COMPARATIVE METABOLIC STUDIES IN OCTOPUS AND RAT BRAIN

A Thesis presented to the University of London for the Degree of Doctor of Philosophy in the Faculty of Science

by

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ABSTRACT

The metabolism of glucose and of amino acids derived from it (glutamate, aspartate, glutamine, alanine and GABA) was studied *in vitro* and *in vivo* in two different parts of the brain of **eledone** , and compared with the rat cortex.

IN VITRO

1. Like the rat brain, octopus brain slices incorporated $U^{14}C$ glucose into CO_2, lactate, glutamate, aspartate, glutamine and alanine; unlike the rat brain, octopus brain did not form GABA, but formed much more alanine, but the proportion of counts going into CO_2 , lactate and glutamate was similar in both animals.

2. Octopus and rat brain was incubated *in vitro* with $U^{14}C$ glucose, and $U^{14}C$ glutamate or $U^{14}C$ alanine in the presence and absence of non-labelled glucose, and the specific activities of the amino acids were determined. Generally the labelling pattern was similar in both animals, except that glucose did not dilute the incorporation of $U^{14}C$ alanine into octopus brain amino acids, contrary to the rat brain.

3. The activities of some enzymes associated with amino acid interconversions were lower in the octopus brain than the rat brain. Octopus brain GAD was present in traces only, but alanine transaminase had an activity comparable to the rat.

4. Octopus brain slices did not accumulate amino acids against a concentration gradient, contrary to the rat brain.

5. Incubation of rat brain slices with $U^{14}C$ alanine in the presence of inhibitors of the citric acid cycle served to confirm known interrelations.

IN VIVO

Injection of U^{14}C glutamate into the octopus brain resulted in the labelling of glutamine, alanine and aspartate but not GABA. After one hour the specific activity of glutamine reached a value four times as high as glutamate. Berl, Lajtha and Waelsch (1961) found this also to be the case in rat brain but only three minutes after injection.

The general conclusion is that apart from quantitative differences in the metabolism of GABA and alanine, there are no major differences between the metabolism of the amino acids in the two animals. The differences found between the optic and the vertical lobe, and between the octopus and the rat brain are speculated upon. The difficulties in comparing data from two different animals is discussed, together with the special role of free amino acids in marine molluscs in maintaining ionic balance; the possibility of a different pathway for the oxidation of alanine in the octopus brain by conversion to succinate is also discussed.

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ABBREVIATIONS

ADP, ATP	Adenosine 5' di- and triphosphate
amino acids	As in the Biochemical Society policy to authors
GABA	gamma amino butyric acid
gline, gln	glutamine
NAD, NADH	Nicotinamide adenine dinucleotide, oxidised and reduced forms
PCr	Phosphocreatine
PEP	Phosphoenolpyruvate
Tris	Tris-hydroxymethyl aminomethane

PUBLICATIONS

Glucose and amino acid metabolism in some invertebrate nervous systems.
Bradford, H.F., Chain E.B., Cory H.T. & Rose S.P.R. (1969) J. Neurochem. 16, 969.

Glucose and amino acid metabolism in octopus optic and vertical lobes *in vitro*.
Cory H.T. & Rose S.P.R. (1969), J. Neurochem. 16, 979.

CHAPTER 1

INTRODUCTION

General

The central nervous system of Octopus vulgaris has been extensively studied from the anatomical and behavioural point of view (Young, 1965). The present work is an attempt to begin to investigate in the octopus brain some of the characteristic aspects of the biochemistry of nervous tissue previously observed in mammalian brain. Glucose and amino acids have been shown to be important metabolites in nervous tissue in particular. In the rat cerebral cortex, radioactivity from U ^{14}C-glucose is rapidly incorporated into glutamate, aspartate, glutamine, alanine and GABA, as well as carbon dioxide and lactate, both *in vitro*, (Beloff-Chain, Catanzaro, Chain, Masi and Pocchiari 1955a) and *in vivo* (Vrba, Gaitonde and Richter, 1962).

This work attempts to investigate some of the metabolic pathways by which these five amino acids are synthesised and interrelated in the octopus brain. They are thought to play an important part in the unique functions of nervous tissue, as well as being derived from glucose, the prime energy substrate of mammalian brain tissue. The metabolism of glucose and of these amino acids was studied in the rat cerebral cortex, and in two parts of the octopus brain.

STRUCTURE AND FUNCTIONS OF THE RAT AND OCTOPUS BRAIN

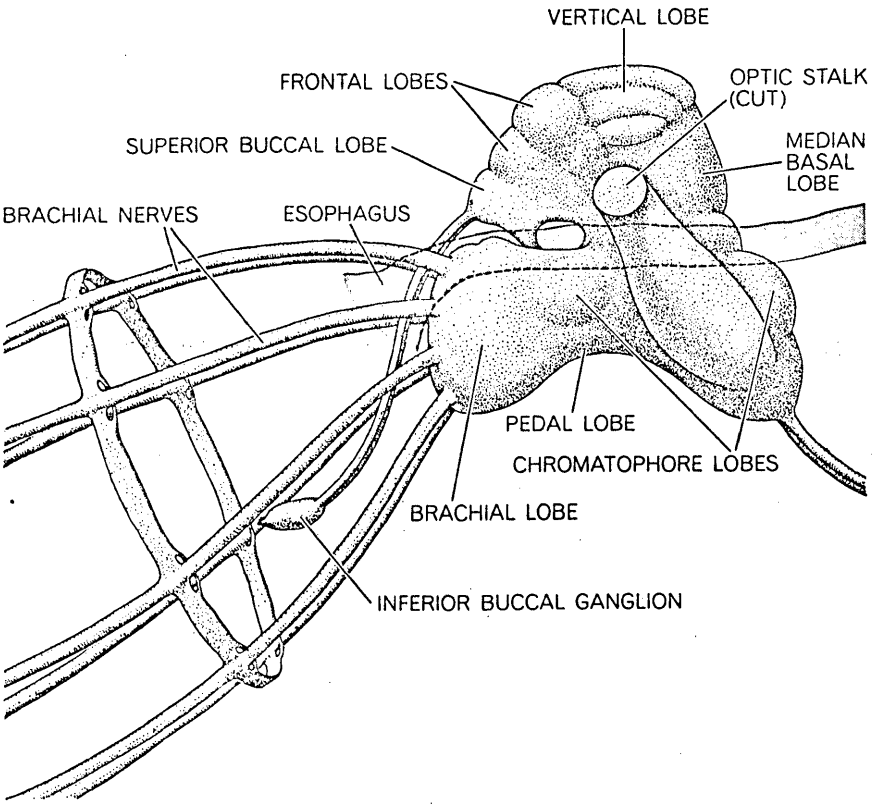
The brains of these two animals are structurally dissimilar. The rat brain, though anatomically compact, is complex: however, the octopus brain, though also complex, consists of several well-defined lobes or ganglia, and this fact makes it convenient to slice different parts of

the brain and compare their metabolism in vitro.

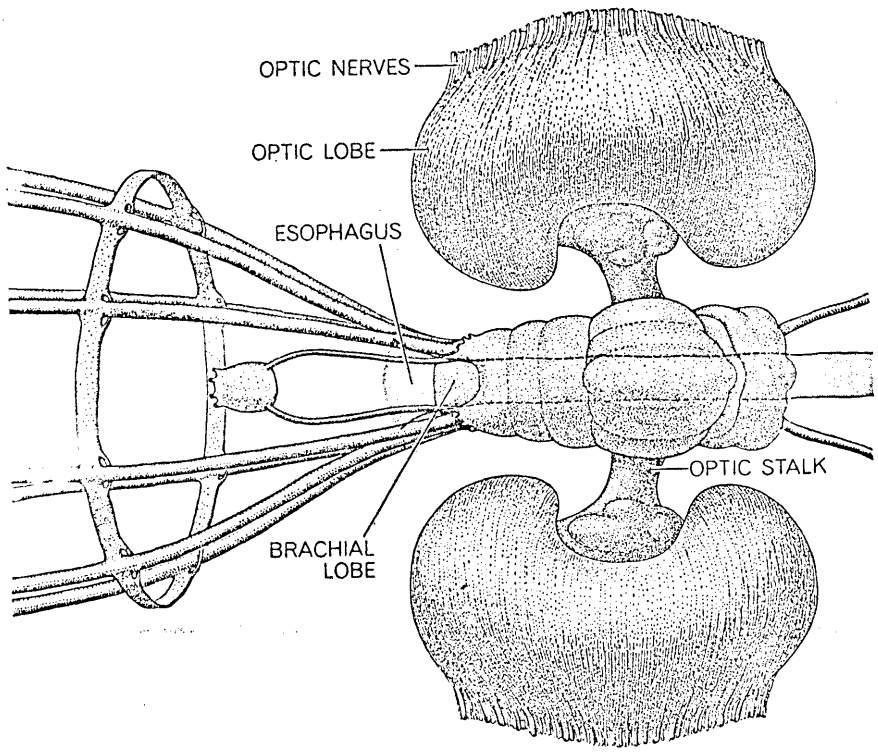
The different physiological functions governed by the various lobes are summarised by Boycott (1965). Figure 1 shows the positions of the lobes in relation to one another, and to the oesophagus and the eyes. The rat cortex comprises the top and front part of the rat brain and is itself structurally complex: within different groups of cells in the cortex, different conscious functions are controlled (Cragg, 1968). Lashley (1950) pointed out the difficulties of locating the sites where memories are stored, that is, where records are kept of information acquired as a result of learning, and he was unable to pin point any sites of this kind by the use of ablation experiments. Young (1964a)has described the advances made in the study of the octopus brain towards locating the sites of information storage and the mechanism by which this occurs, and he has suggested (Young 1967) that mammalian nervous systems contain the same basic elements of organisation that were revealed in the study of the octopus brain. Though the octopus brain is structurally complex, it is divided into lobes and Young was able to use this property to study it.

Examination of figure 1 shows that the central brain surrounds the oesophagus. The two optic lobes lie just behind the eyes, and are attached to either side of the central part by the optic stalks. The central part is itself composed of several lobes, and is encased in cartilage: the oesophagus slides through it, and above it is a cavity containing mucilage. The optic lobes lie outside this casing, but are tucked into the concave sides of the central portion and themselves encased within a tough membrane joining the eye to the central part.

Boycott (1965) describes the functions of these lobes: briefly the suboesophageal lobes are mostly concerned with



a)



b)

FIGURE 1

Diagram of the octopus brain in relation to the oesophagus and arms. a) From the side, with optic lobe removed
b) From the top, showing optic lobes. (Boycott, 1965)

motor actions and reflexes, such as eating, moving, change of colour: the supraoesophageal lobes are involved in learning and memory mechanisms, as is also the optic lobe. The swellings shown on the optic stalks have been shown by Wells & Wells (1969) to be analogous to the pituitary gland in secreting hormones. Animals with their optic and supra-oesophageal lobes removed are unable to exhibit learning and memory, or to seek food for themselves, though they will grasp objects and be sensitive to touch stimuli (Wells, 1962).

Most of the advances in the elucidation of the working of the octopus brain have been made in exhaustive anatomical studies, combined with learning experiments. The most recent comprehensive books are by Wells (1962) and Young (1964a) In the most recent review, Wells (1966) describes many types of learning experiments, but the one most used for the study of learning was to teach the animal to reject or accept their natural prey, crabs, by means of punishment or reward. Boycott and Young (1955) showed the octopus a live crab at a few feet distance, with or without a white square. If the crab was shown alone, the octopus was allowed to keep and eat the crab if it attacked (reward) and if shown with a white square, the octopus was given a mild electric shock if it attacked. They found that after a very few trials the animals, now fully trained, only attacked crabs shown alone, and never with a white square even if hungry.

Boycott & Young (1955) then set out to investigate the effect of specific lesions on the ability of the animal to learn to discriminate in this way. Animals with the optic lobes removed could not be trained to make visual discrimin-ations, since they were effectively blind, the optic nerves being severed from the rest of the nervous system. However they found that animals with the vertical lobe removed, though they could see, could not learn to discriminate visually; but if the trials were spaced less than 30 minutes

apart, each trial being the showing of a crab followed by the appropriate reward or punishment, the animal was able to learn to discriminate, but made more errors or wrong choices than an intact animal. Young (1958) showed that the number of errors was proportional to the percentage of the vertical lobe excised.

Young then examined the role of the vertical lobe in more detail: he compared the number of times an octopus attacked a crab out of a total number of trials, in intact and operated animals, and found that animals with excised vertical lobes attacked more readily than intact animals, and those deprived of the superior frontal lobe less readily (Young, 1964b). This was interpreted as showing that the vertical lobe had a supressor function, and the superior frontal lobe, situated just below and to the front of the vertical lobe, had an amplification function. Figure 2 is a sagittal section of the central brain showing the arrangement of the lobes.

Muntz (1961) revealed another function of the vertical lobe. Normal animals could be trained using one eye only, and if subjected to trials with the other eye, reacted as trained: animals without vertical lobes were unable to transfer learning in this way, even if the optic commissure, a tract of nervous tissue linking the two optic lobes, was intact.

On the basis of these and other experiments, Young (1963a) formulated a theory for the organisation of a visual memory system, in which paired centres in the supraoesophageal lobes regulated and addressed the signals of the results of the action taken in response to certain stimuli i.e. taste or pain as a result of seeing and attacking crabs. Earlier Wells (1959) had discovered that blinded octopus could learn to discriminate by touch, using similar methods to those used



FIGURE 2

Sagittal stained section of the central brain of the octopus. The oesophagus roughly divides the upper and lower halves of the brain. Magnification 15 times.

Reproduced from Boycott,(1965).

by Boycott and Young (1955) in visual training. The animals were able to discriminate by touch between different shaped objects in the presence of lesions that prevented visual learning, and Wells concluded that the visual and touch learning systems operated independently and were located in different parts of the brain. Destruction of the subfrontal and inferior frontal lobes, (located in front of and beneath the superior frontal lobe) abolished tactile learning, and these two lobes function in parallel and in an analogous way to the superior frontal and vertical lobes in the visual system (Wells, 1961). Young (1965) now formulated a theory for the organisation of a memory system based on both the visual and tactile systems of the octopus, studied by means of learning experiments of the type described above, combined with ablative operations, and detailed microscopic examinations to ascertain the extents of all lesions at the end of the experiments. The theory will be described here in simplified terms only, in order to show the relation between the morphology of the optic and vertical lobes, whose biochemistry is to be investigated in later chapters, and their supposed function as postulated by Young (1965).

Figure 3 is a diagram of the basic memory unit or mnemon as seen by Young (1963a). Each one records a single unit of information, namely the record of a particular stimulus and its result, so that learning in effect consists of accumulating sufficient switched mnemons, to produce behaviour for the good of the animal. The optic lobe contains classifying cells that are sensitive to and respond to signals according to the occurrence of illuminating contours in extent and direction. Hubel and Wiesel (1959) have shown that single neurons in the visual centre of the cat brain have receptive fields in the retina. Sutherland (1961) has also shown that there are cells in the octopus retina, connected to the optic lobe, with receptive dendritic fields oriented in the horizontal-vertical direction.

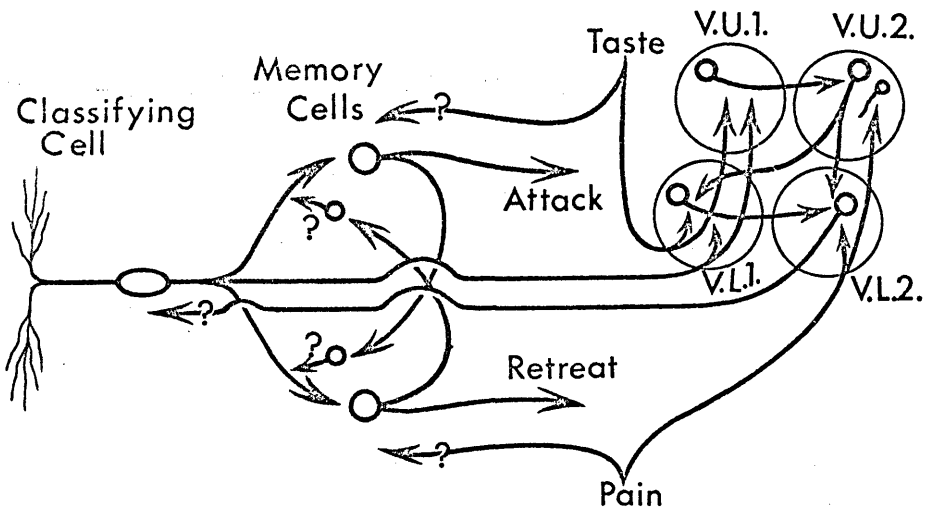


FIGURE 3

Diagram of the visual learning system of the octopus. On stimulation, the classifying cell in the optic lobe signals to the attack or retreat pathway, according to whether the action was previously beneficial to the animal : this closure of one of the pathways has occurred through the action of the visual centres VU and VL 1 and 2 (visual upper and visual lower centres 1 and 2), when stimulated by taste or pain. VU and VL 1 and 2 shown here are the superior frontal and vertical lobes respectively.

After Young, (1963a)

The classifying cell itself, according to Young (1965) is located in the optic lobe and is biaxonal, having two alternative motor outputs, resulting in either attack or retreat. If the animal taking in an unfamiliar object for the first time experiences pain, the pathway leading to attack is shut off so that in the future the other operates in response to the same stimulus. Young (1965) suggests this occurs by means of the amacrine cells present also in the optic lobe. These are small neurons, under 5 μ in diameter, containing electron dense vesicles, and short, stumpy axons; (Young 1962); these cells are somehow stimulated to secrete an inhibitory substance which acts on one of the axons of the classifying cells and inhibits conduction of impulses. In a detailed study of the optic lobe, Young (1962) showed that all the elements were present for such a system: Figure 4 shows cells drawn from a stained preparation of the optic lobe: large biaxonal cells are seen to occur, as well as small amacrines. It is noteworthy that nowhere else in the octopus nervous system were biaxonal cells found, except in a part of the subfrontal lobe, which has been shown by Wells (1961) as being the region containing the classifiers for the tactile system.

Young (1965) describes the role of the visual upper lobes, that is, the superior frontal and vertical lobe, as combining the visual stimulus with the outcome of the action taken in response to it (say taste or pain), and conveying this result back to the optic lobe so that only one possible output operates in the future. The experiments of Young (1964b) described earlier showed that the superior frontal lobe serves to amplify the signal coming from the optic lobe, and the vertical lobe serves to inhibit it. Figure 5 has been drawn from stained preparations, and illustrates schematically the connections between the optic lobes and

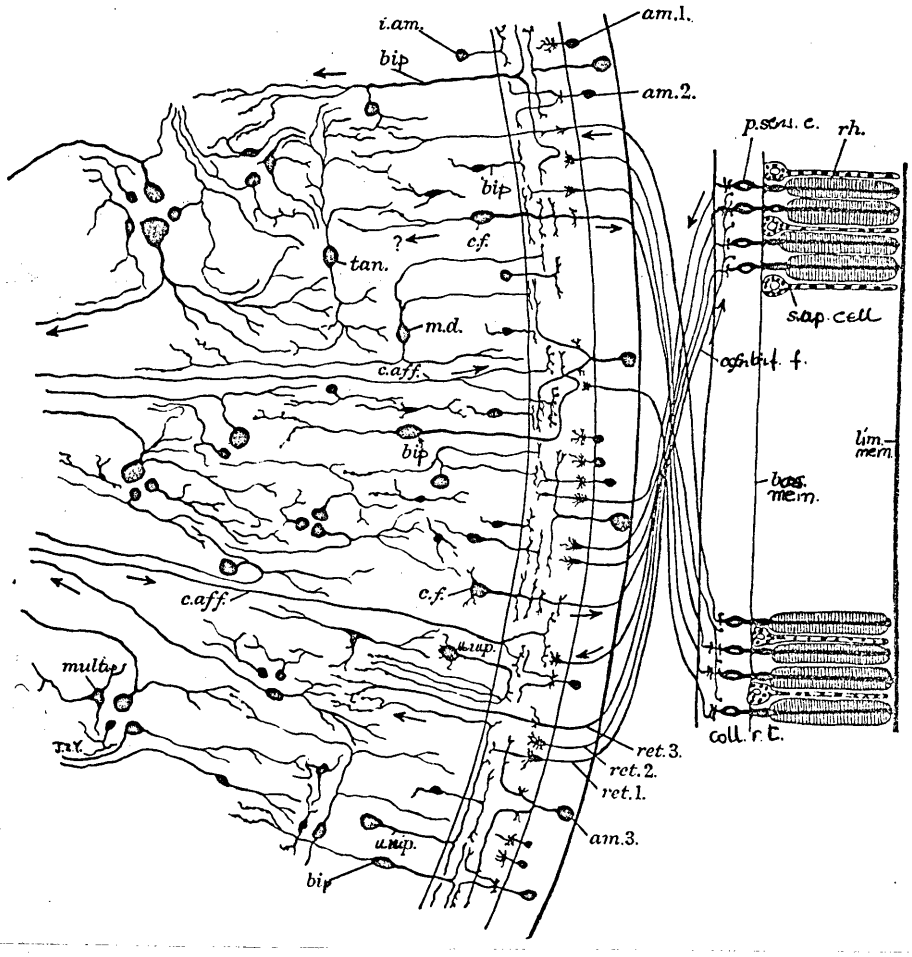


FIGURE 4

Diagram of the connections of the outer portion of the OPTIC LOBE , and the retina, based on stained preparations of the octopus.

amac. = amacrine cells; bas. mem.= basal membrane of retina; bip. = bipolar cell ; c.aff. = afferent cell from central region; centrif. f. = centrifugal fibre entering retina, c.f. = centrifugal cell of optic lobe ; coll.r.c. = collateral of retinal sense cell; in. amac. = inner amacrine cell ; lim. mem.= limiting membrane of retina; m.d. = cells with many dendrites; multip.= small multipolar cell; ret. = retinal nerve fibres ; sens.c. = primary sense cell ; sup. c. = supporting cell; tan. = tangential cell ; unip.= unipolar cell connecting with flexiform zone.

After Young, (1962)

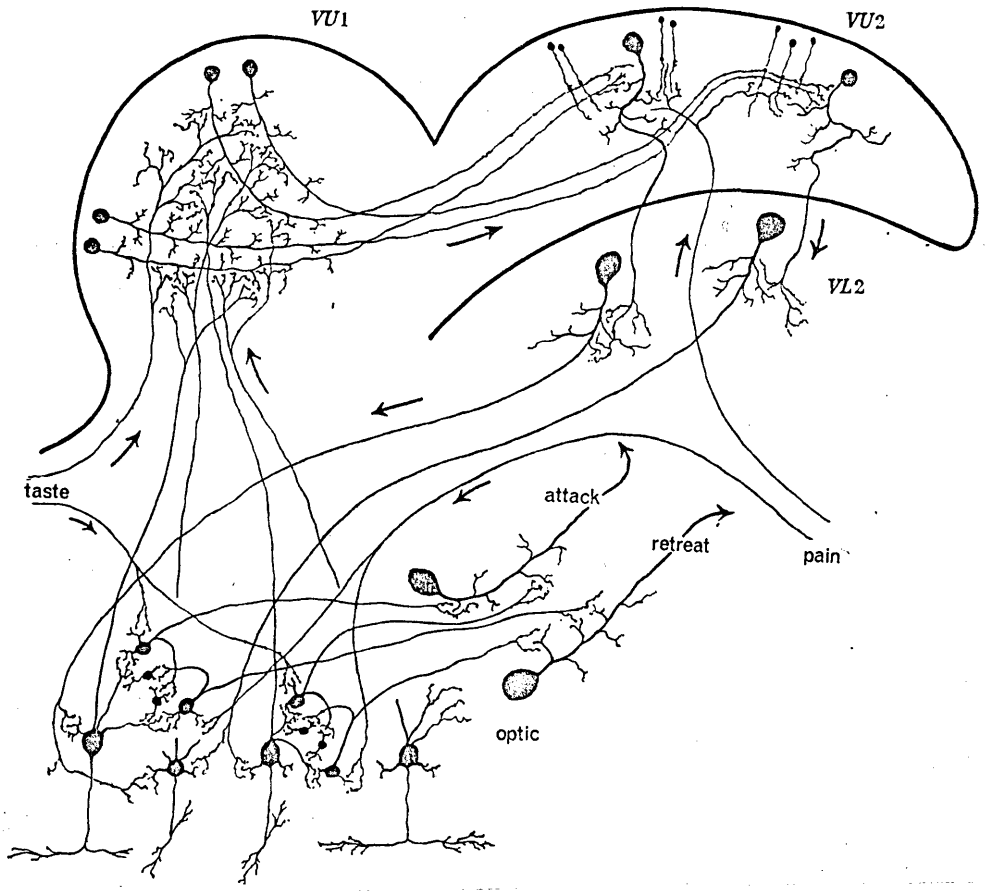


FIGURE 5

Schematic drawing of the connections in the visual memory system of the octopus. Neurons have been identified as shown, but some of the connections are not proved.
VU 1 = superior frontal lobe , VU 2 = vertical lobe
VL 2 = subvertical lobe.

(Young, 1965)

the visual upper lobes: The superior frontal lobe contains conventional synapses and receives afferents from visual and taste fibres, multiplies these and mingles them with the signal from the optic lobe in a plexus of nerve connections. These pass to the vertical lobe: in this lobe fibres come from below, and Young (1965) presumes them to be 'pain' fibres, activated by painful stimuli. Figure 5 shows how these connect with cells that intercept the fibres coming from the superior frontal lobe. Gray and Young (1964) compared the electron microscopic structure of these two lobes: the superior frontal contained conventional synapses with vesicles (presumably filled with synaptic transmitter) on the presynaptic side only. In contrast the vertical lobe contained many amacrine cells filled with vesicles, and synapses had vesicles on both sides. This has been found elsewhere in the nervous system and has been discussed as a characteristic of inhibitory synapses (Gray, 1962). Thus signals amplified in the superior frontal and passing through the vertical lobe may be modified by the action of the amacrine cells, which are stimulated to secrete inhibitory substance if the pain fibres are stimulated.

Young (1965) makes clear which connections have been morphologically demonstrated in connection with his model of the mnemon and those which have not. In the latter category is the connection bearing one of two types of message from the vertical lobe back to the amacrines of the optic lobe, which will induce one of two amacrines in Figure 3 to shut off one of two motor pathways. However, Young (1962) suggests that the dendritic twigs on the large cells in the optic lobe (see Figure 4) supposed to hold the memory store ('memory cells') may receive such signals.

Young (1963a) also stresses that the visual upper lobes are required to ensure delivery of the signals of the results of the action to the correct classifiers, by means

of their complex circuitry, which maintains the classifiers at a lowered threshold until these signals arrive; there is also the necessity for visual signals to be understood wherever they occur, and the role of the visual upper circuit as discovered by Muntz (1961) in the process of generalisation is to spread the information to all the relevant cells.

From the simplified description above, taken mostly from the work of Young, it can be seen that the optic and supraoesophageal lobes form the two components of the visual learning system. In the optic lobe the classifying cells and memory cells keep a record of stimuli and their outcomes and the visual upper lobes contain the mechanism in terms of circuitry and connections for modification, amplification and generalisation of signals, and for the permanent recording of a memory in the optic lobe.

The lobes of the tactile system with its parallel organisation were not studied biochemically. Though similar in arrangement to the visual system (Wells 1961) the memory is not located in a separate lobe, but is at the base of the subfrontal lobe. The amounts of tissue involved are much smaller, and from the point of view of dissection and slicing, the use of the lobes of the visual system was more profitable.

In comparing the types of nerve cell in the octopus and the rat brain, it would appear that the optic and vertical lobe contain a heterogeneous population of cell types. The cellular structure of the rat brain is also heterogeneous, and the cortex itself comprises several layers, each containing a different arrangement of different types of cells. The structure of the mammalian brain is the subject of many detailed text books, but a synopsis of neuroanatomy has recently been written by Matzke and Foltz (1967) which gives a good general account of the brain for non-anatomists. Figure 6 is a diagram showing the arrangement of the cells in the cortex in simplified form. The pyramidal cells

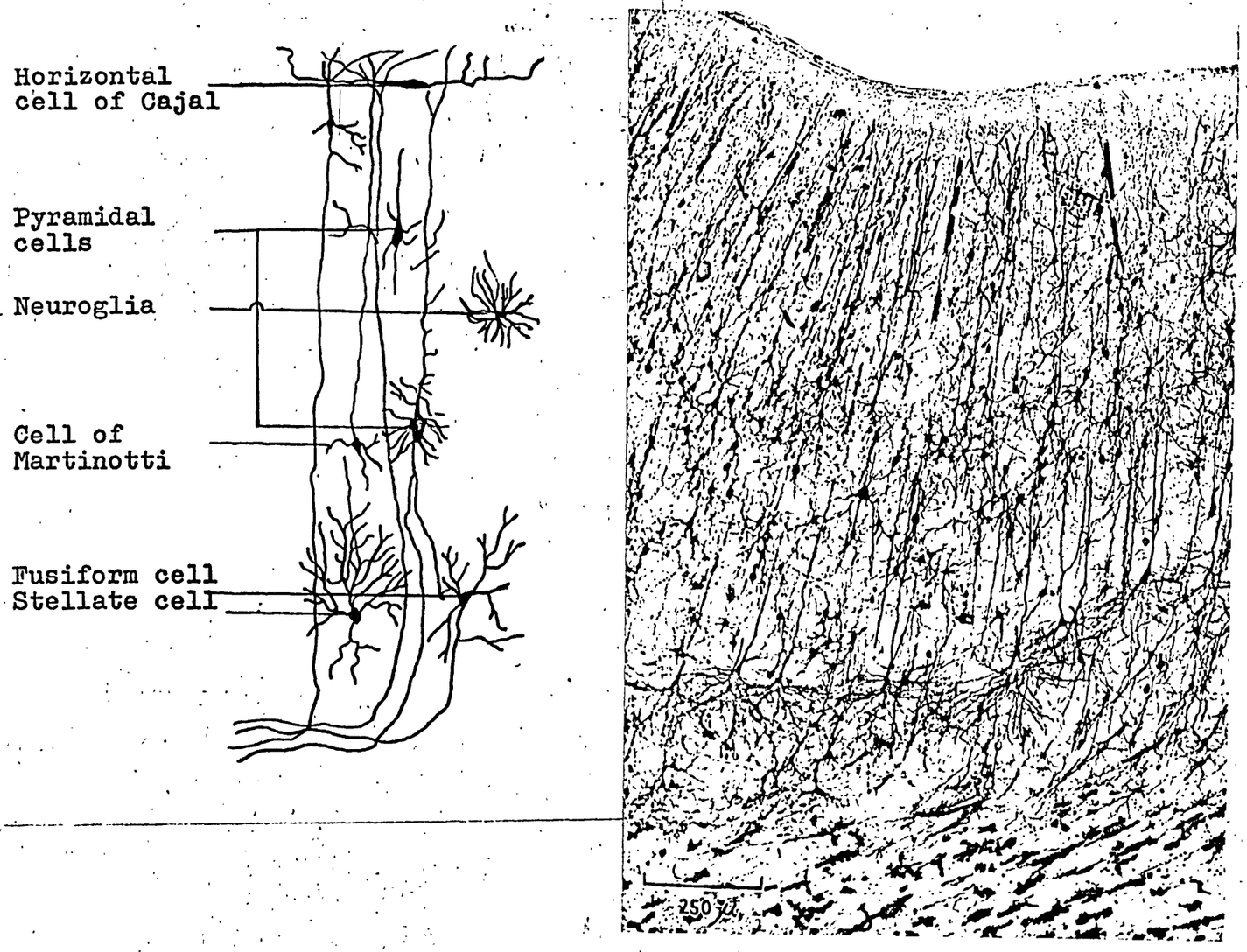


FIGURE 6

THE MAMMALIAN CORTEX

Left: Simplified diagram showing the types of nerve cell in the various layers of the cortex. (After Brodmann, Ranson & Clark, 1953)

Right: Photograph of the corresponding layers in the cortex; stained sagittal section of the cat striate cortex. (After Sholl, 1956)

extend from the surface to the deeper layers of the cortex or to the white matter beneath the cortex. The stellate cells have short axons, and do not connect with parts of the brain outside the cortex. At the cortical surface are the horizontal cells of Cajal, whose axons lie parallel to the surface. The polymorph cells have their bodies in the deeper layers, but their dendrites extend to the upper layers. The cells of Martinotti have short dendrites but a long axon that runs to the surface. As well as these layers, bands of fibres run across the cortex, consisting of the terminal ends of afferent fibres. Loops consisting of bundles of fibres, and passing through the white matter beneath the cortex, serve to connect different parts of the cortex with each other and with other parts of the brain, and are known as association bundles. In addition to these different neuronal types in the cortex alone, there are the glial cells. Cragg (1968) describes the neurons as the "computing centres" of the nervous system and of the whole organism, which connect with a very large number of other neurons, by means of their short dendrites, which receive signals, and their long axon, through which the signal is transmitted to other cells. The glia have no axons: they are intimately connected with the neurons, and are thought to have a supporting function, including the control of entry of substances into the neurons from the blood and the extracellular fluid. Some of the aspects of their interactions with the neurons are as yet unclear (Rose, 1968a). In the mammalian brain, the glia are estimated to outnumber the neurons by as much as ten times in some parts of the brain (Nurnberger, 1958) and range in size from 3 to 50 μ in diameter (Hosokawa, 1963). In contrast, there appear to be very few glia in the octopus brain: this was mentioned by Roberts (1964) and Young does not seem, to discuss glia in his writings.

Just as the optic lobe receives visual stimuli and is the seat of visual memory in the octopus, the mammalian cortex receives stimuli from all the senses, and in it conscious experience is generalised. Functions are localised in cortical areas, but the study of the cortex seems to show less and less evidence of the complete dependence on small areas for specific functions (Ranson & Clark, 1953). The cortex is also the centre of motor control, as well as a sensory receptive centre, and is the seat of conscious sensation and of memory: since this subject is so complex and controversial, it will not be discussed further here. As regards the functional organisation of the mammalian brain, it has only begun to be studied relatively recently, notably by Hubel and Wiesel (1959, 1963, 1965, 1969), who have studied the visual centre of the cat and the monkey, and related the different types of nerve cells and their connections to function. The classification of inputs appears to be complex and occurs at several levels, in the retina, and also at three levels in the cortex, where each functional area is grouped in columnar form. Young (1967) points out that in man, 3 million afferents reach the central nervous system, but there are very many more at the periphery, 100 million in the eye alone. In contrast, the octopus CNS receives afferents from 8 million receptors condensed into half a million channels; thus an important difference between the rat and octopus brains is the far greater condensation of input and complexity of classification found in the mammalian brain. For example, in the octopus brain, classifying cells are found in the optic lobe, but in the mammalian brain, in the retina.

Thus the study of the octopus brain has led to the beginning of the elucidation of the extremely complex working of the mammalian brain, hitherto unfathomable.

Since relatively little study has been made of the biochemistry of the octopus brain however, in contrast to the rat brain, which has been studied more than that of any other species, it seemed worthwhile to study in the octopus the metabolism of substances shown to be of prime importance in the rat.

THE METABOLISM OF GLUCOSE AND AMINO ACIDS IN THE MAMMALIAN BRAIN.

It has been known for a long time that under normal conditions glucose is the prime oxidisable substrate in the brain (McIlwain, 1963, 1966). Himwich (1951) first showed that glucose was the only substance to be taken up from the blood in sufficient quantity to satisfy the energy requirements of the brain tissue. Geiger (1963) showed that glucose is also a substrate for protein and lipid synthesis as well as being a substrate for oxidation, yielding lactate, CO_2 and ATP.

Perfusion of mammalian brain with glucose in situ.

Geiger, Kawakita and Barkulis (1960) perfused cat brain with a glucose-containing saline, and found that the utilisation of glucose was accounted for as CO_2 and lactate. However it seemed that glucose was not the only substrate oxidised. U^{14}C glucose yielded a $^{14}CO_2$ of a specific activity never greater than 30% that of the glucose, implying that other substrates were contributing to the formation of CO_2. During convulsions brought about by drug treatment or electroshock, more CO_2 was formed, though the amount of $^{14}CO_2$ actually decreased: the uptake of glucose into the brain was also decreased, suggesting the increased oxidation of other substrates. More glucose was converted into lactate during convulsions.

After convulsions, the uptake of glucose increased to the normal (non-convulsive), resting value, but the recovery period was associated with an increased protein and glycolipid synthesis, (Barkulis, Kawakita & Geiger, 1960). McIlwain (1963) also discusses in detail the depletion of glycolipid during excessive electrical activity in vitro, and its resynthesis afterwards, requiring glucose.

Barkulis et al. (1960) also measured the incorporation of U ^{14}C glucose into free amino acids isolated from samples of cat cortex after different perfusion times: after one hour, the specific activity of the total amino acid fraction reached 18% that of glucose, and remained constant thereafter. This value was not altered by convulsions, and it was concluded that, like CO_2, amino acids were also formed from non-glucose precursors.

The metabolism of glucose in vivo.

Similar results to the above were obtained by Vrba, Gaitonde & Richter (1962) in vivo in the rat. Intravenous injection of U ^{14}C glucose resulted in the incorporation of radioactivity into brain amino acids: the amino acid fraction contained 72% of the total radioactivity in the soluble brain extract after 20 minutes, whereas it contained only 15% of the total radioactivity of the liver extract. The amino acid fraction also had a higher specific activity in the brain than any other organ. After 12 hours, radioactivity was found to be incorporated into brain proteins, 65% of it being in bound aspartate and glutamate.

Gaitonde, Marchi and Richter (1965) compared the incorporation of glucose carbon in the brain and other organs of the cat. 20 minutes after subcutaneous

injection of $U^{14}C$ glucose the distribution of the label in the brain was: 70% amino acids, 3% unchanged glucose. By contrast, in the liver, muscle and blood, 70 to 90% of the radioactivity was in unchanged glucose, and very little in free amino acids. The specific activity of the total amino acids in the brain was 10 times that in the other organs, 80% of the amino acid activity being in the acidic fraction (glutamate and aspartate), whereas in the other tissues, a higher proportion of counts went into the neutral amino acids. Gaitonde, Dahl and Elliott (1965) showed that only three minutes after injection of $U^{14}C$ glucose the distribution of radioactivity in the brain was as follows: glutamate 37%, aspartate 9%, glutamine 9%, GABA 4% and alanine 2%. The values for the liver were: alanine 30%, glutamate 5%, glutamine 5% and aspartate 3%.

The metabolism of glucose in vitro.

Beloff-Chain, Catanzaro, Chain, Masi and Pocchiari (1955a) incubated rat cortex slices with $U^{14}C$ glucose, and after one hour, found that the glucose was incorporated into the following products: lactate 60%, CO_2 20%, the remaining 20% being mostly in free amino acids: glutamate 9%, GABA 3%, aspartate 2.5%, alanine 1.5%. In contrast Beloff-Chain et al. (1955b) found the incorporation of radioactivity from $U^{14}C$ glucose by rat diaphragm muscle in vitro to be: 14% CO_2, 26% lactate, and the rest as free glucose, oligosaccharides and glycogen, in contrast to the brain where less than 1% of the glucose carbon was incorporated into glycogen.

From this data it is possible to make important comparisons between the incorporation of glucose

^{14}Carbon *in vitro* and *in vivo*, and between the brain and other organs in each case. In the brain as compared to other organs, more glucose carbon is incorporated into amino acids, *in vitro* and *in vivo*: rat brain *in vitro* incorporates more glucose carbon into lactate, less into free amino acids, compared to the *in vivo* situation. Vrba et al. (1962) pointed out a considerable incorporation of glucose carbon into protein and lipid *in vivo*, whereas Beloff-Chain et al. (1955a) found only traces *in vitro*.

The pathway by which glucose is oxidised by the brain is also well documented. Tower (1959), di Pietro and Weinhouse (1959) and Villee and Loring (1961) state that in the cortex glucose is metabolised exclusively through the glycolytic pathway by conversion into pyruvate, which is completely oxidised via the Krebs cycle. Villee and Loring (1961) did point out that in other parts of the brain, the pentose pathway operated to some extent.

The value of *in vitro* preparations.

The differences in the metabolism of brain *in vivo* and *in vitro* pose the question of the value of using slices to obtain information about metabolism *in situ*. This problem is discussed by, among others, McIlwain (1966), Elliott and Wolfe (1962), and Bradford (1968).

In assessing any differences, one must look closely at the structure of the brain *in situ* as compared with the slice *in vitro*. The brain is supplied by a network of blood vessels, and its outer surface and inner epithelia are bathed in cerebrospinal fluid (CSF): the composition of the CSF is not identical with that of the

blood, and the passage of substances between the two appears to be regulated. (Cragg, 1968).

Inside the tissue itself, the closely interconnected neurons and glia are bathed in the extracellular fluid, which is estimated to occupy from 9 to 15% of the whole tissue. It is thought that the glia, which form a layer around the blood capillary surfaces, and surround the neurons may control the entry of substances into the neurons (Rose, 1968a), and the interrelationships between the two cell types may well be metabolic and functional, as well as morphological (Hydén and Pigon, 1960).

In the rat cortex slice, which is cut tangentially to the surface, axons are necessarily severed, since they run perpendicular to the surface. However there is evidence that the cut ends do close up (Bourke and Tower 1966). Neame (1968) points out that substrate concentration is controlled in the slice, whereas <u>in vivo</u> it may be necessary to inject radioactive substrate repeatedly to obtain a fairly constant substrate environment. Perfusion with a known fluid is advantageous in this respect, but Geiger (1962) points out that this only measures gross changes in the entire brain.

A further advantage of the use of slices is that diffusion can occur freely between medium and extracellular space, by-passing the blood capillaries and their surrounding layer of astrocytes, which are presumed to control entry of substances from the blood to the extracellular fluid in the brain <u>in situ</u>, (Hydén, 1960). There is no restriction on the entry of glucose, nor of

amino acids *in vitro* (see the next section). Most important is the fact that cells in the slice have the membrane intact, which is of prime importance in the functioning of the brain.

McIlwain (1963) showed that brain slices respond to electrical pulses *in vitro* by an increased oxygen uptake and rate of glucose utilisation. In the presence of glucose, brain slices synthesise PCr and ATP (Woodman and McIlwain 1961), and the effect of pulses is to reduce their levels. Bradford (1968) discussed the efficiency with which various substrates, sugars and intermediates, maintain the levels of these labile phosphates, as well as support other properties, such as respiration, accumulation of amino acids against a concentration gradient, respiratory response to electrical pulses, and concluded that glucose is the most efficient in all these respects. He also pointed out that lactate formation *in vitro* is not a good guide to the situation *in vivo*; it is formed in excessive amounts due to its ability to diffuse out of the slice into the medium, so that the total amount formed *in vitro* depends on the medium/slice volume ratio, the amount in the slice after incubation being similar to the intracellular concentration *in vivo*, (McIlwain 1956).

Slices are also affected by the ionic composition of the medium. Bourke and Tower (1966) and also Pappius and Elliott (1956) pointed out that a concentration of potassium ions above 20mM causes swelling due to the uptake of water into the slice.

Kini and Quastel (1959) showed that even higher concentrations of potassium (105M) increase slice respiration and alter the metabolic pattern with $U^{14}C$ glucose *in vitro*: more labelled glutamine and GABA are formed from glucose compared with incubation in a saline containing 5mM potassium. Quastel (1962) has suggested that the effect of an increased K^+ / Ca^{++} ratio is to cause more breakdown of ATP and PCr, forming more ADP and inorganic phosphate, which leads to the increased respiration observed, and to the faster turnover of the citric acid cycle, and acceleration of the pacemaking step of pyruvate conversion to Acetyl Coenzyme A. He also points out that electrical stimulation has the same apparent effect on slices, as does a high potassium level in the medium.

The effect on the electron microscopic structure of incubating mammalian cortex *in vitro* under various conditions was investigated by Cohen and Hartmann (1964). In the absence of added substrate, severe damage occurred, cell membranes were ruptured and mitochondria distorted. However, slices incubated with glucose possessed an ultrastructure similar to that of unincubated tissue: the best preservation of structure occurred using a pH of 7.4, the pH of the Elliott-Henderson saline (1948). They also found that, as a rule, tissue was best preserved by conditions where phosphorylation occurred, the only exception to this being with glutamate, which did not support phosphorylation but caused no structural damage, and also potassium ions which at 100mM concentration, supported

phosphorylation and a high respiration, but caused cellular distortion.

For these reasons the choice of media for incubation of the rat cortex and the octopus brain was difficult, as it was not certain that the ions that affected the metabolism of the rat brain slices would affect the octopus brain in the same way. The medium chosen for the rat was a phosphate-buffered saline, similar to that of Elliott and Henderson (1948) containing no calcium, but potassium at the relatively high concentration of 27mM. This concentration was reported by Berl, Hicklas and Clarke (1968) to yield a glutamine *in vitro* of different specific activity compared to 5mM potassium, when radioactive glutamate and aspartate were used as substrates.

Uptake of amino acids by mammalian brain tissue *in vitro* and *in vivo*.

The passage of amino acids into the mammalian brain differs *in vitro* and *in vivo*. *In vivo* raised levels of amino acids in the blood do not result in raised levels in the brain in contrast to other organs, but labelled amino acids injected into the blood exchange very rapidly with those of the brain, (Lajtha, 1962; Neame, 1968).

Stern, Eggleston, Hems and Krebs (1949) first showed that glutamate was accumulated by brain slices *in vitro*, against a concentration gradient: the process required glucose and oxygen and was inhibited by respiratory poisons. Terner, Eggleston and Krebs (1950) further showed that potassium was taken up concommitantly with glutamate and in equivalent amounts. Abadom and

Scholefield (1962) demonstrated the phenomenon *in vitro* with a wide range of amino acids and showed that the amount of amino acid taken up was proportional to the ATP content of the tissue, hence the glucose and oxygen requirement. This limitation *in vivo* to the entry of large quantities of amino acids is one aspect of the so-called blood-brain barrier, and as yet, the mechanism of its action is not fully understood, (Dobbing, 1968), nor is the mechanism by which amino acids are transported into the brain. This is very complex and involves several systems, and a tentative scheme has been outlined by Neame (1968) who believes that this mechanism *in vivo* and *in vitro*, though apparently contrasting, may have a common basis.

The effect of glutamate in vitro on the mammalian brain.

The effect of glutamic acid at 2 to 10mM concentration on the brain slice has been thoroughly investigated by McIlwain's group. McIlwain, Harvey and Rodriguez (1969) showed that the effect of glutamate on the outer surface of the slice was similar to an electrical pulse, in that it resulted in an alteration in the permeability of the membrane to sodium and potassium ions, and the reversal of the membrane polarity at the point of action. Earlier, Woodman and McIlwain (1961) reported that incubation with glutamate in the presence of glucose reduced the levels of PCr and ATP synthesised *in vitro* compared to that with glucose alone. Bradford (1968) suggested this was because more ATP was broken down in the active transport of glutamate into the cells and in the recovery of the membrane after the depolarising effect of the glutamate. The observation of Cohen and Hartmann (1964)

that a slice incubated with glutamate showed as little ultrastructural damage as one incubated with glucose, may also indicate that glutamate does support phosphorylation to the same extent as glucose *in vitro*, the extra ATP being broken down for the reasons stated above, to give the resulting lower levels measured by Woodman and McIlwain (1961).

Metabolism of glutamate in the mammalian brain in vivo.

A widely documented phenomenon which raises difficulties as regards interpretation of data is that of compartmentation, which was first discovered in connection with the metabolism of glutamate *in vivo*. Roberts (1956) found that intracerebral injection of $U^{14}C$ GABA into mice yielded a glutamine of higher specific activity than glutamate, its supposed precursor; in the brain glutamine is synthesised exclusively from glutamate, and is the major means of removal of ammonia. Takagaki, Berl, Clarke, Purpura and Waelsch (1961) found that intracarotid infusion of ^{15}N ammonium acetate yielded in the brain a glutamine whose α-amino nitrogen was ten times more heavily labelled than that of glutamate, from which it was presumably made; in the liver the reverse was the case, the α-amino nitrogen of the glutamate being more heavily labelled. However the α-amino nitrogen of aspartate was higher than that of the glutamate in the liver, but lower in the brain. (Waelsch, 1962a)

Berl, Lajtha and Waelsch (1961) injected $U^{14}C$ glutamate intracisternally into the rat and three minutes after injection isolated a glutamine whose specific activity was 4 to 5 times that of the glutamate; one hour

after injection the specific activity of glutamine fell to 2½ times that of glutamate. The use of U ^{14}C glucose as precursor yielded a glutamine of lower specific activity than the glutamate, but it must be emphasised that it was not injected intracerebrally (Gaitonde et al., 1965). The explanation of these phenomena was given by Berl et al. (1961) and Waelsch (1962 a) as follows: there were two pools of glutamate in the brain, of different sizes, and not readily interchangeable. The injected glutamate came into equilibrium with the smaller pool, in which glutamine synthesis occurred more readily than in the larger pool. During extraction, the high specific activity glutamate was diluted with the large pool to yield a glutamate of apparently lower specific activity than its product, glutamine: but with time, the effect decreased as the two glutamate pools mingled. In the liver, the glutamine was synthesised from the larger pool of glutamate, aspartate from a smaller pool. More recently, Berl et al. (1968) were able to demonstrate this so-called "Waelsch effect" in vitro, thus showing it was an intracellular phenomenon, not dependent on transformations occurring outside the brain.

There has been much evidence from other sources on the existence of separate compartments: Garfinkel (1966) made a computer simulation study (based on the results of the Waelsch group) of the metabolism and compartmentation of glutamate, aspartate and intermediates, and concluded that there were two unequally-sized pools of citric acid cycle intermediates. Van den Berg, Mela and Waelsch (1966) reported findings that suggested glutamine

could be made from glucose in the brain by a route not involving glutamate, but Haslam and Krebs (1963) showed conclusively that interconversions *in vitro* between amino acids in mammalian brain occurred only through known transaminase reactions and citric acid cycle enzymes.

The identification of these compartments is as yet uncertain. Simon, Cohen and Berry (1967) postulated two citric acid cycles located in two distinct pools of mitochondria. Rose (1968b) showed that the answer may be in the different kinds of cell in the brain, when he demonstrated that the metabolism of glucose and glutamate differed in isolated neurons and glia *in vitro*. More recently Rose (1969) reported that intracisternal injection of U^{14}C glutamate into rat brain, followed by bulk separation of neurons and glia, yielded a glial glutamine with twice the specific activity of the glutamate, but the Waelsch effect was not shown by the neuronal fraction. However, though compartmentation would appear to be within the one cell type, the glial fraction is in fact contaminated by nerve-ending particles, so the question remains as yet unanswered. Recent studies by Bradford (1969) reveal that these nerve-ending particles, formed in homogenates from the synaptic portion of the neurons, close up into particulate entities and show biochemical properties not unlike intact cells, rather than isolated organelles such as mitochondria: they support respiration, accumulate potassium, synthesise ATP and PCr, comparably to slices, but unlike brain mitochondria. Further study of their metabolism should be very rewarding and

perhaps reveal that they may be a third type of compartment. There may indeed be up to as many compartments as there are cell types and subcellular organelles in the brain, and Bradford (1968) points out their possible importance in the control of brain metabolism.

The metabolism of pyruvate in vitro.

Certain anomalies have been reported in the metabolism of acetate and pyruvate in the rat brain in vitro, though these substances are derived from glucose, and it is exclusively by conversion to them that glucose is oxidised (Villee and Loring, 1961). Beloff-Chain et al. (1962) found that the metabolic pattern of radioactive acetate and pyruvate in rat cortex slices differed from that of glucose. Cremer (1967) found a similar difference and attributed it to the compartmentalisation of available NADH, giving an apparently different metabolic pattern with substrate pyruvate, and substrate glucose which gives rise to endogenous pyruvate. Abadom and Scholefield (1962) found that the active transport of amino acids in vitro supported by pyruvate was not sensitive to inhibitors which blocked glucose-supported transport, posing the question of the location of ATP formation and availability in the cell, also posed by Balázs, Magyar and Richter (1964) and by Waelsch (1962a) who points out that the control of the passage of ATP from the mitochondria where it is made, to the other parts of the cell, and of ADP back, will in effect control the entire metabolism of the cell, as regards reactions that require ATP: for instance, glutamine synthesis from glutamate,

the phosphorylation of glucose. Lowry and Passoneau (1964) have studied the relationship between substrates and enzymes of glycolysis in brain, and have showed that several enzymes are sensitive to ATP or ADP. One can see how the presence of levels of either of these in a cellular compartment may inhibit or aid metabolic transformations in that compartment alone, and differently to other compartments. In fact Lowry (1966) believes that the unique properties of the brain may not lie so much in its different biochemical properties but in the unique organisation of the same properties possessed by other tissues.

OCCURRENCE AND FUNCTIONS OF FREE AMINO ACIDS IN NERVOUS TISSUE.

Levels in Mammalian brain.

Some amino acids have a concentration in mammalian brain which is higher than any other organ. These are the non-essential amino acids, which are connected with the citric acid cycle, namely aspartate, glutamine, GABA, and especially glutamate (Waelsch 1962 b). Roberts (1963, 1964) points out that GABA is only found in nervous tissue, but that it occurs very widely among vertebrates and invertebrates; each species also has its own particular pattern of ninhydrin reactive constituents. Fallan (1962) in a survey of the amino acid content of vertebrate nervous tissue, points out that glutamate has the highest concentration of any other amino acid in vertebrates as opposed to invertebrate nervous systems, where taurine or aspartate is the most abundant. Table 1 shows the levels of amino acids in rat brain, blood and

TABLE 1

LEVELS OF FREE AMINO ACIDS IN TISSUES OF THE RAT AND SOME INVERTEBRATES

µmoles/g

Animal	GLU	ASP	GLN	ALA	GABA	TAU	GLX	?HRE
Rat (1)								
brain	9.0	2.6	5.0	0.8	2.1	4.9	1.1	0.5
liver	2.8	1.0	2.0	2.5		5.5	2.8	0.6
plasma	0.1	0.02	0.7	0.4		0.2	0.3	0.2
Eledone								
optic lobe	11.0	8.4	2.2	7.7	1.0			
vertical l.	10.4	8.2	3.7	4.8	1.0			
O. Vulgaris								
Mantle (2)	9.0	10.0		3.1			15	40
Loliguncula								
whole (3)	2.0	0.5		15		55	20	
Mytilus								
whole (4)	15	15		41		200	53	
Lobster								
Nerve (5)	25	112		33	0.8	12	35	
Bee								
Brain (6)	50	8		35	11	23	4	

Values expressed on a wet weight basis. Figures quoted from:
(1)- Carver, 1965 (2)-Florkin, 1966
(3)- Simpson et al. (1959) (4)- Allen, 1961
(5)- Lewis, 1952 (6)- Frontali, 1964

liver, as well as those in some invertebrate bodies and nervous systems.

Physiological action of amino acids on mammalian nervous tissue.

There are a great number of chemical substances which are known to mediate synaptic transmission in vertebrate and invertebrate nervous systems; some of these are known to operate as synaptic transmitters in vivo, e.g. acetylcholine (Hebb and Krnjevic, 1962) and catecholamines (Von Euler, 1962). Others are suspected of being transmitters but there is no conclusive evidence for their operation in vivo. The situation is probably much more complex, in that several transmitters may operate simultaneously, though each neuron contains only one transmitter, (Cragg, 1968). The acidic amino acids glutamate and aspartate have such a possible role.

At this stage, it is necessary to give a simplified account of the mechanism of neural transmission, though the subject is described by Katz (1966) among others. All nerve cells show a resting potential of 60 to 70 mV, the inside of the cell being negative with respect to the outside. This is due to a higher level of internal potassium and external sodium, kept as such by a process known as the sodium pump which is energy dependent. The action of excitatory substances is to reverse this polarity by causing a temporary alteration of the membrane's permeability to sodium which enters. This local reversal of the polarisation of the cell membrane is conducted down the nerve axon as an action potential. On reaching the end of the axon, it is transmitted to another nerve cell or to a muscle end-plate, by causing

the release of a chemical across the synapse, which initiates a fresh wave of depolarisation. The action of so-called inhibitory transmitters is to inhibit the transmission across a synapse or nerve-muscle junction, by causing the resting potential to become more negative, thus counteracting its reversal by excitatory transmitters.

It was noted earlier that glutamate and aspartate caused depolarisation in mammalian cortical slices in vitro (Gibson & McIlwain, 1965; Bradford & McIlwain, 1966) and that this effect was similar to that of an electric pulse, (McIlwain, Harvey and Rodriguez 1969). These amino acids have also been found to cause excitatory depolarisation in situ, to cortical surfaces, (Krnjevic and Phillis, 1963) spinal neurons, (Curtis and Watkins, 1960) as well as excised spinal ganglia (Nagata, Yokio and Tsukada, 1966). Conversely GABA has been found to cause hyperpolarisation to cortical cells (Krnjevic and Phillis, 1963) and to spinal cells (Curtis and Watkins, 1960). GABA was first postulated as an inhibitory transmitter when Bazemore, Elliott and Florey (1957) showed that it was present in an extract of mammalian brain, which inhibited the crayfish stretch receptor muscle. However, McLennan (1958) found that the extracts containing this factor did not contain GABA, and supposed that the active substance was split to GABA. The evidence for GABA being an inhibitory transmitter is discussed more recently by Krnjevic and Schwarz (1966). Werman, Davidoff and Aprison (1968) have reported that glycine also behaves as an inhibitory

transmitter in the synapses of cat spinal neurons. Curtis and Watkins (1960) also found that taurine had an inhibitory effect on cat spinal neurons.

Levels of amino acids in invertebrates.

The occurrence of free amino acids and their levels in invertebrate tissue has been reviewed by Awapara (1962) and their levels in the brains of many species by Tallan (1962) and Roberts (1964). Table 1 shows the levels of some free amino acids in the brain or other tissues of invertebrates and molluscs, as well as the rat, for comparison.

As regards amino acid levels in invertebrates, free amino acids are generally higher in invertebrate than vertebrate tissue and represent a high proportion of the non-protein nitrogen content of the tissue, (Awapara, 1962). That this is not entirely due to a marine environment alone was demonstrated by Velankar and Govindan (1958) who showed that fishes contained about five times less amino nitrogen than a selection of marine invertebrates. Also, Duchâteau, Sarlet, Camion and Florkin (1952) showed that amino acid levels in marine invertebrates were higher than in related freshwater species.

Simpson, Allen and Awapara (1959) showed that a feature of a wide range of molluscs was that five amino acids which are connected to the citric acid cycle, glutamate, aspartate, glycine, taurine and alanine have markedly higher levels than the other amino acids, compared with the rat organs other than the brain, where the amino acids have a more even distribution (Awapara, Landua and Fuerst, 1950). They showed also that

taurine (α aminoethane sulphonic acid) was abundant in molluscs, and more in marine than freshwater forms. Its presence in the octopus has long been known (Henze, 1905). Awapara (1962) suggests that its function is to balance the high cationic content of the tissue: the large amounts of amino acids in marine invertebrates have been suggested as contributing to the maintenance of osmotic balance by Florkin (1966) and Robertson (1964), among others: Duchâteau, Florkin and Jeuniaux (1959) found that transferring salt water crabs to a medium of sea water diluted 1:1 caused a drop of the levels of some amino acids to half the value. Lange (1963) found that the concentration of taurine in the mussel (which can be as high as 4%) was proportional to the salinity of the medium. Negus (1968) noted that in the estuarine mollusc, pennant, the alanine content also varied with the salinity. Therefore the presence of high levels of free amino acids in marine invertebrates including molluscs, in all the parts including nervous tissue, represents a characteristic adaptation of the organism to the marine environment. Table 1 shows that in the rat brain, the glutamate level is much higher than that of the liver and muscle, but the amino acids of the optic lobe and the mantle of the octopus are comparable in level. Thus it is hard to decide how far the function of free amino acids in the octopus brain is connected with maintaining ionic balance in the animal as a whole, or, as in the rat brain, with the unique metabolism of the brain.

Physiological action of amino acids on invertebrate nervous tissue.

A great deal of investigation has been carried out on the possible role of glutamate and GABA as excitatory and inhibitory transmitters in the invertebrate brain, particularly in the crustacea, but also in the molluscs. Kerkut and Walker (1966) not only summarise much of the evidence to date, but stress the necessity to bear in mind the occurrence, localisation, synthesis and breakdown of these amino acids as well as their pharmacological action, when considering them as possible transmitters in a particular organism.

There is good evidence for the action of glutamate as an excitatory transmitter at the neuromuscular junction of the crayfish: Takeuchi and Takeuchi (1964) showed that iontophoretic application of glutamate caused contraction of the crayfish muscle, but only if applied to the region of the neuromuscular junction, the rest of the muscle being insensitive to this effect. Kerkut, Leake, Shapira, Cowan and Walker (1965) showed conclusively that glutamate was the transmitter *in vivo*, when they perfused the neuromuscular junction of crab, (as well as snail and cockroach) while electrically stimulating the nerve, and found that a ninhydrin-positive substance was released into the perfusate, in quantities proportional to the amount of stimulation, which they identified as glutamate.

Gerschenfeld and Lasansky (1964) showed that glutamate caused excitatory depolarisation in the central neurons of the snail: Kerkut et al. (1965) as above, demonstrated the role of glutamate as neuromuscular transmitter.

There is a good deal of evidence, summarised by Hall and Kravitz (1967), on the role of GABA as inhibitory transmitter in the lobster nervous system. All the enzymes necessary for the formation and oxidation of GABA are active. Also, the inhibitory nerve fibres contain 100 times the GABA levels of the excitatory fibres. Further, Iversen & Kravitz (1966) showed that GABA is specifically taken up by the lobster stretch receptor, and this would represent a possible mechanism for the inactivation of the transmitter. Finally, Otsuka, Iversen, Hall and Kravitz (1966) showed that GABA is released by lobster nerve-muscle preparations during the stimulation of inhibitory axons (compare Kerkut et al. 1965).

As regards other crustacea, Takeuchi and Takeuchi (1965) demonstrated that GABA duplicated the action of inhibitory transmitter at the crayfish neuromuscular junction. Recently, Kerkut, Pitman and Walker (1969) demonstrated this also in the central nervous system of the cockroach. There appears to be however no evidence for such a role of GABA in molluscs.

Systems for the synthesis and breakdown of glutamate and GABA have been described in most of the cases where these amino acids appear to be in evidence as transmitters. Kravitz (1962) describes such systems for GABA and glutamate in the lobster nervous system. In molluscs, in general the enzymes of glutamate metabolism have been extensively discussed by Goddard and Martin (1966), and have been demonstrated in the brains of cephalopods including the octopus by Drukker and Schadé (1964) and Pascoe and Schadé (1976). Kerkut, Shapira & Walker (1967)

demonstrated most elegantly that in the snail, glutamate, earlier shown to be released at the neuromuscular junction, was synthesised from glucose or alanine in the brain, and moved from the brain to the neuromuscular junction.

Roberts (1962) showed that in a wide range of animal species, GABA levels are related directly to the activity of GAD, the enzyme forming GABA from glutamate. However, Roberts and Simonsen (1962) found that octopus brain contained very low levels of GABA and GAD activity. So it would seem that while there is a good case for glutamate being an excitatory transmitter in some parts of molluscan nervous systems, there appears to be no evidence for GABA as an inhibitory transmitter, while in the crustacea and the insect, there is good evidence for both.

EXPERIMENTS PERFORMED.

The question as to where to start comparing the biochemistry of the octopus and rat brain posed a formidable problem, since there were not only several biochemical aspects of interest, but also several types of cellular preparations to use for study.

Since the previous sections have shown that glucose and its derivatives are interesting and important metabolites in the brain, it was decided to study the metabolism of glucose and amino acids *in vitro*, after Beloff-Chain et al. (1955a), as well as use homogenates to study activities of enzymes involved in some interconversions of the amino acids, after Balázs, Dahl and Harwood (1966), and to begin

to investigate the metabolism *in vivo* of glutamate in the octopus brain, since this yielded such interesting results for the rat brain from Berl et al. (1961).

As stated at the beginning of this chapter, two parts of the octopus brain were compared with each other and the rat brain, and they comprised two parts of the visual learning system. It must be emphasised that the rat cortex slice however contains elements associated with all the senses, and contains different types of neurons depending on whether it is the first or the second slice cut from each particular hemisphere. But since the optic and vertical lobe slices are cut sagitally to the lobe, each slice will have a similar range of cell types.

The following is a summary of the experiments performed on rat cortex and optic and vertical lobe tissue, the results of which are given in Chapters 3 and 4, and discussed in Chapter 5.

1) The determination of the content of free amino acids, proteins and nucleic acid in the three tissues, the last to estimate relative cell sizes and to try to assess the number of glia in the octopus brain.

2) The incubation of slices with radioactive glucose, glutamate, alanine to determine their incorporation into products *in vitro*, and the specific activities and levels of free amino acids after incubation: also to ascertain the effect on these values, by adding non-labelled glucose to the amino acids as substrates.

3) The testing of octopus brain slices for active uptake of certain amino acids, presumed to be involved in its

metabolism, for comparison with the rat brain: glutamate, glutamine, alanine and GABA.

4) The use of homogenates to study the activities of the enzymes involved in amino acid synthesis and interconversions.

5) The injection of U^{14}C glutamate into the octopus brain to determine the specific activities of the free amino acids in the optic and vertical lobe at different times after killing.

6) Further incubations <u>in vitro</u> of rat cortex slices with U^{14}C pyruvate or alanine, to try to clarify the apparent anomalies of pyruvate metabolism in the rat brain described earlier, and with labelled alanine in the presence of competitive inhibitors of citric acid cycle intermediates, to ascertain whether pyruvate or alanine can be converted to amino acids connected with the citric acid cycle, glutamate, glutamine or GABA, without entry into the citric acid cycle via Acetyl Coenzyme A. Unfortunately, lack of animals at the time prevented these potentially very interesting experiments from being carried out on the octopus.

It is understood that the scope of these experiments is severely limited; the observed metabolism will be that of a wide variety of cell types in the rat and the octopus. Also, as regards the octopus brain, knowledge of the gross biochemistry, as contributed in the case of mammalian brain by Himwich, Geiger and McIlwain, is lacking, and no such studies have been performed here. The implications and limitations of those that have been performed will be discussed in Chapter 5.

CHAPTER 2
EXPERIMENTAL METHODS

Three main classes of experiment were carried out as follows:

1. The incubation of octopus optic and vertical lobes and rat cortex slices *in vitro*, with various substrates universally labelled with ^{14}Carbon. After incubation, the slices and media were treated in one of three ways, according to the information required:

a) Ethanol extraction of the slice alone, followed by the separation of metabolites in slice and medium, using paper chromatography, and identification of the spots by autoradiography followed by scanning, for the quantitative determination of the counts incorporated into products.

b) Extraction of the slice with trichloroacetic acid followed by radioactive counting of the extract and medium to test uptake of the labelled substrate.

c) Isolation of free amino acids and other intermediates from the perchloric acid extract of the slice, by means of column and paper chromatography, or by the use of the automatic amino acid analyser, and the determination of the specific activities of these products.

2. Determination of the activity of some of the enzymes involved in the synthesis and interconversions of the amino acids in homogenates of rat cortex, and optic and vertical lobes of octopus.

3. Injection of live octopus with U^{14}C glutamate directly into the brain cavity, followed by killing at different times, and extraction of the free amino acids from the optic and vertical lobes, and determination of their specific activities as in 1c.

MATERIALS.

Reagents.

Radioactive materials: $U^{14}C$ D-glucose, $U^{14}C$ pyruvate, $U^{14}C$ L-glutamate, $U^{14}C$ L-alanine and $1^{14}C$-DL-glutamic acid were obtained from the Radiochemical Centre, Amersham, Bucks. $U^{14}C$ L-glutamine and $U^{14}C$ GABA were from the New England Nuclear Corporation, Boston, Mass. The $U^{14}C$ glucose, glutamate and pyruvate were purified by streaking onto paper and running in butanol-acetic acid-water (40:11:25 by vol.) for 16 hours. The radioactive substance was located by autoradiography over Kodirex X Ray film for 2 to 4 hours, the band cut out and eluted with water, and the eluate evaporated to dryness and redissolved in a small volume of distilled water. The yield was usually over 80%, and depended on the purity of the substance: pyruvate and glutamate in particular tended to be contaminated with their decomposition products.

Enzymes, coenzymes and chemicals: Lactate dehydrogenase and ATP trisodium salt were from Boehringer & Soehne GmbH, Mannheim, W. Germany. Malate dehydrogenase, reduced NAD, ADP, BAL (2,3 dimercapto-propan-1-ol), glutathione in reduced form, glutamohydroxamic acid, and triton-X-100 were from Sigma (London) Chemical Company Ltd., London S.W.6. Pyridoxal Phosphate was from BDH Laboratory Chemicals Ltd, Poole, Dorset.

Resins Zeocarb 225 Resin, (SRC 13) in the sodium form was from Permutit Ltd., London, W.14. It was converted into the hydrogen form by alternate washings of HCl and NH_4OH through 3 or 4 cycles. Dowex AG x 10 (200 to 400 mesh) was from Biorad, Richmond, Calif. This was taken

through several generation cycles with alternate washings of HCl and NaOH, then washed with water until the washings were chloride free; finally CO_2 was bubbled through for one hour to convert the resin to the carbonate form. The final washing was concentrated and tested with ninhydrin. All resins were washed with at least 4 bed volumes of distilled water before used.

Scintillation Chemicals: Hydroxide of Hyamine, p-(diisobutyl-cresoxethoxyethyl)-dimethyl benzyl ammonium chloride, PPO, 2,5 diphenyl oxazole, and POPOP, 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene, were from Packard Instrument Co. Inc., Ill.

Animals.

The rats used were adult male Wistars ('rattus norwegicus') bred in this department, weighing between 100 and 200g each. They were killed by stunning on the back of the head with a blow, their throats cut to exsanguinate, and the brain removed. Exsanguination was found to be a better method than pulling the neck, as the latter in the hands of an inexperienced person invariably resulted in accumulation of blood around the cerebellum and cortex surface.

The species of octopus used was eledone cirrhosa which is closely related to octopus vulgaris, whose anatomy was described in Chapter 1. They are alike in appearance except that eledone has a single row of suckers on the arms and o. vulgaris a double row, and both belong to the order Octopoda, class Cephalopoda, phylum Mollusca. Young (1963b) estimated that the optic lobes contained 65 million neurons each; the vertical lobe contained 25 million cells, which amounted to more

than 80% of the total cells in the supraoesophageal lobes. Of these, over 90% are under 5μ in diameter. It must be emphasised that during excision of the vertical lobe for slicing, some of the subvertical and frontal lobes were sometimes excised as well, but from the figures above, the resulting contamination would be expected to be under 10%.

The animals supplied to us were caught off the Devon and Cornwall coast. Eledone are in fact found in waters all around Britain and in the North Sea. They were caught in trawls by fishermen based in the port of Brixham, Devon, and were placed in tanks of sea water on board. On arrival in Brixham they were transferred to a tank with circulating cooled sea water, on the premises of a local hotelier, Mr. Robert Stephens, who placed them in specially designed plastic transporters for the journey to London. These were designed and built by Mr. Graham Moffatt in this Department but their description has not been published. The tubs were 2' high and 18" wide. At the beginning, they were half-filled with sea water and the animal put in: the lids were kept on by means of a metal ring. The whole was placed into a larger plastic container 2½ft square by 2½ft. high, also containing a small oxygen cylinder clamped to the side of the larger container. A small rubber tube delivered oxygen at a slow rate to the animal throughout the journey through a small hole in the lid of the tub. The square lid of the larger container was clamped down by means of screws at each corner. Later the system was modified to avoid the larger container and the gas cylinder, and the octopus was placed in a polythene bag inside the tub, half-filled

with cooled sea water, and gassed with oxygen for the journey. On arrival at Paddington station they were collected immediately and placed in a tank in the animal house in this Department. The animal survived the journey under these conditions for up to six hours, the average time from Brixham to the laboratory, but if left in the transporters longer than that, they were likely to die from overheating and lack of oxygen.

The tanks in the animal house were also built by Mr. Moffatt and consisted of a series of plastic tubs, each 3' by 2' by 2', and each with inlet and outlet taps. Sea water from Brixham was cooled by means of a refrigeration unit to 8 to 10°C, and circulated through the tanks, and also through a gravel bed and a column of diatomaceous earth, to remove impurities. All parts of the system through which the water flowed were made of plastic, and pump parts were coated with polyurethane paint to avoid corrosion. The arrangement was such that the tanks were in parallel with each other, all filling from and emptying into the pipe system, which contained the filter bed and column in series. The water was kept to a standard density by topping up with distilled water, and changed as often as it became cloudy. The animals could survive up to several weeks in this system and were fed on crabs. The tanks were covered with closely fitting plastic lids held down with half a dozen bricks when occupied, since octopus are capable of squeezing through very small gaps. The best time for reception of the animals was the winter, since <u>eledone</u> tend to die in warm weather, in contrast to <u>o. vulgaris</u>, whose habitat is the much warmer seas. The <u>eledone</u> received

in our laboratory varied in size, but were usually 12" to 18" in arm span, and weighed around 500g.

For killing, one person held the animal while the other severed between the eyes and the mantle with a razor. Accuracy is essential here as the brain lies just behind the eyes, and is made all the more difficult by the movement of the animal. Figure 7 shows the dissection of the octopus brain. The eyes were cut out and the fatty tissues removed, and the optic lobes severed from their stalks. The vertical lobe was reached by slicing across the cartilaginous covering of the central brain until the mucilage-filled cavity above the vertical lobe was exposed. Then four vertical cuts were made and the block of cartilage containing the supraoesophageal was cut out by slicing at the level of the oesophagus. The vertical lobe was cleaned of mucilage and cartilage on a wax block. Optic lobes usually weighed around 150 mg each, vertical lobes around 70 mg.

For enzyme assays, eledone optic and vertical lobes were sent to us frozen solid in a Thermos flask filled with solid carbon dioxide, by courtesy of Dr. G. Cottrell, Gatty Marine Laboratory, St. Andrew's.

INCUBATION PROCEDURE

Preparation of brain slices.

Prior to slicing some workers prechill incubation media and place the excised brains on ice. In these experiments, this procedure was avoided and the following method used: each animal was killed and the brain sliced, and slices were placed into the incubation

Diagram of the octopus brain

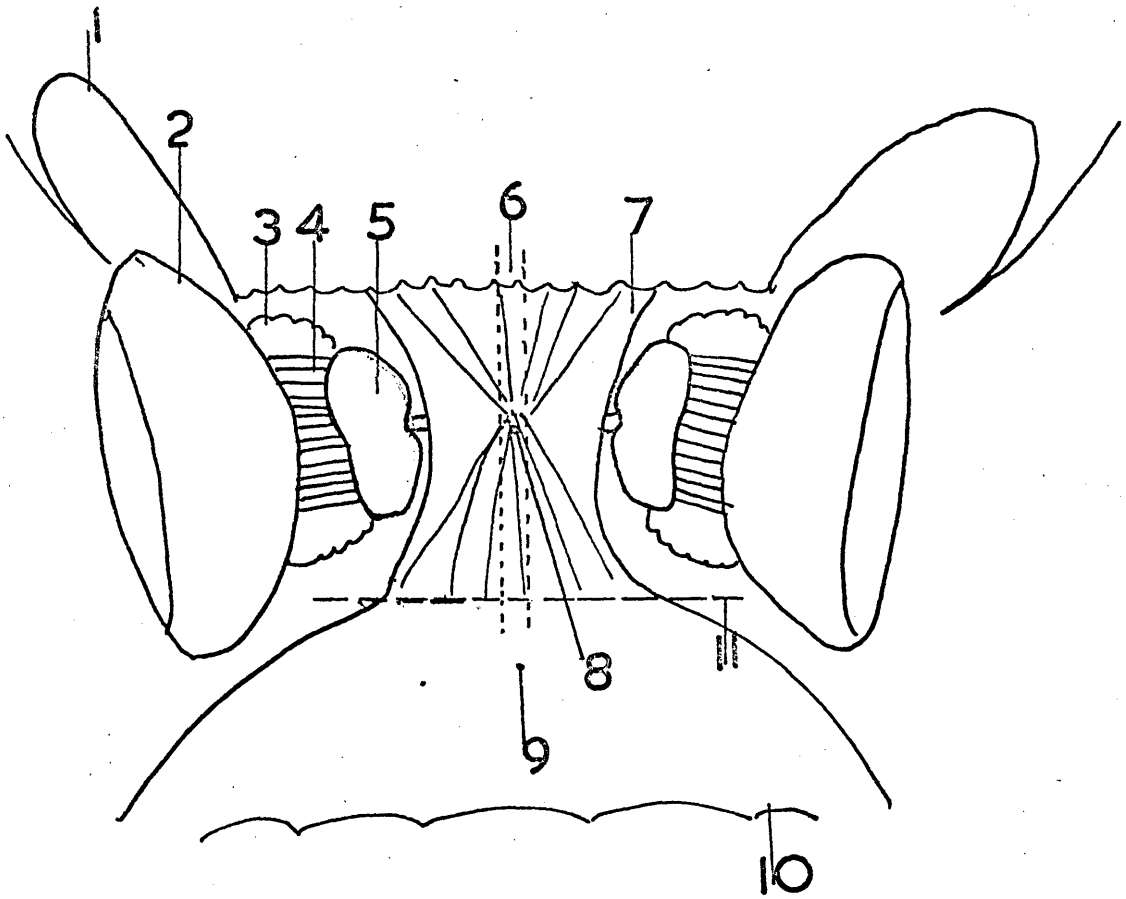


FIGURE 7

DISSECTION OF THE OCTOPUS BRAIN SEEN FROM THE TOP.

Outer layer of skin has been removed, and the parts shown are as follows:

1- Funnel 2- Eye 3- Fatty tissue 4- Optic nerves
5- Optic lobe and stalk 6- site of oesophagus
7- Cartilaginous covering of brain 8- Site of vertical lobe, and point of injection into the brain
9- Site of beak and mouth 10- Mantle and arms
11- Line of decapitation.

Scale: about actual size, for a large animal.

medium in Warburg vessels, attached to the manometers, and gassed with oxygen before killing the next animal, so that not more than 3 minutes elapsed between excision of the brain and its shaking in vitro under oxygen.

The rat brain was divided longitudinally, the cerebellum removed and the cortex cleaned of white matter on a wax block. The cortical shell was placed, pial (top) surface upward, on a filter paper moistened with saline, and sliced with a clean, dry Stadie blade with the aid of a McIlwain guide, of clearance 0.3mm. Two slices of around 70mg each, pial and subpial, were cut from each hemisphere and weighed accurately on a torsion balance before transfer to a Warburg vessel.

Octopus brain slices were made in the same way, but three or four small slices were obtained from an optic lobe, and two from a vertical lobe. An important difference between octopus and rat brain slices is that the latter are cut tangentially to the surface, necessarily severing the axons. Octopus brain slices are cut sagitally to the surface, cells being damaged at the cut end only, so the slices contained a considerable number of intact neurons and axons.

Incubation salines.

The slices were transferred to Warburg vessels for incubation. The most suitable size was found to be those of capacity around 14ml, containing 2 to 3 ml of saline, 3ml being used for tissue weights greater than 100mg. The centre well contained a filter paper wick moistened with hydroxide of hyamine to absorb CO_2: this solvent is miscible with Brays scintillant and is strongly basic.

Though there are several saline in use with rat brain slices, and reasons for choosing and omitting certain ions, (Bourke and Tower, 1966) only one medium was used throughout in these experiments, a high potassium, calcium-free, phosphate-buffered saline similar to that of Elliott and Henderson (1948), of the following composition: 98mM NaCl, 27mM KCl, 2.4mM $MgSO_4$, 0.8mM KH_2PO_4, 3.5mM Na_2HPO_4, pH 7.4. This was the medium used by Beloff-Chain et al. (1955a), Rose (1968b) and Chain, Rose, Masi and Pocchiari (1969), and was used here for ease of comparison with these earlier data on rat brain metabolism in vitro. For the octopus, the medium chosen was that originally used by Hoyle (1953) but its ionic composition is similar to octopus plasma (Robertson 1964) and to a high calcium, tris-buffered Krebs saline, its composition being: Tris-HCl 26mM, NaCl 125mM, KCl 5mM, $MgSO_4$ 1.2mM, $CaCl_2$ 5mM, pH 7.4. This medium was also used for incubation of snail brain and locust brain in vitro by Bradford, Chain, Cory and Rose (1969), and Bradford found that it maintained physiological conditions in giant snail neurons (unpublished results).

Substrates were 10mM in concentration. The reason for this choice, also used by Rose (1968b) and Chain et al. (1969) are discussed in the next chapter. 5 to 10 microCuries of radioactive substrate was generally used per flask.

Temperature. Rat brain slices were incubated at normal body temperature, 37°C, but the choice of a suitable temperature for the octopus brain incubations was difficult, since eledone is cold-blooded, and has no fixed

body temperature. Preliminary incubation of octopus brain was carried out at 37° with glucose as substrate, but a low and erratic oxygen uptake was obtained, and no counts were incorporated into substances other than lactate and $@O_2$ in contrast to the situation when the lower temperature of 25°C was adopted. This was used by Ghiretti-Magaldi, Giuditta and Ghiretti (1958) for incubation of octopus vulgaris tissue in vitro. Even this was relatively high considering eledone inhabits colder waters than o. vulgaris.

Incubation time. Two hours was chosen for both animals, to allow sufficient counts to be incorporated into products in the octopus, whose metabolism was much slower than the rat's. However, not every substrate used maintained a linear oxygen uptake over the two hours.

The flasks were attached by springs to the manometers, using anhydrous lanolin to grease the glass joints. Pure oxygen was passed through the flasks in the shaker water bath for 10 minutes, as described by Umbreit, Burris and Stauffer (1964). This allows maximal solution of oxygen in the medium, sufficient not to be a limiting factor in respiration: the shaking of the bath aids diffusion of substrate and salts into the slice. After gassing the flasks were sealed and allowed to shake for two hours. The shaking of the slice in the substrate-containing medium is said by McIlwain (1966) to be analogous to the flow of blood through the tissue in vivo. Oxygen uptake was measured manometrically at 10 or 15 minute intervals over the two hours, and calculated as described by Umbreit et al. (1964). In each experiment a control flask was run, containing the saline and radioactive

substrate but no slice. It served as thermobarometer and was treated identically to the other flasks. In each run , flasks with different substrates or different tissues were run in triplicate or quadruplicate, and if two different radioactive substrates were used in one run, one control flask was run for each. Several such experiments were run for each substrate or tissue.

QUANTITATIVE DETERMINATION OF METABOLITES.

Extraction of slices and counting procedure.

The filter paper wicks containing the $^{14}CO_2$ were transferred to scintillation counting vials, the centre wells were washed out three times with 0.1 ml of water and the washings added to the vials. 10ml of scintillation fluid were added, of composition similar to that of Bray (1960): Naphthalene 60g, PPO 4g, POPOP 0.2g, 1,2 ethanediol 20ml, toluene 100 ml, methanol 100 ml, made up to 1 litre with dioxan.

Scintillation counting was carried out in the Packard or Beckman liquid scintillation counter. The principles of scintillation counting are described by Birks (1964); very simply, the β particles emitted by ^{14}Carbon are converted to flashes of light by the liquid solvent system of the scintillant in which the radioactivity is suspended. These flashes are converted to pulses in the machine, and are counted. The samples were usually counted for ten minutes, or until an upper set limit of counts had been reached. The disintegrations per minute in each sample were estimated by comparison with a known standard ^{14}C source. However certain additives to the solvent system, such as alkali or

hyamine, cause quenching, that is, a reduction of the efficiency with which disintegrations are converted to scintillations in the fluid, so that the true radioactivity of the sample is underestimated. One of the means of estimating this is the channels ratio: it is known that the quenching agent also causes the curve, number of particles v. energy of particles, to be shifted: the extent of the shift is inversely proportional to the efficiency of counting, and is estimated by counting particles of two different energy ranges simultaneously. The ratio of the counts in each is determined, and the true efficiency found by comparison with a standard curve (prepared with a standard ^{14}C solution and the same scintillant) of efficiency v. channels ratio (the channels being the energy ranges of the particles). An alternative method of assessing efficiency is that of external standardisation, where a standard gamma-ray emitting source is brought up against the sample and the pair counted for one minute. Efficiencies usually agreed whether measured by this or the channels ratio method, but the latter method was used for calculation. Counts were expressed in absolute values of disintegrations per minute.

Immediately after removal of the filter paper wick from the centre well, the rim of the vessel was wiped with a tissue moistened with acetone to remove the grease, and the slice removed for extraction by the method of Beloff-Chain et al. (1955b). It was carefully removed whole by means of a small spatula, washed three times in three separate Petri dishes containing clean saline, blotting in between, and homogenised by hand in

a ground glass homogeniser with 1.0 ml cold 60% ethanol. The homogenate was transferred to a conical centrifuge tube together with two 1 ml washings, chilled for 15 minutes, and spun at 2000 rpm for 15' on the International Centrifuge. The precipitate was washed twice with 1ml of 60% ethanol, then twice with 1ml of water; the combined supernatants were pooled in another conical tube, and evaporated to dryness at 37° on a multisample Buchler rotary evaporator. The slice extract was redissolved in 0.5 ml water and kept deep frozen for chromatography, and an aliquot of the incubation medium was also kept. The ethanol-insoluble residue containing protein, lipid, polysaccharide and nucleic acid was washed a further three times with water, the washings now being discarded, and dissolved at 60°C in 0.2 ml hyamine, then washed into a counting vial with Brays Scintillant.

Chromatography and scanning.

Bidimensional chromatography for the detection of amino acids, sugars and intermediates was carried out using the method of Boloff-Chain et al. (1955b). A total sample of 20 µl of each sample of medium and 50 µl of each sample of extract was spotted onto one corner of a sheet of Whatman no. 1 filter paper; the paper was 46 cm by 52 cm, and was drawn with two margins all the way round, 10cm and 7cm from the edge. The samples were spotted at one of the intersections of the inner margins, and the papers folded for placing onto the solvent-containing trough along one of the outer margins next to the spot. 5 µl was spotted at a time using an Agla micrometer syringe, and allowed to dry in a stream of cold air from a hair dryer before applying the next spot.

The papers were run first in the solvent sec Butanol-formic acid-water (75:13:17, by vol.) for 15 to 18 hours, then dried in a stream of cold air, and run in the perpendicular direction in phenol-water-d 0.88 ammonia (80:20:1, by vol.) for 18 to 20 hours.

Lactate was isolated separately by monodimensional chromatography. 5 samples of a total of 20 µl each were spotted along the inner margin of the same type of paper as above, and run overnight in the solvent n Butanol-acetic acid-water (40:11:25, by vol.) Before drying, the papers were hung in an atmosphere of ammonia vapour for 5 minutes, to fix the labile lactate.

All chromatograms were dried in a stream of cold air (warm air caused phenol-soaked papers to collapse), and placed on Kodirex X Ray film for 3 to 5 days, then developed, to locate the radioactive spots, and to keep a record of the chromatogram.

The papers were scanned by a system first described by Frank, Chain & Pocchiari (1959), modified and built in this department by Mr. A.E. Lowe, and described in detail by Lowe, (1969). Briefly, the papers are held closely and vertically in front of a gas flow counter, which automatically moves along the paper horizontally in steps of 0.4", then one step down, and again horizontally, until the entire area of paper has been scanned. The time for which each area is counted may be set from 10 to 900 seconds according to how heavily the paper is loaded with radioactivity. The results were recorded as punched holes in a tape, which, after processing through the computer, were converted into counts. A print out was obtained showing the numbers

of counts in each spot. All chromatograms were spotted in duplicate and with this system, agreement between the values of the counts was within 5%. The counts in each product were expressed as a fraction of the total counts in the radioactive substrate in the control flask. A small portion of the radioactive substrate was also counted in the liquid scintillation counter, to obtain an absolute value of disintegrations per minute. Also, the sums of the counts recovered in the chromatograms were within 10% of the counts in the substrate of the control flask chromatograms.

Determination of specific activities.

In these experiments, CO_2, amino acids, and non-amino acid metabolites such as glucose, pyruvate and lactate, were separated from each other in bulk before isolation by paper chromatography. Though there is a method for the determination of CO_2 specific activity, precipitation with Barium Hydroxide, planchetting the precipitate, weighing and counting it, (Sellinger, Catanzaro, Chain & Pocchiari, 1962), the amounts of CO_2 produced by the octopus brain from the radioactive substrates used here were too small to be measured gravimetrically. The following attempt was made to measure carbon dioxide: CO2 was collected in 4M NaOH in the centre well of the Warburg vessel, made up to 5ml with freshly boiled (CO_2 free) distilled water and titrated with 0.1M HCl using methyl orange as indicator. It was also titrated with HCl after addition of 1 ml of 0.1M $BaCl_2$ (excess) to precipitate the carbonate, using phenolphthalein as indicator this time. The difference between these two titres represented the carbonate

(Vogel, 1964). An aliquot of the NaOH / Na_2CO_3 solution was taken for counting. Though the counts were reproducible, the titration values were low and erratic, so the determination of the specific activity of CO_2 was abandoned.

Slices and media were extracted together using the method of Balázs, Dahl and Harwood (1966) as modified by Rose (1968). The flask contents were poured into ground glass homogenisers together with two 1ml water washings, and perchloric acid (PCA) added to a final strength of 0.5 to 1M. The homogenate was chilled for 15 minutes, transferred to pyrex test tubes and spun at 2000 rpm for 15 minutes in the International refrigerated centrifuge at 5°C. The supernatants were poured into cooled 100ml beakers, the precipitates washed with 10ml of 0.2M PCA and the washings added to the beakers. The extracts were neutralised with 1M KOH to pH 6.5 to 7.0 on the pH meter, and using a magnetic stirrer: perchlorate ions were precipitated during neutralisation. At this stage, extracts could be left in the deep freeze until chromatography. The precipitates were washed a further three times with 0.2M PCA, dissolved in hyamine and counted, or in 1M NaOH at 70°C, if protein estimation was to be done on the pellet.

Column and Paper Chromatography.

Chromatography was carried out in three stages. The first, to separate the amino acids from the other metabolites; second, to separate the acidic or dicarboxylic amino acids (glutamate and aspartate) from the neutral ones (alanine, glutamine and GABA); the third, to isolate them individually. The extract was poured

through a fluted filter paper onto a column of Zeocarb resin in the acid form (For description of the resin and its regeneration, see the Reagent section at the beginning of this chapter). A 10 cm length of resin on a column of 1 cm diameter was sufficient to hold up to 20 µMoles of amino acid. When 10mM glutamate or alanine was substrate, a 20cm length was used. The non-amino acid intermediates were washed through with 3 bed volumes of distilled water and collected. The eluate was neutralised on the pH meter, an aliquot counted, and a portion of it was concentrated on the rotary evaporator for estimation of lactate and glucose (see later). The amino acids were eluted with 5 bed volumes of 1M ammonia solution into 500ml round-bottomed flasks, and evaporated to dryness at 40°C on a rotary evaporator. Glutamine is liable to decompose above this temperature. The eluate was redissolved in 5 ml water, a small portion taken for counting, and the rest passed through a column of Dowex resin in the carbonate form. Since the resin is very fine, and flow through it is very slow, it was necessary to force the liquid through by using air or nitrogen at 5lb pressure. For this purpose a scaffolding was set up comprising a glass manifold linked to the gas cylinder and connected by quickfit taps to the columns, in which an extra side arm had been fitted to allow introduction of liquid without interrupting the gas flow. The neutral amino acids were washed through with 3 bed volumes of water, and the acidic ones with 5 bed volumes of 1M HCl. When the acid was added it was necessary to increase the air pressure slightly as large

bubbles of CO_2 were released which tended to break up the resin. Collecting vessels were switched when the solvent front was 1" from the bottom of the column, since glutamic acid runs very close to the solvent front. The acidic and neutral fractions were taken to dryness in two stages, first on the rotary evaporator, then transferred quantitatively to conical centrifuge tubes and taken to dryness on the multisample rotary evaporator. The amino acids were taken up in 0.5 ml of water for chromatography. Small portions of these were counted. Table 2 shows the recoveries of the counts in all the fractions after column chromatography, in rat and octopus brain, with the different radioactive substrates used.

Paper chromatography was carried out monodimensionally, streaking 250 µl of the extract in a band on the paper sheet as shown in Figure 8. Razor blade cuts separated the samples from one another and made cutting out easy later. Amino acid markers were also run for identification; a spot (10 to 25 µl) of the acidic (A) and neutral (N) fractions were run, for one of two reasons: first, for estimating the loss of $^{14}CO_2$ from the paper during the colour development, (this will be fully explained below) and secondly, for spotting fractions which contained an amino acid in excess of 1 µmole. This occurred when glutamate or alanine were used as substrates.

The papers were run for 35 to 40 hours in the solvent n Butanol-propionic acid-water (10:5:4, by vol), the bottom of the paper having been trimmed with pinking shears to ensure even flow, since the solvents ran off the edge of the paper.

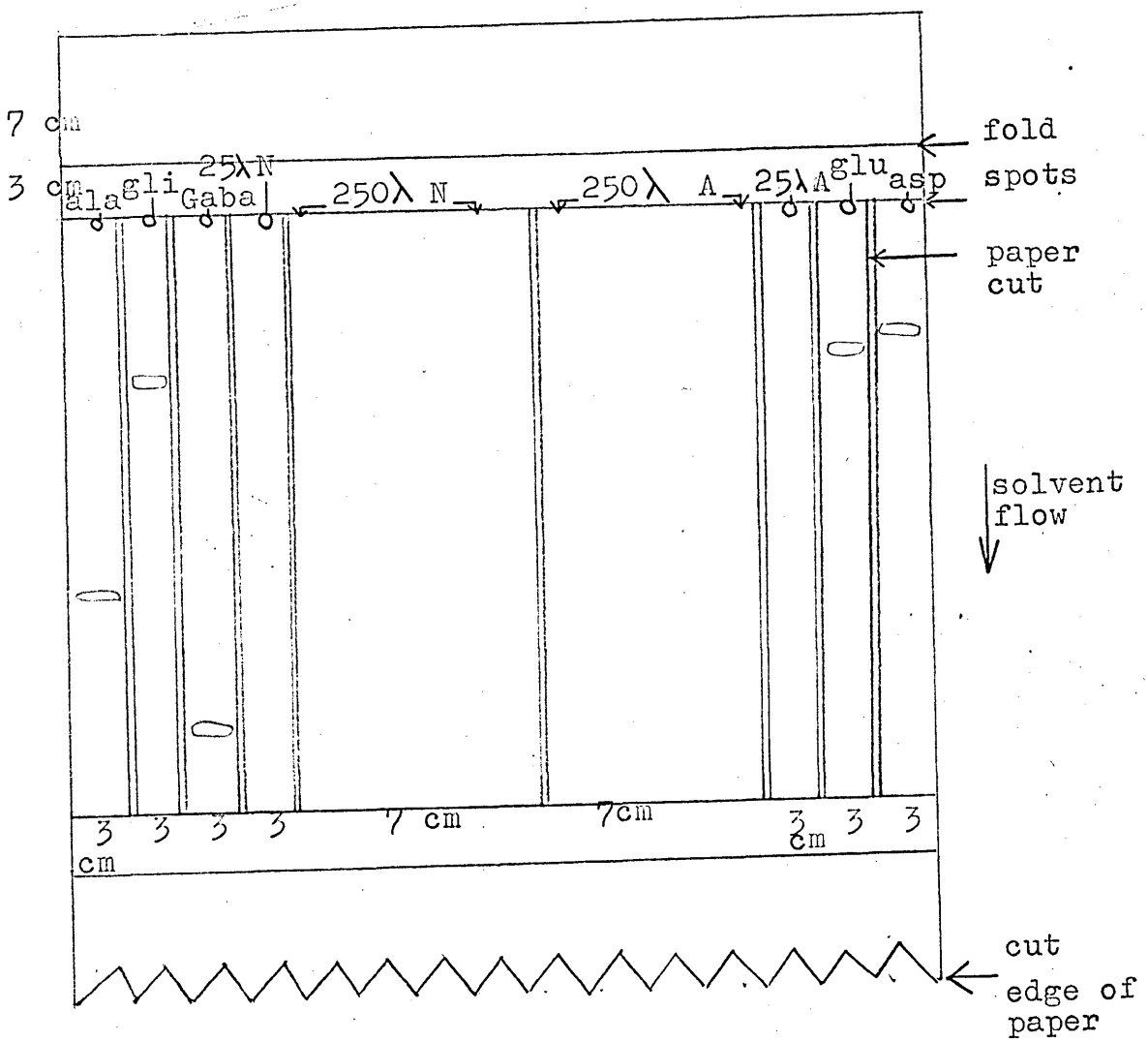


FIGURE 8

Scale diagram of monodimensional chromatogram, as prepared for isolation of amino acids for determination of specific activities as described in the text. The paper is slit with a razor blade where shown. Acidic (A) and neutral fractions (N) streaked as shown (250 µl or more) or spotted (25 µl). The positions of the amino acid spots after the run are also shown.

TABLE 2

YIELDS OF COUNTS OBTAINED AT STAGES IN THE COLUMN CHROMATOGRAPHY OF SLICE EXTRACTS AFTER INCUBATION WITH RADIOACTIVE SUBSTRATES

Percentages of the total counts in the control flask

	mean wt. of slice(mg)	non amino acid eluate	amino acid eluate	N	A
SUBSTRATE					
$U^{14}C$ GLUCOSE					
Optic l.	197 (3)	103%	2.1%	1.5%	0.4%
Vertical l.	123 (3)	101	1.2	1.0	0.2
Rat brain	144 (3)	83	4.0	2.2	1.3
$U^{14}C$ GLUTAMATE					
Optic l.	185 (3)	40%	42%	0.7%	30%
Vertical l.	98 (3)	21	37	1.1	23
Rat brain	128 (3)	4	72	2.0	63
$U^{14}C$ GLUTAMATE + GLUCOSE					
Optic l.	172 (3)	10%	95%	0.9%	81%
Vertical l.	88 (3)	5	72	0.6	63
Rat brain	112 (3)	5	59	1.3	41
$U^{14}C$ ALANINE					
Optic l.	128 (4)	5%	91%	76%	2.5%
Vertical l.	97 (4)	5	93	76	1.6
Rat brain	178 (4)	6	88	75	1.9
$U^{14}C$ ALANINE + GLUCOSE					
Optic l.	188 (4)	7%	92%	67%	4%
Vertical l.	65 (4)	7	94	73	3
Rat brain	150 (4)	13	83	65	3

The table shows the yields of radioactive counts in the Zeocarb and Dowex eluates, after column chromatography of the extracts of optic lobe, vertical lobe and rat brain slices as described in the text. Results expressed as the means of the numbers of slices in brackets. The non amino acid and amino acid eluates theoretically add up to 100%, and the acidic(A) and neutral(N) fractions to the value for the amino acid eluate.

Colour development.

Papers were dried in an atmosphere free of ammonia vapour, then sprayed evenly to saturation with a freshly made 0.25% solution of ninhydrin in acetone. Ninhydrin (Indane Trione Hydrate) reacts with amino groups to give a purple complex. The reaction is quantitative for a wide range of amino acid concentration, but each amino acid has a curve of different slope. Standard curves were made by spotting onto chromatography paper 0.2, 0.4, 0.6, 0.8 and 1.0 µmoles of each of the amino acids glutamate, aspartate, glutamine, alanine and GABA; the papers were dried and sprayed at the same time as the sample chromatograms and with the same ninhydrin solution, and the colour developed by heating the papers hanging in an oven at 70^{0} for 30 minutes. Tsukada and Nagata (1961) found that these conditions allowed maximal colour development. Preliminary experiments showed that there was no loss of colour if the same quantities of each amino acid were run through the solvent, as spotted directly onto paper, so the latter procedure saved running many extra papers. The graph (Figure 9) shows that for these five amino acids, colour development is linear for up to 1.25 µmoles per sample. The standard curve was prepared for every experiment, since there were always small differences in the ninhydrin reagent, or the colour development. The purple bands were identified by comparison with the markers. From Figure 8, it can be seen that in the A fraction glutamate runs faster than aspartate, in the N fraction GABA runs faster than alanine, then

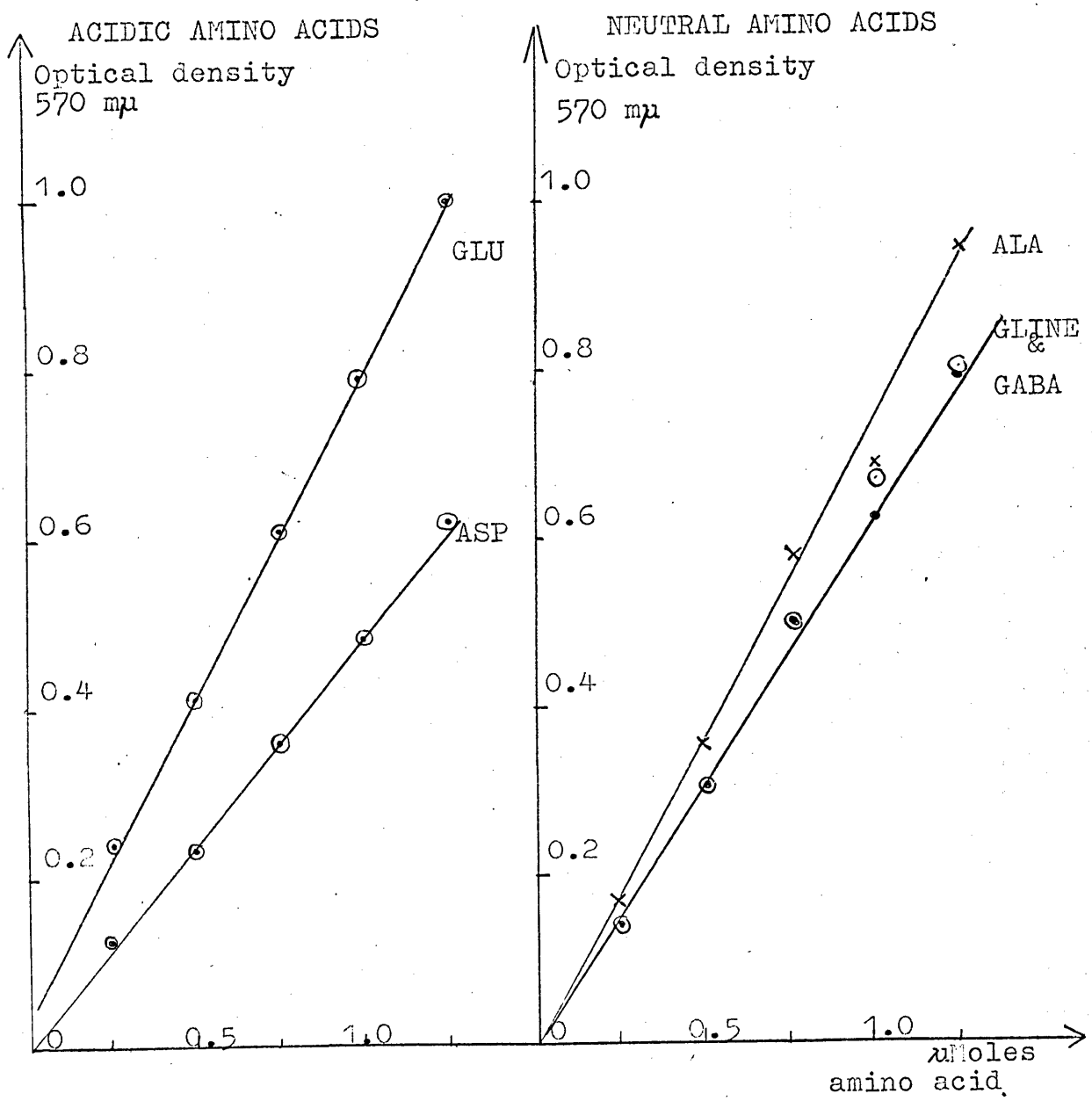


FIGURE 9

STANDARD CURVES FOR THE NINHYDRIN DETERMINATION OF AMINO ACIDS

Typical set of standard curves, optical density v. concentration, for the amino acids glutamate, aspartate, glutamine, alanine and GABA. Curves are not identical, but are linear up to 1.25 μMoles per sample.

glutamine, whose Rf value is very close to those of glutamate and aspartate: if the fractions were mixed before running, these three amino acids would be inseparable. The bands were cut out, also a blank piece of similar area, rolled with a forceps and pushed into test tubes. 10ml of 60% ethanol were added and elution aided by whirling gently on a vortex mixer; shaking the tubes produced fragmentation of the paper which interfered with the reading. The samples and standards were read at 570 mμ on the Unicam SP 500 or SP 600 spectrophotometer fitted with a flow cell. Afterwards 0.1 ml of the radioactive eluates and the paper blank were pipetted into counting vials. Table 3 shows the recoveries of the counts in the individual amino acids, after paper chromatography of the Dowex eluate: yields in this case were poorer than in Table 2, especially with U ^{14}C alanine as substrate.

In the first set of experiments using U ^{14}C glucose as substrate, 25 μl spots of the A and N fractions were also run. The strips shown in Figure 8 were cut out BEFORE spraying and the radioactive spots located by autoradiography. They were then cut out and placed directly into counting vials, and Brays Scintillant was added. This procedure was to estimate the loss of ^{14}C occurring during the colour reaction, which results in an underestimation of the radioactivity, but not of the amount of amino acid, giving an underestimate of the specific activity: the error also varies with each amino acid. The proportion of ^{14}C lost

TABLE 5

YIELDS OF AMINO ACIDS AFTER PAPER CHROMATOGRAPHY

Counts in free amino acids in the ninhydrin eluates expressed as a percentage of those in the Dowex eluates

$U^{14}C$ SUBSTRATE	GLU	ASP	Total A	GLN	ALA	GABA	Total N
GLUCOSE							
Optic l.	54%	6%	60%	36%	35%	-	71%
Vertical l.	40	23	63	48	6	-	54
Rat brain	54	11	65	33	7	11	51
GLUTAMATE							
Optic l.	84%	2%	86%	55%	44%	-	99%
Vertical l.	79	1	80	36	92	16	144
Rat brain	66	7	73	31	3	13	47
ALANINE							
Optic l.	26%	13%	40%	1%	58%	-	59%
Vertical l.	15	15	30	0.5	47	-	47
Rat brain	11	15	26	0.7	64	0.1	65

Yields of radioactive counts in the individual amino acids after paper chromatography of the Dowex acidic (A) and neutral (N) eluates: Incubation of octopus and rat brain slices was carried out with three radioactive substrates, and extracts were worked up and chromatographed as described in the text.
Results shown are the means of three values.

will depend on the carbon chain length of the amino acid in question, and if uniform labelling is assumed, will be equal to the reciprocal of the number of carbon atoms in the amino acid molecule x 100%, which will be 20% for glutamate and glutamine, 25% for aspartate and GABA and 33% for alanine. However if labelling is non-uniform in the carbon skeleton, the loss may range from zero (if C-1 is not labelled) to 100% (if only C-1 is labelled).

By inspection of figure 10, showing the carbon skeletons of the amino acids and intermediates, one may predict the theoretical labelling of the carbon skeletons of each amino acid. $U^{14}C$ glucose gives rise to $U^{14}C$ pyruvate. Alanine would have the same labelling pattern as pyruvate, since in transamination reactions, carbon skeletons do not exchange. C-3 of pyruvate becomes C-1 of α ketoglutarate in the first turn of the citric acid cycle. This carbon atom is lost as CO_2 before reaching oxaloacetate, but this molecule may be formed from PEP or pyruvate by the Malic enzyme, PEP carboxykinase or pyruvate kinase reaction, in which case it is labelled in all but the C-4 position, or fully labelled if it picks up a radioactive CO_2 molecule. Therefore, in many turns of the cycle, one would expect alanine and the other amino acids to be labelled uniformly , or in all carbon atoms except the omega position. Therefore the proportionate loss of radioactivity during the ninhydrin reaction would be 33% for alanine, but would be between 20 and 25% for glutamate, 25 to 33% for aspartate and GABA.

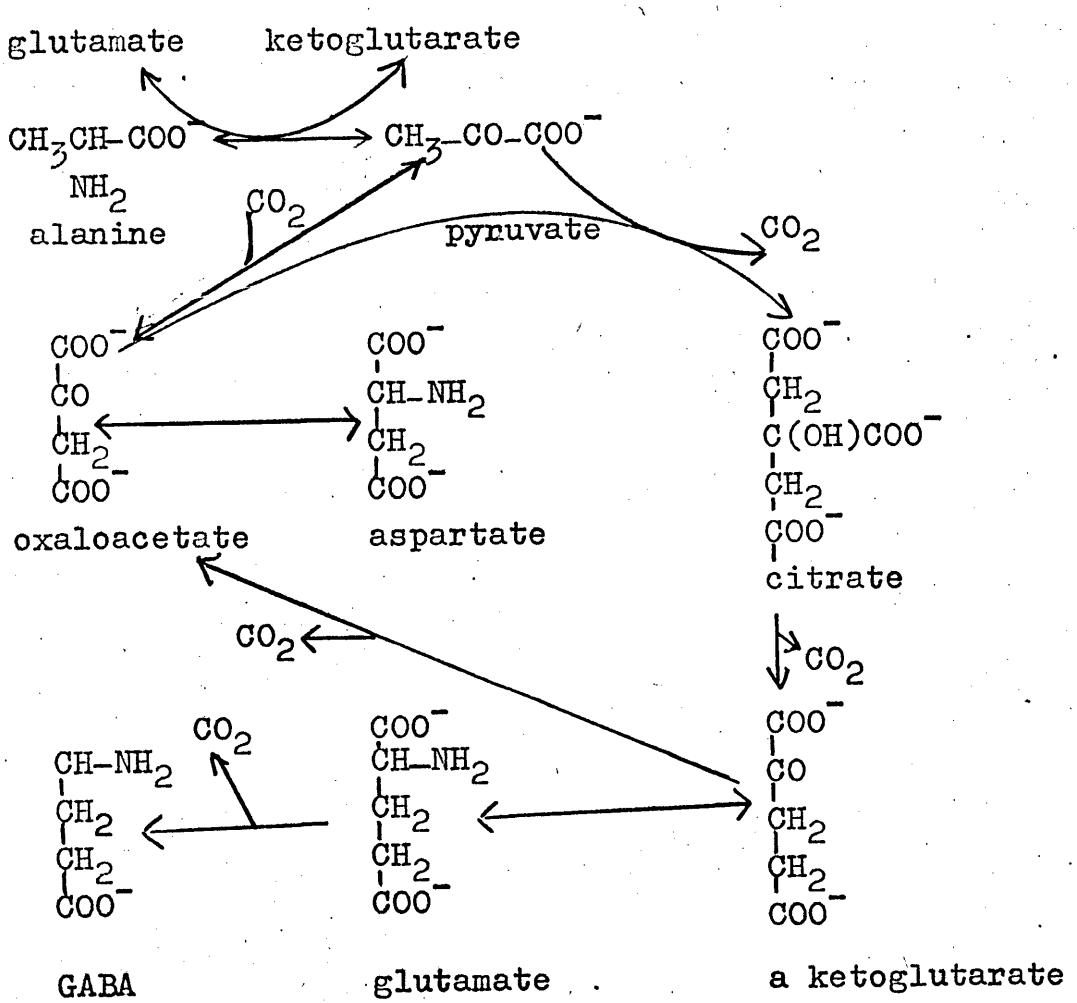


FIGURE 10

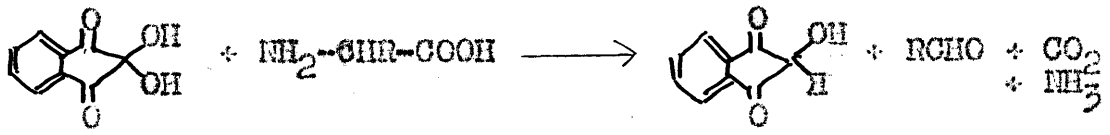
DIAGRAM OF AMINO ACIDS AND CITRIC ACID CYCLE INTERMEDIATES, SHOWING CARBON SKELETONS OF THE MOLECULES.

From this diagram, the labelling pattern in the Carbon skeletons of the amino acids may be predicted as described in the text, when pyruvate, glutamate or alanine is the radioactively labelled precursor.

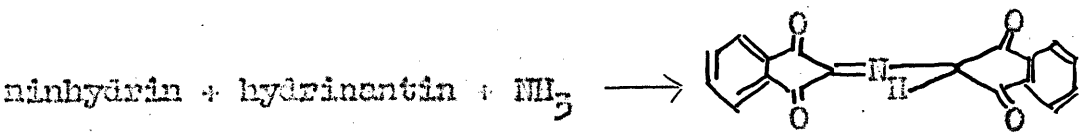
In contrast, incubation with U^{14}C glutamate would yield amino acids uniformly labelled in their carbon skeletons, and the theoretical losses would be as stated in the first instance. The use of ^{14}C alanine or pyruvate would yield a pattern of labelling within the carbon skeleton identical with glucose.

Table 4 compares the yields of counts in the amino acids, in the same amount of extract, from the paper and from the ninhydrin eluate, as described earlier, and a loss would be expected in the eluate counts. Rose (1968b) did find a loss, but estimated it as being below the theoretical loss, and did not make a correction for it. Table 4 shows that only in the case of glutamine and alanine formed from U^{14}C glucose was there a loss, amounting to 30% in each case, but in the other cases there was none, and in the case of glutamate and GABA an actual gain. The reason for this may be the loss of counts from the paper during autoradiography. It was therefore decided to calculate the specific activities from the counts in the eluates alone. Rose (1968b) also made no correction, and pointed out that the underestimation of the true specific activity did not affect the comparison of values of specific activities of the same amino acid with different tissues or substrates.

Specific activities were estimated as disintegrations per minute per molecule, divided by the amount of amino acid in 10^{-9} moles. They were not calculated on the basis of carbon atoms on account



ninhydrin hydrinantin



purple complex

Equation showing the ninhydrin colour reaction , and the loss of CO_2
(After Mahler and Cordes, 1968)

TABLE 4

LOSS OF $^{14}CO_2$ DURING NINHYDRIN REACTION

Comparison of counts in amino acids from ninhydrin eluates and from paper strips.

	Disint/min. in free amino acids x 10^{-3}				
SUBSTRATE	GLU	ASP	GLN	ALA	GABA
U ^{14}C GLUCOSE					
paper	154	46	243	53	51
eluate	213	45	172	36	57
U ^{14}C GLUTAMATE					
Paper	5,210	434	244	-	123
eluate	6,510	476	230	17	124

Total counts in the amino acid fractions were determined from the ethanol eluates of the ninhydrin-stained amino acids, and from the non-stained chromatographed amino acids in the rat cortex slice, after incubation with the radioactive substrates shown, as described in the text. Results are the means of 3 values for U ^{14}C glucose, and of 6 values for U ^{14}C Glutamate.

of the dissimilarity of the labelling pattern within the carbon skeleton with different substrates as described above. Pool sizes were expressed as 10^{-9} moles per mg. protein since the protein content per gram wet weight of optic lobe, vertical lobe and rat brain varied, as described in Chapter 3. Results were expressed ± standard errors or S.E.M.s, and differences tested for significance by means of the student's t test.

Automated amino acid analysis.

In the later experiments, the amino acids were determined by means of an automated amino acid analyser built in the Department by Dr. A.J. Thomas, and described in detail by Thomas, (1969). Its use in the detection of free amino acids in brain tissue is described by Bradford and Thomas, (1969). Briefly, the Zeocarb eluate was dissolved in 10 or 20 ml of 0.1M HCl, so that 1 ml would not contain more than 2 µmoles of a single amino acid. The HCl contained 0.1 µmole norleucine as standard. A 1ml sample was then pumped through a column of sulphonated polystyrene resin, and the amino acid fractions eluted in turn by a gradient of ever-increasing pH. The acidic amino acids were eluted first, followed by the neutral and basic ones. The effluent was then divided into two roughly equal parts: one was mixed with ninhydrin reagent and heated, the colour measured in a colorimeter and recorded on a chart. Figure 10 is a photograph of such a chart. The amino acid fractions in the other stream were collected individually by means of an automatic fraction collector. The fractions were washed into scintillation vials with

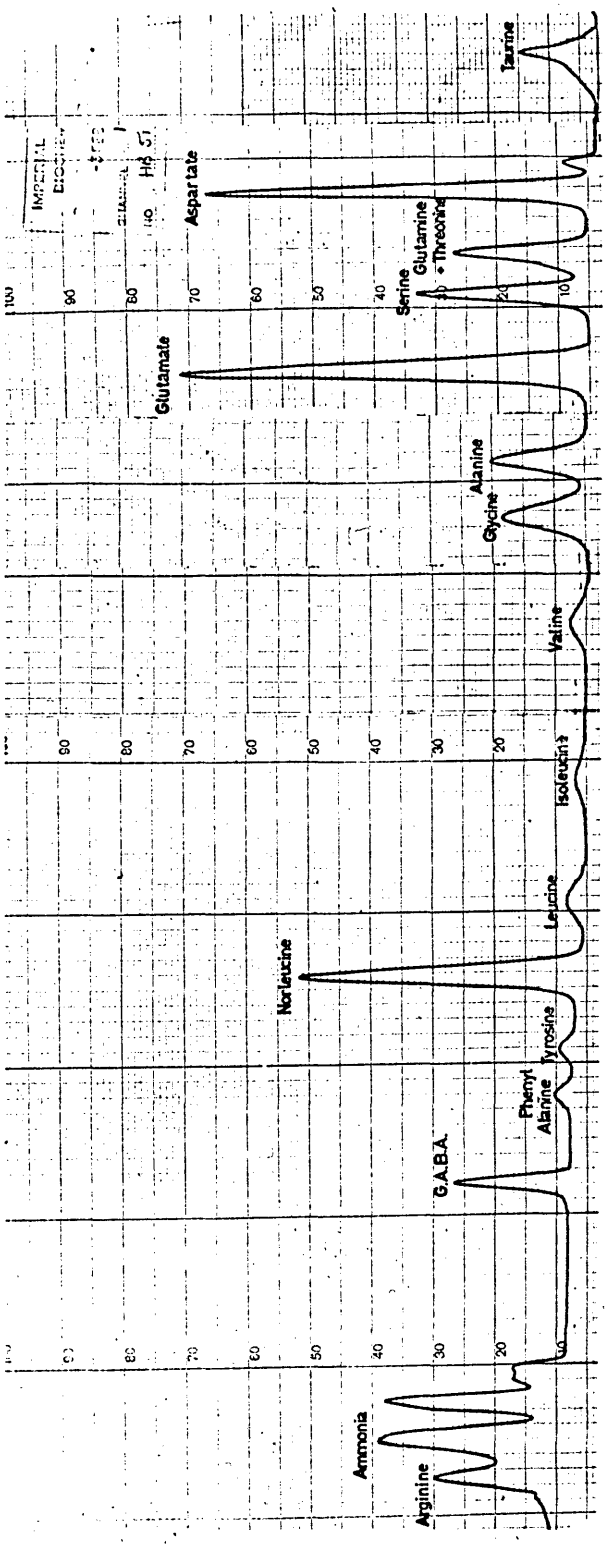


FIGURE 11

PRINT-OUT FROM THE AUTOMATED AMINO ACID ANALYSER, showing peaks corresponding to the amino acids (Photograph reproduced by courtesy Bradford & Thomas, 1969)

scintillant and counted. The areas under the peaks on the chart were proportional to the amounts of amino acid in the sample. An integrator made corrections for changes in the recorder baseline during the run, and calculated and printed out the areas under each peak. Alternatively these may be calculated from the chart itself, the area being taken as the product of ½ base x height of peak. The areas were converted to micromoles of amino acids by expressing them as fractions of the norleucine peak, then multiplying them by a predetermined factor for each amino acid. The use of ^{14}C labelled norleucine enabled the operator to calculate the exact distribution of amino acid between the eluate that was analysed and that which was collected and counted, so as to make a true assessment of the specific activities. The system is able to detect as little as 5×10^{-9} moles of amino acid (Thomas, 1969). Using the paper chromatography method, only 5×10^{-8} moles can be detected and accurately measured.

Comparison of the methods used for separation and detection of the amino acids.

The low recoveries of the amino acids after paper chromatography, shown in Table 3, and the fact that no loss of counts seems to occur after reaction of the amino acids with ninhydrin (Table 4) raise the question of the limitations of the different means of isolating and estimating the amino acids by (a) scanning, (b) column and paper chromatography and (c) automated amino acid analysis.

As stated in the subsection on colour development,

the underestimation of true specific activity did not affect comparison of the values for the same amino acid under different incubation conditions. However, this method is primarily for isolating already known products in bulk, and determining their specific activities, since 250 / 500 µl of the total final amino acid extract is spotted. In contrast the scanning method gives a picture of all labelled intermediates and amino acids, albeit only a small portion (50 / 500 µl) is spotted, and the scanners count at a lower efficiency than the liquid scintillation counter. The automated amino acid analyser would appear to be the best method for determining pool sizes, since the loss of amino acids recovered after paper chromatography does not occur here. On the other hand, the possibility remains that the low sum of the yields seen in Table 3 especially after incubation with U^{14}C alanine may be due to the fact that other labelled amino acids escaped detection, in which case, they would have been detected by the scanning method. Unfortunately, the lack of supply of octopus at this stage made further experiments impossible. The fact that the missing substances were more likely to be amino acid in nature was because the greatest loss became apparent after the stage where amino acid separation occurs, after the Dowex chromatography stage, (Table 3) and not so much before, (Table 2).

Determination of glucose, lactate and pyruvate.

The Zeocarb non-amino acid eluate was concentrated

10 times, and glucose, pyruvate and lactate estimated quantitatively by the following methods:
Glucose was estimated by the glucose oxidase method of Fleming and Pegler (1963), lactate by the spectrophotometric reduction of NAD using lactate dehydrogenase at pH 10 (Method of Hohorst, 1963), pyruvate by the reversal of the lactate dehydrogenase reaction (Bücher, 1963) at pH 7.6. The counts in these metabolites were determined by spotting 100 µl of the concentrated eluate in strips and running them monodimensionally in the butanol-acetic acid-water solvent (40:11:25 by vol.), locating the bands by autoradiography over X ray film for three to five days, and cutting them out and placing them into counting vials with Brays scintillant.

The lowest detectable and measurable levels were as follows: for glucose, 0.005 µ Moles per sample, for pyruvate and lactate, 0.01 µMoles per cuvette.

Determination of proteins and nucleic acids.

Protein was determined in brain homogenates or NaOH digests of tissue by the method of Lowry, Rosebrough, Farr and Randall (1951). Nucleic acids were extracted by the method of Schneider (1945). The homogenate, containing not less than 100mg wet weight of tissue was treated with ice-cold perchloric acid, 0.5 to 1.0M to precipitate the protein, nucleic acids and lipids. The precipitate was washed three times with 0.5M PCA, then defatted with two washings of 80% ethanol followed by one washing with ethanol: chloroform (3:1, v/v). DNA and RNA were extracted from the residue with two washings of 5% PCA at 90°C for 15 minutes. RNA was estimated in the extract by

the orcinol method of Mejbaum (1939) for pentoses. DNA was estimated by the diphenylamine method of Dische (1930) for deoxypentoses, using the modification of Burton (1956) and the correction of Zamenhof, Bursztyn, Rich and Zamenhof, (1964) for greater sensitivity. The lowest levels that were accurately measurable were found to be 50 /ug of RNA per sample, and 10 /ug DNA per sample.

Uptake Experiments.

The uptake of amino acids, characteristic of rat brain slices discussed in Chapter 1 was also tested in octopus brain slices, for glutamate, alanine, glutamine and GABA.

Incubation was carried out in Warburg vessels using 10mM glucose, and 10mM U ^{14}C amino acid, and 1 to 2 microcuries of radioactivity per flask. Incubation was for 1 or 2 hours, and $^{14}CO_2$ was collected and measured. After incubation the slices were reweighed to check loss by fragmentation or gain by uptake of water, then rinsed in medium as described earlier and homogenised in 5.0 ml of 1.0M Trichloroacetic acid (TCA). The homogenate was spun down and the supernatant counted. An aliquot of medium was also counted. Assuming the density of the slice to be equal to that of the medium, i.e. a 100 mg. slice occupied 0.1ml, the ratio, counts per 100mg slice to counts per 100 /ul medium was calculated. If the ratio was greater than unity then active uptake against a concentration gradient was assumed to have occurred. Zero time controls were also run to check adherence of radioactivity to the slice surface.

ENZYME ASSAYS IN HOMOGENATES.

The enzymes assayed in rat cortex and octopus optic and vertical lobe homogenates were the following: glutamate dehydrogenase (GDH), glutamate decarboxylase (GAD), glutamine synthetase (GNS), aspartate aminotransferase (AAT) and alanine transaminase (Ala-AT). The reactions catalysed by these are shown in figure 12. GAD, GDH, GNS, AAT and Ala-AT catalyse the conversion of glutamate into GABA, α-ketoglutarate, glutamine, aspartate and alanine respectively, and the last enzyme also the interconversion of alanine and pyruvate.

Enzyme assays were carried out on homogenates diluted to suitable concentrations which were found experimentally (they are discussed in Chapter 4). 10% homogenates were made of frozen eledone brain, thawed once, and of fresh rat cortex in water or 0.32M sucrose: homogenisation was carried out in a glass homogeniser immersed in a beaker of ice using a motor-driven Teflon pestle, clearance 0.005", for 30 seconds, to obtain maximum breakdown of cell organelles and release of enzymes.

GAD: (E.C. 4.1.1.15.) was assayed by the method of Roberts and Frankel (1950) as modified by Balázs et al. (1966). The following reaction

$$1\text{-}^{14}C \text{ glutamate} = {}^{14}CO_2 + \text{GABA}$$

was followed manometrically in Warburg vessels. Rat brain homogenates were incubated at 37°, and octopus brain at 25°, under nitrogen in 2.5 ml of the following buffer-substrate mixture:

50mM sodium glutamate, containing 1 to 2 μCuries of $1\text{-}^{14}C$

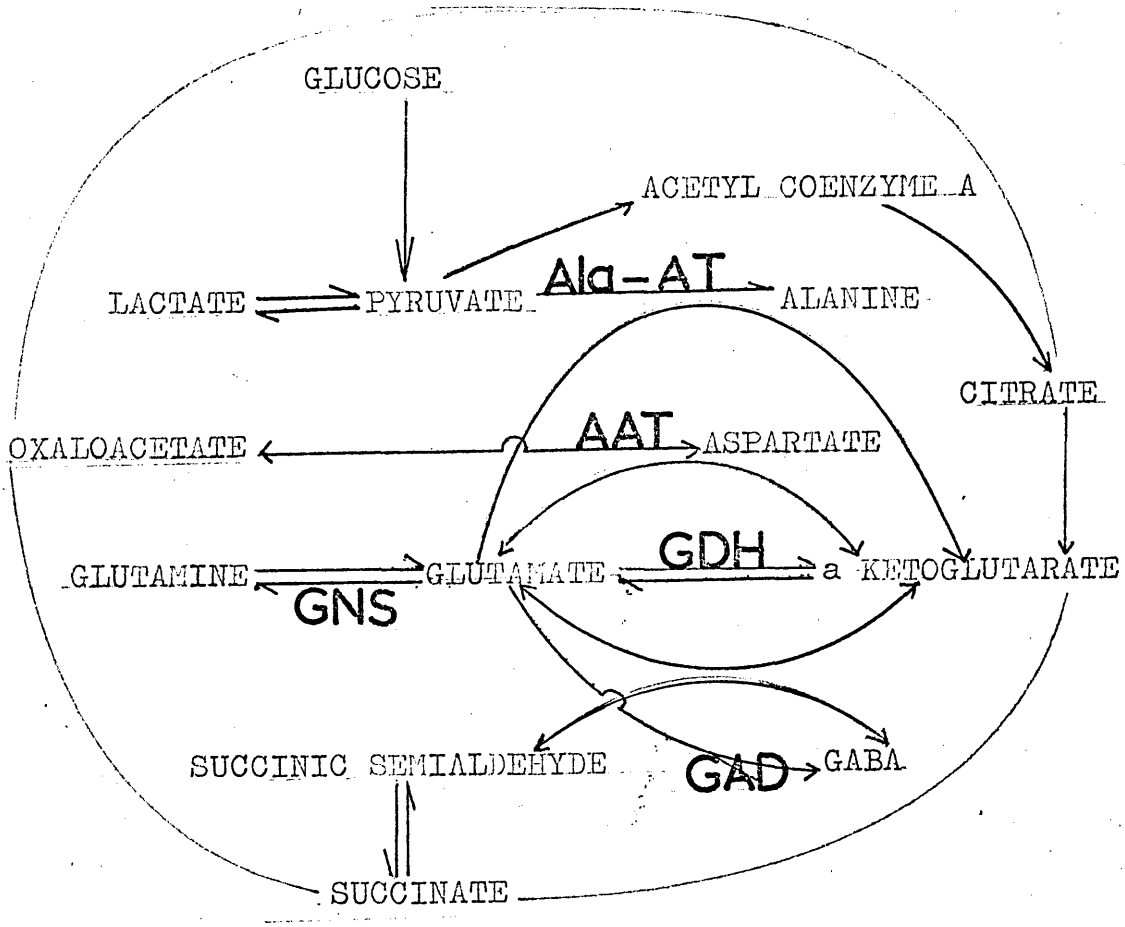


FIGURE 12

DIAGRAM OF SOME OF THE INTERRELATIONSHIPS BETWEEN AMINO ACIDS AND CITRIC ACID CYCLE INTERMEDIATES

Also shown are the points of activity of the enzymes studied, as described in the text.
Diagram partly taken from Roberts, (1962).

glutamic acid, 10mM reduced glutathione, 0.2 mg pyridoxal phosphate, 0.02M phosphate buffer at pH 6.3. Carbon dioxide was collected in the centre well and counted as described earlier in the chapter. A blank was run with no tissue, and at each tissue concentration a flask containing 10mM semicarbazide was run: at this concentration, it completely inhibits the reaction (Roberts and Frankel 1950) by blocking the pyridoxal phosphate. This was to ascertain the amount of glutamate actually being oxidised by conversion into α ketoglutarate. In each run, three different concentrations of tissue were used, as well as different incubation times, from 10 minutes to 1 hour. The experiment was repeated at least twice and results calculated as the means of three such experiments.

GDH: (E.C. 1.4.1.2.) was assayed by the method of Olson and Anfinsen (1952) modified by Balázs, (1965). It was assayed in the direction of glutamate synthesis:

$$\alpha \text{ ketoglutarate} + NH_4^+ + \text{NADH} = \text{glutamate} + \text{NAD} + H_2O$$

The fall in extinction of NADH was measured spectrophotometrically at 340 mμ in the SP800 Unicam spectrophotometer at 25°C. Each cuvette contained brain homogenate in 3.0 ml of the following buffer-substrate mixture: 10mM α ketoglutarate, 15mM NH_4Cl, 0.07mg/ml NADH, 0.5mM ADP, and 0.02M phosphate buffer pH 7.7. Balázs, (1965) found that ADP stimulated the reaction 4 to 5 times. In each experiment three cuvettes were run with different tissue concentrations, against blanks containing tissue in buffer alone. A fourth cell contained all the constituents but no tissue against a blank without

NADH, to check for spontaneous oxidation of NADH, but this was never found to occur. Three such experiments were performed, at least, and the means of these calculated. The amount of substrate converted was calculated using the formula: change of 0.1 optical density units at 340 mμ = 0.0161 μMoles NADH oxidised per ml. Protein was determined in the homogenates, and activities were calculated as micromoles substrate converted /hr/ mg protein.

AAT: (E.C. 2.6.1.1.) was assayed by the method of Boyd (1961). Aspartate was converted into oxaloacetate, which was reduced to malate by addition of excess malate dehydrogenase, coupled to NADH oxidation which was followed spectrophotometrically at 25°C:

α ketoglutarate + aspartate = glutamate + oxaloacetate

oxaloacetate + NADH = NAD + malate

Each cuvette contained brain homogenate in 3.0 ml of the following buffer-substrate mixture:
30 mM aspartate, 0.07 mg/ml NADH, 10mM α ketoglutarate, 0.02M phosphate buffer, pH 7.4. 5 μl of ammonium sulphate suspension of malate dehydrogenase were added to start the reaction. Blanks were run in the same way as for GDH assays, and enzyme activities were calculated in the way described above.

Ala-AT: (E.C. 2.6.1.2.) was assayed by Assay Method II of Segal, Beattie and Hopper (1962), and was also measured spectrophotometrically:

L-alanine + α ketoglutaric acid = L-glutamate + pyruvate

pyruvate + NADH = lactate + NAD

Alanine was converted into pyruvate which was measured by following the extinction at 340 mμ of NADH in the presence of excess lactate dehydrogenase. Brain homogenates were incubated at 25°C in 3.0 ml of the following buffer-substrate mixture:
10mM α ketogluterate, 0.07 mg/ml NADH, 7mM L-alanine, and 0.1M phosphate buffer at pH 7.6. 5 μl of lactate dehydrogenase suspension were added to start the reaction. Blanks were run in the same way as for GDH.

In all the spectrophotometric assays of GDH, AAT and Ala-AT, runs were made at least three times, each time with three tissue concentrations and with a suitable blank. Readings were taken automatically of the optical density every 10 minutes, and at 2 to 5 minutes in the case of AAT which was very active. Cuvettes were inverted every ten minutes to prevent settling of the enzyme.
GNS: (E.C. 6.3.1.2.) was assayed by the method of Sellinger and De Balbian Verster (1963). The reaction was as follows, hydroxylamine here being the amino donor instead of ammonia:

$$\text{Glutamate} + \text{hydroxylamine} + \text{ATP} \xrightarrow{Mg^{++}} \text{ADP} = P_i + \text{glutamohydroxamic acid}$$

Different amounts of tissue homogenate were incubated in capped test tubes at 37°C or 25°C (for the rat and octopus respectively) in a shaking water bath, in 3.0 ml of the following buffer-substrate mixture:
100mM Tris-HCl buffer pH 7.4, 0.05M hydroxylamine hydrochloride freshly neutralised with NaOH, 30mM glutamate, 10mM ATP trisodium salt, 0.02M $MgCl_2$, 0.0005M BAL.
Controls were run by omitting ATP or tissue from the tubes. After 30 minutes of incubation, 6ml of acid ferric chloride

solution (0.5N HCl-10% $FeCl_3$-8% TCA) were added to each tube and a brown complex formed immediately, between the glutamohydroxamate and the ferric ions. The colour was read at 500mμ against a standard curve, prepared by incubating samples of glutamohydroxamic acid ranging from 1 to 5 μmoles identically to the tissue samples as above. Samples were run in triplicate at three different tissue concentrations. Such runs were repeated at least twice, a standard curve being made with every run.

In all the enzyme assays described above except GNS, pretreatment of the homogenate with Triton-X-100 was recommended. This detergent, consisting of a complex mixture of polyoxyethylene ethers and partial anhydrides of sugar derivatives was claimed to stimulate enzyme activities 5 to 20 fold by Balázs et al. (1966), by releasing them from the subcellular particles to which they are attached. A dilution of the homogenate in 0.1% Triton solution was recommended, with gentle mixing, without frothing. Rat and octopus brain homogenates were assayed with and without Triton pretreatment, except for GNS where it was not recommended.

METABOLISM IN THE OCTOPUS BRAIN IN VIVO.

It is possible to inject octopus directly into the brain cavity just above the vertical lobe. I am indebted to Dr. Andrew Packard (Stazione Zoologica, Naples) for his personal communication and instructions for this operation. The operator feels for the ridge of cartilage between the octopus's eyes and injects directly down, through the cartilage into the mucilage above the vertical lobe: the site is shown in Figure 6. Trial injections

were made into dead animals, then into live ones, using Methylene blue as a marker. On dissection, it was found that the injection was nearly always successful. For the experiments, 30 to 50 /uCuries of $U^{14}C$ L-glutamate were injected in not more than 50 /ul of 0.9% saline. Diffusion of radioactivity did occur into the optic lobe. Table 5 shows the counts going into the amino acids of the two optic lobes, expressed as a percentage of the amino acid counts in the vertical lobe, the site of injection. This is seen to increase rapidly with time.

TABLE 5

Counts in optic lobe amino acids / Counts in vertical lobe amino acids x 100%, at different times after injection of $U^{14}C$ glutamate into the octopus.

Time	Percentage
15'	4.6% (2)
30'	118% (2)
60'	240% (5)

Values are means of the numbers of determinations in brackets

After killing, the optic and vertical lobes were excised and kept frozen at -20^{o} until required for the isolation and determination of the specific activities of the amino acids, by means of the automated amino acid analyser method described on page 75.

CHAPTER 3

EXPERIMENTAL RESULTS
METABOLISM IN BRAIN SLICES.

COMPARATIVE PROPERTIES OF OCTOPUS AND RAT BRAIN
Comparison of protein and nucleic acid content

Table 6 shows the protein and nucleic acid content of rat and octopus brain. The protein content of the vertical lobe is substantially lower than that of the optic lobe: this may be as it has mucilage adhering to it. The amount of nucleic acid, based on wet weight and protein content, is around four times greater in the octopus brain than the rat brain, suggesting a greater number of cells of smaller size in the octopus brain. Young (1963b) estimated that over 95% of the cells in the optic lobe and over 99% of the cells in the vertical lobe were under 5/u in diameter, whereas in the rat brain, cells range from 3 to 50/u in diameter, (Hosokawa, 1963). The RNA/DNA ratio is also a useful indication of cell size, when comparing two parts of the brain in the same species, but its value is uncertain in estimating relative cell sizes in different species.

It is possible to calculate the numbers of cells in the nervous system from its DNA content, which is constant for all the haploid cells of a given species (Mirsky and Ris, 1951). Table 6 shows the estimated number of cells per gram wet weight, and per lobe for the octopus. Assuming that the value of DNA per nucleus of Mirsky and Ris (1951) for the squid is similar to the value for the octopus, the estimated number of cells per g. wet weight is 2 to 3 times greater in the octopus brain, again suggesting cells of smaller size. Assuming also that the average weights of the optic and vertical lobes of

TABLE 6

COMPARISON OF PROTEIN AND NUCLEIC ACID CONTENT OF OCTOPUS OPTIC AND VERTICAL LOBE AND RAT BRAIN.

Property	Optic lobe	Vertical lobe	Rat cortex
Rough wet weight per nervous system (cleaned)	120 mg	70 mg	600 mg
mg protein per g wet wt.	97±3 (8)	72±3 (5)	101 (3)
mg RNA/ g wet wt.	5.16 (3)	2.04 (3)	1.58 (3)
mg DNA/g wet wt. mg DNA/100 mg prot.	3.17 (3) 3.27	2.13 (3) 2.96	0.84 (3) 0.83
Ratio RNA/DNA	1.63	0.96	1.88
Estimated no. of cells/g wet wt.	3.52×10^8 (1)	2.37×10^8 (1)	1.27×10^8 (2)
Estimated no. of cells per nervous system: neurons	0.65×10^8 (3)	0.25×10^8 (3)	0.22×10^8 (4)
glia	-	-	0.97×10^8 (4)

(1) Calculation based on a figure of 4.5×10^{-12} g DNA per nucleus for the squid (Mirsky and Ris, 1955)

(2) Calculation based on a figure of 6.6×10^{-12} g DNA per nucleus for the rat (Vendrely, 1955)

(3) Values for octopus vulgaris (Young, 1963b)

(4) Nurnberger, (1958).

Some of the values are expressed as the means of the numbers of experiments in brackets, and ± S.E.M.s in some cases.

O. vulgaris used by Young (1963b) in his estimation are similar to those of eledone used here, it is possible to make a tentative comparison: Young obtained a value of 65 million cells per optic lobe, or 540 million per gram as against 350 million in the Table, and a value of 25 million cells in the vertical lobe or 360 million per gram, as against 250 million in the Table. The figures are, at least, in the same order. However the method of calculation from DNA content gives the total number of nuclei, whereas Young (1963b) only estimated nerve cells. This rules out a glial: neuronal cell number ratio in the order of 10:1, as is the case in the rat brain. The paucacity of glial elements in the octopus brain has already been mentioned in chapter 1, and morphologically speaking, the optic and vertical lobes are more analogous to the ganglia in the rat nervous system than to the cortex. The estimated number of cells per gram wet weight in the Table agrees well with that of Nurnberger (1958), being about 110 million nuclei (neuronal and glial) in the rat cortex.

Comparison of amino acid levels in rat and octopus brain.

Table 7 shows the levels of the amino acids glutamate, aspartate, glutamine, alanine and GABA in the octopus brain and the rat cortex. Many estimates of the values in the rat brain are to be found in the literature, and they are all in the same order. Those quoted in the Table are from Carver (1965), and are calculated on a protein basis for comparison with the values for the octopus brain. From Table 7 it can be seen that, compared with the rat, the level of glutamate is higher, levels of aspartate and alanine much higher, and that of GABA lower in the octopus

TABLE 7

POOL SIZES OF SOME FREE AMINO ACIDS IN UNINCUBATED OCTOPUS AND RAT BRAIN.

	m/umoles/ mg protein		
AMINO ACID	OPTIC LOBE	VERTICAL LOBE	RAT BRAIN(1)
Glutamate	113	144	90
Aspartate	87	114	26
Glutamine	23	51	50
Alanine	79	66	8
GABA	10	14	21

(1) Carver, (1965)

Values for the octopus brain are the means of 3 determinations in each case, made as described in Chapter 2. The range is 15% , except for the GABA values, where the range is 30%.

brain, whereas glutamine is similar in vertical lobe and rat cortex, and half this value in the optic lobe.

METABOLISM OF GLUCOSE IN VITRO.

The importance of glucose as a metabolic and respiratory substrate for brain tissue was discussed in Chapter 1. The experiments of Beloff-Chain et al. (1955a), following the fate of U^{14}C glucose in rat brain slices were repeated and compared with optic and vertical lobe slices.

Concentration curve for glucose.

The effect of the concentration of substrate glucose on the oxygen uptake and CO_2 production was tested in rat cortex slices. They were incubated in Warburg vessels with Elliott-Henderson saline as described in Chapter 2, with U^{14}C glucose, ranging in concentration from 1mM to 20mM, and 1 to 2 /uCuries of radioactivity per flask. Oxygen uptake was measured at 10 minute intervals for one hour and CO_2 collected and counted. Figure 13 is a plot of oxygen uptake and CO_2 production against substrate concentration. The curves are roughly parallel, and they rise to a plateau between a concentration of 5 and 10mM. Therefore 10mM was chosen to be the substrate concentration in all the experiments to follow, for amino acids as well as glucose, to make comparison of results obtained with these different substrates easier; thus substrate availability would not be a limiting factor in these experiments. Rose (1968) also uses this concentration. Moreover Bradford (1968) quotes the mammalian brain's rate of glucose utilisation *in vivo* as being around 20 /umoles/g/hr; therefore for a 100 mg slice in two hours,

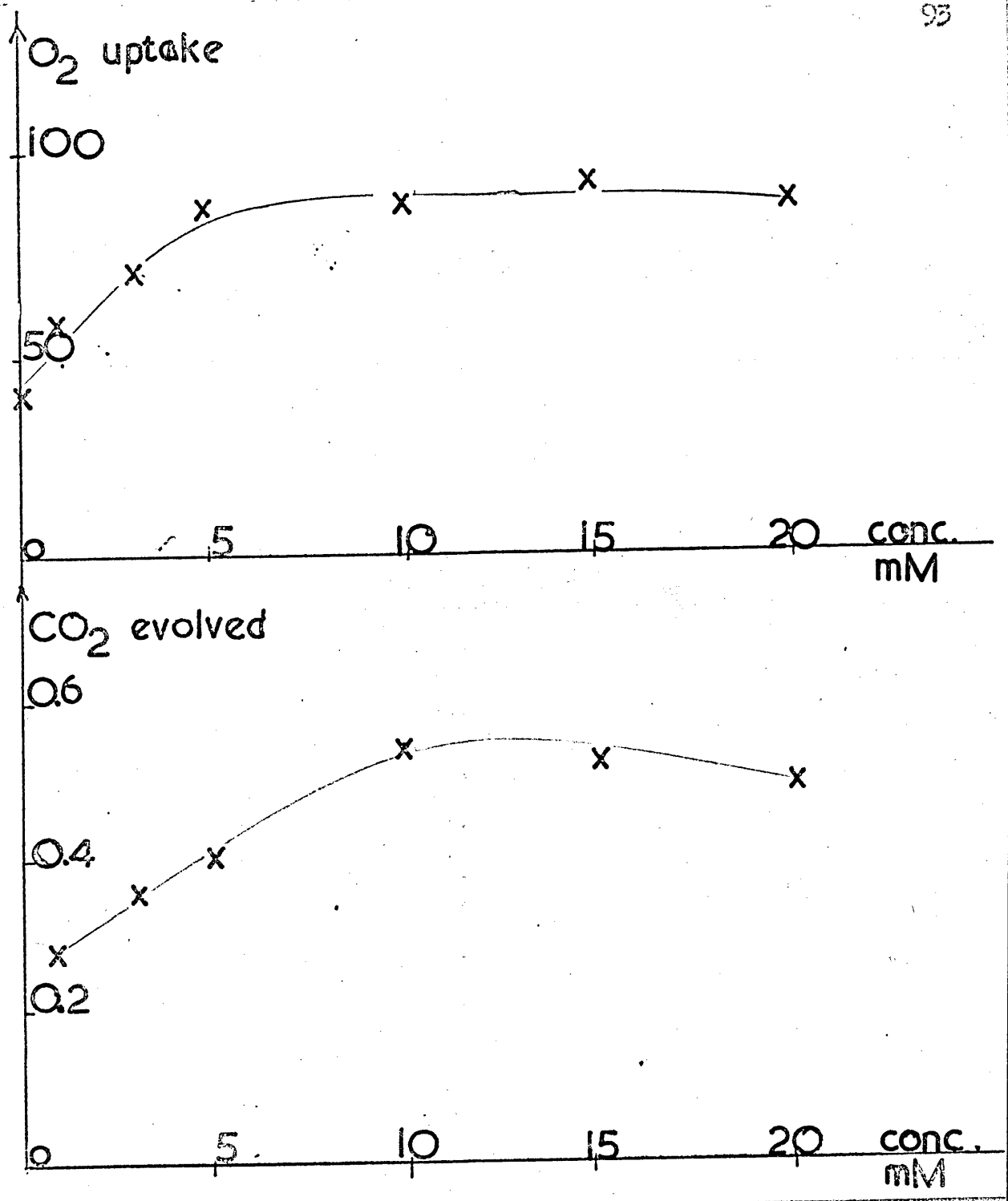


FIGURE 13

OXYGEN UPTAKE (umoles/g/hr) and CO_2 PRODUCTION (counts x 10^{-2}/mg. protein) plotted against concentration of glucose. Rat brain slices were incubated at 37°, and oxygen uptake and CO_2 production calculated as described in Chapter 2. Points are the means of two determinations.

30 µmoles of glucose in 3 ml of medium should be sufficient.

Oxygen consumption and CO_2 production in rat and octopus brain in vitro.

Figure 14 shows the oxygen uptake with time of a 100mg slice of rat cortex, optic and vertical lobe at 37° and 25°C respectively, with 10mM glucose. Figure 15 shows the respiration of the same tissues in the absence of substrate: under these conditions, the slices oxidised endogenous substrates present in the tissue, and the graph shows a non-linear, slow rate of oxygen uptake over the two hours. In the presence of glucose all three slices respired at double the endogenous rate, and oxygen uptake was linear in the rat brain slice though that of octopus brain levelled off after one hour. Table 8 lists the initial rates of oxygen uptake in the presence and absence of glucose.

TABLE 8

INITIAL RATE OF OXYGEN UPTAKE OF BRAIN SLICES IN THE PRESENCE AND ABSENCE OF 10mM GLUCOSE

µMoles/100mg protein/hr

SUBSTRATE	NONE	GLUCOSE	% STIMULATION
Rat cortex	40 ± 1 (6)	90 ± 5 (9)	225%
Optic lobe	22 ± 1 (5)	34 ± 2 (14)	150%
Vertical lobe	15 ± 3 (3)	24 ± 3 (4)	160%

Oxygen uptake was measured manometrically as described in Chapter 2. Values are means ± S.E.M. s of numbers of experiments in brackets.

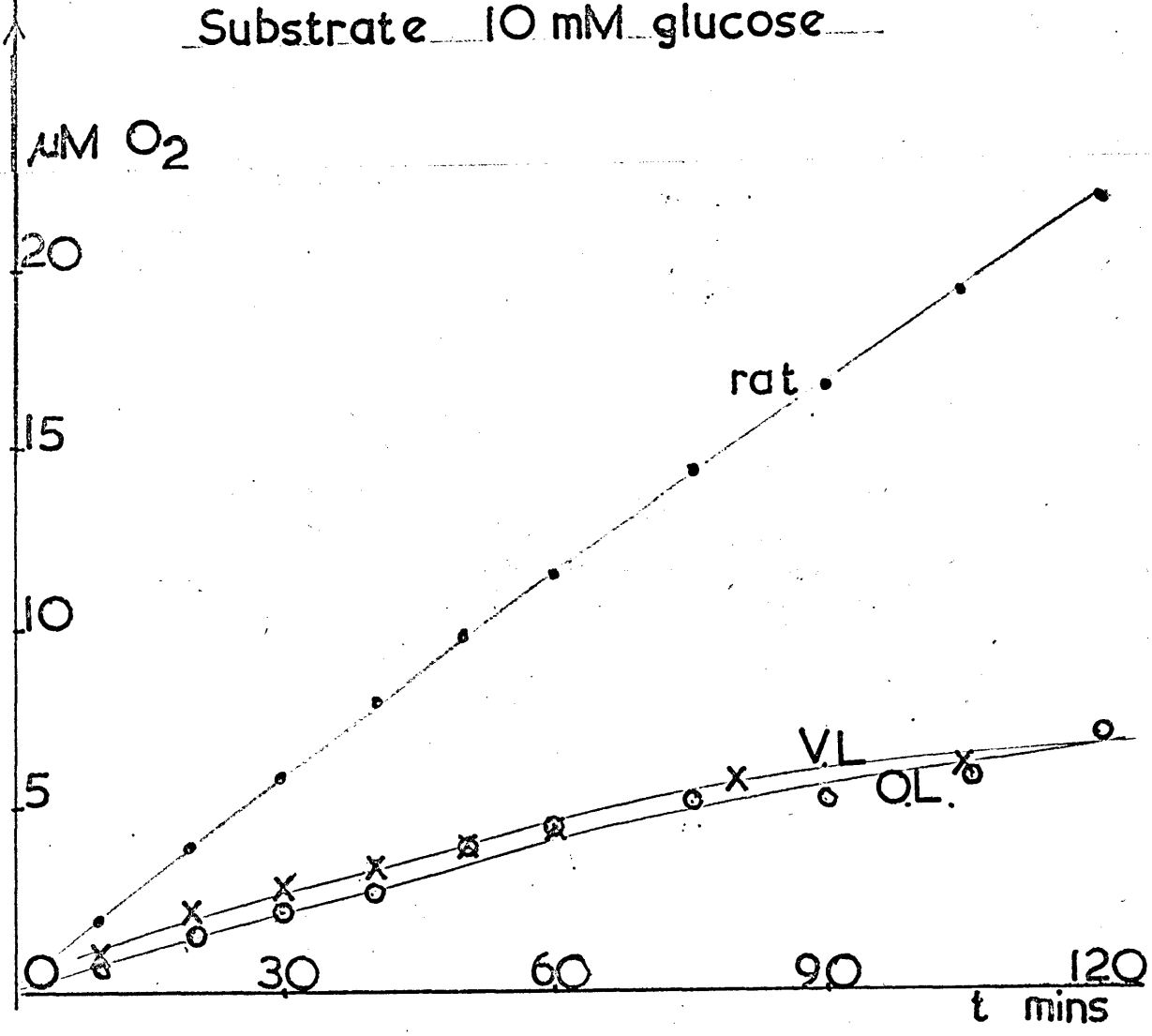


FIGURE 14

Oxygen uptake of a 100mg slice of octopus optic and vertical lobe, and rat cortex. SUBSTRATE 10mM GLUCOSE. For incubation conditions, see chapter 2. Points are the means of 3 values.

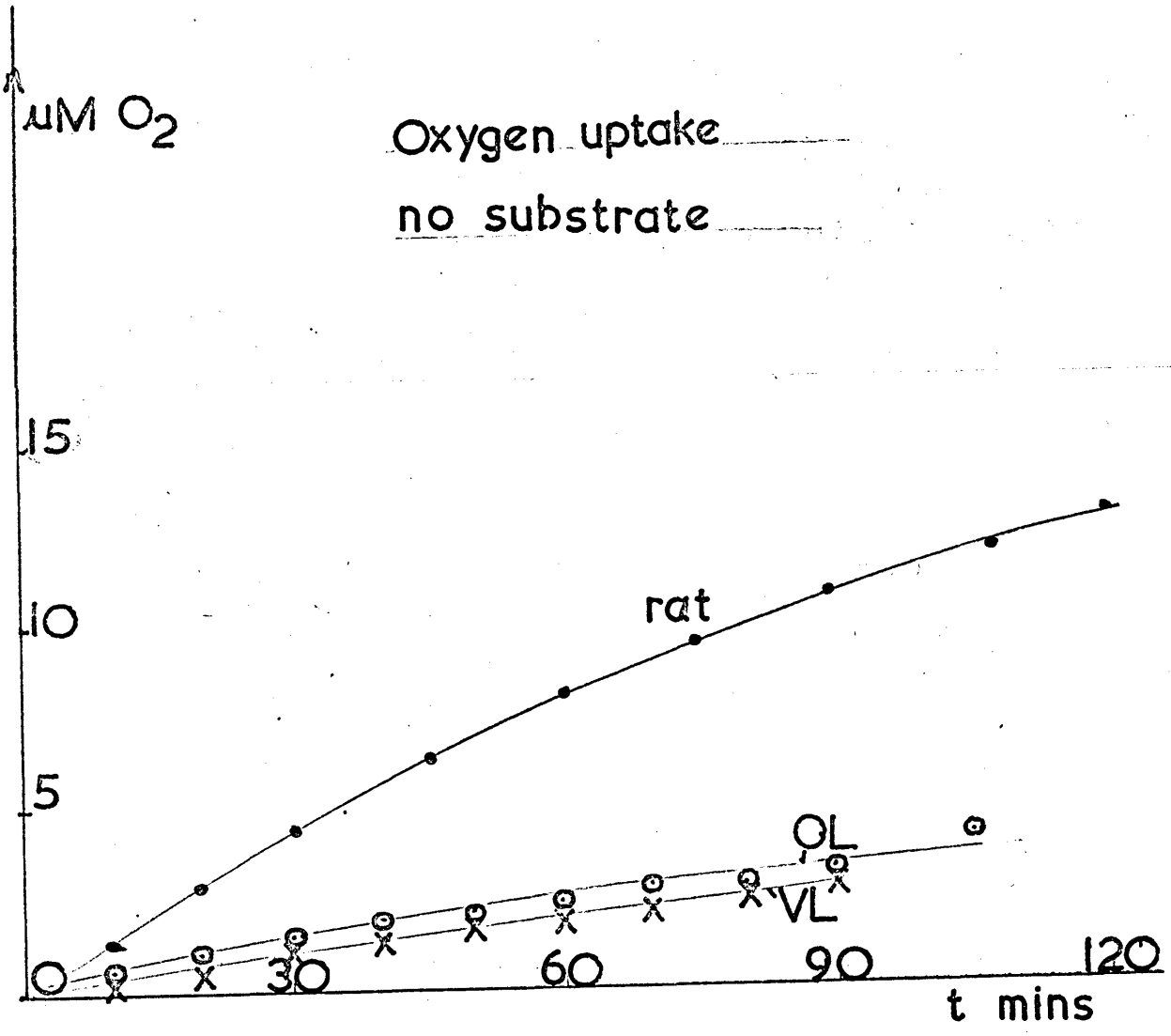


FIGURE 15

Oxygen uptake of a 100mg. slice of rat cortex , vertical and optic lobe at 37° Or 25°C.

NO SUBSTRATE ; Incubation medium saline (for composition see text) Points are means of 3 values.

Metabolic pattern for Glucose in tissue slices.

Slices of rat and octopus brain were extracted after incubation with U ^{14}C glucose, and the counts incorporated into CO_2, lactate and amino acids were determined as set out in Chapter 2, using the scanning technique of Lowe (1969). Table 9 shows the counts incorporated into various metabolites after two hours incubation. A similar pattern was found in the rat and octopus brain, except that no ^{14}C GABA was formed in octopus brain slices; counts incorporated into products by the optic and vertical lobe were generally similar. Comparing the rat and octopus brain on a protein basis, incorporation was generally higher in the rat brain with the important exception of alanine which was 5 fold greater in the octopus brain; on the other hand only traces of aspartate and glutamine were formed in the octopus brain, and no GABA. Figures 16, 17 and 18 are photographs of the autoradiographs of the chromatogrammed extracts of rat cortex, optic and vertical lobe slices after incubation with U ^{14}C glucose. Incidentally, taurine was detected by ninhydrin staining of the octopus brain chromatograms, but it did not appear on the autoradiographs and was therefore not measured. The absence of a GABA spot is also shown on the chromatograms.

Since the differences in absolute values may partly reflect the different incubation temperatures of the octopus and the rat brain, a truer picture may be obtained if the counts are expressed as percentages of the total glucose recovered as metabolites, thus eliminating this effect. Table 9 shows that only 11% and 8% of the total glucose counts were recovered as products in optic and

TABLE 9

INCORPORATION OF $U^{14}C$ GLUCOSE INTO PRODUCTS BY SLICES OF OCTOPUS OPTIC AND VERTICAL LOBE AND RAT CORTEX.

	Counts/mg protein/ 10^6 counts substrate		
PRODUCT	OPTIC LOBE	VERTICAL LOBE	RAT BRAIN
CO_2	688±68 (12)	645±94 (13)	6770
lactate	7340±244 (7)	5710±170 (5)	24240
Insoluble residue	43±11 (7)	64±20 (3)	49
Glutamate	806±137 (7)	842±94 (5)	2324
alanine	1810±352 (7)	1512±193 (5)	316
aspartate	traces	traces	308
glutamine	traces	traces	946
GABA	n.d.	n.d.	424
Total counts recovered as products	11%	8%	35%

Slices of rat and octopus brain were incubated for 2 hours at 37° and 25° respectively, and the products extracted and determined as described in Chapter 2. The substrate was 10mM $U^{14}C$ GLUCOSE.
Values expressed as the means of the numbers of experiments in brackets, ± S.E.M.s , in the case of the octopus.
Values for the rat brain are the means of 4, the range being 15% .



FIGURE 16

Autoradiograph of chromatogrammed extract of OPTIC LOBE SLICE after incubation with U ^{14}C GLUCOSE. Origin is at top left hand corner. gluc = glucose, lac = lactate

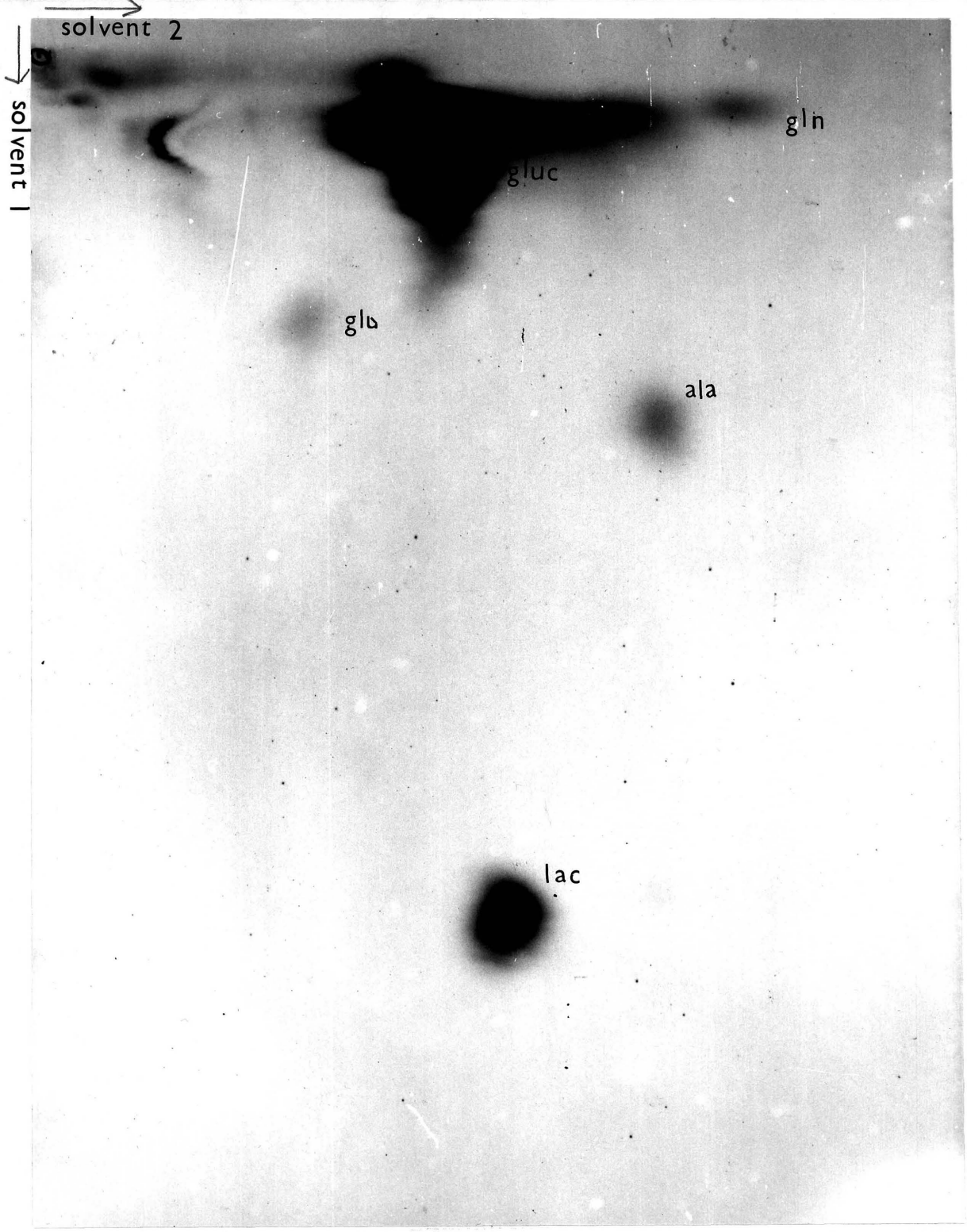


FIGURE 17

Autoradiograph of chromatogrammed extract of VERTICAL LOBE slice after incubation with U ^{14}C GLUCOSE. Origin is at top left hand corner. gluc = glucose, lac = lactate

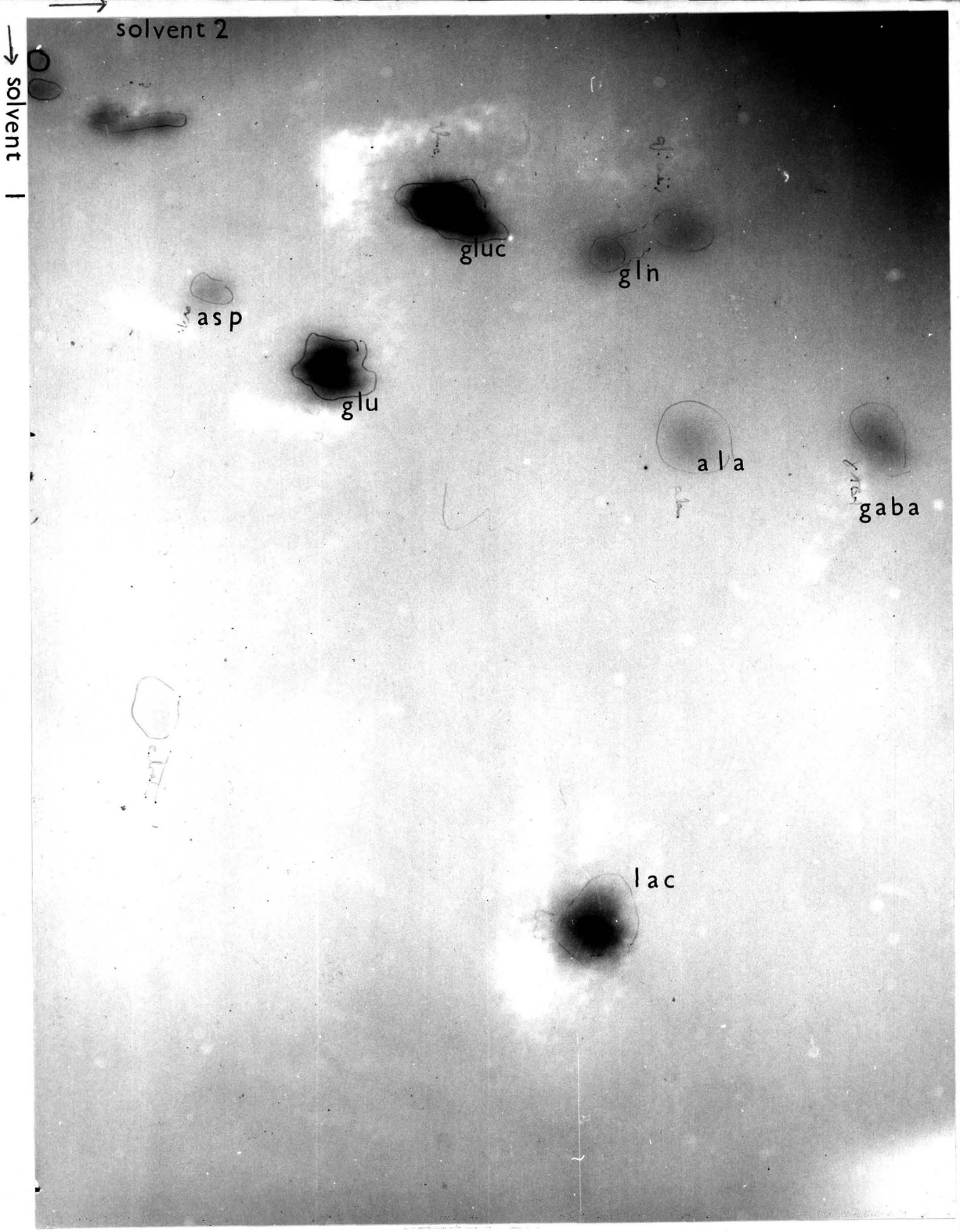


FIGURE 18

Autoradiograph of chromatogrammed extract of RAT CORTEX SLICE after incubation with U ^{14}C GLUCOSE. Origin is at top left hand corner. gluc = glucose, lac = lactate

TABLE 10

PERCENTAGE OF $U^{14}C$ LABEL RECOVERED IN PRODUCTS AFTER INCUBATION OF RAT AND OCTOPUS BRAIN SLICES WITH $U^{14}C$ GLUCOSE

Product	OPTIC LOBE	VERTICAL LOBE	RAT BRAIN
CO_2	6.4	7.3	19
Lactate	69	65	66
Total amino acids	25	26	15
glutamate	7.5	9.5	7.0
alanine	17	17	0.8
aspartate			0.9
glutamine			2.7
GABA			1.2

The values recorded in Table 9 were expressed as a percentage of the total counts transformed into products.

TABLE 11

COMPARISON OF THE METABOLIC PATTERN OF $U^{14}C$ GLUCOSE IN RAT BRAIN AFTER ONE AND TWO HOURS INCUBATION

PRODUCT	Counts/ mg protein / 10^6 counts glucose 1 hour	2 hours
CO_2	3400	6770
lactate	7700	24240
glutamate	1150	2324
alanine	190	316
aspartate	167	318
GABA	220	424
Unchanged glucose in slice	traces	3430

Rat brain slices were incubated for two hours as described in Chapter 2. Data from Chain et al. (1969) for one hour incubation, using substrate of similar specific activity to that for two hours.

vertical lobe respectively, compared with 35% in rat brain. Table 10 shows that on a percentage basis, incorporation into lactate and glutamate was similar in octopus and rat brain; optic lobe and vertical lobe CO_2 were 34% and 38% of the value for the rat, but the total amino acids in octopus brain were double the value in the rat brain. This difference was due to the incorporation into alanine, 20 fold higher in the octopus brain.

It is interesting at this point to compare the values for rat brain in Table 9 with those obtained by Chain et al. (1969) for one hour's incubation (Table 11). The latter generally are around half those found for two hours, but only traces of unchanged radioactive glucose are found inside the slice after one hour: after two hours this accumulated to around 0.3% of the total substrate in the medium. The lactate values were not comparable on the same basis, since lactate was calculated as the sum of that in the slice and that which had diffused into the medium, in contrast to the amino acids which did not leak out of the slices. Bradford (1968) pointed out that the total lactate production <u>in vitro</u> was dependent on the slice to medium volume ratio and this would appear to be the case here; the ratio of lactate counts inside and outside the slice ranged from 1/3 to 1/10 in these experiments, though the total counts as shown in Table 9 did not show this variation.

<u>Specific activities and Pool sizes of amino acids.</u>

Slices of rat and octopus brain were incubated with $U^{14}C$ glucose and the specific activities of the amino acids formed determined as set out in Chapter 2. Table 12 shows the specific activities of the amino acids compared

TABLE 12

SPECIFIC ACTIVITIES OF FREE AMINO ACIDS IN RAT AND OCTOPUS BRAIN SLICES AFTER INCUBATION WITH RADIOACTIVE 10mM GLUCOSE

	Disint/min/mμmole							
AMINO ACID	OPTIC LOBE		VERTICAL LOBE		P	RAT CORTEX		
Glutamate	26±2	(11)	22±3	(8)	n.s.	199±33	(6)	
Aspartate	5±1	(11)	18±5	(8)	<0.01	185±46	(7)	
Alanine	40±2	(11)	34±3	(8)	n.s.	115±29	(6)	
Glutamine	45±2	(4)	43±18	(3)	n.s.	195±20	(6)	
GABA	n.d.		n.d.		n.s.	174±45	(5)	

TABLE 13

POOL SIZES OF FREE AMINO ACIDS IN OCTOPUS AND RAT BRAIN SLICES AFTER INCUBATION WITH 10mM GLUCOSE

	mμmoles /mg protein							
AMINO ACID	OPTIC LOBE		VERTICAL LOBE		P	RAT CORTEX		
Glutamate	124±37	(14)	92±16	(8)	<0.02	130±29	(9)	
Aspartate	44±13	(12)	75±18	(8)	n.s.	36±13	(9)	
Glutamine	24±3	(12)	29±4	(8)	n.s.	120±25	(9)	
Alanine	93±15	(14)	98±8	(8)	n.s.	59±16	(9)	
GABA	n.d.		n.d.		n.s.	57±21	(9)	

Slices of octopus optic and vertical lobe and rat cortex were incubated for two hours , and the pool sizes and specific activities of the free amino acids determined as described in Chapter 2. The substrate was 10mM U ^{14}C GLUCOSE, of specific activity 1000 dpm/mμmole .

Values expressed ± S.E.M. of numbers of experiments in brackets. n.d. = not detected, n.s. = not significant.

to a substrate specific activity of 1000 disint/min/10^{-9} moles. Table 13 lists the levels of these amino acids in the three tissues after incubation. The pool size values are less than their true values since isolation of the amino acids takes place in several stages as set out in Chapter 2, and some is lost at each stage; however this does not affect the specific activity values or the comparison of the pool sizes under different conditions.

In the rat brain the specific activities of glutamate, aspartate, glutamine, alanine and GABA were all in the same order (Table 12), around 10 to 20% of the substrate specific activity, that of alanine being the lowest. By contrast in the octopus, specific activities were generally under 5% that of the substrate, and this is compatible with the lower rate of metabolism and absolute rate of incorporation of U^{14}C glucose seen in the previous section. However the specific activities of the amino acids showed more variation in value compared with each other, than in the case of rat brain where they were all similar: that of alanine was the highest in both optic and vertical lobe, alanine and glutamine were higher than glutamate, and aspartate was lower than glutamate. The specific activity of aspartate in the vertical lobe was four times that in the optic lobe.

There was an increase in the levels of the amino acids after incubation with glucose, compared with the unincubated levels. Table 14 shows the ratios of these values to facilitate comparison and the estimation of increases or reduction of the levels after incubation.

In the rat, glutamate and aspartate levels were raised by around 40%, glutamine and GABA levels by about 150% and

TABLE 14

LEVELS OF FREE AMINO ACIDS IN OCTOPUS AND RAT BRAIN AFTER INCUBATION WITH 10mM GLUCOSE

Pool size after incubation / pool size before incubation.

	GLU	ASP	GLN	ALA	GABA
Optic lobe	1.10	0.51	1.04	1.18	-
Vertical lobe	0.64	0.66	1.76	1.48	-
Rat cortex	1.44	1.38	2.40	7.40	2.70

Values of pool sizes after incubation with glucose, recorded in Table 13, calculated as a fraction of pool sizes before incubation, recorded in Table 7.

the alanine level seven times. Increases were not so marked in the octopus brain where they occurred: aspartate levels were reduced by about half in both lobes, as was vertical lobe glutamate. The largest increases were in vertical lobe glutamine and alanine. The small amount of GABA in unincubated octopus brain dropped to levels below detection after incubation with glucose.

METABOLISM OF GLUTAMATE IN VITRO.

The properties of glutamate in connection with brain metabolism have been discussed in Chapter 1. It is of interest to study its metabolism *in vitro* here, as it was shown to be made from glucose by rat and octopus brain slices in the previous section; Beloff-Chain et al. (1955a) found that radioactive glutamate appeared in the rat cortex slice after only three minutes incubation with U ^{14}C glucose.

Oxygen uptake and CO_2 production.

Figures 19 and 20 show the oxygen uptake of rat and octopus brain slices with 10mM glutamate, and with 10mM glutamate + 10mM glucose. Glutamate stimulated the respiration of rat brain slices above the endogenous value (compare figure 15) as did glucose (compare figure 14), but unlike glucose, did not maintain a linear rate of respiration over two hours. The use of glucose and glutamate as combined substrates (figure 20) supported a respiration which was identical to that with glutamate alone.

The overall oxygen uptake of vertical lobe slices in the presence of glutamate was less than that with no substrate at all (compare figures 15 and 19), and was also identical to the respiration supported by glucose and

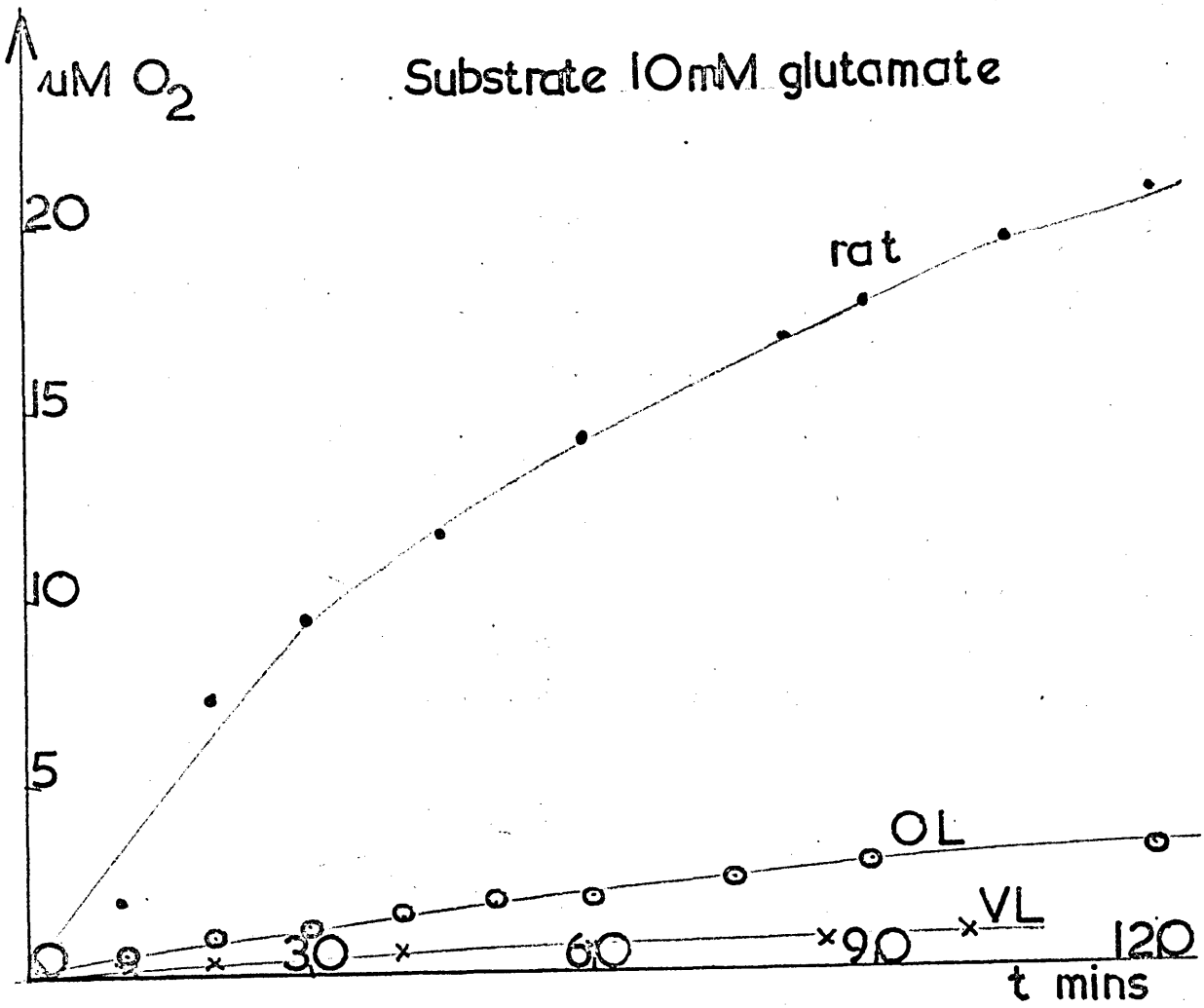


FIGURE 19

Oxygen uptake of a 100 mg slice of octopus optic and vertical lobe, and rat cortex. SUBSTRATE 10mM GLUTAMATE. For incubation conditions, see Chapter 2 . Points are the means of three values .

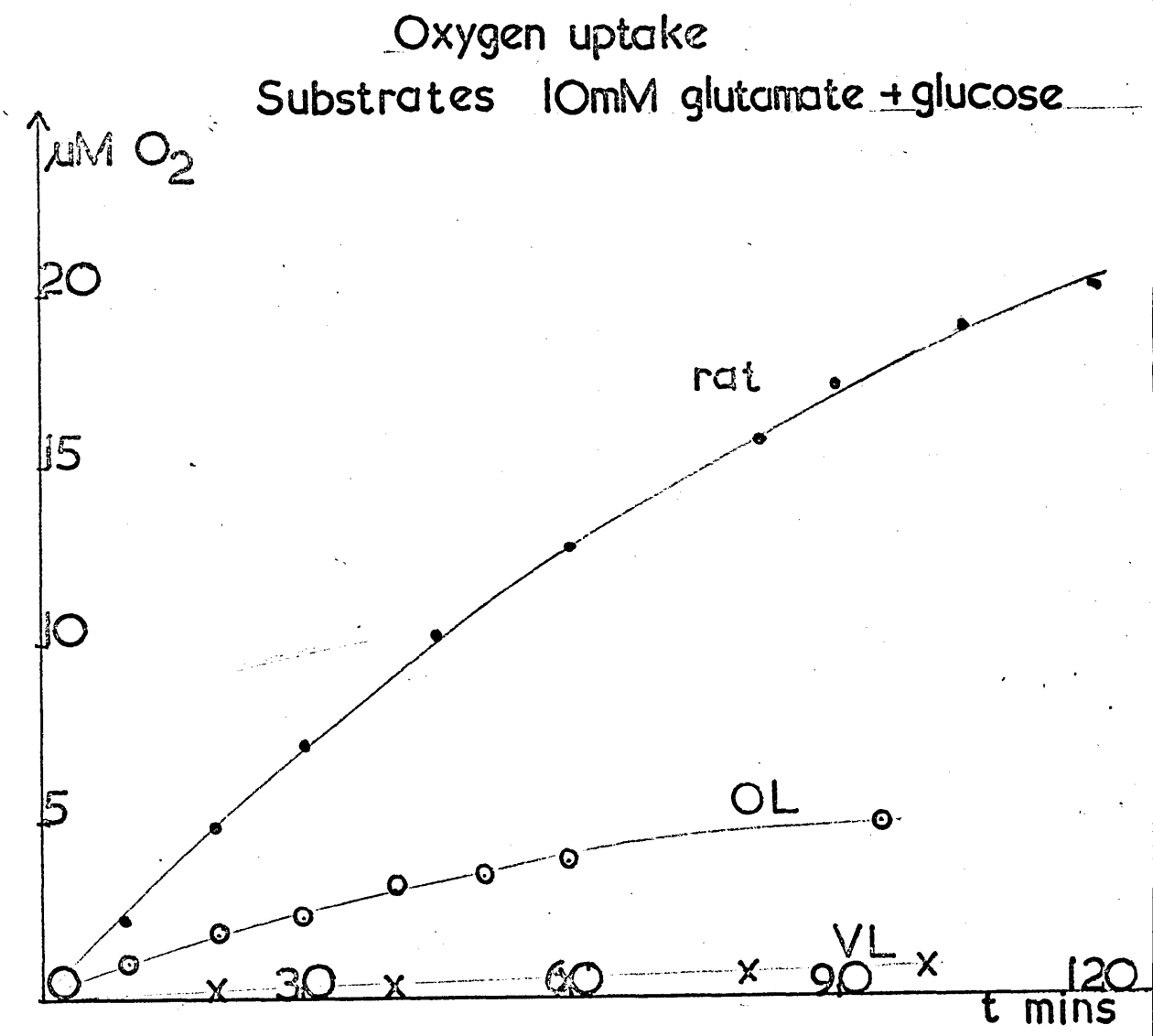


FIGURE 20

Oxygen uptake of a 100 mg slice of optic lobe, vertical lobe, and rat cortex. Substrates 10mM Glutamate + 10mM glucose. For Incubation conditions, see Chapter 2. Points are the means of three values.

glutamate together. This in effect meant that addition of glutamate to glucose reduced vertical lobe respiration *in vitro* to one quarter the value, compared to the situation with glucose alone. In contrast, glutamate did not support in the optic lobe an oxygen uptake higher than the endogenous value, but glutamate and glucose together supported an oxygen uptake similar to glucose alone.

Table 15 lists the initial rates of oxygen uptake of rat and octopus brain, with glucose or glutamate alone or as combined substrates. Initial rates are highest in the rat brain, and higher in the optic lobe than the vertical lobe.

Metabolic pattern of glutamate *in vitro*.

The incorporation of U ^{14}C glutamate *in vitro* into products by the rat cortex slice has been studied by Sellinger et al. (1962), and Rose (1968b) and was not repeated. Rose (1968b) found that the labelled products formed were, in order of labelling: CO_2, aspartate, glutamine and GABA. Lactate and alanine were not detected. With added 10mM glucose, the pattern was changed. Fewer counts went into aspartate but more into glutamine. In contrast, Sellinger et al. (1962) found that addition of unlabelled glucose slightly increased the formation of $^{14}CO_2$ as well as non-labelled CO_2, though the other data was in agreement with that of Rose (1968 b). The drop in aspartate was explained as being due to a stimulation of the production of ATP by the glucose, which accelerated the rate of oxidation of aspartate and glutamate to CO_2.

Table 16 shows the $^{14}CO_2$ production by octopus and rat brain slices incubated with U ^{14}C glutamate in the presence and absence of glucose. The slightly higher $^{14}CO_2$ production

TABLE 15

INITIAL RATES OF OXYGEN UPTAKE IN RAT AND OCTOPUS BRAIN SLICES

10mM Substrate	uMoles / 100 mg protein / hr		
	Optic lobe	Vertical lobe	Rat brain
Glucose	34±2 (14)	24±3 (3)	89±5 (9)
Glutamate	27±2 (12)	21±1 (6)	81±3 (9)
Glutamate + glucose	30±1 (8)	17±2 (7)	87±5 (9)
none	22±1 (5)	15±3 (3)	40±1 (6)

Oxygen Uptake was measured in octopus optic and vertical lobe and rat cortex slices, as described in Chapter 2. Values are the means of numbers of experiments in brackets, ± S.E.M.s .

TABLE 16

CO_2 PRODUCTION BY RAT AND OCTOPUS BRAIN SLICES FROM $U^{14}C$ GLUTAMATE IN THE PRESENCE AND ABSENCE OF GLUCOSE

Cosubstrate	Disint/min/ 10^6 cts. substrate/mg. protein		
	OPTIC LOBE	VERTICAL LOBE	RAT BRAIN
none	850±155 (15)	726±252 (6)	3480±270 (6)
10mM glucose	457± 84 (9)	144±15 (6)	3700±205 (6)
P	< 0.1 (n.s.)	< 0.05	n.s.

Octopus and rat brain slices were incubated with 10mM $U^{14}C$ glutamate in the presence and absence of 10mM glucose, and $^{14}CO_2$ production measured as described in Chapter 2. Results expressed as means ± S.E.M.s of numbers of experiments in brackets.

in the presence of glucose in the rat brain agrees with Sellinger et al. (1962). In the octopus brain the situation was reversed: addition of glucose reduced the incorporation of ^{14}C into CO_2 by half in the optic lobe, and by four fifths in the vertical lobe: therefore, in contrast to the rat brain, added glucose did not stimulate the conversion of glutamate into CO_2.

Specific activities and pool sizes of free amino acids.

Tables 17 to 20 show the specific activities and pool sizes of the free amino acids after incubation with $U^{14}C$ glutamate in the presence and absence of glucose. To understand the results it is necessary to make several comparisons, between the three tissues for a single substrate, and between the same tissues with different substrates.

With $U^{14}C$ glutamate as sole substrate, specific activities of the other amino acids were generally lower in the octopus brain than the rat brain, presumably because of the reasons discussed earlier for the case of glucose, i.e. the slower rate of metabolism in the octopus brain. The specific activity of aspartate was the highest in all cases. In the rat, that of GABA was equal to aspartate, and glutamine was half that value. In the octopus, glutamine and GABA were both less than aspartate, (Table 17). The glutamine specific activity in the octopus was similar to that derived from glucose (Table 12). In the vertical lobe the specific activity of alanine was similar to that of glutamine, and eight times higher than in the optic lobe, where it equalled that of GABA. In the rat brain the specific activity of alanine was the lowest of the amino acids.

TABLE 17

SPECIFIC ACTIVITIES OF FREE AMINO ACIDS IN OCTOPUS AND RAT BRAIN SLICES AFTER INCUBATION WITH U^{14}C GLUTAMATE

Disint/min/ m/umole

Amino acid	Optic lobe	Vertical lobe	P	Rat brain
Glutamate	1000	1000		1000
Aspartate	141±40 (11)	132±31 (9)	n.s.	618±80 (8)
Glutamine	52±2 (8)	36±8 (6)	n.s.	357±140 (7)
Alanine	6±0.5(6)	48±11 (6)	<0.01	104±40 (6)
GABA	9±3 (3)	20±10 (3)	n.s.	730±260 (8)

TABLE 18

POOL SIZES OF FREE AMINO ACIDS IN OCTOPUS AND RAT BRAIN AFTER INCUBATION WITH 10mM GLUTAMATE

m/umoles/mg protein

Amino acid	Optic lobe	Vertical lobe	p	Rat brain
Aspartate	141±27 (11)	204±39 (8)	n.s.	189±54 (9)
Glutamine	37±6 (9)	61±11 (7)	n.s.	117±33 (8)
Alanine	105±11 (11)	160±32 (8)	n.s.	85±30 (7)
GABA	8±1 (3)	19±4 (3)	<0.01	80±30 (8)

Slices of octopus optic and vertical lobe and rat cortex were incubated and pool sizes and specific activities of the free amino acids determined as described in Chapter 2.

Substrate 10mM U^{14}C Glutamate, of specific activity 1000 dpm/ m/umole.

Results expressed as means ± S.E.M.s of numbers of experiments in brackets. n.s. = not significant.

TABLE 19

SPECIFIC ACTIVITIES OF FREE AMINO ACIDS IN OCTOPUS AND RAT BRAIN SLICES AFTER INCUBATION WITH U^{14}C GLUTAMATE IN THE PRESENCE OF NON-LABELLED GLUCOSE

	Disint/min/m/umole			
Amino acid	Optic lobe	Vertical lobe	p	Rat brain
Glutamate	1000	1000		1000
Aspartate	82±10 (15)	195±34 (6)	<0.01	483±63 (9)
Glutamine	53±8 (12)	31±8 (6)	<0.01	450±116(9)
Alanine	6±0.6 (12)	5±0.6 (6)	n.s.	64±16 (9)
GABA	n.d.	12±3 (4)	<0.01	590±190(9)

TABLE 20

POOL SIZES OF FREE AMINO ACIDS IN OCTOPUS AND RAT BRAIN AFTER INCUBATION WITH 10mM GLUTAMATE + GLUCOSE

	m/umoles/mg protein			
Amino acid	Optic lobe	Vertical lobe	p	Rat brain
Aspartate	150±13 (6)	193±25 (6)	n.s.	124±17 (9)
Glutamine	47±8 (6)	101±22 (6)	n.s.	115±25 (9)
Alanine	230±28 (12)	196±25 (8)	<0.05	91±20 (9)
GABA	n.d.	86±26 (4)	<0.01	70±16 (9)

Slices of optic and vertical lobe and rat cortex were incubated and specific activities and pool sizes of the free amino acids determined as described in Chapter 2.

Substrate 10mM U^{14}C Glutamate + 10mM Glucose.

Results expressed as means ± S.E.M.s of numbers of experiments in brackets. n.d. = not detected. n.s.= not significant.

Table 19 lists the specific activities of the amino acids obtained with glucose as cosubstrate to U^{14}C glutamate. The relative specific activities of the amino acids were not altered, except that aspartate was reduced. Table 21 compares the values in the presence and absence of glucose, and only in the case of vertical lobe alanine was there a significant, ten fold reduction of specific activity by the addition of glucose. Table 22 compares the pool sizes of the amino acids formed from 10mM glutamate in the presence and absence of glucose, and Table 23 the pool sizes expressed as fractions of the unincubated values. Compared to preincubation levels, the pool sizes of the amino acids after incubation with 10mM glutamate were altered as follows: all the amino acids were higher in the rat brain after incubation, notably aspartate and GABA, increased 7 fold and 4 fold respectively. In the octopus, the highest increase was in vertical lobe alanine, being 2½ times raised. Addition of 10mM glucose to glutamate as substrate caused a 35% drop in the level of aspartate, and it would seem, as Sellinger et al. (1962) suggest, to be due to the action of glucose in promoting the complete oxidation of glutamate and aspartate. There was no change in the levels of the other amino acids. In the octopus brain, the optic lobe alanine level was doubled, and in the vertical lobe, GABA was raised 4 fold.

For the sake of clarity, Tables 21 and 22 show the results already shown, for comparison of the specific activities and pool sizes with U^{14}C glutamate with and without added glucose, together with tests of significance. The only significant differences (less than 0.01) were

TABLE 21

COMPARISON OF THE SPECIFIC ACTIVITIES OF THE FREE AMINO ACIDS IN OCTOPUS AND RAT BRAIN SLICES, AFTER INCUBATION WITH $U^{14}C$ GLUTAMATE, IN THE PRESENCE OR ABSENCE OF GLUCOSE

	Disint/min/m/umole				
	no glucose		with glucose		p
OPTIC LOBE					
aspartate	141±40	(11)	82±10	(15)	n.s.
glutamine	52±2	(8)	53±8	(12)	n.s.
alanine	6±0.5	(6)	6±1	(12)	n.s.
GABA	9±3	(3)	n.d.		
VERTICAL LOBE					
aspartate	132±31	(9)	195±34	(6)	n.s.
glutamine	36±8	(6)	31±8	(6)	n.s.
alanine	48±11	(6)	5±1	(6)	0.01
GABA	20±10	(3)	12±3	(4)	n.s.
RAT BRAIN					
aspartate	618±80	(8)	483±63	(9)	n.s.
glutamine	357±140	(7)	450±116	(9)	n.s.
alanine	104±40	(6)	64±16	(9)	n.s.
GABA	730±260	(8)	590±190	(9)	n.s.

Slices of optic and vertical lobe were incubated with $U^{14}C$ glutamate in the presence and absence of glucose, as was also rat brain. The table shows the results already given in Tables 17 and 19, with tests of significance.
n.s. = not significant. n.d. = not detected.

TABLE 22

COMPARISON OF POOL SIZES OF FREE AMINO ACIDS IN RAT AND OCTOPUS BRAIN AFTER INCUBATION WITH 10mM GLUTAMATE, IN THE PRESENCE OR ABSENCE OF 10mM GLUCOSE

	m/umoles / mg protein		
	no glucose	with glucose	p
OPTIC LOBE			
aspartate	141±27 (11)	150±13 (6)	n.s.
glutamine	37±6 (9)	47±8 (6)	n.s.
alanine	105±11 (11)	230±28 (12)	<0.01
GABA	8±1 (3)	n.d.	
VERTICAL LOBE			
aspartate	204±39 (8)	193±25 (6)	n.s.
glutamine	61±11 (7)	101±22 (6)	n.s.
alanine	160±32 (8)	196±25 (8)	n.s.
GABA	19±4 (3)	86±26 (4)	< 0.01
RAT BRAIN			
aspartate	189±54 (9)	124±17 (6)	n.s.
glutamine	117±33 (8)	115±25 (9)	n.s.
alanine	85±30 (7)	91±20 (9)	n.s.
GABA	80±30 (8)	70±16 (9)	n.s.

The Table shows the results already given in Tables 18 and 20, with tests of significance. n.s. = not significant, n.d. = not detected.

TABLE 23

POOL SIZES OF FREE AMINO ACIDS IN RAT AND OCTOPUS BRAIN, AFTER INCUBATION WITH 10mM GLUTAMATE IN THE PRESENCE AND ABSENCE OF GLUCOSE.

Pool size after incubation / Pool size before incubation.

No glucose	ASP	GLN	ALA	GABA
Optic lobe	1.62	1.61	1.23	0.80
Vertical lobe	1.79	1.20	2.42	1.30
Rat brain	7.25	2.34	1.06	3.80
With glucose				
Optic lobe	1.73	2.04	2.91	-
Vertical lobe	1.69	1.98	2.97	6.20
Rat brain	4.78	2.30	1.14	3.33

Pool sizes of free amino acids after incubation with 10mM glutamate, recorded in Table 18, or after incubation with 10mM glutamate + glucose, recorded in Table 20, calculated as a fraction of pool sizes before incubation, recorded in Table 7.

TABLE 24

POOL SIZES OF FREE AMINO ACIDS IN RAT AND OCTOPUS BRAIN, AFTER INCUBATION WITH 10mM GLUCOSE, IN THE PRESENCE AND ABSENCE OF GLUTAMATE

Pool size after incubation / Pool size before incubation

No glutamate	ASP	GLN	ALA	GABA
Optic lobe	0.51	1.04	1.18	-
Vertical lobe	0.66	1.76	1.48	-
Rat brain	1.38	2.40	7.40	2.70
With glutamate				
Optic lobe	1.73	2.04	2.91	-
Vertical lobe	1.69	1.98	2.97	6.20
Rat brain	4.78	2.30	11.40	3.33

Pool sizes of free amino acids after incubation with 10mM glucose (Table 13) or 10mM glucose + 10mM glutamate(Table 20) expressed as a fraction of those before incubation (Table 7)

the doubling of optic lobe alanine levels on the addition of glucose, the four fold increase in vertical lobe GABA, and the reduction of the specific activity of vertical lobe alanine.

An interesting comparison can be made, between pool sizes after incubation with glucose alone, and with glucose + glutamate (Tables 13 and 22). Table 24 shows the ratios of the pool sizes of the amino acids to their levels before incubation under these conditions: compared with glucose alone, pool sizes after incubation with both substrates were raised in all cases, the only exception being the glutamine level in rat brain and vertical lobe; apart from these, amino acid levels were generally raised 2 to 3 fold.

It is also worth while to consider the values of the specific activities and pool sizes simultaneously. Differences found in the specific activities were sometimes accounted for as changes in the pool sizes, sometimes not, and the lowering of the specific activity of an amino acid was not always concommitant with an increased pool size. In the rat brain, the specific activity of glutamate-formed aspartate (Tables 17 and 19) fell by 20% with added glucose, but the pool sizes fell by 35% (Tables 18 and 20); this appears to be due to the promotion of the net oxidation of aspartate caused by glucose. The situation in octopus brain was different, in that glucose caused a fall in the specific activity of aspartate without a change in pool size, indicating that this amino acid was formed from glucose as well as from glutamate. The same effect was encountered earlier when considering $^{14}CO_2$ production under the same conditions. Alanine metabolism in the

octopus brain was interesting: addition of glucose doubled the pool size in optic lobe without altering the specific activity, therefore the added glucose may have stimulated net incorporation of glutamate into alanine.

METABOLISM OF ALANINE IN OCTOPUS AND RAT BRAIN IN VITRO.

In a previous section in this chapter it was observed that octopus brain slices incubated with U ^{14}C glucose incorporated many more counts into alanine than any other amino acid, in contrast to the rat brain, and that octopus brain alanine had the highest specific activity of all the amino acids. Therefore the metabolism of U ^{14}C L-alanine was investigated in rat and octopus brain slices *in vitro* in the presence and absence of added non-labelled glucose.

Oxygen Uptake, CO_2 and lactate production.

Figures 21 and 22 show the course of oxygen uptake of a 100mg slice of optic lobe, vertical lobe and rat cortex, with alanine as substrate, in the absence and presence of glucose. In none of these tissues did alanine alone support a linear respiration rate over two hours, and comparison with figure 15 shows that the rates were similar to those obtained with no substrate. However, the addition of glucose stimulated the respiration of rat brain slices to an extent slightly higher than glucose alone (Figure 14), and a linear rate was supported over two hours. Vertical lobe oxygen uptake was also stimulated, but not to the extent of the value with glucose as substrate. Optic lobe slices likewise were stimulated, but to a lesser degree than the vertical lobe. Table 25 compares the initial rates of oxygen uptakes for the three tissues under these

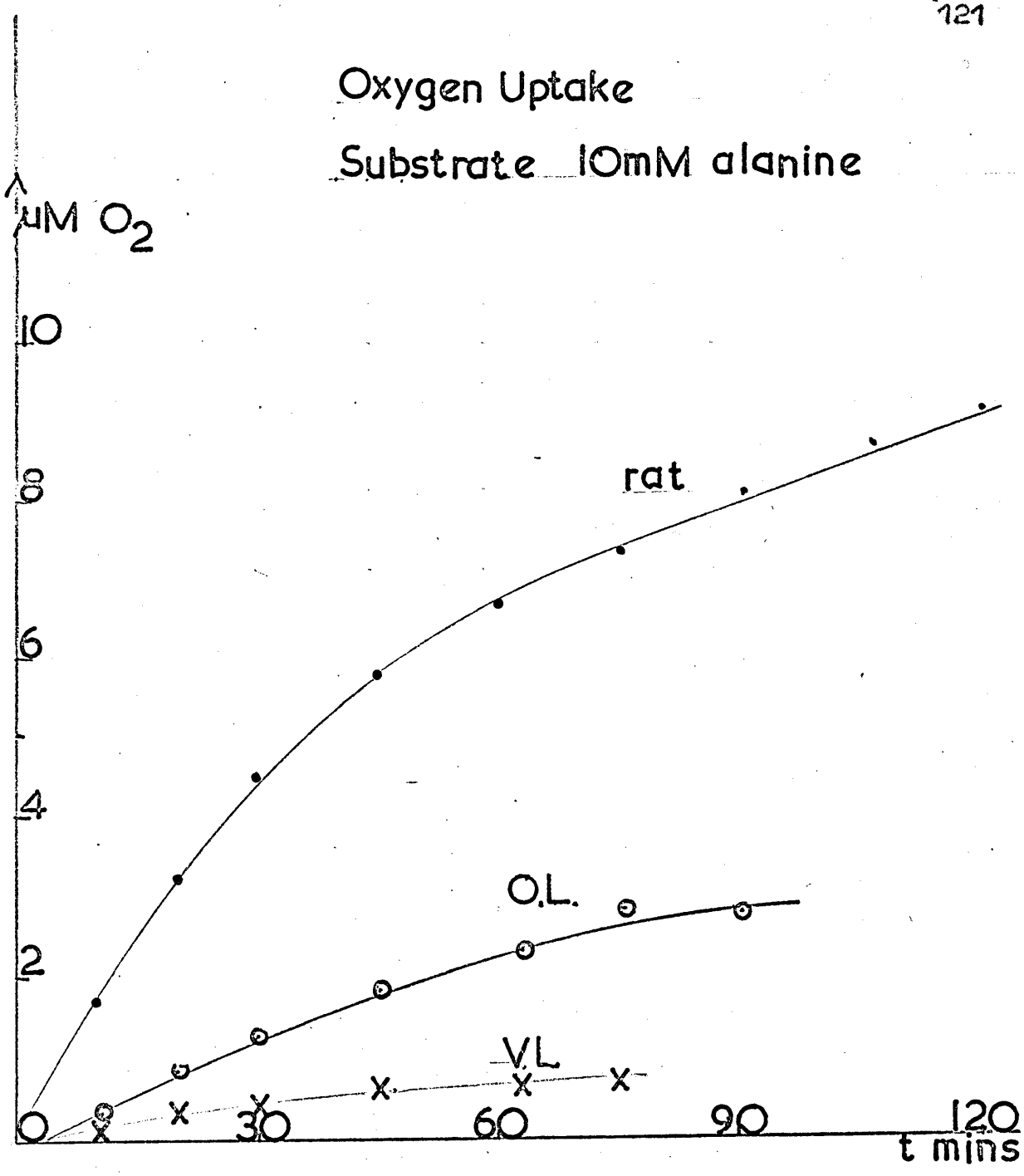


FIGURE 21

Oxygen uptake of optic lobe, vertical lobe, and rat cortex slices incubated with 10mM alanine : incubation conditions were as described in Chapter 2.

Points are the means of three values, for a 100 mg slice.

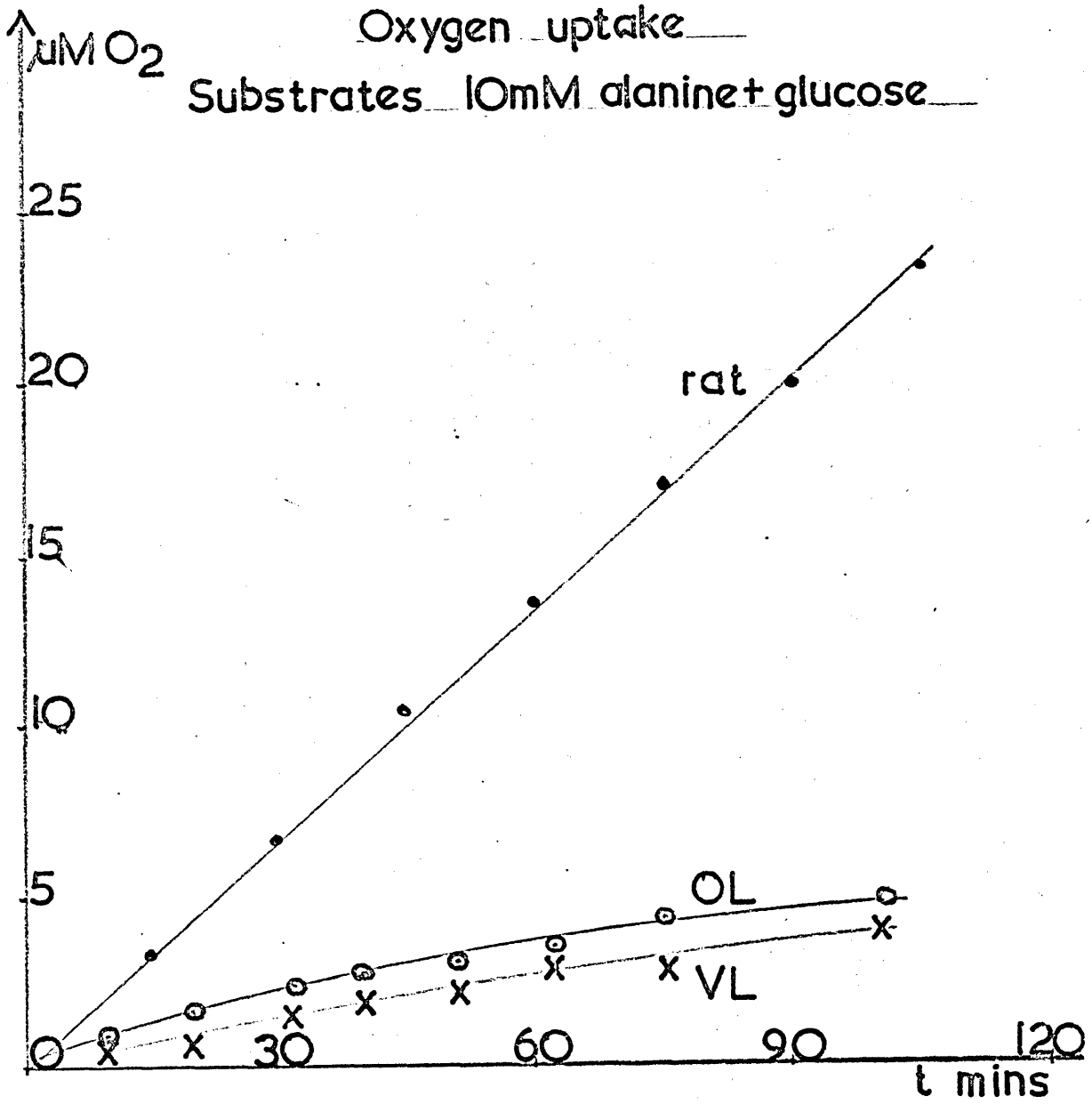


FIGURE 22

Oxygen Uptake of a 100mg slice of optic lobe, vertical lobe and rat brain, incubated with 10mM Alanine + 10mM glucose. Incubation conditions were as described in Chapter 2.

Points are the means of three values.

TABLE 25

INITIAL RATES OF OXYGEN UPTAKE OF OCTOPUS AND RAT BRAIN SLICES INCUBATED WITH 10mM ALANINE IN THE PRESENCE AND ABSENCE OF 10mM GLUCOSE

	µMoles/ 100 mg protein / hr.		
	no glucose	with glucose	p
Optic lobe	27 (1)	27 (1)	-
Vertical lobe	8 (1)	40 (2)	-
Rat brain	65±6 (7)	146±4 (7)	< 0.01

TABLE 26

CO_2 PRODUCTION BY RAT AND OCTOPUS BRAIN SLICES INCUBATED WITH $U^{14}C$ ALANINE IN THE PRESENCE AND ABSENCE OF GLUCOSE

	Disint / min /10^6 cts. substrate/mg prot.		
	no glucose	with glucose	p
Optic lobe	601±100 (4)	218±104 (4)	< 0.05
Vertical lobe	183±44 (4)	84±30 (4)	n.s.
Rat brain	302±35 (12)	370±36 (10)	n.s.

Slices of optic and vertical lobe and rat cortex were incubated with 10mM $U^{14}C$ L-Alanine in the presence and absence of 10mM glucose, and oxygen uptake and CO_2 production determined as described in Chapter 2. Results expressed as means ± S.E.M.s of numbers of experiments in brackets, where possible. n.s. = not significant.

conditions. Table 26 compares the CO_2 production of rat and octopus brain slices under those conditions. $^{14}CO_2$ was formed in octopus and rat brain, and production is comparable in both brains. Addition of non-labelled glucose caused a slight increase in CO_2 production by the rat brain, presumably by the stimulation of the net oxidation of ^{14}C alanine by the glucose. In contrast, octopus brain $^{14}CO_2$ evolution was diminished by the addition of glucose; it would have been useful to have known the specific activity of the CO_2 here, to determine whether more unlabelled CO_2 was formed.

In no case was radioactive lactate detected, in octopus or rat brain, except in the rat brain slice incubated with U^{14}C alanine in the presence of glucose. Beloff-Chain et al. (1962) also found no counts were incorporated into lactate from ^{14}C alanine alone. It is noteworthy that no radioactive lactate was detected with U^{14}C glutamate as sole substrate, also found by Rose (1968b.)

<u>Specific activities and pool sizes of amino acids formed from U^{14}C alanine in the presence and absence of glucose.</u>

Tables 27 to 30 show the pool sizes and specific activities of the free amino acids formed from U^{14}C alanine <u>in vitro</u>, in rat and octopus brain, in the presence and absence of glucose. Specific activities on the whole were about a quarter the values in octopus brain compared with the rat. In the octopus brain glutamate had the highest specific activity, aspartate and glutamine were lower, and GABA was not detected. In contrast, rat brain glutamine, GABA and glutamate all had comparable values, aspartate having half the value of these three.

TABLE 27

SPECIFIC ACTIVITIES OF FREE AMINO ACIDS IN OCTOPUS AND RAT BRAIN SLICES AFTER INCUBATION WITH U ^{14}C ALANINE

	Disint/min/ n/umole			
Amino acid	Optic lobe	Vertical lobe	p	Rat brain
Glutamate	79±20 (4)	57±8 (4)	n.s.	224±36 (14)
Aspartate	56±10 (3)	28 (2)	-	148±19 (14)
Glutamine	54 (1)	12±3 (3)	-	295±70 (9)
Alanine	1000	1000		1000
GABA	n.d.	n.d.		202±54 (8)

TABLE 28

POOL SIZES OF FREE AMINO ACIDS IN OCTOPUS AND RAT BRAIN AFTER INCUBATION WITH 10mM ALANINE

	n/umoles/mg protein			
Amino acid	Optic lobe	Vertical lobe	p	Rat brain
Glutamate	72±13 (4)	150±35 (4)	$<$ 0.05	43±4 (14)
Aspartate	46±6 (4)	55±8 (4)	n.s.	77±7 (14)
Glutamine	19 (2)	15±1 (4)	n.s.	30±5 (13)
GABA	n.d.	n.d.		20±3 (8)

Slices of octopus optic and vertical lobe and rat brain were incubated with 10mM U ^{14}C L-alanine , specific activity 1000 dpm / n/umole, and the pool sizes and specific activities of the free amino acids determined as described in Chapter 2. Results given as means ± S.E.M.s of numbers of experiments in brackets, where possible.

n.d. = not detected, n.s. = not significant.

TABLE 29

SPECIFIC ACTIVITIES OF FREE AMINO ACIDS IN OCTOPUS AND RAT BRAIN SLICES AFTER INCUBATION WITH U ^{14}C ALANINE + GLUCOSE

	Disint/ min / m/umole							
Amino acid	Optic lobe		Vertical lobe		p	Rat brain		
Glutamate	63±18	(4)	102±14	(4)	n.s.	166±13	(11)	
Aspartate	23±10	(4)	62±10	(4)	<0.05	83±8	(7)	
Glutamine	16	(2)	n.d.			71±8	(6)	
GABA	n.d.		n.d.			79±12	(7)	

TABLE 30

POOL SIZES OF FREE AMINO ACIDS IN RAT AND OCTOPUS BRAIN AFTER INCUBATION WITH 10mM ALANINE + 10mM GLUCOSE

	m/umole / mg protein							
Amino acid	Optic lobe		Vertical lobe		p	Rat brain		
Glutamate	135±20	(4)	170±8	(4)	n.s.	90±9	(11)	
Aspartate	76±14	(4)	140±8	(4)	<0.01	32±6	(11)	
Glutamine	26	(2)	n.d.			93±17	(8)	
GABA	n.d.		n.d.			94±14	(11)	

Slices of optic lobe, vertical lobe and rat cortex were incubated with 10mM U ^{14}C L-Alanine, specific activity 1000 dpm / m/umole, together with 10mM glucose: pool sizes and specific activities of the free amino acids were determined as described in Chapter 2. Results expressed as means, ± S.E.M. s of numbers of experiments in brackets, where possible. n.d. = not detected, n.s. = not significant.

Addition of glucose reduced the specific activities of the amino acids in the rat brain, but glutamine and GABA were most reduced, so that glutamate had the highest specific activity.

In the octopus brain, specific activities were not reduced: optic lobe glutamate was unchanged, vertical lobe glutamate and aspartate were doubled. To assess the values of these differences, Table 31 compared the specific activities in the presence and absence of glucose, with tests of significance. Assuming that the alanine enters the citric acid cycle by transamination and conversion into pyruvate, it is difficult to see how addition of glucose caused a rise in the specific activities of glutamate and aspartate in vertical lobe, unless a new pathway for alanine was present for entry into the citric acid cycle, by-passing pyruvate.

Table 33 lists the ratios of the pool sizes recorded in Tables 28 and 30, to the values before incubation. Some surprising features are evident: there was no increase in the pool size of any amino acid in octopus or rat brain after incubation with 10mM alanine, with the exception of rat brain aspartate. The level of glutamate in rat cortex and in the optic lobe was reduced to about a half. Octopus aspartate levels were also halved, though aspartate was doubled in the rat brain. Vertical lobe glutamine fell to one third the preincubated value. It would have been interesting to have obtained values for the total production of CO_2, to see if alanine promoted the oxidation of the amino acids, resulting in their fall in level.

Inspection of Tables 32 and 33 reveals that addition of glucose resulted in an increase of the levels of all the

TABLE 31

COMPARISON OF THE SPECIFIC ACTIVITIES OF THE AMINO ACIDS OF RAT AND OCTOPUS BRAIN AFTER INCUBATION WITH U^{14} C ALANINE, IN THE PRESENCE AND ABSENCE OF GLUCOSE

	Disint/ min/ n/umole no glucose		with glucose		p
OPTIC LOBE					
glutamate	79±20	(4)	63±18	(4)	n.s.
aspartate	56±10	(3)	23±10	(3)	n.s.
glutamine	54	(1)	16	(2)	n.s.
GABA	n.d.		n.d.		n.s.
VERTICAL LOBE					
glutamate	57±8	(4)	102±14	(4)	$<$ 0.05
aspartate	28	(2)	62±10	(4)	n.s.
glutamine	12±3	(3)	n.d.		n.s.
GABA	n.d.		n.d.		n.s.
RAT BRAIN					
glutamate	224±36	(14)	166±13	(11)	n.s.
aspartate	148±19	(14)	83±8	(7)	$<$ 0.05
glutamine	295±70	(9)	71±8	(6)	$<$ 0.05
GABA	202±54	(8)	79±12	(7)	n.s.

The table shows the results already given in Tables 27 and 29, together with tests of significance.

TABLE 32

COMPARISON OF POOL SIZES OF AMINO ACIDS IN RAT AND OCTOPUS BRAIN AFTER INCUBATION WITH 10mM ALANINE IN THE PRESENCE OR ABSENCE OF 10mM GLUCOSE

	m/umoles / mg protein		
	no glucose	with glucose	p
OPTIC LOBE			
glutamate	72±13 (4)	135±20 (4)	< 0.05
aspartate	46±6 (4)	76±14 (4)	< 0.05
glutamine	19 (2)	26 (2)	n.s.
GABA	n.d.	n.d.	n.s.
VERTICAL LOBE			
glutamate	150±35 (4)	170±8 (4)	n.s.
aspartate	55±8 (4)	140±8 (4)	< 0.01
glutamine	15±1 (4)	n.d.	n.s.
GABA	n.d.	n.d.	n.s.
RAT BRAIN			
glutamate	43±4 (14)	90±9 (11)	< 0.01
aspartate	77±7 (14)	32±6 (11)	< 0.01
glutamine	30±5 (13)	93±17 (8)	< 0.01
GABA	n.d.	94±14 (11)	< 0.01

The Table shows the pool sizes already listed in Tables 28 and 30, together with tests of significance.

TABLE 33

POOL SIZES OF FREE AMINO ACIDS IN RAT AND OCTOPUS BRAIN AFTER INCUBATION WITH ALANINE AND/OR GLUCOSE

Pool size after incubation / Pool size before incubation.

SUBSTRATES	GLU	ASP	GLN	ALA	GABA
Alanine					
Optic lobe	0.64	0.53	0.83	-	-
Vertical lobe	1.04	0.48	0.29	-	-
Rat cortex	0.48	2.96	0.60	-	0.95
Glucose					
Optic lobe	1.10	0.51	1.04	1.18	-
Vertical lobe	0.64	0.66	1.76	1.48	-
Rat cortex	1.44	1.38	2.40	7.40	2.70
Alanine + Glucose					
Optic lobe	1.20	0.88	1.13	-	-
Vertical lobe	1.18	1.23	-	-	-
Rat cortex	1.00	1.23	1.86	-	4.48

Pool sizes , recorded in Tables 28, 30 and 14, expressed as a fraction of pool sizes of amino acids before incubation, recorded in Table 7.

amino acids in the three tissues to some extent, with the exception of rat brain aspartate and vertical lobe glutamine which was halved. The most significant increases were rat brain glutamate, glutamine and GABA, optic lobe glutamate and aspartate, and vertical lobe aspartate. The decrease of rat brain aspartate was probably due to the stimulation of the net oxidation of this amino acid by the addition of glucose, since the reduction of the pool size was accompanied by a fall in specific activity, (Table 31). Rat brain glutamine and GABA values were raised 3 to 4 fold by the addition of glucose.

As was mentioned earlier in Chapter 2, there is a possibility that other amino acids have been undetected, and these may well be involved in some of the transformations of alanine into the citric acid cycle intermediates.

FURTHER INVESTIGATION OF THE METABOLISM OF PYRUVATE AND ALANINE IN THE RAT BRAIN IN VITRO.

Comparison of the metabolism of pyruvate and glucose.

In Chapter 1, certain unexpected differences were pointed out in the metabolism of glucose and pyruvate *in vitro*, and reasons were suggested for them. The following is an attempt to help clarify the situation in the rat brain; regrettably, lack of animals made the investigations on octopus brain impossible.

Figure 23 compares the course of respiration of a rat brain slice in the presence of substrate glucose or pyruvate. Pyruvate seemed to support a slightly higher respiration than glucose, as also stated by Bradford (1968), and it maintained a linear respiration rate over two hours.

OXYGEN UPTAKE OF RAT CORTEX SLICE
WITH 10mM SUBSTRATE

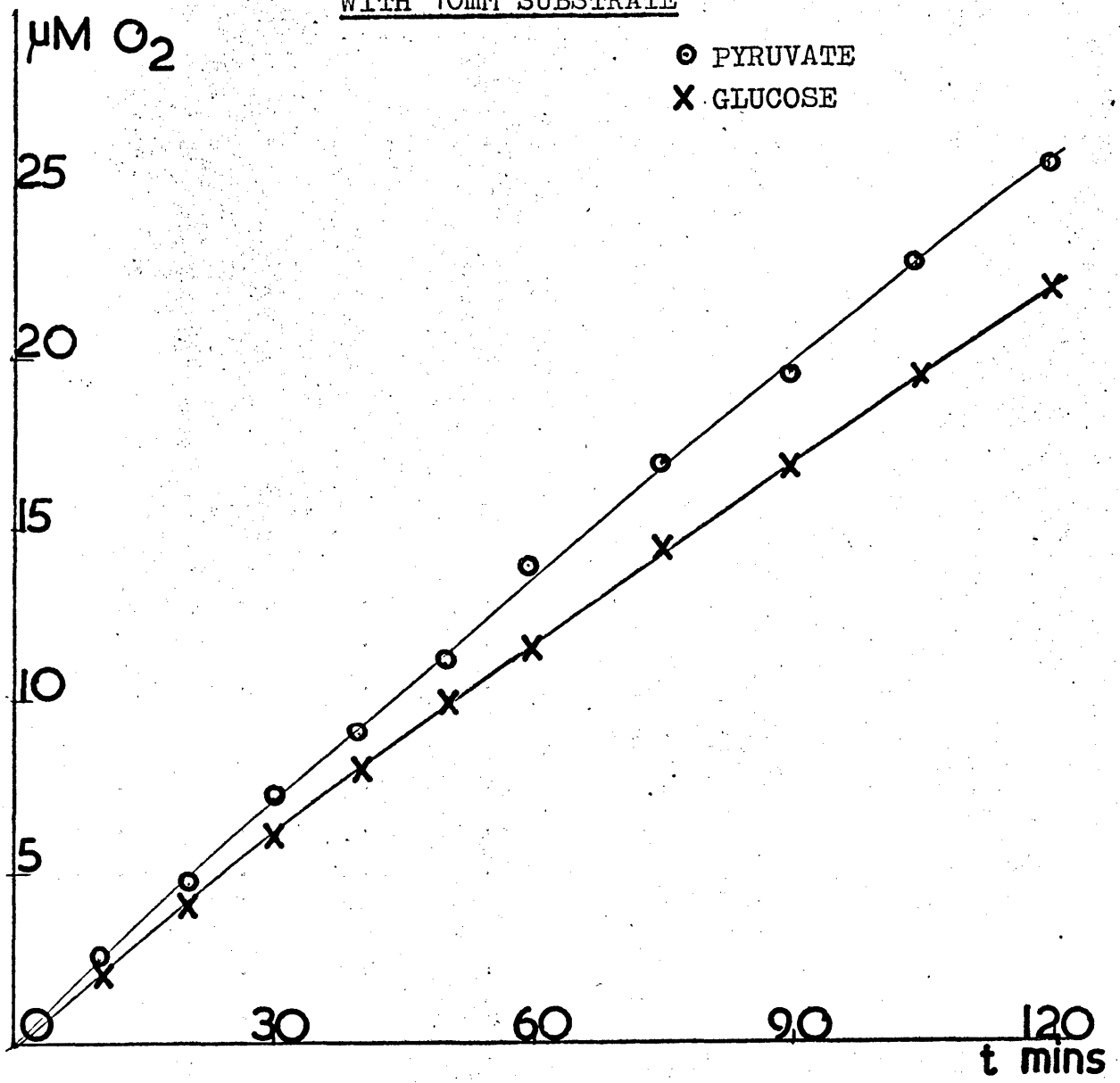


FIGURE 23

Oxygen Uptake of a 100 mg slice of rat cortex. Substrate 10mM Glucose or Pyruvate. Incubation conditions were as described in Chapter 2. Points are the means of 3 values.

Table 34 compares the formation of CO_2 and lactate from U^{14}C pyruvate or glucose in vitro. These were both higher with glucose as substrate, and the results of Chain et al. (1969) are in accordance with this. This is unexpected since Cremer (1967) points out that pyruvate is oxidised to CO_2 twice as fast as glucose, but she also found a higher lactate production from glucose, though the reason for this anomaly is still not apparent.

Chain et al. (1969) compared the metabolic pattern of incorporation of radioactivity from glucose or pyruvate into products, and found that glutamate and GABA production was similar in both cases, but that of aspartate and alanine was less from glucose.

Tables 35 and 36 compare the specific activities and pool sizes of the free amino acids after incubation of rat brain slices with radioactive glucose or pyruvate. The differences were striking and unexpected. U^{14}C pyruvate yielded a glutamate of specific activity ten times that obtained from U^{14}C glucose, though none of the other amino acids showed such a large discrepancy. Specific activities of aspartate and GABA were twice as high after incubation with pyruvate, but those of alanine and glutamine were similar.

The pool sizes of alanine and GABA were similar after incubation with glucose or pyruvate, but that of glutamate was twice the level after incubation with glucose than with pyruvate, and that of glutamine five times.

Effect of non-labelled alanine on the metabolism of pyruvate in vitro.

The effect of the addition of non-labelled alanine on the specific activities and pool sizes of the amino acids

TABLE 34

CO_2 AND LACTATE PRODUCTION BY RAT BRAIN SLICES INCUBATED WITH LABELLED GLUCOSE OR PYRUVATE

	Disint/min/ 10^6 counts substrate/mg protein	
Substrate	CO_2	lactate
$U^{14}C$ glucose	6770±920 (4)	5020±292 (8)
$U^{14}C$ pyruvate	4890±570 (11)	2164±508 (6)

TABLE 35

SPECIFIC ACTIVITIES OF FREE AMINO ACIDS IN RAT BRAIN SLICES AFTER INCUBATION WITH $U^{14}C$ GLUCOSE OR $U^{14}C$ PYRUVATE

	Disint/min/m/umole	
Amino acid	Glucose	Pyruvate
Glutamate	199±33 (6)	1629±380 (10)
Aspartate	185±46 (7)	353±34 (7)
Glutamine	195±20 (6)	112±24 (10)
Alanine	115±29 (6)	191±24 (10)
GABA	174±45 (5)	254±11 (6)

TABLE 36

POOL SIZES OF AMINO ACIDS IN RAT BRAIN AFTER INCUBATION WITH 10mM GLUCOSE OR PYRUVATE

	m/umole / mg protein	
Amino acid	Glucose	Pyruvate
Glutamate	130±29 (9)	69±8 (10)
Aspartate	36±13 (9)	45±9 (10)
Glutamine	120±25 (9)	25±4 (8)
Alanine	59±16 (9)	50±6 (9)
GABA	57±21 (9)	42±9 (8)

Slices of rat cortex were incubated with $U^{14}C$ 10mM glucose or pyruvate, and CO_2, lactate production, and the pool sizes and specific activities of the free amino acids determined as described in Chapter 2. SUBSTRATE SPECIFIC ACTIVITY = $\frac{1000}{\text{dpm / m/umole}}$. Results expressed as means ± S.E.M.s of numbers of experiments in brackets.

formed from $U^{14}C$ pyruvate in vitro by rat brain slices was investigated. This was to ascertain whether pyruvate was incorporated into glutamate, aspartate, glutamine and GABA through a pathway involving alanine, by-passing the conversion of pyruvate to Acetyl CoA and citrate: if so, then non-labelled alanine should considerably dilute this incorporation.

Table 37 shows the respiration of slices with 10mM pyruvate + 10mM alanine was not found to be different to that with pyruvate alone. Also, more CO_2 and less lactate were formed in the presence of alanine, though the differences were not significant.

Tables 38 and 39 show the specific activities and pool sizes of the amino acids after incubation of rat brain slices with $U^{14}C$ pyruvate in the presence and absence of alanine. Only in the case of glutamine was there a significant reduction of specific activity by the addition of the non-labelled substrate, though its pool size remained unaltered: it is hard to interpret this other than alanine competed with the synthesis of glutamine (and not of the other amino acids) from pyruvate, that is, glutamine could be synthesised via alanine, by a route not involving the citric acid cycle. The reader is referred back to Figure 10 in Chapter 2 (page 72) for a diagram of the interrelationships of the amino acids and citric acid cycle intermediates.

Influence of added pyruvate and of inhibitors on the metabolism of alanine in rat brain in vitro.

The above experiments were performed in reverse, that is, $U^{14}C$ alanine was incubated with non-labelled pyruvate. Incubations were also carried out in the presence of

TABLE 37

OXYGEN UPTAKE, CO_2 AND LACTATE PRODUCTION BY RAT BRAIN SLICES INCUBATED WITH $U^{14}C$ PYRUVATE, IN THE PRESENCE AND ABSENCE OF ALANINE

	Pyruvate	Pyruvate+alanine	P
Oxygen Uptake (/umoles/ 100mg protein/hr)	142±9 (9)	137±6 (16)	n.s.
CO_2 production dpm/10^6cts. substrate/mg prot.	4891±570 (11)	5325±650 (11)	n.s.
Lactate production dpm / 10^6 cts. substrate/mg prot.	2164±508 (6)	1483±333 (6)	n.s.

Slices of rat cortex were incubated with $U^{14}C$ 10mM pyruvate in the presence and absence of 10mM L-alanine, and oxygen uptake, CO_2 production and lactate production determined as described in Chapter 2. Results expressed as means ± S.E.M.s of numbers of experiments in brackets. n.s. = not significant.

TABLE 38

SPECIFIC ACTIVITIES OF FREE AMINO ACIDS IN RAT BRAIN AFTER INCUBATION WITH U^{14}C PYRUVATE, WITH AND WITHOUT ALANINE

	Disint/min/m/umole				
Amino acid	No alanine		With alanine		P
Glutamate	1629±380	(10)	1394±105	(9)	n.s.
Aspartate	355±34	(7)	568±142	(8)	n.s.
Glutamine	112±22	(6)	23±6	(5)	< 0.01
Alanine	191±24	(10)	50±6	(13)	-
GABA	254±11	(6)	148±28	(5)	< 0.01

TABLE 39

POOL SIZES OF FREE AMINO ACIDS IN RAT BRAIN AFTER INCUBATION WITH 10mM PYRUVATE, WITH AND WITHOUT 10mM ALANINE

	m/umoles/ mg protein				
Amino acid	No alanine		With alanine		P
Glutamate	69±9	(10)	66±5	(9)	n.s.
Aspartate	45±9	(10)	38±9	(8)	n.s.
Glutamine	25±4	(8)	32±7	(8)	n.s.
GABA	42±9	(8)	60±12	(6)	n.s.

Slices of rat cortex were incubated with U^{14}C 10mM pyruvate in the presence and absence of 10mM alanine. Starting specific activity of pyruvate = 1000 dpm/m/umole. Specific activities and pool sizes of the amino acids were determined as described in Chapter 2. Results expressed as means ± S.E.M.s of numbers of experiments in brackets. n.s.= not significant

inhibitors of the citric acid cycle, to see whether U^{14}C alanine is able to be converted into glutamate and aspartate, by-passing the cycle at two stages. The inhibitors were fluoracetate, which is converted into fluorocitrate and inhibits the enzyme aconitase (Peters, 1952) catalysing the isomerisation of citrate; the other inhibitor acts at a different point of the cycle: malonate is a competitive inhibitor of succinic dehydrogenase (Kini and Quastel, 1959). The method of addition was as follows: the radioactive substrate was tipped from the side arm of the Warburg incubation vessel after the slice had been allowed to equilibrate with the inhibitors during the gassing period of about ten minutes. The concentration used for the inhibitors was 10mM, the same as the substrates, since they are both competitive inhibitors, (Dixon and Webb, 1964).

Figure 24 shows the respiration of rat brain slices with 10mM alanine alone, or in the presence of pyruvate, fluoracetate, and fluoracetate + malonate together. Addition of pyruvate raised respiration, compared with alanine alone, and a linear respiration rate was obtained over two hours. However, the presence of fluoracetate reduced respiration to below the level of that with alanine alone, and the further addition of malonate practically abolished it altogether.

Table 40 shows the production of CO_2 from U^{14}C alanine, in the presence of pyruvate and the inhibitors. Addition of non-labelled pyruvate did not alter the CO_2 production, which is unexpected if alanine is supposed to enter the citric acid cycle via conversion to pyruvate, for conversion into CO_2. Surprisingly, the presence of fluoracetate

OXYGEN UPTAKE OF RAT CORTEX SLICE WITH
10mM L-ALANINE

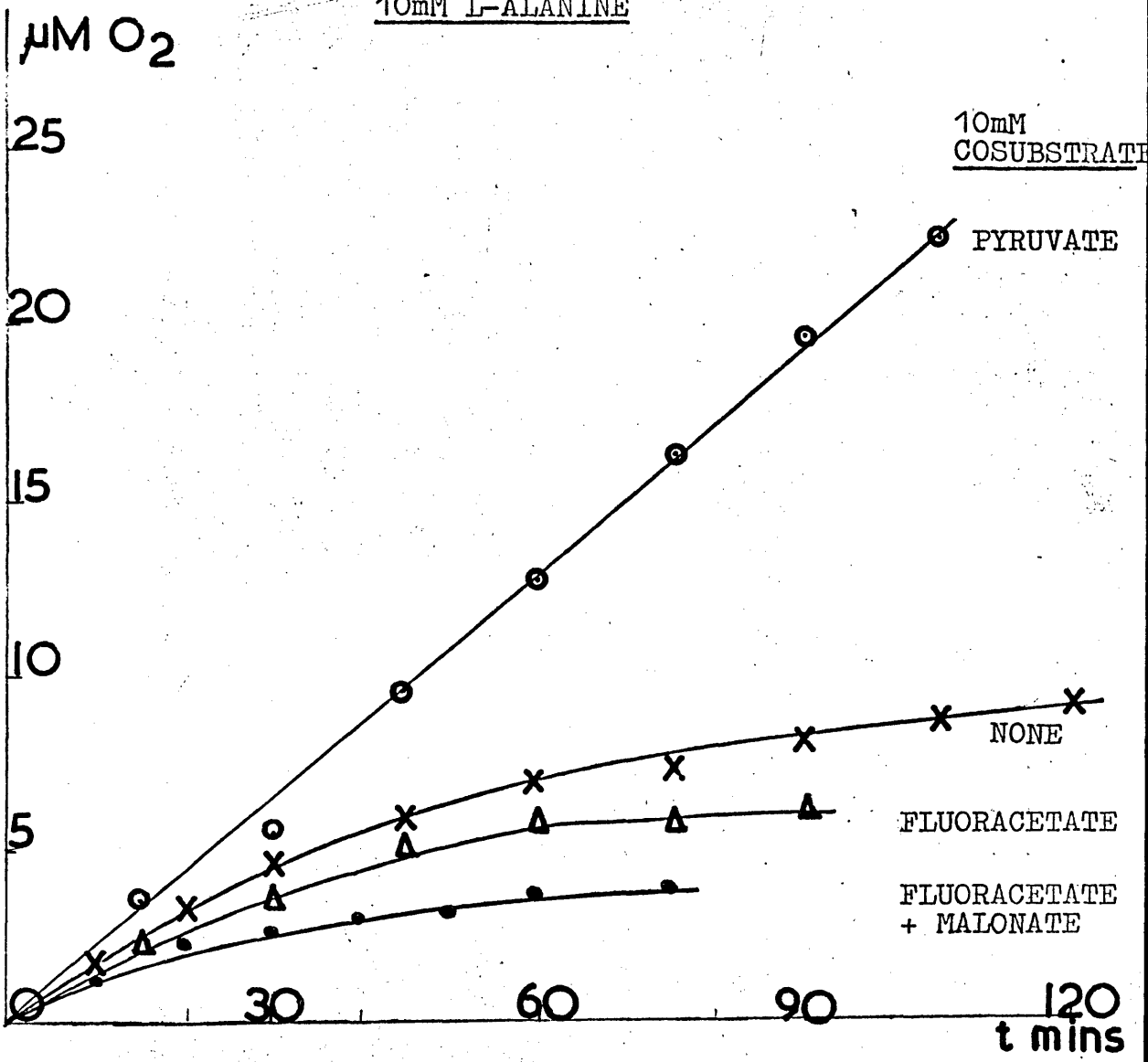


FIGURE 24

Oxygen uptake of a 100 mg rat brain slice incubated with 10mM alanine, in the presence and absence of 10mM cosubstrates and inhibitors: pyruvate, fluoracetate, and fluoracetate + malonate. Incubation conditions as described in Chapter 2, and method of addition of inhibitors as described in Chapter 3. Points are the means of 3 values.

TABLE 40

CO_2 PRODUCTION BY RAT BRAIN SLICES FROM $U^{14}C$ ALANINE IN THE PRESENCE AND ABSENCE OF COSUBSTRATES AND INHIBITORS.

Cosubstrate or inhibitor	Disint/min/ 10^6 cts substrate/ mg protein
none	302±35 (12)
glucose	370±36 (10)
pyruvate	254±61 (10)
fluoracetate alone	249±17 (5)
fluoracetate + malonate	25±5 (6)

CO_2 production was measured in rat brain slices incubated with 10mM $U^{14}C$ L-alanine as described in Chapter 2. Method of addition of inhibitors (10mM) described in Chapter 3. Results expressed as means ± S.E.M.s of numbers of experiments in brackets.

barely altered the CO_2 production, while that of malonate combined with fluoracetate almost reduced it to zero. This may indicate that alanine is able to enter the citric acid cycle after the citrate step, but before the succinate step. (See Figure 10, Chapter 2, page 72). One possible mechanism would be the conversion of alanine to propionyl CoA, which is converted to succinate via propionyl carboxylase; this enzyme has been demonstrated in some molluscan tissues, (Goddard and Martin, 1966). Alternatively, aspartate and alanine are interconvertible through decarboxylation or CO_2 fixation, (Lioret and Moyse, 1963) and this may also be a means of entry of alanine into the cycle, though the formation of glutamate would still be inhibited, as it would involve going round the citric acid cycle in reverse through succinate.

Tables 41 and 42 show the specific activities of the amino acids formed from U^{14}C alanine in the presence of pyruvate and the inhibitors. Addition of pyruvate caused the specific activity of the glutamate to be twice the value compared to incubation with alanine alone, but that of glutamine was reduced to a tenth, and there was no change in the other amino acids. Table 41 shows that the addition of fluoracetate alone as supposed inhibitor gave values for the specific activities of the amino acids similar to those obtained by using glucose as cosubstrate. Glutamate, aspartate and GABA were decreased by around half, compared to incubation with alanine alone. Addition of malonate and fluoracetate to alanine did not, contrary to expectation, decrease the specific activities further, compared to fluoracetate alone. It is surprising that incorporation of radioactivity from alanine occurred at all

TABLE 41

SPECIFIC ACTIVITIES OF FREE AMINO ACIDS IN THE RAT BRAIN SLICE AFTER INCUBATION WITH U^{14}C ALANINE, WITH AND WITHOUT COSUBSTRATES AND INHIBITORS

Disint/ min/m/umole

Cosubstrate or Inhibitor	GLU	ASP	GLN	GABA
none	224±36(14)	148±19(14)	245±70(9)	202±54(8)
glucose	166±13(11)	83±8 (7)	71±8 (6)	79±12(7)
pyruvate	419±55(10)	196±42(7)	23±6 (4)	255±47(7)
fluoracetate	154±25(5)	55±7 (5)	n.d.	56±13(5)
fluoracetate + malonate	56±12(6)	110±14(6)	36±4 (5)	121±16(5)

TABLE 42

POOL SIZES OF FREE AMINO ACIDS IN RAT BRAIN AFTER INCUBATION WITH 10mM ALANINE WITH AND WITHOUT COSUBSTRATES AND INHIBITORS

m/umoles/mg protein

Cosubstrate or Inhibitor	GLU	ASP	GLN	GABA
none	43±4(14)	77±7(14)	30±5(13)	20±3 (8)
glucose	90±9(11)	32±6(11)	93±17(8)	94±14(11)
pyruvate	97±9(10)	54±5(8)	52±8 (9)	29±3 (7)
fluoracetate	52±2(4)	92±4(4)	43±3 (5)	26±2 (5)
fluoracetate +malonate	144±8(6)	28±3(6)	69±4 (6)	20±2 (5)

Slices of rat cortex were incubated with 10mM U^{14}C alanine in the presence and absence of cosubstrates or inhibitors, and specific activities and pool sizes of the free amino acids determined as described in Chapter 2. Starting specific activity of pyruvate = 1000 dpm/m/umole. Results expressed as means ± S.E.M.s of numbers of experiments in brackets. n.d.= not detected.

under these conditions, since the production of CO_2 was practically zero. Furthermore, the poisoned slices appeared pale and crinkled at the end of the equilibration period, before the addition of radioactive alanine, and seemed in no condition to carry out metabolic transformations. It is noteworthy that Kini and Quastel (1959) found that malonate abolished the incorporation of radioactivity from glucose into amino acids. It is clear that interconversions occur which are obscure.

CHAPTER 4

Supplementary experiments were performed to confirm earlier findings on the comparative metabolism of the amino acids in the rat and octopus brain. These consisted in testing for active uptake of ^{14}C amino acids *in vitro* from the medium in the presence of non-labelled glucose to provide energy, and the quantitative assays of some of the enzymes involved in the formation and inter-conversions of the amino acids.

ACTIVE UPTAKE OF AMINO ACIDS BY RAT AND OCTOPUS BRAIN SLICES.

The phenomenon of energy-dependent uptake of amino acids by rat brain slices *in vitro* from the medium was discussed in Chapter 1. Nagata et al. (1966) used uptake as a test for showing that GABA was not involved in the metabolism of isolated rat spinal ganglia. Since in Chapter 3 it was shown that GABA was not formed by octopus brain slices, under conditions where it was formed by rat brain slices, slices of octopus and rat brain were tested for active uptake of GABA. It was also useful to test the other amino acids, since the study of uptake in the octopus could have implications as regards the comparative membrane properties of the two animals.

Optic and vertical lobe and rat cortical slices were incubated with 10mM radioactive glutamate, glutamine, alanine and GABA, in the presence of 10mM glucose, and the concentration of radioactivity per unit volume of slice and medium determined as described in Chapter 2. $^{14}CO_2$ was also collected. Table 43 shows the uptake of the four amino acids by rat and octopus brain slices. The ratio was greater than fourfold in the case of glutamate, glutamine and alanine in the rat brain.

TABLE 43

UPTAKE OF $U^{14}C$ AMINO ACIDS BY OCTOPUS AND RAT BRAIN SLICES

Amino acid	Optic lobe	Vertical lobe	Rat brain
L glutamate	0.83±0.07 (8)	1.03±0.2 (6)	4.13 (3)
L glutamine	0.70±0.06 (10)	1.35±0.13(8)	4.80 (3)
L alanine	0.91±0.11 (4)	1.10±0.07(6)	4.77 (3)
GABA	0.52±0.04 (7)	0.57±0.03(6)	2.01 (3)

Slices of rat and octopus brain were incubated at 37° and 25° respectively for 2 hours, in the presence of 10mM $U^{14}C$ amino acid and 10mM non-labelled glucose. Counts per unit volume medium and slice were determined as described in Chapter 2. Results expressed as ratio counts per unit volume slice / counts per unit volume medium, given as the means ± S.E.M.s of numbers of experiments in brackets, where possible.

In the octopus brain, the ratios barely reached unity after two hours incubation, and the value for GABA was only 0.5; in the rat brain also, the value for GABA was also half that of the other amino acids, but this still amounted to greater than unity. Tsukada, Nagata, Hirano and Matsutani (1963) also found that GABA was taken up by rat cortex slices to a lesser extent than glutamate: however, glutamine was taken up even less than GABA after one hour. Also, Blasberg and Lajtha (1965) found that GABA was taken up by mouse brain slices to a greater extent than glutamate. So it would seem that the results obtained depend on the incubation conditions, and it was hoped that though the values in Table 43 did not correspond exactly with those of other authors, this would not affect the comparison made here between the rat and octopus brain.

Table 44 shows the uptake of glutamate at zero time and in the presence of cyanide, and confirms that the radioactivity found in the slice is only due to surface adhesion to a small extent, and that the accumulation of glutamate is energy-dependent and sensitive to cyanide, though passive diffusion does occur in the presence of cyanide.

TABLE 44

UPTAKE OF GLUTAMIC ACID BY RAT BRAIN IN VITRO

Conditions	slice/medium ratio
Zero time incubation	0.11
1 hour incubation	2.96
1 hour incubation with 5mM KCN	0.80

Results are expressed as in Table 43, and are the means of two values.

Slices of octopus brain were incubated for longer times with U^{14}C glutamate, to see whether active uptake did occur, but more slowly than in the rat brain. Table 45 shows that even after four hours, the slice/medium concentration ratio was not much greater than unity.

TABLE 45

UPTAKE OF U^{14}C GLUTAMATE BY OCTOPUS BRAIN SLICES FOR DIFFERENT INCUBATION TIMES.

Time	Optic lobe	Vertical lobe
1 hour	0.44 (3)	0.68 (1)
2 hours	0.83 (8)	1.03 (6)
3 hours	1.20 (1)	-
4 hours	1.16 (1)	1.62 (1)

Results are expressed as in Table 43, and are the means of the numbers of experiments in brackets.

Table 46 lists the CO_2 production by octopus and rat brain slices after incubation with these same amino acids. The amount of CO_2 formed from glutamine and glutamate by rat brain slices was several times greater than that formed by the octopus brain. Also, octopus brain slices did not convert U^{14}C GABA into $^{14}CO_2$ at all, in contrast to the rat brain. However, alanine gave comparable values in all three tissues. Therefore, in spite of the lower temperature of incubation and slower rate of entry of alanine into the octopus brain in vitro, as shown in the previous section, alanine seemed to be oxidised to CO_2 to a greater extent, comparatively, than in the rat brain. In contrast, GABA is oxidised in the rat brain through the GABA shunt pathway (Roberts, 1962: see also figure 12, Chapter 2, page 82). GABA transaminase converts

TABLE 46

COUNTS INCORPORATED INTO CO_2 BY RAT AND OCTOPUS BRAIN SLICES INCUBATED WITH $U^{14}C$ AMINO ACIDS

Disint/min/ 10^6 cts substrate / mg protein

$U^{14}C$ amino acid	Optic lobe	Vertical lobe	Rat brain
glutamate	457±64 (9)	114±15 (6)	3700±205 (6)
glutamine	950±290(4)	986±185(4)	1435 (3)
alanine	218±104(4)	84±30 (4)	370±36 (10)
GABA	n.d.	n.d.	1470 (3)

Incubation of rat brain slices was carried out as described in Chapter 2, and footnote of Table 43. Results expressed as means ± S.E.M.s of numbers of experiments in brackets, where possible. n.d. = not detected.

GABA and α ketoglutarate to glutamate and succinic semialdehyde; the latter is oxidised by its specific dehydrogenase to succinate which is converted to CO_2 through the citric acid cycle. Therefore it would seem that the inability of the octopus brain to oxidise GABA may be due to the absence of GABA transaminase in the octopus brain. Roberts and Simonsen (1962) have already shown that GAD is virtually absent in octopus brain, and this is confirmed in the following section.

ENZYME LEVELS IN RAT AND OCTOPUS BRAIN.

The enzyme assays were carried out on tissue homogenates as described in Chapter 2. It must be noted that octopus brain homogenates were made from frozen solid, once-thawed tissue, and rat brain homogenates from fresh tissue. All enzymes were assayed with and without pretreatment with Triton X-100 detergent as described in Chapter 2.

It was found that different enzymes were measured best at different tissue concentrations which were first found experimentally. The most sensitive, reproducible and convenient methods were found to be the spectrophotometric methods, since they required the least tissue, and readings at several time intervals could be taken on a single sample. These were GDH, AAT and Ala-AT. Although the GAD assay was a sensitive radiochemical method, greater tissue quantities were required since this was found to be the least active enzyme in rat and octopus brain. The assay of GNS was a relatively crude chemical method.

GAD: glutamate decarboxylase.

Figure 25 shows the time course of $^{14}CO_2$ evolution from 1 ^{14}C glutamate at three different tissue concentrations

TABLE 47

ENZYME ACTIVITIES IN RAT AND OCTOPUS BRAIN

/umoles substrate converted/ mg protein/hr

Enzyme	Treatment with 0.1% Triton	OPTIC LOBE	VERTICAL LOBE	RAT BRAIN
GDH	+	1.82 (3)	0.77 (3)	5.10 (3)
	-	0.13 (7)	0.15 (6)	0.62 (5)
	stimulation	14 times	5.2 times	8.0 times
AAT	+	7.48 (3)	1.54 (3)	31.2 (3)
	-	2.02 (6)	0.95 (5)	10.7 (5)
	stimulation	3.7 times	1.6 times	2.9 times
Ala-AT	+	0.41 (3)	0.20 (3)	3.17 (3)
	-	0.45 (3)	0.31 (3)	0.39 (3)
	stimulation	0.9 times	0.7 times	8.1 times
GAD	+	0.04 (3)	0.03 (3)	0.19 (3)
	-	n.m.	n.m.	0.04 (3)
	stimulation			4.8 times
GNS	-	0.60 (4)	0.58 (3)	1.43 (3)

Enzyme assays were performed as described in Chapter 2 on homogenates of rat and octopus brain with and without pretreatment with 0.1% Triton X 100. All determinations at 25°C except for rat brain GAD and GNS, performed at 37°. Results expressed as the means of numbers of experiments in brackets, the range being $\leq$ 15%. n.m.= not measurable. Also shown are values for stimulation by Triton, i.e. activity with treatment expressed as a fraction of activity without treatment.

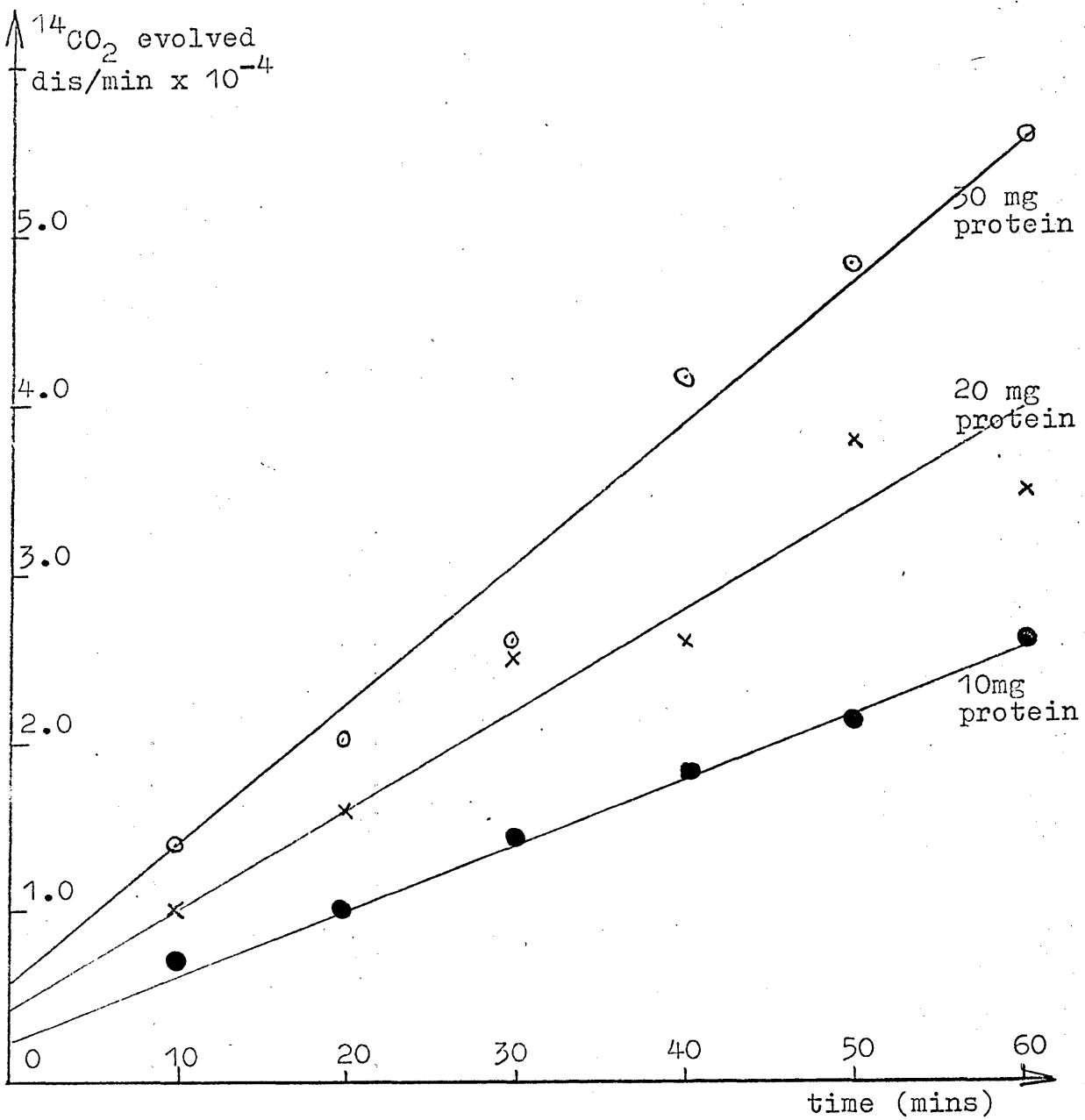


FIGURE 25

GAD ASSAY IN RAT BRAIN

CO_2 evolution by rat brain homogenate incubated with 1-^{14}C glutamic acid: incubation medium as described in Chapter 2. Curves shown for three tissue concentrations.

of rat brain homogenate. The rates were linear with time, and the graph represents one experiment. The result was calculated as the mean of three such runs. Balázs et al. (1966) found the activity to be proportional to tissue concentration up to 250 mg wet weight per vessel for the rat brain. Figure 25 shows that linear rates were obtained for up to 300 mg tissue, but higher concentrations were not tried. Addition of semicarbazide practically abolished $^{14}CO_2$ formation. GAD levels were very low in the rat brain, (Table 47) and Triton stimulated the activity nearly five times. In the case of the octopus brain, GAD was barely detectable, activity being so low that it could not be accurately measured. Addition of Triton stimulated this activity to the unstimulated level found in the rat brain.

GDH: glutamate dehydrogenase.

Figure 26 shows the fall in extinction at 340 mμ with time, at three different tissue concentrations of rat brain homogenate. The rate was linear with time, and was always found to be so, unless the concentration of tissue in the cuvette was too high. In the case of GDH assay, the most suitable range of tissue concentration was found to be between 0.2 to 1.0 mg protein per cuvette. Figure 27 shows a plot of the total substrate a ketogluterate converted against the amount of tissue in the sample, for rat and octopus brain. The activity is directly proportional to tissue concentration in all three tissues, but the slopes of the octopus curves are lower than that for the rat brain. (Figure 27). Table 47 shows that the activity of GDH is stimulated 8 fold by Triton in rat brain, 5 fold in vertical

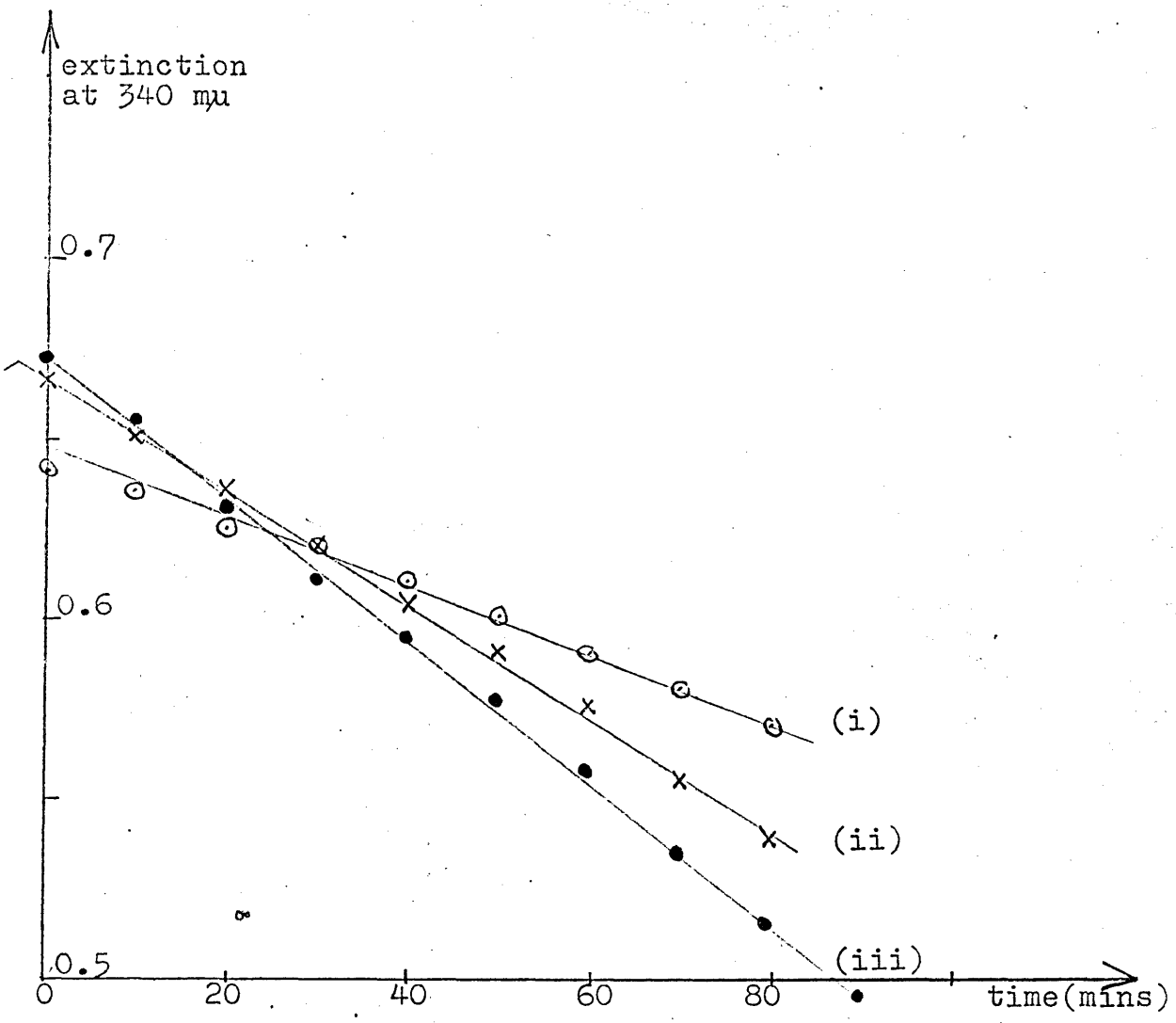


FIGURE 26

GDH ASSAY IN RAT BRAIN

Fall in extinction with time of NADH , due to oxidation by rat brain homogenate of α ketoglutarate. Incubation conditions are as described in Chapter 2. (i) , (ii) and (iii) represent 1, 2 and 3 volumes of homogenate.

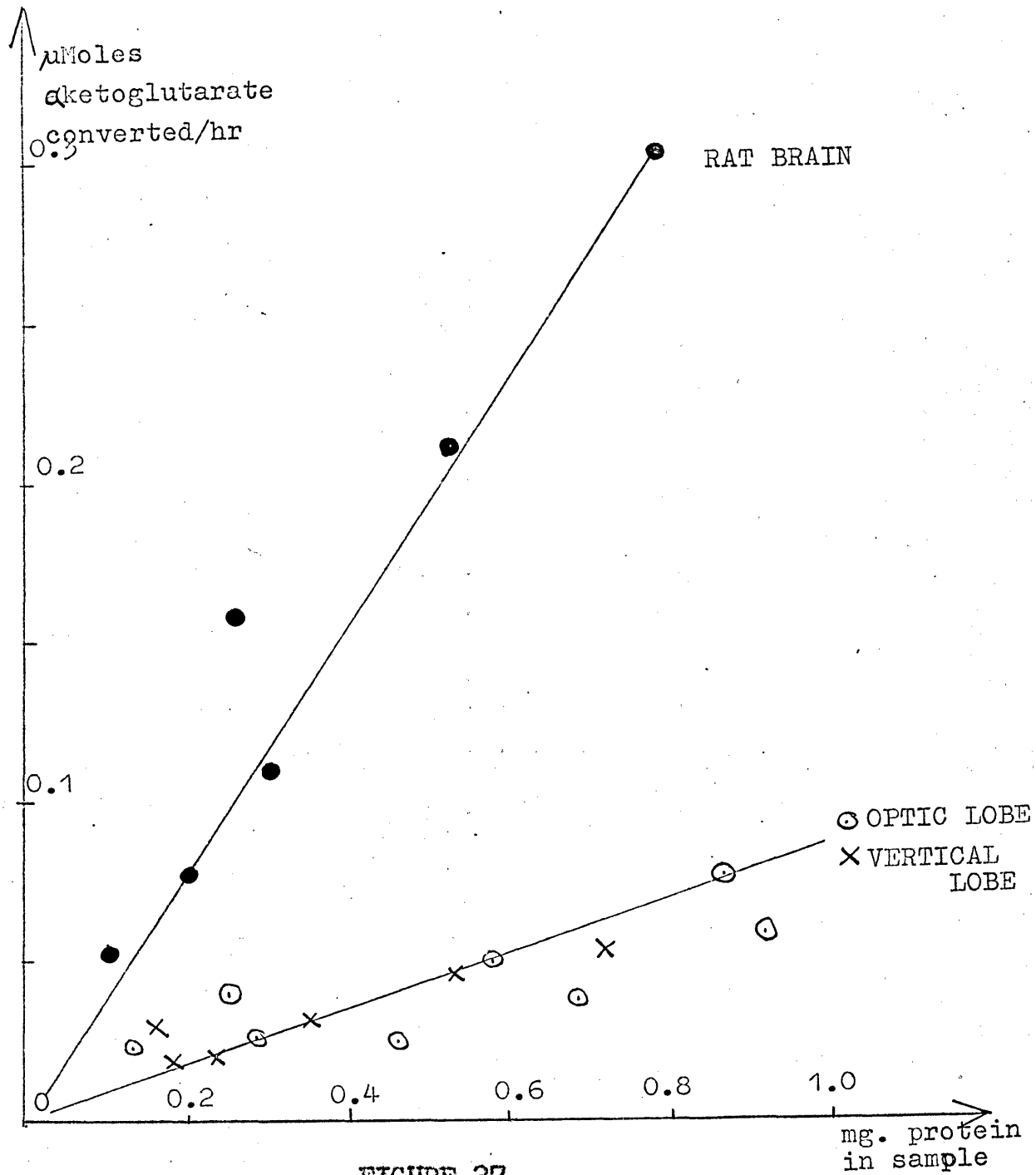


FIGURE 27

GDH ASSAY IN RAT AND OCTOPUS BRAIN

Plot of reduction of α ketoglutarate , against tissue concentration of rat and octopus brain homogenate. Assay conditions were as described in Chapter 2.

lobe and 12 fold in optic lobe homogenates.

AAT: aspertate aminotransferase.

This was the most active enzyme measured, in all three tissue homogenates. The most suitable concentrations of tissue to use ranged from 0.02 to 0.1mg protein per cuvette. Figure 28 shows that like GDH, the amount of substrate transformed was proportional to the amount of tissue in the sample, the slope of the curves for the octopus brain being lower than that of the rat brain. Again, from Table 47 it can be seen that Triton activated the enzyme threefold in octopus and in rat brain.

ALA-AT: alanine transaminase.

This enzyme was less active than GDH or AAT. As in the case of GDH and AAT, the fall in extinction at 340 mu was linear with time and with tissue concentrations up to 3 mg protein per cuvette. (This is not shown graphically). Triton stimulated ala-AT activity in rat brain homogenate tenfold, but that of both optic and vertical lobe was unchanged. This was the only case found where octopus and rat brain differed in this way.

GNS: glutamine synthetase.

This method was the least sensitive and accurate. The blank value was high (0.15 optical density units on the spectrophotometer) and small readings above the blank were obtained, so values were erratic; large amounts of tissue could not be used, as in octopus brain homogenates, substrate conversion was not proportional to tissue concentration above 1.5mg protein per sample, in contrast to the rat, where it was linear up to 4 mg protein per sample. This is shown in Figures 29 and 30. The activity in the octopus brain was calculated on the

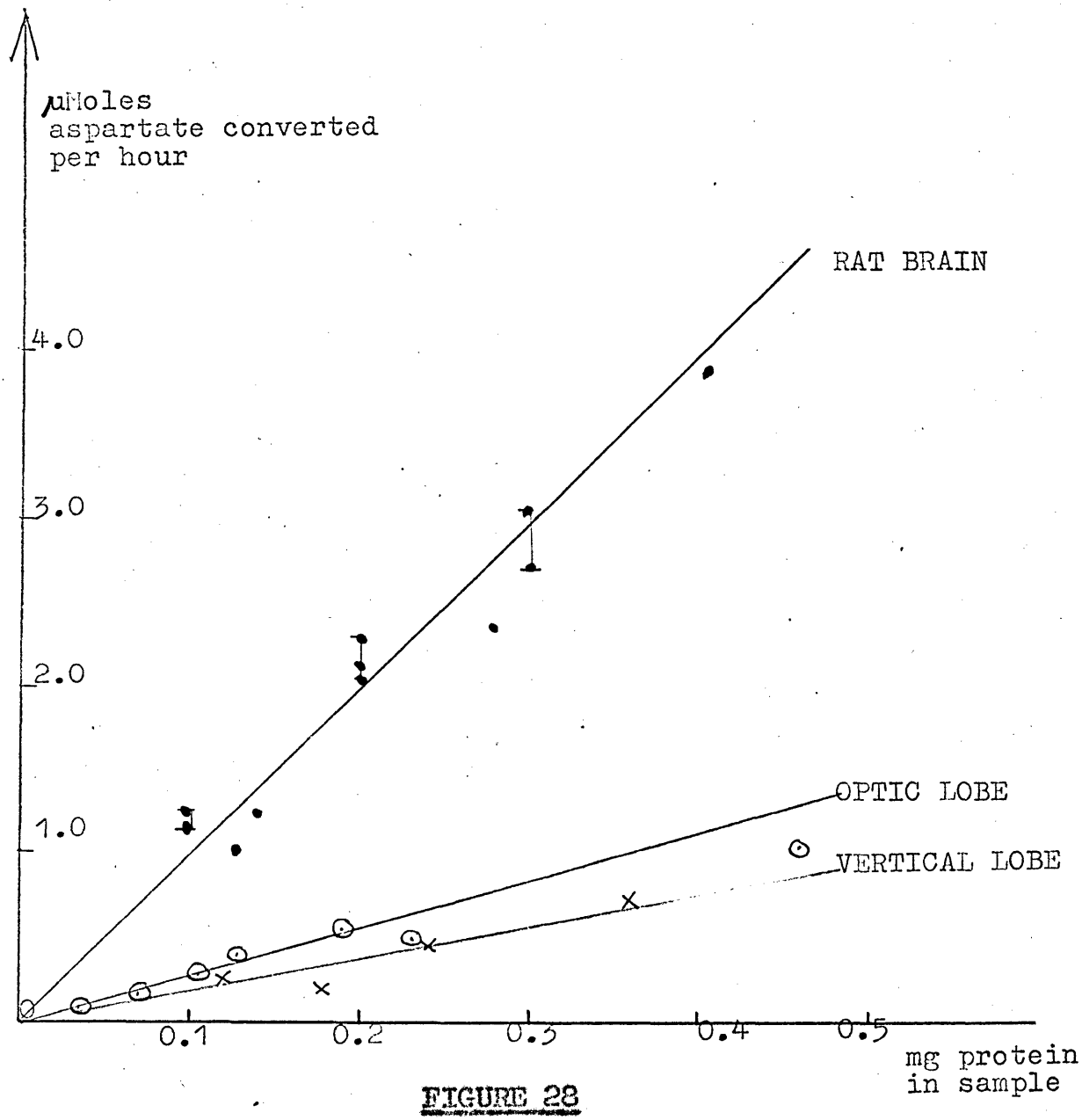


FIGURE 28

AAT ASSAY IN RAT AND OCTOPUS BRAIN

Plot of substrate conversion against tissue concentration of rat and octopus brain homogenates. Assay conditions were as described in Chapter 2.

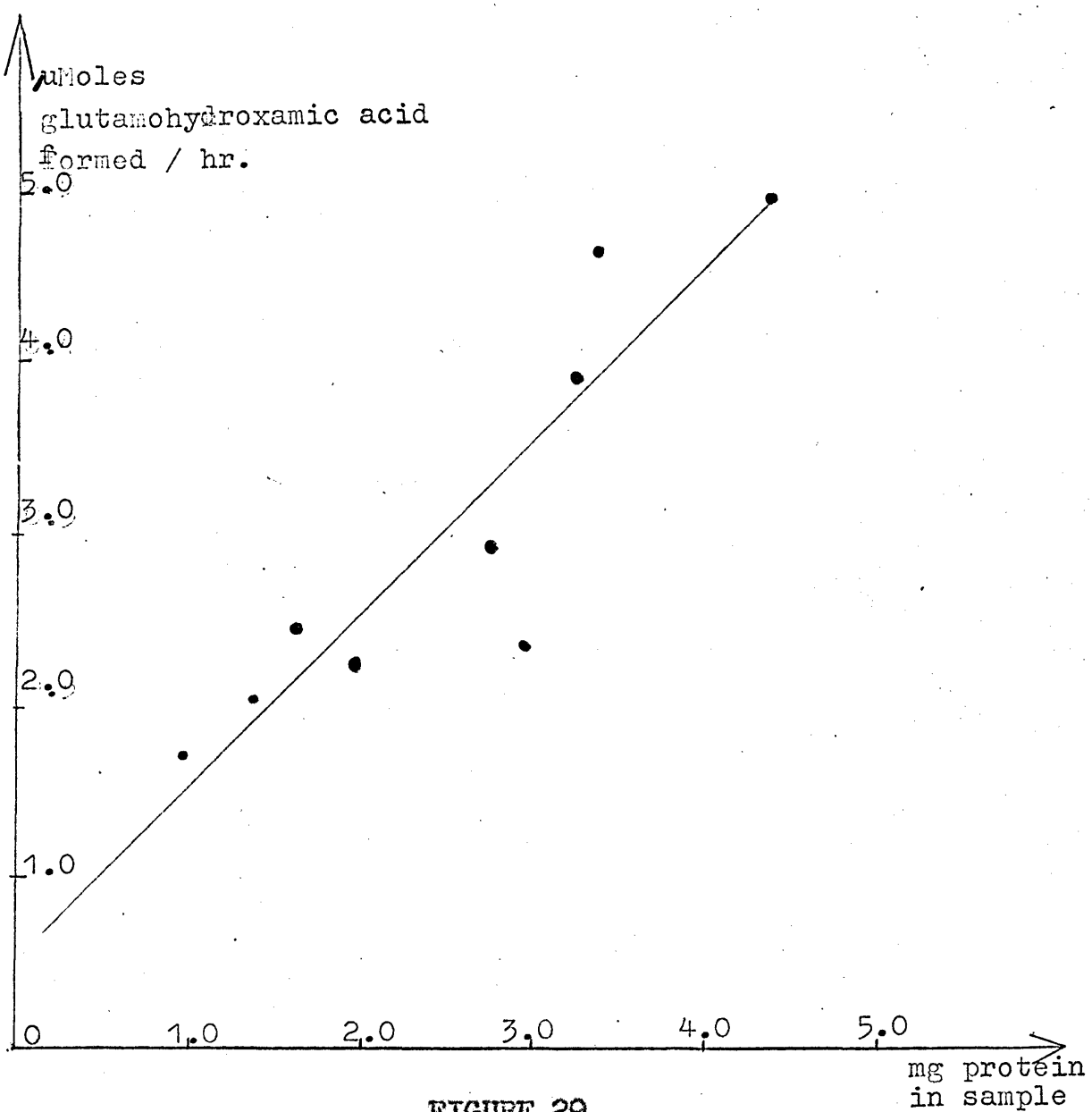


FIGURE 29

GMS ASSAY IN RAT BRAIN

Plot of substrate transformed against concentration of rat brain homogenate. Assay conditions were as described in Chapter 2.

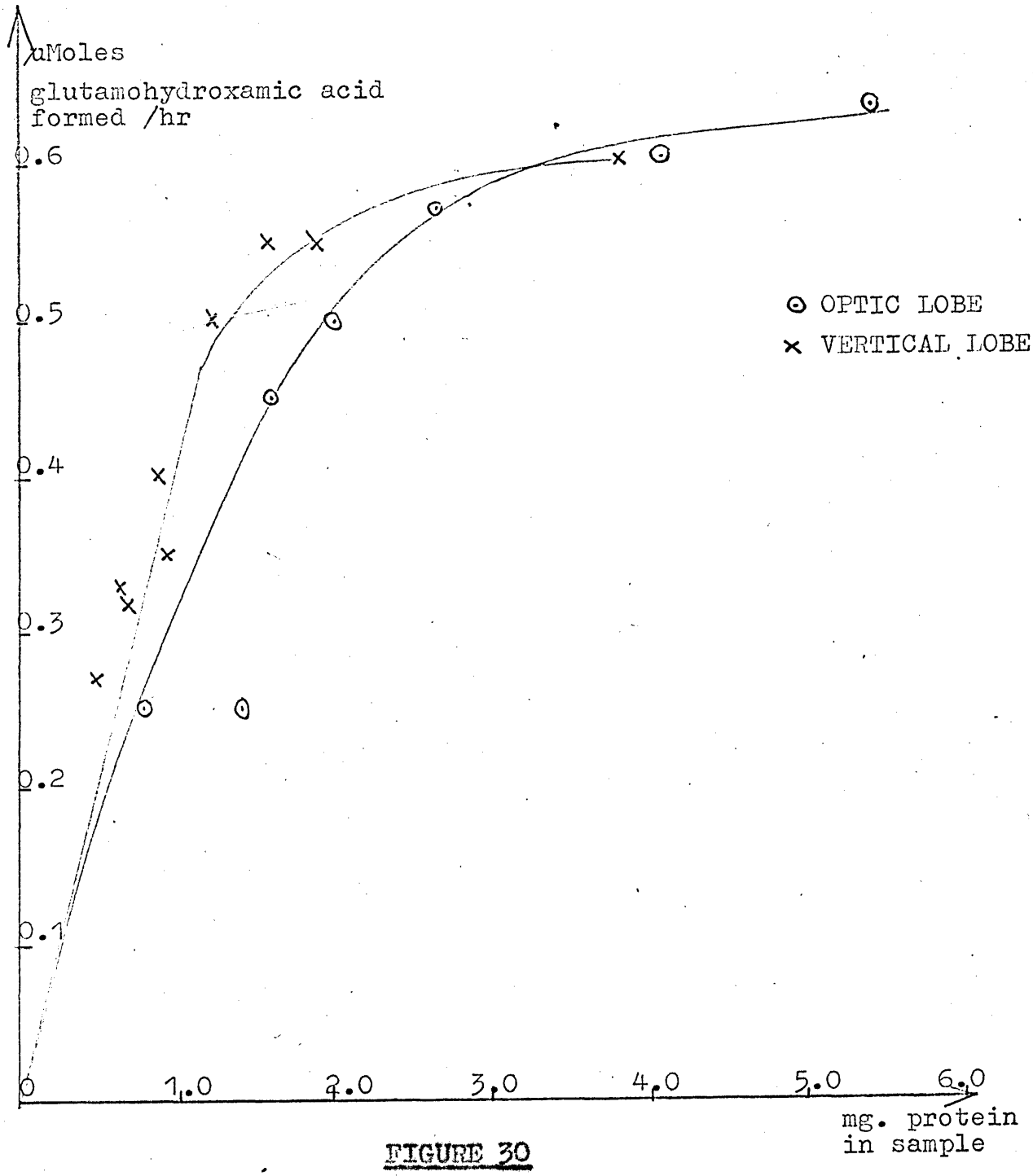


FIGURE 30

GMS ASSAY IN OCTOPUS BRAIN

Plot of substrate converted against concentration of optic and vertical lobe homogenate in sample. Assay conditions were as described in Chapter 2.

basis of the initial linear rates: presumably activity fell off at high tissue concentrations because of the presence of ATPase in the tissue, as suggested by Berl, (1966). GNS was not measured in the presence of Triton.

Activities of the enzymes.

Table 47 shows the activities of all the enzymes measured with and without Triton pretreatment, in the rat and octopus brain, and the percentage activation of the enzymes by Triton. Generally, values were 5 times higher in the rat brain compared to the octopus, with the exception of Triton-unstimulated values of Ala-AT, which were of the same order in both animals. This was yet another indication of the relative quantitative significance of the metabolism of alanine in octopus brain as noted in the previous chapter. GAD in contrast had a very low activity in the octopus brain, in agreement with Roberts and Simonsen (1962). The low GAD activity, together with the absence of CO_2 production from GABA shows that very little formation and metabolism of GABA occurs at all in the octopus brain, though it is of quantitative importance in the rat brain, (Salganicoff & De Robertis, 1965).

The values of the enzyme activities obtained here, for Triton-treated rat brain homogenates are in the same order as those obtained by Balázs et al. (1966), who studied the activities and subcellular localisation of the enzymes concerned with the metabolism of amino acids in brain, especially of GABA. Salganicoff & De Robertis (1965), in a similar investigation, found that GDH was mitochondrial, that AAT and Ala-AT were localised partly in the mitochondria as well as the soluble fraction, and GAD in the nerve-ending particles of the rat brain. They suggested

that Triton treatment released the enzymes located inside these particles, apparently activating them. On this basis, it is possible that Triton did not stimulate Ala-AT because unlike the rat enzyme, it is located entirely in the soluble fraction. However, Table 47 shows that GDH and AAT were stimulated by Triton to the same extent in both brains, implying that their subcellular distribution may be similar in both animals. Salganicoff & De Robertis (1965) also found rat brain GAD was located in the nerve-ending particles. The fact that Triton stimulates the very low GAD activity of the octopus brain, to small but measurable values indicates that octopus brain GAD is also particle-bound.

THE METABOLISM OF GLUTAMATE IN THE OCTOPUS BRAIN IN VIVO.

Since Berl et al. (1961) obtained interesting results by injecting U^{14}C glutamate intracisternally into the rat brain, (these are discussed in Chapter 1), the same experiment was repeated with the octopus. 30 µCuries of high specific activity U^{14}C glutamic acid in 0.05 ml of 0.9% saline was injected into the brain cavity of the octopus using the technique described in Chapter 2. The animals were killed 15 minutes, 30 minutes and one hour after injection and the optic and vertical lobes worked up as described in Chapter 2 for the determination of the specific activities of the amino acids, using the automated amino acid analyser.

The results of the experiments were very interesting. Table 48 shows the specific activities of aspartate, alanine and glutamine at different times, relative to a value of 1000 for glutamate, in optic and vertical lobe,

as well as the data for the rat of Berl et al. (1961). Figures 31 and 32 show the data for the octopus in graphical form, for the sake of clarity. Actual specific activities varied widely for each brain, depending on the time of killing and the directness of the injection. All the results were therefore calculated on the basis of the specific activity of glutamate being equal to 1000 dpm/n /umole. Other non-amino acid products, such as lactate, pyruvate and glucose, were not measured.

The graphs show that in optic and vertical lobe, the specific activities of glutamine, aspartate and alanine were lower than glutamate at 15 and 30 minutes, but rose after one hour. That of glutamine rose sharply, up to four times that of glutamate, and those of alanine and aspartate to levels lower than glutamate. Berl et al. (1961) likewise found the specific activity of glutamine in the rat brain was four times that of glutamate, but only 3 minutes after the injection of U^{14}C glutamate. The effect was attributed to the presence of two pools of glutamate in the brain, not in ready equilibrium with each other; injected glutamate mingled with the smaller pool, in which glutamine was synthesised from glutamate. Extraction thus diluted the glutamate to an apparent specific activity lower than the glutamine, though the latter was in fact formed <u>in situ</u> from a higher specific activity glutamate. The effect fell off with time as exchange occurred between the two glutamate pools.

It is possible to explain the longer time for the effect to manifest itself in the octopus if one remembers the overall slower entry of amino acids into the octopus brain, and the lower body temperature of the octopus.

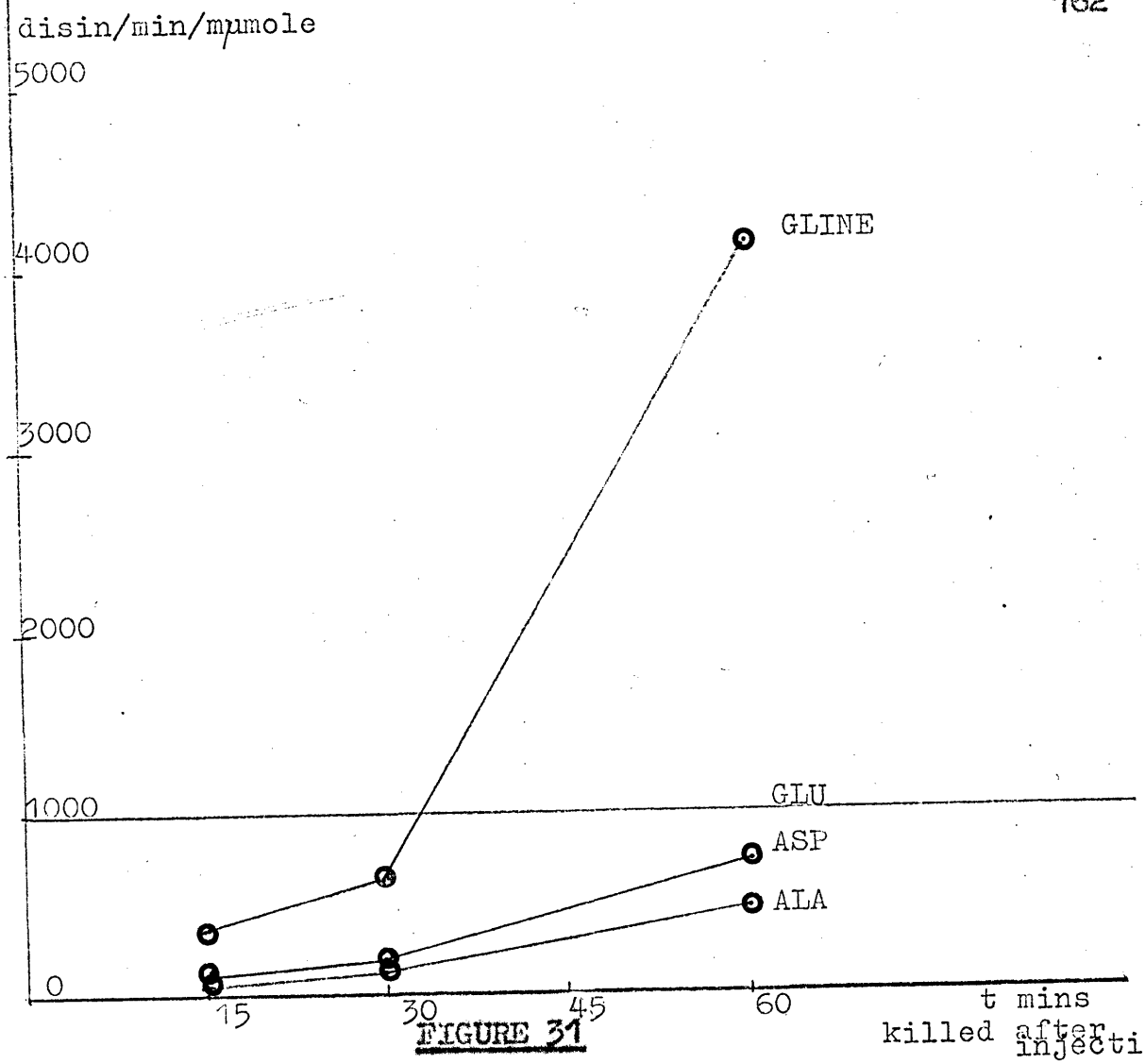


FIGURE 31

GRAPH OF SPECIFIC ACTIVITIES OF FREE AMINO ACIDS IN OPTIC LOBE OF OCTOPUS AFTER INJECTION OF U^{14}C GLUTAMATE IN VIVO, AFTER DIFFERENT TIMES.

For numbers of determinations in each point, and range, see Table 48

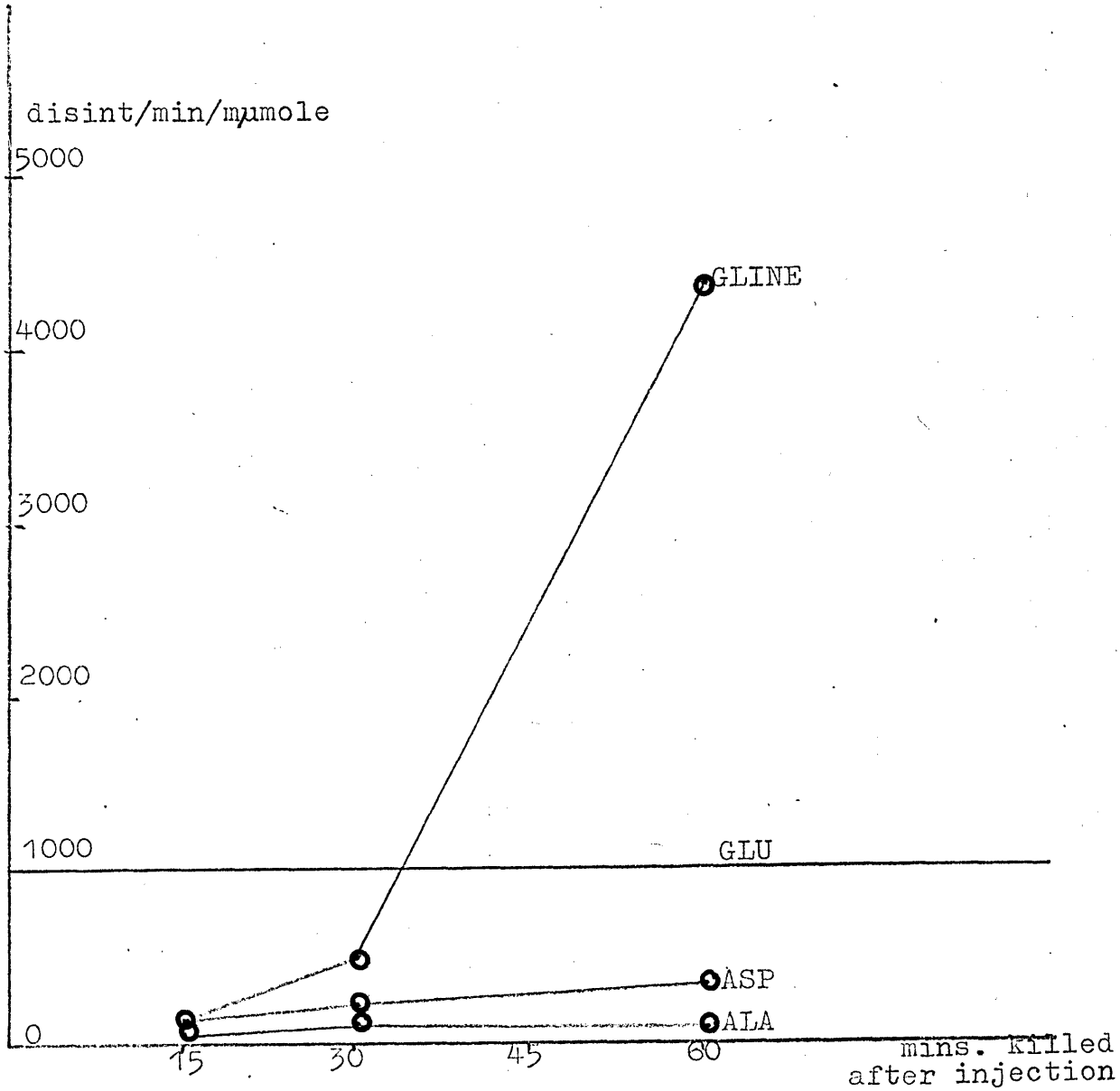


FIGURE 32

SPECIFIC ACTIVITIES OF FREE AMINO ACIDS IN OCTOPUS VERTICAL LOBE , AT DIFFERENT TIMES AFTER IN VIVO INJECTION OF $U^{14}C$ GLUTAMATE.

For numbers of experiments, see Table 48

If killing was delayed for several hours, presumably the specific activity of glutamine relative to glutamate would fall as in the rat, but this was not tested, owing to a shortage of animals. However, this finding does point to a two-compartment system for glutamate metabolism in the octopus brain, like the rat brain.

TABLE 48

SPECIFIC ACTIVITIES OF FREE AMINO ACIDS AFTER INJECTION OF $U^{14}C$ GLUTAMATE INTO RAT AND OCTOPUS BRAIN IN VIVO

Disint/min / m umole

Time after injection	15'	30'	60'
OPTIC LOBE			
GLU	1000	1000	1000
ASP	48 (3)	124±26 (5)	719 (2)
GLINE	372(3)	620±170(5)	4142±450(4)
ALA	49 (2)	184±59 (3)	349 (3)
GABA	70 (2)	509 (3)	-
VERTICAL LOBE			
GLU	1000	1000	1000
ASP	10 (2)	220 (2)	236 (2)
GLINE	113(2)	500 (2)	4370±1000(5)
ALA	78 (2)	110 (2)	40 (2)
GABA	n.d.	n.d.	n.d.
RAT BRAIN *			
GLU	1000	1000	1000
GLINE	5000 (2)	3500 (2)	2600 (2)

* Data from Berl et al. (1961)

$U^{14}C$ glutamic acid was injected directly into the brain and animals killed at different times for determination of the specific activities of the amino acids, as described in Chapter 2. Results are the means of the numbers of experiments in brackets, and are expressed ± S.E.M.s where n exceeds 3. n.d. = not detected.

CHAPTER 5
DISCUSSION

At the moment great advances are taking place in the metabolic area of mammalian brain biochemistry, though this started 30 years ago. Basic metabolic pathways and enzymes have been known since the discoveries of Embden and Meyerhof, and Krebs, but much more knowledge of the distribution of the metabolism within the different types of cells in the brain and the subcellular organelles is essential to understand the working of the brain in every aspect. The connection between the role of the amino acids (particularly those linked with the citric acid cycle which have featured in the foregoing chapters) in carbohydrate and protein metabolism and the excitatory properties of the nerve cell is still unknown: they seem to be involved in the energy metabolism of the nerve cell yet also possibly function as transmitters, and the two properties are unlikely to be completely independent of each other.

There are good reasons for investigating a few of the biochemical properties of the octopus brain. Remarkable advances have been made in the elucidation of the design of the brain by the use of the octopus, which has led to the beginning of the understanding of the actual working and organisation of the mammalian brain which, though far more complex, still possesses (so it is thought at the moment) the same underlying units of organisation, only they are themselves organised in a more complex manner. Similarly it is reasonable to believe that the study of the biochemistry of the octopus brain will unravel properties characteristic of all nervous tissue, which have been elusive in the mammalian brain on account of its greater

complexity. Also, comparative studies in several aspects of biochemistry have revealed that basic, essential properties, such as the glycolytic pathway, the citric acid and urea cycles are common to most of the species studied. In fact, only a very small number of species have actually been studied and tend to be studied, but they do represent a wide range in the animal kingdom.

Differences between the optic and vertical lobe.

In this work, only the aspect of glucose and amino acid metabolism was investigated. It was hoped that the comparison of the metabolism of two such anatomically and functionally different lobes would show fundemental biochemical differences reflecting the functional differences. In fact, only a very few isolated instances of this kind occurred which are as yet impossible to interpret due to the shortage of data, but an attempt will be made to summarise these differences and speculate on their possible meaning.

1. The lower amino acid content of the vertical lobe compared with the optic lobe on a protein basis, with the exception of alanine (Table 7). The relatively higher level of alanine in the vertical lobe may be due to its higher content in a particular type of cell in the vertical lobe rather than all the cells, and this question will only be answered by the separation of the different cell types, perhaps according to their size, in order to study their individual properties and metabolism.

2. The lowering of the oxygen uptake of vertical lobe slices by the addition of glutamate to the incubation medium, even in the presence of glucose. This is a peculiar property and not readily explicable: if the presence of glutamate caused a net breakdown of ATP as in the rat brain slice

(Woodman & McIlwain, 1961), one might expect a rise in respiration. In fact it is not clear why this does not even occur in the rat brain, but the situation is complex and is discussed by Bradford, (1968).

3. Incubation of octopus brain slices with U^{14}C glucose yields an aspartate of specific activity 4 times greater in the vertical lobe than the optic lobe; the level of glutamate is reduced compared to that before incubation in the vertical but not in the optic lobe. Incubation with U^{14}C glutamate yields an alanine of specific activity eight times greater in the vertical lobe compared with the optic lobe, and a GABA level in the vertical lobe twice that in the optic lobe. It is possible that such differences are due to compartmentation differences between the two. The specific activity of an amino acid as measured after incubating a slice with a radioactive substrate will depend not only on the metabolic pathways available for the transformations involved, but, in the case of the octopus where diffusion and metabolic rates are slow, also on the relative speed with which the substrate may diffuse into the compartment (cell type, or subcellular organelle) where that transformation occurs, because the enzymes are located there. Work by Balázs et al. (1966) and Salganicoff & De Robertis (1965), among others, has shown that enzymes catalysing a particular set of reactions tend to be localised subcellularly. Rose (1968 b) also found that levels of the same enzymes in the neurons and the glia were not identical. Therefore differences between the relative specific activities of the products in the optic and vertical lobes must to some extent be due to the different cytology of the lobes. In fact, in view of their dissimilarity, it is

surprising that not more differences were found.

4. The incubation of octopus brain slices with glutamate and non-labelled glucose together yields an aspartate whose specific activity is twice in the vertical lobe the value in the optic lobe; the specific activity of vertical lobe glutamine is half the value of the optic lobe's. Also after incubation, the level of alanine in the optic lobe is twice as high as in the vertical lobe, but the latter contained GABA levels comparable to the rat under the same conditions, while it was not even detected in the optic lobe.

5. GDH, AAT, and Ala-AT activities were lower in the vertical lobe, generally 20 to 40% of the value in the optic lobe. With Triton X-100 treatment, vertical lobe enzymes were stimulated to a slightly greater extent than the optic lobe. It is possible that not all the small cells of the vertical lobe were disrupted, in spite of the vigorous shearing action of the homogenisation, because the clearance was 0.005" or 0.008 cm, whereas most of the cells in the vertical lobe are under 5 /u in diameter. This would also explain the greater stimulating effect of the Triton.

Differences between the octopus and the rat brain.

The differences found between the octopus and rat brain may similarly be summarised and commented upon.

1. The smaller size of the cells in the octopus brain, and the relative paucity of glial cells, as found by circumstantial calculation at the beginning of Chapter 3. Though glial cells are present in the octopus brain, they are not distributed as widely as in the mammalian brain, but rather in certain areas (Horridge, 1965). Cajal (1917) pointed out that there were no glial types in the octopus optic lobe corresponding to mammalian microglia, but only to the astrocytes. Perhaps these are the cells

referred to by Young (1962) as multipolar cells with many dendrites in the optic lobe, and they may have been included in his estimation of the numbers of nerve cells (Young, 1963b).
2. The higher levels of aspartate and alanine in the octopus brain, and the lower level of GABA. Tallan (1962) pointed out that in general aspartate and alanine levels are high in the invertebrate brain, contrary to the mammalian brain, where glutamate has the highest concentration of any amino acid. Also, the absence of GABA seems to be characteristic of molluscs. Huggins, Kerkut and Rick (1967) found that snail and mussel brains incubated for 2 hours with radioactive glutamate failed to produce GABA, contrary to mouse and cockroach brain. Also, no GABA was formed from U^{14}C glucose by the snail brain *in vitro* nor by eledone, unlike the locust and the rat brain (see Bradford et al. 1969).
3. The lower rate of metabolic transformations and of respiration in the octopus brain (at 25°) compared to the rat brain (37°). This was to be expected, and was also found in the locust and snail brain, (Bradford et al. 1969). Apart from this, there was the slower metabolism of glutamate in the octopus brain *in vivo*, and the Waelsch effect did not manifest itself in the octopus brain nearly as rapidly as in the rat. Earlier in this chapter, the relative rates of diffusion of substrate to,and of products from the compartment in which a certain metabolic transformation takes place was given as a possible reason for the differences between patterns of specific activities in the optic and vertical lobe. The same is true when comparing the rat with the octopus brain. Recalling the results of incubation of radioactive glucose with rat or octopus brain slices, the specific activities of the rat brain amino acids were all

in the same order, and there was more variation in the values of those in the octopus brain. Presumably in the rat, intercompartmental diffusion was not a limiting factor, and after two hours, the specific activity pattern was a reflection of the enzymes present. In contrast in the octopus, the pattern was the result determined partly by intercompartmental diffusion, and it would be impossible to determine the extent of this effect. The incubation of rat and octopus brain for different times would clarify this, as well as the use of homogenates, and separated cells.

4. The higher proportion of $U^{14}C$ glucose converted into CO_2 by the rat brain, more than twice that in the octopus brain, and the much greater conversion into alanine of glucose by the octopus brain, more than 20 times that by the rat brain. The snail and the locust are also like the octopus in this respect, (Bradford et al. 1969).

5. The reduction of $^{14}CO_2$ formation by octopus brain from $U^{14}C$ glutamate and alanine, caused by the addition of non-labelled glucose, in contrast to the rat, where a slight increase in $^{14}CO_2$ formation occurs. It is strange that in contrast to the octopus, addition of glucose doubled the formation of radioactive CO_2 from $U^{14}C$ glutamate by snail and locust brain <u>in vitro</u> (Bradford et al. 1969). This is the only case where the snail differs from the octopus.

6. The effect of glucose in reducing aspartate formation from $U^{14}C$ glutamate in the rat cortex was not shown by octopus brain slices, (see Table 23, Chapter 3). The same is true in the snail brain, but the locust brain behaves like the rat in this respect, (see Bradford et al., 1969).

7. $^{14}CO_2$ was not formed by octopus brain slices from radioactive GABA, in contrast to the rat brain. This was interpreted as being due to the absence of the enzyme SSADH in the octopus brain, but this enzyme was not tested.
8. The uptake of some amino acids by octopus brain in vitro against a concentration gradient did not occur for times up to four hours, in contrast to the rat, where uptake was found after one hour. On the other hand, Table 5 in Chapter 1 shows that diffusion did occur from the vertical lobe cavity into the optic lobe.
9. Enzyme activities in the octopus brain were generally about one fifth those of the rat brain, with the exception of Triton-unstimulated Ala-AT. Triton did not stimulate octopus brain Ala-AT, and this was interpreted as the probability that this enzyme was entirely located in the soluble fraction of the cell. GAD levels were barely detectable in the octopus brain. This property has been observed previously (Roberts and Simonsen, 1962). It is interesting to consider the activities of some of the enzymes also in the snail and the locust here: though snail brain, unstimulated by Triton, showed GDH and AAT activities comparable to the rat and much higher than the octopus brain, and an Ala-AT activity 5 times that in the octopus and the rat brain, GAD levels were barely measurable. The activity of GAD in the locust brain was measurable however, being half that of the rat. (Table 49).

So it would seem that generally the aspects of the metabolism of alanine and GABA in the octopus that differ from the rat brain, are also shown by the snail brain, another mollusc, and not by schistocerca (the insect, locust).

TABLE 49

ENZYME ACTIVITIES IN HELIX, SCHISTOCERCA, ELEDONE AND RAT BRAIN

/uMoles/ substrate converted /mg protein/ hr.

	Helix*	Eledone		Schistocerca*	Rat
		Optic lobe	Vertical lobe		
GDH	0.95 (5)	0.13 (7)	0.15 (6)	1.34 (5)	0.62 (5)
AAT	4.82 (2)	2.02 (6)	0.95 (5)	8.49 (3)	10.66 (5)
Ala-AT	2.27 (3)	0.45 (3)	0.31 (3)	5.65 (3)	0.39 (3)
GAD	traces	traces	traces	0.02 (3)	0.04 (3)
GMS	0.12 (3)	0.60 (4)	0.58 (3)	0.85 (6)	1.43 (3)

* Values from Bradford et al. (1969)

Enzyme activities were measured in homogenates without pretreatment with Triton X-100, as described in Chapter 2. Results are the means of the numbers of experiments in brackets.

Complications arising in interpreting data.

The difficulties in comparing results from incubating the brains of two different animals, not even of the same class, were discussed in Chapter 1, and also earlier in this Chapter, with regard to compartmentation. Ions were shown to affect metabolic patterns. Since two different incubation media were used for the two animals, it may have been useful, but perhaps misleading, to have compared the metabolic patterns using different media. However, the saline used for incubation of the octopus brain (Hoyle, 1953) does resemble sea water and octopus plasma in composition (Robertson, 1964), in having a high sodium and chloride content, and a low calcium, potassium magnesium and sulphate content.

In view of the effect that glutamate has on the mammalian brain slice *in vitro* , causing swelling and potassium uptake (Bourke and Tower, 1966), and the depletion of nucleotide phosphates (Woodman & McIlwain, 1961), it may

have been better to have used glutamate at a substrate concentration of less than 1 mM , at a correspondingly higher specific activity. However, as is often the case, the procedures of other workers were automatically followed at the time.

It is also worthwhile to compare the accessibility of ions and nutrients to the brains in situ. Briefly, the mammalian brain is well vascularised, and though there is no barrier to the exchange of amino acids between the brain and the blood, their net entry is restricted by the blood brain barrier: there is however no restriction to the entry of glucose, under normal conditions. The optic lobes in the octopus are bathed in fluid which is analogous to the cerebrospinal fluid in the rat brain in being a low protein filtrate of plasma. The vertical lobe is bathed in the same fluid, also containing gelatinous connective tissue. The octopus brain also has an abundant blood supply, and capillary networks connect the venous and arterial systems, as in the mammalian brain (Horridge, 1965). The octopus has a blood sugar level of 20 mg % as against 180 mg % in the mammal. It would be interesting to know whether glucose is utilised by total conversion into CO_2 and lactate like the rat brain, but no studies on arteriovenous differences seem to have been reported for the octopus brain. Though the results in Chapter 2 have shown that radioactivity injected into the vertical lobe does pass quickly into the optic lobes (Table 5), presumably through the optic commissure, it is not known how far the entry of substances into the plasma filtrate from the blood is restricted in the octopus: Robertson (1964) states that the octopus skin does control the passage of solutes through it, so it is not unlikely that a blood-brain barrier of a sort exists in the octopus.

Summary of metabolic differences between the rat and octopus brain.

As far as these experiments show, there is no evidence to suggest that there are metabolic pathways for the interconversions of the amino acids in the octopus brain which are different from the rat brain. However, these studies have been of a limited nature. Goddard and Martin (1966) have reviewed the extensive studies on carbohydrate metabolism in molluscs. Other sugars beside glucose are utilised, e.g. fructose and galactose. The presence of enzymes of the glycolytic pathway, the citric acid cycle, and those catalysing interconversions between the two (i.e. PEP carboxykinase, pyruvate kinase , and the malic enzyme) has been demonstrated in a wide range of molluscs. However, the tissues examined tend to be other than the brain, such as the hepatopancreas. The only accounts of cephalopod brain enzymes to be found were by Drukker and Schadé, (1964) who demonstrated histochemically the presence of GDH and isocitric dehydrogenase and some esterases in octopus optic lobe, and by Pascoe and Schadé (1967), who measured succinate dehydrogenase in the optic lobe of Sepia.

The most important implication of the work is that concerning the metabolism of alanine in both the octopus and the rat brain. To recapitulate the results of Chapter 3, the addition of non-labelled glucose to U ^{14}C alanine as substrate in vitro did not cause a dilution of the specific activities of the other amino acids in octopus brain slices, contrary to the rat cortex, (Table 31). This must surely indicate that alanine may enter the citric acid cycle by a route by-passing pyruvate and Acetyl CoA. The clue to a possible metabolic route may be obtained from the literature. Alanine does appear to have an unusual metabolism in molluscs: Stokes and Awapara (1965) showed that alanine

and succinate were the equimolar end-products of glucose metabolism in the clam, ATP being formed in the PEP carboxykinase reaction to oxaloacetate, leading to the formation of succinate backward through the citric acid cycle: thus half the pyruvate is converted to alanine, the other half to succinate, without any net oxidation or reduction, but with the formation of ATP. Lionet and Moyse (1963) pointed out that alanine can be made by the amination of propionate in some organisms, and the conversion of propionate into succinate is known to occur in molluscs (Goddard and Martin, 1966). Therefore it is theoretically possible for alanine to be converted to succinate via propionate, and it only remains for this pathway to be proved in the octopus.

The results of the incubations of rat brain slices with inhibitors may be understood if one examines Figures 10 and 12 (pages 72 and 82), as well as Table 41 (pages 142). The addition of fluoracetate as cosubstrate to alanine causes more of the alanine to enter the cycle by conversion to pyruvate or PEP, not via acetyl CoA, but by the malic enzyme, PEP carboxykinase or pyruvate carboxylase. The addition of malonate, which inhibits at the succinate step, will prevent the flux of label backwards through the cycle past that point, or , the labelling of glutamate will be inhibited, but that of aspartate will not be, but will even be a little enhanced. Table 41 shows this to be so. Compared with fluoracetate a alone, the specific activity of aspartate is higher when malonate is added to fluoracetate as inhibitor. It now remains for the effect of these inhibitors to be tested on the metabolism of alanine in the octopus brain <u>in vitro</u>, and differences found will certainly give an answer relating

to the direct conversion of alanine to succinate, without pyruvate.

One might speculate with regard to the uptake phenomenon and its significance. Since the amino acids taken up may have a physiological effect on the nerve cell membrane, causing energy-expensive repolarisation, perhaps uptake is a safety mechanism to move the substance to the inside of the cell membrane where it can do no harm. It is possible that the lack of this property in the octopus brain may be connected with the high levels of the amino acids in the animal as a whole, and whose presence is essential to the survival of all marine invertebrates.

Conclusions and future of the work

The citric acid cycle and the enzymes by which the amino acids are connected with the cycle and with each other occur in the octopus brain as well as the rat brain, and also in the snail and locust brain. There is a possibility that alanine is oxidised by an alternative route in the octopus brain, without transamination to pyruvate, which is converted to Acetyl CoA and citrate. The metabolism of GABA in the octopus brain, like the snail CNS , is quantitatively insignificant, but may be important where it occurs. Other pathways such as the fixing of CO_2, known to be of quantitative importance in the rat brain (Nicklas et al., 1969) have not been investigated in the octopus.

In Chapter 1, the evidence was discussed as to the identity of the compartments suggested by Berl et al. (1961). The question is, since Chapter 4 has shown that the Waelsch effect also occurs in the octopus brain, what is the nature of the compartments in the octopus brain. It remains to isolate the individual types of nerve cells and determine the specific activities of their free amino acids after intracranial injection of U^{14}C glutamate, in the same way

as Rose (1969) did with the rat. The method of Rose (1966) for the bulk separation of neurons and glia was attempted in the octopus brain (unpublished results), but it was not s successful on account of the small size and density of the cells, which all passed through the dense sucrose. Present work in the laboratory of Rose is in progress to separate cells of different sizes and surface properties by means of a column of glass beads. If this technique is successfully applied to the octopus brain, accompanied by thorough cytological studies, it will be possible to determine whether compartmentation is an intracellular phenomenon.

The future of the work therefore seems to lie in the more intensive investigation of certain problems, and in the use of more specialised techniques . A good example of the detail which will have to be used is the determination of the specific activities of every single carbon atom in the free amino acids (and other metabolites), as performed by Hicklas et al. (1969), because it is the only way to obtain really accurate information about the molecular mechanism of interconversions. The experiments described in the foregoing chapters have been relatively crude, and were carried out to obtain an outline of differences and general similarities between two very different animals.

Work is also in progress in the laboratory of Rose to investigate the incorporation of these amino acids into protein, an important aspect of their metabolism which was not examined in this thesis on account of its complexity. It should be interesting to compare protein synthesis and metabolism <u>in vivo</u> during different learning situations in the octopus, in the different parts of the brain which are known from the work of Young to have different functions in learning. The octopus brain promises to be a worthwhile topic for future biochemical and other research for a long time to come.

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