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STUDIES ON THE ANOXIC IMPAIRMENT OF CEREBRAL RESPIRATION

A thesis presented in candidature

for the degree of

Master of Science

by

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October

1974

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ABBREVIATIONS

CNS	Central Nervous System
CPM	Counts per minute
CSF	Cerebro-Spinal Fluid
L-DOPA	L-Dihydroxyphenylalanine
Dopamine	3-Hydroxytyramine
DPM	Disintegrations per minute
DTT	Dithiothreitol
EEG	Electroencephalogram
5-HT	5-Hydroxytryptamine
5-HTP	5-Hydroxytryptophan
PPO	2,5-Diphenyloxazole
POPOP	1,4-Di- 2-(5-phenyoxazolyl) -benzene
TCA	Trichloroaceticacid

SUMMARY

It was shown that when the respiration of adult rat brain slices is measured *in vitro* in calcium containing saline, a period of anoxic preincubation at 37°C in basal saline causes a severe impairment of this respiration. The respiratory defect can, however, be prevented if any of the following conditions exist:

1. The slices are derived from new born (7 day old) rats.
2. The preincubation is carried out at 0°C instead of at 37°C.
3. A supernatant of 50 (w/v) brain homogenate is used as preincubation medium.
4. Entire hemispheres are preincubated, instead of slices.

These conditions suggested that the damage was caused by a loss of material from the slices, and attempts were made to characterise and isolate this material from the brain supernatant. Although these attempts were not totally successful, experiments indicated that the potassium ion content of the supernatant contributed substantially to its protective activity.

The role of potassium ions was confirmed by the fact that the conditions that protected against the anoxic impairment of respiration also maintained the potassium ion concentration of the slices. Conversely, depleting

the potassium ion concentration of the slices under aerobic conditions produced an impairment of respiration similar to that induced by anoxia. Finally the use of buffered solutions of potassium chloride as preincubation media were shown to mitigate the effects of anoxia.

It was also shown that potassium stimulated respiration and the incorporation of amino acids were more vulnerable to anoxia than unstimulated respiration. These results were discussed in relationship to recently published findings that the susceptibility of the brain to anoxia *in vivo* may be affected by extra-neural factors such as a defective post ischaemic circulation.

1.1.0 INTRODUCTION

In advanced industrial societies mental and neurological diseases represent more than half the total burden of medical care. Without assuming an aetiological connection it is only reasonable to expect that with the spread of industrialisation the rest of the world will be faced with similar problems. The understanding of the brain and its malfunction must therefore be one of the most urgent and socially important areas of research. It is perhaps a reflection of the complex nature of the problems that despite much effort, neurochemical research has not yet provided us with much insight into the working of the brain.

1.1.1 CEREBRAL ANOXIA

One of the conditions to which the brain is particularly susceptible is oxygen deprivation. Much attention has been paid to determining the duration of anoxia necessary to produce loss of brain function. A period of anoxia as short as 15 s will result in a loss of consciousness, but restoration of the oxygen supply to the brain will restore consciousness without any apparent permanent impairment of function. After about 5 min. of anoxia it is not possible to restore function as irreversible neurological damage has occurred (Meyer 1963 a).

1.1.2 CLASSIFICATION OF ANOXIA

Oxygen deprivation, anoxia, may be classified in several ways, and the one given below is that of Peters and Van

Slyke (1931).

Anoxic Anoxia: This arises from a lack of oxygen in the blood while the cerebral circulation is maintained e.g. when the p O_2 of the inspired air falls below a critical level.

Ischaemic Anoxia: This is due to a decreased blood flow to the brain and is accompanied by a reduction in glucose supply and a build-up of metabolic end products in the affected area.

Histotoxic anoxia: This is an inability of the cell to utilise oxygen due to a poison such as cyanide.

Such a classification is of limited use in human pathology as the various forms may co-exist. Moreover circulatory defects that arise following anoxia tend to blur the distinction between conditions where there is only a lack of oxygen and those in which the supply and removal of metabolites are also impaired.

1.2.0 ANOXIA AND CEREBRAL METABOLISM IN VIVO

The sequence of events that occurs after the brain has been subjected to various types of anoxia has been studied in live animals.

Denny-Brown and Meyer and their associates produced varying degrees of ischaemic anoxia by the experimental occlusion of the larger arteries (for review see Meyer 1963 b). The electroencephalogram, oxygen tension, cerebral blood flow, pH, blood pressure and sodium and potassium movements in

the cortex were all recorded simultaneously. During nitrogen inhalation a fall in cortical oxygen tension was observed 5 or more seconds before changes appeared in the electroencephalogram. When the pO_2 fell below 8 mm an efflux of potassium into the extracellular space took place. A fall in the pH in the ischaemic zone caused capillary dilation and a reduction of vascular resistance. If the consequent fall in blood pressure persisted longer than 2 mins. microstasis occurred, the capillaries became engorged, endothelial damage developed, haemorrhages appeared and the tissue became swollen. Even at this stage restoration of the blood pressure resulted in a reversal of the damage; but if the pressure remained low the process went on to infarction.

Kaasik and his associates, amongst others, have studied the level of ATP and phosphocreatine in the brains of rats subjected to 'asphyxia' i.e. anoxic anoxia (Kaasik et al 1970 a) and 'stagnant hypoxia' i.e. ischaemic anoxia (Kaasik et al 1970 b). In the first case they found that after 3 mins. of respiratory arrest almost all the phosphocreatine had disappeared and the ATP level dropped by more than 50%. In the second the fall in phosphocreatine and ATP levels was smaller though still significant.

Other studies (Lowry et al 1964, Gurdjian et al 1949) have also shown that anoxia results in a drop in the level of high energy phosphates in brain.

The fall in the energy reserves of the brain during anoxia

suggests that energy requiring processes, such as the synthesis of macromolecules would also be depressed. Yap and Spector (1965) subjected rats to unilateral ischaemic anoxia and showed a reduction in the incorporation of ^{14}C from uniformly labelled glucose into the protein of the affected hemisphere. This depression of protein synthesis persisted after the anoxia was relieved.

1.3.0 ANOXIA AND CEREBRAL METABOLISM IN VITRO

The majority of biochemical investigations into cerebral anoxia have been carried out, not on live animals, but on brain preparations of various types. The behaviour of these preparations *in vitro* is, however, in many ways similar to the situation *in vivo*. It is not intended to give here a comprehensive review of the various effects of anoxia on the brain *in vitro*, but merely to mention a few of the more obvious effects that emphasise this similarity.

1.3.1 ANOXIA AND CEREBRAL ENERGY RESERVES

The main function of respiration is to provide energy in the form of high energy intermediates. The brain is known to have very small reserves of energy and a high metabolic rate. Inhibition of respiration should therefore lead to a rapid depletion of the high energy intermediates. Subjecting brain slices to anoxia is known to deplete their phosphocreatine and ATP (Cohen 1961). It follows from this that processes that require high energy phosphates such as the active transport of ions and the synthesis of macromolecules will also be depressed.

1.3.2 ANOXIA AND CEREBRAL ELECTROLYTES

Slices of rat cerebral cortex when incubated in oxygenated Krebs-Ringer bicarbonate buffered medium accumulate potassium and pump out sodium (Pappius and Elliot 1956). This is thought to occur through ion pumps probably driven by ATP (Deul and McIlwain 1961). Calculations based on electrochemical gradients across the cell membrane (Keesey and Wallgren 1965) suggest that as much as one quarter of all the energy available from respiration would be needed to maintain the optimum sodium and potassium concentrations in brain slices. It is therefore not surprising that anoxic brain slices are unable to maintain a high potassium concentration relative to the incubating medium (Pappius and Elliot 1956).

1.3.3 ANOXIA AND EXCITABILITY

It has long been known that when brain slices are incubated aerobically in the presence of glucose, electrical stimulation increases the uptake of both glucose and oxygen (McIlwain 1951). If there is no glucose in the incubating medium electrical stimulation has little effect on respiration. Subsequent addition of glucose to such a preparation does not restore the normal response to electrical stimulation (McIlwain 1963 a). If the incubation is carried out in the presence of glucose but in the absence of oxygen electrical stimulation depresses glycolysis. Subsequent reoxygenation does not restore the normal response to electrical stimulation (McIlwain 1963 b).

Increasing the potassium ion concentration of the medium

in which brain slices are incubated to 100 mM is also known to bring about a marked increase in their rate of respiration (Dickens and Greville 1935; Ashford and Dixon 1935). It has been suggested that both methods of respiratory stimulation have a common basis (Quastel and Quastel 1961).

1.3.4 ANOXIA AND BIOSYNTHESIS

The effect of anoxia on the biosynthesis of macromolecules in brain slices has many similarities with the effect *in vivo* on the intact brain. Ghosh and Cohen (1974) subjected brain slices to anoxia and showed that the incorporation of ^{14}C from uniformly labelled glucose into proteins, lipids, DNA and RNA decreased progressively to zero within 15 mins. If the slices were reoxygenated they recovered the ability to incorporate ^{14}C into protein, lipids DNA and RNA but only at a very much lower rate than those attained before they were deprived of oxygen. This irreversible aspect of anoxic damage is also described by Yanagihara (1972).

1.3.5 ANOXIA AND CEREBRAL MONOAMINES

Although the prime function of oxygen in cerebral metabolism is to oxidise glucose and yield high energy phosphate intermediates it is also required in the formation of the catecholamines and 5-hydroxytryptamine (5-HT). Tyrosine hydroxylase and tryptophan hydroxylase, the probable rate-limiting enzymes in the synthesis of the monoamines require molecular oxygen for activity since the new hydroxyl groups of dihydroxyphenylalanine (DOPA) and 5-hydroxytryptophan

(5-HTP) are derived from molecular oxygen (Daly et al 1968). Anoxia may therefore be expected to impair the synthesis of monoamines and this has been shown to be so by Davis and Carlsson (1973). They have also suggested that this decreased synthesis is accompanied by a decrease in the turnover of the monoamines so that the level in the brain is not significantly reduced.

1.4.0 ANOXIA AND THE RESPIRATION OF BRAIN SLICES

From what has been said so far it will be clear that the effects of anoxia in vitro generally parallel those in vivo and that in either situation they are much as one would expect: a reduction in, or a cessation of oxidative phorphorylation; the consequent exhaustion of ATP and phosphocreatine reserves, and therefore failure of such energy-requiring processes like ion transport and biosynthesis. There remains the question why in several respects temporary anoxia, of quite brief duration, produces permanent impairment of, for example, the respiratory response to electrical stimulation or the ability to synthesise proteins and nucleic acids.

A possible answer to this question may lie in the suggestion of J.S. Haldane that, "Anoxaemia not only stops the machine but wrecks the machinery". (Barcroft 1920). The findings of Phizackerley and Fixter (1973) are therefore very interesting; for whilst others have also found that a period of anoxia severely and irreversibly impairs the ability of rat brain slices to respire subsequently in glucose containing saline (e.g. Dickens

and Greville 1933; Elliot and Rosenfeld 1968) Phizackerley and Fixter have shown that this impairment is caused by the leakage of material from the slices during anoxic preincubation. If anaerobic preincubation was carried out under conditions which minimised leakage or counteracted its effects the slices retained their ability to respire when their oxygen supply was restored. These conditions included the preincubation of entire hemispheres rather than slices, the preincubation of slices at $0^{o}C$ rather than $37^{o}C$ and the preincubation of slices at $37^{o}C$ in the supernatant of a 50% (w/v) homogenate of cerebral hemispheres in saline. Phizackerley and Fixter also showed that the active constituents of the brain supernatant were L-glutamine and L-aspartate and that the addition of these substances to the preincubation saline made it almost as effective as the brain supernatant in preventing damage during anaerobic preincubation.

1.5.0 THE ROLE OF CALCIUM IONS

There is, however, a fundamental shortcoming in the work of Phizackerley and Fixter. Their incubations were carried out in the phosphate buffered saline of Krebs (1950) which unlike extracellular fluid contain no calcium and in which the phosphate concentration is approximately seven times that in the extracellular fluid.

Now calcium ions are known to have a crucial effect on many aspects of cerebral metabolism. For example they are involved in the release of neurotransmitters (Katz 1969) and regulate axonal conductivity and excitability

(Brink 1954; Grundfest et al 1954). Much more to the present point, they affect the respiration of brain slices. Their absence from the incubating medium stimulates the respiration of the slices in a manner similar to that produced electrically or by the presence of high concentrations of potassium ions (McIlwain and Bachelard 1971). Conversely electrical and potassium stimulation are known to increase the intracellular calcium content of brain slices (Bull and Trevor 1972).

In view of this it was a very serious matter that if Phizackerley and Fixter's experiments were repeated in media containing calcium ions and buffered with Tris instead of phosphate (i.e. in conditions that approximate more closely to those *in vivo*) the addition of L-glutamine and L-aspartate to the preincubation medium did not protect against anoxic damage. (Fixter personal communication.)

1.6.0 THE AIM OF THE THESIS

The work described in this thesis was undertaken to establish whether the experiments with calcium containing media invalidated the theory of Phizackerley and Fixter that the loss of respiratory capacity when brain slices are aerobically incubated is due to loss of material or whether they can be explained by some suitable modification of the original theory.

It would of course be naive to extrapolate results from such a system to the problems of anoxic damage *in vivo*. It is nevertheless hoped, that the exploration of the

mechanism through which anoxic damage occurs in a simple system *in vitro* may eventually provide an insight which will prove useful in the treatment of cerebro-vascular disease or cardiac arrest.

CHAPTER 2

MATERIALS AND METHODS

2.1.0 MATERIALS

2.1.1 SALINES

All the salines used in the experiments were based on those of Krebs (1950) in which constituent solutions were isotonic with 0.154M sodium chloride. The final concentrations of all the substances present in the salines are given in table 2.1. The pH of the salines was adjusted to 7.4 at room temperature.

2.1.2 CHROMATOGRAPHY MATERIAL

Sephadex G25 was obtained in the dry form from Pharmacia, London. AG1-X8 100-200 mesh strongly basic anion exchanger, AG2-X8 200-400 mesh basic anion exchanger and Biorex 70 200-400 mesh weakly acidic cation exchanger were obtained from Bio-Rad Laboratories, St. Albans, Hertfordshire. Dowex-50W 20% cross linked strongly acidic cation exchanger was obtained from Sigma Chemical Company, Kingston-upon-Thames, Surrey.

2.1.3 PROTEOLYTIC ENZYMES

Pronase 45,000 units/g obtained from BDH Chemical Ltd., Poole, Dorset.

Carboxypeptidase A 30 units/mg protein in toluol aqueous solution was obtained from the Sigma Chemical Company.

Table 2.1

Composition of Salines

	Basal Saline (mM)	Glucose Saline (mM)	LiGlucose Saline (mM)
Calcium Chloride	1.0	1.0	1.0
Sodium Bicarbonate	3.5	3.5	0
Tris-HCl	20.0	20.0	20.0
Potassium Chloride	4.7	4.7	4.7
Potassium Dihydrogen Phosphate	1.2	1.2	1.2
Magnesium Sulphate	1.2	1.2	1.2
Glucose	0	11.5	11.5
Sodium Chloride	120.0	114.6	0
Lithium Chloride	0	0	118.1

Papain 0.06 units/mg, insolubilized on to a cellulose support, was obtained from Miles Servac (Pty) Ltd. of Maidenhead, Berkshire.

Trypsin, 7,500 BAEE units/mg was obtained from Koch-Light Laboratories Ltd. of Colnbrook, Buckinghamshire.

2.1.4. ULTRAFILTRATION

Diaflo ultrafiltration cell with stirrer and Diaflo membranes (43.0 mm diameter) were obtained from Amicon Corporation, High Wycombe, Buckinghamshire.

2.1.5 DIALYSIS

Visking tubing 8/x was obtained from the Scientific Instrument Centre Ltd., 1, Leake Street, London W.C. 1.

2.1.6 AMINO ACIDS

Synthetic L-amino acids were obtained from T.J. Sas and Son Ltd. of Holborn, London W.C. 1.

2.1.7 RADIOCHEMICALS

L-Leucine-4, 5-T 100 m Ci/mM and Inulin (carboxylic acid - C14) 5-15 m Ci/mM were obtained from the Radiochemical Centre, Amersham.

2.1.8 MISCELLANEOUS COMPOUNDS

L- β-3,4- Dihydroxyphenylalanine (L-DOPA), 3-Hydroxytyramine.HCl (Dopamine), 5-Hydroxytryptamine (5-HT), 5-Hydroxyl-L-Tryptophan (5-HTP) and Reserpine were all

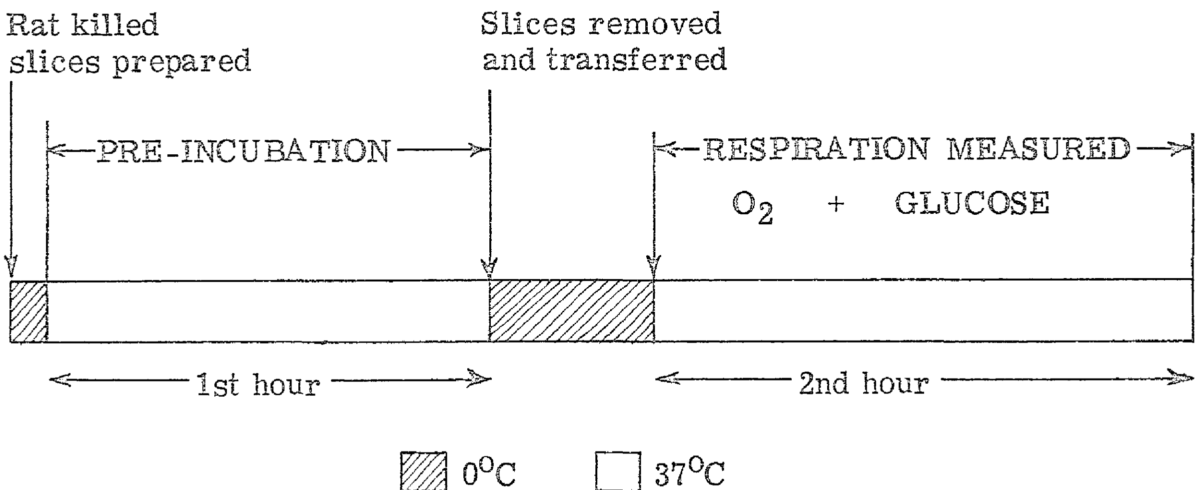


Fig 2.1

obtained from the Sigma Chemical Company. Other chemicals used were either Analar or the purest commercial grade available.

2.1.9 SCINTILLATION FLUID

The Scintillation fluid was made up to contain 0.5% (w/v) of PPO and 0.01% (w/v) of POPOP in a 50% (v/v) solution of Triton X-100 in toluene.

2.1.10 ANIMALS

Wistar rats were obtained from the departmental animal house.

2.2.0 METHODS

2.2.1 PLAN OF EXPERIMENT

The basic experiment was designed in two parts (figure 2.1). The first part involved the anaerobic preincubation of the slices of cerebral cortex in various media and the second part involved their incubation in oxygenated glucose saline so that the rate of respiration of the slices could be measured and compared to the rate of respiration of slices that had been preincubated aerobically in glucose saline. The difference in the rates of respiration during the second period was regarded as a measure of the damage caused to the slices by anaerobic preincubation. When media were assayed for their protective activity this was given in terms of the rate of respiration of brain slices that had been anaerobically preincubated in that particular medium for 60 minutes at 37°C.

2.2.2 PREPARATION OF SLICES FROM CEREBRAL CORTEX

Slices were cut from the brains of rats weighing between 100-140 g. because rats within this weight range have brains that are biochemically indistinguishable from those of mature rats and their skulls are soft and can be easily and rapidly opened. The animals were killed by cervical dislocation and decapitation. The cerebral hemispheres were removed into ice-cold glucose saline. The entire procedure took about 1 minute.

Slices were cut from the pial surface of the hemispheres. The procedure was initially carried out freehand using a recessed guide (McIlwain and Rodnight 1962) but because of the high degree of skill required to produce slices of uniform thickness by this method it was necessary to design a microtome which gave slices of uniform thickness and consistent size with a minimum of manual skill. The microtome was made from perspex in the departmental workshop, and its performance was well up to specification. It was designed to be used with interchangeable recessed guides which had troughs milled to different depths. The guide used routinely was one with a trough of 0.3 mm and with this it was possible to obtain three slices from each hemisphere without penetrating into the underlying white matter.

The guide was fitted into the base with the wire stretched across the top of the trough (figure 2.2). The hemispheres were placed in the trough with the ventral surface uppermost

THE MICROTOME

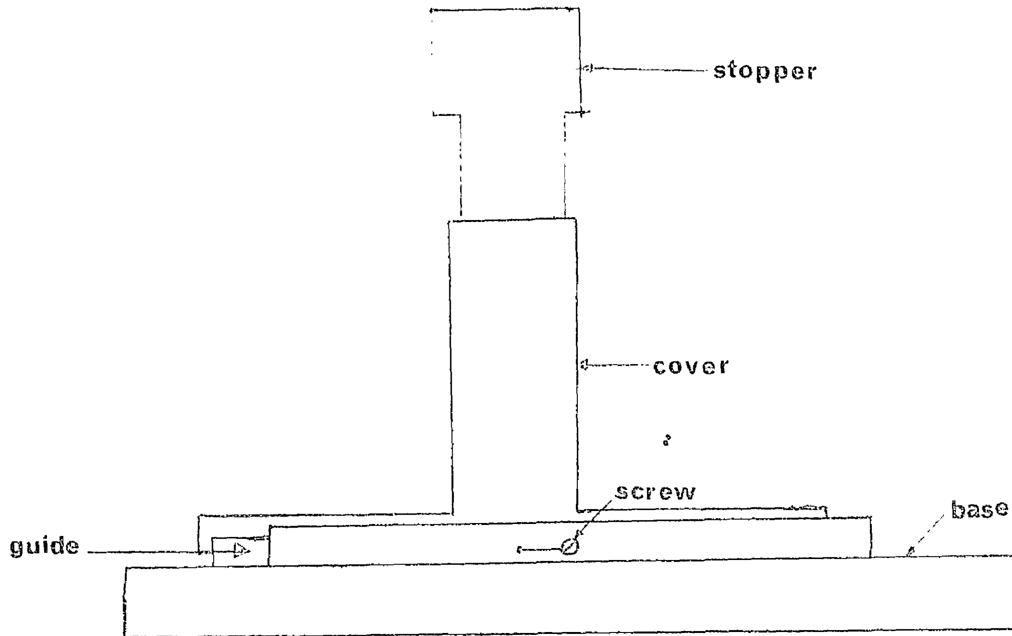
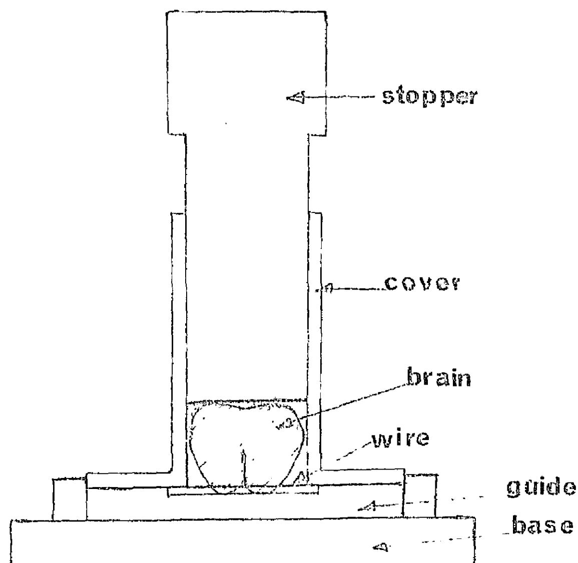


Fig 2.2 b



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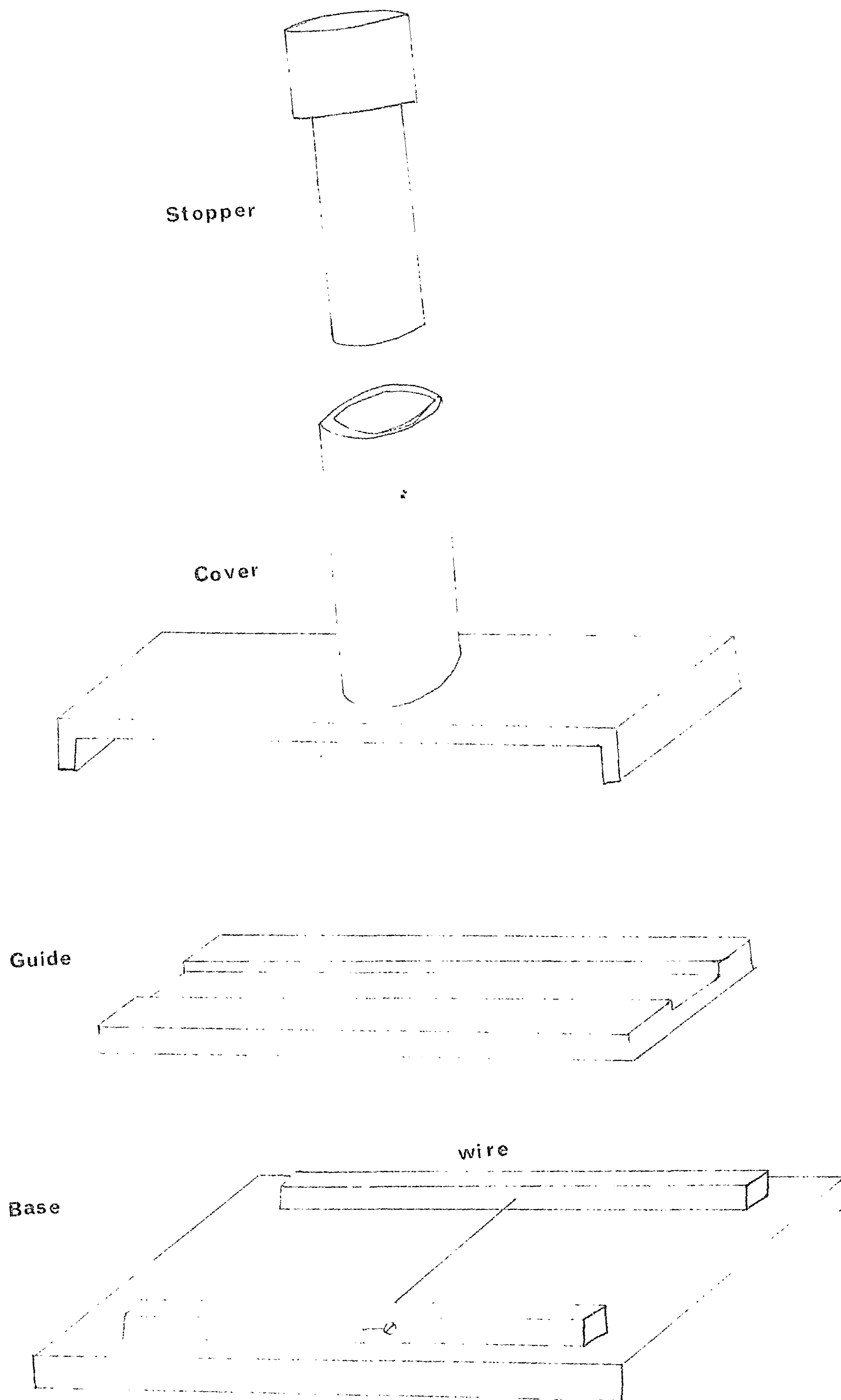


Fig 2.2a

and to one side of the wire. The cover and stopper were placed over the guide so that the wire was pressed taut across the trough and the hemispheres were held in place within the well of the cover. The cover and guide were then moved smoothly so that the hemispheres passed through the wire cutter in the base. The cover was then lifted off and the slices were removed from under the hemispheres with a platinum wire. The process was repeated twice to obtain three pairs of slices.

Each slice was cut in two and so four pieces of tissue were obtained from each of the three levels. Each reaction flask contained a piece from each of the three levels, as the rate of respiration of brain slice depends on the layer of the cortex from which it is obtained. (Elliot and Henderson 1948). Sufficient material for four flasks could be obtained from one animal.

2.2.3 THE PREINCUBATION

This was carried out in Warburg flasks attached to manometers. The main compartment of the flask contained three pieces of tissue in 1.0 ml of preincubation medium. For aerobic preincubation this medium was usually glucose saline and the flasks were gassed with 100% oxygen. In the case of an anaerobic preincubation the flasks were gassed with oxygen free nitrogen and contained a stick of yellow phosphorus in the centre well to remove any residual traces of oxygen. The preincubation medium varied according to the experiment but in the case of the anaerobic control it was basal saline. The preincubation was

carried out at 37°C and lasted 60 minutes.

2.2.4 AEROBIC INCUBATION

Following the preincubation the slices of cerebral cortex were briefly rinsed in ice cold glucose saline and transferred to a fresh set of Warburg flasks which contained 1.0 ml of glucose saline in the main compartment. The centre well contained 1.0 ml of 4M KOH absorbed on to a piece of fluted filter paper. The flasks were gassed with 100% oxygen and placed into the water bath at 37°C. After an initial period of 20 minutes of thermoequilibration the uptake of oxygen was normally measured over a period of 40 minutes.

The slices were then removed, rinsed briefly in ice cold distilled water and transferred to pre-weighed weighing bottles and dried for at least 3 hours at 110°C. The dry weights were then determined using a Mettler ME22 microbalance. The rate of respiration was then calculated in terms of µl of oxygen consumed per h per mg dry weight.

When potassium stimulated respiration was measured, the slices were incubated in 0.9 ml of glucose saline and 0.1 ml of 1.0M potassium chloride was included in the side arm of the flask. After thermoequilibration the potassium chloride was tipped into the main compartment, and the rate of respiration measured as usual.

2.2.5 PREPARATION OF BRAIN SUPERNATANT

The initial medium used to protect the slices during anaerobic

preincubation was the high speed supernatant of a 50% (W/V) homogenate of cerebral hemispheres in basal saline. The cerebral hemispheres were removed into ice cold glucose saline, blotted on filter paper and weighed. An equal amount (w/v) of basal saline was added and the homogenate was prepared using a MSE top drive homogeniser for 3 min. at full speed. The usual amount of homogenate prepared was about 40 ml. The homogenate was centrifuged at 105,400g ($r_{av.}$ 5.9 cm) for 2 h at 4°C and the supernatant removed.

The supernatant could be stored frozen for indefinite periods without loss of activity.

2.2.6 PREINCUBATION OF ENTIRE HEMISPHERES

One of the conditions that protected against anoxic damage was the preincubation of entire hemispheres. The brain was removed from the animal as usual, but instead of cutting slices the cerebrum was divided into its hemispheres by cutting along the central fissure. Each hemisphere was preincubated in a Warburg flask with 0.1 ml of basal saline. Anaerobic conditions were obtained in the usual way by gassing with 100% nitrogen and including a stick of yellow phosphorus in the flask.

After preincubation the hemisphere was removed from the flask and slices were cut from its dorsal surface in the usual manner. The rate of respiration of these slices in oxygenated glucose saline was measured.

2.2.7 PREINCUBATION OF SLICES FROM NEW BORN RATS

Slices were cut from the brains of 7 day old rats in the usual manner but because of the size of their brains only one, or at best two, pair(s) of slices could be obtained from each animal.

2.2.8 HEAT TREATMENT OF SUPERNATANT

The supernatant was added to a stoppered centrifuge tube and placed in a bath of boiling water for 30 min. After 30 min. it was removed from the bath, cooled in running water and centrifuged on a bench centrifuge for 10 min. at full speed.

2.2.9 TREATMENT WITH ACID

The supernatant was made 0.1N in acid by the addition of concentrated hydrochloric acid. This was then placed in a boiling water bath for 30 min., after which it was allowed to cool. The pH was adjusted to 7.4 with a concentrated solution of sodium hydroxide.

2.2.10 TREATMENT WITH ACTIVATED CHARCOAL

Purine and pyrimidine derivatives were adsorbed on activated charcoal. The method was essentially similar to that described by Thomson (1969) except that the supernatant was shaken with the charcoal for 16 h at 0°-$4^{o}C$. The supernatant was recovered by centrifugation.

2.2.11 DIALYSIS OF THE SUPERNATANT

This was carried out using Visking tubing. The tubing

was washed in distilled water and boiled in a solution of 10% (w/v) sodium bicarbonate before use. A volume of the supernatant was enclosed in the tubing and dialysed against a large excess (approximately 100fold) of basal saline at 0°-4°C for 16 h.

2.2.12 GEL FILTRATION WITH SEPHADEX G25.

15 ml of supernatant were applied to a column (28 x 1.5 cms) of sephadex G25. The sephadex was swollen according to the makers instructions in the appropriate amount of basal saline. Twenty five fractions each 5 ml in volume were collected using the basal saline as the eluent.

2.2.13 ULTRAFILTRATION OF THE SUPERNATANT

The supernatant was filtered through various cellulose nitrate membranes. The procedure was carried out at 5°C and the supernatant was kept under pressure with 20 lbs p.s.i. of nitrogen gas.

2.2.14 TREATMENT OF SUPERNATANT WITH PROTEOLYTIC ENZYMES

The proteolytic enzymes used were: Pronase, Carboxypeptidase A, Papain, and Trypsin.

5.0 mg of Pronase were incubated with 2 ml of brain supernatant for 2 h at 37°C. The supernatant was then heated to 100°C for 5 min. to deactivate the enzyme.

0.1 ml of Carboxypeptidase A in Toluol aqueous solution was diluted to 1.0 ml in basal saline and dialised overnight at 4°C against 500 ml of basal saline to remove the toluol.

0.1 ml of the dialysis residue was incubated with 3.0 ml of supernatant for 30 min. at 37°C. After treatment the enzyme was removed from the supernatant by filtering the mixture through a UM05 membrane filter.

Papain was incubated with brain supernatant at a concentration of 1.0 mg/ml for 2 h at 37°C and then separated from it by [illegible]filtration.

Trypsin was incubated with brain supernatant at a concentration of 1.0 mg/ml for 2 h at 37°C. The enzyme was then deactivated by heating the mixture at 100°C for 10 min.

Samples of glucose saline were treated with each of the enzymes in a manner identical with that for the supernatant. By aerobically preincubating slices in these it was possible to determine whether the experimental conditions had any effect on the subsequent respiration of the slices.

2.2.15 MATERIAL OBTAINED FROM LIVE ANIMALS

In order to obtain a supernatant that was depleted of monoamines 3 rats were subcutaneously injected with 0.3 ml of reserpine solution that had been made up to a concentration of 1.0 mg/ml in a 20 (v/v) solution of acetone acidified with acetic acid until 0.1N. The rats were decapitated 4.0 h after the injection and a supernatant prepared from their cerebral hemispheres in the usual way. A control supernatant was prepared from the brains of rats that had been injected with basal saline.

All injections were performed by Dr. R.Y. Thomson who was also responsible for extracting blood from a rat so that rat serum could be used as a preincubation medium.

2.2.16 DETERMINATION OF ELECTROLYTES

After preincubation the slices were dried on a glass plate and weighed on a torsion balance. They were then homogenised in a test tube homogeniser with 5 ml of 6% (w/v) trichloroacetic acid and allowed to stand for 30 min at room temperature. After centrifugation at 3,000 r.p.m. for 10 min the supernatant was removed into 25 ml volumetric flask. The pellet was washed twice with distilled water and the washings added to the flask. The combined supernatants were made up to 25 ml with distilled water. The sodium and potassium were then determined using an EEL flame photometer. All the glassware used was rinsed in 20% (v/v) nitric acid followed by distilled water.

2.2.17 DETERMINATION OF LEUCINE INCORPORATION

After preincubation in the appropriate medium for 60 min. at 37°C the slices were transferred to a set of Warburg flasks containing 1.0 ml of glucose saline with 1.0 µCi of 3H-Leucine and 0.1 µCi of 14C - Inulin dissolved in it. after the usual aerobic incubation for 60 min. the slices were removed from the flasks, blotted dry on a glass plate and weighed on a torsion balance. Each set of slices was then homogenised in a test tube homogeniser with 3.0 ml of 5% (v/v) trichloroacetic acid. The resulting precipitate was recovered by centrifugation, washed three

times with TCA (the third wash was heated for 15 min at 90°C), and extracted with diethylether-ethanol 1:1 (v/v) and diethylether. The dried sample was dissolved in 1.0 ml of 1.0N sodium hydroxide. The TCA supernatant and the TCA washings were pooled and made up to 10.0 ml in a volumetric flask.

1.0 ml of the TCA soluble fraction was mixed with 10.0 ml of scintillation fluid and counted in the ISOCAP/300 Liquid Scintillation System supplied by Nuclear Chicago. The TCA non soluble fraction (0.3 ml) was mixed with 0.7 ml of TCA and 10.0 ml of scintillation fluid and similarly counted; as was 1.0 ml of the incubation medium mixed with 0.9 ml of distilled water and 10.0 ml of scintillation fluid.

CPM for both 14C and 3H were converted to DPM by using the external channels ratio and quench correction curves prepared with single isotope standards. The ratio of the free, or TCA soluble, leucine DPM in the inulin and non-inulin spaces of the slices was determined from the distribution of the 14C inulin.

CHAPTER 3

RESULTS

3.1.0 THE EFFECT OF ANAEROBIC PREINCUBATION ON THE SUBSEQUENT RATE OF RESPIRATION OF BRAIN SLICES.

3.1.1 THE ANAEROBIC IMPAIRMENT OF RESPIRATION

After preincubation at 37°C without glucose and oxygen, the rate of respiration of brain slices in oxygenated glucose saline was less than the rate of respiration of brain slices that had been preincubated aerobically in glucose saline. The time course of the damage to respiration is shown in figure 3.1. It can be seen that in basal saline the impairment of respiration increases with time and is nearly maximal at 40 minutes. Sixty minutes was chosen as the standard time of preincubation to ensure a large difference between the rates of respiration of aerobic and anaerobic slices.

In table 3.1 the anoxic impairment of respiration after 60 minutes preincubation is shown for slices incubated anaerobically with and without glucose. It can be seen that glucose affords a large degree of protection against the anoxic impairment of respiration. The investigation was therefore concerned mainly with the damage caused by the combined lack of oxygen and glucose in order to obtain conditions which produced a large defect. The situation was analogous in some respects to ischaemic anoxia.

EFFECT OF PREINCUBATION ON SUBSEQUENT RESPIRATION

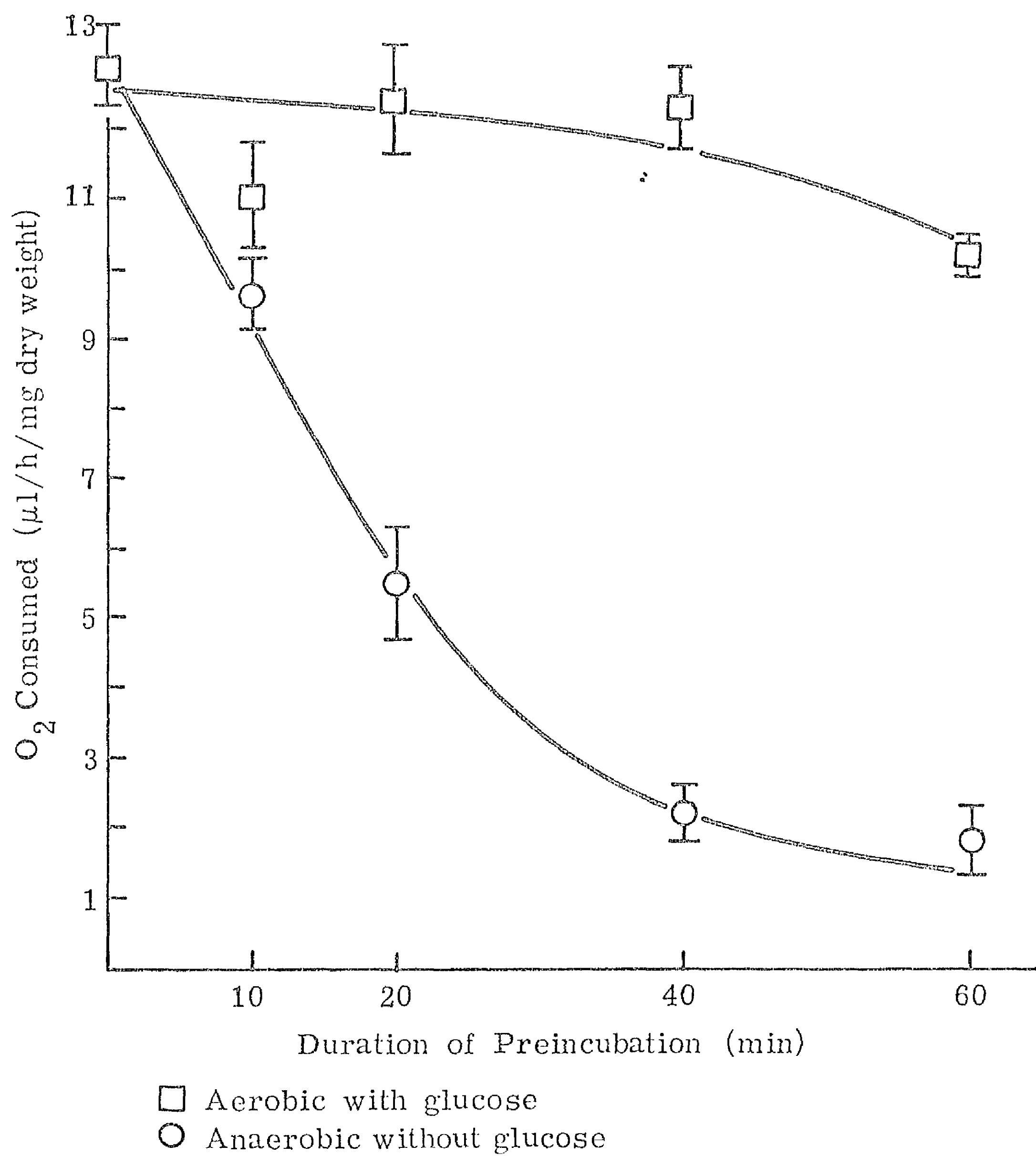


Table 3.1

The Effect of Anaerobic Preincubation for 60 min.

Preincubation Conditions (at 37°C)	Rate of Respiration in 2nd hour (μl/h/mg dry weight)
Glucose Saline, O_2	10.1 ± 0.33 (6)
Basal Saline, N_2	1.8 ± 0.47 (6)
Glucose Saline N_2	5.6 ± 0.52 (6)

Results are given as the mean value ± one SEM. The number of experiments is given in brackets.

Table 3.2

The Greater Resistance of Neonatal (7 day old) Rats to Cerebral Anoxia

Preincubation Conditions (60 min at 37°C)	Rate of Respiration in 2nd hour. (μ l/h/mg dry weight)
Slices from neonat rats in glucose saline + O_2	8.5 ± 0.35 (4)
Slices from neonat rats in basal saline + N_2	5.9 ± 0.36 (4)
Slices from adult rats in glucose saline + O_2	10.1 ± 0.33 (6)
Slices from adult rats in basal saline + N_2	1.8 ± 0.47 (6)

Results are given as the mean value ± one SEM.
The number of experiments is given in brackets.

3.1.2 PROTECTION AGAINST ANOXIC DAMAGE.

In order to determine whether anaerobic preincubation for 60 minutes always results in a severe decrease in the rate of respiration of brain slices, a number of preincubation procedures were examined.

It was found that slices from new born rats were less vulnerable to anoxic damage and that aerobically preincubated slices respired at a rate considerably lower than that for slices from adult rats similarly treated (table 3.2).

Slices from adult rats also respired at a high rate after anaerobic preincubation if the preincubation was modified in one of the following ways.

1. The preincubation was carried out at 0°C rather than at 37°C.
2. Entire hemispheres were preincubated and slices cut from them after the preincubation.
3. A supernatant of a 50% (w/v) homogenate of brain in basal saline was used as the preincubation medium.

Table 3.3 shows the extent of the protection afforded by these conditions.

Since brain supernatant was effective in preventing a decrease in the rate of respiration following anaerobic preincubation, other tissue extracts were also examined to see if they too could protect the slices. Table 3.4 shows the effect of rat liver supernatant and rat serum. Although the liver supernatant showed considerable

The Effect of Anaerobic Preincubation of Brain Slices in Varying Concentrations of Brain Homogenate Supernatant.

(Each point represents the mean of 2 experimental values)

FIG 3.2

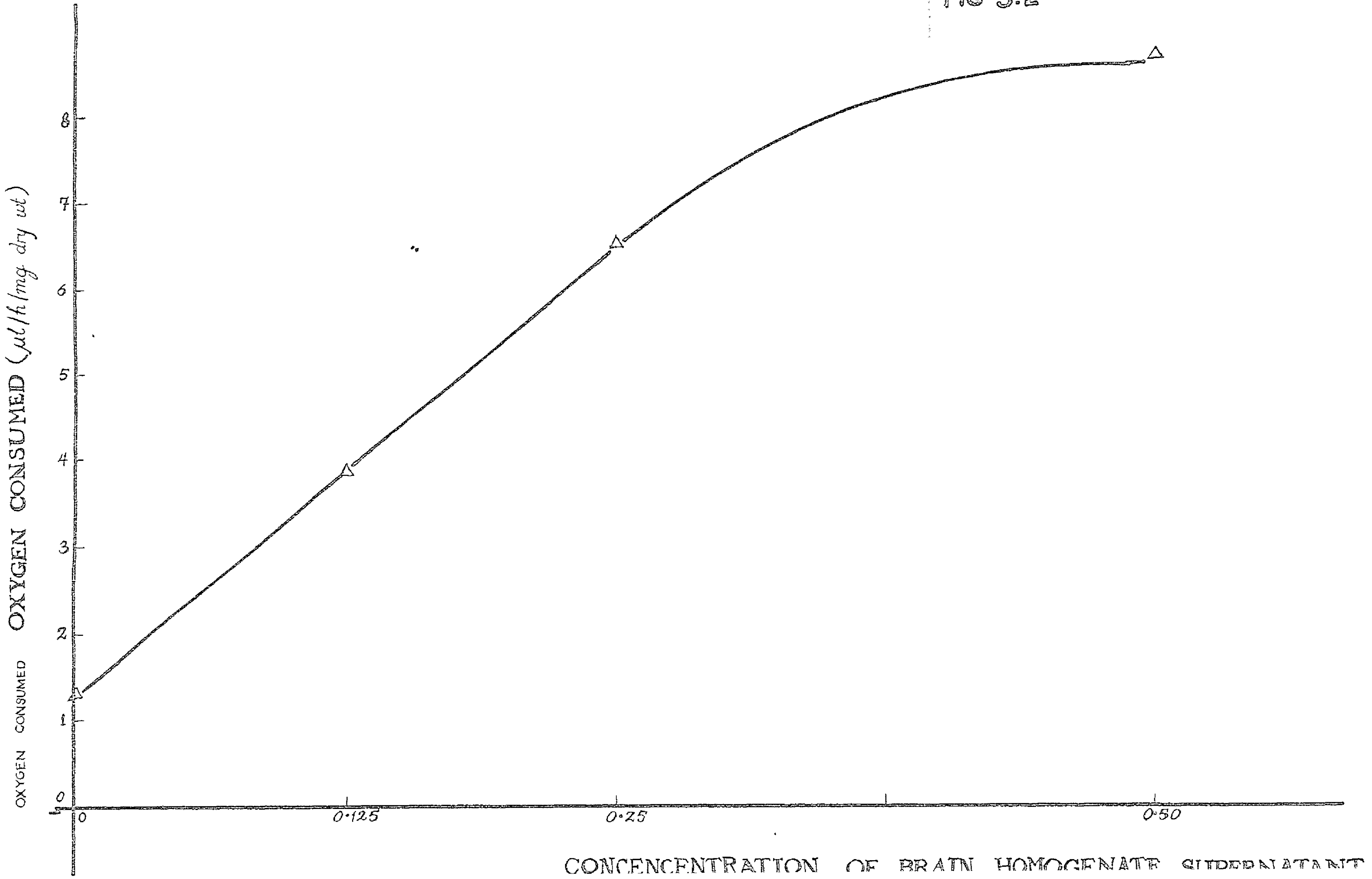


Table 3.3

Conditions that Protect Brain Slices from Adult Rats Against Anaerobic Preincubation

Preincubation Conditions (for 60 min)	Rate of Respiration in 2nd hour (μl/h/mg dry weight)
Glucose Saline + O_2 at 37^oC	10.1 ± 0.33 (6)
Basal Saline + N_2 at 0^oC	9.1 ± 0.31 (4)
Brain Supernatant + N_2 at 37^oC	12.0 ± 0.63 (6)
Entire hemisphere + N_2 at 37^oC	11.9 ± 0.59 (4)
Basal Saline + N_2 at 37^oC	1.8 ± 0.47 (6)

Results are given as the mean value ± one SEM.
The number of experiments is given in brackets.

Table 3.4

Protective Activity of Various Tissue Extracts

Preincubation Conditions (60 min at 37°C)	Protective Activity (μ l/h/mg dry weight)
Glucose Saline + O_2	10.1 ± 0.33 (6)
Brain Supernatant + N_2	12.0 ± 0.63 (6)
Liver Supernatant + N_2	9.1 ± 0.53 (6)
Serum + N_2	2.6 ± 0.54 (4)
Basal Saline + N_2	1.8 ± 0.47 (6)

Results are given as the mean value ± one SEM.
The number of experiments is given in brackets.

The brain and liver supernatants were obtained by centrifugation (100,000 g for 2h) from 50% (w/v) homogenates of tissues in basal saline.

Serum was prepared from blood extracted by cardiac puncture and was diluted 50% (v/v) with basal saline.

protective ability this could be attributed, in part, to the high concentration of glucose in the liver supernatant (table 3.5), whereas brain supernatant had no glucose and so provided a more suitable medium to investigate the protection of slices against the combined deprivation of glucose and oxygen.

3.2.0 ATTEMPTS TO CHARACTERISE THE MATERIAL IN BRAIN SUPERNATANT THAT PREVENTS ANOXIC DAMAGE.

Since the previous experiments suggested that the brain supernatant could protect slices against anoxia damage it was decided to characterise the material that was responsible for the protection. At this stage the presence of the material could only be determined by the bioassay of its protective activity. The protective activity of a preincubation medium was determined in terms of the rate of respiration of slices after anaerobic preincubation in that medium for 60 minutes at 37°C.

3.2.1 DILUTION EFFECT.

In order to bioassay the protective effect of the supernatant it was necessary to study variation in activity of the supernatant with increasing dilution. Figure 3.2 shows that diluting the supernatant into basal saline reduces its activity so that a supernatant containing less than 0.2 g brain/ml would be considerably less active, and the protective activity would be more difficult to detect.

3.2.2 THE EFFECT OF HEAT

Table 3.6 gives the effect of heat treatment for various

Table 3.5

Protective Activity of Various Glucose Solutions

Preincubation Conditions (anaerobic for 60 min at 37°C)	Glucose Concentration (mM)	Protective Activity (μl/h/mg dry weight)
Brain Supernatant	0	12.0
Glucose Saline	11.5	5.6
Liver Supernatant	72.2	7.4
Basal Saline + Glucose	77.0	6.0

The glucose concentrations of rat brain and rat liver supernatants were determined experimentally.

times on the protective effect of the supernatant. It can be seen that even after 30 minutes at 100°C the supernatant still retained most of its activity.

3.2.3 THE EFFECT OF ACID AND ALKALI TREATMENT

Brain supernatant that had been treated with hydrochloric acid (0.1M for 30 min at 100°C) and then neutralised with concentrated sodium hydroxide retained its protective activity. Slices anaerobically preincubated in it for 60 minutes at 37°C later consumed oxygen at a rate of 8.7 µl/h/mg dry weight.

Brain supernatant that had similarly been treated with sodium hydroxide failed to retain its activity so that slices anaerobically preincubated in it could later only consume oxygen at a rate of 0.5 µl/h/mg dry weight.

3.2.4 EFFECT OF CHARCOAL TREATMENT

Although treatment of the brain supernatant with activated charcoal decreased its absorbance at 260 nm by 80% there was no corresponding decrease in its protective activity. Slices anaerobically preincubated in it later consumed oxygen at a rate of 9.5 µl/h/mg dry weight.

3.2.5 EFFECT OF PROTEOLYTIC ENZYMES

Incubating the brain supernatant with various proteolytic enzymes did not appreciably reduce its protective activity. Table 3.7 gives the rate of respiration of slices that had been anaerobically preincubated in brain supernatant after the various enzymic treatments.

Table 3.6

Protective Activity of Brain Supernatant after Heat Treatment

Heat Treatment	Protective Activity after Treatment (μl/h/mg dry weight)
Untreated Supernatant	12.0
80°C for 5 min.	10.0
100°C for 3 min.	8.8
100°C for 30 min.	8.5

Each result is the mean of 4 experimental values

The Protective Activity is expressed in terms of the rate of respiration of brain slices in the 2nd hour after anaerobic preincubation in the appropriate medium in the 1st hour.

Table 3.7

Protective Activity of Brain Supernatant

After Treatment with Various Proteolytic Enzymes

Enzyme	Treatment	Deactivation	Protective Activity (µl/h/mg dry weight)
Papain	160 mg/ml for 2h at 37°C	filtered	7.9
Pronase	2.5 mg/ml for 2h at 37°C	100°C for 5 min.	8.2
Carboxy-peptidase A	0.003 mg/ml for 30 min. at 37°C	filtered	8.4
Trypsin	100 mg/ml for 2h at 37°C	100°C for 10 min.	10.1

Each result is the mean of 4 experimental values

The Protective Activity is expressed in terms of the rate of respiration of brain slices in the 2nd hour after anaerobic preincubation in the appropriate medium in the 1st hour.

3.3.0 DETERMINATION OF MOLECULAR WEIGHT OF PROTECTIVE MATERIAL IN BRAIN SUPERNATANT.

Having established some of the basic properties of the protective activity of the supernatant, it was decided to determine the molecular weight of the material that protected the slices against anoxic damage.

3.3.1 DIALYSIS OF THE SUPERNATANT.

Dialysis of the supernatant into a large excess of basal saline revealed that the non diffusible material retained none of the activity of the supernatant. Slices anaerobically preincubated in it later respired at a rate of 2.7 µl/h/mg dry weight.

3.3.2 ULTRAFILTRATION OF THE SUPERNATANT.

To obtain a more precise value for the molecular weight of the active material, the supernatant was filtered through a series of cellulose nitrate membrane filters. Table 3.8 shows the relationship between the average retentivity of the membrane and the activity of the filtrate and the residue. Although there was a reduction in activity in the filtrates, the residues had no protective effect when dissolved in basal saline. The recombined filtrate and residue did not have an activity higher than the filtrate. Investigations were therefore carried out to determine whether the supernatant was losing activity by air oxidation during filtration. Table 3.9 shows that there was a significant reduction in activity when the supernatant was

Table 3.8

Protective Activity of Brain Supernatant after Ultrafiltration through Cellulose Nitrate Membranes

Filter	Average Retentivity (Daltons)	Protective Activity (μ l/h/mg dry weight)		
		Filtrate	Residue	Recombined
UM05	500	6.2 ± 0.47 (15)	2.9	6.0
UM2	1,000	7.6 ± 0.62 (4)	2.5	7.5
UM10	10,000	6.3 ± 0.22 (7)	2.2	5.3
PM30	30,000	5.8 (2)	1.3	6.7

Variation in results is in terms of $\pm$ one S.E.M.

Figure in brackets indicates number of experiments.

Table 3.9

Protective Activity of Brain Supernatant After Air Oxidation

Experimental Conditions (for 70 h at $5^{o}C$)	Protective Activity (μl/h/mg dry weight)
Supernatant stirred under O_2	7.7
Supernatant stirred under N_2	10.3
Supernatant + 5 mM DTT not stirred	10.8
Supernatant stored frozen	11.9

Each result is the mean of 4 experimental values

The Protective Activity is expressed in terms of the rate of respiration of brain slices in the 2nd hour after anaerobic preincubation in the appropriate medium in the 1st hour.

oxygenated. The addition of the anti oxidation agent dithiothreitol (DTT) appeared to protect against this loss.

3.3.3 ULTRACENTRIFUGATION OF THE SUPERNATANT

As the results on ultrafiltration were difficult to interpret the supernatant was fractionated by ultra-centrifugation into high and low molecular weight fractions. Under conditions that sedimented proteins with a molecular weight larger than 12,000 daltons (i.e. 100,000 g for 66 h) the activity was still totally in the supernatant low molecular weight fraction. Brain slices anaerobically preincubated in the supernatant for 60 minutes at 37°C later consumed oxygen at a rate of 8.8 µl/h/mg dry weight (mean of 9 results).

3.3.4 GEL FILTRATION OF THE SUPERNATANT

A sample of the supernatant was fractionated on a column of Sephadex G25 and the fractions were assayed for reducing substance, ninhydrin positive material and material absorbing at 280 and 260 nm. They were also bioassayed for protective activity. Figure 3.3 shows the relationship between the protective activity and the substances measured. It can be seen that the activity eluted together with the amino acids and the reducing substances as detected by Somogyi's Reagent, so the active material was probably of small molecular weight.

Gel filtration on Biogel P_2 gave better separation of the low molecular weight material butit was not possible to recover activity from it.

Table 3.10

The Amino Acid Composition of Brain Supernatant and the Synthetic Mixtures

Amino Acid	Spnt (mM)	Mix A (mM)	Mix B (mM)
Aspartate	3.5	2.5	2.5
Threonine	0.3	0.5	0.5
Serine	1.0	1.0	1.0
Glutamate		5.0	5.0
Glycine	1.2	1.0	1.0
Alanine	0.6	1.0	1.0
Valine	0.26	0.25	0
Ornithine	0.1	1.0	0
Lysine	.32	1.0	0
Glutamine	4.6	5.0	0
Asparagine	0.22	2.5	0
γ Amino butyrate	1.7	1.5	0

The analysis of the supernatant was carried out on an analyser incapable of distinguishing between glutamate and glutamine.

3.4.0 THE EFFECT OF SYNTHETIC AMINO ACID MIXTURES AND SOME AMINO ACID DERIVATIVES.

ND
SOME AMINO ACID DERIVATIVES.

The experiments with gel filtration had shown that most of the activity of the supernatant fractionated together with substances of small molecular weight. Instead of further fractionating the peak containing the small molecular weight substances it was decided to make up solutions of some of the small molecular weight compounds known to occur in brain and to test the solutions for protective activity.

3.4.1 THE EFFECT OF THE AMINO ACIDS

The amino acid composition of the brain supernatant was determined on an amino acid analyser and a mixture of synthetic amino acids in basal saline was prepared so that it contained approximately the same concentration of the amino acids as the supernatant. (Table 3.10). The mixture was assayed in order to determine whether slices anaerobically preincubated in it for 60 minutes at 37°C would later respire at a rate comparable to slices that had been similarly preincubated in oxygenated glucose saline.

A synthetic mixture of L-aspartate and L-glutamine in basal saline was also examined for protective activity.

Since some evidence had been obtained that air oxidation reduced the activity of the supernatant and that dithiothreitol prevented this loss certain amino acids and their derivatives which are known to be air oxidisable and

Table 3.11

Protective Activity of Synthetic Amino Acid Solutions

Preincubation Conditions anaerobic for 60 min at 37°C	Protective Activity (µl/h/mg dry weight)
Mixture A	3.0
Mixture B	2.8
2mM Aspartate + 4mM Glutamate in Basal Saline	1.3
1mM Cysteine - HCl in basal saline	1.3
1mM Glutathione in basal saline	2.5
5mM DDT in basal saline	2.8

Each result is the mean of 4 experimental values

The Protective Activity is expressed in terms of the rate of respiration of brain slices in the 2nd hour after anaerobic preincubation in the appropriate medium in the 1st hour.

are known to occur in brain were tested for protective activity. Table 3.11 shows that none of the amino acids in the synthetic mixture exerted a protective effect. Aspartate and glutamate were similarly without activity as was the common thiol containing amino acid cysteine and its peptide derivative glutathione.

3.4.2 THE EFFECT OF SOME NEUROTRANSMITTERS AND RELATED COMPOUNDS.

Some of the neurotransmitters are also air oxidisable and since these are derivatives of amino acids and have similar molecular weights it was decided to examine some of these to determine whether they exerted a protective effect. Table 3.12. shows that solutions of synthetic substances in basal saline had no protective effect.

This result was confirmed by determining the protective effect of a supernatant made from the brains of rats that had been treated with reserpine in order to severely diminish their stores of cerebral catecholamines (Iversen and Callingham 1971).

The supernatant from the brains of reserpine treated rats protected brain slices of untreated rats so that after the preincubation the slices respired at a rate of 8.3 µl/h/mg dry weight.

3.5.0 ATTEMPTS AT THE PURIFICATION OF THE SUPERNATANT BY ION EXCHANGE CHROMATOGRAPHY

Although the previous experiments provided some information about the nature of the substances that protect against

Table 3.12

Protective Activity of Some Neurotransmitters and Related Compounds

Preincubation Procedure (anaerobic for 60 min at $37^{o}C$	Protective Activity (μ l/h/mg dry weight)
1.0 mM Adrenaline in basal saline	1.3
1.0 mM L-DOPA in basal saline	0.7
1.0 mM 5-HTP, 1.0 mM 5-HT, 1.0 mM L-DOPA, 1.0 mM Dopamine in basal saline	3.5
Isoprenaline in basal saline (1.5 mg/ml)	3.7
0.5 mM Dopamine + 1.0 mM DTT in basal saline	3.9
5 mM DTT in basal saline	2.8

Each result is the mean of 4 experimental values

The Protective Activity is expressed in terms of the rate of respiration of brain slices in the 2nd hour after anaerobic preincubation in the appropriate medium in the 1st hour.

anoxic impairment they also showed that the procedures involved were not suitable for the isolation of the active material. For example, the gel filtration on Sephadex G25 failed to separate the active constituents from the amino acids, the nucleotides and the reducing substances.

The basic difficulty with ion exchange chromatography was that eluting the activity off the column required a gradient either of salt concentration or of pH. As this would lead to difficulties in preparing the fractions for bioassay in a physiological saline, the approach adopted was to test various ion exchange resins in order to obtain one that removed as much of the non-active material as possible whilst the active material passed through unadsorbed. At physiological pH, however there was not much adsorption on to the resins. The results from this are summarised in table 3.13. On some of the resins the fractionation of the ninhydrin positive material was used as the basis for determining the extent of fractionation undergone by the supernatant. It can be seen that where the activity passed through the column unadsorbed the ninhydrin positive material was also unadsorbed indicating that little fractionation of the supernatant was occurring.

3.6.0 THE ROLE OF SODIUM AND POTASSIUM IONS IN ANOXIC DAMAGE.

3.6.1 THE EFFECT OF VARYING THE SODIUM AND POTASSIUM CONCENTRATIONS IN THE PREINCUBATION MEDIA.

In order to determine whether it might be possible to elute any of the activity adsorbed on to an ion exchange resin

Table 3.13

Protective Activity of Brain Supernatant after Treatment with Various Ion Exchange Resins

Resin Type	Ionic form	Protective Activity of Unadsorbed Material ($\mu l/h/mg$ dry weight)	Ninhydrin Positive Material adsorbed (%)
Dowex 50 strongly acidic cation exchange resin	H^+	3.1 ± 1.41 (5)	10
	Na^+	5.5 ± 2.0 (9)	7
Biorex 70 weakly acidic cation exchange resin	H^+	2.5 ± 2.5 (8)	30
	Na^+	4.0 ± 2.3 (6)	20
AG1 strongly basic cation exchange resin	Cl^-	7.1 ± 0.65 (8)	
	$HCOO^-$	3.5 (1)	
	CH_3COO^-	1.9 (2)	
AG2 basic anion exchange resin	Cl^-	5.2 ± 2.1 (4)	5

Results are given as the mean value $\pm$ 1 SEM. Number of experiments in brackets.

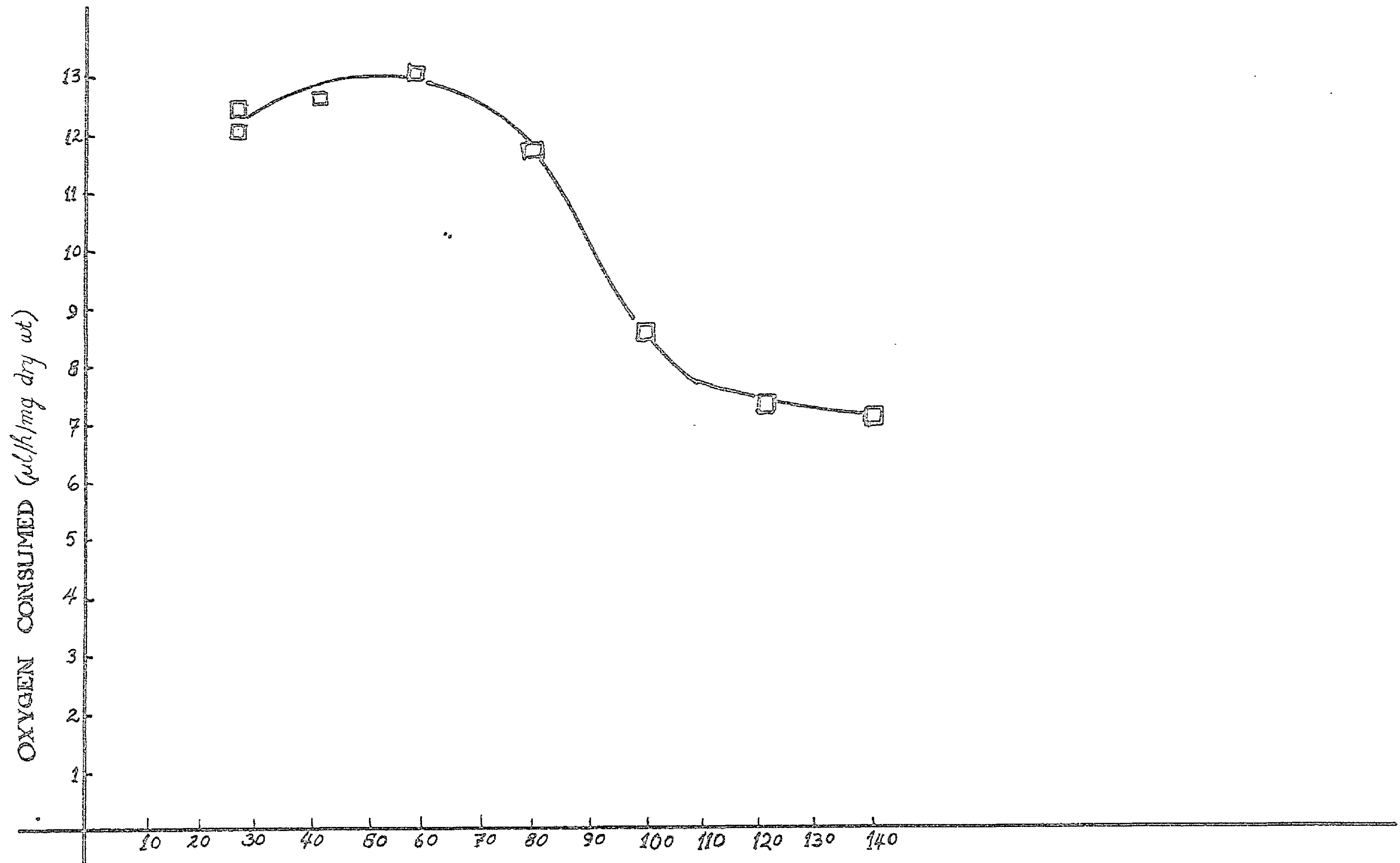
the effect of varying concentrations of sodium chloride on the activity of the supernatant was explored. In order to do this a brain supernatant was prepared in 20 mM Tris-HCl and its activity measured after the addition of varying amounts of sodium chloride. Figure 3.4 shows that there was a fairly rapid decrease in the activity of the supernatant with increasing concentrations of sodium chloride.

Since sodium chloride had an inhibitory effect on the activity of the supernatant other eluting agents were tried. It was found that potassium chloride exerted a protective effect on the brain slices. This prompted an investigation into the effect of the potassium concentration of the brain supernatant. It was found that the potassium concentration of the brain supernatant was 35 mM.

Figure 3.5 shows the variation in the protective effect of isotonic mixtures of potassium chloride and Tris-HCl with increasing potassium concentration. It can be seen that at 35 mM potassium chloride affords considerable protection against anaerobic preincubation.

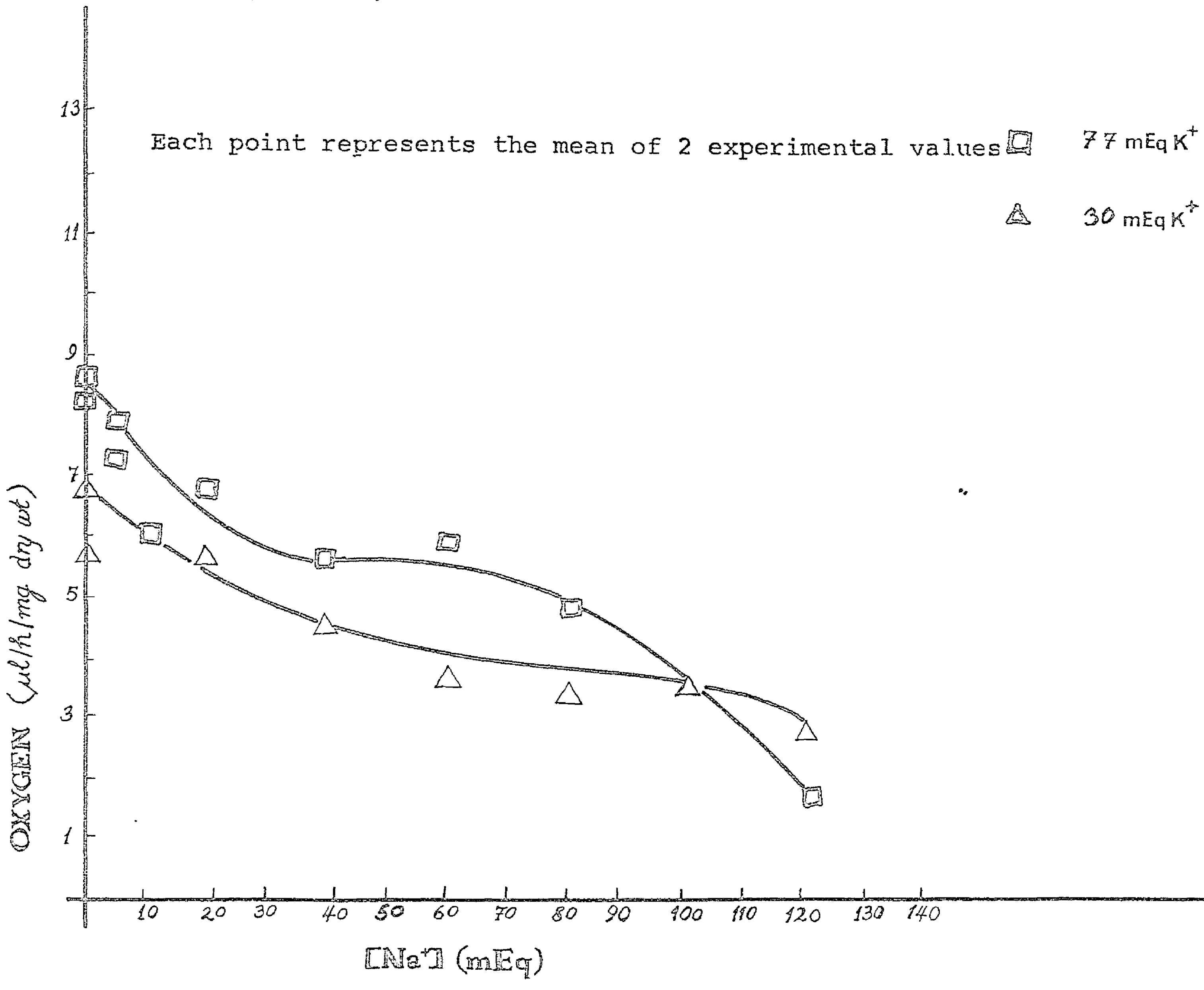
Figure 3.6 shows the effect of increasing concentrations of sodium on the protective effect of mixtures of potassium chloride and Tris-HCl. It can be seen from this that a buffered solution of 77 mM potassium chloride can afford almost complete protection against the damage caused to the respiration of brain slices following an anaerobic preincubation if no sodium is present in the preincubation medium. This protection decreases with increasing concentrations of sodium chloride, and at 100 mM which is

EFFECT OF SODIUM ON THE PROTECTIVE EFFECT OF BRAIN SUPERNATANT FIG 3.4



Each point represents the mean of 2 experimental values

EFFECT OF SODIUM ON THE PROTECTIVE EFFECT OF POTASSIUM IONS FIG 3.6



the concentration present in a supernatant of brain homogenate in basal saline the activity is such that slices later respire only at a rate of 4.0 µl/h/mg dry weight.

3.6.2 THE POTASSIUM CONCENTRATION OF BRAIN SLICES AFTER PREINCUBATION

In order to determine the method by which the high potassium in the preincubation mixture exerted its protective effect, the potassium concentration of the slices was determined after the various preincubation procedures. Table 3.14 shows that those preincubation procedures that protect the subsequent respiration of the brain slices, also maintain high potassium levels in the slices. Slices that have a potassium concentration above 18 mM respire at a level comparable to aerobically preincubated slices. Table 3.14 also shows that when the potassium concentration of the slices was depleted, not by anaerobic preincubation but by substituting all the sodium of the preincubation medium with lithium, the subsequent rate of respiration of the slices was low even though they had been aerobically preincubated. An attempt at depleting the potassium of the slices during aerobic preincubation by the use of the ionophone, valinomycin did not prove successful.

3.7.0 OTHER ASPECTS OF ANOXIC DAMAGE

It was mentioned in the introduction that anaerobic preincubation of brain slices is known to affect not only their subsequent rate of respiration but also their ability to respond to potassium stimulation and to incorporate amino

Table 3.14

<u>The Relationship Between the Rate of Respiration and the Potassium Concentration After Preincubation.</u>

Preincubation Conditions (for 60 min)	Rate of Respiration in 2nd hour (μl/h/mg dry weight)	Slice K^+ after preincubation (μ equiv/g wet weight)
Glucose Saline + O_2 at 37°C	10.1	26.5
Basal saline + N_2 at 37°C	1.8	6.9
Brain supernatant + N_2 at 37°C	12.0	37.0
Basal saline + N_2 at 0°C	9.1	21.3
Li^+Glucose saline + O_2 at 37°C	5.0	13.7
Entire hemisphere + N_2 at 37°C	11.7	31.0

The values for the K^+ are the mean of 4 results.

The figures in the two columns are derived from separate sets of experiments.

acids into their proteins. Experiments were carried out to determine if either the brain supernatant or a buffered solution of potassium chloride could protect the slices against these other aspects of anoxic damage.

3.7.1 THE LOSS OF POTASSIUM STIMULATED RESPIRATION

Table 3.15 shows that after aerobic preincubation the potassium stimulation of brain slices results in a doubling of their rate of respiration. Slices anaerobically preincubated in basal saline did not respond to potassium stimulation. Brain supernatant was not nearly as effective in preserving potassium stimulated respiration as it was in protecting unstimulated respiration. A buffered isotonic solution of 77 mM KCl and Tris-HCl was even less effective in preserving the normal response of brain slices to potassium stimulation.

3.7.2 LEUCINE INCORPORATION INTO BRAIN SLICES AFTER ANOXIA

The amount of leucine incorporated into proteins in 60 minutes in brain slices that had been preincubated for 60 minutes was estimated by calculating the DPM of 3H-leucine in the TCA precipitated fraction of the slices. It was found that after anaerobic preincubation the slices incorporated lower amounts of leucine as compared to aerobically preincubated slices (table 3.14). It can be seen that the use of either the brain supernatant or anisotonic mixture of 77 mM KCl and Tris-HCl as preincubation media did not protect brain slices against this consequence of anaerobic preincubation.

Table 3.15

The Protection of Potassium Stimulated Respiration.

Preincubation Conditions for 60 min. at 37°C	The Rate of Respiration on the 2nd h. (μ l/h/mg dry weight)	
	Unstimulated	Stimulated by 0.1 M KCl
Glucose saline, O_2	10.1	21.0
Basal saline, N_2	1.8	2.0
Brain supernatant, N_2	9.0	13.8
77mM KCl in 77 mM Tris HCl N_2	7.1	9.0

Each value is the mean of 4 experimental results.

Table 3.16

The Effect of Anaerobic Preincubation on the Incorporation of L-(4,5 ^{3}H) Leucine into Proteins of Brain Slices

Preincubation Conditions (60 min at 37^oC)	Leucine Incorporated in 60 min. (DPM/mg protein)	Ratio of free Leucine inside/outside (DPM after 60 min.)
Glucose Saline $+O_2$	9,307	2.23
Basal Saline $+N_2$	2,177	1.96
77 mM KCl/Tris-HCl $+N_2$	2,513	3.43
Brain Supernatant $+N_2$	1,965	2.63

Each value is the mean of 4 experimental results.

Table 3.14 also lists the ratio of the concentration of 3H-Leucine inside and outside the inulin free compartment of the slice and it can be seen that anaerobic preincubation does not affect the ability of the slices to concentrate leucine in the inulin-free compartment.

CHAPTER 4

THE DISCUSSION

4.0.0 ANOXIC IMPAIRMENT IN VITRO AND IN VIVO

Since the initial impetus for this research arose from the work of Phizackerley and Fixter (1973) it is appropriate at this stage to consider the relationship between their work, the research described in this thesis, and the situation *in vivo*. There are two main conclusions that can be drawn from their work. The first is that the anaerobic preincubation of slices from adult rat brain cortex at 37°C decreases their subsequent rate of respiration. In spite of the fact that Phizackerley and Fixter worked with salines that did not contain calcium it is not surprising that a similar result is described in this thesis when calcium containing salines were used. The anoxic impairment of cerebral respiration is a fairly well established phenomenon (Dickens and Greville, 1933; Elliot and Rosenfeld, 1958) and its relevance to the situation *in vivo* can be judged from the finding of Fazekas and Bessman (1951) that in a man in irreversible coma after attempted strangulation, the rate of respiration of the brain measured *in situ* was reduced by 50%.

The second conclusion that can be drawn from the work of Phizackerley and Fixter is that a marked impairment of cellular respiration does not always follow cerebral anoxia *in vitro*. This is of particular interest, because the nature and extent of anoxic damage *in vivo* is now a matter

of controversy following the accumulation of a growing body of evidence which suggests that brain cells may not be as vulnerable to anoxia as had previously been supposed.

Most of the conditions used by Phizackerley and Fixter (1973) to alleviate the effects of anaerobic preincubation were shown to be just as effective when used in conjunction with calcium containing salines, and it is possible that these conditions have a greater relevance to the situation _in vivo_. The protection of slices against anoxic damage either by preincubating them in the supernatant of a brain homogenate or by the preincubation of entire hemispheres in a small volume of saline, suggest that extra-neural factors may be involved.

The work of Hossmann and Ames and their respective co-workers has suggested that the tolerance of the intact brain to ischaemia may be limited by factors related to the composition of the blood and the extracellular fluid, and to the impairment of post ischaemic blood circulation. Ames and Gurian (1963), for example, have pointed out that the isolated retina which is essentially similar to other examples of grey matter is considerably less vulnerable to anoxic damage than the intact brain. Since this preparation is nourished from the incubation medium it is plausible that the greater vulnerability of the brain _in vivo_ is a result of a deficient capillary circulation which prevents adequate re-oxygenation of the ischaemic cells thus effectively depriving them of oxygen

and glucose for a period much greater than the actual time of interruption of blood supply (Webster and Ames, 1965). This has been termed the no-reflow phenomenon and it has been demonstrated that with some common methods used to induce cerebral ischaemia part of the brain fails to become adequately reperfused if ischaemia is prolonged beyond 7-8 min (Ames et al 1968). Initially this was attributed to a narrowing of the vascular lumen caused by the swelling of glial and endothelial cells (Chiang et al, 1968), but blood factors such as thrombocyte aggregation and increased viscosity have also been implicated (Fisher and Ames, 1972).

These ideas are supported by the observation that the flushing of cerebral vessels with saline before the ischaemia reduces tissue damage (Olsson and Hossmann, 1971), and that by preventing blood from stagnating in the vasculature it is possible to show the revival of neurological function (Neely and Youmans, 1963), of EEG and evoked cortical response (Hossmann and Sato, 1970a and b) and of the metabolic state of the tissue (Hinzen et al, 1972; Kleihues et al, 1974) after periods of ischaemia that are considerably longer than those previously believed to be the maximally permissible ones.

There are others, however, (Brierley et al, 1973) who point out that circulatory arrest produces a type of pathogenic result which, although it may vary in extent, is essentially similar to that found after hypoglycaemia and carbon monoxide and cyanide intoxications. In

contrast the no-reflow phenomenon is peculiar to the single situation of circulatory arrest and cannot be invoked as the main cause of brain damage. They point out moreover that the no-reflow phenomenon has not yet been observed in any animal that has resumed spontaneous respiration after circulatory arrest and so they propose that it is probably a post mortem event occurring only after the experimental equivalent of brain death.

It is possible that some of the confusion and controversy arises from the different experimental methods used to induce anoxia, the different criteria used for assessing brain damage and the problems associated with correlating biochemical studies with functional activity. Brierley and his group (Brierley et al, 1973) assessed brain damage by the microscopic examination of brain preparations using the technique of perfusion-fixation in vivo. They followed the appearance of microvacuoles and ischaemic cell change in the neurones. The majority of microvacuoles they believe are expanded mitochondria in varying states of disorganisation. The ischaemic cell change that follows, transform a normal neurone into a more or less naked and very shrunken nucleus that is eventually engulfed by phagocytes. Hossmann and his co-workers (for review see Hossmann and Kliehues, 1973) however measure the restoration of various experimental parameters of the brain such as, EEG waves, ATP concentration, glucose concentration, the incorporation amino acids into proteins, water and electrolyte content besides histologic and morphologic characteristics.

The work described in this thesis deals mainly with the preservation of normal rates of respiration in brain slices and the associated preservation of sodium and potassium concentrations. By using slices in the post-anoxic period it is possible to eliminate problems associated with post-anoxic circulatory impairment. The other extra-neural factor that has been suggested, is the composition of the extra cellular fluid. The protection afforded by preincubating entire hemispheres in a minimal quantity of basal saline or by preincubating slices in brain supernatant suggests that there is a loss of material from the brain during anoxia which must be either prevented or offset, and it is because of this that the presence and composition of extracellular fluid during anoxia assumes a crucial role.

Even the few results, however, that have been presented here on the preservation of potassium stimulated respiration and the incorporation of leucine into proteins reflect the fact that the preservation of one experimental variable does not indicate the preservation of normal cerebral function. Even Hossmann does not claim that a cat showing signs of functional recovery after 60 min of ischaemia will ever catch mice again.

The other methods for the preservation of the respiration of brain slices that were described by Phizackerley and Fixter (1973) and confirmed in this thesis also reflect the situation *in vivo* but are not subject to much controversy.

The lack of sensitivity of slices from neonatal (7 day old) rats to anoxia, for example, is a reflection of the findings of Fazekas and co-workers (Fazekas et al, 1943) that the new born rat is resistant to anoxia _in vivo_ and gradually acquires sensitivity until at around 12 days it is just as sensitive as the adult to anoxia.

It is also not surprising that slices anaerobically preincubated at $0^{o}C$ instead of at $37^{o}C$ did not develop a respiratory defect, in view of the fact that Chetvenkov and Gasteva (1966) have reported that a reduction in body temperature increases the survival time of rats in hypoxic conditions.

4.1.0 CHARACTERISATION OF MATERIAL LOST FROM SLICES DURING ANOXIA

The fact that the use of a supernatant of a brain homogenate as preincubation medium prevented the anoxic impairment of the respiration of brain slices suggests that it contains a material that protects the slices. It is also possible to conclude that it is the loss of this material from the slices that is the cause of the anoxic damage to respiration. The experiment with the entire hemispheres, indicates moreover, that the loss of material involves a leakage from the brain to the medium rather than an irreversible transformation of the material within the brain. The isolation and characterisation of this material was the logical next step.

The material could only be detected at this stage by

determining whether it protected the respiration of brain slices that were anaerobically preincubated in it. A dilution curve of the supernatant revealed that a supernatant containing the equivalent of 30% (w/v) of brain provided substantial protection against the anoxic impairment of cerebral respiration. Such a supernatant must contain less of the active material than the brain, so it appears that brain slices can tolerate some loss of active material before developing a respiratory defect.

It was found that the material was stable to heat and acid treatment but not to alkali. The extraction of purine and pyrimidine derivatives by the use of activated charcoal and the degredation of proteins by the use of proteolytic enzymes revealed that the active material was probably not a nucleotide or a protein.

4.1.1 <u>DETERMINATION OF THE MOLECULAR WEIGHT OF THE MATERIAL</u>

A number of methods were used to estimate the molecular weight of the active material. Dialysis into a large excess of basal saline revealed that it was probably of small molecular weight. Attempts to make a more specific estimate by using cellulose nitrate filters gave inconclusive results. It was thought possible that an active component of the material was being removed on filtration, but recombination of filtrate with residue did not restore activity to a level comparable to the unfiltered extract. It was possible to show, however, that the loss of activity might have been caused by oxidation. In spite of this there was still sufficient

activity in the filtrate to indicate that a substantial component of the active material had a molecular weight less than 500 daltons. This is only an approximate figure because the membrane specification assumes a spherical molecule. The gel filtration with Sephadex G25 revealed that the activity eluted with the peak of ninhydrin positive material. The free amino acids were also found with this peak and it appeared probable that the active material was of a similar molecular weight.

4.2.0 BIOASSAY OF SUBSTANCES FOR PROTECTIVE ACTIVITY

The number of small molecular weight compounds that are found in rat brain is probably around 400. Even after eliminating purine and pyrimidine derivatives and small molecular weight peptides there are still far too many left for all of them to be individually bioassayed. There are some compounds, however, which in the light of the results presented by Phizackerley and Fixter and the preliminary results already discussed in this thesis appeared more probable than others.

4.2.1 BIOASSAY OF AMINO ACIDS

The first group of compounds to be assayed were the amino acids. The concentrations of these in the brain are known to vary with anoxia *in vivo* (Tews et al, 1963), and Phizackerley and Fixter (1973) have shown that it is the leakage of L-glutamine and L-aspartate that is responsible for the anoxic impairment of the respiration of brain slices in media that do not contain calcium. With calcium containing salines, however, none of the amino

acids that commonly occur in brain had any protective activity.

4.2.2 BIOASSAY OF MONOAMINES

The other group of compounds examined for protective activity were the monoamines. Apart from the close structural and functional relationships between some amino acids and them there were other reasons that made them likely candidates for bioassay. In view of the finding of Davis and Carlsson (1973) that hypoxia *in vivo* decreases the rate of synthesis, though not the concentration, of monoamines in brain, it appeared plausible that in brain slices their level would be decreased by loss to the medium. They are also known to be released on depolarisation and anoxia is known to cause depolarisation. Their release from neurones on stimulation is dependent on calcium ions (Katz, 1969) and some of them are known to be easily oxidised. In spite of this circumstantial evidence the monoamines when bioassayed failed to exhibit any substantial protective activity. The experiments with rats treated with reserpine in order to deplete their brain monoamines also confirmed this result.

4.3.0 ATTEMPTS AT PURIFICATION BY ION EXCHANGE CHROMATOGRAPHY

Another approach adopted in order to determine the nature of the protective material was its isolation on ion exchange resins. The difficulties associated with this have already been mentioned in the results section.

Since high concentrations of sodium caused a loss of activity it was not possible to elute the material from the columns with a gradient of sodium concentration. It was also not possible for the same reason to concentrate large volumes of eluate by freeze drying, therefore the only conclusion that could be derived from the experiments was, that the active material behaved as though it were a positively charged species.

4.4.0 THE ROLE OF SODIUM AND POTASSIUM IONS IN ANOXIC DAMAGE

On finding that a high sodium concentration decreased the activity of the supernatant a number of cations were tried in order to determine whether they had any effect on the respiration of brain slices. It was found that potassium exerted a strong protective effect if used in the absence of sodium.

In order to determine whether the protective activity was associated with the prevention of potassium loss from slices the slice potassium was determined after various preincubation procedures. It was found that those conditions that resulted in an impairment of respiration also resulted in a depletion of slice potassium whilst conditions that protect against anoxic damage to respiration also prevented a loss of potassium from the slices.

Pappius and Elliot (1956) have found that the anaerobic incubation of brain slices reduces their potassium content,

but they maintain that this loss can be subsequently restored if the slices are transferred to oxygenated glucose saline, and that the extent of the restoration depends on the period of anaerobic incubation. They also showed that a lack of substrate during the anoxic preincubation interferes with the active extrusion of sodium. They reach the conclusion that anoxia not only results in a loss of potassium but damages the potassium accumulating machinery as well. This is thought to be the sodium-potassium-magnesium dependent ATPase.

The work presented here indicates, however, that anoxia by itself does not damage the potassium accumulating mechanism, it is rather the loss of potassium that causes the damage. This is shown by the fact that anoxia does not always result in potassium loss and that similar damage to respiration can be caused by the depletion of potassium under aerobic conditions.

4.5.0 THE ANOXIC IMPAIRMENT OF POTASSIUM STIMULATED RESPIRATION

Quastel and Quastel (1961) have suggested that the potassium stimulated respiration of brain slices *in vitro* is a closer approximation to the functioning brain, that is, to brain tissue *in vivo* stimulated by sensory impulses. They point out that on stimulation brain slices respire at twice the normal rate and that this is nearer to the estimates of the rate of respiration of the brain *in vivo*. In other respects too, such as its response to drug action, the stimulated slice behaves in a manner more like the intact brain. It is pertinent therefore that the

preservation of slice potassium does not protect potassium stimulated respiration to the same extent as it protects unstimulated respiration. Ruscak and Whitam (1967) have suggested that the metabolic responses of brain cortex slices to high potassium depends on the operation of the sodium pump. From these results, however, it is just as likely that damage to respiration other than that to the ATPase might be caused by anoxia and that this does not manifest itself when slices are not stimulated.

4.6.0 THE ANOXIC IMPAIRMENT OF AMINO ACID INCORPORATION

It was also shown that the ability of brain slices to incorporate leucine into their protein was impaired by a period of anoxia. Similar results have been published by Yanagihara, (1972) and Ghosh and Cohen (1974). It was also seen that conditions that protected against anoxic damage to respiration did not protect against damage to the ability of the brain slice to incorporate amino acids. It is possible therefore that this damage occurs not by loss of material but through some other mechanism.

It has been suggested that a marked disaggregation of polysomes occurs in the ischaemic and post ischaemic phases, (Kleihues and Hossmann, 1971) and this would account for the decreased incorporation. The other obvious site of the defect would be the transport of the amino acids into the cell which might be an energy requiring process (Banay-Schwartz et al, 1971) but this can be ruled out because there was no appreciable difference between the "free leucine" within aerobic or anaerobically preincubated slices.

4.7.0 CONCLUSIONS

In conclusion it can be said that the loss of potassium from brain slices during anaerobic preincubation is probably responsible for a large part of their irreversible respiratory impairment. It is likely that other substances also leak from the slices and these have yet to be identified.

The site of the damage cannot be definitely established by these experiments. If, as Ruscak and Whittam (1967) maintain, the sodium-potassium-magnesium dependent ATPase is responsible for the potassium stimulated respiration, then, that would appear to be a likely site for the damage. It is also possible, that damage occurs at the mitochondria and it should be possible to devise experiments to show if mitochondria are irreversibly damaged by anoxia. This would be interesting because it has been suggested that the mitochondria are not responsible for irreversible brain damage following brief periods of total cerebral ischaemia *in vivo* (Schutz et al, 1973).

Finally, although it is not possible to show from these results that anoxic damage *in vivo* can be prevented by maintaining brain potassium during anoxia, it is feasible that such experiments can be devised and would provide interesting results, especially as anoxia *in vivo* is known to cause a leakage of potassium from the cells into the extra-cellular fluid of the brain and from there into the circulating blood (Meyers, 1973).

REFERENCES

AMES, A. and GURIAN, B.S. (1963) J. Neurophysiol. 26, 617-634.

AMES, A., WRIGHT, R.L., KOWADA, M., THURSTON, J.M. and MAJNO, G. (1968) Amer. J. Path. 52, 437-454.

ASHFORD, C.A. and DIXON, K.C. (1935) Biochem. J. 29, 157-168.

BARCROFT, J. (1920) The Lancet, 2, 485-489.

BRIERLY, J.B., MELDRUM, B.S. and BROWN, A.W. (1973) Arch. Neurol. 29, 367-374.

BRINK, F. (1954) Pharmac. Rev. 6, 243-298.

BULL, R.J. and TREVOR, A.J. (1972) J. Neurochem. 19, 999-1009.

BANAY-SCHWARTZ, M., PIRO, L. and LAJTHA, A. (1971) Archs Biochem Biophys. 145, 199-210.

COCKING, E.C. and YEMM, E.W. (1955) Analyst, 80, 209-213.

COHEN, M. (1961) J. Neurochem. 9, 337-344.

CHIANG, J., KOWADA, M., AMES, A., WRIGHT, R.L. and MAJNO, G. (1968) Amer. J. Path. 52, 455-476.

DALY, J., LEVITT, M., GUROFF, G. and UDENFRIEND, S. (1968) Archs. Biochem. Biophys. 126, 593-598.

DAVIS, J.N. and CARLSSON, A. (1973) J. Neurochem. 21, 783-790.

DEUL, D.H. and McILWAIN, H. (1961) J. Neurochem. 8, 246-256.

DICKENS, F. and GREVILLE, G.D. (1933) Biochem J. 27, 1134-1140.

DICKENS, F. and GREVILLE, G.D. (1935) Biochem J. 29, 1468-1482.

ELLIOT, K.A.C. and HENDERSON, N. (1948) J. Neurophysiol. 11, 437-490.

ELLIOT, K.A.C. and ROSENFELD, M. (1958) Canad. J. Biochem. Physiol. 36, 721-730.

FAZEKAS, J.F. and BESSMAN, A.N. (1953) Am. J. Med. 15, 804-812.

FAZEKAS, J.F., ALEXANDER, F.A.D. and HIMWICH, H.E. (1941) Am. J. Physiol. 134, 281-287.

FISHER, E.G. and AMES, A. (1972) Stroke, 3, 538-542.

GHOSH, S.K. and COHEN, M.M. (1974) Exp. Neurol. 43, 570-579.

GRUNDFEST, H., KAO, C.Y. and ALTAMIRANO, M. (1954) J. Gen. Physiol. 38, 245-282.

GURDJIAN, E.S., WEBSTER, J.E. and STONE, W.E. (1949) Am. J. Physiol 156, 149-157.

HINZEN, D.H., MULLER, U., SOBOTKU, P., GEBERT, E., LANG, R. and HIRSCH, H. (1972) Amer J. Physiol 223, 1158-1164.

HOSSMANN, K.A. and SATO, K. (1970a) Science 168, 357-376.

HOSSMANN, K.A. and SATO, K. (1970b) Z.ges Neurol. Psychiat 198, 33-45.

HOSSMANN, K.A. and KLEIHUES, P. (1973) Archs Neurol. 29, 375-384.

IVERSEN, L.L. and CALLINGHAM, B.A. (1971) in Fundamentals of Biochemical Pharmacology p.299 Ed. Z.M. Bacq Publ. Pergamon Press.

KAASIK, A.E., NILSSON, L. and SIESJO, B.K. (1970a) Acta physiol scand 78, 433-447.

KAASIK, A.E., NILSSON, L. and SIESJO, B.K. (1970b) Acta physiol scand 78, 448-458.

KATZ, B. (1969) in "The Release of Neurological Transmitter Substances" p.33 publ. Liverpool University Press.

KLEIHUES, P., KOBAYASHI, K. and HOSSMANN, K.A. (1974) J. Neurochem. 23, 417-426.

LOWRY, O.H., PASSONNEAU, J.V., HASSELBERGER, F.X. and SCHULZ, D.W. (1964) J. biol. Chem. 239, 18-30.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. and RANDALL, R.J. (1951) J. biol. Chem. 193, 265

McILWAIN, H. (1951) Biochem. J. 49, 382-393.

McILWAIN, H. (1963a) in "Chemical Exploration of the Brain". p.3. Elsevier publishing Co.

McILWAIN, H. (1963b) in "Chemical Exploration of the Brain". p.9. Elsevier publishing Co.

McILWAIN, H. and BACHELARD, (1971) in "Biochemistry and The Central Nervous System". p.81. publ. Liverpool University Press.

McILWAIN, H. and RODNIGHT, R. (1962) in "Practical Neurochemistry". p.112 publ. J. and A. Churchill Ltd., London.

MEYER, A. (1963a) in "Greenfield's Neuropathology" p.239 publ. E. Arnold Ltd., London.

MEYER, A. (1963b) in "Greenfield's Neuropathology". pp.238-239. publ. E. Arnold Ltd., London.

MEYERS, R.E. (1973) Arch. Neurol. 29, 394-399.

NEEYLY, W.A. and YOUMANS, J.R. (1963) J. Amer. med. Ass. 183, 1085-1087.

OLSSON, Y. and HOSSMANN, K.A. (1971) Acta Neuropath (Berl.) 17, 68-79.

PAPPIUS, H.M. and ELLIOT, K.A.C. (1956) Canad. J. Biochem. Physiol. 34, 1053-1067.

PAPPIUS, H.M., ROSENFELD, M., JOHNSON, D.M. and ELLIOT, K.A.C. (1958) Canad. J. Biochem. Physiol. 36, 217-226.

PETERS, J.P. and VAN SLYKE, D.D. in "Quantitative Clinical Chemistry". Vol.1. p.597. publ. William and Wilkins Co., Baltimore, U.S.A.

PHIZACKERLEY, P.J.R. and FIXTER, L.M. (1973) J. Neurochem. 20, 123-134.

QUASTEL, J.H. and QUASTEL, M.J. (1961) in "Chemical Exploration of Brain Metabolism" p.12.

RUSCAK, M. and WHITTAM, R. (1967) J. Physiol. (Lond.) 190, 595-510.

SCHUTZ, H., SILVERSTIEN, P.R., VAPALAHTI, M., BRUCE, A.D., MELA, L. and LANGFITT, T.W. (1973) Arch Neurol 29 408-416.

SOMOGYI, M. (1945) J. biol. Chem. 160, 61-73.

THOMSON, R.Y. (1969) in "Chromatographic and Electrophoretic Techniques" Vol. 1. pp.306-307 ed. I. Smith publ. W. Heinemann Medical Books Ltd., London.

YAP, S.L. and SPECTOR, R.G. (1965) J. Path. Bacteriol. 90, 543-549.

YANAGIHARA, T. (1972) Stroke, 3, 733-738.

"And men should know that from nothing else but from the brain come joys, delights, laughter and jests and sorrows, griefs, despondency and lamentations. And by this in an especial manner, we acquire wisdom and knowledge, and see and hear and know what are foul and what are fair, what sweet and what savory ...... And by the same organ we become mad and delirious and fears and terrors assail us, some by night and some by day, and dreams and untimely wanderings, and cares that are not suitable, and ignorance of present circumstances, desuetude and unskillfulness. All these things we endure from the brain, when it is not healthy, but is more hot, more cold, more moist or more dry than natural or when it suffers any other preternatural and unusual affliction."

Hippocrates "On the Sacred Disease"