STUDIES ON THE EFFECTS OF DRUGS ON THE PROPERTIES OF SYNAPTOSOMES

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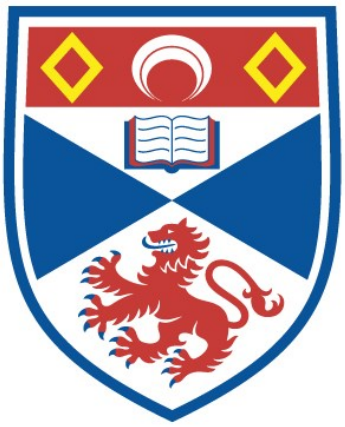
A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1971

STUDIES ON THE EFFECTS OF DRUGS ON THE PROPERTIES OF SYNAPTOSOMES

by

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A thesis

submitted to the University of St. Andrews

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of Philosophy

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D E C L A R A T I O N

I hereby declare that the following thesis is based on work carried out by me, that the thesis is my own composition and that no part of it has been presented previously for a higher degree.

The research was performed in the Department of Biochemistry of the University of St. Andrews, under the direction of Dr. J.C. Gilbert.

C E R T I F I C A T E

I hereby certify that David Balfour has spent nine terms in research work under my direction, and that he has fulfilled the conditions of Ordinance No. 16 (St. Andrews) and that he is qualified to submit this thesis for the degree of Doctor of Philosophy

A C K N O W L E D G E M E N T S

Grateful thanks are extended to Dr. J.C. Gilbert and Dr. Stephen Bayne for their interest and criticism during this work. Thanks are also due to Mr. R. Stuart and Mr. M.I.S. Hunter for assistance with the electron microscopy, Mr. A. Grieve for amino acid analysis and Mr. W.J. Blyth for the photography.

ACADEMIC RECORD

I matriculated at the University of St. Andrews in October 1964, and graduated with the degree of Bachelor of Science, First Class Honours, in Biochemistry in June 1968.

In October 1968, I was awarded a Science Research Council Studentship to undertake research for the degree of Doctor of Philosophy at the University of St. Andrews.

C O N T E N T S

Page

INTRODUCTION

Historical Review

The existance of the nervous system was known in the early 1800s and by the beginning of the twentieth century the main components of the neuron, as we know it today, had been observed and reported. It had been shown that the neuron consisted of a cell body which emitted processes along which, apparently, signals to or from other cells were carried. The characteristic boutons, which occur at the ends of the processes, were first observed at about this time and were called 'synapses' by Sherrington in 1897 (see review by McLennan 1965). Although some workers at this time still believed that there was a cytoplasmic continuity along the whole length of a nervous fibre, the theory of Waldeyer and Cajal, who proposed that the nervous system was made up of morphologically separate cells, was gaining acceptance. The synapses were points of close proximity between neurons and other cells and it was proposed that, at these points, interactions between cells could occur. Recent work on the morphology of the nervous system, especially that using the electron microscope, has proven, beyond doubt, the Waldeyer-Cajal theory. The early work on the study of the nervous system has been reviewed by Shantha, Manocha, Bourne and Kappers (1969), De Robertis

(1964) and McLennan (1965).

Elliot (1904) is believed to be the first person to suggest that impulses might be transmitted from one cell to the next by means of a chemical transmitter, but it was the early work of Loewi and of Dale who first demonstrated the role of a compound, acetylcholine, in the transmission of impulses (Dale 1935, see also De Robertis 1964 and McLennan 1965).

It had been proposed that the neuronal membranes were polarised in 1902 (Bernstein 1902), but it was not until 1939 that Hodgkin and Huxley were able to show, experimentally, the presence of a resting membrane potential, using the squid axon preparation. In 1952 papers by Huxley, Hodgkin and Katz and by Huxley and Hodgkin reported results which demonstrated the order of events as an impulse travelled down a squid axon and also during the subsequent re-polarisation process. They showed that the observed depolarisation was due to a large change in the permeability of the membrane to sodium, followed by a change in the potassium gradient.

The development of microelectrodes capable of measuring synaptic potentials, by Ling and Gerard (1949), and the subsequent development of microiontophoretic techniques,

permitted experiments to be performed which gave a clearer understanding of the mode of transmission at the chemical synapse. Using these techniques, workers, notably Curtis and Eccles, were able to show that certain compounds could hyper- or depolarise synaptic membranes. Acetylcholine was the first compound to be studied in detail, principally using the motor endplate system. While studying this system, Fatt and Katz observed spontaneous small depolarisation of the post-synaptic membrane, even though no impulses were arriving at the junction. They attributed these miniature endplate potentials (mepps) to the spontaneous release of acetylcholine from the pre-synaptic membrane (1952). In further reports, Del Castillo and Katz (1955, 1956) proposed that mepps were caused by the release of packets, or quanta, of acetylcholine and that these quanta were stored in the pre-synaptic region of the neuromuscular junction. Upon the arrival of an impulse it was assumed that a great number of quanta were released, the sum of which caused depolarisation of the motor endplate.

During the 1950s, the improvement in electron microscope techniques enabled workers to use this instrument to study the ultrastructure of the synapse in a large number of preparations. The same basic structure

was found to be a common feature of most types of synapse. The pre-synaptic region contained small mitochondria and vesicles, first observed by De Robertis and Bennett (1955), which are thought to contain the transmitter compounds. De Robertis and Bennett (1955) further suggested that each vesicle contained one quantum of transmitter. The pre-synaptic membrane is separated from the post-synaptic membrane by a small cleft, about 200 - 500Å in width. It is thought that, upon the arrival of an impulse at the synapse, the vesicles fuse with the pre-synaptic membrane in some way such that their contents are released into the synaptic cleft. The transmitters then modify the neighbouring cell membrane by crossing this cleft and binding to receptor sites on the post-synaptic membrane. (Katz (1962) has summarised the events occurring at the synapse upon the arrival of an impulse, using the motor endplate system as a model.) De Robertis (1958) has claimed to have observed vesicles fused with the pre-synaptic membrane such that the interior of the vesicle opened into the cleft, but this observation has not been made by other workers using similar preparations.

Because it is easier to study synapses from the periphoral nervous system, or from invertebrates, under the electron microscope, it was not until 1959 that Gray first published photographs which showed that synapses

from the vertebrate CNS were, essentially, similar to those from other sources. In addition to the general features of the synaptic region, described above, Gray also observed electron dense material within the synaptic cleft which is believed to be intersynaptic filaments which join the pre- and post-synaptic membranes.

The History of the Nerve-Ending Particle

The study of chemical synapses in the CNS has advanced rapidly over the last ten years due to the discovery that nerve-endings may be isolated, as artificial subcellular particles, from homogenates of nervous tissue.

During the 1950s, it had been shown that acetylcholine, acetylcholinesterase and choline acetyltransferase were concentrated in the mitochondrial fraction from brain homogenates (Hebb and Smallman 1956, Aldridge and Johnson 1959). In 1958, Hebb and Whittaker isolated an acetylcholine containing particle by density gradient fractionation of homogenates of cerebral cortex. It was thought, at first, that these particles corresponded to the vesicles, first observed by De Robertis and Bennett (1955), but subsequent work by Whittaker (1959a) and by Gray and Whittaker (1962) showed that, under the electron microscope, these particles closely resembled photographs of intact cerebral cortex synapses (Gray 1959). The particles contained vesicles and small mitochondria and often had small portions of post-synaptic membrane still attached to them. These particles were apparently formed during the mild homogenisation procedure, when the pre-synaptic bouton broke away from the axon and rapidly resealed. Similar

particles have since been isolated from a variety of sources (see Whittaker 1969). Their size varies, depending on the source, but most are about 0.5μm in diameter, although synaptosomes, as these particles have been termed (Whittaker, Michaelson and Kirkland 1964), as large as 10μm in diameter have been isolated from the mossy fibre region of the cerebellar cortex in guinea-pig (Israel and Whittaker 1965).

Further work showed that, when the crude mitochondrial fraction was prepared from homogenates of nervous tissue and subfractionated as described above, a number of the compounds, which some workers consider are transmitters, were concentrated in the synaptosome fraction; acetylcholine, serotonin and nor-adrenalin are particular examples (Whittaker 1959b, 1965 and De Robertis 1964). Whittaker and his colleagues have studied the nerve-ending particle in relation to cholinergic transmission. Acetylcholine and choline acetyltransferase activity is high in the vesicle fraction, which may be isolated from the synaptosomes by rupturing the synaptosome membrane osmotically in water and fractionating the resulting suspension on a sucrose gradient (De Robertis et al 1963, 1967 and Whittaker et al 1964). This work has been reviewed extensively (De Robertis 1964, Whittaker 1969). Whittaker and Sheridan (1965)

attempted to measure the amount of acetylcholine in a single vesicle but, although they found that the quantity was within the limits found by physiological measurements, (reviewed by Katz, 1962) they had to make certain assumptions which qualified the usefulness of their results.

The distribution of other putative transmitter compounds and enzymes associated with their metabolism have also been studied. De Robertis and his coworkers have made attempts to isolate synaptosomes containing different transmitters, using a discontinuous density gradient consisting of more steps than the simple one of Gray and Whittaker (1962). They found that the distribution of nor-adrenalin and serotonin was similar to that of acetylcholine and that they could isolate a fraction, somewhat more dense than that containing nerve-endings rich in acetylcholine, which contained synaptosomes which had high GABA aminotransferase and glutamic acid decarboxylase activity (Salganicoff and De Robertis 1963). They proposed that these synaptosomes had been formed from nerve-endings which had contained GABA, but Whittaker (1965) cast doubt on this conclusion on the grounds that all the differences could be explained by the increased number of mitochondria present in the synaptosomes of this fraction. De Robertis summarised the results which

his group have obtained, concerning the heterogeneity of nerve-endings, in 1964. Subsequently Iversen and Snyder (1968) and Kuhar, Shaskan and Snyder (1971) have shown that if synaptosomes are prepared from cerebral cortex slices which have been preincubated in the presence of catecholamines and GABA, the distribution of the compounds along the density gradient is not uniform. They proposed this as a better way of demonstrating the heterogeneity of the synaptosome preparation.

Synaptosomes contain lactic dehydrogenase, the glycolytic enzymes (Johnson and Whittaker 1963), potassium and free amino acids (Ryall 1964, Mangan and Whittaker 1966). These compounds, which are normally found in the axonal cytoplasm, are released if the synaptosomal membrane is ruptured osmotically, which indicates that synaptosomes have axonal cytoplasm trapped within their membranes and that the membranes are complete and relatively impermeable. Work by Marchbanks (reported by Whittaker 1969) and by Bradford (1969) has shown that synaptosomes are capable of quite high rates of oxygen consumption. The rates are, in fact, comparable to those of cerebral cortex slices when they are suspended in oxygenated Krebs Ringer solution. Further work by Bradford and Thomas (1969) has shown that

the fate of labelled carbon atoms, derived from (U-^{14}C)-glucose, in synaptosomes is similar, but not identical, to the fate of (U-^{14}C)-glucose in cerebral cortex slices. The ability of synaptosomes to respire and metabolise added substrate has led Whittaker to liken the particle to a small anucleate cell (1969). The synaptosomal membrane has an active ATPase (Hosie 1965, Kurokawa, Sakamoto and Kato 1965, Bradford, Brownlow and Gammack 1966) and is capable of forming Na^+ and K^+ gradients across the membrane (Bradford 1969, 1970a). Bradford (1970a,b) has further reported that the synaptosomal membrane may be excitable - that is to say the membrane may be depolarised by an external stimulus such as electrical pulses - and thus the particle could be used as a model system for studying excitable membranes.

The permeability of the synaptosomal membrane has also been widely studied, with special reference to ions and compounds of pharmacological interest, for example: Na^+, Ca^{++} and K^+ (Marchbanks 1967, Blaustein and Weisman 1970, Weinstein and Kuriyama 1970); choline (Marchbanks 1968b, Potter 1968); acetylcholine (Marchbanks 1968a) and the catecholamines (Coyle and Snyder 1969, Colburn et al 1968).

Thus the preparation offers us a means of studying the effects of drugs on synaptic metabolism and on some of the factors which might affect the release of the putative transmitter compounds, such as the fragility or the permeability of the pre-synaptic membrane.

An Out-line of the Proposed Project

The object of the work, to be reported in this thesis, is to use the synaptosome preparation as a means of studying the effects of anticonvulsant drugs on the nerve-ending.

Although drugs with anticonvulsant properties have a wide range of structure and possible modes of action, all must, in some way, prevent the initiation or spread of the neuronal discharges which result in convulsions. Most anticonvulsants seem to alter the permeability of neuronal membranes to ions or maintain the ionic gradients in some other way such that the threshold for depolarisation of the membranes is raised. The variety of metabolic effects, which result from treatment with these drugs, may directly affect the ionic gradients, or may be the result of direct physico-chemical effects of the drugs on the properties, especially the permeability and carrier properties, of the membranes (Tower 1960, Millichap 1965).

Two anticonvulsant drugs, used to treat Grand Mal epilepsy, are tested in this study. The first is acetazolamide, a sulphonamide, which Koch and Woodbury (1958) have shown to inhibit the enzyme carbonic anhydrase in the CNS. The

effect of this is to increase the CO_2 content of the nervous tissue and this is believed to reduce the intracellular Na^+ concentration which results in hyperpolarisation of the neuronal membrane. Increased intracellular CO_2 has also been reported to promote the influx of K^+ (Shanes 1958), thus increasing the hyperpolarisation of the membranes, and this increased intracellular K^+ concentration is also a feature of the action of acetazolamide (Millichap 1965).

The second anticonvulsant tested is the barbiturate phenobarbitone. Possible mechanisms for its anticonvulsant activity are much less well defined. It is difficult to separate the effects which give the drug its anticonvulsant properties from those which result in sedation. Certainly it does not exert its effect by inhibiting carbonic anhydrase (Millichap 1965), but it is believed to 'stabilise' neuronal membranes in some other way. Tower (1960) suggests that the stabilisation could result from direct physico-chemical interaction between phenobarbitone and the neuronal cell membranes, but the nature of this stabilisation is still not clear. Hillman, Campbell and McIlwain (1963) have shown 'in vitro' that the rate of reformation of the Na^+ gradient, across the cell membranes ,

is enhanced by phenobarbitone, which is an interesting observation in view of the fact that phenobarbitone is of particular use in treating epilepsy which results from defective repolarisation mechanisms (Millichap 1965). The clinical uses and biochemical effects of these drugs have been the subject of reviews by Millichap (1965) and Woodbury and Kemp (1970).

In this study, the effects of the anticonvulsants are also compared with the effects of two drugs, pentamethylene tetrazol and strychnine, which may be used to initiate convulsions in experimental animals (Millichap 1965). The mode of action of these compounds is not fully understood, although it seems that strychnine has its effect by blocking the release or the receptors for inhibitory transmitters (Roper, Diamond and Yasargil 1969) in the CNS, whereas pentamethylene tetrazol acts on cholinergic synapses. In addition pentamethylene tetrazol inhibits acetyl-cholinesterase activity (Mahon and Brink 1970). Holz and Westerman (1965) summarise evidence which shows that monoamine oxidase inhibitors protect animals from pentamethylene tetrazol induced seizures but not from those induced by strychnine. This is further evidence to suggest that the two drugs do not exert their effect by

the same mechanism.

Much of the work reported for the effects of anti-convulsants 'in vitro' has been done using cerebral cortex slices whose properties closely resemble those of nervous tissue 'in vivo' (McIlwain 1966). The work of Hillman et al (1963) which demonstrated that phenobarbitone enhanced the rate of reformation of the Na^+ gradient in slices, after depolarisation, has already been mentioned. Some anticonvulsants, however, can also affect the permeability of cerebral cortex slices to non-ionic compounds. The rate of uptake of sugars into brain slices is enhanced by some anticonvulsants, apparently due to modification of the carrier process by which the sugars are transported into the cells (Gilbert, Ortiz and Millichap 1966, Gray and Gilbert 1970).

The cerebral cortex slice system is a good one for studying the effects of drugs on neuronal excitability as a whole, but slices are multicellular systems and some of the effects of the drugs on neurons may be masked by the presence of other cells. Also the components of the neuron - the cell body, axons and dendrites - have different functions and there is no reason to suppose

that the drugs have the same effect on the different parts of the cell. It is useful, therefore, to use the synaptosome preparation as a means of studying the effects of the drugs on the nerve-ending. One must bear in mind, however, that the synaptosome preparation is, itself, heterogeneous and contains nerve-endings from the whole of the cerebral cortex.

With the points which have been out-lined above in mind, it was proposed to study the effects of anti-convulsant drugs on synaptosomes in two ways. In the first instance, the metabolic responses of the particles have been assessed by measuring the effects of the drugs on the oxygen consumption and on the quantity of radio-activity incorporated into the synaptosomes when they were allowed to respire in oxygenated Krebs Ringer solution containing (U-^{14}C)-glucose. There is evidence to suggest that there is more than one TCA cycle operating in the CNS (Clarke, Nicklas and Berle 1970). One of these cycles, which accounts for most of the oxygen uptake, is believed to be an 'energy cycle' geared mainly to providing ATP, while the other cycle, which is smaller, is geared mainly to synthesis of free amino acids. By estimating the effects of the drugs on both the oxygen

uptake and the incorporation of the label, it was hoped to detect a response in either of the cycles.

In the second part of the study, the effects of the drugs on the properties of the synaptosomal membrane were studied, and the problem was approached from three aspects. In the first instance, the effects of the drugs on the mechanical properties of the membranes were assessed by allowing the synaptosomes to swell in hypotonic solution, after pre-treatment with the drugs. Secondly the effects on the permeability of the synaptosome membrane to K^+, Na^+ and xylose have been estimated. The excitable nature of neuronal membranes is dependent upon the ability of the cells to form and maintain gradients of these ions across the cell membranes and, since Tower (1960) has suggested that the cells of epileptogenic tissue are often unable to maintain the ionic gradients, any alteration in the permeability of the synaptosomal membrane to these ions is of importance. The pentose, xylose, appears to be taken up by the cells of the CNS by the same process as glucose (Gilbert 1965) and this uptake is modified by some anti-convulsants (Gilbert, Ortiz and Millichap 1966, Gray and Gilbert 1970). In view of this, the effect of anticonvulsants on the permeability of the synaptosome membrane to this

compound was also examined. Finally the effects of the drugs on the K^+, Na^+, Mg^{++} activated ATPase activity of the synaptosomes were studied. The ATPases are thought to be responsible for providing the energy for the Na^+ and K^+ pumps which are present in the neuronal membranes. If the drugs were to modify the activity of these enzymes, it could be a means by which they altered the membrane permeability to the ions.

E X P E R I M E N T A L

SECTION I

ISOLATION AND CHARACTERISATION OF THE SYNAPTOSOME FRACTION

Introduction

The method of preparation of synaptosomes chosen for this work was, essentially, that of Gray and Whittaker (1962) since it is a relatively simple procedure. In addition, if the method is modified a little, it can be completed within six hours (Bradford 1969). Although other methods of preparation yield synaptosome fractions which may be less badly contaminated with mitochondria and myelin and which contain synaptosomes which have a better appearance under the electron microscope (Whittaker 1965), these procedures are long and tedious or yield too few synaptosomes for the type of experiments planned for this work. Because the preparation of Gray and Whittaker does provide the easiest method of isolating synaptosomes, many other workers have adopted it and, since we wished to characterise our preparation by comparing its properties with those attributed to synaptosomes by others, it was better that we should use the same preparation as far as was possible.

In this section, not only has the preparation been described, but also the experiments which were performed to characterise the fraction. In the first instance, the fractions, isolated from the sucrose gradient, were studied

under the electron microscope since this provides direct evidence of the content of each fraction. But synaptosomes do not withstand the procedures required to prepare specimens for the electron microscope as well as mitochondria, and therefore photographs of the fractions taken under the electron microscope tend to underestimate the number of whole synaptosomes present in the fractions (Whittaker 1965). Further characterisation of the synaptosome fraction was carried out by comparing its enzymic and respiratory properties with results published by other workers.

The enzyme acetylcholinesterase has quite a wide distribution in the CNS, but of that found in the crude mitochondrial fraction, most is bound to the synaptosomal membrane and not to membranes of free mitochondria (Whittaker 1969). The distribution of acetylcholinesterase activity was therefore studied. The distribution of succinic dehydrogenase activity has also been studied because this enzyme is localised on the mitochondrial membrane (Aldridge and Johnson 1959) and the distribution of the activity of this enzyme provides an estimate of the distribution of the mitochondria.

Finally the oxygen consumption of the fraction was

estimated since this seemed to offer the best method of assessing the number of functionally viable synaptosomes present in the fraction. The oxygen uptake was measured in oxygenated Krebs Ringer solution, fortified with glucose as substrate. Under these conditions synaptosomes with intact membranes which have trapped the cytoplasmic enzyme systems and cofactors necessary to oxidise glucose, respire actively and in a stable and linear manner (Bradford 1969). Damaged synaptosomes and free mitochondria, however, are not able to respire to any great extent, or with any stability, under these conditions.

Materials and Methods

Materials

Rabbit Brains: Male rabbit brains were supplied, in the frozen state, by Honee-Bun Farm Products Ltd., Bideford, Devon. (The brains were rapidly frozen to minimise deterioration.)

Guinea-Pigs: Six-week old male albino guinea-pigs (Dunkin-Hartley strain) were supplied by A. Tuck and Son, Rayleigh. Essex.

Acetylcholine Perchlorate: Supplied by British Drug Houses, Poole. Dorset.

Bovine Serum Albumin: Supplied by British Drug Houses, Poole. Dorset.

Methods

Isolation of the Synaptosomes

Synaptosomes were isolated from the cerebral cortex of frozen rabbit brain or fresh guinea-pig brain. The

cortex of the frozen rabbit brain was isolated from the rest of the brain by allowing the brain to thaw slowly at room temperature until the surface of the brain became pliable. In this condition the grey matter could easily be distinguished from the white and the grey matter was scraped away from the brain with a micro-spatula. When guinea-pigs were used they were sacrificed by decapitation and the brains were rapidly removed and put on ice. The cortex was cut away from the rest of the brain, and any adhering white matter was scraped away. The grey matter obtained from rabbits or from guinea-pigs was then treated as described below, all operations being performed at 0 - 5°C.

The grey matter was homogenised in ice-cold 0.32M sucrose (1g of grey matter in 10ml of sucrose solution) in a teflon homogeniser running at 800rpm, for 35 seconds. During the homogenisation, seven up and down strokes of the pestle were made. The homogenate was then subjected to the subcellular fractionation procedure, which is summarised Figure 1 which is essentially the method of Gray and Whittaker (1962), but modified as recommended by Bradford (1969) so that the preparation might be completed within 6 hours.

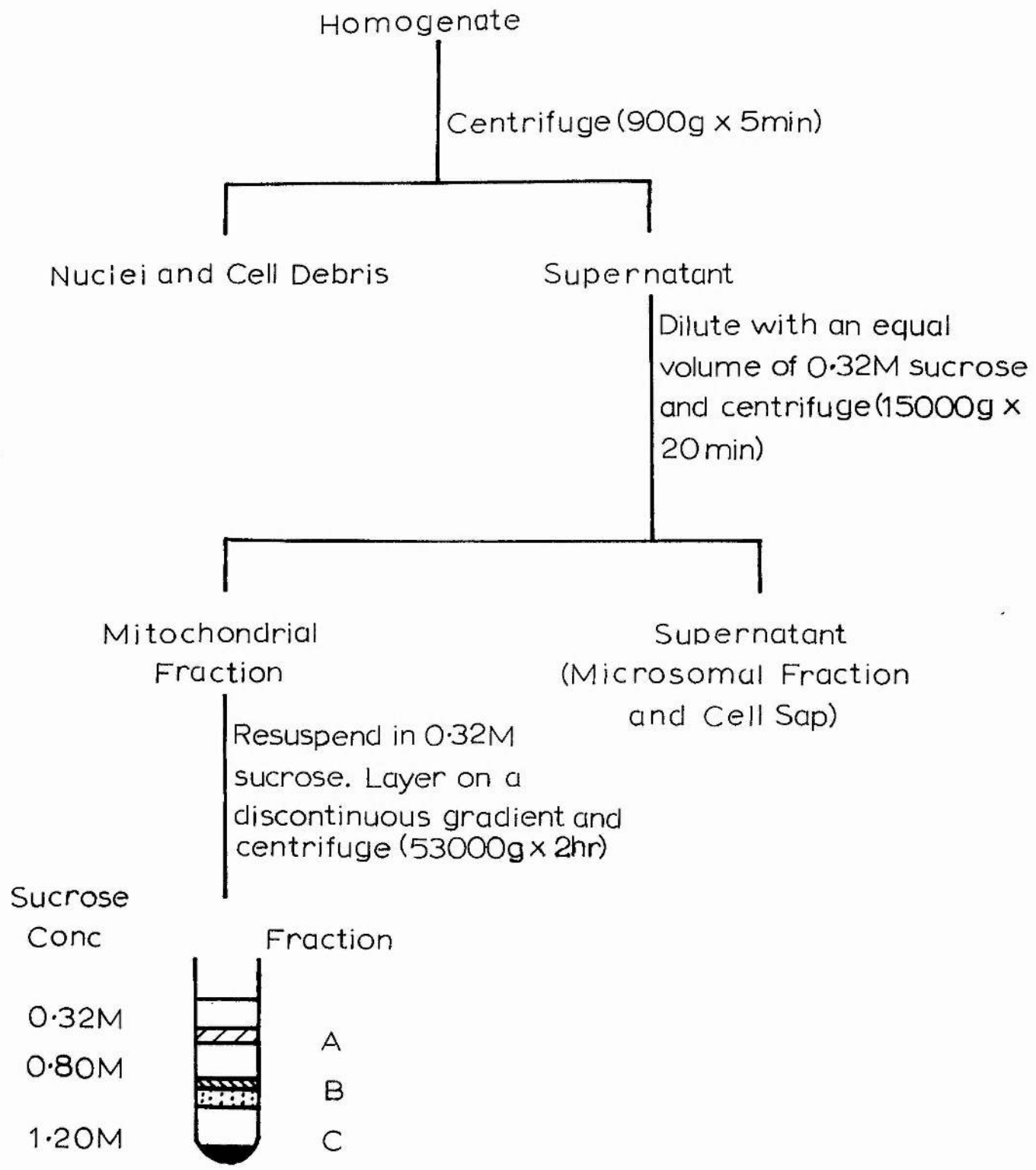
Figure I

Preparative Procedure

A detailed description of the preparative procedure, out-lined in this Figure, is given on p27.

Fig 1

Preparative Procedure



The homogenate was centrifuged at 900g for 15 minutes to remove the nuclei and other large cell debris. It is necessary to remove the nuclei before fractionating the mitochondrial fraction because many synaptosomes are trapped with the nuclei, on the gradient, if the nuclei are not removed. The resulting supernatant was then centrifuged at 15,000g for 20 minutes to sediment the crude mitochondrial fraction. The crude mitochondrial fraction was resuspended in 0.32M sucrose and layered on top of a discontinuous gradient consisting of 5ml bands of 1.2M and 0.8M sucrose. The gradient was centrifuged for 2 hours at 53,000g (23,000rpm in a 3x20ml head on an MSE super 50 or super 65 preparative ultracentrifuge) and the fractions lying between the sucrose bands were collected with a Pasteur pipette and the molarity of the sucrose was returned to 0.32M by the slow addition of 0.16M sucrose. The fractions were centrifuged down at 20,000g for 30 minutes and resuspended in a medium suitable for the proposed experiment.

The preparation was always used, for various experiments, on the day of its isolation.

Characterisation of the Synaptosome Fraction

Electron Microscopy

Fractions were prepared for electron microscopy in two ways.

(i) Negative Staining

Fractions isolated from both frozen rabbit brain and fresh guinea-pig brain were negatively stained using the method of Whittaker, Michaelson and Kirkland (1964). The fractions were resuspended in 0.32M sucrose and were fixed by the addition of an equal volume of ice-cold 10 per cent formaldehyde solution, dissolved in 0.32M sucrose. The pH of the formaldehyde solution had been brought to 7.4, by the slow addition of 0.33N NaOH, before it was added to the suspensions. A drop of the fixed suspension was placed on a collodion-coated grid and the excess moisture removed with filter paper. A drop of 1 per cent phosphotungstic acid, also dissolved in 0.32M sucrose at pH 7.4, was added to the grid and the excess moisture was again removed with filter paper. The grids were dried in a desiccator and then studied under the electron microscope.

(ii) Positive Staining

Only fractions prepared from fresh guinea-pig brain were subjected to this procedure.

The fractions were resuspended in 0.32M sucrose and centrifuged at 70,000g for 1 hour. The plugs were fixed in 2 per cent glutaraldehyde, which was dissolved in 0.1M phosphate buffer (pH 7.4) containing 7.5g of sucrose per 100ml of solution, and in osmium tetroxide. They were then dehydrated in alcohol, embedded in araldite, sectioned and stained with lead citrate and uranyl acetate.

Enzymic Activity

Cholinesterase Activity

In this work, total cholinesterase activity has been estimated, but 95 per cent of the cholinesterase activity in the crude mitochondrial fraction can be attributed to acetylcholinesterase (Aldridge and Johnson 1959).

Cholinesterase activity was assayed manometrically using the method of Aldridge and Johnson (1959). The assay is based on the fact that the enzyme releases acetic acid which displaces CO_2 from the reaction medium, which is saturated with CO_2 prior to the commencement of the assay.

$$(CH_3\overset{O}{\overset{\|}{C}}O(CH_2)_2N(CH_3)_3)ClO_4 \underset{ChE}{\rightleftharpoons} CH_3CO_2H + (HO(CH_2)_2N(CH_3)_3)ClO_4$$

$$CH_3CO_2H + NaHCO_3 \rightleftharpoons CH_3CO_2Na + H_2CO_3$$

$$H_2CO_3 \rightleftharpoons H_2O + CO_2$$

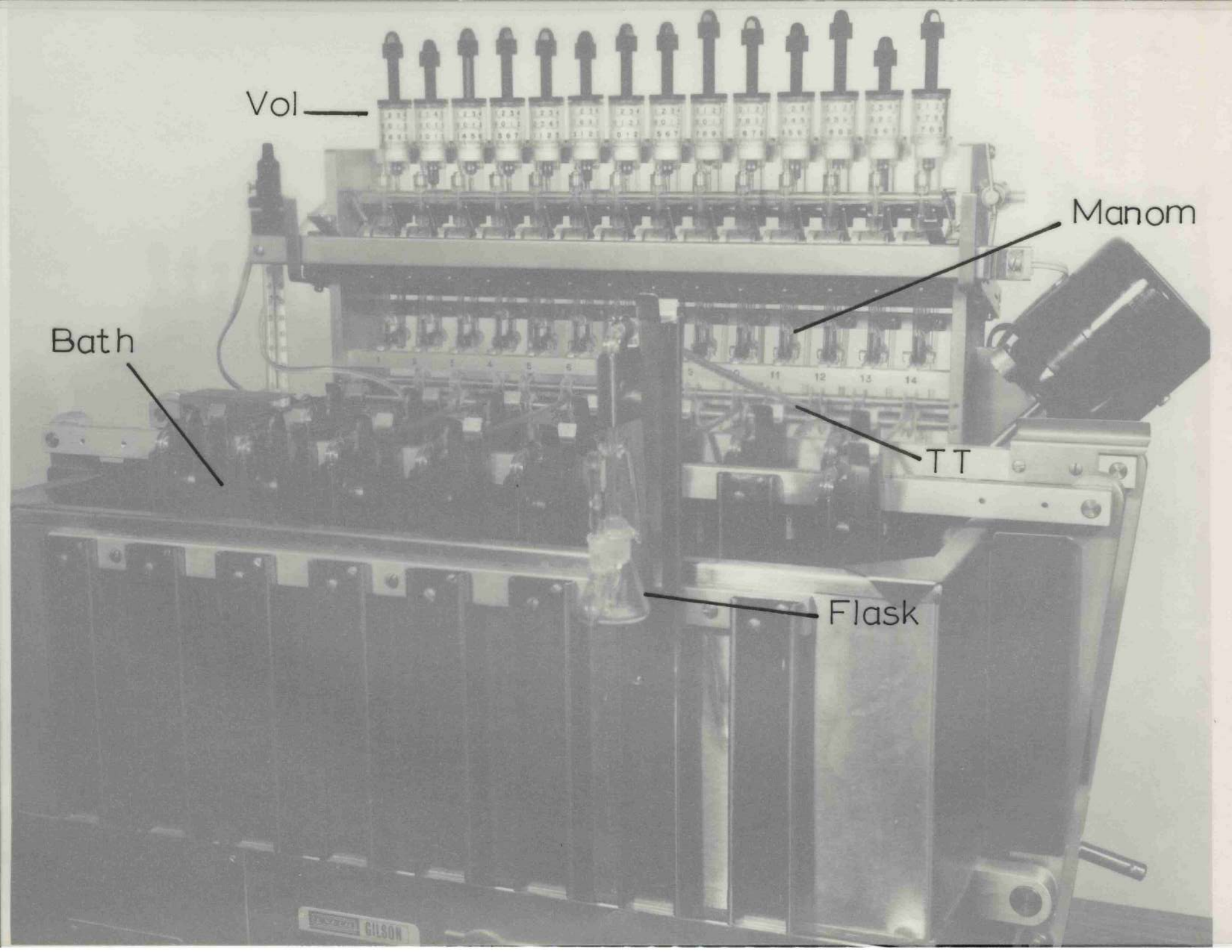
A 0.5ml sample of synaptosomes or mitochondria, suspended in 0.32M sucrose and containing approximately 2mg protein per ml, was pipetted into the side-arm of a manometer flask. 3ml of medium, which contained the following constituents in their final concentrations:- NaCl 130mM, $MgCl_2$ 35mM, $NaHCO_3$ 31mM, acetylcholine perchlorate 14mM, was placed in the main compartment of the manometer flask, which was then connected to a Gilson Differential Respirometer. (This instrument determines gas uptake or output, at constant pressure, and it has several advantages over the conventional Warburg manometer. It is shown in Plate I. Gas exchanges occurring in the manometer flasks attached to the Gilson Respirometer are relayed to the manometers of the instrument by flexible tygon tubes. Changes in the levels of liquid can then be nullified by turning thumb-screws which are graduated in such a way that the changes in gas volumes

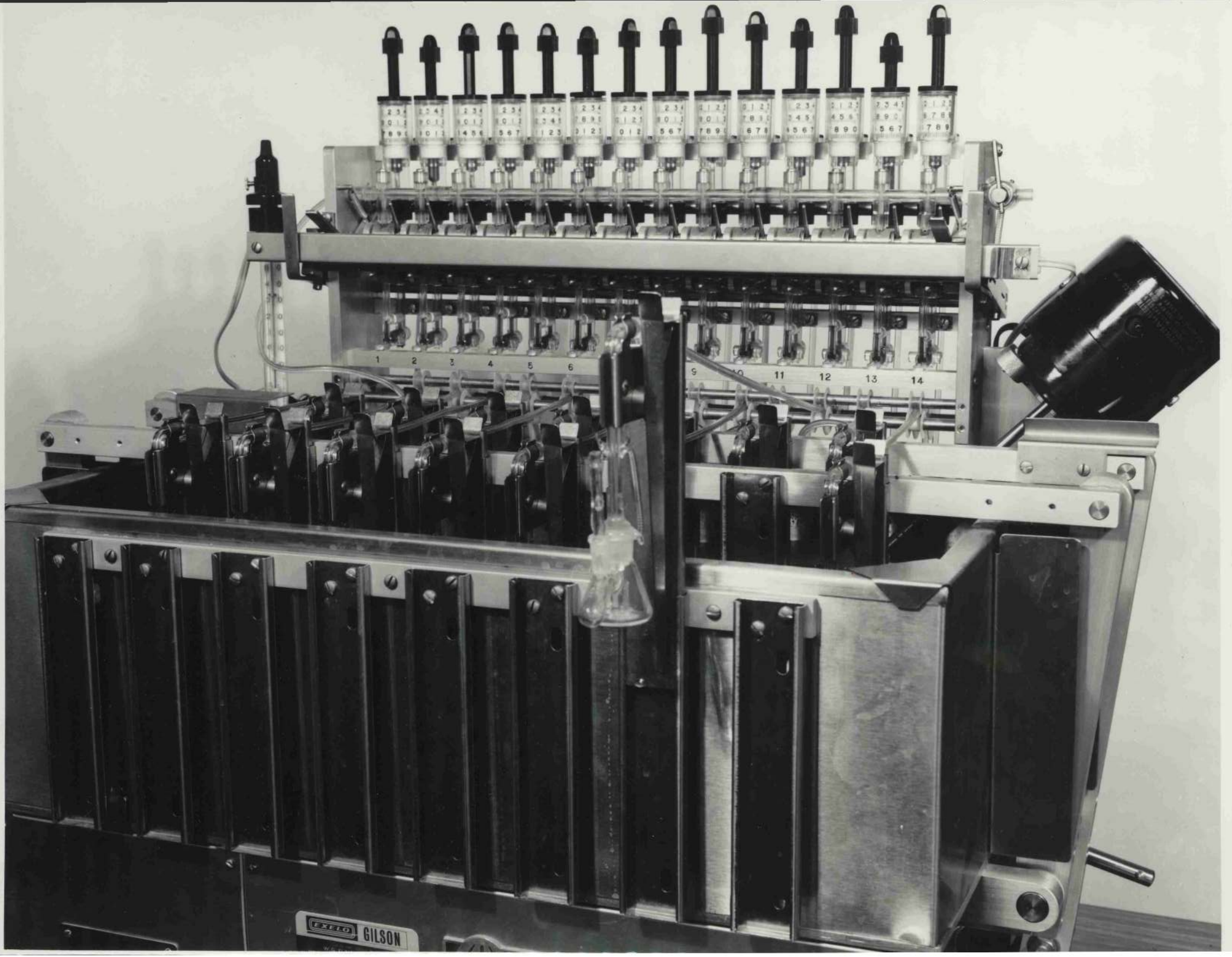
Plate I

Gilson Differential Respirometer

Plate I Gilson Differential Respirometer

This instrument measures gas evolution or uptake at constant pressure. Gas volume changes occurring in the flasks are relayed to the manometers (Manom) by means of flexible tygon tubes (TT). Changes in the levels of the manometers may then be nullified by alteration of the 'volumometer' units (Vol) which read directly in µl. The temperature at which the gas volume changes occur may be controlled by immersing the flasks in the thermostatically controlled bath.


Vol
Manom
Bath
TT
Flask
GILSON


EXELO
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can be read off a 'volumometer' unit on the Respirometer directly in µl.) The system was gassed thoroughly with 5 per cent CO_2 in N_2 and allowed to equilibrate, at 37°C, for 15 minutes. The suspension was tipped into the main compartment and, after 5 minutes further equilibration, the CO_2 evolution was measured, at 4 or 5 minute intervals, for 24 or 30 minutes and the rate of CO_2 evolution estimated. The units of enzyme activity were µl CO_2 evolved per mg protein per hour at 37°C.

Succinic Dehydrogenase Activity

Succinic dehydrogenase activity was assayed using the manometric technique of Quastel and Wheatley (1938) as described and modified by Aldridge and Johnson (1959). The assay procedure is a general one for dehydrogenases and involves the estimation of CO_2, liberated from a medium already saturated with CO_2, by the following series of reactions:-

$$\begin{matrix} CH_2CO_2H \\ | \\ CH_2CO_2H \end{matrix} + 2Fe(CN_6)^{3-} \underset{SDH}{\rightleftharpoons} \begin{matrix} H\text{-}C\text{-}CO_2H \\ \| \\ H\text{-}C\text{-}CO_2H \end{matrix} + 2Fe(CN_6)^{4-} + 2H^+$$

$$2H^+ + 2HCO_3^- \rightleftharpoons 2H_2CO_3 \rightleftharpoons 2H_2O + 2CO_2$$

A 0.5ml sample of synaptosomes or mitochondria, suspended in water and containing approximately 2mg protein per ml, was placed in one of the side-arms of a double-armed manometer flask. 2ml of medium, of the following composition (in final concentrations):- K_2HPO_4 12.5mM, $MgCl_2$ 3.5mM, EDTA 0.6mM, potassium succinate 22.5mM, $NaHCO_3$ 25.0mM, was placed in the main compartment of the manometer flask. (The pH of the medium was brought to 7.4 with KOH before the $NaHCO_3$ was added.) 0.5ml of $K_3Fe(CN)_6$ (12.5mM final concentration) was placed in the other side-arm. The manometer flask was attached to a Gilson Differential Respirometer and gassed with 5 per cent CO_2 in N_2. The flask was allowed to equilibrate for 15 minutes at 37°C and then the contents of the side-arms were tipped into the main chamber and, after a brief re-equilibration, the CO_2 evolution, at 37°C was estimated. If the fractions were isolated from fresh guinea-pig brain, the CO_2 evolution was estimated, at 5 minute intervals, for 30 minutes. The rate of gas evolution during this time was linear (Fig II). The units of enzyme activity were µl CO_2 evolved per mg protein per hour. When the fractions were isolated from frozen rabbit brain, however, it was found to be impossible to maintain the gas evolution for 30 minutes and therefore, in this case, the volume of CO_2 evolved by duplicate

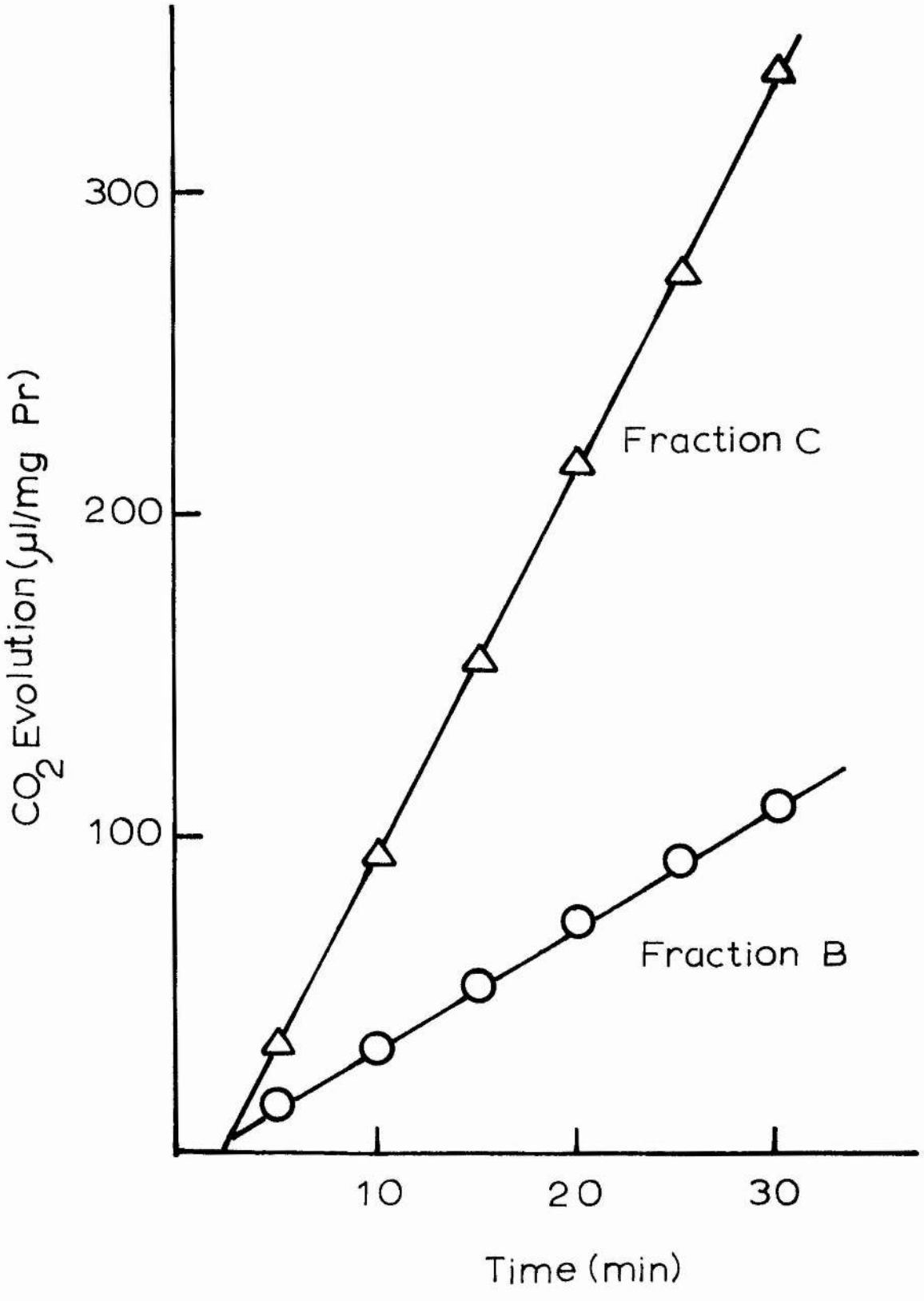
Figure II

Succinic Dehydrogenase Activity of Guinea-Pig Brain Fractions

Fractions B and C were isolated from homogenates of guinea-pig cerebral cortex using the method out-lined in Figure I. The succinic dehydrogenase activity of samples, suspended in water, was estimated in a Gilson Differential Respirometer using the method described on p31. The results represent the means of 3 estimations. The SD's were small and have been omitted.

Fig II

Succinic Dehydrogenase Activity of Guinea-Pig Brain Fractions



samples during the time between the 2nd and 7th minute was measured. (The flask was re-equilibrated during the first two minutes.) The units of enzyme activity, in this case, were µl CO_2 evolved per mg protein per 5 minutes.

Respiration Experiment

Fraction B, which lies between the 0.8M and 1.2M sucrose after the gradient has been centrifuged (Fig I), is the fraction reported to contain most of the synaptosomes (Gray and Whittaker 1962). This fraction was resuspended in saline solution of the following composition:- Na^+ 142mM, K^+ 5mM, Ca^{++} 1mM, Mg^{++} 1mM, Cl^- 142mM, $PO_4^=$ 3mM, $SO_4^=$ 1mM. The medium, which is essentially Krebs Ringer phosphate solution, is buffered to pH 7.4. The medium also contained 10mM D-glucose as substrate.

2ml of suspension, containing about 7mg of protein was placed in the main compartment of a manometer flask which had 0.2ml of 20 per cent KOH in the centre well and a filter paper (to increase the surface area). The flask was attached to a Gilson Differential Respirometer and the system was gassed briefly with O_2 and allowed to equilibrate at 37°C for 15 minutes. The oxygen uptake was then estimated,

at 5 minute intervals, for 45 minutes at 37°C.

Protein Estimation

Protein was estimated using the method of Gornal, Bardawill and David (1949). Samples, containing about 2mg of protein, were added to an equal volume of ice-cold 1N perchloric acid and left, on ice, for 10 minutes to precipitate the soluble protein. The protein was then centrifuged down on a bench centrifuge and washed with ethanol/ether (1:1) and then chloroform/methanol(2:1) to extract the lipid (a procedure which prevents opalescence of the final solution (Aldridge and Johnson 1959)). The remaining solid material was dissolved in hot 0.1N NaOH and the volume made up to 1.2ml. 2.4ml of biuret reagent (0.15%(w/v) $CuSO_4 5H_2O$ and 0.6%(w/v) potassium sodium tartrate dissolved in 0.33%(w/v) NaOH) was added to the samples and mixed thoroughly. After 30 minutes, the extinction at 540nm was measured. Samples of bovine serum albumin were used as standards and it was found that this protein estimation was linear over the range 0 - 4 mg of bovine serum albumin (Fig III).

Figure III

Protein Standards

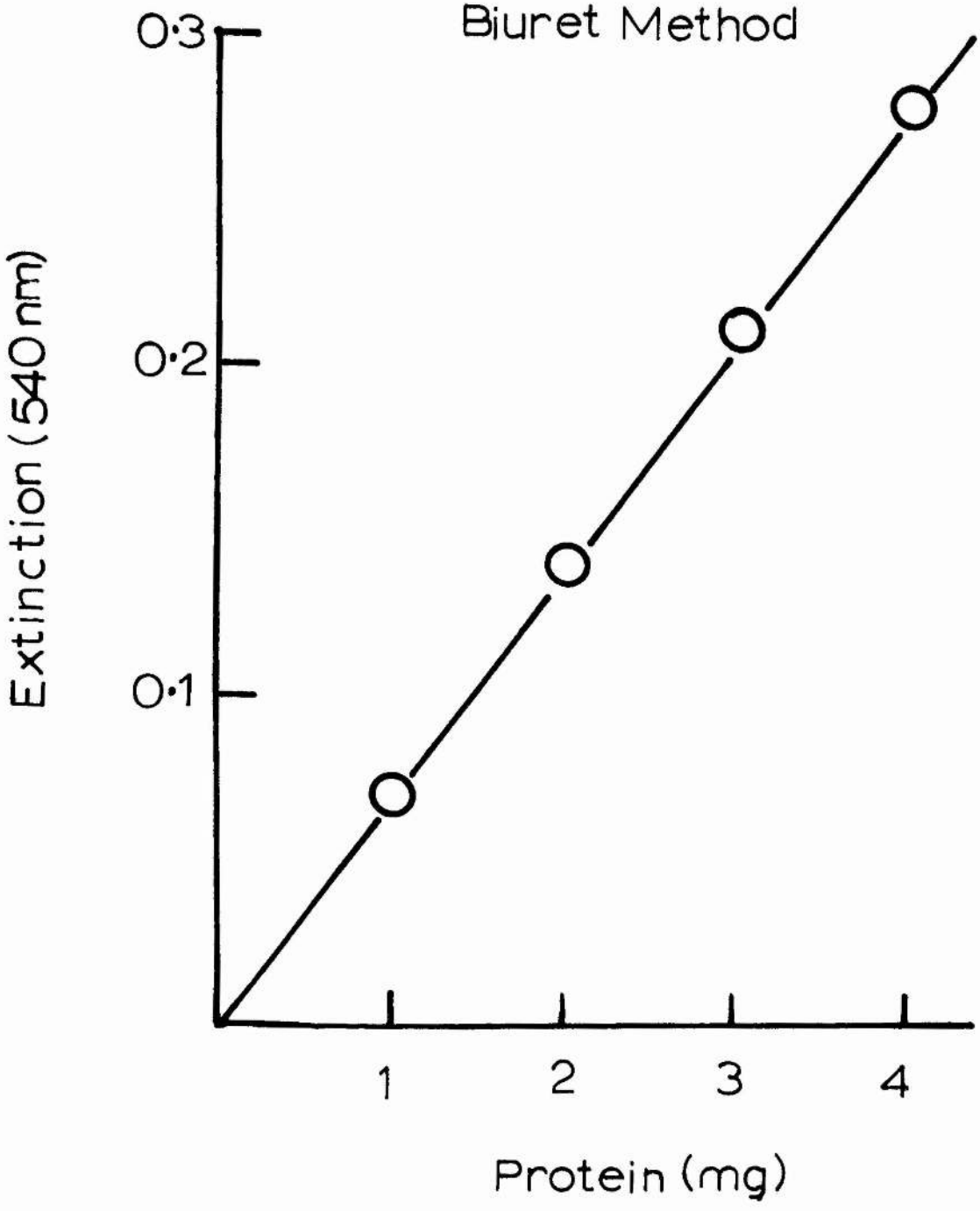
Biuret Method

Standard solutions, containing known weights of bovine serum albumin, were assayed by means of the Biuret Reaction according to the procedure of Gornal *et al* (1949) as described in the methods section (p34).

Fig III

Protein Standards

Biuret Method



Results and Discussion

The appearance of the sucrose gradient, after centrifugation, is presented in diagrammatic form in Figure I. Photographs of sections which had been negatively stained and photographed under the electron microscope were very unsatisfactory so the technique was abandoned in favour of positively stained sections.

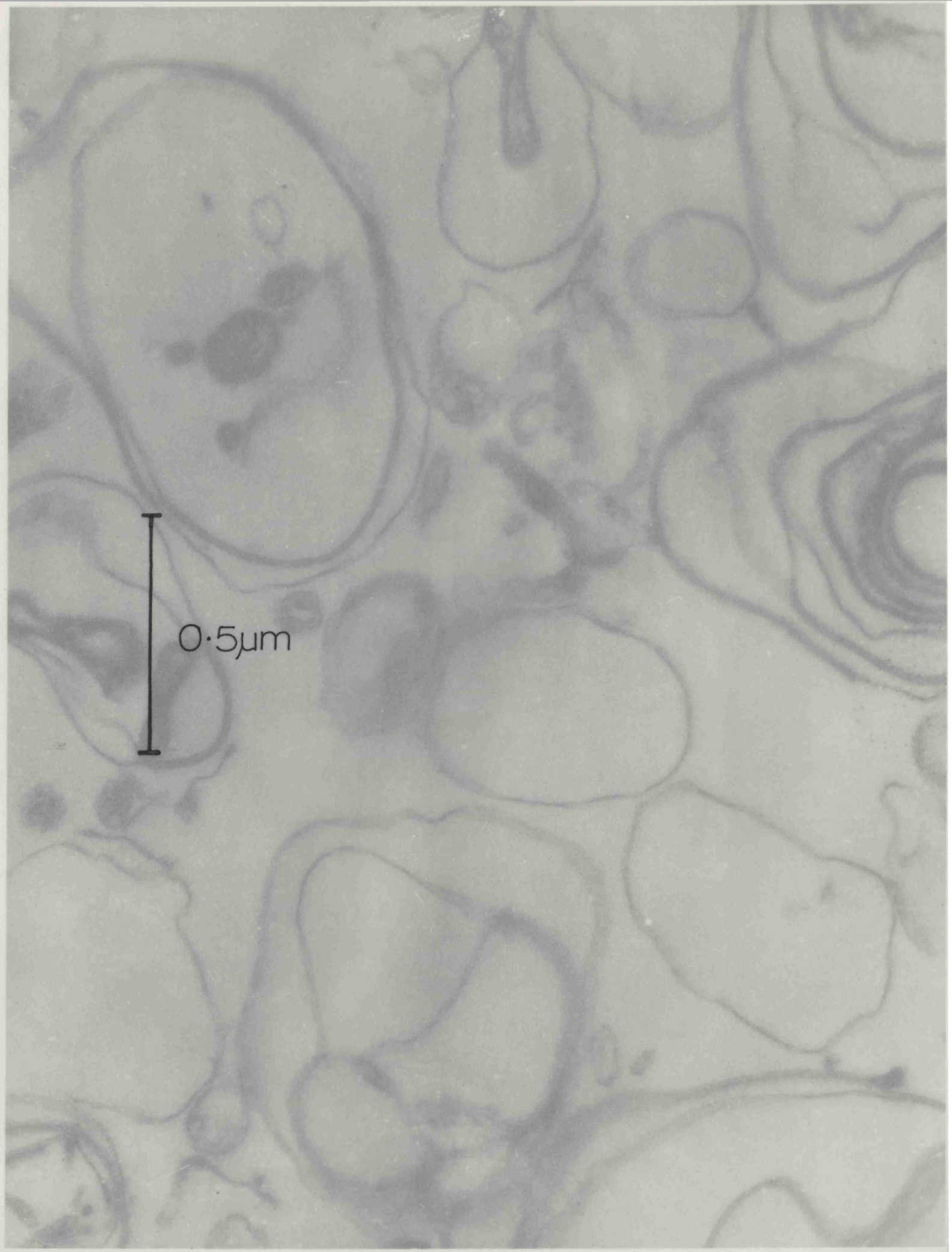
Photographs of the sections after positive staining are presented in Plates II - IV. The fractions have been designated A, B and C in the same way that Gray and Whittaker (1962) labelled their three fractions (see also Fig I). Fraction A (Plate II), which lies above the 0.8M sucrose, consists mainly of myelin and broken membrane fragments. The striking feature about the fraction is the almost total absence of organised structures. Fraction B (Plate III), on the other hand, which lies between the 0.8M and 1.2M sucrose, has many organised structures, most of which are synaptosomes. The appearance of the synaptosomes in this fraction is very similar to pictures of synaptosomes published by other workers (Gray and Whittaker 1962, De Robertis et al 1962). The synaptosomes, which vary between 0.3μm - 1.0μm in diameter, contain vesicles and often have

Plate II

Electron Micrograph of Fraction A

Plate II Electron Micrograph of Fraction A

Fraction A was isolated from homogenates of guinea-pig cerebral cortex using the procedure out-lined in Figure 1. The fraction was positively stained (p29) before being viewed under the electron microscope. The plate shows that fraction A consists predominantly of myelin and broken membrane fragments.


0·5μm

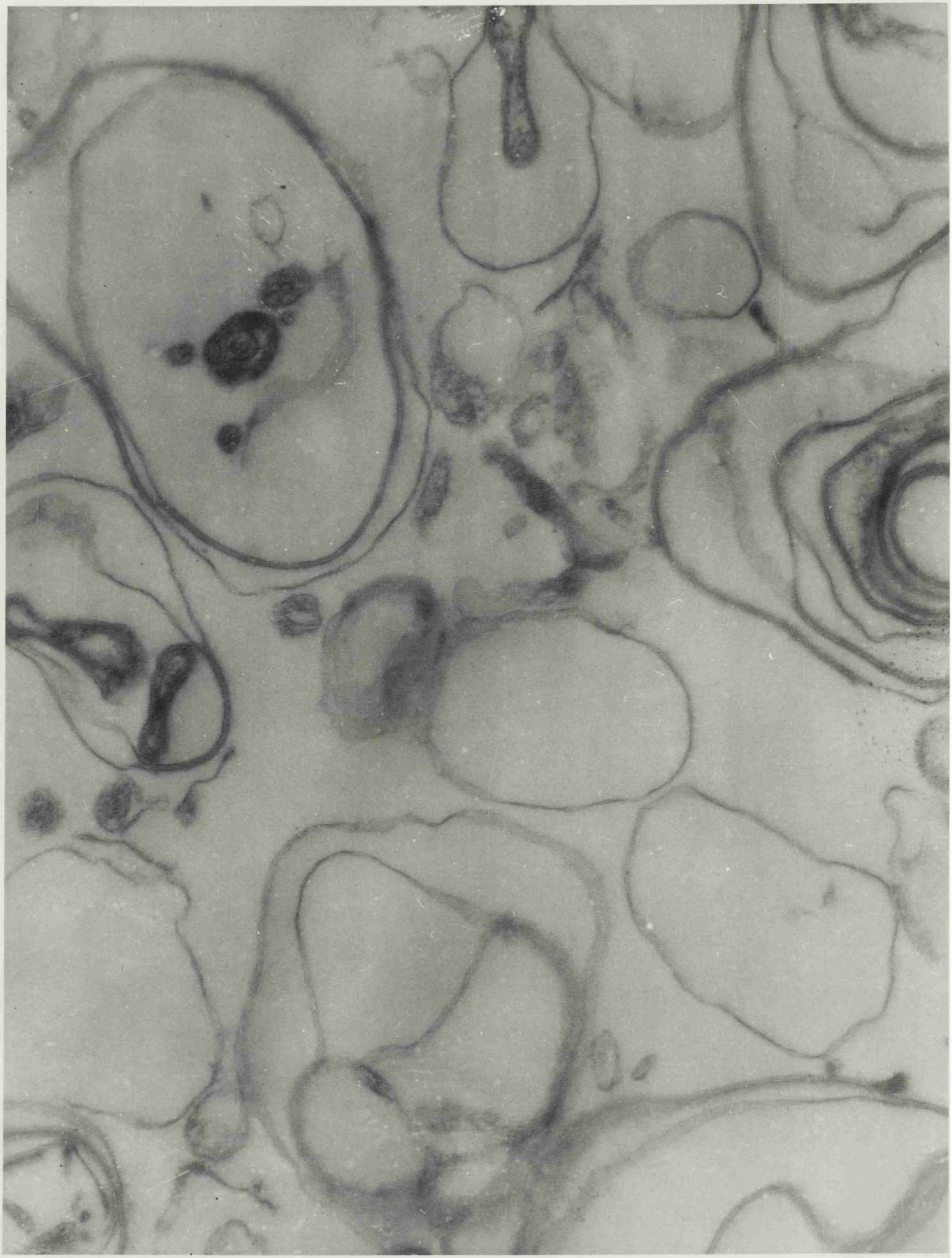


Plate III

Electron Micrograph of Fraction B

Plate III Electron Micrograph of Fraction B

Fraction B was isolated from homogenates of guinea-pig cerebral cortex using the procedure out-lined in Figure I. The fraction was positively stained (p29) and viewed under the electron microscope. The fraction contains a large number of synaptosomes (Syn). The synaptosomal cytoplasm contains vesicles and, in some instances, mitochondria (Mit). The membrane (PSM) lying adjacent to one of the synaptosomes is similar in appearance to photographs of post-synaptic membranes, published by others (Gray and Whittaker 1962), which remain attached to some of the synaptosomes.

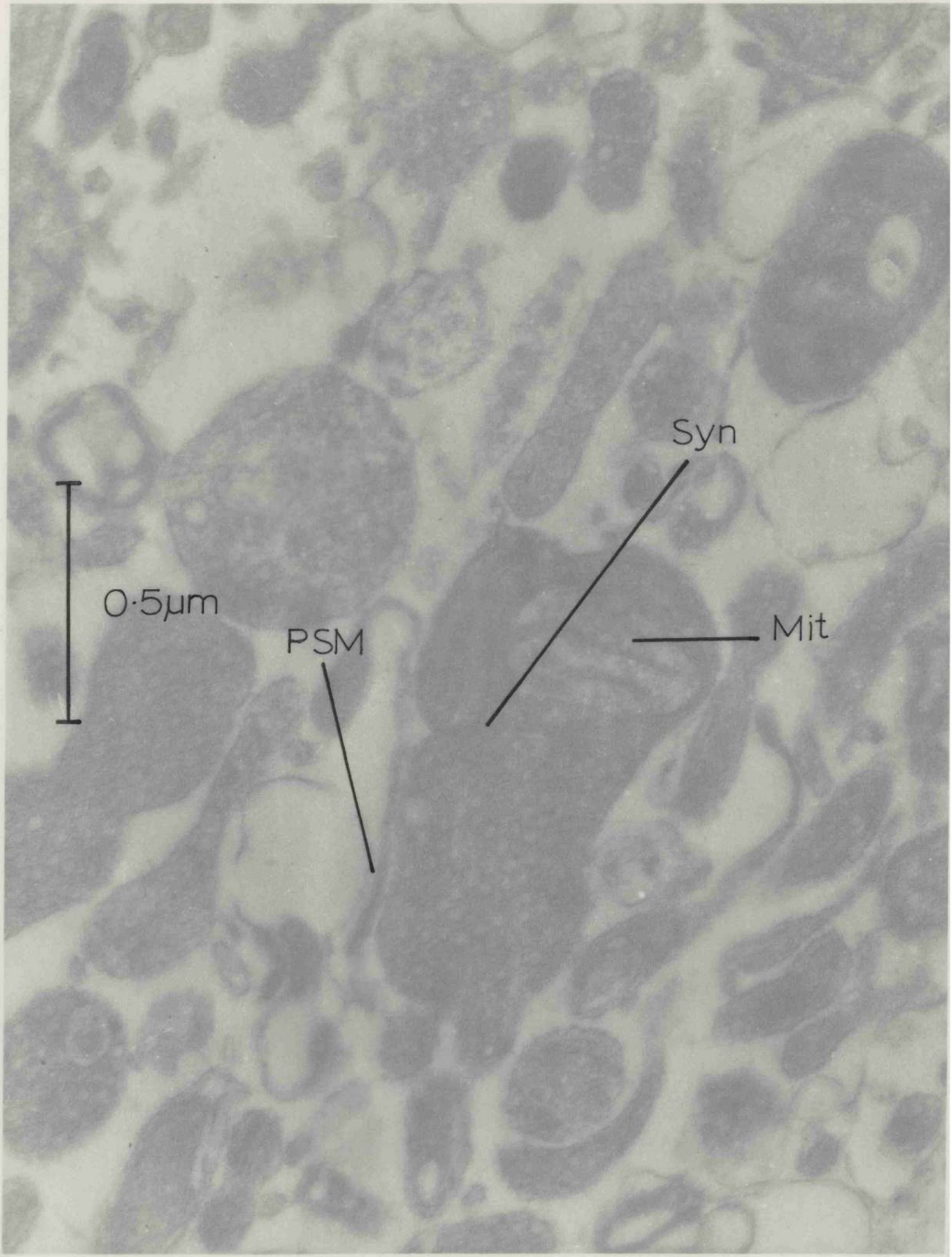
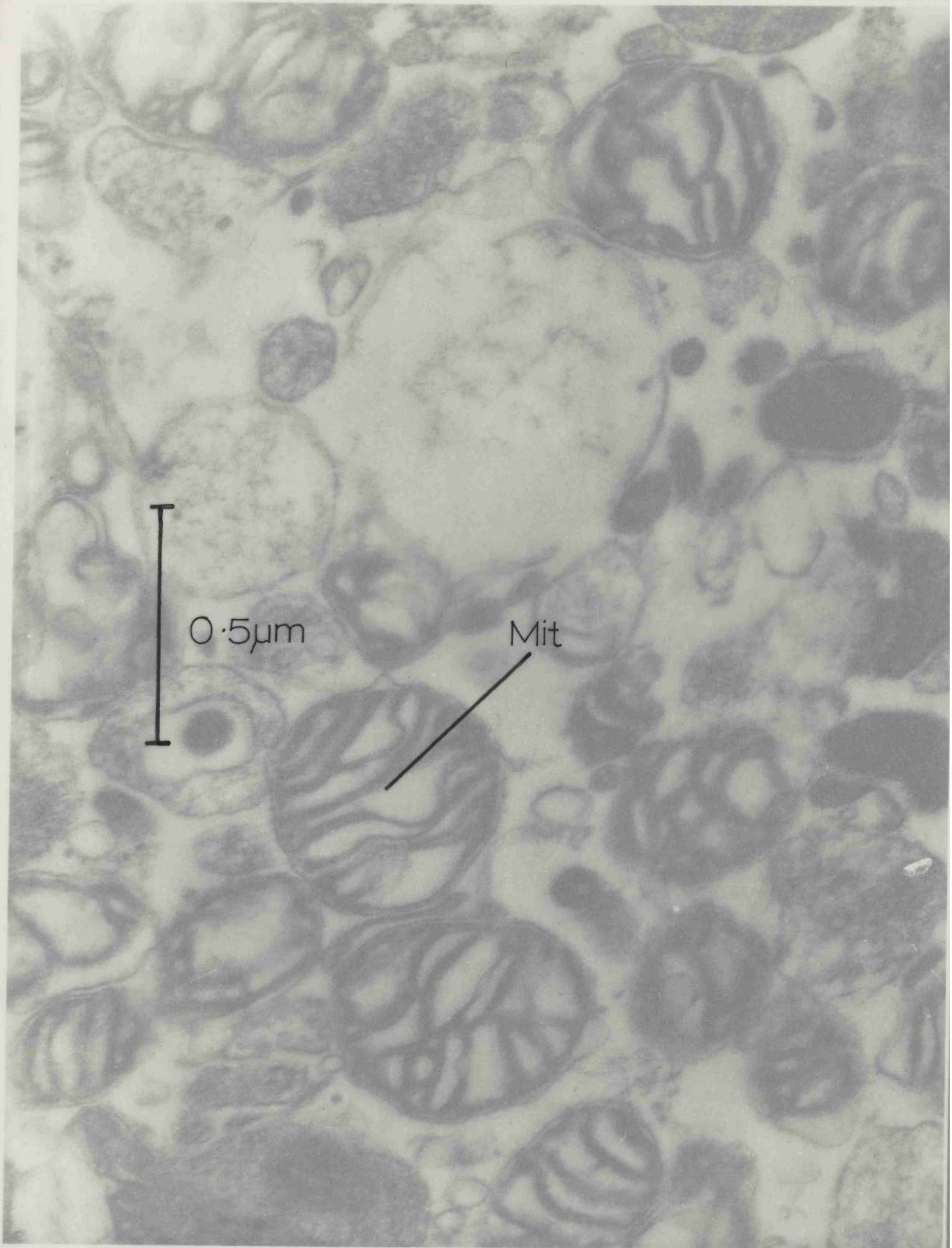

Syn
0·5µm
PSM
Mit

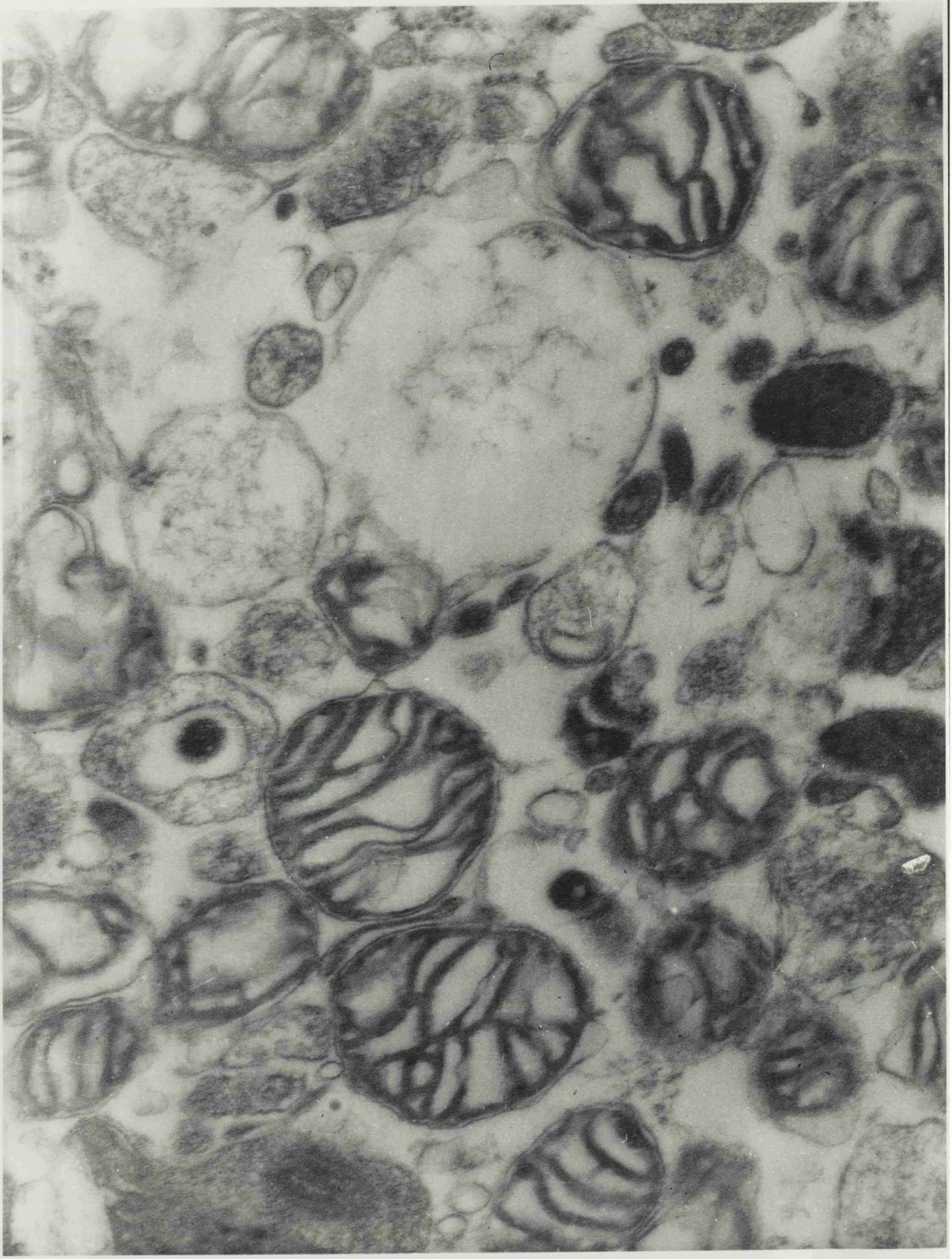
Plate IV

Electron Micrograph of Fraction C

Plate IV Electron Micrograph of Fraction C

Fraction C was isolated from homogenates of guinea-pig cerebral cortex using the procedure out-lined in Figure 1. The fraction was positively stained (p29) and viewed under the electron microscope. The plate shows that the fraction consists mainly of free mitochondria.


0·5µm
Mit



small mitochondria trapped within the synaptosomal cytoplasm. The post-synaptic membrane is still attached to some of the synaptosomes. The fraction is contaminated with some membrane fragments and a few free mitochondria which are small or damaged. This type of contamination was reported by other workers (Gray and Whittaker 1962, Bradford 1969). Fraction B in fact consists of two sub-fractions. After centrifugation, there appear to be two bands lying between the 0.8M and 1.2M sucrose. The top one, which is white, lies directly on top of a yellow band, and it has been suggested that the white band contains synaptosomes lacking mitochondria and the yellow band contains synaptosomes which do contain mitochondria. Fraction C (Plate IV), which sediments to the bottom of the centrifuge tube, contains numerous mitochondria and only a few synaptosomes. The mitochondria of this fraction are approximately 0.4μm in diameter and are only a little larger than those found enclosed within the synaptosomes. This fraction has a tan colour which is to be expected from a fraction containing mitochondria which contain pigmented cytochromes. The appearance of all the fractions was similar to photographs of the fractions published by other workers.

The distribution of enzymic activity between fractions B and C is consistent with the observations made under the electron microscope, namely that the free mitochondria have been separated from the synaptosomes, and are located in fraction C whereas the synaptosomes are to be found in fraction B. The activity of cholinesterase was considerably higher in fraction B than in fraction C (Fig IV) and in terms of units of activity, three times as much cholinesterase activity was found in fraction B compared with fraction C (Table I), a result which is in agreement with results published by De Robertis et al (1962). Although much of the acetylcholinesterase activity found in the CNS is located in the microsomal fraction (De Robertis 1964, Whittaker, Michaelson and Kirkland 1965), the crude mitochondrial fraction retains over half the total activity. When the crude mitochondrial fraction is fractionated further, the acetylcholinesterase activity is located on the synaptosomal membrane (Whittaker 1969).

There was three times as much succinic dehydrogenase activity in fraction C compared with fraction B (Table II) a result which is in agreement with the results of Whittaker (1959a) and Johnson and Whittaker (1964).

Figure IV

The Acetylcholinesterase Activity of Brain Fractions

Fractions B and C were isolated from homogenates of cerebral cortex using the method out-lined in Figure 1. The acetylcholinesterase activity was assayed in a Gilson Differential Respirometer using acetylcholine perchlorate as substrate (p29). The results are the means of 3 estimations. The SEMs were small and have been omitted for clarity.

Fig IV

Acetylcholinesterase Activity of Brain Fractions

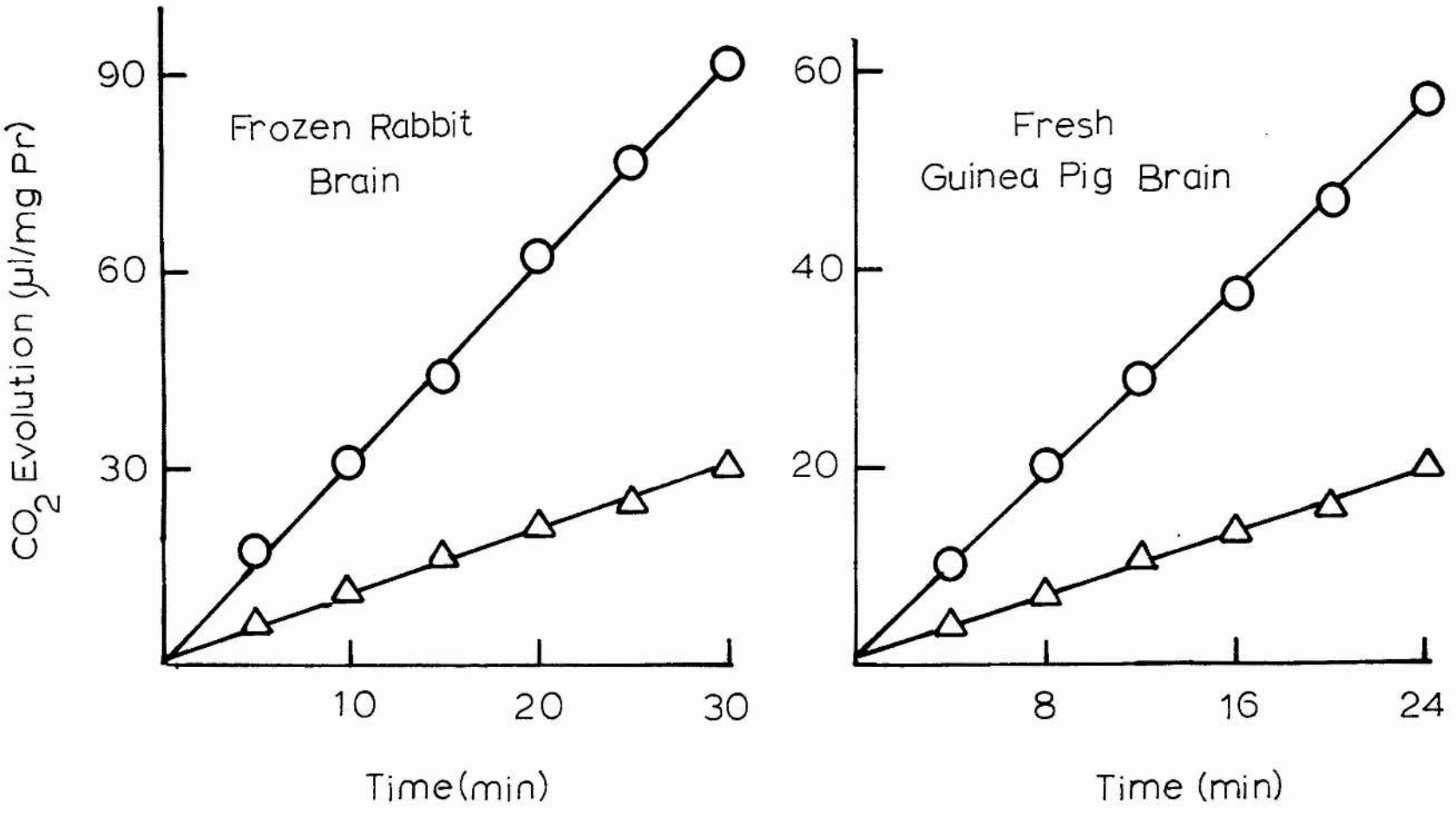


Table I

Cholinesterase Activity in Fractions B and C from the Crude Mitochondrial Fraction of Cerebral Cortex

Source of Enzyme	ChE Activity(Units/mg Pr) Fraction B	Fraction C	Ratio B:C
Frozen Rabbit Brain	183 ± 8	56 ± 5	3.3:1
Fresh Guinea-Pig Brain	148 ± 2	48 ± 1	3.1:1

Fractions B and C were isolated from homogenates of cerebral cortex using the procedure out-lined in Figure 1 and cholinesterase activities were determined in a Gilson Differential Respirometer using acetylcholine perchlorate as substrate (p29). 1 unit of activity is equal to 1µl CO_2 evolved per hour. The results represent the means of 3 expts.

Table II

Succinic Dehydrogenase Activity in Fractions B and C from the Crude Mitochondrial Fraction of Cerebral Cortex

	SDH Activity	
	Frozen Rabbit Brain (μl CO_2/mg Pr/5min)	Fresh Guinea-Pig Brain (μl CO_2/mg Pr/hr)
Fraction B	$2.7 \pm 0.4_{(4)}$	$72 \pm 4_{(3)}$
Fraction C	$9.6 \pm 0.6_{(4)}$	$133 \pm 4_{(3)}$
Ratio B:C	1:3.6	1:3.2

Fractions B and C were isolated from homogenates of cerebral cortex using the procedure out-lined in Figure 1 and the succinic dehydrogenase activities were determined in a Gilson Differential Respirometer (p31). The results represent the means of the number of expts given in the parentheses.

Succinic dehydrogenase is an enzyme which is localised on the mitochondrial membrane (Aldridge and Johnson 1959) and thus the activity of the enzyme is a measure of the number of mitochondria present in a fraction. A large part of the succinic dehydrogenase activity in Fraction B can be attributed to intrasynaptosomal mitochondria, since 30 per cent of the synaptosomal protein is mitochondrial in character (Moore and Lindall 1970). When the synaptosomes are suspended in water, the synaptosomal membrane is ruptured and the mitochondria are released. In hindsight, it becomes clear why mitochondrial preparations from brain have to be assayed for succinic dehydrogenase activity under conditions of hypo-osmolarity whereas this is not necessary if the same fraction is isolated from other tissue. Clearly the synaptosomal membrane must be impermeable to one or more of the reagents necessary for the assay.

The oxygen uptake of samples of fraction B which had been isolated from fresh guinea-pig brain was 65 $\pm$ 2 µmoles of O_2 per 100mg protein per hour (mean of 6 estimations) which is very similar to the rates observed by Bradford (1969) and Marchbanks (personal communication) for synaptosomes allowed to respire under conditions similar to those used here. The rate of oxygen uptake was linear over a 45

minute period (Fig V). If mitochondria or damaged synaptosomes are allowed to respire under the same conditions the oxygen uptake is small and it is not linear (Bradford 1969). When fraction B was isolated from homogenates of frozen rabbit brain, the oxygen uptake was very small and unstable. This result was not unexpected since the freezing, storage and subsequent thawing of the brain could be expected to damage the cell membranes and thus the membranes would, no longer, retain the cytoplasmic factors necessary for glycolysis to occur. This type of storage did not prevent the formation of a fair number of synaptosomes since the distribution of enzymic activity between fractions B and C was similar for preparations from either source (Tables I and II).

7.8 $\pm$ 0.3 mg protein per g of guinea-pig cerebral cortex (mean of 10 estimations) were isolated in fraction B, which is approximately half the maximum yields obtained by other workers. Bradford (1969) demonstrated that about 40 per cent of the synaptosomes formed were centrifuged down with the nuclear fraction and could only be recovered by thoroughly washing this fraction. This washing procedure, however, was not used here since it was desirable to complete the preparation within 6 hours in

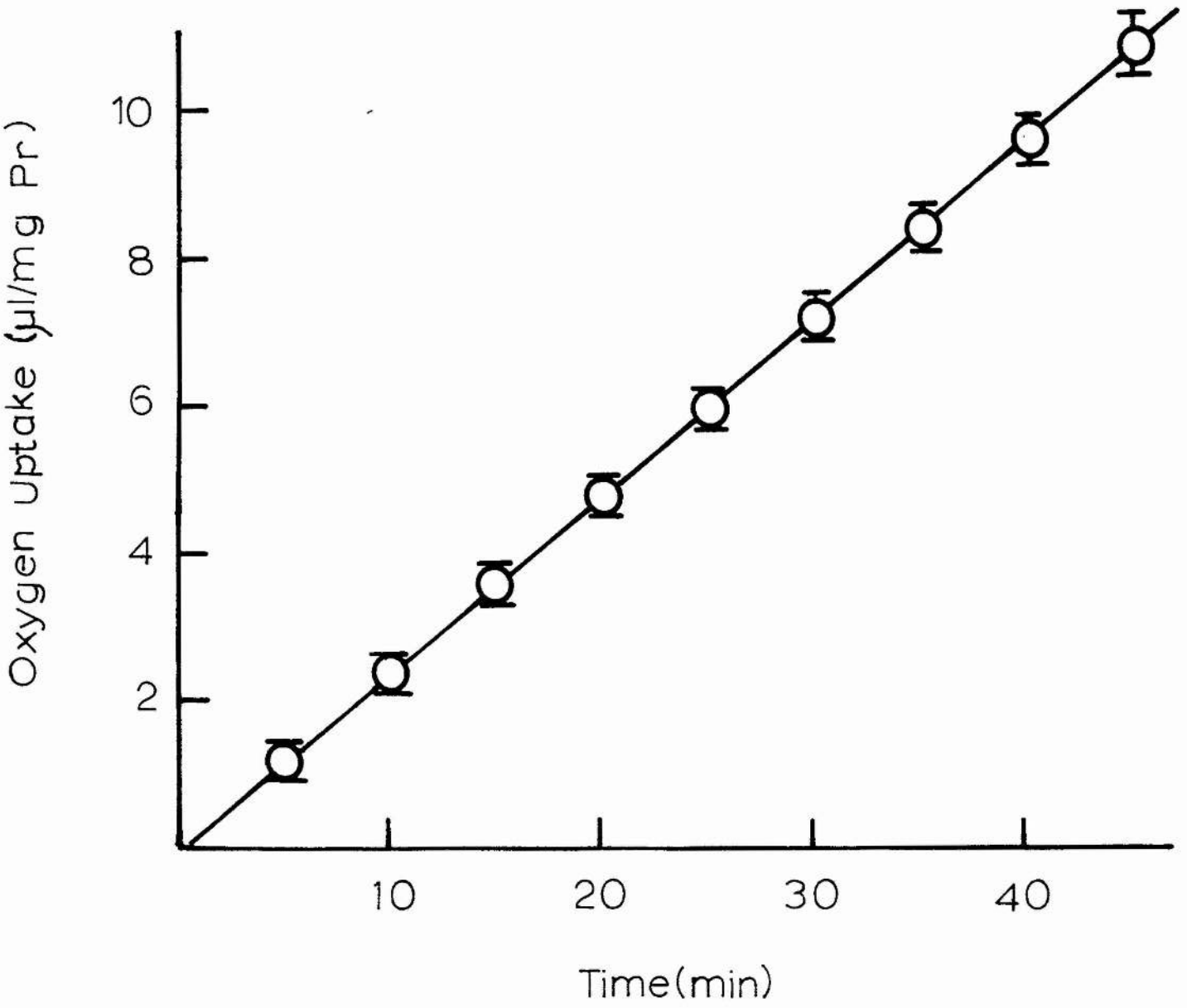
Figure V

Oxygen Uptake by Synaptosomes

The oxygen consumption of synaptosome samples, suspended in oxygenated saline containing 10mM glucose was estimated in a Gilson Differential Respirometer (p33). The results represent the means $\pm$ SEM of 6 estimations.

Fig V

Oxygen Uptake of Synaptosomes



view of the experiments to be subsequently performed. The yield of protein in fraction C was 3.5 ± 0.2 mg per g of guinea-pig cerebral cortex (mean of 3 estimations) which is a little less than half the amount found in fraction B. Other workers (Gray and Whittaker 1962, Johnson and Whittaker 1964, Bradford 1969) have also found that fraction B contained twice as much protein as fraction C. If the fractions were isolated from frozen rabbit brain, then the yields were 1.9 ± 0.4 mg protein per g of brain in fraction B (mean of 4 estimations) and 2.0 ± 0.3 mg protein per g of brain in fraction C (mean of 4 estimations). The yield in both fractions is reduced, but the yield in fraction B is so much reduced that it is now equal to that of fraction C. It would seem therefore that the freezing procedure, applied to rabbit but not to guinea-pig brains, impairs the ability of the brain to form synaptosomes. This is not surprising in view of the observations of Whittaker (1965) who reported that mitochondria withstand rough treatment, such as the treatments necessary for electron microscopy or suspension in hypo-osmotic solution, far better than synaptosomes.

The results of experiments, which were performed on

fraction B isolated from fresh guinea-pig brain and which are to be described in detail in subsequent sections of the thesis, have shown that the electrolyte content and metabolic and permeability properties of the fraction are similar, in all essential respects, to those reported for synaptosomes by other workers. In view of this evidence, therefore, fraction B has been used in subsequent experiments as a synaptosome preparation and will, in future sections of the thesis, be referred to as the synaptosome fraction.

SECTION II

THE EFFECTS OF DRUGS ON SYNAPTOSOMAL METABOLISM

Introduction

The objective of the experiments, which are to be described in this section, was to study the effects of drugs on the stability of synaptosomal metabolism.

Synaptosomes have been compared with anucleate cells because they will actively respire and metabolise added substrate under conditions which normally only support metabolic activity in preparations containing whole cells (Whittaker 1969, Bradford 1969). Bradford (1970a,b) has further claimed that the synaptosomal membrane is polarised and excitable under these conditions - that is to say the membrane is capable of being depolarised by electrical stimuli. If cerebral cortex slices are depolarised by electrical pulses or by altering the ionic content of the medium, the change in the polarised state of the neuronal membrane is reflected by a large increase in the oxygen uptake of the tissue (McIlwain 1966). Bradford (1970a,b) has reported results which suggest that there is a similar respiratory response when the synaptosomal membrane is depolarised. These observations suggest that the synaptosome might be used as a simple model with which

to test the effects of compounds which increase or decrease neuronal excitability, and with which to investigate mechanisms of action of anticonvulsant drugs.

In the present work, therefore, compounds which are known to affect excitable neuronal membranes 'in vivo', have been tested for their effects on the oxygen uptake of synaptosomes. The type of compound tested included K^+ and some of the putative transmitter compounds. Although the putative transmitter compounds are thought to act primarily on the post-synaptic membrane, the interactions of some of these compounds with the pre-synaptic membrane, from which synaptosomes are formed, may be important. In particular, Koelle (1962) has suggested that, at some synapses, acetylcholine, released by the nerve-ending, acts on the pre-synaptic membrane to cause the release of more transmitter which may be acetylcholine itself or another transmitter (Burn and Rand 1962). This pre-synaptic role of acetylcholine has been put forward as one of the explainations of the wide distribution of the compound in the cerebral cortex where only 15 per cent of the synapses are thought to be cholinergic (McLennan 1965). It was then proposed to test the effects of the anti-

convulsant drugs on any response that was observed, and also to test the effects of the drugs, themselves, on synaptosomal respiration.

Brief mention has already been made of the possibility that more than one TCA cycle is present in the CNS (Van Den Berg et al 1969, Clarke, Nicklas and Berle 1970) and that drugs may affect the fate of added substrate without having any significant affect on the oxygen consumption. It is not known whether these different cycles are spacially separated within the cells, or are located in different cells, or if they occur in different mitochondrial fractions which are known to be present in the CNS (Blokhuis and Veldstra 1970). Much of the glucose which is incorporated into free amino acids is retained within the synaptosomes (Bradford and Thomas 1969) and, as a means of assessing the effects of the convulsant and anticonvulsant drugs on the incorporation of glucose into these metabolites, the effects of the drugs on the accumulation of ^{14}C, derived from (U-^{14}C)-glucose substrate, were examined.

Materials and Methods

Materials

Amino Butyric Acid	Supplied by British Drug Houses Ltd
Folin - Ciocalteu Reagent	Poole. Dorset.
L-Glycine	
L-Glutamic Acid	Supplied by Koch - Light
Serotonin	Laboratories, Colnbrook. Bucks.
Acetylcholine Chloride	Supplied by Sigma Ltd. London.
Pentamethylene Tetrazol	
Acetazolamide Sodium	Supplied by American Cyanamid Co. Pearle River. New York.
(U-^{14}C)-Glucose	Supplied by the Radiochemical Centre, Amersham. Bucks.

Phenobarbitone Sodium	Supplied by Pfizer Ltd. Sandwich. Kent
nor-Adrenalin	Generous gift of Dr. Cottrell
Histamine	Gatty Marine Laboratory, St. Andrews.

Methods

Oxygen Uptake

The synaptosomes, isolated from 4 guinea-pig brains, were pooled and resuspended in saline medium (p33) which contained 10mM glucose as substrate. Paired 2ml samples were placed in the main compartments of manometer flasks and 0.25ml samples of saline, with or without drug, were placed in the side-arms. The flasks, which contained 0.2ml of 20 per cent KOH in contact with filter paper wicks in the centre wells, were attached to a Gilson Differential Respirometer, gassed briefly with oxygen, equilibrated and allowed to respire for 20 minutes as described in the previous section. After 20 minutes, the contents of the side-arms were tipped into the main compartments and, after a brief re-equilibration period which lasted 2

minutes, the oxygen uptake for the next 2 minutes was measured. The oxygen uptake was then measured at 5 minute intervals, for a further 20 minutes.

In some experiments, in which the effects of the convulsant and anticonvulsant drugs were examined for their effect on the resting respiratory rate, the drugs were added at the beginning of the experiment and the oxygen uptake was measured over a 45 minute period.

The Incorporation of ^{14}C into Synaptosomes

Synaptosomes, isolated from the brains of 2 guinea-pigs, were pooled and resuspended in saline medium (p33). Paired 2ml samples, taken from the same stock suspension, were placed in the main compartments of manometer flasks and, where necessary, the drugs, dissolved in 0.25ml of saline, were added to the suspensions. 0.5ml of saline containing (U-^{14}C)-D-glucose (75mM to give a final concentration, when added to the contents of the main chamber, of 10mM) with 0.5μCi of radioactivity, were placed in the side-arms. The flasks were gassed with O_2, allowed to equilibrate and then stoppered and incubated for 15 minutes. The contents of the side-arms were then tipped

into the main compartments and the incubation continued at 37°C for the desired time. The incubation was stopped by transferring the contents of the manometer flasks to 10ml conical centrifuge tubes and centrifuging them for 90 seconds on a bench centrifuge. The pellets were resuspended in ice-cold 0.32M sucrose and the synaptosomes separated from extrasynaptosomal compounds, using the gel filtration method of Marchbanks (1967).

Gel Filtration of the Synaptosome Suspensions

Columns (11cm x 0.8cm) were prepared, containing 0.5g of G50 sephadex (bead form). The 0.32M sucrose, in which the gel had been pre-incubated, was allowed to drain down and 0.5ml samples of synaptosome suspension were carefully layered on the tops of the columns and also allowed to drain down. The columns were then eluted with ice-cold 0.32M sucrose. The first clear effluent, to come off the column (about 1.5 - 2.0ml) was discarded, but the first ml of effluent which was milky in appearance was collected, since it contained approximately 80 per cent of the synaptosomal protein which had been applied to the column. This fraction, which was found to contain

only 1 per cent of the extrasynaptosomal glucose, was diluted to 5ml with water and assayed for its ^{14}C and protein content in the manner described below.

Measurement of Radioactivity

The radioactivity in the fractions was assayed on a Packard Tricarb Automatic Scintillation Counter, using the scintillation medium of Bray (1960), which was found to be 55 per cent efficient under the conditions used in these experiments. The efficiency was calculated using an internal ^{14}C standard of known activity and was checked for each estimation by the channels ratio method. The results have been expressed as counts per minute (cpm) per mg protein, since the efficiency of counting remained constant over the range of activities used.

Protein Estimation

Because the concentration of protein in the diluted effluent was too low to be estimated by the method of Gornal et al (1949), the method of Lowry et al (1951) was used in the gel filtration experiments. 0.6ml of sample, containing approximately 100µg of protein per ml, were added to 3ml of alkaline copper reagent (0.01%

$CuSO_4 5H_2O$, 0.02% sodium potassium tartrate and 2% Na_2CO_3 in 0.4% NaOH) and mixed. 0.3ml of Folin-Ciocalteu reagent (stock solution diluted with an equal volume of water) was then added to the solutions and mixed thoroughly and left to stand for 90 minutes. The extinction of the solutions was then read at 500nm and the results compared with standards of bovine serum albumin. The protein estimation was linear over the range 0 - 200µg bovine serum albumin per ml (Fig VI). Bradford et al (1966) showed that, if the protein contents of synaptosome samples were estimated by the method of Gornal et al (1949) and by the method of Lowry et al (1951), then the results were the same. This observation was confirmed in this work.

Figure VI

Protein Standards

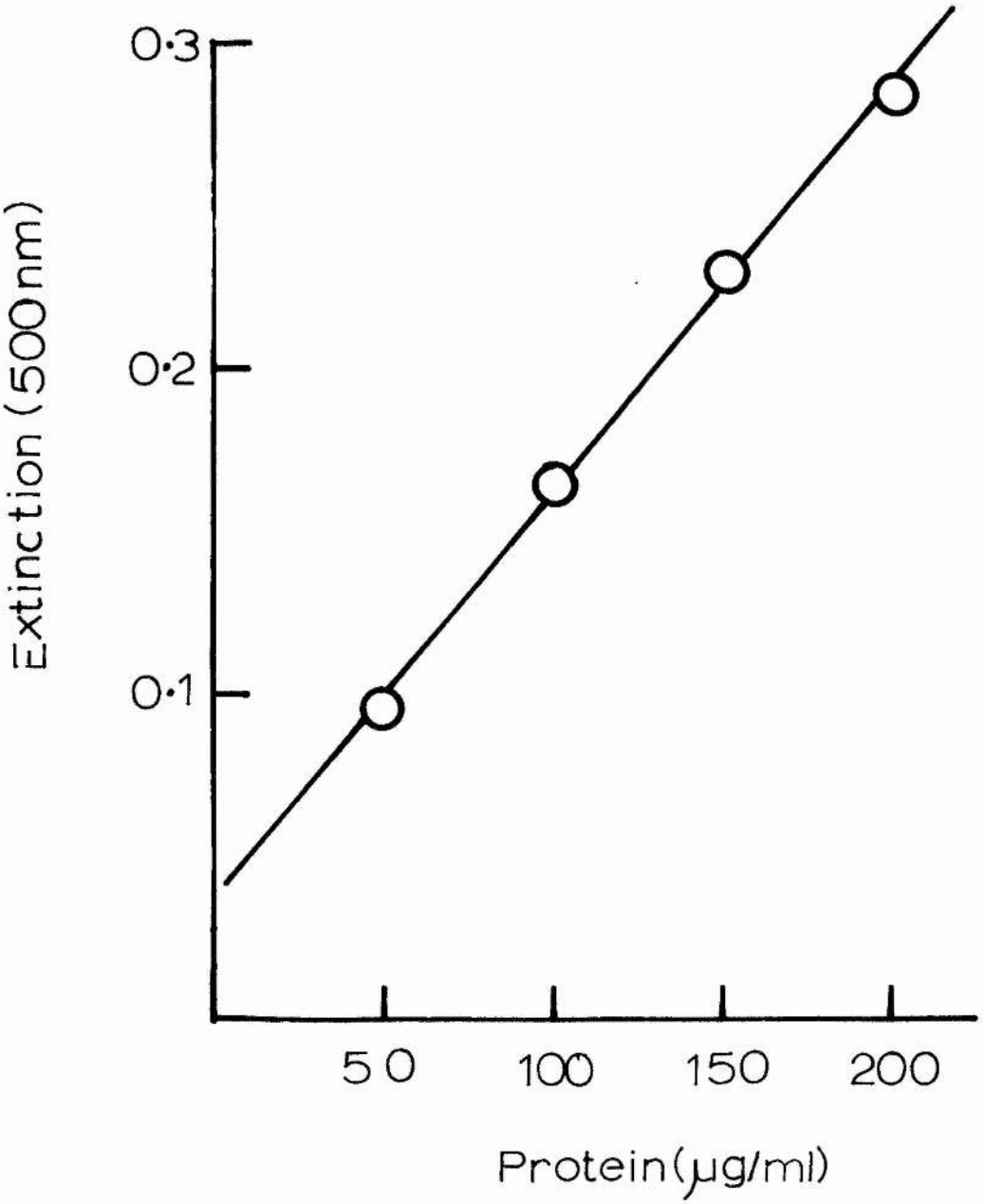
Folin Method

Standard solutions, containing known weights of bovine serum albumin, were assayed by means of the Folin-Ciocalteu Reaction according to the procedure of Lowry *et al* (1951) as described in the methods section (p52).

Fig VI

Protein Standards

Folin Method



Results and Discussion

Oxygen Uptake Experiments

In the previous section, it was shown that synaptosomes respired, in a stable and linear way, over a period of at least 45 minutes (Fig V). The addition of K^+ (15 mequiv per l) to give a final concentration of 21.6 mequiv per l did not alter the rate or stability of respiration of the synaptosomes significantly (Table III, Fig VII). The same result was obtained if the K^+ concentration was raised to 106 mequiv per l. This result is not in agreement with the findings of Bradford (1970b) who has reported that the addition of K^+ produced a small stimulation in the oxygen consumption of synaptosomes. A similar, but more marked, stimulation of respiration is observed if K^+ is added to cerebral cortex slices, respiring under similar conditions, and this effect has been attributed to depolarisation of the neuronal cell membranes by the excess K^+ (McIlwain 1966). It was possible that, in our experiments, the rate of oxygen consumption might have been limited by the rate of diffusion of oxygen through the medium, but it was found

Figure VII

The Effect of K^+ on the Oxygen Uptake of Synaptosomes

Paired samples of synaptosomes were suspended in oxygenate saline medium containing 10mM glucose (p33). The oxygen uptakes were measured, in a Gilson Differential Respirometer, for 20 minutes and then saline, with or without extra K^+ (23.6mequiv per l final concentration), was tipped and the oxygen uptakes measured for a further 20 minutes (p49). The results are the means of 5 estimations. The SEMs were small and have been omitted for clarity.

Fig VII

Effect of K^+ on the Oxygen Uptake of Synaptosomes

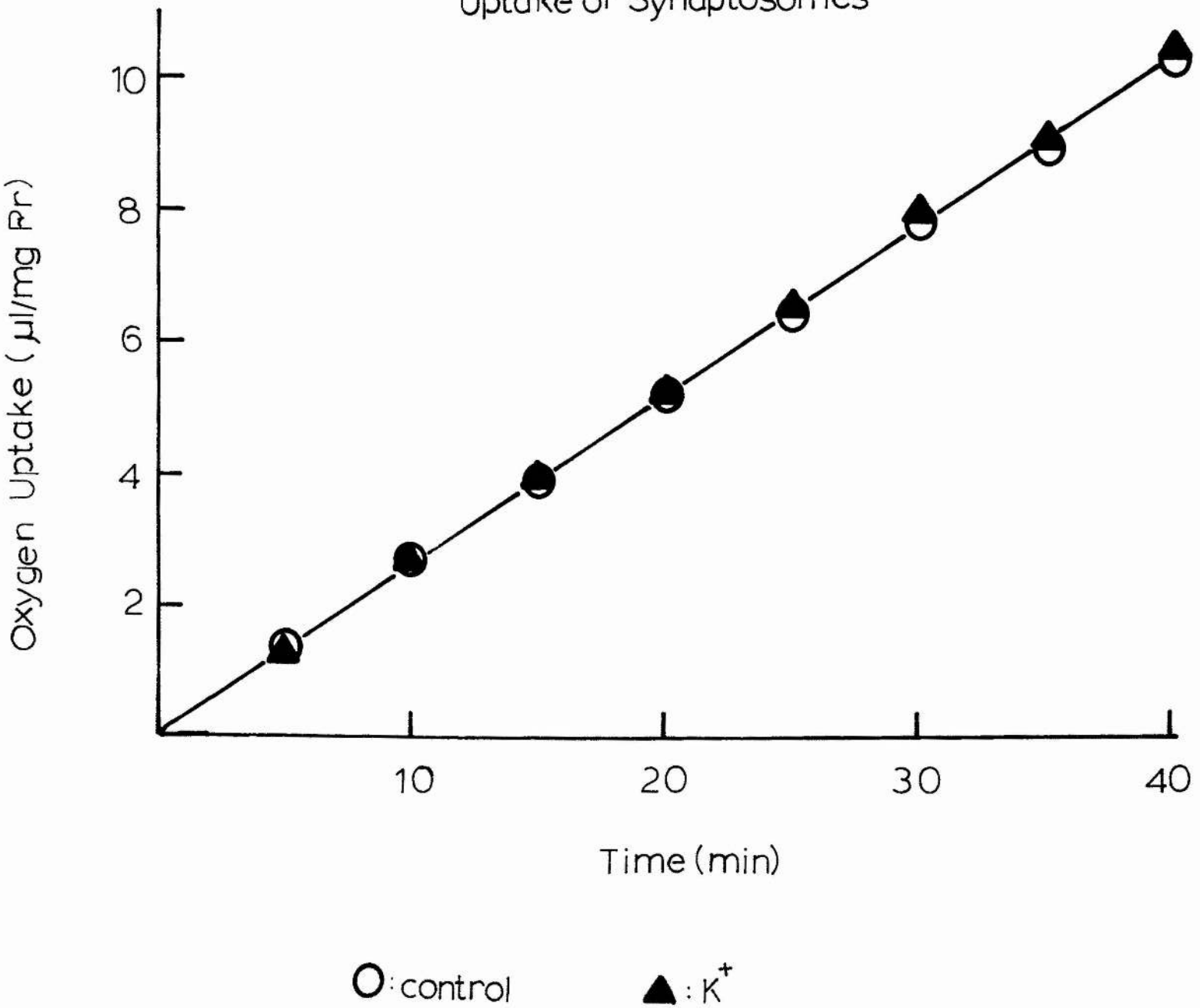


Table III

The Effect of KCl and Phenobarbitone Sodium on the Respiration of Synaptosomes

Flask Contents		Rate Before	Rate After
Main Chamber	Soln Tipped	Tipping $\mu l\ O_2/mg\ Pr/hr$	Tipping $\mu l\ O_2/mg\ Pr/hr$
Suspension	Saline	15.3 ± 0.4	15.1 ± 0.3
Suspension	KCl	15.7 ± 0.9	15.0 ± 0.8
Suspension + Phen	KCl	14.9 ± 1.0	15.1 ± 0.9
Suspension	Saline	15.1 ± 0.6	15.3 ± 0.4
Suspension	Phen	14.5 ± 0.8	14.3 ± 1.0

The rates of oxygen uptake of the synaptosome samples, suspended in oxygenated saline, was estimated over a 20 minute period in a Gilson Differential Respirometer (see p49). The results are expressed as the means of 5 estimations $\pm$ SEM. The rates before and after tipping were statistically compared by the 'students' t-test and no significant differences were found.

Phen: phenobarbitone sodium (2mM final concentration)

KCl: potassium chloride (21.6 mequiv/l)

that if twice the quantity of synaptosomes were suspended in the same volume of medium, there was a twofold increase in the oxygen uptake. It is, therefore, unlikely that the rate of diffusion of oxygen was a limiting factor. It is difficult to reconcile the results, reported in this work, with those of Bradford since, in all other respects, the preparation, used here, was similar to those used by other workers.

It had been anticipated that, if K^+ stimulated the respiration of synaptosomes, the effect might have been reduced by the inclusion of phenobarbitone sodium (2mM) in the medium. This was not the case, however, and phenobarbitone, itself, was without effect (Table III).

In the next series of experiments, compounds which are found to be present in the CNS 'in vivo' and which have been reported, by others, to affect polarised membranes and have been implicated as possible transmitter compounds at CNS synapses, were examined for their effects on synaptosomal respiration. The compounds tested were:- L-glutamic acid, acetylcholine, nor-adrenalin and serotonin all of which, for the most part, depolarise neuronal membranes, and γ-amino butyric acid, L-glycine and

histamine all of which have been proposed as possible inhibitory transmitters of the CNS. (The evidence implicating these compounds in CNS transmission has been summarised by Bradley 1969). The tranquilising drug, reserpine, which causes the release of serotonin and the catecholamines from brain (Pletscher, Shore and Brodie 1956), was also tested. Figure VIII shows that when one source of L-glutamic acid (Koch - Light Laboratories) was examined for its effect on respiration, it caused a marked reduction in the oxygen uptake of the synaptosomes. This effect was not observed if the same L-glutamic acid was added to cerebral cortex slices, prepared by the method of Gilbert (1969). However, if chromatographically pure L-glutamic acid (Boots Pure Drug Co.) was added to the synaptosomes, there was no effect on the respiration (Table IV), a result which is in agreement with that of Bradford (1969). It was concluded, therefore, that the first sample of L-glutamic acid (Koch - Light) contained an impurity which was able to cross the synaptosomal membrane and inhibit oxygen uptake but to which cerebral cortex slices were not so sensitive. Chromatographic analysis failed to reveal any amino acid or sugar contamination and it seems most likely

Figure VIII

The Effect of Impure Glutamate on the Respiration of Synaptosomes

The oxygen consumption of synaptosome samples, suspended in oxygenated saline solution containing 10mM glucose was estimated in a Gilson Differential Respirometer (p33). The oxygen uptake was measured for 20 minutes and then saline, with or without glutamate (10mM final concentration) was tipped and the oxygen uptake measured for a further 20 minutes. The results represent the means of 5 estimations. The SEMs were small and have been omitted for clarity.

Fig VIII

The Effect of Impure Glutamate on the Oxygen Uptake of Synaptosomes

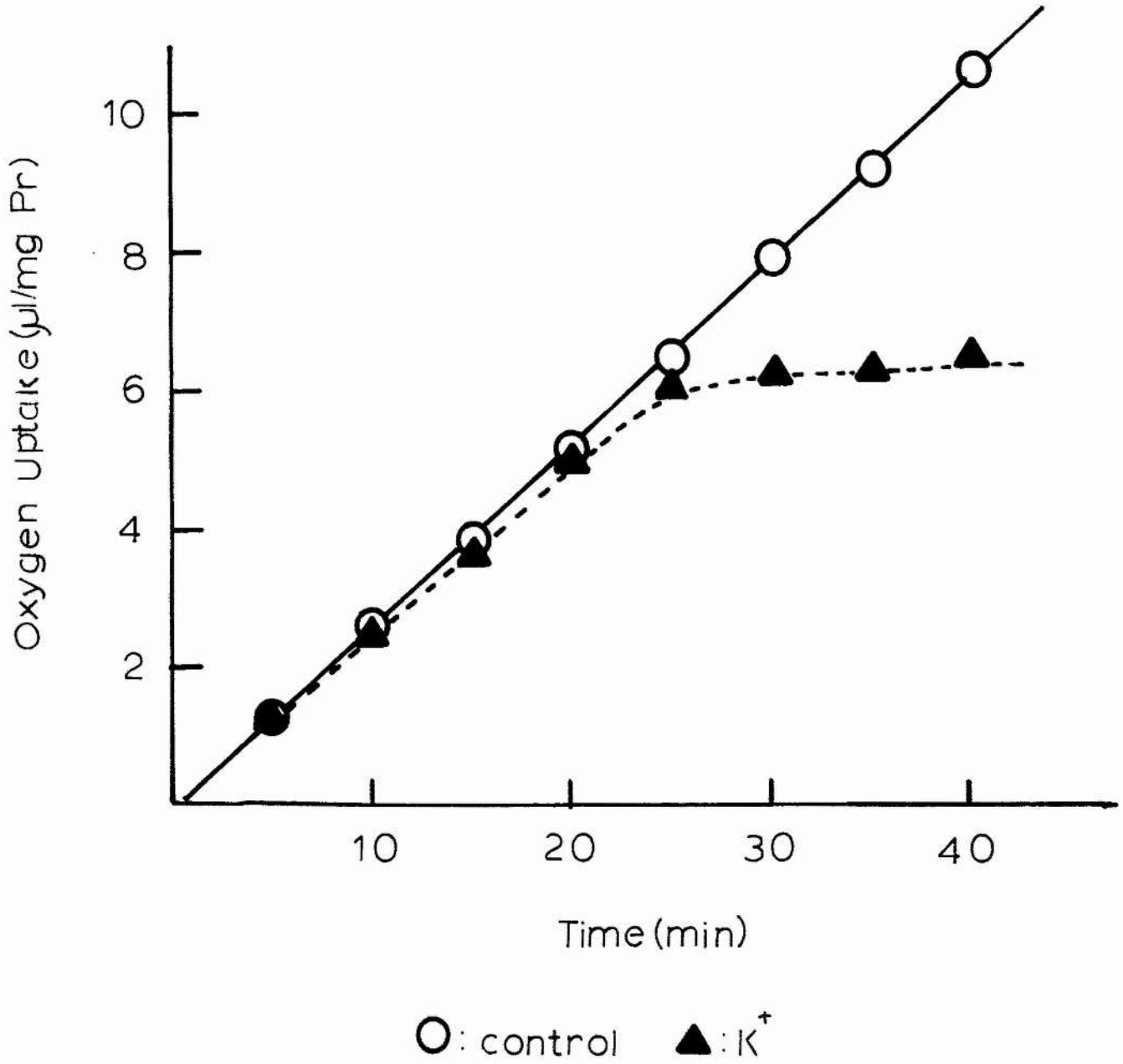


Table IV

The Effect of L-Glutamate, GABA, L-Glycine, Acetylcholine, nor-Adrenalin, Serotonin, Histamine and Reserpine on the Respiration of Synaptosomes

Expt Group	Soln Tipped	Rate Before Tipping $\mu l\ O_2$/mg Pr/hr	Rate After Tipping $\mu l\ O_2$/mg Pr/hr
I	Saline	21.0 ± 0.9	20.6 ± 0.5
	Glut	21.6 ± 0.2	20.1 ± 1.0
II	Saline	17.1 ± 1.3	17.0 ± 1.3
	GABA	16.6 ± 0.8	17.3 ± 1.2
	Gly	15.8 ± 2.1	15.8 ± 1.8
	ACh	15.8 ± 0.5	16.1 ± 0.2
	Res	19.4 ± 2.9	17.6 ± 2.6
III	Saline	17.4 ± 0.8	15.6 ± 1.2
	5-HT	16.2 ± 1.0	15.1 ± 1.0
	Hist	16.0 ± 1.2	15.0 ± 0.7
	nor-A	15.8 ± 1.1	15.4 ± 0.8

The rates of oxygen uptake of the synaptosome samples, suspended in oxygenated saline, were estimated over a 20 minute period in a Gilson Differential Respirometer (see p49).

/The

(Table IV continued)

The results are expressed as the means $\pm$ SEM of 4 estimations (3 for L-glutamate). The results before and after tipping were statistically compared by the 'students' t-test and no significant differences were found

Glut: L-Glutamic Acid (10mM final conc.)

GABA: δ-Amino Butyric Acid (5mM final conc.)

Gly: L-Glycine (2mM final conc.)

ACh: Acetylcholine (250μM final conc.)

Res: Reserpine (8.3μg/ml final conc.)

5-HT: Serotonin (20μM final conc.)

Hist: Histamine (20μM final conc.)

nor-A: nor-Adrenalin (20μM final conc.)

that there was some inorganic impurity, possibly a heavy metal ion, present in the sample.

Table IV shows that none of the compounds which were tested had any significant effect on the rate of oxygen consumption of the synaptosomes. The stability and linearity of respiration was also unaffected by any of these compounds. Readings taken only 2 minutes after the compounds were tipped into the suspensions also showed no effect (Table V). Marchbanks (personal communication) also found synaptosomal respiration to be insensitive to putative transmitter compounds.

It would seem, therefore, that, although synaptosomes may retain certain of the properties of excitable (polarised) membranes, as reported by others (Bradford 1970a,b), under the conditions used in the present experiments, either the polarised state of the membrane is unaffected by the compounds tested, or the polarised state of the membrane is not sensitively geared to the gross respiratory rate as we measure it. The system does not seem to be a suitable model, therefore, for examining the effects of anticonvulsants on the interaction of the putative transmitter compounds with the

Table V

The Effect of Drugs on the Oxygen Uptake of Synaptosomes Immediately after the Addition of the Drugs

Expt Group	Soln Tipped	Oxygen Uptake $\mu l\ O_2/mg\ Pr$	Percent of Control
I	Saline	0.71 ± 0.03	
	Glut	0.70 ± 0.04	97.1 ± 14.0
II	Saline	0.65 ± 0.42	
	GABA	0.77 ± 0.43	118.5 ± 13.9
	Gly	0.50 ± 0.24	77.0 ± 23.0
	Res	0.92 ± 0.69	141.5 ± 27.6
	ACh	0.80 ± 0.10	118.5 ± 20.0
III	Saline	0.52 ± 0.05	
	5-HT	0.52 ± 0.07	100.0 ± 7.0
	Hist	0.50 ± 0.24	96.2 ± 11.4
	nor-A	0.57 ± 0.23	109.6 ± 11.4

The oxygen uptakes of paired samples of synaptosomes were estimated in a Gilson Differential Respirometer over a 2 minute period immediately after the addition of the drugs (see p49). The results, which are the means of

/4

(Table V continued)

4 estimations ± SEM (3 for L-glutamate), were statistically compared, with the corresponding control values, by the 'students' t-test, but no significant differences were found.

Glut: L-Glutamic Acid (10mM final conc.)

GABA: γ-Amino Butyric Acid (5mM final conc.)

Gly: L-Glycine (2mM final conc.)

ACh: Acetylcholine (250µM final conc.)

Res: Reserpine (8.3µg/ml final conc.)

5-HT: Serotonin (20µM final conc.)

Hist: Histamine (20µM final conc.)

nor-A: nor-Adrenalin (20µM final conc.)

pre-synaptic membrane.

In the final series of experiments, the effect, on oxygen uptake, of the anticonvulsant acetazolamide and the convulsants strychnine and pentamethylene tetrazol were tested. Again no significant effect was observed (Table VI). This was not unexpected since these compounds, at the concentrations used, have been found to be inactive on the resting repiratory rate of other brain preparations (Anguiano and McIlwain 1951, Greengard and McIlwain 1955, Forda and McIlwain 1958, Webb and Elliott 1951).

Incorporation of ^{14}C from (U-^{14}C)-Glucose

Figure IX is the elution pattern obtained when a synaptosome suspension, which had been mixed with (U-^{14}C)-glucose just prior to its application to the top of the column, was eluted from the column with ice-cold 0.32M sucrose (p51). The bulk of the synaptosomal material is separated from the extrasynaptosomal glucose. The contamination of the synaptosome fraction was further reduced by collecting only the first ml of synaptosome suspension, which came off the column, since this contained 80 per cent of the protein, but only 1 per

Figure IX

The Separation of Synaptosomes from

Extrasynaptosomal Glucose

A synaptosome sample in ice-cold 0.32M sucrose was mixed with a sample of (U-^{14}C)-glucose and immediately eluted through a column containing 0.5g of G50 sephadex (p51). The effluent was assayed for protein and radioactivity (p52).

Fig IX

Separation of Synaptosomes from Free Glucose

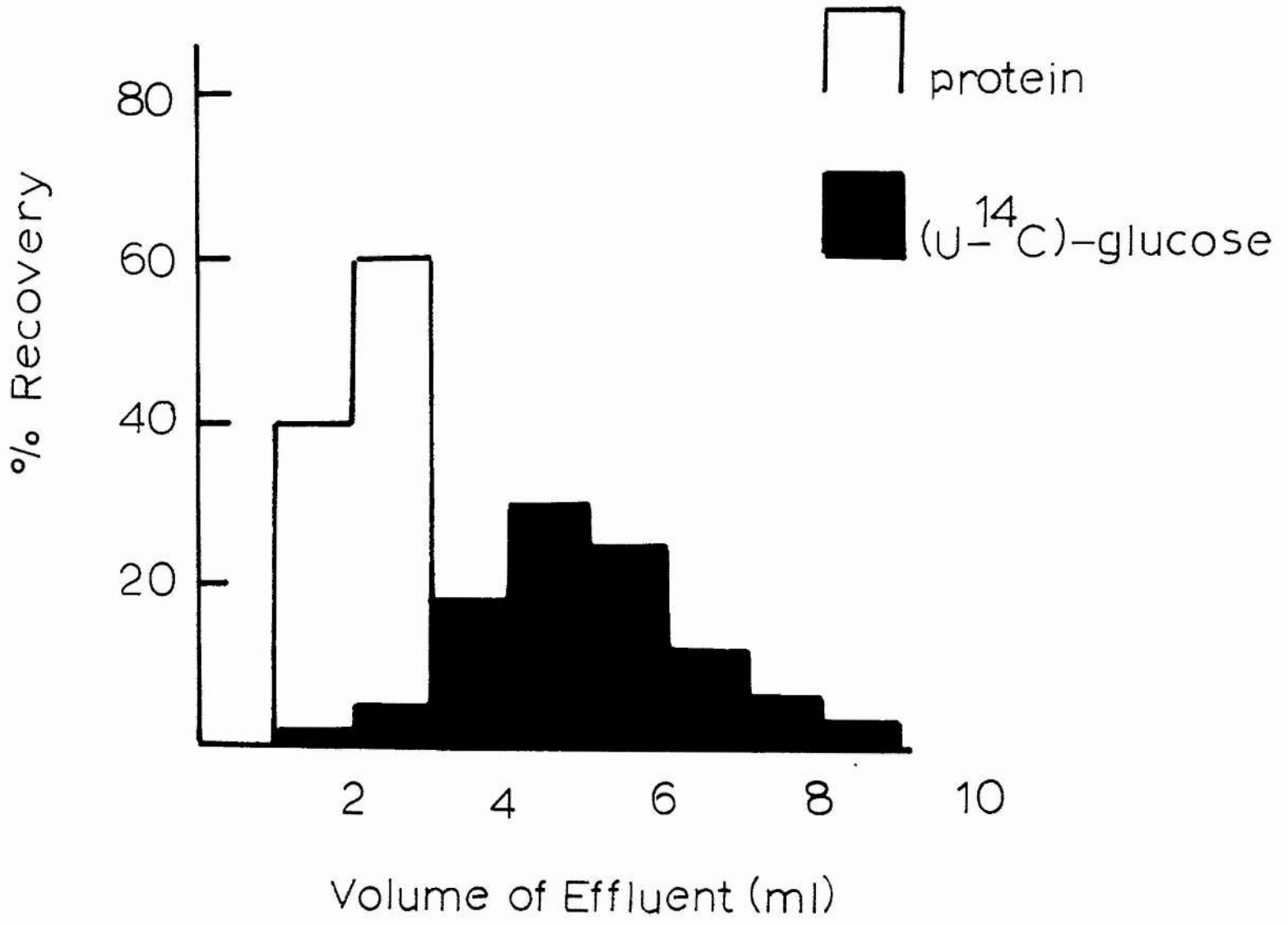


Table VI

The Effect of Convulsant and Anticonvulsant Drugs on the Oxygen Uptake of Synaptosomes

Drug	Oxygen Uptake $\mu l\ O_2/mg\ Pr/hr$	Percent of Control
None	14.4 ± 0.6	
Strych (50µg/ml)	13.7 ± 0.5	98.7 ± 1.7
PMT (50µg/ml)	14.5 ± 0.5	100.3 ± 1.7
PMT (100µg/ml)	14.9 ± 0.4	104.1 ± 2.5
Acet (20µM)	14.2 ± 0.8	99.3 ± 1.6

The oxygen uptakes of paired samples of synaptosomes were measured in a Gilson Differential Respirometer over a 45 minute period (p49) and the results are expressed as the means $\pm$ SEM of 5 experiments. The results were statistically compared, with the corresponding controls, by the 'students' t-test and no significant differences were found.

Strych: Strychnine; PMT: Pentamethylene Tetrazol; Acet: Acetazolamide.

cent of the extrasynaptosomal glucose. The recovery of both protein and radioactivity was 100 per cent in the first 10ml of effluent. These results are in agreement with those of Marchbanks (1967) who first demonstrated the separation of synaptosomes from free low molecular weight compounds by this gel filtration technique.

In Figure X the uptake of ^{14}C, derived from (U-^{14}C)-glucose, by synaptosomes is presented. It can be seen that almost 50 per cent of the total amount of radioactivity, accumulated in a 90 minute period, is taken up in the first 20 minutes. The rest is accumulated, in a linear manner, over the next 70 minutes. The initial rapid uptake of label can be attributed to the uptake of (U-^{14}C)-glucose by the synaptosomal cytoplasm. In experiments to be described in the next section of the thesis, it will be shown that the uptake of the non-metabolised sugar, xylose, by synaptosomes is almost complete after 20 minutes incubation under similar conditions. Gilbert (1965) has shown that the mechanisms of uptake of xylose and glucose by cerebral cortex slices are similar and, therefore, it is not unreasonable to suppose that the uptake of glucose by the synaptosomes

Figure X

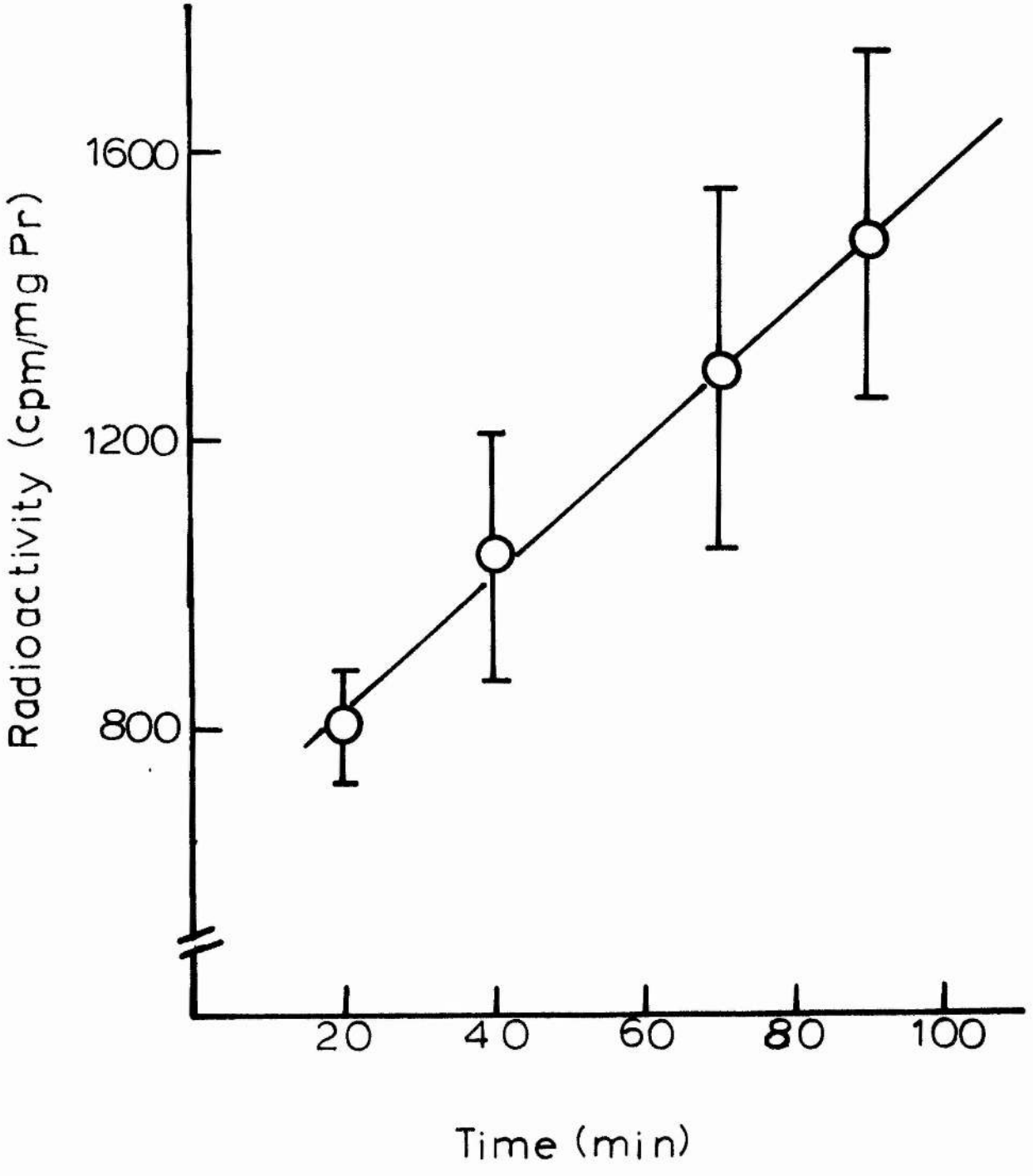
The Incorporation of ^{14}C from (U-^{14}C)-Glucose

by Synaptosomes

Synaptosomes were incubated in saline medium containing 10mM glucose (p33) and also containing (U-^{14}C)-glucose (0.5μCi) as described in the methods section (p50). The radioactivity was assayed in a Packard Tricarb Automatic Scintillation Counter (p52). The results are the means ± SEM of 3 estimations.

Fig X

The Incorporation of ^{14}C from ($U^{14}C$)-Glucose by Synaptosomes



is, for the most part, also complete after 20 minutes incubation.

The slow accumulation of radioactivity during the last 70 minutes of incubation is consistent with the incorporation of labelled carbon atoms into the metabolic pools of the particle. Bradford and Thomas (1969) have shown that, under the same conditions as those used in these experiments, most of the label, derived from (U-^{14}C)-glucose, is incorporated into lactic acid, CO_2 and free amino acids. Bradford and Thomas further reported that the free amino acids are retained within the synaptosomes. (The high rate of incorporation into lactic acid is probably due to the fact that lactic acid seems to cross neuronal and other cell membranes freely and escape into the medium (McIlwain 1966)). In our experiments, the rate of accumulation of label into the synaptosomes, over the 70 minute period during which the uptake was linear, was $4.7 \times 10^{-4} \pm 0.6\mu Ci$ per mg protein, which is approximately equivalent to the rate of incorporation of labelled carbon atoms into the free amino acids which was observed by Bradford and Thomas (1969).

In two experiments, paired samples of synaptosomes

which had been incubated with (U-^{14}C)-glucose and centrifuged as described before (p50), were resuspended, one in 0.32M sucrose and the other in distilled water, a procedure which ruptures the synaptosomal membrane and releases the contents of the cytoplasm (De Robertis et al 1963, Whittaker et al 1964). The sample, suspended in sucrose, was eluted through a sephadex column with 0.32M sucrose. The other sample was eluted through an identical column with water. The radioactive content of the sample, which had been supended in and eluted with water, was only 10 per cent of the activity of the corresponding fraction which had been suspended in and eluted with sucrose. This suggests that most of the radioactivity, inside the synaptosomes, is in the form of low molecular weight compounds which are free in the cytoplasm, a result which is in agreement with the results of Bradford and Thomas (1969).

The convulsant and anticonvulsant drugs were examined for their effects on the quantity of radioactivity incorporated into the synaptosomes during a 45 minute incubation. The drugs were tested at concentrations at which they have their effect 'in vivo'. That is to say,

the concentrations of the convulsants which cause convulsions or, in the case of the anticonvulsants, reduce or prevent the onset of convulsions in experimental animals. The anticonvulsants were also tested at 20 times their effective 'in vivo' concentrations. Table VII shows that neither strychnine or acetazolamide had any significant effect on the incorporation of ^{14}C from (U-^{14}C)-glucose, but that pentamethylene tetrazol and phenobarbitone sodium (2mM) had significant effects. Pentamethylene tetrazol inhibits the incorporation and, at concentrations in excess of 100µg per ml, the drug inhibits the incorporation by approximately 50 per cent (Fig XI). It has already been suggested that a substantial part of the ^{14}C in the synaptosomes, after incubation, is contained in unchanged glucose and that the other part is incorporated into metabolites (p65). Since subsequent work, to be reported in the next section, suggests that this drug does not affect the permeability of the synaptosomal membrane, it is probable that the drug inhibits the formation of compounds from glucose. Free amino acids form the major part of these compounds, which are retained within the synaptosomes, and it seems likely that the site of action of pentamethylene

Figure XI

The Effect of Pentamethylene Tetrazol on the Incorporation of ^{14}C by Synaptosomes

Dose Response Curve

Paired samples of synaptosomes were preincubated in saline solution containing 10mM glucose (p33), with or without drug. (U-^{14}C)-glucose (0.5pCi) was then added to the suspensions and the ^{14}C incorporation during a 45 minute incubation estimated (p50). The inhibition of ^{14}C incorporation which resulted from preincubation in the different concentrations of the drug was compared with the maximum inhibition observed (about 50 per cent of the control values) and the results are expressed as a percentage of this maximum response. Each point is the mean of at least 2 estimations.

Fig XI

The Effect of Pentamethylene Tetrazol on the Incorporation of ^{14}C by Synaptosomes

Dose Response Curve

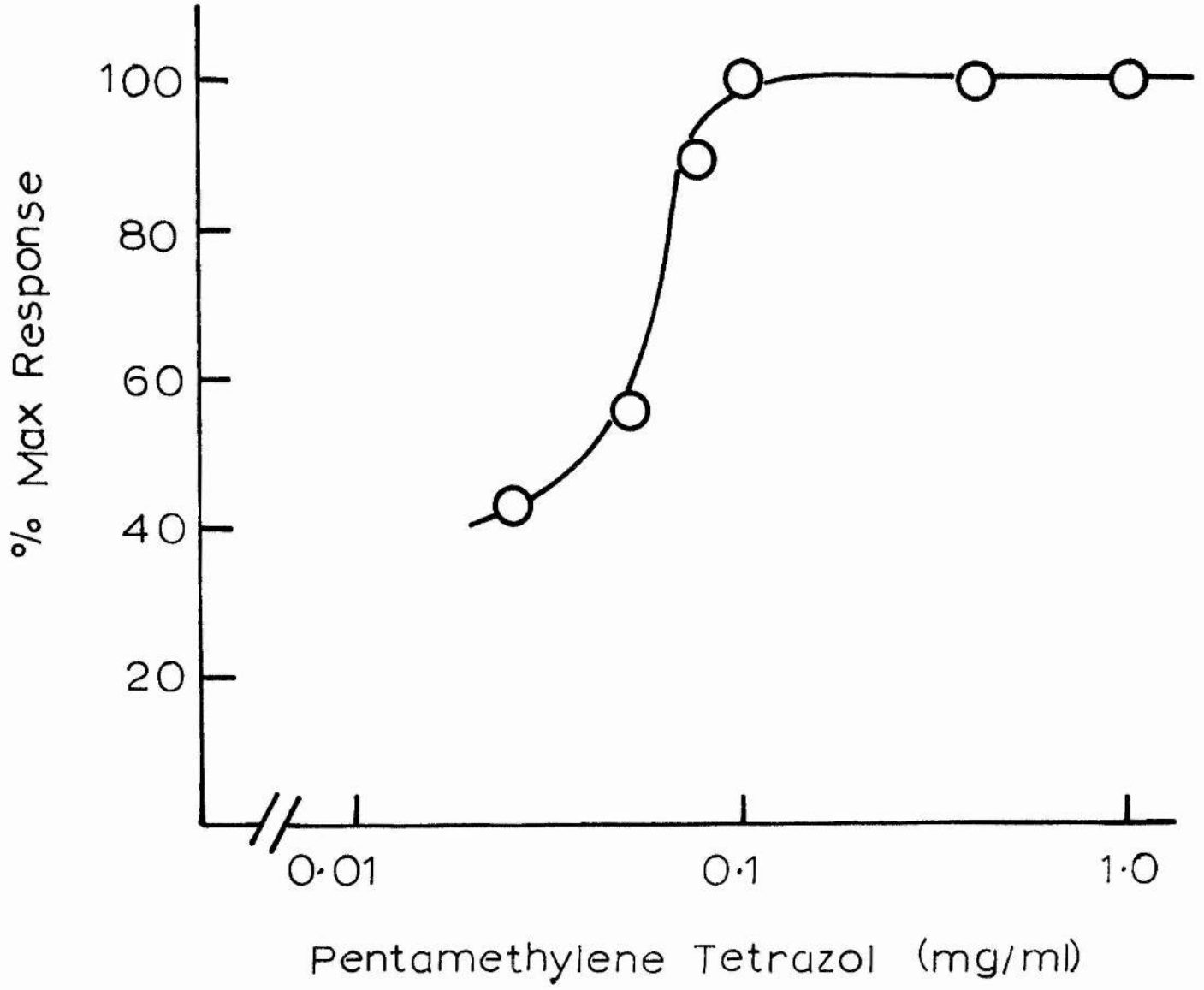


Table VII

The Effect of Convulsant and Anticonvulsant Drugs on the Incorporation of ^{14}C into Synaptosomes

Expt Group	Drug	Radioactivity cpm/mg Pr	Percent of Control	Significance from Control
I	None	$778 \pm 80_{(5)}$		
	Acet (20μM)	$721 \pm 64_{(5)}$	92.2 ± 7.0	n.s.
	Phen (100μM)	$722 \pm 24_{(5)}$	93.4 ± 8.3	n.s.
	Strych (50μg/ml)	$710 \pm 110_{(5)}$	91.3 ± 46.4	n.s.
II	None	$831 \pm 80_{(6)}$		
	PMT (50μg/ml)	$616 \pm 47_{(6)}$	67.8 ± 5.4	$P<0.01$
III	None	$685 \pm 40_{(6)}$		
	Phen (2mM)	$780 \pm 41_{(6)}$	113.8 ± 3.6	$P<0.05$
IV	None	$691 \pm 47_{(5)}$		
	Acet (400μM)	$740 \pm 46_{(5)}$	107.4 ± 6.5	n.s.

Paired samples of synaptosomes were incubated in oxygenated saline medium containing (U-^{14}C)-glucose for 45 minutes, with or without drug, and their radioactive contents were then estimated (p50). The results, which are the means ± SEM of the number of estimations given in the parentheses, were statistically compared by the

/'students'

(Table VII continued)

'students' t-test.

Acet: Acetazolamide

Phen: Phenobarbitone Sodium

PMT: Pentamethylene Tetrazol

Strych: Strychnine

tetrazol is such that it interferes with the formation of these amino acids. It was not the purpose of this work to study the metabolic effects in detail, but the possible implications of these results are discussed, in detail, in the General Discussion at the end of the thesis.

In the next section, results are reported which suggest that phenobarbitone does not affect the permeability of the synaptosomal membrane to sugars. Thus, it seems likely that the small, but significant, increase in the incorporation of ^{14}C from (U-^{14}C)-glucose caused by phenobarbitone (Table VII) is due to an increase in the amount of ^{14}C contained within the metabolites which are formed from glucose and which are retained within the synaptosomes. This effect of phenobarbitone could result from a change in the metabolic processes of the particles or from a change in the permeability of the synaptosomal membrane to the metabolites containing the labelled carbon atoms. These explainations for the effect of phenobarbitone on the incorporation of ^{14}C by synaptosomes will be discussed, at greater length, in the General Discussion, where they may be related to the effects of

the drug on the permeability of the synaptosome membrane which are reported in the next section.

SECTION III

THE EFFECTS OF DRUGS ON THE PROPERTIES OF THE SYNAPTOSOMAL MEMBRANE

Introduction

In this last experimental section, experiments will be described in which the effects of drugs on the permeability and fragility of the synaptosome membrane were examined. Synaptosomes are formed, essentially, from the pre-synaptic membrane, and it is through this membrane that the transmitter compounds are thought to pass. One of the ways, by which anticonvulsants might have their effect, is by stabilising the pre-synaptic membrane so that less transmitter leaks out into the synaptic cleft. The drugs may also stabilise the ionic gradients and increase the threshold for depolarisation and, in this way, reduce the amount of transmitter which leaks out of the nerve-ending.

In previous experiments, the effects of centrally acting drugs on the stability and permeability of erythrocyte membranes (Sheppard, Tsein and Burghardt 1969, Mikilits, Mortora and Spector 1970) or mitochondrial membranes (Spirtes and Guth 1963) have been examined. Although biological membranes have many properties in common, it is probable that each has unique features

which are specific for its biological role. It is, therefore, better to test the effects of centrally acting drugs on neuronal membranes, such as the synaptosome membrane.

Anticonvulsants alter the transport properties of neuronal cell membranes with respect to Na^+ (Hillman, Campbell and McIlwain 1963) and with respect to sugars (Gilbert, Ortiz and Millichap 1966, Gray and Gilbert 1970). The synaptosome membrane also contains an active ATPase (Hosie 1965) and this enzyme is reported to be intimately involved in the active transport of Na^+ across cell membranes (Skou 1957). In this work the effects of the drugs on aspects of the potential active transport systems of the synaptosomal membrane were, therefore, tested. The effects of the drugs on the ATPase activity of the fraction were examined, since a change in ATPase activity could result in a redistribution of ions across the membrane. Secondly, the effects of phenobarbitone (2mM) and pentamethylene tetrazol (50µg per ml) on the rate of xylose uptake were tested. At these concentrations the drugs altered the rate of incorporation of ^{14}C derived from (U-^{14}C)-glucose and it was of interest to know if they also altered the rate of uptake of a

non-metabolised sugar which is taken up, by other CNS preparations, by a similar mechanism to glucose. Gilbert, Gray and Heaton (1971) have proposed that an increased intracellular sugar concentration might stabilise membranes and they have further suggested that some anticonvulsants, including phenobarbitone, may, in part, have their effect in this way. In order to compare the uptake of xylose into synaptosomes with that into cerebral cortex slices, the effect of 2,4-dinitrophenol on the final level of xylose taken up by the synaptosomes was examined, since Gilbert (1966) has shown that this sugar seems to be accumulated against a concentration gradient by the cells of cerebral cortex slices. 2,4-dinitrophenol restricts the availability of ATP by uncoupling the electron transport chain and, thereby, inhibits active transport (Lehninger 1965). Gilbert (1966) found that the inclusion of 2,4-dinitrophenol in the incubation medium prevented the accumulation of xylose by the slices.

In all the experiments, which have been described so far in this thesis, the effects of the drugs have been examined on synaptosomes which were actively metabolising added substrate and absorbing oxygen. In

the last part of the study, the effects of the drugs on the mechanical properties of the synaptosome membrane were examined. These experiments were performed on synaptosome samples suspended in sucrose and under these unphysiological conditions little metabolism is likely to occur. The purpose of these experiments was to discover if the drugs altered the rate at which the constituents of the synaptosomal cytoplasm are lost to the medium, since this is also of great importance when trying to establish a mode of action for the drugs.

Work by Tedeschi and Harris (1955) and by Koch (1961) has shown that the change in the light scattering of a suspension of small particles is a function of the volume changes which may therefore be followed by observing the change in the reciprocal of the extinction of the suspension. In recent work, Keen and White (1970) have shown that synaptosomes perform as osmometers, when they are suspended in NaCl solution, and that changes in the volume may be followed by observing the change in the reciprocal of the extinction, at 520nm, of the suspension. Using this fact, experiments were performed to test the effects

of preincubation with the drugs on the volume of the synaptosomes in NaCl, which was approximately isotonic, and on the mechanical properties of the synaptosome membrane. Some forms of epilepsy may result from imperfect neuronal membranes, caused by errors in the laying down of the membranes at birth, or by subsequent injury and, therefore, some anticonvulsants, especially the barbiturates, may have their action by interacting directly with the cell membranes to alter their mechanical properties. In subsequent experiments, the effect of phenobarbitone on the release of specific constituents of the synaptosomal cytoplasm was examined.

Materials and Methods

Materials

Na_2ATP	Supplied by Sigma Ltd. London
n-Ethylmaleimide	
p-Chloromercurybenzoate	
Ouabain	
Dithiobisnitrobenzoate	Supplied by The Aldrich Chemical Company, Milwaukee. Wis.
NE 220 Scintillation Fluid	Supplied by Nuclear Enterprises, Edinburgh.
2,4-Dinitrophenol	Supplied by British Drug Houses, Poole. Dorset.
(U-^{14}C)-Xylose	Supplied by the Radiochemical Centre, Amersham. Bucks.
Thiopentone	Supplied by Abbot Laboratories, Queenborough. Kent.

Methods

ATPase Assay

ATPase activity was estimated using, essentially, the method of Samson and Quinn (1967). 25ml conical flasks were charged according to the table below:-

Flask	Medium	Water	Drug Soln	Susp	TCA (5%)	Phosphate
Blank	3.0ml	-	-	0.3ml	2.5ml	1.5ml
Control	3.0ml	1.5ml	-	0.3ml	-	-
Test	3.0ml	-	1.5ml	0.3ml	-	-

The medium consisted of NaCl 170mM, KCl 34mM, $MgCl_2$ 10mM, Tris-HCl buffer (pH7.4) 80mM. (The concentrations are those finally attained, once all the solutions have been added to the flasks). The blanks were put on ice, while the remaining flasks were shaken in a Dubnoff shaker, at 30°C for 30 minutes. 0.6ml of 51mM Na_2ATP was then added to the flasks to start the reaction, and the incubation was continued for a further 20 minutes. (It was found that, if the blank was incubated also, the trichloracetic acid reacted in some way to release more inorganic phosphate). After this time the reactions were stopped by the addition

of 2.5ml of 5 per cent trichloracetic acid solution. The mixtures were left on ice for 10 minutes and were then spun down on a bench centrifuge for 5 minutes. The supernatants were kept and assayed for inorganic phosphate. The enzymic activity, for each condition, was assayed in triplicate.

Phosphate Estimation

Inorganic phosphate was estimated using a modification of the method of Fiske and Subbarow (1925). The following solutions were prepared:-

(a) 25g of ammonium molybdate was dissolved in 300ml of 10N H_2SO_4 and made up to 1 litre with distilled water.

(b) 0.125g of aminonaphtholsulphonic acid was dissolved in 195ml of 5 per cent sodium bisulphite solution. 20 per cent sodium sulphite solution was added slowly until the precipitate, which forms, just disappears. The reagent was kept at $4^{\circ}C$ in the dark and filtered before use.

The colour development, with this reaction, is time

dependent and, therefore, the assay was modified for use on a Tecnicon Autoanalyser which enabled us to read the colour of each sample after the same time elapse. The automated system is described in Figure XII. The system analysed 60 samples per hour and the flow rates were:- sample 0.80ml per minute, ammonium molybdate 0.42ml per minute, aminonaphtholsulphonic acid 0.16ml per minute, water 1.20ml per minute, air 0.60ml per minute. The colorimeter was set to read at 662nm. Each sample was run in duplicate and the trace from one of the runs is presented (Fig XIII). In Figure XIV the plot of the standards is shown.

Uptake of (U-^{14}C)-Xylose

2.0ml of synaptosome suspension, in saline solution containing 10mM glucose (p33), was placed in the main compartment of a manometer flask and 0.25ml of saline, with or without drug, was also added. 0.5ml of saline, containing (U-^{14}C)-D-xylose (0.5μCi of radioactivity), were placed in the side-arm. The flask was gassed briefly with O_2 and stoppered and preincubated for 20 minutes at 37°C. The xylose was then tipped into the main

Figure XII

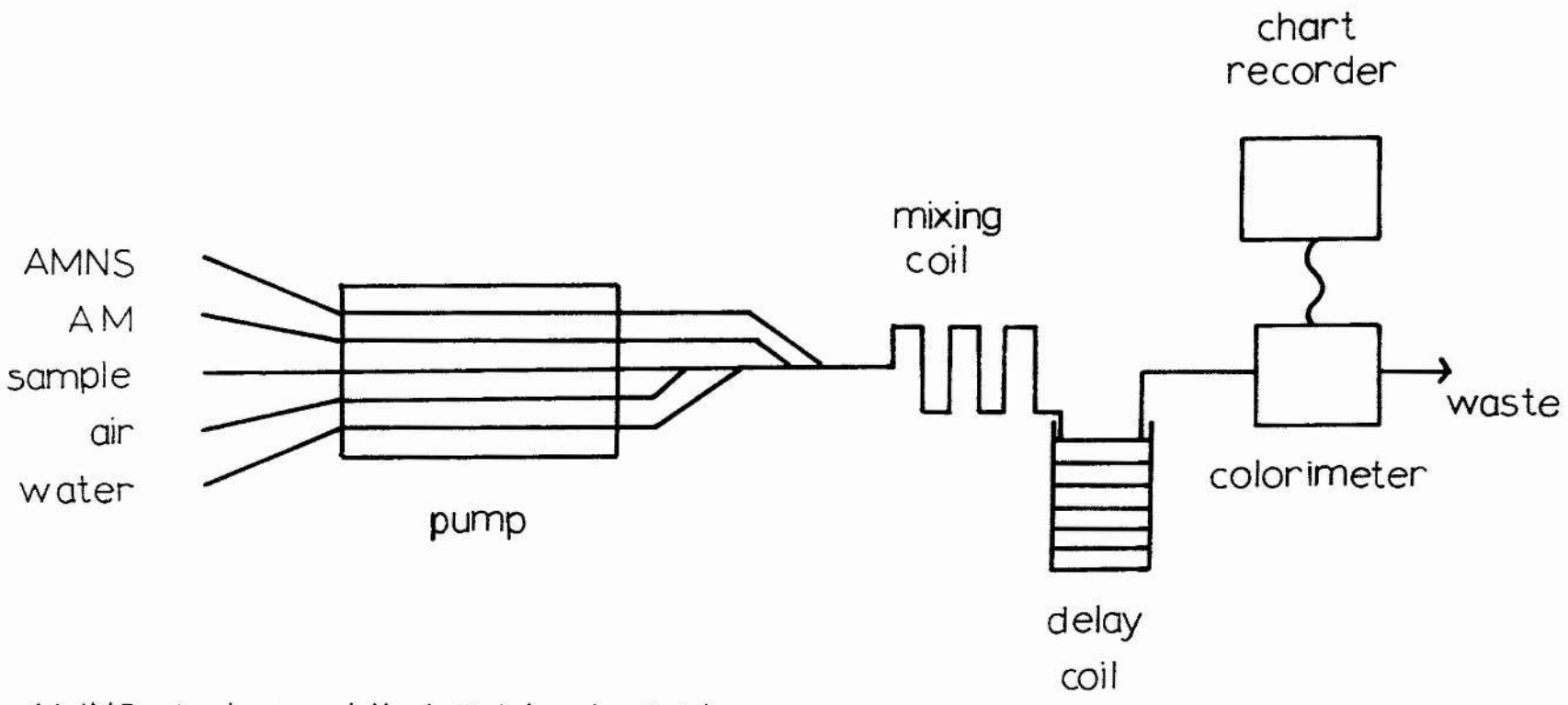
Autoanalyser System to Assay Phosphate

The phosphate assay procedure of Fiske and Subbarow (1925 - see p81) was adapted for use on a Tecnicon Autoanalyser. The residence time in the system was 20 minutes for each sample, the colorimeter was set at 662nm and the system analysed 60 samples per hour. A sample trace is presented in Figure XIII. The flow rates for each of the reagents were:-

Sample:	0.80ml per minute
Ammonium Molybdate:	0.42ml per minute
Aminonaphtholsulphonic Acid:	0.16ml per minute
Water:	1.20ml per minute
Air:	0.60ml per minute

Fig. XII

Autoanalyser System to Assay Phosphate



AMNS : Aminonaphthol Sulphonic Acid

AM : Ammonium Molybdate

Figure XIII

Trace from Autoanalyser

This trace, taken from the Technicon Autoanalyser, represents the results of a phosphate estimation using the modification of the method of Fiske and Subbarow (1925) described on p81. In order to avoid any bias in the results due to a slow drift of the base-line which might occur during the run, each sample was assayed twice, once in the first part and once in the second part of a symmetrical run, and the mean taken. (i.e. The second part of the run was identical to the first, but run in the reverse order). Starting at either end, the first 3 peaks (4 from the left) are phosphate standards, the next 2 peaks are tissue blanks and the remaining peaks, in groups of 3, are the test samples in the following order (see p80 for details):- controls, pentamethylene tetrazol (50µg per ml), strychnine (50µg per ml), ouabain (0.1mM), phenobarbitone sodium (0.1mM) and acetazolamide (20µM).

Technicon• AUTOANALYZER CHART NO. R.0483

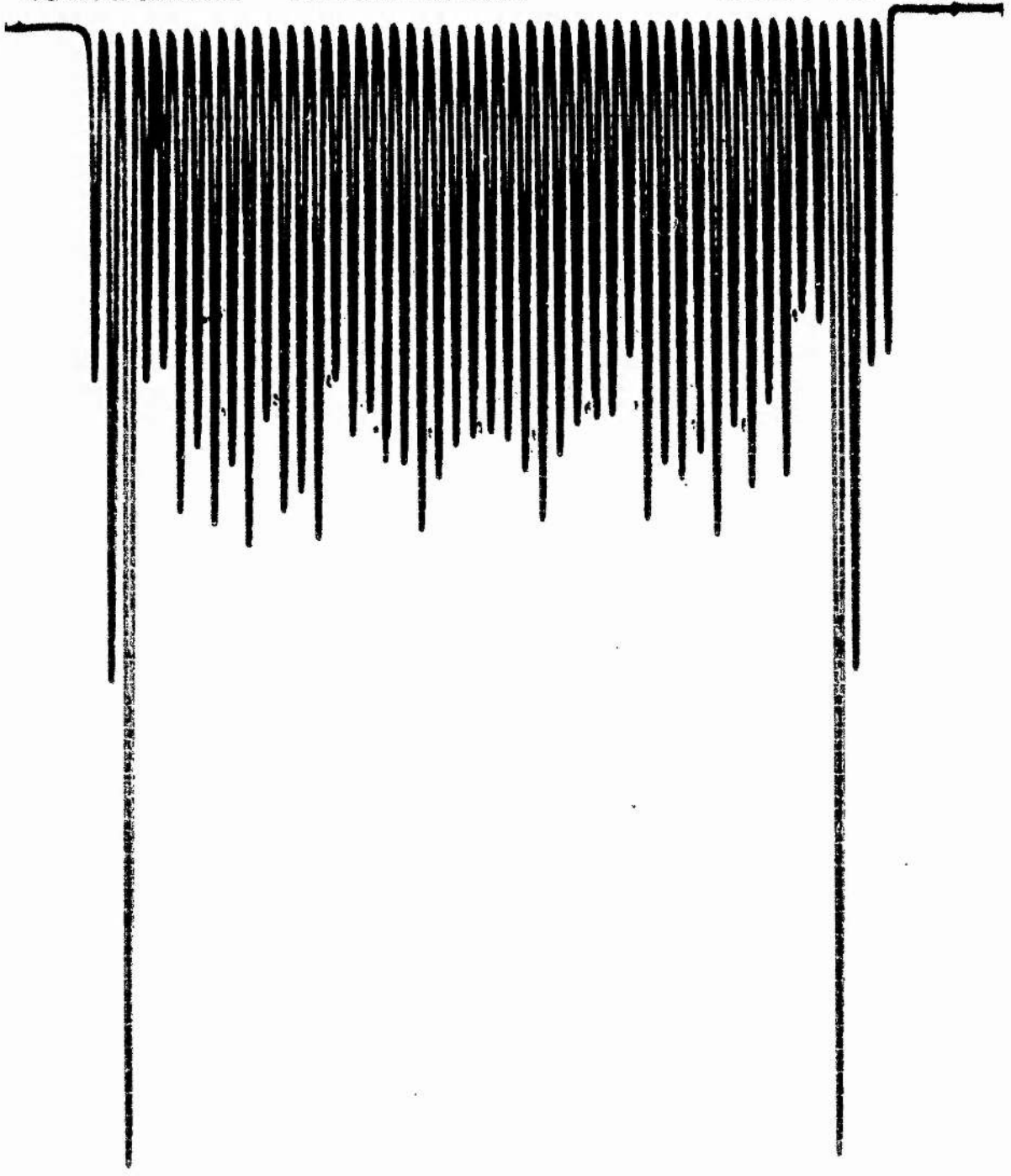


Fig 1E

Figure XIV

Phosphate Estimation

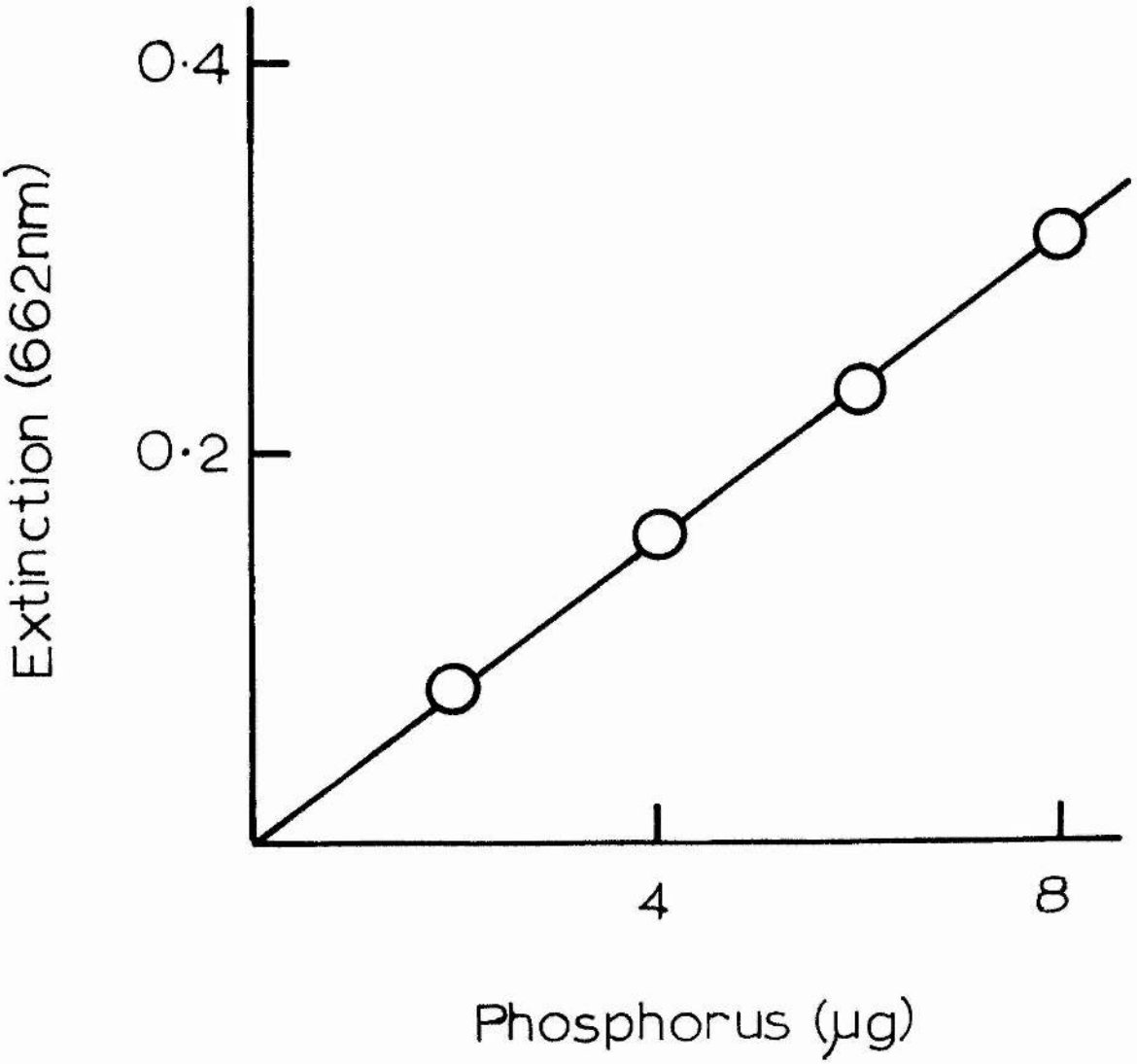
Standard Curve

Standard solutions, containing known weights of inorganic phosphate, were assayed on a Tecnicon Autoanalyser (Fig XII) by the method of Fiske and Subbarow (1925) as described in the methods section (p81).

Fig XIV

Phosphate Estimation

Standard Curve



chamber (final concentration 30mM) and the incubation was continued for the required time. The incubation was stopped by transfering the suspension to a 10ml centrifuge tube and centrifuging for 90 seconds on a bench centrifuge. The synaptosome pellet was rapidly resuspended in ice-cold 0.32M sucrose and separated from the extrasynaptosomal xylose using the gel filtration technique described in an earlier section (p51). The synaptosome fraction, after elution from the sephadex column, was assayed for its protein and radioactive content. In these experiments, the ^{14}C activity was assayed on a scintillation counter (p52) but using NE 220 scintillation fluid which had a counting efficiency of 75 per cent, under the conditions of the experiment. As before the efficiency was always checked using the channels ratio method.

Measurements of Volume Changes in Synaptosomes

The changes in the volumes of the synaptosomes were estimated by following the change in the reciprocal of the extinction of each suspension at 520nm, as described by Keen and White (1970). 0.2ml of synaptosome suspension,

in 0.32M sucrose and containing about 50µg of protein, was pipetted into a cuvette containing 2.8ml of NaCl solution of known molarity. Air was gently bubbled through the mixture until an even suspension had been formed and then the extinction was measured at 520nm. The extinction of duplicate samples was always estimated and the mean taken.

In the first experiments, synaptosomes were suspended in NaCl solution of varying molarities (0.2M to 0.04M) to test the conformity to the van't Hoff Law. In subsequent experiments, paired samples of synaptosomes, taken from the same preparation, were preincubated, in the presence or absence of drug, for 30 minutes at 25°C. 0.2ml samples were suspended 0.2M NaCl and, when required, 0.05M NaCl and the extinctions estimated as described above. The tonicity of 0.2M NaCl is similar to that of the sucrose in which the synaptosomes were suspended, whereas the 0.05M NaCl is relatively hypotonic. In some experiments 0.1ml of 4.5M NaCl solution was added to the cuvettes containing synaptosomes in 0.05M NaCl, to return the molarity of the NaCl to 0.2M. The extinctions were then measured again.

The Effect of Phenobarbitone on the Release of K^+, Na^+ and Xylose from the Synaptosomal Cytoplasm

Paired samples of synaptosomes were preincubated, in the presence or absence of phenobarbitone, for 30 minutes at 25°C. The samples were then eluted through sephadex columns with ice-cold 0.32M sucrose (p51) in order to separate the particles from the extrasynaptosomal K^+ and Na^+. The synaptosome fractions from the columns were then assayed for their protein and ion content. The K^+ and Na^+ content was estimated on an EEL flame photometer. Marchbanks (1967) reported that Na^+ and sucrose did not interfere with the K^+ estimation, but that K^+ interfered, to an extent of 5 per cent, in the Na^+ estimation. We were looking, here, for a change in the ion contents, so the results were compared with standard graphs formed from solutions prepared in the absence of the other ion.

In other experiments, 2ml of synaptosome suspension, in oxygenated saline medium containing 10mM glucose (p33), was incubated at 37°C for 20 minutes. 0.5ml of saline, containing (U-^{14}C)-xylose (30mM final concentration and containing 0.5μCi of radioactivity), was then added

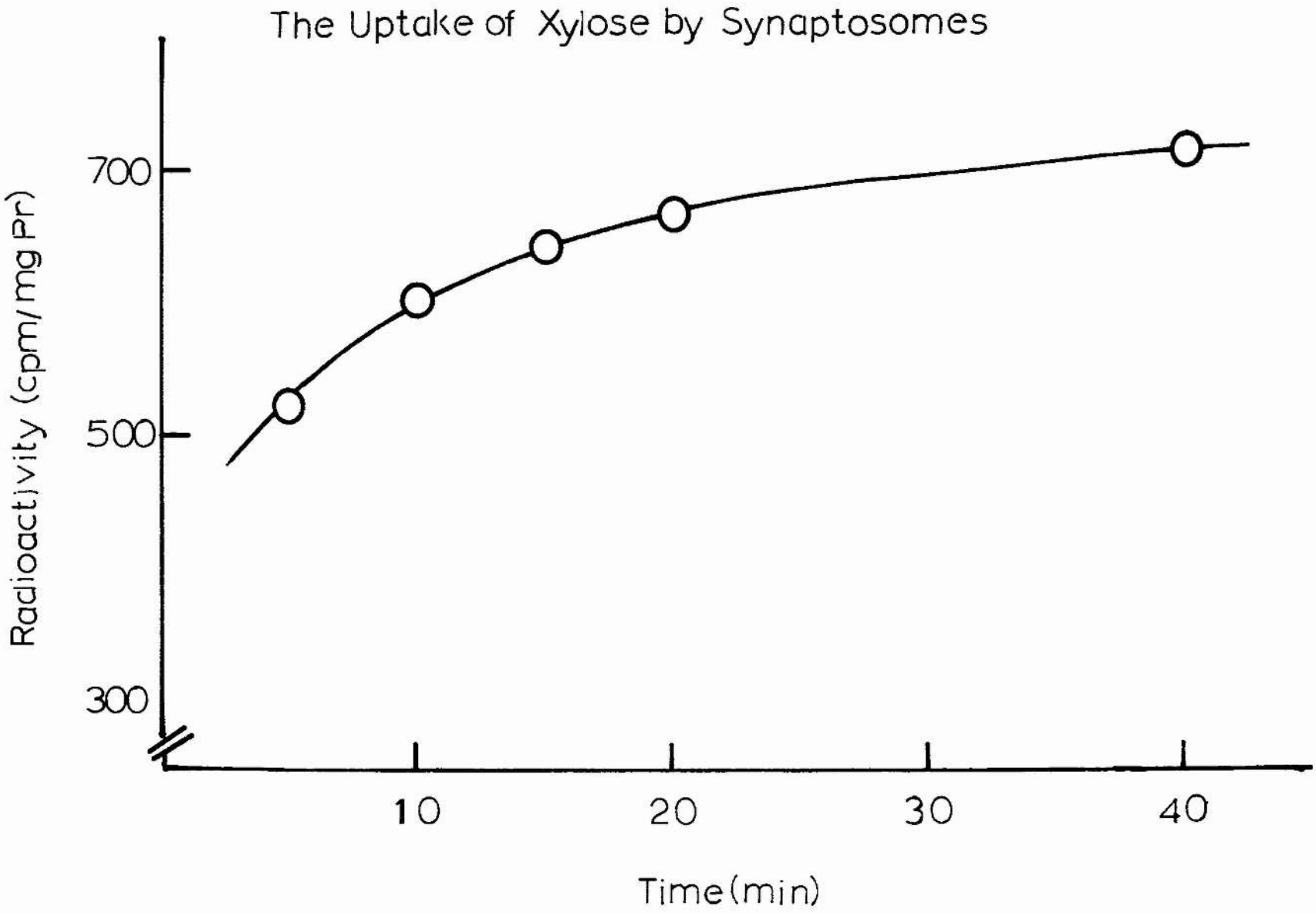
(see p82) and the incubation was continued for a further 30 minutes. The uptake of xylose by the synaptosomes appears to be complete after this time (Fig XV). The synaptosomes were then centrifuged on a bench centrifuge for 90 seconds and paired samples were rapidly resuspended in 0.32M sucrose, with or without drug, and incubated at 25°C for 7 minutes. The synaptosome suspensions were then eluted through G50 sephadex columns (p51) and the synaptosome fractions from the columns were assayed for their ^{14}C and protein content. The radioactivity was assayed on a scintillation counter, using NE 220 scintillation fluid (p83).

Figure XV

The Uptake of Xylose by Synaptosomes

Synaptosome samples were incubated in saline medium containing 10mM glucose (p33) and 30mM (U-^{14}C)-xylose (0.5μCi). The radioactive content of the synaptosomes was estimated in a Packard Tricarb Automatic Scintillation Counter (p82). The results are the means of 2 estimations

Fig XV


The Uptake of Xylose by Synaptosomes
Radioactivity (cpm/mg Pr)
700
500
300
10
20
30
40
Time(min)

Results and Discussion

ATPase Activity

Total ATPase activity has been assayed in this work and, under our conditions, the activity of the enzyme was 9.1 ± 0.2μmoles P_i liberated per mg protein per hour. This result is a little lower than those reported previously (Hosie 1965, Kurokawa et al 1965), but this difference may well be due to the lower temperature used here (Samson and Quinn 1967) or to species differences.

In Table VIII the effects of preincubation in a number of drugs, on ATPase activity, is shown. Ouabain (0.1mM) inhibited the activity by approximately 15 per cent. This effect of ouabain is well known, and the degree of inhibition caused is not only concentration dependent but also dependent upon the species (Rawson and Pincus 1968). Rawson and Pincus also reported that most anticonvulsants do not seem affect the activity of the ATPases. In this work, however, small changes in ATPase activity were observed after preincubation in acetazolamide (20μM) and pentamethylene tetrazol

Table VIII

The Effect of Drugs on ATPase Activity

Expt Group	Drug	ATPase Activity $\mu g\ P_i$/mg Pr/hr	Percent of Control	Significance from Control
I	None	270 ± 12		
	Phen	279 ± 15	101.0 ± 1.0	n.s.
	Ouab	237 ± 15	88.3 ± 1.7	P<0.01
II	None	291 ± 15		
	PMT	300 ± 15	101.8 ± 0.6	P<0.05
	Strych	294 ± 18	102.8 ± 14.4	n.s.
III	None	279 ± 15		
	Acet	270 ± 15	96.8 ± 1.1	P<0.05

Paired samples of synaptosomes were preincubated, in the presence or absence of drugs, for 30 minutes, and then the ATPase activity was assayed at 30°C, using Na_2ATP as substrate, as described in the methods section (p80). The results represent the means ± SEM of the number of experiments given in the parentheses. The results were statistically compared by the 'students' t-test.

/Phen

(Table VIII continued)

Phen: Phenobarbitone Sodium (0.1mM)

Ouab: Ouabain (0.1mM)

PMT: Pentamethylene Tetrazol (50µg/ml)

Strych: Strychnine (50µg/ml)

Acet: Acetazolamide (20µM)

(50µg per ml). Neither strychnine or phenobarbitone sodium had any effect on ATPase activity, at concentrations at which the drugs affect membrane properties in other CNS preparations. Under our conditions, acetazolamide inhibited ATPase activity by 3 per cent, whereas, pentamethylene tetrazol stimulated the activity of the enzyme to a similar degree. These effects are small and probably contribute little to the mode of action of the drugs. During the time that this work was being performed, conflicting reports about the mode of action of diphenylhydantoin, another anticonvulsant drug, were reported. This drug, whose mode of action is thought to be a stimulation of the Na^+ pump (Woodbury 1955), can either stimulate or inhibit the activity of the enzyme, depending on the conditions used (Festoff and Appel 1968, Rawson and Pincus 1968, Formby 1970). Formby further suggested that the preincubation procedure, used to test the effects of these drugs 'in vitro', may give misleading results. In view of these findings and the difficulty in the interpretation of the results, the study of ATPase activity was limited to the results presented in Table VIII.

The Uptake of (U-^{14}C)-Xylose by Synaptosomes

The elution pattern, shown in Figure XVI, demonstrates the ability of a sephadex column to separate synaptosomes from free extrasynaptosomal xylose. As in the case of glucose, the recovery of xylose was 100 per cent in the first 10ml of effluent.

The uptake of xylose was measured under conditions in which the particles were actively metabolising substrate. In previous work Gilbert (1965) has shown that cerebral cortex slices transport xylose into the cells by a carrier mediated system, and that the slices seemed to be able to accumulate xylose against a concentration gradient (Gilbert 1966). Since glucose competed for the xylose carrier, Gilbert used pyruvate as the substrate for the slices, since slices are able to perform equally well on either pyruvate or glucose (Elliott and Wolfe 1962). This does not seem to be true for synaptosomes since, if pyruvate replaces glucose as substrate, there is a 40 per cent increase in the oxygen uptake (Bradford 1969). This is in agreement with an earlier finding, reported by Elliott and Wolfe (1962), that a similar increase in the oxygen uptake was

Figure XVI

The Separation of Synaptosomes from

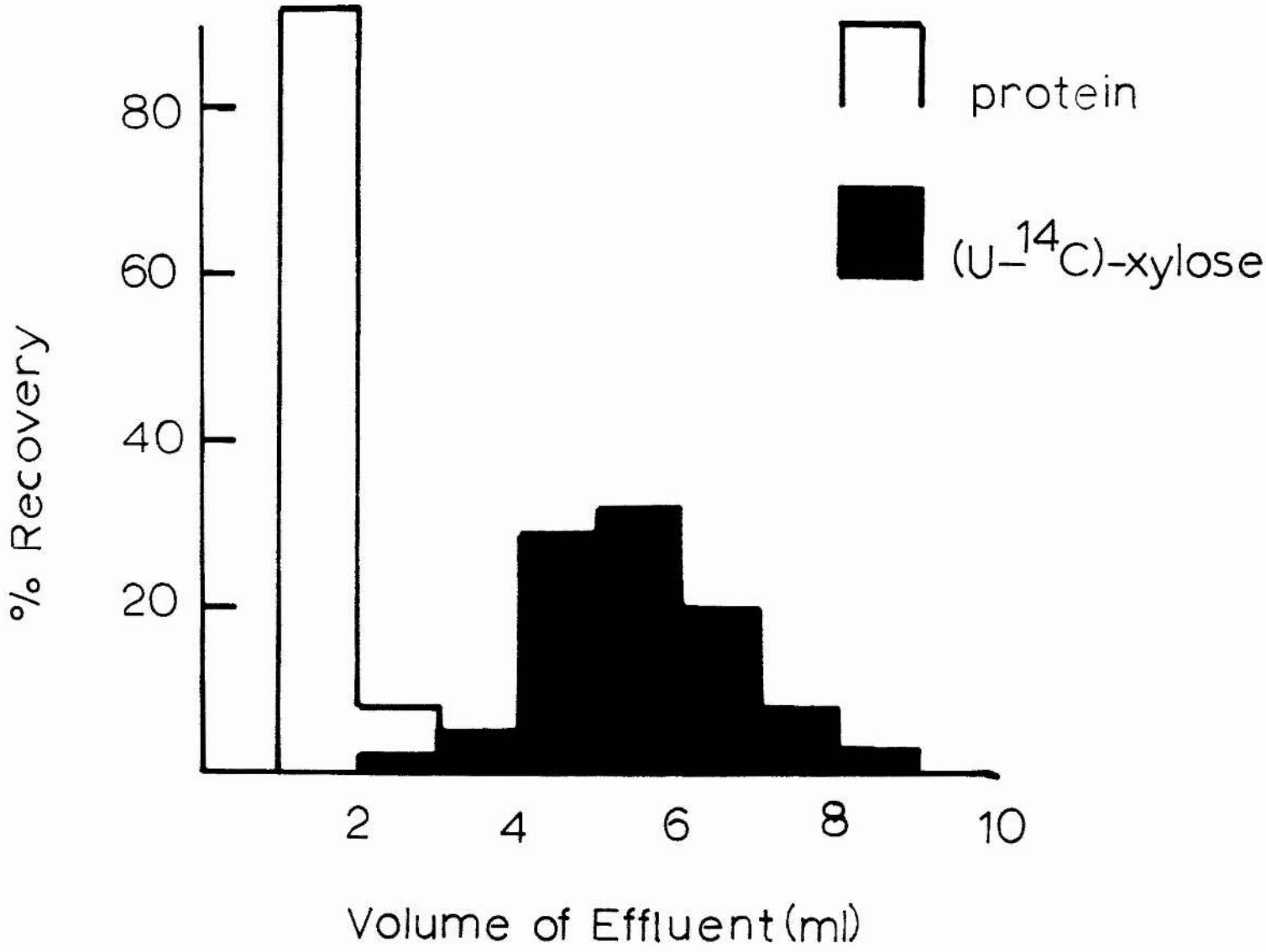
Extrasynaptosomal Xylose

A synaptosome sample in ice-cold 0.32M sucrose was mixed with a sample of (U-^{14}C)-xylose and immediately eluted through a column containing 0.5g of G50 sephadex (p51). The effluent was assayed for protein and radioactivity (p52).

Fig XVI

Separation of Synaptosomes from

Free Xylose



observed if pyruvate replaced oxygen as the substrate for the respiration of brain homogenates, prepared in such a way that synaptosomes would have been present in the homogenates. Although Bradford (1969) reported that the respiratory rate was stable if synaptosomes were incubated with pyruvate as substrate, this was not found to be the case for our synaptosome preparation and, for these reasons, xylose uptake was measured in the presence of 10mM glucose. It was considered that the xylose concentration used (30mM) was sufficiently great to compete for a possible common carrier, but not so great that any accumulation within the particle might have a detrimental affect on the synaptosomes.

The uptake of xylose is shown in Figure XV and it may be seen that the uptake seems to be complete after 20 minutes incubation. To test the possibility that xylose is accumulated against a concentration gradient, as in the case of cerebral cortex slices, the effect of 2,4-dinitrophenol on the final concentration of the sugar attained by the synaptosomes was tested. Table IX shows that this compound had no significant effect on the xylose concentration. In fact there was a small

Table IX

The Effect of 2,4-Dinitrophenol on the Equilibrium Xylose Concentration of Synaptosomes

Condition	Radioactivity cpm/mg Pr	Percent of Control	Significance from Control
Control	661 ± 45		
2,4-DNP	698 ± 36	106.1 ± 5.6	n.s.

Synaptosome samples were incubated in saline medium containing 10mM glucose (p33) and (U-^{14}C)-xylose for 40 minutes, at 37°C, with or without 2,4-dinitrophenol (1mM), as described in the methods section (p82). The results, which represent the means ± SEM of 5 paired experiments, were statistically compared by the 'students' t-test.

increase in the amount of xylose taken up, and this probably reflects changes due to the disturbance of the ionic gradients which also rely on active transport processes (Bradford 1970a).

If we assume that there is no accumulation of xylose against a concentration gradient, and that after 40 minutes incubation the concentration of xylose inside the synaptosomes equals that of the medium, then we may calculate the volume of the synaptosome accessible to xylose.

Let x be the intrasynaptosomal volume (µl per mg protein). We can assume at equilibrium that

$$\text{cpm/µl intrasynaptosomal volume} = \text{cpm/µl medium}$$

$$\text{but cpm/µl intrasynaptosomal volume} = \frac{\text{cpm/mg Pr at equilibrium}}{x}$$

$$\therefore x = \frac{\text{cpm/mg Pr at equilibrium}}{\text{cpm/µl medium}}$$

The volume of the synaptosomes, calculated in this way, is 2.2 ± 0.1µl/mg protein (mean of 5 estimations) which is similar to that found by Marchbanks (personal communication) which was 2.0µl/mg protein. This result

is important since it suggests that our technique is sound and that the assumption that we have made (i.e. that, under our conditions, there is no accumulation of xylose against a concentration gradient) is reasonable. The difference between the value obtained here and that obtained by Marchbanks is probably due to the difference in the conditions under which the volumes were estimated. In this work, the synaptosomes were metabolising substrate and ion gradients were probably present (Bradford 1969, 1970a), but this was not the case in the experiments from which Marchbanks calculated the volume.

In the simplest case, the uptake of xylose by the synaptosomes will occur by means of a simple diffusion process. This process may be presented mathematically in the form of the equation below:

$$\frac{dx}{dt} = kx$$

Integrate:

$$\ln \left(\frac{X_o}{X_o - x}\right) = kt$$

$$\text{or} \qquad \log \left(\frac{X_o}{X_o - x}\right) = \frac{kt}{2.303}$$

X_o is the concentration of xylose in the medium and x is the concentration of xylose in the synaptosomes after time t. In these experiments, where (U-^{14}C)-xylose has been used, the concentration of xylose is directly proportional to the radioactive concentration (cpm per µl). If we assume that the concentration of xylose inside the synaptosomes, after 40 minutes incubation, equals that of the medium (p94) then, if v is the intrasynaptosomal volume, C_e is the radioactive concentration (cpm per mg protein) of the synaptosomes after 40 minutes and c is the radioactive concentration (cpm per mg protein) of the synaptosomes at time t, we may rewrite the equation in the form:

$$\log \frac{\frac{C_e}{v}}{\frac{C_e - c}{v}} = \frac{kt}{2.303}$$

$$\text{or} \quad \log \left(\frac{C_o}{C_o - c}\right) = \frac{kt}{2.303}$$

The logrithmic plot for the uptake of xylose into the synaptosomes (Fig XVII) shows that the points fall on a straight line, but that the line does not pass through the origin as one would expect if the uptake had been a simple diffusion process. This suggests that xylose uptake, by synaptosomes, may be enhanced by interaction with a membrane component. (This possibility is discussed in relation to a theoretical treatment of the results in the appendix).

From the slope of the logrithmic plot, the flux of xylose across the synaptosomal membrane may be calculated (Marchbanks 1968b). Clementi et al (1966) reported that the mean radius of synaptosomes in fraction B was 0.28×10^{-4} cm and Marchbanks (1968b) used this value as a means of calculating the flux F in terms of uumoles per cm^2 per minute. The flux k' in uumoles per mg protein per minute may be obtained by dividing k (which may be obtained from the slope of the logrithmic plot - see equation above) by the specific activity of the (U-^{14}C)-

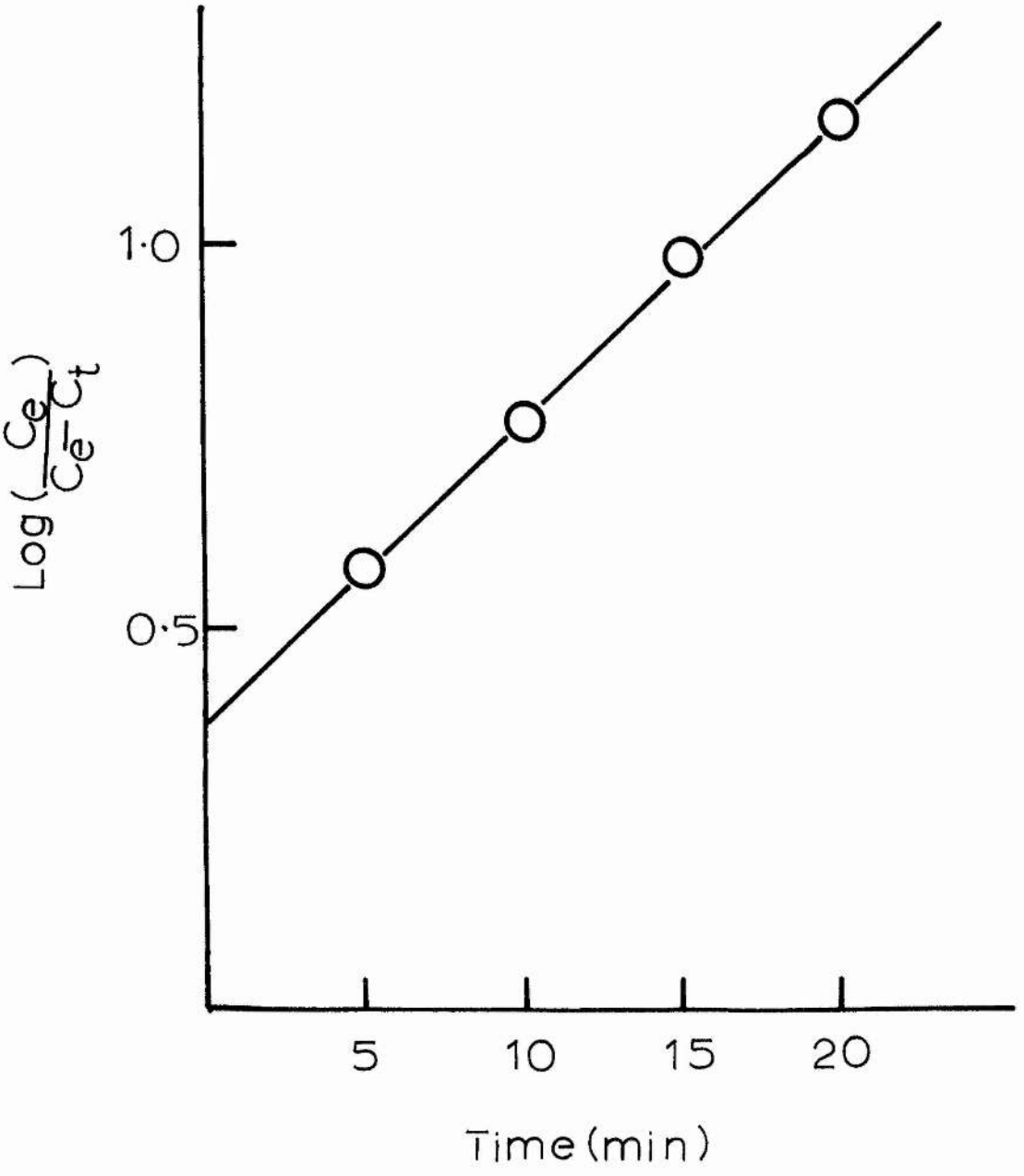
Figure XVII

Log Plot of (U-^{14}C)-Xylose Uptake by Synaptosomes

The uptake of xylose by synaptosomes (Fig XV) has been plotted in a log form. The theoretical basis for this plot is shown on p95. C_e is the radioactive concentration (cpm per mg protein) of the synaptosomes at equilibrium (after 40 minutes incubation) and C_t is the concentration (cpm per mg protein) after t minutes incubation. The results are the means of 2 estimations.

Fig XVII

Log Plot of the Uptake of
(U-^{14}C)-Xylose by Synaptosomes



xylose. If v is the intrasynaptosomal volume (ml per mg protein) and r is the mean synaptosome radius (cm), then F is given by:

$$F = \frac{k'}{v} \times \frac{r}{3}$$

Under our conditions, the flux of xylose into synaptosomes is 0.83μμmoles per cm^2 per minute (mean of 2 estimations).

In Table X the effects of phenobarbitone sodium (2mM) and pentamethylene tetrazol (50μg per ml) on the uptake of xylose by synaptosomes are reported. The results show that neither of these drugs significantly altered the uptake of xylose. There are two possible conclusions that may be drawn from these results in relation to the results reported (Table VII) for the effects of these drugs on the incorporation of ^{14}C derived from (U-^{14}C)-glucose substrate. The first is that, as in the case of cerebral cortex slices, xylose is taken up by a similar mechanism to glucose and that the effects on the incorporation of label derived from (U-^{14}C)-glucose were not due to an increase in the rate of

Table X

The Effect of Phenobarbitone and Pentamethylene Tetrazol on the Uptake of Xylose by Synaptosomes

Drug	Radioactivity cpm/mg Pr	Percent of Control	Significance from Control
None	545 ± 80		
Phen (2mM)	548 ± 80	100.3 ± 10.6	n.s.
PMT (50µg/ml)	551 ± 60	100.5 ± 15.0	n.s.

Paired samples of synaptosomes were incubated in saline medium containing 10mM glucose (p33) and (U-^{14}C)-xylose for 5 minutes at 37°C, with or without drug, as described in the methods section (p82). The results, which are the means ± SEM of 5 estimations, were statistically compared by the 'students' t-test.

Phen: Phenobarbitone Sodium

PMT: Pentamethylene Tetrazol

uptake of the glucose, but rather to a difference in the utilisation of (U-^{14}C)-glucose after it has entered the particles. The alternative conclusion is that the synaptosomal membrane contains a carrier system by which glucose, but not xylose, may be transported into the particles. It is very improbable that either of the drugs could alter the permeability of the synaptosome membrane to the passive diffusion of one of these sugars and not the other, since they have similar solubility properties. If one postulates that the synaptosome membrane contains a carrier system which is specific for glucose and not for xylose one must further postulate that this transport system is different from the carrier system which is present in cerebral cortex slices and which has been characterised by Gilbert (1965). The former of these two alternatives appears to be the more attractive.

The Effects of Drugs on the Volume of Synaptosomes

Evidence was presented, in the introduction, to suggest that changes in the volumes of synaptosomes could be followed by observing the changes in the

reciprocals of the extinctions of synaptosome suspensions. Figure XVIII shows the effect of NaCl molarity on the extinction of synaptosome suspensions. The extinction rises with the increase in the molarity in which the particles are suspended. These results are consistent with the shrinkage of synaptosomes in media of increasing tonicity. The van't Hoff Law predicts that the volume of a particle which performs as an osmometer will vary linearly with the change in the reciprocal of the molarity of the medium in which the particle is suspended. If the results, presented in Figure XVIII, are plotted in the form E^{-1}, which is a measure of the volume (p77), against NaCl (M^{-1}), then the points fall on a straight line (Fig XIX). These results are in agreement with those of Keen and White (1970) and suggest that synaptosomes perform as osmometers in NaCl solution over the range 0.2M to 0.04M.

Synaptosomes were then preincubated in 0.32M sucrose, with or without drugs, and the extinctions estimated in 0.2M and 0.05M, as described in the methods section

Figure XVIII

The Effect of NaCl Concentration on the Extinctions of Synaptosome Suspensions

0.2ml samples of synaptosomes, suspended in 0.32M sucrose, were suspended in 2.8ml of NaCl solution of different molarities and the extinctions measured at 520nm (p85). The results are the means $\pm$ SEM of 3 estimations.

Fig XVIII

The Effect of NaCl Concentration on the Extinction of Synaptosome Suspensions

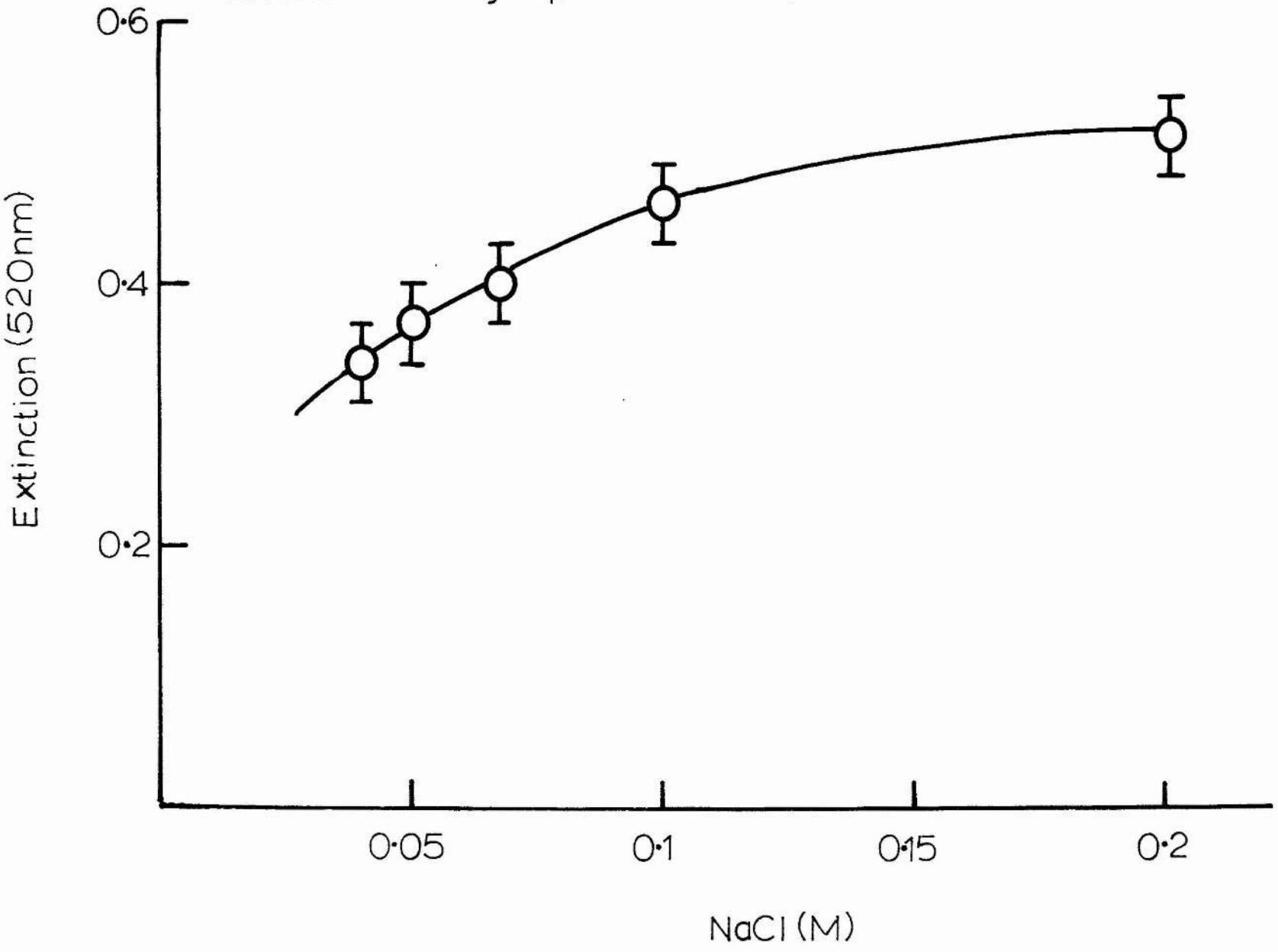


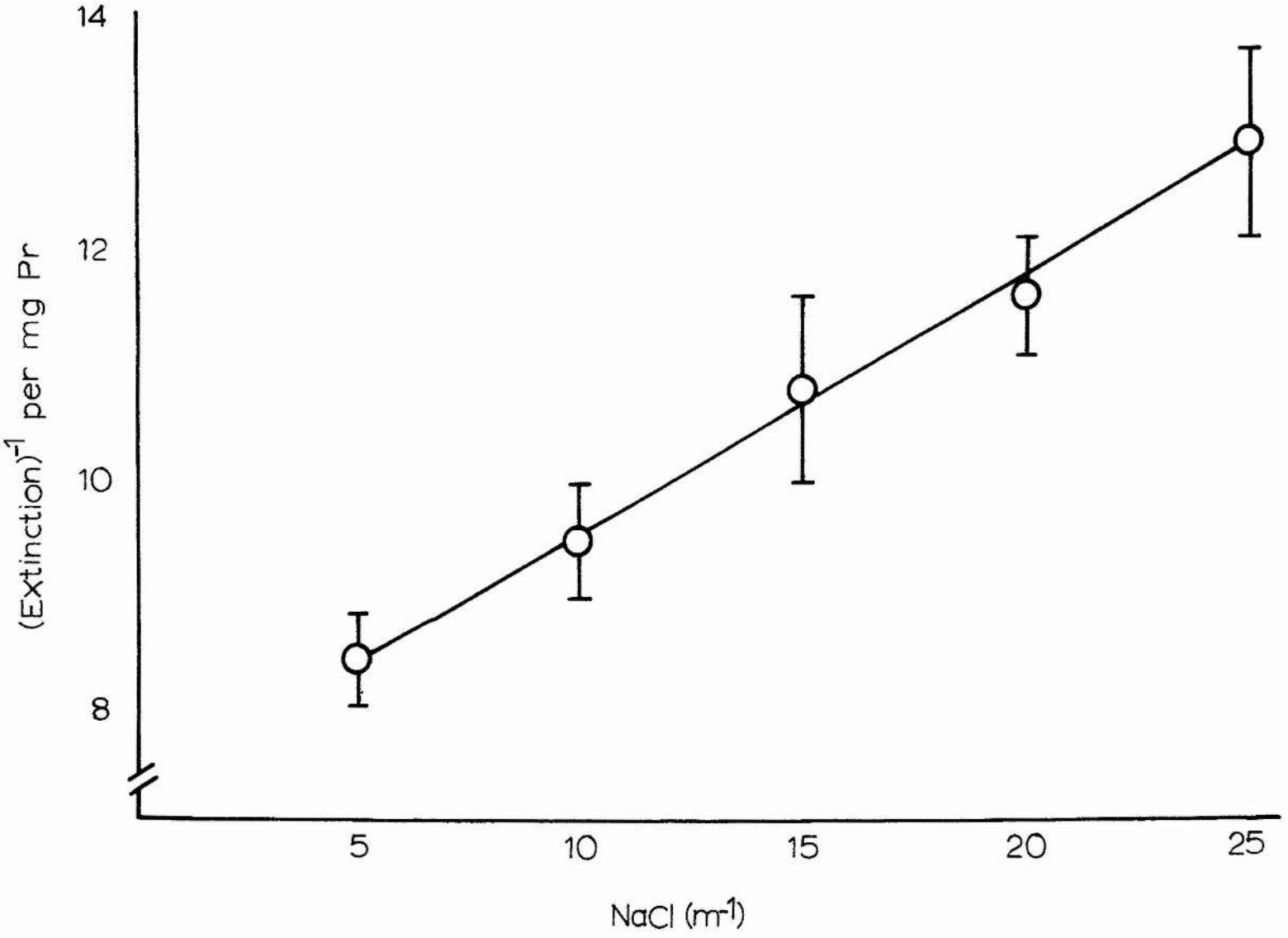
Figure XIX

The Effect of NaCl Concentration (M^{-1}) on $(Extinction)^{-1}$ of Synaptosome Suspensions

The results for the effect of NaCl concentration on the extinction of synaptosome suspensions (Fig XVIII) have been plotted in a reciprocal form to test for conformity to the van't Hoff Law (plOl). The results are the means $\pm$ SEM of 3 estimations.

Fig XIX

The Effect of NaCl Concentration (m^{-1}) on $(Extinction)^{-1}$ of Synaptosome Suspensions



(p83). The results are summarised in Tables XI and XII. Neither of the convulsants, strychnine or pentamethylene tetrazol, had any significant effect on the value of E^{-1} of the synaptosomes in the NaCl solutions, but E^{-1} in both NaCl concentrations was increased by preincubation of the synaptosomes with acetazolamide (400μM) and phenobarbitone sodium (0.1mM and 2.0mM). If we consider first the effect of preincubation with the anticonvulsants on E^{-1} measured in 0.2M NaCl (Table XI), there are two possible explainations:-

(1) Preincubation with the drugs could be causing some of the synaptosomes to burst.

(2) The differences in E^{-1} reflect true differences in the volumes of the synaptosomes.

In the next series of experiments, the effect of varying the length of time of preincubation of the synaptosomes with the drugs was examined and the results are summarised in Figure XX. The value of E^{-1} of suspensions of untreated synaptosomes drops as the incubation time increases, and this is consistent with the shrinkage of the particles which would result from

Figure XX

The Effect of Drugs on the Shrinkage of Synaptosomes

Paired samples of synaptosomes were incubated in 0.32M sucrose, with or without drug, for 30 minutes at 25°C. The extinctions of samples in 0.2M NaCl were then estimated (p83). The results are the means of 2 estimations.

Phen: Phenobarbitone Sodium

Acet: Acetazolamide

Fig XX

The Effect of Drugs on the Shrinkage of Synaptosomes

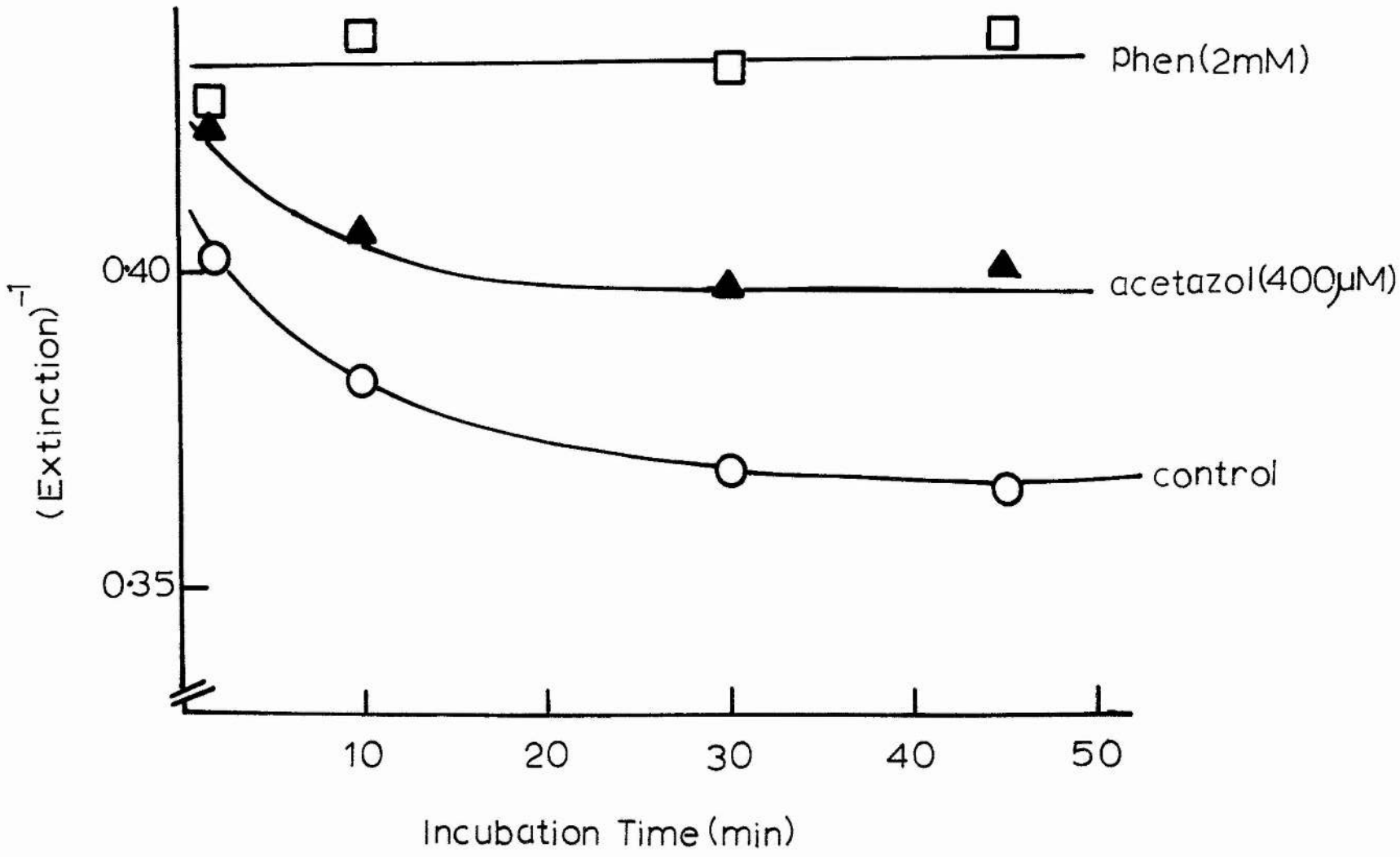


Table XI

The Effect of Preincubation with Convulsant and Anticonvulsant Drugs on E^{-1} Measured in 0.2M NaCl

Expt Group	Drug	E^{-1}	Percent of Control	Significance from Control
I	None	3.0 ± 0.2		
	Phen (2mM)	3.6 ± 0.3	121.7 ± 2.0	P<0.01
	Phen (0.1mM)	3.2 ± 0.3	106.3 ± 2.0	P<0.05
	Acet (400µM)	3.4 ± 0.2	115.0 ± 5.0	P<0.05
	Acet (20µM)	3.1 ± 0.1	104.0 ± 2.0	n.s.
	PMT (1000µg/ml)	3.0 ± 0.1	101.0 ± 2.3	n.s.
	PMT (50µg/ml)	2.9 ± 0.2	99.0 ± 3.0	n.s.
II	None	3.3 ± 0.1		
	Strych (1000µg/ml)	3.3 ± 0.1	100.7 ± 1.4	n.s.
	Strych (50µg/ml)	3.3 ± 0.1	100.3 ± 2.0	n.s.

Paired samples of synaptosomes were preincubated in 0.32M sucrose, with or without drug, for 30 minutes at 25°C. The extinctions were then measured in 0.2M NaCl, as described in the methods section (p83). The results, which are the means ± SEM of 6 estimations, were compared statistically

/by

(Table XI continued)

by the 'students' t-test.

Phen: Phenobarbitone Sodium

Acet: Acetazolamide

PMT: Pentamethylene Tetrazol

Strych: Strychnine

Table XII

The Effect of Preincubation with Convulsant and Anticonvulsant Drugs on E^{-1} Measured in 0.05M NaCl

Expt Group	Drug	E^{-1}	Percent of Control	Significance from Control
I	None	3.9 ± 0.3		
	Phen (2mM)	6.1 ± 0.4	152.5 ± 10.4	P<0.01
	Phen (0.1mM)	4.6 ± 0.3	114.9 ± 1.6	P<0.01
	Acet (400µM)	5.1 ± 0.3	118.8 ± 4.4	P<0.01
	Acet (20µM)	4.2 ± 0.3	102.9 ± 2.9	n.s.
	PMT (1000µg/ml)	4.2 ± 0.3	103.0 ± 3.8	n.s.
	PMT (50µg/ml)	4.0 ± 0.3	100.1 ± 3.3	n.s.
II	None	4.6 ± 0.2		
	Strych (1000µg/ml)	4.7 ± 0.2	102.0 ± 1.9	n.s.
	Strych (50µg/ml)	4.5 ± 0.2	99.1 ± 1.7	n.s.

Paired samples of synaptosomes were preincubated in 0.32M sucrose, with or without drug, for 30 minutes at 25°C. The extinctions were then measured in 0.05M NaCl, as described in the methods section (p83). The results, which are the means ± SEM of 6 estimations, were statistically compared

/by

(Table XII continued)

by the 'students' t-test.

Phen: Phenobarbitone Sodium

Acet: Acetazolamide

PMT: Pentamethylene Tetrazol

Strych: Strychnine

the loss of osmotically active constituents of the cytoplasm into the medium. It is known that, if synaptosomes are suspended in warm medium, this loss of osmotically active constituents, especially K^+ and Na^+, does occur and does indeed result in a reduction in the volume (Marchbanks 1967, Whittaker 1969). However, if phenobarbitone sodium (2mM) is included in the pre-incubation medium, this shrinkage appears to be totally prevented (Fig XX). Acetazolamide (400μM) reduces the amount of shrinkage by approximately 50 per cent. It seems, therefore, that these drugs do not cause lysis of the synaptosomes, nor do they make the membrane permeable to sucrose since, if either of these effects had occurred, the apparent volume of the drug-treated synaptosomes would have increased with preincubation time. It is likely, therefore, that these drugs limit the shrinkage which occurs when synaptosomes are incubated in warm isotonic media, possibly by reducing the permeability of the synaptosome membrane and thus restricting the loss of osmotically active constituents of the cytoplasm.

The dose response curve for the effect of phenobarbitone sodium (Fig XXI) shows that the drug exerts its maximal

Figure XXI

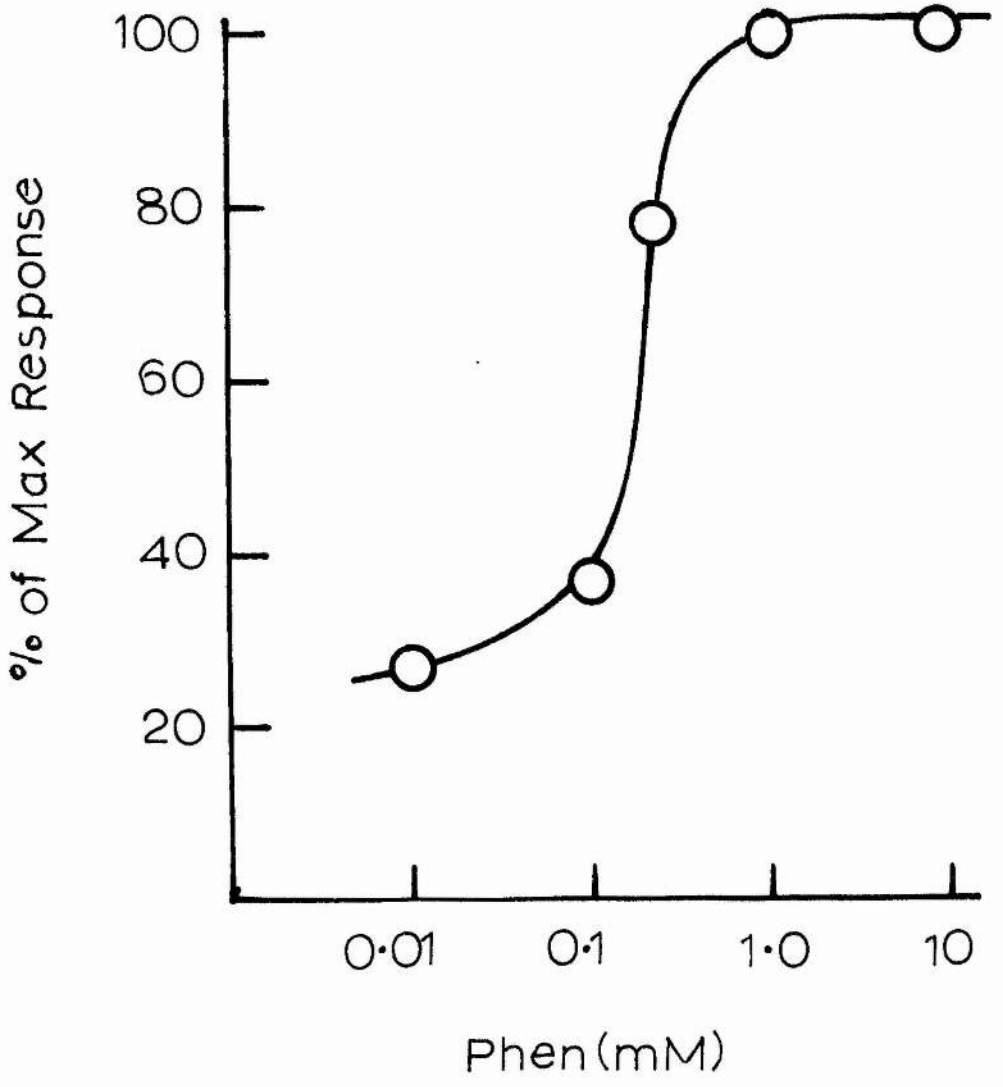
The Effect of Phenobarbitone on the Shrinkage of Synaptosomes

Dose Response Curve

Paired samples of synaptosomes were incubated in 0.32M sucrose, with or without drug, at 25°C for 30 minutes and the extinctions of samples suspended in 0.2M NaCl estimated (p83). The effects of the different concentrations of phenobarbitone on E^{-1} are expressed as a percentage of the maximum difference observed between E^{-1} for drug-treated synaptosomes and E^{-1} for the controls. The maximum response occurred at concentrations of phenobarbitone which prevented shrinkage of the synaptosomes completely. Each point represents the mean of at least 2 observations.

Fig XXI

The Effect of Phenobarbitone on the Shrinkage of Synaptosomes Dose Response Curve



effect at a concentration of approximately 0.5mM, a concentration which is not far removed from the level found in the brains of animals treated with an effective dose of the drug (Millichap 1965). The drug, therefore, has its effect at concentrations similar to those found 'in vivo'.

This is not true for acetazolamide since, as Figure XXII shows, this drug does not exert its maximal effect until the concentration is 2 - 5mM, whereas its effective level 'in vivo' is about 20μM (Millichap 1965). Preincubation in even higher concentrations of acetazolamide result in small, but significant, decreases in the value of E^{-1}, when compared with corresponding controls, and it seems that, at these very high concentrations, the drug increases the permeability of the synaptosome membrane. It is not very surprising that phenobarbitone has an effect at a concentration close to its 'in vivo' level, since barbiturates are thought to have their effects by direct physico-chemical interactions with the cell membranes, but acetazolamide is thought to mediate its anticonvulsant effect by inhibition of the enzyme carbonic anhydrase (Koch and

Figure XXII

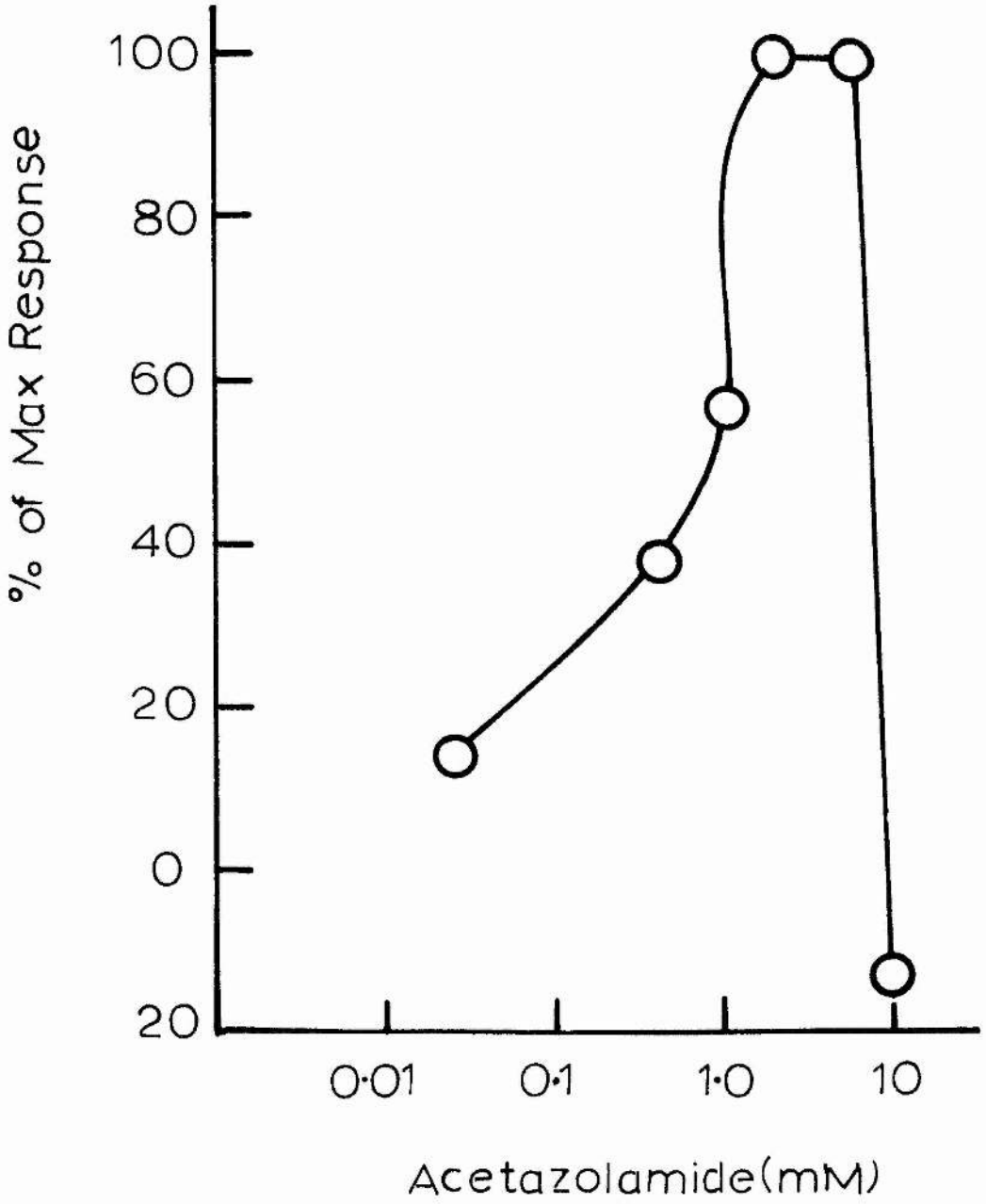
The Effect of Acetazolamide on the Shrinkage of Synaptosomes

Dose Response Curve

Paired samples of synaptosomes were incubated in 0.32M sucrose, with or without drug, at 25°C for 30 minutes and the extinctions of samples suspended in 0.2M NaCl estimated (p63). The effects of the different concentrations of acetazolamide on E^{-1} are expressed as a percentage of the maximum difference observed between E^{-1} for drug-treated synaptosomes and E^{-1} for the controls. The maximum response observed for acetazolamide was the same as that observed for phenobarbitone (Fig XXI). Each point represents the mean of at least 2 observations.

Fig XXII

The Effect of Acetazolamide on the Shrinkage of Synaptosomes Dose Response Curve



Woodbury 1958).

In Tables XI and XII it was shown that the value of E^{-1} in both 0.2M and 0.05M NaCl was significantly greater than corresponding control values if the synaptosomes were preincubated in phenobarbitone sodium or in fairly high concentrations of acetazolamide, but in Table XIII it may be seen that, after preincubation in these effective doses of the anticonvulsants, the synaptosomes appear to swell to a significantly greater extent, relative to their original volume, when suspended in hypotonic solution. The volume of the synaptosomes in 0.2M NaCl, after preincubation in phenobarbitone sodium (2mM), is approximately the same as that of the control synaptosomes suspended in 0.05M NaCl. Therefore, although the control synaptosomes performed as osmometers over the range 0.2M to 0.04M NaCl, it seems probable that synaptosomes, preincubated in phenobarbitone sodium (2mM), could not withstand the increase in volume which occurred when the synaptosomes were suspended in 0.05M NaCl because of the higher concentration of osmotically active compounds which were retained within the cytoplasm. To test this

Table XIII

The Relationship of E^{-1} in 0.2M NaCl to E^{-1} in 0.05M NaCl after Preincubation with Drugs

Expt Group	Drug	$\frac{(E^{-1})_{0.05}}{(E^{-1})_{0.2}}$	Percent of Control	Significance from Control
I	None	1.36 ± 0.03		
	Phen (2mM)	1.68 ± 0.05	124.2 ± 2.2	P<0.01
	Phen (0.1mM)	1.45 ± 0.02	106.6 ± 0.7	P<0.01
	Acet (400μM)	1.52 ± 0.05	111.8 ± 3.6	P<0.01
	Acet (20μM)	1.39 ± 0.03	102.9 ± 2.9	n.s.
	PMT (1000μg/ml)	1.39 ± 0.03	102.2 ± 2.2	n.s.
	PMT (50μg/ml)	1.36 ± 0.03	100.0 ± 2.2	n.s.
II	None	1.40 ± 0.05		
	Strych (1000μg/ml)	1.39 ± 0.03	99.5 ± 2.1	n.s.
	Strych (50μg/ml)	1.35 ± 0.03	98.0 ± 2.3	n.s.

Paired samples of synaptosomes were preincubated in 0.32M sucrose at 25°C for 30 minutes, with or without drug. The extinctions in 0.2M and 0.05M NaCl were then estimated (p83). The results for this table were derived by dividing the value of E^{-1} for synaptosomes suspended in 0.05M NaCl

/(Table XII p105)

(Table XIII continued)

(Table XII p105) by the value of E^{-1} for synaptosomes suspended in 0.2M NaCl (Table XI p103). The results reported in Tables XI and XII were obtained on the same samples of synaptosomes. The results, which are the means $\pm$ SEM of 6 estimations, were statistically compared by the 'students' t-test.

Phen: Phenobarbitone Sodium

Acet: Acetazolamide

PMT: Pentamethylene Tetrazol

Strych: Strychnine

possibility, paired samples of synaptosomes were pre-incubated, with or without drug, and samples were suspended in 0.2M and 0.05M NaCl and the extinctions measured as before (p83). 0.1ml of concentrated NaCl solution was then added to the cuvettes containg the synaptosomes in 0.05M NaCl, to return the molarity of the NaCl to 0.2M, and the extinction was measured again. From these results the ability of the synaptosomes to recover their original volume can be estimated. The control synaptosomes recover by only 50 per cent (Fig XXIII) and this inability to recover fully, after suspension in hypotonic solution, has been reported before (Keen and White 1970). But if the synaptosomes have been preincubated with phenobarbitone sodium (2mM), their ability to recover their original volume is significantly impaired. Suspension of these drug-treated synaptosomes in 0.05M NaCl, therefore, seems to damage the synaptosome membrane and the results are consistent with the synaptosomes being ruptured by the hypotonic treatment.

The effects of drugs on the swelling of synaptosomes in hypotonic solution may, in some instances, be used to test the effects of drugs on the mechanical properties of

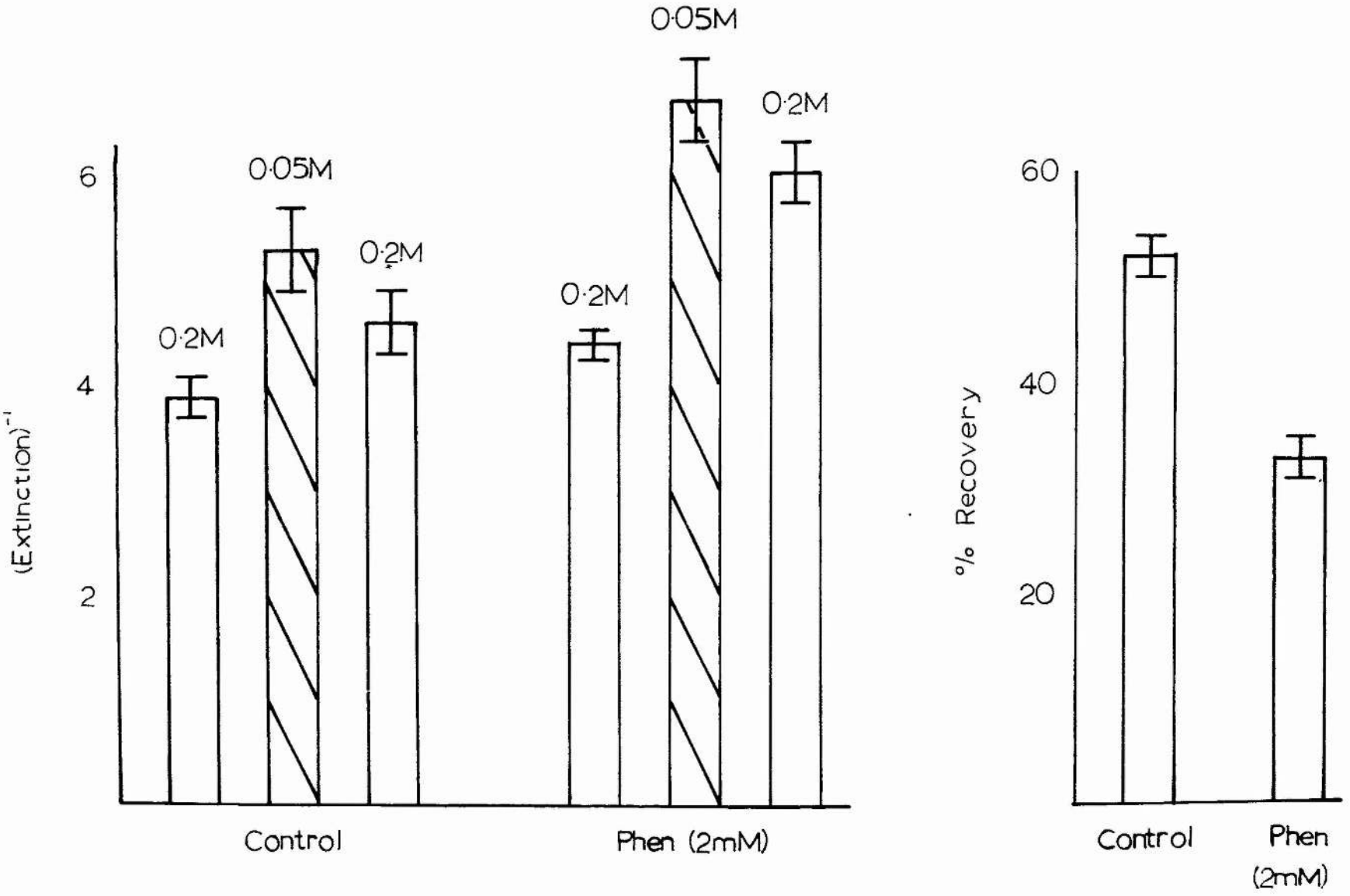
Figure XXIII

The Effects of Phenobarbitone on the Ability of Synaptosomes to Recover their Original Volume After Suspension in Hypo-osmotic Solution

Paired samples of synaptosomes were incubated in 0.32M sucrose, with or without drug, for 30 minutes at 25°C. The extinctions of samples were then estimated in 0.2M and 0.05M NaCl (p83). The $(\text{extinctions})^{-1}$ are represented, in the left-hand part of the Figure, by the clear columns on the left of each group and by the shaded columns in the centre. Concentrated NaCl solution was then added to the synaptosomes in 0.05M NaCl and the extinctions measured again (p84). The reciprocals of these results are represented by the right-hand clear column of each group. In the right-hand part of the Figure the recovery of the synaptosomes is presented (100 per cent recovery equals a full recovery to the original volume in 0.2M NaCl after suspension in 0.05M NaCl). The recovery of the drug-treated synaptosomes was significantly impaired ($P<0.01$) compared with controls. The results, which are the means $\pm$ SEM of 5 estimations, were statistically compared by the 'students' t-test.

Fig XXIII

The Effect of Phenobarbitone on the Ability of Synaptosomes to Recover Their Original Volume after Suspension in Hypo-osmotic Solution



of the membrane, since such effects may alter the ability of the synaptosome to change shape or stabilise the synaptosome membrane against lysis. Effects of this type have been observed before, especially with chlorpromazine, using other preparations (Mikilits et al al 1970). In the experiments reported here, however, effects of this type which result from preincubation with phenobarbitone or acetazolamide would be masked, to a great extent, by the effects of these drugs which are more consistent with an effect on permeability. Also, the convulsants, strychnine and pentamethylene tetrazol, do not seem to affect the mechanical properties of the synaptosome membrane (Table XIII).

In Table XIV the results of experiments in which the effects of preincubation with another barbiturate, thiopentone, are compared with the effects of preincubation with phenobarbitone are reported. Thiopentone is a barbiturate whose main therapeutic use is a general anaesthetic, but which also has anticonvulsant properties (Harrison, Rees and Watson 1971). The effects of pre-incubation with thiopentone are similar to the effects of phenobarbitone at the same concentrations, and the

Table XIV

The Fffect of Preincubation of Synaptosomes with Thiopentone and Phenobarbitone on E^{-1} in 0.2M NaCl

Expt Group	Drug	E^{-1}	Percent of Control	Significance from Control
I	None	3.0 ± 0.2		
	Phen (0.1mM)	3.2 ± 0.3	106.3 ± 2.0	$P<0.05$
	Phen (2mM)	3.6 ± 0.3	121.7 ± 2.0	$P<0.01$
II	None	2.3 ± 0.1		
	Thio (0.1mM)	2.4 ± 0.1	107.0 ± 2.4	$P<0.05$
	Thio (2mM)	2.7 ± 0.1	117.4 ± 2.2	$P<0.01$

Paired samples of synaptosomes were preincubated in 0.32M sucrose at 25°C for 30 minutes, with or without drug. The extinction of samples were then measured in 0.2M NaCl (p83). The results, which are the means ± SEM of 6 estimations, were statistically compared by the 'students' t-test.

Phen: Phenobarbitone Sodium

Thio: Thiopentone Sodium

effect of preincubation in 2mM thiopentone was not significantly different from preincubation in 2mM phenobarbitone (Fig XXIV). The problems involved in trying to separate the different effects of barbiturates and attributing therapeutic roles to these effects has already been mentioned, and these results show that these two barbiturates which have similar, but by no means identical, effects 'in vivo' produce very similar effects 'in vitro'.

Gray and Gilbert (1970) found that the ability of some anticonvulsants to stimulate sugar uptake by cerebral cortex slices could be abolished by compounds which compete for thiol groups on the membranes. It was possible that these compounds would also inhibit the ability of anticonvulsants to reduce the permeability of the synaptosome membrane, by occupying the sites through which the drugs became bound to the membranes. The thiol group inhibitors produced a variety of effects (Table XV). Preincubation with 0.5mM dithiobisnitrobenzoic acid alone had no effect on the volume of the synaptosomes, but if this drug was added to synaptosomes preincubated with phenobarbitone sodium

Figure XXIV

The Effect of Preincubation of Synaptosomes with Phenobarbitone and Thiopentone

Paired samples of synaptosomes were incubated in 0.32M sucrose, with or without drug, for 30 minutes at 25°C and the extinctions measured in 0.2M NaCl (p83). The reciprocals of the extinctions are represented by the columns. E^{-1} for the synaptosomes incubated with phenobarbitone or thiopentone was significantly greater ($p<0.01$) than the corresponding control values, but E^{-1} for thiopentone-treated synaptosomes was not significantly different from E^{-1} for phenobarbitone-treated synaptosomes. The results, which are the means $\pm$ SEM of 6 estimations, were statistically compared by the 'students' t-test.

Phen: Phenobarbitone Sodium

Thio: Thiopentone Sodium

Fig. XXIV

The Effect of Preincubation of Synaptosomes in Phenobarbitone and Thiopentone

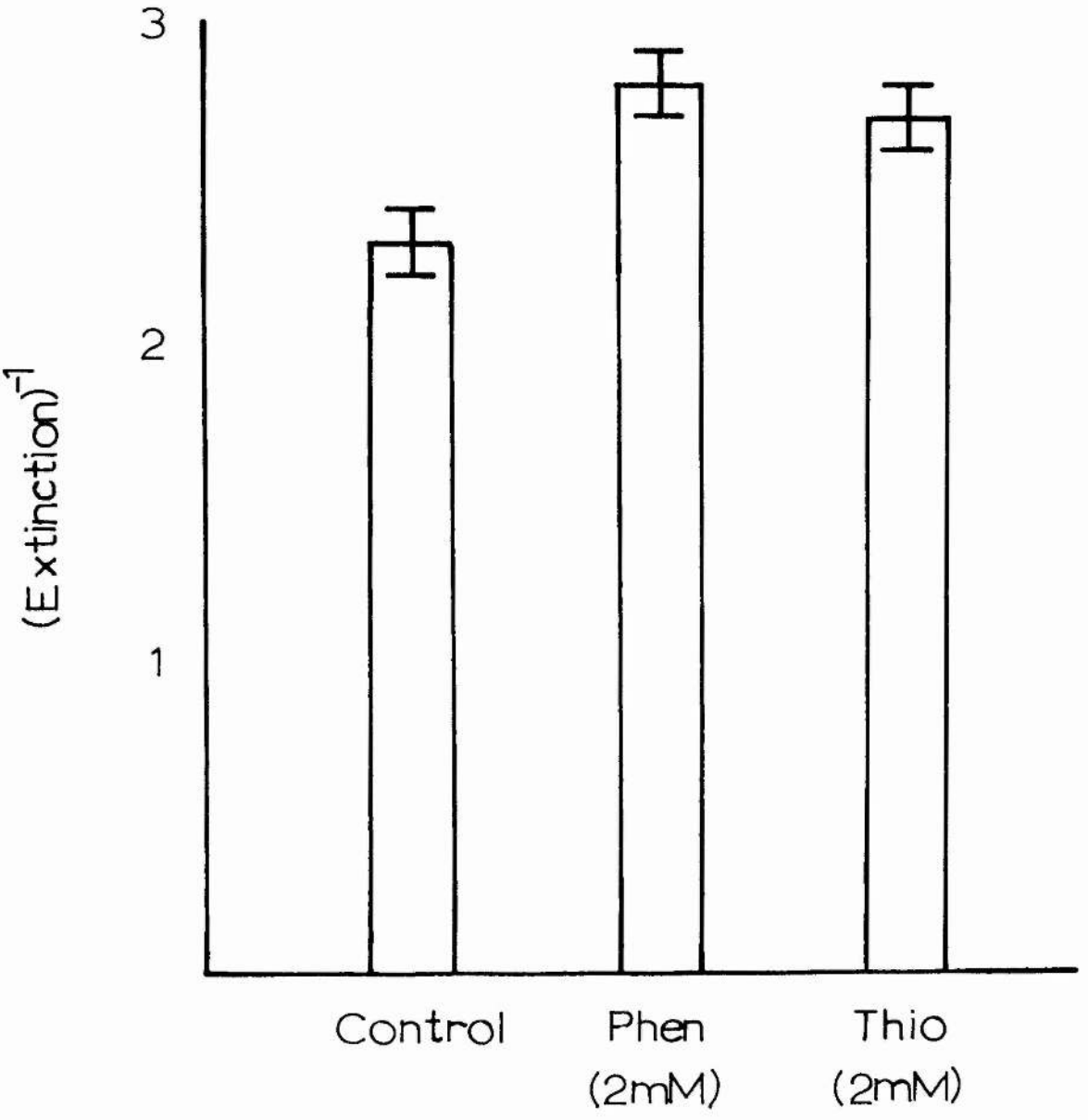


Table XV

The Effects of Thiol Group Inhibitors on the Effects of the Anticonvulsant Drugs on Synaptosomes

Experimental Group I

Drug	E^{-1}	Percent of Control	Percent of Anticon	Significance from Control	Significance from Anticon
None	2.3 ± 0.1				
Phen	2.8 ± 0.1	120.2 ± 1.7		P<0.01	
DTNB	2.3 ± 0.1	100.9 ± 2.7		n.s.	
Phen+DTNB	2.6 ± 0.1	113.9 ± 1.6	93.9 ± 1.8	P<0.01	P<0.05

Experimental Group II

Drug	E^{-1}	Percent of Control	Percent of Anticon	Significance from Control	Significance from Anticon
None	3.3 ± 0.1				
Phen	4.0 ± 0.1	122.7 ± 3.0		P<0.01	
Acet	4.0 ± 0.1	121.8 ± 3.0		P<0.01	
NEM	5.1 ± 0.3	154.5 ± 10.6		P<0.05	
PCMB	3.2 ± 0.1	98.5 ± 1.8		n.s.	
Phen+PCMB	3.9 ± 0.1	120.0 ± 1.2	98.0 ± 2.5	P<0.01	n.s.
Acet+PCMB	4.1 ± 0.1	125.7 ± 2.4	103.2 ± 3.2	P<0.01	n.s.

(Table XV continued)

Paired samples of synaptosomes were preincubated at 25°C for 30 minutes, with or without drug, and the extinctions measured in 0.2M NaCl (p83). The results are the means ± SEM of 6 estimations (3 for NEM). The results were statistically compared by the 'students' t-test with the corresponding controls and, where applicable, with corresponding synaptosome samples preincubated with anticonvulsants alone.

Acet: Acetazolamide (2.5mM)

Phen: Phenobarbitone Sodium (2mM)

DTNB: Dithiobisnitrobenzoic Acid (0.5mM)

NEM: n-Ethylmaleimide (4mM)

PCMB: p-Chloromercurybenzoic Acid (5mM)

(2mM), the value of E^{-1} was significantly less than the corresponding samples preincubated in phenobarbitone alone, suggesting that dithiobisnitrobenzoate might block some of the sites through which phenobarbitone exerts its effect. Dithiobisnitrobenzoate is not very soluble, therefore the effects of two other more soluble thiol group inhibitors, p-chloromercurybenzoate and n-ethylmaleimide, were also tested. Preincubation in p-chloromercurybenzoate was without effect on the volumes of synaptosomes themselves and it also did not modify the effects of either of the anticonvulsants (Table XV). Preincubation in n-ethylmaleimide, however, resulted in a marked increase in E^{-1} and the extinction of synaptosome suspensions, after preincubation in n-ethylmaleimide was independent of the molarity of the NaCl solution. This suggests that this drug bursts synaptosomes and no further experiments were performed with it. If either of the anticonvulsants had their effect by binding to the membrane through thiol groups on the membrane, one would expect both thiol group inhibitors, dithiobis-nitrobenzoic acid and p-chloromercurybenzoate, to inhibit the action of the drugs. This was not the case,

however, and it seems reasonable to suppose that dithiobisnitrobenzoic acid inhibits the action of phenobarbitone, not by binding to the same site, but by occupying sites close to those to which phenobarbitone binds and, thus sterically blocking these sites.

The Effect of Phenobarbitone on the Release of K^+, Na^+ and Xylose from the Synaptosomal Cytoplasm

Synaptosomes retain significant quantities of K^+ and Na^+ during their preparation (Ryall 1964), but if the particles are suspended in warm sucrose, these ions are lost rapidly into the sucrose (Marchbanks 1967). Marchbanks further showed that the synaptosomes may be separated from the ions which have been released by gel filtration. In Figure XXV the elution of a synaptosome fraction through a G50 sephadex column is shown and it may be seen that there are two K^+ peaks. The first of these peaks is eluted with the synaptosomal protein and corresponds to the intrasynaptosomal K^+, while the second peak, which is eluted subsequently, corresponds to the extrasynaptosomal K^+. The elution pattern which is presented (Fig XXV) is similar, in all essential

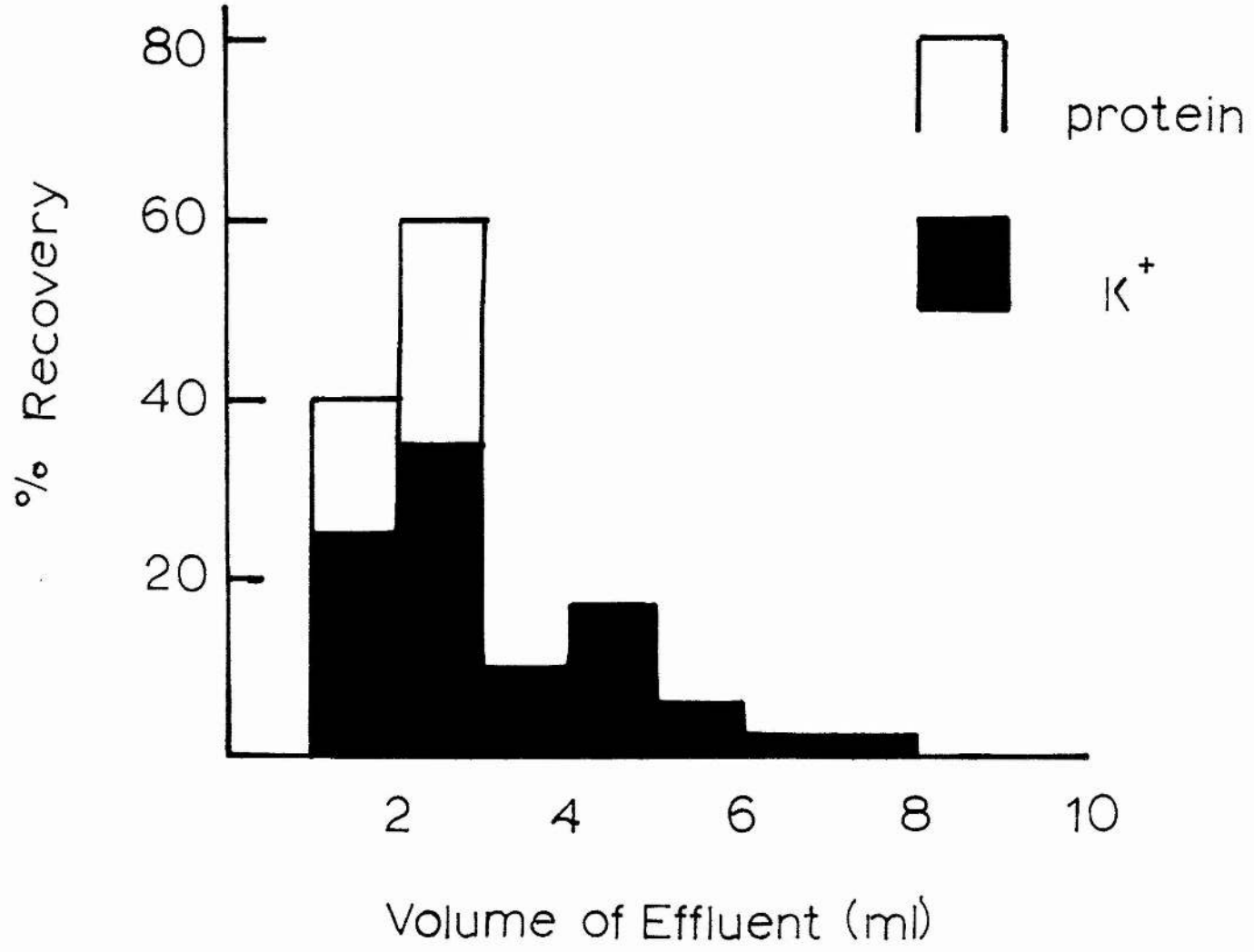
Figure XXV

The Elution of Synaptosomes Through a Sephadex Column

A sample of synaptosomes, suspended in 0.32M sucrose, was eluted through a column, consisting of 0.5g of G50 sephadex, with 0.32M sucrose (p51) and the effluent was assayed for protein and K^+ (p85).

Fig XXV

The Elution of Synaptosomes Through a Sephadex Column



respects, to that first published by Marchbanks (1967).

It seemed likely that the shrinkage which occurred when the synaptosomes were suspended in warm sucrose resulted, to some extent at least, from the loss of these ions. To test this possibility, synaptosomes were preincubated in sucrose, in the presence or absence of 2mM phenobarbitone, and the K^+ and Na^+ content of the synaptosomes was estimated using the methods described earlier (p85). Table XVI shows that if synaptosomes were preincubated in phenobarbitone sodium (2mM), the concentration of these ions in the synaptosome fraction was significantly greater than the corresponding control values. There was a 27 per cent reduction in the K^+ concentration of the synaptosome fraction, after 30 minutes incubation (Table XVII), but this loss of K^+ was very much reduced if phenobarbitone sodium (2mM) was included in the medium. Table XVII also shows that there was a 45 per cent loss in the Na^+ content of the particles during 30 minutes incubation but, if phenobarbitone sodium (2mM) was included in the medium, there was a small increase in the concentration of this ion. This small increase can be explained if phenobarbitone

Table XVI

The Effect of Incubation with Phenobarbitone (2mM) on the Concentrations of K^+ and Na^+ of Synaptosomes

Condition	Ion Concentration (mμequiv/mg Pr) K^+	Na^+
Control	$154 \pm 9_{(9)}$	$72 \pm 8_{(6)}$
Phen (2mM)	$190 \pm 12_{(9)}$	$135 \pm 18_{(6)}$
Percent of Control	123 ± 3	54 ± 12
Significance from Control	$P<0.01$	$P<0.05$

Paired samples of synaptosomes were incubated for 30 minutes at 25°C, with or without drug. The K^+ and Na^+ concentrations were then estimated (p85). The results, which are the means $\pm$ SEM of the number of estimations given in the parentheses, were statistically compared by the 'students' t-test.

Table XVII

The Effect of Phenobarbitone (2mM) on the Release of K^+ and Na^+ from Synaptosomes.

Ion	Ion Concentration (mμequiv/mg Pr) Initially	After Incubation Control	After Incubation Phen (2mM)
K^+	193 ± 3	141 ± 13	179 ± 10
Na^+	148 ± 6	82 ± 11	171 ± 2

The K^+ and Na^+ concentrations of freshly prepared synaptosomes were estimated (p85). Paired samples were then incubated in 0.32M sucrose at 25°C for 30 minutes, with or without phenobarbitone, and the ion concentrations were measured again. The results represent the means ± SEM of 3 experiments.

not only prevents the loss of Na^+ from the synaptosomal cytoplasm but also binds to the synaptosome membrane. The drug was added in the form of its sodium salt and the small increase in the Na^+ content of the drug-treated synaptosomes over that found in freshly prepared synaptosomes could be due to Na^+ bound to the particles through phenobarbitone. If phenobarbitone sodium was eluted through an identical G50 sephadex column to that used to separate synaptosomes from extrasynaptosomal Na^+, in the absence of synaptosomes, there was no increase in the Na^+ content of the fraction which was equivalent to the synaptosome fraction. The results reported above suggest that phenobarbitone does, indeed, reduce the permeability of the synaptosome membrane to K^+ and Na^+.

Marchbanks (personal communication) estimates the volume of the synaptosomal cytoplasm, in sucrose solution, as 2.0ul per mg protein and, using this volume, the concentrations of K^+ and Na^+ in the cytoplasm of freshly prepared synaptosomes may be estimated. The concentration of K^+ was 96 $\pm$ 2mequiv per l (mean of 3 estimations), while that of Na^+ was 64 $\pm$ 2mequiv per l (mean of 3 estimations), if we allow for 5 per cent contamination

of K^+ (see Marchbanks 1967). These results are close to those reported by Marchbanks (1967) for the levels of these ions in synaptosomes.

The results, presented above, suggested that phenobarbitone reduced the permeability of the synaptosome membrane to K^+ and Na^+, but this did not mean that the drug altered the permeability to all ions and molecules. In order to examine the effect of phenobarbitone on the permeability of the synaptosome membrane to a non-ionised compound, synaptosomes were preincubated in (U-^{14}C)-xylose and the effect of the drug on the amount of xylose released, during a 7 minute incubation in warm sucrose, examined (p85). Table XVIII shows that phenobarbitone sodium (2mM) did not alter the amount of xylose released. This result is in agreement with the findings, reported earlier (p99) in the thesis, that phenobarbitone did not alter the uptake of this sugar, although it must be emphasised that the conditions, under which the two experiments were done, were very different. Also there is no reason why influx and efflux of xylose should not be altered independently by phenobarbitone. Phenobarbitone, it seems, does not alter the permeability

Table XVIII

The Effect of Phenobarbitone (2mM) on the Release of Xylose from Synaptosomes

Drug	Radioactivity cpm/mg Pr	Percent of Control	Significance from Control
None	370 ± 80		
Phen (2mM)	387 ± 86	5 ± 16	n.s.

Synaptosomes were incubated with (U-^{14}C)-xylose and then paired samples were transferred to 0.32M sucrose, with or without drug, and incubated at 25°C for 7 minutes (p99). The results, which are the means ± SEM of 5 estimations, were statistically compared by the 'students' t-test. The radioactivity inside the synaptosomes, prior to the 7 minute incubations, was 660 ± 35 cpm per mg protein.

of the synaptosome membrane to all types of compound but, of those examined here, it restricts its effect to K^+ and Na^+.

G E N E R A L D I S C U S S I O N

The purpose of the experiments, which have been described in this thesis, was to use the synaptosome preparation as a means of examining the effects of convulsant and anticonvulsant drugs on the stability of the nerve-ending and, in particular, on the properties of the nerve-ending membrane. Previous studies had suggested that acetazolamide and pentamethylene tetrazol exerted their effects by inhibiting the enzymes carbonic anhydrase and acetylcholinesterase respectively, but that strychnine and phenobarbitone acted on neuronal cell membranes. The effect of phenobarbitone on these membranes seems to be more general than that of strychnine which seems to inhibit the action of glycine (Roper et al 1969) either by restricting the release of the amino acid from the pre-synaptic membrane or by blocking the receptor sites on the post-synaptic membrane.

The effects of the drugs on the synaptosomes were examined under two conditions. The first experiments tested the ability of the drugs to modify or stabilise the synaptosomes under conditions in which the particles were actively metabolising added substrate. It was thought that this state was closer to that found 'in

vivo'. Work by Bradford (1969, 1970ab) and by Bradford and Thomas (1969) had shown that the metabolism and respiratory properties of synaptosomes were very similar to those of cerebral cortex slices and, in particular, the respiratory and metabolic response of the particles to electrical pulses and to the addition of excess K^+ was similar, qualitatively but not quantitatively, to the response of cerebral cortex slices when treated in the same way. Bradford (1969) had reported that synaptosomes could form ion gradients and proposed that the synaptosome membrane might be polarised and capable of being depolarised. It seemed, therefore, that the synaptosome preparation could be used as a model system to test the effects of drugs on excitable neuronal membranes.

In view of the points discussed above, the effects of the addition to synaptosomes of a number of compounds, known to affect neuronal membranes 'in vivo' (K^+ and putative transmitter compounds) were examined, but none of these compounds had any significant effect on the respiratory rate of the particles (Table III - p55, Table IV - p58). These results, which were discussed

in Section II, were disappointing and they were also difficult to reconcile with those reported by Bradford (1970b) in which it was shown that, if the K^+ concentration of the medium was increased, the oxygen uptake of the synaptosomes was also increased.

It was, perhaps, not surprising that the putative transmitters were without effect since their action is primarily on the post-synaptic membrane, but the proposed pre-synaptic role of these compounds may be of importance here. Koelle (1962) has suggested that, at some cholinergic synapses, the first acetylcholine released binds to the pre-synaptic membrane to cause the release of more transmitter. Burn and Rand (1962) have further suggested that acetylcholine has a similar function at non-cholinergic synapses, and that this might explain the wide distribution of acetylcholinesterase in the cerebral cortex, although only 15 per cent of the synapses are cholinergic. We concluded, therefore, that either these putative transmitter compounds did not alter the polarised state of the synaptosomal membrane, or that the changes were not sensitively geared to the gross respiratory rate as we measured it. It was also possible

that the transmitter compounds were metabolised too rapidly by inactivating enzymes and that we should have added compounds which inhibited these inactivating enzymes. But we felt that the addition of such inhibitors might complicate the interpretation of the results and we, therefore, estimated the respiratory response immediately after the addition of the transmitters (Table V p61) with the view of detecting any transitory response. No such response was observed. Whittaker (1969) also found that the respiratory response of synaptosomes to changes in the ion composition of the medium were very small and not similar to those found in brain slices. It must be remembered that cerebral cortex slices are multicellular systems and that the presence of non-neuronal membranes may contribute to the excitable nature of the slices.

When the convulsant and anticonvulsant drugs were examined for their effect on the metabolism of synaptosomes it was found that none of the drugs altered the resting respiratory rate of the particles (Table VI p64). This was not unexpected because although some of the drugs tested, phenobarbitone and pentamethylene tetrazol in

particular, do affect the resting respiratory rate of brain homogenates containing synaptosomes (Webb and Elliott 1951), these effects are only observed at concentrations in excess of those used here.

In Table VII (p69), however, it was shown that phenobarbitone and pentamethylene tetrazol altered the amount of ^{14}C incorporated into synaptosomes from (U-^{14}C)-glucose substrate. Neither of these drugs altered the permeability of the synaptosome membrane to xylose (Table X p99), a non-metabolised sugar whose mode of uptake into other brain preparations is similar to that of glucose. It seems, therefore, that these drugs altered the amount of ^{14}C which was incorporated into metabolites which were formed from glucose and which were retained within the synaptosomes. Most of the ^{14}C which is incorporated into the free amino acids from (U-^{14}C)-glucose is retained within the synaptosomes (Bradford and Thomas 1969). Webb and Elliott (1951) examined the effect of pentamethylene tetrazol on brain homogenates, prepared in such a way that synaptosome formation was favoured, and found that the resting respiratory rate and lactate production of the preparation

were insensitive to the concentrations of pentamethylene tetrazol used in this work. We would therefore suggest that this drug, which reduced the amount of ^{14}C incorporation into the synaptosomes, reduces the formation of free amino acids from glucose in the synaptosome and, since a number of these free amino acids modify excitable neuronal membranes, this effect may contribute, in part, to the convulsive effect of the drug. Synaptosomes seem to be more sensitive to pentamethylene tetrazol than cerebral cortex slices, since the drug only causes small changes in the metabolism of those preparations at the concentrations used (Potter and van Harreveld 1962). This could be explained if the synaptosomal membrane is more permeable to pentamethylene tetrazol than the neuronal membranes, as they exist in cerebral cortex slices, or if the concentration of the drug reaching the neuronal membranes is considerably lowered by the presence of other cells, as one might expect. It would be of value, in any future study of the mode of action of this drug, to study its effect on the metabolism of the nerve-ending in detail.

Phenobarbitone increased the amount of label retained

from (U-^{14}C)-glucose (Table VII p69). In this case, however, effects on the incorporation of carbon atoms from glucose into metabolites may be complicated by the effects of this drug on the permeability of the synaptosome membrane. The results will, therefore, be discussed later in the General Discussion in relation to the effects on permeability.

Preincubation in convulsant and anticonvulsant drugs seemed to have little effect on the potential transport systems of the synaptosomal membrane. Mention has already been made of the difficulty of interpretation of ATPase results (p90). The effects are not only dependent upon the concentration of the drugs, but also upon the conditions under which the enzyme is assayed (Festoff and Appel 1968). The anticonvulsant drug, diphenylhydantoin, is reported to exert its effect by stimulation of the Na^+ pump (Woodbury 1955), but incubation of synaptosomes in media containing the drug results in stimulation of the ATPase (Festoff and Appel 1968) or inhibition (Formby 1970) depending upon the conditions of the experiment. The results of changes in the activity of this enzyme are also difficult to assess since neuronal

membranes seem to actively transport a variety of ions and compounds. No clear pattern of response seemed to emerge and, in view of the controversy over interpretation of the results, the study of this enzyme in this work was limited. It must also be said that none of the convulsant and anticonvulsant drugs tested here have been shown to exert effects by altering the activity of the membrane ATPase.

Under the conditions used here, there appeared to be no active transport of xylose into the synaptosomes (p92) and, in this respect, the synaptosomal membrane seems to differ from the neuronal membranes as they exist in cerebral cortex slices, which do actively transport this sugar (Gilbert 1966). Marchbanks (1968a) has suggested that treatment with 2,4-dinitrophenol need not affect active transport processes since the drug does not reduce the reserves of high energy phosphate in the synaptosomes to zero. But 2,4-dinitrophenol does prevent the active transport of xylose into cerebral cortex slices (Gilbert 1966) inspite of the fact that the high energy phosphate reserves of the slices are not reduced to zero (Joanny and Hillman 1963). The

volume of the synaptosomes, under our conditions, calculated from the equilibrium concentration of xylose was 2.2µl per mg protein (p94). This value is close to that reported by Marchbanks (1967), and this further suggests that xylose is not accumulated against a concentration gradient within the synaptosomes, under our conditions.

If xylose does, indeed, equilibrate evenly across the synaptosomal membrane, such that its concentration in the cytoplasm is the same as that in the surrounding medium, it may be possible to use xylose to measure the synaptosomal volume under conditions in which the active transport of ions is occurring. It would be of value to be able to measure the volume of the synaptosomal cytoplasm under these conditions, since the volume is, in part, dependent upon the activity of the ion pumps and changes in the metabolic state of the particles could be reflected as a change in the volume. A non-metabolised sugar is suitable for such a measurement because it is unionised and, therefore, not affected by the ion gradients and it also diffuses out of the particles slowly enough, in the cold, to enable the

experimenter to separate the synaptosomes from the extrasynaptosomal xylose. It must be emphasised, however, that xylose may be actively accumulated by synaptosomes under different conditions - another buffer system for instance - which favour the active transport process.

The remaining results were obtained using synaptosomes suspended in 0.32M sucrose, a condition which is far from physiological. The object of these experiments was to examine the ability of the convulsant and anticonvulsant drugs to interact with the membranes and, thereby, modify the mechanical or permeability properties of the membranes.

In order to study the effects of drugs on the synaptosomal membrane, it was necessary to establish a method by which these effects could be observed. Many previous studies on the interaction of drugs with cell membranes had been done by testing the ability of the drugs to inhibit the lysis of erythrocytes by detergents or osmotic shock. The results of such experiments are difficult to interpret and also effects observed on erythrocyte membranes are not necessarily

true for neuronal cell membranes. However, once Keen and White (1970) had shown that synaptosomes performed as osmometers in NaCl solution and that changes in the reciprocals of the extinctions of synaptosome suspensions were proportional to changes in the synaptosome volume, it was possible to test the ability of the drugs to modify the elastic properties of the synaptosomal membrane. But these results would also be difficult to interpret in terms of effects on the permeability of the synaptosome membrane. However, if freshly prepared synaptosomes are suspended in warm sucrose, they shrink due to the loss of some of the osmotically active components of the cytoplasm and it was shown, in this work, that this shrinkage could be observed by measuring the extinction of synaptosome suspensions at varying time intervals.

By observing the effects of the drugs on both the shrinkage of the synaptosomes and on the performance of the particles in hypotonic solution, we were able to differentiate between the effects of the drugs on the permeability properties and the effects on the mechanical properties of the synaptosome membrane.

Using these techniques, it was shown here that pre-incubation with either of the convulsant drugs was without effect on the synaptosome membrane (Table XI p103), but that the anticonvulsants, especially the barbiturates, had a marked effect on the volumes of the synaptosomes in 0.2M NaCl, after 30 minutes incubation in warm sucrose. These effects were consistent with an alteration of the permeability properties of the synaptosome membrane which resulted from the inclusion of the drugs in the incubation medium. It was not surprising that phenobarbitone modified the permeability properties of the synaptosome membrane at concentrations close to those found 'in vivo' since previous work had suggested that this drug exerted its effect by interacting directly with neuronal cell membranes (Tower 1960, Millichap 1965).

When thiopentone was examined for its effect on the permeability of the synaptosome membrane, its effects were very similar to those of phenobarbitone at the same concentrations. Thiopentone is used, primarily, as an anaesthetic, but it also has anticonvulsant properties (Harrison, Rees and Watson 1971), thus effects observed

in the present work could contribute to the role of these drugs as anticonvulsants because they were observed for both drugs. It is difficult to separate the different effects of the barbiturates and attribute a physiological response to them since, in an extreme case, barbiturate drugs which cause convulsions may also have a depressant effect in the brain (Domino 1956). It would, however, be of interest, in a future study, to compare the effects of a convulsant barbiturate with those observed here for phenobarbitone and thiopentone. It is not unreasonable to suppose that most barbiturates bind to neuronal cell membranes and modify their permeability properties, and that the variety of other physiological effects which result from treatment with the different barbiturates are a result of differences in the chemical structure of the drugs which enable specific drugs to alter other aspects of neuronal metabolism and stability, or alter the distribution of the drug in the CNS.

Acetazolamide only affected the permeability of the synaptosome membrane at concentrations far in excess of those found 'in vivo' (p108). Other effects of acetazolamide, especially the inhibitory effect of the

drug on the enzyme carbonic anhydrase, occur at concentrations closer to those found 'in vivo' and probably contribute more to its anticonvulsant effect.

In subsequent experiments, the nature of the compounds which leak out of the synaptosomes during suspension in warm sucrose were studied in more detail. It was found that, as a result of the preincubation in warm sucrose, the Na^+ and K^+ concentrations of the synaptosomes were reduced (Table XVII p122). It is interesting that, whereas only 25 per cent of the K^+ is lost to the medium during a 30 minute incubation, nearly 50 per cent of the Na^+ is lost. Marchbanks (1967) and Weinstein and Kuriyama (1970) reported that the permeability of the synaptosome membrane is greater for K^+ than it is for Na^+, therefore it is likely that factors other than those of simple diffusion are involved. The membrane ATPase may be active at 25°C (Weinstein and Kuriyama 1970) and this may favour the expulsion of Na^+ from the synaptosomes in favour of K^+. Weinstein and Kuriyama further suggested that K^+ and Na^+ acted as counter-ions for non-diffusible anions of the cytoplasm, such as free glutamate and aspartate and the cytoplasmic proteins. They proposed that K^+ and Na^+ were,

in part, released through the synaptosomal membrane by a process of ion exchange. The only cation available in the medium was H^+. The salts of weak acids are almost fully ionised, but the acids themselves are ionised only to a small extent, thus, if such an exchange took place, the concentration of osmotically active constituents of the synaptosomal cytoplasm would be reduced and the volumes of the synaptosomes would decrease. This may account for the fact that almost the entire loss of synaptosomal volume, which occurred during 30 minutes incubation, could be accounted for by the amount of K^+ and Na^+ lost during the same time.

In Table XVII (pl22) the effects of preincubation with 2mM phenobarbitone on the amounts of K^+ and Na^+ lost from the synaptosomes during a 30 minute incubation in warm sucrose were reported, and it was shown that the loss of these ions was almost totally prevented by the phenobarbitone. It seems, therefore, that phenobarbitone markedly reduces the permeability of the synaptosome membrane to K^+ and Na^+ and this effect is entirely consistent with the effects of this drug on the shrinkage of synaptosomes in warm sucrose, discussed above (pl39).

This effect of phenobarbitone on K^+ and Na^+ permeability could clearly contribute to the mode of action of this drug as an anticonvulsant, and the results are of special significance in view of the reports by Tower (1960) that one of the features of epileptogenic tissue is its inability to form or maintain ion gradients, and the reports by Millichap (1965) that phenobarbitone is especially useful when treating epilepsies which result from inadequate repolarisation mechanisms. If the pre-synaptic membranes did not provide a sufficiently great permeability barrier to Na^+ and K^+, due to incorrect formation of the membranes or external injury or if the active transport of the ions was impaired, the presence of phenobarbitone could enhance the formation of the gradients since its effect would be to reduce the amount of ions diffusing down the concentration gradient, from the outside to the inside of the cell in the case of Na^+. The drug could, in this way, increase the degree of polarisation of the membrane and thus the threshold for depolarisation. It may also hyperpolarise normal synapses near to the epileptogenic focus and, in this way, inhibit the spread of seizures in the brain.

There are more subtle effects which may result from a change in the rate of flow of K^+ and Na^+ across the pre-synaptic membrane. The leakage of pharmacologically active compounds, such as acetylcholine (Tower 1960) or glutamate (Bradford 1970b), may be accelerated if the K^+ and Na^+ gradients are disturbed. Thus, if phenobarbitone exerts its effect by reducing the passive diffusion of these ions and enhancing the formation of the gradients, it may also reduce the amounts of these pharmacologically active compounds which leak out of the cells.

The results of experiments in which the effects of phenobarbitone on the incorporation of ^{14}C from (U-^{14}C)-glucose were examined, suggested that the incubation with the drug resulted in an increased incorporation of labelled carbon atoms into metabolites formed from glucose (p133). This effect could clearly result from an alteration in the metabolic processes of the particles, but the release of some of the metabolites formed from glucose, notably glutamate, from the synaptosomes seems to be coupled, to some extent, to the flow of K^+ ions out of the particles (Bradford 1970b).

We may now compare the results obtained in this work with those obtained using other preparations. Much of the previous work in which the mode of action of centrally acting drugs has been examined has been performed using brain homogenates or cerebral cortex slices and reference has already been made to the results. From reports of this earlier work, it was clear that acetazolamide inhibited the enzyme carbonic anhydrase and probably alters the distribution of Na^+ across neuronal cell membranes by altering the amount of anion available in the cytoplasm. Phenobarbitone, on the other hand, seems to have its effect by a direct physico-chemical interaction with the neuronal cell membranes. The results reported here seem to confirm the hypothesis that phenobarbitone interacts with membranes to alter their properties.

The advantage of the system used here is that we were able to show a clear effect on membranes which were neuronal in origin, whereas cerebral cortex slices are multicellular systems and the experiments in which slices were used could not differentiate between effects on neuronal and non-neuronal membranes. In the past,

in order to study the effects of drugs on only one type of membrane at a time, workers have resorted to using mitochondria (Spirtes and Guth 1963) or erythrocyte preparations (Sheppard et al 1969, Mikilits et al 1970). Experiments using mitochondria are only truely valid for drugs which act on the mitochondrial membrane, however, since this membrane is unique in character. In other work, the ability of drugs to inhibit the lysis of the erythrocyte membrane, by osmotic shock or detergents, has been examined. The results of these experiments are difficult to interpret, however, since an alteration in the stability of the erythrocyte membrane could be the result of an alteration in the stability of the membrane or in the permeability of the membrane or both. In this work the effects of drugs have been studied on the permeability of the synaptosome membrane directly. This has enabled us to draw conclusions which are a little different from those of Mikilits et al (1970), working on erythrocyte membranes, concerning the mode of action of the anticonvulsants. It has been shown here that the barbiturates, phenobarbitone and thiopentone, reduce the permeability of the

synaptosome membrane to the same degree at the same relatively low concentrations, but that acetazolamide has the same effect only at concentrations which are far greater than those found in the brain 'in vivo'. After incubation with these drugs, at concentrations which reduced the permeability, the synaptosomes seemed to become less stable in hypotonic solution (p109) and these findings are in agreement with those of Mikilits et al (1970) for the effects of these drugs on the erythrocyte membrane. It is our belief that, in our experiments, this instability resulted from a decrease in the permeability of the synaptosome membrane and that any direct effects of the drugs on the stability of the membrane were masked.

The results obtained in this work would suggest that it would be profitable, in some future research, to extend further the studies of the effects of drugs on the permeability of the synaptosome membrane. In particular it would be useful to study the effects of a wider range of drugs, including a convulsant barbiturate, and also to study the effects of the drugs on the permeability of the synaptosome membrane to a wider

range of compounds. For example it would be interesting to know if phenobarbitone altered the rate at which pharmacologically active compounds, such as glutamate or the putative transmitter compounds, escaped through the synaptosome membrane.

S U M M A R Y

1. Synaptosomes have been isolated from guinea-pig cerebral cortex and their appearance and enzymic and respiratory properties found to be similar to those reported for synaptosomes by other workers.

2. The effects of the convulsants, strychnine and pentamethylene tetrazol, and the anticonvulsants, phenobarbitone and acetazolamide, on some of the properties of synaptosomes have been examined.

3. The resting respiratory rate of synaptosomes was found to be insensitive to the convulsants and anticonvulsants and to a variety of other compounds which affect excitable neuronal membranes 'in vivo'. The possible significance of these results has been discussed.

4. The incorporation of ^{14}C into synaptosomes from (U-^{14}C)-glucose was inhibited by pentamethylene tetrazol and enhanced by phenobarbitone.

5. The uptake of xylose, which did not seem to occur by means of a simple diffusion process, was unaffected by phenobarbitone and pentamethylene tetrazol.

6. The concentration of xylose in the synaptosomes, once xylose uptake was complete, was unaffected by 2,4-dinitrophenol.

7. The release of osmotically active constituents from synaptosomes suspended in warm sucrose was very much reduced by phenobarbitone at relatively low concentrations and by acetazolamide at concentrations which were substantially greater than those found 'in vivo'.

8. 45 per cent of the Na^+ and 27 per cent of the K^+ was lost to the medium if freshly prepared synaptosomes were incubated in sucrose at 25°C for 30 minutes. Phenobarbitone prevented the loss of these ions during the incubation.

9. Phenobarbitone had no effect on the release of xylose from synaptosomes which were suspended in warm sucrose.

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A P P E N D I X

A Treatment of the Xylose-Uptake Data for Synaptosomes

Let x = the concentration of xylose in the medium surrounding the synaptosomes, and be constant;

y = the concentration of xylose in the synaptosomes at time t ($y = 0$ when $t = 0$);

$f = y/x$;

K = the apparent dissociation constant of any xylose-carrier complex formed at the synaptosome membrane, and let it be assumed that the same dissociation constant is involved in uptake by, and efflux from, the synaptosome;

V_{max} = maximal transport rate;

t = time over which uptake by the synaptosomes is determined.

(a) Diffusion Kinetics

If there is no interaction between xylose and a carrier during uptake (or if K is large compared to x), then

$$\frac{dy}{dt} = k(x - y)$$

On integration and substitution we can derive

$$\ln \left(\frac{1}{1 - f}\right) = kt$$

or

$$\frac{\ln \left(\frac{1}{1 - f}\right)}{f} = k \cdot \frac{t}{f} \qquad \ldots\ldots(a)$$

(b) Double Michaelis-Menten Kinetics

If xylose interacts with a mobile membrane component during its uptake, and if it can be transported out of the synaptosome by a similar process involving the same membrane component, then

$$\frac{dy}{dt} = \frac{V_{max}x}{K + x} - \frac{V_{max}y}{K + y}$$

On integrating and rearranging this equation we can derive

$$\frac{\ln\left(\frac{1}{1-f}\right)}{f} = \frac{KV_{max}}{(K+x)^2} \cdot \frac{t}{f} + \frac{x}{K+x} \qquad \ldots\ldots(b)$$

(See Fisher, R.B. and Gilbert, J.C. (1970): J. Physiol 210 287)

It will be seen from equations a and b that by calculation $\ln\left(\frac{1}{1-f}\right)$ and $\frac{t}{f}$ from the uptake data, then the plot of the results may provide a means of distinguishing between the general processes involved in xylose uptake, the main distinguishing feature being the point at which the best straight line through the results intersects the y-axis. The results for the xylose uptake by synaptosomes, obtained in this work, have been plotted in this log form (Figure overleaf) and it may be seen that the results are consistent with the plot for double Michaelis-Menton kinetics (equation b).

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Log Plot of Xylose Uptake by Synaptosomes