BINDING AND RELEASE OF AMINO ACIDS IN BRAIN .

by

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INTRODUCTION

Neuroinhibitory characteristics have been ascribed to a number of substances in the central nervous system (McLennan, 1959; McGeer, McGeer and Mclennan, 1961). However, there is strong evidence that all the activity of extracts or Factor 1 from the mammalian central nervous system, which block the sensory discharge of cray fish receptor preparation (Florey, 1954, 1956; Florey and Mclennan, 1955, 1956), can be totally accounted for by gamma-aminobutyric acid (Bazemore, Elliott and Florey, 1956, 1957; Levin, Lovell and Elliott, 1961; Lovell and Elliott, 1963).

Whether or not gamma-aminobutyric acid or its metabolite is an inhibitory transmitter or a modifier of neuronal activity remains to be definitely shown. At present, it is known that gamma-aminobutyric acid is present in large amounts in the central nervous system (Awapara, Landua, Fuerst and Seale, 1950; Roberts and Frankel, 1950; Udenfriend, 1950). It has, at most, only very limited ability to pass the blood brain barrier (Van Gelder and Elliott, 1958); and it is evidently produced in the brain. Part of the Factor 1 or the chemically determined gamma-aminobutyric acid is present in the brain as an easily extractable "Free" form in isotonic saline solutions and immediately active on the cray fish receptor organ. The other part is present in an occluded or "Bound" form which is associated with solid matter and is not active on the stretch

receptor organ nor chemically detectable until it has been released (Elliott and Florey, 1956; Elliott and Van Gelder, 1960). Variations in concentration of gamma-aminobutyric acid or GABA * from one cerebral area to another have been reported (Krzalic, Mandic and Mihailovic, 1962). Also, it has been found that the total amount of GABA extractable from brain that has been frozen in situ at the moment of death is lower than that in brain excised at room temperature (Lovell and Elliott, 1963).

Extensive studies are required for the understanding of the physiological role, factors affecting its formation, storage, release and further metabolism of GABA and other amino acids in the brain. It will be shown in subsequent chapters that numerous factors can rapidly change the proportions of "bound" to "free" GABA in cerebral tissue extracts and that the other amino acids related to the krebs cycle: glutamic acid, glutamine, aspartic acid, alanine and N-acetyl Aspartic acid are similarly bound or occluded and probably undergo similar changes.

Gamma-aminobutyric acid has been implicated in the nervous system as an inhibitory agent (Elliott and Florey, 1956; Elliott and Jasper, 1959); and as a participant in energy metabolism through the GABA shunt pathway (for references see the review

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* Throughout this thesis, the symbol GABA and Ach will be used for the naturally occurring amino acid, gamma-aminobutyric acid or 4-amino-n-butyric acid and acetylcholine respectively.

by Elliott and Jasper, 1959). To obtain further evidence for the physiological role of GABA and other amino acids in the brain, the release of glutamic acid, glutamine, aspartic acid and GABA in the mammalian (cat) cerebral cortex have been measured under a variety of experimental conditions (Chapter 4). The present findings are compatible with the postulated role of GABA as an inhibitory transmitter substance (Edwards and Kuffler, 1959; Dudel and Kuffler, 1960; Kravitz, Potter, and Van Gelder, 1962; Eccles, Schmidt, and Willis, 1963) and of glutamic acid as an excitatory transmitter.

The release of acetylcholine has been measured under "aroused" and "sleep" conditions and the results are presented in Chapter 5. Studies summarized in Chapter 4 and Chapter 5 were undertaken to show to what extent GABA and acetylcholine are involved in the behaviour of sleep and wakefulness and if an antagonism exists between their activities.

Chapter 1

HISTORICAL SURVEY

Most of this historical review will be concerned with the lines of research related to the binding and release of amino acids and their physiological role in the central nervous system. The role of GABA from the neurophysiological and neurochemical point of view has been reviewed recently by a number of authors (Elliott and Jasper, 1959; Roberts, 1962; Elliott, 1965).

Gamma-aminobutyric acid or GABA was for many years known as a putrefaction product or glutamic acid breakdown in plants and bacteria (Ackermann, 1910; Abderhalden, Fromme and Hirsch, 1913). The discovery of the presence of GABA in the brain (Roberts, Frankel and Harman, 1950; Roberts and Frankel, 1950) stimulated biochemical research in the field of its distribution and metabolism in the central nervous system. Udenfriend (1950) confirmed the identification of GABA as a normal component of brain tissue.

In 1959 the metabolic relationships of GABA to the citric acid cycle was shown (Beloff-Chain, Catanzaro, Chain, Masi and Pocchiari, 1955, 1956; Tsukada, Nagata and Takagaki, 1958; and the distribution and properties of the participating enzymes were worked out (Bessman, Rossen and Layne, 1953; Roberts and Bregoff, 1953; Roberts and Baxter, 1958). Isolation

of GABA-alpha ketoglutaric transaminase (Scott and Jakoby, 1958) and succinic semialdehyde dehydrogenase from Pseudomonas fluorescens was an important step towards the study of metabolism and an enzymatic determination of GABA and other related amino acids of the citric acid cycle. Metabolic inhibitors have been extensively used to find out the relationship between the metabolism of GABA and nervous activity (Elliott and Jasper, 1959; Baxter and Roberts, 1960, 1961; Wallach, 1960, 1961; Elliott and Van Gelder, 1960; Ferrari and Arnold, 1961; Maynert and Kaji, 1962; Palm, Balzer and Holtz, 1962).

It has been demonstrated that intravenous injection of GABA does not alter the brain GABA levels (Van Gelder and Elliott, 1958). This can be explained by the existence of blood brain barrier to GABA and, therefore, the presence of GABA in relatively large amounts in the brain is regarded as an indication of its formation and metabolism in cerebral tissues (Dobbing,1961). GABA and other amino acids, and some electrolytes are also strongly taken up by brain slices against concentration gredients by means of an energy requiring transport systems. The absorption of these amino acids and GABA from the surrounding medium by brain slices and tissue may be one of the mechanisms for the active removal of these physiologically significant amino acids (Stern, Eggleston, Hems, and Krebs, 1949; Schwerin, Bessman and Waelsch, 1950; Elliott and Van Gelder, 1958). On the other hand, the blood brain

barrier limits the access of various amino acids to the brain. It has also been suggested that the blood brain barrier and amino acid transport mechanisms in the brain could be a way of restricting physiologically active amino acids from interfering with neuronal function and activity (Lajtha, 1962). Following intravenous injection, Lajtha and Mela (1961) found a rapid exchange of labelled amino acids between plasma and the brain. The rate of exchange increased when the level of the amino acids in the brain was increased. Mechanisms for this sort of exchange do not require external energy.

Recent studies on the interrelationships between carbohydrate and amino acid metabolism have shown that all physiologically active amino acids in the brain are rapidly produced from glucose (Vrba, Gaitonde and Richter, 1962; Gaintonde, Marchi and Richter, 1963, 1964; Gaintonde, Dahl and Elliott, 1964). Five minutes after intravenous injection of glucose-^{14}C to rats, nearly 50% of total radioactivity in the brain was found in alanine, glutamate, asparate, glutamine and gamma-aminobutyric acid. In earlier experiments of Chain et al and Tsukada et al (1955, 1956, 1958) it was also shown that glucose carbon retained in the rat or cat brain and the adminstered labelled glucose was incorporated largely into amino acids. The results of Gaitonde et al (1964) show evidence that GABA is formed by decarboxylation of glutamate of higher specific radioactivity than the average for glutamate for the whole tissue. This finding also suggests that GABA is formed

in a mitochondrial pool in which highly labelled glutamate is derived from alpha-oxoglutarate. Chain et al (1955,1956) followed the path of glucose in different regions of the rabbit brain in Vitro. In the cerebral cortex greater amounts of alanine, aspartic acid, glutamic acid and glutamine were formed than in the hypothalamus while hypothalamic tissue formed more GABA from glucose than did either the cerebral or cerebeller cortex.

Florey (1954) showed that extracts from the central nervous system of mammals contain a substance or factor which reversibly inhibits the generation of impulses by crustacean stretch receptor neurons. The substance or factor resposible for this inhibition has been called "Factor 1" (Elliott and Florey, 1956). In crustaceans purified preparations of Factor 1 block neuromuscular transmission, slow or stop the neurogenic heart-beat, inhibit the spontaneous activity of the intestine, and nullify the effects of acetylcholine on the intestine (Florey, 1954). Partially purified extracts of the central nervous system have been found to inhibit spontaneous and acetylcholine stimulated contractions of guinea pig and rabbit intestine (Florey, 1953). It also blocks synaptic transmission in the inferior mesenteric ganglion of cat and rabbit and in the stellate ganglion of the cat (Florey and Mclennan,1955). If topically applied to the spinal cord of the cat, this blocks the monosynaptic tendon jerk reflex and often enhances the polysynaptic flexor reflex. Bazemore, Elliott and Florey (1957) obtained crystals showing higher Factor 1 activity

which they identified as gamma-aminobutyric acid. Mclennan (1959) insisted that Factor 1 and GABA are not identical. However, Levine, Lovell and Elliott (1961); and Lovell and Elliott (1963) showed clearly that GABA, undoubtedly, present in all brain extracts, can account for all Factor 1 activity. This important finding gave considerable stimulus to neurophysiological studies of GABA. It is likely that GABA and other amino acids are involved in regulating some of physiological activity in the nervous structures and brain (Khan, Jasper, Elliott and Wolfe, 1964).

The studies of the distribution of Factor 1 in the mammalian brain were elaborated by Florey and Florey (1958). Factor 1 was shown to be present in the grey matter of extrapyramidal centers and was absent in the white matter. On the basis of the distribution and different location of Factor 1, they assumed that inhibitory neurons contain Factor 1. Assigning the role of chemical transmitter to Factor 1, these authors also suggested that its presence in neurons causes either a direct inhibition of other centers or that it is contained within the first and last neurons of polysynaptic extrapyramidal pathways.

Kuffler and Edwards (1958) studied the mechanism of action of GABA in the central nervous system and its relation to synaptic inhibition. Their studies present a strong case for a role of GABA as an inhibitory transmitter in crustacean neuromuscular junction. Similar conclusions have been drawn from the studies on amphibians (Diomond, 1963). More recently

Florey and Chapman (1961) have put forth evidence from which they concluded that GABA is not the inhibitory transmitter in the crustacean group. In direct contradiction to these findings of Florey and Chapman (1961) Kravitz, Potter and Van Gelder (1962) have obtained GABA from the leg extensor muscle of the crab. They demonstrated that isolated GABA from the above preparation exerted inhibitory activity on the crustacean. The GABA and related inhibitory substances have been found in large amounts in inhibitory fibers of peripheral nerve of crustacean central nervous system (Kravitz, Kuffler, Potter and Van Gelder, 1963; Kravitz, Kuffler and Potter, 1963).

Curtis and Watkins (1960) observed depressing action of GABA on various types of neurons when it was applied by pressure or iontophoretic injection in the spinal cord and brain. At the same time, there was neither any effect on membrane resting potentials nor on presynaptic fibers. All the experiments mentioned show that GABA and beta-alanine have a nonspecific depressant action on the whole surface membrane of neurons. This is true both for the chemically activated subsynaptic regions and the remaining electrically excited postsynaptic membrane. Furthermore, Curtis and Watkins (1963) have shown that the amino acids studied can be classified into two distinct groups: the excitatory amino acids like glutamic acid, aspartic acid, cysteic acid, cysteinic sulphinic acid and the inhibitory amino acids: beta-alanine, GABA and taurine.

Evidence that gamma-aminobutyric acid or GABA may be an inhibitory transmitter substance in the mammalian central newvous system is accumulating (Khan, Jasper, Elliott and Wolfe, 1964). Eccles, Schmidt and Willis (1963), from their microelectrode studies assume that GABA may be involved in the action of certain presynaptic inhibitory neurons. Iwama and Jasper (1957) showed that topical application of GABA solution causes a variety of changes in electrocortical activity of cats. In the same year Purpura, Girado and Grundfest (1957) confirmed such changes. The surface negative dendritic response to direct electrical stimulation was replaced by a surface positive response soon followed the application of GABA solution. Also the surface negative component of the usual biphasic evoked response to thalamic stimulation was suppressed. This important group of experiments led Purpura and Grunfest (1959) to believe that GABA inactivates excitatory axodendritic synapses in the mammalian brain.

It has been shown (Khan, Jasper, Elliott and Wolfe, 1964) that the cat showing electrocorticogram with aroused pattern after cervical section or local anaesthesia in the waking animal small amount of GABA will leak out of the cortex, provided the pia arachnoid membrane has been pierced. The release GABA, however, is three times more in cats showing sleep pattern with marked spindles following mid brain section. If the aroused pattern was constantly maintained, no GABA release could be detected. Bessman and Fishbein (1963), however, very recently

have found a possible intermediate of gamma-aminobutyric acid metabolism, gamma-hydroxybutyric acid which caused sleep in mammals, mice and rats. In all species, sleep was produced within fifteen minutes and lasted for one and a half to two hours. Preliminary investigations in rats showed that the sleep period corresponds to an increment of gamma-hydroxybutyric acid in the brain of about two millimoles per kilogram weight of the subject under study. Sleep lasted as long as this level was exceeded. Preliminary data on human beings treated with this substance showed sleep to occur while the blood level of gamma-hydroxybutyrate exceeded one millimolar.

Grundfest (1964) has shown recently that GABA causes hyperpolarizing inhibitory postsynaptic potentials in the fibers of a jumping muscle of a grasshopper. He observed that postsynaptic responses were increased during depolarization of the muscle fiber with applied current and were diminished, then reversed, during hyperpolarization. Gamma-aminobutyric acid was found to be the activator of the inhibitory synaptic membrane. Picrotoxin blocked the activation of the inhibitory synapses.

In early work of Elliott and Florey (1956) on the factor or substance in the brain which inhibits the activity of the crustacean stretch receptor neuron, it was observed that part of this factor was not immediately active. It could be released into the active form by heat, weak acid or alkali, hypotonic solutions or organic solvents, freezing, and to a large extent in

salt free sucrose solution (Elliott and Van Gelder, 1960; Lovell and Elliott, 1963). All the bound factor was found in the residue on centrifuging. These authors also found that the ratio of free to bound or occluded Factor 1 in the normal rat brain is fairly constant but is changed by the administration of certain drugs and metabolic inhibitors.

Radioactivity studies using GABA-1-^{14}C have shown some binding of the added GABA only in the insoluble residue of mouse or rat brains. There was no binding by brain acetone powder residues or to any other tissue (Sano and Roberts, 1962). Sano and Roberts (1961) showed little or none of this binding in the absence of sodium chloride, and sodium ion could not be replaced by other electrolytes. Elliott, Dahl and Balazs (1964) have applied the methods of Gaitonde (1964) for separation and estimation of amino acids of the supernatant and residue fractions of brain from rats. Of the total glutamic acid, aspartic acid, glutamine, alanine and GABA in the brain, 25 to 47% radioactivity appeared in the bound centrifuged residue fraction, while 48 to 61% appeared in the case of GABA.

Crude mitochondria from brain were first prepared by Brody and Bain (1952). Recently, the density gredient techniques have been developed to refine brain crude mitochondria into several subcellular fractions in order to study the distribution of important enzymes and physiologically active substances in brain homogenates (Whittaker, 1959; De Robertis, Pellegrino, Rodriguez and Salganicoff, 1962). The preparation of these

subcellular fractions involves homogenization of the tissue in salt free saline solution. Elliott and Van Gelder (1960) showed that much of the bound GABA is released in such solutions. However, Bilodeau (1962) found that significant amounts of GABA and glutamic acid are found in the crude mitochondrial fraction of sucrose homogenates of the rat brain. Subfractionation of the crude mitochondrial fraction into myelin, nerve endings, synaptosomes and pure mitochondria revealed that most of the bound amino acids were in the "nerve ending fraction". Similar results were obtained by Ryall (1962, 1964) and Weinstein, Roberts and Kakefuda (1963). Of the total GABA, 70 to 80% was in the cytoplasmic fraction; thus, only 20 to 30% remained bound in sucrose homogenates of rat brains. Elliott and Van Gelder (1960) had found that only 20 to 30% of Factor 1 activity remained bound under such conditions.

It has been postulated that the transmission across a number of synapses in the central pathway of autonomic and motor neurons occurs through the mediation of acetylcholine. In view of the profuse output of acetylcholine in the cortex, it is definitely of interest to find out whether acetylcholine does alter neuronal activity. Many earlier experiments have put forth suggestions that cortical neurons are excited by acetylcholine (Feldberg, 1943, 1950; Elliott, Swank and Henderson, 1950; MacIntosh and Oborin, 1953; Stone, 1957; Mitchell, 1963).

However, these results have often been in conflict (Schlag, 1956; Nakao, Ballin, and Gellhorn, 1956). Furthermore, it has been suggested that the states of sleep and waking may be due to balanced excitatory and inhibitory synaptic actions of reticular activating system (Pavlov, 1927; Krnjevic and Phillis, 1963; Krnjevic and Silver, 1963; Shute and Lewis,1963).

To increase further understanding, detailed studies have been made. Perfusion chambers have been inserted into the skulls of experimental animals. The fluid in the chambers has been analyzed for quantities of acetylcholine and GABA in (1) intact animals with local anaesthesia and flaxedil, (2) encephale isolé preparations, (3) cerveau isolé preparations, (4) midbrain hemisection of the brain stem. (Jasper, Elliott, Sie, Khan and Wolfe, 1964).

Chapter 2

GENERAL METHODS

PREPARATIONS OF TISSUE EXTRACTS

Rats weighing about 150 g. were decapitated by means of a guillotine. When the head was not frozen, the brain (two hemispheres and brain stem without cerebellum) was quickly removed. Within 2 minutes of decapitation, it was dispersed by means of a Potter-Elvehjem homogenizer in Ringer phosphate, ethanol, or other media, according to the data sought.

When the brain was to be frozen *in situ* the decapitated head was permitted to fall directly into liquid air. The brain was removed in the frozen state and homogenized in absolute ethanol. Rat brain saline homogenates were used when proportions of free to bound amino acids were determined. The saline suspensions were prepared by homogenizing the brain usually in 4 volumes of ice-cold Ringer phosphate solution. Suspensions were centrifuged at 0-4 degrees centigrade at 15,000 x g for 30 minutes. The amino acids found in the supernatant fluids were called "free". Those in the centrifuged residue, corrected for the amounts of free amino acids contained in the fluid trapped in the residue, were called "bound".

To 1 ml of the supernatant fluid 3 volumes of absolute ethanol were added. The residue was suspended in Ringer phosphate to make it up to the original volume and the resultant

suspension was mixed with 6 volumes of 95% ethanol. The alcoholic solutions were centrifuged after standing 30 minutes at 0-4 degrees centigrade.

DETERMINATION OF GABA, GLUTAMIC ACID, GLUTAMINE, ASPARTIC ACID AND ALANINE

Chromatographic Ninhydrin Method

For the determination of GABA alone, the one dimensional chromatographic ninhydrin method of Levin, Lovell and Elliott (1961) as later modified by Lovell, Elliott (1963) was used. The other amino acids: alanine, glutamin, glutamine and aspartic acid were separated by two dimensional chromatographic methods as used by Giatonde (1961). The solvent system consisted of butanol: acetic acid: water - 12:3:5 v/v as first phase and phenol: water - 80:20 w/w as a second phase with normal running time of 18-20 hours was used. Whatman No. 4 paper was used for the glutamic acid and aspartic acid; whereas Whatman No. 1 was used for alanine, glutamine and GABA. Amounts of extract which contained 1-20 ug each of the amino acid in question were applied to the paper. The chromatograms were dried overnight in a fume hood and then for 1 hour in an oven at 40 degrees centigrade. The purpose of this was to remove traces of phenol and butanol. The dried chromatograms were then dipped in 0.25% ninhydrin in acetone and heated in an oven at 65-70 degrees centigrade for 25 minutes. In order to identify the zone that corresponded to a specific amino acid the brain extract and a known amount of the amino acid in question were applied to the paper. Different dilutions of standard solutions

containing 2.50 micromoles per ml of each amino acid were also spotted on separate sheets as standards. The standard spots also helped in locating the amino acid in question. The coloured zones of paper, thus identified, were cut into fragments and treated with 2 ml of freshly prepared 0.5% ninhydrin in 80% v/v ethanol-water at room temperature for 15 minutes. Then 3 ml of 70% v/v acetone-water were added. After 15 minutes, the fluid was transferred to a centrifuge tube. The paper was re-extracted for 15 minutes with a further 3 ml of 70% acetone and the combined fluids were then centrifuged to remove small cuttings of paper. The absorption of this solution was measured at 575 millimicrons in a spectrophotometer against similarly prepared ninhydrin ethanol acetone extract from a part of the paper which showed no colour. Mixtures of the acid and neutral amino acids were similarly spotted, chromatographed, developed, and read against paper blanks. To determine the recovery, standard solutions of each amino acid alone, and with extract, were also applied to different sheets of paper. Resulting spots were eluted and the absorption was measured. The recovery of 5-10 ug of each amino acid applied with 100 ul of extracts was 86-102%. To estimate the total (uncombined plus acetylated) aspartic acid an aliquot of the ethanol extract was mixed with an equal volume of 0.05 N sulphuric acid and heated in a sealed tube for 1 hour on a hot sand bath. An aliquot of this mixture was taken for

chromatography. The amount of aspartic acid found, minus the amount found without hydrolysis was taken as the amount of N-Acetyl aspartic acid.

ENZYMATIC METHOD

In some experiments, the method of Jakoby and Scott (1959), as modified by Jakoby (1962), was used for the enzymatic determination of gamma-aminobutyric acid. When this method was to be used for GABA, the ethanol extracts were dried under a stream of air at 50°C. The resulting residues were taken up in a measured volume of distilled water and centrifuged. The enzyme preparation was made from Psuedomonas fluorescens cells, according to the fractionation procedure used by Jakoby (1962).

The dried psuedomonas cells were gently dispersed with a glass rod in 10 volumes of potassium phosphate buffer solution pH 7.35 containing 0.01% mercaptoethanol, stirred several times, and centrifuged. Then the supernatant was discarded. The residue was suspended in an equal volume of potassium phosphate buffer and dispersed in an ultrasonic disintegrator for 15 minutes. The resultant suspension was diluted with 2 volumes of potassium phosphate buffer and centrifuged at 15,000 g. for 30 minutes. The supernatant was removed and kept cold. The residue was again disintegrated in the ultrasonic disintegrator; the second supernatant was then added to the first. To this crude extract 1% protamine sulphate solution was added. The resulting

precipitate was centrifuged and the residue discarded. To the supernatant 35 g. of ammonium sulphate per 100 ml were added with gentle and constant stirring. The formed precipitate was centrifuged and discarded. A second ammonium sulphate fractionation was then carried out by adding 21 g. of ammonium sulphate per original 100 ml of supernatant at 0 degree centigrade. The precipitate which, salted out, was removed by centrifugation and dissolved in a small volume of phosphate buffer solution. The mercaptoethanol concentration was decreased from 0.1% to 0.01% and the solution poured into 10 volumes of acetone at minus 15 degrees centigrade.

The suspension was filtered with suction to remove the precipitate which was then suspended in a small volume of phosphate buffer. The insoluble material was spun down and discarded. The resulting supernatant was adjusted to a mercaptoethanol concentration of 0.05% and dialysed overnight against 100 volumes of 0.05 M phosphate buffer. After the dialysis, any insoluble material was removed by centrifugation. The supernatant was divided into parts of 1 ml in small sample tubes for storage in the deep freeze.

The enzymatic method of determination of GABA depends on the formation of NADPH, the absorption of which is measured in a spectrophotometer at 340 millimicrons. In the presence of added excess of alpha-keto glutarate, GABA is transaminated to succinic semialdehyde which is then oxidized to succinate, $NADP^+$

serving as the hydrogen ion acceptor. The amount of NADPH is a measure of the GABA originally present in the samples.

The reaction mixture specified by Jakoby (1962) consisted of the following:

M - Tris (hydroxy-methyl-amino-methane chloride pH 7.9) 0.05 ml; 1.5 M sodium sulphate 0.5 ml; NADP (20 mg/ml) 0.05 ml; 0.02 M alphaketoglutarate 0.1 ml; enzyme preparation 0.05 ml - 0.2 ml; and an amount of tissue extract containing 0.5 - 20 micrograms of GABA. The total volume was made up to 1 ml with distilled water. Preliminary runs with standards were carried out with enzyme preparation to determine what volume of the enzyme solution gave maximum O.D. at 340 mu within 15 minutes.

Mercaptoethanol, sodium sulphate, $NADP^+$, water, the buffer and sample, or standard were pipetted first into the 1 ml quartz cells. The enzyme was kept ice-cold and added just before the alpha-keto glutarate. The addition of alpha ketoglutarate initiated the reaction. The absorption of the solutions at 340 millimicrons was then followed with time until the maximum was reached. The maximum optical density was proportional to the GABA content of standard or tissue sample.

Chapter 3

FACTORS AFFECTING THE BOUND GABA AND OTHER AMINO ACIDS IN THE BRAIN

Introduction

Part of Factor 1 or the chemically determined gamma-aminobutyric acid content of the brain is present in a "free" form. It is immediately active on the crayfish stretch receptor preparation and is chemically detectable as GABA in in the supernatant when the brain has been suspended in isotonic saline solutions. The other part is present in an occluded or bound form. It is associated with solid matter and is neither active on the stretch receptor nor chemically detectable until it has been released. The release is achieved by the action of mild agents such as heat, hypotonicity, dilute acid or alkali, freezing, or ethanol (Elliott and Florey, 1956; Elliott and Van Gelder, 1960; Lovell and Elliott, 1963). The nature and subcellular location of this bound GABA is not clear. It has been reported to be present in the mitochondria (Bilodeau, 1962; Ryall, 1962, 1964; Weinstein, Roberts and Kakefuda, 1963). However, it has been found that much of the bound GABA is released by the sucrose solutions which are used for the preparation of mitochondria (Elliott and Van Gelder, 1960). The finding that the total amount of GABA, chemically determined; in brain tissue that has been frozen in situ; is, at the moment of death, lower than in the brain excised at room temperature

(Lovell and Elliott, 1963), suggest a rapid post mortem increase in the total GABA content of the brain. It will be shown in this chapter that other factors can rapidly influence the proportion of bound GABA and amino acids related to Kreb's cycle are similarly bound and follow similar changes.

Methods

Saline extracts for determination of proportions of free to bound GABA and other amino acids were prepared as described in General Methods (Chapter 2). Water extract was obtained by homogenizing rat brain tissue in four volumes of water, heating it in boiling water, and acidifying with acetic acid to pH 5. It was centrifuged. When alcohol extracts were made for the determination of the total amount of GABA and other amino acids, the rat brains obtained after decapitation of the animal were weighed, and immediately homogenized in four volumes of 90% v/v cold ethanol. The tissue water brought the final ethanol concentration of the extract to about 75%. The homogenates were centrifuged at about 30,000 x g for 30 minutes.

The supernatants were spotted, as described in Chapter 2, for the quantitative determination of GABA and other amino acids. When the brain was to be frozen in situ the head was allowed to fall, as it was severed, directly into liquid air. The brain was removed in the frozen state and homogenized in 90% ethanol. The saline extracts were prepared by homogenizing the rat brain in four volumes of ice-cold Ringer phosphate solution and centrifuging the suspension at 30,00 x g for fifteen minutes at 0-4 degrees centigrade.

To an aliquot of the supernatant which contained in the fluid

the free amino acids, 3 volumes of 90% (v/v) ethanol were added. The residue which contained the bound amino acids was weighed and made up to the original volume with Ringer phosphate. To this, 3 volumes of absolute ethanol were added. The two alcoholic solutions were centrifuged clear and amino acids in the supernatant were determined chromatographically. Bound amino acid was taken as the amount of an amino acid in the residue from the saline suspension minus a correction for the free amino acid present in the fluid trapped in the residue. The volume trapped was taken as being approximately equal to the total volume of the residue and its amino acid concentration which was equal to that in the supernatant saline fluid.

RESULTS

In experiments in which the brain was frozen *in situ* with liquid air or excised at room temperature, Lovell and Elliott (1963) have shown that a rapid increase of 35% in the total GABA content of the brain occurs immediately post mortem. Since freezing releases bound GABA, it was impossible to show whether this post mortem increase was due to an increase in the bound or free GABA or both. Fig. 1 shows variations in the proportions of free and bound GABA that are found when the solid excised brain is allowed to stand at room temperature before homogenization in cold saline medium and separation of the free and bound GABA by centrifugation. It is noticeable that

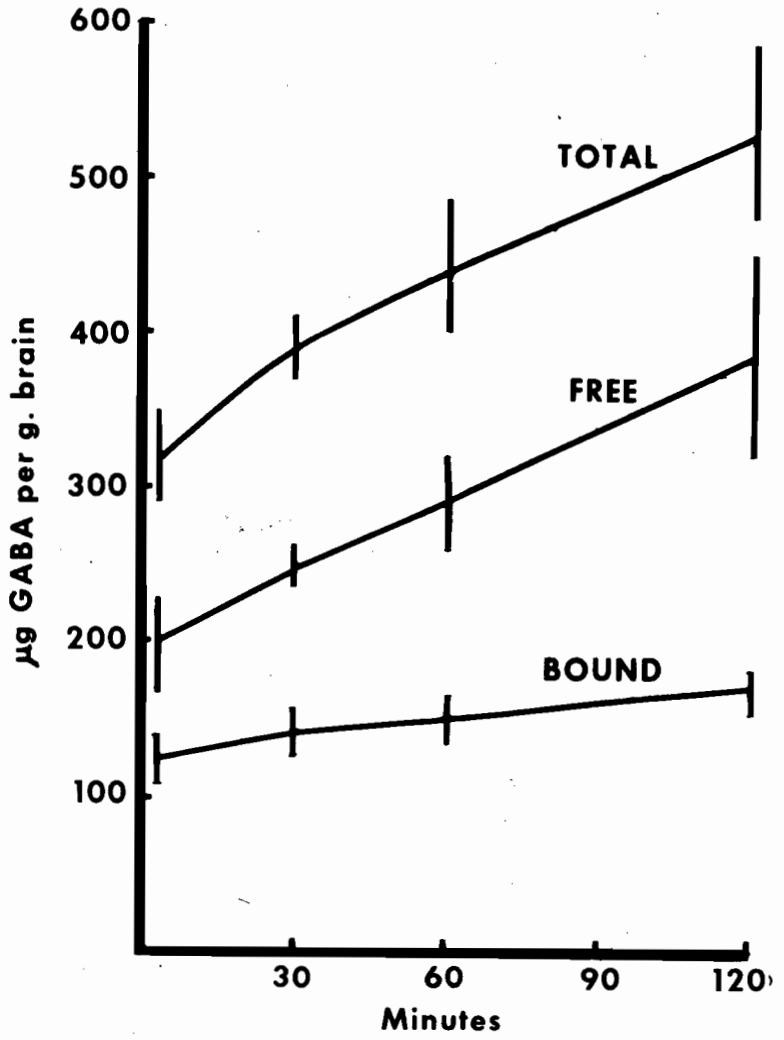

Fig. 1 Changes in free and bound GABA in excised rat cerebral hemispheres on standing at room temperature.

The brains were excised immediately after decapitating the animals and, at the times indicated, homogenised in cold Ringer-phosphate solution and the suspensions were centrifuged at 0°C. Each point indicates the average obtained with 5 to 7 brains and the vertical bars show the standard deviations.

the initial rapid post mortem increase is followed by a steady increase with time. This increase is almost all in the free form.

About 66% of the total Factor 1 activity in the whole brain was reported to be present in the bound form by Van Gelder and Elliott (1958). If correction had been applied for free Factor 1 in the fluid remaining in the centrifuged tissue residue that contained the bound, the figure would have been 58%. In the experiments of Lovell and Elliott (1963) and in the present study, a lower proportion of bound GABA has been obtained which is equivalent to approximately 40% of the total. Such lower figures are obtained when the brain is homogenized in ice-cold saline solution instead of at room temperature as was previously the case.

Effect of Temperature and Different Media

Results summarized in Table 1 show that the proportion of GABA in the bound form is considerably higher in suspensions prepared at room temperature or at 38°C than in suspensions prepared in a cold medium. Fig. 2 shows results of typical experiments showing changes in free and bound GABA in brain suspensions that are prepared in the cold or at room temperature 23°C, and then kept standing cold at room temperature or at 38°C, (also see Fig. 3). It is apparently evident that a lower proportion of bound GABA is present when the suspension is

Table 1

Effect of temperature of homogenization and medium on free and bound GABA.

Homogenization Medium	Temperature of Homogenization	No. of Animals	µg per g. tissue		Percent Bound
			Bound	[Bound + Free] Total	
Ringer-Phosphate	0°	20	131 ± 19	328 ± 33	40 ± 4
0.32 M Sucrose	0°	2	61,67	407,327	15,18
Ringer-Phosphate	23°	11	199 ± 25	346 ± 40	57 ± 4
0.32 M Sucrose	23°	3	65 ± 5	379 ± 60	17 ± 2
Ringer-Phosphate	38°	3	232 ± 39	404 ± 66	57 ± 2

Rat cerebral hemispheres were homogenized in Ringer-phosphate solution at the temperature shown and the suspension was centrifuged at 0°. "Free" means the GABA found in the supernatant, "bound" means the GABA found in the residue corrected for the amount of free GABA present in solution in the fluid remaining in the residue.

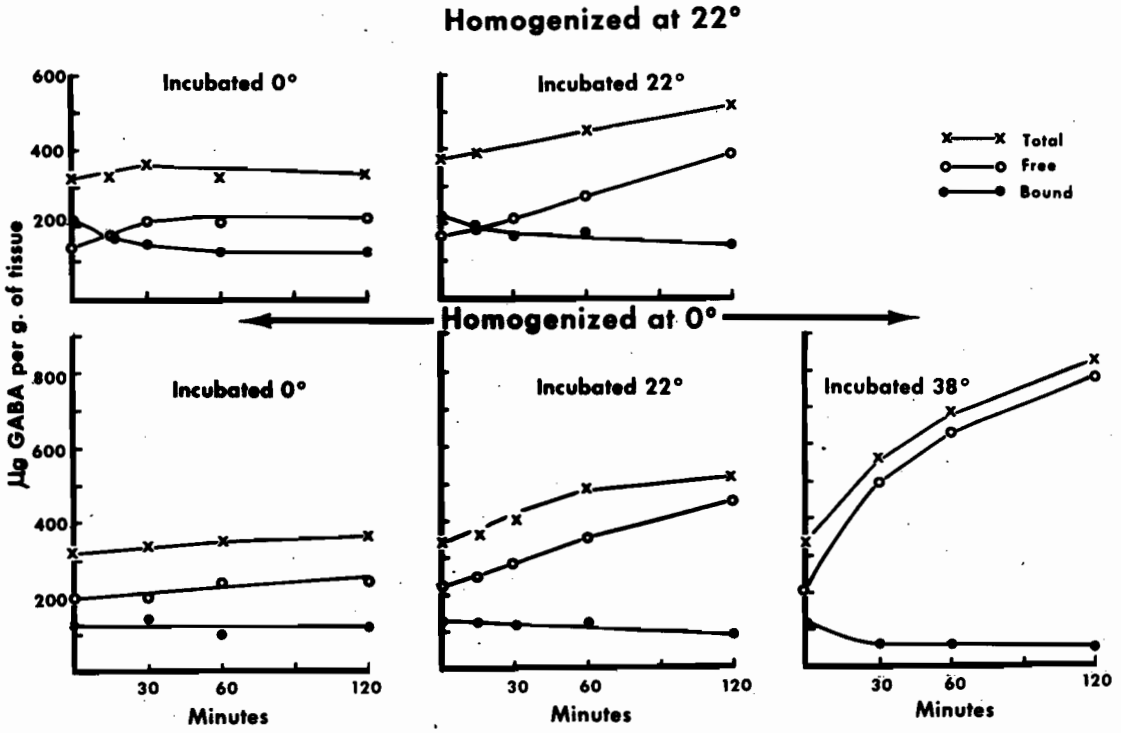

Fig. 2 Effects of temperature on changes in free and bound GABA in rat brain suspensions during incubation.

Cerebral hemispheres were homogenized in Ringer-phosphate solution at room temperature or cold and samples were kept for the times and at the temperatures indicated before being centrifuged. All centrifugations were done at 0°C.

prepared cold. However, once the suspension has been prepared, warming or cooling it does not cause any appreciable immediate change. In suspensions prepared and kept at 0 degrees centigrade the total GABA and the proportions free and bound do not change markedly with time. The extra bound GABA found in suspension prepared at room temperature or at 38 degrees is released with time in the cold. During its standing at 38 degrees, a marked increase in the total GABA occurs. This increase is evident in the free form though it cannot be stated whether it is directly produced free or produced in the bound form and rapidly released.

Effects of Metabolic Conditions

Results illustrated in Fig. 3 show the changes in bound, free, and total GABA in brain suspensions shaken at 38 degrees centigrade. In the presence of oxygen and glucose, the total GABA does not change a great deal although there is a rapid initial release of bound GABA. Under anaerobic conditions (or on standing, Fig. 2) the total GABA increases rapidly, all in the free form. With brain slices in the presence of oxygen, Elliott and Van Gelder (1958) found no change in the total GABA (determined as Factor 1), but they saw that the absence of oxygen there was a decrease rather than an increase in the total GABA and some loss from the slice to the medium. Presumably, loss of glutamate from the slice to the medium under anaerobic conditions prevents its conversion to GABA.

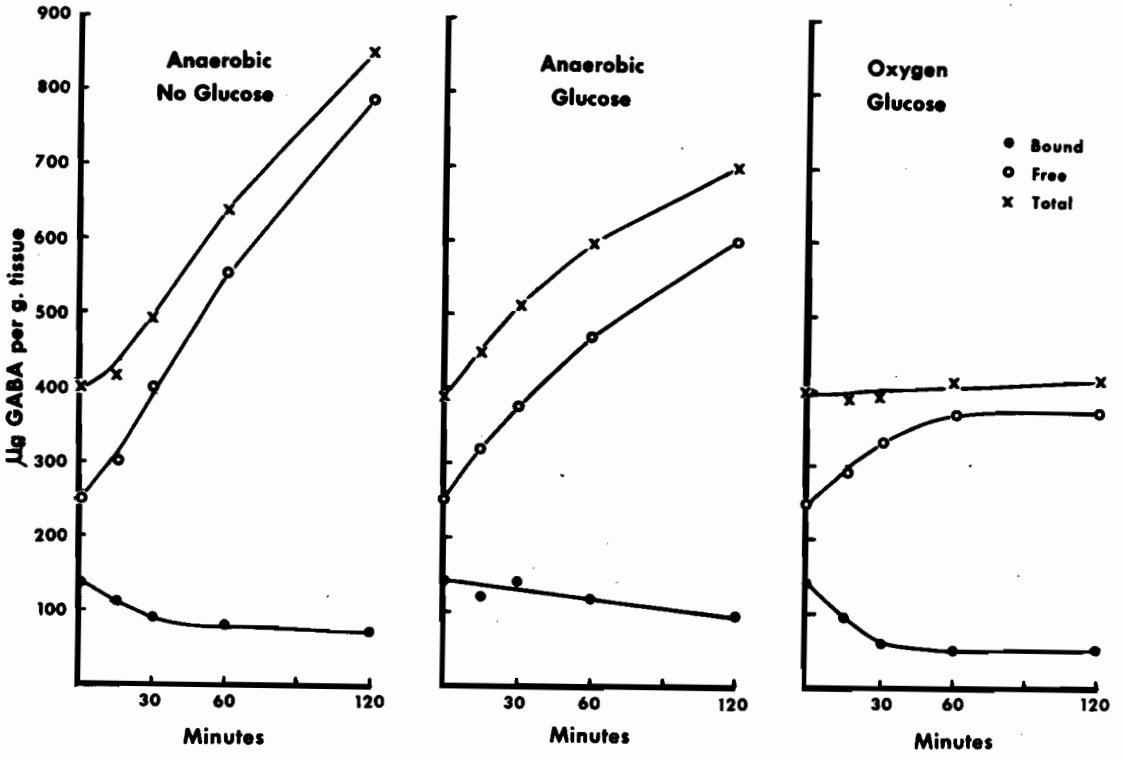

Fig. 3 Effects of metabolic conditions on changes in free and bound GABA in brain suspensions at 38°C.

Cerebral hemispheres were homogenized in cold Ringer-phosphate and samples were shaken at 38°C in the presence of nitrogen or oxygen and glucose.

Effects of pH

Fig. 4 shows that the binding of GABA is most stable at about pH 7. At higher or lower pH values much GABA is rapidly released from the bound form.

Effects of Sodium and Potassium

Fig. 5a shows that, as potassium ions are substituted for sodium ions in the isotonic saline medium in which brain suspensions are prepared, the proportion of GABA left in the bound form is decreased. Elliott and Van Gelder (1960) found that suspension of brain tissue in salt free sucrose solution caused liberation of much of the bound GABA (estimated as Factor 1). Table 1 shows the amounts of GABA found free and bound when the brain is suspended at $0^{o}C$ or at room temperature in saline or in sucrose medium. It is apparent that the amounts of bound GABA, and the proportions of the total GABA that are in this form, are much lower after suspension in 0.32 M sucrose than those found after suspending the tissue in warm or cold saline medium. The temperature of homogenization in sucrose medium makes almost no difference.

Elliott and Van Gelder (1960) reported that the amount of bound GABA found when the brain is suspended in sucrose medium is increased by the presence of some sodium chloride in the medium. As is shown in Fig. 5b, quite low concentrations of sodium chloride are effective. Replacement of the sodium ion by potassium ion decreased the proportion of bound GABA considerably.

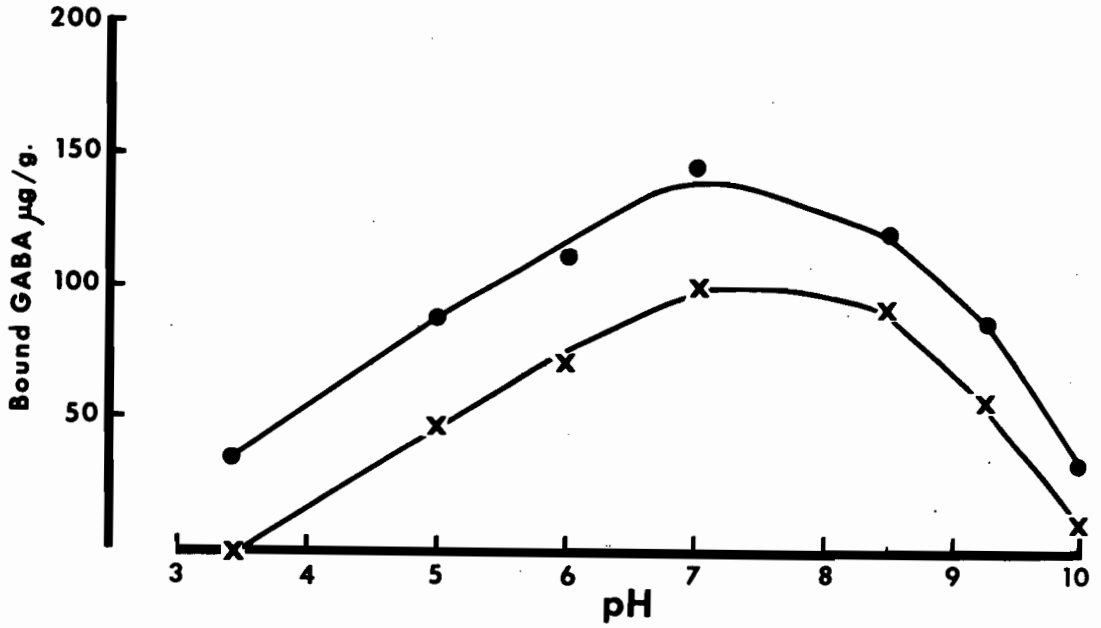

Fig. 4 Effect of pH on the retention of GABA in the bound form in rat brain suspensions. Five brains were homogenized in Ringer phosphate solution at 22°C, the suspension was cooled in ice while portions were adjusted to pH 7 and three acid pH values. Samples were taken immediately and after standing for 30 minutes at 22°C for determination of free and bound GABA. Similar experiments were carried out at pH 7 and alkaline pH values.

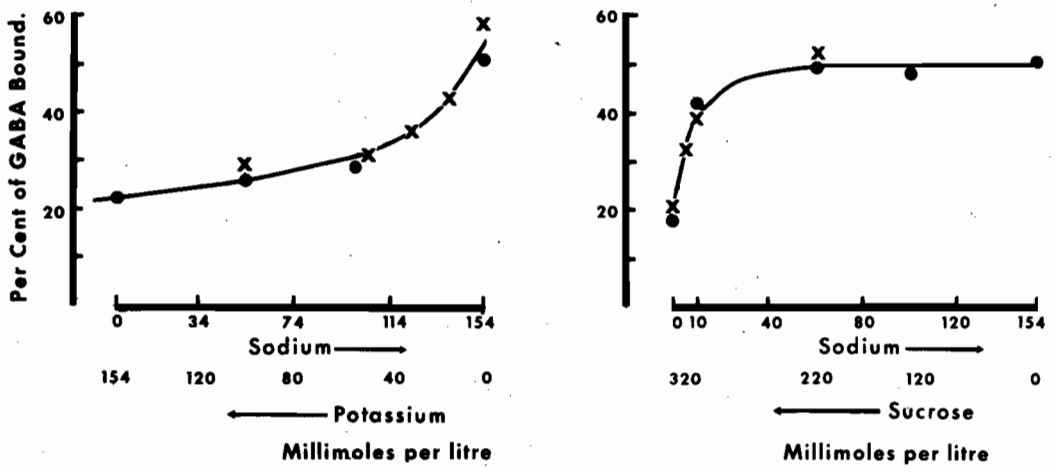

Fig. 5 (a) Fig. 5 (b)

Fig. 5 (a) Effects of sodium and potassium ion concentrations in isotonic saline media on the proportion of GABA in the bound form.

Fig. 5 (b) Effect of sodium ion and sucrose concentrations in isotonic media on the proportion of GABA in the bound form. Rat cerebral hemispheres were homogenized at 24° in isotonic media in which the sodium and potassium concentrations or sodium and sucrose concentrations were reciprocally varied.

For the points marked with dots and crosses the tissue was homogenized in respectively in four times and in nineteen times its volume of saline.

Fig. 5a and 5b also demonstrate the fact that the proportion of GABA which remains in the bound form is independent of the relative volumes of tissue and medium.

Binding of Other Amino Acids

Results summarized in Table 2 show that alanine, glutamic acid, glutamine, aspartic acid and N-acetyl Aspartic acid are also occluded in brain tissue. The amounts of bound glutamic acid and aspartic acid are greater than those of GABA but the proportion of the total, that is, bound is lower. As in the case with GABA, this binding is much less significant in liver and kidney tissue than in brain. The binding of other uncombined amino acids present in liver and kidney tissue is also less marked than in brain.

Table 2 also shows that, like bound GABA, the other bound amino acids are released completely by heat, mostly by homogenization in water, and to a considerable extent by homogenization in 0.32 M sucrose solution.

Table 3 shows that, as is the case with GABA, the total amounts of alanine, glutamic acid, glutamine and aspartic acid found in the brain frozen *in situ* are considerably lower than amounts found if the brain is removed and extracted at room temperature.

Location of Bound Amino Acids

The binding of GABA and other amino acids is not a simple occlusion or artefact within unbroken cells may be concluded from the fact that bound GABA could not be released.

Table 2

Binding of various amino acids by rat tissues

	Alanine	Glutamic acid	Aspartic acid	Acetyl aspartic acid	Glutamine	GABA
Brain homogenate in Ringer-phosphate solution						
Free umoles/g	I3	5.I	I.8	I.3	2.3	I.3
Bound umoler/g	0.52	2.85	0.49	0.52	I.06	2.0
Percent bound	29	36	2I	29	32	6I
Brain homogenized in Ringer-phosphate; suspension then passed through Emanuel-Chaikoff						
Bound umoles/g	0.47	2.2	0.46	--	I.0	I.7
Brain in Ringer-phosphate solution heated I5 mins. at I00° C.						
Percent bound	0	o	0	0	0	0
Brain homogenized in water						
Percent bound	I0	7.5	3.5	9	4	2.4
Brain homogenized in 0.32 M sucrose solution						
Percent bound	I2	I7	9	II	I7	I9
Liver homogenized in Ringer-phosphate solution						
Free umoles/g	3.3	I.83	I.36	0	6.I	0
Bound umoles/g	0.44	0.I2	0.22	0	0.27	0
Percent bound	I2	6	I4	-	4	-

Contd.

Table 2 Contd.

Kidney homogenized in Ringer-phosphate solution

	Alanine	Glutamic acid	Aspartic acid	Acetyl aspartic acid	Glutamine	GABA
Free umoles/g	0.64	8.2	0.89	0	0	0
Bound umoles/g	0.I5	0.32	0.I4	0	0	0
Percent bound	2	4	I4	-	-	-

Each figure is the average of two closely agreeing results and -- means amounts not chemically determined. All homogenizations were done at room temperature. The suspensions were centrifuged at 0^{o} C.

Table 3

Total uncombined amino acid content of brain removed at room temperature or frozen *in situ* before extraction.

	Alanine	Glutamic acid	Glutamine	GABA	Aspartic acid
Brain frozen *in situ*.[1]	0.84±0.1(5)	6.6±0.2(5)	2.5±0.1(5)	1.7±0.1(5)	1.25±0.14 (5)
Brain removed at room temperature.[2]	1.4±0.2(4)	8.7±0.9(4)	3.4±0.3(4)	2.8±0.3(3)	2.3±0.3(4
Percent increase at room temperature	67	32	38	64	83

umoles per g tissue ± standard deviation numbers of determinations in parenthesis.

1. The animal was decapitated and the head dropped immediately into liquid air, the frozen brain was removed, weighed and extracted with 90% ethanol.

2. The animal was decapitated, the brain was removed at room temperature and dispersed in 90% ethanol, within 2-3 minutes of decapitation.

from the brain tissue or by grinding the tissue with sand (Van Gelder and Elliott, 1958). This is also evident from the results of experiments in which saline suspensions were passed through an Emanuel Chaikoff homogenizer which ensured that the suspension contains no particles larger than 27 micro in diameter. As shown in Table 2, the amounts of bound amino acids found in the centrifuged residues were about the same as after simple homogenization.

Bound GABA, as well as other bound amino acids, can be gradually extracted by consecutive washings with saline medium. The brain tissue was suspended and homogenized in a fresh amount of saline after each centrifugation. An aliquot of the supernatant fluid obtained at each centrifugation was mixed with 3 vols. of 90% v/v ethanol (final ethanol concentration 75% v/v) and centrifuged to discard the precipitated proteins. The residue obtained after the centrifugation was also treated with 75% ethanol. A complete separation of the supernatant fluid from the residue obtained after each centrifugation could not be possible. The volume of the solution left with the residue in the bottom of the tube was estimated and a correction was applied to allow for the amount of amino acids left in the supernatant fluid which remained in the residue. Table 4 shows the results of such an experiment.

DISCUSSION

The strange decrease in the proportion of GABA found in the bound form when the tissue is homogenized in the cold is

TABLE 4

Successive extractions of amino acids of brain tissue with potassium free Ringer saline

Successive Extractions		Alanine	Glutamic Acid	Glutamine	GABA	Aspartic Acid
1st	SnF1 ug/ml	15.	159	44	30	31
	ug/g. discarded	60	636	176	119	124
	ug/g. in Residue	69	522	287	181	157
	ug/g. Corr.Bound	54	413	237	151	126
	% Bound	40	94	51	50	44
2nd	SnF2 ug/ml	4	38	20	17	13
	ug/g. discarded	15	152	80	66	54
	ug/g. in Residue	54	428	201	115	103
	ug/g. Corr.Bound	50	382	181	98	90
	% Bound	38	91	40	33	32
3rd	SnF3 ug/ml	3	18	17	10	7
	ug/g. discarded	12	76	68	40	30
	ug/g. in Residue	42	364	133	75	73
	ug/g. Corr.Bound	39	350	116	65	67
	% Bound	30	29	25	22	24
4th	SnF4 ug/ml	2	10	7	5	5
	ug/g. discarded	9	40	28	20	20
	ug/g. in Residue	33	324	105	55	53
	ug/g. Corr.Bound	31	312	98	50	48
	% Bound	24	24	21	17	17
5th	SnF5 ug/ml	2	8	4	5	4
	ug/g. discarded	8	32	16	19	16
	ug/g. in Residue	25	292	89	36	37
	ug/g. Corr.Bound	23	284	85	31	33
	% Bound	18	23	19	10	12
Residue left	ug/g.	27	197	48	20	20
Sum	ug/g.	133	1121	421	289	268
Total found	ug/g.	125	1296	494	312	294

Each figure is the average of two closely agreeing results. For explanation of experimental procedures, see text. SNF stands for free amino acid in the supernatant fluid.

not understood. It is probable that the low proportions of bound GABA and other amino acids found when the tissue is homogenized at 0 degree centigrade in saline medium may represent the proportion *in vivo*. It might be due to an effect of the rate of metabolism of the tissue fragments on the distribution of electrolytes. Fig. 5a shows that the proportion of the bound GABA is sensitive to electrolytes. Also, in the cold there is less metabolism and so more sodium and less potassium ions in cells. Results of Fig. 5b show that potassium releases some of the GABA in the brain. These experiments also suggest that the Na ion is essential for maximum building.

With brain slices in the presence of oxygen and glucose, Elliott and Van Gelder (1958) found no change in the total GABA determined as Factor 1. The rate of removal of GABA must be equal to the rate of formation in the slices. They obtained full recovery even when GABA was added to the medium. This indicates that the reversible transamination reaction between GABA and alpha ketoglutarate, which yields succinic semialdehyde and glutamic acid, is not affected by variations in the concentration of GABA in the medium or absorbed in the tissue. The slices were able to absorb relatively large amounts of GABA from the medium. In the absence of oxygen there was a decrease in the total GABA and some loss from the slice to the medium. Presumably, in the absence of oxidative energy.

production, glutamate diffuses from the slice to the medium and becomes unavailable for conversion to GABA. In brain suspensions, no absorption of GABA from the medium into the tissue particles occurs. The GABA content of a brain suspension remains constant if oxygen and glucose are supplied. In the absence of oxygen, the total amount of GABA increases with time (Fig. 2 and Fig. 3). Evidently, the glutamate present in the tissue suspensions is accessible to the glutamic decarboxylase. It seems that the production of GABA from glutamic acid by the action of glutamic decarboxylase continues in the absence of oxygen; but removal of GABA is checked due to the lack of alpha-keto glutaric acid for the transamination reaction and oxygen for oxidation of succinic semialdehyde which is fed back into the citric acid cycle in the brain suspension. The amount of oxygen which would be required per unit time to oxidize succinic semialdehyde to succinic acid would be equivalent to the amount of GABA that accumulates. This rate of oxygen usage is equal to about 10% of the rate of oxygen uptake of the brain tissue suspension. Thus, 10% would be an estimate of the proportion of the total metabolism that involves the GABA shunt in a brain suspension. Fig. 4 shows that the binding of GABA is most stable at pH 7. At higher or lower pH values much of the bound GABA is rapidly released. This, apparently, suggests that the binding could be enzymatic in nature. However, Elliott (1965) has pointed out in his very

recent review that binding could be of two types - loosely bound and more firmly bound. The effect of pH on the stability of bound GABA provides insufficient evidence to differentiate between the above stated two types of binding. A similar effect of pH on the binding of acetylcholine has been reported (Brodkin and Elliott, 1953). It is probable that virtually all the physiologically active GABA found in the brain is present in more firmly bound form. Acid or alkali, or various other agents, can release it. The physiological role of GABA must, therefore, be closely connected with the relationship between free, loosely bound, and more firmly bound forms.

Table 3 shows that the total amounts of alanine, glutamic acid, glutamine, aspartic acid, and GABA found in brain tissue; frozen in situ; are considerably lower than the amounts found if the brain is removed and extracted at room temperature. Presently, there is insufficient evidence to put forward any detailed hypothesis concerning the mechanism responsible for this rapid increase in the amount of amino acid in the brain. It is known, of course, that the brain contains proteolytic enzymes. Thus, Kies and Schwimmer (1942) have described an active cathepsin; and Ansell and Richter (1954), a proteinase active at pH 7. Disruption of the tissue structure might result in rapid activity by proteolytic enzymes; but this could not account for the sudden increase in GABA, which is not present in proteins, unless the glutamate produced by proteolysis is rapidly available to glutamic decarboxylase. Although it

would seem extremely probable that this general increase in total amino acids in the brain when excised and homogenized at room temperature may really be due to an acceleration of the proteolysis that may be normally concerned with the turnover of nerve cells and myelin protein in vivo.

Present results and those of Elliott and Van Gelder (1960) show that much of the bound GABA is released in salt-free sucrose solution. Thus, there is probably more than one form of bound GABA in the brain. One form, which remains bound in suspensions prepared in sucrose solution, (Table 1), is evidently occluded in subcellular particles, most likely in certain nerve ending particles. Bilodeau (1962) has found that 20-30% of the total GABA and glutamic acid remained in crude mitochondrial fraction in sucrose homogenates of rat brains. The other form is released in the absence of sodium or by the presence of high potassium. Other amino acids also seem to be bound in these two different ways.

Chapter 4

RELEASE OF GABA AND OTHER AMINO ACIDS FROM THE CAT CEREBRAL CORTEX

Introduction

Florey (1954) demonstrated that extracts from the central nervous system show inhibitory properties on the discharges from the slow adapting neurons in the crayfish receptor organ. The inhibitory agent was called Factor 1. Bioassays on the crayfish stretch receptor preparation showed that GABA behaves as Factor 1 and could account for much of the activity in the brain (Bazemore, Elliott, and Florey, (1956, 1957). More recently it was proved that this substance could account for all the Factor 1 activity in the brain (Levin, Lovell, and Elliott, 1961; Lovell and Elliott, 1963).

Several criticisms must be refuted before we can have full confidence in classifying a substance as a synaptic transmitter. The substance must exist in sufficient amounts in the pre-synaptic terminals, the tissue must contain a synthesizing enzyme system, stimulation of the presynaptic nerves must release the substance in adequate quantities from the pre-synaptic terminals. The action of a substance on the post-synaptic cell must be identical to that of the synaptic action. There should be an inactivating enzyme system in the region of the synaptic cleft. When the actions

of drugs are tested by micro-electrophoretic injection, the pharmacology of the synaptic transmission and of the post-synaptic action must be similar.

There are extensive studies on the production, storage, and neurophysiological and neuropharmacological roles of GABA. Eccles, Schmidt, and Willis (1963) are of the opinion that GABA may be involved in the action of presynaptic inhibitory potentials. At present, a transmitter role of GABA has been proved in crustacean stretch receptor neurons (Dudel and Kuffler, 1960; Kravitz, Potter, and Van Gelder, 1962). Curtis and Watkins (1963) have suggested a possible role of glutamate and aspartate as excitatory transmitter substances. To obtain further evidence for the transmitter role of amino acids, quantitative release of gamma-aminobutyric acid, glutamic acid, aspartic acid, and glutamine have been measured.

Methods

These operations were carried out under the expert directorship of Dr. H.H. Jasper, Professor of Experimental Neurology at the Neurophysiological Labratory of the Montreal Neurological Institute. A collaborator was Dr. P.G. Sie.

DESCRIPTION OF THE TECHNIQUE OF PREPARATION OF ANIMALS FOR EXPERIMENTS ON CORTICAL PERFUSATE FOR GABA AND OTHER AMINO ACIDS

There were actually 4 types of preparations used:

(1) The intact animal under flaxedil and artificial respiration:

These animals were prepared with ether anaesthesia for the operative procedures, i.e., placement of the animal in the stereotaxic instrument head holder, and careful injection of local anaesthesia (1% nupercaine), preparation of the scalp and all contact points. Additional precautions against pain were taken by injection of the Gasserian ganglion with nupercaine repeatedly throughout the experiment. Injection of the occipital nerves was also done; and, in addition, the injection of the external auditory meatus was carried out to prevent pain from the ear plugs. The animal was then permitted to recover from anaesthesia and kept on a curarizing dose of flaxedil to prevent movement. The dosage was kept very light in order that slight movements might occasionally occur. The perfusion chambers were made of plastic and sealed into the skull after removal of the dura, beneath the point of insertion. The cross-section of the chambers was approximately 1 cm^2. They rested gently on the surface of the cortex; and the junction with the pia was sealed with a small amount of

agar jelly. The temperature of the animal was maintained at 35-37 degrees centigrade. Care was taken that the solution in the chamber was also heated to this temperature. The area of the cortex used was usually the middle suprasylvian gyrus. Sometimes, the chamber was placed forward on the post central or post cruciate gyrus. The chambers were often placed bilaterally over homologous areas of the two sides and perfusion was carried on simultaneously from the two hemispheres.

(2) The "encephale isolé preparation":

This is the classical brainstem section of Bremer (1936) with the complete section of the cervical cord at the junction with the medulla at C_1. It is performed under ether anaesthesia. Artificial respiration was carried out as described above, but flaxedil was not utilized, as it was unnecessary for this preparation. Other details were similar, with careful local anaesthesia of the scalp and injection of the Gasserian ganglion as well.

(3) The "cerveau isolé preparation":

This is the classical brainstem section of Bremer at the level of the superior colliculus. In experiments conducted here, there were some variations in the section; at times, variation was in existence between the superior and inferior colliculi, which would be a midbrain section somewhat below the standard level of Bremer's cerveau isolé.

In this preparation, due to sectioning of the pain pathways of the head, injection of pain points was not necessary. Control of this section was obtained by recording the surface cortical electrical activity. When complete, the record was composed largely of spindles.

(4)"Hemisection of upper midbrain":

This is a form of hemi-cerveau isolé preparation in which a section is made at the level of the superior colliculus of only one half of the brainstem. This section was made by a blunt disector and was not always complete. It was sometimes slightly extended to the opposite side, so that effects were occasionally seen on both sides. However, a control showed that arousal stimuli such as pinching the tail, blowing puffs of air into the nose, or olfactory stimulation would produce arousal or blocking of the spindle waves on the intact side only. Therefore, in a sense, one half of the brain was asleep and the other side awake; at least when maintained in a waking state by arousing stimuli.

"General Comments on Technique":

The surface electrocorticogram was taken continuously by silver ball electrodes applied inside of the chamber gently resting on the surface of the cortex. These electrodes were about 0.5 mm in diameter and were insulated, except for the very under surface of the ball. This caused short-circuiting

by the fluids to be unimportant in the recording. Until records were taken with Elliott solution (Elliott and Jasper, 1949) in the chamber, and finally, when a sleep tracing or spindle tracing was desired, the animal was left quiet, unstimulated. When it was desired to have a waking tracing, the corticogram was monitored continuously. On the appearance of spindles, a brief puff of air was delivered to the face. This seemed to be adequate for causing arousal in the animal with the intact midbrain. The olfactory route was also extremely useful for this purpose since the olfactory arousal pathways were not sectioned in the encephale isolé and intact preparations.

Sampling and Quantitative Determination of Amino Acids

Measured amounts of 1 ml Elliott solution were placed in the perfusion chambers and were withdrawn after 15 minutes. Fresh solution then replaced this, so that it was an intermittent sampling technique with the solution being changed every 15 minutes. In the preparation for GABA determination, the piarachnoid was punctured only in points relatively free of vessels, in 6-10 places under dissecting microscope control.

In case of slight bleeding, the surface was liberally washed. One waited for coagulation of the bleeding point to occur before proceeding to the perfusion experiments. Unless this penetration or puncture of the pia was done,

little GABA appeared in the fluid. All samples obtained were under artificial respiration. The cerebral cortex was washed with Elliott solution and this solution was left in contact with the cortex for 5-10 minutes. In the cases of encaphale isolé and cerveau isolé preparations, a continuous superfusion technique was used. The rate of flow of Elliott solution into and out of the chamber was less than a drop per minute. In the case of neuraxially intact animals, superfusate was collected from the chamber every 5-10 minutes by careful suction with a polyethylene tube. When the GABA release was small, as in the case of encaphale isolé and neuraxially intact preparations, samples from both sides of the same preparation were pooled. Samples obtained under the various conditions were stored in the deep freeze and later lyophilized overnight. To the lyophilized samples containing inorganic salts, 3 ml of acetone containing 5% 6 N HCl per g. of dry residue was added. The extraction was repeated 3 times and the acetone was drawn off in a flash evaporator. The residue was taken up in 1.0 ml of distilled water and was put on ion exchange cation columns. The H ion form of Dowex 50 was suspended in distilled water and, after stirring, the smallest particles which did not settle rapidly were poured off. The resin was suspended in 4 N HCl so that an equal volume of acid remained above the resin surface. The temperature was

TABLE 5

RELEASE OF GABA FROM THE SURFACE OF THE CEREBRAL CORTEX

Preparation	ECoG Pattern	No. of Samples	GABA Released $\mu g/hr./cm^2$ Average ± S.D.
Neuraxially intact	"Aroused"	4	0.60 ± 0.20
Cervical section "Encephale isole"	"Aroused"	6	0.66 ± 0.26
Mid-brain section "Cerveau isole"	"Sleep"	17	2.09 ± 0.60
Left mid-collicular hemisection			
Right hemisphere	"Aroused"	2	0.80, 1.01
Left hemisphere	"Sleep"	2	2.12, 2.60

These experiments were carried out in collaboration with Dr. H.H.Jasper, Dr. K.A.C.Elliott, Dr. P.G.Bie and Dr. L.S.Wolfe of Montreal Neurological Institute. For explanation of the experimental details see text.

raised to 100 degrees centigrade. After occasional stirring for about 1 hour, the supernatant, which was yellow, was decanted and the process was repeated several times until the supernatant was colourless. The resin was well washed with distilled water. A column (15 cm x 0.5 cm) of resin was prepared. A 1.0 ml sample of the unknown sample was passed through a column and washings with 10-15 ml of distilled water were also passed through. The amino acids were displaced with 2 N NH_3. The eluate was collected and evaporated down to dryness; and the residue was used for quantitative chromatographic analysis for glutamine, glutamic acid, aspartic acid and GABA. In some experiments an enzymatic method for GABA was also used (General Methods, Chapter 2).

Results

Results summarized in Table 5 and Table 6 show no significant differences in the amounts of GABA, glutamine, glutamic acid, and aspartic acid released into the perfusion fluid, between the encephale isolé and the neuraxially intact preparations. Also, no significant differences were observed in the amounts of the various amino acids released from the two sides of the cat cerebral cortex of the same animal preparation, except in the case of midbrain hemisection preparations. In all cases, the chromatograms developed showed that there are other acidic and basic amino acids which are also released in all the above-mentioned neurophysiological

TABLE 6

RELEASE OF AMINO ACIDS FROM THE SURFACE OF THE CEREBRAL CORTEX

Preparation	ECoG Pattern	No. of Samples	Amino Acid Released µg/hr./cm²			
			Glutamic	GABA	Glutamine	Aspartic
Neuraxially intact	"Aroused"	8	9.2 ±0.8	0.7 ±0.2	1.0 ±0.6	2.1 ±1.0
Cervical section	"Aroused"	6	8.6 ±0.2	0.7 ±0.25	1.1 ±0.15	1.8 ±0.4
Mid brain section	"Sleep"	13	5.7 ±1.0	2.0 ± 0.7	1.1 ±0.3	1.7 ± 0.4
Left mid-collicular hemisection						
Right hemisphere	"Aroused"	2	7.2, 7.0	1.1, 0.8	2.0, 2.0	2.5, 2.0
Left hemisphere	"Sleep"	2	5.0, 4.5	2.6, -	2.2, 2.2	2.5, 2.0

These experiments were carried out in collaboration with Dr. H.H.Jasper, Dr. K.A.C.Elliott, Dr.P.G.Sie and Dr. L.S.Wolfe of Montreal Neurological Institute.

For explanation of the experimental details see text.

animal preparations.

To get a more detailed picture, extensive studies would be required for the separation and quantitative measurements of each amino acid under different physiological conditions. The separation of aspartic acid from glycine and alanine required special care and was not entirely satisfactory. Double controls and standards were run. From our results it was apparent that no change occured in the amounts of aspartic acid or glutamine released under the various experimental conditions. Tables 5 and 6 demonstrate that the amount of GABA released from cerveau isolé preparations was three times as rapid as that released from the cerebral cortex of the intact cat. No distinction was made between the gradation of spindling, i.e., good or poor, as this did not significantly affect the release of various amino acids. However, the electrical activity of the cat cerebral cortex was the only criterion for differentiating between the encaphale isolé and the cerveau isolé, or the neuraxially intact and cerveau isolé preparations. In some experiments, when a continuous waking state was maintained by periodic stimulation of the brain stem reticular formation, no measurable GABA was collected in the perfusate. Glutamic acid release was decreased under those conditions under which GABA release was increased.

Discussion

Synaptic transmission consists in the induction of permeability change and consequent ion movements across the post-synaptic cell membrane. The total process consists of the movement of the substances across the post-synaptic membrane. Depending on the area and structure of the pre and post-synaptic membrane, the concentration change of the ions in the synaptic area is brought about by the arriving nerve impulses. It may be sufficient to cause ion movements across the post-synaptic membrane. These, in turn, may be enough to alter the excitability of the post-synaptic cell. Intracellular recordings by Aljure, Gainer, and Grundfest (1962) have confirmed that GABA does not activate inhibitory post-synaptic membrane in the adductor muscle of the dactylus of walking legs of cancer borealis. No effect of GABA on the non-synaptic membrane of the muscle fibers was observed. It did not alter the rates of movement of K^+ and Cl^- as determined from the responses of the membrane potential to changes in outside concentration of the respective ions.

The states of sleep and waking may be due to balanced excitatory and inhibitory actions of the reticular activating system. It could be infered from the results of Table 5 and Table 6 that GABA or a derivative may be involved in the control of apparent inhibitory effects of the sub-cortical origin and thereby perhaps governs the sleep mechanisms.

It also seems likely, therefore, that GABA must have a dual function. This may be involved in activating the inhibitory synaptic membrane and in blocking excitatory post-synaptic potentials. The blocking of excitatory post-synaptic potentials may be caused by interference with the excitatory transmitter or with its release. Purpara and Grundfest (1956) concluded that GABA inactivates excitatory axodendritic synapses in the mammalian brain. Kuffer and Edwards (1958) have demonstrated that the effects are very similar when the inhibitory nerve is stimulated or when GABA is applied to the isolated crustacean stretch receptor. Stimulation of the inhibitory nerve apparently controls the excitability of the stretch receptor neurons. Their studies furnish further support to the theory of the dual role of GABA. Eccles, Schmidt, and Willis (1963) believe that GABA may be involved in the action of certain pre-synaptic inhibitory inter-neurons.

The present results indicate that GABA is released under physiological conditions in the mammalian brain. The two hypotheses of the involvement of GABA in intermediary metabolism and in central inhibition are not necessarily mutually exclusive. A chemical substance may be an intermediary metabolite involved in the energy metabolism and simultaneously be a modulator of functional activity of the central nervous system. The present studies

concerning these roles of GABA in the central nervous system are not sufficiently clear to decide whether one or the other, or both roles, are exercised. The early suggestion that GABA might be a specific transmitter substance at inhibitory synapses could be further supported by the present results. However, it is logical to say that the results summarized in Tables 5 and 6 provide no direct evidence on this. If there is a continuous gradation between neurosecretion and quickly acting and quickly disappearing transmitter, it is necessary that there be a post-synaptic inactivating enzyme for the transmitter, as in the case of acetylcholine. There is, however, no rapidly acting inactivating enzyme present. The only enzyme known to be capable of destroying GABA is GABA-glutamate transaminase. The absorption of GABA from the surrounding medium by slices of brain could perhaps represent a mechanism for the removal of active GABA (Elliott, 1965).

Interest in GABA in crustacea stems from physiological experiments demonstrating that externally applied GABA mimics the effect of the naturally released inhibitory transmitter compound. This has been demonstrated at a synapse of the central nervous system; at peripheral neuromuscular junction (Boistel and Fatt, 1958; Grundfest, Reuben, and Rickles, 1959); and at stretch receptor cells in the crustaceans (Kuffler and Edwards, 1958). In addition, GABA

has a pre-synaptic action causing a reduction in the amount of transmitter released from excitatory nerve terminals at crayfish neuromuscular junctions (Dudel and Kuffler, 1961). Nevertheless, it is doubtful whether it is valid to consider GABA as an antagonist to acetylcholine. The effects of drugs and other pharmacological agents on the proportions of free and bound and the rapid absorption of GABA by brain slices against the concentration gredient suggest that GABA may be physiologically active only in a bound form. Elliott (1965) suggests that the "more firmly bound" GABA in nerve ending particles represents a storage state, and that "less firmly bound" material represents GABA absorbed on receptor sites. Free GABA represents the substance in transition between these sites.

Curtis and Watkins (1963) showed that glutamic acid and aspartic acid may behave as excitatory substances. This suggestion was further supported by the evidence of Krnjevic and Phillis (1963) who believe that these amino acids are excitatory transmitter substances. In our experiments, glutamic acid, aspartic acid, gamma-aminobutyric acid, and glutamine were found to leak from the punctured cortical surfaces. The rate of release of GABA was increased during the E.Co.G.* "sleep" pattern. The rate of glutamic acid was increased about 50% when the E.Co.G. pattern indicated "arousal". This suggests that an excitatory effect of glutamic acid is involved in the production of fast wave

*E.Co.G. signifies Electrocorticogram.

electrocortical activity. There were no obvious changes in the amounts of aspartic acid and glutamine that came out of the cerebral cortex under the different experimental conditions.

Inhibition has been used in historical perspective to describe a variety of neurophysiological processes which retard, decrease, or arrest the activity of neurons and which raise the threshold or block synaptic or neuromuscular transmission of normally adequate excitatory processes. A significant difference exists between the amounts of GABA and glutamic acid released per hour in the cerveau isolé and encaphale isolé preparations. The higher rate of GABA output in the sleeping state seems well correlated with the slow wave spindle activity of E.Co.G. The sleep pattern may be the result of inhibition produced by GABA.

To further increase our understanding concerning GABA and other amino acids, the precise study of the modes of action of neurons in the cortex and brain stem and of the inter-relationships between their activities is vitally important. One can scarcely doubt the metabolic basis of nervous exhaustion that progresses over a period of many hours and which requires, for its elimination, the recovery process of sleep. Nothing, however, could be said for certain pertaining to these metabolic changes. What is the nature of this progressive build up of metabolic exhaustion?

Physiologically speaking, one is aware of the necessity of ion pumps for the maintenance of the ionic compsition of the interior of the nerve cells; and of the necessity for the metabolic replenishment of synaptic transmitters. However, we do not know, why over many hours, these processes should deplete or fail to maintain the integrity and functional activity of the nerve cells and synapses.

Chapter 5

RELEASE OF ACETYLCHOLINE DURING SLEEP AND WAKEFULNESS

Introduction

The central nervous system shows continuous nervous activity, and assuming the role of acetylcholine in synaptic transmission, its continuous release in the absence of external stimuli is to be expected. Therefore, the presence and distribution of acetylcholine and of the cholinesterase enzyme in the central nervous tissue, the ability of such tissue to synthesize acetylcholine and its release under certain conditions, all provide strong evidence in favour of acetylcholine's being a chemical transmitter across a number of synapses in the central nervous system. It has, however, been difficult to obtain direct evidence in support of this view (Feldberg, 1945, 1950; Crossland, 1960; Mitchell, 1963).

Despite existing technical difficulties in experiments on the intact brain, reasonable attempts have been made by several workers to correlate the release of acetylcholine with nervous activity. However, conclusions from studies on the release of acetylcholine in the central nervous system seem to conflict. Feldberg (1945) claims that the release of acetylcholine from the cerebral cortex is independent of electro-cortico activity of the central nervous system. On the other hand, Elliott, Swank, and

Henderson (1950), who studied the amounts of acetylcholine left in the brain tissue and the release of acetylcholine into a plastic cup on the cortical surface, are of the belief that the release of acetylcholine is related to the spontaneous electrical activity of the cortex. MacIntosh and Oborin (1953), also using plastic cups on the cortical surface of cats, have shown more definitely the release of acetylcholine under different experimental physiological conditions. It has been shown recently by means of microelectrodes, that temporary alterations in the resting membrane potential are associated with the excitation and inhibition of neurons in the central nervous system (Curtis, 1961). Single cells in the cerebral cortex have been selectively activated by the iontophoretic application of acetylcholine through micropipettes (Krnjevic and Phillis, 1961).

The release of acetylcholine may be a result of the activity of cholinergic synapses or may be a by-product of cellular metabolism. If we consider the two possibilities, we must expect to find cortical neurons which are clearly either excited or inhibited by acetylcholine. As far as the second possibility is concerned, acetylcholine released by metabolic processes may or may not affect the excitability state of the surrounding neurons.

In view of the output of acetylcholine, it is clearly advantageous to find out whether acetylcholine does alter

neuronal activity. A technique similar to that of Elliott, Swank, and Henderson(1950); MacIntosh and Oborin (1953) has been used in the present experiments to study acetylcholine release from the cerebral cortex of neuraxially intact cats under flaxedil and local anaesthesia; in encephale isole and cerveau isole preparations; and after hemisection of the upper midbrain.

Methods

Cats weighing 2-5 kg were anaesthetized with ether. The trachea was cannulated and a catheter inserted in the femoral vein. The head was fixed in a stearotaxic frame. In experiments with neuraxially intact animals, the animals were paralysed slowly with flaxedil, about 1 ml of 1 mg in 10cc of distilled water. Encephale isole preparation section was done at the C_1-C_2 level. Severe shock was checked by giving intravenous injection of 0.25 cc pitressin and 0.25 cc epinephrine before the section was made. Cerveau isole preparations were made by dissection of the mesencephalon at the intercollicular level. The upper midbrain hemisection preparation was made by section, at the level of the superior colliculus, of only one half of the brain stem. This section was made by a blunt disector. The detailed procedures are given in Chapter 4.

The monopolar electro-corticogram was carefully recorded throughout the experiment with these preparations.

Recording electrodes were placed on the cortex under study as well as on other areas. Burr holes, 12 mm in diameter, were made on both sides of the midline suture. The dura was carefully opened and the posterior part of the lateral, suprasylvian, and electrosylvian gyri were exposed. Cylindrical plastic chambers having volume 2.5 cc, 10.5 mm internal diameter were fitted into the threaded burr holes. The chambers were sealed to the surrounding bone with dental cement. Leakage from the chambers over the cerebral cortex of the brain was checked with gel-foam. Collection of samples was commenced two hours after the animal was anaesthetized. The cerebral cortex was washed with saline solution (NaCl 9.0, KCl 0.42, $CaCl_2$ 0.24, $NaHCO_3$ 0.2, glucose 2.0 g/l). Body temperature of the cats was maintained with an electric blanket at 37 ± 2 degrees centigrade.

Collection, Assay of Acetylcholine

The plastic cups were filled with Ringer solution containing Neostigmine (100 mg/liter) and left twice for 15 minutes before starting the collection of samples. The washings were removed with a polyethylene tube and discarded. The chambers were filled with 2-5 ml of Ringer solution containing fresh Neostigmine, which was left in contact with the cortex for 15 minutes. Then the cortex was removed into graduated centrifuged tubes and stored in the deep freeze till bioassays were done.

Preparation of standard acetylcholine solutions

I. Stock solution A

100 mg of AchCl dissolved in 100 ml distilled water gives concentration 1000 ug/cc.

II. Stock solution B

1 cc of 1000 ug/cc diluted to 100 cc gives concentration 10 ug/cc.

III. Stock solution C

5 cc of 10 ug/cc diluted to 50 cc gives concentration 1 ug/cc.

Routine standard solutions

Stock solution C	ml added	ul	diluted with saline to	ng, nanagram= millimicrogram
1.	0.05	50	4.0 ml	12.5
2.	0.10	100	4.0 ml	25.0
3.	0.20	200	4.0 ml	50.0
4.	0.30	300	4.0 ml	75.0
5.	0.40	400	4.0 ml	100.0
6.	0.50	500	4.0 ml	125.0
7.	0.60	600	4.0 ml	150.0

Bioassays were done as soon as possible: between 12-16 hours after the collection of samples. The frog rectus abdominis muscle was prepared as described by MacIntosh and Perry (1950). The muscle prepared was sensitized for one hour in a 5 ml muscle bath containing Ringer Locke without Neostigmine. The oxygen supply was maintained steady and the solution changed every 15 minutes. The same muscle was again left, for 1 hour, in contact with Ringer Locke containing Neostigmine. Sensitivity of the muscle was checked by adding freshly prepared 4.0 ml standard solution of acetylcholine of varing concentrations: 12.5 ng-50.0 ng. The samples and standards for assay were made up in the same medium. The contraction during the 3 minutes was recorded in ink on a kymograph. Assays were alternately run on each of the two muscle preparations at nearly regular intervals. On each muscle, standards were run initially and after every unknown. The quantity of unknown used was, as far as possible, such as would give a contraction of the same magnitude as the standards. The sensitivity was not always the same for different rates of contraction. Therefore, standards with different concentrations of acetylcholine were run at intervals during the series. Each unknown was assayed

2-4 times. The unknown sample was estimated from the curve of the standards with different concentrations of acetylcholine ; and, from this, the amount of acetylcholine in the sample used was calculated (Crossland, Elliott, and Pappius, 1955). After each bioassay, the muscle was rinsed twice with Ringer Locke without Neostigmine. Six minutes were given to relax the muscle before starting a new unknown sample or standard. All results were expressed in ng per minute (ng, i.e. nanagram=millimicrogram or 10^{-9} g).

In a few experiments where the acetylcholine output was low, samples from similar preparations were pooled and bioassayed. Some samples were treated with 0.1 N NaOH to about pH 13, and allowed to stand at room temperature for 30 minutes. They were then neutralized with 1 N HCl. These samples, when tested on the frog rectus abdominis muscle preparation, were inactive.

Results

As is shown by the results summarized in Table 7 and Table 8, the release of acetylcholine from the encephale isole preparations (Table 7), is considerably higher than that from the cerveau isole preparations (Table 8). The results of these tables verify that

TABLE 7

The release of acetylcholine in encephale isole preparation

Cat No.	Sample No.	Acetylcholine release ng/min. (15 minute samples)
SK-18	I	1.20
SK-21	I	0.78
	II	0.72
	III	0.65
	IV	0.64
	V	0.54
SK-22	I	1.20
	II	0.97
	III	1.30
SK-24	I	0.75
	II	0.70
	III	0.87
	IV	0.65
	V	0.70
	Mean±S.D.	0.84±0.30 (14)

For explanation of experimental procedure, see text. These experiments were carried out in collaboration with Dr. H.H. Jasper, Dr. K.A.C. Elliott, Dr. P.G. Sie and Dr. L.S. Wolfe of Montreal Neurological Institute.

TABLE 8

Acetylcholine output in cerveau isole preparation and values obtained from successive samples.

Cat No.	Sample No.	Ach release ng/min. (15 minute samples)
SK- 31	I	0.21
	II	0.22
SK-32	I	0.51
	II	0.28
	Mean ± S.D.	0.30±0.14 (4)

For explanation of the experimental procedure, see text. These experiments were carried out in collaboration with Dr. H.H. Jasper, Dr. K.A.C. Elliott, Dr. P.G. Sie and Dr. L.S. Wolfe of Montreal Neurological Institute.

TABLE 9

Liberation of acetylcholine during collective periods in encephale isole preparations

		Collection periods			
		10 min.	15 min.	30 min.	
Cat SK (A)	Total Ach ng	7.10	12.60	26.50	ng
(2.8 kg)	ng/min.	0.71	0.84	0.88	ng
Cat SK (B)	Total Ach ng	-	18.00	31.50	ng
(3.0 kg)	ng/min.	-	1.20	1.05	ng
Cat SK (C)	Total Ach ng	8.00	16.00	33.00	ng
(2.9 kg)	ng/min.	0.80	1.07	1.10	ng

For explanation of the experimental procedure, see text.

These experiments were carried out in collaboration with Dr. H.H. Jasper, Dr. K.A.C. Elliott, Dr. P.G. Sie and Dr. L.S. Wolfe of Montreal Neurological Institute.

TABLE 10

Acetylcholine release from the cortex of cats with left hemisection, followed by complete section at the intercollicular midbrain level

Cat No.	Sample No.	Ach release ng/min. (15 minute samples) Neuraxially intact Left	Neuraxially intact Right	Hemisection Left	Hemisection Right	Cerveau isole Left	Cerveau isole Right
SK-41	I	1.50	1.44	0.56	0.83	0.54	0.44
	II	1.46	1.51	0.58	0.83	0.52	0.54
	III	1.50	1.42	0.52	0.83	0.52	0.33
Mean±S.D.		1.48± 0.03	1.46± 0.05	0.55± 0.03	0.83± 0.0	0.53± 0.01	0.44± 0.11

For explanation of experimental procedure, see text.

These experiments were carried out in collaboration with Dr. H.H. Jasper, Dr. K.A.C. Elliott, Dr. P.G. Sie and Dr. L.S. Wolfe of Montreal Neurological Institute.

the variation in acetylcholine output from any preparation between successive 15 minute collection periods is small. Larger variation in acetylcholine release occured from animal to animal and under different neurophysiological preparations. The time course of the accumulation of acetylcholine in the unchanged fluid in the cup was also followed in encephale isole preparations (Table 9). The anaesthesia was maintained at a constant depth for such preparations; and the fluid was left in contact with the cortex for periods of 10, 15, and 30 minutes without being changed in the cup. Table 9 gives the result of 3 such experiments. It demonstrates that during these periods the acetylcholine liberated in the cups increased in an approximately linear manner. Until 30 minutes, no equilibrium was approached between the acetylcholine in the cup and that in the cerebral cortex. The linear release of acetylcholine with time may be simple diffusion. The above experiments also suggest that the acetylcholine recovered in the cup solution was released from the cortical neurons immediately beneath it.

Discussion

From various studies, it has been learned that in the peripheral nervous system, neurons of the same functional

type are neurochemically alike and produce the same transmitter substance. All motor neurons, all pre-ganglionic autonomic neurons, and the post-ganglionic parasympathetic neurons produce acetylcholine. There is sufficient evidence that many central nerve cells do the same. All neurons which contain and release acetylcholine are referred to as cholinergic.

Results of Table 9 suggest that the acetylcholine is liberated from the cortical neurons underneath the cup. This suggestion, however, could be criticized because it is impossible to avoid tissue damage caused by operation. Liberation of acetylcholine has also been suggested by Elliott, Swank, and Henderson (1950); and by MacIntosh and Oborin, (1953), who found that acetylcholine release was abolished from the cortex slabs which had been undercut but had an intact blood supply. They also found that the output of acetylcholine from the surface of the cortex depends on the depth of anaesthesia, and is approximately proportional to the spontaneous electrical activity of the cortex. These findings have been confirmed by present experiments.

Tables 7 and 9 show a steadier increase in the acetylcholine output in the case of the encephale isole and neuraxially intact animals than that in the case of the cerveau isole preparation (Table 8).

The results of the electrocorticogram in our experiments were the bases on which the validity of this increased electrocortical activity was established. From our experiments it is impossible to supply direct evidence as to the postulated role of acetylcholine as the central chemical transmitter substance. However, our results suggest that the states of sleep and waking may be due to the balanced excitatory and inhibitory synaptic actions of the reticular activating system exerted through neurons which release acetylcholine, glutamate, and GABA in the cortex.

Observation of the release of acetylcholine by nerve impulses is one of the principal experimental bases for the hypothesis of cholinergic transmission in the peripheral nervous system. More recently, there has been investigation leading to quantitative measurement of the output of acetylcholine by a single impulse at the motor synapse. The release of acetylcholine from the caudate nucleus in response to stimulation has been studied by Mitchell and Szerb (1962) in experiments on anaesthetized cats. These authours suggest that there are a large number of cholinergic nerve endings in the caudate nucleus which can be activated either directly or by restricted cortical stimulation. Very recently, Mitchell (1963) has shown the spontaneous and evoked release of acetylcholine

from the cerebral cortex. The rate of release was 0.1-5.5 ng Ach/min./cm^2, and was roughly proportional to the electrical activity of the brain cortex. Direct electrical stimulation of the cortex or excitation by transcallosal or peripheral stimulation increased the rate of Ach release from the primary somato-sensory cortex. This increase in the rate of acetylcholine release depended on the frequency of stimulation. This definitely suggests that fewer cholinergic nerve endings were activated by a sensory nerve type stimulation.

It is also possible to conclude from the previous work of Mitchell (1963) and from our results that the cerebral cortex does have many cholinergic nerve endings. These nerve endings may lie in the primary pathways or in the other associated circuits which include cholinergic intracortical synapses. According to Grundfest (1957), a single neuron may possess membranes upon which there are both excitatory and inhibitory chemically sensitive areas. These give rise to graded post-synaptic potentials. Whether one or more chemical substances can bring about these actions at the membrane is still largely a matter of opinion. Eccles (1960) thinks that it may be that a single neuron may possess membranes upon which it generates, in all its potentialities, one sort of transmitter. It is possible,

however, that the secretion of the same transmitter substance by a single neuron may produce depolarization; and, therefore, excitation at one post-synaptic site; whereas, by causing hyperpolarization, it may produce inhibition in other receptor sites.

Previously, it was demonstrated that the ascending reticular activation of the cerebral cortex which produces its typical desynchronization of cortical electrical activity and arousal or alerting responses in behaviour may be mediated by acetylcholine. It has become very evident, however, from our results, that there is another component to the ascending reticular system whose effects upon cerebral cortical activity has been synchronizing as opposed to desynchronizing. This is of inhibitory character rather than of excitatory in its effect. Slow, rhythmic stimulation of this system has been shown to facilitate the sleeping rather than the waking state. It is very likely that the states of sleep and waking may be due to balanced excitatory and inhibitory actions by the reticular activating system. It is,therefore, suggested that excitatory effects of the so-called arousal response of brain stem origin may be cortically mediated by acetylcholine and glutamic acid; while GABA or other similar compounds may be involved in the mediation of apparent inhibitory effects of the subcortical region. This is, perhaps, involved in the sleep mechanism.

Chapter 6

General Discussion

The effect on the proportions of bound and free GABA of the temperature of the saline medium in which the brain is homogenized in the preparation of brain suspensions is remarkable. Van Gelder and Elliott (1960) have reported that the fraction of the total Factor I activity found in the bound condition in the whole brain is 66%. However, in these experiments no correction for free Factor I remaining in the supernatant fluid included in the centrifuged residue had been applied. Upon application of this correction, the figure becomes 58%. More recently, experiments by Lovell and Elliott (1963) and experiments in the present study have given a lower percentage of GABA in the bound form. These findings occurred when the brain was homogenized in ice-cold saline solution instead of at room temperature, which was previously the case. What is the cause for there being more bound GABA at room temperature than at 0 degrees centigrade? It is shown in Fig. 5a and Fig. 5b that the binding of GABA is affected by changes in concentrations of sodium and potassium ions. The different percentage of bound GABA might be due to the different distribution of the electro-

lytes between the medium and tissue particles resulting from the different metabolic rates of the homogenized tissue fragments.

No change was found in the total GABA (determined as Factor 1) in brain slices in the presence of oxygen and glucose(Elliott and Van Gelder, 1958). The rate of formation of GABA from glutamate through glutamic decarboxylase enzyme must, therefore, be equal to the rate of removal of GABA as succinic semialdehyde in slices in the presence of oxygen and glucose. The brain slices could absorb GABA and other amino acids against the concentration gredient from the medium. Under anoxic conditions there was a decrease in the total GABA content, and also, GABA leaked from the slice to the medium. Presumably, in the absence of oxidative energy production glutamate diffuses from the slice into the medium and becomes unavailable for conversion into GABA. In brain suspensions, on the other hand, no absorption of GABA from the medium into the tissue particles occurs. The total GABA and the proportions of free and bound do not change radically with time in suspensions prepared and kept at 0 degrees centigrade (Fig. 2). While this remains standing at 38 degrees, a marked increase in the total GABA occurs, - apparently an increase in the free form. It cannot be said whether this is directly produced free or produced in the bound form and then rapidly released. As Fig. 2 and Fig. 3 show, in the absence of oxygen the total amount of GABA increases with time. The glutamate

present in the tissue suspensions is evidently accessible to the glutamic decarboxylase.

It is demonstrated in present studies concerned with the metabolism of GABA (Fig. 3) that the total GABA content of a brain suspension remains constant if oxygen and glucose are supplied. However, the GABA content increases under anaerobic conditions. Evidently, production from glutamate present in the suspension continues, but removal then stops because of a lack of alpha-ketoglutaric acid for the transamination and oxygen for the oxidation of succinic semialdehyde to succinic acid. It is suggested, taking into account the results of Fig. 3, that the amount of oxygen that would be required per unit time to oxidize succinic semialdehyde to succinate is equal to approximately 10% of the rate of oxygen uptake of the brain tissue suspension. Thus, 10% is an estimate of the proportion of the total metabolism involving the GABA shunt in a brain suspension. These results are in contradiction with McKhann et al (1960) and Albers et al (1961) who claimed as much as 40% of the oxidative metabolism of the brain tissue could go through shunt pathways.

It is shown in the results of Fig. 4 that the binding of GABA is most stable at about pH 7. At higher or lower pH values, much GABA is quickly released in the bound form. Furthermore, these results are very similar to the effect of pH on the

binding of acetylcholine (Brodkin and Elliott, 1953). However, it seems inappropriate to think of GABA simply as an antonym of acetylcholine. There is no mechanism which has been discovered up to the present time for the rapid destruction of GABA comparable to cholinesterase enzymes which destroy acetylcholine.

Previous work and recent work done from this laboratory led Elliott (1965) to believe that in the brain there are at least five states of GABA which should be given consideration: free extracellular, free intracellular, loosely bound, more firmly bound, and convalently combined. There is only a small amount of GABA in the brain which is present in extracellular fluid. However, as has been shown in the present study, GABA can be released into extracellular fluid under certain neurophysiological conditions in vivo.

When brain tissue is homogenized in a saline medium,, only about 50% of the total Factor I activity is free which is immediately active on the crayfish receptor organ or chemically determined in the supernatant fluid. The proportion of the inert or bound form is not affected by variation in the mechanical conditions of the preparation of tissue suspension (Table 2). The GABA found in the solid matter after centrifugation, which is about 50-60% of the total, contains at least two forms of bound GABA - loosely and more firmly bound. When brain tissue is homogenized in 0.32 M sucrose medium only 18-20% of the total

GABA remains bound. The loosely bound fraction that is released in sucrose media in the absence of 10 mM sodium or calcium chloride is, perhaps, in some physicochemical binding with membraneous material. The physicochemical binding could be either hydrogen, Vander Waals forces, or Columb's type. From a comparative point of view, the following observations are worth noting: Virtanen and Miettinen (1953) reported that, in the pea plant, GABA, alanine, and Homoserine are present in bound but soluble fractions. Since proteolytic enzymes did not release these amino acids, therefore, they were not bound in proteins, they were apparently bound to sugars. Gottschalk and Partridge (1950), furthermore, have demonstrated the reactivity of the amino group with reducing sugars; beta and gamma-amino acids were more inclined to be bound to form complex peculiar structures with sugar components of membranes than alpha-amino acids. Results presented in Chapter 3 concerning the change in the proportions of free and bound GABA suggest that changes of this nature would hardly occur if the binding of GABA were artefact, produced upon the disintegration of the tissue. The signifigance of differences in levels of bound and free forms of GABA produced by drugs and other factors are suggestive of the importance of this binding in the physiological role of GABA; and probably of glutamic acid, glutamine, aspartic acid,

and alanine. It appears improbable that the relatively large fractions of amino acids found to be retained by the tissue residue after extraction with saline media would be linked with the "amino acids ATP system". Also, GABA cannot be attached to the amino acid specific ribonucleic acid which is involved in protein synthesis (Lipmann et al, 1959; Zamecnik et al, 1958) because GABA is not known to be a protein constituent. The firmly bound form which remains bound in suspension prepared in sucrose solutions is evidently occluded in subcellular particles most likely in certain nerve ending particles (Bilodeau, 1962, unpublished; Ryall, 1962, 1964; and Weinstein et al, 1963; (Table 2 and Table 4)) also seem to be bound in these two different ways. Also, it is probable that isolated mitochondrial fractions would be capable of absorbing GABA and other amino acids in the presence of sodium or calcium.

The finding by Lovell and Elliott (1963) that the total amount of GABA extractable from brain tissue which has been frozen in situ at the moment of death is lower than that in brain tissue excised at room temperature indicated a rapid post mortem change in the total GABA content. Like GABA, other amino acids in the brain have also been found to increase in amount immediately after death unless the tissue is immediately frozen.

Dis-integration of the tissue structure might result in rapid activity by proteolytic enzymes. However, this alone could not account for the sudden GABA increase which is not present in proteins. Possibly the glutamate produced by proteolysis is especially accessible to glutamate decarboxylase. Results of Table 3 suggest that some protein in the brain is peculiarly susceptible to post mortem hydrolysis.

Much evidence has been put forth concerning the inhibitory phenomenon and probable inhibitory agents in the central nervous system. Eccles et al (1963) has demonstrated that GABA is involved in the action of certain pre-synaptic inhibitory interneurons. Results summarized in Table 5 and Table 6 provide evidence that GABA and other amino acids are released under physiological conditions in the mammal. In cats showing an "aroused" electroencephalographic pattern either with local anaesthesia in the waking animal or following cervical section, only a small amount of GABA will leak out of the cerebral cortex into the perfusion chamber. In some experiments, when a continuous waking state was maintained by periodic stimulation of the brain stem reticular formation, no measurable GABA was present in the perfusate. The release of GABA, however, from the brain of a cat showing a sleep pattern with marked

spindles following midbrain section, is quite marked. Eccles, Schmidt, Willis (1963), and Kravitz, Potter and Van Gelder (1962) find the action of GABA to be similar in many important aspects to that of the normally occurring inhibitory neuronal transmitter. It is thought that GABA blocks by selectively increasing the movements of specific ions across the nerve cell or membrane. However, there is no mechanism for the rapid destruction of GABA in contrast to the cholinestrase enzymes which destroy acetylcholine. Perhaps, where true transmission occurs but with long-lasting effects, the necessity of a rapid-acting destructive enzyme may not exist. There are some biochemical situations in which diffusion or active absorption can account for the inactivation. It is evident that almost any substance will be metabolized in an organism sooner or later. Therefore, the inactivatory system may not be necessary for the defunction of the transmitter. Absorption of GABA into one of the bound forms may serve the same role as enzyme destruction.

There is now considerable evidence supporting the hypothesis that GABA can be given a transmitter role at inhibitory nerve endings to muscle or to stretch receptor neurons in crustacea. In the nerve cells where GABA is intracellularly involved in metabolism it is quite difficult to accept it as a transmitter substance.

However, there may be other neurons or parts of neurons where it could be an end product and could function as a transmitter agent in the central nervous system. The evidence that acetylcholine is a transmitter agent is indirect and inferential. Therefore, one should not set more rigorous criteria for GABA or for other related substances than one can set at present for any other kind of agent which one suspects must mediate synaptic excitation. In our experiments glutamic acid, glutamine, and aspartic acid, as well as GABA, were found to leak from the punctured cortical surface. Whereas the rate of release of GABA was increased during the sleep condition, the rate for glutamate was increased about 50% when the electroencephalographic pattern indicated arousal which suggested that an excitatory effect of glutamate is involved in the production of the waking state. Therefore, glutamate may behave as an excitatory transmitter. No obvious changes have been found in different physiological conditions in vivo in the amounts of aspartate or glutamine that exuded.

GABA and glutamic acid are involved in the oxidative metabolism of the nervous system as well as in neuronal function. There are at least two pathways in the nervous system by which alpha-ketoglutaric acid could be metabolized to succinate. The chemical direct citric acid cycle

pathway via succinyl CoA, and a shunt pathway through glutamic acid, GABA, and succinic semialdehyde. A new concept of the regulation of the function seems to be involved because of the roles of GABA and glutamate in transport mechanisms, metabolism, and their physiological action which are interdependant. All the amino acids related to Kreb's cycle exist in bound and free forms. In conjunction with physiological action, it is reasonable to assume that release or other factors which change the state of the amino acids in the brain might affect the utilization of the amino acids for energy production. Bound forms of these amino acids may be involved in the regulation of their physiological actions.

Inhibitory effects are demonstrated by applying them during excitation of neurons by acetylcholine or by an electrical current. Krnjevic et al (1964) demonstrated a particularly convenient and quick method of testing neuronal excitability in the cortex. The method utilizes the fact that cortical neurons are very readily excited by L-glutamic acid released from a micropipette by iontophoresis. Furthermore, it has been suggested by Krnjevic and Phillis (1963) that the ascending reticular activation of the cerebral cortex which produces its typical desynchronization of cortical electrical activity is likely to play an important role in setting the level of excitability. This excitability is mediated by acetylcholine which is released by cholinergic neurons

in the cortex. The results summarized in Chapter 4 and 5 in which acetylcholine and GABA were analyzed in cortical perfusate from encephale and cerveau isole preparations in the cat cerebral cortex showed that there is an inverse relationship between GABA and acetylcholine release in the above neurophysiological conditions in vivo. There is a higher rate of liberation of acetylcholine in the encephale isole and in the intact animals with a desynchronized activated electrocorticogram, while there was a very low rate of liberation in the cerveau isole state with continuous spindles in the electrocorticogram.

The states of sleep and waking may be due to balanced excitatory and inhibitory actions of the reticular activating system. The present studies suggest that the inhibitory system is exercised by GABA in opposition to the cholinergic excitatory system in the cortex. The cholinergic fibers originating in the brain stem reticular formation, by liberating an increased amount of acetylcholine, may control the phenomena of wakefulness.

Summary

Van Gelder and Elliott (1958) observed previously that the proportion of GABA in the brain in the bound state was about 60% of the total when the brain was homogenized at room temperature. However, we have recently found that when brain tissue in homogenized in ice-cold saline the proportion of bound GABA is only 40%. Once the suspension has been prepared, warming or cooling does not cause any immediate change in free or bound GABA. Under anaerobic conditions or on standing at room temperature, the total GABA in brain suspensions increases steadily with time. This increase is largely in the free form. In the presence of oxygen and glucose, the total GABA does not change appreciably, although there is a rapid initial release of bound GABA. It seems that under anaerobic conditions, loss of glutamate from the slice to the medium prevents its conversion to GABA. The binding of GABA is susceptible to pH changes; and is most stable at pH 7.

It has been demonstrated that in a salt-free isosmotic sucrose solution much of the bound GABA is released. The binding of GABA found when suspensions are made in isosmotic sucrose medium is increased by the presence of the sodium ion. Replacement of the sodium ion by the potassium ion in saline or sucrose

solutions considerably decreases the amount of bound GABA. The proportion of GABA remaining in the bound form is independent of the relative volumes of tissue and medium. Like GABA, alanine, glutamic acid, glutamine, aspartic acid, and N-acetyl-Aspartic acid are also bound or occluded in brain tissue. The amount of bound glutamic and aspartic acids is greater than that of GABA, but the proportion of the total is lower. The binding of these amino acids is less marked in the liver and kidney than in the brain. The bound GABA, as well as the other amino acids, can be gradually released from the binding material by repeated extraction with saline medium. However, approximately 8-10% remained bound even after 5 extractions. This may correspond to the more firmly bound form of these amino acids. GABA and other amino acids are rapidly released from the bound form by heat, hypotonicity, dilute acid or alkali, freezing, or ethanol. The total amounts of GABA, alanine, glutamic acid, glutamine, and aspartic acid found in the brain frozen <u>in situ</u> are a good deal lower than the amounts found if the brain is removed and extracted at room temperature.

GABA and other amino acids are released from the cat cerebral cortex if the pia-arachnoid is punctured. The quantitative release of GABA, glutamic acid, aspartic acid, and glutamine from the cortices of flaxedilized cats was determined in the following experimental

preparation: neuraxially intact, "encephale isole", "cerveau isole", and midcollicular brain stem section. Electrical activity from the cortical surface was recorded. The amino acids were collected in chambers perfused with Elliot's solution. In neuraxially intact cats, 0.60±0.20(4) ug GABA/hr/cm^2 was released as determined chromatographically. In "encaphale isole" cats 0.66±0.26(6) ug GABA/hr/cm^2 was released as determined chromatographcally. and 0.86±0.11(5) as determined by an enzymatic method. In both the above preparations, the electrocortical activity showed mostly an aroused pattern. GABA release was much more lower when the recticular formation was stimulated to maintain constant arousal. In "cerveau isole" cats with good sleep spindles in the E.Co.G there was a marked increase in GABA release: 2.09±0.60(18) ug/hr/cm^2 chromatographically and 2.57±0.44(13) ug/hr/cm^2 enzymatically. After mid-collicular hemisection. GABA release increased on the side of hemisection. Glutamic acid release was decreased under conditions in which GABA was increased ("encephale isole" 8.55±0.20(6) ug/hr/cm^2, "cerveau isole" 5.65±0.99(13) ug/hr/cm^2). No change was found in the amounts of aspartic acid or glutamine released under the various experimental conditions.

In experiments in which acetylcholine and GABA were analysed in cortical perfusate of encephale isole and cerveau isole preparations in the cat cerebral cortex there was an inverse relationship between GABA and ace-

tylcholine release. There was a higher rate of liberation of acetylcholine in encephale isole and in the intact animals, while the reverse is true in the cerveau isole with continuous spindles in the electrocorticogram.

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