AMMONIA METABOLISM IN THE BRAIN

by

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ABSTRACT

It is known that the functional activity of the nervous system is associated with ammonia formation and that the administration of ammonium salts to experimental animals produces convulsions. Mechanisms, therefore, that control ammonia metabolism in the brain are of importance for brain cell function.

The presence of ammonia utilizing mechanisms in the brain maintains the low free cerebral ammonia levels found in vivo. There is, however, a rapid formation of ammonia in the brain on the death of the animal and a further liberation of ammonia takes place when isolated brain cortex is incubated aerobically in the absence of glucose. Studies of these and other aspects of ammonia metabolism form the subject matter of this thesis.

The estimation of ammonia in these studies is based on a modification of the diffusion technique of Conway. Ammonia and amino acid analyses have been carried out using the Beckman amino acid analyzer.

The rapid rate of cerebral ammonia formation that takes place when the brain is removed from the animal is partially arrested by trichloracetic acid (TCA), presumably by the inactivation of cerebral enzymes. Our results rule out the possibility that glutamine, glutamate,

taurine and ATP are significant contributors to the initial or pre-incubation levels of ammonia and the evidence favors the involvement of TCA-insoluble components as precursors of such ammonia.

In the presence of glucose the pre-incubation levels of amino acids of cerebral cortex slices of the rat are maintained during subsequent aerobic incubation at 37°C for one hour. In the absence of glucose, however, we have found marked changes in the pre-incubation levels of amino acids of cerebral cortex slices under these experimental conditions. A considerable rise of ammonia also occurs in the absence of glucose and this can be largely accounted for by a net loss of $-NH_2$-groups of the amino acid pools of brain slices. The significant fall in the cerebral levels of glutamate and glutamine under these conditions indicates that for short periods of incubation (one hour), these amino acids may serve as major sources of ammonia formation by respiring brain cortex slices.

Our findings of a marked suppression of ammonia formation by cerebral cortex slices incubated for one hour either anaerobically, or aerobically, in a glucose-free medium in the presence of amytal, D-glutamate or α-methylglutamate, implicate the oxidative deamination of cerebral glutamate as a major mechanism for ammonia liberation. D-glutamate also acts by inhibiting the hydrolysis of glutamine.

In the presence of glucose aerobic incubation with 2, 4-dinitrophenol, iodoacetate, malonate, hydroxylamine or D-glutamate, increases the rate of ammonia formation by cerebral cortex slices. This is doubtless due to

diminished activity of cerebral glutamine synthetase required for ammonia fixation to occur.

We find that the level of ammonia in the brain tissue itself is not markedly affected by the presence or absence of glucose. The increased quantity of ammonia formed by cerebral cortex slices in the absence of glucose is found largely in the incubation medium. This fact points to the formation of ammonia in specific compartment(s), and the retention of ammonia within such compartment(s) up to a certain level. Above this level there is efflux of ammonia into the incubation medium. Such a conclusion helps to explain the apparent high concentration ratio (tissue:medium) of NH_4^+ obtained either aerobically (viz. 42) or anaerobically (viz. 12) at the end of one hour incubation in the presence of glucose. There is some accumulation of NH_4^+ ions in rat brain cortex slices against a concentration gradient.

Our finding that ouabain stimulates ammonia formation in respiring cerebral cortex slices is in accord with the fact that ouabain inhibits the utilization of NH_4^+ for the biosynthesis of glutamine, presumably by affecting the transport of NH_4^+ to the site of glutamine synthesis. Ouabain has no effect on the cerebral glutaminase of the rat.

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ABBREVIATIONS

TCA	Trichloracetic acid
AMP	5'-Phosphate of adenosine
ADP	5'-Diphosphate of adenosine
ATP	5'-Triphosphate of adenosine
Pi	Orthophosphate
DNP or 2, 4-DNP	2, 4-Dinitrophenol
NAD+, NADH	Oxidized and reduced forms of nicotinamide-adenine dinucleotide.
γABA	γ-amino butyric acid

1. INTRODUCTION

1.1 Ammonia formation in nerve tissue (other than brain).

As early as 1922, Tashiro (1) demonstrated an evolution of ammonia from nerve fibres. He also showed that while electrical stimulation increased ammonia liberation, mechanical injury depressed it. Winterstein and Hirschberg (2, 3) not only confirmed these findings, but also further established the suppressive effects of anoxia and anaesthesia on ammonia formation. Observations on the effects of stimulation were made by a number of workers (4-7). For example, frog retina irradiated with sunlight increased ammonia formation when compared to controls left in the dark (5). Isolated nerve on stimulation, electrically or mechanically, by thermal or osmotic pressure changes and by various chemical agents, gave similar results (6, 7). These observations led to the conclusions that ammonia formation occurs as a reaction of nerve fibres to stimulation.

The central nervous system, like peripheral nerves, has been shown to react to stimuli by increased formation of ammonia. Articles reviewing ammonia metabolism by the central nervous system have appeared (8-11).

1.2 Ammonia levels in the brain in vivo.

Interest in brain ammonia levels stems from the fact that ammonia is a powerful cerebral irritant. The administration of ammonium salts

causes convulsions in animals and it has long been thought that ammonia may play a role in the precipitation of epileptic seizures (11-16).

Richter and Dawson (16) investigating the ammonia levels in the brain of young rats killed by immersion in liquid air confirmed the low values found by previous workers (12, 13, 17, 18). By this freezing technique tissues are rapidly fixed and post-mortem changes are avoided. Using this technique values of 0.15 - 0.36 micromoles per g. fresh weight brain tissue have been obtained in different species (Table I). These values are considerably lower than those obtained when the severed head of the decapitated animal is dropped into liquid air. For example, there is about a twofold increase in ammonia content when the head is frozen one second after decapitation (16). Cat brain gave 2.0 micromoles per g. fresh weight three minutes after death while sheep brain gave values of 2.7 and 1.7 micromoles per g. fresh weight in grey and white matter respectively when freezing was done within five minutes after death (19). Weil-Malherbe and Green (20) obtained 5.0 micromoles per g. fresh weight in slices of brain cortex fixed twenty minutes after death, which is in good agreement with values obtained by other workers (21, 22) and with results reported in this thesis.

1.3 Factors affecting ammonia formation in the brain.

A. In vivo studies

The ammonia content of the central nervous system, analyzed after rapid fixation in liquid air or liquid nitrogen, is not constant

TABLE I. *In vivo* levels of ammonia in the brain

Species	Amount of ammonia µmoles/g fresh wt.	Reference
Rat	0.16	16
	0.36	23
Dog	0.26	24
	0.20	25
Rabbit	0.18	26
Mice	0.32	27
Garden Dormice	0.35	28

but depends on the state of activity of the brain at the time of fixation.

(i) Conditions producing low brain ammonia levels.

When Tashiro (1) observed a greater formation of ammonia during electrical stimulation of nerve fibres, he speculated that anaesthesia would depress it. This was shown to be true by Winterstein and Hirschberg (2), and later workers confirmed this observation (3, 16, 29). Richter and Dawson (16), for example, showed a significant decrease in brain ammonia levels (0.033 micromoles per g. wet weight) when rats under prolonged nembutal narcosis (thirty minutes or longer) were killed in liquid air. Vladimirova obtained similar effects with urethane and sodium bromide (30).

Ammonia concentration of rat brain decreases by 50 per cent during sleep (31). During hibernation, too, there is a 50 per cent lowering of ammonia levels in the brain (28).

Thus, a reduction in functional activity is associated with a reduced concentration of free ammonia in the brain.

(ii) Conditions producing high brain ammonia levels.

In confirmation of earlier reports (1 - 3) with nerve, a number of workers have established the induction of ammonia formation in the brain during electrical stimulation (16, 23).

This is consistent with the view that any method that increases cerebral irritability will produce greater amounts of ammonia in the brain. Thus, a number of drugs capable of producing convulsions, e.g., camphor (30), picrotoxin (16, 32), telodrin (33), pentamethylene tetrazole (34) and bemigride (35), when injected into animals resulted in increased brain ammonia levels. In some instances the rise occurred in the preconvulsive state (16, 35). This effect was marked in the brain of dogs after injections of fluoracetate, where the cerebral ammonia level rose and reached a maximum at the time of the convulsions (24). This increase by fluoracetate on ammonia formation has also been demonstrated in vitro on incubation of brain cortex slices in the presence of glucose (36). However, it is not known definitely whether an increase in brain ammonia is a result or the cause of convulsions. Injections of ammonium chloride in the rat caused convulsions when the brain ammonia level reached 9.0mg per cent (about 5.0 micromoles per g. fresh weight) (16). Both anoxia (16) and high oxygen pressure (35) result in increases in ammonia levels in the brain.

Milder stimulation of the central nervous system, like injection of amphetamine (37) or corticotropin (38), painful electric shock to the extremities (23, 31) or certain conditioned reflexes (23, 30), also elevated ammonia levels. Though emotional excitement caused by tumbling in a revolving drum

had a similar effect (39), Richter and Dawson found no marked difference in rats excited by allowing them to drop from side to side in a glass beaker (16), while Vrba (9) found no change in rats made to undergo physical exercise. Cerebral ammonia increases may not occur in some cases of physical or emotional excitement owing to increased formation of glutamine (9-11, 40, 41); a rise in cerebral glutamine usually accompanies a rise in blood ammonia.

B. In vitro studies

Work on the formation of ammonia in brain slices and the factors affecting its formation has been carried out to throw light on the following problems:

(i) The origin of ammonia in the brain;

(ii) The mechanism of its formation;

(iii) The mode of utilization of the ammonia.

How far these problems have been solved will be discussed later in this thesis.

Ammonia formation by cerebral cortex slices in vitro has two components (20) as follows:

(1) Spontaneous ammonia formation following decapitation.

(2) Ammonia formation that depends on

(i) structural integrity of the tissue (e.g., brain homogenates produce ammonia less efficiently than brain slices though they

give higher initial values (20));

(ii) Metabolic activity of the tissue (e.g., ammonia formation in the brain is inhibited (a) by the presence of glucose and lactate (42-45); (b) by the presence of substances such as cyanide or arsenite that inhibit electron transport, or 2:4 dinitrophenol that inhibits oxidative phosphorylation (20, 46,47); and (c) by anaerobiosis (20, 46).

In the presence of certain inhibitors, e.g., 2:4-dinitrophenol (46), fluoracetate (36) and iodoacetate (47), the suppressive effects of glucose on ammonia formation is less marked.

1.4 Ammonia formation in the brain: Origin and utilization mechanisms.

(i) 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 free ammonia (9, 40). However, it was shown that under such conditions free glutamine levels are increased in the brain (40) with concomitant decreases in free glutamate (41, 48) and protein bound amide nitrogen (49, 52). Similar results are obtained during oxygen intoxication (9) or carbon disulphide poisoning (52). Even during electrically induced

convulsions, glutamine levels are elevated (53). Acute telodrin intoxication, too, results in an increase in glutamine content with a concomitant decrease in glutamate levels in the brain (33). In the early stages of acute telodrin intoxication the content of free ammonia in the brain remains unchanged, but an increase is observed later in the seizure pattern. According to Hathway and Mallinson (33), 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. These results would lend support to the view that ammonia is formed from protein sources and is converted to glutamine by fixation with glutamate.

Such findings are also substantiated by in vitro studies with brain homogenates (9) or cerebral cortex slices (54) in incubations of long duration (3-4 hours). Besides, under such conditions Weil-Malherbe et al., on considering the six deaminating enzymes known to occur in the brain, dismissed them as major factors in the process of ammonia formation. The enzymes they considered were: glutaminase, glutamic dehydrogenase, adenylic and adenosine deaminases, amine oxidase (20) and hexosamine deaminase (55). Thus, according to Weil-Malherbe and Green (20), small molecules

like amino acids from the free pool may not be involved significantly as ammonia precursors. From these considerations it would appear that ammonia formation occurs by splitting part of the protein amide bonds, perhaps from glutamine moieties of the brain proteins, as brain protein is relatively rich in glutamine (16). However, Vrba reported that only 25 per cent of the ammonia formed from guinea pig cerebral cortex slices in the absence of substrate can be accounted for by the splitting of protein amide bonds in a four hour incubation (49, 54). Weil-Malherbe and Green (20) suggested that ammonia formation is linked with proteolysis because both ammonia and non-protein nitrogen were increased under similar conditions. Vrba (56), too, obtained evidence showing that the TCA insoluble fraction of brain slices during substrateless incubations lost some amide groups with concomitant significant increases in non-protein and lipid nitrogen. However, Vrba does not consider ammonia formation simply as a consequence of ordinary proteolytic processes.

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

When rats physically exhausted (by prolonged swimming) are allowed to rest, their cerebral glutamine levels decrease with a concomitant increase in their cerebral protein nitrogen (51, 56). It should be mentioned here that glutamine has been implicated in the synthesis of polypeptides and proteins (9, 57 - 60). Thus,

changes that occur in the free glutamine levels in the brain on the application of stimuli to the whole animal, or the removal of the applied stimuli, appear to be directly associated with brain protein metabolism.

1.5 Enzyme systems involved in the utilization of ammonia.

Ammonia in excess of the normal is toxic to the central nervous system (11-16, 61, 62). The mechanisms by which excessive ammonia is reduced in amount centre around glutamic acid metabolism. This is understandable when it is realized that glutamic acid is one of the few amino acids that occur in appreciable concentrations in the brain and accounts for close to 50 per cent of α-amino nitrogen of protein free brain tissue filtrates (8, 19, 63, 64). Also, it is known that glutamic acid decreases during strychnine convulsions (65), in hypoglycemic coma (66), and in epileptogenic foci of cat cortex produced in vivo by freezing (67). Moreover, glutamate injections inhibit ammonium chloride convulsions (68), while the infusion of ammonium salts leads to marked increases in protein amide groups (69) and in the glutamine concentrations of rat, cat and dog brain (70 - 72). These and other facts mentioned earlier implicate glutamine as an end product of cerebral ammonia utilization.

The main enzymes implicated in ammonia utilization are as follows:

(i) glutamic dehydrogenase

(ii) glutamine synthetase

(iii) glutamate transaminases

(i) Glutamic dehydrogenase:

This enzyme specifically catalyzes the oxidative deamination of glutamic acid (the only amino acid oxidized to any appreciable extent in the brain (42, 73), according to the following equations (8):

$$\text{glutamic acid} + NAD^+ \rightleftharpoons \alpha\text{-iminoglutaric acid} + NADH + H^+ \quad \ldots(1)$$

$$\alpha\text{-iminoglutaric acid} + H_2O \underset{\text{enzymic}}{\overset{\text{non}}{\rightleftharpoons}} \alpha\text{-ketoglutaric acid} + NH_3 \quad \ldots\ldots\ldots\ldots(2)$$

In the presence of ammonium salts the equilibrium of reaction (1) is much in favor of glutamic acid, only 1.4 per cent being oxidized at pH 7.4. In the absence of ammonium salts, iminoglutaric acid hydrolyzes spontaneously to α-ketoglutarate and ammonia. Thus, the supply or removal of ammonia can condition the oxidation of glutamic acid. α-ketoglutaric acid inhibits ammonia formation, which is due to the fact that the enzyme favors the reductive amination of α-ketoglutarate (42). Glutamic acid thus appears to be particularly adapted for the synthetic reaction which occurs spontaneously as soon as sufficient ammonia is available (74).

(ii) Glutamine synthetase:

This enzyme (42, 73) catalyzes the endergonic formation (75, 76) of glutamine according to the following equation:

$$\text{glutamic acid} + NH_3 + ATP \xrightarrow{Mg(Mn)} \text{glutamine} + ADP + Pi \quad \ldots\ldots(3)$$

Oxidizable substrates such as glucose suppress ammonia formation (42 - 45) by exerting a 'sparing action' on endogenous oxidizable nitrogenous materials, by checking autolytic breakdown of adenine nucleotides (8) and by forming glutamine (77, 78). Brain slices can bring about a disappearance of added ammonia in the presence of glucose (42). However, any method that inhibits ATP synthesis (e.g., 2, 4-DNP (20, 46), fluoracetate (36), and thereby lowered glutamine formation) concomitantly increases ammonia output. Glutamine presumably is an end product of the ammonia binding mechanism. It may serve as a storage or transport form of ammonia and may be hydrolyzed at the sites of ammonia utilization (79). In this connection it is perhaps pertinent to note that glutamine passes much more freely through the blood brain barrier than glutamate (80).

(iii) Glutamate transaminases:

Transamination reactions (8) utilizing glutamate as the amino group donor, and the keto-acids, pyruvate and oxaloacetate as acceptor molecules (to yield alanine and aspartic acid respectively)

serve as auxiliary mechanisms assisting ammonia utilization in the brain. The reactions shown below are the only transaminations that occur to a considerable extent in brain tissue (81, 82), and are catalyzed by specific transaminases which require pyridoxine derivatives as coenzymes (83).

glutamic acid + pyruvate $\rightleftharpoons$ α-ketoglutaric acid + alanine.....(4)

glutamic acid + oxaloacetate $\rightleftharpoons$ α-ketoglutaric acid + aspartate....(5)

When the formation of free ammonia is high, the reductive amination of α-ketoglutarate mediated by glutamic dehydrogenase occurs and a rapid synthesis of glutamic acid results. If the rate of ammonia formation exceeds the glutamine synthesizing capacity, then glutamic acid transaminates with pyruvic or oxaloacetic acids and the α-ketoglutarate regenerated can again accept ammonia so that a steady flow of ammonia into the transamination system is ensured. In the absence of ammonia, α-ketoglutarate accepts an amino group from alanine or aspartate and the keto acid (pyruvate or oxaloacetate) released re-enters the citric acid cycle (8). Thus, these amino acids (alanine and aspartate) act as temporary storehouses for excess ammonia ensuring gradual disposal during periods of restitution.

Early reports claimed that oral administration of glutamic acid decreased petit mal seizures, increased mental alertness and

aroused patients from insulin coma (84, 85). However, no convincing evidence of a relationship between these clinical effects and glutamic acid metabolism in the central nervous system has yet been put forward (85, 86).

1.6 Scope of the present work.

The work presented here is a preliminary study of cerebral ammonia metabolism and factors affecting its formation in vitro, in attempts to throw further light on the unsettled origin and obscure mechanism of cerebral ammonia formation.

2. MATERIALS AND METHODS

2.1 Animals

Adult rats of the Wistar strain and 2-day old infant rats were used in these studies. Male rats were usually employed. Josan et al., (22) observed no marked difference between the ammonia contents and rates of formation of adult male or female rat brains.

2.2 Sacrifice and brain removal

Adult rats were killed by decapitation. The whole brain was rapidly excised from the severed head and placed in chilled Ringer solution. With infant rats the head was cut off with scissors before the brain was removed. In some cases, particularly during the determination of initial ammonia levels in infant brain, the animals were immersed in liquid nitrogen. This procedure froze the animals in a few seconds and produced a rapid fixation of metabolites in the brain (16). Before the sacrifice of adult rats, in some instances intraperitoneal injections were made in attempts to prevent the initial bursts of ammonia, details of which will be mentioned below.

2.3 Tissue preparation

The brain was removed from the chilled Ringer medium and the cerebellum was dissected free from the hemispheres. Cerebral cortex slices prepared by a Stadie-Riggs microtome, were placed on petri

dishes chilled externally with ice. Care was taken that the slices were not more than 0.3mm thick, but not so thin that they tended to disintegrate when shaken in the Warburg manometric apparatus.

Cortex slices from each hemisphere of adult rat brain (two in number) were weighed quickly on a torsion balance (average weight 80-100mg wet weight) and suspended into chilled manometric vessels containing the incubation media.

2.4 Media composition and incubation conditions (87)

(i) Ringer-phosphate medium:

The basal medium had the following composition:

Ringer solution: NaCl, 142.6mM; KCl, 5.7mM; $CaCl_2$, 3.06mM; and $MgSO_4 . 7H_2O$, 1.43mM.

Phosphate buffer: Na_2HPO_4, 10mM made to pH 7.4 with NHCl.

Incubation in a Ringer-phosphate medium was carried out in a conventional Warburg manometric apparatus at 37°C for one hour unless otherwise stated. The gaseous phase was pure oxygen. The centre well contained a small glass tube, closed at one end, containing 0.2ml of 20 per cent KOH and a roll of filter paper. Oxygen was passed through the apparatus for five minutes after which the manometers were placed in the Warburg apparatus and thermally equilibrated for seven minutes.

(ii) Ringer-bicarbonate medium:

The basal medium had the following composition:

Ringer solution: NaCl, 141.8mM; KCl, 5.65mM; $CaCl_2$, 3.02mM; $MgSO_4.7H_2O$, 1.42mM; KH_2PO_4, 1.42mM.

Bicarbonate buffer: $NaHCO_3$, 28mM.

Incubation in a Ringer-bicarbonate medium was carried out at 37°C for one hour. Gassing with a N_2/CO_2 (95/5%) mixture was carried out for five minutes before thermal equilibration at 37°C for seven minutes.

The incubation media were five times the strength of the final solutions and were diluted after additions, or modifications, as mentioned in the text. The final volume of all incubation media was always 3.0ml. Glucose, when present, was 5.0mM unless otherwise stated. All solutions were made to pH 7.4 with NaOH before use.

2.5 Procedures adopted

Estimations were carried out to determine the initial ammonia content and the rates of ammonia formation during one hour incubation under various conditions. Homogenization was carried out in ice-cold Ringer medium for about one minute in a Potter-Elvehjem homogenizer. The ammonia content of the tissue, at the time of gassing, was taken as the initial ammonia content. This did not vary beyond the

experimental error between 10-30 minutes under the conditions of estimation used here.

Initial ammonia values. The tissues were placed in Warburg vessels containing 3.0ml Ringer-phosphate medium. The flasks were stoppered and K_2CO_3 from the side arm added immediately.

Ammonia content of the tissue. The slices were removed after incubation and quickly washed by dipping in a chilled Ringer medium and placed in another Warburg vessel containing 3.0ml water or Ringer-phosphate medium, immediately stoppered and K_2CO_3 from the side arm was added.

Ammonia content of the incubation media. This was estimated after the slices were removed. The flasks were stoppered and K_2CO_3 from the side arm was added into the main compartment.

Total ammonia values. These were the values found in the flasks after incubation was completed without removing the tissue. These were also the sums of the ammonia contents of tissue and medium as obtained separately.

2.6 Ammonia estimation

A modified method of Braganca et al., (21) was used, the principle being the same as the micro diffusion method of Conway (88). The Warburg manometric vessel was used, ammonia

liberated by K_2CO_3 being absorbed by acid in the centre well.

For initial (tissue, media or total) ammonia estimations, the centre well of a Warburg vessel contained 0.2ml N H_2SO_4 and a roll of filter paper. The side arm contained 0.3ml K_2CO_3 (saturated) solution. The vessels were tightly closed with rubber stoppers, and the K_2CO_3 solution from the side arm was quickly added into the main compartment and mixed. The pH rose to 10.5. In some cases 0.1ml of 100g per cent TCA was added prior to K_2CO_3.

Under the above conditions ammonia present in the test solutions diffused in the centre well where it was absorbed by the acid on the filter paper. The vessels were allowed to stand at room temperature overnight. This was found to be sufficient for complete ammonia diffusion and absorption. Ammonia estimations could be duplicated with accuracy, experimental deviation being $\pm$ 5 per cent.

After the overnight incubation in the presence of K_2CO_3, the roll of filter paper was taken out of the centre well of the flask and placed in graduated tubes. The centre well was washed carefully a number of times using Pasteur pipettes. The washings were added to the graduated tubes containing the filter paper.

Color was developed by adding 1.0ml Nessler's reagent (87) and 2.0ml of 2N NaOH in a total volume of 10.0ml. After standing

for twenty minutes, the color intensity was determined at 425mμ on a Bausch and Lomb colorimeter or a Beckman spectrophotometer and the ammonia content computed from standard ammonia values treated similarly and obtained concurrently with all experiments.

For the reagent blank a Warburg vessel containing 3.0ml distilled water and no tissue preparation was treated under the same conditions as in the experimental vessels.

Whenever a new test substance was used for the first time two Warburg vessels containing all solutions together with the test substance (in the absence of tissue) was set aside and treated identically to the experimental vessel. This was done in order to ascertain

(i) whether the test substance contained traces of ammonia,

(ii) the lability of the test substance to alkali used during estimation. For example, glutamine was hydrolyzed 10-12 per cent (see also ref. 21), and methionine sulfoximine is also partially deaminated. Corrections were always made for such effects.

2.7 Amino acid analyzer estimations

Sample preparation.

All samples were homogenized in the presence of 3 per cent TCA in a Potter-Elvehjem homogenizer for one minute, then transferred to graduated centrifuge tubes and centrifuged for fifteen minutes. The debris was separated from the supernatant by

decantation, washed with distilled water, recentrifuged and the washings added to the supernatant. The supernatant was made up to a known volume and extracted thrice with equal volumes of ether. This removed much of the TCA and some lipid material. Traces of ether left behind were evaporated by carefully blowing in filtered air and the volume readjusted to its original level. Aliquots were then put on appropriate columns of a Beckman 120B Amino acid analyzer. The estimations are based on the method of Spackman et al., (89) using the principle of ion exchange chromatography developed by Moore and Stein (90). The details of the reagents and column preparations, the operation of the Beckman 120B, and the calculation of results are as given in the Beckman Instruction Manual AIM-2. The essential conditions used for the acidic and neutral amino acids, and ammonia determinations, are as follows:

Acidic and neutral amino acids analyses.

The amino acids, viz. taurine, aspartic acid, glutamic acid, glutamine, glycine and alanine, that were estimated in the studies reported in this thesis, were separated on a column (50 x 0.9cm) sulphonated polystyrene - 8% divinyl benzene copolymer ion exchange resin (Type 50A-particle size, 25-31μ) at 50°C by elution with a 0.2N sodium citrate buffer at pH 3.28. The eluate then mixed with ninhydrin in a mixing manifold. The mixture then flowed through a long length of very small bore tubing in the boiling water of the reaction bath for

fifteen minutes during which time the ninhydrin reaction takes place. In the presence of amino acids, ammonia and certain amines blue diketohydrindylidene - diketohydrindamine is formed (a yellow color being formed with imino acids like proline). By maintaining constant environmental conditions the color intensity can be made proportional to the quantity of amino acid present. Using a colorimeter containing three photometer units each consisting of a light source, a lens, an interference filter, a slit, the cuvette and the photovoltaic cell, an electrical current is generated proportional to the density of color in the effluent-ninhydrin mixture. The electrical current is then used to drive a multipoint recorder which plots the results of the analysis as absorbance versus time. As the diketohydrindylidene-diketohydrindamine from each amino acid passes through the colorimeter, light to the photovoltaic cell is reduced, resulting in a reduction of electrical output and a movement on the recorder pen. Three multipoint curves are plotted simultaneously consisting of a series of peaks, each peak corresponding to a specific amino acid.

The height-width method was used to integrate these peaks. Here the height of the peak is multiplied by the width which is measured at half height. The width of a peak is measured in terms of time by counting the number of dots printed above the half height of the peak. The constant per micromole (i.e. the area of the peak per micromole) for each amino acid is obtained from an amino acid profile of a standard amino acid mixture. From these values the

concentration of each amino acid is computed. The constants were invariably obtained for each new batch of buffer or ninhydrin reagent prepared.

After the estimation of the acidic and neutral amino acids in a sample was completed, the 50cm column was regenerated with 0.2 N NaOH and equilibrated with a 0.2 N sodium citrate buffer at pH 3.28, before re-use.

Ammonia estimation.

Ammonia was estimated by elution at room temperature with a 0.35N sodium citrate buffer at pH 5.28 from a 15cm column (resin Type 15A, particle size 19-25 microns).

2.8 Dry weight estimation

Weighed cerebral cortex slices were dried on a steam bath (at about 100°C) or an electric oven at 110° C to constant weights.

2.9 Protein estimation (91)

The Lowry method (92) using the Folin-Ciocalteu reagent (93) was employed for protein estimation, the blue color being read at 750mμ. Albumin was used as standard for expressing the results.

2.10 Expression of Results

The results obtained are expressed as micromoles ammonia per gram fresh weight tissue under the given conditions, unless otherwise stated. Medium ammonia concentration is either expressed

as mM ammonia obtained on incubation with 100mg tissue, or as micromoles of ammonia that would be obtained on incubation with a gram fresh weight tissue.

The mean and standard deviation values were calculated. Most results are averages of not less than three experiments.

$$\text{Standard deviation} = \sqrt{\frac{\Sigma(x - \bar{x})^2}{n - 1}}$$

where x = experimental value

$\bar{x}$ = mean of experimental values obtained under similar conditions

n = number of experiments

3. INITIAL CONCENTRATION OF AMMONIA IN RAT BRAIN

3.1 The total solids, protein and ammonia levels present initially in two-day old infant brain, and in adult brain, cortex slices.

The results of these determinations are summarized in Table II. It is seen that the initial ammonia content under the experimental conditions used is about 5.0 micromoles per gram wet weight. Ammonia was estimated by the modified method of Braganca et al.(21) and TCA was not used here. Similar values were obtained by Weil-Malherbe and other workers with adult rats (20 - 22). However, experiments designed to give values close to in vivo levels gave extremely low quantities of ammonia in the brain of rat and other species (0.2-0.3 micromoles per gram wet weight: see Introduction). Experiments that follow are therefore attempts to control and understand the high values obtained under these experimental conditions.

It should be mentioned that on a dry weight basis (dry weight infant brain equals 13.15 per cent wet weight; dry weight adult brain equals 20.4 percent wet weight), the adult initial ammonia is lower (35 percent) than that of infant brain.

3.2 Effects of intraperitoneal injections of glucose, amytal and iproniazid on initial ammonia contents.

(i) Glucose: (dose : 100mg/100g body weight) was used because it is known to inhibit the in vitro formation of ammonia (42 - 45).

TABLE II. Initial--total solids, protein and ammonia contents of 2-day old infant and adult brain.

	% Total Solids	Protein content		Ammonia content	
		mg/g wet wt	mg/g dry wt	µmoles/g wet wt	µmoles/g dry wt
Infant	13.15 ± 0.68	69.55 ± 2.90	529.0 ± 21.0	5.06 ± 0.49	38.50 ± 3.72
Adult	20.40 ± 0.68	114.90 ± 1.30	564.0 ± 6.4	5.10 ± 0.29	25.00 ± 1.42

TABLE III. The effects of intraperitoneal injections of glucose, amytal and iproniazid on initial ammonia of adult rat cerebral cortex slices.

Test Substance	Dose per 100g body wt (mg)	Ammonia Content		
		Control Animals		Animals injected with test substance
		Not injected	Injected with physiological saline	
Glucose	100.0			5.80 ± 0.35
Amytal	12.5	5.35 ± 0.17	5.68 ± 0.18	6.15 ± 0.77
Iproniazid	1.0			4.91 ± 0.46

The animals were sacrificed 30 minutes after injections.

(ii) Amytal (94) was used because it has been established that anaesthesia results in low initial brain ammonia levels (2, 16). The dose given was 12.5mg/100g body weight and the animals were sacrificed about 30 minutes later when they were anaesthetised.

(iii) Iproniazid (dose : 1mg/100g body wt) was tried because it is an effective inhibitor of ammonia liberation from cerebral amines (95, 96). The animals were sacrificed 30 minutes after injection.

The results summarized in Table III show that these injections had no effect on the initial ammonia levels under our experimental conditions.

3.3 The effects of liquid nitrogen and TCA on initial ammonia in infant brain.

When intraperitoneal injections of known cerebral ammonia inhibitors failed to reduce the initial ammonia level, other means to achieve this aim were tried. Infant rats were dropped in liquid nitrogen and, when completely frozen, were removed. The brain was dissected while it was still frozen and ammonia estimations, with potassium carbonate, were carried out on weighed portions. In another set of experiments the effects of homogenizing infant brain

TABLE IV. The effects of liquid nitrogen and TCA on initial ammonia of infant brain (2-day old).

Condition	Ammonia Content
Cerebral tissue (control)	5.19 ± 1.17
Cerebral tissue from frozen animal	5.39 ± 0.51
Cerebral tissue homogenized in TCA (3%)	2.35 ± 0.12
Cerebral tissue from frozen animal homogenized in TCA (3%)	2.07 ± 0.17

TABLE V. Initial ammonia levels in TCA inactivated cerebral cortex slices.

Total ammonia estimated in	Ammonia Content
Tissue slice (control)	6.40 ± 0.48
TCA inactivated tissue slice	4.30 ± 0.15

Total NH_3 values include ammonia contents of the incubation (3 mins.) media.

tissue in TCA (3%) from frozen or unfrozen animals on ammonia content were studied.

Freezing in liquid N_2 followed by TCA extraction of the frozen brain is a method that has been employed by other workers investigating *in vivo* ammonia levels in brain (16, 23-28). Using our method of estimation, preliminary treatment with liquid nitrogen was without any effect on the ammonia content, but when TCA was used, there was a marked reduction of the initial ammonia level amounting to about 50 percent. These results are summarized in Table IV).

3.4 Initial ammonia levels using TCA to inactivate the cerebral cortex slice prior to ammonia estimation.

Weighed brain cortex slices were inactivated by dipping in a Ringer-phosphate medium containing 3 percent TCA, while the controls were dipped in a Ringer-phosphate medium devoid of TCA. After three minutes standing the slices were removed, placed in flasks ready for ammonia estimation, corked and potassium carbonate added. The ammonia in the media was also estimated. Total ammonia values are given in Table V. It is seen that TCA inactivated slices gave values of ammonia, 68 percent that of the control.

3.5 Effects of various substrates on ammonia estimation with K_2CO_3

Using cerebral cortex slices, the effects of the following substrates on the initial ammonia levels were then tried. This was done to observe whether such substances affected ammonia formation during the procedure of potassium carbonate estimation of ammonia. Results are given in Table VI.

(1) L-Glutamine.

The suppression of initial ammonia by TCA was thought at first to be due to suppression of glutaminase activity. Though the pH optimum for this enzyme in brain is 8.8 (8, 73), it seemed possible that under the conditions of ammonia estimation used here (pH 10.5) some glutaminase activity is retained and the glutamine present in the tissue is hydrolyzed to give ammonia. In vivo the glutamine level in rat brain is about 5.5 micromoles per gram wet weight.((16, also see Table XIII). However, both with slices and homogenates no appreciable increase in ammonia occurred on addition of 1.0mM glutamine.

(2) D- and L-Glutamate

If glutaminase was indeed responsible for the high initial ammonia inspite of the high pH, then inhibitors of this enzyme may be expected to lower ammonia levels. It is known (73) that brain glutaminase, active at pH 9.0, is strongly inhibited by

TABLE VI. Effect on ammonia formation by added substrates during the estimation of ammonia in the presence of K_2CO_3.

	Test substances	Condition of cerebral tissue	Ammonia formation during estimation			
			Controls Tissue		Tissue + test substance	
			-	3% TCA	-	3% TCA
(1)	L-Glutamine (1mM)	Slice	5.94 ± 0.26	3.22 ± 0.20	7.20 ± 0.27	3.80 ± 0.38
		Homogenate	5.30 ± 0.30	3.30 ± 0.00	5.27 ± 0.42	3.43 ± 0.71
(2)	D-Glutamate (5mM)	Slice	6.15 ± 0.52	2.60 ± 0.38	6.50 ± 0.45	2.29 ± 0.83
	L-Glutamate (5mM)	Slice	6.15 ± 0.52	2.60 ± 0.38	7.15 ± 0.05	2.24 ± 0.50
	D- + L-Glutamate (5mM each)	Slice	6.15 ± 0.52	2.60 ± 0.38	7.78 ± 1.18	2.25 ± 1.25
(3)	AMP (2mM)	Slice	6.00 ± 0.04	3.13 ± 0.27	7.53 ± 0.75	3.23 ± 1.14
(4)	Taurine (5mM)	Slice	5.78 ± 0.07	2.31 ± 0.18	5.36 ± 0.22	3.06 ± 0.08

both isomers of glutamate. The effects of D- and L-glutamate separately, or together, in the presence or absence of TCA, were then investigated. Experiments showed that ammonia levels do not diminish under these conditions (Table VI). Moreover, glutamic dehydrogenase seems to be inactive under these conditions.

(3) Adenosine monophosphate

The possibility that nucleotide deaminases (97, 98) are active under these conditions had to be investigated. The nucleotide pool is relatively small in the brain (20, 99). Added AMP (2.0mM) gives little or no increase in ammonia formation under the given experimental conditions.

(4) Taurine

A possible deamination of taurine under these conditions seems not to occur.

It should also be mentioned here that glucose does not affect ammonia formation under these conditions.

3.6 Formation of ammonia from exogenous substrates on aerobic incubation with brain tissue.

In addition to a study of the effects of substrates on the initial cerebral ammonia level estimated by K_2CO_3, studies were carried out to demonstrate their possible effects on ammonia

formation in vitro. These studies are described below and the results appear in Tables VII - X.

(1) L-Glutamine

Experiments were carried out to observe the effects of the addition of glutamine in the presence of whole brain homogenates under physiological pH at room temperature. Incubation times were nil and four hours, after which K_2CO_3 was added from the side arms for the ammonia estimation. One set of flasks had TCA (3%) present prior to the addition of homogenates. Glutamine was present at a concentration of 1.0mM. The results are given in Table VII. In the presence of TCA the formation of additional ammonia both in the presence and absence of exogenous glutamine is prevented. Though there are increases in ammonia, estimated after 4 hours incubation at room temperature, both in the presence or absence of glutamine, only an insignificant amount can be attributed to that coming from added glutamine. However, brain slices incubated at 37°C for one hour under similar medium conditions, produce ammonia from added glutamine.

(2) D-Glutamate.

Results in Table VIII show that cerebral cortex slices incubated at 37°C for one hour in a Ringer-phosphate-glucose

TABLE VII. The apparent absence of glutaminase activity at pH 7.4 and room temperature incubation using brain homogenates.

Additions to a Ringer-Phosphate Medium	Ammonia formation Incubation time, after which K_2CO_3 was added	
	0	4 hours
-	5.30 ± 0.30	13.45 ± 0.25
TCA (3%)	3.30 ± 0.00	3.12 ± 0.40
Glutamine (1mM)	5.27 ± 0.42	14.78 ± 0.83
Glutamine (1mM) + TCA (3%)	3.43 ± 0.71	3.21 ± 1.41

TABLE VIII. The deamination of glutamine (1mM) by cerebral cortex slices and the inhibition of the deamination by D-Glutamate.

Additions to a Ringer-Phosphate-Glucose Medium	Total NH_3 formation
Nil	6.30 ± 0.15
D-Glutamate (5mM)	8.71 ± 0.72
L-Glutamine (1mM)	15.55 ± 0.95
L-Glutamine (1mM) + D-Glutamate (5mM)	11.70 ± 0.70

Incubation one hour at 37°C.

medium produce ammonia from added glutamine. A significant inhibition of this glutaminase activity by addition of D-glutamate (70%) takes place.

(3) AMP and ATP

AMP has no substantial effect on initial ammonia (Table VI). Using cerebral cortex slices in a Ringer-phosphate-glucose medium and incubation at 37°C for one hour, both AMP and ATP bring about increases in ammonia formation (Table IX). That AMP is deaminated to a greater extent than ATP (Table IXa) may imply hydrolysis prior to deamination, since adenylic deaminase only attacks the monophosphate of adenosine (100). Weil-Malherbe and Green (20) using dialyzed or washed brain suspension, have reported significant ammonia formation from ATP, ADP and adenosine.

(4) Taurine.

Taurine is found in high concentrations in brain, rat brain containing 5.0 micromoles free taurine per gram fresh weight (101 , also see Table XIII). Taurine is converted to isethionic acid in rat brain (102) and heart muscle (103). This involves a replacement of an amino by a hydroxyl group. The mechanism of the conversion has not yet been elucidated; however, transamination is indicated. The possibility that

TABLE IX. The deamination of ATP and AMP by cerebral cortex slices in a Ringer-Phosphate-Glucose medium.

Nucleotide Concentration (mM)	Total ammonia formation from	
	ATP	AMP
Nil	8.95 ± 0.22	7.23 ± 0.50
1.0	16.30 ± 1.90	21.80 ± 1.20
2.0	20.90 ± 0.40	26.80 ± 3.80
4.0	31.65 ± 3.75	42.30 ± 0.50

TABLE IXa. Percentage deamination of ATP and AMP: analysis of Table IX.

Nucleotide Concentration (mM)	Theoretical yield for complete deamination μ moles	Amount of ammonia produced from nucleotide & percentage deamination			
		ATP	Percentage Deamination	AMP	Percentage Deamination
1.0	3.0	0.611	20.4	0.985	32.80
2.0	6.0	0.920	15.3	1.200	20.00
4.0	12.0	1.870	15.6	2.330	19.40

TABLE X. The effects of taurine on ammonia formation by cerebral cortex slices incubated for one hour at 37°C.

Additions to a Ringer-Phosphate Medium	Total ammonia formation
Nil	17.10 ± 1.50
Taurine (5mM)	17.35 ± 0.95
Glucose	7.60 ± 0.35
Glucose + taurine (5mM)	7.68 ± 0.88

taurine can yield free ammonia *in vitro* in the presence of cerebral cortex slices was then studied. However, from Table X it can be seen that taurine is without any effect on ammonia formation when added to the incubation medium in the presence or absence of glucose.

3.7 The source of the extra ammonia produced during potassium carbonate estimation in the absence of TCA.

Experiments were carried out to explore the source of the ammonia found in the brain, without incubation.

The following preparations were made:

Set a: Homogenates of cerebral cortex slices in a Ringer-phosphate medium.

Set b: Supernatant of Set a, prepared by centrifuging under chilled conditions for 15 minutes. The supernatant contained the soluble cell components including soluble proteins.

Set c: Supernatant of Set b after precipitation with TCA (final concentration 3%) followed by centrifuging off the precipitate.

Set d: Homogenates of cerebral cortex slices in a Ringer-phosphate medium containing 3% TCA.

TABLE XI. Initial ammonia values from cerebral cortex tissue examined under different conditions.

Set	Condition: (Tissue in Ringer-Phosphate medium)	Initial ammonia values estimated by	
		K_2CO_3 method	Amino acid analyzer
a.	Homogenate	5.35 ± 0.36	-
b.	Supernatant of a (includes soluble components)	4.47 ± 0.38	-
c.	Supernatant of b after precipitation of certain soluble components with TCA	2.54 ± 0.16	2.76 ± 0.12
d.	Homogenate prepared in TCA	3.43 ± 0.39	-
e.	Supernatant of d.	2.50 ± 0.14	3.08 ± 0.07

Set e: Supernatant of Set d.

Results summarized in Table XI show that the removal of TCA insoluble components results in a lower initial ammonia level. The addition of TCA to brain homogenates (Set d) substantially but not totally (70%) suppresses ammonia formation. It seems from these experiments that TCA may be immobilizing an enzymatic breakdown of TCA insoluble components. However, further work is required to throw more light on the process.

3.8 Kinetics of ammonia formation

Using cerebral cortex slices, the aerobic formation of ammonia with time of incubation, was carried out in a Ringer-phosphate medium at 37°C in the presence or absence of glucose. Values given in Table XII compare well with those of other workers (20, 25).

The effects of the addition of TCA (3%) to experimental flasks, on the completion of incubation, were also investigated. From Table XII it is seen that TCA, when added to a Ringer-phosphate medium devoid of glucose, has little or no effect on the total amount of ammonia present in the incubation flask.

However, in the presence of glucose, the addition of TCA at the end of one hour incubation gives a markedly lower value (4.23 micromoles per gram wet weight) than control flasks (7.03 micromoles

TABLE XII. Ammonia formation with time.

Time of incubation (hours)	Total ammonia formation			
	---		Glucose	
	--	TCA	--	TCA
0.25	9.26 ± 1.17	-	-	-
0.50	14.10 ± 0.82	-	-	-
*1.00	17.90 ± 0.05	2.63 ± 0.35	6.65 ± 0.40	2.75 ± 0.01
1.00	17.00 ± 0.19	16.50 ± 1.25	7.03 ± 0.17	4.23 ± 0.07
2.00	21.68 ± 0.68	22.50 ± 0.07	6.83 ± 0.30	7.30 ± 0.97
3.00	22.93 ± 0.17	23.20 ± 0.23	7.59 ± 0.21	8.05 ± 0.36
4.00	24.57 ± 0.36	25.57 ± 1.02	7.02 ± 0.75	7.80 ± 0.47

TCA (final concentration 3%), when added, was at the end of the incubation period, except for * when it was added before incubation commenced.

per gram wet weight). This difference, viz. (7.03 - 4.23) = 2.8 micromoles per gram wet weight, is smaller than the ammonia difference obtained when TCA is added to the flask before the one hour incubation commences, viz. (6.65 - 2.75) = 3.9 micromoles per gram wet weight, and completely disappears in 2, 3 and 4 hour incubations. This implies that during incubation in the presence of glucose, ammonia formation continues up to a point, being only prevented by the addition of TCA. In other words, it appears that there are at least two mechanisms (or sources) by which ammonia formation takes place. One, that is dependent on time of incubation, occurs not only in the presence of glucose but even to some extent in the presence of K_2CO_3 and is inhibited by TCA. The other, is suppressed by glucose and also on the addition of K_2CO_3 or TCA.

Since the final addition of TCA neither affects the glucose-sensitive ammonia formation, nor the ammonia formation in a medium devoid of glucose, its use has been discontinued in the experiments that follow.

A graphical representation of ammonia formation with time of incubation in the presence or absence of glucose, with or without terminal addition of TCA, appears in Figure 1.

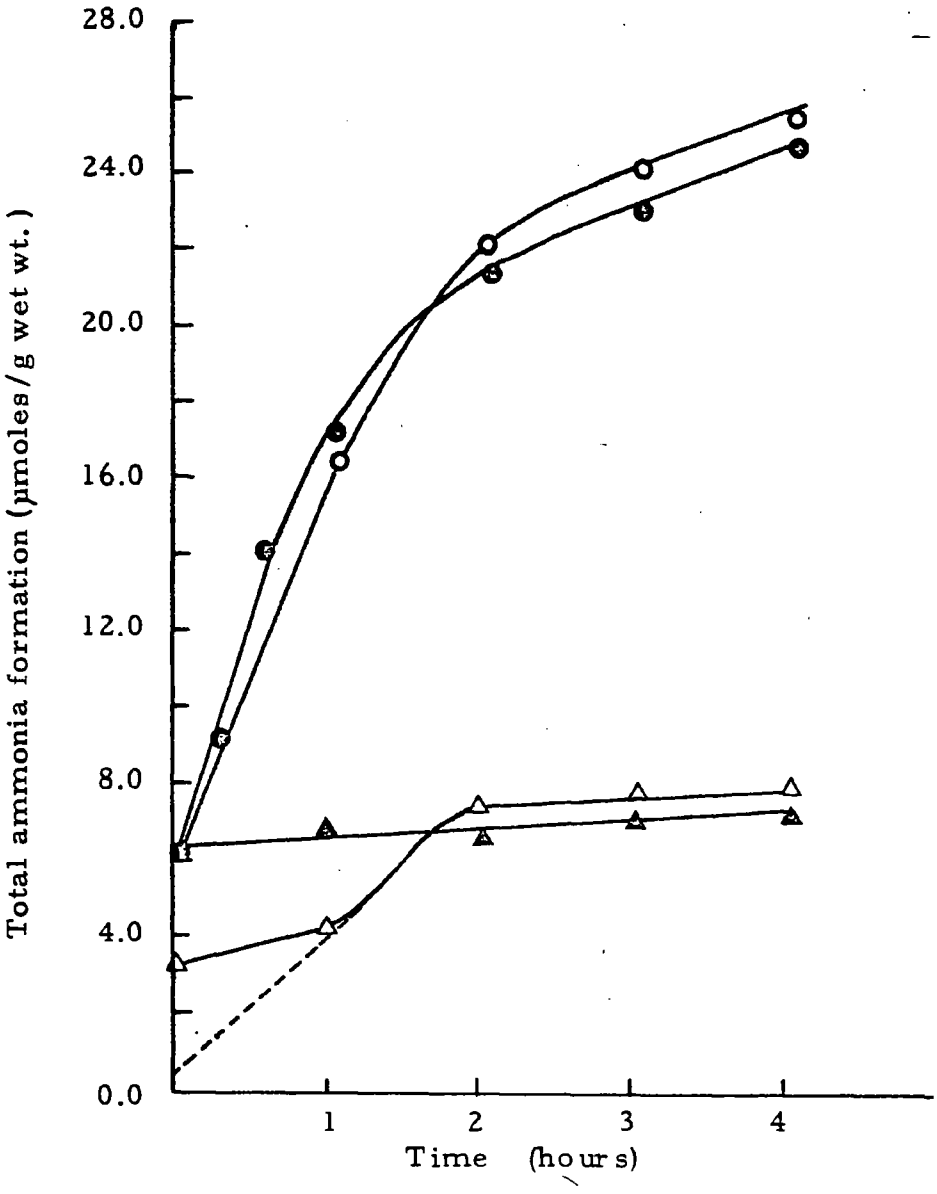

FIGURE 1. Time course of the formation of ammonia by rat cerebral cortex slices at 37°C in a Ringer-phosphate medium in the presence or absence of glucose.

● : Ammonia formation in the absence of glucose

○ : Same as (●) except that TCA (3%) was added at the end of the incubation

▲ : Ammonia formation in the presence of glucose (5mM)

△ : Same as (▲) except that TCA (3%) was added at the end of the incubation

---- : Extrapolation to the in vivo ammonia content of brain (0.3μ moles/g wet weight).

3.9 Amino acid content of cerebral cortex slices.

Initial values of amino acid content of cerebral cortex slices were determined. Parallel studies on the total (tissue plus medium) amino acid content at the end of one hour incubation at 37° C in the presence or absence of glucose, were also carried out. The results are given in Table XIII. They show that the initial values are not substantially changed on incubation of slices with glucose. In the absence of glucose however, the values of taurine, glutamate and glutamine levels fall, while the aspartate and alanine levels rise. Glutamate is known to be converted to aspartate either via the citric acid cycle (104) or by a pathway independent of it (105, 106). Oxaloacetate and pyruvate are converted to aspartate and alanine respectively by transamination reactions with glutamate (8, 80-82). Taurine is converted to isethionic acid on losing an amino group, presumably by a transamination reaction (101-103).

Under the following experimental conditions of ammonia and amino acid analysis, viz.

(1) initial,

(2) one hour incubation at 37° C,

TABLE XIII. The total, ammonia and amino acid content of cerebral cortex slices initially and on incubation in a Ringer-Phosphate medium for one hour at 37°C.

Compound estimated	μmoles per gram wet weight				
		Incubation: one hour at 37°C			
	Initial	+Glucose	*Change	No glucose	*Change
Ammonia	3.08 ± 0.07	5.33 ± 0.67	+ 2.25	16.90 ± 2.40	+13.82
Taurine	5.05 ± 0.69	5.00 ± 0.41	- 0.05	2.93 ± 0.09	- 2.12
Aspartate	2.97 ± 0.48	3.00 ± 0.12	+ 0.03	7.33 ± 0.28	+ 4.36
Glutamine[x]	5.42 ± 0.52	4.86 ± 0.21	- 1.12	2.00 ± 0.30	- 6.84
Glutamate	11.35 ± 0.86	10.02 ± 0.84	- 1.33	2.87 ± 0.13	- 8.48
Glycine	1.30 ± 0.13	1.50 ± 0.17	+ 0.20	1.05 ± 0.16	- 0.25
Alanine	0.60 ± 0.12	0.62 ± 0.21	+ 0.02	1.10 ± 0.11	+ 0.50
Total $-NH_2$ present in the free pool	35.19 ± 3.42	35.19 ± 2.85	0.00	36.18 ± 3.77	- 0.99

* Change in amide and α-amino nitrogen over initial values.

x Glutamine values taken twice for obtaining total $-NH_2$ present in the free pool.

All estimations carried out with the amino acid analyzer.

(3) one hour incubation at 37°C in the presence of glucose, it is seen that the amount of $-NH_2$ groups present in the amide and alpha amino groups of the main amino acids in brain, when added to the ammonia content, give values which are remarkably equal. In other words, the loss of amino groups is about equal to the ammonia formation. Thus, it is justifiable to conclude that, under these conditions, rise of ammonia is due to loss of amino acid nitrogen.

Conclusion

It may be concluded from these results that there are at least two mechanisms by which ammonia formation occurs in brain tissue. One that occurs immediately after death of the animal, presumably from TCA-insoluble material. Its formation is inhibited by TCA. The other occurs during aerobic incubation of brain cortex slices in a glucose-free medium. Evidence obtained shows that ammonia formation by cerebral cortex slices incubated at 37°C for one hour can largely be accounted for by the loss of amino acid nitrogen of cerebral amino acids.

4. CONDITIONS AFFECTING AMMONIA FORMATION IN BRAIN DURING INCUBATION

4.1 Ammonia formation by infant and adult rat cerebral cortex slices.

Estimations were made of the rate of ammonia formation by two day old infant and adult rat cerebral cortex slices in the presence or absence of glucose, on incubation aerobically at 37°C for one hour. Studies were also made of the rate of ammonia formation by adult rat cerebral cortex slices under anaerobic conditions. Results of these experiments on the basis of wet weight and dry weight are given in Table XIV. With reference to estimates given in terms of wet weight tissue, it should be noted that the water content of the brain changes during growth; the concentration of solids nearly doubles (107, Table II). On a dry weight basis the protein contents in infant brain and cerebral cortex slices of adult rats are found to be very similar (Table II).

It can be seen from Table XIV that, on a wet weight basis, aerobic incubation with glucose gives similar values for the rate of ammonia formation in either infant or adult cortex slices, whereas in the absence of glucose infant brain gives lower values than the adult.

On a dry weight basis, however, the reverse is true. The rate of ammonia formed in the absence of glucose is about the same within experimental error, while, with glucose, adult brain cortex

TABLE XIV. Total ammonia formation in infant and adult cerebral cortex slices in one hour at 37°C in the presence or absence of glucose (5mM).

Conditions	Total ammonia production μmoles/g wet wt.	Total ammonia production μmoles/g dry wt.	Ammonia formation on dry weight basis (Final - Initial)
I Aerobic			
A. Infant			
(i) -	10.55 ± 1.65	80.5 ± 12.5	42.0
(ii) Glucose	7.20 ± 0.70	54.7 ± 5.3	16.2
B. Adult			
(i) -	17.80 ± 1.00	87.4 ± 4.9	62.4
(ii) Glucose	8.00 ± 0.50	39.2 ± 2.4	14.2
II Anaerobic			
A. Adult			
(i) -	8.55 ± 0.74	41.9 ± 3.62	16.9
(ii) Glucose	8.52 ± 0.27	41.7 ± 1.32	16.7

Initial values obtained from Table II, i.e. Infant = 38.5 ± 3.72
Adult = 25.00 ± 1.42

slices give lower values. On subtracting initial (non-incubation) values of ammonia from those after one hour incubation, it is seen:

(1) The rate of ammonia formation in one hour by infant brain aerobically, in the absence of glucose, is 65 percent that of adult cerebral cortex slices (under similar conditions) on a dry weight basis. (On a wet weight basis the percentage is about 45.)

(2) About 16 micromoles ammonia per gram dry weight are produced in one hour when

(i) infant or adult cerebral cortex slices are incubated aerobically in the presence of glucose; or,

(ii) adult cerebral cortex slices are incubated anaerobically in the presence or absence of glucose.

4.2 Tissue and incubation medium ammonia content of adult rat cerebral cortex slices on incubation at 37°C for one hour.

Values of tissue and medium content of ammonia on incubation, aerobically or anaerobically, in the presence or absence of glucose, are given in Table XV. Values of total ammonia produced aerobically in the presence of glucose, or anaerobically in the presence or absence of glucose, are about the same. While the tissue contents of ammonia formed aerobically, in the absence or presence of glucose, are not

TABLE XV. Tissue and media concentrations of ammonia produced under aerobic and anaerobic conditions in the presence and absence of glucose from adult rat cerebral cortex slices.

Incubation conditions	Ammonia formed--µmoles/g wet weights			
	Total	Tissue	Media	(*)
I Aerobic				
(i) -	18.23 ± 1.25	3.47 ± 0.13	14.76 ± 1.12	(0.492)
(ii) Glucose	7.95 ± 1.28	3.52 ± 0.35	4.43 ± 0.93	(0.148)
II Anaerobic				
(i) -	8.55 ± 0.74	2.47 ± 0.26	6.08 ± 0.48	(0.210)
(ii) Glucose	8.52 ± 0.27	2.97 ± 0.26	5.55 ± 0.21	(0.185)

(*) Media ammonia in mM, produced by 100mg fresh weight tissue.

different, the concentration of ammonia in the incubation medium in the absence of glucose is threefold that in its presence. This results in the total ammonia formation in the absence of glucose being at least twice the total ammonia formation in its presence.

Anaerobically, there is a greater leakage of ammonia into the incubation medium, thereby leaving the tissue with a lower ammonia content.

4.3 Effects of amino acids, dipeptides and urea on the rate of ammonia formation in cerebral cortex slices of adult rat.

The addition of urea or glycine only slightly increases the aerobic formation of ammonia, when added to a Ringer-phosphate medium, and that of taurine, γABA, L-aspartate or glycyl-L-aspartate is without effect. There is only a slight diminution in the rate of ammonia formation in the presence of added L-alanine, D-aspartate or L-arginyl-L-glutamate. The presence of L-glutamate has a larger inhibitory effect on the rate of ammonia formation under similar conditions, and D-glutamate is even more effective. The results are given in Table XVI.

4.4 The effects of glucose, glycolytic and citric acid cycle intermediates on the rate of ammonia formation in cerebral cortex slices.

It may be seen from Table XVII that either lactate or pyruvate added to a Ringer-phosphate medium at concentrations of 5mM or

TABLE XVI. The effects of amino acids, dipeptides and urea on the formation of ammonia in brain cortex slices.

Addition to a Ringer-Phosphate Medium (5.0mM)	Total ammonia formation
Nil	17.37 ± 0.34
Glucose	8.18 ± 0.51
Glycine	18.36 ± 0.16
L-alanine	15.50 ± 0.30
Taurine	17.35 ± 0.95
γ-amino butyrate	17.18 ± 0.32
L-glutamate	14.64 ± 0.20
D-glutamate	11.44 ± 1.00
L-aspartate	17.10 ± 0.90
D-aspartate	16.62 ± 0.63
Glycyl-L-aspartate	17.60 ± 0.65
L-arginyl-L-glutamate	15.50 ± 0.97
Urea	19.38 ± 0.22

TABLE XVII. The effects of glycolytic and the citric acid cycle intermediates on ammonia formation in cerebral cortex slices.

Substrate(s) added to Ringer - Phosphate Medium		Total ammonia formation		
		Controls		Test Substrate
		Nil	Glucose	
Pyruvate	(5mM)	17.57 ± 0.73	8.31 ± 0.03	7.30 ± 0.27
DL-lactate	(5mM)	17.37 ± 0.55	8.22 ± 0.28	8.30 ± 0.80
DL-lactate	(10mM)	17.37 ± 0.55	8.22 ± 0.28	8.32 ± 0.28
Acetate	(5mM)	17.57 ± 0.73	8.31 ± 0.03	16.70 ± 0.63
Pyruvate (5mM) + Acetate	(5mM)	17.57 ± 0.73	8.31 ± 0.03	7.31 ± 0.24
Citrate	(5mM)	18.09 ± 0.61	6.30 ± 0.15	15.00 ± 0.90
α-ketoglutarate	(5mM)	18.09 ± 0.61	6.30 ± 0.15	16.25 ± 0.35
Oxaloacetate	(5mM)	17.05 ± 0.85	6.43 ± 0.02	11.03 ± 1.67

greater, suppresses the rate of ammonia formation to about the same extent as that due to 5mM glucose (see also Figure 2). With 5mM oxaloacetate, the inhibition of ammonia formation is not as marked. Citrate only slightly suppresses the rate of ammonia formation and acetate or α-ketoglutarate has little or no effect.

4.5 Tissue ammonia content under different experimental conditions.

Results given in Table XVIII show that the ammonia contents of cerebral cortex slices, incubated aerobically for one hour at 37°C in a Ringer-phosphate medium containing different substrates, are the same within experimental error. This implies that the variation in the rate of ammonia formation, obtained aerobically under a variety of media conditions, is seen only in the ammonia contents of the incubation medium.

Anaerobic incubations, it should be noted (Table XV), give slightly lower tissue ammonia contents, due to the greater leakage of ammonia into the incubation medium under these conditions.

4.6 The effects of glucose and pyruvate concentrations on ammonia formation from rat brain cerebral cortex slices.

Experiments were carried out to find the lowest (threshold) concentration of glucose and pyruvate that would be inhibitory to ammonia formation in an incubation of one hour at 37°C. It is seen

TABLE XVIII. Ammonia content of cerebral cortex slices after one hour incubation at 37°C in a Ringer-Phosphate medium.

Additions to a Ringer-Phosphate Medium (5mM)	Ammonia in tissue
Nil	3.87 ± 0.13
Glucose	3.74 ± 0.30
2-deoxy glucose	4.00 ± 0.10
Pyruvate	3.72 ± 0.60
Citrate	3.37 ± 0.17
α-ketoglutarate	3.90 ± 0.36
Anaerobic (Ringer HCO_3 medium)	3.16 ± 0.16

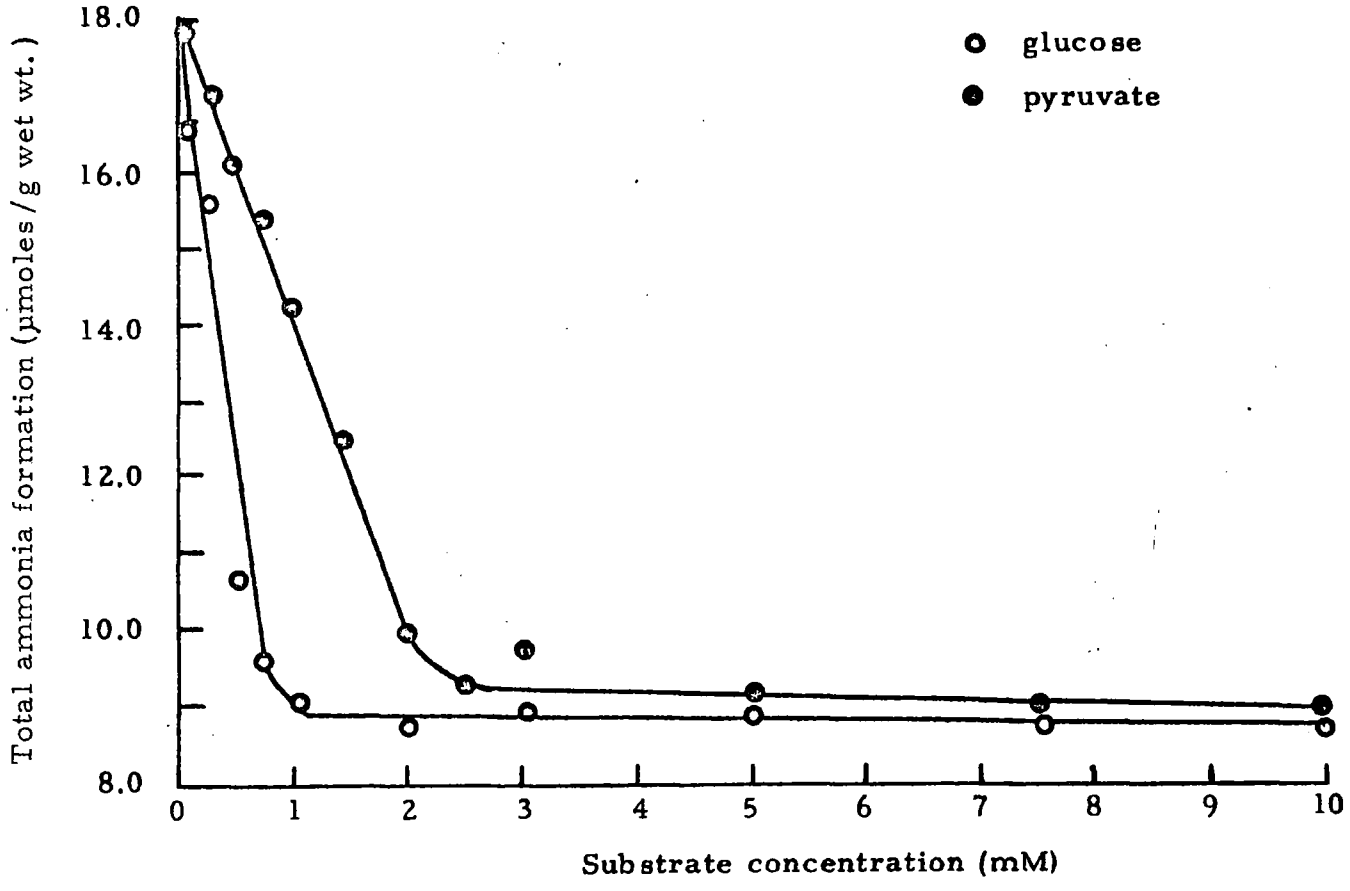

FIGURE 2. The effects of glucose and pyruvate concentrations on the rate of ammonia formation by rat brain cortex slices on incubation in a Ringer-phosphate medium for one hour at 37°C.

from Figure 2 that 1.0mM glucose is about the minimum concentration required for maximal suppression of ammonia formation, while for pyruvate the concentration is about 2.5mM. Above these concentrations the rate of ammonia formation is not further depressed. These threshold levels (viz. 1.0mM for glucose and 2.5mM for pyruvate) are utilized in further experiments carried out to obtain a further insight into the mechanisms of ammonia formation.

4.7 The effects of 0.1mM iodoacetate on the suppression of ammonia formation by glucose and pyruvate.

The increase in the rate of ammonia formation by cerebral cortex slices in an incubation medium containing glucose and iodoacetate (47), presumably occurs by the inhibition of glucose metabolism. The inhibition of phosphoglyceraldehyde dehydrogenase by iodoacetate decreases the supply of pyruvate formed from glucose and thereby the oxidation of pyruvate through the citric acid cycle, whereby intermediates (e.g. α-ketoglutarate) are formed resulting in ammonia fixation. With this in mind, the effect of iodoacetate on the rate of ammonia formation in the presence of pyruvate was then tried. It is seen from the results in Table XIX that, while iodoacetate increases the rate of ammonia formation by cerebral cortex slices in an incubation medium containing 5mM glucose, it has little or no effect when 5mM pyruvate is the substrate.

TABLE XIX. The effects of 0.1mM iodoacetate on the suppression of ammonia formation by glucose and pyruvate in rat brain cortex slices.

Additions to a Ringer - Phosphate Medium	Total ammonia formation
Nil	17.60 ± 0.40
Iodoacetate (0.1mM)	16.77 ± 1.33
Glucose (5mM)	9.00 ± 0.17
Glucose (5mM) + Iodoacetate (0.1mM)	13.48 ± 0.23
Pyruvate (5mM)	9.92 ± 0.35
Pyruvate (5mM) + Iodoacetate (0.1mM)	10.18 ± 0.15

4.8 The effects of high potassium ion concentration on the suppression of ammonia formation by different glucose and pyruvate concentrations.

The results for glucose and pyruvate are given in Tables XX and XXI respectively.

In the presence of 5mM glucose, the addition of 100mM KCl to a Ringer-phosphate medium has no effect on the rate of ammonia formation. This is consistent with the observed effects of high potassium concentrations on ammonia formation in the presence of glucose using guinea pig brain slices in a four hour incubation (9, 54, 108). This is also found to be true in the presence of 5mM pyruvate and high potassium in experiments reported here (Table XXI).

In the presence of the threshold level of glucose (1mM), a high potassium ion concentration strikingly elevates the rate of ammonia formation in one hour. The result is consistent with the conclusion that in the presence of a high concentration of KCl there is a marked increase in the operation of the citric acid cycle (77, 78) so that the intermediates from glucose are oxidized too fast to allow for the normal rate of ammonia fixation. As expected on this view, the threshold level of pyruvate (2.5mM) gives only very small increases in the rate of ammonia formation over control values when incubation is carried out in the presence of a high potassium ion concentration.

TABLE XX. The effects of high K^+ on the suppression of ammonia formation by different glucose concentrations in rat cerebral cortex slices.

Additions to a Ringer-Phosphate Medium	Total ammonia formation
Nil	16.88 ± 0.12
KCl (100mM)	16.46 ± 0.36
Glucose (0.5mM)	11.75 ± 0.35
Glucose (0.5mM) + KCl (100mM)	15.11 ± 0.71
Glucose (1.0mM)	7.71 ± 0.53
Glucose (1.0mM) + KCl (100mM)	13.55 ± 0.45
Glucose (5.0mM)	8.68 ± 0.00
Glucose (5.0mM) + KCl (100mM)	8.24 ± 0.55

TABLE XXI. The effects of high K^+ on the pyruvate inhibition of ammonia formation in cerebral cortex slices.

Additions to a Ringer-Phosphate Medium	Total ammonia formation
Nil	17.50 ± 0.23
Pyruvate (2.5mM)	10.15 ± 0.30
Pyruvate (2.5mM) + KCl (100mM)	11.91 ± 0.47
Pyruvate (10.0mM)	9.61 ± 0.68
Pyruvate (10.0mM) + KCl (100mM)	9.12 ± 0.73

TABLE XXII. The effects of inhibitors of oxidative phosphorylation on ammonia formation in rat cerebral cortex slices.

Inhibitor Used	Total Ammonia Formation			
	Additions to a Ringer-Phosphate Medium			
	Nil	Inhibitor	Glucose (5mM)	Glucose (5mM) and Inhibitor
DNP (0.1mM)	18.10 ± 0.72	13.94 ± 1.00	9.18 ± 0.47	13.98 ± 0.86
Salicylate (5mM)	17.75 ± 0.05	14.80 ± 0.10	8.80 ± 0.20	11.70 ± 0.00

In the absence of glucose high potassium is only slightly inhibitory to the rate of ammonia formation in a one hour incubation. However, for a longer incubation period (4 hours), Vrba (108) reported a considerable lowering of ammonia formation by high potassium ion concentrations, while at the same time there was a diminution in the rate of oxygen uptake.

4.9 The effect of inhibitors of oxidative phosphorylation on the rate of ammonia formation in brain cortex slices.

Results in Table XXII confirm the fact that 2,4-dinitrophenol (DNP) inhibits the rate of ammonia formation in the absence of glucose (20, 46). In the presence of glucose, DNP elevates the rate of ammonia formation, presumably by inhibiting ATP synthesis and thereby the conversion of glutamate to glutamine. Similar results are obtained here with Na-salicylate (5mM), known to uncouple oxidative phosphorylation (109. However, with salicylate the increase in the rate of ammonia formation in the presence of glucose is not so marked.

4.10 The effects of amytal (1mM), and malonate (5mM) on ammonia formation by rat cerebral cortex slices in the presence of low glucose concentrations.

In vivo, the ammonia level in the brain is depressed by nembutal narcosis (16). However, intraperitoneal injections of

TABLE XXIII. The effects of amytal and malonate on ammonia formation in cerebral cortex slices of rat in the presence of low glucose concentrations.

Additions to a Ringer-Phosphate Medium	Total ammonia formation
Nil	17.90 ± 0.20
Glucose (0.25mM)	16.68 ± 0.08
Amytal (1mM)	11.50 ± 0.35
Glucose (0.25mM) + Amytal (1mM)	11.10 ± 0.00
Glucose (1mM)	8.41 ± 1.01
Glucose (1mM) + Amytal (1mM)	8.90 ± 0.32
Glucose (1mM) + Malonate (5mM)	11.68 ± 0.33

of amytal (12.5mg/100g body wt) do not lower initial ammonia under our experimental conditions (Table III). Its effect in vitro was then investigated. It is seen from Table XXIII that 1mM amytal strongly inhibits the rate of ammonia formation in the absence of glucose, presumably by blocking the oxidation of endogenous substances that liberate ammonia. Amytal (1mM) does not affect the reduced rate of ammonia formation obtained in the presence of 1mM glucose. This may be understood if glucose oxidation still proceeds sufficiently rapidly to produce intermediates resulting in ammonia fixation.

Malonate, as may be expected, by its suppression of the citric acid cycle, elevates the rate of ammonia formation in the presence of 1.0mM glucose. Malonate in the absence of glucose has been shown to be without effect on both the rate of ammonia formation and the rate of oxygen uptake (47), indicating that in the absence of glucose the rate of operation of the citric acid cycle is greatly reduced.

4.11 Effect of glutamate derivatives and of hydroxylamine on the rate of ammonia formation by cerebral cortex slices.

The results of these studies carried out in a Ringer-phosphate medium in the presence or absence of glucose are given in Table XXIV.

(a) In the absence of glucose.

It is seen from Table XXIV that, while L-glutamate, L-glutamate-γ-methylester or L-glutamate-γ-ethyl ester added at

5mM levels inhibit the rate of ammonia formation, the inhibition is more pronounced with DL-α-methylglutamate or D-glutamate. In the presence of hydroxylamine (5mM), however, the rate of ammonia formation is elevated under the experimental conditions used. This is contrary to the results reported by Weil-Malherbe and Green (20) where a 42 percent inhibition of ammonia formation was shown to occur in a four hour incubation with guinea pig brain slices. However, the concentration of hydroxylamine they used was 1mM in the one experiment they carried out.

(b) In the presence of glucose.

Among the glutamate analogs tried, D-glutamate is the only one that elevates the rate of ammonia formation in a Ringer-phosphate glucose medium. This is understandable as D-glutamate is known to inhibit glutamine synthetase (73,110). In the presence of 5mM hydroxylamine, a considerable increase in the rate of ammonia formation occurs, which compares well with the rate of ammonia formation in a substrate free incubation medium. Under these conditions there is observed a concomitant drop in the rate of oxygen uptake by cerebral cortex slices to that value seen in the absence of glucose. A fall in the rate of oxidation of glucose (111) brought about by hydroxylamine, would result in an increased rate of ammonia formation. It is also possible that hydroxylamine competes with ammonia in the glutamine synthetase reaction (112).

TABLE XXIV. The effects of glutamate, its analogs and hydroxylamine on ammonia formation by cerebral cortex slices of rat in the presence or absence of glucose.

Experiments using the test substance	Total ammonia formation			
	Additions to a Ringer-Phosphate Medium			
	Controls		+(5mM) Test Substance	
	-	Glucose	-	Glucose
L-glutamate	17.89 ± 0.41	8.48 ± 0.32	15.16 ± 0.64	7.41 ± 0.13
D-glutamate	18.09 ± 0.61	6.30 ± 0.15	12.23 ± 1.37	8.71 ± 0.82
DL-alpha-methyl glutamate	17.05 ± 0.85	6.43 ± 0.02	12.18 ± 1.32	4.17 ± 1.02
L-glutamate-gamma-ethyl ester	17.95 ± 0.20	7.45 ± 0.10	15.16 ± 0.39	5.22 ± 0.49
L-glutamate-gamma-methyl ester	17.20 ± 0.02	7.57 ± 0.23	14.87 ± 1.10	6.32 ± 0.19
Hydroxylamine	17.15 ± 0.05	7.59 ± 0.23	19.20 ± 1.12	17.93 ± 0.91

4.12 The effects of pre-incubation in the presence of ammonium ions on the rate of ammonia formation in a Ringer-phosphate medium.

It was thought that a one hour pre-incubation of rat cerebral cortex slices at 37°C in a Ringer-phosphate medium containing NH_4Cl (5mM), might result in a reversal of the process of ammonia formation by ammonia producing endogenous substrates. This idea stems from the knowledge that exogenous NH_4^+ in the incubation medium increases glutamine and protein amide groups of brain tissue (113, 114).

The details of the experiment were as follows:

Rat brain cortex slices were pre-incubated for one hour at 37°C in the following media:

Medium A1 : Ringer-phosphate medium.

Medium B1 : Ringer-phosphate-NH_4Cl (5mM) medium.

Medium C1 : Same as B1.

After the one hour incubation was completed the slices were removed from the Warburg manometric vessels, washed by dipping momentarily and successively in three beakers containing 25ml Ringer-phosphate medium. The tissues from media A1, B1 and C1 were placed respectively in Warburg manometric vessels containing fresh media A2, B2 and C2, as described below, and allowed to incubate for one hour at 37°C. The time lapse between the end of the first and the

TABLE XXV. The effect of a one hour pre-incubation in the presence of 5mM NH_4Cl on ammonia formation by cerebral cortex slices of the rat.

Conditions	Ammonia formation
Pre-incubation control medium.	
Medium A1 - (Ringer-phosphate)	14.40 ± 0.80
Total ammonia formation. *	
Medium A2 - Tissue incubated in Ringer-phosphate, transferred to a Ringer-phosphate medium.	8.52 ± 0.29
Medium B2 - Tissue incubated in Ringer-phosphate-NH_4Cl, transferred to a Ringer-phosphate medium.	15.11 ± 0.11
Medium C2 - Tissue incubated in Ringer-phosphate-NH_4Cl, transferred to a Ringer-phosphate-TCA medium.	10.21 ± 0.09

* Subsequent incubation — 1 hr. at 37° C.

beginning of the second incubation was 15 minutes.

Medium A2 : Ringer-phosphate medium.

Medium B2 : Same as A2.

Medium C2 : Same as A2 + 3% trichloracetic acid.

Ammonia estimations were carried out on medium A1. Total ammonia was determined in A2, B2 and C2. The results are given in Table XXV. For NH_4^+ reversal to occur a value of 24.6 (i.e. 14.4 + 10.2) µmoles/g wet wt. (Table XXV) should be obtained in the second incubation; however, the value obtained is only 15.11 micromoles per gram wet weight. Hence, the results do not indicate the inhibition by added NH_4^+ of the breakdown of ammonia-forming endogenous substances.

4.13 Studies on the mechanism of the inhibitory action of glucose on ammonia formation.

Since the formation of ammonia by cerebral cortex slices is inhibited in the presence of glucose (42 - 44), it was thought that the inhibition of ammonia formation by a one hour pre-incubation of cerebral cortex slices at 37°C in an incubation medium containing glucose might be reversed on placing slices treated in such a manner in a glucose free incubation medium. The following experiment was carried out.

Rat cerebral cortex slices were pre-incubated in a Ringer-phosphate-glucose (5mM) medium for one hour at 37°C. The tissue was then separated from the pre-incubation medium, washed by dipping momentarily and successively into two beakers containing 25ml Ringer-phosphate medium, placed into Warburg manometric vessels containing fresh Ringer-phosphate medium with or without glucose, and incubated for one hour at 37°C. The time lapse between the end of the first and the beginning of the second incubations was 20 or 15 minutes, depending respectively upon whether or not a 5 minute gassing was done using pure oxygen. Josan et al. (22) observed no difference in the ammonia producing capacity of cerebral tissue when either air or oxygen were used as the gas phase.

Pre-incubation media ammonia and (total) ammonia, formed after the completion of the second incubation, are given in Table XXVI. For a complete "sparing action" of glucose on ammonia-forming substrates to occur, about 18 micromoles ammonia per gram wet weight should be produced in the second incubation. However, about 9-11 micromoles NH_3 per gram wet weight is produced. The results indicate that inspite of the inhibition of ammonia formation during pre-incubation in a glucose medium, the rate of formation of ammonia under these conditions corresponds to the rate of ammonia formation in the second hour of a two hour incubation in a glucose free incubation medium.

TABLE XXVI. The effect of one hour pre-incubation in the presence of glucose and oxygen on the ammonia formation by cerebral cortex slices in the presence or absence of glucose.

Conditions	Ammonia formation	
	Air	Oxygen
Pre-incubation medium estimated after one hour incubation.		
Ringer-phosphate-glucose medium	4.60 ± 1.05	4.12 ± 0.5
Total ammonia formation in the second hour. (incubation using either air or oxygen as the gaseous phase)		
Ringer-phosphate medium	9.58 ± 0.29	10.90 ± 1.83
Ringer-phosphate-glucose medium	4.58 ± 0.82	5.40 ± 0.41

TABLE XXVII. The effect of a one hour anaerobic pre-incubation at 37°C on the aerobic formation of ammonia by cerebral cortex slices in the presence and absence of glucose.

Conditions	Ammonia formation
Pre-incubation medium estimated after one hour. (anaerobic incubation)	
Ringer-HCO_3-glucose (5mM) medium	6.78 ± 0.75
Total ammonia estimated after the second hour incubation.	
Ringer-phosphate medium	5.65 ± 0.67
Ringer-phosphate-glucose medium	5.83 ± 0.43

4.14 The irreversible effects of anaerobic pre-incubation on ammonia formation.

A one-hour pre-incubation of cerebral cortex slices in a Ringer-bicarbonate medium was carried out. The slices were then separated from the medium, washed free from adhering Ringer-bicarbonate (by dipping momentarily and successively into two beakers containing 25ml of a Ringer solution), resuspended in fresh Ringer-phosphate medium contained in Warburg vessels and incubated after a 5 minute oxygenation for one hour at 37°C. The time lapse between the end of the first and the beginning of the second incubation was 20 minutes. The pre-incubation medium ammonia, and ammonia found after the completion of the second incubation, were estimated. Results are given in Table XXVII. It can be seen from these results that a one hour anaerobic pre-incubation at 37° C completely and irreversibly inhibits the ammonia-forming mechanism operating on incubation.

Conclusion

It may be concluded from the results in this section that the rate of formation of ammonia depends upon the proper functioning of electron transport and oxidative phosphorylation processes, and is inhibited when ammonia utilizing processes are operative. While the tissue concentration of ammonia is maintained at a constant level, the variations in the rate of ammonia formation are seen for the most part in the incubation medium.

5. TRANSPORT OF AMMONIUM IONS INTO BRAIN

Studies on ammonium ion uptake were first carried out with erythrocytes, where swelling and hemolysis commonly occur (115 - 118). The transport of ammonium ions in erythrocytes have been assumed to depend upon the permeability of the red cell membrane to anions (chloride, bicarbonate) (115, 116). In fact, Orskov (115) showed that the rate of hemolysis of mammalian erthrocytes in solutions of ammonium chloride is increased many-fold by the addition of bicarbonate. Thus, the active transport of ammonium (NH_4^+) itself cannot be measured because NH_4^+ ions pass through the membrane rapidly as shown by Jacobs and co-workers (116-118). However, an indirect method has been employed by Post and Jolly (119). They showed that the addition of ammonium ions to Na-filled red cells induces maximal active sodium transport in the absence of external potassium. Saturating the transport system with potassium ions (by incubating with external potassium) produces only a small increase in sodium transport by ammonium ions. However, ammonium ions do not facilitate potassium ion transport in low sodium cells and, presumably, cannot substitute for sodium. According to their results, NH_4^+ ions appear to substitute directly for K^+ (in erythrocytes) and require a concentration 3 - 7 times greater than potassium to produce a comparable effect.

In brain, though the ammonia content in vivo is very low in the normal state, during convulsions it is markedly increased. The administration of ammonium salts, without affecting the ATP levels (120) brings about experimental convulsions in animals due to a rise in brain ammonia. This implies the passage of ammonia across the blood brain barrier (121).

Work on the influx of NH_4^+ ions in cat brain cortex slices has been carried out by Tower and colleagues (114) and their studies demonstrate a competition of ammonium ions with potassium ions for transport into the cell.

Work reported here is a preliminary study on the uptake of ammonium ions by rat brain cortex slices.

5.1 The effect of exogenous NH_4^+ on the accumulation of ammonia in cerebral cortex slices.

These studies were carried out under the following conditions:

1) aerobically in the presence of glucose (Table XXVIII) where the percentage swelling of the brain slice is taken as 16.8 (122);
2) aerobically in the absence of glucose (Table XXIX) where the percentage swelling is taken as 44 (123);
3) anaerobically in the presence of glucose (Table XXX) where the percentage swelling is taken as 50 (122 - 124).

It is seen from the results recorded in Tables XXVIII, XXIX and XXX that, if the tissue ammonia content in the absence of added NH_4^+ to the medium, is not deducted from the tissue ammonia contents obtained at the end of the one-hour incubation with external NH_4^+, there appears a marked uptake of ammonia against a concentration gradient. For example, in Table XXVIII, 2, 5, 10, 20 and 30mM concentrations of NH_4Cl added to a Ringer-phosphate-glucose medium give tissue: medium concentration ratios of 3.74, 2.33, 1.87, 1.55 and 1.31 respectively (where the tissue water content is taken as 80% (Table II), all values being corrected for swelling (section 5.2)). It should be mentioned that the concentrations of ammonia is taken, after incubation is completed, as the added NH_4^+ concentration. These values do not significantly change especially at the high NH_4^+ concentration used. Since the tissue ammonia contents under a variety of media conditions at the end of one-hour incubation at 37^oC are about the same (Table XVIII), the tissue value with no added NH_4^+ in the incubation medium is subtracted from the tissue ammonia values found when incubation is carried out in the presence of external NH_4^+. For example, in Table XXVIII, the tissue ammonia concentrations (mM) when deductions are made, are 3.36, 7.54, 14.59, 26.89 and 35.09, for external NH_4^+ concentrations of 2, 5, 10, 20 and 30mM respectively. The deduction of the control NH_4^+ value (i.e., the tissue NH_4^+ concentration found with no added NH_4^+ in the incubation

TABLE XXVIII. The effect of ammonium ions added to a Ringer-phosphate-glucose medium on its accumulation in rat cerebral cortex slices in an incubation of one hour at 37°C and an atmosphere of pure oxygen.

NH_4Cl added to the incubation medium mM	Weight of cerebral cortex slices mg	Tissue ammonia at the end of the incubation µmoles	Tissue ammonia after correction for 16.8% swelling calculated as mM	$\frac{\text{Tissue}}{\text{medium}}$	Tissue (x) ammonia content after control ammonia value deducted mM	$\frac{\text{Tissue (x)}}{\text{medium}}$
0 (control)	67 ± 12	0.259 ± 0.028	4.11	41.6	--	--
2	72 ± 13	0.520 ± 0.071	7.47	3.74	3.36	1.68
5	71 ± 12	0.802 ± 0.026	11.65	2.33	7.54	1.51
10	75 ± 8	1.352 ± 0.026	18.70	1.87	14.59	1.45
20	89	2.670	31.00	1.55	26.89	1.35
30	79	2.948	39.20	1.31	35.09	1.17

The control medium contained 0.099 ± 0.025mM ammonia at the end of the one hour incubation. With 10mM NH_4+ the amount of swelling did not change and is 16.9% (122).

medium) seems to be a logical procedure as there is no evidence that the bulk of the autogenous ammonia is cytoplasmic and can therefore be used in assuming a concentration gradient for externally applied ammonia. In fact, the evidence already secured (Table XVIII) indicates that the autogenous tissue ammonia is remarkably constant under a variety of conditions, a fact most easily understood if the autogenous ammonia is formed in one (or a limited number) compartment of the cell and that above a limiting concentration ammonia leaks into the cytoplasm and thence into the incubation medium. Furthermore, a tissue:medium concentration ratio of 12 is obtained in an anaerobic incubation of cerebral cortex slices in the absence of added NH_4^+ (Table XXX). This is a very unlikely value as there is no case of so high a concentration ratio being obtained in brain cells under anaerobic conditions. This implies that a major portion of the ammonia is not locked up in the cytoplasm. On the other hand, if ammonia is formed in specific compartments, from which ammonia leaks above a constant concentration, the result is understandable.

TABLE XXIX. The effect of ammonium ions added to a Ringer-phosphate medium on its accumulation aerobically in rat cerebral cortex slices incubated for one hour at 37°C.

NH_4Cl added to the incubation medium mM	Weight of cerebral cortex slices mg	Tissue ammonia at the end of the incubation µmoles	Tissue ammonia after correction for 44% swelling calculated as mM	$\frac{\text{Tissue}}{\text{medium}}$	Tissue (x) ammonia content after control ammonia value deducted mM	$\frac{\text{Tissue (x)}}{\text{medium}}$
0 (control)	61 ± 3	0.236 ± 0.008	3.03	10.10	--	--
2	68 ± 11	0.490 ± 0.046	5.80	2.90	2.77	1.36
5	62 ± 5	0.716 ± 0.025	9.32	1.86	6.29	1.26
10	62 ± 5	1.100 ± 0.026	14.30	1.43	11.27	1.13

The control medium contained 0.30 ± 0.02mM ammonia at the end of the one hour incubation.

TABLE XXX. The effect of ammonium ions added to a Ringer-bicarbonate-glucose medium on its accumulation anaerocially in rat cerebral cortex slices incubated at 37°C for one hour.

NH_4Cl added to the incubation medium mM	Weight of cerebral cortex slices mg	Tissue ammonia at the end of the incubation µ moles	Tissue ammonia after correction for 50% swelling calculated as mM	$\frac{\text{Tissue}}{\text{medium}}$	Tissue (x) ammonia content after control ammonia deducted mM	$\frac{\text{Tissue (x)}}{\text{medium}}$
0 (control)	90 ± 6.0	0.237 ± 0.026	2.02	12.24	--	--
2	67 ± 3.0	0.427 ± 0.059	4.89	2.45	2.87	1.44
5	70 ± 5.0	0.758 ± 0.076	8.33	1.66	6.31	1.26
10	78 ± 8.0	1.340 ± 0.040	12.90	1.29	10.88	1.09

The control medium contained 0.165 ± 0.004mM ammonia at the end of the one hour incubation.

The work of Tower and his colleagues (114) shows that an aerobic incubation of cat cerebral cortex slices with 10mM NH_4Cl in the medium gives a tissue: medium ratio of 1.67 (tissue values being corrected for swelling and assuming 80% water content of the brain tissue). This uptake of NH_4^+ against a concentration gradient has indicated to them an active transport of ammonium ions, which they say, competes with potassium ions for the transport carrier. The tissue: medium ratio obtained in our studies with rat brain cortex slices incubated in a medium containing 10mM NH_4Cl is 1.45 (Table XXVIII), which is greater than the ratio 1.13 obtained aerobically in the absence of glucose (Table XXIX), or the ratio 1.09 obtained anaerobically (Table XXX), both with 10mM external NH_4Cl. The gradient obtained aerobically in the absence of glucose, or anaerobically, may be attributed to passive diffusion of ammonium ions into the brain slice. Thus, though some uptake of ammonium ions against a concentration gradient is indicated here, an energy-dependent transport of ammonium ions into brain cells cannot be confirmed.

5.2 Correction for swelling.

Taking, for example, the control experiment of Table XXX, the correction for tissue NH_3 concentrations due to swelling is made as follows.

Assuming the water content of adult cerebral cortex slice to be 80% (Table II), then 90mg wet weight cerebral cortex slices contain 72µl of water.

Under anaerobic conditions a 50% tissue swelling (122 - 124) increases the tissue water by 45µl to a value of 117µl at the end of one hour incubation, at which time the cerebral tissue ammonia content is 0.237µmoles and the incubation medium ammonia content is 0.165mM.

Thus, ammonia concentration in the cerebral tissue corrected for swelling equals $\frac{0.237}{0.117}$ µmoles/ml, which equals 2.02mM. Hence, the tissue:medium concentration ratio equals $\frac{2.02}{0.165}$, which equals 12.24.

Conclusion

Results given in this section point to a compartmentation of autogenous ammonia formation in cerebral cortex slices.

6. EFFECTS OF OUABAIN ON AMMONIA METABOLISM IN VITRO.

The work of Gonda and Quastel (125) using cerebral cortex slices of the rat, showed that ouabain at a concentration not inhibitory to respiration (10^{-5}M), inhibits the formation of labelled glutamine from labelled glucose. However, when a glutamine synthetase system isolated from rat brain was used, no inhibition of glutamine synthesis by ouabain was found. It was concluded from these studies that ouabain may inhibit the transport of NH_4^+ into the mitochondria, a major site of glutamine synthesis.

6.1 Effects of ouabain (0.01mM) on the rate of ammonia formation in the presence and absence of glucose.

It is seen from Table XXXI that ouabain, at a concentration (0.01mM) not inhibitory to the respiration of cerebral cortex slices (125), increases the rate of ammonia formation at the end of one hour at 37°C in a Ringer-phosphate-glucose medium.

In the absence of glucose, ouabain only slightly inhibits ammonia formation, when compared to the corresponding controls.

TABLE XXXI. The effects of 0.01mM ouabain on ammonia formation in the presence and absence of glucose using cerebral cortex slices.

Additions to a Ringer-phosphate medium	Total ammonia formation
Nil	17.78 ± 0.22
Ouabain	16.94 ± 0.27
Glucose	8.57 ± 0.17
Glucose + ouabain	10.28 ± 0.06

6.2 Effects of ouabain (0.01mM) on the rate of ammonia formation in an incubation medium containing glutamine in the presence or absence of glucose.

In the presence of glucose.

To support the contention that ouabain, by blocking the resynthesis of glutamine in cerebral cortex slices elevates the ammonia formation, the following experiments were carried out. One- and four-hour incubations were carried out and the effects of ouabain were studied using a Ringer-phosphate-glucose medium containing L-glutamine. The results given in Table XXXII are analyzed in Table XXXIIa. From these results it is seen that, ouabain in the presence of glutamine, substantially increases the rate of ammonia formation. However, in a four-hour incubation, glutamine is still not quantitatively deaminated.

In the absence of glucose.

It is seen from Table XXXIII that ouabain does not contribute to the increase of ammonia from glutamine in the absence of glucose. This result shows that ouabain does not activate glutaminase. If it did, it would work in the absence of glucose. However, more ammonia is formed from 0.5mM glutamine in one hour in the absence of glucose than in its presence (about

TABLE XXXII. The effect of 0.01mM ouabain on the formation of ammonia from cerebral cortex slices in the presence of glucose and glutamine.

Additions to a Ringer-phosphate-glucose medium	Total ammonia formation	
	1 hour	4 hours
Nil	7.88 ± 1.03	10.95 ± 1.10
Ouabain	9.45 ± 1.85	21.50 ± 2.09
Glutamine (0.5mM)	9.92 ± 0.96	--
Glutamine (0.5mM) + ouabain	12.48 ± 0.90	--
Glutamine (1.0mM)	14.94 ± 1.44	18.24 ± 1.00
Glutamine (1.0mM) + ouabain	18.55 ± 0.75	29.34 ± 3.69

TABLE XXXIIa. Analysis of Table XXXIII: Percentage deamination of added glutamine by brain slices.

Ringer-phosphate-glucose medium	Theoretical ammonia from added glutamine	Ammonia obtained from added glutamine	Percentage Deamination
	micromoles	micromoles	
One hour incubation			
Glutamine (0.5mM)	1.5	0.117	7.8
Glutamine (0.5mM) + ouabain	1.5	0.230	15.3
Glutamine (1.0mM)	3.0	0.279	9.3
Glutamine (1.0mM) + ouabain	3.0	0.462	16.1
Four hour incubation			
Glutamine (1.0mM)	3.0	0.452	15.1
Glutamine (1.0mM) + ouabain	3.0	1.031	34.4

TABLE XXXIII. The effects of 0.01mM ouabain on ammonia formation in cerebral cortex slices in a Ringer-phosphate medium containing glutamine (0.5mM) (and no glucose).

Additions to a Ringer-phosphate medium	Total ammonia formation
Nil	19.23 ± 0.25
Ouabain	18.60 ± 0.10
Glutamine	23.36 ± 0.21
Glutamine + ouabain	22.89 ± 0.29

TABLE XXXIV. The effects of 0.01mM ouabain on ammonia formation in cerebral cortex slices in the presence of 1.0mM glucose and high potassium.

Additions to a Ringer-phosphate medium	Total ammonia formation
Glucose	8.56 ± 0.68
Glucose + ouabain	13.53 ± 0.85
Glucose + potassium*	13.47 ± 0.47
Glucose + potassium* + ouabain	13.32 ± 0.22

* as KCl (100mM)

4μmoles/g wet weight compared to 1.6μmoles/g wet weight).

6.3 Effects of ouabain (0.01mM) on the rate of ammonia formation in the presence of 1mM glucose and high potassium ion concentrations.

From the results in Table XXXIV it is seen that both ouabain or high potassium reverses partially the ammonia formation by 1mM glucose to about the same extent, but together their effects are not additive. This can be explained on the basis that high potassium greatly accelerates 1mM glucose utilization so that, in effect, the system becomes a no-glucose one--i.e., ouabain will have no effect. However, while high potassium in the presence of 5mM glucose does not reverse even partially the suppression by glucose of ammonia formation, ouabain in the presence of 5mM glucose increases ammonia formation by 1.6μmoles/g wet weight (Table XXXI) when compared to a value of about 5μmoles/g wet weight obtained under otherwise similar conditions (Table XXXIV).

Conclusion

From the results in this section it may be concluded that ouabain inhibits the synthesis (but not the breakdown) of glutamine in cerebral cortex slices, presumably by inhibiting the transport of NH_4^+ into the site(s) of glutamine synthesis.

7. DISCUSSION

7.1 Initial cerebral ammonia levels.

Ammonia level in the brain prior to incubation (called here, initial level) is around 5.0μmoles/g wet weight tissue (Table II). Such a level is high when compared to the ammonia content of brain obtained by rapidly freezing the animal in liquid air or nitrogen, followed invariably by extracting the frozen brain with TCA before the estimation of ammonia by the alkali diffusion method of Conway (88). The cerebral ammonia levels of a number of species (Table I) obtained by such treatment (0.2 - 0.3μmoles/g wet weight) are assumed to be *in vivo* levels, and it is possible, as has been suggested (8), that in reality free ammonia is absent in cerebral tissue. However, Weil-Malherbe and other investigators (20, 22) obtained values around 5.0μmoles/g wet weight under conditions similar to those used in this study. Attempts have therefore been made (section 3) to account for the ammonia concentration of the brain and to devise methods affecting rates of cerebral ammonia formation.

Intraperitoneal injections of glucose, amytal or iproniazid failed to lower initial ammonia levels (Table III). Freezing infant rats in liquid nitrogen also failed to lower the initial levels. However, these values are significantly reduced (about 50 percent) on treating the tissue with 3 percent TCA prior to the alkali estimation of ammonia either

with or without fixation with liquid nitrogen (Table IV). Similar low values are obtained using the Beckman amino acid analyzer (Table XI) where K_2CO_3 is not needed. In this method the sample preparation calls for ether extraction to remove the TCA used for deproteinization. Thus the results for the initial ammonia content of the brain indicate that, in the absence of TCA treatment, an increased rate of ammonia formation takes place. That TCA itself if not an interfering factor in the estimation is shown by the results obtained by

(1) removing the cerebral cortex slices from an incubation medium containing TCA (after tissue inactivation) whereby the NH_4^+ is not affected (Table V);

(2) addition of 0.1ml, 100 g. % TCA to 0.3ml saturated K_2CO_3 in the side tube of a Warburg vessel (a value 6.10$\pm$ 0.25µmoles/g wet weight is obtained as compared with the control 5.88$\pm$0.63 where no TCA was used).

The possibility of some enzyme activity (liberating ammonia) occurring at pH 10.5 (which is the pH at which the brain is incubated in the presence of K_2CO_3), but destroyed on TCA treatment, was considered. For example, brain glutaminase having a pH optimum at 9.0 (73) can still retain some activity at pH 10.5. However, very small increases are obtained on addition of L-glutamine to the tissue immediately prior to the estimation with K_2CO_3. Also, the initial levels are not depressed either by D-glutamate or L-glutamate known

inhibitors of glutaminase. While AMP and taurine do not contribute any ammonia to initial values, glucose does not prevent its formation (initially) (Table VI).

Parallel studies using these substrates were also carried out. Homogenates left standing at room temperature for four hours produce 8μmoles/g wet weight, with an increase of only 1μmole/g wet weight, of ammonia from added L-glutamine (1mM) (Table VII). However, a greater rate of ammonia formation occurs in the presence of L-glutamine using cerebral cortex slices after one hour incubation at 37°C. This rate is strongly inhibited (70%) by D-glutamate (Table VIII). Rat brain cortex slices also form considerable amounts of ammonia from ATP and AMP when they are added to the incubation medium (Table IX). These results are consistent with the occurrence of adenylic deaminase in brain, but it should be noted that Takagaki (126) failed to observe deamination of AMP in the presence of guinea pig brain cortex slices. Taurine is known to occur in high concentrations in brain (101, Table XIII). It is converted to isethionic acid (102), but it has no effects on the rate of ammonia formation in the presence or absence of glucose (Table X).

TCA extraction of frozen brain results in a suppression of the rapid initial rate of ammonia formation, before incubation at 37°C is allowed to take place. Presumably this occurs by an inactivation of enzyme system(s) that directly, or indirectly, contribute(s) to the

ammonia formed post-mortem. According to Richter and Dawson (16), the ammonia burst after death is neither derived from glutamine nor from nucleotides, and from evidence reported in Table VI, it is seen that neither glutamine nor AMP contribute ammonia to initial ammonia levels. The increase in the initial ammonia during the K_2CO_3 estimation is seen to arise from TCA-insoluble cell components, for removal of cell debris and TCA-insoluble material gives lower initial ammonia values (Table XI). In this connection it should be noted that the existence of a very unstable neutral proteinase in brain (which could not be extracted) active for only a short time after death, has been reported by Ansell and Richter (127). Based on experiments carried out in vivo, Vrba et al.(48, 50, 51) conclude that the source of ammonia is an amidic functional group of cerebral proteins.

A kinetic study of the process of ammonia formation in the presence or absence of glucose and of the effects of TCA addition on the completion of the incubation was then carried out (Table XII, Figure 1). TCA has little or no effect when it is added at the end of incubations when the bathing medium was devoid of glucose. However, with glucose present in the medium a one hour incubation gives lower ammonia values with TCA than in the absence of TCA; while the ammonia values without TCA after two, three or four hours, are about the same as the ammonia values with TCA. This implies that ammonia formation during incubation in the presence of glucose continues up to a

point, and during its formation is only prevented by the addition of TCA.

Further experiments show that ammonia formed in one hour on subtracting the initial values, is about 16μmoles/g dry weight under the following conditions (Table XIV).

(1) Aerobic incubation of either infant or adult cerebral cortex slices in the presence of glucose.

(2) Anaerobic incubation of cerebral cortex slices in the presence or absence of glucose.

From the work of Weil-Malherbe and Drysdale (55) it is evident that the anaerobic formation of ammonia occurs up to a point (2 hours), after which no further ammonia formation occurs. Thus, the ammonia formed during aerobic incubation in the presence of glucose, may seem to be related to, or originate from, the same source as the ammonia formed during anaerobic incubation.

It may be concluded from these results that there are at least two distinct mechanisms (or processes) by which ammonia formation occurs. One that occurs immediately after death of the animal. Its formation is inhibited by TCA, but occurs to some extent in the presence of K_2CO_3. It possibly arises from amide groups of cerebral proteins. However, further work is required for complete characterization. The other occurs during aerobic incubation in a medium devoid of added substrate. Its formation is inhibited during anaerobic incubations, or during aerobic incubations in the presence of glucose.

7.2 Evidence for the compartmentation of autogenous ammonia formation.

The ammonia contents of cerebral cortex slices at the end of one hour aerobic incubation at 37°C in a variety of incubation media are remarkably constant (Table XVIII). The differences in the rates of ammonia formation, therefore, appear to be manifested in the incubation medium. For example, the incubation medium concentration of ammonia in the absence of glucose is three times that in its presence (Table XV). These facts are most easily understood if the autogenous ammonia is formed in one (or a limited number) compartment of the cell and above a limiting concentration leaks into the cytoplasm and thence into the incubation medium. This would explain why a major proportion (75-80%) of the ammonia formed by cerebral cortex slices during endogenous respiration is found in the medium while the tissue maintains a constant ammonia level.

Concomitant with ammonia formation in the absence of glucose there is a continuous efflux of NH_4^+ when the cell ammonia reaches an optimum level. This optimum limit of cell ammonia is also reached in the presence of glucose and since the rate of ammonia formation is diminished under these conditions, the efflux of NH_4^+ is greatly reduced. Thus the constant tissue ammonia value represents the limit beyond which the efflux of NH_4^+ occurs from specific cellular compartment(s) in which it is formed. It should be noted that, though the formation of ammonia is suppressed anaerobically, there is a greater ammonia leakage from

the cerebral cortex slice into the incubation medium compared to an aerobic incubation in the presence of glucose (Table XV). Consequently, the tissue ammonia content obtained at the end of an anaerobic incubation is consistently found to be slightly lower than its aerobic counterpart.

Further evidence in support for the compartmentation of ammonia formation in brain cells is obtained from tissue:medium concentration ratios of ammonia at the end of an anaerobic incubation. At the end of one hour incubation at 37°C a tissue:medium ratio of about 12 is obtained (Table XXX). This rules out the presence in the cytoplasm of a major portion of the autogenous tissue ammonia since there is no case of so high a concentration ratio being obtained in brain cells under anaerobic conditions. On the other hand, these results can be explained if ammonia is formed in specific compartment(s) from which ammonia leaks above a constant concentration. Thus, it is seen that the results point favourably to the compartmentation of autogenous ammonia formation.

7.3 Changes in the amino acid spectrum and the formation of ammonia in brain cortex slices (for incubation periods of short duration).

The origin of ammonia.

The origin of ammonia in respiring brain slices, during their incubation in a glucose free incubation medium, is a subject of much controversy.

According to Weil-Malherbe and co-workers (20), none of the six deaminating brain enzymes are likely to be involved. Glutamic dehydrogenase, they maintain, is strongly in favour of the reductive amination of α-ketoglutarate, while glutaminase can at the most account for only a small proportion of the ammonia formed. Since hexosamine, catecholamines and adenosine nucleotides in brain occur only in small amounts, the enzymes that mediate their deamination have been ruled out as contributors to ammonia formation. These workers suggest that ammonia formation occurs by a reaction closely linked to proteolysis, since guinea pig brain slices incubated for five hours give significant increases in non-protein nitrogen. In their studies they assume a single origin from which free ammonia is derived.

On the other hand, according to Vrba et al.(49) ammonia formation is too complex a process to be explained on the basis of simple proteolysis. These workers showed that not more than 25 percent of the ammonia formed by guinea pig brain slices in

four hours can be accounted for as coming from protein amide nitrogen, and consider the source of the larger part of ammonia as still being unknown. They did not, however, consider the possible formation of some of the ammonia from the entire amino acid pool present in brain slices.

In the studies of Takagaki et al. (47) the incubations of guinea pig brain slices were restricted to one hour because it was felt that ammonia formed at the end of four hours might be a result of a series of highly complicated reactions, the greater part being due to proteolysis. Glutamate (estimated by squash decarboxylase) decreases in the brain concomitant with an increase in ammonia formation when incubations are carried out in a glucose free medium; and, according to these workers, the decrease in glutamate concentrations, accounts for 50 percent of the endogenous oxygen uptake. However, it seems that their estimations of glutamate and ammonia were made in the cerebral tissue, while the incubation medium contents were not considered.

However, studies of the concentration of amino acids show the following changes in amino acids in one hour in the absence of glucose. Glutamate (-8.48μmoles), glutamine (-3.42μmoles), aspartate (+4.36μmoles), alanine (+0.50μmoles), taurine (- 2.12μmoles) and glycine (-0.25μmoles), all expressed per gram wet weight. The

net change in amino-nitrogen (refer to Table XIII) justifies our conclusion that for short incubation periods ammonia formation can be attributed to a loss of amino acid nitrogen. Thus, though incubations in a substrateless medium result in increases in the aspartate and the alanine levels, the levels of taurine, glutamine and glutamate markedly drop. That only an insignificant amount of γABA appears on the decarboxylation of glutamate in the absence of glucose was demonstrated by Haber (111) who obtained only small increases of labelled γABA from labelled L-glutamate in rat cerebral cortex slices. Thus, it is seen that the changes in amino acid nitrogen account substantially for the increase of ammonia in short incubation periods.

In the light of these results, the formation of ammonia in respiring brain slices for short incubation periods (e.g., up to one hour) will now be discussed.

Carbohydrate metabolism and ammonia formation.

Even in the absence of glucose oxygen is absorbed by brain slices and CO_2 is evolved (128) with a concomitant liberation of ammonia (Table XVII). Endogenous respiration falls with time and this is accompanied by a fall in the rate of ammonia formation (Table XII). Glucose is known to maintain cerebral respiration and at the same time suppresses ammonia formation

(Table XVII). Experiments with brain suspensions (129) and slices (130) show that under these conditions glucose is being used instead of endogenous substrates. Though there is some evidence that both protein and lipid may be utilized to support endogenous brain respiration (131), results (for short incubation periods) reported in this thesis (Table XIII) and by other workers (47) implicate free amino acids particularly glutamate, as substrates for endogenous oxidations.

Let us consider the changes taking place in the brain in the absence of glucose. Glutamate transaminates with oxaloacetate and pyruvate to give increases in aspartate and alanine (Table XIII) and the α-ketoglutarate formed in these reactions enters the citric acid cycle enabling oxygen consumption to occur from α-ketoglutarate. α-Ketoglutarate is also formed under these conditions by the operation of glutamic dehydrogenase and ammonia is liberated in the reaction (Reaction a). Ammonia is also liberated from glutamine by glutaminase (Reaction b) and glutamate formed in the reaction can undergo further degradation to give α-ketoglutarate and ammonia (Reaction a). Aspartate and alanine through transaminations with α-ketoglutarate (Reaction c), followed by the action of glutamic dehydrogenase, can give rise to ammonia (Reaction a).

a. glutamate $\longrightarrow$ α-ketoglutarate + NH_3

b. glutamine $\longrightarrow$ glutamate + NH_3

c. aspartate, (alanine) $\xrightarrow{\text{α-ketoglutarate}}$ glutamate
$\downarrow$
α-ketoacids

Since ammonia formed in these reactions cannot be utilized due to a lack of both citric acid cycle intermediates and high energy compounds, it accumulates and diffuses into the incubation medium (Table XV).

Now let us consider reactions in the presence of glucose. Glucose, as a source for citric acid cyle intermediates and a rich energy source, can either directly prevent the breakdown of glutamate or, if metabolism is viewed as a dynamic process, remove ammonia formed by the glutamine synthetase reaction. This is borne out by the fact that the addition of glucose to the incubation medium prevents ammonia liberation while simultaneously maintaining cerebral free amino acid concentrations to pre-incubation levels (Table XIII).

Lactate has also been shown to have a similar effect to glucose in preventing the fall of free glutamate levels (47).

Thus, with glucose, pyruvate or lactate where the rate of operation of the citric acid cycle is high, and with a continued high rate of formation of α-ketoglutarate and thereby glutamate

by transamination and of glutamine by fixation of ammonia in the presence of optimal ATP, the suppression of ammonia liberation is the greatest (Table XVII). The minimum levels (threshold) for this suppression to occur is around 1mM and 2.5mM for glucose and pyruvate respectively (Figure 2), which is consistent with the fact that glycolysis of one molecule of glucose yields two of pyruvate.

Iodoacetate only slightly inhibits ammonia formation (Table XIX), but this may be due to a slight diminution in oxygen uptake (47). In the presence of glucose iodoacetate however affects both glycolysis and oxygen uptake (132, 133) by its attack on triose phosphate dehydrogenase. Thus, the citric acid cycle is impeded by diminution of pyruvate formation and hence fixation of ammonia does not occur. The results show that in the presence of glucose, but not pyruvate, iodoacetate increases ammonia formation by more than 50%.

Acetate is only oxidized feebly by slices (134, 135) and hence has little or no effect on ammonia fixation. Similarly, citrate and α-ketoglutarate have little effect in replacing glucose owing to permeability barrier (136) and like acetate do not suppress ammonia formation. However, oxaloacetate affords high respiratory rates (137) and, consequently, inhibits quite strongly ammonia formation. This is due to relatively rapid influx into the brain cell and participation in the citric acid cycle.

Malonate, an inhibitor of the citric acid cycle (138) at the succinic dehydrogenase stage, increases ammonia formation with 1mM glucose (Table XXIII), but was shown to be without any effect in the absence of glucose (20, 47), presumably because it does not affect endogenous respiration (47). In the presence of glucose malonate elevates the rate of ammonia formation by inhibiting the citric acid cycle and, consequently, suppressing the utilization of ammonia. Lahiri and Quastel (36) have reported a similar effect on ammonia formation when the citric acid cycle is inhibited by fluoracetate in the presence of glucose.

Electron transfer, oxidative phophorylation and ammonia formation.

In the absence of glucose the oxidation of the ammonia-forming precursors in adult cerebral cortex slices results in a greater yield of ammonia than infant cerebral slices (62 vs. 42 μmoles/g dry weight) (Table XIV), presumably because oxygen uptake by infant brain is lower. Milstein et al. (139) showed that the oxygen consumption per mg mitochondrial protein increases with the age of the rat.

A number of investigators have reported a suppression of the rate of ammonia formation by electron transport inhibitors (20, 47). Thus, azide, arsenite, cyanide, etc., inhibit ammonia formation in a substrateless incubation medium. Amytal, a barbiturate that inhibits electron transfer between NADH and the cytochrome system,

strongly suppresses the rate of ammonia formation in the absence of glucose, and whatever little ammonia is formed under these conditions is suppressed in the presence of glucose (Table XXIII). The mode of action of amytal in suppressing ammonia formation may be due to lack of NAD+, which has to be present for activity of glutamic dehydrogenase.

Ammonia formation has been shown to be dependent on the structural integrity of the brain tissue, since the rate of ammonia formation in cell dispersions in much depressed (20).

Further evidence on the dependence of ammonia formation on the proper functioning of the electron chain is obtained from pre-incubation experiments. Though there is little change in the amino acid spectrum of brain cortex slices incubated for one hour in the presence of glucose, the rate of ammonia formation by slices, pre-incubated in a glucose medium for one hour and transferred to a glucose free medium, is lower than if no pre-incubation was carried out (Table XXVI). That this may be due to an impairment of oxidative processes leading to ammonia formation is substantiated by the observation that an anaerobic pre-incubation of cerebral cortex slices results in a complete and irreversible damage to the ammonia forming system (Table XXVII). In this connection it should

be noted that a sixty minute exposure of rabbit brain cortex slices to anoxia results in an irreversible damage to the mitochondria and phosphorylating mechanisms, and a diminution in ATP and creatine phosphate levels (140).

A direct consequence of the inhibition of the respiratory chain is a diminished rate of ATP synthesis. Both DNP and salicylate, (109) compounds that reduce cellular ATP levels, suppress the endogenous formation of ammonia. In the presence of glucose the suppression of ammonia formation is not complete in the presence of either of these inhibitors of ATP formation. These uncouplers diminish the yield of labelled glutamine from labelled glucose in the presence of rat brain cortex slices, a process well known to depend on ATP (42, 73, 77, 78). However, salicylate exerts a slighter effect than DNP (141) which may be reflected in its lower capacity to accumulate ammonia (Table XXII).

The effect of a high concentration of potassium ions on ammonia formation may be explained on the basis of its effect on the cellular energy and the respiration of rat brain cortex slices. In a glucose free medium, oxygen uptake in an incubation period of one hour, and therefore the rate of ammonia formation, are only slightly impaired in the presence of high K+ (100mM). However, in a four hour incubation Vrba and Folberger (108) reported a marked inhibition of the rate of ammonia formation at the same time

as a substantial diminution of oxygen uptake. Kini and Quastel (77, 78) demonstrated a marked increase in the operation of the citric acid cycle in K^+-stimulated brain cells, so that in the presence of 1mM (but not 5mM) glucose the intermediates are oxidized too fast to allow for the normal rate of ammonia fixation (Table XX). High K^+ ion concentration also speeds up the rate of formation of lactate from glucose. This probably explains why the K^+-effect on ammonia formation in the presence of threshold levels of pyruvate is not so marked (Table XXI).

Amino acid effects on ammonia formation.

In view of the fact that there is a profound change in the amino acid spectrum of cerebral cortex slices after aerobic incubation in a glucose-free incubation medium, it may be thought that addition of amino acids to the incubation medium might affect the formation of ammonia by cerebral cortex slices. Thus, addition of glutamate might be expected to increase the rate of ammonia formation. However, the oxidation of L-glutamate by brain slices results in a decrease in the rate of ammonia formation (Table XXIV), with a concomitant increase in amide formation (42, 73). The reaction has been formulated as follows:

$$2 \text{ glutamic acid} + \tfrac{1}{2}O_2 \longrightarrow \text{glutamine} + \alpha\text{-ketoglutaric acid} + H_2O$$

It indicates that the process of deamination of glutamate also involves the process of glutamine synthesis. Thus, addition of L-glutamate

does not necessarily involve ammonia liberation if energy is available to enable fixation of ammonia to take place with formation of glutamine. Moreover, it is a well known fact that both isomers of glutamate are inhibitors of the glutaminase reaction (73). The presence therefore of glutamate both in the incubation medium and in the cerebral tissue bathed in such a medium, may retard the further deamidation of glutamine.

Tower has pointed out that glutamate may be concentrated in the mitochondria (142). It seems probable in view of results reported in this thesis that the formation of ammonia is confined to the oxidative mechanisms present and its rate may depend on the structural integrity of the mitochondria. Formation of ammonia from glutamine and glutamate by mitochondria of rat liver and kidney has recently been reported (143). However, evidence on the compartmentation of glutamic acid metabolism in brain slices has recently appeared (144). That is to say, glutamate exists in brain in at least two distinct pools. One of these pools of glutamate is particularly concerned with the formation of glutamine, and in such a pool the breakdown of glutamine formed from external L-glutamate would be inhibited by the presence of excess L-glutamate.

The inhibition of glutamine breakdown by glutamate analogs and derivatives (viz. L-glutamate-γ-methyl ester, L-glutamate-γ-ethyl ester, and DL-α-methyl glutamate) results in a further

suppression of ammonia formation in the presence of glucose (Table XXIV). With α-methyl glutamate, Braganca et al. (145) showed, that in addition to the inhibition of glutamine breakdown, this compound is capable of forming α-methyl glutamine in a reaction analogous to the glutamine synthetase reaction. In the absence of glucose the glutamate analogs all inhibit the rate of ammonia formation, the inhibition being greatest with D-glutamate and α-methyl glutamate. Though Weil-Malherbe and Green (20) have reported that D-glutamate is without any effect on ammonia formation in a glucose-free medium, our results on the marked suppression of ammonia formation by D-glutamate is in agreement with those of Takagaki et al. (146), who also obtained a concomitant inhibition of oxygen uptake with this inhibitor. It is possible that D-glutamate and α-methyl glutamate (and to a lesser extent the other glutamate derivatives) in addition to inhibiting the glutaminase reaction also inhibit glutamic dehydrogenase.

There is no marked change in the rate of ammonia formation on the addition of glycine, L-aspartate, taurine or γABA (Table XVI). However, glutamine added to the incubation medium is deamidated by brain slices and there is more ammonia formed by the operation of this reaction in the absence of glucose than in its presence. This suggests that resynthesis of glutamine occurs in the presence of glucose since citric acid cycle intermediates and the necessary

energy supply are available. The inhibition of the formation of labelled glutamine from labelled glucose by ouabain, has been shown to occur in cerebral cortex slices (125). The addition of glutamine to a Ringer-phosphate-glucose medium in the presence of ouabain gives a marked increase in the rate of ammonia formation over the control (i.e., in the absence of ouabain) (Table XXXII). It is concluded that ouabain inhibits the resynthesis of glutamine in the presence of glucose in brain cortex slices. It may be noted that ouabain does not activate glutaminase, for if it did, it would also act on glutamine in the absence of glucose (Table XXXIII). Since ouabain has been shown to be without any effect on glutamine synthetase isolated from brain tissue (125), it is considered that ouabain affects glutamine biosynthesis by changing the fluxes of ammonium ions at the membranes of either the mitochondria or the microsomes, in which compartments glutamine synthetase is held to be active (141, 147).

7.4 The formation of ammonia in brain slices. (for incubation periods of long duration.

Most of the work reported in this thesis has involved studies of ammonia formation during incubation periods of one hour; during longer periods of incubation in a glucose-free incubation medium larger quantities of ammonia are liberated (9, 20).

As cerebral proteins contain a large proportion of cerebral cellular nitrogen, they may serve as a source of ammonia after the intracellular free amino acid pools (particularly those of glutamate and glutamine) have diminished in quantity. In a four hour incubation 25% of the total ammonia formed can be accounted for on the basis of protein amide nitrogen (49). Protein metabolism in the brain is in a dynamic state (148). In the presence of glucose, protein (114) and amino acids levels (Table XIII) remain constant since both are synthesized as fast as they are utilized. In the absence of glucose, however, there is a net catabolism of protein (149). Non-protein nitrogen has been shown to increase in an incubation of four hours in a glucose-free medium (20, 49). Amino acids, particularly glutamate and glutamine, that form during protein catabolism, can serve as sources of ammonia in the way they serve during incubation periods of short duration. Brain protein metabolism is closely related to that of peripheral nerve, where it has

been shown that stimulation is associated with an increased activity of the neutral proteinase, a decreased protein content, increased utilization of glutamate and liberation of ammonia (150).

There seems to be a relation between the rate of protein breakdown and that of ammonia formation. Conditions that inhibit protein catabolism are apparently identical with the conditions that inhibit the subsequent formation of ammonia. A neutral proteinase of whole rat brain is more active in an atmosphere of oxygen than in an atmosphere of nitrogen; and the requirement of energy for protein catabolism indicates a process different from simple hydrolysis (148). Conditions that inhibit incorporation of amino acids into proteins, or diminish cell energetics, such as anaerobiosis or the addition of cyanide, azide, DNP, as well as amino acid analogs, also inhibit protein breakdown (148). Weil-Malherbe and Drysdale (55) showed that after an initial lag (about two hours), anaerobiosis, or the presence of DNP in an aerobic incubation, completely inhibits the subsequent formation of ammonia. Moreover, though protein synthesis occurs in the microsomes, protein catabolism in brain tissue has been shown to occur in the mitochondria (148). With an irreversible damage to the mitochondria, ammonia formation does not take place, possibly due to the inhibition of the protein breakdown system.

8. SUMMARY

1. The initial (pre-incubation) ammonia content of adult rat cerebral cortex slices is about 5μmoles/g wet weight tissue. This value was obtained by a diffusion technique in which K_2CO_3 was used to liberate the ammonia from the tissue.

2. Intraperitoneal injection of glucose, amytal or iproniazid into adult rats, or the rapid freezing of infant rats in liquid nitrogen, is without effect on the initial ammonia content of cerebral tissue. However, if the tissue is treated with trichloracetic acid (TCA, 3%), the initial ammonia content is diminished by 50% giving initial ammonia values, by the alkali assay, comparable with those obtained by use of the Beckman amino acid analyzer in which ammonia is measured by the ninhydrin reaction.

3. The estimation of the initial ammonia content of cerebral cortex slices is not significantly affected by the addition of L-glutamine, D- or L-glutamate, AMP, taurine or glucose.

4. A 50% diminution of the initial ammonia content occurs when the TCA-insoluble components are removed from the tissue prior to ammonia estimation.

5. It is suggested that TCA immobilizes an enzyme system(s) of cerebral tissue partly responsible for the high initial ammonia values.

6. The addition of TCA at the end of the experimental incubation period is without significant effect on the assay of cerebral ammonia formation when brain slices are incubated either in the presence or absence of glucose.

7. The pre-incubation concentrations of the amino acids of rat cerebral cortex slices are not significantly changed on incubation of the tissue for one hour at 37°C in the presence of glucose.

8. In the absence of glucose marked changes in the pre-incubation levels of amino acids of cerebral cortex slices occur during subsequent incubation. These are as follows: taurine (-2.12 μmoles/g wet weight), glutamine (-3.42 μmoles/g wet weight), glutamate (-8.48 μmoles/g wet weight), glycine (-0.25 μmoles/g wet weight), aspartate (+4.36 μmoles/g wet weight) and alanine (+0.5 μmoles/g wet weight), there being a change of (-12.83 μmoles NH_2 - N/g wet weight). A considerable rise in ammonia formation (+13.82 μmoles/g wet weight) also occurs under these conditions. This can be largely accounted for by the net loss of amino-nitrogen groups of the amino acid pools in the brain slices.

9. Certain substrates that support cerebral respiration inhibit the rate of cerebral ammonia formation. Glucose, lactate or pyruvate at 5mM levels suppress the rate of ammonia formation by rat cerebral cortex slices to about the same extent. 5mM oxaloacetate is less inhibitory and acetate, citrate or α-ketoglutarate has little or no effect on the rate of ammonia formation owing

probably to permeability barriers.

10. The threshold level for the maximum suppression of the rate of ammonia formation by rat cerebral cortex slices is 1.0mM for glucose and 2.5mM for pyruvate. This is consistent with the fact that glycolysis of one molecule of glucose yields two of pyruvate.

11. The rate of ammonia formation by cerebral cortex slices of the rat is elevated by 0.1mM iodoacetate in the presence of glucose but not in the presence of pyruvate. These facts indicate that cerebral oxidation of pyruvate may be responsible for the suppression of the rate of ammonia formation. In the absence of added substrate, iodoacetate is only slightly inhibitory to the rate of ammonia formation which may be due to a slightly diminished oxygen uptake.

12. In the absence of glucose the yield of ammonia, in one hour, from adult rat cerebral cortex slices (on a dry weight basis) is greater (by about 50%) than that from infant rats. This is consistent with the known fact that the rate of cerebral oxidation of glucose in the adult rat is greater than that in the infant rat.

13. The addition of 100mM KCl to a Ringer-phosphate medium increases the rate of ammonia formation by rat brain cortex slices in the presence of 1mM glucose (threshold level), but not in the presence of 5mM glucose. This is probably because high K+ ion

concentration increases the rate of glucose breakdown in brain slices, so that with initially 1mM glucose, the brain tissue soon becomes deprived of glucose. High K+ ion concentration also speeds the rate of formation of lactate from glucose. This probably explains why K+ ions affect ammonia formation in the presence of 1mM glucose but not in the presence of 2.5mM pyruvate. In the absence of added substrate high K^+ ion concentration is only slightly inhibitory to the rate of ammonia formation in one hour.

14. The inhibition of the citric acid cycle by malonate (5mM) in the presence of 1mM glucose, increases the rate of ammonia formation by cerebral cortex slices by suppressing the formation of citric acid cycle intermediates, required for ammonia fixation to occur.

15. Amytal (1mM) diminishes the rate of ammonia formation by cerebral cortex slices in the absence of glucose. This presumably occurs because amytal suppresses the oxidation of glutamate.

16. In the presence of glucose, uncouplers of oxidative phosphorylation (e.g. DNP, 0.1mM; or salicylate, 5mM) increases the rate of ammonia formation by brain cortex slices by inhibiting the synthesis of glutamine (an ATP dependent process). In the absence of glucose there is a diminution in the rate of ammonia formation by these inhibitors.

17. Anaerobiosis suppresses the rate of ammonia formation, probably by suppressing the oxidation of glutamate. It irreversibly damages the ammonia forming system present in cerebral cortex slices.

18. With the exception of glutamine, none of the naturally occurring amino acids (e.g. alanine, glycine, γABA, taurine, L-aspartate) of cerebral tissue when added to incubated cerebral cortex slices significantly affects the rate of ammonia formation. The glutamate derivatives (DL-α-methyl glutamate, L-glutamate-γ-methylester, and L-glutamate-γ-ethylester) at 5mM levels inhibit the rate of ammonia formation both in the presence of glucose (by suppressing glutamine hydrolysis) and in the absence of glucose (by suppressing glutamate oxidation). Hydroxylamine (5mM) increases the rate of ammonia formation both in the presence or absence of glucose, presumably by competing with ammonia for glutamine synthetase.

19. D-glutamate has the following effects on the ammonia metabolism of cerebral cortex slices: a) it suppresses the rate of ammonia formation in the absence of glucose, doubtless by inhibiting glutamic dehydrogenase; b) it elevates the rate of ammonia formation in the presence of glucose by inhibiting glutamine synthetase; c) it suppresses the rate of ammonia formation from L-glutamine in the presence of glucose by inhibiting glutaminase.

20. AMP or ATP in the presence of brain cortex slices brings about an increase in the rate of ammonia formation.

21. Results obtained point to a possible compartmentation of the site of ammonia formation and the retention (within the compartment(s)) of ammonia up to a limiting level, above which the efflux of ammonia into the incubation medium takes place. Such a conclusion helps to explain the fact that the brain tissue ammonia is not markedly affected by the presence or absence of glucose, as the increased quantity of ammonia formed in the absence of glucose is found largely in the incubation medium. It also explains the apparent high concentration ratio (tissue:medium) of NH_4^+ at the end of one hour incubation in the presence of glucose, under aerobic or anaerobic conditions where the concentration ratio (tissue:medium) is 42 or 12 respectively.

22. Evidence obtained from the examination of NH_4^+ influx from the incubation medium, shows some accumulation of NH_4^+ ions in rat brain cortex slices against a concentration gradient.

23. Ouabain, at a concentration of 0.01mM which has no effect on the rate of respiration of rat brain cortex slices, a) increases the rate of ammonia formation in the presence of glucose but has little effect in the absence of glucose, b) increases the rate of ammonia formation from added glutamine in the presence of glucose but it does not affect the rate of ammonia formation from added glutamine in the absence of glucose. These results are consistent with the

known fact that 0.01mM ouabain inhibits the rate of biosynthesis of glutamine by rat cerebral cortex slices respiring in a Ringer-phosphate medium containing glucose. It is suggested that ouabain affects this process by altering the fluxes of NH_4^+ ions at the membranes of either the microsomes or the mitochondria in which compartments glutamine synthetase is held to be active. Ouabain has no effect on the cerebral glutaminase of the rat.

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