BRANCHED CHAIN AMINO ACID METABOLISM IN BRAIN IN HYPERAMMONEMIA

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This is to certify that I, **JESSY JOHN** have carried out the work embodied in the thesis 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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ABBREVIATIONS

AAA - Aromatic amino acids

AAT - Aspartate aminotransferase

AcCoA - Acetyl CoA

AlAT - Alanine aminotransferase

ATP - Adenosine triphosphate

Amm.Ac. - Ammonium acetate

BBB - Blood brain barrier

BCAA - Branched.chain amino acid

BCAA-T - Branched chain amino acid aminotransferase

BCKA - Branched chain keto acid

BCKA-DC - Branched chain keto acid decarboxylase

BCKA-DH - Branched chain keto acid dehydrogenase

BSA - Bovine serum albumin

CNS - Central nervous system

2,4 DNP - 2,4 Dinitro phenylhydrazine

$Fe(CN)_6$ - Ferricyanide

GABA - Gamma amino butyric acid

GS - Glutamine synthetase

HE - Hepatic encephalopathy

HEPES - N-2-Hydroxy ethyl piperazine N'-2-ethane sulphonic acid

α-KG - α-Ketoglutaric acid

α-KGDH - α-Ketoglutaric acid dehydrogenase

α-KIC - α-Ketoisocaproic acid

α-KIV - α-Ketoisovaleric acid

αC-KMV - αC-Ketomethylvaleric acid

LDH - Lactate Dehydrogenase

MDH - Malate dehydrogenase

MSO - Methionine sulphoximine

NAA - Neutral amino acids (Leucine, Isoleucine, Valine, Phenylalanine, Tyrosine and Tryptophan).

NAD^+ - Nicotinamide adenine dinucleotide (oxidised)

NADH - Nicotinamide adenine dinucleotide (reduced)

Na^+,K^+-ATPase - Magnesium dependant, Sodium, Potassium stimulated adenosine triphosphatase

NH_3 - Ammonia

NH_4^+ - Ammonium ion

OAA - Oxaloacetate

PCA - Perchloric Acid

PDH - Pyruvate dehydrogenase

PEP - Phosphoenol pyruvate

PEPCK - Phosphoenol pyruvate carboxykinase

PFK - Phosphofructokinase

PMS - Phenazine methosulphate

POPOP - 2,2'-p-phenylene-bis (4-methyl-5-phenyloxazole)

PPO - 2,5 Diphenyl oxazole

PYR - Pyruvate

SDH - Succinate dehydrogenase

TCA - Trichloroacetic Acid

TCA cycle - Tricarboxylic acid cycle

TPP - Thiamine pyrophosphate

CONTENTS

INTRODUCTION

Ammonia, a by product of nitrogen metabolism, is a universal constituent of all organisms. It is a universally accepted fact that ammonia in high concentrations is toxic to most of the organisms irrespective of their evolutionary status. Though animals are highly vulnerable to toxic concentrations of ammonia, there is a wide range of tolerance. Because of its toxic nature, there are several mechanisms which strictly guard and maintain very low concentrations of ammonia in the tissue and body fluids. However, failure of these mechanisms, which usually occurs in the pathological states, would result in an enhancement of ammonia levels in tissues and body fluids. A wide variety of neurological symptoms are seen under such conditions which may or may not be fatal. Because of its direct bearing on the health of the organism, especially of humans, several investigations were made in the past to understand the mechanism of ammonia toxicity and to evolve suitable therapeutic measures. Despite intensive investigations spanning more than half a century, the mechanism of ammonia toxicity remains enigmatic. Since hyperammonemia causes neurological dysfunction , it is appropriate to present an overview of the relationships between ammonia and cerebral metabolism.

ORIGIN OF CEREBRAL AMMONIA

Blood ammonia is the chief source for cerebral ammonia (Onstad and Zieve, 1979). In addition, there are several

endogenous reactions that also generate ammonia in brain (Cooper and Plum, 1987). Thus, brain ammonia levels are governed by blood ammonia levels, the rates of influx and efflux of ammonia to and from the brain, the rate of ammonia production from the endogenous reactions and the rate of endogenous reactions utilizing ammonia.

BLOOD AMMONIA

Though several tissues produce ammonia, not much of this ammonia enters into the blood under normal physiological conditions. Major portion of the blood ammonia is derived from the bacterial metabolism of dietary nitrogenous compounds in the gut (Onstad and Zieve, 1979). In addition, ammonia is also produced by the intestinal metabolism of glutamine (Weber and Veach, 1979; Onstad and Zieve, 1979) and it has been shown that much of this ammonia along with that generated by the bacterial metabolism is released into the hepatic portal vein (Onstad and Zieve, 1979). This ammonia is converted to a large extent to urea in the liver and then eliminated. However, in certain pathological states such as cirrhosis, fulminant hepatic failure and in disorders of urea cycle enzymes, gut derived ammonia escapes the detoxification process in liver and enters into systemic circulation.

TRANSPORT OF AMMONIA ACROSS THE BLOOD BRAIN BARRIER

Ammonia is freely permeable across the BBB, which maintains a selective permeability for the passage of

substances from blood into brain. A large body of evidence indicates that ammonia enters the brain largely by diffusion and not by saturable transport (Cooper and Plum, 1987). Ammonia exists in two forms i.e., the unprotonated gaseous form (NH_3) and protonated ionic form (NH_4^+). At physiological pH (7.4), about 97% of the ammonia exists in the protonated form (S.C.O.A., 1979). It has been shown that the protonated ionic form of ammonia is impermeable while the unprotonated form of ammonia, due to its high lipid solubility is freely permeable across the membranes of the blood vessels and brain (Milne et al., 1958; S.C.O.A., 1979; Cooper and Plum, 1987). Existing evidence indicates that ammonia enters brain in unprotonated form by a process of simple diffusion. It has also been reported that the brain and blood pH would have a regulating influence on the diffusion of ammonia from blood into brain (Stabenau et al., 1959; Warren et al., 1960; Warren, 1962; Moore et al., 1963; Phelps et al., 1977; Lockwood et al., 1980; Raichle and Larson, 1981). Ammonia content in brain is higher than that of blood, yet an efflux of ammonia from brain into blood has not been conclusively demonstrated in normoammonemic states while such an efflux was demonstrated in hyperammonemic animals (Cooper and Plum, 1987). Though some studies have indicated that the blood derived ammonia does not mix freely with the endogenously produced ammonia, there are evidences to indicate that atleast a portion of the former is utilised in brain (Cooper

and Plum, 1987).

ENDOGENOUS PRODUCTION OF AMMONIA

An insight into the metabolic compartmentation of glutamate, which is very closely involved in the production and utilisation of ammonia in the brain, is essential to understand ammonia metabolism in brain. Several studies have shown that there is a large pool and a small pool of glutamate in the brain which do not intermix with each other. The small pool is associated with the production of glutamine from glutamate while the large pool is associated with the hydrolysis of glutamine to glutamate. There are evidences to indicate that the small pool of glutamate is localised in astrocytes (Martinez-Hernandez et al., 1976; Norenberg and Martinez-Hernandez, 1979) and is synthesised from various substrates like glucose, ketone bodies, BCAA etc. The large pool of glutamate is localised in the neurons and is majorly synthesised from glucose and glutamine. It was concluded that the small pool of glutamate is involved in ammonia detoxification and the large pool with ammonia production (Berl et al., 1962; Benjamin and Quastel, 1972; 1975). Knowledge pertaining to the precise contribution of these to the cerebral ammonia pool is yet to be investigated. Of the several reactions involved in ammonia production, three are supposed to be more important than the rest. It must be mentioned that glutamate family of amino acids (glutamate, GABA, aspartate, alanine and glutamine) are intimately

associated with the production of ammonia. Following are some of the major reactions involved in the production of ammonia in brain.

GLUTAMINASE

This enzyme produces ammonia by hydrolysis of the amido group of glutamine.

Glutamine + H_2O ---> Glutamate + NH_4^+

Two isozymes of this enzyme are known to be present in mammalian tissues, the maleate activated glutaminase and phosphate activated glutaminase. The former is present in the brain but its function remains a matter of contradiction. It was found to be very similiar to γ-glutamyl transferase. The phosphate activated glutaminase is present in the outer and inner leaflets of the inner mitochondrial membrane (Kvamme, 1983). Cellular distribution of this enzyme in brain is, a subject of much controversy. Subsequent to the theory of metabolic compartmentation of glutamate, it was postulated that this enzyme might be associated to a large extent or even exclusively with the large compartment of glutamate which is supposed to be localised in the neurons. It was also postulated that this enzyme might be absent in the small compartment of glutamate, supposed to be localised in the astrocytes (Benjamin and Quastel, 1972;1975). However, equivocal results were obtained when this hypothesis was experimentally verified. Some of the earlier investigators reported an exclusive localisation of this enzyme in the

synaptosomal fraction but not in the astrocytes (Bradford and Ward, 1976; Bradford et al., 1978). However, Subbalakshmi and Murthy, (1985b) demonstrated a substantial activity of this enzyme in the astrocytes isolated from adult rats. A similar report was made by Schousboe et al., (1979) and Kvamme et al., (1982) in astrocytes in primary cultures. This controversy, on the localisation of this enzyme, bears an important relation in deciding the site of production of ammonia in the brain. If the postulates of metabolic compartmentation are true, then the ammonia production is associated with the neurons but not with the astrocytes. On the contrary, since this enzyme is also localised in the astrocytes, ammonia is produced both in neurons and astrocytes. Astroglial and synaptosomal glutaminases were shown to have different properties (Kvamme et al., 1982). Astroglial enzyme was shown to be inhibited by glutamate but not by ammonia while synaptosomal enzyme is inhibited by both. As glutamate concentration in the brain is very high , probably under physiological condition this enzyme is inhibited atleast to a certain extent. It has been estimated that this enzyme must be expressing approximately 5-10% of its activity under in vivo condition (Kvamme, 1983). If this were to be true then the reaction mediated by this enzyme may not be a major source of ammonia. It is worth mentioning that the results obtained by Lai and Clark (1976) does not support this concept as they observed no differences in the

properties of glutaminase in the synaptosomal and nonsynaptosomal preparations.

GLUTAMATE DEHYDROGENASE

The enzyme, glutamate dehydrogenase, mediates a reversible reaction. In one direction it oxidatively deaminates glutamate producing α -KG and ammonia. In the reverse direction it brings about the reductive amination of α -KG with ammonia resulting in the formation of glutamate.

$$\text{GLUTAMATE} \underset{\text{NADH}}{\overset{\text{NAD}^+}{<\!\!-\!\!-\!\!-\!\!-\!\!-\!\!-\!\!-\!\!-\!\!-\!\!>}} \alpha\text{-KG} + NH_3$$

Studies on subcellular localisation indicated this enzyme to be localised in the mitochondrial matrix. This is contested by the studies of Lai et al., (1987) who reported the presence of this enzyme in the nucleus. It was proposed that the combined action of aminotransferases and glutamate dehydrogenase can bring about an effective degradation of any amino acid and this pathway has been referred to as the transdeamination pathway (Martin et al., 1983). Earlier observations suggested that atleast 35-50% of cerebral ammonia is produced in this reaction (Cooper and Plum, 1987). It was postulated that this enzyme in the large compartment of glutamate (neurons) may be involved in the degradation of glutamate and production of ammonia (Benjamin and Quastel, 1972; 1975). However, in the small compartment of glutamate (astrocytes) it is supposed to be involved in the synthesis of glutamate from α-KG (reverse reaction)(Berl, 1974; 1983;

Benjamin and Quastel, 1972; 1975). However, results obtained by Yu et al., (1982) contradicted this hypothesis by demonstrating that the rate of $^{14}CO_2$ production from [1-^{14}C] glutamate was much higher in the astrocytes than in the neurons. Moreover, production of $^{14}CO_2$ from [1-^{14}C] glutamate in the astrocytes was observed to be resistant to amino-oxyacetic acid suggesting that transamination plays a very minor role in this reaction. Subbalaksmi (1984) and Subbalakshmi and Murthy (1985b) also reported that the activity of this enzyme (when measured in the direction of glutamate synthesis) was much higher in the synaptosomal enriched fraction than in the astrocytic fraction. Studies of Cooper et al., (1979; 1985) also support the concept that this enzyme may mediate a net breakdown of glutamate rather than its synthesis.

PURINE NUCLEOTIDE CYCLE

This cycle comprises of three reactions and is supposed to play a major role in the production of ammonia in tissues like muscle. The sequence of the reactions are as follows:

1. AMP ----------> IMP + NH_3
2. IMP + ASPARTATE + GTP ------> ADENYLOSUCCINATE + GDP + Pi
3. ADENYLOSUCCINATE ----------> AMP + FUMARIC ACID

From the above reactions, it is quite evident that the net effect of this cycle is the oxidation of the aspartate to fumarate and ammonia consequent to the hydrolysis of GTP to

GDP and Pi. There are very few experimental evidences to assess the actual contribution of this cycle to cerebral ammonia pool.

In addition to these three major reactions, ammonia is also produced by the action of monoamine oxidase on catecholamines, in the glutamine transaminase-W-amidase pathway, glycine synthase, serine and threonine dehydratases. Despite the redundancy in the reactions generating ammonia, very low levels of ammonia are maintained in brain. This suggests there must be an efficient mechanism for the removal and/or utilization of ammonia.

DETOXIFICATION OF AMMONIA

Unlike liver where a major portion of ammonia is converted to urea, ammonia detoxification in brain does not occur by way of urea formation. This is due to the fact that the complete complement of the urea cycle enzymes is not present in the brain (Sadasivudu and Rao, 1974; 1976). Hence, it was postulated that in brain ammonia is detoxified by its conversion to glutamate (glutamate dehydrogenase reaction) and to glutamine in the reaction mediated by glutamine synthetase.

GLUTAMATE DEHYDROGENASE

As was discussed earlier, this enzyme can synthesise glutamate from α-KG and ammonia by reductive amination. Earlier studies, especially those on metabolic compartmentation, postulated that this enzyme in the astro-

cytes may be involved in the synthesis of glutamate from ammonia (Berl and Clarke, 1983). This hypothesis was very well accepted because of the fact that blood ammonia enters rapidly into the astrocytes, and that the enzyme glutamine synthetase is also localised in the same cellular compartment (Martinez-Hernandez et al., 1977). Berl (1974) observed that the ^{15}N label from ^{15}N-ammonia is preferentially incorporated into the α-amino group and amido groups of glutamine thus supporting the above concept. However, results obtained by Cooper et al., (1979; 1985) were contradictory to this concept. These investigators observed that when ^{13}N labelled ammonia was administered, much of the label was found in the amido group of glutamine than in the α-amino group of glutamate and glutamine. Based on these results they proposed that the reaction mediated by glutamate dehydrogenase may not be the major pathway for ammonia detoxification. In support of this concept, Yu et al., (1982) observed that the major metabolic fate of glutamate in primary cultures of astrocytes is oxidative deamination. Yudkoff et al., (1983a,b) also observed that the incorporation of ^{15}N label into the α-amino group of glutamate decreases in the presence of high concentration of ammonia in explant cultures. Similarly, Subbalakshmi and Murthy (1983b) and Subbalakshmi (1984) observed that the administration of ammonium salts suppressed the activity of glutamate dehydrogenase in isolated astrocytes. Thus, the contribution of this enzyme to the glutamate

pool in the brain and particularly in the astrocytes and its significance in ammonia detoxification is yet to be clarified.

GLUTAMINE SYNTHETASE

The enzyme glutamine synthetase mediates the synthesis of glutamine from glutamate and ammonia and this reaction requires ATP.

Glutamate + ammonia + ATP --------> Glutamine + ADP + Pi

Du Ruisseau et al., (1957) reported that ammonia is metabolised majorly to glutamine in the brain and this has been repeatedly confirmed. Tracer experiments with ^{15}N and ^{13}N ammonia revealed that a major portion of the label is incorporated into the amido group of glutamine thus supporting the above concept (Berl, 1974; Cooper et al., 1979; 1985; Yudkoff et al., 1983a,b). Initial studies revealed an exclusive localization of this enzyme in the astrocytes, (Martinez-Hernandez et al., 1977; Norenberg and Martinez-Hernandez, 1979). However, presence of this enzyme in the neuronal components isolated from the brain such as synaptosomes and neuronal perikarya was also reported (Subbalakshmi, 1984; Rani et al., 1983; Subbalakshmi and Murthy, 1985 a,b). Evidence in support of this observation has not been obtained in neurons in primary cultures.

In addition to these two major reactions, ammonia is also utilised to a smaller extent for the biosynthesis of asparagine and glycine. Net contribution of these two

pathways in the detoxification of ammonia is yet to be assesed.

NEUROTOXIC EFFECTS AMMONIA AND HYPERAMMONEMIC CONDITIONS

There is a large amount of clinical and experimental evidence to implicate ammonia in the etiology of various neurological and neuropsychiatric disturbances in the central nervous system. As the blood and brain ammonia levels equilibrate very rapidly, any increase in the blood ammonia level will be reflected in the brain. Hence, conditions associated with an increase in blood ammonia levels are usually associated with an elevation in the brain and CSF ammonia levels. Clinically, majority of the hyperammonemic states are always associated with hepatic dysfunction such as cirrhosis of liver, hepatitis etc. In addition to these, there are congenital disorders especially those associated with the disturbances in the metabolism of amino acids and of urea cycle enzymes which would lead to hyperammonemia. In their review, Cooper and Plum (1987) have listed out about 20 human illnesses associated with hyperammonemic states. A brief description of the acquired and congenital hyperammonemic disorders is given below.

ACQUIRED HYPERAMMONEMIA

As liver is the major site for detoxifying the systemic ammonia load, any dysfunction of the liver is inevitably associated with an enhancement of ammonia levels in the blood. A direct relationship has been established between

hepatic functioning and blood ammonia levels. A wide spectrum of neurological, neuropsychiatric and neuromuscular symptoms have been observed in various states of hepatic dysfunction. These changes have been termed as Hepatic encephalopathy (Schenker et al., 1974). In the early stages of HE, mental, personality and emotional changes were found to be very subtle and could be observed only by those who are close to the patient. As the condition deteriorates, hypothermia, hyperventillation, confusion and drowsiness are seen. Further deterioration leads to a comatose state. In addition to these symptoms neuromuscular changes such as asterixis, hyper-reflexia, unsustained clonus and deterioration of pupillary and corneal reflexes were also observed. Convulsions, decerebrate rigidity and decortical posture are not uncommon (Schenker et al., 1974).

The comatose condition observed in the HE has been categorised into grade I to V depending upon the rapport between the environment and the patient, (Polli, 1974). Hepatic encephalopathy due to liver dysfunction is usually seen in cirrhosis of liver and due to acute necrosis of hepatocytes. In the former condition portal hypertension develops due to lipid accumulation in the liver leading to formation of anastamosis of the portal blood vessels. The portal blood bypasses the liver and enters the systemic circulation without being detoxified. A similar situation, i.e., the entry of portal blood into systemic circulation is

also seen during the acute necrosis of the liver cells, in hepatitis and during the ingestion of hepatotoxins. The degree of neurological dysfunction and cerebral pathology is highly variable and depends upon the extent of hepatic dysfunction (Schenker et al., 1974). One of the prominent histopathological change in hepatic encephalopathy is cerebral edema (Schenker et al., 1974). Other pathological changes are more prominent in astroglia than in neurons. A condition known as Alzheimer type II astrocytosis, wherein astrocytes proliferate, enlarge and finally undergo hydropic degeneration is seen in hyperammonemia of various etiologies The proliferation of the glial cells was supposed to be by endomitosis without the formation of spindle fibres. In this state the glial cells are swollen, accumulate glycogen granules and the glial fibrillary acidic protein disappears. Prior to degeneration, astrocytes undergo hypertrophy and hyperplasia. Occasionally neurons tend to show demyelination and degeneration. Histopathological changes observed in the astrocytes are believed to be due to the detoxification of ammonia by the formation of glutamate and glutamine which depletes the energy stores in these cells. It has also been postulated that the swelling of the protoplasmic astrocytes is due to the changes in the ionic composition of the cell, brought about by the altered ionic fluxes (Kline et al., 1971; Cole et al., 1972; Zamora et al., 1973; Gibson et al., 1974; Norenberg and Lapham, 1974; Cavanaugh and Kyu, 1974;

Guiterrez and Norenberg, 1975; 1977; Cavanaugh, 1974; Putnam et al., 1976; Norenberg, 1976; 1977; 1981; Taylor et al., 1979).

However, the etiology of the histopathological and neurological changes observed during HE is not clearly understood. Enhanced blood and CSF ammonia levels are a common laboratory finding in experimental animals and humans with HE. However, there is no corelation between blood ammonia levels and the neurological status (Schenker et al., 1974). As a result, the concept implicating ammonia in the etiology of HE has been doubted and other mechanisms were proposed.

False neurotransmitter hypothesis has been proposed to explain the etiology of HE (James et al., 1979). Due to the changes in the ratio of AAA to BCAA in the blood, it was proposed that the AAA flood the the nervous system. Hence, aromatic amino acid content in brain would be enhanced beyond the saturation limits of both tyrosine and tryptophan hydroxylases. Excess of AAA would be decarboxylated directly (bypassing the initial hydroxylation step) resulting in the formation of tyramine, tryptamine and phenylethanolamine. These amines displace the catecholamines (epinephrine, norpinephrine and dopamine) and serotonin from the synaptic vesicles and are released into the synaptic cleft under appropriate condition as false neurotransmitters. Enhanced levels of AAA, aromatic amines and their derivatives in brain, in hepatic encephalopathy, lent credence to this

hypothesis. Moreover, intravenous perfusion of BCAA or BCKA, which restores the ratio of BCAA/AAA and thereby their transport into the nervous system, was observed to improve the neurological status in the experimental animals and humans with HE (Fischer et al., 1976; Jellinger et al., 1978; Morgan et al., 1978; Chase et al., 1978; James et al., 1979; Jeppson et al., 1980; Marchesini et al., 1982; Mans et al., 1984; Schafer et al., 1985 and Hilgier et al., 1985). However, in some of the recent studies no such beneficial effects were observed upon the perfu sion of BCAA in HE (Erikson et al., 1982; McGhee et al., 1983; Wahren et al., 1983; Mans et al., 1984).

Mercaptans, which are derived from the metabolism of the sulphur containing amino acids by the intestinal flora, acting synergistically with ammonia and short chain fatty acids were also proposed to be responsible for the neurological dysfunction in HE (Zieve et al., 1974 a,b). It has also been proposed that a synergistic action of more than one toxin may be responsible for the etiology of HE.

The most recent hypothesis deals with the abnormalities with the functioning of the GABAergic neurotransmission. It is believed due to alteration in the BBB, GABA produced (by the bacteria) in the intestine crosses the BBB and floods the nervous system. As a result, GABAergic neurotransmission is enhanced and thus the cerebral functioning is altered (Baraldi and Zeroli, 1982; Schaffer and Jones, 1982).

Despite the controversies in the etiology of HE and the multiplicity of the hypothesis explaining the pathophysiology, ammonia is still believed to be the major neurotoxin (Cooper et al., 1984).

CONGENITAL DISORDERS INVOLVING HYPERAMMONEMIA

Congenital hyperammonemia may be due to the derangement in the production or the utilization of ammonia. There are two disorders associated with glutamate dehydrogenase (GDH). Reye's syndrome is a congenital disorder with lowered levels of GDH in liver. It has been reported that an acute insult by unknown factors rupture the mitochondria in the liver (Reye et al., 1963). Mitochondrial enzymes like carbamoyl synthetase and GDH are lost, the capacity to detoxify ammonia decreases and hyperammonemia ensues. Another disorder involving cerebral glutamate dehydrogenase was recently reported (Plaitakis et al., 1982; 1984).

No reports are available on congenital deficiency of glutamine synthetase either in human or in laboratory animals. This may be due to the premature death of the embryos, as glutamine participates not only in the detoxification of ammonia but also in the synthesis of nucleic acids and other vital compounds (Martin et al., 1983). Several congenital disorders of urea cycle enzymes associated with hyperammonemia and neurological defects have been reported. The degree of derangement and the clinical status of the patient ranges from very mild to very severe, (Hsia,

1974). Hyperammonemia was also observed in congenital disorders of amino acid metabolism such as Maple Syrup Urine Disease, hypervalinemia, hyperlysinemia and derangements in ornithine metabolism (Hsia, 1974; S.C.O.A, 1974).

MECHANISM OF AMMONIA TOXICITY

Several hypotheses were proposed to explain the mechanism of ammonia toxicity. These hypotheses may be classified into two categories; those which deal with (1) ionic fluxes and (2) metabolic fluxes. A brief description and dicussion of these hypotheses are given below.

EFFECTS OF AMMONIA ON IONIC FLUXES:

Maintainance of ionic gradients is essential for cerebral activity. It has been observed that about one-third of the energy synthesised in the brain is utilised for maintainance of the ionic gradients especially by Na^+,K^+-ATPase (Berl, 1974). Changes in the ionic composition also has a wide range of effects on the cerebral metabolism (Lowry and Passonneau, 1966). Transport of several neurotransmitters is dependent on the availability of Na^+ ions. In addition to these metabolic effects, ionic gradients also have a vital role in the release of the neurotransmitter and on the resting membrane potential of neuron. It was shown that the ionic radii of the ammonium and K^+ ions are similar and thus NH_4^+ ion can replace K^+ ions (Skou, 1960). Elevation of cerebral Na^+,K^+-ATPase activity was observed in hyper-ammonemic states, which would alter the ionic gradients and

the resting membrane potential (Hawkins et al., 1973; Sadasivudu et al., 1977, 1978; Subbalakshmi and Murthy, 1981). As this enzyme is also involved in the release of neurotransmitters, any changes in the activity of this enzyme by NH_4^+ might also affect the release of neurotransmitters. Extracellular NH_4^+ ion was also shown to inhibit the outwardly directed chloride pump which results in the disinhibition of the post synaptic neurons (Lux et al., 1970; Llinas et al., 1974; Raabe and Gunmit, 1975). Benjamin et al., (1978) reported an influx of Cl^- ions into the brain slices in the presence of NH_4^+ ions. It is possible that these changes in the ionic fluxes may be associated with the recently reported changes in the fluidity of the neuronal membranes in the presence of NH_4^+ (O'Connor et al., 1984).

EFFECTS OF AMMONIA ON CEREBRAL ENERGY METABOLISM

One of the very old and controversial concepts is that ammonia affects the cerebral energy metabolism. Acute adminisration of ammonium salts enhances the A/V difference for glucose across BBB suggesting an increase in the cerebral utilization of glucose in hyperammonemic states (Hawkins et al., 1973). Under these conditions, a fall in cerebral glycogen levels was observed while in chronic hyperammonemia there was an increase in the same (Norenberg, 1981). Ammonia is known to stimulate the activity of PFK, a rate limiting enzyme in the glycolytic pathway thus channelling more glucose into this pathway (Lowry and Passoneau, 1966).

Hawkins et al., (1983) reported an increase in the levels of the glycolytic intermediates in brain in acute hyperammonemic condition. Pathophysiological concentration of ammonia was observed to elevate cerebral lactic acid both in vivo and in vitro conditions (Hawkins et al., 1973; Benjamin et al., 1978). It was postulated that the enhanced glucose utilization might account for the enhanced lactic acid production (Hawkins et al., 1973). However, studies in primary cultures of astrocytes and neurons indicated that NH_4^+ in pathophysiological concentration (3mM) had no effect on the glucose oxidation, (Lai J.C.K., Murthy Ch.R.K., Cooper, A.J.L., Hertz, L., personal communication).

Effects of ammonia on the operation of citric acid cycle is a subject of much controversy. It was postulated by Bessman and Bessman (1955) that ammonium ion promotes the synthesis of glutamate in the reaction mediated by GDH and thereby depletes cerebral ∝- KG levels and thus impairs the operation of the TCA cycle and energy production in brain. In support of this hypothesis Eiseman et al., (1953) and Schenker and his associates (1967) observed a decrease in the cerebral ∝-KG levels especially in the brainstem. McCandless and Schenker (1981) reported a decrease in the cerebral ATP levels in the microdissected areas of reticular activating system of the brain stem in hyperammonemic states. A similar observation was made by Bessman and Pal (1976). McKhan and Tower (1961) reported that high concentration of ammonia

suppressed the oxidation of pyruvate and ∝-KG in the cerebral mitochondrial preparation. Additional support was provided by the observation of Lai and Cooper (1986) that ammonium ion inhibits the activity of ∝-KGDH in the mitochondrial preparations.

Despite these evidences, this hypothesis is not accepted equivocally because of other contradictory observations. Hindfelt and Siesjo (1971) observed no changes in the contents of ∝-KG and ATP in brain in hyperammonemic states. Varagara et al., (1974) reported an increase in ∝-KG content in brain under these conditions. A similiar observation was made by Hawkins et al., (1973). It is interesting to note that the oxidation of pyruvate was unaffected by the presence of pathophysiological concentrations of ammonia in primary cultures of astrocytes and neurons in the presence of glutamine (Hertz et al., 1987). Observations made in this laboratory also indicate that the activities of PDH and the enzymes of TCA cycle are enhanced by the acute, subacute and drug induced hyperammonemic states suggesting an increased flux of the carbon skeleton through the TCA cycle (Ratnakumari et al., 1985; 1986). Thus, the effects of ammonia on the TCA cycle and energy metabolism are yet to be resolved. These discrepancies on the effects of ammonia might be due to the differences in the experimental animals used in these studies, mode of inducing hyperammonemia, methods adopted to arrest post-mortem changes and the regions selected for the

analysis. Though controversial results were obtained in the levels of the metabolites of citric acid cycle and of ATP, it is quite possible that changes might occur in distinct cellular compartments such as astrocytes and neurons.

EFFECTS OF AMMONIA ON THE MALATE-ASPARTATE SHUTTLE

Another hypothesis proposed to explain the neurotoxic effects of ammonia is its effects on the operation of the malate-aspartate shuttle (Hindfelt, 1975). This shuttle is responsible for the transport of reducing equivalents across the mitochondrial membrane. Of the several mechanisms regulating carbohydrate metabolism, the redox state of the cell, expressed as ratio of reduced pyridine nucleotides to oxidised pyridine nucleotides (NADH/NAD), is of prime importance. Several dehydrogenases participate in the oxidation of glucose, both in cytosol and mitochondria, require a continuous supply of NAD^+. It was shown that their activities are suppressed by excess of NADH, hence it is imminent that NADH generated must be reoxidised to NAD^+. In the mitochondria, NADH is oxidized in the electron transport chain and thereby NAD^+ is generated under aerobic conditions. Due to the lack of electron transport chain in the cytosol, NADH cannot be oxidized directly to NAD^+. Moreover, NADH is not exported into mitochondria as the mitochondrial inner membrane is impermeable to these and other nucleotides. Therefore, it was proposed that NADH will be converted to NAD^+ in the cytosol itself and the reducing equivalents will

be transported into the mitochondria in the form of metabolites. Several such shuttle mechanisms have been proposed for the transport of reducing equivalents across the mitochondrial membrane and of these malate-aspartate shuttle is the major one in brain (Siesjo, 1978). This shuttle involves the participation of cytosolic and mitochondrial isozymes of AAT, MDH, the mitochondrial dicarboxylic acid carriers and glutamate-aspartate carrier.

Operation of this shuttle is as follows. NADH generated in the cytosol is utilized for the production of malate from OAA by the cytosolic MDH. Malate, produced in the cytosol, is transported into mitochondria in exchange for ∝-KG. As a result, malate is transported into mitochondria while ∝-KG into cytosol. Mitochondrial MDH acts on malate and produces OAA which undergoes transamination with glutamate to produce ∝-KG and aspartate. ∝-KG transported into the cytoplasm undergoes transamination with aspartate in a reaction mediated by cytosolic AAT. As a result, OAA is regenerated in the cytosol concomitant to the production of glutamate. Cytosolic glutamate is transported into mitochondria in exchange for mitochondrial aspartate and this process is mediated by the appropriate carrier protein (Siesjo, 1978). Importance of this shuttle for cerebral glycolysis has been repeatedly demonstrated. It has been shown that inhibition of AAT activity with B-methylene aspartate would interfere with cerebral glucose metabolism, (Fitzpatrick et al., 1983).

Elevation of $^{14}CO_2$ production from [1-^{14}C]pyruvate by aspartic acid in the primary cultures of astrocytes supports the concept that cytosolic OAA serves as precursor for mitochondrial MDH (Murthy and Hertz, 1988). It has been postulated that in the presence of excess of ammonia cytosolic pool of glutamate is diverted from the malate-aspartate shuttle towards glutamine synthesis. Consequently operational rates of this shuttle will be affected and thus the cerebral glucose metabolism (Murthy and Hertz, 1988). Accumulation of malate (Hindfelt and Siesjo, 1970) and decrease in levels of glutamate in brain (Cooper and Plum, 1987) in hyperammonemic states is in accordance with this concept. Similarly an increase in lactate production, lactate/pyruvate ratio, and cytosolic NADH/NAD ratios provides support to this hypothesis (Hindfelt and Siesjo, 1970; Hawkins et al., 1973; Polli et al., 1974). It was also shown recently that the production of $^{14}CO_2$ from [1-^{14}C]-pyruvate in primary cultures of astrocytes was suppressed by the addition of pathophysiological concentrations of ammonium acetate (3mM) in the absence of glutamate or glutamine. This effect was abolished by the addition of glutamate, aspartate and glutamine (Murthy and Hertz, 1988). Thus, precursors which replenish the cytosolic glutamate pool in hyperammonemic states are capable of supporting the operation of malate-aspartate shuttle even in the presence of ammonia. Thus, it appears to be true that ammonia affects cerebral

energy metabolism, but the mechanism is different from that proposed by Bessman and Bessman (1955).

The present investigation is aimed towards the understanding of the role of BCAA in hyperammonemic states. It was observed by Cooper et al., (1979) that the incorporation of ^{13}N -NH_3 into brain glutamate is only about 0.3% of that incorporated into the amide group of glutamine in normal and hyperammonemic states. These observations indicate that ammonia detoxification in brain is primarily by way of glutamine synthesis rather than by way of reductive amination of α-KG in the reaction mediated by GDH. These results also indicate that that glutamate required for glutamine synthesis is not provided by the GDH reaction and is derived from other sources. This concept is not in accordance with that proposed by Berl (1974). Subbalakshmi and Murthy (1983) reported that GDH activity in isolated cortical astrocytes is suppressed by about 30-40% in acute hyperammonemic states. A similar result was also observed in cerebellar astrocytes (Rao, V.L.R., personal communication). The suggestion made by these investigators with respect to the source of glutamate for the synthesis of glutamine is in agreement with that of Cooper et al., (1979). It was also observed that the oxidation of [1-^{14}C]- and [U-^{14}]- glutamate in primary cultures of astrocytes was suppressed by the pathophysiological concentrations of ammonia (Yu et al., 1984). Studies with ^{15}N ammonia in cerebellar explants carried out by Yudkoff et al., (1983 a,b) also supported this observation. Further, it was observed that the GDH in the extracts of

primary cultures of astrocytes was suppressed by ammonia, albeit at a high concentration (Hertz et al., 1986). Duffy and Plum (1982) postulated that the glutamate required for the synthesis of glutamine might be generated in the transamination reaction rather than reductive amination of ∝-KG.

Of the several transaminases in the brain AAT exihibits the highest activity (Benuck et al., 1971; 1972). However, this enzyme appears to play a minor role in providing the glutamate for glutamine biosynthesis for following reasons. 1. The equilibrium constant of this reaction is close to one suggesting that the direction of reaction depends upon the concentration of the reactants and products. As glutamate concentration exceeds that of aspartate, it is reasonable to assume that this enzyme is involved more in synthesis of aspartate than in the direction of glutamate. 2. If aspartate is to be utilized as a substrate for the production of glutamate by transamination, a continuous supply of aspartate is required to provide amino groups. There is no other reaction, except that of AAT, in which aspartate is synthesized. Next to AAT, AlAT expresses the highest activity and the reasons mentioned above holds good for considering alanine as a major precursor for the synthesis of glutamate. Hence, Duffy and Plum (1982) suggested that glutamate required for glutamine synthesis may arise by transamination of ∝-KG in the small pool with amino acids that are taken up by the brain from the blood. They further

suggested that "the BCAA, (leucine, isoleucine and valine) by transaminating with the small pool of α-KG reduces the neurotoxicity of ammonia by promoting the synthesis of glutamate in the small pool (astrocytic) of cerebral ammonia metabolism."

It well known that brain is one of the extrahepatic tissues capable of metabolizing the BCAA. The levels of BCAA in brain are low when compared to the levels of glutamate family of amino acids (McIlwain and Bachelard, 1971). As in any other tissue, the levels of these amino acids in brain depend on their transport from blood and their utilization in brain. As these are essential amino acids, they are not synthesized endogenously either in brain or in any other tissue. Hence, diet is the chief source of these amino acids for mammalian tissues. Brain depends upon the blood for the supply of BCAA. The transport of BCAA across the BBB is by a saturable carrier mediated mechanism. This carrier transports not only leucine, isoleucine and valine but also aromatic amino acids (phenylalanine, tyrosine and tryptophan) and the sulphur containing amino acid, methionine. Hence, the rate of transport of BCAA across BBB depends not only on the absolute concentration of these amino acids in the plasma but also on the relative concentration of AAA i.e., the ratio of BCAA/AAA. Changes in the concentration of BCAA or AAA alters this ratio and would affect the transport process. In addition to transport, degradation of endogenous proteins

also provides BCAA required for the tissues. Muscle is said to be major tissue, especially under pathological conditions, which releases amino acids into the blood.

In mammalian tissues BCAA are utilised either for protein synthesis or oxidised to produce CO_2. The rate of incorporation into proteins varies with the rate of protein synthesis in the tissue and the type of protein synthesised. Initial step in the metabolism of BCAA is the transamination with ∝-KG resulting in the lting in the production of BCKA and glutamate. This reaction is mediated by BCAA-T. BCKA thus formed are oxidatively decarboxylated by BCKA-DH, which requires NAD, CoA and thiamine pyrophospate. It has been shown that the kinetics and structure of this enzyme are similar to that of PDH and ∝-KGDH. This enzyme is a complex of three enzymes.∝-keto acid dehydrogenase, lipoate-acetyl transferase and lipomide dehydrogenase. Wohleuter and Harper (1970) proposed that all the three BCKA are handled by a single enzyme complex in rat liver while Bowden and Connelly, (1968) provided evidence to indicate that keto-isovaleric dehydrogenase may be a seperate entity. Unlike the transaminases for which no regulatory mechanisms have been suggested, dehydrogenase is regulated by phosphorylation -dephosphorylation mediated by cAMP cascade system (Randle, 1971). Phosphorylation inactivates the enzyme while dephos-phorylation stimulates the same. Though the BCKA-DH has not been purified or characterised from the brain tissue, such

studies have been carried out in other tissues. Most of these studies indicate that this reaction is the rate limiting step in the metabolism of BCAA in the brain. Further metabolism of BCAA proceeds in several discrete steps (Fig 1) and, the end product varies with the type of BCAA (Fig 1). Thus, leucine yields acetoacetyl CoA and acetyl CoA, isoleucine yields acetyl CoA and propionyl CoA and valine yields only propionyl CoA. Acetyl CoA from BCAA will be finally oxidised in the TCA cycle. Propionyl CoA is converted to succinyl CoA thus anaplerotically replenishing the TCA cycle intermediates. An overall view of the metabolism of BCAA indicates that CO_2 production occurs in two discrete steps viz. one molecule of CO_2 is produced when BCAA are decarboxylated by BCKA-DH and the rest of the CO_2 is produced in the TCA cycle. Further, glutamate is also produced in two discrete steps viz. by transamination of BCAA with KG where only the α-amino group is transferred and the other from valine and isoleucine where the propionyl enters into the TCA cycle as succinyl CoA which will be converted to α- KG. The latter (i.e. β-KG) will be converted to glutamate either by transamination or by reductive amination. Strictly speaking glutamate is not synthesised from the carbons of leucine, as leucine forms acetyl CoA. However, leucine carbon are shown to be incorporated into glutamate.

Metabolism of BCAA in mammals is initiated in extrahepatic tissues especially in skeletal muscle. The activity

of the BCAA-T was observed to be the rate limiting factor for the metabolism of BCAA in liver .It has been shown that skeletal muscle plays a major role in the degradation of BCAA. The α-amino group of these amino acids is transferred to α-KG resulting in the formation of glutamate and BCKA. The glutamate is then converted to either to glutamine or transaminated to alanine in the skeletal muscle. These compounds ie. BCKA, glutamine and alanine are released into circulation and transported to liver. In this tissue, BCKA are oxidised while glutamine and alanine are converted to glucose in gluconeogenetic pathway . This cycle of reactions is known as glucose - alanine cycle. Next to muscle brain is the tissue that is actively involved in the metabolism of BCAA. As most of this information is available in general biochemistry books, no specific reference is given. However, it is interesting to note that compared to the muscle our understanding of the BCAA metabolism in brain is limited.

Earliest report on the metabolism of BCAA in the brain is that of Roberts et al., (1965) who has shown that brain has the capacity to oxidise BCAA. This report was later confirmed by Schepartz (1961); Swaiman and Milstein (1965); Odessey and Goldberg (1972); Chaplin et al., (1976); Shinnick and Harper (1976) and Brand, (1981) . It has been shown that the oxidition of leucine may account for 4-10% of the total CO_2 produced in brain. Patel and Balazs (1970) reported that the primary metabolic fate of BCAA in brain is

its incorporation into protein. However, studies of Chaplin et al., (1976) indicated that the production of CO_2 from leucine is about 23 times more rapid than its incorporation into protein. Later studies demonstrated that the carbon of BCAA is also incorporated into amino acids of the glutamate family (Cremer et al., 1975). Recent studies demonstrated that the nitrogen of BCAA is also incorporated into glutamate family of amino acids and infact this may account for about 30% of total glutamate synthesised by the brain (Berl, 1974; Yudkoff et al., 1983a, b).

Studies on metabolic compartmentation indicate that these amino acids are primarily metabolised in the astrocytes (Cremer et al., 1975). However, studies by Chaplin et al., (1976), Wiggins et al., (1978) and Buse et al., (1975) indicated that neuronal preparation have the capacity to oxidise BCAA. These results are in accord with those of Murthy and Hertz, (1987a,b) who have shown that all the three BCAA (both [U-^{14}C] and [1-^{14}C] are oxidised in neurons in primary cultures. These authors have also indicated that the rate of BCAA oxidation in the astrocytes in primary cultures is much higher than that observed in the neurons. Both in astrocytes and neurons, it was observed that the decarboxylation of [1-^{14}C]leucine occurs at a much higher rate than that of [U-^{14}C]leucine. There may be two explanations for this observation viz., the rate of decarboxylation of BCKA by BCKA-DH is much higher than the rate of subsequent reaction

and/or the dilution and distribution of the label in several of the intermediates of the BCAA metabolism of citric acid cycle and related metabolites (for eg., glutamate, glutamine, etc.) might result in lowering the amount of $^{14}CO_2$ produced from the ^{14}C labelled BCAA. Whatever the reason may be, this observation is consistent with the reports in white matter on higher activity of BCAA-T than that of gray matter of brain (Brosnan et al., 1985; Jessy and Murthy, 1985). This observation is consistent with the concept that these amino acids serve as precursors for the synthesis of lipids (Wiggins et al., 1978).

Studies have also been conducted on the effects of hyperammonemia on BCAA metabolism. An increase in the incorporation of ^{3}H label from BCAA into glutamate family of amino acids was observed in hyperammonemic states which suggests of an increase in the metabolism of BCAA (Cremer et al., 1985). Murthy and Hertz (1987b) demonstrated that the production of CO_2 from BCAA decreased by the acute addition of pathophysiological concentration of ammonia to primary cultures of astrocytes. Chronic exposure of these cultures to ammonia enhanced production of $^{14}CO_2$ from [U-^{14}C]leucine and abolished the suppresisve effects of ammonia on the CO_2 production from [1-^{14}C] labelled BCAA (Murthy and Hertz, 1987a).

THERAPEUTIC ROLE OF BCAA IN HYPERAMMONEMIC STATES

It is a common observation that the plasma and brain levels of BCAA are decreased in HE and during the infusion of

ammonia salts into normal animals (Eriksson et al., 1982). Mans et al., (1982) observed an enhanced transport of BCAA across the BBB in portacaval shunted rats while Shiota (1984) observed that $^{14}CO_2$ production from [1-^{14}C]leucine was enhanced in hyperammonemic states. It was argued by Fischer and Baldessarini (1976) that changes in the plasma composition of BCAA in HE would result in an altered ratio of BCAA/ AAA. As a result, the transport of these amino acids across the BBB will be lowered. Hence, it was felt that normalization of the plasma concentration of these amino acids might restore the clinical symptoms to normalcy, restored (Fischer et al., 1976). Indeed, they observed that the infusion of synthetic mixture of BCAA into dogs with portacaval anastamosis has improved the clinical status. Following this several reports have appeared in the literature on the efficacy of BCAA for ameliorating the neurological studies in hepatic encephalopathy. Not all these reports are unequivocal as some of them are contradictory. Recently it was suggested that this therapeutic regime may be effective in treating latent and mild hepatic encephalopathy than in the advanced state (Eriksson et al., 1982).

The main aim of the present study is to understand the role of BCAA and their metabolism in hyperammonemic states. Initially, activities of the two enzymes involved in the initial phase of BCAA metabolism i.e., BCAA-T and BCKA-DH were determined in different regions of brain in normal and

in hyperammonemic states of various etiologies. Further the distribution of these enzymes and the changes in their activities was studied in different subcellular and cellular fractions of brain in normal and hyperammonemic states. In the second phase of this study, the uptake and CO_2 production from BCAA were studied in different cellular and subcellualr fractions isolated from normal and hyperammonemic animals. Such a study would yeild information regarding the metabolic fate of these amino acids in hyperammonemic states. Further it will also provide information on the role of these amino acids in therapy of hyperammonemic states.

METABOLISM OF BRANCHED-CHAIN AMINO ACIDS

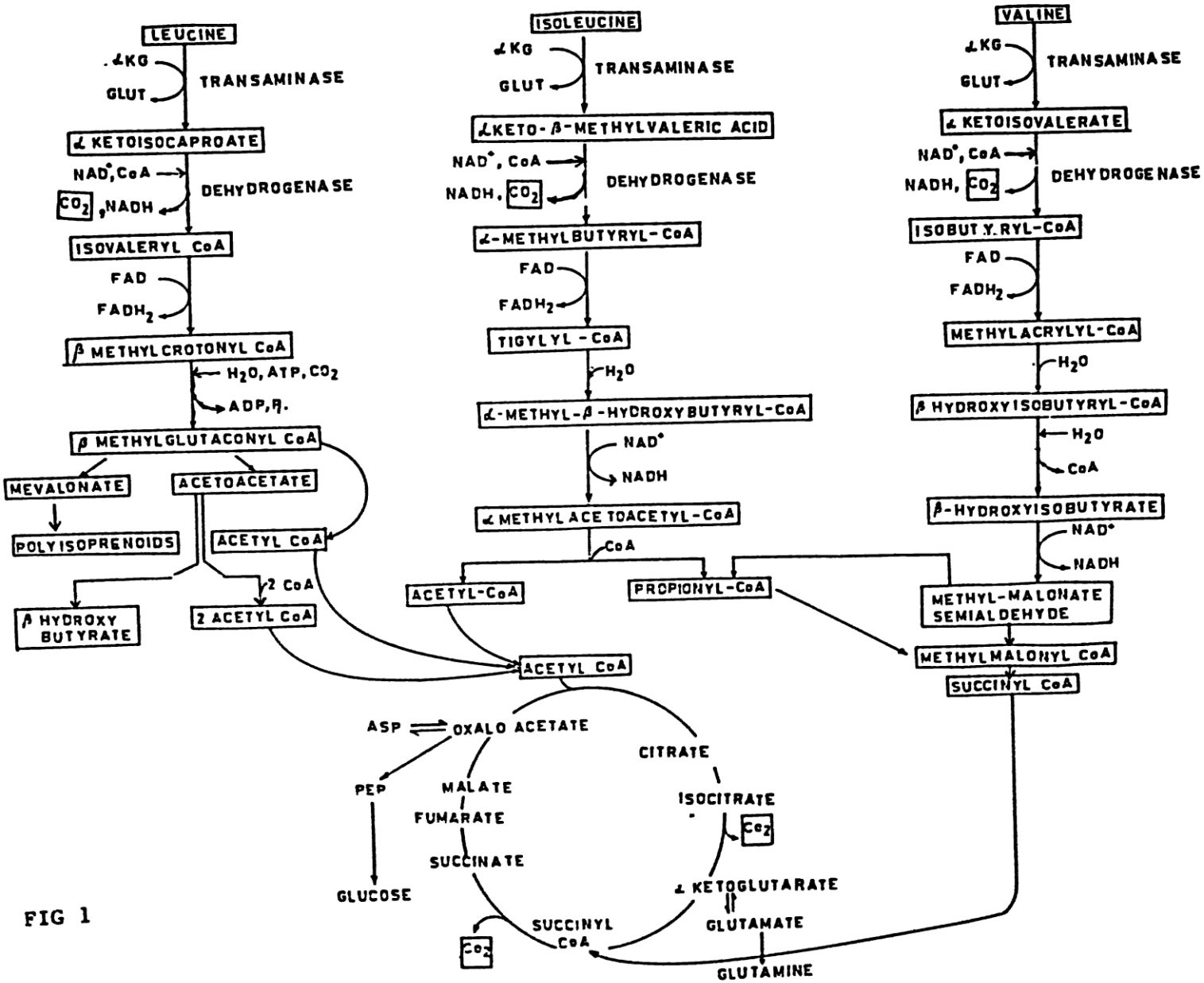


FIG 1

MATERIALS AND METHODS

MATERIALS

Nylon screens were purchased from Nybolt Co., Zurich; [U-^{14}C]leucine, [U-^{14}C]isoleucine, [U-^{14}C]valine, [^{3}H]leucine were supplied by Bhabha Atomic Research Centre, Bombay, India; Ficoll-400 was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

Acetylated trypsin, soybean trypsin inhibitor, leucine, isoleucine, α-KG, pyridoxal-5-phosphate, sodium pyruvate, sodium succinate, PPO, POPOP, HEPES, PMS were obtained from Sigma Chemical Co., St. Louis (U.S.A). Hank's balanced salt solution was purchased from Himedia, India. Hyamine hydroxide was obtained from BDH Chemicals Ltd., Poole, England. All other chemicals were of AnalaR or GR grade and procured locally. L-Methionine-DL-Sulfoximine was a kind gift from Prof.S.L.N.Rao, Osmania University, Hyderabad.

Spectrophotometric measurements were made with Gilford Spectrophotometer model 250 equipped with thermoprogrammer. For centrifugation, Remi C-24, Beckman J2-21 refrigerated centrifuges and Beckman L-8M ultracentrifuge were used. Microscopic observations were made with Zeiss photo-microscope. Radioactive measurements were made with Beckman liquid scintillation counter LS-1800.

ANIMALS

Adult albino rats from an inbred colony of Wistar strain were used. Animals were of either sex weighing 250-300 gms.

They were maintained under natural light and dark conditions. Food (balanced pellet diet from Pragathi Animal Feeds, India) and water were given ad libitum.

INDUCTION OF HYPERAMMONEMIA

I. Ammonium Acetate treatment

Ammonium acetate (2.5 mmoles/100gms body wt) was administered intraperitoneally using saline as a carrier, while the controls received none. Treated animals were decapitated during the preconvulsive (at the end of 20 min) or convulsive (at the end of 30 min) phases.

II. L-Methionine-DL-sulphoximine treatment (MSO)

L-Methionine-DL-sulphoximine was administered intraperitoneally using saline as a carrier. To induce acute toxicity, experimental animals were administered with a dose of 300 mg of MSO/kg body weight and the animals were sacrificed at the end of 3.5 hrs. For the subacute treatment the animals were administered 150 mg of MSO/kg body weight and were decapitated at the end of 17.5 hrs.

III. Partial hepatectomy

Animals were anaesthetized by exposing them to ether. An incision was made on the abdomen and the liver gently squeezed out of the abdomen through the incision and approximately 2/3 of the liver, thus exposed, was removed. Remaining portion of the liver was pushed back into the abdominal cavity. The incision was sutured and the animal was left to recover. For sham operated, an incision was made, liver was

squeezed out and was pushed back into abdominal cavity. Later the incision sutured. The animals were decapitated 8 hrs after the surgery was performed.

PREPARATION OF HOMOGENATES

Brains were quickly removed and washed with ice-cold saline. Cerebral cortex, cerebellum, brainstem, hippocampus and corpus striatum were separated at 4°C, the rest of the brain, designated as midbrain which includes thalamus, hypothalamus and related structures. Using a Potter Elvehjem homogeniser with a teflon pestle, a 10% homogenates (w/v) were prepared in 0.32 M sucrose.

PROCEDURE FOR ISOLATION OF SUBCELLULAR FRACTIONS

Subcellular fractions i.e., synaptosomes, mitochondria and cytosol were prepared by the method of Cotman (1974). Gray matter of the cerebral cortex was homogenised in 0.32M ice-cold sucrose (pH 7.0) in a Potter-Elvehjem type homogenizer with a teflon pestle to obtain a 10% (w/v) homogenate. The homogenate was centrifuged at 1,000g for 5 min and pellet (P_1) was discarded. The supernatant (S_1) obtained was further centrifuged at 15,000g for 12 min. The supernatant (S_2) was subjected to a centrifugation at 100,000g for 1 hr. The supernatant (S_3) was taken as cytosol and the pellet (P_3) contained the microsomal fraction. The pellet (P_2) obtained at 15,000g was suspended in 5 ml of 0.32M sucrose and layered on top of a preformed discontinous density gradient with 5 ml of 4%, 6% and 13% Ficoll in 0.32 M

sucrose. The pellet (P_4) obtained after centrifugation at 63,500g for 45 min was taken as mitochondria (nonsynaptic). The interface between 6% and 13% Ficoll was diluted 4 times with 0.32 M sucrose and centrifuged at 50,000g for 20 min. The pellet (P_5) contained synaptosomes. The synaptosomal and mitochondrial pellets were washed once with 0.32 M sucrose and resuspended in 0.32 M sucrose and protein concentration was adjusted to 1mg/ml (Fig 2.1).

CELL ISOLATION PROCEDURES

Neurons and Astrocytes

Both astrocytes and neurons were isolated using the method of Farooq and Norton (1978) with a few modifications. Cell isolation medium consisted of 8% (w/v) glucose, 5% (w/v) fructose and 2% (w/v) Ficoll in 10mM KH_2PO_4-NaOH buffer pH 6.0. Additional Ficoll was added to this medium to prepare solutions of increasing densities. Thus, a 7% Ficoll solution contained a total of 9% (w/v) Ficoll and so on. Cerebral cortex was isolated from the rats soon after decapitation. Grey matter was separated from the white matter and gently pressed on a wet Whatman No.1 filter paper to remove blood capillaries. Free hand slices of approximately equal thick ness were prepared and incubated at 37°C with constant shaking in 5 ml of isolation medium consisting of 0.1% acetylated trypsin. After 60 min, trypsin containing medium was aspirated and the tissue was washed once with the isolation medium and then with 5 ml of ice-cold isolation

medium containing 0.1% soybean trypsin inhibitor. This suspension was cooled to 4°C for 10 min and the tissue was washed once again with cell isolation medium. Tissue slices were then placed on 300 μ nylon screen and the cells were dissociated by gently tapping the tissue with a glass rod. The nylon screen was washed with isolation medium and the dissociated cells were collected. The crude cell suspension was then passed twice successively through monofilament nylon screens of pore diameter 300, 105, 80 and 55 μ to remove debris and broken processes. The crude cell suspension was centrifuged at 720g for 15 min at 4°C. The pellet (P_1), enriched with cells, was suspended in 7% Ficoll and centrifuged at 720g for 10 min to separate a neuronal cell enriched pellet (P_2). Supernatant (S_1) was diluted 1:1.25 with the isolation medium and centrifuged at 1,120g for 20 min to get a pellet enriched with astrocytes (P_3). Both these cell enriched pellets P_2 and P_3 were suspended seperately in cell isolation medium and layered seperately on two different preformed density gradient of 15% and 28% Ficoll and centrifuged at 8,500g for 10 min. The pellet contained relatively pure fraction of neurons and the interface between 15% and 28% Ficoll contained astrocytes. The interface was aspirated and diluted with medium and centrifuged to obtain a pure astrocytic pellet. Both the neuronal and astrocytic pellets were washed with 0.32M sucrose and were suspended in 0.32M sucrose (Fig. 2.2).

Cells from the experimental animals were isolated in a similar manner with slight modifications. The time for incubation with acetylated trypsin was reduced to 50 min and the crude cell suspension after the disaggregation step was passed through 300, 105, 80 μ nylon meshes. The time for each differential centrifugation was increased by an additional 5 min. Cell purity was determined by phase contrast microscopy. For enzyme assays and biochemical determinations the cells were suspended in 0.32 M sucrose and subjected to a cycle of freezing and thawing, while for metabolic studies, the isolated cells were suspended in 0.32M sucrose and used directly.

Isolation of oligodendrocytes

Oligodendroglia were prepared essentially by the method of Snyder et al., (1980) with slight modifications. Cell isolation medium was Hank's balanced salt solution containing 25mM Hepes and the pH of the final solution was adjusted to 7.2 with NaOH. Normal and experimental animals were decapitated and the cerebral hemispheres were isolated. After the removal of blood capillaries as described above, slices of gray matter were incubated with 0.1% acetylated trypsin for 30 min at 37^{o}C in Hanks balanced salt solution. After incubation, trypsin containing incubation medium was decanted, the tissue washed once with isolation medium and latter suspended in 5 ml of medium containing 0.1% soybean trypsin inhibitor and chilled for 5 min on ice. The tissue

was placed on 300 μ nylon mesh stretched over a porcelain Hirsh funnel and the tissue gently tapped with a glass rod to aid dissociation of cells. The nylon screen was washed with isolation medium and the dissociated cells were collected. Crude cell suspension was then passed twice successively through monofilament nylon screens of pore diameter 300, 105, 80, 50 and 30 μ . The final cell suspension was centrifuged at 1,120g for 10 min. The pellet enriched with cells obtained was suspended in 35% sucrose and was layered over a discontinuous sucrose gradient of 8 ml of 53% sucrose and 5 ml of 45% sucrose in medium. The tubes were centrifuged at 3,065g for 15 min in a swinging bucket rotor. Oligodendroglial cells were obtained in 53% sucrose gradient. The pellet and 53% sucrose gradient was diluted five fold with isolation medium and centrifuged to sediment cells. Oligodendroglial cells were suspended in 0.32 M sucrose, the purity of the preparations was monitored by phase contrast microscopy (Fig 2.3).

Cell number

An aliquot (10-20ul) of the cell enriched preparation was stained with methylene blue and the cell number was determined using a haemocytometer. Cells were counted at random and the cell number was calculated using the formula

$$\frac{\text{Number of cells/square X 1000}}{0.00625}$$

Neurons, Astrocytes and Oligodendroglia were counted using this method. Synaptosomal number could not be determined by this method as their size was beyond the resolution of the light microscope.

Cell viability

Viability of the isolated cells was determined by trypan blue exclusion method.

BIOCHEMICAL DETERMINATIONS

Protein determination

Protein content in the homogenates was determined by the method of Varley (1969) using biuret reagent. Aliquots ranging from 0.1 to 0.5 ml of the 10% homogenate was taken and made up to 1 ml with 1 N NaOH. Biuret reagent was added and absorbance was read at 540 nm. Bovine serum albumin was used as standard and the protein content was expressed as mg protein/gm wet wt. tissue.

Protein in the cellular and subcellular fractions was estimated by the method of Lowry et al., (1951). An aliquot of the subcellular fraction in 0.32 M sucrose was taken and the protein content estimated with Folin-Ciocalteau reagent (1N) using BSA as standard. The sample was diluted to 1 ml with 0.1 N NaOH and 5 ml of solution A (2% Na_2CO_3 in 0.1 N NaOH 48 ml, 1% NaK Tartarate 1 ml, 0.5% $CuSO_4 \cdot 5H_2O$ 1 ml) was added. After 10 min, 0.5 ml of Folin-Ciocalteau reagent (1N) was added. After 30 min at room temperature, optical density of the sample was read at 670 nm. Protein content in cell

preparations was expressed as pg/cell and for subcellular fractions as mg/gm wet wt. tissue.

Ammonia determination

Ammonia levels were determined in the whole brain homogenates. Rats were decapitated and the heads were allowed to fall directly into liquid nitrogen and were fixed for 10 min. Brains were quickly chiseled out and powdered in a stainless steel mortor pre-cooled with liquid nitrogen. Tubes, containing 5 ml of 5% (w/v) PCA, were weighed and the tubes were re-weighed after transferring the powdered tissue. Tissue was homogenized and centrifuged at 5,000 rpm for 10 min. Supernatant was used for determining ammonia content after neutralization with 2 N potassium bicarbonate.

Blood was collected from neck wound soon after decapitation and serum was seperated. To 0.5 ml of serum, one ml of 5% (w/v) ice cold PCA was added. The tubes were kept in ice for 30 min and centrifuged for 10 min at 5000 rpm. Supernatants were neutralised with 2 N $KHCO_3$ to pH 7.0 and centrifuged.

Ammonia was determined using phenol-hypochlorite reagent. To 1 ml of supernatant solution A (phenol 50 gms and sodium nitroprusside 250 mg/3.75 l water) and 2 ml of solution B (8.4 gms NaOH, 89.2 gms disodium hydrogen phosphate and 10 ml of 5% NaOCL/lit) were added. After 20 min the blue color was read at 630 nm. Ammonium chloride (0.1-1 µmole) was taken as standard. Ammonia content was expressed

as μmoles ammonia/gm wet wt. of tissue and the blood levels were expressed as μmoles of ammonia/ml of serum.

ENZYME ASSAYS

I.Lactate dehydrogenase (E.C.1.1.1.27)

This enzyme was used as a cytoplasmic marker by Johnson and Whittaker (1963). Lactate dehydrogenase was assayed by following the oxidation of NADH as described by Bergmeyer and Bernt (1974).

Pyruvate + NADH.H -------> Lactate + NAD^+

Reaction mixture (250 μl) contained 12 μmoles of potassium phosphate buffer (pH 7.5), 15 μmoles of sodium pyruvate, 0.045 μmoles NADH and 2 μg of enzyme protein. After a preincubation period of 5 min, pyruvate was added and NADH oxidation at 37°C recorded for 5 min at 340 nm. Enzyme activity was expressed as μmoles of NADH oxidised/mg protein/hr.

II. Succinate dehydrogenase (E.C.1.3.99.1)

Succinate dehydrogenase was assayed by following the reduction of INT as mentioned by Nandakumar et al.,(1973). It was assayed to detect the mitochondrial integrity as this enzyme is tightly bound to the inner membrane of the mitochondria.

Succinate + FAD ----------> Succinyl CoA + $FADH_2$

The reaction mixture contained 100 μmoles of Tris-phosphate buffer pH 7.6, 5 μmoles of INT, 1 μmole of PMS and

enzyme protein in a final volume of 0.5 ml. Substrate (40 µmoles of succinate) was added and incubated for 15 min. Blanks were run without the addition of substrate. The reaction was stopped by the addition of 2 ml glacial acetic acid. To this 5 ml of toluene was added, vortexed thoroughly and the tubes were centrifuged. Organic layer was taken and absorbance read at 500 nm.

Branched Chain Amino Acid Aminotransferase (E.C.2.6.1.6)

Branched chain amino acid aminotransferase was assayed using leucine, isoleucine and valine as substrates by the method of Taylor and Jenkins (1966) with a few moditications (Jessy and Murthy, 1985). The 2,4-dinitrophenylhydrazones of the corresponding∝-keto acids were extracted into cyclo-hexane from the acidic solution. The colour developed depends on the amount of BCKA produced.

BCAA +∝-KG -----> BCKA + Glutamate

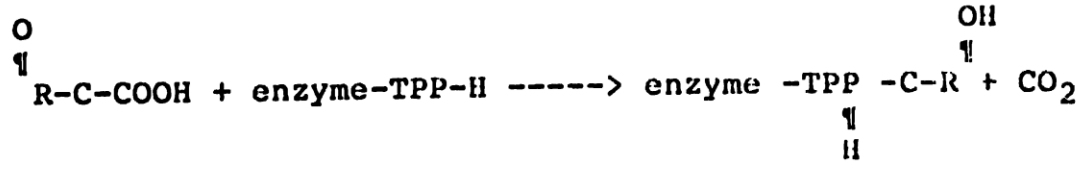
Reaction mixture (2.9 ml) contained 50 µmoles of potassium pyrophosphate buffer (pH 8.3), 30 µmoles of either leucine, isoleucine or valine, 10 µmoles of∝-KG, 1 µmole of pyridoxal-5-phosphate, and 1 µmole of 2-mercaptoethanol. Reaction was initiated by the addition of 0.1 ml of the homogenate and incubated for 15 min at 37°C. Substrate blanks were run simultaneously. Reaction was terminated by the addition of 1 ml of 2,4-dinitrophenylhydrazine (in 1 N HCl) and the hydrazone formation was allowed to proceed for 10 min at room temperature. Dinitrophenylhydrazones, thus formed,

were extracted into 4 ml of cyclohexane by vigorous shaking for 20 sec. Organic and aqueous phases were separated by centrifugation and the upper organic phase was transferred to 1.5 ml of 2% (w/v) sodium carbonate and the hydrazone was extracted from cyclohexane by vigorous shaking. Phase separation was aided by centrifugation at 5000 rpm for 5 min and the sodium carbonate layer was transferred to 3 ml of 1 N NaOH. Absorbance of aquoeus phase was measured at 440 nm after 5 min.

While assaying these enzymes in cellular and sub-cellular fractions, the volume of the reaction mixture was reduced to 500 µl and the incubation time was increased to 30 min. Volume of cyclohexane added was reduced to 1.5 ml, and the organic layer was transferred to 0.75 ml sodium carbonate which was later added into 1.5 ml 1 N NaOH for color development. Activities were expressed as umoles of keto acid formed/mg protein/hr or pmoles of keto acid formed/cell/hr.

α-Ketoacid Decarboxylase (E.C.1.2.4.4.)

The α-ketoacid decarboxylases have been assayed by the method of Gubler (1961) Development and characterisation of the ferricyanide assay has been reported previously by Sullivan et al.,(1976). The stoichiometry is $Fe(CN)^{-3}_6$ reduced : ketoacid oxidised =2:1.



$$\text{Enzyme-TPP-C(OH)(H)-R} + H_2 + 2Fe(CN)_6^{-3} \longrightarrow$$

$$R\text{-COOH} + 2Fe(CN)_6^{-4} + 2H^+ + \text{ENZYME-TPP-H}$$

Reaction mixture containing 100 μmoles of potassium sodium phosphate buffer (pH 7.4), 0.002 μmoles of thiamine pyrophosphate, 4.4 μmoles of freshly prepared potassium ferricyanide, 0.1% (v/v) Triton X-100 and enzyme in a final volume of 250 μl was transferred into 0.3 ml spectrophotometer cuvettes and preincubated for 10 min for stabilisation.Reaction was initiated by the addition of 15 μmoles of substrate (α-ketoisocaproic acid or α-ketomethylvaleric acid or α- ketoisovaleric acid) and change in absorbancy at 420 nm was recorded for every 30 seconds for 3 min. Correction for endogenous activity was made with a substrate blank. The ferricyanide reduced was calculated using molar extinction coefficient of 1.05×10^3 M^{-1} cm^{-1}.

Metabolism of [U-^{14}C] Branched Chain Amino Acids

Metabolism of BCAA (leucine, isoleucine and valine) is initiated by transamination during which the corresponding α-ketoacids are formed. These α-ketoacids are subsequently oxidised in a series of reactions, including those of the TCA cycle, resulting in the production of carbon dioxide. Thus, measurement of [$^{14}CO_2$] produced from [^{14}C]BCAA gives an idea on the metabolism of these amino acids. Hence,tissue preparations from normal and hyperammonemic rats were

incubated with [U-^{14}C]BCAA and the liberated [^{14}C] carbon dioxide was trapped into hyamine hydroxide.

Production of $^{14}CO_2$ from [U-^{14}C]Branched Chain Amino Acids

Reaction mixture (0.5 ml) consisting of 75 µmoles of potassium phosphate buffer (pH 7.4); 50 µmoles of sodium chloride; 25 µmoles of potassium chloride, 50 µmoles of glucose, 100 nmoles of either [U-^{14}C]leucine, or [U-^{14}C]isoleucine or [U-^{14}C]valine (specific activity of 240, 264, and 282mci/-mmole) was placed in vials closed with Kontes rubber stoppers from which center wells with a strip of Whatman no. 1 filter paper were suspended. Vials were sealed with parafilm and the reaction was initiated by the addition of the cellular/subcellular fractions. The reaction mixture was incubated with shaking for 30 min at 37^{o}C. To zero time controls, PCA was added before commencing incubation. Reaction was terminated by injecting 0.2 ml of 3 M PCA into the reaction mixture and 0.2 ml of hyamine hydroxide was injected into the presuspended centre wells. The reaction mixture was further incubated for 1 hr. with shaking at 37^{o}C. At the end of incubation, the center wells were removed and introduced into scintillation vials containing 10 ml of Bray's Scintillation fluid (240 gms Napthalene, 15 gms PPO, 150 mg POPOP in 1 ltr. each of toulene, alcohol and dioxane). Medium (100 µl) was also counted to determine the specific activity of the added amino acids. Radioactivity was determined with Beckman liquid

scintillation counter. The contribution of volatile keto acids formed during incubation to total radioactivity in hyamine was also determined. The reaction was carried out as mentioned above but leaving the vials open during initial incubation. After 30 min PCA was added and the vials were closed after 5 min with a stopper and later the radioactivity trapped by hyamine hydroxide was determined. The radioactivity trapped in hyamine after the metabolic reaction would be due to the volatile ketoacids formed. This was observed to be less than 5% of the radioactivity due to the produced $^{14}CO_2$.

Production of ^{14}C-Keto Acids from [U-^{14}C]BCAA

Reaction mixture used was the same as above. After the removal of center wells, 2,4-dinitrophenylhydrazine reagent (1 N HCl) was added to the remaining reaction mixture and after 10 min at room temperature 1 ml of cyclohexane was added. The tubes were thoroughly vortexed and centrifuged. The organic layer was transferred to Bray's Scintillation fluid and radioactivity was determined.

Efflux of [U-^{14}C] keto acids

The reaction mixture was identical to that used for the decarboxylation of BCAA. [U-^{14}C] Amino acids were added to the vials with the reaction mixture and incubated in a shaking water bath at 37°C for 30 min after which the tubes were centrifuged in cold at 5,000 rpm. The pellet and supernatant were separated. To each of the fractions, 1 ml of 2,4-

dinitrophenylhydrazine was added and left for 10 min. One ml of cyclohexane was added, vortexed and centrituged. The cyclohexane layer with [U-^{14}C]ketoacidhydrazones was transferred to Bray's scintillation fluid and and the radioactivity was determined. Blanks were run for the same period of incubation at 0^{o}C and processed similarly.

Amino Acid uptake studies:

The cellular/subcellular fractions were incubated in Krebs Ringer-Phosphate glucose medium containing labelled amino acids (U-^{14}C)-leucine, isoleucine, valine or [^{3}H]leucine for 4 min in a shaking water bath at 37^{o}C. Uptake was terminated by placing the tubes immediately in ice and centrifuging for a three min at 5000 rpm. The pellet was washed 3-4 times with ice-cold nonradioactive medium containing the amino acid under study. The tubes were inverted and the supernatant was left to drain tor half-an hour at room temperature. The pellet was suspended in 0.1 N NaOH (100 µl) which was transferred to Bray's Scintillation fluid and counted. Radioactivity in 100 µl of the medium was also determined to calculate the specific activity. Non specific uptake was measured in a similiar fashion but the incubation was carried out at 0^{o}C.

Purity of [^{14}C]-Amino Acids

Purity of commercial [^{14}C] amino acids, used in metabolic studies, was checked by paper chromatography and autoradiography. Commercial [U-^{14}C]-leucine, isoleucine and

valine were diluted with water and spotted on one half of a Whatman no. 1 paper (10"x12") along with standard amino acids on the other half and were chromatographed with Butanol : acetic acid : water (65:15:25 v/v) solvent for twelve hours at room temperature. Chromatogram was dried and the portion containing standard amino acids was sprayed with ninhydrin (0.25% in acetone with 1% pyridine) and the colour was developed. In the other half, containing [^{14}C] amino acids, areas corresponding to standard leucine, isoleucine and valine were cut from the chromatogram. In addition, areas of 1"x1" were also cut above and below the identified spots. These were transferred into scintillation vials containing 10 ml of Bray's scintillation fluid and the radioactivity was determined.

Another chromatogram was developed in a similiar fashion and was placed against an X-ray film and covered with black paper. After storing for one month at -80^{o}C, the X-ray films were developed.

Purity of [^{14}C] Keto Acids

The reaction was carried out similiar to that of the experiment for determining the production of [^{14}C]-keto acids from [U-^{14}C]BCAA. The cyclohexane layer was evaporated and the residue was redissolved in a known volume (0.05 ml) of cyclohexane. An aliquot (10 µl) of this was used for determining radioactivity while the remaining 40 µl was spotted on a Whatman no.1 filter paper and was chromato-

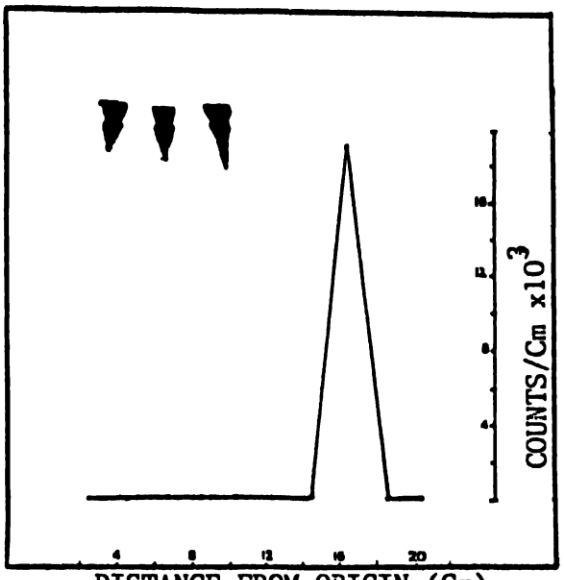
graphed with Butanol : acetic acid : water (65:15:25 v/v) solvent at room temperature. Chromatogram was dried and areas corresponding to the keto acid hydrazones (which can be distinguished by their yellow colour) were cut from the chromatogram. In addition, areas of 1"x1" were also cut from above and below the identified spots. These were transferred to scintillation vials containing 10 ml of Bray's scintillation fluid and the radioactivity was determined.

Another chromatogram was developed in a similar fashion and was placed against X-ray film and covered with black paper. After 2 months at -80°C, the X-ray films were developed.

Statistical analysis

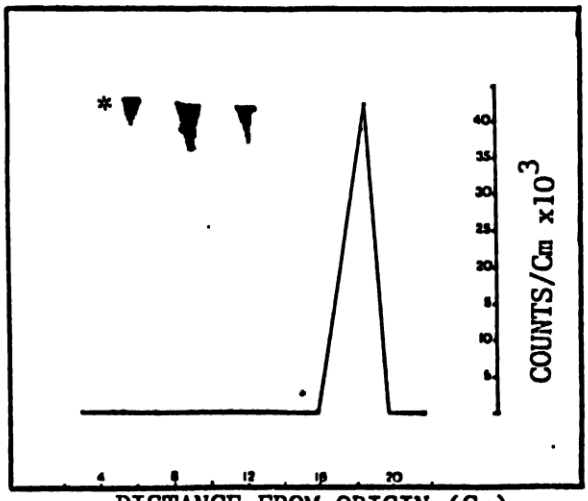
Statistical analysis was carried out by Student's 't' test and Neuman Keul's Multiple range test. Statistical estimation of enzyme kinetics was performed by the method of Wilkinson (1961) for which a computer programme was developed.

PURITY OF COMMERCIAL [U-^{14}C]BRANCHED-CHAIN AMINO ACIDS



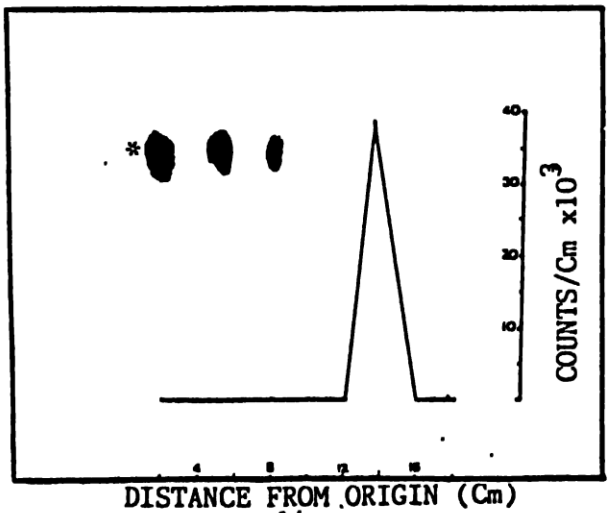
DISTANCE FROM ORIGIN (Cm)

[U-^{14}C]LEUCINE



DISTANCE FROM ORIGIN (Cm)

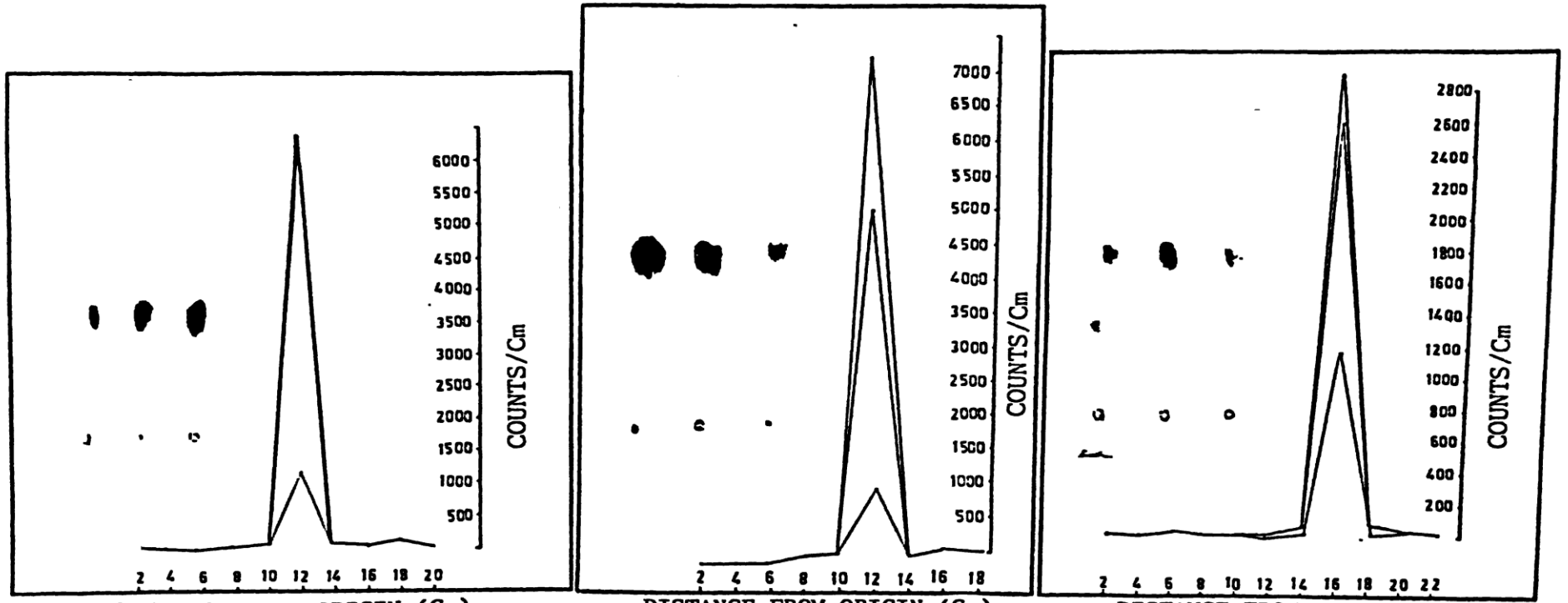
[U-^{14}C]ISOLEUCINE



DISTANCE FROM ORIGIN (Cm)

[U-^{14}C]VALINE

* SPOTS IN AUTORADIOGRAPHY



PURITY OF [^{14}C]-BRANCHED-CHAIN KETO ACID HYDRAZONES

SYNAPTOSOMES FROM CEREBRAL CORTEX OF NORMAL AND EXPERIMENTAL ANIMALS.

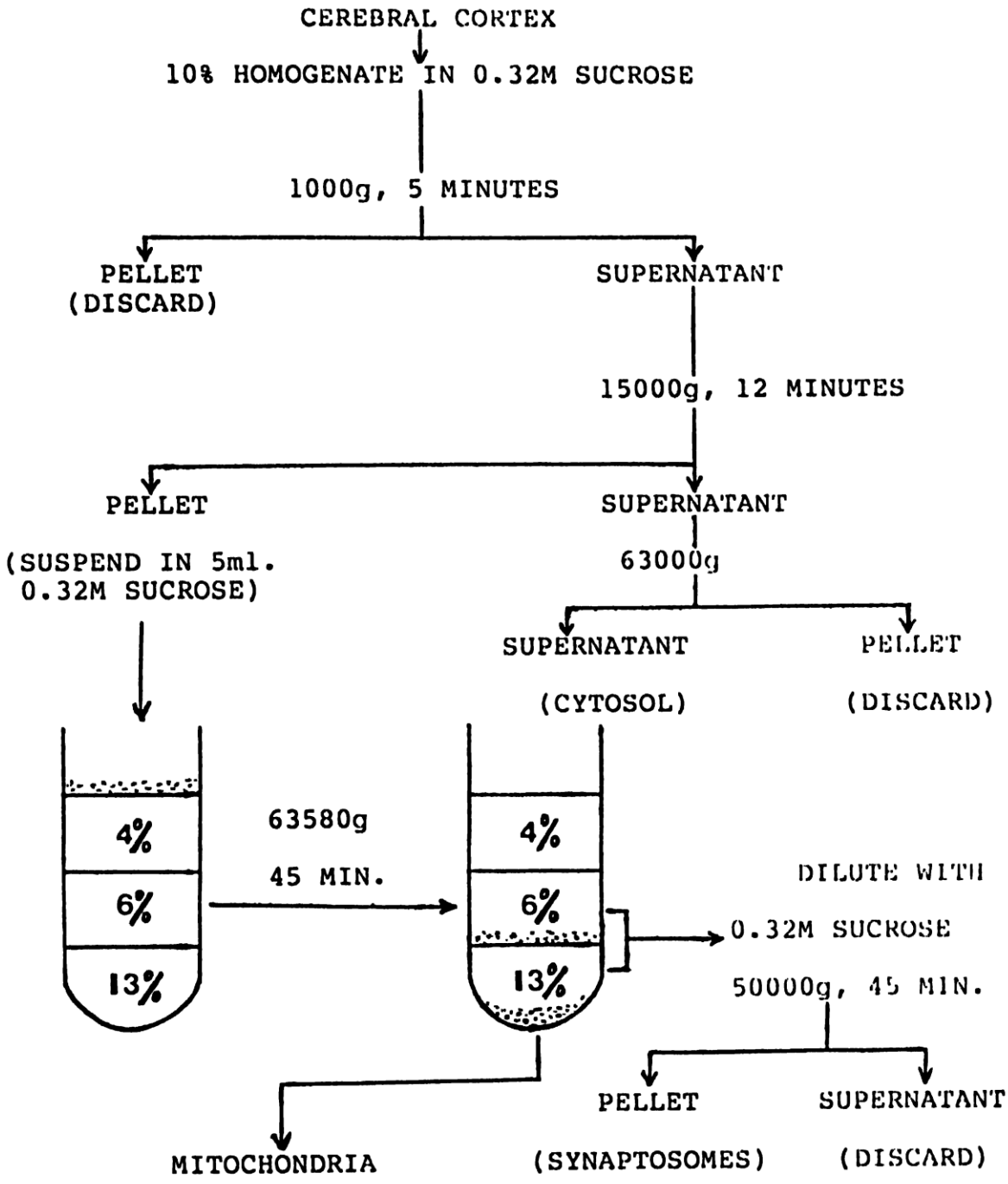


FIG 2.2

FLOW DIAGRAM FOR THE ISOLATION OF

ASTROCYTES & NEURONS FROM RAT BRAIN

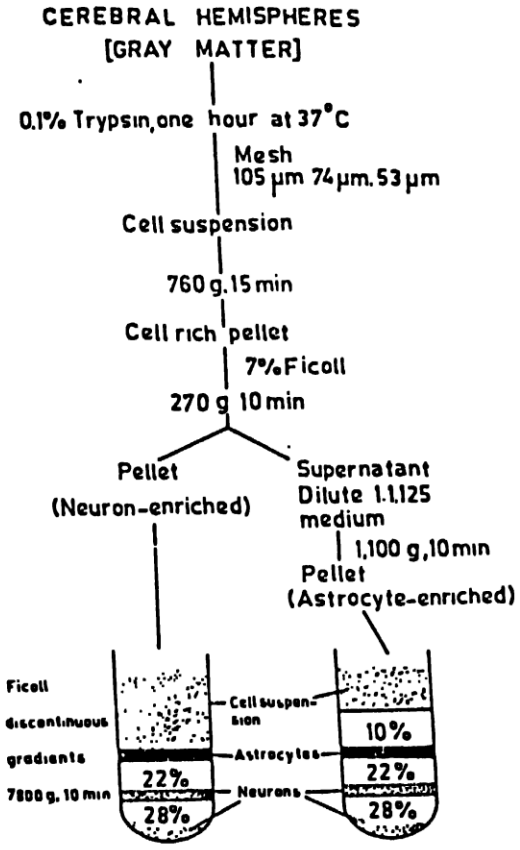


FIG 2.3

FLOW DIAGRAM FOR THE ISOLATION

OF OLIGODENDROGLIA FROM RAT BRAIN

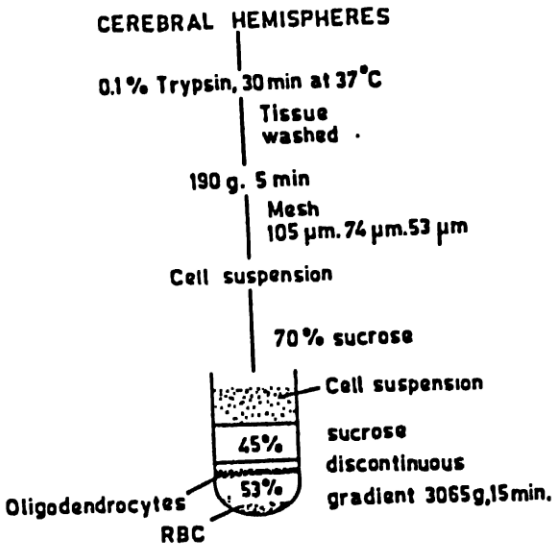
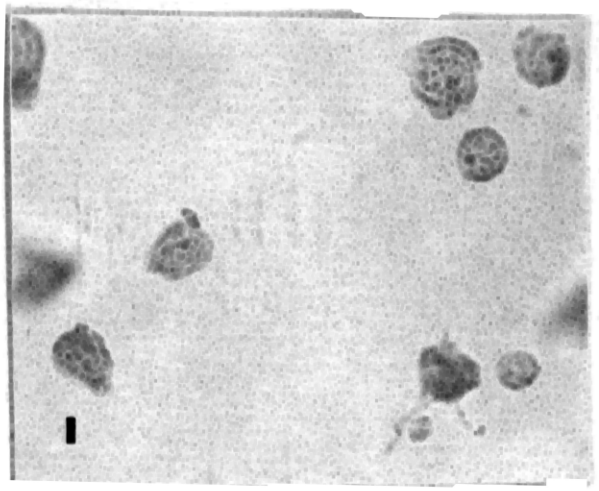


FIG 2 4

1. Light microscope photomicrographs of neurons isolated from rat cerebral cortex.

2. Light microscope photomicrographs of astrocytes isolated from rat cerebral cortex.

3. Light microscope photomicrographs of oligo cells isolated from rat cerebral cortex.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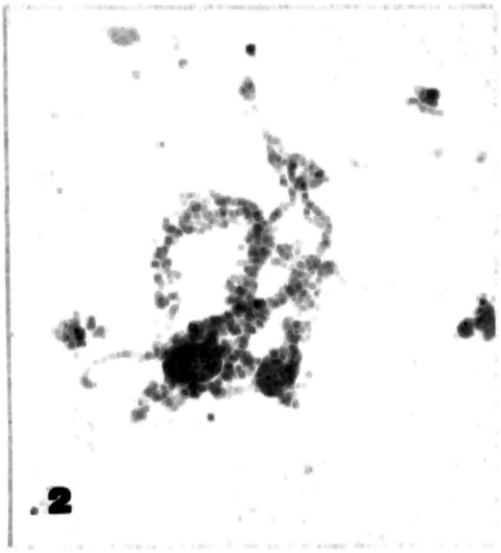
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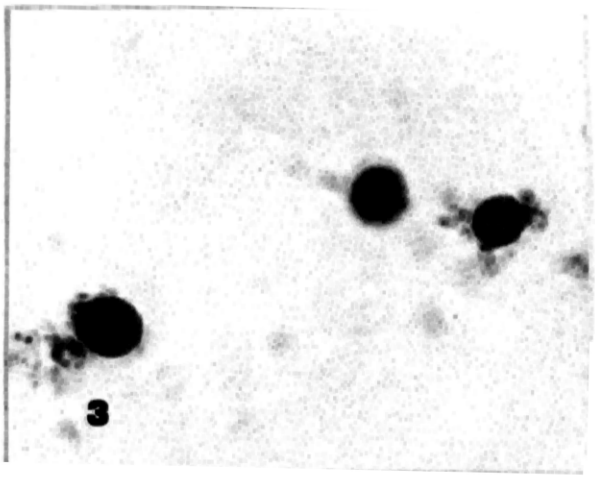
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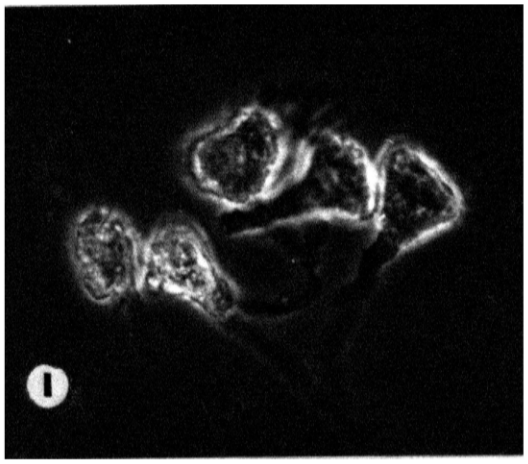
1. Phase contrast photomicrographs of neurons isolated from rat cerebral cortex. Scale = 20 u.

2. Phase contrast photomicrographs of astrocytes isolated from rat cerebral cortex. Scale = 20 u.

3. Phase contrast photomicrographs of oligo cells isolated from rat cerebral cortex. Scale = 20 u.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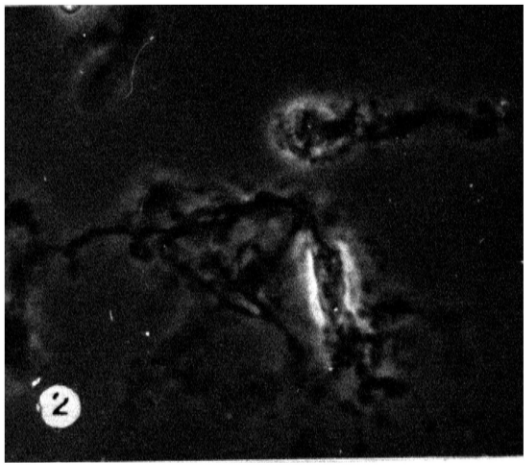
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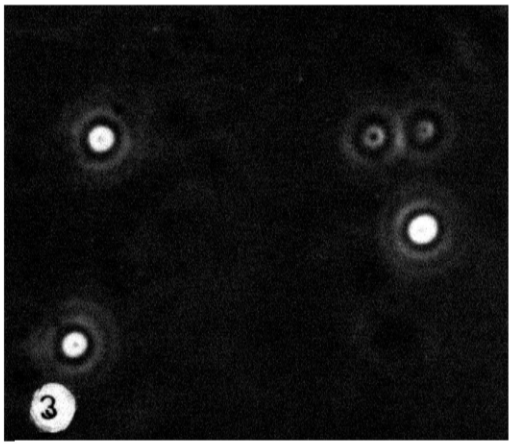

3

ENZYMATIC STUDIES

TRANSAMINASES IN HOMOGENATES

Initial reaction in the metabolic breakdown of amino acids is the removal of amino group. This process is usually by transamination wherein the amino group of the amino acid to be metabolised is transferred to an α-keto acid without any modification. As a result, the amino acid is converted to a keto acid and the carbon skeleton of the amino acid is either decarboxylated (thus carbons are eliminated as CO_2) or converted to a suitable form to be utilised mostly in the TCA cycle. Transamination reactions are mediated by amino-transferases (transaminases) which are specitic for their substrates and also require pyridoxal-5-phosphate as a cofactor. In biological systems only three keto acids viz., α-KG, OAA and PYR are the major acceptors of amino group in transamination reactions. On acceptance of the amino group they are converted to glutamate, aspartate and alanine respectively. It has been observed that α-KG participates in a majority of transamination reactions when compared to OAA and PYR (Lehninger, 1981; Cooper and Meister, 1985).

Aminotransferases are present both in cytosol and mitochondria of animal tissues (Cooper and Meister, 1985). Though all the mammalian tissues are endowed with aminotransferases, metabolism of amino acids is usually initiated in the liver (Miller, 1961, ; Cooper and Meister, 1985; Odessey and Goldberg, 1972). This is understandable as the portal blood enriched with dietary amino acids first encounters

liver. However, metabolism of BCAA is an exception to this and is initiated in the peripheral tissues like skeletal muscle (Miller, , 1961, McMenamy et al., 1962, 1965; Manchester, 1965; Odessey and Goldberg, 1972).

Metabolism of BCAA is initiated by the transamination of these amino acids with α-KG, resulting in the formation of glutamate and BCKA in a reaction mediated by BCAA-α-KG amino-transferase (BCAA-T). The nature of BCKA formed in this reaction depends on the nature of BCAA, thus leucine gives rise to α-KIC, valine to α-KIV and isoleucine to α-KMV (Rodwell, 1969; Lehninger, 1981). Studies on the tissue distribution of BCAA-T indicated that it is present in all tissues including brain (Roswell, 1956; Manchester, 1965; Goodman, 1963; Ichihara and Koyama, 1966; Dawson et al., 1967; Dancis, 1974; Ichihara et al., 1981). Activity levels of these enzymes in brain are higher than that of skeletal muscle and liver and lower than those of heart and kidney (Taylor and Jenkins, 1966; Ichihara et al., 1981).

In brain, BCAA-T is both cytosolic and mitochondrial. Of the three BCAA, valine was shown to be the least preferred substrate for the enzyme as the Km for valine is 5-10 times higher than that of leucine and isoleucine (Ichihara et al., 1981). Benuck et al., (1971) reported that this enzyme can transfer the amino group to other keto acids such as OAA, PYR, and phenylpyruvic acid, but maximum activity was obtained with α-KG. Brosnan et al., (1985) reported highest

activity of this enzyme in brainstem than in other brain regions. Very few studies have been conducted on the activity of this enzyme in brain in pathological states. One such report is that of Brosnan et al., (1985) in which no change in the activity of this enzyme was demonstrated in brain in streptozotocin induced diabetes. In the present study, BCAA-T is determined in different regions of the brains of normal and hyperammonemic rats. Some of these results of have already been published (Jessy and Murthy, 1985, 1988).

Preliminary studies on the standardisation of BCAA-T was carried out with 10% (w/v) homogenates of cerebral cortex. With all the three BCAA as substrates, enzyme activity was linear up to 600 ug of protein (Fig. 3.1). Activity of this enzyme was highest with valine as substrate and lowest with leucine as substrate. Activity of BCAA-T, with all the three amino acids as substrates, was found to be linear up to 60 min of incubation (Fig 3.2). Hence, in the routine assays, 15 min was selected as the time of incubation.

An increase in the enzyme activity was noticed with increasing concentration of all three amino acids as substrates (Fig 3.3). Substrate [S] vs velocity [V] plots revealed an early saturation of the enzyme with valine when compared with leucine and isoleucine. Calculated Km and Vmax values of the enzyme for the amino acid substrates are given in Table 3.1.

TABLE 3.1

KINETIC CONSTANTS OF BCAA-T ACTIVITY IN HOMOGENATES WITH LEUCINE, ISOLEUCINE AND VALINE AS SUBSTRATES.

AMINO ACID	Km	Vmax
LEUCINE	24±6	132±19
ISOLEUCINE	19±5	126±15
VALINE	13±5	124±19

Km was expressed as mM substrate. Vmax was expressed as μmol of amino acid/gm wet weight tissue/hr.

Similarly, enzyme activity also increased with increasing concentration of α-KG (Fig 3.4). However, increase in α-KG concentrations beyond the optimal concentration, inhibited the enzyme activity, similar to the inhibition reported for several enzymes such as acetylcholinesterase, catalase etc. Due caution must, however, be exerted before arriving at such a conclusion. Being a keto acid, α-KG reacts with 2,4-DNP. At higher concentrations α-KG might interfere in the formation and extraction of the BCKA-hydrazones. Hence, the effect of increasing concentrations of α-KG were studied on the formation and extraction of hydrazones from commercial BCKA in the absence of added enzyme (Fig.3.6). It is evident that, at higher concentrations, α-KG does interfere in the formation and extraction of BCKA-hydrazones and due corrections were made for this effect and the curves were redrawn (Fig 3.5). From these results it may be concluded that α-KG does not exert any inhibition on the activity of

TABLE 3.2

KINETIC CONSTANTS FOR BCAA-T ACTIVITY IN HOMOGENATES USING α-KG AS SUBSTRATE.

AMINO ACID	Km	Vmax
LEUCINE	2.73±0.9	86.7±8.87
ISOLEUCINE	1.89±0.4	146±5.87
VALINE	2.37±0.7	90±7.49

Km was expressed as mM substrate. Vmax was expressed as μmol of keto acid/gm wet weight tissue/hr.

BCAA-T at higher concentrations. Calculated Km and Vmax values (Table 3.2) were found to be closer to the values reported by Taylor and Jenkins, (1966).

REGIONAL DISTRIBUTION OF BCAA-T

Brain contains three major cell types, viz., neurons, astrocytes, and oligodendrocytes. As there is structural and functional heterogenity in these cells, it is possible that their metabolic properties might also be different. Depending on the relative preponderance of these cells in different regions, BCAA-T activity may vary from one region to the other in brain. Hence, distribution of BCAA-T was determined in six different regions of the brain.

In general, BCAA-T activity with valine and isoleucine as substrates was found to be higher than with leucine as substrate in all the regions of brain. Activity of this enzyme was higher in the brainstem (pons and medulla) and in midbrain than in other regions. In cerebral cortex,

TABLE 3.3

REGIONAL DISTRIBUTION OF BCAA-T IN THE BRAINS OF NORMAL RATS

REGION	LEUCINE	ISOLEUCINE	VALINE
CC	1.20±0.10(8)	1.79±0.20(6)	2.08±0.19(5)
CE	1.18±0.19(8)	1.79±0.19(6)	1.59±0.29(5)
BS	3.59±0.25(6)	6.93±0.90(6)	6.00±0.70(5)
HC	1.10±0.13(8)	1.53±0.20(5)	1.89±0.45(5)
CS	1.05±0.13(8)	1.68±0.18(6)	1.75±0.36(6)
MB	2.15±0.28(5)	5.58±0.47(6)	4.79±0.46(5)

CC: cerebral cortex; CE: cerebellum; BS: brainstem; HC: hippocampus; CS: corpus striatum; MB: midbrain.
Values are Mean±S.D. Activity is expressed as μmoles of branched chain keto acid formed/gm wet wt.tissue/min. Numbers in parenthesis indicate the number of experiments.

cerebellum, hippocampus and corpus striatum, activity levels of BCAA-T, with all the three BCAA as substrates, were similar. Activity levels and the profiles of regional distribution observed in the present study were found to be similar to the reports of Brosnan et al., (1985). Results obtained in this study are also in agreement with the results of (Rao et al., 1982), though the activity levels reported by them were much higher than the values obtained in the present study. Similarly, a difference in the activity levels were observed when the results of our study were compared with that of Shinnick and Harper (1976) and Benuck et al., (1972). These variations may be due to the differences in the assay methods.

A higher activity of BCAA-T in brainstem might result in formation of more BCKA, which would be metabolised to Ac·Ac

CoA (leucine, isoleucine) and propionyl CoA (valine, isoleucine). Acetyl CoA, thus formed, might be oxidized in the TCA cycle or used for lipid biosynthesis. Propionyl CoA might be metabolized in a similar fashion. In addition to this, HMG CoA, an intermediate in the metabolism of leucine, might serve as a precursor for cholesterol synthesis (Stillway et al., 1979). It has been shown that the leucine carbons are incorporated into the lipids and into cholesterol in skeletal muscle, adipose tissue and in aorta. Stillway et al., (1979) demonstrated that leucine may be the major precursor for the biosynthesis of cholesterol and phosphotidylcholine in the sciatic nerve. They have also observed that carbons from isoleucine are incorporated into several lipids, especially neutral lipids albeit at a lesser rate than leucine. But Patel & Balazs (1970) and Patel & Owen (1978) demonstrated that in developing brain less than 2% of the radioactivity from leucine is incorporated into lipids. However, there are evidences to indicate that leucine serves as a better precursor for the synthesis of myelin lipids in developing brain.

Thus, higher activities of BCAA-T in brainstem, may be related to the higher lipid content of this region, where Ac CoA formed from the metabolism of BCKA may be diverted for lipid biosynthesis. In addition to the BCKA, glutamate is also formed in greater amounts from BCAA in brainstem due to a very high activity of BCAA-T. In other words, BCAA-T in

the brainstem has a dual role i.e., synthesis of glutamate and the synthesis of precursors for lipid biosynthesis. With the exception of midbrain, in all the other regions of the brain where the lipid content is lower than brainstem, BCAA-T activity is also low and may be geared towards production of glutamate. Acetyl CoA formed from BCKA, in these regions, might be utilised in the TCA cycle.

CHANGES IN BCAA-T IN HYPERAMMONEMIC STATES

As was mentioned, glutamate is formed from BCAA in two different reactions. In the initial reaction i.e., transamination of BCAA with ∝-KG results in the formation of glutamate, but this would not represent net (de novo) synthesis of glutamate. Similarly, incorporation of carbons of Ac CoA produced from leucine does not represent a net synthesis of glutamate. However, valine and isoleucine can bring about the net synthesis of glutamate as they are converted to succinyl CoA which enters the TCA cycle as succinate. In other words, though glutamate is produced from BCAA, net synthesis can occur only from keto acids of isoleucine and valine (Murthy and Hertz, 1987b).

Using ^{15}N-leucine Yudkoff et al., (1983 a,b) demonstrated that the amino group from leucine is incorporated into the ∝-amino group of glutamate, glutamine and aspartate. They suggested that atleast 30% of the total glutamate and glutamine in brain may be generated by transamination of BCAA. It has been demonstrated that the carbons

from BCAA (including leucine) are incorporated into the carbons of glutamate family of amino acids (Berl and Frigyesi, 1969). Summarising the results of several investigators Duffy and Plum (1982) and Cooper et al., (1983) proposed that glutamate generated from BCAA might serve as a precursor for the synthesis of glutamine in brain in hyperammonemic states.

It is important to determine which of the above two reactions in the synthesis of glutamate from BCAA in brain serves as a major contributor for the synthesis of glutamine in the brain. Though this problem appears to be trivial, it has a physiological significance which can be understood by looking at the subcellular localisation of the enzymes involved in the metabolism of BCAA and glutamine biosynthesis. It is worth recalling that GS is cytosolic while BCAA-T is both cytosolic and mitochondrial. Enzymes of TCA cycle and BCKA-DH are exclusively mitochondrial in their localization (Randle et al., 1981). If the site of formation of glutamate from BCAA is cytosol, then glutamate formed from BCAA might be freely available for the synthesis of glutamine. Then, the rate limiting factor will be the availability of α-KG in the cytosol. Rate of transport of dicarboxylic acids from mitochondria regulates the availability of α-KG in the cytosol and thus the transamination of BCAA in this subcellular compartment. In the latter case i.e., if glutamate generated in the mitochondria is the

precursor for glutamine synthesis then export of glutamate from mitochondria to cytosol, by the glutamate - aspartate carrier or glutamate-OH^- carrier, would act as a regulating factor. In addition to this, rate of carbon flow through the TCA cycle might be having a modulatory effect on this process. In the present study this problem is initially addressed by determining the BCAA-T activity in hyperammonemic states of various etiologies. Blood and brain ammonia levels have been determined to ensure that the adopted method has indeed induced hyperammonemia (Table 3.4). Regional protein content in the brain was determined both in normal and hyperammonemic animals to ensure that any change in BCAA-T activity was not due to changes in protein content (Table 3.5)

TABLE 3.4

AMMONIA LEVELS IN BRAIN AND BLOOD OF NORMAL AND HYPERAMMONEMIC RATS.

CONDITION	SERUM	BRAIN
CONTROL	0.1±0.01(3)	0.4±0.09(3)
AMM.ACETATE	1.8±0.08(4)*	2.6±0.52(4)*
MSI (ACUTE)	0.8±0.10(5)*	2.7±0.48(5)*
MSI (SUBACUTE)	0.7±0.10(5)*	2.1±0.20(5)*
SHAM OPERATED	0.5±0.10(5)*	0.7±0.20(5)
HEPATECTOMISED	0.8±0.10(5)*	2.2±0.50(5)†

Ammonia levels in serum are expressed as μmoles/ml and in brain umoles/gm wet weight of tissue. Values are expressed as Mean±S.D. Number in parenthesis indicate the number of experiments. All the values are significant †$p<0.005$ *$p<0.001$

TABLE 3.5

PROTEIN CONTENT IN DIFFERENT REGIONS OF BRAIN ISOLATED FROM NORMAL AND HYPERAMMONEMIC RATS.

	control I	Amm. Ac. II	MSI Acute III	MSI sub Acute IV
CC	100±9 (5)	104±8 (5)	96±8 (6)	96±8 (6)
CE	99±12(5)	103±5 (5)	95±12(5)	128±14(5)
BS	107±22(5)	78±38(5)	96±13(5)	169±4 (5)
HC	91±10(6)	78±12(5)	71±6 (5)	101±13(6)
CS	98±9(4)	103±10(5)	102±17(5)	88±10(5)
MB	107±12(5)	109±11(5)	116±17(6)	115±24(5)

Protein content is expressed as mg protein/gm wet weight tissue. Values are Mean±S.D. Number in the parenthesis indicate the number of experiments. None of the changes are statistically significant. CC - cerebral cortex; CE - cerebellum; BS - brainstem; HC - hippocampus; CS - corpus striatum and MB - mid brain.

BCAA-T ACTIVITY IN HEPATECTOMISED RATS

Major portion of the ammonia formed in the gut (which serves as a source for blood ammonia) is detoxified in the liver, where it is converted to urea (Onstad and Zieve, 1979). Hence, hyperammonemia can be induced by reducing the liver mass. This can be achieved either by administering hepatotoxins or by surgical techniques. In the present study partial hepatectomy was performed and about one third of the liver was removed in the rats to induce hyperammonemia. In sham operated animals which served as controls, a similar surgical procedure was performed but for the removal of the liver. Preliminary studies revealed that serum ammonia levels reach a peak value around 8 hrs after hepatectomy. Hence, this time period was chosen for detecting changes, if any, in

the activity of BCAA-T in sham as well as in hepatectomised rats (Tables 3.6 - 3.8).

TABLE 3.6

BCAA-T ACTIVITY WITH LEUCINE AS SUBSTRATE IN DIFFERENT REGIONS OF BRAINS OF NORMAL, SHAM OPERATED AND HEPATECTOMIZED RAT.

REGION	CONTROL	SHAM	HEPATECTOMY
CC	1.20±0.10(8)	0.76±0.11(4)$^{a+}$	2.42±0.26(5)^{a*b*}
CE	1.18±0.19(8)	0.78±0.10(4)^{a*}	1.76±0.15(5)^{a*b*}
BS	3.59±0.25(6)	1.78±0.18(5)^{a*}	3.28±0.23(5)^{b*}
HC	1.10±0.13(8)	0.85±0.11(5)	1.68±0.21(5)^{a*b*}
CS	1.05±0.13(8)	0.58±0.08(5)$^{a+}$	1.60±0.25(5)^{a*b*}
MB	2.15±0.28(5)	1.98±0.33(5)	3.28±0.05(4)^{a*b*}

CC: cerebral cortex; CE:cerebellum; BS: brain stem; HC: hippocampus; CS: corpus striatum; MB: mid brain.
Values are Mean±S.D. Activity is expressed as μmoles of α-keto-isocaproic acid formed/gm wet wt./min. Numbers in parenthesis indicate the number of experiments. *p<0.01, +p<0.05
a-comparison with control; b-comparison of sham operated and partially hepatectomised.

With leucine as substrate a statistically significant decrease in the activity of BCAA-T was observed in all the regions of brain in sham operated rats except in the midbrain. When the activity of this enzyme was compared with that of the controls, in hepatectomised rats it was elevated in all the brain regions, except brain stem, of hepatectomised rats. Comparison with sham operated animals revealed a higher magnitude of elevation in hepatectomised rats which was statistically significant in all the regions of brain (Table 3.6).

A similar pattern of change was noticed when valine was

used as a substrate except that the activity was unchanged in the cerebellum of the sham operated rats (Table 3.7).

With isoleucine as substrate, changes observed in BCAA-T activity were similar to those mentioned above (Table 3.8). These changes in BCAA-T in hepatectomized rats have been reported elsewhere (Jessy et al., 1988).

TABLE 3.7

BCAA-T ACTIVITY WITH VALINE AS SUBSTRATE IN DIFFERENT BRAIN REGIONS OF NORMAL, SHAM OPERATED AND HEPATECTOMIZED RATS

REGION	CONTROL	SHAM	HEPATECTOMY
CC	2.08±0.19(5)	1.30±0.16(4)^{a*}	3.18±0.28(4)^{a*b*}
CE	1.59±0.29(5)	1.50±0.20(5)^{a*}	2.83±0.12(5)^{b*}
BS	6.00±0.70(5)	2.62±0.42(5)^{a*}	5.35±0.43(4)^{a*b*}
HC	1.89±0.45(5)	1.27±0.21(5)^{a*}	2.18±0.41(4)^{b*}
CS	1.75±0.36(6)	1.22±0.16(4)	2.57±0.15(4)^{a*b*}
MB	4.79±0.46(5)	3.05±0.20(4)^{a*}	6.80±0.40(4)^{a*b*}

CC: cerebral cortex; CE:cerebellum; BS: brain stem; HC: hippocampus; CS: corpus striatum; MB: mid brain.Values are Mean±S.D. Activity is expressed as μmoles of α-ketoisovaleric acid formed/gm. wet wt/min. Numbers in parenthesis indicate the number of experiments. *p<0.01; +p<0.05. a-comparison with control, b-comparison between sham operated and partially hepatectomised animals.

Decrease in the activity of BCAA-T in sham operated animals may be due to the surgical stress. Significant alterations in protein and amino acid metabolisms were observed during injury. Utilisation of BCAA by the skeletal muscle was found to be the major component of response to injury, (Blackburn et al., 1981; Freund et al., 1981). It has been shown that proteolysis occurs in the muscle

TABLE 3.8

BCAA-T ACTIVITY WITH ISOLEUCINE AS SUBSTRATE IN DIFFERENT BRAIN REGIONS OF NORMAL, SHAM OPERATED AND HEPATECTOMIZECD RATS

REGION	CONTROL	SHAM	HEPATECTOMY
CC	1.79±0.20(6)	1.18±0.16(5)$^{a+}$	2.52±0.28(6)^{a*c*}
CE	1.79±0.19(6)	1.47±0.16(5)^{a*}	2.23±0.16(5)^{c*}
BS	6.93±0.90(6)	1.97±0.37(5)^{a*}	3.92±0.58(4)^{a*c*}
HC	1.53±0.20(5)	0.95±0.16(4)^{a*}	2.60±0.51(6)^{c*}
CS	1.68±0.18(6)	1.28±0.22(4)	2.08±0.25(5)$^{a+c*}$
MB	5.58±0.47(6)	2.50±0.26(5)^{a*}	5.15±0.28(6)$^{a+c*}$

CC: cerebral cortex; CE:cerebellum; BS: brainstem; HC: hippocampus; CS: corpus striatum; MB: mid brain. Values are Mean±S.D. Activity is expressed as μmoles of α- ketomethylvaleric acid formed/gm.wet wt/min. Numbers in parenthesis indicate the number of experiments. *p<0.01; +p<0.05.

immediately after surgery and plasma BCAA levels are elevated. Due to hyperinsulinemia, which is prevalent under these conditions, non-protein fuels like glucose and fatty acids fail to satisfy the energy demands of skeletal muscle. Ketone bodies also fail to meet the energy requirements of the peripheral tissues. Plasma content of these compounds (ketone bodies) is decreased due to a fall in the activity of hepatic carnitine acyltransferase activity. It has been postulated that this deficiency in the fuel supply is satisfied by the oxidation of BCAA (Blackburn et al., 1981). Decreased activity of BCAA-T in brain under these conditions might spare the much required BCAA to the skeletal muscle where the energy demand is greater. However, it must be mentioned that not much literature is available on the

effects of surgery of the peripheral tissues, on brain metabolism.

Removal of liver would affect the metabolism of several nitrogenous compounds including BCAA. It was shown that the plasma and brain levels of BCAA are increased in hepatectomised rats (Astre et al., 1984). As a result, availability of these amino acids to brain is enhanced.

It has been repeatedly been demonstrated that the reductive amination of ∝-KG decreases in hyperammonemic states due to the inhibition of the enzyme GDH (Yu et al., 1982; Subbalakshmi and Murthy, 1983b). Studies with ^{15}N and ^{13}N ammonia indicated that this reaction plays a minor role in ammonia detoxification (Cooper et al., 1979, 1985; Yudkoff et al.,1983b). Similarly, a suppression in the activities of AAT and AlAT was observed in hyperammonemic states (Subbalakshmi and Murthy, 1983b; Ratnakumari et al., 1986). However, an increase or no change in the levels of ∝-KG were reported in brain in hyperammonemic states, (Shorey et al., 1967; Hindfelt and Siesjo, 1971; Vargara et al., 1973; Hindfelt et al., 1977; Hawkins et al., 1983). These results suggest that glutamate required for glutamine biosynthesis may be generated in reactions other than that mediated by GDH. Other possibilities to generate glutamate are (1) breakdown of glutamine or (2) transamination of amino acids (other than those of the glutamate family) with ∝-KG. The latter possibility appears to be in operation in hyper-

ammonemic states as glutaminase activity was shown to be inhibited by its product ammonia (Matheson and Van Den Berg, 1975). Increased activities of BCAA-T, observed in hepatectomised animals in the present study, suggest that these amino acids may be involved in the production of glutamate required for the synthesis of glutamine in hyperammonemic states. Earlier observations of increased utilisation of BCAA in hepatectomised animals and an increase in the contents of these amino acids in brain (Astre et al., 1984) in these conditions strongly supports this concept.

Though hepatectomy induces hyperammonemia, it has other side effects such as hypoglycemia (Weinbren and Dowling, 1972). These might be responsible either singly or in conjunction with other parameters in increasing the activity of BCAA-T in brain. In order to confirm whether hyperammonemia is the factor responsible for stimulation of BCAA-T, an acute dose of ammonium acetate was administered prior to the determination of BCAA-T activity in brain. These results have already been published (Jessy and Murthy, 1985).

EFFECTS OF AMMONIUM ACETATE ON BCAA ACTIVITY

When leucine was used as substrate, an elevation in the activity of BCAA-T was observed in all regions of brain in preconvulsive states, except in cerebellum, brainstem and hippocampus where the activity was unaltered. During convulsions, enhanced activity of this enzyme was noticed in

all regions of brain, except in brainstem where it remained unchanged (Table 3.9).

TABLE 3.9

BCAA-T ACTIVITY WITH LEUCINE AS SUBSTRATE IN DIFFERENT REGIONS OF BRAIN OF NORMAL AND HYPERAMMONEMIC RATS

		AMMONIUM ACETATE TREATED	
REGION	CONTROL	PRECONVULSIONS	CONVULSIONS
CC	1.20±0.10(8)	1.67±0.15(7)*	1.67±0.10(5)*
CE	1.18±0.19(8)	1.37±0.18(6)+	1.33±0.25(6)+
BS	3.59±0.25(6)	3.45±0.43(6)	3.38±0.27(5)
HC	1.10±0.13(8)	1.08±0.18(5)	1.47±0.17(4)*
CS	1.05±0.13(8)	1.37±0.30(5)*	1.73±0.30(5)*
MB	2.15±0.28(5)	2.72±0.25(6)*	2.95±0.25(5)*

CC: cerebral cortex; CE:cerebellum; BS: brainstem; HC: hippocampus; CS: corpus striatum; MB: mid brain. Values are Mean±S.D. Activity is expressed as μmoles of α-keto-isocaproic acid formed/gm. wet wt/min. Numbers in parenthesis indicate the number of experiments. * $p<0.005$, + $p<0.05$.

Following the administration of an acute dose of ammonium acetate, BCAA-T activity with isoleucine as substrate was elevated in all regions of brain during preconvulsive and convulsive states, except in the brainstem where it exhibited either a decrease or was unaltered. In general, the magnitude of change was less in convulsive than in the preconvulsive phase (Table 3.10).

An elevation in the activity of BCAA-T with valine as substrate was noticed in the preconvulsive state in cerebellum and hippocampus. Activity of the same was, however, decreased in brainstem and corpus striatum under these con-

TABLE 3.10

BCAA-T ACTIVITY IN DIFFERENT BRAIN REGIONS WITH ISOLEUCINE AS SUBSTRATE IN NORMAL AND HYPERAMMONEMIC RATS

REGION	CONTROL	AMMONIUM ACETATE TREATED	
		PRECONVULSIONS	CONVULSIONS
CC	1.79±0.20(6)	3.00±0.38(6)*	2.20±0.28(6)*
CE	1.79±0.19(6)	2.10±0.18(5)	1.97±0.33(5)
BS	6.93±0.90(6)	5.50±0.60(5)+	6.87±1.22(4)
HC	1.53±0.20(5)	2.00±0.42(5)+	2.28±0.22(5)*
CS	1.68±0.18(6)	2.90±0.37(4)*	2.12±0.33(5)
MB	5.58±0.47(6)	4.78±1.00(6)+	6.38±0.77(5)

CC: cerebral cortex; CE:cerebellum; BS: brain stem; HC: hippocampus; CS: corpus striatum; MB: mid brain. Values are Mean±S.D. Activity is expressed as μmoles of α-keto-methylvaleric acid formed/gm wet wt./min. Numbers in parenthesis indicate the number of experiments. * $p<0.005$, + $p<0.05$

ditions. During convulsions, activity of this enzyme was either decreased or remained unaltered in most regions except brain stem where a marginal increase was observed (Table 3.11).

Results obtained in the present study indicate that pathophysiological concentrations of ammonia are capable of stimulating the activity of BCAA-T atleast in some of the brain regions. This is in accordance with the above made suggestion that these amino acids may provide glutamate required for glutamine biosynthesis in hyperammonemic states. Increased influx of these amino acids across the BBB, elevation in their content in brain were reported earlier (Strom et al., 1984; Hawkins et al., 1984; Mans et al., 1982, 1983;

TABLE 3.11

BCAA-T ACTIVITY WITH VALINE AS SUBSTRATE IN DIFFERENT REGIONS REGIONS OF BRAINS OF NORMAL AND HYPERAMMONEMIC RATS

REGION	CONTROL	AMMONIUM ACETATE TREATED	
		PRECONVULSIONS	CONVULSIONS
CC	2.08±0.19(5)	2.08±0.30(4)	0.98±0.17(6)*
CE	1.59±0.29(5)	1.85±0.23(4)+	1.38±0.20(5)
BS	6.00±0.70(5)	5.17±0.72(4)+	7.05±0.28(4)*
HC	1.89±0.45(5)	2.58±0.18(4)*	1.13±0.17(5)*
CS	1.75±0.36(6)	1.12±0.17(5)*	1.83±0.33(5)
MB	4.79±0.46(5)	4.45±0.38(4)	4.58±0.38(5)

CC: cerebral cortex; CE:cerebellum; BS: brainstem; HC: hippocampus; CS: corpus striatum; MB: mid brain. Values are Mean±S.D. Activity is expressed as μmoles of α-keto-isovaleric acid formed/gm wet wt./min. Numbers in parenthesis indicate the number of experiments. * $p<0.005$, + $p<0.05$.

Ede et al., 1984a, b; Jonung et al., 1984). An elevation in BCAA-T activity in brain in hyperammonemic states (as observed in the present study) might enhance the utilization of BCAA. However, it must be mentioned that the changes in the activities of BCAA-T observed in the different regions of the brain of rats injected with ammonium acetate were not exactly similar to those observed in hepatectomised animals. For instance a small decrease in the activity of this enzyme was observed in the brainstem of rats injected with ammonium acetate while an elevation was noticed in the same region in hepatectomised animals. This may be due to the difference in the induction of hyperammonemia and the differences in the time course of increase in blood and brain ammonia levels.

In hepatectomised animals, rise in ammonia level is much slower than in the animals injected with ammonium acetate. As a result, brain is exposed to a persistently increasing ammonia levels for prolonged periods in the earlier group of animals. Duration of exposure of brain to ammonia is much shorter in the animals injected with an acute dose of ammonium acetate. Such differences would naturally affect the reactivity of the tissue to the toxic insult.

It has been shown that the brainstem is highly vulnerable to acute hyperammonemia (Schenker et al., 1967; Hindfelt and Siesjo, 1971; Hindfelt et al., 1977; McCandless and Schenker, 1981; Sadasivudu et al., 1977). This may be due to the lack of change in the BCAA-T activity in this region. Consequently replenishment of glutamate required for the synthesis of glutamine would be inadequate. As a result, there might be an early depletion of glutamate pools in this region in hyperammonemia. This may affect the operation of the malate-aspartate shuttle (in which glutamate is a participant) and the neurotransmitter function of glutamate in this region. This suggestion is in concurrance with the reported decrease in ATP levels in this region in hyperammonemia (c.f. above references).

In order to verify the suggestion that BCAA may provide glutamate required for glutamine synthesis in brain in hyperammonemic states, the effects of the drug MSO were studied on BCAA-T activity. MSO is known to inhibit

glutamine synthesis by acting as a competitive inhibitor of GS and induces hyperammonemia (Sellinger and Weiler, 1963; Ronzio et al., 1969; Rao and Murthy, 1988). As the effect of this drug depends on the amount of drug injected, two different doses were administered, (Subbalakshmi and Murthy, 1981, 1983a; Ratnakumari et al., 1985). A dose of 300 mg/kg body wt, hereafter referred to as **acute dose**, induced hyperammonemia and other changes in the behaviour which terminate in convulsions and death within a span of 3.5 hours. A **subacute dose** of 150 mg/kg body wt induces similar changes in behaviour over a prolonged period of 17.5 hours. Due to these differences in the time course of drug action, reactivity of the brain would also vary with the dose of the drug administered. Results obtained on the effects of MSO on BCAA-T have been published, (Jessy and Murthy,1988).

EFFECTS OF METHIONINE SULPHOXIMINE ON BCAA-T ACTIVITY

Administration of an acute dose of MSO suppressed BCAA-T activity with leucine as substrate in all the regions of brain except in hippocampus, corpus striatum and midbrain where it was unaltered, while on administration of a subacute dose of MSO, an elevation in the activity of this enzyme was observed in all the regions of brain except in corpus striatum (Table 3.12).

When isoleucine was used as substrate, a statistically significant decrease in the activity of of BCAA-T was

TABLE 3.11

BCAA-T ACTIVITY WITH LEUCINE AS SUBSTRATE IN THE BRAINS OF NORMAL AND METHIONINE SULFOXIMINE TREATED RATS

REGION	CONTROL	METHIONINE SULFOXIMINE TREATED	
		ACUTE	SUBACUTE
CC	1.20±0.10(8)	1.03±0.12(6)$^{+}_{*}$	1.81±0.14(6)$^{*}_{*}$
CE	1.18±0.19(8)	0.82±0.14(5)*	1.69±0.19(5)*
BS	3.59±0.25(6)	1.78±0.25(6)	2.60±0.43(5)$^{+}$
HC	1.10±0.13(8)	1.21±0.08(5)	1.32±0.19(6)
CS	1.05±0.13(8)	1.83±0.27(5)	1.47±0.16(5)*
MB	2.15±0.28(5)	2.15±0.03(6)	3.92±0.49(5)

CC: cerebral cortex; CE:cerebellum; BS: brain stem; HC: hippocampus; CS: corpus striatum; MB: mid brain. Values are Mean±S.D. Activity is expressed as μmoles of ∝-keto-isocaproic acid formed/gm wet wt./min. Numbers in parenthesis indicate the number of experiments. * $p<0.005$, + $p<0.05$.

TABLE 3.12

BCAA-T ACTIVITY WITH ISOLEUCINE AS SUBSTRATE IN THE BRAINS OF NORMAL AND METHIONINE SULFOXIMINE TREATED RATS.

REGION	CONTROL	METHIONINE SULFOXIMINE TREATED	
		ACUTE	SUBACUTE
CC	1.79±0.20(6)	1.65±0.23(5)	5.00±1.00(5)*
CE	1.79±0.19(6)	1.72±0.34(5)*	6.70±1.20(5)$^{+}_{*}$
BS	6.93±0.90(6)	4.53±0.40(5)$^{+}$	8.58±0.33(4)*
HC	1.53±0.20(5)	1.86±0.18(4)$^{+}$	1.51±0.51(5)*
CS	1.68±0.18(6)	2.15±0.37(6)$^{+}$	4.79±0.28(6)*
MB	5.58±0.47(6)	4.64±0.60(5)$^{+}$	11.40±0.62(4)

CC: cerebral cortex; CE:cerebellum; BS: brain stem; HC: hippocampus; CS: corpus striatum; MB: mid brain. Values are Mean±S.D. Activity is expressed as μmoles of ∝-ketomethyl-valeric acid formed/gm wet wt./min. Numbers in parenthesis indicate the number of experiments. * $p<0.005$, + $p<0.05$.

observed in brainstem and midbrain regions of rats administered with an acute dose of MSO. Activity of this enzyme was elevated in hippocampus and corpus striatum under these conditions. In animals treated with a subacute dose of MSO, an elevation in the activity of this enzyme was observed in all regions of brain. Changes noticed in the activity of this enzyme were of greater magnitude with isoleucine as substrate than those found with leucine (Table 3.12).

With valine as substrate, BCAA-T activity showed a decrease substantially in all regions of the brain except cerebellum (unchanged), and in midbrain (elevated) in animals injected with an acute dose of MSO. On administration of a subacute dose of MSO, activity of this enzyme was enhanced in all regions of brain (Table 3.13).

TABLE 3.13

BCAA-T ACTIVITY WITH VALINE AS SUBSTRATE IN THE BRAINS OF NORMAL AND METHIONINE SULFOXIMINE TREATED RATS.

		METHIONINE SULFOXIMINE TREATED	
REGION	CONTROL	ACUTE	SUBACUTE
CC	2.08±0.19(5)	1.52±0.18(4)*	2.80±0.36(5)*
CE	1.59±0.29(5)	1.79±0.23(4)*	2.45±0.37(5)*
BS	6.00±0.70(5)	3.64±0.18(5)*	8.10±0.64(4)
HC	1.89±0.45(5)	0.80±0.08(6)*	2.37±0.39(6)*
CS	1.75±0.36(6)	0.88±0.14(5)*	2.52±0.22(6)*
MB	4.79±0.46(5)	6.59±0.39(5)*	6.55±1.00(4)+

CC: cerebral cortex; CE:cerebellum; BS: brainstem; HC: hippocampus; CS: corpus striatum; MB: mid brain. Values are Mean±S.D. Activity is expressed as μmoles of ∝-ketoisovaleric acid formed/gm wet wt./min. Numbers in parenthesis indicate the number of experiments.

As was mentioned earlier, during MSO toxicity glutamine synthesis is suppressed but hyperammonemia prevails in the organism. It has been shown that under these conditions excess ammonia would be detoxified by the reductive amination of ∝-KG in GDH reaction (Guiterrez and Norenberg, 1977, 1979; Subbalakshmi and Murthy 1983a, 1985a). In such a situation, requirement to replenish the glutamate pool (required for glutamine synthesis), will be minimal and the emphasis would be shifted towards the replenishment of ∝-KG required for GDH reaction. Decreased activity of BCAA-T under these conditions would lower the production of glutamate by transamination of BCAA and spare ∝-KG required for the GDH reaction.

However, in animals administered with a subacute dose of MSO, BCAA-T activity was found to be enhanced in several brain regions. At the outset it may appear that this observation contradicts the above hypothesis. However, it has been shown that the GS activity recovers in the subacute group of animals (Rao and Murthy, 1988) and as a result ammonia is detoxified to a small extent in this pathway. Under these conditions, the need to replenish the glutamate pool arises once again and thus changes seen in the BCAA-T activity seems to be appropriate under these conditions.

TRANSAMINASES IN SUBCELLULAR FRACTIONS

It is well known that there are several distinct membrane bound, physically separate compartments, such as mitochondria, endoplasmic reticulum, nucleus etc., in the cell. Some of the metabolic reactions are restricted to specified subcellular compartments. For eg., localisation of the TCA cycle in the mitochondria and the glycolytic pathway in the cytosol. Though these compartments are physically seperated, a dynamic interaction involving an exchange of substrates, precursors and products between these compartments has been postulated. Hence, changes in metabolic processes in one subcellular compartment might affect related metabolic processes in other compartments. Studies on stress induced alterations of metabolism will never be complete unless these changes are attributed to a particular subcellular compartment. In order to evaluate such a localisation, these subcellular compartments are to be separated before studying the alterations in the metabolic process.

Subcellular fractionation of several tissues yield majorly five fractions - nuclei, mitochondria, cytosol, microsomes and lysomes. Unlike these tissues, brain yields two more fractions i.e., myelin and synaptosomes (Whittaker and Barker, 1972). The latter fraction comprises of the detached nerve terminals. It has been shown that when brain is homogenized under controlled conditions, axonal nerve

endings will be severed from the neuron and are resealed to form membrane bound particles which are called synaptosomes. Each synaptosome comprises of a membrane enclosing mitochondria, cytoplasm (synaptoplasm) and synaptic vesicles (Whittaker and Barker, 1972). As the name itself indicates, myelin fraction consists of myelin membranes.

Earlier studies on subcellular localization of BCAA-T in several tissues including brain revealed that this enzyme is present both in cytosol and mitochondria (Taylor and Jenkins, 1966; Roswell and Turner, 1963; Ichihara and Koyama, 1966; Kadawaki and Knox, 1982; Benuck et al., 1972). There are three isozymes of BCAA-T i.e., Type I, II and III. Of these, isozyme II is leucine specific and present in the liver while isozymes I and III are more ubiquitious in their distribution. Studies on the subcellular distribution of these isozymes in brain revealad that the mitochondria contained both type I and III isozymes while the cytosol has predominantly type III isozyme. Myelin and synaptosomal fraction were found to have only type III isozyme. Kinetic studies revealed no differences in the affinity of cytosolic and mitochondrial type III isozyme for BCAA and α-KG. Both type I and type III were, however, shown to be immunologically different forms and the latter isozyme is a characteristic feature of undifferentiated cells. Type II isozyme of BCAA-T is absent in the brain (Kadawaki and Knox, 1982).

As was mentioned earlier, BCAA-T is localised in cytosol

and in mitochondria. Preparation of homogenates in sucrose and the addition of detergents, such as Triton X-100, during the assay of transaminases, abolishes the constraints imposed by subcellular compartmentation, thereby creating a homogenous system. Hence, increased activity of BCAA-T observed in hyperammonemic states (c.f. chapter 3.1) could not be assigned to any of the subcellular compartments of the brain.

Standardisation studies carried out on this enzyme with subcellular fractions isolated from cerebral cortex indicated that, with the three BCAA as substrates, the activity of this enzyme is linear upto 40 µgms protein in cytosol, mitochondria and synaptosomes (Fig 3.7). Activity of BCAA-T was found to be linear upto 60 min of incubation (Fig 3.11A). Hence, in the routine assays an incubation period of 30 min was selected. Enzyme activity was found to increase with increasing concentration of α-KG till the point of saturation is reached (Fig 3.9). Calculated Km values for α-KG, leucine, isoleucine and valine are given below (Table 3.12).

With leucine as substrate, no differences were seen in the activity levels of BCAA-T in the mitochondrial, cytosolic and synaptosomal fractions isolated from normal animals (Table 3.13). A similar profile was also observed by (Brosnan et al., 1985) but for a higher activity in their cytosolic preparation than that observed in the present study. This variation could be due to the differences in the isolation procedures adopted. Activity levels of BCAA-T with

TABLE 3.12

KINETIC PARAMETERS OF BCAA-T IN SUBCELLULAR FRACTIONS ISOLATED FROM THE CEREBRAL CORTEX OF NORMAL RATS

FRACTION		LEUCINE	ISOLEUCINE	VALINE
Cytosol	Km	3.55	2.95	1.45
	Vm	2.89	6.93	4.97
Mitochondria	Km	2.49	1.75	3.49
	Vm	2.30	6.58	4.75
Synaptosomes	Km	2.58	2.00	0.80
	Vm	2.35	5.19	3.90

Km values are in mM
Vm values are in μmoles of α-keto acid formed/mg protein/hr

isoleucine as substrate was more or less same in all the three subcellular fractions. With valine as substrate, activity of this enzyme was more in mitochondria than in the other two subcellular fractions (Table 3.13).

TABLE 3.13
SUBCELLULAR DISTRIBUTION OF THE BCAA-T ACTIVITY IN BRAINS OF NORMAL RATS

FRACTION	SUBSTRATE		
	LEUCINE[a]	ISOLEUCINE[b]	VALINE[c]
MITOCHONDRIA	2.3±0.4(5)	5.5±0.9(9)	5.6±1.2(4)
CYTOPLASM	2.9±0.2(5)	5.0±0.7(7)	3.6±0.2(5)
SYNAPTOSOMES	2.7±0.4(5)	4.3±1.2(7)	2.3±0.9(5)

Values are Mean±S.D. Numbers in parenthesis indicate the number of experiments.
a: μmoles of α-ketoisocaproic acid formed/mg protein/hr.
b: μmoles of α-ketomethylvaleric acid formed/mg protein/hr.
c: μmoles of α-ketoisovaleric acid formed/mg protein/hr.

Studies on subcellular distribution of these enzymes revealed certain interesting aspects. Higher activity observed with isoleucine and valine than with leucine may be due to the differences in the permeability of these amino acids across the mitochondrial and synaptosomal membrane or it may be because of the fact that these may be preferred substrates for the enzyme or there may be different isozymes with different substrate specificities. In the present study, differential permeabilities may not play a major role as the membranes have been permeabilised with addititon of Triton X-100 to the reaction mixture. Another interesting feature of the present study is the presence of this enzyme in the synaptosomal fraction.

Based on the results obtained from the studies on metabolic compartmentation, it was proposed that the BCAA are used exclusively in the small pool of glutamate, localised in the astrocytes (Cremer et al., 1977). The large compartment of glutamate represented primarily by the neuronal elements, is devoid of the mechanisms to metabolise BCAA (Cremer et al., 1975, 1977; Cavanaugh, 1974). However, Chaplin et al., (1976) have shown that the synaptosomes are capable of producing $^{14}CO_2$ from both [1-^{14}C] and [U-^{14}C] leucine. Production of CO_2 from the latter indicates that leucine is metabolised in the synaptosomes beyond C-1 decarboxylation. This is in accordance with the present observations. Further,

Murthy and Hertz (1986, 1987a,b) reported that primary cultures of neurons are capable of oxidising all the three BCAA. As synaptosomes are derived from neurons, results of the present study are in agreement with their results. While interpreting the results with synaptosomal enriched preparation, contamination with gliosomes must be given due importance. However, earlier studies indicate that the synaptosomal fraction isolated by the method adopted in the present study is atleast 80-85% pure (Subbalakshmi and Murthy, 1985), thus the possibility of a major contribution of gliosomes to this reaction seems to be remote.

Metabolic fate of the BCAA may vary in each subcellular compartment. In the mitochondria glutamate, produced due to the transamination of BCAA, may be utilised as a substrate in transamination with other keto acids or may be deaminated and α-KG will be regenerated. However, in the latter process α-amino group will be eliminated as ammonia in the mitochondria. Moreover, BCKA produced during transamination of BCAA might be oxidised in the mitochondria, producing CO_2 and thus helping in the production of ATP. In the cytosol, due to the absence of GDH, glutamate formed in the transamination of BCAA will be used majorly as a substrate in other transamination reactions. This pool of glutamate (formed from BCAA) may also be utilised for the synthesis of glutamine in the cytosol. Alternately glutamate, may be transported into the mitochondria by the aspartate-glutamate

carrier and thus participate in the the malate-aspartate shuttle.

Studies of Yudkoff et al., (1983b) reveal that major fraction of label from ^{15}N-leucine enters into glutamate and glutamine and a very small amount into aspartate and other amino acids. This suggests that the glutamate formed by the transamination of BCAA is majorly utilised for the synthesis of glutamine. As synaptosomes contain both cytosol and mitochondria, the fate of the glutamate formed in the transamination of BCAA may follow the same path as described above.

However, it must be mentioned that there are controversies regarding the synthesis of glutamine and the presence of GS in the synaptosomes. Though the results of Martinez-Hernandez et al., (1977) and Norenberg and Martinez-Hernandez (1979) corroborate the concept of metabolic compartmentation (that GS is present exclusively in the astrocytes), Some of the recent results demonstrate the presence of GS in synaptosomes and other neuronal elements (Subbalakshmi and Murthy, 1985b; Rani et al., 1983). Unless this issue is resolved it will be difficult to asses the involvement of synaptosomes in glutamine synthesis.

ACUTE EFFECTS OF AMMONIA ON BCAA-T IN SUBCELLULAR FRACTIONS

Similar to the homogenates, a generalised increase in the activities of BCAA-T was observed in subcellular fractions following the administration of ammonium acetate except that

the activity of BCAA-T in the mitochondrial fraction remained unaltered when isoleucine and valine were used as substrates. The magnitude of elevation appears to be more in the cytosol with all the three BCAA as substrates. With leucine as substrate, BCAA-T activity was enhanced in all the sub-cellular fractions isolated from hyperammonemic rats. Decrease in the activity of this enzyme in the mitochondria, with valine as substrate, was not statistically significant. The BCAA-T activity using isoleucine as substrate remained unaltered in mitochondria, while an elevation was observed in the synaptosomes and cytosol. The profile of changes in BCAA-T activity with leucine as substrate was mitochondria > synaptosomes > cytosol; with valine and isoleucine it was synaptosomes > cytoplasm > mitochondria (Table 3.14).

These results are in accordance with earlier proposal that the glutamate formed from the BCAA-T is utilised for the synthesis of glutamine. In the mitochondrial fraction glutamate produced from BCAA may not be deaminated as GDH is suppressed by pathophysiological concentration of ammonia (Subbalakshmi and Murthy, 1983b; Yu et al., 1984). Hence, glutamate in this subcellular may be transaminated with OAA to produce aspartate. Aspartate may be exchanged with the cytosolic glutamate through the glutamate-aspartate carrier and thus participate in the malate-aspartate shuttle. Branched chain keto acids, produced during transamination, may be utilised in the mitochondria for the production of

TABLE 3.14

BRANCHED-CHAIN AMINO ACID AMINOTRANSFERASE ACTIVITY IN SUBCELLULAR FRACTIONS ISOLATED FROM NORMAL RATS AND THOSE TREATED WITH AMMONIUM ACETATE

CONDITION	MITOCHONDRIA	SYNAPTOSOMES	CYTOPLASM
		LEUCINE AS SUBSTRATE[A]	
NORMAL	2.3±0.4(5)**	2.7±0.4(5)**	2.9±0.2(5)**
AMM.AC	7.2±1.6(5)**	6.1±1.1(4)**	7.9±0.8(5)**
		VALINE AS SUBSTRATE[B]	
NORMAL	5.6±1.2(4)	2.3±0.9(5)**	3.6±0.2(5)**
AMM.AC	4.2±0.8(6)	4.2±0.8(6)**	5.3±0.6(4)**
		ISOLEUCINE AS SUBSTRATE[C]	
NORMAL	5.5±0.9(9)	4.3±1.2(7)**	5.0±0.7(7)**
AMM.AC	5.4±1.2(5)	7.3±1.1(6)**	7.5±1.5(6)**

Values are Mean±S.D. ** $p<0.001$.
A) μmoles of α-ketoisocaproic acid formed/mgprotein/hr;
B) μmoles of α-ketoisovaleric acid formed/mg protein/min.
C) μmoles of α-ketomethylvaleric acid formed/mg protein/min. Numb
in parenthesis indicate the number of experiments.

energy. However, BCKA produced in the cytosol may be transported either into the mitochondria or out of the cell. As similar changes were observed in the activity of BCAA-T in the synaptosomes the metabolic pathway may be the same as above. It must be mentioned at this juncture that the theory of metabolic compartmentation predicted no substantial changes in the metabolism of the neuronal components (Benjamin and Quastel, 1974). However, earlier studies from this laboratory has demonstrated that synaptosomes may be involved in the process of ammonia detoxification

(Subbalakshmi and Murthy, 1985a,b). Results of the present study are in accordance with these results.

EFFECTS OF MSO ON BCAA-T IN SUBCELLULAR FRACTIONS

As mentioned earlier, MSO induces hyperammonemia by blocking glutamine synthesis. By virtue of this, this drug may be used as an excellent tool to study the interrelation between glutamine synthesis and BCAA metabolism in hyperammonemia. Hence, in the present study MSO has been used to gain further insights into the role of BCAA in hyperammonemia.

Following the administration of an acute dose of MSO, BCAA-T activity was suppressed in all the three subcellular fractions, irrespective of the amino acid used as substrate. The magnitude of suppression was greatest with isoleucine as substrate in the three subcellular fractions and was least with leucine as substrate. On administration of a subacute dose of MSO, activity of this enzyme with leucine as substrate was unaltered in mitochondria and cytosol while it was suppressed in the synaptosomes. Magnitude of suppression in the activity of this enzyme was lesser in subacute than in acute condition in synaptosomes. Similarly, the activity of this enzyme with valine and isoleucine as substrate was suppressed to a lesser degree in all the subcellular fractions. Only exception to this is in synaptosomes where the percent change in activity of this enzyme as valine with was higher in subacute than in acute condition.

TABLE 3.14

BCAA-T ACTIVITY IN SUBCELLULAR FRACTIONS ISOLATED FROM NORMAL RATS AND THOSE TREATED WITH METHIONINE SULPHOXIMINE

CONDITION	MITOCHONDRIA	SYNAPTOSOMES	CYTOPLASM
	LEUCINE AS SUBSTRATE[A]		
NORMAL	2.3±0.4(5)*	2.7±0.4(5)**	2.9±0.2(5)**
MSO(ACUTE)	1.5±0.2(5)*	1.4±0.2(5)*	1.0±0.1(5)**
MSO(SUBACUTE)	2.6±0.6(5)	1.9±0.2(5)*	3.1±0.9(5)
	VALINE AS SUBSTRATE[B]		
NORMAL	5.6±1.2(4)**	2.3±0.9(5)	3.6±0.2(5)**
MSO(ACUTE)	1.2±0.1(5)**	1.0±0.2(5)+	1.7±0.3(5)**
MSO(SUBACUTE)	3.3±0.6(5)++	0.6±0.2(4)*	3.1±0.2(4)++
	ISOLEUCINE AS SUBSTRATE[C]		
NORMAL	5.5±0.9(9)**	4.3±1.2(7)**	5.0±0.7(7)**
MSO(ACUTE)	0.5±0.1(5)**	1.3±0.2(5)**	1.2±0.2(5)**
MSO(SUBACUTE)	3.0±0.7(5)**	2.5±0.5(5)++	3.6±0.9(5)+

Values are Mean±S.D. A) μmoles of α-ketoisocaproic acid formed/mg protein/hr; B) μmoles of α-ketoisovaleric acid formed/mg protein/hr; c) μmoles of α-ketomethylvaleric acid formed/mg protein/min. Numbers in parenthesis indicate the number of experiments. **p<0.001, *p<0.005, ++p<0.01, p<0.02.

These changes in the activity of BCAA-T in MSO toxicity are similar to those observed in homogenates. Various aspects of this phenomena have already been discussed . These changes are in concurrance with the proposed role of BCAA in hyperammonemia.

TRANSAMINASES IN CELLULAR FRACTIONS

Studies on the subcellular localization of BCAA-T revealed that the metabolism of BCAA might take place in both neuronal and non-neuronal fractions. As the mitochondria which were isolated from brain might have been derived from either neuronal or the glial cells it would be difficult to localise BCAA-T in the three different cell types. It is quite possible that these enzymes are totally absent in the nerve terminals under in vivo condition, but might have been acquired during the isolation procedure. Hence, an attempt is made, in the present study, to isolate the different cell types from normal and hyperammonemic animals. Before presenting the results of this study, it may be worthwhile to review the earlier work done in this aspect. Berl and Frigyesi (1969), van den Berg and van den Velden (1970), Cremer et al., (1975, 1977), studied the incorporation of leucine carbon into glutamate and glutamine and observed that specific radioactivity of the glutamine was higher than that of glutamate. They concluded that the BCAA are metabolised in the compartment with a rapidly turning over small pool of glutamate where glutamine is synthesized. This was later shown to be localized in the astrocytes. Hence, it was predicted that BCAA are metabolised in the astrocytes but not in neurons. Moreover, van den Berg and van den Velden (1970) demonstrated that the pool of glutamate formed from leucine and incorporated into glutamine expands in hyper-

ammonemic states induced by MSO. Similarly, Cremer et al., (1975, 1977), reported an increased incorporation of ^{3}H label from leucine into glutamate in the brains of rats subjected to portacaval shunt. This change was found to occur at the time when astrocytes undergo an Alzheimer type II astrocytic change. Identical results were also obtained by Cavanaugh (1974). All these studies led to a firm belief that BCAA are metabolized in the astrocytes.

It is surprising that while formulating this concept, reports on the incorporation of leucine carbon into myelin lipids (Wiggins et al., 1979) and oxidation of these amino acids in the peripheral nervous system (Buse et al., 1975) were ignored. Similarly, not much importance was given to the works of Chaplin et al., (1976), demonstrated $^{14}CO_2$ production from [1-^{14}C] and [U-^{14}C]-leucine in synaptosomes and suggested that the BCAA may be metabolized in neuronal components.

With the availability of primary cell cultures the problem of cross contamination with other cells has been eliminated to a large extent. Murthy et al., (1986) and Murthy and Hertz (1987 a,b) reported that neurons as well as astrocytes in primary cell cultures are capable of producing CO_2 from all the three BCAA. They observed that the metabolism of BCAA in primary cultures of neurons was lower than that of astrocytes. Thus, these results indicates that the entire pathway for the metabolism of these amino acids is

present both in astrocytes and in neurons. In addition, they also observed that these amino acids are also incorporated into proteins of these cells. Further, studies indicated that pathophysiological concentrations of ammonia suppressed the oxidation of these amino acids atleast in the astrocytes in primary cultures.

CELLULAR DISTRIBUTION OF BCAA-T IN NORMAL ANIMALS

Preliminary studies revealed the presence of BCAA-T in all the three cell fractions viz., neurons, astrocytes and oligo cells. Enzyme acitivity was linear in all the preparations upto 80 µg of protein (Fig 3.10), hence 50 µg of protein was used in subsequent studies. Linearity was observed in enzyme activity upto 60 min of incubation time (Fig 3.11B), hence 30 min was selected as the time of incubation. Michealis-Menten kinetics were observed in the

TABLE 3.15

KINETIC CONSTANTS OF BCAA-T IN VARIOUS CELLULAR FRACTIONS ISOLATED FROM NORMAL ANIMALS.

FRACTION		LEUCINE	ISOLEUCINE	VALINE
ASTRO-CYTES	Km	8.5	2.71	1.56
	Vmax	2.95	0.47	0.30
NEURONS	Km	3.49	4.12	3.77
	Vmax	0.67	0.64	0.43
OLIGO CELLS	Km	1.84	1.38	2.66
	Vmax	0.60	0.60	0.58

Km is expressed as mM amino acid. Vmax is expressed as µmol amino acid/mg protein/hr.

enzyme activity with respect to the concentration of BCAA and the Km and Vmax are given in the accompanying table (Table 3.15).

Two different modes have been adopted to express the enzyme activity i.e., activity/mg protein (specific activity) and activity/cell (cellular activity). This becomes essential as the protein content per cell varies with the cell type, for example astrocytes have much higher protein compared to neurons and oligo cells.

TABLE 3.16

PROTEIN CONTENT OF DIFFERENT CELLULAR FRACTIONS ISOLATED FROM NORMAL AND EXPERIMENTAL ANIMALS.

FRACTION	NORMAL	AMM.ACETATE	%OVER CONTROL
NEURONS	391±54(4)	561±37(4)*	+43
ASTROCYTES	614±32(4)	794±54(4)*	+29
OLIGOCELLS	167±35(4)	131±31(6)	-21

Values expressed as pg/cell. Number in parenthesis indicate number of experiments. *p<0.001

As the enzyme protein is only a part of several different proteins in the cell representation of activity per mg protein might be misleading if the ratio of the enzyme protein to other proteins varies from one cell to the other or within the same cell in pathological states. Such differences in the enzyme makeup of different cells in the same tissue is quite well known. For eg., in the brain the activity of GS is higher in glia than in neurons. In fact there is an enhancement in the astrocytic and neuronal

proteins and a decrease in the protein content of the oligo cells in hyperammonemia (Table 3.16). A change in the total protein content does not necessarily indicate a change in the enzyme protein, hence mere representation of the enzyme activity as specific activity might be misleading under these conditions. Change in enzyme protein with out any change in total protein content might complicate the situation. If the enzyme activity is regulated by parameters other than its content, such as, as phosphorylation - dephosphorylation or allosteric modulators are associated with these changes in total or enzymic proteins, then the situation becomes much more complicated.

TABLE 3.17

DISTRIBUTION OF BRANCHED-CHAIN AMINOACID AMINOTRANSFERASE ACTIVITY DIFFERENT CELLULAR FRACTIONS ISOLATED FROM NORMAL RATS

FRACTIONS	AMINOTRANSFERASE ACTVITIY		
	LEUCINE·	VALINE·	ISOLEUCINE·
	NMOLES/MG. PROTEIN/HOUR		
NEURONS	340+030(5)^{b*c*}	470+080(5)^{b*c*}	180+040(5)^{b*c*}
ASTROCYTES	680±170(6)	700±180(4)	560±040(4)$^{c+}$
OLIGOCYTES	600±090(7)	640±170(7)	430±070(7)
	PMOLES/CELL/HOUR X 10^{-3}		
NEURONS	138±013(5)	180±030(5)	080±020(5)
ASTROCYTES	530±100(4)^{a*c*}	430±100(4)^{a*c*}	540±020(4)
OLIGOCYTES	100±018(8)	104±027(7)	072±010(7)

Values are Mean±S.D. *$p<0.001$; +$p<0.01$
Numbers in parenthesis indicate the number of experiment.
a-comparison with neurons, b-comparison with astrocytes,
c-comparison with oligo cells.

A comparison of the activity of BCAA-T in the three different cell fractions isolated from normal animals reveal no differences in the specific activity of this enzyme in astrocytes and oligo cells (Table 3.17). However, the specific activity in the neurons was 2-3 fold lesser than that of astrocytes. Cellular activity of BCAA-T was similar in neurons and oligocells and lower than that of astrocytes. These studies reveal that the metabolism of these amino acids is not restricted to the astrocytes alone as was predicted in the concept of metabolic compartmentation. Results of the present study are in agreement with the reports on the presence of this enzyme in the synaptosomes and neurons in primary cultures. This evidently indicates that the BCAA-T activity detected in the synaptosomes may not due to the contamination with gliosomes or acquired during the isolation procedure. It is possible that BCAA-T is synthesised in the neuronal perikarya may be transported down to the nerve terminals by axonal transport.Higher activity of BCAA-T in astrocytes is in accordance with the concepts of metabolic compartmentation.

Presence of BCAA-T in neurons suggest that atleast some amount of glutamate may be formed from BCAA. BCKA produced during transamination might be utilised in neuronal energy metabolism, or released into the extracellular space, which may be utilized by astrocytes. Glutamate derived from BCAA in the astrocytes may be utilised for the synthesis of

glutamine, aspartate or alanine. The fate of BCKA in these cells might be similar to that of neurons but they may also be released into the blood.

Presence of BCAA-T in the oligo cells suggest that these cells also participate in the metabolism of these amino acids with the subsequent production of glutamate. However, it is difficult to envisage the metabolic fate of this glutamate as no information is available on the role of these cells in glutamate metabolism in brain. The ketoacids generated from the BCAA in these cells may be further metabolised to generate acetyl CoA which may be utilised for the synthesis of myelin lipids. This concept is in accordance with the earlier observation that the labelled carbon from BCAA is incorporated into the myelin lipids (Wiggins et al., 1979). Thus, the present results on the cellular distribution of these enzymes suggest that all the three cell types in the brain might participate in the metabolism of BCAA and that it is not an exclusive property of any single cell type. The fate of glutamate, formed in these cells during the transamination of BCAA, might vary according to the cell type.

ACUTE EFFECTS OF AMMONIA ON THE ACTIVITY OF BCAA-T IN CELLS

Having established the presence of this enzyme in all the three cell types, it would be worthwhile to check the response of BCAA-T to administered ammonium salts in these cell types.

In the neuronal perikarya isolated from hyperammonemic rats both the specific and cellular activities of this enzyme were suppressed (Table 3.18). The magnitude of suppression was highest when leucine was the substrate and least when valine was substrate. A suppression of similar magnitude was observed in the cellular activity of BCAA-T when leucine and

TABLE 3.18

BRANCHED-CHAIN AMINO ACID AMINOTRANSFERASE ACTIVITY IN NEURONAL PERIKARYA ISOLATED FROM NORMAL RATS AND THOSE TREATED WITH AMMONIUM ACETATE

CONDITION	AMINOTRANSFERASE ACTVITIY		
	LEUCINE[1]	VALINE[2]	ISOLEUCINE[3]
	NMOLES/MG. PROTEIN/HOUR		
NORMAL	340±30(5)**	180±40(5)**	470±80(5)**
AMM.AC	160±10(6)	110±10(6)	270±50(5)
	PMOLES/CELL/HOUR X 10^{-3}		
NORMAL	138±13(5)**	80±20(5)**	180±30(5)+
AMM.AC	075±005(6)	52±08(6)	140±30(6)

Values are Mean±S.D. Activity expressed as (1)nmoles α-keto-isocaproic acid (2)nmoles α-ketoisovaleric acid (3)nmoles α-ketomethyl valeric acid. **p<0.001, +p<0.02.
Numbers in parenthesis indicate the number of experiment.

valine were substrates. With isoleucine as substrate though there was a suppression in the cellular activity, it was statistically less significant when compared to that with leucine and valine.

The magnitude of suppression in the BCAA-T activity with isoleucine and valine as substrates was more or less same irrespective of the mode of expression of the activity. An

execption to this was that of isoleucine wherein the suppression of the specific activity was much higher than that of the cellular activity.

TABLE 3.19

BRANCHED-CHAIN AMINO ACID AMINOTRANSFERASE ACTIVITY IN ASTROGLIAL CELLS ISOLATED FROM NORMAL RATS AND THOSE TREATED WITH AMMONIUM ACETATE

	AMINOTRANSFERASE ACTVITIY		
CONDITION	LEUCINE[1]	VALINE[2]	ISOLEUCINE[3]
	NMOLES/MG. PROTEIN/HOUR		
NORMAL	680±170(5)**	560±40(4)**	700±180(4)
AMM.AC	500±060(5)**	340±30(7)**	680±080(6)
	PMOLES/CELL/HOUR X 10^{-3}		
NORMAL	530±100(4)**	340±20(4)**	430±100(4)*
AMM.AC	390±050(6)**	280±20(6)**	540±050(6)*

Values are Mean±S.D. Activity expressed as (1)nmoles of ∝-ketoisocaproic acid (2) nmoles of ∝-ketoisovaleric acid (3) nmoles of ∝-ketomethyl valeric acid formed. **p<0.001, *p<0.05. Numbers in parenthesis indicate the number of experiment.

In the astrocytes isolated from hyperammonemic rats both cellular and specific activities of BCAA-T were suppressed when leucine and valine were used as substrates (Table 3.19). With isoleucine as substrate, there was no statistically significant change in the specific or cellular activities of this enzyme.

Changes in the specific and cellular activities of BCAA-T in oligodendroglia obtained from hyperammonemic rats were

TABLE 3.20

BRANCHED-CHAIN AMINO ACID AMINOTRANSFERASE ACTIVITY IN OLIGODENDROGLIA ISOLATED FROM NORMAL RATS AND THOSE TREATED WITH AMMONIUM ACETATE

CONDITION	AMINOTRANSFERASE ACTVITIY		
	LEUCINE[1]	VALINE[2]	ISOLEUCINE[3]
	NMOLES/MG. PROTEIN/HOUR		
NORMAL	600+090(7)	430+070(7)**	640+170(7)
AMM.AC	600±100(6)	230±030(5)**	600±100(5)
	PMOLES/CELL/HOUR X 10^{-3}		
NORMAL	100+018(8)	072+010(7)**	104+027(7)
AMM.AC	086±017(5)	031±005(5)**	110±010(4)

Values are Mean+S.D. Activity expressed as (1)nmoles ∝-keto-isocaproic acid (2) nmoles ∝-ketoisovaleric acid (3) nmoles ∝-ketomethylvaleric acid. **$p<0.005$.
Numbers in parenthesis indicate the number of experiment.

statistically not significant when leucine and isoleucine were used as substrates. With valine as substrate a 50% fall in both specific and cellular activities in the same cellular fraction in hyperammonemia (Table 3.20).

In brief, the results obtained indicated that there is a generalized suppression in BCAA-T activity in the neuronal perikarya and to a small extent in astrocytes. In oligodendroglia the activity of this enzyme remained unaltered except when valine was the substrate. Such differential behaviour of the enzyme with respect to its substrate in hyperammonemic states is difficult to explain if it is assumed that there is a single enzyme catalysing this reaction. There are evidences in literature to indicate the

existance of multiple molecular forms of this enzyme in brain i.e., Type I and Type III, (Kadawaki and Knox, 1982). It is quite possible that there is a differential distribution of this enzyme in the three different cell population of the brain. Further studies in this direction are required to clarify this aspect. Results obtained in the cellular preparations were rather surprising. In the studies with the homogenates and subcellular preparations it was observed that the activity of this enzyme (BCAA-T) is enhanced in hyperammonemic states. As these preparations are derived from one or the other cell types, it is quite natural to expect an enhanced BCAA-T activity in the cellular preparations. However, the results obtained were contradictory to this assumption. Inhibition in the activity of BCAA-T may be either real or an experimental artifact as the assay method involves the estimation of residual keto acids. An enhanced utilisation of these keto acids in cell preparations from hyperammonemic rats, would decrease the amount of keto acids that remain for estimation. Thus, an apparent decrease in activity would be seen. If this suppression in the activity is true then there would be a decrease in the amount of glutamate formed in the neuronal perikarya from the BCAA. In both the glial cells, changes in the activity of this enzyme were either statistically insignificant or of lesser magnitude. This suggests that the transamination of leucine and isoleucine are not affected while that of valine is

inhibited, as a result the synthesis of glutamate might still continue at normal rates in these cells in hyperammonemic states. Glutamate thus formed in the glial cells might be used for the synthesis of glutamine under these conditions.

In summary studies on BCAA-T revealed the possibility of these amino acids may be acting as precursors for the synthesis of glutamate in hyperammonemic states. However, it must be mentioned that the BCKA-DH and not BCAA is the rate limiting enzyme in the metabolism of BCAA. Hence changes in the activity of BCKA-DH in hyperammonemic states will be described in the following section.

BRANCHED CHAIN KETO ACID DECARBOXYLASES IN SUBCELLULAR FRACTIONS

As was mentioned earlier transamination of BCAA with ∝-KG results in the production of BCKA both in cytosol and mitochondria. The nature of the BCKA depends on the substrate oxidised, thus ∝-KIC is produced from leucine, ∝- KMV from isoleucine and ∝-KIV from valine. Further metabolism of the keto acids is supposed to take place in the mitochondria (Randle et al., 1981) i.e., the second step in the metabolism of BCKA, C-1 decarboxylation, which requires the presence of CoA, NAD^+ and TPP, resulting in the formation of branched chain ketoacyl CoA. Decarboxylation of all the three BCKA is mediated by a single enzyme complex, BCKA-DH, which is present in all mammalian tissues (Parker and Randle, 1978; Petit et al., 1978; Danner et al., 1979). Activity of this enzyme varies from tissue to tissue (Connelly et al., 1968; Wolheuter and Harper, 1970). Highest activity of this enzyme is in liver and lowest in muscle (Shinnick and Harper, 1976). This profile is opposite to that observed for BCAA-T. Based on these studies, it was proposed that the rate limiting process in the metabolism of BCAA in the liver is transamination while in extra hepatic tissues the rate limiting step is decarboxylation (Shinnick and Harper, 1976). It has been shown that about 91% of the BCKA-DH in the body is present in the liver, about 6% in the kidney, 1.3% in brain and about 0.2% in the heart (Shinnick and Harper, 1976;

Wohlhueter and Harper, 1970). Activity of this enzyme is modulated to suit physiological demands. For eg., its activity is enhanced during fasting or when the animals are supplied with a high protein diet (Gillim et al., 1983; Paul and Adibi, 1981; Wohlheuter and Harper, 1970). It was shown that it is an inducible enzyme and the induction is by cycloheximide sensitive and by cycloheximide insensitive mechanisms (Wohlheuter and Harper, 1970).

It was shown that brain is capable of oxidising the [1-^{14}C]BCKA, which suggests the presence of BCKA-DH in this tissue (Brand, 1981). However, it is interesting to note that virtually no literature is available on the cerebral BCKA-DH. Much of the available information is on the liver enzyme. Hence, a brief description on the properties and the mechanism of action of liver enzyme are given below.

Branched chain keto acid dehydrogenase is a multienzyme complex and is similar to that of PDH and α-KGDH (Ishikawa et al., 1966; Connelly et al., 1968; Pettit et al., 1978). It is mitochondrial in its localisation and consists of three subunits (Pettit et al., 1978). It comprises of a decarboxylase (E1), an acyl transferase(E2), and dihydrolipoamide dehydrogenase (E3). The E2 serves as the core of the complex on which E1 and E3 are arranged. In addition to these, an intrinsic kinase is also present which phosphorhylates E1 component of the complex. The E1 has subunits while E2 is a monomer with a single polypeptide chain. The E2 component of

the complex has a higher mol wt. than the E1 component (Pettit et al., 1978; Danner et al., 1979; Fatiana et al., 1981; Lau et al., 1982; Odessey, 1982; Lawson et al., 1983; Randle et al., 1981; Cook et al., 1985). The activity of this complex is regulated by feedback inhibition exerted by the products and Ac CoA. Under in vivo conditions, its activity is also regulated by the ratio of NAD to NADH (redox state) and by the ratio of reduced to oxidised CoA (Danner et al., 1978; Parker and Randle, 1978; Pettit et al., 1978; Randle et al., 1981).

Another important regulatory parameter is phosphorylation - dephosphorylation of the enzyme. While the inhibition exerted by the earlier two factors are non-convalent type of regulation, the phosphorylation-dephosphorylation system involves the covalent modification of the enzyme. As was mentioneed earlier, it is the E1 subunit of the enzyme which is subjected to phosphorylation. The E1 component is phosphorylated at two sites which are closely grouped on the α- subunit of the E1 component. There is a distinct homology in the sequence of amino acids at these phosphorylation sites of BCKA-DH with those of PDH. Phosphorylation of the E1 component changes its behaviour and results in the formation of E1b which is distinct from the i.e., non-phosphorylated E1 (E1a). The subunit E1a has greater affinity towards the E2 core than E1b. It is interesting to note that the kinase which phosphorylates E1 is associated with E2 rather is its

substrate i.e., E1. The α- subunit of the E1 component is phosphorhylated only in the presence of E2 and hence it is believed that the E2-kinase complex acts on the α- subunit of the E1 rather than the kinase alone. This suggests that the phosphorylation of the E1 component may take place at specific sites on the E2 subunit for which an intracore movement of E1 is a prerequisite. Though structurally and kinetically BCKADH and PDH are similar, there is no cross reaction between the E1 and E2 components of these two complexes. However, it is believed that there may be a common dihydrolipoamide dehydrogenase component (E3) for these two complexes (Randle, 1983; Cook et al., 1985).

Kinetics and mechanism of action of this enzyme is similar to that of PDH and α-KGDH. Oxidative decarboxylation of BCKA is achieved in four distinct reactions mediated by the decarboxylase, acyl transferase and dihydrolipoamide dehydrogenase.The first step mediated by BCKA-DC involves the formation of an active branched chain keto aldehyde-TPP complex, with the elimination of CO_2 from the BCKA. In the second stage, the active branched chain aldehyde-TPP complex reacts with lipoic acid forming an acyl lipoate and the TPP is regenerated. The acyl lipoate reacts with reduced CoA and is converted to branched chain keto acyl CoA and reduced lipoate is released. These two reactions are mediated by the acyl transferase component of the BCKA-DH complex. The reduced lipoate is oxidised in the presence of NAD to lipoic acid by

the dihydrolipoamide dehydrogenase (E3 component of the complex). The reducing equivalents are transferred to NAD. Not all the intermediates formed in the course of the reaction are released, instead they are transferred from one enzyme to the other in the multienzyme complex (Randle et al., 1981).

Several methods have been evolved to assay BCKA-DC. Initially, the CO_2 produced from the BCKA was measured by manometric techniques using Warburg apparatus. This method involves the measurement of the activity of the entire complex. Later, spectrophotometric determination of $Fe(CN)_6^{-3}$ reduction in the presence of BCKA was used as the assay procedure. In this procedure by ommitting CoA and NAD in the assay mixture, it is possible to measure decarboxylase, while a change in the absorbancy at 340 nm in the presence of NAD and CoA (without $Fe(CN)_6^{-3}$) is used as a measure of activity of the entire multienzyme complex (Gubler, 1961; Connelly et al., 1970). This has been replaced by a method in which [1-^{14}C]BCKA are used as substrates and the amount of liberated $^{14}CO_2$ indicates the activity of the enzyme (Wohlheuter and Harper, 1970). Though the latter method is highly sensitive, due to the non-availability of [1-^{14}C]BCKA, it was not adopted in this study. In the present study $Fe(CN)_6^{-3}$ reduction was used to assay BCKA-DC. The stoichiometry of $Fe(CN)_6^{-3}$ reduction to keto acid oxidised is 2:1 (Connelly et al., 1970). It has been shown that this complex even when

purified retains adequate quantities of co-factors required for its activity.

As was mentioned earlier, very little work has been done on the BCKA-DH in brain. The presence of this enzyme in brain was demonstrated (Khatra et al., 1977), and it was shown that the total activity in the brain is not very high but it is higher than that of the skeletal muscle and is similar to that of heart. It was also shown that in under *in vivo* condition atleast 60% of the enzyme is in active state in brain while in liver about 98% of the enzyme is active. When compared to other tissues brain has the highest amount of the active enzyme next to the liver (Wagenmakers et al., 1984). However, measurement of the transamination to decarboxylation ratio in different tissues indicated that transamination of BCKA is the predominant pathway in brain whereas decarboxylation is the predominant pathway for BCKA in liver (Shinnick and Harper, 1976; Brand, 1981). Regional distribution of this enzyme in brain was reported for the first time by Rao et al., (1982) and Brosnan et al., (1985). It was observed that the BCKA-DH activity was highest in the telencephalon and lowest in the brainstem regions. This is in contrast to transaminase, for which the highest activity occured in brainstem and the lowest in the telencephalon regions (Brosnan et al., 1985). However, these results do not agree with those of Rao et al., (1982), who reported similar activity level of BCKA-DC with α-KIC as substrate in cerebral

cortex, cerebellum, and brainstem of rat brain.

Studies on the subcellular distribution of this enzyme confirmed its mitochondrial localization (Bronsnan et al., 1985). They also reported its presence in the synaptosomes which is in conformity with the results of Chaplin et al., (1976) who demonstrated production of $^{14}CO_2$ from [1-^{14}C] and [U-^{14}C]BCAA in this fraction. These two observations are not in accordance with the theory of metabolic compartmentation that the BCAA metabolism occurs in astrocytes.

No studies were made on cellular distribution of this enzyme but the results of Murthy and Hertz (1987a,b) on the primary cultures of neurons and of astrocytes suggest that this enzyme may be present in both the cell types. The BCKA are not only produced endogenously in brain, but are also transported from blood into brain by a monocarboxylate transport system present in BBB (Conn and Steele, 1982). Branched chain keto acids that are produced in the cytosol are transported into the mitochondria by a specific carrier which is distinct from that of PYR (MacKay and Robinson, 1981; Hutson and Rannels, 1985). Based on the results of several investigators, Brosnan et al., (1985) suggested that the telencephalic regions are capable of completely oxidising the BCAA that are supplied through the blood while the brainstem regions can partially oxidise the BCAA. In all these studies, the main constraint is the low activities of the BCKA-DC.

In the present study, activity of this enzyme is measured only in the cellular and subcellular fractions but not in homogenates. It was observed that a large amount of tissue extract has to be added to obtain a detectable and meaningful reduction of the $Fe(CN)_6^{-3}$ in the homogenates. However, such an addition would result in the development of turbidity which affected the measure of absorbancy. In fact, Connelly and Bowden (1968) faced a similar a problem and concluded that there is no BCKA-DH in the brain.

CEREBRAL BCKA-DH IN NORMAL ANIMALS

Preliminary results and standardisation of BCKA-DC with the three BCKA as substrates are presented in Fig 3.12. Enzyme activity was found to be linear with respect to sub-cellular protein upto 50µgms. Hence, 20µgms protein was chosen for routine assays (Fig 3.13). Activity of BCKA-DC was also determined at various concentration of the three BCKAs and Km and Vmax were determined and the values are given in Table 3.21.

It is observed that the enzyme has low affinity towards ∝-KIV and high affinity towards ∝-KMV in the mitochondrial fraction. In the synaptosomes, the Km values for ∝-KIC and ∝-KIV were similar and higher than that of ∝-KMV. In the cytosol the profile of Km values were ∝-KIV>∝-KMV>∝-KIC. In both synaptosomes and mitochondria, Vmax values for this enzyme were higher with ∝-KIC and ∝-KMV than with ∝-KIV. In

TABLE 3.21

KINETIC CONSTANTS OF BRANCHED CHAIN KETO ACID DECARBOXYLASE IN SUBCELLULAR FRACTIONS

FRACTIONS		∝-KIC	∝-KMV	∝-KIV
SYNAPTOSOMES	Km	2.93	0.94	2.33
	Vmax	6.40	4.60	2.80
MITOCHONDRIA	Km	2.55	1.88	6.09
	Vmax	5.75	5.16	2.95
CYTOPLASM	Km	0.59	2.33	3.90
	Vmax	3.34	3.90	4.70

Km expressed as mM keto acid. Vmax expressed as μmol keto acid/mg protein/hr.

the cytosol, Vmax values were more or less the same with all the three keto acids.

Studies on the distribution of BCKA-DC in the three subcellular fractions indicated that the activity of this enzyme was more or less same in all the three subcellular fractions (Table 3.22).

Values obtained for the BCKA-DC in the present study in the mitochondrial fraction are higher than those reported by Brosnan et al., (1985), despite the fact that in their preparation the enzyme was activated prior to the assay. This disparity in the results may be due to the differences in the methods used for the isolation of the subcellular fractions and the methods of assay employed. It must be mentioned that the values given above are given as umoles of $Fe(CN)_6^{-3}$

TABLE 3.22

DISTRIBUTION OF BRANCHED CHAIN KETO ACID DECARBOXYLASE ACTIVITY IN SUBCELLULAR FRACTIONS ISOLATED FROM NORMAL RATS.

	DECARBOXYLASE ACTIVITY		
FRACTION	α-KIC	α-KMV	α-KIV
MITOCHONDRIA	2.5±0.6(7)	2.3±0.7(6)	3.4±0.8(9)
CYTOPLASM	3.2±0.5(9)	3.4±0.9(6)	4.0±1.0(5)
SYNAPTOSOMES	3.4±0.7(6)	2.3±0.6(6)	2.1±0.6(8)^{a*b*}

Values are Mean± S.D. Activity expressed as μmoles of ferricyanide reduced/mg. protein/hr.Number in parenthesis indicate the number of experiments. *p<0.01. a-comparison with mitochondria; b-comparison with cytosol.

reduced and this can be converted to umole of keto acid oxidised by dividing the values by two. Presence of this enzyme in the synaptosomal fractions is also in accordance with the results of Brosnan et al., (1985), though the activity levels were lower in the present study due to the reasons mentioned above. These results are also in conformity with the production of labelled of CO_2 from [1-^{14}C]-leucine in synaptosomal fraction reported by Chaplin et al., (1976).

Presence of this enzyme in the cytosolic fraction was surprising, as this enzyme is supposed to be mitochondrial in its localization and the cytosol is supposed to be devoid of it. Brosnan et al., (1985) reported ten times lesser activity of this enzyme in cytosol than that in mitochondria. However, in the present study more or less similar activities were

obtained in all the three fractions. This could be due to the contamination of cytosol with mitochondria. However, this possibility may be eliminated as the activity of SDH (marker for mitochondria) is 70 times higher in mitochondria than in cytosol (Table 3.23).

TABLE 3.23

LEVELS OF SDH AND LDH IN SUBCELLULAR FRACTIONS ISOLATED FROM NORMAL AND EXPERIMENTAL ANIMALS

		CYTOSOL	MITOCHONDRIA	SYNAPTOSOMES
SDH	NORMAL	0.04±0.003(4)	2.8±0.19(4)	0.53±0.04(4)
	EXPTL	0.33±0.009(4)	5.8±1.00(4)	0.90±0.10(4)
LDH	NORMAL	385±72 (4)	79±3 (4)	121±9 (4)
	EXPTL	145±29 (4)	34±7 (4)	108±4 (4)
SDH (%)	NORMAL	1.4	100	19
	EXPTL	5.6	100	16
LDH (%)	NORMAL	100	20.5	31
	EXPTL	100	23.0	75

Activity expressed as SDH-µmoles of formazan formed/mg protein/hr. LDH-µmoles of NADH oxidized/mg protein/hr. Exptl = subcellular fractions from ammonium acetate injected rats. Numbers in parenthesis indicate the number of experiments.

In other words, contamination of the cytosol with mitochondria is not more than 1-2%. If the activity of BCKA-DC in the cytosol is due to mitochondrial contamination, then the activity in the cytosol should not be more than 1-2% of the mitochondrial activity. Further, production of CO_2 form BCAA

was also observed when cytosol was incubated with labelled BCAA (see chapter IV). If the enzyme is exclusively mitochondrial as was proposed, the only possibility appears to be the presence of small mitochondria with a different byonant density. Existence of such a heterogenity in mitochondrial population has been reported earlier (Van den Berg, 1974). Physiological significance of the presence of this enzyme in the cytosol cannot be assesed at this stage. It is not clear from these studies whether the entire BCKA-DH complex or only the BCKA-DC is present in the cytosol. Moreover, it is also not clear whether all the enzymes of BCAA metabolism are also present in this fraction or only this particular enzyme. If all the enzymes of the BCAA metabolism are present in the cytosol then Ac CoA and propionyl CoA might be produced in this subcellular compartment. In such a case, these compounds may be utilised for the lipid biosynthesis in brain as fatty acid synthesis occurs in the cytosol. This would then bypass the need for transporting carbon units (Ac CoA) across the mitochondrial membrane for this purpose. If only BCKA-DH is present in the mitochondria then it is difficult to envisage how the branched chain keto acyl CoA esters are transported across the mitochondrial membrane. Further studies in this direction are required to clarify these issues.

EFFECTS OF AMMONIA ON BCKA-DH ACTIVITY

With∝-KIC as substrate, BCKA-DH activity was enhanced significantly in all the three subcellular fractions isolated

TABLE 3.24

BRANCHED CHAIN KETO ACID DECARBOXYLASE ACTIVITY IN SUBCELLULAR FRACTIONS ISOLATED FROM NORMAL AND HYPERAMMONEMIC RATS.

FRACTION		DECARBOXYLASE ACTIVITY		
		α-KIC	α-KMV	α-KIV
MITOCHONDRIA	NORMAL	2.5±0.6(7)	2.3±0.7(6)†	3.4±0.8(9)
	AMM.AC	6.9±1.2(6)*	4.2±1.2(5)	5.8±1.0(5)*
CYTOSOL	NORMAL	3.2±0.5(9)	3.4±0.9(6)	4.0±1.0(5)
	AMM.AC	4.2±0.7(5)*	5.6±1.3(5)*	4.3±1.3(5)
SYNAPTOSOMES	NORMAL	3.4±0.7(6)	2.3±0.6(6)	2.1±0.6(8)
	AMM.AC	5.0±1.3(5)†	4.3±0.8(5)+	4.2±0.8(6)*

Values are Mean± S.D. Activity is expressed as the μmoles ferricyanide reduced /mg protein/hr. Number in parenthesis indicate the number of experiments. *$p<0.001$; +$p<0.005$; †$p<0.02$

from rats injected with an acute dose of ammonium acetate. The magnitude of elevation was highest in the mitochondria. With α-KIV as substrate, activity of this enzyme was enhanced both in mitochondria and in synaptosomes. The magnitude of elevation was higher in synaptosomes than in mitochondria. With α-KIV as substrate, changes observed in the activity of this enzyme in the cytosol were statistically not significant. When α-KMV was used as substrate, BCKA-DH activity was enhanced in all three subcellular fractions.

These changes in the activity of BCKA-DH are more or less parallel to the changes in BCAA-T activity. As was

mentioned earlier, increased activity of the transaminase would increase the production of BCKA. Stimulation of BCKA-DH activity under these conditions might enhance the utilisation of these keto acids. As a result, there might be an increase in the production of Ac CoA and succinyl CoA which might be utilised in the TCA cycle for the production of energy. Moreover, utilization of succinyl CoA may act as an anaplerotic mechanism for the replenishment of TCA cycle intermediates in hyperammonemic states. Moreover, α-KG is also regenerated in this process to compensate for its loss due to the enhanced BCAA-T activity. Though the above discussion holds good for the changes occuring in mitochondria and synaptosomes, it is very difficult to explain the significance of similar changes observed in the cytosol. As was mentioned earlier, further studies are required to determine the extent to which the metabolism of BCAA occurs in the cytosol.

BRANCHED CHAIN KETO ACID DECARBOXYLASE IN MSO TOXICITY

As was mentioned earlier, MSO was used to ascertain whether these changes in BCKA-DC are related to the elevation of glutamine synthesis in hyperammonemic states. Administration of acute doses of MSO had no effect on the activity of BCKA-DC in the mitochondrial and synaptosomal fractions while it enhanced the enzyme activity in the cytosol when α-KIC was the substrate. With α-KMV as substrate the activity of this

TABLE 3.25

BRANCHED CHAIN DECARBOXYLASE ACTIVITY IN SUBCELLULAR FRACTIONS ISOLATED FROM NORMAL RATS AND THOSE TREATED WITH METHIONINE SULPHOXIMINE.

FRACTIONS		DECARBOXYLASE ACTIVITY		
		∝-KIC	∝-KMV	∝-KIV
MITOCHONDRIA	NORMAL	2.5±0.6(7)	2.3±0.7(6)**	3.4±0.8(9)
	ACUTE	2.9±0.6(4)*	3.9±0.7(4)	3.8±0.7(5)
	SUBACUTE	4.6±1.4(6)	3.3±0.1(5)**	2.5±0.8(4)
CYTOSOL	NORMAL	3.2±0.5(9)	3.4±0.9(6)	4.0±1.0(5)
	ACUTE	7.7±1.4(4)**	3.3±1.3(6)	3.6±0.6(5)
	SUBACUTE	3.3±0.1(5)	3.5±0.9(4)	3.1±1.1(6)
SYNAPTOSOMES	NORMAL	3.4±0.7(6)	2.3±0.6(6)	2.1±0.6(8)
	ACUTE	4.0±0.8(5)	4.2±0.9(4)**	3.9±0.8(6)**
	SUBACUTE	4.9±1.2(6)++	3.7±1.2(5)++	7.0±1.8(4)**

Values are Mean±S.D. Activity is expressed as μmoles of ferricyanide reduced/mg. protein/hr. Number in parenthesis indicate the number of experiments. **p<0.001, *p<0.005, ++p<0.02, +p<0.01.

enzyme was enhanced in the mitochondria and synaptosomes and remained unaltered in the cytosol. With ∝-KIV as substrate in acute MSO toxicity there was very little change in the activity of BCKA-DC in the mitochondrial and cytosolic fractions while there was an enhancement of the same in the synaptosomal fractions. Under these conditions it is interesting to note that the activity of BCAA-T was suppressed in all the subcellular fractions.

Administration of a subacute dose of MSO enhanced the activity of BCKA-DC in the mitochondria and synaptosomes with ∝-KIC and ∝-KMV as substrates. With ∝-KIV as substrate,

activity of this enzyme was enhanced only in the synaptosomal fraction. With all the three keto acids as substrates there was very little change in the activity of this enzyme in the cytosol under these conditions. While discussing the results on transaminases, it was mentioned that in acute MSO toxicity there will be very little synthesis of glutamine, hence the requirement to replenish glutamate required for glutamine biosynthesis is minimal. Decrease in the transamination of BCAA in acute MSO toxicity would result in the production of less amounts of keto acids, hence the need for increased utilisation of these keto acids does not arise. So more or less parallel changes observed in the activity of these two enzymes involved in the initial two stages of BCAA metabolism. This is in concurrance with the concept that these amino acids might serve as precursors for glutamate.

Though the activity of GS is suppressed immediately after the administration of subacute doses of MSO, it recovers by atleast 30% in the later stages (Rao and Murthy, 1988). Under such conditions, requirement for the replenishment of the cytosolic pool of glutamate (required for glutamine biosynthesis) arises only in the later stages. Hence, there is a recovery of the BCAA-T activity in the cytosol and the keto acids produced in this fraction may be transported into the mitochondria where they may be readily oxidised. Though the oxidation of BCKA can take place in the cytosol, availability of NAD^+ may be a rate limiting factor

in this subcellular compartment.

Present studies on the BCKA-DC in the subcellular fractions are in accordance with the concept that the BCAA may provide the glutamate required for biosynthesis of glutamine in hyperammonemic states.

DECARBOXYLASE IN CELLULAR FRACTIONS

Studies on metabolic compartmentation indicated that the metabolism of BCAA occurs exclusively in the astrocytes. However, several evidences, including results of the present work, indicated that the metabolism of BCAA is not an exclusive property of the astrocytes. This is evident from the results of the earlier chapter on transaminases. As was mentioned earlier, decarboxylation of BCAA is the rate limiting enzyme in the metabolism of these amino acids and this reaction is mediated by branched chain keto acid dehydrogenase complex. As the transaminases are present in the three major cell types of brain, it would be interesting to study whether other enzymes required for further metabolism of BCAA are present in these cells. Hence, a study was conducted on the distribution of BCKA-DC in the three major cell types of brain of normal and hyperammonemic rats.

Data on the standardisation of this enzyme in the cell preparations are presented in Fig. 3.14. Amount of cellular protein selected for the assay of BCKA-DC (25 μg) is in the linear portion of the enzyme concentration curve. Km and Vmax values of BCKA-DC for the three BCKA in neurons, astrocytes and oligo cells are given in Table 3.26. In neurons, Km values for all the three BCKA similar. However, Vmax values for α-KIV were higher than those with the other two keto acids. In astrocytes, Km values for α-KIC and α-KIV were

TABLE 3.26

KINETIC CONSTANTS OF BRANCHED CHAIN KETO ACID DECARBOXYLASE IN CELLULAR FRACTIONS ISOLATED FROM NORMAL ANIMALS.

FRACTION		α-KIC	α-KMV	α-KIV
NEURONS	Km	2.04	4.12	2.02
	Vmax	2.35	4.22	1.93
ASTROCYTES	Km	1.24	6.30	1.56
	Vmax	2.95	5.50	2.25
OLIGOCELLS	Km	2.66	8.90	4.87
	Vmax	2.88	4.81	3.23

Km is expressed as mM substrate. Vmax is expressed as μmol $Fe(CN)_6^{\cdot}$/mg protein/hr.

lower than that for α-KMV. These values were lower in astrocytes cytes when compared to those in the neurons and oligo cells. Vmax values obtained with three keto acids in the astrocyte preparation were similar to those in neurons. In oligo cells, Km and Vmax values were more or less same for the three keto acids.

Results on the distribution of BCKA-DC in the three cell preparations are given below (Table 3.27). With α-ketoisocaproic acid as substrate, specific activity of BCKA-DC was higher in astrocytes and oligo cells than in neurons. With α-KMV as substrate a higher activity was observed in neurons and astrocytes than in oligo cells. With α-KIV as substrate, specific activity of this enzyme was higher in astrocytes than in neurons and oligo cells which had similar activities.

TABLE 3.27

DISTRIBUTION OF BRANCHED CHAIN KETO ACID DECARBOXYLASE ACTIVITY IN CELLULAR FRACTIONS ISOLATED FROM NORMAL RATS.

FRACTION	∝-KIC	∝-KMV	∝-KIV
	μmoles $Fe(CN)_6$ REDUCED/MG PROTEIN/HR.		
NEURONS	1.7±0.3(5)^{b*c*}	3.2±0.6(5)	2.4±0.2(5)
ASTROCYTES	2.6±0.6(5)	3.1±0.7(4)	3.3±0.5(4)
OLIGOCELLS	2.1±0.2(5)	2.4±0.3(5)$^{a++}$	2.4±0.3(5)
	pmoles $Fe(CN)_6$ REDUCED/MG PROTEIN/HR.		
NEURONS	0.7±0.1(5)$^{c+}$	1.3±0.2(5)	0.9±0.1(5)^{b*c*}
ASTROCYTES	1.6±0.4(5)^{a*c*}	1.9±0.4(4)^{a*b*}	2.0±0.3(4)^{c*}
OLIGOCELLS	0.4±0.02(5)	0.4±0.05(5)	0.4±0.04(5)

Values are Mean±S.D. Number in the parenthesis indicate the number of experiments. a:Neurons, b:Astrocytes, c:Oligocells. *p<0.01, ++p<0.02, +p<0.05.

A similar profile was obtained for the cellular activity of the enzyme (with all the three keto acids as substrates) i.e., astrocytes > neurons > oligo cells. Comparison of the specific activity of the enzyme in each cell fraction, revealed that the rates of decarboxylation of the three BCKA were more or less the same in astrocytes and oligo cells. In neurons the profile for the decarboxylation of the three keto acids was ∝-KMV>∝-KIV>∝-KIC. When the activity was expressed per cell, the profile obtained for the rate of decarboxylation of the BCKA remained same in neurons and in oligo cells. In astrocytes, there were marginal differences in the rate of oxidation of the three BCKA. In summary, it may be concluded

that the decarboxylase activity may be higher in the astrocytes compared to that of the other two cellular fractions.

Presence of BCKA-DC in all the three major cell types of brain, as was shown above, indicated that the BCAA metabolism occurs not only in the astrocytes but also in neurons and oligo cells. However, in keeping with the concept of metabolic compartmentation, activity profiles obtained for this enzyme indicate that the metabolism of these amino acids occur to a greater extent in the astrocytes. These results are also in accordance with the observations of Murthy and Hertz (1987a,b) on the production of $^{14}CO_2$ in primary cultures of astrocytes and neurons with both [1-^{14}C]- and [U-^{14}C]- BCAA. These investigators observed the rate of production of $^{14}CO_2$ from BCAA was higher with astrocytes than with neurons. Results obtained in the present study with BCKA-DC are in concurance with these observations. Similarly. results of the present study are also in accordance with those of Chaplin et al., (1976), on the production of $^{14}CO_2$ from both [1-^{14}C]- and [U-^{14}C]- leucine by neuronal preparations (synaptosomes). Presence of BCKA-DC in the oligo cells suggests that these keto acids are decarboxylated and the resultant branched chain keto acyl CoA may metabolized and their carbons may incorporated into myelin lipids. This concept is in accordance with the observations on the incorporation of carbons of BCAA into myelin lipids (Wiggins et al., 1979).

TABLE 3.28

BRANCHED CHAIN DECARBOXYLASE ACTIVITY IN CELLULAR FRACTIONS ISOLATED FROM NORMAL AND HYPERAMMONEMIC RATS.

FRACTION		α-KIC	α-KMV	α-KIV
		μmoles/mg protein/hr.		
NEURONS	NORMAL	1.7±0.3(5)	3.3±0.6(5)	2.4±0.2(5)
	EXPTL	3.45±0.8(5)[b]	4.7±1.0(5)[a]	4.0±0.5(5)[c]
ASTROCYTES	NORMAL	2.6±0.6(5)	3.1±0.7(4)	3.3±0.5(4)
	EXPTL	4.9±1.2(5)[b]	6.5±1.4(5)[b]	7.1±2.0(5)[c]
OLIGOCELLS	NORMAL	2.1±0.2(5)	2.4±0.3(5)	2.4±0.3(5)
	EXPTL	2.7±0.2(5)[c]	3.4±0.5(5)[b]	2.8±0.4(5)
		pmoles/cell/hr		
NEURONS	NORMAL	0.665±0.1	1.27±0.2(5)	0.9±0.08(5)
	EXPTL	1.94±0.4(5)[c]	2.60±0.6(5)[c]	2.2±0.3(5)[c]
ASTROCYTES	NORMAL	1.6±0.4(5)	1.9±0.4(4)	2.0±0.3(4)
	EXPTL	3.9±0.9(5)[c]	5.1±1.1(5)[c]	5.1±1.6(5)[c]
OLIGOCELLS	NORMAL	0.35±0.03(5)	0.39±0.05(5)	0.40±0.04(5)
	EXPTL	0.3±0.2(5)	0.45±0.06(4)	0.39±0.07

Activity is expressed as units of ferricyanide reduced.
Number in parenthesis indicate the number of experiments.
Values are Mean±S.D. a = $p<0.02$; b = $p<0.005$; c = $p<0.001$.

When the activities of transaminases and decarboxylases are compared, it is evident that the rate of transamination of these amino acids is far in excess of the rate of

decarboxylation in all the cell types. If the cellular activity of these two enzymes are taken together, it is evident that the metabolism of these amino acids might be higher in the astrocytes than in neurons and oligo cells. This is in accordance with the observation that incorporation of labelled carbon from BCAA into the small glutamate pool is associated with the astrocytes (Roberts and Morelos, 1965; Cavanaugh, 1974; Cremer et al., 1975).

ACUTE EFFECTS OF AMMONIA ON BCKA-DC ACTIVITY

Changes observed in the activity of BCKA-DC in the cellular fractions isolated from hyperammonemic rats are shown in the table 3.28. There was an increase in the specific activity of this enzyme in all the three major cell types of hyperammonemic rats though the magnitude of change was much less in the oligo cells. Cellular activity of BCKA-DC was enhanced only in neurons and astrocytes isolated from hyperammonemic rats while in the oligo cells it was unaltered. Further details on the changes in the cellular activity of this enzyme were similar to those described for specific activity.

In summary, results obtained indicate enhanced decarboxylation of BCKA in astrocytes and neurons in hyperammonemic states. These results are in accordance with the observation of Shiota (1984), on the enhanced decarboxylation of [1-^{14}C]-leucine in the homogenates prepared from the brains of rats treated with carbon tetrachloride. However,

results of the present study are not in agreement with those of Murthy and Hertz (1987a,b) on the acute effects of ammonia on the production of $^{14}CO_2$ from BCAA in neurons and astrocytes in primary cultures. These authors observed a decrease in the rates of decarboxylation of BCAA in the presence of pathophysiological concentrations of ammonia. This discrepancy might be due to differences in substrates and cell preparations used in both these studies. This may also be due to an interplay of several regulating factors in the intact cells in culture. While discussing the changes in BCAA-T activity in these cell preparations isolated from hyperammonemic rats, it was suggested that the decrease observed in the activity may be due the increased utilization of these keto acids, which appears to be true. Due to the increased decarboxylation of keto acids, lesser amount of keto acids will be left over in the assay mixture to react with 2,4-DNP. Hence, the observed suppression in the activity of BCAA-T in these cell fractions in hyperammonemic states may be an artifact.

Increased activity of decarboxylases might facilitate the removal of these keto acids and thereby making the transaminase reaction irreversible. This would increase the formation of glutamate from BCAA in the transaminase reaction. Moreover, enhanced metabolism of keto acids would lead to the production of more of acetyl CoA and succinyl CoA from BCAA which may be utilized in the citric acid cycle. As

the citric acid cycle intermediates, especially ∝-KG and OAA are in equilibrium with glutamate family of amino acids (Balazs et al., 1970), the carbons from BCAA may thus be incorporated into glutamate family of amino acids. This is in agreement with the observations of Cremer et al., (1975).

Thus, in hyperammonemic states there will be an increased incorporation of both nitrogen and carbon from BCAA due to the enhancement in the activities of both transaminase and decarboxylases. As was mentioned earlier, this glutamate may be used to replenish the cytosolic pool of glutamate which could be utilized for the production of glutamine.

Activities of the enzymes measured under optimal conditions need not represent the situation under in vivo conditions. Hence, studies were conducted on the metabolism of BCAA in intact cells and subcellular fractions by measuring the production of $^{14}CO_2$ from labelled BCAA and by measuring the production of labelled BCKA. Results of these studies will be presented in the subsequent chapter.

BRANCHED-CHAIN AMINO ACID TRANSAMINASE ACTIVITY IN THE HOMOGENATES OF CEREBRAL CORTEX

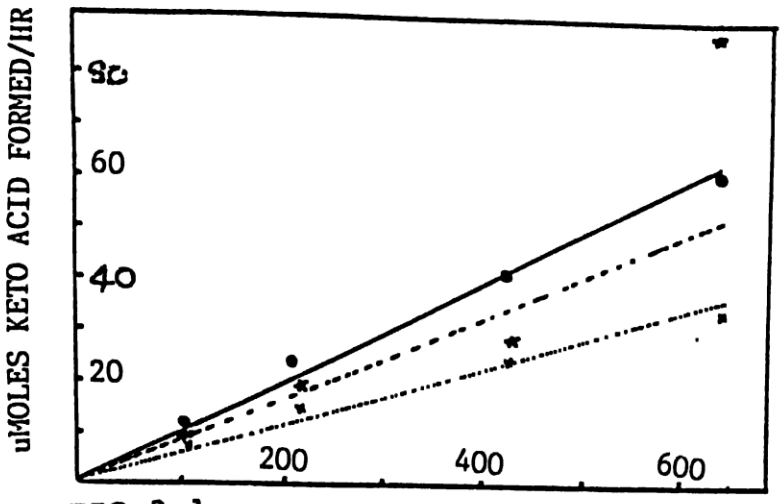


FIG 3.1

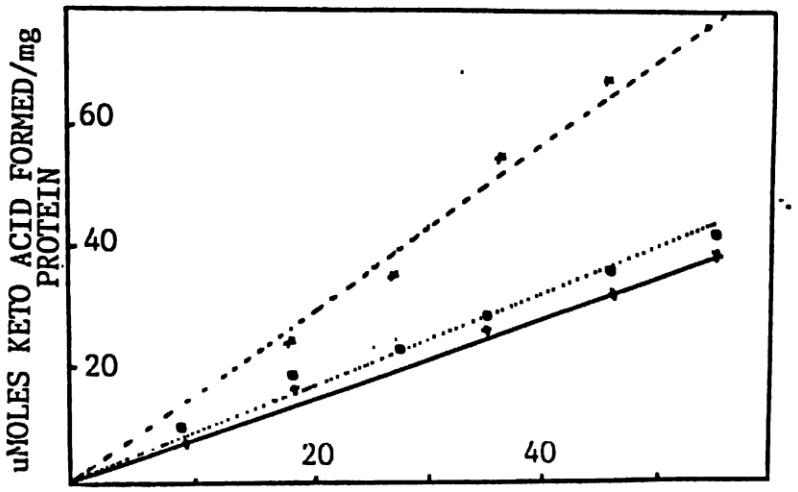


FIG 3.2

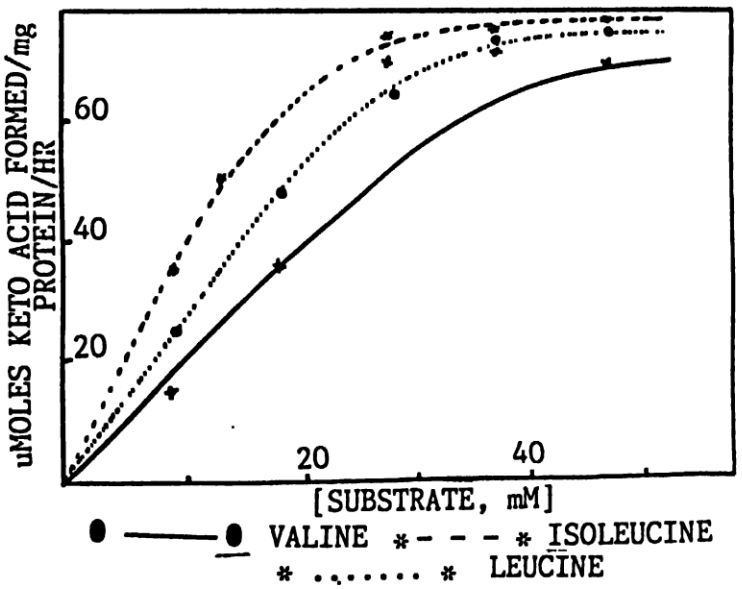


FIG 3.3

OF DIFFERENT CONCENTRATIONS OF α- KETOGLUTARATE ON BRANCHED-CHAIN AMINO ACID AMINOTRANSFERASE ACTIVITY IN CORTICAL HOMOGENATES (BEFORE CORRECTION)

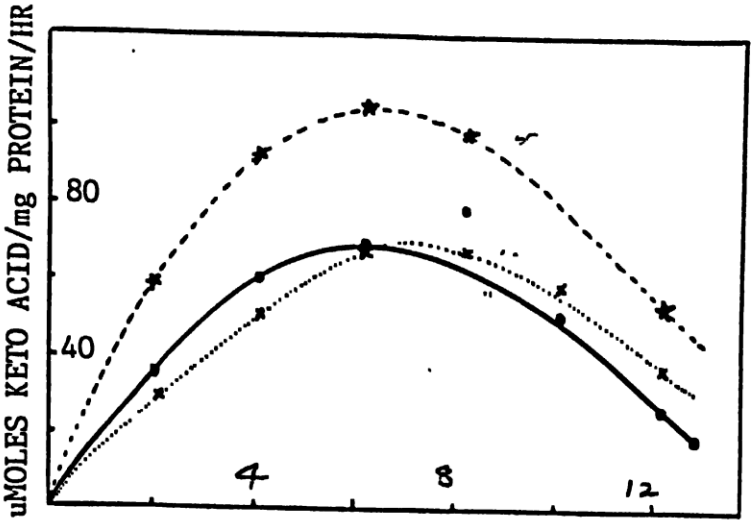


FIG 3.4

(AFTER CORRECTION)

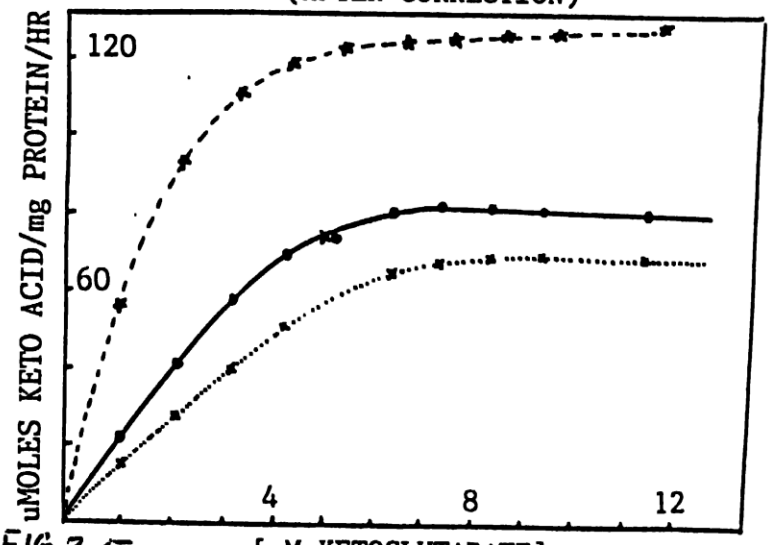
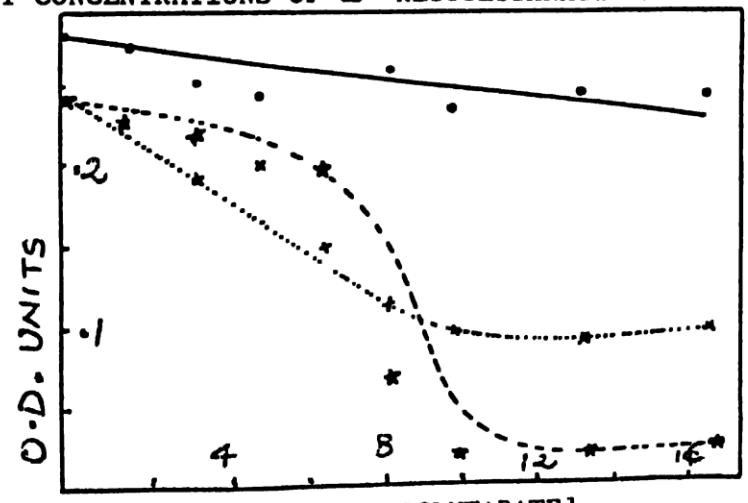


FIG 3.5

T OF DIFFERENT CONCENTRATIONS OF α -KETOGLUTARATE ON BRANCHED-CHAIN KETOACID EXTRACTION



X ..... X LEUCINE X - - - - X ISOLEUCINE

● ——— ● VALINE

FIG 3.6

BRANCHED-CHAIN AMINO ACID TRANSAMINASE ACTIVITY IN SUBCELLULAR FRACTIONS :
ACTIVITY Vs PROTEIN

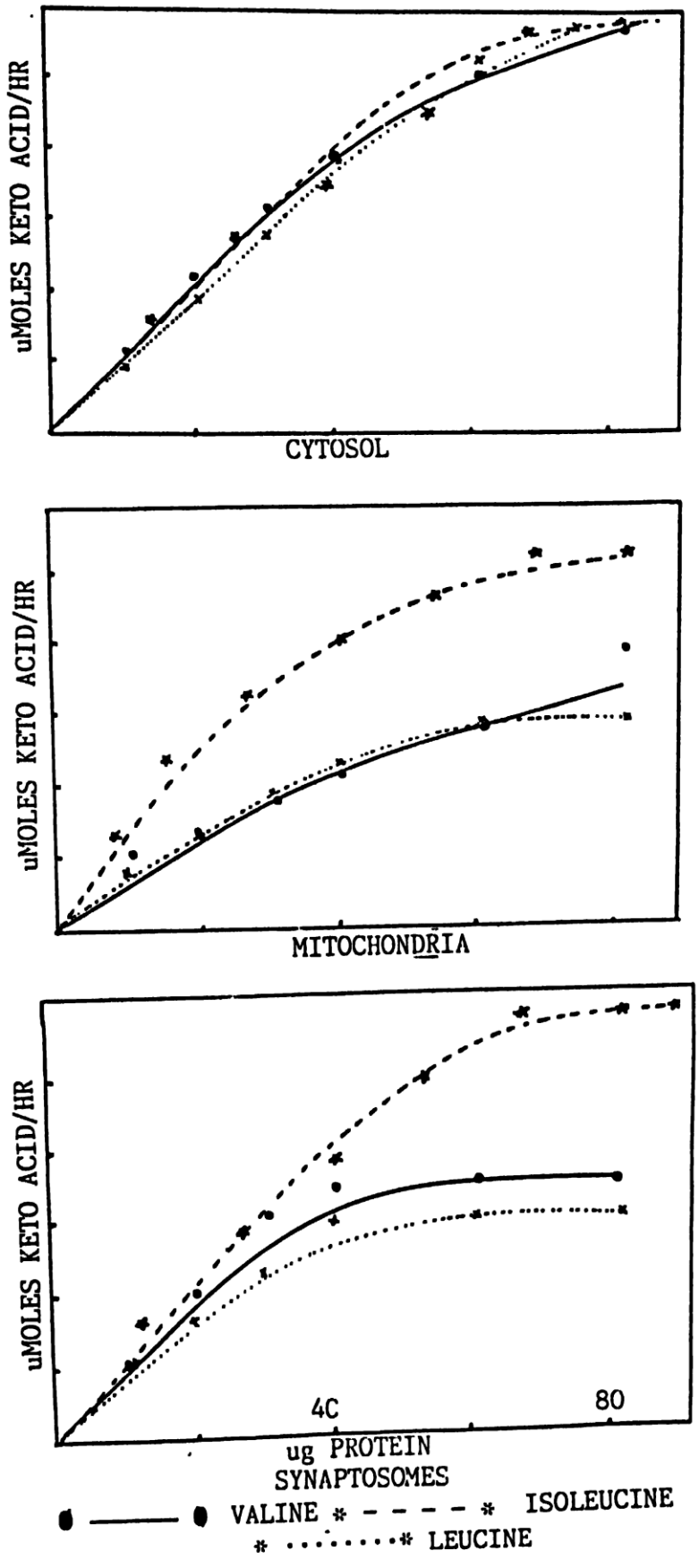


FIG 3.7

α-KG CONCENTRATION CURVE
IN CELLULAR AND SUBCELLULAR FRACTIONS

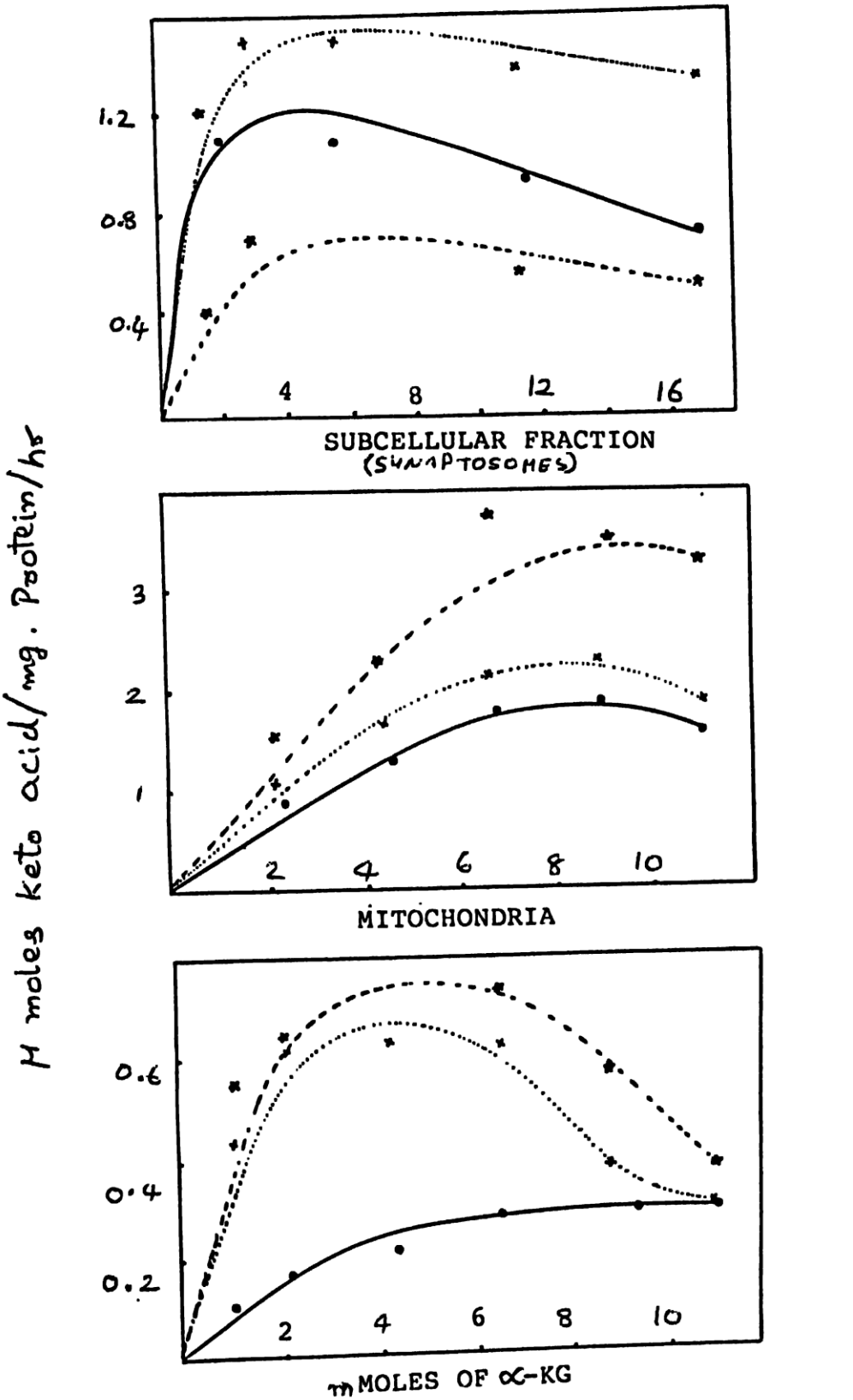


FIG 3.9

BRANCHED-CHAIN AMINO ACID AMINOTRANSFERASE ACTIVITY IN CELLS :
ACTIVITY Vs PROTEIN ADDED

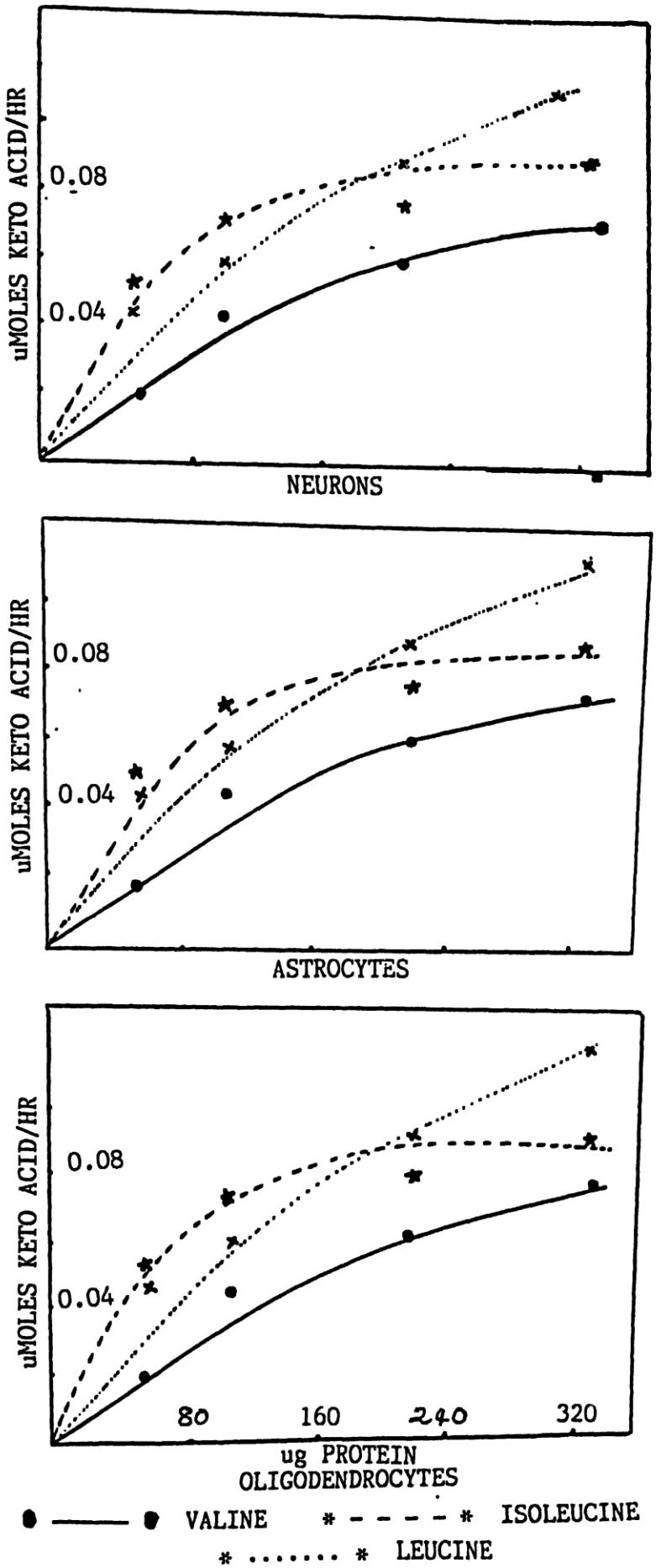


FIG 3.10

SUBCELLULAR FRACTIONS

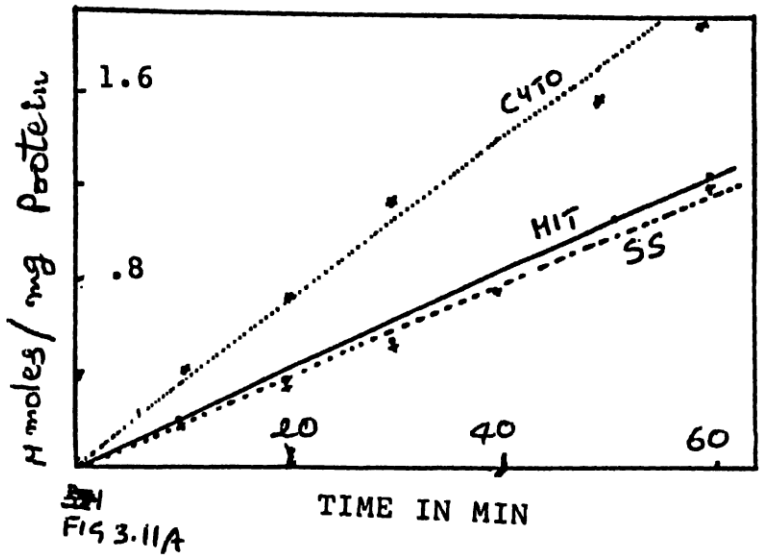
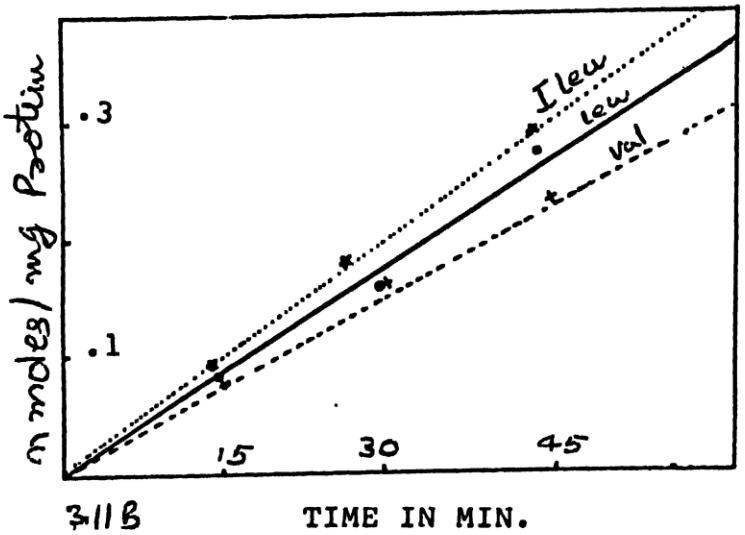


FIG 3.11A

ASTROCYTES



3.11B

FIG 3.11 BCAA-T TIME CURVE

BRANCHED-CHAIN AMINO ACID TRANSAMINASE ACTIVITY IN CELLS :
[SUBSTRATE] Vs ACTIVITY

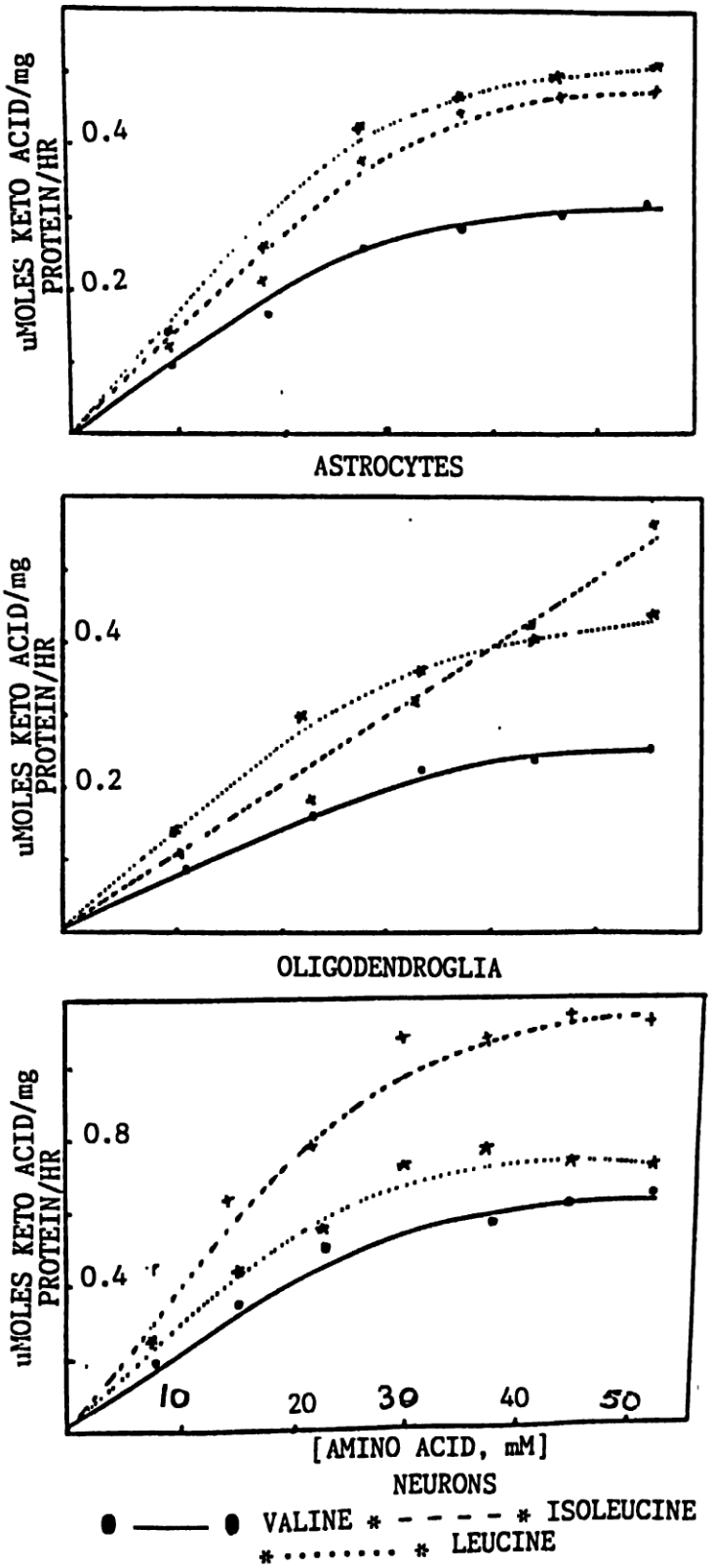
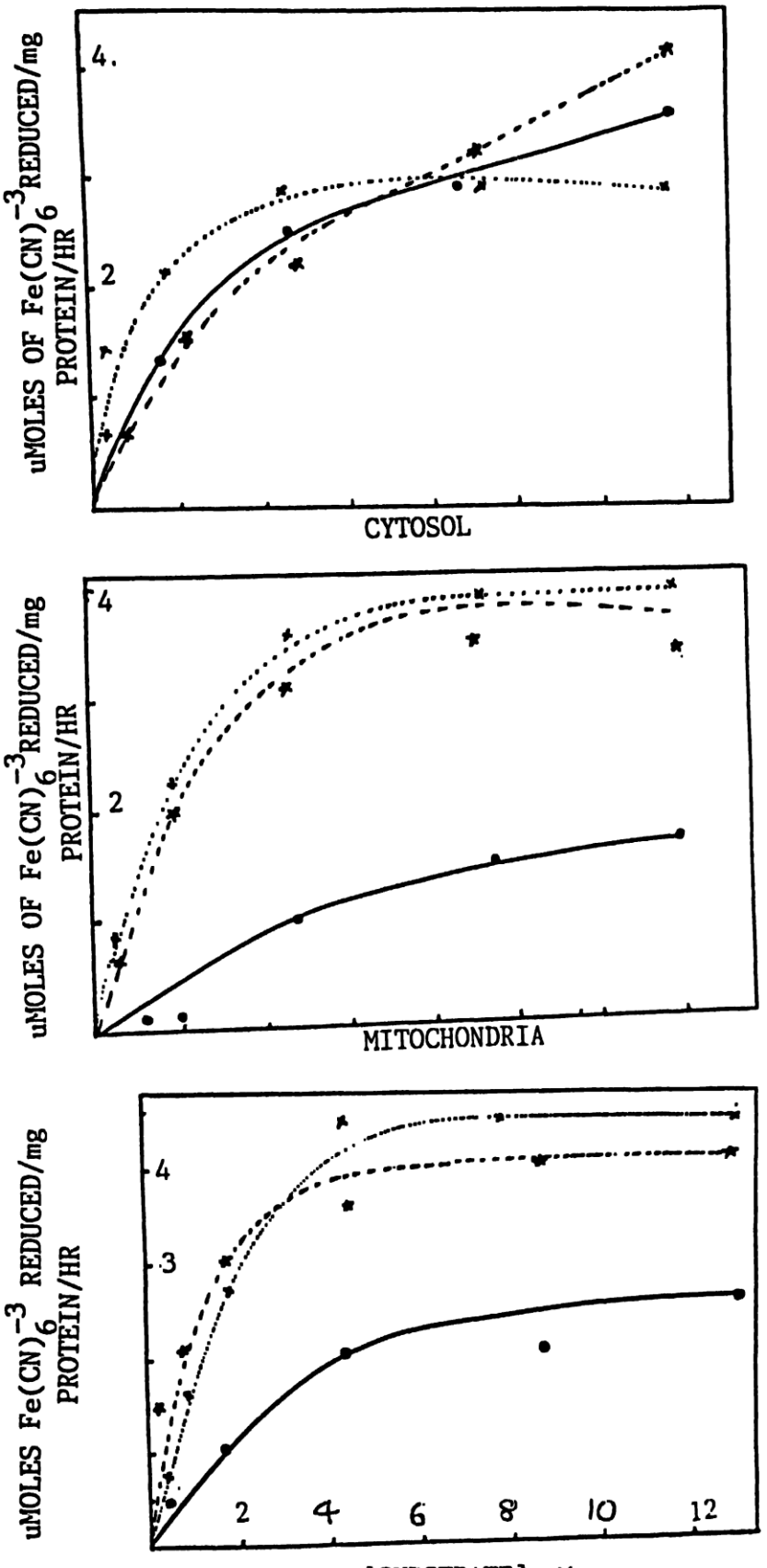


FIG 3.11a

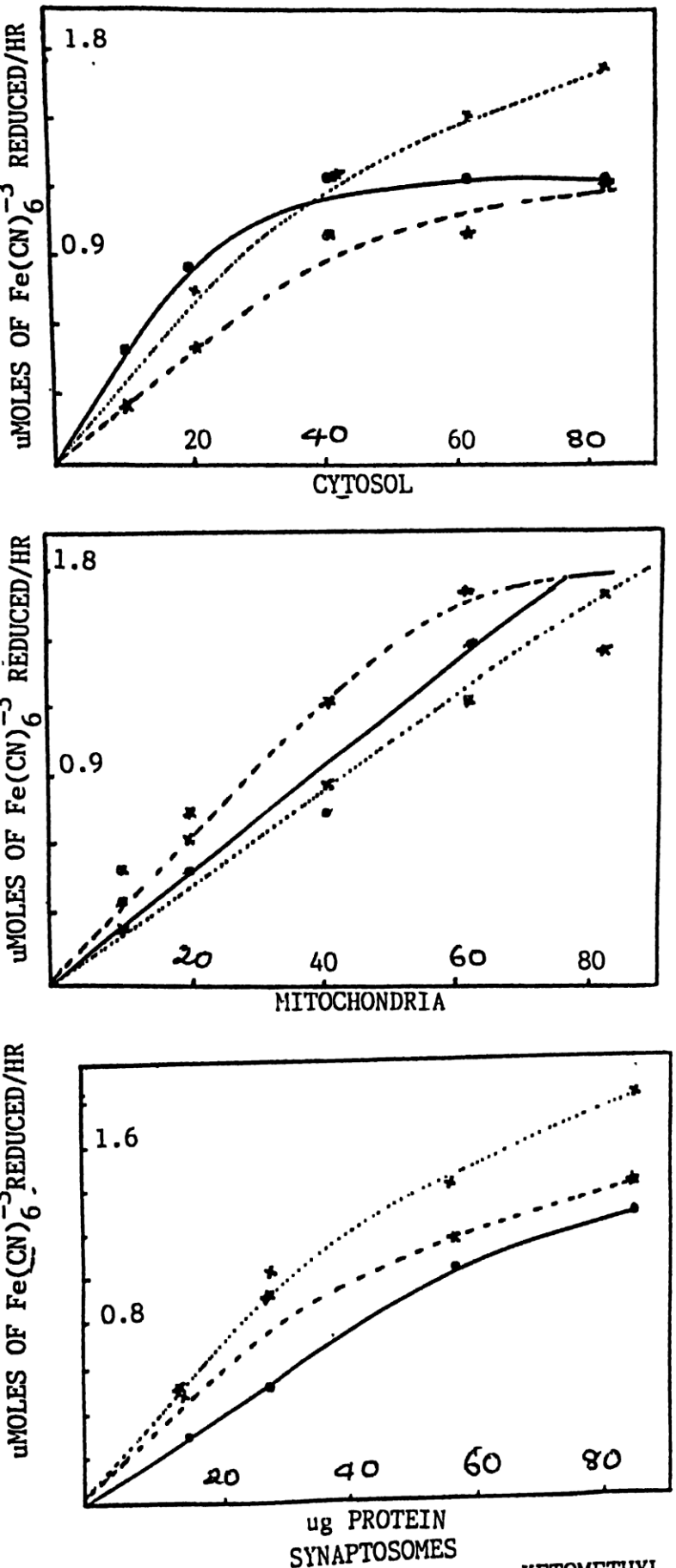
BRANCHED-CHAIN KETO ACID DECARBOXYLASE ACTIVITY :
SUBSTRATE CONCENTRATION Vs ACTIVITY



●—● KETO ISOVALERIC ACID * - - -* KETO METHYLVALERIC ACID
*..... * KETO ISOCAPROIC ACID

FIG 3.12

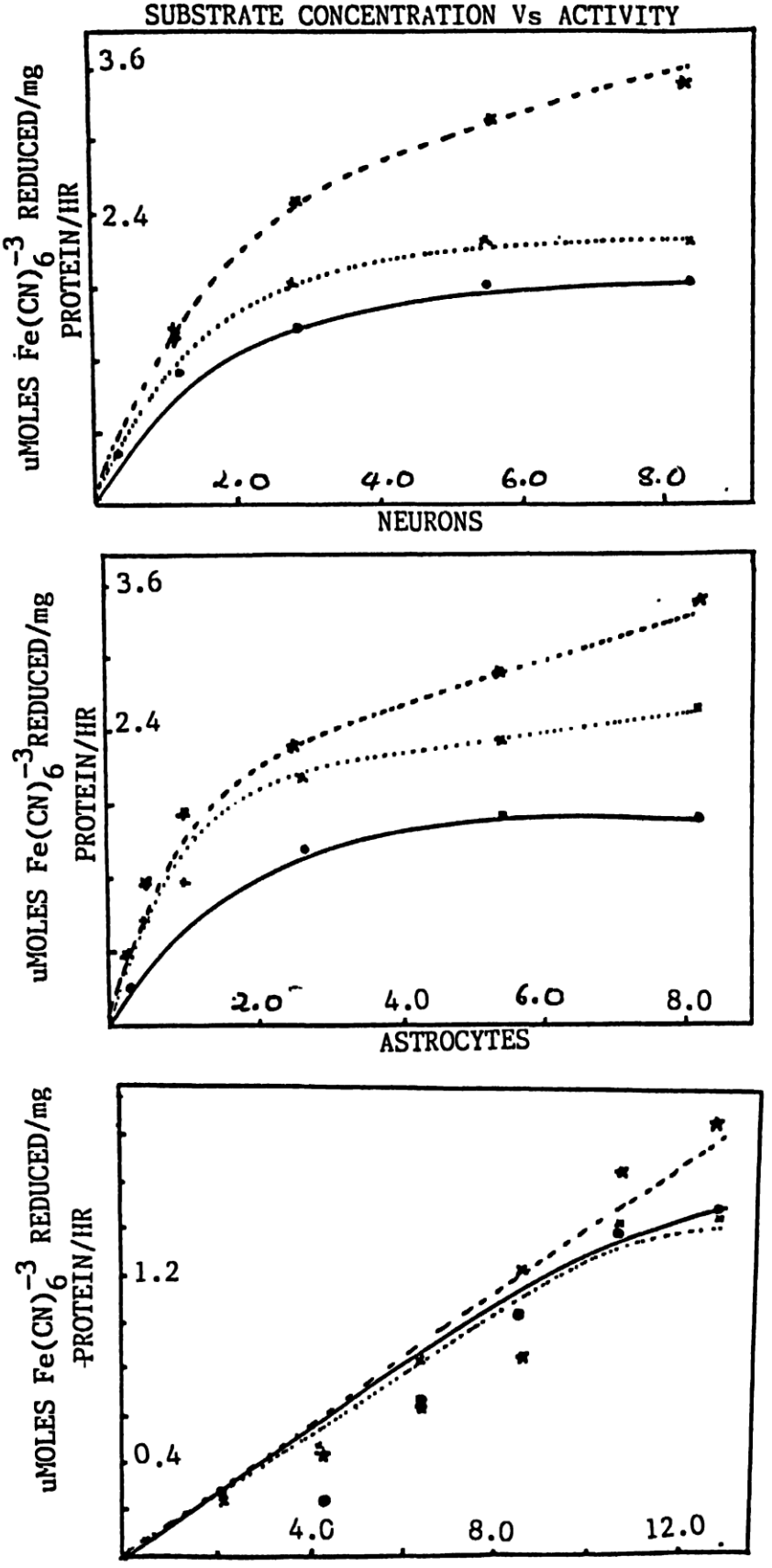
BRANCHED-CHAIN KETO ACID DECARBOXYLASE IN SUBCELLULAR FRACTIONS :
ACTIVITY Vs PROTEIN ADDED



● ——— ● KETO ISOVALERIC ACID * - - - -* KETOMETHYL VALERIC ACID
* ..... * KETO ISOCAPROIC ACID

FIG 3.13

:CHED-CHAIN KETO ACID DECARBOXYLASE ACTIVITY IN CELLULAR FRACTIONS :
SUBSTRATE CONCENTRATION Vs ACTIVITY



●——● KETO ISOVALERIC ACID * - - - - * KETO METHYLVALERIC ACID

..... KETO ISOCAPROIC ACID

FIG 3.14i

TRANSPORT

CEREBRAL TRANSPORT OF BCAA INTO SUBCELLULAR (SYNAPTOSOMES AND MITOCHONDRIA) AND CELLULAR FRACTIONS (NEURONS, ASTROCYTES AND OLIGO CELLS).

Metabolism of any compound in the cell depends on its effective concentration near the site of metabolism. Unless the compound is produced endogenously, it has to be provided either by the cells surrounding the target cell or by blood. In either case the substances should cross the plasma membrane of the target cell. It is well known that not all the substances are freely permeable across the plasma membrane. There are special proteins in the plasma membrane to transport these substances to or out of the cell, known as "carrier proteins". Such carrier mediated transport for a variety of substances has been shown in the animal tissues including brain. These carriers usually exhibit typical Michealis-Menten kinetics i.e., they exhibit substrate saturation like that of an enzyme. Carrier mediated transport may or may not utilise ATP. In the latter case, this process is known as facilitated diffusion. Some of the carriers utilise the phosphate bond energy of ATP directly and some of them indirectly. Most of these carriers transport an ion along with the substrate and the ion is usually Na^+. In such cases Na^+ accumulate inside the cells and energy is spent in pumping out these ions by the Na^+ pump (Na^+, K^+ATPase). Interference with this process would abolish the gradient for Na^+ across the plasma membrane and the transport of the substance

is inhibited. Depending upon the ionic requirement for the transport, these carriers are classified into two categories. The Na^+ independent system and Na^+ dependent system (Oxender and Christensen, 1963).

Unlike other tissues brain is uniquely protected by a barrier i.e., BBB which restricts the free entry of several substances from blood to brain. The BBB is endowed with several carrier systems which transport some of the blood borne substances into the brain and from brain to blood. Very few substances are freely permeable across the BBB (Bradbury 1979). Even after crossing the BBB the substances do not have free access to various cell types of the brain. This is because of the fact that the astrocytes surround the capillaries and communicate with them through their end feet (Bradbury 1979, 1985). Hence, once they cross the BBB, the substances have to encounter the membrane of astrocytes and escape their metabolism to gain access to the other cells. Astrocytes do release some of the substances either in the native or modified state into the extracellular space. Later, these substances successfully encounter the membrane of the target cells. In other words,to gain access into the cells of the brain a substance should be transported atleast across three different membranes. Carrier proteins present in these membranes have different properties and may exhibit different kinetics for the same substance. For example there are two types of carrier mediated mechanisms, one being high affinity

(low capacity) and the other is low affinity (high capacity), for neurotransmitter amino acids such as glutamate.

Keeping these facts in view, a brief account on the cerebral transport of BCAA is given below. BCAA are not freely permeable across the BBB and they are transported by a specific carrier mediated saturable transport mechanism (Cohen and Lajtha (1972); Pardridge, 1977). This carrier, known as the large neutral amino acid carrier, has a very broad substrate specificity. It transports not only the three BCAA but is also responsible for the transport of AAA (Phenylalanine, Tyrosine and Tryptophan) and also for the transport of methionine (Cohen and Lajtha, 1972; Oldendorf and Szabo, 1976). As a result, there will be a competition between these amino acids for the transport sites (Oldendorf, 1971). Hence, the transport of BCAA across the BBB depends not only their content in blood but also on the ratio of BCAA to other competing amino acids (Fernstrom and Wurtman, 1972). As a result, changes in the content of any of these amino acids would affect the transport of BCAA across the BBB (Fischer and Baldessarini, 1976). It was proposed that this transport may be bidirectional and there may be an exchange of these amino acids with other neutral amino acids in blood such as glutamine across the BBB (James et al., 1979; Cangiano et al., 1983; Jonung et al., 1984). In addition, any change in the properties of the BBB would also affect the transport of these amino acids. In fact, it has been shown

that in hyperammonemic states the properties of the BBB is altered and there is enhanced transport of these amino acids across the BBB (Sarna et al., 1977, 1979; Mans et al., 1982; 1983; Cangiano et al., 1984; Watanbe et al., 1983; Hawkins et al., 1981 ; James et al., 1979). A decrease in the content of BCAA and an increase in AAA content in blood was also observed (Fischer et al., 1975; 1976; Rosen et al., 1977; Erikkson et al., 1982; Jonung et al., 1983; Beaubernard et al., 1984; Rigotti et al., 1985). Hence, it was proposed that the AAA impede the transport of BCAA and the AAA flood the CNS (Fischer et al., 1975; 1976; Fischer and Balderssarini, 1976). It was also suggested that glutamine produced in the brain may exchange with BCAA in blood across the BBB (Cangiano et al., 1983; 1984; Rigotti et al., 1985).

Once the BCAA cross the BBB, they encounter plasma membrane of various cell types in the brain. Two transport systems have been identified for the transport of the BCAA, an 'A' type and a 'L' type. The former system is sodium dependant, co-transporting the amino acids and the Na^+ ions. The 'L' type system is sodium independant and is responsible for the homo and heteroexchange of these amino acids (Sershen and Lajtha, 1979; Betz and Goldstein, 1978). The homo and heteroexchange differs in that the former does not alter the the amino acid concentration on either side of the cell while the latter mediates exchange transport of two different amino

acids, and is capable of bringing about drastic changes in the amino acid composition of the cell(Pajari, 1984).

Transport of BCAA was shown to be mainly by the 'L' system while the 'A' system may be responsible to a very small extent for the transport of valine (Sershen and Lajtha, 1979; Betz and Goldstein, 1978). Age dependant variations have also been demonstrated in the transport of BCAA. Peterson and Raghupathy (1973) reported greater rates of accumulation of BCAA in the synaptosomes isolated from immature animals than in the adults. Pajari (1984) reported that the steady state accumulation of BCAA increased with age in brain slices. He has also reported mutual inhibition in the transport of BCAA into the brain slices, the inhibition being more in young than in old animals. Transport of BCAA was shown to be inhibited by BCKA. Phenylalanine, tyrosine and histidine adversely affected this process (Vahvelainen and Oja, 1975; Pajari, 1984). Amino acids such as glycine, glutamate, glutamine and aspartate exerted minimal effects while GABA stimulated the same.

Several reports are availablle on the transport of BCAA across the BBB in hyperammonemic states. It has been shown that the carrier for the transport of neutral amino acids in the BBB is selectively disrupted in hyperammonemia resulting in an increased influx of BCAA across the BBB (James et al., 1978; Sarna et al., 1977, 1979; Hawkins et al., 1981; Watanbe et al., 1983; and Cangiano et al., 1983; 1984). Cangiano et

al.,(1983) showed that the increased influx of BCAA into the brain in hyperammonemia is due to the enhanced levels of glutamine which is exchanged for the BCAA by the carrier protein in the BBB. Suppression of glutamine synthesis with MSO suppressed the influx of BCAA into brain supporting the concept that BCAA are exchanged for glutamine across the BBB (James et al., 1979; Jonung et al., 1983; and Rigotti et al., 1985). However, Mans et al.,(1983) demonstrated that a time course of the uptake processes are different, hence may not be related to each other , thus contradicting this hypothesis. The carrier protein for neutral amino acids in the BBB was shown to have very low affinity for glutamine when compared to other amino acids. However, it is generally accepted that the transport of neutral amino acids including BCAA is enhanced in hyperammonemia. Though literature is available on the transport of these amino acids across the BBB, no information is available on their transport into cells or subcellular fractions in hyperammonemic states.

Results of the present study indicated an enhancement in transamination and decarboxylation of BCAA in the cellular and subcellular fractions of the brain. This increased metabolism can be sustained only when there is enhanced availability of these amino acids. As it was shown that the transport of these amino acids across the BBB increased, it will be interesting to study the transport of these amino acids into various subcellular and cellular components of brain in

hyperammonemic states.

TRANSPORT OF BCAA INTO SUBCELLULAR FRACTIONS OF BRAIN:

As cytosol cannot be used to study transport processes, presently studies on transport of these amino acids was carried out only with mitochondrial and synaptosomal preparations. Preliminary studies indicated that the transport process was linear upto 0.8 mg of mitochondrial and 0.5 mg of synaptosomal proteins (Fig 4.1). Similarly, uptake process was linear from 0-30 min in these two fractions (Fig 4.2). In order to minimise the metabolism of these amino acids in the subcellular fractions, incubation time was set at 3 min. Using these parameters, kinetics of BCAA transport into synaptosomes and mitochondria and the kinetic constants with increasing concentration of the BCAA were determined (Fig 4.3.and 4.4).

TABLE 4.1
KINETIC CONSTANTS FOR THE TRANSPORT OF BRANCHED CHAIN AMINO ACIDS INTO THE SUBCELLULAR FRACTIONS ISOLATED FROM BRAINS OF NORMAL RATS.

FRACTION		LEUCINE	ISOLEUCINE	VALINE
SYNAPTO-SOMES	Km	1.6±0.20(2)^{b*c*}	5.0±0.80(2)	2.8±0.03(2)
	Vmax	60±14(2)	60±01(2)	62±01(2)
MITOCHON-DRIA	Km	3.1±0.11(2)^{b*}	9.0±1.40(2)^{c**}	3.6±1.54(4)
	Vmax	126±31(2)^{c**}	133±20(2)$^{c+}$	72±12(4)

Km is expressed as mM concentration of the substrate. Vmax is expressed as µmol of amino acid/mg protein/hr. Number in parenthesis indicate the number of experiments. * - $p<0.05$, ** - $p<0.02$, + - $p<0.01$. a-leucine, b-isoleucine, and c-valine.

In synaptosomes isolated from normal animals there was no difference in the Vmax of the transport process for all the three amino acids, while the Km was significantly higher for isoleucine and lower for leucine (Table 4.1).

In the mitochondria isolated from brains of normal animals, Vmax for transport was highest with isoleucine and lowest with valine. These differences between transport of isoleucine and valine, and leucine and valine were statistically significant, whereas the difference in the Vmax with leucine and isoleucine was statistically not significant. The Km values for the uptake of leucine and valine were similar and significantly lower than that of isoleucine.

Several studies have been made on the transport of BCAA across the BBB and in brain slices by different investigators. However, the kinetic parameters reported for the transport of these amino acids across the BBB cannot be compared with those reported in the present study due to differences in the preparations. Cohen and Lajtha(1972) reported a Km of 0.5mM for the transport of leucine in brain slices, while Vahvelainen and Oja (1972) reported 0.4mM for this amino acid. Benjamin et al., (1980) reported a Km of 1.86mM for leucine, 2.24mM for valine and 2.14mM for isoleucine. The values reported by us for leucine and valine in the synaptosomal preparations are much closer to the values reported by Benjamin et al.,(1980). The disagreement with other values may be because of the differences in the two

preparations.

The presence of the uptake process in the synaptosomes is in accordance with that reported by Peterson and Raghupathy (1973). They have shown that the synaptosomes from immature rat brains accumulate both valine and leucine and this uptake process differs from that seen synaptosomes isolated from adult animals. Presence of uptake process in the synaptosomes duly confirms the suggestion that the metabolism of BCAA also takes place in these neuronal elements. The difference in the kinetic properties of this uptake process between synaptosomes and mitochondria is understandable as they have been measured in two different membrane preparations. The synaptic membrane representing the plasma membrane, the structure, function and chemical composition of which is entirely different from that of mitochondria. Lower Km values in the synaptosomes for the uptake of the three BCAA indicates that the carrier(s) might be saturated at low extracellular concentration of these amino acids while in the mitochondria, where the Km is higher the affinity will be low and the carrier protein(s) will be saturated only when their is a increase in the content of these amino acids in the cytosol. Lower Km and Vmax in the synaptosomes compared to that of mitochondria indicates that the carrier proteins in the synaptosomes is (are) high affinity, low capacity system while that in the mitochondria is (are) low affinity, high capacity system. The higher Km

values of the uptake process in the mitochondria may have a physiological significance. As a result of the low affinity for the amino acid, this carrier protein will not function efficiently when the cytosolic concentration are low. This would allow utilization of these amino acids for other cytosolic reactions, such as transaminases and protein synthesis.

TRANSPORT OF BCAA INTO CELLULAR FRACTIONS OF BRAIN:

Preliminary standardisation studies indicated that the uptake process in the astrocytes was linear upto 400 µgms of protein (Fig 4.5) and upto 200 µgms of protein in the oligodendrocytes (Fig 4.6). Hence, 100 µgms of protein was used for routine assays. The uptake process was found to be linear upto 8 min in all the cellular fractions (Fig 4.7), similar

TABLE 4.2

KINETIC CONSTANTS FOR THE UPTAKE OF BCAA IN DIFFERENT CELLULAR FRACTIONS ISOLATED FROM BRAINS OF NORMAL RATS.

FRACTIONS		LEUCINE	ISOLEUCINE	VALINE
ASTRO-CYTES	Km	2.42±0.90(3)	2.12±0.66(3)	1.20±0.22(3)
	Vmax	72±09(3)$^{b+c+}$	160±19(3)	177±14(3)
NEURONS	Km	1.20±0.03(2)$^{b+}$	3.4±0.26(3)$^{c+}$	2.34±0.60(4)
	Vmax	22±02(2)$^{b+c+}$	83±05(3)^{c**}	59±28(4)
OLIGO-CELLS	Km	2.92±0.16(3)^{c**}	3.04±0.60(4)$^{c+}$	4.56±0.60(4)
	Vmax	29±0.02(3)$^{b+c+}$	86±08(4)	203±20(4)

Km is expressed as mM amino acid. Vmax is expressed as µmol of amino acid/mg protein/hr. Number in the parenthesis indicate the number of experiments. (a) leucine, (b) isoleucine and (c) valine. *-$p<0.05$ **-$p<0.02$ and +-$p<0.01$.

to the subcellular fractions the incubation time was set at 3

min. Using these parameters the kinetics for the uptake of these amino acids into the neurons, astrocytes and oligo cells isolated from normal animals is given in Table 4.2 (see also Figs 4.8, 4.9, 4.10).

In astrocytes, though, Km for valine was lower than that of isoleucine and leucine, this difference was stastically insignificant. In neuronal preparations, Km for the uptake process was same for isoleucine and valine which were higher than that of leucine. The highest rate of uptake process was seen in these preparations with valine as the substrate and the lowest with leucine as substrate. These differences in the Vmax of the uptake of BCAA in the neuronal preparations were statistically significant. In the oligo cells, Km and Vmax value for the uptake of valine were higher than those of leucine. In this cell preparation, Vmax for the transport of isoleucine was significantly higher than that of leucine. Expressed in a different fashion the rate of uptake of isoleucine and leucine were much higher in astrocytes than in neurons and oligo cells while that of valine was higher in oligo cells than in neurons and astrocytes.

As was mentioned earlier, virtually no reports are available on the uptake of BCAA in different cell types in brain except that of Hannumeini and Oja (1981) on the kinetics of transport of leucine in astrocytes and neurons isolated in bulk from cerebral cortex of 3 week old rat. They reported a Vmax value of 13 and 24 μmoles/hr/kg cells for the

uptake of leucine into neurons and astrocytes respectively. These values cannot be compared with the values of the present study as the activities are represented/kg cells and they cannot be converted to µgms protein since neither protein content per cell nor weight of cells/mg protein are given in their report. It must be mentioned that the values given in the present study for the cellular fractions might have been modified due to the treatment with proteolytic enzymes prior to their isolation. The results obtained indicated that the three major cell types in the brain participate in the utilisation of BCAA, which is understandable as these amino acids are required not only for the metabolic processes but also for the synthesis of proteins and to a certain extent for the synthesis of lipids. It has been reported earlier that both neurons and oligocells are actively engaged in protein synthesis when compared to astrocytes. The present study on transaminases and decarboxylases in cells indicated the presence of the enzyme responsible for their utilization. In general, results obtained with uptake studies suggested that astrocytes have a low affinity, high capacity uptake system when compared to the other two cell types. In other words, when the extracellular concentration of these amino acids are low the neurons and oligo cells, in which the rate of metabolic degradation of these amino acids is low, will preferentially take up these amino acids and utilize them probably for protein/lipid synthesis. When the

extracellular concentration are high, these amino acids are taken up by all the cell types including the astrocytes. In astrocytes, they may be used both for protein synthesis and for metabolic degradation. High Vmax values observed in the uptake of the BCAA in astrocytes are in accordance with the proposed role of these cells in the metabolism of BCAA.

CEREBRAL SUBCELLULAR BCAA UPTAKE IN HYPERAMMONEMIA

Changes observed in the kinetics of the uptake process between the synaptosomes and mitochondria isolated from brains of rats injected with an acute dose of ammonium acetate are presented in Table 4.3.

TABLE 4.3
KINETIC CONSTANTS OF THE UPTAKE OF BCAA INTO SYNAPTOSOMES AND MITOCHONDRIA ISOLATED FROM BRAINS OF NORMAL AND HYPERAMMONEMIC RATS.

CONDITION		LEUCINE	ISOLEUCINE	VALINE
			SYNAPTOSOMES	
CONTROL	Km	1.6±0.20(2)	5.0±0.80(2)	2.8±0.03(2)
	Vmax	60±14(2)	60±01(2)	62±01(2)
EXPERI-	Km	3.3±0.34(2)*	8.0±0.8(2)	6.0±1.58(2)
MENTAL	Vmax	84±09(2)	182±16(2)+	195±04(2)++
			MITOCHONDRIA	
CONTROL	Km	3.1±0.11(2)	9.0±1.40(2)	3.6±1.54(4)
	Vmax	126±31(2)	133±20(2)	72±12(4)
EXPERI-	Km	4.2±0.03(2)+	9.0±0.20(2)	8.1±1.00(4)*
MENTAL	Vmax	94±07(2)	279±17(2)**	274±34(4)+

Km is expressed as mM amino acid. Vmax is expressed as µmol of amino acid/mg protein/hr. Number in the parenthesis indicate the number of experiments. *-$p<0.05$, **-$p<0.02$, +-$p<0.01$ and ++-$p<0.005$.

A generalised increase was observed both in the Km and

Vmax of the uptake process for all the three amino acids in the synaptosomal preparations in hyperammonemia. Increase in the Km of the transport of leucine and valine were of higher magnitude than that of isoleucine. There was an increase in the Vmax of BCAA uptake under these conditions, but the increase observed for isoleucine and valine were of higher magnitude than that of leucine. In the latter case, a change in the Vmax was not statistically significant.

In the mitochondrial preparations isolated from brains of hyperammonemic rats, a statistically significant increase was noticed in the Km of the uptake of leucine and valine while the Km for isoleucine remained unaltered under these conditions. Similarly, increase in the Vmax for the uptake of isoleucine and valine was statistically significant while the decrease in the Vmax for the uptake of leucine was statistically insignificant.

CEREBRAL CELLULAR BCAA UPTAKE IN HYPERAMMONEMIA:

A comparison of the kinetic properties of the uptake of BCAA in the three major cell types isolated from normal and hyperammonemic rats is given in the accompanying table 4.4.

From the results it is evident that in the astrocytes isolated from hyperammonemic rats, Km values for the uptake of all the three amino acids and the Vmax values for the uptake of isoleucine and valine were unaltered in hyperammonemic states. However, Vmax for the uptake of leucine showed a statistically significant increase by about two

fold. In contrast, Km for the uptake of isoleucine and valine decreased by about 20% in neurons of hyperammonemic rats, while the Km for the uptake of leucine increased by about five fold. There was a decrease in the maximal rates of

TABLE 4.4

KINETIC CONSTANTS OF THE UPTAKE OF BCAA INTO VARIOUS CELLULAR FRACTIONS ISOLATED FROM BRAINS OF NORMAL AND HYPERAMMONEMIC RATS.

CONDITION		LEUCINE	ISOLEUCINE	VALINE
			ASTROCYTES	
CONTROL	Km	2.42±0.90(3)	2.12±0.66(3)	1.20±0.20(3)
	Vmax	72±09(3)	160±19(3)	177±14(3)
EXPERI-	Km	2.62±0.30(3)	2.30±0.26(4)	1.80±0.40(3)
MENTAL	Vmax	168±15(3)$^{++}$	196±28(4)	190±13(3)
			NEURONS	
CONTROL	Km	1.20±0.03(2)	3.40±0.26(3)	2.34±0.60(4)
	Vmax	22±02(2)	83±05(3)	159±28(4)
EXPERI-	Km	6.60±1.60(3)$^{+}$	2.80±0.46(3)**	1.80±0.16(3)**
MENTAL	Vmax	142±24(3)$^{++}$	120±16(3)	94±04(3)
			OLIGOCELLS	
CONTROL	Km	2.92±0.16(3)	3.04±0.60(4)	4.56±0.60(4)
	Vmax	29±02(3)	86±08(4)	203±20(4)
EXPERI-	Km	2.72±0.50(3)	3.24±0.60(3)	1.90±0.20(3)$^{++}$
MENTAL	Vmax	112±12(3)$^{++}$	210±12(3)$^{++}$	200±16(3)

Km expressed as mM amino acid. Vmax expressed as μmol of amino acid/mg protein/hr. Number in the parenthesis indicate the number of experiments. **p<0.005, ++p<0.001, +p<0.01.

uptake of valine while there was a significant increase in the rate of uptake of isoleucine and leucine. In the oligo cells isolated from hyperammonemic rats, no changes were noticed in the affinity of the carrier protein for leucine and isoleucine. There was an increased affinity for valine

(decrease in the Km) by about 60%. Under these conditions, Vmax for the uptake of leucine and isoleucine was enhanced while that of valine remained unaltered.

There are several reports to indicate that the transport of BCAA across the BBB is enhanced in HE, James et al., (1978); Sarna et al., (1979); Hawkins et al., (1981); Mans et al., (1982); Watanbe et al., (1983); and Cangiano et al., (1984). Perfusion of the animal with pathophysiological concentrations of ammonium salts were also adequate enough to elicit the above mentioned response in the transport of BCAA (Mans et al., 1983; Cangiano et al., 1984). Cangiano et al., (1984) demonstrated that perfusion of the brain with ammonium salts enhanced the cerebral leucine uptake. These studies have conclusively demonstrated that the enhanced synthesis of glutamine in brain in hyperammonemia is associated with an enhanced transport of these amino acids into the brain. As a result of an enhanced transport of these amino acids across the BBB, there might be an increase in the extracellular concentrations of these amino acids. Unless corresponding changes take place in the uptake of these amino acids in the different cell types in brain, these amino acids may not be utilised in the brain. Perusal of the results obtained in the present study reveal an interesting change in the cellular uptake of BCAA during hyperammonemia. The uptake rate (Vmax) was unaltered in the experimental condition if the Vmax in the normal condition is already high. This is evident in the

transport of isoleucine and valine in the astrocytes and that of valine in the oligocells. However, when the rate of transport is low in normal animals, then there is a large increase in the Vmax values following the administration of ammonium acetate. This is quite evident in the transport of leucine in the astrocytes, isoleucine and leucine in neurons and oligo cells. As a result of these differential changes the rate of transport of the three BCAA into these cellular fractions will be more or less same in hyperammonemic states. Such a change would probably abolish any competition in the transport among these amino acids. Such a change will also ensure equal availability of the BCAA for further metabolic reactions.

It is usually assumed that the Vmax represents the number of transport sites while the Km represents the properties of these sites. In such a case, a change in the Km represents alteration in the properties of the transport sites while a change in the Vmax represents the alteration in the number of transport sites (either by synthesis of new proteins or by degradation of existing proteins). In the present study very few changes have been observed in the Km in cellular preparation while there was substantial changes in the Vmax. This suggests that in hyperammonemic states the number of transport sites (membrane bound carrier proteins) are increased. This will be difficult to comprehend as the time period between administration of ammonium salts and the

cannot be definitely concluded that the new carrier protein molecules are synthesised and inserted into the membrane in such a short span unless otherwise proved. Moreover, this may not be an experimental artifact due to the trypsin treatment, as the treatment is similar in both normal and hyperammonemic animals and there are few changes in the Km. This suggests that due to some changes in the membrane properties (such as membrane fluidity) during hyperammonemia more number of transport sites are exposed or unmasked. Such changes in the membrane properties in hyperammonemia have already been reported (Pappas et al., 1982; Baraldi and Zanrol, 1982; O'Connor et al., 1984).

Increase in the transport of these amino acids into synaptosomal preparations reemphasises the role of these subcellular organelles in ammonia detoxification. In this fraction, an increase in the transport of valine and isoleucine was also observed. It has been repeatedly shown that the synaptosomes do contain a small amount of GS and thus may contribute to ammonia detoxification to some extent (Rani et al., 1983; Subbalakshmi and Murthy, 1985).

In summary, results on the transport studies indicates an enhanced transport of BCAA which may trigger their utilisation for the purpose of ammonia detoxification in brain.

UPTAKE OF [U-^{14}C]LEUCINE IN MITOCHONDRIA AND SYNAPTOSOMES : RATE OF UPTAKE Vs PROTEIN.

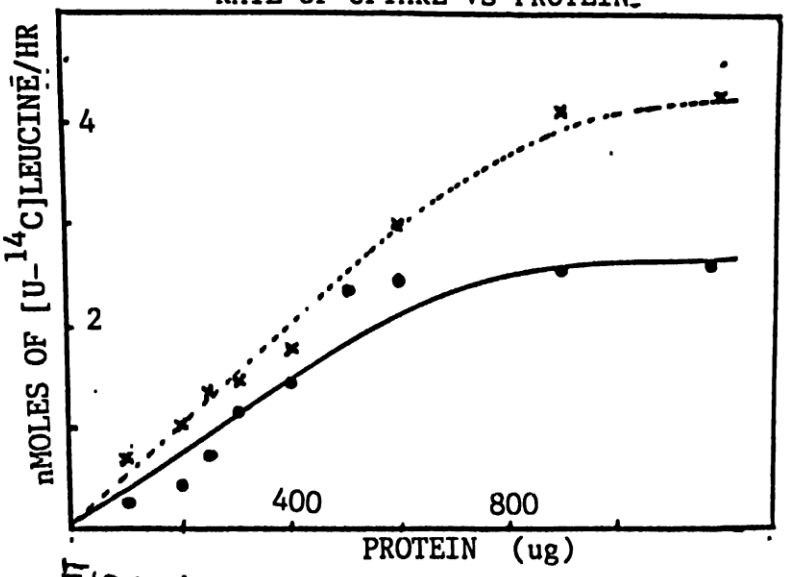


FIG 4.1

TIME COURSE OF [U-^{14}C]LEUCINE UPTAKE IN MITOCHONDRIA AND SYNAPTOSOMES

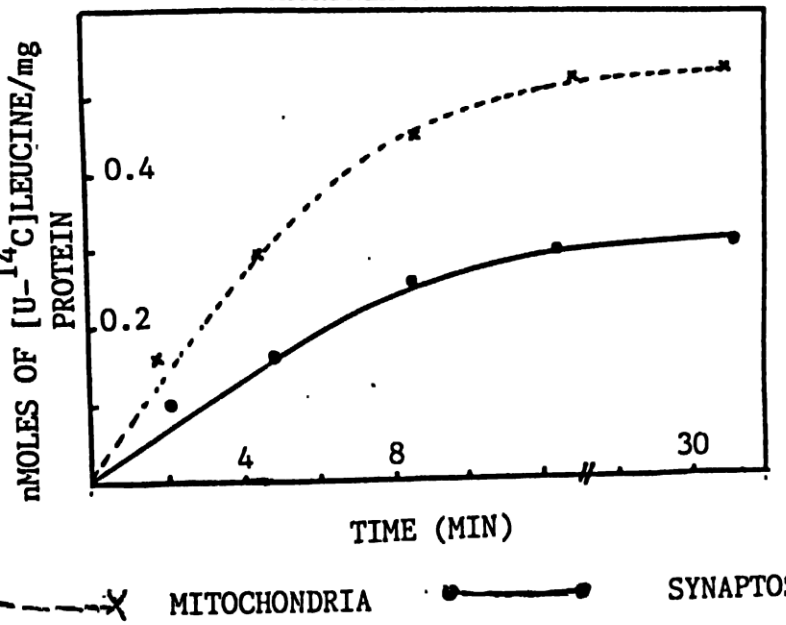


FIG 4.2

KINETICS OF [U-^{14}C]BRANCHED-CHAIN AMINO ACID UPTAKE IN SYNAPTOSOMES

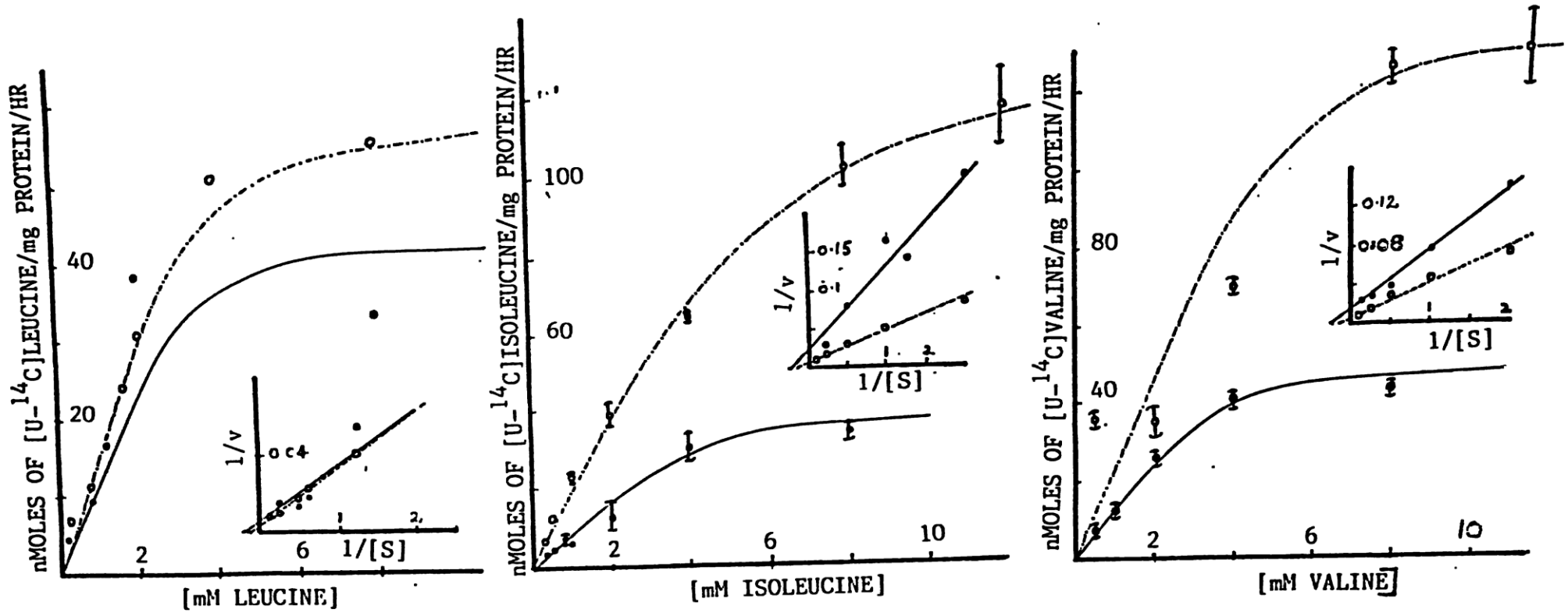
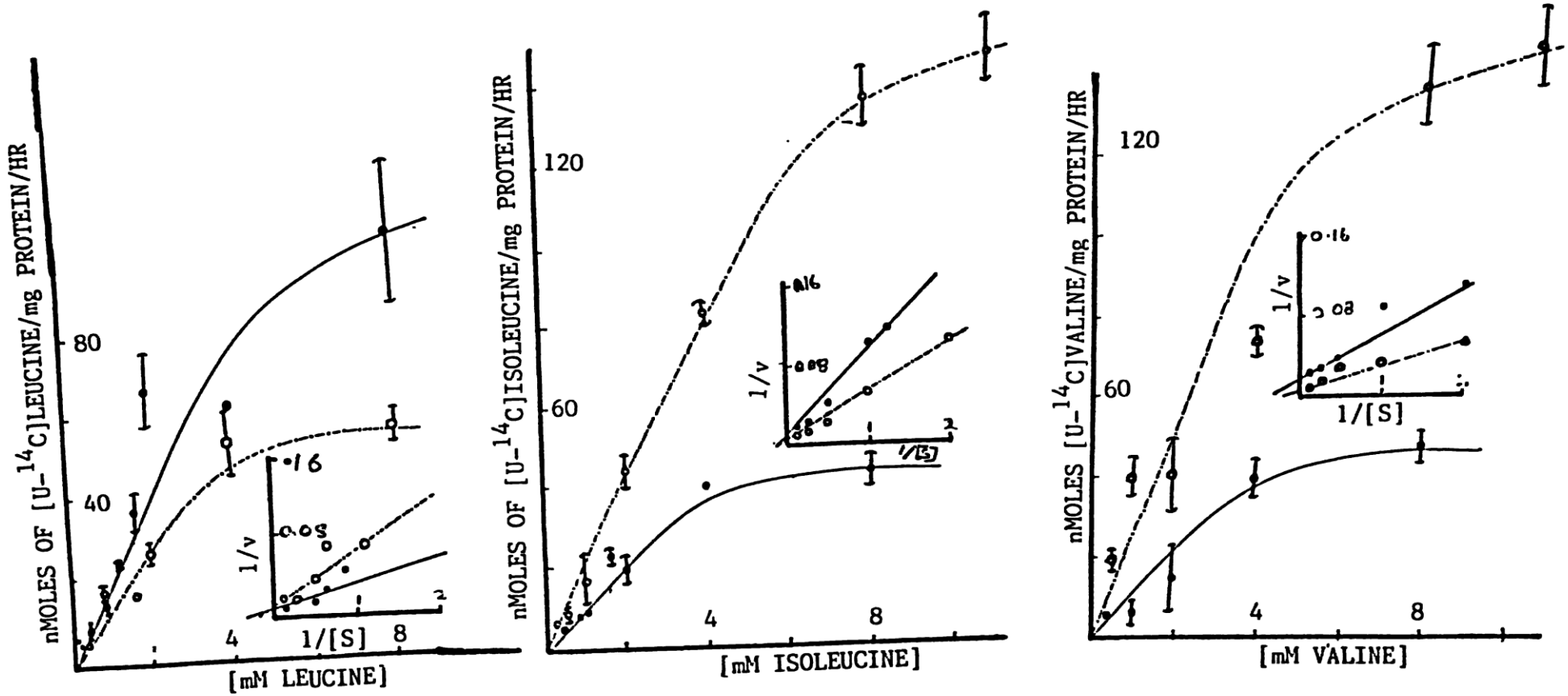


FIG 4.3

VALUES ARE MEAN ± S.D OF 2 TO 3 EXPERIMENTS

KINETICS OF [U-^{14}C]BRANCHED-CHAIN AMINO ACID UPTAKE IN MITOCHONDRIA



● —— ● CONTROLS 0 -·-·-·-·- 0 EXPERIMENTAL

FIG 4.4

VALUES ARE MEAN ± S.D OF 3 EXPERIMENTS

UPTAKE OF [U-^{14}C]LEUCINE IN ASTROCYTES :
RATE OF UPTAKE Vs ug PROTEIN

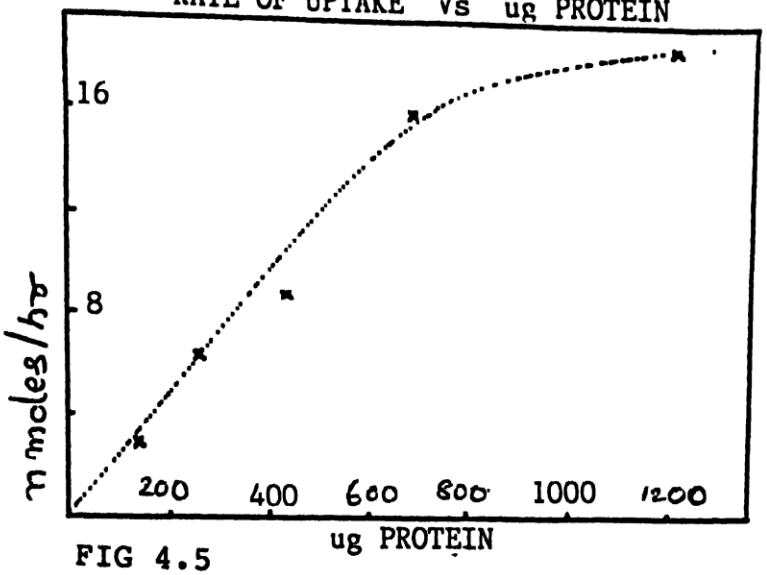


FIG 4.5

UPTAKE OF [U-^{14}C]LEUCINE IN OLIGODENDROCYTES :
RATE OF UPTAKE Vs ug PROTEIN

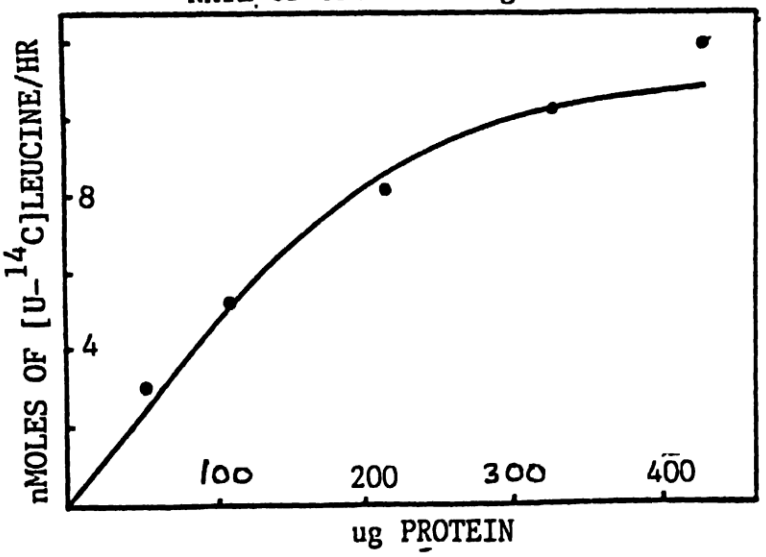


FIG 4.6

UPTAKE OF [U-^{14}C]LEUCINE IN CELLS : TIME COURSE

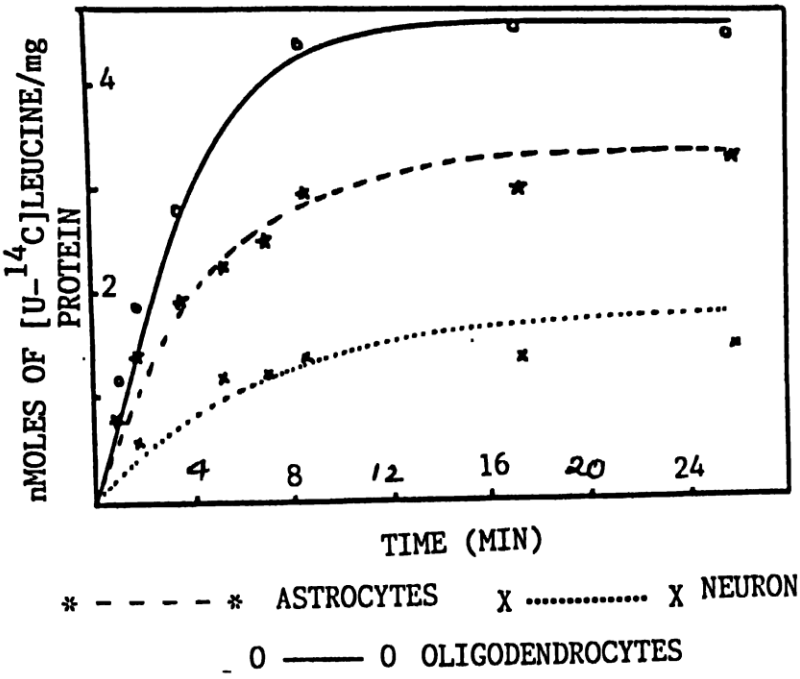


FIG 4.7

[U-^{14}C]BRANCHED-CHAIN AMINO ACID UPTAKE IN NEURONAL PERIKARYA : KINETICS

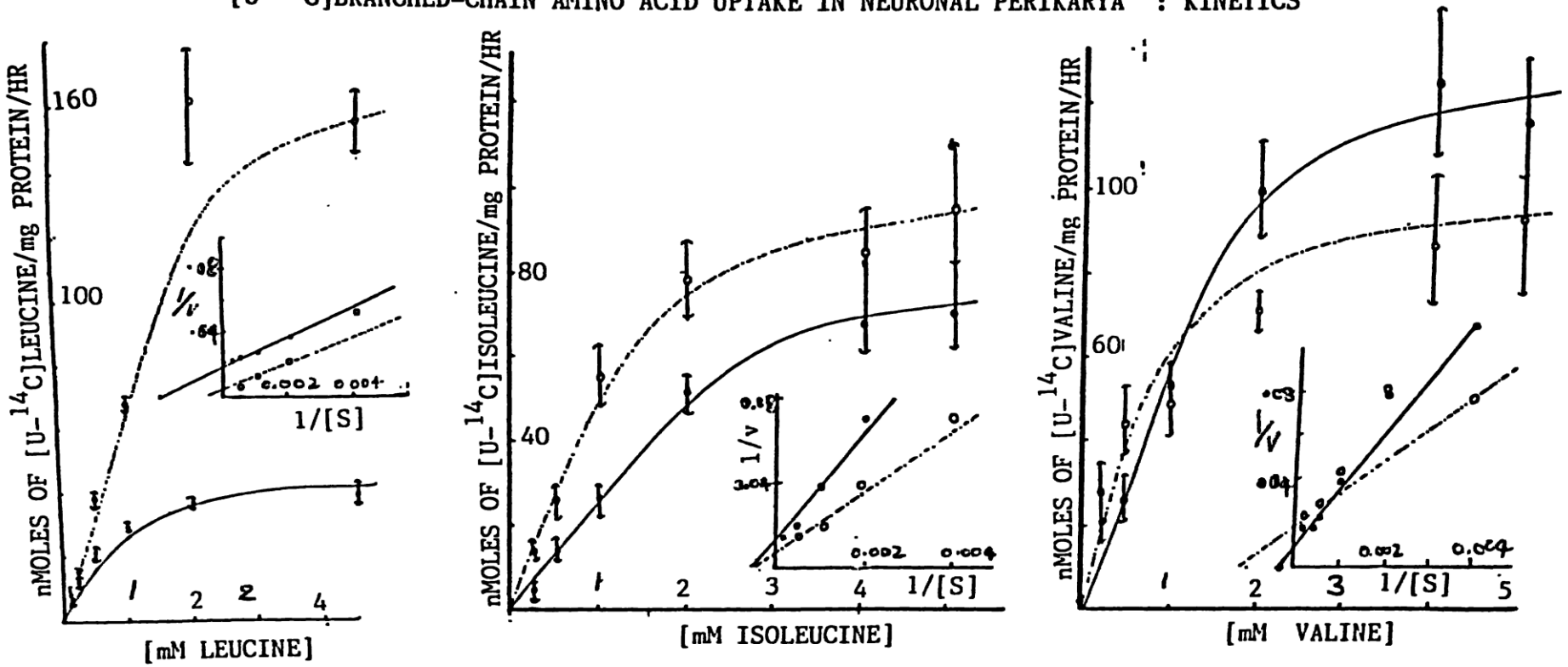
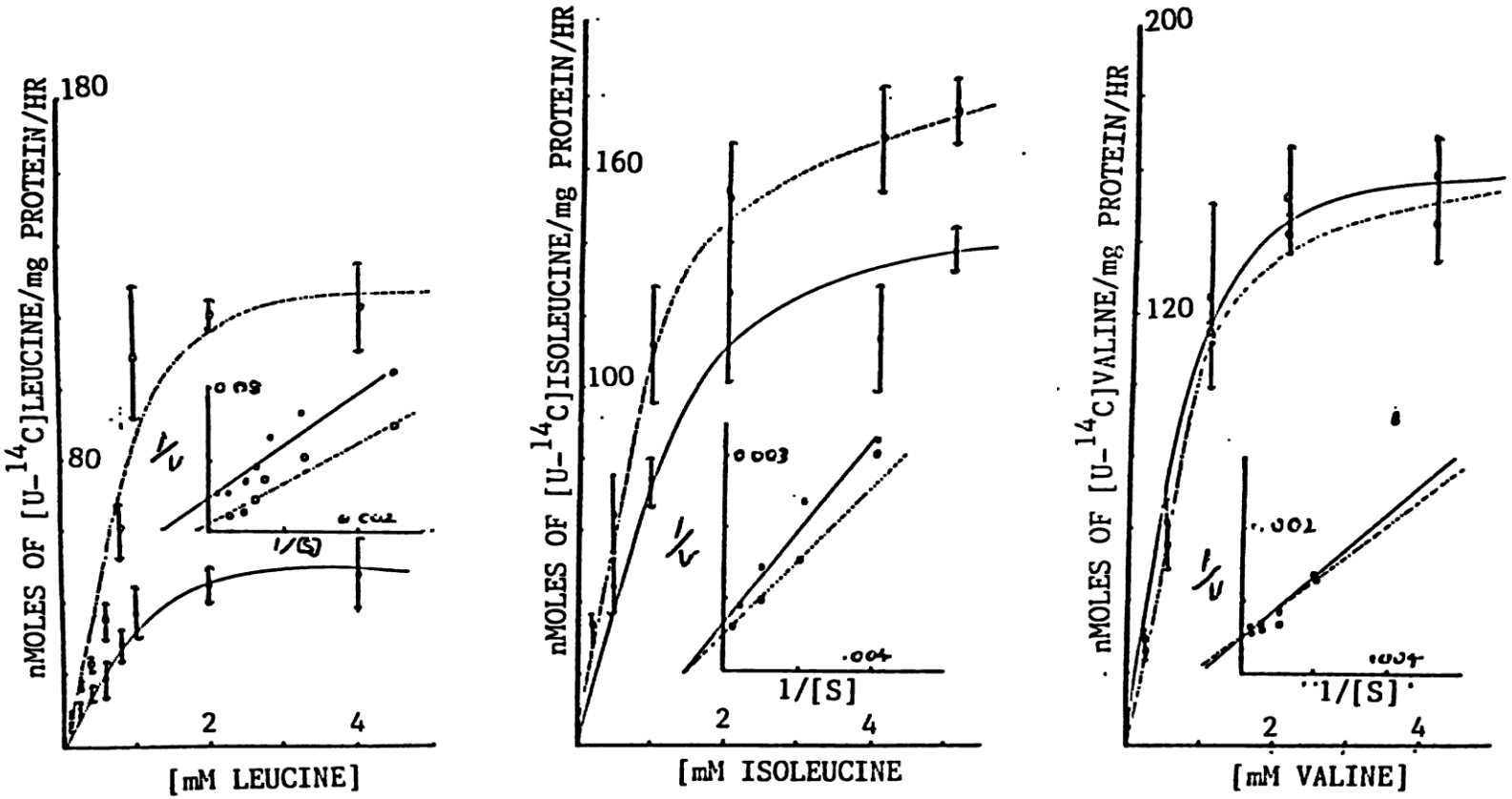


FIG 4·8

EACH VALUE IS MEAN ± S.D. OF 3-4 EXPERIMENTS

KINETICS OF [U-^{14}C]BRANCHED CHAIN AMINO ACID UPTAKE IN ASTROCYTES

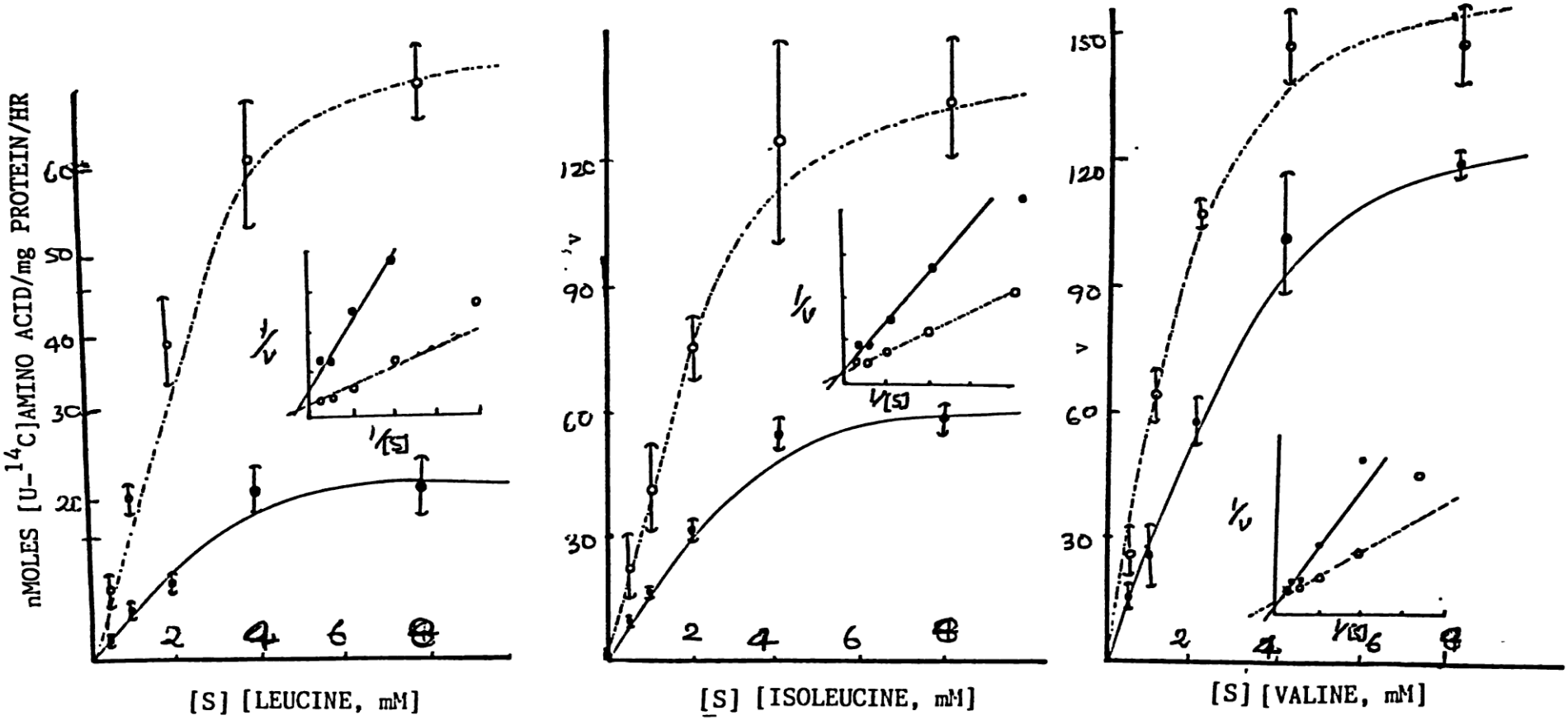


● ——— ● CONTROLS O -.-.-.- O EXPERIMENTAL

FIG 4.9

EACH VALUE IS MEAN ± S.D OF 3 - 4 EXPERIMENTS

BRANCHED-CHAIN AMINO ACID UPTAKE IN OLIGODENDROCYTES



●———● CONTROLS O- - - -O EXPERIMENTAL

Each value is mean±s.d of 4 values.

FIG 4.10

METABOLIC STUDIES

METABOLIC STUDIES IN SUBCELLULAR FRACTIONS

In the present study results obtained so far indicate the positive role played by the BCAA in replenishing the glutamate pool required for the synthesis of glutamine in hyperammonemic states. Studies on enzyme systems also indicated an enhanced transamination, and decarboxylation of these amino acids. Some of the intermediates formed in these reactions, such as acetyl CoA and succinyl CoA, might be used in the TCA cycle and modulate the flux of carbon through this pathway and the energy production. However, enzymatic studies in isolated systems especially under optimal assay conditions may not reflect the actual changes in the in vivo system. The reason being that during the assay of an enzyme, concentrations of the required substrates, cofactors and pH are optimal and the barriers which regulate the entry and exit of these compounds are also destroyed by the process of homogenisation or by the addition of detergents such as Triton X-100. Moreover, other enzymes which share substrates along with the enzymes under study may not be functional in these conditions. In in vivo conditions, most of the enzymes are never fully saturated with their substrates and co-factors, nor is the pH optimal. In addition, they have to share their substrates with other enzymes. Moreover, products generated in other reactions might have a regulatory effect on these enzymes. For eg. Ac CoA generated from pyruvate oxidation might have an inhibitory effect on the decarboxy-

lation of BCAA. However, studies with enzyme systems would give information on the optimal capacity of the system, though not of the operational capacity. Hence, in the present study, the increased activities of transaminases and decarboxylases of BCAA may or may not be occuring under in vivo conditions. This aspect has been studied presently by measuring the production of ^{14}C-BCKA and $^{14}CO_2$ from [U-^{14}C]BCAA in normal and hyperammonemic states.

As was mentioned earlier, CO_2 production from BCAA occurs by C-1 decarboxylation of the BCKA (produced in the transamination reaction) and the oxidation of Ac CoA or succinyl CoA generated from the branched chain keto acyl CoA. The later part of CO_2 production occurs in the TCA cycle. These two processes can be recognised by using [1-^{14}C] and [U-^{14}C]-BCAA. When [1-^{14}C]BCAA are used as substrates, labelled carbon is removed by the decarboxylase whereas when [U-^{14}]BCAA are used as substrates the labelled carbons are removed by the BCKA-DC and enzymes of TCA cycle. Use of differentially labelled compounds would provide information about various stages of regulation in the metabolism of these amino acids. However, in the present study, only [U-^{14}C]BCAA were used to elucidate the changes induced in the metabolism of these amino acids in hyperammonemia.

Survey of the literature revealed only two reports on the production of carbon dioxide from BCAA in hyperammonemic states. Shiota (1984) reported an increase in the production

of $^{14}CO_2$ from [1-^{14}C]leucine in rats treated with carbon tetrachloride. Murthy and Hertz (1987b) reported a fall in the production of $^{14}CO_2$ from both [1-^{14}C]-leucine and valine and [U-^{14}C]isoleucine in the differentiated astrocytes in primary cultures in the presence of 3mM NH_4Cl. More or less a similar effect was observed by them in primary cultures of undifferentiated astrocytes except the changes in the production of $^{14}CO_2$ from valine which were not statistically significant. In contrast to these effects in the astrocytes, they observed no differences in the production of $^{14}CO_2$ in the presence and absence of ammonia in the primary cultures of neurons.

These authors also compared acute and chronic effects of exposure to pathophysiological concentrations of ammonia on these processes using primary cultures (Murthy and Hertz, 1987a). They reported that following chronic exposure decarboxylation of [U-^{14}C]leucine was significantly enhanced while the suppression of $^{14}CO_2$ production from [1-^{14}C]leucine as seen in the acute experiments was abolished. However, the differences in the CO_2 production from [1-^{14}C]valine and [U-^{14}C]- valine, isoleucine were moderate both in acute and chronic experiments. Chronic exposure of neurons in primary cultures to ammonia resulted in a significant inhibition in the oxidation of [U-^{14}C]-valine and isoleucine. However, if the incorporation of isotopically labelled carbon and hydrogen atoms from BCAA into glutamate is taken as a measure

of processing the carbon skeleton of these amino acids in the TCA cycle, then the reports of Cremer et al., (1975, 1977) suggest an enhanced flux of BCAA carbon through this pathway.

PRODUCTION OF $^{14}CO_2$ IN SUBCELLULAR FRACTIONS

Data on the preliminary standardisation of $^{14}CO_2$ production in the subcellular fractions is shown in Fig.5.1. These results indicate that the amount of protein added during the assay i.e.,150 µgms is within the linear range of the curve. The time course of $^{14}CO_2$ production was carried out with [U^{14}C]valine shows that the activity is linear upto 60min (Fig.5.2). Hence, 30 min was used as the time of incubation for routine assay. Kinetic constants such as Km and Vmax were not determined, as CO_2 production from [U-^{14}C]BCAA does not occur in a single reaction but involves multiple reactions. In such a case, determined Km and Vmax values represent an aggregate value of all these reactions, the significance of which will be difficult to asses. Hence, all the assays were performed at a fixed substrate concentration of 100µM of BCAA (i.e. 50 nmoles/0.5 ml of assay mixture containing of glucose).

With all the three amino acids as substrates (Table 5.1), rate of CO_2 production was much less in cytosol when compared to mitochondria and synaptosomes. This difference was statistically significant all the cases. Production of $^{14}CO_2$ from [U^{14}-C]leucine was lesser in mitochondria than in synaptosomes while a reverse profile was obtained with valine

TABLE 5.1

PRODUCTION OF [$^{14}CO_2$] FROM LABELLED BRANCHED CHAIN AMINO ACIDS IN SUBCELLULAR FRACTIONS ISOLATED FROM NORMAL RATS.

FRACTION	LEUCINE	ISOLEUCINE	VALINE
MITOCHONDRIA[a]	630±030(5)[c+]	980±100(5)[c*]	570±066(5)
CYTOSOL[b]	220±020(4)[a*c*]	300±050(5)[a*c*]	240±040(5)[a*c*]
SYNAPTOSOMES[c]	720±070(6)	570±070(4)	510±070(5)

Values are Mean±S.D. Activity expressed as pmoles [$^{14}CO_2$] liberated/mg. protein/hr. Number in parenthesis indicate the number of determinations. *=$p<0.05$ +=$p<0.01$

and this difference was statistically significant only when isoleucine was the substrate.

These studies on the production of CO_2 in the subcellular fraction from BCAA revealed two interesting aspects viz., 1) the production of CO_2 from BCAA by synaptosomal fraction and 2) production of CO_2 in the cytosol. This observation in synaptosomes is in agreement with reports of Chaplin et al.,(1976) on CO_2 production from [1-^{14}C]- and [U-^{14}C]-leucine in this fraction. These results support the presence of BCAA-T and BCKA-DC as well as uptake of these amino acids by synaptosomes which are reported in the present study. Moreover, they also suggested that the neuronal components participate in the BCAA metabolism. In this respect, these results are not in agreement with the concept of metabolic compartmentation.

The second observation, i.e., production of CO_2 in cytosol is surprising. It is generally assumed that the enzymes responsible for the production of CO_2 from BCAA. BCKA-DC and the enzymes of TCA cycle are localised in the mitochondria. In such a case, there should not be any production of CO_2 from the BCAA with cytosolic preparations. However, production of CO_2 in the cytosolic fraction, albeit at low rates, strongly support the presence of BCKA-DH in this fraction. One possibility for the presence of BCKA-DC and the production of CO_2 from BCAA in the cytosol, is the contamination of this fraction with mitochondria. However, determination of SDH, (a marker enzyme for mitochondria) revealed <2% of the activity in the cytosol (Table 3.23). This does not correspond to the amount of $^{14}CO_2$ produced from BCAA in this fraction which is about 30-40% of the rate observed in the mitochondria. In such a case, it may be assumed that either BCKA-DC or the entire BCKA-DH complex is present in the cytosol and TCA cycle enzymes are absent. Then, it may be expected that only C-1 decarboxylation of BCKA is taking place to a larger extent in the cytosol while in mitochondria both C-1 decarboxylation and the oxidation of the branched chain keto acids might be the major pathways for production of CO_2 from BCAA.

Transamination of BCAA is the prerequisite for the oxidation of BCAA. Though the presence of BCAA-T has been demonstrated in the cytosol, the question on the source of

∝-KG for this process has to be considered. In the present study, glucose was added as a source of carbon atoms in the assay mixture. As TCA cycle is absent in cytosol, ∝-KG should have been produced in the cytosol from alternate sources. Under in vivo condition, the major source of ∝-KG in the cytosol is the malate-aspartate shuttle. However in the present experimental conditions, this shuttle may not be a major source as the mitochondrial components of the shuttle are missing in the cytosolic preparation. Under these conditions, it is quite possible that the ∝-KG might have been generated in the cytosol by transamination of glutamate with either OAA or PYR. As the enzyme, PEPCK is not cytosolic, OAA might not have been formed from PEP in the cytosol. Hence, due to the paucity of OAA, the reaction between glutamate and OAA may not take place at rates adequate enough, to generate required amount of ∝-KG in the cytosol. Pyruvate generated in the glycolytic pathway might participate in transamination with glutamate thereby generate the required ∝-KG. Comparing the values given in the literature for this enzyme it is evident that the activity of AlAT is much higher than that of BCAA-T. Hence, this reaction might provide the required ∝-KG. It is also possible that BCAA-T might be utilising PYR as the keto acid instead of ∝-KG. However, it must be mentioned that the transamination of BCAA with PYR occurs at a lesser rate than with ∝-KG (Benuck et al., 1971). Irrespective of the fact that ∝-KG or PYR is used for trans-

amination, the reaction is bound to proceed at a lesser rate.

From these studies, it is not very clear whether the entire complex of BCKA-DH or only the BCKA-DC is present in the cytosol. In case only the latter enzyme is present then it must be assumed that the enzyme-TPP-BCKA complex that is formed in the course of decarboxylation reaction is hydrolysed spontaneously. If the entire dehydrogenase complex is present then there should have been adequate amounts of CoA, which is required for this reaction, in the cytosol. Even if all the enzymes of BCAA metabolism are present, the final products of this metabolism i.e., Ac CoA and succinyl CoA may not be oxidised in the cytosol due to the absence of TCA cycle in this fraction.

This suggest that most of the CO_2 produced from BCAA in the cytosol may be due to C-1 decarboxylation. Using [1-^{14}C] leucine as substrate Brosnan et al., (1976) reported that 0.04 nmoles/mg/min of $^{14}CO_2$ is produced in the cytosol and the same in mitochondria to be 0.4 nmoles/mg/min. Thus, they noticed a ten fold difference in the activity of BCKA-DC in these two fractions. Activities obtained in the present study if converted to same units as those reported by Brosnan et al., will be 3.7 nmoles for cytosol and 10.5 nmoles for mitochondria. Despite the differences in the activity levels in these two studies, it is interesting to note that BCKA are decarboxylated in the cytosol albeit at a lower rate compared

to mitochondria.

Production of $^{14}CO_2$ from BCAA in mitochondria is not surprising as both BCKA-DH and TCA cycle are present in this organelle. Higher rate of $^{14}CO_2$ production was seen from isoleucine and valine than leucine which may be due to the differences in the transport of these amino acids or in the preference of the enzymes, involved in the metabolism of these amino acids. As was mentioned earlier, transamination is the prerequisite for the metabolism of BCAA and α-KG is required for this process.

In the assay conditions employed in the present study, glucose is provided as the carbon source. It is well known that glucose is not metabolised in the mitochondria as the glycolytic enzymes are localised in the cytosol. Hence, it is possible that the endogenous α-KG pool in isolated mitochondria, which survived the isolation procedure, may be adequate enough to intiate the metabolism of BCAA. This pool of α-KG, that participates in the transamination of BCAA would be converted into glutamate. Glutamate so formed should further metabolised to regenerate the α-KG lost in the transamination of BCAA. This regenaration of α-KG from glutamate may be either due to transamination or oxidative deamination of glutamate. It is worth mentioning at this stage that leucine acts as an allosteric regulator for GDH (Martin et al., 1983). The BCKA that are produced in the transamination of BCAA may be further metabolised to Ac CoA

or succinyl CoA depending on the nature of the BCAA. These compounds may be finally oxidised in the TCA cycle.

PRODUCTION OF CARBON DIOXIDE FROM BCAA IN SUBCELLULAR FRACTIONS IN HYPERAMMONEMIC STATES

In the mitochondria isolated from cerebral cortex of hyperammonemic rats, production of $^{14}CO_2$ from [U-^{14}C]-leucine and isoleucine was suppressed. The magnitude of suppression with isoleucine as substrate was much higher that that with leucine. In contrast to this, the production of $^{14}CO_2$ from [U-^{14}C]valine was enhanced in the cortical mitochondria of hyperammonemic rats (Table 5.2).

TABLE 5.2

PRODUCTION OF [$^{14}CO_2$] FROM LABELLED BCAA IN SUBCELLULAR FRACTIONS ISOLATED FROM NORMAL AND HYPERAMMONEMIC RATS.

FRACTION		LEUCINE	ISOLEUCINE	VALINE
MITOCHONDRIA	NORMAL	630±060(5)*	980±100(5)*	570±066(5)
	EXPTL	420±030(5)*	360±090(6)*	970±250(4)+
SYNAPTOSOMES	NORMAL	720±070(6)*	570±070(4)*	510±070(5)*
	EXPTL	290±040(6)*	370±080(7)*	370±060(5)*
CYTOSOL	NORMAL	220±020(4)*	300±050(5)*	240±040(5)*
	EXPTL	550±070(5)*	540±090(6)*	500±100(4)*

Values are Mean±S.D. Activity expressed as pmoles of $^{14}CO_2$ liberated/mg. protein/hour. Number in parenthesis indicate the number of determinations. * $p<0.001$, + $p<0.01$.

In the synaptosomes isolated from cerebral cortex of hyperammonemic rats, production of $^{14}CO_2$ from all the three BCAA suppressed. The magnitude of suppression was much

higher with leucine than with the other two BCAA (Table 5.2). The observed profile of changes in the production of $^{14}CO_2$ from BCAA in the cytosol was opposite to that of synaptosomes. There was a two fold increase in the prodution of CO_2 from all three BCAA in the cytosol isolated from hyperammonemic rats. Changes observed in the cytosol under these condition were statistically significant.

Enhanced transamination and decarboxylation observed in the present study in hyperammonemic states is not in accordance with the changes observed in the oxidation of BCAA in the mitochondrial fraction except for isoleucine. This discrepancy, however, supported the concept that measurement of enzyme activities need not represent the process occuring in the in vivo state.

Decrease in the oxidation of these amino acids in the mitochondria and synaptosomes isolated from hyperammonemic rats may not be due to the deficiency of the substrate. This is evident from earlier results on the transport of these amino acids obtained under similar conditions. Reduction in the endogenous pools of α-KG in the mitochondria may be a reason for the decreased CO_2 production from BCAA in hyperammonemic states. However, it must be mentioned that whole brain α-KG levels are increased in hyperammonemia. Till the subcellular α-KG compartment in which these changes are taking place is known, paucity of α-KG may not be ruled out as a reason for decreased CO_2 production in hyperammonemic

states. However, this may not be true in synaptosomes as both TCA and glycolytic pathways are present. Moreover, glucose is provided as the carbon source along with leucine. Interaction between the metabolisms of BCAA and glucose may be ruled out as it was shown that these amino acids have no effect on glucose oxidation in cortical slices (Palaiologos et al., 1979). It has also been reported that glucose stimulates the oxidation of both [1-^{14}C] and [U-^{14}C]-leucine by about 35% in the same preparation.

Suppression of glucose metabolism by ammonia might affect the carbon flux through TCA cycle and the levels of its intermediates. This may have an adverse affect on BCAA metabolism. Available evidences on the effect of ammonia on glucose metabolism are controversial. Increased cerebral glucose utilization in acute hyperammonemic states was reported by Hawkins et al., (1983). However, they showed that increase in glucose consumption was accounted by an increase in lactate production. These authors also observed an increase in the contents of all TCA cycle intermediates including α-KG. This was supported by Vargara et al., (1974) and Duffy et al., (1974). Ratnakumari et al., (1985, 1986) reported an increase in the activities of PDH and enzymes of TCA cycle (except MDH) in the brain homogenates and in mitochondria and synaptosomes isolated from the cortex of rats administered with the same amount of ammonium acetate.

Moreover, no changes were observed in the production of $^{14}CO_2$ from [U-^{14}C]glucose and [2-^{14}C]pyruvate from neurons and astrocytes in primary cultures (Hertz et al., 1987), while Murthy and Hertz, (1988) made a similar observation in neurons and in astrocytes with [1-^{14}C]pyruvate in the presence of glutamine. Contradicting these observation are the reports of McKhan and Tower (1961) wherein 10 mM ammonium salts were found to inhibit the oxidation of PYR and ∝-KG in the mitchondria. Similarly, Lai and Cooper (1986) observed suppression of ∝-KGDH by ammonium ions. However, it must be mentioned that in these two studies mitochondria used were isolated from normal animals. Inhibition of ∝-KGDH by ammonia prevents ∝-KG utilization in TCA cycle and consequently ∝-KG levels would increase. Under these conditions, there might be adequate amounts of ∝-KG which is required for the transamination of BCAA.

From the above discussion it is evident that the suppression of BCAA oxidation by ammonia cannot be explained by any of the reasons when each one is considered individually. Hence, another approach was made to this problem keeping several observations in view. This assumption is based on the following observations. 1. increase in BCAA transport into brain, into cells and into subcellular fractions (Mans et al., 1983; Present study) 2. increased ∝-KG content in brain (Hawkins et al., 1973; Vargara et al., 1974) 3. increased activity of BCAA-T in cells and sub-

cellular fractions (Present study) 4. suppression of malate-aspartate shuttle (Hindfelt, 1975; Ratnakumari et al., 1985; Murthy and Hertz, 1988) 5. BCKA-DH activity is subjected to feed back inhibition by acetyl CoA (Randle et al., 1981) and 6. Non hepatic tissues, like muscle, release BCKA into blood/medium (Odessy and Goldberg, 1972).

It is postulated that in hyperammonemic states there might be an enhanced transamination of BCAA, due to increased availability of BCAA (enhanced transport) and ∝-KG (c.f. above discussion) and increased transaminase activity, resulting in the production of glutamate and BCKA. Initially these BCKA are metabolised and Ac CoA or succinyl CoA are formed. However, due to the suppression of MDH activity and malate-aspartate shuttle (c.f. above), adequate amounts of OAA may not be available for the condensation of Ac CoA or for the continuation of the TCA cycle. Hence, Ac CoA produced from BCKA or PYR would accumulate and exerts a feedback inhibition on BCKA-DH thus lowering the production of CO_2 and further processing of BCKA.

If this concept were to be true, then BCKA would accumulate in the system. Reamination of BCKA may be prevented by the removal of glutamate as glutamine and/or by efflux of BCKA. If these postulates were to be true, then it should be possible to demonstrate an increase in the content of BCKA in the subcellular fractions isolated from the brains of hyperammnoemic rats. Experiments were carried

out to test this hypothesis under conditions that were identical to those described for the oxidation of BCAA. The amount of BCKA in the assay system (tissue + medium) was estimated after their conversion to 2,4-DNPhydrazones and extraction into cyclohexane.

In normal animals, amounts of BCKA produced from all the three BCAA was more or less the same in the mitochondria and synaptosomes (Table 5.3).

TABLE 5.3

LABELLED KETO ACID CONTENT IN SUBCELLULAR FRACTIONS ISOLATED FROM NORMAL AND HYPERAMMONEMIC RATS.

FRACTION		α-KIC	α-KMV	α-KIV
MITOCHONDRIA	NORMAL	1.68±0.5(5)	2.15±0.3(8)	1.80±0.4(6)
	EXPTL	1.70±0.6(5)	2.67±0.5(5)	2.20±0.6(6)
SYNAPTOSOMES	NORMAL	1.26±0.3(4)	2.37±0.1(6)	1.19±0.3(8)
	EXPTL	2.99±0.5(7)*	3.74±0.37(6)*	3.93±0.51(5)*

Values are expressed as Mean±S.D. Content is expressed as nmoles of [^{14}C]BCKA/mg protein/hr. *p<0.001.

Following the administration of an acute dose of ammonium acetate, the contents of the three BCKA i.e. β-KIC, α-KIV, and α-KMV were enhanced significantly in the synaptosomal fraction. The magnitude of elevation in α-KIC and α-KIV was higher than that of α-KMV. However, in the mitochondrial fraction contents of α-KIC, α-KIV and α-KMV remained unaltered in hyperammonemia.

Increase in the contents of all the three keto acids in the synaptosomal fraction corroborates with the suggested postulate. However, unaltered concentrations of the three BCKA in the mitochondria is not in accordance with the proposal that the transamination of these amino acids is enhanced while the decarboxylase is suppressed in brain in hyperammonemic states.

It is quite possible that the keto acids are formed in mitochondria are in excess of their utilisation and instead of being accumulated in the organelle they may be transported out so that their contents inside the organelle would decrease or be unaltered. To verify this possibility mitochondria and synaptosomes were incubated with each BCAA seperately under the conditions mentioned in the above two experiments. Incubation was terminated by centrifuging the tubes to seperate the tissue and medium. BCKA were extracted as 2,4-DNPhydrazones and were determined in the pellet and supernatant (Table 5.4).

These results indicate that in the mitochondrial pellet there is no change in the contents of α-KIC and α-KIV but there was an increase in the contents of α-KMV. In the medium in which the mitochondria were incubated, the content of all the three keto acids were enhanced. In the synaptosomal pellet, the contents of α-KIV and α-KMV decreased, while that of α-KIC increased. In the medium, in which the synaptosomes were incubated, there is a statistically significant increase in

TABLE 5.4

BCKA PRODUCTION FROM LABELLED BCAA IN SUBCELLULAR FRACTIONS ISOLATED FROM NORMAL AND HYPERAMMONEMIC RATS.

FRACTION		∝-KIC	∝-KMV	∝-KIV
		SYNAPTOSOMES		
PELLET	NORMAL	120±030(3)*	060±009(3)++	220±050(3)**
	AMM.AC	520±100(4)	043±008(3)	080±025(3)
SUPER-NATANT	NORMAL	1200±300(3)**	2250±160(3)+	1100±130(3)**
	AMM.AC	2800±220(3)	3700±540(3)	3210±420(3)
		MITOCHONDRIA		
PELLET	NORMAL	120±040(2)	110±030(4)**	190±030(3)
	AMM.AC	140±040(4)	680±110(4)	160±030(4)
SUPER-NATANT	NORMAL	1500±175(2)++	1700±500(4)++	1810±600(4)+
	AMM.AC	2800±500(5)	2850±400(4)	2700±300(4)

Values are Mean±S.D. BCKA content is expressed in pmoles /mg protein/hour in the pellet and in nmoles/mg protein/hr. in the supernatant. Number in parenthesis indicate the number of determinations. **p<0.001, *p<0.005, ++p<0.025, +p<0.01.

the contents of all the three BCKA. In general, these results indicate an increase in the efflux of these keto acids fron the organelles into the medium during hyperammonemia. This is in accordance with the suggestion that the keto acids are formed far in excess of their utilisation and are accumulated due to the inactivation of their metabolism and these keto acids are transported from the organelles into the medium. Efflux of keto acids may be by a carrier mediated mechanism as the presence of such a carrier in the mitochondria has already been reported (Mackay and Robinson,1981: Hutson and Rannels, 1985). Moreover, the increased keto acid content in

the medium indicate enhanced transamination resulting in an increased production of glutamate from BCAA in hyperammonemia.

As was mentioned earlier studies with subcellular fractions do not provide the precise cellular localisation of this process hence it will be interesting to study whether these changes occur in the cells in hyperammonemia.

METABOLIC STUDIES IN CELLULAR FRACTIONS AND THEIR RESPONSE TO HYPERAMMONEMIA

Preliminary studies have indicated that all the three cell types i.e., neurons, astrocytes and oligo cells are capable of producing CO_2 from BCAA. The rate of production of CO_2 is linear with respect to the amount of cell protein added and for routine assays, 100 µgms of astroglial or neuronal protein was added while for oligo cells only 12 µgms of protein was used per assay (Fig 5.3). A linear relationship was observed between the amount of CO_2 produced and the time of incubation upto 1hr (Fig 5.4). Hence, 30min was chosen as the time of incubation for routine assays. The relationship between substrate concentration and CO_2 was not established as mentioned earlier, as the process involves more than one reaction, such as transport and the activities of enzymes of BCAA metabolism and the TCA cycle. A fixed concentration of 100 µM BCAA was used throughout. The amount of CO_2 produced is expressed both per mg protein and per cell. The reasons for expressing the activity in these two modes has already been discussed.

In the neuronal perikarya isolated from normal animals greater amount of CO_2 was produced from valine, than for isoleucine or leucine. Irrespective of the mode of expression of activity a similar profile was obtained in astrocytes and oligo cells. Comparison of the amount of CO_2 produced from each amino acid by the different cell types revealed that the

oligo cells are more active in oxidising all the three BCAA than astrocytes and neurons. There were no statistically significant differences in the amount of CO_2 produced from BCAA by neurons and astrocytes except in the case of valine where neurons were much more active than astrocytes (Table 5.5).

TABLE 5.5

PRODUCTION OF $^{14}CO_2$ FROM LABELLED BCAA IN DIFFERENT CELLULAR FRACTIONS IN THE BRAIN OF NORMAL RATS.

FRACTION	LEUCINE	VALINE	ISOLEUCINE
	nmoles $^{14}CO_2$/mg protein/hr.		
NEURONS	0.60±0.09(6)	4.56±0.09(7)	1.52±0.08(5)
ASTROCYTES	0.55±0.08(6)	2.68±0.38(6)	1.23±0.60(6)
OLIGOCELLS	2.70±0.80(5)	4.80±0.80(6)	2.50±0.34(4)
	pmoles $^{14}CO_2$/cell/hr. X 10^{-3}		
NEURONS	0.24±0.03(5)	1.80±0.30(5)	0.59±0.12(6)
ASTROCYTES	0.34±0.05(6)	1.62±0.20(6)	0.63±0.06(4)
OLIGOCELLS	0.50±0.10(4)	0.81±0.12(6)	0.42±0.05(4)

Values are Mean±S.D. Number in the parenthesis indicate the number of determinations.

Production of CO_2 from ^{14}C-BCAA in neurons and oligocells is in accordance with the results obtained on the enzyme and transport systems in the present study. This also negates the possibility of glial contribution to the CO_2 production in the synaptosomes. As was mentioned, these enzymes may be synthesised in the neuronal perikarya and transported to the nerve terminals by axonal transport. The present observation on the neuronal involvement in BCAA

metabolism is in accordance with the reports of Murthy and Hertz (1987a,b) in the primary cultures of neurons. These authors also observed that the neurons are capable of oxidising both [1-^{14}C]- and [U-^{14}C]-BCAA. Differences in the rates may be due to the differences in the assay conditions and the cells used. The role of oligo cells in the BCAA metabolism has not been reported so far despite the fact that leucine carbon is incorporated into myelin lipids (Wiggins et al.,1979). Higher rates of CO_2 production in the oligo cells are in accordance with the greater activity of transaminases in the brainstem and also with the fact that the Ac CoA generated from the BCAA in these cells may be incorporated into lipids and also suggest that they might have a significant role in the metabolism of BCAA than astrocytes or neurons. However, their net contribution to cerebral BCAA metabolism may be less than the other two cell types as oligo cells are few in number. In astrocytes and neurons, carbons from BCAA may be incorporated into the amino acids of the glutamate family or it may be oxidised in the TCA cycle. It must be mentioned, however, that not all these observations are in accordance with the postulates of metabolic compartmentation.

PRODUCTION OF CO_2 FROM BCAA IN CELLULAR FRACTIONS IN HYPERAMMONEMIC STATES

In neuronal perikarya isolated from brains of hyperammonemic rats, production of $^{14}CO_2$ from [U-^{14}C]leucine was completely suppressed. With [U-^{14}C]isoleucine as substrate

TABLE 5.6

PRODUCTION OF $^{14}CO_2$ FROM LABELLED BCAA BY VARIOUS CELLULAR FRACTIONS FROM BRAINS OF NORMAL AND HYPERAMMONEMIC RATS.

CONDITION		LEUCINE	VALINE	ISOLEUCINE
Activity expressed as nmoles/mg protein/hr.				
NEURONS	NORMAL	0.60±0.09(6)	4.56±0.09(7)*	1.52±0.08(5)
	AMM.AC	N.D.(4)	0.63±0.10(5)*	1.31±0.17(5)
ASTRO-CYTES	NORMAL	0.55±0.08(6)*	2.68±0.38(6)	1.23±0.60(6)*
	AMM.AC	1.08±0.07(5)*	2.14±0.68(4)	0.30±0.10(5)*
OLIGO-CELLS	NORMAL	2.70±0.80(5)*	4.80±0.80(6)*	2.50±0.34(4)
	AMM.AC	1.35±0.35(4)*	2.64±0.50(4)*	0.50±0.10(4)
Activity expressed as pmoles/cell/hour X 10^{-3}				
NEURONS	NORMAL	0.24±0.03(5)*	1.80±0.30(5)*	0.59±0.12(6)
	AMM.AC	N.D.(4)*	0.39±0.04(6)*	0.72±0.11(4)
ASTRO-CYTES	NORMAL	0.34±0.05(6)*	1.62±0.20(6)	0.63±0.06(4)*
	AMM.AC	0.85±0.05(5)*	1.50±0.30(4)	0.20±0.04(4)*
OLIGO-CELLS	NORMAL	0.50±0.10(4)*	0.81±0.12(6)*	0.42±0.05(4)*
	AMM.AC	0.18±0.04(4)*	0.35±0.06(4)*	0.06±0.02(4)*

Values are Mean±S.D. Number in parenthesis indicate the number of experiments. N.D. Not detectable. *-$p<0.001$

there was a small but significant decrease in the rate of $^{14}CO_2$ production. A similar decrease in $^{14}CO_2$ production was also seen with [U-^{14}C] valine as substrate. The above mentioned changes were observed irrespective of the mode of expression except for isoleucine where there was no change in the rate of CO_2 production (Table 5.6). In contrast to neuronal perikarya, oxidation of [U-^{14}C]leucine was enhanced in astrocytes isolated from hyperammonemic rats. The decrease

in CO_2 production from [U-^{14}C]isoleucine observed in these cell preparations is similar to that of neurons though of a higher magnitude. Unlike the neuronal perikarya $^{14}CO_2$ production with valine as substrate remained unaltered. Once again these changes were unchanged irrespective of the mode of expression of activity.

The profile of changes observed in the oxidation of [U-^{14}C]BCAA in oligo cells was more or less similar to that of neuronal perikarya. The only exception is the higher magnitude of suppression in the production of $^{14}CO_2$ from [U-^{14}C] isoleucine. The observed suppression of CO_2 production from BCAA in the cellular fractions(except in few cases) is similar to that obtained in the subcellular fractions especially mitochondria and synaptosomes. These results are in accordance with the reports of Murthy and Hertz (1987a,b) with astrocytes and neurons in primary cultures. The report of Shiota (1984) on the enhancement of CO_2 fron leucine is in agreement with the enhanced oxidation of leucine in astrocytes isolated from hyperammonemic rats. The earlier suggestion that the suppression of CO_2 production due to their efflux was established by measuring the keto acid content in the medium and tissue. This argument could not be applied to the cellular fractions as the activity of the transaminases are suppressed in hyperammonemia. In fact, this could be the reason for the fall in the CO_2 production from labelled BCAA. If this were to be true the keto acid content should not

change or should decrease on incubation of the cellular fractions with BCAA. Such a situation would not support the increased transport of these amino acids into the cellular fractions. In order to clarify this issue total keto acid content and the content of keto acids in the tissue and medium were measured.

TABLE 5.7
TOTAL BRANCHED CHAIN KETO ACID CONTENT IN VARIOUS CELLULAR FRACTIONS ISOLATED FROM NORMAL AND EXPERIMENTAL RATS.

FRACTION		∝-KIC	∝-KMV	∝-KIV
		nmoles keto acid/mg protein/hr.		
NEURONS	NORMAL	0.99±0.16(4)*	1.26±0.16(4)	0.68±0.07(4)
	EXPERIMENTAL	1.32±0.18(4)*	1.62±0.23(4)	0.74±0.04(4)
ASTROCYTES	NORMAL	1.23±0.19(4)**	1.82±0.09(4)**	0.87±0.08(4)
	EXPERIMENTAL	1.89±0.14(4)**	1.3±0.16(4)**	0.87±0.04(4)
OLIGOCELLS	NORMAL	1.30±0.33(4)	0.87±0.05(4)	1.48±0.21(4)+
	EXPERIMENTAL	1.25±0.15(4)	1.27±0.11(4)++	1.05±0.03(4)+
		pmoles of keto acid/cell/hr.X10^{-4}		
NEURONS	NORMAL	3.88±0.60(4)++	4.94±0.64(4)**	2.65±0.27(4)
	EXPERIMENTAL	7.38±1.01(4)++	9.08±1.28(4)**	4.15±0.25(4)++
ASTROCYTES	NORMAL	7.53±1.16(4)	11.2±0.53(4)	5.30±0.45(4)**
	EXPERIMENTAL	14.9±1.14(4)++	10.3±1.28(4)	6.89±0.32(4)**
OLIGOCELLS	NORMAL	2.15±0.16(4)	1.43±0.09(4)*	2.43±0.43(4)
	EXPERIMENTAL	1.62±0.21(4)	1.66±0.13(4)*	1.37±0.04(4)++

Number in parenthesis indicate the number of experiments.
**p<0.005, *p<0.05, ++p<0.001, +p<0.01. Values are Mean±S.D.

In the neuronal perikarya the total keto acid content as well as the content in tissue and medium were enhanced with leucine as substrate. With isoleucine as substrate there was

TABLE 5.8

EFFLUX OF BRANCHED CHAIN KETO ACIDS FROM DIFFERENT CELLULAR FRACTIONS ISOLATED FROM NORMAL AND EXPERIMENTAL ANIMALS.

FRACTION		∝-KIC	∝-KMV	∝-KIV
		nmoles of BCKA/mg protein/Hr;		
NEURONS				
CONTROL	PELLET	0.02±0.004(4)	0.04±0.004(4)	0.02±0.007(4)
EXPTL.		0.03±0.004(4)	0.05±0.009(4)	0.02±0.005(4)
CONTROL	SUPER-	0.98±0.16(4)	1.23±0.17(4)	0.61±0.10(4)
EXPTL.	NATANT	1.29±0.18(4)	1.58±0.24(4)	0.67±0.05(4)
ASTROCYTES				
CONTROL	PELLET	0.04±0.006(4)	0.056±0.009(4) *	0.028±0.004(4)
EXPTL.		0.06±0.012(4) *	0.035±0.01(4)	0.023±0.005(4)
CONTROL	SUPER-	1.15±0.204(4) **	1.770±0.092(4) **	0.835±0.070(4)
EXPTL	NATANT	1.82±0.140(4)	1.27±0.164(4)	0.845±0.041(4)
OLIGOCELLS				
CONTROL	PELLET	0.033±0.007(4)	0.015±0.005(4) **	0.043±0.006(4) **
EXPTL		0.037±0.01(4)	0.035±0.005(4)	0.026±0.004(4)
CONTROL	SUPER-	1.13±0.16(4)	0.85±0.11(4) **	1.23±0.19(4)
EXPTL	NATANT	1.23±0.14(4)	1.24±0.12(4)	1.02±0.04(4)
		PMOLES OF BCKA/CELL/HR.; SUPERNATANTX10^{-4}; PELLETX10^{-6}		
NEURONS				
CONTROL	PELLET	7.72±1.47(4) **	15.6±1.40(4)	6.06±2.70(4)
EXPTL		14.6±2.00(4)	26.5±5.00(4) +	8.8±2.75(4)
CONTROL	SUPER-	3.81±0.60(4) **	4.79±0.65(4) **	2.39±0.41(4) **
EXPTL.	NATANT	7.20±1.00(4)	8.84±1.30(4)	3.90±0.30(4)
ASTROCYTES				
CONTROL	PELLET	23.5±3.28(4)	34.2±5.74(4)	17.2±2.59(4)
EXPTL.		46.1±9.20(4) +	28.0±7.63(4)	18.3±4.18(4)
CONTROL	SUPER-	7.04±1.24(4) **	10.8±0.57(4)	4.63±0.70(4)
EXPTL	NATANT	14.5±1.09(4)	10.2±1.14(5)	6.7±0.33(4) ++
OLIGOCELLS				
CONTROL	PELLET	5.46±1.10(4)	2.42±0.80(4)	7.18±1.03(4)
EXPTL.		4.18±0.93(4)	4.60±0.68(4) ++	3.41±0.56(4) ++
CONTOL	SUPER-	1.87±0.25(4)	1.41±0.18(4)	2.05±0.30(4)
EXPTL	NATANT	1.60±0.17(4)	1.62±0.15(4) †	1.34±0.05(4) ++

Values are Mean±S.D. Number in parenthesis indicate the number of experiments. Exptl-experimental. **p<0.005, *p<0.025, ++p<0.001, +p<0.01, †p<0.05.

an enhanced total keto acid content when expressed per cell but remained unaltered when expressed per mg protein. A similar result was obtained with valine as substrate. In astrocytes isolated from hyperammonemic rats the total keto acid content in the pellet and medium was enhanced with leucine as substrate. With isoleucine as substrate, it was observed that the keto acid content decreased when expressed per mg protein but remained unaltered when expressed per cell. The keto acid content was unchanged with valine as substrate when expressed per mg protein but was enhanced when expressed per cell.

In the oligo cells when leucine was used as substrate the keto acid content was unaltered irrespective of the mode of expression. With isoleucine as substrate there was an enhanced keto acid content in the tissue and medium while with valine there was a fall in the keto acid content. These results on the keto acid content apparently show some discrepancy depending upon the mode of expression. For eg. in neurons with valine as substrate the keto acid content per mg protein remained unchanged while a significant increase was observed when expressed per cell. Similarly in astrocytes, with isoleucine as substrate, a decrease was observed when the keto acid content was expressed per mg protein but remained unaltered when expressed per cell. However, in the oligo cells no such discrepancies were observed. The reason for such discrepancies might be due to the changes in the

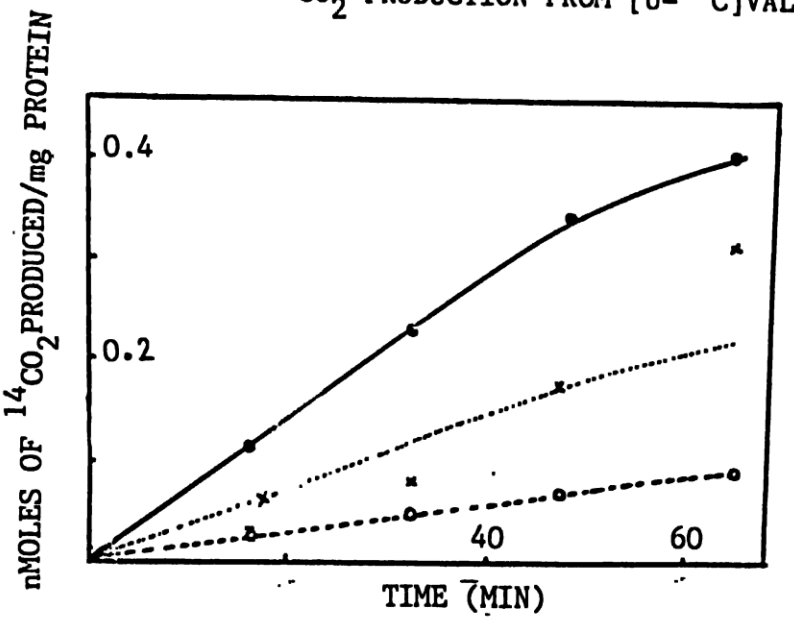
protein content as there was a statistically significant increase in protein contents of neurons and astrocytes in hyperammonemia whereas in the oligo cells there was a small and statistically insignificant decrease (Table 5.8). Any change in this parameter would give a lower keto acid content. This problem would not arise when the keto acid content is expressed per cell as the cell number is unaltered in hyperammonemic rats. It may be said that there is an increase in the keto acid content in the astrocytes and neurons isolated from hyperammonemic rats when they are incubated with BCAA. The apparent decrease noticed in the oxidation or CO_2 production under certain conditions may be due to the non-availability of the keto acid to the decarboxylase. The enhanced keto acid content may be due to increased transamination of BCAA, atleast in astrocytes and neurons in hyperammonemia. As a result of this, there may be an increase in the formation of glutamate by the transamination of these amino acids. Moreover, as was suggested for the subcellular fraction, the accumulated keto acids may be transported into the blood rather than being decarboxylated.

It has been repeatedly been emphasised that utilisation of the BCKA and their oxidation in the TCA cycle might support the energy metabolism in hyperammonemia. Efflux of keto acids from the cell, as is observed in the present study, is not in accordance with the role of BCKA in cerebral energy metabolism. This is understandable as the primary requirement

of the brain in hyperammonemic state is the production of glutamate for glutamine biosynthesis. The energy required for the synthesis of glutamine may be derived from the oxidation of glucose rather than BCKA. This because of the fact that leucine generates AcCoA, for the oxidation of which OAA is required. It has been repeatedly demonstrated that the malate-aspartate shuttle is suppressed in hyperammonemic states due to the lack of OAA. Hence oxidation of leucine requires additional OAA which would further strain the malate-aspartate shuttle. Similarly, valine oxidation produces succinyl CoA, the metabolism of which result in the production of malate. As MDH is suppressed in hyperammonemic states (Ratnakumari et al., 1986) malate would be accumulated instead of participating in the TCA cycle. This might upset the operation of the malate-aspartate shuttle by promoting malate efflux from the mitochondria which is the reverse of the malate-aspartate shuttle. Under such conditions, the required energy for the synthesis of glutamine is to be derived from the oxidation of glucose. This notion is supported by the unaltered levels of ATP, unchanged rates of glucose and PYR oxidation and enhanced activity of TCA cycle enzymes except MDH. Efflux of BCKA, rather than their accumulation , prevents their reamination. Moreover, it is well known that BCKA in large concentrations have adverse effects on the metabolism, such as inhibition of PYR oxidation (Land and Clark, 1974).

These studies suggest that the primary function of BCAA is to provide glutamate for the production of glutamine and this glutamate is derived chiefly by transamination not by oxidation.

TIME COURSE OF $^{14}CO_2$ PRODUCTION FROM [U-^{14}C]VALINE



O ——— O CYTOPLASM ● ——— ● MITOCHONDRIA

X ····· X SYNAPTOSOMES

FIG 5.2

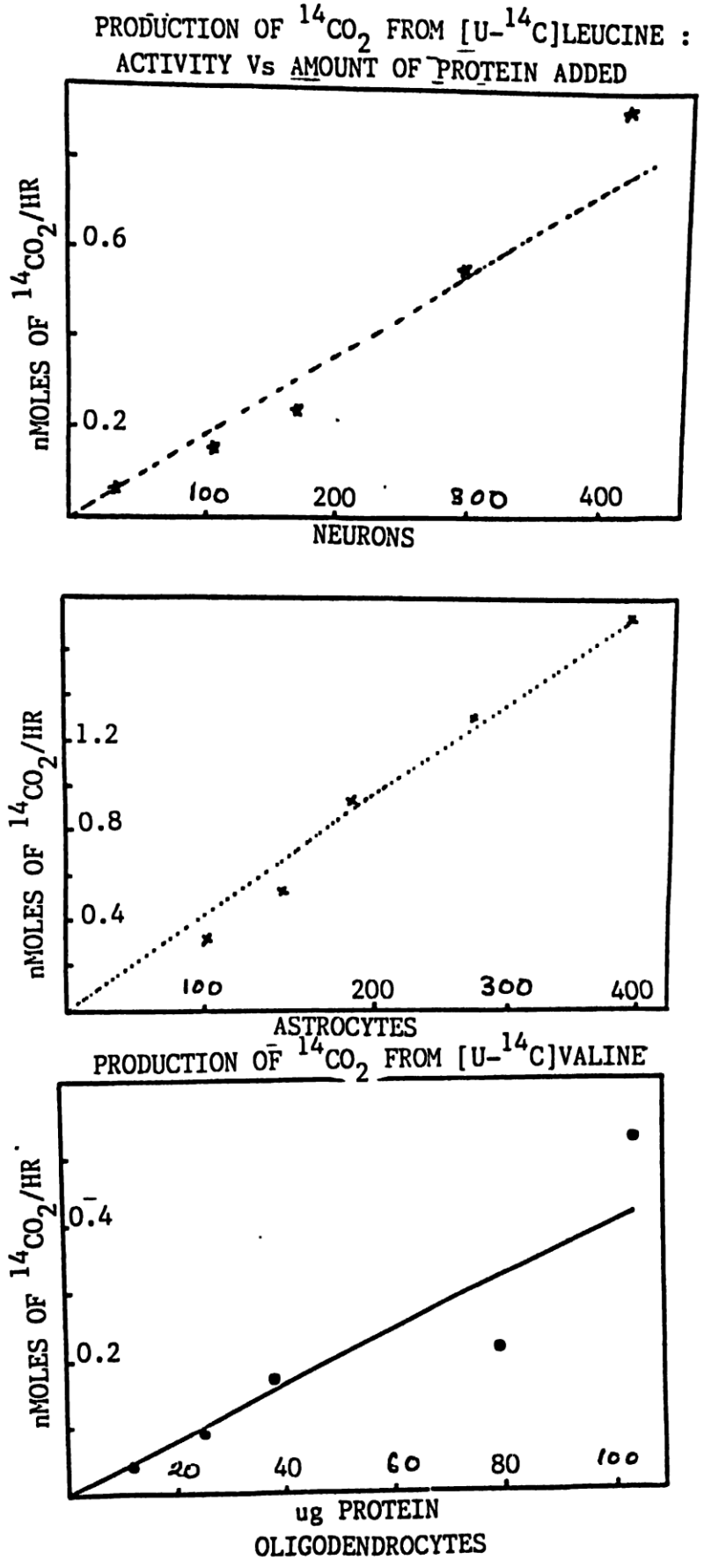
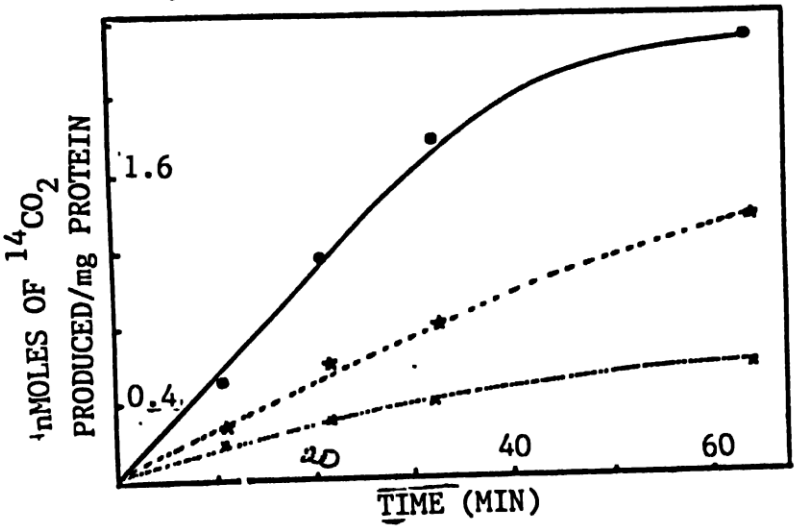

PRODUCTION OF $^{14}CO_2$ FROM [U-^{14}C]LEUCINE :
ACTIVITY Vs AMOUNT OF PROTEIN ADDED
nMOLES OF $^{14}CO_2$/HR
0.6
0.2
100
200
300
400
NEURONS
nMOLES OF $^{14}CO_2$/HR
1.2
0.8
0.4
100
200
300
400
ASTROCYTES
PRODUCTION OF $^{14}CO_2$ FROM [U-^{14}C]VALINE
nMOLES OF $^{14}CO_2$/HR
0.4
0.2
20
40
60
80
100
ug PROTEIN
OLIGODENDROCYTES

FIG 5.3

TIME COURSE OF $^{14}CO_2$ PRODUCTION FROM
[U-^{14}C]BRANCHED-CHAIN AMINO ACIDS IN ASTROCYTES



● —— ● [U-^{14}C]VALINE

* - - - - * [U-^{14}C]ISOLEUCINE X ····· X [U-^{14}C]LEUCINE

FIG 5.4

SUMMARY

SUMMARY

1. Activities of BCAA-T and BCKA-DC along with the uptake, oxidation of BCAA and production of BCKA were studied in different preparations of brains of normal and hyperammonemic rats.
2. Hyperammonemia was induced by administration of an acute dose of ammonium acetate (2.5 mmoles/100 gm body weight) or MSO (30 mg/100gm body wt.) or a subacute dose of MSO (15mg/100 gm body wt.). In addition, partial hepatectomy was performed in a seperate group of animals to induce hyperammonemia.
3. In the brain and serum of animals subjected to the above treatments, there was an increase in ammonia content, indicating the patency of the method.
4. Homogenates were prepared from six different regions of the brain of normal and hyperammonemic rats and BCAA-T activity was determined. In general there was an increase in the activity of this enzyme in the brains of animals subjected to partial hepatectomy and animals administered with ammonium acetate. Based on these results it is suggested that the glutamate formed in the BCAA-T reaction would be utilised for the synthesis of glutamine in hyperammonemic states.
5. To check the validity of the above argument, hyperammonemia was induced with MSO (which blocks GS). In the animals administered with an acute dose of MSO activity

levels of this enzyme were unaltered suggesting a relationship between the synthesis of glutamine and the increase in BCAA-T activity.However, in animals injected with a subacute dose of MSD, there was a mild decrease in the activity levels of BCAA-T which may be due to the recovery of glutamine synthesis.

6. Subcellular fractions were isolated from brains of normal and hyperammonemic rats. Studies on subcellular distribution indicated that BCAA-T enzyme is present in the cytosol, mitochondria and synaptosomes. Activity of this enzyme was elevated in all these three fractions in animals administered with an acute dose of ammonium acetate. Results obtained for this enzyme in the subcellular fraction isolated from rats treated with MSD were similar to those described earlier. (c.f. 5).

8. In addition to the transaminase activity levels of BCKA-DC were also determined in the subcellular fractions isolated from normal and hyperammonemic animals. In both these groups measurable activity was observed in all the three subcellular fractions. In hyperammonemic states activity of this enzyme was enhanced in the mitochondria, cytosol and synaptosomes.

9. Three different cell types i.e., astrocytes, neurons and oligo cells were isolated from normal and hyperammonemic rats. Purity of these preparation was assesed by light microscopic examination.

10. Activities of BCAA-T and BCKA-DC were determined in these cellular fractions in normal and hyperammonemic states. Activity levels were expressed per mg protein and per cell. In the astrocytes and neurons, the BCAA-T activity was suppressed while that of BCKA-DC was elevated in hyperammonemic states.
11. Studies on the transport of BCAA indicated that these amino acids are transported by a saturable carrier mediated mechanism into synaptosomes, mitochondria, neurons, astrocytes and oligo cells. This phenomenon was observed to increase in all the cellular and subcellular fractions in hyperammonemic states.
12. Production of $^{14}CO_2$ and ^{14}C-BCKA from [U-^{14}C]BCAA was studied in the cellular and subcellular fractions isolated from normal and hyperammonemic rats.
13. In hyperammonemic states, production of $^{14}CO_2$ from [U-^{14}C]BCAA was suppressed in the cytosol, mitochondria and synaptosomes. However, the content of [U-^{14}C]-BCKA was enhanced in synaptosomes but not in the mitochondria isolated from brains of hyperammonemic rats. It was observed that the efflux of these keto acids from synaptosomes and mitochondria into medium was enhanced in hyperammonemic states.
14. In the cellular fractions $^{14}CO_2$ was produced from [U-^{14}C]BCAA in the astrocytes, neurons and oligocells indi-

cating that all these cell types are capable of metabolising BCAA. In hyperammonemic conditions, the protile of changes observed in the production of $^{14}CO_2$ and [^{14}C] BCKA from [U-^{14}C]BCAA was more or less similar to those observed in the subcellular fractions.

CONCLUSIONS:

Results obtained in the present study suggest that brain has the capacity to metabolise BCAA. As a result of this, glutamate may be generated from BCAA. These studies also suggest that the major cellular elements of the brain i.e., astrocytes, neurons and oligo cells, participate in the metabolism of these amino acids. Further, it is also suggested that the rate of transamination of these amino acids is far in excess of the rate of utilisation of the keto acids and as a result these keto acids are either accumulated or transported into a different subcellular/cellular compartment. They may also be transported across the BBB from the brain into blood. In the latter case these keto acids will be oxidised in the liver. These results also indicate that though the rate of metabolism per cell is high in the oligo cells compared to the other two cell types, their net contribution to the cerebral metabolism of BCAA will be lesser than that of astrocytes or neurons as they are few in number in adult brain. These results do not exclude the possibility of incorporation of the carbons of BCAA into glutamate family of amino acids but they suggest that this would be lesser than

the amount of BCAA nitrogen incorporated into the glutamate family of amino acids. It is also suggested that under normal physiological conditions this process would be limited by the availability of the BCAA to brain. The major rate limiting factor would be the transport of these amino acids across the BBB which is regulated not only by the content of the amino acids in the blood, but also by the relative amounts of other neutral amino acids such as phenylalanine, tyrosine, tryptophan and methionine.

In hyperammonemic states, there is an increase in metabolism of BCAA, particularly the transamination and as a result more amount of glutamate will be generated from BCAA under these conditions. The glutamate so formed may be utilised for the synthesis of glutamine. As a result of enhanced transamination the production of BCKA is also increased. Further metabolism of BCKA is, however, suppressed in hyperammonemic states. It is suggested that the suppression of BCKA metabolism in hyperammonemic states is beneficial as it would spare the much needed OAA for the operation of the malate-aspartate shuttle. However, this would result in the accumulation of the BCKA which is known to have deleterious effects on brain metabolism (as seen in Maple Syrup Urine Disease). Under these conditions there might be an enhanced efflux of these keto acids from the brain. These results, suggest that in hyperammonemic states

the glutamate required for the synthesis of glutamine might be produced by the transamination of BCAA. The fall in the glutamate content, inspite of its production from BCAA in hyperammonemic states, might be due to the limited availability of BCAA. As a result of enhanced utilisation of BCAA their content decreases in the plasma and alters (increases) the ratio of AAA/BCAA in the blood in hyperammonemia. This would further limit the availability of BCAA to the brain under these conditions. However, on BCAA infusion, as therapeutic measure, the ratio of AAA/BCAA would decrease and thus enhance the availability of these amino acids to the brain. This may be probably one of the mechanisms operative in the therapeutic action of BCAA in hyperammonemia.

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