CELLULAR AND SUBCELLULAR TRANSPORT AND METABOLISM OF BRANCHED-CHAIN AMINO ACIDS IN YOUNG. ADULT AND AGED BAT BRAIN

A THESIS SUBMITTED FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

BY

K. VERKATA RAMA RAO

DEPARTMENT OF ANIMAL SCIENCES
SCHOOL OF LIFE SCIENCES
UNIVERSITY OF HYDERABAD
HYDERABAD - 500 046
INDIA

1995 JUNE

ENROLMENT No. LL-2947

DEPARTMENT OF ANIMAL SCIENCES
SCHOOL OF LIFE SCIENCES
UNIVERSITY OF HYDERABAD
HYDERABAD

Dated 22 June 95'

DECLARATION

I, K. VENKATA RAMA RAO, declare that the work presented in my thesis has been carried out by me under the supervision of Dr. Ch. R. K Murthy, and has not been submitted for any degree or diploma of any other University.

K. Venkata Rama Rao
Candidate

Dr. Ch. R. K Murthy,
Supervisor

DEPARTMENT OF ANIMAL SCIENCES
SCHOOL OF LIFE SCIENCES
UNIVERSITY OF HYDERABAD
HYDERABAD

Dated -22/6/95

CERTIFICATE

This is to certify that Mr. K, Venkata Rama Rao, has carried out the research work embodied in the present thesis entitled " Cellular and Subcellular Transport and Metabolism of Branched-Cham Amino Acids in Young. Adult and Aged Rat Brain" under my supervision and guidance for the full period prescribed under the Ph.D ordinance of this University. I recommend his thesis for the submission for the degree of Doctor of Philosophy of this University.

Dr. Ch. R. K Murthy
Supervisor

Dr. Ch. R. K. Murthy
Head, Department of Animal Sciences

22.6.95
Prof. A. R Reddy
Dean, School of Life Sciences

THIS WORK IS

DEDICATED

TO

MY

MOTHER, FATHER AND UNCLE.

ACKNOWLEDGEMENTS

It is a great pleasure for me to record my deep sense of gratitude to Dr. Ch. R. K Murthy, for giving me an opportunity to cany out my research in the fascinating field of brain research. His constant encouragement, stimulating discussions, valuable suggestions, critical analysis of the results and up on that his immense patience really helped me gain insight into my research field. I sincerely thank him for his care, advice and help in both academic and non-academic matters.

I am grateful to Dr. Mohan. C. Vemuri, for his advice and also for providing me financial assistance in the form of ICMR-SRF during my critical period of stay in the school.

I am grateful to Prof. A. R Reddy, Dean, School of Life Sciences, Prof. P. R. K Reddy and Prof. N. C Subramanyam former Deans of school of Life Sciences for providing me necessary facilities for this work.

I am also thankful to Dr. Aparna Dutta-Gupta for her constant encouragement and help.

1 wish to thank Prof. T. Suryanarayana, Dr. R.V Thampan and Dr. M N. V Prasad for their encouragement during my stay in the school.

I sincerely thank Mr. Pattabhi Raman, for his help in many ways.

I sincerely thank my colleagues Dr. Raghavendra Rao, *Dr.* Ratna Kumari, Reeba, Rukmini, Shanavas, Jyothi, Dr. Uma and Narasa Raju for their help and ad vice.

I wish to thank my friends Mr. K. S. G Haviryaji, Dr. K Murali Krishna, Mr. V. Srinivas, Dr. Varsha, Dr. Narasaiah, Dr. K. L. N Rao, Mr. Venkatesh. S, Mr. Raju, Mr. Kashyapa, Mr. Venkat, Mr. Naresh, Mr. Bhaskar, Dr. Prem Kumar, Ms. Nirmala, Mr. Karthikeyan, Dr. Anuradha, Dr. Kiran Kumar, Mr. R. Srinivas, Mr. K. Srinivas, Dr. Vijaya Bhaskar

Reddy, Dr. K. Seshagirrao, Dr. Narender, Dr. Ranga Rao, Dr. Giridhar Rao, Dr. Usha who made my stay memorable in this campus.

I am very much thankful to Mrs. Jayasree Murthy, for her constant affection towards me during my stay in campus.

I am deeply indebted to my parents, uncle and other members of my family for the encouragement given to me through out my career.

The financial assistance from UGC and ICMR, India is greatly acknowledged.

I thank Mr. Narasinga Rao for his assistance in the laboratory.

I sincerely thank the members of the non-teaching staff for their help.

-Rama Rao

CONTENTS

ABBREVIATIONS

CHAPTER 1 GENERAL INTRODUCTION AND SCOPE OF THE PRESENT INVESTIGATION

CHAPTER 2 MATERIALS AND METHODS

CHAPTER 3 TRANSPORT OF BRANCHED-CHAIN AMINO ACIDS

FIGURES OF TRANSPORT STUDIES

CHAPTER 4 MEIABOLISM OF BRANCHED-CHAIN AMINO ACIDS

FIGURES FOR METABOLIC STUDIES

SUMMARY OF THE THESIS

REFERENCES

PUBLICATIONS

ABBREVIATIONS

AAA	Aromatic Amino Acids
ATP	Adenosine Triphosphate
BBB	Blood Brain Barrier
BCAA	Branched-Chain Amino Acids
BCAA-T	Branched-Chain Amino Acid Aminotransferase
BCKA	Branched-Chain Ketoacids
BCKA-DH	Branched-Chain Ketoacid Dehydrogenase
BSA	Bovine Serum Albumin
CNS	Central Nervous System
GABA	y-Amino Butyric Acid
HEPES	N-2-Hydroxy Ethyl Piperazine N-2-Ethane Sulphonic acid
α-KG	α-Ketoglutaric Acid
α-KGDH	α-Ketoglutaric Acid Dehydrogenase
α-KIC	tt-Ketoisocaproic Acid
α-KMV	α-Keto-β-Methyl Valeric Acid
α-KIV	α-Keto-β-Methyl-Isovaleric Acid
LDH	Lactate Dehydrogenase
MDH	Malate Dehydrogenase
MSO	Mathionine Sulphoxamine
NAA	Neutral Amino Acids
NAD^+	Nicotinamide Adenine Dinucleotide (Oxidized)
NADH	Nicotinamide Adenine Dinucleotide (Reduced)
PDH	Pyruvate Dehydrogenase
POPOP	2-2'-P-Phenyl-bis (4-Methyl-5-Phenyloxazole)
PPO	2,5 Diphenyloxazole

TCA Cycle	Tricarboxylic Acid Cycle
TPP	Thiamine Pyrophosphate.

INTRODUCTION

Branched-chain amino acids, (BCAA; leucine, isoleucine and valine) are three of the eight essential amino acids, that are not synthesized in animal tissues. Hence, they are called essential amino acids and are indispensable. They are obtained from diet. BCAA comprise about 50% of the total essential amino acids in the tissue.

BCAA are given a special place in amino acid biochemistry due to the multi-functional roles performed by them in the health and disease of animals. Earlier, functional importance of BCAA was revealed in terms of their incorporation into proteins. This concept further gained importance by observations that neither growth nor positive nitrogen balance were maintained without adequate supply of diet having BCAA. This brought new dimensions in the treatment of certain diseases such as hepatic encephalopathy, muscle wasting disorders, sepsis, trauma and diabetes (Herlong et al., 1980, Sapir et al., 1983, Freud et al., 1978, Blackburn et al., 1979 and Brosnon et al., 1984).

Very small quantities of BCAA are found in a free state in the tissue, while large amounts are present as components of proteins. Dietary proteins are the major source of these amino acids although small amount of BCAA are derived from the degradation of proteins within the tissues. Ingested dietary proteins are degraded by intestinal proteases and free amino acids are released into the intestinal lumen. From the lumen, these amino acids are transported into portal blood by a carrier mediated transport that requires energy and sodium for its function (Wiseman 1968). Carriers for the transport of BCAA have been identified on the luminal side of the intestinal epithelia (Larsen et al., 1964). These carriers are called Neutral Amino Acid Carriers as they transport other neutral amino acids such as phenylalnine, tyrosine, tryptophan besides BCAA. All these amino acids

(including BCAA) compete for a common binding site on the carrier protein and both *in vivo* and *in vitro* competitive inhibitions were reported for the transport (Mathews & Lasten 1965; Adibi & Gray 1967). Thus the relative concentrations of each amino acid with respect to other neutral amino acids determines the rate of transport. This carrier has a very low affinity for neutral amino acids (Km ~ 20 mM) (Bronk & Leesel 1974) suggesting that it is saturated at very high concentrations of amino acids in the intestinal lumen. This is advantageous as the quantity and quality of food (with respect to neutral amino acids) consumed by animals is never consistent. If the need arises, such a carrier would be able to handle large amounts of neutral amino acids. Besides this, Meister, (1973) proposed that some of these amino acids might also be transported by γ-glutamyl cycle. Amino acids are transported through portal circulation (all the amino acids including neutral amino acids) to, liver which is the primary site for the catabolism of most of the amino acids. The unique feature in the catabolism of BCAA in animals is that the process is initiated in extra-hepatic tissues primarily in muscle. This is in contrast to the catabolism of other essential amino acids which occurs primarily in liver. This process might be a beneficial adaptation as it facilitates the maintenance of optimum levels of BCAA in peripheral blood for their utilization in vital metabolic reactions (such as protein synthesis) in other tissues (Krebs 1972).

In earlier years, BCAA were thought to be mainly utilized in the synthesis of tissue proteins. However, later studies indicated additional metabolic roles for BCAA in several tissues.

Metabolic roles of BCAA :

(I) Protein Synthesis and Degradation :

Branched chain amino acids, especially leucine and valine are

abundant constituents of tissue proteins. They are incorporated into primary structure of proteins and impart hydrophobicity to the peptide chain. Interaction between hydrophobic amino acid plays a pivotal role in protein conformation and in integrating the proteins into membranes.

In addition, substantial evidence exists to suggest that BCAA and their keto analogues have anabolic effects on protein metabolism in muscle and other peripheral tissues including brain. Experiments conducted primarily on rat muscle indicated that BCAA suppress protein degradation and stimulate protein synthesis (Buse & Reid 1975). *In vivo* studies on muscle also indicated an enhanced protein degradation and a decline in the total muscle protein content when the supply of BCAA was restricted. This was reported to occur especially in conditions of starvation, malnutrition etc., (Paul and Adibi, 1980). These studies confirmed the involvement of BCAA in protein metabolism.

(II) Glucose-Alanine Cycle :

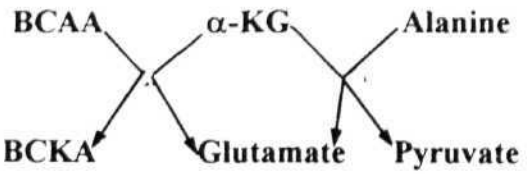
For the sustenance and survival, tissues like brain and RBC have absolute requirement for glucose. Though it is cunently believed that brain has the ability to use alternate metabolic fuels (such as ketone bodies, glutamate and BCAA), glucose is till considered as the primary substrate for cerebral energy metabolism (Hawkins et al., 1971).

Under conditions of starvation, glucose supply to brain would be lowered which adversely affects its function. Under such conditions, muscle metabolism of BCAA plays a pivotal role in sparing glucose to brain (Odessy et al., 1974). When blood glucose levels are lowered (as in starvation), hepatic gluconeogenesis is stimulated under the influence of hormones such as glucagon. Primary substrates for this process are alanine and glutamine. These amino acids are synthesized in skeletal muscle, re-

leased into blood and transported to liver for gluconeogenesis.

During starvation, muscle converts glucose to pyruvate which gives rise to alanine after undergoing transamination with glutamate.

To maintain a continuous supply of glutamate, muscle transaminates BCAA with α-KG. This results in the production of BCKA besides replenishing the tissue glutamate pools. This was evidently shown earlier by Felig and Wahren (1971), Manchester (1965). Some of this glutamate is converted to glutamine. Both glutamine and alanine along with BCKA are released into blood and carried to liver. In this tissue, carbon skeletons of alanine and glutamine are converted to glucose and their nitrogens are incorporated into urea. BCKA, supplied by muscle, are further metabolised either to CO_2 or to produce ketone bodies (from the BCKA of leucine and isoleucine). Glucose, synthesized in liver, is released into blood stream and are supplied to other tissues which depend exclusively on glucose. This cycle is called *glucose-alanine cycle.* Schematic diagram of the roles of BCAA in glucose-alanine cycle is shown below.



(III) Role of BCAA in Lipid Metabolism :

β-hydroxy-β-methyl-glutarylCoA, produced during the metabolism of leucine, might act as a precursor for the synthesis of cholesterol.

Several studies both in man and rats indicated that cholesterol is synthesized from leucine in tissues such as adipose tissue and muscle (Rosenthal et al., 1974), aorta (Stillway et al., 1977) and brain (Dopeshwarker et al., 1979; Stillway et al., 1978). In addition, end products BCAA metabolism such as acetyl CoA and propionyl CoA might serve as precursors for the aniso- and anteiso- fatty acids (Smith 1974).

(IV) Role of BCAA in the synthesis of Glutamate :

In brain, BCAA were also found to be involved in the synthesis of glutamate. Both carbons and nitrogens of BCAA were reported to be incorporated into glutamate family of amino acids (glutamate, aspartate, alanine, glutamine and GABA; Sadasivudu & Lajtha 1970). Yudkoff et al., (1983) reported that nitrogen of BCAA is incorporated into glutamate through transamination and 30% of brain glutamate might be derived through this process. Cremer et al., (1975) demonstrated that carbons of BCAA are also incorporated into glutamate family of amino acids. Though the carbons of leucine are incorporated into glutamate family of amino acids, this is not considered as net synthesis. This is because of the fact that acetyl CoA (produced from leucine) enters into TCA cycle. In the TCA cycle, carbons of acetyl CoA are distributed into various intermediates. As both α-KG and oxaloacetate are in equilibrium with amino acids of glutamate family, the labelled carbons (derived from acetyl CoA which in turn produced from leucine) are incorporated into glutamate family of amino acids. However, when isoleucine and valine are used as the substrates, then there is a net synthesis of glutamate family of amino acids. This is because of the fact that these amino acids generate succinyl CoA which are anaplerotically replenish the intermediates of TCA cycle.

Roles of BCAA in health and disease :

It is interesting to note that BCAA play a dual role in human health - i.e., they are implicated in the aetiology of diseases such as maple syrup urine disease and also involved in the therapy of certain diseases such as hepatic encephalopathy.

In nlaple syrup urine disease (MSUD), a congenital disorder of BCAA metabolism, activity of BCKA-DH is lower or defective in its functional activity in patients than normal subjects. As a result, BCKA formed during the transamination reaction, accumulate and exert toxic effects. BCKA, in large amounts are known to suppress the oxidation of pyruvate and α-KG. Under these conditions, BCKA also undergo transamination with aromatic amino acids, especially with phenylalanine, to form compounds such as phenyl pyruvate. Patients with MSUD exhibit various neurological symptoms including mental retardation (Nyhan, 1984).

In conditions of hepatic encephalopathy, surgical trauma and sepsis, BCAA were shown to have beneficial effects. In the conditions of hepatic encephalopathy, due to imbalance of insulin/glucagon ratio, blood levels of BCAA are decreased while those of aromatic amino acids (AAA) are elevated (Fischer and Baldessarini, 1976). This adversely affects the cerebral functioning as both BCAA and AAA are transported across BBB by the same carrier. Due to the increase in the AAA concentration in blood, these amino acids are transported in large amounts in brain. Ultimately, their concentration exceeds the Km of the hydroxylases for tyrosine, phenylalanine and tryptophan. Under such conditions, these amino acids are directly decarboxylated to form aromatic amines such as octopamine and phenyl ethylamine. These aromatic amines act as false neurotransmitters and displace the biogenic amines such as catecholamines from their nerve

terminals. Moreover, when released, they bind to the receptors of monoamines and mimic their action. These effects of false neurotransmitters are supposed to involve in the aetiology of cerebral dysfunction of hepatic encephalopathy. Keeping this mechanism in view, it was suggested that normalization of plasma levels of BCAA and restoration of ratio of BCAA/AAA would have beneficial effects. Indeed, such beneficial effects were observed upon the infusion of BCAA to patients with hepatic encephalopatliy. (Fischer and Baldessarini, 1976). Some investigators suggested that infusion of BCKA would be more beneficial than BCAA as it reduces the amino acid load on the tissue and promotes the synthesis of glutamate and glutamine (Walser, 1978; Fischer, 1981; Egberts et al., 1981; Blackburn et al., 1981; Freund et al., 1981). However, controversial results are reported in latter studies.

Tissue Metabolism of BCAA :

Transport as a prerequisite for metabolism:

As it was mentioned earlier that, BCAA are essential amino acids and are not synthesized in animal tissue. Hence, transport of these amino acids from the systemic circulation into various tissues is the sole mechanism for their availability to tissues. Survey of literature indicated that BCAA are transported into various tissues, including brain, by a carrier mediated mechanism which is common for almost all the tissues. However, the affinities and rates of transport of these carriers differ in different tissues. These amino acids are transported by a neutral amino acid carrier that transports not only BCAA, but also other neutral amino acids such as tyrosine, phenylalanine, glutamine with different affinities and rates (Sershan & Lajtha 1979, Christensen & Cullen 1969). This transport system called L-system (leucine preferring system) is a non-concentrative,

Saturable transport system that maintains a dynamic equilibrium between blood and tissue levels of these amino acids (Pajari, 1984).

Literature on hepatic transport of neutral amino acids, especially BCAA is not abundant due to the nature of the transport system. Studies on neutral amino acid transport in liver indicated that the carrier has very low affinity when compared to other tissues and requires very large amounts of amino acids to saturate the system (>100 mM) (Pardridge et al 1975). Moreover, this system is characterized by the absence of competitive inhibition among these amino acids (Mortimore, et al., 1972). As the amino acids derived from the diet are transported directly to portal blood, it was suggested that the portal blood is rich in several neutral amino acids including BCAA. However, their concentrations in the portal blood rarely reach to the level where it can saturate the liver carrier. Hence, in normal physiological conditions, BCAA transport into liver would be minimal as the carrier sites are never saturated. This would ensure minimal metabolism of BCAA in liver and acts as an additional regulatory mechanism to provide adequate amounts of BCAA to extra-hepatic tissues. Lack of competitive inhibitions by other neutral amino acids is also meaningful as such high concentrations of amino acids are not achieved (in portal blood) usually and the catabolism of neutral amino acids is known to occur primarily in liver (Lehninger, 1981). Moreover, transamination is the rate limiting reaction in liver. However, the second enzyme in the metabolism of BCAA i.e., BCKA- dehydrogenase was found to be highly active in liver. Hence, the keto acids produced in extra-hepatic tissues (large amounts from muscle) will be released into the blood. In liver, they are transported into mitochondria by a dicarboxylic acid carrier which also transports other keto acids such as pyruvate and α-KG (Makay

and Robinson. 1981). Since liver depends on other tissues, especially muscle, for the supply of BCKA, inter-organ interaction exists among liver and other extra hepatic tissues (Harper and Zapalowski, 1981). Accordingly. BCKA levels in blood would regulate the metabolism of BCAA in liver. In other words, higher levels of BCKA in blood (due to increased transamination in peripheral tissues) would stimulate the BCKA oxidation in liver. This is essential as the accumulation of BCKA in blood was found to exert deleterious effects.

Transport of BCAA and other neutral amino acid into muscle was reported to be mediated by a low affinity high capacity system as evidenced by the studies using α-aminoisobutyric acid, a marker for the low affinity transport system (Christensen, 1969,. Guoff et al., (1962) reported no mutual inhibition of BCAA transport in muscle in the presence of large doses *of* aromatic amino acids indicating non-competitive nature of transport. This may have a physiological significance as muscle is the site for initiating the catabolism of BCAA but not other neutral amino acids. It might enable muscle to transport and utilize BCAA even when their concentrations are low in blood plasma. This is particularly important in conditions where relative concentrations of these of amino acids in blood plasma are altered, e.g. hepatic encephalopathy.

In kidney, neutral amino acids were found to be transported by low affinity high capacity transport systems (Christensen and Cullen, 1969).

As mentioned earlier, BCAA are transported into brain across Blood Brain Barrier (BBB) by a neutral amino acid carrier. The physiological and pathological significance of such a transport has been already mentioned.

Enzymes of BCAA metabolism :

Initial reaction in the metabolic breakdown of BCAA is the transfer of amino group from BCAA to another keto acid usually a-KG. This process is mediated by BCAA-a-KG aminotnuisferase (BCAA-T) wherein the amino group of BCAA is transferred to a-KG. As a result, BCAA will be converted to their corresponding a-keto acids and a-KG becomes glutamate. Besides a-KG, pyruvate can also act as an amino group acceptor to become alanine at least in *in vitro* assay conditions. However, the affinity for pyruvate was reported to be lower than that of a-KG (Benuck et al., 1971). Thus a-ketoisocaproic acid, (α-KIC), α-keto-β methyl valeric acid (a-KMV) and α-ketoisovaleric acid (α-KIV) are generated from leucine, isoleucine and valine respectively (Lehninger 1981). As any other transamination reactions, this also requires pyridoxal-5-phosphate as a cofactor. BCAA-T is both cytosolic and mitochondrial (Cooper and Meister, 1985). Earlier studies on this enzyme indicated that it is present in all the tissues including brain (Roswell, 1956; Manchester, 1965; Ichihara et al., 1981). Activity levels of this enzyme in brain were higher than that of skeletal muscle and liver and lower than heart and kidney (Taylor and Jenkins, 1966; Ichihara and Koyama, 1966; Ichihara et al., 1981). Though activity levels of this enzyme is low per unit mass of the muscle, total contribution of muscle to BCAA-t is very high as muscle represents 90% of the tissue mass in the total body. Activity levels of this enzyme were lowest in liver (Krebs 1972). Three isozymes of BCAA-T have been reported. Isozyme-I is present in all animal tissues but remarkably low in liver. Isozyme-III was reported to occur only in brain and placenta while the isozyme-II is exclusively present in liver (Ichihara et al., 1973). Isozymes-I and III were observed to catalyze the transamination

of all the three BCAA while isozyme-II was specific for leucine. This enzyme was found to be regulated through dietaiy and honnonal changes in liver, hence it was suggested to be a rate limiting enzyme in liver (Krebs, 1972).

Branched-chain keto acid dehydrogenase complex (BCKA-DH) is the next enzyme involved in the degradation of BCAA. This enzyme catalyzes the irreversible decarboxylation of branched chain keto acids (BCKA). This enzyme requires CoA, NAD^+ and thiamine pyrophosphate (TPP) as cofactors. It mediates C-1 decarboxylation of all the three BCKA resulting the production of branched-chain ketoacyl CoAs with one carbon atom less than the BCKA. Decarboxylation of BCKA is mediated by the single enzyme complex which is present in all the mammalian tissues (Parker and Randle, 1978; Petit et al., 1978; Danner et al., 1979). Activity of this enzyme has been reported to be highest in liver and lowest in muscle (Shinnick and Harper, 1976). Based on the enzyme profiles in various tissues, it was proposed that this enzyme would be rate limiting in all extra-hepatic tissues while in liver, the rate limiting enzyme was BCAA- transaminase. Hence, it was suggested that BCKA produced especially in muscle would be transported to liver for further metabolism (Shinnik and Harper, 1976). It has been reported that 91% of total activity of BCKA-DH was found in liver, about 6% in kidney, 1.3% in brain and 0.2% in heart (Wohlhueter and Harper, 1970).

BCKA-DH, is a multi-enzyme complex and is similar to that of pyruvate and a-KG dehydrogenases complexes in its structure and function (Ischiwaka et al., 1966, Petit et al., 1978). It is localized in inner membrane of the mitochondria, consists of three subunits, a decarboxylase (E_1component), an acyltransferase (E2 component) and a dihydrolipoamide

dehydrogenase (E3) component. E2 and E3 are common for PDH and a-KGDH and are supposed to be shared by all three enzymes (Randle, 1983). The E_2 serves as a core of the complex on which E_1 and E3 are arranged. BCKA-DH requires Ca^+ and Mg^+ for its optimum activity as both Ca^{++} and Mg^{++} might be required for the activation of loosely bound phosphatase (Patel and Olson, 1982). In addition, an intrinsic kinase and a loosely bound phosphatase are also reported to be present along with the enzyme. These two respectively phosphorylate and dephosphorylate the E1 component of the enzyme complex. BCKA-DH was reported to be activated and inactivated by phosphorylation and dephosphorylation respectively (Danner et al., 1979). BCKA-DH activity was also shown to be regulated by feed back inhibition by the end products of BCAA such as acetyl CoA and succinyl CoA. Under *in vivo* conditions, its activity was reported to be regulated by ratio of NAD to NADH (Randle et al., 1981). Johnson and Conelly (1972) reported that this enzyme was also inhibited by ATP and activated by ADP.

Kinetics and mechanism of action of BCKA-DH were found to be similar to that of PDH and u-KGDH. Oxidative decarboxylation of BCKA is achieved in four distinct reactions mediated by decarboxylase, acyltransferase and dehydrogenase, thus producing branched chain acyl CoAs with one less carbon atom than that of BCKA.

$$\text{R.CO.COOH} + \text{TPP-E}_1 \rightarrow (\text{R.C.OH}) \cdot \text{TPP-E}_1 + \text{CO}_2$$

$$(\text{R. C. OH}) \cdot \text{TPPE,} + (\text{Oxidised Lipoate}) \cdot \text{E}_2 \leftrightarrow \text{TPPE}_1 + (\text{R.CO.hydrolipoamide}) \cdot \text{E}_2$$

$$(\text{R. CO. hydrolipoamide}) \cdot \text{E}_2 + \text{CoA} \leftrightarrow \text{R. CO. CoA} + (\text{dihydrolipoyl}) \cdot \text{E}_2$$

$$(\text{dihydrolipoyl}) \cdot \text{E}_2 + \text{NAD} \cdot \text{E}_3 \leftrightarrow (\text{oxidised lipoate}) \cdot \text{E}_2 + \text{NADII2.E3}$$

Subsequent enzymatic reactions involved in BCAA metabolism have not been investigated in detail in animal tissues. However, the end

products of BCAA catabolism were identified to be acetyl CoA (generated from leucine and isoleucine) and succinyl CoA (isoleucine and valine). These products enter into TCA cycle and subsequently generate energy. In addition, end products of BCAA catabolism are also involved in synthesis *of* polar and non-polar fatty acids (Stillway **et al., 1977,** 1978, Polgar et al., 1971; Dopeshwarker et al, 1979).

Much of the work on tissue metabolism of BCAA has been done in liver and muscle. Inter-organ interactions in the BCAA metabolism between muscle and liver has already been described (cf. glucose-alanine cycle).

Very few reports are available on the metabolism of BCAA in other tissues such as heart, kidney and diaphragm. Activity levels of BCAA-aminotransferase was found to be higher in heart and kidney than in liver and muscle (Ichihara and Koyama, 1966). BCKA dehydrogenase activities in these tissues were also reported to be as high as liver (Harper & Zapalowski 1981). In heart, BCKA-DH activity was found to be inhibited by clofibric acid and this inhibition was due to inactivation of BCKA dehydrogenase kinase (Paxton and Harris, 1982). Hormonal influences on the metabolism of BCAA was studied in isolated rat heart and diaphragm (Buse et al., 1973). They reported that, epinephrine and glucagon markedly stimulated the oxidation of BCAA in both heart and diaphragm during fasting and this stimulation was found to be suppressed in presence of pyruvate.

Cerebral Metabolism of BCAA :

Literature available on this aspect is very scanty. The earliest report on the cerebral metabolism of BCAA was that of Schepartz (1963) who reported that brain has capacity to oxidize BCAA. This was later confirmed by Swaiman and Milstein (1965); Roberts & Morelos(1965); Odessy and Goldberg (1972); Shinnik and Harper (1976), Chaplin et al.,

(1976) and Brand, (1981); Jessy and Murthy, (1985, 1988 i. ·); Murthy and Hertz, (1987a, 1987b), Jessy (1988); Jessy and Murthy (1989, 1993). It has been shown that oxidation of leucine accounts for 4-10% of total CO_2 produced in brain (Brand, 1981). Patel and Balazs (1970) reported that the primary metabolic fate of BCAA in brain is its incorporation into proteins. However, studies of Chaplin et al., (1976) indicated that the production of CO_2 from leucine was found to be 23 times higher than its incorporation into proteins. Cremer et al., (1975) demonstrated that the carbons of BCAA were also incorporated into glutamate family of amino acids while Yudkoff et al., (1983) reported that the nitrogen of BCAA were also incorporated into glutamate family of amino acids which accounts for 30% of total glutamate synthesized by brain. Extensive studies on BCAA metabolism in brain have been carried out by Jessy (1988). She reported that the activities of BCAA-T was higher in brain stem than in other regions. She also carried out studies on the sub-cellular and cellular distribution of BCAA-T and reported that the activities of BCAA-T were highest with isoleucine as a substrate in synaptosomcs, mitochondria and cytosol while the activities of the same were highest in astrocytes followed by neurons and oligodendroglial cells. Activities of BCKA-decarboxylase in sub-cellular and cellular preparations showed no substrate specificity . Studies carried out by her clearly indicated that metabolism of BCAA occur in all the three major cell types Viz., neurons, astrocytes and oligodendrocytes. In addition she also conducted studies on the oxidation of BCAA in different cell types and also in nerve tenninals and non-synaptic mitochondria and reported that BCAA are oxidized in all these preparations with highest oxidation being found in astrocytes. Interestingly she reported that the production of BCKA was considerably higher than the rate of their

oxidation and excess BCKA do not accumulate in the cell but are transported out. Murthy and Hertz (1987a, 1987b) studied the oxidation of BCAA in primary' cultures of neurons and astrocytes under acute and chronic ammonium acetate treated cultures.

Studies of metabolic compartmentation indicated that BCAA are primarily metabolized in glial cells (Cremer et al., 1975). However, Chaplin et al., (1976), Wiggins et al., (1979) and Buse & Reid (1975) indicated that neuronal preparations have the capacity to oxidize BCAA. This was further supported by Jessy (1988), Jessy and Murthy (1989) Murthy and Hertz, (1987) who demonstrated the oxidation of BCAA in neuronal perikarya, synaptosomcs isolated from adult rat brain and in primary cultures *of* neurons. These authors also demonstrated that the rate of BCAA oxidation in primary cultures of astrocytes and in astrocytes isolated from adult rat brain were much higher than neurons.

Studies were also conducted on the incorporation of BCAA carbons into cerebral lipids. Stillway et al., (1978), Dopeshwarker et al, (1979) demonstrated the incorporation of carbons of BCAA into different classes of cerebral lipids.

Survey of literature on the cerebral metabolism of BCAA under pathological conditions indicated that studies were mostly conducted in hepatic encephalopathy of various aetiologies. Cremer et al., (1975) observed an increased incorporation of the label from BCAA into glutamate family of amino acids under hyperammonemic conditions and suggested an increase in the metabolism of BCAA under these conditions. Murthy and Hertz (1987) reported that chronic exposure of ammonia to the primary cultures of neurons and astrocytes enhanced the production of $^{14}CO_2$ from [U-^{14}C] leucine and abolished the suppressive

effects of ammonia on $^{14}CO_2$ production. Jessy and Murthy (1985), reported that the transamination of BCAA was enhanced in hyperammonemic conditions and they suggested enhanced transamination of BCAA might replenish glutamate pool involved in the synthesis of glutamine there by excess ammonia might be detoxified.

Jessy (1988) extensively studied the metabolism of BCAA in normal and hyperammonemic conditions. Results of her study indicated that the transamination of BCAA was enhanced under hyperammonemic conditions in homogenates of 6 different regions of rat brain and in various sub-cellular preparations such as synaptosomcs, mitochondria and cytosol, while the same was decreased in neurons, astrocytes and oligodendrocytes. BCKA decarboxylase activities were found to be elevated under these conditions in cellular as well as sub-cellular preparations. Transport of BCAA into synaptosomcs, mitochondria, neurons and astrocytes were found to be enhanced under hyperammonemic states. Production *of* $^{14}CO_2$, an intermediate in the metabolism of BCAA was found to be suppressed under hyperammonemic conditions in all the cellular and sub-cellular preparations.

The above information on the metabolism of BCAA in brain suggest that brain is one of the extra-hepatic tissues capable of oxidizing BCAA. Though studies have been carried out on the metabolism of these amino acids in brain, most of the work is related to alterations in pathological conditions. However, no studies have been performed on this aspect under brain development and aging.

Many biochemical changes are expected to occur during the growth, development and aging of an animal. Metabolic demands of the

organs might not be uniform during the entire life span of an organism. For example, the energy demands of the animal will be very high daring growth and maturation stages for proper development and functioning of different organs. As the animal reaches adulthood, most of the growth and development will be completed and most of the energy might be utilized for the maintenance of the organs to function efficiently and a new steady state will be attained in the metabolism of various substrates. As the aging progresses, especially to the terminal stages of life, efficiency of different organs gradually declines as a result of which, the turn over and metabolism of various active substrates might also be altered. Hence, during entire life span, the metabolism of a particular substrate might be modulated according to the age dependent functions and requirements and also to the conditions under which the animal survives.

Aging :

Aging is a multi-factorial phenomenon, associated with a gradual decline in the normal performance of the organs with a concomitant loss in the genetic, biochemical and physiological mechanisms of the cell (Shock, 1962). Aging has been a subject of interest and speculation to biologists not only as an academic curiosity but also due to its social and ethical implications. During the last 3 decades, research on aging and age associated disorders has progressed to a considerable extent in many modern ways. However, the causes and effects of aging is still a subject of debate and discussion. This might be due to the universality of the aging process in every cell that constitutes an organ and as such it tantalized biologists and led to the formulation of multiple theories. Of the biological theories of aging, "Error Catastrophe Hypothesis" of Orgel (1963), Gene Regulation Theory (Kanungo 1975), DNA Repair Theory

(Hart and Setlow, 1974), DNA Damage Theory (Gensler and Bernstein, 1981) have attracted considerable attention. In addition to the genetic theories, Free Radical Theory has also been able to explain the mechanism of aging process to certain extent (Harman, 1981). These theories are briefly discussed in the following section.

Error Catastrophe Hypothesis :

Orgel (1963), suggested that the progressive decrease in the accuracy of protein synthesis might be one of the factors contributing to the process of aging. He suggested that metabolic errors are natural and inevitable although most of them are unlikely to be important. However, some might lead to further errors in the protein synthetic machinery. For example, any error in coding information in DNA for a particular mRNA would lead to synthesis of defective mRNA populations and as a result of which the proteins synthesized from that particular mRNAs might behave abnormally.

In addition to this, DNA also contains the information for the production of ribosomal RNAs and transfer RNAs and some of the proteins which are part of the translational machinery (e.g. ribosomal proteins, RNA polymerase involved in transcription, and the amino acyl tRNA synthetase etc.,). Any alterations in coding of mRNAs for these proteins might also lead to cumulative errors in the synthesis of target proteins that might lead to the catastrophe of the cells.

Gene Regulation Theory of Aging :

" Studies of Kanungo et al., (1976-till to date) on the biochemistry and molecular biology of aging process lead to the genetic theory of aging. They extensively studied the induction of enzymes, structural and functional changes in chromatin and expression of genes responsible

for aging (Chainy and Kanungo, 1978, Chaturvedi and Kanungo, 1985a and 1985b, Das and Kanungo, 1982, Kanungo, 1975). Their earlier studies indicated that enzymes of neurotransmitter synthesis such as choline acetyl transferase, (involved in the synthesis of acetylcholine), pyruvate kinase, (involved in energy metabolism) were found to be reduced in rats of older age groups. Several proteins responsible for either synthesis or functioning the neurotransmitters in brain such as tyrosine hydroxylase (for the synthesis of dopamine), β-adrenergic receptors were found to be reduced in rats of older age groups.

Significant changes in the chromatin were also reported by these investigators. These changes include the increased compaction of the chromatin due to tight association of the histones to the DNA thus preventing the transcription and translation process. Increased methylations in the DNA isolated from older brains were also noticed (Rath and Kanungo, 1989).

Reduced expression of Nerve Growth Factor (NGF) an important protein in the nervous system was due to increased methylation of the gene for the synthesis of the same (Kanungo, 1980). These changes in some of the genetic factors were suggested to be responsible for the deviation from the homeostasis functions of an organism and ultimately aging.

DNA Repair and Damage Hypothesis :

Both DNA repair and DNA damage theories proposed by (Hart and Setlow, 1974; Gensler and Bernstein, (1981) respectively, may be combined into a single theory. This theory suggests that DNA damage might occur due to endogenous and exogenous factors which result in loss or modifications of DNA base pairs, production of mismatched base pairs,

strand breaks, DNA-DNA cross links and cross links between DNA and other cellular constituents (Friedberg, 1990; Bernstein and Bernstein, 1991). Many of these modifications seem to be deleterious to the cell. Endogenous damage to DNA might be due to the constant hydrolysis of DNA base pairs leading to depurination and deamination of the base pairs (Lindhal and Nyberg, 1972). Moreover, free radical damage of the DNA is also a potential factor in the DNA damage. During old age, there would be excessive generation of free radicals in normal course *of* metabolic reactions. This is due to reduced antioxidant mechanisms. Free radicals act on the DNA and produce a variety of adducts (Harman, 1981). These changes in the structure and intactness of the DNA might in turn lead to the altered structure and function of chromatin during aging. In fact, such changes in the altered chromatin structure were reported to be responsible for reduced transcriptional abilities in the nuclei of neurons and astrocytes isolated from older rats (Venugopal and Rao, 1991). Recently, Bhaskar and Rao (1994) reported increased single and double strand breaks in DNA *of* neurons and astrocytes isolated from older rats when compared to the younger age groups. They suggested that the increased strand breaks were indicative of increased DNA damage in older age groups.

Free Radical Theory of Aging :

Free radicals generated during the process of normal metabolic reactions, escape anti-oxidant mechanisms during old age (due to reduced activities of the enzymes of xenobiotic metabolism), and damage the intra-cellular components such as DNA and RNA. In addition to this, these free radicals also act on and inflict damage in the cellular membranes (Harman,

1981). Such changes might alter the lipid compositions (increased cholesterol and decreased phospholipid content) of the membrane. This might affect the orientation of the proteins present on the membranes and alter the physico-chemical properties of the membranes such as fluidity and viscosity. These changes in turn lead to altered biochemical functions such as transport of metabolites across the plasma membranes. In fact, such alterations in the transport of amino acid neurotransmitters such as glutamate and GABA were reported (Strong et al., 1984).

Biochemical Changes in Aging :

Literature surveyed and incorporated here so far on the aging indicated that genetic factors greatly influence the process of aging. These changes in the genomic organization would obviously lead to alterations in the normal biochemical functions of the cell during aging. This could be due to changes in the structure and functioning of DNA which might lead to alterations in the protein synthesis as a result of which many biochemical functions such as protein synthesis, neurotransmitter functions, energy metabolism would be altered in brain. Survey of literature on these parameters are described here briefly.

Aging and Protein Synthesis :

Protein synthesis, an important constitutive biochemical process was reported to be reduced during aging by more than one mechanism. Reff , (1985) reported a decrease in the rates of RNA and protein synthesis while Petricivic et al., (1983) reported a decrease in the total RNA content in aged rat brain. Yarannel et al., (1977) reported decrease only in mRNA levels without any changes in the other RNA species. Seismi et al., (1982) reported a 26% reduction in polyA$^+$ mRNA in cerebral cortex of older age groups of rats. Translational abilities of different mRNA

species were also reported to be reduced in older rats and decreased protein synthesis in aging was suggested to be due to decreased aggregation of ribosomes into polysomes, a pre-requisite for translation. All these studies, suggested that during aging, functional efficiency of cellular synthetic machinery might be decreased which would eventually decrease the synthesis of various macromolecules.

Aging and Neurotransmitter Functions :

Aging is known to decrease the neurotransmitter functions in brain. This was reported to be due to both reduced activities of the enzymes responsible for the synthesis *of* the transmitter substances and alterations in the membrane receptors. Activities of tyrosine hydroxylase and dopamine hydroxylase (for the synthesis of biogenic amines such as dopamine, noradrenaline and adrenaline), catecholamine O-methyl transferase, choline acetyl transferase (for the synthesis of acetyl choline) were reported to be reduced during aging (Lloyd et al., 1972; McGeer and McGeer, 1975, 1976, James and Kanungo, 1976, 1978).

Changes in the receptor density, affinity and its functional coupling with the ligand were also reported to be altered during aging. Decreased receptor densities for several neurotransmitters such as dopamine, norepinephrine, GABA were reported in aging (Memo et al., 1980; Govoni et al., 1980).

Aging and Energy Metabolism :

Age associated changes in the energy metabolism were also reported in brains of different animal species. Rapoport et al., (1982) reported a biphasic response in regional cerebral metabolic rates for glucose. The metabolic rate increased between 1 month and 3 months in rats and declined between 3 and 12 months old age and remained constant

at that level up to 24 months age. Smith et al., (1980) reported a 25% reduction in the glucose utilization in cerebral cortex of 24 months old rats and this was more pronounced in other brain areas. Hoyer , (1981) reported cerebral glucose utilization decreased 1.5 times in rats between 6 and 12 months age and no further changes were observed in later age groups. They also reported a decline in the levels of glucose, glucose-6-phosphate, pyruvate, lactate, malate, creatinine and ATP levels while ADP levels were reported to be increased in brains of older age groups of rats. Patel (1977) reported a diminished oxidation of glucose (by 40%) in cerebral cortex of rats aged 24 months. Decreased activities of LDH, MDH in older rat brains were reported. Iwangoff et al., (1979) reported a moderate decline in the glycolytic enzymes in different regions of rat brain during aging. Taken to gather, all these changes suggest that cerebral energy metabolism might be reduced at least moderately if not completely.

Aging and Lipid metabolism :

It has been well reported that lipid composition of brain undergoes distinct changes during growth, development and aging due to the structural changes in the nervous system. Quarto et al., (1964) reported an increased cholesterol, phosphoiipid and cerebroside contents in rat brain during aging while Norton and Poduslo (1973) reported decrease in phosphotidylcholine and increase in plasmalogens during aging. Cerebrosides, a major component of myelin were reported to be decreased in aging rat (Horrocks, 1973). Singh and Rao (1979) reported an increase in the total lipid, cholesterol, glycolipids and phospholipids as a function of age in chick cerebrum and cerebellum. They also reported increase in the individual glycolipids such as cerebroside, sulphatide and phospho-

lipids such as phosphotidylcholine, phosphotidyl ethanolamine, phosphotidylserine in aged chick brain. Studies on the synaptic membrane lipid composition in different age groups of rats indicated decrease in phosphotidylcholine and an increase in ethanolamine plasmalogen and phosphotidyl ethanolamine levels (Hitzemann and Johnson, 1983). Increased cholesterol content in the synaptosomal membranes isolated from **the** older rats was reported by Nagy et al., (1983). They suggested that these changes in the lipid compositions on the cerebral membranes might be responsible for the altered membrane properties.

The above information confirm alterations in the cerebral biochemical mechanisms during cellular aging.

Aim and Scope of the Present Investigation :

Presently, studies were carried out on BCAA metabolism in various cerebral preparations such as neuronal perikarya and astrocytes, synaptosomcs and non-synaptic mitochondria. The rationale in selecting these two cellular preparations is obvious as the structure, function and metabolism of neurons and astrocytes are different. Due to inherent differences in the metabolic requirement, it is envisaged that metabolism of BCAA will also be different in these two major cell types of brain. The term neuronal perikarya is assigned to neuronal cell preparations which are devoid of processes such as axons and dendrites. Astrocytes prepared in the present investigation retained their cellular processes. Although there are different neuronal cell types in brain, in the present study pyramidal cells were predominant over other neuronal cell types. Similarly, protoplasmic astrocytes were dominant glial cell types. While studying neuronal preparation a clear distinction was made between neuronal perikarya and nerve terminals despite the fact that these might be compo-

nents of the same cell type. Such distinction became essential due to the functional heterogeneity in these two cellular compartments. Neuronal perikarya are chiefly involved in the reception of the nerve impulses while nerve terminals are involved in the onward transmission *of* impulses. Such functional heterogeneity implies an inherent structural heterogeneity. This is evident from the fact that nerve terminals have specialized structures (synaptic vesicles) for storage and release of neurotransmitters while neuronal perikarya are devoid of such structures. Besides structural heterogeneity, functional heterogeneity is always associated with biochemical heterogeneity. This is evident from the fact that nerve terminals are enriched with the biochemical systems required for the synthesis, storage, reception and inactivation of neurotransmitters. Hence, it is not surprising to assume that metabolism of BCAA would be different in these two different preparations belonging to the same cell type. The term non-synaptic mitochondria refers to the mitochondria present in the neurons (excluding nerve terminals), astrocytes and other cell types in brain. Mitochondria (both synaptic and non-synaptic) are supposed to be sites for the oxidation of BCAA as the rate limiting enzyme i.e., BCKA-DH is primarily a mitochondrial enzyme. In addition, the TCA cycle enzymes which are involved in the oxidation *of* acetyl CoA and succinyl CoA, generated from the BCAA, are also mitochondrial.

In the present investigation, studies were carried out on the transport and metabolism of BCAA in the above said cerebral preparations isolated from three different age groups. Studies on the transport are essential as this is the only mechanism through which cellular pools of BCAA are constantly replenished (besides endogenous proteolysis). More-

over, alterations in the transport can affect the further metabolic fate of BCAA in brain. A comparison was also made between mitochondrial transport of BCAA and that into other cell types (neurons, astrocytes) and nerve terminals. This is essential as the latter three preparations are endowed with plasma membrane which is in direct contact with the external milieu while the mitochondria are always in contact with cellular cytoplasm but never with external environment. These differences in membrane micro environment might influence the transport of nutrients across these two membranes.

Besides transport, studies were also conducted on the metabolic fate of BCAA. These include production of keto acids from BCAA (first reaction of BCAA metabolism) and production of CO_2 from BCAA (the final stage of BCAA metabolism). In addition, incorporation of carbons from BCAA into other amino acids especially glutamate was studied as this constitutes an alternate fate for the carbons of BCAA. In addition to this, studies were also carried out on the incorporation of BCAA carbons into cerebral lipids. These studies were aimed to fill the lacuna in the knowledge of age associated changes in BCAA metabolism.

MATERIALS AND METHODS

Chemicals :

Acetylated trypsin, adenosine triphosphate (ATP), alanine, 2-amino-2-norborane carboxylic acid, bovine serum albumin, 2,5-diphenyl oxazole (PPO), glutamate, glutamine, γ-aminobutyric acid, N-[2-hydroxyethyl])piperzine-(N-[2-ethanesulfonic acid]) (HEPES), hyamine hydroxide, imidazole, L-isoleucine, α-ketoglutarate, α-ketoisocaproic acid, α-keto-β-methyl valeric acid, α-ketoi so valeric acid, D-leucine, L-leucine, 1-4-bis[5-phenyl-2-oxozolyl]benzene (POPOP), pyridoxyl-5-phosphate, soybean trypsin inhibitor and L-valine, were purchased from Sigma Chemical Co., MO, USA. Ficoll-400 was purchased from Pharmacia Fine Chemicals, Sweden. Ninihydrin was purchsed from Fluca Biochemicals, Switzerland. Rest of the chemicals used were reagent grade and were locally purchased. [^{3}H]-leucine (12 Ci/mmole), [^{3}H]-isoleucine (9.2 Ci/mmole), [U^{14}C]-leucine (282 mci/mmole), [U^{14}C]-isoleucine (180 mci/mmole), [U^{14}C]-valine (180 mci/mmole) were purchased from Bhaba Atomic Research Centre, Bombay, India. [^{35}S]-methionine (1017 Ci/mmole) was purchased from American Radiolabelled Chemicals. Inc. St.Louis. MO, USA. Monofilament nylon screens were purchased from Nybolt Co., Zurich, Switzerland.

Animals :

Inbred albino rats of Wistar strain were used through out this study. Animals belonging to different age groups were maintained under natural light and dark cycles (12 hr light and 12 hr dark) in groups of 4-6 in a cage at 21 ± 2°C. Food (balanced pellet diet supplied by Hindustan lever Ltd., India) and water were provided *ad libitum.*

Cerebral Cannulation :

To study the in vivo incorporation of [^{3}H]-leucine into cerebral

lipids, cerebral cannulation was done to rats of 30, 90, and 750 days old. However, in 10 day-old rats, [^{3}H]-leucine was directly injected into the brain as described by Dopeshwarker et al (1979). Rats of other age groups were anesthetized with an intraperitoneal injection of sodium pentobarbitone (40 mg/kg body weight; dissolved in saline). A middle line incision was made on the skull at the parietal region (near bregma) and the skull was exposed. A burr hole was made at the parietal region (2 mm left to bregma) with a dental drill. A 22 gauge stainless steel cannula of suitable length was implanted. The cannula was fixed to the skull with dental cement and the incision on the skin was sutured. Tip of the cannula was sealed with parafilm till the commencement of the experiment. Rats were allowed to recover from the surgical stress for 72 hr. All the surgical operations were done under strictly sterile conditions in a laminar flow hood. [^{3}H]-leucine (5 μCi/kg body weight) was injected with a Hamilton syringe through the cannula.

Preparation of Homogenates :

Immediately after decapitation, brains were removed from cranial vault and transferred into ice cold saline. They were gently pressed between two wet filter papers (Whatman No. 1) to remove excess of water and meninges. Different regions of the brain (cerebral cortex, cerebellum, brain stem, hippocampus and striatum) were quickly dissected out . A 10% or 20 % (w/v) homogenates of different regions was prepared in 0.32 M sucrose using a motor driven Potter- Elvehjem homogenizer with Teflon pestle at 800 rpm with 6-8 up and down strokes at 0°-2°C. Homogenates were either directly used for enzyme assays or used for the isolation of sub-cellular preparations such as synaptosomcs and mitochondria.

Isolation of Synaptosomcs and Mitochondria :

Synaptosomcs and mitochondria were prepared from the cerebral cortex of rats of 3 different age groups Viz . 1 month, 3 months and 24 months, by the methods of Cotman (1974) as described by Rao and Murthy, (1991). Cerebral cortex was isolated as described above and homogenized in 0.32 M sucrose to obtain a 10% homogenate (w/v). Homogenates were centrifuged at 1100 x g for 5 min to obtain a pellet (P_1) consisting of broken cells, cell debris, crude nuclei and blood capillaries. Supernatant obtained (S_1) was again centrifuged at 21000 x g for 15 min to obtain a pellet (P_2) containing synaptosomcs, mitochondria and myelin. This pellet was suspended in a small volume of 0.32M sucrose and layered on top of a pre-formed discontinuous Ficoll density gradients (consisting of 10 ml each of 4%, 6% and 13% Ficoll-400 dissolved in 0.32 M sucrose) and centrifuged at 63,500 x g for 45 min This resulted in the separation of myelin (4% Ficoll layer), synaptosomcs (at the interphase of 6% and 13% Ficoll) and mitochondria (pellet). Myelin was discarded and the synaptosomcs were carefully aspirated with a Pasteur pipette and diluted with 5 volumes of 0.32 M sucrose. This was centrifuged at 21000 x g for 30 min. The final pellet was suspended in (Krebs-Ringer-HEPES-phosphate-glucose medium pH 7.4 supplemented with 110 mM sucrose) and used for biochemical studies. All the centrifugations were carried out at 2°- 4°C in a Beckman J2-21 refrigerated centrifuge and density gradient centrifugations were done in Beckman L8-80M ultracentrifuge using SW-28 rotor.

Isolation of Neurons and Astrocytes :

Astrocytes and neurons were isolated from the cerebral cortex of rats of 3 different age groups viz., 1 month, 3 months and 24 months, by

the method of Farooq and Norton (1978) as described by Rao and Murthy (1992). Cerebral cortex was isolated as described above. Each half of the cortex was made into small pieces using sterile surgical blade, washed with cell-isolation medium (8% glucose, 5% fructose, 2% Ficoll-400 in 10 mM KH_2PO_4 -NaOH buffer (pH 6) and transferred into Erlenmeyer flasks. Incubation was carried out in constant shaking water bath in 5 ml of cell-isolation medium containing acetylated trypsin (0.1% final concentration) at 37°C for 45 min with continuous oxygenation. After incubation, the flasks were transferred to an ice-bath and the slices were allowed to settle down. The medium was decanted and an equal volume *of* cell isolation medium containing 0.1% soybean trypsin inhibitor was added and incubated for 10 min at 0°C. The medium was discarded and the slices were washed 3 times with fresh cell-isolation medium. Cells were triturated with Pasteur pipette. Crude cell suspension was passed twice successively through monofilament nylon meshes with pore sizes of 300μ, 105μ, 80μ and 50μ respectively. The cell suspension was centrifuged at 750 x g for 15 min to obtain a pellet (P_1) and the supernatant (S_1) was discarded. Pellet (P_1) was suspended in 7% Ficoll- 400 (dissolved in cell isolation medium having 2% Ficoll) and centrifuged at 270 x g for 10 min to obtain pellet (P_2) rich in neurons and supernatant (S2). Supernatant (S_2) was diluted to 1:1.125 with ice-cold cell-isolation medium and centrifuged at 1,120 x g for 20 min to obtain pellet (P3) rich in astrocytes. Both the pellets P_2 and P3 were layered separately on top *of* discontinuous Ficoll-400 density gradients (28%, 22% and 10%) and centrifuged at 8500 x g for 30 min. The interphase between 10% and 22% contained astrocyte fraction and the pellet was enriched with neuronal perikarya. Both the fractions were diluted with 3 to 4 volumes of the

medium and centrifuged at 8500 x g for 20 min. Final pellets containing neurons and astrocytes separately, were suspended in Krebs-Ringer-glucose-HEPES medium supplemented with 110 mM sucrose and used for further biochemical studies.

Cell Viability Test :

Cell viability was determined using the dye exclusion method employing 0.4% trypan blue and the cells that were not taken up the dye were considered as viable cells while cells that were taken up the dye were considered as non-viable.

Cell Purity Test :

Purity of the cell preparation were routinely checked with light microscope and by assaying the marker enzymes for the respective cell populations.

Protein Estimation :

Protein content in cellular and sub-cellular preparations was assayed by the method of Lowry et al. ,(1951), while in homogenates it was assayed by biuret method (Varley, 1969)

Branched-Chain Amino Acid aminotransferase :

Branched-chain amino transferase in homogenates of 6 different regions of rat brain was assayed by the method of Jessy and Murthy (1985) with few modifications. Reaction mixture (1.5 ml) contains 250 μmoles of sodium pyrophosphate buffer pH 8.3, 40 μmoles of amino acid, 10 μ moles of a-ketoglutaric acid , 1 μmole of pyridoxal-5-Phosphate, 1 μmole of β-mercaptoethanol and .01% Triton X 100. Reaction was started by the addition of 50 μl of 20% homogenate and incubated for 30 min at 37°C. Reaction was terminated by the addition of 1 ml of 2,4- dinitrophenyl hydrazine and the branched-chain keto acid hydrozones were selectively

extracted into 3 ml of cyclohexane by vortexing. Cyclohexane layer was transferred into another test tube. To this 1.5 ml of 2% sodium carbonate and 3 ml of 1 N NaOH were added and vortexed. The cyclohexane layer was carefully aspirated and discarded. Absorbency of the colour developed in the carbonate layer was measured at 440λ. Parallel blanks were run with out amino acid. Activities were calculated using the standard curve constructed for 3 different keto acids.

Transport of BCAA :

Transport of leucine and isoleucine into synaptosomcs, mitochondria, neurons and astrocytes was studied by the method of Rao and Murthy (1994). Well oxygenated Krebs-Ringer-phosphate-glucose-HEPES medium (120 mM NaCl, 2.5 mM KCl; 10 mM glucose; 1.3 mM $MgSO_4$; 10 mM disodium hydrogen orthophosphate; 1 mM $CaCl_2$ and 10 mM HEPES (pH 7.4) was used as an incubation medium. Transport studies were carried out within 3 hours from the isolation of either sub-cellular or cellular preparations . Reaction mixture (0.5 ml) containing different concentrations of non- radioactive amino acid under study and 0.4 μCi of [^{3}H]-labelled amino acid was incubated at 37°C for 5 min prior to the commencement of uptake for thermal equilibration. Uptake was initiated by the addition of sub-cellular or cellular preparations under study. Incubations were carried out at 37°C for 5 min in shaker water bath. Uptake was terminated by rapidly cooling the samples to 2°C. Samples were rapidly centrifuged at 15000 x g in cold (4°C). Supernatant was carefully removed and the pellet was immediately dissolved in 0.1 N NaOH and transferred to scintillation vials containing 3 ml of scintillation fluid. Radioactivity was determined using Beckman LS 1800 liquid scintillation spectrometer with automated quench correction. Prior to these experi-

ments, optimum amount of protein and time of incubation were standardized using different protein concentrations (25 to 800 μgm protein fraction) and different time periods (0 to 60 min) of incubation. In all these experiments, incubations were carried out in duplicates.

Determination of Kinetic Constants :

Kinetics of transport was studied using different concentrations of amino acids (indicated in the text) and the saturation isotherms were subjected to Scatchard analysis. Kinetic constants (Km and Vmax) were determined using Sigma Plot program subjecting the data obtained by the saturation isotherms to Eadie-Scatchard analysis. A computer program (Km-Vm) was developed by us using the least square equations to cross check the graphical analysis of the data.

[$^{14}CO_2$] Production from [U-^{14}C]Branched-chain amino acids

Amount of carbon dioxide produced from the carbon labelled amino acids was estimated by the method of Jessy (1988) with minor modifications. Reaction mixture (0.5 ml) consists of Krebs-Ringer-phosphate-glucose-HEPES medium (composition described in uptake studies) was used. 100 μM of either leucine, isoleucine or valine [0.1 μ Ci/assay] were used. Reaction was carried out in 10 ml injection vials closed with Kontes rubber stoppers from which the center wells with a strip of Whatman No. 1 filter papers were suspended. Vials were tightly sealed with parafilm and vacuum grease was applied externally to ensure no leakage. Reaction was started by injecting externally 100 μgm of either sub-cellular or cellular protein and incubated for 30 min with constant shaking at 37^{o}C. Reaction was terminated by injecting 0.2 ml of 3M PCA into the vial and 0.2 ml of hyamine hydroxide into the center well. To zero time controls, 0.2 ml of PCA was added into the vial prior to

the incubation. Vials were further incubated with constant shaking for another 60 min at 37°C to trap the carbon dioxide released from the cellular and subcellular preparations. At the end of incubation, the center wells were carefully removed and dropped into the scintillation vials having Bray's scintillation fluid and the radioactivity was determined in Beckman LS-1800 spectrometer with 75% efficiency for carbon label. For the determination of the specific activities of the added amino acid, radioactivity in an aliquot of the incubation medium was determined.

Extraction of Cerebral Lipids :

One hour after intra-cannular injection of [^{3}H]-leucine, brains were dissected and washed with ice-cold saline. Total lipids were extracted from the whole brain employing the method of Phillips et al., (1978) . Brains were homogenized in 5 ml of 0.25 % acetic acid and centrifuged at 6000 x g for 15 min. This was repeated twice with the pellets obtained and the supernatants were discarded. The final pellet was homogenized in 40 ml of ice-cold chlorofonn : methanol mixture (1:1; v/v) and allowed to stand for 5 min to ensure complete extraction of lipids. Tubes were centrifuged at 6000 x g for 10 min. This was repeated thrice and the supematants were pooled. A final wash was given with chloroform : methanol (1:2; v/v). Pooled chlorofonn phases were evaporated under reduced pressure. Residual lipid extract was dissolved in a known volume of chloroform : methanol mixture (1:2; v/v) and was used for further analysis.

Estimation of Total Cholesterol :

An aliquot (30μL) of total lipid fraction was evaporated under nitrogen to estimate the cholesterol content using the method of Natelson (1971). To the residual lipid extract, 2 ml of glacial acetic acid was

added followed by 2 ml of acetic anhydride : concentrated sulphuric acid (10:1; v/v). Tubes were incubated at room temperature for 20 min and the optical density was measured at 625λ against acetic anhydride : H_2SO_4 blank. Cholesterol (100-1000 μgm) was used as standard.

Estimation of Total Phospholipids :

An aliquot of total lipid (10 μl) fraction was evaporated under nitrogen and the phospholipid content was determined by the method of Summer, (1944) by estimating the inorganic phosphorus. To the residual lipid extract, 1 ml of digestion mixture (50 ml distilled water, 20 ml conc. H_2SO_4, 25 ml of 70% PCA) was added and the samples were heated at 200°C for 2 hr to convert organic phosphate to inorganic phosphate. Tubes were cooled after digestion and 1 ml of distilled water was added to convert pyrophosphate to orthophosphate. To this, 1 ml of 50% sodium acetate (trihydrate) was added and the volume was made to 10 ml with distilled water. 1 ml of 2.5% ammonium molybdate was added and mixed well. To this, 1 ml of Metol (p-methylaminophenol sulphate; 1 gm in 100 ml of 3% sodium sulphate) was added. Absorbency of the blue colour was measured at 700λ. Different concentrations (10-100 μgm corresponding phosphorous content) of potassium dihydrogen phosphate was used as standard.

Estimation of Glycolipids :

Carbohydrate content of glycolipids was estimated by the method of Dubois et al (1956). Lipid extract (10 μl) was evaporated under nitrogen and 1 ml of distilled water and 40 μl of 80% distilled phenol and 2.5 ml of concentrated H_2SO_4 was added. Tubes were cooled for 20 min, and the absorbency of the colour was measured at 490 λ after 20 min, against H_2SO_4 blank. Galactose (5-50 μgm) was used as standard.

Thin Layer Chromatography of Labelled Lipids :

To determine the incorporation of radioactivity into different lipid classes, total lipid fraction was subjected to thin layer chromatography using pre-coated silica gel plates (Kieselgel 60 F_{254}, Merck, W. Germany). Cholesterol was separated using hexane : ether (85:15; v/v), phospholipids with chloroform : methanol : ammonia (35:15:10; v/v), and glycolipids by n-propanol : water (70:30; v/v) and known standards were chromatographed along with the lipid samples. After the separation, cholesterol, phospholipids and glycolipids were localized using specific localization reagents. The spots were scraped from the silica gel plate and extracted into 1 ml of chloroform. Radioactivity in these lipids was determined using Beckman LS 1800 liquid scintillation spectrometer.

Estimation of [^{14}C]-KIC Produced from [U^{14}C]-Leucine :

[^{14}C]-KIC produced from [U^{14}C]-leucine was determined by the method of Jessy and Murthy (1993). 100 μM of [U^{14}C]-leucine (0.5 μCi) in 0.5 ml of KRPG-HEPES medium was incubated at 37^{o}C for 30 min with 0.5 mg of cellular or subcelular preparations. Reaction was arrested by the addition of 1 ml of 2,4-dinitrophenyl hydrazine (in 1N HC1). After 10 min. incubation, 1 ml of cyclohexane was added and vortexed to extract the hydrozone. Tubes were centrifuged at 3,000 rpm for 5 min to separate the two phases. Cyclohexane phase was carefully aspirated and added into scintillation vial having 5 ml of scintillation fluid (8 gm PPO, 200 micrograms POPOP in 1 litter of toluene). Radioactivity present in the cyclohexane was determined using liquid scintillation spectrometer.

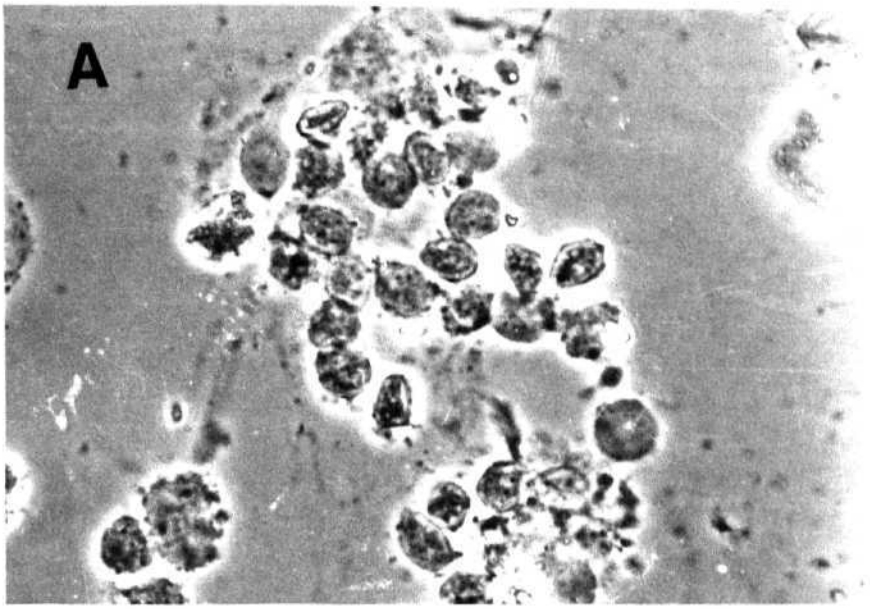
Estimation of [^{14}C]-Glutamate from [U^{14}C]-leucine :

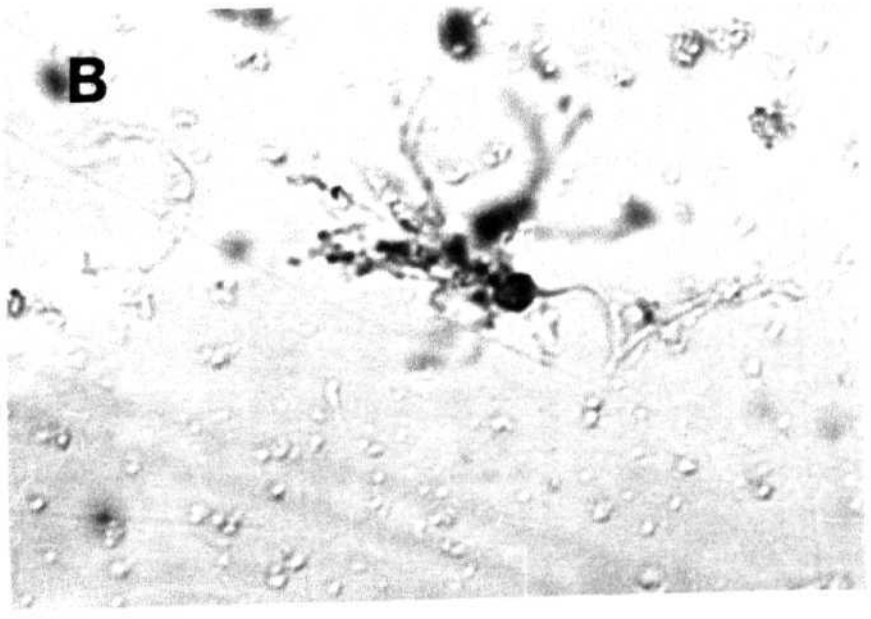
Incorporation of leucine carbons into glutamate was determined essentially by the same method followed for the determination of labelled

KIC except that the reaction was terminated by adding 1 ml of 2N HC1. Sample was passed through a column of Dowex 50X (H^+ form, 8% cross linked; 200-400 mesh, activated and packed in 5 ml bed volume) to separate the organic acids from the glutamate fraction. Amino acids were eluted from the column with 30% (v/v) ammonia solution and the eluate was concentrated through lyophilization. Residue was dissolved in a small aliquot of distilled water and amino acids were separated by ascending chromatography on precoated silica gel TLC plates (Merck, Germany) using n- butanol: acetic acid: water (65:15:25). Unlabelled authentic amino acid standards (50 nmoles each of leucine and glutamate) were co-chromatographed. After the separation, ninhydrin was sprayed on the area containing standards. Areas corresponding to these two spots were cut from the chromatogram and transferred to vials containing 5 ml of scintillation fluid. Radioactivity in glutamate and leucine spots was determined.

Statistical Analysis of Data :

Neuman-Keuls Multiple Range test was used for the statistical analysis of the data. A computer program was developed in basic language for this purpose.





PHASE CONTRAST PHOTOMICROGRAPHS OF CELLS ISOLATED FROM RAT CEREBRAL CORTEX. A: NEURONAL PERIKARYA (X 400) B: PROTOPLASMIC ASTROCYTES (X 400).

TRANSPORT OF BRANCHED-CHAIN AMINO ACIDS

Substrate/ionic transport into brain cells is a very complicated process. In extra cerebral tissues, cells have direct access to blood. In such tissues, blood bom substances arc transported across the plasma membrane either by a simple diffusion or by a carrier mediated mechanism. In brain, such a direct contact of brain cells with plasma would lead to uncontrolled activity, since many substances present in plasma might affect and damage the brain tissue. To prevent this, tight junctions between the endothelial cells of cerebral capillaries form a barrier, called blood-brain barrier **(BBB).** This hairier restricts free entry of all blood born substances and is selectively permeable to substances essential for cerebral metabolism. It is endowed with several carriers - each with its own specificity. Different carrier mediated transport mechanisms have been identified on the BBB (Pardridge and Oldendorf, 1975) These include transporters for hexoses, carboxylic acids, amino acids, nucleic acids and amines. Among these, transport of amino acids has been well studied (Chirstensen 1969; Sershen & Lajtha 1979).

Amino acid transport systems have been further classified into three major systems depending on the nature and properties of the amino acid transported (Christensen 1969; 1973; 1984) viz., neutral, basic and acidic amino acid transport systems. Carriers for these three groups of amino acids have also been identified on the BBB and their properties have been studied (Pardridge and Oldendorf, 1975). These transport systems are Saturable and stereo-specific. These systems transports more than one amino acid in the specified group (acidic or basic or neutral) and have different affinities for individual amino acids within the same group (Oldendorf, 1973). Interestingly, affinities of these transport systems for different amino acids were found to be close to their blood concentrations (Sershen and Lajtha, 1979).

Hence, it was suggested that the absolute as well as relative concentration of an amino acid in a particular group would affect its transport across BBB. Hence, competition always exists in the same group of amino acids.

Transport of neutral amino acids (leucine, isoleucine, valine, methionine, glutamine, phenylalanine, tyrosine and tryptophan) across the BBB was reported to be mediated by two types of transport systems. A sodium independent L-system (leucine preferring system; Christensen, 1969) capable of transporting large neutral amino acids with different affinities. This was shown to be present on the luminal side of the blood capillaries. A sodium dependent A-system (alanine preferring system) has been reported to be present on the anti-luminal side of the blood capillaries (Betz et al., 1981). L-system was reported to be a non-concentrative, but Saturable transport system that maintains a dynamic equilibrium between blood and brain levels of these amino acids (Pajari, 1984). Branched-chain amino acids were supposed to be transported across the BBB by L-systcm along with other neutral amino acids such as phenylalanine, tyrosine and tryptophan. Km of this transport system for BCAA was reported to be $\simeq$ 0.5 mM, which is close to their concentration in the blood.

Once BCAA cross the blood brain barrier, they encounter the plasma membranes of various cell types present in the brain. Sershen and Lajtha, (1979) reported that transport of BCAA and other neutral amino acids across the plasma membranes of brain cells was mediated by the L- type of system. Affinities of this system for BCAA was reported to be very low (1.5 to 3 mM). Hence, it was suggested that this system might be saturated only at very high concentrations (mM range) of these amino acids. In other words, this system functions as a low affinity - high capacity transport system and is capable of transporting large amounts of BCAA from extra-

cellular space into cells. L- systems were found to be similar in their structure and function for both the membranes of BBB and cell. These systems were shown to transport large neutral amino acids from extra-cellular fluid in exchange with intracellular glutamine (Cangiano et al., 1983). This concept was further supported by using methionine sulphoximine, a drug that inhibits glutamine synthesis. James et al., (1979) and Jonung et al., (1984) reported a suppression in the transport of BCAA into the brain when glutamine synthesis was suppressed.

Several studies were performed on BCAA transport under pathological conditions such as hyperammonemia (Jessy, 1988; James et al., 1978; Hawkins et al., 1981), portal systemic encephalopathy (James et al., 1979), hyperinsulinemia (Brosnon et al., 1984) etc. In hyperammonemia, enhanced transport of BCAA was reported in cellular and sub-cellular preparations of rat brain (Jessy, 1988; James et al., 1978). Jessy (1988) suggested that such an enhanced transport of BCAA, under acute hyperammonemic conditions, would replenish the glutamate pools required for the synthesis of glutamine. Though studies were performed on the transport of BCAA under above said conditions, few reports are available on their transport as a function of age. Pajari et al., (1984) reported that steady state accumulation of BCAA in brain slices increased with age. They also reported that mutual inhibition of transport of these amino acids was more pronounced in younger age groups than in the older age groups. Peterson et al., (1973) reported greater accumulation of BCAA in synaptosomcs in younger age groups than the older ones. Moreover, they also reported that sodium dependent transport of BCAA was present only in immature rats and it ceases to exist in the adult groups. In all these studies, physiological mechanisms of the changes in the transport of BCAA as function of

age were not explained satisfactorily.

Presently, transport of BCAA (leucine and isoleucine) was studied in sub-cellular (mitochondria and synaptosomcs) and cellular (neurons and astrocytes) preparations isolated from the cerebral cortex of 1, 3 and 24 months aged rats. Though synaptosomcs were isolated as a sub-cellular particles, they do not exactly satisfy the definition of a sub-cellular particle as they are bound by plasma membrane which encloses mitochondria and other membrane bound structures. The rationale in selecting these age groups has already been explained.

Transport of BCAA into Synaptosomcs :

Preliminary standardization of the uptake was performed in synaptosomes isolated from 3 months old rats. Transport of leucine was linear up to 200 μgm of protein, while isoleucine uptake was linear up to 600 μgm of protein (Figure 1 A). However, to maintain a favourable tissue to medium ratio during uptake, in all the subsequent studies only 100 μgm of protein was used. Studies on the time dependency of the transport indicated that this process was linear up to 10 min of incubation with both leucine and isoleucine (Figure 1 B). To minimize the metabolism of BCAA, a time period of 5 min was selected. Earlier reports indicated that the transport of BCAA in brain slices and in C1300 cell line, was a Saturable process (Hannuniemi et al., 1987). This aspect was further studied by measuring the uptake of BCAA into subcellular and cellular preparations at different concentrations of leucine and isoleucine .

Saturation isotherms, for both leucine and isoleucine as a function of amino acid concentration were observed to be biphasic. The first phase of uptake was linear up to 50 μM of both leucine and isoleucine. Between 50 and 100 μM there was a perceptible change in the transport of BCAA.

Increasing the concentration of these amino acids beyond 100 μM in the incubation medium, resulted in a rapid increase in the rate *of* transport up to 6 mM for both leucine and isoleucine (Figure 2), thus representing the second phase of uptake and the constructed Scatchard plot showed the curvilinear plot (Figure 3). This bi-phasic nature of the saturation isotherms for the transport of BCAA was not reported by earlier investigators (Jessy, 1988). This may be due to the fact that these investigators have used a concentration ranging from 1 mM to 5 mM (Jessy, 1988; Benjamin and Quastel, 1980), while the concentration range used in the present study was 0.5 μM to 10 mM.

Biphasic nature of the transport process might be due to (a) co-operativity among the transporter sites present on the same protein molecule akin to haemoglobin (Stryer, 1981) or (b) two types of transporter sites with no co-operativity but with different affinities. In the former conditions, the transporter protein might be having more than one ligand binding site. Binding of the ligand to one of the sites might increase (positive co-operativity) or decrease (negative co-operativity) the affinity of the adjacent sites (homotropic effect). Under such conditions, the saturation isotherms for the transport process would usually be sigmoidal instead of a normal rectangular hyperbole. True existence of more than one independent transporter sites for the same ligand can be confirmed by applying Hill equation to the initial velocity values. Hill coefficient (h) having the value of >1 indicates positive co-operativity among the ligand binding sites or 1 for non-co-operative independent sites with different affinities for the same ligand. Presently, Hill coefficient (h) derived from the slope of Hill plot was ~ 1 in synaptosomcs (Figure 4 A&B) indicating the non-cooperativity among the transporter sites and true existence of independent transporter sites with

different affinities for the transport of BCAA.

Scatchard analysis of the saturation isotherms, obtained in these studies, clearly indicated the presence of two different transport systems in synaptosomcs- a high affinity and low capacity (low Km and low Vmax) and a low affinity but high capacity (high Km and high Vmax) systems (Figure 4 C&D). These transport systems were found to differ several folds in their Km and Vmax values for BCAA (Table **1)**

TABLE 1

	HIGH AFFINITY		LOW AFFINITY	
AMINO ACID	Km (μM)	Vm (nmoles/mg protein/hr)	Km (mM)	Vm (nmoles/mg protein/hr)
LEUCINE	44 ±3	40 ±3	6.2 ±0.4	371 ±32
ISOLEUCINE	62 ±2	60 ±3	4.4 ±0.4	270 ±38

Transport of leucine and isoleucine in synaptosomcs of 3 months-old rat. Values arc mean ± S.D of 4 individual experiments done in duplicates. Kinetic constants were calculated from the Eadie-Scatchard plot drawn individually for each experiment. Significance: $p < 0.01$.

Existence of high affinity and low affinity transport systems for the transport of glutamate, GABA, aspartate, tyrosine and tryptophan has been reported earlier (Shank and Campbell, 1984; Hanneunami & Oja., 1981; Knapp and Mandel, 1972)). Besides differences in Km and Vmax of the high and low affinity transport systems, earlier investigators have also reported differences in the sodium dependency of these two types of transport systems. High affinity uptake system was shown to be sodium dependent while low affinity uptake system was sodium independent (Dreger et al., 1982). Hence, studies were carried out on the sodium dependency of high and low affinity BCAA transport in synaptosomcs isolated from 3 months old rats. Uptake was performed in a medium in which sodium

concentration was varied from 0 to 120 mM and ionic balance was maintained with the addition of the same concentration of Tris to a final concentration of 130 mM (pH 7.4). Rest of the conditions for incubation were similar to those described above. In all these studies, 100 μM and 1 mM leucine were selected as concentrations required for high and low affinity transport systems respectively. Results of this study indicated that high affinity uptake of leucine was affected by Na^+ in a concentration dependent manner. Reduction in the sodium ion concentration in the medium suppressed the transport of leucine by high affinity transport systems in synaptosome by 75% when sodium ions were completely replaced with Tris in the uptake medium (Figure 5A). Lack of 100% suppression in the high affinity transport under these conditions might be due to the presence of residual endogenous sodium. Sodium dependency was not noticed for the low affinity uptake system wherein no suppression in the uptake was found even when sodium was completely replaced with Tris ions (Figure 5 A). These studies clearly indicated that (i) high affinity transport systems for BCAA in synaptosomcs differ from the low affinity transport systems not only by their affinities but also on their sodium dependency (ii) high affinity uptake in synaptosomcs is mediated by carriers but not by a simple homoexchange of the label.

Stereo-specificity of both high and low affinity transport systems was assessed by studying the transport in presence of D-leucine and leucine analogue, 2-amino-2-norborane carboxylic acid (Zaleski et al., 1986). Results obtained in this study indicated that leucine transport by high and low affinity transport systems was unaffected by both these compounds suggesting a highly stereo-specific nature of this transport (Figures 5 B&C).

Survey of the literature indicated that existence *of* two carrier systems with different affinities for the transport *of* BCAA (especially leucine) was reported in several preparations such as synaptosomcs, neurons, astrocytes and neuroblastoma C1300 cell lines (Hanneunami et al., 1987). Surprisingly, no physiological function was assigned to these transport systems by earlier investigators.

By convention, high affinity transport systems are supposed to be involved in the termination of neurotransmitter activity of the amino acid neurotransmitters such as glutamate and GABA (Erecinsca and Silver 1990). In fact, presence of high affinity transport system is taken as a criteria for the substance to be considered as a neurotransmitter (Fonnum et al., 1980). However, as of today, no neurotransmitter activity has been reported for BCAA.

Scrutiny of the literature also indicated that high affinity transport systems may be involved in providing precursors for neurotransmitters - eg. choline for acetylcholine synthesis and tyrosine and tryptophan for the synthesis of biogenic amines such as dopamine, adrenaline and noradrenaline (Simon et al., .. , 1976; Knapp and Mandel, 1972). It has been reported that BCAA provide both carbon and nitrogen for the synthesis of glutamate. As was mentioned earlier, BCAA contribute to 30% total glutamate present in brain (Berl, 1974). However, studies from several laboratories indicated that glutamate is synthesized from several sources. Of these glutamine is a better precursor for neurotransmitter pool of glutamate. It is however; not known whether glutamate synthesized from BCAA would enter the neurotransmitter or metabolic pool of glutamate. Under such conditions, the physiological significance for the existence of both high and low affinity transport systems for BCAA remains to be elucidated.

In this context, it is worth mentioning here that BCAA are essential amino acids. Hence, they are not synthesized in the body and diet is the sole source for the cerebral pool of these amino acids. In general, content of these amino acids in proteins is low and is variable. Depending on the quality and quantity of the food consumed, BCAA levels in blood might fluctuate over a wide range. Cerebral BCAA content is influenced by the levels of these amino acids in blood. In such a case, under conditions of malnutrition, undernutrition and starvation brain may not receive adequate quantities of these amino acids leading to the depletion of cellular stores of BCAA. Such a condition would adversely affect the functioning of the tissues. For example, it has been reported that tissue proteolysis sets in if minimal concentration of BCAA are not maintained in the tissue (Paul and Adibi, 1980). Inadequate levels of BCAA would also affect the growth and development of brain (Patel and Balzas, 1970). In addition, there are several pathological conditions which would alter the levels of these amino acids in blood. For example, blood levels of BCAA were reported to decrease in hepatic encephalopathy and normalization of the levels of these amino acids in blood (by way of infusion of either BCAA or BCKA) was found to improve the clinical status of the patients (Beaubernnard et al., 1984).

Under such conditions, if brain is endowed only with low affinity transport system, availability of these amino acids would be restricted when blood levels of BCAA are low. However, it has been reported that even when rats were fed with leucine free diet, brain leucine content was reduced only by 30% while the same in the serum was reduced by 75% (Fernstrom & Faller 1978). Hence, it was suggested that alterations produced in the serum levels of essential amino acids would be poorly reflected in brain. In other words, brain accumulates these amino acids even when their levels in blood

are sub-optimal. This would be possible only when brain is endowed with a high affinity transport system. This would enable the tissue to maintain minimal quantities of BCAA, adequate to sustain the essential needs even under adverse conditions. Hence, existence of high affinity system (in addition to low affinity system) for the transport of BCAA and other essential amino acids would ensure a continuous supply of these essential amino acids even in adverse conditions.

For the above tenet to be true, it should be possible to demonstrate the existence of high affinity transport systems not only for BCAA but also for other essential amino acids such as tryptophan, threonine, methionine, phenylalanine and lysine. Existence of such high affinity transport systems were reported for tryptophan (Knap and Mandel 1972), threonine (Peterson and Raghupathy, 1978), lysine (Hannuneimi and Oja, 1981). Peterson and Raghupathy (1978) reported that sodium dependent high affinity transport systems for serine and threonine were found only in the synaptosomcs of immature brain but not in the synaptosomcs of adult brain. However, no such high affinity transport systems have been reported for the transport of methionine. Presently, an attempt was made to verify the above tenet.

For these studies, 0.5 μM to 10 mM [^{35}S]methionine (0.4 μCi/assay) was used. Conditions and the method adopted for the uptake was similar to that of BCAA. Saturation isotherms obtained in these studies were also biphasic as described for leucine and isoleucine and Scatchard plots were curvilinear indicating the existence of a two affinity transport system. A Hill constant of 1.04 suggested lack of co-operativity among these transporter sites and true existence of the dual transport systems for the transport of this amino acid (Figures 6 A&B). Results of the present study on the transport of BCAA and methionine in conjunction with the results of earlier

investigators on the transport of tryptophan, threonine, phenylalanine, lysine, strongly support the existence of high affinity transport systems for essential amino acids in cerebral membrane preparations. Hence, it is suggested that an additional function of transporting essential amino acids (besides the termination of post synaptic activity of neurotransmitter amino acids and supply of precursors for the synthesis of neurotransmitter amino acids) may be assigned to high affinity transport systems.

When a comparison was made on the kinetic parameters for these amino acids in synaptosomcs of 3 months old rats, the rank order for Km of high affinity transport systems was methionine > isoleucine > leucine. Vmax, representing the rate of accumulation of these amino acids, was in the order of isoleucine > leucine > methionine (Table 2).

TABLE 2

	HIGH AFFINITY		LOW AFFINITY	
AMINO ACID	Km (μM)	Vm (nmoles/mg protein/hr)	Km (mM)	Vm (nmoles/mg protein/hr)
LEUCINE	44 ± 3^a	40 ± 3^a	6.2 ± 0.4^a	371 ± 32^a
ISOLEUCINE	62 ± 2^b	60 ± 3^b	4.4 ± 0.4^b	270 ± 38^b
METHIONINE	84 ± 10^c	12 ± 3^c	21 ± 3^c	1558 ± 500^c

Comparison of kinetic constants of leucine, isoleucine and methionine transport in the synaptosomcs of 3 months-old rat. Values are ± SD of 4 different experiments done in duplicates. Kinetic constants (Km and Vm) were calculated from the Eadie-Scatchard plot for each experiment. Significance: a→ between leucine and isoleucine, b → between isoleucine and methionine, c → between leucine and methionine.

From these results, it is suggested that the transport pattern for all these amino acids might be different from one another. This might be due to the presence of different transporter sites for each amino acid or same transporter with different affinities.

An attempt was also made to compare the kinetic parameters (Km and Vmax) obtained in the present investigation with the earlier reports in which these high affinity transport systems were reported. However, very few studies were done in the past on high affinity transport system. A Km of 48 μM was reported by Hannuneimi and Oja (198 V) for high affinity transport of leucine in neuroblastoma C1300 cell line. The Km value obtained in the present investigation (44 μM) was found to be well within the range of the reported value. However, difficulties were encountered while comparing the Vmax values of present study with that of Hannuneimi et al., (1987). These investigators represented the Vmax values in terms of μmoles/kg cells/second. As the protein content of these cells was not known, it was difficult to calculate the Vmax per mg protein per unit time.

When a comparison was made in the kinetic parameters for the transport of these BCAA and methionine by low affinity transport systems, the rank order *of* Km and Vmax for the transport of these amino acids was observed to be methionine > leucine > isoleucine. Vmax for the transport of methionine was observed to be different from that of leucine and isoleucine (4 to 6 fold for Km and 7 to 10 fold for Vmax) (cf. Table 2). A comparison of the kinetic parameters of low affinity transport systems with that of high affinity transport systems indicated the Km and Vmax values of the former system were greater by approximately 3 orders of magnitude when compared to the later.

Kinetic parameters obtained by the earlier investigators were different from the values obtained presently. Cohen and Lajtha, (1972) reported a Km of 0.5 mM for leucine transport in brain slices while Vahlevan and Oja, (1975) reported a value of 0.4 mM in the same preparations. Benjamin **et** al., , (1980), reported a Km *of* 1.86 mM for leucine, and 2.14 mM for

isoleucine in crude synaptosomal (P_2) fraction . Jessy, (1988) reported a Km of 1.6 mM for leucine and 3 mM for isoleucine in synaptosomcs prepared from the cerebral cortex of 6 months old rats. Values obtained presently could not be compared with the reports of Cohen and Lajtha, (1972), or Benjamin and Quastel, (1980) since in the former investigators used brain slices. In this preparation, rate of transport depends not only on the concentration of the amino acid but also on several other factors such as size and thickness of the slice. Moreover, brain slice contains neurons and different types of glial cells as well as nerve endings. In such a preparation, the obtained Km value would be an algebraic sum of Km of all the cell types and nerve endings. Benjamin and Quastel used crude synaptosomal fraction, which contains a mixture of synaptosomcs, mitochondria, and contaminants such as myelin. Hence, the above argument would be applicable for this preparation also. Values obtained by Jessy (1988) for synaptosomcs were some what similar to present results but the age group she selected was different from the age groups of present study.

Transport of BCAA into mitochondria :

In addition to synaptosomcs, studies were also performed on the transport of leucine and isoleucine into mitochondria isolated from the cerebral cortex. BCAA are known to be actively metabolized in brain mitochondria (Jessy, 1988). Initial enzyme in the metabolism of BCAA viz., BCAA-amino transferase is present in both mitochondria and cytosol (Cooper and Meister, 1985). Moreover, the rate limiting enzyme for the catabolism of BCAA i.e., BCKA-dehydrogenase is exclusively localized in mitochondria. Previous studies on cerebral BCAA metabolism indicated that the activities *of* these enzymes were higher in mitochondria than in synaptosomes and cytosol (Jessy, 1988). It is implicitly understood **that the**

metabolism of any substrate in a membrane bound compartment would be regulated by its availability (in that particular compartment) which in turn would be regulated by its transport across the membrane. This tenet is applicable to the BCAA metabolism in cerebral mitochondria and very few studies have been conducted on this aspect.

Preliminary standardization was done in mitochondria isolated from the cerebral cortex of 3 months old rats. In these studies, non-specific uptake was observed to be 20-25% of the total uptake. Results of the present study indicated that leucine transport was linear up to 400 μgm of mitochondrial protein while the isoleucine uptake was linear up to 800 |igm of protein and 20 min of incubation time (Figures 7 A&B). To minimize the metabolism of these amino acids, 100 μgm of mitochondrial protein and 5 min of incubation time were selected for subsequent experiments. Kinetics of transport were studied using 0.1 mM to 10 mM of BCAA.

Saturation isotherms obtained for leucine and isoleucine transport were found to be monophasic, unlike in synaptosomes (wherein saturation isotherms were biphasic). Transport was Saturable around 6 mM for both leucine and isoleucine indicating the presence of only one type of system for the transport of BCAA in these preparations (Figure 8 A&B). This aspect was further confirmed in the Scatchard analysis of the data obtained from the saturation isotherms. Scatchard plots for both leucine and isoleucine indicated no curvilinearity and the data could be fitted into a first order linear regression curve (Figure 8 C&D) - confirming the presence of single type of Saturable transport system. Kinetic parameters (Km and Vmax) derived from the Scatchard plots, indicated that BCAA were transported into mitochondria by a low affinity, high capacity (high Km and Vm) system which operates in mM concentration range (Table 3).

TABLE 3

AMINO ACID	LOW AFFINITY	
	Rm (mM)	Vm (nmoles/mg protein/hr)
LEUCINE	26 ± 1	902 ± 30
ISOLEUCINE	12 ±3	557 ±51

Mitochondrial transport of leucine and isoleucine in brain Values are mean ± S.D of 4 individual experiments done in duplicates Kinetic constants (Km and Vm) were calculated from Eadie-Scatchard plots drawn individually for each experiment. Significance $p < 0.001$

Rm and Vmax values, obtained presently, for BCAA transport in mitochondrial preparations were in accordance with the earlier reports of Jessy (1988). In this context, it is worth mentioning here that apart from their incorporation into cerebral proteins, BCAA might be involved in cerebral energy metabolism, as end products of BCAA metabolism (acetyl CoA and succinyl CoA) are oxidized in TCA cycle. Earlier reports indicated that i) rate of production of CO_2 from BCAA was more than their rate of incorporation into cerebral proteins (Chaplin et al., 1976) and ii) enzymes responsible for the oxidation of BCAA to be localized in mitochondria (Olson, 1989). Under such conditions, complete catabolism of BCAA occurs in mitochondria. High rates of metabolism of these amino acids in mitochondria would require a higher rate of their transport which is possible only through a low affinity uptake system.

As mentioned earlier, unlike synaptosomcs, mitochondria seemed to be endowed with only low affinity transport systems for BCAA. Presence of only low affinity transport systems for BCAA in the mitochondria is understandable as these amino acids are available to mitochondria only when the cytosolic pool for these amino acids is saturated. In other words, mitochondria have no direct access to the extracellular environment and

obtain BCAA only from cytosol. Under normal conditions, the cytosolic pools for the BCAA would be maintained al optimal levels. However, the intracellular levels of these amino acids might be below the Km of the mitochondrial low affinity transport systems for BCAA. ** Hence, the mitochondrial carrier might not be saturated at these intracellular concentrations and operates at sub-optimal levels. This is understandable as the mitochondrial metabolism of BCAA include their oxidation to CO_2, with an irreversible loss of the carbon skeleton of the essential amino acids. Moreover, the presence of only low affinity transport systems in mitochondria enables the cell to maintain adequate amounts of the amino acids in the cytosol which is required for protein synthesis and other such vital phenomenon.

Transport of BCAA into Neurons and Astrocytes :

Preliminary standardization of BCAA transport, in neurons and astrocytes isolated from 3 months old rats, indicated that transport of BCAA was linear up to 400 μgm of protein and 10 minutes of incubation time (Figures 9&10 A to D), However, to minimize the metabolism of these

---

** *An approximation of the intra-cellular pool size of amino acid can be calculated from the saturation point on time cun>es. It is usually assumed that on prolonged incubation, the intracellular amino acid pool (unlabelled) would be completely replaced by the labelled amino acid present in the medium. Hence, the amount of radioactivity present in the cells after prolonged incubation represents the size of the intracellular amino acid compartment. Hence, any saturation point on time curve might be due to equilibration of intra and extracellular pools. With 'his assumption and also assumung that cell water content is -70%, the intra-cellular pool size of leucine isoleucine might be around 0.66 to J mM. It must, however be mentioned that this value is the size total of the intra-cellular pool vhich includes cytosol and mitochondria.*

amino acids in cells, a protein concentration of 100 μgm and a time period of 5 minutes were selected. Kinetics *of* transport of BCAA was performed in neurons and astrocytes as described earlier. Amino acid concentrations ranging from 0.5 μM to 8 mM of BCAA were used.

Saturation isotherms obtained from these studies indicated that BCAA transport into these preparations was biphasic in both astrocytes and neurons having two phases of saturation for leucine and isoleucine (Figure 11&12). Hill coefficient for the uptake of leucine and isoleucine in neurons and astrocytes was close to 1 indicating no-cooperativity among the transporter sites and true existence of two independent transporter sites with different affinities (Figure 13 A&B; Figure 14 A&B). This aspect was further verified by subjecting the data obtained from the saturation isotherms to Scatchard analysis. The curvilinear nature of the Scatchard plot confirmed the presence of dual affinity transport systems (Figure 15&16 A - P). These studies indicated that BCAA transport into neurons and astrocytes might be mediated by a high affinity (low Km and Vmax) and a low affinity (high Km and Vmax) transport systems. The Km and Vmax values for these two transport systems differ by several order of magnitude (Tables 4&5).

A comparison of these transport systems between neurons and astrocytes indicated that the Km and Vmax values of low affinity transport systems for leucine and isoleucine were higher in astrocytes than in neurons. Km of high affinity transport system for leucine in neurons was slightly higher than astrocytes while, Km of the same was significantly higher for isoleucine than leucine. Vmax for the this system for both leucine and isoleucine was much higher in astrocytes than neurons (Tables 4&5).

TABLE 4

	HIGH AFFINITY		LOW AFFINITY	
AMINO ACID	Km (μM)	Vm (nmoles/mg protein/hr)	Km (mM)	Vm (nmoles/mg protein/hr)
LEUCINE	58 ± 3	2.2 ± 0.4	3.8 ±0,6	214 ±40
ISOLEUCINE	84 ± 9[a]	3.7 ± 0.4[a]	2.8 ± 0.7	233 ±24

TABLE 5

	HIGH AFFINITY		LOW AFFINITY	
AMINO ACID	Km (μM)	Vm (nmoles/mg protein/hr)	Km (mM)	Vm (nmoles/mg protein/hr)
LEUCINE	41 ±8	4.6 ± 1	12 ±0.4	812 ± 97
ISOLEUCINE	61 ± 9[a]	5.4 ±0.8	12 ± 2	660 ± 126[a]

Leucine and Isoleucine transport into neurons and astrocytcs in 3 months-old rats Values are mean ± SD of 4 individual experiments done in duplicates Rest of legend is same as that of table 3. Table 4 is uptake into neurons and Table 5 uptake into astrocytes, **respectively.** $p < 01$

A comparison of these transport systems in neurons, astrocytes and synaptosomcs indicated that the Km for low affinity transport systems was in the rank order of astrocytes > synaptosomcs > neurons while the Km for high affinity transport systems was in the order of neurons > astrocytes = synaptosomcs. Such differences in the transport of BCAA among different cellular and sub-cellular preparations could be due to differences in the nature of these transporter proteins present on the membranes of different cell types. It is interesting to note that the Km of high affinity transport system was lower in synaptosomcs than in neuronal perikarya, while Vmax of this system was about 200 fold higher in synaptosomes than neuronal perikarya. In other words, the affinity of the transporters to its substTate as

well as number of transporter sites were greater in synaptosomcs than neuronal perikarya. Such differences in the affinity could be due to the differences in the membrane microenvironment in these two regions of neuron. Similarly, the greater Vmax (indicating the number of transporter sites) of the transport in synaptosomcs might be due to preferential accumulation of these proteins in the nerve terminals than in cell bodies.

In contrast to the high affinity transport systems, Km of the low affinity transport was higher in synaptosomcs than in neuronal perikarya while the difference in Vmax was not as great as high affinity transport systems. In fact, a greater Vmax in synaptosomcs of low affinity transport system than neuronal perikarya was observed only for leucine but not for isoleucine.

Preferential accumulation of high affinity transporters and their higher affinity of transporters in the nerve terminals than cell bodies suggest that the high affinity transport systems might be for the synthesis of neurotransmitter pool of glutamate while low affinity transport systems might be associated with the oxidative path way of BCAA. This is further supported by the observation that the Vmax and Km of the low affinity transport systems was much higher in astrocytes than the other two preparations. In addition, absence of high affinity transport systems in mitochondria is also in accordance with this suggestion.

Results of this present investigation are in accordance with the theory of metabolic compartmentation proposed by Berl et al., (1969). According to this theory, BCAA are mainly metabolized in small pool of glutamate supposed to be localized to the glial cells. Higher rates of BCAA uptake indicate greater rate of metabolism in astrocytes than in other cell types of brain. It was reported earlier that activities of the enzymes respon-

sible for BCAA metabolism were higher in astrocytes than in neurons (Jessy, 1988). Murthy & Hertz 1987) also reported a higher rate of oxidation of BCAA in primary cultures *of* astrocytes than in neurons. Transport of BCAA into neurons and synaptosomcs is also in accordance with the reports of Chaplin et al., (1976), who reported that the metabolism of BCAA might also occur in neurons and nerve endings.

Results of the present investigation indicated that BCAA would be transported into all these cell types by two kinds of transport systems - a high affinity low capacity transport system and a low affinity high capacity transport system.

Survey of literature on BCAA transport into neurons and astrocytes indicated that very few studies have been carried out on this aspect and no reports are available on the high affinity transport of BCAA in these preparations. However, Hannunemi & Oja (1981) reported that leucine and lysine were transported into neurons and astrocytes 3 week-old rat brain by high affinity transport systems. They reported a Km of 9 μM for leucine in neurons and 4 μM in astrocytes. Vmax values reported by them could not be compared with the present values as it was represented per kg cells and neither the amount of protein/cell nor the number/weight of the cells per mg protein were given in their reports.

BCAA Transport in Subcellular Fractions as a Function of Aging :

After establishing the existence of transport systems for BCAA in synaptosomcs, mitochondria, neuronal penkarya and astrocytes isolated from the cerebral cortex of 3 months aged rats, these studies were extended to the other age groups of rats viz., young (1 month), adult (3 months) and aged (24 months) rat brain cortices. Age dependent alterations were

observed in both high and low affinity transport systems in synaptosomes and in the low affinity transport systems in mitochondria.

In synaptosomcs, Km of high and low affinity transport systems for leucine and isoleucine were lowered in 24 months old rats when compared with 1 month-old rats. Km of high affinity transport system for leucine was lower in adult rats than young rats while no such change was observed in the case of isoleucine. Changes observed in the Km of low affinity transport system for these amino acids in these age groups were opposite to those observed for high affinity transport system in that changes of greater magnitude were observed for isoleucine. When a comparison was made between adult and old rats, there was a statistically significant difference in the Km of high affinity transport *of* isoleucine and low affinity transport *of* leucine (Tables 6&7).

Similar to changes in Km, Vmax values for leucine and isoleucine were also decreased in the 24 months old rats when compared to 1 month old rats. The magnitude of decrease was between 47 to 64% (Tables 6&7). When a comparison was made between young and adult rats, Vmax for low affinity transport system in cortical synaptosomes for these two amino acids was lower in the later age group than in the earlier age group. In contrast, Vmax of high affinity transport system of isoleucine was higher in 3 months age group than in one month age group of rats, while no such difference was noticed in Vmax of high affinity transport system for leucine. A comparison between adult and old rats revealed a fall in the Vmax of both high and low affinity transport systems of these two amino acids in synaptosomes with the exception of low affinity system for isoleucine (Tables 6&7).

Similar to changes in synaptosomes, the Km and Vmax values were altered in the cerebral mitochondria isolated from the rats of different age groups. Km for leucine transport was found to be highest in mitochondria

TABLE 6

	HIGH AFFINITY		LOW AFFINITY	
AGE	Km (μM)	Vm (nmoles/mg protein/hr)	Km (mM)	Vm (nmoles/mg protein/hr)
1 MONTH	70 ± 10	45 ± 5	7.6 ± 2	770 ± 174
3 MONTHS	44± 3[a]	40 ± 3	6 ± 0.4[a]	371 ± 32[a]
24 MONTHS	43 ± 2[c]	25 ± 4[b,c]	3 ± 0.2[bc]	278 ± 12[bc]

TABLE 7

	HIGH AFFINITY		LOW AFFINITY	
AGE	Km (μM)	Vm (nmoles/mg protein/hr)	Km (mM)	Vm (nmoles/mg protein/hr)
1 MONTH	66 ± 4	41 ± 3	8± 1	638 ± 123
3 MONTHS	62± 2	60 ± 3[a]	4 ±0.4[a]	270 ± 38[a]
24 MONTHS	28 ± 1[bc]	16 ± 1[b,c]	4 ± 0.5[c]	313 ± 26[c]

Age dependent variations in the kinetics of transport of leucine and isoleucine in synaptosomes. Values are mean ± SD of 4 individual experiments done in duplicates. Kinetic constants (Km and Vm) were calculated from Eadie-Scatchard of each experiment. Significance a —> 1 and 3 months, b → 3 and 24 months, c → 1 and 24 months

isolated from adult rats. Similar to changes in synaptosomcs, Km and Vmax for leucine transport into mitochondria were significantly decreased in 24 months aged rats when compared to young and adult rats. Km and Vmax for leucine transport were significantly higher in adult rats than young rats. A comparison between young and aged rats revealed a significant decrease in both Km and Vmax in leucine transport in mitochondria isolated from the latter age group of rats (Table 8).

With isoleucine, changes in Km and Vmax values were exactly

opposite to that of leucine transport. There was a significant increase in both Km and Vmax values for isoleucine in older rats when compared with other two age groups. A comparison between 1 and 3 months rats, showed an increase in both Km and Vmax values in 3 montls rats than 1 month rats. However, a comparison between 3 and 24 months rats, showed no significant changes in the same in these 2 age groups (Table 8).

TABLE 8

	LEUCINE		ISOLEUCINE	
AGE	Km (mM)	Vm (nmoles/mg protein/hr)	Km (mM)	Vm (nmoles/mg protein/hr)
1 MONTH	8 ±0.7	644 ±60	5 ±0.6	379 ±25
3 MONTHS	26 ± 3[a]	902 ± 30[a]	12 ± 3[a]	557 ± 51[a]
24 MONTHS	6 ± 0.9[bc]	337 ± 45[bc]	14 ± 3[bc]	582 ±65[bc]

Age dependent variations in the mitochondrial transport of leucine and isoleucine. Values are mean ± SD of 4 different experiments done in duplicates Kinetic constants were calculated from Eadie-Scatchard plot drawn for individual experiments. Significance: a→between 1 and 3 months, b→between 3 and 24 months, c→between 1 and 24 months

Transport of BCAA into Neurons and Astrocytes :

Studies on the transport of BCAA were also carried out in neurons and astrocytes isolated from cerebral cortex of young, adult and aged rats. Results of this study indicated that both Km and Vmax for the transport of BCAA decreased significantly in neurons and astrocytes of old rats when compared to younger age groups.

Km and Vmax of high affinity transport systems for leucine were significantly decreased with increasing age in both neurons and astrocytes. Though there was an over all decrease in the Km of high affinity transport systems between young and old rats, the magnitude of decrease was not uniform. Decrease in the Km of high affinity transport system in neurons

was only 19% in adult rats when compared to young rats while the same was 40% between adult and old rats. In contrast, magnitude of decrease in the Vmax of high affinity transport system for leucine was uniform (-40%) between young and adult and adult and older age groups of rats. In astrocytes, magnitude of decrease in the Km of high affinity transport system for leucine was more between young and adult (-45%) than between adult and older age groups (-27%). Profile of age dependent changes in Vmax of high affinity transport of leucine in astrocytes was exactly opposite to that observed for Km (-29% between young and adult and - 54% between adult and older rats; Table 9& 11).

TABLE 9

	HIGH AFFINITY		LOW AFFINITY	
AGE	Km (uM)	Vm (nmoles/mg protein/hr)	Km (mM)	Vm (nmoles/mg protein/hr)
1 MONTH	74 ±6	6.5 ±0.5	22 ± 4	1401 ± 303
3 MONTHS	41 ± 8[a]	4.6 ± 1	121 1[a]	812 ± 97[a]
24 MONTHS	30 ± 1[b,c]	2.1 ± 0.6[b,c]	8.4 ± 1[b,c]	576 1 147[b,c]

Age dependent variations in the kinetic constants of leucine uptake in neurons. Legend same as in Table 8

TABLE 10

	HIGH AFFINITY		LOW AFFINITY	
AGE	Km (μM)	Vm (nmoles/mg protein/hr)	Km (mM)	Vm (nmoles/mg protein/hr)
1 MONTH	99 ± 16	6 1 1.2	20 ± 6	1251 1283
3 MONTHS	61 ± 9[a]	5.4 ± 0.8	12 ± 2[a]	660 ± 126[a]
24 MONTHS	33 1 4[b,c]	3.4 ± 0.5[c]	8 1 2[b,c]	462 1 84[b,c]

Legend same as in Table 8 Table 10 represents **the** kinetic constants of isoleucine uptake in neurons.

In the case of isoleucine, there was a significant decrease (-38%) in the Km of high affinity transport system in neurons isolated from rats aged

24 months when compared with 1 month aged rats. No such change was observed between 1 and 3 months old rats. Km of the high affinity transport of isoleucine in astrocytes showed a consistent decrease with increasing age. Changes observed in Vmax of high affinity transport for isoleucine in neurons were similar to the changes observed in the Vmax for leucine. Vmax of high affinity transport for this amino acid decreased with increasing age in neurons while in astrocytes. It was less in rats aged 24 months when compared to 1 month and 3 months old rats while between 3 and 24 months age groups no significant change in the Vmax was observed (Table 10&12).

TABLE 11

	HIGH AFFINITY		LOW AFFINITY	
AGE	Km (μM)	Vm (nmoles/mg protein/hr)	Km (mM)	Vm (nmoles/mg protein/hr)
1 MONTH	72 ±8	5.3 ± 1	4.1 ±0.6	253 ± 16
3 MONTHS	58 ± 3[a]	2.2 ± 0.4[a]	3.8 ±0.6	214 ±40
24 MONTHS	35 ± 6[b,c]	1.4 ± 0.2[b,c]	2.5 ± 0.7[c]	164 ± 7[c]

Age dependent variations in the klinetic constants for leucine uptake in astrocytes. Legend same as in Table 8

TABLE 12

	HIGH AFFINITY		LOW AFFINITY	
AGE	Km (uM)	Vm (nmoles/mg protein/hr)	Km (mM)	Vm (nmoles/mg protein/hr)
1 MONTH	87 ± 10	9 ±0.5	3.1 ±0.5	272 ± 10
3 MONTHS	84 ±9	3.7 ± 0.4[a]	2.8 ±0.7	233 ±24
24 MONTHS	55 ± 9[b,c]	2 ± 0.2[b,c]	2 ± 0.1[c]	166 ± 14[b,c]

Age dependent variations in the kinetic constants for isoleucine uptake in astrocytes. Legend same as in Table 8

Km of low affinity transport for both leucine and isoleucine showed a statistically significant decrease in neurons of the adult when compared with young rats while no statistically significant difference was found between adult and older age groups. In astrocytes the Km and Vmax values of low affinity transport for both leucine and isoleucine were significantly decreased with increasing age and the magnitude of decrease was found to be -60% in both Km and Vmax for leucine and isoleucine (Tables 9 - 12).

A comparison of the kinetic parameters of leucine transport between neurons, astrocytes and synaptosomcs isolated from cerebral cortex of rats of above said age groups, revealed an interesting pattern. There was no statistically significant difference between neuronal perikarya and astrocytes in the Km of high affinity transport for leucine in rats of same age groups except in 3 months old rats where the Km in neurons was higher than that of astrocytes. In contrast to this, Km of high affinity transport for isoleucine was higher in neurons than astrocytes in adult and older age groups of rats while it was more or less same in 1 month age group. Unlike the changes in Km, Vmax values of high affinity transport for both leucine and isoleucine were more or less same in neurons and astrocytes of rats of same age groups except in 3 months age where the Vmax in astrocytes was higher than that of neurons.

A comparison of the Km of low affinity transport in neurons and astrocytes revealed that it was higher in astrocytes than neurons in all the three age groups. A similar profile was also observed for Vmax values. Vmax of high affinity transport of both leucine and isoleucine was higher in synaptosomcs than neurons in all the age groups of rats. Km of this transport system for isoleucine was lower in synaptosomcs than in neurons in all the age groups. In the case of high affinity transport systems for leucine,

Km was more or less same in both neurons and synaptosomcs at the early ages. However, in 24 months age group, Km of synaptosomal transport system was higher than that of neurons. In the case of low affinity transport systems, both Km and Vmax of synaptosomal transport were higher than that of neurons.

In brief, with few exceptions, studies on the transport of BCAA in different age groups showed an over all decrease in Km and Vmax with increasing age. Part of these results have already been published (Rao and Murthy, 1995).

Changes observed in Km and Vmax might be due to (i) alterations in the synthesis and degradation of the carrier proteins and/or (ii) synthesis of carrier proteins with altered conformations and/or (iii) alterations in the membrane microenvironment such as membrane fluidity and viscosity.

Earlier studies on the mechanisms responsible for aging indicated that an overall decrease in cerebral protein synthesis due to alterations in both transcription and translation process in aged animals. Reff (1985) reported decreased rates of RNA and protein synthesis while Petricivic et al., (1983) reported a decrease in total RNA content in aged rat brain. Yarannel et al., (1977) reported only decrease in the mRNA levels without any changes in the other RNA species. Seismi (1982) reported a 26% reduction in polyA$^+$ mRNA in the cerebral cortex of aged rats. Alterations in the translational abilities of different mRNA species were also reported to be reduced in aged rat brain (Yaranell et al., 1977). It was also reported that the decreased protein synthesis in aged brain might be due to decreased aggregation of ribosomes into polysomes a pre-requisite for the translational process. All these studies suggested that during aging, cellular synthetic machinery might be altered which would eventually

decrease the synthesis of the macro molecules.

It was mentioned earlier that the transporter proteins are synthesized in the cellular cytoplasm, sorted and transported to the target sites such as membranes. Alterations either in the sorting or targeting of the proteins in aged rats might also affect the membrane transporters. In neurons, proteins are synthesized in the cell body and transported to nerve terminals by slow and fast axoplasmic transport processes (Agranoff et al., 1993). Amino acid carrier proteins were reported to be transported by fast axoplasmic transport (Agranoff et al., 1993). However, a reduction in the rate of fast axoplasmic transport has been reported during the process of aging (Mc Martin, 1979). Alterations in the rate of synthesis and transport of proteins in aging could affect only the Vmax of the amino acid carriers but not their Km. Hence, it is suggested that changes in the Km may be due to alterations in the amino acid sequence of these synthesized proteins. Such an event would result in an abnormal folding of the synthesized protein and/or its insertion and orientation in the membrane which would eventually influence the affinity of these carrier proteins.

Such a possibility of the synthesis of proteins with altered or abnormal conformations was predicted by Orgel (1963) in the Error Catastrophe Hypothesis. This hypothesis predicts, that the progressive decrease in accuracy of the protein synthesis might be one of the factors responsible for the process of aging and age related functional changes in the cellular macro molecules. This decrease in the accuracy of the protein synthesis was assumed to be due to (a) alterations in the coding sequences of DNA that might lead to the synthesis of defective mRNA molecules and translation of these defective mRNAs might result in the synthesis of proteins with altered functional domains (b) defective synthesis of rRNA and tRNA molecules

since DNA also codes for the synthesis of these RNA molecules. As a result of the defective synthesis of these RNA molecules, synthesis of essential regulatory proteins such as initiation and elongation factors for translational processes might be altered. This process would exert a cumulative effect on the synthesis of target proteins in the cell. Taken together, such alterations of the genomic organization of the cells might result in the decreased accuracy in protein synthesis.

In addition to this, reports of the earlier investigations also indicated that aging enhanced the generation *of* free radicals which damage cellular membranes. In aging animals, free radicals escape anti-oxidant mechanisms and react with the fatty acids present in the plasma membrane and alter the physical environment *of* the membrane (Harman, 1981). Such changes might alter the lipid composition (increase in cholesterol content and decrease in phospholipid content) of the membrane. This might affect the orientation of the proteins in the membrane. If the membrane protein happens to be a transporter protein, then changes in lipid-protein interactions and their orientation in the membrane would affect the affinity of the carrier for its ligand and also on the overall rate of transport process. In fact, such alterations in the oxidation of —SH groups in proteins in the synaptosomal membranes isolated from the aged rats were suggested to be responsible for the altered conformation of the integral proteins of the membrane which might alter the affinities of carriers for the ions and amino acids (Nagy et al., 1983). Above said alterations in membrane lipid composition (i.e., increased cholesterol and decreased phospholipid contents) would also affect the physical parameters of the membrane such as fluidity. It has been reported that changes in the cholesterol content has a biphasic influence on the fluidity of the membrane - an initial increase

followed by a decrease in the membrane fluidity while the nature of the fatty acids especially their degree of unsaturation directly influences membrane fluidity. Free radical induced oxidation of unsaturated fatty acids in membranes was therefore assumed to alter the membrane fluidity.

As was mentioned above, in the carrier mediated transport of any substance across the membrane, binding of the ligand brings a conformational change in the transporter protein. Such conformational changes would be facilitated in fluid environment and are adversely affected in a rigid frame work. Increased cholesterol and decreased phospholipid contents are known to increase the viscosity (decreased fluidity) of the membrane and affect membrane associated processes such as transport (Nagy et al., 1983).

Using spin labelled probes in the synaptosomal membranes of young, adult and aged rat brain cortex, Nagy et al., (1983), reported decrease in the synaptosomal membrane fluidity and increased membrane viscosity in aged animals. They reported that the lipid bilayer of the synaptosomal membrane was rigid in aged rats. As a result, fluidity of the membrane was decreased. They indicated that such changes in the fluidity might affect the functioning of carrier proteins in membranes. Moreover, conformational changes induced in the carrier proteins by binding of ligand involves lipid-protein interactions. Alterations in lipid composition might result in an unfavourable environment where such lipid-protein interactions might be unduly interfered.

Decrease in both Km and Vmax for high and low affinity transport systems for BCAA in all these preparations as a function of age might have a physiological significance. Increased affinities (decreased Km) of these transporters in cellular and sub-cellular preparations of aged rats might be

helpful to transport these essential amino acids when their extra-cellular concentrations are sub-optimal. It is generally believed that the food consumption declines in senescent rats due to more than one mechanism such as (a) loss of certain normal cognitive functions in these rats might reduce the exploratory behaviour for food and water (b) The decreased metabolism might possibly increase the half life of these amino acids in cellular cytoplasm which would exert a feed back inhibition on the animals food consumption (Harper 1983). As a result of the declined food consumption availability of essential amino acids to brain might also be lowered. Under such conditions, membrane transporters with lower affinities (high Km) might not be efficient in transporting these amino acids from the extracellular environment. In this situation, the transporters with much higher affinities (low Km) might serve the purpose of transporting the essential amino acids at suboptimal levels to meet the basal requirements of these amino acids especially when the extra-cellular levels are sub-optimal. Thus, modifications in the affinities of these transporters might be a beneficial adaptation in the senescent period.

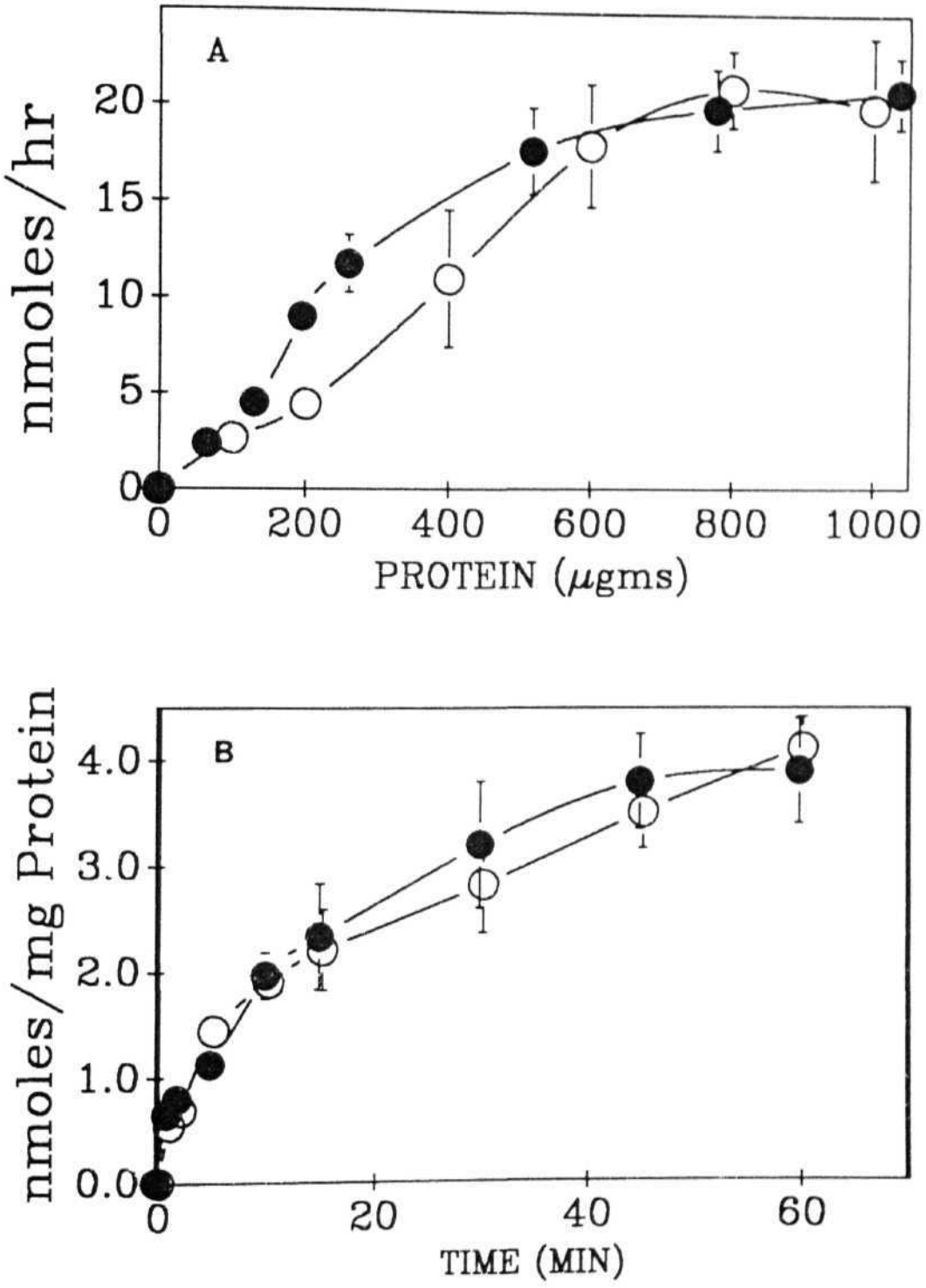


Figure 1: Transport of [^{3}H]-leucine and isoleucine into synaptosomes as a function of protein concentration (A) and time of incubation (B). Values are mean ± SD. of 4 individual experiments done in duplicates. O— O represents leucine transport and •—• isoleucine transport.

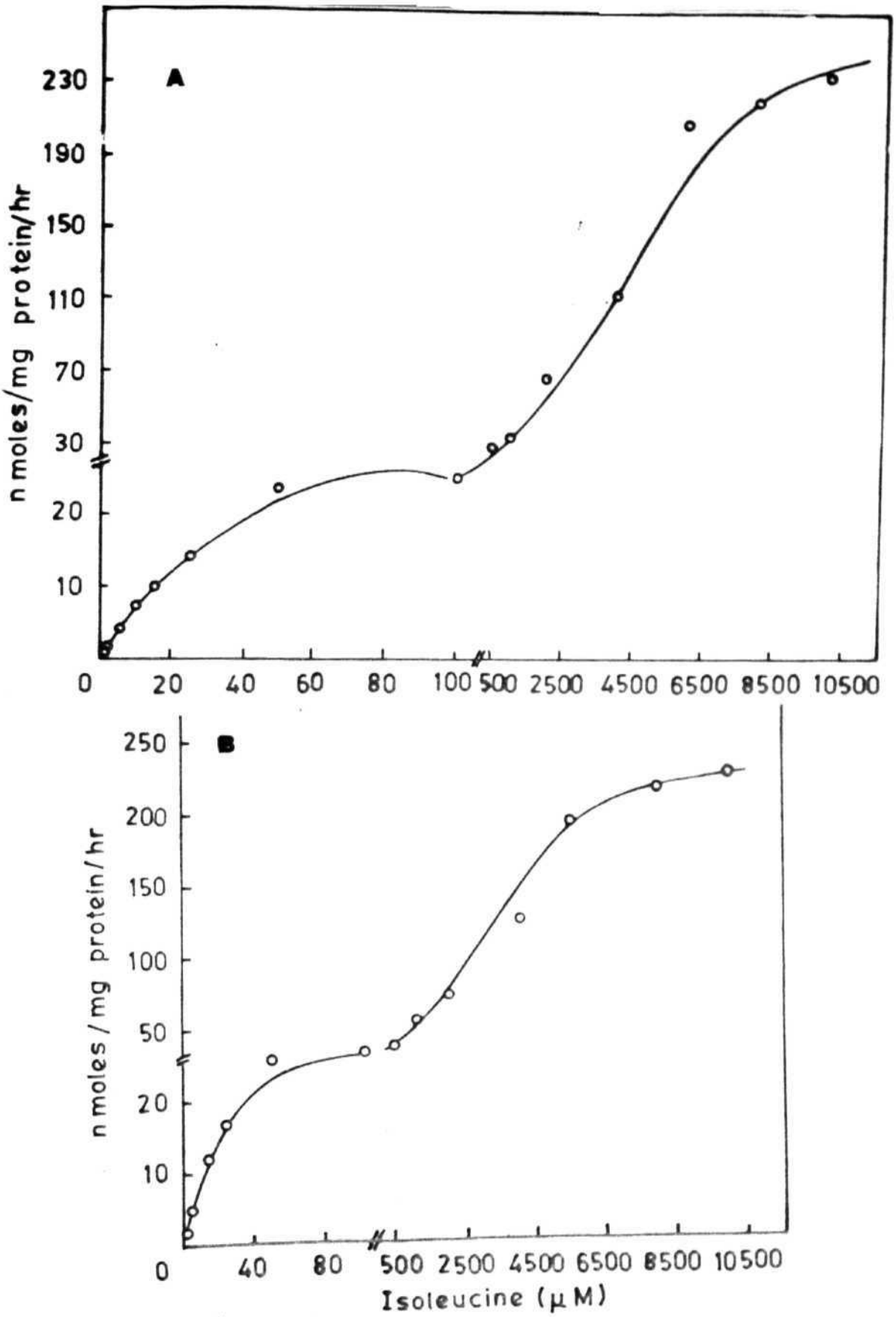


Figure 2: Biphasic saturation isotherms of leucine and isoleucine transport into synaptosomes. Values are mean ± S.D of 4 individual experiments done in duplicates.

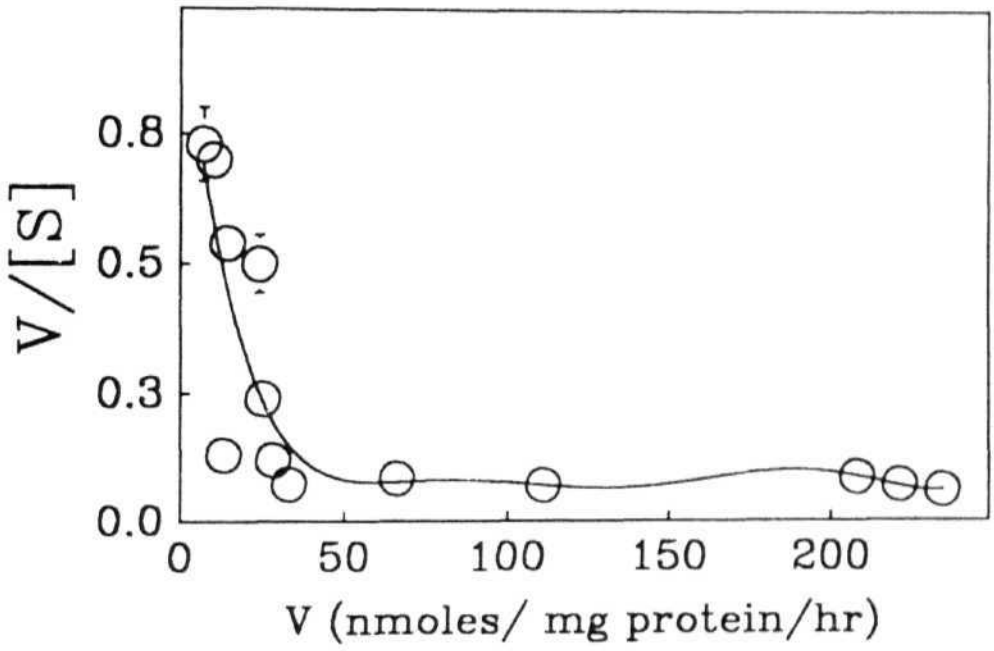


Figure 3: Curvilinear Scatchaid plot of leucine transport into synaptosomes. Scatchaid Plot was constructed subjecting the data of saturation isotherms to Scatchaid analysis. Values are mean ± S.D of 4 individual experiments done in duplicates.

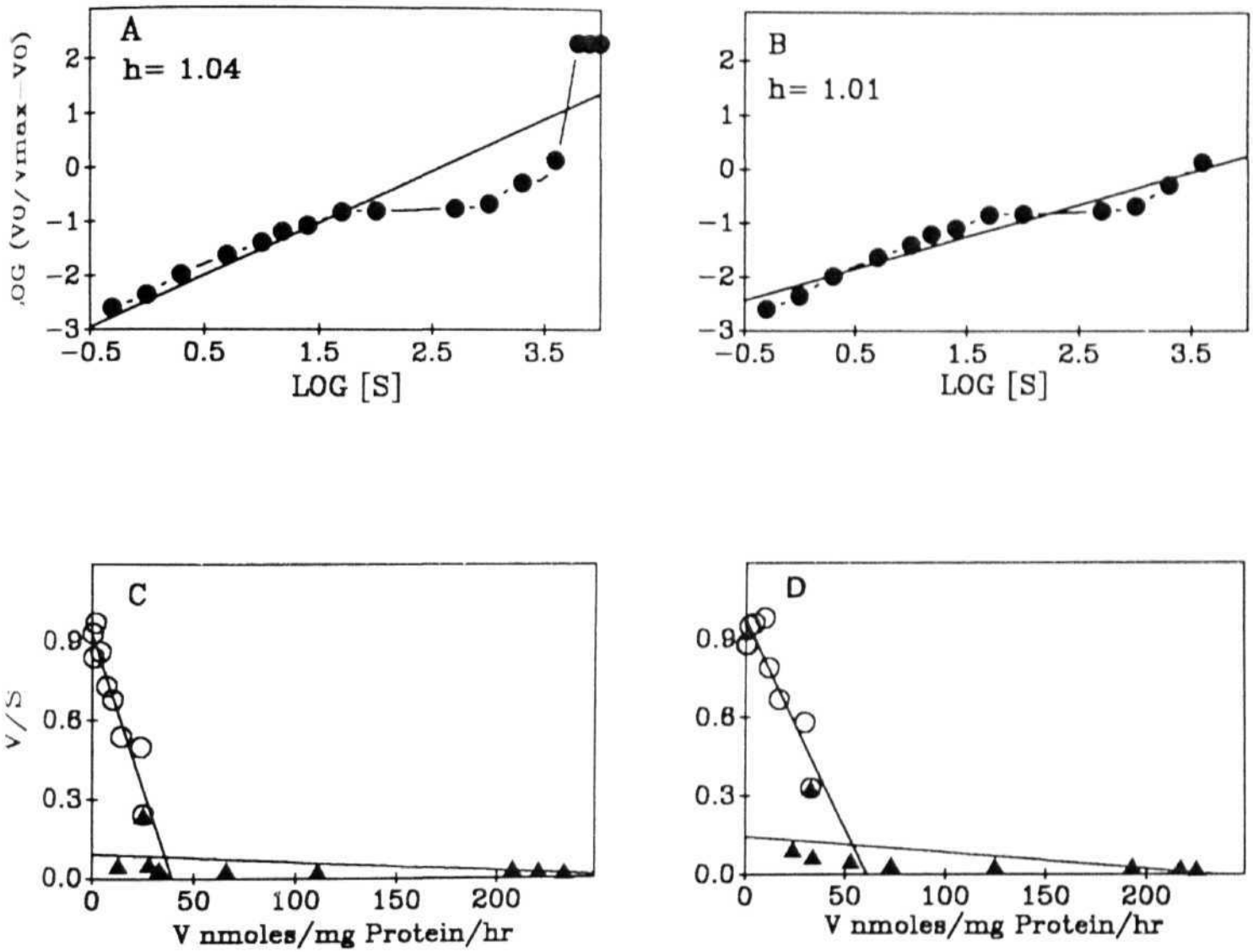


Figure 4: Hill plots of [^{3}H]-leucine (A) and isoleucine (B) in synaptosomcs showing non-cooperativity of the transporter sites. Scatchard plots (C and D) of [^{3}H]-leucine and isoleucine transport respectively in synaptosomcs showing two affinity transport systems. Values are mean ± S.D of 4 individual experiments done in duplicates.

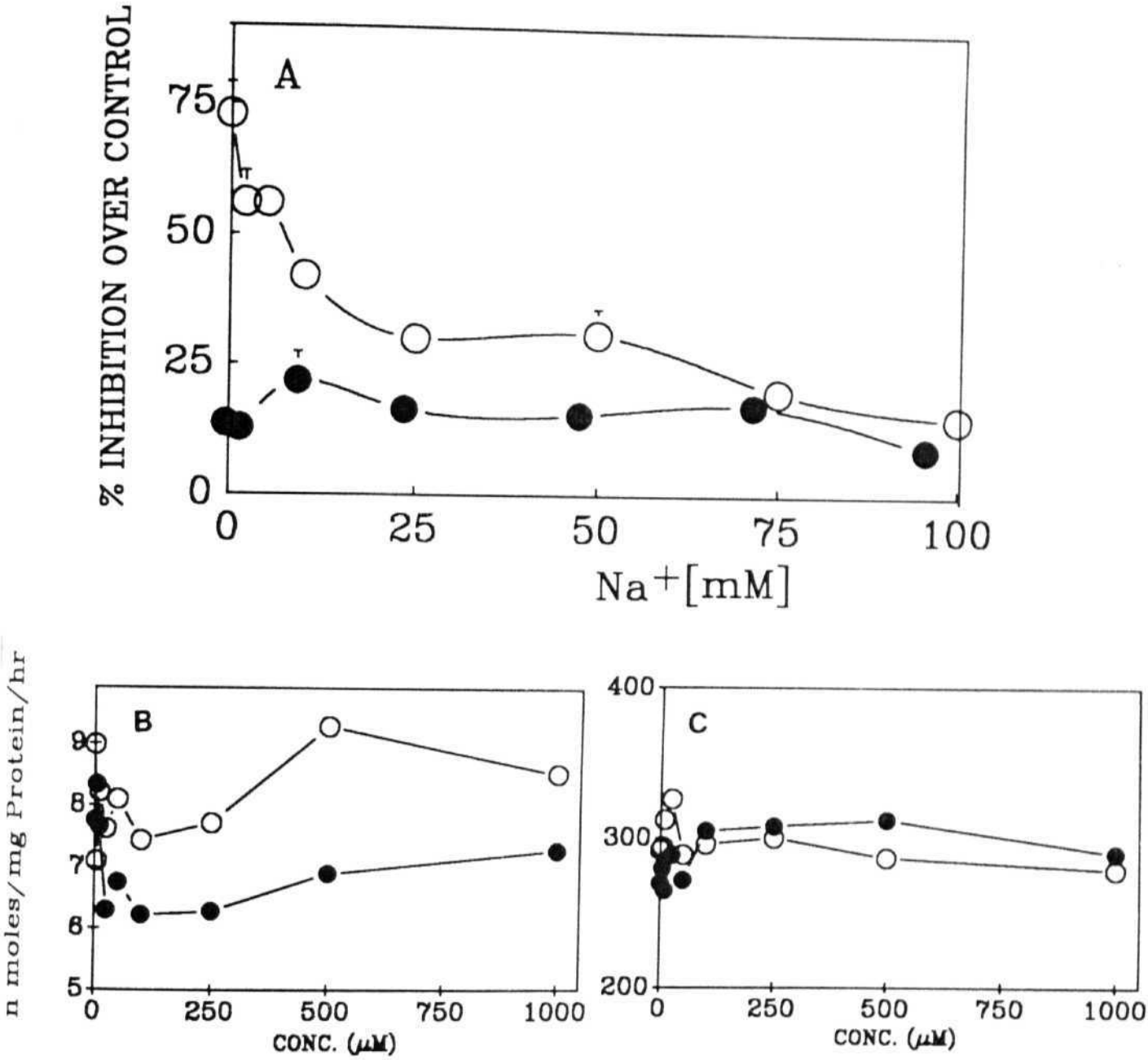


Figure 5: A : Sodium effect on high and low affinity transport of[^{3}H]-leucine. Sodium concentration was varied from 0 to 130 mM. B and C : Effect of(2-amino-2-norborane carboxylic acid) and D-leucine on the stereo-specificity of [^{3}H]-leucine transport into synaptosomcs. (2-amino-2-norborane carboxylic acid) and D-leucine concentrations were varied from 0 to 1000 μM. In all the experiments 50 μM and 1 mM leucine concentration was selected for high and low affinity transport respectively. Values are mean ± S.D of 4 individual experiments done in duplicates.

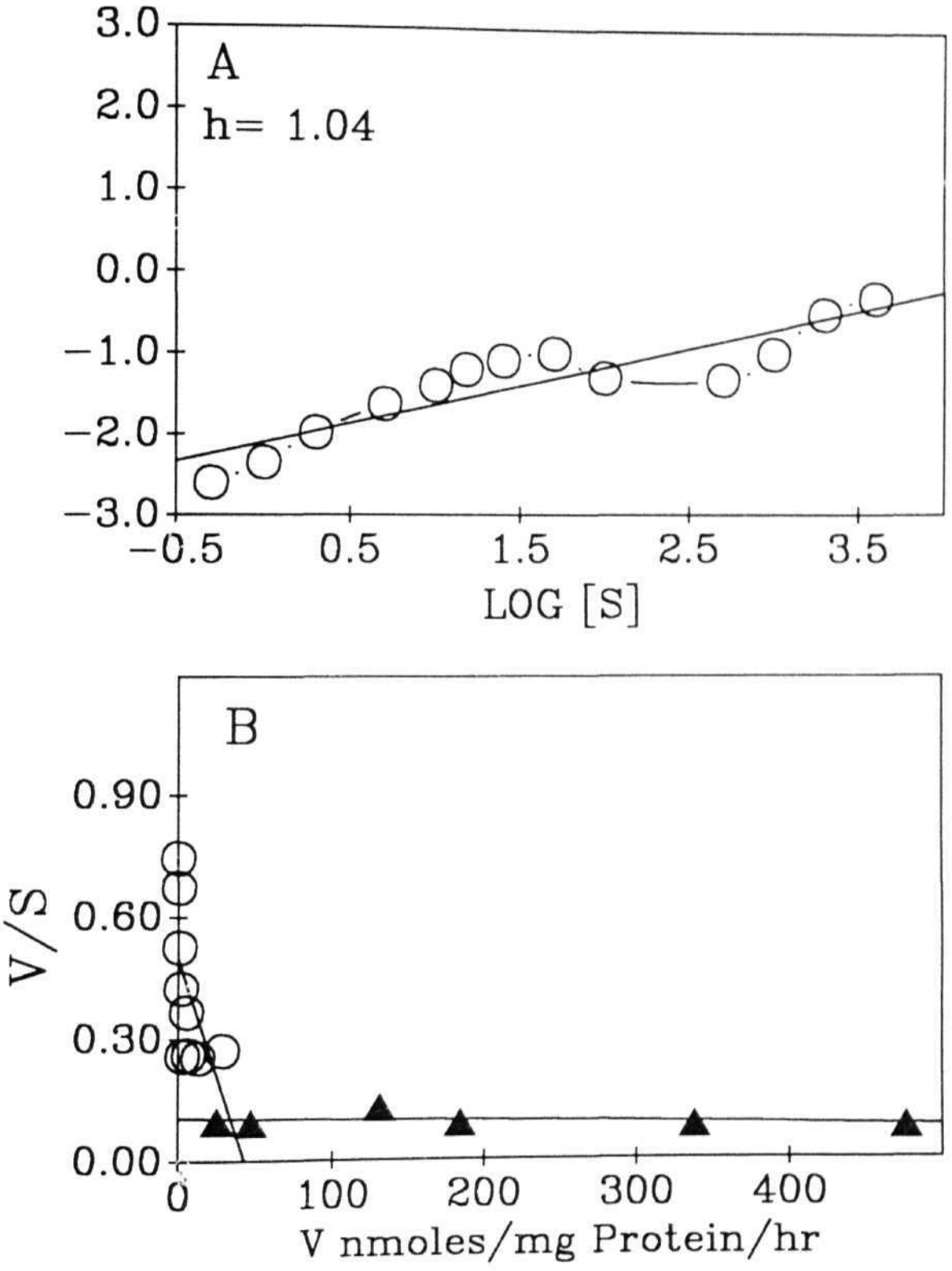


Figure 6: A : Hill plot of [^{35}S]-methionine transport in synaptosomcs showing non-coopcrativity of transporter sites. B: Scatchard plot of [^{35}S]-methionine transport indicating high and low affinity transport systems. Values are mean ± S.D of 4 individual experiments done in duplicates.

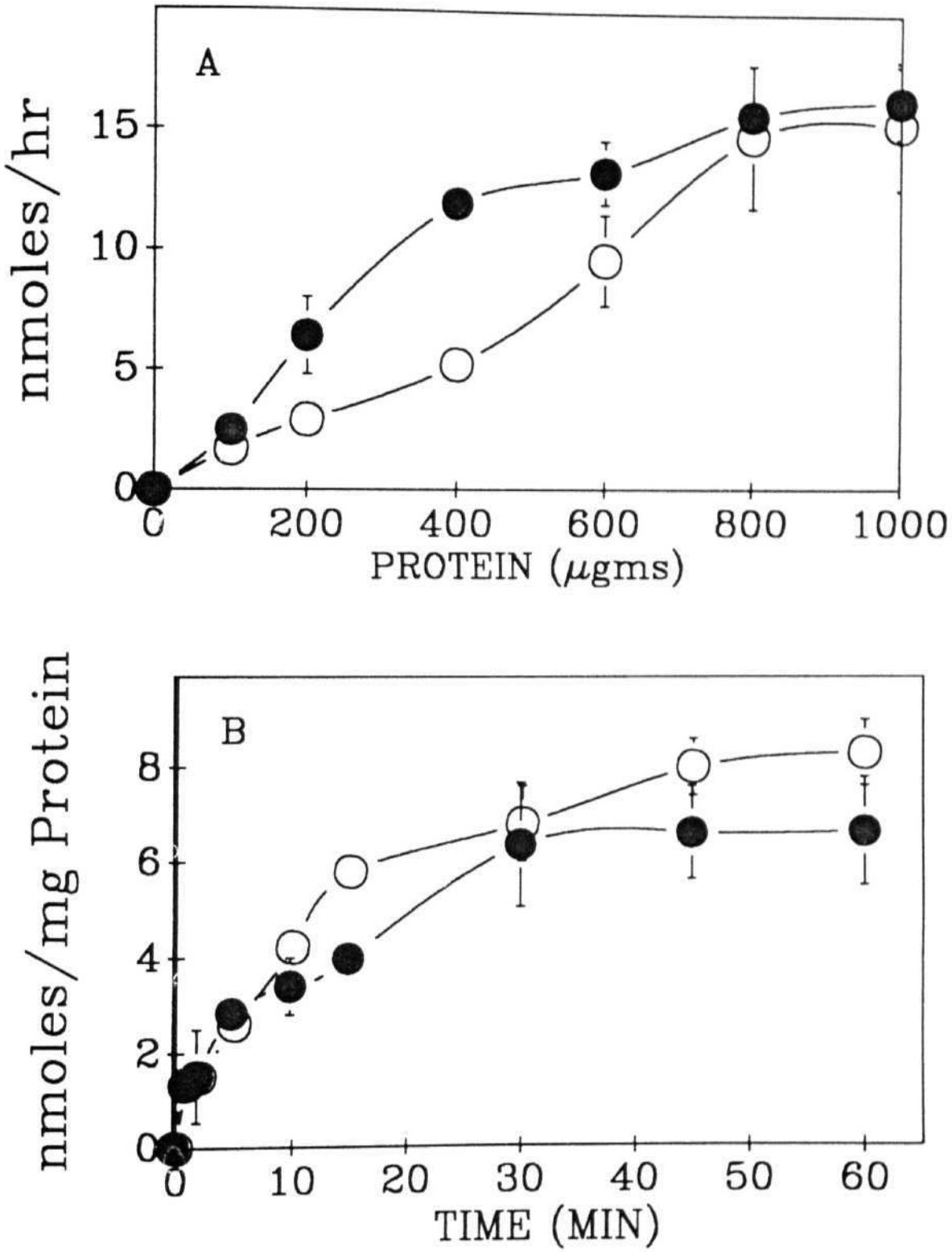


Figure 7: A : Transport of [^{3}H]-leucine and isoleucine into mitochondria as a function of protein concentration. B: Transport of [3H]-leucine and isoleucine into mitochondria as a function of time *of* incubation Values are mean ± SD of 4 individual experiments done in duplicates. O—O represents leucine and •—• represents isoleucine.

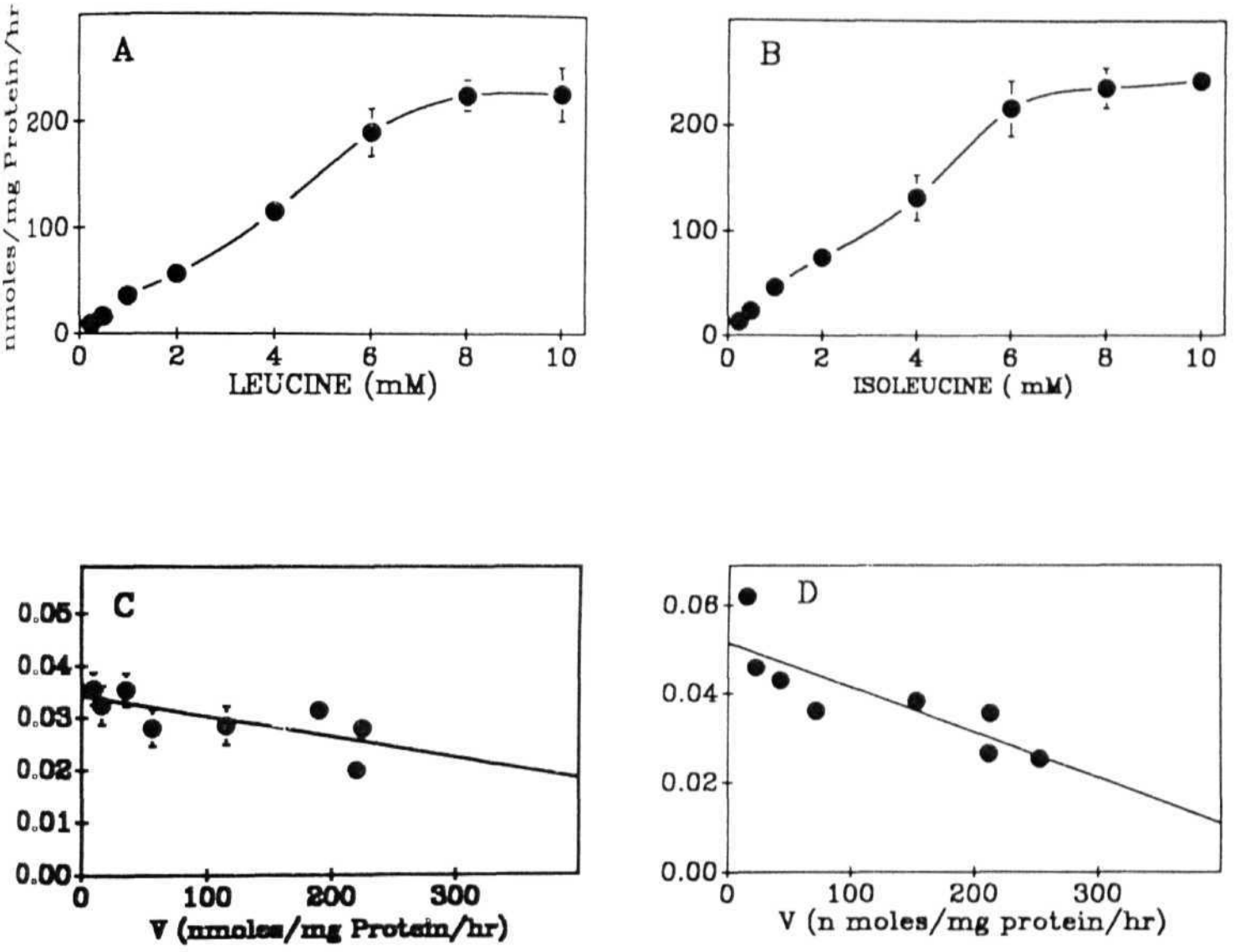


Figure 8: Saturation isotherms of [^{3}H]-leucine (A) and isoleucine (B) transport into mitochondria. C and D: Scatchard analysis of [^{3}H]-leucine and isoleucine transport into mitochondria. Values are mean ± SD of 4 individual experiments done in duplicates.

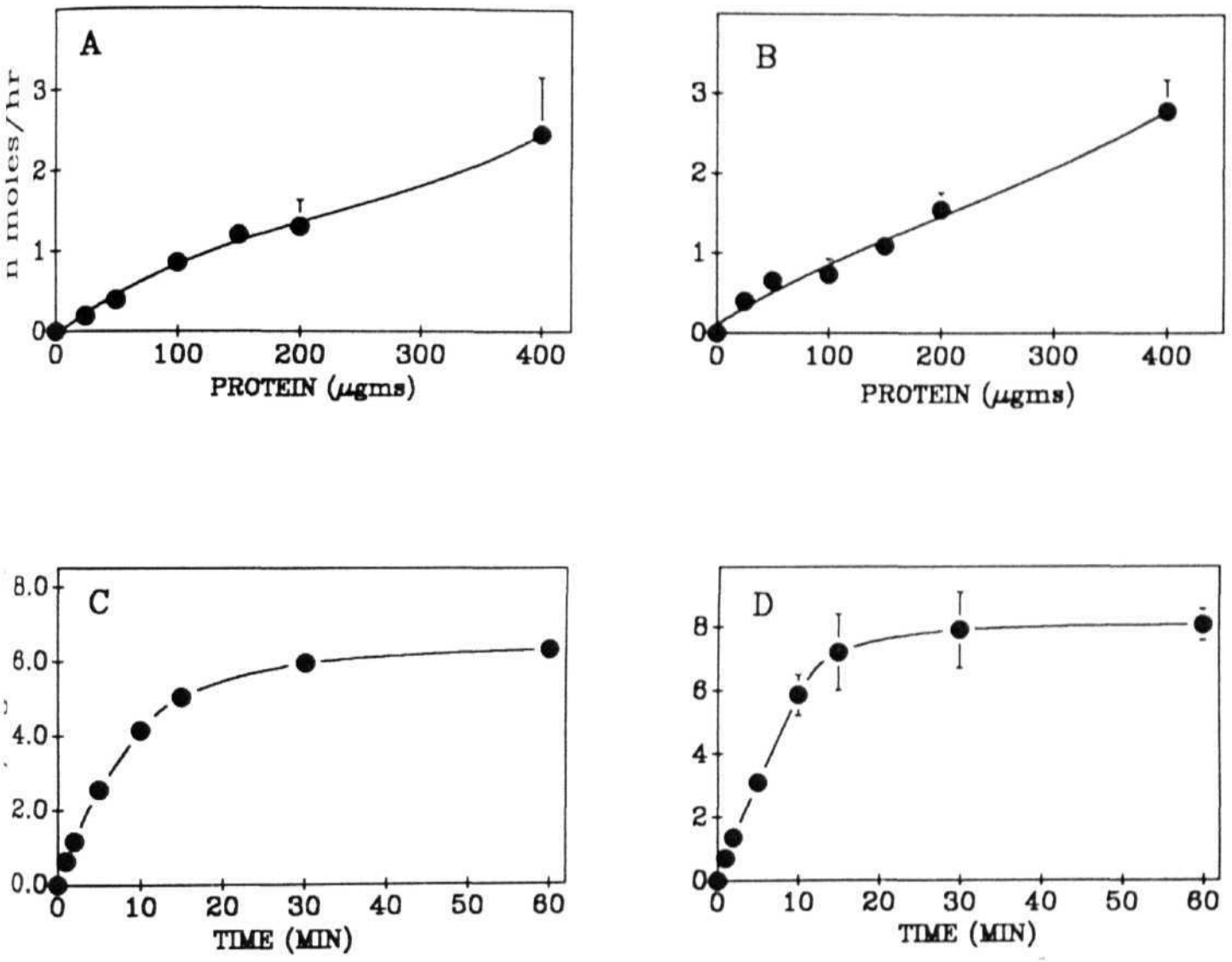


Figure 9: Transport of [^{3}H]-leucine (A) and isoleucine (B) into neurons as a function of protein concentration. (' and D: Transport of [^{3}H]-leucine (c) and isoleucine (D) into neurons as a function of time of incubation. Rest of the legends are same as figure 7.

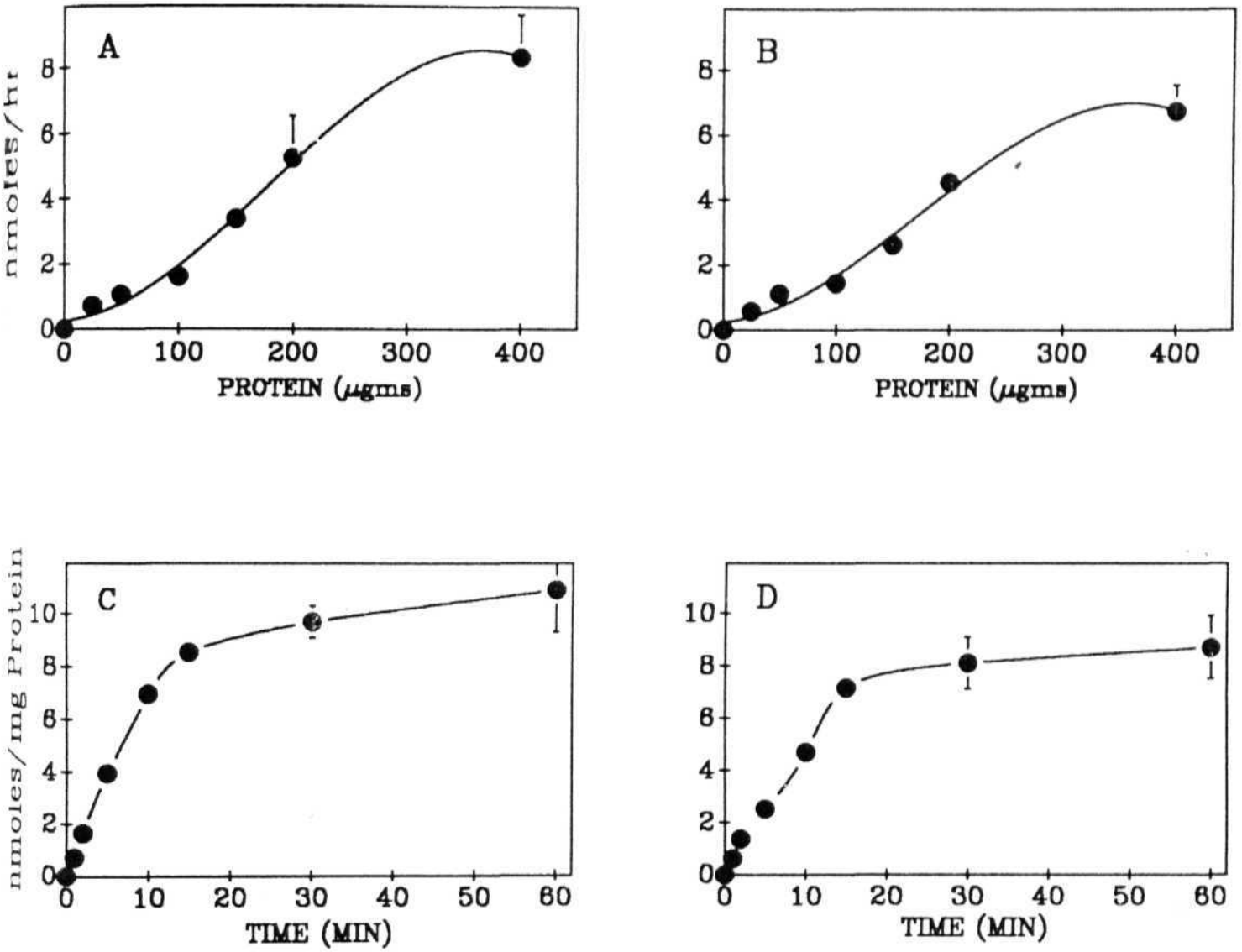


Figure 10: Transpoit of [^{3}H]-leucine (A) and isoleucine (B) into astrocytes as a function of protein concentration. C and D: Transport of [^{3}H]-leucine (C) and isoleucine (D) into astrocytes as a function of time of incubation. Rest of the legends are same as figure 7.

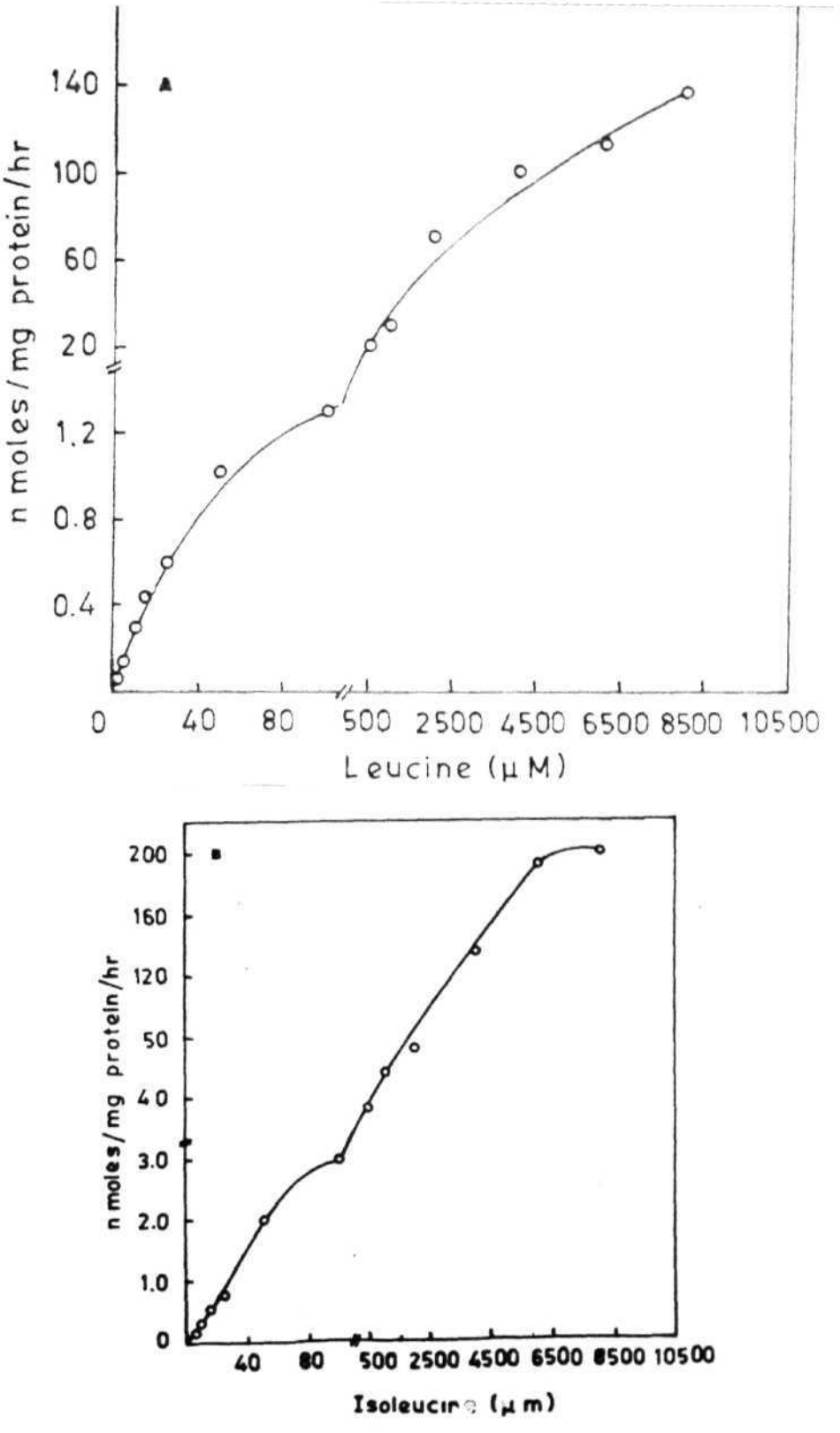


Figure 11: Biphasic saturation isotherms of [3H]-leucine and isoleucine transport into neurons. Values are mean ± S.D of 4 individual experiments done in duplicates.

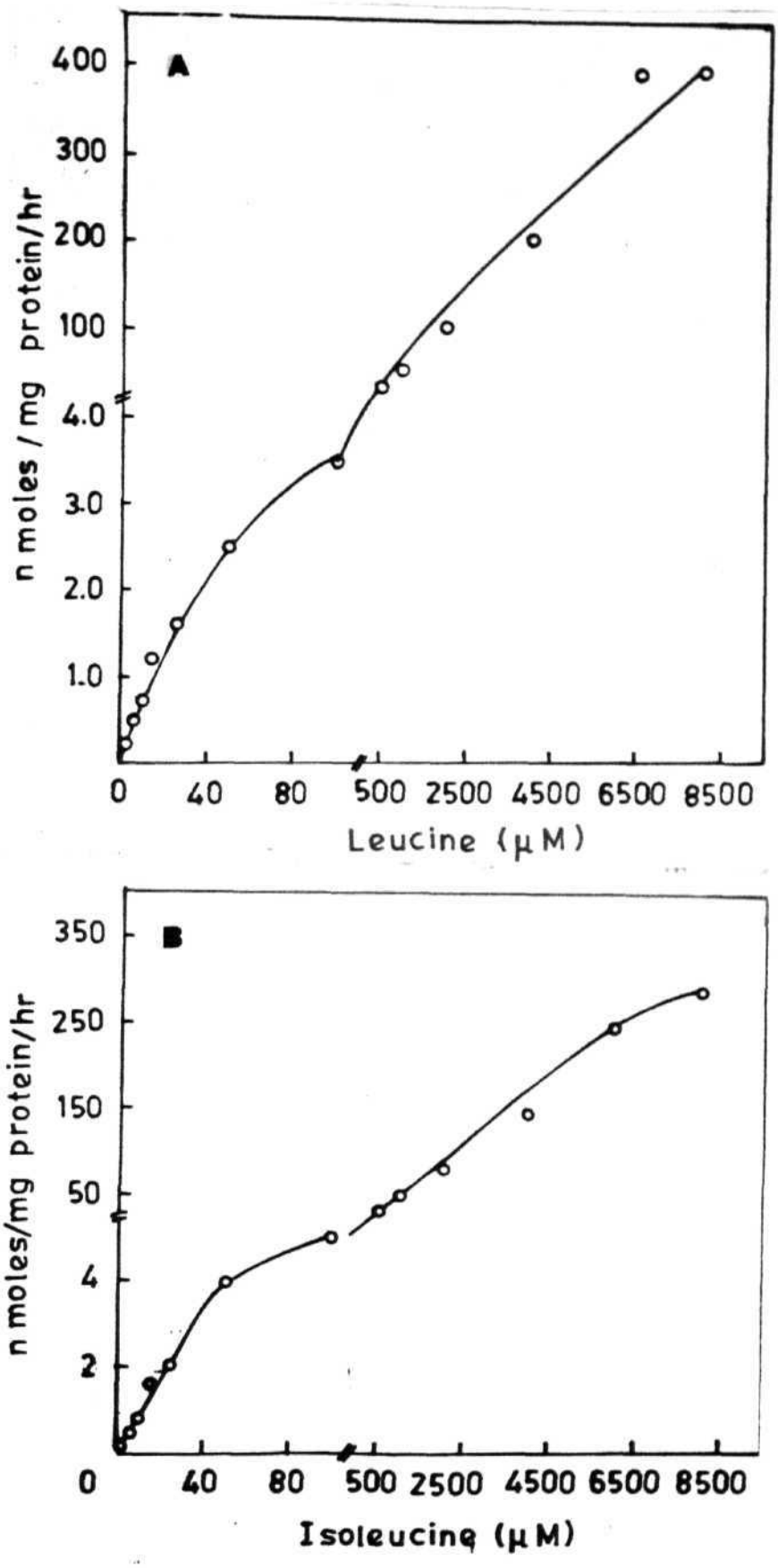


Figure 12: Biphasic saturation isotherms of [3H]-leucine and isoleucine transport into astrocytes. Values are mean ± SD of 4 individual experiments done in duplicates.

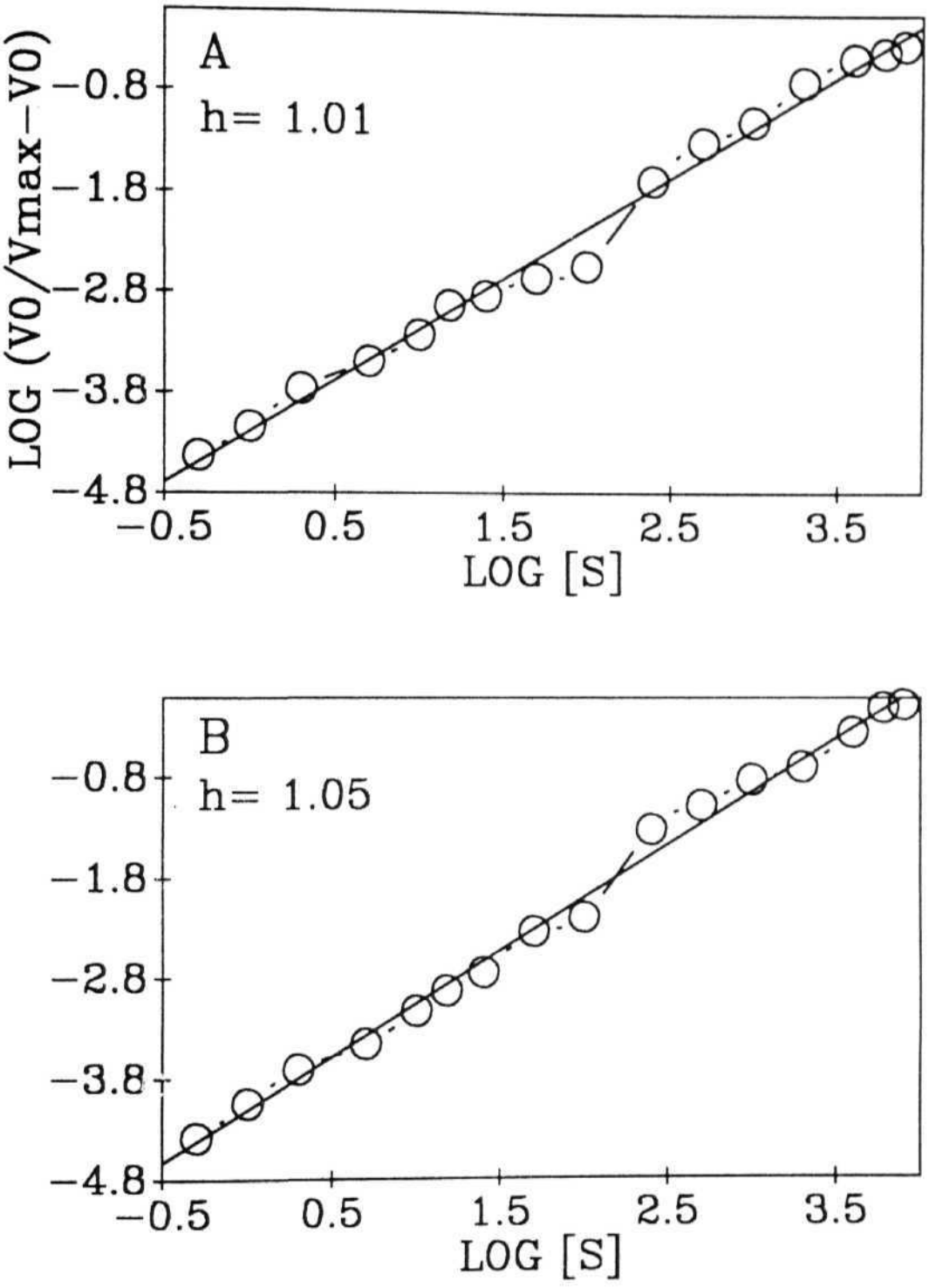


Figure 13: Hill plots of [^{3}H]-leucine (A) and isoleucine (B) transport in neurons respectively showing non-cooperativity of transporter sites. Values are mean ± SD of 4 individual experiments done in duplicates.

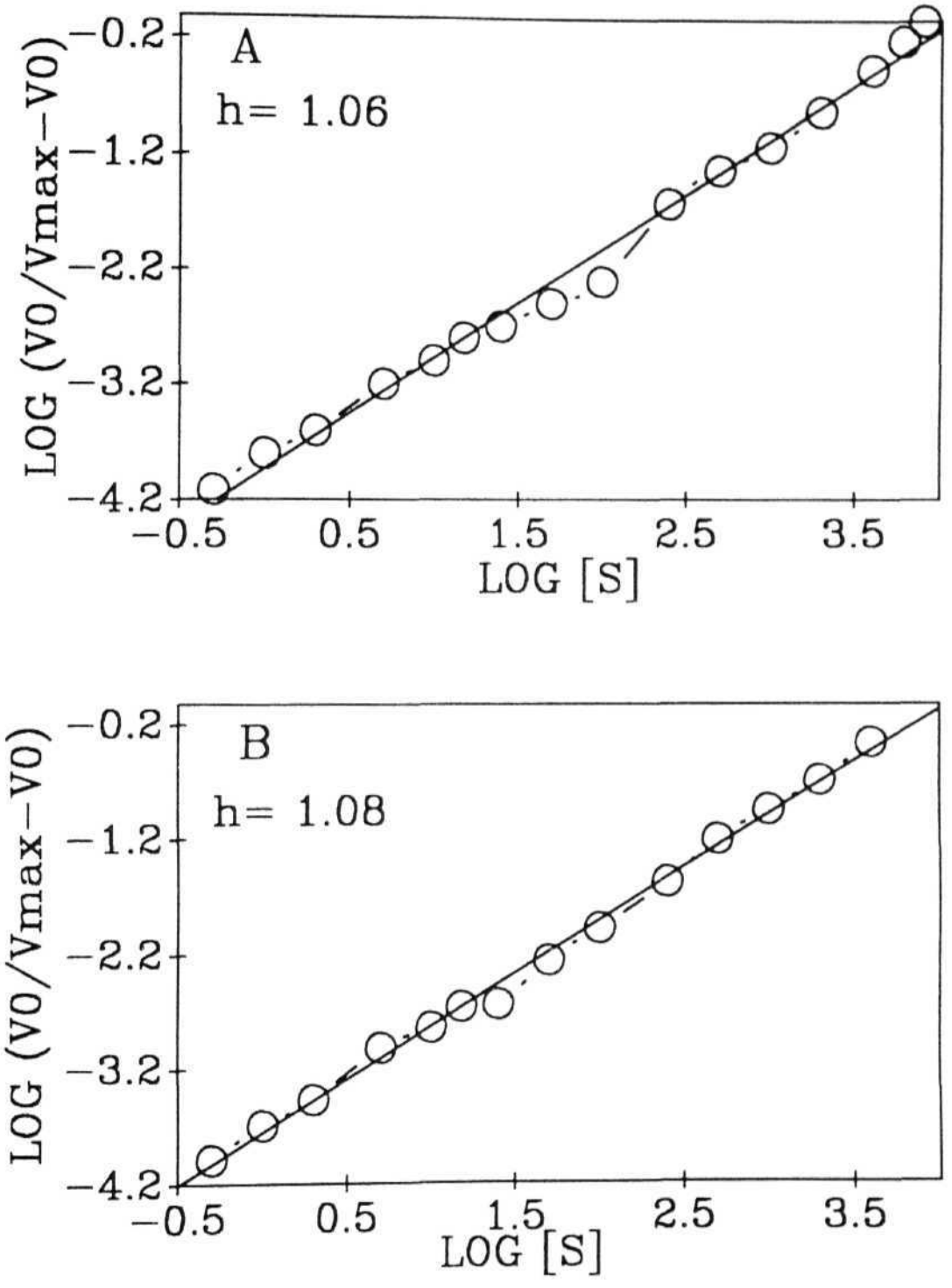


Figure 14: Hill plots of [^{3}H]-leucine (A) and isoleucine (B) transport in astrocytes respectively showing non-cooperativity of transporter sites. Values are mean ± S.D of 4 individual experiments done in duplicates.

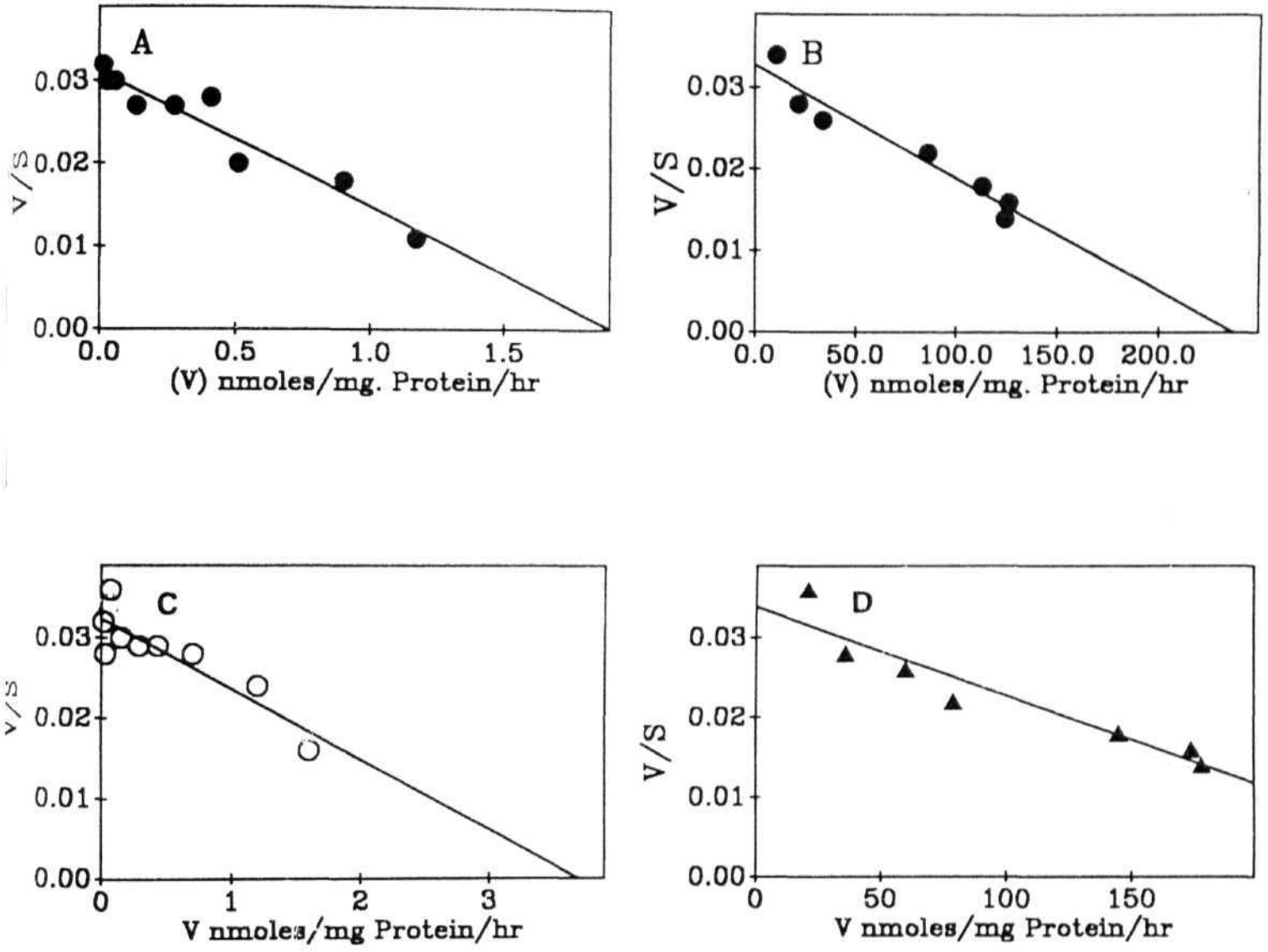


Figure 15: Scatchard plots of high (A) and low affinity (B) transport of[^{3}H]-leucine into neurons. C and D: Scatchard plots of high and low affinity transport of[^{3}H]-isoleucine into neurons. Values are mean ± S.D of 4 individual experiments done in duplicates.

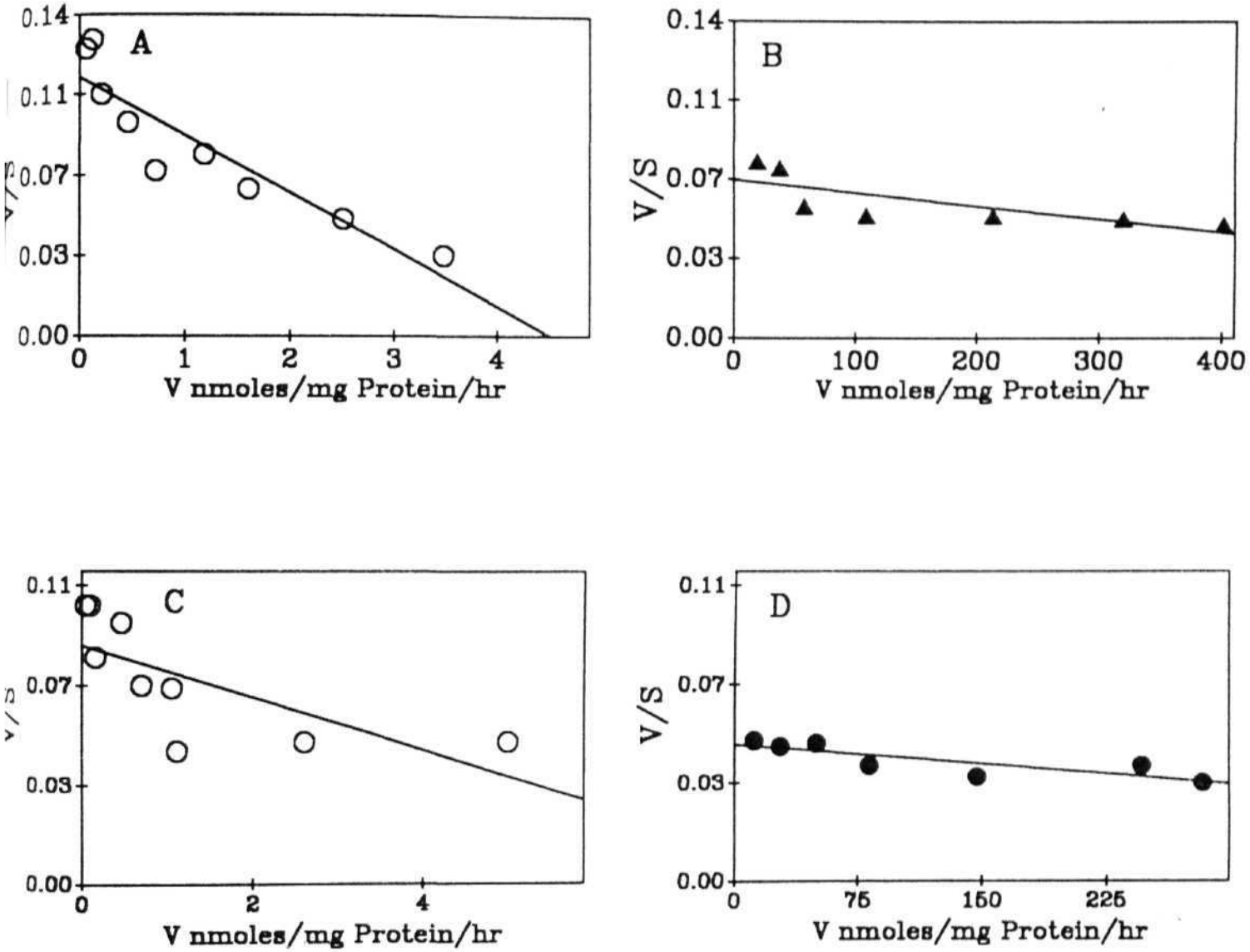


Figure 16: Scatchard plots of high (A) and low (B) affinity transport of [^{3}H]-leucine into astrocytes. C and D: Scatchard plots of high (C) and low (D) affinity transport of [^{3}H]-isoleucine into astrocytes. Values are mean ± S.D of 4 individual experiments done in duplicates.

METABOLISM OF BRANCHED-CHAIN AMINO ACIDS

Results from the previous chapter indicated that BC A A are transported into cellular and sub-cellular preparations of the brain over a wide range of concentrations and age dependent variations were also observed in the transport of BCAA. Subsequent to their transport, these amino acids are actively metabolized in the tissue. Enzymes involved in the metabolism of BCAA have been identified in brain and it has been reported that brain might be the major site of metabolism of these amino acids after skeletal muscle.

Studies on the *in vitro* activities of the enzymes involved in the metabolism of amino acids may not indicate their actual *in vivo* metabolism of those substances. The reason being that during the *in vitro* assay of an enzyme, the concentration of the substrate, cofactors and pH would be optimal, while in *in vivo* conditions the concentrations of the substrates and other cofactors, pH are optimal. Moreover, during the process of homogenization the barriers for the entry and exit of various substances and other regulatory mechanisms involved in the metabolism of compounds might be destroyed. In addition, enzymes of different metabolic pathways would be acting on the same substrate under *in vivo* conditions and the products generated may some times modulate the operational rates of either the same or different metabolic pathways utilizing the same substrate. Hence, studies merely on the enzyme reactions might give information on the optimal capacity for the utilization of substrate by the system but not the operational rate. In order to study the actual turn over of various compounds, it is always suggested to study some of the metabolic reactions in *in vivo* conditions. This is possible by using labelled substrates and measuring the intermediates or terminal products of the pathway. Such studies might give information regarding the actual rate of a metabolic pathway.

Hence, in the present investigation, production of labelled $^{14}CO_2$ front the [U-^{14}C] BCAA was studied in various cellular and sub-cellular preparations isolated from the cerebral cortex of different age groups of rats.

Rate of utilization of amino acids depends on (i) their availability at the site of utilization (ii) need for the tissue to utilize these amino acids, viz., in the synthesis of proteins and (iii) their involvement in energy metabolism. All the 20 biologically active amino acids are known to undergo metabolic degradation in tissues by various enzymes and the resulting end products are utilized for the synthesis of various other substances such as neurotransmitters (dopamine from tyrosine and phenylalanine, taurine from methionine) or gain entry into citric acid cycle and generate ATP. Depending on the nature of their end products, amino acids are classified as ketogenic and glucogenic amino acids. BCAA are either ketogenic (leucine) or glucogenic (valine) or both (isoleucine).

Extensive studies have been conducted on the metabolism of BCAA in extra-cerebral tissues. However, very few studies have been conducted on the metabolism of these amino acids in brain. It was believed earlier that BCAA are primarily utilized for protein synthesis (Patel & Balazs 1970) . Later studies have indicated that BCAA carbons are oxidized at a rate higher than their incorporation into proteins (Cremer et al., 1975). Moreover, studies on metabolic compartmentation in brain suggested that astrocytes might be the primary sites of BCAA metabolism (Berl & Clarke 1969). It was further suggested that the carbons from these amino acids might label the small pool of glutamate. However, later studies have revealed that neurons as well as oligodendroglial cells also have the capacity to oxidize BCAA to CO_2 (Chaplin, 1976., Jessy, 1988).

Very few studies have been done on the cerebral metabolism of BCAA under altered physiological or pathological states while several studies were conducted on extra cerebral tissues such as muscle, heart and diaphragm (Morgan et al., 1981; HutsonRannels1985; Tischler & Goldberg 1981). Jessy (1988) reported that $^{14}CO_2$ production from BCAA was suppressed in various cellular and sub-cellular preparations in hyperammonemic conditions. Shiota (1984) reported an increase in the cerebral production of $^{14}CO_2$ from [1-^{14}C]leucine in rats treated with carbon tetrachloride. Murthy et al., (1987b) reported a fall in $^{14}CO2$ production from [U^{14}C]- isoleucine and -valine in both undifferentiated and differentiated astrocytes in primary cultures in presence of 3 mM ammonium salts. They also reported an enhanced production of $^{14}CO_2$ from [U^{14}C]leucine under chronic hyperammonemic conditions while the $^{14}CO_2$ production from [1-^{14}C]leucine was suppressed in acute conditions of ammonium acetate treatment. In neurons, chronic exposure of ammonia lead to the suppression of $^{14}CO_2$ production from [U-^{14}C]-valine and isoleucine. Jessy (1988) reported a fall in the production of $^{14}CO_2$ fr°m [U^{14}C]-BCAA in synaptosomcs and neurons while the same was slightly enhanced in astrocytes. In mitochondria, production of $^{14}CO_2$ was suppressed from [U^{14}C]leucine and isoleucine while the same was enhanced in the case of valine. Oxidation of leucine as indicated by the production of $^{14}CO_2$ was reported to be enhanced in diabetic conditions (Brosnan et al, 1985).

Though studies were done on the oxidation of BCAA under the above said conditions, no reports are available on this aspect in aging. Though BCAA are functionally important essential amino acids constituting 45% of the total essential amino acids, involved in many vital meta-

bolic reactions such as protein synthesis and energy production. In the present investigation, an attempt was made to study the oxidation of BCAA and production of [$^{14}CO_2$] from labelled BCAA in sub-cellular and cellular preparations isolated from young (1 month), adult (3 months) and aged (24 months) rat brain cortex.

Production of CO_2 from the BCAA occurs in two stages Viz. (a) C-1 decarboxylation of BCKA mediated by BCKA-DH (b) further oxidation of acetyl CoA and succinyl CoA in TCA cycle. These two stages of CO_2 production can be determined independently by using [^{14}C]-BCAA differentially labelled at different positions. If [1^{14}C]-BCAA are used as substrates, the $^{14}CO_2$ produced during C-l decarboxylation will be labelled and the rest of the products will be unlabelled. If [U^{14}C]-BCAA are used as substrates, all the products distal to the decarboxylation reaction are also labelled. Thus, [$^{14}CO_2$] released from [U^{14}C]-BCAA would be the total sum of [$^{14}CO_2$] produced from the decarboxylation reaction and from citric acid cycle. In the present investigation, [U^{14}C]-BCAA were used to study the production of $^{14}CO_2$.

Several lines of evidence indicated that initial decarboxylation *of* BCKA (produced in the transamination reaction) is the regulatory step in the metabolism of BCAA and the enzyme BCKA-DH is subjected to activation and inactivation by dephosphorylation and phosphorylation respectively and is also regulated by several modulators (Randle, 1983). It appears that, of the total amount of BCKA produced, only a small quantity is oxidized in brain while the rest are either reaminated or transported out of the cell. Jessy & Murthy 1993) reported that less than 10% of the BCKA produced in mitochondria will be retained and oxidized in them while the rest of the BCKA are transported out and this

efflux of BCKA was enhanced in hyperammonemic conditions. They suggested that the efflux of BCKA would help in preventing excessive oxidation and loss of carbon skeletons of BCAA. Brand (1981) reported that 30% of the BCKA produced in brain are oxidized to CO_2 while the 70% of BCKA are reaminated to BCAA, thus the carbon skeleton of these essential amino acids is conserved.

$^{14}CO_2$ Production in subcellular preparations:

Production of $^{14}CO_2$ was studied in mitochondria and synaptosomcs isolated from different age groups of rats.

Before giving the details of the results obtained in the present study, it is important to mention here that the expression of the rate of production of [$^{14}CO_2$] obtained through out the studies were only the nominal rates but not the absolute rates. This distinction between the two terms is essential since the rate of CO_2 production was calculated based on the specific radioactivity of BCAA in the incubation medium but not on the specific activity of the endogenous pools of BCAA either in subcellular or cellular preparations. At the initial time periods of incubation, when the endogenous pools of unlabelled BCAA are not replaced by the labelled BCAA (from the incubation medium), much of the $^{14}CO_2$ produced would be unlabelled. As the time of incubation progresses, equilibration of endogenous unlabelled pools of BCAA with the labelled BCAA from the incubation medium also increases till the endogenous pools are completely replaced by the exogenous labelled pools of BCAA. Time required for this process varies greatly for different types of cellular and sub-cellular preparations due to the variations in the size of the endogenous pools (larger the pool size, greater the time required for the replacement). Moreover, the rate of transport of BCAA also determines

the time required for the equilibration of these amino acids as the *de nova* synthesis of these amino acids is negligible or almost absent. In addition, the endogenous pools of amino acids undergoing different metabolic transformations such as transamination, decarboxylation are segregated in different sub-cellular compartments. For example, transamination of BCAA occurs both in mitochondria and cytosol while the rest of the metabolism of BCAA occurs in mitochondria. Under such conditions different sub-pools (compartments) of these amino acids might be existing within the cellular compartment and determination of which might be difficult as intermixing of these different sub pools (especially during homogenization) cannot be ruled out. Hence, the calculation of the absolute rate of production of [$^{14}CO_2$] may not be possible under these conditions.

Preliminary standardization of [$^{14}CO_2$] production from [U-^{14}C]-BCAA was carried out in sub-cellular preparations from the cerebral cortex from rats aged 3 months. Results of these studies indicated that production of [$^{14}CO_2$] was linear up to 200 µgrams of protein in both synaptosomal and mitochondrial preparations with all the three BCAA (leucine, valine and isoleucine). Similarly, [$^{14}CO_2$] production was linear up to 60 min of incubation time in both these preparations with all three BCAA. Hence, in subsequent studies, a protein equivalent of 100 µgrams and a time period of 45 min were selected (Figures 17 & 18 A-C).

Kinetics of [$^{14}CO_2$] production could not be studied in these preparations as the [$^{14}CO_2$] production from [U^{14}C]-BCAA does not occur by a single reaction but by several reactions (C-1 decarboxylation and subsequent production of CO_2 in TCA cycle). Under these conditions, the measured rate of [$^{14}CO_2$] production might be an average of all the reactions and the significance of the results might be difficult to assess.

Hence, in the present study, 100 μM BCAA was used along with glucose.

With all the three BCAA, [$^{14}CO_2$] production was found to be higher in mitochondria (+79% with leucine,+135% with isoleucine and +170% with valine) than in synaptosomcs. Results of the present study indicated, mitochondria have the capacity to oxidize all three BCAA. However, the rate of oxidation was observed to be different with different BCAA. The rank order of the [$^{14}CO_2$] production from BCAA was found to be valine > isoleucine > leucine, suggesting that valine is oxidized at a greater rate compared to isoleucine and leucine (Table 13).

TABLE 13

AGE	LEUCINE	ISOLEUCINE	VALINE
SYNAPTOSOMES	1.31 10.2	1.3710.2	1.3 10.3
MITOCHONDRIA	2.4 ± 0.5[a]	3 1 0.4[a]	3.5 ± 0.7[a]

Production of [$^{14}CO_2$] from [U-^{14}C]-BCAA in synaptosomcs and mitochondria of 3 months-old rats. Values are Mean ± S.D of 4 individual experiments done in duplicates. [$^{14}CO_2$] production was expressed as nmoles/ mg. protein/hr.
a : p< 0.001

Such differences in the rate of oxidation of the BCAA might be due to differences in the pool sizes and /or the nature of the intermediates which enter into TCA cycle. Earlier studies on the transport (chapter 1) indicated that rate of transport of leucine was at least 1.5 fold greater than that of isoleucine, though the rate of [$^{14}CO_2$] production was higher with isoleucine than leucine. This suggested that the differences in the pool sizes of these amino acids might be responsible for differences in the rate of oxidation. The mitochondrial pool sizes of these amino acids was not determined in the present study as these amino acids might be lost due to leakage during the isolation process. However, pool sizes of these

amino acids can be estimated from the uptake studies. It is assumed that at longer incubation times, the endogenous pool of the amino acid will be completely equilibrated and replaced by the exogenously added amino acid, which gives an approximation of the pool size. From these studies, the pool size of isoleucine was found to be twice that of leucine.

Production of [$^{14}CO_2$] in synaptosomal preparations was in agreement with earlier reports of Chaplin et al., (1976) and Jessy (1988). Chaplin et al., (1976) reported that in addition to glial cells, neurons and nerve terminals were also involved in the oxidation of BCAA. This aspect was further verified and supported by Jessy (1988) who reported the presence of both BCAA-T and BCKA-DH in neuronal perikarya and nerve terminals.

Production of [$^{14}CO_2$] in mitochondrial preparation is not surprising as the enzymes involved in the BCAA metabolism were reported to be localized in mitochondria. Presence of complete TCA cycle in mitochondria might also facilitate the oxidation of the intermediates of BCAA metabolism. Moreover, higher rate of [$^{14}CO_2$] production in these preparations would ensure that BCAA are completely oxidized in mitochondria.

As it was mentioned earlier, that the transamination is the prerequisite for the oxidation of BCAA and a-KG is required for this process. Thougli it was already mentioned that glucose (10 mM) was provided in the incubation medium, glucose can not be metabolized in mitochondria due to the absence of the enzymes of glycolysis in mitochondria. In such a condition, the source of a-KG for the transamination reaction is questionable. One possibility might be that a-KG pool present in mitochondria might have survived the isolation procedures and was

adequate enough to initiate the transamination of BCAA to synthesize BCKA and glutamate. The glutamate so formed in the transamination reaction should be able to generate the α-KG lost in the transamination reaction by oxidative deamination (GDH reaction).

In this context, it is worth mentioning here that Williamson et al., (1981) using isolated rat hepatocytes estimated that only 15% of acetyl CoA produced from KIC enters into TCA cycle. In other words, contribution of acetyl CoA moieties derived from leucine catabolism was only 15% in the operation of one TCA cycle. This suggests that very less amounts of acetyl CoA derived from BCAA are utilized for energy production. Precise contribution of acetyl CoA generated from BCAA to TCA cycle has not been determined for brain preparations. However, if the same were to be true in the case of brain, then there would be an accumulation *of* acetyl CoA derived from BCAA in brain. In brain, lipid synthesis was higher at least in younger animals when compared to other tissues, and part of acetyl CoA might be utilized for the synthesis of cerebral lipids.

[$^{14}CO_2$]Production from [U-^{14}C]BCAA in Synaptosomcs and Mitochondria as a Function of Age:

Production of $^{14}CO_2$ from [U^{14}C]-BCAA was studied in synaptosomes and mitochondria isolated from the cerebral cortex of rats aged 1, 3 and 24 months. Results of these studies revealed several changes in the production of [$^{14}CO_2$] as a function of age.

There was an over all decrease in the production of [$^{14}CO_2$] with increasing age in synaptosomcs isolated from the cerebral cortex. This decrease was observed with all the three BCAA. A comparison between 1 and 3 months age groups revealed a 49% reduction in the [$^{14}CO_2$]

production with leucine, 28% with isoleucine and 52% with valine. A comparison between 3 and 24 months age groups showed a 47% reduction in the [$^{14}CO_2$] production with leucine as a substrate and no statistical significance was noticed in the case of isoleucine and valine. The profile of changes observed between 1 and 24 months age groups were statistically significant with a 73% fall in the production of [$^{14}CO_2$] with leucine, 44% reduction with isoleucine and 68% decrease with valine in cortical synaptosomcs (Table 14)

TABLE 14

AGE	LEUCINE	ISOLEUCINE	VALINE
1 MONTH	2.7±0.3	1.8 ± 0 3	2.8 ±0 7
3 MONTHS	1.36 ± 0.14[a]	1.3 ± 0.2[a]	1.4 ± 0 2[a]
24 MONTHS	0.7±0 01[b,c]	1.01 ± 0.06[c]	0.9 ± 0 1[b]

Production of [$^{14}CO_2$] from [U-^{14}C]-BCAA in synaptosomes as a function of age. Values are Mean ± S D of 4 individual experiments done in duplicates [$^{14}CO_2$] production was expressed as nmoles/ mg protein/hr Significance : a → 1 and 3 months, b → 3 and 24 months, c → 1 and 24 months,

A comparative account of [$^{14}CO_2$] production from the three BCAA within the same age group revealed an interesting pattern. In one month age group, the rank order of [$^{14}CO_2$] production was found to be valine > leucine > isoleucine while in 3 and 24 months age group no statistical significance was found in the oxidation of BCAA among the three amino acids (Table 14).

Results of the present investigation in synaptosomcs isolated from different age groups of rats indicated that (a) age dependent alterations in [$^{14}CO_2$] production from [U^{14}C]BCAA were more pronounced between 1 and 24 months age groups with the highest reduction being found when leucine was used as a substrate followed by valine and

isoleucine (b) greater rates of oxidation were observed in the production of [$^{14}CO_2$] in younger age group (1 month) with valine and leucine as substrates, lowest with isoleucine, while no such differences were noticed in latter age groups.

In mitochondria, production of [$^{14}CO_2$] was found to be much higher than synaptosomcs with all the three BCAA. The profile of age dependent changes in the production of [$^{14}CO_2$] were found to be different in mitochondria when compared to synaptosomcs. A comparison between 1 and 3 months age group showed no statistically significant changes in the production of [$^{14}CO_2$] with all the three amino acids. A comparison between 1 and 24 months and 3 and 24 months of age groups showed a statistically significant decrease (50% and 60%) in the [$^{14}CO_2$] production with all the three amino acids (Table 15).

TABLE 15

AGE	LEUCINE	ISOLEUCINE	VALINE
1 MONTH	3 ±0.3	2.62 ±0.3	3 ±0.4
3 MONTHS	2.36 ± 0.5[a]	3 ± 0.4	3.5±0.7
24 MONTHS	1.2 ± 0.2[b,c]	1.3 ± 0.2[b,c]	1.6 ± 0.3[b,c]

production of [$^{14}CO_2$] from [U-^{14}C]-BCAA in mitochondria as a function of age. Values are Mean ± S.D of 4 individual experiments done in duplicates. [$^{14}CO_2$] production was expressed as nmoles/ mg. protein/hr. Significance : a → 1 and 3 months, b → 3 and 24 months; c → 1 and 24 months.

In brief, results of the present investigation indicated that there was a significant reduction in the production of [$^{14}CO_2$] from all the three [U^{14}C]BCAA in both synaptosomes and mitochondria in rats aged 24 months when compared to younger age groups. These results on age dependent alterations in the production of [$^{14}CO_2$] in sub-cellular preparations of the brain could not be compared with the reports of earlier

investigators as there were no reports on the age associated changes in the oxidation of BCAA in brain.

Studies on the oxidation of BCAA in these preparations indicated a differential metabolism of BCAA in different brain preparations. Nerve terminals, being the part of neuronal cells, have their own synaptoplasm and synaptic mitochondria. Mitochondria used in the present study were mostly of non synaptic origin, derived from neuronal cell bodies or glial cells. Hence, it was felt worth to study the oxidation of BCAA in whole cell preparations having all the sub-cellular components, to gain more insight into the metabolism of these amino acids. Hence, studies on the oxidations of BCAA were extended to neurons and astrocytes isolated from the cerebral cortex of rats aged 1, 3 and 24 months.

[$^{14}CO_2$] Production from [U-^{14}C]BCAA in Neurons and Astrocytes :

Preliminary standardization of [$^{14}CO_2$] production was carried out in cortical neurons and astrocytes 3 months-old rats. With all the three amino acids, [$^{14}CO_2$] production was linear up to 250 µgrams of neuronal protein and 50o µgrams of astrocytic protein and till 60 min of incubation time in both neurons and astrocytes (Figures 19 & 20 A-C). In subsequent studies, protein equivalent of 250 µgrams and 45 min of incubation time were selected.

Production of [$^{14}CO_2$] was found to be higher in astrocytes than in neurons with all the three BCAA. In both neurons and astrocytes, [$^{14}CO_2$] production from isoleucine and valine was higher from leucine (Table 17).

TABLE 17

AGE	LEUCINE	ISOLEUCINE	VALINE
NEURONS	0.9 ± 0.1	1.43 ± 0.2	1.8 ± 0.2
ASTROCYTES	1.7 ± 0.2[a]	2.2 ± 0.2[a]	2.24 ± 0.17

Production of [$^{14}CO_2$] from [U-^{14}C]-BCAA in neurons and astrocytes in 3 month-old rats. Values are Mean ± S.D of 4 individual experiments done in duplicates. [$^{14}CO_2$] production was expressed as nmoles/ mg protein/hr p < 0.01

An over all comparison of [$^{14}CO_2$] production from [U^{14}C]BCAA in neurons, astrocytes, synaptosomcs revealed an interesting pattern. [$^{14}CO_2$] production was highest in astrocytes followed by synaptosomcs and neurons with all the three BCAA used. The rank order of [$^{14}CO_2$] production with leucine was found to be astrocytes > synaptosomes > neurons, while with isoleucine it was found to be astrocytes > neurons = synaptosomcs. Valine showed an order of astrocytes > neurons > synaptosomcs. Higher rate of production of [$^{14}CO_2$] astrocytic preparation than the rest of the two preparations might be due to either preferential oxidation of BCAA in these preparations than neurons and nerve terminals. Moreover, according to the theory of metabolic compartmentation BCAA are preferentially oxidized in the small pool of glutamate supposed to be localized in astrocytes and the activities of the enzymes of BCAA metabolism were reported to be high in these preparations (Berl & Clarke 1969; Jessy, 1988). Lower rate of oxidation in neurons might be due to the less availability of these amino acids than the other two preparations. This was evident from the studies of previous chapters that the rate of transport of BCAA were lower in neurons than astrocytes and synaptosomcs.

[$^{14}CO_2$]Production in Neurons and Astrocytes as a function of age :

Age dependent alterations were observed in the production of [$^{14}CO_2$] inneurons and astrocytes. There was an overall decrease in the production of [$^{14}CO_2$] from all the three [U-^{14}C]BCAA in rats aged 24 months when compared to younger age groups.

In neurons, a 30-50% reduction was observed in the production of $^{14}CO_2$ with all the three BCAA between 1 and 3 months ;: I 3 and 24 months age. A similar reduction in [$^{14}CO_2$] production was observed in astrocytes of these age groups. A comparison between 1 and 2 1 months age groups showed a more pronounced decrease in the [$^{14}CO_2$] production with a magnitude of 60-70% in both neurons and astrocytes with three BCAA as substrates (Tables 18 & 19).

Overall changes in both cellular and sub-cellular preparations in the production of [$^{14}CO_2$] revealed interesting results. A comparison between 1 and 3 and 3 and 24 months age groups for all the three amino acids showed an average reduction of 40 ± 10% in all the cellular and subcellular preparations with the exception of isoleucine in mitochondria.

A comparison between 1 and 24 months age groups showed a further decline in [$^{14}CO_2$] production and the average magnitude of decrease was found to be 65 ± 15% with a highest reduction being 80% for leucine in the mitochondria of 24 months age. This overall comparison in [$^{14}CO_2$] production indicated that, in all the preparations, the magnitude of decrease is more or less similar with very few exceptions (Tables 14 & 15; 18 & 19).

TABLE 18

AGE	LEUCINE	ISOLEUCINE	VALINE
1 MONTH	1.49 ± 0.18	2.04 ± 0.09	2.62 ± 0.18
3 MONTHS	0.87 ± 0.11	1.43 ± 0.16[a]	1.8 ± 0.2[a]
24 MONTHS	0.5 ± 0.1[b,c]	0.88 ± 0.13[c]	0.9 ± 0.1[b,c]

Production of **[$^{14}CO_2$]** from **[U-^{14}C]-BCAA** in neurons as a function of age. Values are Mean ± S.D of 4 individual experiments done in duplicates. [$^{14}CO_2$] production was expressed as nmoles/ mg protein/hr. Significance : a → 1 and 3 months, b → 3 and 24 months, c → 1 and 24 months

TABLE 19

AGE	LEUCINE	ISOLEUCINE	VALINE
1 MONTH	2.6 ± 0.4	3.9 ± 0.3	3.6 ± 0.12
3 MONTHS	1.7 ± 0.16[a]	2.17 ± 0.23[a]	2.24 ± 0.2[a]
24 MONTHS	0.9 ± 0.1[b,c]	1.33 ± 0.13[b,c]	1.5 ± 0.01[b,c]

production of **[$^{14}CO_2$]** from **[U-^{14}C]-BCAA** in astrocytes as a function of age. Values are Mean ± S.D of 4 individual experiments done in duplicates. [$^{14}CO_2$] production was expressed as nmoles/ mg protein/hr. Significance ; a —> 1 and 3 months, b→ 3 and 24 months, c→ 1 and 24 months

Survey of the literature on the cerebral metabolism of BCAA is rather disappointing. Many reports are available on BCAA metabolism in extra cerebral tissues such as heart, liver, diaphragm and skeletal muscle whose metabolism is different from brain. Earlier studies by Jessy (1988) indicated that synaptosomcs, mitochondria. neurons and astrocytes are capable of oxidizing BCAA. Values obtained by Jessy (1988) were found to be lower in all the preparations (except the values obtained with valine in neurons) than the values of the present study. Such discrepancies in the values might be due to (a) she studied [$^{14}CO_2$] production in 6 months old rats where as presently studies were carried out in 1, 3 and 24 months age groups and age dependent differences in the production of [$^{14}CO_2$] can not be ruled out. (b) In the present study, medium and the cellular and subcellular preparations were thoroughly oxygenated prior to the

experiments while this was not carried out by Jessy.

Decreased production of [$^{14}CO_2$] from [U^{14}C]BCAA in older age groups might be due to (a) decreased availability of these amino acids at the site of metabolism (due to decreased transport of BCAA) as the replenishment of tissue BCAA pool is solely due to the transport of BCAA from the extracellular environment and not by the *de nova* synthesis of BCAA (b) decreased activities of the enzymes involved in the metabolic conversion of BCAA, either by way of decreased synthesis of enzyme molecules or increased degradation (c) decreased availability of substrates other than BCAA (such as α-KG and oxaloacetate) and cofactors (such as pyridoxal-5-phosphate, thiamine pyrophosphate) etc. which are required for the metabolism of BCAA (d) suppression of glucose metabolism as a result of which availability of TCA cycle intermediates would be reduced and (e) reduced operational rates of TCA cycle to oxidize the products such as acetyl CoA and succinyl CoA generated in the metabolism of BCAA.

It was mentioned previously BCAA are transported into brain both by high and low affinity carrier mediated transport systems. It was also mentioned earlier that the former system is a low capacity transport systems operating at μmolar concentrations and the latter systems were high capacity system transporting relatively large amounts of BCAA into brain along with other neutral amino acids. High affinity transport systems were suggested to have a role in transporting minimal quantities of BCAA which might be utilized for essential functions such as protein synthesis (Rao and Murthy, 1994). Amount of BCAA transported by high affinity transport systems might not be adequate for channelling them into the degradative pathway in brain. The low affinity system that transports

BCAA in large amounts was shown to be present in all the brain preparations including mitochondria. This system was suggested to be involved in supplying BCAA required for the metabolic conversion of these amino acids. Presence of low affinity transport systems for BCAA in mitochondria also supports such a concept. Different roles suggested for high and low affinity transport systems of BCAA was akin to that assigned for other substances such as choline wherein the high affinity transport systems were involved in the synthesis of acetylcholine and the low affinity transport systems were primarily involved in the synthesis of lipids (Simon et al., 1976).

Previously, it was shown that the transport of BCAA (both high and low affinity transport) was decreased in older age groups of rats when compared to younger age groups in all the brain preparations. Under such conditions, availability of BCAA to tissue to carry out normal metabolic functions might also be lowered. This might lead to the reduced production of BCKA and further oxidation of BCKA might also be lowered. Hence, the decreased rates of production of [$^{14}CO_2$] from BCAA in the brains of older rats might be related to the lowered rates of their transport in these animals.

Decreased activities of enzymes might be responsible for the metabolic conversion of BCAA into keto acids and their further oxidation to CO_2. It was mentioned earlier that transamination was a pre-requisite for the production of BCKA in brain. Preliminary studies performed on the activity levels of BCAA-transaminase in homogenates of 6 different brain regions was found to be reduced with age (Tables 20- 22). This might lead reduced production of BCKA, hence their further oxidation. Decreased activity of this enzyme in older age groups might be due to decreased syn-

thesis of new enzyme molecules or synthesis of inactive or less active enzyme molecules.

TABLE 20

AGE	CC	CB	BS	HC	CS
10 DAYS	336 ± 20	226 ±45	515 ±45	442 ±27	347 ± 16
1 MONTH	370 ±30	341 ± 45[b]	687 ± 41[b]	374 ± 15[b]	345 ± 22
3 MONTHS	330 ±40	397 ±60[a]	740±35[a]	302±31[a]	342 140
24 MONTHS	253 ±30[ab]	192 ±11[b]	393 ±24[ab]	204 ±30[ab]	182 ±20[ab]

TABLE 21

AGE	CC	CB	BS	HC	CS
10 DAYS	295 ±23	260±18	624±62	350±26	467±26
1 MONTH	377±34[b]	385±37[b]	738±17[b]	291±44[b]	288±20[b]
3 MONTHS	450±30[a]	423±32»	876±40[ab]	394±18[b]	546±13[ab]
24 MONTHS	241±43[b]	310±25[b]	447±42[ab]	260±24[ab]	231±20[ab]

TABLE 22

AGE	*CC*	CB	BS	HC	CS
10 DAYS	379±51	432±39	1015±70	606±53	633±32
1 MONTH	483±22[b]	523±54[b]	1102±43	580±13	551±61
3 MONTHS	659±31[ab]	550±47[a]	1220±40[a]	563±70	708±45[ab]
24 MONTHS	290±40[ab]	307±30[ab]	824±60[ab]	380±28[a]	391±30[ab]

Age dependent changes in branched-chain amino acid **aminotransferase** activity with leucine (Table 20), isoleucine (Table **21)** and valine **(Table 22).** Values are Mean ± S.D of 4 individual experiments done in duplicates. Enzyme was expressed as nmoles/gm. wet. wt/min. SIGNIFICANCE: **a** = when compare to 10 days; b = when compared to previous age group. CC= Cerebral Cortex, CB= Cerebellum, BS= Brain Stem, HC= Hippocampus, CS= Carpus Striatum

Several studies have been conducted in the past by measuring the activity of this enzyme in homogenates (Rao et al., 1982; Jessy and Murthy, 1985; Jessy, 1988) or in sub-cellular preparations (Brosnon et al., 1985, Jessy, 1988). However, it must be mentioned that mere measurement of enzyme activities might not represent the actual flux of substrares through a metabolic pathway. This is due to the fact that the conditions (substrate concentration, pH and cofactors) employed for the enzyme assay are always maintained at optima! level. Such a situation might not exist in a cell. Under *in vivo* conditions, the concentrations of substrate and cofactors are always sub-optimal and their availability is governed by the operational rates of the transport systems, pool sizes and metabolic pathways that share a common substrate.

Another approach which is more close to the *in vivo* state is to incubate a cellular or subcellular preparation with the substrate and determination the enzyme activity by incubating the samples with a known concentration of labelled BCAA and the estimation of the labelled BCKA produced . This method would be close to the *in vivo* activities of the enzyme as the barriers for the entry and exit of substrate might be intact and the reactions utilizing other substrates and cofactors are also functional (Brand 1981). Very few studies were done in the past along these line in brain tissue though most of the *in vivo* studies were conducted on the extrahepatic tissues such as muscle (Wolfe et al., 1981, Nissen & Morey 1981).

Hence, in the present investigation, studies were also conducted on the production of [^{14}C-KIC] from [U^{14}C]leucine in subcellular (synaptosomcs and mitochondria) and cellular preparations (neurons and astrocytes) isolated from the cerebral cortex of 3 age groups of rats.

Production of [^{14}C-KIC] from [U^{14}C]leucine showed interesting

results in cellular and subcellular preparations. A comparison between the rate of [^{14}C-KIC] production and its subsequent oxidation and production of [$^{14}CO_2$] indicated that the rate of [^{14}C-KIC] production was found to be higher than its oxidation as indicated by the rate of [$^{14}CO_2$] production in all the above preparations. This indicates that keto acids from BCAA are produced far in excess than their oxidation which in turn indicates that the rate of transamination of BCAA might be very high when compared to the subsequent enzymatic reactions in the BCAA catabolism. An approximate ratio of transamination to oxidation of BCAA (rate of CO_2 production) in the present study indicated between 20-25% in synaptosomes, neurons and 40% in mitochondria and astrocytes. These results on the ratio of keto acid production to its oxidation, is in accordance with that of Brand (1981) who reported that 30% of BCKA produced in brain from transamination of BCAA might be oxidized while the 70% of BCKA might be transported out of the brain and/or reaminated to BCAA (Jessy and Murthy, 1993).

TABLE 23

AGE	SYNAPTOSOMES	MITOCHONDRIA
1 MONTH	8.03 ±0.8	6.21 ± 0.84
3 MONTHS	7.52 ± 1.2	6.28 ±0.48
24 MONTHS	$4.17 \pm 0.55^{b,c}$	$3.98 \pm 0.3^{b,c}$

Production of [^{14}C]KIC from [U-^{14}C]leucine in synaptosomcs and mitochondria in young, adult and old rats. Values are Mean ± S.D of 4 individual experiments, [^{14}C-KIC] production was expressed as nmoles/mg. protein/hr. Significance: a → between 1 and 3 month, b → between 3 and 24 months c → between 1 and 24 months.

Age dependent changes in the [^{14}C-KIC] production in synaptosomcs and mitochondria indicated an overall reduction in 24 months

age group of rats when compared to 1 and 3 months. A comparison between 1 and 3 months age groups showed no significant changes in the [^{14}C-KIC] production in both synaptosomcs and mitochondria while, a comparison between 3 and 24 months and 1 and 24 months showed a fall ~ 45% in synaptosomcs and a reduction of -37% in mitochondria (Table 24).

TABLE 24

S. NO	AGE	NEURONS	ASTROCYTES
1	1 MONTH	4.34 ± 1.1	10.9 ± 1
2	3 MONTHS	3.24 ± 0.5	11 ± 1.2
3	24 MONTHS	1.9 ± 0.24^{b,c}	8.7 ± 1.05^{b,c}

Production of (^{14}C]KIC from [U-^{14}C]leucine in neurons and astrocytes in young, adult and old rats. Values are Mean ± SD of 4 individual experiments. [^{14}C-KIC] production was expressed as nmoles/mg protein/hr. Significance: a → between 1 and 3 month, b → between 3 and 24 months c → between 1 and 24 months.

Similar to the above results in subcellular preparations, a reduction in the production of KIC in both neurons and astrocytes was observed in older age group of rats when compared to younger age groups. A comparison between young and adult age groups showed no significant changes in [^{14}C-KIC] production in astrocytes, while it was slightly reduced in neurons. A comparison between 1 and 24 months showed a fall in the production of KIC in both the preparations (Table 25).

Results of the [^{14}C-KIC] production in all these preparations indicated decreased production of the same in older age groups of rats when compared to younger age groups. These results were in concurrence with the measured activities of BCAA-T (Table 20-22; p. 89).

Another possibility suggested (cf. p 87) for the decreased production of [$^{14}CO_2$] from BCAA was that the activities of the enzymes and the operational rates of TCA cycle might be reduced resulting in reduction in the processing of acetyl CoA (generated from leucine) and the availability of α-KG for the metabolism (transamination) of this amino acid. Though the activities of the enzymes and the operational rate of TCA cycle were not measured in the present study, there are evidences that these two are decreased in aging.

Recently, Turpeenoja et al., (1988) reported both quantitative and qualitative changes in cerebral mitochondrial proteins during aging. They also reported that these changes were more pronounced in the inner membranes of mitochondria which synthesize enzymes involved in the metabolism of various amino acids. They suggested that the decreased synthesis of the inner membrane proteins might be due to the alterations in nuclear and/or mitochondrial genome. Since the BCKA-DH, a rate limiting enzyme in BCAA metabolism, is also localized in the inner membrane of mitochondria, such age associated changes in mitochondrial proteins might also alter the synthesis of this enzyme.

It was already mentioned earlier that production of CO_2 from BCAA occurs at two stages i.e., (a) C-1 decarboxylation of BCKA by BCKA-DH and resulting branched-chain acylCoAs which are further metabolized to acetyl CoA (leucine and isoleucine) and succinyl CoA (isoleucine and valine) (b) these compounds enter into the TCA cycle and are oxidized. Hence, the second stage of CO_2 production occurs in TCA cycle. Under these conditions, any alterations in the operational rates of TCA cycle might also affect the CO_2 production from BCAA.

Earlier reports indicated age associated changes in the operational

rates of TCA cycle and levels of TCA cycle intermediates in brain. Rapoport et al., (1982) reported that regional cerebral metabolic rate for glucose had a biphasic response in which the metabolic rate increased between 1 and 3 months age and declined between 3 and 12 months age and remained constant up to 24 months age. Smith et al., (1980) reported a 25% reduction in the glucose utilization in cerebral cortex and this was more pronounced in other areas of brain. Hoyer (1981) reported that cerebral glucose utilization decreased 1.5 times in rats between 6 and 12 months age and no further significant change in Cerebral Metabolic Rate for glucose (CMRglu). They also reported a decline in the levels of glucose, glucose-6- phosphate, pyruvate, lactate, malate, creatinine, and ATP levels while ADP levels were reported to be increased in brains of older age groups of rats. Patel (1977) reported a diminished oxidation of glucose by 40% in cerebral cortex of rats aged 24 months. Benzi et al., (1979) reported decreased activities of LDH, MDH, NADH-Cytochrome reductase in aged rat brain while Iwangoff et al., (1979) reported a moderate decrease in the glycolytic enzymes in different regions of rat brain during old age.

All the above reports indicated a decrease in the operational rates of cerebral glucose metabolism. This might result in diminished operation of TCA cycle which might be due to the synthesis of inadequate levels of intermediate compounds or diminished activities of enzymes of TCA cycle. It was mentioned earlier, that metabolism of BCAA is closely coupled to glucose metabolism and glucose is known to stimulate BCAA oxidation. Moreover, end products of BCAA metabolism (acetyl CoA and succinyl CoA) might modulate the flux of carbons through TCA cycle (Odessy et al., 1974). Under such conditions, alterations in the glucose

utilization in older age groups *of* animals would affect BCAA oxidation and production of CO_2. As a result, acetyl CoA and succinyl CoA produced from BCAA might not be efficiently oxidized in TCA cycle. This might even lead to the accumulation of these intermediates. It is well known that accumulation of these metabolites might exert feed back inhibition not only on BCKA-DH but also on other related dehydrogenascs such as PDH and α-KGDH and suppress oxidation of keto acids including those produced from BCAA (Randle 1983).

As mentioned above, no direct measurements were done on the activities of the TCA cycle enzymes or the operational rates of this cycle. However, two parameters viz. incorporation *of* label from leucine into lipids and into glutamate were determined which provide information on the operational rates of TCA cycle.

It was mentioned earlier that the reduced production of CO_2 from BCAA in aged animals might be due to the decreased activities of the enzymes involved in their metabolism. It has been shown that BCAA-T, the initial enzyme of the degradative pathway, was decreased resulting in the lowered production *of* BCKA. However, this does not reveal the status of the down stream degradation *of* KIC to acetyl CoA in aged animals. In order to study this aspect, incorporation of acetyl CoA, the end product of KIC degradation, into lipids was determined.

It must be mentioned that the important precursor for the synthesis of cerebral lipids is glucose (Dopeshwarker et al., (1979). However, alternate sources for the synthesis of cerebral lipids have been identified by several investigators. These include, ketone bodies such as acetoacetate and β-hydroxy butyrate (Owen, 1967), branched-chain amino acids (Stillway et al 1978).

Studies of Dopeshwarker et al., (1979), Miettenin et al.. (1968, Stillway et al., (1978), Grigor et al., (1970), Smith (1974) indicated that branched-chain amino acids are major alternate precursors for the synthesis of brain lipids. These authors reported that carbons of branched chain amino acids especially, leucine were incorporated into cholesterol, free fatty acids, phospholipids and to some extent glycolipid. P-Hydroxy-methyl glutaryl CoA (HMG CoA), an intermediate in the catabolism of leucine was found to be a direct precursor for the synthesis of cholesterol in brain (Stillway et al., 1978).

Incorporation of [^{3}H]Leucine into Cerebral Lipids :

Presently incorporation of label from [^{3}H]leucine into cerebral lipids was studied in 4 different age groups of rats. Preliminary studies with intraperitoneal injection of label into the animal, especially into adults, resulted in negligible incorporation of the label into brain lipids. This might be due to the fact a large portion of the injected label was being utilized by muscle and very small amount of the label might be reaching the brain. Under such circumstances, the acetyl CoA generated from leucine might be diluted with the acetyl CoA from glucose. Studies with intravenous injection, though were feasible with adult animals, were difficult to perform with young/neonatal animals. Hence, to maximize the availability of label to the brain, 10 μCi/100 gm body wt. of [^{3}H]leucine was injected into the brain through a canula implanted into the skull. Different classes of lipids (cholesterol, phospholipids and glycolipids) were extracted and the amount of label incorporated was determined along with their content.

Results obtained in the present study on the content of cholesterol, phospholipids and glycolipids (Tables 25-27) were found to be similar to that of earlier investigators (Cabezas et al., 1991).

Studies on the incorporation *of* label into these lipids indicated that the label from leucine was equally distributed into cholesterol, phospholipids and glycolipids at a given age group, except in glycolipids in 10 days old rats (Tables 25-27). This was in accordance with the earlier reports of Dopeshwarker et al., (1979).

Age dependent alterations were observed in both the content as well as incorporation of label into all these lipids. Cholesterol content was increased consistently with increasing age, while no significant changes were observed in the content *of* phospholipids. Glycolipid content was very low in 10 days old rats, while it consistently increased in latter age groups of rats. These results indicated that alterations observed as a function of age were different for different classes of lipids (Tables 25-27).

TABLE 25

AGE	CONTENT (mg / gm. wt	SPECIFIC ACTIVITY dpm/ mg. cholesterol
10 DAYS	4.65±0.88	367 ± 96
1 MONTH	7.96 ± 0.5^{b}	284 ± 15
3 MONTHS	10.6 ± 0.2^{ab}	304 ± 6
24 MONTHS	20. 8 ± 3.6^{ab}	162 ± 22^{ab}

TABLE 26

AGE	CONTENT (mg / gm. wt)	SPECIFIC ACTIVITY dpm/ mg phospholipid
10 DAYS	20 ± 1.4	235 ± 32
1 MONTH	18 ± 1.3	161 ± 20^{b}
3 MONTHS	25 ±3.0	161 ± 25
24 MONTHS	23 ± 1.0	140 ± 23^{a}

Age dependent changes in the cholesterol (Table 25) and phospholipid content (Table 26) and the incorporation of leucine carbon into these **lipids.** Values are Mean ± S.D of 4 different experiments. Significance : a → when compared to 10 days animals; b → when compared to previous age group.

Results of the present investigation, corroborates with the results of Singh and Rao (1979) who reported increased cholesterol and phospholipid contents in older chick brains. Increased cholesterol content in rats of 24 months age groups was also reported earlier by Nagy et al., (1983). They suggested that the increased cholesterol and decreased phospholipid contents might alter the cholesterol phospholipid ratio of the cerebral membranes as a result of which the physico-chemical properties of the membranes might be altered. They suggested that under such conditions membrane fluidity is decreased while its viscosity is increased. Moreover, altered contents of cholesterol and phospholipid also affect the lipid-protein interactions and the orientation of proteins in th membrane. Such changes in the membrane would alter the membrane associated phenomenon such as transport. One of the reasons for the decrease in the transport of BCAA in the aged animals, observed in the present study, might be due to the changes in the cholesterol to phospho-lipid ration of the membranes in the aged animals.

TABLE 27

AGE	CONTENT (mg / gm. wt)	Sp. ACTIVITY (dpm/ mg glycolipid)
10 DAYS	1.47 ± 0.2	953 ± 55
1 MONTH	5.98± 1[b]	257 ± 58[b]
3 MONTHS	9.5 ± 1.4[ab]	246 ± 18[a]
24 MONTHS	13.1 ± 1[ab]	269 ± 33[a]

Age dependent changes in the glycolipid content and **the** incorporation of leucine carbon into glycolipid. Values are Mean ± S.D of 4 different experiments. Significance : a → when compared to 10 days animals; b → when compared to previous age group.

Results of the incorporation of label from leucine into cholesterol revealed a reduction in older age groups of rats when compared to younger age groups. Incorporation of label into phospholipids was higher in 10 days old rats and decreased in the remaining age groups. Interestingly, the incorporation of label into glycolipids was very high in 10 days old rats while it decreased in the remaining age groups. Overall decrease in the incorporation of label into lipids con finned the reduced metabolism of BCAA and the production of acetyl CoA in older age groups when compared to younger age groups.

It is well known that acetyl CoA, irrespective of the source, once formed also enters the TCA cycle where its carbons are distributed into various intermediates and are finally oxidized. However, it has been shown that the carbons of TCA cycle intermediates, especially of α-KG, are in equilibrium with the carbons of the glutamate family (glutamate, aspartate, glutamine, alanine and GABA) of amino acids in brain (Balazs et al., 1970). It has been reported earlier that the carbons of leucine are incorporated into glutamate (Cremer et al., 1975). This is possible only if the carbons of leucine are converted to acetyl CoA, enter into TCA cycle and are distributed among various intermediates of the cycle including α-KG.

It has been reported that the operational rates of TCA cycle (results of earlier investigators; cf. above) and production of CO_2 from BCAA (present study) are decreased in aged animals. Under such conditions, the entry of labelled carbon from leucine into glutamate should also be reduced. To verify this tenet, synaptosomcs, were isolated from the cerebral cortex of 3 different age groups of rats and incubated with 100 μM [U^{14}C]-leucine (0.5μCi) for 30 minutes. Incorporation of label from

leucine into glutamate was determined as described in methods.

Before describing the results of this study, it must be mentioned that incorporation of labelled carbons from leucine into glutamate does not represent net synthesis while the same process from valine and isoleucine represent the net synthesis. This is due to the fact that the carbons of acetyl CoA (generated from leucine) are merely distributed into the intermediates of citric acid cycle and they do not alter the levels of these intermediates. However, the latter two amino acids produce succinyl CoA and thus add to the net flow of carbons in the citric cycle.

It is well known that in brain, glutamate exists in two pools - small pool (located in glial cells) and large pool (located in neurons).

Incorporation of carbons of leucine into glutamate:

Results *of* the present study indicated that the carbons of leucine were incorporated into glutamate in nerve terminals (synaptosomcs) indicating the precursor nature of leucine for the synthesis of glutamate in nerve terminals. However, incorporation of carbons of leucine into glutamate family of amino acids were shown earlier (Cremer et al., 1975) but present results may not provide any information whether the label from leucine was incorporated into releasable or non-releasable pool of glutamate as studies were not conducted under depolarizing conditions.

Age dependent decline in the incorporation of leucine carbons into glutamate was more pronounced in 24 months age groups when compared to younger age groups in nerve terminals . A comparison between 1 and 24 months showed a significant decrease (-54%) in the incorporation while a comparison between 1 and 3 and 3 and 24 months age groups showed no significant changes in the incorporation in synaptosomal preparations (Table 26).

TABLE 26

AGE	Sp. ACTIVITY dpm/mg. protein/hr
1 MONTH	7116 ± 1000
3 MONTHS	5600 ±650
24 MONTHS	3938 ± 253[b,c]

Values are Mean ± S.D of 3 different experiments done in duplicates. Significance : a→ between 1 and 3 months, b → between 3 and 24 months, c→ between I and 24 months.

Decreased incorporation of label from leucine into glutamate might be explained in terms of reduced production of BCKA (thus, acetyl CoA) and its subsequent utilization in TCA cycle. This might be due to the reduced synthesis/activities of the enzymes and levels of intermediates of TCA cycle, as described above.

It is well known that in brain, glutamate exists in two pools - a small pool (possibly localized in astrocytes) and a large pool possibly localized in the neurons. The latter pool is known to have another sub-pool from which glutamate is released on neuronal depolarization. This pool is called the releasable (neurotransmitter) pool while the rest of the large pool is known as metabolic pool (Berl & Clarke 1969; Van den Berg, 1976 Benjamin and Quastel, 1974). It was earlier suggested that leucine is preferentially used in the small pool (astrocytic) as a precursor for the production of glutamate. However, results of the present study indicated that the synaptosomcs, which form a part of the large compartment of glutamate, were also capable of producing glutamate from leucine. This is in conformity with the results of the present study and those of earlier investigators (Chaplin et al., 1976;

Jessy, 1988; Jessy and Murthy, 1989) that neuronal preparations are capable of oxidizing leucine and other BCAA. However, results *of* the present study fail to indicate the precise localization of this glutamate in the neuronal (large) compartment, i.e., releasable pool or metabolic pool.

Results of the present study indicated that transport and metabolism of the BCAA are reduced in aging. This might be physiologically significant as the energy demands in senescent period might be lower when compared to younger age groups due to cessation of most of the activities such as reproduction, exploratory behavior etc. Such progressive degeneration of normal performance *of* organs during senescent penod might impose less demand on energy stores. Under such conditions, there might be an inherent negative feed back on the catabolism of compounds such as glucose and amino acids. This might prevent precursors undergoing excessive metabolic conversions and spare them for essential pathways.

In addition, it was shown earlier that BCAA transport was also decreased with age. Hence, less amount of these amino acids would be available to the tissue. Under such conditions, if the rate of oxidation is not decreased, then most of these amino acids entering the cell would be oxidized and might not be available for essential functions. Thus, it appears that the reduced oxidation of BCAA in aging might be a physiological adaptation to the lowered intake of food and availability of essential amino acids to the tissue.

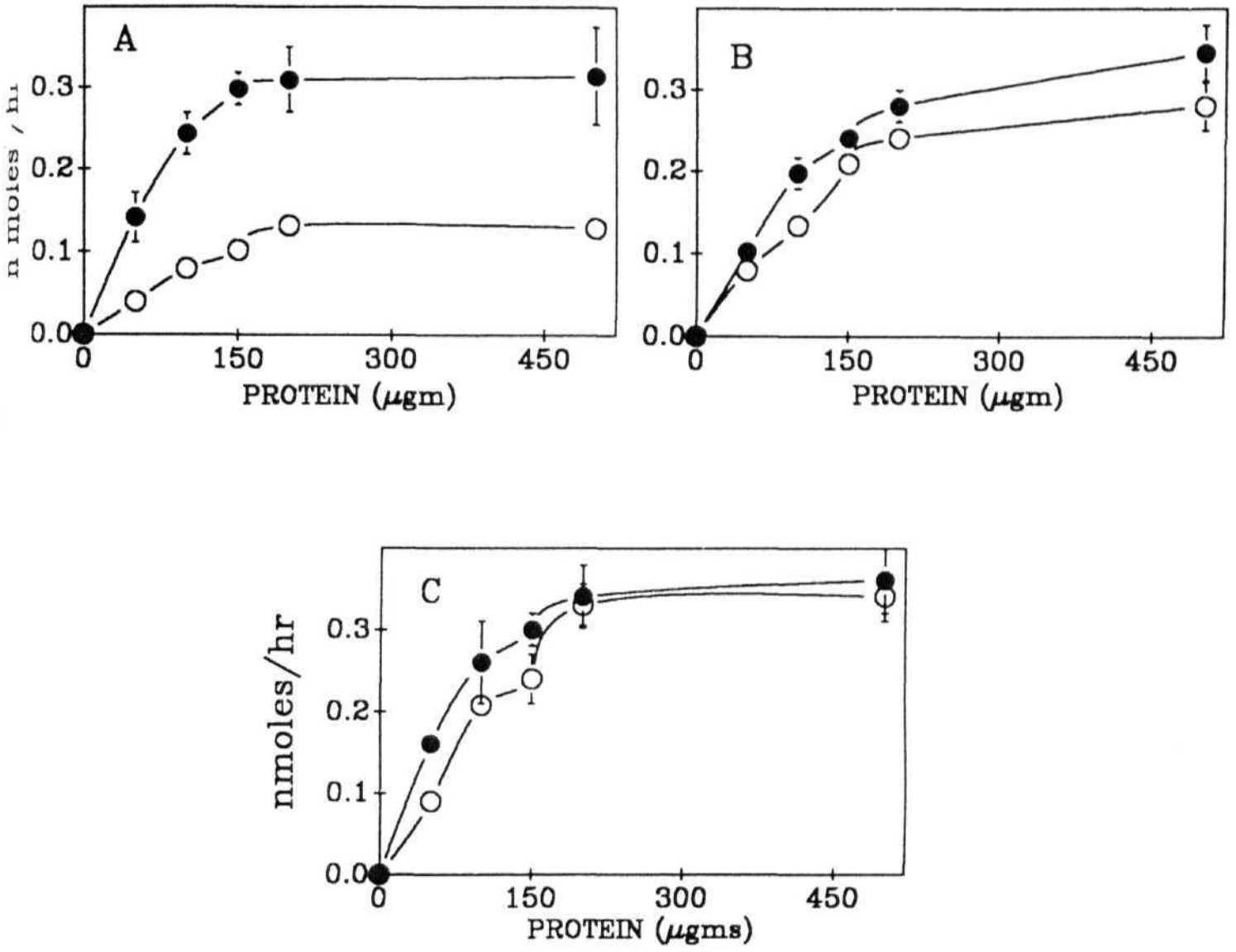


Figure 17: [$^{14}CO_2$] production from [U-^{14}C]-leucine (A), isoleucine (B) and valine ((') respectively in synaptosomes and mitochondria as a function of protein concentration. Values are mean ± S.D of 4 individual experiments done in duplicates. O—O represents synaptosomes and ●—● represents mitochondria.

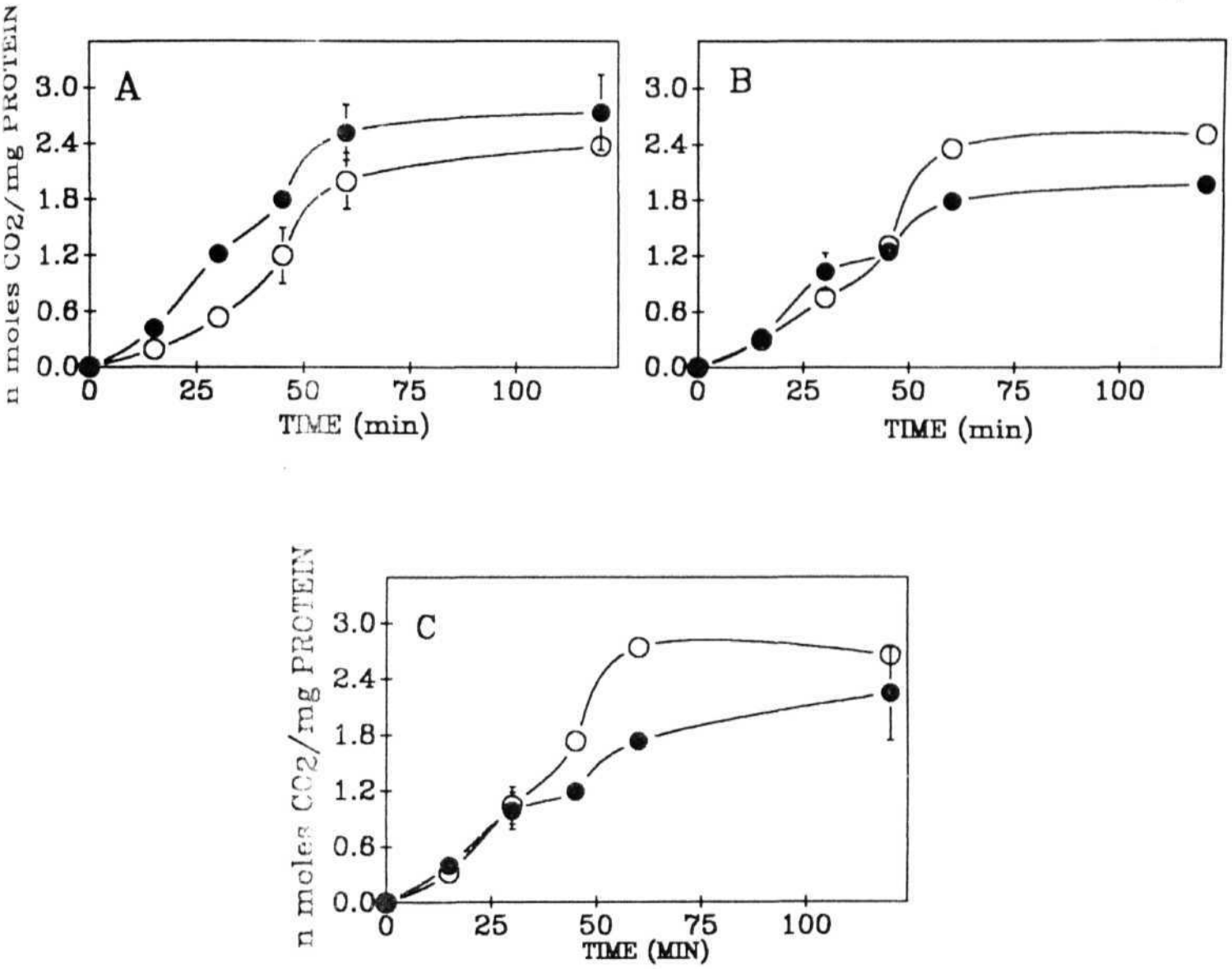


Figure 18: [$^{14}CO_2$] production from [U-^{14}C]-leucine (A), isoleucine (B) and valine (C) respectively in synaptosomes and mitochondria as a function of time of incubation. Values are mean ± S.D of 4 individual experiments done in duplicates. O—O represents synaptosomes and •—• represents mitochondria.

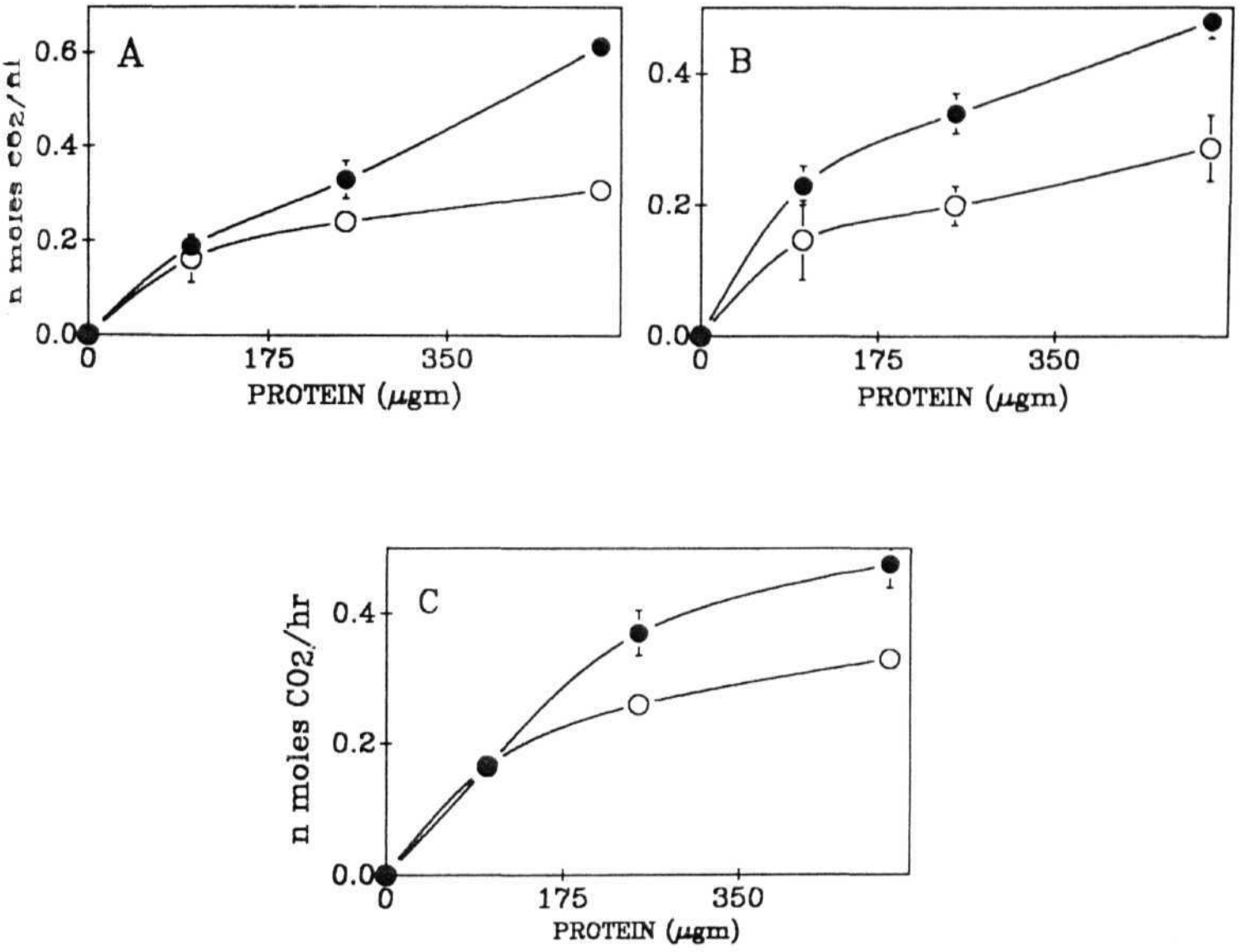


Figure 19: [$^{14}CO_2$]production from [U-^{14}C]-leucine (A) isoleucine (B) and valine (C) in Neurons and Astrocytes as a function of protein concentration. Values are mean ± S.D of 4 individual experiments done in duplicates. O—O represents neurons and ●—● represents astrocytes.

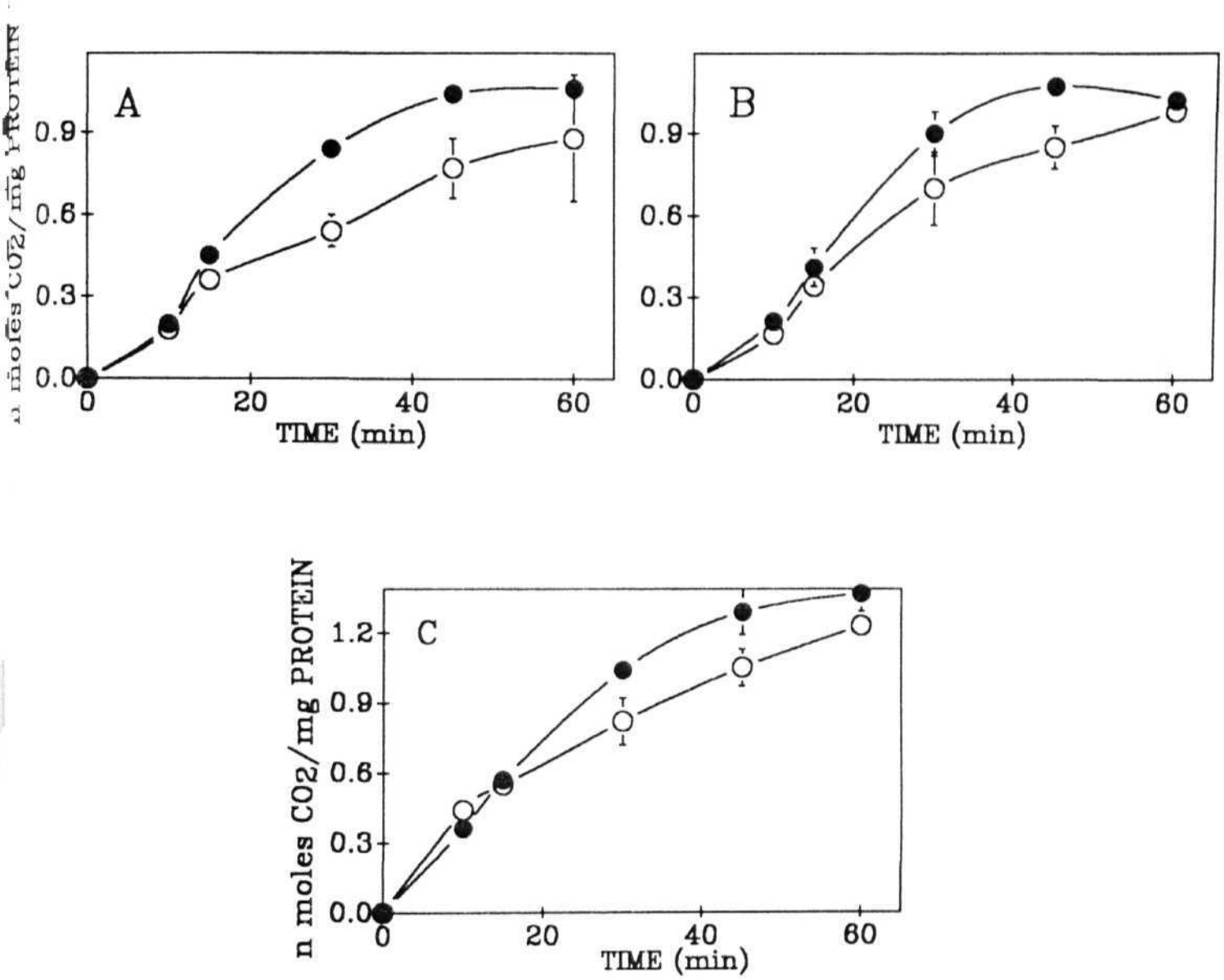


Figure 20: $[^{14}CO_2]$ production from [U-^{14}C]-leucine (A), isoleucine (B) and valine (C) in Neurons and Astrocytes as a function of time of incubation. Values are mean ± S.I) of 4 individual experiments done in duplicates. O—O represents neurons and • — • represents astrocytes.

SUMMARY

(1) BCAA metabolism was studied with respect to aging in various cerebral preparations such as nerve terminals (synaptosomcs), mitochondria (non-synaptic), neurons and astrocytes.

(2) Results of this study revealed the presence of a novel high affinity, low capacity transport system for BCAA in nerve terminals, neurons and astrocytes but not in mitochondria.

(3) In addition to the above, a low affinity high capacity system for the transport of these amino acids was identified in all the cerebral preparations including mitochondria.

(4) Both these systems were further characterized. High affinity transport systems had a low Km (2 to 3 order of magnitude) less than the low affinity transport system, suggesting that this carrier would be saturated at very low concentrations of BCAA.

(5) The rate of transport (Vmax) of the high affinity transport system was very low (100 fold) when compared to that of low affinity transport system. In other words, the high affinity system acts as a low capacity system while low affinity system acts as a high capacity system for the transport of BCAA.

(6) High affinity transport system requires sodium for the transport while low affinity transport system is not sodium dependent.

(7) Both these transport systems are stereo-specific as indicated by the lack of effect of BCH (a leucine analogue) and D-leucine.

(8) It is suggested that high affinity transport systems of BCAA might be involved in maintaining adequate amounts of essential amino acids in tissues when their supply is very low (as seen in prolonged starvation, undernutrition and malnutrition). Thus, an additional

function has been assigned to the high affinity transport systems which were thought to be involved in the termination of action of amino acid neurotransmitters (glutamate and GABA).

(9) The tenet that presence of high affinity transport systems for the transport of essential amino acids (BCAA) was further confirmed by demonstrating the presence of a similar system for methionine.

(10) A comparison of the transport indicated that the rate of this process is high in mitochondria than in synaptosomcs. Such high rates of transport into former preparations may be required to support the greater rates of metabolism of these amino acids.

(11) A similar comparison between neurons and astrocytes revealed that the rate of transport was higher in the latter cell types than in the former which is commensurate with the concept that astrocytes (representing the small pool of glutamate) are seats for the metabolism of BCAA.

(12) Age dependent studies indicated that Km and Vmax of the transport process declined with age in all the preparations.

(13) It is suggested that the decreased Km (increased affinity) in older age groups might be an adaptation to decreased food intake (thus lowered supply of BCAA) in aged animals. Under such conditions, transporters with low Km (high affinity) would be more efficient in transporting these essential amino acids than a system with a high Km (low affinity).

(14) Decreased rate of transport in aged animals is in concurrence with the reduction in the rate of metabolism of BCAA (see below).

(15) To study the metabolism of BCAA, CO_2 production from [U-^{14}C]-BCAA was measured in these cerebral preparations. Higher rate of

$^{14}CO_2$ production in mitochondria was in agreement with the localization of enzymes of BCAA metabolism in these preparations. Similarly, higher rate of CO_2 production in astrocytes than in neurons was in agreement with the theory of metabolic compartmentation.

(16) Production of $^{14}CO_2$ in all these preparations was reduced in aged animals. this might be due to (a) decreased transport (b) decrease in the production of BCKA and their further metabolism and (c) decrease in the operational rates *of* TCA cycle. Reduced rate *of* transport has already been reported.

(17) Activities of BCAA-T, initial enzyme of BCAA metabolism responsible for the production of BCKA, were found to decrease in the brains of aged animals.

(18) Production of BCKA from labelled BCAA with respect to aging was studied. It was observed that BCKA production was far in excess of their rate of oxidation. Hence, it was suggested that BCKA may be transported out of the cell and utilized by other cells of brain or other tissues (if transported out of the tissue).

(19) Rate of production of BCKA was also decreased with age.

(20) Down stream of degradation of BCKA, resulting in the production of acetyl CoA was measured by following the fate of acetyl CoA in brain. In brain, acetyl CoA is utilized in (a) TCA cycle (resulting CO_2 production) (b) incorporation into lipids (c) other minor reactions (such as synthesis of acetylcholine). It has already been shown that CO_2 production from BCAA, thus the oxidation of acetyl CoA produced from BCAA was reduced in aging.

(21) Incorporation *of* BCAA carbons into cerebral lipids as well as into amino acids such as glutamate was reduced in aged animals.

(22) Above studies are indicative of reduced metabolism of BCAA in aged animals. As suggested, this might be a physiological adaptation as it spares these essential amino acids for other vital reactions such as protein synthesis. This is especially important when the in take of food (thus BCAA) is reduced in senescent animals.

REFERENCES

Adibi, S. A (1980) *J. Lab. Clinic .Med.* 95, 475

Adibi, S.A and Gray, S.J (1967) *Gastroenterology* 52, 837-845.

Agranoff, B. W., Siegcl. G. J., Albers, R. \V and Molinoff, P. B (1993) *Basic Neurochemistry.* Fifth edition, Raven Press, New York. 545.

Balazs, R., Machiyama., Y., Hammond, B. J., Julian, T and Richtcr, D (1970) *Biochem. ,/.* 116, 445.

Beaubernnard , C, Delorme, M-L., Opolon, P et al., (1984) *Hepatology.* 4, 288-294.

Benjamin, A. M and Quastel, J. H (1974)./. *Neurochcm.* 23, 457-464.

Benjamin, A. M, Verjee, H and Quastel, J. H. (1980) *J. Ncurochcm.* 35, 67-77.

Bcnuk, M., Stern, F and Lajtha, A (1971)./. *Ncurochcm.* 18, 1555-1557.

Bcnzi, G., Arrigoni, E., Dagani, E., Marsatico, F., Curti, D., Manzini, A and Villa, R. F (1979) *Biochcm. Pharmacol.* 28, 2703-2708.

Berl, S and Clarke, I). D (1969) In *"Handbook of Neurochemistry"*(A. Lajtha ed) Plenum Press. New York . Vol 2, pp 447.

Berl, S **(1974)** *Exptl. Biol. Med.* 4, 71-84.

Bernstein, C and Bernstein, H (1991) *Age ing, Sex, and DNA repair.* Academic Press, Sandiago. CA, USA.

Betz, A. L and Goldstein, G. W. (1981) *Science.* 202, 225-227.

Bhaskar, M. .S. and Subba Rao, K (1994) *Biochem. Mol. Biol. Intl.* 33, 377-389.

Blackburn, G.L., Moldawer, L.L., Usui, S., et. al., (1979) *Surgery* 86,

Blackburn, G. L., Desai S. P.. Keenan, R. A., Bentley, B. T. (1981) In *"Metabolism and Clinical Implications of Branched Chain Amino and Ketoacids"* (Walser, M and Williamson, J. R eds) Elsevier/NorthHolland, pp 521-526.

Brand, K (1981) *Biochim. Biophys. Ada* 677, 126-132.

Bronk, J.R and Leese, H.j (1974) *J. Physiol* (Lond). 241, 271-286.

Brosnon, J. T., Robert, G. P. F and Brosnon, M. E (1984) *Am. J. Physiol.* 247, C 450-453.

Brosnon, M. E., Lowry, A., Yasmin, W et al., (1985) *Can. J. Physiol. Pharmacol.* 63, 1234-1238.

Buse, M.G., Biggers, J. F., Drier, C and Buse J. F. (1973)./. *Biol. Chem.* 248, 697-706

Buse, M.G and Reid, S. S (1975) *J. Clin. Invest.* 56, 1250

Cabezas, J. A., Andres, R., Hueso, P et al., (1991) *Neurochem. Res.* 7, 781-785.

Cangiano, C., Cangiano, P. C, James, J. H et al., (1983)./ *Biol. Chem.* 258, 8949-8954.

Chainy, G. B. N and Kanungo, M. S (1976) *Biochim. Biophys. Res. Commun.* 72, 777-781.

Chainy, G. B. N and Kanungo, M. S (1978)./. *Neurochem.* 30, 419-427.

Chaplin, E. R., Goldberg , A and Diamond, J (1976) *J. Neurochem.* 26, 701-708.

Chaturvedi, M. M and Kanungo, M. S (1985a) *Mol. Biol. Rep.* 10,

Chaturvedi, M. M and Kanungo, M. S (1985b) *Biochim. Biophys. Res. Commun.* 127, 604-609.

Christensen, H and Handlogten, M.E (1979) *J. Neural. Transm. Suppl.* 15, 1-13.

Christensen, H.N and Cullen, A.M (1968) *Biochim. Biophys. Acta.* 150, 237-252.

Christensen, H.N and Cullen, A.M (1969) *J. Biol. Chem.* 244, 1521-1526.

Christensen, H. N (1969) *Adv. Enzymol.* 32. 1-20.

Christensen, H. N (1973) In *"Membrane Structure and Mechanisms of Biological Energy Transductions"* (Avery J. ed) Plenum Press London, pp 238-263.

Christensen, H. N(1984) *Biochim. Biophys. Acta.* 779, 255-269.

Cohen, R and Lajtha, A (1972) In *"Handbook of Neurochemistry"* (A. Lajtha, ed) First ed. Raven Press, New York, 2, 543-572.

Cooper, A. J. L and Meister, A (1985), In *"Transaminases"* (Christen, P and Metzler, D.E. eds). John Wiley and Sons. New York, pp 533-563.

Cotman, C. W (1974) In *"Methods in Enzymol"* (S. Fleisher and L. Packer eds). Academic Press. New York, Vol XXXI. pp 445.

Cremer, J. E., Heath, D. F., Patel, A. J and Balazs, R (1975) In *"Metabolic Comparmentation and Neurotransmission"* (Berl, S., Clarke, D . D and Schneider, D eds) Plenum Press, New York, pp 461-478.

Danner, D. J., Leman, S .K., Besharse, J. C. and Elas L. J (1979) *J. Biol.Chem.* 254, 5522-5526.

Das, R and Kanungo, M. S (1982) *Exptl. (ierontol.* 17,95-103.

Dopeshwarker, GA. and Subramanian, C. (1979) *Lipids.* 14, 47-51.

Dreger, J., Larsson, 0. M. and Schomsboe, A (1982) *Expt. Brain Res.* 47, 259-269

Dubois, M., Gillis, K. A., Hamilton, J. K and Rebers, P. A (1956) *Anal. Chem.* 28, 350-356.

Egberts, E. H., Hamster, W and Jurgens, P (1981). In *"Metabolism and Clinical Implications of Branched Chain Amino and Ketoacids"*(Walser, M and Williamson , J. R eds) Elsevier/North Holland, pp 453-464.

Erecinsca, M and Silver, I. A (1990) *Prog. Neurobiol.* 35, 245-296.

Farooq, M and Norton, W. T (1978) *J. Neurochem.* 31, 887.

Feigein , R. P., Biescl. W. R and Wannemacher, R. W Jr **(1971)** *Am. J. Cl in. Nutr.* 24, 329-341.

Felig, P and Wahren, J (1971)./. *Clin. Invest,* 50, 2703-2714.

Fernstrom, J. D. and Faller, D. V(1978) *./. Neurochem.* 80, 1531-1538.

Fischer, J. E. and Balderssarini, R (1976) *Prog.in Liver Disease.* 5, (Grune and Stratton Inc., New York) pp 363-399.

Fischer, J. E (1981) In *"Metabolism and Clinical Implications of Branched Chain Amino and Ketoacids"* (Walser, M and Williamson, J.R. eds) Elsevier/North Holland pp 441-446.

Fonnum, F., Lund-Karl son, R., Mathe-Sorensen, D., Stern, S and Walaas, I (1980) In " *The Cell Surface and Neuronal Function"* (Cotman. C. W., Poste, G and Nicholson, G eds) Elsevier/North-Holland Biomedical Press, Amsterdam pp 455-504.

Freund, H., Yoshimira, N., Lunetta, L and Fishcer, J. E (1978) *Surgery.* 83, 611-618

Freund, H. R., Lapidot, A and Fischer, J. E, (1981) In *"Metabolism and Clinical Implications of Branched Chain Amino and Ketoacids "* (Walser, M and Williamson, J. R) Elsevier/NorthHolland, pp 527-532.

Friedberg, E. C (1990) *Mutation Res.* 236, 145-312.

Gensler, H. L and Bernstein, H (1981) *Q. Rev. Biol.* 56, 279-303.

Govoni S., Memo, M., Saiani L., Spano, P. F.(1980) *Mech.Aging. Dev.* 12, 39-46.

Grigor, M. R., Dunkley, G. G. and Purves, H.D (1970) *Biochim. Biophys. Acta.* 218, 389

Guoff, G and Udenfriend, S (1962) J. *Biol Chem.* 237, 803-806.

Hannumiemi, R and Oja, S. S.(1981) *Neurochem. Res.* 6, 873-884.

Hannuniemi, R, Backas, P. M.,Oja 0. S. and Oja S. S (1987) *Acta.Physiol Scand.* 131,575-581.

Harman, D (1981) *Proc. Natl. Acad. Sci.* USA 78, 7124-7128.

Harper, A. E and Zapalowski, C (1981) In *"Metabolism and Clinical Implications of Branched Chain Amino and Ketoacids"* (Walser, M and Williamson J. R. eds.) Elsevier North Holland, Inc. pp 195-204.

Harper, A. E. (1983) *Experimentia.* 44, 107-34.

Hart, R. W and Setlow, R. B. (1974) *Proc. Natl. Acad. Sci.* USA 71,

Hawkins, R. A ., Williamson, D. H and Krebs, H. A (1971) *Biochem. J.* 122, 13.

Hawkins, R. A., Mans, A. M. and Biebuyek, J. F. (1981) *./. Cerebral Blood Flow, Metabol.* 1, 5385-5386.

Herlong, H. F., Maddrey, W. C and Walser, M (1980) *Annals of Internal Medicine.* 93, 545-550

Hitzemann, R. J and Johnson, D. A (1983) *Neurochem. Res.* 8, 121-131.

Horrocks, L. A (1973) *Prog. Brain. Res.* 40, 384-395.

Hoyer, S (1981) In *"Brain Aging: Pathology and Neuropharmacology"* (J. Servos-Navarro, H-I Sarkander eds) Raven Press, New York, pp 289-300.

Hutson, S. M. and Rannels, S. L (1985)./ *Biol. Chem.* 260, **14189-14193.**

Ichihara, A and Koyama, E (1966) *./. Biochem.* 59, 160-169.

Ichihara, A., Noda, C and Ogawa, K (1973) *Advan. Enz. Regulation.* 11, 155-166.

Ichihara, A., Noda, C and Goto, M (1975) *Biochem. Biophys. Res. Commun.* 67, 1313-1318.

Ichihara, A., Noda, C and Tanaka, K (1981) In *"Metabolsm and Clinical Implications of Branched Chain Amino and Ketoacids"* **(Walser, M and** Williamson, J. R. eds) Elsevier/ **North Holland pp** 227-232.

Ishikawa, A., Oliver, R. M. and Reed, L. J. (1966) *Proc. Natl. Acad. Sci.* U.S.A. 56, 534-541.

Iwangoff, P., Reichlmeier, K., Enz, A and Meier-Ruge, E (1979) In *"Inter Disciplenary Topics in Gerontology"* Kargel Basel 15, 13-33.

James, T. C. and Kanungo, M. S (1976) *Biochem. Biophys. Res. Commun.* 72, 170-175.

James, T. C. and Kanungo, M. S. (1978) *Biochim. et. Biophys. Acta.* 538, 205-211.

James, J. H., Escourrou, J and Fischer, J. E (1978) *Science* (Washington. D.C). 200, 1395-1397.

James J. H., Ziparo, V., Jcppsson, B and fischcr, J. E.(1979) *Lancet.* 2, 772-776.

Jcsssy. J and Murthy, Ch. R. K (1985) *Neurochem. Intl.* 7, 1027-1031

Jessy, J (1988) *Branched-Chain Amino Acid Metabolism in Hyperammonemia.* Ph. D Thesis, submitted to University of Hyderabad.

Jesssy. J and Murthy, Ch. R. K (1988) *Biochem. Intl.* 16,245-251.

Jesssy. J and Murthy, Ch. R. K (1988) In *"The Bichemical Pathology of Astrocytes"* (Leif Hertz, Elling Kvarnme., Edith G. McGeer and Arne Schouboe eds) Alan R. Liss., Inc., New York, pp 449-450.

Jesssy. J and Murthy, Ch. R. K (1989) In *"Hepatic Encephalopathy"* (Roger. F. Butterworth and Gilles Pomeier Layrargues eds) Humana Press, Inc., Clifton, NJ, pp 99-111.

Jesssy. J and Murthy, Ch. R. K (1989) *Annals. New York. Acad. Sci.*

Jesssy. J and Murthy, Ch. R. K (1993) *Neurosci. Lett.* 149, 221-224.

Johnson, W. A and Conclly, J. A (1972) *Biochemistry.* 11, 1967-1973.

Jonung, T., Rigoth, P., Jeppson, B., et al., (1984) *J. Surg. Res.* 36,

Kanungo, M.S. (1975)./. *Theor. Biol.* 53, 253-261.

Kanungo, M S. (1980) *Biochemistry of Ageing.* Academic Press. London.

Knap, S and Mandell, A. J. *(1912) Science.* 177, 1209-1211.

Krebs, H..A (1972) *Advances in Enzyme Regulation* 10,397-420

Larsen, P. R., Ross, J. E and Tapley, D. F (1964) *Btochim. Biophys. Ada.* 88, 570-577.

Lehninger (1981), In *"Biochemistry"* Worth Publishers Inc. Newyork.

Lindhal, T and Nyberg, B (1972) *Biochemistry,* 11, 3610-3618.

Lloyd, K. G and Hornykie Wiez, O (1972)./. *Neurochem.* 19, 1549-1559.

Lowry, O. H., Rosenbrogh, N. J., Farr, A. L and Radall, R. J (1951) *J. Biol. Chem.* 193, 265.

Makay, N and Robinson, B (1981) In *"Metabolism and Clinical Implications of Branched Chain Amino and Ketoacids"* (Walser, M and Williamson, J. R. eds.) Elsevier North Holland, Inc. pp 55-60.

Manchester, K. L (1965) *Biochim. Biophy. Ada* . 100, 295-298.

Mathews, D. M and Laster, L (1965) *Am. J. Physiol.* 208, 601-606.

McGeer, E. G and Mc Geer, P. L (1975) *Neurobiol. Ageing* (R.D.Terry and S.Gershon eds) Raven Press, New York pp 384-403.

McGeer. E. G and Mc Geer, P. L (1976) *./. Neurochem.* 26, 65-76.

Mc-Martin , D. N and O' Conner, J. A Jr (1979) *Mech. Aging. Dev.* 10, 241-248.

Meister, A (1973) *Science.* 180, 33?39.

Memo, M., Lucchi, L., Spano P. F. and Trabucchi, M (1980) *Brain Res.* 202, 488-492.

Miettinen, T. A and Penttila, I. M (1968) *Acta. Med Scand.* 184, 159.

Morgan, H. E., Chua, B. H., Boyd, T. *A* and Jefferson, L. S (1981) In *"Metabolism and Clinical Implications of Branched Chain Amino and*

Ketoacids" (Walser, M and Williamson, J. R. eds.) Elsevier **North Holland,** Inc. pp 217-226.

Mortimore, G. E., Woodside, K. H and Henry, J. E (1972)./. *Biol. Chem.* 247, 2776-2784.

Murthy, Ch. R. K and Hertz, L (1987) *Neurochem. Res.* 13, 57-61

Murthy, Ch. R. K and Hertz, L (1987a) ./. *Neurosci. Res.* 17, 271-276.

Murthy, Ch. R. K and Hertz, L (1987b) ./. *Neurochem.* 49, 735-741.

Nagy K., Simon, P and Nagy, I. Z (1983) *Biochem. Biophys. Res. Commun.* 117, 688-694.

Natelson, S (1971) In *"Techniques of Clinical Chemistry"* (Charles. C Thomas publishers) Springfield, Illinois.

Nissen, S and Morey, W. H (1981) In *"Metabolism and Clinical Implications of Branched Chain Amino and Ketoacids"* **(Walser,** M and Williamson, J. R) Elsevier/NorthHolland, pp 301-306.

Norton, W. T. and Poduslo, S. E. (1973) ./. *Neurochem.* 21, 759-773.

Nyhan, L. W (1984) In *"Abnormalities in Amino Acid Metabolism in Clinical Medicine"* (William. L. Nyhan, eds) Appleton-Century-Crofts/ New Walk, Connecticut. pp 21-35.

Odessy, R and Goldberg, A.. L. (1972) *Am. ./. Physiol.* 223, 1376-1383

Odessy, R., Khairallah, E. A and Goldberg A L. (1974)./. *Biol. chem.* 249, 7623

Oldendorf, W. H (1973) *Am. J. Physiol.* 224, 967-969.

Olson, M. S (1989) *Annals. New York. Acad. Sci.* 218-229.

Orgel, L. E. (1963) *Proc. Natl. Acad Sci.* U.S.A. 49, 517-521.

Owen, O. E., Morgan, A. P., Kemp, H. G., Sullivon, J. M and Cahill, G. F (1967) *J. Cl in. Invest.* 46, 1589-1595

Pajari, M (1984) *Acta Physiol.Scand.* 122, 415-420.,

Pardridge, W.M and Jefferson, L.S (1975) *Am. J. Physio!.* 228, 1115-1161.

Pardridge, W. M. and Oldendorf, W. H. (1975) *Biochim. et. Biophys. Acta.* 401, 128-136.

Pardridge, W. M. and Oldendorf, W. H. (1977) *J. Neurochem,.* 28, 5-12.

Parker, P. J and Randle, P. J (1978) *Biochem. J.* 171, 751-757.

Patel, A. J and Balazs, R (1970) *./. Neurochem.* 17, 955-71.

Patel, A. J and Olson (1982) *Biochemistry.* 21, 4259-4265.

Patel, M. S (1977) *J. Gerontol.* 32,643-646.

Paul, H. S. and Adibi, S. A (1980) *./. Clin. Invest.* 65, 1285-1293.

Paxton, R. and Hams, R. A. (1982) *J. Biol. Chem.* 257, 14433-14439.

Peterson, N. A. and Raghupathy, E, (1973) *./. Neurochem.* 21, 97-110.

Peterson, N. A, and Raghupathy, E (1978) *./. Neurochem.* 31, 989-996.

Petriciric M., Denko, C. W and Messineo, L (1983) *Intl. J. Biochem.* 15, 751-753.

Pettit, F. H., Feaman, S. J. and Reed, L. J (1978) *Proc. Natl. Acad. Sci.* U.S.A., 75,4881-4885.

Phillipis, F and Privett, O. S (1978) *Lipids.* 14, 590-595.

Polgar, A (1971) In *"Topics in Lipid Chemistry"* (F. D.Gunstone, eds) vol 2, John Wiley and Sons, Inc. pp 207.

Quarto di palo, F. M., Mombell, L. E. and Gastaldi, L (1964) *Gerontologia,* 10, 161-166.

Randle, P. J., Lay, K. S and Parker, R. S (1981) In *"Metabolism and Clinical Implications of Branched Chain Amino and Ketoacids"* (Walser, M and Williamson, J. R) Elsevier/NorthHolland, pp 13-22.

Randle, P. J. (1983) *Philos. Tran. R Soc.* London Ser. B. 302, 47-57.

Rao, T. I., Rao, G. N., Swamy, M and Sadasivudu, B (1982),/ *Neurosci. Res.* 7, 387-395.

Rao, K. V. R and Murthy, Ch. R. K (1994) *Neurosci. Lett.* 175, 103-106.

Rao, K. V. R.. Mohan C. Vemuri and Murthy, Ch. R. K (1995) *Neurosci. Lett.* 184, 137-140.

Rao, V. L. R and Murthy. Ch. R. K (1991) *Neurosa. Lett.* 130. 49-54.

Rao, V. L. R and Murthy, Ch. R. K and Burterworth, R. F (1992) *Metab Barm. Dis.* 7, 1-20.

Rapoport, S. I., London, E. D and Takei, H (1982) In, *"Aging Brain. Physiological and Pathological Aspects"* (Hoyer, S. ed) Exp. Brain Res. Suppl 5, Springer, Berlin pp 86-101.

Rath, P. C. and Kanungo, M.S. (1989), *FEBS. Letts* 244, 193-198.

Reff. M. E. (1985) In *"Handbook of Biology of Ageing* (C. E. Finch and E. L.Schneider eds) Van Nostrand Reinhold, New York pp 225-249.

Roberts, S and Morelos, B (1965)./. *Neurochem.* 12, 373-387.

Rosenthal, J., Angel, A and Farkas, J (1974) *Am. J. Physiol.* 226, 411

Rosewell, E. V(1956) *Biochem. J.* 64, 235-245.

Sadasivudu, B and Lajtha, A (1970)./ *Neurochem.* 17, 1299-1311.

Sapir, D. G., Stewart, P. M., Walser, M., Moreadith et.al., (1983) *Lancet.* 1, 1010-1014.

Schepartz, B (1963) *J. Neurochem.* 10, 825-829.

Seismi (1982) *Arch. Gerontol. Geriatrics.* 1, 29-42.

Sershen, H and Lajtha, A (1979) ./. *Neurochem.* 32, 719-726.

Shank, R. P and Campbell, G. L_M (1984) *J. Neurochem.* 42, 1162-1169.

Shinnick, F. L and Harper, \. E (1976) *Biochim. et. Biophys. Acta*437,

Shiotä, T (1984) *ActaMed. Okayama.* 38, 219-226.

Shock, N. W (1962) *Sci. Am.* 206, 100.

Simon, J. R., Atweh, S and Kuhar, M (1976)./ *Neurochem.* 26, 909-922

Singh, K. N and Subba Rao, K (1979) *Ind. J. Biochem. and Biophys.* 16, 349-353.

Smith, A.E (1974) *J. Neurochem.* 23, 435-440.

Smith, C. B., Goochee, C, Rapoport S. 1 and Sokoloff L (1980) *Brain* 103, 351-365.

Stillway, L. W and Weigand, D. A., Riefler J. F and Buse, M. G (1977) *Lipids.* 12, 1012.

Stillway L.W., Weigand, D.A. and Ricffler, J.F (1978) *Lipids,* 14,

Strong, R., Samorajski, T and Gottesfeld, Z (1984) *J. Ncurochcm.* 43, 1766-1768.

Stryer (1981) *Biochemistry.*

Summer, J. 13 (1944) *Science.* 100, 413.

Swaiman, K and Milstein, J. M.(1965) *J. Neurochem.* 12,981-986

Taylor, R. T. and Jenkins, W. T (1966)./. *Biol.Chem.* 241, 4391-4395

Tischler, M. E and Goldberg, A. L (1981) In *"Metabolism and Clinical Implications of Branched Chain Amino and Ketoacids"* (Walscr. M and Williamson, J. R) Elsevier/NorthHolland, pp 283-288.

Turpccnoja, L., Villa, R. F., Magri, G and Stella, G. A. M (1988) *Neurochem. Res.* 13, 859-866.

Vahvelainen, M. L., and Oja, S. S (1975) *J. Neurochcm.* 24, 885-892.

Van den Berg, C. J., Reguierse, G. L. A., Blochnis, G. G. C, Kron, M. C., Randa, G., Clarke, D. D and Garfinkel (1976) In *"Metabolic Compartmentation and Neurotransmission"* Relation to Brain Structure and Function (Berl, S., Clarke, D. D and Schneider, S eds) Plenum Press. New York, pp 515.

Varley, M (1969) In *"Practical Clinical Chemistry".* ELBS and William Heinemann Medical Books Limited. London, U. K.

Venugopal, J and Subba Rao, K (1991) *J. Neurochem.* 56, 812-817.

Walser, M (1978) *Nephron.* 21, 57-74.

Wiggins, R. C, Fuller, G. N and Bell, M. E. (1979) *J. Neurochem.* 32, 1579-1582.

Williamson, J. R., Martin-Requero, A., Corkey, B. E.. Brandt, M and Rothman, R (1981) In *"Metabolism and Clinical Implications of Branched Chain Amino and Ketoacids"* (Walser, M and Williamson, J. R eds) Elsevier/NorthHolland pp 105-118.

Wiseman, G (1968) In *"Handbook of Physiology"* (Code, C.F eds) American Physiological Society, Washington, DC. vol 3, pp 1277-1307.

Wolfe, B. M, Tsai, M. U., Rogers, Q. R. and Heni, L. J (1981) In *"Metabolism and Clinical Implications of Branched Chain Amino and Ketoacids"* (Walser, M and Williamson J. R.eds) Elsevier/North Holland pp 301-306.

Wolhuetcr, R. M. and Harper, A. E(1970) *J. Biol. Chem.* 2245,

Yaranell, A., Schumm, D. E. and Webb, T. E (1977) *Mech. Ageing. Dev.* 6, 259-264.

Yudkoff, M., Nissumi, I., Kim, S., Pleasure, D., Hummeler, K and Segal, S (1983) *Biochem. Biophys. Res. Commun.* 115, 174-179

Zalenski, J., Wilson, D. F and Erecinska, M (1986) *J. Biol. Chem.* 261, 14091-14094.

ELSEVIER

Neuroscience Letters 175 (1994) 103–106

NEUROSCIENCE LETTERS

Synaptosomal high affinity transport systems for essential amino acids in rat brain cortex

K.V. Rama Rao, Ch.R.K. Murthy*

Department of Animal Sciences, School of Life Sciences, University of Hyderabad, Hyderabad, 500 134 India

Received 2 December 1993; Revised version received 28 April 1994; Accepted 28 April 1994

Abstract

High affinity uptake systems have been identified for the transport of leucine, isoleucine and methionine in synaptosomcs hut not in the mitochondria of rat cerebral cortex. These systems were found to be different from the conventional low affinity uptake systems in terms of their affinity, sodium dependency and the rale of transport. As these amino acids have no neurotransmitter function, it is suggested that high affinity uptake systems might be involved in the transport of essential amino acids and maintain a minimal level in brain when the concentrations of these amino acids arc low in blood (as in starvation, malnutrition). As some of these amino acids serve as precursors for neurotransmitters, such as glutamate (leucine, isoleucine), taurine (methionine), it is also suggested that high affinity uptake systems for the essential amino acids might also replenish the precursor pools of neurotransmitter amino acids.

Key words: Essential amino acid; High affinity transport; Isoleucine; Leucine; Methionine; Synaptosome

Neutral amino acids (leucinc, isoleucine, valinc, methionine, glutamine, phenylalanine, tyrosine and tryptophan) are known to be transported from blood to brain by (i) a sodium independent common transport mechanism (L-system; leucine preferring system), and (ii) a sodium dependent bidirectional transport system ('A' type; alanine preferring), supposed to be present on the antiluminal side of the blood capillaries in brain [2,3]. The latter system was reported to have a higher affinity towards neutral amino acids than the L-type system present on both the luminal and antiluminal side of the capillaries. Though high and low affinity uptake systems have been reported for the transport of neutral amino acids across brain capillaries, reports on the transport of these amino acids into the brain cells seem to be contradictory. Earlier reports indicated that neutral amino acids were transported into brain cells by a low affinity uptake system [16]. Recently, both high and low affinity uptake systems have been implicated for the transport of threonine and leucine in neuroblastoma, synaptosomcs, neurons and astrocytes [7,8,12]. Since neurotransmitter function has not been demonstrated for the above mentioned neutral amino acids, physiological significance of the high affinity uptake systems for these amino acids has not been satisfactorily explained.

Results of the present study indicated that the transport of leucine, isoleucine and methionine into cortical synaptosomcs was mediated by high and low affinity uptake systems, while the same was mediated in mitochondria by a low affinity uptake system. Based on the results of the present study and those of earlier investigators, on the presence of high affinity uptake systems for the transport of tryptophan, leucine, lysine and threonine [7,8,10,12], it is suggested that essential amino acids may be transported into brain cells by high and low affinity uptake systems. Such a process would help to maintain a minimal amount of these amino acids in brain during starvation, malnutrition and pathological states which alter the availability of these essential amino acids to brain [4-6]. As the carbons and nitrogens of some of these amino acids are incorporated into the neurotransmitter amino acids (such as glutamate, taurine etc.), it is suggested that these essential amino acids might be re-

* Corresponding author. Fax: (91) 040-253145.
E-mail: chrkmsl@uohyd.ernet.in

SSDI 0304-3940(94)00372-H

shing the precursor pool required for the synthesis urotransmitter amino acids.

bred rats of Wistar strain of 3 months age, maind at 21 ± PC and on standard pellet diet (Hindu- Lever Ltd., India) and water, were used presently. tion of synaptosomcs and non-synaptic mitochonfrom the cerebral cortex using Ficoll density gradiand the purity of the preparations, (as monitored by er enzymes) have been described earlier [13,14].

stake of [^{3}H]leucine, isoleucine and [^{35}S]methionine °C was studied in synaptosomcs and mitochondria he method of Rao and Murthy [14]. Arnino acid entration in the incubation medium (well oxygen- Krebs-glucose-phosphate-HEPES medium) was d from 0.5 //M to 10 mM, and *0.4* μCi of the radive amino acid was added to each tube. Uptake was inated by rapid centrifugation at 10,000 x *g* at 4°C. t was briefly washed twice with ice-cold medium ng same concentration of non-radioactive amino under study. Final pellet was dissolved with contin-. vortexing in 100 μl of 0.1 N NaOH and radioactivas determined. Non-specific uptake was determined ncubating the sample at 0°C and due corrections made. Kinetic constants (A'_m and V_{max}) were calcufrom Eadie-Scatchard plots. In some experiments ion was terminated by rapid filtration through Mile membrane filters (0.45 μm) and filters were ed twice as described above. The values obtained by tion and centrifugation methods were more or less ame (data not shown). Hence, in subsequent experts, uptake was terminated by centrifugation.

all the uptake studies, non-specific uptake was t 20–25% of the total uptake. Amino acid uptake found to be linear upto 200 μg of synaptosomal ein and 500 fg of mitochondrial protein (data not n). The uptake was linear upto 10 min of incubation gh concentrations and for 5 min al low concentrations of leucine (Fig. 1E). In the subsequent studies, 100 f protein and 5 min of incubation time was used. Use wer incubation times (< 1 min), though preferable cially at lower concentrations of substrate, gave ervalues. This might be due to problems associated thermal equilibriation. Under such conditions, uprates may have an underestimate since the uptake performed almost at the end of initial velocity pe- This was overcome by using the tangent extrapolamethod from the progressive time curve [17], for the ective amino acids and the true rates were estimated. observed and true rates are given in the Table 1. a problem was not encountered at high substrate entrations (millimolar range).

order to determine the K_m and V_{max} of transport, ne uptake was studied using 0.1 to 10 mM of the o acid. All the data points in the Eadie-Scatchard ; for mitochondrial preparation could be fitted into gle linear regression curve indicating the presence of a single uptake system (data not shown). However, in synaptosomal preparation, Eadie-Scatchard plots were curvilinear indicating the likely presence of more than one uptake system. Hence, a concentration range of 0.5 μM to 10 mM was used in subsequent experiments with synaptosomcs. This confirmed the presence of two uptake systems with different affinities in synaptosomcs – a high affinity system with a K_m in μM range and a low affinity uptake system with a K_m in mM range (Fig 1A; Table 1). The two uptake systems differed by several fold in their transport rates and in their amino acid.

As high affinity uptake systems have a stringent requirement for the presence of sodium ions in the mediurn [6], sodium dependency of the high and low affinity leucine uptake was studied in synaptosomcs. Replacement of sodium ions in the medium with Tris ions suppressed the high affinity uptake system in a concentration dependent manner and the maximal suppression was by 75% when sodium ions in the medium were completely replaced with Tris. However, the low affinity uptake system (Fig. 1B) was not affected under these conditions. These results indicated that the high affinity uptake might be mediated by a specific carrier, but not by homo-exchange of the label into synaptosomcs. D-Leucine or 2-amino-2-norborane carboxylic acid (leucine analog) had no significant effect on the high affinity uptake system for leucine in synaptosomcs (data not shown) indicating the stereospecificity of the transport process.

It is believed that high affinity uptake systems are usually involved in the termination of the neurotransmitter activity of amino acids such as glutamate and GABA [15]. As of today, no neurotransmitter activity has been demonstrated for leucine. However, as leucine is an es-

Table 1
Kinetic constants of high and low affinity uptake of [^{3}H]leucine, isoleucine and [^{35}S]methionine in mitochondria and synaptosomcs of rat cerebral cortex

	Mitochondria	Synaptosomcs	
		LAUS	HAUS
V_{max} (nmol. protein^{-1} • h^{-1})			
Leucine	903 ± 13	371 ± 32	16 ± 1.3
Isoleucine	556 ± 51	272 ± 39	24 ± 1.2
Methionine		1558 ± 500	12 ± 3.0
A'_m	mM	mM	μM
Lcucine	26 ± 1	6.2 ± 0.4	44 ± 4
Isoleucine	12 ± 3	2.9 ± 0.5	62 ± 2
Methionine		21 ± 6.0	84 ± 10

Values are mean ± S.D. of 4 experiments done in duplicate. Kinetic constants (K_m and V_m) were calculated from the Eadie-Scatchard plots drawn individually for each experiment. Non-specific uptake was determined by carrying out parallel incubations at 0°C and due corrections were made. When the V_{max} values of high affinity uptake systems were adjusted for underestimate (see text), V_{max} for leucine was 38 ± 3; isoleucine 60 ± 8; methionine 30 ± 3. HAUS: high affinity uptake system; LAI'S: low affinity uptake system; N.D.: not determined.

plenishing the precursor pool required for the synthesis of neurotransmitter amino acids.

Inbred rats of Wistar strain of 3 months age, maintained at 21 ± 1°C and on standard pellet diet (Hindustan Lever Ltd., India) and water, were used presently. Isolation of synaptosomcs and non-synaptic mitochondria from the cerebral cortex using Ficoll density gradients and the purity of the preparations, (as monitored by marker enzymes) have been described earlier [13,14].

Uptake of [^{3}H]leucine, isoleucine and [^{35}S]methionine at 37°C was studied in synaptosomcs and mitochondria by the method of Rao and Murthy [14]. Amino acid concentration in the incubation medium (well oxygenated Krebs-glucose-phosphate-HEPES medium) was varied from 0.5 μM to 10 mM, and 0.4 μCi of the radioactive amino acid was added to each tube. Uptake was terminated by rapid centrifugation at 10,000 × g at 4°C. Pellet was briefly washed twice with ice-cold medium having same concentration of non-radioactive amino acid under study. Final pellet was dissolved with continuous vortexing in 100 μl of 0.1 N NaOH and radioactivity was determined. Non-specific uptake was determined by incubating the sample al 0°C and due corrections were made. Kinetic constants (A_{m} and V_{max}) were calculated from Eadie-Scatchard plots. In some experiments reaction was terminated by rapid filtration through Millipore membrane filters (0.45 μm) and filters were washed twice as described above. The values obtained by filtration and centrifugation methods were more or less the same (data not shown). Hence, in subsequent experiments, uptake was terminated by centrifugation.

In all the uptake studies, non-specific uptake was about 20–25% *of* the total uptake. Amino acid uptake was found to be linear upto 200 μg of synaptosomal protein and 500 fg *of* mitochondrial protein (data not shown). The uptake was linear upto 10 min of incubation al high concentrations and for 5 min at low concentrations of leucine (Fig. 1E). In the subsequent studies, 100 μg of protein and 5 min of incubation time was used. Use of lower incubation times (< 1 min), though preferable especially at lower concentrations *of* substrate, gave erratic values. This might be due to problems associated with thermal equilibriation. Under such conditions, uptake rates may have an underestimate since the uptake was performed almost at the end *of* initial velocity period. This was overcome by using the tangent extrapolation method from the progressive time curve [17], for the respective amino acids and the true rates were estimated. Both observed and true rates are given in the Table 1. Such a problem was not encountered at high substrate concentrations (millimolar range).

In order to determine the K_m and V_{max} *of* transport, leucine uptake was studied using 0.1 to 10 mM of the amino acid. All the data points in the Eadie-Scatchard plots for mitochondrial preparation could be fitted into a single linear regression curve indicating the presence of a single uptake system (data not shown). However, in synaptosomal preparation, Eadie-Scatchard plots were curvilinear indicating the likely presence of more than one uptake system. Hence, a concentration range of 0.5 μM to 10 mM was used in subsequent experiments with synaptosomcs. This confirmed the presence of two uptake systems with different affinities in synaptosomcs – a high affinity system with a K_m in μM range and a low affinity uptake system with a K_m in mM range (Fig 1A: Table 1). The two uptake systems differed by several fold in their transport rates and in their amino acid.

As high affinity uptake systems have a stringent requirement for the presence of sodium ions in the medium [6], sodium dependency of the high and low affinity leucine uptake was studied in synaptosomcs. Replacement *of* sodium ions in the medium with Tris ions suppressed the high affinity uptake system in a concentration dependent manner and the maximal suppression was by 75% when sodium ions in the medium were completely replaced with Tris. However, the low affinity uptake system (Fig. 1B) was not affected under these conditions. These results indicated that the high affinity uptake might be mediated by a specific carrier, but not by homo-exchange of the label into synaptosomcs. D-Leucine or 2-amino-2-norborane carboxylic acid (leucine analog) had no significant effect on the high affinity uptake system for leucine in synaptosomcs (data not shown) indicating the stereospecificity of the transport process.

It is believed that high affinity uptake systems are usually involved in the termination of the neurotransmitter activity of amino acids such as glutamate and GABA [15]. As of today, no neurotransmitter activity has been demonstrated for leucine. However, as leucine is an es-

Table 1
Kinetic constants of high and low affinity uptake of [^{3}H]leucine, isoleucine and [^{35}S]methionine in mitochondria and synaptosomcs of rat cerebral cortex

	Mitochondria	Synaptosomcs	
		LAUS	HAUS
V_{max} (nmol. protein^{-1} • h^{-1})			
Leucine	903 ± 13	371 ± 32	16 ± 1.3
Isoleucine	556 ± 51	272 ± 39	24 ± 1.2
Melhionine		1558 ± 500	12 ±3.0
K_m	mM	mM	μM
Leucine	26 ± 1	6.2 ± 0.4	44 ± 4
Isoleucine	12 ± 3	2.9 ± 0.5	62 ± 2
Methionine		21 ± 6.0	84 ± 10

Values are mean ± S,D. of 4 experiments done in duplicate. Kinetic constants (K_m and V_m) were calculated from the Eadie-Scatchard plots drawn individually for each experiment. Non-specific uptake was determined by carrying out parallel incubations at 0°C and due corrections were made. When the V_{max} values of high affinity uptake systems were adjusted for underestimate (see text), V_{max} for leucine was 38 ± 3; isoleucine 60 ± 8; melhionine 30 ± 3. HAUS: high affinity uptake system; LAUS: low affinity uptake system; N.D.: not determined.

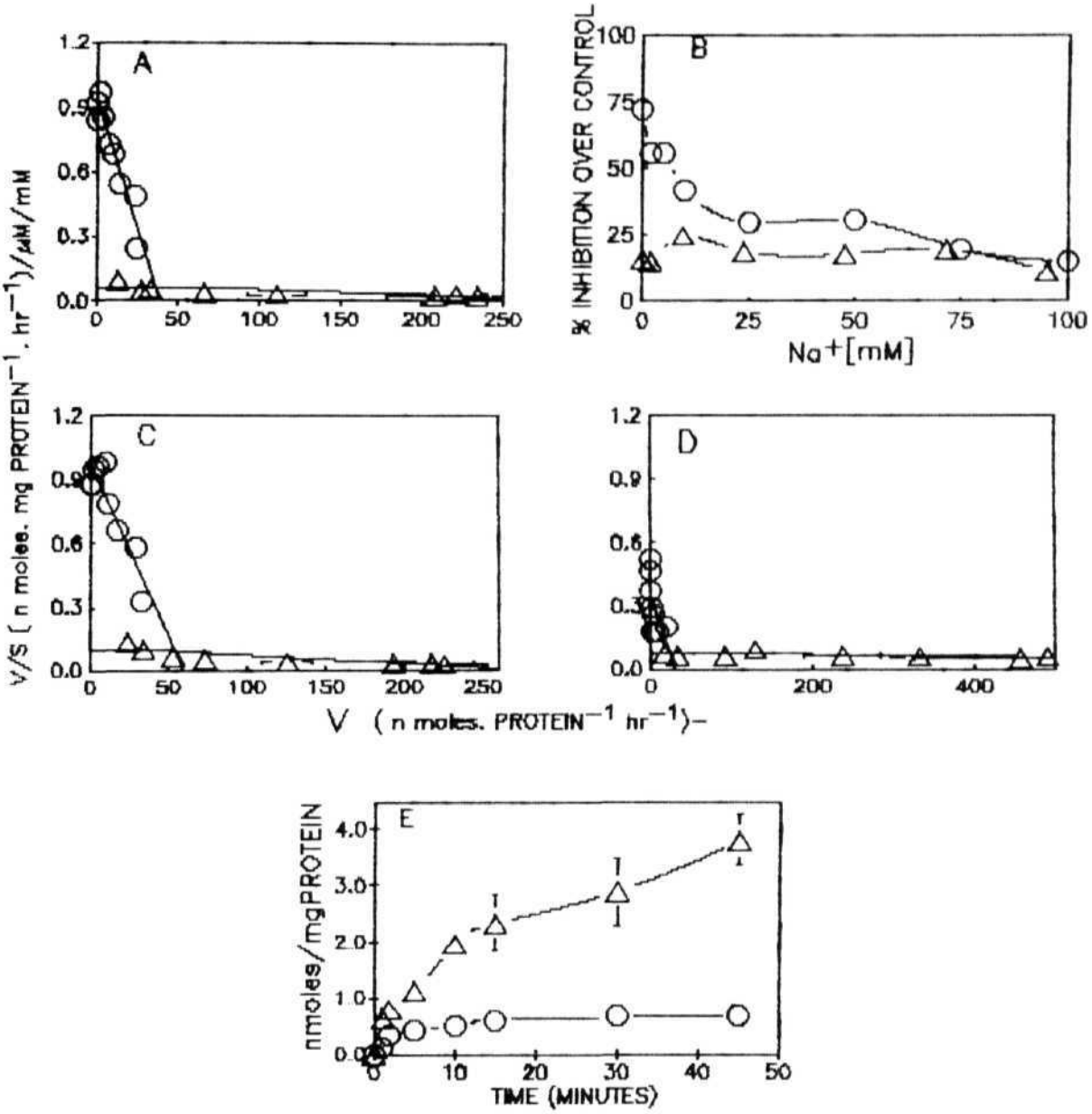


Fig. 1. A: Eadie-Scatchard plots of the high and low affinity uptake of [^{3}H]leucine into synaptosomes of rat cerebral cortex. B: percent inhibition over control of high and low affinity uptake of [^{3}H]leucine in cortical synaptosomes with respect to varying concentrations of sodium ions in the medium. Leucine concentration was 50 μM for high affinity system and 5 mM for low affinity system and 0.4 μCi of labelled leucine was added to each tube. Sodium ions were replaced with Tris in Krebs-Ringer-glucose medium. C D: Eadie-Scatchard plot of high and low affinity uptake of [^{3}H]isoleucine and [^{35}S]methionine in cortical synaptosomes. E: time course of leucine uptake at high affinity (100 μM) and low affinity (I mM) concentrations into synaptosomes. Each value in all the plots is mean ± S.D. of 4 individual experiments done in duplicate. In all the plots, open circles denote high affinity uptake while open triangles denotes low affinity uptake system, The plots were drawn after calculating the true initial velocities as described in the text.

sential amino acid, it was felt that high affinity uptake systems might also be involved in the transport of essential amino acids. Identification of high affinity uptake systems for isoleucine and methionine (Fig. 1C,D), two other essential amino acids, supported this concept. Such high affinity uptake systems have been demonstrated for the transport of other essential amino acids, tyrosine, tryptophan, lysine and threonine [7,8,12].

Presence of a high and low affinity uptake systems for the transport of essential amino acids in brain is understandable as these amino acids are not synthesized in the tissue and arc to be supplied through diet. Depending on the qualitative and quantitative aspects of diet, levels of these amino acids in blood may fluctuate over a wide range [5]. If the tissue is endowed only with a low affinity uptake system, then availability of these essential amino

acids to cells would be restricted when the animal consumes inadequate amounts of food or food poor in essential amino acids. It was reported earlier that brain leucine content decreased only by 30% while that in the serum decreased by 75% when rats were fed with leucine free diet [5]. This suggests that a minimal amount of these amino acids would be maintained in brain even when they arc not supplied in the diet. This is essential as the depiction of essential amino acids adversely affects brain development, functioning and results in tissue proteolysis [11]. Existence of both high and low affinity uptake systems would ensure the transport of essential amino acids into cells at a wide range of extracellular concentrations. Thus, high affinity uptake systems for essential amino acids might be responsible for maintaining a minimal level of these amino acids in brain. Moreover, some of these amino acids arc known to serve as precursors for the synthesis of neurotransmitters, such as glutamate (leucine and isoleucine) [18], and taurine (methionine) [I], Hence, these high affinity uptake systems might also be involved in the maintenance of the precursor pool required for the synthesis of tliese neurotransmitters.

Interestingly, high affinity uptake systems were observed only in synaptosomcs, but not in mitochondria. In the latter, only low affinity uptake systems for the transport of leucine and isoleucine were present. Existence of only low affinity uptake system in mitochondria is understandable as a minimal concentration of essential amino acids will always be available in the cytosol for mitochondria. Presence of a low affinity uptake system in mitochondria would ensure that these amino acids are transported into mitochondria only when the cytosolic pool is saturated. This is essential as cerebral mitochondria are capable of oxidizing the carbons *of* BCAA to *CO*, [9], resulting in an irreplaceable loss of these amino acids.

Financial assistance to K.V.R. Rao in the form *of* UGC-SRF is acknowledged. Authors wish to thank ICMR and CSIR for providing financial assistance.

[I] Awapara, J., The metabolism of taurine in animals. In R. Huxtable and A. Barbeau (Eds.), Taurine, Raven. New York, 1976, pp. 1–19,

[2] Bel/. A.L and Goldstein. G.W., Polarity of blood brain barrier. Neutral amino acid transport into isolated brain capillaries, Science, 202 (1978) 225-227.

[3] Christensen, H.N.. Some special kinetic problems of transport. In F.F. Nord (Eds.), Advances in Enzymology, Vol. 32, Wiley, New York, 1969, pp. 1-20.

[4] Ericksson, L.S., Peterson, A. and Wahren, J., Branched chain amino acids in the treatment of chronic hepatic encephalopathy, Gut. 23 (1982) 801–806.

[5] Fernstrom, J.D. and Faller, D.V., Neutral amino acids in the brain. Changes in response to food and ingestion, J. Neurochem., 30(1978) 1531.

[6] Fonnum, F., Lund, R.. Karlsen, D., Sorrenson. M., Sterri, S. and Walaas, I., High affinity uptake systems and their role in Transmitter action. In C.W. Cotman and G. Poste (Eds.). The Cell Surface and Neuronal Function. Elsevier/North-Holland, Amsterdam, 1980. pp. 455–504.

[7] Hannuniemi, R., Pajari-Backas, M and Oja, S.S., Transport of leucine, lysine, glycine and aspartate in neuroblastoma C1300 and glioma C cells. Acta Physiol. Scand., 131 (1987) 575-581.

[8] Hannuniemi, R. and Oja, S.S., Uptake of leucine, lysine, aspartic acid into isolated neurons and astrocytes. Neurochem, Res.. 6 (1981) 873–884.

[9] Jessy, A. and Murthy, Ch.R.K., Alterations in mitochondrial branched chain amino acid metabolism in brain in acute hyperammonemia, Neurosci. Lett., 149 (1993) 221–224.

[10] Knapp, S. and Mundell, A.J., Narcotic drugs: effects on the serotonin biosynthetie systems of brain. Science, 177 (1972) 1209–1211.

[11] Paul, H.S. and Adibi, S.A., Leucine oxidation and protein turnover in clolibrate induced muscle protein degradation in rats, J. Clin. Invest.. 65 (1980) 1285–1293.

[12] Peterson, N.A. and Raghupathy, F.. Evidence for strictly Na dependent accumulation of serine and threonine in brain cortical synaptosomcs from new born rats, J. Neurochem., 31 (1978) 989–996.

[13] Rao. V.I..R. and Murthy, Ch.R.K., Hyperammonemic alterations in the uptake and release of glutamate and aspartate by rat cerebellar preparations, Neurosci. Lett., 130(1991)49–54.

[14] Ratna Kumari. L. and Murthy, Ch.R.K.. Activities of pyruvate dehydrogenase, enzymes of citric acid cycle and amino transferases in sub cellular fractions of cerebral cortex of normal and hyperammonemic rats, Neurochem. Res., 14 (1985) 221-228.

[15] Roskoski, R.. Net uptake of L-glutamate and GABA by high affinity synaptosomal transport systems, J. Neurochem., 31 (1978) 493–498.

[16] Sershen, H. and Lajtha, A.J., Inhibition pattern by analogs indicates the presence of ten or more transport systems for amino acids in brain cells. J. Neurochem., 32 (1979) 719–726.

[17] Wong, T.J., In: Kinetics of Enzyme Mechanisms, Academic, London. 197$, pp. 1-13.

[18] Yudkoff, M., Nissim, 1.. Kim, S.. Pleasure. D., Hummeler, K, and Segal. S.. [^{15}N]Leucine as a source of [^{15}N]glutamate in organotypic cerebellar explants. Biochem. Biophys. Res. Commun., 115 (1983) 174–179.

ELSEVIER

Neuroscience Letters 184 (1995) 137–140

NEUROSCIENCE LETTERS

Synaptosomal transport of branched chain amino acids in young, adult and aged rat brain cortex

K.V. Rama Rao, Mohan C. Vemuri, Ch.R.K. Murthy*

Department of Animal Sciences, School of Life Sciences, University of Hyderabad, Hyderabad 500134, India

Received 20 September 1994; revised version received 22 November 1994; accepted 24 November 1994

Abstract

Uptake of branched chain amino acids (BCAA, leucine and isoleucine) was studied in synaptosomes prepared from the cerebral cortex of rats of 1, 3 and 24 months of age. In addition to the conventional low affinity sodium independent transport system, a high affinity sodium dependent stereospecific transport system for the transport of BCAA was **identified** in synaptosomes pa-pared from the cerebral cortex of the above three age groups. There was an overall decrease in K_m and V_{max} of both high and low affinity transport systems for leucine and isoleucine in the cortical synaptosomes of 24-month-old rats when compared with younger age groups. This study indicates that the non-neurotransmitter essential amino acids are transported by high and low affinity transport systems and these systems undergo age-dependent alterations. These changes might be due to the altered synthesis of these transporter proteins and/or synthesis of transporters with altered conformation and/or changes in the physical properties (fluidity) of the membrane. The decrease in the transport of BCAA is on a par with the decrease in the overall metabolism of BCAA in brain. As food consumption decreases in the older age groups of animals, the availability of essential amino acids to the tissues might also be lowered. Under such conditions, it is suggested that the observed increase in the affinity (decreased K_m) of the carrier might be helpful in the supply of essential amino acids.

Keywords: Aging; Branched chain amino acids; High affinity; Isoleucine; Leucine; Synaptosomes; Uptake

Aging is a multifactorial phenomenon associated with a gradual decline in the normal performance of the organs with a concomitant loss in the genetic, biochemical and physiological mechanisms of the cells [18]. Brain is no exception to these changes, wherein a reduction in overall metabolism (such as protein synthesis), neurotransmitter functions, transport of the metabolites were reported [3,5,8]. Several theories have been proposed to explain the mechanism of the aging: (i) alterations in gene expression [13]; (ii) the enzymes responsible for the transcription and translation process [20,22]; and (iii) free radical induced alterations on the protein and lipid compositions of the cell membranes [7].

It was proposed earlier that alterations in membrane composition would affect the fluidity of the membrane [10]. Such changes in membrane architecture might change the conformation of membrane proteins with a concomitant decrease in the transport of ions and amino acids [3]. Although studies were performed on the age dependent alterations in the transport of neurotransmitter amino acids such as glutamate and aspartate [19], no information is available on the transport of essential amino acids such as branched chain amino acids (BCAA) in brain with respect to aging.

BCAA (leucine, valine and isoleucine) are essential amino acids and are not synthesized in brain. Hence, their levels in the tissue depend on the availability of these amino acids in the extracellular environment. These amino acids are involved in many of the metabolic functions in brain such as protein synthesis [2], alternative sources of energy [12], and synthesis of glutamate [21]. Hence, in the present investigation, in vitro transport of BCAA (leucine and isoleucine) was studied in synaptosomes prepared from the cerebral cortex of rats of different age groups.

Earlier reports indicated that BCAA are transported into the brain by a sodium independent common neutral

* Corresponding author, Tel.: +91 40 253901 4562; Fax: +91 40 253145; Email: chrkm-sl@uohyd.ernet.in.

0304-3940/95/$09.50
SSDI 0304-3940(94)11189-M

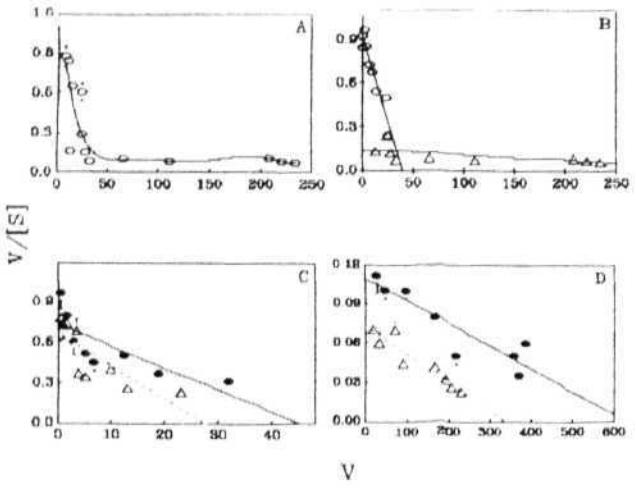


Fig. 1. (a) Eadic-Scatchard plot representing the curvilinear points of the data obtained from the saturation isotherms of leucine transport, indicating the two affinity transport systems (high and low affinity). (b) Eadie–Scatchard plot representing both high and low affinity transport systems for leucine in synaptosomcs prepared from 3-month-old cerebral cortex. ○ represents high affinity uptake, while △ represents low affinity uptake. (e) Eadie–Scatchard plot representing the high affinity transport system for leucine into synaptosomcs of young (1 month) and old (24 months) rat brain cortex. (d) Eadie–Scatchard plot representing the low affinity transport system for leucine into synaptosomcs of young (1 month) and old (24 months) rat brain cortex. In (c) and (d), ○ represents (he 1 month age group, while A represents the 24 months age group. In all t he plots, V = nmol (mg protein)$^{-1}$ h^{-1}, VAS, nmol (mg protein)$^{-1}$ h^{-1}/μM/mM.

amino acid carrier (L-systcm) [4], In addition, a sodium dependent, bidirectional, high affinity transport system has been identified on the antiluminal side of the blood capillaries [1]. Recent studies from our laboratory indicated that essential amino acids are transported into synaptosomcs by both high affinity (sodium dependent) and low affinity (sodium independent) transport systems [14]. It was observed that both high and low affinity transport systems were present in cortical synaptosomcs of the rats of all the age groups studied. There was an overall decrease in K_m and V_{max} for both high and low affinity BCAA uptake into the synaptosomcs prepared from rats aged 24 months when compared to younger age groups. It is suggested that the decrease in the transport of BCAA might be due to a decrease in the synthesis of the carrier proteins or altered conformation of these carriers due to the alterations in the membrane architecture (membrane fluidity). These changes might facilitate the transport of BCAA into synaptosomcs even at further lower concentrations of these amino acids in the extracellular medium which might occur due to decreased food intake in older animals.

Male Wistar rats aged 1, 3 and 24 months, maintained under 12 h light and 12 h dark cycles at 21 ± 1°C were used. A nutritionally balanced diet (Hindusthan lever Ltd, India) and water were given ad libitum.

Synaptosomcs were prepared from the cerebral cortex of rats of the above age groups and their purity was determined as described earlier [14,15]. Protein content was determined by the method of Lowry et al. [9].

Both high and low affinity uptake of [^{3}H]leucine and isoleucine was performed as described earlier [14]. Non-specific transport was determined by incubating the samples at 0°C and due corrections were made.

The kinetic constants K_m and V_{max} were determined by Eadie-Scatchard analysis of the data obtained from the saturation isotherms using the Sigma Plot program.

Preliminary standardization of the transport was done in synaptosomcs prepared from the rats aged 3 months as described earlier [14]. Initial studies on transport were performed using a concentration range of 0.1–10 mM BCAA. Scatchard analysis of the data showed a curvilinear plot (Fig. 1a), indicating the presence of more than one transport system. This was confirmed using a concentration range of 0.5 μM to 10 mM. Scatchard plots of these data confirmed the presence of two types of transport system, one with high affinity and low capacity, and the other with low affinity and high capacity (Fig. 1b). Further studies revealed the presence of two types of uptake system in all the age groups studied. In all the age groups studied, K_m for the high affinity uptake system was 2-3 orders less than that of the low affinity uptake system, while the V_{max} for the former, was several fold less than the latter (Tables 1 and 2). The physiological significance of such high affinity transport systems for essential amino acids in the brain was previously discussed [14].

Presently, age-dependent changes in the kinetic parameters (K_m and V_{max}) of both high and low affinity transport systems into cortical synaptosomcs were reported. The K_m for both high and low affinity transport systems of leucine and isoleucine into cortical synaptosomcs of 24 month-old rats was lower than that of 1 month-old rats. The K_m of the high affinity transport systems for leucine was lower in 3-month-old rats than in 1-month-old rats while no such difference was noticed

Table 1

Age dependent variations of the kinetic parameters of [^{3}H]-leucine transport into cortical synaptosomcs

Age	High affinity		Low affinity	
	K_m (μM)	V_{max} (nmol (mg protein)$^{-1}$ h^{-1})	K_m (mM)	V_{max} (nmol (mg protein)$^{-1}$ h^{-1})
1 month	70 ± 10	45 ± 5	7.6 ± 2	770 ± 174
3 months	44 ± 3[a]	40 ± 3	6.2 ± 0.4	371 ± 32[a]
24 months	43 ± 7	25 ± 4[a,b]	3.2 ± 0.2[a,b]	278 ± 12[a,b]

K_m and V_{max} were derived from the Eadie-Scatchard plot of the data obtained from the saturation isotherms of four individual experiments done in duplicate.

Table 2

Age dependent variations of the kinetic parameters of [^{3}H]isoleucine transport into cortical synaptosomcs

Age	High affinity		Low affinity	
	K_m (μ)M)	V_{max} (nmol (mg protein)$^{-1}$ h^{-1})	K_m (mM)	V_{max} (nmol (mg protein)$^{-1}$ h^{-1})
1 month	66 ± 4	41 ± 3	8.0 ± 1	638 ± 123
3 months	62 ± 2	60 ± 3[a]	4.4 ± 1[a]	270 ± 38[a,b]
24 months	28 ± 1[a,b]	16 ± 1[a,b]	4.0 ± 0.5[a]	313 ± 26[a]

K_m and V_{max} were derived from the Eadie–Scatchard plot of the data obtained from the saturation isotherms of four individual experiments done in duplicates. In all these studies, statistical significance was calculated using Neuman–Keul's multiple range test.

[a] Significant when compared to 1 month, $P < 0.01$.

[b] Significant when compared to 3 months, $P < 0.05$.

for isoleucine. Changes observed in the K_m of low affinity transport systems for these two amino acids in 1- and 3-month-old rats were opposite to those described for the high affinity transport system. When a comparison was made between 3- and 24-month-old rats, a statistically significant difference was noticed in the high affinity transport systems of isoleucine and low affinity transport systems of leucine only (Fig. lc,d) (Tables 1 and 2).

Similar to the changes in K_m, the V_{max} of these two transport processes was decreased in 24 month old rats when compared to 1-month-old rats. The magnitude of decrease was between 47 and 64%. When a comparison was made between 1- and 3-month -old rats, the V_{max} for the low affinity transport system in cortical synaptosomcs of these two amino acids was lower in the later age groups than the earlier age groups. In contrast, the V_{max} of the high affinity transport systems of isoleucine was higher in 3-month-old rats than in 1-month-old rats, while no difference was observed in the V_{max} of the high affinity transport system for leucine. A comparison between 3- and 24-month-old animals revealed a fall in the V_{max} of both high and low affinity transport systems into cortical synaptosomcs for these two amino acids with the exception of the low affinity system for isoleucine (Tables 1 and 2).

The results of the present investigation indicated an overall decrease in the K_m and V_{max} of both high and low affinity transport systems into cortical synaptosomcs prepared from rats aged 24 months. Such a change could be due to: (a) alterations in the rate of synthesis and degradation of carrier proteins and/or (b) synthesis of carrier proteins with altered conformations and/or (c) alterations in the membrane micro-environment (such as membrane viscosity and membrane fluidity). Earlier studies have indicated a decreased turnover of brain proteins and also synthesis of proteins with altered or abnormal conformation in the rats of older age groups [10,11]. Moreover, decreased fluidity and increased viscosity of the synaptic membranes in the rats aged 24 months have already been reported [10]. As the membrane fluidity is dependent on the lipid composition of the membranes, such a change would suggest alterations in the lipid composition. This would affect the hydrophobic interactions between lipids and integral proteins of membranes (such as carrier proteins) and the degree of intercalation of these proteins [10]. Such changes might alter the conformation of the carrier proteins, thus altering their affinity for the substrate and availability of the transporter sites (thus change the V_{max} of the transport process).

An age-dependent decrease in the transport of BCAA might have a physiological significance. Branched chain amino acids are essential amino acids and are not synthesized endogenously in tissue. Hence, diet is the sole source of these amino acids. As it is generally believed that food consumption declines in the older age groups of rats than in growing rats, availability of these essential amino acids to the brain (and thus their extracellular concentration in the brain) might also be lowered. Under such conditions, carriers with low affinities might not be efficient in transporting these amino acids from the extracellular environment into the cells. Lowering of K_m (and thus increase in affinity) would result in the saturation of these carriers even when the concentrations of BCAA in the extracellular environment are low. This might be helpful in maintaining adequate amounts of these amino acids required for the basal metabolism in the brain of aged rats. Moreover, it has been reported that metabolism of these amino acids (i.e. incorporation into proteins and oxidation to CO_2) were lowered in the brain of older animals (data not shown). Under such conditions, transport of these amino acids at higher rates (high V_{max}) would lead to their accumulation in the brain. A large excess of branched chain amino acids is known to exert toxic effects in the brain [17].

Financial assistance to K.V.R. Rao, from U.G.C, India, is acknowledged. The authors wish to acknowledge CSIR and ICMR for financial support and Ms. K.V. Reeba and Mr. A. Shanavas for preparing this manuscript.

[1] Betz, L. and Goldstein, W., Polarity of blood brain barrier: neutral amino acid transport into isolated brain capillaries, Science, 202 (1978) 225–227.

[2] Muse, M.G. and Reid, S.S., Leucine: a possible regulator of protein turnover in muscle, J. Clin. Invest, 56 (1975) 1250–1261.

[3] Brauhler, J.M., Lipid peroxidation induced inhibition of γ-amino butyric acid uptake in rat brain synaptosomcs: protection by glucocorticoids, J, Neurochem., 44 (1985) 1282–1288.

[4] Christensen, H.N., Some special kinetic problems of transport. In F.F. Nord (Ed.), Advances in Enzymology, Vol. 32, Wiley, New York, 1969, pp. 1–20.

[5] Corsellis, J.A.N., Neuronal loss in aging brain. In Proc. 10th Int. Congress on Gerontol., Vol. I, 1975, p. 109.

[6] Fernstrom, J.D. and Faller, I) V., Neutral amino acids in the brain. Changes in response to food and ingestion, J. Neuroehem., 30 (1978) 1531.

[7] Harman, D., The free radical theory of aging. In H.R. Warner,

R.N. Butler, R.L. Sprott and E.L. Schneider (Eds.). Modern Biological Theories of Aging, Raven Press, New York.

[8] Ingvar, M.C., Maeder, P., Sokoloff, L. and Smith, C.B , Effects of aging on local rates of cerebral protein synthesis in Sprague-Dawley rats, Brain, 108 (1985) 155–170.

[9] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., Protein measurements with Folin phenol reagent, J. Biol. Chem., 193 (1951) 265–275.

[10] Nagy, K., Simon, P. and Nagy, I.Z., Spin label studies on synaptosomal membranes of rat brain cortex during aging, Biochem. Biophys. Res. Commun., 117 (1983) 688-694.

[11] Orgel, L.E., Aging of clones of mammalian cells, Nature, 243 (1973) 441.

[12] Paliologas, G., Koivisto, V.A. and Felig, P., Interaction of leucine, glucose and ketone body metabolism in rat brain in vitro, J. Neurochem., 32 (1979) 67.

[13] Petricivic, M., Denko, C.W. and Messineo, L., Age related changes in total DNA and RNA and incorporation of thymidine and uridine in rat brain, Int. J. Biochem., 15 (1983) 751-753.

[14] Rao, K.V.R. and Murthy, Ch.R.K... Synaplosomal high affinity transport systems for essential amino acids in rat brain cortex, Neurosci. Lett., 175 (1994) 103–106.

[15] Ratna Kumari, L. and Murthy, Ch.R.K., Activities of pyruvate dehydrogenase, enzymes of citric acid cycle and amino transferases in sub cellular fractions of cerebral cortex of normal and hyperammonemic rats, Neurochem. Res., 14 (1985) 221-228.

[16] Reff, M.E., RNA and protein metabolism. In C.E. Finch and E.L. Schneider (Eds.), Handbook of Biology of Aging, Van Nostrand Reinhold, New York, 1985, pp. 225-249.

[17] Silberman, J., Daneis, J. and Feigin, I., Neuropathological observations in maple syrup urine. branched chain ketoaciduria, Arch. Neural., 5 (1961) 351-363.

[18] Shock, N.W., The physiology of aging, Sci. Am., 206 (1962) 100.

[19] Strong, R., Samorajski, T. and Gottesfeld, Z., High affinity uptake of neuro-transmitters in rat neostriatum: effects of Aging, J. Neurochem., 43 (1984) 1766–1768.

[20] Yaranell, A., Schumm, D.E. and Webb, T.E., Age dependence of nuclear RNA processing, Mech. Aging Dev., 6 (1977) 259-264.

[21] Youdkoff, M., Nissim, I., Kim, S., Pleasure, D., Hummeler, K. and Segal, S., [^{15}N]Leucine as a source of [^{15}N]glutamate in organotypic cerebellar explants, Biochem. Biophys. Res. Commun., 115 (1983) 174–179.

[22] Zang, C, Lu, H., Wang, X., Chen, L.T. and Wang, Q., Age related changes in amino acyl t-RNA synthetase activities of mouse brain, Shengwu Huaxc Zahi. I (1985) 75-78.