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THE APPLICATION OF A TECHNIQUE FOR COUNTING SYNAPTOSOMES IN AN INVESTIGATION OF THE EFFECT OF HYPOTHYROIDISM ON THE NUMBER OF SYNAPSES IN CERTAIN REGIONS OF THE RAT BRAIN

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SUMMARY

A technique for enumerating synaptosomes first developed by Clementi Whittaker and Sheridan (1966) was investigated. Various modifications were examined but none proved more effective than the original method.

The technique was used to determine the number of synapses in the developing rat brain and in the brains of rats subjected to thyroidectomy. It was shown that the technique, when used with care and attention to detail, could be successfully applied to this type of investigation.

Some results obtained parallel those achieved by other workers using different techniques, whilst other results provide new information on the effect of thyroidectomy on synaptogenesis. The pros and cons of the Clementi technique, and of other possible procedures for the enumeration of cortical synapses, was critically discussed.

The possible significance of the effect of thyroidectomy on the mitochondria, structure and function of synapses was considered in relation to the mental deficiency associated with cretinism.

CONTENTS

1. INTRODUCTION

There are two principal systems concerned with the co-ordination of the animal body: the endocrine system and the nervous system. The first acts by the release of chemical substances into the vascular system which exert their effect at some more or less remote site in the body; the second acts by the transmission of impulses along nerve fibres and the release of chemical substances at either intermediate stages and/or the site of action. Whereas the nervous system is largely concerned with the control and co-ordination of muscular activities - the endocrine system is primarily, but not exclusively, cencerned with the co-ordination of vegetative functions; for example, growth, reproduction, etc. It is not surprising that two systems with such allied and complementary functions should interact with each other. Thus it is well known that the hypothalamus exerts a controlling influence over the adenohypophysis of the pituitary and consequently over many other endocrine glands and conversely the thyroid gland exerts a controlling influence on the growth and development of the entire brain.

The mammalian brain is the most complex structure known to science. It can, however, be analysed by studying its various sub-units of which one of the most important is the synapse. It is only at these junctions that communication between neurons and other recipient cells can occur. It is, therefore, not unreasonable to assume that behavioural complexity may be related to synaptic complexity.

Synaptic complexity is, of course, a difficult concept to define. However, in later sections of this thesis, aspects of the morphology, size, and biochemical activity of synapses will be discussed as well as the variation of these parameters induced by changes in the environment. In this project only one parameter has been experimentally investigated: number.

The number of synapses in unit volume of animal brain has been investigated by several workers. The technique employed has involved fixing and sectioning brain tissue and examining the result either in the optical or electron microscope. In the first case, synaptic spines have been counted; in the second, synapses have been identified and enumerated.

In 1966 Clementi, Whittaker and Sheridan published a technique for counting synaptosomes derived from the homogenisation and subsequent fractionation of entire cortices. This project was designed to test and, if possible, improve Clementi's technique and to use it to examine the influence of thyroidectomy on synaptic numbers in the rat brain.

2. REVIEW OF THE LITERATURE

2.1 THE CNS AND INTER-NEURONAL COMMUNICATION

2.11 Types of Neurones

Histological examination of the CNS, using specialised staining and silver impregnation methods (Eayrs and Goodhead, 1959), reveals that there are various types of neurones and glial cells differing from each other in both structure and function. Thus neurones, which may vary considerably in size, can be identified and differentiated by the axon and dendritic processes seen to arise from the cell body (Fig. 1).

Neurones described as unipolar may be one of two types; true unipolar (Fig. 1A), which are rare and, in humans, found in the mesencephalic nucleus of the fifth cranial nerve (Bradbury, 1973); and pseudo-unipolar (Fig. 1B), in which the single process which arises from the perikaryon divides some distance from the cell body. These latter cells are located in the sensory ganglia and are concerned with such sensory perceptions as pain, temperature, light touch, pressure, proprioception, vibratory sense. The bipolar cells, so described because of their axon and single dendrite, are found in the visual, auditory and olfactory systems. The third major type of neurone is the multipolar cell having a single axon but more than one dendrite. These may be further divided on the morphology of the cell body; fusiform, basket, stellate, and pyramidal. These cells constitute the majority of neurones to be found in the CNS, particularly in the motor cortex.

FIGURE 1.

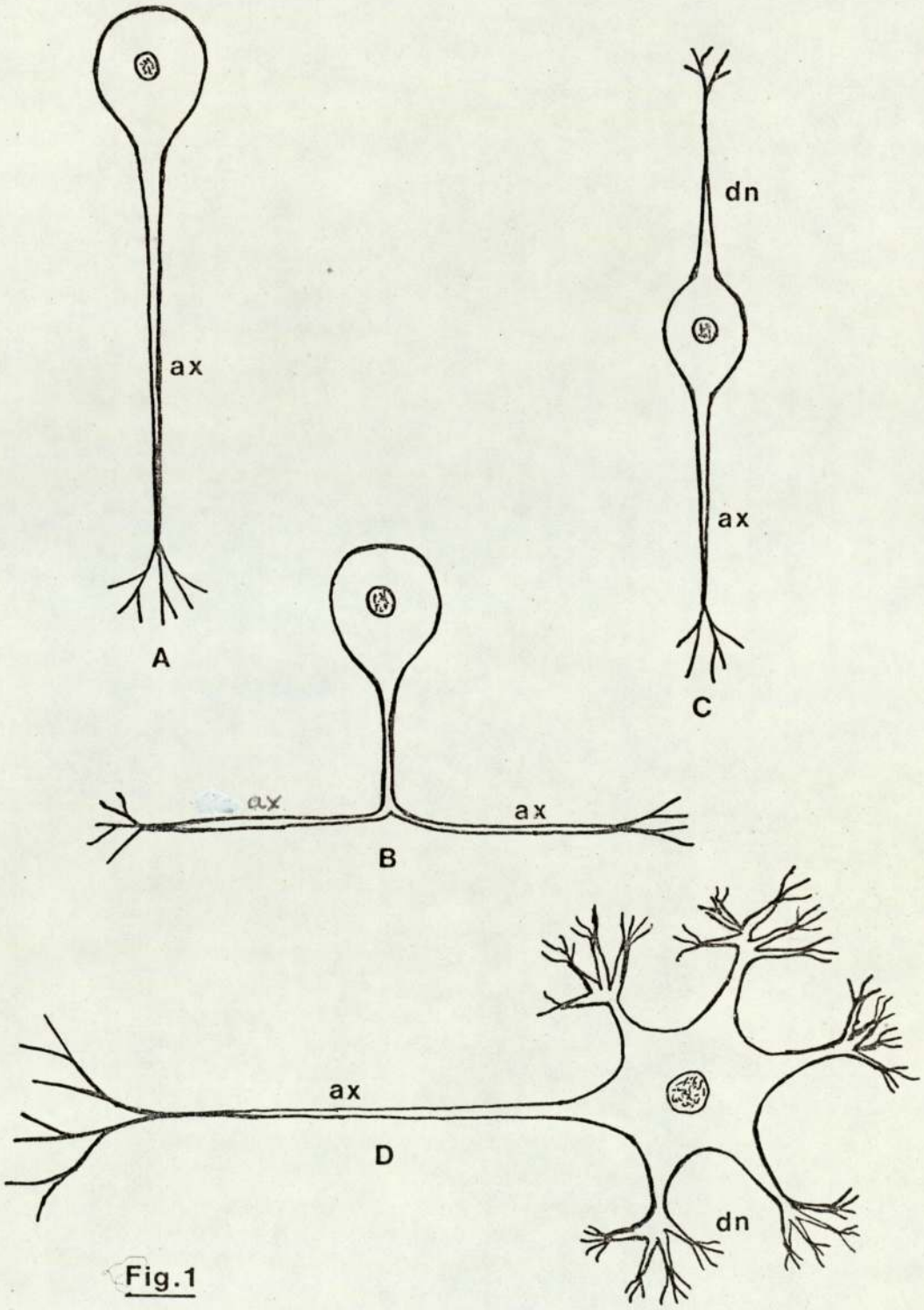


Fig.1

2.12 Types of Glial Cells

Glial cells constitute the brain substance, providing the supporting tissue and service facilities to the neurones. (The term glia means glue.) They may be differentiated from neurones by the lack of a prominent nucleolus. Glial cells may be differentiated into three main types; astrocytes, oligodendroglia and microglia (Fig.2), on their morphology and function.

The astrocyte, as can be seen in Fig. 2B, is a relatively large cell with many convoluted processes which either attach to the fibres of the nerve cells, or form foot-like attachments almost completely enclosing the blood capillaries. Since neurones have no direct contact with the circulatory system it is believed that the astrocytes act as transport cells for the movement of nutrients to the neurones and perhaps removal of waste materials to the blood. Oligodendroglia (Fig. 2C) are more numerous in the white matter of the CNS and are consequently thought to be concerned with the formation of myelin, a proteolipid material which forms an insulating sheath around the axons. Microglia (Fig. 2A) are migratory and spread throughout the CNS. They can be compared with the polymorphonuclearcytes of the circulatory system in that they are phagocytic and perhaps function as scavenger cells.

2.13 The Neurone

Detailed histological examination of the CNS, together with the information gained from such experiments as the electrical stimulation of specific areas of the cortex during surgical procedures, reveals that groups of cells can be associated with specific functions. This information has formed the basis of architectonics, a technique developed by such workers as Bailey, Brodman, Vogt, Von Economo and Von Bonin (Schadé, 1967) to show cellular relationships and differences, and thus provide a form of

FIGURE 2.

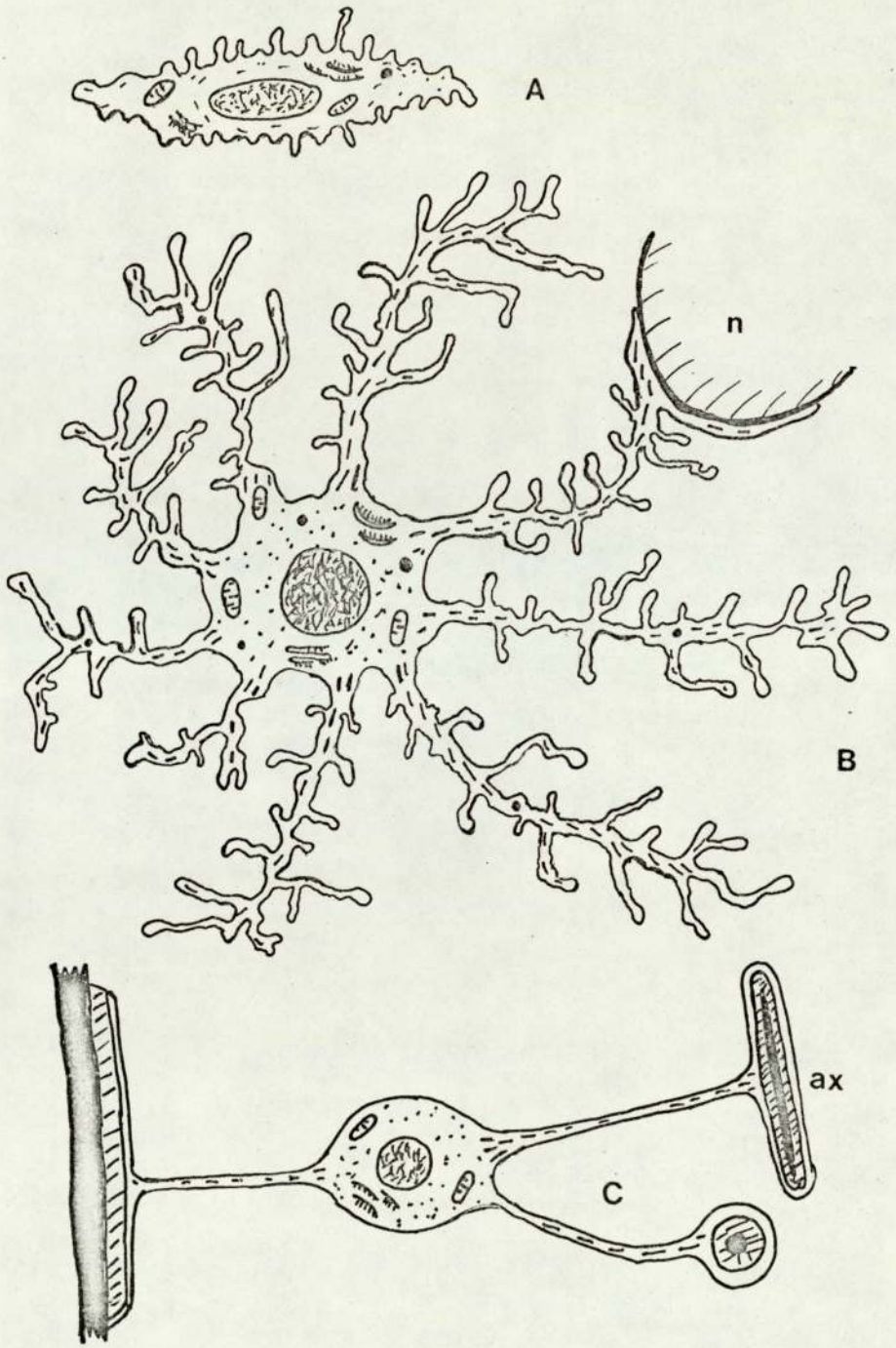


Fig. 2

mapping which would allow functional localisation to be related to cell types. Since such information shows a complex inter-relationship between neurones, within the various parts of the CNS, the necessity to completely understand the structure and functional activity of the single neurone is the key to the eventual understanding of the overall neurophysiology of the brain.

The neurone is the basic functioning unit of the nervous system since these cells possess the particular property, following stimulation, of relaying an impulse and, as a consequence, are constructed accordingly. Essentially the neurone consists of the perikaryon containing the organelles common to all cells; a nucleus containing a nucleolus and bounded by a nuclear membrane, golgi appartus, and endoplasmic reticulum. In addition, mitochondria and various cytoplasmic inclusions; vacuoles, glycogen, crystalloids, etc. may be found. (Fig. 3 and 4). Neurones also possess neurofilaments which are fine structures measuring 10 nm* in diameter and neurotubules 23 nm in diameter, the function of which is not clearly understood. Such structure, when examined by light microscopy, following routine histological fixation and processing, are seen as aggregations and are referred to as neurofibrils. In electron micrographs the neurofilaments are seen to be present in both the axon and dendrites, and extending into the cell body. Another characteristic of neurones is Nissl substance so named after Nissl, the neurohistologist who first demonstrated this material. Electron micrographs of Nissl substance (Palay and Palade, 1955) reveals that it is composed of aggregates of narrow tubes which are flattened cisternae, vesicles or tubules of the endoplasmic reticulum and are frequently covered

* All submicroscopical measurements in nm (x 10 = A units)

FIGURE 3.

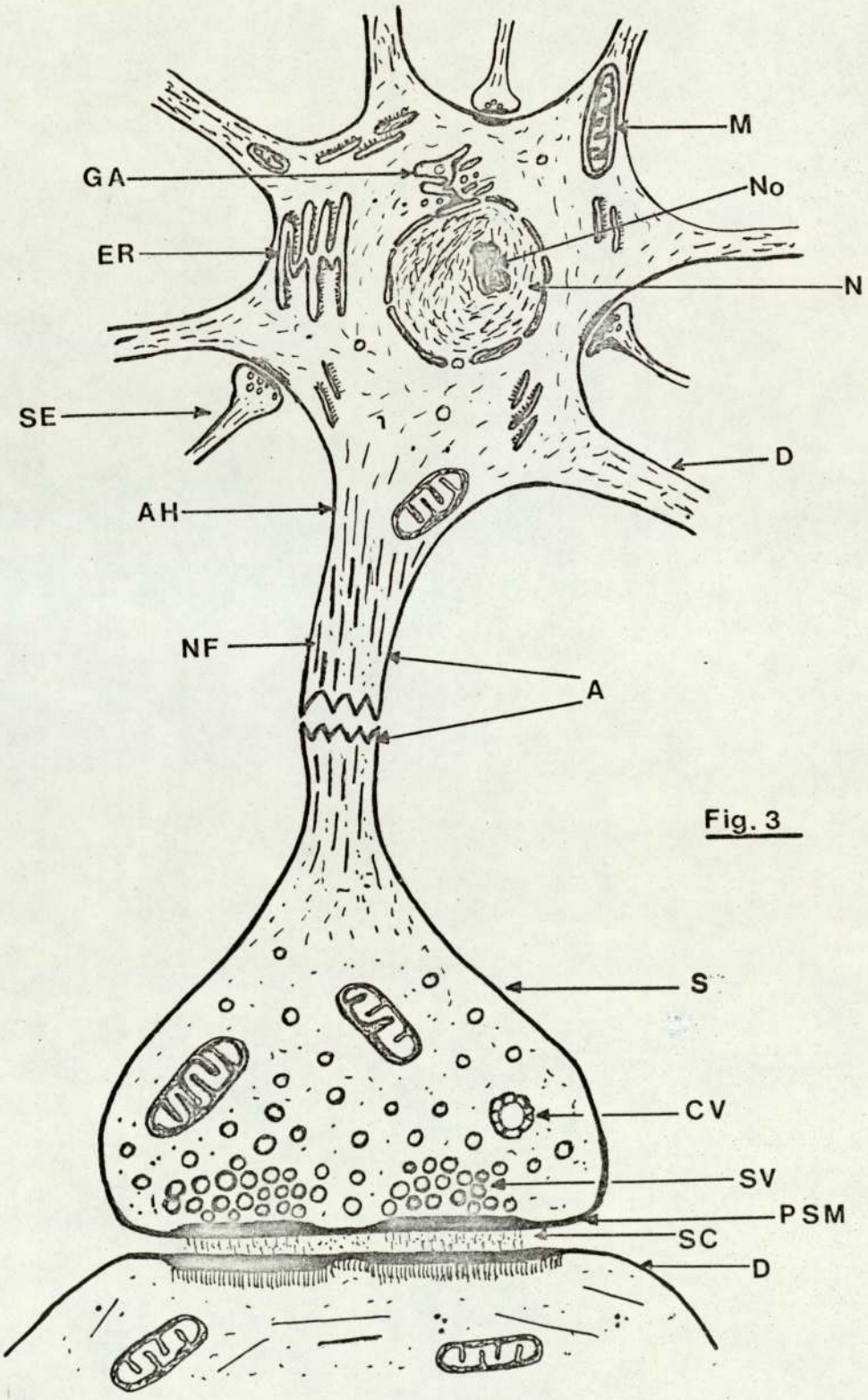


Fig. 3

FIGURE 4.

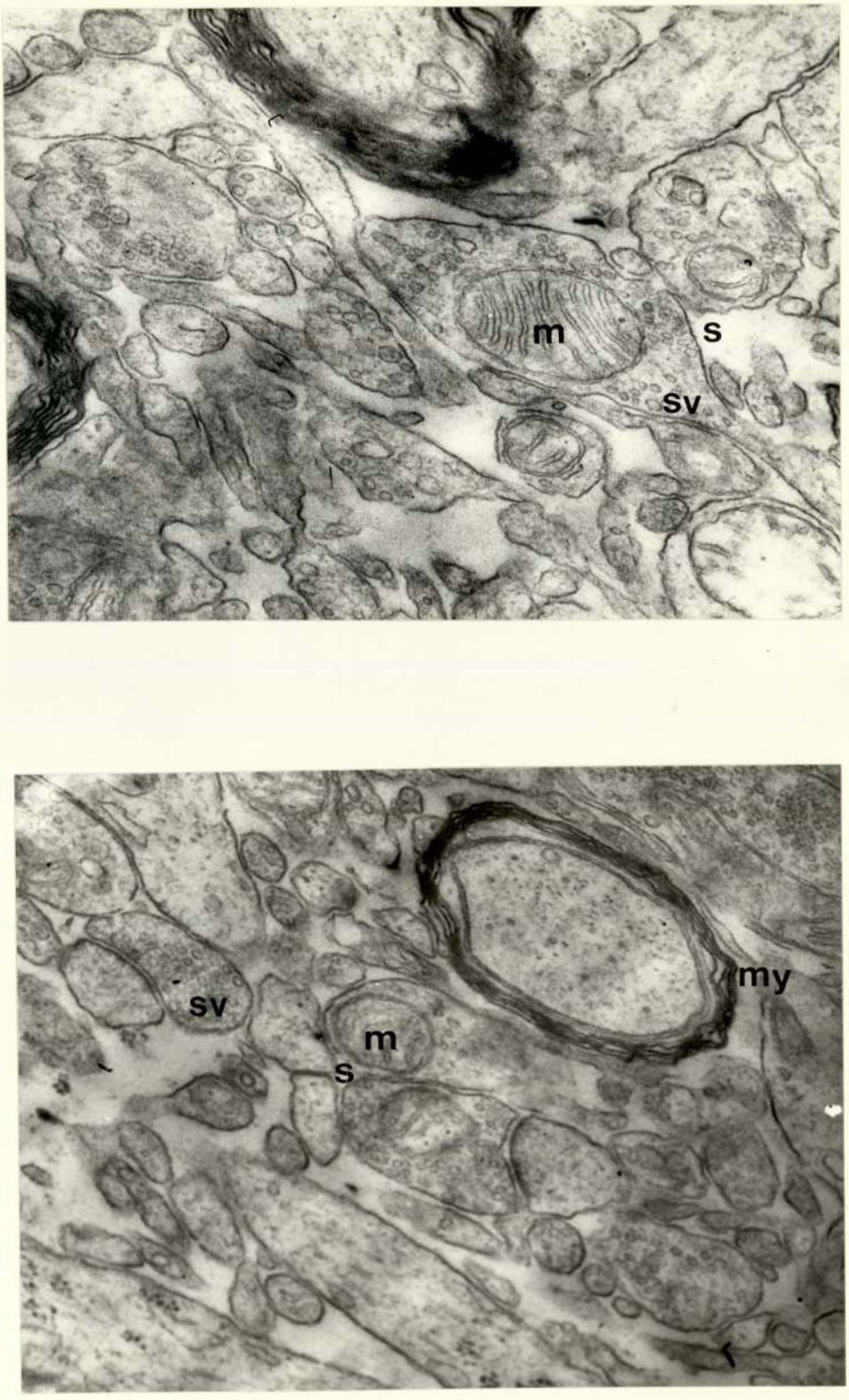


Fig. 4

with granules. The specific staining reactions of these granules, which allowed Nissl to demonstrate them, is due to their composition which is essentially RNA and associated protein (RNA-P). Nissl substance is only found in the cell body and dendrites, and its demonstration allows clear identification of neurones.

The dendrites can be considered as extensions of the cell body cytoplasm through which extensive communication with the axons of other neurones is made possible. Such communication can take place through the synaptic junction vide infra formed at any point on the dendrite, cell body, or axon initial segment. The surface area of the dendrite is sometimes greatly enlarged by the existence of small projections (spines), e.g. cerebral pyramidal cells and cerebellar Purkinje cells. In the case of the Purkinje cells, this area enlargement may be as much as 90,000 μm^2 (Schadé and Ford, 1967). In addition, extensive branching of the dendrite allows a greater number of synaptic contacts between several cells. For example, in the case of the human adult motor cortex neurone, the number of branching points may exceed forty with a total length of nearly 7,000 μm. The dendrites are thus afferent processes allowing the message received, from the axon of another neurone, to be transmitted to the cell body and hence down the efferent axon process of the recipient cell.

The axon, like the dendrites, is an extension of the neurone perikaryon and is constructed in the form of a tube which contains the axoplasm. The axoplasm provides the necessary ionic composition for transmission of the impulse vide infra. There has been disagreement as to whether the axoplasm flows from the perikaryon, down the axon, to the synapse thus facilitating, in the view of the protagonists, the migration of the manufactured proteins. Droz (1963) has clearly shown, using tritrium labelled leucine and

methionine, and timed autoradiography, that these amino acids are incorporated into proteins manufactured in the neurone perikaryon. Following manufacture some proteins can, at sequential intervals, be found in the axon hillock and at various distances down the axon.

The axon of the CNS neurone is insulated from the outside by the oligodendroglia and astrocytes. In the case of the peripheral nerves, insulation is provided by the cytoplasmic folds of Schwann cells. The method of Insulation of the CNS neurones has only been discovered in recent years by the aid of the electron microscope. The location of the cell body in relation to the dendrites and axon endings, together with other structural characteristics, can reveal differences which may be related to neurone type and function (Copenhaver, Bunge and Bunge, 1964). It will be observed from Fig. 1 that many axons do not branch until just proximal to the synaptic junction. An exception to this is the interneuron of the CNS.

2.14 The Synapse

2.141 Types of Synapses: Morphological

Sections of the brain stained by the silver impregnation methods of Golgi reveal various structural specialisations at the axon terminals which, at the low magnification of the light microscope, appear as spherical bodies. Electron micrograph studies of these structures reveals a morphology which can now be related to the function of these synaptic junctions. Since the neurone conducts in only one direction, it is not surprising that the synapse has an asymmetrical shape. It is generally described as being flask-like in shape (Fig. 3) although morphology can vary in both shape and size. The synapse is referred to by a number of descriptive terms including: axon terminal, pre-synaptic ending, and bouton. Since synapses, in the CNS, are regions of apposition between two or more

neurones, they may be found on any part of the neurone. As a consequence they may be described in terms which indicate their location, for example: axo-dendritic synapses are made between axons and dendrites, these are perhaps one of the most commonly encountered types; axo-somatic synapses are made between axons and the neurone perikaryon, these are also commonly encountered; less frequently encountered synapses include axo-axonic synapses which are made between axons.

Dendrites may also synapse with other dendrites, dendro-dendritic. But in view of the differences between the inter-dendritic apposition and axo-dendritic apposition, together with the apparent lack of synaptic vesicles vide infra and a similar distribution of mitochondria, the significance of these functions is not understood (Van der Loos, 1963). A recent report by Lieberman and Webster (1972) provides evidence of still further intercellular contacts with the observation of neurones with pre-synaptic dendrites. They show that, in the rat dorsal lateral geniculate, dendrites may be found which are synapsing with other dendrites and cell processes. The synapsing dendrites are identified by the structure containing granular endoplasmic reticulum, ribosomes, other organelles typical of neuronal perikaryon and proximal dendritic segments. That the dendrite process is synapsing is shown by the presence of disc shaped synaptic vesicles which are in accordance with Dennisons category 4 vide infra. Dowling and Chappell (1972) have also shown that, in the median ocellus of the dragonfly, in addition to the receptor terminals synapsing with other receptor axon terminals and dendrites, the dendrites also synapse with not only other dendrites but also the receptor terminals (axons).

Other types of synapses include dendro-somatic, dendro-axonic and somato-dendritic; these appear to be restricted to regions of complex interaction between the larger neurones of sensory pathways and microneurones (Warwick and Williams, 1973).

As would be expected, the pattern of axon terminations varies, thus an axon may only synapse with one other neurone, e.g. the climbing fibres ending on the Purkinje cells of the cerebellum, or more frequently with more than one neurone. Synapses may also be arranged in groups; this provides a complex inter-relationship between the various neurones. A simple example of this is shown in Fig. 5D in which there is an axo-dendritic (excitatory) synapse and an axo-axonic (inhibitory) synapse. In the same diagram a reciprocal synapse (Fig. 5E) is shown; this allows transmission between two processes in both directions. This arrangement, as indicated, is frequently a staggered synapse involving both excitatory and inhibitory actions vide infra.

More complicated arrangements can be observed where several synapses interact. Examples of these include synaptic glomeruli (Fig. 6) in which various types of synapses are involved including: excitatory, inhibitory, reciprocal, 'boutons de passage'. In other instances several axo-dendritic endings synapsing in a small region of the dendrite are isolated by glial cells to form a synaptic cartridge (Fig. 7). Finally, synapses may be identified by inter-synaptic structures which indicate their possible function. Reference has already been made to two examples, excitatory and inhibitory (Fig. 5) and their significance, in addition to the synapses containing dense cored synaptic vesicles, will be dealt with later in this section.

FIGURE 5.

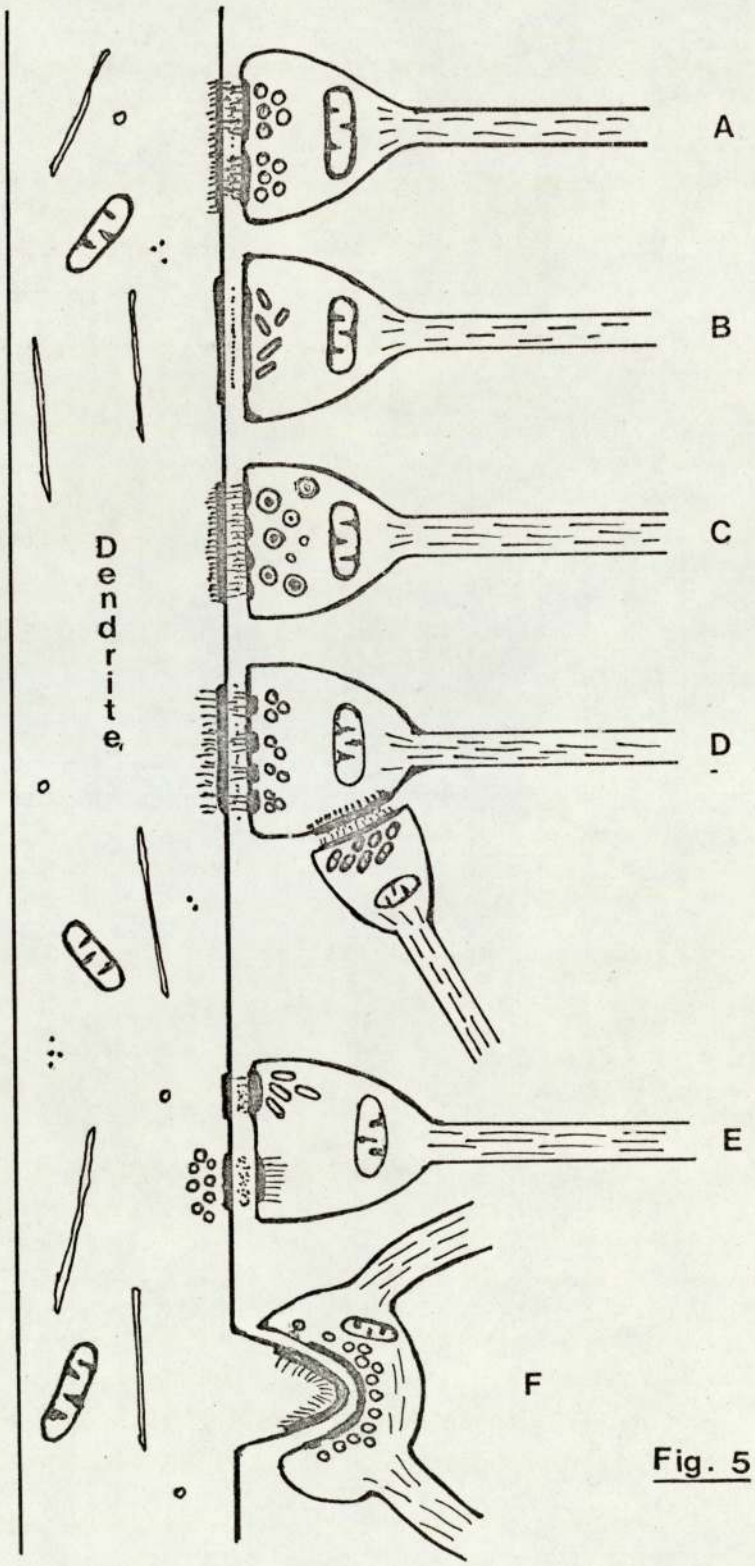


Fig. 5

FIGURE 6.

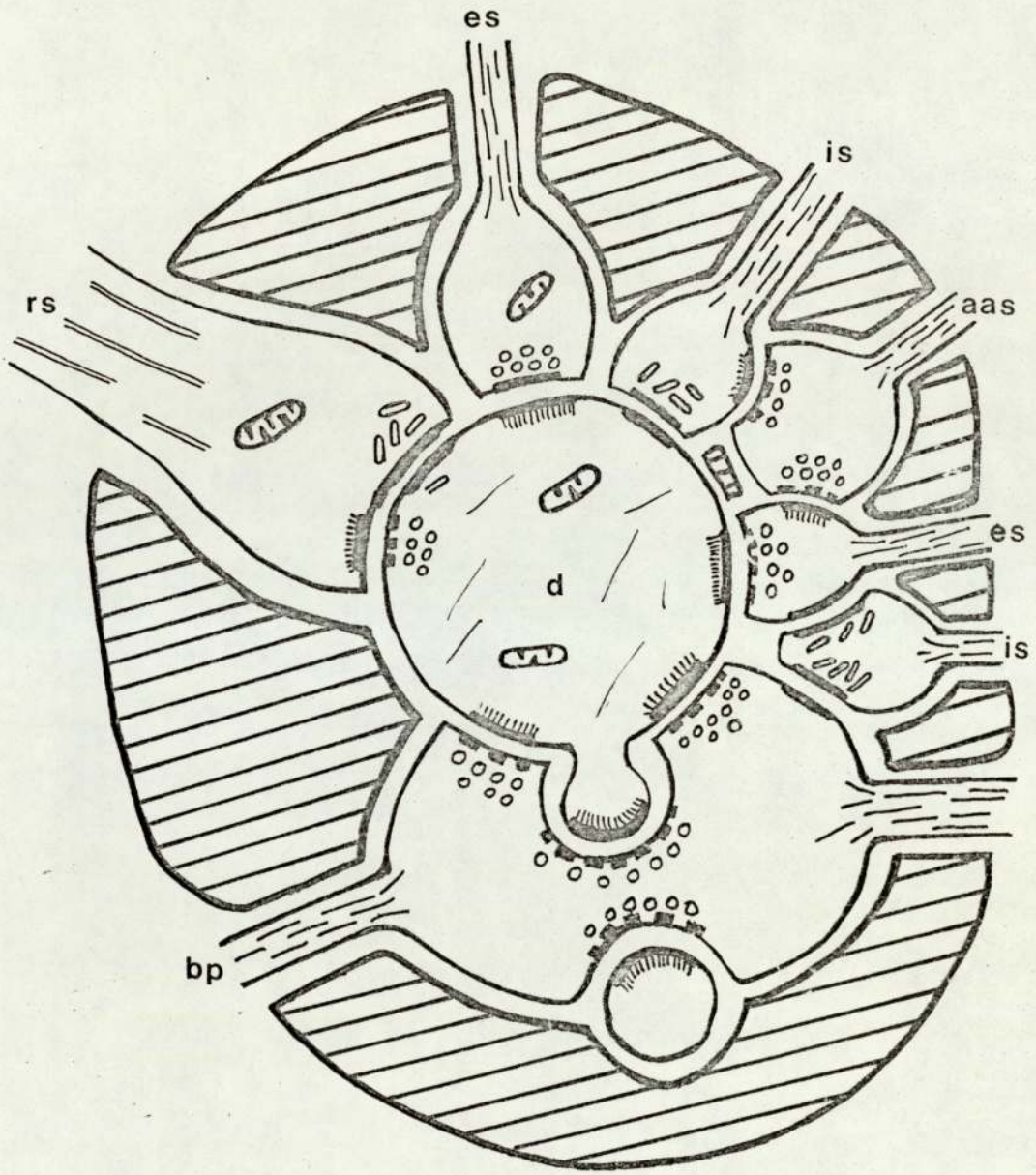


Fig. 6

FIGURE 7.

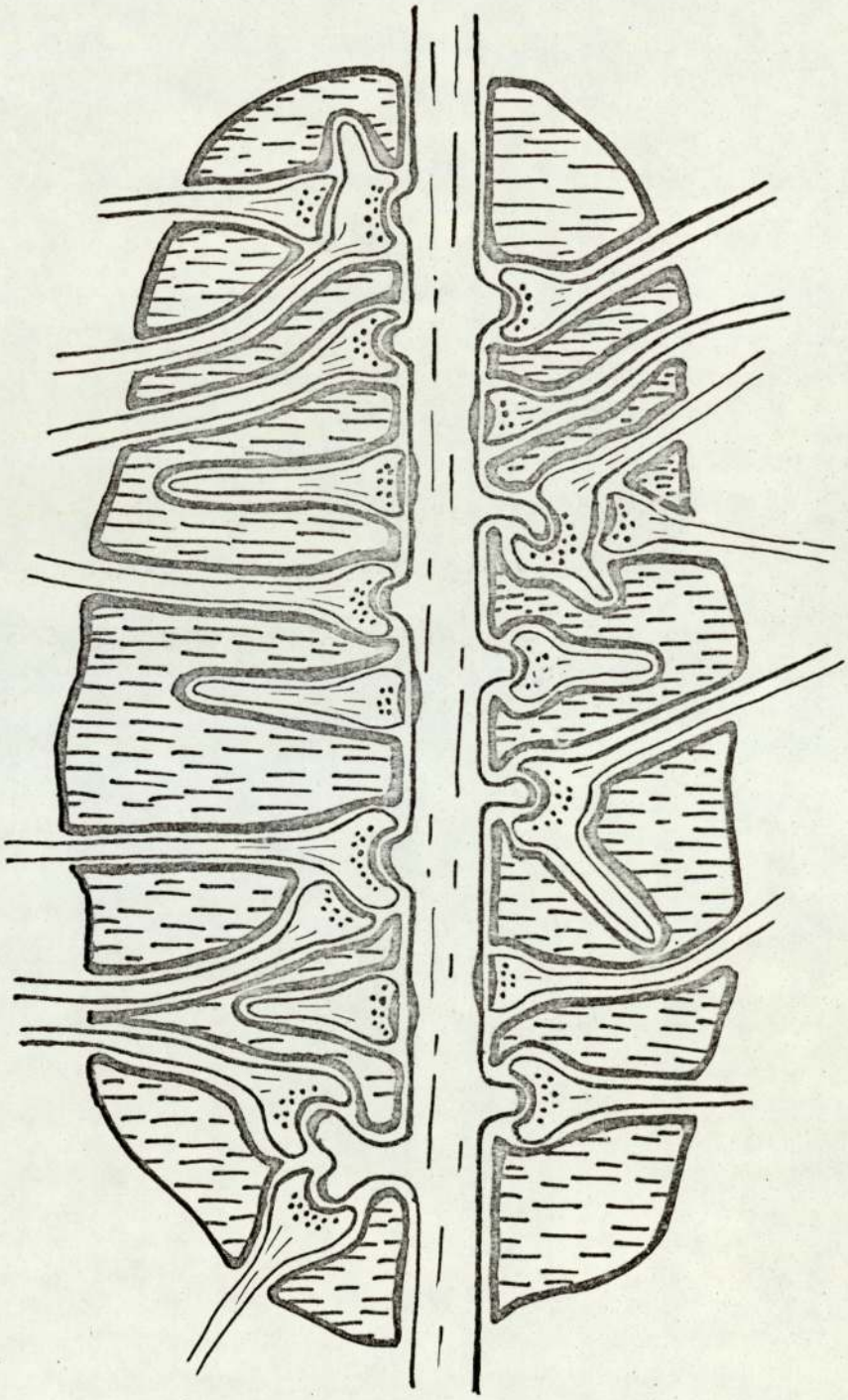


Fig. 7

In addition to interneuronal synapses, axons form synapses with muscle fibres, and such cellular relationships have provided the experimental vehicle for investigation into neurophysiology, particularly in the early development of this subject.

2.142 Magnitude of Synapses:

The basic structural appearance of the synapse in mammals is similar for most interneuronal functions (Palay, 1956; Mugnaini, 1970) in that the pre-synaptic fibre ends in a swollen knob varying in size from 0.5 - 4 μm (Whittaker and Gray, 1962) and best described as being flask shaped. Illis (1964) investigating synapses in cats using the light microscope suggested the grouping of synapses based on size. Thus Group 1, of which there were over 90%, consisted of synapses measuring less than 2 μm, Group 2 were between 2 - 4.8 μm (8.7%), and Group 3, of which there were only 0.5%, contained synapses measuring in excess of 4.8 μm. Hamlyn (1963), investigating pyramidal neurones in the Ammons horn of the rabbit, has shown that the appearance and arrangement of the synapses is related to their location. Thus those found on the perikaryon (axo-somatic) are larger than those found on main branches of apical dendrites. Whilst single synapses are found on the perikaryon, as previously described clusters of synapses may be found on the dendrites. Myelin or glial cell substance may, or may not, be found surrounding the bouton and normally does not interpose between the pre- and post-synaptic membranes.

Not all synapses present the rather classical appearance in the mammalian central nervous system. For example, some synapses found in the dragonfly ocellus may have presynaptic

membranes which are adjacent to post-synaptic membranes of two cell processes thus forming a triangular junction (Dowling and Chappell, 1972). The portion of the axon membrane in close proximity to the other neurone processes is referred to as the pre-synaptic membrane, whilst the proximal membrane of the recipient neurone is described as the post-synaptic membrane.

Contained within the bouton are mitochondria, the cell organelles concerned with respiration and consequential energy production in the synapse (Bradford, 1969). The number of mitochondria varies throughout the neurone but are more numerous in areas of high activity. For example, they have been shown to accumulate in areas close to axon damage (Schadé and Ford, 1967) presumably because of protein synthesis concerned with repair. As would be expected, mitochondria are found to accumulate in the bouton, although according to van der Loos (1964) they are less frequently encountered in the axo-somatic synapse than in the axo-dendritic synapse. Gray (1959) has also shown that, in the visual area of the occipital cortex of adult rats, the number of mitochondria per synapse varies. He found 47% did not contain mitochondria, 43% contained one, 8% contained two and 1% contained four. The latter number was the maximum observed. Morphologically the mitochondria are similar to those found in other cells, although longitudinal cristae have been observed (Gray, 1959).

2.143 Numbers of Synapses:

The number of synapses and neurones present in the CNS will provide an indication of the degree of intercommunication between the neurones. Such communication can, theoretically, reflect the possible degree of intelligence and there is

evidence vide infra that the number of synapses increase following certain types of stimulation. The determination of synaptic densities is therefore important and several workers have contributed to this topic, for example Cragg (1967, 1970, 1972), Clementi et al (1966), Aghajanian and Bloom (1967), Rebière and Legrand (1972), Nicholson and Altman (1972). Table 1 (Appendix) provides the data reported by these workers. With the exception of Clementi's data, the determination of synaptic numbers was achieved by sectioning selected areas of the cerebral cortex and therefore exact comparisons are not possible. However, the results show that the number of synapses in a specific region of the brain which has a possible limit to the degree of function does not vary between higher animals of different species. For example, the visual cortex contains synaptic densities of the following magnitude: $6.6 \times 10^8/mm^3$ - mouse, $6.2 \times 10^8/mm^3$ - monkey, $3.2 \times 10^8/mm^3$ - rat, and $3.4 \times 10^8/mm^3$ - cat. If this data is expressed as the number of synapses per neurone the results again show a close similarity between the animals, e.g. 7×10^3/neurone - mouse, 5.6×10^3/neurone - monkey, 8×10^3/neurone- cat. Thus, since these animals represent quite different Families it can be construed that the expected density of synapses in the visual cortex of animals is $3 - 7 \times 10^8/mm^3$ or $5 - 8 \times 10^3$/neurone.

Examination of the data from the motor cortex indicates that the number of synapses/mm^3 is very similar for mice ($8.5 \times 10^8/mm^3$), monkeys ($9.6 \times 10^8/mm^3$), and rats ($1.2 \times 10^9/mm^3$). However, of particular significance is the conversion of this data to the number of synapses per neurone. This shows that the monkey has a far higher density of interneuronal

connections (60 x 10^3/neurone) than the mouse (13 x 10^3/ neurone) or the guinea-pig (2.7 - 7.6 x 10^3/neurone). It should be noted that the latter result was obtained by a method (Clementi et al, 1966) different to that used for the other results.

Such findings support the belief that intelligence may be related to the synaptic density since it can be reasoned that the monkey is endowed with a greater degree of intelligence than the mouse or guinea-pig.

Finally, information on the number of synapses, in the rat cerebellum, obtained by two groups of workers, shows a sufficiently close similarity (Table 1) to indicate that the density is approximately 200 - 300/500 μm^2.

2.144 Presynaptic Membrane and Related Structures:

In synapses of the lower thoracic and upper lumbar region of the adult rat and cat spinal cord, Gray (1963) has shown that the pre-synaptic membrane possesses electron dense triangular shaped structures projecting into the axon (Fig. 8, drawn to scale). These structures, 80 nm from the base to the apex, are located 100 nm from each other. If sections are cut parallel with the synapse, these structures form a hexagonal arrangement. The view that they are organised structures is supported by their regular patterning and detection in different animals; Gray and Pease (1971) report similar projections in the synapses of retinal receptors in the guinea-pig and rat retina.

Pfenninger, Sandri, Akert and Eugster (1969) investigated Gray's presynaptic projections using a new staining method employing bismuth iodide block impregnation and uranyl acetate and lead hydroxide for contrast. Their results show that the

FIGURE 8.

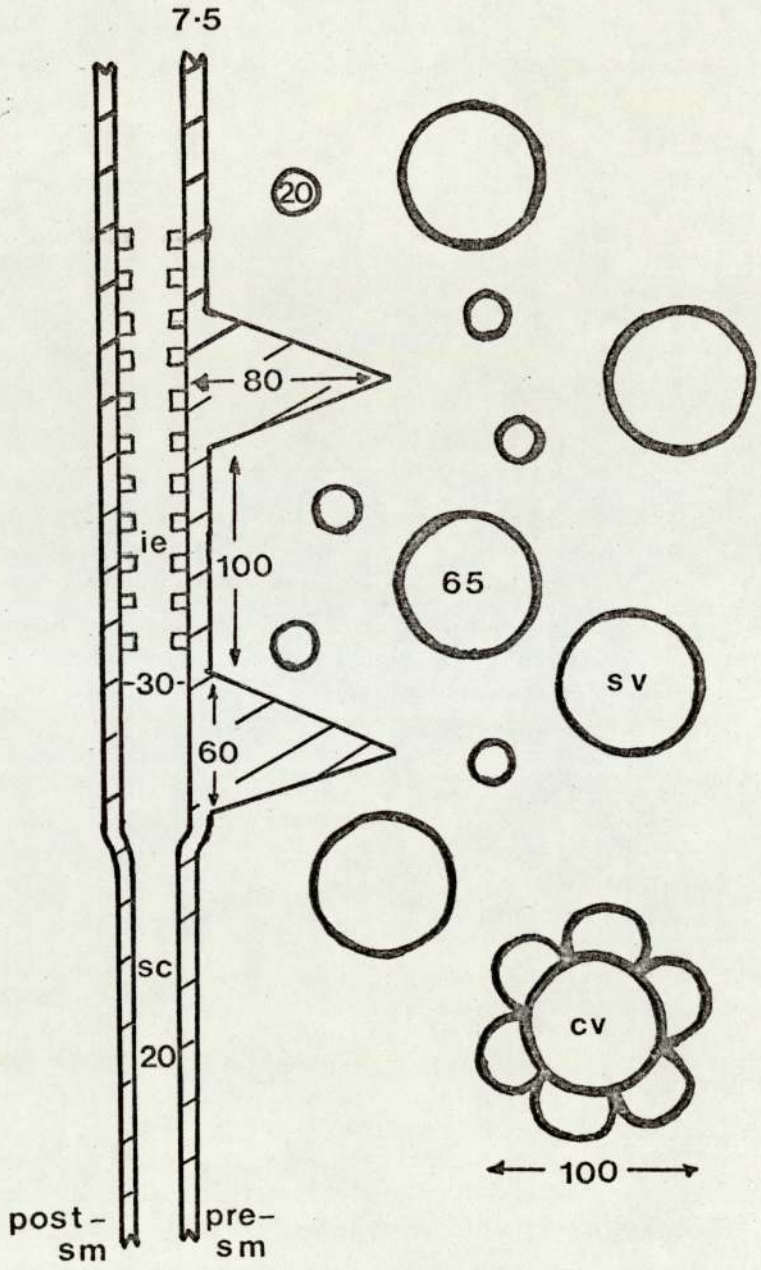


Fig. 8

projections consist of electron dense oval shaped structures 55 x 60 nm spaced at 80 nm intervals and linked together with bars to form a hexagonal grid. In addition, they observed vesicles containing material which stained in a similar manner to the projections. These they refer to as dark cored vesicles and suggest that in view of the staining reactions they could be concerned with the transport and deposition of the dense material which forms the triangular projections.

In cells of the special sense organs associated with vision and hearing, other bodies have been observed in the boutons (Thorn, Schinko and Wetzstein, 1970). These structures vary in shape and appear as bars, laminae, cones and spheres. For example, in the synapse of retinal rods of the guinea-pig, such structures were first observed and described by Sjöstrand (1953). Since then they have been found in other sensory cells and animals, e.g. human retina, frog photo-receptor cells, reptile pineal, chicken, guinea-pig and cat hair cells, and the efferent part of the synapses found in the guinea-pig organ of corti. (Thorn, Schinks and Wetzstein, 1972). The significance of these various structures is not clear although it has been suggested (Gray, 1963) that they may contribute to the direct movement of the synaptic vesicles vide infra.

2.145 Synaptic Junction:

The junction or synaptolemma (Figs. 3 and 8) formed by the bouton and the recipient neurone dendrite or axon etc. consists of a thickened area formed by the pre-synaptic membrane (bouton) and the post-synaptic membrane (recipient neurone). The extent and shape of the synaptolemma varies (van der Loos, 1964) in that it may appear as a straight or curved section, or

may completely encircle a small dendrite. The space between the pre- and post-synaptic membranes is referred to as the synaptic cleft vide infra (Figs. 3 and 8).

2.146 Synaptic Vesicles:

An important identifying feature of the bouton is the presence of synaptic vesicles (Figs. 3, 4 and 8) which were reported and described by Sjöstrand (1953), Palade and Palay (1954), and De Robertis and Bennett (1954). These structures, also called neurovesicles, although scattered throughout the bouton, tend to accumulate at the synaptic cleft. Synaptic vesicles vary in size from 20 - 65 nm in diameter and also in shape (Palay and Palade, 1954; and Palay, 1956).

In examination of synapses from frog and earthworm material, De Robertis and Bennett (1954) report that some of the synaptic vesicles seem to perforate the presynaptic membrane so that portions of the vesicles lie in the synaptic cleft and come into direct contact with the post-synaptic membrane.

In 1961 Gray, investigating the mossy synapses and Purkinje spine synapses of the rat cerebellum, found what he described as complex vesicles. These vesicles consist of a central sphere measuring 60 - 80 nm surrounded by a shell formed from a closely packed layer of small vesicle bodies each measuring 15 - 20 nm. Thus the total diameter of these complex vesicles varies from 90 - 120 nm. (Figs. 3 and 8).

The origin of these synaptic vesicles is one of dispute. In 1970 Gray and Willis, following investigation into synapses of the rat cerebral cortex, suggested that vesicles are formed from the surface membranes of the axon as initially complex vesicles. The vesicles then emerge from the complex vesicles

as plain vesicles. At some stage during this process they become charged with the transmitting agent vide infra. Examination of synapses reveals the presence of all three types of structures; complex, plain vesicles, and empty shells. Gray and Willis further suggest that the dense projections on the pre-synaptic membrane may be partly or wholly composed of the fragments remaining from the complex vesicles.

Stelzner (1971) suggests that the synaptic vesicles are produced by the immature neurone in the Golgi apparatus and smooth endoplasmic reticulum. Whilst Tennyson (1970) suggests that the accumulation of synaptic vesicles takes place after the formation of the synaptic junction.

Gray and Pease (1971) substantiate the original report of Gray and Willis by reporting the presence of complex and plain vesicles together with empty shells in the synapses of the retinal receptor of rats. They suggest that the pre-synaptic projections may guide the vesicles to the pre-synaptic membrane.

Needless to say both theories have aspects to them that still leave the question of how and where synaptic vesicles are formed open to further investigations and debate. Not least of all, the fact that there are variously shaped vesicles and that they may increase in number following certain types of stimulation vide infra.

In biochemical investigations using radioactive materials the work of von Hungen, Mahler and Moore (1968), and Rodriguez de Lores Aenaiz, Alberici de Canal and de Robertis (1971) provides information which supports neither hypothesis and in fact raises again the whole question of the origin of synaptic vesicles. The investigations of von Hungen et al indicates

that both the synaptic vesicles and the adjacent membranes are in a dynamic steady state with respect to protein synthesis. Furthermore, there is no evidence that vesicles are appreciably broken down as a consequence of electrical activity of the neurone. Rodriguez de Lores Arnaiz et al, using labelled L- ^{14}C -leucine, in which the half life duration was determined, have shown that the protein structure of the synaptic vesicles is different to that of the membranes of the bouton.

In 1965 Walberg reported another type of vesicle which was cylindrical in shape measuring 17 - 20 x 50 nm, whilst Uchinzone (1969) also reported differences in the synaptic vesicles of the cat cerebellum and accordingly placed them into the following designation: Type 'S' for spherical vesicles and Type 'F' for flattened vesicles (Fig. 5). Uchizone also suggested that these morphological differences may be related to function, thus the S type vesicle would be those found in excitatory synapes whilst the F type were characteristic of inhibitory synapses. However, Bodian (1970) has reported that the morphology of synaptic vesicles is affected by the method of fixation in that poorly fixed tissue resulted in flattened vesicles. Dennison (1971) using electron stereoscopy has shown that there is a variation in the morphology of synaptic vesicles and proposes that they be placed in the following categories: (1) spherical vesicles, (2) cylindrical vesicles, (3) cylindrical with curved ends and (4) disc shaped. She further reports that in the methods of processing employed there was no difference in the appearance of the vesicles. Lieberman and Webster (1972) have used this categorising of vesicles to identify synapses

in the rat dorsal lateral geniculate nucleus. They describe the boutons as being R type if they possess spherical vesicles, F for those containing cylindrical, and P for synapses with discord vesicles. Using this form of designation they were able to show that P and R types are found in equal numbers, whilst F were rarely found. P boutons synapse upon: conventional dendrites, other P boutons, and occasionally R boutons. They are post-synaptic to R, F and P boutons.

Yet another type of vesicle arrangement has been reported by Dowling and Chappell (1972) in the ocellus of the dragonfly. They describe a button like synapse in which an electron dense area is surrounded by a cluster of vesicles. Such synapses are found in both axon terminals and dendritic processes.

Although there is disagreement on the effect of fixation on the appearance of synaptic vesicles, de Iraldi and Suburo (1970) have reported differential effects with mixtures of osmium tetroxide and various iodides. It is suggested that these differences indicate variation in chemical composition which in turn would suggest the presence of different transmitting agents (Fig. 5).

Froesch and Martin (1972) using similar fixing and staining reagents, namely zinc iodide-osmium mixture, to those of de-Israldi and Suburo, found that the population of synaptic vesicles in the squid giant fibre system was varied. If the staining reactions together with the size of synaptic vesicles were combined, the results showed that there were four significantly different classes of vesicles. They suggest that these morphological variations could reflect chemical differences and it is consequently conceivable that there could be four different transmitting agents.

Following the finding, by Uchizono (1969), of both spherical and flattened vesicles in the same tissue, other workers, for example: Dennison, de Iraldi and Suburo, Froesch and Martin, have also shown that there are different morphological forms of vesicles, therefore the siginificance of these variations is important and cannot be ignored. Uchizono reports of 2,000 synapses examined in the cat cerebellum, 95% were excitatory whilst 5% were inhibitory. The nature and significance of transmitting agents and their relationship with synaptic vesicle variation is commented upon in the section dealing with synaptic physiology (2.15).

2.147 Inter-lemmal Elements

The space between the presynaptic membrane and the post-synaptic membrane referred to as the synaptic cleft is approximately 20 nm (Palay, 1956). The width of the synaptic cleft together with its length is of physiological importance, and also has a bearing on the type of synapse, vide infra. Whilst the presence of a synaptic cleft supported the neuro-histologists' concept of contiguity without direct contact between neurones, there is now evidence that the synaptic cleft is not a void but contains structures which, either fully or partially, bridge the two membranes. Thus van der Loos (1963a, 1964) has shown that there is variation in the structural form of the synaptic cleft, and that this variation may be associated with the type of synaptic junction. For example, in the case of axo-dendritic synapses, there may be found, at regular intervals of 16.5 nm, small units designated as inter-lemmal elements, orientated perpendicular to the synaptolemma. Some of these inter-lemmal elements entirely bridge the synaptic cleft, whilst others originating from the

post-synaptic membrane end halfway in a spherule or knoblet. The thickness of these bridges is approximately 7 nm and the diameter of the spherules is in the region of 16.5 nm. De Roberts (1964) describes the inter-lemmal elements as being a series of parallel filaments measuring approximately 5nm and spaced at 10 nm intervals.

The function of the inter-lemmal element is one of conjecture; van der Loos (1964) suggests that there are three possibilities: they could be depositions of the transmitting chemicals, or their physical presence may prevent the diffusion of the transmitting chemicals in a direction parallel to the synaptolemma, or thirdly, they may simply provide a physical contact thus locating the two structures to within a close proximity of each other.

That the two structures are closely attached to each other is born out by the work of Cotman and Taylor (1972) who homogenised rat brain and using sucrose gradients, Triton X-100, and differential centrifugation separated the pre- and post-synaptic membranes from the remaining portions of the bouton and recipient neurone. The two membranes were joined by the synaptic cleft. They further reported the presence of a series of projections arising from the post-synpatic membrane and projecting halfway into the synaptic cleft. Thus whilst the synaptic cleft is thought of as a gap, there must be attachment points between the two membranes otherwise they could not remain attached during the processing treatment.

The third possibility suggested by van der Loos that the projections provide a means of location for the pre- and post-synaptic membranes would seem the most attractive. That neurones make specific contact with only certain cells is a

well established fact (Rose, 1973). Indeed, special methods to prevent regeneration are necessary where such a reaction needs to be avoided (Illis, 1973). Furthermore, synaptic contacts are developed during maturation of the brain as is shown by this research project and other workers, (Woodward, Hoffer, Siggins and Bloom, 1971; Kornguth, Anderson and Scott, 1968), and that such contacts are made haphazardly is highly unlikely. In addition, specific acceptance or rejection of cells by each other can be shown by the separation of cells from a tissue or organ using mild enzymic digestion (trypsin), and resuspending the cells in tissue culture media. Following this treatment it is found that like cells will clump together within a short space of time. Cell recognition is a feature of not only the cells of the kidney and liver (as experienced by myself) but apparently also of the CNS according to comment by Rose (1973).

Such accumulation of data suggests a capacity of cells to recognise and where appropriate accept or reject other cells. That such a relationship should exist between neurones is highly probable and that the interlemmal structures participate in this recognition seems more than likely.

Finally it should be appreciated that the appearance of the synaptic junctions varies with the age of the animal. Thus, in the rat, Aghajanian and Bloom (1967) have shown that the synaptic junctions may evolve through several stages and they put forward a tentative morphogenetic scheme. In the immature brain the pre-synaptic membrane projections are smaller and consist of a series of connected peaks. In the mature brain the projections adopt a polygonal shape and are clearly separated from each other. An intermediate stage

has also been observed in which, whilst most of the pre-synaptic membrane projection is continuous, there is usually at least one well-formed pre-synaptic projection.

2.148 Post-synaptic Membrane and Related Structures

Palay (1956), like other workers, draws attention to the dense area around the synaptolemma and particularly the area within the vicinity of the post-synaptic membrane. Van der Loos (1964) has shown that this dense area is not due to thickening of the post-synaptic membrane but due to what he believes to be a sub-synaptic organelle related to, but distinguishable from, the membrane. The organelle consists of an agglomeration of dot- and thread-like electron dense structures that extend from the post-synaptic membrane for 20 - 67 nm into the neurone cytoplasm.

In most preparations of the central nervous system, the dense area of the synaptic junction appears to be continuous. However, Peters and Kaiserman-Abramof (1969) investigating the synapses on the dendritic spines of the small pyramidal neurones of the rat cerebral cortex have shown that the dense area may be discontinuous. There may be as many as four dense areas, the shapes of which vary with the level and angle of sectioning. As a consequence, curved and ring synapses may be formed as reported by van der Loos (1964). Peters and Kaiserman-Abramof further report that the distribution of the synaptic vesicles follows the arrangement of the synaptolemma in that the vesicles are more closely packed in the areas of the dense projections. Peters and Kaiserman-Abramof did not observe the interlemmal elements reported by van der Loos.

The post-synaptic structure, as previously mentioned, may vary and can be: part of the neurone perikaryon (axo-somatic);

a dendritic stalk or spine (axo-dendritic); an axon (axo-axonic) etc. Recognition of axo-somatic synapses can present little difficulty because of their large size and the presence of a nucleus or other cell organelles associated with the perikaryon e.g. Nissl granules, ribosomes, endoplasmic reticulum. The axo-somatic bouton generally contains fewer mitochondria and the synaptic vesicles are less obviously clustered compared to axo-dendritic synapses (van der Loos, 1964). The synaptic cleft also shows structural differences in that the interlemmal elements are less regularly spaced and also are found further apart.

There is no evidence of the post-synaptic organelles seen in the axo-dendritic synapses (van der Loos , 1964).

In the axo-axonic type of junction van der Loos (1964) has shown that whilst the junctions are narrower than those previously described they still contain interlemmal elements. In addition, he has observed processes, presumably originating from glial cells, which interpose between the membranes forming the junction.

A fourth type of junction referred to as dendro-dendritic presents problems of identification since there is little evidence of synaptic vesicles in those observed in the mammalian CNS or an accumulation of mitochondria. However, there is evidence of interlemmal elements which closely resemble in size, and distance apart, those found in axo-somatic synapses. Such elements completely bridge the two membranes.

The significance of dendro-dendritic junctions is subject to a great deal of speculation. In view of the large number of these junctions, van der Loose (1964) has calculated that

they are in the order of 1500 - 2500 per pyramidal cell of the rabbit cerebral cortex, their contribution to neuronal interaction must be significant. That it is possible for the pre-ephatic* neurone to modify the membrane potential of the post-ephatic neurone is more than likely. Such influences could assist the action potential created by other synapses, for example, axo-somatic, and/or play a role in the spread of certain type of waves through neural masses and in the synchronization of rhythmic neurone activity. (van der Loos, 1964).

It is worth noting that Dowling and Chappell (1972) have reported the presence of synaptic vesicles in dendro-dendritic junctions in the median ocellus of thedragonfly. In this instance it can also be assumed that these junctions are synaptic in the neurophysiological sense and concerned with transmission of the impulse through chemical mediation.

2.149 Inhibitory and Excitatory Synapses:

In sections 2.141, 2.142 reference was made to classification of synapses on morphological grounds. In addition a further division was made based on the structural form of the synaptic vesicles (section 2.146, and Figs. 5 and 6), namely, those with spherical vesicles (excitatory) and those with flattened vesicles (inhibitory), Figs. 5 and 6.

Thus synapses may be further classified on their functional criteria:

* See section on electrical transmission 2.149 for further information and definition.

1. Chemical transmission.

   (a) excitatory

   (b) inhibitory

2. Electrical transmission.

The significance of the two types of chemical transmitting synapses is indicated in Figs. 5 and 6. An axon will usually produce only one type of synapse either excitatory (hypopolarisation) or inhibitory (hyperpolarisation) irrespective of whether it possesses one or many boutons. Each bouton can synapse, as previously indicated, on any part of a recipient neurone. However, in the case of the axo-axonic synapse, the bouton may synapse with either an inhibitory or excitatory axon terminal. Thus an excitatory bouton synapsing on an inhibitory axon terminal will provoke an inhibitory transmission on the dendrite (Fig. 6 aas/is). Similarly, the inhibitory bouton which makes an axo-axonic synapse on an excitatory axon-terminal will inhibit the transmission of the excitatory synapse (Figs. 5D and 6). Inhibitory synapses are frequently encountered on the axon-initial segment (Fig. 6) where they can more effectively suppress the excitatory impulses being received by the dendrites and perikaryon. The firing of a neurone may therefore depend on the net effect of all the synapses received.

The axo-somatic and axo-dendritic synapses following osmium tetroxide fixation and phosphotungstic acid staining can be divided into Type 1 and Type 2 synapses (Gray, 1959) on the following criteria; Type 1 synapses, which occur on dendritic trunks and dendritic spines, show a large percentage, between 70 - 100%, of the apposed membranes to be thickened and with a greater electron density. The post-synaptic thickening is more pronounced, in the order of 40 nm, compared to the pre-synaptic thickening. The synaptic cleft is wider

in the areas of the thickened membranes 30 nm and within this area an intermediate band of material can be observed.

In the Type 2 synapse the length of thickening is less than 40% whilst the synaptic cleft remains a constant width of 20 nm. The pre- and post-synaptic thickenings are similar to those observed with Type 1. but there is no evidence of an intermediate band within the cleft.

Colonnier (1968) found in formalin fixed tissue, from the cat visual cortex, results similar to Gray. However, he felt that there were sufficient differences to support an alternative terminology for synapses, based on the synaptic membrane. He proposed the terms "asymmetrical" which corresponds to Gray's Type 1, and "symmetrical" which is similar to Type 2. In a previous paper Colonnier and Guillery (1964) reported two types of synapses observed in the monkey lateral geniculate nucleus and distinguished by their size and staining intensity of the mitochondria. These they designated LP and SD, presumably for large and pale, small and dark.

Examination of the cerebral cortex shows that the synapses on the dendritic spines are always Type 1 whilst those on the cell body are always Type 2. Both types may be present on the dendritic trunks.

Eccles (1964) believes that these two types of synapses reflect their functional activity in that the Gray Type 1 is an excitatory synapse and Type 2 is inhibitory. The location of Type 2 synapses is in keeping with an inhibitory effect.

The synapses so far described all have a number of common features, mainly: the presence of a cleft which physically separates the two neurones although the evidence of interlemmal elements makes this aspect less definite, the presence of

vesicles in the pre-synaptic terminal, and delay between the commencement of the excitation of the pre-synaptic terminal and the response of the post-synaptic structure; this latter aspect supports the evidence of an intermediate stage between the two neurones. Examination of invertebrate animals and the nervous system of some non-mammalian vertebrates reveals a synaptic mechanism which depends upon electronic rather than chemical means for transmission. Such junctions are referred to as tight junctions because the cleft is sufficiently narrow to be almost indiscernable. Further identifying features include either an absence of synaptic vesicles or an even distribution of them in both the pre- and post-synaptic terminals. Synaptic vesicles are not exclusive to the pre-synaptic terminal in the electronic synapse. The functional advantages of the electrical synapse are twofold; in the first instance they are more rapid in action, and secondly, they are immune from chemical antagonists, unlike the chemical synapse which can be affected by toxins, drugs, etc. vide infra. It is evident that these electrically transmitting junctions may be uni-directional, in which case they are, by definition, true synapses, or they may be bi-directional or multi-directional (McLennan, 1970). That in some nervous systems one neurone may, without apparent synaptic contact, affect the excitability of other neurones is recognised and the term ephapse introduced by Arvanitaki is used to describe this functional relationship (McLennan, 1970). As previously mentioned (section 2.148), van der Loos (1964) believes that the dendro-dendritic junctions observed in the mammalian CNS may have a transmitting significance which could be electrical. Evidence of electrical coupling between pre-synaptic and post-

synaptic junctions in the vertebrate is provided by Martin and Pilar (1964) in their investigations of the ciliary ganglion of chicks.

2.15 Synaptic Physiology

The impulse travelling down the axon eventually reaches a synapse where it is now necessary to transfer the signal from one cell (neurone) to the next cell which may be either a neurone or an effector cell. The structural nature of the synapse is such that the conductivity at the synaptic junction is so poor that in most case the impulse cannot proceed beyound the pre-synaptic membrane (Katz, 1966). The signal must therefore be transferred by a different mechanism.

The involvement of chemical transmission was demonstrated by Loewi (1935) who showed the effect of acetylcholine (Fig. 9) on frog's heart muscle. Following the discovery of acetylcholine, other possible transmitting agents have been discovered including the catecholamines, for example, noradrenaline (norepinephome NE) (Fig. 10) (Carlsen, Falck and Hillarp, 1962), dopamine (3,4-dihydroxy-phenyl-methylamine). In addition to excitatory transmitting agents, inhibitory substances have also been described including gamma amino butyric acid (GABA) (Dudel and Kuffler, 1961), 5-hydroxtryptamine (5-HT) and glycine.

The mechanism by which transmission of the impulse is effected has not yet been fully resolved. Furthermore, the system must vary according to whether the synapse is excitatory or inhibitory. However, knowledge gained from investigation into the functional relationship between neurones and muscle cells, at the motor end plates, provides a sound insight into the main principles concerned with the continuance of the signal. Contained within

FIGURE 9.

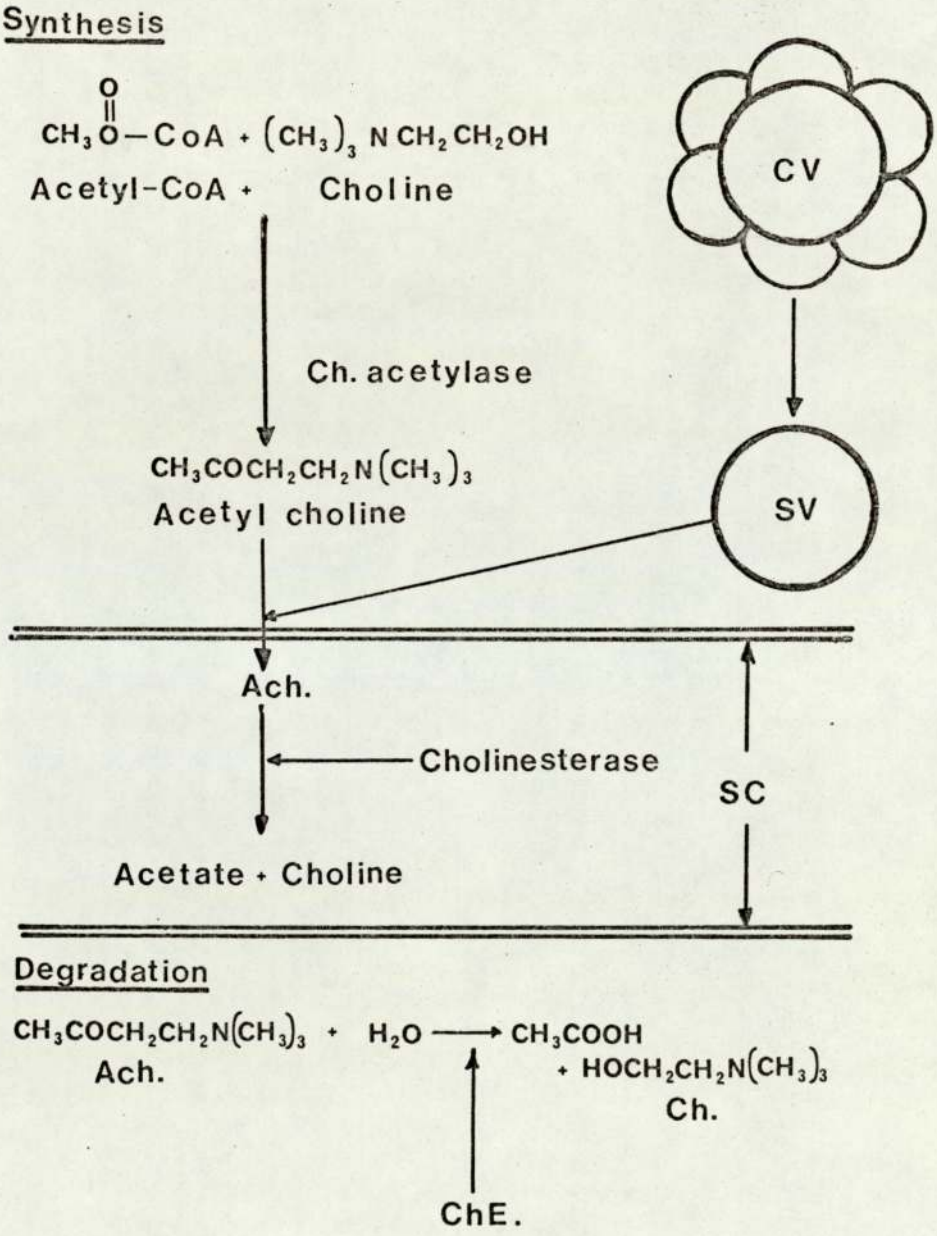
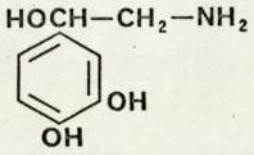

Synthesis
CH3O—CoA + (CH3)3 N CH2 CH2OH
Acetyl-CoA + Choline
CV
Ch. acetylase
CH3COCH2CH2N(CH3)3
Acetyl choline
SV
Ach.
Cholinesterase
SC
Acetate + Choline
Degradation
CH3COCH2CH2N(CH3)3 + H2O → CH3COOH
Ach.
+ HOCH2CH2N(CH3)3
Ch.
ChE.

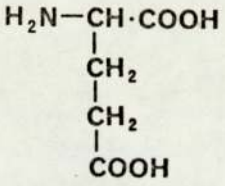
Fig. 9

FIGURE 10.

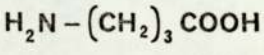
Nor-adrenalin (nor-epinephrine)



Glutamic acid



Gamma amino butyric acid



Serotonin

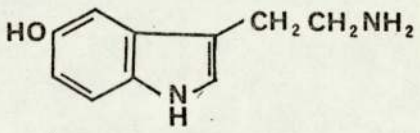


Fig. 10

the excitatory synapses are the previously described synaptic vesicles (Figs. 3 and 8); these were believed to contain the chemical transmission agent acetylcholine. Such a hypothesis was subsequently proved by Whittaker and Sheridan (1965) who isolated the synaptic vesicles and demonstrated quantitatively their acetylcholine content.

In 1963 Pellegrino de Iraldi, Farini-Duggan and De Robertis demonstrated that the synapses from the rat hypothalamus contained two types of vesicles; one, consisting of approximately 80% of the total number, were small, 51 nm, while the other type was larger measuring 130 nm and containing a dense deposit of osmium which was thought to be reserves of catecholamines (Fig. 5C). This was substantiated in 1965 by De Robertis, Pellegrino de Iraldi, Rodriguez de Lores Arnaiz and Zieher who reported the presence of nor-epinephrine in synaptic vesicles isolated from the rat hypothalamus. In 1967 De Robertis quantitatively estimated the concentration of nor-epinephrine and showed that there was five to six times more in the hypothalamus than in similar fractions obtained from total brain hemispheres. The hypothalamus fraction also contained numerous granular vesicles; thus this morphological form may be a means of identifying synapses containing nor-epinephrine.

The synaptic vesicles are in close proximity to the pre-synaptic membrane as storehouses of acetylcholine, in the case of excitatory synapses, and some appear to be attached to the pre-synaptic membrane (De Robertis and Bennett, 1954). Actual fusion of the synaptic vesicles with the pre-synaptic membrane is questioned by Lapetina, Soto and De Robertis (1968) in view of the differences in protein and lipid composition of the two membranes.

The arrival of an impulse results in release of the acetylcholine into the synaptic junction. This has the effect of

depolarising the post-synaptic membrane. The depolarisation is believed to be due to a selective permeability to sodium, potassium and chloride ions which move down their concentration gradients. The net result of this ionic change is alteration of the potential which decreases towards zero. The essential difference between this mechanism and that which creates the action potential is that, in the latter, the selective permeability is to sodium only. How acetylcholine brings about the permeability is not fully understood. That it attaches to specific proteolipid (Dikstein, 1973) receptor sites has been shown by the use of radio-labelled ^{14}C curare (Waser, 1961), which is known to cause paralysis of muscle cells. Autoradiography reveals that the ^{14}C curare locates in the motor end plates in concentrations equivalent to the amount of acetylcholine which activates the change in membrane potential. It is interesting to note that curare is used medically to paralyse muscles to counter the effect of toxin produced by Clostridium tetani in cases of tetanus. Tetanus toxin, like strychnine, would appear to block the inhibitory synapses and it is suggested that these substances may be sterically related to the transmitting agent and thus compete for the post-synaptic receptor sites (Eccles, 1964). Kryzhanovsky (1973) reports the accumulation of elongated synaptic vesicles following tetanus intoxication. This would suggest that the effect of the tetanus toxin is to prevent the release of the transmitting agent. The observation on the morphology of the synaptic vesicles supports the contention that boutons containing elongated vesicles are inhibitory synapses. Kryzhanovsky further reports that the post-synaptic membrane retains the ability to respond to the inhibitory transmitter during tetanus. This would seem to negate the view that the toxin acts directly on the receptor sites.

Similarly, the exotoxin of Clostridium botulinum would appear to block the excitatory cholinergic synapses (Eccles, 1964) resulting in paralysis of the muscles and the classical symptoms of botulism.

The duration of acetylcholine activity is very brief since contained within the synaptic cleft is cholinesterase which splits the transmitter substance into acetic acid and choline (Fig. 9).

Since the firing of a neurone depends on the summation of the excitatory and inhibitory synapses it must be assumed that the inhibitory synapse must counter the effect of the depolarisation of the excitatory transmitting agent. Eccles' work on the inhibitory synapse, which was to earn him the Nobel Prize in 1964, substantiated this hypothesis. His work, involving the implantation of microelectrodes into neurones, revealed that the inhibitory transmitting agent instead of inducing a depolarisation of approximately 20 mV, as in the case of the excitatory post-synaptic potential (EPSP), in fact produced a hyperpolarisation where the inside of the axon showed a potential of -75 mV. Since this change in the resting potential would make the creation of an action potential more difficult, it is referred to as an inhibitory post-synaptic potential (IPSP). Eccles and other workers, using electrophoretic injections of anions into cat motoneurones, showed that the creation of the IPSP could be due to an alteration in the permeability of the chloride ions. The effect of chloride ions was also shared by other anions, and the remarkable feature is that the anions either could or could not effect an IPSP. In addition, with minor exceptions, those anions which could induce an IPSP had an ionic size, in the hydrated state, less than that of Na^+. (Na^+ = 0.56 nm, K^+ = 0.38 nm, Cl^- = 0.36 nm). It is therefore believed that the effect of inhibitory transmitting

agents such as gamma amino butyric acid (GABA) vide infra is to convert the inhibitory zones of the post-synaptic membrane into a sieve-like structure that allows small hydrated ions to pass and blocks all larger ones. In addition to the movement of chloride ions, since potassium ions are of a similar hydrated size, these must also pass along their electrochemical gradients. However, the migration of both ions results in a potential which seldom exceeds -80 mV.

The change in the membrane potential at the post-synaptic membrane does not directly produce an action potential. The action potential is only activated at the axon hillock. At this point the membrane potential is as previously stated the summation of the EPSP and IPSP. That this is the case is supported by various collaborative concepts on neurone physiology including the realisation that each neurone receives large numbers of synapses. Cragg (1967) has estimated that the number of synapses per neurone is in the order of 5.6×10^3 monkey visual cortex, 7×10^3 mouse visual cortex, 6×10^4 monkey motor cortex and 1.3×10^4 mouse motor cortex (Table 1). These will be distributed along the dendrites and perikaryon. In addition to accepting that there is a mixture of excitatory and inhibitory synapses, it is reasonable to assume that those situated on the axon hillock, and the axo-axonic synapses, are of an inhibitory nature (Figs. 5 and 6).

There are a growing number of chemicals which have been detected in the CNS that could act as transmitting agents. These include 5-hydroxytryptamine (serotonin), amino acids such as glycine, glutamic acid, aspartic acid and cysteic acid, and GABA. Only acetylcholine, GABA and glycine have been clearly established as transmitting agents. Since it is believed that both EPSP and IPSP could be initiated by Ach, the antagonistic effects must be

due to differences in the receptor sites on the post-synaptic membranes. Such differences would determine the type of response.

2.16 The Synaptosome

Synapses have been examined both qualitatively and quantitatively in a wide variety of animals; for example, rat (Armstrong, 1970), guinea-pig (Clementi, 1966), monkey (Cragg, 1968), cat (Voeller, 1962), toad (Pessacq, 1973), crayfish (Atwood and Morin, 1970), squid (Froesch, 1972), octopus (Jones, 1970), dragonfly (Dowling and Chappell, 1972), mouse (Cragg, 1967). In addition, synapses have been observed in a wide variety of lesser animals, including the more primitive ones; for example, coelenterates (Westfell, 1970). References to synapses in other animals may be found in books by Eccles (1963, 1972), McLennan (1970) and Smith, C.U.M. (1972).

The techniques employed fall broadly into two categories; the first is concerned with the study of synapses in situ using either light or electron microscopy, the second involves the separation of the synapse from the axon, by homogenisation, to form a structure referred to as a synaptosome.

2.161 The Nature of the Synaptosome

The technique to separate the synapse was developed by Gray and Whittaker (1962). In this method the brain substance is carefully homogenised under controlled conditions so that not only are the cells ruptured releasing their organelles but the nerve endings are separated from their axon trunks. The membrane of the severed bouton immediately unites to form an intact, spherical, sub-cellular particle called by Whittaker a Synaptosome (Figs. 25 and 26). The synaptosome may be recognised by a number of features including shape,

size, possession of synaptic vesicles, mitochondria, and a single intact membrane. In addition to retaining their morphological integrity, the synaptosomes remain metabolically active thus providing an excellent facility for studying their biochemical features. A more recent advance in which special staining methods vide infra were used has revealed the presence of both the pre- and post-synaptic membranes, thus it would seem that the synaptic junction may also be separated intact with the synaptosomes.

The demonstration of in-situ synapses has provided the opportunity for the development of specific staining methods; for example, the technique developed by Bloom and Aghajanian (1966) in which glutaraldehyde fixed tissue is stained with an ethanolic solution of phosphotungstic acid, to specifically demonstrate the synaptic cleft. Aghajanian and Bloom (1967) used this technique to quantitatively estimate the number of synapses in the developing rat brain. Synaptic vesicles may also be stained by a modified Champy Maillet fixative containing osmium tetroxide and a soluble iodide (de Iraldi and Suburo, 1970).

The examination of synapses in whole tissue has the obvious advantage of clearly showing not only the inter-neuronal relationships, but also the exact location of the synaptic junctions. In addition, several workers, Aghajanian and Bloom (1967), Cragg (1967, 1968), Armstrong James (1969) have used this method to estimate the numbers of synapses and Table 1 (Appendix) provides a summary of the available data. The disadvantage of this method for estimating the numbers of synapses per unit area is that the technique must, by its very nature, produce a result which only indicates

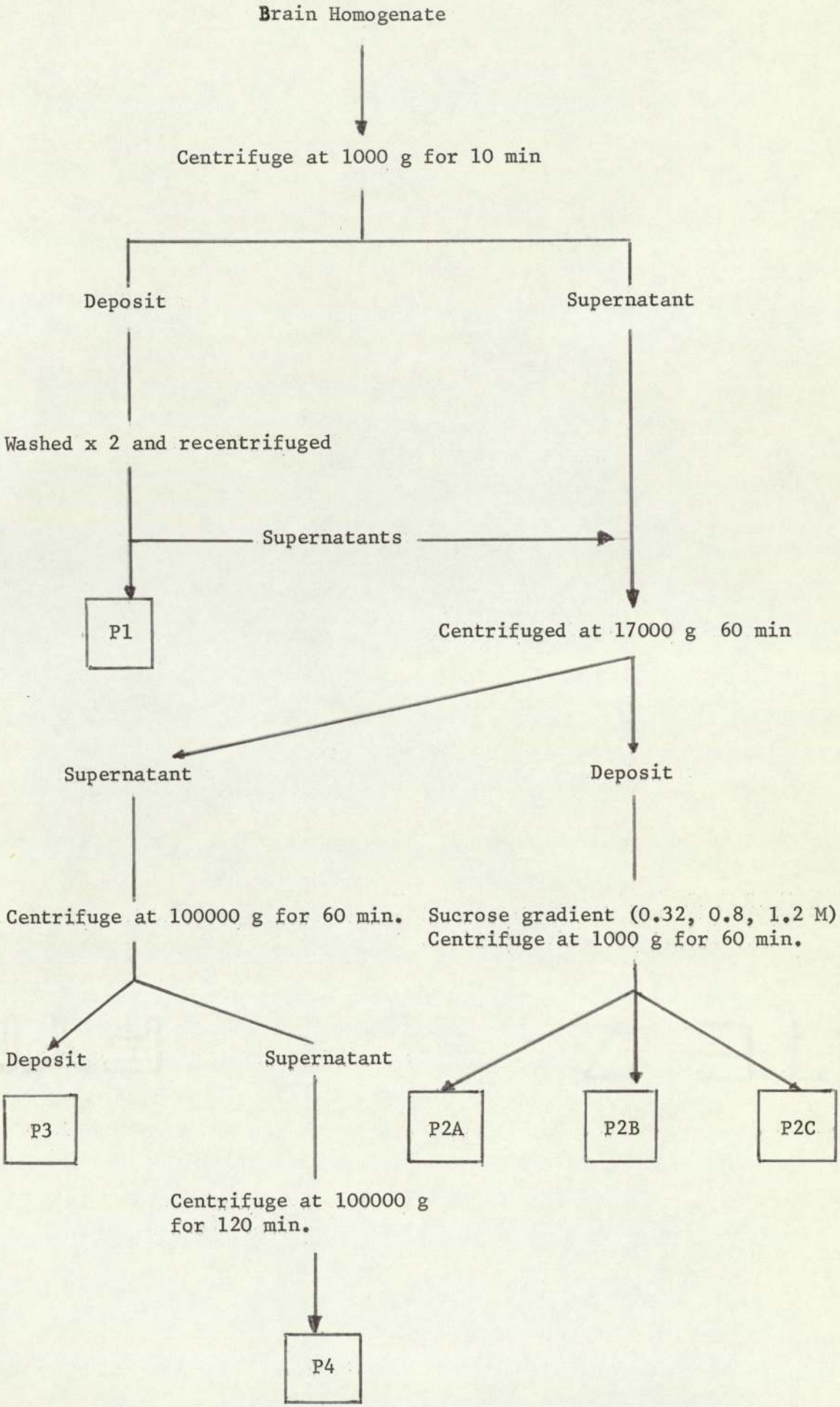
the number within the limited area sampled. To reduce this problem, Cragg (1967) took random samples from three sections of a selected cortical area. Nevertheless the method still confines the results to the areas examined.

In-situ biochemical investigations are performed by histochemical staining methods which although benefitting from the microscopical precision are restricted by the available methods and technical problems. To determine biochemical values brain extracts are required, prepared using techniques which minimise inactivation of the material.

2.162 The Techniques of Isolation

The carefully controlled homogenisation of brain tissue in a solution of sucrose, using the technique described by Gray and Whittaker (1962), results in a homogenate of sub-cellular particles. The sub-cellular particles are then separated by differential centrifugation, using sucrose gradients, in a high speed ultracentrifuge. Since the introduction of this technique various modifications have been introduced in recent years, including the use of Ficoll sucrose gradients (Abdel-Latif and Abood, 1966), more complex sucrose gradients (Shevtsov, Poydnyakov, Musin and Glebov, 1972), and the application of electrophoresis in a U tube containing a mixture of sodium chloride and sucrose in a tris-EDTA buffer solution (Trachenko, 1972). A review of the methods prior to 1972 is provided by Garcy, Harper, Best and Goodman (1972) in which they investigated the use of Ficoll, in comparison to the use of sucrose by other workers, for the isolation of synaptosomes and synaptic junctions. The fractions obtained (Fig. 11) were shown by Gray and Whittaker to contain sub-cellular particles in accordance with their density;

FIGURE 11.


Brain Homogenate
Centrifuge at 1000 g for 10 min
Deposit
Supernatant
Washed x 2 and recentrifuged
Supernatants
P1
Centrifuged at 17000 g 60 min
Supernatant
Deposit
Centrifuge at 100000 g for 60 min.
Sucrose gradient (0.32, 0.8, 1.2 M)
Centrifuge at 1000 g for 60 min.
Deposit
Supernatant
P3
P2A
P2B
P2C
Centrifuge at 100000 g
for 120 min.
P4

P1 nuclei, P2(A) myelin fragments, P2(B) nerve ending particles and P2(C), P3 microsomes, P4 ribosomes.

Synaptosomes were identified by the presence of synaptic vesicles, and in some instances the additional presence of mitochondria. Whilst the former are essential to the identification of synaptosomes, the latter are not.

The importance of controlled conditions, in the preparation of the brain homogenate, was shown by Whittaker and Dowie (1965) in experiments in which they compared different methods of homogenisation. From the results obtained it was apparent that the method employed affected the yield of synaptosomes as determined by acetylcholine estimation. Of the homogenisers tested, they found that devised by Aldridge (1960) (Fig. 12) gave the best results with a hand glass/stainless steel modified pestle and mortar giving very close results.

Further separation can yield synaptic vesicles which have been investigated for their chemical transmitter content (Whittaker and Sheridan, 1965).

Synaptic complexes, consisting of the pre-synaptic membrane and post-synaptic membrane joined by the synaptic cleft together with the post-synaptic density, which is located on the inner surface of the post-synaptic membrane, may also be isolated. To achieve this the synaptosome fraction is exposed to Triton-X-100 and further differential centrifugation (Cotman and Taylor, 1972). The complexes may be stained by the use of Bloom and Aghajanian's method employing ethanolic phosphotungstic acid which selectively demonstrates these structures (Davies and Bloom, 1973).

The employment of sucrose instead of isotonic saline, in the homogenisation of the brain, is recommended because of the

FIGURE 12.

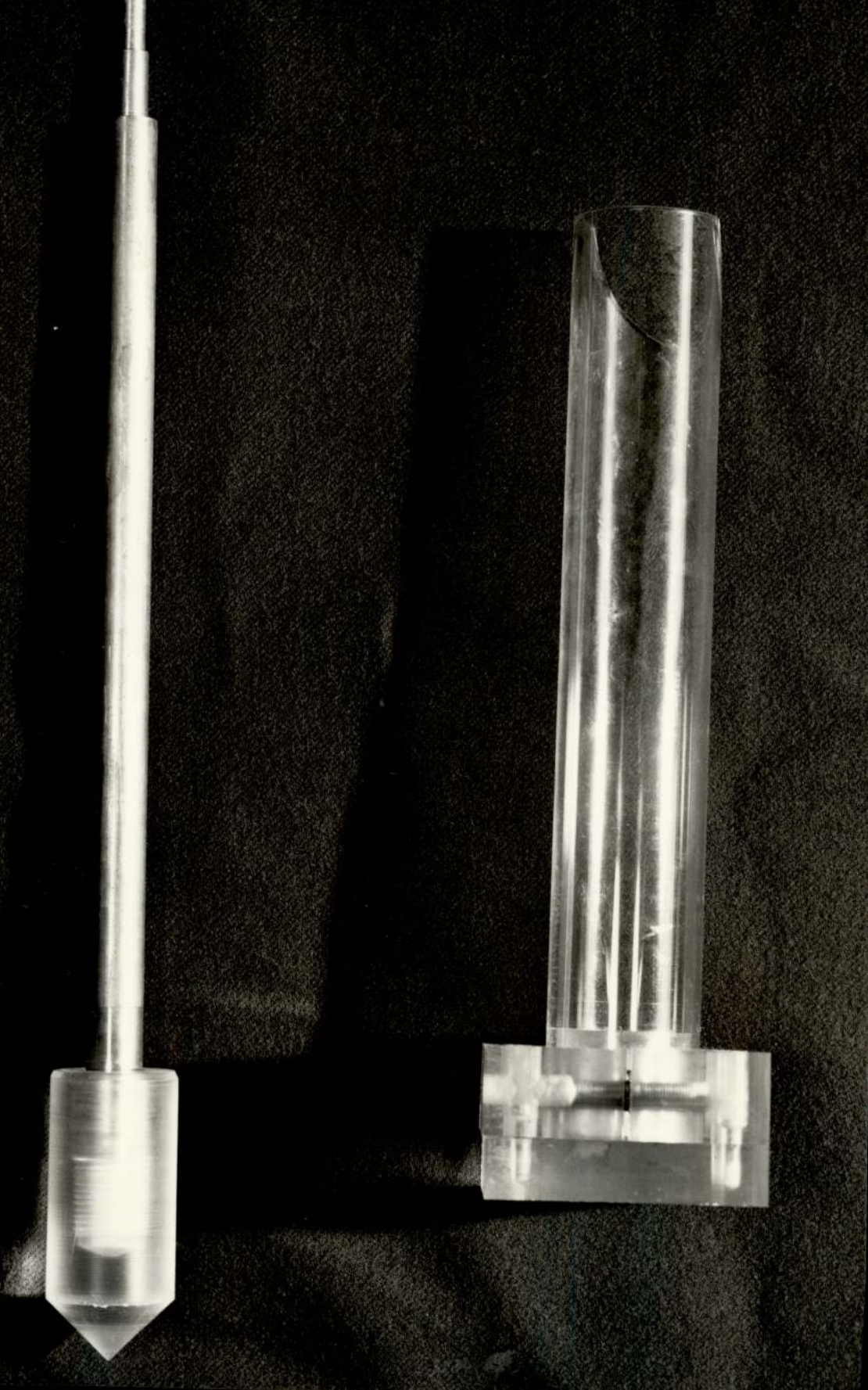


Fig. 12

higher yield of synaptosomes despite some adverse effect on the structural appearance of the mitochondria (Gray and Whittaker, 1962). The sucrose is believed to render more soluble the extracellular cement and thus aid the shearing effect of the homogeniser.

The separation of sub-cellular particles into a reasonably pure fraction has provided a means by which biochemical analysis can be performed on these structures. Furthermore, using the technique of Clementi, Whittaker and Sheridan (1966), synaptosomes may be estimated quantitatively using known numbers of latex particles.

2.17 Effects of Various Factors on Synapses and Synaptosomes

With the various techniques of neurohistology, biochemistry, and electron microscopy now available, considerable research has, and still is taking place, on a comparison of values in the CNS between normal and experimental animals subjected to various conditions.

The investigation into synapses or synaptosomes and their inclusions can be divided broadly into those concerned with biochemical analysis or with structural form, either qualitatively or quantitatively. Since in the rat the brain at birth is not fully developed (Rose, 1973), it is possible to observe the final stages of neurone and neuroglia development. For example, Gonatas, Antilio-Gambetti, Gambetti and Shafer (1971) have investigated the morphological and biochemical changes in the post-natal development of synaptosomes. Using discontinuous Ficoll gradients, they obtained 5 distinct bands from the crude mitochondrial fraction. On examination of these bands, prepared from the brain homogenates of animals at different ages, the material was found to be

dissimilar in both morphology and biochemistry. At one day of age the synaptosomes were found in fractions lighter than the normal synaptosome fraction. These 'immature' synaptosomes were very granular and contained few, if any, synaptic vesicles. Gonatas et al quantitatively showed that the number of synaptosomes based on a percentage comparison with other structures increased with age.

The type of conditions which animals may be subjected to include those of a stimulatory type; for example, the effect of light on synapses (Cragg, 1963), electrical stimulation on synaptic vesicles (Perri, 1972) and synaptosomes (Jones and Bradford, 1971), the effect of sound (Fehér, Joó and Halasz, 1972), environmental complexity (Møllgaard, Diamond, Bennett, Rosenzweig and Lindner, 1971), (West and Greenough, 1972). The effect of malnutrition on synapses was investigated by Cragg (1972), on synaptosomes by Gambetti, Gambetti, Gonatas, Shafer and Steiber (1972). Finally the effect of pharmaceutical drugs, poisons and microbial toxins vide infra.

2.171 Light

The investigations of Cragg (1967, 1968 and 1969) on the size and density of synapses in the rat visual cortex and lateral geniculate nucleus revealed differences in dark reared animals compared to those exposed to light. In the visual cortex the diameter of the synapses varied from 4 - 14% between dark and light exposed rat. In the superficial cortex light exposed rats had larger synapses whilst in the deeper half they had smaller synapses.

In the lateral geniculate nucleus the diurnal weaned rats subsequently exposed to seventy seven days of darkness had synapses which were larger in diameter than the littermstes

continued in diurnal lighting. However, the light reared animals had a greater density of synapses than the dark exposed rats. No apparent differences were detectable in either the synaptic junctions or the synaptic vesicles. Complementary results were obtained for animals born and weaned in the dark and then either continued in the dark or transferred to diurnal lighting.

2.172 Sound

In experiments in which waking cats were acoustically stimulated (clicks at 20/S for 30 - 45 min.) Fehér et al showed this had a marked effect on the number of synaptic vesicles. The results varied with the size of the axon terminal, thus those of 0.1 and 0.3 sq.μm showed an increase in vesicular density whilst those of 0.2 and 0.4 - 0.6 sq.μm showed a decrease. From these results it was concluded that the number of synaptic vesicles would rise or fall depending on the functional state of the synapse. The number of synaptic vesicles was also affected by the degree of stimulation in that moderate stimulation resulted in increased numbers, probably due to over compensation in producing new vesicles, whilst exhaustive stimulation always resulted in considerable loss of vesicles.

Fehér et al also conclude that the effects of acoustic impulse on the CNS are mediated by both the specific afferents and the separate brain stem reticular formation. Since the reticular system is a diffuse activating one, which excites all cortical areas and is concerned with the general wakeful condition, it is unlikely that acoustic stimulation will exhaust these synapses of their synaptic vesicles. However, it can be assumed that continued stimulation will exhaust

the synapses of the specific afferents. Fehér et al therefore suggest that the axon terminals, e.g. 0.2 and 0.4 - 0.6 sq.μm, represent the cortical endings of the specific thalamocortical pathway, whilst those with elevated vesicle counts, e.g. 0.1 and 0.3 sq.μm, may belong to the reticulocortical neurons.

These findings were further supported by the results of other experiments performed on cats in pentobarbital anaesthesia. Fehér et al found, as previous workers had shown, that this form of anaethesia depresses the brain stem reticular formation so that there was no increase in vesicular density following acoustic stimulation, whereas there was a decrease in synaptic vesicles in other categories of synapses.

Anaesthesia with chloralose produces a somewhat different effect in that there is a decrease in synaptic vesicle density of the 0.1 and 0.3 sq.μm, and axons in the 0.2 and 0.3 sq.μm categories show opposite responses to stimulation when compared with pentobarbital anaesthesia.

2.173 Electrical Stimulation

In addition to sound affecting the numbers of synaptic vesicles, Jones and Bradford (1971) have also shown that electrical stimulation has an effect on certain types of vesicles. Morphological examination of electrically stimulated synaptosomes, obtained from rat brain homogenates, showed that their appearance and size, including the contained mitochondria and synaptic vesicles, was identical to non-stimulated synaptosomes. Quantitative examination showed that whilst the number of synaptic vesicles did not decrease following stimulation there was a significant decrease of complex vesicles. This could be explained if Gray's hypothesis on synaptogenesis is correct in that he believes

plain synaptic vesicles originate from complex vesicles. If this is the case then the stimulation of the synaptosomes resulted in utilisation of the synaptic vesicles and consequently their replacement from complex vesicles. This resulted in depletion of the complex vesicles.

Contrary to the findings of Jones and Bradford, Perri, Sacchi, Ravisla and Ravioli (1972) showed that the numbers of synaptic vesicles, present in the axon terminals of the superior cervical ganglion of the rat, decreased in numbers following electrical stimulation. In addition, the vesicles migrated towards the pre-synaptic membrane. However, it should be noted that the results obtained by these two groups of workers were on different forms of axon terminals, and the results obtained on synaptosomes must be considered to be obtained under an artificial - in-vitro environment.

2.174 Diet

Both Cragg (1972) and Gambetti et al (1972) found that whilst body and brain weights in starved rats were reduced by 30 - 57% and 18 - 23% respectively, there was litte effect on the density of axon terminals. However, Cragg has calculated that in view of the reduced neuropil and increased neurone density the numbers of synapses per neurone is reduced by 38 - 41%. This is not supported by Gambetti since he reports that the post-natal development of synapses is unaffected by starvation. It is possible, according to Gambetti, that malnutrition may cause changes in the axon terminals which could result in an altered distribution in the homogenate fractions. This is a hypothesis put forward to account for the higher yields in protein, acetyl cholinesterase and choline acetyl transferase obtained in the synaptosome fraction of

starved rats. It has been shown by McGovern et al (1973) that there are differences in synaptosomal densities which can be used to separate adrenergic and cholinergic synaptosomes in Ficoll density gradients. Since Gambetti used the method of Autilio et al (1968) which employs Ficoll gradients it is possible for starvation to have had a differential effect on synaptosome densities.

2.175 Environmental Complexity

Møllgaard et al (1971) subjected young (25 days old) rats to an environment which was designed to stimulate their CNS. The design of the experiment was such that one group of test animals (EC - enriched condition) were exposed to a varying set of toys daily over a period of 30 days. From similar experiments conducted by Diamond (1967) such exposure resulted in animals with larger neuronal cell bodies, longer dendrites, and more glial tissue. The increase in the size of the nerve cell perikaryon of environmentally enriched rats varied with the level of the cortex examined. Such variation ranged from 7% in the middle cortex levels to 17.7% in the upper cortex. The greatest increase in brain dimensions was found in the visual region. A difference not surprising if there are differential changes following stimulation. Altman and Das (1964) have also shown that an enriched environment also stimulates the growth of neuroglial cells of the rat cerebral cortex particularly in the cortical radiation and corpus callosum. In the experiments of Møllgaard, a comparison of E C animals with a group which had not been been affected to a stimulating environment, but one which was impoverished (I C - Impoverished Condition), showed differences in both size and number of synapses in the

occipital cortex region. The synapses measured were of the asymmetrical axo-dendritic type in the neuropil of layer III. E C animals displayed synaptic junctions that averaged 52% greater in length when compared with I C littermates. However, the synapses were only 67% as numerous. Whilst the E C rats had larger synapses than the I C animals they also had fewer small synapses. The total area of synapse was 40% greater in the E C animal compared to the I C. Measurements in thickness of the cortex showed a 4% increase in the E C animal. West and Greenough (1972) performed similar experiments and achieved results which were comparable with those of Møllgaard. West and Greenough showed that the diameter of the synapses in the visual cortex, as determined by the method introduced by Cragg (1967), of E C animals was within experimental limits the same as the I C rats. Their measurements, of the synaptic junction of the Gray Type I synapses, showed that the E C animals had statistically significant larger thickened areas than those of the I C animals, particularly in the region approximating layer 4 and to a lesser extent layer 1. No detectable difference was found in layer 6. This confirms the findings of Møllgaard et al that environmental complexity stimulates the CNS to the extent that the synaptic junctions increase in size.

2.176 Age

Several groups of workers, Aghajanian and Bloom (1967), Gonatas et al (1971) have shown the increase in numbers of synapses in selected areas of developing brains, similarly Hasan and Glees (1973) have shown a reduction of axo-somatic synapses in the hippocampal neurones of senile rats. They also review other changes in neurones and neuroglia observed

by other workers in the ageing brain.

2.18 Biochemistry of Synapses and Synaptosomes

Biochemical investigations of synapses, following Gray and Whittaker's (1962) demonstration that they could be separated in the form of synaptosomes, has resulted in a growing accumulation of information on their biochemical and biophysical properties.

2.181 Transmitters

Whittaker, Michaelson and Kirkland (1964) isolated synaptic vesicles by rupturing the synaptosomes with water containing escerine phosphate (physostigmine), and then subjecting the extract to centrifugation at 53000 g for 120 min in a continuous sucrose density gradient. The synaptic vesicle layer was found to contain acetylcholine, choline acetyltransferase, 5-hydroxytryptamine and nor-adrenaline. The detection of the latter two substances supports the view that transmitting agents other than acetylcholine may be active at the synapse, a concept for which there is much other evidence including the histochemical evidence of Carlson, Falck and Hillarp (1962).

2.182 Metabolism

The demonstration that synaptosomes remain metabolically active following their isolation was shown by: Abdel-Latif and Abood (1964), Abdel-Latif (1966) and by Bradford (1969), in a series of respiration experiments. Bradford showed that the metabolism of synaptosomes was in variance with that of mitochondria in that synaptosomes were capable of respiration with both glucose and pyruvate, whilst the mitochondria would only metabolise the pyruvate in a medium lacking Na^+ but

containing high K^+ and phosphate. Moreover, the respiration rate of synaptosomes was higher than that of mitochondria with either the glucose or pyruvate and the concentration of Na^+ or K^+ did not affect metabolism.

Exposure of synaptosomes to a hypotonic medium resulted in loss of lactate dehydrogenase and soluble protein. A consequential low uptake of oxygen, similar to that of mitochondria, suggests that the synaptosomal membrane provides a barrier between the medium and the mitochondria contained within the synapses. Both the ATP and phosphocreatine were found to be synthesised by synaptosomes. A finding which is not surprising in view of the importance of ATP. For example, it is essential for the functioning of the sodium-potassium pump, a process by which the axon membrane maintains the asymmetrical distribution of sodium and potassium ions, against their concentration gradients, in the axo-plasm compared to the external environment.

2.183 Effect of drugs

Whilst the overall effect of such drugs as those inducing stimulation or depression of the CNS is well known, as indeed is the action of other pharmacological agents, for example: the psychotropic drugs: tranquilizers, energizers, or halucinogens: and those acting as anticonvulsants and convulsants, little is understood. of the effect of these agents at the cellular or sub-cellular level (Rodriguez de Lores Arnaiz and de Robertis, 1973). However, the extraction of synaptosomes by the technique of Gray and Whittaker, and other workers previously mentioned, has provided a preparation on which these agents can be tested. Thus, for example, the effect of phenobarbitone on synaptosomes has been investigated

by Balfour and Gilbert (1971), the toxic effect of the antibiotic Gramicidin by Keen and White (1971). The review by Rodriguez de Lores Arnaiz and de Robertis, in Dikstein's book on cell pharmacology, suggests that drugs which are antagonistic to synapses may have an effect in several ways and could include any of the following: inhibition of the synthesis of the transmitting agent, displacement by an analogue, alteration of the storage mechanism, destruction of the storage sites, effect on receptors, inhibition of the enzymes concerned with degradation of the transmitting agent in the synaptic cleft.

In addition to the considerable evidence of interference in the metabolism and action of the transmitting agents there is additional information that such activity can be reflected in the cell structure. For example, methione sulphoximine, which produces convulsions in experiment animals such as rats, cats and rabbits, by interference with the inhibiting transmitting agent GABA, causes not only swelling of the glial cells but also swelling of nerve endings. In addition there is almost complete loss of synaptic vesicles of the non-aminergic synaptosome fraction. Allylglycine, another convulsive drug, produces similar effects resulting in swelling of nerve endings and loss of synaptic vesicles.

Whilst several drugs, including the psychotominetic drugs, are known to interfere with the cholinergic synapses there is no evidence of structural changes. Unlike the drugs which affect the catecholamines (NE, DA and 5HT), for example, rats treated with resurpine, 6-hydroxydopamine and 5-hydroxydopamine have in addition to reduced levels of NE, DA and 5HT also show disappearance of the small granulated synaptic vesicles associated with high levels of NE referred to

previously.

2.2 HORMONES AND THE CENTRAL NERVOUS SYSTEM

2.21 General Introduction

The inter-relationship of the endocrine system and the central nervous system is a well established fact. A casual comparison between males and females shows the contrast between the emotional patterns of behaviour of the two sexes, for example, the aggressive male reaction to that of the more docile! female. That there are grades of masculinity and feminity, for example, the effeminant male and the dominant female, can be explained in the development of the CNS and the effect of sex hormones on the nervous system (Harris, 1964).

Essentially, all vertebrate nervous systems are female and only display male characteristics if the appropriate hormones, androgens, in the correct proportions, affect the system during early foetal development. Needless to say, other aspects such as inherited tendences and environment also play their part.

2.22 Hypothalamus and Pituitary

At the physiological level the most obvious interaction between the endocrine and nervous systems is in the relationship between the hypothalamus and the hypophysis (pituitary) (Fig. 13). This relationship provides the answer to many of the body's reactions to both the internal and external environment resulting in the influence, or rather control, of such patterns of behaviour as: mood, emotion, pleasure, sexual activity, as well as regulating the levels of a number of substances, thus contributing to homeostasis. In addition, control of the autonomic nervous system originates from the hypothalamus and as a consequence this

FIGURE 13.

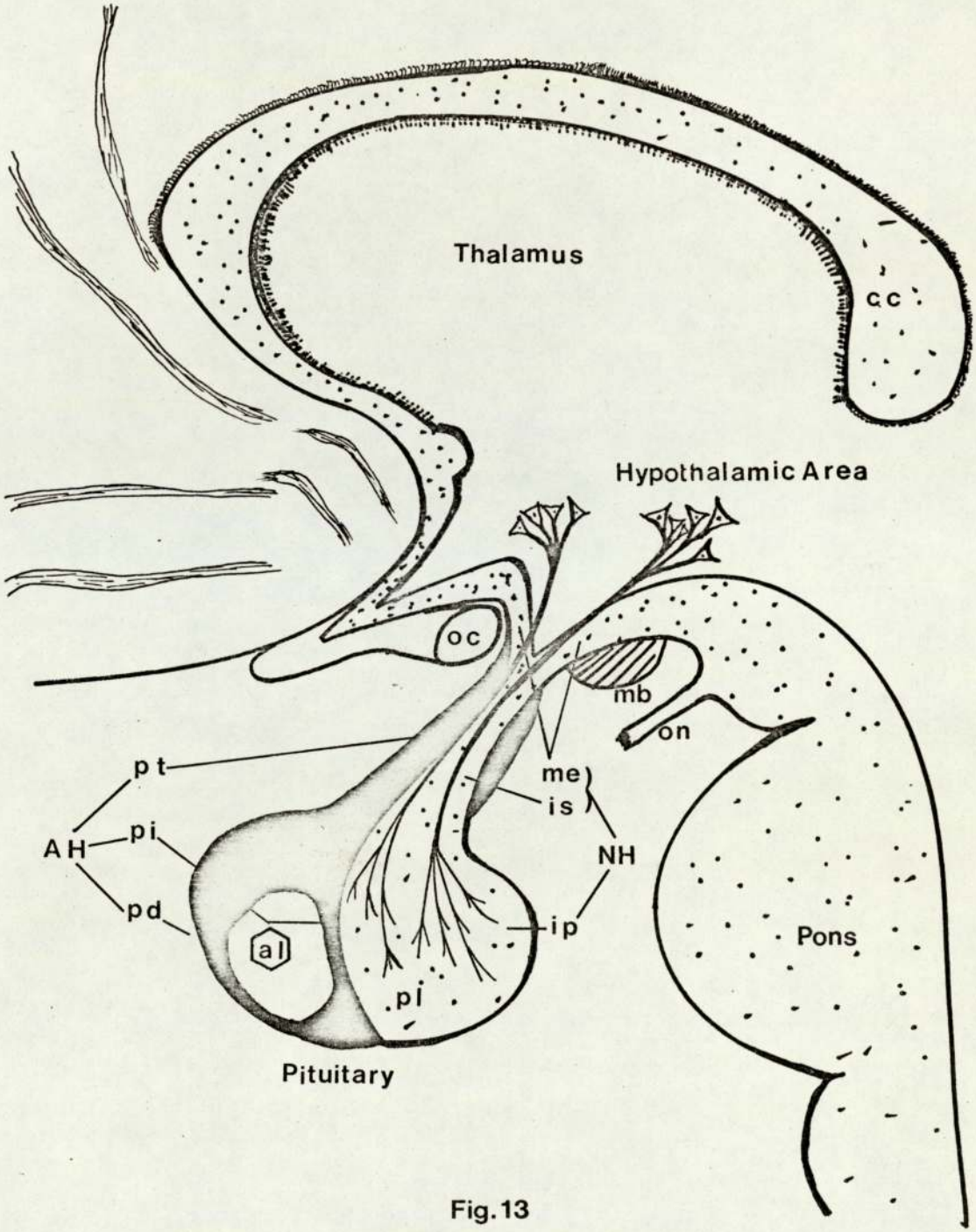

Thalamus
cc
Hypothalamic Area
oc
mb
on
pt
AH
pi
pd
me
is
NH
ip
al
pi
Pons
Pituitary

Fig. 13

part of the CNS influences such activities as respiration, sleep, blood pressure, water conservation, hunger, etc.

Since the pituitary has direct control over the whole of the endocrine system it can be considered that the hypothalamus has indirect control over the hormone regulation of the body vide infra.

The development of the hypothalamus indicates clearly the intimate relationship between the CNS and the endocrine system. The embryonic development of the forebrain results in the formation of the diencephalon (thalamencephalon) which may be divided into a number of regions: two large nuclear masses termed the thalami from which eventually outgrowths arise and form the retinae and optic nerves; the epithalamus (roof of the thalamencephalon) from which arise the pineal and parietal bodies; and the hypothalamus. A more detailed description of the embryonic development of the brain is provided by Warwick and Williams (1973), Schadé and Ford (1967).

From the hypothalamus there develops a downgrowth which unites with an upgrowth from the roof of the pharynx to form the hypophysis. The hypophysis therefore consists of two main parts, the neurohypophysis originating from the hypothalamus and the adenohypophysis which has developed from the roof of the pharynx. An immediate structural relationship between the CNS and the endocrine system is apparent.

It is perhaps important to appreciate the significance of the thalamencephalon in the overall involvement of the CNS in the body's activities. Most authors aptly describe the thalamencephalon as a staging post. Within this region neuronal perikarya are concentrated into discrete areas referred to as nuclei. In addition, these areas contain fibres originating from sensory cells located in different parts of the body. For example, in

the thalamus the areas defined as the lateral geniculate nuclei contain fibres originating from the retina which synapse with neurones which will relay the information to the visual cortex.

In addition, in the hypothalamus other nuclei may be found; these are concerned with a number of activities which are related to; the hypophysis, the reticular formation of the brain stem, and the limbic system. This latter system involves the behavioural reactions of the animal towards the external environment based on information received from all sensory receptors. The reactions include those related to "fight and flight", feeding and reproduction (Schadé and Ford, 1967; Rose, 1973).

The relationship between the hypothalamus and the hypophysis originates from the neurosecretory cells, situated in the former, whose axons terminate either in the neurohypophysis or on the capillary loops of the hypothalamus. The hypophysial portal venules convey blood to the sinusoides of the adenohypophysis. The importance of this direct connection between the neurosecretory cells and the cells of the anterior lobe lies in the liberation of "multiple releasing factors" by the former. Such factors control the release of the anterior pituitary hormones which in turn control the release of hormones produced by the other endocrine glands.

The pituitary is the source of at least nine hormones which affect the adrenals, sex organs, kidneys, vascular system, bones and soft tissues, pigment cells and the thyroid.

2.23 The Thyroid

The control of the adenohypophysis over the thyroid, the gland with which this research is concerned, is accomplished through the release of the thyrotrophin hormone - thyroid stimulating hormone

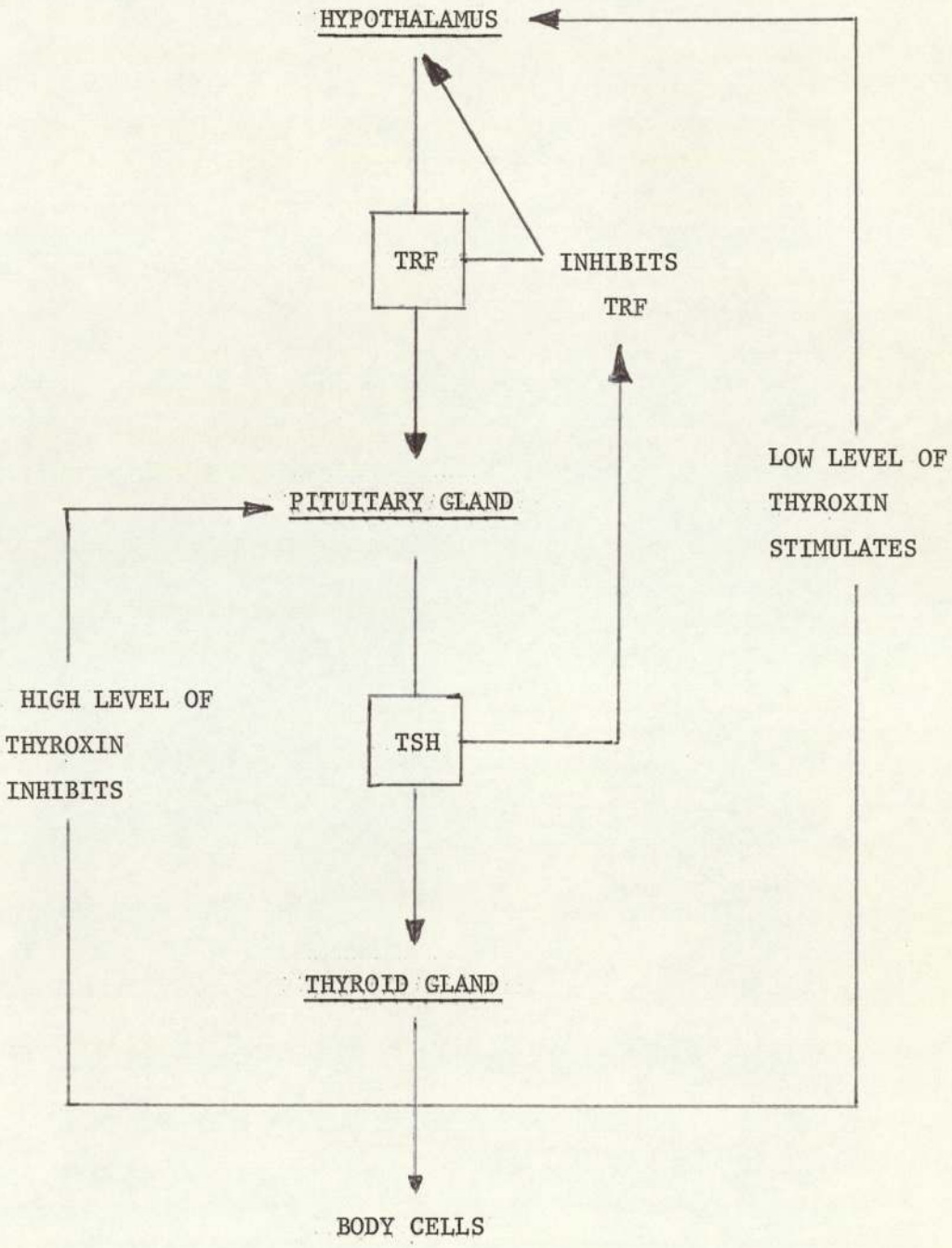
(TSH).

There is well documented experimental evidence (Harris, 1964) that the release of TSH is influenced by two factors; one is the result of feedback action of thyroxine (T_3) on the anterior pituitary and possibly at the hypothalmic level (Reichlin, 1964). The second factor is the release, from the median eminance tissue of the hypothalamus, of a thyrotrophin releasing factor (TRF). Thus the thyrotrope cells of the anterior pituitary are subject to two interacting chemical influences, thyroxine which would inhibit TSH production and TRF which would stimulate TSH synthesis. In addition, thyroid hormone production would be influenced by the rate of thyroxine synthesis, release and degradation in the body (Fig. 14).

The thyroid gland is situated on either side of the upper third of the trachea, and consists of two main lobes which are joined over the anterior surface of the trachea. The thyroid may also contain, or have located on its surface, depending on the animal, the parathyroid glands which are responsible for the elaboration of the "Parathyroid Hormone" (PTH). The size of the thyroid is influenced by a number of factors including: state of diet, age, and the physiological state with particular reference to normal or pathological conditions. Thus the states of chronic 'hyper' and 'hypo' thyroid activity may influence the size of the thyroid, as witnessed in such syndromes as "Endemic Goitre".

Microscopically the thyroid is one of the more easily recognised tissues, since the arrangement of the secretory epithelial cells is in spherical or oval follicles which vary in size. The follicles, in normal animals, contain a homogeneous acidophilic staining material. This substance, referred to as "colloid", together with the cellular arrangement of the gland, provides the easily identifiable histological picture. The amount

FIGURE 14.


HYPOTHALAMUS
TRF
INHIBITS
TRF
LOW LEVEL OF
THYROXIN
STIMULATES
PITUITARY GLAND
HIGH LEVEL OF
THYROXIN
INHIBITS
TSH
THYROID GLAND
BODY CELLS

of colloid varies with the activity of the gland, for example in the hypo-active thyroid the colloid tends to accumulate whilst in the hyper-active state the colloid store is reduced. The colloid is composed mainly of thyroglobulin with a number of enzymes concerned with its hydrolysis. This enzyme activity results in the formation of a number of iodinated amino acids; monoiodotyrosine, diiodotyrosine, 3,3'-triiodothyronine, 3,3',5'-triiodothyronine, and 3,5,3',5'-tetraiodothyronine (thyroxine). Of these, only thyroxine (T_4) and 3,5,3'-triodothyronine (T_3) are active in the peripheral tissues and it is these thyronines which are designated as "Thyroid Hormone" (TH). (Fig. 15). Investigations into the effect of TH show that it has a variety of activities which may be initially summarised as affecting the metabolism of the animal. The exact form in which the TH is active is not clearly understood. However, it is known that triiodothyronine is more active than thyroxine and that the latter can be converted to the former by the kidney. Furthermore, mitochondria are capable of converting thyroxine and triiodothyronine to their respective acetic acid analogues; tetraiodothyroacetic acid (TETRAC) and triiodothyroacetic acid (TRIAC). Both derivatives are active in the mitochondrial processes concerned with energy metabolism, in that they are capable of uncoupling oxidative phosphorylation reactions resulting in the reduced production of adenosine triphosphate (ATP). Such processes result in an increased oxygen uptake, a phenomenon associated with TH activity (Turner, 1966).

Located with the thyroid gland, in the case of many mammals, are the parathyroid glands which, microscopically, are distinguishable from the thyroid tissue. The function of the parathyroid glands is to secret "parathyroid hormone" (PTH) which, acts upon bone, kidney, and possibly the intestines, to control the

FIGURE 15.

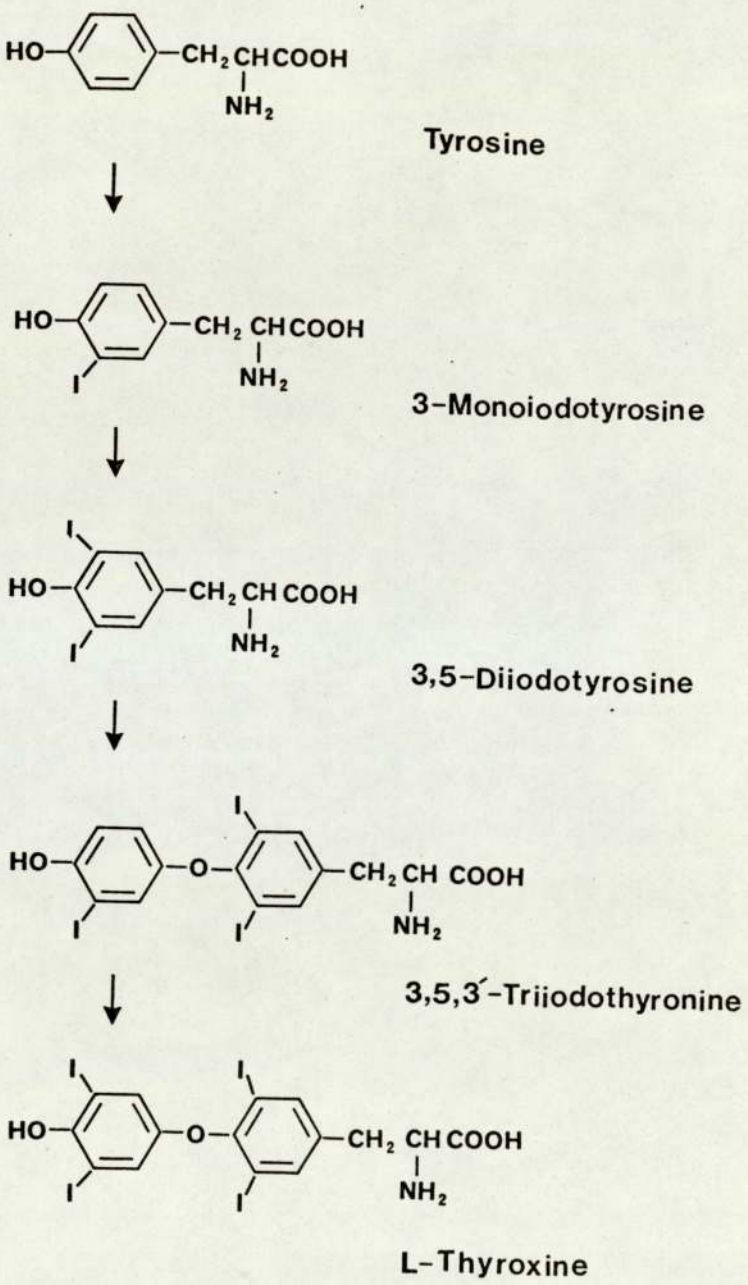

HO
CH2 CHCOOH
NH2
Tyrosine
HO
CH2 CHCOOH
I
NH2
3-Monoiodotyrosine
I
HO
CH2 CH COOH
I
NH2
3,5-Diiodotyrosine
I
HO
O
CH2 CH COOH
I
I
NH2
3,5,3´-Triiodothyronine
I
I
HO
O
CH2 CHCOOH
I
I
NH2
L-Thyroxine

Fig.15

metabolism of calcium and phosphate.

From the evidence now available it is clear that the CNS controls the endocrine system principally through its control of the pituitary gland. The reverse control also occurs as is shown with the obvious differences between males and females, which is reflected in the CNS response to given situations. Rose (1973) in fact reports on experiments in which the hypothalamus of physically and sexually immature male rats were briefly exposed to oestrogens with the result that they continued a female sexual behaviour pattern into adult life. This indicates that in one case at least the endocrine system affects the operation of the CNS. In addition it is reasonable to assume that the activity of the thyroid hormones are equally effective on the CNS as they are on the remaining tissues of the body. Such an assumption is supported by the evidence of the effect of hypothyroidism in both animals and man.

2.24 Hypothyroidism

The state of cretinism is well documented as being due to the malfunction of the thyroid during early development.

2.241 General Effects

Hypothyroidism is characterised by a lowering of excitability, responsiveness, and alertness (Benda, 1949). In addition, mental retardation occurs ranging from imbecility to moderate subnormality. This effect cannot be due entirely to a reduced metabolism but must be related to a combined developmental abnormality with reduced metabolism (Eayrs, 1971).

Reduction in mentation of the human cretin, according to Benda (1949), runs parallel to the physical manifestation

and develops according to age at the time of onset. Thus in sporadic cretinism in which the child is affected by congenital thyroid aphasia, not only is there the classical physical abnormalities, e.g. reduced stature, facial bone alterations resulting in a uniform appearance, etc., but also severe mental retardation as indicated by failure to learn to speak and feeblemindedness. In acquired hypothyroidism the effect, although often varying from individual to individual, nevertheless produces a uniform effect in the younger person and the earlier the onset the more uniform the "cretinoid" appearance of the child (Benda, 1949). In addition, Smith, Blizzard and Wilkins (1957) have shown that the success of hormone therapy is related to the age at which treatment is commenced, which implies that more permanent damage to the CNS, of either a structural or functional nature, may result from hypothyroidism. Eayrs (1971) has also indicated in his review of thyroid and the developing brain that there may be "an irreversible component related to the maturation of as yet only a partially identified enzyme system".

That there is a critical period in the development of the CNS at which thyroid abnormality can have its effect has been shown by a number of workers. Thus Eayre (1961) has shown that, if rats are thyroidectomised before 10 days of age, there is a considerable increase of error scores in the Hebb-Williams closed field test. However, there is no effect on the score errors of rats thyroidectomised between the age 10 - 24 days. Gomez (1971) investigating the effect of thyroidectomy on rats, together with replacement T_3 therapy, has shown that providing therapy is commenced before 10 days of age, the levels of DNA, RNA, succinate dehydrogenase and GABA transaminase are restored to normal. However,

replacement therapy is ineffective if it is delayed to the 15th day.

Evidence produced by earlier workers (Goodkind and Higgins, 1941) was such that they could not correlate the effect of therapy on the degree of recovery, which suggested that other factors may influence the final outcome of hypothyroidism.

Experimental hypothyroidism in animals was first performed on sheep in 1895 by Von Eiselberg and subsequently in several other animals: rabbits (Tatum, 1913), sheep (Simpson, 1913), and rats (Salmon, 1938).

These early investigations initiated considerable research into the effects of both hypo and hyperthyroidism from either a general response or to the effects on specific tissues or organs. The overwhelming evidence, from cases of cretinism, that the thyroid can affect intelligence in the developing animal, resulted in the development of research into the influence of the thyroid on the CNS. A number of avenues presented themselves for investigation; these involved the effect of the thyroid on: learning capacities, structural form, physiology.

2.242 Methods of Establishing Experimental Hypothyroidism

Creation of a hypothyroid state in animals can be achieved by several methods including the surgical removal of the thyroid gland which is difficult in small laboratory animals, particularly at the immediate post-natal stage. As a consequence, the administration of radioactive iodine (^{131}I) was introduced by Goldberg (1949) as an alternative method. Since iodine is an essential element of the thyroid hormones, thyroxine and triiodothyronine (Fig. 13), the

thyroid requires a relatively large proportion, in the order of a third, of the iodine absorbed by the body (Th. van der Werff, 1966). Accumulation of radioactive iodine in the thyroid results in hypoplasia and degeneration of the gland. Hypothyroidism may also be induced by the administration of thiouracil (Hughes, 1944). Studer and Greer (1967) employed propylthiouracil and iodine deficient diet to induce hypothyroidism in animals. Thiouracil and other antithyroid agents (thiourea) prevent the organic combination of the iodide thus inhibiting thyroid hormone synthesis (Bell, Davidson and Scarborough, 1968). Injections of thyroxine will reverse this condition and produce a hyperthyroid effect.

2.243 Effect on the Brain: Macroscopical and Microscopical (Optical)

Investigations show that the retardation of body development, following hypothyroidism, is accompanied by a corresponding reduction in brain weight. The brain is not only reduced in size but also abnormally shaped, being disproportionately wide in relation to its length (Eayrs and Taylor, 1951; Eayrs, 1971). At the microscopical level, the reduction in brain size is apparently due to a number of abnormalities including a reduction in the size of the perikaryon and a closer packing of the neurones (Eayrs and Taylor, 1951). Neurone processes also show changes in that axon density is significantly less (Eayrs and Horn, 1955) whilst the basal dendrites, although remaining the same in number, are shorter and branch less. Horn (1955) suggests that the difference in brain weights between normal and hypothyroid animals is due to the changes in the fibres since the number of neurones remains the same. The

reduction in axon density is not proportional in all the laminae but selective to laminae 4. (Eayrs, 1955).

Hamburgh (1968) proposes a hypothesis that the effect of thyroxine is to 'push' cells into the differential phase by pulling them out of the proliferative or mitotic phase. It is known that thyroxine can exert opposite effects in that it may accelerate the growth of some cells and stimulate mitotic activity whilst initiating differentiation in other cells. Such a hypothesis is supported by the proliferation of such cells as granule cells and their retarded development vide infra coupled with the reduced neuronal development.

Eayrs (1968) has shown that there is a correlation between dendritic patterning as influenced by thyroidectomy, and the learning ability of rats when subjected to maze tests. A most interesting observation made by Eayrs, in the same article, is that whilst the neurohistological changes are largely reversed by medication, irrespective of the age at which it commenced, the impairment of behaviour still persists.

Additional evidence of the influence of the thyroid on the maturation of the CNS is provided by the effect on the vacularity of the cerebral cortex (Eayrs, 1954). Despite their increase in size, the number of blood vessels is sufficiently reduced to affect the available surface area for metabolic exchange (Eayrs, 1971).

Within the cerebellum of thyroidectomised animals the microscopical changes include retardation of granule cell development (Hajós, et al. 1973) and increased numbers of granule cells (Nicholson and Altman, 1972). Hyperplasia of the glia cells in the hypothyroid rat cerebellum was also observed by Clos, Rebière and Lebrans (1973). Although hypothyroidism

did not affect the number of Purkinje cells there was an overall delay in cell differentiation of about 5 days (Nicholson and Altman, 1972); this evidence again supports the hypothesis of Hamburgh.

2.244 Metabolic Changes

Investigation into the localisation and activity of enzymes at the cellular level has been pursued using histochemical methods (Robinson and Eayrs, 1968). It would appear that none of the twelve enzymes investigated, $NADH_2$-diaphorase dehydrogenase, α glycerophosphate dehydrogenase, succinate dehydrogenase, glucose 6 phosphate dehydrogenase, acid phosphatase, alkaline phosphotase, Ca^{2+}- ATPase, monoamine oxidase, acetylcholinesterase, Mg^{2+}-ATPase, 5-nucleotidase, thiamine pyrophosphatase, showed significant changes in thyroidectomised animals although slight reductions were noted.

At the sub-cellular level research has followed two main lines, namely, the influence of the thyroid on ultrastructure, and secondly, its effect on biochemical parameters. In the latter, use has been made of the homogenisation and fractionation techniques introduced by Gray and Whittaker (1962). A review of the effects of thyroxine at the metabolic level, provided by Wolff and Wolff (1964), shows that its activity is not limited to one specific process but affects a variety of enzymes and substrates. Amongst the enzymes which may be either inhibited or stimulated, the following are included: dehydrogenases (e.g. malic, lactic, glyceraldehyde 3-phosphate, glucose 6-phosphate, glutamic, etc.), phosphatases (e.g. acyl, ascorbic acid, carbomyl, etc.), peroxidase, dehydrases (e.g. serine, threonine). Substrates affected

include those in which oxidation is increased by hyperthyroidism (e.g. glucose, pyruvate, α ketogluterate, succinate, etc.) and those in which oxidation is reduced (e.g. lactate, β-hydroxybutyrate, tyrosine, etc.). It is interesting to note that some of the results of Robinson and Eayrs (1968) would appear to be at variance with the data quoted by Wolff and Wolff (1964), e.g. in hypothyroidism, glucose 6-phosphate is not affected whereas in hyperthyroidism it is utilised at a higher rate. However, the circumstances of the experiments differ, the former being conducted with 'in vivo' tissues and utilising histochemistry, whilst the latter was conducted 'in vitro'.

In addition to reduction in activity, following hypothyroidism, of such enzymes as succinic dehydrogenase, glutamate decarboxylase, etc. (Hamburgh and Flexner, 1957), (Garcia Argiz, Pasquini, Kaplan and Gomez, 1967), (Szijan, Chepelinsky and Piras, 1970), amino acid levels are also affected (Guglielmone and Gómez, 1966). Interference of amino acid transport by thyroxine is thought to be responsible for the effect on protein synthesis (Geel, Valcana and Timiras, 1967) which would account in part for the reduction in brain neuropil. Geel and Timiras (1967a) also report reduced levels of acetylcholine esterase and choline esterase in the rat brain. Such findings have also been found by Ling (1970). An investigation into nucleic acid levels of cerebral cortex of neonatal rats by Geel and Timiras (1967b) shows significantly higher levels of DNA/unit mass of brain in hypothyroid animals whilst RNA levels were reduced. It is consequently assumed that hypothyroidism delays both body and brain development by interference with protein synthesis. This view is supported by the work of Balázs, Kovacs,

Teichgraber, Cocks and Eayrs (1968) who have shown that hypothyroidism reduces the relative concentrations of protein and RNA to that of DNA. Since the concentration of DNA remains at normal levels the results suggest that thyroidectomy does not interfere with the numbers of cells, but in their size. Legrand and Rabié (1972) have also shown the effect of the thyroid on the developing rat brain in that hypothyroidism and hyperthyroidism respectively cause a decrease and increase in protein concentrations within the synaptosome fraction.

In addition to the effect on proteins, enzymes and RNA, neonatal thyroidectomy also influences myelination (Balázs, Brooksbank, Davison and Wilson, 1969). The evidence presented by these workers shows that in the rat brain there is a 40% reduction in cerebrosides in addition to reductions in phospholipids and cholesterol. Raveglia, Gomez and Ghittoni (1972) extended the work of Balázs et al and have shown, in addition to decreases in cerebrosides, gangliosides and sphingomyelin, there are decreases in all lipids, in both the cortex and cerebellum, of developing rats, which have been thyroidectomised at birth. Although it would appear that both these areas of the brain are affected, the cerebrum shows significantly greater decreases in all lipids compared to the cerebellum.

2.245 Ultrastructural Changes

Whilst a considerable amount of attention has been paid to the effect of either hyper or hypothyroidism on the biochemistry of the brain, less experimental work has been reported on the response of the CNS ultrastructure to thyroid hormones. Eayrs and co-workers initiated investigations

into the effect on cell structure of the CNS, as previously described, and their findings of retardation of development has been supported by other workers. For example, Hajós, Patel and Balázs (1973) have shown that in the cerebellum the somatic spine synaptic sites of the climbing fibres disappear from the Purkinje cells in normal rats between 8 and 16 days after birth. In the hypothyroid animal these synaptic junctions of the climbing fibres persist up to the 27 day. In addition there was retardation of the granule cells, as indicated by the failure of the dendrites of these cells to divide, and a continued formation of multiple synapses with the small mossy fibre "rosette". Nicholson and Altman (1972) have also shown retardation of synaptogenesis in the rat cerebellum. Whilst in the normal rat synaptogenesis is complete by 30 days, in the hypothyroid animal it is not complete by the 55th day. Other evidence of cerebellar retardation includes prolonged cell proliferation in the external granular layer and retarded disappearance of this layer. In the molecular and internal granular layers there is also retarded cell differentiation, and in the case of specific cells there is a terminal increase in granule cells and astrocytes, and a decrease in basket cells.

In the visual cortex Cragg (1970) has shown findings similar to those found by Eayrs et al in the sensory motor cortex, namely, the neurones are more densely packed in the hypothyroid rat than in the normal animal. Cragg also showed that whilst the axon terminals in the neurophil of thyroidectomised animals were of a similar size and frequency to that of normal animals, because of the increased number of neurones per cm^3 the actual number of axon terminals per neurone was reduced by 20%. Also reported in the same

article was the presence of membranous bodies in the superficial layers of the visual cortex in thyroidectomised animals. Such structures, varying in diameter from 0.3 - 1.5 NM, were unevenly distributed in the glial processes, dendrites, neuronal cytoplasm and synapses.

Since such structures were observed in three separate experiments in which thyroidectomy was achieved by the administration of either ^{131}I or methyl-thiouracil, their presence must be due to the influence of thyroxine deficiency. Cragg postulates that the presence of these membranes suggests abnormal membrane synthesis due to either, excess lipid metabolism, or reduced protein synthesis which results, in part, of the membrane production becoming superfluous.

In a more recent report by Rebière and Legrand (1972), in which they investigated the number of synapses in the molecular zone of the cerebellum of normal and thyroidectomised animals, there would appear to be a significant difference between the two types of animals. Following daily administration of propylthiouracile (PTU) for varying periods of time, synaptic densities were determined by sectioning the brains of animals at 14, 21, 28 and 35 days and counting over 420 sq.µm. Results indicated that whilst there was a progressive increase in numbers of synapses in both normal and hypothyroid animals, the numbers were significantly less in the latter type of animal than in the former. The degree of effect caused by daily administration of PTU varied with the duration of treatment. In animals treated post-natally for 14 days, the number of synapses continued to increase so that at the adult stage the difference between these animals and the normal controls was

only approximately 20%. There would appear to be a significant increase between 14 and 21 days in the numbers of synapses detected in both normal and hypothyroid animals. Adult numbers of synapses were reached at 35 days. If the treatment was continued post-natally for 35 days, the number of synapses at the adult stage was little different from the numbers seen at 21 days in normal animals. This effect was irreversible, in that discontinuation of the treatment at 35 days, thus allowing thyroid refunction, did not change significantly the numbers of synapses measured at the adult stage. Thus it can be deduced that if hypothyroidism is continued from birth to 35 days there is an irreversible effect on synaptogenesis of the molecular zone of the rat cerebellum.

2.25 Conclusion

The evidence in the literature, published prior to the commencement of this research, showed that the thyroid hormone influences the development of the CNS. Deficiency of the hormone results in retardation of this development which is macroscopically apparent from alterations in the size and shape of the brain. Evidence from microscopical examinations shows development deficiencies at the cellular level which would account for the changes in brain structure.

Of particular interest is the evidence of Eayrs and Horn (1955) that the cellular changes are not uniform throughout the cortex but more apparent in those areas which receive the main weight of thalamic projections. The reduction in the size of the perikaryon, in dendritic arborisation, and axon density would account significantly towards the reduced brain size.

In view of the reduced dendritic arborisation it is possible that there is also a reduction in the number of synapses, a point raised by Eayrs (1955). Whether or not this could be responsible for mental retardation depends upon the degree of effect. Since commencement of this research, Cragg has shown a reduction in the numbers of synapses/neurone in the visual cortex of thyroidectomised rats. Whilst Rabière and Legrand have shown a permanent reduction in the number of synapses in the cerebellum of hypothyroid animals.

It is possible that mental retardation could be due to either a reduced number of synapses in specific areas of the brain, or to an overall reduction.

This research was initiated to determine whether or not there was an overall effect of thyroxine on synaptogenesis and at what stage in development hypothyroidism was effective.

To achieve figures which would represent the numbers of synapses in the whole of the cerebral cortex, techniques alternative to those of tissue sectioning were required. Homogenisation of the cortex was the first pre-requisite, to be followed by a method for determining the numbers of resulting synaptosomes. Two methods presented themselves as possibilities, the first was employment of an electronic counter (Coulter counter), and the second was by visual recognition and manual counting. The first method was quickly discarded following preliminary tests vide infra. This left the second method which was adopted using essentially the methods of Gray and Whittaker (1962) and Clementi, Whittaker and Sheridan (1966).

3. MATERIALS AND METHODS

3.1 ANIMALS

Three groups of rats varying in age from suckling animals to adults not exceeding 6 months were used in these experiments. Two of the groups were descendent from a Wistar line which had been established from breeding pairs supplied by SPF Ltd. (Cherry Garden Lane, Ash, Canterbury, Kent). These animals were used to establish a normal range of synaptosome numbers and for the induction of hypothyroidism by the administration of ^{131}I.

The third group of animals were white CFHB males, obtained from Carworth Europe (Alconbury, Huntingdon), in which half were thyroidectomised at 21 days of age by surgery, and the other half were sham operated.

3.2 THYROIDECTOMY OF RATS USING ^{131}I

Six female rats, between 6 and 12 months of age, were placed with male rats for three days, Monday - Wednesday, inclusive. The limited exposure of the females to the males enabled the time of conception to be controlled, thus guaranteeing birth within known dates. This ensured that the ^{131}I was at its maximum level of radiation simultaneous with the birth of the rats.

Radioactive iodine in the form of 1 millicurie of sodium iodide (^{131}I) contained in 1 cm^3 of a sterile isotonic phosphate buffered solution containing 0.08 mg/mCi of sodium thiosulphate (Code No. 1BS 2P) was ordered from the Radiochemical Centre (Amersham, Buckinghamshire) to be delivered on the Tuesday 21 days after animal mating. In all three series of experiments, births took place within 24 hours of receiving the ^{131}I.

Litters of 12 suckling rats (excess numbers were removed from the

litter) born no earlier than 12 hours before the commencement of the experiment were selected. Trial intraperitoned injections of 0.15 cm^3 of phosphate buffered saline solution (Dulbecco A, Paul, 1970) showed a considerable tendency towards leakage, from the suckling rats, if the site of injection was in the mid-line region of the abdomen. Less leakage occurred if the injection was made lateral to the mid-line of the animal where there was less abdominal pressure and a reasonable amount of slack skin.

Because of the radiation hazard involved with the use of ^{131}I special precautions and procedures were adopted. The experiments were carried out in a laboratory side room of the nuclear suite of the City of Birmingham Polytechnic. Access to this room was restricted and the animals and all waste matter were retained in the room for a minimum period of 6 weeks. This allowed the level of radiation to return to normal. In addition, all waste material, cages, animal carcasses and apparatus used in this stage of the experiment were tested with a radiation monitor (Mini Monitor, Model S, Chandos Products Ltd., Stockport, Cheshire: Fig. 16) before disposal or cleaning.

Although benches were covered with an impervious plastic Formica type covering, additional precautions to safeguard against spillage or leakage were adopted. Materials, including animals, were handled in plastic trays lined with 'Benchkote', a paper material in which the top coat, being of absorbent material, would absorb spillage whilst the bottom plastic layer would prevent penetration and minimise contamination of the tray.

Using a sterile disposable 1 cm^3 'tuberculin' syringe, fitted with a 25G x $\frac{5}{8}$ (Gillette) sterile needle, six of the suckling rats (Wistar) were injected with 0.15 cm^3 of 10^3 $\mu Ci/cm^3$ ^{131}I. Thus to induce hypothyroidism each animal received 150 μCi, a dose used by

other workers (Goldberg, 1949; Cragg, 1970). The remaining six suckling rats of the litter were used as controls and injected with 0.15 cm^3 of sterile phosphate buffered saline (Dulbecco A). The control animals were identified by marking their backs with 1% crystal violet in alcohol.

Since the greatest radiation danger was during the initial stages of injecting the animals, particularly in charging the syringe, a technique was devised to reduce this risk to the minimum. At no time was the glass vial of ^{131}I removed from its lead container. The top of the lead container was removed and replaced with a transparent plastic shield measuring 100 mm x 100 mm x 10 mm with a 3 mm hole located in the middle. The container was clamped, in the inverted position, to a retort stand so that the vial top rested on the plastic shield with the rubber cap aligned over the hole of the shield (Fig. 16). The syringe needle was inserted into the vial through the hole in the plastic shield, thus reducing exposure of the hands to the ^{131}I radiation.

To reduce the hazard of inhalation of radioactive contaminated material the inoculated animals and the mother were housed in plastic cages lined with Benchkote instead of the conventional sawdust. The cages were placed in a stainless steel fume cupboard with a partially closed glass front (Fig. 17). Exhaust fans were used so that the air could be changed prior to handling the animals. Cages were not cleaned until after radiation checks had shown that they were harmless. Animals were transferred every 2 - 3 days to fresh cages.

Protection and control of individuals throughout this part of the experimental work was achieved by the wearing of: a white coat, the use of which was restricted to the radiation experiments; a radiation monitoring badge; a monitoring tape (Thermo Luminescent Detector: TCD) worn on the right forefinger during the injection procedures; and

FIGURE 16

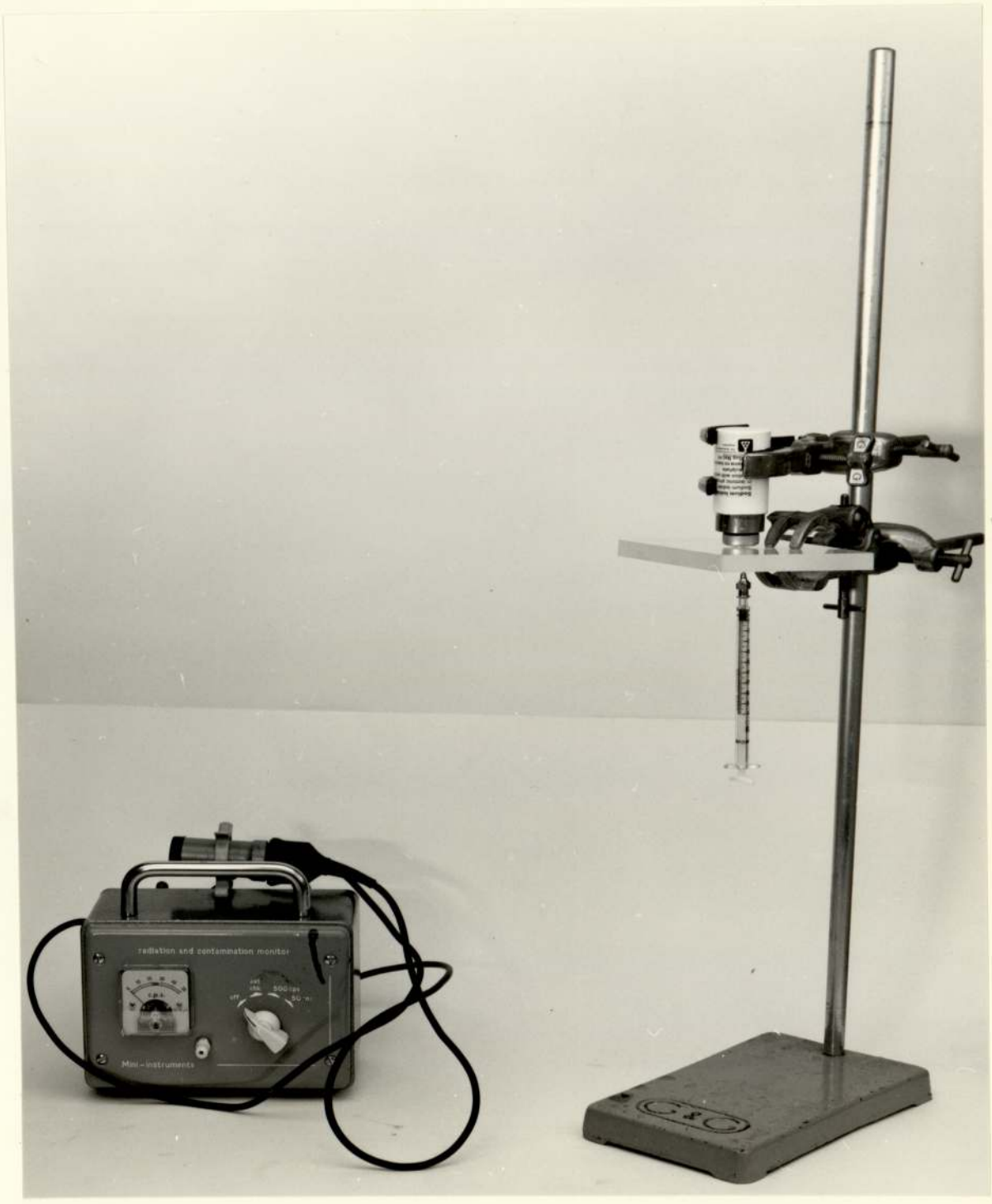


Fig. 16

FIGURE 17.

Fig. 17

plastic disposable gloves.

3.3 PREPARATION OF SYNAPTOSOME EXTRACTS

The techniques employed were based on those developed by Clementi, Whittaker and Sheridan (1966).

The rats were killed either by gasing with coal gas or in the case of suckling rats by severance of the spinal cord at the region of the upper cervical vertebrae. The animals were stretched out, dorsal side uppermost, onto a dissecting board and held by pins. The head was moistened with alcohol to reduce fur contamination. A mid-line incision was made through the skin with scissors, above the superior sagital suture and extending to the neck and snout. The skin was pared away exposing the whole of the upper skull. Using either fine straight iris scissors or fine bone cutters, an incision was made in the inter-parietal bone and extended in both directions through the parietal and frontal bones. This allowed the removal of an oval section of the skull exposing the brain which was carefully removed intact with the aid of flat curved stainless steel spatulas.

The brain was washed in normal saline at $4^{o}C$ to remove blood, gently blotted with fine filter paper (Whatman No. 50) and weighed. The cerebellum, medulla oblongata, remaining spinal cord fragment, olfactory lobes, pituitary, pons and other brain structures were then removed from the cerebrum. As much as possible of the white matter was also removed and the remaining cerebral cortex was weighed.

Following the report by Rebière and Legrand (1972) and Legrand and Rabie (1972) that there was variation in the protein concentration and numbers of synaptosomes in the molecular zone of the cerebellum of thyroidectomised rats compared with normal rats, the cerebellum of some of the animals, used in the later stages of the experiment work, were also weighed and processed.

After weighing, the cortex or cerebellum was homogenised in a freshly prepared 0.32 M-sucrose solution at 0 - 4°C. This temperature was maintained by the use of a Grant cooling unit placed in a stainless steel water bath with a heating unit set at 0°C. The bath was filled with a rock salt solution. Following malfunction of this equipment it was found that the temperature was not critical to a few degrees and could be adequately maintained by the simple expedient of using ice in plastic containers. Control over the temperature of reagents was part of a general policy in controlling and investigating all stages of the technical procedures. This became necessary following precipitation of the potassium permanganate fixative (Luft, 1956) at the final stage of the synaptosome preparation. As a consequence various modifications vide infra were tried to eliminate this problem, including the employment of freshly prepared reagents, and the careful control of reagent temperature. Although the cause of preciptiation was eventually determined vide infra, the use of freshly prepared reagents and controlled temperature was continued since the developed technique, now being described, was relatively problem free.

Using a Quickfit and Quartz all glass hand grinder, homogenisation of the brain tissue was commenced with 6 - 10 up and down rotating strokes which reduced the material to small fragments. These were then transferred to a Thomas hand homogeniser (Grade B) consisting of a teflon pestle with stainless steel shank and glass grinding chamber. The clearance between the pestle and mortar was 0.127 - 0.178 mm. This was a closer clearance than that recommended by Whittaker and Dowe (1965), which was 0.25 mm, but was justified since their technique employed shearing at a pestle speed of 840 rpm.

A number of preliminary experiments were performed in which variation in the degree of homogenisation was tried by using from 4 to 20 downward rotating strokes. The results showed that satisfactory

preparations were achieved using 10 - 12 strokes. Less than this number resulted in the presence of myelin fragements and little evidence of synaptosomes whilst increased homogenisation resulted in apparent disruption of the synaptosomes.

Experiments by Whittaker and Dowe (1965) also showed the need to standardise the homogenisation process and they recommended the use of an Aldridge homogeniser (Aldridge, 1960) with a 0.25 mm bore at a speed of 800 rev/min for 12 up and down strokes. An Aldridge homogeniser connected to an electric drill with a rheostat to reduce the speed was tried (Fig. 18) but preliminary experiments showed that insufficient shearing was taking place resulting in the presence of myelin (Fig. 27). Since homogenisation, with the Thomas Teflon grinder, was proving to be reliable by achieving reproducable results in the estimation of synaptosome numbers in adult rat brain, the Aldridge homogeniser was not adopted for these experiments.

Following homogenisation the extract was centrifuged, at 2000 rev/min for 10 min in an MSC Multex-centrifuge, to remove any intact RBC's or large particles of debris. The supernatant was collected and stored ar $0^{o}C$. The pellet was resuspended in chilled 0.32 M-sucrose and the process repeated three times. The combined supernatants were then centrifuged to deposit the synaptosomes in a Griffin and Christ ultracentrifuge using an 8 x 35 cm^3 angle head rotor at 12500 g for 60 min at $4^{o}C$.

Following centrifugation the supernatant was discarded and the pellet resuspended in fresh 0.32 M-sucrose. Since in addition to the synaptosomes other cell organelles, including mitochondria, ribosomes and myelin fragements, are deposited, further separation in a sucrose gradient is required. The volume of sucrose solution used for resuspension depended on the original weight of the homogenised brain tissue which in turn influenced the pellet size. For example; the

weight of cortex from an adult rat brain was generally in the region of 0.65 g, for this size of extract 0.9 cm^3 of 0.32 M-sucrose was added. Resuspension was carefully performed by mixing up and down in a 1 cm^3 graduated pipette. It was essential that mixing was carried out meticulously since the centrifuging caused the extracted cell particles to clump. This was more pronounced in those experiments where the pH of the sucrose solution was controlled to pH 7 by the use of Sorensen's M_{15} phosphate buffer (Silverton and Anderton, 1961). The control of pH was one of the experiments designed to determine the cause and hence elimination of fixative precipitation. Control of pH by this method proved unsuccessful because of the inability to produce even suspensions of synaptosomes.

Separation of the synaptosomes from other sub-cellular organelles was achieved in sucrose gradients which were prepared by layering approximately 2 cm^3 of a 0.8 M-sucrose solution onto 2 cm^3 of a 1.2 M-sucrose solution in a 5 cm^3 transparent polypropylene (ultracentrifuge) tube. It was found that these solutions layered more easily if their viscosity was increased by cooling to 0^oC. (Fig. 19).

A volume of the resuspended extract equivalent to not more than 0.2 g of the original brain tissue was layered on top of the 0.8 M-sucrose. The restriction of the extract to the equivalent weight of 0.2 g was necessary because, in experiments to resolve the problem of fixative precipitation, it was found that if the concentration of the extract was increased beyond this factor when using a total volume of 4 cm^3 of fixative, excess precipitate occurred. Providing this ratio, or less, of tissue extract to fixative was maintained, precipitation of the fixative did not occur vide infra.

The prepared density gradient tubes were placed in a 3 x 5 cm^3 swing out rotor and centrifuged in the ultracentrifuge at 30000 g for 120 min at 4^oC. After centrifugation the gradient tubes

showed three bands or layers (Fig. 19), a top band (A) consisting of small myelin fragments, the middle band (B) which contained mainly synaptosomes with some free mitochondria and other spherical cell organelles which were probably axon remnants (Fig. 25 and 26), and the third and last band (C), found at the base of the tube, which contained mitochondria.

The layer above the (B) band was carefully removed with a pasteur pipette and discarded. The (B) band was removed with a fresh pasteur pipette and transferred to a 5 cm^3 polypropylene ultracentrifuge tube. A minimum amount of fluid was removed with the (B) layer to prevent excess dilution of the fixative.

Estimation of the synaptosome density was achieved by counting the number present and calculating the final figure against a known number of latex particles added to the synaptosome extract. The latex particles used were 1.099 μm diameter ± 5.9 nm and were obtained in a 10% suspension from Serva, Feinbiochemica, Heidelberg (agent for The Don Chemical Company).

The concentration of latex particles is critical since too few could result in inaccurate estimations, similarly excess numbers, whilst not contributing to a statistical error, can result in inaccuracy due to possible coalescing. The optimum volume of latex to be added to a known concentration of brain homogenate therefore required to be determined. Serial 10 fold dilutions of the supplied 10% suspension in the range 10^{-1} - 10^{-3} and suspensions containing a total of from 10^7 - 10^{12} latex particles were prepared in addition to the use of varying volumes of neat latex suspension. These were added to equal volumes of the brain homogenate (Experiments 5 and 9). The results of these experiments showed that the optimum concentration of the supplied latex suspension was 0.1 - 0.15 cm^3 per 0.2 g of brain cortex homogenised. In addition to these preliminary experiments

alternative tagging materials were tried in case the latex proved difficult to identify. Such alternatives included suspension of known numbers of Escherichia coli, Saccharomyces and Serratia marcescens (Experiments 6, 7 and 8). Such alternatives proved unnecessary since the latex was easily recognised once the optimum concentrations had been determined.

Addition of the optimum volume of latex to the fraction (B) was followed by careful mixing with a pasteur pipette. Freshly prepared Luft's potassium permanganate fixative at 4°C was then added to just below the top of the polypropylene tube and the contents mixed. The relative proportions of brain homogenate approximately 0.6 cm^3 (equivalent to 0.2 g cortex), latex suspension 0.1 cm^3 and fixative approximately 4 cm^3, were critical. If the concentration of cortex exceeded 0.2 g by more than 50%, heavy precipitation occurred. This resulted in a final pellet which became easily fragmented in the subsequent processing. In addition the synaptosomes and latex particles were too widely distributed to provide sufficient numbers for counting purposes.

Fixation was carried out at 4°C for 45 minutes, after which the material was again mixed and then centrifuged at 5000 g for 10 min in the ultracentrifuge. The supernatant was discarded and the tube rinsed with chilled 50% alcohol in water.

3.4 PROCESSING FOR ELECTRON MICROSCOPY

The prepared pellet with a label showing a number code was dehydrated, in the polypropylene tube, through ascending grades of chilled alcohol commencing with 50% alcohol for 30 minutes, followed by 75% alcohol at 4°C overnight. The following day the pellet was processed through a single change of 90% alcohol at 4°C followed by 3 changes of 74 OP spirit, 60 min for each treatment. The last

FIGURE 18.

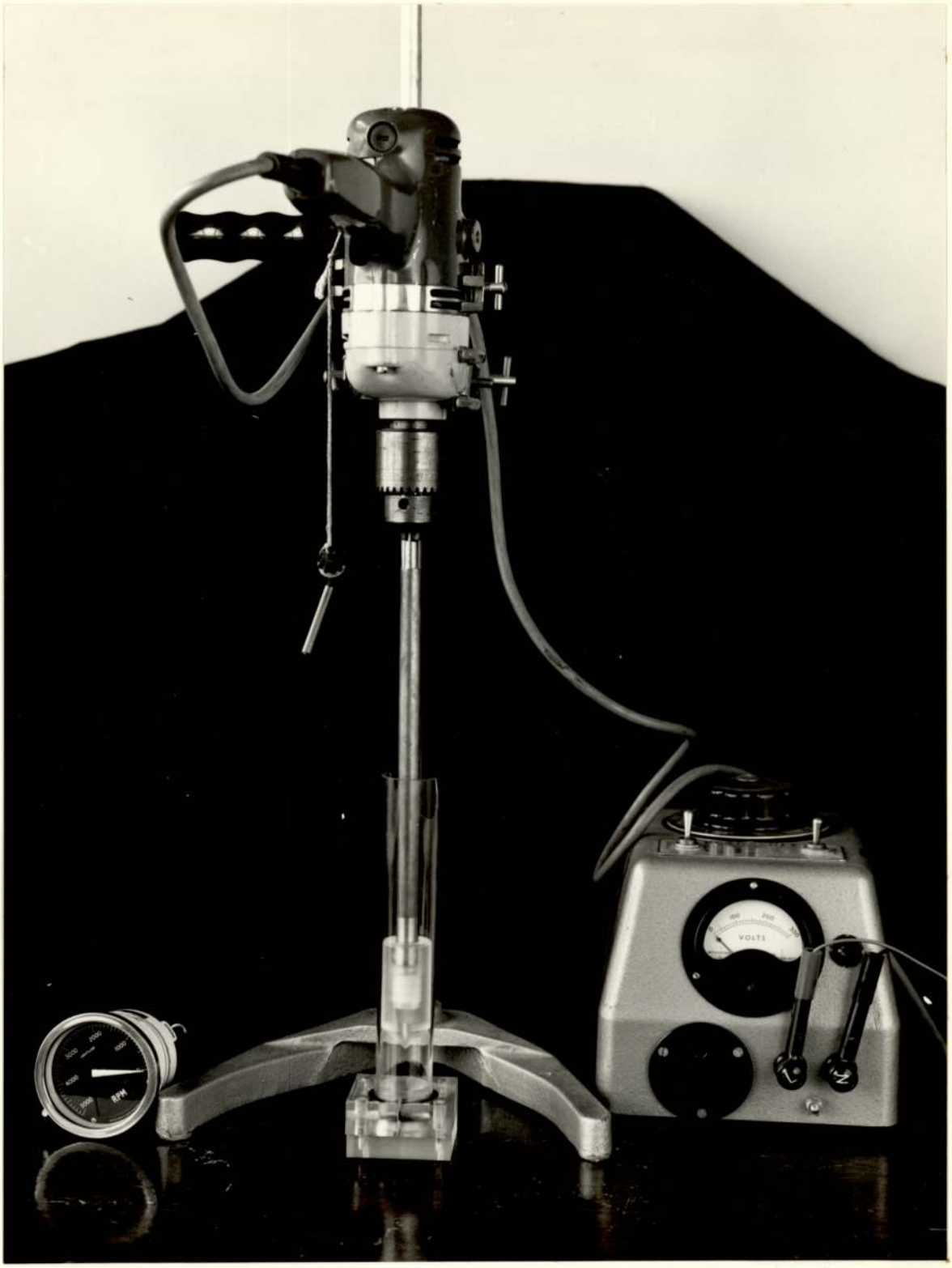


Fig. 18

FIGURE 19.

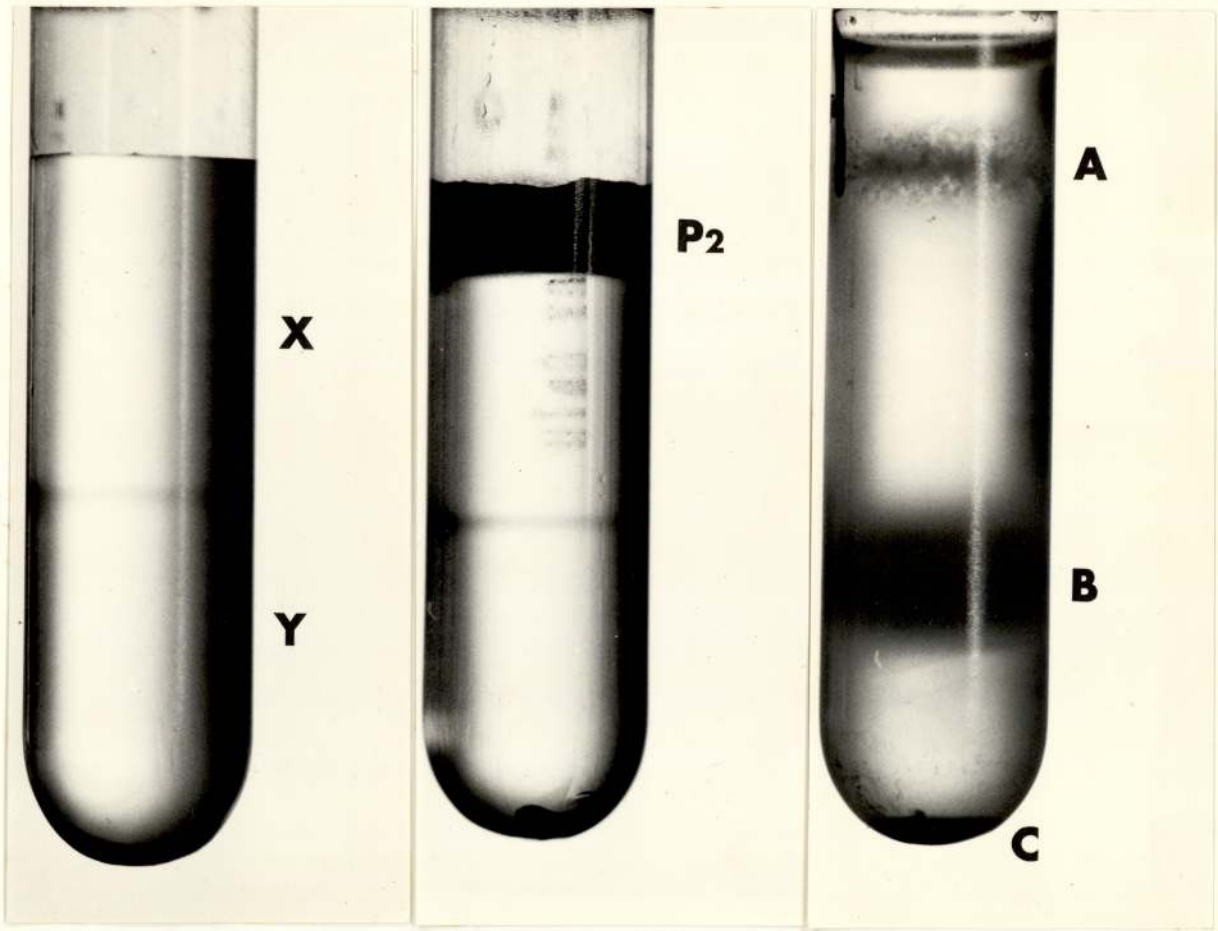


Fig. 19

alcohol treatment and subsequent processing was conducted at room temperature or at the temperature indicated.

To prepare the pellet for embedding it was transferred to a screw capped bijou bottle and treated with 2 changes of epoxypropane, 45 min duration for each treatment. This was followed by 3 hours in a mixture of epoxypropane and the embedding resin (Epon) in equal volumes.

The Epon resin was prepared according to Luft (1961) in which 7.0 cm^3 of Mixture A (Epon 812 62 cm^3 and dodecenyl succinic anhydride 100 cm^3) was added to 3 cm^3 of Mixture B (Epon 812 100 cm^3 and methyl nadic anhydride 89 cm^3) and 0.15 cm^3 of the accelerator benzyl dimethylamine. Stock mixtures of A and B were prepared in appropriate volumes and stored at -20°C.

Small gelatin capsules (Eli Lilly Co.) were filled with the resin mixture and supported in plasticine. The processed pellet was distributed, in fragments between 4 of the gelatin capsules, in such a way that samples were taken from various aspects of the pellet. The fragments, which were placed on the top of the resin, sedimented to the bottom of the capsule during embedding which was carried out at 56°C for 3 days.

In a number of experiments variation in the hardness of the blocks was encountered. To minimise this, careful attention was paid to the preparation of the resin, particularly the mixing prior to embedding. In addition, removal of the gelatin capsule, by washing in warm water, was omitted.

3.41 Section Cutting

Blocks were trimmed with a razor blade to form a pyramid shape with the tissue fragment located in the cut off apex. The blocks and subsequent sections in addition to the experimental number also received a colour code. The code was designed to eliminate unintentional sub-conscious bias during the counting of the

synaptosomes.

For section cutting glass knives were prepared with an LKB Type 7801A glass knife making machine. Troughs to contain the floating out mixture of 1% alcohol in water were manufactured from plastic insulation tape and sealed with dental impression wax. The blocks were rough cut, on the LKB ultramicrotome (Ultrotome Type 4801A), to provide a section face of 1 - 2 mm square. Sections were initially cut with an interference colour of gold, silver and grey. Such sections are reputed to be respectively 90 - 150, 60 - 90, and < 60 nm thick. Examination of sections, from early experiments, revealed that section thickness was important in that sections of a definite gold colour were unsuitable because their thickness resulted in densely packed synaptosomes, and other cell particles, making identification of the latex particles and synaptosomes difficult. Sections of a silver colour, whilst being ideal for high resolution, reduced the number of potentially countable particles. As a consequence of these findings, sections of a pale gold colour (approximately 100 nm) were cut from all subsequent blocks since such sections were found to produce a maximum countable number of particles coupled with a resolution which made synaptosomes and latex particles identifiable.

Sections were floated out, and those of the required colour infringence were collected onto carbon coated grids. These were prepared in an AEI vacuum unit for carbon coating. However, following breakdown of this coating machine, grids coated with formvar were tried. Whilst these proved to be acceptable, in preliminary tests to determine their suitability, non-coated grids were also tried. Providing grids of a 200 - 400 mesh size were used loss of sections was not noticed with the non-coated grids.

3.42 Section Staining

Sections were stained with Millonig's (1961) lead solution which was prepared by the addition of 0.5 cm^3 of Millonig's Sol. II (20 g sodium hydroxide and 1 g potassium sodium tartrate in 50 cm^3 distilled water) to 2.5 cm^3 of a 20% aqueous solution of lead acetate. The mixture was then diluted with glass distilled water to a total volume of 15 cm^3 and filtered through Whatman No. 50 filter paper.

Staining was accomplished by inverting the grids onto the surface of the staining solution which had been placed in the bottom of a petri dish. Division of the petri dish into 8 segments, with a felt pen, allowed sections from 8 blocks to be stained simultaneously. Following staining, the sections were washed by dipping the grids several times in three successive changes of glass distilled water and finally dried on Whatman No. 50 filter paper. To minimise lead precipitation all reagents were freshly prepared, in minimum quantities, for each batch of staining. Particular attention was also paid to the cleanliness of the apparatus, e.g. petri dishes, pipettes, beakers, etc. Such measures reduced the problem of precipitation which was experienced in earlier experiments.

3.5 ELECTRON MICROSCOPY

Sections were examined in an AEI (EM6B) electron microscope by firstly scanning the grid at low magnification to locate suitable sectionsand then observing at 15000X and 20000X magnification which with the binocular microscope attachment gave a total magnification of 150000 - 200000X. Sections were examined for the presence of suitably stained synaptosomes, precipitate free, and in which the distribution of the synaptosomes and latex would allow satisfactory counting. The latter aspect, as previously mentioned, related to the

thickness of the section.

Suitable sections were photographed, at 7500X magnification, on four Ilford EM5 $3\frac{1}{4}$ x $3\frac{1}{4}$ photographic plates in such a manner that the photographed areas did not overlap. The areas were random selected and included the outer and inner regions.

The plates were developed in Ilford ID2 (commercially supplied) and fixed in Ilford Ilfofix (commercially supplied). Prints were produced on Ilford Ilfobrom (IB21P) No. 3 or No. 1 glossy single weight paper at an enlargement size of twice that of the plate thus giving a final magnification of 15000X. The papers were developed in Kodak D163 (2.2 g metol, 75 g sodium sulphite anhydrous, 17 g hydroquinone, 65 g carbonate anhydrous, 2.8 g potassium bromide, 1000 cm^3 water, and diluted one in four for use), acid stopped in 1% acetic acid, and fixed in an acid hardening bath (Jacobson and Mannheim, 1969) consisting of 10 parts of: 80 g sodium sulphite anhydrous, 80 g potassium alum, 80 cm^3 glacial acetic acid, 1000 cm^3 of distilled water and 1 part of: 20 g sodium thiosulphate, 1.5 g potassium metabisulphite, 100 cm^3 distilled water.

The total number of photographs to be examined per brain homogenate was sixteen (4 blocks : 4 photographs from each block).

3.6 ESTIMATION OF NUMBERS OF SYNAPTOSOMES.

Identification of synaptosomes was based on the following criteria; an intact single membrane, spherical in structure, containing not less than two recognisable synaptic vesicles. In some instances mitochondria were also observed in the synaptosome in which case a separate score for this type of synaptosome was recorded. The reason for the separate score was in case thyroidectomy affected the mitochondria of cerebral cortex synapses. For the estimation of total numbers of synaptosomes both types were combined.

The structural form of the membrane was important since this was often the only way possible to distinguish synaptosomes from what were assumed to be mitochondria.

Latex particles were easily detectable by their even staining density and shape. The effect of section cutting was to change the appearance of the latex particle from that of a sphere to an oval shape. This distortion was due to the compression of the block by the knife. Since all particles were affected in the same manner their long axis was always in the same direction. Thus irrespective of the plane in which they were cut, the consequential variation in size did not present problems in recognition, since this depended less on size and more on shape and direction of the long axis.

Whilst identification did not present difficulties, evidence of coalescing was observed. Such clumping resulted in latex masses which could not be used for comparative estimations of latex and synaptosomes. careful attention was therefore paid to mixing of the stock solution of latex before adding to each brain homogenate. Satisfactory mixing was accomplished by placing on a Matburn Mixure for 2 hours at $4^{o}C$ followed by vigorous hand shaking prior to dispensing. Calculation of the number of latex particles per cm^3 was based on the following formulae which was derived as follows:

$$\text{Volume of one latex particle} = \frac{4}{3}\pi r^3 = \frac{4}{3}\pi\left(\frac{d}{2}\right)^3$$

$$= \pi d^3/6$$

$$d = 1.099\ \mu$$

$$\therefore \text{ volume of one latex particle} = \frac{\pi \times (1.099\ \mu)^3}{6}$$

Now 1 μ = 10^{-4} cm

$\therefore$ volume of one latex particle = $\frac{\Pi \times (1.099 \times 10^{-4})^3 \text{ cm}^3}{6}$

$= \frac{\Pi \times (1.099)^3 \times 10^{-12}}{6}$

$\therefore$ $\frac{6 \times 10^{12}}{(1.099)^3 \Pi}$ particles occupy 1 cm^3

$\therefore$ A 10% suspension will contain $\frac{6 \times 10^{11}}{(1.099)^3 \Pi}$ particles/cm^3

$= \frac{1.910 \times 10^{11}}{1.099^3}$

$\therefore$ Number of particles/cm^3 = $\underline{1.738 \times 10^{11}}$

Calculation of the number of synaptosomes cm^{-3} was arrived at as follows:

$$\left\{ \left(\left[(1.738 \times 10^{11}) \times K \right] \times \frac{s}{L} \right) \times \frac{1}{t} \right\} \quad cm^{-3}$$

where K = volume of latex suspension added (cm^3)

s = number of synaptosomes counted

L = " of latex particles counted

t = weight of tissue processed (g)

The photographic prints were examined with an illuminated magnifying lens which increased the 15000X magnification sufficiently to make identification of the synaptosomes possible. The relatively large area sampled with the 7500X negative provided reasonable numbers of synaptosomes and latex per photograph. Actual numbers varied with the samples examined but normally ranged from 10 - 100 for both latex and synaptosomes.

Since the identification of synaptosomes, and their differentiation from other cell structures, e.g. mitochondria, together with recognition

of the latex particles required experience, photographs were examined in batches. In addition to marking the synaptosomes and latex with a colour code, to prevent counting the same structure twice, all photographs were read on two separate occasions before the final figure was obtained for the calculations.

3.7 ALTERNATIVE FIXATIVES AND STAINING METHODS

3.71 Gluteraldehyde and Osmic Acid Fixation

Although latex particles presented little difficulty in identification, synaptosomes could be confused with mitochondria, and in instances where the synaptosomes were less obvious, because of cutting through at a small plane, they could be mistaken for fragments of axons and vice versa.

In an attempt to overcome these problems and those encountered in the earlier experimentsdue to precipitation of the potassium permanganate fixative a number of alternative methods were tried. Gluteraldehyde and osmium tetroxide fixation was used in duplicated samples from experiments 29, 30, 31 and 34; the other portion of the cortex homogenate was fixed in potassium permanganate as previously described. Following differential centrifugation in sucrose gradients the fraction B obtained was suspended in approximately 4 cm^3 of 5% gluteraldehyde in 0.3 M-phosphate buffer pH 7.4 (Aghajanian and Bloom, 1967) at $4^{o}C$ for 45 - 120 min. Latex was added in concentrations previously described. The suspension of synaptosomes in gluteraldehyde fixative was then centrifuged at 5000 g for 10 min. The gluteraldehyde solution was removed and the tube rinsed with 13% sucrose in 0.3-phosphate buffer pH 7.4. The pellet was left in the sucrose-phosphate solution overnight. The following morning the sucrose solution was replaced with fresh reagent and left for 30 min. The pellet

was then removed from the polypropylene centrifuge tube, and transferred to a screw capped bijou bottle containing Caulfield's veronal buffered osmium tetroxide sucrose fixative (Kay, 1967). This secondary fixation was carried out for varying periods of time from 1½ to 6 hours and was followed by washing in two changes of 0.3 M-phosphate buffer pH 7.4 for 30 min per change. Dehydration and processing for section cutting followed the same procedure as described previously. Sections were examined unstained and following treatment with freshly prepared 2% aqueous uranyl acetate. In addition sections were stained with Millonig's lead solution.

3.72 Selective Staining of Synaptic Vesicles

Following the report of Iraldi and Suburo (1970) that synaptic vesicles can be stained by the Champy-Maillet technique homogenates of brain cortex were subjected to the following procedures (Experiment 45). Three adult rats, 41 days old, were sacrificed, two normal animals acted as untreated controls whilst the third had been thyroidectomised at birth with ^{131}I. Their brains were removed and cortex homogenates prepared using the techniques previously described. Following separation of the B fraction in a sucrose gradient the synaptosome suspensions were divided as follows:

Control (A) - one half fixed in potassium permanganate (Clementi et al, 1966), the other in gluteraldehyde and osmium tetroxide as previously described;

Control (B) - one half fixed for 60 min in 4 cm^3 of fresh zinc iodide and osmium tetroxide solution prepared as follows; 1 cm^3 of 2% aqueous osmium tetroxide added to 3 cm^3 of a solution of zinc iodide (3 g of zinc powder were mixed with 1.25 g of bisublimated iodine in a flask, 50 cm^3 of distilled water was

then added and the mixture shaken for the first 5 minutes and then intermittently for 30 min (the supernatant was removed and filtered). The other half of the synaptosome fraction was fixed for 60 min in a mixture of 3 cm^3 of 3% aqueous potassium iodide and 1 cm^3 of 2% aqueous osmium tetroxide.

The synaptosome fraction from the thyroidectomised animals was divided into three fractions each being fixed in one of the three fixatives used for the control animals, namely, potassium permanganate, zinc iodide-osmium tetroxide, and potassium iodide-osmium tetroxide. To all samples, latex was added in proportion to the concentration of cortex.

3.8 DETERMINATION OF THE ATHYROID STATE

In view of the slight differences in the effect of ^{131}I on the gross development of the animals vide infra (Table 2, Fig. 22) thyroidectomised rats were examined to determine the effect of ^{131}I on the thyroid.

Following removal of the brain for estimations of synaptosomes an incision above the oesophagus was made through the skin and the area exposed to reveal and locate the thyroid. No macroscopical evidence of the presence of thyroid tissue was found. As a consequence a section of the oesophagus including the pharynx was removed and fixed in 10% phosphate buffered formalin. The tissue was then processed, in a histokine, through ascending grades of alcohol, cleared in chloroform and embedded in paraffin wax. Sections 5 μm thick were cut on a Leitz sledge microtome and transferred to glass slides.

Sections were dewaxed with xylene and transferred via alcohol to water. They were then stained with Erhlich's haematoxylin and 1% aqueous eosin. In the first set of sections examined thyroid tissue was not detected. As a consequence tissue taken from other animals was orientated to enable longitudinal sections to be cut. The

blocks were then cut at different levels and sample sections taken and stained by the same method.

3.9 PREPARATION OF BRAIN TISSUE FOR FIGURE 4.

An adult mouse was anaesthetised by the intraperitonal injection of Nembutol. The animal was stretched out on a board and the head moistened with alcohol. A mid-line incision was made through the skin, with scissors, above the sagittal suture extending from the neck to the snout. Liquid nitrogen was poured through a plastic funnel onto the exposed surface of the skull until the bone was hard frozen. The skull bone was removed with scissors exposing the brain. A 0.5 mm horizontal slice was removed from the cerebral cortex and transferred to 2% gluteraldehyde in 0.3 M-phosphate buffer pH 7.4 for 2 hours. The tissue was cut into small fragments and washed for 2 hours in three successive changes of the 0.3 M-phosphate buffer on a Matburn mixer. Secondary fixation was carried out in Caulfield's buffered osmium tetroxide solution for 1½ hours. The tissue was then washed three times in 0.3 M-phosphate buffer for 2 hours. The tissue was then dehydrated in alcohol and embedded in Epon as previously described. The block was prepared and silver sections cut on the LKB ultramicrotome. These were transferred to grids and stained with Reynold's lead citrate (Kay, 1967) for 20 minutes. After rinsing in distilled water the sections were then treated with 0.5% uranyl acetate for 30 minutes, and finally rinsed in distilled water and dried. The manipulative techniques were the same as those previously described for staining sections by Millonig's method (section 3.42).

4. RESULTS

4.1 AFFECT OF THYROIDECTOMY ON GROSS BODY DEVELOPMENT

The administration of ^{131}I to the young rat resulted in levels of activity at the body surface which exceeded the maximum measurable with the available monitor (Fig. 16). The radioactivity recorded from the control animals also showed an increase which reached a maximum by the fourth day and persisted to the sixth, when in parallel with the test animal, it started to decline (Fig. 20). The demonstration of radioactivity on the control animals is presumably due to contamination with excreta from the test animal. It was assumed that the surface contamination, of the control animals, had no effect on their development since their growth followed the normal pattern in contrast to the growth of the test animals.

Table 2 (Appendix) contains the body weight of rats either thyroidectomised by operation or by the administration of ^{131}I together with those of normal and control animals. When the average body weights are plotted against age in days the effect of thyroidectomy (whether produced surgically or induced by ^{131}I) is immediately apparent (Figs. 21, 22 and 23). Figure 21 shows two rats, both of the same age, one is a control animal having been injected with phosphate buffered saline and the other has been rendered hypothyroid by the administration of ^{131}I. Figure 22 shows two groups of animals, both groups contain control and hypothyroid (^{131}I) animals. The weight of the two types of animals, e.g. control and hypothyroid, have been averaged and plotted against age. Whilst both groups exhibit the same disproportionate rate of growth, between the controls and the hypothyroid animals, it can be noted that not all animals would appear to respond equally to the administration of ^{131}I. As a consequence animals were examined macroscopically, and in some instances microscopically, to ascertain

the state of the thyroid gland. Whilst in the control animals the thyroid gland was readily detectable, in the test animals this was not the case. At no time was it possible to detect the thyroid gland in the thyroidectomised (^{131}I) animal.

Table 2 (Appendix) contains the weights of: the brain, the cerebellum and the cerebral cortex, for both control and test animals. Additional information to be found in this table includes: age of the animals, type of experiment, and the number of synaptosomes estimated per gram of cerebral cortex.

If the brain weights of the three types of animals, normal (control), thyroidectomised by surgical removal of the thyroid and by the administration of ^{131}I, are plotted against age (Fig. 24), the results show that the brain is also retarded in growth by the suppression of the thyroid. However, examination of Fig. 24 shows that at 48 days of age the maximum brain weight achieved, in normal animals and those thyroidectomised, was approximately 1.4 g. However, it would appear from Fig. 24 that in the earlier stages, e.g. before 40 days of age, that brain growth in irradiated animals is retarded. Furthermore, the effect of irradiation is variable inasmuch that both brain weights and body weights for animals of a similar age are not always the same (Exp. 61 and 62, Table 2). Table 2 also provides data which indicates that the brain in normal animals continues to increase to 1.6 - 2 g. The latter weight is exceptional since the majority of brain weights above 70 days of age are in the order of 1.6 - 1.8 g. Only one result is available from an irradiated animal (Exp. 47, Table 2) and this indicates considerable suppression of growth since the weight of the brain was only 0.9 g. Such a result cannot be ignored and it must be assumed that this is a further example of variation in the effect of the administration of ^{131}I.

In the animals that had been thyroidectomised (surgically) at 21

days of age (Fig. 24) the brain weight was again in the order of 1.4 g at 48 days of age. The brains examined in this group of animals also achieved weights close to 2 g, e.g. 1.8 at 112 days. Comparison of body weights (Fig. 23), at 48 days of age, shows a considerable difference between the two types of animals, 160 g (thyroidectomised), 260 g (sham operated). On a comparable basis the brain weights theoretically should be 1.4 g (thyroidectomised) and 2.4 g (control) or 0.8 g and 1.4 g. It must therefore be presumed that thyroidectomy at 21 days of age has less effect on brain development than body development.

Similarly, thyroidectomy at birth (^{131}I) would seem to have less effect on the brain than on the body although there is evidence of initial brain retardation. The exceptional result of Exp. 47 (Table 2) would suggest that thyroidectomy (^{131}I) at birth may have a profound effect, although it should be noted that the monitoring of the body weight of this rat showed that the animals having achieved a weight peak of 30 g at 35 days failed to show any further increase and lost weight. Comparison of the body weight (29 g) and brain weight (0.9 g) of this animal with that of a similar aged animal (Exp. 77; body weight 324 g and brain weight 1.6 g) shows that again the brain is markedly less affected than the body.

It must be presumed that thyroidectomy at 21 days of age has less effect on brain development than on body development. Furthermore, comparison of brain and body weights of animals at 21 days of age and older indicates that at 21 days of age the brain is structurally at a more advanced stage than the body. This does not mean that developmental aspects, which take place during the post 21 day period, perhaps the final stages of synaptogenesis could not be inhibited vide infra.

As previously indicated although there would appear to be retardation of brain development following thyroidectomy at birth, the brains of these animals can achieve a weight comparable to those of

normal animals despite considerable reduction in body weight. However, as already commented upon, irradiated animals can show a reduced brain weight indicating a retarded development. It is therefore of interest to see if these variations are reflected in the number of synaptosomes vide infra.

These findings on the development of brain and body weights are supported by similar data reported by other workers (Eayrs, 1971).

FIGURE 20.

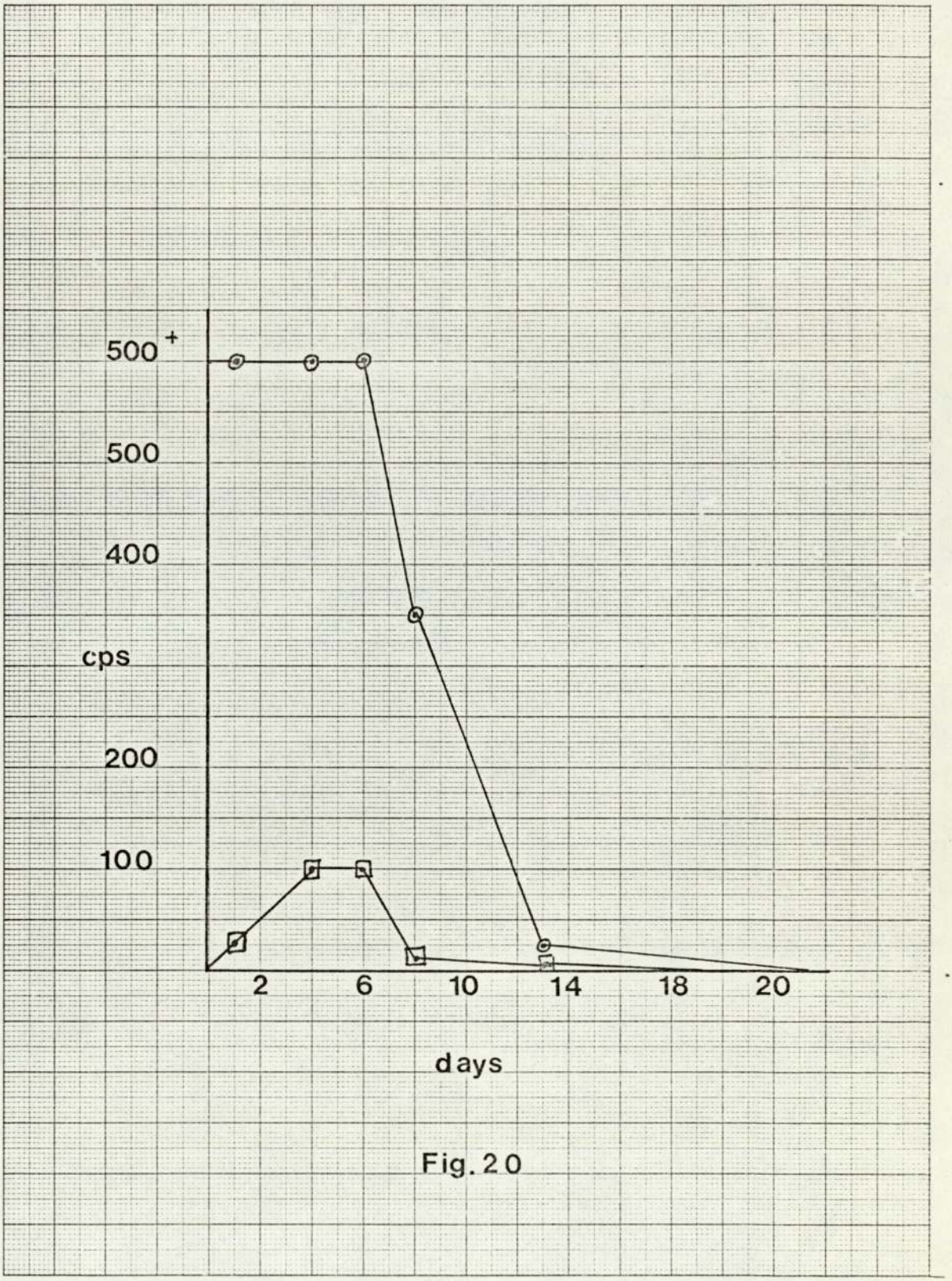


Fig. 20

FIGURE 21.

Fig. 21

FIGURE 22.

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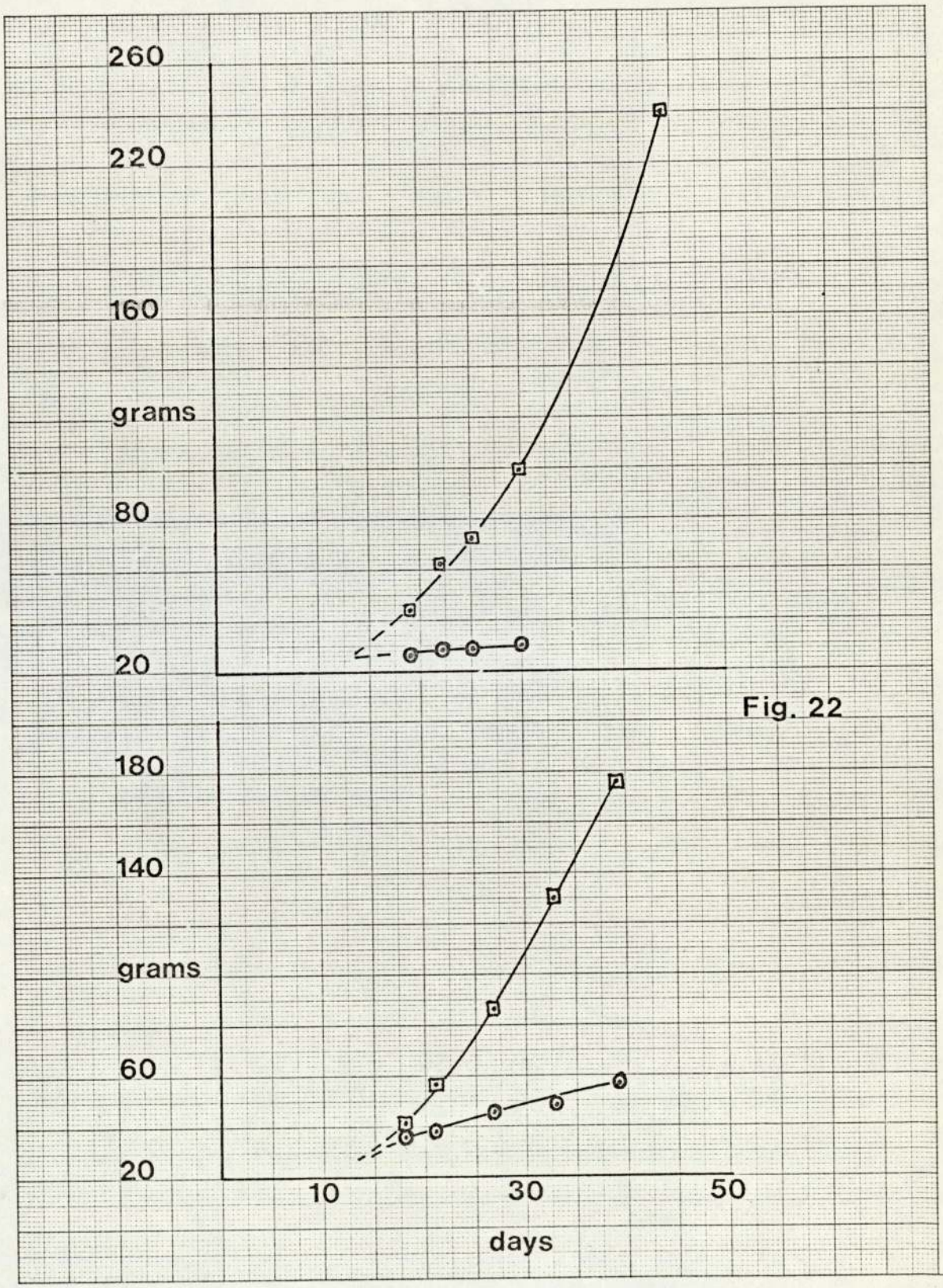

Fig. 22

FIGURE 23.

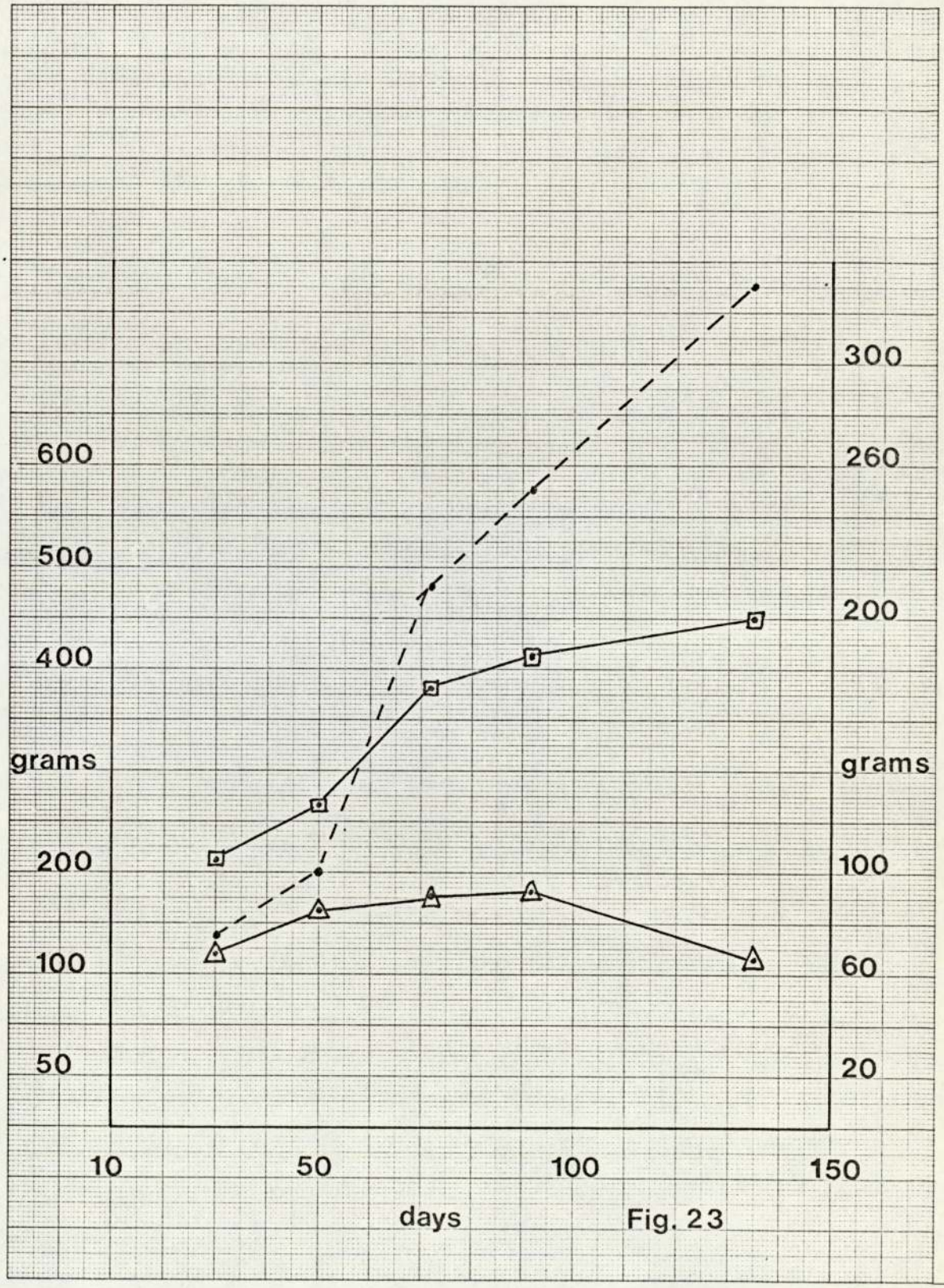

Fig. 23

FIGURE 24.

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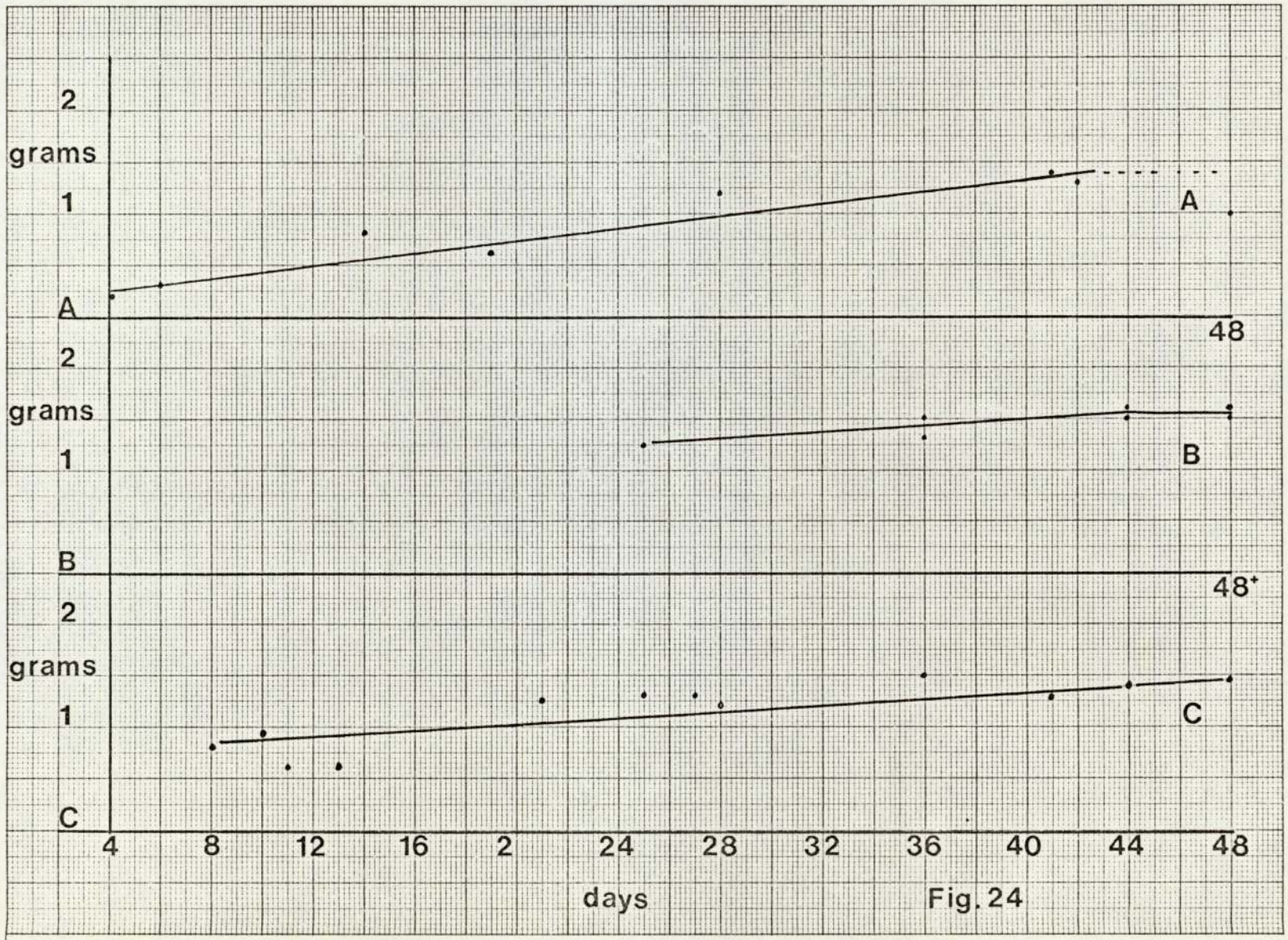


Fig. 24

4.2 PREPARATION OF SYNAPTOSOME EXTRACTS

4.21 Identification of Synaptosomes

Homogenisation of brain tissue and the strict following of the technical procedure outlined in Section 3 (Materials and Methods) will result in clearly identifiable synaptosomes. Figures 25 and 26 show that the synaptosomes (S) vary in size. This is due both to an actual variation in size and also to sections being cut at different levels. The synaptosomes can be seen to contain mitochondra (m) and synaptic vesicles (v). Free mitochondria may be observed and in some instances the mitochondrial cristae resemble synaptic vesicles. However, differentiation between mitochondria and synaptosomes can be made on the grounds that the former contain a double membrane whilst the latter possess only a single membrane. At least four of the synaptosomes in Fig. 25 contain two mitochondria, examples of these are indicated with an X. Identification of synaptosomes is based on the following criteria: spherical-ellipsoidal shape, single boundary membrane, inclusion of two or more spherical bodies resembling synaptic vesicles. Thus those structures indicated with an (e) in Fig. 26 would not be included in a count, whilst the structures (i) in Figs. 25 and 26 would be included.

FIGURES 25 and 26

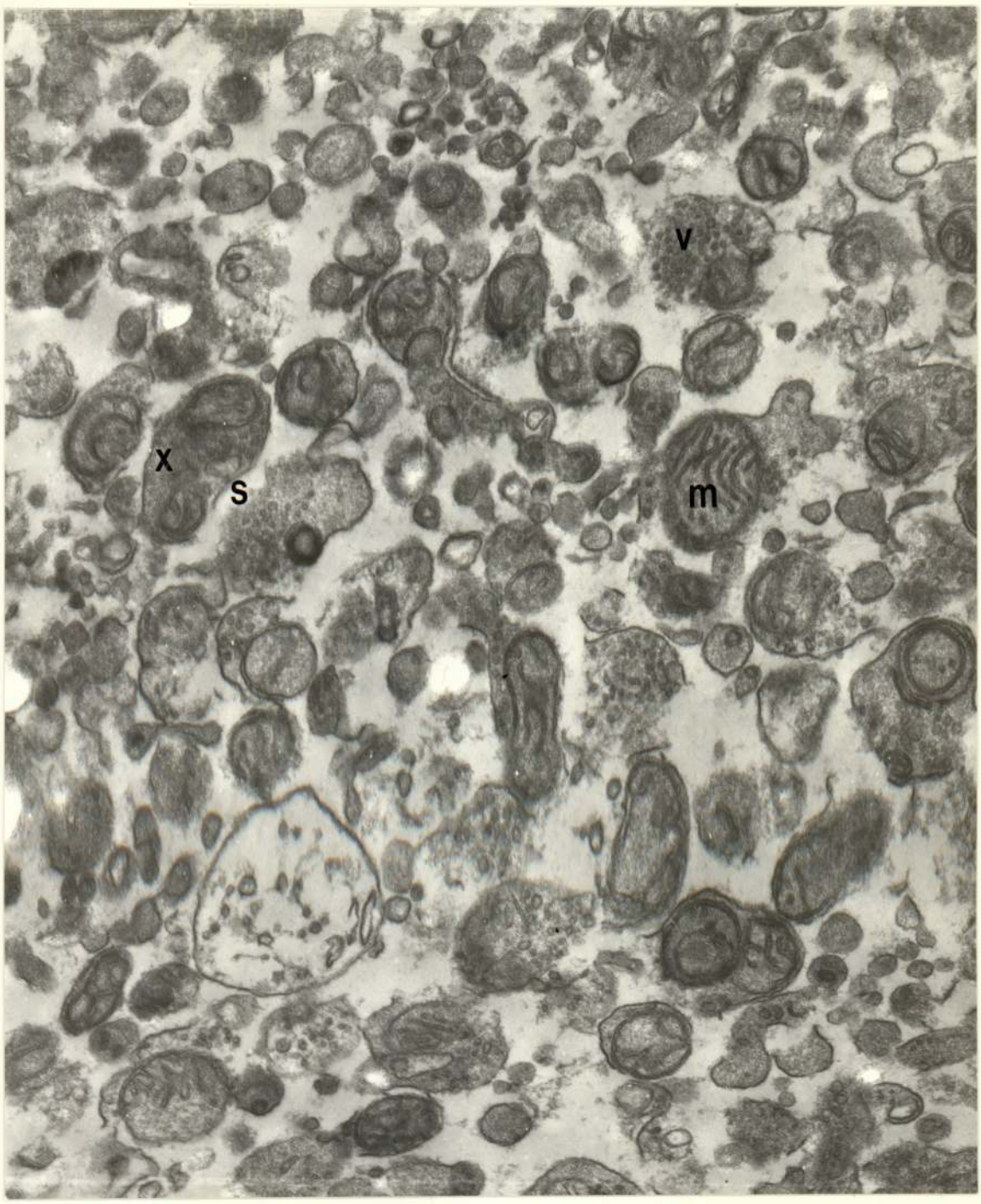


Fig. 25

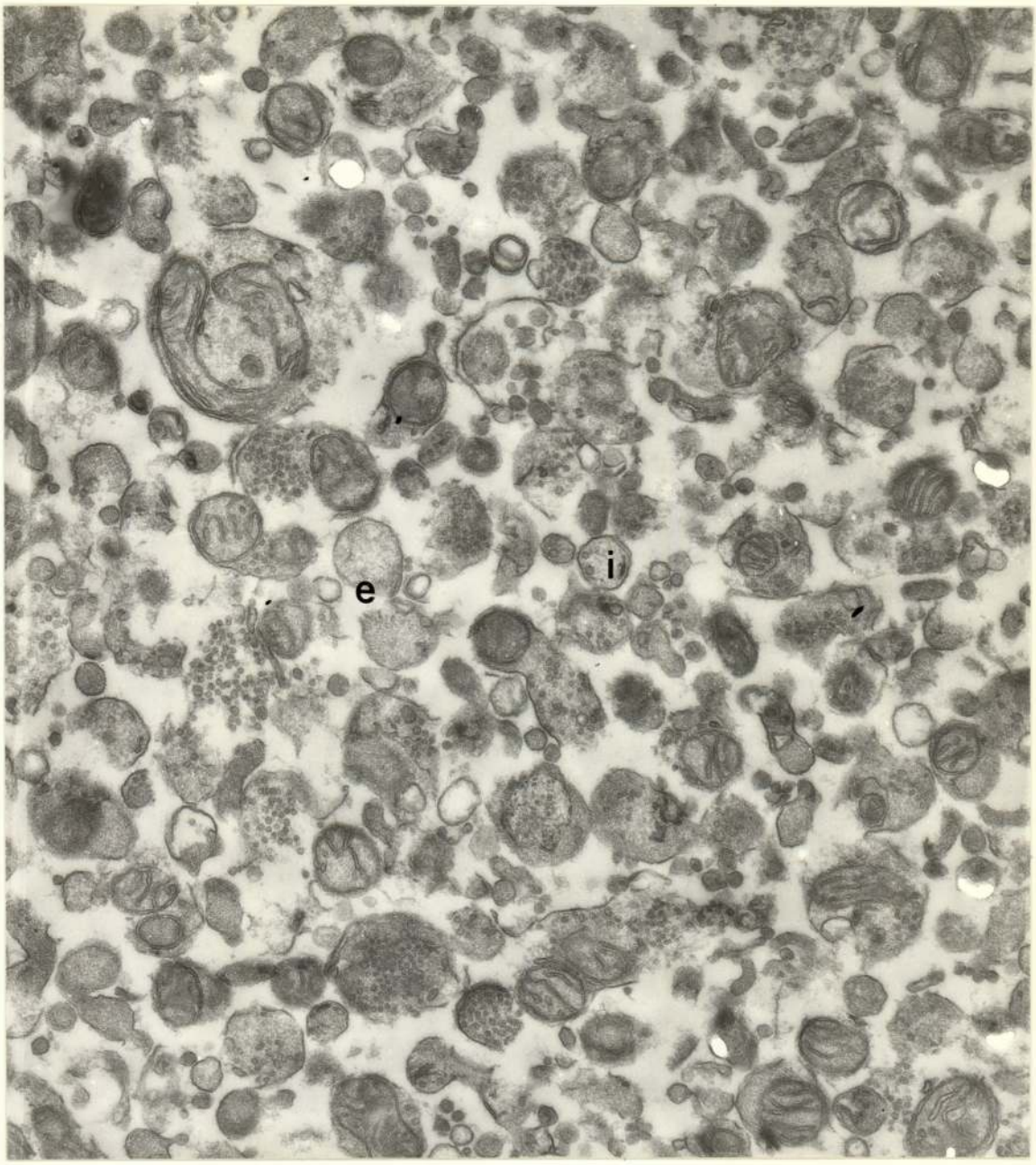


Fig. 26

4.22 Homogenisation

The technique for obtaining synaptosomes has a number of difficulties. The most important of these include: tissue homogenisation, the concentration of and occasional coalescence of the latex particles, the distribution and fixation of synaptosomes, and their identification (vide supra Sections 2, 3 and 4.21).

Taking these in the order indicated, homogenisation can, as Whittaker and Dowe (1965) have shown, affect the efficiency of synaptosome separation. Inadequate homogenisation results in the presence of axon and myelin fragments combining with the synaptosomes. This is clearly seen in Fig. 27 where it can be noted that the synpatosomes are surrounded by axon and myelin fragements (my). As would be expected, excessive homogenisation can result in disruption of many of the synaptosomes (Fig. 28). In this photograph whilst some structures can be identified as synaptosomes it is clear from the fragmented appearance of the material that this preparation is not suitable for estimating numbers of synaptosomes.

Whilst accepting the findings of Whittaker and Dowe that the Aldridge homogeniser (Figs. 12 and 18) was superior to the modified Dounce type stainless steel pestle and glass mortar, and to the Emanue-Chaikoff homogeniser, it was found in this research that the Thomas teflon glass homogeniser produced a satisfactory homogenate providing that the procedure was standardised in accordance with the methodology recommended in the Materials and Methods Section 3. The basis of this assessment, which was arrived at in the early experiments designed to develop the technique and evaluate its precision, was the appearance of the synaptosomes (Figs. 25 and 26) and the relatively close results obtained as shown in Experiments 14, 16, 18 and 19 (Table 3: Appendix). However as can be seen

with Experiment 30, some results can vary and whilst it can be argued that closer results would be desirable, this is not possible in view of the techniques involved.

4.23 Latex Distribution

Mention has been made, in the Materials and Methods Section 3, of experiments designed to determine the optimum volume of latex particle suspension to add to the brain extract. Figure 29 clearly shows the even distribution of latex throughout the extract. Note the oval shape of the latex due to pressure created in cutting through the Epon block. Variation in size of the latex is due to the particles being cut at different levels. A particular problem associated with the latex was its tendency to coalesce resulting in irregular shaped masses which varied in size. Whilst these could be easily recognised and hence not confused with synaptosomes, it did mean that the number of latex particles could not be determined and as a consequence synaptic densities could not be estimated. Fig. 28 provides an example of mild coalescing whilst Figs. 30 and 31 show excessive coalescing. Needless to say all three preparations were unsuitable for the determination of synaptosome numbers.

FIGURE 27

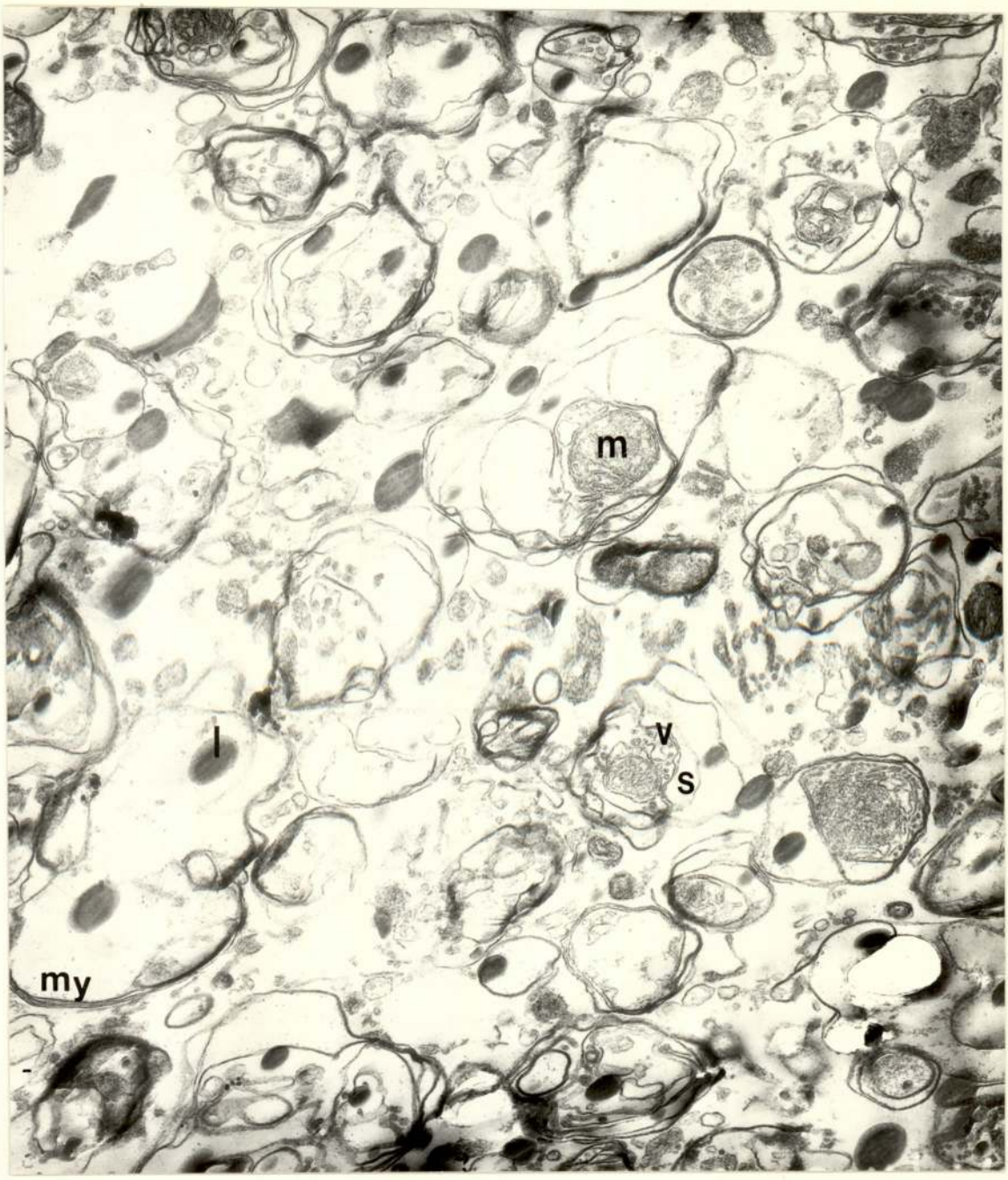


Fig.27

FIGURE 28.

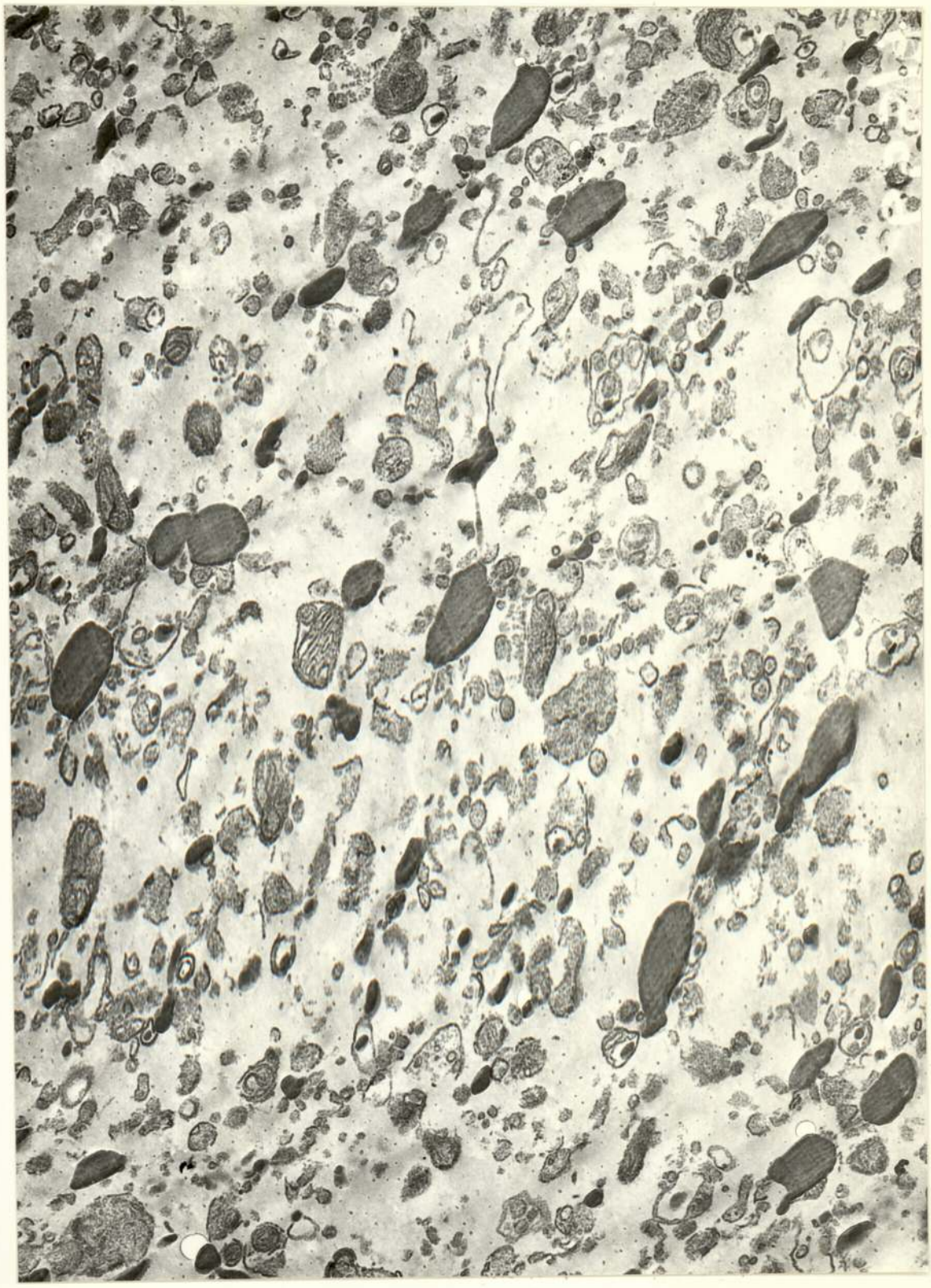


Fig.28

FIGURES 29, 30 and 31

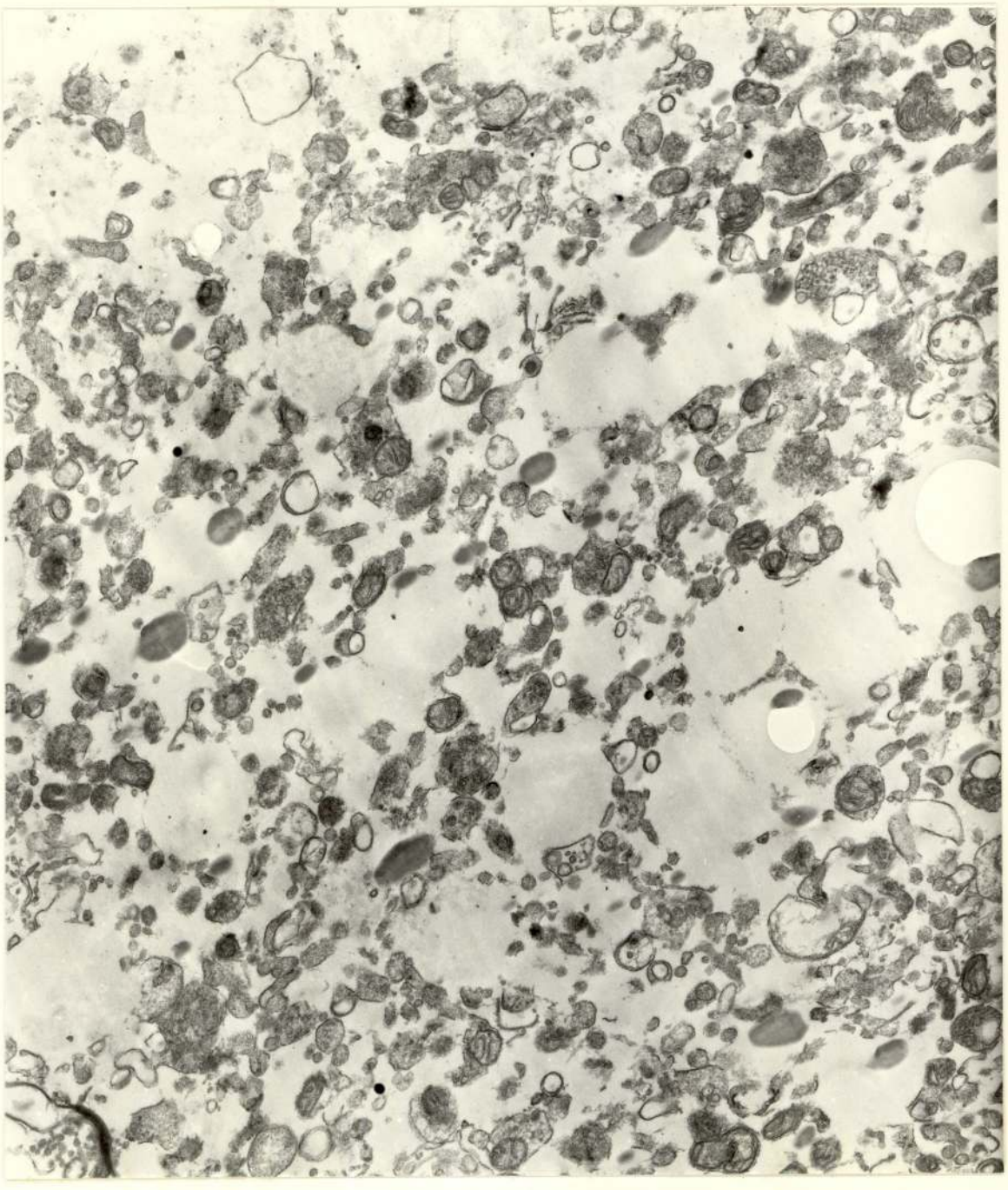


Fig. 29

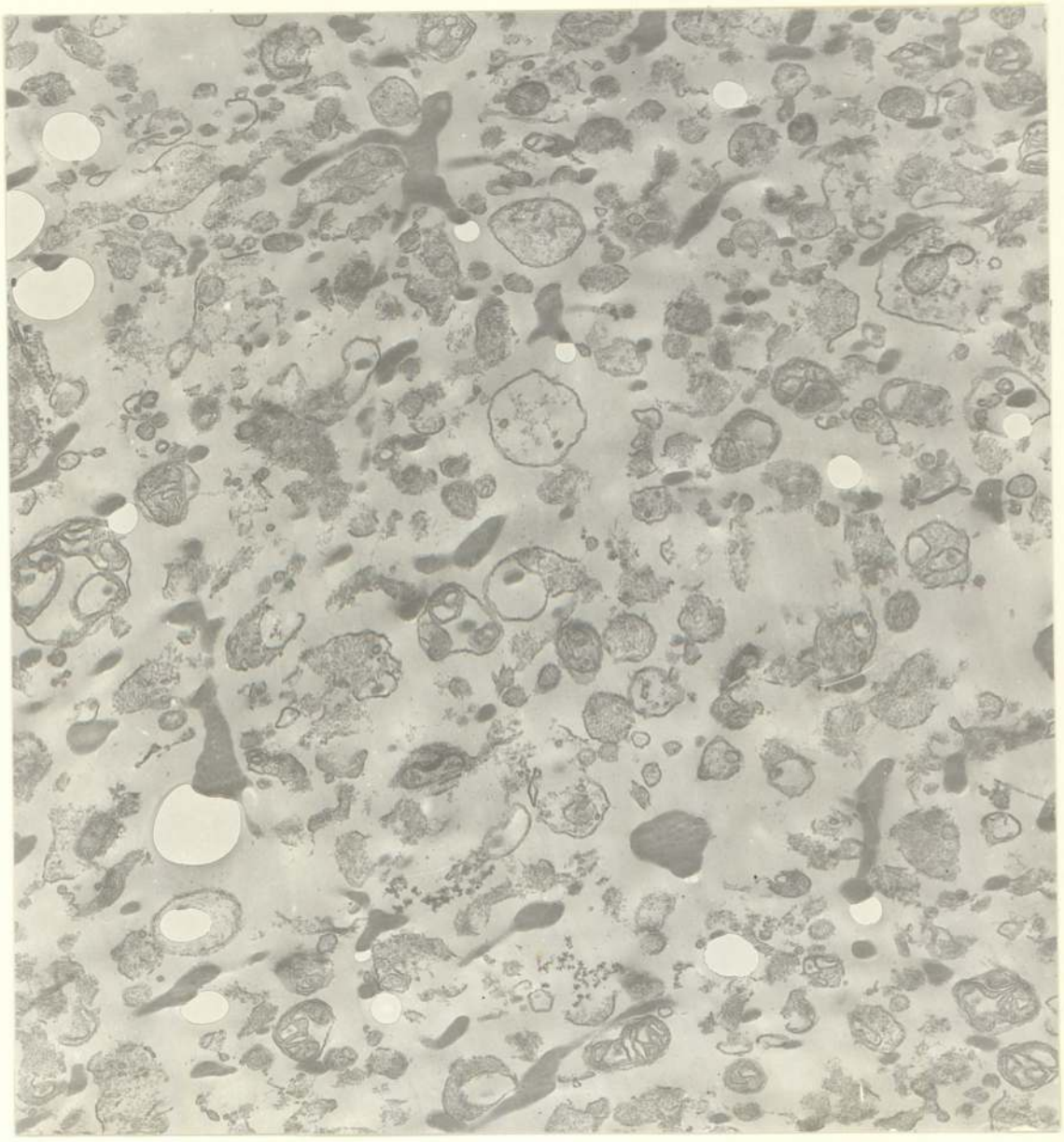


Fig. 30

Fig.31

4.24 Alternative Fixatives

It was found that when potassium permanganate was used as a fixative a precipitate occurred which adversely affected the preparations. As a consequence, proceeding concurrently with investigations into this problem, a number of alternative fixatives were tried. These included gluteraldehyde and osmic acid which, on occasions, appeared to be satisfactory (Fig. 32). However, more frequently the results were such that synaptosomes could not be identified. Since the technique for homogenisation had been standardised and proven satisfactory, it was concluded that the failure to obtain identifiable material was because of inadequate fixation.

In addition zinc and other metallic iodides together with osmic acid were tried based on de Iraldi and Suburo's (1970) technique for synaptic vesicles (Sections 2.145 and 3.7). Of the iodides only zinc iodide produced a block which could be cut, and where there was some evidence of fixation (Fig. 33). However, the material was so poorly demonstrated that it could not be identified.

In view of the disappointing results obtained with the gluteraldehyde, osmic acid, and iodide fixatives, and the eventual solution of the potassium permanganate problem, referred to in Section 3.3, it was decided that there was little purpose in pursuing this aspect of the experimental work. The main purpose of testing other fixatives was in case the problem of precipitation proved unsolvable. There was no question of faulting the potassium permanganate for its ability to fix the tissue extract and allow adequate staining. On the contrary, as will be observed from Figs. 25 and 26, this fixative is excellent and has

the advantage, according to Clementi *et al*, of being reduced to manganese oxide which acts as an excipient thus preventing the synaptosomes and other particles becoming tightly packed. This feature allows easier counting.

FIGURES 32 AND 33.

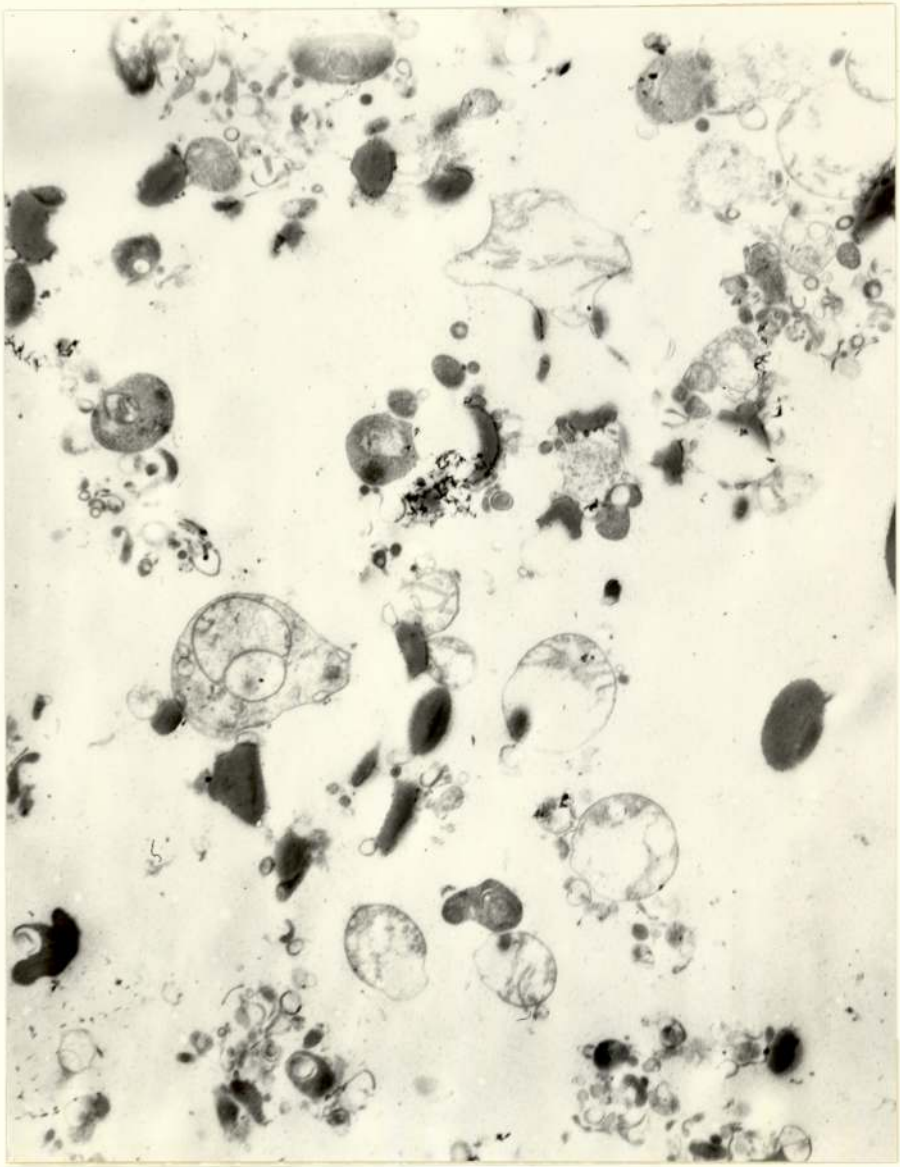


Fig. 32

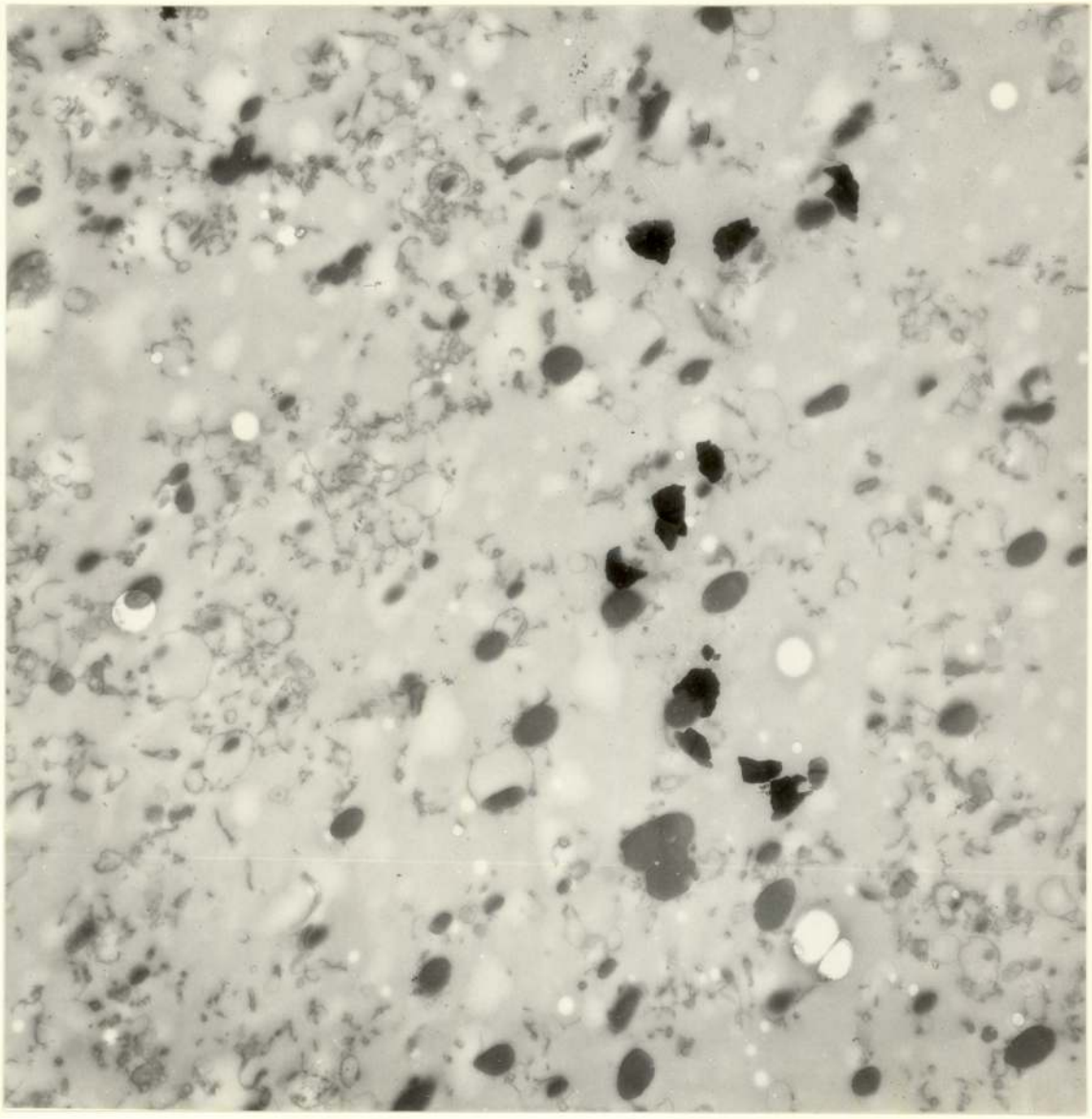


Fig.33

4.25 Staining

As in all investigations involving morphological examination of cells, or their ultrastructures, staining reactions are crucial to the success of the investigation. It was not surprising that variation in staining was encountered and Figs. 34 and 35 show the two common faults met. In Fig. 34 inadequate staining results in a preparation in which the synaptosomes are unidentifiable because synaptic vesicles and mitochondria are not detectable. On first encountering the effect shown in Fig. 35 it was thought that the latex particles had stained with outstanding success! However, on closer examination, and it would seem more apparent in photographs, the structures are obviously artefacts due to staining precipitate. Fig. 35 shows both large and fine precipitate together with latex particles. The synaptosomes, or at least what are assumed to be synaptosomes, are poorly demonstrated.

FIGURES 34 AND 35.

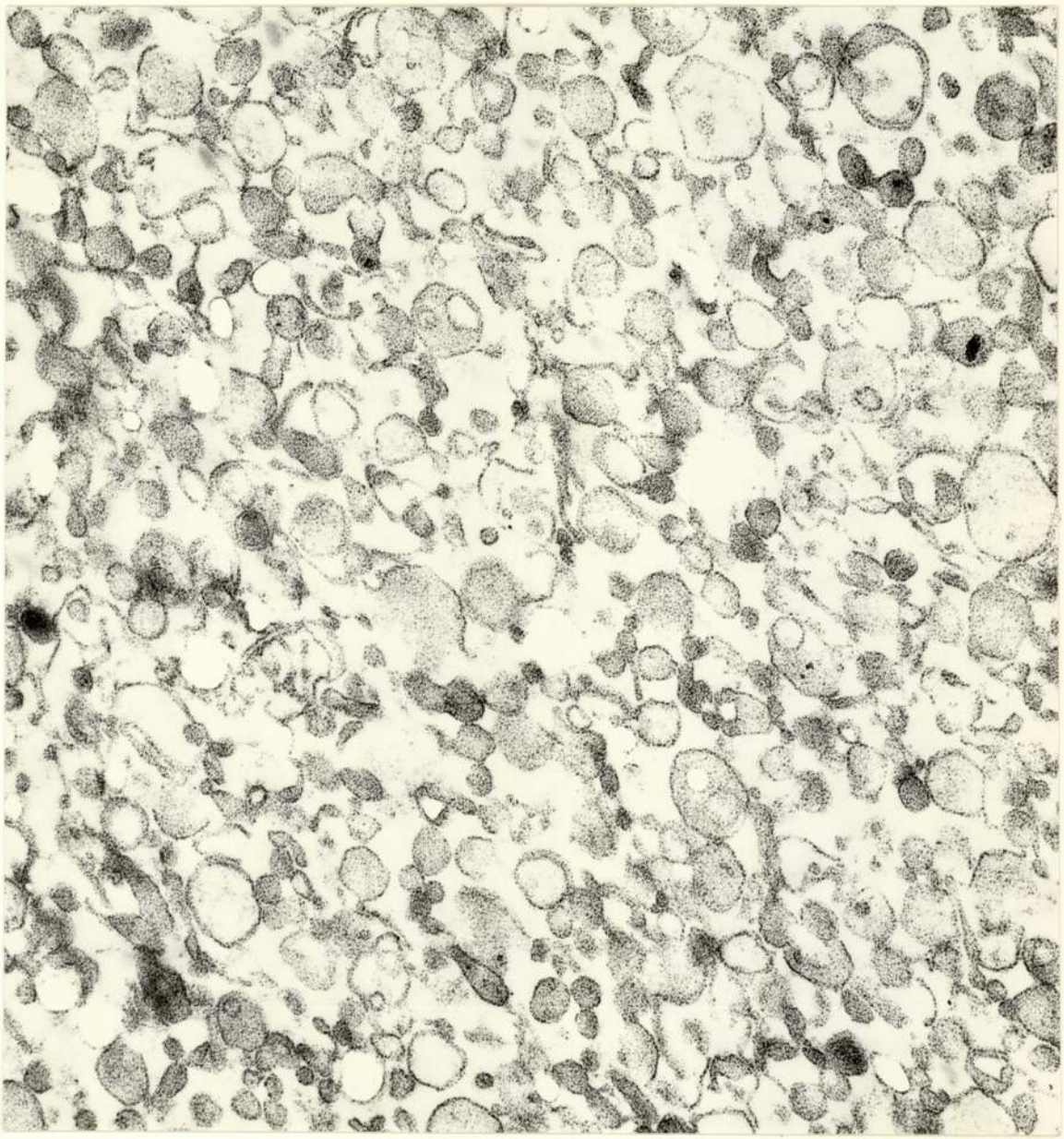


Fig. 34

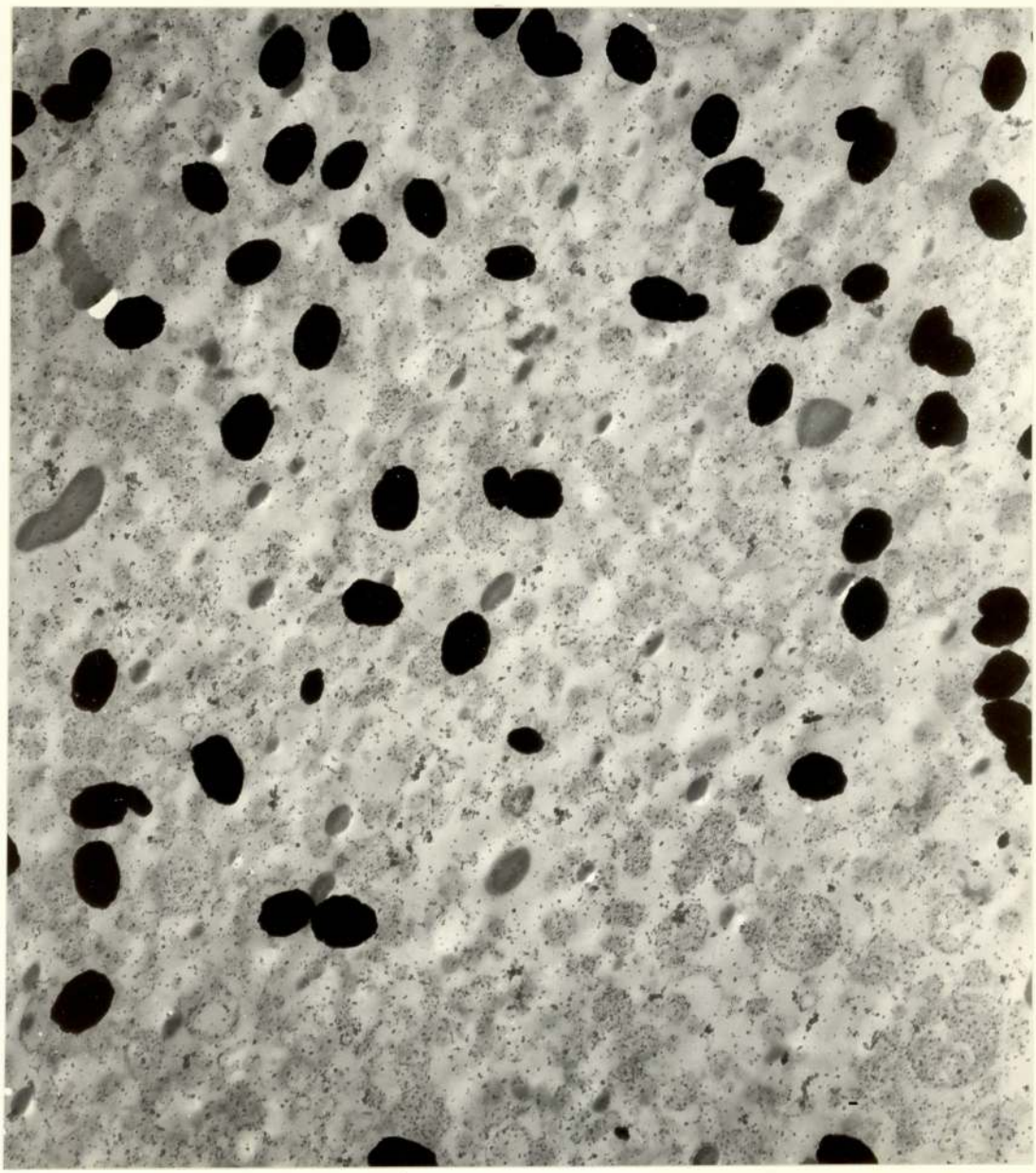


Fig.35

4.3 SPECIAL MORPHOLOGICAL APPEARANCES

4.31 Vacuolated Structures and Mitochondria

Examination of photographs from early experiments involving irradiated animals revealed that a number of synaptosomes contained structures best described as vacuoles (Figs. 36, 37 and 38). These are similar in appearance to mitochondria deficient in cristae. The three photographs show that these vacuoles may be found as single structures (Fig. 36), in pairs (Fig. 37), and in addition to mitochondria (Fig. 38). The vacuoles were thought to be abnormal and possibly due to one of several possibilities including the deficiency of thyroxine which either prevented maturation of the mitochondria or induced some form of degeneration because of failure to maintain hormonal stimulation. A third possibility, which was considered, was that they were an artefact vide infra.

Following the original observation of these structures the number of synaptosomes containing mitochondria (referred to as Type 1) were recorded together with those containing vacuoles (referred to as vacuolated). The numbers were determined per gram of cerebral cortex and also expressed as a percentage of the total number of synaptosomes (Table 4). From the results obtained (Figs. 39, 40, 41 and 42) it can be seen that the percentage of synaptosomes containing mitochondria ranges from 12 - 53 with an average of 35. Nearly half of the results are contained within the range 30 - 40%. It is significant that the number of synaptosomes containing mitochondria should fall within a limited range. The lower and upper end of the percentage scale are obtained with single results. If these are excluded the range is narrowed considerably from 21 - 48%. Figure 39 shows the spread of results related to types of animals, e.g. normal and

thyroidectomised (surgically and irradiated). Although the results show that the synaptosomes of irradiated animals contain mitochondria within the broad average range of 25 - 45%, there is a definite indication (Fig. 41) that these animals contain fewer synaptosomes with mitochondria than normal animals. It was considered that normal, sham operated and surgically created hypothyroid animals were similar in the distribution of mitochondria, and the comparison of the irradiated animals was made against these three types of animals. Of course age is another factor which must be considered and Table 5 relates age to the percentage of synaptosomes with mitonchondria. Examination of this Table together with Fig. 39 shows that the younger animals, e.g. those below 25 days of age, would appear to have fewer synaptosomes with mitochondria than those above this age level. The percentage of synaptosomes containing mitochondria, in normal animals above 25 days of age, appears to stabilise at between 30 and 50%. Thus age and hypothyroidism is of particular significance and is commented upon more fully in the Discussion.

The number of synaptosomes containing vacuoles is shown in Figs. 40 and 42 and these reveal a number of factors. The first is that vacuoles are not exclusive to synaptosomes from hypothyroid animals. They are therefore not specifically induced by this form of hormonal disturbance. Their presence in normal animals increases the problems of deciding the origin of these structures vide infra. The spread of results Figs. 40 and 42 shows that between 3 and 20% of synaptosomes possess these "vacuoles". Further examination of Fig. 42 reveals that a higher percentage of synaptosomes from irradiated animals contain vacuolated structures than normal animals. In addition, there is some suggestion, although limited, that age, particularly in the hypothyroid animal,

may influence the percentage of synaptosomes containing vacuoles, in that the older the hypothyroid animal, the greater the frequency of vacuolated synaptosomes encountered.

FIGURES 36, 37 AND 38.

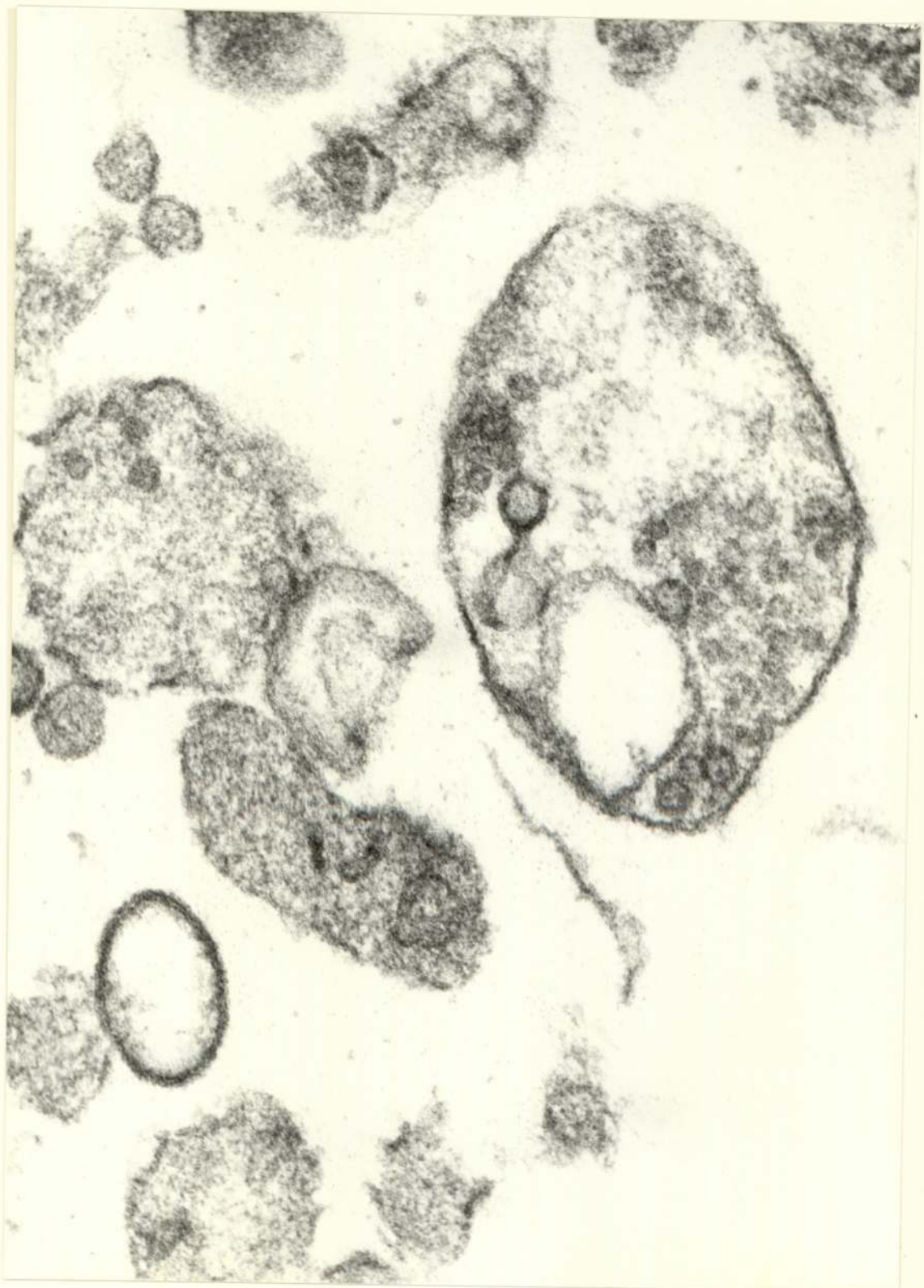


Fig.36

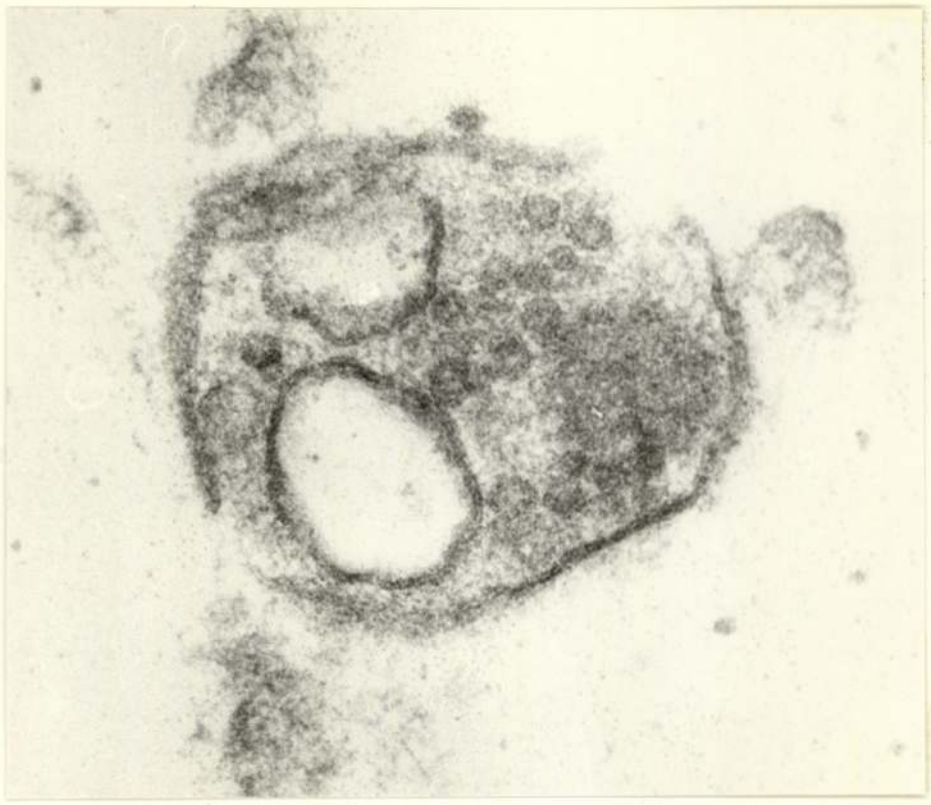


Fig.37

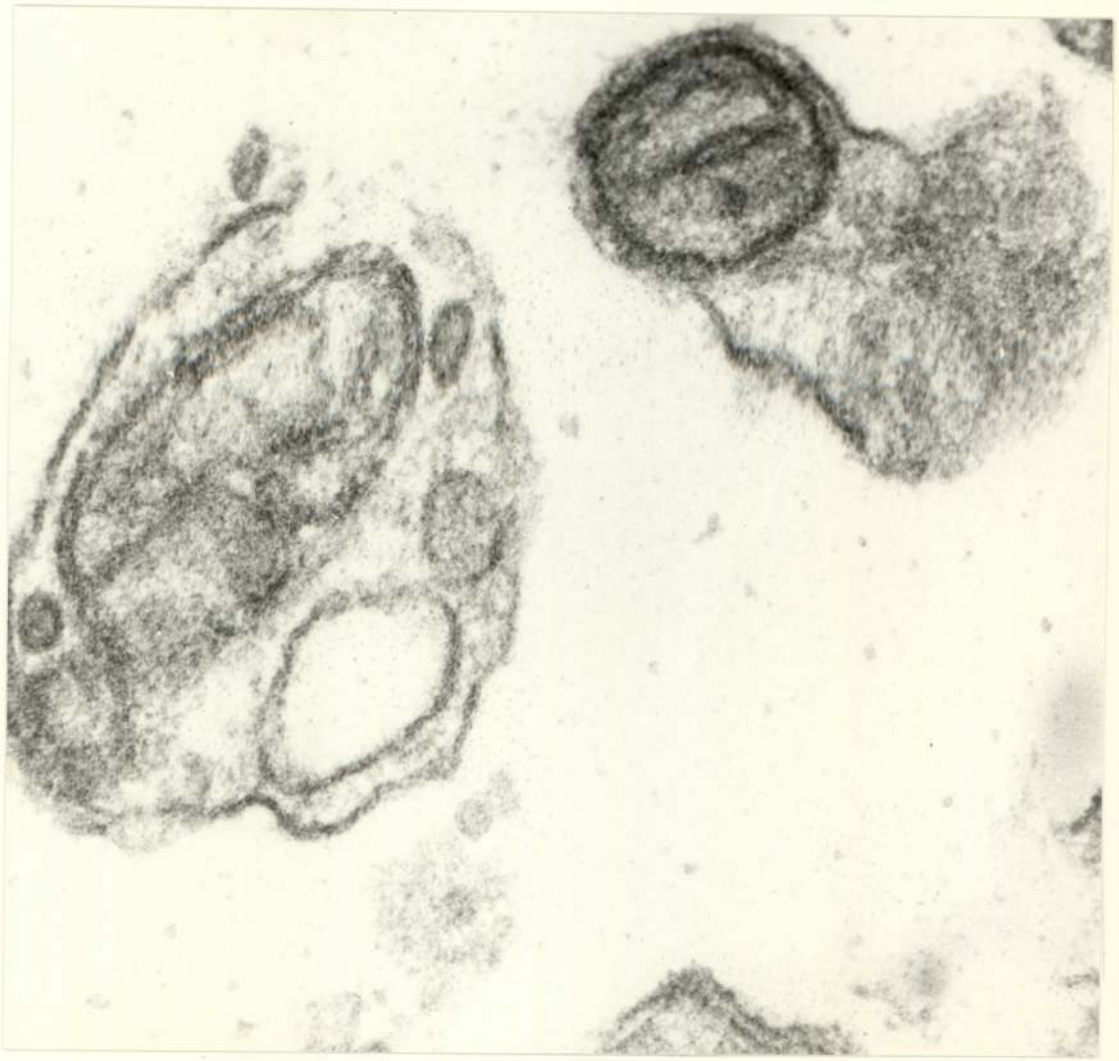


Fig.38

FIGURES 39, 40, 41 AND 42

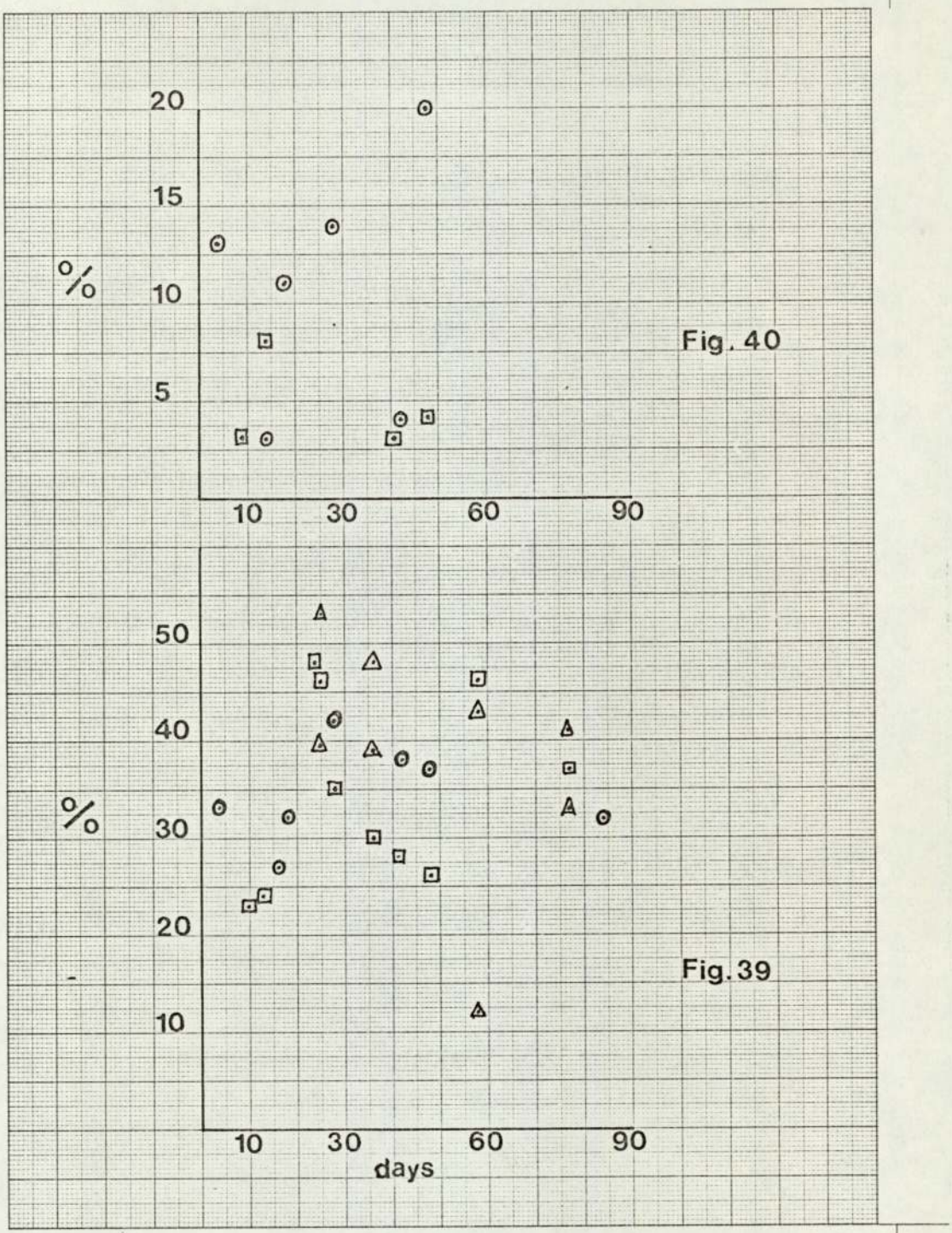


Fig. 40

Fig. 39

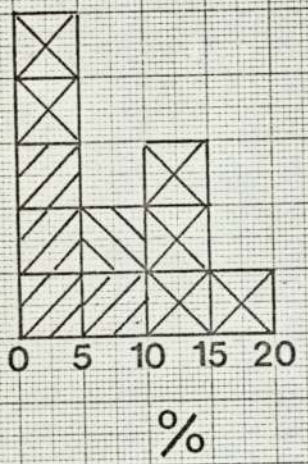


Fig. 42

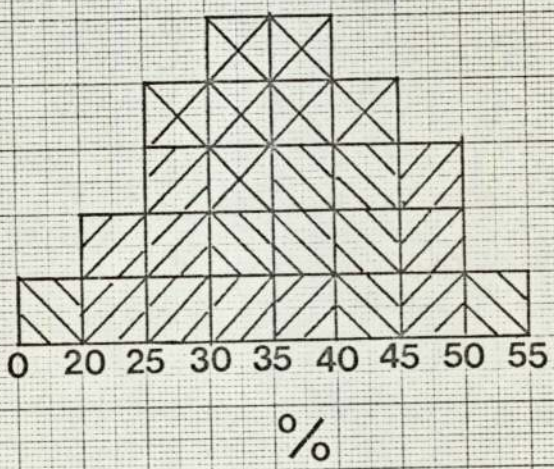


Fig. 41

4.32 Large Unusual Strctures Found in Cerebellum Extracts

Extracts prepared from the cerebellum revealed the presence of large unusually shaped structures (Figs. 43, 44 and 45) which at first were considered to be synaptosomes. An alternative identification proposed is that they were the spiny branchlets of Purkinje cells since their curved and hooked shape resembles closely these structures as shown in the electron photomicrographs of Eccles et al (1967).

These large structures contained: mitochondria, vesicles varying in size and density, and vacuolated bodies which varied in shape and were much larger than the synaptic vesicles. The morphology of the vacuolated bodies does not support the notion that the structures are synaptosomes. Indeed a feature of the Purkinje cell spiny branchlet, and one used as a criterion for identification (Eccles et al, 1967), is a vacuolar system similar to that seen in Figs. 43, 44 and 45. Variation in the size of the vesicles is of less significance since this is to be expected vide supra Section 2.146. Further, Figs. 36 and 38 show vesicles larger than those normally expected.

It is unlikely that the varied shapes of these large structures are artefacts since they were not observed in extracts from the cerebral cortex. Therefore it is presumed that the morphological appearance, as indicated in Figs. 43 and 45, is characteristic for a particular type of structure, either more commonly encountered, or exclusive to, the cerebellum. The curved and hooked shape would, it is presumed, be necessary to allow contact with a number of sub-adjacent neurone processes, a requirement of the Purkinje spiny dendrites or perhaps synapses, reference Figs. 6 and 7.

It is most probable that these structures are pinched-off Purkinje cell spiny branchlets and not morphologically complex synapses.

FIGURES 43, 44 AND 45

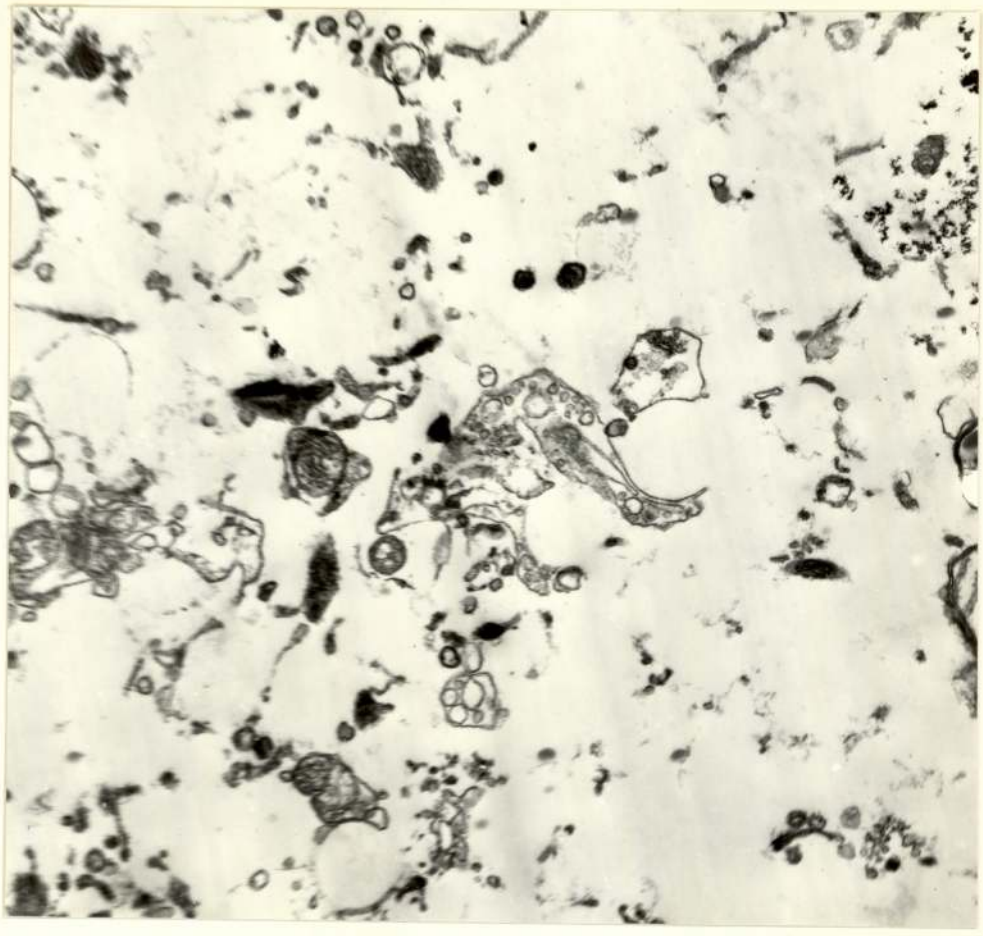


Fig.43

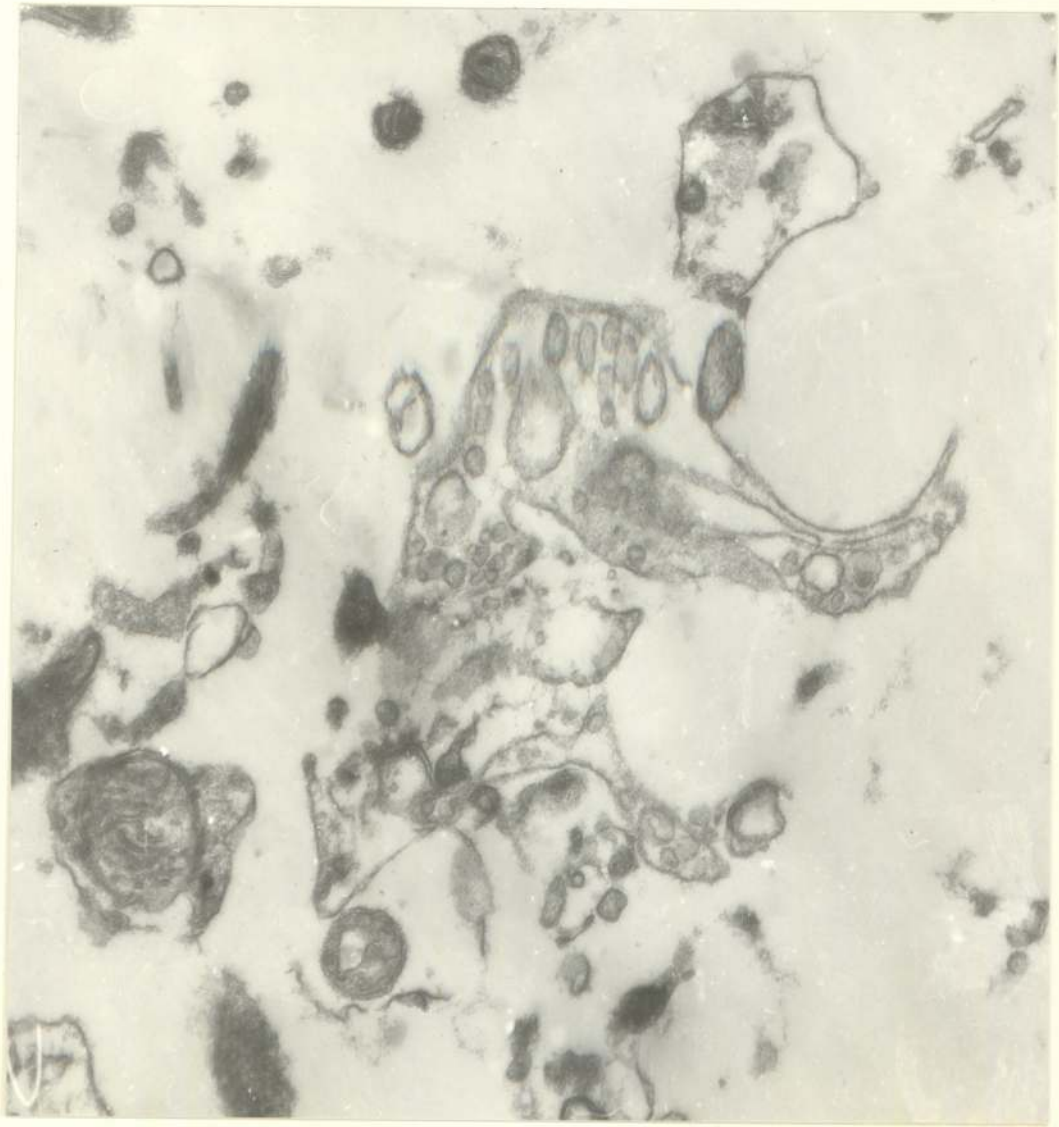


Fig.44

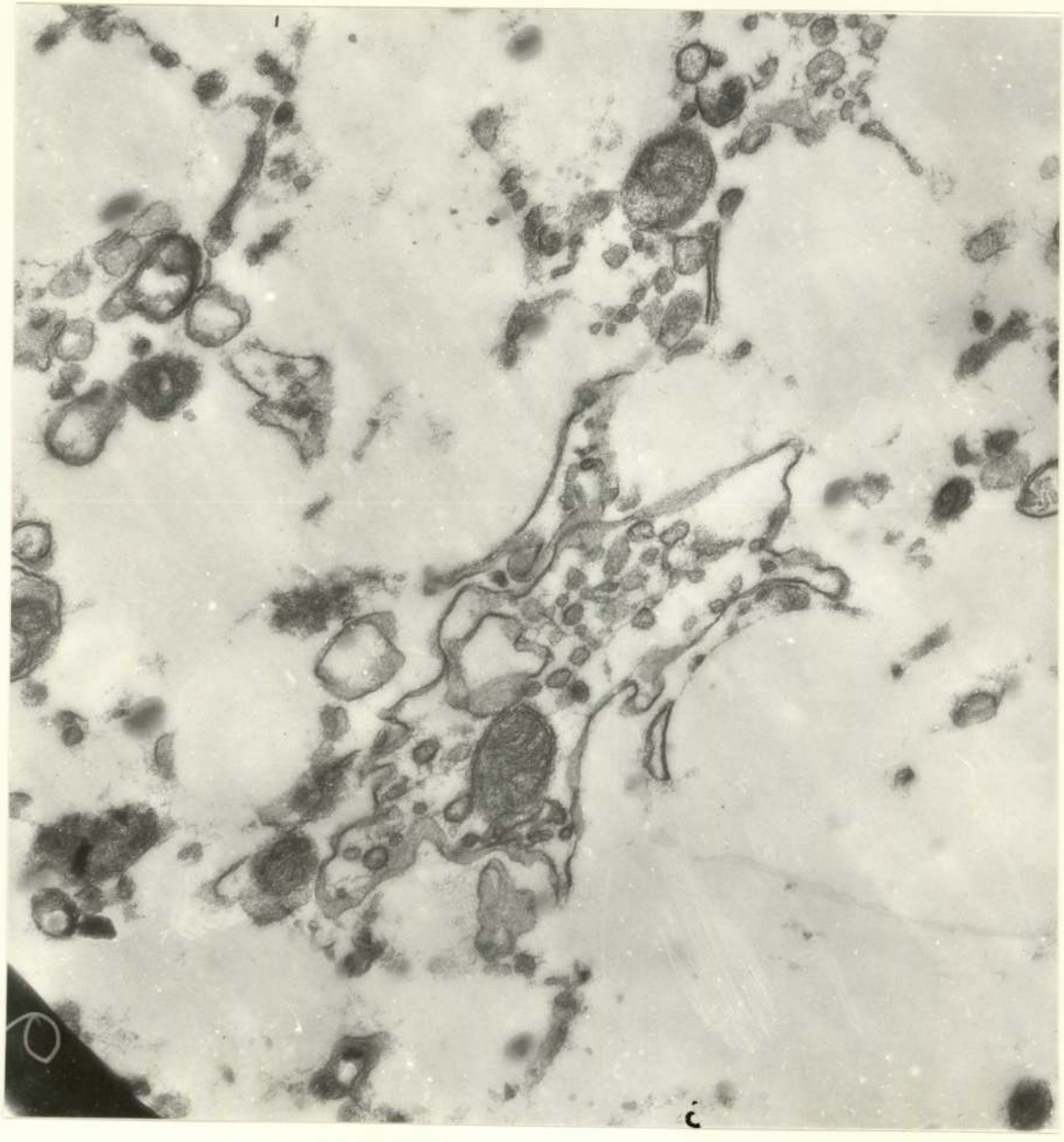


Fig. 45

4.4 NUMBERS OF SYNAPTOSOMES PER GRAM AND THE EFFECT OF HYPOTHYROIDISM

4.41 In the Cerebral Cortex

Table 2 (Appendix) provides the data on the numbers of synaptosomes per gram of cerebral cortex. Figure 46 shows the distribution of synaptosomes in normal rats against age. If the results are averaged, e.g. where more than one result is obtained within a three day period, some indication of increasing numbers of synaptosomes with age is obtained (Fig. 47). If the results from all animals including thyroidectomised are expressed in the same manner, thus providing a larger sample, the progressive increase in numbers of synaptosomes related to age can be seen (Figs. 48 and 49). The results in Figs. 47 and 49 show a rapid increase over the first 42 days reaching a plateau with an average total number of synaptosomes/g in the normal adult rat cerebral cortex of 1.2×10^{11}. In irradiated animals (Fig. 50) the total number of synapses would appear to be in a similar range although the time taken to achieve the total number takes longer, in the order of 58 - 66 days.

The suppression of synaptogenesis in the irradiated animals can be more clearly shown by determining the coefficient of linear correlation of the numbers of synaptosomes at a specified age. For example, if the results for both the normal and irradiated animals (Table 2) are analysed by an Olivetti 101 electronic desk computer using the Bravais-Pearson formulae, an appreciable difference between the two types of animals can be shown (Fig. 53). The results expressed in Fig. 53 are based on the following calculations in which an arbitrary time of 10 days has been taken to determine the slope.

Normal Animals

Age in Days	9	10	13	25	27	28	28	36	41	48
Number of Synaptosomes x 10^{10}	7.1	1.9	7.9	8.4	9.4	6.6	5.3	9.4	5.1	19

r = correlation coefficient

Y (Number of Synaptosomes) = a (intercept) + b (constant) X

where X = time, e.g. days.

From this data the following was obtained:

r = 0.6073 a = 2.4794 and b = 0.2087

using approximated figures

$\therefore$ Y = 2.4794 + (0.2087)X

where X (days) = 0

Y = 2.4794 + (0.2087 x 0)

= 2.48

where X = 0

Y = 2.4794 + (0.2087 x 10)

= 4.58

Animals Thyroidectomised with ^{131}I

Age in Days	4	6	14	19	28	41	42	48
Number of Synaptosomes x 10^{10}	5.6	2.6	4.9	6.8	11.0	5.0	6.8	11.0

r = 0.5843 a = 4.1597 and b = 0.1011

using approximated figures

where X = 0

Y = 4.1597 + (0.1011 x 0)

= 4.16

where X = 10

Y = 4.1597 (0.1011 x 10)

= 5.16

The inference that at zero time the two groups of animals would start at different levels of synaptic densities is, of course, incorrect. It is the result of extending the time to a theoretical zero time, in just the same way extension of the time beyond 10 days would suggest there is no limit to synaptogenesis.

FIGURES 46 - 52

Figs. 46 - 51

abscissa - age in days

ordinate - log numbers of synaptosomes

Fig. 52 Number of synaptosomes/g in the cerebral cortex of normal rats whose age exceeds 70 days but less than one year.

abscissa - numbers of synaptosomes

ordinate - accumulative number of samples

Median (indicated with hatched lines) = $1.0 \times 10^{11}/g$

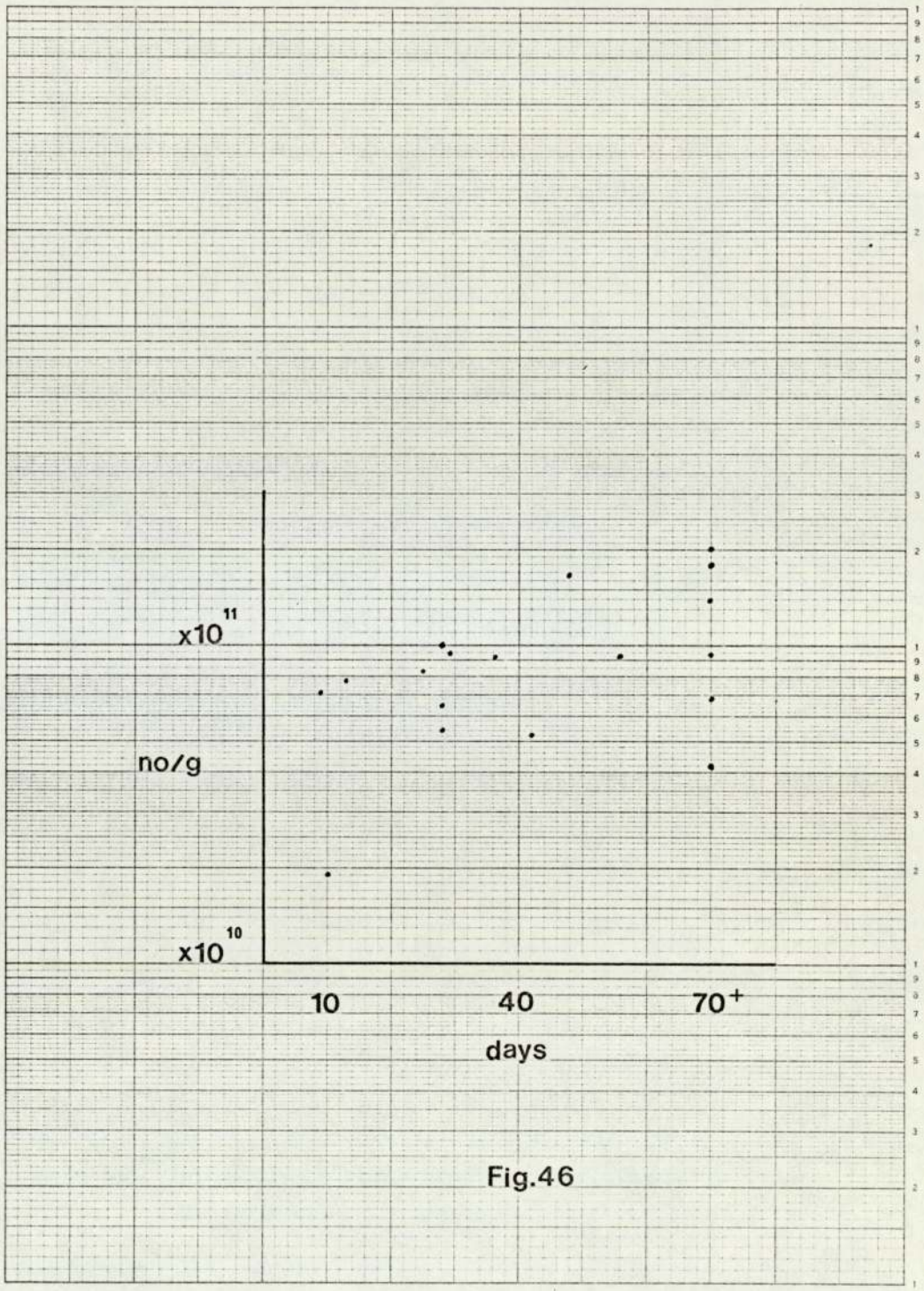


Fig.46

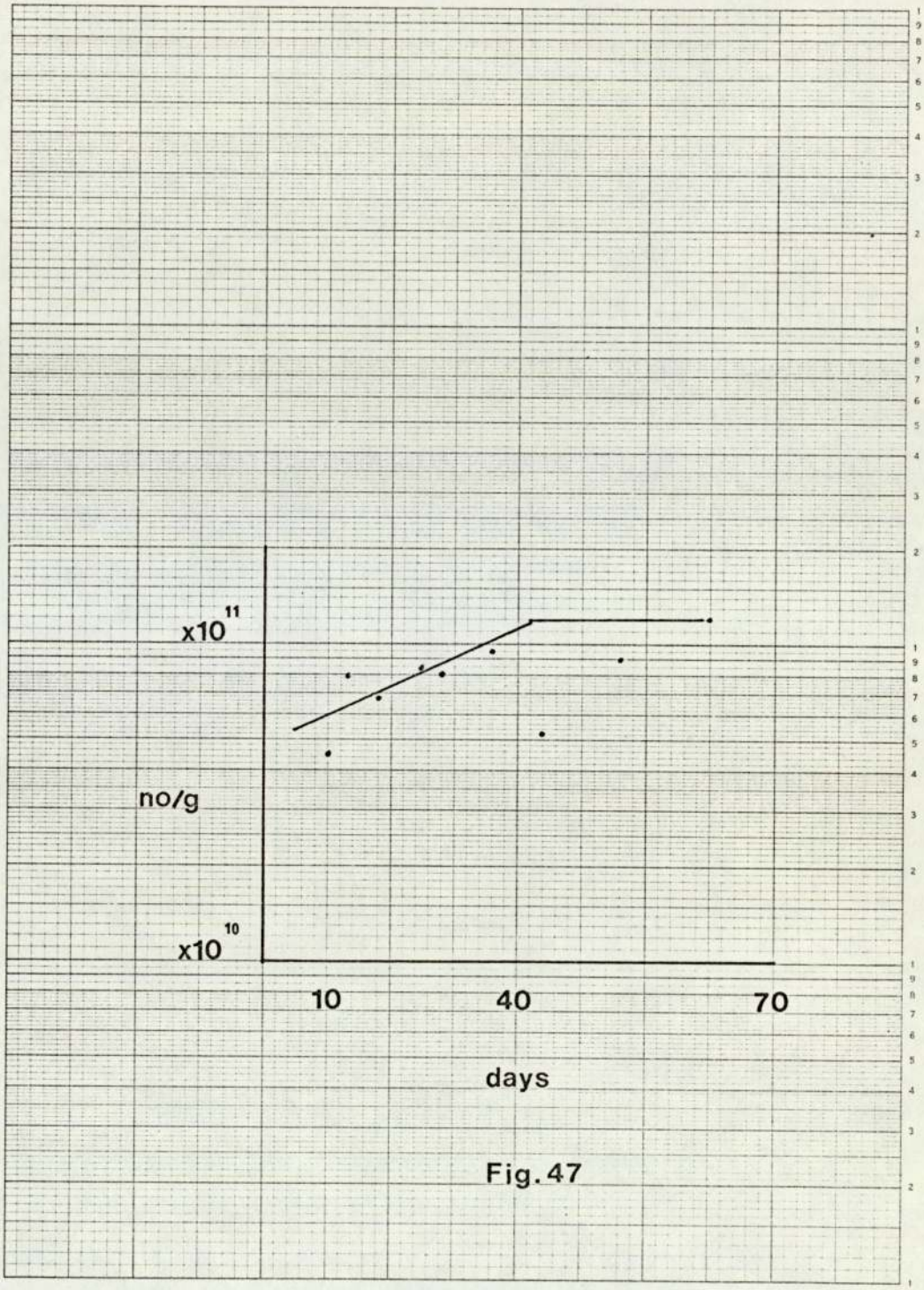


Fig. 47

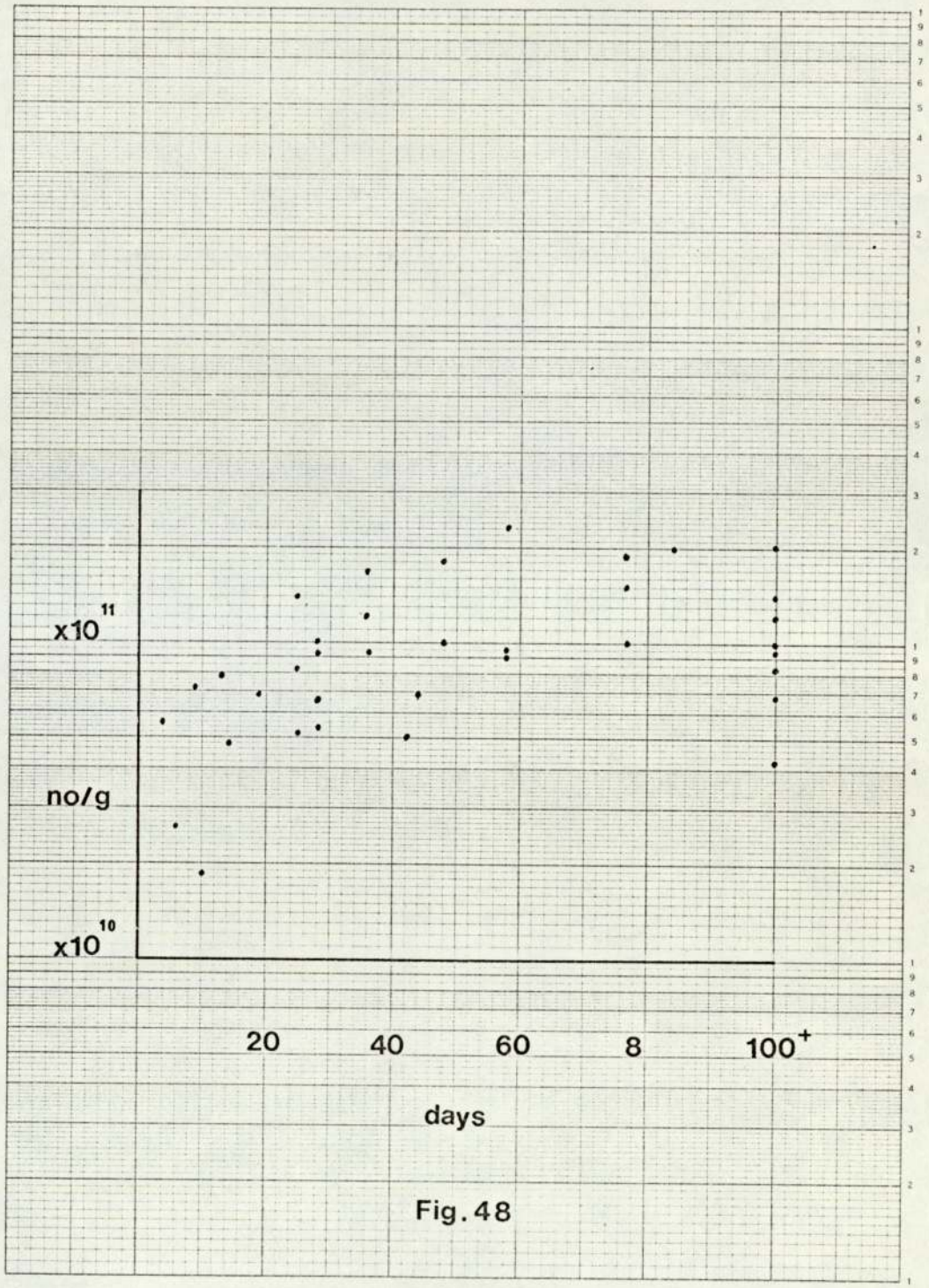

x10^11
no/g
x10^10
20
40
60
8
100+
days
Log 4 Cycles x 10th, ½ and 1 inch
CHART
WELL
Graph Data Ref. 5545

Fig. 48

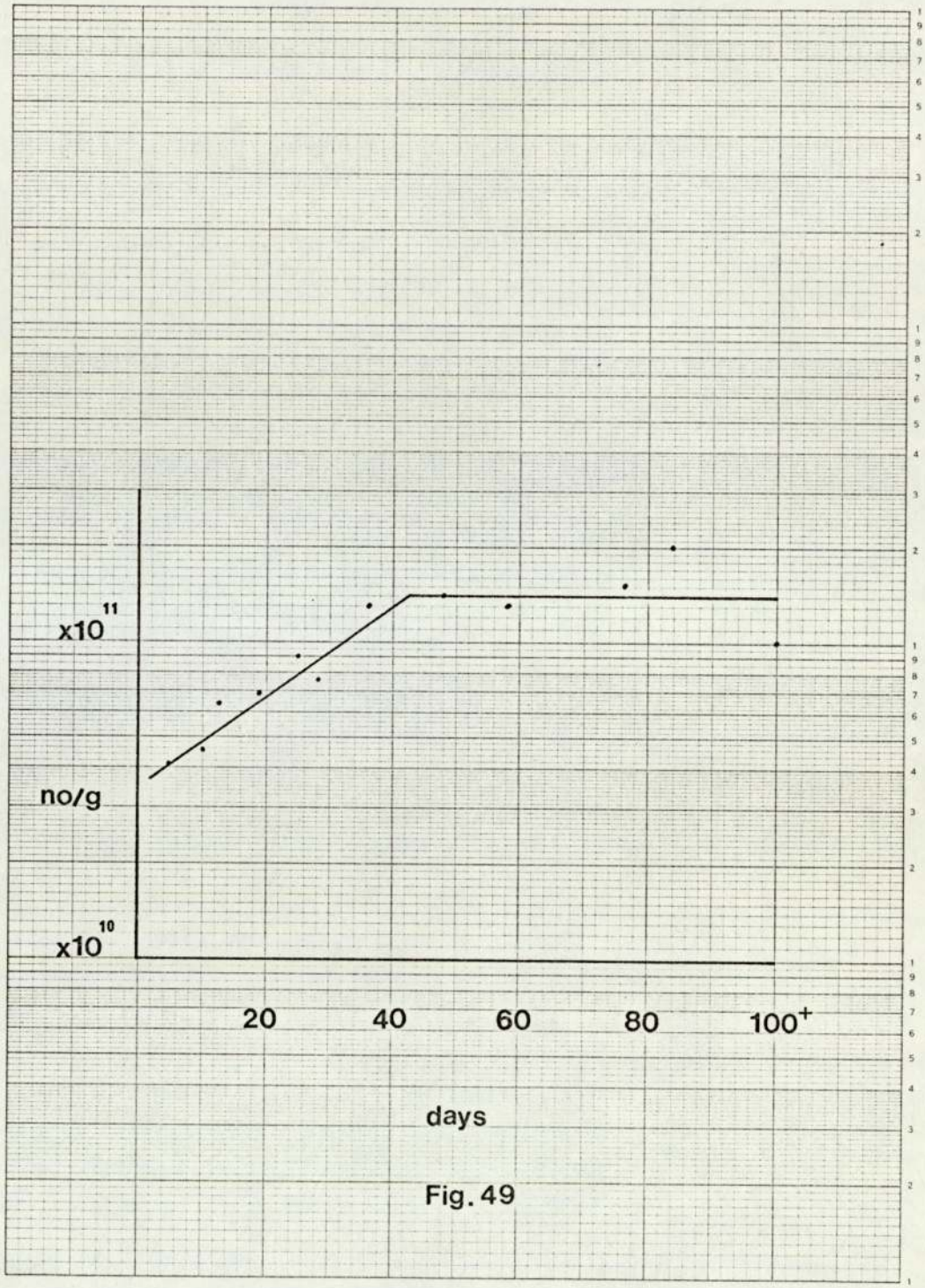


Fig. 49

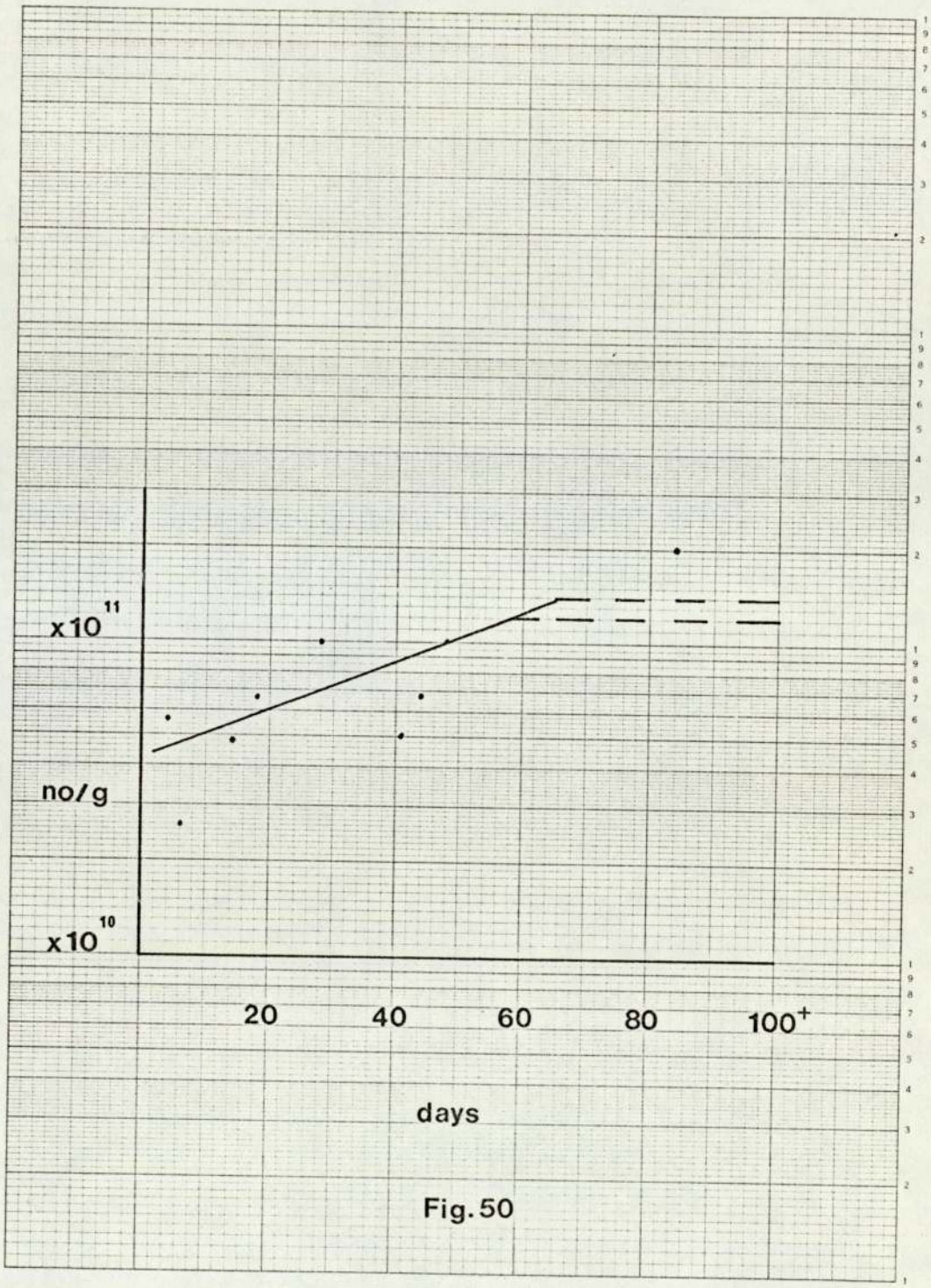


Fig. 50

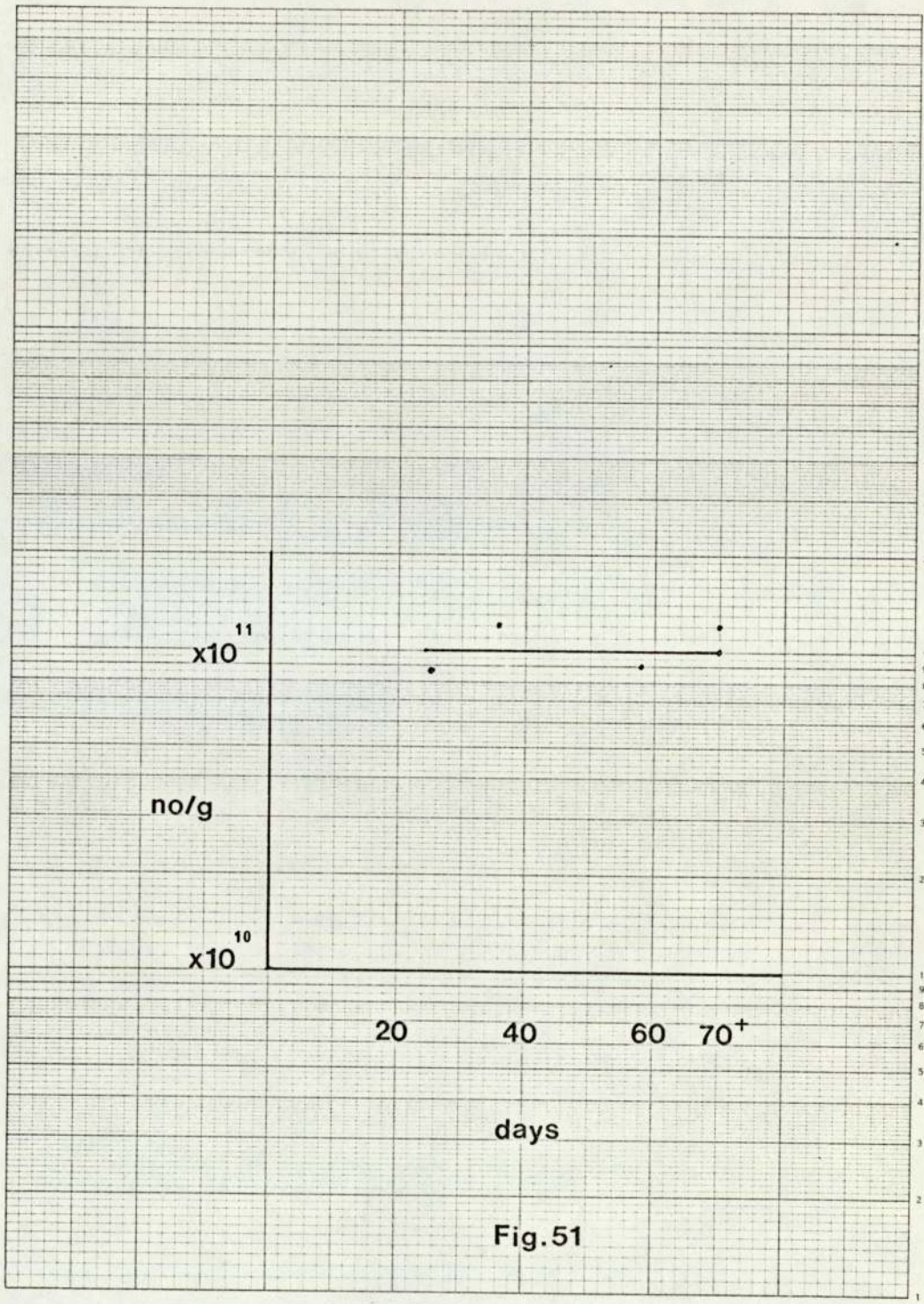


Fig.51

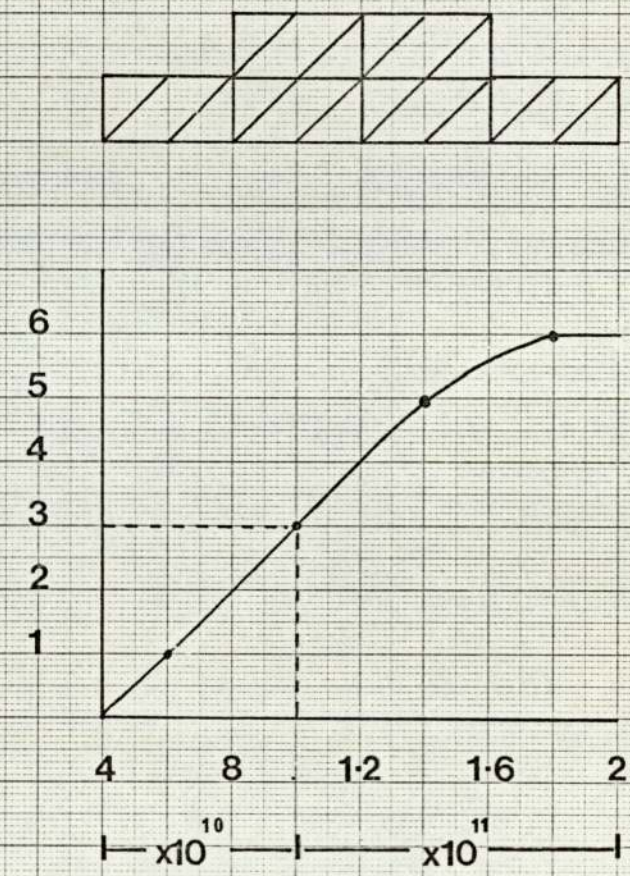


Fig. 52

FIGURE 53.

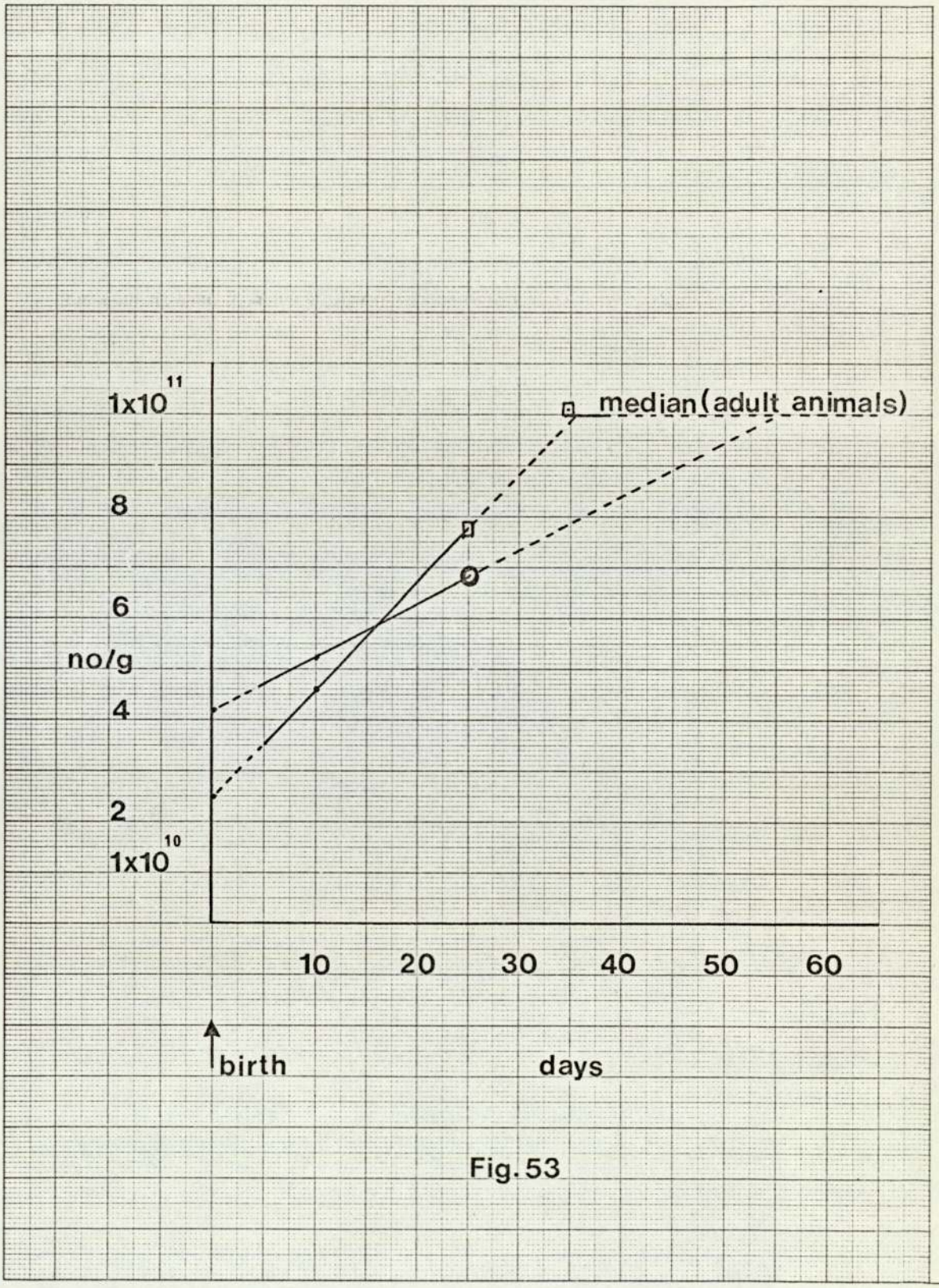


Fig. 53

4.42 In the Cerebellum

This aspect of the research was initiated in the later stages because of the reports by Rebière and Legrand (1972) and Legrand and Rabie (1972) in which they reported that hypothyroidism reduces both protein levels in the synaptosomal fraction and the number of synapses in the molecular zone.

Unfortunately the number of animals which could be investigated was limited to a total of twelve. These included seven normal animals and five in which hypothyroidism was induced by irradiation with ^{131}I. In such circumstances experiments where the results are dubious can have an inordinate effect on the overall picture. For example, the results of experiments 49, 53 and 61 do not conform to the pattern which analysis of the other results would suggest vide infra.

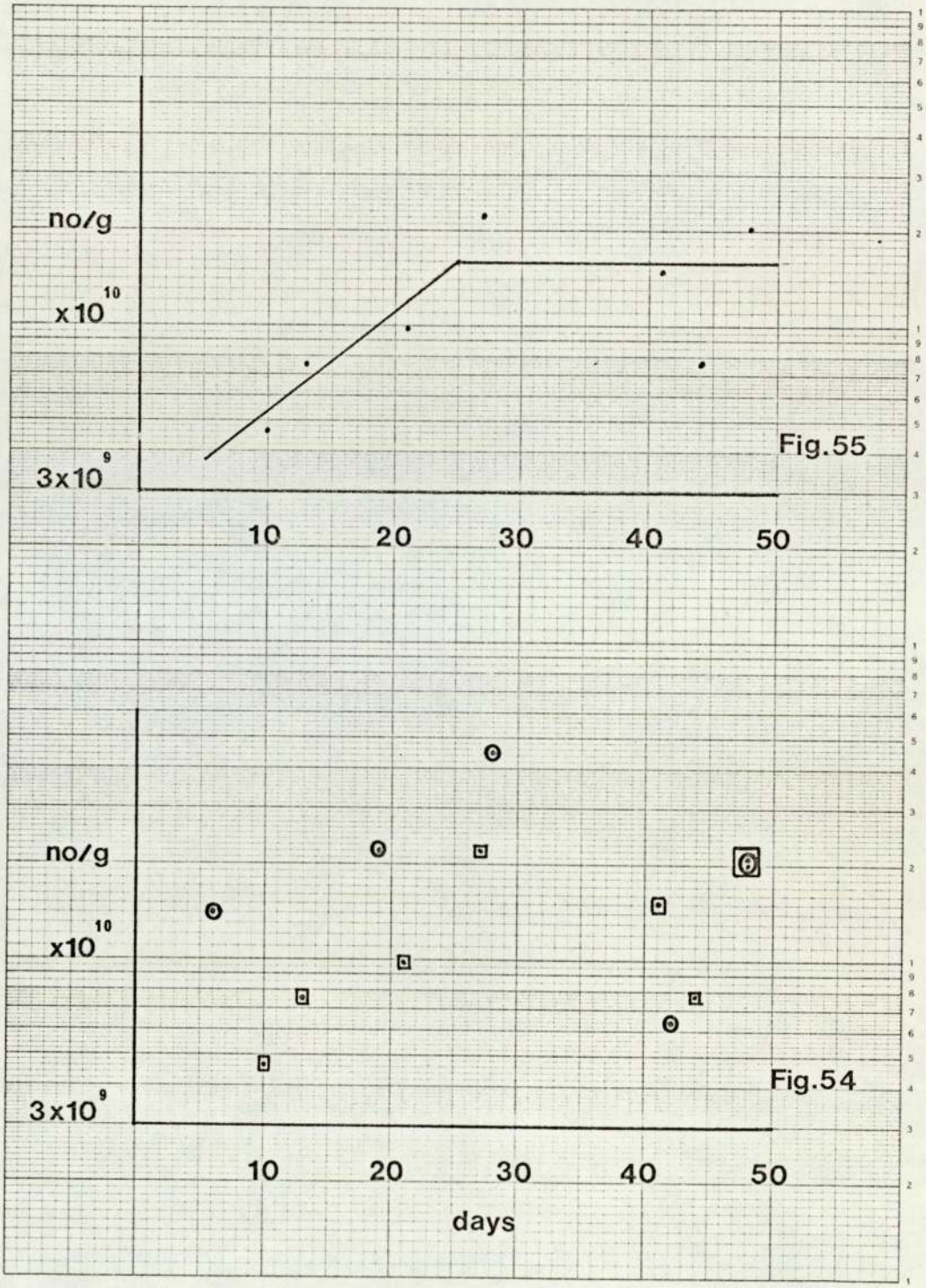
Table 2 contains the relevant data, which has been extracted and shown in Table 6 for ease of reference.

In Figure 54 the number of synaptosomes per gram of cerebellum from both normal and experimental animals has been shown against age in days. Figure 55 contains the number of synaptosomes/gram from normal animals. From these Figures it is possible to draw two conclusions. The first is that the average number of synaptosomes/g of cerebellum in adult animals is approximately 1.7×10^{10}. This number is considerably less, almost tenfold, than the density of synaptosomes to be found in extracts from the cerebral cortex. The second observation is that synaptogenesis in the cerebellum would appear to reach a maximum stage by 25 days. This is earlier than in the cerebral cortex. There are insufficient results to draw any conclusions on the effect of hypothyroidism on synaptogenesis.

TABLE 6. NUMBERS OF SYNAPTOSOMES IN EXTRACTS OF THE CEREBELLUM

Exp. No.	Type of Animal	Age (Days)	Number of Synaptosomes per gram	Weight of Cerebellum (grams)
49	Normal	10	4.7×10^9	0.08
54	"	13	7.6×10^9	0.15
57	"	21	9.9×10^9	0.18
59	"	27	2.2×10^{10}	0.12
60	"	41	1.5×10^{10}	0.08
49	"	44	7.6×10^9	0.15
62	"	48	2.0×10^{10}	0.11
53	Irradiated	6	1.4×10^{10}	0.07
56	"	19	2.2×10^{10}	0.05
59	"	28	4.5×10^{10}	0.12
61	"	42	6.4×10^9	0.16
62	"	48	2.1×10^{10}	0.11

FIGURES 54 AND 55.



4.5 NUMBERS OF SYNAPTOSOMES IN THE WHOLE OF THE CEREBRAL CORTEX AND THE EFFECT OF HYPOTHYROIDISM

The number of synaptosomes has so far been expressed per gram of cerebral cortex. However, from measurements of brain weight it is apparent that hypothyroidism affects the growth of the brain (Fig. 24). It was considered, therefore, that it was important to correlate brain weights and the number of synaptosomes/g from both normal and thyroidectomised (^{131}I) animals and thus calculate the total number of synaptosomes in the cerebral cortex. This would indicate more precisely the effect of hypothyroidism on the brain.

Table 7 shows the distribution of brain weight to age. The age in days has been arranged in blocks of 5 day periods and the results for normal and hypothyrpid separated. It can be seen that there is a definite suppression of brain growth in the hypothyroid animal during the first 20 days.

Age (days)	Brain Weight in gram(s)	
	Normal Animals	Thyroidectomised (^{131}I)
0-5		0.2
5-10	0.8 0.9	0.3
10-15	0.6	0.8
15-20		0.6 0.6
20-30	1.3 1.2 1.2 1.3 1.4 1.3 1.2 1.2	1.2 1.2
30-40	1.3 1.5 1.5	
40-50	1.4 1.3 1.3 1.4	1.3 1.3 1.0
50-70	1.5 1.5 1.6	
70-100	1.6 1.6 1.6	0.9
100+	1.4 1.4 1.5 1.8 2.0 1.9 1.6 1.5 1.6	

TABLE 7. WEIGHT OF BRAIN RELATED TO AGE

The data relating to brain weights for the experiments associated with the irradiation of animals, including controls, has been extracted and compiled to form Table 8.

Exp. No.	44	47	49	51	53	54	55	56	57	59	60	61	62	63
Cortex Wt.	0.6	0.3	0.4	0.1	0.1	0.3	0.5	0.4	0.6	0.7	0.6	0.6	0.5	0.7
Brain Wt.	1.4	0.9	0.9	0.2	0.3	0.6	0.8	0.6	1.2	1.2	1.3	1.3	1.0	1.4
Type of Animal	E	E	C	E	E	C	E	E	C	E	C	E	E	C

E : experimental animals C: control (normal) animals

TABLE 8. BRAIN AND CORTEX WEIGHTS IN NORMAL AND EXPERIMENTAL (^{131}I irradiated) ANIMALS

From Table 8 the following can be calculated:

All animals (experimental and controls)

Cerebral cortex wt. = 6.4 g $\therefore$ Ratio = $\frac{1}{2}$ (approx.)

Brain wt. = 13.1 g

Experimental animals (^{131}I)

Cerebral cortex wt. = 3.8 g $\therefore$ Ratio = $\frac{1}{2}$ (approx.)

Brain wt. = 7.7 g

Control (normal) animals

Cerebral cortex wt. = 2.6 g $\therefore$ Ratio = $\frac{1}{2}$ (approx.

Brain wt. = 5.4 g

It will be noted that in all three calculations the ratio of cerebral cortex to brain is in the order of 1 : 2. Since the measurement of brain weight was more accurate than the disection and measurement of cortex, the ratio of 1 : 2 was used to estimate the

probale weight of cortex and the total number of synaptosomes. The results obtained by these calculations are expressed in Table 9.

Exp. No.	Age (Days)	Synaptosomes	Type of Exp.	Exp. No.	Age (Days)	Synaptosomes	Type of Exp.
14	A	9.8×10^{10}	N	37	112	7.6×10^{10}	O
16	27	6.1×10^{10}	N	"	112	4.2×10^{10}	S
17	9	2.84×10^{10}	N	"	112	6.5×10^{10}	N
18	A	1.4×10^{11}	N	41	168	7.4×10^{10}	S
19	28	3.96×10^{10}	N	41	168	1.0×10^{11}	O
19	28	3.18×10^{10}	N	44	41	3.5×10^{10}	^{131}I
30	25	3.3×10^{10}	O	47	84	9.0×10^{10}	^{131}I
"	25	9.8×10^{10}	O	49	10	8.5×10^{9}	N
"	25	5.5×10^{10}	S	51	4	5.6×10^{9}	^{131}I
31	36	7.8×10^{10}	O	53	6	3.9×10^{9}	^{131}I
"	36	1.2×10^{11}	O	54	13	2.37×10^{10}	C
"	36	7.0×10^{10}	S	55	14	1.96×10^{10}	^{131}I
34	58	6.9×10^{10}	S	56	19	2.0×10^{10}	^{131}I
"	58	6.9×10^{10}	O	59	28	6.0×10^{10}	^{131}I
"	58	2.1×10^{11}	O	60	41	3.3×10^{10}	C
35	77	1.2×10^{11}	O	61	42	4.4×10^{10}	^{131}I
"	77	8.0×10^{10}	O	62	48	5.0×10^{10}	^{131}I
"	77	1.5×10^{11}	S	63	48	1.3×10^{11}	C

N = normal animal

A = adult animal age between 6 and 12 months

O = thyroidectomised by surgical operation

S = sham operated

^{131}I = thyroidectomised by irradiation with ^{131}I

C = control

TABLE 9. TOTAL NUMBER OF SYNAPTOSOMES IN THE CEREBRAL CORTEX OF RATS

When the information compiled for Table 9 is plotted against age for both normal and experimental animals (Fig. 56), it can be observed that there is a definite increase in the number of synaptosomes with maturation of the brain; a result which is similar to that obtained in calculations of synaptosomes per gram cerebral cortex (Figs. 46 - 52). If the data from Fig. 56 is averaged (Fig. 57) the resulting graph provides an indication of the growth pattern. The number of synaptosomes in the cerebral cortex is 1.0×10^{11} which is slightly less than the average number of synaptosomes to be expected in normal animals 1.1×10^{11} (Fig. 58), but this is to be expected since the results from the hypothyroid animals would reduce the number obtained in the normal animals. The results shown in Fig. 58 also indicate that synaptogenesis is complete by approximately 42 days of age. If the results of irradiated animals are expressed in a similar manner (Fig. 59) it can be seen that there is a delay in synaptogenesis. This conclusion is based on the extension of the development of numbers of synaptosomes to the level expected in normal animals. N.B. There are insufficient results to show the levelling off of synapse production in the irradiated animals. It would seem that synaptogenesis is unlikely to be completed before 54 days, which is a delay in the order of at least 12 days or 25%. This retardation of synaptogenesis is more clearly demonstrated in Fig. 60 where the average number of synaptosomes formboth normal and irradiated animals is shown.

Using the data provided by Brizee et al (1963) that the number of neurones in the rat cerebral cortex of animals of 100 days of age is $85.9 \times 10^3/mm^3$, this would be equivalent to $85.9 \times 10^6/cm^3$. Assuming an equivalence of brain volume and weight, the total number of neurones in the cerebral cortex is $(85.9 \times 10^6) \times 0.8$ g (average weight of brain divided by the ratio of 1 : 2 ref. Tables 7 and 8) which is 70.7×10^6. The average number of synapses per neurone in the rat cerebral cortex

is therefore $1.1 \times 10^{11} \div 7.07 \times 10^{7}$, ($1.1 \times 10^{11}$: total number of synaptosomes per cerebral cortex ref. Fig. 58), which is approximately 1600.

The results expressed in Figures 56 - 60 support the findings ref. Fig. 47 that synaptogenesis in normal animals proceeds at a rapid rate during the first approximately 42 days (42 days in Fig. 47 and 42 days in this section ref. Fig. 58). After this period no further increase in numbers can be detected. In hypothyroid animals synaptogenesis also proceeds at a rapid rate but it is quite evident that it is suppressed in comparison with normal rats (Fig. 60).

The data obtained on numbers of synaptosomes per gram of cerebral cortex and total number in the cerebral cortex was analysed on the City of Birmingham computer to determine the best curve using the Bravais-Pearson formulae. The curve fitting was achieved by using a logarithmic transformation of the data and gave a best curve of the form

$$Y = ax^{b}$$

where Y : number of synaptosomes, a and b : constants, and x : number of days.

The actual numbers of synaptosomes were then analysed using the best curve ($Y = ax^{b}$) and the estimated numbers of synaptosomes related to age were determined. Tables 10 - 13 (Appendix) are copies of the computer (line-out machine) data sheets: Table 10 contains the results using the number of synaptosomes per gram of cerebral cortex in normal animals, and Table 11 from hypothyroid (^{131}I) animals; Table 12 contains the results using the number of synaptosomes, in the whole of the cerebral cortex, from normal animals, and Table 13 from hypothyroid (^{131}I) animals.

If the estimated numbers of synaptosomes related to age are plotted (Figs. 61 and 62) it is clear that although hypothyroidism suppresses synaptogenesis the brain eventually produces the same number of synapses as the normal animals. This conclusion is more obvious in Fig. 62 where

the number of synaptosomes/total cerebral cortex has been plotted, than in Fig. 61 synaptosomes/gram cerebral cortex.

FIGURES 56 - 60

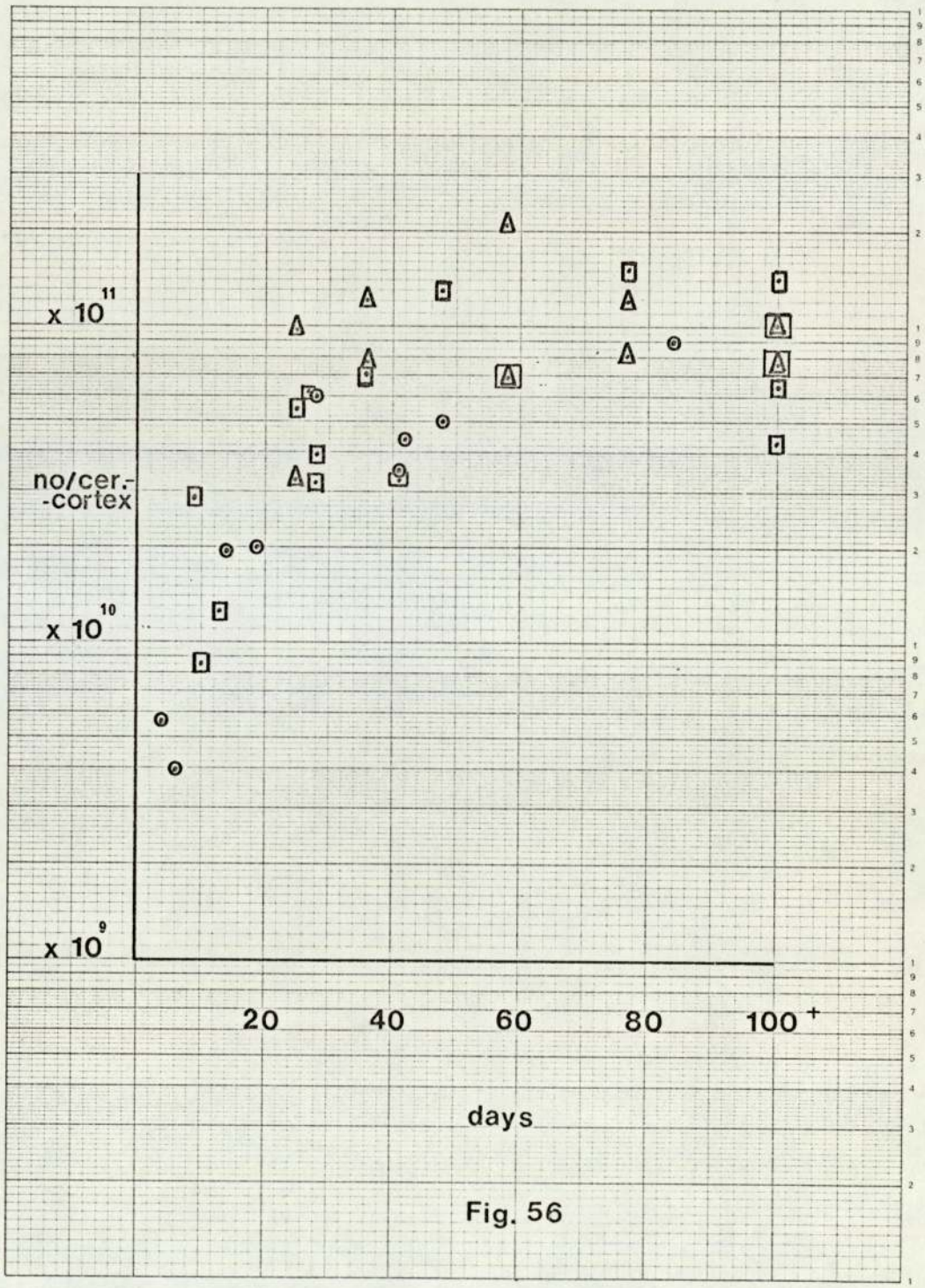


Fig. 56

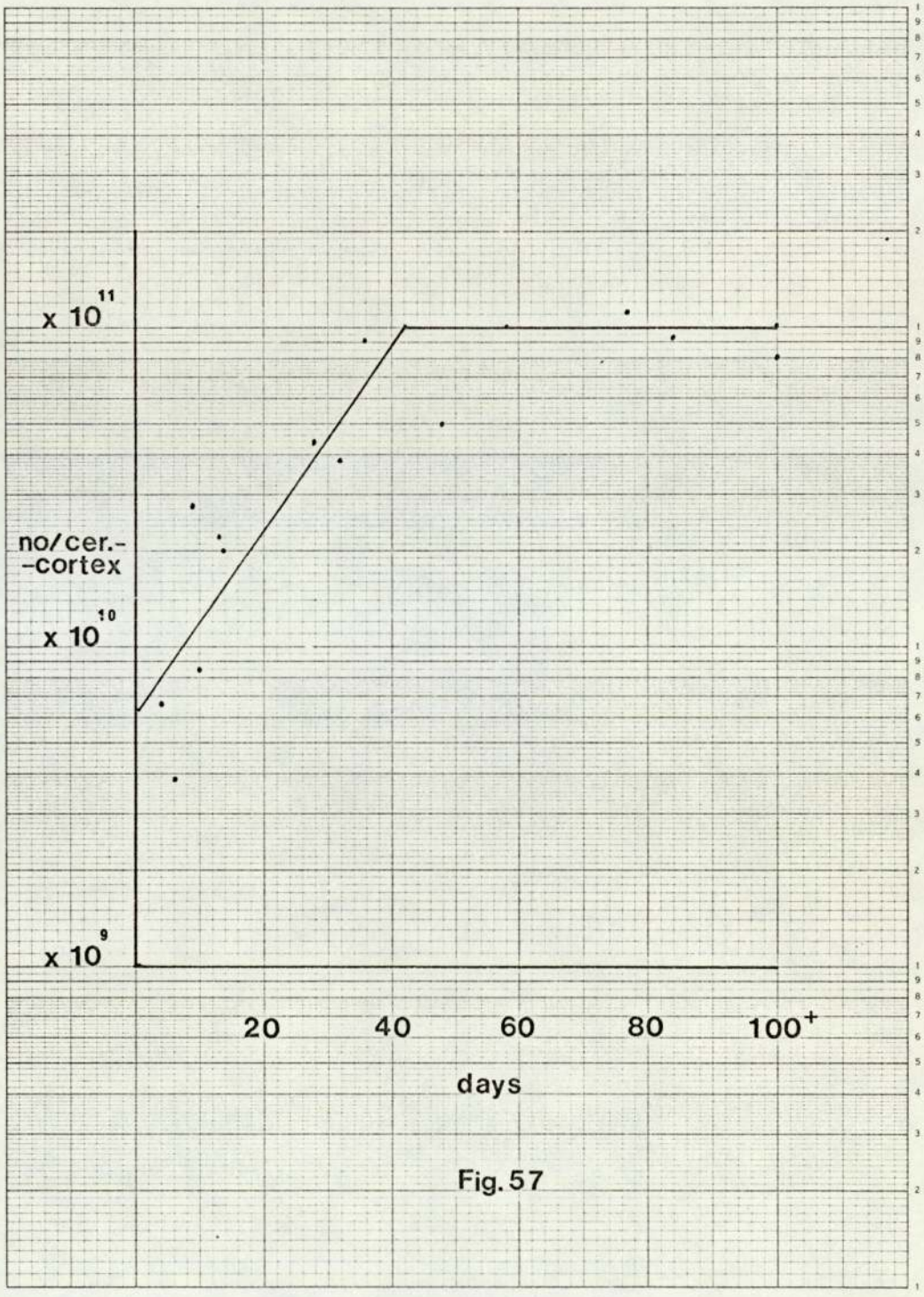


Fig. 57

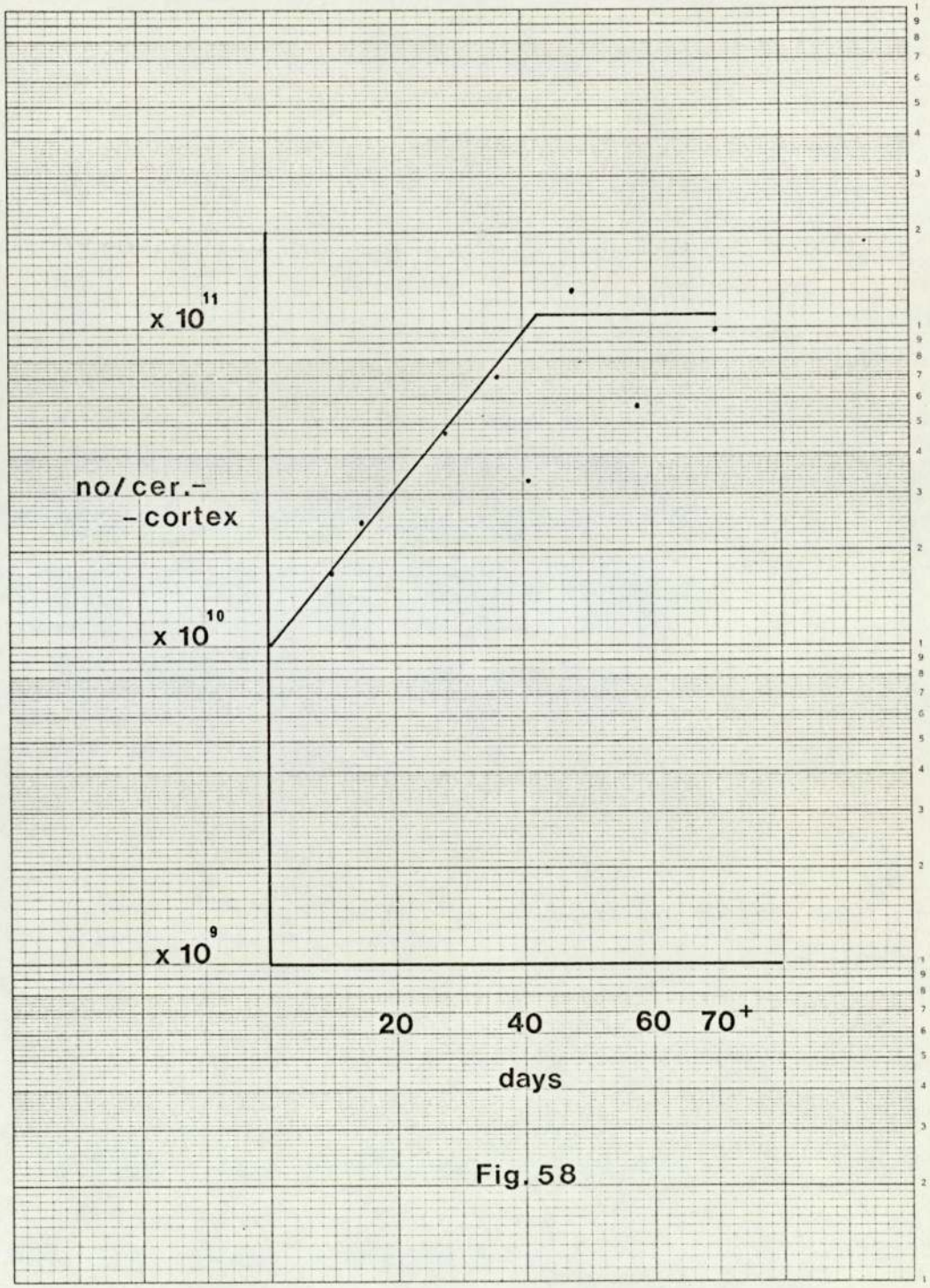

x 10^11
no/cer.-
-cortex
x 10^10
x 10^9
20
40
60
70+
days
Log 4 Cycles x 10th, ½ and 1 inch
Graph Data Ref. 5545
WELL

Fig. 58

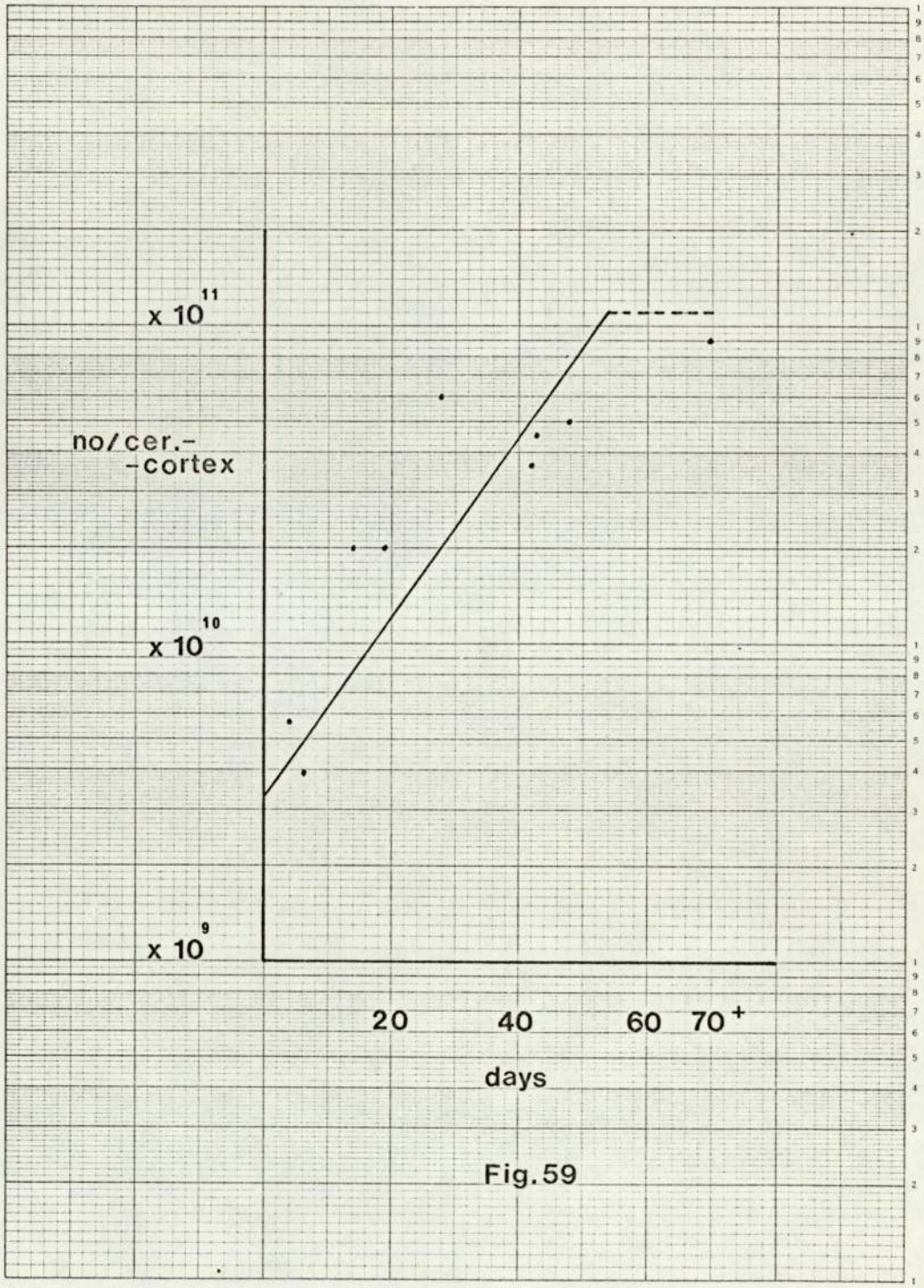


Fig. 59

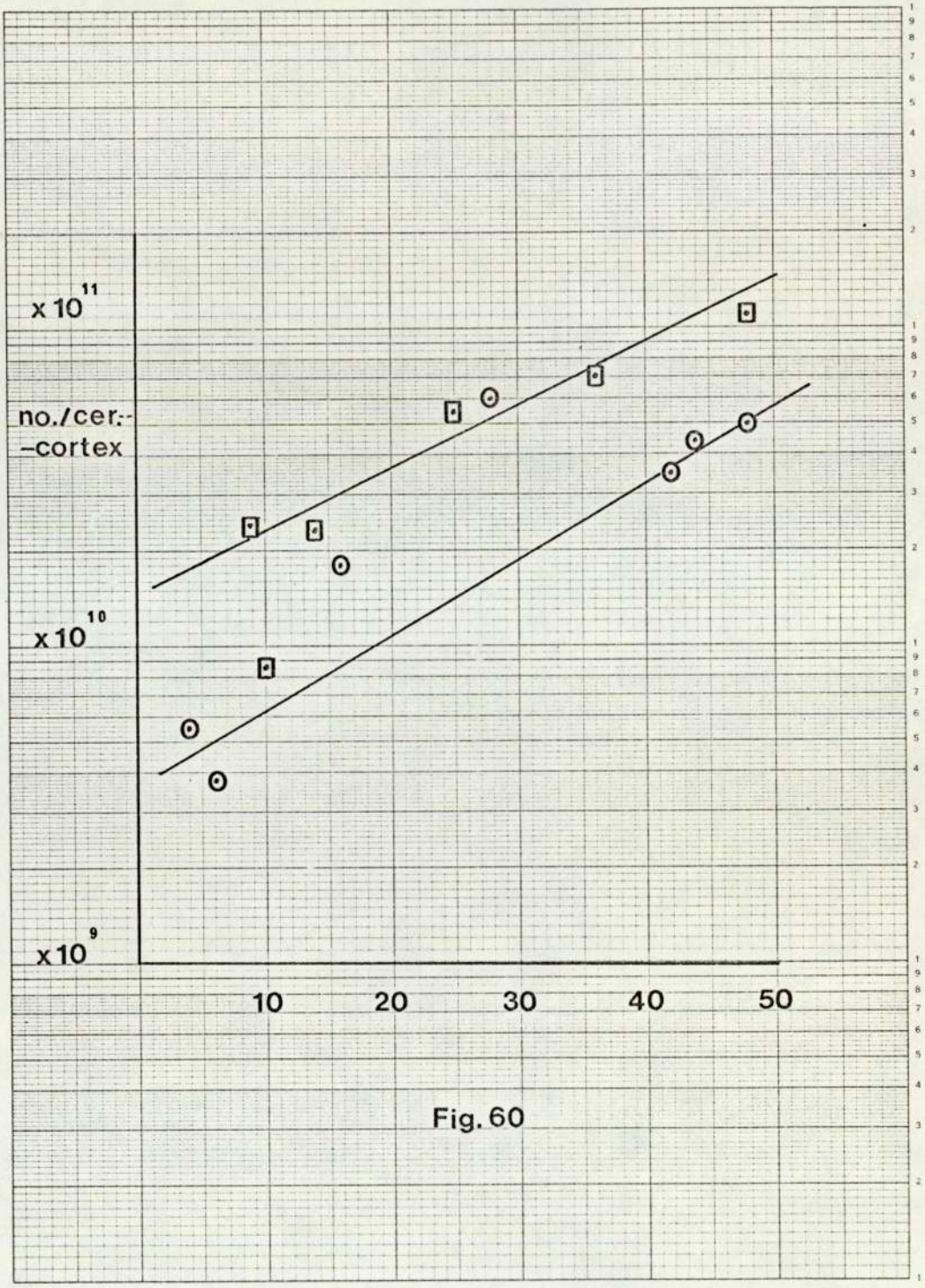

x 10^11
no./cer.-
-cortex
x 10^10
x 10^9
10
20
30
40
50
Log 4 Cycles x 10th, ½ and 1 inch
Graph Data Ref. 5545
CHART WELL

Fig. 60

FIGURES 61 and 62

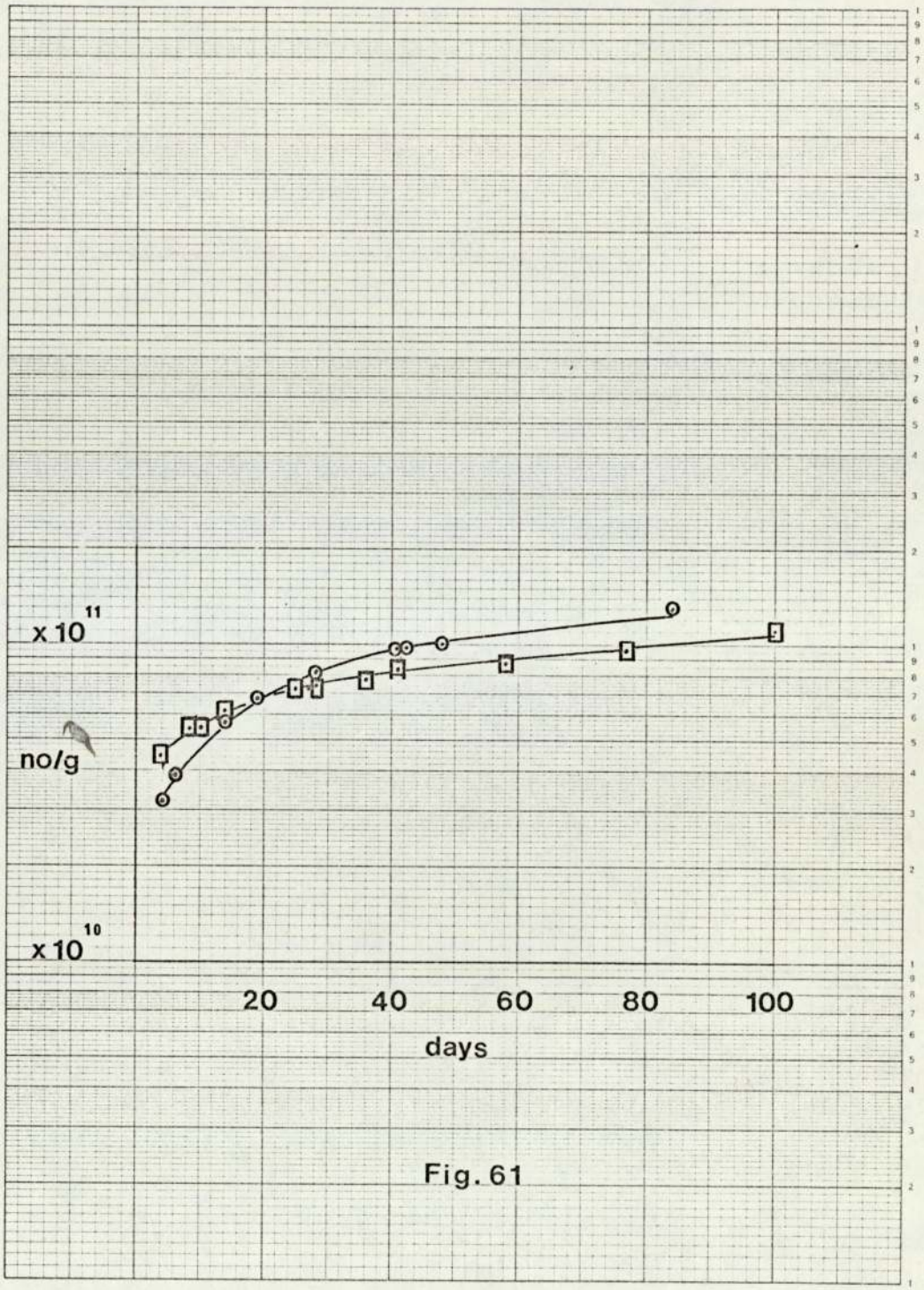


Fig. 61

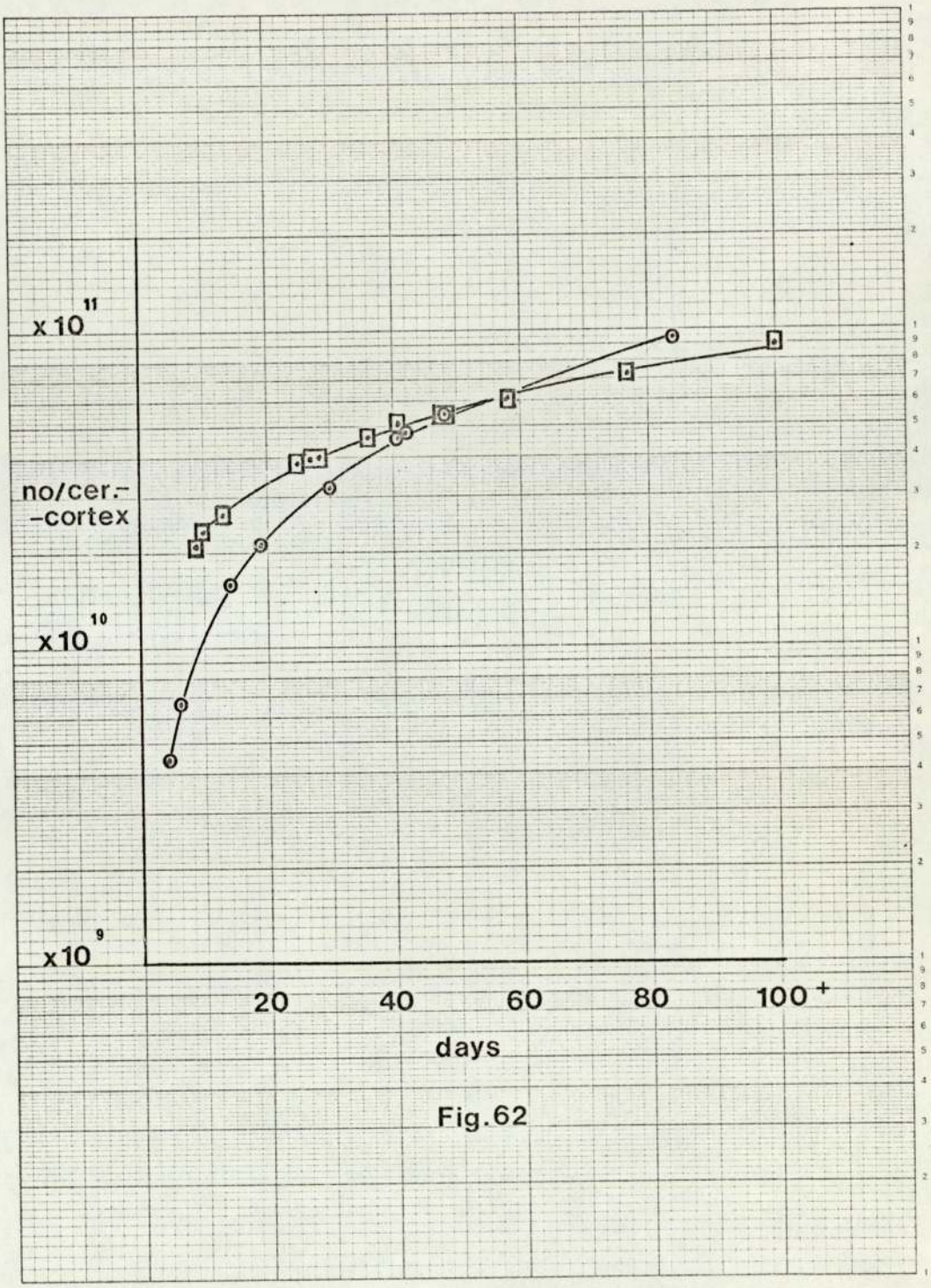


Fig.62

5. DISCUSSION

5.1 EVALUATION OF METHODOLOGY

The method for enumerating synaptosomes introduced by Clementi et al (1966) does not appear to have been much used by subsequent workers. Hence the claim by Clementi et al that the method could be used to determine numbers of synapses requires critical analysis.

Whilst the technique would, according to the reported methodology, appear straightforward experience has shown that considerable care must be exercised in the protocol. The main problems and their solution have been referred to in previous sections (2.162, 3.3, 3.4, 3.5, 3.6 and 4.2) of this thesis and there is no necessity to repeat them here.

However, it is important to distinguish between synapses and synaptosomes. The latter are, of course, artefacts, in that they do not exist in the living brain. Therefore one of the major considerations in this thesis, or at least this part of the thesis, was to examine the isolation and quantification of synaptosomes, obtained by the Clementi method, with the number of synapses in the original brain. Clementi's method, designed to collect and enumerate all the extractable synaptosomes, evokes a number of fundamental questions which include: 1. do all synapses form synaptosomes? 2. are all the extractable synaptosomes found in the specified band, following ultracentrifugation in a sucrose density gradient? 3. are the methods of sampling and identification of synaptosomes satisfactory? The first question is almost impossible to answer. It is quite possible that some synapses do not form synaptosomes, and whether or not this is the case can only be resolved by comparison of data from the Clementi's method with that obtained by another technique. To date there is only this method for determining total numbers of synapses, therefore the only

comparison possible is between the results obtained by sectioning selected areas of the brain with the data published by Clementi *et al* on guinea pigs or the results of this research *vide infra*.

Questions 2 and 3 relate to the efficiency of the method and it should be noted that when the method is followed with the precision recommended there are still occasions when the appearance of the preparation is such that it must be disregarded, if scientific accuracy is to be maintained within the limits of the method. With reference to the distribution of synaptosomes in the density gradients, Whittaker and Dowe (1964) in their analysis of homogenisation techniques reported the presence of acetylcholine in fractions other than the synaptosomal. They used the detection of this transmitting agent to determine the efficiency of the shearing forces used for homogenisation. Clementi *et al* (1966) also paid particular attention to the distribution of acetylcholine in the other fractions. They estimated that only approximately 50% remained in the synaptosomal fraction and on this basis multiplied their original estimation $1.64 - 1.94 \times 10^{11}$ synaptosomes/g of guinea pig cerebral cortex by a factor of 2 giving $3.28 - 3.88 \times 10^{11}$/g. These results are remarkably close to those obtained in this work (1.2×10^{11}/g normal adult rat cerebral cortex) which if multiplied by the same factor gives 2.4×10^{11} synaptosomes/g which, in addition to changing the total number of synaptosomes/g, would also affect the calculation of the number of synaptic contacts per neurone by increasing them from 1600 to 3200. Whether the distribution of acetylcholine in the other fractions reflects actual distribution of synaptosomes is questionable. Examination of any photograph of synaptosomes will reveal that not all of them are entire. It must therefore be presumed that the synaptic vesicles, and/or their contents, lost from such synaptosomes could be distributed in the other fractions. Of course, acetylcholine is only one of a growing number of recognised transmitter agents (*vide supra* Section 2.151) which are known or believed to be functional in the CNS.

Consequently, if these are also distributed between the fractions to the same extent as acetylcholine, then the number of synapses could theoretically be increased by a factor of 2n where n = the number of transmitting agents. Such speculation, whilst being noted, should be ignored until it is clearly established that synaptosomes can be detected in the other fractions.

Comparison of results between two different methods employed by various workers is often unsatisfactory. Thus to compare, for example, the results of Cragg (Table 1, Appendix) with those of Clementi et al and this research is not entirely satisfactory for several reasons including the important point that Cragg's results are on a selected area of the brain, unlike those of Clementi's and this research. However, the results are sufficiently comparable to suggest that the Whittaker et al method must be extracting the majority of synapses.

In the analysis of results, obtained by one investigator, from one set of animals compared with those obtained from another set, e.g. normal vis à vis hypothyroid animals, the essential prerequisite to acceptable data is the standardisation of the method. The acceptability of the method following standardisation, then, relates to its reproducability rather than its accuracy. Here accuracy is defined in the biochemical sense as the ability to detect the exact amount of a material present in the sample. The distribution of results (Table 3, Appendix) suggests that the method is not as reproducable as, for instance, the determination of a biochemical parameter in which reproducability is well established. But to expect such reliability is unrealistic in view of the technical procedures involved. It is therefore essential that adequate samples are taken at the appropriate stages, e.g. after final centrifuging for epon processing, at various levels in the epon block, and at the stage when the synaptosomes are visualised in the electron microscope.

A critical evaluation of a method should also take into account alternative techniques to compare their respective values since the rationale is to determine if the method under investigation is the most suitable. There is, as will be appreciated, a limited choice of method for determining the number of synapses. The simplest and obvious method, assuming technical feasibility, would be to homogenise the brain and determine by electronic counting (Coulter Counter) the number of synaptosomes. However, until a method can be devised to remove extraneous particles of a similar size, e.g. mitochondria, axon fragments, etc., this method is not suitable. Tkayehko (1972) has reported a method employing electrophoresis to separate synaptosomes. If this technique is sufficiently specific then it may provide the means for employing rapid electronic counting on pure fractions of synaptosomes.

A second group of methods involves the use of 'markers'. These include specific staining methods for synaptic structures, e.g. synaptic vesicles (already tried without success), and pre-synaptic junctions. Such methods possess the technical problems associated with the method used in this research. Furthermore, using the criteria indicated in Section 4.21, little difficulty was encountered in identifying synaptosomes once sufficient experience had been gained. Therefore little support can be given to this type of approach. The biochemical determination of a substance(s) specific to the synapses would also seem to be an attractive solution to the problem, and the detection of transmitter agents fits such a requirement. Unfortunately, the identification of CNS transmitter agents is still an area of incomplete knowledge. Their estimation, always assuming that they can be measured accurately, would only provide a part answer because not all transmitting agents have been identified and furthermore, synapses are likely to contain varying amounts of neurotransmitter substance. Hence estimation of synaptic numbers from neurotransmitter quantities

would be extremely difficult.

There appears to be only two other methods available; either fixing whole tissue and location of synapses in specific areas, or the preparation of synaptosomes from whole brain regions. In the case of the former method it is well established that the number of neurones and their processes, found within the cerebral cortex, varies between the layers (Schafer, 1920). Furthermore, it is not unreasonable to assume that there is an uneven distribution of neurones between all the various architectonic areas. Indeed Cragg (1967) has shown variation in the number of neurones in the visual cortical area compared to the motor areas. In addition he has also showed variation in the number of synapses in these two areas. (It should be noted that a similar variation is found in a number of animals; Table 1, Appendix. Therefore it is not unreasonable to assume that both neurone and synaptic densities will vary throughout the brain. On this premise, to determine total number of synapses, and the effect of some influencing agent, would require sectioning through all layers and regions of the brain, a rather time consuming process. An exception would be where there are good grounds for assuming that the effect of the influencing agent would be local, e.g. light on the occipital regions, sound on the auditory area of the temperal lobe. It is therefore not surprising that many authors have chosen this type of investigation (Section 2.17).

The remaining method is to employ the technique used in this research. Appreciation of the major sources of error; homogenisation, differential centrifugation, latex distribution, and identification of synaptosomes will result in particular attention being given to these stages with resulting reduction in potential error. If, in addition, adequate samples are taken, the method, as indicated by the compatible results of Clementi et al and this research, would seem suitable for estimating synaptic densities. Finally it should be appreciated that this

method is also laborious and consequently more fitted to a research team than the limited resources of a single investigator.

The creation of a hypothyroid state is also deserving of comment at this stage. From experience gained in the administration of ^{131}I it is most unlikely that this method would be used again. Grounds for this decision are various and one reason is dealt with further on in this section, but others include: the loss of animals through the lethal effect of ^{131}I which reduces the experimental samples; some variation in the degree of effect in the survivors; the unnecessary use of a hazardous reagent; and the need to synchronize the reception of the ^{131}I with the birth of the animals which, although not difficult, is restrictive in overall planning of research work. Alternative methods of inducing hypothyroidism are indicated in the Introduction (2.242) and although lacking experience in them the choice would be to administer methyl -thiouracil which would appear to be rather more specific than ^{131}I vide infra.

5.2 SYNAPTOGENESIS

Quantitative determinations on synaptogenesis in selected areas of the brain have been reported, for example, in the molecular layer of the parietal cortex of rats (Aghajanian and Bloom, 1967), in the superficial motor cerebral cortex of rats up to the age of 26 days (Armstrong-James and Johnson, 1970), and the molecular layer of the rat cerebellar cortex (Nicholson and Altman, 1972). However, this thesis contains the first data, as far as is known, on the total number of synapses in the rat cerebral cortex at various stages during and after the completion of synaptogenesis.

The experimental data clearly indicates that rats are born with

established neuronal connections albeit they are not as extensive as in the adult animal; at birth they are in the order of 3.5×10^{10}/g rising to between $1 - 2 \times 10^{11}$/g cerebral cortex in the adult animal, 1.0×10^{10} (at birth) rising to 1.1×10^{11} (adult animal) in the whole of the cerebral cortex. These latter figures indicate a tenfold increase during the final stages of brain development. This increase follows an "explosive" growth spurt during the first 42 days (post-natal) and Fig. 58 indicates a logarithmic growth pattern. These findings bear a close similarity to those obtained in the rat parietal cortex by Aghajanian and Bloom (1967). In their report, the maximum number of synapses was reached in 35 days and the pattern of development mirrors that found in this investigation. Similary Cragg (1972) found that synaptogenesis in the cat visual cortex was complete between 27 and 35 days of age. Whilst it is accepted that the whole of the CNS does not develop uniformly and that certain areas may be in advance of others, the findings of Nicholson and Altman (1972) and Rebière and Legrand (1972) that synaptogenesis in the molecular layer of the cerebellum is concluded by 35 days still further supports the findings of this research. Further support, of a general nature, is provided by Davison and Jobbing (1968) who showed that the rat brain achieves its greatest rate of growth during the first twenty days after birth, with a significant growth spurt between the third and tenth day of post-natal life.

It might be argued that the increase in numbers of synaptosomes with age is due to some factor other than an increase in the number of synapses in the brain. For instance, it might be due to a change in the fragility of the synaptosomes which allows the more mature synapse to withstand the rigours imposed by the extraction and processing method. That the synapse is relatively fragile is evident from the difficulties encountered with the method which resulted, on occasions, with a homogenate that was unsatisfactory because of

disruption of the synaptosomes. Indeed there were occasions when the prepared homogenates were sufficiently unsatisfactory to support such a concept. However, in view of the satisfactory preparations obtained from some animals and the findings of Aghajanian and Bloom, and other workers previously mentioned, the evidence strongly supports the view that synaptogenesis follows a development path indicated in Figs. 47 and 58.

The estimation of 3.2×10^3 synapses/neurone in the cerebral cortex of adult rats referred to earlier in this section is very close to the 3.6×10^3 synapses/neurone estimated by Clementi et al for neurones in the cerebral cortex of guinea pigs. These figures are certainly less than the maximum number of synapses/neurone detected in the visual cortical regions of the rat (51×10^3/neurone) by Cragg (1970). N.B. This latter figure of 51×10^3 synapses/neurone in the rat visual cortex is considerably higher than the numbers determined for the mouse, monkey or cat visual cortex by the same worker (Table 1). Cragg (1967) has also estimated that there may be as many as 60×10^3 synapses/neurone in the monkey motor cortex (Table 1). Of course there is no reason to believe that the number of synapses should be the same in all animals. On the contrary, if the degree of communication between neurones influences or reflects intelligence, then it must be assumed the more mentally advanced the animal the great the synaptic density. Therefore a monkey would be expected to possess a higher synapse-neurone ratio than the rat. The results of Clementi et al are extraordinarily close to those obtained in this research, and although the animal species are different they are nevertheless of a similar body and brain size. Whilst it is well recognised that rats can be trained to perform maze tests etc. and guinea pigs are rarely, if ever, used for such experiments, it is probable that their brain capacities in terms of intelligence are not greatly different. Therefore it is presumed that the figures of

1.6 (3.2) x 10^3 synapses/neurone, 1.2 x 10^{11} synapses/g of cerebral cortex, and 1.1 x 10^{11} synapses/total cerebral cortex, for the normal adult rat, are realistic figures and consequently acceptable data for comparative purposes.

5.3 EFFECT OF HYPOTHYROIDISM

5.31 Gross Body Changes

The gross retardation of body development observed in the experimental animals was in accordance with those reported by other workers. In addition to a reduction in body weight and to a considerably less extent, brain weight, there appeared to be a reduced level of calcium in the bone structures. This observation was based on the ease with which the skull bones of the experimental animals were cut with ordinary dissecting scissors. Whilst this is hardly a scientific means of assessing calcium levels, the difference from normal animals was sufficiently pronounced to deserve comment. The interplay between the parathyroid hormone (PTU) and calcitonin, produced by the thyroid, (the site of calcitonin production is controvertial and may be either the thyroid or the parathyroid), maintains the calcium levels in the body together with the rates of absorption and excretion. Parathyroid-ectomy results in various effects including reduced levels of calcium in the serum particularly the diffusable fraction (Turner, 1966). The implication of the reduced ossification is that the administration of ^{131}I affected calcium metabolism. This could be due either to impairment of the thyroid or the parathyroids or both glands. Calcium ions in addition to their important role in muscular contractions etc. are also essential for the emission of acetyl choline from the synaptic vesicles into the synaptic cleft (Eccles, 1973). In view of this latter fact it could be

construed that a reduced calcium level in the serum would result in a similar reduction in the CNS with a consequential effect on the release of acetylcholine. It is not suggested that a reduction in calcium levels could be the cause of cretinism, since this condition can be induced in a number of ways which need not affect the parathyroids. However, it is conceivable that the administration of ^{131}I may have more than one effect, and to attribute, for example, impaired learning ability solely to the effect of thyroxine deficiency on the CNS may exclude other contributory factors.

5.32 Mitochondria

Before dealing with the quantitative effects of hypothyroidism on synaptogenesis some comment is necessary on the structural appearances of the synaptosomes. In view of the role of thyroxine and other thyroid hormones on energy metabolism, as noted by the physical responses of hypothyroid patients and animals, it is not surprising that interference in thyroid function can affect mitochondrial activity (Tata, 1971). Indeed the evidence shows that not only are the respiratory enzymes affected, but also the number and proportion of cristae and cristae-associated enzymes. Furthermore, it has been shown that thyroxine in excess can cause swelling of mitochondria on some tissues (Ford, 1968).

The observation, prior to the appreciation of the effect of thyroxine on mitochondria of "vacuolated" structures within the synaptosomes of experimental animals raised the possibility that neonatal hypothyroidism could induce cretinism by interfering with mitochondria, thus reducing energy levels with consequential impairment of neuronal function. It is well established that ATP levels in the brain are reduced in hypothyroidism (Table 14, Appendix).

Such interference could affect transmitter agent metabolism and subsequently neuronal communication. Again, there is evidence that some transmitter agents are depressed in hypothyroidism (Table 14, Appendix). The preliminary evidence on both the number of synaptosomes containing mitochondria and the percentage of vacuolated mitochondria would seem, despite the limited number of results, not to invalidate this hypothesis.

When the vacuolated structures were first observed it was considered that they could be: immature mitochondria, degenerate ones, or artefacts. The latter idea was at first dismissed since they had been found in both normal and experimental animals. The possibility that they were degenerate mitochondria was thought unlikely since they were observed in animals as young as 4 days (Experiment 51). This left the third possible answer that they were immature mitochondria. Such a concept required an appreciation of mitochondrial development which, regretably, is an aspect of cell development that is still obscure. Although difficult to substantiate, it was considered that the cristae may develop as a secondary stage of mitochondrial maturation and the vacuolated structures represented an earlier stage.

Alternative possibilities included the idea that the vacuolated structures could be related to synaptic vesicle formation, but this was considered to be most unlikely for the following reasons: differences in the width of the membrane; no intermediate structures; there is no similarity between these vacuolated structures and the complex synaptic vesicles reported by other workers, (Section 1.45). Yet another possibility is that they could be structures containing metabolic reserves but such a proposal must be dismissed on the following grounds: there is no structural evidence that they are metabolically active; if they are not active then they must be

produced elsewhere in the cell and transported down the axon. No report has been found, in the literature, in which such structures have been observed in either the axon or the perikaryon.

Finally, it was recalled that Gray and Whittaker (1962) reported in the description of their technique for producing sub-cellular particle extracts from the brain that sucrose could have a deleterious effect on some particles. They reported both swelling and shrinkage of the mitochondria present in fraction C. This fraction contains free mitochondria, hence they are directly exposed to the sucrose solution. The photographs published show the appearance of the various sub-cellular particles and reveal that the cristae of the mitochondria appear disrupted. The processing technique of Gray and Whittaker employed osmium tetroxide and phosphotungstic acid for fixation and staining, and the results, according to the published photographs, do not have the clarity and definition which would seem possible with potassium permanganate fixation, as indicated in the photograph in Section 4, Figs. 25 and 26, etc. The mitochondria in Gray and Whittaker's photographs did not in any way resemble the vacuolated structures recorded in this thesis. An article on the synapse by Whittaker and Gray (1962) contains information and photographs, not surprisingly, very similar to the paper by Gray and Whittaker (1962). Of interest, though, is the use of the word vesication to describe the mitochondria present in fraction C. Although, as previously commented, they do not resemble the vacuolated structures, the term vesication aptly describes, according to the Oxford English Dictionary, the vacuolated structures observed in this research.

Until there is evidence to the contrary, it must be presumed that the structures observed are vacuolated mitochondria. If these structures had only been found in irradiated animals the

reason for their presence would have been obvious. That they are present in normal animals does present difficulties in providing an acceptable explanation. The effect of sucrose on mitochondria cannot be ignored, although it must again be stressed that the abnormalities recorded by Gray and Whittaker were observed in mitochondria directly exposed to the sucrose, and not protected by the synaptosome as in the instance of the vacuolated mitochondria. That there is a higher percentage in irradiated animals unquestionably suggests that the effect of sucrose, if any, is not the only factor involved. A case could be made, because of the effect of thyroxine deficiency on lipid metabolism (Table 14, Appendix) and on the induction of membraneous body formation in synapses (Cragg, 1970), that the mitochondrial membranes are so affected that they are more sensitive to the action of sucrose. This would imply that hypothyroidism could affect the integrity of the mitochondrial membranes with a possible consequential repercussion on function and, in turn, neuronal metabolism and activity. Even if it assumed that sucrose has no effect there still remains the implication that hypothyroidism may have an effect on mitochondria to induce vacuolated forms.

A similar problem of explanation is also presented by the data which shows that not all synaptosomes contain mitochondria. Several other workers have reported both variation in numbers and size of mitochondria in synapses (Gray, 1959; Colonnier and Guillary, 1964; Schadé and Ford, 1967). The need for an explanation presupposes a basic question which is: "Why should only approximately one-third of the synaptosomes possess mitochondria whilst it would appear that the remainder do not?" If all synapses were of the same size, and all mitochondria located in the same position, and if the sections cut were one-third the thickness of the synaptosome, an explanation would indeed be possible! But the general opinion

is that synapses vary in size (Section2.141) and in any case many synapses have two or more mitochondria. It is therefore presumed that the regularity with which only 25 - 45% of synaptosomes are encountered containing mitochondria indicates, quite simply, that not all synapses contain mitochondria. Perhaps the possession of mitochondria and their number indicates the degree of activity of the synapse and there is evidence that there is an increase in numbers of mitochondria in areas of high activity (Schadé and Ford, 1967, Section 2.141). Thus the presence of 2^{+} mitochondria would indicate a synapse which was highly active. Correspondingly, a synapse with only one mitochondrion would be used less frequently. Synapses without mitochondria would depend for their ATP requirements on the perikaryon of the neurone. Such circumstances could indicate either close proximity of the two neurones, hence a short axon length, or a synapse which was used infrequently. If such a hypothesis has substance, then it is conceivable that by regular or serial sectioning of brain tissue, and the estimation of numbers of mitochondria per synapse, areas of high and low activity could be identified and located.

The results also indicate that the synapses from irradiated animals are less likely to have mitochondria than normal rats. If this finding is substantiated, and the present data is such that confirmation would need to be obtained, the significance is important and has a bearing on neuronal function. A lower number of mitochondria would result in a reduced energy level with a possible low threshold of activity. Such a situation could well be reflected in the dull responses of the cretin.

5.33 Synaptogenesis

To turn to aspects of hypothyroidism more directly concerned with brain development and function. Unquestionably the effect

of thyroxine deficiency is various and Table 14 (Appendix) provides a selection of current information.

The data produced by this research provides an insight into the effect of hypothyroidism on synaptogenesis which is contrary to that expected. The first observation to be made is that the effect of hypothyroidism is related to the time of its commencement; this supports the findings of other workers (Eayrs, 1961). For example, it was found that animals which had had their thyroids removed at 21 days of age, although showing the expected deficiency of body weight, nevertheless had a similar number of synapses to that of normal animals (Fig. 51). This is not surprising in view of the rate of neuronal development in the suckling animal. It is clear from the findings of other workers (Aghajanian and Bloom, 1967), in addition to this research, that the development of neurone connections is well advanced by 21 days of age. Furthermore, it is possible that the levels of thyroxine may be sufficient at this stage of the animal's development to sustain the continued rate of synaptogenesis to its final stage which is concluded by approximately 42 days.

To create the greatest effect of thyroxine deficiency the thyroid must be inactivated at the earliest post-natal date. This was achieved by the administration of ^{131}I at birth. In this instance it would appear that there is an immediate response, that is within 4 - 6 days which is the earliest time at which numbers of synaptosomes were determined. The numbers of synaptosomes are appreciably less in the irradiated animals compared to the controls. However, the reduced number is a transient phenomenon since by approximately58 days (Figs. 50 and 59) the numbers of synaptosomes would appear to have reached almost normal levels. This is of particular interest as there are reports in the literature that

hypothyroidism causes reduced numbers of synapses/neurone. For example, Cragg (1970) showed that there was a 22% reduction in boutons /neurone between 21 and 35 days of age in the rat cortex overlying the dorsal hippocampal commissure. Similarly at 35 days of age it was found, in the present research, that the number of synaptosomes in the normal rat cerebral cortex was 7.0×10^{10} whilst in the thyroidectomised (^{131}I) animal it was 2.5×10^{10} which is equivalent to a 60% reduction. This reduction in synaptic numbers disappears however in the adult animal. This raises the question of whether Cragg's findings reflect a permanent reduction in synaptic numbers/ neurone. Rebière and Legrand (1972) also report a reduction of approximately 70% at 35 days in the rat cerebellum. They also report a similar reduction in "adult" animals although the actual age is not given.

Figure 60 is interesting since it suggests that the rate of synaptogenesis is faster in hypothyroid than normal animals. Such an overall effect was initially surprising since it was expected, if anything, that there would be a slower growth leading to a permanent reduction in synapses. Although admittedly there were no grounds for this supposition other than it was expected that this would be the effect of hypothyroidism. Explanations for this situation are necessary and the following hypothesis is offered. At birth the development of the rat brain has reached an advanced but not complete stage. That this is the case is supported by the following observations: all neurones have been formed; as this research has shown, it is probable that a large number of the synaptic connections have been formed; and, finally, reflex and other bodily activity shows that nerve circuits are functioning. On these grounds it is surmised that the cerebral cortex at birth, has nearly concluded its development and has commenced the formation of

neuronal circuits for the storage of acquired information. If, therefore, at birth the thyroid is suddenly inactivated with the consequential loss to the body, including the brain, of thyroxine, this specific hormonal starvation may have a markedly immediate effect of halting a highly dynamic process. It could be argued that at the end of the first week of post-natal life the development of cell connections was reaching a maximum. Such a developmental stage is feasible since Eayrs and Goodhead (1959) have shown that the maximum axon density occurs between 6 and 18 days. It might therefore be the case that thyroxine starvation halts this process at a critical stage. This retardation would appear as a reduced number of synaptic junctions/neurone. Since hypothyroidism does not completely halt body growth in the initial stages of its effect (Fig. 22) but certainly slows the rate of growth, it is believed that after the initial setback of thyroxine deficiency the maturation of the brain continues at a slower rate. Assuming, as the evidence suggests, that the brain of the hypothyroid animal eventually achieves the normal number of synapses then at some stage there would be an artificial increase in the rate of synaptogenesis (Fig. 62) because the final connections are being made in a shorter period of time.

5.4 CONCLUSION

This thesis was designed to investigate a number of inter-related topics, namely: the value of a method for determining the number of synapses within the cerebral cortex; the effect of throidectomy on synaptogenesis and whether cretinism could be due to a reduced number of inter-neuronal connections.

That hypothyroidism, if induced in the early development of the animal, affects intelligence is without dispute. The results of

experimental work with hypothyroid rats by other workers provides ample evidence of impaired learning ability which supports the symptoms observed in athyroid patients of a sluggish dull state of behaviour. However, hypothyroidism, according to the results of this research, does not affect the final numbers of synapses. Therefore this aspect of neuronal development does not provide the obvious answer to this particular type of mental sub-normality. Alternative answers must be sought.

It is clear from the large numbers of reports that the thyroid hormones exert their effect on cells in a variety of ways, and whilst it would appear that the brain does not always respond in the same way as other tissues, e.g. neuronal mitochondria do not swell, protein concentrations do not increase in hyperthyroidism, it is quite apparent that the brain is influenced at both the structural and metabolic levels.

Reasons for impaired intelligence based on this research and the data in the literature could include the following: intelligence is clearly linked to the establishment of neuronal circuits; if there is any interference in such developments, particularly at the highly sensitive period of the first 40 days, then this could interfere with the order in which neuronal connections are made. That there is order in the establishment of neuronal circuits is, of course, unknown but in view of the orderly way in which the body achieves maturity it would seem logical that the development of the neuronal connections may also follow an orderly manner rather than a haphazard process. The completion of one set of neuronal circuits may therefore initiate the commencement or completion of others. If during this period the process is temporarily halted, the out of phase development may affect the formation of neuronal circuits and consequently, learning ability. That a physiological upset during the critical phase of growth can cause a permanent regression in brain development has been shown by Dickerson

et al (1966) in their work on the effect of malnutrition in the post-natal development of pigs.

A method for assessing neuronal function, in cretinised rats, is to hold them upside down and drop them from a height. In the normal animal the rat will revert and land on its feet, the cretinised animal will fail this test. Since such a test reflects a highly efficient CNS it can only be assumed that either the neurones in the hypothyroid rat are not firing sufficiently quickly, or the circuits which control such physical action are non-existent. Further support to the concept of impaired neuronal circuitry is the pronounced unsteady gait of the hypothyroid animal.

An obvious and probably more realistic solution to the problem is the effect of thyroxine deficiency on metabolism. A considerable number of cellular enzymes, in addition to RNA values, synaptosomal proteins (cerebellum), and myelination are reduced in amounts following thyroid-ectomy (Table 14, Appendix A). Such reductions must affect the functional efficiency of neurones. Obvious examples include reduction in ATP. A more striking and direct interference with synaptic activity could be the reduction in transmitting agents such as GABA and glutamic acid, and the enzymes associated with acetylcholine metabolism. The reduction in synthesis of RNA, following thyroidectomy, could be of considerable importance in view of the work of, for example, Glassman et al and Rose et al (Rose, 1973) who have shown that the rate of RNA synthesis in the brain increases following stimulation or exposure to a learning situation. Many of the changes observed in the CNS, following thyroidectomy, could cause a reduction in neuronal efficiency, or as this research has shown, interfere with the development pathway along which the brain proceeds. However, it is highly probable that the solution to cretinism may not be found in the alteration of one parameter but may be due to the summation of several of the various changes which have been

observed (Table 14) together with those yet to be discovered.

This research has provided new information on the effect of hypothyroidism on synaptogenesis in that the reduction in numbers of synapses, shown by both this research and other workers, may not be a permanent reduction. In addition, it has drawn attention to the possibility that thyroxine deficiency may also have an effect on the mitochondria of synapses. As a consequence of these findings and others discussed in this section, a number of new research lines emerge. These include the following:

1. A more detailed investigation into the effect of hypo- and hyper-thyroidism on neuronal mitochondria, particularly those to be found in the synapses.
2. An investigation into the number of mitochondria present in synapses and the possibility that this could reflect the degree of neurone-synapse activity.
3. The continuing need for a simpler and more reliable technique for counting synapses. Perhaps the use of electrophoresis and a more accurate counting technique using an electronic counter may provide the answer.
4. To investigate modifications to the Clementi et al method which would reduce the adverse shearing effects by, for example, fixation of the brain, prior to homogenisation.

APPENDIX

TABLE 1.

Number of Synapses/Neurone (Normal Animals)

Mouse	visual cortex	7×10^3	Cragg	1967
"	motor "	13×10^3	"	"
Monkey	visual "	5.6×10^3	"	"
"	motor "	60×10^3	"	"
Cat	visual "	8×10^3	"	1972
Guinea pig	cerebral "	$2.7\text{-}7.6 \times 10^3$*	Clementi et al	1966
Rat	visual "	$19\text{-}51 \times 10^3$	Cragg	1970

Number of Synapses/mm^3 (Normal Animals)

Rat	molecular layer parietal cortex	1.4×10^9	Aghajanian et al	1967
Mouse	visual cortex	6.6×10^8	Cragg	1967
"	motor "	8.5×10^8	"	"
Monkey	visual "	6.2×10^8	"	"
"	motor "	9.6×10^8	"	"
Rat	visual "	3.2×10^8	" (Gray)	"
"	motor "	1.4×10^9	Armstrong-James et al	1970
Rat	cerebral "	2.3×10^9	Cragg	1970
Cat	visual "	3.4×10^8	"	1972
Rat	lateral geniculate nucleus	1.5×10^9	"	1970

Numbers of Synaptosomes/g. (Normal Animals)

Guinea pig	cerebral cortex	3.6×10^{11}*	Clementi et al	1966

Numbers of Synapses Using Other Measurements

Rat	cerebellum	148/420 μm^2	Rebière and Legrand	1972
"	"	40/96 μm^2	Nicholson and Altman	1972
"	"	32 synaptic profiles/cell	" " "	"

TABLE 2.

Exp.No.	Age (Days)	Type of Animal	Body Wt/g	Brain Wt/g	Cerebral Cortex Wt/g	Cerebellum Wt/g	No. of Synapses g.	CO/CE
14	A	N		1.4	0.8		1.4×10^{11}	CO
16	27	N	65	1.3	0.8		9.4×10^{10}	"
17	9	N	18.5	0.8	0.5		7.1×10^{10}	"
18	A	N	289	1.4	0.6	0.06	2.0×10^{11}	"
19	28	N	78	1.2	0.6		$6.6. \times 10^{10}$	"
"	28	N	76	1.2	0.6		5.3×10^{10}	"
30	25	T	54	1.3	0.6		5.1×10^{10}	"
"	25	T	61	1.4	0.8		1.4×10^{11}	"
"	25	S	55	1.3	0.6		8.4×10^{10}	"
31	36	T	80	1.3	0.9		1.2×10^{11}	"
"	36	T	93	1.5	0.9		1.7×10^{11}	"
"	36	S	110	1.5	0.8		9.4×10^{10}	"
34	58	S	233	1.5	0.7		9.2×10^{10}	"
"	58	T	155	1.5	0.8		9.2×10^{10}	"
"	58	T	130	1.6	0.7		2.7×10^{11}	"
35	77	T	131	1.6	0.5		1.5×10^{11}	"
"	77	T	217	1.6	0.6		1.0×10^{11}	"
"	77	S	324	1.6	0.6		1.9×10^{11}	"
37	112	T	154	1.8	0.7		8.4×10^{10}	"
"	112	S	422	2.0	0.8		4.2×10^{10}	"
"	112	N	522	1.9	0.6		6.9×10^{10}	"
41	168	S	452	1.6	0.5		9.3×10^{10}	"
"	168	T	137	1.5	0.6		9.8×10^{10}	"
"	168	T	109	1.6	0.7		1.3×10^{11}	"

Exp.No.	Age (Days)	Type of Animal	Body Wt/g	Brain Wt/g	Cerebral Cortex Wt/g	Cerebellum Wt/g	No. of Synapses g.	CO/CE
44	41	^{131}I	56	1.4	0.6		5.0×10^{10}	CO
47	84	^{131}I	29	0.9	0.3		2.0×10^{11}	"
49	10	N	22	0.9	0.4		1.9×10^{10}	"
"	10	N	22	0.9		0.08	4.7×10^{9}	CE
"	44	N	208	1.4		0.16	7.6×10^{9}	"
51	4	^{131}I	4	0.2	0.1	0.05	5.6×10^{10}	CO
53	6	^{131}I	6	0.3	0.1		2.6×10^{10}	"
"	6	^{131}I	6	0.3		0.07	1.4×10^{10}	CE
54	13	C	11	0.6	0.3		7.9×10^{10}	CO
"	13	C	11	0.6		0.06	4.6×10^{9}	CE
55	14	^{131}I	19	0.8	0.5	0.06	4.9×10^{10}	CO
56	19	^{131}I	9	0.6	0.4		6.8×10^{10}	"
"	19	^{131}I	9	0.6		0.05	2.2×10^{10}	CE
57	21	C	85	1.2	0.6	0.18	9.9×10^{9}	"
58	27	C	49	1.2		0.12	2.2×10^{10}	"
59	28	^{131}I	50	1.2	0.7		1.0×10^{11}	CO
"	28	^{131}I	50	1.2		0.12	4.5×10^{10}	CE
60	41	C	111	1.3	0.6		5.1×10^{10}	CO
"	41	C	111	1.3		0.08	1.5×10^{10}	CE
61	42	^{131}I	92	1.3	0.6		6.8×10^{10}	CO
"	42	^{131}I	92	1.3		0.16	6.4×10^{9}	CE
62	48	^{131}I	32	1.0	0.5		1.0×10^{11}	CO
"	48	^{131}I	32	1.0		0.11	2.1×10^{10}	CE
63	48	C	148	1.4	0.7		1.9×10^{11}	CO
"	48	C	148	1.4		0.17	2.0×10^{10}	CE

Exp. No.	Age (Days)	Type of Animal	Number of Synaptosomes x 10^{10}
16	27	Normal	9.4
19	28	"	6.6
19	28	"	5.3
14	Adult (6 months)	"	14.0
18	" "	"	20.0
30	25	Sham Operated	14.0
30	25	" "	8.4
30	25	" "	5.1

TABLE 3

Numbers of synaptosomes in normal or sham operated animals within the age group 25 - 28 days.

TABLE 4.

Exp.No.	Exp.Type	Age of Animal Days	Type 1 No/g	% Type 1	Vacuolated No/g	% Vacuolated
19	N	28	2.4×10^{10}	35	-	-
30	O	25	2.8×10^{10}	53	-	-
30	O	25	5.6×10^{10}	39	-	-
30	S	25	3.9×10^{10}	46	-	-
31	O	36	4.6×10^{10}	39	-	-
31	O	36	7.7×10^{10}	45	-	-
31	S	36	2.8×10^{10}	30	-	-
34	S	58	4.3×10^{10}	46	-	-
34	O	58	4.2×10^{10}	44	-	-
34	O	58	3.3×10^{10}	12		8.5
35	O	77	6.1×10^{10}	41	-	-
35	O	77	3.6×10^{10}	33	-	-
35	S	77	7.0×10^{10}	38	-	-
41	S	24	4.6×10^{10}	48	-	-
47	^{131}I	84	6.2×10^{10}	31	-	-
49	N	10	4.0×10^{9}	21	6.3×10^{8}	3
51	^{131}I	4	1.8×10^{10}	32	7.6×10^{9}	13
54	N	13	1.9×10^{10}	24	6.4×10^{9}	8
55	^{131}I	14	10	26	1.8×10^{9}	3
56	^{131}I	19	2.2×10^{10}	32	7.4×10^{9}	11
59	^{131}I	28	4.4×10^{10}	42	1.4×10^{10}	14
60	N	41	1.0×10^{10}	27	1.3×10^{9}	3
61	^{131}I	42	2.5×10^{10}	38	2.8×10^{9}	4
62	^{131}I	48	3.6×10^{10}	36	1.9×10^{10}	20
63	N	48	5.3×10^{10}	27	7.9×10^{9}	4

Age Days	Type 1 x 10^{10}/g	Type 1 %	Vacuolated x 10^{10}/g	Vacuolated %	Type of Animal
0- 5	118	32	0.76	13	^{131}I
6-10	0.4	21	0.63	3	N
11-15	1.9	24	0.64	8	N
"		26	0.18	3	^{131}I
15-20	2.2	32	0.74	11	^{131}I
26-30	4.4	42	1.4	14	^{131}I
41-45	1.0	27	1.3	3	N
"	2.5	38	0.28	4	^{131}I
46-50	3.6	36	1.9	20	^{131}I
"	5.3	27	0.79	4	N

TABLE 5.

Number of Type 1 synaptosomes and synaptosomes containing vacuolated structures per gram of cerebral cortex and % of total number of synaptosomes.

N = Normal animals

^{131}I = Thyroidectomised by the administration of ^{131}I

TABLES 10 - 13.

```
OLD SIXCRS£***

FILE NOT SAVED--SIXCRS

OLD SIXCR£***

READY.

100 176,14,27,9.4,9,7.1,176,20,28,6.6,28,5.3,25,8.4,36,9.4,58,9.2,77,
110 19,112,4.2,112,6.9,168,9.3,10,1.9,4,5.6,13,7.9,41,5.1,48,19
RUN



SIXCR£    10:08   G265 C 12/06/74

VERSION OF MARCH 26, 1970

XMEAN: 63.7778                    YMEAN  9.35

NUMBER          CURVE          INDEX          A              B

  1             Y=A+B*X        .223313        6.67057        .042012
  2             Y=A*EXP(B*X)   .202828        5.06232        4.43125 £3
  3             Y=A*X↑B        .25162         3.02013        .266689
  4             Y=A+(B/X)      .150164        10.9145        -34.1391
  5             Y=1/(A+B*X)    .126389        .189331        -6.46007 £4
  6             Y=X/(A*X+B)    .116285        .11999         .614042

FOR WHICH CURVE ARE DETAILS DESIRED (NUMBER) ? 3

COEFFICIENTS:

          EXPECTED VALUE     95PCT CONFIDENCE LIMITS

     A:       3.02013          1.18627          7.68898
     B:       .266689          2.23479 £-2      .511029

TYPE 1 FOR CONFIDENCE LIMITS ON ESTIMATED Y,
OR   2 FOR PREDICTION LIMITS ON OBSERVATIONS OF Y.
WHICH ? 2


X-ACTUAL        Y-ACTUAL        Y-ESTIM          95PCT PREDICTION LIMITS

  4               5.6            4.37107          1.2313           15.5171
  9               7.1            5.42639          1.64604          17.8888
 10               1.9            5.58102          1.70573          18.2607
 13               7.9            5.98551          1.85976          19.264
 25               8.4            7.1259           2.27325          22.3374
 27               9.4            7.27367          2.32427          22.7626
 28               6.6            7.34456          2.34852          22.9687
 28               5.3            7.34456          2.34852          22.9687
 36               9.4            7.85368          2.51843          24.4916
 41               5.1            8.13086          2.60774          25.3518
 48               19             8.47995          2.717            26.4665
 58               9.2            8.9189           2.84935          27.9175
 77               19             9.61902          3.04906          30.3456
112               6.9            10.6299          3.31402          34.0959
112               4.2            10.6299          3.31402          34.0959
168               9.3            11.8438          3.59894          38.9767
176               14             11.9916          3.63138          39.599
176               20             11.9916          3.63138          39.599

FOR WHICH CURVE ARE DETAILS DESIRED (NUMBER) ? STOP

USED     13.17 UNITS.
```

TABLE 10.

```
100 41,5,84,20,4,5.6,6,2.6,14,4.9,19,6.8,28,10,42,6.8,48,10,
110 48,19,←
RUN


SIXCR£     10:19    G265 C 12/06/74

VERSION OF MARCH 26, 1970

XMEAN: 33.4                          YMEAN  9.07

NUMBER          CURVE              INDEX          A              B

  1             Y=A+B*X            .64734       2.51657        .196211
  2             Y=A*EXP(B*X)       .62611       3.83192        2.04266 £E
  3             Y=A*X↑B            .543956      1.68641        .473864
  4             Y=A+(B/X)          .255886      11.6644        -3E.2537
  5             Y=1/(A+B*X)        .483926      .249249        -2.77985 £3
  6             Y=X/(A*X+B)        .34923       .106737        .732286

FOR WHICH CURVE ARE DETAILS DESIRED (NUMBER) ? 3

COEFFICIENTS:

          EXPECTED VALUE     95PCT CONFIDENCE LIMITS

     A:       1.68641          .522567          5.44232
     B:       .473864          .119378          .82835

TYPE 1 FOR CONFIDENCE LIMITS ON ESTIMATED Y,
OR   2 FOR PREDICTION LIMITS ON OBSERVATIONS OF Y.
WHICH ? 2

X-ACTUAL        Y-ACTUAL        Y-ESTIM        95PCT PREDICTION LIMITS

  4               5.6            3.2528          .920463          11.495
  6               2.6            3.94186         1.19106          13.0457
 14               4.9            5.88941         1.94389          17.8431
 19               6.8            6.80641         2.2765           20.3502
 28               10             8.17936         2.73978          24.4187
 41               5              9.79948         3.2327           29.7057
 42               6.8            9.91203         3.26489          30.0923
 48               10             10.5595         3.44521          32.3646
 48               19             10.5595         3.44521          32.3646
 84               20             13.7661         4.23037          44.7962

FOR WHICH CURVE ARE DETAILS DESIRED (NUMBER) ?
```

TABLE 11.

```
                                                    STOP

USED     11.00 UNITS.


100 176,9.8,27,6.1,9,2.8,176,14,28,3.96,28,3.18,25,5.5,36,7,58,6.9,
110 77,15,112,4.2,112,6.5,168,7.4,10,0.85,13,2.37,41,3.3,48,13
RUN


SIXCR&     10:28    G265 C 12/06/74

VERSION OF MARCH 26, 1970

XMEAN: 67.2941                    YMEAN  6.58

NUMBER          CURVE             INDEX          A                  B
  1             Y=A+B*X           .318621        3.89904            3.98394 &8
  2             Y=A*EXP(B*X)      .348028        3.24598            7.28514 &8
  3             Y=A*X↑B           .538249        .641994            .554469
  4             Y=A+(B/X)         .397464        9.32473            -81.1688
  5             Y=1/(A+B*X)       .208075        .38783             -2.00163 &8
  6             Y=X/(A*X+B)       .54272         5.37279 &-2        5.89643

FOR WHICH CURVE ARE DETAILS DESIRED (NUMBER) ? 3

COEFFICIENTS:

          EXPECTED VALUE     95PCT CONFIDENCE LIMITS

     A:       .641994            .211394          1.9497
     B:       .554469            .271162          .837776

TYPE 1 FOR CONFIDENCE LIMITS ON ESTIMATED Y,
OR   2 FOR PREDICTION LIMITS ON OBSERVATIONS OF Y.
WHICH ? 2

X-ACTUAL        Y-ACTUAL          Y-ESTIM          95PCT PREDICTION LIMITS
  9               2.8             2.17084          .642728          7.33213
 10               .85             2.30144          .688838          7.68922
 13               2.37            2.66181          .816229          8.68048
 25               5.5             3.82512          1.22247          11.9688
 27               6.1             3.99188          1.27957          12.4535
 28               3.18            4.07319          1.30729          12.6911
 28               3.96            4.07319          1.30729          12.6911
 36               7               4.68222          1.51213          14.4983
 41               3.3             5.03233          1.62757          15.5596
 48               13              5.49195          1.77652          16.9779
 58               6.9             6.09954          1.96885          18.8965
 77               15              7.13726          2.28563          22.2872
112               4.2             8.78534          2.76055          27.959
112               6.5             8.78534          2.76055          27.959
168               7.4             11.0001          3.35126          36.1063
176               14              11.2875          3.4244           37.2058
176               9.8             11.2875          3.4244           37.2058

FOR WHICH CURVE ARE DETAILS DESIRED (NUMBER) ? STOP

USED     12.17 UNITS.
```

TABLE 12.

```
100 41,3.5,84,9.0,4,,←0.56,6,0.39,14,1.96,19,2,28,6,42,4.4,48,5
RUN


SIXCR£     10:42    G265 C 12/06/74

VERSION OF MARCH 26, 1970

XMEAN: 31.7778                    YMEAN  3.64556

NUMBER          CURVE          INDEX          A                B

  1             Y=A+B*X        .848346        .413449          .10171
  2             Y=A*EXP(B*X)   .697995        .80817           3.52948 £8
  3             Y=A*X↑B        .900729        .109746          1.00778
  4             Y=A+(B/X)      .534609        5.47417          -25.035
  5             Y=1/(A+B*X)    .452087        1.43817          -2.30517 £8
  6             Y=X/(A*X+B)    .772273        2.32981 £-2      9.3418

FOR WHICH CURVE ARE DETAILS DESIRED (NUMBER) ? 3

COEFFICIENTS:

          EXPECTED VALUE     95PCT CONFIDENCE LIMITS

     A:       .109746          4.16218 £-2      .289373
     B:       1.00778          .708176          1.30739

TYPE 1 FOR CONFIDENCE LIMITS ON ESTIMATED Y,
OR   2 FOR PREDICTION LIMITS ON OBSERVATIONS OF Y.
WHICH ? 2


X-ACTUAL        Y-ACTUAL        Y-ESTIM        95PCT PREDICTION LIMITS

  4               .56            .443746          .157843          1.24751
  6               .39            .667723          .250799          1.77774
 14              1.96           1.56833           .632292          3.89005
 19              2               2.13351          .868077          5.24361
 28              6               3.15362          1.28095          7.76404
 41              3.5             4.63152          1.85111          11.5882
 42              4.4             4.74538          1.8938           11.8907
 48              5               5.42893          2.1467           13.7296
 84              9               9.54209          3.56921          25.5103

FOR WHICH CURVE ARE DETAILS DESIRED (NUMBER) ? STOP

USED     11.50 UNITS.
800
```

TABLE 13.

TABLE 14.

UNAFFECTED	INCREASE	DECREASE
Synapse size [4]	Diameter blood vessels [6]	Brain size [1,4]
Synapses/cm^3	Neurones/cm^3 [4]	Neuronal perikaryon [2]
	Triiodothyronine [14]	Selective axon density [3]
		Synapses/neurone [4]
		Dendrite arborisation [5]
		Myelination [7]
	DNA [8]	RNA/Unit DNA [8]
		Lipids [9]
		Acetylcholine esterase [10]
		Cholinesterase [10]
		Phospholipids [7]
		Cholesterol [7]
		Succinate dehydrogenase [11]
		Glutamate decarboxylase [11]
		GABA transaminase [11]
		Mg-ATP [11]
		Na^+-K^+-ATP [11]
		GABA* [12]
		Glutamic acid * [12]
		Synaptosomal proteins † [13]

* Transient effect; † Cerebellum

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LOCATION OF FIGURES