Running Title:

THE BINDING AND UPTAKE OF AMINO ACIDS BY BRAIN TISSUES

M.Sc. ABSTRACT BIOCHEMISTRY

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by

Pak-chung Shiu

When rat brain is homogenised in plain sucrose solution about 20-35% of tissue GABA, L-glutamic acid, L-aspartic acid, L-alanine, L-glutamine and glycine are found to be occluded by brain particles. An extra 30% for GABA and 10-15% for the other five amino acids are bound if the medium contains 40 mM NaCl. These sodium-dependent bound forms are in rapid equilibrium with the free amino acids in the medium. The binding characteristics of L-glutamine resemble that of GABA.

Both ouabain and protoveratrine inhibit the binding(sodium dependent) of these amino acids to brain particles. Their effects are greatest on GABA. Exchange, in contrast to binding, is not affected by these drugs.

Brain cortical slices incubated in normal saline medium can accumulate all these amino acids against their concentration gradients. The net uptake of GABA is the highest and that of L-glutamic acid is the least.

Ouabain is very effective in inhibiting the uptake of neutral amino acids but less effective in inhibiting the two acidic amino acids. Protoveratrine can also inhibit the uptake of all the amino acids. It actually causes leakage of L-glutamic acid from slices. Tetrodotoxin has no effect alone but it reverses the inhibition caused by protoveratrine but not that caused by ouabain. Exchange is unaffected by all these three drugs.

To my mother

THE BINDING AND UPTAKE OF AMINO ACIDS BY BRAIN TISSUES

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Master of Science.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Å	Angstrom unit
ACh	Acetylcholine
AChE	Acetylcholinesterase
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BBB	Blood-brain-barrier
°C	Degree Centigrade
C	Carbon
Ca	Calcium
Cl	Chloride
CNS	Central Nervous System
cpm	Counts per minute
CSF	Cerebrospinal fluid
DNS	1-Dimethylaminonapthalene-5-sulphonyl
DNP	Dinitrophenol
L-DOPA	L-Dihydroxyphenylalanine
dpm	Disintegrations per minute
g	Gravitational force
GABA	Gamma-aminobutyric acid
GABA-T	Gamma-aminobutyric acid-α-ketoglutarate transaminase
GAD	Glutamic acid decarboxylase
GOT	Glutamic-oxaloacetate transaminase
GPT	Glutamic-pyruvate transaminase
K	Potassium
μCi	Micro curie
μl	Microliter
μM	Micromolar
μmole	Micromole
M	Molar
mM	Millimolar
mg	Milligram
ml	Milliliter
N	Normal
Na	Sodium
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate(reduced)
Protov.	Protoveratrine(A+B)
RSA	Relative specific activity
S.A.	Specific activity
SSA-D	Succinic semialdehyde dehydrogenase
TCA	Trichloroacetic acid
TTX	Tetrodotoxin
U(U.L.)	Uniformly labelled

INTRODUCTION AND STATEMENT OF PROBLEMS STUDIED

Neurochemistry is the study of the biochemical events which take place in the nervous system and its ultimate goal is to find out how the nervous system works. In conjunction with the equally exciting and intriguing work carried out simultaneously in other fields(e.g., neurophysiology and neuroanatomy), one may gain more insight into the processes by which messages are transmitted from cell to cell, and finally, the mechanism of storing messages, or memory! Hence those substances within the nervous system which enable nerve cells to carry out their unique functions as excitable and conducting cells are worth studying, especially as to how and to what extent the metabolism of these substances may regulate their physiological functions.

Ever since the discovery of acetylcholine(ACh) as a neuromuscular excitatory transmitter(Dale,1914) numerous substances had been reported to function either as excitatory or inhibitory transmitters. Among the most intensely studied recently are the free amino acids in nervous tissues and these are the subject of this present study. More and more evidence has indicated that gamma-aminobutyric acid(GABA) is a neuroinhibitory substance in crustacean neuromuscular junctions as well as in the brains of mammals(see reviews by Elliott and Jasper, 1959; Florey, 1960; Roberts, 1962; Curtis and Watkins, 1965; Elliott, 1965 and Baxter, 1970). The parent compound of GABA, glutamic acid, and other dicarboxylic amino acids like

aspartic acid and cysteic acid are capable of depolarizing neurones (Curtis, Phillis and Watkins, 1960; Krnjevic and Phillis, 1963; Curtis and Watkins, 1965; Krnjevic, 1970), and glutamic acid is believed to be a physiological excitatory transmitter in addition to ACh. Other omega amino acids like β-alanine and glycine(GABA itself is an omega amino acid) have been shown to posses strong inhibitory property in the central nervous system(CNS)(Hayashi, 1959,1960; Curtis and Watkins, 1965). In fact, glycine is the most likely candidate for an inhibitory role in the spinal cord of cat (see review by Aprison, Davidoff and Werman, 1970). α-alanine was shown to block the excitatory effect of ACh and transmission through autonomic ganglia in the peripheral nervous system(Damjanovich, Feher, Halasz and Mechler, 1960).

The interest of studying the above amino acids lies in the fact that "..... we are dealing with a set of amino acids which include excitatory as well as inhibitory substances and which are related through the keto-acids to glucose and fatty acid metabolism and so are linked to energy metabolism " (Elliott, 1967). Indeed, glutamic acid, GABA and succinic semialdehyde constitute the " GABA shunt " to the Krebs cycle. Intravenous injection of radioactive glucose resulted in the rapid labelling of the amino acids closely related to the Krebs cycle in the brain(Gaitonde, Dahl and Elliott, 1965; Yoshino and Elliott, 1970). Glutamine is involved in the metabolism of ammonia which is also related to the neuronal activity of the brain(Elliott, 1965). The biochemical and physiological

interests of these amino acids seem to merge at this point-the energy yielding biochemical metabolism is intimately connected with the production and removal of these highly physiologically active substances.

" The elementary criteria of a transmitter substance are that, in the relevant tissue at appropriate times, it must be produced, stored, released, exert an appropriate action and be removed" (Elliott, 1965). GABA has been shown to be produced from glutamate via glutamic acid decarboxylase (GAD) activity(Sisken, Roberts and Baxter, 1960) and be released from surface of cerebral cortex of cat which was in the "sleep" state while glutamic acid was released during the "aroused" state(Jasper, Khan and Elliott, 1965). A specific GABA uptake mechanism, possibly involved in transmitter inactivation, was described in crayfish stretch receptor(Edwards and Kuffler, 1958, 1959; Sisken and Roberts, 1964) and in lobster nerve muscle(Iversen and Kravitz, 1966). In vitro experiments have shown that brain slices are capable of taking up amino acids from the incubating medium(Elliott and van Gelder, 1958; Abadom and Scholefield,1962; Lajtha and co-workers, 1965a, 1965b, 1966, 1968, 1969, 1970). A binding mechanism of GABA has been demonstrated in brain homogenate and in brain subcellular particles (see reviews by Elliott, 1965, 1967; Roberts, 1968; Baxter, 1970). Binding studies for the other physiologically active amino acids have been lightly touched upon(Gaitonde, Dahl and Elliott, 1964; Elliott, Khan, Bilodeau and Lovell, 1965; Strasberg, 1967).

A variety of neurotropic drugs can exert tremendous

effects on the nervous system and many of these substances have been shown to affect amino acid metabolism(see review by Himwich and Agrawal, 1969; Baxter, 1970). Ouabain is known to affect brain functions such as amino acid transport and ionic transport(Quastel, 1965). The anti-hypertensive agent, Protoveratrine, has been shown to affect amino acid-carbohydrate metabolism in brain(Kini and Quastel, 1960) and to inhibit the uptake of dihydroxyphenylalanine(DOPA)(Yoshida, Kannike and Namba, 1963) and GABA(Gottesfeld and Elliott, 1971) by brain slices. The neurotoxin, tetrodotoxin, also affects the excitability of nerve cells(Kao, 1966) and brain metabolism concerning ionic relationships (McIlewain et al, 1969; Okamoto and Quastel, 1970). All these substances are capable of affecting the specific uptake and binding of GABA by brain tissues(Gottesfeld and Elliott, 1971) and their powerful effects on neuronal activities may indicate that they can interfere with the behaviour of neurotransmitters.

The present work was undertaken to elucidate further the nature of the processes involved in the binding and uptake of physiologically active amino acids by brain tissues and the factors which affect these processes. Such an attempt might shed light on the roles of GABA, glutamic acid, aspartic acid, alanine and glycine in brain and their relationships to the excitatory and inhibitory processes.

CHAPTER ONE

HISTORICAL SURVEY

A. OCCURRENCE OF FREE AMINO ACIDS

Amino acids other than GABA are building blocks of proteins and are found in living organisms throughout the plant and animal kingdoms. Almost a hundred different amino acids have been identified in plants(Fowden and Gray, 1962). They have also been found in micro-organisms(Holden, 1962). GABA has been reported to be found in the nervous tissues of a number of insects(Frontali, 1961; Ray, 1964) but has not yet been identified in protozoa(Loefer and Scherbaum, 1962). Pasantes et al(1965) reported that the nervous system of the insect Lethocerus angustipes contains more than 3 micromoles of GABA per gram of fresh tissue. The unique occurrence of GABA in nerve tissues has been demonstrated in many species of invertebrates including earthworm and especially the crustaceans. In fact, a considerable amount of work on the physiology of GABA and related amino acids is being done on nerve receptors, nerve fibers and neuromuscular preparations from lobster and crayfish(major references are being given in the review by Curtis and Watkins, 1965). The presence of GABA in the mammalian nervous system was first demonstrated by Roberts and Frankels and Awapara and co-workers in 1950, and vigorously confirmed in the same year by Udenfriend. Small amounts of GABA, not more than 10 micrograms per gram of tissue, have been detected in liver, pancreas, kidney,

gastocnemius, bladder, plasma, urine, gastric juice and saliva.

Free amino acids are quite ubiquitous in nature from the simplest to the most complex forms of organisms. GABA seems to be present in appreciable amount only in nerve tissues of higher forms but it appears in plants and other lower organisms without nervous systems without special localisation. This might suggest a specific role for GABA in higher organisms.

B. QUANTITATIVE DETERMINATION OF AMINO ACIDS IN BRAIN TISSUES.

Free amino acids can be extracted quantitatively from brain tissues by homogenisation in agents which are mild enough to denature proteins but not to cleave covalent bonds. These reagents include 80% ethanol, 1% aqueous picric acid, 5-10% trichloroacetic acid, perchloric acid and heat coagulation. The efficiencies of extraction by these agents seem to vary and hence different levels of amino acids in brain have been reported in literature. However, all values reported were within a reasonable range.

Quantitatively, amino acids can be determined by paper chromatography. Although this method is somewhat out of date it is still widely used. It has the advantage when measuring as little as one microgram of substance. However, it cannot afford the separation desirably of more than two or three amino acids in a single application in most cases. The commonest reagent used for staining the spots after they have been separated on the paper by using various solvents is the ninhydrin reagent. The subject has been reviewed quite extensively (Block et al, 1955; Smith, 1960).

Column chromatography by using ion-exchange resins for separating amino acids followed by color reaction was also used (Tallan, Moore and Stein, 1954). From these the amino acid autoanalyser has been developed(Spackman, Stein and Moore, 1958). Various modified methods to improve separation by using the analyser are available (Moore and Stein, 1963; Benson, Gordon and Patterson, 1967; Peters et al, 1968; Atkin and Ferdinand, 1970). A fully automated amino acid analyser can separate 40-50 amino acids in one single application in about 12 hours.

A recently developed quantitative method of amino acid analysis is the thin layer chromatography which involves solvent separation of amino acids or their derivatives on silica gel or cellulose coated on glass plates. This method is rapid and sensitive to measure less than one microgram of substance(see review by Stahl, 1965). 1-dimethylaminonaphthalene-5-sulphonyl(DNS) derivatives of amino acids(Morse and Horecker, 1966) and dinitrophenol derivatives (Shank and Aprison, 1970) have been used sucessfully.

Within the last couple of years there have been reports on the separation and determination of amino acids as their methyl-n-trifluoroacetyl or n-butyl-n-trifluoroacetyl derivatives by gas chromatography(Gehrke et al, 1965a, 1965b, 1966a, 1966b, 1967, 1968; McBride and Klingman, 1968; Darbe and Islam, 1968).

Apart from these general methods, a specific enzymic method for the determination of GABA was found(Jakoby and Scott,1959; Scott and Jakoby, 1959; Baxter, 1961; Jakoby, 1962). An enzyme found

in the bacterium Pseudomonas fluorescens can convert GABA to succinate via a transamination reaction with α-ketoglutarate to give succinic semialdehyde. Succinic semialdehyde is then oxidised to succinate with the formation of NADPH which can be measured spectrophotometrically. This method was improved recently (Graham and Aprison, 1966) by converting the NADPH to a highly fluorescent product by alkaline hydrogen peroxide. This permits a measurement of 10^{-11} moles of GABA.

C. METABOLISM OF GABA AND RELATED AMINO ACIDS IN MAMMALIAN BRAIN.

I. BIOCHEMICAL PATHWAYS:

There is general agreement that the brain in situ under normal physiological conditions respires almost exclusively at the ultimate expense of glucose(Gibbs et al, 1942; Elliott and Wolfe, 1962). Brain slices and suspensions in a suitable medium provided with glucose will respire at a nearly constant rate for hours. The respiratory quotient(RQ) of slices approaches untiy, the theoretical value for complete combustion of carbohydrate(Elliott et al, 1937). The glucose that is utilised directly in the mammalian brain is metabolised almost entirely through the Embden-Meyerhof pathway and under normal conditions the direct oxidative pathway(hexosemonophosphate shunt) is of little importance(Bloom, 1955; Sacks, 1957). In 1936 Greville demonstrated the tricarboxylic acid cycle in brain (Greville, 1936, 1962). L-Glutamic acid can support respiration of brain tissue in the absence of glucose and appears to be the only amino acid which could do so(Krebs, 1935; Weil-Malherbe, 1936; Woodman and McIlwain, 1961).

Intravenous injection of radioactive glucose in rats resulted in the rapid labelling of free glutamic acid, aspartic acid, alanine, glutamine and GABA in brain(Gaitonde, Marchi and Richter, 1963, 1964; Gaitonde, Dahl and Elliott, 1965; Yoshino and Elliott, 1970). All these observations suggested an intimate relationship between the metabolism of these five amino acids and that of carbohydrates. The metabolic pathway through glutamate, GABA and succinic semialdehyde is known as the " GABA shunt ". This pathway have been described in many reviews (Elliott and Jasper, 1959; Elliott, 1965; Baxter, 1970) and is shown in Figure I.

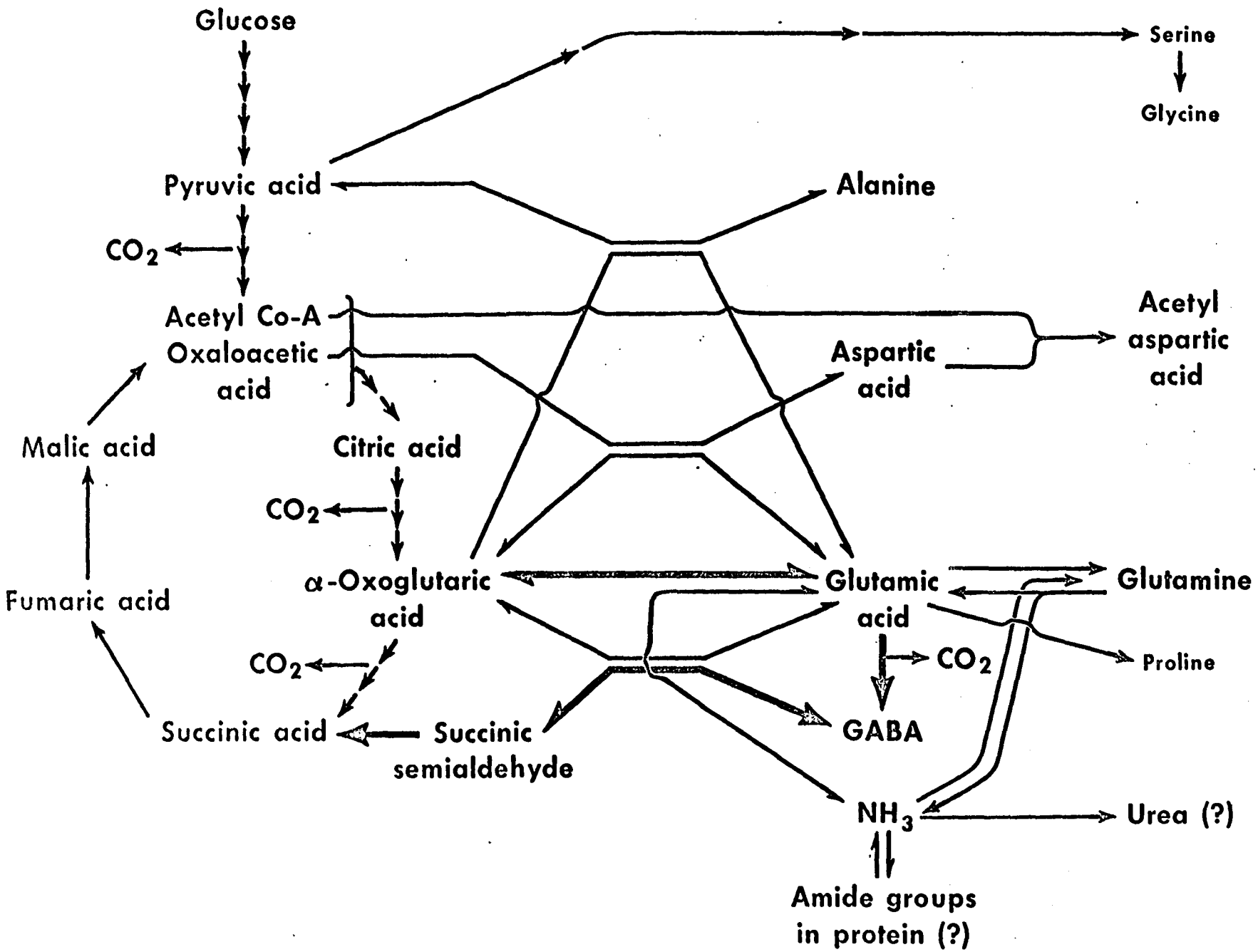
Briefly, GABA is formed by the decarboxylation of glutamic acid via glutamic acid decarboxylase(GAD) activity(Roberts and Frankel, 1953; Roberts and Simonsen, 1963; Susz, Haber and Roberts, 1966). GABA transaminates with α-ketoglutarate(intermediate of Krebs cycle) to give glutamic acid and succinic semialdehyde. This reaction is carried out by the enzyme GABA-α-ketoglutarate transaminase (GABA-T)(Bessman et al, 1953; Baxter and Roberts, 1958; Albers and Jakoby, 1960; Waksman and Roberts, 1965). Succinic semialdehyde is oxidised to succinate(another Krebs cycle intermediate) by succinic semialdehyde dehydrogenase (SSA-D) (Albers and Koval, 1961; Embree and Albers, 1964; Hall and Kravitz, 1967). All the above reactions require pyridoxal phosphate as cofactor. As an overall reaction mechanism, the shunt pathway is pulled in the direction of succinate. Since glutamate is continually regenerated in the transamination reaction, the net effect of the "GABA shunt" is to

FIGURE I

Relation of the metabolism of amino acids and ammonia to that of glucose in brain.

Note: Parallel lines represent transamination reactions.

Reproduced from Elliott, 1965.


Glucose
Serine
Glycine
Pyruvic acid
Alanine
CO_2
Acetyl Co-A
Oxaloacetic acid
Acetyl aspartic acid
Aspartic acid
Malic acid
Citric acid
CO_2
α-Oxoglutaric acid
Glutamic acid
Glutamine
Fumaric acid
CO_2
CO_2
Proline
GABA
Succinic acid
Succinic semialdehyde
NH_3
Urea (?)
Amide groups in protein (?)

convert α-ketoglutarate to succinate with the evolution of carbon dioxide and the reduction of NADP. Carbon atoms of α-ketoglutarate are thus shunted around the portion of the Krebs cycle sequence which involve the enzyme α-ketoglutarate dehydrogenase(two enzymes) and succinyl thiokinase. Energetically, however, the Krebs cycle pathway is the more efficient, producing four ATP equivalents instead of the three ATP equivalents generated with the GABA shunt. The shunt pathway has been estimated to constitute about 10% of the total metabolism of glucose in brain(Haslem and Krebs, 1963; Elliott, 1965; Myles and Wood, 1969; Balazs et al, 1970).

Glutamic acid can combine with ammonia to yield glutamine through the activity of glutamine synthetase(Krebs, 1935; Wu, 1966). It has been suggested that this reaction might be important in the regulation of ammonia metabolism as well as neuronal activities in brain(Vrba, 1955; Elliott, 1965).

Many transamination reactions occur among these various amino acids. Glutamic-pyruvic transaminase(GPT) catalyses transamination between glutamic acid and pyruvate to produce alanine and α-ketoglutarate. Glutamic-oxaloacetic transaminase(GOT) catalyses transamination between glutamate and oxaloacetate to form aspartate and α-ketoglutarate(Meister, 1962). Transamination reactions outlined above would result in the appearance of carbon-14 atoms from C-14-glucose rapidly in the amino acids concerned.

Minor metabolic pathways lead to the formation of various dipeptides of these amino acids, especially that of glutamic

acid and GABA. However, in spite of vigorous analytical work, the physiological significance of the peptides in the diverse functional activity of the central nervous system remains obscure(Himwich and Agrawal, 1969; Pisano, 1969).

II. POSTMORTEM CHANGES IN FREE AMINO ACID LEVELS IN BRAIN:

There have been slight discrepancies concerning the amounts of certain amino acids in brain reported in the literature. The situation became clearer in 1963 when Lovell and Elliott reported that lower and presumably more valid figures for some amino acid contents of brain tissue were obtained if the brain is frozen *in situ* at the moment of death than if it is excised at room temperature before extraction.Conclusive results have been obtained for the rapid postmortem increases in the levels of GABA and alanine(Yoshino and Elliott, 1970). Moreover, the increase of GABA (about 60%) is mainly in the'free' form while the ' bound' form remains almost unchanged (Elliott *et al*, 1965)(The concept of free and bound forms of amino acids will be discussed in later sections). The cause(s) of these postmortem changes is(are) still unclear. It has been suggested that perhaps anoxic condition 'activates' glutamic acid decarboxylase(GAD) activity as well as glutamic-pyruvate transaminase(GPT) activity but 'inhibits' that of GABA-T(Strasberg, 1967). Ruscak and Macejova(1965) found increases in GABA and alanine levels in brain slices when incubated in acid medium. Such would be the condition of brain after death due to the accumulation of lactic acid. Quite recently ATP was found to inhibit

GAD activity(Tursky, 1970) and the ATP level is known to drop rapidly under anoxic conditions such as death. Perhaps the fall in ATP level may allow increased activity of GAD with resultant rise of GABA level postmortem. No rapid postmortem changes occur in amino acids other than GABA and alanine(Yoshino and Elliott, 1970).

III. COMPARTMENTATION OF AMINO ACID METABOLISM IN BRAIN

"Metabolic compartmentation" refers to the presence in a tissue of more than one distinct pool of a given metabolite. These separate pools are not in rapid equilibrium with each other but maintain, more or less, their own integrity, turnover rates and flux rates. In a single-compartment system, following the administration of tracer quantities of an isotopically labelled compound serving as precursor to a metabolic product, as the specific activity of the precursor falls, that of its product rises. With time the specific activity of the product becomes equal to or slightly greater than that of its precursor(Zilvermit, Entenman and Fishler, 1943). However, if the specific activity of the product rises within minutes to values 4-5 times as great as that of its precursor, it is most unlikely to be a one-compartment system. In the area of amino acid metabolism heterogeneous functional pools have been postulated for micro-organisms (Cowie and McClure, 1959), plants(MacLennan, Beevers and Harley, 1963) and animal tissues(Kipnis, Reiss and Helmreich, 1961). Compartmentation of amino acid metabolism in mammalian brain has been demonstrated both by *in vivo* and *in vitro* experiments using glucose and non-glucose

sources of carbon and nitrogen as well as metabolic inhibitors(see the excellent review article by Berl and Clarke, 1969).

It has been mentioned that intravenously administered U-^{14}C-glucose led to rapid appearance of labels in GABA, glutamic acid, glutamine and aspartic acid. The specific activity of glutamate of brain is always greater than that of glutamine(Cremer, 1964; Gaitonde, 1965). In addition these investigators reported that when glucogenic precursors, such as glucose, lactate or glycerol were used, the specific activity of glutamine was always less than that of glutamate and, conversely, when glutamate, glutamine or ketogenic precursors, such as acetate, butyrate or propionate were used, the specific activity of glutamine was always greater than that of glutamic acid. ^{14}C-Glutamic acid or ^{14}C-aspartic acid scarcely enters the brain if administered intravenously due to a blood-brain barrier effect (Schwerin, Bessman and Waelsch, 1950; Lajtha, Berl and Waelsch, 1959). Intracisternal administration of these compounds resulted in a ratio of the specific activity(S.A.) of glutamine to that of glutamate as high as 5(Lajtha, Berl and Waelsch, 1960; Berl, Lajtha and Waelsch, 1961; Berl and Clarke, 1969). It was suggested that the data could be explained best by assuming two pools of tricarboxylic acid cycle intermediates, both accessible to pyruvate but only one accessible to metabolites that show compartmentation.

Further support for the compartmentation of the glutamate-glutamine system came from the study with ^{15}N-ammonium acetate and ^{14}C-sodium bicarbonate. After infusion of labelled ammonium

acetate into the carotid artery the ^{15}N content of the amide group of cerebral glutamine was higher than that of liver or blood. This indicated that glutamine was synthesised in the brain. The α-amino group of brain glutamine had ten times the S.A. found in brain glutamate (Berl et al, 1962; Takagaki et al, 1961; Berl and Clarke, 1969). Apparently, the glutamine must have been derived from a compartment of glutamic acid that was not in equilibrium with the total content of this acid unless glutamine was synthesised appreciably by a pathway that did not require glutamic acid as the immediate precursor. In contrast to the brain, the α-amino group of glutamine in liver had a lower ^{15}N concentration than that of the α-amino group of glutamic acid (Berl, 1962). Following the infusion of ^{14}C-sodium bicarbonate solution the S.A. of glutamine was again higher than that of glutamic acid(Berl et al, 1962; Rossi et al, 1962; Waelsch et al, 1964). The S.A. of glutamine also was high relative to that of oxoglutarate. This suggests that the oxoglutarate is also compartmented into at least two pools(Berl and Clarke, 1969). A similar conclusion was reached by Gaitonde as a result of studies of the metabolism of ^{14}C-glucose in rat brain(Gaitonde, 1965). Balaz et al(1970), using 1-^{14}C-GABA as tracer metabolite, and Berl and Frigyesi(1969), using ^{14}C-leucine, were able to demonstrate the compartmentation of the glutamate-glutamine system in brain.

The paradoxical finding that fluoroacetate, the specific inhibitor of the enzyme aconitase of the citric acid cycle, did not affect the rate of respiration, hence the production of carbon

dioxide from glucose, of brain tissue(Lahiri and Quastel, 1963) but did inhibit the production of carbon dioxide from acetate(Gonda and Quastel, 1966) and also inhibited the formation of glutamine(Nicklas et al, 1968) suggested that there are two citric acid cycles operating in the brain. Since the mitochondria are the sites of operation of the citric acid cycle, the finding that rat brain mitochondria consisted of heterogenous populations with regard to enzyme distributions(Salganicoff and De Robertis, 1965) and metabolic pathways(Neidle et al, 1969) provide further evidence for the existence of two Krebs cycles in brain. Berl and Clarke(1969) suggested that one cycle is composed of relatively larger pools of intermediates that interchange rapidly with a large pool of glutamic acid and a smaller pool of glutamine. The other cycle, composed of relatively smaller pools of intermediates, interchanges rapidly with the small pool of glutamic acid that equilibrates with the large pool of glutamine. The former cycle is called the 'energy' cycle which is mainly involved in the production of energy (e.g., the production of ATP and creatine phosphate) while the latter cycle is called the 'synthetic' cycle which is mainly involved in the synthesis of glutamine and possibly other physiologically active amino acids, in protein synthesis and in ammonia detoxification. The above mentioned experimental observations made in the study with fluoroacetate would be explainable if we assume that the 'synthetic' cycle is inhibited by this substance but not the'energy' cycle.

As to how these compartmentations come about is still unknown. It may be considered that the compartments consist of two or

more cell types, one of which contains a low level of glutamic acid but rapidly converts the amino acid to glutamine. The other cell type would contain a substantial amount of glutamic acid and little glutamine, with little capacity to make it and thus would dilute the isotopic glutamic acid to a greater extent. Compartmentations can also consist of different types of subcellular particles. The heterogeneity of mitochondria is a good indication of this possibility. Therefore, these different cell types and(or) different types of subcellular particles would allow certain metabolites to enter but restrict the entry of others; and(or) these different structures contain a heterogeneous distribution of the enzyme which utilise the tracer metabolites administered. The finding that mitochondria isolated from nerve endings were rich in GAD but contained little GABA-T activity(Salganicoff and De Robertis, 1963) might be a good support for this assumption. Hence the work on mitochondrial heterogeneity could be a promising beginning in the elucidation of metabolic compartmentations.

D. PHYSIOLOGY AND PHARMACOLOGY OF γ-AMINOBUTYRATE, GLUTAMATE AND RELATED AMINO ACIDS IN NERVE TISSUE:

Amino acids had been considered as intermediates in cellular metabolism and as building blocks of proteins, and it was said that " simple amino acids do not show startling drug actions". Recently there has been a considerable awakening of interest in these substances, particularly because of the neurochemical and neuropharmacological concern with GABA and related compounds.

In 1950, Roberts and his co-workers found considerable amount of GABA in brain. Florey(1954) reported that extracts from mammalian central nervous tissue exerted inhibitory and excitatory effects on the discharges from the 'slow-adapting' neurone in the crayfish stretch receptor organ. The substance or factor responsible for the inhibitory action was called 'Factor I'(Elliott and Florey, 1956). In crustaceans preparations of Factor I blocked neuromuscular transmission, slowed and even stopped neurogenic heart-beat, antagonised the excitatory effect of acetylcholine(Florey, 1953, 1954). It also blocked synaptic transmission in the inferior mesenteric ganglion of cat and rabbit and in the satellate ganglion of the cat(Florey and McLennan, 1955). This factor was later purified and identified as γ-aminobutyric acid(GABA)(Bazemore, Elliott and Florey, 1956,1957). Topical application of GABA solution caused a variety of changes in the electrical activity of the cerebral cortex of the cat(Iwama and Jasper, 1957; Purpura, Girado and Grundfest, 1957). GABA applied iontophoretically showed depressant actions on various types of neurones(Curtis,Phillis and Watkins, 1959). The powerful effect of GABA was later shown to be indistinguishable from synaptic inhibition, that is, it hyperpolarises membrane potentials(Krnjevic, 1970). Comparable observations on neurones in Deiter's nucleus make it probable that GABA is the inhibitory transmitter released in the medulla by the endings of the cerebellar Purkinje cells(Obata et al, 1967).

Electrophysiological studies have shown that other short-chain omega amino acids and derivatives of GABA possess inhibitory

property(see review by Curtis and Watkins, 1965). Some of these substances are:

$H_2N.CH_2COOH$	glycine(Aprison et al,1970)
$H_2N.CH_2CH_2CH_2COOH$	GABA(Elliott,1965)
$H_2N.CH_2CH_2COOH$	β-alanine(Curtis and Watkins,1965)
$H_2N.CH(CH_3)COOH$	α-alanine(Curtis and Watkins, 1965)
$H_2N.CH_2CH_2SO_3H$	taurine(Curtis and Watkins, 1965)
$HN=C(NH_2)NHCH_2CH_2COOH$	β-guanidinopropionic acid (Curtis and Watkins, 1965)
$H_2N.CH_2CH_2CH_2(C=O)OCH_2CH_2\overset{+}{N}(CH_3)_3$	γ-aminobutyrylcholine (Kuriaki et al, 1958)
$H_2N.CH_2CH_2CH_2COOH$	delta-amino-N-valeric acid (Curtis and Watkins, 1965)
$H_2N.\ CH_2CH(OH)CH_2COOH$	γ-amino-β-hydroxybutyric acid(GABOB) (Hayashi, 1958)

Among these compounds, GABA is thought to be the natural inhibitor in the brain while glycine is the natural inhibitor in the spinal cord. According to Eccles and his co-workers, physiological inhibition by GABA would be presynaptic since its action is strychnine-resistant and depressed by picrotoxin. Hence glycine is a postsynaptic inhibitory substance in the spinal cord since its action is depressed by strychnine but not by picrotoxin(Eccles et al, 1963).

Other excitatory transmitter suspects are generally acidic amino acids and glutamic acid is the one which has been studied most. The excitatory action of glutamate was first apparent in Hayashi's experiment on cerebral cortex(Hayashi, 1956). More elaborate work by Krnjevic and his co-workers have demonstrated that glutamate works by

depolarising the neuronal membrane at the synaptic region since it will not depolarise spinal ganglia and neuroglia(Krnjevic and Phillis, 1963; Krnjevic and Schwartz, 1967; Krnjevic, 1970). L-Aspartic acid, the other dicarboxylic acid found in brain, has an excitatory action comparable to that of glutamate. The excitatory action depends critically on the presence of one amino and two acidic groups- thus oxoglutarate, glutamine and N-acetylaspartic acid are practically inactive(Krnjevic and Phillis, 1963; Krnjevic, 1970).

Apart from neurophysiological studies, regional distribution study of some amino acids in cat spinal cord by Aprison and his co-workers showed that the distribution patterns of GABA, glycine, glutamate and aspartate fitted their expected roles as neurotransmitters(Graham, Jr., Shank, Werman and Aprison, 1967). Subcellularly, GABA and glutamate were shown to be present in nerve ending particles, just like ACh which is present almost exclusively in the same fraction(Bilodeau, 1962; Johnson and Whittaker, 1963). Salganicoff and De Robertis(1965) reported that GAD(the enzyme which makes GABA from glutamate) is localised in the ACh-poor nerve ending particles, suggesting a difference in the distribution of GABA, glutamate and ACh.

Others said that the above studies might be irrelevant to brain function <u>in situ</u>. One problem is that in the intact animal brain amino acids levels could be raised or lowered without producing clear-cut and reproducible behavioral effects. Perhaps the experiment by Jasper, Khan and Elliott(1965) and subsequent work by Jasper and his co-workers might have shed light on the problem.

They were able to show, by measuring the amino acids released from the perforated pial cortex of the cat in different EEG states of 'arousal', that glutamate and aspartate were released during the 'aroused' state while GABA was released during the 'sleep' state. Free, released GABA is probably more significant for function than the total level of GABA which is mostly in an inactive, storage situation. This may also be true for glutamate,aspartate and glycine as well.

E. THE UPTAKE AND BINDING OF AMINO ACIDS BY BRAIN TISSUE:

In 1958, Kuffler and Edwards, while studying the inhibitory effect of GABA on the crayfish stretch receptor, found that the effect of GABA quickly disappeared and the GABA in the bathing medium disappeared too. Fresh GABA had to be added to the medium in order to restore inhibitory action. This was the first indication that there might be a very active mechanism to inactive GABA. The same phenomenon was later found in lobster nerve muscle (Iversen and Kravitz, 1966). As Krnjevic and Schwartz(1966) pointed out, GABA is virtually absent in cerebral spinal fluid despite its high concentration in the brain. It is only released into the extracellular fluid where it exerts its effect. Elliott and Florey(1956) found that when brain tissue was homogenised in isosmotic saline solution and centrifuged, the supernatant fluid contained only part of the Factor I activity. Part of the Factor I(GABA) remained in the sediment and it could be extracted by heat, dilute alkali or acid, hypotonic medium, freezing or ethanol treatments(Elliott and van Gelder,

1958,1960; Lovell and Elliott, 1962). Radioautographic studies showed that GABA was bound to isolated abdominal stretch receptors of crayfish during brief incubation in suitable salt solution(Sisken and Roberts, 1964). In vitro experiments have shown that brain slices are capable of accumulating amino acids from the incubation medium, with GABA, glutamic acid, glycine and aspartic acid most actively taken up (Elliott and van Gelder, 1958; Abadom and Scholefield, 1962; Lajtha et al , 1965-1970).

I. BOUND AND FREE AMINO ACIDS IN BRAIN HOMOGENATES:

The binding of GABA had been studied most thoroughly. When brain is homogenised in isotonic(0.32 M) sucrose solution at room temperature by using a Potter-Elvejhem homogeniser and the homogenate is then centrifuged at 15,000xg for 15-30 minutes at 0°C, about 60-65% of GABA is found in the supernatant and the remaining 35-40% is associated with the sediment. The GABA that is found in the supernatant is called 'free' and that found in the sediment is called 'bound' (Elliott and Florey, 1956; Elliott and van Gelder, 1960; Lovell and Elliott, 1963). However, when the homogenising medium contains some sodium chloride, the amount of GABA bound increases to 60-65% . The extra GABA bound in the presence of NaCl is termed the 'loosely bound'form.and the original 30-35% GABA found in the residue in the presence of plain sucrose solution is called the 'firmly bound' form or the 'occluded' form. The loosely bound form is decreased if the brain is homogenised at 0°C instead of at room temperature or at 37°C.

The optimum pH for binding is 7.3-7.5, in the vicinity of 7.38 which is the isoelectric point of GABA. Sodium ion is necessary for extra binding and a sodium concentration of 40 mM is optimal for binding. Other cations are less effective(Elliott et al, 1965; Strasberg and Elliott, 1967; Gottesfeld and Elliott, 1971). The anion, Cl^-, is apparentlynot important since Na_2SO_4 is as effective as NaCl(Sano and Roberts, 1963). If potassium ion is used, no extra binding can be observed(Strasberg and Elliott, 1967; Gottesfeld and Elliott, 1971).

Using C^{14}GABA as tracer, it was shown that the 'loosely' bound GABA is readily exchangeable with the 'free' form and the exchange is sodium as well as temperature dependent(Strasberg and Elliott, 1967). The occluded form cannot exchange rapidly with the free form but some exchange can be promoted by sodium between the occluded and loosely bound forms.

From the study with brain homogenates it was concluded (Elliott, 1965) that GABA could exist in at least five states in brain:

1. Free extracellular GABA—apparently very little exists extracellularly; there is practically none in the cerebrospinal fluid(Dickinson and Hamilton, 1966).

2. Free intracellular GABA—that GABA which is not bound in saline suspensions at 23°C.

3. Loosely bound GABA which exists in the presence of NaCl and is readily exchangeable with free GABA.

4. Firmly bound GABA—that amount found associated with

particles in plain sucrose medium.

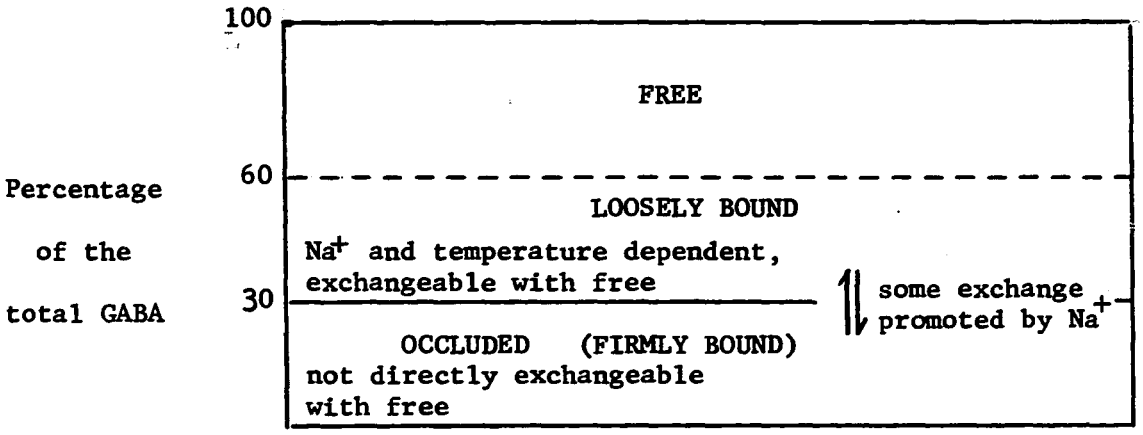
5. GABA which is covalently linked to another amino acid to form a dipeptide, e.g., homocarnosine, GABA-choline and γ-aminobutyrylcholine. The amounts of these substances in brain are small and their significance is still unknown. The different forms of GABA are illustrated in Figure II.

Grinding the tissue with sand(Elliott and van Gelder, 1960) or passing saline suspensions through an Emanuel-Chaikoff homogeniser with a clearance of 27 microns(Elliott et al, 1965) had no effect on the binding of GABA and other amino acids, thus excluding the possibility that GABA was being occluded in unbroken cells. Microsomes and nerve ending fragments(but not mitochondria) were shown to possess this binding property(Varon et al, 1965; Elliott,1967).

The binding phenomenon had been further characterised by Roberts and his co-workers who showed that the affinity of GABA for the sedimentable fraction is organ specific and presumably the result of selective binding sites on membranes of subcellular particles. The process is non-enzymatic and requires no energy source. The binding site is quite specific as judged from inhibitor studies and sulfhydril reagents, surface active agents and GABA analogues inhibit binding. Low concentrations(1×10^{-3}M) of chlorpromazine, α-γ-diaminobutyric acid and imipramine inhibit the binding of GABA to synaptic vesicles by more than 75% (Sano and Roberts, 1963; Varon et al, 1965; Roberts and Kuriyama, 1968; Kuriyama et al, 1968).

Preliminary binding studies on the other putative

Figure II. Diagrammatic illustration of different forms of GABA:*



* Reproduced from Elliott, 1967.

transmitter amino acids had been carried out by Elliott and his co-workers(1965) and a summary of their finding is presented in Table I.

Table I Binding of amino acids by brain homogenates:

	Percentage of total amino acid bound				
Medium	L-alanine	L-aspartate	L-glutamate	L-glutamine	Investigator
0.32 M sucrose	12	9	17	4	Elliott et al, (1965)
(23°C)	23	33	23	20	Gaitonde et al, (1965)
Ringer-phosphate	29	21	36	32	Elliott et al, (1965)
(23°C)	27	44	30	30	Gaitonde et al, (1965)

II. UPTAKE OF AMINO ACIDS BY BRAIN SLICES:

A given substance may cross a membrane by a number of ways. Substances of lipid nature can permeate a membrane by free diffusion which is driven by the concentration difference of the substance on the two sides of the membrane. Water and small water-soluble molecules may permeate through hypothetical channels(4-8 Å) in the membrane. A substance can also cross a membrane by means of pinocytosis in which the substance is engulfed within the membrane by a vesiculative effect, and is then released on the other side. The passage of amino acids through a membrane is by means of carrier-mediated transport.

The uptake of amino acids by brain tissue is quite complex and is energy dependent(Elliott and van Gelder, 1958; Abadom and Scholefield, 1962; Battistin, Grynbaum and Lajtha, 1969). This process may lead to a concentration in the tissue often against a diffusion gradient. A molecule may also cross a membrane by exchange process, carrier mediated or otherwise, between molecules on one side of the membrane and molecules on the other side without net transfer.

The mechanism by which various substrates enter the brain is largely unknown. Many substances such as electrolytes, metabolites, colloids, dyes and even amino acids(e.g., GABA and glutamic acid) do not penetrate the CNS from the blood. The brain remains free of them or accumulates only a small amount even in the presence of high plasma levels when other organs become easily saturated(Bakay, 1956). This phenomenon of normally restricted permeability has given rise to the term ' blood-brain-barrier'(BBB). This barrier system emerges as a complex of morphological and metabolic components, a system of membrane and transport processes(Lajtha, 1962). However, it is to be distinguished from a CSF-brain barrier because a substance, which cannot pass through the BBB, may well enter the brain when it is introduced intracisternally. Hence the <u>in vitro</u> uptake of amino acids which cannot pass the BBB <u>in situ</u> is still relevant to the mechanism operated in brain <u>in situ</u> although the controlling factors may be different or missing in the former situation(Lajtha, 1964).

In general, all amino acids can be taken up by brain slices against their concentration gradients. Glucose(as energy source)

and sodium ions are essential for uptake. GABA, glutamate and glycine are the ones that are taken up most actively(see also below). The uptake is rapid initially, followed by a slower rate and finally reaches a steady state when the efflux is equal to the influx(Blasberg and Lajtha, 1965). The substrate specificity of transport was studied by measuring the inhibition of uptake of individual amino acids by related compounds. It was found that there are six transport sites, each one utilised by a group of amino acids of similar charge and structure. The six classes are: (1) small neutral, (2) large neutral, (3) small basic, (4) large basic, (5) acidic and (6) GABA. A particular amino acid seems to have an affinity to more than one transport site, a high affinity to its primary site of transport and a lower affinity to a secondary site of entry(Blasberg and lajtha, 1965).

III. THE EFFECTS OF NEUROTROPIC DRUGS ON THE BINDING AND UPTAKE OF AMINO ACIDS BY BRAIN TISSUE:

Since amino acid uptake is an active process utilising energy it is natural to assume that any substance which depletes cells of energy supply will inhibit amino acid uptake. Hence cyanide, dinitrophenol and ouabain are all inhibitors. However, the situation seems to be more complicated than this. Dinitrophenol(DNP) at concentrations which uncouple oxidative phosphorylation does not inhibit amino acid uptake(Berger, 1957). Higher concentrations become effective(Tsukada et al, 1963). Also ouabain at concentrations which do not affect neuronal respiration or glycolysis and the

production of ATP inhibits amino acid uptake(Quastel, 1965; Okamoto and Quastel, 1970). Ouabain is known to be an inhibitor of the Mg^{++} dependent Na^{+}-K^{+} activated adenosine triphosphatase(ATPase). It is postulated that ouabain inhibits amino acid transport by competing with these substances for the carrier sites(Gonda and Quastel, 1962) which require ATP for activity. Such an hypothesis makes it possible to link ATPase to transport. Ouabain also inhibits the sodium-dependent binding of GABA to brain suspensions(Gottesfeld and Elliott, 1971).

Protoveratrine also inhibits the sodium-dependent binding of GABA to brain particles(Gottesfeld and Elliott, 1971) and the uptake of GABA(Gottesfeld and Elliott, 1971) and L-DOPA (Yoshida et al, 1963) by brain slices. Both protoveratrine and ouabain promote influx of sodium and water into brain slices(Okamoto and Quastel,1970). Tetrodotoxin(TTX), a substance which inhibits the sodium-carrier(Moore and Narahashi, 1967) and therefore the influx of sodium ions into cells, can release the inhibitory effect of protoveratrine but not that of ouabain in the binding and uptake of GABA(Gottesfeld and Elliott, 1971).

Thus, at least in part, cerebral passage of amino acids is carrier-mediated and has an active energy-requiring component. The work which is to be presented in this thesis concerns the elucidation of the effects of these three drugs, ouabain, protoveratrine and TTX, on the binding and uptake of GABA and the other amino acids related to the Krebs cycle by brain tissue.

CHAPTER TWO

MATERIALS AND METHODS

I. ANIMALS

Adult male white rats of the Sprague-Dawley strain, weighing 150-200 grams, were obtained from the Canadian Breeding Laboratories in St. Constant, Quebec, Canada.

II. MATERIALS

Radioisotopes were purchased as follows: L-aspartic-C^{14}(U) acid, L-glutamic-C^{14}(U) acid, L-glutamine-C^{14} (U), L-alanine-C^{14} (U), glycine-C^{14}(U) and inulin-carboxyl-C^{14} from New England Nuclear Corporation, Boston, Mass.; GABA-1-C^{14} from California Corporation for Biochemical Research, Los Angeles, California.

Unlabelled amino acids were purchased as follows: L-glutamic acid and GABA from California Corporation for Biochemical Research, Los Angeles, California; glycine and L-alanine from British Drug House Ltd., Poole, England; L-glutamine and L-aspartic acid from Nutritional Biochemicals Corporation, Cleveland, Ohio; and calibration amino acid mixture, type 1, for the amino acid analyser from the Spinco Division of Beckman Instruments Inc., Palo Alto, California.

Resins, type PA-28, PA-35 and UR-30, and other accessories for the Beckman Model 120C automatic amino acid analyser were purchased from Beckman Instruments Inc., Palo Alto, California;

reagents needed for the same sytem(ninhydrin, methyl cellosolve and thioglycol) were purchased from Pierce Chemical Co., Rockford, Illinois; other materials for making amino acid analysing buffers were purchased from Matheson Coleman and Bell, Norwood, Ohio.

All reagents for scintillation counting were bought either from the Packard Instrument Co., Downers Grove, Illinois or from the Matheson Coleman and Bell, Norwood, Ohio.

Dowex 2-X8 resin, Cl^- form, trichloroacetic acid and picric acid were obtained from J.T. Baker Chemical Co., Phillipsburg, New Jersey.

Drugs were obtained as follows: ouabain from Sigma Chemical Co., St. Louis, Missouri; protoveratrine(A + B) from the Mann Research Laboratory, New York, N.Y. and tetrodotoxin, crystalline 3X form, was manufactured by the Sankyo Company Ltd., Tokyo, Japan.

All other chemicals used were analytical grade reagents purchased from either the Fisher Scientific Co. or the Canadian Laboratory Supplies, Montreal, Quebec.

The water used for all purposes was de-ionised distilled water from the McIntyre Medical Sciences Building, McGill University, Montreal and was allowed to pass through another column of ion-exchanger, produced by the Illinois Water Treatment Company, Rockford, Illinois, before use.

III. EXPERIMENTAL DESIGN FOR BINDING STUDIES WITH BRAIN HOMOGENATES:

Rats were decapitated with a guillotine, their brains

removed and divided into two hemispheres. Each hemisphere was weighed and homogenised in 9 volumes solution containing either 0.32 M sucrose or 0.24 M sucrose-0.04 M NaCl. The homogeniser used consisted of a bodine motor(Greiner Scientific Corporation, New York) which turned a chuck into which the shaft of a pestle could be securely locked. The teflon pestles and glass homogenisers(with a clearance of 0.15 mm) were obtained from A.H.Thomas(Philadelphia, Penn.). This type of homogeniser is generally known as the Potter-Elvejhem homogeniser.

When the effects of drugs were studied, the drugs were added to the homogenising media to a concentration of 1 mM. Tracer of labelled amino acids were added as follows: 1.1×10^5 dpm of each of C^{14}-amino acid(U.L.)— L-alanine(4.1×10^{-4}μmoles), L-glutamate (2.5×10^{-4}μmoles), L-glutamine(2.4×10^{-4}μmoles), L-aspartate (2.9×10^{-4}μmoles) and 1-C^{14}-GABA(4.0×10^{-4}μmoles).

Homogenisation was usually carried out for one minute at room temperature(23°C). Either immediately or after standing at room temperature for 30 minutes, the suspension was centrifuged at 15,000g for 30 minutes at 0°C in a tared tube.which has been weighed.

After centrifugation, the supernatant was carefully taken out with a Pasteur pipette. The sides of the tube were gently wiped with a Q-tip and the tube together with the sediment were weighed. To the supernatant an equal volume of saturated picric acid solution(strength less than 2%) was added. The same volume of 1% picric acid solution was added to the sediment and homogenised. Both picric acid mixtures were centrifuged at about 10,000g for 10 minutes.

The amino acids contained in the clear(protein-free) picric acid extract obtained from the supernatant of the initial homogenisation are referred to as ' free ' while those extracted from the sediment are referred to as ' bound '. The picric acid extracts were subjected to further treatments as described in section V.

IV. EXPERIMENTAL DESIGN FOR UPTAKE STUDIES WITH BRAIN CORTEX SLICES:

(A) PREPARATION OF BRAIN CORTEX SLICES:*

Immediately after decapitation, the brain of the rat was carefully removed from the skull with an Elliott brain scoop. It was then introduced into a humid chamber which was continually flushed with air saturated with water. Cerebral cortical slices, not more than 0.5 mm thick, were cut with a modified Stadie-Riggs slicer (Elliott, 1955, 1969). Four dorsal and four lateral slices were obtained from each brain. Slices were put in petri dishes resting on ice in order to keep the slices cold and preventing anaerobic swelling(Pappius, Klatzo and Elliott, 1962). All the above manipulations were carried out in the humid chamber.

(B) INCUBATION OF BRAIN SLICES:

Pairs of slices, one dorsal and one lateral, were weighed on a torsion balance(Roller-Smith, Bethlehem, Pa., U.S.A.). Each pair of slices was then transferred to a 25 ml Erlenmyer flask

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* For detailed discussions on this aspect, see Elliott, 1955, 1969.

containing 4.8 to 5.0 ml(depending on the volume of amino acid solution to be added later) of ice-cold bicarbonate-buffered balanced salt solution containing glucose. The solution used had the following composition(Elliott, 1955, 1969):

ION	mM	
Na^+	147.0	
K^+	3.4	
Ca^{++}	1.3	
Mg^{++}	1.2	
Cl^-	128.0	
HCO_3^-	24.5	
$H_2PO_3^-$	0.4	
$SO_3^=$	1.2	
Glucose	10.0	
Osmolarity	316.0	(pH=7.4)

Each flask contained already 0.1 ml of a 0.5 mM solution of each drug to be tested. The flasks were filled with 95% O_2- 5% CO_2(gassed for 30 seconds), stoppered, and then placed in a water bath at 37°C and shaken. Preincubation of the slices with the drugs(same for control flask without drugs) was allowed for 10 minutes. Then 0.1 ml(or 0.2 ml for L-aspartic acid and L-glutamic acid due to solubility problem for these two amino acids) of radioactive amino acid solution was added to each flask to make up a final volume of 5 ml. The final concentration of amino acid in the medium was 2 mM and that of each drug was 10μM. The specific activity of each amino acid was 0.02 μCi per μmole. The flasks were then re-gassed with the O_2-CO_2 mixture, stoppered, and incubation was carried on for another 30 minutes. At the end of the

latter incubation period, the slices were collected on a perforated porcelain disc, drained and reweighed.

The tissue was then homogenised in 2 ml of 1% picric acid and the homogenate was centrifuged at 10,000g for 10 minutes. The supernatant from each tube was separated and the sediment was re-extracted by homogenisation in 1 ml of water, centrifuged, and this supernatant was added to the first lot.

V. PREPARATION OF AMINO ACID EXTRACTS FROM PICRIC ACID SOLUTIONS:

Protein-free picric acid extracts of amino acids obtained either from the brain homogenates in binding studies or from brain slices in uptake studies were treated by the method described in the Instruction Manual for the Beckman Model 120C amino acid analyser.

To the picric acid extract(yellow in color) was added 1-2 grams of Dowex 2-X8 resins, 200-400 mesh, Cl^- form. It was then stirred on a magnetic stirrer(micro-magnetic bar was used due to the small volume of the extract) for ten minutes. The slurry was filtered through a wetted filter paper(Whatman, No. 40, ashless) and the resin washed three times with 0.02 N HCl . The washing materials were added to the filtrate(now colorless).

Due to the relatively small amounts of endogenous amino acids extracted in binding studies, the large volume of filtrate obtained had to be reduced to about 5 ml by flash evaporation. This step was not necessary, however, when working on uptake of amino acids by brain slices due to the large amount of exogenous amino acids taken

up by the slices.

The pH of each clear extract was adjusted to 7.2-7.5 with 1 N NaOH. 0.1 ml of sodium sulphite(0.5 M) solution was added and the solution was usually left overnight open to air. This step was necessary in order to convert any glutathione which, if present, would interfere the chromatography of the other amino acids,* to glutathione-S-sulphonate. The pH of the solution was then adjusted back to 2.0-2.2 and its final volume noted. Solutions thus prepared were now ready for analysis.

VI. QUANTITATIVE ANALYSIS OF AMINO ACIDS:

Quantitative analysis of aminoacids was carried out using a Beckman Automatic Amino acid Analyser, Model 120C. The operation principles of this instrument have been fully described elsewhere(see Beckman's Instruction Manual). However, a couple of important points and some modifications are summarised in Table II.

VII. DETERMINATION OF RADIOACTIVITIES:

The radioactivities of the amino acids were determined by using a flow-cell system linking the amino acid analyser and a Packard scintillation spectrometer. The eluates from the resin column(s) were directed through a Packard 2 ml flow-cell packed with anthracene crystals. The flow-cell was inserted into the counting chamber(after

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* This treatment would also convert cystine and cysteine to cysteine-S-sulfonate. Since these two amino acids were not taken into consideration in the present study, methods for their analysis are not given here.

Table II Operation conditions for the analysis of amino acids by the automatic amino acid analyser.

Amino acid analysed	Resins		Eluting buffers		
	type	height	pH	ionic strength	Temp.*
GABA	PA-35	23 cm	4.25	sodium citrate(0.38N)	44°C
L-glutamate L-glutamine L-aspartate	UR-30	56 cm	2.80	lithium citrate** (0.3N Li , 0.16N cit.)	44°C
	or				
L-alanine Glycine	PA-28	56 cm	3.25	sodium citrate(0.2N)	44°C

* This temperature was higher than that recommended in the Instruction Manual in order to separate completely the peaks for glutamine and glutamic acid when using lithium citrate as the eluting buffer. In doing so, the enormous glutamic acid peak overlapped the asparagine peak. However, the amount of asparagine in brain tissue is about 1% that of glutamic acid(Tallan, Moore and Stein, 1954) and the error introduced into the calculation of the latter is negibible.

**Lithium citrate buffer was used for the separation of asparagine from glutamine(Benson, Gordon and Patterson, 1967).

some modifications had been made in the scintillation counter) where a counting vial is normally situated. The radioactive peaks, when passing through the flow-cell, were registered. The counter was set to print out one minute counts. The eluates were then directed back to the ninhydrin system of the amino acid analyser and their constituents were then quantitatively determined.

This printout technique enabled total counts for any substance to be determined by summing the individual printouts that occurred during the passage of the radioactive material through the flow-cell and then multiplying this sum by the factor:

$$\frac{\text{counting time + printout time(dead time)}}{\text{counting time}}$$

With the flow rate of the eluate being 50 ml per hour the counting efficiency was about 40% and the value was about 32% when the flow rate was increased to 70 ml per hour.

VIII. RECOVERY EXPERIMENTS:

Since absolute values were important for the present studies, especially for binding studies, it was necessary to estimate the recovery of all amino acids in the various steps of the procedures. Known amounts of pure amino acids(usually close to the amounts found in natural tissue) were dissolved in water and put into homogenisers. They were subjected to treatments which were identical to that used

for the handling of brain tissues. Results are given in Table III. Since recoveries for all amino acids were close to 100%, no attempts had been made to account for experimental loses.

IX. ESTIMATION BY ' INULIN SPACE ' OF EXTRAPARTICULATE FLUID IN CENTRIFUGED PELLETS(AS IN BINDING EXPERIMENTS) AND EXTRACELLULAR FLUID IN INCUBATED BRAIN SLICES:

(A) CENTRIFUGED PELLETS:

In binding studies which involved homogenising brain tissue in sucrose solution and then centrifuging the homogenate, the pellet thus obtained contained a considerable amounts of free amino acids in the medium which was trapped in the sediment. The composition of this trapped fluid would be expected to be identical to that of the supernatant fluid. In order to account for the'trapped' amino acids in the pellets, the 'inulin space' method was employed.

Inulin has a molecular weight of about 6,000 and an effective hydrodynamic radius at 25°C of 15.3 Å(Bourke and Tower, 1966). Since an average cell membrane pore radius measures 4-8 Å, inulin can be used safely as an extracellular fluid marker. In this particular case the inulin space is a measure of the extra-particulate space rather than the extracellular space, but mitochondria, nerve ending particles and other subcellular particles can be assumed to be impermeable to inulin.

The inulin space of pellets was determined by homogenising the brain tissue(normally one cerebral hemisphere which weighed about 0.5 gram) in 9 volumes of medium containing 0.2 μCi (0.1 mg) of

Table III. Recoveries of amino acids after going through all steps in the extraction procedures.

Amino acid	% Recovery
GABA	99 ± 1
L-Glutamic acid	96 ± 2
L—Glutamine	97 ± 3
L-Aspartic acid	100 ± 2
L-Alanine	99 ± 1

values represent mean ± S.D. for 3-5 experiments.

carboxyl-C^{14}-inulin. The sediment obtained after centrifugation was weighed and then re-homogenised in 2 ml of 6% trichloroacetic acid. The precipitated proteins were centrifuged down and 0.5 ml of this protein-free TCA extract was pipetted into a counting vial. Also 0.5 ml of the clear supernatant obtained from the initial homogenisation step was pipetted into another vial. 10 ml of Bray's counting fluid was added into each vial and the radioactivities were determined in a Packard Scintillation Spectrometer. Since TCA quenched to a small extent, the counts obtained for the pellet was multiplied by a factor of 1.1(determined by counting known amounts of standards in the presence and absence of 6% TCA) to account for quenching.

Inulin space was calculated according to the formula;

$$\frac{\text{cpm per gram wet pellet}}{\text{cpm per ml medium}} \times 100\%$$

and values were expressed as percentage of fresh tissue weight. Inulin spaces of pellets for a number of homogenising conditions are given in Table IV.

(B) INCUBATED BRAIN CORTEX SLICES:

Swelling of brain slices upon incubation is a well known phenomenon(Pappius and Elliott, 1956; Pappius, Klatzo and Elliott, 1962). Much of the swelling is extracellular. This extracellular fluid has the same composition as that of the incubation medium. Determination of inulin spaces of slices was therefore necessary to correct for substance that was not really absorbed by the tissue but trapped in the

Table IV Inulin spaces of pellets obtained in various experimental conditions.

Homogenising medium	sediment wt.(gm) per gm fresh tissue	Inulin space, % of fresh wt.	Solid + Intracell. fluid in grams.
Plain sucrose	1.13 ± 0.03 (4)	62 ± 2 (4)	0.43
Sucrose + NaCl	1.01 ± 0.03 (6)	53 ± 2 (5)	0.47
Sucrose + KCl	1.09 ± 0.02 (8)	57 ± 5 (7)	0.47
Sucrose + $CaCl_2$	0.90 ± 0.02 (4)	53 ± 2 (2)	0.42

Concentration of plain sucrose solution was 0.32 M; all salts were present in 40 mM with sucrose reduced to 0.24 M.

All values represent mean ± S.D. but average ± range for two determinations; with number of determinations in parenthesis.

Values were unchanged when drugs(ouabain, protoveratrine or tetrodotoxin) were present, data not shown.

extracellular fluid upon swelling and adherent to the surfaces of the slices.

The inulin spaces for slices were determined by incubating the slices under conditions identical to those used in the actual experiments except that carboxyl-C^{14}-inulin was added into the incubation medium to a final concentration of 0.08 μCi (0.04 mg) per ml and that 2 mM of non-radioactive amino acid was used instead of the radioisotope. After incubation, the slices were filtered, drained, re-weighed, homogenised in 2 ml of 6% TCA, and the suspension centrifuged. Of the clear TCA extract, 0.5 ml was counted in the presence of 10 ml of Bray's solution. A measured volume(usually 1 ml) of the incubation medium was added an equal volume of 10% TCA, and the precipitated proteins centrifuged down. Of this extract 0.5 ml was used for counting. The inulin spaces(in μl per 100 mg fresh slices) were calculated according to the following formula:

$$\frac{\text{total cpm in slices} \times 10^2 \times 10^3}{\text{cpm per ml medium} \times \text{fresh weight of slices, mg}}$$

The intracellular(non-inulin space) and extracellular volumes (inulin spaces) of brain slices incubated under various experimental conditions are shown in Table V.

X. CALCULATION OF RESULTS:

The calculation for the amount of amino acids bound by brain particles for homogenate experiments was straight and simple:

Legends to:

Table V Effects of amino acids and drugs on the swelling and inulin spaces of incubated brain cortex slices.

[1] mg per 100 mg fresh weight

[2] µl per 100 mg fresh weight

[3] The non-inulin (or intracellular) space is taken as the final weight after incubation(per 100 mg fresh tissue) minus the inulin space(or extracellular space, per 100 mg fresh tissue) and minus the final dry weight which taken as 19 mg per 100 mg fresh tissue (Pappius, Klatzo and Elliott, 1962).

Table V Effects of amino acids and drugs on the swelling and inulin spaces of incubated brain cortex slices.

Addition to medium	Final concentration	[1] Final weight after incubation	[2] Inulin space	[2,3] Non-inulin space	No. expts.
None	—	132 ± 5	45 ± 5	68	10
GABA	2 mM	134 ± 3	46 ± 4	69	3
L-Alanine	2 mM	130 ± 1	39 ± 1	72	2
L-Glutamine	2 mM	132 ± 0	41 ± 0	72	2
Glycine	2 mM	129 ± 2	37 ± 3	73	3
L-Aspartic acid	2 mM	143 ± 3	39 ± 3	85	3
L-Glutamic acid	2 mM	141 ± 1	32 ± 2	90	2
Ouabain	10 μM	138 ± 3	39 ± 4	80	3
Protoveratrine	10 μM	151 ± 2	32 ± 1	100	2
Tetrodotoxin	10 μM	126 ± 0	41 ± 0	66	2
Ouabain + TTX		126 ± 3	41 ± 1	66	3
Protov + TTX		125 ± 2	41 ± 1	65	3
L-Glutamic acid plus TTX	2 mM 10 μM	138 ± 0	36 ± 1	83	2
L-Glutamic acid plus ouabain	2 mM 10 μM	142 ± 3	38 ± 2	88	3
L-Glutamic acid plus protov.	2 mM 10 μM	156 ± 3	34 ± 2	103	2
L-Aspartic acid plus TTX	2 mM 10 μM	135 ± 3	43 ± 2	73	3

the amount bound was obtained by subtracting the amount trapped in the inulin space in the pellet from the total amount found in the pellet. In the case of uptake experiments by brain slices, the calculations are more complicated and a simple equation has been derived to facilitate easier calculations. The derivation runs as follows:

Let T = total amount of amino acid, in μmoles, in incubated slices.

C = concentration of amino acid in medium, in μmoles per ml, at the end of the incubation period.

I = inulin space, μl per 100 mg fresh tissue

N = non-inulin space, μl per 100 mg fresh tissue

W = fresh weight of slices

Since the volume of extracellular fluid = $\frac{W}{100}$ x I μl ,

the amount of extracellularly trapped amino acid, in μmoles,

$$= C \times \frac{W}{100} \times I$$

therefore the amount, in μmoles, of amino acid actually taken up by the slices into the intracellular space

$$= T - C \times \frac{W}{100} \times I$$

when expressed as μmoles per 100 mg fresh wt., the equation becomes

$$\frac{\left(T - C \times \frac{W}{100} \times I \right) \times 100}{W}$$

re-arranging, it becomes:- $$\left[\frac{100T}{W} - (C \times I) \right]$$

when expressed as μmoles per μl of intracellular fluid, it becomes:-

$$\frac{\left[\frac{100T}{W} - (C \times I) \right]}{N}$$

therefore, expressed as μmoles per ml of intracellular fluid, the final equation becomes:-

$$\text{μmoles per ml of intracellular fluid} = \left[\frac{100T}{W} - (C \times I) \right] . \frac{10^3}{N}$$

The same equation gives the uptake of radioactivity by substituting T and C terms by counts per minute(cpm) instead of μmoles.

CHAPTER THREE

THE BINDING OF AMINO ACIDS BY BRAIN HOMOGENATES

RESULTS:

In preliminary experiments it was found that if a tracer amount of one of the radioactive amino acid was added to the sucrose-NaCl medium in which the brain was homogenised, and the the homogenate was centrifuged in the cold, most of the radioactivity was found in the original amino acid as show in Table VI. Of the total radioactivity 85% or more was recoveried for aspartic acid, glutamic acid, alanine and GABA. Only 60% of the radioactivity was recovered from glutamine. However, only very small amounts of labels from glutamine was found to appear in the other amino acids. This indicated that there was little metabolism for glutamine during the brief exposure to the brain tissue. On examining the labelled glutamine from the vial in which it was shipped*, it was found that about 30% of the radioactivity was not in glutamine but in other compounds. Probably about 10% of the radioactivity was in the cyclised product of glutamine, pyrrolidone carboxylic acid, which is known to be formed readily in acidic medium from glutamine(Tallan, Moore and Stein, 1956) (glutamine was dissolved in dilute HCl when it was shipped). Of the

* Known amount(5.5×10^5dpm) of radioactive material from the original vial was chromatographed together with a cold amino acid mixture, which acted as carriers, in an analysing column of the amino acid analyser, and the radioactive peaks were identified and determined.

Table VI Distribution of C^{14} labels from tracer amounts of labelled amino acids in brain homogenate immediately after homogenisation.

[1] Labelled amino acid used	Percentage of radioactivity recovered in:				
	L-aspartate	L-glutamine	L-glutamate	L-alanine	GABA
L-aspartate	85	0.3	1.4	0	0
L-glutamine	3	[2] 60	8	0.1	0.1
L-glutamate	8	0.8	85	0.2	0.2
L-alanine	0	1	0.5	84	0.4
GABA	0.8	0.2	0.2	0.5	98.2

1.1×10^5 dpm of one labelled amino acid was homogenised with one rat brain hemisphere in 9 volumes of sucrose-NaCl medium for one minute at room temperature. The homogenate was treated with saturated picric acid and the amino acids were extracted according to the procedures given in chapter 2. The radioactivity in the original compound as well as in other compounds was determined using the amino acid analyser coupled to the scintillation counter.

[1]Labelled glycine was not tested since the radioisotope was not available until later experiments with brain slices.

[2]Corresponds to 85% recovery from actual labelled glutamine.

remaining 20% label, 2% was found to be associated with glutamic acid, 2% with aspartic acid, and the remainder was present in compounds which did not interfere with the patterns of the other known amino acids. Of the radioactivity actually in glutamine, 85% was recovered. Hence it was permissible to add tracer amounts of several amino acids together to the homogenisation medium and to study the immediate distribution of these between solution and particles in the same tissue suspension.

Table VII summarises the results for binding by brain homogenates of a number of physiologically active amino acids. As has been shown previously by this laboratory, the binding of GABA was increased in the presence of sodium chloride. The amount of GABA bound was doubled in the presence of 40 mM NaCl. The firmly bound form, that is, the amount bound in the absence of NaCl, was almost unlabelled as shown by the small(0.02) relative specific activity(RSA) value. Slow exchange between the occluded form and the labelled amino acid in the medium probably occurred as judged from the increase,after 30 minutes, in RSA value from 0.02 to 0.13. All the salt-dependent bound form was probably labelled because the RSA increased to 0.41 in the presence of NaCl. Upon standing at room temperature for 30 minutes, the RSA increased further to 0.67, which indicated exchange between the loosely bound, salt-dependent, or the free form and the occluded form.

The next most effectively bound amino acid was L-glutamic acid. However, the percentage bound decreased after standing. This might have been due to a rise in total level upon standing, the extra glutamic

Legends to:

Table VII The binding and exchange of amino acids by brain homogenate.

[1] R.S.A. (relative specific activity) = $\frac{\text{specific activity of bound form}}{\text{specific activity of free form}}$

[2] Final concentration of ouabain or protoveratrine = 1 mM

Note: All values are expressed as mean ± S.D., but average ± range where there were only two determinations. Results, which show that both drugs had no effect when brain was homogenised in plain sucrose solution, are not shown in order to simplify the table.

Table VII The binding and exchange of amino acids by brain homogenate

Amino acid	Homogenising condition	Total μmoles per gm.		% Bound		R.S.A.[1]	
		0 min.	30 min.	0 min.	30 min.	0 min.	30 min
GABA	No NaCl	3.0 ± 0.4(19)	3.6 ± 0.4(19)	37 ± 1(3)	33 ± 7(4)	0.02 ± 0.01(4)	0.13 ± 0.04(4)
	NaCl(40 mM)			61 ± 5(8)	56 ± 4(9)	0.41 ± 0.06(8)	0.67 ± 0.08(8)
	NaCl + ouabain[2]			59 ± 3(3)	40 ± 3(3)	0.42 ± 0.02(2)	0.63 ± 0.08(3)
	NaCl + protov.[2]			56 ± 1(3)	29 ± 4(3)	0.38 ± 0.01(2)	0.54 ± 0.10(3)
L-glutamine	No NaCl	3.8 ± 0.4(17)	2.5 ± 0.3(15)	16 ± 1(3)	22 ± 3(3)	0.02 ± 0.03(4)	0.13 ± 0.04(4)
	NaCl ,			28 ± 5(8)	25 ± 3(9)	0.23 ± 0.10(5)	0.38 ± 0.10(8)
	NaCl + ouabain			25 ± 3(3)	20 ± 5(3)	0.23 ± 0.06(2)	0.27 ± 0.00(2)
	NaCl + protov.			28 ± 2(3)	17 ± 3(3)	0.32 ± 0.11(2)	0.51 ± 0.01(2)
L-glutamic acid	No NaCl	10.8 ± 0.8(19)	12.4 ± 1.2(19)	36 ± 2(4)	22 ± 6(4)	0.31 ± 0.03(4)	0.63 ± 0.13(4)
	NaCl			50 ± 2(9)	35 ± 3(9)	0.40 ± 0.09(8)	0.65 ± 0.10(9)
	NaCl + ouabain			49 ± 1(3)	30 ± 1(3)	0.43 ± 0.05(2)	0.64 ± 0.08(3)
	NaCl + protov.			51 ± 5(3)	31 ± 2(3)	0.45 ± 0.05(2)	0.64 ± 0.09(3)
L-aspartic acid	No NaCl	3.2 ± 0.3(19)	3.5 ± 0.5(19)	29 ± 3(3)	25 ± 5(4)	0.43 ± 0.04(4)	0.48 ± 0.07(4)
	NaCl			40 ± 5(9)	35 ± 2(9)	0.61 ± 0.04(8)	0.66 ± 0.08(9)
	NaCl + ouabain			43 ± 2(3)	30 ± 2(3)	0.62 ± 0.03(2)	0.61 ± 0.12(3)
	NaCl + protov.			47 ± 4(3)	30 ± 2(3)	0.54 ± 0.05(2)	0.58 ± 0.07(3)
L-alanine	No NaCl	0.8 ± 0.1(19)	1.0 ± 0.2(19)	26 ± 2(4)	21 ± 8(4)	0.33 ± 0.03(3)	0.46 ± 0.00(4)
	NaCl			38 ± 3(9)	32 ± 1(9)	0.46 ± 0.07(9)	0.49 ± 0.05(8)
	NaCl + ouabain			38 ± 4(4)	25 ± 3(3)	0.42 ± 0.05(3)	0.38 ± 0.01(2)
	NaCl + protov.			37 ± 4(4)	25 ± 1(3)	0.42 ± 0.06(3)	0.35 ± 0.07(3)
glycine	No NaCl	1.0 ± 0.1(16)	1.3 ± 0.2(19)	31 ± 1(2)	23 ± 7(4)		
	NaCl			40 ± 2(8)	32 ± 3(9)		
	NaCl + ouabain			42 ± 2(3)	29 ± 2(3)		
	NaCl + protov.			40 ± 3(3)	26 ± 1(3)		

acid that was formed being mainly in the free form. The firmly bound form was considerably labelled and it had an high RSA value. The presence of salt did not increase this greatly. After 30 minutes standing, the RSA values increased to the same level in the presence and absence of salt. Like GABA, the binding of all other amino acids was promoted by NaCl but to a considerably smaller extent. Upon standing, the amounts of all the amino acids remaining bound decreased somewhat.

The firmly bound, salt-independent, forms of aspartate, glutamate and alanine were highly labelled but that of glutamine behaved like GABA, which in the absence of salt, has a RSA value of only 0.02. The extra glutamine bound in the presence of salt was labelled and could exchange freely with that in the medium.

In a preceding paper from this laboratory(Gottesfeld and Elliott, 1971) it was reported that ouabain and protoveratrine prevented the sodium-dependent binding of GABA. In the present study this effect was observed only when the suspension had been allowed to stand for 30 minutes. The possible causes for this difference will be discussed in a later section. In the present study, ouabain and protoveratrine had no obvious effect initially on the sodium-dependent increase in binding of any one of the amino acids studied. Some inhibition was apparent in all cases after standing but in no case was this as marked as with GABA. The drugs had no obvious effect on the exchange of any of the bound amino acids with the corresponding radioactive amino acids in the medium.

DISCUSSION:

Ever since the discovery of the extensive binding of GABA by brain tissue, numerous studies had been carried out to elucidate the nature of this phenomenon(Elliott and co-workers and Roberts and co-workers). The present study confirms the existence of different conditions of GABA in brain tissue. Homogenised brain particles contain one GABA pool that equilibrates rapidly with the GABA in the external medium. This pool is referred to as the loosely bound form of GABA. The size of this pool obtained from the present study was about 25% of the total GABA in the tissue, which is consistent with the previous reported value from this laboratory as well as from Roberts' group(Varon et al, 1965). The remaining GABA within the particles equilibrates more slowly with that in the external medium; this pool is referred to as the occluded form. In the presence of NaCl, the sodium-dependent bound(same as the loosely bound) GABA can exchange with the occluded form. We have shown here that the sodium-dependent binding can be inhibited by ouabain and protoveratrine without affecting the occlusive binding(Table VII) which is consistent with previous findings(Gottesfeld and Elliott, 1971).

Since the process of binding is not energy-requiring, non-enzymatic and organ specific, a model of a carrier-mediated diffusion process had been proposed to account for the behaviour of GABA pools(Weinstein et al, 1965). The model proposes that there is a specific site on the membrane where an amino acid(GABA) molecule and probably two sodium ions bind to form a complex. The formation of

this complex on the outer surface of the membrane is governed by a certain equilibrium constant. When the external sodium concentration is high, lots of these complexes are formed and they can diffuse across the membrane to reach the inner surface of the membrane. In contact with the intraparticulate content, the complex is then governed by another equilibrium constant of smaller magnitude than the first one. Since the sodium content inside a particle of brain tissue is low compared to the external surroundings, the complexes dissociate into its components, and the protein-lipid carriers may travel back to the outer surface of the membrane where they form new complexes.

The findings of the present study seem to support this carrier-mediated diffusion model. Firstly, this model would explain why external sodium is necessary for the extra-binding of GABA. When brain is homogenised in plain sucrose solution, according to the model, no such amino acid-sodium-carrier complexes can be formed and the only form of GABA in the particles is represented by the occluded form which is unable to get out under those experimental conditions. Secondly, when tracer amount of radioactive GABA is present in the medium, the radioisotopes would be carried into the occluded compartment in exchange for some unlabelled ones. This seems to explain the observation that, in the presence of external sodium, the occluded form of GABA becomes labelled. Thirdly, ouabain is an inhibitor of the Mg^{++}dependent, K^{+}-Na^{+} activated ATPase which is important in maintaining the sodium pump. When the ATPase is inhibited,

the outside to inside sodium concentration cannot be maintained, resulting in the accumulation of sodium inside the particles. As a result, more complexes are formed on the inside of the membrane then on the outside and GABA is thus 'leaked' out together with a efflux of sodium until an equilibrium is reached at which no net diffusion of GABA occurs but exchange is still possible. This would explain the fact that ouabain does not affect binding at $0^{o}C$ for at this temperature the activity of the ATPase is not activated(Samson and Quinn, 1967). Also, it would probably explain the observation that ouabain was not effective if the brain tissue was homogenised in the absence of NaCl and the homogenate was centrifuged immediately but was effective if the brain tissue was homogenised in salt solution and the homogenate was allowed to stand at room temperature for 30 minutes to allow equilibration of sodium contents between inside and outside of the membrane. Furthermore, this is also consistent with the fact that ouabain would only inhibit extra(sodium-dependent)-binding but has no effect on the exchange of GABA(Table VII).

Protoveratrine is a drug known to increase the permeability to sodium ion(Okamoto and Quastel, 1970). It therefore promotes the influx of sodium into the brain particles. The effect of this drug could also be explained in a similar manner to that of ouabain. The only difference between the actions of these two drugs is that they destroy the sodium gradient via different mechanisms, for protoveratrine is not known to be an inhibitor of the ATPase. The actions of these two drugs can be separated in later experiments with

brain slices.

Results in Table VII show that the binding property of GABA and glutamine seems to deviate from that of the others. The occluded forms of these two amino acids in the absence of sodium were almost completely unlabelled. In the presence of sodium the bound forms became highly labelled, with 10 and 20-fold increase in the RSA values for glutamine and GABA respectively while the increase of RSA values for the other amino acids was of the order of 1.3 folds. Even after standing at room temperature for 30 minutes, the RSA values for glutamine and GABA in the presence of sodium were still many times greater than that in the absence of the salt. On the other hand, in the case of glutamate, aspartate and alanine, the corresponding RSA values at 30 minutes in the presence and absence of sodium were almost the same. The amounts of these three amino acids that were bound in the presence of sodium after 30 minutes were significantly greater than that without sodium. This seems to indicate that complete exchange between the bound forms and free forms could still occur even though sodium was absent, as long as enough time was available for this exchange process to take place.

The extra-binding and exchange of glutamine and GABA were therefore highly dependent on sodium ions while that for the other amino acids studied were less sensitive to sodium. Could these data imply that perhaps GABA and glutamine are occluded in particles which could be different from those in which the other amino acids are occluded ? In light of the recent findings which indicate that amino

acids are 'compartmentalised' in brain tissues(for more detailed information on the compartmentation of amino acid metabolisms,refer to chapter one), the present finding may perhaps be interpreted that glutamine and GABA are in 'compartments' different from the other amino acids. Indeed, both in vivo and in vitro experiments have shown that glutamine and glutamate are situated in different compartments (Berl and Clarke, 1969). Likewise alanine has been shown to exist in at least two pools in the brain(Yoshino and Elliott, 1970). As mentioned in chapter one, compartmentation may be the manifestation of restrictive entrance of certain substances into certain subcellular particles, this property would well reside in the membranes of these particles. Binding is believed to be the first step in 'transporting' a substance into particles and different types of membranes with different properties might restrict the passages of certain substances but at the same time allow other substances to pass through. The almost absolute requirement for sodium for loose binding of glutamine and GABA may indicate that the particles in which these two amino acids are contained require a sodium gradient for binding and only under this situation can these substances enter the particles. Amino acids which show less dependency on sodium for binding may enter particles, which are different from those that contain glutamine and GABA in permeabilities, more readily.

Since several of the amino acids studied so far are believed to be possible neurotransmitters and their regional distributions in nervous tissue have reflected their respective physiological functions

(Aprison et al, 1970), there is no reason to expect that thet should have identical subcellular distributions. Different cell types in nervous tissues have shown to contain different types of neuro-transmitters. The membrane from one particular cell type may be specific for binding of that particular transmitter only. A brain homogenate contains a very heterogeneous population of membrane-bound particles derived from a heterogeneous population of cell types. Therefore, the binding properties exhibited by different amino acids may indicate that they are bound, transported into and stored in membrane-bound systems of different properties. Glutamine is not known to be a neurotransmitter; the significance of the fact that it is bound similarly to GABA is not at present understood.

As can be seen in Table VII, the total amounts of all amino acids after standing for 30 minutes went up except that of glutamine which went down. The extra amounts might have arisen from metabolism which occurred during standing. The extra-glutamic acid was probably derived from glutamine. All the extra amino acids seem to be in the free forms because the amounts bound remained the same even after standing(absolute amounts bound after 30 minutes standing are not shown in table) and as a result, the percentage bound decreased.

A question has been raised whether the fact that more amino acids found in the sediment in the presence of salt than in its absence might not be due to specific binding but was the result of more complete sedimentation in the presence of salt. Table IV(page 42) shows that the solid plus intracellular fluid weights per gram of

fresh weight for sediment obtained in the presence or absence of salts were almost the same, which indicates that more complete sedimentation was not sufficient to account for the extra amounts of amino acids found associated with the sediments.

CHAPTER FOUR

THE UPTAKE OF AMINO ACIDS BY BRAIN CORTICAL SLICES

RESULTS:

Brain slices absorb considerable amounts of the solution in which they are incubated. In a normal balanced salt solution the fluid taken up is mainly extracellular provided that the slices have been kept cold until they are supplied with adequate oxygen and glucose(Pappius, Klatzo and Elliott, 1962). In the presence of glutamate(or high potassium) considerably more fluid is absorbed intracellularly(Pappius et al, 1962). Furthermore, various substances, drugs for example, may also affect the uptake of water by brain slices(Okamoto and Quastel, 1970; and present study). Therefore, in order to compare the uptake of different amino acids by slices, it was necessary to estimate the extracellular fluid under the various conditions studied and to correct for this in each case. The extracellular fluid is assumed to equal the inulin space and to have the same composition as the medium in which the slices have been incubated. The non-inulin space is assumed to equal the intracellular fluid.

Results summarised in Table V (page 44) show that addition to the incubation medium of glycine, L-alanine, GABA and L-glutamine have no appreciable effects on the intracellular space of the slices but L-glutamic acid and , to a lesser extent, L-aspartic acid,

caused intracellular swelling of the slices. These observations are consistent with previous work (Pappius and Elliott, 1956). Ouabain and,more strongly, protoveratrine caused intracellular swelling. Tetrodotoxin alone had no effect but it prevented the effects of ouabain, protoveratrine and aspartic acid but not that of glutamic acid. When other amino acids were mixed with the drugs there were no obvious potentiations or reversals of effects of the drugs alone(data not shown in Table V).

For uptake experiments, one amino acid at a time was studied because amino acids tend to inhibit the uptake of each other (Blasberg and Lajtha, 1965, 1966), and considerable metabolism occurs during the incubation.

Results summarised in TableVIIIshow that all amino acids studied were taken up and accumulated by slices during the 30 minutes incubations in the glucose-saline medium(see page 34). GABA has the highest uptake rate as well as the greatest net uptake per unit of intracellular fluid. The next highest concentration that could be achieved occurred with L-glutamic acid. However, because the endogenous glutamate content in the slices is high , the actual net uptake of glutamate was the least among the amino acids tested. Earlier studies by this laboratory(Elliott and van Gelder, 1958) and subsequent studies by Lajtha and his co-workers(Blasberg and Lajtha, 1965) had shown that at 30 minutes GABA uptake is still active while uptake of L-glutamate has nearly reached its maxium with a final concentration no more than double its endogenous content(Stern et al, 1949). The highest ratio

Legends to:

Table VIII Effects of drugs on the uptake and exchange of amino acids by rat brain cortex slices.

[1]Values are expressed as mean ± S.D. when there are three or more determinations; but average ± range when there are only two determinations; individual values are given when there is only one determination; numbers in parenthesis represent numbers of determinations.

[2]R.S.A.(Relative Specific Activity) is the ratio of the specific activity of amino acid in tissue slices to the specific activity of amino acid in the medium after incubation.

[3]R.S.A. values in brackets for L-alanine are calculated assuming that all the extra L-alanine has the same specific activity as in the medium and no simple exchange and no metabolism occurs. Similarly calculated R.S.A. values for all other amino acids were lower than the corresponding observed values(data not shown in order to simplify table).

Table VIII Effects of drugs on the uptake and exchange of amino acids by rat brain cortex slices.

Addition to medium	μmoles per ml intracellular space[1] (R.S.A.[2] values underlined)					
	GABA	L-alanine	glycine	L-glutamine	L-glutamate	L-aspartate
No addition	4.2±0.5 (2)	1.3±0.1 (5)	1.7±0.1 (5)	3.6±0.2 (3)	13.5±0.5 (5)	4.5±0.4 (4)
Amino acid (2 mM)	21.4±1.3 (2)	12.6	16.1	12.1	21.0±1.0 (2)	15.3
	0.75±0.04	0.73(0.90)[3]	1.01	0.79	0.59±0.00	0.78
Amino acid plus ouabain(10 μM)	5.4±0.6 (2)	6.2	6.6	4.9	18.0±2.0 (4)	9.5
	0.72±0.05	0.63(0.78)	0.87	0.89	0.58±0.08	0.83
plus protov.(10 μM)	7.0±1.0 (2)	5.5	5.1	5.8	8.0±0.7 (3)	4.2
	0.78±0.03	0.57(0.76)	0.60	0.92	0.46±0.04	0.76
plus TTX (10 μM)	23.2±0.5 (2)	19.2	17.1	12.8	23.3±0.8 (2)	19.5
	0.82	0.50(0.93)	0.93	0.77	0.65±0.07	0.81
plus ouabain + TTX	9.0±1.0 (3)	8.1	8.9	6.6	20.7±0.0 (2)	14.1
	0.78±0.05	0.64(0.84)	0.95	0.88	0.56±0.05	0.79
plus protov. + TTX	25.0±4.0 (2)	14.8	16.6	12.4	25.0±1.0 (2)	16.6
	0.81±0.0	0.58(0.92)	0.99	0.70	0.54±0.03	0.91

of uptake to endogenous content occurred with alanine and glycine.

Protoveratrine inhibited the uptake of all amino acids and it actually caused loss of endogenous L-glutamic acid from slices(Benjamin and Quastel reported similar finding, 1971). Ouabain was most effective in preventing the uptake of L-alanine, GABA, glycine and L-glutamine, less effective for L-aspartic acid and L-glutamic acid. Tetrodotoxin alone had no effect. It reversed the inhibition caused by protoveratrine but not that caused by ouabain. However, the uptake of both L-glutamate and L-aspartate seemed not to be affected if both ouabain and tetrodotoxin were present together.

In all cases, except L-alanine, the ratio of the specific activity of the amino acid in the intracellular fluid of slices to that in the medium(RSA) was higher or equal to, but never less than(within experimental errors) what would be expected for the amount of labelled amino acid taken up and mixed with the endogenous unlabelled amino acid. This observation was most marked when uptake was inhibited by ouabain or protoveratrine. This indicates that exchange between endogenous and exogenous amino acids was still active although net uptake was prevented. Similar results were reported above for binding of these same amino acids in brain suspensions.

DISCUSSION :

Detailed studies on the entry of radioactive amino acids into brain slices have been made previously by Lajtha and his colleaques(1965-1970). However, they studied amino acid uptake by

measuring only the total radioactivity in the slices after incubation without actually separating the amino acids concerned and without measuring the total amounts of the amino acids chemically. Although they have shown that over 85% of the amino acids taken up by the slices were in the original forms after incubating the slices for even as long as 70 minutes, considerable metabolism of added amino acids do occur in slices. In fact, Balaz et al (1970) had shown that only 50% of the added 1-C^{14}-GABA could be recovered from brain slices after 30 minutes incubation and we have found that, in the present study, about 30% of added 1-C^{14}- GABA was metabolised in the same period of time. Therefore, uptake values reported previously would likely to be exaggerated. In the present study, the radioactivities as well as the absolute amounts of amino acids were determined. In doing so, we obtained more meaningful data. Besides net uptake, exchange of amino acids between slices and medium is apparently very active(Table VIII).If the amino acid in the incubation medium is labelled, this exchange process would increase the radioactivity in the slices without actually affecting the net uptake. Therefore, determination of radioactivity alone in the slices does not give a measure of the true uptake but tends to give exaggerated uptake values. Moreover, the D isomers of many amino acids were previously used to study amino acid uptake by many groups because the D forms are not likely to be metabolised by brain tissues. However, we are interested in the physiologically significant amino acids and therefore the L forms were studied. On specific points where the results can be properly compared we confirm

the previous work of the Lajtha group.

Battistin et al(1969) were able to differentiate various types of amino acid uptakes in brain slices by studying the sensitivities of uptake to various inhibitors. They showed that the degree of sensitivity to a decrease in energy supply was not the same for all amino acids and it also varied with the inhibitors used. Some correlation for classes of amino acids existed in the sensitivity to a decrease energy supply, in that the small neutral and the acidic amino acids and GABA were more sensitive than the large basic and the large neutral amino acids. The present study by using ouabain and protoveratrine seems to furnish similar and consistent results in that the uptake of L-glutamic acid and L-aspartic acid is less sensitive to ouabain but that of L-glutamine, L-alanine, glycine and GABA(small neutral amino acids) is very sensitive.

Amino acid uptake by brain slices was shown to be dependent on ionic gradient, especially that of sodium, existing between the slices and the incubation medium. However, the presence of a physiological sodium gradient is not an absolute requirement (Margolis and Lajtha, 1968). In other words, the uptake of some amino acids is highly dependent on a physiological sodium gradient and any substance(e.g., ouabain and protoveratrine) which tends to upset this gradient will likely to cause inhibition of their uptake. Uptake of some other amino acids, on the other hand, is less dependent on this physiological sodium gradient and therefore is less sensitive to drugs which destroy the gradient. L-glutamine, L-alanine, glycine and GABA

are shown to belong to the former group while L-glutamic acid and L-aspartic acid belong to the latter group. The L and D forms of glutamic acid were accumulated to approximately the same extent under normal conditions, differences in uptake were observed in sodium-deficient medium, with the L form less affected by the depletion of sodium in the medium(Margolis and Lajtha, 1968). Here we have an example which shows that physiologically active form(L form) of an amino acid may behave differently from the form(D form) which is not found in brain tissue under physiological conditions.

Apparently, the inhibition of amino acid uptake by ouabain is not due to the depletion of energy supply in the slices. Ouabain inhibits amino acid uptakes at concentrations(similar to those used in the present study) that do not affect neuronal respiration, glycolysis or the production of ATP(Quastel, 1965; Margolis and Lajtha, 1968; Okamoto and Quastel, 1970). Ouabain is known to be an inhibitor of the Mg^{++} -dependent, Na^{+}-K^{+} activated ATPase, an enzyme believed to couple amino acid transported with the Na^{+}-pump. It is postulated that ouabain inhibits amino acid transport by competing with these substances for the carrier sites(Gonda and Quastel, 1962). The activity of the ATPase is apparently maximal when the external sodium concentration is high. Sodium somehow facilitates the binding of amino acids to the carrier at the membrane and if ouabain competes with an amino acid molecule for the same site, resulting in the formation of a ouabain-Na^{+}-carrier complex instead of a amino acid-Na^{+}-carrier complex, and the amino acid uptake is thus inhibited. Since ouabain

has little inhibitory effect on the uptake of L-glutamate(Table VIII). it is apparent that L-glutamate and ouabain do not share the same binding site on the carrier. The fact that uptake of L-glutamate still takes place in sodium-deficient medium(Margolis and Lajtha, 1968) is consonant with the observation that ouabain has little effect on the inhibition of the uptake of L-glutamate.

Both ouabain and protoveratrine are capable of causing accumulation of sodium inside a cell(Okamoto and Quastel,1970). However, these two drugs seem to work on different mechanisms. Protoveratrine is not known to inhibit ATPase activity. Like ouabain, protoveratrine inhibits the uptake of amino acids by brain slices. It actually causes an increase in efflux of L-glutamate from slices (Benjamin and Quastel, 1971; see also Table VIII).Tetrodotoxin, a substance which is believed to be a specific inhibitor of the sodium-carrier(Moore and Narahashi, 1967) and which prevents influx of sodium ions (McIlwain et al, 1969; Okamoto and Quastel, 1970), reverses the inhibition of amino acid uptake caused by protoveratrine but not that by ouabain. This shows that ouabain and protoveratrine exert their effects by different mechanisms. Perhaps tetrodotoxin prevents the effect of protoveratrine by blocking the influx of sodium promoted by protoveratrine(Gottesfeld and Elliott, 1971). The normal concentration gradient of sodium established by the sodium pump would thus tend to be preserved. This gradient is believed to be necessary for the transport of the amino acid-sodium-carrier across the membrane, at least for certain classes of amino acids(

Crane, 1965; Margolis and Lajtha, 1968).

On examining Table VIII, one can see that the RSA values for all amino acids, except for L-alanine, are higher than the values to be expected if the radioactivity of the amino acid in the incubated tissue was due only to net uptake of labelled amino acid from the medium and no exchange had taken place(see footnote under Table VIII). Evidently exchange does occur. With L-alanine, all the observed RSA values are lower than the expected values. This may indicate that perhaps there are two pools of L-alanine. Some of the L-alanine derived from exogenous source in one pool is preferrably metabolised and the content of this pool is replaced by some unlabelled L-alanine derived from glucose source. In fact, Yoshino and Elliott(1970) had indicated that there are more than one pool of L-alanine in brain tissue. Sadasivuda and Lajtha(1970) had actually shown that L-alanine has the highest rate of metabolism in incubated brain slices. This observation supports the two-pool idea for L-alanine.

CHAPTER FIVE

GENERAL DISCUSSION

Detailed studies of the binding and uptake of amino acids by brain tissues(mainly by Elliott and co-workers, Roberts and co-workers and Lajtha and co-workers; also the present study) have indicated that the two phenomena are probably closely related. Interactions of various inhibitors with binding and uptake of amino acids may suggest that they are successive processes in transporting amino acids into brain tissue. The 'transport' phenomenon probably consists of an initial non-enzymatic, energy-independent, but sodium-dependent binding of amino acids to carriers on the surface of membrane. Under the operation of the sodium pump which requires energy, the amino acids are then actively transported across the membrane into the inside where the amino acids are either metabolised or stored. At the same time, exchange, whether carrier-mediated or otherwise, between endogenous and exogenous amino acids in the free medium takes place to some extent providing that sodium is present extracellularly.

As has been mentioned before, several of the amino acids under investigation are putative neurotransmitters, with GABA and glycine being strong candidates as neuroinhibitors in the brain and spinal cord respectively while glutamate is believed to be an neuroexcitatory transmitter substance. These substances have been shown to be produced, released at appropriate times, and to exert their effects.

The process of uptake of these amino acids is believed to be a mode for the inactivation of these substances after they have exerted their effects and as a way of storing these substances for future use. There is another possibility that the initial sodium-dependent binding of amino acid may actually be on effector sites. However, this cannot be appreciated from the present study.

Acetylcholine(ACh), once released into the synapses, is rapidly destroyed by the enzyme acetylcholinesterase(AChE) and this is a very efficient way of removing the transmitter substance. However, there are no known ways to destroy these amino acids in the synaptic regions. GABA-T, the enzyme that destroys GABA and other enzymes that metabolise glutamate or glycine, are not present in synapses. Most of these enzymes are probably found in mitochondria which are not present in synapses. Mechanisms of some sort have to exist for the removal of these powerful neurotropic substances. An uptake mechanism for GABA was described(Elliott and van Gelder, 1958; Kuffler and Edwards, 1958; Sisken and Roberts, 1964; Iversen and Kravitz, 1966). Since then, numerous uptake experiments were performed *in vitro* in order to obtain more insight as to how these substances are removed under physiological conditions.

The correlation between the *in vitro* binding and uptake and the physiological removal of these amino acids in brain *in situ* can be visualised from the following picture. The free extraneuronal amino acid that is liberated into the extracellular region of the synapse binds to the highly mobile sites on the membrane(pre- and post-

synaptic and possibly glial) when the external sodium concentration is high. Under normal physiological conditions extracellular fluid in brain has a high sodium concentration. The requirement for sodium for binding and uptake is thus met. The amino acid bound on the outer surface of the membrane equilibrates with that in the extracellular fluid. The amino acid on the inside of the membrane can equilibrate with that in solution intracellularly. The binding sites are partially restricted to one or the other side of the membrane by a barrier. The frequency with which the binding sites traverse the barrier may be dependent upon various asymmetries on the sides of the membrane (redox, degree of phosphorylation, sodium, potassium and chloride ion concentrations, levels of sugars, amino acids and nucleotides, etc.; Kuriyama et al, 1968). Under metabolic conditions, because of the lower concentrations of sodium ions present intraneuronally than extraneuronally, the amino acid would tend to dissociate from the carrier on the intraneuronal side and to become available for mitochondrial metabolism. Thus, the asymmetric concentration of sodium ions would set up the conditions for a rapid removal of the amino acid from the extraneuronal synaptic enviroment into the intraneuronal enviroment and a rapid metabolism of the amino acid therein.

Varon et al(1965) have shown that mitochondria-containing subcellular particles metabolised GABA rapidly provided that a high sodium content existed in the outside medium. As far as GABA is concerned, most of what is taken up is metabolised, probably

in glial and endothelial cells where 80% of GABA-T activity seemed to be present(Salganicoff and De Robertis, 1963). The lack of change in membrane conductance in glial cells(but not in neurones) in response to GABA and glutamate(Krnjevic and Schwartz, 1966) suggested that perhaps these two amino acids are taken up from the synaptic regions (assuming they are indeed transmitter substances) into glial cells where they are metabolised.

Therefore, the binding and uptake processes being studied are compatible with the physiological significances of these amino acids.

CHAPTER SIX

SUMMARY

The present work confirms previous findings that GABA is effectively bound to particles of brain homogenates. When the homogenising medium is plain sucrose, only about 35% of the tissue GABA was found to be associated with the particles and this portion is referred to as the 'firmly bound' or 'occluded' form of GABA. When the medium contained sodium chloride, 30% extra-binding of GABA to particles was observed and this portion is referred to as the 'loosely bound' or sodium-dependent bound form. The loosely bound GABA exchanges rapidly with the free form. Some exchange between the occluded GABA and the extra-bound GABA as well as the free GABA can take place if the suspension is allowed to stand at room temperature or if the medium contains sodium.

L-glutamic acid, L-aspartic acid, L-glutamine, L-alanine and glycine also exist in bound and free forms. However, they are not bound to the same extent as GABA. The binding and exchange of L-glutamine resemble that of GABA.

Both ouabain and protoveratrine inhibit the binding of these amino acids to brain particles. Again, their effects are greatest on GABA. Exchange between bound and free amino acids, in contrast to binding, is not affected by these drugs.

Brain slices are capable of accumulating all these

amino acids against their concentration gradients when the slices are incubated in normal salt solutions. The net uptake of GABA is the highest and that of L-glutamic acid is the least.

Ouabain is very effective in inhibiting the uptake of GABA and other neutral amino acids but less effective in inhibiting the uptake of the two dicarboxylic amino acids. Protoveratrine can also inhibit the uptake of all these amino acids and it is more powerful than ouabain in this respect. It actually causes the leakage of L-glutamic acid from brain slices. Tetrodotoxin alone has no effect on the uptake of amino acids by slices. However, it reverses the inhibition caused by protoveratrine but not that caused by ouabain. This suggests that that the latter two drugs exert their effects via two different mechanisms.

The study of the effect of drugs on amino acid uptake by slices reveals that different amino acids show different degree of sensitivities towards the drugs. Results tend to confirm the existence of different classes of amino acid transport in brain tissue as suggested by Lajtha and his colleaques.

The physiological significance of binding and uptake of amino acid by brain tissue is probably concerned with the removal of these physiologically active amino acids from where they exert their effects into intracellular compartments either for storage or metabolism.

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