METABOLISM OF NERVE ENDINGS ISOLATED FROM CEREBRAL CORTEX AND THEIR RELEASE OF PHYSIOLOGICALLY ACTIVE SUBSTANCES INDUCED BY DEPOLARIZATION

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ABSTRACT

The metabolic properties of synaptosome beds (deposits of mammalian cerebral cortex synaptosomes between nylon gauzes) and their response to depolarizing influences were studied. When synaptosome beds were incubated in physiological medium containing glucose as substrate, they respired, glycolysed, produced ATP and phosphocreatine and metabolised (U-^{14}C) glucose to glutamate, aspartate, alanine and GABA at similar rates to synaptosome suspensions. Metabolic inhibitors caused massive loss of amino acids from the beds.

Synaptosome beds also responded metabolically to alternating electrical pulses (respiration and lactate production increasing 40%) and elevated medium potassium (56 mM) by an increased lactate production. Differential release of glutamate, aspartate and GABA occurred during electrical stimulation, maximal release being after 10 - 15 min stimulation. This differential release also occurred when medium potassium was raised. The stimulation induced release of glutamate, aspartate and GABA was greatly reduced by omitting calcium from the medium and was totally abolished by including EGTA to complex endogenous calcium. Including amino acid analogues (β-aminobutyric acid, α,γ -diaminobutyric acid and N-acetyl glutamate) in the incubation medium changed the patterns of amino acid present in the medium, indicating that under normal conditions active amino acid uptake processes are occurring in synaptosomes. Tetrodotoxin and ouabain also interferred with amino acid release without greatly affecting the response to stimulation. Cerebral cortex slices incubated between gauzes also showed a glycolytic response to electrical stimulation but GABA was the only amino acid showing a significant increase in amount released with both electrical and potassium stimulation of slices.

Electrical and potassium stimulation also caused a significant increase in the release of acetylcholine to the medium from synaptosome beds which was dependent on the presence of calcium, in the same way as shown for amino acids. This stimulation also led to an increase in the total acetylcholine suggesting that increased synthesis had occurred.

Synaptic vesicles, isolated from synaptosomes and purified by gel filtration, were examined for their amino acid content at $0^{\circ}C$ and after incubation in medium at $37^{\circ}C$. Taurine was found to be present in synaptic vesicles in significant amounts and was the only identified amino acid present.

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ABBREVIATIONS

The standard abbreviations adopted by the Biochemical Journal are used in this thesis. In addition the following abbreviations are used:-

∝KG	∝ keto glutarate
ACh	acetyl choline
AChE	acetyl choline esterase
BABA	β-aminobutyric acid
DABA	∝,γ-diaminobutyric acid
EGTA	ethylene glycol bis-(2 amino-ethyl) tetra-acetic acid
GAD	glutamic acid decarboxylase
5-HT	5-hydroxy-tryptamine
mepps	miniature end plate potentials
NAG	N-acetyl glutamate
NE	noradrenaline
PCA	perchloric acid

PUBLICATIONS

1) Metabolism of beds of mammalian cortical synaptosomes : response to depolarizing influences.
by J. de Belleroche and H.F. Bradford
J. Neurochem. 19, 585-602 (1972).

2) Synaptosome Beds : a Method for the study in vitro of the metabolism and function of nerve endings.
by J. de Belleroche and H.F. Bradford
Biochem. J. 127, 21 P (1972).

3) The stimulus-induced release of acetylcholine from synaptosome beds and its calcium dependence.
by J. de Belleroche and H.F. Bradford
J. Neurochem. 19, 1817-1819 (1972).

CHAPTER 1

GENERAL INTRODUCTION

Introduction

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CHAPTER 1 GENERAL INTRODUCTION

Introduction

The present thesis describes an *in vitro* study of the metabolic and physiological properties of nerve terminals obtained from mammalian cerebral cortex. Nerve terminals, the proximities of neuronal axons and dendrites represent the presynaptic portion of the synapse. When the terminal membrane is depolarized, a chemical transmitter is released from the nerve terminal and crosses the synaptic gap (200 Å) to the post-synaptic membrane, part of an adjacent neuronal cell body or dendrite, and triggers off either depolarization or hyperpolarization of the post synaptic membrane. The former occurs in response to an excitatory transmitter and the latter with an inhibitory one. When the threshold of depolarization is reached post synaptically, an action potential is generated and the impulse travels further along the neuronal pathway. Apart from being responsible for transmitter release, nerve terminals also provide metabolic energy for maintainance of ionic gradients and turnover of cytoplasmic components, which may for example involve the synthesis of transmitter.

Electrophysiological studies have provided much knowledge about impulse conduction and the postsynaptic response of neurones to natural transmitters or transmitter-like substances, and this forms the basis for a biochemical study of transmitter release itself in which the presynapse alone is used. Nerve endings, obtained by homogenisation of rat cerebral cortex tissue under controlled conditions, followed by differential and density gradient centrifugation, were incubated in physiological medium in the form of a deposit of nerve endings between two pieces of nylon gauze. Two important considerations in choosing an *in vitro* preparation for metabolic studies are whether the preparation is autonomous under the conditions of study and secondly, whether its structure and functional capabilities are similar enough to those *in situ*, for the preparation to be of value. These aspects,

together with the principles behind the method of preparation, will be discussed below.

Four compounds were chosen for particular consideration: glutamic acid, aspartic acid, GABA and acetyl choline, since they are all physiologically active on cerebral neurones and would be likely to be released from the presynapse if they were in fact neuro-transmitters in the mammalian CNS. The evidence concerning the possible transmitter role of these compounds will also be discussed in this chapter, together with an outline of the experimental approach used in this study.

The word synaptosome has been coined by Whittaker et al (1964) to describe nerve endings rounded off from their axons and this word together with 'nerve endings' and 'nerve terminals' will be used synonymously in this thesis.

A. Synaptosomes as an in vitro preparation.

(i) The choice of an in vitro preparation. By homogenisation and centrifugation techniques, it is possible to prepare a reasonably pure fraction of nerve endings, which although separate from other parts of the brain are very active metabolically. The use of an in vitro preparation to observe the activities of the presynapse has the advantage over an in vivo system in that the gross changes measured occur in one component, the nerve endings, and are not due to a mixed population of cells, neuronal and non neuronal. Certain techniques utilized in vivo are not applicable to measurements on nerve endings, for example micropipettes (used for electro physiology measurements) are as yet too large (tip diameter 0.5 - 1.0 μ) to penetrate the nerve ending (0.5 - 1.0 μ diameter) without destroying it. An in vitro system has the added advantage that it allows specification of the environment

surrounding the nerve ending and this can be altered to suit the needs of the experiment. Since the physical components of nerve endings are virtually the same as those in situ, as judged by electron microscopy and the biological performance is similar to a living cell, this preparation would seem very useful for a biochemical study, which can be correlated with the living situation.

(ii) The continuity of the synaptosomal membrane. During the homogenisation procedure, nerve endings are torn away from axons. There are indications that resealing has occured, from the fact that nerve endings prepared in this way retain their soluble cytoplasmic components, such as enzymes, e.g. lactate dehydrogenase (Johnson & Whittaker 1963), co-enzymes, e.g. pyridine nucleotides (Lindall & Franz 1967), ions, e.g. K^+ (Marchbanks 1967) and metabolites, e.g. acetyl choline and amino acids (Mangan & Whitt 1966; Bradford & Thomas 1969). The presence of a wide range of these components is further shown by the number of energy-producing and synthetic reactions demonstrated by nerve endings when incubated in simple media.

As shown by processes such as pinocytosis, the cell membrane would seem capable of sealing the gap in the nerve ending membrane caused by severence of the axon, especially since the diameter of the axon is small (1/5 - 1/10μ) compared to that of the nerve ending particle (1μ). Indeed, slices of mammalian cerebral cortex and other regions of brain, which have inevitably been prepared with much severence of axons are able to maintain high membrane potentials (Hillman & McIlwain 1961; Gibson & McIlwain 1965; Bradford & McIlwain 1966) and respond to depolarization with action potent generation (Richards & McIlwain 1967; Yamamoto & Kawai 1967; Richards & Sercombe 1968). Other preparations in which axons and dendrites have bee lost, such as fractions enriched in neuronal perikarya (Rose 1967; 1968) a

capable of glucose oxidation. It would thus seem feasible that neuronal tissue is able to overcome loss of axons, without observable deleterious effect.

The osmotic properties of synaptosomes by which swelling and shrinkage occur in media of different tonicity also indicate the presence of a continuous membrane (Marchbanks 1967; Keen & White 1970).

(iii) Synaptosomal Morphology. Electron microscopy of synaptosomes shows that they have the morphological features of the synaptic complex (Figure 1), containing a continuous membrane, numerous synaptic vesicles and usually displaying one or more mitochondria (Jones & Bradford 1971). The post-synaptic thickening is seen on some structures but may be present on a larger proportion and be lost on sectioning, since De Robertis et al (1967) were able to obtain a large yield of post-synaptic thickenings on dissolving away other structures with detergent. Jones & Brearly (1972; 1972a) recently have made a detailed morphological comparison of synaptosomes with nerve endings in situ, and have found that the differences are minimal.

When synaptosomes have been incubated in physiological medium, they show a more spherical structure than when fixed directly in the sucrose with which they have been prepared (Jones & Bradford 1971), which may be due to a slight shrinkage which occurs in sucrose.

B. Metabolic Properties of Synaptosomes.

(i) Oxygen and glucose utilization. Suspensions of cerebral cortex nerve endings in Krebs-Ringer medium buffered with tris-HCl, glycylglycine or phosphate containing 10mM glucose are able to take up oxygen in a linear fashion at about 60-67 μ moles O_2/100mg protein/h (Bradford 1969; Whittaker 1969; Balfour & Gilbert 1970), which is a similar rate to that shown by

Figure 1 Electron Micrographs of Synaptosomes

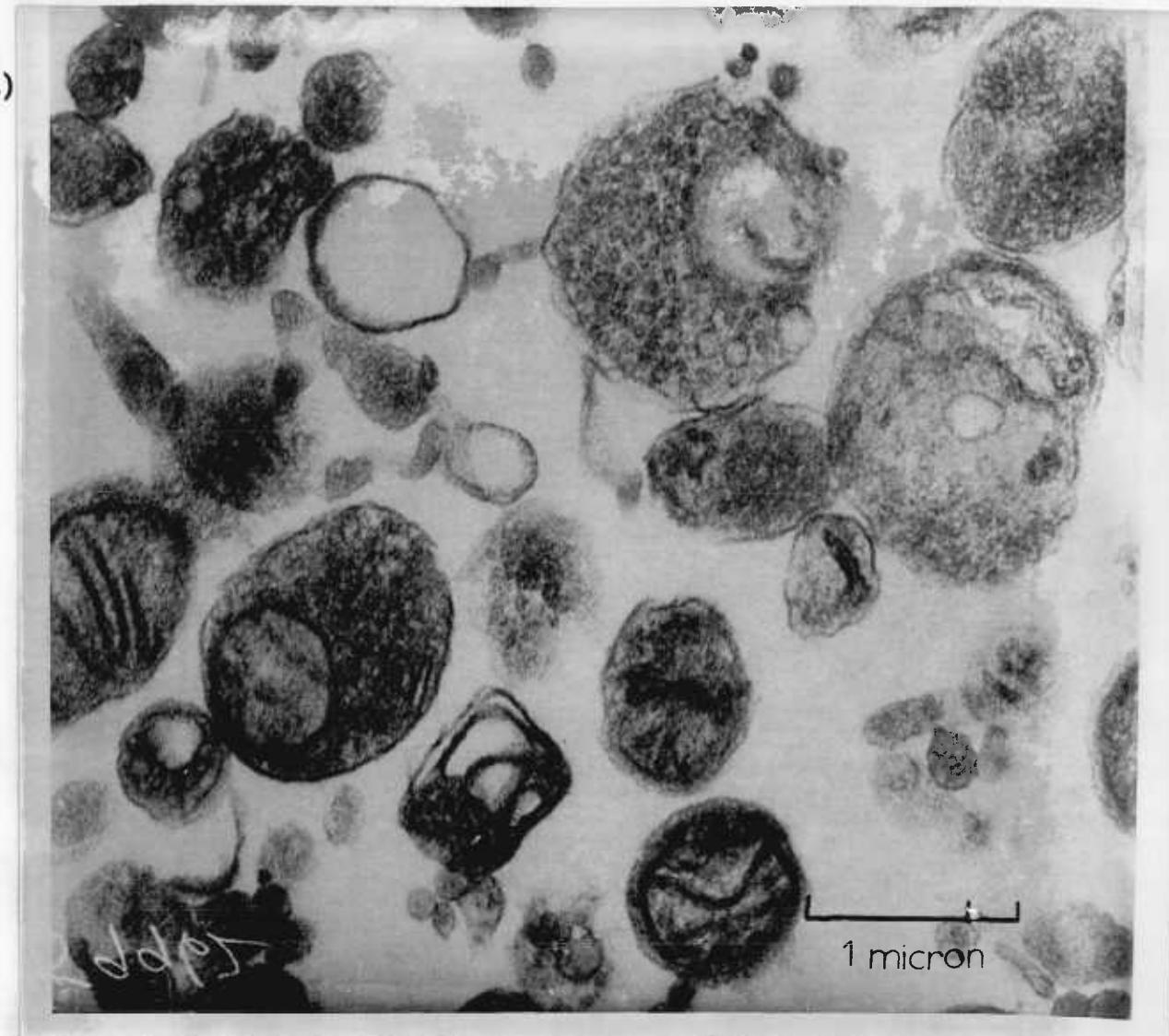
Synaptosome suspensions were fixed in buffered OsO_4, dehydrated in ethanol and embedded in araldite for sectioning and electron microscopy.

a) Electron micrograph of sucrose suspension immediately after the centrifugation procedure, showing the synaptosomes as dark, slightly irregular bodies.

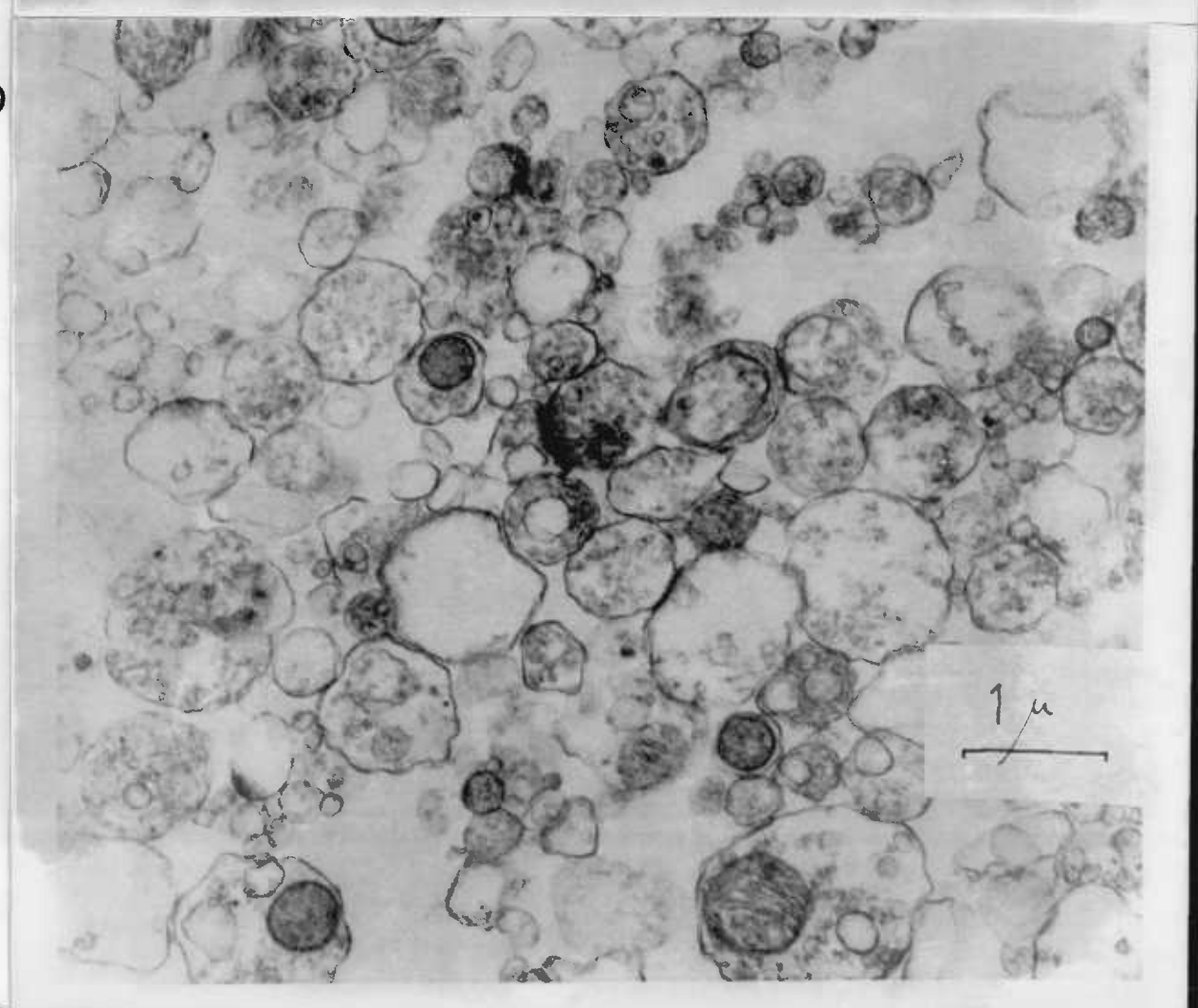
b) Electron micrograph of synaptosomes after incubation in Krebs phosphate medium for 1 h at 37°C, showing the synaptosomes as lighter spherical and sometimes more vacuolated bodies.

Electron microscopy was carried out by Dr. D.G. Jones, Department of Anatomy, University of Western Australia.

a)



b)



slices from the same region (McIlwain & Bachelard 1971). A predominantly linear rate of respiration continues for up to 4 h, and for up to 24 h in culture the morphology of synaptosomes is preserved (Bradford, Booher & Jones 1972).

The presence of the full enzyme and co-enzyme complement necessary for complete glycolysis and oxidation of glucose linked to ATP production is clearly indicated by the considerable lactate production (16 μ moles/100mg protein/h; Bradford & Thomas 1969) and formation of ATP and phosphocreatine (Bradford 1969). Isotope studies show that in parallel, glucose carbon is being converted into intermediary metabolites such as oxaloacetate and oxoglutaric acids subsequently giving rise to labelled glutamate, aspartate, GABA and alanine (Bradford & Thomas 1969). The distribution of metabolic enzymes follows the pattern found in whole tissue, glycolytic enzymes being in the soluble cytoplasm and citric acid cycle and oxidative phosphorylation systems in the intrasynaptosomal mitochondria. The mitochondria no doubt play a central role in synaptosomal metabolism, since they contribute a considerable proportion of the total protein (30%; Moore & Lindall 1970) and occupy a large proportion of the synaptosome volume (25%; Whittaker 1969).

There is a wide range of enzymes which have been found in synaptosome-enriched fractions as indicated in Table 1 and Figure 2, which shows their great metabolic potential. Synaptosome fractions are however contaminated to varying degrees by free mitochondria and membrane fractions and enzyme activity in a synaptosomal fraction may be present in a contaminant. For example, although succinate dehydrogenase (SDH) activity has been demonstrated in the mitochondria obtained from rupture of a synaptosomal fraction (Whittaker 1965), the absence of SDH <u>in situ</u> in nerve terminal mitochondria has been shown by means of electron microscope histochemistry (Hajos & Kerpel Fronius 1971). This demonstration lies in the oxidative phosphorylation

Table 1 SYNAPTOSOMAL ENZYMES

Enzyme	Distribution and Comments	Reference
(i) Glycolytic enzymes	Shown by lactate production of synaptosomes	1
Hexokinase E.C.2.7.11.		2
Lactate dehydrogenase E.C.1.1.1.27.	Used as a synaptosomal cytoplasm marker. The most basic isoenzyme becomes attached to particulate fractions at low ionic strength.	3 4
(ii) Kreb's cycle, oxidative phosphorylation and associated enzymes.		
	Presence indicated by respiratory capacity of synaptosomes.	5
Citrate synthase E.C.4.1.3.7.		6
Fumarate hydratase E.C.4.2.1.2.		6
Succinate dehydrogenase E.C.1.3.99.1.	Associated with mitochondrial membrane of synaptosome.	7, 8 (but see 36)
$NADH_2$ dehydrogenase E.C.1.6.99.3.	Soluble cytoplasm and mitochondria	9
$NADH_2$ cytochrome C reductase E.C.1.6.2.1.	Mitochondria and microsomes	9
Cytochrome oxidase E.C.1.9.3.1.	Mitochondria	9
(iv) Enzymes of hexose monophosphate shunt.	Present and operating in synaptosomes	10
(v) Amino acid metabolising enzymes		

Glutamic acid dehydrogenase		
E.C.1.4.1.2.	Mitochondrial matrix of synaptosome	8, 7, 11
Glutamic acid decarboxylase		
E.C.4.1.1.15.	40 - 90% of whole brain GAD activity present in cytoplasm of synaptosomes.	7, 35
	Originally GAD found particularly in synaptosomes of 1.2M sucrose or greater.	7
	Other workers find GAD peak at 1.1 to 1.17M sucrose.	12, 13
	Ca^{2+} dependent binding of GAD to vesicles not easily solublized.	
Succinic acid semi-aldehyde dehydrogenase		
E.C.1.2.1.6.	Matrix of synaptosomal mitochondria	7, 8
GABA aminotransferase	"	7, 8, 11, 14
E.C.2.6.1.c.		
Glutaminase 1	Membrane of synaptosomal mitochondria	7, 8
E.C.3.5.1.2.		
Aspartate amino -transferase	Bimodal distribution in soluble cytoplasm and synaptosomal mitochondria.	7, 11, 12
E.C.2.6.1.1.		
Alanine aminotransferase	As Aspartate aminotransferase	7
E.C.2.6.1.2.	"	

(vi) Enzymes involved in the synthesis of putative transmitters.

a) Acetyl choline: choline acetylase E.C.2.3.1.6.	Probably found in the synaptosome cytoplasm at the high ionic strengths in vivo, although non specially bound to membranes at the low ionic strengths used in preparation.	4
b) Noradrenaline		
Tyrosine hydroxylase.	Found in both synaptosome and synaptic vesicle fractions.	15, 16, 18
E.C.1.14.3a		
Dihydroxy, phenylalanine decarboxylase	About 30% of the total enzyme activity is found in synaptosomes in the soluble fraction.	17,
E.C.4.1.1.26.		
c) 5-Hydroxytryptamine:		
Tryptophan 5 hydroxylase		19, 20
E.C.1.99.1.4.		

5-Hydroxytryptophan decarboxylase	About 50-60% of this enzyme activity is found in synaptosomes and negligible activity in synaptic vesicles.	17
E.C.4.1.1.28.		
d) Taurine; cysteine sulphinate decarboxylase.	50% of whole brain activity is in synaptosomes.	33
E.C.4.1.1.29.		
(vii) Membrane bound enzymes		
Na^+/K^+ ATPase.		21, 22, 23, 24
E.C.3.6.1.4.		
Mg dependent ATPase	Synaptosomes and vesicle membranes	22, 25, 26
Na^+/Ca^{2+} and Mg^{2+} dependent phosphatases	Synaptosomes and vesicles	22, 27
A Ch E		32, 21
E.C.3.1.1.7.		
(viii) Enzymes associated with Cyclic AMP		
Adenylate kinase		24
E.C.2.7.4.3.		
Adenyl cyclase	Enriched in synaptosomes and synaptic membranes.	24, 28, 29, 30, 31
Cyclic AMP dependent protein kinase	Synaptosome plasma membranes contain 70% of particulate bound enzyme. Also present in synaptic vesicles.	28 34
Cyclic AMP phosphodiesterase		29, 30
E.C.3.1.4.d.		

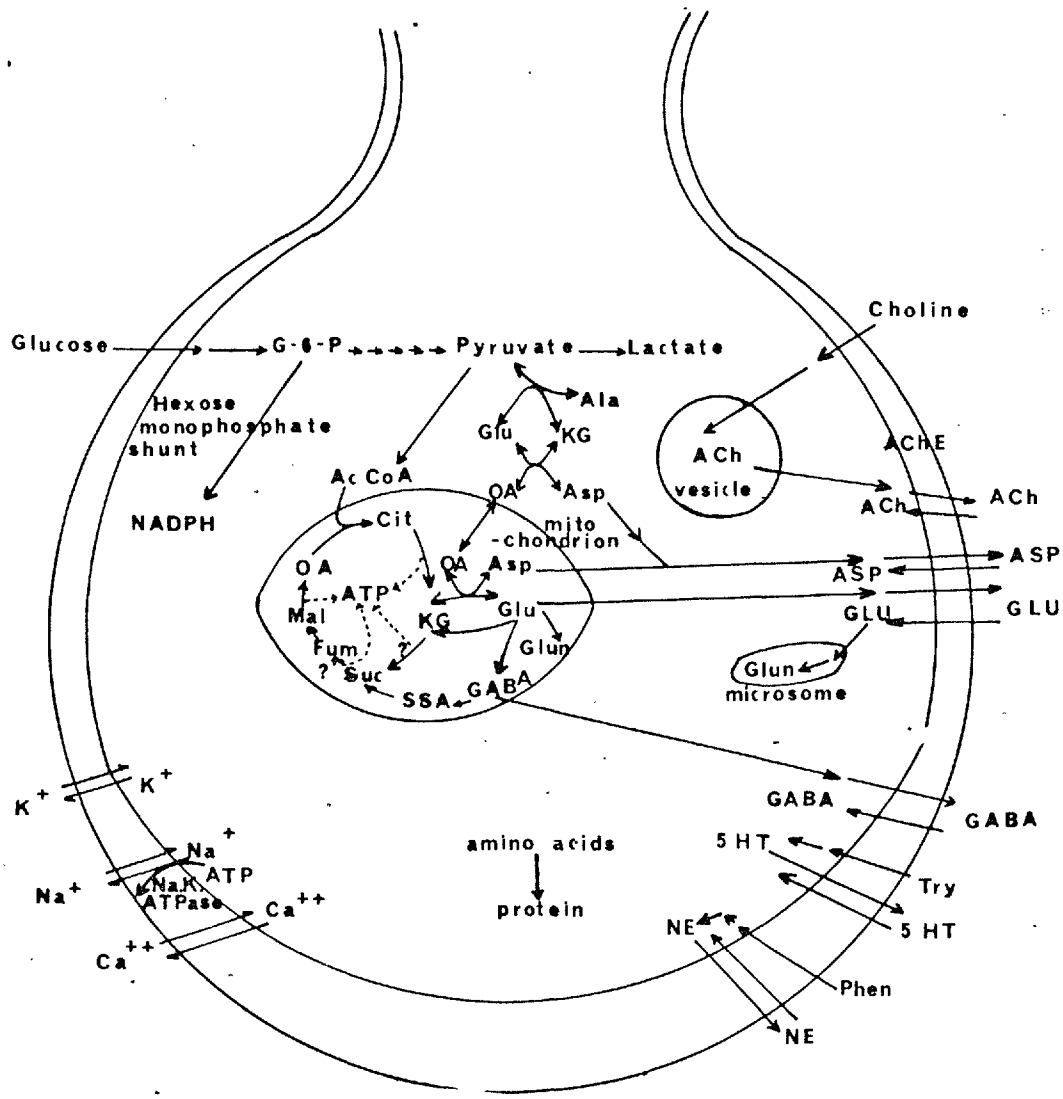
KEY TO TABLE

1. Bradford & Thomas 1969;
2. Abood 1969.
3. Johnson & Whittaker 1963;
4. Fonnum 1967.
5. Bradford 1969;
6. Tucek 1967.
7. Salganicoff & De Robertis 1963;
8. Salganicoff & De Robertis 1965.
9. Moore & Lindall 1970;
10. Appel & Parrot 1970.
11. Balazs et al 1966;
12. Fonnum 1968.
13. Kornguth et al 1971;
14. Waksman et al 1968.
15. Fahn et al 1969;
16. Karobath & Baldessarini 1972.
17. Rodniguez, De Lores Arnaiz & De Robertis 1964;
18. McGeer et al 1965.
19. Grahame-Smith 1967;
20. Stjarne & Lishajko 1967.
21. Bradford et al 1966;
22. Albers et al 1965.
23. Abdel-Latif et al 1967;
24. Abdel Latif et al 1970.
25. Germain and Proulx 1965;
26. Hosie 1965.
27. Kadota et al 1965;
28. Gaballah & Popoff 1971.
29. Gaballah & Popoff 1971;
30. De Robertis et al 1967.
31. Cheung & Salganicoff 1967;
32. De Robertis et al 1962.
33. Agrawal et al 1971;
34. Johnson et al 1971.
35. Fonnum 1965;
36. Kerpel, Fronius & Hajos 1971.

linked accumulation (inhibited by KCN or DNP) of Sr^{2+} by mitochondria which results in the appearance of an electron dense reaction product inside the mitochondria (Kerpel Fronius & Hajos 1970). When brain slices are incubated in medium containing ∝KG, pyruvate, succinate, glutamate, isocitrate or malate, Sr^{2+} is accumulated by mitochondria of neuronal perikarya and dendrites but terminal mitochondria only accumulated Sr^{2+} in the presence of pyruvate, glutamate, isocitrate or malate (Kerpel Fronius & Hajos 1971), the latter confirming that the respiratory chain is effectively coupled. These results might simply reflect some unexpected impermeability property of the terminal membrane to succinate and ∝KG but if SDH should be reduced in amount or absent from these mitochondria the implications are rather interesting. Thus, there would be a need for a by-pass of the block in the citric acid cycle at this point which could be mediated by CO_2 fixation with pyruvate to form oxaloacetate, a process which has been demonstrated in nervous tissue (Cheng 1967). However, since axoplasmic flow studies (Zalená 1968; 1969) and time-lapse cinematography (Pomerat 1964) show that bidirectional movement of mitochondria occur between cell body and nerve terminal it would be difficult to attribute special properties to terminal mitochondria.

(ii) The presence of putative transmitters. The nerve ending is the tissue region where compounds directly involved in nervous inhibition and excitation are most likely to be concentrated and synaptosomes may therefore provide a rich source of such compounds. Certain physiologically active compounds are concentrated in synaptosomes such as Acetyl choline (Gray & Whittaker 1962; De Robertis et al 1962), noradrenaline and 5-hydroxytryptamine (5 HT) (Michaelson & Whittaker 1963; Inouye et al 1963; Levi & Maynert 1964; Snyder et al 1965). Confirmation of the localization of 5 HT in certain presynaptic endings in situ has come from fluorescent histochemistry

Figure 2 _ Diagramatic representatation of processes occurring in Synaptosomes



(Dahlstrom & Fuxe 1964; 1965). Most of the bound dopamine, which represents 40% of the dopamine in whole brain, is also associated with synaptosomes (Laverty et al 1963). More impressive however is the large proportion (60 - 90%) of total brain acetyl choline which is recovered in synaptosomal fractions (Whittaker 1959; Kurokawa et al 1965).

Amino acids are a dominant compositional feature of synaptosomes as indeed they are in whole brain, glutamate, GABA and aspartate being the most predominant, together with the related compounds, taurine (Agrawal et al 1971) and N-acetyl glutamate (Reichett & Fonnum 1969). Although amino acids are not particularly concentrated in synaptosomes, biochemical and autoradiographic studies have led to the conclusion that GABA is specifically associated with nerve terminals of the amacrine cells of the retina (Graham 1972; Ehinger & Falck 1971) and basket cells of the cerebellum (Hökfelt & Ljungdahl 1972) and glycine with the nerve terminals of spinal cord (Hökfelt & Ljungdahl 1971; Matus & Dennison 1971).

(iii) Further synthetic processes. Enzymes synthesising key products such as possible central transmitters are present in synaptosomes and have been shown to be synthesised in the isolated nerve ending by incorporation of label from radioactive precursors. This has been shown for amino acids (Bradford & Thomas 1969), acetyl choline (Schubeth et al, 1970), noradrenaline (McGeer et al 1967; Baldessarini & Karobath 1972) and 5-hydroxytryptamine (Grahame-Smith 1967).

NADPH is produced in nerve endings via the hexose monophosphate shunt (Appel & Parrot 1970), and together with ATP is probably used in the various synthetic processes carried out there such as synthesis of nucleotides, phosphoproteins and phospholipids (Abdel-Latif, Yamaguchi, Yamaguchi & Chang 1968). Phosphatidyl inositol shows a high rate of production in

vitro (Lunt & Lapetina 1970), which is surprisingly much greater than the rate of formation of phosphatidyl ethanolamine and phosphatidyl choline which are important constituents of the nerve ending membrane.

Although some synaptic proteins are synthesised in the cell body and pass down to the nerve ending by axoplasmic flow (Weiss & Hiscoe 1948), synaptosomes are able to incorporate amino acids into protein *in vitro* (Austin & Morgan 1967). About 25% of this incorporation is due to the synaptosomal mitochondria as shown by inhibition by chloramphenicol, and the rest occurs in the cytoplasm and can be inhibited by cycloheximide (Autillo, Appel, Pettis & Gambetti 1968; Morgan & Austin 1969). This synaptosomal protein synthesis is not dependent on exogenous substrate or sources of energy such as ATP, but is influenced by ion concentrations, 100mM Na^+ and 10mM K^+ result in a 4 fold increase in protein synthesis above control values. Since ouabain is a potent inhibitor (Autillo *et al* 1968), it might be the case that this process is mediated by the activation of the Na^+/K^+ ATPase present in synaptosomes.

(iv) *Permeability properties and uptake processes.* When synaptosomes are incubated under physiological conditions, there is virtually no loss of LDH (Bradford 1971), but in hypo-osmotic solution most of the LDH is lost to the medium, and similarly most of the LDH, K^+, Na^+ are lost from association with the particulate fraction when synaptosomes are eluted with water from a Sephadex column (Marchbanks 1967). These cytoplasmic components are thus normally maintained within the synaptosome by an osmotically sensitive barrier and not merely attached to the surface in a non specific fashion. Synaptosomes show specific permeability to certain ions, which leads to swelling. This has been shown by Keen & White (1970) using a light scattering technique since swelling leads to an increase in light scattering which

can be monitored spectrophotometrically. The changes occur in accordance with Boyle & van't Hoff's Law and are largely reversible. At 5°C in 0.8M sucrose, conditions where there is unlikely to be active transport, passive diffusion of Na^+ and K^+ in and out of the synaptosome occurs, efflux and influx occuring at the same rate (Marchbanks 1967).

The presence of an active Na^+, K^+ dependent ATPase in nerve endings (Hosie 1965; Bradford, Brownlow & Gammack 1966) gives rise to the possibility that the isolated nerve ending maintains a membrane potential. The demonstration of this by ion flux, as the nerve ending is too small for direct potential measurement, would be good evidence for the presence of a functional membrane.

Substitution of the synaptosomal concentrations of Na^+ and K^+ after incubation, obtained from measurement of ion content and the volume of the non-inulin space, into the modified Nernst equation (Hodgkin 1958):

$$Em = \frac{R\,T}{F} \ln \frac{(K)o - b\,(Na)\,o}{(K)i - b\,(Na)\,i}$$

where o = extracellular concentration
i = intracellular concentration
b = apparent permeability of membrane to Na relative to K, taken as 0.04
Em = membrane potential

gives the value of -27 mV (Bradford 1971), which is not unrelated to the neuronal membrane potential of -60 to -70 mV. The value obtained represents a minimum, since there are amorphous fragments present in a synaptosome fraction and these would increase the non-inulin space and also the mitochondria and synaptic vesicles occupy a considerable volume of this space. Following application of electrical pulses to synaptosome suspensions, Bradford (1971) found that the calculated membrane potential changed from -27 to -7 mV, which shows that treatments which are known to depolarise

membranes are capable of displacing the synaptosome membrane potential. It would seem that an incomplete membrane would not be capable of these properties.

When synaptosomes are incubated under metabolic conditions, active transport of various ions and molecules can occur in addition to the passive diffusion processes mentioned above and these active processes are dependent on the availability of energy and prevented by metabolic inhibitors. Active transport occurs of ions such as Na^+ (Ling & Abdel-Latif 1968), K^+ (Escueta & Appel 1969) and Ca^{2+} (Lust & Robinson 1968) however little association of anions such as Cl^- occurs (Kuriyama & Roberts 1971).

A number of compounds are taken up by synaptosomes by Na^+ dependent processes such as choline (Marchbanks 1968a; Potter 1968; Diamond & Kennedy 1969), GABA (Weinstein, Varon, Muhleman & Roberts 1965; Kuriyama, Weinstein & Roberts 1969), noradrenaline (Bodganski, Tissari & Brodie 1968; Colburn, Goodwin, Murphy, Bunney & Davis 1968; Kuriyama & Speken 1970), tryptophan (Grahame-Smith & Parfitt 1970) and serotonin (Marchbanks 1966; Bogdanski, Tissari & Brodie 1968; Kuriyama & Speken 1970), and the uptake of glutamate and aspartate occuring by a high affinity process (Logan & Snyder 1971).

C. The case for glutamate and GABA being central transmitters.

Introduction. In this section will be presented the evidence for glutamate and GABA being central transmitters, together with a less detailed discussion with respect to aspartate for which there is less evidence. It is a direct consequence of this evidence that these particular amino acids were chosen for study of their possible transmitter role in this thesis. From a definition of the role of a transmitter, an evaluation will be made of the criteria

which need to be fulfilled by a compound in order to give it transmitter status. Then the available evidence will be presented in terms of these criteria, with assessment of the case for glutamate and GABA being central transmitters.

What is a Transmitter? A transmitter is a chemical mediator of synaptic transmission. The nervous system is a network of interconnecting pathways spread throughout the body and controlled by co-ordinating centres, the brain and spinal cord. A pathway is activated, for example, by a change in the external environment detected by a sensory nerve cell of the peripheral nervous system, and a nerve impulse is transmitted along this pathway, which may contain several neurones. A nerve impulse is propagated along the length of the neurone as a wave of depolarisation in which the normally negatively charged membrane becomes temporarally positive. When the impulse reaches the terminals of the nerve cell a chemical is released which travels across the gap between the nerve cells, the synapse, and interacts with specific post synaptic receptors, with a subsequent development of a change in permeability of the post synaptic membrane. This may either be an increased permeability to Na^+ and another ion, leading to excitation of the post synaptic membrane by depolarisation or an increased permeability to Cl^- or K^+ or both causing membrane hyperpolarisation and inhibition. The former process is initiated by an excitatory transmitter and the latter by an inhibitory one. Transmitter action is terminated by enzyme inactivation or removal from the synapse by uptake.

Thus a transmitter is a chemical which is present in presynaptic nerve terminals, is released from nerve endings as a result of excitation of a neural pathway and is capable of either depolarising or hyperpolarising the post synaptic membrane. The effects of a proposed transmitter should be identical with the action of natural transmission and both processes should

be affected in the same way by a potentiating or blocking substance. Thus the role of glutamate and GABA as transmitters will be studied from four main aspects, first their action on the neuronal membrane, secondly whether they are released from nerve terminals during transmission, thirdly their distribution and fourthly, possible ways in which they may be inactivated.

(i) Application of glutamate and GABA (a) in vivo. The first observation of the physiological action of glutamate was made by gross application of glutamate to the surface of exposed brain which was found to give rise to spreading depression (Hayashi 1956). This is a slowly moving wave of intense neuronal depolarisation and inactivation, abolishing all spontaneous and evoked activity (Van Harreveld 1959). Topical application of GABA causes a surface positive response (Iwama & Jasper 1957; Purpura, Girado & Grundfest 1957). The depression is immediate and reversible. With electrodes 1mm deep in the cortex, the normal negative wave is unaffected by GABA, indicating that GABA has a selective depressant effect on superficial layers of cortex.

Some of the most convincing evidence that glutamate and GABA are central transmitters has come from iontophoretic studies, whereby small quantities are applied directly onto individual neurones. Glass micropipettes with tip diameter 0.5 - 1.0μ containing the dissolved compound in charged form are used for this purpose. The retention or ejection of the compound is controlled by appropriate electrical currents applied to each pipette. With the aid of a micromanipulator (moving in 1μ steps), the micropipette may be placed in single neurones in specific regions of the brain. Multibarreled micropipettes may be made, with as many as 6 drug containing pipettes, the whole assembly having an overall tip diameter of less than 5μ.

One pipette may serve as a microelectrode, permitting the recording of both extracellular spike potentials and intracellular potentials at the same time as administering the drug. The advantages of this technique are that it interferes little with brain function and allows several drugs to be tested on one neurone and also many neurones to be assayed in succession in one animal, utilizing only small quantities of the drug.

There are however two uncertain aspects of this method; one is that the exact drug concentration affecting the neurone is not known, since some may diffuse away and secondly it is difficult to ensure that the added substance is acting on the same area of membrane as is involved in the synaptic process. Effects may be caused indirectly by excitation or depression of synaptically linked neurones. Nevertheless much very useful information has been obtained using the technique of microelectrophoresis and further details can be found in reviews by Curtis (1964) and Salmoiraghi & Bloom (1964).

Iontophoretic studies by Krnjević & Phillis (1963), showed that out of 200 neocortical neurones tested, all responded to small amounts of glutamate. The evoked discharge was rapid in onset and termination and was associated with depolarisation of the membrane. Other cells such as neuroglia were not depolarised by glutamate (Krnjević & Schwartz 1967a) and neurones of spinal ganglia were found not to respond either, suggesting that sensitivity to glutamate depends on the presence of synaptic contacts (Nishi, Soeda & Koketsu 1965). The excitatory action of glutamate on the mammalian central nervous system has been shown to be widespread from tests carried out on cat, rat and rabbit. Thus glutamate excites neurones of the pericruciate cortex (Crawford & Curtis 1964), hippocampus, thalamus and subthalamic area (Steiner & Ruf 1966; 1966a), pyriform cortex (Legge, Randic & Straughan 1966), lateral geniculate nucleus (Curtis & Davis 1962; Curtis

& Watkins 1963), cuneate nucleus (Galindo, Krnjević & Schwartz 1967), midbrain (Curtis & Koizumi 1961) and cerebellar cortex (Crawford & Curtis 1964; Crawford, Curtis, Voorhoeve & Wilson 1963). Spinal interneurones, motor neurones and Renshaw cells are also depolarised by glutamate (Curtis, Phillis & Watkins 1959; 1960; Curtis & Watkins 1960; 1963). In most of these studies, the same neurones were also found to be depressed by GABA.

The depolarisation of spinal neurones by glutamate does however not exactly reproduce an excitatory post synaptic potential (EPSP), the equilibrium potential (i.e. the membrane potential at which there is no net movement of ions) for glutamate being at a slightly lower level of depolarisation (Curtis 1962). This result, however, is not incompatible with glutamate depolarisation occuring by the same ionic mechanism as an EPSP, for in these experiments the EPSP is generated by an intracellular microelectrode probably located in soma, from which numerous nerve endings at the extremities of the dendrites may be triggered, whereas the effect of the amino acid is restricted to a limited region of membrane. An affirmative demonstration of this kind would be weighty evidence for glutamate being a transmitter.

The depressant action of GABA on cortical neurones was first shown by Hayashi (1956) and later with the use of intracellular recordings the remarkable similarity between GABA action and synaptic inhibition (IPSP) became evident (Krnjević & Schwartz 1966; 1967; Dreifuss, Kelly & Krnjević 1969). In both there is hyperpolarisation, a similar reversal potential and a large decrease in membrane resistance, chiefly attributable to an increase in chloride permeability. The action of GABA is rapid and complementary to glutamate (Krnjević & Phillis 1963). Apart from cerebral neurones, GABA has also been shown to produce hyperpolarisation and conductance increase in spinal motor neurones (Curtis, Hosli, Johnston & Johnston 1968), and

Deiters' nucleus (Bruggencate & Engberg 1971; Obata, Ito, Ochi & Sato 1967).

Elevation of intracellular chloride ion concentrations converts both IPSPs and GABA hyperpolarisations of neocortex (Krnjevic & Schwartz 1967) and spinal motor neurones into depolarisations. Experiments carried out with a series of anions of different hydrated diameter indicate that motor-neurone membrane permeability changes induced by GABA are identical with those occuring during inhibition (Curtis, Hosli, Johnston & Johnston 1968).

The specific prevention of both GABA and natural inhibition by bicuculine and picrotoxin is further substantial evidence in favour of GABA being an inhibitory transmitter in the CNS. Blockage of both GABA induced and natural post synaptic inhibition by these two agents has been shown in Deiters' neurones (Curtis, Duggan & Felix 1970; Obata, Takeda & Shinozake 1970), hippocampus (Curtis, Felix & McLennan 1970), olfactory bulb (Nicoll 1971), occulomotor neurones (Obata & Highstein 1970; Ito, Highstein & Tsuchiya 1970) and spinal motor neurones (Kellerth & Szumski 1966).

Recently, it has been shown that GABA exerts distinguishable effects on the primary afferent terminals and post synaptic membranes of the cuneate nucleus of rat. GABA exerts a depressant action on post synaptic membranes while exciting the primary afferent terminals (Davidson & Southwick 1971; Davidson & Reisine 1971). The depolarization of the primary afferent terminals indicates that GABA is acting at the same receptor site as the presynaptic inhibitory transmitter. Further, GABA has been shown to overcome the depressant action of picrotoxin on presynaptic inhibition.

Thus the evidence is in favour of GABA being both a post synaptic and presynaptic inhibitory transmitter. However,certain central inhibitory processes are not mediated by GABA and may be distinguished by the fact that strychnine blocks their action such as in spinal motor neurones (Curtis,

Hosli, Johnston & Johnston 1968). Inhibition of neocortical, pyramidal (hippocampus) and Purkinje neurones is not sensitive to strychnine (Andersen, Eccles, Loyning & Voorhoeve 1963; Crawford, Curtis, Voorhoeve & Wilson 1963).

The case for glutamate being a central transmitter is not so clear cut as for GABA, partly because of the absence of evidence from specific inhibitors of glutamate action. However,recently Haldeman et al (1972) have found that DLα methyl glutamate and L glutamic acid diethyl ester, themselves not excitatory agents, can block the excitation of thalmic neurones produced by L glutamate. Synaptic excitation of cells of the ventrobasal thalamus evoked by electrical stimulation of hind limb nerves was also reduced by these compounds.

Curtis & Watkins (1960; 1961), carried out iotophoretic studies on spinal motor neurones with structurally similar compounds to glutamate and GABA and found that many of the compounds used had similar or even more potent effect when applied iotophoretically (Table 2). Crawford (1970) has pointed out that there is no difference in the sensitivity of different classes of cerebral cortex neurone to glutamate, and other acidic amino acids (D glutamate, D & L aspartate, N methyl aspartate and D & L homocysteate), although the various amino acids were of different potencies (Table 2). Glutamate also exerts facilitatory action indiscriminatingly on primary afferent terminals and post synaptic membranes, increasing the excitability of the former and causing postsynaptic inhibition in the latter. This general non specific action of glutamate and other acidic amino acids on all neurones could be explained by these compounds being ubiquitous central transmitters or alternatively as being agents which cause a non specific excitation, by interacting with a structural component present in all central neuronal membranes, perhaps as an adjunct to synaptic transmission.

Table 2 Relative potencies of compounds applied electrophoretically to spinal interneurones

Excitatory Activity	L-glutamate	3
	L-aspartate	3
	L-cysteic acid	3
	N methyl D aspartate	20 - 30
	D-homocysteic acid	9 - 18
	L-homocysteic acid	4 - 5
	D-aspartate	2
	D-glutamate	2
	N methyl L aspartate	2
	β hydroxy glutamic acid	2
	α amino malonic acid	1
	α amino adipic acid	1
	α amino pimelic acid	1
	N methyl glutamic acid	0
Depressive Activity	GABA	3
	β alanine	3
	Taurine	3
	γ amino β hydroxy butyrate	2
	N methyl β alanine	2
	Glycine	2
	δ amino valeric acid	2
	ε amino caproic acid	1
	3 amino propane sulphonic acid	6 - 15

These values were obtained by Curtis & Watkins 1960; 1961. Excitation was indicated by an increase in frequency of discharge spikes and depression by a reduction in frequency. The larger numbers denote greater excitatory activity in the upper group and greater depressive activity in the lower group.

However, some variation in the response of neurones has been demonstrated, for example, thalamic neurones vary markedly in their responsiveness to glutamate (McLennan et al 1968) only a few units of the olfactory bulb are responsive to glutamate (Von Baumgarten et al 1963) and large pyramid neurones of the cerebral cortex have a very high threshold of action (Krnjević 1964). This does indicate a certain degree of specificity which is further shown by the fact that glutamate must be iontophoresed onto an external portion of membrane for depolarization to occur (Takeuchi & Takeuchi 1964).

Indirect evidence of glutamate action has been shown by microinjections of glutamate into specific areas of the hypothalamus of cat, which was found to mimic the effect of electrical stimulation in these areas in producing attack and flight behaviour (Brody, De Feudis & De Feudis 1969).

(ii) Application of glutamate and GABA (b) in vitro. When glutamate is added to cerebral cortex slices incubated in metabolic medium, there is a drop in membrane potential from (-50 to -60 mV) to (-20 to -40 mV) which represents a significant drop of 26% in the resting potential (Hillman & McIlwain 1961). The illustration of this depolarisation phenomenon enabled a comparison in vitro of the ion fluxes occuring with glutamate, with those occuring during natural excitation processes. Within a minute of adding glutamate to a brain slice, there is an increase in the Na^+ and a decrease in the K^+ of the non-inulin space, which is used as an index of the intracellular volume (Harvey & McIlwain 1968; Bradford & McIlwain 1966). Subsequently there is continued Na^+ influx accompanied by K^+ and water influx, the latter preventing a rise in internal osmolarity. The initial loss of K^+ and gain of Na^+ is the same as that occuring with the depolarisation of an EPSP and also following electrical stimulation of cerebral slices (Batchelard, Campbell & McIlwain 1962). In addition to glutamate, aspartate, cysteine, D, L homocysteine and α amino adipate caused membrane depolarisations (Bradford & McIlwain 1966) and all

amino acids produced a simultaneous decrease in phosphocreatine and increase in inorganic phosphate. Application of the Nearst equation to the data obtained, led to the conclusion that glutamate increased 5 fold the permeability of the tissue to Na^+ relative to K^+.

Glutamate also causes an increased calcium uptake (Ramsey & McIlwain 1970), parallel to that of Na^+, but smaller, the two rates being 0.8 and 36.0 μ equivalents/g/tissue/10 minutes for Ca^{2+} and Na^+ respectively after incubation in 5mM glutamate. The Na^+ uptake occuring with stimulation is in fact accelerated by low external Ca^{2+} conditions produced by addition of calcium chelators such as EDTA, EGTA and ATP. However, the effect of glutamate is not likely to be due to the chelation of calcium, as the degree of chelation is considerably less than the action implies, i.e. glutamate excites neurones more powerfully than stronger chelators (Krnjević & Phillis 1963; Curtis, Perrin & Watkins 1960). Glutamate uptake itself occurs and is slightly faster in calcium free medium than one containing 2.7mM Ca^{2+} (Takagaki, Hirano & Nagata 1959). Both the glutamate stimulated Ca^{++} entry and neural excitation are inhibited by tetrodotoxin (Ramsay & McIlwain 1970; Pull & McIlwain 1970).

(c) Action of glutamate and GABA in invertebrate tissue. From studies of the simpler nervous systems of crustaceans and insects, GABA has been shown to be almost certainly an inhibitory transmitter. GABA was first identified as a possible inhibitory transmitter, when Elliott and others (Bazemore, Elliott & Florey 1957; Levin, Lovell & Elliott 1961) identified GABA as an active agent in mammalian brain extracts which had an inhibitory action on crayfish stretch receptors (Florey 1954; Elliott & Florey 1956). This inhibitory action was later found to be indistinguishable from synaptic inhibition (Kuffler & Edwards 1958; Takeuchi & Takeuchi 1965; 1966a; 1966; Dudel 1965; 1965a). GABA action also duplicates inhibitory transmitter

action in that both are blocked by picrotoxin in crustacean muscle (Curtis & Watkins 1965) and both reduce the number of quanta released from the excitatory nerve (Dudel & Kuffler 1961).

Invertebrate muscles contract in response to electrophoretically applied glutamate as shown in crayfish (Takeuchi & Takeuchi 1964) and locust at 6×10^{-7}M (Usherwood & Machili 1966) and by perfusion with glutamate in the cockroach (Kerkut, Shapira & Walker 1965), the response increasing with concentration. Application induces depolarisation and is blocked by GABA (Usherwood & Machili 1966; Kerkut & Walker 1967).

(ii) Release of glutamate and GABA. In vivo studies have been carried out on the release of amino acids from the surface of exposed cerebral cortex, or into perfused ventricles following direct and indirect stimulation of neuronal pathways. In vitro release has been measured by depolarisation of brain slices. However, by all these methods the place of collection is remote from the site of release. It is not certain where the released amino acids have come from, whether they have been released from nerve terminals by a physiological process or produced as a result of metabolism. Assuming that the amino acids have been released from nerve terminals it is still not known whether they are directly associated as a transmitter in the pathway which has been activated, or indirectly. Another difficulty which might be encountered is that the released substance is either enzymically inactivated, or removed by uptake before measurement is made. Despite these problems, measurable amounts of amino acid have been detected and correlations have been made with neuronal activity.

(a) Release of glutamate and GABA in vivo. By using a superfusion chamber fixed in the skull over the surface of cat cerebral cortex, the rate of release of glutamate into the superfusate has been found to be reduced

to one half in the sleeping state, as compared to the aroused animal. GABA showed an opposite effect, increasing 2-3 fold in sleeping animals. Other amino acids showed no detectable change (Jasper, Kahn & Elliot 1965). In similar cerveau isolé preparations the rate of release of glutamate during electrical stimulation of the midbrain reticular formation was increased, together with that of ACh, taurine and aspartate. An increase in the release of ACh due to mesial thalamic stimulation produced no increase in glutamate, showing that the glutamate and ACh pathways that overlapped in the midbrain were separate in the thalamus (Jasper & Koyama 1969). GABA was released in largest amounts during slow wave sleep or following lesion of the midbrain reticular formation.

Release of GABA has been shown by Mitchell & Srinivasan (1969) to occur in vivo following cortical inhibition. In these experiments, perspex cylinders containing Krebs bicarbonate medium were placed over the posterior lateral gyrus of cat, the occipital lobes having been exposed. After preloading with H^3 GABA, an initial rapid efflux of GABA was observed. Stimulation of the geniculate nucleus caused inhibition of firing of the posterior lateral gyrus and a significant release of H^3 GABA. An increased release of endogenous GABA (by 3-7 times) has also been demonstrated during cortical inhibition produced by stimulation of the lateral geniculate nucleus or cortical surface (Iversen, Mitchell & Srinivasan 1971). Removal of calcium did not effect the resting release but increased the release evoked by stimulation. Under these experimental conditions, there was no difference in the rates of release of other amino acids during stimulation. However, it would seem possible that some cortical excitation might occur during this stimulation, and glutamate has been shown to be released by Jasper & Koyama (1969). Local superfusion of monkey brain has also shown GABA release (De Feudis et al 1970). While it is still possible that the released amino acid may have come from a non neural source,

the levels of GABA in blood and CSF are negligible. However, release of glutamic acid from retina appears to be attributed to a blood source (van Harreveld & Kooiman 1965).

An increased release of GABA from perfused 4th ventricle follows repetitive stimulation of the cerebellar cortex (Obata & Takeda 1969), perhaps indicative of liberation from Purkinje cell terminals in Deiters' and cerebellar nuclei lying close to the ventricle.

(b) Release of glutamate and GABA in vitro. Accelerated release of H^3 GABA from preloaded brain slices has been shown following electrical stimulation, elevated medium K^+ (Katz, Chase & Kopin 1969; Srinivasan, Neal & Mitchell 1969) and addition of glutamate (10mM) to the medium (Arnfred & Hertz 1971), the potassium stimulated release being Ca^{2+} dependent (Srinivasan et al 1969). About 30% of the total GABA present in cortex slices was released by elevated K^+ (Machyama, Balazs, Hammond, Julian & Richter 1970) but in the same experiments, aspartate and glutamate were only increased 3-5% by elevated K^+. However, glutamate release was detected from corpus striatum slices by both electrical and K^+ stimulation after preloading, though the release was similar for lysine, leucine and taurine and unaffected by absence of Ca^{++} (Katz, Chase & Kopin 1969). Glutamate and aspartate have also been shown to be released from cerebral cortex slices with K^+ stimulation (Arnfred & Hertz 1971). A more specific release has been shown using frog sciatic nerve which, when pre-incubated in the presence of a labelled amino acid (one of the following:- glycine, aspartate, glutamate, lysine, leucine, GABA or glutamine), only the rate of release of glutamate was found subsequently to increase with electrical stimulation (Wheeler, Boyarsky & Brooks 1966). Similarly, glutamate has been shown to be released by stimulation of cockroach leg muscle (Kerkut, Leake, Shapira, Cowan &

Walker 1965) and GABA released by stimulation of lobster inhibitory nerve fibre (Otsuka, Iversen, Hall & Kravitz 1964).

(iii) Distribution of glutamate, GABA and aspartate. Glutamate, GABA and aspartate have a wide distribution in the CNS; whole mammalian brain contains 9-13 μ moles/g glutamate; 1.5-4 μ moles/g GABA and 2 μ moles/g aspartate (Tallan 1962). In mammals there is an almost exclusive occurrence of GABA in the CNS, and small amounts are also found in the kidney (Whelan, Scriver & Mohyuddin 1969). In vertebrates there is more glutamate in brain than any other tissue and in the brain it is the amino acid present in the highest amount. The most abundant amino acid in invertebrates is either aspartate or taurine, the latter also being found in considerable amounts in mammals.

Distribution studies can yield information about possible transmitter function at 2 levels, firstly by showing an enhanced concentration of amino acid to be associated with a region enriched in specific neurone types, and secondly by finding an enhanced concentration of amino acid in a neuronal subfraction such as the nerve terminal fraction with a possible role in transmission. Distribution studies on different regions of the cat CNS (Johnson & Aprison 1970; 1971) show that glutamate is present in larger amounts in grey matter than white and the cortex shows the highest concentrations (see Table 3). Within the regions of the cerebral cortex there is variation, which is not reflected by differences in total amino acids, since this remains fairly constant.

(a) Regional distribution. In the spinal cord, there is a concentration of glutamate in the dorsal root fibres and dorsal grey quadrant, the site of termination of the majority of primary afferent fibres (Graham, Werman

Table 3 Free glutamate in various regions of the cat CNS (μ moles/g)

Sympathetic ganglion*	3.0
Optic nerve	3.5
Dorsal root ganglion*	4.3
Corpus Callosum	5.9
Grey Lumbrosacral cord*	6.9
Superior/inferior colliculus	8.1
Thalamus	8.9
Somatic/auditory cortex/ cerebellum	10.6
Caudate nucleus	11.8
Pyriform lobe	12.4
Sylvian-ectosylvian gyri	13.0

Data from Johnson & Aprison (1971) except * from Johnson & Aprison (1970)

et al 1967; Aprison et al 1965), which is where one would expect to find a concentration of excitatory transmitter. The ventral grey matter has the second highest concentration of glutamate which would be in keeping with some dorsal root fibres continuing into the ventral grey matter to form monosynaptic connections with motor neurones. An excitatory transmitter released by spinal interneurones (in the dorsal grey) with terminals in the ventral grey matter would be more concentrated in ventral grey matter, followed by dorsal grey matter and this is the pattern to which aspartate conforms. Moreover, there is a correlation between loss of aspartate and disminished interneurone cell count, following interneuronal lesions of feline spinal cord caused by asphyxiation. Glutamate is also reduced under these conditions but with no similar correlation with cell count (Davidoff, Graham, Shank, Werman & Aprison 1966). Of the free amino acids found in the feline spinal roots, dorsal root ganglia and peripheral nerves, glutamate is the only amino acid showing disproportionately high concentrations in the part of the dorsal root between the ganglion and spinal cord (Duggan & Johnston 1970), which only contains central processes of dorsal root ganglion cells (all types of sensory fibre, cutaneous, autonomic and afferents from muscles and joints). This distribution is consistent with glutamate being an excitatory transmitter at the terminals of primary afferent fibres.

In the same way that inhibitory activity is correlated with a high concentration of GABA in lobster inhibitory nerves, being a hundred times more concentrated than in excitatory nerves (Kravitz, Molinoff & Hall 1965), distribution studies in mammalian cerebellum and retina lead to similar conclusions. Purkinje cells, which are inhibitory in function, have a high concentration of GABA, higher than the cerebellum as a whole or any other cerebellar region (Hirsch & Robins 1962; Kuriyama, Haber, Sisken &

Roberts 1966). In addition to this, the Deiters neurones with which the Purkinje cells synapse are hyperpolarised by externally added GABA (Obata, Ito, Ochi & Sato 1966). These two corroborative pieces of evidence can only point out the likelihood that GABA is the inhibitory transmitter released from Purkinje cells. A clearer demonstration of GABA distribution has been shown by Hökfelt & Ljungdahl (1972), who employed autoradiography combined with electron microscopy. They were able to show that labelled GABA became clearly associated with the nerve terminals of basket cells. These cells are inhibitory interneurones synapsing onto the cell bodies of Purkinje cells which were themselves found to have low activity. The high levels of GABA in Purkinje cells may partially be attributed to a high GABA concentration in the surrounding inhibitory basket cells, where GABA is likely to be present in order to function as an inhibitory transmitter.

GABA is a known constituent of the retina (Kojima, Mizuno & Miyazaki 1958; Yamamoto, Aono & Kunimitsu 1964), found particularly in the innermost layers together with its synthesising enzyme, GAD (Graham, Lolley & Baxter 1968; Graham, Baxter & Lolley 1970; Kuryiama, Sisken, Haber & Roberts 1968). By a combination of electrophysiological autoradiography and more specific distribution evidence, the action of GABA as an inhibitory transmitter in the retina has become established. Autoradiographic studies by Ehinger & Falck (1971) on the uptake of H^3 GABA by rabbit retina tissue <u>in vivo</u> after intravitreal injection or after <u>in vitro</u> incubation show an association of GABA particularly with a proportion of amacrine cells (perhaps a subgroup) and also variable activity with ganglion cells. This shows that it is a specific property of certain neurones to take up GABA. These results agree with the electrophysiological evidence that GABA has an inhibitory effect on retinal responses (Ames & Pollen 1969; Straschill

& Perwein 1969; Scholes & Roberts 1964; Kramer, Shermen & Seifter 1967; Kishida & Naka 1967). Analysis of GABA content and GAD activity of 6 layers of the retina (Graham 1972), which are indicated in the diagram (Figure 3), show that the inner synaptic layer (layer 5) contains the most GABA and by far the greatest GAD activity. These results coincide with this layer containing the greatest proportion of amacrine nerve endings. The layers on either side contain the next highest amounts, and are layer 6,rich in ganglion cells and amacrine nerve endings and layer 4,rich in amacrine cell bodies and bi-polar cells.

When these autoradiographic techniques were carried out using labelled glutamate and aspartate and incubating retina both in vivo and in vitro (Ehinger & Falck 1971), radioactivity occurred in all layers including the pigmented epithelium. The retinal neuronal cells were only slightly radioactive and the general pattern of labelling with these compounds fits in well with the glial Müller cells, which have their cell bodies in the middle of the inner nuclear layer and send their processes to the inner and outer limiting membrane. This ,however, does not correspond with the fact that the inner synaptic layer is especially sensitive to glutamate (De Robertis 1964) and that glutamate causes an increased rate of firing in ganglion cells, when injected into the ganglion layer in vivo (Noell 1958). Uptake may thus be only regional specific for GABA and not for glutamate.

In vitro studies show that cortex slices and nerve endings (Srinivasan et al 1969; Kuriyama et al 1969) readily take up labelled GABA and the labelled GABA readily mixes with endogenous GABA (Neal & Iversen 1969) and since there is no significant metabolism, the uptake of H^3 GABA can be combined with electron micrograph studies to localise the uptake structures. Bloom & Iversen (1971) found that most of the label was recovered in the nerve endings of both cerebral slices and homogenates / and other structures such

Figure 3. Distribution of retinal cells

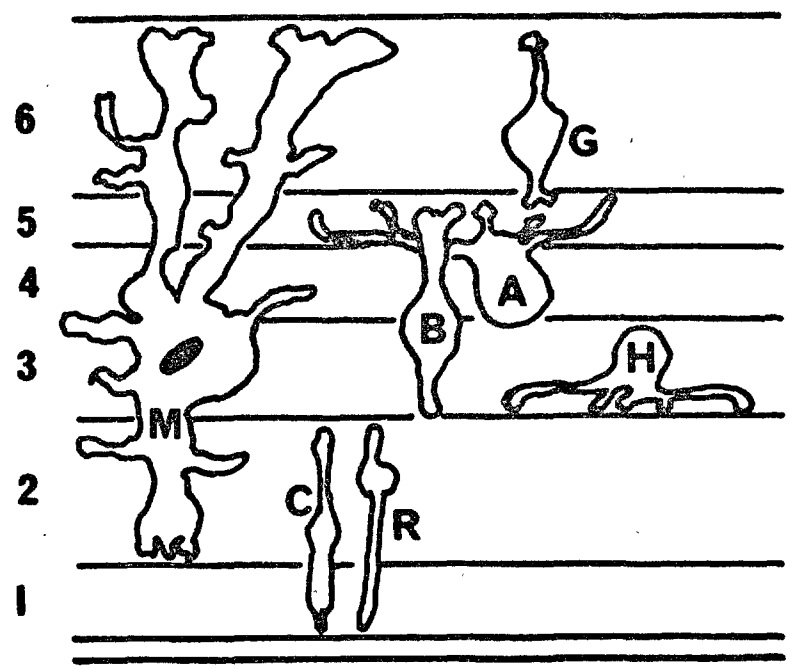


Diagram to show the spatial distribution of retinal cells and layers as dissected by Graham (1972). Key to cell types G-ganglion cell; A-amacrine cell; M-Muller cell; B-Bipolar cell; H-Horizontal cell; R-Rod; C-Cone.
Layer 1 is the pigmented epithelium; Layer 2, the photoreceptor layer; Layer 3, the outer synaptic layer; Layer 4, the bipolar layer; Layer 5, the inner synaptic layer and Layer 6 the ganglion layer.

as myelin and mitochondria did not tend to take up GABA. They estimated that about 30% of the nerve endings were labelled which gives an idea of the abundance of GABA in the cerebral cortex.

(b) Distribution in synaptosomes and their subfractions. Many amines of postulated transmitter function such as ACh, 5HT, noradrenaline and dopamine have been found to be concentrated in synaptosomes obtained by density gradient centrifugation (as discussed in previous section). In view of this type of distribution, the amounts of glutamate and GABA in various subcellular fractions were measured (Weinstein, Roberts & Kakefuda 1963; Ryall 1964; Mangan & Whittaker 1966). The results showed that about 10% of the total amino acids were recovered in the synaptosome fraction and that this distribution followed that of other cytoplasmic constituents such as lactate dehydrogenase (LDH) and K^+. There was also found to be no significant difference in distribution between pharmacologically active amino acids and inactive amino acids (Mangan & Whittaker 1966).

Osmotic rupture of the synaptosome fraction yields synaptic vesicles, cytoplasm, membranes and undisrupted synaptosomes which can be separated by density gradient centrifugation (Whittaker et al 1964) and about 20% of the amino nitrogen is found in the vesicles and 30% is found in the undisrupted fraction (Mangan & Whittaker 1966). These preliminary results on the distribution of amino acids do not show an obvious concentration in the nerve endings or synaptic vesicles and it should be pointed out that the vesicle fraction referred to above contained a large proportion of cytoplasmic material. However, the amounts of amino acid present appears to be sufficient to act as transmitter. Krnjević & Whittaker (1965) applied aqueous extracts of synaptic vesicles by electro-osmosis from micropipettes to cortical neurones and found that the material exerted a powerful depressant action similar to that of GABA. Whittaker also calculated the amount of

glutamate and GABA present in one synaptosome from the amounts present in the synaptosomal fraction and from the estimation by polystyrane tagging (Clementi, Whittaker & Sheridan 1966) that 2×10^{11} synaptosomes were obtained per g fresh guinea pig cortex. The values obtained were that each synaptosome contained 0.4×10^{-17} moles glutamate, although this value would be higher if only a portion of the synaptosomes contained these amino acids. Since Krnjević & Phillis (1963) found that 10^{-15} moles of amino acid is sufficient to fire cortical neurones, then 250 nerve endings would be required to empty their contents in order to have a similar effect. This is not an abnormally high figure as there are over 8,000 nerve endings on a single neurone of cat sensorimotor cortex (Armstrong & Young 1957).

The distribution of amino acids in synaptosomes and their subfractions will be further considered in Chapter 7.

(iv) Uptake Mechanisms; a means of inactivating a transmitter.

A method of termination of the potent action of a transmitter would seem another prerequisite at the synapse as in the case of ACh, where large amounts of a degrading enzyme, AChE, are present, mostly bound to the synaptic membrane. For other transmitter systems there are specific uptake mechanisms (Iversen 1967; Shasken & Snyder 1970; Roberts & Kuriyama 1968; Blackburn, French & Merrils 1967; Iversen & Neal 1969), which in the case of noradrenaline in the peripheral nervous system has been shown to provide a method of transmitter inactivation (Iversen 1967). If uptake is a method of inactivation one would expect a high affinity transport for physiologically active amino acids. Logan & Snyder (1971) examined the uptake of amino acids by synaptosomes of rat cerebral cortex and spinal cord and found that all amino acids were accumulated by low affinity saturatable systems as in brain slices (Blasberg 1968). However, glutamate and aspartate in the cerebral

cortex and glycine additionally in the spinal cord had unique high affinity uptake systems as well. Double reciprocal plots for alanine, arginine, leucine, serine and glycine were straight lines in the cortex, showing a single mode of uptake with K_M = 2-15 x 10^{-4}M, whilst those obtained for glutamate and aspartate were curved and could be resolved into 2 components, one low affinity and one high affinity (K_M = 0.26 x 10^{-4}M). Similarly in the spinal cord all amino acids showed a low affinity uptake (K_M = 10-26 x 10^{-4}M) and in addition aspartate, glutamate and glycine showed a high affinity uptake (K_M = 0.22 x 10^{-4}M). Glutamate and aspartate showed mutual competitive inhibition not affected by GABA. This high affinity uptake has also been shown in rat spinal cord slices for glutamate (Hammerschlag et al 1971), and glycine (Neal & Pickles 1969; Johnston & Iversen 1971).

The property of nerve endings to take up amino acids has been used successfully by Snyder and his co-workers to separate nerve endings which accumulate different amino acids. It has been shown that there is a population of nerve endings in the cerebral cortex which selectively accumulates glutamate and aspartate, and equilibrates at a different density to GABA accumulating or catecholamine accumulating synaptosomes (Wofsey et al 1972; Kuhar et al 1970). At the moment this is only a qualitative separation, but if it could be adapted to a preparative scale, it would have useful application as a functionally homogeneous fraction. The fact that some synaptosomes may be specialized to take up glutamate and aspartate in preference to other amino acids or catecholamines supports the idea that glutamate and aspartate have a specific function in some synaptosomes which is thus separate from their widespread metabolic involvement.

Another physiologically active amino acid : Glycine. There is good evidence that glycine is an inhibitory transmitter in the spinal cord, reviewed by Curtis & Watkins (1965), Hebb (1970) and Aprison, Davidoff & Werman (1970). The case for this amino acid is more convincing as it is off the main metabolic route. The action of electrophoretically applied glycine on spinal motorneurones is indistinguishable from the natural inhibitory transmitter. Both produce hyperpolarization, increase membrane conductance, have the same reversal potential for hyperpolarization and their actions are blocked by strychnine (Curtis, Hosli, Johnston & Johnston 1968; Werman, Davidoff & Aprison 1967). The distribution of glycine is also suggestive that it is an inhibitory transmitter, autoradiography showing the localization of glycine in vesicles of spinal cord synapses (Matus & Dennison 1971) and the spinal cord content of glycine being highest in ventral grey matter where inhibitory interneurones synapse with motorneurones (Graham, Shank, Werman & Aprison 1967).

Although glycine has been shown to have a depressant action on cerebral cortex neurones (Krnjevic & Phillis 1963; Curtis, Hosli & Johnston 1968), GABA is a more potent depressant in this region. Glycine is not particularly enriched in the cerebral cortex, the pons medulla region containing levels, three times higher (Shaw & Heine 1965). This fact together with the reduced potency in the cerebral cortex indicate that glycine is not the major inhibitory transmitter in this region.

D. An outline of the experimental approach.

Synaptosomes, being metabolically active and potentially capable of measuring physiological processes, were used for the present study. In order to reproduce the in situ condition more closely than in the form of a suspension, synaptosomes were used as a

deposit between nylon gauzes termed a synaptosome bed. This could be rapidly transferred to and away from incubation medium.

A study was first made of the metabolic properties of synaptosome beds in order to test that the system allowed suitable penetration of oxygen and substrate and was capable of the same high rates of metabolic processes shown by suspensions of synaptosomes (Chapter 3). The metabolic parameters measured were respiration as determined by oxygen consumption, glycolysis by lactate production, phosphorylation by ATP and phosphocreatine production and synthetic ability by the incorporation of radioactive label from glucose into amino acids.

The effect of depolarizing influences such as application of electical pulses or elevated potassium were then measured on the metabolic parameters mentioned above. At the same time the release of amino acids to the medium was measured (Chapter 4). This release process was then studied in greater detail, its variation with time and the simultaneous occurrence of uptake processes as demonstrated by ouabain and amino acid analogue. Release was further shown to be sensitive to alterations in ion concentrations, for example, the absence of calcium completely inhibiting release (Chapter 5). Acetylcholine release in response to the same stimuli was also analysed and compared to amino acid release under identical conditions (Chapter 6).

From measurements of the specific radioactivities of amino acids released in response to stimulation as compared to those in the synaptosome bed, an indication of the origin of the released amino acid was obtained (Chapter 4). This was followed up by measuring the amino acid content of subfractions of synaptosomes, with special emphasis on the content of synaptic vesicles (Chapter 7).

CHAPTER 2

METHODS AND ASSAY PROCEDURES

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   (b) Removal of cerebral cortex
   (c) Homogenisation
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   (e) Modifications of procedure
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   (i) Respiration of synaptosome beds
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4. Preparation and purification of synaptic vesicles
   Principle
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11. Potassium determination

Appendix : A comparison between the respiration of synaptosome beds prepared by a long and short centrifugation procedure

METHODS AND ASSAY PROCEDURES

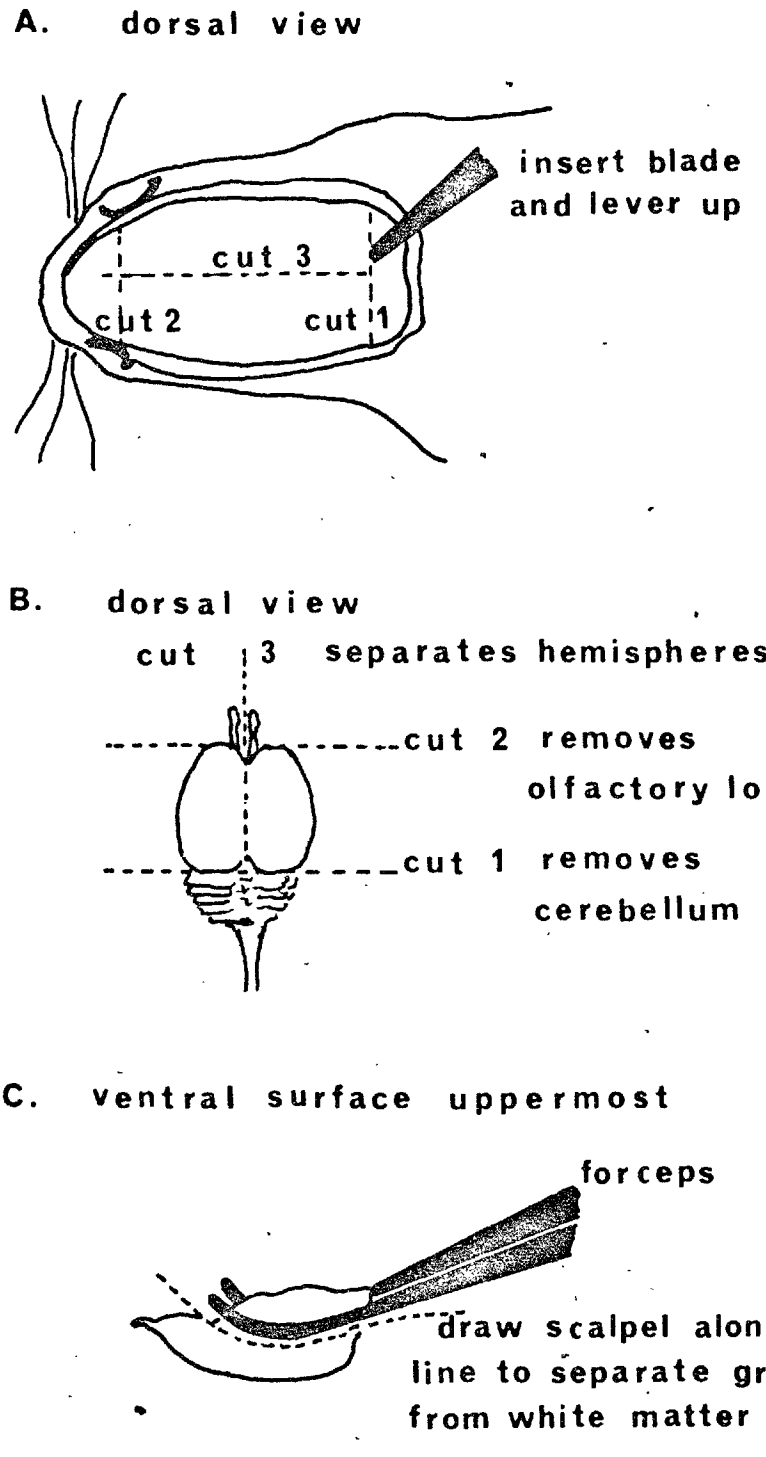
1. Preparation of Synaptosomes

(a) Principle. Brain tissue was homogenised in iso-osmotic sucrose under defined conditions of shear in which the cell bodies were fragmented and the nerve terminals were torn away from the axons and formed subcellular particles which appear to retain the morphology of the nerve terminal region. These particles known as synaptosomes can be separated from other cell components by means of differential centrifugation followed by discontinuous sucrose density gradient centrifugation. The method used was that of Gray & Whittaker (1962) with modifications as described by Bradford (1969).

All procedures were carried out at 0° - 4°C. Rotors were pre-cooled and glass vessels to be used in the experiment were kept on crushed ice. All glassware was thoroughly washed and rinsed in tap water followed by several washings of deionised distilled water. Sucrose solutions were kept refrigerated and made up within 1-3 days of use, as bacterial growth was found to develop very rapidly.

(b) Removal of cerebral cortex. Adult Sprague-Dawley female rats in the weight range 200-250 g were used. The animal was stunned by a blow on the cervical spine just sufficient to cause unconsciousness. The carotid arteries were cut and the animal exsanguinated by massaging the thorax, to reduce cerebral haemorrage. The ears and scalp were removed with scissors and three cuts were made, one cutting the spine at the base of the cranium, one between the two eye orbits and another along the sagittal sutre (Figure 4a). In making the last cut, the lower blade should be kept fairly close to the cranium in order not to destroy the cerebral tissue beneath. This enabled the dorsal surface

Fig. 4 - Dissection of cerebral cortex



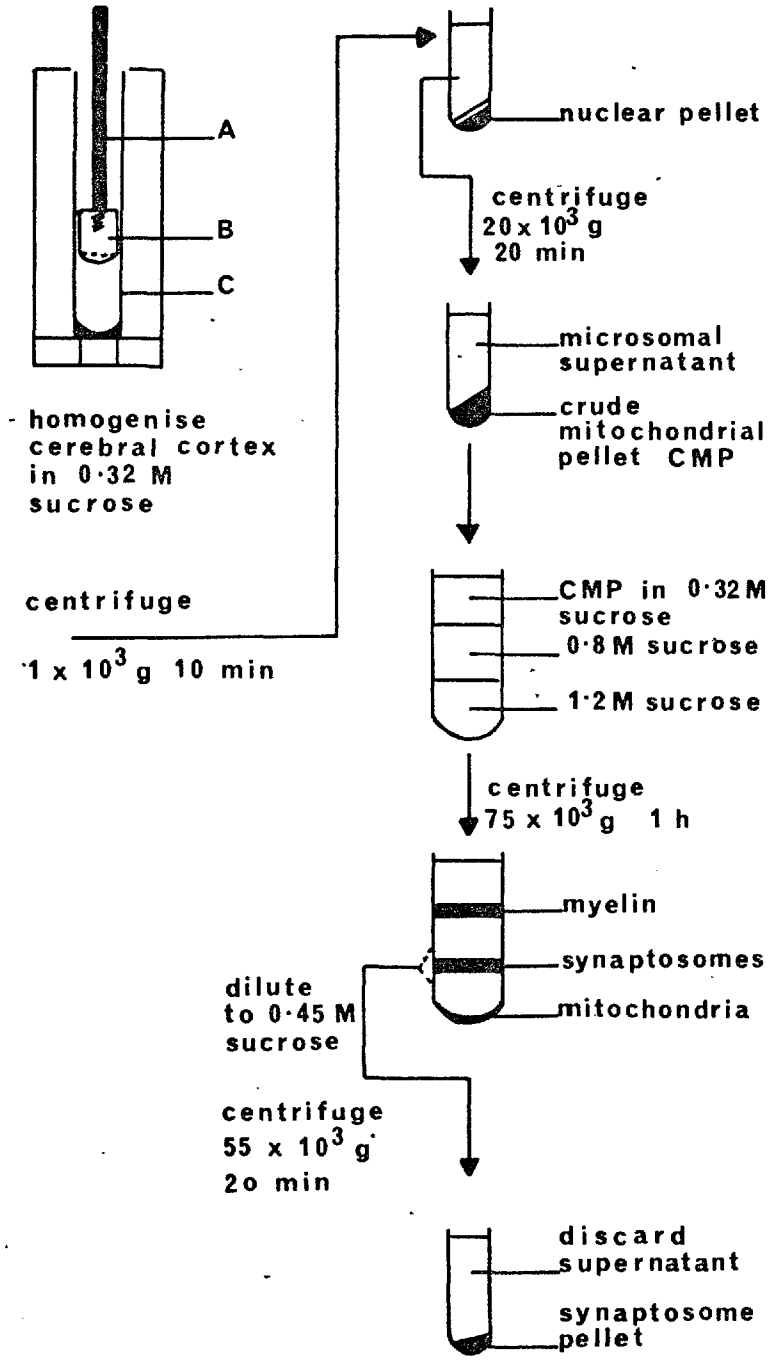
(roof) of the cranium to be levered up each side of the original longitudinal cut, thus exposing the brain. The whole brain was scooped up using a spatula and placed on a surgical block covered with a damp piece of filter paper. A cut was made separating the cerebellum and posterior parts of the brain from the cerebrum. Another cut was made separating the two cortices (Figure 4b). The white matter ventral to the cortex was gripped with a pair of forceps and separated from the grey matter by a movement of scalpel blade between the grey matter and the forceps (Figure 4c). The same was done to the other hemisphere, and the two were placed in a tared 50ml beaker kept on crushed ice. The whole procedure from the stunning of the rat to the placing of the cleaned cortex in the beaker was done as rapidly as possible and took less than 1 minute. The cerebral cortex was then similarly removed from 9 other rats. The total weight of cortex was obtained by reweighing the beaker containing the tissue.

(c) Homogenisation. A 10% suspension (w/v) was then made by adding 0.32M sucrose (85-90ml) to the cerebral tissue. From 10 rats, about 8.9g of tissue was usually obtained and 89ml 0.32M sucrose was added to this to make a 10% homogenate. The suspension was divided approximately into half, each half being placed in a 500ml conical flask kept on ice. This size flask with a large base exposed to cooling enabled the liquid contents to be rapidly cooled. Half of the suspension was placed in a glass homogeniser, homogenised by 3 up and down strokes of a perspex pestle, rotating at 900 rev./min., and returned to the flask in ice. Each up and down stroke occupied about 6 seconds. The perspex and glass tissue homogeniser had a radial clearance (i.e. the difference between the external radius of the pestle and the internal radius of the homogeniser) between the pestle and mortar of 250 microns (Aldridge,

Emery & Street, 1960). The other half of suspension was similarly homogenised and cooled,and the procedure repeated until each half of suspension had been homogenised by 4 groups of 3 strokes. The up and down strokes should be carried out evenly and not too slowly, in which case local heating is likely to occur. Cooling is also essential between successive periods of homogenisation.

(d) Centrifugation. A summary of the centrifugation procedure is given in Figure 5. Centrifugations were carried out in Beckman L2 or L2 65B Spincos. The 10% homogenate was centrifuged at 1,000g (4,000 r.p.m. in a 30 rotor) for 10 minutes. The resulting supernatant was removed carefully with a pipette (5ml) in order not to disturb the loose sediment of the nuclear pellet. This pellet from the first centrifugation contained mainly nuclei, large myelin fragments, blood vessels, glial cells and small amounts of synaptosomes. The supernatant was recentrifuged at 19,620g for 60 minutes (15,000 r.p.m. in Beckman Spinco rotor No. 30). The resulting microsomal supernatant was discarded and the pellet termed the crude mitochondrial pellet was resuspended in 0.32M sucrose (45ml) by 3 strokes using the perspex and glass homogeniser used in the initial homogenisation. The crude mitochondrial pellet contained small myelin and glial fragments, synaptosomes and mitochondria. The crude mitochondrial suspension was then carefully layered onto a sucrose gradient which had been previously made up. This gradient consisted of a layer of 0.8M sucrose (20ml), above 1.2M sucrose (20ml) in a centrifuge tube (for a Beckman Spinco SW 25.2 rotor). When adding sucrose of lighter density to a more dense solution it is necessary to tilt the tube containing the dense sucrose at a slight angle and to run in slowly the lighter density sucrose down the side of the tube in order to obtain a sharp interface between the layers. If the sucrose is run

Fig. 5 – PREPARATION OF SYNAPTOSOMES



in too fast the layers will mix. The sucrose gradient was centrifuged at 75,460g for 120 minutes (25,000 r.p.m. in a Beckman Spinco SW 25.2 rotor). Three fractions were obtained from this centrifugation, one at the 0.32/0.8M sucrose interface (A) containing myelin, one at the 0.8/1.2M sucrose interface (B) containing synaptosomes and small amounts of membrane fragments and the pellet (C) containing mitochondria. The fractions were removed by hand using a pasteur pipette. In removing the synaptosome band (B), equal amounts of 0.8 and 1.2M sucrose either side of the interface were taken in order to assume the final sucrose concentration in this fraction to be 1.0M. Cold distilled water was then added to this fraction dropwise and with continual shaking to bring the final concentration of the sucrose to 0.45M sucrose, which, to allow for entry of some sucrose into the synaptosomes during the fractionation, made the sucrose concentration approximately iso-osmotic with the contents of the synaptosomes. Synaptosomes are extremely sensitive to osmotic rupture and so the addition of water at this stage was done as gently as possible. The diluted suspension of synaptosomes was then centrifuged at 54,500g for 20 minutes (25,000 r.p.m. in a Spinco rotor No. 30). The resulting pellet was taken up in metabolic medium and used for preparation of synaptosome beds.

(e) Modifications of procedure. In later experiments it was found sufficient to carry out the 2nd centrifugation for 20 minutes and the 3rd (gradient) centrifugation step for 60 minutes, without loss of significant amounts of synaptosomal material. This method will be referred to later as the short centrifugation procedure and the previously described method as the long centrifugation procedure. A partial subfractionation of the nerve ending fraction was carried out in some experiments by inserting an extra step in the sucrose gradient

of 1M sucrose between 0.8M and 1.2M sucrose.

(f) Preparation of synaptosomes using a Ficoll Gradient. The same procedure as described above was followed except that the gradient was made up with discontinuous steps of Ficoll: 17.5%, 13%, 3%. The homogenate of the crude mitochondrial pellet was made up as usual and placed on top of the 3% Ficoll.

Before use, the Ficoll (Pharmacia Ltd) was made up in 0.32M sucrose at a concentration of 23% and dialysed against 0.32M sucrose at 0°C with stirring for 88h. Dialysis tubing of diameter 26mm (No. DL 620) was used. After dialysis there was an increase in volume giving a 17.5% Ficoll solution which was diluted with 0.32M sucrose, to maintain isotonicity, to obtain the other concentrations of Ficoll required in the experiment.

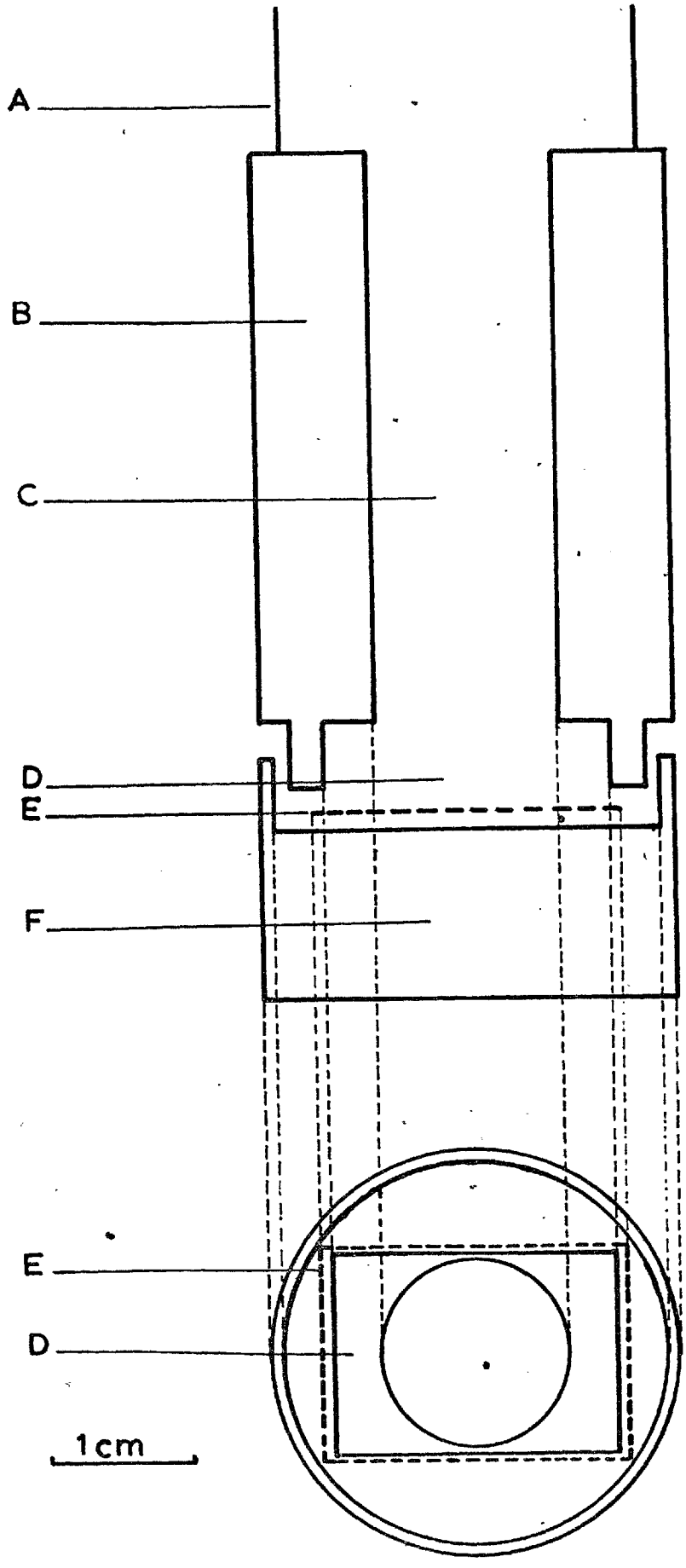
2. Preparation of Synaptosome beds

(a) Principle and preparation of apparatus. Synaptosome beds were prepared by gently sedimenting a suspension of nerve endings in medium, onto nylon gauze (aperture size 120μ and 110 mesh). The nerve endings aggregate under the ionic conditions of the physiological medium and form a deposit on the gauze. Another piece of gauze was placed on top of this to form a sandwich, which was then held between the jaws of a quick transfer holder. By means of this system, the synaptosome bed can be immersed in medium and be rapidly removed and fixed at the end of the incubation period.

Sedimentation of the nerve endings onto nylon gauze was carried out using a perspex cell, which is shown in Figure 6. This consists of a hollow cylindrical upper part (B), which makes a close fit with the lower part a solid removable base (F). The cell contains a

Figure 6 Cell for making synaptosome beds

A, metal rod to facilitate removal of cell from centrifuge bucket; B, hollow perspex cylinder; C, central cavity; D, rectangular cavity; E, nylon gauze; F, solid perspex base with push pull connection.

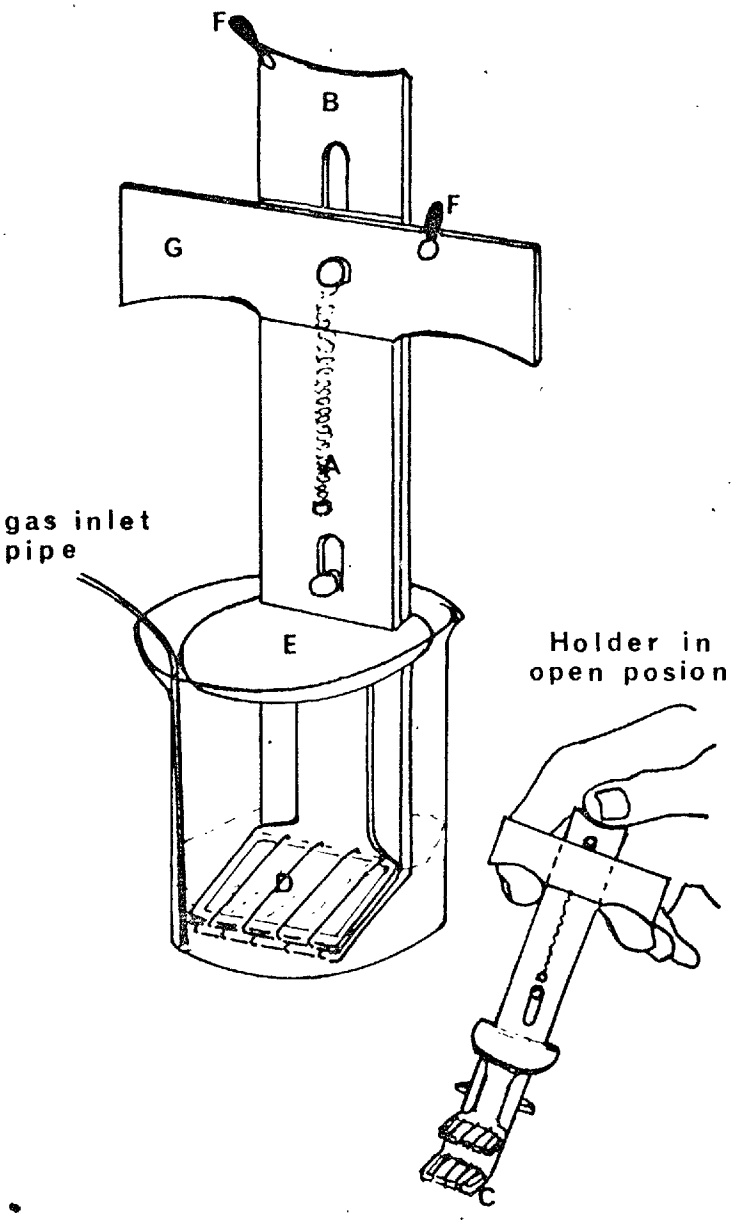


cylindrical cavity (C), which is continuous with a rectangular cavity (D). Two metal rods (A) in the top of the assembly facilitate the removal of the cell from the centrifuge bucket.

When preparing the cell for use, it was first thoroughly cleaned and a piece of nylon gauze was inserted (E) between the upper and lower part to overlap the rectangular cavity on all sides by 1-2mm. In order to do this the two parts (B and F) were separated, the upper part (B) was inverted, a piece of gauze was cut, just larger than the cavity, the corners were trimmed off and it was put in place. The top was carefully put on top and pressed down, without displacing the gauze from completely covering the cavity. The cell was then ready for use.

A quick transfer holder (described by McIlwain & Rodnight, 1962) consists of 2 strips of perspex, bent at right angles at one end to form two jaws as illustrated in Figure 7. The two strips are held together by a spring (A), which enables one part to slide down when depressed at the top (B) by a finger, while at the same time the other remains fixed(G).In the open position, a portion of tissue, such as a cerebral cortex slice or synaptosome bed may be rested on the lower jaw (C), then on release, the jaws close holding the tissue in place. The jaws consist of rectangles of perspex whose central part has been cut out. In the quick transfer holders used in the present experiments, silver wire (D) was wound round these rectangular frames about 4 times, and thus each jaw functioned as an electrode. The wire used for this purpose is coated with lacquer and so can be exposed only at the sides which come in contact with the material to be electrically stimulated, in all other regions the lacquer acts as a layer of insulation. A circle of sponge (E) functions as a lid, when the holder is placed in a 25ml beaker, in order to keep the system closed. Leads from a power

Figure 7_ A Quick Transfer Holder
(after McIlwain & Rodnight 1962)



source are connected to the electrodes at the terminals (F).

When a voltage is applied across the two electrodes of a quick transfer holder, held in a conducting fluid such as the medium and also under the conditions of an experiment, a current flows between the electrodes and a black deposit is formed on the surface of the exposed wire, which is probably Ag or AgO. Before using again, this layer was gently scraped off with a scalpel blade.

(b) Sedimentation of a suspension of nerve endings onto nylon gauze. Pellets from the nerve ending fraction were resuspended in metabolic medium (composition defined below) under mild homogenisation conditions using 7 strokes of a teflon pestle in a glass homogeniser of loose fit (radial clearance 180μ). All precedures in which the synaptosomes were in contact with metabolic medium, prior to incubation were carried out at room temperature. This prevents loss of synaptosomal contents, which occurs rapidly in ionic medium in the cold. Volumes (5ml) of this suspension, containing approximately 2-3mg protein/ml, were placed in each prepared cell and the cells were centrifuged at 550g for 10 minutes in an IEC International Centrifuge. The time and speed of centrifugation were critical in order to produce a substantial synaptosome bed without loss of material through the gauze, onto the perspex lid, which occured at higher g forces than those used in this preparation.

(c) Incubation procedure. Two types of incubation media were used, Krebs-phosphate and Krebs-bicarbonate medium (Keesey, Wallgren & McIlwain, 1965). The medium used in the individual experiments is indicated in the tables and legends. The composition of these media is as follows. with the concentration in mM in parentheses.

Krebs-phosphate Medium: NaCl (124); KCl (5); Na_2HPO_4 (20); KH_2PO_4 (1.2); $MgSO_4$ (1.3); $CaCL_2$ (0.75); p.H. 7.5 containing glucose (10) as substrate.

Krebs-bicarbonate Medium: NaCl (124); KCl (5); $NaHCO_3$ (26), KH_2PO_4 (1.2); $MgSO_4$ (1.3); $CaCl_2$ (0.75); p.H. 7.5 containing glucose (10) as substrate.

At the end of centrifugation the perspex cells were lifted out using the metal rods and the supernatant was decanted. The supernatant was fairly opalescent but did not account for much synaptosomal loss. Holding the cell upright, the lid was pulled straight off. The gauze remained adhering to the lid, with a creamy-white deposit of nerve endings evenly spread over the gauze, (about 2mm thick) with 1mm of gauze exposed all round. Using forceps to grip an uncovered corner of the gauze, the bed was lifted up and placed (synaptosomes uppermost) on the lower jaw of an open quick transfer holder. Then another piece of nylon gauze of the same size as the original, wet with medium was picked up with forceps and placed on top of the bed to form a sandwich. Care was taken to exclude air bubbles. In placing the second piece of gauze, first a corner of the gauze was aligned with an equivalent corner of the original and placed on it, then gently working along the length of the bed, the gauze was let down to make contact with the nerve endings. The jaws of the holder were closed without squeezing the synaptosomal material out of place. The synaptosome bed and the relevant portions holding the bed were immersed in 5ml medium contained in a 25ml beaker, held in a water bath maintained at 37°C. The medium was gassed by means of a gas inlet tube, dipping beneath the surface of the medium. O_2 gas was used in equilibrium with phosphate medium and 95% O_2/ 5% CO_2 was used with the Krebs-bicarbonate medium. This system

gives rapid distribution of a drop of aqueous methylene blue which indicated that the method of circulation and agitation was sufficient. The whole procedure described in this paragraph takes 30-40 seconds to complete for one synaptosome bed. This procedure was then repeated for the remaining synaptosome beds.

No additions were normally made during the first 30 minutes of incubation. After this period, stimulation where appropriate was begun by application of electrical pulses or addition of K^+, or the effects of added compounds were studied by making their additions at this point. (The details of this are described below).

The compositions of various media used in the experiments are summarised in Table 4.

Table 4 Composition of Incubation Media

Medium	Composition (mM)						
	KCl(5) KH_2PO_4(1.2) $MgSO_4$(1.3) Glucose(10)	Na_2HPO_4 (20)	$NaHCO_3$ (26)	$CaCl_2$ (0.75)	NaCl (124)	EGTA	Sucrose (124)
Phosphate medium	*	*		*	*		
Bicarbonate medium	*		*	*	*		
No Ca^{2+}/bicarbonate	*		*		*		
No Ca^{2+}/EGTA/ bicarbonate	*		*		*	*	
Low Na^+/phosphate	*	*		*			*

Key: * indicates that this component is present

(d) Termination of incubation. At the end of the incubation period, (i.e. specific periods between 40 and 60 minutes of total incubation) the quick transfer holder was lifted out of the incubation medium, and a few drops of fluid adhering were allowed to drain. Then the quick transfer holder was opened, the bed lifted out with forceps and put into 5ml 1.0M PCA and mixed by vortex. This fraction was later analysed for protein and amino acids. Extraction of ATP and phosphocreatine is described below.

The incubation medium was fixed in a similar way, by placing in 3.0ml M PCA, and mixing by vortex. When lactate measurements were to be made, the supernatant was first rapidly centrifuged, to remove the small amount of protein, sometimes present in the medium, at 800g for 1 minute, and then frozen and stored at -22°C until used. The amount of protein lost from the bed to the medium was not more than 5% of the total bed protein.

These steps, described so far, represent the procedure referred to during the thesis for the preparation of synaptosome beds, except when oxygen uptake is measured, in which case the method is adapted as indicated below (Section 2 (i)).

(e) Electrical stimulation. After 30 minutes preincubation of synaptosome beds, square wave pulses of 10 volts, 0.4 ms duration and alternating in polarity were applied to either quick transfer electrodes or grid electrodes at a frequency of 100/s for 5-30 min. as indicated in the text, the average continuous current being 35 mA through tissue and medium. Square wave pulses were applied using an A.F. signal generator (Type H I. Advance, England) and a power amplifier. An oscilloscope (Type 502 A Dual beam - Tektronix, Guernsey Ltd.)

was used to measure the voltage and current passing through the apparatus.

(f) Potassium stimulation. Synaptosome beds held in quick transfer holders were incubated in medium for 30 minutes after which 0.25ml 1.0 M KCl made up in medium was injected into the medium to give a final concentration of K^+ in the medium of 56mM. Incubation was continued for a further 10 minutes before removal of the synaptosome bed and extraction as described above. Medium containing extra NaCl equivalent to the KCl, was added to the controls or in some experiments, Krebs-bicarbonate medium alone was added to the controls. The procedure used in the particular experiment will be indicated in the tables and legends.

(g) Incubation with ouabain and amino acid analogues. Synaptosome beds were incubated as described above for 30 min. before addition of ouabain to a final concentration of 0.05mM, or analogues (BABA, DABA and NAG) to a final concentration of 1mM. Medium containing the analogue or ouabain was injected into the 5ml of incubation fluid at the point of gassing in order to ensure rapid mixing and distribution.

In order to assess the stability of analogues, BABA, DABA and NAG were incubated separately at 1mM in medium at 37°C for 1 hr., in the absence of tissue. The media were analysed for amino acid content. There was found to be no conversion to other amino acids (e.g. GABA or glutamate) and where the analogue was found to be ninhydrin positive as in the case of DABA, its position on ion exchange chromatography did not obscure other amino acids.

(h) Incubation in the presence of ($U^{-14}C$) glucose, tetrodotoxin or citrate. Additions of these compounds were made before incubation commenced and the amounts used are summarised in Table 5.

Table 5 Additions made to the Incubation Medium

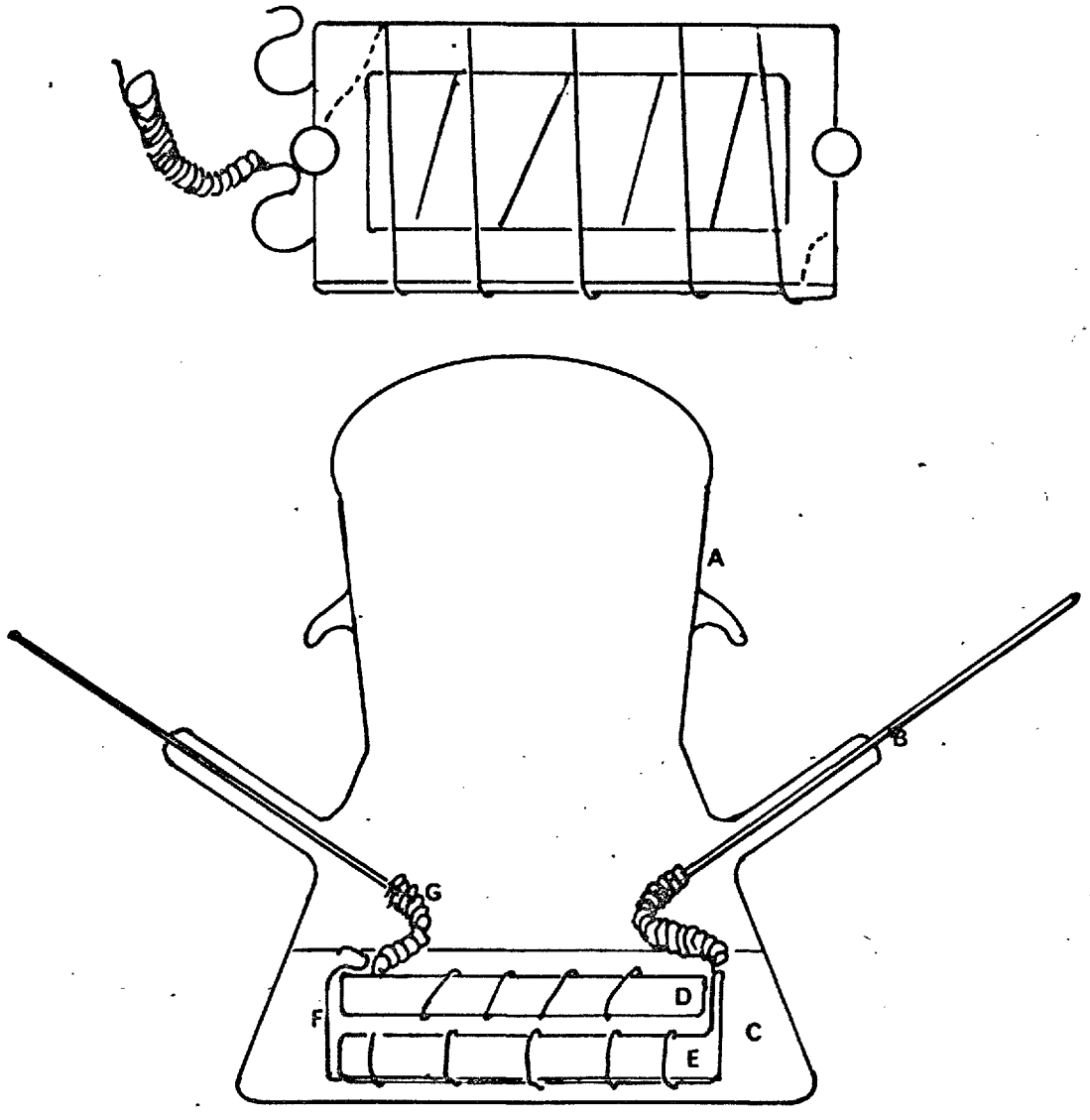
Compound	Amount added	Final Concentration
a) Additions made after 30 min incubation		
KCl	250µl 1.0M made up in medium	56mM (K^+)
NaCl	" "	214mM (Na^+)
DABA	50µl 100mM	1mM
BABA	"	"
NAG	"	"
Ouabain	10µl 10mM	0.05mM
b) Additions made just prior to incubation		
Citrate	50µl of 100mM	1mM
Tetrodotoxin in citrate buffer	160µl of 3mM	100µM
($U^{-14}C$) glucose	100µl of 250u Ci/ml	5µ Ci/ml

(i) Measurement of respiration of synaptosome beds. In order to measure oxygen uptake, a closed system is required, and for this purpose a smaller synaptosome bed was prepared with dimensions 0.7 by 1.5cm which could be placed inside a Warburg flask. Synaptosome beds of this size were prepared in a similar way to that described

above except that the perspex cell used had a smaller rectangular cavity with these dimensions, and the cerebral cortices of 5 rats only, were required for preparation of 4 synaptosome beds. This small synaptosome bed was then placed between the valves of grid electrodes (McIlwain & Rodnight, 1962), which consist of perspex rectangles (A), round which is wound silver wire in a similar fashion to the quick transfer electrodes, and as illustrated in Figure 8. There are two "S" shaped clips to hold the 2 valves together (A), and two perspex guides on each side of the lower valve which fit into corresponding recesses on the upper valve serving this purpose too. From each electrode comes a coiled lead (C), which fits over a gold stub, fused into the glass wall of a Warburg flask (volume about 9ml) forming a connection through which a current may be applied.

When the synaptosome bed had been placed between grid electrodes, the assembly was placed inside a Warburg flask to lie flat, with the coils of wire protruding upwards. Then using a small pair of forceps, the coils were pulled over the gold stubs attached to the wall of the flask. The bed was covered with 2ml of medium and a wick of filter paper soaked with hyamine (Packard Instruments, Illinois, U.S.A.) solution (1:1 with H_2O) and damped dry, was placed inside the well (D) of the flask to absorb carbon dioxide. A rod was placed in the side arm of the flask and the flask was attached to a manometric arrangement, using a spring and lanolin to grease the connections and the flask was immersed in a water bath at 37°C. The flask was first gassed with oxygen for 5 minutes with the rod air inlet open, then the oxygen inlet and rod air inlet were closed. The system was allowed to equilibrate for 5 minutes and then manometric readings were taken of the contraction

Figure 8 Grid Electrodes



in volume of the oxygen of the system due to the uptake of oxygen by the synaptosome bed.

Electrical stimulation was carried out as described above in Section 2 (e). When the current was turned on, there was an initial expansion in gas volume, and the accompanying manometric change was a characteristic of the Warburg flask. This change was determined by incubating 2ml of medium in a Warburg flask of the type used in these experiments at 37°C with a hyamine wick in the centre well. A thermal barometer was also incubated to check for atmospheric changes. After oxygenation and 20 minutes preincubation, electrical pulses were applied of the same magnitude as used in the metabolic experiment. The manometric change reached a maximum 2.5 minutes after application of pulses and thereafter remained a constant value for the rest of the incubation. This value was subtracted from all manometric changes occuring during electrical stimulation.

(j) Respiration of synaptosome suspensions. Synaptosomes were prepared as described in section 1 (a) - (d) of this chapter. The pelleted synaptosomes were taken up in Krebs-phosphate medium (18ml of medium for the synaptosomes obtained from the cerebral cortices of 10 rats), and resuspended at room temperature with a loose fitting teflon pestle and glass mortar (radial clearance 180μ). Volumes (3ml) of this suspension were pipetted directly into Warburg flasks (about 15ml total volume). Hyamine soaked wicks were placed in the central wells and the flasks were placed in a water bath at 37°C and gassed and equilibrated as described in section 2 (i). Some flasks possessed 2 gold ring electrodes with fused connections through the wall of the flask, which could be used for electrical stimulation of the synaptosome suspension. The procedure for stimulation and the

characteristics of the electrical pulses applied are described in section 2 (e). At the end of incubation the suspensions were removed from the flasks and centrifuged at 96,600g for 5 minutes (4,000 r.p.m. in a Beckman Spinco rotor No. 50), in order to separate the particular fraction from the medium.

(k) Apparatus suppliers

Nylon Bolting cloth 110 mesh, i.e. 110 threads/inch aperture size 120μ from J. Stanier Ltd., Manchester 3, Lancs.

Quick Transfer Holders from Specialized Laboratory Equipment Ltd., 20 Sydenham Road, Croydon, Surrey.

Perspex Cells from Alpha Neurochemistry Research Apparatus, 56 Abbotts Road, Mitcham, Surrey CR4 1JU.

3. Preparation of Cerebral Cortex Slices

Principle. Cortex slices were cut from the outer surface of the whole cerebral cortex using a strip of razor blade and a glass guide recessed to give slices 350-400μ in thickness and weighing 50-100mg. The details of this procedure are described by McIlwain & Rodnight (1962). It is necessary to cut a thin slice in order to allow oxygen to reach all cells in the slice and to carry out the operation rapidly so that the slice may reach oxygenated medium as soon as possible.

Procedure. The cerebral cortex was removed as described earlier for preparation of synaptosomes, the two cortices separated and the ventral white matter scooped out with a scalpel. One cortex was placed on an operating table, which was covered with a piece of filter paper moistened with distilled water, and the other was placed in a moisture chamber

which consisted of a petri dish, with moist filter paper on the inner surface of the lid. The guide was rested on the dorsal surface of the first cortex, recessed surface nearest the cortex, and the blade held at right angles to the guide was advanced through the tissue keepting contact with the guide. Thus the portion of cortex fitting into the recess on the guide was separated from the rest of the tissue. In carrying this out, the guide was moved through an angle following the curvature of the cortex, to give a maximal area for the slice. The blade was also held at an angle to the guide to prevent excess contact with the tissue. The slice which adhered to either the blade or guide was removed to a bent rider, with which it was weighed on a torsion balance. The slice was then floated in Krebs-Ringer medium and wafted between the jaws of an open quick transfer holder. The slice was evenly spread out over the grid, the holder shut and the slice immersed in medium and incubated as indicated for synaptosome beds (Section 2 (c)). In some experiments, the slices were sandwiched between two pieces of nylon gauze before placing in the quick transfer holder. For respiration studies, grid electrodes positioned in Warburg flasks were used in place of quick transfer holders.

4. Preparation and purification of Synaptic Vesicles

Principle. Synaptic vesicles were prepared by the method of Whittaker, Michaelson and Kirkland (1964), with modifications as used by Marchbanks (1968). Synaptic vesicles were obtained by hypo-osmotic rupture of the crude mitochondrial pellet, followed by density gradient centrifugation. Purification was then carried out by passing the resulting vesicle fraction through a Sephadex gel (Marchbanks 1968). The purified vesicle fraction appears in the void volume, and the free cytoplasmic molecules are held back by the gel.

Rupture of nerve ending particles was carried out by adding distilled water containing 5mM tris-EDTA to the crude mitochondrial pellet and mixing. The presence of EDTA removes divalent cations which otherwise would cause clumping of the vesicles, in which case they would sediment at a higher density. The amount of ACh found in the vesicle fraction could be increased four fold by including EDTA by this method (Marchbanks 1968).

Protein and amino acid analyses were carried out on the fractions eluted from the Sephadex columns and also on the various fractions of the gradient used to prepare the synaptic vesicles.

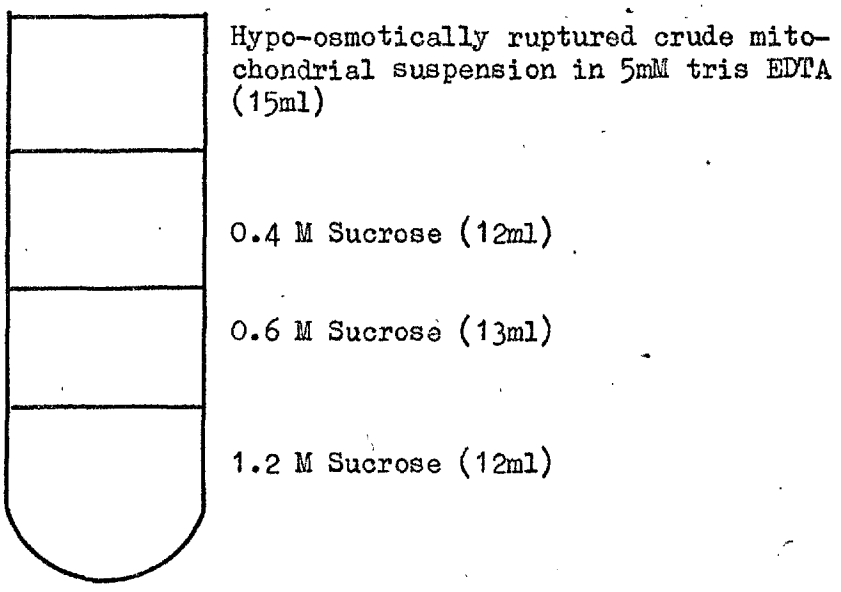
Procedure

a) Preparation of synaptic vesicles. A crude mitochondrial pellet was prepared from cerebral cortex, by the method used for preparation of synaptosomes (Section 1 (a) to (d) of this chapter). To summarise, this consists of centrifuging a 10% homogenate of rat cerebral cortices from 15 rats in 0.32M sucrose at 1,000g for 10 minutes, and centrifuging the resulting supernatant at 19,000g for 20 minutes, to give the crude mitochondrial pellet. To the pellet was added 31ml 5mM tris - EDTA p.H. 6.5 (approximately 2ml per g of fresh tissue) and this was mixed by sucking the fluid up and down a pipette several times (12). This fraction of ruptured nerve endings was then layered onto a gradient containing 1.2/0.6/0.4M sucrose in the quantities indicated in figure 9 and centrifuged at 75,000g for 120 minutes using a Beckman SW 25.2 rotor.

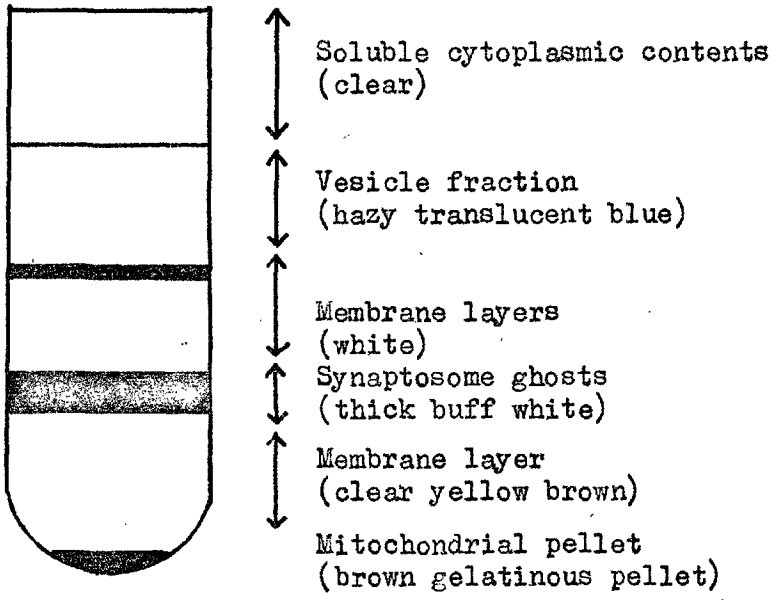
After centrifugation the gradient has the appearance indicated in figure 9. Each layer was removed in the cold, with a pasteur pipette. All layers, except the vesicle layer (0.4M sucrose), were placed straight into 1.0M PCA and stored for amino acid and protein determination.

Figure 9 Preparation of Synaptic Vesicles

Sucrose gradient used for preparation of synaptic vesicles



Appearance of gradient after centrifugation



b) Passage of synaptic vesicles through Sephadex G-50. Twelve glass columns (11 x 0.8cm) were filled with 0.5g Sephadex G-50 (medium) and the Sephadex equilibrated with 0.4M sucrose. One ml of the vesicle fraction (0.4M sucrose layer) was added to the top of each column, allowed to drain down to the top of the column, and eluted with 0.5ml volumes of 0.4M sucrose. The eluates were pooled from each 0.5ml fraction and were used for amino acid and protein determinations.

This purification stage was carried out at 4°C.

5. Amino acid analysis

Principle. Amino acids were analysed by means of automated ion exchange chromatography. The tissue extracts were first passed through cation exchange columns and eluted with NH_3 to obtain a partially purified fraction of amino acids, which was then applied to a second cation exchange column, forming part of the automated system. Separation and quantification of each amino acid was completely automatic. After electronically programmed elution of amino acids from the column with an acid to base buffer gradient, fractions of the eluent were mixed with ninhydrin, the intensity of the reaction measured colorimetrically and displayed by means of a logarithmic recorder. Zeocarb 225 was used for both chromatography columns. It is a cross-linked polystyrene-divinyl benzene cation exchange resin with a strongly acidic active group SO_3.

Tissue extraction for column chromatography. Synaptosome beds or cerebral cortex slices were added to 5ml 1.0M PCA, mixed thoroughly and kept at 0-4°C for at least 30 minutes. The extract was centrifuged at 800g for 15 minutes and the pellet further extracted with 3ml 0.5M PCA, centrifuged as before and the two supernatants pooled. The perchloric acid precipitated protein was frozen and kept for protein analysis.

Similarly PCA extracts were made of the media in which the synaptosomes had been incubated (referred to as incubation media). The incubation media were placed in 3ml M PCA to precipitate soluble protein, centrifuged as above and kept for amino acid analysis.

The stability of amino acids in PCA under the conditions of the extraction was tested and no significant change was found. Long periods in PCA brought about considerable loss of glutamine.

Primary purification on cation exchange columns. Columns (10 cm. x 0.5 cm) were filled with Zeocarb 225 (8% cross linked) and turned into the H^+ form by passage of 8ml 1.0M HCl through the columns. The inlet tubes passed between the plates of a peristaltic pump producing an even flow rate of 2.0ml/5 min. A water wash (8ml) was then passed through the columns to remove excess acid. Each amino acid extract was applied to a column in the acid form, washed with water (8ml) and eluted with 1M NH_3 (8ml). The first 5 minutes of elution could be discarded and thereafter collection tubes were placed under the columns. The eluate was taken to dryness using a Rotary Evapomix (Buchler Instruments, Fort Lee, New Jersey, U.S.A.), with the water bath at 30-40°C. The dried extract was taken up in 0.025M HCl containing 50 nmol/ml nor leucine as an internal standard. Nor leucine was chosen as a standard as it is not found in mammalian tissue and is ninhydrin positive. The volumes of HCl added to the dried sample were chosen to contain about 100-160 nmol/ml of the most predominant amino acid, thus extracts from synaptosome beds (approx. 10mg) were normally taken up in 5ml, and the incubation medium in 1.5ml 0.025M HCl. In this form the extracts could be applied to the automated amino acid analyser. The columns were regenerated by passing 8ml water, 8ml 1.0M HCl and a final wash of 8ml water. Recovery from these columns was 98-100%.

Automated amino acid analysis. The system used was that developed by Thomas (1969), and consists of the following components:

1. Automated Sampling Device, such as Sampler II (Technicon Instruments Ltd.) which loads the sample from cups on a turntable and is programmed to do this at the beginning of each analytical cycle. Between sampling, the sampler rests in a reservoir.

2. Chromatography Column. This is a column packed with Zeocarb 225, a cation exchange resin, (microsperical beads 8% cross linked) ultrafractionated to give a fraction of known diameter, sizes of 8-10μ or 13μ have been used. The sample is loaded onto the column from the sampler via a chromatography pump, which gives a defined flow rate and column pressure of 600 - 750 lb/sq. inch. The column is maintained at 60-70°C, which allows this pressure to be used.

3. Elution System. The elution buffer is generated from an acid (0.05M citric acid and 0.2M NaCl) and basic (0.05M citric acid and 0.2M NaOH) buffer by an electronic programmer and system of solenoids. This programmer gives a precise control, with elution times reproducible to 1-2 minutes, making collection of individual amino acids relatively easy.

4. Analytical System. Analysis was carried out by a flowing stream method. The eluate was separated into discrete portions by regularly placed gas segments (air or argon), which prevent mixing. All tubing and glassware is of known and regular internal diameter, so that constant flow rates are obtained, which control the time allowed for reaction. The portions of eluate are mixed with ninhydrin and buffer (Thomas, Evans, Siriwardene and Robins, 1966). All reagent inlet tubes pass through the plates of a peristaltic pump. Colour development takes 10 minutes and is carried out at 90°C in an oil bath.

5. Detecting System. The colour intensity is detected by a photo-electric cell (Mullard) fitted with a 550mμ interference filter and a stabilized 6 volt light bulb as light source. Colour changes are monitored by means of a logarithmic recorder, and quantitated by means of an integrator.

6. Fraction Collection. The eluate from the column can be divided 60:40, to allow the larger fraction to pass to a fraction collector (LKB type 7000 from Produkter AB, Stockholm, Sweden). The fraction collector is signalled to start at appropriate times by the gradient programmer and collects 1ml samples during the period of amino acid elution.

Two versions of this system were used for analysis and the flow diagram for one, the microsystem is shown in Figure 10. It has a small column, requires only 0.1ml samples and takes 1 hour for a complete cycle. The earlier model used (not illustrated) possesses a larger column (75 x 0.6cm) and since it has faster flow rates, of about 1ml/min. through the column, also requires larger volumes of sample, about 1ml, the whole cycle taking about 110 minutes. The other main difference between the systems is that in the larger version, the segmenting bubbles are removed before calorimetric analysis, which has the disadvantage of giving a slower response time than the "Hold and sample" electronic technique used in the microsystem. Apart from minor details, the two systems are similar in other respects. Both systems are highly reproducible, specific, rapid, and give high sensitivity resolution. The measurement range for each amino acid is 10 - 200 nmol/ml with detection down to 1 - 3 nmol.

An example of the amino acid separation of a standard by the microsystem is shown in Figure 11.

Figure 10. Flow diagram for automated amino acid analytical microsystem

A, B, C, D, E & F are solenoids.

Buffer compositions are as follows:

Acidic 1- 0.05M citric acid, 0.2M NaCl, 0.03% Brij 35, 0.25% triodoglycol

Acidic 2- 0.05M citric acid, 0.2M NaCl, 10% methanol, 0.03% Brij 35, 0.25% triodoglycol.

Basic- 0.05M citric acid, 0.2M NaOH, 0.03% Brij 35.

Column dimensions: length 33cm, diameter 2mm.

Pressure pump : 600 lb/sq.in.

Column water jacket at 70 C.

Flow rates :	Argon	0.32ml/min
	Ninhydrin	0.23ml/min
	Hydrazine buffer	0.1 ml/min
	Sample	0.16ml/min
	Buffers (entering reservoir)	0.23ml/min

Figure 10

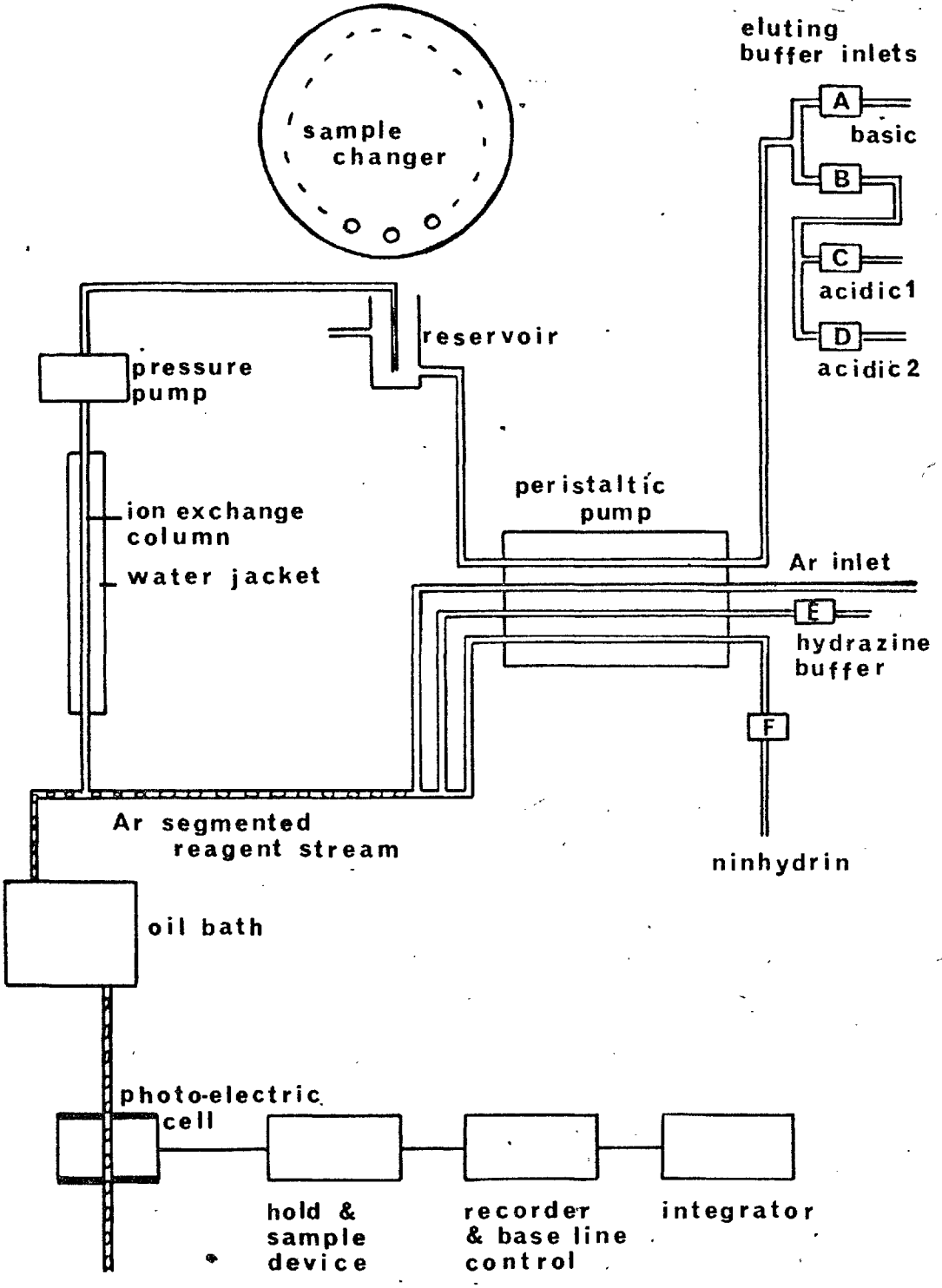
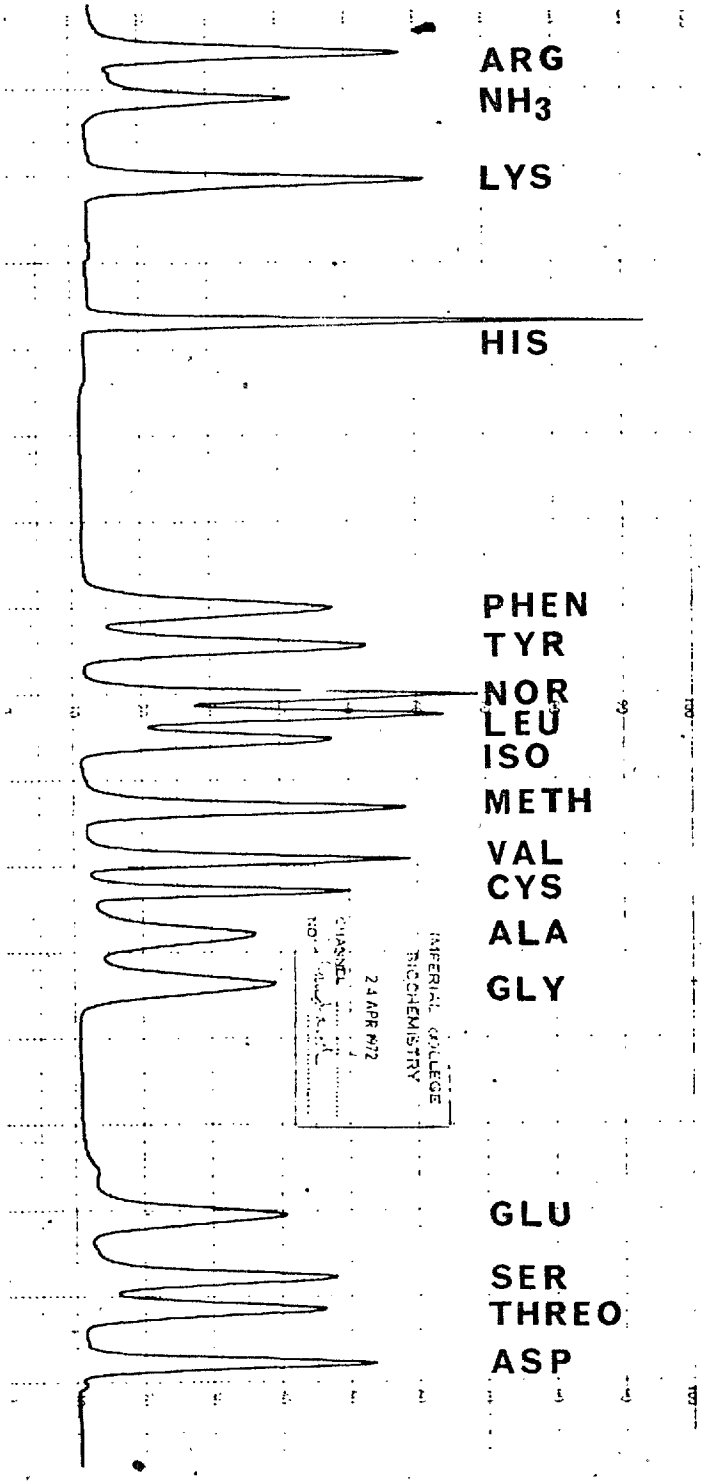

sample changer
eluting buffer inlets
A
basic
B
C
acidic1
D
acidic2
reservoir
pressure pump
peristaltic pump
ion exchange column
water jacket
Ar inlet
E
hydrazine buffer
F
Ar segmented reagent stream
ninhydrin
oil bath
photo-electric cell
hold & sample device
recorder & base line control
integrator

Fig.11 - Chromatogram of amino acids



Calculation of results. The amounts of amino acid in each sample were calculated on the basis of the peak area, with reference to norleucine which had been introduced to each sample at the known concentration of 50nmol/ml. The amino acid peak areas were indicated by a number of integrations or were worked out by hand, using height times width at mid height as a formula for peak area. As can be seen in Figure 11, not all amino acids react with ninhydrin to the same extent, and amino acid colour factors (F) were calculated. These were found by determining the peak sizes obtained from accurately made up standard amino acid solutions, 50nmol/ml with respect to all amino acids. The peak size for norleucine was set at 1 and all the others were related to this, i.e. an amino acid giving a smaller degree of colour with ninhydrin will have a colour factor greater than 1 and vice versa. A commercially produced amino acid standard is available (Technicon Instruments Ltd.) which contains 18 common amino acids and is normally used for this purpose together with a separate standard for glutamine and GABA, which are absent from the former preparation. The colour factors for one particular system do not vary greatly but standards were run with each experimental group of samples to detect changes due to slight variation in reagents.

An example of the estimation of one amino acid concentration follows:

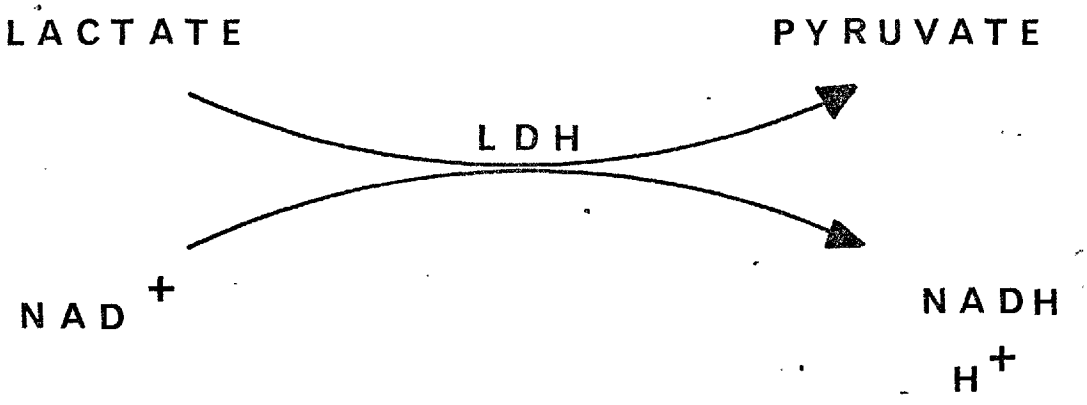
With a volume of sample = V ml containing norleucine as an internal standard at a concentration of 50 nmol/ml and after column chromatography giving a peak size of amino acid = A, and one of norleucine = N, then the total amount of amino acid present in the sample

$$= \frac{50\ A\ .\ F\ .\ V}{N} \quad \text{nmol}$$

In subsequent results the amino acid concentration is normally related to the amount of protein, as nmol/100mg protein.

6. Lactate determination

Principle. Lactate was determined by the method of Bergmeyer (1965) by measuring the production of NADH by a sample containing an unknown quantity of lactate, lactate dehydrogenase (E.C.1.1.1.27) and excess NAD. The following reaction occurs:



NADH has a characteristic absorption at 340mμ and the change in absorption at this wavelength produced by LDH addition to the sample is monitored. The amount of NADH formed is equivalent to the lactate used up on a molecular basis and thus the original amount of lactate can be calculated from the absorption changes produced by standard amounts of lactate.

Readings were taken before addition of LDH, to assess the amount of endogenous NADH, which value was subtracted from subsequent readings. Internal lactate standards were added to each sample after the unknown lactate had reacted, to characterise the response of that particular system to the standard, since the presence of certain tissue components may modify the reaction.

Procedure. Hydrazine sulphate buffer was made up as follows:- 30g glycine, 20.8g hydrazine sulphate and 0.8g EDTA made up to 200ml with distilled water. This could be stored at room temperature for

several weeks.

Reaction buffer was made up, just prior to the assay from 14.7ml hydrazine buffer, 4ml 1% NAD and 22ml water. 15.3ml 2 N. NaOH/ Spectrophotometer cells, either quartz or glass, of 1cm path length were thoroughly cleaned and dried. One cell was required per sample to be measured and was filled with 2.8ml reaction buffer and 0.2ml tissue sample. The cell was covered, mixed by shaking, and placed in a spectrophotometer (Unicam SP 500) and the absorbance at 340mμ measured (R 1). A cell containing distilled water was used to calibrate the zero reading. Then 10μl of LDH (pig heart LDH from Boehringer) was added to each cell, the contents mixed and a stop watch started. This volume of LDH was also added to a cell containing 2.8ml reaction buffer and 0.2ml water (reagent blank) to measure any change in absorbance due to the addition of the enzyme suspension ($\Delta 1$) and this value was subtracted from all samples. After 40 minutes, the absorbance was measured (R 2). Then 0.2ml 0.5mM Lactate was added to each cell, the contents mixed and a stop watch started. After a further 40 minutes, the cells were again placed in the spectrophotometer and the absorbance measured (R 3).

The unknown amount of lactate was calculated as follows:

Change in absorbance due to unknown amount of lactate

$$= (R\,2 - R\,1 - \Delta 1) \quad = \Delta 2$$

Change in absorbance due to 100n moles of standard lactate

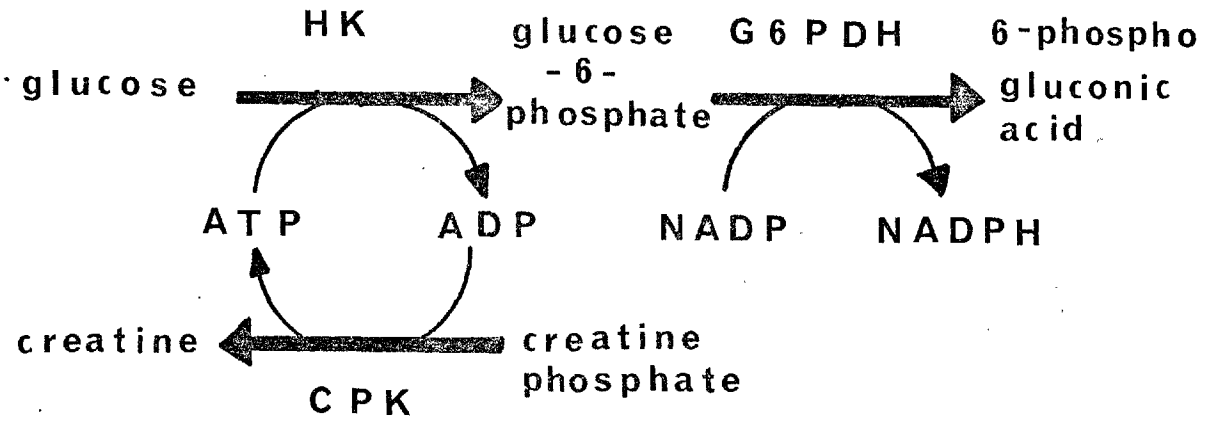
$$= (R\,3 - R\,2) \qquad = \Delta 3$$

Therefore the amount of lactate present in 0.2ml of sample

$$= 100 \times \frac{\Delta 2}{\Delta 3} \text{ n moles.}$$

7. ATP and Phosphocreatine assay

Principle. ATP and phosphocreatine were assayed by the method of Lowry, Passoneau, Hasselburger & Schultz (1964), using a fluorometric assay in which the final product measured is NADPH. If hexokinase (E.C.2.7.1.1.), Glucose-6-phosphate dehydrogenase (E.C.1.1.1.49), NADP and glucose are added to a tissue extract containing ATP and phosphocreatine, then NADPH will be produced by the following set of reactions:



The amount of NADPH produced will be equivalent to the ATP originally present, on a molecular basis. When creatine phosphokinase (E.C.2.7.3.2.) is then added, the ADP will be used up to produce ATP, which will be cycled by the same set of reactions, producing more NADPH, which will be equivalent to the phosphocreatine present.

Procedure. a) Extraction of ATP & phosphocreatine. After incubstion of synaptosome beds, they are immediately transferred to 1ml of 1.0 M PCA containing 200mM Tris base, to inactivate Na^+/K^+ATPase. After leaving

on ice for 30 minutes with frequent mixing, the samples are centrifuged at 800g for 10 minutes in the cold. The supernatants were retained and the pellets mixed with a further 0.5ml 1.0 M PCA containing 200mM Tris base and recentrifuged as before. The pooled supernatants were adjusted to pH 7.0 with a probe pH electrode using 1 M KOH and 1 M PCA. The precipitate of potassium perchlorate was removed by centrifugation, and the supernatants quick frozen with an acetone-dry ice mixture and stored at $-22^{\circ}C$ until required.

b) ATP & phosphocreatine assay. A spectrofluorometer (Mark 1 made by Farrand, New York, U.S.A.) was used for this assay, with Xenon lamp power supply and potentiometer recorder. A clean fluorometer cell was filled with 0.3ml tissue sample and 1.7ml buffer containing the following constituents: 30ml 0.2 M Tris buffer pH 7.5; 1.5ml 1% NADP: 3.0ml 1.0 M $MgCl_2$; 1.5ml 0.1 M glucose.

The cell contents were mixed and the cell placed in the spectrofluorometer and a steady base line obtained. The following additions were made, after each of which the cell contents were mixed and the change in fluorescence monitored with a recorder until the reaction ceased and a line parallel with the original base line was obtained.

1) 5µl glucose-6-phosphate dehydrogenase (0.15mg protein/100ml)
This uses up any endogenous glucose to provide a new base line.

2) 10µl Hexokinase
This will give a change equivalent to the ATP in the sample

3) 10µl 1mM ATP (6.05mg/10ml)
This will give the response to a standard from which the unknown amount of ATP can be calculated.

4) 20µl 25mM ADP tri sodium salt (.02g/2ml). (To ensure an excess of ADP is present)

5) 10µl Creatine phospho-kinase (5mg/ml)

This will give a change equivalent to the creatine phosphate in the extract.

6) 10µl 1mM Creatine phosphate (3.63mg/10ml)

Standard from which to calculate unknown creatine phosphate.

Thus, the ATP present in the sample is represented by the absorption change (2) - (1) and was calculated from the response to standard ATP (3) - (2). Similarly the creatine phosphate caused an absorption change (5) - (4) and the standard creatine phosphate (6) - (5).

8. Protein determination

Protein measurements were made of the synaptosome beds, and of the various sub-fractions obtained in the fractionation procedure such as synaptic vesicles. The method used was that of Lowry, Rosebrough, Farr & Randall (1951).

Principle. The protein sample is treated with a copper solution in alkaline solution and this is then used to reduce an aqueous solution of a phosphomolybdic-phosphotungstate reagent known as Folin Ciocalteau reagent. A blue coloured complex is produced which has an absorption maximum at 750mµ. The degree of colour development is related to the protein concentration and has a linear relationship at low concentrations and it is in this range that the assay was used.

The coloured complex is thought to be a co-planar quadridentate chelate (Chou & Goldstein 1960), formed between the phenol group of the phosphomolybdic-phosphotungstate reagent and functional groups of amino acids. In the absence of copper, Folins Ciocalteau reagent gives

a complex formation dependent on the presence of tyrosine, tryptophan, cysteine and histidine residues. Copper increases the blue intensity, the peptide bonds being responsible for some of this colour and some amino acid sequences being more chromogenic than others. A dipeptide containing histidine and either arginine or glutamate is particularly chromogenic. The chromogenic groupings responsible for the reaction are discussed in more detail by Chou & Goldstein (1960). Copper acts as an efficient catalyst in transferring electrons from the chromogenic groupings of the protein to the phenol reagent.

This method can be used for measuring both soluble and insoluble proteins. In the latter case, the material was first solublised in alkaline solution (Lowry et al 1951).

PCA precipitation of protein. After incubation of synaptosome beds, the beds were removed, placed in 5ml 1.0M PCA and mixed by vortex. After leaving at $4^{\circ}C$ overnight, the PCA extract was mixed thoroughly and centrifuged at 1,000g for 10 minutes in a UV International centrifuge at $4^{\circ}C$. The supernatant was removed and a further 3ml of 0.5M PCA was added to the pellet which was mixed and centrifuged as before. The pellet thus obtained was ready for protein determination and the combined supernatants were used for amino acid analysis of the synaptosome bed.

Solubilisation of Protein. To the PCA precipitated protein was added 3ml of distilled water, which was mixed by vortex to suspend the protein. Then 6ml 4.5M NaOH was added to this suspension and further mixing was carried out. This was left for 60 minutes. Standard bovine serum albumen (BSA) (obtained as a lyophilised powder from Sigma) was treated in a similar way, i.e. equal volumes of BSA containing 2mg by weight per ml water and 6N NaOH were mixed and left for 60 minutes.

Spectrophotometry. The copper reagent was made up fresh for each assay and contained 50ml 2% Na_2CO3 and 1ml 0.5% $CuSO_4$ in 1% Na/K Tartarate. Each solution was stable independently for several months but in combination the mixture only lasts for up to 3 hours.

Assay tubes were filled with the following:

3.0ml copper reagent

0.1ml protein extract

1.1ml distilled water

The alkaline protein extract was thoroughly mixed before taking a sample to ensure using a representative portion of the suspension for analysis. After completion of the above additions, the mixture was mixed and left to stand for 10 - 15 minutes, to equilibrate. Standards were made up by adding 20, 50, 100, 150, 200 and 250 µl volumes of the BSA solution (containing 1mg/ml), in turn to 3ml of copper reagent and making up the final volume to 4.2ml with distilled water. All samples and standards were done in duplicate.

An aqueous solution of Folin Ciocalteau reagent was made by diluting this reagent 1 : 1 with distilled water, and 0.3ml volumes of this were added to each assay tube. The addition was made rapidly followed by immediate mixing. After 40 minutes, the colour developed was measured at 500mµ in a Unicam SP 600, using a continuous flow cell.

The optical densities were measured against a reagent blank, containing 3ml copper reagent and 1.2ml water, which was set at zero. From the values obtained with standards, a standard curve was drawn and the unknown amounts taken from this. The amounts of sample used above are appropriate for measuring the protein of a synaptosome bed, but where other fractions are being measured, different quantities may be needed in order to utilize the linear part of the standard curve.

9. Radioactivity measurements

Radioactivity studies were carried out on the incorporation of radioactive label from (U-^{14}C) glucose into amino acids, and the effect of stimulation by means of electrical pulses and elevated K^+ on this incorporation. Both synaptosome beds and the incubation media were analysed. Incubation was carried out as described in section 2 c) in the presence of medium containing 3-5μCi/ml of D-(U-^{14}C) glucose, which had been purified by paper chromatography. Extraction and analysis of amino acids was carried out as described in section 5. For these experiments the column effluent was stream split before ninhydrin admixture and a proportion (60%) was collected in 1.0ml samples using a fraction collector, the remaining effluent (40%) entered the analytical system. About 60 fractions were obtained per sample and these were blended with Bray's scintillation medium and counted in a Packard Liquid Scintillation Counter.

Purification of (U-^{14}C) glucose. This was carried out by paper chromatography on Whatman No. 1 paper with the following solvent : butanol: acetic acid:water 40:11:25. The position of glucose was located by means of autoradiography and the glucose eluted with water. The activity of the purified material was determined by counting a small sample of it.

Radioactivity Counting. The scintillant mixture was made up of the following components:- 5 parts toluene P.B.D. : 4 parts 2-methoxy-ethanol. Toluene P.B.D. contained 250g naphthalene A.R., 30g scintillator butyl P.B.D. (Ciba made up to 5 litres with toluene A.R.).

Each eluate from the column was blended with 19ml scintillant medium, in which the aqueous phase could be dissolved. An initial

series of 1 minute counts was made of all fractions using a Beckman LS-200B Liquid Scintillation Counter and the samples with counts at or below background level were discarded. All the other fractions were counted for 10 minutes in a Packard Tricarb liquid scintillation spectrometer with automatic standardization. The resulting data from the scintillation counter was fed into a PDB-9 digital computer, calibrated for ^{14}C measurement and the scintillation medium used in these experiments, which yielded results in μμ curies. (1-^{14}C) norleucine was included in all samples with the non-isotopic norleucine before analysis (1nCi/ml) to measure radioactivity recoveries and to serve as an aid in identifying peaks. Recoveries of radioactivity were greater than 90%. Chromatograms from the amino acid analysis were compared with radioactivity data and the number of counts assigned to each amino acid peak. Results were expressed as specific activities μμ curie/n.mol amino acid.

10. Bioassay of acetyl choline

Principle. ACh present in tissue extracts is usually estimated by bioassay since the various chemical (Fellman 1969) and enzymic (Feigenson & Saelens 1969) methods with the exception of gas chromatography do not have the requisite sensitivity and specificity. Bioassays are carried out by comparing the contractions of a suitable loaded muscle, evoked by unknown amount of extract with those obtained by known amounts of ACh. Doses of the standard or unknown are given at regular intervals for accurately timed test periods, followed by periods of washing the muscle free of test solution and bringing about relaxation. A sigmoid dose response curve is obtained with ACh standards and the linear portion defines the working range of the preparation. Doses of extract are

preceded and followed by bracketing doses of ACh. Other components of the extract may potentiate or inhibit the muscle contraction such as K^+, choline, histamine or phosphate and for the characterization of activity as due to ACh, the following criteria should be obeyed. The activity of the extract should be inactivated by alkaline solution (p.H 9 for 10 min. at 100°C), be increased by AChE inhibitors, be decreased by AChE, and be antagonised by an ACh blocker such as atropine or nicotine in the same way that ACh activity would be affected. As a further precaution against interferring substances ACh standards are made up in tissue extracts in which the ACh has been inactivated.

The frog rectus abdominis muscle was used for bioassay as it is a stable preparation giving a rapid and reproducible response and has a sensitivity within the requirements of the present study. The use of this preparation was first described by Riesser (1921) and later more comprehensively by Chang & Gaddum (1933), Feldberg (1950) and Hebb & Silver (1970).

The contraction of the muscle was measured by a linear motion transducer used in conjunction with a potentiometer recorder (Gaddum 1953). By this method muscle contraction caused movement of an axial core passing through the bore of a cylindrical coil assembly which produced a voltage change in the output proportional to the displacement, which was passed on as a DC voltage to the recorder.

ACh was measured in synaptosome beds and the effect of depolarizing influences on its release into the incubation medium was also studied.

Extraction of ACh. All sucrose solutions and media used for the preparation, incubation and extraction of synaptosome beds contained

neostigmine bromide (Therapharm. Kingston, Surrey) at a final concentration of 10μg/ml (0.33mM) to inactivate AChE.

After incubation synaptosome beds were placed in 1.25ml volumes of frog Ringer and mixed by vortex. Frog Ringer solution has the following composition (mM) : NaCl (110); KCl, (4); $CaCl_2$, (1.2); $NaHCO_3$, (4.25); glucose, (4). The ACh was extracted from the synaptosomes by heating the 1.25ml suspensions in frog Ringer at p.H 4 for 10 minutes at 100C by the method of Hebb & Whittaker (1958). The solution was neutralized and centrifuged at 800g for 10 minutes to remove the precipitated protein. The supernatant was removed and stored at -22°C until used for ACh assay and the pellet was kept for protein determination.

The 5ml volumes of medium in which the synaptosome beds had been incubated were freeze dried, in order to concentrate the ACh in the sample. Immediately before use, 1.5ml of frog Ringer solution was added to the dried extract for assay.

Bioassay of ACh using the rectus abdominis muscle of Rana temporaria.

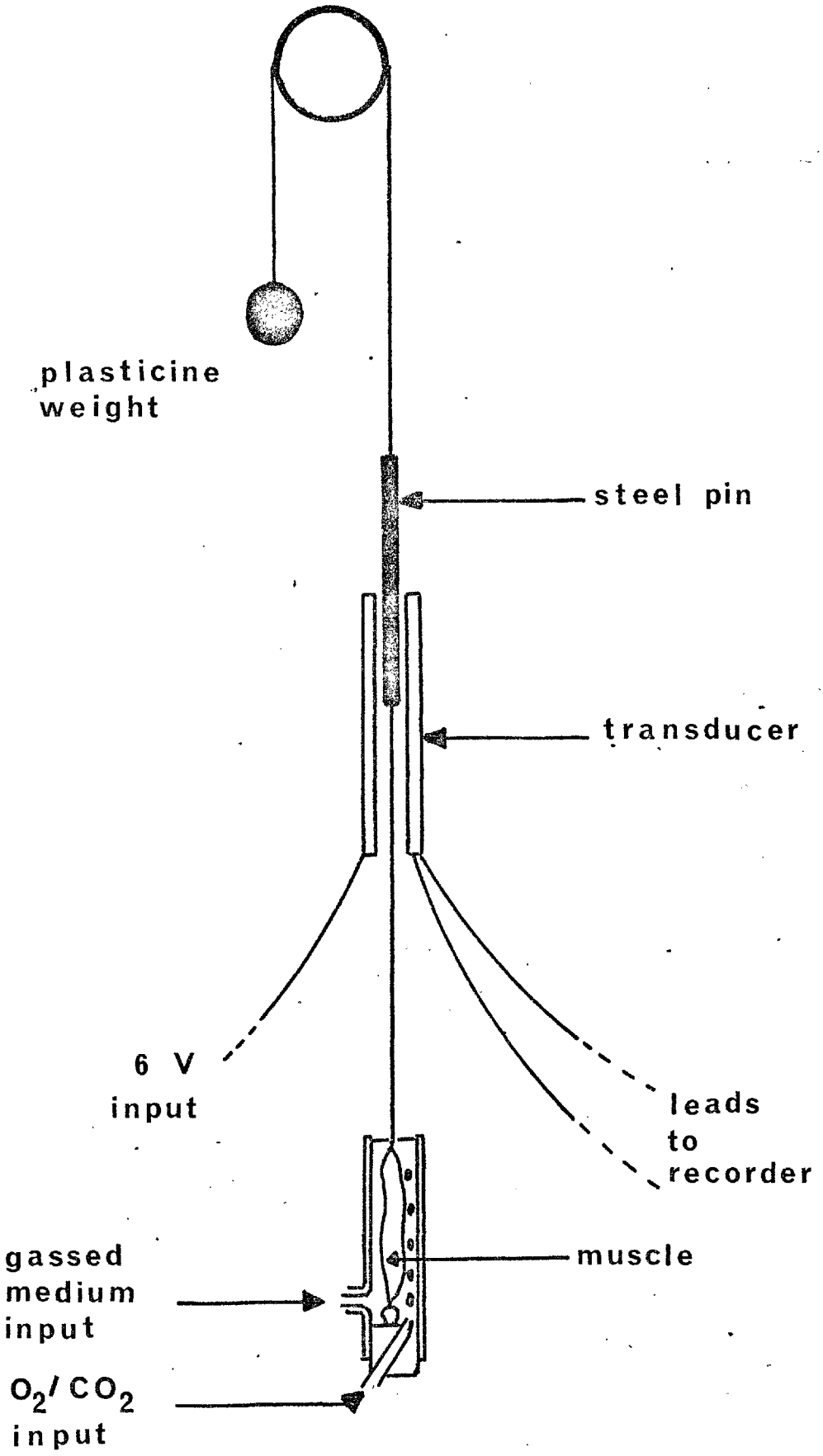
The frog was stunned and killed by severage of the spinal cord at the base of the brain. The skin was removed from the ventral body wall exposing the rectus abdominis muscles. Then one muscle of the pair was separated from the other by a cut along the ventral midline and also separated from lateral muscles by a parallel cut. The muscle was cut as close to the point of insertion as possible, the tendinous pelvic attachment posteriorly and a cut was made through the xiphisternum of the pectoral girdle anteriorly leaving a portion of the later attached to the upper end of the muscle. During dissection the muscle was frequently washed with frog Ringer solution. Both muscles were dissected out, one

for immediate use and the other was stored at 4°C in frog Ringer solution for use the following day. In practice a fresh frog was normally used each day.

The muscle was immersed in frog Ringer solution in a petri dish and a piece of cotton thread was tied with two knots as close as possible to each end of the muscle using forceps. The posterior piece of cotton was tied to a hook in a rubber bung, forming the base of the incubation bath. The bung was inserted in the bath, which was then filled with frog Ringer solution and the muscle was thus suspended in the bath. The incubation bath was gassed with 95% O_2/5% CO_2 via a hyperdermic needle inserted in the rubber bung. The anterior piece of cotton was attached to a steel pin which passed through the transducer (Hewlett-Packard Co., Massachusetts, U.S.A., Model 70 CDT -500 with D.C. 6V input by Coutant Electronics Ltd., Reading) and was loaded with plasticine (about 5g), by a thread passing over a pulley(Fig12).With the load on the muscle, the muscle was allowed to relax for 15 minutes. The load was such to allow adequate relaxation and prevent spontaneous unwanted activity. The bath was then flushed with frog Ringer containing neostigmine and the muscle left to relax for a further 30 minutes.

A suitable base line was found, by adjustment of the position of the transducer. Samples (100µl) were assayed by addition to the centre of the incubation bath, using a hyperdermic syringe with rubber tubing over the tip of the needle. At the moment of release of sample, a stop watch was started and movement of the recorder (Smiths-Servoscribe RE 511) paper started. After 90 seconds, the degree of contraction monitored by the recorder was noted and the bath was flushed out with frog Ringer solution. A period of 240 seconds was allowed for the muscle to relax, which was aided by gentle stroking of a thread attached to the anterior end of the

Figure 12. Bioassay of Acetyl choline



muscle. When the response to ACh standards was established, the unknown samples were measured using 0.1ml samples bracketing them between standard ACh samples of similar response, assay being carried out in duplicate.

Tissue extracts were made alkaline and boiled to inactivate ACh (Feldberg 1950) and ACh atandards were incorporated into these inactivated samples to test for potentiating or inhibiting substances in the extract. The effect of concentrating the ions of the medium by freeze-drying was tested by incorporating ACh standards into portions of medium which had been treated in exactly the same way as the medium in which the synaptosome beds had been incubated. Normal bicarbonate medium and medium containing EGTA and no calcium, with ACh standards included, were incubated at 37°C for 40 minutes, either alone or with elevated K^+ or electrical pulses in the last 10 minutes of incubation. These samples were then freeze-dried and taken up in 1.5ml of frog Ringer solution and assayed for ACh. The final concentration to which the rectus muscle was exposed did not cause a potentiating effect or illicit contraction on its own. Similarly the presence of EGTA and no calcium were without effect when included with the standards and the samples assayed.

11. Potassium determination.

Potassium was measured by flame photometry, using an EEL flame photometer (Evans Electroselenium Ltd., Halstead, Essex).

Procedure. Extraction of K^+ from synaptosome beds was carried out by placing the bed in distilled water (9ml) and mixing by vortex. After removal of the particulate fraction by centrifugation, the supernatant could be used directly for K^+ determination. A K^+ filter (730-850mμ) was used in the flame photometer and the zero calibrated with water and 100% deflection with

500μM K^+/1,000μM Na^+. Using known K^+ concentrations, a standard curve was plotted of % deflection against K^+ concentration, and was used to calculate unknown K^+ concentrations present in the synaptosome bed extracts.

APPENDIX

A comparison between the respiration of synaptosomes prepared by the long and short centrifugation procedures.

Introduction. In an attempt to reduce the time involved in the preparation of synaptosomes, a shorter centrifugation was tried out. The efficiency of this shorter procedure was tested by measuring the respiratory activity of synaptosomes prepared by this method and comparing the rate with that of synaptosomes prepared by the long centrifugation method. Two possible disadvantages of the short process are that fewer light nerve endings might sediment in the second centrifugation and that some nerve endings may not have reached their equilibrium density during the shorter density gradient centrifugation. The two centrifugation procedures only differed in the time of centrifugation and not in technique and are summarised as follows:

Force of centrifugation	Time of centrifugation	
	Short method	Long method
1. 1,000 g	10 min	10 min
2. 19,600 g	20 min	1 h
3. 75,500 g	1 h	2 h
4. 54,500 g	20 min	20 min

A sucrose gradient was used with an extra sucrose step of 1.0M sucrose between 0.8 and 1.2M sucrose, in order to detect variations in the respiratory activity of synaptosomes of different density. The fraction obtained at the 0.8/1.0M sucrose interface was termed the B layer and that at the 1.0/1.2M sucrose interface the C layer. The pelleted nerve endings were taken up in Krebs phosphate medium and incubated in Warburg flasks for 1 h at 37° C.

Results. Using both methods of preparation, suspensions of synaptosomes from the B and C layer showed linear respiration for the 1 h period of incubation (Figure 13 and Table 6). There was no significant difference in the rates of oxygen uptake of either the B or C layer under the different conditions of centrifugation. In both methods, the respiratory rate of the B layer was lower, probably reflecting the greater contamination of this layer with microsomes, since this would reduce the value of oxygen uptake relative to protein. The respiratory rate of microsomal fractions obtained by pelleting the microsomal supernatant from the second centrifugation at 54,500 g for 1 h and incubating as for synaptosomes, was found to be very low and irregular. Approximate rates of 7.2 and 11 μmol/100mg protein/h were obtained from a short and long centrifugation procedure respectively. Thus the presence of microsomes would contribute protein but negligible respiratory activity. When the microsomal fraction referred to above was placed on top of the four step gradient used in these experiments and centrifuged for 2 h at 75,500 g, a large protein band appeared at the 0.32/0.8 M sucrose interface and a smaller band at the 0.8/1.0 M sucrose interface. This further indicated that microsomal contamination of the B layer was possible.

Both B and C layer synaptosomes obtained from both forms of preparation responded to the application of electrical pulses by increased rates of respiration. From a preliminary study, the range of increased rates of respiration was 40 - 80%.

From these results, it was concluded that the shorter centrifugation procedure did not lead to reduced respiratory activity of synaptosomes obtained between 0.8 and 1.2M sucrose and would provide a convenient method of preparation. Electron microscopy studies also verified that this fraction was rich in synaptosomes.

Table 6 Respiration of synaptosomes in the B and C layer obtained from a four step sucrose gradient

	B layer 0.8 / 1.0 M sucrose interface	C layer 1.0 / 1.2 M sucrose interface
Short centrifugation	33.1 ± 6.2 (7)	46.5 ± 2.6 (17)
Long centrifugation	24.1 ± 1.4 (4)	48.6 ± 1.6 (17)

Values are given in μ mols oxygen consumed per 100mg protein per h ± SEM with the number of experiments in parenthesis. The synaptosomes were incubated as suspensions in Krebs phosphate medium at 37°C for 1 h.

Figure 13 Respiration of synaptosome and microsome subcellular fractions (shown on previous page). Synaptosomes were prepared by the long centrifugation method and incubated as described in Table 6. Respiration of the B layer of synaptosomes is denoted by filled circles and the C layer by filled triangles. The microsomal supernatant from the second centrifugation at 19600 g was sedimented and incubated in a similar way and the respiration indicated by unfilled circles. Respiration was measured as u mol of oxygen per 100 mg protein consumed with time.

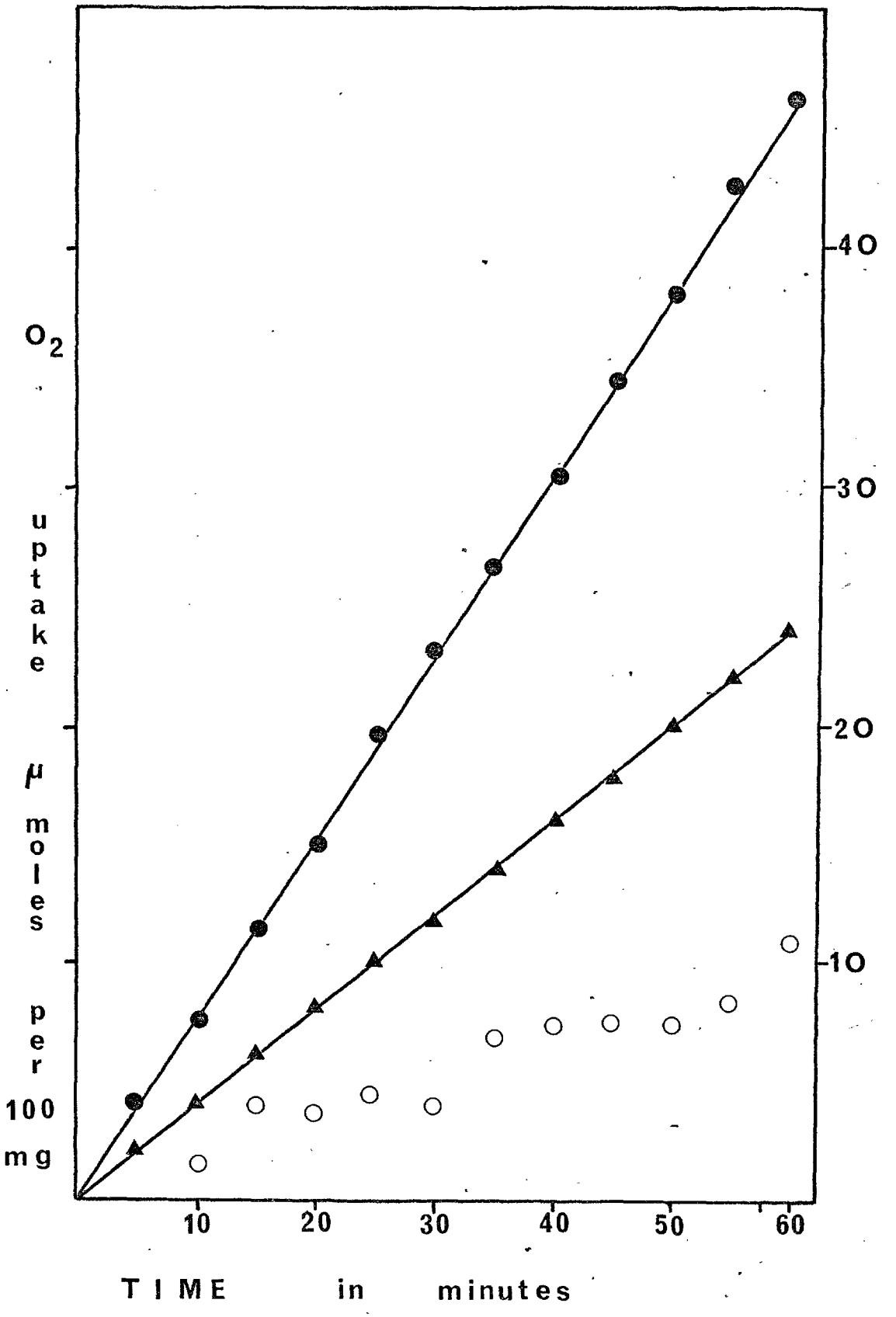


Figure 13 - Respiration of synaptosome subfractions

CHAPTER 3

THE SYNAPTOSOME BED PREPARATION

Introduction

The advantages of the synaptosome bed preparation

Formation of synaptosome beds

Results

Lactate formation and oxygen uptake

Incorporation of (U-^{14}C) glucose into amino acids

ATP and phosphocreatine levels

Effect of hypertonicity and added cyanide

Loss of protein from the synaptosome bed

Electron microscopy

Conclusions

CHAPTER 3 THE SYNAPTOSOME BED PREPARATION

Introduction. Synaptosome beds were made by depositing a suspension of synaptosomes in metabolic medium, onto a rectangle of nylon gauze and covering this deposit with a second piece of gauze. The two pieces of gauze adhere together in the presence of moisture and the sandwich thus formed can be held in a holder of the McIlwain quick transfer type (Figure 7) or between a pair of grid electrodes (Figure 8). By means of the holder, the bed can be rapidly immersed in medium and at the end of incubation be rapidly removed.

Advantages of the synaptosome bed preparation. Beds of synaptosomes were used as an alternative preparation to the simpler method employing suspensions. In a suspension of synaptosomes there is a high fluid to tissue ratio, and in Warburg flasks the synaptosomes are continually agitated and washed by fluid movements induced by oscillations. Since these conditions are far removed from those *in situ*, a more static system with a low fluid to tissue ratio would seem more appropriate for the study of synaptosomes. In the form of a synaptosome bed, circulation and agitation of the medium only, is brought about by gas bubbling. The presence of gauze has the added advantage of reducing the direct contact between synaptosomes and electrodes to a minimum and thus, the complications introduced by any local heating or electrode products are avoided. Rapid fixation is possible at the end of incubation since the synaptosome bed can be immediately removed from the surrounding medium. This contrasts with suspension studies where centrifugation or passage through a millipore is required to separate the synaptosomes from the surrounding medium. Thus a synaptosome bed is a particularly useful preparation where sampling of compounds released from nerve endings are

to be made or where rapid fixation is required.

Formation of synaptosome beds. In the presence of cations, as for example in Krebs-Ringer medium, aggregation of synaptosomes occurs and it is this fact that makes synaptosome bed formation possible. It was found possible to use a wide mesh gauze, on which to deposit the synaptosomes which could also allow adequate availability of oxygen and substrate. Gauze was used with apertures of 120 μ which are considerably larger than the diameter of the synaptosome (0.3 - 1 μ). This state of aggregation existed throughout the period of incubation, since only small losses (less than 2%) of the synaptosome bed protein occured during incubation.

Once synaptosomes have come into contact with medium, the ensuing procedures are carried out at 37°C and the centrifugation at room temperature. Sedimentation of the synaptosomes into a bed is carried out using a bucket shaped perspex cell (Figure 6), which has a removable base. Before centrifugation, a piece of nylon gauze is placed on the floor of the cell and the synaptosome suspension in medium poured on top. At the end of centrifugation, the base can be removed with the deposit of synaptosomes forming a layer on the gauze. The gauze is lifted up with forceps and a second piece of gauze placed on top. Two sizes of synaptosome bed were made, one for use with McIlwain quick transfer holders (1.4 x 2.2cm) and the other for use with grid electrodes (0.7 x 1.5cm).

Initially studies were carried out using the synaptosome bed, in order to see whether this particular arrangement impaired the metabolic properties of synaptosomes compared with suspensions of synaptosomes. The adequate availability of substrate and oxygen would be reflected in parameters, such as oxygen uptake, lactate production and ATP and phosphocreatine synthesis with glucose as substrate. Utilization of glucose by synaptosome beds was

also tested by measuring its incorporation into amino acids. The structural integrity of the synaptosome bed was further tested by an examination of the amount of protein loss to the medium during incubation and the effect of hypertonicity and metabolic inhibitors on retention of amino acids.

RESULTS

Lactate formation and oxygen uptake. The lactate accumulated in the medium during 60 minutes incubation is shown in Table 7, alongside a value obtained for lactate production by synaptosome suspensions. These values are similar, the suspensions showing a faster rate of lactate production which may reflect the larger volume of fluid in contact with synaptosomes in the form of a suspension. McIlwain & Rodnight (1962) found that the lactate production by cerebral cortex slices varied with the volume of medium in which they were incubated, a smaller volume being associated with a slower rate of lactate production which in fact approaches the in vivo rate of lactate production more closely.

The rate of oxygen uptake by synaptosome beds was measured by inserting small synaptosome beds (0.7 x 1.5cm) between grid electrodes and placing this assembly in a small Warburg flask, with Krebs phosphate medium containing 10mM glucose. The rate of respiration was linear for the first 35-45 minutes of incubation (Figure 14) and at a comparable rate to synaptosome suspensions (Table 8). After this period of time, there was a small decrease (15%) in respiratory rate during the final 15-25 minutes of incubation. This may be due to the fact that when the first respiratory readings were made for synaptosome beds, they had already been in contact with medium for 30 minutes longer than occurs when preparing a

Table 7 Lactate production by synaptosome beds and suspensions

	Lactate produced (μmol/100mg protein/h)	
	Beds	Suspensions*
Unstimulated	11.8 ± 0.6 (13)	16 ± 2 (20)
Stimulated	16.2 ± 1.0 (15)	25 ± 2 (20)

Synaptosome beds or suspensions were incubated for 1 h at 37°C in Krebs-phosphate medium containing 10mM glucose. Electrical pulses were delivered during the last 30 min. of incubation. Values are means ± S.E.M. for the number of experiments in brackets.

* Data from Bradford (1970a).

Table 8 Oxygen uptake by synaptosome beds and suspensions

	Oxygen uptake (μmol O_2/100mg protein/h)	
	Beds	Suspensions*
First 30 min. of incubation	53.2 ± 2.4 (22)	60 ± 3 (20)
Second 30 min. of incubation	45.0 ± 2.0 (10)	
Electrical stimulation during 30 min of incubation	63.0 ± 5.4 (10)	98 ± 4 (20)

Synaptosome beds or suspensions were incubated for 1 h at 37°C in Krebs-phosphate medium containing 10mM glucose. Values are means ± S.E.M. for the number of experiments in brackets.

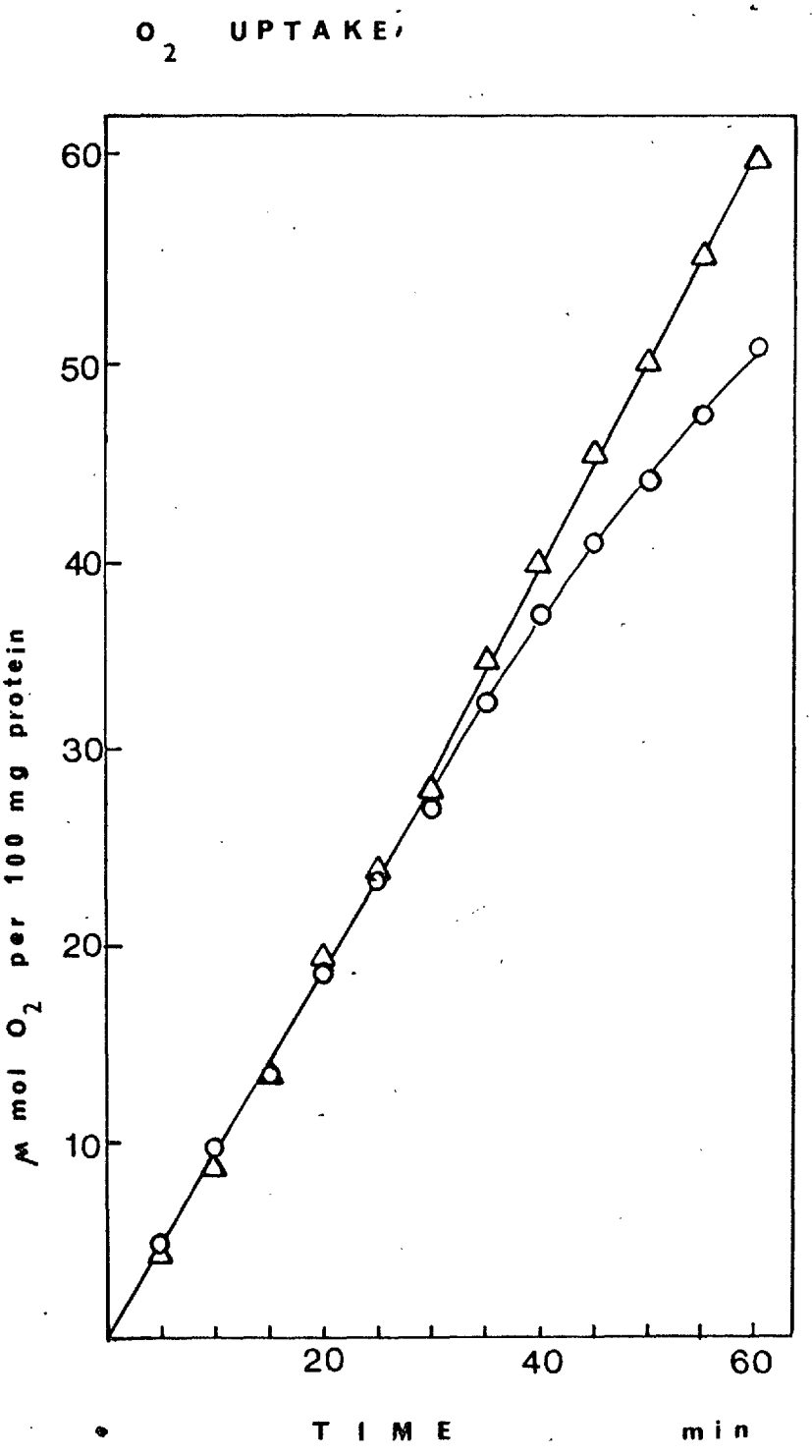
* Data from Bradford (1970a).

Figure 14 Respiration of synaptosome beds

Small synaptosome beds were incubated at 37°C in Krebs-phosphate medium for 1 h ; ○——○ unstimulated samples.

△——△ samples stimulated with electrical pulses after 30 min preincubation and continued to the end of incubation. Values plotted represent the mean rates from 10 (stimulated) and 22 (control) experiments. Values for SEMs are given in Table 8.

Figure 14


O_2 UPTAKE
60
50
40
30
20
10
μ mol O_2 per 100 mg protein
20
40
60
TIME
min

suspension. The extra time is required for centrifugation of synaptosome suspensions onto nylon gauze and manipulation of 4 small synaptosome beds between grid electrodes, to be inserted into flasks. In this case the falling off in respiration is in fact occurring after a period of incubation comparable to 65-75 minutes incubation of synaptosome suspensions, during which time they also show a slight falling off of respiratory rate. In studies using quick transfer electrodes, manipulations are simpler and very rapid, incubation begins 15 minutes after contact with medium, so studies under these conditions are likely to be during the linear period of respiration.

From the values of lactate production and oxygen utilization by synaptosome beds, it was estimated that about 11% of the oxidised glucose was converted to lactate, assuming that all the oxygen taken up by the bed was utilized for glucose oxidation.

Incorporation of (U-^{14}C) glucose into amino acids. In order to obtain a further measure of the adequacy of substrate availability within the synaptosome beds, the extent of incorporation of labelled glucose into amino acids was measured. During 60 minutes incubation in phosphate medium containing 10mM (U-^{14}C) glucose, considerable amounts of radioactivity were incorporated into glutamate, aspartate, alanine and GABA (Table 9), and the final specific radioactivities on a molecular basis, of these amino acids in the synaptosome bed were one fifth to one third of that of the glucose in the incubation medium. This is a similar picture to that found for suspensions of synaptosomes where amino acids are labelled to this extent over the same incubation period.

ATP and Phosphocreatine levels. The presence of glucose or pyruvate at 10mM in the incubation medium caused a large production of ATP and

Table 9 Specific activities of amino acids in synaptosome beds after incubation with (U-^{14}C) glucose

	Control	Stimulated for last 30 min of incubation with electrical pulses
Aspartate	88 ± 4 (12)	114 ± 10 (9)*
Glutamate	126 ± 10 (11)	183 ± 21 (9)*
Alanine	123 ± 6 (11)	95 ± 12(10)**
GABA	122 ± 7 (13)	139 ± 15 (9)

Synaptosome beds were incubated in 5ml of Krebs-phosphate medium at 37°C for 1 h in the presence of 10mM glucose and 25μCi of (U-^{14}C) glucose. Initial specific radioactivity of medium 428 nCi/μmol. Values are means ± S.E.M. for the number of experiments in brackets. Units are nCi/μmol. Values significantly different from control: * $P < 0.02$; ** $P < 0.05$.

Table 10 Levels of ATP and phosphocreatine in synaptosome beds incubated with glucose or pyruvate and without substrate

Substrate	ATP (nmol/100mg protein)	Phosphocreatine (nmol/100mg protein)
10mM glucose	267 ± 23 (8)	394 ± 58 (8)
10mM pyruvate	368 ± 59 (3)	414 ± 32 (3)
No substrate	106 ± 17 (8)	135 ± 28 (7)

Synaptosome beds were incubated at 37°C for 1 h in Krebs phosphate medium with 0.75mM-Ca^{2+} and substrate as indicated. Mean values ± S.E.M. are given for number of experiments in brackets.

phosphocreatine in synaptosome beds, (Table 10). The levels are similar to those produced by synaptosome suspensions in tris medium containing Ca^{2+} (2.8mM), but smaller than those produced in a tris medium containing NaH_2PO_4 (21mM) and no Ca^{2+} (Bradford 1969). In the absence of substrate there was a large decrease in the levels of ATP and phosphocreatine (60-71%). ATP production was found to be greater in the presence of pyruvate as a substrate, than glucose. This might be correlated with the observation that the rate of respiration of synaptosomes is 25% greater with pyruvate than glucose (Bradford 1969).

Effect of hypertonicity and added cyanide. Amino acid retention by the beds appeared to be dependent on their structural integrity and on glycolytic and respiratory activity. Thus when synaptosome beds were incubated in Krebs-phosphate medium, potassium cyanide at 1mM together with iodoacetate at 10mM caused almost total loss of amino acids to the medium (Table 11), under conditions previously shown to cause large drops in the levels of energy rich phosphates (Bradford 1969). Incubation in water instead of medium caused massive loss of amino acid and was accompanied by disintegration of the beds, the synaptosomes being readily washed off the gauze by the fluid movements. The amino acids were recovered in the medium after sedimentation of synaptosomes.

Incubation in water led to a significant increase in total amino acid pool sizes ($P<0.01$) for aspartate, glutamine, serine, glutamate, glycine and GABA; the alanine content showed a small decrease. With 1mM-KCN and 10mM-iodoacetate, there was a significant increase in aspartate and GABA ($p<0.01$) and a significant decrease in alanine ($P<0.01$). The total amounts of glutamine, serine and glycine also decreased in this situation.

TABLE 11 AMINO ACID CONTENT OF SYNAPTOSOMES INCUBATED IN THE PRESENCE OF METABOLIC INHIBITORS OR IN WATER

	Amino acid content (nmol/100 mg protein)								
	No inhibitors			Cyanide plus iodoacetate			Water only		
	Supernatant	Bed	Total	Supernatant	Bed	Total	Supernatant	Bed	Total
Aspartate	88	996	1084	1709	341	2050	3036	26	3062
Glutamine	331	213	544	463	39	502	753	17	770
Serine	387	314	701	495	180	675	856	56	912
Glutamate	187	2822	3009	2843	811	3654	4566	29	4595
Glycine	386	381	767	487	115	602	751	177	928
Alanine	438	334	772	436	54	490	670	25	395
GABA	21	497	518	686	170	865	1477	10	1487

Synaptosome beds were incubated in Krebs–phosphate medium or in water for 1 h at 37°C. Potassium cyanide (1 mM) and sodium iodoacetate (10 mM) were included in the Krebs–phosphate medium as indicated. Values are means for four experiments, Values of S.E.M. were approx. 10 per cent.

Loss of protein from the synaptosome bed. During a one hour period of incubation only a small percentage of protein was lost to the medium. This was found to be 2% ± 0.6 (mean value ± S.E.M. for 14 experiments). This may be partially due to loss of soluble proteins, but is unlikely to be very deleterious to the preparation in such small amounts.

Electron Microscopy. Both positive and negative stained electron micrographs of the synaptosome preparation used in these studies showed the appearance to have similar characteristics to synaptosomes prepared by other workers. In most cases the synaptosome membrane was intact and the average diameter of the synaptosomes was 0.8μ. (Figure 1).

CONCLUSIONS

The basic features of the performance of synaptosomes in suspension could be reproduced in synaptosome beds. Oxygen uptake, lactate production, glucose utilization for synthesis of amino acids and ATP and phosphocreatine occurred at similar rates in both suspensions and beds of synaptosomes. Thus, there was adequate availability of oxygen and substrate in synaptosome beds. When synaptosome beds were incubated in medium they retained their structural integrity but this was lost under hypertonic conditions. The synaptosome bed would thus appear to be a valid preparation and was chosen for the present studies, where use could be made of the advantageous rapid fixation and small fluid space in the immediate environment of the synaptosome.

CHAPTER 4

APPLICATION OF DEPOLARIZING INFLUENCES TO SYNAPTOSOME BEDS

Introduction

The depolarizing action of electrical pulses and elevated potassium

(i) Physiological effects

(ii) Metabolic effects

(iii) Ion effects

Experimental

Results

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Metabolic effects of electrical stimulation

The effect of elevated potassium on lactate production

The effect of gauze on the response to stimulation

B. Release of amino acids in response to stimulation

The effect of electrical stimulation of amino acid release and its variation with time

The effect of elevated K^+ on amino acid release

Comparison of amino acid release with electrical and K^+ stimulation

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The effect of stimulation of cerebral cortex slices

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The specific activities of amino acids released by K^+ stimulation

C. The effect of ouabain and amino acid analogues on amino acid accumulation in the medium

Ouabain : Introduction

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Evidence that depolarization of synaptosome beds occurs

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Amino acid release in response to depolarization

Proportions of amino acids lost in response to stimulation

Uptake of amino acids

The site of release of amino acid

CHAPTER 4 APPLICATION OF DEPOLARIZING INFLUENCES TO SYNAPTOSOME BEDS

Introduction. In order to study the processes occuring at the synapse during transmission, it is necessary to simulate the arrival of an impulse at the nerve terminal. When an impulse arrives at the terminal, depolarization of the terminal membrane occurs which leads to transmitter release. Agents such as electrical pulses and elevated K^+, cause depolarization and have been used both *in vivo* and *in vitro* to produce the same effects as a natural impulse. The evidence supporting the action of these agents in causing depolarization is discussed below. Electrical and K^+ stimulation were used in the present studies and their effects on metabolic processes and release of amino acids were investigated.

The depolarizing action of electrical pulses and elevated external potassium

(i) Physiological effects. Application of electrical pulses *in vivo* to single neurones causes generation of action potentials and depolarization of adjacent neurones in excitatory circuits. These effects are the same as those occuring after indirect stimulation of a neural pathway or during natural transmission. In the brain slice, electrical stimulation can lead to conducted responses, depolarization and spike potentials. This has been demonstrated by various preparations which possess a preserved neural input, such as slices of lateral olfactory tract (Yamamoto & McIlwain 1966; Campbell, McIlwain, Richards & Sommerville 1967), piriform cortex (Richards & Sercombe 1968), hippocampus (Yamamoto & Kawai 1967) and optic tract (Kawai & Yamamoto 1968). Stimulation of incubated tissue with silver electrodes, for example at the anterior end of the olfactory tract,yields post synaptic potentials and spike potentials in the posterior region of the tract which can be monitored with a second electrode.

When isolated muscles are soaked in Ringer solution containing excess K^+, there is large increase in the frequency of the mepps which is

attributable to depolarization of the presynaptic terminal (Liley 1956; Furukawa, Furukawa & Takagi 1957; Birks, Huxley & Katz 1960; Takeuchi & Takeuchi 1961). With an external K^+ concentration of greater than 10mM, the membrane potential falls by 26 mV for each 2.7 fold rise in concentration which also increases the mepp frequency by 55 times (2.7 x 4). This defined action of K^+ at the presynaptic terminal is a clear demonstration of its depolarizing effect. Topical application of KCl (1 drop of 1%) gives rise to spreading depression (reviewed by Marshall 1959). This is a depolarization which moves in a surface negative wave, with depression of cortical activity, together with additional reactions such as fast spikes. Iontophoretic application of K^+ to cerebral neurones (post sigmoid gyrus) only causes a relatively slow and irregular discharge (Krnjevic & Phillis 1963). However, in slices of mammalian cerebral cortex incubated in oxygenated medium, in which a resting membrane potential has been established, addition of K^+ (15 - 50mM) causes a depolarization of the tissue (Hillman & McIlwain 1961) from which the tissue is capable of recovering in fresh medium (6mM K^+).

(ii) Metabolic effects. Metabolic effects also accompany electrical and potassium stimulation and are similar to *in vivo* responses to neural activity which further validates the use of these types of stimulation (reviewed by Hertz 1969 and Quastel 1969). The oxygen consumption of brain slices is increased by electrical pulses (McIlwain 1951; 1953) and 20mM K^+ or greater and giving a maximal response at 50mM (Ashford & Dixon 1935; Hertz & Skou 1962). The maximal electrical response is equivalent to that produced by K^+ and the presence of Na^+ and the same substrates support the increased respiration, i.e. glucose, lactate, pyruvate, but not succinate, αKG or fumarate with electrical (McIlwain 1953a;

Kratzing 1953) and K^+ stimulation (Ghosh & Quastel 1954). This is a comparable phenomenon to the increased O_2 consumption of brain *in vivo* under conditions of increased neural activity (Klein & Olsen 1947), and has also been found to occur with electrical stimulation of isolated ganglia (Larrabee & Posternak 1952) and isolated frog spinal cord (Winterstein 1929). The electrical response of cerebral slices is proportional to frequency and voltage of the applied electrical pulses (McIlwain & Joanny 1963) and requires the presence of Na^+ (Gore & McIlwain 1952; Bachelard et al 1962) and K^+ (Cummins & McIlwain 1961).

Lactic acid formation also occurs during neural activity, for example during convulsions (Klein & Olsen 1947), and *in vitro* on electrical stimulation of isolated autonomic ganglia (Larrabee 1958) and cortex slices (McIlwain 1955; McIlwain & Tresize 1956).

Both electrical stimulation and elevated K^+ cause a depletion of energy rich compounds in slices such as ATP and phosphocreatine (McIlwain 1955; Heald & McIlwain 1956; Gore & McIlwain 1952; Heald 1954; 1960) which are synthesised during incubation, breakdown occuring at rates as large as 1.4mmol/g/h. The connection between loss of ATP and excitation would appear to be due to the Na^+ which enters during depolarization, being actively pumped out by means of the Na/K ATPase of the membrane.

Elevated K^+ leads to a wide range of additional metabolic effects such as an increase in the glutamate, GABA and glutamine formation from glucose or glutamine formation from glutamate in brain slices (Quastel 1959; Kini & Quastel 1959; 1960; Lahiri & Quastel 1963; Berl et al 1968), decreased glycogen synthesis (Kleinzeller et al 1957) and increased phospholipid turnover (Heald 1960). Indeed a large number of enzymes are activated by K^+ in animal, plant and bacterial cells, such as 6 enzymes of glycolysis

and also ChAc (Lubin 1964). Thus the increased oxygen uptake with stimulation may be of use for processes other than extrusion of Na^+, which is in agreement with the estimation made by Whittam (1962) that about 40% of the respiration of brain is concerned with providing energy for the salt pumping mechanisms.

(iii) Ion effects. On the whole, K^+ and electrical stimulation have similar effects on metabolism, causing an increased respiratory rate which is affected by the same agents and to the same degree, indicating that these processes have the same basis of action. The ion fluxes occurring during electrical stimulation and natural transmission are similar, an initial Na^+ influx, followed by an active extrusion of Na^+ and gain of K^+ in the recovery period but those occurring in the presence of high external K^+ are less well understood. A difference has been observed in Na^+ flux, electrical stimulation causes an increased conductance of Na^+ ions across the membrane (Keesey, Wallgren & McIlwain 1965), which can be blocked by a low concentration of tetrodotoxin (3×10^{-6}M), an inhibitor of action potential generation and the electrically stimulated respiration increase (Chan & Quastel 1967). However, K^+ stimulated respiration is unaffected by tetrodotoxin, which is not surprising since tetrodotoxin does not affect K^+ conductance across the membrane.

Experimental

Synaptosome beds were incubated in either Krebs phosphate or bicarbonate medium and stimulated by elevating the medium K^+ concentration from 6mM to 56mM or by application of electrical pulses. Rectangular pulses were used because they provide a defined form of stimulation and have a fast rise and fall in voltage which is superior to that produced by sine waves. Alternating pulses of 10 V amplitude, 0.4ms duration and delivered at the rate of 100/s were used. Alternating positive and negative pulses prevent the formation of electrolytic products and the development of a back e.m.f. The voltage amplitude was chosen since cerebral cortex slices in grid electrodes give a maximal respiratory response under these conditions (McIlwain & Joanny 1963).

Firstly, the metabolic responses of synaptosomes in the form of a bed to electrical pulses were confirmed. The effects, being generally similar to those of suspensions of synaptosomes, are briefly described. The stimulation system was further investigated by measuring the glycolytic response of cerebral cortex slices incubated in quick transfer holders with and without nylon gauze. Since the evidence was in favour of the occurence of metabolic phenomena associated with depolarization, the characteristics of amino acid release under these conditions were then studied. Amino acid release in response to electrical pulses and K^+ stimulation of synaptosome beds was measured and compared to that of cerebral slices and non-neural tissue. The amount and origin of released amino acid were studied by time course and radioactivity experiments and the presence of uptake processes by incubation with amino acid analogues and ouabain.

Results

A. Metabolic Responses to Electrical Stimulation and 50mM K^+.

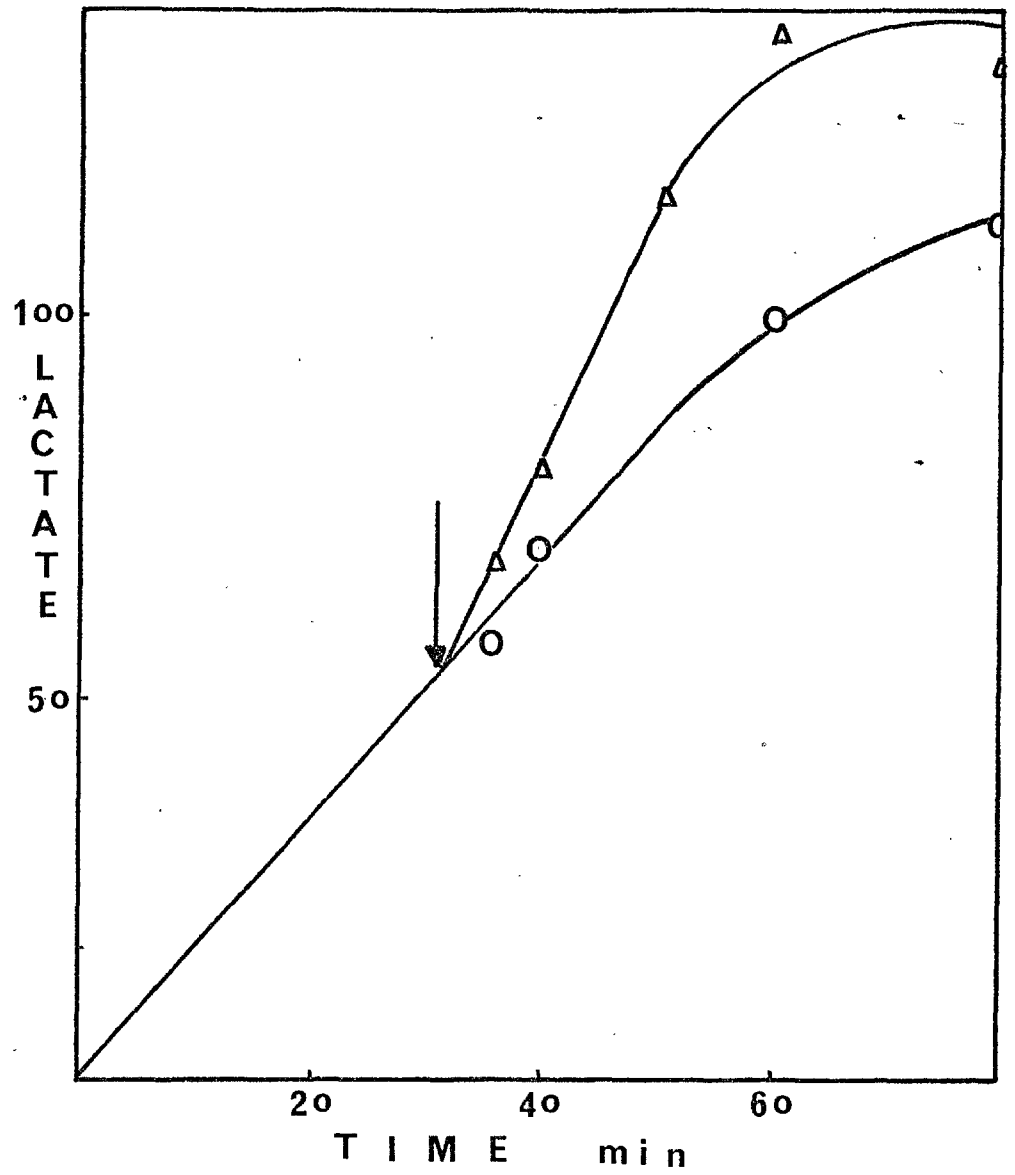
Metabolic effects of electrical stimulation of synaptosome beds.

Electrical pulses applied through quick transfer holders during the last 30 min of 1 h incubation caused a substantial increase (33%) in lactate formation (Table 7) compared to unstimulated beds. Passage of the same pulses through grid electrodes in Warburg flasks brought about an increase in oxygen uptake of a similar magnitude (Table 8 and Figure 14). The lactate accumulation in the medium was found to occur in a linear fashion for at least an hour (Figure 15), in a parallel manner to oxygen uptake.

Electrical stimulation in the presence of (U-^{14}C) glucose caused increases in the specific radioactivities of glutamate (44%), aspartate (10%) and GABA (23%), but not that of alanine which showed a small decrease (Table 9). In the absence of stimulation the specific activities of glutamate, alanine and GABA were similar, being about 29% of the initial specific activity of glucose. However, after electrical stimulation, the specific activity of glutamate became 43% of the original glucose, which was the greatest increase, with GABA showing the second largest change. This would imply that stimulation caused an increased synthesis of glutamate and GABA from the added glucose. The decreased specific activity of alanine is likely to be due to a dilution effect with the unlabelled alanine present in the bed and associated with no net synthesis of alanine.

When synaptosome beds were incubated for 40 min in Krebs phosphate medium and electrically stimulated for the last 10 min of incubation,

Figure 15 The variation of lactate production by synaptosome beds with time and the effect of electrical stimulation



Synaptosome beds were incubated in Krebs phosphate medium at 37°C for various periods of time from 36-80 min O—O ; electrical stimulation was started at 30 min as denoted by the arrow and continued to the end of incubation Δ—Δ . The lactate accumulation in the medium is represented on the ordinate as a percentage of lactate produced during 60 min incubation, i.e. 11.8 μmol/100mg protein (Table 7). Each point is the mean of 2-4 values except the 60 min values which are the mean of 13-15 values. S.E.M. are less than 10%.

there was a significant decrease (26%) in the K^+ content of the bed (Table 12). Potassium was determined by hypo-osmotic rupture of the synaptosome bed in 9ml distilled water, to release all the soluble components of the bed. Soluble protein was found to represent 27.5 ± 4.6% (± S.D. for 35 values) of the total synaptosomal protein.

The effect of elevated potassium on lactate production by synaptosome beds. Elevated medium K^+ produced an increase (28%) in the lactate accumulation in the medium, which was moderately significant ($p<0.05$). Synaptosome beds incubated in Krebs-bicarbonate medium for 40 min produced 11.6 ± 1.0 nmol lactate/100mg protein (± S.E.M. for 7 values), while those in which medium K had been raised by 50mM 10 min before the end of incubation 14.8 ± 1.6 nmol lactate/100mg protein (± S.E.M. for 8 values).

Table 12 Potassium content of synaptosome beds

	Potassium		Number of experiments
	µmol K^+/100mg insoluble protein	µmol K^+/100mg total protein	
Control	46.9±2.3	34.0	12
Electrical stimulation	34.8±1.9	25.2	8

Synaptosome beds were incubated in Krebs phosphate medium for 40 min. Electrical stimulation was for the last 10 min of incubation. Values are means ± S.E.M.

The effect of gauze on the response to stimulation. The nylon gauze could be expected to act as a resistance in the stimulation circuit and effectively reduce the current and therefore the degree of stimulation. However, cerebral cortex slices showed a substantial glycolytic response during application of electrical pulses and insertion of nylon gauzes between the slice and each electrode surface reduced the response by only 20-25%, indicating the extent to which the pulses applied through the gauze remained effective (Table 13).

Table 13 Lactate production by cortex slices in the presence and absence of nylon gauze

	μmol lactate accumulated/g wet wt/h	
	Control	Electrical Stimulation
No Gauze	109±5	144±10.
Gauze present	82±4	115±2

Cortex slices were incubated in Krebs-bicarbonate medium at 37°C for 1 h by means of quick transfer holders. Two rectangles of nylon gauze were positioned on either side of the cortex slices as indicated. Electrical stimulation was for the last 30 minutes of incubation. The values are means ± S.E.M. from six experiments.

B. Release of amino acids in response to stimulation by application of electrical pulses and elevated medium K^+.

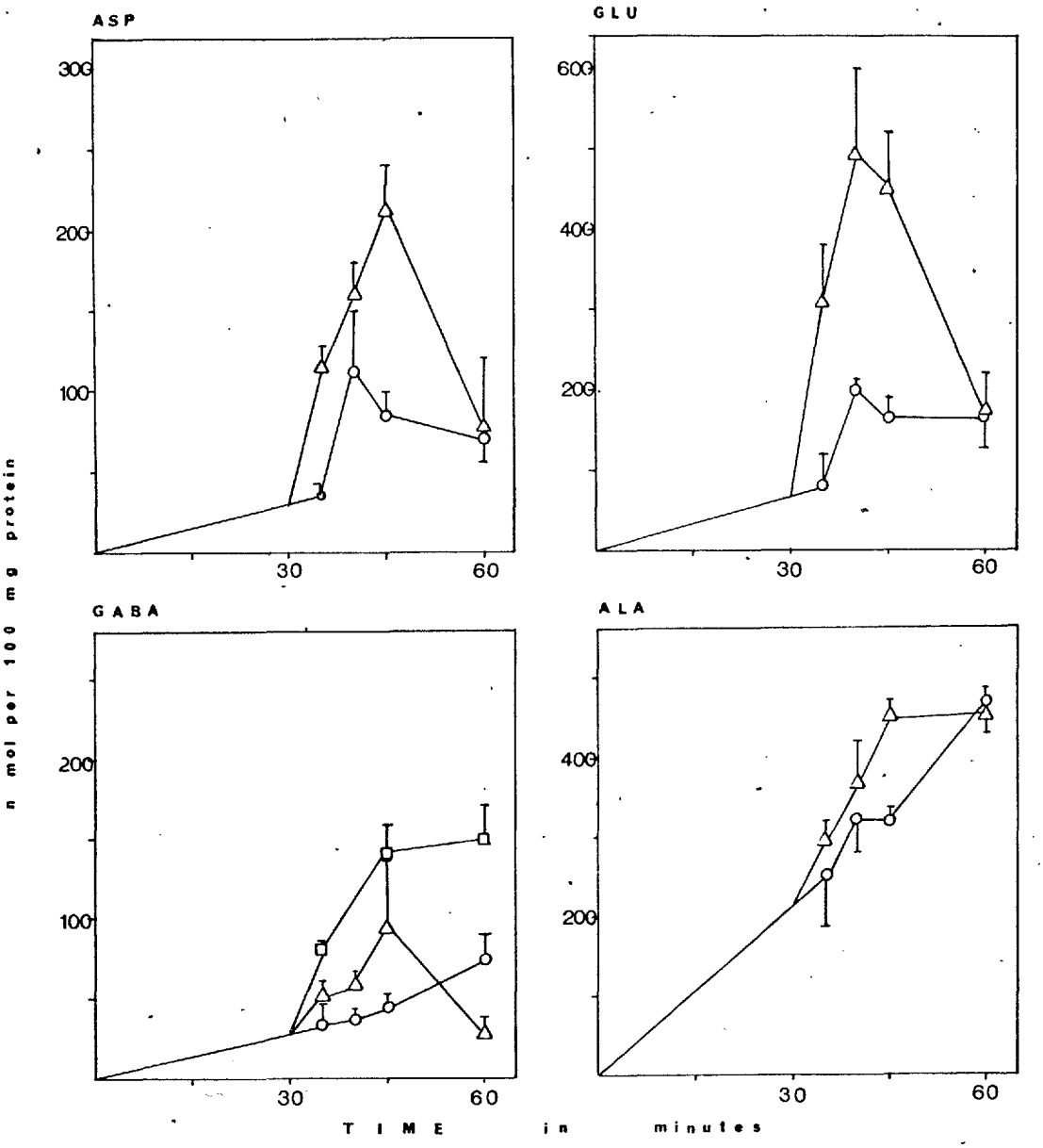
The effect of electrical stimulation on amino acid release and its variation with time. When synaptosome beds were incubated in Krebs phosphate medium for periods between 40 minutes and 1 h and electrical pulses were applied by means of quick transfer holder electrodes after the first 30 minutes, a large increase in the amounts of glutamate, aspartate and GABA released to the medium was observed. The time course of amino acid release with stimulation for these three amino acids showed that the effect was maximal after 10-15 minutes stimulation and thereafter diminished, being only a few per cent from control values after 30 min of pulses (Figure 16). The other amino acids studied, i.e. glutamine, serine glycine and alanine showed a different pattern of behaviour, gradually accumulating in the medium with time and electrical stimulation had little effect on their rates of release. The pattern of alanine release is shown in Figure 16 as being typical of these 4 amino acids.

The effect of elevated K^+ on amino acid release. When synaptosome beds were incubated in Krebs bicarbonate medium for 40 min, and the medium K^+ raised by 50mM after 30 min, there was found to be a large increase in the amounts of glutamate, GABA and aspartate released to the medium. This is illustrated by means of chromatograms of the amino acids in the incubation media of a control and K^+ stimulated synaptosome bed (Figure 17) where the change in glutamate, GABA and aspartate is evident. The values of amino acid released related to the protein content of the bed is given in Figure 18 and is represented in terms of % increase in amino acid release in Figure 19. K^+ stimulation caused a small increase in release of all amino acids (up to 40%), but the increased release of glutamate, GABA and aspartate was considerable, being 130 - 190%. A similar pattern of amino acid release

Figure 16 The effect of the duration of electrical stimulation on amino acid release from synaptosome beds

Synaptosome beds were incubated at 37°C in Krebs-phosphate medium for periods between 35 min and 1 h ; ○——○ Unstimulated samples ; △——△ Samples stimulated with electrical pulses after 30 min preincubation for 5, 10, 15 or 30 min periods, after which time incubation was terminated ; □——□ Samples stimulated as above but with DABA added at the same time as stimulation was commenced. The vertical bars represent the S.E.M. values for 3-7 experiments. Alanine (d) is represented as being typical of the pattern shown also by glutamine, serine and glycine.

Figure 16


ASP
GLU
GABA
ALA
300
200
100
600
400
200
100
400
200
30
60
n mol per 100 mg protein
TIME in minutes

Figures 17, 18 and 19 The effect of elevated medium K^+ on amino acid release to the medium

Synaptosome beds were incubated in Krebs-bicarbonate medium at 37°C for 40 min (control). Potassium stimulation was carried out by raising the medium K^+ by 50 mM, after 30 min incubation (potassium stimulated). The amino acid accumulation in the medium from control and K^+ stimulated beds is expressed in three ways:

Figure 17 - Chromatograms of amino acids released to the medium from control and K^+ stimulated beds.

Figure 18 - Amino acid release to the medium expressed as n mol/100 mg protein in the synaptosome bed. Control value histograms (light) are superimposed on the K^+ stimulated values (dark).

Figure 19 - The percentage increase in the amino acids accumulated in the medium due to K^+ stimulation.

Figure 17 -

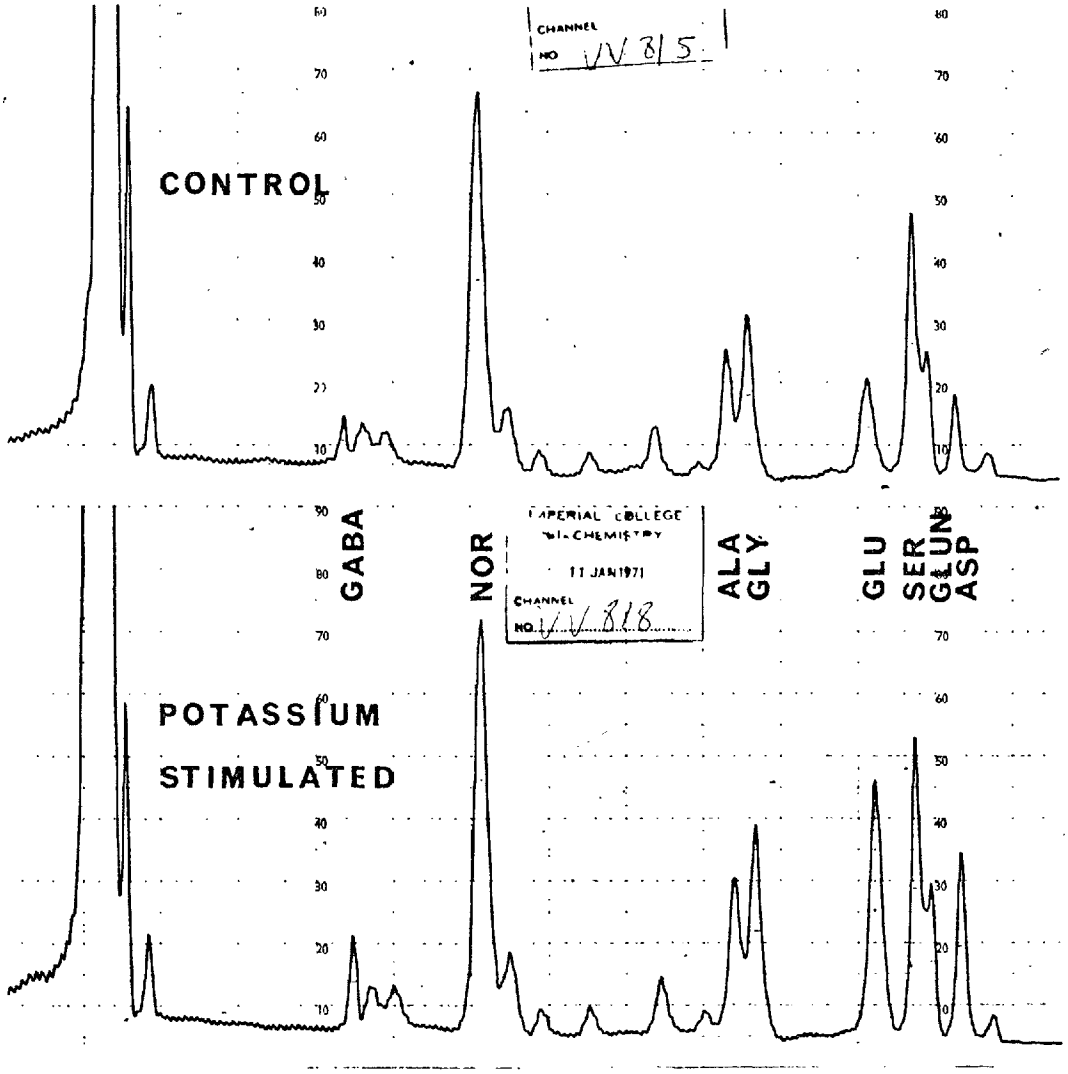

CONTROL
CHANNEL NO VV 815
POTASSIUM STIMULATED
GABA
NOR
IMPERIAL COLLEGE BIOCHEMISTRY 11 JAN 1971 CHANNEL NO VV 818
ALA
GLY
GLU
SER
GLUN
ASP

Figure 18

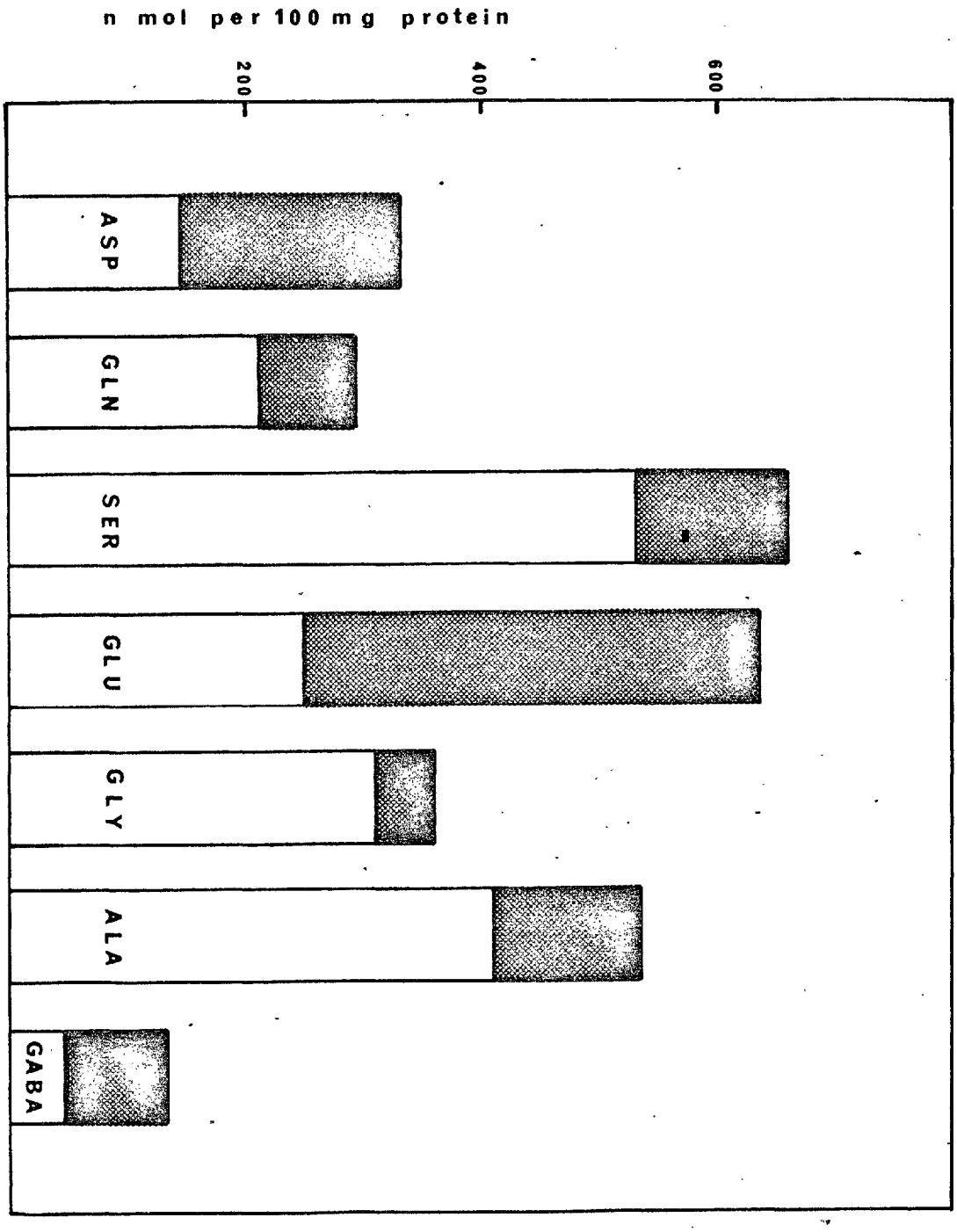
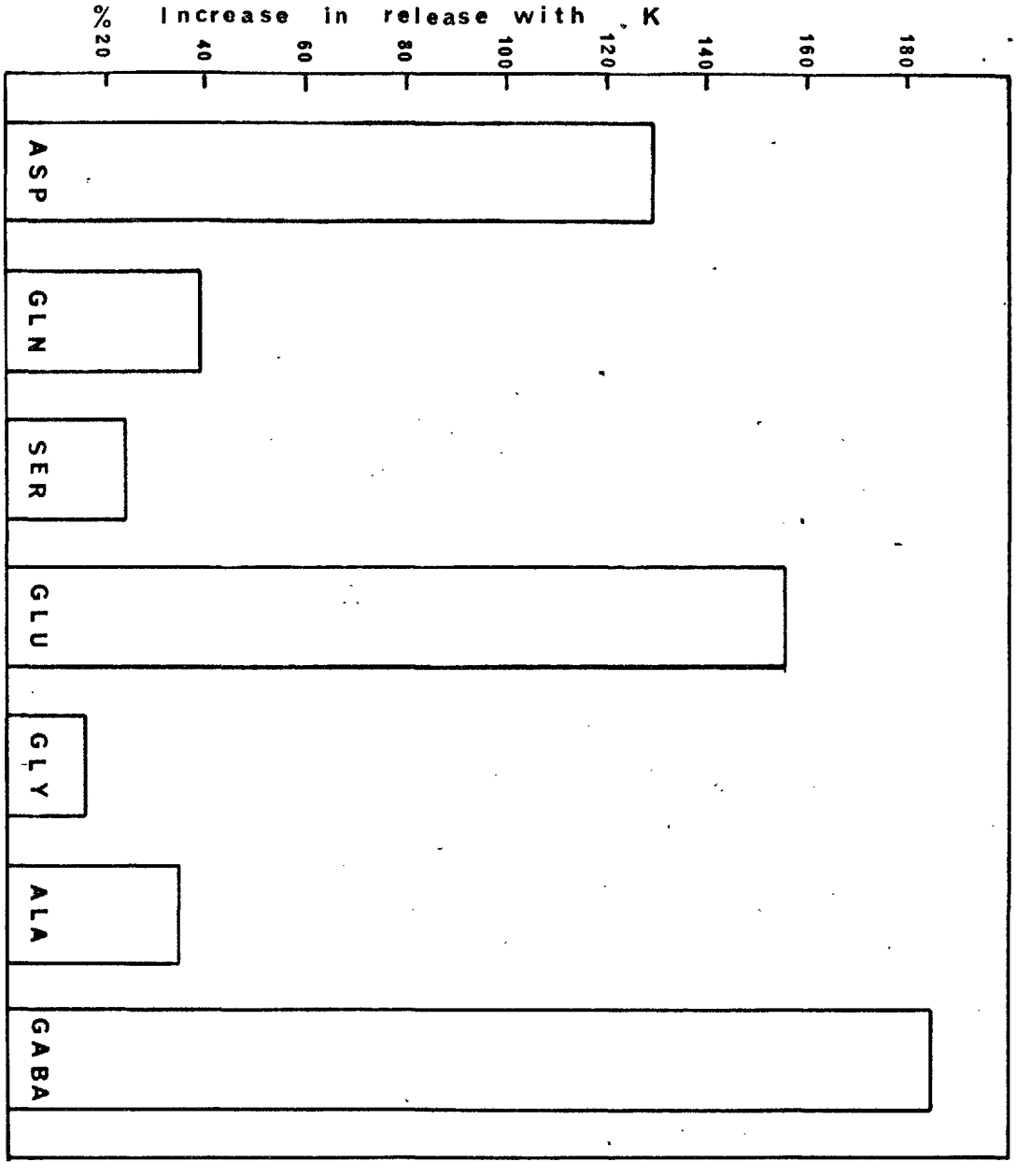

n mol per 100 mg protein
200
400
600
ASP
GLN
SER
GLU
GLY
ALA
GABA

Figure 19


% Increase in release with K
20
40
60
80
100
120
140
160
180
ASP
GLN
SER
GLU
GLY
ALA
GABA

in Krebs phosphate medium was observed (Figure 20), in which K^+ stimulation caused a 160 - 190% increase in the release of glutamate, GABA and aspartate and only small changes in the release of other amino acids. In this medium however, the actual amounts of amino acid found in the medium of all samples were found to be smaller, which may reflect the effect of phosphate in stimulating amino acid uptake (discussed later).

Comparison of amino acid release with electrical and K^+ stimulation. A comparison was made of the effect of both types of stimulation under identical conditions of medium and time of incubation (Table 14). At the time of these experiments, a more sensitive amino acid analysis was available and a wider range of amino acids could be detected. Incubation was carried out in Krebs bicarbonate medium for 40 min with stimulation occuring in the last 10 min of incubation. Electrical and K^+ stimulation produced qualitatively similar patterns of release (Table 14), but the effect of electrical stimulation was smaller. Potassium stimulation caused a more than 100% increase in the aspartate, glutamate and GABA released to the medium as shown above. There were smaller increases in the amounts of the other amino acids, glutamine, serine, phenylalanine, tryptophan and lysine increased by 5 - 23% and methionine sulphoxide, glycine, alanine, isoleucine, leucine and histidine showed a 27 - 38% increase. Electrical stimulation caused a 44 - 90% increase in the glutamate, GABA and aspartate released to the medium and smaller increases of 2 - 12% in methionine sulphoxide, serine, glycine, alanine, leucine, tyrosine, phenylalanine, histidine, tryptophan, and lysine and 17 - 20% increases in glutamine and isoleucine. Methionine sulphoxide is likely to be an oxidation product of methionine which is formed during the analytical procedure.

Table 14 Amino acid accumulation in the medium after stimulation of synaptosome beds with electrical pulses or elevated potassium

	Amino acid content of the incubation medium n mol/100mg protein		
	Control	Electrical Stimulation	Potassium Stimulation
Methionine Sulphoxide	39 ± 3	40 ± 4	54 ± 8
Aspartate	86 ± 13	124 ± 25	184 ± 19
Glutamine	400 ± 28	481 ± 46	471 ± 26
Serine	515 ± 37	573 ± 52	616 ± 67
Glutamate	256 ± 31	379 ± 46	570 ± 50
Glycine	383 ± 21	393 ± 15	505 ± 21
Alanine	391 ± 20	417 ± 18	539 ± 32
Isoleucine	68 ± 4	79 ± 5	94 ± 5
Leucine	180 ± 10	192 ± 12	230 ± 12
Tyrosine	87 ± 4	90 ± 4	107 ± 7
Phenylalanine	105 ± 5	110 ± 5	120 ± 6
GABA	24 ± 30	45 ± 8	99 ± 20
Histidine	78 ± 6	85 ± 5	105 ± 3
Tryptophan	50 ± 5	56 ± 7	61 ± 14
Lysine	246 ± 18	270 ± 15	257 ± 15
Arginine	176 ± 8	161 ± 13	-
Number of experiments	16	11	6

Synaptosome beds were incubated in Krebs bicarbonate medium for 40 min at 37°C. Electrical stimulation was carried out by application of pulses for the last 10 min of incubation and K^+ stimulation by raising the medium K^+ to 56mM 10 min before the end of incubations.
Values are means ± S.E.M.s

The effect of elevated K^+ on amino acid pool sizes. Elevated K^+ also increased the amino acid pool sizes of synaptosome beds for glutamate (26%), aspartate (36%) and GABA (10%) as shown in Table 15. The glycine content of the bed did not change but that of alanine decreased. Calculation from Table 15 and Figure 18 shows that the proportions of total amino acids released after 10 min K^+ stimulation were 18–22 per cent for aspartate, glutamate and GABA and 8–12 per cent in the absence of stimulation.

The effect of stimulation of cerebral cortex slices with potassium or electrical pulses. In order to compare the properties of synaptosomes and whole cortex tissue under similar conditions, the release of amino acids from cerebral cortex slices during K^+ and electrical stimulation was studied. Cortex slices were held between nylon gauze rectangles in Quick Transfer holders. Electrical pulses applied for the last 10 min of 40 min incubation caused a significant increase in the loss of only GABA to the incubation medium (Table 16). However, this period of electrical stimulation of synaptosomes caused maximal loss of aspartate, glutamate and GABA (Figure 16). Cerebral slices did show additional release of glutamate when stimulated electrically in the absence of gauze, though the results were not consistent, release only occuring in half the cases. Thus for all the experiments glutamate accumulation in the medium of control slices (40 min incubation) was 816 ± 124 nmol/g fresh weight ($\pm$ S.E.M.; $n = 5$), whilst 10 min stimulation increased this value to 1186 ± 205 ($n = 5$).

Raising the medium K^+ produced a similar effect to that of electrical stimulation (Table 16), causing little change in the amino acid loss to the medium. There was however a significant increase (440%) in the GABA

Table 15 Amino acid pool sizes of synaptosome beds

	Amino acid content of Synaptosome Beds (nmol/100 mg protein)	
	Control	Potassium Stimulated
Aspartate	872 ± 62 (8)	1188 ± 113 (14)
Glutamate	2023 ± 128 (7)	2543 ± 227 (14)
GABA	533 ± 32 (6)	587 ± 17 (14)
Glycine	221 ± 18 (5)	194 ± 15 (7)
Alanine	402 ± 56 (5)	270 ± 42 (7)

Synaptosome beds were incubated in Krebs-bicarbonate medium for 40 minutes at 37°C. Potassium stimulation began at 30 minutes by elevation to 56mM potassium and incubation was continued for a further 10 minutes. Values are means ± S.E.M. with the number of experiments in parenthesis.

TABLE 16 AMINO ACID POOL SIZES IN CORTEX SLICES AND IN THE INCUBATION MEDIUM FOLLOWING STIMULATION WITH POTASSIUM OR WITH ELECTRICAL PULSES

	Amino acids (nmol/g wet wt.)						
	Control			Potassium stimulation			Electrical stimulation
	Medium	Total	% Released into medium	Medium	Total	% Released into medium	Medium
Aspartate	116	876	13·2	131	1209	10·8	107
Glutamine	1632	3539	46·1	1637	3820	42·8	1315
Serine	548	1453	37·7	590	1713	34·4	530
Glutamate	591	6826	8·6	747	8980	8·3	496
Glycine	381	1826	20·8	374	1591	23·5	419
Alanine	397	1552	25·5	484	1499	32·2	381
GABA	26	3418	0·7	140*	3864	3·6	62†

Cortex slices held between two pieces of nylon gauze, were incubated in Krebs–bicarbonate medium at 37°C for 40 min. Potassium stimulation was by elevation of K^+ to 56 mM in the medium at 30 min. Electrical stimulation was commenced at 30 min and continued to the end of the incubation and amino acids were only measured in the medium. Values are means for four experiments with the S.E.M. approx. 10 per cent.

* Values significantly different from the control: * $P < 0{\cdot}1$; † $< P\ 0{\cdot}1$.

The differences shown by the other amino acids were not statistically significant.

present in the medium. With both potassium and electrical stimulation of cortex slices, very low proportions of glutamate, GABA and aspartate were recovered in the medium, (0.7 - 13%), which is close to the equally low levels of these amino acids recovered in the medium from incubation of synaptosomes. In addition, K^+ led to an increase in the total amino acid pool sizes, aspartate and glutamate increasing 32-38% and GABA, serine and glutamine increasing 8-18%. The total pool sizes of glycine and alanine decreased (3-15%).

Values of amino acid are expressed as nmol/g fresh weight, using the weight of slice before contact with medium, in order to draw a comparison with the synaptosomal values which are expressed as nmol/100mg protein, since fresh cerebral cortex contains approximately 10% protein. The protein content of a series of cortex slices was measured and found to be 9.5 $\pm$ 0.4% ($\pm$ S.E.M. for 6 determinations).

Electrical stimulation of chopped liver slices. Slices of rat liver were cut using a recessed guide and blade and the slices were chopped into squares of about 1-3 mm length. The pieces of liver were sedimented in Krebs bicarbonate medium onto nylon gauze by the method used for preparing synaptosome beds and with a second piece of gauze on top were incubated in medium for 40 min. The amino acids present in the largest amounts in liver were alanine, glycine, glutamine and glutamate compared to glutamate, GABA and glutamine in cerebral slices. GABA was not detectable in liver. A large proportion of all amino acids (50-80%) was lost with incubation alone which suggests that the tissue is fairly damaged under these conditions and unable to retain amino acids. Electrical stimulation produced no significant changes in the amino acid release or total amino acid pool sizes (Table 17).

Table 17 Amino acid pool sizes in chopped liver slices and in the incubation medium following stimulation with electrical pulses

	Amino acids (n mol/g wet wt.)					
	Control			Electrical Stimulation		
	Medium	Total	%Released into medium	Medium	Total	%Released into medium
Aspartate	113	331	34	162	366	44
Glutamine/ Threonine	1156	1465	79	1352	1721	78
Serine	406	621	64	380	608	63
Glutamate	713	1395	51	715	1384	52
Glycine	926	1504	61	995	1594	63
Alanine	1561	2375	66	1979	2973	67
Valine	484			528		
Isoleucine	430	574	75	447	557	80
Leucine	711	805	89	761	935	81
Tyrosine	72			113	162	70
Phenylalanine	149			227	292	78
Lysine	281	371	76	303	405	75

Chopped liver slices held between 2 pieces of nylon gauze, were incubated in Krebs-bicarbonate medium at 37°C for 40 min. Electrical stimulation was by application of pulses for the final 10 min of incubation. Values are means for 4 experiments with the S.E.M.s 5-10%.

The specific radioactivities of amino acids released by K^+ stimulation

Specific radioactivities of amino acids present in synaptosome beds and released into the medium after 40 min incubation in Krebs-bicarbonate medium, and also when stimulated by elevated medium K^+ for the last 10 min of incubation showed much similarity (Table 18). After this period, the specific radioactivities of aspartate, glutamate, alanine and GABA had reached 10-17% of that of the original glucose in the medium. On the assumption that incorporation of label from glucose into amino acids is linear over the first 60 min incubation, as might be implied from respiratory and glycolytic studies, then the specific radioactivity of aspartate would have reached 15%, GABA 21% and glutamate and alanine 28-30% of the original glucose. By comparison with the specific activities reached after 60 min incubation in Krebs-phosphate medium (Table 8), Krebs-bicarbonate medium shows values which are 14-29% lower.

From the values of specific radioactivities in the medium, the following calculation was used to determine the specific radioactivity of the actual amino acid released in response to stimulation;

let r_c and r_s counts be the values of radioactivity of the amino acid in the medium of control and K^+ stimulated synaptosome beds respectively,

let x_c and x_s be the amounts of amino acid in the medium of control and K^+ stimulated synaptosome beds respectively.

Then the specific activity of released amino acid $= \dfrac{r_c - r_s}{x_c - x_s}$

The specific radioactivities of the released amino acid could thus be compared to those remaining in the synaptosome bed. For glutamate, the stimulation released amino acid had a specific activity of 45 which is similar to that of the bed (49), released aspartate had a specific activity

Table 18 Specific activities of amino acids in synaptosome beds and medium after incubation with (U-^{14}C) glucose

	Medium		Beds	
	Control	Potassium Stimulated	Control	Potassium Stimulated
Aspartate	48 ± 5 (9)	36 ± 3 (9)	29 ± 2 (9)	30 ± 1 (9)
Glutamate	45 ± 3 (8)	45 ± 3 (8)	49 ± 3 (9)	50 ± 2 (9)
Alanine	34 ± 1 (9)	34 ± 2 (9)	48 ± 3 (9)	47 ± 2 (9)
GABA	34 ± 5 (9)	28 ± 2 (9)	39 ± 3 (9)	42 ± 3 (9)

Synaptosome beds were incubated in Krebs-bicarbonate medium for 40 min at 37°C, in the presence of 10mM glucose and 16.5 µCi of (U-^{14}C) glucose. Potassium stimulation began at 30 min by elevation to 56mM potassium and incubation was continued for a further 10 min. Initial specific radioactivity of the medium = 297 nCi/µmol. Values are means ± S.E.M. with the number of experiments in parenthesis. Units are nCi/µmol.

of 27, compared to 30 in the bed, released alanine had a specific activity of 34, slightly less than that of the bed (47). The largest difference ($p<0.1$) was shown for GABA which showed a specific activity of 28 in the released amino acid, compared to 42 in the bed. When synaptosome beds were incubated in Krebs-phosphate medium for 1 h and stimulated by electrical pulses for the last 30 min incubation, the glutamate and its associated radioactivity were measured in the medium. The specific radioactivity of glutamate in the medium was 32.5 ± 5.6 (± S.E.M. for 8 experiments) for the control and 75.0 ± 14.4 (± S.E.M. for 9 experiments) with stimulation. This gives a specific activity of 205 for the amino acid released due to electrical stimulation, and this is once again similar to the bed (183 given in Table 8).

The effect of ouabain and amino acid analogues on amino acid accumulation in the medium

Introduction. In view of the time course of amino acid release shown with electrical stimulation (Figure 16), in which the amount of amino acid in the medium decreases after 15 minutes stimulation, a process of uptake would seem evident. If this was indeed the case then interference with the uptake process would show whether this was actually happening. Two methods were used, firstly the use of ouabain, which is known to inhibit amino acid uptake by slices through its action on the Na^+/K^+ dependent ATPase and secondly the inclusion in the incubation medium of analogues of amino acids to compete for uptake sites.

In brain slices various amino acids are taken up and concentrated from the medium by Na^+ and energy dependent, saturable processes, inhibited by metabolic inhibitors such as 2, 4 -dinitrophenol and occuring more actively

than in other tissues (Neame 1968). Ouabain has been shown to inhibit uptake of GABA, D and L glutamate and glycine (Gonda & Quastel 1962; Iversen & Neal 1969; Tsukada et al 1963; Nakazawa & Quastel 1968). From the evidence of ouabain action on the Na^+/K^+ ATPase of erythrocytes, (Glynn 1957) and squid axon (Caldwell & Keynes 1959) and the presence of the enzyme in crab nerve (Skou 1960), ouabain is shown to compete for the K^+ binding sites of this enzyme on the external membrane surface. This would imply that in the presence of ouabain, energy cannot be made available at the membrane by breakdown of ATP. This would also explain the decreased respiration of brain slices (Ca^{2+} 0.75mM) in the presence of ouabain, since ADP is not made available by ATPase action in order to enter in the reactions of glycolysis and oxidative phosphorylation. Respiratory inhibition occurs at 10^{-4}M ouabain, while uptake processes are inhibited at even lower concentrations of 10^{-5} to 10^{-6} M. Ouabain also abolishes the electrical (Wallgren 1963) and K^+ stimulated (Gonda & Quastel 1962) increased respiration of slices, and leads to a reduction in creatine phosphate levels (Swanson 1968).

Results - The effect of ouabain on amino acid accumulation

Synaptosome beds were incubated in phosphate medium for a total period of 40 minutes. Addition of ouabain to a final concentration of 0.1mM or 0.05mM was made after the first 30 minutes of incubation. K^+ stimulation was carried out as described above. In the presence of ouabain, the accumulation in the medium of glutamate, aspartate, GABA and alanine was markedly increased while the other amino acids only showed slight increase (Figure 20). Potassium stimulation produced its established pattern of amino acid release in the presence of ouabain with glutamate and aspartate showing even larger changes than in the absence of ouabain.

Figures 20, 21 and 22 The effect of ouabain and amino acid analogues on amino acid release from synaptosome beds

Synaptosome beds were incubated in Krebs-phosphate medium at 37°C for 40 min. Potassium stimulation was carried out by raising the medium K^+ from 6 mM to 56 mM after 30 min of incubation. In the controls, the medium concentration of Na^+ was similarly elevated by 50 mM. Additions of ouabain, BABA, DABA and NAG were made at 30 min also, where indicated. The amounts of amino acid present in the medium at the end of incubation, related to the protein of the synaptosome bed (n mol/100 mg protein) are represented in the figures as histograms. The bars denote the value of the SEMs and the explanation of the histogram code is given below with the number of experiments in brackets.

Figure 20 - The effect of ouabain on amino acid release

C	(open histogram) Control (6)
K	K^+ stimulated (9)
O	Control + ouabain (5)
K + O	K^+ stimulated + ouabain (5)

Figure 21 - The effect of BABA and DABA on amino acid release

C	Control (6)
K	K^+ stimulated (9)
BABA	Control + BABA (3)
K+BABA	K^+ stimulated + BABA (4)
DABA	Control + DABA (4)
K+DABA	K^+ stimulated + DABA (6)

Figure 22 - The effect of NAG on amino acid release

C	Control (6)
K	K^+ stimulated (9)
NAG	Control + NAG (8)
K+NAG	K^+ stimulated + NAG (10)

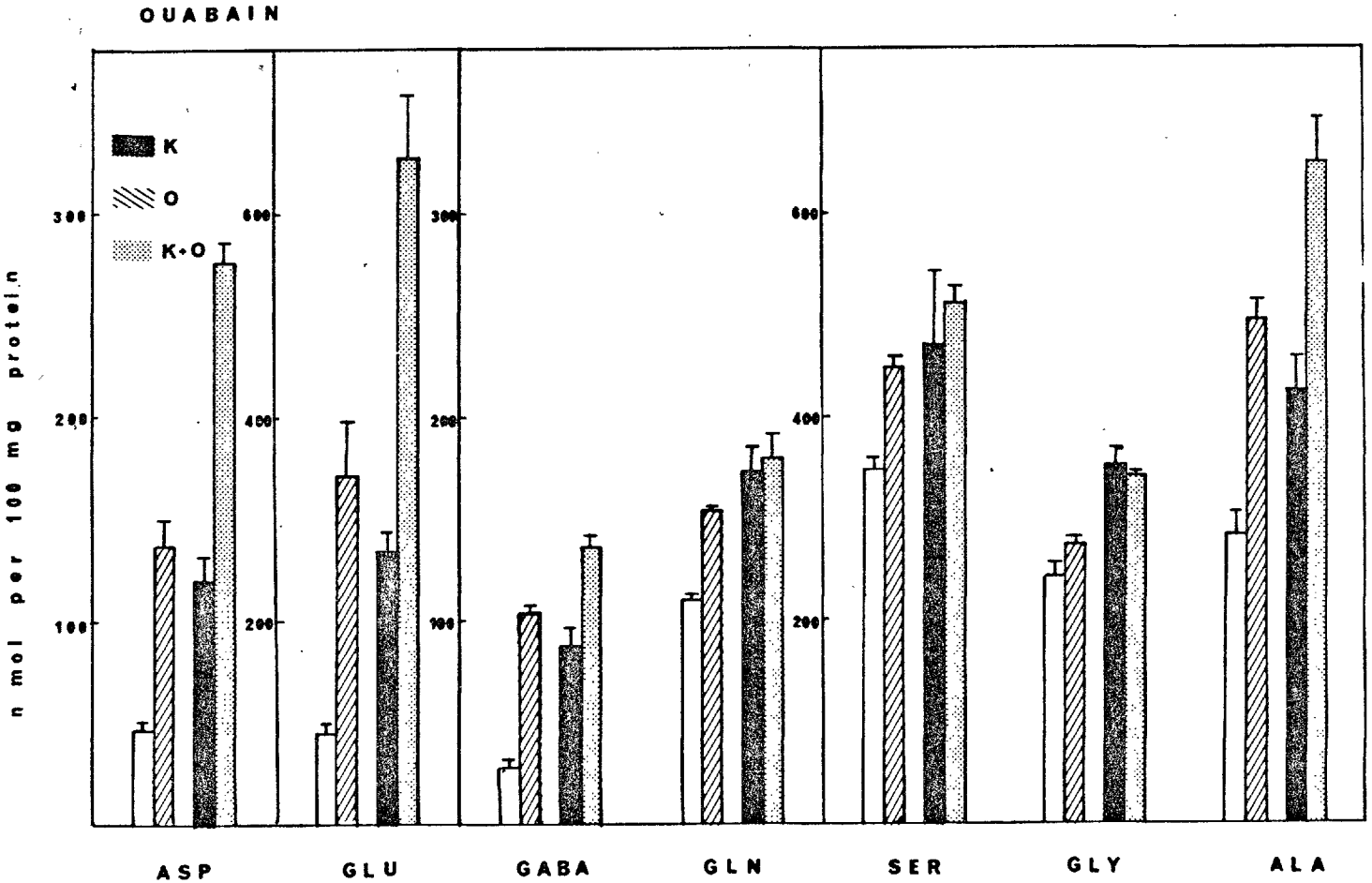

OUABAIN
K
O
K+O
n mol per 100 mg protein
ASP
GLU
GABA
GLN
SER
GLY
ALA

Figure 20 -

The additional amino acid found in the medium with ouabain is likely to represent the amino acid normally taken up by the synaptosomes but prevented by inhibition of the Na^+ pump. It could also be amino acid which has leaked out due to a membrane interaction with ouabain. Amino acid accumulation in the medium also occurs with slices (Gonda & Quastel 1962), but in this case glutamine, alanine and glutamate occur to the greatest extent in control slices and glutamate, GABA, alanine and aspartate in the presence of ouabain.

If stimulation leads to generalised leakage of amino acids, the same percentage of each amino acid would have leaked out in response to stimulation which can be detected in the presence of ouabain(which inhibits amino acid uptake). This is based on the assumption that there is one generally accessible pool of amino acids and that there is no significant amino acid fraction associated with synaptic vesicles or synaptosomal membranes which seems to be the case (see Chapter 7). In order to make this calculation, the amount of each amino acid present in the bed at 30 minutes incubation was estimated (by extrapolation from Table 11) together with the amount of amino acid subsequently released due to stimulation in the presence of ouabain to correct for uptake (Figure 20 columns 2 & 4). By this calculation the percentages of each amino acid released in the last 10 minutes of incubation due to K^+ stimulation are given in brackets after each amino acid as follows: aspartate (13.4); glutamine (6.9); serine (12.8); glutamate (10.7); glycine (12.2); alanine (28.5); GABA (6.3). These results are not consistent with a leakage due to structural change, since approximately equal percentages of total amino acid are not released.

From a calculation of the percentage increase of each amino acid in the medium with ouabain, as compared to the control, it can be seen that

the greatest increases are shown for glutamate, GABA and aspartate, followed next by alanine. The values are given in brackets as follows: glutamate (277%); GABA (259%); aspartate (194%); alanine (102%); glutamine (40%); serine (29%); glycine (14%).

Since glutamate, aspartate and GABA are accumulated most in the medium with ouabain, it would appear that uptake of these amino acids occurs at high rates and the extra amino acid present with ouabain represents a fraction that would have been resorbed. Thus it can be deduced from the values in Figure 20 that minimal rates of amino acid uptake shown under stimulated conditions are 936 nmol aspartate/100mg protein/h and 2328 nmol glutamate/100mg protein/h.

Amino acid analogues. Two analogues of GABA were used to block uptake of GABA, 2, 4 -diamino butyric acid (DABA) and β amino butyric acid (BABA). Both these amino acids have been shown to block the uptake of GABA by a crude mitochondrial pellet which is rich in nerve endings (Sano & Roberts 1963) and DABA to inhibit GABA uptake by mouse brain slices (Blasberg & Lajtha 1965). In the presence of GABA at 10^{-5} M and the analogue at 10^{-3} M, DABA was found to cause a 65% inhibition and BABA a 61% inhibition of GABA uptake, by a crude mitochondrial pellet (Sano & Roberts 1963). However, BABA was found not to cause inhibition of GABA uptake by rat cerebral cortex slices (Iversen & Neal 1969) and lobster nerve muscle preparations (Iversen & Kravitz 1968). There is a very high rate of active uptake of DABA by synaptosomes especially the De Robertis D layer (Navon & Lajtha 1969) and also Ehrlich mouse ascites carcinoma cells (Riggs, Coyne & Christensen 1954) as compared to other amino acids.

DABA occurs naturally in liver, semen, bacteria and spider secretions

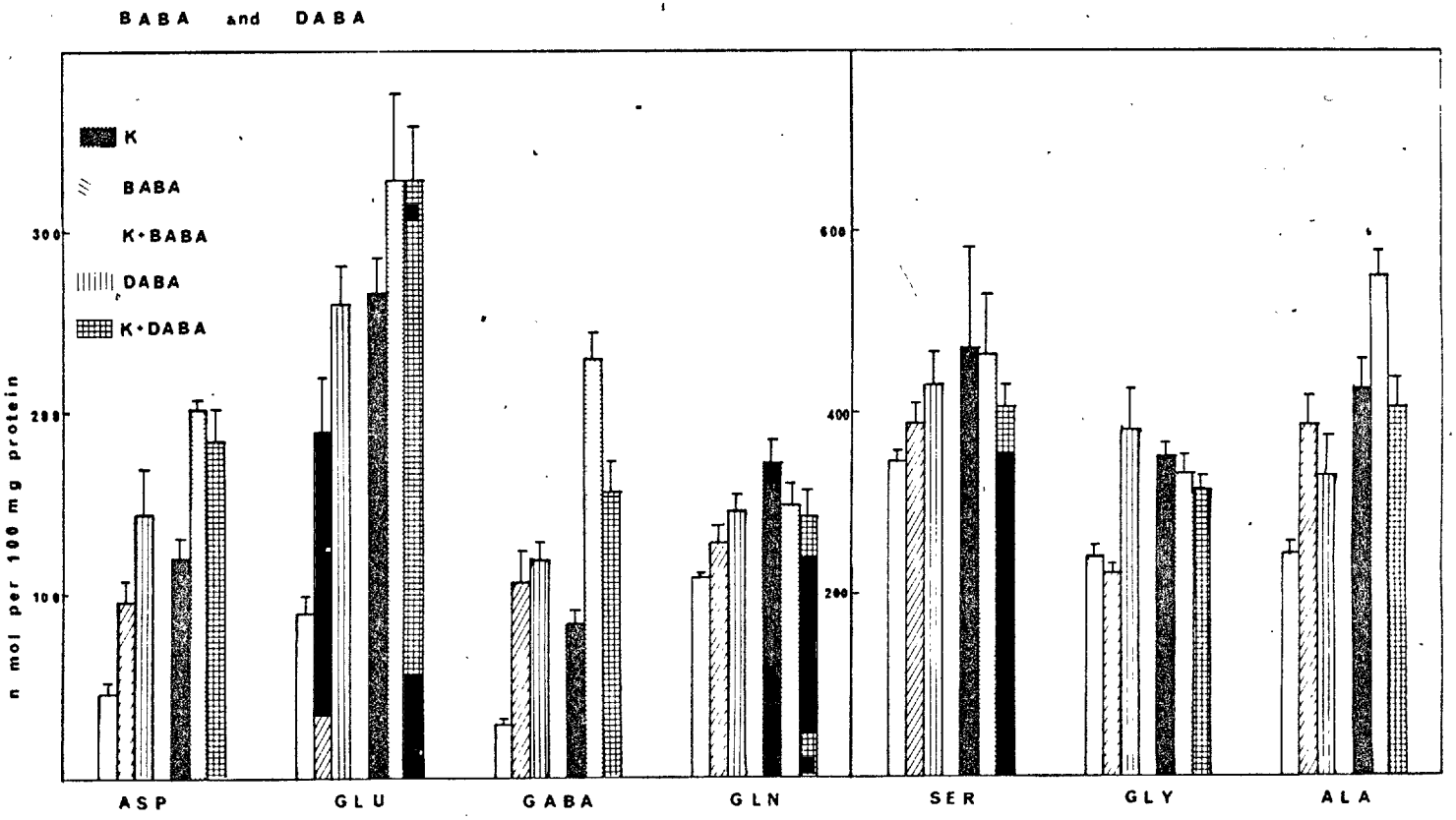
and has been characterised from bovine brain (Nakajima, Wolfgram & Clark 1967), present at 3 μg/Kg wet weight of tissue. It is toxic, a dose of 7mM/Kg subcutaneously administered leads to preconvulsive, convulsive states and death (Vivanco, Ramos, Jimenez-Diaz 1966; Riggs, Coyne & Christensen 1954), small doses lead to degenerative changes in the cerebellum and cerebral cortex. Although other GABA analogues such as δ amino β-hydroxy butyric acid can like GABA, block the surface negative response (due to depolarizing excitatory synapses) of electrocortical activity of cat cerebral and cerebellar cortex evoked by surface stimulation, DABA only has minimal effect (Purpura, Girado, Smith, Callan & Grundfest 1959).

N-acetyl glutamate was used as an analogue of glutamate. This compound is itself present in brain in considerable amounts, for example Reichett and Fonnum (1969) found that rat brain homogenates contained 5.2 μmol NAG/g compared to 9.9 and 3.4 μmol/g for glutamate and aspartate respectively. It has no known function beyond providing a source of acetyl groups for acetylation processes, being a very active co-factor in the formation of carbamyl phosphate (Marshall et al 1961).

Results.

When incubation of synaptosome beds in the presence of BABA or DABA was carried out at 1mM for the last 10 min of a 40 min period of incubation, the amount of GABA accumulating in the medium showed a 300% increase (Figure 21). This increased GABA is most likely to be due to inhibition of uptake of GABA which would normally occur during incubation, and reflects the fact that even in the control situation GABA uptake and loss is actively occuring. Large increases of glutamate and aspartate in the media were also found in the presence of these analogues, BABA causing an approximate doubling and DABA a trebling of the amino acid accumulated. It is not unexpected that DABA

Figure 21


BABA and DABA
K
BABA
K+BABA
DABA
K+DABA
n mol per 100 mg protein
300
200
100
600
400
200
ASP
GLU
GABA
GLN
SER
GLY
ALA

would cause a greater inhibition of uptake, so leading to greater amino acid accumulation in the medium as it is also an α amino acid, unlike BABA, and might thus show greater structural similarity at the site of uptake. Similar inter-relationships between acidic amino acids, GABA and DABA uptake processes havebeen shown in mouse brain slices (Blasberg & Lajtha 1965). Less marked effects were shown for glycine and alanine, DABA increasing glycine control levels and BABA increasing alanine values. Here again it appears that structurally similar molecules could compete for the same uptake site, for BABA and alanine are similar amino acids both possessing a methyl group attached to a carbon bearing an amino group.

During potassium stimulation in the presence of analogues there was a further increase in GABA and smaller increases of glutamate and aspartate. Using DABA, the GABA measured was approximately equal to the GABA released during control incubation with the analogue together with the extra GABA illicited by stimulation. However with BABA, there was a larger amount of GABA indicating that BABA was a more efficient inhibitor of uptake and that greater uptake of GABA occurs during stimulation.

With electrical stimulation, DABA produced a similar pattern of change in amino acid accumulation and GABA again showed by far the greatest change (Table 19). From the time course study of GABA accumulation in the presence of DABA, with different periods of electrical stimulation (Figure 16), GABA was found to increase with time of stimulation, and not show the maximum at 15 minutes. This further indicated that fairly substantial uptake of GABA occurred during stimulation.

Effect of N-acetyl glutamate on amino acid accumulation. Glutamate accumulation in the incubation medium of control samples was increased

Table 19 The effect of DABA on the amino acid release with electrical stimulation

	Amino acid content of supernatant n mol/100mg protein		
	Control	Electrical Stimulation	Electrical Stimulation with DABA
Aspartate	85 ± 18	213 ± 65	131 ± 26
Glutamine	116 ± 6	140 ± 36	136 ± 15
Serine	353 ± 47	436 ± 60	408 ± 58
Glutamate	164 ± 30	451 ± 77	322 ± 72
Glycine	258 ± 27	303 ± 20	275 ± 48
Alanine	321 ± 18	453 ± 14	386 ± 37
GABA	44 ± 9	95 ± 10	132 ± 16

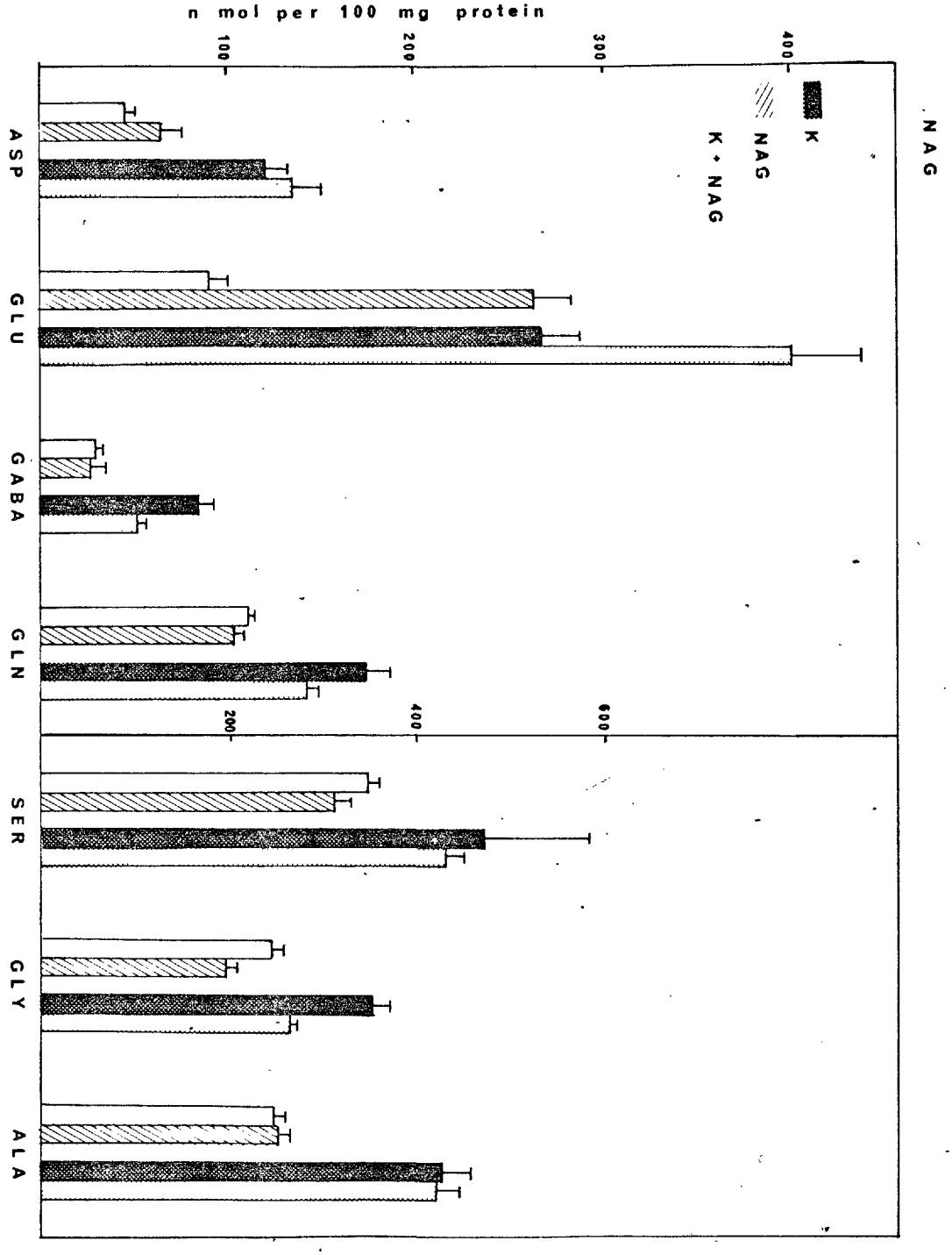
Synaptosome beds were incubated in Krebs-phosphate medium for 45 minutes at 37°C. Electrical stimulation and DABA addition to a final concentration of 1mM was started at 30 minutes and continued for a further 15 minutes. Values are means ± S.E.M. for 3 experiments.

(250%) by N-acetyl glutamate, other amino acids were virtually unaffected (Figure 22). The additional glutamate released during K^+ stimulation in the presence of this agent was equivalent to that released in its absence.

Discussion

Evidence that depolarization of synaptosomes occurs. In brain slices, elevated medium K^+ and electrical pulses cause membrane depolarisation and furthermore the latter gives rise to conducted responses such as action potentials. This is associated with Na^+ entry and K^+ loss, increased rates of respiration and glycolysis, followed by recovery. Thus by application of the same mode of electrical pulses and concentration of K^+ to the synaptosome, one would expect deploarization of the synaptosome to occur, if the existence of a membrane potential has persisted through the fractionation procedure. Unfortunately the synaptosome is too small (1μ) for insertion of a microelectrode (0.5 μ), so that the direct measurement of membrane depolarization is not possible. However synaptosomes respond to depolarising influences by increased rates of respiration and glycolysis in the same way as slices. Electrical stimulation leads to K^+ efflux from synaptosomes (Table 12) accompanied by Na^+ entry (Bradford 1970a), which suggests that lowering of transmembrane potential is occurring and the glycolytic and respiratory response is of the same magnitude as that occurring in slices. Since experiments on synaptosome suspensions and beds show similar effects in response to electrical and K^+ stimulation, as do cortex slices under similar conditions of incubation, it would seem a fair assumption to make that depolarization is also occuring in synaptosomes.

Figure 22


n mol per 100 mg protein
100
200
300
400
K
NAG
K + NAG
NAG
ASP
GLU
GABA
GLN
SER
GLY
ALA
200
400
600

Electrical stimulation : Heating as a factor. An important consideration in evaluating electrical stimulation is whether the passage of a current causes a heating effect which could be responsible for membrane damage leading to amino acid loss. This possibility has been largely ruled out by use of a micro thermocouple to measure temperature change during stimulation which was found to be a rise of 0.5 to 0.8°C in the medium and 1.0°C at the electrode surfaces and secondly by measuring the effect of raising the incubation temperature 2 to 5°C, which did not illicit amino acid release from synaptosome suspensions (Bradford & Thomas 1972). It is also possible to calculate the temperature change possible, during passage of electrical pulses between quick transfer holder electrodes, with a knowledge of the average current (I in amps) passing during the period of stimulation (t in seconds) and the resistance of the system (R in ohms).

Since the heat generated by an electric current $= \frac{R.\ I^2.\ t.}{4.18}$ calories

Then heat generated during 10 min stimulation wich quick transfer electrodes

$= \frac{(0.032)^2\ (20)(600)}{4.18} = 2.93$ calories.

Since Q = m.s.t., then 2.93 calories could raise the temperature of 5ml of medium with the assumed specific heat of 1 (s) and density of 1 by

$\frac{2.93°C}{(5)(1)} = 0.58°C.$

This is a small temperature change and is unlikely to cause damage, except where heat generated causes local hot spots on the electrode, but these were not detected by the thermocouple. This is also likely to be an over-estimation as no account has been taken of heat loss during the

10 min of stimulation. A further indication that there is no heating effect due to electrical pulses is the similarity of response of synaptosome beds to both electrical and K^+ stimulation (Table 14), the former being slightly attenuated. In addition to this the amino acid synthesis which occurs with incubation is unlikely to occur in damaged tissue.

The effect of the gauze on the degree of electrical stimulation. There is likely to be a reduction in the current flow through the tissue caused by the presence of the gauze, as judged by the capacity of gauzes to reduce the glycolytic response of cortex slices (Table 13) though the response of slices remains substantial. However the current flow between the electrodes of quick transfer holders when incubating a synaptosome bed is about double that between ring electrodes in a Warburg flask containing a synaptosome suspension, on application of identical pulses. This would imply that there is a smaller resistance in the former case which would allow adequate current flow.

Amino acid release in response to depolarization. Electrical and K^+ stimulation caused a large and significant release of glutamate, GABA and aspartate from synaptosome beds to the medium. These three amino acids are physiologically active when applied iontophoretically to the cerebral cortex (Krnjević & Phillis 1963). In all conditions the amount released of these compounds more than doubled with 10 min stimulation. The quantitative amino acid release varied in the different media used, smaller resting release occuring in phosphate medium which may be due to a phosphate stimulated uptake of amino acids which has been shown to occur in cortex slices

Stimulation with elevated K^+ or electrical pulses caused a much smaller proportional release of the other amino acids studied, i.e. glutamine, serine, glycine and alanine. A comparison between electrical and K^+ stimulation

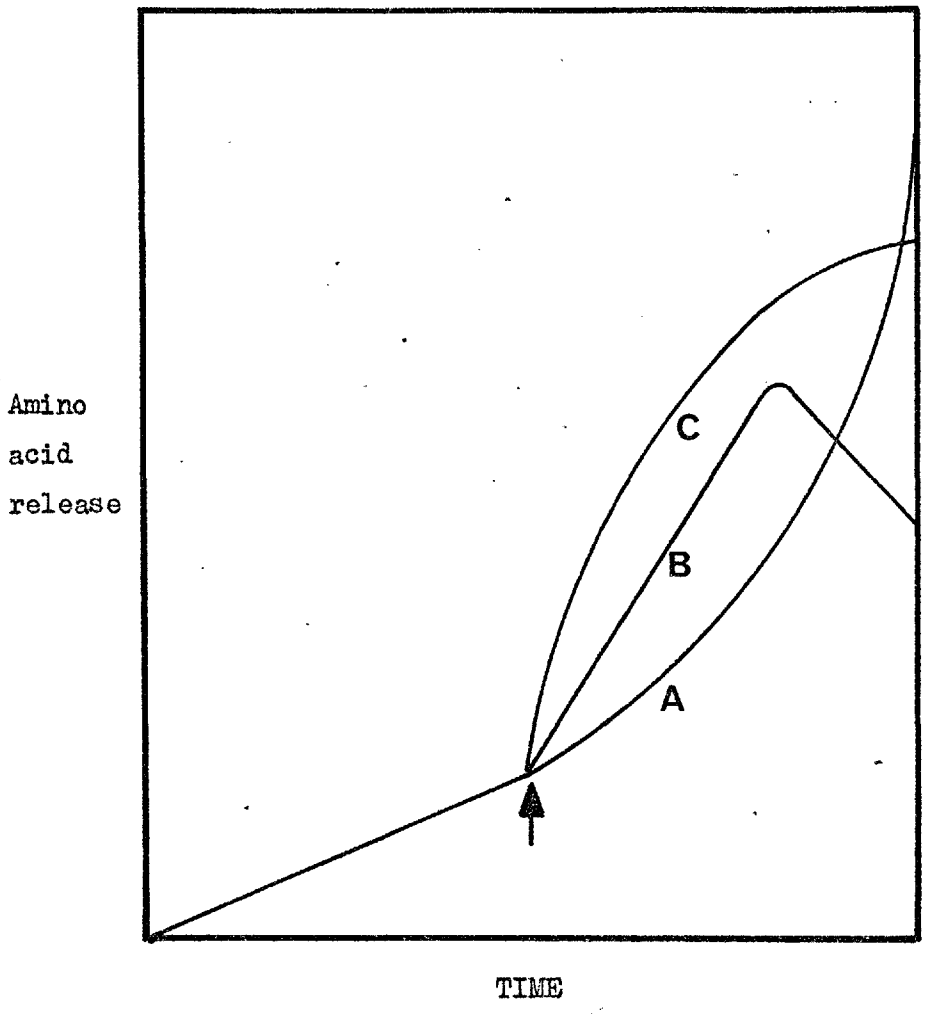
(Table 14) showed the two types of stimulation to cause qualitatively similar patterns of release, though the electrical effect was smaller.

Later studies showed that the specific glutamate, GABA and aspartate release resembled in situ release processes for transmitters by being calcium dependent (Chapter 5), and was also comparable to the calcium dependent release of acetyl-choline (Chapter 6). Further support for this release being like a physiological process comes from the time course study of release (Figure 16). If the release was due to membrane damage for example, as the stimulation continued so would the damage and hence an exponential effect would be expected as represented diagramatically by curve A in Figure 23. However what in fact occurs is an initial increase in release followed by a decrease (curve B) which effect is exaggerated in the presence of an analogue (curve C), to prevent uptake.

GABA was the only amino acid showing significant release from cerebral cortex slices held between rectangles of nylon gauze and incubated and stimulated as for synaptosome beds, though there was a detectable release of glutamate. The release of GABA with stimulation is consistent with similar reports of electrically stimulating cortex using grid electrodes and 40mM K^+ (Srinivasan, Neal & Mitchell 1969; Machiyama, Balazs & Richter 1967; Arnfred and Hertz 1971). The different patterns of release shown by nerve endings and cerebral cortex slices presumably reflect the differences in the basic properties of the cellular elements predominating in the two preparations. It is possible that the additional presence of neuronal perikaryia and glia in the slice, take up released compounds before they reach the bathing medium.

Depolarization with electrical pulses of non-neural tissue, such as liver showed no specific amino acid release with stimulation, although a similar study on chopped cortex slices shows the release of large amounts of GABA glutamate and aspartate (Bradford 1970a).

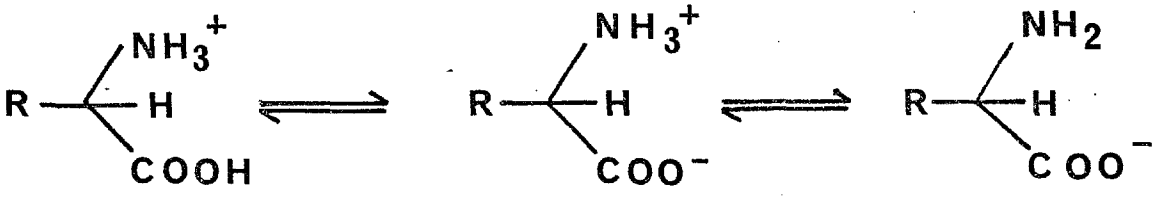
Figure 23 The nature of amino acid release with time



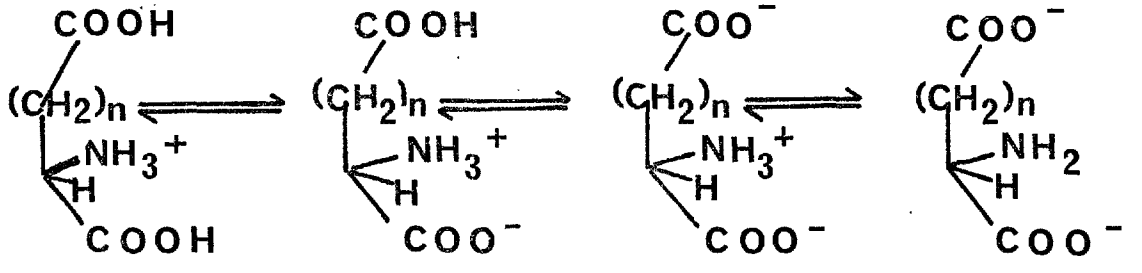
Three types of release pattern in response to electrical stimulation are shown above. The beginning of stimulation is denoted by the arrow. Curve A represents a hypothetical condition in which the effect of stimulation continues throughout the period of application. Curve B represents the observed pattern of release of physiologically active amino acids from synaptosome beds. Curve C is the same condition as B, except that amino acid analogues are present and partially inhibit amino acid uptake.

Proportions of amino acids lost in response to stimulation. It is interesting that the amounts of glutamate, GABA and aspartate released by electrical and K^+ stimulation of synaptosome beds as a percentage of their total are small (12-25%). In fact larger percentages of the remaining amino acids in the synaptosome bed are released with unstimulated incubation (25-40%) and are not greatly affected by stimulation. This large proportion lost may be due to passive diffusion of these amino acids from the high concentration in the synaptosome bed to the medium. At the pH. of the synaptosome which is assumed to be pH. 7.4, these amino acids, serine, glutamine, glycine, alanine, leucine, isoleucine, valine, tryptophan, tyrosine, phenylalanine and methionine have a p.Ka (carboxyl) of about 2 and a p.Ka (amino) of about 9, and are thus electrically neutral. GABA has a pK_1 of about 4 and pK_2 of 10.5 and would also be electrically neutral at p.H. 7.4. Neutral amino acids are unlikely to be retained by an ionic mechanism and in the absence of specific compartmentation will leak out, passing down a concentration gradient. Glutamate and aspartate, however, are negatively charged at this p.H. (p.Ka α carboxyl 2; β/γ carboxyl 4; amino group 9.8), and might need an active process for their loss from synaptosomes.

Ionization of amino acids neutral at p.H. 7.4



Ionization of aspartate and glutamate



Compared to suspensions of synaptosomes, which release 58-62% of their glutamate, GABA and aspartate on electrical stimulation, (Bradford 1970a) the release by synaptosome beds is smaller. This release from suspensions represents a sizeable component of the synaptosome, about 39% of its total amino acid fraction and about 4% of its dry weight, and orders of magnitude greater than acetyl choline loss by synaptosomes. Two possible explanations might exist to explain the difference in release between beds and suspensions of synaptosomes, firstly the bed may be in a more favourable condition to respond to depolarization as it does in situ (discussed above), and so release what would appear to be more physiological levels of transmitter comparable to that of ACh (see Chapter 6). Suspensions of synaptosomes are mechanically shaken in Warburg flasks and are in direct contact with their electrodes. Secondly in the synaptosome bed, the amino acid released is likely to be held by capillarity between adjacent synaptosomes, and would thus represent a high local concentration of amino acid which could be taken up by the high affinity uptake processes known to exist in synaptosomes for glutamate and aspartate (Logan & Snyder 1971). A combination of these possibilities might contribute to an explanation, in which larger amounts of amino acid are released by beds than detected in the medium as deduced from the analogue studies, but that these amounts would still be considerably smaller than the amounts released by suspensions.

Uptake of amino acids. The high activity of amino acid uptake processes was shown by including ouabain or amino acid analogues in the incubation medium, which led to increased amounts of amino acid in the medium. The amount of amino acid present in the medium would thus be the difference between that which has been released and the fraction which has been taken up by the synaptosome. The GABA analogues not only inhibited GABA uptake,

but also affected structurally similar amino acids, indicating a stereo-specific uptake site for GABA.

In the presence of ouabain, larger amounts of glutamate and aspartate were found in the medium, which showed an even larger increase than normal with K^+ stimulation, suggesting that ouabain has inhibited amino acid uptake to a greater extent than the other agents. The proportions of all amino acids studied which were released by stimulation in the presence of ouabain were considerably different ranging from 6 to 29%, which is not consistent with general leakiness induced by stimulation. It is not clear from these results whether inhibition of amino acid uptake by ouabain is a direct consequence of Na/K ATPase inhibition or not. In the case of a direct effect, it could be envisaged that Na^+ movements, for example, induced by depolarization could influence amino acid uptake. Peterson & Raghupathy (1972) observed that ouabain inhibited uptake of aspartate by synaptosomes only occured after a lag phase of 5 minutes which occurs similarly for NE and 5HT uptake (Tissari, Schönhöfer, Bogdanski & Brodie 1969), and indicates an indirect effect. However the medium used for these studies was sucrose containing 10mM tris-HCl/15mM Mg^{2+}/5mM Na^+ (Peterson & Raghupathy 1972) but without substrate. Further, since ouabain was added after 20 min pre-incubation there would have been a reduced amount of ATP present to act as a substrate of the ATPase.

The site of release of amino acid in response to stimulation. The observation that the specific radioactivities of aspartate, glutamate and GABA released by K^+ and electrical stimulation were close in value to those of the amino acids remaining in the tissue, suggests that each released amino acid was coming from its main pool within the synaptosome, and not principally from any smaller pools such as vesicles. A parallel observation has been

made with respect to the site of release of acetyl choline from synaptosomes (Richter & Marchbanks 1971a).

However the GABA released with K^+ stimulation showed a slightly lower specific radioactivity than the bed indicating that it was coming from a pool not rapidly equilibrating with the newly synthesised GABA.

During 40 min control incubation (Table 18), the specific activity of the medium amino acids are close to those of the synaptosome bed. This is surprising since there is initially no amino acid in the medium and subsequently it is released from the bed whose initial specific radioactivity is 0 and a medium specific activity of about 56% of that of the bed would be expected if the bed contained one pool of amino acid which was lost in a linear manner (Figure 20). For glutamate and GABA the medium specific activity is about 90% of the bed and for aspartate, it is about 165% of the bed, which implies that the lost amino acid is probably coming from a recently synthesised pool of amino acid. This is also true of the amino acids released due to stimulation, which also have specific activities of this order.

CHAPTER 5

THE EFFECTS OF IONS ON THE RELEASE OF AMINO ACIDS

Introduction

The widespread involvement of Ca^{2+} in release mechanisms

The effect of Ca^{2+} in transmitter release in the CNS

Involvement of Na^{+} in transmitter release

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Results

The effect of Ca^{2+} on transmitter release

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Results

The effect of tetrodotoxin

Experimental

Results

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The effect of tetrodotoxin

CHAPTER 5 THE EFFECT OF IONS ON THE RELEASE OF AMINO ACIDS

Introduction. In response to depolarization, synaptosome beds show a significant differential release of three physiologically active amino acids, which action may be taken as partial evidence for these amino acids being central transmitters. If indeed this is a physiological process, alterations in Na^+ and Ca^{2+} concentrations which are known to affect transmitter release are likely to modify release from synaptosome beds. This section contains results of studies on amino acid release in the presence of reduced Na^+ and Ca^{2+} concentrations and in the presence of agents which change the concentrations of these ions.

The widespread involvement of Ca^{2+} in release mechanisms. The presence of Ca^{2+} is required in an extensive range of release processes of hormones and transmitters, such as the release of insulin from the pancreas (Grodsky & Bennett 1966), vassopressin and oxytocin from the neurohypophysis (Douglas & Poisner 1964; Dicker 1966), TSH and LH from the adenohypophysis (Vale & Gullemin 1967), catecholamines from the adrenal medulla (Douglas & Rabin 1961; Poisner & Douglas 1966) and the release of noradrenaline from sympathetic nerves (Burn & Gibbons 1964).

In addition to this, calcium has been shown in squid axon and the neuromuscular junction to have a complex involvement in transmission. Calcium is involved in the initiation of the action potential and its conduction (Frankenhauser & Hodgkin 1957; 1957a), the release of transmitter at the terminals (Katz & Miledi 1965), reaction of the transmitter at the post synaptic membrane (Takeuchi & Takeuchi 1962; Nastuk & Liu 1966) and action potential generation in the muscle and its contraction (Sandow 1965). The presence of a low but constant concentration of Ca^{2+} in the extracellular bathing fluid of nerve cells and fibres is important, high external Ca^{2+}

leading to increased nerve fibre resistance (Cole 1949) and an increased firing threshold (Frankenhauser & Hodgkin 1957) and very low Ca^{2+} leading to a lowered threshold of excitation and spontaneous activity. The essential nature of Ca^{2+} in transmitter release is shown at the neuromuscular junction which when placed in a Ca^{2+} free medium and electrically stimulated will fail to produce end plate potentials (epps) in the muscle fibre, although the nerve impulse reaches the nerve terminals. However, if calcium is iontophoretically applied to the surface of the nerve terminals, there is localized ACh release and a large increase in the quantal components of the epps (Katz and Miledi 1964; 1967).

The effect of calcium on transmitter release in the CNS. In the guinea pig piriform cortex, it is possible to measure excitatory post synaptic potentials (EPSPs), which are developed directly in response to the release of excitatory transmitter. The amplitude of EPSPs developed after lateral olfactory tract stimulation has been found to increase proportionally to $(Ca^{2+})^n$ external, where n is approximately 3 (Richards & Sercombe 1970). Similar results were obtained for hippocampus (Bliss & Richards 1971). Thus release of excitatory transmitter is directly linked to Ca^{2+} concentration, no release of transmitter occuring in the absence of Ca^{2+}.

In vivo, absence of Ca^{2+} prevents the release of GABA from superfused cortex following cortical stimulation (Iversen, Mitchell & Srinivasan 1971) and reduces by 40-65% the ACh release under similar conditions (Hemsworth & Mitchell 1969). In vitro, the absence of Ca^{2+} prevents the K^+ (Baldessarini & Kopin 1967) and electrically stimulated release of preloaded, labelled noradrenaline from corpus striatum slices (Katz & Kopin 1969) and the K^+ but not electrically stimulated release of GABA from cerebral cortex slices (Srinivasan, Neal & Mitchell 1969).

Involvement of sodium in transmitter release. During transmission, depolarization of nerve terminals occurs and Na^+ entry is a vital part of this process, although other cations such as Ca^{2+} have been shown to carry some of the inward current in squid giant synapse (Katz & Miledi 1969). In the subsequent step involving Ca^{2+}, Na^+ may also play an important role, either directly or indirectly via a coupling system with Ca^{2+} transport (some evidence of the latter is mentioned in the discussion). The effect of a low external Na^+ concentration was measured on amino acid release from synaptosomes, and also the effect of an inhibitor of Na^+ conductance, tetrodotoxin. However, the effects of low Na^+ on amino acid accumulation in the medium are likely to be complex, since Na^+ is also important in the transport of amino acids.

The action of Tetrodotoxin. Tetrodotoxin, a potent neurotoxin found in Japanese puffer fish, the Californian newt and elsewhere (Kao 1966), inhibits action potentials at concentrations of 10^{-7}M by blocking Na^+ conductance without affecting K^+ fluxes (Goldman & Blaustein 1966). This has been demonstrated in a variety of excitable tissues, such as frog myelinated nerve fibres (Furukawa et al 1959), lobster and squid giant axons (Narahasi et al 1964) guinea pig prepiriform slices (Richards & Ter Keurs 1971) and eel electroplaques (Nakamura 1964), suppression of action potentials occurring, but resting potentials remaining unaffected. Tetrodotoxin has been successfully used in frog neuromuscular junction, and the giant synapses of squid stellate ganglion by Katz & Miledi (1966; 1967b; 1969) to eliminate the propagation of extraneous nerve impulses, allowing mepps and the effects of locally applied depolarizing pulses on presynaptic terminals to be studied.

The associated metabolic responses occurring in brain slices with electrical stimulation such as increased oxygen uptake, lactate production and

oxidation via acetate are also inhibited in the presence of tetrodotoxin (McIlwain 1967; Chan & Quastel 1967). Similarly, the increased respiration that occurs in the absence of Ca^{2+} (Chan & Quastel 1967) and part of the increased respiration occuring in 5mM glutamate (McIlwain et al 1969) is inhibited by tetrodotoxin. However the increased respiration and increased oxidation via acetate shown by slices in high K^+ (100mM) are unaffected by tetrodotoxin (Chan & Quastel 1967), implying that K^+ stimulation does not necessarily involve Na^+ entry at the same site as occurs during electrical stimulation.

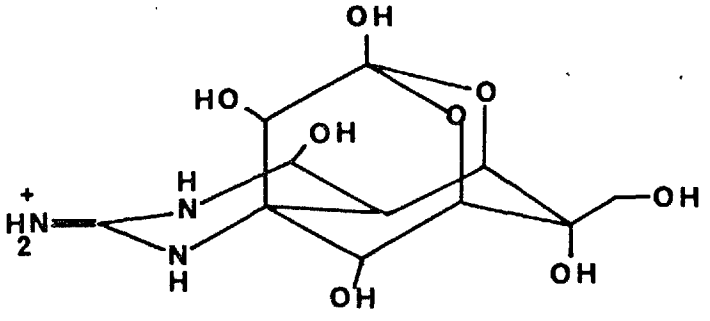
Although the major site of tetrodotoxin action is on Na^+ conductance, metabolic effects occuring as a consequence, this toxin has also been found able to suppress action potentials in medium containing either only divalent cations (Watanable et al 1967) or in lithium sea water (Moore et al 1966). Influx of Ca^{2+} in both control and glutamate stimulated neocortex slices is also reduced by tetrodotoxin (Ramsay & McIlwain 1970). Thus tetrodotoxin, acting as a cation, may act by binding strongly to the external membrane surface and hinder rapid exchange of small ions such as Na^+ or Ca^{2+}. Tetrodotoxin can exist as a zwitterion (B^+) or one of two cations (A^+ & B^+) as shown in Figure 24, and the cations have been found to be the most potent (Camougis et al 1967). Moore et al (1966) have estimated that there are fewer than 13 channels for Na^+ per μ^2 of axon from measurements of the amount of tetrodotoxin taken up by lobster nerve trunk during inhibition of action potentials.

Results

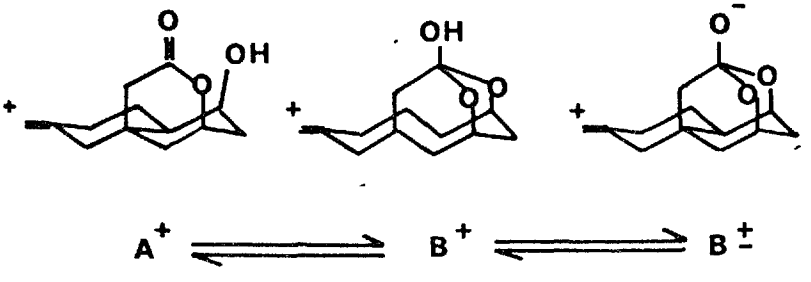
The effect of calcium on transmitter release

Experimental. Synaptosome beds were incubated and stimulated with elevated medium K^+ and electrical pulses as described before, using medium in which

Figure 24_Structure of Tetrodotoxin



Ionic species of Tetrodotoxin



the Ca^{2+} had been omitted. In some experiments EGTA was also included to complex any endogenous Ca^{2+} that might be released during incubation. EGTA specifically binds Ca^{2+} ions, binding them 10^5 times more effectively than Mg^{2+} ions.

Results

When synaptosome beds were incubated in Ca^{2+} free medium containing EGTA, the increased release of glutamate, GABA and aspartate usually induced by K^+ stimulation was completely inhibited (Table 20). Under similar conditions of absence of calcium, the effects of electrical stimulation were greatly reduced though not totally as in the case of K^+ stimulation. By only omitting Ca^{2+} from the medium, an intermediate position was obtained where the response to elevated K^+ concentration was partially reduced.

Potassium and electrical stimulation alone are thus not capable of producing the specific glutamate, GABA and aspartate release and Ca^{2+} ions are a necessary part of the release process.

Incubation of beds in a Ca^{2+} free medium containing EGTA led to a significantly reduced control release of glutamine, serine and alanine which is similarly shown by ACh (see Chapter 6). However even though this level was lower, there was no change with stimulation.

The effects of lowering medium sodium

Experimental. A Krebs-phosphate medium was used for incubation of synaptosome beds, which contained Na^+ at a concentration of 40mM present as Na_2HPO_4, the NaCl being replaced by sucrose to maintain isotonicity. Amino acid accumulation in the medium was measured at low and elevated K^+ concentrations.

Results. Lowering the medium Na^+ concentration from 164mM to 40mM caused an overall increase (100-134%) in the accumulation of glutamine, serine,

TABLE 20 THE EFFECT OF OMMISSION OF CALCIUM FROM THE INCUBATION MEDIUM ON THE AMINO ACIDS ACCUMULATION IN THE MEDIUM

	Amino acid content of supernatant (nmols/100 mg protein)					
	Calcium-containing medium		Medium without calcium containing EGTA			without EGTA
	Control	Potassium-stimulation	Control	Potassium-stimulation	Electrical-stimulation	Potassium-stimulation
Aspartate	146	333	134	103	195	245
Glutamine	211	294	118	118	145	186
Serine	530	660	287	329	230	484
Glutamate	249	635	256	259	346	484
Glycine	310	359	255	281	210	288
Alanine	410	535	261	244	249	386
GABA	47	133	41	45	50	79
No. of samples	6	6	5	6	6	4

Synaptosome beds were incubated in Krebs–bicarbonate medium in the presence of 10 mM-glucose at 37°C for 40 min. Medium with calcium contained 0·75 mM-calcium. EGTA was present at 0·5 mM where indicated. After the first 30 min of incubation, potassium or electrical stimulation was started as indicated and continued for the remaining 10 min of incubation. Values given are mean values, standard errors were about 10 per cent of the mean. In the controls, an equivalent volume of medium was added to the incubation fluid after 30 min, and the high potassium was added to the potassium stimulation samples.

glycine and alanine in the medium (Table 21), after 40 min incubation. GABA showed a large increase, a six fold change, whilst the glutamate and aspartate levels were only slightly larger in the low Na^+ medium. With K^+ stimulation, aspartate and glutamate showed their characteristic patterns of significantly increased release, while the other amino acids showed very little or no extra release.

The effect of Tetrodotoxin

Experimental. Synaptosome beds were incubated in Krebs bicarbonate medium containing tetrodotoxin (Sankyo Co. Ltd., Tokyo) for 40 min and the effect of K^+ stimulation in the presence of this agent was measured. The toxin was obtained as a crystalline powder containing one part by weight of tetrodotoxin and five parts of citric acid-sodium citrate buffer and therefore parallel incubations were carried out in which citrate buffer alone was added to the incubation medium. This procedure was to check whether the observed effects were due to tetrodotoxin itself or the citrate present in the mixture.

Results.

Incubation of beds in the presence of tetrodotoxin reduced the control release of glutamate, GABA and aspartate by 47-60%, alanine levels decreased 28% while the other amino acids were present in similar amounts as in the absence of tetrodotoxin (Table 22). With K^+ stimulation glutamate, GABA and aspartate release increased 2 to 3 fold in similar amounts as in the absence of tetrodotoxin. The final amounts of amino acid present in the medium after stimulation were less in the presence of tetrodotoxin.

Since tetrodotoxin was used in a citrate buffer, the effect of adding citrate buffer alone was examined. The effect of citrate would be expected

Table 21 Effect of lowering the sodium concentration of the incubation medium on amino acid release

Amino acid content of supernatants after incubation (nmol/100mg protein)			
		Low sodium medium	
	Control	No addition	Potassium stimulated
Aspartate	112 ± 39	126 ± 14	265 ± 21
Glutamine	121 ± 9	269 ± 25	305 ± 11
Serine	350 ± 6	688 ± 74	760 ± 10
Glutamate	200 ± 8	298 ± 59	617 ± 73
Glycine	206 ± 7	459 ± 40	456 ± 11
Alanine	319 ± 50	734 ± 50	813 ± 119
GABA	36 ± 5	247 ± 42	288 ± 22

Synaptosome beds were incubated at 37°C for 40 min in Krebs-phosphate medium in which the sodium of the Krebs has been replaced by 0.248 M-sucrose. Controls were in normal medium. Potassium stimulation was started at 30 min and continued for 10 min. Values are mean ± S.E.M. for four experiments. An equivalent volume of medium containing no potassium was added to the controls after 30 min incubation.

TABLE 22 EFFECT OF TETRODOTOXIN AND CITRATE ON AMINO ACID RELEASE

	Amino acid content of supernatant after incubation (nmol/100 mg protein)					
	No addition		Tetrodotoxin		Citrate	
	Control	Potassium stimulation	Control	Potassium stimulation	Control	Potassium stimulation
Aspartate	161	277	65	194	136	153
Glutamine	176	291	144	253	155	208
Serine	484	681	448	592	566	552
Glutamate	306	582	153	422	280	363
Glycine	292	421	249	300	261	249
Alanine	332	496	240	365	348	414
GABA	46	102	25	62	48	78
No. of experiments	2	4	3	3	4	4

Synaptosomes were incubated in Krebs–bicarbonate medium for 40 min at 37°C. Tetrodotoxin (100 μM) or citrate (1 mM) were added prior to the incubation. Tetrodotoxin was in a citrate buffer which gave a final citrate concentration of 0·64 mM. Potassium stimulation began at 30 min and was continued for 10 min. The values are means for the number of experiments shown, S.E.M. were approx. 10 per cent of the mean. An equivalent volume of medium containing no potassium was added to the controls after 30 min incubation.

to reflect the high affinity of citrate for Ca^{2+}. Incubation of synaptosomes in bicarbonate medium containing 1mM citrate had virtually no effect on levels of amino acids present in the medium (Table 22). However the additional release of amino acids due to K^+ stimulation was much less in the presence of citrate. The suppression of release was most marked for glutamate and aspartate. From a comparison of the effects produced by citrate alone and tetrodotoxin in a citrate buffer, the action of tetrodotoxin evidently allowed release to occur as usual with a small reduction due to the presence of depressed Ca^{2+} concentrations.

The results obtained with citrate further show the importance of Ca^{2+} in amino acid release and together with the results shown in Table 20 can be used to find a relationship between Ca^{2+} concentration and the degree of amino acid release. The data in Table 20 shows the amino acid release due to K^+ stimulation under three different Ca^{2+} concentrations, firstly (column 4 - 3) with no Ca^{2+} in the medium and the endogenously Ca^{2+} complexed by EGTA ($Ca^{2+}_{ext} = 0$), secondly with no Ca^{2+} in the medium (column 6 - 1), but with Ca^{2+} released from the endogenous pool present ($Ca^{2+}_{ext} = X$) and thirdly (column 2 - 1), where Ca^{2+} is present in the medium (0.75mM) and may also be released from the endogenous store ($Ca_{ext} = X + 0.75mM$). The data in Table 22 shows the amino acid release under two further Ca^{2+} concentrations, firstly (column 6 - 5), where citrate is present at 1mM and Ca^{2+} present at 0.75 mM together with a contribution from the endogenous pool ($Ca_{ext} = X - 0.25mM$) and secondly (column 4 - 3) where citrate is present in the tetrodotoxin buffer at 0.64mM and by a similar calculation gives $Ca_{ext} = X + 0.75 - 0.64 = X + 0.11mM$. In the last two calculations the amount of free Ca^{2+} was estimated from the knowledge that Ca^{2+} and citrate form a complex (Ca:citrate of 1:1) and the log K of the reaction is 3, from which can be assumed that the amount of complex formed will equal the component present in the smallest amount.

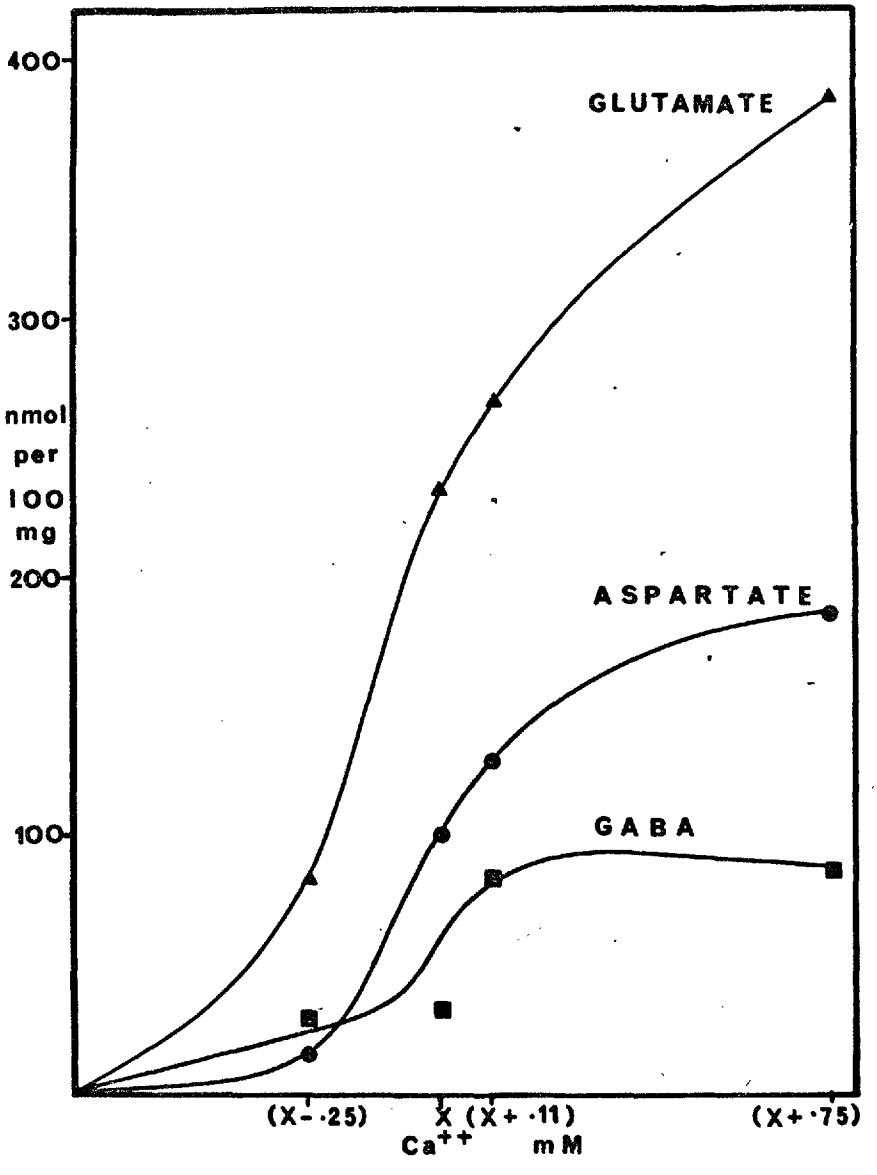
A plot of Ca_{ext} against amino acid release due to K^+ stimulation is shown in Figure 25, for glutamate, GABA and aspartate and all three can be seen to give a sigmoid shaped curve. The appearance of this type of curve implies that there is an exponential effect of Ca^{2+} on amino acid release and this might be related to the postulated co-operative action of 4 Ca^{2+} ions being necessary for the release of one quantum at motor end plates (Dodge & Rahamimoff 1967).

Discussion

The membranes of neural tissue have defined properties of Na^+ and K^+ permeability which are altered during transmission. The exchanges of both these ions has been found to be coupled to Ca^{2+} transport which would indicate that Ca^{2+} is the link between depolarization of nerve terminals and transmitter release, which is further substantiated by the fact that absence of Ca^{2+} prevents transmitter release in a number of cases.

The release of physiologically active amino acids from synaptosome beds in response to depolarization was found to be dependent on the presence of calcium and the amount of calcium present affected the degree of release. This can be usefully correlated with the results of Blaustein & Wiesmann (1970), who found that Ca^{2+} uptake by synaptosomes was directly proportional to the potassium concentration in the surrounding medium after a threshold level of K^+ of 15mM and above had been reached. This threshold of K^+-stimulated uptake would theoretically depolarize the synaptosome membrane by 30 mV, which is also the level of depolarization of presynaptic terminals above which there is a post synaptic response in squid giant synapse (Katz & Miledi 1967a). Thus with an external K^+ concentration of 50mM as used in the present experiments, Ca^{2+} uptake would be expected to occur accompanying depolarization. The existence of a relationship between external Ca^{2+}

Figure 25_ Variation of amino acid release due to K^+ stimulation with Ca^{++} concentration



concentration and amino acid release due to K^+ stimulation (Figure 25) is evidence for the K^+ stimulated uptake of Ca^{2+}, the Ca^{2+} dependent amino acid release and the K^+ stimulated amino acid release from synaptosomes being part of the same process and therefore indicating the mechanism of release. A possible sequence might be high external K^+ stimulating Ca^{2+} entry into the nerve terminal and the Ca^{2+} triggering release.

The manner in which external Ca^{2+} concentration affects the quantity of transmitter released is similar to that occurring at the neuromuscular junction (Katz & Miledi 1965) and the piriform cortex (Richards & Sercombe 1970). The last authors measured the amplitude of EPSPs under different Ca^{2+} concentrations and obtained a sigmoid curve also, the steepest rise occurring between about 0.4 and 0.8mM external Ca^{2+}. This part of the curve is equivalent to the steep rise shown in Figure 25, occurring between X-0.25 and X-0.11mM Ca^{2+}, giving an estimation of X of 0.65mM Ca^{2+}. Since the linear parts of the curves obtained for glutamate, GABA and aspartate were approximately parallel to each other, the same release mechanism would be implicated in each case.

Dodge & Rahamimoff (1967) have examined the non-linear dependence of the end plate potential on external Ca^{2+} concentration by postulating that a co-operative action of 4 Ca^{2+} ions is needed for the release of one packet of transmitter. This hypothesis was based on the observation that the amplitude of the end plate potential was proportional to the fourth power of the Ca^{2+} concentration. Richards & Sercombe (1970) similarly found the amplitude of EPSPs was proportional to $(Ca^{2+}_{ext})^n$ where n was in the range 1.5 to 4.3.

Two important aspects of Ca^{2+} involvement in transmitter release mechanisms are firstly, the site and mode of action of Ca^{2+} and secondly, the way

in which this is controlled, such as,by interactions with other ions.

Calcium uptake by synaptosomes. If specific Ca^{2+} movements were part of the process translating a state of depolarization into a process of transmitter release, these movements would be expected to occur at the site of transmitter release. Calcium uptake has in fact been shown to occur in incubated nerve endings, against a concentration gradient in the presence of Mg^{2+} and either ATP or a substrate such as glucose (Yoshida et al 1966; Lust & Robinson 1968; 1970; Blaustein & Wiesmann 1970; Diamond & Goldberg 1971). As shown in squid axon and brain slices, there is a Na^{+} stimulated efflux which is insensitive to ouabain (Lust & Robinson 1968). However Ca^{2+} uptake is not specific to synaptosomes and has been shown to occur at similar rates in brain and liver microsome fractions (Yoshida et al 1966; Diamond & Goldberg 1971) and at greater rates by iso-osmotically prepared mitochondria (Lust & Robinson 1970; Diamond & Goldberg 1971). Hyperosmotically prepared mitochondria take up Ca^{2+} at slower rates than synaptosomes (Yoshida et al 1966; Blaustein & Wiesmann 1970) and the difference in uptake rates of mitochondria confirmed by Lust & Robinson 1970, indicates that mitochondria may be damaged under hyperosmotic conditions. The high rates of Ca^{2+} uptake that can be shown by mitochondria could be sufficient to account for the Ca^{2+} uptake by synaptosomes, suggesting that the intrasynaptosomal mitochondria are the sites of calcium accumulation (Lust & Robinson 1970). Furthermore, on subfractionation of synaptosomes the bulk of the labelled Ca^{2+} taken up by the synaptosomes was found associated with mitochondria.

It is not certain whether ATP is in fact necessary for Ca^{2+} uptake by subcellular particles, since ATP dependence was found under conditions of hypotonic incubation, i.e. 50mM tris buffer/3 mM Mg/3 mM ATP/0.1 mM Ca^{2+} (Lust & Robinson 1970) and incubation under these conditions was found to lead to

rupture of synaptosomes and also to increased rates of Ca^{2+} accumulation by microsomes compared to iso-osmotic conditions (Diamond & Goldberg 1971). Other substrates such as succinate and β hydroxybutyrate were found to support Ca^{2+} uptake (Lust & Robinson 1970a) and were not inhibited by oligomycin, indicating that ATP was not essential.

The effect of Na^+ and K^+ on calcium transport. Under conditions which imitate depolarization with respect to relative Na^+ concentrations, i.e. leading to a high internal Na^+, or low external Na^+, there is a stimulated Ca^{2+} influx into squid axon (Baker et al 1969) and crab nerve (Baker & Blaustein 1968). This Na^+ -Ca^{2+} counter transport is further demonstrated by the coupling of Ca^{2+} efflux to Na^+ influx (Blaustein & Hodgkin 1969) and the presence of a component of Na^+ efflux which is stimulated by Ca^{2+} and insensitive to ouabain (Baker et al 1969). Consistent with these findings are the observations in brain slices that electrical stimulation (Lolley 1963), glutamate (Ramsay & McIlwain 1970) and high K^+ (Fujisawa et al 1965), conditions which lead to depolarization and increased internal Na^+, cause increased Ca^{2+} influx, without necessarily increasing the Ca^{2+} pool. An associated Ca^{2+} uptake with increased internal Na^+ is also produced by incubation of brain slices in the presence of ouabain, an increased loss of K^+ occurring at the same time (Stahl & Swanson 1969; 1971). A Ca^{2+} pumping system is thus closely connected with the Na^+ and K^+ movements ensuing from depolarization. The ionized Ca^{2+} of nerve cells is 10^{-5} M (Hodgkin & Keynes 1957; Portzehl et al 1969) but if Ca^{2+} was distributed passively across the membrane according to the Nernst equation, intracellular levels of Ca^{2+} would be 0.1 - 1.0 M (Blaustein & Hodgkin 1969) which is a further indication of an active Ca^{2+} transport system, pumping Ca^{2+} out against a concentration gradient. The concentration of Ca^{2+}

from medium into brain slices is greater than could be explained by diffusion alone indicating an auxillary metabolic process, but Ca^{2+} uptake can occur in various metabolic states such as in a nitrogen atmosphere and in the absence of substrate (Lolley 1963; Charnock 1963), which these authors have proposed as being due to Ca^{2+} binding in the tissue.

The dependence of Ca^{2+} uptake on Na^{+} in synaptosomes has not been comprehensively studied but it has been shown by Diamond & Goldberg (1971) only to occur in the absence of ATP.

Loss of amino acids in the presence of low concentrations of sodium. All amino acids, except aspartate and glutamate showed a large loss to the medium from synaptosome beds in 40mM Na^{+}, GABA showing the greatest loss. It is interesting that the two potent excitants, aspartate and glutamate should be retained so firmly, which would be a vital property in situ. In the case of the remaining amino acids, diffusion down a concentration gradient is a likely process to occur in the absence of an active mechanism, such as one requiring Na^{+}.

The dependance of amino acid transport systems on sodium is well known (Lahiri & Lajtha, 1964). In synaptosomes the sodium dependance of GABA uptake has been shown by Varon et al (1965). It was therefore not surprising that lowering the medium sodium concentration from 164 to 40mM caused a much larger amount of GABA than other amino acids to accumulate in the incubation medium. On the other hand, increasing medium sodium by 50 to 214mM caused a fall specifically in aspartate and glutamate levels in the medium. This different sensitivity to sodium concentrations is evidence for the existence of different transport processes for the two groups of amino acids, possibly occurring in different populations of

synaptosomes. Since decrease in sodium in these experiments was accompanied by decreased chloride (124 - OmM), the effects observed could be, at least partially, due to this. This relative insensitivity of aspartate and glutamate uptake mechanisms to low sodium has also been shown in cortex slices (Margolis & Lajtha 1968) and therefore appears to be a general property of neural systems.

Amino acid release may be very closely linked to ion fluxes, for example at low external Na^+ large amounts of GABA may be released as shown in the present experiments in the presence of low Na^+, and conversely following depolarization of the terminal, when the internal Na^+ is high, glutamate and aspartate may be pumped out as counter ions with the Na^+ ions.

Low Na^+ did not increase or decrease the amount of amino acid released with stimulation. On the other hand, at the motor end plate a reduced external Na^+ concentration produces a large increase in epp magnitude and quantal content (Kelly 1965). However, Na^+ ions do not change the power relationship between Ca^{2+} and the quantal content, where four Ca^{2+} ions are required for a co-operative effect (Rahamimoff & Colomo 1967) and Na^+ may be acting at the terminal membrane or even at the post synaptic membrane. The latter explanation would fit in with the present results where there was a lack of enhanced response to stimulation in the presence of low Na^+.

The effect of Tetrodotoxin. Tetrodotoxin did not affect the release of glutamate, GABA and aspartate from synaptosome beds with stimulation. In a similar way, at the neuro-muscular junction, tetrodotoxin does not reduce ACh release during depolarization. Thus reduction of Na^+ levels by interference with Na^+ conductance does not alter the levels of release. It would appear that although depolarization triggers the transmitter release process, the latter is fairly stable to changes in sodium concentration. Ca^{2+} flux

is also blocked by tetrodotoxin (Pull et al 1970) in cerebral slices, the same degree of inhibition being produced as for Na^+ conductance, suggesting that Na^+, K^+ and tetrodotoxin act at the same site. Thus, although Ca^{2+} may be involved in this tetrodotoxin sensitive part of transmission probably at the axonal membrane, the Ca^{2+} dependent process at the terminal is unaffected by tetrodotoxin.

The Na^+ involved in release and coupled to Ca^{2+} would seem to be part of a different system to the Na^+ taking part in impulse conduction.

CHAPTER 6

THE STIMULATED RELEASE OF ACETYLCHOLINE FROM SYNAPTOSOME BEDS AND ITS DEPENDENCE ON CALCIUM

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The physiological action of ACh on neurones

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The effect of elevated medium K^+ and electrical pulses on ACh release from synaptosome beds

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Discussion

The induced release of ACh from synaptosome beds and the analogy with release of amino acids under similar conditions

The control levels of ACh

The levels of ACh in synaptosomes

CHAPTER 6 THE STIMULATED RELEASE OF ACETYL CHOLINE FROM SYNAPTOSOME BEDS AND ITS DEPENDENCE ON CALCIUM

Introduction. A well established transmitter in the peripheral and autonomic nervous system is acetyl choline, and since it is present in sizeable amounts in the CNS, particularly in nerve endings, would also be likely to have a central transmitter function ~~centrally also~~. Thus the release of ACh from synaptosome beds was studied under the same conditions used to study amino acid release and a comparison made between the release processes with particular respect to the calcium dependence.

The physiological action of ACh on neurones. ACh is the transmitter at parasympathetic post ganglionic nerve endings, all preganglionic nerve endings of the autonomic nervous system and at the neuromuscular junction of somatic motor nerves. At the neuromuscular junction, del Castillo, Katz, Miledi and others (summarised by Katz & Miledi 1966a) have shown that the end plate potential evoked by a nerve impulse and the spontaneous miniature end plate potentials (0.5 mV) from which they are composed have the same pharmacology as the potentials produced by local application of ACh. For example, the size of all three is increased by AChE inhibitors and decreased by curare. The post synaptic response to stimulation of this nerve muscle preparation does not occur in the absence of Ca^{2+}, although conduction of the impulse to the terminal can occur as demonstrated by the appearance of a presynaptic spike (Katz & Miledi 1965b).

In the CNS, iontophoretic application of ACh causes depolarization and even hyperpolarization of some neurones. About 14% of the cerebral neurones tested by Krnjevic & Phillis (1963) were found to be cholinoceptive, the deep pyramidal cells of the cortex showing greatest sensitivity (Krnjevic & Phillis 1963a), but the response was found to develop more slowly

than in the spinal cord. Here iontophoretic application of ACh to Renshaw cells causes excitation which is the same as that produced by motor nerve stimulation and furthermore the effects of drugs are similar for both types of stimulation (Curtis 1963), indicating a predominance of nicotinic receptors over muscarinic ones.

Other regions of the CNS, which have been found to be sensitive to ACh are the piriform cortex (Legge et al 1966; Randic & Straughan 1965), brain stem (Bradley & Wolstencroft 1962; Bradley et al 1966), medulla (Salmoiraghi & Steiner 1963), lateral geniculate nucleus (Curtis & Davis 1962), ventrobasal hypothalamus (Andersen & Curtis 1964), hypothalamus (Bloom et al 1963), caudate nucleus (Bloom et al 1964), hippocampus (Stephanis 1964) and cerebellum (Crawford et al 1966). The action of ACh is either blocked as in the neocortex by atropine, which blocks muscarinic sites, or by dihydroβerythroidine, which blocks nicotinic sites and which occurs in parts of the paleocortex.

ACh has also been shown to depress firing of central neurones, and this action can be induced by cholinimetic agents and antagonised by atropine and dihydroβerythroidine. These antagonists were also found to depress the long duration synaptic inhibitions of cortical neurones evoked by direct stimulation of cortex or reticular formation. Phillis & York (1968) found that out of 600 neurones tested in pericruciate, auditory and visual cortex, 46% showed depressed firing with ACh and in the pons and medulla, 22% of a further 600 neurones were also depressed by ACh (Bradley et al 1966).

Release of ACh. Several demonstrations of ACh release have been made in vivo, by superfusing areas of cortex covered by a collecting cup, by using push-pull cannulae or more specifically from the regions of single neurones by means of concentric micropipettes, the central barrel recording electrical

activity and fluid being gently removed through the outer barrel (reviewed by Mitchell 1966). The resting release of ACh has been shown to be depressed by deepening anaethesia which could be correlated with electrographic patterns (MacIntosh & Oborin 1953). Increased release of ACh into perfusion fluid has been shown following direct stimulation of cerebral cortex and after peripheral stimulation (Mitchell 1963; Collier & Mitchell 1966; Neal, Hemsworth/1968; Bartolini & Pepeu 1967). Stimulation of mesencephalic reticular formation has also been found to lead to increased liberation of ACh (Kanai & Szerb 1965; Celesia & Jasper 1966; Szerb 1967; Collier & Murray-Brown 1968). The implication being that there are cholinergic pathways ascending from the site of reticular formation to the cerebral cortex and that the site of ACh action is the synapse since lowering calcium in the perfusing medium reduces spontaneous and evoked outputs. Histochemical localization of AChE suggests that the reticular activating system terminating on the cortical and striate neurones is largely cholinergic (Shute & Lewis 1966). By interrupting certain pathways with stereotaxically placed lesions, the presence of cholinergic pathways is implied by decreased distal AChE and proximally increased AChE. However, AChE is not a perfect marker as it does exist in non-neural tissue and does not always parallel ACh and ChAc regional distribution.

There are further gross correlations between ACh levels and cerebral activity, for example ACh levels in rapidly fixed cortex are influenced by the state of the animal being increased by convulsions (Richter & Crossland 1949). ACh is not normally found in the CSF but it is found there after epileptic seizures or convulsions indicating that it has been used or consumed during these activities.

In vitro, ACh release has been evoked from cortex slices by electrical stimulation (Rowsell 1954) and by elevated medium K^+ (Mann et al 1939;

Richter & Marchbanks 1971), which stimulus is also effective in perfused ganglia (Birks 1963).

Since nerve endings contain a large proportion of the total brain ACh (about 70%) together with its synthesizing enzyme ChAc (Hebb & Whittaker 1958; Gray & Whittaker 1962) and inactivating enzyme AChE (Whittaker 1965), the nerve ending would seem a likely site for the release of ACh. Superfusion or brain slice experiments cannot however give direct information about this but it is possible by using a synaptosome preparation. Increased release of ACh has indeed been shown to occur from a crude mitochondrial preparation, which is rich in synaptosomes, in response to elevated medium K^+ (Haga 1971) and the same stimulus brings about a decrease in the ACh content of synaptosomes isolated from K^+ stimulated cortex slices (Richter & Marchbanks 1971a).

In the present studies release of ACh from synaptosome beds was measured together with the ACh remaining in the beds, under conditions of stimulation which cause amino acid release. Since Ca^{2+} has been found essential for the release of ACh at the neuromuscular junction (Katz & Miledi 1964) and from superfused auditory cortex (Hemsworth & Mitchell 1969), the requirement for this ion was examined in the present study.

Experimental.

Incubation and stimulation of synaptosome beds was carried out as described in Chapter 2 and discussed in the foregoing chapters. The only amendment to these methods being that neostigmine bromide, an AChE inhibitor, was included in all preparative sucrose solutions and in the incubation media. ACh was measured by bioassay using the frog rectus abdominis muscle of Rana temporaria, precautions being taken to detect substances in the tissue extract or incubation medium that would potentiate or inhibit the response of the frog

muscle to ACh (Chapter 2, section 10). In order to concentrate the 5ml of incubation medium, it was first freeze dried and then taken up in frog Ringer (1.5ml) and 0.1ml samples used for assay. This would result in a concentration of K^+ ions especially in medium containing elevated medium K^+, and which if present in high enough concentrations would itself cause muscle contraction. However, medium alone containing elevated K^+ and treated in the same way as incubation medium was found not to illicit muscle contraction and was also calculated to give a final concentration of less than 13 mM in the assay system.

Results.

The effect of elevated medium K^+ and electrical pulses on ACh release from synaptosome beds. Elevation of medium K^+ by 50 mM in the last 10 min of a 40 min incubation period led to a significant ($p<0.001$) increase of 45% in the ACh released to the medium and a significant ($p<0.001$) decrease in the ACh remaining in the synaptosome bed (Table 23). There was an increase of 13% in the total ACh ($p<0.01$), which is likely to be due to an increase in ACh synthesis.

Application of electrical pulses for the last 10 min of the 40 min incubation period also produced a significant ($p<0.01$) increase in the release of ACh, a significant ($p<0.001$) decrease in the ACh of the synaptosome bed and an increase in the total ACh. However, the effects of electrical stimulation were somewhat smaller.

The effect of absence of calcium from the medium on ACh release. Incubation of synaptosome beds in the absence of Ca^{2+} and in the presence of 0.5 mM EGTA to complex endogenous tissue calcium prevented the ACh release evoked by both types of stimulation (Table 23). There was no significant diminution in the synaptosomal ACh but the control levels of ACh in the medium

Table 23 Acetylcholine in synaptosome beds and incubation medium following electrical and potassium stimulation

	Acetylcholine content (nmol/100 mg protein)	
	Synaptosome bed	Incubation medium
Calcium medium		
Control	60.9 ± 1.04 (23)	69.8 ± 2.57 (16)
Potassium stimulation	46.2 ± 2.02 (11)	101.0 ± 4.96 (10)
Electrical stimulation	52.8 ± 2.05 (15)	83.7 ± 5.02 (10)
Calcium-free medium (+ EGTA)		
Control	64.2 ± 2.45 (9)	27.5 ± 4.72 (6)
Potassium stimulation	55.8 ± 3.0 (7)	34.9 ± 2.49 (7)
Electrical stimulation	57.7 ± 3.0 (5)	29.6 ± 5.52 (4)

Synaptosome beds were incubated for 40 min at 37°C in Krebs-bicarbonate medium (calcium medium) or in Krebs-bicarbonate medium containing no calcium and 0.5 mM EGTA, as indicated. Potassium stimulation was by elevation of medium K^+ by 50 mM 10 min before the end of incubation and electrical stimulation by application of electrical pulses for the last 10 min of incubation. Values are means ± S.E.M. with the number of experiments in brackets.

were about half of the control values in the presence of Ca^{2+}.

Discussion.

The induced release of ACh from synaptosome beds and the analogy with release of amino acids under similar conditions. Potassium (50 mM) and electrical pulses stimulated release of ACh from synaptosome beds. The release was found to be dependent on the presence of Ca^{2+} and the effect of K^+ was about twice as great as that of pulses. The characteristics of ACh release are thus analogous to glutamate, GABA and aspartate release under similar conditions. The fact that the synaptosome bed preparation shows the same properties of ACh release as occurs *in vivo* and in more intact preparations, i.e. Ca^{2+} dependent release in response to depolarization, indicates that it is able to demonstrate transmitter release as it occurs physiologically and other compounds which are released in a similar way such as amino acids may also be transmitters.

A difference between release of physiologically active amino acids and ACh lies in the proportions released; release of about 2/3 of the total ACh occurs under K^+ stimulation and only about 1/5 of these amino acids. This difference reflects that amino acids have additional functions to transmission which may be the sole function of ACh.

In terms of the amounts of ACh and glutamate released due to K^+ stimulation, there are 31 and 386 nmol/100 mg protein released of ACh and glutamate respectively, under similar conditions (values taken from Tables 23 and 20), which shows that glutamate release is about 13 times greater than that of ACh. This is a greater proportion of glutamate than would be expected from the findings of Krnjevic & Phillis (1963), who found that 14% of neurones tested in the cerebral cortex were sensitive to ACh, and 100% were sensitive to glutamate.

The control levels of ACh. The control levels of ACh in the medium containing no Ca^{2+} and EGTA were about half the control levels of those in the presence of Ca^{2+}. Presumably, in the presence of Ca^{2+}, ACh is being continuously lost from cholinergic synapses as evidence by miniature end plate potential generation. This could partly account for the losses of ACh from the controls seen in the present experiments.

The levels of ACh in synaptosomes. The literature levels for the ACh content of synaptosomes do not vary greatly, De Robertis et al (1962) found 3.43 nmol Ach/g cortex in nerve endings isolated from rat cerebral cortex (combining the B and C layer between 0.8 and 1.2 M sucrose equivalent to the fraction used in the present studies). Barker, Dowdall, Essman and Whittaker (1970) found 3.2 nmol ACh/g in synaptosomes isolated from guinea pig cerebral cortex (P2B). Conversion of the values of ACh given in Table 23 to the units quoted above gives the value of 3.58 nmol ACh/g original cerebral cortex for control incubated synaptosome beds and 2.72 nmol ACh/g for synaptosome beds incubated in the presence of elevated potassium. Richter and Marchbanks (1971a) found comparable levels of 2.7 and 2.1 nmol ACh/g in synaptosomes isolated from incubated control, and K^+ stimulated guinea pig cerebral slices, respectively, using dorsal muscle of leech for bioassay. The present results are also consistent with the findings of Haga (1971), who, following the release of (^{14}C) ACh from crude mitochondrial pellet, found a 25 per cent fall, in tissue levels of ACh with incubation in elevated K^+ and confirms that the ACh may be recovered in the incubation medium. However, from these present studies, ACh release has a clear dependence on the presence of calcium. Haga (1971) did not find this, probably because he did not complex the endogenous calcium of his preparations. Thus, when synaptosome beds, incubated in physiological medium are stimulated by elevated potassium or electrical pulses there is a significant increase in the release of ACh to the medium, which is dependent on the presence of calcium. Such stimulation also leads to an increase in the total ACh, suggesting that increased synthesis is occurring.

CHAPTER 7

THE AMINO ACID CONTENT OF SYNAPTIC VESICLES

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Estimation of the taurine content of individual synaptic vesicles

CHAPTER 7 THE AMINO ACID CONTENT OF SYNAPTIC VESICLES

Introduction.

Synaptic vesicles in sizeable numbers are universally found in nerve terminals. Interest in synaptic vesicles has arisen since Fatt & Katz (1950) defined the release of transmitter at the neuromuscular junction as being quantal. The evidence (reviewed by Del Castillo & Katz 1956; Katz 1969) showed conclusively that miniature end plate potentials (mepps) are produced by ACh liberated from the presynaptic terminals in packets or quanta of remarkably uniform size for several thousand molecules. It was then thought that there might be some connection between the transmitter content of a vesicle and a quantum. Indeed,the presence of transmitters such as ACh and NE has been demonstrated in synaptic vesicles. Morphological studies have shown the presence of associated structures, possibly involved in transmission,and there is some evidence of vesicles fusing with the nerve ending membrane, which could provide a mechanism for the release of transmitter into the synapse.

In view of the implication of vesicles in transmission either involved directly in the release processes or indirectly as a store for transmitters, it seemed important to make a study of the amino acid content of vesicles, as part of the investigation into amino acids as transmitters.

Synaptic vesicles. Synaptic vesicles are usually isolated by first preparing a crude mitochondrial pellet (P2B) and rupturing this fraction hypoosmotically to release vesicles which are then separated from other components by differential centrifugation (De Robertis et al 1963), discontinuous sucrose gradient centrifugation (Whittaker, Michaelson & Kirkland 1964; Lapetina et al 1967; 1968) or by continuous sucrose gradient centrifugation (Eichberg, Whittaker & Dawson 1964). However, Whittaker et al (1964) have reported that synaptic vesicles prepared by the first method are contaminated with a large proportion of membrane fragments and whole nerve endings,also

evident from the presence of typical membrane constituents in this fraction such as AChE, Na^+/K^+ ATPase and gangliosides (De Robertis et al 1963; Germain & Proulx 1965). Using the method of fixation used by De Robertis (resuspension of osmic acid treated pellet), Whittaker, Michaelson & Kirkland (1964) showed that free vesicles would be yielded. Synaptic vesicles have thus been isolated from guinea pig cerebral cortex (Whittaker, Michaelson & Kirkland 1963; 1964), guinea pig whole brain (Kadota & Kanaseki 1969), rat cerebral cortex (De Robertis et al 1963; 1964; Rodriguez De Lores Arnaiz et al 1967), rat whole brain (Trotter & Burton 1969), mouse whole brain (Kuriyama, Roberts & Kakefuda 1969), rat hypothalamus (De Robertis 1964), supraoesophageal lobes of octopus (Jones 1970), rabbit brain stem (Seguwa et al 1968) and electric organ of the Torpedo fish (Israel et al 1968; 1970).

The size and morphology of synaptic vesicles. When fixatives such as osmium or permanganate are used to heighten the opacity of membranes, synaptic vesicles become conspicuous in nerve terminals. The mean diameter of vesicles isolated from central mammalian nerve terminals is about 500 Å, with only slight variation due to methods of fixation. Using formaldehyde and phosphotungstic acid (PTA), guinea pig isolated vesicles have a mean diameter of 469 Å or 471 Å when viewed within synaptosomes (Whittaker, Michaelson & Kirkland 1964). Rat cerebral cortex vesicles prepared by differential centrifugation and fixed with OsO_4 and PTA have a mean diameter of 500 Å (De Robertis et al 1963). In other species vesicles of different sizes are found, for example, octopus has synaptic vesicles in the range 200-1400 Å (Jones 1970) and Torpedo has vesicles of 800 Å diameter (Israël et al 1970), both being stained with $KMnO_4$/Pb citrate.

Uchizono (1965) has suggested that there is a differentiation between

the vesicles of excitatory and inhibitory nerve endings on a morphological basis. For example, the inhibitory Gray Type 2 nerve terminals (on cell bodies of Purkinje cells) have flat vesicles whereas Gray Type 1 terminals (found on dendritic spines) have spherical vesicles. However, there is not a lot of evidence to support this theory and vesicles are known to be very labile; for example, placing vesicles in buffer for 30 minutes before fixation leads to the appearance of flat vesicles (Robertson 1970). Differential morphological types of vesicle have been found in isolated fractions which have been termed hollow, dense cored and complex on the basis of their appearance in electron micrographs after various staining techniques.

Although artifacts of fixation are likely to occur, for example, the formation of spherical bodies such as lipoprotein micelles, synaptic vesicles have been visualised under a large variety of fixation and preparative conditions and are not produced when a tissue such as liver is treated in the same way (Whittaker, Michaelson & Kirkland 1964).

Associated presynaptic structures. Associated with vesicles are other presynaptic components which have been suggested as being involved in transmission. For example, tubular connections between isolated vesicles and tails on vesicles, have been seen in preparations from cerebellum of guinea pig (Israël & Whittaker 1965; Whittaker 1966), octopus (Jones 1970) and mouse brain (Kuriyama, Roberts & Kakefuda 1968). These have been suggested as being part of a tubular system connecting vesicles which may play a part in transmission guiding the vesicles to the dense projections and the synapse (Jones 1970).

In vertebrate synapses, Gray & Willis (1970) have observed single vesicles surrounded by polyhedral shells and termed these structures complex vesicles. Unbuffered material is used to visualize these most clearly, under which

conditions Kanaseki & Kadota (1969) reported the appearance of 'coated' vesicles from guinea pig. It is thought that the presynaptic dense projections may be derived from the shell or basket of the complex vesicles (Gray & Willis 1970), and the process by which complex vesicles become associated with the dense projections appears to be accentuated by electrical stimulation (Jones & Bradford 1971a).

A presynaptic vesicular grid has also been observed by means of freeze etching techniques (Akert & Pfenninger 1969; Akert et al 1969; Pfenninger et al 1969) which may be equivalent to the presynaptic network found by Jones and co-workers. Thus many varieties of staining and fixing techniques have been used,which raises the problem of integration of the results obtained by all these methods.

The acetyl choline of synaptic vesicles. When synaptosomes are burst hypoosmotically, a certain amount of ACh is released but some still remains bound and a significant part of this bound ACh is found in the fraction(0.4 M sucrose layer), which is shown by electron microscopy to contain a homogeneous fraction of synaptic vesicles (Whittaker, Michaelson & Kirkland 1964). The presence of bound ACh in vesicles has also been shown by De Robertis and co-workers (1962; 1964). The distribution of ACh was summarised by Whittaker (1965) as follows: 70% of the total ACh of brain tissue survives homogenisation and is presumed to be in nerve endings, half of this being in synaptic vesicles and the other half in the synaptosomal cytoplasm. Partial localisation of NE in vesicles has been shown by Michaelson, Whittaker, Laverty & Sharman (1963) and De Robertis et al (1964).

Measurement of the content of synaptic vesicles. There are technical difficulties in obtaining a high yield of synaptic vesicles; Richter & Marchbanks (1971a) estimated that their yield of vesicular ACh was 2.4%

of the total tissue ACh from which the vesicles were derived. The vesicular ACh was 0.85 nmol/g original tissue. In preparation of vesicles from sucrose gradients, the layer containing synaptic vesicles is adjacent to the layer containing free cytoplasmic contents and in order to make an accurate measurement of the ACh actually in synaptic vesicles it is necessary to separate the vesicles from the surrounding sucrose which may contain cytoplasmic ACh. This may be done in three ways, either by sedimenting the vesicles at fairly high g (Whittaker & Sheridan 1965), or by inserting an additional layer of 0.2 M sucrose between the water and 0.4 M sucrose to reduce diffusion (Richter & Marchbanks 1971a), or by passing the vesicular fraction through a column of Sephadex which retards free material and allows the vesicles to pass out uncontaminated in the initial void volume (Marchbanks 1968). By the first method using centrifugation, Whittaker & Sheridan obtained 0.67 nmol ACh/g of original tissue (a yield of 17% from the crude mitochondrial pellet); passage through Sephadex gave a slightly smaller value. If eserine was included in the density gradient, Whittaker & Sheridan (1965) found 2.71 n mol ACh per g original tissue in the vesicular fraction. Normally eserine would be expected to block AChE acting in free fractions, but here it is acting by preventing a leakage of ACh or alternatively there may be a fraction of ACh bound to the outside of the vesicles, which is destroyed in the absence of eserine.

The foregoing discussion shows the problems involved in the measurement of the ACh content of synaptic vesicles resulting from the low yield and the apparent leakage of ACh from vesicles. An additional problem arises when measuring the amino acid content of vesicles, for the colorimetric methods available for detection of amino acids are not as sensitive (limit of sensitivity 1-2 nmol) as the bioassays available for ACh, for example, the dorsal muscle of leech has a threshold of sensitivity of 0.2 - 2.0 p mol/ml. In

order to ascertain the feasibility of using the available method of amino acid analysis for measurement of vesicle amino acid content, the following estimate was made of the least concentration of amino acid that could be detected by this method in vesicles.

Allowing for the vesicle membrane having similar dimensions to a bi-molecular leaflet, the internal diameter of a vesicle would be 500-140 Å and hence its internal volume would be 0.61×10^{-17} cc. (volume of a sphere = $1/3\ \pi r^3$). Since the yield from 1 g of guinea pig cortex is 3.8×10^{12} vesicles (Whittaker & Sheridan 1965), then the total vesicle volume from 10 g original cerebral cortex would be $10 \times 3.8 \times 10^{12} \times 0.61 \times 10^{-17}$ cc $= 2.32 \times 10^{-4}$ cc. The least amount that the colorimetric amino acid estimation could detect unequivacably would be 5 n mol/ml or about 2 n mol/ 0.3 ml as this volume is all that is required for analysis. Thus if the total synaptic vesicle fraction from 10 g cerebral cortex tissue contained 2 n mol of an amino acid such as glutamic acid, then the concentration of that amino acid in the vesicles would be $\frac{2 \times 10^{-6} \times 10^{3}}{2.32 \times 10^{-4}} = 8.6$ mM.

However, allowing for the assay to be carried out in duplicate, and for the fact that leakage occurs during passage through Sephadex, a minimum concentration of 20 mM amino acid would be required for detection. For glutamic acid, the amino acid which occurs in highest concentration, i.e. about 10 mM in nervous tissue, it would seem quite feasible that, if it were concentrated in vesicles, it would be present there in a concentration of at least 20 mM.

A preliminary study on the amino acid content of synaptic vesicles has been made by Mangan & Whittaker (1966), in which they measured the amino nitrogen in the vesicle fraction from guinea pig brain (2 experiments), before and after pelleting the vesicles. The values were 0.84 and 0.23 µg

atoms N/g original tissue respectively, which is equivalent to 60 and 16 nmol amino acid/g original tissue. The value of 60 nmol/g of amino acid in the unpelleted vesicle fraction is considerably larger than the equivalent amount of ACh in this fraction, which is 0.7 nmol/g. However, these authors did not measure the amounts of individual amino acids in the pelleted vesicle fraction. This is probably due to the limited sensitivity of their amino acid detection system which required 7 µg atoms N, i.e. 500 nmol, for a complete analysis.

Although small amounts of amino acid were recovered in the vesicle fraction, Krnjević & Whittaker (1965) directly demonstrated by electro-endo-osmosis of vesicle extracts from micropipettes that there was a sufficient GABA concentration (about 10^{-15} nmoles) to produce depressant effects on single guinea pig neurones. Binding of GABA to vesicles has been shown to occur (Kuriyama, Roberts & Katefuda 1968; Kuriyama, Roberts & Vos 1968) which might reflect concentration of this amino acid in vesicles.

Outline of experimental procedure. In the following experiments, synaptic vesicles were prepared from rat cerebral cortex by the method of Whittaker, Michaelson & Kirkland (1964) with modifications as used by Marchbanks (1968). The method involves the hypo-osmotic rupture of the crude mitochondrial pellet, which is placed on a discontinuous sucrose gradient for centrifugation. The modification is that 5 mM tris/EDTA p.H. 6.5 is used for the rupture. The presence of EDTA binds divalent ions which tend to aggregate the vesicles and draw them through the gradient, depleting the 0.4 M sucrose layer and reducing the yield. Mechanical disturbance during this treatment, carried out by sucking the suspension up and down a pipette aids the rupture process preventing resealing of synaptosomes and clotting of the vesicles.

After preparation of the vesicles, they were passed through Sephadex to separate the vesicles from the surrounding cytoplasm. The amino acid content of the purified vesicles was examined after elution through a Sephadex column with either 0.4 M sucrose, in which the vesicles had been prepared, water or with medium after incubation of vesicles in Krebs-bicarbonate medium containing a range of amino acids. Vesicle contents were extracted with PCA and also dilute acid.

Results.

The preparation of synaptic vesicles. Synaptic vesicles were prepared by discontinuous sucrose density gradient centrifugation of the hypo-osmotically ruptured crude mitochondrial pellet in 5 mM tris-HCl-EDTA pH. 6.5. The lysed fraction (H_2O layer) was placed on top of a 1.2/0.6/0.4 M sucrose gradient and at the end of centrifugation the 0.4 M sucrose layer had a hazy, slightly translucent appearance due to the presence of synaptic vesicles (nomenclature of the vesicle gradient is given in Figure 9).

Electron microscopy of the vesicle fraction (0.4 M sucrose) after staining in OsO_4 and embedding in araldite showed that it contained a fairly homogeneous population of synaptic vesicles (Figure 26), together with a few membrane fragments.

Distribution of protein and amino acids in the vesicle gradient. The distributions of protein and amino acids in the vesicle gradient are shown in Table 24 and Figure 27 respectively. The protein of the membrane fractions and synaptosome ghosts represented over half of the total protein and the vesicle fraction was about 9% of the total protein.

The unpurified vesicle fraction (0.4 M sucrose) was found to have the same relative amino acid distribution as the soluble cytoplasmic fraction

Figure 26 Electron micrograph of synaptic vesicles (x 46,000)

A suspension of the synaptic vesicle fraction was fixed in buffered OsO_4, dehydrated in ethanol and embedded in araldite for sectioning and electron microscopy.

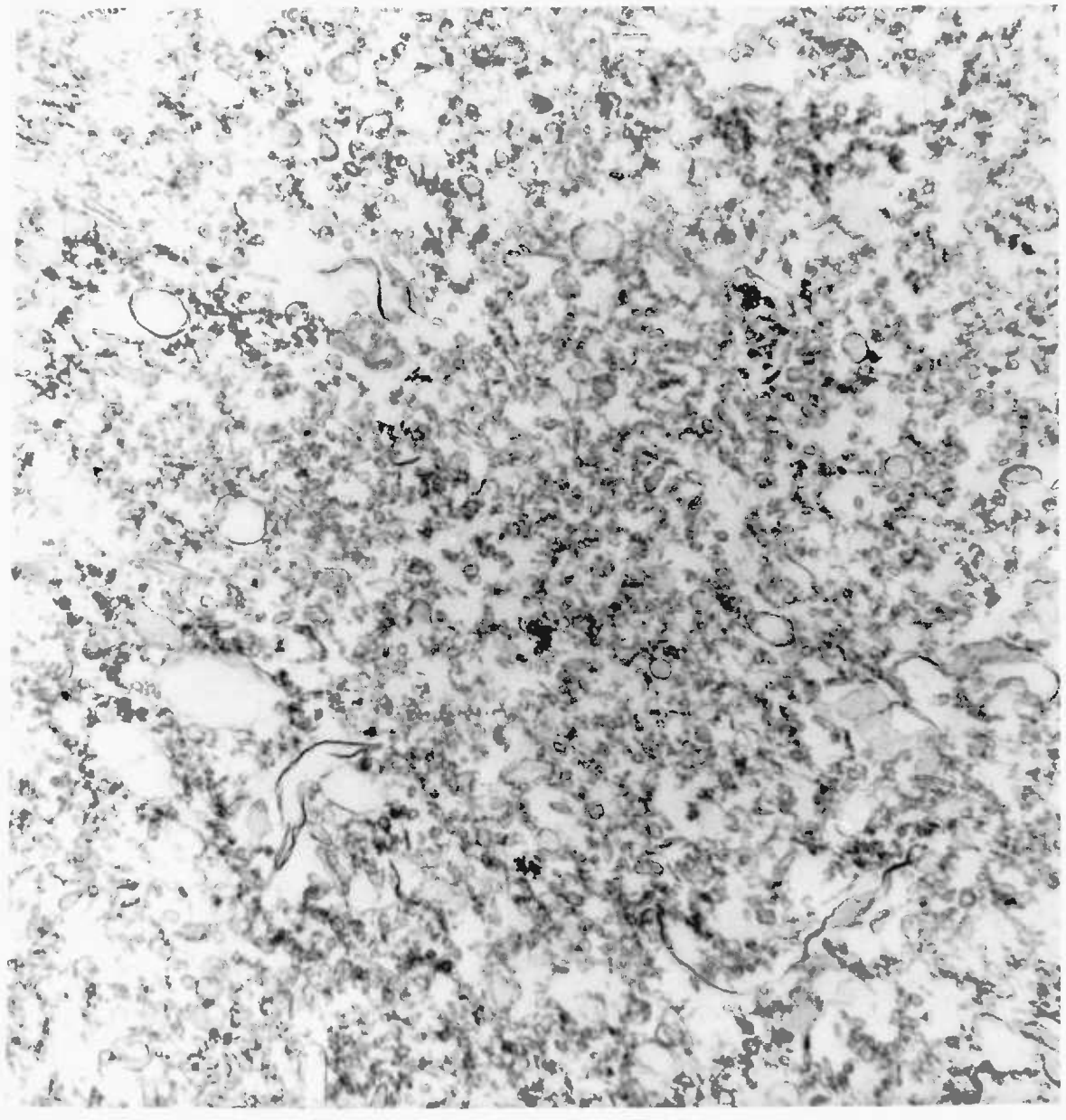
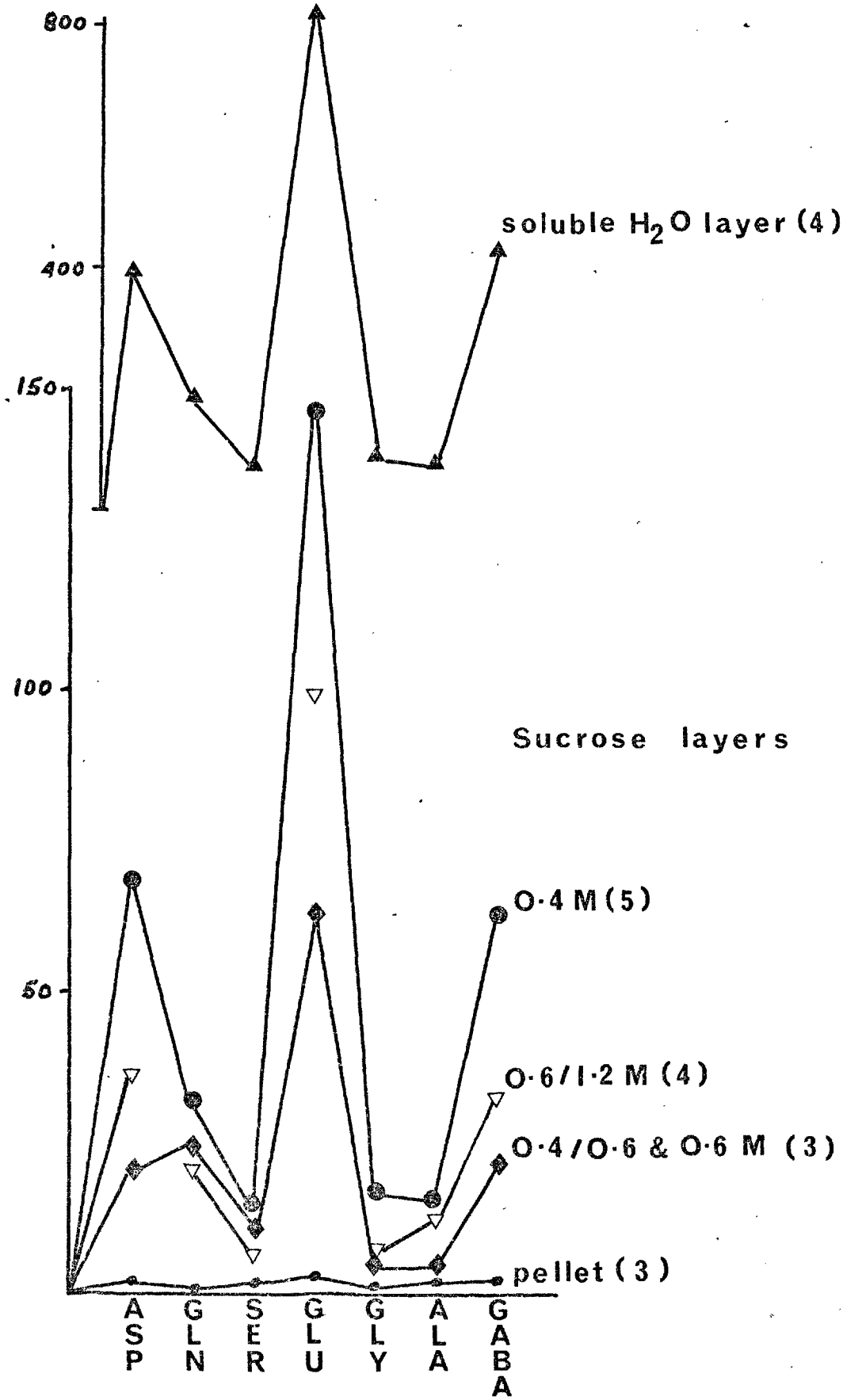


Table 24 Distribution of protein in the vesicle gradient

Fraction	Protein content in mg.
1. H_2O layer soluble cytoplasm	38.2 ± 5.4 (5)
2. 0.4 M sucrose vesicles	21.8 ± 2.0 (7)
3. 0.4/0.6 M sucrose interface and 0.6 M sucrose membranes	77.0 ± 5.7 (4)
4. 0.6/1.2 M sucrose interface synaptosome ghosts and unruptured synaptosomes	81.0 ± 9.8 (4)
5. 1.2 M sucrose layer mitochondria	10.5 ± 0.8 (5)
6. Pellet mitochondria	26.0 ± 0.6 (4)

The cerebral cortex tissue from 15 rats (about 13.8 g wet tissue) was used to prepare a crude mitochondrial pellet which was then hypo-osmotically ruptured with 31 ml of 5 mM tris-EGTA p.H. 6.5 and placed on a discontinuous sucrose gradient of 1.2/0.6/0.4 M sucrose. After centrifugation at 75,000 g for 2 h, protein analysis was carried out on the fractions as indicated above. Values are means ± S.E.Ms. for the number of experiments in brackets.

Figure 27 _ Distribution of amino acids in the vesicle gradient in n mol / g
No of expts in brackets S.E.M.s ~ 10 %



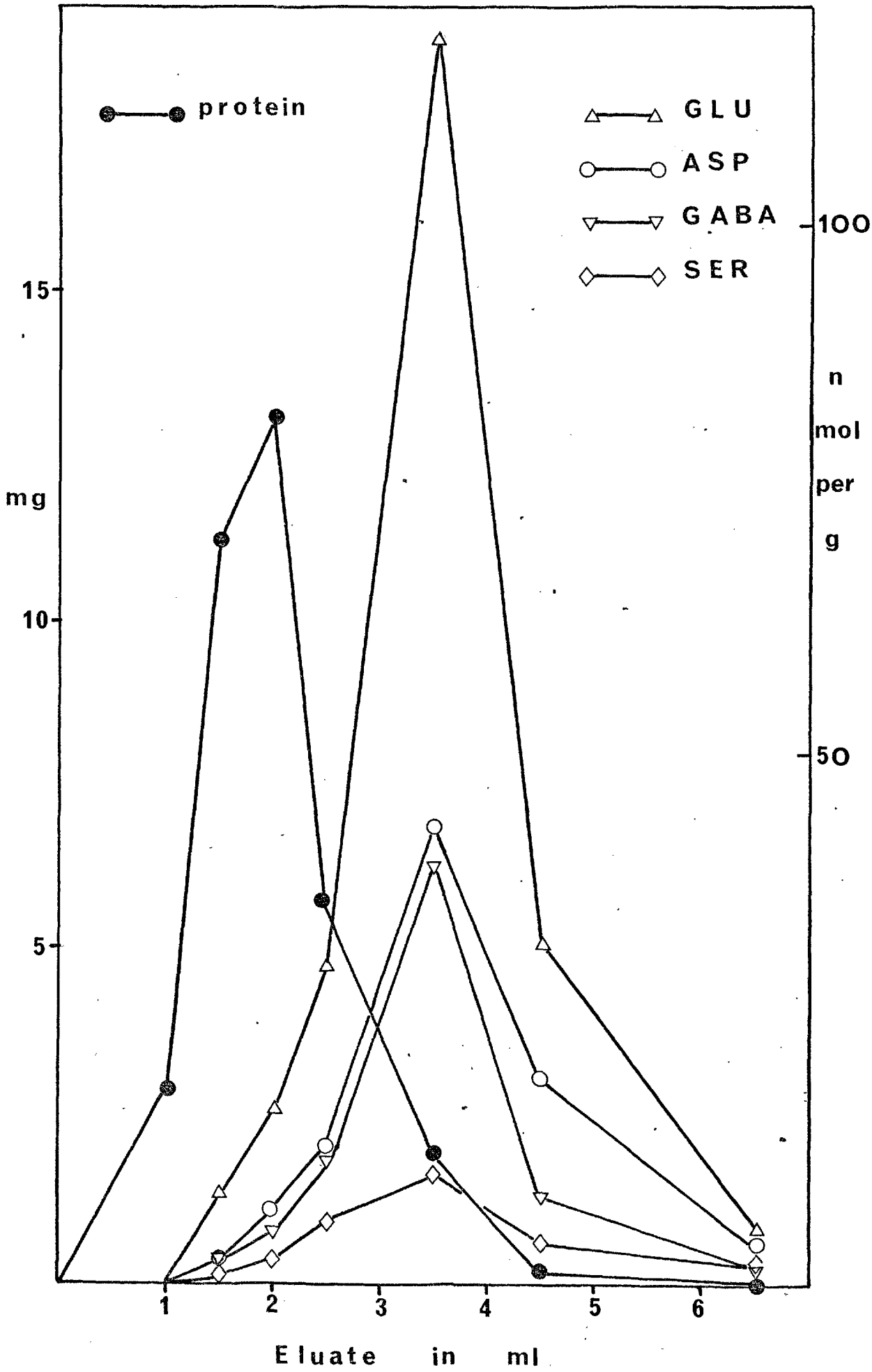
(H_2O layer), but this was later found to be due to contamination of this vesicle fraction with soluble contents of amino acids. The same distribution in the 0.4/0.6 M sucrose fraction may also be due to a similar type of contamination. About 66-77% of the aspartate, glutamate, GABA and glycine of the total vesicle gradient was found in the soluble fraction (H_2O layer) and about 58-60% of the glutamine, serine and alanine were present in this fraction. The 1.2 M sucrose fraction and the mitochondrial pellet were found only to contain about 1% of the total amino acid content of the vesicle gradient, the content of hydrophobic amino acids being significant in the latter. The fact that the 0.6/1.2 M sucrose fraction containing ruptured synaptosomes was fairly low in amino acids indicates the high degree of rupture that had occurred by this method.

Purification of synaptic vesicles. Synaptic vesicles were purified by passing them through Sephadex G-50 medium (Pharmacia Ltd.) by the method used by Marchbanks (1968) to measure the ACh content of vesicles. One ml samples of the vesicle fraction in 0.4 M sucrose were applied to each of twelve small columns equilibrated in 0.4 M sucrose, to enable rapid purification. The vesicles passed through in the void volume, leaving the free amino acids in the gel. Passage of the appropriate quantities of amino acid standards through the columns showed that there was no contamination of the void volume with free amino acids and that their recovery from the columns was high (87 to 106% for all amino acids giving a mean value of 96%).

The Sephadex columns were eluted with 0.4 M sucrose and fractions of the eluate were analysed for protein and amino acid content and the results of one such experiment are shown in Figure 28.

The amino acid content of sequential eluates is shown for glutamate, aspartate, GABA and serine by open symbols and the right hand vertical scale

Figure 28 _ Passage of vesicles down Sephadex



and similarly the protein is indicated by filled circles and the scale shown on the left hand ordinate. The elution patterns of glutamine, glycine and alanine were very close to that of serine and were omitted from the figure for clarity. The results shown on this figure are typical of those from four experiments. The major fractions containing protein corresponded to the purified vesicles and did not contain any amino acid. The amino acids of the crude vesicle fraction were eluted in the position of free amino acids. However, in the presence of vesicles, the upward rise of the amino acid peak started earlier with some overlap with the protein containing fractions. This may have been due to a drag effect, the protein particles trapping free amino acids and pulling them through into the void volume. This point was investigated further later.

The possibility existed that the small amount of amino acid present in the later protein fractions could represent a peak which was obscurred by the free amino acids. This was tested firstly by passing the vesicle fraction through Sephadex twice to reduce the free amino acids, but no change was found in the elution pattern. Secondly, the vesicles were passed through Sephadex and eluted with water to release the vesicle contents, but once again the same amino acid pattern was shown with no association of amino acids with vesicles and with some overlap of protein fractions with the amino acid peak, confirming the possibility of a drag effect.

Incubation of vesicles in Krebs-bicarbonate medium containing amino acids. All the previous purification steps were carried out at 0 to 4° C, under non metabolic conditions. If amino acids are in fact present in vesicles, they may have been lost during the preparative procedure, in the same way that ACh is lost in the presence of sucrose at 0°C (Whittaker 1965). In

order to see if this was the case, the vesicles taken from the gradient in 0.4 M sucrose were mixed with an equal volume of Krebs-bicarbonate medium containing glucose and amino acids. The final concentration in this mixture of glucose was 10 mM, the amino acids (aspartate, glutamate, glutamine, glycine, alanine, valine and GABA) were 5 mM and Ca^{2+} was used at a final concentration of both 0.75 mM and 1.5 mM. The remaining bicarbonate medium components had a final concentration of half that of normal and the sucrose was 0.2 M. This mixture was incubated at 37°C for periods between 30 min and 1 h in an O_2/CO_2 atmosphere and then portions of it were applied to a large Sephadex column (24 x 1.8 cm) containing Sephadex G-50, equilibrated in Krebs-bicarbonate : 0.4 M sucrose, 1 : 1 and kept at room temperature. Fractions were collected, extracted twice with PCA and analysed for amino acid and protein. No amino acids were found associated with protein fractions under any condition of time of incubation or Ca^{2+} concentration. A large column was used to obtain a good separation of vesicles from the high amino acid content of the incubation medium.

Extraction with dilute acid. In all the previous experiments, vesicle contents were obtained by extraction twice with PCA and before amino acid analysis the extract was partially purified by passage through a cation exchange column and eluted with NH_3. To ensure complete extraction from vesicles, the method of Hebb & Whittaker (1958) to extract ACh from vesicles was used. This method involved heating the vesicle fraction in a boiling water bath at p.H. 4 for 10 min, followed by neutralization and centrifugation. In this case the primary purification on cation exchange columns was omitted. This method had the advantage that taurine, the second most abundant amino acid in brain, would not be lost in this process.

This method of extraction was applied to the purified vesicle fractions

and the protein and amino acid contents subsequently determined. Once again none of the previously studied amino acids was found in the purified vesicle fractions; however, chromatography showed the presence of three ninhydrin positive components. All three were eluted with acid prior to aspartic acid, which implied that they were either only loosely held by the ion exchange resin or that they were strongly acidic. The largest component was the second to be eluted (II), the first being present in slightly smaller quantities (I) and the third being about 1/4 of the size of II and possessing a broad peak.

Confirmation of the presence of taurine (i) Ion Exchange Chromatography. Chromatography of taurine standards showed that it was a ninhydrin positive compound, eluted in the same position as the component II of vesicles and possessing the same peak shape, with a sharp rise and slightly trailing descent. This amino acid, being strongly acidic, is one of the first to be eluted with acid from a cation exchange resin together with other strongly acidic amino acids such as cysteic and homocysteic acid. However, cysteic acid and homocysteic acid standards and mixtures of these with taurine showed distinct separation. Sucrose and its products of hydrolysis, fructose and glucose, mixtures of which are present in the vesicle extract, do give a ninhydrin reaction(red) and are found in the same region as taurine but a 3-5 M solution is required to give barely detectable peaks. Extracts containing sucrose were not used for assay of taurine with quantities of sucrose as large as this. On the assumption that component II was taurine and would have the same amino acid colour factor, then the concentration of taurine in the purified vesicle fraction was calculated to be 3292 ± 394 nmol/100 mg protein ($\pm$ S.E.M.) from 4 separate experiments.

Confirmation of the presence of taurine (ii) Colour reaction with -phthalaldehyde and urea. The presence of taurine was shown by a reaction which is fairly specific to taurine and allows a quantitative measurement to be made. The reaction occurs between the amine group of taurine and phthalaldehyde with urea and phosphate ions present and acidification with acetic acid gives a purple product with an extinction maximum at 560 nm. The experimental conditions were those described by Gaitonde & Short (1971) for optimal results. These authors examined the effect of 60 amino compounds, including all the common amino acids, in the phthalaldehyde reaction and only found 14 which in similar quantities gave an extinction of greater than 10% of that of taurine under similar conditions. These compounds were homotaurine, hypotaurine, glycine, lysine, hydroxylysine, GABA, alanine, ethanolamine, ornithine, cysteamine, crystamine, tyramine, dopamine, phenylethylamine and tryptamine. However, all of these compounds, except homotaurine and hypotaurine, have been largely ruled out on the basis of their elution, or likely position of elution in cation exchange chromatography not coinciding with the elution pattern of the vesicle component.

Assay of a portion of the purified vesicle fraction after passage through Sephadex, together with taurine standards by the phthalaldehyde reaction, gave a value of 163 nmol taurine and assay of the same sample by column chromatography (assuming the component of vesicles to have the same colour factor as taurine) gave a value of 186 nmol taurine. The similarity of these results indicated that component II of vesicles detected by column chromatography was indeed taurine.

Further evidence that the ninhydrin positive peak present in vesicles is taurine comes from analysis of a 10% homogenate of rat cerebral cortex, which also contains this component, where it is present at 8.9 μmol per g

fresh tissue (value extrapolated from Table 25). By comparison, Gaitonde & Short (1971) found that after removal of interferring substances by a double ion exchange column system, the phthaladehyde reaction gave a value of 6.8 μmol/g for whole rat brain. The smaller value of the latter is to be expected from a whole brain preparation.

Distribution of amino acids including taurine. The amino acid content of various cerebral cortex fractions and subfractions are shown in Table 25. These values were obtained after extraction of amino acids from tissue by boiling at p.H. 4 (100°C) for 10 min. Under these conditions there was found to be high recovery (93 - 108%) of aspartate, serine, glutamate, glycine, alanine and GABA but there was almost total loss of glutamine, some glutamate being formed but not on a stoichiometric basis (about 10% of the glutamine was onverted glutamate) and no other ninhydrin positive compound being formed apart from ammonia.

The distribution of amino acids in the 10% sucrose homogenate, the microsomal fraction (the supernatant after centrifugation at 19,000 g for 20 min) and the crude mitochondrial pellet were qualitatively similar, glutamate being the most abundant followed by taurine, aspartate, GABA, serine, glycine and alanine. However,in the synaptosomal fraction taken directly from the sucrose gradient, taurine was the most abundant amino acid. In the sucrose fraction immediately under this in the sucrose gradient, i.e. the layer of 1.2 M sucrose, taurine was by far the most abundant amino acid also, which may be due to this fraction containing a proportion of synaptosomes since De Robertis et al (1962) have prepared a fraction of nerve endings at a 1.2/1.4 M sucrose interface. Thus taurine shows a relatively enhanced concentration in fractions containing nerve endings compared with other amino acids which show a similar distribution

Table 25 Distribution of amino acids in fractions of cerebral cortex

	n mol amino acid / g original tissue		
	10% cerebral cortex homogenate in 0.32M sucrose	Microsomal supernatant	Crude mitochondrial pellet
Taurine	8868 ± 758	4421 ± 226	1441 ± 79
Aspartate	6922 ± 531	3592 ± 64	1151 ± 92
Serine	1781 ± 228	773 ± 39	233 ± 26
Glutamate	12927 ± 181	6852 ± 132	1798 ± 77
Glycine	1238 ± 38	585 ± 27	147 ± 23
Alanine	799	348 ± 17	56
GABA	2358 ± 99	1310 ± 48	354 ± 34
No of experiments	5 (except alanine)	5	7 (except alanine)
	Synaptosomes	Myelin	1.2 M sucrose
Taurine	974 ± 57	834 ± 6	102 ± 10
Aspartate	417 ± 41	446 ± 27	23 ± 1
Serine	115 ± 13	108 ± 17	-
Glutamate	720 ± 70	871 ± 75	41 ± 4
Glycine	48 ± 10	99 ± 5	-
GABA	128 ± 12	147 ± 7	7 ± 0
No of experiments	7	2	4

cont...

Table 25 cont.

Samples of different fractions obtained in the preparation of synaptosomes were extracted with dilute HCl at p.H. 4.0 for 10 min at 100°C. The values are means ± S.E.M.s for the number of experiments indicated. The synaptosome, myelin and 1.2 M sucrose fractions are those taken directly from the discontinuous sucrose gradient before sedimentation. All the above amino acids were found to be stable under the conditions of extraction; however, glutamine was found to be totally lost, 10% being converted into glutamate. Thus the value for glutamate will also include extra glutamate obtained from about 10% of the total glutamine.

CMP = crude mitochondrial pellet (P_2B)

in all cytoplasmic containing fractions, as found by Mangan & Whittaker (1966). The data from Table 25 shows that 5.5 to 6.7% of the aspartate, glutamate, serine and GABA of the 10% sucrose homogenate was found in the synaptosomal fraction whereas 10.7% of the total taurine was found in this fraction.

In the synaptosome fraction, components I and III of the vesicle fraction were also present in approximately the same ratio as occurs in the purified vesicle fraction.

In Table 26, the amino acid content of cerebral cortex fractions is related to the protein content of the particular fraction. The microsomal supernatant containing the free cytoplasmic contents released during homogenisation showed the highest content per 100mg protein as was to be expected. The concentration of aspartate, glutamate, and GABA were similar in the 10% homogenate as in the synaptosomal fraction and also similar but slightly less in the crude mitochondrial pellet. However, the amount of taurine is shown to double in concentration in the synaptosomal fraction compared to the 10% homogenate and crude mitochondrial pellet, once again showing an enhancement of taurine in the synaptosome fraction.

Discussion

When a synaptic vesicle fraction was purified by gel filtration, 3 ninhydrin components were found to be present and these were present in significant amounts. The position and elution pattern of one of these components after ion exchange chromatography were identical with taurine and distinct from other acidic amino acids such as homocysteic acid and cysteic acid which appear in a similar position after chromatography.

Table 26 Amino acid distribution in fractions of cerebral cortex related to protein concentration

	n. mol amino acid / 100mg protein					
	10% Homogenate	Microsomal Supernatant	CMP	Synaptosomes	Myelin	1.2 M Sucrose
Taurine	9283	2622	6509	16739	14602	15422
Aspartate	7073	2221	5255	7220	7798	1577
Serine	1820	4683	1055	1992	1890	-
Glutamate	13211	41539	8154	12439	15249	2850
Glycine	1265	3548	666	834	1736	-
Alanine	816	2112	254	-	841	-
GABA	2410	7940	1607	2214	2573	454

The values on this table were derived from Table 25, where the experimental details are given.

The purified vesicle fraction also exhibited a colour reaction with σ phthalaldehyde and urea which is typical for taurine and a few closely related compounds and which gave a quantitative value of the taurine concentration very close to the values obtained from the ion exchange chromatography. The possibility has not yet been ruled out that other compounds such as homotaurine and hypotaurine are responsible for both the ninhydrin and phthalaldehyde reaction.

A number of methods were carried out to verify that the physiologically active amino acids, aspartate, glutamate and GABA shown in these studies to be released from nerve terminals by depolarization, were not present in vesicles in amounts detectable by the methods used. Purification was carried out at both 0°C and room temperature, in 0.4 M sucrose and after incubation in medium containing amino acids and glucose. However, no affinity of the amino acids aspartate, glutamate, glutamine, glycine, alanine, serine, valine or GABA was shown for synaptic vesicles. At the time of carrying out this experiment, similar results were obtained by Rassin (1972) by passing a vesicle fraction through both Sephadex and Biogel, except that taurine was not found concentrated in vesicles. The reason for this is not clear, though even in the homogenate of guinea pig cortex used as the starting material, Rassin only found taurine to be the 7th most abundant amino acid although the amounts of the other amino acids were found to be present in similar amounts to those obtained in these experiments (Table 25) A factor which might have led to a loss of taurine in Rassin's experiments could have been the use of water alone to burst the CMP, rather than tris/EDTA which Marchbanks (1968) found increased the yield of vesicular ACh four fold.

Kuriyama, Roberts & Kakefuda (1968) have found an association of labelled GABA to a vesicle rich fraction obtained by differential centri-

fugation which occurs at 0°C and only in the presence of Na^+. From the present results it seems possible that this was either due to non specific adsorption of GABA molecules which could be easily lost on passage through Sephadex, since this process was found to occur in a similar manner in microsomal and synaptosomal fractions. Alternatively, GABA may have been lost in the period (about 10 min) when the vesicles were being passed through the Sephadex column.

The observation that the glutamate, GABA and aspartate released in response to electrical and K^+ stimulation appeared to be coming from the major metabolic pool in the synaptosome (see Chapter 4) was a preliminary indication of the absence of a small amino acid pool particularly associated with transmission. Thus, the demonstration of a lack of amino acids in vesicles is in keeping with a process by which physiologically active compounds are released from the main cytoplasmic pool. This has also been found to be true of the ACh released from synaptosomes (Richter & Marchbanks 1971a). In addition to this, the ACh present in vesicles does not appear to be in equilibrium with the cytoplasmic ACh (Marchbanks 1968), which would tend to indicate the lack of involvement of vesicles in synaptic transmission.

Concentration of taurine. Considerable variation exists for the taurine concentration of brain; Tallan (1962) gives a range of values for rat brain of 2.3 to 8.5 μmol/g fresh weight and Gaitonde & Short (1971) by means of the o-phthalaldehyde reaction give a value of 6.8 μmol/g for whole rat brain. Higher values have been obtained for rat cerebral hemispheres of 7.6 μmol/g (Himwich and Agrawal 1969) and for mouse brain 9.13 μmol/g (Agrawal et al 1968). Thus the value of 8.9 μmol/g found in the present studies in a 10% homogenate of dissected cerebral cortices is in keeping

with this value in view of the fact that regional distribution of taurine in rat brain shows that cerebral cortex contains about 2 to 3 times the concentration of taurine as midbrain pons, medulla and about 1 1/2 times that of cerebellum (Shaw & Heine 1965; Agrawal et al 1971). The values quoted above refer to adult animals.

The distribution of taurine. These studies indicate that taurine is concentrated in nerve endings to a greater degree than any other amino acid. Associated with this is the observation that about one third of the total activity of the enzyme that synthesizes taurine from cysteine via taurine, cysteinesulphinic decarboxylase is found in nerve endings (Agrawal et al 1971). Although these authors prepared synaptic vesicles as part of a distribution study of taurine in rat brain, no taurine appears to have been found in vesicles.

The physiological action of taurine. The concentration of taurine in nerve endings above that of other amino acids and its presence in vesicles in sizeable amounts is a parallel distribution to that of ACh and leads to the implication of taurine in transmission. Indeed, taurine has been found to have a depressive activity, reducing spike activity when applied iontophoretically to cortex cells (Crawford & Curtis 1964), Renshaw cells and spinal interneurones (Curtis & Watkins 1960). This activity is almost as potent as that of GABA and is similar in that it antagonises activity produced by application of glutamate, aspartate or cysteic acid. Taurine action is also antagonised by strychnine as occurs for glycine (Curtis, Hosli and Johnston 1968). In brain slices, K^+ uptake is increased in the presence of taurine (Neame 1964) which further shows a link with the ion fluxes occurring during transmission, this being in a reverse direction to the initial K^+ efflux occurring on depolarization.

Estimation of the taurine content of individual synaptic vesicles. A very rough estimate of the taurine content of a single vesicle can be made by assuming that the vesicle yield from rat cortex is the same as that from guinea pig cortex. The latter has been obtained by Clementi et al (1966) by a polystyrene bead tagging method and shown to be 3.8×10^{12} vesicles per g fresh tissue. In the prsent experiments a value of 3.3 μmol taurine/100mg protein was obtained in the purified vesicle fraction and a yield of 21.8 mg protein in the vesicle fraction from 13.8 g cerebral cortex (Table 24). Hence, the taurine content of a single vesicle would be $\frac{3.3 \times 21.8 \times 10^{-6} \times 6 \times 10^{23}}{100 \times 13.8 \times 3.8 \times 10^{12}}$ molecules = 8230 molecules. If only a fraction of the vesicles contained taurine, however, this would be an under-estimation. This value is, in fact, in the likely range of 1,000 to 10,000 molecules of ACh estimated by Katz (1969) and Miledi (1961) for the ACh content of a single quantum. Similarly the ACh content of vesicles obtained from a purely cholinergic tissue, the electric organ of Torpedo was found to be about 5,000 molecules per vesicle (Marchbanks 1971). Assuming an internal vesicle volume of 0.6×10^{-17} cc, the taurine concentration in the vesicle would be 2.26 moles/litre, which represents a very concentrated form.

The presence of taurine in vesicles in amounts compatible with the 'quantal' hypothesis, i.e. each vesicle containing a quantum of transmitter, would indicate that taurine was a possible inhibitory transmitter, especially since it has a depressive effect on the activity of central neurones. The taurine content of brain does, however, decrease during postnatal development to the adult (Agrawal et al 1971) which is not apparently in keeping with taurine being functional in the adult. The decrease may be due to a more widespread need of an inhibitory transmitter in the immature brain

where neuronal sensitivity to transmitter is over the whole surface (Harris & Dennis 1970). However, a 6-7 fold increase in the release of taurine from cerveau isolé preparations occurs on stimulation of reticular activating pathways which is a similar increase as shown by glutamate (Jasper & Koyama 1969). Another interesting parallel with glutamate is that homotaurine (3-amiho propane sulphonic acid) and homocysteate, related compounds of taurine and glutamate, show far more potent neural action than their parent compounds.

CHAPTER 8 CONCLUDING REMARKS

The aim of this research, in general terms, has been to further the knowledge about biochemical aspects of transmission at central mammalian synapses. To date, a lot of information on this subject has been deduced from studies on the peripheral nervous system and from preparations of non-mammalian tissue. Tissues of this kind are more easily handled than central nervous tissue due to factors such as cellular size, accessability, and identification of cells and pathways involved. It is not unlikely that the basic mechanisms of neurotransmission are universal and with this background assumption, information can be obtained from a wide range of preparations. Action potentials are the results of identical ion fluxes in a wide range of Phyla and the site of synaptic transmission, i.e. the nerve terminal and synapse, are remarkably similar from octopus to man. Nevertheless, although basic mechanisms may be universal, the details can clearly be seen to be different as shown by the range of sensitivities shown by different classes of neurone to different putative transmitters.

Iontophoretic studies on central tissue have provided a body of data about key putative transmitters (Chapter 1) but the evidence is often contraversial. This has, however, provided a useful basis for the studies on release of transmitter, undertaken in this thesis, where post synaptic action cannot be measured. The preparation chosen for this study was one consisting almost exclusively of central nerve endings, which has the advantage over *in vivo* superfusion techniques, that the source of the released compounds is known. Although an *in vitro* preparation, isolated nerve endings are capable of behaving metabolically as whole tissue (Chapter 3).

The depolarization induced release of glutamate, GABA, aspartate (Chapter 4) and acetyl choline (Chapter 6) from synaptosomes was interesting not only because these compounds are physiologically active on cerebral neurones but also because these compounds are specifically released in vivo from superfused mammalian cortex by stimulation (discussed in Chapter 1). This evidence lends support to the observations that superfusion methods provide evidence about the processes occurring at the synapse and in a reciprocal way gives support to the validity of using a synaptosomal preparation since it performs at least qualitatively as an in vivo system.

The essential requirement of Ca^{2+} for synaptic transmission was borne out in the release of glutamate, GABA, aspartate and ACh from synaptosomes and the nature of this dependence was similar to that of an EPSP in a brain slice. The demonstration of a similar mechanism of release for these amino acids as ACh, which has a known transmitter function in the peripheral nervous system, lends weight to the proposal that these amino acids are central transmitters. The widespread appearance of amino acids and the almost universal sensitivity of neurones to them has, however, given rise to ideas that they, together with the catecholamines, function as "modulators" of transmitter action (discussed by Florey 1967). A modulator would be a substance released at the same time as a transmitter and which is able to facilitate or defacilitate depolarization to a certain degree and perhaps stimulate energy producing processes. If this were the case for the amino acids then a term such as "co-transmitter" would be more apt in view of their potency of action and their transmitter-like mechanisms of release.

An important question to ask about released amino acid, is its origin within the nerve terminal. Specific radioactivity measurements in this

study showed that the actual released amino acid had a similar specific activity to that remaining in the synaptosome (Chapter 6). A single pool of each amino acid would appear to exist, readily accessible to recently synthesised amino acid and available for release. The absence of these amino acids from purified synaptic vesicles, under conditions in which ACh is retained within vesicles supports the idea that released material is coming from the cytoplasm and not the vesicles. Stimulated release of radioactively labelled ACh also shows that the ACh is likely to be coming from a recently synthesised pool in the synaptosomal cytoplasm and not the vesicles which have low radioactivity (Richter & Marchbanks 1971a). It has been found possible to detect a small highly labelled radioactive pool of ACh associated with vesicles of the electric organ of Torpedo (Marchbanks & Israel 1971) after incubation of the tissue in radioactive choline. Although this pool is unattacked by AChE in the tissue, it is lost when the vesicles are passed through Sephadex and the bulk of the ACh is found as bioassayable ACh within the vesicular component. The general conclusion from the foregoing studies is that the released amino acid or ACh does not come from a stable major vesicle pool, but from the cytoplasm or a transient superficial vesicular pool, probably in equilibrium with the cytoplasmic pool.

The idea that vesicles are involved in transmission is embodied in the vesicular hypothesis of Katz & Miledi (summarised by Katz 1969) which explains the vesicles as the most likely means of providing identical packets of transmitter, which when released at the synapse to give rise to mepps and in large amounts EPSPs. The evidence is,of course, only circumstantial but together with the presence of complex networks associated with vesicles near the presynaptic membrane, (discussed in Chapter 7),

provides a strong case for some kind of vesicular involvement in synaptic transmission.

Alternatively, a mechanism for quantal transmitter release could be postulated without the involvement of vesicles. Depolarization itself might be quantized, the resulting influx of ions initiating chemical or physical changes in a quantized way. If pores in the presynaptic membrane were open for a constant period, a quantized amount of small molecular weight compound could be lost.

An interesting outcome of the vesicle study was finding large amounts of taurine in the vesicles. Although taurine is the second most abundant amino acid in the cerebral cortex it has been greatly ignored in neurochemical studies, despite its central depressive activity, antagonism by strychnine and release in similar amounts to glutamate in cerveau isole preparations (Chapter 7). Thus the question arises as to the reason for the presence of taurine in vesicles, whether it is there as, an inhibitory transmitter, an anion to bind cationic molecules such as ACh, to form a source of metabolites such as cysteine or for any other reason. This question raises many points for further investigation.

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SHORT COMMUNICATION

The stimulus-induced release of acetylcholine from synaptosome beds and its calcium dependence

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ACETYLCHOLINE is now well established as an excitatory transmitter in the autonomic and peripheral nervous system. In the CNS also, ACh shows many transmitter properties, in that it causes depolarization of neocortical neurons (KRNJEVIĆ and PHILLIS, 1963*a*, *b*) and Renshaw cells (CURTIS, 1963) and is released from superfused cerebral cortex in response to direct electrical and to peripheral stimulation (MITCHELL, 1963) and in response to reticular formation and mesial thalamic stimulation (JASPER and KOYAMA, 1969). In addition to this, ACh is largely found in nerve endings together with its synthesizing enzyme ChAc (HEBB and WHITTAKER, 1958) and inactivating enzyme AChE (WHITTAKER, 1965). Release of ACh *in vitro* from cortex slices has been found to be increased in response to elevated K^+ (MANN, TENNENBAUM and QUASTEL, 1939; RICHTER and MARCHBANKS, 1971*a*) and is released by the same stimulus from perfused ganglia (BIRKS, 1963) and from a crude mitochondrial pellet which is rich in nerve endings (HAGA, 1971). Release of ACh from cortex slices has also been shown in response to electrical stimulation (ROWSELL, 1954). In the present studies it was decided to measure the release of ACh from incubated synaptosome beds under conditions of stimulation by elevated K^+ and electripulses. Synaptosome beds are deposits of nerve endings positioned between nylon gauzes and held in grid electrodes. This method has been used previously for the study of a calcium-dependent amino acid release which occurs in response to stimulation of synaptosomes (DE BELLEROCHE and BRADFORD, 1972). Since calcium has been found to be essential for the release of ACh at the neuromuscular junction (KATZ and MILEDI, 1964), and from superfused auditory cortex (HEMSWORTH and MITCHELL, 1969) the requirement for this ion was examined in the present study.

METHODS

Synaptosomes were prepared from cerebral cortex of Sprague–Dawley rats by the method of GRAY and WHITTAKER (1962) with modifications as described by BRADFORD (1969). Synaptosome beds were then prepared by the method of DE BELLEROCHE and BRADFORD (1972), in which a suspension of synaptosomes in Krebs–Ringer bicarbonate medium is sedimented onto nylon gauze by centrifuging at 550 *g* for 10 min at room temperature. Another piece of nylon gauze is placed on top forming a sandwich, which can be easily manipulated by holding it in a McIlwain quick transfer holder. From the cortices of 15 rats, eight synaptosome beds could be prepared, each containing about 10 mg of protein. Synaptosome beds were incubated for 40 min at 37°C in Krebs–bicarbonate medium of composition (mM): NaCl, 124; KCl, 5; KH_2PO_4, 1·2; $CaCl_2$, 0·75; $MgSO_4$, 1·3; $NaHCO_3$, 26; glucose, 10; pH 7·4. The medium was gassed with 95% O_2/5% CO_2. In experiments with no calcium in the medium, ethylene glycol tetra-acetic acid (EGTA) was added at a final concentration of 0·5 mM. Potassium stimulation was by elevation of medium K^+ by 50 mM at 10 min before the end of incubation, which was done by adding 250 μl of 1·0 M-KCl made up in medium, at the point of gassing to give rapid mixing. A similar volume of medium only, was added to the controls. Electrical stimulation was carried out for the last 10 min of incubation by application of square wave pulses of 10 V amplitude, 0·4 ms duration and alternating in polarity. At the end of incubation, the synaptosome beds were removed from the quick transfer holders, using forceps, and placed in 1·25 ml vol. of frog Ringer solution medium, mixed by vortex and kept at 4°C. The medium in which the synaptosome beds had been incubated was then frozen and stored at −22°C. The frog Ringer solution used was of the following composition (mM): NaCl, 110; KCl, 4; $CaCl_2$, 1·2; $NaHCO_3$, 4·25; glucose, 4. All sucrose solutions and media used in the preparation and incubation contained neostigmine bromide at a final concentration of 10 μg/ml (0·33 mM).

Abbreviation used: EGTA, ethylene glycol tetra-acetic acid.

ACh was extracted from the synaptosomes by heating the 1·25 ml suspensions in frog Ringer solution at pH 4 at 100°C for 10 min (HEBB and WHITTAKER, 1958). The solution was then neutralized and centrifuged at 800 *g* for 10 min to remove the precipitated protein. The supernatant fluid was stored at −22°C and later used for assay of ACh and the pellet was kept for protein determination. The 5 ml volumes of medium in which the synaptosome beds had been incubated were freeze-dried. Immediately before use, 1·5 ml of frog Ringer solution containing neostigmine was added to the dried extract for assay. ACh was assayed by the method of CHANG and GADDUM (1933), using the frog rectus abdominis muscle of *Rana temporaria*, with ACh perchlorate as the standard. A bracketing method was employed, 0·1 ml samples of the extracts being alternated with standards giving similar responses, assay being in duplicate. Tissue extracts were made alkaline and boiled (FELDBERG, 1950) to inactivate ACh, and ACh standards were incorporated into these inactivated samples to test for potentiating or inhibiting substances in the extract. The effect of concentrating the ions of the medium by freeze-drying was tested by incorporating ACh standards into portions of medium which had been treated in exactly the same way as the media in which the synaptosome beds had been incubated. Normal bicarbonate medium and medium containing EGTA and no calcium, with ACh standards included, were incubated at 37°C for 40 min, either alone, with elevated K^+ or with electrical pulses in the last 10 min of incubation. These samples were then freeze-dried and taken up in 1·5 ml of frogs Ringer solution containing neostigmine, when ready for assay. The final concentration of K^+ to which the rectus muscle was exposed did not cause a potentiating effect or illicit contraction on its own. Similarly the presence of EGTA and no calcium in the medium were without effect when included with the standards and the samples assayed. Protein determination was carried out by the method of LOWRY, ROSEBROUGH, FARR and RANDALL (1951) with bovine serum albumin as standard.

RESULTS AND DISCUSSION

Elevation of the medium K^+ by 50 mM in the last 10 min of a 40 min incubation period led to a significant ($P < 0·001$) increase of 45 per cent in the ACh released to the medium and a significant ($P < 0·001$) decrease of 24 per cent in the ACh remaining in the synaptosome bed (Table 1). There was an increase of 13 per cent in the total ACh, ($P < 0·01$), which is likely to be due to an increase in

TABLE 1.—ACETYLCHOLINE IN SYNAPTOSOME BEDS AND INCUBATION MEDIUM FOLLOWING ELECTRICAL AND POTASSIUM STIMULATION

	Acetylcholine content (nmol/100 mg protein)	
	Synaptosome bed	Incubation medium
Calcium medium		
Control	60·9 ± 1·04 (23)	69·8 ± 2·57 (16)
Potassium stimulation	46·2 ± 2·02 (11)	101·0 ± 4·96 (10)
Electrical stimulation	52·8 ± 2·05 (15)	83·7 ± 5·02 (10)
Calcium-free medium (+ EGTA)		
Control	64·2 ± 2·45 (9)	27·5 ± 4·72 (6)
Potassium stimulation	55·8 ± 3·0 (7)	34·9 ± 2·49 (7)
Electrical stimulation	57·7 ± 3·0 (5)	29·6 ± 5·52 (4)

Synaptosome beds were incubated for 40 min at 37°C in Krebs–bicarbonate medium (calcium medium) or in Krebs–bicarbonate medium containing no calcium and 0·5 mM-EGTA, as indicated. Potassium stimulation was by elevation of medium K^+ by 50mM 10 min before the end of incubation and electrical stimulation by application of electrical pulses for the last 10 min of incubation. Values are means ±S.E.M. with the number of experiments in brackets.

ACh synthesis. Application of electrical pulses (Table 1) for the last 10 min of the 40 min incubation also gave a significant ($P < 0·01$) increase in the release of ACh, a significant ($P < 0·001$) decrease in the ACh of the synaptosome bed and an increase in the total ACh. However these effects were somewhat smaller.

Incubation of synaptosome beds in the absence of calcium and the presence of 0·5 mM EGTA and the effect of stimulation were studied. EGTA binds calcium 10^5 times more effectively than it binds

magnesium, two molecules of calcium being bound per molecule of EGTA. In the absence of calcium and with EGTA present to complex any endogenous tissue calcium, there was no significant diminution (Table 1) in the synaptosomal ACh and no release of ACh evoked by either type of stimulation. The control levels of ACh in this medium were about half of the control values in the presence of calcium. Presumably, in the presence of calcium, ACh is being continuously lost from cholinergic synapses as evidenced by miniature endplate potential generation. This could partly account for the losses of ACh from the controls seen in the present experiments.

The literature values for the ACh content of synaptosomes do not vary greatly, DE ROBERTIS, RODRÍGUEZ DE LORES ARNAIZ, SALGANICOFF, PELLEGRINO DE IRALDI and ZIEHER (1962) found 3·43 nmol ACh/g cortex in nerve endings isolated from rat cerebral cortex (combining the B and C layer between 0·8 and 1·2 M-sucrose equivalent to the fraction used in the present studies). BARKER, DOWDALL, ESSMAN and WHITTAKER (1970) found 3·2 nmol ACh/g in synaptosomes isolated from guinea pig cerebral cortex (P2B). Conversion of the values of ACh given in Table 1 to the units quoted above gives the value of 3·58 nmol ACh/g original cerebral cortex for control incubated synaptosome beds and 2·72 nmol ACh/g for synaptosome beds incubated in the presence of elevated potassium. RICHTER and MARCHBANKS (1971*b*) found comparable levels of 2·7 and 2·1 nmol ACh/g in synaptosomes isolated from incubated control, and K^+ stimulated guinea pig-cerebral slices, respectively, using dorsal muscle of leech for bioassay. The present results are also consistent with the findings of HAGA (1971), who, following the release of [^{14}C]ACh from crude mitochondrial pellet, found a 25 per cent fall, in tissue levels of ACh with incubation in elevated K^+, and confirms that the ACh may be recovered in the incubation medium. However, from our studies, ACh release has a clear dependence on the presence of calcium. HAGA (1971) did not find this, probably because he did not complex the endogenous calcium of his preparations. Thus, when synaptosome beds, incubated in physiological medium are stimulated by elevated potassium or electrical pulses there is a significant increase in the release of ACh to the medium, which is dependent on the presence of calcium. Such stimulation also leads to an increase in the total ACh, suggesting that increased synthesis is occurring.

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METABOLISM OF BEDS OF MAMMALIAN CORTICAL SYNAPTOSOMES: RESPONSE TO DEPOLARIZING INFLUENCES

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Abstract—The metabolic properties of synaptosome beds (deposits positioned between nylon gauzes) were studied. They respired, glycolysed, produced ATP and phosphocreatine, and metabolized [U-^{14}C]glucose to glutamate, aspartate, alanine and GABA at similar rates to synaptosome suspensions. Metabolic inhibitors caused massive loss of amino acids from the beds. Synaptosome beds also responded metabolically to electrical pulses; respiration and lactate production increasing by 40 per cent. Differential release of glutamate, aspartate and GABA occurred during electrical stimulation, maximum release being after 10–15 min of stimulation. This differential release also occurred when medium potassium was increased. Omitting and chelating calcium reduced or abolished this response with both forms of stimulation. Including amino acid analogues (β-aminobutyric acid, α,γ-diaminobutyric acid and *N*-acetyl glutamic acid) in the incubation medium changed the patterns of amino acids present in the medium, indicating that under normal conditions active amino acid uptake processes are occurring in synaptosomes. Tetrodotoxin and ouabain also interfered with amino acid release without greatly affecting the response to stimulation. Cerebral cortex slices incubated between gauzes also showed a glycolytic response to electrical stimulation. GABA was the only amino acid showing a significant increase in the amount released with both potassium and electrical stimulation of the slices.

PREVIOUS papers from this laboratory and elsewhere have presented evidence that synaptosomes from mammalian cerebral cortex, when incubated in appropriate media, show a wide range of metabolic properties and accumulate a variety of ions against a concentration gradient. (BRADFORD, 1967, 1969; BRADFORD and THOMAS, 1969; LING and ABDEL-LATIF, 1968; MARCHBANKS, 1968, 1970; ESCUETA and APPEL, 1969).

Application of electrical pulses or increased medium potassium causes increased metabolic rates, loss of K^+, and differential loss of physiologically active amino acids (BRADFORD 1970*a*, 1970*b*, 1970*c*; BRADFORD and THOMAS, 1972), and this pattern of reponse has been taken as evidence for the existence of a synaptosomal membrane potential (BRADFORD, 1970*a*; 1971*b*).

In all of these studies suspensions of synaptosomes were employed, and such preparations necessarily entail a very high fluid: tissue ratio. This paper describes another approach which provides conditions under which this ratio is low, and closer to that in the whole tissue. At the same time it provides a means of rapid fixation of the synaptosomes following various treatments. This approach involves positioning a deposit of synaptosomes between nylon gauzes, and we have termed these preparations, synaptosome beds.

Abbreviations used: PCA, perchloric acid; EGTA, ethylene glycol bis-(2 amino-ethyl) tetra-acetic acid; BABA, β-aminobutyric acid; DABA, α,γ-diaminobutyric acid; NAG, *N*-acetyl glutamic acid.

MATERIALS AND METHODS

Preparation of synaptosomes. Female Sprague–Dawley rats (200–250 g) were used; synaptosomes were prepared from cerebral cortex tissue by the method of GRAY and WHITTAKER (1962) modified as described by BRADFORD (1969).

Preparation and incubation of synaptosome beds. Pellets from the nerve-ending fraction were gently resuspended in either a Krebs–phosphate medium (Krebs–Ringer) of composition: (mM); NaCl, 124; KCl, 5; Na_2HPO_4, 20; KH_2PO_4, 1·2; $MgSO_4$, 1·3; $CaCl_2$ 0·75; pH 7·5 containing 10 mM-glucose or a Krebs–bicarbonate medium of composition: (mM); NaCl, 124; KCl, 5; KH_2PO_4, 1·2; $MgSO_4$, 1·3; $CaCl_2$, 0·75; $NaHCO_3$, 26 mM; pH 7·5 containing 10 mM-glucose. (The medium used is indicated on the tables and legends to the figures). Volumes (5 ml) of this suspension (approx. 2 mg protein/ml) were placed in a perspex cell which consisted of a hollow cylindrical upper part on a solid removable base. The cell contained a rectangle of gauze (1·7 × 2·4 cm) held between upper and lower parts. The cross-section of the chamber in the upper part was rectangular (1·5 × 2·2 cm) thus allowing the gauze to overlap by 0·5–1·0 mm at the edges (Fig. 1). The nylon gauze (110 mesh) could be inserted by removing the base of the cell, placing the gauze symmetrically over the rectangular space and replacing the base of the cell, which was secured by a push-fit. For experiments where oxygen uptake was measured, smaller beds were prepared (1·5 × 0·7 cm) in a smaller cell (3 ml volume). The cells

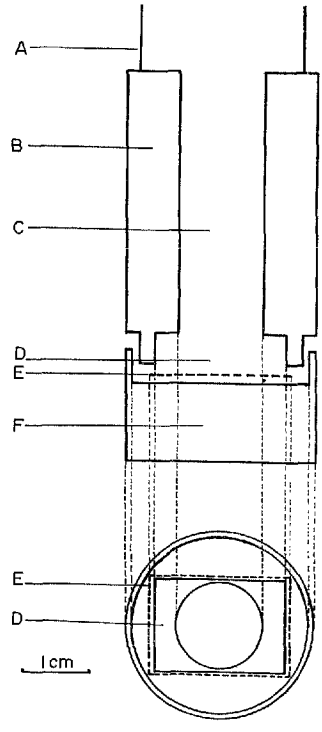


FIG. 1.—Cell for making synaptosome beds. A, metal rod to facilitate removal of cell from centrifuge bucket; B, hollow perspex cylinder; C, central cavity in which synaptosome suspension is placed; D, rectangular shaped cavity, E, nylon gauze just larger than D; F, solid perspex base piece of cell with push-fit connection.

were then centrifuged at room temperature at 550 *g* for 10 min. The nerve-endings which aggregate in the medium were deposited on the gauze, forming a bed whose protein content was arranged to be similar to that of a slice of equivalent dimensions. The fluid supernatant was then poured off and the perspex base, bearing the gauze, was carefully removed from the cell. The gauze was then transferred using forceps to the lower jaw of a Quick Transfer Holder (MCILWAIN and RODNIGHT, 1962). A second piece of gauze, wet with medium, was placed over the synaptosome bed to form a sandwich

on which the jaws of the Quick Transfer Holder were closed. This system was then placed in a 25 ml beaker containing 5 ml of the Krebs–Ringer medium (Krebs–phosphate medium), gassed with oxygen or with 95% O_2: 5% CO_2 for Krebs-bicarbonate medium and incubated in a water bath at 37°C. After incubation synaptosomes were washed from the gauze into 5 ml of 1 M-PCA by vortex mixing. The medium was centrifuged briefly at room temperature to remove the very small amount of material (less than 5 per cent) washed from the gauze. Samples of this supernatant were taken for lactate determination and the remainder was mixed with 1 M-PCA for amino acid extraction.

Preparation and incubation of cortex slices. Cortex slices were cut from the outer surface of the whole cerebral cortex using a strip of razor blade and a glass guide recessed to give slices 350–400 μm in thickness (McILWAIN and RODNIGHT, 1962). These were incubated in Quick Transfer Holders either sandwiched between two pieces of nylon gauze or without the gauze, as indicated. For respiration studies, grid electrodes positioned in Warburg flasks were used in place of Quick Transfer Holders (McILWAIN and RODNIGHT, 1962).

Respiration of synaptosome beds. The small synaptosome beds (0·7 × 1·5 cm) described above were used. Each bed was placed between a pair of silver and perspex grid electrodes and immersed in Krebs–phosphate medium (2 ml) in Warburg flasks of the type described by McILWAIN and RODNIGHT, 1962, but containing gold metal stubs to convey pulses through the silver grid electrodes (for further details see BRADFORD, 1971*a*). The Warburg flasks were then incubated at 37°C for 50–70 min. After incubation, the beds were removed from the grid electrodes and treated as described for those removed from Quick Transfer Holders.

Electrical stimulation. After 30 min preincubation, square-wave pulses of 10 V, 0·4 ms duration and alternating in polarity were applied to both types of electrode at a frequency of 100/s for 30 min or as indicated, the average continuous current being 35 mA through tissue and medium.

Potassium stimulation. Synaptosome beds held in Quick Transfer Holders were incubated in the Krebs–phosphate medium for 30 min after which 0·25 ml of 1·0 M-KCl was injected into the medium to give a final concentration of 56 mM. Incubation was continued for a further 10 min before removal and extraction of synaptosomes and medium as described above. Medium containing extra sodium chloride equivalent to the potassium was added to controls. In some experiments, Krebs–bicarbonate medium was used as indicated for incubation and in this case, medium only was added to controls.

Incorporation of labelled glucose into amino acids. Synaptosome beds in Quick Transfer Holders were incubated for 60 min in Krebs–phosphate medium containing 5 μCi/ml of D-[U-^{14}C]glucose. Electrical stimulation was applied as described above.

Incubation with amino acid analogues. Synaptosome beds were prepared and incubated as described above at 37°C for 30 min before addition of amino acid analogues (BABA, DABA and NAG) to a concentration of 1 mM. Medium (0·05 ml) containing the analogue at 100 mM was injected into the 5 ml of incubation fluid at the point of gassing in order to ensure rapid mixing and distribution of analogue. In order to assess the stability of analogues, BABA, DABA and NAG were incubated separately in medium at 1 mM at 37°C for 1 h, in the absence of tissue. The media were analysed for amino acid content. There was found to be no conversion to other amino acids (e.g. GABA or glutamate).

Extraction and analyses of amino acids. Suspensions of synaptosomes in 1 M-PCA were further extracted in the cold with 0·5 M-PCA. Perchloric acid was added to the medium to precipitate soluble protein. These extracts were then passed through 10 × 0·5 cm ion exchange columns in the acid form (Zeocarb 225, 52–100 mesh, 8 per cent cross-linked) followed by a water wash. Amino acids were eluted from the columns with 1 M-NH_3 and the eluate evaporated down to dryness before dissolving in 0·025 M-HCl containing norleucine standard. These samples were then analysed using a rapid automated ion exchange column analysis (BRADFORD and THOMAS, 1969) modified to employ a shorter analysis time (83 min) (see BRADFORD and THOMAS, 1972 for details). When amino acid radioactivity was measured, the column effluent was stream-split before ninhydrin admixture and a proportion (60 per cent) was collected in 1·0 ml samples and mixed with Bray's (BRAY, 1960) scintillation medium. These mixtures were then counted in a Packard Tri-carb liquid scintillation counter. Recoveries of radioactivity were greater than 90 per cent. In these experiments [1-^{14}C]norleucine (1 nCi/ml) was included with the non-isotopic norleucine.

Other determinations. Protein determination was carried out on protein precipitates by the method of LOWRY, ROSEBROUGH, FARR and RANDALL (1951). Lactic acid was measured enzymically by the method of BERGMEYER (1965).

Extraction and assay of ATP and phosphocreatine. After incubation, the synaptosome beds were immediately transferred to 1 ml of 1 M-PCA containing 200 mM-tris base and mixed to inactivate Na–K ATPase. After leaving on ice for 30 min with frequent mixing, the samples were centrifuged at 800 *g* for 10 min in the cold. The supernatants were collected and the pellets mixed with a further 0·5 ml 1 M-PCA containing 200 mM-tris base and recentrifuged as before. The supernatants were pooled. The pH was adjusted approx. to pH 7·0 with a probe pH electrode. The precipitate of

potassium perchlorate was removed by centrifugation and the supernatants were quick-frozen with an acetone-dry ice mixture before storage at −22°C.

The quick frozen extracts were thawed just before assay and analysed for ATP and phosphocreatine by the method of LOWRY, PASSONEAU, HASSELBURGER and SCHULTZ (1964) using a Farrand Model MK-1 spectrofluorometer (Farrand, New York, U.S.A.).

Materials. Analar grade chemicals were used throughout. [U-^{14}C]glucose was from the Radiochemical Centre, Amersham. Nylon gauze was 110 mesh (i.e. 110 threads/in., aperture size 130 μm) from J. Staniar Ltd., Manchester. Amino acid analogues were the crystalline salts from Sigma Chemical Co. Crystalline Tetrodotoxin was obtained from Sankyo Co. Ltd., Tokyo, and the preparation contained one part by wt, of tetrodotoxin and five parts of citric acid–sodium citrate buffer.

RESULTS

Lactate formation and oxygen uptake. The data of Tables 1 and 2 and Fig. 2 show that beds of synaptosomes respired and produced lactate at rates of the same order as those measured for suspensions of synaptosomes. The respiratory rates showed only a small decrease (15 per cent) during 60 min of incubation (Fig. 2).

TABLE 1.—LACTATE PRODUCTION BY SYNAPTOSOME BEDS AND SUSPENSIONS

	Lactate produced (μmol/100 mg protein/h)	
	Beds	Suspensions†
Unstimulated	11·8 ± 0·6 (13)	16 ± 2 (20)
Stimulated	16·2 ± 1·0 (15)	25 ± 2 (20)

Synaptosome beds or suspensions were incubated for 1 h at 37°C in Krebs–phosphate medium containing 10 mM-glucose. Electrical pulses were delivered during the last 30 min of incubation. Values are means ± S.E.M. for the number of experiments in brackets.

† Data from BRADFORD (1970*a*).

TABLE 2.—OXYGEN UPTAKE BY SYNAPTOSOME BEDS AND SUSPENSIONS

	Oxygen uptake (μmol O_2/100 mg protein/h)	
	Beds	Suspensions*
First 30 min of incubation	53·2 ± 2·4 (22)	60 ± 3 (20)
Second 30 min of incubation	45·0 ± 2·0 (10)	
Electrical stimulation during 30 min of incubation	63·0 ± 5·4 (10)	98 ± 4 (20)

Synaptosome beds or suspensions were incubated for 1 h at 37°C in Krebs–phosphate medium containing 10 mM-glucose. Values are means ± S.E.M. for the number of experiments in brackets.

* Data from BRADFORD (1970*a*).

When the first respiratory readings were taken, these synaptosome beds had already been in contact with medium for 30 min longer than occurs when preparing a

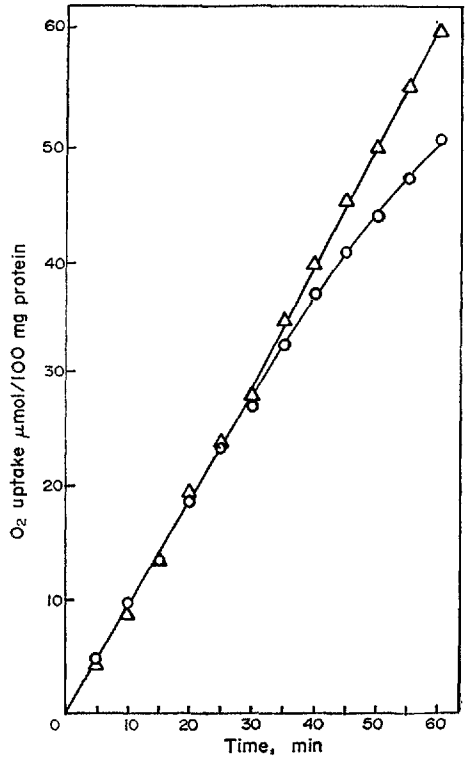


FIG. 2.—Respiration of synaptosome beds. Small synaptosome beds were incubated at 37°C in Krebs–phosphate medium for 1 h; ○——○. Unstimulated samples; △——△. Samples stimulated with electrical pulses after 30 min preincubation and continued to the end of incubation. Values plotted represent the mean rates from 10 (stimulated) or 22 (control) experiments. Values for S.E.M. are given in Table 2.

suspension. This may partly explain the falling-off of respiration in the beds after 30–40 min incubation. In studies using Quick Transfer Holders, incubation began about 15 min after contact with the medium.

Incorporation of [U-^{14}C]glucose into amino acids. In order to obtain a further measure of the adequacy of substrate availability within the synaptosome beds, the extent of incorporation of labelled glucose into amino acids was measured. During 60 min incubation in phosphate medium containing 10 mM-[U-^{14}C]glucose, considerable amounts of radioactivity were incorporated into aspartate, glutamate, alanine and GABA (Table 3) and the final specific radioactivities of these amino acids, on a molecular basis, were one fifth to one third of that of the glucose in the incubation medium. This is a similar picture to that found with suspensions of synaptosomes where the amino acids were also labelled to this extent over the same incubation period.

ATP and phosphocreatine levels. The levels of ATP and phosphocreatine measured in synaptosome beds after incubation in Krebs–phosphate medium at 37°C for 1 h (Table 4) appear to be slightly less than those of synaptosome suspensions (BRADFORD, 1969). In the absence of substrate there were large decreases (60–71 per cent) in the levels of the ATP and phosphocreatine.

TABLE 3.—SPECIFIC ACTIVITIES OF AMINO ACIDS IN SYNAPTOSOME BEDS AFTER INCUBATION WITH [U-^{14}C]GLUCOSE

	Control	Stimulated for last 30 min of incubation with electrical pulses
Aspartate	88 ± 4 (12)	114 ± 10 (9)*
Glutamate	126 ± 10 (11)	183 ± 21 (9)*
Alanine	123 ± 6 (11)	95 ± 12 (10)†
GABA	122 ± 7 (13)	139 ± 15 (9)

Synaptosome beds were incubated in 5 ml of Krebs–phosphate medium at 37°C for 1 h in the presence of 10 mM-glucose and 25 μCi of [U-^{14}C]-glucose. Initial specific radioactivity of medium 428 nCi/μmol. Values are means ± S.E.M. for the number of experiments in brackets. Units are nCi/μmol. Values significantly different from control: * $P < 0{\cdot}02$; † $P < 0{\cdot}05$.

TABLE 4.—LEVELS OF ATP AND PHOSPHOCREATINE IN SYNAPTOSOME BEDS INCUBATED WITH GLUCOSE OR PYRUVATE AND WITHOUT SUBSTRATE

Substrate	ATP (nmol/100 mg protein)	Phosphocreatine (nmol/100 mg protein)
10 mM-glucose	267 ± 23 (8)	394 ± 58 (8)
10 mM-pyruvate	368 ± 59 (3)	414 ± 32 (3)
No substrate	106 ± 17 (8)	135 ± 28 (7)

Synaptosome beds were incubated at 37°C for 1 h in Krebs–phosphate medium with 0·75 mM-Ca^{2+} and substrate as indicated. Mean values ± S.E.M. are given for number of experiments in brackets.

Effect of hypotonicity and added cyanide. Amino acid retention by the beds appeared to be dependent on their structural integrity and on respiratory and glycolytic activity. Thus potassium cyanide at 1 mM together with iodoacetate at 10 mM caused almost total loss of amino acids to the medium (Table 5) under conditions previously shown to cause large falls in the levels of energy-rich phosphate (BRADFORD, 1969). Incubation in water instead of medium also caused massive loss of amino acids and was accompanied by disintegration of the beds, the synaptosomes being readily washed off the gauze by the fluid movements. The amino acids were recovered in the medium after sedimentation of synaptosomes (Table 5).

Incubation in water led to a significant increase in total amino acid pool sizes ($P < 0{\cdot}01$) for aspartate, glutamine, serine, glutamate, glycine and GABA, the alanine content showed a small decrease. With 1 mM-KCN and 10 M-iodoacetate, there was a significant increase in aspartate and GABA ($P < 0{\cdot}01$) and a significant decrease in alanine ($P < 0{\cdot}01$). The total amounts of glutamine, serine and glycine also decreased in this situation.

Response to electrical stimulation. Electrical pulses, applied through Quick Transfer Holders, caused a substantial increase (37 per cent) in lactate formation compared with the controls (Table 1), and passage of the same pulses through grid electrodes in

TABLE 5.—AMINO ACID CONTENT OF SYNAPTOSOMES INCUBATED IN THE PRESENCE OF METABOLIC INHIBITORS OR IN WATER

	Amino acid content (nmol/100 mg protein)								
	No inhibitors			Cyanide plus iodoacetate			Water only		
	Supernatant	Bed	Total	Supernatant	Bed	Total	Supernatant	Bed	Total
Aspartate	88	996	1084	1709	341	2050	3036	26	3062
Glutamine	331	213	544	463	39	502	753	17	770
Serine	387	314	701	495	180	675	856	56	912
Glutamate	187	2822	3009	2843	811	3654	4566	29	4595
Glycine	386	381	767	487	115	602	751	177	928
Alanine	438	334	772	436	54	490	670	25	395
GABA	21	497	518	686	170	865	1477	10	1487

Synaptosome beds were incubated in Krebs–phosphate medium or in water for 1 h at 37°C. Potassium cyanide (1 mM) and sodium iodoacetate (10 mM) were included in the Krebs–phosphate medium as indicated. Values are means for four experiments, Values of S.E.M. were approx. 10 per cent.

Warburg flasks brought about an increase in oxygen uptake of similar magnitude (Table 2 and Fig. 2).

There were also increases in the specific radioactivities of glutamate (44 per cent), aspartate (20 per cent) and GABA (23 per cent), but not in that of alanine, which showed a small decrease (Table 3). Electrical stimulation caused increased amounts of aspartate, glutamate and GABA to be released to the medium (Fig. 3), whereas the other amino acids measured did not increase significantly, as exemplified by the results for alanine shown in Fig. 3. The time-course of release of glutamate, aspartate and GABA, showed that the effect was maximal after 10–15 min stimulation and thereafter diminished, being only a few per cent from control values after 30 min of pulses (Fig. 3). The other amino acids showed a quite different pattern of behaviour, gradually accumulating in the medium with time and electrical stimulation had little effect on their rates of release. The behaviour of alanine is also shown in Fig. 3, and is typical of the other amino acids.

The effect of the gauze on the response to stimulation. The nylon gauze could be expected to act as a resistance in the stimulation circuit and effectively reduce the current, and therefore the stimulation. The situation is complicated by the uncertainty of the extent to which current may be short-circuited around the beds, through the medium. However cortex slices showed a substantial glycolytic response during application of pulses and the insertion of nylon gauzes between the slice and each electrode surface reduced this response by only 20–25 per cent, indicating the extent to which the pulses applied through the gauze remained effective (Table 6).

In order to compare the properties of synaptosomes and whole cortex tissue under similar conditions. the release of amino acids from cortex slices during electrical stimulation was also studied. Table 7 shows that pulses applied for 10 min caused a significant extra loss of only GABA to the incubation medium. However, this period of electrical stimulation of synaptosomes caused maximal release of aspartate, glutamate and GABA (Fig. 3). Cortex slices, however, did appear to show release of glutamate and GABA when stimulated electrically in the absence of gauze, though the

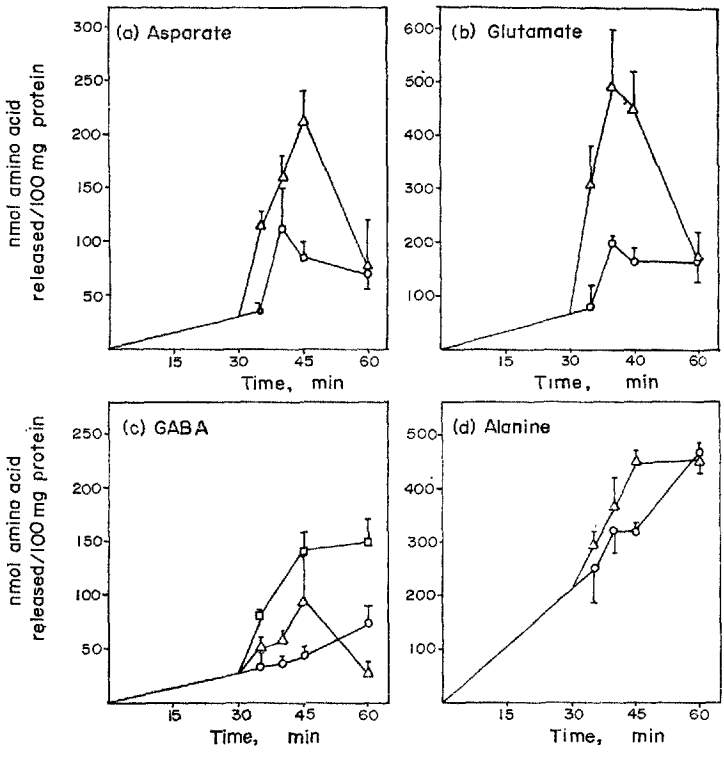


FIG. 3.—The effect of the duration of electrical stimulation on amino acid release from synaptosome beds. Synaptosome beds were incubated at 37°C in Krebs–phosphate medium for periods between 35 min and 1 h; ○——○. Unstimulated samples; △——△. Samples stimulated with electrical pulses after 30 min preincubation for 5, 10, 15 or 30 min periods, after which time incubation was terminated; □——□. Samples stimulated as above but with DABA added at the time same as stimulation was commenced. The vertical bars represent the S.E.M. values for 3–7 experiments. Alanine (d) is represented as being typical of the pattern shown also by glutamine, serine and glycine.

TABLE 6.—LACTATE PRODUCTION BY CEREBRAL CORTEX SLICES IN THE PRESENCE AND ABSENCE OF NYLON GAUZE

	Lactate accumulated (μmol/g wet wt./h)	
	Control	Electrical stimulation
No Gauze	109 ± 5	144 ± 10
Gauze present	82 ± 4	115 ± 2

Cortex slices were incubated in Krebs–bicarbonate medium at 37°C for 1 h in Quick Transfer Holders. Two rectangles of nylon gauze were positioned on each side of the cortex slices as indicated. Electrical stimulation was for the last 30 min of incubation. The values are means ± S.E.M. from six experiments.

TABLE 7.—AMINO ACID POOL SIZES IN CORTEX SLICES AND IN THE INCUBATION MEDIUM FOLLOWING STIMULATION WITH POTASSIUM OR WITH ELECTRICAL PULSES

	Amino acids (nmol/g wet wt.)						
	Control			Potassium stimulation			Electrical stimulation
	Medium	Total	% Released into medium	Medium	Total	% Released into medium	Medium
Aspartate	116	876	13·2	131	1209	10·8	107
Glutamine	1632	3539	46·1	1637	3820	42·8	1315
Serine	548	1453	37·7	590	1713	34·4	530
Glutamate	591	6826	8·6	747	8980	8·3	496
Glycine	381	1826	20·8	374	1591	23·5	419
Alanine	397	1552	25·5	484	1499	32·2	381
GABA	26	3418	0·7	140*	3864	3·6	62†

Cortex slices held between two pieces of nylon gauze, were incubated in Krebs–bicarbonate medium at 37°C for 40 min. Potassium stimulation was by elevation of K^+ to 56 mM in the medium at 30 min. Electrical stimulation was commenced at 30 min and continued to the end of the incubation and amino acids were only measured in the medium. Values are means for four experiments with the S.E.M. approx. 10 per cent.

* Values significantly different from the control: * $P < 0{\cdot}1$; † $< P\ 0{\cdot}1$.

The differences shown by the other amino acids were not statistically significant.

results were not consistent, release occurring in only half the cases. Thus, from all experiments, glutamate accumulation in the medium of control slices (40 min incubation) was 816 ± 124 nmol/g fresh wt. (S.E.M.; n = 5), whilst 10 min stimulation increased this value to 1186 ± 205 (n = 5). This is a similar result to that reported previously for electrical stimulation of small prisms of cortex suspended in medium between ring-electrodes (BRADFORD, 1970*a*) and between grid electrodes (SRINIVASAN, NEAL and MITCHELL, 1969).

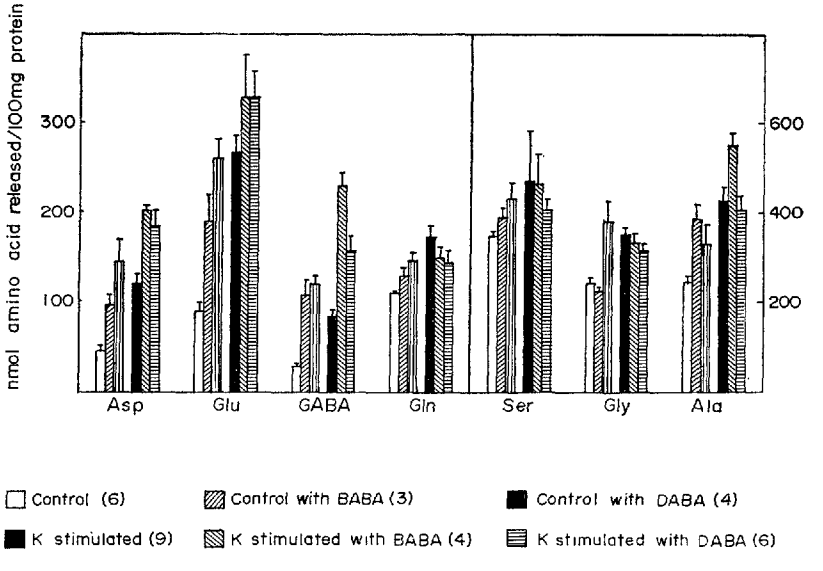


FIG. 4a

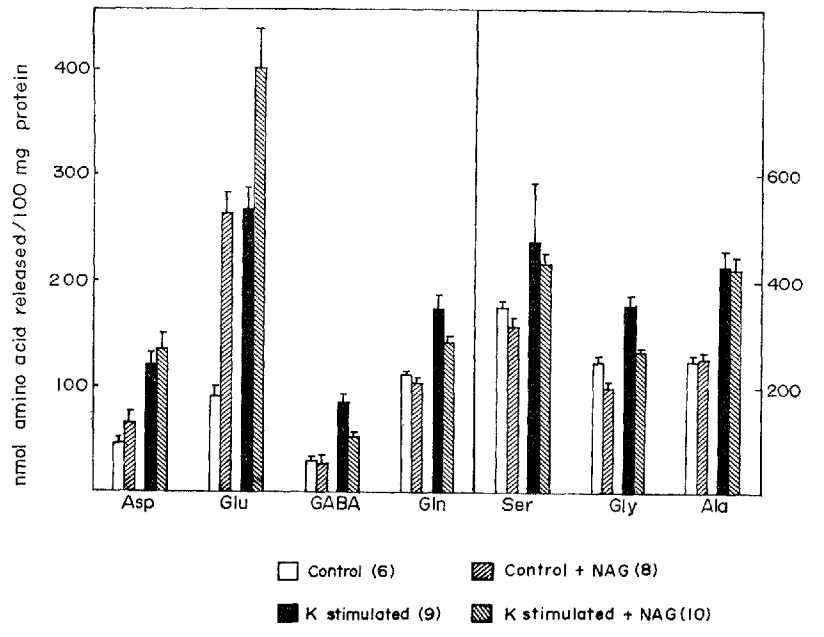


FIG. 4b

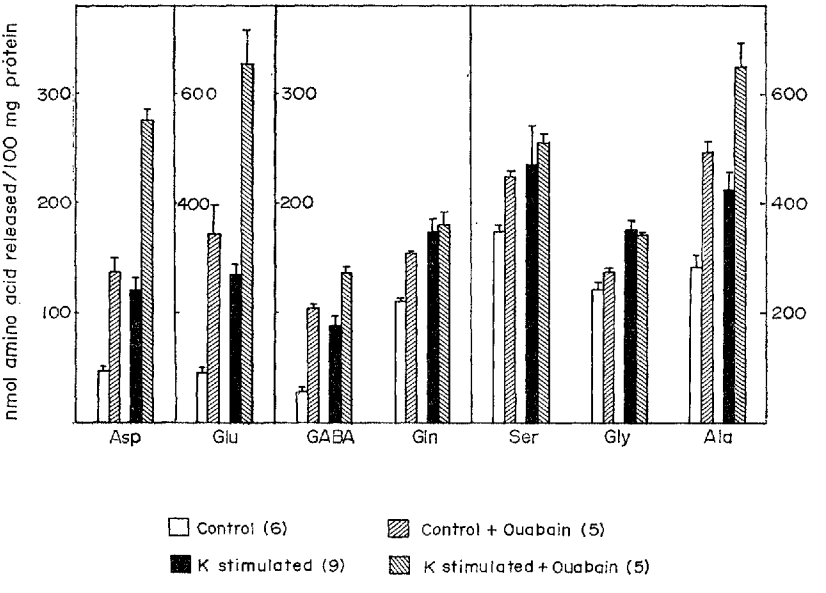


FIG. 4c

FIG. 4.—The effect of amino acid analogues and ouabain on amino acid release from synaptosome beds with stimulation by elevated potassium in the medium. Synaptosome beds were incubated in Krebs–phosphate medium at 37°C for 40 min. Potassium stimulation was by elevating the medium potassium by 50 mM at 30 min and continuing the incubation for a further 10 min. In the controls, the medium concentration of sodium was similarly elevated by 50 mM. Additions of BABA, DABA, NAG and ouabain were made at 30 min also. The bars represent the values of the S.E.M. of the number of experiments given in brackets.

The effect of elevated potassium on amino acid release from synaptosome beds. Increased medium potassium concentration, from 6 to 56 mM, caused a large increase in the proportion of aspartate (160 per cent), glutamate (190 per cent) and GABA (190 per cent) released to the medium; other amino acids showed much smaller changes (Fig. 4). Elevation of medium sodium by 50 mM in the controls was without effect, since the amino acid values were similar to those of controls where sodium was not increased (Fig. 4). Increase of the potassium concentration to 56 mM in the medium also yielded an increase in the amino acid pool sizes of the synaptosome beds for glutamate (26 per cent), aspartate (36 per cent) and GABA (10 per cent) (Table 8).

TABLE 8.—AMINO ACID POOL SIZES OF SYNAPTOSOME BEDS

	Amino acid content of synaptosome beds (nmol/100 mg protein)	
	Control	Potassium stimulated
Aspartate	872 ± 62 (8)	1188 ± 113 (14)
Glutamate	2023 ± 128 (7)	2543 ± 227 (14)
GABA	533 ± 32 (6)	587 ± 17 (14)
Glycine	221 ± 18 (5)	194 ± 15 (7)
Alanine	402 ± 56 (5)	270 ± 42 (7)

Synaptosome beds were incubated in Krebs–bicarbonate medium for 40 min at 37°C, Potassium stimulation began at 30 min by elevation of K^+ to 56 mM and incubation was continued for a further 10 min. Values are means ± S.E.M. with the number of experiments in brackets.

The glycine content of the bed did not change but that of alanine decreased. Omission of calcium from the medium, and the inclusion of EGTA to complex any endogenous calcium, completely inhibited the effects of increased amino acid release with elevated potassium concentration (Table 9). Under similar conditions of absence of calcium, the effects of electrical stimulation on amino acid release were greatly reduced. By only omitting calcium from the medium, an intermediate position was obtained where the response to elevated potassium concentration was partially reduced.

Calculation from Tables 8 and 9 shows that the proportions of total amino acids released after 10 min potassium stimulation were 18–22 per cent for aspartate, glutamate and GABA and 8–12 per cent in the absence of stimulation.

The effect of raising medium potassium concentration on loss of amino acids from cortex slices held between nylon gauze rectangles in Quick Transfer Holders was much less than with synaptosomes. The main change was a 440 per cent increase in GABA concentration ($P < 0{\cdot}01$) in the medium (Table 7). With both potassium and electrical stimulation of cortex slices, the very low proportions of total aspartate, glutamate and GABA recovered in the medium (0·7–13 per cent; Table 7) were close to the equally low levels of these amino acids recovered in the medium from incubation of synaptosomes.

The specific radioactivities of aspartate, glutamate, GABA and alanine were determined in both tissue and medium after potassium stimulation. These measurements were made in an attempt to determine whether the released amino acids were

TABLE 9.—THE EFFECT OF OMMISSION OF CALCIUM FROM THE INCUBATION MEDIUM ON THE AMINO ACIDS ACCUMULATION IN THE MEDIUM

	Amino acid content of supernatant (nmols/100 mg protein)					
	Calcium-containing medium		Medium without calcium containing EGTA			without EGTA
	Control	Potassium-stimulation	Control	Potassium-stimulation	Electrical-stimulation	Potassium-stimulation
Aspartate	146	333	134	103	195	245
Glutamine	211	294	118	118	145	186
Serine	530	660	287	329	230	484
Glutamate	249	635	256	259	346	484
Glycine	310	359	255	281	210	288
Alanine	410	535	261	244	249	386
GABA	47	133	41	45	50	79
No. of samples	6	6	5	6	6	4

Synaptosome beds were incubated in Krebs–bicarbonate medium in the presence of 10 mM-glucose at 37°C for 40 min. Medium with calcium contained 0·75 mM-calcium. EGTA was present at 0·5 mM where indicated. After the first 30 min of incubation, potassium or electrical stimulation was started as indicated and continued for the remaining 10 min of incubation. Values given are mean values, standard errors were about 10 per cent of the mean. In the controls, an equivalent volume of medium was added to the incubation fluid after 30 min, and the high potassium was added to the potassium stimulation samples.

coming from the main pools within the synaptosomes, or from small compartments, consisting, for instance, of the synaptic vesicles. In fact, the data of Table 10 show the specific activity of each amino acid in the bed was similar to that recovered in the medium after potassium stimulation.

Effects of GABA analogues on amino acid release. Compared with suspensions, the amino acid release evoked by stimulation of beds is much attenuated, especially when

TABLE 10.—SPECIFIC ACTIVITIES OF AMINO ACIDS IN SYNAPTOSOME BEDS AND MEDIUM AFTER INCUBATION WITH [U-^{14}C]GLUCOSE

	Medium		Beds	
	Control	Potassium stimulated	Control	Potassium stimulated
Aspartate	48 ± 5 (9)	36 ± 3 (9)	29 ± 2 (9)	30 ± 1 (9)
Glutamate	45 ± 3 (8)	45 ± 3 (8)	49 ± 3 (9)	50 ± 2 (9)
Alanine	34 ± 1 (9)	34 ± 2 (9)	48 ± 3 (9)	47 ± 2 (9)
GABA	34 ± 5 (9)	28 ± 2 (9)	39 ± 3 (9)	42 ± 3 (9)

Synaptosome beds were incubated in Krebs–bicarbonate medium for 40 min at 37°C, in the presence of 10 mM-glucose and 16·5 μCi of [U-^{14}C]glucose. Potassium stimulation began at 30 min by elevation of K^+ to 56 mM and incubation was continued for a further 10 min. Initial specific radioactivity of the medium was 297 nCi/μmol. Values are means ± S.E.M. with number of experiments in brackets. Units are nCi/μmol.

compared with the metabolic responses to stimulation which were similar in the two preparations. It seemed possible therefore that re-uptake of released amino acids was occurring in beds. This was already indicated by the time course of accumulation of aspartate, glutamate and GABA. This showed a maximum after 10 min and subsequently declined. Investigation of the re-uptake mechanisms was made by including amino acid analogues in the medium with the purpose of interfering with the re-uptake mechanisms. BABA and DABA at 1 mM caused measurable increase in medium glutamate, aspartate, alanine, glycine, and GABA, but GABA accumulation was elevated to the greatest extent (300 per cent) with glutamate and aspartate showing the second largest change (Fig. 4a). During potassium stimulation in the presence of analogues, there was a further release of GABA. In the case of DABA, the extent of the release was similar to that occurring in the absence of the analogue, but with BABA present, the release of GABA was much greater than the normal response to stimulation.

TABLE 11.—THE EFFECT OF DABA ON AMINO ACID RELEASE WITH ELECTRICAL STIMULATION

	Control	Electrical stimulation	Electrical stimulation with DABA
Aspartate	85 ± 18	213 ± 65	131 ± 26
Glutamine	116 ± 6	140 ± 36	136 ± 15
Serine	353 ± 47	436 ± 60	408 ± 58
Glutamate	164 ± 30	451 ± 77	322 ± 72
Glycine	258 ± 27	303 ± 20	275 ± 48
Alanine	321 ± 18	453 ± 14	386 ± 37
GABA	44 ± 9	95 ± 10	132 ± 16

Synaptosome beds were incubated in Krebs-phosphate medium for 45 min at 37°C. Electrical stimulation and DABA addition, to a final concentration of 1 mM, was begun at 30 min and continued for a further 15 min. Values are means ± S.E.M. for three experiments.

With electrical stimulation, DABA produced a similar pattern of change in amino acid accumulation and GABA again showed by far the greatest change (Table 11).

Effect of N-*acetyl glutamate on amino acid accumulation.* Glutamate accumulation in the incubation medium of control samples was increased (250 per cent) by *N*-acetyl glutamate. The additional glutamate released during potassium stimulation in the presence of this agent was equivalent to that released in its absence (Fig. 4b).

Effect of ouabain on amino acid accumulation. Ouabain at 0·1 mM was included in the incubation medium with the purpose of interfering with the active transport of amino acids to the medium (GONDA and QUASTEL, 1962). This agent was effective in changing the pattern of accumulation in the medium of glutamate, aspartate, GABA and alanine and had less effect on the other amino acids. The levels in the medium of glutamate and aspartate were elevated to the greatest extent by this agent with GABA and alanine showing lesser effects (Fig. 4c). Potassium stimulation produced its established pattern of amino acid release in the presence of ouabain with aspartate and glutamate showing even larger changes than in the absence of ouabain.

Effect of tetrodotoxin. The amounts of glutamine, serine and glycine which accumulated in the medium after incubation of beds in the presence of 0·1 mM-tetrodotoxin were virtually unchanged (Table 12). However, with aspartate, glutamate GABA and alanine, there were decreases of 60, 50, 47 and 28 per cent respectively in the absence of stimulation. With potassium stimulation these decreases were smaller, ranging from 26 to 33 per cent. However the total amounts of aspartate, glutamate, GABA and alanine released from the synaptosomes by potassium stimulation was the same with or without tetrodotoxin present.

TABLE 12.—EFFECT OF TETRODOTOXIN AND CITRATE ON AMINO ACID RELEASE

	Amino acid content of supernatant after incubation (nmol/100 mg protein)					
	No addition		Tetrodotoxin		Citrate	
	Control	Potassium stimulation	Control	Potassium stimulation	Control	Potassium stimulation
Aspartate	161	277	65	194	136	153
Glutamine	176	291	144	253	155	208
Serine	484	681	448	592	566	552
Glutamate	306	582	153	422	280	363
Glycine	292	421	249	300	261	249
Alanine	332	496	240	365	348	414
GABA	46	102	25	62	48	78
No. of experiments	2	4	3	3	4	4

Synaptosomes were incubated in Krebs–bicarbonate medium for 40 min at 37°C. Tetrodotoxin (100 μM) or citrate (1 mM) were added prior to the incubation. Tetrodotoxin was in a citrate buffer which gave a final citrate concentration of 0·64 mM. Potassium stimulation began at 30 min and was continued for 10 min. The values are means for the number of experiments shown, S.E.M. were approx. 10 per cent of the mean. An equivalent volume of medium containing no potassium was added to the controls after 30 min incubation.

Since the tetrodotoxin employed was in citrate buffer it was necessary to examine the effects of adding citrate alone. These could be expected to reflect the high affinity of citrate for calcium. Incubation of synaptosome beds in bicarbonate medium containing 1 mM-citrate had virtually no effect on levels of amino acids present in the supernatant (Table 12). However, the additional release of amino acids due to potassium stimulation was much less in the presence of citrate.

Effects of lowering medium sodium. Since sodium is known (LAHIRI and LAJTHA, 1964) to be important in the transport processes of amino acids, the effect of lowering medium sodium concentration on amino acid accumulation in the medium was studied.

Lowering the sodium from 164 to 40 mM whilst maintaining isotonicity with sucrose caused an overall increase (100–134 per cent) in the accumulation of glutamine, serine, glycine and alanine in the medium (Table 13). The largest increase, a six-fold change, was seen in GABA accumulation. Aspartate and glutamate contents increased only slightly.

The release due to potassium stimulation remained unchanged except for GABA which showed very little or no extra release.

TABLE 13.—EFFECT OF LOWERING THE SODIUM CONCENTRATION OF THE INCUBATION MEDIUM ON AMINO ACID RELEASE

	Amino acid content of supernatants after incubation (nmol/100 mg protein)		
		Low sodium medium	
	Control	No addition	Potassium stimulated
Aspartate	112 ± 39	126 ± 14	265 ± 21
Glutamine	121 ± 9	269 ± 25	305 ± 11
Serine	350 ± 6	688 ± 74	760 ± 10
Glutamate	200 ± 8	298 ± 59	617 ± 73
Glycine	206 ± 7	459 ± 40	456 ± 11
Alanine	319 ± 50	734 ± 50	813 ± 119
GABA	36 ± 5	247 ± 42	288 ± 22

Synaptosome beds were incubated at 37°C for 40 min in Krebs–phosphate medium in which the sodium of the Krebs has been replaced by 0·248 M-sucrose. Controls were in normal medium. Potassium stimulation was started at 30 min and continued for 10 min. Values are mean ± S.E.M. for four experiments. An equivalent volume of medium containing no potassium was added to the controls after 30 min incubation.

DISCUSSION

The synaptosome bed preparation. The synaptosome bed preparation, obtained by depositing nerve endings onto nylon gauze, provides an alternative method for the study of the properties of nerve endings *in vitro*. The gauze apertures (120 μm) are very much larger than the diameter of synaptosomes (0·3–1·0 μm) indicating the extent to which synaptosomes are aggregated by the ions of the incubation medium, thus making bed formation possible. The preparation shares the active metabolic properties of synaptosome suspensions and possesses additional advantages. Synaptosome beds are able to respire, produce lactate, ATP and phosphocreatine and metabolise glucose to amino acids at rates similar to those shown by suspensions of synaptosomes, (BRADFORD, 1969; BRADFORD and THOMAS, 1969) showing that the presence of gauze does not reduce the access of oxygen and glucose from the medium to the synaptosomes. The bed sandwiched between two pieces of gauze can be easily manipulated and rapidly fixed whilst positioned in the jaws of a Quick Transfer Holder. Direct contact between the synaptosomes and the electrodes is prevented by the gauze and therefore the complications introduced by any local heating or electrode products are limited. There is however, likely to be a reduction in the current flow through the tissue as judged by the capacity of gauzes to reduce the glycolytic response of cortex slices (Table 6), though the response of slices remains substantial. The preparation also has a low fluid:tissue ratio which approximates more closely than suspensions to the *in vivo* environment of the nerve endings.

Response of synaptosome beds to electrical stimulation and elevated K^+ concentration. Electrical stimulation produced a significant increase in the rates of oxygen uptake, lactate production, and incorporation of radioactivity from glucose into aspartate and glutamate. Both electrical and potassium stimulation brought about a large in-

crease in the amounts of glutamate, GABA and aspartate released to the medium, though the other amino acids measured (glutamine, serine, glycine and alanine) showed little change above the resting values. It is interesting that the maximal amounts of glutamate, GABA and aspartate released by electrical and potassium stimulation of beds is small (12–26 per cent), compared to values (58–62 per cent) for electrical stimulation of synaptosome suspensions (BRADFORD 1970*a*). This reduction may be seen as a consequence of the low fluid:tissue ratio, within the preparation. Thus the amino acids lost from the synaptosomes would exist in a higher concentration in the smaller extracellular space of the bed and therefore accelerate concentration-dependent amino acid transport processes. The extent to which amino acids accumulate in the medium would therefore be represented by the difference between the loss and the greatly increased re-uptake. The high activity of these re-uptake processes was demonstrated by including amino acid analogues in the medium which interfered with specific processes; BABA, for instance resulted in greater accumulation of GABA in the medium during potassium stimulation. A sodium-dependent, rapid accumulation of GABA by synaptosomes has already been demonstrated by VARON, WEINSTEIN, KAKEFUDA and ROBERTS (1965), and this GABA uptake at 0–4°C is inhibited by the analogues BABA and DABA (SANO and ROBERTS, 1963). BABA and DABA also appeared to interfere with the uptake of other structurally-similar amino acids, whereas NAG affected only glutamate. In the presence of ouabain, greater amounts of glutamate and aspartate were released by potassium stimulation, which suggests that ouabain has also inhibited to a certain degree the amino acid transport mechanism which normally occurs.

The observation that the specific radioactivities of aspartate, glutamate and GABA released by potassium stimulation were close in value to those of the amino acids remaining in the tissue, suggests that each released amino acid was coming from its main pool within the synaptosome, and not principally from any smaller pools such as the vesicles.

Cortex slices positioned between gauzes showed a substantial glycolytic response to electrical stimulation. Both electrical and potassium stimulation caused a significant increase in the release of GABA only. A small, statistically insignificant, increase in glutamate release occurred with potassium stimulation. The different patterns of release shown by nerve endings and cortex slices presumably reflect the differences in the basic properties of the cellular elements predominating in the two preparations.

Changes in the ionic composition of the medium. Omission of calcium from the medium together with the inclusion of EGTA totally inhibits the differential release of glutamate, GABA and aspartate in response to potassium stimulation, without influencing the release observed in the absence of stimulation. Omission of calcium from the medium without addition of chelating agent reduces amino acid release but does not prevent it. Amino acid efflux due to electrical stimulation is greatly decreased under these circumstances. It seems, therefore, that in the absence of added calcium, membrane-bound, endogenous calcium is sufficient for the release to occur. Thus, calcium appears to be intimately involved in the release of all three physiologically-active amino acids from synaptosomes, just as it is involved in the potassium-stimulated release of GABA from rat whole cortex tissue *in vitro* (SRINIVASAN *et al.*, 1969), and from the cat visual cortex *in vivo* (IVERSEN, MITCHELL and SRINIVASAN, 1971). The need for calcium is well established in the release of acetylcholine from the neuromuscular

junction (KATZ and MILEDI, 1965*a*), sympathetic ganglion (HARVEY and MACINTOSH, 1940), or superfused cortex *in vivo* (HEMSWORTH and MITCHELL, 1969), and in release of catecholamines from a variety of organs (IVERSEN, 1967) and tissue slices (BALDESSARINI and KOPIN, 1967), and in other secretory processes such as oxytocin and vasopressin release from posterior pituitary *in vitro* (DOUGLAS and POSINER, 1964).

Synaptosomes actively concentrate calcium from the incubation medium (BLAUSTEIN and WIESMANN, 1970; DRACHMAN and FANBERG, 1969), in amounts which increase with the extent of depolarization caused by K^+ (BLAUSTEIN and WEISMAN, 1970). Where calcium is essential for release of amino acid, its restricted availability during depolarization could therefore be expected to lead to blockage of this release.

The dependance of amino acid transport systems on sodium is well known (LAHIRI and LAJTHA, 1964). In synaptosomes the sodium dependance of GABA uptake has been shown by VARON *et al.* (1965). It was therefore not surprising that lowering the medium sodium concentration from 164 to 40 mM caused a much larger amount of GABA than other amino acids to accumulate in the incubation medium; glutamate and aspartate showed the least effect. On the other hand, increasing medium sodium by 50–214 mM caused a fall specifically in aspartate and glutamate levels in the medium. This different sensitivity to sodium concentrations is evidence for the existence of different transport processes for the two groups of amino acids, possibly occurring in different populations of synaptosomes. Since decrease in sodium in these experiments was accompanied by decreased chloride (124–0 mM), the effects observed could be, at least partially, due to this.

This relative insensitivity of aspartate and glutamate uptake mechanisms to low sodium has also been shown in cortex slices (MARGOLIS and LAJTHA, 1968) and therefore appears to be a general property of neural systems.

Incubation in phosphate medium rather than bicarbonate medium led to reduced levels of all seven amino acids studied and is a further indication of the sensitivity of amino acid transport to different ions. The high binding affinity of citrate for calcium to form an unionized complex, suggests that citrate is likely to act through lowering calcium levels. The much reduced amino acid release seen in the presence of citrate could therefore be due to this effect, particularly as more than sufficient citrate was present to bind the calcium present.

The experiments in which tetrodotoxin was included in the incubation medium were complicated by the presence of citrate buffer used as carrier. This was present at a final concentration of 0·64 mM with tetrodotoxin at 0·1 mM. At this relatively high concentration, the effect of tetrodotoxin in reducing the levels specifically of aspartate, glutamate and GABA in the incubation medium could result equally from increased uptake or diminished efflux. The observation that tetrodotoxin did not change the extra glutamate, aspartate or GABA released by potassium stimulation may be compared with the ineffectiveness of tetrodotoxin in reducing acetylcholine release during depolarization of the neuro-muscular junction (KATZ and MILEDI, 1965*b*).

Synaptosome beds therefore provide a valuable preparation for the *in vitro* study of the biochemical properties of nerve endings, and their response to depolarizing agents. Thus, since both electrical and potassium stimulation lead to qualitatively similar patterns of amino acid release and metabolic response (BRADFORD and THOMAS, 1972), and since low concentration of calcium limit the extent of release in both cases, it seems likely that depolarization is the main influence responsible for the observed effects.

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Synaptosome Beds: a Method for the Study *in vitro* of the Metabolism and Function of Nerve Endings

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The functional unit of chemical transmission in the nervous system is the nerve ending. When cortex tissue is homogenized in iso-osmotic sucrose, the nerve endings seal off from the axon and may be prepared by sucrose-density-gradient centrifugation (Gray & Whittaker, 1962). Suspensions of nerve endings or synaptosomes exhibit the metabolic properties of cellular material, being able to carry out active glycolysis and oxidation of added glucose, linked to ATP and phosphocreatine production (Bradford, 1969).

When applied iontophoretically to the mammalian cerebral cortex, small concentrations of glutamate and aspartate cause excitation and γ-aminobutyrate causes inhibition of neuronal membranes (Krnjević & Phillis, 1963). Treatment of suspensions of synaptosomes with electrical pulses or elevated K^+ concentrations leads to a specific release of these three physiologically active amino acids (Bradford, 1970).

To decrease the large fluid/tissue ratio of the suspension to something more closely resembling that *in situ*, synaptosomes were sedimented on to nylon gauze, and a sandwich was formed by placing another piece of gauze on top. The resulting bed of synaptosomes could be easily manipulated and rapidly fixed at the end of incubation. This preparation showed similar metabolic properties to synaptosome suspensions, glycolysing and oxidizing glucose with parallel formation of ATP and phosphocreatine. Application of electrical pulses stimulated O_2 uptake and glycolysis by 40% and oxidation of glucose to amino acids. Both electrical stimulation and elevated K^+ concentrations in the medium led to a significant increase in the release of glutamate, γ-aminobutyrate and aspartate into the medium, whereas the concentrations of other amino acids were not greatly affected.

On the inclusion of amino acid analogues of glutamate or γ-aminobutyrate in the incubation medium, increased concentrations of these amino acids were found in the medium, which indicated that uptake of these amino acids had occurred under normal conditions when the analogues were not present.

In the absence of Ca^{2+}, the release of glutamate, γ-aminobutyrate and aspartate in response to depolarizing influences was greatly decreased. When ethanedioxybis(ethylamine)tetra-acetate was included in the incubation medium containing no Ca^{2+}, the K^+-stimulated release was totally inhibited. The presence of Na^+ at a concentration greater than 40mM was found to be essential for maintaining the normal concentration of γ-aminobutyrate found in the synaptosome bed.

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