AMMONIA AND AMINO ACID METABOLISM AND TRANSPORT IN BRAIN IN VITRO

by

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ABSTRACT

Studies have been made of the factors controlling the formation, transport and utilization of ammonia in the brain and its effects on brain metabolism, of the processes promoting amino acid fluxes in brain under a variety of conditions, especially those leading to increased nerve activity, and on the specific locations, and sites of formation, of amino acids in the brain.

By using tetrodotoxin (2 μM) to suppress partly the neuronal efflux of amino acids brought about by the joint action of protoveratrine (5 μM) and ouabain (0.1 mM), the former drug being used to promote neuronal efflux of amino acids, and the latter being added to diminish re-uptake of amino acids, it has been shown that the major pools of glutamate, aspartate, glycine, serine and probably γ-aminobutyrate, are in the neurons. However, the major pool of glutamine appears to be in the glia. Glutamine formation takes place in the glia and is a process partly controlled by the concentrations of cations (K^+, Na^+, Ca^{++}) within these cells. Fluoroacetate (3 mM) acts mainly in the glia as it suppresses glutamine synthesis, but not the protoveratrine-stimulated brain respiration. Malonate (2 mM) acts mainly in the neurons since it suppresses the protoveratrine-stimulated respiration but not the synthesis of glutamine.

The amino acids, particularly glutamate, γ-aminobutyrate, aspartate and glycine, are released from brain cortex slices under conditions associated with brain cell excitation. The release processes are partly or wholly blocked by tetrodotoxin (2 μM). Tetrodotoxin does not affect the release of glutamine nor does protoveratrine accelerate it. This result is in accord with the conclusion that the main depot of glutamine lies not in the neurons but in the glia. Protoveratrine brings about an increased rate of formation of glutamine in incubated brain slices, suggesting that glutamate released from the neurons is taken up by the glia and there converted to glutamine. L-Glutamine is more

effective than L-glutamate as a precursor of γ-aminobutyrate in brain slices. As glutamic acid decarboxylase is localized in the neurons, it is concluded that glutamine released from the glia is taken up by the neurons and there converted to glutamate and γ-aminobutyrate.

Changes in the contents of NH_4^+ in incubated brain slices are accompanied by quantitatively equivalent changes in the amino acid contents of the tissue. Amytal (1 mM) suppresses endogenous glutamate oxidation and enhances the neuronal contents of glutamate and γ-aminobutyrate. It diminishes ammonia liberation. Ammonia is formed aerobically by brain cortex slices in a glucose-free medium largely by endogenous glutamate oxidation within the neurons, and also by glutamine hydrolysis. External L-glutamate is taken up against a concentration gradient largely by the glia and is less effective than endogenous glutamate as a source of ammonia in brain. Ammonium ions are not accumulated in brain slices against a concentration gradient. They are presumably formed, up to a limiting concentration, in the neurons independently of the external NH_4^+ concentration.

Ammonium ions affect both neuronal and glial metabolism and the brain cell transport of Na^+ and K^+ in the incubated brain slices. The decrease of K^+ is partly due to exchange with NH_4^+. The exchange process is most marked in infant rat brain. The effects of NH_4^+ in inhibiting respiration, diminishing ATP concentrations, and changing the cationic concentrations at the brain cell membrane are more pronounced in the stimulated than in the unstimulated brain tissue. It is concluded that the effects of NH_4^+ on brain metabolism and cation transport may be explained by its inhibitory effect on ATP formation in the neurons, by removal of α-ketoglutarate and hence by partly blocking the operation of the citric acid cycle. This may be one of the reasons for ammonia toxicity in the central nervous system.

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ABBREVIATIONS

AMP	adenosine 5'-monophosphate
ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
ATPase	adenosine triphosphatase
Acetyl-CoA	acetyl-coenzyme A
cpm	counts per minute
dpm	disintegrations per minute
rpm	revolutions per minute
DNP, 2, 4-DNP	2, 4-dinitrophenol
EGTA	ethylene glycol-(diaminoethyl) tetra acetic acid
EDTA	ethylene diamine tetra acetic acid
GABA	γ-aminobutyric acid
NAD^+, NADH	oxidized and reduced forms of nicotinamide adenine dinucleotide
$NADP^+$, NADPH	oxidized and reduced forms of nicotinamide adenine dinucleotide phosphate
Pi	orthophosphate
QO_2	oxygen consumption
TCA	trichloroacetic acid
Tris	tri (hydroxymethyl) amino methane
T/M	tissue to medium concentration ratio
TTX	tetrodotoxin
v/v	volume/volume
w/v	weight/volume
wt	weight

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1. INTRODUCTION

1.1 Formation of ammonia by nerve tissue

The first demonstration of the liberation of ammonia by nerve fibres was by Tashiro in 1922 (1). He and other workers (1-3) showed that electrical stimulation enhanced the rate of ammonia evolution and that mechanical injury depressed it. Winterstein and Hirschberg (2, 3) demonstrated the suppressive effects of anoxia and anesthesia on ammonia formation by the nervous system. Similar observations were made by a number of other workers (4-7). Frog retina, for example, when irradiated with sunlight exhibited increased ammonia formation when compared with that of controls left in the dark (5). Similar results were also reported when isolated nerves were stimulated, not only electrically or mechanically, but also by thermal or osmotic changes, or by chemical agents (6, 7). These studies led research workers in the field to conclude that nerve fibres liberate ammonia as a reaction to stimulation. Like peripheral nerves, the central nervous system reacts to stimuli by an increased rate of ammonia formation. There are detailed review articles on these and other aspects of ammonia metabolism in the central nervous system (8-16).

1.2 Factors affecting the in vivo levels of ammonia in the brain

(i) Ammonia content of brain in vivo

Abnormally high levels of ammonia in the brain are suspected to be involved in the precipitation of epileptic seizures (17-22) and in hepatic coma (23-27), as it is well known that the administration of ammonium salts to experimental animals causes convulsions, coma and death (22, 23, 28-32). For example, Richter and Dawson (22) showed that injection of ammonium chloride in the rat caused convulsions when the brain ammonia level had risen to 9 mg% (about 5 μmole/g). The knowledge that ammonia is a powerful cerebral irritant has stimulated interest in the study of brain

ammonia levels in vivo (18, 19, 22, 33, 34). Determinations of the levels of ammonia in the brain of animals in vivo have to be carried out with great caution as ammonia is a common laboratory contaminant. Moreover, the presence of labile substances like glutamine which may give rise to ammonia, must be taken into consideration, especially when the alkali diffusion method for the assay of ammonia is employed. It is well known that soon after death there is a rapid release or "burst" of ammonia in the brain (8, 36). Thus, in order to avoid post-mortem changes, the animals are often sacrificed by immersion in liquid air or liquid nitrogen. Using this freezing technique, values of about 0.15 - 0.36 μmole/g fresh wt brain have been obtained in the rat (22, 35, 36), dog (37, 38), rabbit (39), mouse (4), and garden dormouse (41). Such low values have led to the suggestion that there may be complete absence of free ammonia in the brain of the living animal (8). It may be argued that even rapid freezing in liquid air or nitrogen is not completely instantaneous (the time of freezing varies directly with the size of the animal (282)). Ammonia may be produced between the time of immersion and the time of complete freezing of the brain. The explosive formation of ammonia in the brain after death, possibly due to anoxia, becomes evident when heads of decapitated animals are dropped into liquid air. For example, there is about a twofold increase in ammonia content when the head is frozen one second after decapitation (22). Three minutes after death, cat brain gave 2 μmole/g fresh wt. Sheep brain gave values of 2.7 and 1.7 μmole/g fresh wt in grey and white matter respectively when freezing took place 5 min after death (42). Weil-Malherbe and Green (43) obtained a value of 5 μmole/g fresh wt in guinea pig brain cortex slices 20 min after death, a value which is in good agreement with those obtained in other studies (44, 45). In this thesis values of about 1.6 μmole/g initial wet wt are reported for brain cortex of rats taken a few minutes after death (Table 2).

(ii) Factors producing low brain ammonia levels in vivo

The ammonia content of the central nervous system, analyzed after

rapid freezing, is not constant but seems to depend on the state of activity of the brain at the time of the freezing. For example, a reduction in functional activity seems to be associated with a reduced concentration of free ammonia in the brain. Thus, Richter and Dawson (22) showed a significant decrease in brain ammonia levels (to 0.033 μmole/g fresh wt) when rats under prolonged nembutal narcosis were killed in liquid air. Vladimirova (46) obtained similar effects with urethane or sodium bromide. These reports followed the earlier suggestions and observations on the depression of ammonia formation by nerve fibres under anesthesia (1 - 3). Ammonia content of rat brain decreases by about 50 per cent during sleep (47). During hibernation, too, there is a 50 per cent diminution in the brain ammonia levels of garden dormice (41).

(iii) Factors producing high brain ammonia levels in vivo

Like peripheral nerves (1 - 3), the brain forms ammonia following electrical stimulation (22, 35, 36). This is consistent with the view that any method that enhances cerebral irritability will also enhance the rate of cerebral ammonia formation. Thus, the administration of drugs capable of producing convulsions, like camphor (30), picrotoxin (22, 46), telodrin (49), pentamethylene tetrazole (38, 50), bemigride (38), fluoroacetate (37, 51) or methionine sulfoximine (52) results in increased brain ammonia levels. In some instances the rise in the brain content of ammonia occurs in the preconvulsive state (22, 37, 52). However, even though ammonium salts are known to cause convulsions when administered to animals (22, 23, 28-32), it is still uncertain whether the increase in brain ammonia levels occuring from endogenous sources, is the cause or the result of convulsions. Anoxia (22) and high oxygen pressure (51) enhance ammonia levels in brain. Mild stimulation of the central nervous system, like injection of amphetamine (53) or corticotropin (54), painful electrical shock to the extremities (35, 36, 47), or certain conditioned reflexes (35, 47), also elevates brain ammonia levels. Though excitement caused by tumbling animals in a revolving drum has a similar

effect (55), Richter and Dawson (22) found no marked difference in rats excited by allowing them to drop from side to side in a glass beaker, while Vrba (9) found no change in brain ammonia levels of rats made to undergo physical exercise. Cerebral ammonia increases may not occur in some cases of physical or emotional excitement, owing to an increased rate of glutamine formation in the brain (9 - 11, 56, 57). Increased blood ammonia levels also result in enhanced glutamine contents in the brain (22).

1.3 (i) Some chemical changes that occur in the brain on the application of stimuli to the whole animal.

Increased cerebral activity produced in rats by physical exertion does not result in an enhanced brain concentration of ammonia (9, 56). However, there occurs an enhanced cerebral glutamine level (56) with concomitant decreases in the amounts of free glutamate (57, 66) and protein bound amide nitrogen (67 - 70). Similar results were obtained during oxygen intoxication (9) and carbon disulphide poisoning (70). Even during electrically induced convulsions, glutamine levels are elevated (71). Acute telodrin intoxication also results in an increase in glutamine content with a concomitant decrease in the glutamate levels in the brain (49). In the early stages of acute telodrin intoxication the content of free ammonia in the brain remains unchanged, but an increase is observed later when seizures occur. According to Hathway and Mallinson (49), telodrin causes liberation of ammonia in the brain and this occurs before the onset of convulsions and throughout their course. α-Ketoglutarate and glutamate are utilized in an ammonia binding mechanism which later becomes overwhelmed and free ammonia accumulates in the cerebral tissue.

It was long suspected that the glutaminyl amide - N of cerebral proteins represented a source of endogenous cerebral ammonia (57, 67, 74, 75). This was lately found to be true (76). According to Wherrett and Tower (76), 16 per cent of the glutaminyl residues of cerebral cortical proteins are readily

deaminated in situ.

These results lend support to the view that ammonia is formed from protein sources and is converted to glutamine by condensation with glutamate. Brain protein metabolism seems closely related to that of peripheral nerve, where stimulation increases the activity of the neutral proteinase, decreases protein content, and increases utilization of glutamate and liberation of ammonia (72).

(ii) Recovery processes that occur in the brain on the removal of the stimuli applied to the whole animal

When rats physically exhausted (by prolonged swimming) are allowed to rest, their cerebral glutamine levels decrease with concomitant increases in their cerebral protein nitrogen (69, 73).

Amidation of free carboxylic groups of cerebral proteins after the administration of NH_4Cl seems to take place (80, 81), but confirmation using ^{15}N labelling is needed (76). Wherrett and Tower (76) were unable to obtain reamidation of cerebral proteins in vitro by increasing free pools of ammonia and glutamine of cat cortex slices. In spite of this, they consider that protein deamidation is a reversible process as suggested by Mycek and Waelsch (77) as a result of their studies on transglutaminase. Transglutaminase is a calcium-activated enzyme which catalyzes (a) the exchange of protein amide groups with primary amines (like cadaverine, putrescine, histamine, serotonin, glycinamide, etc.); and (b) the hydrolytic deamidation of protein bound amide residues (78 - 79).

(iii) Convulsions and brain energy levels.

No alteration in the cerebral cortical ATP levels of rats was observed with ammonium acetate induced convulsions (82), or in hypoxic mice at the onset of seizures (83). Similarly, no change in the ATP or phosphocreatine

levels were observed in the brains of mice undergoing methionine sulfoximine induced seizures (52). Such results do not support the postulate of several authors (84, 85) that ATP is a factor in the induction of convulsions. The apparent absence of cerebral ATP depletion does not, however, rule out the possible depletion of a small localized pool of ATP that may be vital for normal brain function.

1.4 Factors affecting the formation of ammonia in brain tissue in vitro

The results of our earlier work (62) on the factors concerned with the rate of ammonia liberation by rat brain cortex slices incubated in oxygen at 37°C for one hour in Krebs-Ringer phosphate medium, were explained by changes in amino acid metabolism, particularly those of glutamate and glutamine (see section 1.5 (ii) (b)). A summary of our observations is given below.

(i) Carbohydrate metabolism and ammonia formation

Ammonia formation is suppressed by various exogenous substrates that are capable of supporting cerebral respiration. Thus, with glucose, lactate (58 - 62) or pyruvate (62), where the rate of the operation of the citric acid cycle is high, there is a maximal suppression of ammonia liberation. The minimum (threshold) concentration for this suppression is about 1mM for glucose and about 2.5 mM for pyruvate (62). This may be correlated with the fact that glycolysis of one molecule of glucose yields two molecules of pyruvate.

In the presence of glucose but not of pyruvate, the addition of iodoacetate (0.1 mM), by its suppressive effect on triose phosphate dehydrogenase, impedes the operation of the citric acid cycle and thereby the energy dependent glutamine synthesis. It enhances the output of ammonia by more than 50 per cent (62). In the absence of glucose, iodoacetate only slightly inhibits endogenous ammonia formation (62, 64); this may be due to a small diminution

in endogenous oxygen consumption (64).

Acetate is only oxidized feebly by brain cortex slices (226) and hence has little or no diminishing effect on ammonia formation. Similarly, citrate or α-ketoglutarate (62), acetoacetate or succinate (our unpublished observations), do not suppress the formation of ammonia. However, oxaloacetate causes high respiratory rates (227) and is capable of partially suppressing ammonia liberation. This is presumably due to its relatively rapid influx into the brain cell and to its participation in the citric acid cycle.

In the presence of certain inhibitors, e.g., 2, 4-dinitrophenol (62, 64), fluoroacetate (63), methionine sulfoximine (65) or iodoacetate (64), the suppressive effects of glucose is less marked. Malonate (5 mM), an inhibitor of the citric acid cycle at the succinic dehydrogenase stage (228), enhances ammonia liberation only in the presence of threshold concentrations (1 mM) of glucose. This is also found to be true with 100 mM KCl (62). Kini and Quastel (213, 229) demonstrated a marked stimulation in the operation of the citric acid cycle in K^+-stimulated cells so that in the presence of 1 mM (but not 5 mM) glucose, the intermediates in the breakdown of glucose are oxidized too fast to allow for the normal rate of (energy requiring) ammonia fixation.

(ii) Electron transport, oxidative phosphorylation and ammonia formation.

In the absence of glucose, adult cerebral cortex slices yield greater amounts of ammonia than those of the infant brain (62), presumably because oxygen uptake by infant brain is lower. Milstein et al., (230) showed that the oxygen consumption per mg mitochondrial protein increases with the age of the rat.

Electron transport inhibitors, e.g., azide, arsenite or cyanide, suppress autogenous ammonia formation (43, 63, 64). Amytal (1 mM) inhibits electron transport between NADH and the cytochrome system (219 - 221) and

concomitantly suppresses ammonia formation in a glucose-free medium (62). The electron transport inhibitors presumably act by suppressing the regeneration of NAD^+ required for glutamate oxidation.

A direct consequence of the inhibition of the respiratory chain is a diminished rate of ATP synthesis. Both 2, 4-dinitrophenol (0.1 mM) (62, 63), or sodium salicylate (5 mM) (62), suppress the endogenous formation of ammonia, but enhance the liberation of ammonia in the presence of glucose, presumably by inhibiting the ATP-requiring glutamine synthesis.

The rate of ammonia production is suppressed under anoxia (43, 62, 244), doubtless by an inhibition of the oxidation of endogenous glutamate (62).

(iii) Effects of exogenous amino acids on ammonia formation.

The addition of L-glutamate to brain cortex slices respiring in a normal Krebs-Ringer phosphate medium results in a diminished rate of ammonia formation (62, 103), accompanied by an enhanced rate of glutamine synthesis (58, 103). Five mM D-glutamate (62, 138, 231), or 5 mM α-methyl glutamate inhibits ammonia liberation in a glucose-free medium, presumably by inhibiting endogenous glutamate oxidation. In the presence of glucose, D-glutamate enhances the rate of ammonia liberation (62) by inhibiting glutamine synthetase (103).

The rate of autogenous ammonia formation is unaffected by taurine, γ-aminobutyrate (GABA), L- or D-aspartate glycine or glycyl-L-aspartate. However, with L-alanine or L-arginyl-L-glutamate there seems to be a small inhibition (62).

L-Glutamine is deamidated by brain slices. Net deamidation occurs to a greater extent in the absence of glucose than in its presence, as glutamine resynthesis is promoted by the presence of glucose (62).

1.5 Ammonia formation and utilization mechanisms in the brain

(i) Urea

(a) Urea as a source of ammonia.

Urea is present in mammalian brain at a concentration of about 5.0 mM (41, 88, 100). Can it serve as a source of ammonia in the brain? Godin et al., (41) have shown that in hibernating garden dormice, both the ammonia and urea levels fall to about 50 per cent of the control (awake) animals. According to these workers, the decreased brain ammonia level in hibernating animals is due to reduced functional activity. During hibernation, the internal body temperature, the blood flow, the oxygen consumption and the utilization of different metabolic substrates are all reduced. It is conceivable that brain urea level falls due to a fall in the blood level of urea, as a result of a drop in ammonia formation, arising partly through diminished bacterial action in the intestine and partly due to lowered nitrogen metabolism in the hibernating animals. Thus, lowered cerebral urea is due to lowered blood urea levels (see section 1.5 (i) (b)); it is not accompanied by an increased ammonia level in the brain.

Moreover, urea seems not to be the source of ammonia in brain during telodrin-induced seizures, since urea levels remain unchanged under these conditions (49).

Furthermore, in vitro studies showed that the addition of urea to respiring brain cortex slices of the rat incubated in oxygen for one hour at 37°C in a normal Ringer phosphate medium (glucose free) had little or no effect on the rate of ammonia formation (62).

Additionally, urease seems not to be present in mammalian tissues so far examined (86).

It seems unlikely, therefore, that urea is a source of ammonia in brain.

(b) Possible formation of urea as a mechanism for ammonia utilization.

Urea is not synthesized in brain from NH_4^+ and CO_2 (100). Some of the intermediates of the Krebs-Henseleit urea cycle (87) are present in brain. Contents of these intermediates in cat brain calculated in terms of μmole/g initial wet wt, from the data of Tallan, Moore and Stein (given as mg/100 g) (88), are as follows: ornithine, 0.046; citrulline, 0.23; arginine, 0.2; and urea, 4.2. Carbamyl phosphate has not yet been detected in brain. Several of the enzymes that participate in the synthesis of urea cycle metabolites (namely, arginino succinate synthetase, arginino succinase and arginase) are also present in crude preparations of brain (89, 90, 100). It seems that part of the urea cycle and its mediating enzymes occur in brain. Metabolic studies in vivo show that L-proline is converted to glutamic acid, arginine and ornithine (91); and L-arginine (92), or citrulline (93) to urea. However, the enzyme that catalyzes the formation of citrulline from carbamyl phosphate and ornithine (viz., ornithine transcarbamylase) does not operate in the central nervous system (93, 100). Carbamyl phosphate synthetase, the enzyme that fixes ammonia to CO_2 in the presence of ATP, is likewise absent in the brain (100). It is doubtful therefore, whether the Krebs-Henseleit cycle per se occurs in brain at any rate to any significant extent.

The relatively high concentration of urea in brain is probably due to passive diffusion from the blood. A brain to plasma concentration ratio of about one (0.8 as calculated from the data of Tallan et al.(88) see Table 1), has been found (see also reference 281). It may be pointed out that in the liver the concentration of urea is not much different from that of brain (5.16 μmole/g for liver versus 4.5 μmole/g for brain (100)). According to Blass (see reference 49), brain synthesizes urea at a maximum rate of 1 μmole/g of tissue/hr, whereas the rate for glutamine synthesis is 10-20 μmole/g of tissue/hr (95).

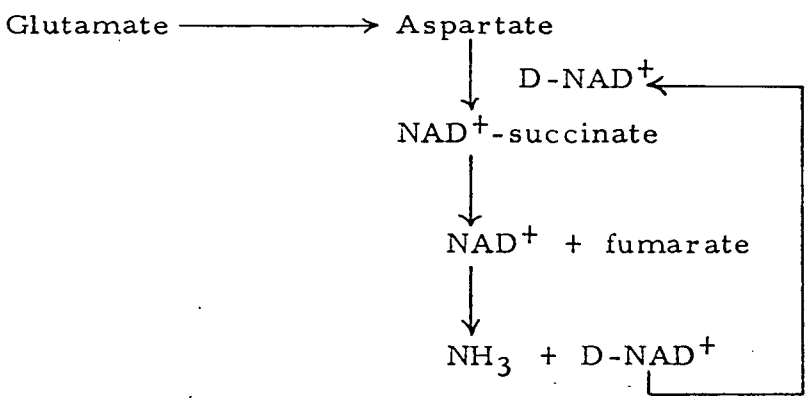
The above considerations lead to the conclusion that removal of ammonia in the brain does not occur by urea formation. The observation that hepatectomy in the dog leads to cessation of urea production points to the liver as possibly the only major source of urea in the mammalian organisms (96, 97).

(c) Possible importance of urea synthesis in the brain (86, 95, 100).

The metabolism of citrulline and argininosuccinate in the brain via urea formation seems to be important for brain development. Relatively large amounts of citrulline and argininosuccinate are present in the brain, cerebrospinal fluid, and plasma, and are excreted in the urine in 'citrullinuria' and 'argininosuccinic aciduria', respectively. These diseases of the central nervous system are due to the relevant enzymes being absent in brain. The conditions are accompanied by mental retardation.

(ii) (a) Ammonia formation from amino acids via aspartate channelled through NAD^+ or AMP.

According to Buniatian and co-workers (14), ammonia is formed in brain and other organs from amino acids. They have proposed a mechanism by means of which amino acids through aspartate supply amino groups to desamino-NAD^+ (D-NAD^+). NAD^+ thus formed on deamination yields ammonia and desamino-NAD^+. The sequence of reactions proposed is as follows:



This process is a variation of the scheme suggested by another Russian worker. According to Kometiani (81, 98), the reamination of IMP by aspartate and the deamination of AMP thus formed, is a mechanism by which ammonia is produced from amino acids. The results leading to this hypothesis were obtained using mitochondrial preparations (14).

The amount of ammonia formed from NAD^+ and aspartate by mitochondria of rat brain (14) seems to be insufficient to account for the formation of ammonia by brain tissue in vitro. This is evident from the following calculations (see reference (14) for values): 5.28 ± 0.3 µg ammonia/mg protein/2 hr at 37°C, was obtained on the addition of NAD^+ (1.4 µmoles) and aspartate (26 µmoles) to mitochondria. The control value was 3.07 ± 0.44. With aspartate or NAD^+ present alone, the values recorded were 3.45 ± 0.3, or 3.63 ± 0.3, respectively.

Taking standard deviations into consideration, it seems that a little over 1 µg ammonia/mg protein is formed in two hours by brain mitochondria from NAD^+ and aspartate. This amounts to 0.03 µmole ammonia formed per mg mitochondrial protein/hr. Even if the protein content of brain was wholly mitochondrial, then only 3 µmole ammonia/100 mg protein and hence per g wet wt brain would be formed under these conditions. This value is too small to account for the liberation of ammonia by brain under a variety of incubation conditions that will be recorded in this work. Moreover, the addition of L-aspartate (5 mM) to respiring brain cortex slices was without any effect on the rate of ammonia liberation (62). Again, raising the endogenous tissue level of aspartate by incubating cerebral cortex slices in a glucose free medium containing 105 mM KCl, or lowering it by the use of metabolic inhibitors such as malonate or amino oxyacetic acid, does not affect the rate of ammonia formation. These experiments will be described in greater detail in Chapter 3.

With guinea pig brain slices, Takagaki (99) obtained no decrease in

endogenous levels of adenylic compounds, and concluded that the conversion of adenylic acid to inosinic acid by adenylic deaminase is quantitatively a relatively minor process. It may be mentioned that free adenylic compounds are present at relatively low levels in the brain and therefore cannot be considered as major sources for cerebral ammonia formation (43).

(ii) (b) Ammonia formation from amino acids via terminal glutamate oxidation.

The origin and mechanism of ammonia formation is brain slices respiring in a glucose-free incubation medium is still a subject of much controversy.

According to the early work of Weil-Malherbe and co-workers (43, 75) none of the six deaminating brain enzymes present in brain (e.g., glutamic dehydrogenase, glutaminase, amine oxidase, adenylic deaminase, adenosine deaminase or hexosamine deaminase) can account for ammonia formation. These workers suggested that ammonia formation occurs largely by a reaction closely linked to proteolysis, since guinea pig brain slices incubated for 5 hrs gave significant increases in non-protein nitrogen. In their studies, they assumed a single origin from which free ammonia is largely derived.

On the other hand, according to Vrba et al. (67, 101), ammonia formation is too complex a process to be explained on the basis of simple proteolysis. These workers showed that not more than 25 per cent of the ammonia formed by guinea pig brain slices in incubations of 4 hrs and 6 hrs duration can be accounted for as coming from protein amide nitrogen, and consider the source of the larger part of ammonia as still being unknown. In their studies they also observed some increases in non-protein nitrogen and lipid nitrogen, and some decreases of nitrogen in the nucleic acid and protein fractions.

That some ammonia may possibly be derived from protein amide

nitrogen is supported by the demonstration that 16 per cent of the glutaminyl residues of cerebral cortical proteins are readily deamidated (76). However, even this does not account for the major portion of ammonia formed *in vitro* by brain cortex slices (67, 76, 101).

In the studies of Takagaki *et al.* (64), the incubation of guinea pig brain slices was restricted to one hour because it was felt that ammonia formed at the end of 4 hrs might be the result of a series of highly complicated reactions, much of which involved proteolysis. Glutamate decreases in the brain concomitant with an increase in ammonia formation when incubations are carried out in a glucose-free medium; according to these workers, the decrease in glutamate concentration accounts for 50 per cent of the endogenous oxygen uptake. However, it appears that their estimations of glutamate and ammonia were confined to the cerebral tissue, the contents of these substances in the incubation medium not being considered. Moreover, the possible conversion of glutamate to other amino acids such as aspartate, γ-aminobutyrate, and alanine, were not taken into account.

In earlier work (62, 65) we have shown that the total production of ammonia (i.e., in both tissue and incubation medium) by brain slices incubated for short periods of time in the absence of glucose, is largely due to amino acid metabolism, in which endogenous glutamate oxidation is the terminal process. These studies showed how the individual levels of ammonia and amino acids varied on incubating rat brain cortex slices in oxygen for one hour at 37°C in the presence or absence of glucose. They indicated that the diminution in the glutamate and glutamine levels in the brain cortex slices accounts largely for the liberation of ammonia. Changes in the observed rates of ammonia formation, on incubating rat brain cortical slices for one hour under a variety of conditions, could be explained on the basis of glutamate oxidation and glutamine synthesis. This has already been mentioned in section 1.3. Subsequent to our work (62, 65), a recent report by Weil-Malherbe and Gordon (102) supports our view that changes in the levels of

amino acids (particularly glutamate) in guinea pig brain cortex slices, can account for the liberation of ammonia. However, the contents of amino acids found initially in their slices are about 30 - 50 per cent of the values reported in the literature (102, 233). Also, their rate of ammonia formation is 40 - 50 per cent of the values they reported in 1955 (43).

The origin, mechanism and factors affecting ammonia formation in incubated brain cortex slices, will be dealt with in Chapter 3, in which an attempt will be made to characterize the compartments in the brain tissue mainly responsible for ammonia production (Chapter 10).

(iii) Ammonia utilization processes in brain

There appear to be at least three mechanisms of ammonia utilization in the brain.

(a) Urea formation. As mentioned earlier, conversion of ammonia to urea in brain occurs either not at all or only at a very small rate.

(b) Amidation of proteins. According to a number of workers, deamidation of glutaminyl residues in protein (9-12, 57, 67, 74, 75) catalyzed by the calcium requiring transglutaminase (78, 79) occurs in brain (see section 1.4 (ii)). Though there is general consensus among workers in the field that the reamidation process (utilizing either NH_4^+ ions or glutamine, should take place, the actual demonstration of its occurrence is still a matter of doubt (76, 80, 81).

(c) Glutamine synthesis. The formation of glutamine seems to be the major process of ammonia utilization in brain. Increased glutamine synthesis occurs in vivo during increased functional activity (9-11, 56, 57) brought about by physical exercise, convulsive agents, or infusion of ammonium salts. Ammonia

liberation by brain tissue is inhibited under conditions leading to high rates of glutamine synthesis; e.g., in the presence of glucose (58-62) or glutamate (103). One of the objectives of our work is to understand in greater detail the control mechanism for glutamine synthesis and its actual location in the brain cells.

1.6 Transport of amino acids in brain.

Transport of amino acids in brain has usually been investigated by the use of tissue slices as they are able to accumulate amino acids against a concentration gradient. In the intact animal, however, the brain is only able to concentrate amino acids to a small extent or not at all. The difference between *in vivo* and *in vitro* experiments led to the concept of a blood brain barrier. Recently it has been found that, though there is but little increase in the amounts of amino acids in brain on increasing blood amino acid concentrations, there is a rapid movement of amino acids into and out of the brain (15, 104-107) as shown by the use of labelled amino acids.

The supply of amino acids to the brain from the blood is of obvious importance for the normal functional activity of the brain. In addition to their incorporation into protein, they are needed for the synthesis of biogenic amines whose uptake is much smaller than the corresponding amino acid giving rise to them (104).

Passive diffusion leads only to a concentration within the cell no higher than that outside, except under circumstances where binding of the substance to a cell constituent takes place in the cell or in a cell compartment (104). Active transport, on the other hand, requires energy, being less in the absence of oxygen and glucose, and also in the presence of metabolic inhibitors like cyanide and 2, 4 dinitrophenol. It utilizes ATP and involves movement against a concentration gradient. It is subject to saturation kinetics, the rate of uptake reaching a limit with increase in the concentration of amino acid available, owing to saturation of carrier sites. Competition also occurs

between amino acids during transport into brain slices. Amino acids may compete with each other for a common carrier but may bring about the suppression of each other's transport indirectly by diminishing the ATP levels (104, 106, 113). Unlike competitive inhibition, non-competitive inhibition of transport processes does not involve a specific carrier. It can be produced, for example, by interference with metabolic processes within the cell upon which energy for transport is dependent, or by damage to the cell membrane, and is unrelated to the concentration of substrate amino acid (106).

The uptake of amino acids is a measurement of the net transport and has been based essentially on the amount by which the concentration of a particular free amino acid of the tissue has increased over a defined period (usually one hour). Both influx and efflux processes occur when the steady state is reached. Either of these processes may be produced by passive diffusion or by active transport or processes such as facilitated diffusion (107). Passive diffusion may also occur at the same time as active transport (108). There may be a factor in the influx and efflux system that involves counter transport (106, 109) or exchange diffusion (110, 111) in which movement of substrate in one direction causes movement at the same time in the opposite direction. This latter process may be of special interest in brain as it may be related to the blood brain barrier phenomenon in which net transfer is impeded in vivo inspite of rapid interchange between blood and brain (106).

Active transport of amino acids in brain is sodium dependent (113, 104, 105). High potassium inhibits amino acid uptake (114) by diminishing ATP levels (115). Absence of calcium retards the rate of amino acid uptake, presumably by lowering ATP levels. Inhibition also occurs with high calcium salts (10 mM), presumably due to the inhibition of Na^+, K^+-stimulated ATPase. Ouabain inhibits both the active transport of amino acids and the Na^+, K^+-stimulated ATPase. (section 1.9 (v)). Ouabain presumably acts by its affinity for the Na^+ transport carrier (at the K^+-site) and thus blocks transport coupled with this carrier (section 1.9 (v)). Ouabain has little or no effects on

cerebral ATP levels (115). Ouabain (112, 280), anoxia, Na^+ lack and respiratory chain inhibitors, enhance the rate of amino acid efflux from brain (104, 105, 205).

More information on transport processes for individual amino acids and other compounds of biochemical interest in brain and in other tissues of a variety of species may be obtained from the reviews by Quastel (104, 105) and Naeme (106).

Table 1 gives brain/plasma concentration ratios of amino acids calculated from the data (for the cat) of Tallan, Moore and Stein (88, see also 95, 116).

In our work, our interest in transport processes has been chiefly confined to the transport of amino acids (particularly that of glutamate), and that of ammonia in rat brain cortex slices.

1.7 (i) Amino acid metabolism in brain.

Glutamic acid occupies a central position in brain metabolism, in view of its high concentration, its involvement in many biochemical reactions, its relatively high rate of oxidation, and its possible role as a transmitter. Glutamate, together with the closely related amino acids, viz., glutamine, aspartate, GABA and also alanine, contribute up to 70 per cent of the total amino acid nitrogen of adult brain. In this respect the free amino acid pool of brain is unlike that of other animal tissues. While special attention will be given to the above mentioned group of amino acids, the amino acids glycine, serine, threonine,taurine and N-acetyl aspartate, that together make up much of the remaining amino acid nitrogen of the free amino acid pool, will also be dealt with in studies reported in this thesis.

TABLE 1. Some brain to plasma concentration ratios of amino acids for the cat.

These calculations were made from the data of Tallan, Moore and Stein (88) given as mg%. Conversion of μmole/g to μmole/ml tissue water was done by assuming the tissue water content to be 80% that of the initial fresh weight brain.

	Brain		Plasma mM	Brain/ Plasma
	μmole /g	μmole /ml		
Glutamate	8.74	10.92	0.123	89.0
Glutamine	> 3.42	>4.27	>0.427	10.0
Aspartate	2.23	2.79	0.0076	368.0
Asparagine	0.20	0.25	0.068	3.7
GABA	2.27	2.84	< 0.02	> 142.0
Alanine	0.95	1.19	0.845	1.4
Glycine	1.35	1.69	0.307	5.5
Serine	0.73	0.91	0.20	4.6
Threonine	0.22	0.27	0.118	2.3
Valine	0.18	0.22	0.21	1.1
Leucine	0.14	0.17	0.123	1.4
Isoleucine	0.09	0.11	0.092	1.2
Methionine	0.11	0.14	0.034	4.1
Arginine	0.08	0.10	0.08	1.2
Ornithine	0.045	0.06	0.015	4.0
Citrulline	< 0.023	<0.03	<0.0057	< 5.3
Urea	4.17	5.21	6.67	0.8

(a) Initial cerebral contents of amino acids

The initial levels of amino acids, determined in these studies and given in Tables 2 and 4 for adult and infant (2 day old) rat brain, are in close agreement with those reported in the literature (125-131). Infant rat brain has high concentrations of taurine and phosphoethanolamine (see reference 130, 131), but have low levels of glutamate and its metabolically derived amino acids. The activities of the enzymes involved in the formation and interconversion of these amino acids are also low (15, 163). Adult values of amino acids and of their related enzymes are reached as the brain matures and at about the time of the complete laying down of myelin (129, 130). Animals such as the guinea pig, in which the infant stage shows many mature characteristics, show only small variations in amino acid levels with age (129).

(b) Pathways of glutamate metabolism

A detailed account of the synthesis and catabolism of amino acids is beyond the scope of this thesis. Only the main aspects pertinent to the present study will be discussed. For more detailed information there are many useful reviews (8, 11, 12, 15, 86, 95, 106, 117-120).

Glutamate is closely related to the citric acid cycle due to its formation by transamination of α-ketoglutarate.

Glutamate transaminases: Glutamate can reversibly transaminate with the keto acids, oxaloacetate and pyruvate to yield aspartate and alanine respectively. These reactions, shown below, are quantitatively the dominant transaminations occurring in brain tissue (122, 123). They are catalyzed by specific transaminases requiring pyridoxal phosphate as co-enzyme (124).

$$\text{glutamate} + \text{oxaloacetate} \rightleftharpoons \alpha\text{-ketoglutarate} + \text{aspartate} \qquad (1)$$

$$\text{glutamate} + \text{pyruvate} \rightleftharpoons \alpha\text{-ketoglutarate} + \text{alanine} \qquad (2)$$

When the rate of formation of free ammonia is high, reductive amination of α-ketoglutarate, mediated by glutamate dehydrogenase, occurs and a rapid synthesis of glutamate takes place, followed by the synthesis of glutamine. Glutamate, however, transaminates with pyruvate and oxaloacetate forming alanine and aspartate, and regenerating α-ketoglutarate which again undergoes reductive amination in presence of excess ammonia. Thus, a flow of ammonia into amino acids (glutamate, glutamine, alanine and aspartate) takes place. When the amount of ammonia formed is low, α-ketoglutarate is mainly converted to glutamate by transamination with alanine or aspartate forming pyruvate or oxaloacetate which enter the citric acid cycle.

Glutamate can also transaminate directly with α-ketoglutarate, each compound being converted to the other. This allows the rapid exchange of labelled carbon between glutamate and members of the citric acid cycle (121).

The GABA shunt. Glutamate is also formed from α-ketoglutarate and GABA by transamination (by the enzyme GABA transaminase). Succinic semialdehyde formed in the reaction enters the citric acid cycle on conversion to succinate by the NAD^+ requiring enzyme succinic semialdehyde dehydrogenase. These reactions including the decarboxylation of glutamate by glutamate decarboxylase constitute the GABA shunt (116, 119). It represents an alternate pathway of α-ketoglutarate oxidation in the metabolism of carbohydrate in the central nervous system of mammals or the peripheral nerves of anthropods. According to Balazs et al. (116), 8 per cent of the α-ketoglutarate of the citric acid cycle may be diverted to such a route. Haber (279) had already concluded that less than 10 per cent of carbohydrate oxidation in brain took place via the GABA shunt. The reactions constituting the GABA shunt are depicted below (Reactions 3, 4, 5).

$$\text{Glutamate} \longrightarrow \text{GABA} + CO_2 \quad (3)$$

$$\text{GABA} + \alpha\text{-ketoglutarate} \rightleftharpoons \text{Glutamate} + \text{Succinic semialdehyde} \quad (4)$$

$$\text{Succinic semialdehyde} + NAD^+ \overset{H_2O}{\rightleftharpoons} \text{Succinate} + NADH + H^+ \quad (5)$$

The sum of the reactions:

$$\alpha\text{-ketoglutarate} + NAD^+ + H_2O = \text{Succinate} + NADH + H + CO_2$$

Glutamate dehydrogenase. Glutamate is formed reversibly from α-ketoglutarate and ammonium ion by the enzyme glutamate dehydrogenase according to the following equations (8, 95) (Reactions 6 and 7).

$$\text{Glutamate} + NAD^+ \rightleftharpoons \alpha\text{-iminoglutarate} + NADH + H^+ \quad (6)$$

$$\alpha\text{-iminoglutarate} + H_2O \underset{\text{non-enzymic}}{\rightleftharpoons} \alpha\text{-ketoglutarate} + NH_3 \quad (7)$$

In the presence of ammonium salts the equilibrium of reaction (6) is much in favour of glutamate formation only 1.4 per cent being oxidized at pH 7.4 (8). In the absence of ammonium salts, α-iminoglutarate spontaneously hydrolyzes to α-ketoglutarate and ammonia. Thus the supply or removal of ammonia may regulate the rate of oxidation of glutamate (8, 133). The presence of α-ketoglutarate inhibits cerebral ammonia formation (42). These conclusions were reached from reactions in homogenous solutions derived from brain (8, 42, 132). In brain slices in the presence of glucose, the reductive amination of α-ketoglutarate is favoured (102). However, in the absence of glucose, as endogenous substrates of incubated brain cortical slices are depleted, the $NAD^+/NADH$ ratio increases while the ATP/ADP ratio decreases. Increase of ADP results in increase of NAD^+ ($\because$ NADH + ADP + Pi $\xrightarrow{O}$ NAD^+ + ATP). These changes favour the oxidation of

glutamate by glutamate dehydrogenase and ammonia and α-ketoglutarate are formed in the reaction.

Glutamine synthetase. Glutamate combines with the ammonium ion, through the activity of the enzyme glutamine synthetase (58, 103) in the presence of ATP (134, 136) according to the following reaction (Reaction 8).

$$\text{Glutamate} + NH_4^+ + \text{ATP} \xrightarrow{Mg^{++}(Mn^{++})} \text{Glutamine} + \text{ADP} + \text{Pi} \qquad (8)$$

Oxidizable substrates like glucose lactate and pyruvate, suppress ammonia formation in brain both by competition with endogenous oxidizable nitrogenous material and by the formation of glutamine (58-62). The presence of glucose brings about a disappearance of ammonia with incubated brain slices (58). Inhibition of ATP synthesis (e.g., by 2, 4 dinitrophenol (62, 64) or fluoroacetate (63)) and thereby diminished glutamine synthesis causes increased ammonia output.

Glutamine synthetase is localized in the microsomal fraction of brain cells (15). For the brain enzyme the Km for L-glutamate is 2.5 mM; for ATP, 2.3 mM; and for NH_4^+, 0.18 mM (95). The low value for NH_4^+ is significant in relation to the role of glutamine synthetase in the cerebral utilization of ammonia.

Glutaminase. Glutamate is formed from glutamine with the release of ammonia by the mitochondrial enzyme glutaminase (15). The process is as follows (Reaction 9).

$$\text{Glutamine} \xrightarrow{H_2O} \text{Glutamate} + NH_4^+ \qquad (9)$$

Glutaminase is strongly inhibited by D- or L-glutamate (103). Phosphate and other polyvalent anions such as arsenate and sulphate activate the enzyme (137, 138). In the absence of phosphate, glutaminase activity of guinea pig brain homgenates is strongly enhanced by the presence of tri-

carboxylic acids like citrate, less strongly by that of dicarboxylic acids, and slightly, if at all, by that of monocarboxylic acids like lactate or propionate (138). The enzyme from brain and kidney has allosteric properties (15, 138, 139).

(ii) Compartmentation of amino acid metabolism in brain

The concept of metabolic compartmentation is the outcome of observations (116, 141-148) that cannot be explained by the simple precursor-product relation as described by Zilversmit et al. (140). It implies the presence of two or more distinct pools of a given metabolite in a given tissue not necessarily in equilibrium with each other, each maintaining quantitatively and/or qualitatively its own patterns of metabolism. Metabolic compartmentation in brain was first described for the glutamate-glutamine system. Berl and Clarke (141) have recently reviewed the relevant literature.

Early work on this subject was carried out in vivo by Waelsch and co-workers (141-143). They studied the effects of intravenously, intracerebrally and intracisternally administered ^{14}C-glutamate for short time intervals (2-5 min, in order to avoid equilibration of isotope through all metabolic pools) and found that the specific activity of glutamine isolated from brain tissue was greater than that of glutamate. This observation was also found to be true for intravenously administered ^{15}N-ammonium acetate or ^{14}C labelled bicarbonate. On the assumption that glutamate is the only precursor of glutamine, these observations indicated that glutamine must be derived from a compartment of glutamate not in equilibrium with the rest of the glutamate present in the brain. In other words, glutamate exists in at least two distinct pools, one (the smaller) pool being particularly disposed to the formation of glutamine.

Measurements of the ratio of the specific radioactivity of glutamine to that of glutamate showed that labelled ketogenic precursors such as acetate, butyrate, propionate and acetoacetate give rise to a ratio greater than one.

With labelled glycogenic substances such as glucose, pyruvate, lactate or glycerol, the specific activity of glutamate is greater than that of glutamine. These effects are accounted for by assuming the presence of at least two pools in the brain cells, in which the citric acid cycle operates, which differ in their accessibilities to substrates that show metabolic compartmentation (144).

Compartmentation of glutamate metabolism has also been demonstrated in vitro using brain cortex slices prepared and incubated under certain conditions (145). The amino acids lysine, leucine, GABA, proline and phenylalanine and also ethanol exhibit metabolic compartmentation in the formation of glutamate and glutamine (146, 148, 283, 284).

The assumption of the presence of two pools of glutamate in brain necessitates the presence of two pools of α-ketoglutarate from which they are derived. As the physical location of these pools has not been characterized, Berl and Clarke (141) have called the process (involving the citric acid cycle) that generates a large glutamate pool but only a small glutamine pool, "the energy cycle". The process (also involving the citric acid cycle) which is predominantly active in the synthesis of glutamine, they have called "the synthetic cycle". "The energy cycle" is presumably responsible for a larger pool of ATP.

A search for metabolic inhibitors that selectively affect one of these pools is beginning to prove successful. Lahiri and Quastel (63) showed that while fluoroacetate has little effect on oxygen consumption, it strongly inhibits the synthesis of glutamine and concluded that at low (convulsive) concentrations it affects cerebral ammonia metabolism. According to Clarke et al. (150), the phenomenon can be explained if fluoroacetate inhibits operations of "the synthetic cycle" with relatively no effect on those of "the energy cycle". Work on the action of malonate and neurotropic agents like protoveratrine, on the operations of the two pools, will be presented in this thesis (Chapter 8).

Metabolic compartmentation has not yet been satisfactorily defined in structural or cellular terms. Mitochondria, heterogenous with respect to their enzymes (232), may offer a possible explanation to this problem. But mitochondria may be derived from different cell types (e.g., neuron and glia) or different portions of the same cell (e.g., nerve cell body and nerve ending). Some evidence in favour of neurons and glia as responsible for metabolic compartmentation has been presented by Rose (159) using his neuronal and glial preparations. However, at the present time fractionation procedures for the separation of cell types such as neurons and glia are somewhat drastic and cannot be expected to give pure preparations. Moreover, it is uncertain how far the procedure affects the properties of the fractions. Metabolic compartmentation of the glutamate-glutamine system is not manifest in immature rat brain (149), or in octopus brain that contains little glia (160). Compartmentation of amino acids following neuronal degeneration has been reported (161). Briefly stated, such indirect results indicate that the "large" glutamate compartment may consist of neuronal processes (116, 149, 154-156), while the "small" glutamate pool associated with high glutamine content is thought to consist of glial tissue (161, 162). In studies reported in this thesis more definitive attempts will be made to characterize the compartments of amino acids in brain cortex slices of the rat using pharmacological agents, rather than fractionations, for localization of the compartments.

1.8 Propagation of nerve impulse

Neurophysiological aspects of excitation have been extensively reviewed (164-168, 176, 262). Only material relevant to this work will be presented here.

(i) Brain cell types (168, 169)

The cells of the central nervous system may be divided into two classes on the basis of whether they are excitable (neurons) or not (glia). Each

neuron is made up of (1) dendrites which are covered by synaptic processes; (2) the cell body responsible for the proper functioning of the neuron; and (3) an axon whose terminal branches form synaptic connections with other neurons. Glia consists of three types, namely, astrocytes, oligodendroglia and microglia, and are capable of proliferation. They act as insulators because of their high resistance membrane and are essential for the laying down and maintenance of the myelin sheath. It is thought that they offer structural support, supply nutrition from the blood, and maintain the chemical environment of the neuron (305).

There are about ten times more glial cells than neurons in the brain (171). The volume occupied by the glial cells, however, is about the same as that occupied by the neurons (95). There is agreement that the neurons are responsible for much of the oxygen consumption of brain (175, 264, 268-271). In the immature brain neuronal processes are not developed. The growth of such processes coincides with myelination.

(ii) Resting potential (164-168)

When a membrane separating two solutions is permeable to one ionic species a potential difference, E, is set up across the membrane whose magnitude is given by the following Nernst equation (at 38°C and for a monovalent ion):

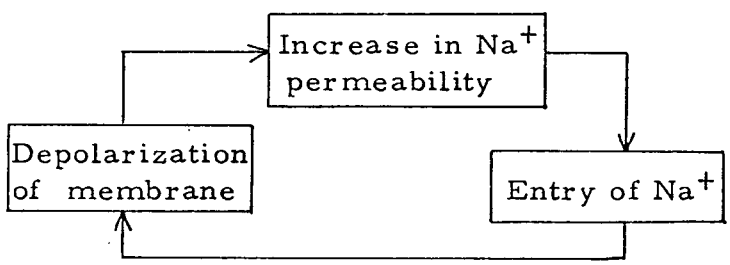
$$E = 61.5 \log_{10} \frac{[\text{penetrating ion outside}]}{[\text{penetrating ion inside}]} \text{ millivolts}$$

Under resting conditions the interior of nerve and muscle fibres is 69-75 mV negative with respect to the outside. This is caused by the selective permeability of the cell membrane to the flow of K^+. Under these conditions the membrane pore size of the neuron is intermediate (3Å) between the hydrated diameter of K^+ (2.2 Å) and that for Na^+ (3.4 Å) and Na^+ is practically impermeable (164). The nature of the charge on the membrane is another factor controlling permeability of ions (164).

The K^+ concentration inside the cell is greater than that in the body fluids outside, and the converse is true for Na^+. Thus, for cat motoneurons the concentration of K^+ in the neurons is 27 times higher than that outside, while the concentration for Na^+ is 10 times higher outside than inside (165).

(iii) Action potential (164-168)

Action potentials are generated in an all or none process when the membrane potential falls to the threshold level. Thus, when the membrane is depolarized by an outward flow of current, its Na^+ permeability immediately rises and there is a net inward movement of Na^+ ions down the sodium concentration gradient. If the initial depolarization is large enough (i.e., the threshold potential for firing is reached), Na^+ enters faster than K^+ can leave and this causes the potential to drop still further increasing the Na^+ permeability even more, in the following manner:



The entry of sodium is halted when the membrane potential reaches a level close to (E_{Na}), the equilibrium potential for sodium, when the net inward driving force acting on sodium ions becomes zero. It is still uncertain whether Na^+ and K^+ traverse the same channels (166). However, the Na^+ and K^+ channels can be differentially blocked; e.g., by tetrodotoxin (190, 193, 194) and tetraethylammonium ions (167, 172) respectively.

(iv) Sodium-Pump (164-168)

After the conduction of the nerve impulse, the sodium-pump re-establishes the resting potential by ejecting Na^+ from and returning K^+ to

the nerve tissue. ATP is utilized as the source of energy. The sodium-pump is believed to be operated by the Na^+, K^+-stimulated Mg^{++}-dependent ATPase present in cell membranes. These and other aspects of the sodium-pump will be dealt with in a later section (section 1.9 (v)).

(v) Synaptic transmission (164-168, 170)

Intercellular communication at the synaptic or nerve ending is achieved, it is presently understood, through the action of chemical transmitters. For a substance to be considered a transmitter, the following criteria should be satisfied. It must be synthesized and stored in the presynaptic nerve terminal (not necessarily in vesicles). It should be released in adequate quantities on the stimulation of the presynaptic nerves. It must be capable of interacting with receptors on the post-synaptic neuron or effector cell and bring about transient alterations in the permeability of the post synaptic membrane towards ions in the immediate environment. Its action on post synaptic structures when applied directly should be identical with the normal transmitter action. In some cases at least, an inactivating enzyme should be present in the synaptic cleft. Blocking and competitive agents should affect its normal action, and its action on direct application in a similar manner. Acetylcholine, and the biogenic amines, noradrenaline, dopamine and serotonin, are strongly implicated in central transmission.

(vi) Amino acids as putative transmitters (164, 165, 168, 170, 233)

Glutamate, and related substances such as aspartate, excite many central neurons when iontophoretically applied. Since glutamate is involved in metabolism of cortical neurons, enzymes for its synthesis are obviously present in the brain, and although there is no evidence for the presence of a destructive enzyme, analogous to choline esterase in the synaptic cleft, a rapid uptake of excess glutamate into neighbouring nerve cells may serve to ensure the necessary brevity of its transmitter action. Like L-glutamate

and L-aspartate, DL-homocysteate and N-methyl aspartate reduce the threshold for firing and are therefore considered to be excitatory amino acids (164, 170). GABA and glycine are inhibitory amino acids, raising the threshold for firing, by hyperpolarization caused by an increase in the post synaptic intake of K^+ and/or chloride (170). Curtis and Johnston (170) have reviewed the evidence for the participation of amino acids as transmitters in the vertebrate and invertebrate nervous system. The mechanism of action of amino acids as transmitters is not known. It is believed that they cause changes in the ionic permeability of the post synaptic membrane by reactions with specific receptor sites.

(vii) Ca^{++} ions and excitation

There is a twenty-fold increase in calcium permeability accompanying the propagation of impulses along giant nerve fibres. The inflow of Ca^{++}, however, is too small to convey appreciable current or to modify the character of the action potential (172).

Nerve fibres fire spontaneously when external Ca^{++} is reduced. Conversely, a rise in external Ca^{++} tends to stabilize the nerve membrane and to raise the threshold for excitation (166-168). Ca^{++} may achieve this by blocking transmembrane channels to Na^+ by binding with the membrane phospholipid layer; dislocation of Ca^{++} from this bond might then permit free passage of Na^+ (174).

Ca^{++} is known to be required for transmitter release (173). It causes an increased release of acetylcholine at the neuromuscular junction. Mn^{++} and Mg^{++} reduce Ca^{++} entry and inhibit transmitter release (172, 173).

(viii) High K^+ ion concentration and excitation

High external K^+ causes depolarization (177). However, if the high K^+ is applied simultaneously over the whole surface of a fibre, excitability

is usually depressed or abolished. A maintained depolarization, produced by high K^+, raises the threshold for excitation and greatly affects the permeability properties of the nerve tissue to sodium and potassium ions (168). Sometimes the application of K^+ to excitable cells make them generate pulses. Stimulatory effects of this kind arise if the applied solutions do not penetrate uniformly into the tissue and one part of the nerve is depolarized much more than another (168).

In addition, to its depolarizing effect, K^+ appears to have a potentiating action on transmitter release (156, 178).

(ix) Cerebral cortex slices and excitation phenomena

The use of cerebral cortex slices to study excitation phenomena is rapidly gaining support. Li and McIlwain (179) used microelectrodes to demonstrate the maintenance of resting membrane potentials of -55 to -60 mV in cerebral cortex slices of the guinea pig, values approaching those observed in vivo. Gibson and McIlwain (180) have been able to correlate potential differences across cortical cell membranes, as calculated from the Nernst equation from electrolyte analysis, with potential differences measured directly by microelectrodes inserted into the incubated slices. Moreover, cortical cells are depolarized in vitro by an increased external K^+ or the application of electrical pulses, without losing the ability to repolarize on the removal of the applied stimuli (180). Yamamoto and McIlwain (181) obtained spike potentials in the isolated piriform cortex of guinea pig brain, which are said to be akin to action potentials (224, 264). The tetrodotoxin sensitivity of such evoked potentials in cerebral slices (265) lends support to this view. Similarly, changes in the tetrodotoxin-sensitive ionic balance of rat brain cortex slices (presumably at the neuronal membrane) on incubation under certain conditions, led Okamoto and Quastel (182) to suggest that action potentials may be generated in incubated brain slices.

Slices of brain actively accumulate serotonin (183), norepinephrine (184), acetylcholine (185) and amino acids (104-106, 113, 114, 231, 252, 295) against concentration gradients. Presumably, this may be one method for the removal of released transmitter substance from their site of action. Many studies have demonstrated that the stimulation of brain electrically or chemically (e.g., with protoveratrine) depolarizes the nerve membrane and markedly enhances the release of label from the brain slice pre-loaded with label transmitter substrate (e.g., 186, 187). Our earlier studies showed that the activation of the sodium current system in brain slices under certain conditions of incubation (182), concomitantly affects the release of endogenous amino acids from the tissue (188). These and later studies are described in detail in this thesis.

The above considerations (and others that will be apparent in due course) support the validity of employing the isolated brain slice for the study of nerve function and metabolism. Elliot, in his review (234) on "the use of brain slices", has described the usefulness of the tissue slice technique in the discovery and elucidation of metabolic processes.

1.9 Some properties of drugs and metabolic inhibitors used as tools for this investigation

Only a brief account of the properties of some of the chemical substances used as tools in these studies will be presented here.

(i) Tetrodotoxin (TTX)

Tetrodotoxin, a potent non-protein neurotoxin found in Japanese puffer fish and Californian newts, suppresses at concentrations as low as 0.3 μmolar the generation of action potentials in a variety of tissues including frog myelinated nerve fibres and lobster and squid giant axons, and hence the associated influx of Na^+ ions (190, 193, 194). Its mechanism of action

resembles that of local anesthetics but it has a potency more than a 100,000 times that of cocaine (189). It acts on the outer, rather than the internal, surface of the membrane selectively blocking the inward movements of Na^+ ions accompanying the generation of action potentials. It seems to have no effect on K^+ ion movements (195, 196).

Earlier studies of TTX concerned with its chemistry, pharmacology (in in vivo studies) and its electrophysiological effects using isolated nerve and muscle have been reviewed by a number of workers (189-192). Only recently has TTX been used in the study of metabolism and cationic transport processes in isolated brain tissue by Quastel and his co-workers, working mainly with rat brain (182, 188, 192, 197, 198, 205) and by McIlwain's group using chiefly the brain of the guinea pig (199, 201-204). While TTX has no effect on the rate of respiration of rat brain cortex slices incubated in a physiological glucose saline medium, it completely blocks the stimulated respiration due to the application of electrical impulses (197, 199) or the presence of protoveratrine (182), or when calcium ions are omitted from the incubation medium (197). However, it has no effect on the potassium stimulated respiration (197).

Application of electrical impulses (182, 202) or the presence of protoveratrine (182) brings about a TTX-sensitive influx of Na^{++} in brain cortex slices. The K^+ content of incubated electrically stimulated slices is greater in the presence of TTX than in its absence (199, 200). TTX has a similar effect with protoveratrine stimulated slices (182).

Chan and Quastel (198) showed that the inhibition of acetate oxidation by rat cerebral cortex slices is completely suppressed by TTX. They concluded that TTX exerts its action on acetate metabolism indirectly by its effects on Na^+ movement.

It is well known that L-glutamate excites nervous tissue (section 1.8

(vii)). An influx of Na^+ ions into incubated cerebral cortex slices takes place when 5 mM sodium L-glutamate is added to the incubation medium (95, 182, 201, 263). This is only partly inhibited by TTX (182) during a short initial period of incubation (202). According to Ramsay and McIlwain (203), low concentrations of TTX are capable of inhibiting Ca^{++} influx both in the presence or absence of L-glutamate. It also causes a detectable diminution in Ca^{++} efflux (203). Chelating agents, e.g., 5 mM EDTA bring about increases in the influx of Na^+ into, and efflux of K^+ from, incubated guinea pig cerebral cortex slices and these changes are partially prevented by TTX (204).

Recent experiments of Okamoto and Quastel (182) have shown that under a variety of incubation conditions, the increase of Na^+ influx and of water uptake that take place in rat brain cortex slices are suppressed either wholly or partially by small concentrations of TTX (3 μM). Their experiments led to the conclusion that action potentials, or activation of the Na^+ current are generated in brain cortex slices in vitro. This occurs for example, in the absence of glucose, or in the presence of 0.1 mM ouabain or of 10 μM protoveratrine or on the application of electrical impulses or according to Shankar and Quastel (192, 205) at the onset of anoxia. Under these conditions TTX affects both the cationic fluxes and water uptake. However, TTX has no effect on the enhanced Na^+ influx and water uptake in the presence of 30 μM 2, 4-dinitrophenol or of 100 mM KCl (182).

Recently, Shankar and Quastel (192, 205) have shown that TTX (2 μM) enhances the rate of anaerobic glycolysis in rat cerebral cortex slices when added before the onset of anoxia. They also showed that the effects of TTX on anaerobic glycolysis are specific for mature cerebral tissue and require the integrity of the brain cell for its action. The stimulatory effect of TTX on anaerobic glycolysis is indirect. It suppresses Na^+ influx into, and K^+ efflux from, cerebral cortex slices that occur at the onset of anoxia. The enhanced K^+/Na^+ concentration ratio activates the rate limiting pyruvate

kinase thereby stimulating the rate of anaerobic glycolysis. The effect of TTX on anaerobic glycolysis is reduced or abolished in the presence of protoveratrine, high K^+, NH_4^+ or L-glutamate (192).

In studies reported here, TTX will be used to inhibit Na^+-movement into brain cortex slices when these are incubated in such a manner as to bring about the activation of the sodium current at the cell membrane or the generation of action potentials.

(ii) Local anesthetics (166, 176)

Local anesthetics reversibly block the nerve impulse by reducing the membrane permeability to sodium ions. Increased extracellular sodium decreases anesthetic action. Most local anesthetics will depolarize the membrane at concentrations higher than are needed for blocking action, and will also slightly reduce the potassium permeability of the membrane. Lidocaine is said to be a hundred times more effective on Na^+ channels than on K^+ channels (176).

Local anesthetics behave like Ca^{++}, stabilizing the nerve membrane, raising the threshold for excitation, abolishing spontaneous activity, and blocking conduction without depolarization. Feinstein (206) states that local anesthetics may act primarily by inhibiting release of Ca^{++} from sites to which it is bound to the membrane. They thereby prevent secondary changes in the Na^+ and K^+ permeabilities, and consequently suppress the generation and propagation of the nerve impulse. The suggestion has been made that both local anesthetics and Ca^{++} act competitively on the system carrying Na^+ through the membrane. For example, increased extracellular Ca^{++} decreases procaine effects, whereas procaine completely reverses the depolarization caused by removal of external Ca^{++} (167, 176).

Local anesthetics are said to compete with acetylcholine for receptor sites on the post-synaptic membrane (176).

The results described above have been obtained chiefly in studies employing isolated nerve fibres. However, studies have also been carried out with brain cortex slices with similar results. Thus, Chan and Quastel (198) showed that cocaine (0.2 mM), lidocaine (0.5 and 1 mM), and procaine (1 and 3 mM) block the increased influx of Na^+ due to electrical stimulation without affecting either the content of Na^+ found in unstimulated brain tissue, or the activity of membrane Na^+, K^+-ATPase. These local anesthetics were also found to abolish the depressed oxidation of (1-^{14}C) acetate to $^{14}CO_2$, and the enhanced oxygen uptake brought about by the application of electrical pulses. Such effects were not obtained with the unstimulated slices at the concentrations studied. This led to the conclusion that local anesthetics at low concentrations act on sodium channels that become available following the application of electrical impulses. They inhibit electrically stimulated respiration presumably by blocking the influx of Na^+ ions and thereby the stimulated activity of the Na^+ sensitive membrane ATPase. This leads to suppression of the rate of formation of ADP that partly controls the rate of mitochondrial respiration. Thus, like TTX, local anesthetics exert their effects on metabolism indirectly, by their action on cationic movements in stimulated nervous tissue.

In studies reported here, lidocaine (0.5 mM) will be used as a representative of the local anesthetic group of drugs, for suppressing the activation of the Na^+ current system generated in brain slices incubated under specific conditions.

(iii) Protoveratrine

It is well known that veratrine alkaloids, of which protoveratrine is a member, generate action potentials in nervous tissue (167, 207). Shanes has shown that in resting nerve, veratrine alkaloids cause a net release of K^+ ions and an uptake of an equivalent amount of Na^+ ions (208). However, the mode of action of veratrine drugs is not yet clear. There is some evidence

suggesting that the basic effect of veratrine compounds is a displacement of Ca^{++} ions from the cell surface (209), thus affecting the stability and consequently the permeability of the excitable cell membrane to the flow of ions.

Wollenberger (211) showed that protoveratrine at low concentrations (about 2 μM) stimulates respiration and aerobic glycolysis, and inhibits anaerobic glycolysis in guinea pig brain cortex slices incubated in a physiological glucose saline medium. Under these conditions it also brings about a TTX-sensitive influx of Na^+ ions into, and efflux of K^+ ions from, rat cerebral cortex slices (182, 210). Such cationic fluxes are accompanied by an enhanced oxygen consumption (182). The enhanced uptake of oxygen due to protoveratrine is inhibited by TTX (182), cocaine (213) or malonate (226). Protoveratrine may also stimulate the increased respiration due to electrical stimulation (212). It increases yields of labelled glutamate, glutamine, GABA and aspartate from labelled glucose in incubated brain slices, and these effects are reversed by cocaine (213).

As protoveratrine has effects on incubated brain slices closely resembling those of electrical impulses (211, 182), and as it is known to generate action potentials in nerve tissue (167, 207), it will be used extensively in studies reported in this thesis.

(iv) Barbiturates

There are at present two schools of thought concerning the mode of action of barbiturates on nerve function. According to one group of workers, barbiturates exert their effects by "diminishing ionic movements of excitation" (214), or "on membrane parameters" (215), or by "cutting down inward sodium leakage" (216). However, another group of workers have obtained results not clearly explicable by such statements. With the barbiturates amytal (0.25 and 0.5 mM) and pentothal (0.1 and 0.2 mM) at concentrations that do not affect the content of $^{22}Na^+$ or the respiration of unstimulated

rat brain cortex slices incubated for one hour, Chan and Quastel (198) found no abolition of the enhanced influx of $^{22}Na^+$ following electrical stimulation. Hillman et al. (217) similarly found that phenobarbital was without effect on the Na^+ influx obtained by the application of electrical impulses to guinea pig cerebral cortex slices. Thus, the effects of barbiturates under the given experimental conditions were different from those of local anesthetics or of TTX which completely block the increased influx of $^{22}Na^+$ that occurs on the application of electrical impulses. Like local anesthetics or TTX, barbiturates at small concentrations nevertheless suppress electrically stimulated brain respiration. None of these drugs at these concentrations affects the activity of membrane bound Na^+, K^+-ATPase (198).

Unlike the local anesthetics or TTX, barbiturates do not alleviate the depressed oxidation of (1-^{14}C) acetate to $^{14}CO_2$ brought about by the influx of Na^+ and the efflux of K^+ due to the application of electrical impulses (198). However, in contrast to the local anesthetics and TTX, the barbiturates inhibit the rate of (1-^{14}C) acetate oxidation to $^{14}CO_2$ in unstimulated rat brain slices. Chan and Quastel (198) have concluded that barbiturates (at the concentrations quoted and under the incubation conditions studied) do not directly affect the movement of Na^+ and K^+ across the brain cell membrane; rather, they act by suppressing cell energetics. Such suppressing action also explains the inhibitory effects of barbiturates on the stimulation of rat brain cortex respiration brought about by the application of electrical impulses or by the presence of high K^+ concentrations (218), or with protoveratrine, or in a medium devoid of Ca^{++} (210). It is well known following the work of Michaelis and Quastel (219), and Ernster and co-workers (220, 221), that the barbiturate, amytal, as well as certain other hypnotics at low concentrations, suppress the oxidation of NADH and hence the generation of ATP in the cell.

In view of the great amount of work which has been carried out with amytal, both in neurochemical and neurophysiological studies, it has been used in the studies reported in this thesis as representative of barbiturates

in work on the transport and metabolism of amino acids and ammonia in brain slices incubated under conditions leading to the activation of the sodium current or to the generation of action potentials.

(v) Cardiac glycosides - Ouabain

The process whereby Na^+ is extruded from the cell and K^+ accumulated against a concentration gradient is usually referred to as the "Sodium-Pump". This carrier mediated process maintains the polarity of non-excitable membranes, and restores that of excitable ones which have undergone depolarization, during the generation of nerve impulses. Repolarization is achieved at the expense of metabolic energy derived from aerobic metabolism. Thus, inhibition of ATP formation by anoxia, cyanide, DNP, or in the absence of glucose, causes a redistribution of cations eliminating their concentration gradients (104, 105). Active uptake of K^+ is Na^+ dependent. Moreover, the effects of increased K^+ on the metabolism of intact tissue (e.g. increased respiration) requires the presence of Na^+ (104, 218). Thus, the kinetics of brain metabolism are greatly influenced by the operation of the sodium-pump which controls the cellular levels of Na^+ and K^+. It is known, for example, that increased Na^+ suppresses acetate oxidation by its inhibitory effect on acetate conversion to acetyl CoA (198), and that increased K^+ enhances pyruvate kinase activity (192, 262, 299, 300) and the oxidation of acetate (198).

Skou (222, 223) reported that a membrane bound enzyme (ATPase) that hydrolyzes ATP to ADP and Pi, requires Na^+ and K^+ for activation and is dependent on the presence of Mg^{++}. This Na^+, K^+-activated Mg^{++} dependent ATPase and the active transport of Na^+ and K^+ across the membrane, have many features in common, namely, their location at the cell membrane, their activation by the simultaneous presence of both Na^+ and K^+, their requirement for energy, and inhibition by ouabain (104, 105, 223).

Ouabain is held to act (see references 115, 262) by inhibiting the K^+-activated dephosphorylation (Reaction 11) of an intermediate formed from ATP and the enzyme ATPase (En) in the presence of Na^+ (Reaction 10).

$$En + ATP + Na^+_{in} \longrightarrow (En \sim P)\ Na + ADP \qquad (10)$$

$$(En \sim P)\ Na \xrightarrow{K^+out} En + Pi + Na^+_{out} + K^+_{in} \qquad (11)$$

Much of the cellular energy is said to involve the operation of the sodium-pump (see references 104, 224). ADP released by the action of the Na^+, K^+-activated ATPase controls energy production by enhancing respiration. This it does through the reaction: $NADH + ADP + Pi \longrightarrow NAD^+ + ATP$. However, in the presence of ouabain, when both cation transport and the ATPase activity associated with it are inhibited, there is not necessarily a concomitant diminution in the respiration of incubated cerebral cortex slices (112, 182, 224). Inhibition may be observed in incubations of lengthy durations (as observed in our studies). In an experiment of one hour there is only a small drop in the ATP levels of rat brain cortex slices incubated in presence of 0.1 mM ouabain (115). Presumably, when high energy levels fall below a certain level respiratory inhibition by ouabain takes place (225). This view is supported by the observations that ouabain suppresses the stimulated respiration due to the absence of Ca^{++} (224) or the presence of high K^+ (112) or on the application of electrical impulses (200) and in our studies in the presence of 5 μM protoveratrine (by 48%). Such conditions are known to bring about a fall in the cellular levels of high energy compounds.

Tower (224) has shown that, in a medium devoid of Ca^{++}, the calcium content of cat cortex slices incubated for one hour falls to 30 per cent of the control levels whether or not ouabain is present. In the presence of Ca^{++}, however, ouabain significantly enhances the tissue level of Ca^{++} by 36 per cent — which primarily occurs in the mitochondrial compartment. This could

be due either to an increased membrane permeability to Ca^{++} or to an inhibition of the active transport mechanism for the extrusion of calcium.

It is a well known fact that ouabain inhibits the active transport of many compounds of biological interest (104, 105). It has been mentioned already that ouabain inhibits the accumulation of amino acids against concentration gradients (section 1.6). This is so because active transport of materials across membranes is a process dependent on the operation of the sodium-pump (104). One result of the inhibition of active transport by ouabain or by Na^+ lack, is leakage of amino acids and other substances from the brain (104, 112, 280).

The sodium influx and the water uptake that occur in brain cortex slices of the rat incubated in the presence of 0.1 mM ouabain is partly suppressed by TTX (182). A concomitant retention of K^+ may occur with TTX under these conditions.

In the work presented in this thesis ouabain is mainly used

(a) to throw light on the control mechanism for glutamine synthesis in brain cortex slices (Chapter 4);

(b) to study transport processes of ammonia and amino acids in isolated brain (Chapters 5 and 6); and

(c) to block the re-uptake process of amino acids that are released from brain slices incubated under conditions leading to the generation of action potentials or the activation of sodium current at the brain cell membrane (Chapters 7 and 8).

(vi) Miscellaneous

Additional metabolic inhibitors have also been used in the studies reported in this thesis.

(a) Malonate, well known to inhibit the operation of the citric acid cycle by competition at the succinic dehydrogenase stage (228).

(b) Fluoroacetate, which forms fluorocitrate and inhibits the operation of the citric acid cycle by inhibition of aconitase (63, 150).

(c) Methionine sulfoximine, which inhibits the synthesis of glutamine by competitive inhibition of glutamine synthetase (15, 65, 146).

(d) Amino oxyacetate, which inhibits enzyme systems requiring pyridoxal derivatives as co-enzymes. It is used in these studies mainly for suppressing the conversion of glutamate to aspartate by transamination (147, 279, 304).

(e) 2, 4-Dinitrophenol, well known to uncouple oxidation from phosphorylation, and which leads to low ATP levels (115).

(f) Ethane dioxybis (ethylamine) tetra acetate (EGTA), which specifically chelates calcium ions and is used for complete removal of Ca^{++} from the incubation medium (306, 307).

1.10 Objectives of the present work

The aim of the present investigation has been to throw further light on the processes controlling ammonia formation and accumulation in brain and its effects on brain metabolism, on the processes concerned with amino acid fluxes in brain under various conditions associated with increased nerve activity, and on the specific locations of amino acids in the brain.

2. MATERIALS AND METHODS

2.1 Animals

Adult rats (usually male) of the Wistar strain, weighing 150-200 g, were used and were obtained from the Vivarium, Department of Zoology, or from the Animal Unit, Faculty of Medicine, University of British Columbia. In some experiments 2-day old infant rats of the same strain were used. All animals had free access to food and water. Infant rats were separated from their mothers before the start of the experiment.

2.2 Chemicals

All common laboratory chemicals were of "reagent grade" and were used without further purification.

Sodium (U-^{14}C) glutamate was obtained from Volk Radiochemical Co., Illinois, U.S.A., and ^{22}NaCl was obtained from the Radiochemical Centre, Amersham, England.

Glucose-6-phosphate dehydrogenase (E.C.1.1.1.49) (from yeast), and hexokinase (E.C.2.7.1.1.) (from yeast) were obtained from Calbiochem, Los Angeles, California.

Tetrodotoxin and sodium fluoroacetate were obtained from Calbiochem; lidocaine (base) from Astra Pharmaceuticals; amytal from Eli Lilly Co., Montreal; protoveratrine from K&K Laboratories, Plainview, N.Y.; ouabain from Nutritional Biochemicals Coporation, Cleveland, Ohio; amino oxyacetic acid from Eastman Kodak Co., Rochester, N.Y.; disodium malonate from Matheson Coleman and Bell, Norwood, N.J.; EGTA from Koch and Light, Colnbrook, Buckinghamshire, U.K.; DL-methionine-DL-sulfoximine from California Coporation for Biochemical Research, Los Angeles; triethanolamine and tris ATP from Sigma Chemical Co., St. Louis, Mo.,; and $NADP^+$ from Calbiochem.

2.3 Tissue preparation

Rats were killed by decapitation. The brains were removed and cerebral cortex slices were prepared using a Stadie-Riggs tissue slicer. In some experiments, rat kidney cortex slices were prepared in a similar manner. One dorsal and one lateral brain slice (first slices) weighing a total 80-100 mg initial wet weight and not more than 0.4 mm thick, were used for incubation. The slices were quickly weighed on a torsion balance (to give the initial wet weight) and suspended in chilled manometric vessels containing the appropriate incubation media.

Infant (2-day old rat) brain cortex slices were prepared by cutting by hand, only slices of the temporal-parietal portion of each hemisphere being used. As the infant brain is small, it was found expedient to use slices up to 1 mm thick. According to Itoh and Quastel (235), the rates of oxygen consumption or $^{14}CO_2$ formation from ^{14}C-labelled substrates by infant rat brain cortex slices are not significantly lower with thickness of 0.9-1 mm than those with thicknesses of 0.4 - 0.5 mm, which is probably largely due to the fact that the rate of respiration in infant rat brain slices is sufficiently low to allow adequate oxygenation of the brain cells even with slices 1 mm thick (237).

2.4 Media compositions and incubation procedures

(i) Krebs-Ringer phosphate medium

This had the following composition unless otherwise stated:

128 mM - NaCl; 5 mM - KCl; 2.8 mM - $CaCl_2$; 1.3 mM - $MgSO_4$; and 10 mM - Na_2HPO_4 adjusted to pH 7.4 with HCl. Glucose when added was 10 mM. In a K^+-free medium KCl was omitted. In a Ca^{++}-free medium $CaCl_2$ was omitted. To ensure complete absence of free calcium ions in the medium during incubation, 3 mM EGTA was added when stated. Various substances were added to the incubation medium in a final volume of 3 ml. Cups

containing rolls of filter paper moistened with 0.2 ml 20% KOH were placed in the centre wells to absorb evolved CO_2.

The incubation was carried out in a conventional Warburg manometric apparatus in an atmosphere of O_2 at 37°C for a period of usually one hour. The flasks were oxygenated for 5 minutes and thermally equilibrated for 7 minutes prior to the commencement of the incubation. Readings of the rates of oxygen consumption were routinely taken to ensure that the brain slices under investigation exhibited normal respiratory activities.

(ii) Krebs-Ringer bicarbonate medium

This had the following composition:

128 mM - NaCl; 5 mM - KCl; 2.8 mM - $CaCl_2$; 1.3 mM - $MgSO_4$; 1.4 mM - KH_2PO_4; and 28 mM - $NaHCO_3$; in a final volume of 3 ml. Gassing was carried out for 5 minutes either with O_2/CO_2 (95/5%) or N_2/CO_2 (95/5%) mixtures, depending on whether the incubation was carried out aerobically or anaerobically respectively. After 7 minutes thermal equilibration the incubation was carried out for one hour at 37°C. It should be stated that no significant differences were found in the amino acid patterns, or the cationic fluxes, of rat brain cortex slices incubated aerobically in a Krebs-Ringer medium whether this was buffered with phosphate ions or with bicarbonate ions.

(iii) Medium II

This medium was a Na^+-rich, Ca^{++} and K^+-free medium having the following composition:

178 mM - NaCl; 1.3 mM - $MgSO_4$; 10 mM - Na_2HPO_4 adjusted to pH 7.4 with HCl.

2.5 Amino acid analyzer estimations

(a) Sample preparation

(i) Tissue sample preparation

At the end of the period of incubation, the Warburg manometric vessels were placed in ice, the tissue quickly removed, and adhering medium drained off by blotting with filter paper. The slices were quickly weighed (to give the final wet weight) and then homogenized in 3 ml of 5% TCA. The debris, separated from the supernatant by centrifugation and decantation, was washed with 5% TCA, recentrifuged, and the washings added to the supernatant. The supernatant was then extracted three times with equal volumes of diethyl ether to remove TCA. Traces of ether left behind were evaporated by carefully blowing in nitrogen gas (bubbled through NH_2SO_4 to ensure complete absence of possible contaminating ammonia) and the samples were made up to a known volume (4-6 ml).

(ii) Medium sample preparation

At the end of the incubation 2.0 or 2.5 ml of the medium were mixed with 2 or 2.5 ml of 10% TCA and centrifuged. The supernatant was extracted with diethyl ether, the ether removed as described earlier, and samples were made up to known volumes (4-6 ml).

(b) Sample analysis

Amino acids present in aliquots (1-2 ml) of the samples were separated on appropriate columns of a Beckman 120B, Amino Acid Analyzer, operated as described in the Beckman manual. The accelerated method was used to separate the amino acids which were detected by the ninhydrin reaction.

(i) Acidic and neutral amino acid analyses

The amino acids, taurine, aspartic acid, threonine, glutamine and serine, glutamic acid, glycine, and alanine, were separated on a 50 x 0.9 cm column of sulfonated polystyrene−8% divinyl benzene copolymer ion exchange resin (Type 50A - particle size 25-31 μ) at 50°C by elution with 0.067 M sodium buffer pH 3.28. Under these conditions glutamine, asparagine and serine co-elute. The free asparagine pool in brain is very small (0.1 - 0.2 μmole/g initial wet wt) when compared with those of glutamine and serine (22, 88, 239), and was not estimated. Values for serine were obtained in a number of experiments by the following procedure. The sample containing glutamine and serine was heated with 10% TCA at 75°C for 75 minutes (22). This brings about hydrolysis of the glutamine while the serine is unaffected. TCA is removed with diethyl ether and the sample prepared as described earlier. Glutamine values are obtained by estimating either (1) the difference between the areas of the peaks, at the glutamine-serine position of the amino acid profiles, obtained before and after hydrolysis, or (2) the increase in the area of the ammonia peak. It should be mentioned that glutamine hydrolyzed by this method yields ammonia and pyrrolidone carboxylic acid. A small proportion of this cyclic compound is further hydrolyzed to glutamic acid (238). Thus, we find that about 22 per cent of the glutamine hydrolyzed appears as glutamic acid under these conditions. This is in accord with the results of Dobkin and Martin (236).

(ii) γ-Aminobutyric acid and ammonia

An aliquot (usually 1 ml) of the sample was eluted with 0.127 M sodium citrate buffer pH 4.26 at 50°C from a 5.5 x 0.9 cm column of sulfonated polystyrene - 8% divinyl benzene copolymer (Beckman resin, Type 15A - particle size 19-25 μ). The advantage of this method for ammonium assay is the separation of labile ammonia producing components, like glutamine, from free ammonia in the sample. There is also total recovery of ammonia from the column on elution with buffer resulting in greater accuracy in the

ammonia estimation.

(iii) N-Acetylaspartate

This amino acid derivative is measured as aspartate after hydrolysis with 0.5 volume of 5.5 NHCl at 100°C for 30 minutes, followed by removal of HCl. Under these conditions, glutamine is recovered as glutamate and ammonia. The amino acids, taurine,aspartate, glutamate,glycine and alanine, were unaffected by this procedure. HCl is removed by evaporating the sample to dryness. The dried sample is dissolved in 0.067 M sodium citrate buffer pH 2.2, and analyzed with the Amino Acid Analyzer.

(iv) Calculations

The areas of the amino acid peaks were measured by the height-width method. Here the height of the peak was multiplied by the width which is measured at half height. The width of the peak was measured by counting the number of dots printed above the half height of the peak. The constant (i.e., area of the peak) per μmole for each amino acid was obtained from an amino acid profile of a standard amino acid mixture. From these values the concentration of each amino acid was computed. The constants and peak positions were invariably determined for each new batch of buffer or ninhydrin reagent prepared.

After the estimation of amino acids in a sample was completed, the 50 cm column (and sometimes the short column) was regenerated with 0.2 N NaOH and equilibrated with the appropriate buffer before reuse.

2.6 Water uptake by brain cortex slices

Immediately after incubation the slices were removed from the media and drained. They were spread without folding on an ice-cold clean glass surface and excess of fluid around the slices and on the glass was absorbed

with strips of filter paper. The difference between the final wet weight and the initial wet weight gives a measure of the increase of water uptake.

The total water content of the tissue is equal to the sum of the water taken up by the tissue during the period of incubation and the amount of water originally present (section 2.8).

2.7 Oxygen uptake by brain cortex slices

Oxygen uptakes (Q_{O_2} were calculated as given in "Manometric Techniques" (240) in terms of µl/mg dry wt tissue, and converted to µmole/g initial wet wt on multiplication by a factor which for the adult rat was 200/22.4 and for the infant rat 120/22.4.

2.8 Dry weight of brain cortex slices

Weighed cortex slices were dried at 110°C to constant weights. For the adult rat, dry wt = 20% wet wt; for 2-day old infant rat, dry wt = 12% wet wt. with a standard deviation $\pm$ 5%.

2.9 $^{22}Na^+$ influx into brain cortex slices.

In experiments on $^{22}Na^+$ fluxes, 0.5 µCi of $^{22}Na^+$ was placed in the main compartment of the Warburg manometric vessel with 3 ml of the incubation medium. Sodium ion concentration in the medium was usually 148 mequiv/l unless otherwise stated. After the incubation, the brain slices were quickly removed, lightly blotted with strips of filter paper to remove adhering fluid, weighed to estimate swelling, and homogenized in 3 ml of 5% TCA. After standing in the cold for two hours with occasional mixing, the homogenate was centrifuged.

For the estimation of radioactivity due to $^{22}Na^+$ in the medium at the

end of the incubation period, 0.4 ml medium was mixed with 1.6 ml 5% TCA and centrifuged.

0.5 ml aliquots of the TCA extracts of tissue or medium was mixed with 10 ml scintillation liquid and assayed for $^{22}Na^{+}$ using the appropriate settings on a Nuclear Chicago Model Mark I liquid scintillation counter. The value of counts per minute (cpm) was converted to that of disintegrations per minute (dpm) by multiplication with the factor 100/percentage efficiency of counting. Percentage efficiency was obtained by the channel ratio method and was generally about 30 per cent for $^{22}Na^{+}$.

The concentration of $^{22}Na^{+}$ in the tissue slice was calculated from the radioactivity found in unit weight of the tissue slice (dpm/g) divided by the specific radioactivity of $^{22}Na^{+}$ in the incubation medium (dpm/μ equiv sodium in 1 ml incubation medium).

2.10 Experiments with sodium L-[U-^{14}C] glutamate

In experiments with labelled glutamate, L-[U-^{14}C] glutamate of specific activity 0.067 μCi/μmole (or 122,300 cpm/μmole) and concentration of 5 mM was placed in the main compartment of Warburg manometric vessels containing 3 ml incubation medium. At the end of the incubation period, the brain slices were removed and counting was carried out on the TCA extracts of tissue and medium as mentioned earlier in section 2.9. Counting efficiency for ^{14}C was found to be 83 per cent.

2.11 Scintillation liquid composition

This consisted of a mixture of equal volumes of toluene, dioxane and 95% (v/v) ethanol containing 2, 5 diphenyloxazole (5g/l), 1, 4-bis-(4-methyl-5-phenyloxazol-2-yl)-benzene (0.05 g/l) and napthalene (80 g/l).

2.12 Na^+ and K^+ assay by flame-photometry

The tissue contents of unlabelled Na^+ and K^+ were determined as follows. Brain cortex slices, either initially or at the end of the incubation period, were homogenized in 3 ml 5% TCA after removing adhering fluid by strips of filter paper and weighing to determine the extent of water uptake. After standing in the cold for two hours the homogenates were centrifuged and the supernatants were diluted with distilled water to give final cation concentrations of (0.05 - 0.1 μ equiv/ml). The atomic absorptions (% absorbance) were measured at 294.3 and 383.3 nm for Na^+ and K^+ respectively, with a Perkin-Elmer model 303 atomic absorption spectrophotometer. The cation concentrations were computed from standard plots of known concentrations of Na^+ and K^+ versus Absorbance, carried out simultaneously with each set of determinations. Generally good agreement was found between Na^+ contents of a sample obtained using the flame photometer and that obtained by measuring ^{22}Na (also 182).

2.13 Determination of ATP concentrations

ATP was determined according to the method of Greengard (241). The tissue slices at the termination of the experiment were extracted with 2 ml 6% (w/v) perchloric acid and centrifuged. One ml of the supernatant was neutralized with K_2CO_3 and made up to 4 ml. 0.3 ml aliquots were taken for each assay in a final volume of 2 ml containing 0.215 mmole glucose; 9.4 nmole $NADP^+$; 3 μmole $MgCl_2$; 1.2 μmole EDTA (pH 7.4); 24 μmole triethanolamine buffer (pH 8.0); 0.5 units hexokinase; and 1 unit glucose-6-phosphate dehydrogenase. Incubation was carried out at room temperature for 30 minutes. NADPH was measured with an Aminco-Bowman spectrofluorimeter with excitation wavelength 365 nm and fluorescence wavelength 460 nm. One unit of hexokinase was that amount of enzyme catalyzing the conversion of 1 μ mole of glucose per minute at pH 8.5 at 25°C. One unit of glucose-6-phosphate

dehydrogenase was that amount of enzyme catalyzing the reduction of 1 μmole of $NADP^+$ per minute at pH 7.4 at 25°C.

2.14 Assay of Na^+, K^+-ATPase of brain homogenates

(i) Medium composition

The medium used for the assay of Na^+, K^+-ATPase had the following composition: 0.1 mM - disodium EDTA; 1 mM - $MgCl_2$; 58 mM - NaCl; 15 mM - KCl; 3 mM - tris ATP; 95 mM - tris - Cl (pH 7.6); whenever the concentration of Na^+ or K^+ was changed, the tonicity of the medium was maintained by altering an equivalent amount of tris - Cl in the medium.

(ii) Incubation procedure

Incubation was carried out in centrifuge tubes, each containing 3 ml of the medium, with an amount of brain cortical homogenate in cold distilled water equivalent to 1 mg of the original wet tissue-cortex slice. The tubes were shaken in a water bath shaker at 37°C for one hour.

(iii) Estimation

Na^+, K^+-ATPase activity was measured in terms of the amount of phosphate produced by the hydrolysis of ATP by the enzyme preparation. The hydrolysis was brought about by two components: (1) a Mg^{++}-activated ATPase; (2) a Na^+, K^+-stimulated ATPase requiring Mg^{++} for activity. The incubation was terminated by the addition of 0.2 ml 100% (w/v) TCA, and the mixture was centrifuged. To 0.5 ml of the supernatant was added 2.0 ml distilled water and 2.5 ml of a colour reagent prepared by dissolving 4.0 g $FeSO_4$ in 100 ml of 1% ammonium molybdate in 1.15 N H_2SO_4 according to the method of Bonting et al.(242). The resulting blue colour was read within 2 hours at 700 nm on a Beckman Model D U spectrophotometer. Substrate and enzyme blanks were included in each determination, together with phos-

phate standards to convert optical density to μmole Pi (phosphate) released by one mg initial wet wt tissue/hour at 37°C. The Na^+, K^+-ATPase activity was estimated as the difference between the activity of brain homogenate preparation incubated in the presence of Na^+, K^+ and Mg^{++} and that incubated in the absence of Na^+ and K^+. In the following text, only mean values for Pi liberation are given, since the deviations from the mean were never greater than ± 5 per cent.

2.15 Isolation of synaptosomes from rat brain cortex

Synaptosomes (or pinched-off nerve terminals) were isolated essentially by the method of Gray and Whittaker (243). Brain cortex of the rat was homogenized in 0.32 M sucrose (10% w/v), in a teflon homogenizer revolving at 1725 rpm (5 up-down strokes), and centrifuged at 1,000 g for 10 minutes. The crude nuclear pellet separated from the supernatant-S_1, was washed twice by resuspension in 0.32 M sucrose and recentrifuged as before. The pellet - P_1 obtained in the final washing containing large myelin fragments, nuclei and cell debris, was discarded. The washings were mixed with supernatant-S_1 and centrifuged at 17,000 g for one hour. The supernatant-S_2 containing microsomes and soluble cell constituents, was discarded. The crude mitochondrial pellet-P_2 (containing small myelin and membrane fragments, mitochondria and synaptosomes) was suspended in 0.32 M sucrose and centrifuged at 100, 000 g for 45 minutes into a density gradient consisting of equal volumes of 0.8 M and 1.2 M sucrose. The following fractions were obtained.

A. A fraction floating between 0.32 and 0.8 M sucrose (small myelin fragments). This was discarded.

B. A fraction floating between 0.8 and 1.2 M sucrose (SYNAPTOSOMES). This fraction was carefully removed with a pasteur pipette diluted and centrifuged in isotonic sucrose (0.32 M). The pellet obtained

was suspended in isotonic Krebs-Ringer phosphate medium and used.

C. A pellet, below 1.2 M sucrose, containing mitochondria was discarded.

The incubation procedure for synaptosomes was the same as that described for brain cortex slices (section 2.4 (i)). However, the Warburg manometric vessels were shaken very slowly to maintain the integrity of the synaptosome. At the end of the incubation period, TCA was added to give a final concentration of 5 per cent, and samples were prepared for amino acid analysis as described in section 2.5. Prior to incubation, protein was measured, in an aliquot of the synaptosomal suspension used for incubation, as follows.

2.16 Protein estimation

The method of Lowry et al. (249) using the Folin-Ciocalteu reagent (250) was employed for estimating protein, the blue color formed being read at 750 nm on a Beckman Model DU Spectrophotometer. Bovine albumin was used as a standard.

2.17 Reproducibility of results

Each experiment was carried out at least four times and the results found to be reproducible. Controls were always run simultaneously. In our work results are expressed as means of the values obtained and the standard deviations from the mean are given.

2.18 Explanation of various terms used in this thesis

(i) Initial values

"Initial values" are the values (of NH_4^+, Na^+, K^+ or amino acids) obtained prior to incubation.

(ii) Total values

The term "total" has been used as follows:

(a) Total values (i.e., those of tissue + medium) are the sums of the tissue and medium concentrations of a particular substance (e.g., ammonia or amino acid) expressed as μmole/g initial wet wt.

(b) Sometimes it has been used to express the sum of a number of constituents in a given tissue or medium; e.g., total tissue NH_4^+-N + amino acid-N, or total tissue $Na^+ + K^+$ contents. These expressions of total values are made clear in the text.

(iii) Adjustment

"Adjustment" is a term used to signify a change made to maintain the isotonicity of the incubation medium. For example, (a) when substances in the form of their sodium salts (e.g., sodium L-glutamate) are added to the incubation medium, the medium Na^+ ion concentration is maintained at 148 μ equiv per ml by appropriate reductions in the medium NaCl; (b) when increasing concentrations of KCl are added to the medium, the medium is "adjusted" by an equivalent reduction in the NaCl content, thus maintaining the total medium ($Na^+ + K^+$) ion concentration at 153 μequiv per ml.

(iv) Ammonia and ammonium ion

Since the pKa for the reaction

$$NH_4^+ \rightleftharpoons NH_3 + H^+$$

is 9.3 at physiological pH (7.4) at which our experiments are carried out, a large proportion (97%) of the total (NH_3 + NH_4^+) is present in the form of the ammonium ion (278). When the terms "ammonia" and "ammonium ions" are used in the following text, it is understood that the former term represents ammonium ions.

(v) Flux

The terms "influx" and "efflux", in this thesis, are used to describe, respectively, the "net" inward or outward movement of a substance from the tissue in a given interval of time.

(vi) Action potentials

The "generation of action potentials" is used interchangeably with the "activation of the Na^+-current system". It is presumed to occur in brain cortex slices under certain conditions of incubation and is identified by sensitivity to TTX (section 1.8 (viii)).

3. AMMONIA FORMATION IN BRAIN IN VITRO

3.1 Ammonia and amino acid contents of rat cerebral cortex slices initially, and after incubation in the presence or absence of glucose

Values of the contents of amino acids in rat brain cortex slices, freshly prepared and immediately prior to incubation, are given in Table 2. These values are in agreement with those already recorded in the literature (125-131).

Total (tissue + medium) values for ammonia and amino acids found on incubating brain slices in Krebs-Ringer phosphate medium in O_2 at 37°C for various periods of time, with or without the addition of 10 mM glucose, are also recorded in Table 2. The following conclusions may be made.

(i) Changes in the NH_4^+ and amino acid contents of brain slices incubated in the presence and absence of glucose.

The formation of (16.81 - 6.33) = 10.48 μmole ammonia/g tissue is suppressed in the presence of glucose in a one hour incubation period. This suppression by glucose is reflected (1) in an enhanced glutamate content, viz., (10.11 - 3.55) = 6.56 μmole/g, and (2) in an enhanced glutamine content, viz., (6.35 - 1.79) = 4.56 μmole/g (corresponding to 9.12 μatom amino acid-N/g) which together gives an increase of 15.68 μatom amino acid-N/g. On subtracting from this value, the value due to a diminished aspartate level, viz., (9.13 - 4.14) = 4.99, a value of 10.69 μatom amino acid-N/g is obtained. This completely accounts for the suppression of ammonia production by glucose. There are relatively small changes in the levels of other amino acids under these conditions. Therefore, it seems that the fall in the level of NH_4^+ due to glucose is about equal to the rise in glutamate plus the rise in glutamine minus the fall in aspartate levels. A similar conclusion may be drawn on considering the ammonia, glutamate, glutamine and aspartate levels of brain cortex slices incubated for four hours. These calculations are given in Table 2A.

TABLE 2. Ammonia and amino acid changes in adult rat brain cortex slices on incubation in the presence or absence of glucose.

Rat brain cortex slices were incubated in Krebs-Ringer phosphate medium with or without glucose (10mM) in O_2 at 37°C for varying periods of time. Initial values (i.e. values prior to incubation) of ammonia and amino acids and the total (tissue + medium) values on incubation are expressed as μmole/g initial wet wt.

	INITIAL	INCUBATION PERIOD					
		15 min.	30 min.	One hour		Four hours	
		No Glucose	No Glucose	No Glucose	Glucose	No Glucose	Glucose
Glutamate	11.83 ± 1.09	8.10 ± 0.06	5.58 ± 0.18	3.55 ± 0.17	10.11 ± 0.36	2.57 ± 0.24	6.44 ± 0.51
Glutamine	4.40 ± 0.15	3.47 ± 0.10	2.07 ± 0.15	1.79 ± 0.20	6.35 ± 0.18	2.10 ± 0.20	11.40 ± 0.50
GABA	2.01 ± 0.10	2.56 ± 0.26	2.95 ± 0.07	2.32 ± 0.32	2.81 ± 0.08	1.40 ± 0.15	1.75 ± 0.18
Aspartate	3.36 ± 0.34	8.14 ± 0.10	8.70 ± 0.05	9.13 ± 0.47	4.14 ± 0.46	8.47 ± 0.54	2.49 ± 0.14
Alanine	0.61 ± 0.08	0.86 ± 0.04	0.90 ± 0.05	0.73 ± 0.05	1.29 ± 0.01	1.67 ± 0.10	1.04 ± 0.10
Glycine	0.79 ± 0.06	1.00 ± 0.03	1.13 ± 0.02	1.47 ± 0.03	1.53 ± 0.11	2.15 ± 0.19	1.68 ± 0.15
Serine	1.25 ± 0.05	1.55 ± 0.09	1.88 ± 0.14	2.23 ± 0.03	1.82 ± 0.08	3.32 ± 0.19	2.27 ± 0.16
Threonine	0.52 ± 0.11	0.74 ± 0.01	0.89 ± 0.03	0.95 ± 0.05	0.67 ± 0.04	1.51 ± 0.15	1.10 ± 0.20
Taurine	5.81 ± 0.71	5.62 ± 0.28	6.02 ± 0.13	5.57 ± 0.39	5.44 ± 0.24	6.13 ± 0.50	5.72 ± 0.29
Lysine	0.2	0.25	0.25	0.35 ± 0.11	0.25 ± 0.03	1.50 ± 0.30	0.80 ± 0.10
Ammonia	1.58 ± 0.14	8.32 ± 0.28	11.90 ± 0.40	16.81 ± 0.07	6.33 ± 0.45	24.20 ± 1.26	6.82 ± 0.45
Total: NH_4^+-N + amino acid -N	36.76	44.08	44.28	46.69	47.09	57.12	52.91

TABLE 2A. Changes in the glutamate, glutamine, aspartate and ammonia contents of brain cortex slices incubated in the presence or absence of glucose.

Total (tissue+medium) values of ammonia and amino acids (μmole/g initial wet wt.) of brain cortex slices used for these calculations were taken from TABLE 2.

	One Hour			Four Hour		
	No Glucose	Glucose	Δ-NH_2	No Glucose	Glucose	Δ-NH_2
Glutamate	3.55	10.11	-6.56	2.57	6.44	-3.87
Glutamine	1.79	6.35	-9.12	2.10	11.40	-18.60
Aspartate	9.13	4.14	+4.99	8.47	2.49	+5.98
Ammonia	16.81	6.33	+10.48	24.20	6.82	+17.23
Total -NH_2	33.07	33.28	-0.21	39.44	38.55	+0.89

From the above considerations, it is evident that much of the ammonia turnover in incubated brain slices is due to changes in brain amino acids.

(ii) Changes in the initial NH_4^+ and amino acid contents of brain cortex slices on incubation.

In the absence of glucose, the rise in ammonia from brain slices incubated for one hour is accompanied by significant falls in the glutamate and glutamine levels from their initial values. There is an accompanying rise in the level of aspartate. Thus, in the absence of glucose there is a liberation of (16.81 - 1.58) = 15.23 μmole ammonia/g tissue/hour. Concomitantly, glutamate levels fall by (11.83 - 3.55) = 8.28 μmole/g, and glutamine falls by (4.40 - 1.79) = 2.61 μmole/g. The diminution in the levels of these amino acids could account for about 90 per cent (or 13.5 μatoms-N/g) of the total ammonia liberated. However, it is probable that some of the aspartate formed, i.e., (9.13 - 3.36) = 5.77 μmole/g is derived from glutamate by transamination. It is therefore evident that at least (13.50 - 5.77) = 7.73 μmole/g, i.e., 50 per cent, of the total ammonia liberated, is accounted for by breakdown of glutamate and glutamine in the brain tissue (assuming no protein breakdown takes place).

In the absence of glucose, ammonia is continuously being liberated, though it is evident that the rate of its formation diminishes with the time of incubation. For example, less than 8 μmole ammonia/g initial wet wt tissue is liberated in the three hours incubation subsequent to the first hour incubation. compared to 15 μmole/g in the first hour. There seems to be a direct relationship between the rate of ammonia formation and the rate of oxygen consumption. The rate of the liberation of ammonia by rat brain cortex slices incubated in a glucose-free medium parallels the rate of its oxygen consumption. While the rate of oxygen consumption by the slices is almost constant in the presence of 10 mM glucose, even at the end of a four hour incubation period, the rate falls with time in the absence of glucose (Table 15). The drop in the rate of oxygen

uptake in a glucose-free medium probably coincides with the fall in the levels of endogenous substrates, including such substrates as glutamate, capable of liberating ammonia on oxidation.

In the presence of glucose, there is only a small diminution in the initial level of glutamate of brain slices incubated for one hour, but the content of glutamine is increased. In a four hour incubation period, the rate of endogenous glutamate oxidation in brain tissue is diminished in the presence of glucose and ammonia is utilized in the synthesis of glutamine. Glucose maintains high levels of glutamate and glutamine in incubated slices by generating α-ketoglutarate (315) which forms glutamate, either by reductive amination with NH_4^+, or by transamination with amino acids such as aspartate. Moreover, glucose oxidation generates NADH, which favors reductive amination of α-ketoglutarate over the reverse reaction of glutamate oxidation. Furthermore, glucose generates ATP required for glutamine synthesis. These facts account for the inhibitory effect of glucose on ammonia formation in brain slices during incubation.

(iii) Possible protein breakdown in incubating brain slices.

In order to obtain information concerning the kinetics of the rise in aspartate level accompanying the fall in glutamate level, rat brain cortex slices were incubated aerobically at 37°C in Krebs-Ringer phosphate glucose-free medium for 15 minutes and 30 minutes. Results of the total (tissue + medium) ammonia and amino acid changes are given in Table 2. From this table it may be calculated that 83 per cent (or 4.78 µmole/g) of the total rise in the aspartate level occurring in one hour, takes place in the first 15 minutes, and 92 per cent (or 5.24 µmole/g) at the end of 30 minutes. Only 43 per cent (or 3.73 µmole/g) of the total diminution in the level of glutamate, that is obtained in one hour, occurs in the first 15 minutes and 73 per cent (or 6.25 µmole/g) at the end of 30 minutes incubation. These and other results in Table 2 indicate that, if the bulk of the aspartate is derived from endogenous glutamate, by transamination with oxaloacetate, then under these conditions

glutamate from some precursor source (possibly proteins) directly and/or following glutamine hydrolysis, enters its initial free pool, undergoes oxidation and liberates ammonia in the process. Possibly, some aspartate may appear as a result of protein breakdown. That protein breakdown may indeed occur in incubating brain slices is implicated by the continuous rise in the levels of certain amino acids such as glycine, serine, threonine or lysine with increasing times of incubation (0 to 4h). It is to be noted, however, that the level of glutamate (and glutamine) continues to fall with time of incubation indicating its (glutamate) continuous oxidation.

The value of the total amino acid-N, including the value for ammonia, is enhanced from the initial level by about 10 μatoms/g initial wet wt on incubation for one hour, either in the presence or absence of glucose. This possibly reflects cerebral protein breakdown on incubation. It is to be noted that the level of taurine (an amino acid derivative not a constituent of proteins) remains, within experimental error, unchanged during incubation of brain slices in the presence or absence of glucose, even in four hour incubations. Increases in the amino acid content of incubated brain tissue are in accord with the observations made by Weil-Malherbe and Gordon (102), and Jones and McIlwain (267).

It should be mentioned that the initial level of N-acetyl aspartate (5.72 ± 0.24 μmole/g) in rat brain cortex slices is unaffected by incubation in the presence or absence of glucose, which is in agreement with many results reported in the literature (247, 248, 291). Therefore, the rise in the aspartate content of brain slices incubated in a glucose-free medium is not due to a fall in the N-acetyl aspartate content of the tissue. However, this view is opposed to that of Buniatian et al., (273) who obtained a diminution in N-acetyl aspartate of brain tissue incubated in the absence of glucose.

These studies lend support to the view that the bulk of the ammonia formed in brain cortex slices incubated in glucose-free media occurs from

glutamine and by terminal glutamate oxidation.

3.2 Ammonia formation in anoxia.

The total (tissue + medium) ammonia and amino acid contents of rat brain cortex slices incubated at 37°C for one hour in Krebs-Ringer bicarbonate medium with or without the addition of 10 mM glucose in an atmosphere of N_2/CO_2 mixture (95%/5%), are given in Table 3. From these results it is seen that the rate of ammonia formation in a glucose-free medium is suppressed in the absence of oxygen (compared with values obtained on incubation in the presence of oxygen - Table 2). This is in accord with earlier findings (43, 244). Such results support our view (62) that ammonia formation in brain depends greatly on glutamate oxidation.

The value of total amino acid-N, including the value for ammonia, is enhanced by anaerobic incubation and is almost equal in one hour to that obtained at the end of one hour aerobic incubations (Tables 3 and 2). This enhancement is likely to be due to protein breakdown because there are small increases in the amounts of most amino acids apart from glutamine (Table 3A). The net fall in the level of glutamine obtained in anoxia, may presumably be due to the fact that the rate of its hydrolysis by glutaminase exceeds the rate of its formation by the ATP requiring glutamine synthetase. This presumably accounts for a portion of the ammonia liberated anaerobically.

A balance sheet representing the changes in the total (tissue + medium) ammonia and amino acid contents of brain slices incubated (with or without glucose) in the presence or absence of oxygen, is given in Table 3A. From this Table it is evident that much of the ammonia turnover in brain is due to changes in the brain amino acids. Moreover, it is evident that, in the absence of glucose, the enhanced glutamate content of brain slices incubated anaerobically over the tissue value of glutamate obtained aerobically, nearly accounts for the diminished rate of ammonia formation under anoxia. Furthermore, the accompanying increase in GABA content, and perhaps part of the increase

TABLE 3. Effects of anoxia on the ammonia and amino acid contents of rat brain cortex slices incubated in the presence or absence of glucose.

Rat brain cortex slices were incubated in Krebs-Ringer bicarbonate medium at 37°C for one hour in an atmosphere of N_2/CO_2 (95% : 5%). Total (tissue + medium) values of ammonia and amino acids are expressed as μmole/g initial wet wt.

	Additions to the incubation medium	
	No Glucose	Glucose
Glutamate	12.40 ± 0.22	13.43 ± 0.27
Glutamine	2.88 ± 0.18	3.08 ± 0.10
GABA	3.06 ± 0.19	3.63 ± 0.34
Aspartate	4.34 ± 0.03	4.30 ± 0.15
Alanine	1.36 ± 0.18	1.88 ± 0.30
Glycine	1.66 ± 0.13	1.73 ± 0.15
Serine	2.76 ± 0.16	2.89 ± 0.07
Threonine	0.76 ± 0.05	0.77 ± 0.04
Taurine	5.89 ± 0.11	5.86 ± 0.09
Ammonia	7.10 ± 0.31	6.59 ± 0.80
Total: NH_4^+-N + amino acid -N	45.09	47.34

TABLE 3A. Ammonia and amino acid changes of brain cortex slices incubated in the presence or absence of O_2.

Total (tissue + medium) values of ammonia and amino acids (μmole/g initial wet wt.) used for these calculations were taken from TABLE 2 (for O_2) and TABLE 3 (for N_2).

	No Glucose			Glucose		
	O_2	N_2	Δ-NH_2	O_2	N_2	Δ- NH_2
Glutamate	3.55	12.40	-8.85	10.11	13.43	-3.32
Glutamine	1.79	2.88	-2.18	6.35	3.08	+6.54
GABA	2.32	3.06	-0.74	2.81	3.63	-0.82
Aspartate	9.13	4.34	+4.79	4.14	4.30	-0.16
Alanine	0.73	1.36	-0.63	1.29	1.88	-0.59
Glycine	1.47	1.66	-0.19	1.53	1.73	-0.20
Serine	2.23	2.76	-0.53	1.82	2.89	-1.07
Threonine	0.95	0.76	+0.19	0.67	0.77	-0.10
Ammonia	16.81	7.10	+9.17	6.33	6.59	+0.26
Total -NH_2	40.77	39.20	+1.57	41.40	41.38	+0.02

in alanine, must occur by glutamate metabolism.

These data further substantiate the dependence of aerobic ammonia formation largely on endogenous glutamate oxidation.

3.3 Ammonia formation by infant (2-day-old) rat brain cortex slices

In earlier studies, we have shown (62) that the rate of ammonia formation by 2-day-old rat brain cortex slices, incubated for one hour in a glucose-free Krebs-Ringer phosphate medium, is lower than that of the adult. In view of the possibility that formation of ammonia by adult rat brain cortex is dependent on terminal endogenous glutamate oxidation, a study was made of the changes in the total (tissue + medium) ammonia and amino acid levels of infant rat brain incubated in the presence or absence of glucose. Results reported in Table 4 show the following facts.

1. Values of the contents of amino acids in infant cortex, freshly prepared and prior to incubation are in agreement with those reported in the literature (11, 130, 131, 311). The total initial amino acid-N including the value for ammonia, are remarkably close to that found for the adult (Table 2). However, in agreement with the results of other workers (129-131), the lower levels of glutamate (35% of the adult), aspartate (60% of the adult), and glutamine (65% of the adult) are compensated for by the high levels of taurine (300% of the adult). The amino acids, closely related to the citric acid cycle, are considerably lower in the infant brain.

2. In the absence of glucose, the rate of ammonia formation in infant rat brain is 40 per cent that of the adult. There is a rise in the aspartate level accompanying the falls in glutamate and glutamine levels.

3. In the presence of glucose, the initial content of glutamate is little changed in infant cortex incubated for one hour. Unlike the adult

TABLE 4. Ammonia and amino acid changes in infant rat brain cortex on incubation in the presence or absence of glucose.

Infant (2-year old) rat brain cortex slices were incubated in Krebs-Ringer medium with or without glucose in O_2 at 37°C for one hour. Initial values (i.e. values prior to incubation) of ammonia and amino acids and the total (tissue + medium) values are expressed as μmole/g initial wet wt.

	INITIAL	ON INCUBATION	
		No Glucose	Glucose
Glutamate	4.15 ± 0.05	1.91 ± 0.12	4.00 ± 0.23
Glutamine	2.91 ± 0.05	0.34 ± 0.07	0.85 ± 0.15
GABA	1.76 ± 0.12	1.65 ± 0.05	2.16 ± 0.30
Aspartate	2.02 ± 0.10	5.62 ± 0.06	2.52 ± 0.02
Alanine	1.26 ± 0.02	0.93 ± 0.30	1.25 ± 0.20
Glycine	1.01 ± 0.03	1.63 ± 0.19	1.71 ± 0.03
Serine	1.30 ± 0.04	2.26 ± 0.20	2.37 ± 0.40
Threonine	0.40 ± 0.15	1.08 ± 0.18	0.95 ± 0.12
Taurine	17.60 ± 0.30	19.03 ± 1.20	18.91 ± 2.34
Lysine	0.31 ± 0.03	0.59 ± 0.02	0.42 ± 0.11
Ammonia	0.76 ± 0.02	7.04 ± 0.75	5.34 ± 0.64
Total: NH_4^+-N + amino acid -N	36.49	42.42	41.33

brain, however, infant brain glutamine level falls. Ammonia utilization, by glutamine synthesis, seems to occur only feebly in the incubated brain tissue of infant rat. This presumably is not only due to the low activities of the relevant enzymes involved in glutamine synthesis (15, 163), but may also be due to the relatively low rate of the operation of the citric acid cycle, known to occur in the infant rat brain (235), and the resultant low rate of formation of α-ketoglutarate and, therefore, of glutamate.

4. It was also found that the oxygen consumption in the infant brain (per unit wet wt tissue is considerably lower than in the adult (see also reference 235). For example, in the presence of 10 mM glucose, the value for the infant brain is 46 ± 2 µmole/g initial wet wt/hour, and that for the adult is 102 ± 4. In the absence of glucose, the values for the infant and adult brain cortex slices are 42 ± 3, and 68 ± 3, respectively.

5. Compared to the initial, the total amino acid-N including the value for ammonia, is enhanced (by about 5-6 µatoms/g initial wet wt) on incubation for one hour either in the presence or absence of glucose. This is possibly due to gradual protein breakdown (but at a rate lower than that in the adult - Tables 4 and 2). There are enhanced levels of some amino acids, e.g., glycine, serine, threonine or lysine, in infant rat brain incubated in the presence or absence of glucose, which may also reflect some protein breakdown during incubation.

3.4 Is there a direct role of aspartate in the process of ammonia formation in the brain?

As mentioned earlier (section 1.5 (ii) a), some workers (14, 46, 98) studying the origin and mechanism of ammonia formation, have in their model assigned a central role to aspartate. According to this model, all ammonia

liberated emanates from aspartate through a cyclic set of reactions involving either desamino-NAD or inosinic acid. The contribution of other amino acids to ammonia production occurs through aspartate subsequent to transamination with oxaloacetate. In other words, other amino acids play the role of a "reservoir", maintaining aspartate levels. In view of the fact that incubation of brain cortex slices of the rat, in a medium devoid of glucose results in enhanced aspartate levels accompanying ammonia formation, experiments were carried out to observe whether aspartate actually plays a direct role in the formation of ammonia in the incubated brain tissue.

When adult rat brain cortex slices are incubated in O_2 for one hour in Krebs-Ringer phosphate glucose-free medium in the presence of 100 mM KCl, the total (tissue + medium) level of aspartate is enhanced by 40 per cent (Table 5). This increase in aspartate is not accompanied by a parallel decrease in the glutamate level, nor is it accompanied by an enhanced rate of ammonia formation. In fact, high K^+ invariably causes a small but significant fall in the rate of ammonia liberation. This fall accompanies a fall in the rate of respiration under these conditions, which may, in turn, be due to the loss of some endogenous glutamate to the incubation medium (i.e., a loss of 2.75 ± 0.03 μmole/g compared with 0.90 ± 0.07 for the control). Total (tissue + medium) glutamine levels fall under these conditions, while GABA levels (not shown) are unaffected. There is a small but significant rise in the level of alanine (i.e., a rise of 0.32 ± 0.02 μmole/g over the value of 1.01 ± 0.05 for the control). Increasing the time of incubation under these conditions to four hours does not further enhance the total (tissue + medium) aspartate level, nor does it affect the liberation of ammonia. These results show that an increase in the endogenous aspartate level does not necessarily accompany, or result in, stimulation of the liberation of ammonia in brain tissue. It may be mentioned that there was no fall of N-acetylaspartate to compensate for the rise of aspartate under these conditions.

The inhibition of aspartate formation, from endogenous sources, in

TABLE 5. Effects of sodium malonate and amino oxyacetate on aspartate synthesis and ammonia formation in rat brain cortex slices incubated in a glucose-free medium.

Rat brain cortex slices were incubated aerobically in (glucose-free) Krebs-Ringer phosphate medium at 37°C for one hour in the presence or absence of KCl (100mM) with or without the addition of sodium malonate (5mM) or sodium amino oxyacetate (5mM). QO_2 values are expressed as μ mole oxygen consumed per g initial wet wt. Total (tissue + medium) ammonia and amino acids are expressed as μ mole/g initial wet wt.

Additions to the incubation medium	QO_2	Aspartate	Glutamate	Glutamine	Ammonia
Nil	71.2 ± 4.8	8.58 ± 0.54	3.20 ± 0.26	2.49 ± 0.34	17.23 ± 0.11
Malonate	60.3 ± 3.9	7.31 ± 0.11	3.44 ± 0.27	2.58 ± 0.38	16.80 ± 0.38
Amino oxyacetate	63.6 ± 3.0	5.30 ± 0.47	3.58 ± 0.07	3.58 ± 0.07	17.71 ± 0.11
With KCl	54.5 ± 6.2	12.20 ± 0.18	3.85 ± 0.05	1.22 ± 0.12	16.04 ± 0.16
KCl + malonate	45.6 ± 1.0	8.60 ± 0.64	4.87 ± 0.04	1.33 ± 0.22	15.25 ± 0.07
KCl + amino-oxyacetate	52.6 ± 1.0	5.06 ± 0.28	4.98 ± 0.11	3.69 ± 0.27	16.96 ± 1.01

brain slices is another way of studying the effects of aspartate on the rate of ammonia liberation. Aspartate formation in rat brain cortex slices incubated in Krebs-Ringer phosphate (glucose-free) medium may be suppressed by metabolic inhibitors such as malonate or amino-oxyacetate (Table 5).

1. Malonate (5 mM), by inhibiting the rate of operation of the citric acid cycle, limits the supply of oxaloacetate required for aspartate formation by transamination. This is specially evident in the presence of 100 mM KCl when the rate of aspartate formation is considerably suppressed. However, there is no concomitant suppression of the rate of ammonia formation under these conditions.

2. Amino-oxyacetate (5 mM) inhibits aspartate formation both in the presence or absence of 100 mM KCl (in a glucose-free medium) presumably by directly inhibiting the pyridoxal phosphate requiring transaminase reaction (147, 279, 304). The inhibition in the rate of aspartate formation is not accompanied by any inhibition of the rate of ammonia formation.

   It seems, therefore, that the evidence does not support the view that aspartate is a major source of ammonia in rat brain cortex slices under our experimental conditions.

   In view of the lack of supporting evidence for the role of aspartate as an immediate ammonia precursor in brain slices, in our experiments, and in view of the positive evidence favoring glutamate (+ glutamine) as the immediate precursor, it seems unnecessary to consider aspartate as playing a major role in ammonia formation. Nevertheless, these results do not constitute evidence that aspartate plays no role in the process of cerebral ammonia formation.

3.5 Effects of metabolic inhibitors on the rate of ammonia formation from endogenous amino acids of rat cerebral cortex slices incubated in a medium devoid of glucose.

Cerebral cortex slices of the rat were incubated in O_2 in Krebs-Ringer phosphate medium at 37°C for one hour with and without the addition of 2, 4-dinitrophenol (DNP) (0.1 mM) or sodium amytal (1 mM). Results are given in Table 6.

In the presence of DNP there is a small diminution (3 μmole/g/hour) in the rate of ammonia formation, which is chiefly reflected in accompanying enhanced levels of glutamate (by 1.83 μmole/g) and GABA (by 1.46 μmole/g). DNP seems not to impair the breakdown of protein, since the total amino acid nitrogen (including the value for ammonia) is not affected when compared with the value of the control. It was also found that the rate of oxygen consumption, is diminished from the control value of 70 ± 7 μmole/g to 50 ± 2 μmole/g initial wet wt tissue in the presence of 0.1 mM DNP.

With 1 mM amytal, the rate of ammonia formation is suppressed by 5.36 μmole/g/hour. This is accompanied by enhanced levels of glutamate (by 3.31 μmole/g) and GABA (by 1.83 μmole/g). Under these conditions, however, there seems to be a small diminution in the breakdown of protein since the total amino acid-nitrogen (including the value for ammonia) is slightly lower than that for the control. It was also found that amytal (1 mM) lowered the rate of oxygen consumption from the control value of 68 ± 5 μmole/g to the value of 41 ± 3 μmole/g initial wet wt tissue.

These data on the rates of NH_4^+ formation, which are in agreement with our earlier results on the inhibitory effects of DNP or amytal by brain tissue incubated in a glucose-free medium (62), are not wholly explained by increased glutamate levels. An elevated GABA content also takes place due, obviously, to glutamate decarboxylation taking place at a greater rate when the endogenous glutamate concentration is increased.

TABLE 6. Effects of sodium-amytal and 2, 4-dinitrophenol on the total ammonia and amino acid contents of rat brain cortex slices incubated in a glucose free medium.

Rat cerebral cortex slices were incubated in Krebs-Ringer phosphate medium in O_2 at 37°C for one hour in the presence or absence of amytal (1mM) or 2, 4-dinitrophenol (0.1mM). Total (tissue + medium) values are expressed as μ mole/g initial wet wt.

	Addition to the incubation medium		
	Nil	Amytal	2, 4 dinitrophenol
Glutamate	3.62 ± 0.26	6.93 ± 0.25	5.45 ± 0.26
Glutamine	2.22 ± 0.14	1.97 ± 0.12	1.80 ± 0.10
GABA	1.92 ± 0.02	3.75 ± 0.17	3.38 ± 0.08
Aspartate	9.07 ± 1.00	7.45 ± 0.08	9.08 ± 1.08
Alanine	0.84 ± 0.03	0.93 ± 0.09	1.38 ± 0.05
Glycine	1.40 ± 0.03	1.27 ± 0.08	1.58 ± 0.03
Serine	2.21 ± 0.05	2.09 ± 0.10	2.34 ± 0.18
Threonine	0.86 ± 0.05	0.78 ± 0.02	0.87 ± 0.04
Taurine	6.16 ± 1.04	6.10 ± 0.52	6.20 ± 0.82
Ammonia	17.16 ± 0.16	11.80 ± 0.20	14.17 ± 1.16
Total: NH_4^+-N + amino acid -N	47.68	44.94	47.85

3.6 Effects of ouabain on the rate of ammonia formation in rat brain cortex slices incubated in a glucose-free medium

If endogenous glutamate oxidation is a major factor in the cerebral production of ammonia, its depletion from cerebral cortex slices (by inhibition of its re-uptake mechanism) should bring about a reduced rate of ammonia formation and a concomitant inhibition in the rate of respiration in a glucose-free medium. This in fact occurs when rat brain cortex slices are incubated in O_2 for one hour at 37°C in Krebs-Ringer phosphate medium in presence of ouabain (0.1 and 1.0 mM). Results given in Table 7 show the following facts.

1. The content of amino acid-N (including the value for ammonia) is the same in the presence or absence of ouabain, indicating that ouabain does not interfere with the protein breakdown process. This is further supported by the fact that the total (tissue + medium) amounts of amino acids such as glycine, serine or threonine, are unaffected by ouabain, though their rates of release into the incubation medium are considerably enhanced.

2. The rate of ammonia formation is inhibited by 4.74 and 6.74 μmole/g initial wet wt tissue/hour with 0.1 and 1.0 mM ouabain respectively. With 0.01 mM ouabain the value is 1.75 μmole/g (not shown).

3. The inhibition of the content of ammonia formation by ouabain can be accounted for by the gain of glutamate and the amino acids derived from it, viz., aspartate and GABA. There is considerable loss of these amino acids from the tissue and most of the gain takes place in the medium.

4. It was also found that the loss of tissue glutamate and its metabolites under these conditions is accompanied by a drop in the rate of oxygen consumption from control values of 75 ± 4 μmole/g initial wet wt tissue to values of 49 ± 2 and 42 ± 1 μmole/g with 0.1 mM and 1.0 mM ouabain respectively.

TABLE 7. The effects of ouabain on the rate of ammonia formation, and the release of amino acids from brain cortex slices incubated in a glucose-free medium.

Rat brain cortex slices were incubated in a Krebs-Ringer phosphate medium devoid of glucose in O_2 at 37°C for one hour in the presence or absence of ouabain. Tissue values are expressed as μmole/g initial wet wt. and medium values as μ mole/g initial wet wt./3 ml.

	Addition to the incubation medium								
	NIL			OUABAIN (0.1mM)			OUABAIN (1.0mM)		
	TISSUE	MEDIUM	TOTAL	TISSUE	MEDIUM	TOTAL	TISSUE	MEDIUM	TOTAL
Glutamate	1.78 ± 0.33	0.78 ± 0.05	2.56	0.90 ± 0.09	3.60 ± 0.12	4.50	0.65 ± 0.03	4.42 ± 0.06	5.07
Glutamine	0.17 ± 0.04	2.08 ± 0.11	2.25	0.50 ± 0.07	1.75 ± 0.05	2.25	0.41 ± 0.01	1.74 ± 0.21	2.15
GABA	1.22 ± 0.14	0.10 ± 0.08	1.32	0.43 ± 0.02	2.20 ± 0.08	2.63	0.41 ± 0.01	2.27 ± 0.05	2.68
Aspartate	7.27 ± 0.30	1.91 ± 0.58	9.18	3.96 ± 0.38	7.10 ± 0.15	11.06	2.81 ± 0.06	7.21 ± 0.21	10.02
Alanine	0.18 ± 0.02	0.55 ± 0.05	0.73	0.09 ± 0.06	1.09 ± 0.01	1.18	0.10 ± 0.02	1.08 ± 0.03	1.18
Glycine	0.77 ± 0.14	0.67 ± 0.04	1.44	0.30 ± 0.06	1.34 ± 0.01	1.64	0.28 ± 0.02	1.33 ± 0.02	1.61
Serine	0.86 ± 0.16	1.18 ± 0.06	2.04	0.33 ± 0.06	1.97 ± 0.01	2.30	0.27 ± 0.01	1.95 ± 0.03	2.22
Threonine	0.23 ± 0.03	0.53 ± 0.06	0.76	0.14 ± 0.05	0.66 ± 0.03	0.80	0.12 ± 0.03	0.74 ± 0.03	0.86
Taurine	2.99 ± 0.26	2.83 ± 0.21	5.82	1.94 ± 0.16	4.21 ± 0.11	6.15	1.55 ± 0.04	4.54 ± 0.04	6.09
Ammonia	2.17 ± 0.14	15.71 ± 1.35	17.88	2.02 ± 0.24	11.12 ± 0.02	13.14	1.85 ± 0.15	9.29 ± 0.51	11.14
Total: NH_4^+-N + amino acid-N	17.81	28.42	46.23	11.11	36.79	47.90	8.86	36.31	45.17

5. At least 4.3 μmole ammonia/g tissue, must be derived from glutamine under these conditions (assuming no glutamine directly derived from proteins enters the free amino acid pool on incubation) as the difference between the initial and incubation values is 4.40 - 2.25 = 2.15 (Tables 2 and 7).

6. In spite of the ouabain induced amino acid release from the tissue, marked changes in glutamate and aspartate contents (tissue + medium) take place. Thus, considerable conversion of glutamate to aspartate and GABA occurs in presence of ouabain.

3.7 The role of Ca^{++} in the formation of ammonia from L-glutamate by rat brain cortex slices incubated in a medium devoid of glucose.

The evidence so far supports the role of endogenous glutamate oxidation as a major factor in the aerobic generation of ammonia by brain tissue. Experiments were then carried out to observe whether added L-glutamate enhances the rate of ammonia formation. In confirmation of our earlier studies (62) and those of others (58, 103), the addition of L-glutamate (2.5 mM) to respiring brain cortex slices in a normal Krebs-Ringer phosphate medium (containing Ca^{++}) is found not to enhance the amount of free ammonia formed in one hour. In fact, a diminution takes place (Table 8).

Results given in Table 8 show that L-glutamate addition results in accompanying increases in the rate of glutamine synthesis. This is in accord with earlier findings (58, 103).

We now find that, if Ca^{++} is omitted from the incubation medium, the rate of ammonia formation from L-glutamate is substantially enhanced above that in a Ca^{++} containing medium and that of glutamine is diminished (Table 8).

The addition of L-glutamate to respiring brain cortex slices in the presence of Ca^{++} enhances the control rate of oxygen consumption from 70 $\pm$

TABLE 8. Effects of L-glutamate on the formation of ammonia and amino acids by rat brain cortex slices incubated in a medium devoid of glucose in the presence and absence of Ca^{++}.

Rat brain cortex slices were incubated aerobically in Krebs-Ringer phosphate medium for one hour at 37°C in the presence or absence of 2.8mM $CaCl_2$ with or without the addition of 2.5mM sodium L-glutamate. Total (tissue + medium) values are expressed as μ mole/g initial wet wt. Tissue values only of glutamate* are given.

	Additions to the incubation medium			
	Nil		Sodium L-glutamate (2.5mM)	
	Ca^{++}- free	Ca^{++}- containing	Ca^{++}- free	Ca^{++}- containing
Glutamate	*1.62 ± 0.12	*2.03 ± 0.16	*4.48 ± 0.02	*6.36 ± 0.08
Glutamine	1.46 ± 0.13	2.22 ± 0.14	3.22 ± 0.50	8.24 ± 0.38
Aspartate	10.01 ± 0.24	9.07 ± 1.00	20.31 ± 0.45	20.00 ± 0.30
GABA	1.87 ± 0.09	1.92 ± 0.02	2.22 ± 0.32	2.17 ± 0.20
Alanine	0.75 ± 0.11	0.84 ± 0.03	0.90 ± 0.03	1.03 ± 0.13
Glycine	1.23 ± 0.05	1.40 ± 0.03	1.38 ± 0.02	1.53 ± 0.12
Serine	1.75 ± 0.09	2.21 ± 0.05	1.96 ± 0.03	2.22 ± 0.10
Threonine	0.68 ± 0.08	0.86 ± 0.05	0.77 ± 0.02	0.94 ± 0.03
Taurine	6.28 ± 0.20	6.61 ± 1.04	6.64 ± 0.30	6.20 ± 0.43
Ammonia	17.15 ± 0.43	17.16 ± 0.26	19.24 ± 0.62	12.68 ± 0.28

2 μmole/g initial wet wt/hour to a value of 97 ± 3, and in the absence of Ca^{++} from the control rate of 68 ± 2 to a value of 88 ± 2. That L-glutamate can support brain respiration is a well known fact.

These results point to the profound effects of Ca^{++} on glutamine synthesis in brain (see Chapter 4). The increased liberation of ammonia in the absence of Ca^{++} is due to a diminished ability of the tissue to synthesize glutamine under these conditions.

In the presence of external L-glutamate, Ca^{++} has little or no effects on the total (tissue + medium) levels of all amino acids determined except (as mentioned above) that of glutamine. In the presence of L-glutamate, Ca^{++} enhances (1) the tissue content of glutamate (Table 8); (2) the rate of respiration; and (3) the tissue ATP content (Table 71A). A probable increase in glutamine synthetase activity takes place, resulting in an enhanced glutamine and a diminished ammonia content.

3.8 Ammonia formation by rat cerebral cortex slices from exogenous L-glutamine in the absence of glucose.

Results summarized in Table 9 show that, in the absence of glucose, the rate of ammonia formation from 5 mM L-glutamine by incubated rat brain cortex slices, is about 17-18 μmole/g initial wet wt tissue in one hour, in the presence or absence of Ca^{++}, or in the presence of 0.1 mM ouabain, or in the Na^{+}-rich K^{+} and Ca^{++}-free incubation medium - Medium II. The fact that a constant rate of ammonia formation occurs from externally-added L-glutamine implies that the activity of glutaminase is unaltered under such varying media conditions.

It is evident, from the results presented so far, that both endogenous and external L-glutamine can support high rates of ammonia formation in brain tissue incubated in glucose free-media.

TABLE 9. Ammonia formation from exogenous L-glutamine by rat cerebral cortex slices incubated in glucose-free media.

Cerebral cortex slices of the rat were incubated aerobically in a variety of media in the presence or absence of 5mM L-glutamine for one hour at 37°C. Total (tissue + medium) values of ammonia are expressed as μ mole/g initial wet wt.

Medium	Medium L-Glutamine	Total Ammonia	Ammonia formed from L-glutamine
Krebs-Ringer phosphate	-	17.16 ± 0.26	17.54
	+	34.70 ± 4.90	
Krebs-Ringer phosphate + 0.1mM ouabain	-	13.14 ± 0.22	18.65
	+	30.20 ± 4.00	
Ca-free Krebs-Ringer phosphate	-	17.15 ± 0.43	17.06
	+	35.80 ± 5.60	
Medium II	-	14.90 ± 0.80	17.10
	+	32.00 ± 2.20	

3.9 Effects of tetrodotoxin, lidocaine and protoveratrine on the rate of ammonia formation by rat brain cortex slices incubated in a glucose-free medium.

Rat brain cortex slices were incubated in O_2 for one hour at 37°C in Krebs-Ringer phosphate glucose-free medium. The effects of neurotropic drugs on the rate of ammonia formation under these conditions were studied. Results are recorded in Table 10.

The addition of protoveratrine (5 μM) diminishes the rate of ammonia formation, some of which appears in an enhanced glutamine level. These effects of protoveratrine are completely abolished by the presence of tetrodotoxin (2 μM) or of lidocaine (0.5 mM), and occur in the presence, but not in the absence, of calcium. The implication of these findings will be dealt with later. It is to be noted that TTX or lidocaine themselves have little or no effects on the rates of glutamine and NH_4^+ formation under these conditions.

3.10 Changes in the ammonia and amino acid contents of isolated rat brain cortical synaptosomes on incubation.

Amino acid contents of synaptosomes isolated from the brain cortex of the rat, freshly prepared and immediately prior to incubation, are given in Table 11. Also given in this Table are the contents of ammonia and amino acids found on incubation in O_2 at 37°C for one hour in Krebs-Ringer phosphate medium with or without the addition of 10 mM glucose. The following facts are evident from the results.

1. At the end of the incubation period, either in the presence or absence of glucose, the total amino acid-N, including the value for ammonia, is increased by about 6 μatom/100 mg synaptosomal protein. This is probably due to protein breakdown. Proteolysis is also reflected in the increased levels of certain amino acids such as glycine, serine, threonine or lysine.

TABLE 10. Effects of neurotropic drugs on the formation of ammonia, glutamate and glutamine in rat brain cortex slices respiring in a glucose-free medium.

Rat brain cortex slices were incubated aerobically in Krebs-Ringer phosphate medium at 37°C for one hour in the presence or absence of neurotropic drugs. Total (tissue + medium) values are expressed as μmole/g initial wet wt.

Additions to the incubation medium	Ammonia	Glutamate	Glutamine
Nil	17.10 ± 0.58	2.93 ± 0.21	2.25 ± 0.21
+ TTX (2μM)	16.80 ± 0.39	3.61 ± 0.23	1.81 ± 0.02
+ lidocaine (0.5mM)	19.41 ± 0.11	2.84 ± 0.17	1.01 ± 0.05
+ protoveratrine (5μM)	11.79 ± 0.28	3.44 ± 0.20	3.78 ± 0.08
+ protoveratrine (5μM) + TTX (2μM)	13.01 ± 0.25	3.10 ± 0.04	2.04 ± 0.07
+ protoveratrine (5μM) + lidocaine (0.5mM)	18.51 ± 0.19	2.94 ± 0.02	1.83 ± 0.14

TABLE 11. Ammonia and amino acid changes in isolated rat brain cortex synaptosomes incubated in the presence or absence of glucose.

Synaptosomes from rat brain cortex, isolated by the method of Gray and Whittaker (243), were incubated in O_2 at 37°C for one hour in Krebs-Ringer phosphate medium with or without the addition of glucose (10mM). Initial values (i.e. prior to incubation) and the total (cells + medium) values of ammonia and amino acids obtained on incubation are expressed as µmole/ 100 mg synaptosomal protein.

	INITIAL	ON INCUBATION	
		No glucose	Glucose
Glutamate	5.54 ± 0.21	0.92 ± 0.24	3.77 ± 0.17
Glutamine	1.30 ± 0.16	0.72 ± 0.13	0.70 ± 0.14
GABA	1.26 ± 0.13	0.75 ± 0.13	1.27 ± 0.07
Aspartate	2.34 ± 0.18	3.51 ± 0.37	2.83 ± 0.22
Alanine	0.21 ± 0.07	0.79 ± 0.04	0.91 ± 0.05
Glycine	0.27 ± 0.03	0.70 ± 0.08	0.69 ± 0.02
Serine	0.39 ± 0.08	0.98 ± 0.08	0.97 ± 0.07
Threonine	0.20 ± 0.07	0.64 ± 0.10	0.62 ± 0.02
Taurine	2.41 ± 0.08	2.44 ± 0.20	2.43 ± 0.11
Lysine	-	0.40 ± 0.10	0.45 ± 0.01
Ammonia	2.31 ± 0.37	11.21 ± 0.16	8.01 ± 0.19
Total: NH_4^+-N + amino acid -N	17.52	23.78	23.35

2. In the absence of glucose, the rise in the level of ammonia is accompanied by significant falls in the glutamate, glutamine and GABA levels, part of which may account for the rise in the content of aspartate.

3. In the presence of glucose, the total α-amino + ammonia nitrogen after one hour incubation is the same as that in the absence of glucose. However, there are rises in the levels of glutamate and GABA which are balanced by falls in those of aspartate and NH_4^+.

4. Synaptosomes are unable to form glutamine from liberated ammonia in the presence of glucose. In this respect synaptosomes do not behave in a manner similar to brain cortex slices incubated under identical conditions. It is known that there is little or no glutamine synthetase activity in isolated nerve endings (245, 246).

5. It was also found that the rates of oxygen uptakes by synaptosomes incubated in the presence and absence of glucose are 64 ± 2 and 24 ± 1 μmoles/100 mg synaptosomal protein respectively.

3.11 Summary

1. When the oxygen consumption of rat brain cortex slices respiring in glucose-free media is suppressed (as, e.g., in the presence of 0.1 mM DNP, or 1 mM amytal, or 0.1 mM or 1.0 mM ouabain, or in the absence of oxygen), there is an accompanying diminution in the loss of endogenous glutamate and a diminished rate of ammonia formation. Infant rat brain, which respires at a lesser rate than adult per g fresh wt, also shows smaller losses of glutamate and lower rates of ammonia formation.

2. Although endogenous glutamate seems to be the major source of aerobic ammonia formation in brain slices incubated in a normal Krebs-Ringer phosphate glucose-free medium, exogenous L-glutamate diminishes the rate of ammonia formation and increases that of glutamine synthesis.

3. When Ca^{++} is omitted from the incubation medium, the rate of ammonia formation in the presence of L-glutamate is substantially enhanced above that in a Ca^{++} containing medium, and that of glutamine is diminished.

4. Both endogenous and exogenous L-glutamine can serve as sources of ammonia in brain slices respiring in glucose-free media.

5. The aspartate content of brain slices, incubated in a glucose-free medium, is enhanced by over 40 per cent in the presence of 105 mM KCl. It is diminished in the presence of 5 mM malonate or 5 mM amino oxyacetic acid with or without high potassium ion concentrations. These conditions have little or no effects on the rate of ammonia liberation. These results do not support the view that aspartate is a direct precursor of tissue ammonia.

6. Protoveratrine (5 μM), brings about a diminished formation of ammonia together with an enhanced synthesis of glutamine in brain slices incubated in a glucose-free medium containing Ca^{++}. This process is TTX (2 μM) and lidocaine (0.5 mM) sensitive.

7. Brain cortex synaptosomes form ammonia aerobically, largely from their pools of glutamate and glutamine. Unlike brain cortex slices, synaptosomes do not seem to synthesize glutamine in the presence of glucose.

8. Proteolysis seems to occur in incubated rat brain cortex slices under all our incubation procedures.

4. CONTROL MECHANISMS FOR GLUTAMINE SYNTHESIS IN RAT BRAIN CORTEX IN VITRO

Gonda and Quastel (112) have shown that ouabain at concentrations (e.g., 0.01 mM), not inhibitory to respiration inhibits the synthesis of labelled glutamine from labelled glucose. The inhibition of glutamine synthesis (Table 12, Condition II) is accompanied by an enhanced liberation of ammonia (62) into the incubation medium (Table 22). However, it was also shown (112) that ouabain does not inhibit glutamine synthetase isolated from brain tissue. NH_4^+ partially reversed the inhibitory effect of ouabain on glutamine synthesis in brain cortex slices. This led to the suggestion that ouabain suppresses the transport of NH_4^+ to the site of glutamine synthesis (112). However, it later became clear that ammonium ions are not accumulated against a concentration gradient (see Chapter 6). In the light of this new information, the mechanism of ouabain action on the synthesis of glutamine in intact tissue still remained to be found. Further experimental work was therefore carried out and the results are given below.

4.1 Effects of varying sodium ion concentration on the rate of glutamine synthesis in rat brain cortex slices

One of the consequences of the inhibition of the sodium pump by ouabain is an enhanced sodium ion concentration. However, tissue Na^+ concentration may also be changed by altering the sodium concentration of the incubation medium. (Tissue/ Medium concentration ratio for Na^+ was found to be 0.79, within wide ranges of medium Na^+ and K^+ concentrations. See Table 67). Thus, we were able to raise the Na^+ content of brain cortical slices (without resorting to the use of ouabain) by incubating them in Krebs-Ringer phosphate glucose medium containing a high concentration of NaCl.

Rat brain cortex slices were incubated in O_2 at 37°C for one hour in glucose containing media varying in NaCl concentrations. The results obtained

(Table 12, Condition III) show that while the respiratory rates and water uptakes are little affected by changes in the salt concentrations, the synthesis of glutamine is inhibited at high concentrations of NaCl in the incubation medium. There is also an accompanying diminution in the Tissue/Medium concentration ratio of glutamine under these conditions.

4.2 Effects of varying potassium ion concentration on the rate of glutamine synthesis in rat brain cortex slices.

Another consequence of the inhibition of the sodium pump by ouabain is a diminished tissue potassium ion concentration. It is possible to raise the level of K^+ in cortical slices by incubating them in a medium containing high K^+. However, Gonda and Quastel (112) were unable to reverse the inhibitory effect of ouabain on the synthesis of glutamine, with 100mM KCl. In fact, high K^+ enhanced the inhibitory effect of ouabain on glutamine synthesis. This is undoubtedly due to a fall in the tissue ATP level known to occur with 100 mM KCl (115), and although ATP levels in slices are not known to diminish markedly with ouabain (112, 115), the additional presence of 100 mM KCl may greatly reduce the ATP concentration owing to the depleted phosphocreatine reserve (251). It should also be noted that 100 mM KCl enhances the uptakes of Na^+ and water by the tissue (182).

Incubating rat brain cortex slices for one hour in O_2 at 37°C in Krebs-Ringer phosphate glucose medium, devoid (initially) of KCl, results in a fall of tissue K^+ (see Table 66) and ATP levels (115), with little accompanying effects on oxygen and water uptakes. There is a diminution in the rate of glutamine synthesis due to absence of K^+ (Table 12, Condition IV). Higher concentrations of medium KCl than 5 mM, do not affect the amount of glutamine synthesized in one hour (Table 12), although the Tissue/Medium concentration ratio of glutamine is much increased. The effect of K^+ on glutamine retention in the tissue is in accord with the recent results of Machiyama et al. (156).

TABLE 12. Effects of the cationic contents of the incubation medium on the rate of glutamine synthesis in rat brain cortex slices.

Rat brain cortex slices were incubated in O_2 at 37°C for one hour in incubation media of varying composition with glucose present. The tissue and total glutamine levels (expressed as μmole/g initial wet wt.) and that for the medium (expressed as μmole/g initial wet wt./3 ml.) are given. Oxygen uptakes are expressed as μmole/g initial wet wt. and water uptakes as μl/100 mg initial wet wt.

Condition	MEDIUM COMPOSITION (mM)					Oxygen Uptake	Water Uptake	Glutamine Content			Total Content of glutamine % Control
	NaCl	KCl	$CaCl_2$	EGTA	Ouabain			TISSUE	MEDIUM	TOTAL	
I (Control)	128	5	2.8	-	-	101 ± 6	14.0 ± 1.5	3.45 ± 0.30	2.92 ± 0.28	6.37	100
II	128	5	2.8	-	0.01	98 ± 3	19.2 ± 1.0	0.31 ± 0.05	1.86 ± 0.12	2.17	34
	128	5	2.8	-	0.1	96 ± 2	38.0 ± 1.1	0.71 ± 0.01	1.64 ± 0.07	2.35	37
III	78	5	2.8	-	-	90 ± 3	22.2 ± 0.4	4.38 ± 0.15	2.97 ± 0.15	7.35	115
	178	5	2.8	-	-	95 ± 2	15.9 ± 1.1	1.96 ± 0.21	3.01 ± 0.20	4.97	77
IV	128	-	2.8	-	-	110 ± 1	18.3 ± 0.5	1.58 ± 0.05	2.70 ± 0.20	4.28	67
	128	30	2.8	-	-	122 ± 2	17.5 ± 1.4	4.06 ± 0.40	2.40 ± 0.30	6.46	101
	128	55	2.8	-	-	126 ± 2	31.8 ± 3.0	4.93 ± 0.05	1.29 ± 0.07	6.22	98
	128	105	2.8	-	-	144 ± 2	38.1 ± 0.5	5.38 ± 0.22	1.43 ± 0.25	6.81	107
V	128	5	-	-	-	128 ± 5	18.0 ± 2.0	1.96 ± 0.20	2.41 ± 0.14	4.37	68
	128	5	-	3	-	134 ± 5	19.3 ± 0.5	1.05 ± 0.04	1.96 ± 0.07	3.01	47
VI	178	-	-	3	-	105 ± 3	26.9 ± 1.1	0.56 ± 0.01	1.42 ± 0.07	1.98	31

4.3 Effects of calcium on the rate of glutamine synthesis by rat brain cortex slices

Glutamine synthetase, a Mg^{++}-requiring enzyme, is competitively inhibited by Ca^{++}(135). However, results given in Table 12 (Condition V), show that the incubation of rat brain cortex slices in a Krebs-Ringer phosphate glucose medium containing Ca^{++} brings about a higher net glutamine synthesis than in a medium devoid of the cation. The diminished synthesis due to the absence of Ca^{++} is even more marked by the presence of EGTA in the incubation medium.

In the absence of Ca^{++} the tissue gains Na^{+} and loses K^{+} (see Table 66). As a consequence of such ionic changes, the sodium pump is stimulated. There is a fall in the ATP level (see Table 74). A diminished ATP content of the tissue, due to the absence of calcium ions, may possibly be the rate limiting factor in the synthesis of glutamine. On this view, the effect of calcium in maintaining high ATP levels in the tissue may be responsible indirectly for the apparently anomalous behavior of Ca^{++}.

Results reported earlier (Chapter 3) are further evidence for the important role of Ca^{++} in the synthesis of glutamine in the intact brain tissue. These results obtained with rat brain cortex slices incubated in a glucose free medium, demonstrate the requirement of Ca^{++} for the synthesis of glutamine from externally added glutamate (section 3.7), and for the stimulatory effect of 5 μM protoveratrine on glutamine synthesis with an accompanying suppression of ammonia formation (section 3.9). All these results are understandable on the basis of Ca^{++} being necessary to maintain a sufficiently high ATP concentration in the brain tissue to bring about an optimal rate of glutamine synthesis.

4.4 Glutamine synthesis in a Na^+-rich, K^+-free, Ca^{++}-free, incubation medium.

From the above considerations, it would be expected that the inhibition in the rate of glutamine synthesis in brain slices would be most evident in a Na^+-rich, K^+ and Ca^{++}-free Krebs-Ringer phosphate glucose medium (Medium II) for absence of K^+ (115) or Ca^{++} (Table 74) leads to a marked fall in the ATP concentration. This is, in fact, what occurs as shown in Table 12 (Condition VI). Under these conditions, the water uptake is enhanced over the control value (Condition I), but the oxygen consumption is little affected .

4.5 Glutaminase activity and glutamine synthesis

We have seen that, when brain cortex slices are incubated in Krebs-Ringer phosphate glucose medium in the absence of Ca^{++}, or in the presence of ouabain, or in glucose containing Medium II, there is an inhibition of the rate of glutamine synthesis as shown by the diminished total (tissue + medium) levels of glutamine at the end of the incubation period. Evidence was presented earlier (Chapter 3) showing that the incubation of brain tissue in such media does not promote glutamine breakdown by enhancement of glutaminase activity (section 3.8). The fact that the rate of ammonia formation from 5 mM L-glutamine, added to glucose free media containing ouabain or devoid of Ca^{++} is constant (17-18 μmole/g initial wet wt/hr), demonstrates that brain glutaminase activity is unaffected by these conditions.

4.6 Effects of sodium L-glutamate and NH_4Cl on the inhibition of glutamine synthesis in rat brain cortex slices incubated with glucose either in Medium II, or in the presence of ouabain

Results given in Table 13 show that in the presence of 5 mM L-glutamate, or of 5 mM NH_4Cl, the rate of glutamine synthesis by rat brain cortex slices

TABLE 13. Effects of NH_4Cl and sodium L-glutamate in reversing the suppressed rates of glutamine synthesis in rat brain cortex slices due to ouabain or changed media composition.

Rat brain cortex slices were incubated in O_2 at 37°C for one hour in the presence of glucose under media conditions given below. NH_4Cl or L-glutamate when present were 5mM and Ouabain 0.1mM. Tissue glutamate levels, the total and tissue glutamine levels are expressed as μ mole/g initial wet wt. and those for the medium as μmole/g initial wet wt./3 ml. Oxygen uptakes (QO_2) are given as μmole/g initial wet wt. and water uptakes as μl/100 mg. initial wet wt. Medium II was a Na^+ rich, K^+ and Ca^{++} free medium (see Section 2.4 (iii)).

MEDIA COMPOSITION	QO_2	Water Uptake	Tissue Glutamate	Glutamine Content			Total Content of glutamine % Control
				TISSUE	MEDIUM	Total	
Krebs-Ringer phosphate-glucose (Control)	101 ± 6	14.0 ± 1.5	8.44 ± 0.21	3.45 ± 0.30	2.92 ± 0.28	6.37	100
+ ouabain	98 ± 3	38.0 ± 1.1	4.95 ± 0.23	0.71 ± 0.01	1.64 ± 0.07	2.35	37
+ L-glutamate	117 ±10	34.7 ± 0.7	23.50 ± 0.17	5.67 ± 0.17	4.86 ± 0.19	10.34	163
+ L-glutamate + ouabain	98 ± 1	37.9 ± 1.5	12.48 ± 1.12	1.20 ± 0.06	4.76 ± 0.22	5.96	94
+ NH_4Cl	111 ± 1	14.6 ± 0.1	5.98 ± 0.16	4.78 ± 0.19	5.10 ± 0.21	9.88	155
+ NH_4Cl + ouabain	88 ± 2	49.5 ± 0.3	3.71 ± 0.11	1.68 ± 0.03	4.92 ± 0.20	6.68	104
+ L-glutamate + NH_4Cl	104 ± 3	35.9 ± 0.7	14.52 ± 0.18	5.16 ± 0.15	7.37 ± 0.84	12.53	197
+ L-glutamate + NH_4Cl + ouabain	84 ± 3	41.2 ± 1.2	4.82 ± 0.14	0.73 ± 0.04	5.01 ± 0.43	5.74	90
Medium II - glucose	105 ± 3	26.9 ± 1.1	9.09 ± 0.68	0.56 ± 0.01	1.42 ± 0.07	1.98	31
+ L-glutamate	106 ± 2	37.5 ± 0.3	18.77 ± 0.63	2.82 ± 0.38	4.62 ± 0.14	7.44	116
+ NH_4Cl	108 ± 1	30.5 ± 1.5	7.85 ± 0.13	2.79 ± 0.41	4.17 ± 0.14	6.96	109
+ NH_4Cl + L-glutamate	109 ± 1	43.5 ± 1.5	13.70 ± 0.40	2.51 ± 0.33	5.05 ± 0.40	7.56	119

incubated in O_2 for one hour at 37°C in a normal Krebs-Ringer phosphate glucose medium, is enhanced. This is also found to be true when the incubation is carried out in glucose containing Medium II. Both L-glutamate and NH_4^+ cause some elevation of the rate of respiration of the brain slice.

Glutamate and/or NH_4^+ only partially reverses the inhibition caused by ouabain or by incubation in Medium II. However, the reversal seems to be greater in Medium II, than in the medium containing ouabain. For example, when both L-glutamate and NH_4^+ are present, the percentage increases in glutamine synthesis (calculated on the basis of L-glutamate + NH_4^+ free controls) are (90 - 37) = 53 in the ouabain containing incubation medium and (119 - 31) = 88 in Medium II.

From these and other results reported in Table 13 it seems that the rate of glutamine synthesis is dependent on the ouabain-sensitive transport of NH_4^+ and/or glutamate to the site of glutamine synthesis. Since the Km for NH_4^+ is extremely low, 0.18 mM (95) and also since NH_4^+ are not actively accumulated in brain slices (Chapter 6), it is reasonable to conclude that the transport of glutamate, which is known to be impeded by ouabain (Table 18), may be a rate-limiting factor. Another factor limiting glutamine synthesis is the cellular level of ATP, not only required for active transport (of glutamate) but also for the synthesis of glutamine. Since ouabain affects the tissue ATP levels only slightly (115), and since the addition of L-glutamate does not completely reverse the inhibition of glutamine synthesis by ouabain, it follows that inhibition of glutamate transport cannot be the whole explanation for the ouabain effect. It seems likely that there is an ouabain induced fall of ATP level in the specific compartment of glutamine synthesis as a result of the changed tissue Na^+/K^+ concentration ratio.

Studies were carried out with a variety of metabolic inhibitors to throw further light on this phenomenon.

4.7 Comparative effects of metabolic inhibitors (methionine sulfoximine, ouabain, fluoroacetate, malonate, DNP and amytal) on rates of oxygen uptakes, glutamine synthesis and amino acid contents of rat brain cortex slices incubated in the presence of glucose

The results of a study of the effects of metabolic inhibitors on the total (tissue + medium) rates of glutamine synthesis and oxygen uptakes of rat brain cortex slices incubated in Krebs-Ringer phosphate glucose medium are given in Tables 14 and 15, respectively. Also given in Table 14 are the total (tissue + medium) values for ammonia and amino acids obtained on incubation of the brain tissue in the presence of the inhibitors. The results are summarized below.

1. Methionine sulfoximine (5 mM), a known inhibitor of glutamine synthetase, is without any effects on oxygen consumption, but inhibits the formation of glutamine and brings about an increased liberation of ammonia. Glutamate and other amino acid contents are little affected.

2. Ouabain (0.01 mM), has no effect on oxygen consumption in one hour, but depresses it by 25 per cent in four hours. It suppresses the rate of glutamine synthesis and concomitantly enhances ammonia output in one and four hours. GABA levels are elevated in one hour, but not significantly in 4 hour incubation, while alanine levels are enhanced in both the one and four hour incubation periods. Glutamate and other amino acid levels do not change significantly under these conditions.

3. Fluoroacetate (3 mM), does not affect respiration but inhibits the formation of glutamine in one hour and elevates the rate of ammonia formation. The GABA content is elevated but the aspartate level falls to a small extent. Glutamate, and other amino acid contents seem not to be affected with fluoroacetate.

TABLE 14. Effects of metabolic inhibitors on the rate of glutamine synthesis and amino acid contents of incubated rat brain cortex slices.

Rat brain cortex slices were incubated in Krebs-Ringer phosphate glucose medium in O_2 at 37°C, in the presence or absence of ouabain (0.1mM), DL-methionine DL-sulfoximine (5mM), sodium malonate (2mM), sodium fluoroacetate (3mM), sodium amytal (1mM), and DNP (0.1mM). Total (tissue + medium) values are expressed as μmole/g initial wet wt.

Addition to the Incubation Medium	Glutamate	Glutamine	GABA	Aspartate	Alanine	Glycine	Ammonia
One hour incubation							
Nil	10.11 ± 0.30	6.35 ± 0.18	2.81 ± 0.12	3.84 ± 0.21	1.29 ± 0.02	1.53 ± 0.11	5.12 ± 0.35
+ methionine sulfoximine	10.91 ± 0.18	2.50 ± 0.20	2.81 ± 0.10	4.03 ± 0.17	1.41 ± 0.07	1.41 ± 0.07	11.20 ± 0.20
+ ouabain	11.29 ± 0.14	2.35 ± 0.08	4.32 ± 0.29	3.88 ± 0.27	1.92 ± 0.08	1.61 ± 0.11	10.92 ± 0.29
+ fluoroacetate	11.46 ± 0.32	3.64 ± 0.12	3.63 ± 0.15	3.23 ± 0.23	1.35 ± 0.02	1.47 ± 0.03	10.61 ± 0.21
+ malonate	8.75 ± 0.26	6.00 ± 0.28	3.98 ± 0.12	1.20 ± 0.03	1.17 ± 0.10	1.23 ± 0.06	6.67 ± 0.44
+ amytal	10.62 ± 0.42	2.94 ± 0.20	6.03 ± 1.11	2.50 ± 0.04	1.53 ± 0.09	1.15 ± 0.19	8.00 ± 0.14
+ DNP	9.69 ± 0.40	3.48 ± 0.15	4.50 ± 0.38	2.59 ± 0.25	2.47 ± 0.12	1.53 ± 0.06	11.43 ± 1.01
Four hour incubation							
Nil	6.82 ± 0.51	11.40 ± 0.50	1.85 ± 0.18	2.49 ± 0.14	1.04 ± 0.01	1.68 ± 0.10	6.66 ± 0.25
+ methionine sulfoximine	7.95 ± 0.80	1.26 ± 0.40	1.95 ± 0.08	3.76 ± 0.08	3.40 ± 0.11	1.62 ± 0.13	20.32 ± 1.02
+ ouabain	6.52 ± 0.33	1.96 ± 0.24	2.25 ± 0.23	2.64 ± 0.05	2.76 ± 0.19	1.74 ± 0.14	18.20 ± 0.30
+ amytal	10.82 ± 0.42	2.41 ± 0.21	7.75 ± 0.32	2.38 ± 0.30	2.96 ± 0.09	2.16 ± 0.11	9.92 ± 0.58
+DNP	8.42 ± 0.14	3.42 ± 0.12	4.15 ± 0.56	2.53 ± 0.05	3.52 ± 0.02	2.13 ± 0.08	16.20 ± 0.50

TABLE 15. Effects of ouabain , methionine sulfoximine, KCl, 2,4 dinitrophenol, amytal, and glucose absence, on the rates of oxygen consumption in rat cerebral cortex slices.

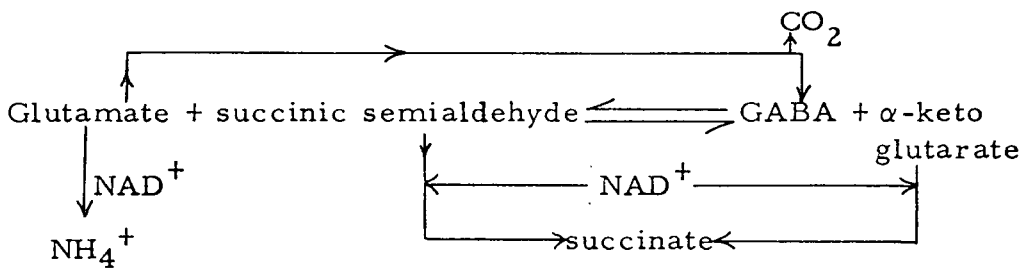
Cerebral cortex slices of the rat were incubated in Krebs-Ringer phosphate glucose medium at 37°C for various periods of time. Control values for oxygen consumption (QO_2) are expressed as μmole/g initial wet wt. Other values of QO_2 are given as percentages of the control, with standard deviations from the mean not exceeding ± 5%.

Time (min)	Control QO_2	QO_2 - % Control					
		Ouabain (0.1mM)	Methionine Sulfoximine (5mM)	KCl(100mM)	2,4 - DNP (0.1mM)	Amytal (1mM)	Glucose free medium
15	24 ± 2	108	98	123	96	72	89
30	46 ± 3	106	101	124	93	55	83
45	71 ± 1	102	102	123	90	48	73
60	96 ± 2	103	102	128	85	43	66
90	141 ± 5	-	103	127	79	-	55
120	192 ± 7	88	103	127	71	26	47
180	283 ± 12	88	104	121	55	20	38
240	376 ± 14	75	101	-	41	16	31

4. Malonate (2 mM), which strongly inhibits respiration, has little or no effect on the rate of glutamine synthesis or ammonia formation. It strongly inhibits aspartate formation and enhances that of GABA. There are diminutions in glutamate and glycine levels. Further experiments carried out with malonate and fluoroacetate will be described later (Chapter 8).

5. DNP at a concentration of 0.1 mM, which inhibits oxygen uptake, inhibits glutamine synthesis and at the same time enhances ammonia, GABA and alanine levels in one and four hour incubation periods. Glutamate is little affected but aspartate levels fall during the one hour incubation. DNP (0.01 mM) stimulates respiration with no effects on glutamine and ammonia levels.

6. Amytal (1 mM), brings about a strong inhibition of oxygen consumption, accompanied by a suppressed rate of glutamine synthesis, which is only partially accountable for by increased ammonia production. There is a substantial increase in the GABA level. The content of glutamate is unaffected in one hour but a higher level is obtained in four hours. The inhibition of the rate of glutamine synthesis is doubtless partly due to the inhibition of the NAD^+-linked glutamate oxidation, through suppression of NADH oxidation, and therefore of ammonia release from glutamate, and partly due to the suppressed generation of ATP. An elevated endogenous glutamate concentration results in an enhanced GABA content according to the following scheme.



Since both α-ketoglutarate and succinic semialdehyde require NAD^+ for oxidation, the operation of the citric acid cycle and its GABA by-pass are both suppressed in the presence of amytal, due to a greatly reduced $NAD^+/NADH$ concentration ratio. Interconversion of glutamate and GABA may reversibly occur, but it is the irreversible decarboxylation of glutamate that trips the balance in favor of GABA accumulation.

4.8 Effects of tetrodotoxin on ouabain inhibition of glutamine synthesis in rat brain cortex slices.

Chan and Quastel (198) have demonstrated that the inhibition of acetate oxidation in incubated rat brain cortex slices due to sodium influx induced by electrical stimulation, is reversed by TTX. Okamoto and Quastel (182) have shown that the influx of Na^+ that occurs on incubating brain slices in the presence of ouabain (0.1 mM) is partially suppressed by TTX. Such effects of TTX may be presumed to be due to its action on the sodium current system at the brain cell membrane and therefore confined to the neurons. Since we have shown that the tissue Na^+ and K^+ are factors controlling the synthesis of glutamine, and also since ouabain is known to affect the cationic concentrations of brain tissue (see Table 65), the question arises to whether TTX will reverse (at least partially) the inhibition of glutamine synthesis due to ouabain. Results obtained with 0.01 mM ouabain are given in Table 16. They show that TTX (2 μM) has little or no effect on the ouabain suppressed synthesis of glutamine in rat brain cortex slices incubated in O_2 at 37°C for one hour in Krebs-Ringer phosphate glucose medium. (This was also found to be true when 0.1 mM ouabain was used.) The tissue levels of glutamate are not diminished by 0.01 mM ouabain, so that glutamate and NH_4^+ concentrations are not likely to be limiting factors in the synthesis of glutamine.

The above results lead to the suggestion that glutamine synthesis occurs

TABLE 16. Effects of tetrodotoxin on the ouabain suppressed glutamine synthesis of rat brain cortex slices.

Rat brain cortex slices were incubated in a Krebs-Ringer phosphate glucose medium containing (0.01mM) ouabain with or without the addition of tetrodotoxin (2μM) at 37°C for one hour. Tissue values are expressed as μmoles/g initial wet wt. and medium values as μmoles/g initial wet wt./ 3 ml. medium.

	Ouabain			Ouabain + Tetrodotoxin		
	Tissue	Medium	Total	Tissue	Medium	Total
Glutamate	9.93 ± 0.53	0.95 ± 0.07	10.88	9.41 ± 0.03	0.72 ± 0.22	10.13
Glutamine	0.23 ± 0.04	2.11 ± 0.27	2.34	0.43 ± 0.06	2.36 ± 0.08	2.79
Aspartate	3.76 ± 0.40	0.50 ± 0.10	4.26	3.63 ± 0.33	0.45 ± 0.05	4.08
GABA	2.47 ± 0.47	0.19 ± 0.01	2.66	2.83 ± 0.59	0.10 ± 0.06	2.93
Glycine	0.85 ± 0.03	0.62 ± 0.03	1.47	0.95 ± 0.10	0.49 ± 0.08	1.44
Alanine	0.70 ± 0.05	1.14 ± 0.09	1.84	0.78 ± 0.05	1.10 ± 0.05	1.88
Ammonia	1.35 ± 0.08	8.03 ± 0.83	9.38	1.40 ± 0.02	8.19 ± 0.13	9.59

in those brain cells that are unaffected by TTX, viz., the glia, but is by no means proof. This suggestion will be considered again when considering the effects of TTX on ouabain induced release of amino acids other than glutamine from brain slices (Chapter 7).

4.9 Summary

1. The synthesis of glutamine by rat brain cortex slices incubated in the presence of glucose is suppressed under a variety of conditions that enhance the tissue Na^+/K^+ concentration ratio, e.g., the presence in the incubation medium of ouabain (0.1 or 1.0 mM), or metabolic inhibitors such as DNP (0.1 mM), amytal (1 mM), or fluoroacetate (1 mM), or when Ca^{++} is omitted. Under these conditions there is an enhanced rate of ammonia formation. This is unlikely to be due to an enhanced glutaminase activity as the rate of ammonia liberation from added L-glutamine is unaffected by these inhibitors.

2. Although Ca^{++} is known to inhibit competitively the Mg^{++} requiring glutamine synthetase isolated from brain tissue, it enhances the rate of glutamine synthesis in brain cortex slices incubated in the presence of glucose or L-glutamate.

3. Results suggest that ouabain inhibits the synthesis of glutamine by suppressing the transport of glutamate to the site of glutamine synthesis and by inducing a localized drop in the energy level of the compartment concerned with much of the synthesis of glutamine in the brain.

4. The inhibition of glutamine synthesis in the presence of DNP (0.1 mM), methionine sulfoximine (5 mM), fluoroacetate (3 mM), or ouabain (0.1 mM) results in an enhanced ammonia formation, under conditions where there is little or no change in the glutamate

content of the tissue. This suggests that glutamine synthesis occurs in a compartment separate from that containing the main bulk of the endogenous glutamate of the brain tissue.

5. Although tetrodotoxin (2 μM) is known to diminish the enhanced tissue Na^+/K^+ concentration ratio due to ouabain (0.01 or 0.1 mM), it has little or no effect on the ouabain induced suppression of glutamine synthesis. This suggests that glutamine synthesis occurs mainly in a TTX-insensitive compartment of the brain.

5. TRANSPORT OF L-GLUTAMATE INTO BRAIN IN VITRO

It is a well known fact that cerebral cortex slices respiring in physiological glucose saline medium accumulate L-glutamate against a concentration gradient (106, 231, 252, 295). We have shown earlier that the oxidation of endogenous glutamate accounts to a large extent for the liberation of ammonia and the respiration of brain slices incubated in the absence of glucose (Chapter 3). However, even in the absence of Ca^{++} when the utilization of ammonia (through the synthesis of glutamine) is seriously impeded, rat brain slices give only relatively small increases in the rate of ammonia formation when L-glutamate is added to a glucose-free medium (see Table 8). This could be due to the slow transport (or penetration) to the site of ammonia formation where presumably the major pool of endogenous glutamate is present. We have carried out experiments in an attempt to throw light on the actual site in the brain of the major pool of exogenous L-glutamate taken up by the tissue.

5.1 Transport of L-glutamate into incubated rat brain

Results of the effects of varying the medium L-glutamate concentrations on the accumulation of this amino acid into rat brain cortex slices respiring in O_2 at 37°C for one hour in Krebs-Ringer phosphate glucose medium are given in Table 17. The tissue values of glutamate obtained, in terms of μmole/g initial wet wt., are corrected for swelling so as to give values of concentrations in the tissue water. For initial medium glutamate concentrations of 2.5, 5, 10, 20 and 30 mM, values for the tissue to medium concentration ratios for glutamate are 9.0, 5.7, 2.9, 1.9 and 1.6 respectively. It is, therefore, evident that exogenous glutamate is accumulated against a concentration gradient in rat brain cortex slices. It may be noted from values in Table 17 that the active component of glutamate transport has reached its saturation level at an external glutamate concentration between 2.5 and 5.0 mM. Increments of glutamate in the medium above this concentration

TABLE 17. Transport of sodium-L-glutamate into brain cortex slices of the rat.

Rat brain cortex slices were incubated in O_2 at 37°C for one hour in Krebs-Ringer phosphate glucose medium containing increasing concentrations of sodium-L-glutamate. The medium Na^+ concentration was adjusted to 148 μequiv/ml by appropriate reductions of medium NaCl. Values of tissue glutamate obtained, as μmole/g initial wet wt. (Tg), were converted to μmole/ml tissue water (T) by taking tissue swelling into consideration. (Tg-E) values were obtained by subtracting the endogenous tissue glutamate (E=8.44 μmole/g) from the glutamate contents of slices incubated with external glutamate; these values were then converted to μmole/ml tissue water (T*) taking swelling into consideration.

Medium L-glutamate (mM)	μmole/g initial wet wt.		Water Uptake μl/100 mg initial wet wt.	Tissue glutamate μmole/ml		Medium glutamate (mM) after incubation	Tissue glutamate / Medium glutamate	
	Tissue glutamate Tg	Net glutamate uptake (Tg - E)		T	T*	M	T/M	T*/M
Nil	8.44 ± 0.58	-	14.8 ± 0.6	8.9	-	0.018	493.00	-
2.5	16.60 ± 1.00	8.16	33.8 ± 0.4	14.6	7.2	1.62 ± 0.07	9.01	4.44
5.0	23.50 ± 0.70	15.06	34.0 ± 0.7	20.5	13.2	3.59 ± 0.03	5.71	3.66
10.0	27.60 ± 2.00	19.16	35.2 ± 1.9	24.0	16.6	8.25 ± 0.10	2.90	2.01
20.0	38.00 ± 2.80	29.56	31.8 ± 2.9	34.0	26.4	18.00	1.89	1.47
30.0	49.20 ± 1.40	40.76	31.2 ± 0.8	44.3	36.7	27.50	1.61	1.33

only result in passive inflow of glutamate into the tissue. For example, an increase in the medium glutamate concentration from 5 to 30 mM (i.e., by 25 mM), enhances the tissue concentration by about the same value.

Values are also given in Table 17, for the net increases of tissue glutamate. These are obtained by subtracting the tissue concentrations of glutamate on incubation in the absence of external glutamate, from those obtained on incubation with externally added glutamate. The tissue to medium concentration ratios for glutamate, using net uptake values, are given in Figure 1 in which a comparison is made of the uptake of glutamate with that of NH_4^+.

5.2 The uptake of L-[U-^{14}C] glutamate by rat cerebral cortex slices

Rat cerebral cortex slices were incubated at 37°C in O_2 under a variety of conditions in the presence or absence of 5 mM, L-[U-^{14}C] glutamate (of specific activity 1.223×10^5 cpm/μmole). At the end of the incubation period, the glutamate contents of the tissue were measured by the following two methods:

1. Liquid scintillation counting. This method gives a measure of the radioactive glutamate taken up by the tissue.
2. Amino Acid Analyzer estimations. This method gives a measure of the total glutamate content of the tissue by the ninhydrin reaction.

The specific activity of the medium glutamate was little affected at the end of the incubation period by the incubation procedure, though there were falls in the medium concentrations of glutamate. Results given in Table 18, show that the tissue to medium concentration ratios for labelled glutamate are considerably lower than those for total glutamate content of the tissue. However, it is evident that, if only net glutamate uptakes are

TABLE 18. Uptake of sodium L-(U-^{14}C) glutamate by rat brain cortex slices.

Rat brain cortex slices were incubated in O_2 at 37°C in glucose containing media under conditions given below. Medium II was a Na^+-rich K^+ and Ca^{++}-free medium. Ouabain when added was (0.1mM) and sodium L-(U-^{14}C) glutamate was 5mM of specific activity 1.223×10^5 cpm/μmole. Medium Na^+ concentrations was adjusted to 148 μequiv/ml. Labelled and Total tissue glutamate determined respectively by liquid scintillation counting and the Amino Acid Analyzer, as μmole/g initial wet wt., were converted to μmole/ml tissue water (T), taking tissue swelling into consideration. Net glutamate uptake in μmole/g initial wet wt. was obtained by subtracting the tissue value of glutamate after incubation in the absence of external L-glutamate, from that value obtained at the end of the incubation period in the presence of external L-glutamate; these values were then converted to μmole/ml tissue water (T*) taking tissue swelling into consideration.

Conditions of incubation	Water Uptake μl/100mg initial wet wt.	Tissue Glutamate						Medium glutamate (mM) after incubation M	T/M		T*/M
		Labelled		Total					Labelled	Total	
		μmole/g	μmole/ml. T	μmole/g	μmole/ml. T	Net glutamate Uptake μmole/g	μmole/ml. T*				
A. Krebs-Ringer phosphate glucose											
(i) 30 min. incubation											
Control	-	-	-	9.39 ± 0.23	-	-					
+ L-(U-^{14}C) glutamate	23.6 ± 0.5	12.50 ± 0.42	12.10	20.60 ± 0.02	19.90	11.21	10.82	3.92 ± 0.08	3.1	5.1	2.8
(ii) 60 min. incubation											
Control	-	-	-	8.44 ± 0.33		-					
+ L-(U-^{14}C) glutamate	34.0 ± 0.7	15.40 ± 0.30	13.42	23.50 ± 0.70	20.48	15.06	13.14	3.59 ± 0.03	3.7	5.7	3.7
+ ouabain	-	-	-	4.95 ± 0.26	-	-					
+ ouabain + L-(U-^{14}C) glutamate	37.9 ± 1.9	7.73 ± 0.95	6.50	12.48 ± 1.12	10.60	7.53	6.42	3.65 ± 0.09	1.8	2.9	1.8
B. Medium II - glucose											
60 min. incubation											
Control	-	-	-	9.09 ± 0.68	-	-					
+ L-(U-^{14}C) glutamate	37.8 ± 0.3	11.20 ± 0.20	9.50	18.77 ± 0.63	15.98	9.68	8.24	3.22 ± 0.08	3.0	5.0	2.6

used to calculate the tissue to medium concentration ratios for glutamate, the tissue to medium ratios for both labelled and total glutamate are identical. These results were obtained in glucose containing media during incubation periods of 30 or 60 minutes, either in the presence of ouabain (0.1 mM), or when the brain slices are incubated in a Na^+-rich, K^+ and Ca^{++}-free medium (Medium II).

The results suggest that glutamate taken up from the medium accumulates within the tissue in a compartment distinct from another which contains the bulk of the endogenous glutamate. Results given later will indicate that the major pool of endogenous glutamate lies in the neurons (Chapter 8), while that of exogenous glutamate lies in the glia.

5.3 Apparent absence of an exchange process between external L-[U-^{14}C] glutamate and endogenous glutamate of rat brain cortex slices.

Experiments were carried out to observe the extent of exchange taking place between the bulk of the glutamate initially present and that taken up from the incubation medium.

Brain cortex slices of the rat were incubated at 37°C for 30 minutes in Krebs-Ringer phosphate glucose medium with or without the addition of 5 mM, L-[U-^{14}C] glutamate (of specific activity 1.223×10^5 cpm/μmole). At the end of the incubation period the tissue contained 1.53×10^6 cpm/g initial wet wt. There was also present 20.6 μmole glutamate/g initial wet wt tissue. This corresponds to a specific activity 0.75×10^5 cpm/μmole, assuming that the entire radioactivity is located in the glutamate. The amount of glutamate absorbed by the tissue from the incubation medium must then be $(1.53 \times 10^6 / 1.223 \times 10^5) = 12.5$ μmole/g, as the specific activity of the incubation medium is 1.223×10^5 cpm/μmole. Therefore, $20.6 - 12.5 = 8.1$ μmole glutamate/g do not exchange with medium glutamate because, had there been free exchange between labelled glutamate taken up by the tissue and the pool

of endogenous glutamate in the tissue, the specific activity of glutamate within the tissue would equal that outside it. Now, in the absence of external glutamate, we find that the total glutamate 10.16 μmole/g initial wet wt tissue is distributed between tissue (9.39 ± 0.23 μmole/g) and medium (0.77 ± 0.08 μmole/g/3 ml). Therefore, it appears that (8.1 x 100)/9.39, or about 87 per cent, of the endogenous tissue glutamate does not exchange with labelled glutamate added to the incubation medium. Actually, the percentage is higher because the total tissue radioactivity was used to estimate the labelled glutamate in the tissue, and the relatively small amounts of radioactivity contributed by glutamate metabolites were not taken into account. The specific activity of the incubation medium was but little changed by the incubation with cortex slices for 30 minutes.

5.4 Location of exogenous L-glutamate uptake.

Rat brain cortex slices were loaded with radioactive L-glutamate by pre-incubation in O_2 at 37°C for 30 minutes in Krebs-Ringer phosphate glucose medium containing 5 mM L-[U-^{14}C] glutamate of specific activity 1.223×10^5 cpm/μmole. At the end of the incubation period, the slices were quickly removed, lightly blotted to soak up adhering fluid, weighed on a torsion balance to measure water uptake, and incubated in O_2 at 37°C for one hour in one of the following incubation media:

A. Krebs-Ringer phosphate glucose medium.

B. Medium A, containing protoveratrine (5 μM) and ouabain (0.1 mM).

C. Medium B, containing TTX (2 μM).

The presence of protoveratrine ensures the activation of the sodium current in brain slices (182) with resultant effluxes of amino acids (310), while that of ouabain blocks the re-uptake of the released amino acids against a concentration gradient (104, 112). The presence of TTX by its selective action on

the neurons suppresses the neuronal efflux of amino acids from brain cortex slices brought about by the joint action of protoveratrine and ouabain.

Examination of the results in Table 19 show the following facts.

1. The apparent specific activity of glutamate in the incubation medium during the one hour incubation following the loading of the tissue with labelled glutamate, is much higher than that of the glutamate in the original incubation medium. This is due to the fact that, during the one hour incubation period, the labelled glutamate taken up originally by the tissue has been converted to other labelled amino acids (e.g., aspartate, alanine, glutamine, (Table 20), which also appear in the incubation medium (Table 21). The apparently high specific activity is illusory, as only a small proportion of the radioactivity in the medium is due to glutamate.

2. The presence of protoveratrine and ouabain brings about release of a relatively large quantity of radioactivity from the tissue, together with a considerable release of glutamate. The apparent specific activity of glutamate in the incubation medium is much lower than that found in the absence of the drugs. Presumably this is due to the release by the drugs of glutamate (both labelled and unlabelled) from its location in the tissue.

3. Addition of TTX suppresses release of glutamate, in presence of protoveratrine and ouabain, from the tissue, without affecting the release of radioactivity. This would indicate that TTX is blocking the release of unlabelled glutamate. This presumably lies in the neurons as TTX is believed to act only upon the neurons. This evidence indicates that a larger pool of endogenous glutamate lies in the neurons. It will be shown later that this pool is actually the major pool of glutamate (Chapter 8).

TABLE 19. Effects of protoveratrine ouabain and tetrodotoxin on the radioactivity of brain cortex slices preloaded with sodium L-(U-^{14}C) glutamate.

Rat brain cortex slices were preincubated aerobically in Krebs-Ringer phosphate glucose medium with 5mM sodium L-(U-^{14}C) glutamate of specific activity 1.223×10^5 cpm/μmole at 37°C for 30 min. The slices were then incubated aerobically in a Krebs-Ringer phosphate glucose medium at 37°C for one hour, in the presence or absence of protoveratrine (5μM) ouabain (0.1mM) and TTX (2μM). The contents of radioactive glutamate were estimated with the Liquid Scintillation Counter and the amounts of glutamate were estimated with the Amino Acid Analyzer.

Incubation Conditions	TISSUE (glutamate)			MEDIUM (glutamate)		
	Total radioactivity cpm/g ($\times 10^6$)	Amount μ mole/g	cpm per μ mole ($\times 10^6$)	Total radioactivity cpm/g/3ml ($\times 10^6$)	Amount μmole/g/3ml	cpm per μmole ($\times 10^6$)
Pre-incubation	1.53 ± 0.05	20.6 ± 0.2	0.075			
Incubation: Additions to Krebs-Ringer phosphate glucose medium						
A. Nil	0.57 ± 0.03	9.0 ± 0.6	0.063	0.509 ± 0.05	1.53 ± 0.03	0.332
B. protoveratrine + ouabain	0.19 ± 0.01	4.1 ± 0.4	0.046	0.902 ± 0.01	10.50 ± 0.34	0.086
C. protoveratrine + ouabain + TTX	0.38 ± 0.01	6.9 ± 0.1	0.055	0.898 ± 0.02	5.25 ± 0.01	0.171

4. TTX brings about a retention of radioactivity in the brain cortex slices, incubated in presence of protoveratrine and ouabain, equal to (0.38 - 0.19)(100)/1.53 = 12.4 per cent of the radioactivity originally taken up (Table 19). This amounts to 1.55 μmole glutamate/g initial wet wt tissue, if it is assumed that the radioactivity retained by TTX is wholly due to glutamate. The amount will be smaller as the value must include radioactivity due to glutamate metabolites. This result indicates that TTX has relatively little affect in suppressing the release of labelled glutamate from the tissue and, therefore, that much of the labelled glutamate lies not in the neurons, but in the non-excitable cells, i.e., the glia.

5.5 Effects of increasing external sodium L-glutamate concentrations on the tissue and medium concentrations of amino acids in incubated rat brain cortex slices.

In addition to the work on the uptake of external L-glutamate by incubated rat brain slices, studies were also made of the effects of increasing medium concentrations of L-glutamate (up to 30 mM) on the concentrations of amino acids in the tissue (Table 20), and in the medium (Table 21). The medium NaCl was adjusted to give a medium sodium concentration of 148 μequiv/ml.

The results given in Tables 20 and 21 show that the tissue and medium and, hence, the total levels of aspartate, GABA, alanine, and glutamine, are enhanced by increasing external L-glutamate concentrations. Other amino acids, e.g., glycine (shown), taurine, serine, or threonine (not shown), are little affected under these conditions. Neither are the concentrations of ammonia in tissue or medium affected. Most of the change in the concentration of glutamine, alanine or aspartate, is brought about by the lowest medium concentrations (i.e., 2.5 mM) of L-glutamate investigated. Increasing

TABLE 20. Effects of increasing medium sodium L-glutamate concentrations on the amino acid contents of incubated rat brain cortex slices.

Brain cortex slices of the rat were incubated aerobically in Krebs-Ringer phosphate glucose medium containing increasing medium sodium L-glutamate concentrations at 37°C for one hour. The medium sodium was adjusted to 148 μequiv/ml by appropriate reductions of medium NaCl. Amounts of amino acids in the tissue are expressed as μmole/g initial wet wt.

Medium L-glutamate (mM)	Glutamate	Glutamine	GABA	Aspartate	Alanine	Glycine
Nil	8.44 ± 0.58	3.07 ± 0.26	2.44 ± 0.04	2.72 ± 0.14	1.09 ± 0.11	1.06 ± 0.01
2.5	16.60 ± 1.00	5.27 ± 0.35	3.51 ± 0.04	4.28 ± 0.86	1.92 ± 0.25	1.02 ± 0.02
5.0	23.50 ± 0.70	5.67 ± 0.17	3.64 ± 0.07	5.03 ± 0.10	1.90 ± 0.04	0.98 ± 0.05
10.0	27.60 ± 2.00	5.06 ± 0.80	4.62 ± 0.02	3.91 ± 0.23	1.87 ± 0.02	1.05 ± 0.01
20.0	38.00 ± 2.80	5.44 ± 0.38	4.70 ± 0.10	4.30 ± 0.32	1.61 ± 0.08	0.96 ± 0.04
30.0	49.20 ± 1.40	4.97 ± 0.58	6.60 ± 0.20	4.39 ± 0.71	1.55 ± 0.11	0.93 ± 0.20

TABLE 21. Effects of increasing medium sodium L-glutamate concentrations on the release of amino acids from incubated rat brain cortex slices.

The experimental conditions were as described in Table 20. Amounts of amino acids in the medium are expressed as μmole/g initial wet wt. tissue/3ml. medium.

Medium L-glutamate (mM)	Glutamate	Glutamine	GABA	Aspartate	Alanine	Glycine
Nil	0.63 ± 0.10	3.34 ± 0.01	0.03 ± 0.01	0.54 ± 0.01	0.60 ± 0.05	0.34 ± 0.04
2.5	1.62 ± 0.07 mM	4.66 ± 0.16	0.03 ± 0.01	3.88 ± 0.73	1.39 ± 0.03	0.38 ± 0.04
5.0	3.59 ± 0.03 mM	4.87 ± 0.19	0.15 ± 0.03	4.12 ± 0.02	1.75 ± 0.16	0.44 ± 0.04
10.0	8.25 ± 0.10 mM	5.42 ± 0.12	0.22 ± 0.01	5.64 ± 0.30	1.64 ± 0.23	0.37 ± 0.07
20.0	-	5.86 ± 0.06	0.45 ± 0.02	7.36 ± 0.40	2.24 ± 0.31	0.53 ± 0.02
30.0	-	7.10 ± 0.45	-	5.01 ± 0.08	2.46 ± 0.17	0.46 ± 0.02

medium glutamate concentrations, enhances the GABA levels in the tissue and effects a small release of GABA into the incubation medium. With 2.5 mM, external L-glutamate, there is about a twofold increase in the tissue, or medium, content of alanine; the tissue and medium contents of aspartate are increased by 50 per cent and 700 per cent, respectively.

5.6 Summary

1. There is an apparent absence of a free exchange process between endogenous glutamate and labelled L-glutamate taken up by brain cortex slices against a concentration gradient. Less than 13 per cent of the endogenous glutamate appears to have free access to labelled L-glutamate taken up by the tissue.

2. Addition of TTX suppresses the neuronal release of glutamate brought about by the joint action of protoveratrine and ouabain from brain slices pre-loaded with labelled L-glutamate without affecting the release of radioactivity. This suggests that the endogenous pool of glutamate lies in TTX-sensitive compartment(s), i.e., the neurons, while L-glutamate taken up by the tissue is largely present in a TTX-insensitive compartment(s), i.e., the glia. Under these conditions TTX brings about a maximal retention of 12.4 per cent of the radioactivity originally taken up by the tissue amounting to a value for tissue glutamate no larger than 1.55 μmole/g initial wet wt.

3. Results on the effects of increasing external L-glutamate concentrations on the amino acid content in, and release from, incubated brain cortex slices, are given.

6. TRANSPORT OF AMMONIUM IONS IN BRAIN IN VITRO

According to Jacobs et al. (253, 254), the rate of transport of ammonium ions into erythrocytes cannot be measured by present methods as ammonia passes the membrane so rapidly. However, Post and Jolly (255) used an indirect method in their studies and showed that the addition of NH_4^+ to Na^+-filled red cells brings about a high rate of Na^+ transport (outward). Saturating the transport system with K^+ (by incubating with external K^+) produces only a small increase in Na^+ transport by NH_4^+ ions. They concluded that NH_4^+ substitutes directly for K^+ in the sodium pump of erythrocytes, but require a concentration 3-7 times that of K^+ to produce a comparable effect.

Tower and his colleagues (256, 257) studied the transport of NH_4^+ in cat brain cortex slices. They incubated the tissue at 37°C for one hour in the presence of 10 mM NH_4Cl and, since they obtained a higher internal (i.e., tissue) than external (i.e., medium) NH_4^+ concentration at the end of the incubation period, they concluded that there was an "active" or energy-dependent uptake of free NH_4^+. In a typical experiment cited (256), the NH_4^+ content (corrected for swelling) was 11.35 μmole/g while the medium concentration at the end of the incubation was 8.52 μmole/ml. This gave an apparent "NH_4^+ space" in the tissue of 133 per cent, which they said was clearly an impossible value unless interpreted to mean an "active" concentration of NH_4^+ in the intracellular spaces of the tissue.

The work with rat brain cortex slices reported in this chapter is in agreement with the values of NH_4^+ obtained by Tower and coworkers, but our interpretation differs considerably from theirs. We conclude that NH_4^+ ions are not taken up against a concentration in brain cortex slices, but endogenous NH_4^+ is formed within specific compartments in the brain tissue. independently of the concentration of NH_4^+ in the incubation medium. The sum of the tissue endogenous NH_4^+ and that which has entered by passive

diffusion, gives the appearance of "active" NH_4^+ transport. Recently published data of Wherett and Tower (76) support our interpretation. The results of experiments directed to throw light on NH_4^+ transport into brain are described below.

6.1 Tissue and medium contents of NH_4^+ of rat brain cortex slices incubated under varying conditions.

Some of the studies on the distribution between tissue and medium of the ammonia formed, when brain cortex slices of the rat are incubated in Krebs-Ringer phosphate medium under varying incubation conditions, are given in Table 22. It is evident from the results in this Table, that the ammonia contents of the tissue remain approximately constant under many different incubation conditions. The results show that the variation in the rates of ammonia formation by rat cerebral cortex slices incubated in media of differing contents, are largely confined to the NH_4^+ contents of the incubation medium. For example, the incubation medium concentration of NH_4^+ in the absence of glucose is more than three times that in its presence. Moreover, the inhibition of ammonia utilization in the presence of glucose (largely due to the inhibition of the synthesis of glutamine) by ouabain, methionine sulfoximine, fluoroacetate, and 2, 4-dinitrophenol, manifests itself in increased media NH_4^+ contents, while the tissue concentrations remain more or less the same. These facts are understandable if ammonia is formed in specific compartment(s) of the brain cells and above a limiting concentration in such compartments, NH_4^+ is released into the cytoplasm and thence into the incubation medium. This interpretation would explain why a major proportion of the ammonia formed by cerebral cortex slices during respiration under various conditions is found in the medium while the tissue retains a constant ammonia level.

TABLE 22. The tissue and medium concentrations of NH_4^+ on incubating rat brain cortex slices under a variety of conditions.

Rat brain cortex slices were incubated at 37°C in Krebs-Ringer phosphate medium in the presence or absence of glucose or metabolic inhibitors for periods of time as given below. Tissue NH_4^+ contents are expressed as μmole/g initial wet wt. tissue and medium NH_4^+ contents as μmole/g initial wet wt. tissue/3 ml.

Additions to the incubation medium	Tissue NH_4^+	Medium NH_4^+	Total
Nil; one hour incubation	2.66 ± 0.27	14.60 ± 0.32	17.26
+ ouabain (0.01mM)	2.65 ± 0.15	13.85 ± 0.25	16.50
+ methionine-sulfoximine (5mM)	2.95 ± 0.11	16.76 ± 0.05	19.71
Ca^{++}-free; two hour incubation	3.14 ± 0.13	19.35 ± 0.18	22.49
Glucose (10mM); one hour incubation	1.89 ± 0.16	4.13 ± 0.28	6.02
+ ouabain (0.01mM)	2.15 ± 0.05	7.55 ± 0.36	9.70
(0.1mM)	2.12 ± 0.15	8.80 ± 0.44	10.92
+ methionine-sulfoximine (5mM)	2.06 ± 0.11	9.36 ± 0.07	11.42
+ sodium fluoroacetate (1mM)	2.22 ± 0.23	8.62 ± 0.58	10.84
+ ouabain (0.1mM) + fluoroacetate (1mM)	2.38 ± 0.26	8.89 ± 0.65	11.27
+ 2,4 DNP (0.1mM)	1.96 ± 0.22	9.90 ± 0.55	11.86
+ sodium malonate (2mM)	2.30 ± 0.34	5.40 ± 0.20	7.70
Glucose (10mM); four hour incubation	1.73 ± 0.05	4.93 ± 0.10	6.66
+ methionine-sulfoximine (5mM)	2.90 ± 0.42	17.42 ± 0.50	20.32

6.2 Tissue/Medium concentration ratios for NH_4^+ in the incubated brain slice.

Further evidence in support of the concept of compartmentation of ammonia formation in brain cells is obtained from a study of the Tissue/Medium concentration ratios for ammonia at the end of one hour incubation periods. The Tissue/Medium concentration ratios are larger than one, under all incubation conditions tested. Results of a few typical examples are given in Table 23. These results are calculated from values reported in Table 22, taking swelling of the tissue into consideration. From these results it can be seen that even in the presence of 10 μM ouabain (a potent inhibitor of active transport processes) in a glucose containing medium, the ratio is as high as 9. If the Tissue/Medium concentration ratios were due to accumulation of NH_4^+ against a concentration gradient, all evidence (104) indicates that the presence of ouabain would reduce this to near unity. Anaerobic incubations (see Table 26) or incubation for lengthy durations (e.g., 2 hours), have little effects on the tissue ammonia content, and the Tissue/Medium concentration ratios for NH_4^+ are considerably larger than one on incubation of the slices with 0.1 mM DNP (T/M = 5.2), or with 2 mM sodium malonate (T/M = 12.3), or in the absence of glucose (T/M = 4.0). Such conditions are known to reduce energy dependent transport against a concentration gradient. The fact that high values are still reached is evidence against active transport.

The relatively high Tissue/Medium concentration ratios of NH_4^+ for brain cortex slices incubated with glucose (T/M = 14.5) is held to be due to the fact that while the tissue NH_4^+ concentration is little affected, the total rate of NH_4^+ formation is much diminished, so that relatively little NH_4^+ is released into the incubation medium. This results in a high Tissue/Medium ratio for ammonia.

TABLE 23. The tissue to medium concentration ratios for NH_4^+ in rat brain cortex slices incubated in a variety of media.

Tissue values of ammonia are obtained from Table 22 and expressed as μmole/ml tissue water, taking into consideration the swelling of the tissue at the end of the incubation period. Medium values of ammonia, from Table 22, are expressed as μ mole/ml taking the average initial wet wt. of the tissue investigated to be 100 mg. The medium NH_4^+ concentration (μmole/ml = mM) is the medium value (Table 22) divided by 3 x 10 = 30.

Additions to Krebs-Ringer phosphate medium	Tissue NH_4^+ μmole/g initial wet wt.	Tissue NH_4^+ μmole/ml T	Medium NH_4^+ mM M	$\frac{T}{M}$
Nil; one hour incubation	2.66	2.13	0.49	4.4
+ ouabain (0.01mM)	2.65	2.10	0.46	4.6
+ methionine-sulfoximine (5mM)	2.95	2.36	0.56	4.2
Ca^{++}-free; two hour incubation	3.14	2.12	0.64	3.3
Glucose (10mM); one hour incubation	1.89	2.02	0.14	14.5
+ ouabain (0.01mM)	2.15	1.96	0.25	7.8
(0.1mM)	2.12	1.80	0.29	6.2
+ methionine-sulfoximine (5mM)	2.06	2.15	0.31	6.9
+ sodium fluoroacetate (1mM)	2.22	2.30	0.29	7.9
+ ouabain (0.1mM) + fluoroacetate (1mM)	2.38	2.06	0.30	6.9
+ 2,4-DNP (0.1mM)	1.96	1.70	0.33	5.2
+ sodium malonate (2mM)	2.30	2.23	0.18	12.3

6.3 Exogenous NH_4^+ accumulation in cerebral cortex slices of the rat

Having demonstrated the existence of a concentration of NH_4^+ in cerebral cortex slices incubated in various media, experiments were carried out to examine the accumulation of NH_4^+ within rat brain cortex incubated for one hour at 37°C in the presence of varying concentrations of NH_4Cl added to the incubation medium.

These studies were carried out under the following incubation conditions, the water uptakes by the tissue being measured at the end of the incubation.

1. Aerobically in the presence of 10 mM glucose (Table 24).
2. Aerobically in the absence of glucose (Table 25).
3. Anaerobically in the presence of 10 mM glucose (Table 26).

It is seen from results given in Table 24, that when the NH_4^+ content of the tissue found in the absence of added NH_4Cl is subtracted from the tissue NH_4^+ contents obtained at the end of the one hour incubation with externally added NH_4^+, the Tissue/Medium concentration ratios are approximately unity.

The value of unity is also obtained when the incubation is carried out aerobically in the absence of glucose (Table 25), or even anaerobically in the presence of glucose (Table 26). This evidence indicates that there is only passive diffusion of NH_4^+ from the incubation medium into the slice. The initial concentrations of NH_4^+ in the incubation medium were used in the calculations of the Tissue/Medium concentration ratios. This is permissible since the utilization of NH_4^+ is sufficiently low not to affect within experimental error, the medium NH_4^+ concentration.

TABLE 24. Uptake of NH_4^+ by rat brain cortex slices incubated in the presence of glucose.

Rat brain cortex slices were incubated in O_2 at 37°C for one hour in Krebs-Ringer phosphate glucose medium containing increasing concentrations of NH_4Cl. Values of tissue NH_4^+ obtained as μ mole/g initial wet wt. (Tg) were converted to μ mole/ml tissue water (T) by taking tissue swelling into consideration. (Tg-E) values are obtained by subtracting the endogenous tissue NH_4 content (E = 2.5 μmole/g) from the NH_4 contents of slices incubated with external NH_4^+; these values were then converted to μ mole/ml tissue water (T*) taking swelling into consideration.

Medium NH_4Cl (mM)	μmole/g initial wet wt.		Water Uptake μl/100 mg initial wet wt.	Tissue NH_4^+ μ mole/ml tissue water		Medium NH_4Cl (mM) after incubation	Tissue NH_4^+ / Medium NH_4^+	
	Tissue NH_4^+ Tg	Net NH_4^+ uptake (Tg - E)		T	T*	M	T/M	T*/M
Nil	2.50 ± 0.20	-	11.6 ± 1.3	2.75	-	0.13 ± 0.01	21.20	-
2.0	4.14 ± 0.19	1.64	14.6 ± 0.3	4.48	1.74	1.81 ± 0.10	2.48	0.96
5.0	6.68 ± 0.40	4.18	14.6 ± 0.2	7.06	4.42	4.40 ± 0.10	1.60	1.00
10.0	12.42 ± 0.12	9.92	26.1 ± 1.1	11.70	9.32	9.23 ± 0.21	1.27	1.01
20.0	26.30 ± 0.50	23.80	40.0 ± 4.0	21.90	19.80	19.40 ± 0.40	1.13	1.02
30.0	40.40 ± 0.20	37.90	47.8 ± 0.6	31.70	29.70	29.20 ± 1.00	1.09	1.02

TABLE 25. Uptake of NH_4^+ by rat brain cortex slices incubated in the absence of glucose.

Rat brain cortex slices were incubated in O_2 at 37°C for one hour in glucose-free Krebs-Ringer phosphate medium containing increasing concentrations of NH_4Cl. Calculations were done as described in Table 24. The medium NH_4^+ concentration was essentially unchanged at the end of the incubation period. E = 3.66 μmole/g.

Medium NH_4Cl M(mM)	μmole/g initial wet wt.		Water Uptake μl/100 mg initial wet wt.	Tissue NH_4^+ μmole/ml tissue water		Tissue NH_4^+ / Medium NH_4^+	
	Tissue NH_4^+ Tg	Net NH_4^+ uptake (Tg - E)		T	T*	T/M	T*/M
Nil	3.66 ± 0.18	-	45.2 ± 1.2	2.92	-	-	-
2	6.19 ± 0.15	2.53	44.2 ± 0.8	4.98	2.03	2.49	1.01
5	10.10 ± 0.22	6.44	48.2 ± 1.2	7.88	5.02	1.58	1.00
10	16.00 ± 1.20	12.34	43.0 ± 1.3	13.00	10.05	1.30	1.01
20	31.40 ± 1.40	27.74	53.7 ± 2.0	23.50	20.76	1.18	1.04
30	40.10 ± 2.70	36.44	42.0 ± 1.4	33.60	29.98	1.12	1.00

TABLE 26. The uptake of NH_4^+ by rat brain cortex slices in anoxia.

Rat brain cortex slices were incubated at 37°C for one hour in Krebs-Ringer bicarbonate glucose medium containing increasing concentrations of NH_4Cl in an atmosphere of N_2/CO_2 (95%/5%). Water uptake was 50 ± 3 µl/100 mg initial wet wt. under these conditions. The medium concentration of NH_4^+ was essentially unchanged at the end of the incubation period. T* = (T - 2.02) µmole/ml. tissue water.

Medium NH_4Cl M(mM)	Tissue NH_4^+		T* µmole/ml.	Tissue NH_4^+/Medium NH_4^+	
	µ mole /g initial wet wt.	µmole/ml T		T/M	T*/M
Nil	2.63 ± 0.26	2.02	-	12.24	-
2	5.28 ± 0.59	4.06	2.04	2.03	1.02
5	9.20 ± 0.76	7.06	5.04	1.41	1.01
10	15.42 ± 0.40	11.82	9.84	1.18	0.98

6.4 Comparison of the transport processes for NH_4^+ and L-glutamate into brain cells.

Experiments were carried out to compare the uptake of NH_4^+ ions with that of an amino acid known to be accumulated against a concentration gradient. Glutamate was chosen for the following reasons:

1. It is present in high concentrations in brain cortex slices and its level does not substantially change on incubation for one hour in a glucose containing medium (Table 2).

2. We have demonstrated that exogenous labelled L-glutamate only slowly exchanges with the bulk of the glutamate (endogenous) present in the tissue (section 5.3). This is in accord with the results of experiments carried out in this laboratory (258) showing that tissue glutamate labelled with radioactive glucose does not exchange freely with cold glutamate in a subsequent incubation.

3. We have also demonstrated that the uptake of labelled glutamate by cerebral cortex slices is less than the total tissue glutamate measured by the ninhydrin reaction using the Amino Acid Analyzer. The difference between these two values is about equal to the tissue glutamate content found at the end of the incubation period with no glutamate added to the incubation medium (section 5.2).

The results given in Figure 1 show that, in contrast to NH_4^+, there is evidence of accumulation of glutamate against a concentration gradient, even when the endogenous content of glutamate is subtracted from the final tissue values after incubation.

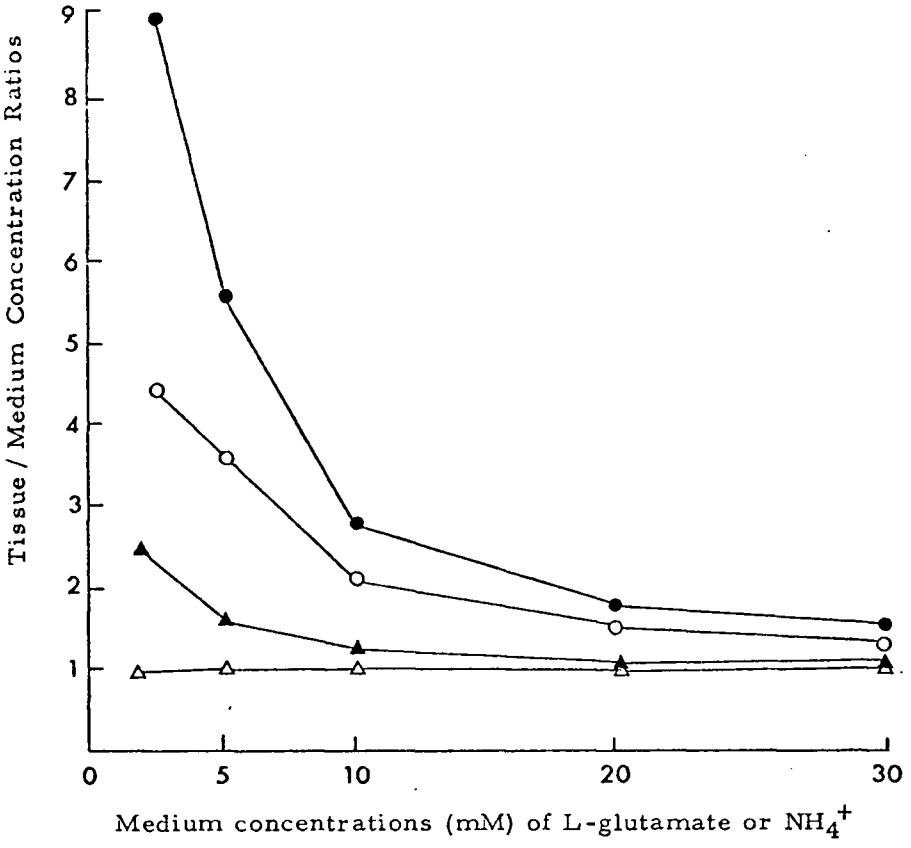


FIGURE 1. Effects of increasing sodium L-glutamate or NH_4^+ concentrations on their Tissue/Medium concentration ratios of incubated rat brain cortex slices.

Rat brain cortex slices were incubated in O_2 at 37°C for one hour in Krebs-Ringer phosphate glucose medium in the presence of increasing concentrations of sodium-L-glutamate or NH_4Cl. Values of Tissue/Medium concentration ratios (T/M) for L-glutamate and NH_4^+ are taken from Tables 17 and 24 respectively. Medium concentrations (mM) of glutamate or NH_4^+ are the concentrations at the beginning of the incubation.

- ● Values for L-glutamate: total tissue glutamate contents used to derive T/M concentration ratios for glutamate.
- ○ Values for L-glutamate: net glutamate uptake values used to derive T/M concentration ratios for glutamate.
- ▲ Values for NH_4^+: total tissue NH_4^+ contents used to derive T/M concentration ratios for NH_4^+.
- △ Values for NH_4^+: net NH_4^+ uptake values used to derive T/M concentration ratios for NH_4^+.

6.5 Rates of NH_4^+ and glycine uptakes by brain cortex slices.

The kinetics of the passive (?) accumulation of NH_4^+ were studied and compared with the kinetics of the active uptake of glycine in rat brain cortex slices incubated in O_2 at 37°C in Krebs-Ringer phosphate glucose medium. Glycine (5 mM) was used instead of glutamate because in brain it undergoes relatively little metabolic change under the given experimental conditions (114, 128, our observation).

From the results given in Table 27, it is seen that, while the amount of glycine taken up by brain cortex slices against a concentration gradient increases with time, that of NH_4^+ reaches its maximum before the commencement of the incubation. This is further evidence for a rapid movement of NH_4^+ into cerebral cortex slices. It is to be noted that the concentration of glycine in the tissue (like that of NH_4^+) very rapidly attains that in the incubation medium, before the incubation commenced. This is obviously due to a rapid passive component of (glycine) uptake.

6.6 Effects of NH_4^+ on glycine uptake.

It is a well known fact that the active transport of one substrate into brain tissue may be competitively inhibited by another substrate, especially when they share the same carrier system (113, 114).

Using labelled glycine (2 mM), Nakazawa and Quastel (114) showed that NH_4^+ inhibits the active transport of glycine into cerebral cortex slices. We have confirmed this finding and have likewise shown the inhibition occurs also with cationic (KCl, 30 mM) stimulated slices (Table 28). This effect of NH_4^+ has been presumed to be due to a fall in the ATP levels, and indeed a small drop in the cellular ATP levels does occur with 10 mM NH_4Cl (see Table 71), even though the synthesis of glutamine is enhanced in the presence of NH_4^+ ions (section 6.10).

TABLE 27. Rates of NH_4^+ and of glycine uptake by rat brain cortex slices.

Incubations were carried out in O_2 at 37°C for various periods of time in Krebs-Ringer phosphate glucose medium in the presence either of 10mM NH_4Cl or of 5mM glycine. QO_2 values are expressed as μmoles/g initial wet wt. tissue. Tissue contents of ammonia or of glycine are expressed as μ mole/g initial wet wt. and μmole/ml. tissue water.

Period of Incubation	Tissue NH_4^+ content of rat brain cortex slices at the end of the incubation period in 10mM NH_4Cl (QO_2 = 114 ± 4)		Tissue glycine content of rat brain cortex slices at the end of the incubation period in 5mM glycine (QO_2 = 113 ± 2)	
	μmole/g	μmole/ml	μmole/g	μmole/ml
After (5 min) oxygenation	8.80 ± 0.10	10.04	2.02 ± 0.22	2.30
After (7 min) equilibration (i.e. at zero time)	11.05 ± 0.57	12.30	6.41 ± 0.77	7.00
15 min	11.73 ± 0.01	12.76	13.60 ± 1.40	14.41
30 min	11.15 ± 0.15	11.84	23.55 ± 2.35	24.30
45 min	12.05 ± 0.55	11.99	24.80 ± 1.20	25.00
60 min	11.64 ± 0.32	11.05	30.00 ± 0.22	27.83

TABLE 28. The inhibitory effects of NH_4^+ on the active transport of glycine into rat brain cortex slices.

Incubation of rat brain cortex slices was carried out in O_2 at 37°C for one hour in Krebs-Ringer phosphate glucose medium containing 5mM glycine with or without the addition of 10mM, NH_4Cl or of 25mM KCl. Oxygen uptakes (QO_2) are expressed as μmole/g initial wet wt. tissue. Tissue and medium contents of glycine determined at the end of the incubation are given below.

Additions to Krebs-Ringer Phosphate Glucose Medium	QO_2	Tissue Glycine Content		Medium Glycine after incubation M(mM)	$\frac{T}{M}$
		μmole/g initial wet wt.	μmole/ml. T		
Glycine	113 ± 9	30.0 ± 0.2	27.8	4.72 ± 0.13	5.9
Glycine + NH_4Cl	108 ± 8	20.0 ± 1.2	17.2	5.33 ± 0.15	3.2
Glycine + KCl	146 ± 2	28.7 ± 2.8	24.4	4.67 ± 0.05	5.2
Glycine + KCl + NH_4Cl	140 ± 4	17.8 ± 1.6	14.7	4.81 ± 0.27	3.1

6.7 Effects of glycine on NH_4^+ uptake.

The effects of glycine (5 mM) on NH_4^+ transport (NH_4Cl, 10 mM) in rat brain cortex slices in the presence or absence of 30 mM KCl are given in Table 29. It is evident that glycine does not affect the uptake of NH_4^+ into the tissue. Possibly this is because the transport of NH_4^+ is a passive process.

6.8 Uptake (into brain cortex slices) of exogenous L-glutamate and NH_4^+ when present together in the incubation medium.

This study carried out with L-glutamate, is similar to the work described above for glycine (sections 6.6 and 6.7). Results obtained are shown in Table 30. Thus, while NH_4Cl (5 mM) strongly inhibits the uptake of L-glutamate (5 mM) from the incubation medium, L-glutamate has no effect on the uptake of NH_4^+. The NH_4^+ inhibition is attributed to the fall in cell ATP. Increased tissue utilization of glutamate to form glutamine is insufficient to account for the fall in tissue glutamate concentration (Table 13).

6.9 Effects of metabolic inhibitors on the uptake of NH_4^+ by incubated rat brain cortex slices.

Experiments were carried out to observe effects of metabolic inhibitors on NH_4^+ uptake into the brain. In Table 31, the effects of various metabolic inhibitors on glycine and NH_4^+ transport into rat brain cortex slices are shown. Glycine was used to test the potency of some of the inhibitors on the process of active transport. The addition of fluoroacetate (1 mM), or malonate (2 mM), or DNP (0.1 mM), or ouabain (0.1 mM), has little or no effect on the NH_4^+ concentrations of brain cortex slices incubated in O_2 at 37°C for one hour in Krebs-Ringer phosphate glucose medium containing 10 mM NH_4Cl. On the other hand, there is a marked inhibition of glycine uptake in the presence of DNP (0.1 mM)

TABLE 29. Absence of an inhibitory effect of glycine on the uptake of ammonium by rat brain cortex slices.

Incubation of rat brain cortex slices was carried out in O_2 at 37°C for one hour in Krebs-Ringer phosphate medium containing 10mM NH_4Cl. Glycine and KCl when added were 5 mM and 25mM respectively. Tissue contents of ammonium (μmole/g) at the end of the incubation are given below.

Additions to Krebs-Ringer phosphate glucose medium	Tissue Ammonium	
	μmole/g initial wet wt.	μmole/ml
NH_4Cl	12.42 ± 0.12	11.70
NH_4Cl + Glycine	13.35 ± 0.10	11.60
NH_4Cl + KCl + Glycine	14.00 ± 0.40	11.64

TABLE 30. Effects of the simultaneous presence of exogenous sodium L-glutamate and NH_4Cl on the transport processes for ammonium and glutamate into rat brain cortex slices.

Incubation of rat brain cortex slices was done in O_2 for one hour at 37°C in Krebs-Ringer phosphate glucose medium with or without the addition of NH_4Cl (5mM) or sodium L-glutamate (5mM). Tissue contents of ammonium or glutamate at the end of the incubation period are expressed as μmole/g initial wet wt. tissue.

Additions to Krebs-Ringer phosphate glucose medium	Tissue Contents Of	
	Ammonium	Glutamate
NH_4Cl	6.68 ± 0.40	-
L-Glutamate	-	23.70 ± 0.70
L-Glutamate + NH_4Cl	6.65 ± 0.07	14.52 ± 0.18

TABLE 31. Effects of metabolic inhibitors on the transport of NH_4^+ and of glycine into rat brain cortex slices.

Rat brain cortex slices were incubated in O_2 at 37°C for one hour in Krebs-Ringer phosphate glucose medium with either 10mM NH_4Cl or 5mM glycine. Metabolic inhibitors were added as indicated. Tissue contents of ammonia or glycine are given in μmole/g initial wet wt. tissue and μmole/ml. tissue water.

Additions to Krebs-Ringer phosphate glucose medium	Tissue NH_4^+ contents of rat brain cortex slices at the end of one hour incubation in 10mM NH_4Cl		Tissue glycine contents of rat brain cortex slices at the end of one hour incubation in 5mM glycine	
	μmole/g	μmole/ml	μmole/g	μmole/ml
No inhibitor	12.42 ± 0.12	11.70	29.80 ± 0.20	27.00
+ sodium fluoroacetate (1mM)	12.45 ± 0.05	11.80	-	-
+ sodium malonate (2mM)	12.76 ± 0.20	11.18	-	-
+ 2,4-DNP (0.1mM)	13.72 ± 0.44	11.45	10.28 ± 0.14	8.72
+ ouabain (0.1mM)	14.51 ± 0.31	11.66	7.39 ± 0.14	6.33
Glucose absent	16.00 ± 1.20	13.00	10.68 ± 0.10	8.68

or glucose-lack, and a nearly complete inhibition of the active transport component of glycine uptake with 0.1 mM ouabain.

The above results support the conclusion that NH_4^+ uptake into brain is by passive diffusion.

6.10 Effects of increasing NH_4^+ concentrations on amino acid content in, and release from, incubated rat brain cortex slices.

Experiments were also carried out to observe the effects of increasing medium NH_4Cl concentrations (tissue, medium and total) of rat brain cortex slices incubated in O_2 at 37°C for one hour in Krebs-Ringer phosphate glucose medium.

It is evident from results given in Tables 32 and 33 that NH_4^+ ions have profound effects on amino acid metabolism. As expected, there is a rise in glutamine of both the tissue and medium. The rate of glutamine synthesis, however, seems to have reached its maximum value with the lowest level of NH_4Cl concentration (2 mM) used. There are substantial falls in the tissue levels of glutamate and aspartate, the falls increasing with increasing concentrations of external NH_4^+. Total (tissue + medium) GABA levels are enhanced with increasing external NH_4^+ concentrations.

The release of amino acids to the incubation medium is little affected by external NH_4Cl concentrations of 10 mM or lower. At concentrations of 20 or 30 mM NH_4Cl, significant increases in the medium concentrations of all amino acids measured occur. The release of amino acids to the incubation medium coincides with relatively large falls in respiration (Table 58), and ATP levels (Table 71) of the tissue. It would appear that the effects of relatively high levels of NH_4^+ in the incubations medium are largely due to the fall of cell ATP accompanying glutamine synthesis. There appears, however, to be a specific effect on the formation and release of GABA.

TABLE 32. Effects of increasing medium NH_4Cl concentrations on the amino acid contents of incubated rat brain cortex slices.

Cerebral cortex slices of the rat were incubated in O_2 at 37°C for one hour in Krebs-Ringer phosphate glucose medium containing increasing medium NH_4Cl concentrations. Tissue contents of amino acids are expressed as μmole/g initial wet wt. tissue.

Initial Medium NH_4Cl (mM)	Glutamate	Glutamine	GABA	Aspartate	Glycine	Alanine	Taurine
Nil	8.48 ± 0.24	3.26 ± 0.09	1.80 ± 0.09	2.93 ± 0.01	0.90 ± 0.06	0.90 ± 0.08	4.49 ± 0.15
2.0	6.37 ± 0.25	4.82 ± 0.12	1.86 ± 0.03	1.98 ± 0.24	0.89 ± 0.06	0.89 ± 0.10	4.04 ± 0.04
5.0	5.98 ± 0.16	4.78 ± 0.19	2.13 ± 0.14	1.86 ± 0.10	0.89 ± 0.04	0.79 ± 0.17	4.18 ± 0.11
10.0	5.67 ± 0.15	5.32 ± 0.23	2.30 ± 0.14	1.66 ± 0.06	0.81 ± 0.03	0.95 ± 0.06	3.21 ± 0.06
20.0	4.34 ± 0.18	4.07 ± 0.17	2.60 ± 0.15	1.18 ± 0.04	0.52 ± 0.11	0.41 ± 0.10	2.31 ± 0.48
30.0	2.71 ± 0.07	3.98 ± 0.11	3.10 ± 0.12	0.89 ± 0.03	0.42 ± 0.05	0.42 ± 0.02	1.75 ± 0.20

TABLE 33. Effects of increasing medium NH_4Cl concentrations on the release of amino acids from incubated rat brain cortex slices.

The experimental conditions were as described in Table 32. Medium contents of amino acids are expressed as μmole/g initial wet wt. tissue/3 ml. medium.

Initial Medium NH_4Cl (mM)	Glutamate	Glutamine	GABA	Aspartate	Glycine	Alanine	Taurine
Nil	0.54 ± 0.09	3.13 ± 0.14	0.04 ± 0.02	0.32 ± 0.02	0.44 ± 0.20	0.50 ± 0.03	1.96 ± 0.02
2.0	0.44 ± 0.05	5.23 ± 0.16	0.04 ± 0.03	0.35 ± 0.01	0.38 ± 0.06	0.65 ± 0.05	2.16 ± 0.08
5.0	0.53 ± 0.07	5.10 ± 0.21	0.08 ± 0.04	0.34 ± 0.02	0.38 ± 0.06	0.76 ± 0.11	2.06 ± 0.02
10.0	0.63 ± 0.14	4.41 ± 0.13	0.09 ± 0.03	0.37 ± 0.04	0.57 ± 0.15	0.99 ± 0.17	3.01 ± 0.11
20.0	1.39 ± 0.17	5.50 ± 0.09	0.22 ± 0.08	0.58 ± 0.06	0.73 ± 0.04	1.11 ± 0.04	3.44 ± 0.32
30.0	2.72 ± 0.10	6.51 ± 0.22	0.34 ± 0.10	1.04 ± 0.04	0.89 ± 0.06	1.15 ± 0.21	4.58 ± 0.28

6.11 Summary

1. The NH_4^+ concentration of incubated rat brain cortex slices is little affected in the presence or absence of glucose, or in the presence of metabolic inhibitors (such as sodium fluoroacetate, sodium malonate, ouabain, DNP or methionine sulfoximine), or under anoxia.

2. The enhanced rates of NH_4^+ formation by incubated brain tissue in the absence of glucose, or in the presence of metabolic inhibitors, are reflected largely in the enhanced NH_4^+ concentrations of the incubation medium.

3. The NH_4^+ concentration of brain cortex slices incubated with external NH_4^+ is little affected in the absence of glucose or of oxygen, in the presence of ouabain or of amino acids such as glycine or sodium L-glutamate, or in the presence of metabolic inhibitors such as sodium fluoroacetate, sodium malonate or DNP. The Tissue/Medium concentration ratios for NH_4^+ become unity when the ammonium concentration of the tissue found in the absence of added NH_4^+ is subtracted from the tissue NH_4^+ concentrations obtained with externally added NH_4^+.

4. It is concluded that NH_4^+ ions are not taken up against a concentration gradient in brain cortex slices, but endogenous NH_4^+ is formed within specific compartments in the brain tissue independent of the concentration of NH_4^+ in the external medium.

5. NH_4^+ ions inhibit the active transport of glycine or L-glutamate presumably by lowering the ATP concentration of the tissue.

6. Results on the effects of increasing external NH_4^+ concentrations on the amino acid content in, and release from, incubated brain cortex slices, are given.

7. EFFECTS OF NEUROTROPIC DRUGS ON THE RELEASE OF AMINO ACIDS FROM THE BRAIN IN VITRO

The generation of action potentials, or an activation of the Na^+-current at the brain cell membrane, is presumed to occur in incubated brain cortex slices under certain conditions. It manifests itself in an enhanced tissue level of Na^+ and a net efflux of tissue K^+. The criterion used to establish its occurrence is its abolition by neurotropic drugs that block the Na^+-current system. An important tool for this purpose is tetrodotoxin (TTX). TTX appears not to affect non-excitable cells and only affects cells in the state of excitation. TTX is not known to have any direct effects on metabolic events in the brain. It does not itself affect the ionic, and as we will show, amino acid contents of brain slices incubated in a normal Krebs-Ringer glucose medium (Introduction, Section 1.9 (i)).

The TTX-sensitive Na^+-current system is activated in brain cortex slices in the presence of protoveratrine, or ouabain, or in the absence of glucose or of Ca^{++}, or on the application of electrical pulses (182), or at the onset of anoxia (192, 205). It should be noted that, while the activation of the TTX-sensitive Na^+-current system results in altered tissue ionic concentrations, not all alterations in the tissue levels of cations (as, for example, due to DNP, or high K^+ or L-glutamate (182)) are the outcome of an activated Na^+-current system that is TTX-sensitive.

It will now be demonstrated that some incubation conditions and a number of neurotropic drugs have profound effects on the release of amino acids from incubated brain tissue. The results of these experiments and their possible implications in our understanding of the fluxes of cerebral amino acids are described below.

7.1 Effects of protoveratrine, ouabain and tetrodotoxin on amino acid content in, and release from, rat brain cortex slices incubated in glucose-saline media.

Results recorded in Tables 34 and 35 show the values of the amino acids present in the slices (Table 34) and in the incubation medium (Table 35) after incubation of the slices in a physiological saline-glucose medium in O_2 for one hour. It will be observed that the incubation brought about relatively small changes in the total (i.e., tissue and medium) quantities of amino acids. There was a rise in glutamine content from 4.40 μmole/g to 6.37 μmole/g, and a fall of glutamate content from 11.83 μmole/g to 9.97 μmole/g. There were small rises in the contents of aspartate, γ-aminobutyrate, glycine and alanine. In view of the fact that glutamate is oxidized under these conditions (112), giving rise to substantial quantities of aspartate, it is evident that a steady state is achieved in the brain tissue during aerobic incubation in the glucose medium whereby glutamate loss by oxidation is compensated by its gain by other processes; e.g., transamination of α-ketoglutarate by other amino acids in the tissue. The steady state is presumably such that efflux of amino acids from the tissue to the incubation medium is balanced by active uptake processes from medium to tissue.

Addition of protoveratrine (5 μM), known to affect the kinetics of glucose metabolism in incubated brain slices (211, 212, 213), and to generate action potentials in nervous tissue (167, 207) brought about significant falls in the tissue contents of glutamate and aspartate (Table 34) with no corresponding increases in the medium (Table 35), and very small changes (if any) in the quantities of the other amino acids investigated. The changes due to protoveratrine were abolished by the presence of tetrodotoxin (2 μM) which, itself, caused no changes in the amino acid content of tissue and medium (Tables 34 and 35).

The changes, brought about by protoveratrine, in the tissue contents

TABLE 34. Effects of protoveratrine, ouabain, lidocaine and tetrodotoxin on the contents of amino acids in incubated rat brain cortex slices.

Amounts of amino acids (expressed as μ mole/g initial wet wt. tissue) present in rat brain cortex slices incubated in O_2 for one hour at 37°C in Krebs-Ringer phosphate medium containing 10 mM glucose in the presence or absence of protoveratrine (5 μM), ouabain (0.1mM), lidocaine (0.5mM) and tetrodotoxin (2 μM).

Additions to the incubation medium	Glutamate	Glutamine	GABA	Aspartate	Glycine	Alanine
Nil	9.20 ± 0.14	3.45 ± 0.30	2.56 ± 0.20	3.45 ± 0.23	1.03 ± 0.15	0.75 ± 0.05
Tetrodotoxin	8.82 ± 0.01	2.88 ± 0.11	2.58 ± 0.16	3.64 ± 0.20	0.88 ± 0.03	0.82 ± 0.02
Protoveratrine	6.00 ± 0.83	4.10 ± 0.39	2.18 ± 0.27	2.15 ± 0.12	1.01 ± 0.06	0.85 ± 0.06
Protoveratrine + Tetrodotoxin	8.83 ± 0.01	3.46 ± 0.03	2.60 ± 0.16	3.51 ± 0.15	1.10 ± 0.10	0.75 ± 0.02
Ouabain	4.95 ± 0.26	0.71 ± 0.05	1.39 ± 0.06	1.75 ± 0.32	0.59 ± 0.03	0.42 ± 0.02
Ouabain + Tetrodotoxin	6.94 ± 0.36	0.53 ± 0.04	1.71 ± 0.06	3.32 ± 0.02	0.82 ± 0.03	0.45 ± 0.03
Initial values prior to incubation	11.83 ± 1.09	4.40 ± 0.15	2.01 ± 0.10	3.36 ± 0.34	0.79 ± 0.06	0.61 ± 0.08
Lidocaine	8.86 ± 0.27	3.23 ± 0.26	2.25 ± 0.14	3.12 ± 0.28	0.98 ± 0.11	0.73 ± 0.13
Protoveratrine + Lidocaine	9.21 ± 0.27	4.11 ± 0.04	2.40 ± 0.02	3.08 ± 0.21	1.08 ± 0.03	0.75 ± 0.02

TABLE 35. Effects of protoveratrine, ouabain, lidocaine and tetrodotoxin on the release of amino acids from incubated rat brain cortex slices.

Amounts of amino acids (μmole/g initial wet wt. tissue) in the incubation medium (3 ml.) after incubation of rat brain cortex slices in O_2 for one hour at 37°C in 3 ml Krebs-Ringer phosphate medium containing 10 mM glucose in the presence or absence of protoveratrine (5 μM), ouabain (0.1mM), lidocaine (0.5mM) and tetrodotoxin (2 μM).

Additions to the incubation medium	Glutamate	Glutamine	GABA	Aspartate	Glycine	Alanine
Nil	0.77 ± 0.13	2.92 ± 0.28	0.03 ± 0.02	0.43 ± 0.05	0.36 ± 0.06	0.44 ± 0.04
Tetrodotoxin	0.87 ± 0.02	2.62 ± 0.13	0.04 ± 0.03	0.38 ± 0.06	0.27 ± 0.02	0.38 ± 0.02
Protoveratrine	0.76 ± 0.08	2.83 ± 0.66	0.10 ± 0.04	0.42 ± 0.01	0.36 ± 0.08	0.47 ± 0.09
Protoveratrine + Tetrodotoxin	0.60 ± 0.06	3.65 ± 0.03	0.05 ± 0.01	0.40 ± 0.04	0.19 ± 0.05	0.37 ± 0.09
Ouabain	6.34 ± 0.40	2.41 ± 0.12	2.93 ± 0.35	2.13 ± 0.05	1.12 ± 0.08	1.50 ± 0.06
Ouabain + Tetrodotoxin	2.18 ± 0.36	1.65 ± 0.08	2.42 ± 0.26	0.95 ± 0.26	0.84 ± 0.16	1.15 ± 0.03
Lidocaine	0.84 ± 0.13	2.44 ± 0.12	0.04 ± 0.02	0.50 ± 0.11	0.49 ± 0.10	0.35 ± 0.11
Protoveratrine + Lidocaine	0.62 ± 0.02	2.77 ± 0.08	0.10 ± 0.05	0.36 ± 0.04	0.29 ± 0.02	0.37 ± 0.10

of glutamate and aspartate could not be due to diminished rates of conversion of glucose to glutamate and aspartate as it has been shown (213) that the yields of labelled glutamate and aspartate from labelled glucose by incubated rat brain cortex slices are enhanced by the presence of protoveratrine (5 μM). Possibly the diminished tissue contents of glutamate and aspartate were due to their removal by oxidation during the process of enhanced respiration that takes place in brain tissue in the presence of protoveratrine (211-213). The fact that no marked changes in the concentrations of amino acids in the incubation medium occurred in the presence of protoveratrine may only reflect the fact that the release processes were balanced by the uptake processes.

Experiments were, therefore, carried out with ouabain partly because this substance is thought to activate the Na^+-current system at the membrane in rat brain slices incubated in a physiological saline glucose medium (182) and partly because it blocks the active transport of amino acids into brain slices (112, 114). Results, given in Tables 34 and 35, show that incubation in the presence of ouabain brought about extensive changes in the amount of amino acids in tissue and medium. There were large falls in the tissue contents of all the amino acids investigated and corresponding rises in the amino acid concentrations in the incubation medium. The total (tissue and medium) contents of glutamate (11.29 μmole/g) or of aspartate (3.88 μmole/g) in presence of ouabain were not markedly changed from the values (9.97 μmole /g and 3.88 μmole/g respectively) obtained in the absence of ouabain. However, the total (tissue and medium) content of glutamine was diminished (from 6.37 μmole/g to 3.12 μmole/g) and that of GABA was increased from 2.59 μmole/g to 4.32 μmole/g.

Addition of TTX (2 μM) brought about a substantial reduction of the stimulating effect of ouabain on the release of glutamate and aspartate, but had only minor effects on the release of the other amino acids (Table 35).

These results lead to the inference that action potentials generated by ouabain and suppressed by TTX (182) bring about the release of glutamate and aspartate from rat brain cortex slices incubated in a physiological saline glucose medium. This becomes evident under the given incubation conditions because the uptake processes are blocked by ouabain.

7.2 Effects of protoveratrine and tetrodotoxin on amino acid content in, and release from, rat brain cortex slices incubated in a glucose-free medium.

In view of the fact that action potentials appear to be generated, or the Na^+-current system activated, in Krebs-Ringer phosphate medium devoid of glucose (182), experiments were carried out to observe the effects of lack of glucose on the amino acid contents of brain tissue and on their release from the tissue in an incubation period of one hour. Results given in Tables 36 and 37 show that the tissue contents of all amino acids investigated, save aspartate, were markedly reduced from the normal (Tables 34 and 35) and that the contents of all amino acids in the incubation medium, save glutamine, were increased, particularly with GABA, aspartate and glycine. The fall in the tissue concentration of glutamate was to be expected, as this amino acid forms a major fuel of the brain in the absence of organic substrates from the incubation medium (see Chapter 3). Moreover, as glutamate oxidation is accompanied by marked production of aspartate (Table 2), the rise in the tissue concentration of aspartate was also to be expected. It was evident that, with every amino acid investigated, the ratio of tissue concentration to the medium concentration is greatly diminished from the normal by incubation of the tissue in the glucose-free medium. This result is perhaps partly due to the fact that there is depletion of tissue ATP in the absence of glucose with consequent suppression of active uptake of the amino acids.

The possibility that the generation of action potentials (on an activation of the Na^+-current system) plays a significant role in the release of amino acids

TABLE 36. Effects of protoveratrine, tetrodotoxin and lidocaine on contents of amino acids in rat brain cortex slices incubated in glucose-free media.

Amounts of amino acids (μmole/g initial wet wt. tissue) present in rat brain cortex slices incubated in O_2 for one hour at 37°C in Krebs-Ringer phosphate medium in the absence of glucose with or without the addition of protoveratrine (5μM), lidocaine (0.5 mM) and tetrodotoxin (2μM).

Additions to the glucose-free incubation medium	Glutamate	Glutamine	GABA	Aspartate	Glycine	Alanine
Nil	2.03 ± 0.33	0.51 ± 0.20	1.38 ± 0.22	7.79 ± 0.27	0.62 ± 0.12	0.17 ± 0.04
Tetrodotoxin	3.14 ± 0.34	0.48 ± 0.20	2.04 ± 0.05	8.60 ± 0.04	0.91 ± 0.11	0.20 ± 0.03
Protoveratrine	1.12 ± 0.10	1.17 ± 0.10	0.65 ± 0.14	5.68 ± 0.42	0.36 ± 0.10	0.07 ± 0.02
Protoveratrine + Tetrodotoxin	2.57 ± 0.01	0.34 ± 0.03	1.20 ± 0.02	8.43 ± 0.01	0.82 ± 0.02	0.08 ± 0.01
Lidocaine	2.36 ± 0.11	0.38 ± 0.10	1.45 ± 0.12	8.41 ± 0.21	0.86 ± 0.04	0.21 ± 0.11
Protoveratrine + Lidocaine	1.88 ± 0.30	0.45 ± 0.04	0.90 ± 0.18	8.14 ± 0.74	0.70 ± 0.06	0.12 ± 0.03

TABLE 37. Effects of protoveratrine, tetrodotoxin and lidocaine on the release of amino acids from rat brain cortex slices incubated in glucose-free media.

Amounts of amino acids (μmole/g initial wet wt. tissue) present in the incubation medium (3 ml.) after incubation of rat brain cortex slices in O_2 for one hour at 37°C in the absence of glucose with or without the addition of protoveratrine (5μ M), tetrodotoxin (2 μM) and lidocaine (0.5mM).

Additions to the glucose-free incubation medium	Glutamate	Glutamine	GABA	Aspartate	Glycine	Alanine
Nil	0.96 ± 0.12	1.74 ± 0.10	0.41 ± 0.08	1.98 ± 0.13	0.70 ± 0.10	0.52 ± 0.10
Tetrodotoxin	0.47 ± 0.11	1.33 ± 0.22	0.35 ± 0.04	0.78 ± 0.06	0.52 ± 0.12	0.36 ± 0.09
Protoveratrine	2.32 ± 0.30	2.61 ± 0.18	0.93 ± 0.07	4.31 ± 0.32	1.02 ± 0.10	0.72 ± 0.03
Protoveratrine + Tetrodotoxin	0.53 ± 0.03	1.79 ± 0.04	0.42 ± 0.02	1.21 ± 0.07	0.47 ± 0.01	0.33 ± 0.02
Lidocaine	0.48 ± 0.06	0.81 ± 0.05	0.32 ± 0.05	0.89 ± 0.10	0.61 ± 0.07	0.53 ± 0.05
Protoveratrine + Lidocaine	1.06 ± 0.32	1.28 ± 0.10	0.63 ± 0.02	1.86 ± 0.52	0.65 ± 0.06	0.54 ± 0.06

from the brain tissue, incubated in a glucose-free medium, was shown by the effects of the addition of TTX (2 μM) to the incubation medium. TTX brought about significant retention in the tissue of glutamate, GABA, aspartate and glycine (Table 36), and reduced concentrations of amino acids, particularly glutamate and aspartate, in the incubation medium (Table 37). The amino acid concentration ratio (tissue to medium) was significantly increased, with glutamate, GABA, aspartate and glycine, when TTX was present in the incubation medium.

Addition of protoveratrine (5 μM) to the glucose-free incubation medium brought about an even more pronounced lowering of the tissue concentration of glutamate, GABA, and glycine (Table 36). The tissue aspartate concentration was also diminished, but the tissue concentration of glutamine was enhanced. These changes in tissue concentrations of amino acids, brought about by protoveratrine, were accompanied by notable increases in the concentration of amino acids, particularly glutamate, GABA and aspartate in the incubation medium (Table 37). That these effects of protoveratrine were largely due to the generation of action potentials was shown by the fact that addition of TTX either abolished, or greatly reduced, the action of protoveratrine in promoting amino acid release from the tissue into the incubation medium (Tables 36 and 37). The amino acid concentration ratio (tissue to medium) found in the presence of protoveratrine, was enhanced by the addition of TTX from 14 to 143 with glutamate, from 21 to 86 with GABA, from 39 to 209 with aspartate, from 10 to 52 with glycine; it fell, however, from 13 to 5 with glutamine (Table 42). A noteworthy effect of the presence of protoveratrine was the considerable rise in the total content of glutamine. A value of 2.25 μmole/g (0.51 + 1.74) found in the absence of protoveratrine rose to 3.78 μmole/g (1.17 + 2.61) in the presence of the drug (Tables 36 and 37; see also Table 10). Such a marked rise did not occur when TTX was present; nor was it so clearly evident when glucose was present in the incubation medium (Tables 34 and 35).

7.3 Effects of protoveratrine and tetrodotoxin on amino acid content in, and release from, rat brain cortex slices incubated in calcium-deficient media.

Absence of Ca^{2+} from the glucose-containing incubation medium brought about relatively small changes from the normal in the amino acid contents of the tissue slices after aerobic incubation for one hour (Tables 34 and 38), though there seemed to be a significant fall in the tissue content of glutamine. A series of experiments was carried out with EGTA added to the calcium-deficient incubation medium in order to secure as complete a removal as possible of calcium ions from the medium. The results showed no significant changes of amino acid contents in either the tissue or the incubation medium from those found in the absence of EGTA.

Addition of protoveratrine (5 μM) to the calcium-deficient medium brought about changes in the tissue contents of amino acids similar to those found in the normal incubation medium (Tables 34 and 38), but it caused substantial increases in the efflux of glutamate, GABA, and aspartate (Tables 35 and 39). These effects of protoveratrine were abolished by the addition of TTX (Table 39). The amino acid concentration ratios (tissue:medium) found in the presence of protoveratrine, in the calcium-deficient media, rose, on addition of TTX with glutamate from 80 to 468, with GABA from 86 to 950, with aspartate from 102 to 226, with glycine from 32 to 76 (Table 42). No significant changes due to TTX occurred with glutamine or alanine.

The effects of protoveratrine in promoting TTX-sensitive amino acid effluxes in incubated rat brain cortex slices were considerably greater in the calcium-deficient medium than in the normal incubation medium, the amino acids most markedly affected being glutamate, GABA, aspartate and glycine.

The total quantity of glutamine found in the tissue and medium, at the end of the incubation period of one hour, was greater in the presence of proto-

TABLE 38. Effects of protoveratrine, tetrodotoxin, lidocaine and of sodium L-glutamate on the contents of amino acids in rat brain cortex slices incubated in calcium-deficient media.

Amounts of amino acids (μmole/g initial wet wt. tissue) in rat brain cortex slices incubated in Krebs-Ringer phosphate medium containing 10 mM glucose in O_2 for one hour at 37°C but with Ca^{2+} omitted. When present protoveratrine was 5μM; tetrodotoxin, 2μM; lidocaine, 0.5mM; and sodium L-glutamate, 2.5mM;

Additions to the Ca^{2+} deficient incubation medium	Glutamate	Glutamine	GABA	Aspartate	Glycine	Alanine
Nil	9.75 ± 0.34	1.96 ± 0.02	2.30 ± 0.05	2.95 ± 0.19	0.88 ± 0.17	0.73 ± 0.23
Tetrodotoxin	10.87 ± 0.31	2.00 ± 0.02	2.44 ± 0.10	3.21 ± 0.28	0.76 ± 0.12	0.68 ± 0.27
Protoveratrine	7.07 ± 0.68	2.53 ± 0.27	1.55 ± 0.06	2.43 ± 0.35	0.65 ± 0.11	0.55 ± 0.10
Protoveratrine + Tetrodotoxin	9.37 ± 0.09	1.50 ± 0.20	2.56 ± 0.48	3.70 ± 0.48	0.91 ± 0.02	0.85 ± 0.02
Protoveratrine + Lidocaine	10.25 ± 0.44	1.83 ± 0.20	2.70 ± 0.12	3.47 ± 0.06	0.81 ± 0.03	0.82 ± 0.12
L-Glutamate	20.17 ± 0.09	3.81 ± 0.20	3.21 ± 0.34	5.69 ± 0.07	0.81 ± 0.03	0.98 ± 0.04
L-Glutamate + Tetrodotoxin	22.61 ± 1.39	3.82 ± 0.21	3.85 ± 0.02	6.30 ± 0.30	0.99 ± 0.08	1.06 ± 0.12

TABLE 39. Effects of protoveratrine, tetrodotoxin, lidocaine and of sodium L-glutamate on the release of amino acids from rat brain cortex slices incubated in calcium-deficient media.

Amounts of amino acids (μmole/g initial wet wt. tissue) present in the calcium deficient incubation medium (3 ml.) after incubation of rat brain cortex slices in O_2 for one hour at 37°C. When present protoveratrine was 5 μM; tetrodotoxin, 2 μM; lidocaine, 0.5mM; and sodium L-glutamate 2.5mM;

Additions to the Ca^{2+} deficient incubation medium	Glutamate	Glutamine	GABA	Aspartate	Glycine	Alanine
Nil	1.17 ± 0.11	2.41 ± 0.14	0.05 ± 0.03	0.45 ± 0.05	0.44 ± 0.10	0.70 ± 0.21
Tetrodotoxin	0.72 ± 0.04	2.46 ± 0.19	0.03 ± 0.02	0.31 ± 0.01	0.34 ± 0.03	0.80 ± 0.06
Protoveratrine	2.63 ± 0.30	3.09 ± 0.34	0.55 ± 0.05	0.71 ± 0.04	0.60 ± 0.10	0.76 ± 0.14
Protoveratrine + Tetrodotoxin	0.60 ± 0.04	2.21 ± 0.04	0.08 ± 0.04	0.49 ± 0.13	0.36 ± 0.10	0.83 ± 0.21
Protoveratrine + Lidocaine	0.83 ± 0.06	2.46 ± 0.03	0.05 ± 0.02	0.51 ± 0.01	0.32 ± 0.01	0.68 ± 0.02
L-Glutamate	1.73 ± 0.06 mM	3.39 ± 0.18	0.26 ± 0.04	2.73 ± 0.03	0.38 ± 0.07	1.34 ± 0.50
L-Glutamate + Tetrodotoxin	1.73 ± 0.05 mM	3.69 ± 0.11	0.16 ± 0.02	2.73 ± 0.05	0.26 ± 0.02	0.50 ± 0.22

veratrine than in its absence (Tables 38 and 39). The values of 4.37 μmole/g was increased in the presence of protoveratrine to 5.62 μmole/g. It was the only amino acid, among those investigated, that showed a rise in total value on incubation in the presence of protoveratrine. The rise was abolished by the addition of TTX.

Experiments were carried out to observe whether the addition of sodium L-glutamate (2.5 mM) to the calcium-deficient medium would result in increased effluxes of amino acids from the incubated brain slices. Results given in Tables 38 and 39 show that there were significantly increased amounts of glutamine, GABA, and aspartate, both in the tissue and the medium, after incubation for one hour, but that these amounts were not markedly affected by the presence of TTX.

7.4 Effects of protoveratrine and tetrodotoxin on amino acid content in, and release from, rat brain cortex slices incubated in glucose-free, calcium-deficient, media.

Absence of both glucose and Ca^{2+} from the incubation medium brought about a markedly high efflux of amino acids, particularly glutamate, GABA and aspartate from brain cortex slices incubated aerobically for one hour (Table 41), the efflux being greater than that found in an incubation medium devoid only of glucose (Table 37) or of Ca^{2+} (Table 39). The rates of release of the amino acids were greatly diminished by the presence of TTX (Table 41). Amino acid concentration ratios (tissue:medium) rose, on addition of TTX (Tables 40 and 41) with glutamate from 23 to 117, with GABA from 23 to 168, with aspartate from 49 to 210, and with glycine from 12 to 34 (Table 42).

Addition of protoveratrine to the glucose-free, Ca^{2+}-deficient medium, increased the efflux of all amino acids except glutamine and alanine (Table 41), and the efflux was diminished by TTX. Amino acid concentration ratios (tissue:medium), found with protoveratrine present, rose on addition of TTX (Table 42) with glutamate from 17 to 56, with GABA from 6 to 114, with

TABLE 40. Effects of protoveratrine, tetrodotoxin, lidocaine and of sodium L-glutamate on the contents of amino acids in rat brain cortex slices in glucose-free, calcium-deficient media.

Amounts of amino acids (μmole/g initial wet wt. tissue) in rat brain cortex slices incubated in Krebs-Ringer phosphate media in O_2 for one hour at 37°C with Ca^{2+} omitted and in absence of glucose. When present protoveratrine was 5μM; tetrodotoxin, 2μM; lidocaine, 0.5mM; and sodium L-glutamate, 2.5mM;

Additions to the incubation medium	Glutamate	Glutamine	GABA	Aspartate	Glycine	Alanine
Nil	1.62 ± 0.12	0.22 ± 0.06	0.80 ± 0.17	6.39 ± 0.42	0.33 ± 0.02	0.07 ± 0.05
Tetrodotoxin	3.01 ± 0.38	0.17 ± 0.03	1.73 ± 0.29	8.04 ± 0.06	0.62 ± 0.13	0.21 ± 0.04
Lidocaine	2.56 ± 0.12	0.10 ± 0.02	1.84 ± 0.07	8.02 ± 0.12	0.79 ± 0.05	0.11 ± 0.01
Protoveratrine	1.48 ± 0.07	0.37 ± 0.02	0.29 ± 0.03	6.63 ± 0.37	0.27 ± 0.02	0.02 ± 0.01
Protoveratrine + Tetrodotoxin	2.39 ± 0.08	0.27 ± 0.07	1.45 ± 0.01	7.67 ± 0.37	0.50 ± 0.02	0.05 ± 0.01
L-Glutamate	5.14 ± 0.02	0.75 ± 0.09	1.37 ± 0.30	9.65 ± 0.20	0.51 ± 0.07	0.12 ± 0.03
L-Glutamate + Tetrodotoxin	9.33 ± 0.60	0.75 ± 0.07	2.52 ± 0.23	12.05 ± 0.20	0.82 ± 0.02	0.22 ± 0.03

TABLE 41. Effects of protoveratrine, tetrodotoxin, lidocaine and of sodium L-glutamate on the release of amino acids from rat brain cortex slices incubated in glucose-free, calcium deficient media.

Amounts of amino acids (μmole/g initial wet wt. tissue) present in the incubation medium (3 ml.) after incubation of rat brain cortex slices in O_2 for one hour at 37°C. When present protoveratrine was 5μM ; tetrodotoxin, 2μM; lidocaine, 0.5mM; and sodium L-glutamate, 2.5mM;

Additions to the incubation medium	Glutamate	Glutamine	GABA	Aspartate	Glycine	Alanine
Nil	2.08 ± 0.19	1.24 ± 0.19	1.07 ± 0.08	3.92 ± 0.18	0.80 ± 0.03	0.60 ± 0.06
Tetrodotoxin	0.77 ± 0.12	0.75 ± 0.14	0.31 ± 0.05	1.15 ± 0.10	0.54 ± 0.04	0.45 ± 0.07
Lidocaine	0.97 ± 0.13	0.90 ± 0.04	0.41 ± 0.08	1.61 ± 0.10	0.58 ± 0.05	0.55 ± 0.09
Protoveratrine	2.68 ± 0.42	0.97 ± 0.01	1.39 ± 0.01	4.81 ± 0.06	0.93 ± 0.05	0.47 ± 0.04
Protoveratrine + Tetrodotoxin	1.29 ± 0.19	0.80 ± 0.10	0.38 ± 0.06	2.02 ± 0.16	0.57 ± 0.03	0.51 ± 0.01
L-Glutamate	2.15 ± 0.02 mM	3.01 ± 0.18	1.25 ± 0.13	12.07 ± 0.22	0.74 ± 0.02	0.49 ± 0.01
L-Glutamate +Tetrodotoxin	1.96 ± 0.07 mM	2.41 ± 0.26	0.70 ± 0.17	6.02 ± 0.51	0.37 ± 0.08	0.84 ± 0.29

aspartate from 41 to 114, and with glycine from 9 to 26. No significant changes occurred with glutamine or alanine.

Addition of sodium L-glutamate to the glucose-free, calcium-deficient, medium, led to increases in the tissue contents of amino acids, particularly glutamate, glutamine, GABA and aspartate after an incubation period of one hour, and also in the contents of these amino acids, particularly aspartate, in the incubation medium (Tables 40 and 41). The presence of TTX led to some retention of the amino acids (apart from glutamine) in the tissue and to diminished values of the contents of these amino acids in the incubation medium. Qualitatively, similar results were obtained when Ca^{++} was present in the glucose-free medium.

7.5 Effects of lidocaine on the release of amino acids from rat brain slices incubated in a variety of media.

Lidocaine and other local anesthetics are known to have suppressive effects similar to those of TTX on the increased respiration and the altered ionic balance that occurs in brain slices when the Na^{+}-current system is activated. It was, therefore, not surprising to find that the TTX-sensitive release of amino acids from rat brain cortex slices incubated in O_2 at 37°C for one hour in certain media, was also lidocaine-sensitive. The relevant results are given in Tables 34 - 41 and are briefly summarized as follows:

1. Incubation in a normal Krebs-Ringer phosphate glucose medium. Like TTX, lidocaine (0.5 mM) does not affect amino acid contents in, or release from, rat brain cortex slices. The diminution of tissue contents of glutamate and aspartate brought about by the addition of protoveratrine (5 μM) is reversed by lidocaine (Tables 34 and 35).

2. Incubation in a glucose-free Krebs-Ringer phosphate medium. Like TTX, lidocaine enhances the tissue contents of amino acids and inhibits their release in glucose-free medium in the presence or absence of protoveratrine (Tables 36 and 37). The increase of the total content of glutamine due to protoveratrine does not occur in the presence of lidocaine.

3. Incubation in Ca^{++}-deficient Krebs-Ringer phosphate glucose medium. The changes in the tissue contents of amino acids and the increased release of glutamate, GABA and aspartate from brain cortex slices brought about by the addition of protoveratrine are abolished in the presence of lidocaine (Tables 38 and 39).

4. Incubation in a Ca^{++}-deficient glucose-free Krebs-Ringer phosphate medium. The release of amino acids, particuarly glutamate, GABA and aspartate, is greatly diminished by lidocaine (Table 41), and this is accompanied by increased tissue contents of these amino acids (Table 40).

7.6 Effects of tetrodotoxin on the tissue to medium concentration ratios of amino acids of rat brain cortex slices incubated in a variety of media.

It is seen from the results given in Table 42, that incubation of rat brain cortex slices for one hour in a variety of media that bring about activation of the Na^{+}-current or the generation of action potentials (182) gives Tissue/Medium concentration ratios for glutamate, GABA, aspartate and glycine that are greatly enhanced by the presence of TTX (2 μM). Lidocaine behaves in a similar manner to TTX. However, it is noteworthy that the values for glutamine are unaffected by the presence of these drugs. These drugs, therefore, do not affect the release of glutamine under various incubation conditions. Moreover, the Tissue/Medium concentration ratio for glutamine is not diminished by the presence of protoveratrine (5 μM). This

TABLE 42. Effects of tetrodotoxin on the tissue to medium concentration ratios of amino acids of rat brain cortex slices incubated under a variety of conditions.

Brain cortex slices of the rat were incubated in O_2 at 37°C for one hour in a variety of Krebs-Ringer phosphate medium that activates the sodium current system. Ratios of the concentrations of amino acids in the tissue (μmole/g initial wet wt.) to those in the incubation medium (μmole/ml) are given below. They were calculated from the values of amino acid concentrations in the tissue given in Tables 34, 36, 38 and 40 and from the amounts of amino acids released, from the quantity of tissue investigated (100mg) into the total volume (3ml) of the incubation medium (Tables 35, 37, 39 and 41).

Incubation Conditions	TTX (2μM)	TISSUE / MEDIUM				
		Glutamine	Glutamate	GABA	Aspartate	Glycine
Glucose medium	-	36	354	2560	246	86
	+	33	306	2580	289	99
Glucose medium + protoveratrine (5μM)	-	43	236	66	150	84
	+	40	440	156	278	174
Glucose, Ca^{++}-free medium	-	25	250	1353	197	60
	+	25	453	2440	311	68
Glucose, Ca^{++}-free medium + protoveratrine (5μM)	-	25	80	86	102	32
	+	25	468	950	226	76
Glucose-free medium + protoveratrine (5μM)	-	13	14	21	39	10
	+	5	143	86	209	52
Glucose-free, Ca^{++}-free medium	-	12	23	23	49	12
	+	7	117	168	210	34
Glucose-free, Ca^{++}-free medium + protoveratrine (5 μM)	-	10	17	6	41	9
	+	10	56	114	114	26
Glucose medium + ouabain (0.1mM)	-	9	23	14	25	16
	+	10	95	21	104	29
Glucose medium + ouabain (0.1mM) + protoveratrine (5μM)	-	9	9	6	12	6
	+	8	88	20	91	25

means that protoveratrine does not accelerate the release of glutamine from brain slices. It is inferred that the main depot of glutamine lies in TTX and protoveratrine insensitive brain cells, viz., glia.

It is to be noted that TTX has no enhancing effect on the Tissue/Medium concentration ratios of rat brain cortex slices, incubated in a normal physiological glucose saline medium.

7.7 Effects of sodium amytal on the release of amino acids from rat brain cortex slices.

As mentioned in the Introduction (Section 1.9 (iv)), the anesthetic effect of barbiturates is believed either to be brought about directly by a block in the Na^+-current system or to be the result of their effects on cell energetics. This latter view was further supported when Chan and Quastel (198) failed to observe a suppressive effect of amytal or pentothal, on the ^{22}Na influx into electrically stimulated brain slices though the increased rate of respiration under these conditions was inhibited. Shankaran and Quastel (210) similarly found that the ^{22}Na influx into slices of the rat brain cortex due to protoveratrine, or to glucose-lack is unaltered by these barbiturates. These workers, however, found that the influx of $^{22}Na^+$ into brain tissue due to (10 μM) protoveratrine (added to a glucose saline medium) is partially suppressed by amytal if Ca^{++} is completely omitted from the incubation medium (i.e., Ca^{++}-free + (3 mM) EGTA). They obtained further evidence supporting the concept that barbiturates act by suppressing the generation of ATP in the mitochondria. They thereby inhibit the mitochondrial ATP-dependent uptake of free Ca^{++} from the cytoplasm or release Ca^{++} already bound to mitochondria (308). Increase of cytoplasmic Ca^{++}concentration may then affect the kinetics of Na^+ and K^+ movements through the brain cell membrane.

As it has been shown earlier in this chapter, that the release of amino

acids from incubated brain tissue accompanying the activation of the sodium current system at the brain cell membrane, is both TTX and lidocaine sensitive, we have carried out experiments to investigate the effects of amytal on the efflux of amino acids. The concentration of 0.25 mM amytal was chosen as this is the anesthetic concentration of amytal for the rat (309).

Rat brain cortex slices were incubated in O_2 at 37°C for one hour, with or without the addition of 0.25 mM amytal, in the following media:

A. Glucose-free Krebs-Ringer phosphate medium (with or without Ca^{++} present). In this condition ^{22}Na influx into brain slices is unaffected by amytal (210).

B. Ca^{++}-free (3 mM EGTA containing) Krebs-Ringer phosphate medium containing protoveratrine (10 μM). In this condition 0.25 mM amytal partially suppresses the ^{22}Na influx into brain slices (210).

Tables 43 and 44 show the amino acid contents of tissue and medium at the end of the incubation period. It is evident from these results that only under condition B is there some suppression of the glutamate, GABA and aspartate release. Total (tissue + medium) glutamine levels are lowered in the presence of amytal, presumably due to a lowered ATP content, accompanying a diminished rate of respiration (210). Amytal has no effect on the efflux of amino acids in the Ca^{++}-free EGTA containing glucose saline medium (results not shown).

It was thus evident that amytal, at its anesthetic concentration, depresses the efflux of amino acids from brain slices under the condition that it depresses the influx of sodium ions; i.e., when an activation of the sodium current system at the brain cell membrane has occurred by the presence of protoveratrine in a Ca^{++}-free incubation medium.

TABLE 43. Effects of sodium amytal on the contents of amino acids in rat brain cortex slices incubated in a variety of media.

Rat brain cortex slices were incubated in O_2 at 37°C for one hour in a variety of media in the presence or absence of 0.25mM sodium amytal. Tissue contents of amino acids are expressed as μmole/g initial wet wt.

Incubation Media	Medium Amytal	Glutamate	Glutamine	GABA	Aspartate	Glycine	Alanine	Taurine
Glucose-free Krebs-Ringer phosphate	-	2.03 ± 0.33	0.51 ± 0.20	1.38 ± 0.22	7.79 ± 0.27	0.62 ± 0.12	0.17 ± 0.04	2.97 ± 0.24
	+	2.01 ± 0.06	0.23 ± 0.02	1.45 ± 0.09	7.37 ± 0.05	0.64 ± 0.06	0.22 ± 0.04	3.00 ± 0.16
Glucose-free Ca^{++}-free Krebs-Ringer phosphate	-	1.62 ± 0.12	0.22 ± 0.06	0.80 ± 0.17	6.39 ± 0.42	0.33 ± 0.02	0.07 ± 0.05	2.39 ± 0.16
	+	1.80 ± 0.05	0.24 ± 0.02	0.92 ± 0.08	6.08 ± 0.48	0.45 ± 0.05	0.09 ± 0.05	2.47 ± 0.11
Ca^{++}-free Krebs-Ringer phosphate-glucose + 3mM EGTA + protoveratrine (10μM)	-	5.90 ± 0.14	2.15 ± 0.17	1.43 ± 0.18	3.12 ± 0.10	0.53 ± 0.01	0.61 ± 0.02	3.05 ± 0.48
	+	7.67 ± 0.33	1.77 ± 0.13	2.37 ± 0.29	3.00 ± 0.17	0.67 ± 0.03	0.70 ± 0.04	3.19 ± 0.21

TABLE 44. Effects of sodium amytal on the release of amino acids from rat brain cortex slices incubated in a variety of media.

Amount of amino acids (μmole/g initial wet wt./3 ml.) present in incubation media after incubation of rat brain cortex slices in O_2 for one hour at 37°C in the presence or absence of 0.25mM sodium amytal.

Incubation Media	Medium Amytal	Glutamate	Glutamine	GABA	Aspartate	Glycine	Alanine	Taurine
Glucose-free Krebs-Ringer phosphate	-	0.96 ± 0.12	1.74 ± 0.10	0.41 ± 0.08	1.98 ± 0.13	0.70 ± 0.10	0.52 ± 0.10	2.71 ± 0.15
	+	1.06 ± 0.09	1.39 ± 0.02	0.46 ± 0.04	2.20 ± 0.15	0.94 ± 0.10	0.86 ± 0.05	2.84 ± 0.12
Glucose-free Ca^{++}-free Krebs-Ringer phosphate	-	2.08 ± 0.19	1.24 ± 0.19	1.07 ± 0.08	3.92 ± 0.18	0.80 ± 0.06	0.60 ± 0.08	3.28 ± 0.30
	+	2.27 ± 0.16	1.09 ± 0.04	1.14 ± 0.06	3.88 ± 0.06	0.90 ± 0.14	0.71 ± 0.05	3.19 ± 0.20
Ca^{++}-free Krebs-Ringer phosphate-glucose + 3mM EGTA + protoveratrine (10 μM)	-	3.34 ± 0.17	2.34 ± 0.09	0.82 ± 0.13	1.16 ± 0.02	0.61 ± 0.08	0.88 ± 0.05	3.62 ± 0.62
	+	2.22 ± 0.23	1.71 ± 0.08	0.35 ± 0.09	0.83 ± 0.08	0.54 ± 0.06	0.90 ± 0.04	3.10 ± 0.32

7.8 Effects of increased K^+ and of tetrodotoxin on amino acid content in, and release from, incubated rat brain cortex slices.

Incubation of rat brain cortex slices in Krebs-Ringer phosphate medium containing 10 mM glucose, in which K^+ was increased to 50 mequiv/l, brought about a significant increase in the tissue content of GABA and an increased efflux of GABA into the incubation medium (Tables 45 and 46). There was some retention of glutamine in the tissue slices. Less definite changes occurred with the other amino acids examined. The increased efflux of GABA due to increased K^+ was reduced to some extent by TTX (Table 45).

In a calcium-deficient medium, increased K^+ brought about no significant changes in the glutamate content of tissue or medium in contrast to the effects of protoveratrine (Tables 38, 39, 45, 46). It would appear, therefore, that the changes in amino acid fluxes in rat brain cortex slices incubated with increased K^+ may differ markedly from those brought about by the presence of protoveratrine.

When glucose was absent from the incubation medium, increased K^+ promoted an increased efflux of glutamate, GABA, aspartate, glycine and alanine. The efflux of glutamine seemed not to be changed (Tables 37 and 46). Accompanying changes took place in the tissue contents of amino acids (Tables 36 and 45) and the total (tissue + medium) content of aspartate was considerably increased. The addition of TTX caused no significant changes in the contents of amino acids both in tissue and medium, except for a small reduction of the efflux of GABA.

7.9 Effects of tetrodotoxin on the efflux of amino acids from kidney cortex slices incubated in the presence of ouabain.

Results given in Table 47 show that the efflux of amino acids from kidney cortex slices of the rat incubated in O_2 at 37°C for one hour in Krebs-Ringer phosphate glucose medium, containing 0.1 mM ouabain, was not

TABLE 45. Effects of increased K^+ and of tetrodotoxin on the amino acid content in rat brain cortex slices incubated in various media.

Amounts of amino acids (μmole/g initial wet wt. tissue) present in rat brain cortex slices incubated in O_2 for one hour at 37°C in Krebs-Ringer phosphate medium with or without 10 mM glucose. When present, tetrodotoxin was 2μM.

Additions to the incubation medium	Glutamate	Glutamine	GABA	Aspartate	Glycine	Alanine
45 mM KCl	8.38 ± 0.49	4.93 ± 0.05	3.98 ± 0.04	2.31 ± 0.13	1.03 ± 0.05	1.13 ± 0.06
45 mM KCl + Tetrodotoxin	8.31 ± 0.13	5.94 ± 0.18	3.80 ± 0.40	2.12 ± 0.16	1.00 ± 0.04	1.13 ± 0.02
Ca^{2+} absent						
45 mM KCl	9.45 ± 0.19	1.45 ± 0.09	2.93 ± 0.34	2.33 ± 0.08	0.67 ± 0.03	0.57 ± 0.04
45 mM KCl + Tetrodotoxin	9.37 ± 0.17	1.06 ± 0.10	2.74 ± 0.03	2.42 ± 0.23	0.71 ± 0.05	0.48 ± 0.04
Glucose absent						
45 mM KCl	1.29 ± 0.27	0.30 ± 0.09	0.72 ± 0.36	7.08 ± 0.32	0.48 ± 0.07	0.18 ± 0.04
45 mM KCl + Tetrodotoxin	1.27 ± 0.16	0.34 ± 0.08	0.76 ± 0.28	7.78 ± 0.06	0.49 ± 0.07	0.17 ± 0.04

TABLE 46. Effects of increase K^+ and of tetrodotoxin on the release of amino acids from incubated rat brain cortex slices.

Amounts of amino acids (μmole/g initial wet wt. tissue) in the incubation medium after incubation of rat brain cortex slices in O_2 for one hour at 37°C in 3 ml Krebs-Ringer phosphate medium with or without 10 mM glucose. When present, tetrodotoxin was 2μM.

Additions to the incubation medium	Glutamate	Glutamine	GABA	Aspartate	Glycine	Alanine
45 mM KCl	0.88 ± 0.08	1.29 ± 0.07	0.63 ± 0.02	0.43 ± 0.01	0.67 ± 0.08	0.77 ± 0.03
45 mM KCl + Tetrodotoxin	0.60 ± 0.02	0.88 ± 0.05	0.32 ± 0.01	0.41 ± 0.02	0.47 ± 0.01	0.67 ± 0.02
Ca^{2+} absent						
45 mM KCl	1.20 ± 0.13	3.77 ± 0.07	0.34 ± 0.04	0.57 ± 0.06	0.54 ± 0.11	1.04 ± 0.01
45 mM KCl + Tetrodotoxin	1.24 ± 0.09	3.82 ± 0.18	0.21 ± 0.01	0.59 ± 0.02	0.59 ± 0.06	1.05 ± 0.02
Glucose absent						
45 mM KCl	2.63 ± 0.21	1.45 ± 0.09	0.74 ± 0.05	6.07 ± 0.93	1.16 ± 0.06	1.00 ± 0.02
45 mM KCl + Tetrodotoxin	2.52 ± 0.04	1.34 ± 0.04	0.53 ± 0.12	5.33 ± 0.50	1.10 ± 0.07	0.91 ± 0.05

TABLE 47. Effects of tetrodotoxin on the contents of amino acids in, and release from, rat kidney cortex slices incubated in the presence of ouabain.

Rat kidney cortex slices were incubated at 37°C for one hour in Krebs-Ringer phosphate glucose medium containing ouabain (0.1mM) in the presence or absence of TTX (2μM). Tissue values are expressed as μ mole/g initial wet wt. and medium values as μmole/g initial wet wt./3ml.

	Ouabain		Ouabain + Tetrodotoxin	
	Tissue	Medium	Tissue	Medium
Glutamate	1.37 ± 0.04	2.09 ± 0.06	1.39 ± 0.16	2.22 ± 0.07
Aspartate	1.05 ± 0.07	0.44 ± 0.03	1.08 ± 0.01	0.45 ± 0.01
Alanine	0.38 ± 0.03	2.31 ± 0.25	0.42 ± 0.16	2.15 ± 0.04
Glycine	0.60 ± 0.05	2.28 ± 0.12	0.54 ± 0.01	2.14 ± 0.08
Threonine	0.21 ± 0.01	1.56 ± 0.15	0.20 ± 0.04	1.46 ± 0.02
Taurine	2.65 ± 0.29	3.92 ± 0.32	2.55 ± 0.28	4.15 ± 0.20
Glutamine + Serine) + Asparagine)	0.90 ± 0.01	6.95 ± 0.75	0.82 ± 0.14	6.79 ± 0.15
Ammonia	1.97 ± 0.05	13.06 ± 0.06	2.15 ± 0.16	13.74 ± 0.50

TABLE 48. Total ammonia and amino acid contents of rat kidney cortex slices initially present and on incubation in the presence or absence of glucose.

Rat kidney cortex slices were incubated at 37°C for one hour in Krebs-Ringer phosphate medium in the presence or absence of glucose. Initial values (i.e. values prior to incubation) of ammonia and amino acides and the total (tissue + medium) values are expressed as μmoles/g initial wet wt.

	INITIAL	ON INCUBATION	
		No Glucose	Glucose
Glutamate	5.85 ± 0.09	3.48 ± 0.03	3.91 ± 0.04
Aspartate	1.76 ± 0.09	1.00 ± 0.03	1.24 ± 0.05
Alanine	0.79 ± 0.04	2.29 ± 0.03	2.22 ± 0.17
Glycine	2.00 ± 0.12	3.65 ± 0.68	2.63 ± 0.51
Threonine	0.75 ± 0.04	2.18 ± 0.05	2.10 ± 0.10
Taurine	6.92 ± 0.28	6.53 ± 0.25	6.68 ± 0.27
Glutamine + Serine + Asparagine	2.35 ± 0.01	8.83 ± 0.21	8.72 ± 0.19
Lysine	0.87 ± 0.03	2.38 ± 0.12	2.57 ± 0.06
Ammonia	3.90 ± 0.06	17.89 ± 0.04	16.31 ± 0.09

affected by the presence of TTX (2 μM). This is in direct contrast to the effects of TTX on the efflux of amino acids from rat brain cortex slices incubated under similar medium conditions (see Tables 34 and 35). This supports the conclusion that TTX-sensitive effluxes of amino acids are confined to excitable tissues.

It may be noted that GABA was not detected in kidney cortex slices. Moreover, the oxygen uptake (92 ± 9 μmole/g initial wet wt), the total (tissue + medium) amino acid contents, and the rate of ammonia formation, were little affected by the presence of glucose (Table 48). Kidney cortex slices do not take up water on incubation even in the presence of 0.1 mM ouabain, or in the absence of glucose. However, large increases in the concentration of glycine, threonine and lysine, occur on incubation, possibly due to protein breakdown (Table 48).

7.10 Effects of tetrodotoxin on the amino acid contents in, and release from, rat brain cortex slices incubated in the presence of 2, 4 dinitrophenol, NH_4^+ or 105 mM KCl.

Okamoto and Quastel (182) have shown that the increases in water uptake, ^{22}Na influx, and the rate of respiration of brain cortex slices, found in the presence of DNP (0.03 mM) or KCl (105 mM), are unaffected by TTX (3 μM). The increases in uptakes of water and Na^+, and the loss of K^+ from brain cortex slices incubated in the presence of NH_4Cl (10 mM), are also unaffected by TTX (3 μM) (Chapter 9). Under these conditions, therefore, action potentials or activation of the sodium current seem not to be generated. If the view is correct that amino acids are released from neurons only when action potentials are generated, or when TTX-sensitive activations of the sodium current system occur, then the release of amino acids brought about by the addition of DNP, KCl (high) or NH_4Cl to the incubation medium, should not be affected by TTX. Experiments show that this is the case.

TABLE 49. Effects of tetrodotoxin on the contents of amino acids in rat brain cortex slices incubated with 2, 4 dinitrophenol, NH_4Cl or KCl (100mM).

Amounts of amino acids (μmole/g initial wet wt. tissue) present in rat brain cortex slices incubated in O_2 at 37°C for one hour in Krebs-Ringer phosphate-glucose medium. Tetrodotoxin when present in the medium was 2 μM.

Addition to the incubation medium	TTX	Glutamate	Glutamine	GABA	Aspartate	Glycine	Alanine	Taurine
DNP (0.1mM)	-	4.87 ± 0.27	0.89 ± 0.10	3.67 ± 0.09	1.14 ± 0.11	0.65 ± 0.09	0.83 ± 0.06	1.92 ± 0.18
	+	4.81 ± 0.14	0.84 ± 0.12	4.10 ± 0.25	1.14 ± 0.02	0.57 ± 0.03	0.75 ± 0.04	2.07 ± 0.20
NH_4Cl (30mM)	-	3.09 ± 0.31	3.27 ± 0.26	4.31 ± 0.26	1.26 ± 0.08	0.68 ± 0.08	0.73 ± 0.07	1.88 ± 0.15
	+	3.80 ± 0.20	2.99 ± 0.10	4.65 ± 0.02	1.35 ± 0.03	0.76 ± 0.02	0.71 ± 0.02	1.92 ± 0.01
KCl (100mM)	-	7.77 ± 0.19	5.38 ± 0.22	3.40 ± 0.06	1.95 ± 0.03	0.80 ± 0.01	1.57 ± 0.01	3.31 ± 0.05
	+	7.72 ± 0.18	4.49 ± 0.29	3.66 ± 0.30	1.62 ± 0.06	0.78 ± 0.03	1.37 ± 0.04	3.48 ± 0.38

TABLE 50. Effects of tetrodotoxin on the release of amino acids from brain cortex slices incubated with 2, 4 dinitrophenol, NH_4Cl or KCl (100mM).

Amounts of amino acids (μmole/g initial wet wt./3 ml.) in the incubation medium after incubation of rat brain cortex slices in O_2 for one hour at 37°C in Krebs-Ringer phosphate glucose medium. Tetrodotoxin when present in the medium was 2μM.

Addition to the incubation medium	TTX	Glutamate	Glutamine	GABA	Aspartate	Glycine	Alanine	Taurine
DNP (0.1mM)	-	3.44 ± 0.22	2.47 ± 0.32	1.40 ± 0.08	1.06 ± 0.12	0.84 ± 0.03	1.59 ± 0.09	3.33 ± 0.17
	+	3.14 ± 0.06	2.37 ± 0.19	1.07 ± 0.03	0.90 ± 0.03	0.75 ± 0.06	1.47 ± 0.05	3.15 ± 0.05
NH_4Cl (30mM)	-	2.22 ± 0.16	6.58 ± 0.42	0.34 ± 0.04	1.02 ± 0.09	1.00 ± 0.07	1.46 ± 0.07	4.03 ± 0.05
	+	2.22 ± 0.12	5.37 ± 0.16	0.31 ± 0.06	0.92 ± 0.02	0.90 ± 0.05	1.40 ± 0.02	4.06 ± 0.14
KCl (100mM)	-	1.05 ± 0.06	1.43 ± 0.25	0.78 ± 0.03	0.47 ± 0.04	0.60 ± 0.01	1.16 ± 0.06	3.89 ± 0.19
	+	0.96 ± 0.02	1.14 ± 0.10	0.85 ± 0.03	0.36 ± 0.02	0.56 ± 0.01	1.08 ± 0.01	4.06 ± 0.23

In order to ensure adequate efflux of amino acids from rat brain cortex slices incubated in O_2 at 37°C for one hour in Krebs-Ringer phosphate glucose medium, relatively high concentrations of DNP (0.1 mM), or NH_4Cl (30 mM), or KCl (105 mM), were used. Results are recorded in Tables 49 and 50.

From these results it is seen that TTX (2 μM) has no effect on the release of amino acids, from the tissue, due to 30 mM NH_4Cl (Table 50). In the presence of 0.1 mM DNP, the net efflux of amino acids, with the possible exception of GABA, is also unaffected by TTX. The efflux of amino acids is further enhanced by 105 mM KCl, when compared with that brought about by 50 mM KCl (Tables 46 and 50). An increase in the efflux of GABA, and a retention in the tissue of glutamine, is also evident with 105 mM KCl. However, the release of amino acids from the tissue by 105 mM KCl is unaffected by TTX. It was also found that the enhanced release of amino acids from incubated brain cortex slices in a glucose-free medium in the presence of this concentration of KCl, is also not affected by TTX.

7.11 Summary

1. Amino acids, particularly glutamate, γ-aminobutyrate, aspartate, and glycine, are released from rat brain cortex slices under specific incubation conditions; e.g., in the presence of protoveratrine (especially in a Ca^{++}-deficient medium), or of ouabain, or in the absence of glucose. The processes of release are partially or wholly suppressed by tetrodotoxin. It is inferred that, during the generation of action potentials or the activation of the sodium current system at the cell membrane in brain slices, there is a release of glutamate, GABA, aspartate, and glycine from the neurons.

2. Tetrodotoxin does not affect the release of glutamine under various incubation conditions, nor does protoveratrine accelerate it. It is

inferred that the main depot of glutamine lies not in the neurons, but in the glia.

3. Protoveratrine brings about an increased rate of formation of glutamine in incubated brain slices.

4. Increased K^+ (50 mequiv/ml) in the incubation medium leads to a release of GABA, the process being partly suppressed by TTX.

5. Incubation of brain slices in medium devoid of glucose leads to an increase production of aspartate but to diminished tissue contents of glutamate, glutamine and glycine.

6. The effects of lidocaine on the release of amino acids from slices are qualitatively similar to those of tetrodotoxin.

7. Tetrodotoxin has little or no effect on the ouabain induced release of amino acids from kidney cortex slices (unlike brain cortex slices).

8. Tetrodotoxin does not affect the release of amino acids from slices incubated in the presence of NH_4Cl (30 mM), DNP (0.1 mM), or KCl (105 mM).

9. Amytal feebly suppresses the release of glutamate, GABA and aspartate from brain cortex slices incubated with protoveratrine in a physiological glucose saline medium devoid of Ca^{++}.

10. It is suggested that drugs which suppress the TTX-sensitive activation of the sodium current at the cell membrane concomitantly suppress the outward movement of amino acids from the brain tissue.

8. LOCATIONS OF AMINO ACIDS IN BRAIN CORTEX SLICES OF THE RAT

8.1 Location of the glutamate-glutamine system.

Compartmentation of the glutamate-glutamine system, in brain, first became evident from in vivo studies with certain labelled precursors, when it was found that the specific activity of isolated glutamine was greater than that of glutamate. Later, this phenomenon was observed in isolated brain cortex slices. The phenomenon is manifested when labelled acetate, but not labelled glucose, is present as precursors (see Chapter 1.7 (ii)). Using the radioactive amino acid data of Gonda and Quastel (259) obtained with labelled acetate, and that of Lahiri and Quastel (63) obtained with labelled glucose, and using also our values concerning the contents of amino acids in brain cortex slices, incubated under identical conditions, we have made calculations of the specific activities of glutamate and glutamine derived from labelled acetate or labelled glucose.

In these experiments, brain cortex slices of the rat were incubated in O_2 at 37°C for one hour in Krebs-Ringer phosphate medium containing 5 mM glucose in the presence of either [1 - ^{14}C] acetate (specific activity, 2×10^5 cpm/μmole), or [U -^{14}C] glucose (specific activity, 4×10^4 cpm/μmole). Labelled amino acids were separated by two dimensional paper chromatography and their radioactivities were estimated (63, 259). The contents of the amino acids were measured by the Amino Acid Analyzer. Results of the calculations given in Table 51 show that the specific activity of glutamine derived from labelled acetate is greater than that of glutamate (280 compared to 58 mμg atom C/μmole). The reverse is true when labelled glucose is used as substrate, i.e., the specific activity of glutamine is lower than that of glutamate (674 compared to 934 mμg atom C/μmole). In other words, the ratio of the specific activities of glutamine to glutamate is greater than one (viz., 4.8) when labelled acetate is employed, but lower than one (viz., 0.72) when labelled

TABLE 51. Specific activities of glutamine and glutamate of rat brain cortex slices derived from sodium (1-^{14}C) acetate and (U-^{14}C) glucose.

Rat brain cortex slices were incubated at 37°C for one hour in Krebs-Ringer phosphate glucose (5mM) medium, in the presence of either 1mM sodium (1-^{14}C) acetate (specific activity, 2×10^5 cpm/μ mole) or (U-^{14}C) glucose (specific activity, 4×10^4 cpm/μmole). Radioactivity data for acetate were obtained from the values given by Gonda and Quastel (259) and for glucose from the values given by Lahiri and Quastel (63). Contents of amino acids were estimated by the Amino Acid Analyzer.

	Radioactivity mμg atom of C/g	Content μmole/g,	Specific Activity mμg atom of C/μmole	Relative Specific Activity Glutamine / Glutamate
(1-^{14}C) Acetate				
Glutamine	970 ± 80	3.45 ± 0.30	280	4.80
Glutamate	530 ± 70	9.20 ± 0.14	58	
(U-^{14}C) Glucose				
Glutamine	2320 ± 110	3.45 ± 0.30	674	0.72
Glutamate	8580 ± 200	9.20 ± 0.14	934	

glucose is used. These results are in accord with those already reported (see section 1.7 (ii)) and may be taken to imply the presence of two pools of citric acid cycle intermediates both accessible to glucose but only one freely accessible to acetate. The latter pool of citric acid cycle intermediates has evidently a relatively high rate of glutamine synthesis and presumably maintains only a relatively small pool of the total tissue glutamate.

8.2 Effects of protoveratrine on the specific activities of amino acids from rat brain cortex slices incubated in the presence of [U-^{14}C] glucose.

Calculations were also made, as described above, to obtain specific activities of amino acids of rat cerebral cortex slices incubated in the presence or absence of protoveratrine, with the radioactive amino acid data of Kini and Quastel (213), and with our values of amino acid contents of cortex slices incubated under identical conditions. Slices of cerebral cortex were incubated at 37^{o}C for one hour in Krebs-Ringer phosphate medium containing glucose (5 mM and specific activity 2 x 10^{5} cpm/μmole) in the presence or absence of protoveratrine (5 μM). Analyses for amino acid radioactivities and contents were carried out as mentioned in section 8.1.

Results given in Table 52 show that accompanying an increased respiration in the presence of protoveratrine (211-213, see Chapter 9), there is an increased utilization of glucose reflected partly in enhanced specific activities of glutamate (117%), GABA (124%), and aspartate (105%). With glutamine, however, the increase in the specific activity is only small in comparison (24%). These results may indicate that the major portion of the amino acids, glutamate, GABA and aspartate, but not glutamine, derived from labelled glucose, is neuronal as protoveratrine is assumed to act only on excitable cells. However, this experiment gives no clue as to the size of the neuronal pool of these amino acids. It is to be noted that protoveratrine does not inhibit glutamine synthesis; in fact, it enhances it. This occurs

TABLE 52. Effects of protoveratrine (5μ M) on the specific activities of amino acids of rat cerebral cortex slices incubated in the presence of (U-^{14}C) glucose.

Slices of rat brain cortex were incubated for one hour at 37°C in Krebs-Ringer phosphate-glucose (5mM) medium. The specific activity of glucose was 2 x 10^5 cpm/μmole. Radioactivity data were calculated from values given by Kini and Quastel (213) and are expressed as cpm/ml tissue water. Contents of amino acids are expressed as μmole/ml tissue water and specific activities as cpm/μmole.

	Glutamate	Glutamine	GABA	Aspartate
Control				
Radioactivity	70.0 x 10^3	16.2 x 10^3	12.1 x 10^3	16.9 x 10^3
Amino Acid Content	11.5	4.25	3.2	4.31
Specific Activity	6.0 x 10^3	3.8 x 10^3	3.8 x 10^3	3.9 x 10^3
Protoveratrine				
Radioactivity	98.6 x 10^3	24.4 x 10^3	22.9 x 10^3	21.6 x 10^3
Amino Acid Content	7.5	5.12	2.72	2.69
Specific Activity	13.1 x 10^3	4.7 x 10^3	8.5 x 10^3	8.0 x 10^3
% Change of Specific Activity from Control	+117	+24	+124	+105

in spite of a diminished tissue ATP content in the presence of protoveratrine (see Table 74). The fall in ATP due to protoveratrine presumably occurs in the neurons as the protoveratrine induced Na^+ influx results in an increase in the activity of the neuronal Na^+ pump, with an accompanying increase in the rate of ADP controlled mitochondrial respiration. The undiminished rate of the ATP dependent glutamine synthesis in the presence of protoveratrine may imply that the neurons are not the major compartment of glutamine synthesis.

8.3 Characterization of amino acid compartments in brain.

The results obtained above are in accord with those of investigations that have shown the existence of at least two separate pools in brain tissue that differ in their contents of amino acids and in their amino acid flux rates. Segregation of glutamate and GABA, and other amino acids into "small" or "large" compartments occurs as demonstrated by the results of a variety of studies of amino acid metabolism in both *in vitro* and *in vivo* (116, 141-158). The "small" compartment seems to be associated with a relatively small content of glutamate, but possesses the major amount of glutamine. The "large" compartment appears to contain the major amount of glutamate. However, the physical boundaries of these compartments have not been established. Indirect evidence has suggested that the "large" compartment may consist of neuronal structures (116, 149, 154-156), while the "small" pool associated with high glutamine content may consist of glial tissue (161, 162).

In Chapter 7 we have shown that, under specific incubation conditions where action potentials or activation of the sodium current system at the cell membrane are thought to be generated in rat brain cortex slices (182), there is an increased efflux of amino acids from the tissue into the medium. This release is blocked partially or wholly by the presence of TTX.

As it is known that glia are not electrically excitable cells and do not generate action potentials (260, 261), it is inferred that the effects of TTX,

which abolishes the activation of the sodium current system at the neuronal membrane (190, 193, 194), are confined to the neurons.

We have carried out experiments to determine the precise incubation conditions that are most effective in releasing amino acids from incubated rat brain cortex slices and we have estimated how much of this release is abolished by the presence of TTX. The amino acid that is retained in the brain tissue by the addition of TTX is considered to be located in the neurons. Its amount represents a minimum value of the amino acid content of the neurons in the incubated brain cortex slices. Experiments carried out on these lines may give, therefore, measurements of minimum values of the contents of a variety of amino acids in the neurons.

The results of these experiments are described below.

(i) Effects of the combined presence of protoveratrine and ouabain on amino acid release.

It was found, after a series of experiments utilizing various incubation conditions, that the optimal condition for the release of amino acids from rat brain cortex slices, incubated under aerobic conditions at 37°C for one hour, was to use an incubation medium consisting of Krebs-Ringer phosphate medium containing 10 mM glucose together with protoveratrine (5 μM) and ouabain (0.1 mM). The presence of protoveratrine ensured the generation of action potentials in the brain slices with resultant efflux of amino acids (see Chapter 7), while that of ouabain blocked the re-uptake of the released amino acids against a concentration gradient (104, 112). Results are shown in Table 53, which records values of the contents of amino acids in the tissue and in the incubation medium at the end of the incubation period.

It will be observed that the presence of protoveratrine and ouabain caused a loss from the brain tissue of 26.10 - 7.74 = 18.36 μmole/g of the total amino acids investigated, i.e., a loss of 70 per cent. At the same time, the

TABLE 53. Effects of protoveratrine, ouabain and tetrodotoxin on amino acid content in, and release from, incubated rat brain cortex slices.

Rat brain cortex slices were incubated in 3 ml Krebs-Ringer phosphate medium containing 10mM glucose in O_2 at 37°C for one hour in the presence or absence of mixtures of protoveratrine (5μM), ouabain (0.1mM) and tetrodotoxin (2 μM) as shown below. Tissue values of amino acid are expressed as μmole/g initial wet wt. and medium values as μmole/g wet wt./3 ml.

Amino Acid	No Addition			Tetrodotoxin			Protoveratrine + Ouabain			Protoveratrine + Ouabain + Tetrodotoxin		
	Tissue	Medium	Total	Tissue	Medium	Total	Tissue	Medium	Total	Tissue	Medium	Total
Glutamate	9.20 ± 0.14	0.77 ± 0.14	9.97	8.82 ± 0.01	0.87 ± 0.02	9.69	2.52 ± 0.11	8.33 ± 0.48	10.85	7.14 ± 0.12	2.42 ± 0.16	9.56
Glutamine	3.45 ± 0.30	2.92 ± 0.28	6.37	2.88 ± 0.11	2.62 ± 0.13	5.50	0.74 ± 0.04	2.56 ± 0.41	3.30	0.53 ± 0.03	2.07 ± 0.25	2.60
GABA	2.56 ± 0.20	0.03 ± 0.02	2.59	2.58 ± 0.16	0.04 ± 0.03	2.62	0.63 ± 0.02	3.23 ± 0.15	3.86	1.47 ± 0.13	2.18 ± 0.25	3.65
Aspartate	3.45 ± 0.23	0.43 ± 0.05	3.88	3.64 ± 0.20	0.38 ± 0.06	4.02	1.20 ± 0.07	2.92 ± 0.18	4.12	3.28 ± 0.19	1.09 ± 0.02	4.37
Glycine	1.03 ± 0.15	0.36 ± 0.06	1.39	0.88 ± 0.03	0.27 ± 0.02	1.15	0.25 ± 0.01	1.22 ± 0.04	1.47	0.66 ± 0.03	0.81 ± 0.05	1.47
Alanine	0.75 ± 0.05	0.44 ± 0.04	1.19	0.82 ± 0.02	0.38 ± 0.02	1.20	0.26 ± 0.01	1.59 ± 0.14	1.85	0.46 ± 0.05	1.28 ± 0.13	1.74
Taurine	4.10 ± 0.35	1.89 ± 0.08	5.99	3.92 ± 0.01	2.05 ± 0.01	5.97	1.76 ± 0.13	4.49 ± 0.71	6.25	2.07 ± 0.50	3.89 ± 0.35	5.96
Serine	1.12 ± 0.13	0.82 ± 0.05	1.94	0.96 ± 0.12	0.67 ± 0.09	1.63	0.28 ± 0.01	1.76 ± 0.15	2.04	0.72 ± 0.02	1.33 ± 0.06	2.05
Threonine	0.44 ± 0.06	0.28 ± 0.05	0.72	0.48 ± 0.03	0.26 ± 0.02	0.74	0.10 ± 0.01	0.66 ± 0.01	0.76	0.21 ± 0.01	0.60 ± 0.04	0.81
TOTAL	26.10	7.94	34.04	24.98	7.54	32.52	7.74	26.76	34.50	16.54	15.67	32.21

incubation medium (3 ml) gained amino acids to the extent of 26.76 - 7.94 = 18.82 μmole/g, a value approximately equal to that lost by the tissue. The tissue losses incurred by the individual amino acids were as follows: glutamate (73%), glutamine (78%), GABA (75%), aspartate (65%), glycine (74%), alanine (65%), taurine (57%), serine (75%), threonine (77%). Thus, all the tissue amino acids suffered substantial losses, taurine being rather less affected than the others.

The total (tissue + medium) quantity of amino acids was not significantly affected by the combined action of protoveratrine and ouabain. However, the total (tissue + medium) value for glutamine fell by 48 per cent, whereas that for GABA increased by 49 per cent, that for alanine increased by 55 per cent, and that for glutamate increased only slightly (9%) (Table 53).

(ii) Effects of tetrodotoxin

Tetrodotoxin (2 μM) exercised little or no effects, within the range of experimental error, on the contents of amino acids both in the tissue and in the incubation medium under the normal incubation conditions. It brought about, however, large changes in the presence of protoveratrine and ouabain.

Results given in Table 53 show that, on the addition of TTX, the total quantity of amino acids in tissue, namely 24.98 μmole/g, was reduced by the combined presence of protoveratrine and ouabain to 16.54 μmole/g, i.e., a loss of 8.44 μmole/g equivalent to 34 per cent of the tissue content. The gain in amino acids in the medium was 15.67 - 7.74 = 8.13 μmole/g, approximately equal to the loss from the tissue. Therefore, TTX reduced a loss of 70 per cent of amino acids from the tissue, brought about by protoveratrine and ouabain, to 34 per cent. The implication is that at least 36 per cent of the tissue content of amino acids is located in the neurons. This value is a minimum value, as it is unlikely that the TTX causes a complete block of the neuronal efflux of amino acids, or that the block is equally effective with all amino acids or with all conditions that are presumed to generate action potentials.

(iii) Alterations in the contents of individual amino acids.

(a) Glutamate

Considering some of the amino acids individually, it is seen from Table 53 that, with glutamate, the tissue content was reduced by protoveratrine and ouabain in presence of TTX from 8.82 μmole/g to 7.14 μmole/g, i.e., a loss of 1.68 μmole/g or 19 per cent of the tissue content of glutamate. Since the loss amounted to 73 per cent in the absence of TTX, it follows that the difference, i.e., 73-19 = 54 per cent, represents the percentage of the tissue glutamate located in the neurons. This constitutes good evidence that at least half of the glutamate in the brain cortex slices is present in the neurons. Considerations of the glutamate contents in the incubation medium (Table 53) give rise to the same conclusion. Increase of the concentration of glutamate in the medium, due to the presence of protoveratrine and ouabain, amounted to 8.33 - 0.77 = 7.56 μmole/g, while with TTX added, the increase amounted to 2.42 - 0.87 = 1.55 μmole/g. The difference, 6.01 μmole/g, represented the amount retained by the neurons and which, therefore, constituted (6.01 x 100)/(9.20) = 65 per cent of the glutamate present in the tissue at the end of the incubation period.

(b) Aspartate

The tissue content of aspartate was reduced by protoveratrine and ouabain, in presence of TTX, from 3.64 μmole/g to 3.28 μmole/g, i.e., a loss of 0.36 μmole/g or of 10 per cent of the tissue content of aspartate (Table 53). As the loss amounted to 65 per cent in the absence of TTX, it appears that the difference, i.e., 65 - 10 = 55 per cent, represents the percentage of the tissue aspartate located in the neurons. Increase of the concentration of aspartate in the medium in the presence of protoveratrine and ouabain amounted to 2.49 μmole/g (Table 53), while, with TTX added, the increase amounted to 0.71 μmole/g. The difference, 1.78 μmole/g, represents the amount retained by the neurons and therefore constitutes (1.78 x 100)/(3.45) = 51 per cent of

the amount of aspartate normally present in the tissue under the given experimental condition.

It seems, therefore, that as with glutamate, the neurons are the site of the major pool of aspartate.

(c) GABA

Calculations similar to those given above, from the values recorded in Table 53, indicate that at least 32 per cent of the tissue content of GABA is located in the neurons. The actual amount must be considerably greater than this because results, recorded in the preceding Chapter 7, of the effects of TTX on GABA efflux brought about by protoveratrine under certain incubation conditions, indicated a more complete block of the release of GABA.

(d) Glycine and Serine

Calculations made in the manner described above from the results in Table 53 show that at least 50 per cent of the tissue content of either glycine or serine lies in the neurons. Presumably, therefore, the neurons are locations of the major pools of glycine and of serine.

(e) Taurine, Alanine and Threonine

Similar calculations show that at least 21 per cent of the tissue content of alanine, 10 per cent of the tissue content of taurine, and 6 per cent of the the tissue content of threonine lie in the neurons. It is not possible to decide, from these values, whether the neurons are the locations of the major pools of these amino acids.

(f) Glutamine

Turning to a consideration of glutamine, it is seen (Table 53) that the percentage fall in tissue glutamine in presence of protoveratrine and ouabain

was (3.45 - 0.74)(100)/(3.45) = 78 per cent, and the percentage fall, with TTX added, was (2.88 - 0.53)(100)/(2.88) = 81 per cent. Thus, the presence of TTX had no diminishing effect on the proportion of glutamine released from the brain tissue in presence of protoveratrine and ouabain. It appears, therefore, that glutamine is not retained in the brain tissue by TTX under conditions where the release of glutamate and other amino acids is greatly affected.

Results, recorded in Table 54, show, moreover, that the presence of protoveratrine (5 μM) has no effect on the tissue content of glutamine under conditions where it causes significant falls in the tissue contents of glutamate, GABA, aspartate, glycine and alanine.

8.4 Effects of sodium malonate and sodium fluoroacetate on cerebral amino acid content and release in the presence of protoveratrine.

It has been suggested (150) that the suppression of cerebral glutamine synthesis by low concentrations of fluoroacetate (63) is due to the localization of its effect in a special compartment where glutamine synthesis takes place. The fluoroacetate is considered to block the operation of the citric acid cycle in this compartment, thereby reducing the amount of ATP available for glutamine synthesis.

Experiments were, therefore, carried out to observe the effects of two citric acid cycle inhibitors, sodium malonate and sodium fluoroacetate, on both the respiration and on the contents of amino acids of brain slices incubated in a medium containing protoveratrine. It is well known that protoveratrine brings about stimulation of brain respiration. The stimulated respiration is abolished by TTX (182) and is considered, therefore, to be associated with the neurons.

Results given in Figure 2 show that the protoveratrine stimulated respiration was blocked by malonate (2 mM) but not by fluoroacetate (3 mM).

TABLE 54. Effects of protoveratrine on amino acid content in rat brain cortex slices incubated in presence of ouabain.

Rat brain cortex slices were incubated in 3 ml Krebs-Ringer phosphate media containing 10mM glucose in O_2 at 37°C for one hour in the presence of protoveratrine (5 μM) or of ouabain (0.1mM) as shown below. Values of amino acids are given as μmole/g initial wet wt.

	Ouabain	Ouabain and Protoveratrine
Glutamate	4.95 ± 0.26	2.52 ± 0.11
Glutamine	0.71 ± 0.04	0.74 ± 0.04
GABA	1.39 ± 0.06	0.63 ± 0.02
Aspartate	1.75 ± 0.22	1.20 ± 0.07
Glycine	0.59 ± 0.03	0.25 ± 0.01
Alanine	0.42 ± 0.02	0.26 ± 0.01

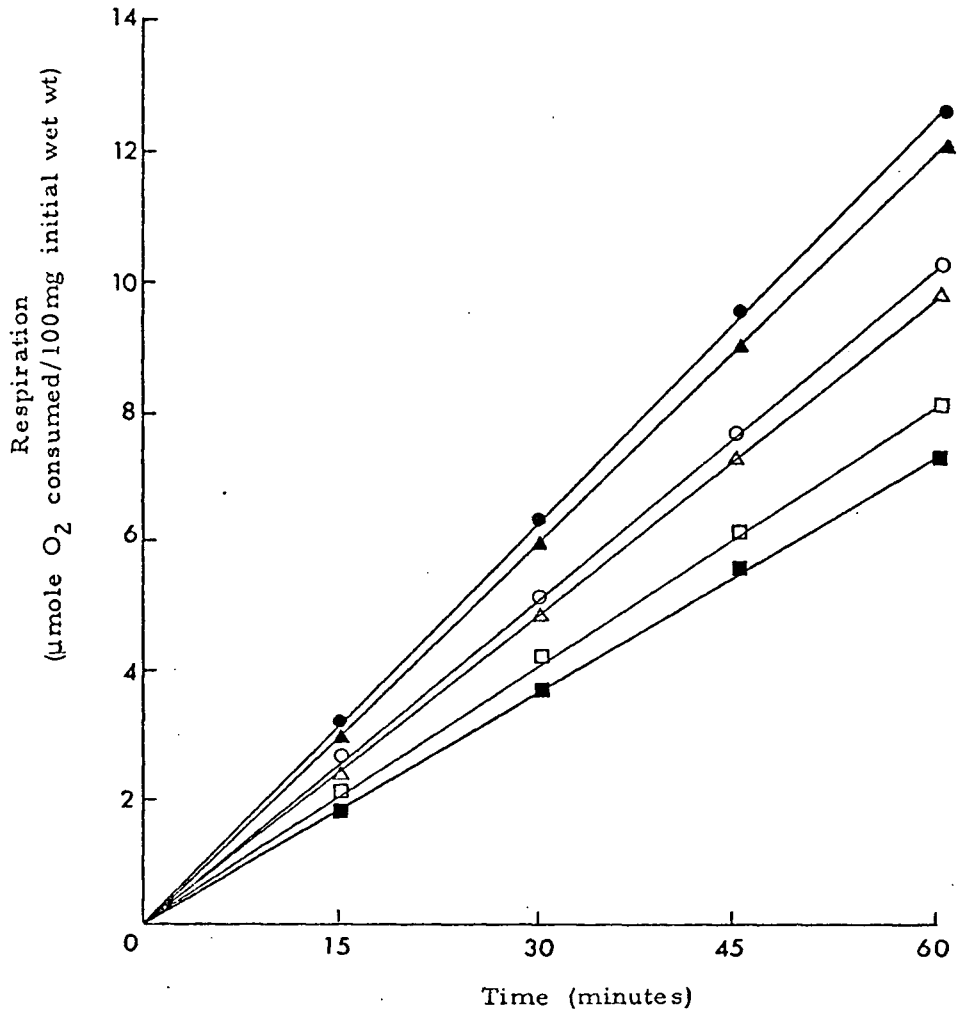


FIGURE 2. Effects of sodium fluoroacetate and sodium malonate on the protoveratrine-stimulated respiration of brain cortex slices.

Respiration of rat brain cortex incubated in O_2 at 37°C in Krebs-Ringer phosphate glucose for various periods of time in the presence or absence of protoveratrine (5μM), sodium fluoroacetate (3mM) or sodium malonate (2mM).

○ No additions
□ With malonate
△ With fluoroacetate
● Protoveratrine added
■ Protoveratrine + malonate added
▲ Protoveratrine + fluoroacetate added

This result is consistent with the conclusion that fluoroacetate, in contrast to malonate at the concentrations investigated does not suppress the operation of the citric acid cycle in the neurons.

Results given in Table 55 indicate that malonate (2 mM) and fluoroacetate (3 mM) brought about different effects on the amounts of amino acids in, and on their release from, brain slices incubated in the presence of protoveratrine.

The major effect of fluoroacetate (3 mM) was to bring about a diminution in the total (tissue + medium) amount of glutamine, amounting to 2.45 μmole/g, accompanied by an increase in that of glutamate amounting of 2.45 μmole/g. There was an increase in the amount of GABA amounting to 1.71 μmole/g, but a slightly diminished value of aspartate. There were minor changes in the other amino acids. These results with fluoroacetate resemble those obtained in earlier studies of the amount of labelled amino acids formed from labelled glucose (63). Fluoroacetate caused a significant rise in tissue, but not medium, glutamate and a significant fall in tissue, but not medium, glutamine. The rise in GABA was mostly confined to the tissue.

The major effect of malonate was to diminish the total (tissue + medium) amount of aspartate by 1.59 μmole/g, and to increase that of GABA by 1.92 μmole/g. There were no statistically significant effects on the total amounts of glutamine or other amino acids (Table 55). Malonate caused significant rises in the medium contents of glutamate and glutamine with accompanying falls in the tissue contents of these amino acids. It brought about a large fall in the tissue value of aspartate with a relatively small change in the medium content of aspartate. There occurred a large rise in tissue GABA with a small, but significant, change in the medium content of GABA. The tissue content of alanine was also significantly diminished by malonate. Changes that took place in the other amino acids investigated were not statistically significant.

TABLE 55. Effects of sodium malonate or sodium flouroacetate on amino acid content in, and release from, incubated rat brain slices in the presence of protoveratrine.

Rat brain slices were incubated in 3 ml Krebs-Ringer phosphate medium containing 10 mM glucose in O_2 at 37°C for one hour in the presence of protoveratrine (5μM) with or without sodium malonate (2mM) or sodium fluoroacetate (3mM). Tissue values of amino acids are expressed as μ mole/g initial wet wt. and medium values as μmole/g wet wt./3ml.

Amino Acid	Protoveratrine			Protoveratrine + Malonate			Protoveratrine + Fluoroacetate		
	Tissue	Medium	Total	Tissue	Medium	Total	Tissue	Medium	Total
Glutamate	7.04 ± 0.06	0.78 ± 0.06	7.82	6.07 ± 0.37	1.22 ± 0.01	7.29	9.31 ± 0.69	0.96 ± 0.10	10.27
Glutamine	3.56 ± 0.07	2.73 ± 0.25	6.29	2.77 ± 0.54	3.88 ± 0.09	6.65	0.91 ± 0.17	2.93 ± 0.59	3.84
GABA	2.92 ± 0.37	0.06 ± 0.03	2.98	4.65 ± 0.01	0.25 ± 0.03	4.90	4.58 ± 0.47	0.11 ± 0.02	4.69
Aspartate	1.98 ± 0.10	0.51 ± 0.01	2.49	0.53 ± 0.15	0.37 ± 0.05	0.90	1.39 ± 0.04	0.53 ± 0.08	1.92
Glycine	0.89 ± 0.07	0.30 ± 0.03	1.19	0.79 ± 0.07	0.53 ± 0.07	1.32	0.98 ± 0.07	0.48 ± 0.03	1.46
Alanine	1.07 ± 0.01	0.59 ± 0.06	1.66	0.66 ± 0.04	0.79 ± 0.01	1.45	1.04 ± 0.01	0.86 ± 0.07	1.90
Taurine	3.61 ± 0.28	2.40 ± 0.10	6.01	2.98 ± 0.32	3.17 ± 0.05	6.15	3.52 ± 0.08	3.10 ± 0.12	6.62
Serine	0.97 ± 0.08	0.70 ± 0.01	1.67	0.86 ± 0.07	0.99 ± 0.13	1.85	1.07 ± 0.08	0.95 ± 0.05	2.02
Threonine	0.51 ± 0.02	0.25 ± 0.02	0.76	0.37 ± 0.06	0.38 ± 0.02	0.75	0.35 ± 0.03	0.37 ± 0.03	0.72
TOTAL	22.55	8.32	30.87	19.68	11.58	31.26	23.15	10.29	33.44

TABLE 56. Effects of L-glutamate and L-glutamine on amino acid content in, and release from, rat brain cortex slices.

Rat brain cortex slices were incubated in 3 ml Krebs-Ringer phosphate medium containing 10mM glucose, but in absence of Ca^{++}, at 37°C for one hour with or without L-glutamate (2.5mM) or L-glutamine (2.5mM). Tissue values of amino acids are expressed as μ mole/g initial wet wt., and medium values as μmole/g initial wet wt./3ml.

Amino Acid	Control			L-Glutamate			L-Glutamine		
	Tissue	Medium	Total	Tissue	Medium	Total	Tissue	Medium	Total
Glutamate	9.75 ± 0.34	1.17 ± 0.11	10.92	20.17 ± 0.09	(1.73 ± 0.06 mM)		10.10 ± 0.08	2.27 ± 0.28	12.37
Glutamine	1.96 ± 0.02	2.41 ± 0.14	4.37	3.81 ± 0.20	3.39 ± 0.18	7.20	12.22 ± 0.09	(1.62 ± 0.06 mM)	
GABA	2.30 ± 0.05	0.05 ± 0.03	2.35	3.21 ± 0.34	0.26 ± 0.04	3.47	4.04 ± 0.06	0.03 ± 0.01	4.07
Aspartate	2.95 ± 0.19	0.45 ± 0.05	3.40	5.69 ± 0.07	2.73 ± 0.03	8.42	2.96 ± 0.23	0.63 ± 0.01	3.59
Ammonia	1.47 ± 0.09	5.69 ± 0.63	7.16	1.48 ± 0.45	4.88 ± 0.36	6.36	1.51 ± 0.12	9.93 ± 0.49	11.44

8.5 Effects of L-glutamine and sodium L-glutamate on cerebral amino acid content and release.

Experiments were carried out to observe the effects of the addition of L-glutamine (2.5 mM) to the incubation medium on the amino acid contents of rat brain cortex slices. Krebs-Ringer phosphate solution containing 10 mM glucose, but devoid of Ca^{2+}, was used as the incubation medium, as with this medium the addition of L-glutamate was found to promote the efflux of GABA and aspartate. Results are shown in Table 56, which also gives comparable results obtained with sodium L-glutamate (2.5 mM). They indicate that the addition of L-glutamine gave rise to an increased value of the tissue content of GABA but no increased efflux of GABA, to an increased quantity of ammonia and to a small rise in the medium concentration of glutamate. There was no increased formation of aspartate. Addition of glutamate (2.5 mM), however, caused a lesser increase of tissue GABA but an increased efflux of GABA, increased formation and efflux of both aspartate and glutamine, and a somewhat diminished formation of ammonia. Thus, the addition of glutamine and of L-glutamate to the incubation medium brought about significantly different effects both in amino acid content and amino acid effluxes in rat brain cortex slices.

8.6 Summary

1. By using tetrodotoxin to suppress the neuronal efflux of amino acids from rat brain cortex slices brought about by the joint action of protoveratrine and ouabain, the latter being added to diminish re-uptake of amino acids, it is shown that the major pools of glutamate, aspartate, glycine, serine and probably γ-aminobutyrate, are in the neurons.

2. The major pool of glutamine lies not in the neurons but in the glia.

3. The citric acid cycle inhibitors, fluoroacetate and malonate, exert different effects on amino acid contents in, and on amino acid releases from, brain slices incubated in presence of protoveratrine. Fluoroacetate (3 mM) diminishes the content of glutamine, increases that of glutamate and γ-aminobutyrate, and does not affect respiration. Malonate (2 mM) diminishes aspartate and γ-aminobutyrate content, suppresses respiration, and does not affect glutamine content.

4. Glutamine is a more effective precursor of γ-aminobutyrate than glutamate.

9. EFFECTS OF NH_4^+ ON BRAIN METABOLISM AND TRANSPORT IN VITRO

It is a well known fact that ammonium ions exert profound effects on brain cell metabolism and function. The maintenance of extremely low concentrations of free NH_4^+ by an efficient utilization mechanism in the normal animal ensures the proper operation of such processes. Injection of ammonium salts may lead to convulsions. This has been attributed to an imbalance of ammonia metabolism in the central nervous system, but no satisfactory explanation is yet available. Diseases like hepatic coma and epilepsy are known to be accompanied by elevated blood and brain levels of ammonia.

We have already considered some aspects of the formation, transport and utilization of NH_4^+ in the brain. In this chapter, the results of studies of the effects of NH_4^+ on metabolism and transport will be described. Their possible bearing on ammonia toxicity will be considered.

9.1 Initial contents of Na^+ and K^+ in the infant (two-day-old) and adult rat brain cortex.

Results in Table 57 show the Na^+ and K^+ contents of two day old rat brain cortex slices freshly prepared and found prior to incubation. Values also given for the brain cortex slices of adult rat are in accord with data in the literature (263, 264). The infant rat brain cortex content of Na^+ expressed in terms of μequiv/ml tissue water is about the same as that of the adult but the K^+ concentration is lower. The sum of the Na^+ and K^+ concentrations for the infant brain is about the same as that used in the incubation medium (viz., 153 μequiv/ml), but that of the adult is 20 per cent higher.

TABLE 57. Initial Na^+ and K^+ contents of 2-day old and adult rat brain cortex slices.

Na^+ and K^+ contents of infant and adult rat brain cortex slices freshly prepared, and found immediately prior to incubation are given below and are expressed as µmole/g initial wet wt. and as µmole/ml. tissue water. Water content of 2-day old cortex of rat brain is 88%, whilst that of adult rat brain cortex slices is 80% of the initial fresh wt.

Rat Brain Cortex	Na^+ Content		K^+ Content		$Na^+ + K^+$
	µequiv/g	µequiv/ml	µequiv/g	µequiv/ml	µequiv/ml
2-day	60.8 ± 0.8	69.1	77.2 ± 1.9	87.6	156.7
Adult	53.6 ± 3.0	67.0	91.4 ± 1.6	114.0	181.0

9.2 Effects of increasing NH_4^+ concentration on water and oxygen uptakes, and cationic fluxes of incubated rat brain cortex slices.

When rat brain cortex slices are incubated in O_2 at 37°C for one hour in a normal Krebs-Ringer phosphate glucose medium, they take up water and Na^+ from, and lose K^+ to, the surrounding medium (Table 58, compare with initial values in Table 57). Increasing the concentration of NH_4Cl in the medium further enhances the tissue content of Na^+ and concomitantly diminishes that of K^+. The water uptake by the brain slices increase with medium NH_4Cl concentrations above 5 mM. Since we have shown that NH_4^+ is not concentrated against a concentration gradient (Chapter 6), the loss of tissue K^+ due to medium NH_4^+, cannot be explained simply by NH_4^+-K^+ exchange. For example, with 10 mM NH_4Cl in the incubation medium, there is a tissue loss of about 32 μequiv K^+/ml tissue water/hour for a gain of 10 μequiv NH_4^+/ml/hour. Moreover, simple NH_4^+-K^+ exchange would not account for the alterations in Na^+ and water contents of the brain slices.

As NH_4^+ can replace K^+ in the activation of the Na^+, K^+-ATPase (223, section 9.3), and as NH_4^+ inhibits the active transport of amino acids such as glycine or glutamate (Chapter 5), it seems possible that NH_4^+ ions may act by uncoupling ATPase activity from transport processes. Evidence to be presented below, however, shows that this possibility is unlikely.

It is to be noted that increase of NH_4^+ in the incubation medium to high levels (e.g., 20 or 30 mM) leads to significant falls in brain respiration.

9.3 ATPases of rat cerebral cortical homogenates

In view of the fact that NH_4^+ ions exert profound effects on the cationic contents of incubated rat cerebral cortex slices (Table 58), studies were carried out on the effects of various cations including NH_4^+ on the ATPase activities of adult rat brain cortical homogenates.

TABLE 58. Effects of increasing NH_4Cl concentrations on oxygen and water uptakes and Na^+, K^+ contents of incubated rat brain cortex slices.

Rat brain cortex slices were incubated in O_2 at 37°C for one hour in Krebs-Ringer phosphate glucose medium with increasing concentrations of NH_4Cl. Tissue concentrations of Na^+ or K^+ at the end of the incubation are given as μequiv/g initial wet wt. tissue and as μequiv/ml tissue water. Oxygen uptake (QO_2) is expressed as μ mole/g initial wet wt. tissue and water uptake as μl/100mg initial wet wt.

Medium NH_4Cl (mM)	QO_2	Water Uptake	Tissue Contents					
			(μequiv/g)			(μequiv/ml)		
			Na^+	K^+	Total	Na^+	K^+	Total
Nil	101 ± 6	14.0 ± 1.5	109.3 ± 1.3	58.5 ± 3.8	167.8	116.3	62.2	178.5
1	104 ± 3	14.6 ± 2.6	116.0 ± 6.0	44.8 ± 7.4	160.8	122.6	47.4	170.0
2	111 ± 3	14.8 ± 1.7	118.1 ± 4.5	42.7 ± 5.1	160.8	124.6	45.0	169.6
5	107 ± 3	18.6 ± 2.5	128.4 ± 0.4	35.5 ± 2.2	163.9	130.2	36.0	166.2
10	107 ± 6	24.4 ± 1.6	135.0 ± 1.7	31.5 ± 2.8	166.5	129.3	30.2	159.5
20	90 ± 1	42.4 ± 0.4	177.2 ± 1.8	19.0 ± 0.4	196.2	144.8	15.5	160.3
30	78 ± 1	50.8 ± 3.3	192.1 ± 3.3	16.3 ± 0.1	208.4	146.9	12.5	159.4

1. Mg^{++}-ATPase. None of the chloride salts of the cations tried (viz., Na^+, K^+, NH_4^+, Li^+, Rb^+, or Cs^+) has any significant effect on the Mg^{++}-ATPase activity (Table 59).

2. Na^+, K^+, (Mg^{++})-ATPase. Results in Table 60 show the dependence of the activity of this ATPase on Mg^{++}, Na^+ and K^+. At 15 mM (chloride) salt concentrations, NH_4^+, Li^+, Rb^+, or Cs^+ can substitute for K^+ in the presence of Na^+ though Li^+ has a relatively small effect. None of these cations can substitute for Na^+ in the presence of K^+. These results are in accord with those of Skou (222, 223). Ca^{++} and ouabain inhibit the Na^+, K^+-ATPase but have no effect on the Mg^{++}-ATPase (222, 223). Protoveratrine, DNP, and TTX have no effect on the Na^+, K^+-ATPase while only protoveratrine may have a small inhibitory effect on the Mg^{++}-ATPase (Table 61). The affinity constants (K_m) for Na^+, K^+-ATPase obtained in our studies is 0.7 μequiv K^+/ml and 3.7 μequiv/ml when NH_4^+ is substituted for K^+.

9.4 Effects of tetramethyl ammonium chloride, and the chloride salts of Li^+, Rb^+ and Cs^+ on the oxygen and water uptakes, and the Na^+, K^+ fluxes in incubated rat brain cortex slices.

Since LiCl, RbCl and CsCl, like NH_4Cl, can replace KCl in activating the Na-K^+-Mg^{++}ATPase, it was of interest to compare the effects of these chloride salts (Li^+, Rb^+, Cs^+) on the cationic fluxes at the brain cell membrane, with those brought about by the presence of NH_4Cl.

Rat brain cortex slices were incubated in O_2 at 37°C for one hour in Krebs-Ringer phosphate glucose medium in the presence of $(CH_3)_4NCl$, LiCl, RbCl or CsCl. From the results given in Table 62, it is seen that the tissue K^+ content is diminished in the presence of these salts. However,

TABLE 59. Effects of cations on the Mg^{2+}-ATPase activity of homogenates of adult rat brain cortex.

Incubations of rat brain cortical homogenates were carried out for one hour at 37°C in the following assay system: EDTA, 0.1mM; Tris-Cl buffer (pH 7.6), 95mM; Tris-ATP, 3mM; (See Methods, Section 2.14). When present $MgCl_2$ was 1mM; $CaCl_2$, 2.8mM; NaCl, 58mM; LiCl, RbCl, CsCl, NH_4Cl, KCl, 15mM. Mg^{2+}ATPase activities (i.e. activities in the absence of combined Na^+ and K^+) given below, are means of 4 determinations, with standard deviations not greater than ± 5%; they are expressed as μ mole Pi released from ATP per hour per mg wet wt. brain cortex.

Additions to the assay system	μ mole Pi/h/mg wet wt.
Nil	0.04
Mg^{2+}	0.76
Mg^{2+} + Ca^{2+}	0.69
Mg^{2+} + Na^+	0.86
Mg^{2+} + K^+	0.73
Mg^{2+} + NH_4^+	0.71
Mg^{2+} + Li^+	0.77
Mg^{2+} + Rb^+	0.75
Mg^{2+} + Cs^+	0.75

TABLE 60. Effects of cations on the Na^+-K^+-Mg^{2+} ATPase activities of homogenates of adult rat brain cortex slices.

Incubations of rat brain cortical homogenates were carried out for one hour at 37°C in the following assay system: EDTA, 0.1mM; Tris-Cl, buffer (pH 7.6), 95mM; Tris-ATP, 3mM; (See Methods, section 2.14). When present $MgCl_2$ was 1mM; $CaCl_2$, 2.8mM; NaCl, 58mM; LiCl, RbCl, CsCl, NH_4Cl, KCl, 15mM; Values given below are means of 4 determinations with standard deviations not greater than ± 5% and are expressed as μmole Pi released from ATP per hour per mg wet wt. brain cortex.

Additions to the assay system	μmole Pi/h/mg wet wt.
$Na^+ + K^+$	0.04
$Mg^{2+} + Na^+ + K^+$	2.36
$Mg^{2+} + Na^+ + K^+ + Ca^{2+}$	0.76
$Mg^{2+} + Na^+ + NH_4^+$	2.45
$Mg^{2+} + NH_4^+ + K^+$	0.78
$Mg^{2+} + Na^+ + K^+ + NH_4^+$	2.51
$Mg^{2+} + Na^+ + K^+ + NH_4^+ + Ca^{2+}$	0.76
$Mg^{2+} + Na^+ + Li^+$	1.34
$Mg^{2+} + Li^+ + K^+$	0.76
$Mg^{2+} + Li$(58 equiv/l) $+ K^+$	0.76
$Mg^{2+} + Na^+ + K^+ + Li^+$	2.45
$Mg^{2+} + Na^+ + Rb^+$	2.10
$Mg^{2+} + Rb^+ + K^+$	0.81
$Mg^{2+} + Na^+ + K^+ + Rb^+$	2.36
$Mg^{2+} + Na^+ + Cs^+$	2.00
$Mg^{2+} + Cs^+ + K^+$	0.80
$Mg^{2+} + Na^+ + K^+ + Cs^+$	2.41

TABLE 61. Effects of protoveratrine, tetrodotoxin, ouabain and 2,4 dinitrophenol on the ATPase activities of rat cerebral cortical homogenates.

Incubations of rat cortical homogenates were carried out at 37°C for one hour in the following assay system for the estimation of Mg^{2+} ATPase activity: EDTA, 0.1mM; Tris-Cl buffer (pH 7.6), 95mM; Tris ATP, 3mM; and $MgCl_2$, 1mM. For the determination of the Na^+, K^+-ATPase + Mg^{2+} ATPase activity NaCl (58mM) and KCl (15mM) were also present. Na^+, K^+-ATPase is obtained by the difference of the two estimations. Results are means of at least 4 determinations with standard deviations not greater than ± 5%. Values are given as μmole Pi released from ATP per hour per mg wet wt. brain cortex.

Additions to the assay system	Mg^{2+}-ATPase	Na^+, K^+-ATPase
Nil	0.76	1.67
Protoveratrine (5μM)	0.56	1.88
Tetrodotoxin (3μM)	0.73	1.71
Ouabain (0.01mM)	0.64	0.94
(0.1mM)	0.62	0.70
DNP (0.01mM)	0.73	1.70
(0.1mM)	0.76	1.67
$CaCl_2$ (2.8mM)	0.72	0.14
$CaCl_2$ (2.8mM) + Protoveratrine (5μM)	0.52	0.02

TABLE 62. Effects of NH_4^+, $(CH_3)_4N^+$, Li^+, Rb^+ and Cs^+ on the oxygen and water uptakes, and the Na^+, K^+ contents of incubated rat brain cortex slices.

Rat brain cortex slices were incubated in O_2 at 37°C for one hour in Krebs-Ringer phosphate glucose medium containing the chloride salts of NH_4^+, $(CH_3)_4N^+$, Li^+, Rb^+ and Cs^+ at concentrations given below. Oxygen uptake (QO_2) is expressed as μmole/g initial wet wt. tissue and water uptake as μl/100mg initial wet wt.

Additions to the incubation medium		QO_2	Water Uptake	Tissue Contents (μequiv/g)	
				Na^+	K^+
Nil	(Control)	103 ± 2	14.5 ± 1.6	110.6 ± 1.4	59.2 ± 4.3
NH_4Cl	5	107 ± 3	18.6 ± 2.5	128.4 ± 0.4	35.5 ± 2.2
	10	108 ± 6	24.4 ± 1.2	135.0 ± 1.7	31.5 ± 2.8
	20	90 ± 1	42.4 ± 0.4	177.2 ± 1.8	19.0 ± 0.4
$(CH_3)_4NCl$	10	105 ± 2	12.6 ± 2.5	110.9 ± 2.1	49.6 ± 3.1
	20	104 ± 2	10.6 ± 2.2	110.7 ± 4.3	40.6 ± 4.4
LiCl	5	107 ± 9	13.6 ± 2.9	109.8 ± 4.0	53.4 ± 4.4
	10	111 ± 4	14.5 ± 0.9	112.0 ± 3.0	48.7 ± 3.1
RbCl	5	105 ± 6	13.2 ± 1.8	114.0 ± 4.7	32.0 ± 0.1
	10	105 ± 2	13.5 ± 1.6	113.6 ± 0.4	27.9 ± 0.2
CsCl	5	105 ± 2	9.9 ± 1.0	108.1 ± 2.7	38.0 ± 1.8
	10	131 ± 5	12.5 ± 1.4	110.8 ± 0.4	32.3 ± 1.2

the diminution in tissue K^+ levels due to LiCl or $(CH_3)_4NCl$ may be simply due to an exchange of Li^+ or $(CH_3)_4N^+$ for K^+, as there is no evidence that Li^+ or $(CH_3)_4N^+$ is actively accumulated in the tissue. The fall in the tissue K^+ levels due to Rb^+ or Cs^+ resembles that due to NH_4^+, but although Rb^+ (unlike NH_4^+) is actively transported into brain cells (224), it is not known whether this is the case with Cs^+ ions. However, neither Rb^+, Cs^+, Li^+ or $(CH_3)_4N^+$ (at 5 or 10 mM), unlike NH_4^+, brings about increase in the water uptake or the Na^+ content of the incubated brain tissue. It is therefore evident that the effects of NH_4Cl are different from those of the alkali metal salts (Li^+, Rb^+, or Cs^+) and of $(CH_3)_4NCl$. None of these salts affect the rate of respiration, except CsCl at 10 mM, which causes a stimulation.

9.5 Effects of increasing concentrations of NH_4^+ on cationic fluxes in incubated two-day-old rat brain cortex slices.

Infant (two-day-old) rat brain cortex slices were incubated in O_2 at 37°C for one hour in Krebs-Ringer phosphate glucose medium containing increasing concentrations of NH_4Cl. From the results given in Table 63, it is seen that the loss of K^+ from the tissue is equivalent to the concentration of NH_4Cl in the medium. This may simply be explained as due to an exchange of NH_4^+ for K^+ in the infant brain. There is no evidence of accumulation of NH_4^+ against a concentration gradient in infant brain.

Water is not taken up by incubated infant rat brain cortex slices in the presence or absence of NH_4^+ in the incubation medium. The content of water in two day old rat brain is, however, high (about 88%) so that increased water uptake would not be expected. The respiratory rate of infant brain cortex slices is unaffected by NH_4^+ in the incubation medium.

The brain Na^+ content in incubated infant rat cortex is enhanced to some extent with medium NH_4Cl concentrations of 20 and 30 mM. Com-

TABLE 63. Effects of increasing NH_4Cl concentrations on the oxygen and water uptakes and Na^+, K^+ contents of infant (2-day old) rat brain cortex slices.

Incubation of two-day old rat brain cortex slices were carried out in Krebs-Ringer phosphate glucose medium in O_2 at 37°C for one hour with increasing medium NH_4Cl concentrations. Tissue contents of Na^+ or K^+ at the end of the incubation are expressed as μequiv/g initial wet wt. tissue, and as μequiv/ml tissue water. Oxygen uptake (QO_2) is given in terms of μmole/g initial wet wt. tissue. Water uptake was found to be (0.0 ± 2.0) μl/100 mg initial wet wt. and water content 88%.

Medium NH_4Cl (mM)	QO_2	Tissue Contents					
		(μequiv/g)			(μequiv/ml)		
		Na^+	K^+	Total	Na^+	K^+	Total
Nil	72 ± 4	82.3 ± 0.5	55.7 ± 0.5	138.0	93.5	63.3	156.8
2.0	73 ± 4	82.0 ± 1.6	53.9 ± 1.1	135.9	93.2	61.3	154.5
5.0	68 ± 5	84.7 ± 3.5	50.3 ± 0.7	135.0	96.3	57.2	153.5
10.0	79 ± 5	84.0 ± 2.0	48.9 ± 1.1	132.9	95.5	55.6	151.5
20.0	82 ± 6	91.1 ± 1.3	36.3 ± 2.9	127.4	103.5	41.3	144.8
30.0	81 ± 1	98.5 ± 2.6	22.7 ± 0.6	121.2	111.9	28.1	137.7

pared with the value for incubated adult brain tissue (Table 58), the increase is relatively small. For example, with 20 mM NH_4Cl in the incubation medium, the increases in the Na^+ contents of incubated infant and adult rat brain cortex slices is 9 and 68 μequiv/g/hour, respectively.

9.6 Quantitative aspects of the effects of NH_4^+ on the Na^+, K^+ and water contents of incubated rat brain cortex slices.

If NH_4^+ ions exert their effects on Na^+ and K^+ contents of incubated adult brain, by uncoupling the Na^+, K^+-ATPase activity from active transport processes, it would be expected that they would act without time lag. Experiments were carried out to observe whether NH_4^+ ions act with or without a time lag.

Rat brain cortex slices were incubated in O_2 at 37°C in Krebs-Ringer phosphate glucose medium with or without 10 mM NH_4Cl for various periods of time. At the end of the incubation periods, the Na^+, K^+, NH_4^+ and water contents of the tissue were analyzed. Results given in Table 64 show that the increase in water and Na^+ contents of the tissue, due to NH_4^+, only occurs after about 30 minutes incubation. While the tissue NH_4^+ content reaches its maximum level within a few minutes of incubation, tissue K^+ content falls significantly (i.e., beyond the value expected by K^+-NH_4^+ exchange) only after 15 minutes incubation and then continues to fall. Such results are only to be expected if the effect of NH_4^+, on K^+ release from the brain, is due to a time-consuming metabolic process and are not consistent with an immediate action of NH_4^+ at the cell membrane leading to an "uncoupling" of ATPase activity from cationic transport.

9.7 Effects of increasing NH_4^+ concentrations on the cationic contents of rat brain cortex slices incubated in the presence of 0.1 mM ouabain or in the absence of glucose.

The fact that NH_4^+ exerts its effects by metabolic changes, is shown

TABLE 64. Kinetics of the alterations in the tissue water, Na^+, K^+ and NH_4^+ contents of rat brain cortex slices incubated in an NH_4Cl containing medium.

Rat brain cortex slices were incubated in O_2 at 37°C for one hour in Krebs-Ringer phosphate glucose medium with or without 10mM NH_4Cl. Water uptake is expressed as µl/100 mg. initial wet wt.

Time	Water Uptake	Tissue Contents					
		µequiv/g initial wet wt.			µequiv/ml tissue water		
		Na^+	K^+	NH_4^+	Na^+	K^+	NH_4^+
After Oxygenation (5 min)							
Nil	5.3 ± 0.1	101.5 ± 1.9	62.4 ± 7.2		119.0	73.2	
+ NH_4Cl	7.6 ± 0.2	95.6 ± 7.0	60.0 ± 4.7	8.8 ± 0.1	109.1	68.5	10.0
After Equilibration (7 min) (i.e. zero time)							
Nil	6.2 ± 1.8	105.5 ± 0.5	54.3 ± 4.5		122.4	63.0	
+ NH_4Cl	9.7 ± 0.7	104.6 ± 8.4	45.3 ± 1.5	11.1 ± 0.6	116.6	50.5	12.3
15 min. incubation							
Nil	8.2 ± 1.4	100.6 ± 0.6	58.0 ± 2.5		114.1	65.8	
+ NH_4Cl	11.9 ± 0.2	99.8 ± 7.4	43.0 ± 2.8	11.7 ± 0.1	109.0	46.8	12.8
30 min. incubation							
Nil	11.9 ± 0.8	108.5 ± 4.5	58.5 ± 1.5		118.1	63.7	
+ NH_4Cl	14.2 ± 0.4	110.5 ± 4.5	40.3 ± 0.9	11.2 ± 0.2	117.3	42.8	11.8
45 min. incubation							
Nil	12.3 ± 1.5	104.9 ± 4.7	60.3 ± 3.9		113.7	65.3	
+ NH_4Cl	20.5 ± 0.3	122.0 ± 1.0	36.6 ± 2.2	12.1 ± 0.6	121.4	36.4	12.0
60 min. incubation							
Nil	14.9 ± 0.7	110.0 ± 0.8	57.4 ± 1.1		115.9	60.5	
+ NH_4Cl	25.3 ± 1.5	133.0 ± 1.6	30.5 ± 3.3	11.6 ± 0.3	126.3	29.0	11.1

also by the results of experiments carried out in the presence of 0.1 mM ouabain (Table 65). Like ouabain, NH_4^+ is known to inhibit active transport processes (Tables 28, 30 and 31), but, unlike ouabain, it stimulates Na^+, K^+-ATPase (Tables 61 and 60). From the results given in Table 65, it is evident that the drop in tissue K^+ levels due to 0.1 mM ouabain is further potentiated by NH_4^+. Thus, with 2 mM NH_4Cl in the medium, the drop in K^+, namely, (19.8 - 14.1) = 5.7 μequiv/ml tissue water is greater than that allowed for by the 1:1 NH_4^+ for K^+ exchange. The effect is also evident with 5 mM NH_4Cl, but the tissue has under this circumstance (i.e., with 5 mM medium KCl) almost reached its minimum concentration of K^+, so that further increase of the medium NH_4^+ concentration cannot further diminish the K^+ content of the tissue. The tissue Na^+ concentrations (μequiv/ml tissue water) with or without added NH_4Cl, does not change as it has reached in the presence of 0.1 mM ouabain, the Na^+ concentration of the incubation medium.

Similar results were obtained on incubation of brain cortex slices in O_2 for one hour at 37°C in glucose-free Krebs-Ringer phosphate medium containing increasing concentrations of NH_4Cl (Table 65). Thus, a fall of (19.9 - 13.5) = 6.4 μequiv K^+/ml tissue water occurs with a concomitant gain, by the tissue, of only 2 μequiv NH_4^+/ml tissue water. The Na^+ level in the tissue has already attained the medium concentration of Na^+, so that further increase of tissue Na^+ cannot occur.

9.8 Effects of changing medium ionic composition on the Na^+, K^+ contents of incubated rat brain cortex slices.

(i) Effects of Ca^{++} and K^+.

Results given in Table 66 show that rat brain cortex slices incubated in O_2 for one hour at 37°C in a physiological glucose saline medium in the absence of K^+ or Ca^{++}, gain Na^+ and lose K^+ to the incubation medium. In the absence from the medium of both Ca^{++} and K^+, the tissue K^+ content is

TABLE 65. Effects of increasing NH_4Cl concentrations on the oxygen and water uptakes and the Na^+, K^+ contents of rat brain cortex slices incubated in the presence of 0.1mM ouabain or in the absence of glucose.

Contents of Na^+ and K^+ in rat brain cortex slices incubated in O_2 for one hour at 37°C in the presence of increasing NH_4Cl concentrations in Krebs-Ringer phosphate medium: A) with glucose and 0.1mM ouabain present; B) without glucose.

Additions to the incubation medium	QO_2 µ mole/g initial wet wt.	Water Uptake µl/100mg initial wet wt.	Tissue Contents µ mole/g initial wet wt.		µmole/ml tissue water	
			Na^+	K^+	Na^+	K^+
A) Glucose + Ouabain	94 ± 5	38.7 ± 1.7	172.6 ± 2.1	23.5 ± 0.9	145.4	19.8
+ NH_4Cl, 2mM	100 ± 2	42.8 ± 0.1	179.7 ± 0.5	17.3 ± 1.0	146.3	14.1
5mM	94 ± 8	40.4 ± 3.5	178.4 ± 1.4	13.1 ± 0.1	148.2	10.9
10mM	85 ± 3	43.3 ± 1.1	183.0 ± 6.8	12.5 ± 0.5	148.4	10.1
20mM	69 ± 5	43.2 ± 3.6	181.8 ± 6.4	11.8 ± 0.2	147.6	9.5
B) Nil	69 ± 3	43.4 ± 0.4	190.9 ± 2.5	24.6 ± 0.1	154.7	19.9
+ NH_4Cl, 2mM	64 ± 1	46.6 ± 0.1	198.6 ± 8.6	17.1 ± 1.0	156.9	13.5
5mM	58 ± 1	48.2 ± 1.2	198.2 ± 4.7	17.5 ± 0.4	154.6	13.7
10mM	53 ± 2	43.6 ± 1.3	189.7 ± 0.6	16.4 ± 0.1	153.5	13.3
20mM	50 ± 2	53.7 ± 0.5	204.4 ± 4.2	16.1 ± 0.1	152.9	12.0

TABLE 66. Effects of medium cation contents on the oxygen and water uptakes, and Na^+, K^+ levels in incubated rat brain cortex slices.

Incubations of rat brain cortex slices were carried out in O_2 at 37°C for one hour under media conditions given below. When adjustments in the incubation media were made, the concentration of NaCl was diminished by an amount equal to the concentration of KCl added. Tissue Na^+ and K^+ contents at the end of the incubation are expressed as µmole/g initial wet wt., oxygen consumption (QO_2) as µmole/g initial wet wt. and water uptake as µl/100 mg initial wet wt.

Changes made to a Krebs-Ringer phosphate glucose medium	Medium Na^+ µequiv/ml	QO_2	Water Uptake	Tissue Content (µmole/g)	
				Na^+	K^+
Control	148	101 ± 6	14.0 ± 1.5	109.3 ± 1.3	58.5 ± 3.8
K^+-free	148	94 ± 3	22.6 ± 2.6	146.0 ± 1.0	35.5 ± 6.5
Ca^{++}- free	148	128 ± 5	18.0 ± 2.0	136.6 ± 3.3	37.4 ± 4.9
K^{++}-free, Ca^{++}-free	148	138 ± 2	19.2 ± 1.0	144.8 ± 5.4	26.6 ± 6.2
KCl (+25mM)					
Adjusted	118	147 ± 2	22.7 ± 2.7	94.4 ± 3.0	80.5 ± 2.3
	123	141 ± 3	22.3 ± 0.4	100.7 ± 1.5	73.4 ± 3.3
Not adjusted	148	127 ± 1	15.1 ± 2.9	110.7 ± 3.9	92.6 ± 2.8
KCl (+50mM)					
Adjusted	98	131 ± 1	40.4 ± 0.4	91.2 ± 0.3	107.3 ± 1.3
Not adjusted	148	133 ± 2	35.8 ± 1.4	135.5 ± 1.2	120.0 ± 4.0
KCl (+100mM)					
Not adjusted	148	137 ± 3	38.0 ± 2.0	138.8 ± 2.4	-

further diminished while the tissue Na^+ concentration approaches that of the incubation medium.

It is to be noted that media, initially free of K^+ ions, gain K^+ from the tissue on incubation. For example, the loss of (9.2 - 3.5) = 5.7 μequiv K^+/100 mg initial wet wt tissue (for values see Tables 57 and 66) which occurs in a one hour incubation period to 3 ml media, is equivalent to a concentration of KCl in the incubation medium of 1.9 mM at the end of the incubation period.

Increasing the medium KCl concentration (to 30 or 50 mM) enhances the tissue levels of K^+.

(ii) Effects of increasing NH_4^+

With the following incubation media:

(a) Krebs-Ringer phosphate glucose, Ca^{++}-free medium;

(b) Krebs-Ringer phosphate glucose, K^+-free medium;

(c) Krebs-Ringer phosphate glucose, K^+ and Ca^{++}-free medium;

and (d) Krebs-Ringer bicarbonate (28 μequiv/ml) glucose medium,

the addition of increasing NH_4Cl concentrations (up to 30 mM) to the incubation medium brings about losses of K^+ from cerebral cortex slices (incubated at 37°C for one hour) that are greater than can be explained by a 1:1 gain of tissue NH_4^+ by K^+-NH_4^+ exchange. With increasing medium KCl, however, it seems that these effects of increased medium NH_4^+ are much reduced (Section 9.9).

(iii) Tissue/Medium concentration ratios for Na^+ and K^+

Table 67 gives the values of tissue to medium concentration ratios for Na^+ and K^+, for cerebral cortex slices incubated in glucose media containing normal Ca^{++} and Mg^{++} concentrations, but with Na^+ concentrations varying between 98 and 148 μequiv/ml and K^+ concentrations varying between 5 and 105 μequiv/ml. From the results it is evident that, while the tissue to medium

TABLE 67. Tissue/Medium concentration ratios for Na^+ and K^+ in rat brain cortex slices incubated in media of varying sodium, potassium and ammonium concentrations.

Tissue to medium concentration ratios of sodium and potassium are calculated for rat brain cortex slices incubated at 37°C for one hour in Krebs-Ringer phosphate glucose medium of varying Na^+, K^+ and NH_4^+ ion concentrations.

Medium concentration μequiv/ml		Medium NH_4Cl (mM)					Medium NH_4Cl (mM)				
		-	5	10	20	30	-	5	10	20	30
Na^+	K^+	Tissue Na^+/Medium Na^+					Tissue K^+/Medium K^+				
148	5	0.79	0.87	0.91	0.98	0.97	12.40	7.20	6.00	3.10	2.50
148	30	0.79	0.83	0.90	0.97	1.01	3.24	2.95	2.43	1.74	1.60
123	30	0.80	0.86	0.91	-	-	2.35	2.00	1.79	-	-
118	30	0.78	0.83	0.89	0.97	1.00	2.62	2.02	1.77	1.59	1.29
148	55	0.79	0.86	0.92	-	-	1.88	1.67	1.54	-	-
98	55	0.78	0.88	0.91	-	-	1.63	1.54	1.40	-	-
148	105	0.79	-	-	-	-	-	-	-	-	-

concentration ratios for K^+ vary considerably according to the medium Na^+ or K^+ concentrations, those for Na^+ appear to be remarkably constant (0.78 - 0.80) under these conditions. On the addition of NH_4Cl to the medium, the tissue to medium concentration ratios for Na^+ approach unity. This also applies for the tissue to medium concentration ratios for K^+.

The tissue to medium concentration ratio for Na^+ is unaffected by medium concentrations of $(CH_3)_4NCl$, LiCl, RbCl, or CsCl, but that of K^+ is diminished, the effect being greatest with Rb^+ and Cs^+ salts (results may be calculated from data in Table 62).

9.9 Effects of high medium K^+ concentrations on the NH_4^+ induced changes in the Na^+, K^+ contents of incubated rat brain cortex slices.

Results are given in Tables 68 and 69 of experiments carried out to observe the effects of 30 mM and 55 mM medium KCl, on the NH_4^+ induced changes in the Na^+ and K^+ contents of brain cortex slices incubated in O_2 at 37°C for one hour in glucose containing media.

The results are summarized below taking into consideration the NH_4^+ for K^+ exchange that occurs in the incubated tissue.

(a) In a normal Krebs-Ringer phosphate glucose medium (i.e., medium Na^+/K^+ = 148/5 = 29.6) tissue K^+ loss due to 10 mM NH_4Cl = (62.2 - 30.2 - 10) = 22.0 μequiv/ml (Table 58).

(b) In a medium containing 30 mM KCl, not adjusted for Na^+ (i.e., medium Na^+/K^+ = 148/30 = 4.9) tissue K^+ loss due to 10 mM NH_4Cl = (97.4 - 72.9 - 10) = 14.5 μequiv/ml (Table 68).

(c) In a medium containing 55 mM KCl, not adjusted for Na^+ (i.e., medium Na^+/K^+ = 148/55 = 2.7) tissue K^+ loss due to 10 mM NH_4Cl =(103.6 - 84.2 - 10) = 9.4 μequiv/ml (Table 69).

TABLE 68. Effects of 30mM KCl on the NH_4Cl induced cationic changes in incubated rat brain cortex slices.

Rat brain cortex slices were incubated in O_2 at 37°C for one hour in Krebs-Ringer phosphate glucose medium containing 30mM KCl. The concentration of sodium in the medium was either : A) adjusted (to contain 123 μequiv/ml); or B) not adjusted (and contained 148 μequiv/ml). NH_4Cl when present was 5 or 10mM. Tissue contents of Na^+ or K^+ are expressed as μmoles/g initial wet wt. tissue and as μmoles/ml tissue water.

Medium NH_4Cl (mM)	QO_2 μmole/g initial wet wt.	Water Uptake μl/100mg initial wet wt.	Tissue Contents (μequiv/g)		Tissue Contents (μequiv/ml)	
			Na^+	K^+	Na^+	K^+
A) Medium adjusted						
Nil	141 ± 3	22.3 ± 0.4	100.7 ± 1.5	73.4 ± 3.3	98.4	71.7
5	133 ± 4	31.5 ± 1.9	116.6 ± 4.6	66.7 ± 0.6	104.6	59.8
10	136 ± 1	35.8 ± 1.5	127.0 ± 3.2	64.3 ± 2.2	109.7	55.5
B) Medium not adjusted						
Nil	127 ± 1	15.1 ± 2.9	110.7 ± 3.9	92.6 ± 2.8	116.4	97.4
5	139 ± 9	19.9 ± 3.2	119.5 ± 5.3	88.6 ± 4.5	119.6	88.7
10	117 ± 4	16.1 ± 1.0	126.5 ± 0.8	70.1 ± 2.9	131.6	72.9

TABLE 69. Effects of 55mM KCl on the NH_4Cl induced cationic changes in incubated rat brain cortex slices.

Rat brain cortex slices were incubated in O_2 at 37°C for one hour in Krebs-Ringer phosphate glucose medium containing 55mM KCl. The concentration of sodium in the medium was either: A) adjusted (to contain 98 μequiv/ml); or B) not adjusted (and contained 148 μequiv/ml). NH_4Cl when present was 5 or 10mM. Tissue contents of Na^+ or K^+ are expressed as μ moles/g initial wet wt. and as μ moles/ml tissue water.

Medium NH_4Cl (mM)	QO_2 μmole/g initial wet wt.	Water Uptake μl/100mg initial wet wt.	Tissue Contents (μequiv/g)		Tissue Contents (μequiv/ml)	
			Na^+	K^+	Na^+	K^+
A) Medium adjusted						
Nil	132 ± 1	40.2 ± 0.4	91.2 ± 0.3	107.3 ± 1.3	75.6	89.3
5	134 ± 2	37.0 ± 3.2	98.4 ± 1.9	99.3 ± 1.7	84.1	84.9
10	124 ± 1	39.8 ± 4.0	101.3 ± 6.0	91.5 ± 6.7	84.6	76.4
B) Medium not adjusted						
Nil	134 ± 2	35.8 ± 1.4	135.5 ± 1.2	120.0 ± 4.0	117.0	103.6
5	135 ± 4	28.9 ± 2.3	137.3 ± 1.4	102.6 ± 5.8	126.1	94.2
10	141 ± 5	36.3 ± 0.7	153.9 ± 3.1	97.9 ± 2.6	132.3	84.2

(d) In a medium containing 30 mM KCl, adjusted for Na^+ (i.e., medium Na^+/K^+ = 123/30 = 4.1) tissue K^+ loss due to 10 mM NH_4Cl = (71.7 - 55.5 - 10) = 6.2 μequiv/ml (Table 68).

(e) In a medium containing 55 mM KCl, adjusted for Na^+ (i.e., medium Na^+/K^+ = 98/55 = 1.8) tissue K^+ loss due to 10 mM NH_4Cl = (89.3 - 76.4 - 10) = 2.9 μequiv/ml (Table 69).

It is to be noted that an increased medium K^+, with a diminished medium Na^+, diminishes the effects of NH_4^+ on the loss of K^+ from the tissue. However, only small, if any, accompanying diminution in the tissue Na^+ contents occurs under these conditions.

It is evident that sufficient loss of tissue K^+, brought about by increasing medium NH_4^+ concentrations, may impair enzyme systems (e.g., pyruvate kinase, acetyl thio kinase (262)) that are normally stimulated by K^+. If these reactions mediated are involved in energy-yielding processes, loss of tissue K^+ and gain of Na^+ may be directly due to impairment of the sodium pump which is ATP dependent. NH_4^+ may also lower ATP levels (Table 71) by its utilization for glutamine synthesis (Table 13). Moreover, utilization of NH_4^+ by reductive amination of α-ketoglutarate (to form L-glutamate) may cause diminished activity of the citric acid cycle, owing to removal of α-ketoglutarate, and hence a diminished rate of ATP formation.

9.10 Effects of increasing Na-L-glutamate concentrations on water, Na^+, and K^+ contents of incubated rat cerebral cortex slices.

Experiments were carried out to compare the effects of L-glutamate on cationic fluxes with those of NH_4^+. Rat brain cortex slices were incubated in O_2 at 37°C for one hour in Krebs-Ringer phosphate glucose medium containing increasing concentrations of monosodium-L-glutamate. Medium NaCl was adjusted so that medium Na^+ ion concentration (with added Na-L-glutamate) was maintained at 148 μequiv/ml. Results given in Table 70 show the

TABLE 70. Effects of increasing sodium L-glutamate concentrations on oxygen and water uptakes, and Na^+, K^+ contents of incubated rat brain cortex slices.

Rat brain cortex slices were incubated in O_2 at 37°C for one hour in Krebs-Ringer phosphate glucose medium containing increasing concentrations of sodium L-glutamate. Medium Na^+ ion concentration was adjusted to 148μequiv/ml by appropriate reductions of medium NaCl. Tissue contents of Na^+ or K^+ at the end of the incubation are expressed as μequiv/g initial wet wt. tissue and as μequiv/ml tissue water. Oxygen uptake (QO_2) is expressed as μmole/g initial wet wt. and water uptake as μl/100 mg initial wet wt.

Medium L-glutamate (mM)	QO_2	Water Uptake	Tissue Contents					
			(μequiv/g)			(μequiv/ml)		
			Na^+	K^+	Total	Na^+	K^+	Total
Nil	105 ± 1	13.6 ± 1.5	110.4 ± 1.2	58.3 ± 2.4	168.7	117.9	62.3	180.2
2.5	112 ± 5	28.2 ± 2.8	129.5 ± 2.0	56.1 ± 4.7	185.6	119.7	51.8	171.5
5.0	109 ± 2	29.4 ± 1.6	132.6 ± 4.1	52.5 ± 5.0	185.1	121.2	48.0	169.2
10.0	105 ± 3	39.4 ± 1.0	149.2 ± 2.4	46.7 ± 2.5	195.9	125.0	39.1	164.1
20.0	103 ± 2	40.5 ± 3.4	158.0 ± 9.0	39.0 ± 3.9	197.0	131.0	32.4	163.4
30.0	104 ± 4	38.8 ± 2.1	159.7 ± 8.3	36.8 ± 3.0	196.5	134.4	31.0	164.4

well known increase in the tissue Na^+ and water contents due to the presence of external L-glutamate. There is only a small diminution in the tissue K^+ content (expressed in terms of μequiv/g initial wet wt) with low external glutamate concentrations, but on the basis of μequiv/ml tissue water, the tissue K^+ content is diluted further. L-glutamate is more effective than NH_4^+ for equivalent concentrations (Table 58) in bringing about increased water uptakes. The tissue content of Na^+ expressed in terms of μequiv/ml tissue water, is greater with external NH_4Cl than with external L-glutamate at equivalent concentrations.

An important fact is the fall in the tissue K^+ contents that occurs with relatively high external concentrations of L-glutamate, but which reaches a limit with 20 mM L-glutamate in the incubation medium (Table 70). The oxygen consumption is unaffected under these conditions. This concentration of tissue K^+ (i.e., 30 μ equiv/ml tissue water) is attained with 10 mM NH_4Cl. However, further substantial reductions in the tissue K^+ can be attained by increased external concentrations of NH_4Cl and these are accompanied by diminution in the rates of oxygen uptakes (Table 58).

We have already indicated that the uptake of L-glutamate occurs largely in the glial cells of the brain tissue (Chapter 5). It seems reasonable to conclude that much of the cationic changes and water uptake will also occur in these cells accompanying the glial utilization of ATP for glutamate uptake and glutamine synthesis (Chapters 4 and 5). It may be noted that these fluxes due to glutamate are but little affected by TTX (Tables 72 and 73).

9.11 Effects of increasing concentration of NH_4^+ on ATP contents of rat cerebral cortex slices.

Results given in Table 71 show that the ATP levels of rat brain cortex slices incubated in O_2 at 37°C for one hour in Krebs-Ringer phosphate glucose medium are diminished with increasing medium concentrations of NH_4Cl.

TABLE 71. Effects of increasing NH_4^+ on ATP concentrations of incubated rat brain cortex slices.

Amounts of ATP (μmole/g initial wet wt.) in rat brain cortex slices incubated in O_2 at 37°C for one hour in Krebs-Ringer phosphate glucose medium with increasing concentrations of NH_4Cl.

Medium NH_4Cl mM	Tissue ATP Content
Nil	1.69 ± 0.02
2.0	1.50 ± 0.01
5.0	1.48 ± 0.05
10.0	1.33 ± 0.06
20.0	0.84 ± 0.05
30.0	0.60 ± 0.09
Initial (prior to incubation)	0.82 ± 0.02

TABLE 71A. ATP concentrations of incubated rat brain cortex slices under a variety of media conditions.

ATP contents (recorded below) of rat brain cortex slices incubated for one hour are taken from the published data of Okamoto and Quastel (115) and are expressed as μmole/g initial wet wt.

Additions to Krebs-Ringer phosphate medium		ATP Content
Glucose 10mM (Control)		1.63 ± 0.03
+ sodium-L-glutamate	0.5mM	1.66 ± 0.06
	2.0mM	1.47 ± 0.07
	5.0mM	1.32 ± 0.04
	10.0mM	1.05 ± 0.11
+ 2,4 dinitrophenol	0.01mM	1.26 ± 0.02
	0.1mM	0.62 ± 0.02
+ sodium malonate	2mM	1.13 ± 0.04
+ sodium D-glutamate	10mM	0.75 ± 0.02
+ ouabain	0.1mM	1.35 ± 0.04
+ KCl	100mM	1.20 ± 0.05
K^+-free medium		0.91 ± 0.09
Glucose free medium		0.59 ± 0.04
+ sodium-L-glutamate	10mM	0.74 ± 0.02
Infat rat (2-day old)	None	0.92 ± 0.02
	+ glucose	2.49 ± 0.04
Electrical pulses (10mM glucose)	None	1.57 ± 0.03
	4V	1.05 ± 0.06

Accompanying the diminished tissue ATP contents is an enhanced water uptake seen with 10, 20 and 30 mM NH_4Cl and a diminished rate of oxygen consumption at 20 or 30 mM NH_4Cl (Table 58).

ATP levels *in vivo* (about 2 μmole/g (282)) are known to fall following decapitation of the animal, but are regained on incubation in O_2 in the presence of glucose (128). In agreement with this observation, it is seen (Table 71) that the tissue ATP content of 0.82 μmole/g is enhanced to 1.69 μmole/g on incubation in O_2 for one hour in the presence of glucose.

ATP contents of rat brain cortex slices incubated in a variety of media are given in Table 71A. These values have a bearing on the results of this thesis, and are taken from the publication of Okamoto and Quastel (115), whose work was carried out in this laboratory under essentially similar incubation conditions to those described here.

9.12 Effects of neurotropic drugs on the oxygen and water uptakes, and Na^+, K^+ fluxes of rat brain cortex slices incubated in the presence of NH_4Cl or of Na-L-glutamate.

Rat brain cortex slices were incubated in Krebs-Ringer phosphate glucose medium in O_2 at 37°C for one hour, with or without the addition of the following substances: TTX (3 μM); protoveratrine (5 μM); ouabain (0.1 mM); NH_4Cl (10 mM); and Na-L-glutamate (10 mM). Results given in Tables 72 and 73 are summarized below.

(i) Incubation in Ca^{++}-containing media (Table 72)

TTX has no effects on the oxygen or water uptakes, or Na^+, K^+ contents of brain slices incubated in a normal Krebs-Ringer medium. However, it suppresses the enhanced oxygen and water uptakes of protoveratrine stimulated brain cortex slices. These observations are in accord with those in published reports (182, 211-213). The increased K^+ efflux due to protoveratrine (182) is

TABLE 72. Effects of tetrodotoxin, protoveratrine, ouabain, NH_4Cl and sodium-L-glutamate on the oxygen and water uptakes, and the Na^+, K^+ contents of rat brain cortex slices incubated in a Ca^{++}-containing medium.

Rat brain cortex slices were incubated in O_2 at 37°C for one hour in Krebs-Ringer phosphate glucose medium, with or without the addition of TTX (3 μM), protoveratrine (5μM), ouabain (0.1mM), NH_4Cl (10mM) and sodium-L-glutamate (10mM). Tissue concentrations of Na^+ or K^+ at the end of the incubation are given as μequiv/g initial wet wt. tissue and as μequiv/ml tissue water. Oxygen uptake (QO_2) is expressed as μmole/g initial wet wt. tissue and water uptake as μl/100mg initial wet wt.

Additions to the medium	QO_2	Water Uptake	Tissue Contents			
			(μequiv/g)		(μequiv/ml)	
			Na^+	K^+	Na^+	K^+
Nil	101 ± 6	14.0 ± 1.5	109.3 ± 1.3	58.5 ± 3.8	116.3	62.2
+ TTX	100 ± 3	12.1 ± 2.9	107.5 ± 3.3	55.7 ± 2.5	116.7	60.5
+ protoveratrine	136 ± 4	19.5 ± 0.6	140.8 ± 3.8	38.4 ± 1.1	141.5	38.6
+ protoveratrine + TTX	99 ± 4	11.5 ± 0.9	116.5 ± 5.5	49.5 ± 1.3	127.3	54.1
+ NH_4Cl	107 ± 6	24.4 ± 1.6	135.0 ± 1.0	33.4 ± 2.7	129.3	32.0
+ NH_4Cl + TTX	104 ± 2	24.1 ± 2.0	140.7 ± 2.5	31.5 ± 1.2	134.8	30.1
+ protoveratrine + NH_4Cl	95 ± 5	29.8 ± 2.1	160.0 ± 6.6	21.3 ± 1.9	145.7	19.4
+ protoveratrine + NH_4Cl + TTX	101 ± 4	29.9 ± 1.4	150.0 ± 4.0	27.8 ± 0.4	136.5	25.3
+ L-glutamate	99 ± 5	39.4 ± 1.0	149.2 ± 2.4	46.7 ± 2.3	125.0	39.1
+ L-glutamate + TTX	98 ± 4	35.5 ± 4.1	142.0 ± 4.0	47.3 ± 3.9	122.9	41.0
+ ouabain	101 ± 3	38.0 ± 0.7	172.6 ± 2.1	23.5 ± 0.9	146.2	19.9
+ ouabain + TTX	97 ± 2	30.0 ± 1.0	136.6 ± 3.4	-	124.0	-
+ ouabain + NH_4Cl	86 ± 5	44.5 ± 1.0	182.7 ± 5.0	-	146.7	-
+ ouabain + NH_4Cl + TTX	85 ± 6	38.7 ± 0.9	170.0 ± 2.0	-	143.4	-

partially suppressed by the presence of TTX. This observation supports the view of Okamoto and Quastel (182) based on theoretical considerations.

The Na^+, or water, uptake due to 10 mM sodium-L-glutamate is not significantly reduced by TTX, confirming the observation of Okamoto and Quastel (182). There is also no effect of TTX on the efflux of K^+ due to the addition of L-glutamate to the incubation medium.

The increased water and Na^+ influx into, and efflux of K^+ from, brain cortex slices respiring in the presence of 10 mM NH_4Cl is unaffected by TTX. NH_4Cl further enhances the water and Na^+ influx into, and K^+ efflux from, the slices in the presence of protoveratrine.

A noteworthy effect of NH_4^+ is its inhibition of the protoveratrine stimulated respiration of brain cortex slices. A similar inhibitory effect of NH_4^+ on electrically stimulated respiration was observed by Nakazawa and Quastel (114).

The effects of TTX in enhancing tissue K^+, and diminishing tissue Na^+, contents of protoveratrine-stimulated slices, are much reduced in the presence of external NH_4Cl. Similarly, the TTX-sensitive component of ouabain-induced Na^+ influx into brain slices seems to be greatly diminished in the presence of NH_4Cl.

(ii) Incubation in Ca^{++}-free media (Table 73).

In accordance with the report of Chan and Quastel (197), TTX suppresses the enhanced respiration of cerebral cortex slices brought about by the omission of Ca^{++} from the incubation medium. However, the increased uptake of Na^+ into, and release of K^+ from, brain cortex slices seem not to be affected by the presence of TTX. It is possible that there is a TTX-sensitive component of the cationic fluxes due to the absence of Ca^{++}, for the following reasons: TTX in the absence of Ca^{++}

TABLE 73. Effects of tetrodotoxin, protoveratrine, NH_4Cl and sodium-L-glutamate on the oxygen and water uptakes, and the Na^+, K^+ contents of rat brain cortex slices incubated in a Ca^{++}-free medium.

Rat brain cortex slices were incubated in O_2 at 37°C for one hour in Krebs-Ringer phosphate glucose medium devoid of Ca^{++}, with or without the addition of TTX (3 μM), protoveratrine (5 μM), NH_4Cl (10mM) and sodium-L-glutamate (10mM). Tissue concentrations of Na^+ or K^+ at the end of the incubation are given as μ equiv/g initial wet wt. tissue and as μ equiv/ml tissue water. Oxygen uptake (QO_2) is expressed as μmole/g initial wet wt. tissue and water uptake as μl/100mg initial wet wt.

Additions to the medium	QO_2	Water Uptake	Tissue Contents			
			(μequiv/g)		(μequiv/ml)	
			Na^+	K^+	Na^+	K^+
Nil	131 ± 3	18.0 ± 2.0	136.6 ± 3.3	37.4 ± 4.9	139.4	38.2
+ TTX	112 ± 3	17.2 ± 1.5	135.0 ± 3.0	37.4 ± 3.1	138.9	38.5
+ protoveratrine	149 ± 4	25.5 ± 2.5	160.0 ± 5.1	23.5 ± 0.9	151.7	22.3
+ protoveratrine + TTX	121 ± 3	21.6 ± 1.9	149.1 ± 8.0	26.6 ± 1.9	146.8	26.2
+ NH_4Cl	123 ± 3	31.2 ± 2.6	156.3 ± 4.2	21.0 ± 1.7	140.6	18.9
+ NH_4Cl + TTX	114 ± 2	25.9 ± 1.1	150.7 ± 4.4	20.7 ± 1.2	142.0	19.5
+ protoveratrine + NH_4Cl	116 ± 2	38.3 ± 3.0	181.6 ± 6.6	16.2 ± 0.5	153.5	13.6
+ protoveratrine + NH_4Cl + TTX	127 ± 4	24.5 ± 1.0	146.7 ± 7.7	27.1 ± 3.6	140.4	25.9
+ L-glutamate	126 ± 4	41.5 ± 3.1	169.3 ± 4.3	32.0 ± 0.8	139.3	26.3
+ L-glutamate + TTX	121 ± 2	37.3 ± 1.6	169.3 ± 1.3	31.9 ± 1.5	144.5	27.2

(a) suppresses the increased respiration;

(b) inhibits the enhanced release of amino acids (Chapter 7);

(c) partially reverses the fall in the ATP contents of brain cortex slices (Table 74).

These results would indicate that TTX may affect cationic fluxes in the neurons due to Ca^{++}-lack. The fact that there is no apparent change in the Na^+ and K^+ contents of the tissue may perhaps be due to changes in glial contents of Na^+ and K^+ that balance those in the neurons. This is a matter for further investigation.

The increased Na^+ and water uptakes, and release of K^+ from the tissue occurring in the presence of external NH_4^+, are unaffected by TTX. These results are similar to those obtained with a Ca^{++}-containing medium (Table 72).

Protoveratrine further enhances the oxygen, water and Na^+ uptakes, and K^+ release, from the tissue due to the absence of Ca^{++}. Whereas TTX inhibits the tissue respiration under these conditions, it has only small effects on the Na^+ or K^+ contents of the tissue.

TTX has no effects on the changes in water, Na^+ and K^+ contents of the tissue induced by external L-glutamate in the absence of Ca^{++}. These results are similar to those obtained in the presence of Ca^{++} (Table 72).

9.13 Effects of neurotropic drugs on the ATP contents of incubated cerebral cortex slices.

It is seen from Table 74 that the protoveratrine-stimulated respiration of brain cortex slices is accompanied by a fall in tissue ATP content. A similar diminution in the ATP content of incubated brain tissue occurs during electrical stimulation (Table 71A). Under these conditions, changes in the cationic fluxes at the neuronal cell membrane result in stimulation of the activity of the ATP-requiring sodium pump, with an increased rate of ADP formation. Mitochondrial respiration and glucose utilization are consequently enhanced. If this view is correct, then the inhibition by TTX, of the enhanced Na^+ influx into, and K^+ efflux from, protoveratrine-stimulated slices should result in a diminution in the enhanced activity of the sodium pump and thereby reduce the rate at which ATP is utilized. Experiment shows that TTX enhances the content of ATP in protoveratrine incubated slices (Table 74).

The increased activity of the sodium pump due to protoveratrine is diminished by ouabain, a well known inhibitor of the sodium pump. Ouabain (0.1 mM) consequently brings about a considerable diminution (48%) in the protoveratrine-stimulated rate of respiration (Table 72). The addition of NH_4^+ to protoveratrine-stimulated brain cortex slices results in a diminution of their ATP contents (Table 74). The ATP content falls, in fact, so low that it may become rate limiting for the initial phosphorylation of glucose (see reference 205), necessary for its further oxidation.

9.14 Effects of sodium fluoroacetate, sodium malonate, tetrodotoxin and NH_4Cl on the Na^+, K^+ and water contents of rat brain cortex slices incubated in the presence of glucose.

Results given in Table 75, show that sodium fluoroacetate (1 and 3 mM) enhances the Na^+, and diminishes the K^+, contents of rat brain cortex slices incubated in O_2 at 37°C for one hour in Krebs-Ringer phosphate glucose

TABLE 74. Effects of NH_4^+, protoveratrine, Ca^{++}-lack and tetrodotoxin on the ATP contents of incubated rat brain cortex slices.

Rat brain cortex slices were incubated in O_2 at 37°C for one hour in Krebs-Ringer phosphate glucose medium in the presence or absence of protoveratrine (5 μM), tetrodotoxin (3 μM) and NH_4Cl (10mM). Oxygen consumption (QO_2) is expressed as μmole/g initial wet wt. and water uptake as μl/100 mg initial wet wt.

Additions to the incubation medium	QO_2	Water Uptake	Tissue ATP Content μmole/g initial wet wt.
Control	102 ± 4	17.1 ± 0.3	1.64 ± 0.03
+ tetrodotoxin	104 ± 3	16.2 ± 0.4	1.58 ± 0.03
+ protoveratrine	139 ± 3	20.0 ± 0.2	1.02 ± 0.04
+ protoveratrine) + tetrodotoxin)	105 ± 2	12.1 ± 1.7	1.36 ± 0.02
+ protoveratrine) + NH_4Cl)	100 ± 2	34.2 ± 0.5	0.70 ± 0.02
Ca^{++}-free medium	136 ± 2	18.9 ± 1.5	0.99 ± 0.04
+ tetrodotoxin	117 ± 2	17.2 ± 1.0	1.12 ± 0.02

TABLE 75. Effects of sodium fluoroacetate, sodium malonate, tetrodotoxin and NH_4Cl on the oxygen and water uptakes, Na^+ and K^+ contents of incubated rat brain cortex slices.

Rat brain cortex slices were incubated in O_2 at 37°C for one hour in Krebs-Ringer phosphate glucose medium with or without sodium fluoroacetate, sodium malonate, NH_4Cl and tetrodotoxin (TTX).

Additions to the incubation medium	Oxygen Uptake µmole/g initial wet wt.	Water Uptake µl/100mg initial wet wt.	Tissue Contents µequiv/g initial wet wt.	
			Na^+	K^+
Nil	100 ± 5	14.2 ± 0.4	110.4 ± 1.5	58.1 ± 3.0
+ NH_4Cl (10mM)	104 ± 4	25.5 ± 1.1	138.8 ± 2.3	29.8 ± 2.0
+ sodium fluoroacetate (1mM)	97 ± 5	16.5 ± 1.7	121.5 ± 4.0	49.6 ± 0.2
+ sodium fluoroacetate (1mM) + NH_4Cl (10mM)	102 ± 2	25.6 ± 4.6	153.0 ± 3.1	27.9 ± 0.2
+ sodium fluoroacetate (3mM)	95 ± 1	18.3 ± 1.0	130.8 ± 4.6	51.4 ± 5.2
+ sodium fluoroacetate (3mM) + TTX (3µM)	95 ± 4	15.2 ± 0.6	127.5 ± 3.9	51.5 ± 6.2
+ sodium malonate (2mM)	73 ± 2	20.5 ± 0.1	139.1 ± 2.5	40.7 ± 2.1
+ sodium malonate (2mM) + NH_4Cl (10mM)	64 ± 5	34.2 ± 2.1	169.2 ± 1.6	24.1 ± 1.4
+ sodium malonate (2mM) + TTX (3µM)	75 ± 2	16.6 ± 0.2	130.2 ± 3.3	42.9 ± 0.8

medium. These effects are TTX-insensitive. The tissue content of water is only slightly enhanced by fluoroacetate. Sodium malonate (2 mM) has quantitatively larger effects on the Na^+, K^+ and water contents of the incubated slices; these effects of malonate are also TTX-insensitive.

Addition of NH_4Cl (10 mM) potentiates the effects of both fluoroacetate and malonate.

9.15 Summary

1. Increasing medium NH_4^+ enhances the water and Na^+ contents and diminishes the K^+ and ATP contents of incubated adult rat brain cortex slices. NH_4^+ ions replace K^+ in activating the Na^+, K^+-ATPase of brain cortex homogenates.

2. Unlike infant rat brain cortex slices, those of the adult rat lose more K^+ than can be accounted for by a 1:1 exchange of K^+ for NH_4^+.

3. A time lag in the occurrence of the effects of NH_4^+ on the cationic contents and water uptake by incubated adult rat brain cortex slices rules out the concept of an 'uncoupling' of active transport process from Na^+, K^+-ATPase activity. Presumably, such effects of NH_4^+ are the outcome of interference with energy yielding processes in incubated brain slices.

4. Diminishing medium Na^+/K^+ concentration ratios (by increasing external K^+) partly inhibits the NH_4^+ induced release of K^+ from the tissue, with little or no effect on the NH_4^+ induced Na^+ uptake.

5. Unlike NH_4Cl, $(CH_3)_4NCl$ and the chloride salts of Li^+, Rb^+ and Cs^+ do not affect the Na^+ and water contents of incubated cortex slices.

6. Increasing medium NH_4Cl and sodium L-glutamate concentrations differ in their effects on the Na^+, K^+ fluxes, and oxygen and water uptakes by incubated brain cortex slices.

7. Changes in the medium concentration of Na^+ (between 98 and 148 μequiv/ml) and of K^+ (between 5 and 105 μequiv/ml) have little or no effect on the tissue to medium concentration ratio for Na^+ (T/M = 0.79).

8. Omission of Ca^{++} and/or of K^+ from the incubation medium enhances the tissue Na^+ concentration and diminishes that of K^+. TTX inhibits the increase respiration due to the absence of Ca^{++} with little change in the cationic contents of the tissue.

9. TTX has little or no effect on the enhanced tissue contents of Na^+ and diminished tissue contents of K^+ brought about by the presence of NH_4^+, or of sodium malonate, or of sodium fluoroacetate, or of sodium L-glutamate. Presumably, such changes in the tissue cationic concentrations are not the outcome of an activation of the Na^+-current system at the brain cell membrane.

10. NH_4 inhibits the protoveratrine stimulated respiration of brain cortex slices. It enhances the Na^+ content of the tissue and diminishes the K^+ and ATP contents.

11. The diminished ATP content of the protoveratrine-stimulated slices is partially reversed by TTX. This effect of TTX is presumably due to a reduced utilization of ATP accompanying a diminished activity of the sodium pump as a result of a reduced sodium concentration in the neurons.

10. DISCUSSION

10.1 Ammonia formation in rat brain cortex slices

The results recorded in Chapter 3 show that changed ammonium ion contents in incubated rat brain cortex slices are associated with, or directly due to, accompanying changed contents of brain amino acids (Tables 2A and 3A). For example, in an aerobic incubation of one and four hours' duration, it is seen that the fall in NH_4^+ concentration due to glucose metabolism is about equal to the rise in concentration of glutamate and glutamine minus the fall in aspartate concentration (Table 2A). We have already reported (62, 65) that cerebral ammonia formation is intimately associated with amino acid metabolism and occurs aerobically, in a glucose free medium, largely as a result of terminal glutamate oxidation. Very recently, Weil-Malherbe and Gordon (102), using guinea pig brain cortex slices, have reported data in accord (qualitatively) with this view. We have carried out studies to throw further light on the processes governing ammonia formation in the isolated brain.

(i) Endogenous glutamate — the possible major source of aerobic ammonia formation.

When rat brain cortex slices are incubated in a glucose-free medium, the initial tissue concentrations of most free amino acids increase with the time of incubation. However, the glutamate (and glutamine) contents are diminished concomitant with the enhanced output of ammonia (Table 2). In the presence of glucose, endogenous glutamate oxidation is largely diminished and the rate of ammonia formation is suppressed (Table 2). Moreover, the diminished rate of glutamate oxidation in incubated brain cortex slices, occurring either in the absence of oxygen (Table 3), or in the presence of metabolic inhibitors such as amytal (1 mM), or DNP (0.1 mM) (Table 6) results in a diminution in the rate of ammonia formation. The conclusion that oxidation

of endogenous glutamate is necessary for supporting high rates of ammonia formation is supported by the fact that ouabain inhibits cerebral ammonia liberation. Ouabain (1 mM), by suppressing re-uptake processes, releases endogenous tissue glutamate to the incubation medium, thus making it unavailable for oxidation and hence for ammonia liberation (Table 7). There is also a diminished rate of cerebral ammonia liberation when endogenous glutamate oxidation is specifically inhibited by D-glutamate (62, 231). An inhibition of the rates of oxygen consumption accompanies the suppressed rates of ammonia formation under these glucose-free conditions. Furthermore, in incubated infant rat brain cortex slices in which there are low concentrations of glutamate (Tables 2 and 4), low activities of the relevant enzymes dealing with glutamate metabolism (15, 163), and a low rate of operation of the citric acid cycle (235), the rate of ammonia formation is correspondingly low.

It is therefore evident from these considerations that aerobic ammonia formation in brain cortex slices incubated in a glucose-free medium is largely dependent on terminal glutamate oxidation.

(ii) Exogenous L-glutamate — as a possible source of cerebral ammonia.

Although endogenous glutamate oxidation seems to be largely responsible for the aerobic formation of ammonia in vitro (section 10.1 (i)), the addition of L-glutamate (2.5 mM) to respiring rat brain cortex slices in glucose-free media, inhibits rather than enhances the rate of ammonia liberation (58, 103, Table 8). This is accompanied by an enhanced rate of glutamine formation. However, we find that when Ca^{++} is omitted from the incubation medium there seems to be a small (2 μmole/g/hour) but significant enhancement of the rate of ammonia formation together with a decreased rate of glutamine synthesis (Table 8). There is a still further increase in the rate of ammonia formation of about 2 μmole/g/hour in the presence of TTX (2μM) in a Ca^{++}-free medium. The action of TTX in increasing the tissue content of glutamate in rat brain

cortex slices incubated in Ca^{++}-free, glucose-free, medium containing 2.5 mM L-glutamate (Table 40), indicates that some of the exogenous L-glutamate taken up by the tissue, under these conditions enters the neurons. The bulk of the exogenous L-glutamate taken up by the tissue in a glucose medium enters by a TTX-insensitive process (Chapter 5). The bulk of the endogenous glutamate of brain tissue, however, is located in brain cells that are affected by TTX (Chapter 8). It seems likely, therefore, that much of the external L-glutamate taken up by the tissue is not located at the site of ammonia formation.

(iii) Glutamine – a source of ammonia in brain.

As mentioned earlier, the initial glutamine concentration of rat brain cortex slices is diminished on incubation in the absence of glucose. The fall in glutamine concentration of incubated brain tissue also occurs in the absence of oxygen (Table 3), or in the presence of 1 mM amytal or 0.1 mM DNP (Table 6), or of 0.1 mM or 1.0 mM ouabain (Table 7). As ammonia is still formed under conditions of suppressed glutamate oxidation (Tables 3, 6 and 7), it is possible that a portion of the ammonia liberated is caused by glutamine breakdown.

Synthesis of glutamine in the brain partly accounts for the inhibitory effect of glucose on ammonia formation (Table 2). The liberation of ammonia caused by suppression of glutamine synthesis in glucose containing media, by the presence of 5 mM methionine sulfoximine, 0.1 mM DNP, 1 mM fluoroacetate, or 0.1 mM ouabain (Table 14), indicates that ammonia normally produced in the brain, is largely removed by its utilization in the synthesis of glutamine. The view that, aerobically, ammonia is formed continuously in brain cortex slices incubated in a glucose-containing medium, is supported by the fact that amytal, by suppressing NADH oxidation, blocks the oxidation of endogenous glutamate, thus diminishing the liberation of ammonia. Consequently, the cerebral concentrations of glutamate and GABA are enhanced, and that of

glutamine diminished (Table 14). The diminution in the rate of glutamine synthesis is greater than that corresponding to the accompanying liberation of ammonia (Table 14). Presumably, this is simply because the formation of ammonia by endogenous glutamate oxidation precedes its utilization by the synthesis of glutamine.

Endogenous glutamine seems to be a relatively good source of ammonia in incubated infant rat brain cortex slices (Table 4) or synaptosomes prepared from brain cortex of the adult rat (Table 11). However, under both these conditions, glucose has little effect in enhancing the total (tissue + medium) concentration of glutamine.

Externally added L-glutamine supports high rates of ammonia formation in brain cortex slices, especially in the absence of glucose (62), where glutamine resynthesis is impaired, presumably due to low concentrations of ATP (Table 71A).

It is therefore evident that either endogenous or exogenous L-glutamine can serve as a source of ammonia in incubated rat brain slices.

(iv) Mechanism of ammonia formation in brain <u>in vitro.</u>

(a) Reactions in the absence of glucose

It is well known that, in the absence of glucose, the concentration of ATP in incubated brain tissue falls (Table 71A). This is partly due to deprivation of readily oxidizable substrate, and possibly partly due to the enhanced NAD^+/NADH concentration ratio. Ammonia may be liberated from glutamate under these conditions by the NAD^+ requiring glutamate dehydrogenase reaction (Reaction a) with the accompanying formation of α-ketoglutarate. Glutamate concentration falls (Table 2), α-ketoglutarate enters the citric acid cycle and forms oxaloacetate. Oxaloacetate may accumulate under these conditions, due to the depleted concentration of acetyl-CoA in the absence of glucose and lead

to an enhanced aspartate formation (Table 2) by transamination (Reaction b). Aspartate itself appears to play no major role in ammonia formation (Section 3.4).

Ammonia may also be liberated by glutaminase (Reaction c). The glutamine concentration falls (Table 2), and glutamate formed in the reaction undergoes further degradation to give α-ketoglutarate and ammonia. As ammonia formed directly in reactions a and c, and indirectly

a. $\text{Glutamate} \xrightarrow{\text{NAD}^+} \alpha\text{-ketoglutarate} + \text{NH}_4^+$

b. $\text{Glutamate} + \text{oxaloacetate} \longleftrightarrow \alpha\text{-ketoglutarate} + \text{aspartate}$

c. $\text{Glutamine} \longrightarrow \text{Glutamate} + \text{NH}_4^+$

in reaction b, cannot be utilized at its normal rate (i.e., as in presence of glucose), due to the falls in the concentrations of pyruvate, of α-ketoglutarate, and of ATP, it accumulates and diffuses into the incubation medium (62, 65, Table 22). Aerobic ammonia formation is in this manner dependent on the NAD^+-dependent oxidation of glutamate and α-ketoglutarate. When NADH oxidation is impaired, as under anoxia or in the presence of amytal, glutamate oxidation (Tables 3, 6, and 14), and the operation of the citric acid cycle, are also impaired. Suppression of ammonia formation and of oxygen consumption follows (Chapter 3).

Aerobic incubation of rat brain cortex slices in a medium devoid of glucose has marked effects on the contents of amino acids, both in the tissue and in the incubation medium. There are considerable increases in the tissue and medium contents of aspartate, and falls in the tissue contents of glutamate, GABA, glutamine and glycine (Tables 36 and 37). Precisely similar results have been obtained in the brains of rats suffering from insulin hypoglycemia (288-290), or in rat brains perfused with a glucose-free saline medium (291). The results, therefore, obtained with incubated rat brain cortex slices, reflect,

at least qualitatively, the results obtained with rat brain in vivo, so far as brain amino acids are concerned.

(b) Reactions in the presence of glucose

Glucose, by its generation of pyruvate and of acetyl-CoA, under aerobic conditions, brings about increased rates of formation of α-ketoglutarate (315) and high energy compounds such as ATP (Tables 71 and 71A). The NADH/NAD^+ concentration ratio in the brain tissue is also relatively high. These conditions favour the formation of glutamate from α-ketoglutarate by reductive amination with ammonia, and also by transamination, e.g., with aspartate. They also favour the utilization of ammonia by ATP-dependent glutamine synthetase. These conclusions are supported by the facts that the addition of glucose to respiring brain cortex slices suppresses aspartate formation and ammonia liberation, and at the same time maintains glutamate and glutamine concentrations considerably higher than those obtained in brain slices incubated in a glucose-free medium (Table 2).

In glucose-containing medium the high concentrations of endogenous amino acids are maintained against large concentration gradients. The amino acid concentration ratios (tissue to medium) are in the order: GABA > glutamate > aspartate > glycine > glutamine (Table 42). The retention of GABA by the tissue is greatest while that of glutamine is the lowest. These results are in accord with the recent observations of Balazs and co-workers (116). It may be added that the ratios observed in vitro are apparently similar to those found in vivo, i.e., to the ratios of the concentration of amino acids in the brain to those in the cerebrospinal fluid (116).

(v) Neurons – the possible site of aerobic ammonia formation.

The brain tissue content of ammonia is little affected by the incubation of brain slices in the presence or absence of glucose, the large increase of ammonia due to the absence of glucose being found only in the incubation medium (Table 22). Inhibiting the synthesis of glutamine in a glucose medium (by

methionine sulfoximine, ouabain, fluoroacetate or DNP (Table 14)) enhances the liberation of ammonia by brain slices, but the tissue content of ammonia is little affected (Table 22). Moreover, under these conditions the tissue content of glutamate is also but little affected (Table 14). In an earlier presentation (62, 65) we have suggested that ammonia formation by glutamate oxidation takes place largely in a specific compartment of the brain from which it rapidly diffuses after attaining a critical concentration there. Evidence now indicates that the neurons may be the major site, or compartment, of ammonia formation. Some of the evidence for this view is as follows.

1. Location of endogenous glutamate. Using TTX, we have found that more than half of the endogenous tissue glutamate is present in the neuronal compartment (Chapter 8). Since we have shown that aerobic ammonia formation is dependent on endogenous glutamate oxidation (Section 10.1 (i)), the site of the major pool of endogenous glutamate is also likely to be the location of aerobic ammonia formation. Enzyme activities required for glutamate oxidation seem to be relatively higher in the neurons than in the glia (274, 276, 277). For example, Pope and co-workers (274) working with cerebral cortex of the rat, conclude that cytochrome oxidase and NAD^+ are localized mainly in the bodies and dendrites of neurons. Lowry and co-workers (277) give evidence indicating that brain respiratory metabolism occurs in the neurons and takes place largely in the dendrites. According to Rose (305), cytochrome oxidase is 80 per cent, and glutamate dehydrogenase is four times more active in the neurons than in the glia. According to Hamberger (296), glutamate is oxidized at a high rate by nerve cells, but α-ketoglutarate is used at about the same rate by both neuronal and glial cells. The considerations lead to the conclusion that the major pool of glutamate and the activities of enzymes for glutamate oxidation occur in the neurons. These facts suggest

that neurons constitute the site at which the bulk of the aerobic ammonia is formed in the brain.

2. Synaptosomes (as part of the neurons) produce ammonia from endogenous glutamate and glutamine, but the rate of glutamine synthesis in the presence of glucose is relatively small or negligible (Table 11).

3. The inhibition of aerobic ammonia formation in the brain by 1 mM amytal, in the presence or absence of glucose (Tables 6 and 14), results in an enhanced glutamate and GABA level. The increase in concentration of GABA must lie in the neurons as glutamic acid decarboxylase is confined to the neurons (212, 213, 246). The increase in concentration of GABA is presumably due to the increased concentration of glutamate, as has been shown (Tables 20 and 21) that externally applied L-glutamate increases GABA levels, and also it is known that labelled glutamate leads to the formation of labelled GABA (63, 112). The increase of glutamate concentration in the neurons is evidently due to the suppression of its oxidation by amytal; it follows, therefore, that aerobic ammonia formation is due to neuronal glutamate oxidation.

4. Major compartment of oxygen consumption in brain tissue. Evidence (175, 264, 268-272) indicates that a major portion of oxygen consumed by brain cortex may be attributed to the neurons. Thus, Heller and Elliot (268) conclude that about 85 per cent of the respiration of brain cortex of the cat or dog is due to neurons. The data of Hess (269) shows that 90-95 per cent of the respiration of cortical tissue may be attributed to the neurons, the neurons respiring 16-50 times the rate of the glial cells. The data of Epstein and O'Connor (270) suggest that the respiration rate of glia is at least an order of magnitude lower than that of the neurons on a volume basis. Hertz (272) showed that on a fresh weight basis nerve cells respire at a rate 6.5 times that of

neuroglia. Korey and Orchen (271) concluded that 77 per cent of the respiration of lamb cortex is due to the neurons. These results imply that the rate of operation of the citric acid cycle in the neurons considerably exceeds that in the glia (volume for volume). This may account for the fact that the glutamate concentration (derived from α-ketoglutarate) is higher in the neurons than in the glia.

10.2 Tetrodotoxin-sensitive fluxes of amino acids in the brain in vitro.

The results recorded in Chapter 7 show that, under a variety of incubation conditions (the presence of ouabain, or of protoveratrine, particularly in a medium deficient in Ca^{2+}, or the absence of glucose), there is an increased release of a number of amino acids from rat brain cortex slices into the medium and that the amino acid concentration ratios between tissue and medium are diminished. The amino acids most markedly affected in this manner are glutamate, GABA, aspartate and glycine. Moreover, the processes involved in the increased efflux of these amino acids, or in bringing about diminished concentration ratios, have TTX-sensitive components (Table 42). As TTX sensitivity, according to electrophysiological evidence (190) is associated with the suppression of action potentials, it follows that the generation of action potentials or the activation of the Na^{+}-current system in incubated rat brain cortex slices is accompanied by the release, from the tissue, of glutamate, GABA, aspartate and glycine. This result has obvious physiological significance as all the amino acids in question are considered to possess transmitter properties in the central nervous system (170).

As the activation of the Na^{+}-current system is presumably confined to the electrically-excitable cells of the nervous system, i.e., the neurons, it follows that the TTX-sensitive efflux of amino acids is also confined to the neurons. It may, therefore, be concluded that the amino acids glutamate, GABA,

aspartate and glycine, released during the generation of action potentials, or the activation of the Na^+-current system, emanate from the neurons.

It has been pointed out by Gottesfeld and Elliott (186), that rat brain cortex slices loaded with GABA by prior incubation in the presence of this substance, lose this amino acid, on incubation in a GABA-free medium, at a rate which is accelerated by protoveratrine, and that the protoveratrine effect is abolished by TTX. Presumably, this phenomenon is analogous to that recorded above. It may, therefore, be inferred that exogenous GABA is taken up by the neurons and is released on the development of action potentials. Results by Bowery and Brown (285) support the conclusion that there is neuronal uptake of GABA.

The TTX-sensitive release of glycine (Tables 36, 38, 40), though small, is significant. Electrical stimulation seems not to affect the rate of glycine uptake (114), nor does it affect the release of glycine from brain cortex slices that have been allowed to take up glycine (286). However, these results may only reflect the fact that the rate of uptake of glycine keeps pace with its increased efflux. Processes of efflux and influx obviously control the actual levels of tissue amino acids under various incubation conditions (287).

Although either ouabain or protoveratrine alters the ionic concentration at the brain cell membrane (Table 72) in incubated rat brain slices, the responses of these two drugs towards TTX are very different. TTX brings about only a partial suppression of the activity of ouabain in stimulating amino acid efflux, or of Na^+ influx (182) into brain slices. It brings about, however, a complete abolition of the activity of protoveratrine with either of these processes. This phenomenon may be explained by the conclusion that protoveratrine, like electrical stimuli, acts only on the neurons, whereas ouabain affects both neurons and glia by suppression of Na^+-K^+-ATPase and its dependent amino acid uptake mechanism. If an amino acid, therefore, is concentrated for the most part in the glia, it will respond to ouabain but its efflux may have no TTX-sensitive component.

The results given above show that, in contrast to the fluxes of glutamate, GABA, aspartate and glycine, the efflux of glutamine is but little affected by incubation conditions associated with the TTX-sensitive alterations in the cationic concentrations at the cell membrane. Even with ouabain, which suppresses the synthesis of glutamine from glucose (112) and brings about a greatly diminished concentration ratio between tissue and medium after one hour incubation, the addition of TTX has no significant effect on this concentration ratio (Tables 16, 34, 35, 42). Such results lead to the conclusion that the main depot of glutamine in normal brain tissue lies not in the neurons but in the glia, a conclusion in accord with previous observations and suggestions (161, 162). It may be noted that synaptosomes form little or no glutamine in the presence of glucose (Table 11). This is in accord with the finding that glutamine synthetase activity appears not to be present in isolated nerve ending particles (245, 246).

Protoveratrine, when added to an incubation medium devoid of glucose (Tables 10, 36, 37), or of Ca^{++} (Tables 38, 39) brings about, in the incubated rat brain cortex slices, a significant increase in the total (tissue + medium) quantity of glutamine. The total quantities of the other amino acids investigated are not significantly changed. Moreover, the total quantity of ammonia liberated in the absence of glucose is reduced (Table 10). The protoveratrine stimulation of total glutamine content in the incubated brain is abolished by the presence of TTX. An explanation for this finding is that release of glutamate from the neurons by protoveratrine results in an increased concentration of glutamate in the intracellular space, with consequent increased uptake of glutamate by the glia and possibly, therefore, an increased amount of conversion, in the glia, of glutamate into glutamine. On this view, glial uptake of glutamate may represent a mechanism for the reduction of the extracellular concentration of glutamate released from neurons during excitation. This suggested movement of glutamate from neuron to glia during stimulation with protoveratrine, resembles the movement of K^+ that occurs under the same conditions (182).

Results obtained with 50 mM KCl in the incubation medium (Tables 45 and 46), indicate relatively small changes in the contents of glutamate and aspartate in rat brain cortex slices incubated in a glucose saline medium, but they show an increased efflux of GABA which is only partially suppressed by the addition of TTX. With 105 mM KCl, the efflux of GABA is little affected by the addition of TTX (Table 50). It has been reported (155, 156, 292) that the effect of increased K^+ is to accelerate the release of GABA from incubated brain tissue. Our results are in accordance with this finding.

When the concentration of K^+, however, is increased in a medium devoid of glucose, there occur considerable increases in the efflux of glutamate, aspartate, GABA and glycine (Tables 37 and 46), and these increased rates of release of the amino acids are but little affected by TTX (Table 46). The lack of effect of TTX may be expected because the large depolarization due to high concentrations of K^+ (177, 180, 261) should prevent the activation of the Na^+-current system or the generation of action potentials (293) and prevent any major effect of TTX. A possible explanation for the apparent K^+ stimulation of amino acid release is that there is a fall in the tissue ATP concentration (Table 71A), resulting in a further suppression of the amino acid uptake processes occurring in a glucose-free incubation medium.

Similarly, TTX has little or no effect on the release of amino acids brought about by the presence of 0.1 mM DNP, or 30 mM NH_4Cl (Table 50) in a glucose medium, presumably because the influx of Na^+ due to DNP (182), or NH_4Cl (Table 72), is also TTX-insensitive. The fall in the tissue ATP levels due to these substances (Tables 71 and 71A) may account for the enhanced release of amino acids from the brain slice.

Results obtained with lidocaine (0.5 mM) show that the TTX-sensitive release of amino acids from brain slices is also lidocaine-sensitive (Tables 34-41). This is to be expected since lidocaine, like TTX, is known to suppress the influx of Na^+ into nervous tissue (198) during excitation.

Amytal at its anesthetic concentration for the rat (0.25 mM) (309), exerts a diminishing effect on amino acid release (Table 44) only when it diminishes the Na^+ influx under certain incubation conditions (as, for example, in a Ca^{++}-free EGTA and protoveratrine-containing glucose medium (210)). However, its effects at 0.25 mM are not as large as those of TTX and lidocaine. Amytal does not diminish the release of amino acid from the tissue respiring in a glucose-free medium.

The release of amino acids by 0.1 mM ouabain from kidney cortex slices (Table 47), unlike that of brain, is unaffected by the presence of TTX. This points to a specific effect of TTX on the release of amino acids from excitable tissues.

10.3 Locations of amino acids in the brain.

Experiments carried out to identify the actual locations of glutamate and glutamine and other amino acids in incubated rat brain cortex slices, lead to the conclusion that the major pools of glutamate, aspartate, glycine, serine and probably GABA, are in the neurons. The major pool of glutamine, however, lies not in the neurons but must lie, therefore, in the glia. These results have been obtained using TTX as a means for the suppression of the increased neuronal efflux of amino acids brought about by the joint action of protoveratrine and ouabain. The results found with glutamate and aspartate definitely indicate that over 50 per cent of the tissue contents of these amino acids are located in the neurons. The inference as to the glutamine location depends on the observation that TTX has no retarding effect on the release of this amino acid under circumstances where it has significant retarding effects on the release of glutamate, aspartate, glycine and GABA.

This conclusion as to the glial location of the major pool of glutamine is supported by results obtained with the use of the citric acid cycle inhibitors, fluoroacetate (3 mM) and malonate (2 mM). It was found that, while malonate blocks the increased brain tissue respiration brought about by protoveratrine,

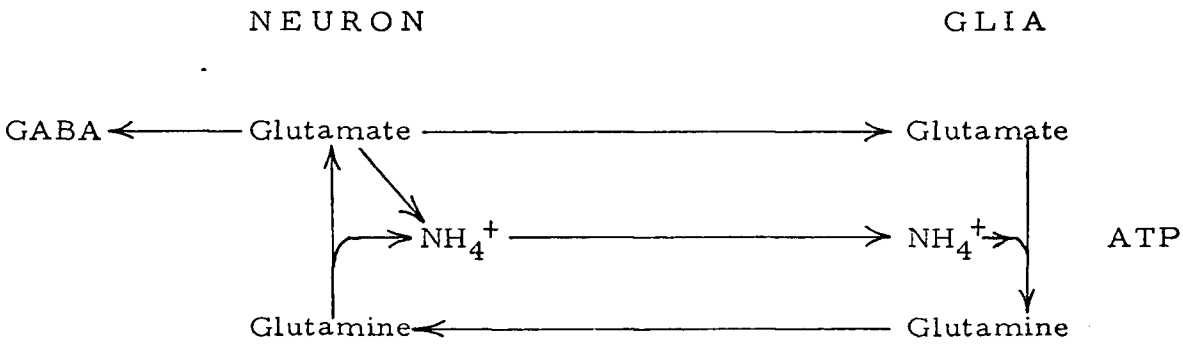
fluoroacetate has no such effect (Figure 2). The lack of action of fluoroacetate, at a concentration that suppresses cerebral glutamine synthesis, is consistent with the conclusion that it acts, at 3 mM, on the operation of the citric acid cycle in a compartment other than that in the neurons, i.e., in the glia. This conclusion is in accord with the findings of Clarke and co-workers (150), who concluded that the fluoroacetate effect is confined to the "small" pool in which glutamine synthesis takes place. Such a conclusion is further supported by the observation that malonate and fluoroacetate exert different effects on the contents of amino acids of rat brain cortex slices where respiration has been stimulated by the presence of protoveratrine. Thus, while fluoroacetate suppresses glutamine formation at the expense of glutamate, whose content in the tissue rises, malonate has no such action on these amino acids, but it suppresses the formation of aspartate. The malonate effect is explicable as it will block the cerebral conversion of succinate to aspartate (294). Its lack of action on glutamine biosynthesis, at the relatively small concentration necessary to suppress the protoveratrine stimulated respiration, is consistent with the conclusion that its main effect is in the stimulated neurons where it will have no significant effect on tissue glutamine formation if the major site of the latter operation is in the glia.

10.4 Neuron-glia interrelations.

The results recorded in this work lead to the conclusion that the neuron is the site of the major pool of glutamate in brain cortex slices, and that during the generation of action potentials glutamate is released from the neurons. Part of it is taken up by the glia and there converted to glutamine as the glia seem to be the site of the major pool of glutamine. Glutamine, however, is also released from the tissue by extracellular glutamate. The extracellular glutamine is taken up by the neurons. This is shown by the results in Table 56 which indicate that glutamine gives rise to an increased

formation of GABA. Glutamic acid decarboxylase, whose activity is responsible for the formation of GABA, seems to be confined to the neurons (246, 312, 313), so that an increase in tissue GABA on incubation with glutamine leads to the inference that glutamine can penetrate the neurons. Presumably the neuronal entry of glutamine is followed by hydrolysis, with release of ammonia (Table 56) and decarboxylation of glutamate. The fact that glutamine appears to be a more effective precursor of GABA than glutamate (Table 56) in incubated rat brain cortex slices indicates that hydrolysis of glutamine takes place inside the neuron rather than outside it (see also 143).

The facts, therefore, indicate a cycle of events in which neurons and glia are coupled to allow part of the glutamate, released from the neurons during excitation, to be withdrawn from the extra-neuronal space and to be returned to the neurons eventually in the form of glutamine. Ammonia, formed in the neurons by glutamine hydrolysis or by glutamate oxidation, on release from the neurons, enters the glia where it is utilized in the synthesis of glutamine. These movements of amino acids and NH_4^+ are shown in the scheme below.



Van den Berg and Garfinkel (314) have carried out a computer study of the possible locations, in the brain, of a total of 39 reactions involving 19 substances. Their model for this study assumes the existence of two compartments, a "large" and a "small", and it postulates a movement of glutamine from the "small" to the "large" compartment and the movement of GABA

in the reverse direction. This postulate is considered to account best for the experimental data. Our studies, using TTX, indicate that the actual location and site of formation of the "large" pool of glutamate is in the neurons and that of glutamine is in the glia. Moreover, they indicate the fact that transfer of glutamine takes place from the glia to the neurons, and that of glutamate in the reverse direction.

10.5 Control of ammonia utilization by glutamine synthesis in brain in vitro.

Glutamine synthetase isolated from brain, is unaffected by ouabain (112), but ouabain inhibits the synthesis of glutamine in rat brain cortex slices incubated in a physiological glucose saline medium (112, Table 12). There is a concomitant liberation of ammonia under these conditions (Table 22). This increase in ammonia is not due to a stimulation of cerebral glutaminase, since ouabain does not accelerate the rate of ammonia formation from L-glutamine by slices incubated in a glucose-free medium (Table 9). However, ouabain brings about profound changes in the intracellular cationic concentrations (182, Table 72) as a consequence of its inhibitory action on the Na^+-pump. Increased influx of Na^+ into, and efflux of K^+ from, rat brain cortex slices occurs under the influence of ouabain. We have found (Table 12) that increasing medium Na^+ concentration results in an inhibition of glutamine synthesis, and the absence of K^+ or of Ca^{++} (especially in presence of EGTA) has a similar effect. Absence of Ca^{++} also diminishes the rate of synthesis of glutamine from exogenous L-glutamate in a glucose-free medium (Table 8). It is therefore evident that, under conditions where there is an enhanced tissue Na^+/K^+ concentration ratio, the rate of glutamine synthesis is diminished. This increased ratio, found in presence of ouabain, is diminished by TTX (182) which, however, does not affect the depressed glutamine synthesis. This may be expected if glutamine biosynthesis is largely confined to glia which are not affected by TTX.

Reversal studies, carried out by the addition of glutamate and/or NH_4^+ to respiring brain slices in presence of ouabain (Table 13), led us to the conclusion that the control of glutamine synthesis is dependent on at least the following two factors.

1. The rate of transport of glutamate to the site of glutamine synthesis (i.e., in the glia). Ouabain is known to suppress active uptake of glutamate (104-106, Table 13). NH_4^+ is not actively accumulated by brain slices (Chapter 6) and ouabain has little or no effect on its transport (Table 31).

2. Active transport processes require ATP. So, also, does the synthesis of glutamine. The concentration of ATP in the glia must be the second factor controlling the rate of glutamine formation. It follows that inhibition of glutamine synthesis may occur under incubation conditions where the oxygen consumption by the tissue is little affected (as with ouabain or fluoroacetate), because the contribution of glia to the oxygen consumption of the brain slice is considered to be small (175, 264, 268-272). Moreover, as the ATP concentrations of glia, according to Cummins and Hyden (275), is relatively small, there may not be any considerable fall in the ATP concentrations of brain cortex slices under these conditions. Furthermore, a relatively large diminution in tissue ATP concentration may not necessarily be accompanied by a diminished rate of glutamine synthesis. For example, with malonate or protoveratrine, there is a large fall in the ATP content of incubated brain cortex slices (Tables 71A and 74), while glutamine synthesis is either little affected, as with malonate (Table 14), or is further enhanced, as with protoveratrine (Tables 38 and 39). This is simply because the glial ATP concentration does not necessarily vary with tissue ATP concentration and vice versa.

It is known that K^+ enhances (192, 262, 299, 300), while Na^+ diminishes (299, 301-303) the rate of conversion of phosphoenol pyruvate to pyruvate. Moreover, K^+ is known to accelerate the rate of acetyl-CoA formation in brain tissue (299). Therefore, an enhanced K^+/Na^+ concentration ratio may accelerate the operation of the citric acid cycle operating in the glia and, as a result, of an enhanced rate of ATP generation, enhances the rate of glutamine synthesis there. This would account for the observed effects of Na^+ and K^+ on the rate of cerebral glutamine synthesis. The enhanced glial K^+ in brain slices incubated in glucose saline medium containing protoveratrine (182) may be partly responsible for the enhanced synthesis of glutamine in presence of this drug (Tables 38 and 39).

10.6 Transport of NH_4^+ into brain cortex slices.

Contrary to a report in the literature (256, 257), we have found that NH_4^+ is not accumulated in rat brain cortex slices against a concentration gradient (Chapter 6). The level of ammonia in the incubated tissue is slightly greater than that in the incubation medium surrounding it (e.g., Table 24), but the difference represents the constant tissue ammonia, presumably confined to the compartment of its formation (62). When this value is subtracted from the tissue content of ammonia, the Tissue/Medium concentration ratio for NH_4^+ invariably becomes unity (Tables 24-26).

Further support for only passive diffusion of NH_4^+ in brain cells is obtained from the following data.

1. The rate of uptake of NH_4^+ is extremely rapid, the concentration of NH_4^+ reaching its maximum (and about equal to the medium concentration of NH_4^+) even before the incubation has commenced (Table 27).

2. Restricting ATP formation has no effect on the accumulation of NH_4 in the tissue. Thus, incubation in a glucose-free medium (Table 25), or under anoxia (Table 26), or in the presence of metabolic inhibitors such as 0.1 mM DNP (Table 31), has little effect on the tissue content of ammonia.

3. Ouabain, 0.1 mM, has little or no effect on the NH_4^+ concentration of the tissue incubated in an NH_4^+ containing medium (Table 31).

4. While NH_4^+ inhibits the active accumulation of glutamate or glycine in slices incubated in a medium containing glutamate or glycine respectively, these amino acids are without any effect on the ammonium uptake from a medium containing NH_4^+ (Tables 28, 29, 30).

The above observations support our conclusion concerning the passive nature of NH_4^+ transport in the brain in vitro. It should be noted that, according to the data of Richter and Dawson (22), the brains of rats injected intraperitoneally with NH_4Cl, have ammonia contents (8.9 mg%) about equal to that in their blood (9.4 mg%).

10.7 Transport of L-glutamate into brain cortex slices.

An estimate of the distribution of L-glutamate found in the tissue at the end of one hour incubation in a physiological glucose saline medium containing 5 mM labelled L-glutamate shows that a relatively small fraction, possibly 15 per cent (i.e., about 1.6 μmole/g) of the total labelled amino acid present in the tissue, is in the neurons. The rest is present in TTX-insensitive compartments, i.e., the glia (Section 5.6). Under these conditions the Tissue/Medium concentration ratio for glutamate obtained by radioactivity measurements is smaller than that obtained when (total tissue) glutamate is measured by the

Amino Acid Analyzer. The disparity in the result is resolved if only net uptake (i.e., the difference between the final tissue concentrations of glutamate with and without external L-glutamate) is used for calculating the Tissue/Medium ratio for glutamate (Table 18). External radioactive L-glutamate does not exchange freely with the bulk of the endogenous tissue glutamate (Section 5.4). These results suggest that L-glutamate is taken up largely in a compartment distinct from another containing the bulk of the endogenous glutamate. This view gains support from the work of Okamoto and Quastel (258), showing that the tissue glutamate labelled by pre-incubation with radioactive glucose, does not exchange with cold L-glutamate in a subsequent incubation, while pre-incubation with labelled L-glutamate brings about free exchange with unlabelled L-glutamate (in a subsequent incubation).

As the bulk of the endogenous pool of glutamate is neuronal (Chapter 8) our results suggest that glia actively accumulate L-glutamate from the incubation medium. This conclusion helps to explain why exogenous L-glutamate does not support high rates of ammonia formation which we have shown to occur largely by endogenous glutamate oxidation in the neurons (Section 10.1 (v)). Moreover, as it is known that glia (but not neurons) take up water when brain cortex slices are incubated in presence of L-glutamate (see 182), this conclusion also helps to account for water uptake known to accompany the uptake of exogenous L-glutamate into the brain slice. Again, it is reasonable to conclude that some of the cationic changes occurring under these conditions (Table 72) will take place in the glia accompanying the glial utilization of ATP for glutamine biosynthesis. These cationic fluxes are but little affected by TTX (Tables 72 and 73) as would be expected if they take place mainly in the glia.

10.8 Effects of NH_4^+ on brain metabolism.

A number of reports (see Introduction) implicate ammonia as a factor in the production of convulsions in hepatic coma and epilepsy. The possible involvement of ammonia in such neurological disorders is shown by increases in free cerebral ammonia levels in experimentally-produced convulsions in animals. These are brought about by administration of convulsive agents such as picrotoxin, strychnine, pentamethylene tetrazole, telodrin and ammonium salts. Furthermore, the cerebral ammonia level of animals can be lowered by administering anti-convulsive drugs such as barbiturates. Under normal conditions, ammonia is believed to be continuously formed in the brain, but its usually low (normal) level is maintained by effective cerebral utilization processes. Mechanisms, therefore, that control cerebral ammonia metabolism are of utmost importance for normal brain cell function.

Bessman (13) has suggested that, with hepatic coma, the accompanying enhanced blood ammonia levels due to failure of the liver to remove it from the portal blood, by urea synthesis (see 13), are due to an increased synthesis of glutamine in the brain and a progressive fall of brain α-ketoglutarate (13) due to reductive amination. This will result in an impaired operation of the citric acid cycle, diminished oxygen consumption (13) and, hence, a lowered rate of ATP synthesis. However, Tower and co-workers (256, 257) believe that ammonia has a direct toxic effect on the decarboxylation of pyruvate and α-ketoglutarate. Their work was carried out with cerebral cortical mitochondria of the cat.

We have found that brain cortex slices gain Na^+ and lose K^+ to the incubation medium when incubated in the presence of NH_4^+ (Table 58). Owing to a possible NH_4^+-K^+ exchange process, a loss of K^+ may be compensated for by a gain of NH_4^+. But more K^+ leaves the tissue than can be accounted for by 1:1 exchange for NH_4^+ in the brain tissue. For example, there is a loss of 32 µequiv K^+/g initial wet wt/hour for a gain of 10 µequiv NH_4^+/g/hour. With

2 day old infant rats, simple K^+ for NH_4^+ exchange (1:1) is demonstrable with little or no increase in tissue concentration of Na^+ (Table 63). It is known that, with infant rat brain the Na^+, K^+-ATPase activity is very low or absent (297, 298) and that the operation of the citric acid cycle proceeds very slowly compared with that in the adult rat brain (235). It is also known that NH_4^+ can replace K^+ in activating the Na^+, K^+-ATPase in nervous tissue (222, 223, Table 60). The possibility that NH_4^+ may act by uncoupling the enzymic activity of ATPase from its carrier properties (as the Na^+ pump) is made unlikely by the fact that NH_4^+ ions do not act immediately. A time interval that may extend to 30 minutes is required before the effects of NH_4^+ are seen (Table 64). It is more likely that NH_4^+ may act by a metabolic process, e.g., by reduction of the ATP concentration (Table 71). This suggestion that NH_4^+ may act, by its effects on cell energetics, is supported by the fact it fails to change Na^+ transport in infant brain (Table 63). Further support for this view is obtained by using TTX. Like the metabolic inhibitors DNP (182), sodium fluoroacetate or sodium malonate (Table 75), NH_4^+ ions bring about TTX-insensitive alterations in the ionic balance at the cell membrane (Table 72). Presumably, NH_4^+ ions do not activate the Na^+ current system at the cell membrane since TTX does not suppress the enhanced Na^+ influx under these conditions. It may be added that Tower and co-workers (256) reported no change of resting potentials from control levels when guinea pig cortex slices were incubated in the presence of NH_4^+ (5 mM).

Comparison of the effects of NH_4^+ with those brought about by other alkali metal salts (Table 62) showed that Rb^+ or Cs^+ chlorides (5 or 10 mM) lower tissue K^+ by about the same extent as does NH_4Cl at the same concentration, but unlike NH_4Cl, they are without any effect on the tissue Na^+ levels. It seems possible that, unlike NH_4^+, Cs^+ like Rb^+ (224) is accumulated against a concentration gradient at the expense of K^+. Li^+ and $(CH_3)_4NCl$ do not affect tissue Na^+. The reduced tissue K^+ is suggestive of a purely exchange process. Unlike NH_4^+, none of these salts (at 10 mM) enhanced the water uptake by the

tissue. It is evident that the effects of NH_4Cl are distinct from those of the alkali metal salts and $(CH_3)_4 NCl$.

The results discussed so far were obtained with the unstimulated brain slices which may be likened to the comatose brain in vivo. Nakazawa and Quastel (114) have shown that NH_4Cl inhibits the increased respiration due to electrical stimulation. We have observed a suppression of the protoveratrine stimulated respiration by 10 mM NH_4Cl (Table 72). This is accompanied by a fall in the tissue ATP content (Table 74). The ATP content falls so low that it may, in fact, become rate limiting for the initial phosphorylation of glucose necessary for its further oxidation (see 192, 205). The tissue cationic contents are considerably altered under these conditions.

Exogenous NH_4^+ affects both glial and neuronal metabolism. NH_4^+ must affect glial metabolism because it stimulates glutamine synthesis and inhibits the uptake of L-glutamate both processes shown to be predominantly occurring in the glia. The inhibition of glutamate uptake may be due partly to a diminished tissue ATP level (Table 71) as a result of the enhanced rate of glutamine synthesis. NH_4^+ must also affect neuronal metabolism because it inhibits the increased respiration due to protoveratrine, whose effects are confined to the neurons.

Like malonate (Table 14), NH_4^+ (Tables 32 and 33) diminishes the total (tissue + medium) aspartate and glutamate concentrations of incubated slices. These effects may presumably be larger in stimulated brain tissue, because the rate of operation of the citric acid cycle is much enhanced under conditions of stimulation.

Our results favour the view that NH_4^+, at relatively high concentrations, causes removal of α-ketoglutarate by reductive amination and so acts as an inhibitor of the citric acid cycle. This is particularly important in the neuron (especially in the stimulated state) where the operation of the citric acid cycle

is rapid. The consequent lowering of ATP results in disturbances of the Na^+ pump and a diminished rate of respiration. The stimulation of aerobic glycolysis by NH_4^+ (256, 257) may then simply be due to diminished oxidation of pyruvate and increased reduction to lactate.

11. GENERAL RESULTS AND CONCLUSIONS

1. An experimental method has been devised whereby it is possible to assess the minimum contents of certain amino acids in the brain. This depends on the combined use of protoveratrine (which stimulates the release of amino acids) and of ouabain (which blocks the re-uptake of amino acids). Added together, protoveratrine and ouabain cause extensive release of amino acids from incubated brain slices into the incubation medium. When tetrodotoxin is added with these drugs, to the incubation medium, there is considerable retention of certain amino acids. The amount retained must be present in the neurons as tetrodotoxin acts only on excitable cells, i.e., the neurons. The amount of retention gives a minimum measure of the content of these amino acids in the neurons.

2. The major locations of glutamate, aspartate, glycine, serine, and probably GABA, are in the neurons. Glutamine is present largely in the glia.

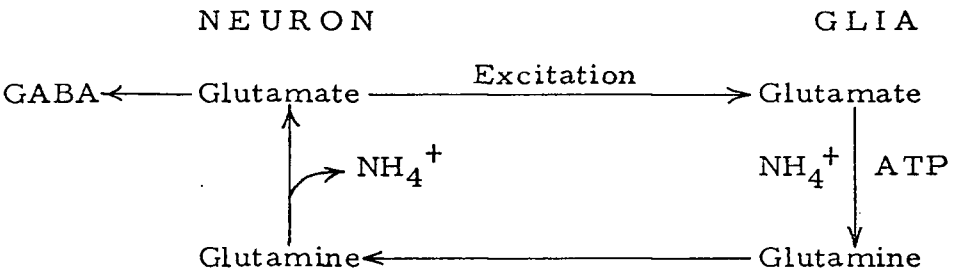
3. The partial, or complete, suppression by tetrodotoxin of the release of amino acids, particularly glutamate, GABA, aspartate and glycine from brain cortex slices, leads to the inference that during excitation (the generation of action potentials or the activation of the sodium current system in brain cortex slices), these amino acids are released from the neurons.

4. A study of the inhibitory effects of ouabain on glutamine synthesis leads to some understanding of the control mechanism for the synthesis of glutamine in brain cortex slices. Glutamine synthesis seems to occur largely in the glia and is partly controlled by the concentrations of cations within these cells. The cationic contents of glia in turn regulate the glial ATP concentration which influences both the active transport of glutamate

to the site of glutamine biosynthesis and the activity of the ATP-dependent glutamine synthetase.

5. Protoveratrine brings about a TTX-sensitive increased rate of glutamine synthesis in brain slices. This fact is consistent with the conclusion that glutamate released from the neuron, during excitation, is partly taken up by the glia and there converted to glutamine.

6. L-Glutamine is a more effective precursor in incubated brain slices of GABA than is L-glutamate. It is inferred that glutamine enters neurons more readily than glutamate. It is hydrolyzed there to liberate ammonia and then decarboxylated to form GABA. Thus, glutamine, formed in the glia from glutamate released from the neurons during excitation, is returned to the neurons where it is converted to glutamate and GABA. The facts point to the movements of glutamate and glutamine in brain cells occurring according to the following scheme.



7. The differential effects of the citric acid cycle inhibitors, sodium fluoroacetate and sodium malonate, on the respiration of, amino acid contents in, and release from, stimulated brain cortex slices, suggest that malonate (2 mM) acts mainly in the neurons and fluoroacetate (3 mM) acts mainly in the glia.

8. In the presence of 1 mM sodium amytal the concentrations of glutamate and GABA are enhanced. This is consistent with the conclusion that the block of endogenous glutamate oxidation leads to enhanced neuronal contents of glutamate and GABA.

9. Ammonia is formed aerobically by brain cortex slices in a glucose-free medium largely by endogenous glutamate oxidation within the neurons, and also by glutamine hydrolysis.

10. External L-glutamate is largely transported into brain cells against a concentration gradient by a tetrodotoxin-insensitive process. This fact would be explained if exogenous L-glutamate is largely taken up by the glia. This conclusion is supported by the fact that exogenous L-glutamate is less effective than endogenous glutamate as a source of ammonia in brain under aerobic conditions.

11. Ammonium ions are not transported against a concentration gradient in brain cortex slices. They are presumably formed up to a limiting concentration in the neurons independently of the external NH_4^+ concentration. Above this limiting concentration, ammonia is liberated into the extracellular spaces and the incubation medium.

12. Ammonium ions affect both neuronal and glial metabolism. They diminish neuronal ATP concentrations, presumably by inhibiting the operation of the neuronal citric acid cycle due to removal of α-ketoglutarate. The formation of aspartate and glutamate is impaired and the tissue contents of Na^+ and water are enhanced. The content of K^+ is diminished partly as a result of K^+-NH_4^+ exchange and partly as an impairment of the Na^+ pump. The exchange process seems to dominate in infant brain. The effects of NH_4^+ in inhibiting respiration, diminishing ATP concentration, and changing the ionic concentration at the brain cell membrane, are more pronounced in the stimulated than unstimulated brain tissue. This may be a major reason for NH_4^+ toxicity.

REFERENCES

1. Tashiro, S., Am. J. Physiol. 60, 519 (1922).

2. Winterstein, H., and Hirschberg, C. Biochem. Z. 156, 138 (1925).

3. Winterstein, H., Naturwissenschaften 21, 22 (1933).

4. Gerard, R.W., and Meyerhoff, O., Biochem. Z. 191, 125 (1927).

5. Rosch, H., and Tekamp, W., Z. Physiol. Chem. 175, 158 (1928).

6. Pravdicz-Neminsky, V.V., Z. Biol. 92, 465 (1932).

7. Halter, K., Biochem. Z. 257, 313 (1933).

8. Weil-Malherbe, H., Physiol. Rev. 30, 548 (1950).

9. Vrba, R., Rev. Czechoslov. Med. 3, 1 (1957).

10. Weil-Malherbe, H., in "Neurochemistry" (ed. Elliot, K.A.C., Page, I.H., and Quastel, J.H.), Thomas, C.C., Springfield, Ill., p. 321 (1962).

11. Waelsch, H., Advan. Protein Chem. 6, 229 (1951).

12. Strecker, H.J., in "Metabolism of the Nervous System" (ed. Richter, D). Pergamon Press, N.Y., p. 459 (1957).

13. Bessman, S.P., in "Chemical Pathology of the Nervous System" (ed. Folch-Pi, J.). Pergamon Press, N.Y., p. 370 (1961).

14. Buniatian, H.C., in "Handbook of Neurochemistry" (ed. Lajtha, A.). Plenum Press, N.Y., 3, 399 (1970).

15. Van den Berg, C.J., in "Handbook of Neurochemistry" (ed. Lajtha, A.). Plenum Press, N.Y., 3, 355 (1970).

16. Seegmiller, J.E., Schwartz, R., Davidson, C.S., J. Clin. Invest. 33, 984 (1954).

17. Ribeling, C., Klin. Wochschr. 10, 554 (1931).

18. Ribeling, C., Klin. Wochschr. 13, 1422 (1932).

19. Harris, M.M., J. Nerv. Ment. Dis. 102, 466 (1945).

20. Hamilton, P.B., J. Biol. Chem. 158, 397 (1945).

21. Brain, R., Proc. Roy. Soc. Med. 20, 861 (1927).

22. Richter, D., and Dawson, R.M.C., J. Biol. Chem. 176, 1199 (1948).

23. Najarian, J.S., and Harper, H.A., Am. J. Med. 21, 832 (1956).

24. Manning, R.T., and Delp, M., New Engl. J. Med. 258, 55 (1958).

25. Sherlock, S., Am. J. Med. 24, 805 (1958).

26. Zieve, L., Arch. Internal Med. 118, 211 (1966).

27. Eiseman, B., Fowler, W.G., White, P.J., and Clarke, G.M., Surg. Forum 6, 369 (1955).

28. Warren, K.S., J. Clin. Invest. 37, 497 (1958).

29. Roberge, A., and Charbonneau, R., Rev. Can. Biol. 27, 321 (1968).

30. Greenstein, J.P., Winitz, M., Gullino, P., Birnbaum, S.M., and Otey, M.C., Arch. Biochem. Biophys. 64, 342 (1956).

31. Clarke, G.M., and Eiseman, B., New Engl. J. Med. 259, 178 (1958).

32. Navazio, F., Gerritsen, T., and Wright, G.J., J. Neurochem. 8, 146 (1961).

33. Schwartz, H., and Dibold, H., Biochem. Z. 251, 190 (1932).

34. Bulow, M., and Homes, E.G., Biochem. Z. 245, 459 (1932).

35. Tsukada, Y., Takagaki, G., Sugimoto, S., and Hirano, S., J. Neurochem. 2, 295 (1958).

36. Tsukada, Y., in "Progress in Brain Research" (ed. Tokizane, T., and Schade, J.P.). Elsevier Publishing Co., Amsterdam, 21A, 268 (1966).

37. Benitez, D., Pscheidt, G.R., and Stone, W.E., Am. J. Physiol. 176, 488 (1954).

38. Whistler, K.E., Tews, J.K., and Stone, W.E., J. Neurochem. 15, 215 (1968).

39. Thorn, W., and Heiman, J., J. Neurochem. 2, 166 (1958).

40. Nathan, D.G., and Warren, K.S., Arch. Biochem. Biophys. 81, 377 (1959).

41. Godin, Y., Mark, J., Kayser, C.H., and Mandel, P., J. Neurochem. 14, 142 (1967).

42. Krebs, H.A., Eggleston, L.V., and Hems, R., Biochem. J. 44, 159 (1949).

43. Weil-Malherbe, H., and Green, R.H., Biochem. J. 61, 210 (1955).

44. Braganca, B.M., Quastel, J.H., and Schucher, R., Arch. Biochem. Biophys. 52, 18 (1954).

45. Josan, V., Tewari, S., and Krishnan, P.S., Indian J. Biochem. 4, 225 (1967).

46. Vladimirova, E.A., Physiol. Zh. SSR 25, 848 (1938).

47. Vladimirova, E.A., Dokl. Akad. Nauk. SSSR 95, 905 (1954).

48. Hathway, D.E., Mallinson, A., and Akintonwa, D.A.A., Biochem. J. 94, 676 (1965).

49. Hathway, D.E., and Mallinson, A., Biochem. J. 90, 51 (1964).

50. Torda, C., J. Pharm. Exper. Therap. 107, 197 (1953).

51. Gershenovich, Z.S., and Krichevskaya, A.A. Chem. Abst. 48, 8909 (1954).

52. Folbergrova, J., Passonneau, J.V., Lowry, O.H., and Schulz, D.W. J. Neurochem. 16, 191 (1969).

53. Torda, C., and Wolff, H.G., Fed. Proc. 11, 163 (1952).

54. Lusenko, V.S., Fiziol. Zh. Kiev 2, 53 (1956).

55. Gray, I., Johnson, J.M., and Spearing, C.W., Fed. Proc. 15, 265 (1956).

56. Vrba, R., Physiol. Bohemoslov. 3, 394 (1954).

57. Vrba, R., J. Neurochem. 1, 12 (1956).

58. Weil-Malherbe, H., Biochem. J. 30, 665 (1936).

59. Warburg, O., Posener, K., and Negelein, E., Biochem. Z. 152, 309 (1924).

60. Loebel, R.O., Biochem. Z. 161, 219 (1925).

61. Dickens, F., and Greville, G.D., Biochem. J. 27, 1123 (1933).

62. Benjamin, A.M., M.Sc. Thesis, University of British Columbia, Canada (1969).

63. Lahiri, S., and Quastel, J.H., Biochem. J. 89, 157 (1963).

64. Takagaki, G., Hirano, S., and Tsukada, Y., Arch. Biochem. Biophys. 68, 196 (1957).

65. Benjamin, A.M., and Quastel, J.H., Proc. Canad. Fed. Biol. Soc. 13, 24 (1970).

66. Vrba, R., Nature 176, 1258 (1955).

67. Vrba, R., Folbergrova, J., and Kanturek, V., J. Neurochem. 2, 187 (1958).

68. Vrba, R., Physiol. Bohemoslov. 4, 397 (1955).

69. Vrba, R., Nature 176, 117 (1955).

70. Vrba, R., in "Metabolism of the Nervous System" (ed. Richter, D.) Pergamon Press, N.Y. (1957).

71. Richter, D., Biochem. Soc. Symp. 8, 62 (1952).

72. Jakoubek, B., Gutmann, E., Hajek, I., and Syrouy, I., Physiol. Bohemoslov. 12, 553 (1963).

73. Vrba, R., Physiol. Bohemoslov. 5, 265 (1956).

74. Vladimirova, G. Ye., and Vladimirova, Ye. A., in "Structure and Function of the Cerebral Cortex" (ed. Tower, D.B., and Schade, J.P.). Elsevier Publishing Co., Amsterdam. p. 405 (1960).

75. Weil-Malherbe, H., and Drysdale, A.C., J. Neurochem. 1, 250 (1957).

76. Wherrett, J.R., and Tower, D.B., J. Neurochem. 18, 1027 (1971).

77. Mycek, M.J., and Waelsch, H., J. Biol. Chem. 235, 3513 (1960).

78. Clarke, D.D., Mycek, M.J., Neidle, A., and Waelsch, H., Arch. Biochem. Biophys. 79, 338 (1959).

79. Mycek, M.J., Clarke, D.D., Neidle, A., and Waelsch, H., Arch. Biochem. Biophys. 84, 528 (1959).

80. Martinson, E.E., and Tyakhepyl'd, L. Ya., Biokhimiya 26, 984 (1961).

81. Kometiani, P.A., Klein, Ye.E., Gvalia, N.V., and Gotsiridze, Ye.G., J. Neurochem. 17, 1331 (1970).

82. Schenker, S., and Mendelson, J.H., Am. J. Physiol. 206, 1173 (1964).

83. Nelson, S.R., and Mantz, M-L., Life Sciences 10, 901 (1971).

84. Sellinger, O.Z., and Weiler, P., Biochem. Pharmacol. 12, 989 (1963).

85. Tower, D.B., "Neurochemistry of Epilepsy". Thomas, C.C., Springfield, Ill. (1960).

86. Meister, A., "Biochemistry of the Amino Acids", Academic Press, N.Y. (1965).

87. Krebs, H.A., and Henseleit, K., Z. Physiol. Chem. 210, 33 (1932).

88. Tallan, H.H., Moore, S., and Stein, W.H., J. Biol. Chem. 211, 927 (1954).

89. Tomlinson, S., and Westall, R.G., Nature 188, 235 (1960).

90. Ratner, S., Morell, H., and Carvalho, E., Arch. Biochem. Biophys. 91, 280 (1960).

91. Sporn, M.B., Dingman, W., and Defalco. A., J. Neurochem. 4, 141 (1959).

92. Sporn, M.B., Dingman, W., Defalco, A., and Davies, R.K., J. Neurochem. 5, 62 (1959).

93. Kemp. J.W., and Woodbury, D.W., Biochem. Biophys. Acta 111, 23 (1965).

94. Schurr, P.E., Thompson, L.E., Henderson, L.M., Williams, J.N., and Elvehjem, C.A., J. Biol. Chem. 182, 39 (1950).

95. McIlwain, H., "Biochemistry and the Central Nervous System", 3rd ed., Little Brown & Co., Boston (1966).

96. Bollman, J.L., Mann, F.C., and Magath, T.B., Am. J. Physiol. 69, 371 (1924).

97. Baldwin, E., "Dynamic Aspects of Biochemistry", 3rd ed., Cambridge University Press, p. 308 (1957).

98. Kometiani, P.A., Ukrain. Biokhim. Zh. 37, 721 (1965).

99. Takagaki, G., J. Biochem. (Japan) 42, 131 (1955).

100. Buniatian, H.C., and Davtian, M.A., J. Neurochem. 13, 743 (1966).

101. Vrba, R., and Folbergrova, J., J. Neurochem. 4, 338 (1959).

102. Weil-Malherbe, H., and Gordon, J., J. Neurochem. 18, 1659 (1971).

103. Krebs, H.A., Biochem. J. 29, 1951 (1935).

104. Quastel, J.H., Proc. Roy. Soc. B. 163, 169 (1965).

105. Quastel, J.H., Neurosciences Res. 3, 1 (1970).

106. Neame, K.D., in "Applied Neurochemistry" (ed. Davison, A.N., and Dobbing, J.), Blackwell Scientific Publications, Oxford, p. 119 (1968).

107. Christensen, H.N., "Biological Transport", Benjamin, W.A., N.Y. (1962).

108. Smith, S.E., J. Neurochem. 14, 291 (1967).

109. Wilbrant, W., and Rosenberg, T., Pharm. Rev. 13, 109 (1961).

110. Johnstone, R.M., and Scholefield, P.G., Adv. Cancer Res. 9, 143 (1965).

111. Heinz, E., and Walsh, P.M., J. Biol. Chem. 233, 1488 (1958).

112. Gonda, O., and Quastel, J.H., Biochem. J. 84, 394 (1962).

113. Abadom, P.N., and Scholefield, P.G., Canad. J. Biochem. Physiol. 40, 1575, 1591, 1603 (1962).

114. Nakazawa, S., and Quastel, J.H., Canad. J. Biochem. 46, 356, 363, 543 (1968).

115. Okamoto, K., and Quastel, J.H., Biochem. J., 120, 25 (1970).

116. Balazs, R., Machiyama, Y., Hammond, B.J., Julian, T., and Richter, D., Biochem. J. 116, 445 (1970).

117. Strecker, H.J., in "Handbook of Neurochemistry" (ed. Lajtha, A.) Plenum Press, N.Y. 3, 173 (1970).

118. Gaitonde, M.K., in "Handbook of Neurochemistry" (ed. Lajtha, A.) Plenum Press, N.Y. 3, 225 (1970).

119. Baxter, C.F., in "Handbook of Neurochemistry" (ed. Lajtha, A.) Plenum Press, N.Y. 3, 289 (1970).

120. Aprison, M.H., Davidoff, R.A., and Werman, R., in "Handbook of Neurochemistry" (ed. Lajtha, A.), Plenum Press, N.Y. 3, 381 (1970).

121. Balazs, R., and Hanslam, R.J., Biochem. J. 94, 131 (1965).

122. Awapara, J., and Seale, B., J. Biol. Chem. 194, 497 (1952).

123. Green, D.E., Leloir, L.F., and Nocito, V., J. Biol. Chem. 161, 559 (1945).

124. Bonavita, V., J. Neurochem. 4, 275 (1959).

125. Roberts, S., and Morelos, B.S., J. Neurochem. 12, 373 (1965)

126. Flock, E.V., Pyce, G.M., and Owen, C.A., J. Neurochem. 13, 1389 (1966).

127. Carver, M.J., J. Neurochem. 12, 45 (1965).

128. Piccoli, F., Grynbaum, A., and Lajtha, A., J. Neurochem. 18, 1135 (1971).

129. Himwich, W.A., and Agrawal, H.C., in "Handbook of Neurochemistry" (ed. Lajtha, A.) Plenum Press, N.Y., 1, 33 (1969).

130. Oja, S.S., Ann. Acad. Sci. Pennicae Series A, 125, 34 (1966).

131. Vernadakis, A.D., and Woodbury, D.M., Am. J. Physiol. 203, 748 (1962).

132. Copenhaver, J.H., McShan, W.H., and Meyer, R.K., J. Biol. Chem. 183, 73 (1950).

133. Strecker, H.J., Arch. Biochem. Biophys. 46, 128 (1953).

134. Elliot, W.H., Nature 161, 128 (1948).

135. Elliot, W.H., Biochem. J. 49, 106 (1951).

136. Speck, J.F., J. Biol. Chem. 179, 1387, 1405 (1949).

137. Greenstein, J.P., and Leuthhardt, F.M., Arch. Biochem. Biophys. 17, 105 (1948).

138. Weil-Malherbe, H., J. Neurochem. 16, 855 (1969).

139. Kramme, E., Tveit, B., and Svenneby, G., Biochem. Biophys. Res. Commun. 20, 566 (1965).

140. Zilversmit, D.B., Entenman, C., and Fishler, M.C., J. Gen. Physiol. 26, 325 (1943).

141. Berl, S., and Clarke, D.D., in "Handbook of Neurochemistry" (ed. Lajtha, A.). Plenum Press, N.Y., 2, 447 (1970).

142. Waelsch, H., Berl, S., Rossi, C.A., Clarke, D.D., and Purpura, D.P., J. Neurochem. 11, 717 (1964).

143. Berl, S., Lajtha, A., and Waelsch, H., J. Neurochem. 7, 186 (1961).

144. O'Neal, R.M., and Koeppe, R.E., J. Neurochem. 13, 835 (1966).

145. Berl, S., Nicklas, W.J., and Clarke, D.D., J. Neurochem. 15, 131 (1968).

146. Van den Berg, C.J., and Van den Velden, J., J. Neurochem. 17, 985 (1970).

147. Berl, S., Clarke, D.D., and Nicklas, W.J., J. Neurochem. 17, 999 (1970).

148. Van den Berg, C.J., J. Neurochem. 17, 973 (1970).

149. Patel, A.J., and Balazs, R., J. Neurochem. 17, 955 (1970).

150. Clarke, D.D., Nicklas, W.J., and Berl, S., Biochem. J. 120, 345 (1970).

151. Berl, S., Nicklas, W.J., and Clarke, D.D., J. Neurochem. 17, 1009 (1970).

152. Nicklas, W.J., Clarke, D.D., and Berl, S., J. Neurochem. 16, 549 (1969).

153. Van den Berg, C.J., Krzalic, L.J., Mela, P., and Waelsch, H., Biochem. J. 113, 281 (1969).

154. Cocks, J.A., Balazs, R., Johnson, A.L., and Eayrs, J.T., J. Neurochem. 17, 1275 (1970).

155. Machiyama, Y., Balazs, R., and Richter, D., J. Neurochem. 14, 591 (1967).

156. Machiyama, Y., Balazs, R., Hammond, B.J., Julian, T., and Richter, D., Biochem. J., 116, 469 (1970).

157. Berl, S., and Frigyesi, T.L., J. Neurochem. 16, 405 (1969).

158. Van den Berg, C.J., and Garfinkel, D., Biochem. J. 123, 211 (1971).

159. Rose, S.P.R., J. Neurochem. 17, 809 (1970).

160. Rose, S.P.R., and Cory, H.T., J. Neurochem. 17, 817 (1970).

161. Margolis, R.K., Heller, A., and Moore, R.Y., Brain Research 11, 19 (1968).

162. Tower, D.B., in "Neurochemistry of Neucleotides and Amino Acids" (ed. Brady, R.O., and Tower, D.B.), John Wiley and Sons, N.Y., p. 197 (1960).

163. Bayer, S.M., and McMurray, W.C., J. Neurochem. 14, 695 (1967).

164. Talwar, G.P., and Singh, U.B., in "Handbook of Neurochemistry" (ed. Lajtha, A.), Plenum Press, N.Y. 6, 29 (1971).

165. Eccles, J.C., "The Physiology of Nerve Cells", The John Hopkins Press, Baltimore (1968).

166. de Jong, R.H., "Physiology and Pharmacology of Local Anaesthetics", Thomas, C.C., Springfield, Ill. (1970).

167. Shanes, A.M., Pharmacol. Rev. 10, 59, 165 (1958).

168. Bell, G.H., Davidson, J.N., and Scarborough, H., "Textbook of Physiology and Biochemistry", 7th edition, E. and S. Livingstone Ltd., London (1968).

169. Cragg, B.C., in "Applied Neurochemistry" (ed. Davison, A.N., and Dobbing, J.), Blackwell Scientific Publications, Oxford, p. 3 (1968).

170. Curtis, D.R., and Johnston, G.A.R., in "Handbook of Neurochemistry" (ed. Lajtha, A.), Plenum Press, N.Y., 4, 115 (1970).

171. Hyden, H., and Lange, P.W., in "Handbook of Neurochemistry" (ed. Lajtha, A.), Plenum Press, N.Y., 4, 221 (1970).

172. Barker, P.F., Hodgkin, A.L., and Ridgeway, E.B., J. Physiol. 218, 709 (1971).

173. Rubin, R.P., Pharmacol. Rev. 22, 389 (1970).

174. Goldman, D.E., and Blaustein, M.P., Ann. N.Y. Acad. Sci. 137, 967, (1966).

175. Rose, S.P.R., in "Applied Neurochemistry" (ed. Davison, A.N., and Dobbing, J.), Blackwell Scientific Publications, Oxford, p. 344 (1968).

176. Dettborn, W-D., in "Handbook of Neurochemistry" (ed. Lajtha, A.), Plenum Press, N.Y., 6, 423 (1971).

177. Hodgkin, A.L., and Horowicz, P., J. Physiol. 148, 135 (1959).

178. Parsons, R.L., Hofmann, W.W., and Feigen, G.A., Nature, 208, 590 (1965).

179. Li, C-L., and McIlwain, H., J. Physiol. 139, 178 (1957).

180. Gibson, I.M., and McIlwain, H., J. Physiol. 176, 261 (1965).

181. Yamamoto, C., and McIlwain, H., J. Neurochem. 13, 1333 (1966).

182. Okamoto, K., and Quastel, J.H., Biochem. J. 120, 37 (1970).

183. Schanberg, S.M., J. Pharm. Exp. Ther. 139, 191 (1963).

184. Dengler, H., Michaelson, I., Spiegel, H., and Titus, E., Int. J. Neuropharm. 1, 23 (1962).

185. Liang, C.C., and Quastel, J.H., Biochem. Pharmacol. 18, 1169, 1187, (1969).

186. Gottesfeld, Z., and Elliot, K.A.C., J. Neurochem. 18, 683 (1971).

187. Katz, R.I., and Kopin, I.J., Biochem. Pharmacol. 18, 1935 (1969).

188. Benjamin, A.M., and Quastel, J.H., Proc. Canad. Fed. Biol. Soc. 14, 75 (1971).

189. Mosher, H.S., Fuhrman, F.A., Buchwald, H.D., and Fischer, H.G., Science 144, 1100 (1964).

190. Kao, C.Y., Pharmacol. Rev. 18, 997 (1966).

191. Evans, M.H., Brit. Med. Bull. 25, 263 (1969).

192. Shankar, R., Ph.D. Thesis, University of British Columbia (1971).

193. Nakamura, Y., Nakajima, S., and Gruendfest, H., J. Gen. Physiol. 48, 985 (1965).

194. Narahashi, T., Moore, J.W., and Scott, W.R., J. Gen. Physiol. 47, 965 (1964).

195. Moore, J.W., and Narahashi, T., Fed. Proc. 26, 1655 (1967).

196. Narahashi, T., Anderson, N.C., and Moore, J.W., Science 153, 765 (1966).

197. Chan, S.L., and Quastel, J.H., Science 156, 1752 (1967).

198. Chan, S.L., and Quastel, J.H., Biochem. Pharmacol. 19, 1071 (1970).

199. McIlwain, H., Biochem. Pharmacol. 16, 1389 (1967).

200. Swanson, P.D., Biochem. Pharmacol. 17, 129 (1968).

201. Harvey, J.A., and McIlwain, H., Biochem. J. 108, 269 (1968).

202. McIlwain, H., Harvey, J.A., and Rodriguez, G., J. Neurochem. 16, 363 (1969).

203. Ramsay, R.L., and McIlwain, H., J. Neurochem. 17, 781 (1970).

204. Pull, I., McIlwain, H., and Ramsay, R.L., Biochem. J. 116, 181 (1970).

205. Shankar, R., and Quastel, J.H., Biochem. J. 126, 851 (1972).

206. Feinstein, M.B., J. Gen. Physiol. 47, 151 (1963).

207. Ulbricht, W., Ergebnisse der Physiologie 61, 18 (1969).

208. Shanes, A.M., Ann. N.Y. Acad. Sci. 55, 1 (1952).

209. Gordon, H.T., and Welsh, J.H., J. Cell. Comp. Physiol. 31, 395 (1948).

210. Shankaran, R., and Quastel, J.H., Biochem. Pharmacol. (in press) 1972.

211. Wollenberger, A., Biochem. J. 61, 68 (1955).

212. Wollenberger, A., Biochem. J. 61, 77 (1955).

213. Kini, M.M., and Quastel, J.H., Science 131, 412 (1960).

214. McIlwain, H., in "Enzymes and Drug Action", Ciba Foundation Symposium (ed. Mongar, J.L., and deReuck, A.V.S.), Little Brown and Co., Boston, p. 180 (1962).

215. Toman, J.E.P. in "Neurochemistry" (ed. Elliot, K.A.C., Page, I.H., and Quastel, J.H.). Thomas, C.C., Springfield, Ill., p. 752 (1962).

216. Mullins, L.J., Pharmacol. Rev. 17, 215 (1965).

217. Hillman, H.H., Campbell, W.J., and McIlwain, H., J. Neurochem. 10, 325 (1963).

218. Quastel, J.H., Br. Med. Bull. 21, 49 (1965).

219. Michaelis, M., and Quastel, J.H., Biochem. J. 35, 518 (1941).

220. Ernster, L., Low, H., and Lingberg, O., Acta Chem. Scand. 9, 200 (1955).

221. Jalling, U., Lindberg, O., and Ernster, L., Acta Chem. Scand. 9, 198 (1955).

222. Skou, J.C., Biochem. Biophys. Acta 23, 394 (1957).

223. Skou, J.C., Physiol. Rev. 45, 596 (1965).

224. Tower, D.B., in "Handbook of Neurochemistry" (ed. Lajtha, A.), Plenum Press, N.Y., 1, 1 (1969).

225. Swanson, P.D., and McIlwain, H., J. Neurochem. 12, 877 (1965).

226. Elliot, K.A.C., and Wolfe, L.S., in "Neurochemistry" (ed. Elliot, K.A.C., Page, I.H., and Quastel, J.H.), Thomas, C.C., Springfield, Ill., p. 177 (1962).

227. Woodman, R.J., and McIlwain, H., Biochem. J., 81, 83 (1961).

228. Quastel, J.H., and Wheatly, A.H.M., Biochem. J. 25, 119 (1931).

229. Kini, M.M., and Quastel, J.H., Nature 184, 252 (1959).

230. Milstein, J.M., White, J.G., and Swaiman, J.F., J. Neurochem. 15, 411 (1968).

231. Takagaki, G., Hirano, S., and Nagata, Y., J. Neurochem. 4, 124 (1959).

232. Neidle, A., Van den Berg, C.J., and Grynbaum, A., J. Neurochem. 16, 225 (1969).

233. Rassin, D.K., J. Neurochem. 19, 139 (1972).

234. Elliot, K.A.C., in "Handbook of Neurochemistry" (ed. Lajtha, A.), Plenum Press, N.Y., 2, 103 (1969).

235. Itoh, T., and Quastel, J.H., Biochem. J. 116, 641 (1970).

236. Dobkin, J., and Martin, M., J. Neurochem. 17, 231 (1970).

237. Warburg, O., Biochem. Z. 142, 317 (1923).

238. Waelsch, H., Advan. Enzymol. 13, 237 (1952).

239. Waelsch, H., in "Neurochemistry" (ed. Elliot, K.A.C., Page, I.H., and Quastel, J.H.), Thomas, C.C., Springfield, Ill., p. 288 (1962).

240. Umbreit, W.W., Burris, R.H., and Stauffer, J.F., in "Manometric Techniques", Burgess Publishing Co., Minneapolis, Minn. (1964).

241. Greengard, P., in "Methods of Enzymatic Analysis" (ed. Bergmeyer, H.U.), Academic Press, N.Y., p. 551 (1960).

242. Bonting, S.L., Simmons, K.A., and Hawkins, M.N., Arch. Biochem. Biophys. 95, 416 (1961).

243. Gray, E.G., and Whittaker, V.P., J. Anat. 96, 79 (1962).

244. Rybova, R., J. Neurochem. 4, 304 (1959).

245. Bradford, H.F., and Thomas, A.J., J. Neurochem. 16, 1495 (1969).

246. Salganicoff, L., and DeRobertis, E., J. Neurochem. 12, 287 (1965).

247. McIntosh, J.C., and Cooper, J.R., J. Neurochem. 12, 825 (1965).

248. Fleming, M.C., and Lowry, O.H., J. Neurochem. 13, 779 (1966).

249. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J., J. Biol. Chem. 193, 265 (1951).

250. Folin, O., and Ciocalteu, V., J. Biol. Chem. 73, 627 (1927).

251. Swanson, P.D., J. Neurochem. 15, 57 (1968).

252. Lassanova, M., and Brechtlova, M., Physiol. Bohemoslov. 20, 235, 241 (1971).

253. Jacobs, M.M., and Parpart, A.K., J. Cell. Comp. Physiol. 11, 175, (1938).

254. Jacobs, M.M., and Stewart, D.R., J. Gen. Physiol. 25, 539 (1942).

255. Post, R.L., and Jolly, P.C., Biochim. Biophys. Acta 25, 118 (1957).

256. Tower, D.B., Wherrett, J.R., and McKhann, G.M., in "Regional Neurochemistry" (ed. Kety, S.S., and Elkes, J.), Pergamon Press, N.Y., p. 65 (1961).

257. McKann, G.M., and Tower, D.B., Am. J. Physiol. 200, 420 (1961).

258. Okamoto, K., and Quastel, J.H., Biochem. J. (in press) (1972).

259. Gonda, O., and Quastel, J.H., Biochem. J. 100, 83 (1966).

260. Kuffler, S.W., and Nicholls, J.G., Ergebn. Physiol. 57, 1 (1966).

261. Kuffler, S.W., Proc. R. Soc. B. 168, 1 (1967).

262. Kernan, R.P., "Cell K", Butterworth's, Washington (1965).

263. Bradford, H.F., and McIlwain, H., J. Neurochem. 13, 1160 (1966).

264. Marchbanks, R.M., in "Membranes and Ion Transport" (ed. Bittar, E.E.), Wiley Interscience 2, 145 (1970).

265. Richards, C.D., and Terkeurs, W.J., Brain Res. 26, 446 (1971).

266. Bourke, R.S., and Tower, D.B., J. Neurochem. 13, 1071, 1099, 1119, (1966).

267. Jones, D.A., and McIlwain, H., J. Neurobiol. 2, 311 (1971).

268. Elliot, K.A.C., and Heller, I.H., in "Metabolism of the Nervous System" (ed. Richter, D.), Pergamon Press, N.Y., p. 286 (1957).

269. Hess, H. H., in "Regional Neurochemistry" (ed. Kety, S.S., and Elkes, J.), Pergamon Press, N.Y., p. 200 (1961).

270. Epstein, M.H., and O'Connor, J.S., J. Neurochem. 12, 389 (1965).

271. Korey, S.S., and Orchen, M., J. Neurochem. 3, 277 (1959).

272. Hertz, L., J. Neurochem. 13, 1373 (1966).

273. Buniatian, H. Ch., Hovhannissian, V.S., and Aprikian, G.V., J. Neurochem. 12, 695 (1965).

274. Pope, A., Hess, H.H., Ware, J.R., and Thomson, R.H., J. Neurophysiol. 19, 259 (1956).

275. Cummins, J., and Hyden, H., Biochim. Biophys. Acta 60, 271 (1962).

276. Pope, A., and Hess, H.H., in "Metabolism of the Nervous System" (ed. Richter, D.), Plenum Press, N.Y., p. 72 (1956).

277. Lowry, O.H., Roberts, N.R., Leiner, K.Y., Wu, M.L., Farr, A.L., and Albers, R.W., J. Biol. Chem. 207, 39 (1954).

278. Christensen, H.N., "Body fluids and the Acid-Base Balance". W.B. Saunders & Co., p. 456 (1964).

279. Haber, B., Canad. J. Biochem. 43, 865 (1965).

280. Cremer, J., Biochem. J. 104, 223 (1967).

281. Coxon, R.V., in "Chemical Pathology of the Nervous System" (ed. Folch-Pi, J.), Pergamon Press, N.Y. p. 551 (1961).

282. Mandel, P., Harth, S., and Rebel, G., in "Chemical Pathology of the Nervous System" (ed. Folch-Pi, J.), Pergamon Press, N.Y. p. 551 (1961).

283. Baxter, C.F., in "Progress in Brain Research" (ed. Lajtha, A., and Ford, D.A.), Elsevier Publishing Co., Amsterdam, 29, 429 (1968).

284. Mushahwar, I.K., and Koeppe, R.E., Biochem. J. 126, 467 (1972).

285. Bowery, N.G., and Brown, D.A., J. Physiol. 218, 32p (1971).

286. Mitchell, J.F., Neal, M.J., and Srinivasan, V., Brit. J. Pharm. Chemotherap. 36. 201p (1969).

287. Cherayil, A., Kandera, J., and Lajtha, A., J. Neurochem. 14, 105 (1967).

288. DeRopp, R.S., and Snedeker, E.H., J. Neurochem. 7, 128 (1961).

289. Dawson, R.M.C., Biochim. Biophys. Acta 11, 548 (1953).

290. Cravioto, R.O., Massieu, G., and Izquierdo, J.J., Proc. Soc. Exp. Biol. Med. 78, 856 (1951).

291. Mukherji, B., Turinsky, J., and Sloviter, H.A., J. Neurochem. 18, 1783 (1971).

292. Srinivasan, V., Neal, M.J., and Mitchell, J.F., J. Neurochem. 16, 1235 (1969).

293. Hodgkin, A.L., and Huxley, A.F., J. Physiol. 116, 497 (1952).

294. Gonda, O., and Quastel, J.H., Nature, 193, 138 (1962).

295. Stern, J.R., Eggleston, L.V., and Krebs, H.A., Biochem. J. 44, 410 (1949).

296. Hamberger, A., J.Neurochem. 8, 31 (1961).

297. Samson, F.E., and Quinn, D.J., J. Neurochem. 14, 1143 (1967).

298. Abdel-Latif, A.A., Brody, J., and Ramahi, H., J. Neurochem. 14, 1133 (1967).

299. Quastel, J.H., in "Neurochemistry" (ed. Elliot, K.A.C., Page, I.H., and Quastel, J.H.), Thomas, C.C., Ill., p. 226 (1962).

300. Axelrod, B., in "Metabolic Pathways" (ed. Greenberg, D.M.), Academic Press, N.Y., 1, 112 (1967).

301. Utter, M.F., J. Biol. Chem. 185, 499 (1950).

302. Utter, M.F., Ann. N.Y. Acad. Sci. 72, 387 (1959).

303. Muntz, J.A., and Hurwitz, J., Arch. Biochem. 32, 124, 137 (1951).

304. Baxter, C.F., and Roberts, E., J. Biol. Chem. 236, 3287 (1961).

305. Rose, S.P.R., in "Handbook of Neurochemistry" (ed. Lajtha, A.), Plenum Press, N.Y. 2, 183 (1969).

306. Reilley, C.N., Schmid, R.W., and Sadek, F.S., J. Chem. Edu. 36, 555 (1959).

307. Wynn, J.E., Vant-Reit, B., and Borzelleca, J.F., Toxic. Appl. Pharmac. 16, 808 (1970).

308. Chappell, J.R., and Crofts, A.R., Biochem. J. 95, 378 (1965).

309. Reading, H.W., and Wallwork, J., Biochem. Pharmacol. 18, 2211 (1969).

310. Benjamin, A.M., and Quastel, J.H., Biochem. J. (in press) (1972).

311. Gaitonde, M.K., and Kelly, R., J. Neurochem. 19, 377 (1972).

312. Neal, M.J., and Iversen, L.L., J. Neurochem. 16, 1245 (1969).

313. Balazs, R., Dahl, D., and Harwood, J.R., J. Neurochem. 13, 897, (1966).

314. Van den Berg, C.J., and Garfinkel, D., Biochem. J. 123, 211 (1971).

315. Richter, D., in "Protein Metabolism of the Nervous System" (ed. Lajtha, A.), Plenum Press, N.Y., p. 244 (1970).