protein contents were assumed to be responsible for the synthesis of new proteins to circumvent the physiological stress in the hyperammonemic state.

13. The activities of all the enzymes of glutamate metabolism (GDH, GS, AAT, AlAT and GLNase) were found to increase in the neuronal perikarya and the magnitude of change was greater during convulsions than in the comatose condition.

14. In contrast to the changes in neuronal perikarya, the activity of GDH was found to be depressed in the astrocytes. But for an increase in GS, the activities of rest of the enzymes of glutamate metabolism were either unaltered or suppressed in acute ammonia toxicity. The magnitude of elevation in the activity of GS in astrocytes was 5-7 fold less than that of the neuronal perikarya. The only significant positive change observed in the aminotransferase activity was that of AlAT, especially during convulsions.

15. In synaptosomes, the profile of changes in the activities of enzymes of glutamate metabolism were almost similar to that of astrocytes. GDH activity was suppressed totally (-93/.) and the activity of GS was elevated in comatose condition and returned to normal levels during convulsions. GLNase was elevated in comatose condition and suppressed in convulsive state.

16. The changes observed in the present investigation suggested that the neuronal perikarya encompassing the large compartment of glutamate would play a major role in ammonia detoxification by way of glutamate and glutamine formation when compared to the astrocytes as evidenced from the magnitude of changes in the activities of GDH and GS. The increase in the activity of AAT in this cell compartment under these conditions might help in the maintenance of a favourable redox state in the mitochondria for the synthesis of glutamate. In addition, it might favour regeneration of α-ketoglutarate lost in the glutamate formation and also in the transport of glutamate from the mitochondria for the synthesis of glutamine. A similar role might be envisaged for the AlAT, the activity of which was also elevated in the neuronal perikarya under these conditions.

17. The suppression of GDH in the astrocytes indicated that the major pathway of ammonia detoxification in the

glial cells would be glutamine formation and the glutamate required for this process might have an exogenous origin as GDH was suppressed and AAT was unaltered. The derangement of metabolism in ammonia toxicity appeared to be minimal in the astrocytes compared to the neuronal perikarya.

18. The observed changes in the activities of enzymes of glutamate metabolism in the synaptosomes suggested that this subcellular compartment was not involved to a major extent in ammonia detoxification but for an initial phase of glutamine formation. The total suppression of GDH and the reduction in AAT activity were in support of such an observation. It was proposed that the nerve endings would accumulate ammonium ions, which would alter the membrane potentials, consequently their electrical activity and neurotransmitter release under these conditions.

19. The swelling observed in astrocytes under these conditions, inspite of a minimal metabolic derangement in the detoxification process, was proposed to be due to the changes in membrane permeability especially to chloride ions.

20. The increase in the Mg^{++}-ATPase activity in the . neurons was greater in magnitude when compared to the changes in the glia or synaptosomes. As this enzyme was supposed

to be heterogenous in nature and its function is yet to be understood, it was proposed to be a representative of non-specific ATP utilization, most likely the initial stages of glycolysis. Increased activity of this enzyme would then represent increased glucose phosphorylation in the neurons and the entry of glucose carbon into citric acid cycle would be enhanced under these conditions. This would supply the necessary carbon skeleton for the synthesis of glutamate in this cellular compartment.

21. Na+ , K+ -ATPase activity was elevated both in comatose and convulsions in the neuronal perikarya, while in the astrocytes such an elevation was observed only in the convulsive phase and was suppressed in comatose condition. The changes in the synaptosomal enzyme were opposite to those observed in glial cells. Such changes in the activity of Na^+, K+,-ATPase in the glia and synaptosomes would influence the ionic gradients across the membranes which might be responsible for the neurological dysfunction.

22. An effort was also made to understand the cellular mechanisms involved in the toxicity of the hyperammonemic drug, methionine sulfoximine (MSI). The pattern of changes in wet weight, dry weight and water content due to MSI administration was more or less similar to that observed in acute ammonia toxicity, but it was of greater magnitude

in the astrocytes. The increase in the water content was very prominent in these cells suggesting a greater swelling of the astrocytes, especially in the subacute state of MSI toxicity.

23. The changes in DNA, RNA and protein in MSI toxicity were also similar to those observed in acute ammonia toxicity, but for their greater magnitude in the astrocytes compared to neurons. A marginal increase in the DNA was observed in both the states of MSI toxicity in neuronal perikarya when compared to the astrocytes where the changes were more prominent in the subacute state. The increase in the protein content in the neuronal perikarya was more or less same in the acute and subacute states of MSI toxicity but in the glia it was more in acute state than in subacute state. The changes in the DNA content especially in the glial cells under these conditions was considered to be an index of the cell proliferation. It was suggested that this change might be akin to endomitosis. The changes in the RNA and protein would help in the proliferation of the cell organelles under these conditions.

24. The activities of GS and A1AT were inhibited under these conditions both in the neurons and astrocytes, a result expected from the earlier studies on the effect of MSI on these enzymes. GDH was elevated in MSI toxicity

and the magnitude of elevation was greater in neurons than the astrocytes both acute and subacute states. The activity of AAT was elevated in the glial cells while the changes in neuronal perikarya were statistically not significant.

25. The changes in the activities of these enzymes in the synaptosomes were more or less similar to those in acute ammonia toxicity as all the enzymes of glutamate metabolism were inhibited to a greater extent excepting glutaminase in subacute state and MAT in acute state.

26. Due to an increase in GDH activity ammonia would be detoxified by way of glutamate formation in neurons and astrocytes and in fact this would be a major mechanism in the absence of GS. In this process, α-ketoglutarate would be depleted from citric acid cycle. In the neuronal perikarya glucose would replenish the loss of dicarboxylic acids while in the astrocytes it would be replenished by AAT. The changes observed in the AAT activity would provide support to this observation. Unaltered activity of this enzyme in neuronal perikarya during MSI toxicity may be understood in the light of above suggestion and also by its role in transport of glutamate for the synthesis of glutamine which was suppressed by MSI. These observations supported the earlier contention that the neuronal perikarya

have major role in the process of ammonia detoxification than the astrocytes.

27. The lack of changes in glutamate metabolism in synaptosomes would lead to a metabolic state similar to that described in the acute ammonia toxicity (cf.No.18).

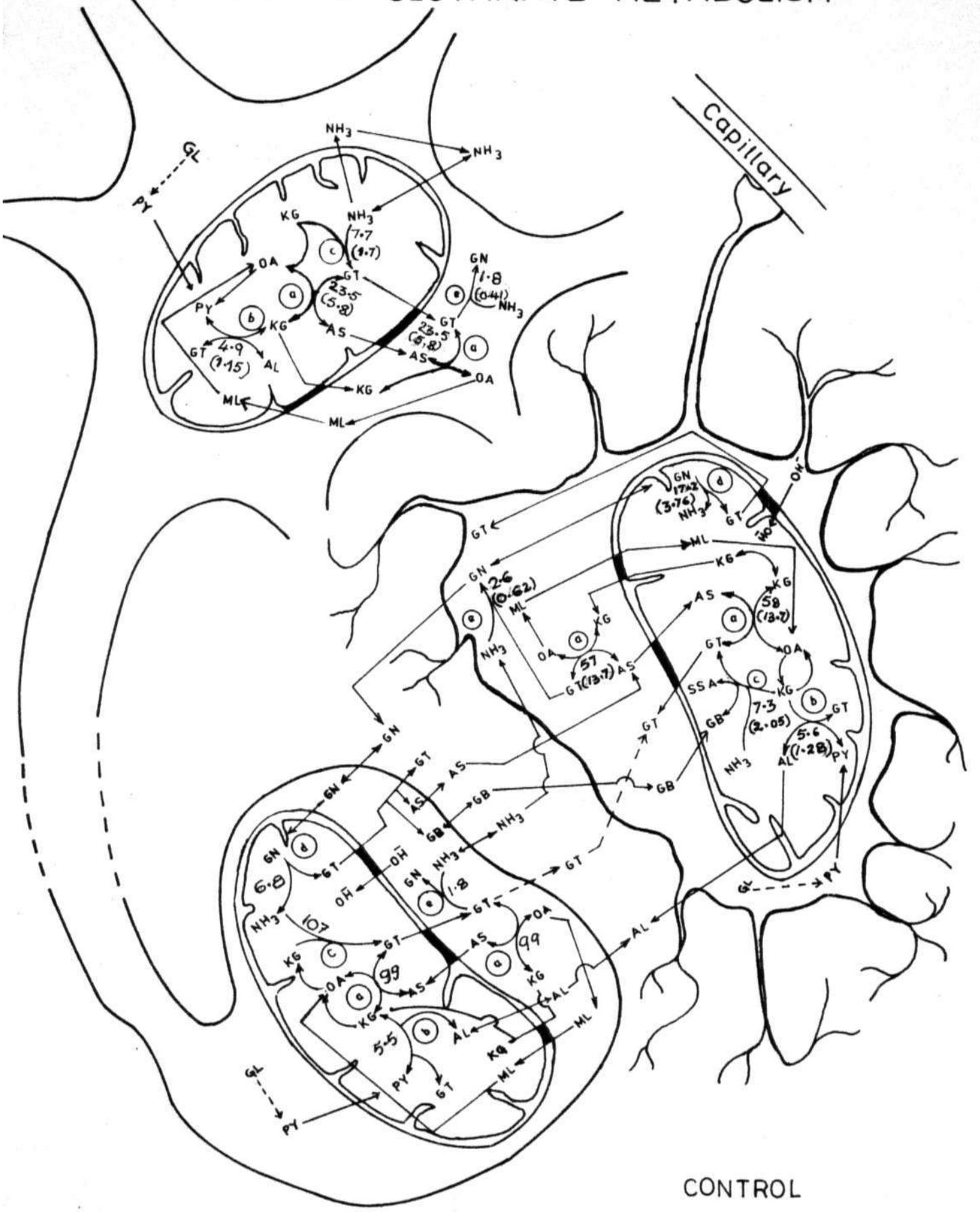
28. The changes in Mg^{++}-ATPase once again suggested a greater magnitude of glucose oxidation in the neuronal perikarya both in acute and subacute states and also in the glial cells under acute condition. As said above, glucose might be providing the skeleton for the glutamate synthesis.

29. The magnitude of the changes in the activity of Na^{+}, K^{+}-ATPase were more or less similar in the astrocytes and synaptosomes and were of greater magnitude when compared to those of neuronal perikarya. As mentioned earlier these changes might be responsible in the alterations in the ionic gradients in the respective cell compartments which might play a major role in the etiology of the neurological symptoms.

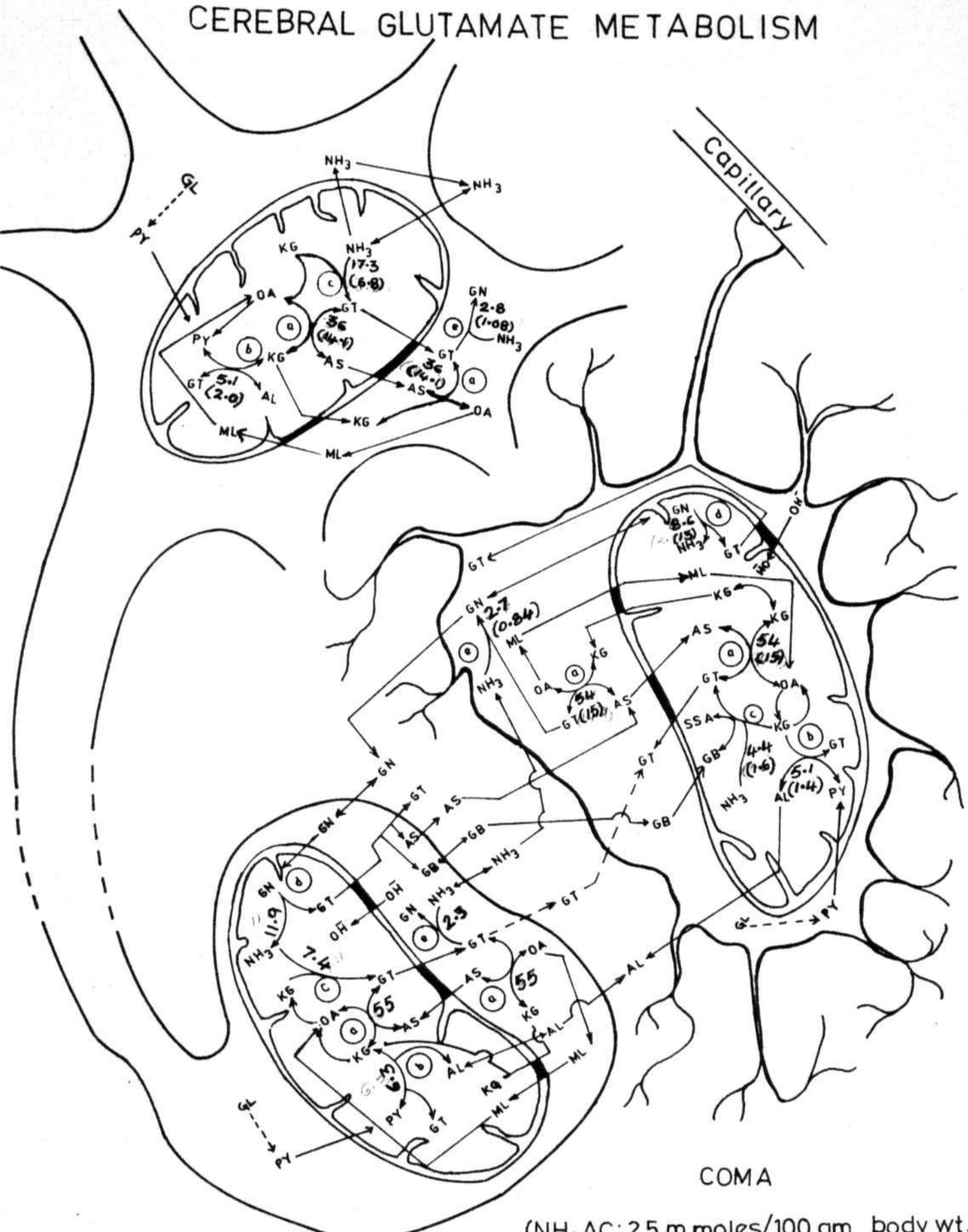
30. The results of present investigation suggested a major role of the neuronal perikarya in the process of ammonia detoxification when compared to the astrocytes.

It was observed that the metabolic derangement due to detoxification was also minimal in the nerve endings leading to the accumulation of ammonia which alters the electrical activity by influencing the ionic gradients. In conclusion, the present investigation would help in filling up the lacunae in understanding the pathophysiology of ammonia toxicity and in the involvement of different cellular populations in the detoxification process. This study would be of importance, as hyperanmonemic states are associated with several metabolic derangements (both acquired and inherited) of amino acid metabolism, urea cycle disorders and in hepatic failure.

CEREBRAL GLUTAMATE METABOLISM



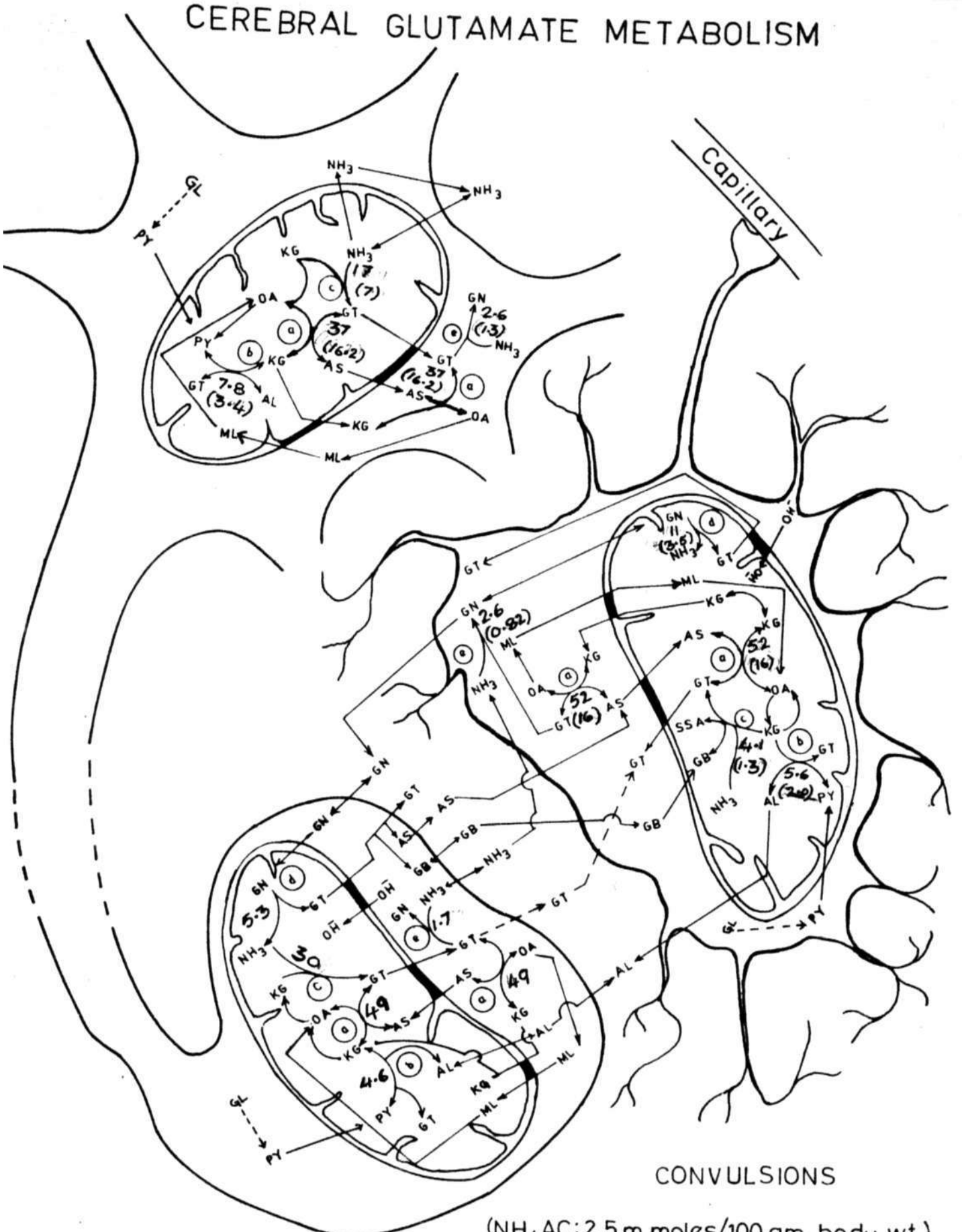
CEREBRAL GLUTAMATE METABOLISM



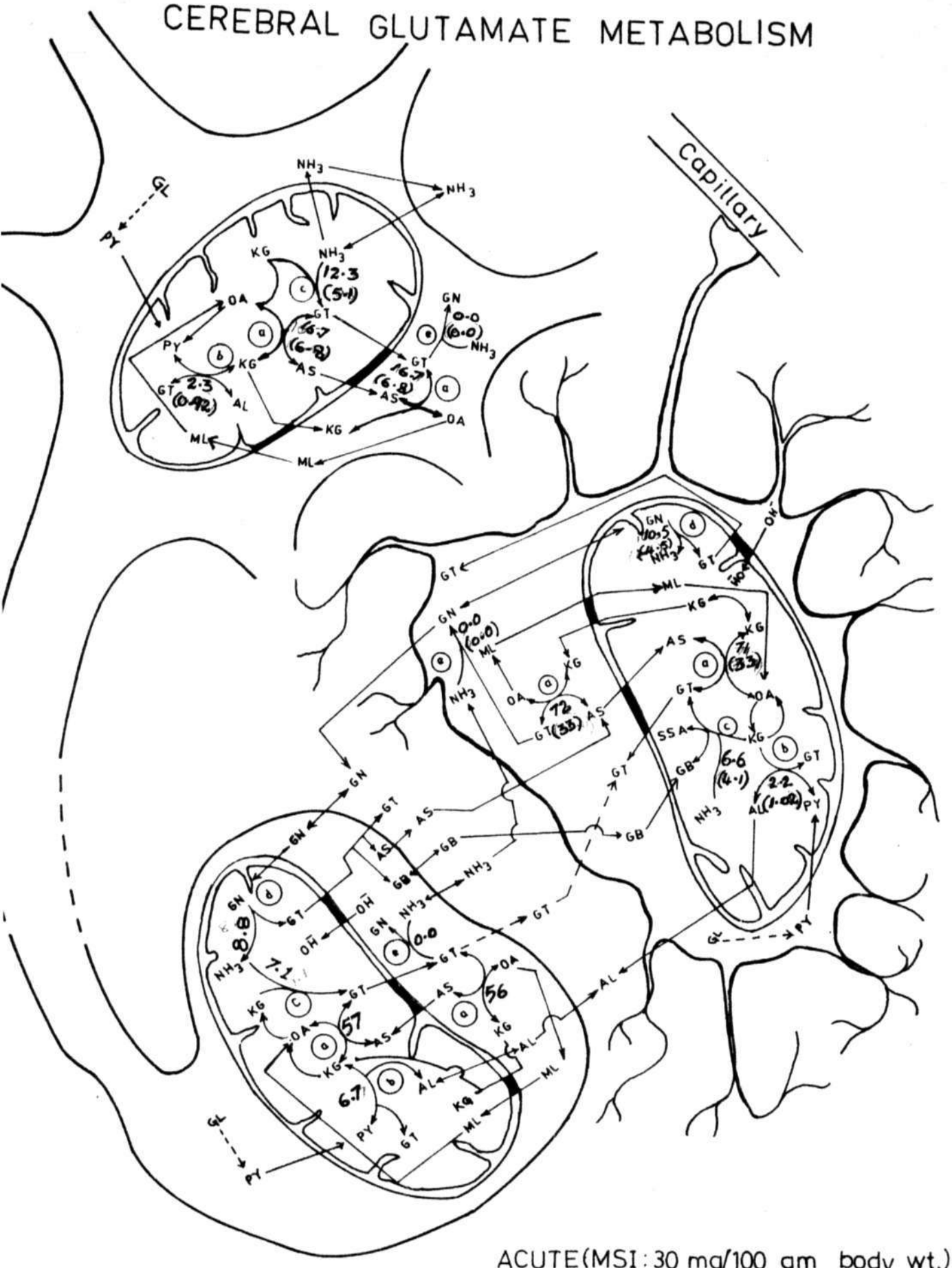
COMA

(NH_4AC: 2.5 m moles/100 gm body wt.)

CEREBRAL GLUTAMATE METABOLISM

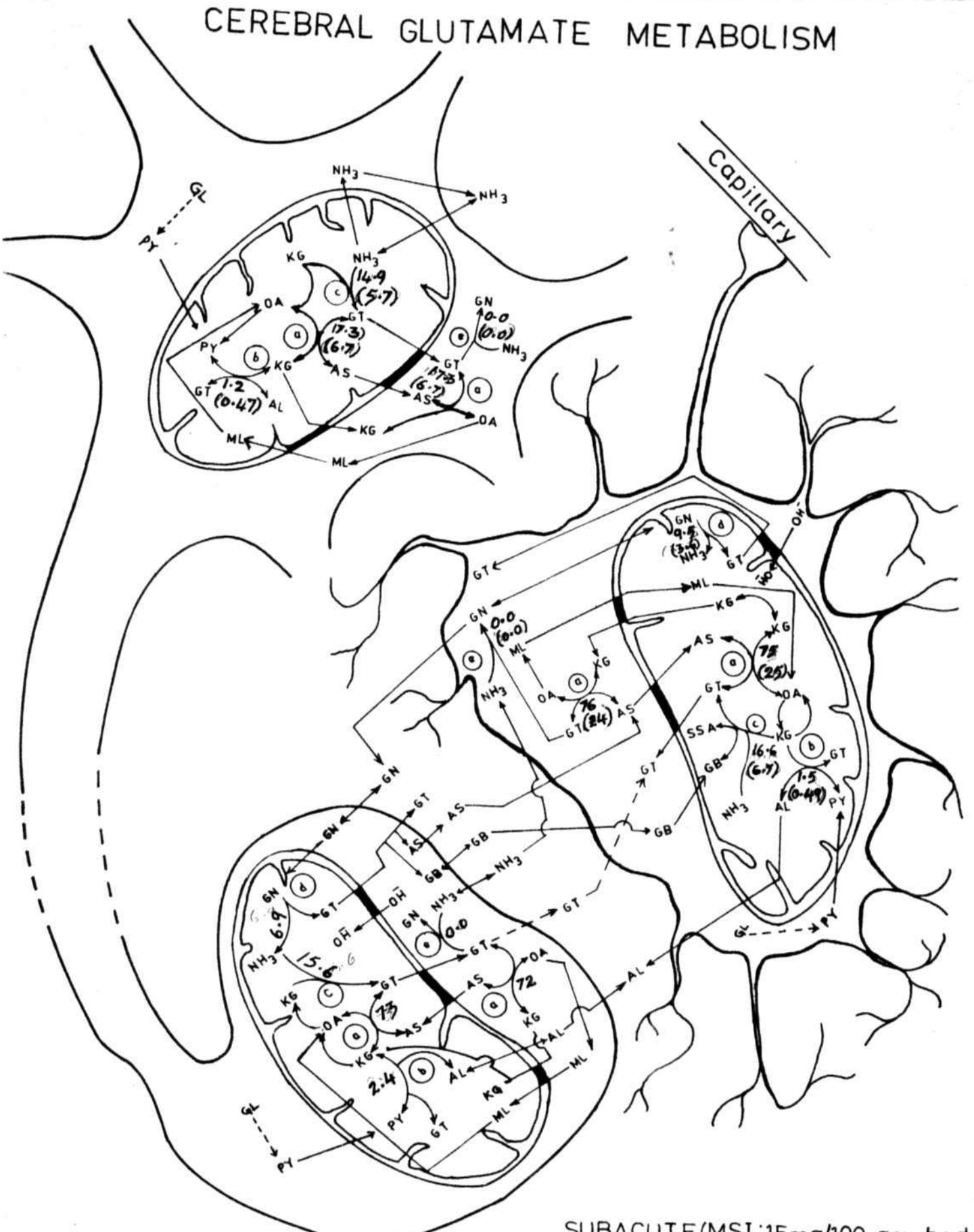


CEREBRAL GLUTAMATE METABOLISM



ACUTE (MSI : 30 mg/100 gm body wt.)

CEREBRAL GLUTAMATE METABOLISM



SUBACUTE (MSI: 15mg/100 gm body wt.)

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