COMPARATIVE NEUROCHEMICAL STUDY OF TWO BEHAVIOURAL STRAINS OF MICE

By

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ABSTRACT

Two behaviourally different strains of mice were used in this work. The first is non-reactive strain C57/BL characterized by high locomotor activity and lack of reaction to mild stress. It is also used as a "control" strain in seizure experiments with mice due to its resistance to audiogenic seizure stimulation. The second is the reactive strain A2G, which is characterized by its susceptibility to audiogenic seizure and low spontaneous locomotor activity within a constant environment. It has been used as a "seizure strain".

Three neurochemical parameters were studied in both strains. The first is the rate of γ-aminobutyric acid (GABA) production *in vivo* and *in vitro* in the cerebral cortex using U-^{14}C glucose as a substrate. The rate of production was measured as GABA/glutamate ratios in terms of both relative amounts of ^{14}C incorporated into each amino acid and specific activities. The results show that the C57/BL strain has a higher rate of GABA production both *in vivo* and *in vitro*.

The second parameter measured was the activity of the enzyme acetylcholinesterase in the cerebral cortex of the brain. The A2G strain had the higher activity.

The third parameter was the estimation of the levels of the biogenic amines dopamine,noradrenaline and adrenaline in the brain stem.A significant difference was shown in the dopamine concentration which is higher in the C57/BL strain.

These biochemical data are tentatively correlated to the known differences in behavioural reactivity between the strains.

C O N T E N T S

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CHAPTER I

CRITICAL SURVEY OF PREVIOUS PERTINENT WORK.

INTRODUCTION

The work in this thesis is concerned with the study of the comparative production of Y-aminobutyric acid (GABA), and glutamic acid (glu), using U-^{14}C glucose as precursor, in vitro and in vivo in the brain of two behavioural strains of mice, non-reactive C57/BL and reactive A2G. These strains differ in behaviour and susceptibility to audiogenic seizures.

At the same time the activity of the enzyme acetylcholinesterase (AChE) in the cerebral cortex of the two inbred strains of mice C57/BL and A2G was studied. Also, the biogenic amines levels in the brain stem and mid brain of C57/BL and A2G were estimated.

The cholinesterase and the acetylcholine (ACh), level in the brain are of a great importance in the regulation of the behaviour of the animals. The animals that show high activity of AChE and ACh, learn more rapidly than the animals of lower AChE and ACh activity, (Fehmi & McGaugh, 1961). Animals raised in rich

environments show significant difference in brain AChE activity from those raised in isolation. Moderate reduction in AChE activity may increase behavioural efficiency, while large reduction shows a decrease in efficiency. (Reeves, 1966; Carlton, 1963).

Recent investigations show that animals with high brain AChE activity can be correlated with high activity of the enzyme glutamic acid decarboxylase (GAD), which is involved in the production of GABA. (Nick, Morris & Kerkut, 1968; Geller, Yuwiler & Zolman, 1965).

BEHAVIOUR OF MICE

Behaviour of the mice differ from strain to strain. This difference in behaviour was studied by many workers from different viewpoints. Barnett and Scott, (1964), studied two strains of mice C57/BL and A2G which have different behaviour. The C57/BL strain shows higher activity than that of the A2G strain. The comparison of activity depends upon the nest-building, gnawing, phenotypes and the effect of environmental change such as low temperature (at $-3^{\circ}C$) between the mentioned strains of mice. The response of mice to low temperatures was found to vary with different strains. The differences are reflected in mortality, growth, number of young born and weaned. The mortality increased while the growth and number of young born and weaned decreased. (Barnett & Manly,1956).

The social behaviour and fearfulness in C57/BL strain and other five strains of different genotypes were studied by Lindzey, Winston & Roberts (1965). The C57/BL strain compared to the other strains always appeared much lower in open field fearfulness than the other strains. It also appeared to be the most sociable strain among the other mice strains.

When a mouse is subjected to a sudden ringing sound for a few seconds, different reflexes appear. (Smith, 1941; Frings & Frings, 1953). This technique recently has been used on a large scale to study the susceptibility of inbred and hybrid strains of animals to audiogenic seizures.

Schlesinger & Doggan, (1965), have studied the factors affecting the susceptibility of different strains of mice to audiogenic seizures. They found that C57/BL mice were resistant to audiogenic seizures. They found that following factors affect this susceptibility:

1. The importance of genetic factors.
2. The age of the animals at the time of testing is of great importance.

Henry, (1967), tested the audiogenic seizures for C57/BL strain of mice of 21 and 28 days old. The occurrence of the successive stages of the audiogenic seizures-syndrome was recorded for each animal. If wild running is considered as a mild form of seizure

even with the C57/BL mice, 50 percent of the strain at four weeks of age are liable to the first stage of such a seizure. The C57/BL has been used as a "control" strain in seizures experiments with mice. The A2G is a "seizure strain" (Fuller & Sjursen, 1967; Schlesinger & Boggan, 1965).

Presensitized mice of A2G strain were subjected to the sound provided by electric doorbell. The animal responded with a phase of running behaviour beginning 10 seconds after the onset of the auditory stimulant and lasting about 40-50 seconds. This phase was followed by general tonic-clonic convulsions, catatonic state and recovery. The concentration of amino acids is disturbed significantly during the running behaviour phase and a further decrease occurred at the moment of maximal convulsions. The GABA level decreased, while the glutamic acid and aspartic acid increased. The levels of the amino acids restored to the normal levels during the recovery phase (Pasquini, Salomane & Gomez, 1968).

The levels of GABA in the central nervous system of the animals is an important factor in some kinds of seizures. Its level was found to be reduced in cases of seizures. This reduction was found to be due to the inhibition of the enzymes involved in its metabolism system and the coenzyme vitamin B6. Some drugs were found to affect these enzymes and led to the production of convulsions in the animals (Kamrin & Kamrin, 1961; Killam and Bain, 1957).

Hydroxylamine inhibits the enzyme γ-aminobutyric-α-ketoglutarate transaminase (GABA-T), with slight effect on the activity of the enzyme glutamic acid decarboxylase (GAD) in some cases. This in turn led to the elevation of GABA content in the brains of rats, cats and monkeys (Baxter & Roberts, 1961). The increase in GABA concentration was due to the continual feeding of GABA pool by the decarboxylation of glutamic acid, which is not interrupted by the hydroxylamine (Baxter & Roberts, 1959). However, Baxter et al (1961) have reported that hydroxylamine does not increase GABA in the mouse brain, in fact, there is a small decrease in its concentration. The administration of amino-oxyacetic acid (AOAA) causes inhibition of GABA-T enzyme of the brain which led to the increase in GABA concentration in the brain of rat (Rindi & Ferrari, 1959; Wallach, 1961 and Baxter et al 1961). AOAA administered in low concentration inhibits both GABA-T and GAD enzymes (Baxter & Roberts, 1958). High doses were found to cause convulsions in several species of animals. The toxicity has been associated with production of vitamin B6 deficiency. Pretreatment of mice with AOAA reduce the seizures susceptibility to the electric shock seizures were found to be reduced at 1.5 hrs and 4 hrs. after the treatment with AOAA (Kuriyama, Roberts & Rubinstein, 1966).

Vitamin B6 is an important cofactor in the GABA-glutamic acid system. There is a correlation between this system and the

onset of convulsions due to its involvement in the neuronal activity. The role of pyridoxine deficiency, or of its antagonists, in the seizure mechanism has been well established by Tower, (1956, 1957) and Killam & Bain, (1957). Toxopyrinidine was found to inhibit GAD enzyme and pyridoxine which led to a decrease in GABA content and the production of epileptic seizures. Administration of pyridoxamine to the epileptic rats stopped the convulsions in about one hour, although the increase in concentration of cerebral GABA reached the normal level within three hours. (Rindi et al 1959; Makino, Kinoshita, Sasaki, Shioi, 1954 and Makino et al 1954). Another vitamin B6 antagonist, 4-methoxy-methyl pyridoxine (MOB6), caused convulsions in mice after 20-40 minutes after their injection. This compound drops GABA level to one half that of the control. Pyridoxine in doses three times that of MOB6 prevents the convulsions. (Kamrin & Kamrin, 1961).

INCORPORATION OF LABELLED CARBON ATOMS INTO AMINO ACIDS

The previous paragraphs give a short review of the behaviour of the two strains of mice and their susceptibility to seizures.

The following paragraphs are concerned with the incorporation of labelled carbon atoms from U-^{14}C-glucose into the amino acids, especially glutamic acid and GABA in the cerebral cortex of the

two strains of mice C57/BL and A2G.

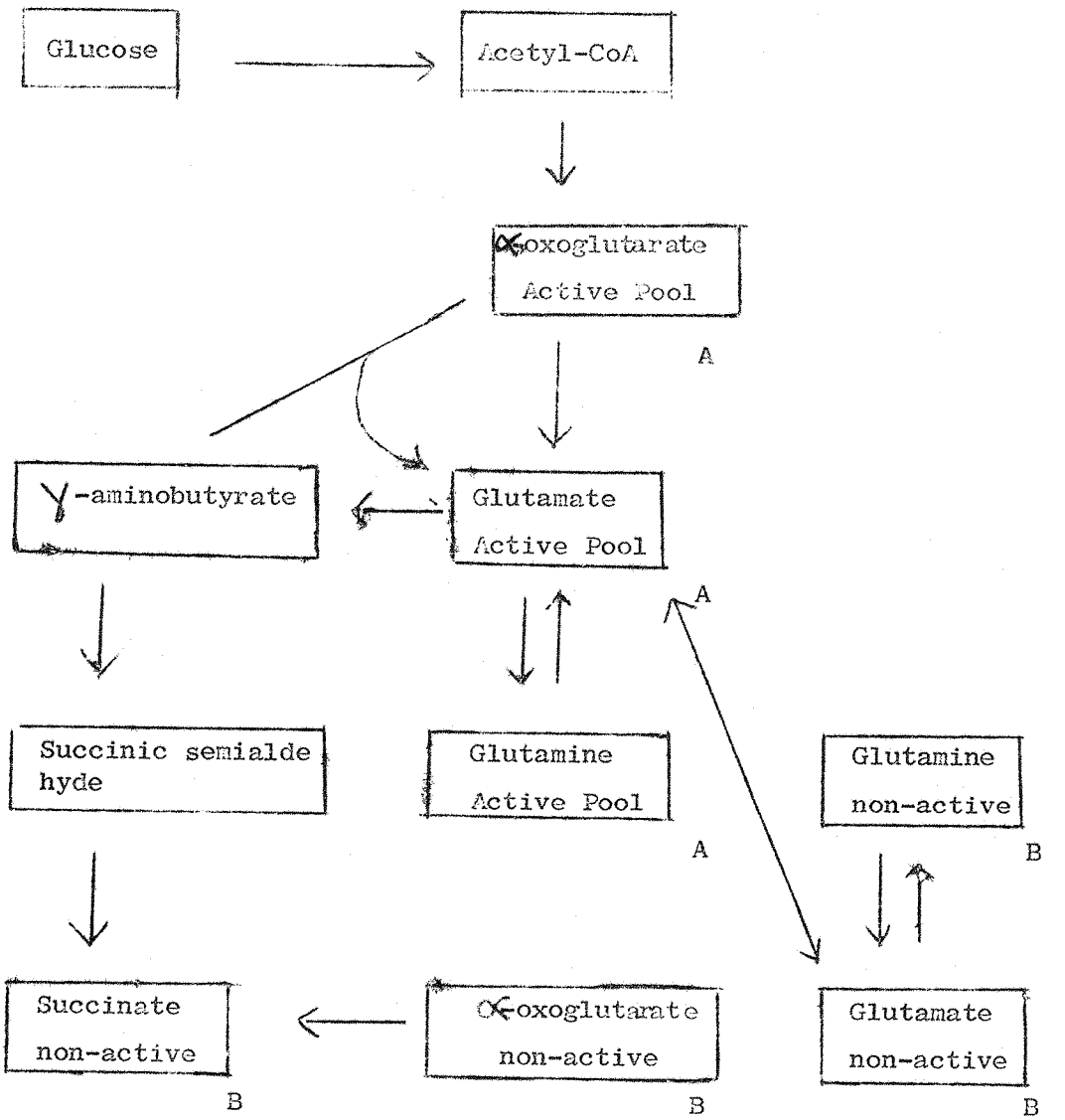
The incorporation of labelled carbon atoms from U-^{14}C-glucose into the protein amino acids was first studied *in vitro* by Rafelson, Winzler & Pearson in 1951. They incubated minced brain of a one day old mouse with U-^{14}C-glucose. This led to the incorporation of ^{14}C atoms into all protein amino acids except proline and threonine. The incubation time was 24 hours. In 1952, Winzler, Moldave, Rafelson & Pearson, studied the incorporation of ^{14}C from U-^{14}C-glucose into the brain amino acids *in vitro* and *in vivo*. The authors suggested that protein bound amino acids are in equilibrium with the free amino acids, which are in turn in equilibrium with essential and non essential precursors. The non essential precursors are in equilibrium with intermediates derived from radio glucose. In the *in vivo* experiments they found that most of the radio-activity of protein fraction was in aspartic acid, glutamic acid and alanine. These results were confirmed by Beloff-Chain, Catanzaro, Chain, Masi & Pocchiari (1955). They found that a considerable amount of the glucose metabolized by the brain slices is transformed into aspartic acid, glutamic acid, GABA and alanine. Also Beloff-Chain *et al* (1955) showed that glutamic acid is the first amino acid formed, within three minutes after the incubation. Similarly, on the injection of 1-^{14}C-glucose and 6-^{14}C-glucose, rapid formation of labelled amino acids in the brains of adult rats was shown to occur, (Busch, Fujiwara & Koer, 1960). U-^{14}C-glucose

was injected into the tail veins of rats which were decapitated at intervals of 1,2,5,10 and 20 minutes after the injection. Two minutes after the injection 41% of the radio-activity in the brain was found to occur in the α-amino acids and this percentage rose to 75% after 30 minutes. The conversion of glucose into amino acids proceeds in the brain at a rate several times that in liver, kidney, heart, lungs, spleen, skeletal muscle or blood. The dicarboxylic amino acids were shown to be labelled several times higher than mono carboxylic acids. (Vrba 1962). Subcutaneous injection of U-^{14}C-glucose, led to the labelling of amino acids of the rat brain. Within 20 minutes approximately 72% of ^{14}C was combined with the free amino acids. It was found that dicarboxylic amino acids contained 80% of the ^{14}C,neutral amino acids 19% and basic amino acids 1% . In the blood the labelling was distributed nearly equally between the dicarboxylic and the neutral amino acids. So glucose, as could be expected, is an excellent precursor of the dicarboxylic amino acids, (Vrba, Caitonde & Richter, 1962). O'Neal & Koeppe, (1966), found that glucose and lactate and possibly glycerol are the best precursors of the amino acids of the brain. Within 10 minutes only 1% of the injected 2-^{14}C-glucose was converted to glutamate, aspartate and glutamine of the brain. The high percentage of labelling observed in the carbon atom number 5 of the glutamate of the brain emphasized the fact that the major route of pyruvate metabolism is the conversion to acetyl-CoA. The high labelling of carbon atom number 5 of glutamate also occurs after

the use of 2-^{14}C-glycerol, 1-^{14}C-acetate or 3-^{14}C-butyric acid. This is because they enter the brain without previous metabolism in the liver, because the liver metabolizes them into 3-4-^{14}C-glucose, which in turn labels carbon atom number one of the glutamate. The subcutaneous injection of U-^{14}C glucose to the rat has shown that 60-72% of the total radioactivity in the brain was transferred to the amino acids, in a period of 30-120 minutes. In another finding 90-110% of the radio-activity was found to occur in glutamate, aspartate, GABA and alanine. The glutamate which is the highest labelled amino acid contained 37% of the radio-activity. The labelling of GABA indicates that active glutamate in the brain was rapidly decarboxylated. The initial specific activity of GABA and aspartate is higher than that of glutamate. The same results were obtained in the labelling pattern of amino acids, after the intravenous injection of U-^{14}C-glucose. The rapidity of labelling is much higher in the case of the intravenous injection than in subcutaneous (Gaitonde, Dahl & Elliott, 1965).

In the brain the specific activity of lactate and alanine run closely parallel to that of the U-^{14}C-glucose injected subcataneously. At 60 minutes they reached the same value. The specific activity of pyruvate after a 5 minutes interval was found to be lower than that of lactate and alanine. Also the specific activity of α-oxoglutarate was lower than that of pyruvate, after a 5 minutes interval. For pyruvate, α-oxoglutarate and glutamate,

the specific activities reach the same value within 10 minutes. Later the specific activity of glutamate exceeded that of α-oxoglutarate. Two hours after the injection, radio-active equilibrium between the α-oxoglutarate and the glutamate was reached. The labelling of the carbon atoms of the amino acids after the injection of U-^{14}C-glucose was only expected to be uniform for the alanine. If all carbon atoms of the amino acids were labelled uniformly, the percentage of labelling of α-carbon atoms of glutamate and glutamine should be 20%, and aspartate it should have been 25%. Experimental results showed different percentages of carbon atoms, proving the expected non-uniformity. The low specific activity of α-oxoglutarate compared with that of glutamate, indicates that there are at least two metabolic pools, one active (A) and the other non-active (B) of α-oxoglutarate corresponding to the two metabolic pools A and B of glutamate. This suggests a metabolic compartmentation between α-oxoglutarate and glutamate as shown in Scheme 1. (Gaitonde, 1965). After 5 and 10 minutes post injection the pyruvate and α-oxoglutarate were significantly more highly labelled than the free amino acids. After 30 minutes they were significantly lower than those of glutamate, glutamine, GABA and alanine. Alanine was shown to be even more labelled, 5 minutes after injection, than glutamate. There was no difference in the labelling of alanine and glutamate at 10 and 30 minutes. The specific activity of glutamate, glutamine and GABA were the same and their results provide no evidence for the occurrence of compartmentation between glutamate, glutamine and GABA (Lindsey & Bachelard, 1966).



SCHEME 1

The Metabolic Compartmentation
between α-oxoglutarate and glutamate

THE BIOSYNTHESIS OF AMINO ACIDS

GLUTAMIC ACID

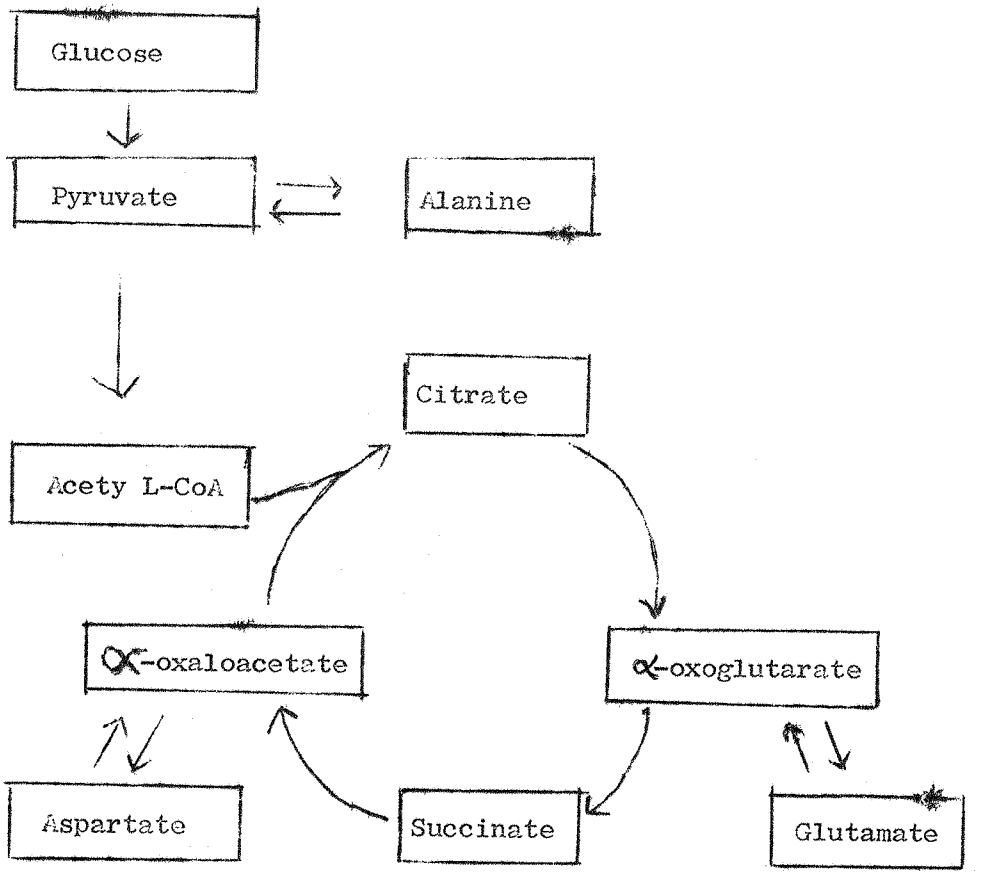
After the discussion of the incorporation of the carbon atoms ^{14}C of the labelled glucose into the system of glutamic acid-γ-aminobutyric acid, their biosynthesis will be considered. It has long been known that α-ketoglutaric acid can serve as a precursor to glutamate by transamination pathway. The mentioning of the Krebs Cycle (tri-carboxylic acid cycle, TCA-cycle), is very important. The Krebs cycle is one of the possible routes to glutamate biosynthesis, as shown in the following reaction:

$$\alpha\text{-ketoglutarate} + \text{alanine} \xleftrightarrow{\text{transaminase}} \text{glutamate} + \text{pyruvate}$$

Many workers have been studying the possible biosynthesis of glutamic acid . The amino acids glutamic acid, aspartic acid and alanine are readily formed by the transamination of the α-keto acids intermediate of the citric acid cycle as shown in Scheme 2.

γ-AMINOBUTYRIC ACID

GABA is formed in the central nervous system of vertebrate animals to a large extent, if not entirely from L-glutamic acid. The reaction is catalyzed by glutamic acid dicarboxylase (GAD), an enzyme found in mammals only in the central nervous system, largely in the gray matter.



SCHEME 2

Transamination pathways between α-keto acids and correspondent amino acids

Until now no convincing evidence has been adduced for the formation of significant amounts of GABA *in vivo* by any reaction other than the decarboxylation of L-glutamic acid. The co-enzyme pyridoxal phosphate (PYP) is required for this reaction as a co-factor for optimum activity.

$$\text{Glutamate} \xrightarrow[\text{PYP}]{\text{GAD}} \text{GABA} + CO_2$$

DISTRIBUTION, ENZYMES AND METABOLISM OF GABA AND GLUTAMATE

DISTRIBUTION

The amino acids are not specifically located in particular fractions or subfractions. Glutamate and GABA were chiefly located in the cytoplasmic fraction. However a small proportion was found in the nerve-ending fraction and this could be attributed to the volume of cytoplasm trapped within the nerve ending (Ryall, 1964).

Kravits & Potter (1965) studied excitatory and inhibitory fibres and found that GABA is unequally distributed. They found also that the concentration of GABA in inhibitory fibres is about one hundred times more than that in the excitatory fibres of the lobster axons. The GABA content of the excitatory axons is

0.64 m-moles/L. of axoplasm, while the inhibitory axons is 100 m-moles/L. axoplasm. The inhibitory nerve fibres contain a very similar concentration of GABA in spite of considerable differences in axon size. This asymmetry led to the suggestion that GABA has a function specifically related to inhibitory neurons. Motor fibres contain no GABA, but it may be present in immeasurable amounts (Kravits et al 1963, 1965 and 1967). Amino acids are formed in two ways, either free or bound. Most of GABA (factor-1) in the brain was shown to have occurred in bound form (Elliott&VanGelder,1960).

GABA is found only in the nervous tissue, while other amino acids are found to occur in the blood, liver, kidney, intestine as well as in the nervous tissue.

THE ENZYMES

The following enzymes are involved in the metabolism of the GABA-glutamic acid system.

1. GLUTAMIC ACID DECARBOXYLASE (GAD)

This enzyme is found in the mitochondrial fraction and soluble parts, and is more concentrated in the acetylcholine-poor fraction. It is suggested that this enzyme is localized in the axoplasm of the nerve ending. It is a soluble enzyme. GAD is found only in the nervous tissue. GAD is inhibited by anions, the halide

ions inhibiting the enzyme in the order $I^- > Br^- > Cl^- > F^-$; the inhibition by chloride ion was shown to be competitive with the substrate.

2. γ-AMINOBUTYRIC ACID →α-KETOGLUTARATE-TRANSAMINASE (GABA-T)

The GABA-T is contained in mitochondria of all neuronal regions, but it seems to be richer in the mitochondria of those neuronal sites on to which GABA might be liberated. In general, the enzyme follows the distribution of succinic acid dehydrogenase (SDH).

3. SUCCINIC SEMIALDEHYDE DEHYDROGENASE (SSADH)

It is a mitochondrial-localized enzyme which is less concentrated in synaptic complex than SDH.

4. GLUTAMIC ACID DEHYDROGENASE (GDH)

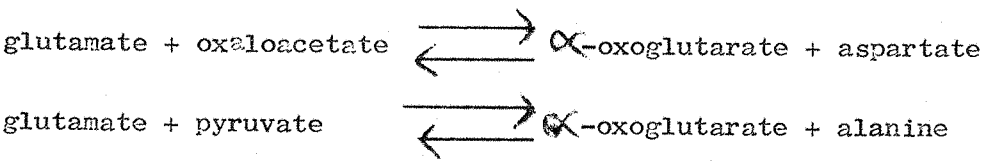
This enzyme is a mitochondrial enzyme which follows a pattern of distribution similar to that of succinic acid dehydrogenase, being concentrated in mitochondria. (Shatunova, Sytinsky, 1964; Van Kempen, Van Den Berg, Van Der Helm & Veldstra, 1965; Salganicoff & De Robertis, 1965 and Balazs, Dahl & Harwood, 1966).

METABOLISM OF GLUTAMIC ACID AND GABA

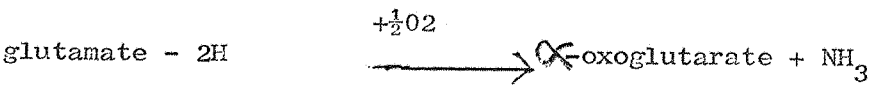
GLUTAMIC ACID

Glutamic acid is a highly active amino acid in the cerebral cortex. It can be converted to GABA by decarboxylation with GAD, transaminated and dehydrogenated to α-oxoglutarate. It can be metabolized to glutamine and other substances. The following equations show the main pathways of its metabolism:

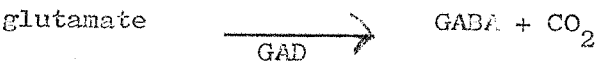
A. Transamination



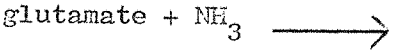
B. Dehydrogenation



C. Decarboxylation



D. Glutamine synthetase



The process of transamination of glutamate to aspartate is inhibited by the addition of malonate to the medium and the formation of CO_2 is reduced as well. There are exchange reactions

between the amino acids and the corresponding α-keto acids which can be explained by Scheme 2 (Haslam & Krebs, 1963).

When glutamate is incubated with homogenates of different tissues, such as liver, kidney cortex and breast muscle, the oxygen consumption is increased and the oxidized glutamate is recovered as aspartate. The conversion could be explained by the following reactions:

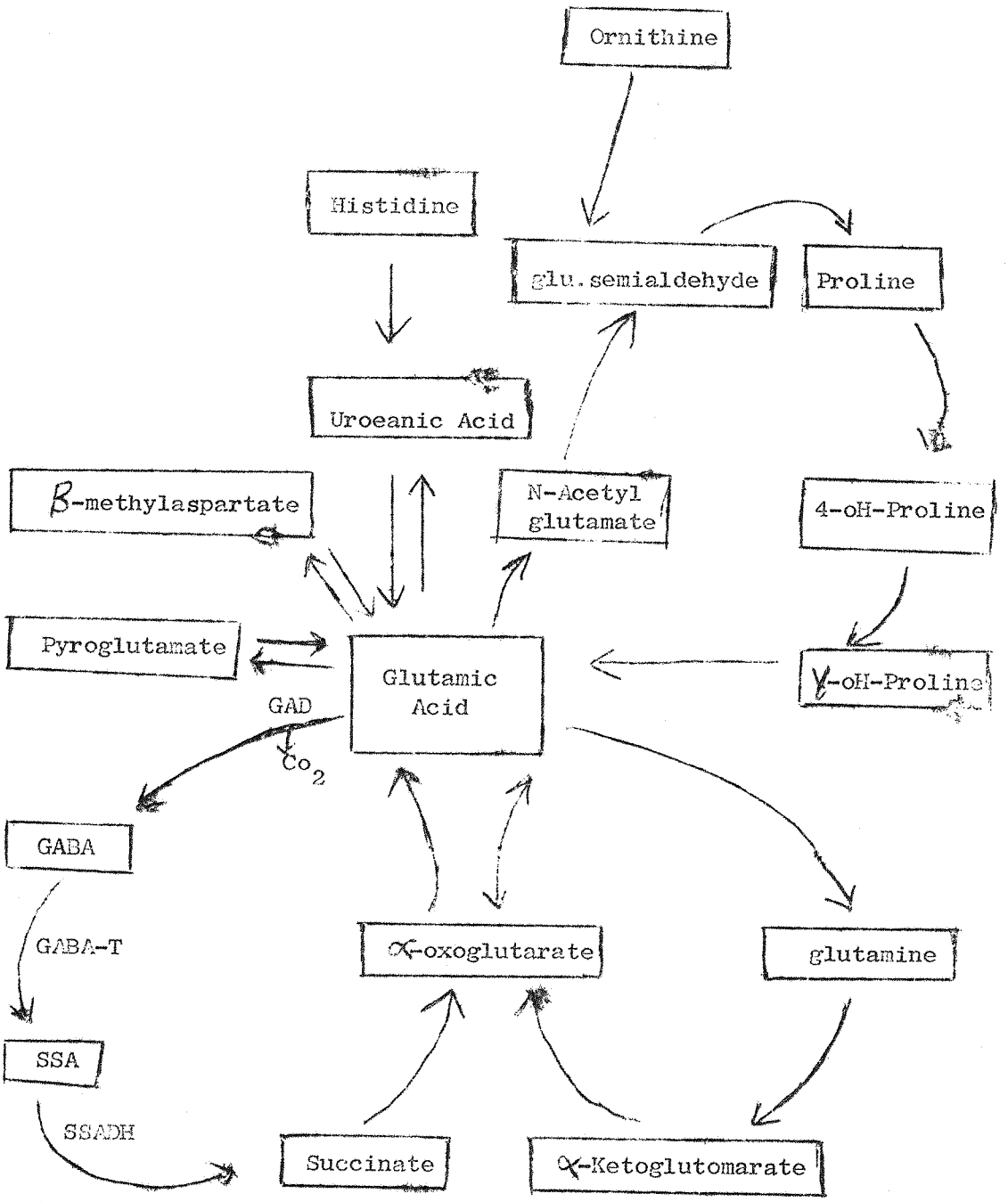
glutamate + oxaloacetate $\longrightarrow$ aspartate + α-oxoglutarate

α-oxoglutarate + $1\frac{1}{2}$ O_2 $\longrightarrow$ oxaloacetate + CO_2

Overall

glutamate + $1\frac{1}{2}$ O_2 $\longrightarrow$ aspartate + CO_2

The amount of aspartate formed is equal to the amount of glutamate removed. But in the rat brain mitochondria, 20-30% of the glutamate removed is converted to aspartate. (Krebs & Bellamy, 1960). In a brain preparation the transamination pathway accounted for 80-90% of the oxidation of 10 mM 1-^{14}C-glutamate in the resting and active state of respiration. The addition of 10 mM malonate will inhibit the uptake of glutamate by 40% and removal of the residual glutamate is due to processes other than transamination. Alpha-oxoglutarate when added to the medium containing glutamate reduces the removal of glutamate by 40-50% and reduces the formation of aspartate as well, (Bala'zs & Haslam, 1965; Bala'zs, 1963).



SCHEME 3

Metabolic Pathways of Glutamic Acid.

The addition of ADP to the medium containing glutamate in a mitochondrial preparation increases the uptake of glutamate and aspartate formation. The oxidation of glutamate by dehydrogenation is increased by the addition of malonate which blocks the oxidation of glutamate by transamination. The activity of GDH enzyme is very low in brain mitochondria and other tissues compared with the transaminase activity. Scheme 3 explains the metabolism of glutamate in general.

GAMA -AMINOBUTYRIC ACID

GABA is synthesised from L-glutamic acid by decarboxylation. The reaction is carried out by a specific enzyme GAD, which is vitamin B6 dependent for optimal activity. GAD exists uniquely in the nervous tissue of vertebrate and invertebrate organisms. GABA re-enters the TCA-cycle via succinic semialdehyde. This complete pathway has been termed by Albers (1958), "The GABA Shunt". The possibility that GABA fitted into an alternative energy cycle within the nerve cell has initiated a number of investigations. The enzymes necessary for the GABA shunt have been shown to occur in nervous tissues of various species of vertebrate and invertebrate. The gray matter of the central nervous system was found to contain these enzymes (Roberts, 1956; Albers & Brady 1959).

The transamination reaction is the rate-limiting step in

the degradation of GABA, (Van Gelder, 1966). 10% of the glucose oxidation through the TCA-cycle turnover is through the GABA Shunt, (Elliott, 1965; Machiyama, Bala'zs & Julian,1965).

GABA is metabolized in the central nervous system via the succinic semialdehyde to succinate by the following reactions: (Van Gelder, 1965).

$$\text{GABA} + \alpha\text{-oxoglutarate} \xrightarrow[\text{PYP}]{\text{GABA-T}} \text{Glutamate} + \text{SSA}$$

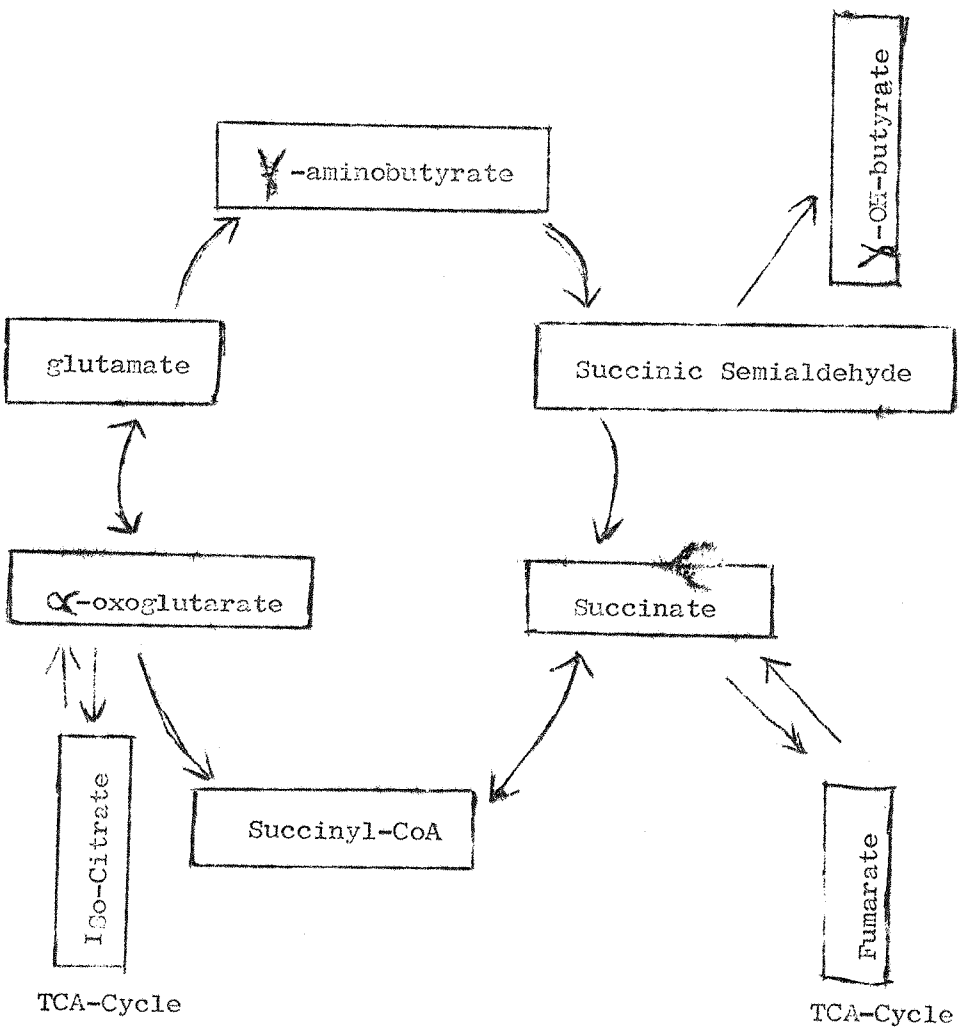
$$\text{SSA} + \text{NAD}^{+} \xrightarrow{\text{SSADH}} \text{Succinate} + \text{NADH}$$

Use of the histochemical technique, by Van Gelder in 1965, led to reports that mouse nervous tissue metabolizes GABA more rapidly than that of rabbit. Kravitz, Potter & Van Gelder, (1963) found that production of succinate from GABA was shown to be due to the action of two enzymes, GABA-T and SSADH, which are found in the central nervous system of the lobster and mammals. For optimal activities, the two enzymes need co-factors, for GABA-T pryidoxal phosphate and thiol; NAD^{+} and alkaline pH for SSADH.

Recently Roth & Giarman (1969), reported that GABA converted <u>in vivo</u> to γ-hydroxy-butyric acid in the rat brain after the administration of tritiated ^{3}H -GABA as shown in the following reaction:

$$\text{GABA} + \alpha\text{-oxoglutarate} \xrightarrow{\text{GABA-T}} \text{Glutamate} + \text{SSA}$$

$$\text{SSA} + \text{NAD}^{+} \longrightarrow \gamma\text{-hydroxy-butyric acid} + \text{NADH}$$



SCHEME 4

Metabolic Pathways of γ-aminobutyrate

The possible importance of the GABA Shunt in the energy production of the nerve cell could be related to the possible role of GABA as transmitter, (Elliott, 1965). Scheme 4 explains the GABA Shunt.

FUNCTION OF GLUTAMATE AND GABA

Free amino acids are present in the central nervous system in a considerable concentration, especially glutamate, aspartate glutamine and GABA. Some amino acids are thought to be possible chemical transmitters. This theory needs more investigation and evidence. Amino acids are divided into two categories according to their action on the neurons.

1. Excitatory Amino Acids

This category includes glutamate and aspartate and some of their derivatives.

The anions of glutamate and aspartate have a potent excitatory action upon spinal neurons, (Curtis & Watkins, 1960). Glutamate as an excitant causes depolarization of the spinal neurons, and consequently it or related substances may be an excitatory transmitter, (Curtis, Phillis & Watkins, 1960). This action was antagonized by the action of depressant amino acids. According to Wiechert & Herbest, in 1966, injection of glutamate causes tonic-clonic cerebral

convulsions depending on the dose. Application of glutamate, aspartate and cysteic acid ions iontophoretically to the external surface membrane of neurons within the spinal cord of cats, lightly anaesthetized with pentobarbital sodium, led to the excitation of interneurons, Renshaw cells and motor neurons by producing membrane depolarization. Their action is non specific and unrelated to excitatory synaptic transmitter action, (Curtis et al 1960). Glutamate, aspartate and their derivatives excite different types of neurons, choline-receptive and choline non-receptive cells in the spinal cord.

Curtis & Eccles (1958), found that the excitant amino acids are effective in concentrations which are similar to those of acetylcholine required to excite central cholinoceptive neurons. These excitatory acids have similar effects on the neurons of the brain stem (Curtis & Koizumi, 1961), cerebral cortex (Curtis & Watkins, 1961) and on the crustacean muscle fibre, (Robbins, 1959). Injection of glutamate in concentrations between 10^{-7} g/ml and 10^{-4} g/ml causes contraction in the leg of cockroach. Concentration above 10^{-4} g/ml diminish this effect. The D-glutamate isomer was found to be less effective than the L-glutamate isomer, (Kerkut, Leake, Shapira, Cowan & Walker, 1965).

2. Inhibitory Amino Acids

This category includes GABA, α-glycine and β-alanine.

Depressant substances, especially GABA, inhibit the contraction of the leg of cockroach caused by excitatory substances as glutamate or by electrical stimulation to thoracic ganglia. The delay in response depends on the concentration of GABA applied. Mixing GABA and glutamate of the same concentration will neutralize each others effect (Kerkut et al, 1965). Beta-alanine and GABA are strong depressants. They produce no change in membrane potential and diminish both excitatory postsynaptic potential (EPSP) and inhibitory postsynaptic potential (IPSP), (Curtis, Phillis & Watkins, 1961). GABA, as glutamate, affects many types of neurons; Kravitz, Kuffer, Potter & Van Gelder (1963), studied the blocking compounds in the peripheral nervous systems of the crab and lobster. They found that GABA was physiologically the most active. In the same year they found that motor fibres contain no GABA while inhibitory nerves contain a high concentration of it, so they strongly suggest that GABA has specific physiological rule combined to inhibitory neurons. Ash & Tucker, in 1966, demonstrated that GABA in concentration as low as 0.5 µg/ml produced a rapid reversible inhibition of the ascaris muscle. Intravenous administration of GABA to cats does not cause cardiovascular action, while in dogs it produces hypertension and bradycardia similar to that of intra-ventricularly administered GABA. The inhibition of central sympathetic neurons is probably responsible for this action (Bhargava & Srivastava, 1964).

The role of GABA in the central nervous system can be summarized by the following statements: (Tewari & Baxter, 1969).

1. GABA as an inhibitory transmitter substance in the crustacean nerve is well documented, and evidence is accumulating that it may have a similar function in some parts of the central nervous system in vertebrates (Takeuchi & Takeuchi, 1966; Otsuka *et al* 1966; Krnjevic & Schwartiz, 1966; Kuriyama, 1966 & Graham et al 1967).

2. In brain-tissue GABA is a route of a metabolic pathway which bypasses part of the TCA-cycle (Elliott, 1965).

3. It was shown that GABA together with the nitrogeneous compounds can participate in the osmotic regulation of a nervous tissue (Baxter & Ortiz, 1966).

4. GABA added to a ribosomal system from immature rat's brain, stimulates the incorporation of ^{14}C-lucine (Campbell, Mahler, Moore & Newari, 1966).

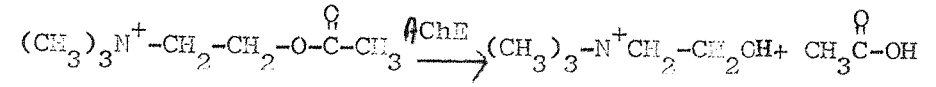
ACETYLCHOLINE AND CHOLINESTERASE

ACETYLCHOLINE

Acetylcholine (Ach), is the acetic acid ester of the choline which is present in the brain and peripheral nerve fibres. Ach is also found in other parts of the body in relatively large quantities. Ach concentration varies from part to part of the brains of different animals. Its concentration is between 0.5-30m moles per gram of brain tissue. Sympathetic nerves or ganglia may contain up to 200m moles per gram. The cerebral hemispheres and the cord gray matter contain much more Ach than that of the white matter. (Feldberg & Vogt, 1948). Hypoglycaemia lowered the quantity of Ach in the brain. This led to the suggestion that glucose metabolism is needed for its maintenance, (Crossland *et al* 1955). Acetylcholine decreases in cases of excitation and convulsions. The cerebrospinal fluid normally contains no appreciable amount of Ach, but this substance has been detected there after epileptic attacks, (McIlwain, 1966). Acetylcholine has been firmly established and characterized as a neurohumoral transmitting agent of the central and peripheral nervous system. The enzymatic system involved in its synthesis and destruction is fully characterized. Choline acetylase (ChAc) and acetyl-cholinesterase (AChE), are the enzymes responsible for the synthesis and hydrolysis of Ach respectively. (Koelle, 1961).

ACETYLCHOLINESTERASE

Acetylcholinesterase (AChE), is the enzyme responsible for the hydrolysis of acetyl-choline, to choline and acetate, as shown in the reaction:



There are two classes of cholinesterase. The first is called the true cholinesterase (AChE), and the second is the pseudo-cholinesterase, (Alles & Hawes, 1940). AChE is even more widely distributed than Ach or its choline analogues, AChE being found in different tissue where neither Ach nor ChAc are present. AChE is present in plasma, liver, smooth muscle and red blood corpuscles which contain the AChE, but no Ach. They can hydrolyze non choline esters. In 1914, Dale suggested that the rapid disappearance of Ach from the blood and tissue is due to the action of an esterase. Matthes (1930), demonstrated the presence of the esterase enzyme. In 1932, Stedman & Easson, prepared the enzyme AChE from the horse serum which hydrolyzed Ach. Mendel & Mundel, (1943), found that this enzyme is not AChE, because the enzyme responsible for the hydrolysis of Ach in the nervous system is much more active than that of the serum, which hydrolyze benzoyl choline, on which AChE has little action. The two classes of the enzyme can also be differentiated by their different susceptibility to inhibitory agents (McIlwain, 1966). The pseudo-ChE is mainly

localized at the walls of the blood vessels and some glial cells, while AChE is highly concentrated in caudate nucleus and hypothalamus. (Abrahams & Pickford, 1956). The gray matter of the brain contains more Ach and AChE than the white matter. The cerebrospinal fluid contains no AChE (Stedman & Stedman, 1935).

The AChE found in the microsomal fraction, contains 35-40%, the heavy mitochondrial fraction contains 27%, the light mitochondrial fraction contains 14%, the supernatant fraction contains 9% and the nuclear fraction contains 6%. The pseudo-ChE activity on the other hand is highest in the nuclear fraction 36%, and 27% is found in the supernatant fraction (Aldridge & Johnson, 1959). The spinal cord contains both AChE and pseudo ChE, the AChE found in the anterior and lateral horns and motor neurons. The cholinergic neurons contain high concentration of the AChE. The adrenergic and sensory neurons appeared to be practically without this enzyme, but recently the results of other workers demonstrated the presence of AChE in all type of peripheral neurons. High concentration in the cholinergic neurons and lower concentration in the sensory and adrenergic neurons were found (Whittaker, 1961).

BIOGENIC AMINES

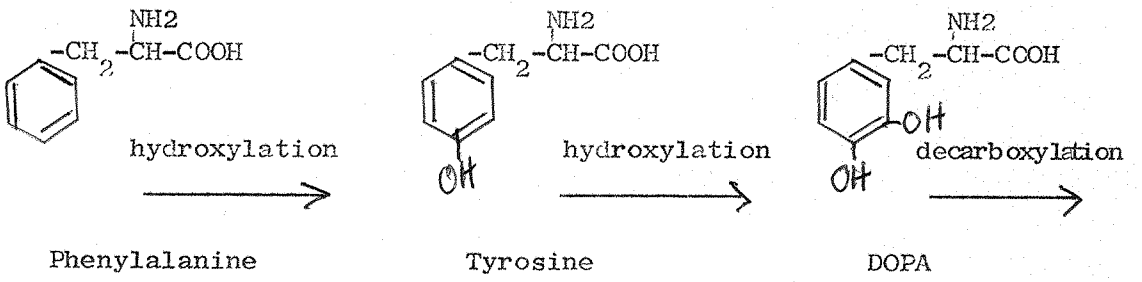
Dopamine, noradrenaline and serotonin (5-hydroxy-tryptamine 5-HT), can be considered to be important factors in controlling the behaviour of animals. The discovery of catecholamines within the central nervous system, and their response to a large number of psychotropic drugs have suggested that these amines have a central as well as a peripheral role in various affective states (Vogt, 1954; Raab, 1943).

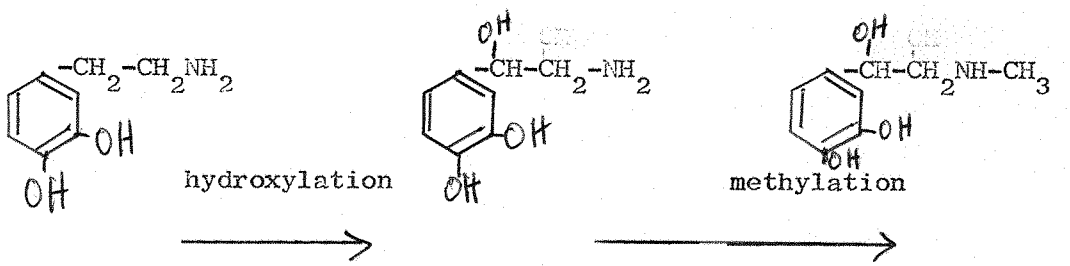
Noradrenaline and adrenaline occur in relatively high concentration in the hypothalamus while dopamine is concentrated in the corpus striatum (Bertler, 1961, 1963; Feldberg, 1966 & Glowinski and Iversen, 1966). The distribution and concentration of catecholamines in the central nervous system were found to differ in various areas of the brain. The cerebral part contains the lowest concentration, while corpus striatum and hypothalamus contain the highest concentration (Carlsson, 1959; Montagu, 1956 & 1963). The richest areas are the parts of the brain connected with activity of the sympathetic nervous system.

BIOSYNTHESIS OF CATECHOLAMINES

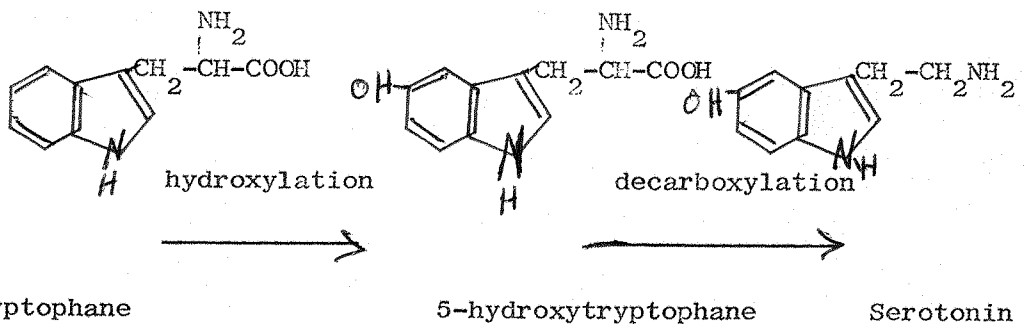
Some of the amino acids in the brain can serve as good precursors for the biosynthesis of the catecholamines. Dopamine, noradrenaline and adrenaline are formed from the amino acids

phenylalanine and tryosine via the dihydroxyphenylalanine (DOPA), according to the following pathways:





The amino acid tryptophane can serve as precursor for serotonin. Serotonin was first isolated in 1948 by Rapport, Green & Page. The occurrance of serotonin in the central nervous system was first demonstrated by Amin, Crawford and Gaddum, (1954) & Twarog and Page, (1953). The biosynthesis of serotonin is as follows:



FUNCTION

The level of dopamine, noradrenaline and serotonin in the brain were found to decrease in the clinical cases of Parkinsonism (Hornykiewicz, 1966).

Injection of L-Dopa intravenously to Parkinson's patient improved the symptoms. The reason behind the use of L-Dopa rather than the direct use of catecholamines is that catecholamines administered by injection intra-peritonealy or intra-venously does not penetrate to the brain due to the blood brain barrier.

Injection of noradrenaline into the cerebral ventricles, or directly into the anterior hypothalamus produced a slowing of respiration, a cessation of shivering, reduction in tone of a skeletal muscle, skin vasodilatation and a fall in body temperature, while serotonin, when similarly applied, produced opposite effects. These facts led to the theory that temperature control was due to the release of adrenaline, noradrenaline and serotonin in the hypothalamus (Feldberg & Myers 1965). Noradrenaline has two effects on the sensitive neurons, either facilitation or depression of their activity, depending on the region to which it is applied and the doses used. Dopamine mainly depresses caudate nucleus neurons and also depresses units in the cortex, lateral geniculatel body and ventrobasal complex of the thalamus. There is evidence that both noradrenaline and dopamine have some control over behaviour.

Wada & McGeer (1966) found that in monkeys general alertness and vigilance is associated with high catecholamine levels. Also Wada, Wrinch, McGeer and McGeer (1963) found that intraventricular injection of these substances gave rise to excitation and a deterioration of avoidance learning.

Serotonin prolonged the hypnosis when injected after an ineffective dose of barbiturate (Fornaroli & Koller, 1954; 1955). It produced hypothermia in mice and rats, (Fuhrman, 1957). Serotonin has sedative or depressive effects on mice. The spontaneous motor activity and aggressivity was reduced after the intraperitoneal injection of serotonin (Brown, 1957; Kobinger, 1958). After the injection of serotonin to mice the aggressive behaviour towards other mice of the same sex was found to be reduced (Yen, Stanger and Millman, 1959). The level of serotonin and noradrenaline was found to be lower in mice susceptible to audiogenic seizures (DBA/2J), than that of the mice resistant to audiogenic seizures (C57/BL) (Schlesinger, Boggon & Freedman, 1965).

CHAPTER II

ESTIMATION OF GAMMA-AMINOBUTYRATE, GLUTAMATE AND ACETYLCHOLINESTERASE IN THE CEREBRAL CORTEX AND BIOGENIC AMINES IN BRAIN STEM

MATERIALS AND METHODS

Animals

The mice were all at least 6 weeks old at the time of the experiment and weighed 20-27 g. The C57/BL mice were obtained from Animal Suppliers Ltd., 685 High Street, North Finchley, London. The A2G mice were obtained from Laboratory Animal Centre, Woodmanster Lane, Carshalton, Surrey. The non-reactive C57/BL strain mice were characterized by resistance to audiogenic seizures while the reactive A2G strain mice were characterized by susceptibility to audiogenic seizures.

GABA Production

In vitro

Incubating Medium

The incubating medium consists of 0.5 ml of the buffer solution of pH 7.4 (0.1M Phosphate buffer, it was prepared by mixing the solution Na_2HPO_4, 9.45 g/L and the solution KH_2PO_4, 4.55 g/L.

The mixture was then adjusted to the correct pH)and 2.5 μC of U-^{14}C-glucose (supplied by The Radio-Chemical Centre, Amersham, England). Numbered test tubes containing the above medium were mounted in a previously prepared shaking water bath at 37^{o}C. The test tubes containing the incubating medium were left in the bath for sufficient time to ensure that the medium reached the temperature of the bath. Two of these were used as control (i.e. no tissue incubated) to check the purity of the labelled glucose.

Dissection of the mice

The mice were sacrificed by a quick blow. The heads were decapitated and dissected. The brains were removed, and the cortex of each brain hemisphere was cut as quickly as possible, weighed, (40-70 mg) and incubated. After 30 minutes of incubation the test tubes were removed from the bath. The incubation was terminated by the addition of 0.2 ml of 80% v/v alcohol and then boiled for 1-2 minutes in a boiling water bath.

Extraction and spotting

In addition to termination of the reaction, boiling enhances the extraction of soluble substances. Full extraction needs about 12 hours during which the test tubes were kept in a refrigerator. The test tubes were centrifuged. The supernatant was brought to

dryness in vacuo in a warm water bath. The residue was dissolved in 0.5 ml 80% v/v alcohol, and spotted on the corner of chromatographic paper by evaporation on a small area, three inches away from each edge. Whatman No.4 chromatographic Grade Papers (46 x 57 cm), were used for the two-dimensional descending paper chromatography.

Chromatography

The papers were placed on the troughs and 70 mls of the mixture solvent added to each trough. The arrangement is shown in figure (1). The operation consists of two runs. The first run uses 70 mls of the isobutyric acid mixture. The mixture was used for the long axis. It should be prepared 24 hours before use. This period of time was needed because esterification equilibrium appeared to be established between the alcohols and the isobutyric acid. The ammonium-isobutyric acid buffered the mixture at a pH 4. Storing the mixture for weeks did not affect its properties. The mixture was prepared according to the following proportions (Crowley, Moses & Ullrich, 1963):

Ethylenediamine tetracetic acid (EDTA)	1.2 g
17N ammonia solution	100 mls
distilled water	950 "
n-propanol	350 "
iso-propanol	75 "
n-butanol	75 "
iso-butyric acid	2500 "

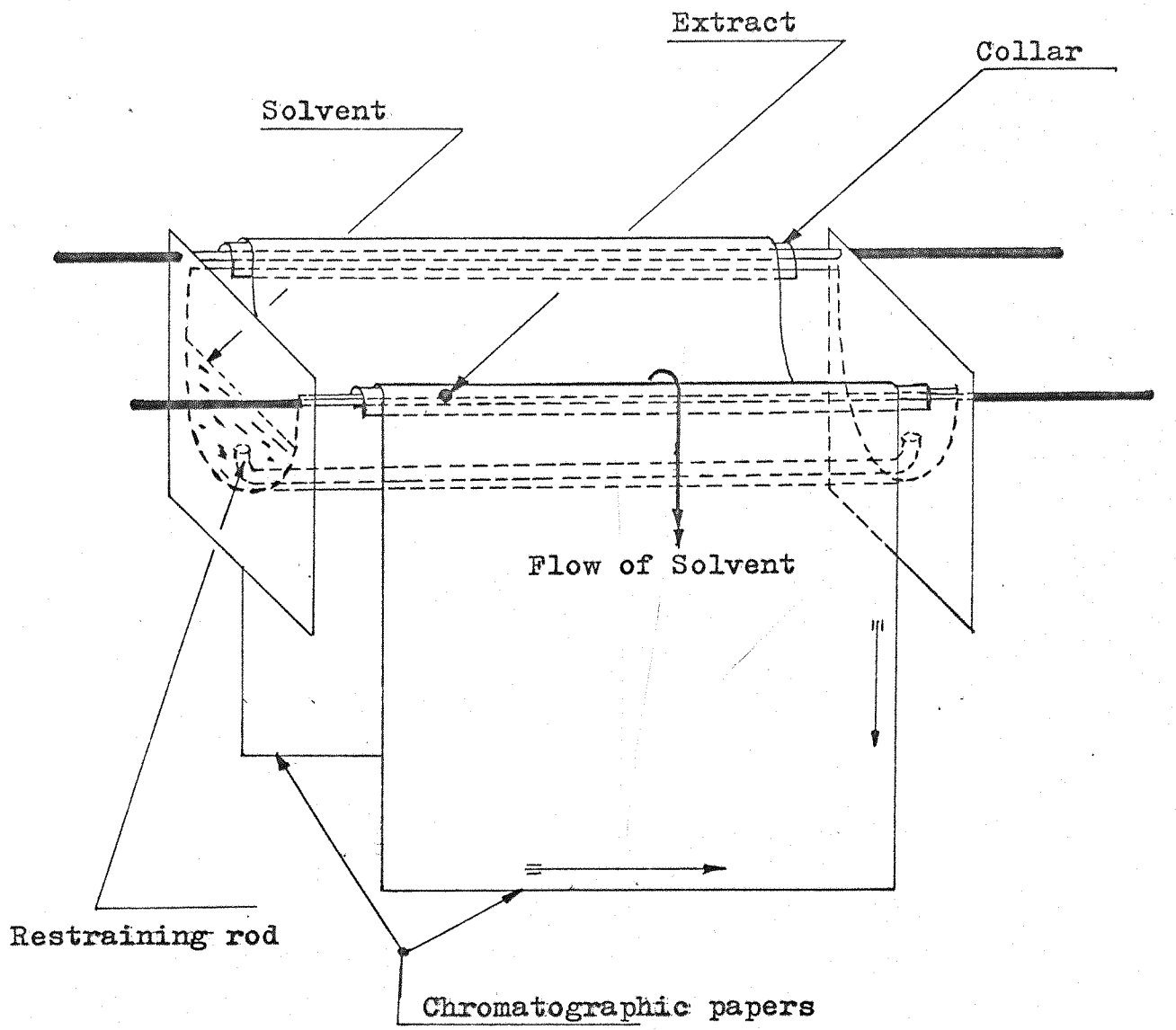


Fig.1 Chromatographic papers in position on the trough

The volume of solvent used is just sufficient to reach the bottom edge of the paper, running time 12-16 hours. Once it reaches the edge the papers were put in a chamber to dry. In the second run the dried papers were hung along the short axis. 70 mls of butanol-propionic acid-water mixture was added. It was a mixture of equal volumes of two solvents, which should be mixed just before use. The first was n-butanol, 919 mls + distilled water, 81 mls. The second was propionic acid, 469 mls + distilled water, 531 mls. The solvent needs 6-10 hours to reach the bottom edge. The papers were then hung in the [redacted] chamber to dry.

Visualization

Each chromatographic paper was folded over a used background x-rays film to act as a support. The paper was unsymmetrically marked with a coloured radioactive solution on two of its corners. These papers were fixed on Blue Brand x-ray films (Kodak Ltd), carrying the same number as the respective paper. The process was carried out in the dark room. The papers so mounted on the films were kept in light proof boxes for two weeks.

Processing of the Films

After the two weeks period the films were opened in the

dark room, mounted on film frames and immersed in Kodak DX 80 developing solution (Kodak Ltd), for 5-7 minutes. This developing time is enough to easily identify the radioactive metabolites. The films were washed in running water for 1-2 minutes and immersed in the first fixing solution (FX-40 Kodak Ltd), for 3-5 minutes. Then the films immersed again in the second fixer 3-5 minutes and finally washed in running water and left to dry.

Identification of the Spots

The films showed the exact location of the various metabolite spots. These spots were located back on the chromatographic paper. Each paper was attached to the corresponding film making use of the coloured indicating spots. Light was transmitted through the set and the spots were marked with a pencil on the chromatographic paper.

Measurement of the Radioactivity Incorporated

The radioactivity of each spot (metabolite) on the chromatographic paper was counted by an automatic scaler counters described under the apparatus paragraph. The spots that showed high radioactivity and large areas were divided into small areas in order to meet the capacity of the counter head. From these data obtained with the counter, the percentage of the radioactivity

incorporated of each metabolite was calculated.

In vivo

The production of GABA in vivo experiments were done according to the following method.

Injection of Mice

In these experiments both sexes of the A2G strain and C57/BL strain were used. Each mouse was injected intraperitonealy (i.p.), with 0.2 ml of $U^{14}C$-glucose solution in saline having the concentration of 20 μC(100 μC/ml).

Freezing in Liquid Nitrogen

After a period of 30 minutes from the injection with the labelled glucose each mouse totally immersed in the liquid nitrogen until frozen.

Dissection and Homogenization

Each mouse was dissected while still frozen and the brain removed, weighed and homogenized with 0.5 ml of distilled water. (The homogenizer used is TRI-R-STIR-R Instruments, of a variable

speed having a maximum of 5000 RPM, Model S63). Then 0.2 ml of 80% v/v alcohol was added and boiled for 1-2 minutes, to terminate the reaction. This process should be done as quickly as possible.

Spotting

Each sample was centrifuged and the supernatant evaporated under reduced pressure to dryness. The residue was dissolved in 0.5 ml of 80% v/v alcohol, then evaporated on the Whatman No.4 chromatographic paper. The same technique of two-dimension chromatographic papers used *in vitro* experiments was applied for these experiments.

Visualization

Each chromatographic paper was treated in the same way as that of the *in vitro* experiments. The films were processed and the radioactive metabolites were counted following the technique explained previously.

Measurement of Amino Acid Pool Sizes

Two standard solutions were prepared, the first solution was glutamic acid of known concentration, and the second solution

was GABA of known concentration.Two mg/L. from each solution, known amounts (10-200 ml) were spotted on Whatman No.4 chromatographic paper, and run in one solvent (butanol-propionic-water mixture), dried and stained with the ninhydrin-cadmium acetate reagent. This reagent should be prepared freshly. It was used to stain the amino acids spots developed on the chromatographic paper. The reagent consists of the following chemicals:

Ninhydrin	0.75 g
Cadmium mixture	12 ml
Acetone	350 ml

The cadmium mixture was prepared according to the following proportions:

Cadmium acetate	0.05 g
Glacial acetic acid	5 ml
Acetone	50 ml

The mixture was shaken until all precipitates completely dissolved. (Atfield & Morris, 1961).

Each coloured spot was cut into small strips and transferred to numbered covered test tubes, which have the same number on the chromatographic paper. To each test tube 10 ml of methanol was added and set in a shaking machine and left for one hour. The intensity of the colour developed in each test tube

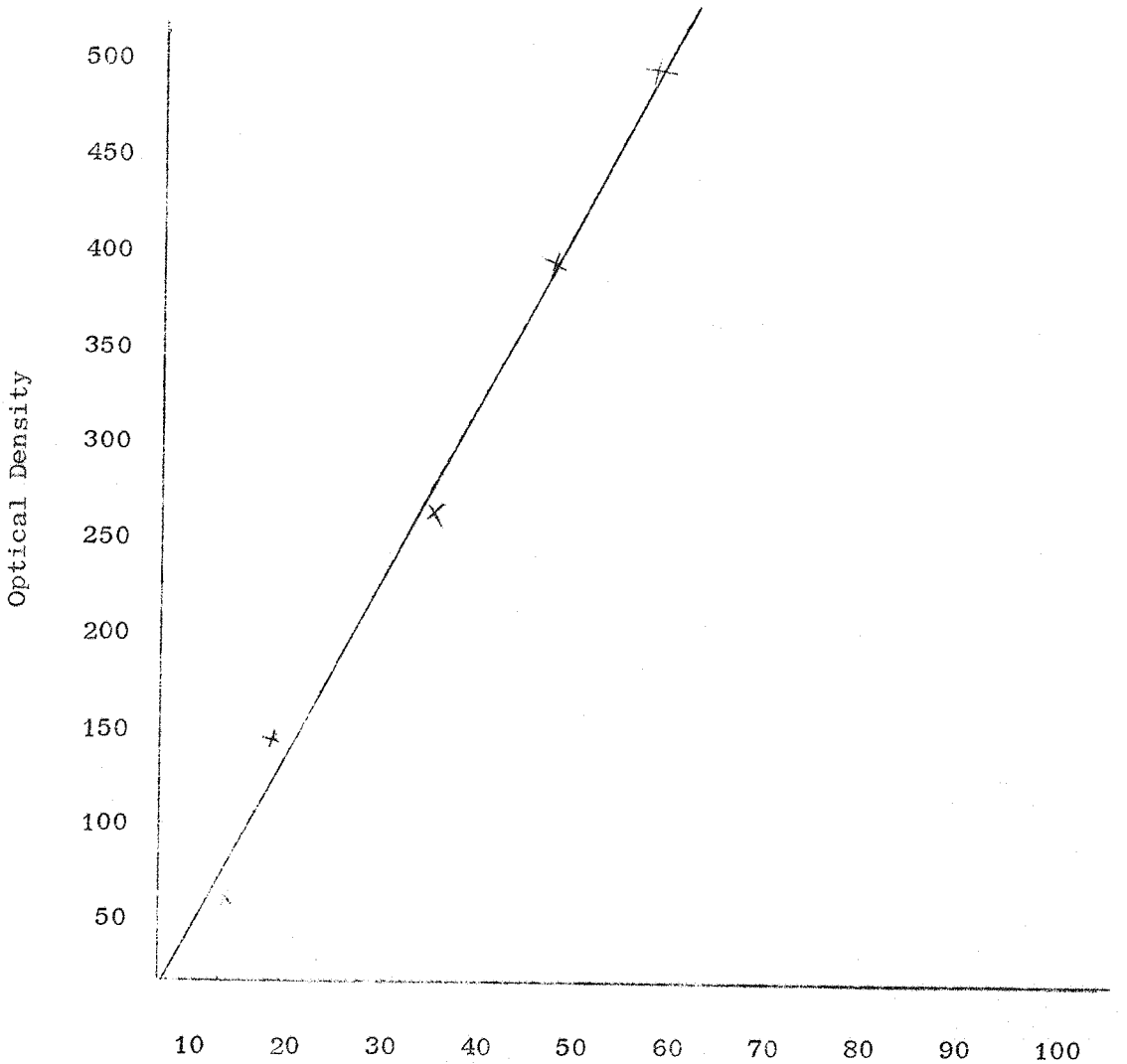
was measured by the SP-600 spectrophotometer set on the 510μm wave length. From the reading the standard curve was drawn (i.e. the concentration against the intensity of the colour developed). This was done for both GABA and glutamate. The figures 2 and 3 show the standard graphs.

Acetylcholinesterase Activity

The Auto-analyzer, made by Technicon Instrument Corp., Chauncey, N.Y., was employed to estimate the enzyme AChE activity following the method described by Gage & Litchfield, (1966). Portions of cerebral cortex (20-30 mg), were homogenized in 0.5 ml buffer solution of the pH 7.4 for each observation. Twenty-four observations were made per each strain of mice C57/BL and A2G. Non-specific cholinesterase activity was assessed using a butyryl choline substrate and a correction made for each observation. The activity was measured in terms of micro-moles of acetate released per milligram of the tissue per minute.

$$(CH_3)_3\text{-}N^+\text{-}CH_2\text{-}CH_2\text{-}O\text{-}\overset{O}{\overset{\|}{C}}\text{-}CH_3 \xrightarrow{AChE} (CH_3)_3\text{-}N^+\text{-}CH_2\text{-}CH_2\text{-}OH + CH_3\overset{O}{\overset{\|}{C}}OH$$

Fig. 2.


Optical Density
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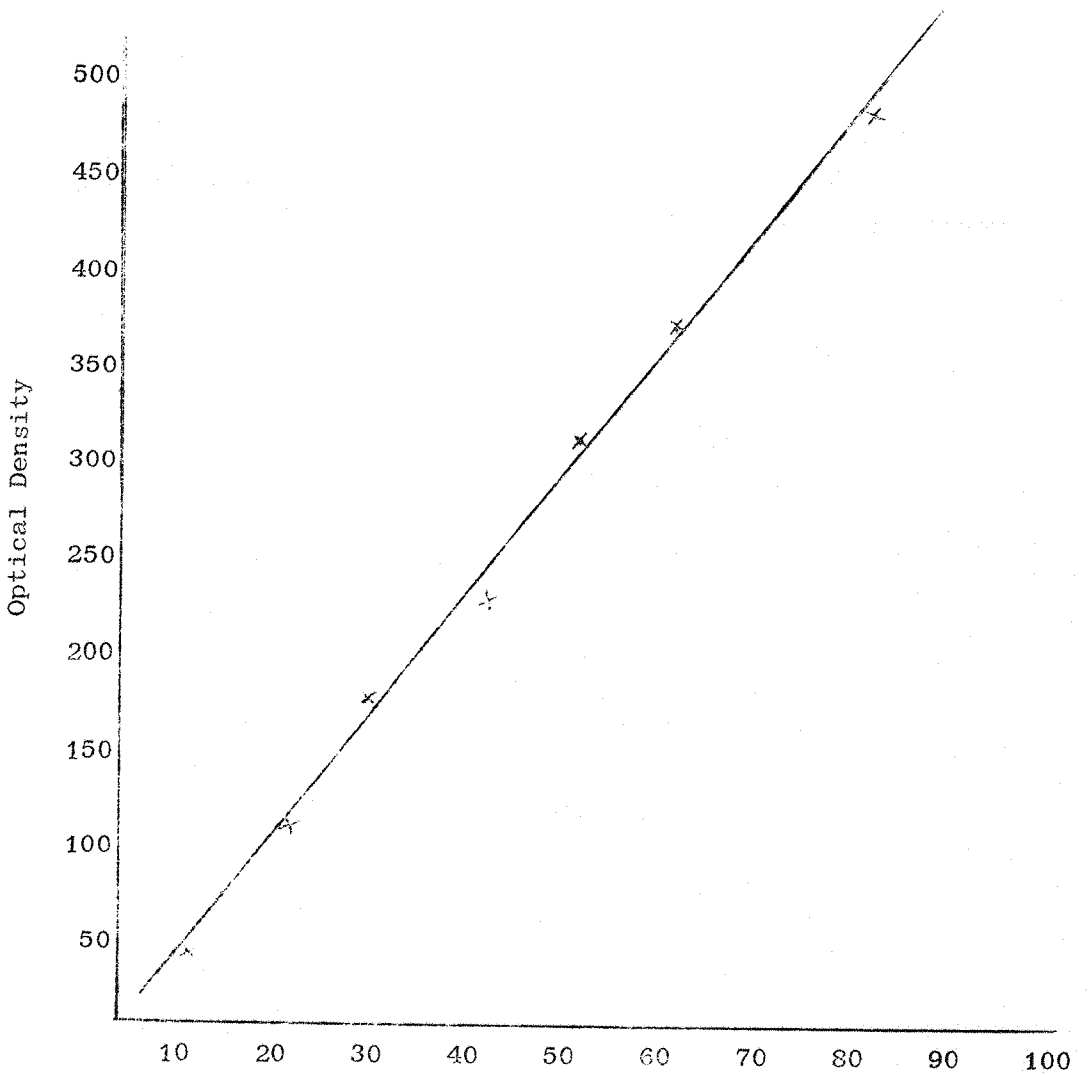
100
(μg) concentration of glutamic acid

Fig. 3.


Optical Density
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(μg) concentration of GABA

Biogenic Amines

The method used in these observations was that described by Brownlee & Spriggs, (1965). The mid brain and brain stem from three mice were used for each set of determinations. Each set of determinations was replicated six times. Both strains of mice C57/BL and A2G were used. The tissue dropped into liquid nitrogen, weighed and homogenized with butanol reagent to which was added 0.01N hydrochloric acid and Na Cl. The suspension was shaken in stoppered bottle to extract the tissue amines into the butanol phase, then centrifuged. The required volume of butanol layer was withdrawn and added to n-heptane plus 7 mls of 0.01 N-HCL and shaken for a period of time to pass the amines from the organic phase into the aqueous phase. The system was then centrifuged and the aqueous phase recovered was used for the estimation of dopamine, noradrenaline and serotonin. An Aminco-Bowman spectrophotofluorimeter was used to determine the intensity of emitted fluorescent radiations.

Extraction of the Radio-Active Glucose (U-^{14}C)

The radio-active glucose U^{14}-C, was supplied spotted on a piece of folded filter paper inside a vacuum tube. The glucose was eluted from the filter paper with distilled water. The filter paper was held between two glass slides one end of which was

dipped in the distilled water. The water absorbed by the filter paper flowed into a small beaker dissolving the glucose away from the paper. Sufficient time was allowed to ensure that all the radio-active glucose was eluted. A hand Geiger detector was used to detect that a negligible amount of radio-active glucose remains on the paper. The collected solution was evaporated to dryness under reduced pressure. The residue was dissolved in distilled water or in saline to the concentration required in the particular experiment. These solutions were stored at $-10^{\circ}C$.

APPARATUS

1. Chromatographic Tanks

Two portable tanks (3ft.6ins x 2ft.6ins x3ft.) made of hard wood were constructed in the department. Each tank has a glass window through which the movement of the solvent along the papers could be watched easily. 200 mls of corresponding mixture was kept in 250 mls beaker inside the tank. The lid of the tank was firmly fixed by means of four screws. This would ensure a saturated atmosphere inside the tank. Each tank contained six stainless steel portable U-shaped troughs. Two collars and one restraining rod were mounted in order to hold the papers in position. The collars and the rod are made of stainless

steel as shown in figure 1.

2. The Radioactivity Counter

Two types of radioactivity counters were used for counting the radioactivity in each metabolite.

a. Automatic Scaler Type N530F, Ekco Electronic Ltd., England.

b. Panax Automatic Counter, Auto Scalers, Type P7702

PANAX EQUIPMENT LTD.

3. UNICAM SP-600 SPECTROPHOTOMETER

This apparatus made by Unicam Instruments Ltd., England. The intensity of the colour developed from the reaction of the amino acids with the ninhydrin reagent was measured by this spectrophotometer.

CHAPTER III

RESULTS

GABA Production

A duration of 30 minutes were selected for all experiments carried out *in vivo* and *in vitro*. The patterns of the incorporation of radioactive carbon atoms (^{14}C), into soluble metabolites, the free amino acids and the intermediate substances of the TCA-cycle of the cerebral cortex in the brain of two strains of mice, the reactive A2G and the non-reactive C57/BL are shown in figures 4 and 5.

The data in Tables 1 and 2 show that the percentage of incorporation of carbon atoms (^{14}C) into the amino acids was significantly higher in the *in vivo* experiments (65-85%), than in the *in vitro* experiments (30-40%). The low incorporation of radioactive carbon atoms into the amino acids in the *in vitro* experiments was probably due to the lack of oxygen indicated by the high accumulation of lactic acid. Labelled lactic acid reaches 60% of the radioactivity of the U-^{14}C glucose metabolized (Table 1, Fig.5). A negligible amount of lactic acid was formed in the *in vivo* experiments (Table 2, Fig. 4).

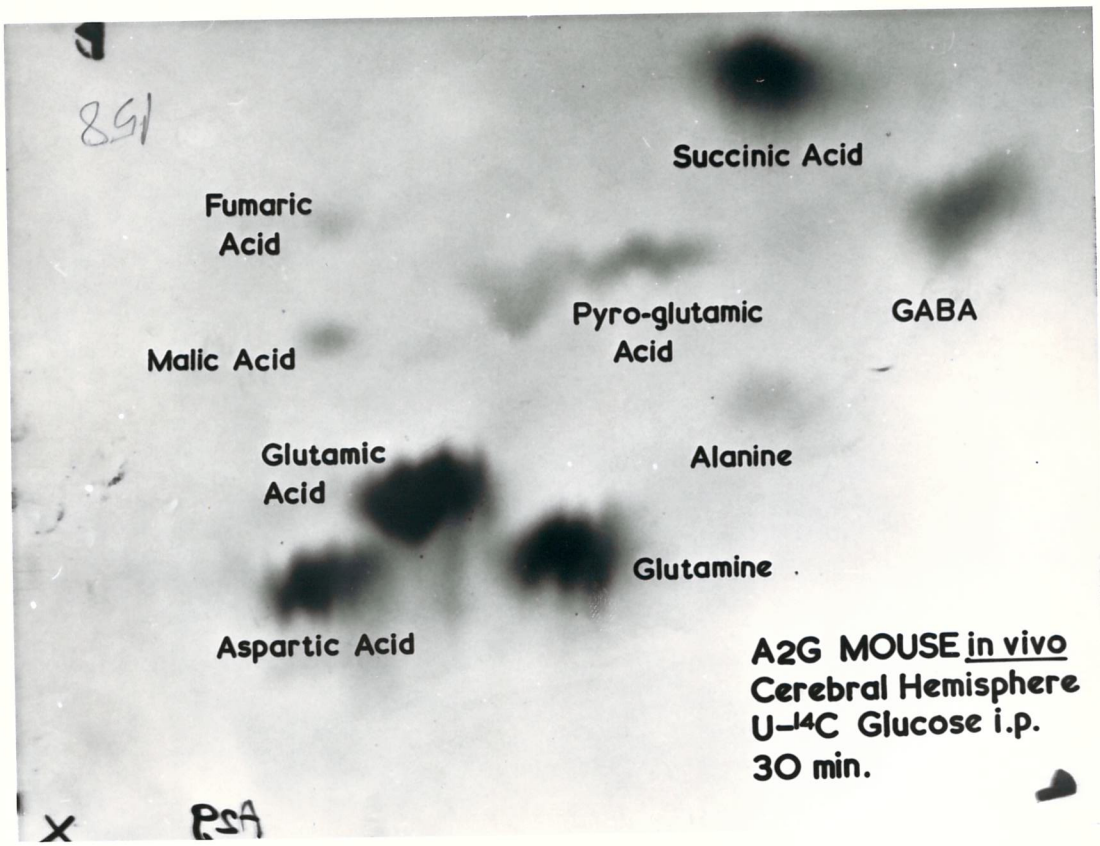

Fig.4 Two-dimensional radioautogram of the soluble intermediates in A2G mouse brain *in vivo* from U-^{14}C-glucose.

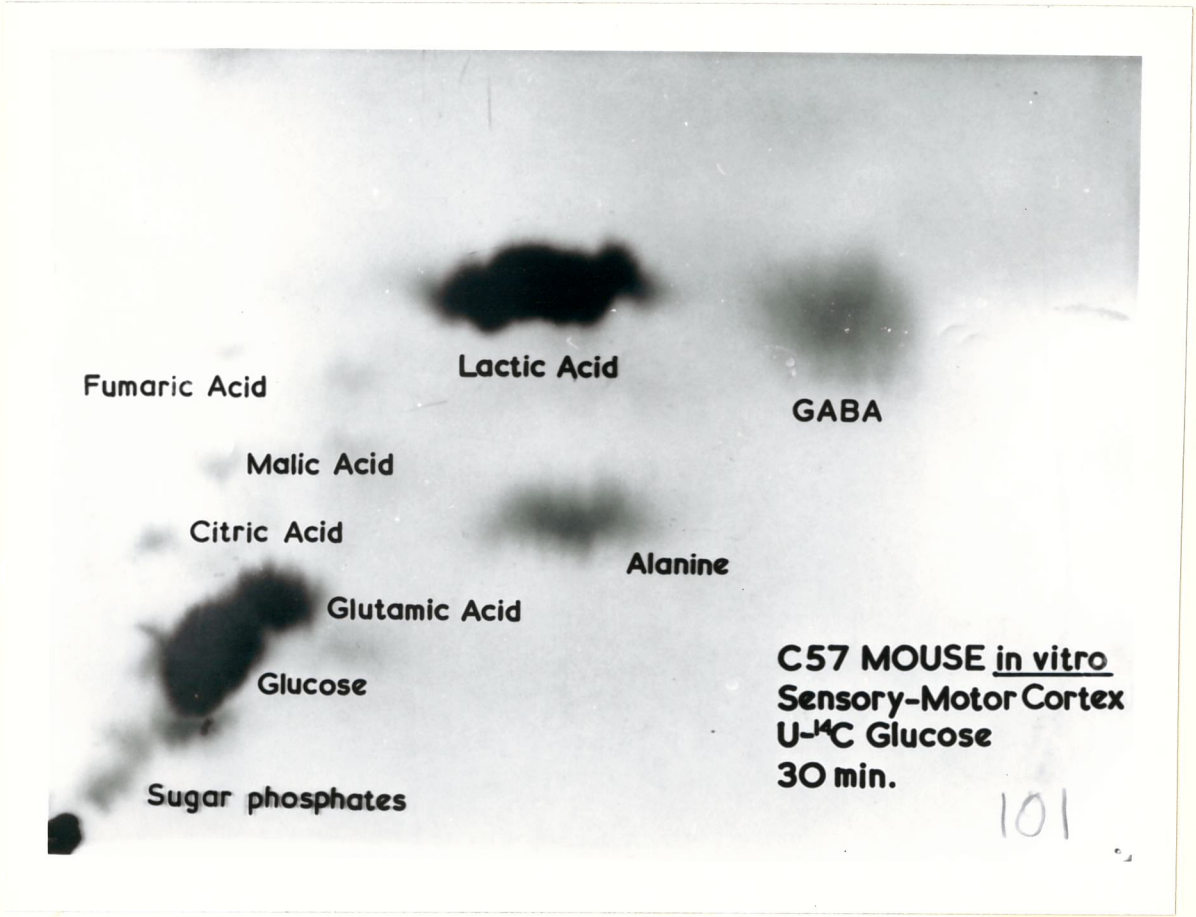

Fig.5 Two-dimensional radioautogram of the soluble intermediates in C57/BL mouse brain invitro from U-^{14}C-glucose.

TABLE 1

The percentages of the radioactive carbon atoms incorporated into the cerebral cortex from U-^{14}C-glucose *in vitro*.

Strains & sex	GABA	glu.	Ala.	gluNH2	lact.
* A2G M & F	5.08	27.31	3.85	0.95	59.0
* C57/BL M & F	5.24	18.3	4.08	0.51	60.2

* 12 animals were used from each strain.

TABLE 2

The percentages of the radioactive carbon atoms incorporated into the soluble metabolites of the brain after i.p. injection of U-^{14}C-glucose. (*in vivo*)

Strains & sex	GABA	glu.	Ala.	gluNH2	Asp.
* A2G M & F	16.59	22.86	2.99	13.37	2.52
* C57/BL M & F	25.02	28.07	3.21	18.88	6.17

* 12 animals were used from each strain.

In this series of experiments the radioactivity incorporated, pool size and the specific activities of GABA and glutamic acid were calculated.

TABLE 3

Radioactivity incorporated into and specific activities of glutamic acid and Y-amino-butyric acid pools in the cerebral cortex of C57/BL and A2G mice from U-^{14}C glucose in vivo and in vitro .

	C57 BL		A2G	
	Glutamate	GABA	Glutamate	GABA
Radioactivity Incorporated (cpm/g wet wt/hr)				
In Vivo	3,446 ± 1,435	1,511 ± 312	2,190 ± 784	1,798 ± 490
In Vitro	7,550 ± 462	2,543 ± 80.5*	10,210 ± 798	2,050 ± 140
Specific Activity (cpm/μ mole)				
In Vivo	577 ± 311.2	424 ± 45.2*	341 ± 36.4	277 ± 45.2
In Vitro	1,664 ± 171	157 ± 16.1*	1,670 ± 175	107 ± 13.7

* $P < .05$

Each result is the mean ± s.e.m. of twelve observations.

In vivo: 20 μc, U-^{14}C glucose (307 mc/mmole) i.p. 30 minutes prior to killing by total immersion in liquid nitrogen.

In vitro: 2.5 μc U-^{14}C glucose in 0.5 ml phosphate buffer, pH 7.4, incubation 30 minutes at 37°C

Note the comparatively large s.e.m's under in vivo conditions, reflecting the high variation in radioactive glucose administered i.p. being taken up by the brain.

Table 4 reports the GABA/glutamate ratios *in vitro* and *in vivo* for the incorporated radioactivity and specific activity of the two strains of mice, the reactive A2G and the non-reactive C57/BL, which shows that the C57/BL strain has the higher incorporated radioactivity and specific activity. The use of the GABA/glutamate ratios were to avoid the experimental error in the case of the *in vivo* experiments due to the variation in the quantity of the radioactive glucose reaching the brain tissues.

TABLE 4

GABA/glutamate ratios *in vivo* and *in vitro* for radioactivity incorporated and specific activity in the two mouse strains.

	Radioactivity Incorporated	Specific Activity
C57/BL	0.930 ± 0.158	1.300 ± 0.192
In vivo		
A2G	0.705 ± 0.067	0.859 ± 0.179
C57/BL	0.417 ± 0.053	0.161 ± 0.116
In vitro		
A2G	0.240 ± 0.018 *	0.101 ± 0.014

* $P < 0.002$

Conditions and abbreviations as per TABLE 1.

Acetylcholinesterase (AChE) Activity

Table 5 reports the activity of the enzyme acetylcholinesterase in the sensori-motor area of the cerebral cortex. The estimation of the enzyme was measured on terms of acetate liberated and was corrected for pseudo-cholinesterase activity in the cerebral cortex of the two strains, non-reactive C57/BL and reactive A2G. The reactive strain A2G shows higher activity than that of the non-reactive strain C57/BL. The technique applied was that described by Gage & Litchfield, (1966).

TABLE 5

Acetylcholinesterase activity in the cerebral cortex of C57/BL and A2G mice.

C57/BL	519.5 $\pm$ 9.94	moles/g cortex/hr.
A2G	593.5 $\pm$ 10.8	moles/g cortex/hr. ***

*** $P < 0.001$

Results are the means $\pm$ s.e.m. for 24 observations per strain. Activity is measured in terms of the acetate liberated.

The Biogenic Amines

The method used in these observations was that described by Brownlee & Spriggs, (1965). Two gross parts of the brain were analyzed together, the mid brain and the brain stem. In each determination these brain parts from three mice were pooled. The results in Table 6 show the level of dopamine, noradrenaline and serotonin in these areas for the two strains of mice C57/BL and A2G. The level of dopamine was significantly higher in the non-reactive strain C57/BL, while the level of noradrenaline and serotonin showed no consistent differences.

TABLE 6

Biogenic amine levels in the brain stem of C57/BL and A2G mice.

	C57/BL	A2G
Dopamine	7.52 ± 0.197	5.90 ± 0.178 ***
Noradrenaline	2.45 ± 0.140	2.04 ± 0.102
Serotonin	4.10 ± 0.089	4.42 ± 0.124

*** $P < 0.001$

The tissue used in each observation included the cerebellum, caudate and hippocampus. The results are the means of six observations $\pm$ s.e.m. Expressed in terms of nmoles/g of tissue.

CHAPTER IV

Discussion.

The behaviour of animals is controlled by many different factors. One important factor is the level of the neurochemical compounds and their metabolites in the central nervous system. The choice of the two behaviourally-inbred strains of mice, reactive A2G and non-reactive C57/BL, was to study the possible relationship between the known differences in these strains' overt behaviour and the biochemical parameters which, for the reasons discussed in Chapter I, it is argued might influence such behaviour. The parameters chosen were: the production of GABA, the activity of the enzyme acetylcholinesterase (AChE) and the levels of the biogenic amines, noradrenaline, dorpamine and serotonin in the central nervous system.

The two strains of the mice differ in their behavioural activities. The reactive strain A2G characterized by its susceptibility to audiogenic seizure, low spontaneous locomotor activity within a constant environment and heightened reaction to mildly stressed stimulations. It has been used as a "seizure strain" mouse. The non-reactive strain C57/BL has the reverse characteristics, it shows resistance to audiogenic seizure

stimulation, high locomotor activity and lack of reaction to mild stress. It has been used as a "control" strain in seizure experiments with mice. (Pasquini et al 1968; Barnett et al 1964; Fuller & Sjursem 1967; Schlesinger et al 1965 and McClearn 1961).

The data reported in Table 4, shows that the ratios of GABA/glutamate for the incorporated radioactivity and the specific activity from the U-^{14}C-glucose are significantly higher ($P < 0.002$) for the non-reactive strain C57/BL mice than for the reactive strain A2G mice, in both *in vitro* and *in vivo* experiments. The GABA production was measured in terms of ratios to avoid the experimental error in the case of the *in vivo* condition reflecting the high variation in radioactive glucose, administered intraperitoneally, reaching the cerebral cortex in each individual case. This means that the rate of production of GABA in the cerebral cortex, which may act as an inhibitory agent in the context of brain activity is higher in the non-reactive strain, C57/BL. This significant difference ($P < 0.002$) in the rate of production of GABA explains one of the possible factors for the protection of the non-reactive strain C57/BL against the audiogenic seizure. Also these results could reflect the susceptibility to seizure of the A2G strain, due to the high level of excitatory transmitter compounds and lower ability to produce the inhibitory transmitter compounds in the cerebral cortex of these animals.

These results are in agreement with the previous finding of in vitro experiments for rats, Maudsley reactive and Maudsley non-reactive strains. Rick, Huggins & Kerkut, (1967) found a significant metabolic difference between the two strains, when the tissues were incubated with U-^{14}C-glucose. The GABA production was shown to be high in the non-reactive strain, compared to the more reactive strain. Also GABA/glutamate ratio measurements have been reported in other studies using behavioural parameters. Jasper, Khan & Elliott (1965) have shown that the GABA/glutamate ratio varies systematically with states of activation or "arousal" of the electrocorticogram, high ratios being concomitant with sleep EEG patterns and low ratios with "arousal" EEG patterns.

The difference in the rate of production of GABA is controlled by two enzymes involved in the "GABA Shunt" particularly the activity of the enzyme glutamic acid decarboxylase (GAD), which is unique to the nervous tissues and the enzyme GAMA-aminobutyric acid transaminase (GABA-T). The activity of enzymes has been shown to be reduced or inhibited by certain drugs (e.g. hydroxyl-amine, amino-oxy acetic acid (AOAA) and Toxopyrimidine) complexing the co-factor vitamin B6, leading to the significant reduction in the level of GABA (Baxter & Roberts 1961; Rindi & Ferrari, 1969 and Makino et al 1954). The correlation between the two enzymes' activity and the presence of cofactor controls the level of GABA

in the nervous tissues which was shown in scheme 4, Chapter I.

The activity of the enzyme AChE, as shown in Table 5, shows a significant difference ($P < 0.001$), between the two strains. The A2G mice exhibit higher AChE activity, than that found in the C57/Bl mice in the cerebral cortex. This result is not in agreement with that of recent investigations reported by Rick, Morris & Kerkut 1968 and Geller, Yuwiler & Zolman 1965. Both these groups of workers established that GABA production measured in terms of GABA/glutamate ratios or in terms of the activity of the enzyme GAD, may be positively correlated with the activity of the enzyme AChE. This means the higher the activity of AChE the higher the production of GABA or the activity of the enzyme GAD.

The work completed by Rick et al and Geller et al was for rat brain and the present results may reflect a species difference in brain chemistry or possibly a chemical abnormality in the A2G strain, in that the hightened presence of acetylcholine, indicated by the activity of AChE is not counter-balanced by the productive capacity for GABA in the tissue. Such an abnormality might well increase seizure susceptibility. Further to this latter interpretation, the level of Ach is known to change during emotional excitement or convulsions (Richter and Crossland 1949).

The data for the level of biogenic amines, dopamine, noradrenaline and serotonin are shown in Table 6. The results show that there are no significant differences in the concentration of noradrenaline and serotonin in both strains C57/BL and A2G mice, but the dopamine level differs significantly ($P < 0.001$) between the two strains. The non-reactive strain C57/Bl mice have a higher concentration of the dopamine compared to that of the reactive strain A2G mice.

This is consistent with the evidence by Hornykiewicz (1966), that dopamine has an inhibitory effect in the central nervous system of the lower animals and mammals. Hornykiewicz also suggests that Parkinsonism is a result of lack of inhibition in the brain by dopamine. This suggestion is in agreement with the present observation concerned with the high concentration of dopamine in the seizure resistant strain C57/BL mice compared to the low concentration of dopamine in the seizure susceptible strain A2G mice. Tunnicliff, Rick & Connolly (In Press), report a lower dopamine level in a highly active Drosophila melanogaster strain, and a higher level in a low active strain compared to a control strain of the fly. However, there is contradictory evidence put forward by Van Rossum (1964), who suggests that dopamine is the significant agent for producing increased motor activity when stimulators are administered to mice. The dopamine level is specifically increased by administration

of GAMA-hydroxy-butyric acid (Gessa, Crabai, Vargin & Spano, 1968), a substance which affords protection against several forms of chemically induced seizure (Laborit 1964).

The relatively high brain serotonin and noradrenaline levels found in the seizure susceptible strain of mice at 21 days of age, compared to the C57/BL strain (Schlesinger et al, 1965), were not substantiated in the present experiments. (Table 6). However, the difference in the Schlesinger et al (1965) study was only found in mice at 21 days, and the mice in the present study were all at least six weeks old.

In conclusion, it is argued that these findings reflect the heightened seizure susceptibility of the A2G strain due to a relatively high excitatory transmitter and low inhibitory transmitter capacity of the cerebral cortex of these mice, compared to that of the C57/BL mice - a seizure resistant strain. These results give evidence for the importance of the rate of production of GABA in the central nervous system in controlling the seizure susceptibility due to its inhibitory effect. The activity of AChE and the biogenic amines dopamine, noradrenaline and serotonin were estimated. These biochemical parameters suggest line of future work for establishing the biochemical processes that may explain further the differences in behaviour between the species.

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