

NEUROCHEMICAL STUDIES ON AMINO ACID TRANSMITTERS

by

Les Philip Davies

Being a thesis submitted to the
Australian National University
for admission to the
Degree of Doctor of Philosophy

Department of Pharmacology
John Curtin School of Medical Research
February, 1975

DECLARATION

I hereby declare that the work recorded in this thesis has been performed by myself, except where otherwise acknowledged, and that it has not been submitted in any previous application for a degree.

AP Davies

TABLE OF CONTENTS

Abstract	iv
Acknowledgments	vi
Papers Published	vii
I. GENERAL INTRODUCTION	1
<u>PART ONE: GLYCINE AND γ-AMINOBUTYRIC ACID</u>	
II. SERINE HYDROXYMETHYLTRANSFERASE IN THE CAT AND RAT CENTRAL NERVOUS SYSTEM	4
III. POSTNATAL CHANGES IN GLYCINE LEVELS AND ACTIVITIES OF SERINE HYDROXYMETHYLTRANSFERASE AND GLYCINE:2-OXOGLUTARATE IN THE RAT CENTRAL NERVOUS SYSTEM	14
IV. POSTNATAL CHANGES IN THE HIGH AFFINITY UPTAKE OF GLYCINE AND GABA IN THE RAT CENTRAL NERVOUS SYSTEM	22
V. POSTNATAL CHANGES IN THE POTASSIUM-STIMULATED, CALCIUM-DEPENDENT RELEASE OF RADIOACTIVE GABA AND GLYCINE FROM SLICES OF RAT CENTRAL NERVOUS SYSTEM	34
VI. THE EFFECT OF SOME DRUGS ON AMINO ACID LEVELS IN 10-DAY RAT SPINAL CORD	48
<u>PART TWO: D- AND L-ASPARTATE</u>	
VII. THE UPTAKE AND RELEASE OF D- AND L-ASPARTATE BY RAT BRAIN SLICES	52
VIII. D-ASPARTATE OXIDASE ACTIVITY IN EXTRACTS OF MAMMALIAN CENTRAL NERVOUS SYSTEM	67
IX. D-ASPARTATE IN THE CENTRAL NERVOUS SYSTEM: PRESENCE OR ABSENCE?	77
Appendices	92
References	94

ABSTRACT

Several neurochemical correlates of synaptically-active amino acids, including high affinity, sodium-dependent uptake into brain slices, calcium-dependent potassium-stimulated release from brain slices and convulsions induced by antagonists of postsynaptic action, were examined with respect to glycine and GABA in the CNS of young rats. Results suggest that these compounds are functioning as inhibitory transmitters in rats of 1-day postnatal age, the earliest developmental stage studied.

Two enzymes, serine hydroxymethyltransferase and glycine:2-oxoglutarate aminotransferase were measured in various regions of rat and cat CNS, and compared with glycine levels in these areas. While no correlation was found between the activity of either enzyme and glycine levels in five regions of the rat CNS, there was some correlation between the activity of serine hydroxymethyltransferase and glycine levels within cat spinal cord. Measurement of the levels of serine hydroxymethyltransferase in rats at different stages of development indicates that this enzyme, which decreases in activity from birth to maturity, correlates with cell growth rate.

Glycine is the only amino acid found in significantly higher concentration in the spinal cord than any other region of 10-day-old rats. The increasing rostro-caudal gradient of glycine levels, so prominent in adult animals, is apparent in 10-day-old animals.

D-aspartate is accumulated by rat brain slices. Results indicate that uptake is by that system specific for the dicarboxylic amino acids. The similarity of uptake

of D- and L-aspartate and the observation that D-aspartate can be released from brain slices by a depolarizing stimulus suggest that D-aspartate may be a useful compound in studying the neurotransmitter role of L-aspartate, particularly as it is only slowly metabolized in the CNS.

D-aspartate oxidase activity has been demonstrated in the mammalian brain. When assayed in vitro, with molecular oxygen as electron-acceptor, this enzyme is far more active on D-aspartate than D-glutamate and has no activity on those D-amino acids which are oxidised by D-amino acid oxidase. Levels of D-aspartate oxidase in the CNS are much lower than in the kidney.

No D-aspartate could be detected in extracts of cat and rat CNS. If present, it would have to be so at a level of less than 1% of the L-isomer.

ACKNOWLEDGMENTS

I am extremely grateful to my supervisor, Dr G.A.R. Johnston, for his close interest in all aspects of my work, as well as his continual encouragement and good-humoured support. My thanks also go to Professor D.R. Curtis and Dr M.L. Uhr for helpful advice throughout the course of this work.

I wish to express my appreciation to Mr L.B. James, of the Department of Biochemistry, for amino acid analyses, and to Dr J. Davies, Dr C.J.A. Game, Mr R.M. McCulloch and Mr J.G. Hall for dissection of feline CNS tissue.

To the members of the neurochemistry laboratory, Mr B. Twitchin, Mrs A.L. Stephanson and Mr V.J. Balcar, I express thanks, both for their companionship and assistance with experiments.

For their help in the preparation of this thesis, I thank Mrs A. Lamberts, Mrs H. Walsh and my wife, Alison.

The award of an Australian National University Ph.D. Scholarship is gratefully acknowledged.

During the tenure of my Australian National University Research Scholarship the following papers have been published or submitted for publication:

DAVIES L.P. & JOHNSTON G.A.R. (1973) Serine hydroxymethyltransferase in the central nervous system: regional and subcellular distribution studies. Brain Res. 54, 149-156.

JOHNSTON G.A.R. & DAVIES L.P. (1974) Postnatal changes in the high affinity uptake of glycine and GABA in the rat central nervous system. J.Neurochem. 22, 101-105.

DAVIES L.P. & JOHNSTON G.A.R. (1974) Postnatal changes in the levels of glycine and the activities of serine hydroxymethyltransferase and glycine:2-oxoglutarate aminotransferase in the rat central nervous system. J.Neurochem. 22, 107-112.

DAVIES L.P. & JOHNSTON G.A.R. (1974) D-aspartate oxidase activity in extracts of the mammalian central nervous system. Accepted for publication.

DAVIES L.P., JOHNSTON G.A.R. & STEPHANSON A.L. (1974) Postnatal changes in the potassium-stimulated, calcium-dependent release of radioactive GABA and glycine from slices of rat central nervous system. Accepted for publication.

I. GENERAL INTRODUCTION

A great volume of research has centred on the involvement of acetylcholine and the monoamines, noradrenaline, dopamine and serotonin, in peripheral transmission and in the central regulation and control of behaviour, emotion and basic drives such as hunger, thirst, aggression and sleep. Major advances have been made in biochemical, histochemical and pharmacological techniques for studying these substances. These studies have led to the widespread assumption that they are quantitatively major transmitters in the mammalian CNS. However, the biogenic amines and acetylcholine together probably account for transmission at only a small portion of central synapses (SNYDER, YOUNG, BENNETT & MULDER, 1973). Thus, in the corpus striatum, the brain region richest in dopamine, only about 15% of nerve terminals appear to contain catecholamines, while in the hypothalamus, which contains the highest levels of noradrenaline, only about 3-5% appear to contain catecholamines (HÖKFELT, 1970). About 5% of all synaptosomes in cerebral cortex homogenates appear to be noradrenergic (IVERSEN & SCHON, 1973). Since the brain contains less serotonin than catecholamines, this compound may account for yet a lower percentage of synapses. Acetylcholine has been shown to be the transmitter released at terminals of motor neuron axon collaterals onto Renshaw cells (ECCLES, FATT & KOKETSU, 1954), but no specific cerebral pathway has been conclusively shown to act by release of acetylcholine; it is probably only associated with a small percentage (about 10%) of nerve terminals (McLENNAN, 1970).

On recent evidence it appears that the amino acids, particularly GABA, glycine, glutamate and aspartate, account for transmission at a substantial proportion of central synapses. With more experimental evidence, taurine and

proline may eventually be added to this list. Based on the assumption that the neuronal uptake of amino acid transmitter candidates by high affinity uptake systems is specific for those neurons using them as transmitters, autoradiographic studies of CNS tissue after either in vivo or in vitro radioactive amino acid labelling have been performed to determine which neuron terminals accumulate them. The proportion of terminals labelled with [³H]GABA in cerebral cortex slices was 27%. In slices prepared from other regions this value varied from 14% in the molecular layer of the cerebellum to 51% in the substantia nigra (IVERSEN & SCHON, 1973). Very similar results were obtained with the proportion of synaptosomes labelled after incubation of homogenates with GABA. In spinal cord homogenates 25% of pinched-off nerve terminals were labelled. [³H]glycine labelled 28% of synaptosomes in spinal cord homogenates, while incubation with both [³H]GABA and [³H]glycine resulted in 51% of terminals being labelled (IVERSEN & BLOOM, 1972). To date, no electron microscope autoradiography of [³H] aspartate or [³H]glutamate has been reported (IVERSEN & SCHON, 1973).

Amino acids have only recently been proposed as possible CNS neurotransmitters. The evidence for certain amino acids acting as synaptic transmitters pertains to the following criteria for transmitter identification (CURTIS & JOHNSTON, 1970):

1. The substance, and possibly the enzymes for its synthesis, should be present in or accumulated by the appropriate nerve endings.
2. The substance should be released following impulses in the appropriate nerve fibres.
3. The postsynaptic action of the substance should be identical with that of the natural transmitter.

4. Compounds which block synaptic transmission by a specific postsynaptic effect must also antagonize the action of the suspected transmitter.
5. The processes which inactivate or remove the released transmitter should also terminate the action of the artificially administered substance.

These criteria for transmitter identification have generally proved acceptable in the identification of transmitters in the autonomic and peripheral nervous system. Because of the anatomical complexity of the mammalian CNS and because amino acids have general metabolic functions in addition to any transmitter role, investigations are complicated by experimental difficulties which necessitate compromise in the rigid application of this 'check-list'.

The first section of this thesis describes work on the inhibitory amino acid transmitters, GABA and glycine. A major part represents the application of several neurochemical correlates of synaptically active amino acids, including high affinity sodium-dependent uptake, potassium-stimulated, calcium-dependent release from brain slices and antagonism of post-synaptic action by systematically administered drugs, to the problem of the stage during postnatal development in the rat at which glycine and GABA are first used as transmitters. The second section reports neurochemical studies on aspartic acid, with particular emphasis on the D-isomer of the natural L-aspartate transmitter.

PART ONE: GLYCINE AND γ -AMINOBUTYRIC ACID

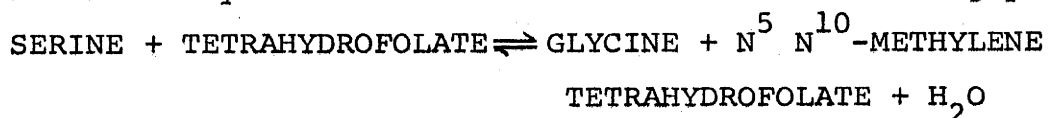
II. SERINE HYDROXYMETHYLTRANSFERASE IN THE CAT AND
RAT CENTRAL NERVOUS SYSTEM

1. INTRODUCTION

Electrophoretic studies of CURTIS & WATKINS (1960, 1961) showed the rapid and reversible depressant action of glycine on spinal neurones. The high levels of glycine in the ventral horn of the spinal cord, together with the comparatively low content in the ventral root fibres (APRISON & WERMAN, 1965; GRAHAM, SHANK, WERMAN & APRISON, 1967) suggested an association between glycine and spinal inhibitory interneurones. The proposition that glycine was the transmitter released by these neurones gained support from a correlation between the decreased levels of glycine in the dorsal and ventral grey matter and the loss of interneurones after aortic occlusion (DAVIDOFF, GRAHAM, SHANK, WERMAN & APRISON, 1966; DAVIDOFF, SHANK, GRAHAM, APRISON & WERMAN, 1967). The levels of γ -aminobutyric acid (GABA), an amino acid which also had a depressant action when applied microelectrophoretically to spinal neurones and which was more concentrated in the dorsal grey matter than in the ventral grey matter, were not significantly affected by this operation.

The accumulated evidence that glycine is a major inhibitory transmitter in certain areas of the mammalian central nervous system, including the spinal cord and medulla oblongata, has been well documented (APRISON, DAVIDOFF & WERMAN, 1970; CURTIS & JOHNSTON, 1970). The major pathways whereby glycine is metabolized in nervous tissue are, however, still unknown.

The enzyme serine hydroxymethyltransferase (L-serine-tetrahydrofolate 5, 10-hydroxymethyltransferase, EC 2.1.2.1) which catalyses the interconversion of serine and glycine,



has been purified from bovine brain (BRODERICK, CANDLAND, NORTH & MANGUM, 1972) and there is evidence to suggest that the enzyme plays an important role in glycine metabolism (SHANK & APRISON, 1970; SHANK, APRISON & BAXTER, 1973).

The observation that glycine levels vary from one CNS region to another (APRISON & WERMAN, 1965; SHAW & HEINE, 1965; SHANK & APRISON, 1970) suggested that the regional distribution of serine hydroxymethyltransferase activity might reveal important information about the possible role of this activity in glycine metabolism. The regional and subcellular distribution of serine hydroxymethyltransferase in extracts of rat and cat central nervous tissue was investigated. Some structural analogues of glycine and serine and various centrally-active drugs were tested as potential inhibitors of serine hydroxymethyltransferase activity.

2. METHODS AND MATERIALS

Tissue preparation and extraction. Adult Wistar rats (150-200g) were killed by decapitation, the brains and spinal cords rapidly removed and chilled, and the various regions quickly separated. Each brain was divided into the following regions: cerebral cortex, midbrain (including the diencephalon and mesencephalon), cerebellum and pons-medulla. The spinal cords were freed from spinal roots and the dura removed before homogenization. For cat spinal cord segments, the lumbosacral spinal cord was removed from cats (4-5 kg) under pentobarbitone anaesthesia and cut transversely into 4-6 mm pieces. Individual segments were placed on a freezing microtome stage (Pelcool, MSE, London) and sectioned into the following regions; dorsal white, ventral white, dorsal grey and ventral grey. The tissue samples were then weighed and homogenized in 0.1 M

potassium phosphate buffer (pH 7.4) containing 0.1 w/v Triton X-100 (to solubilize membrane-bound and occluded enzyme), 2mM EDTA (to limit the action of divalent-cation dependent proteases) and 0.1mM dithiothreitol (to stabilize the enzyme against oxidation during homogenization and dialysis). The 10% w/v homogenates were dialysed for 5 h against several changes of phosphate buffer containing EDTA and dithiothreitol, to remove endogenous glycine and serine. No loss of activity was observed after 16 h of dialysis. It is not known whether pentobarbitone anaesthesia has any effect on glycine levels or the activities of its metabolising enzymes in the spinal cord.

Protein concentrations in the tissue extracts were measured by the biuret method (GORNALL, BARDAWILL & DAVID, 1949) or the method of LOWRY, ROSEBROUGH, FARR & RANDALL, 1951), removing lipid by ether extraction immediately prior to reading in the spectrophotometer.

Assay of hydroxymethyltransferase activity. The rate of production of radioactive formaldehyde from radioactive serine was assayed by the method of TAYLOR & WEISSBACH (1965). The reaction mixtures contained 0.5 mM [$3\text{-}^{14}\text{C}$]serine, 0.5 mM pyridoxal phosphate, 2 mM tetrahydrofolate and 4 mM dithiothreitol in 100 mM potassium phosphate buffer (pH 7.4) in a final volume of 2 ml. Reactions, carried out at 37°C in a shaking water bath, were initiated by the addition of 0.5 ml of extract. At various times 0.4 ml aliquots were removed, and reaction terminated by addition to a mixture of 0.3 ml of 1 M sodium acetate solution (pH 4.5) and 0.2 ml of 0.1 M formaldehyde. The [^{14}C]formaldehyde, formed during the reaction, was trapped with dimedone, extracted into toluene and counted in a toluene-based scintillator (0.01% w/v 1, 4-di-2-(phenyloxayolyl) benzene and 0.4% 2, 5-diphenyloxazole in toluene) (see Appendix I). The enzyme activities measured in this way were linear for at least 40 min. Each assay provided four time aliquots and the rate of production of formaldehyde was calculated from the slope of the line of best fit through a plot of radioactivity versus time, by a least squares analysis using a PDP-8L computer.

Subcellular distribution of serine hydroxymethyltransferase.

Tissue was removed as described and homogenized in 0.32 M sucrose at a concentration of 10% w/v using a stainless steel mortar and Perspex pestle (rotating at 870 rev/min. clearance 0.25 mm difference in diameter). Subcellular fractions were prepared by differential and density gradient centrifugation as described by JOHNSTON, VITALI & ALEXANDER (1970), with the exception that the A₁ myelin layer and the upper S₂ layer were not separated prior to the next centrifugation. Pellets were resuspended in phosphate buffer containing EDTA and dithiothreitol and dialysed overnight against several changes of this buffer. The soluble fraction was concentrated prior to assay in an ultrafiltration apparatus (Amicon, UM-10 Diaflo ultrafilters).

Chemicals. 5-Azaindole was the gift of Professor A. Albert (Canberra), and O-glycyl-L-serine the gift of Dr J.R. Dice (Parke Davis, Detroit). Tetrahydrofolate was prepared by Dr G.A.R. Johnston from folic acid by catalytic hydrogenation as described by HATEFI, TALBERT, OSBORN & HUENNEKENS (1960). Hydrazinoacetic acid was prepared by published procedures (CARMİ, POLLAK & YELLIN, 1960; DODGSON, LLOYD & TUDBALL, 1961).

L-[3-¹⁴C]Serine, specific activity 48 mCi/mmole, was purchased from the Radiochemical Centre, Amersham. The specificity of the [¹⁴C] label was checked by sodium periodate oxidation of the radioactive serine. Other chemicals were purchased as follows: amino-oxyacetic acid, 3-mercaptopropionic acid and thiocarbohydrazide (Eastman); DL-C-allylglycine, D-cycloserine and pyridoxal phosphate (Sigma); 5-aminotetrazole, dimedone, kojic acid, and L-serine (Fluka); thiosemicarbazide and L-threonine (B.D.H.); glycine and sucrose (Mann); aminopterin and

DL-2-methylserine (Calbiochem); N-acetylglycine (Light); DL-3-phenylserine (Nutritional Biochemical Corp.) and strychnine (T. and M. Smith).

3. RESULTS

The regional distribution of serine hydroxymethyltransferase activity in the extracts of rat central nervous tissue is presented in Table 2.1, together with the concentrations of glycine and serine in the various regions as determined by SHANK & APRISON (1970). No significant differences in enzymic activity were observed between the five regions examined, whereas the concentrations of both glycine and serine show appreciable variation.

The distribution of serine hydroxymethyltransferase activity in the four regions of cat spinal cord is given in Table 2.2, together with the distribution of glycine and serine as determined by JOHNSTON (1968). The values for activity in extracts of grey matter are almost double those in white matter. Activity in the ventral grey matter extracts is significantly higher than in the dorsal grey matter extracts at $P < 0.05$ based on wet weight of tissue, and at $P < 0.025$ based on protein, using Student's t-test.

The subcellular distribution of serine hydroxymethyltransferase activity in homogenates of rat cerebral cortex and spinal cord was very similar (Table 2.3). Activity was concentrated in the mitochondrial, soluble and nuclear fractions. The activity observed in the nuclear fraction may be due to contamination with mitochondria and unbroken cells and thus no definite conclusion can be drawn as to the occurrence of the enzyme in or associated with nuclei.

Under the experimental conditions used, a number of substances significantly inhibited the serine

Table 2.1. Regional distribution of serine hydroxymethyltransferase activity in extracts of adult rat central nervous tissue

Region	Serine hydroxymethyltransferase activity (μmol formaldehyde produced/hr/g wet tissue or /mg protein)	Glycine concentration ($\mu\text{mol/g}$ wet tissue)	Serine concentration
Cerebral cortex	2.36 \pm 0.19	0.67 \pm 0.02	1.03 \pm 0.02
Cerebellum	2.42 \pm 0.18	0.63 \pm 0.03	0.60 \pm 0.02
Pons	2.64 \pm 0.32	2.93 \pm 0.15	0.40 \pm 0.01
Medulla		4.02 \pm 0.11	0.44 \pm 0.01
Diencephalon	2.32 \pm 0.37	0.93 \pm 0.02	0.61 \pm 0.01
Mesencephalon		1.63 \pm 0.04	0.42 \pm 0.01
Spinal cord	2.53 \pm 0.23	4.36*	0.46*

*Average of values for grey and white matter.

Serine hydroxymethyltransferase activity is expressed as the means \pm S.E.M. of values from 4 experiments. The glycine and serine concentrations are those of Shank and Aprison (1970).

§Throughout this thesis, the term 'adult' in reference to experiments carried out by the author on rat CNS means young adult, 13-weeks postnatal age.

Table 2.2. Regional distribution of serine hydroxymethyltransferase activity in extracts of cat spinal cord

Region	Serine hydroxymethyltransferase activity (μmol formaldehyde produced/hr /g wet tissue or /mg protein)	Glycine concentration ($\mu\text{mol/g}$ wet tissue)	Serine concentration
Dorsal white	2.46 \pm 0.06	2.92 \pm 0.10	0.58 \pm 0.16
Dorsal grey	4.29 \pm 0.20	5.42 \pm 0.14	0.92 \pm 0.26
Ventral grey	4.97 \pm 0.07	6.51 \pm 0.17	0.92 \pm 0.27
Ventral white	2.47 \pm 0.12	4.55 \pm 0.09	0.61 \pm 0.27

Hydroxymethyltransferase activities are expressed as the means \pm S.E.M. of values from each of 3 cats. The concentrations of glycine and serine are those reported previously (JOHNSTON, 1968).

Table 2.3. Subcellular distribution of serine hydroxymethyltransferase activity in homogenates of adult rat cerebral cortex and spinal cord

Subcellular fraction	Serine hydroxymethyltransferase activity (μ mol formaldehyde produced/hr/g wet tissue)	
	Cerebral cortex	Spinal cord
Nuclear pellet	0.18 \pm 0.07 (23)	0.20 \pm 0.09 (29)
Myelin layer	0.04 \pm 0.02 (5)	0.02 \pm 0.01 (3)
Synaptosomes, vesicles, etc.	0.05 \pm 0.01 (6)	0.03 \pm 0.02 (4)
Mitochondrial pellet	0.33 \pm 0.13 (41)	0.30 \pm 0.11 (44)
Soluble fraction	0.16 \pm 0.01 (20)	0.12 \pm 0.04 (18)
Microsomal pellet	0.04 \pm 0.02 (5)	0.02 \pm 0.01 (2)

Results are means \pm S.E.M. of values from each of 3 rats. Values in brackets refer to the percentage of the total recovered activity.

hydroxymethyltransferase activity in extracts of rat spinal cord (Table 2.4), the most effective being hydrazinoacetic acid, thiocarbohydrazide, amino-oxyacetic acid and cycloserine.

4. DISCUSSION

No significant differences were found in the serine hydroxymethyltransferase activity in extracts of various regions of the rat central nervous system. As there are regional differences in serine and glycine content, it seems from these results that serine hydroxymethyltransferase is not rate determining with respect to either glycine or serine metabolism. The rate controlling step for the formation of glycine from glucose may be the conversion of D-glycerate to hydroxypyruvate, catalysed by D-glycerate dehydrogenase (see Fig. 2.1), since the enzyme from rat brain is inhibited by glycine in a non-competitive manner suggestive of end product inhibition (UHR & SNEDDON, 1971) and, in the cat at least, its activity correlates with the regional distribution of glycine (UHR & SNEDDON, 1972). An attempt was made to measure the activities of D-glycerate dehydrogenase and 3-phosphoglycerate dehydrogenase in five regions of the rat CNS; although both activities were present in extracts of all regions examined including cerebral cortex, midbrain (diencephalon and mesencephalon), cerebellum, pons-medulla and spinal cord, the formation of NADH was not linear with time and activities were not quantified.

A recent report (DALY & APRISON, 1974) has measured serine hydroxymethyltransferase in five regions of rat brain. Utilizing somewhat different conditions and concentrations of substrate and cofactors, they found the levels in the telencephalon and midbrain to be approximately three-quarters of the levels in cerebellum, pons-medulla and spinal cord. When these values were expressed per

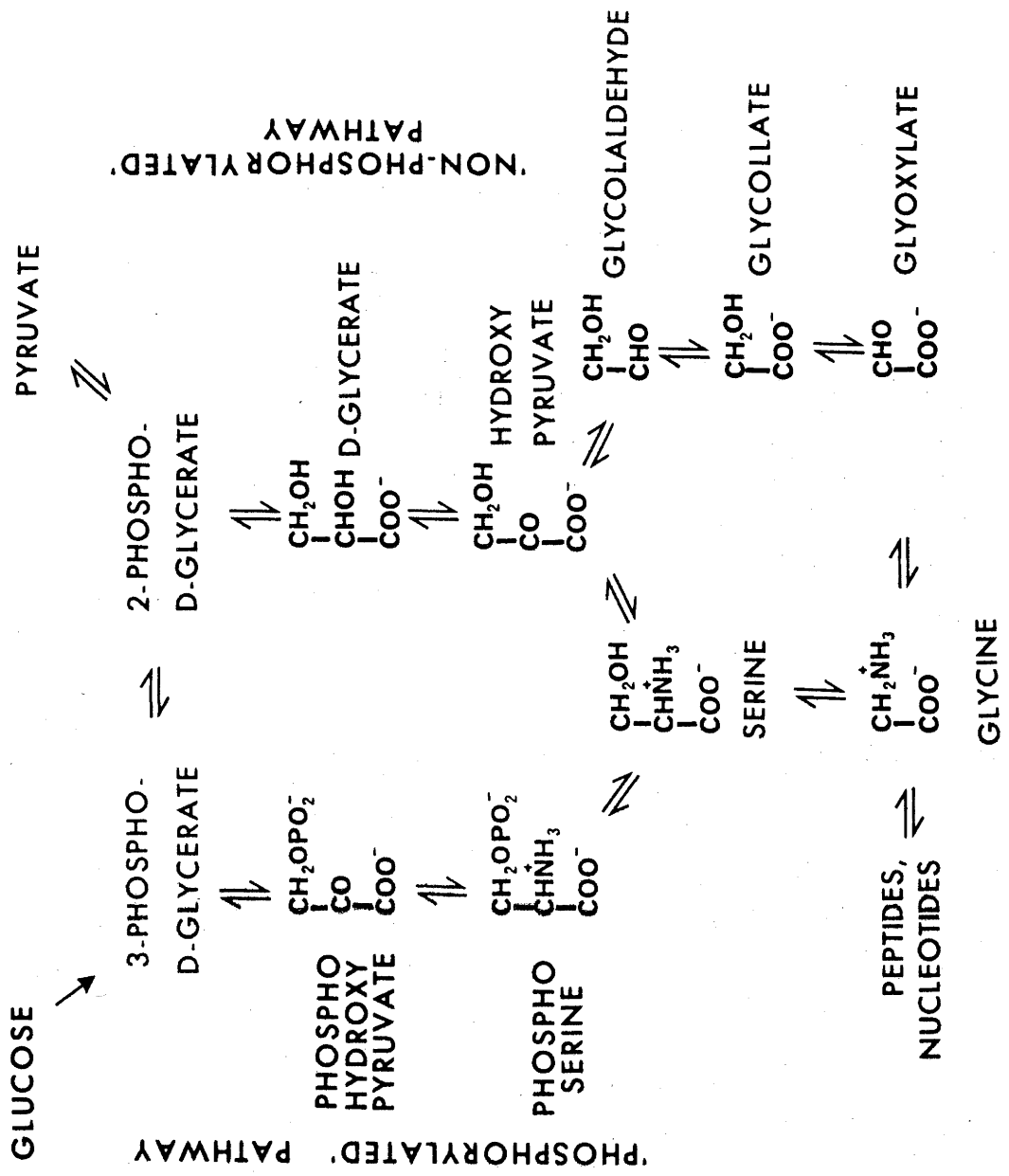
Table 2.4. Inhibitors of serine hydroxymethyltransferase activity in extracts of adult rat spinal cord

Inhibitor (0.5 mM)	% Inhibition
Hydrazinoacetic acid	94 \pm 8
Thiocarbohydrazide	93 \pm 4
Amino-oxyacetic acid	83 \pm 1
D-Cycloserine	76 \pm 2
Thiosemicarbazide	49 \pm 2
O-Glycyl-L-serine	37 \pm 2
Aminopterin	30 \pm 4
N-Acetylglycine	22 \pm 2
DL-Serine-O-sulphate	21 \pm 3
Kojic acid	20 \pm 2
3-Mercaptopropionic acid	17 \pm 2
Threonine	15 \pm 1
DL-2-Methylserine	13 \pm 1
Nikethamide	5 \pm 0.5

No significant inhibition: DL-C-allylglycine, 5-aminotetrazole, 5-azaindole, DL-3-phenylserine, strychnine

Values are means \pm S.E.M. of three determinations. Inhibitors were tested in the standard assay.

Fig. 2.1. Possible routes of glycine metabolism in the CNS (from CURTIS & JOHNSTON, 1974). Possible routes of glycine synthesis from glucose via the 'phosphorylated' and 'non-phosphorylated' pathways are given. Not all reactions represented here have been shown to occur in the CNS e.g. the hydroxypyruvate: α -alanine transaminase is very low or absent (dog, ox: WALSH & SALLACH, 1966; rat, cat: UHR, unpublished results).



relative number of mitochondria in each region (as estimated by measuring the levels of succinate dehydrogenase), there was a correlation with glycine levels. The role of serine hydroxymethyltransferase in non-transmitter metabolism (i.e. in the supply of folates, active carbons and/or 'metabolic' glycine) appears to account for the largest portion of activity measured (see Section III.4). The fact that these regional variations were not apparent in this study may be due to the fact that the concentration of the substrate, serine, used in the assay (0.5 mM) was of the order of the Michaelis constant for the enzyme ($K_m = 0.62$ mM for the brain enzyme; DALY & APRISON, 1974; $K_m = 0.54$ mM for liver enzyme; NAKANA, FUJIOKA & WADA, 1968) and thus activity measured was only about half of that possible with saturating serine concentrations. Significant regional differences might have been more readily observed had maximal in vitro activities been obtained by the use of saturating serine concentrations (e.g. 4 mM).

Serine hydroxymethyltransferase, like other amino acid metabolizing enzymes including D-amino acid oxidase (DeMARCHI & JOHNSTON, 1969), glutamine synthetase, glutaminase, GABA:2-oxoglutarate aminotransferase, aspartate:2-oxoglutarate aminotransferase, glutamate decarboxylase, glutamate dehydrogenase (GRAHAM & APRISON, 1969) and glycine:2-oxoglutarate aminotransferase (JOHNSTON, VITALI & ALEXANDER, 1970), was found to be more concentrated in the grey matter than in white matter of cat spinal cord. Serine hydroxymethyltransferase and glutamate decarboxylase (GRAHAM & APRISON, 1969) are the only ones to show dorsoventral differences within the spinal grey matter. Serine hydroxymethyltransferase and glycine are more concentrated in the ventral grey matter than in dorsal grey matter, while the reverse is true for glutamate decarboxylase, glutamate and GABA. Dorsoventral differences in enzymic activity were not apparent in the white matter, whereas glycine is more concentrated in the ventral than in dorsal regions of both the grey and white matter (APRISON & WERMAN, 1965; JOHNSTON, 1968). The correlation coefficient for the hydroxymethyltransferase activity (per mg protein) when compared with glycine levels in

in the four regions (JOHNSTON, 1968) was 0.90.

Under the assay conditions employed, the measured rate of glycine production from serine by the hydroxymethyltransferase activity in the cat spinal cord extracts was of similar order to the rate of glycine production from glyoxylate by glycine:2-oxoglutarate activity in extracts of the same regions (JOHNSTON, VITALI & ALEXANDER, 1970). The activities are fairly low in comparison with many other amino acid metabolising enzymes, but in as much as enzyme activities measured in vitro can be related to those in vivo, they appear to be quite adequate to account for the estimated rates of glycine and serine formation from glucose in vivo (SHANK & APRISON, 1970).

The subcellular distribution of hydroxymethyltransferase activity was at least bimodal in the homogenates of rat cerebral cortex and spinal cord, being concentrated in the mitochondrial and soluble fractions. A similar subcellular distribution has been found in homogenates of rat liver (NAKANO, FJUIOKA & WADA, 1968). No differences were observed in the subcellular distribution of hydroxymethyltransferase activity in the cerebral cortex, where glycine is unlikely to be a synaptic transmitter, and in the spinal cord, where a transmitter role is highly probable. These findings provide no evidence to suggest that this enzyme is specifically associated with synaptosomes produced on homogenization of nerve endings which released glycine as a transmitter.

However, the total enzyme activities recovered in the subcellular fractionation study were low compared with the activity in Triton X-100 treated homogenates (about 30% for rat cortex; Table 2.3 c.f. Tables 2.1) because these fractions were assayed in the absence of the detergent. Thus particulate enzyme was not fully accessible to substrates during the enzyme assay. This would result in underestimation of the enzyme activity in particulate fractions and on the basis of the

percentage recoveries of activities, lead to an overestimate of the proportion of enzyme in the soluble fraction. Subfractionation of five brain regions by DALY & APRISON (1974) and subsequent assay in the presence of Triton X-100 showed that between 50-63% of the recovered enzyme activity was in the crude mitochondrial pellet (mitochondria, synaptosomes and myelin) and only 1-4% in the soluble fraction. Further fractionation of the crude mitochondrial pellet showed that 77-82% of recovered activity was in the mitochondria and 10-19% in the synaptosomal fraction. RASSIN & GAULL (1975) found 74% of serine hydroxymethyltransferase activity in the crude mitochondrial fraction and 5% in soluble form. Subfractionation of the crude mitochondrial fraction showed that 78% was in mitochondria and 15% in synaptosomes. Thus the enzyme is essentially mitochondrial in nature. It is interesting to note that further results of RASSIN & GAULL (1975) suggest that the enzyme is not associated to any great degree with those mitochondria that are present in synaptosomes. McBRIDE, DALY & APRISON (1974) found no obvious correlation between the rate of conversion of serine to glycine by crude synaptosomal pellets (containing myelinated axons, synaptosomes and mitochondria) prepared from the spinal cord, medulla oblongata, telencephalon and cerebellum of the rat and the levels of glycine measured in the same areas.

Most of the substances found to inhibit serine hydroxymethyltransferase activity in extracts of rat spinal cord are known to inhibit other pyridoxal-dependent enzymes (hydrazinoacetic acid, thiosemicarbazide, thiocarbohydrazide, amino-oxyacetic acid, D-cycloserine, 3-mercaptopropionic acid) and/or are structural analogues of glycine and serine (hydrazinoacetic acid, amino-oxyacetic acid, D-cycloserine, O-glycyl-L-serine, N-acetylglycine, DL-serine-O-sulphate, L-threonine, DL-2-methylserine). The inhibitors include certain convulsants (thiocarbohydrazide, thiosemicarbazide, kojic acid, 3-mercaptopropionic acid) whereas other convulsants (DL-C-allylglycine, 5-azaindole and strychnine) had no effect on hydroxymethyltransferase activity. Glycine levels in rat cerebellum are known to be reduced during convulsions induced

by 3-mercaptopropionic acid. Inhibition of serine hydroxymethyltransferase may contribute to this reduction in glycine level.

Suckling rats, whose mothers were fed on vitamin B₆-deficient diet from parturition, had levels of pyridoxal phosphate which were reduced by two-thirds when compared with control animals. Analysis of amino acids in these animals at 19 days postnatal age (KURTZ, LEVY & KANFER, 1972) showed large changes in levels. Of these, glycine levels were increased nearly three times, while serine levels were reduced by one-quarter. These changes may in part reflect diminished activity of serine hydroxymethyltransferase, although studies of its activity in pyridoxine-deficient rats have not been reported.

In conclusion, these studies did not show any correlation between the activity of serine hydroxymethyltransferase in extracts prepared from different regions of the rat CNS and the glycine or serine content of those regions. On the basis of in vivo studies using radioactive glucose SHANK & APRISON (1970) suggested that glycine appeared to be derived predominantly by de novo synthesis from glucose through serine, but that there appeared to be no correlation between the content of glycine and its rate of synthesis from serine. Within the cat spinal cord, some degree of correlation between glycine levels and hydroxymethyltransferase was apparent, with higher levels of both in ventral as compared with dorsal grey matter. In view of this finding, it would be worthwhile to do regional and subcellular distribution studies of serine hydroxymethyltransferase in feline CNS, particularly since larger amounts of CNS tissue are available from cats than from rats. These studies were not done because of the difficulty at that time of obtaining animals. Subcellular distribution studies on rat brain cerebral cortex and spinal cord reported here suggest that the enzyme is located predominantly in the mitochondria, and to a lesser extent, the cytoplasm.

Data showing levels of this enzyme in rat CNS at several stages postnatally is presented in the following chapter.

III. POSTNATAL CHANGES IN THE LEVELS OF GLYCINE AND
THE ACTIVITIES OF SERINE HYDROXYMETHYLTRANSFERASE
AND GLYCINE:2-OXOGLUTARATE AMINOTRANSFERASE
IN THE RAT CENTRAL NERVOUS SYSTEM

1. INTRODUCTION

In general, the mammalian brain shows a caudal-rostral development, with spinal cord systems maturing morphologically, biochemically and functionally earlier than higher CNS centres (TIMIRAS, VERNADAKIS & SHERWOOD, 1968). The rat is a good choice for developmental studies because the brain is comparatively immature at birth, undergoes a period of accelerated growth during the first three postnatal weeks and reaches maturity at completion of myelination in about 60 days (VERNADAKIS & WOODBURY, 1969).

Using an ethanolic-phosphotungstic acid (E-PTA) stain which renders morphologically specialized junctions electron-opaque (BLOOM & AGHAJANIAN, 1966), the formation of synaptic junctions has been followed in rat parietal cortex molecular layer (AGHAJANIAN & BLOOM, 1967) and in cerebellar cortex molecular layer (folia VII and VIII; WOODWARD, HOFFER, SIGGINS & BLOOM, 1971). Few E-PTA staining synaptic junctions are evident in the molecular layer of cortex on the 12th or 13th postnatal day, but between the 14th and 26th day there is a rapid increase to normal adult levels. In the molecular layer of the cerebellar folia VII and VIII, the latest folia to mature, synapses are first evident at day three, with an increase in two or more slow phases, reaching near adult levels by the end of the third postnatal week. There appears to be a close relationship between morphological development and development of electrical activity.

Electron-microscopic observations on synaptic development in foetal rat lumbar spinal cord (MAY & BISCOE, 1973) have shown the appearance of axodendritic and axosomatic synapses over foetal days 14-18 (gestation period is 21 days). The appearance of these immature

synapses correlates with observed changes in the mobility of the foetus, as foetal movements begin during this period.

As well as adding to knowledge of brain development, neurochemical studies of developmental changes can provide a useful variable which may lead to the better understanding of certain aspects of mature brain function. For both reasons, the developmental studies presented in the following three chapters were performed. Firstly, an examination of levels of serine hydroxymethyltransferase and glycine: 2-oxoglutarate aminotransferase was carried out at several stages of development in an attempt to clarify their roles in the maintenance of different regional glycine levels. Furthermore, the time during ontogenetic development when glycine and GABA are first used as inhibitory transmitters was of interest, in view of the fact that their inhibitory transmitter function in the adult mammalian CNS is relatively well established.

Following the suggestion of APRISON & WERMAN (1965) that glycine was a likely inhibitory transmitter released by some spinal interneurons, it was found that glycine levels in the medulla oblongata and spinal cord of five mammalian species were 3-5 times higher than the levels in supra-medullary structures and cerebellum (APRISON, SHANK, DAVIDOFF & WERMAN, 1968). This increasing rostro-caudal gradient was most evident in cat and rat.

As an initial approach to the problem of glycine neurotransmitter development, therefore, the regional distribution of glycine was determined in 10-day-old animals. In addition, levels of other amino acids were quantified; nearly all studies on the developmental pattern of amino acid levels in the mammalian CNS have been confined to whole brain levels rather than a number of discrete regions, while spinal cord tissue has been little studied.

For the following studies, rats of 1 day, 10 days and 13 weeks postnatal age were used. The brains of 1-day-old rats are quite immature and represent a convenient stage at which tissue can be quickly and conveniently removed and dissected into the following regions; cerebral hemispheres, cerebellum, midbrain and pons-medulla. The brains of 10-day-old rats are undergoing a weight growth 'spurt' (see DISCUSSION) and are at a stage, just prior to the onset of myelination, when differentiation of neuronal and glial cell types has taken place in much of the brain, and neuronal cell processes have proliferated extensively. Thus during the period from birth to 10 days the rat CNS undergoes extensive maturation, although in most regions apart from the cerebellum there is little increase in the total cell numbers over this time. Thirteen-week-old animals were taken to represent a mature stage of brain development. These 'young adults' have obtained about 80% of their maximum body weight (DOBBING, 1968) and from an anatomical point of view, have a mature CNS (ADINOLFI, 1971). 'Young adults' at about this stage of development are commonly used for biochemical studies.

2. METHODS AND MATERIALS

Tissue preparation and extraction. Wistar rats of 1 day, 10 days or 13 weeks postnatal age were killed by decapitation. Removal of CNS tissue, separation into regions and homogenization in buffer was carried out as described (Section II.2). For amino acid analysis, the tissue (100-500 mg wet weight) was homogenized in 2 ml of ice-cold 1% aqueous picric acid with a glass-teflon homogenizer. The homogenate was allowed to stand for 30 min at 0°C and sufficient Dowex 2 x 10 resin (chloride form; 200-400 mesh) added to discharge the yellow colour. The resin was sedimented by centrifugation, washed with 2 x 10 ml volumes of 0.02 M hydrochloric acid, and the combined supernatant and washings evaporated to dryness in vacuo. The residue was dissolved in a known volume of pH 2.2 citrate buffer, centrifuged (27,000g for 10 min) to remove any particulate matter and the supernatant fluid analysed on a modified Technicon Auto Analyser (JAMES, 1972), using the two-buffer system of SPACKMAN, STEIN & MOORE (1958). β -Thienylalanine and α -amino- β -guanidinopropionic acid were added to the initial picric acid homogenates to act as internal standards for the recovery of the natural amino acids.

Assay of Serine hydroxymethyltransferase. This enzyme was assayed as described previously (Section II.2).

Assay of Glycine:2-oxoglutarate aminotransferase. Enzymic formation of radioactive glycine from [2-¹⁴C]glyoxylate and L-glutamate was assayed essentially as described previously (JOHNSTON & VITALI, 1969) by incubating 0.5 ml samples of CNS extract with 0.5 ml of a solution of 60 mM [2-¹⁴C]glyoxylate and 5 mM EDTA, adjusted to pH 7.4 with NaOH, 0.5 ml of 20 mM monosodium L-glutamate and 0.5 ml of 100 mM potassium phosphate buffer, pH 7.4, at

37°C in a shaking water bath. At various times, 0.4 ml samples were removed and chromatographed on columns (7 x 50 mm) of Dowex 1 x 8 resin (acetate form; 100-200 mesh), eluting with distilled water. The first 5 ml of eluent from each column was collected and its radioactivity determined by liquid scintillation using 1 ml samples in 10 ml of dioxan-based scintillator (0.5% (w/v) 2,5-diphenyl-oxazole and 10% (w/v) naphthalene in Dioxan). These columns retained more than 99% of radioactive glyoxylate.

Chemicals. β -Thienylalanine and α -amino- β -guanidinopropionic acid were purchased from Pierce Biochemicals. The source of other chemicals used has been given (Section II.2).

3. RESULTS

Amino acid levels. The levels of amino acids in five regions of the CNS of 10-day rats are given in Table 3.1. As in the adult rat and cat (APRISON, SHANK, DAVIDOFF & WERMAN, 1968), glycine is present in higher concentrations in the spinal cord and pons-medulla than in higher brain regions. In fact, glycine is the only amino acid found in significantly higher concentration in the spinal cord than in any other CNS region of the 10-day-old rats. Spinal amino acid levels from 1-day, 10-day and 13-week-old rats are shown in Table 3.2. The glycine content of spinal cord from 1- and 10-day animals did not differ significantly from that of adult cord.

As observed for whole brain (BAYER & McMURRAY, 1967; AGRAWAL, DAVIS & HIMWICH, 1966) the spinal levels of taurine, serine, proline, α -alanine, valine, leucine, tyrosine, phenylalanine and histidine decreased with increasing age, while the level of aspartate increased, and the levels of lysine, isoleucine and cystathionine showed

Table 3.1. Amino acid levels, expressed as $\mu\text{mol/g}$ wet tissue, in extracts of five CNS regions of 10-day-old rats

Amino acid	Cerebrum	Cerebellum	Midbrain	Pons-Medulla	Spinal cord
Taurine	14.69 \pm 0.74	9.16 \pm 0.50	9.84 \pm 0.52	6.57 \pm 0.64	4.90 \pm 0.42
Aspartate	2.06 \pm 0.08	1.89 \pm 0.25	1.89 \pm 0.21	1.63 \pm 0.12	1.63 \pm 0.10
Serine	0.61 \pm 0.08	0.93 \pm 0.10	0.83 \pm 0.13	1.03 \pm 0.31	0.77 \pm 0.13
Glutamine	3.38 \pm 0.32	4.39 \pm 0.54	3.67 \pm 0.13	4.54 \pm 0.36	4.76 \pm 0.51
Proline	0.40 \pm 0.06	0.31 \pm 0.03	0.23 \pm 0.03	0.23 \pm 0.03	0.39 \pm 0.05
Glutamate	5.27 \pm 0.48	4.39 \pm 0.85	4.32 \pm 0.24	4.47 \pm 0.55	3.06 \pm 0.18
Glycine	1.10 \pm 0.07	1.48 \pm 0.48	1.63 \pm 0.19	2.05 \pm 0.10	3.22 \pm 0.33
α -Alanine	0.88 \pm 0.11	1.08 \pm 0.44	3.65 \pm 0.97	0.50 \pm 0.05	1.93 \pm 0.48
Valine	0.13 \pm 0.01	0.20 \pm 0.01	0.26 \pm 0.04	0.14 \pm 0.02	0.15 \pm 0.03
Cystathionine	0.29 \pm 0.04	0.46 \pm 0.08	0.30 \pm 0.08	0.29 \pm 0.07	0.34 \pm 0.05
Isoleucine	0.10 \pm 0.01	0.11 \pm 0.02	0.14 \pm 0.05	0.08 \pm 0.03	0.14 \pm 0.01
Leucine	0.16 \pm 0.01	0.24	0.23 \pm 0.06	0.11, 0.15	0.19 \pm 0.03
Tyrosine	0.32 \pm 0.04	0.11	0.31 \pm 0.03	0.08, 0.14	0.10 \pm 0.01
Phenylalanine	0.10 \pm 0.01	0.11	0.11 \pm 0.03	0.08, 0.14	0.10 \pm 0.01
GABA	1.03 \pm 0.09	0.94 \pm 0.08	3.95 \pm 0.51	1.25 \pm 0.05	1.59 \pm 0.23
Lysine	0.29 \pm 0.02	0.48 \pm 0.03	0.90 \pm 0.15	0.21, 0.29	0.65 \pm 0.03
Histidine	0.13 \pm 0.01	0.41 \pm 0.02	0.48 \pm 0.12	0.51, 0.61	0.39 \pm 0.05

Values are means \pm S.E.M. of 3 - 5 determinations, unless individual values are given. Because of the lack of separation between the serine and glutamine peaks in the system used, the values given for serine are only approximate.

Table 3.1a. Amino acid levels, expressed as $\mu\text{mol/g}$ wet tissue, in extracts of four CNS regions of adult rats (data of KANDERA, LEVI & LAJTHA, 1968).

Amino acid	Cerebrum	Cerebellum	Midbrain	Pons-Medulla
Taurine	7.55	5.57	2.81	1.96
Aspartate	2.44	1.97	2.45	2.68
Serine & Asparagine	1.16	0.936	0.537	0.630
Glutamine & Threonine	2.35	2.30	1.53	1.64
Glutamate	11.57	10.33	7.50	6.85
Glycine	0.629	0.627	1.56	3.62
α -Alanine	0.666	0.591	0.396	0.560
Valine	0.083	0.065	0.080	0.083
Cystathionine	0.015	0.268	0.025	0.053
Isoleucine	0.025	0.028	0.028	0.034
Leucine	0.064	0.065	0.071	0.064
Tyrosine	0.073	0.070	0.062	0.059
Phenylalanine	0.050	0.052	0.055	0.050
GABA	2.33	1.54	3.79	1.77
Lysine	0.207	0.366	0.363	0.435
Histidine	0.066	0.062	0.059	0.044

Values are the means of 4 determinations. The brain areas were rapidly dissected, frozen, weighed and homogenised in 3% perchloric acid. Analysis was performed on a modified Technicon AutoAnalyser.

Table 3.2. Amino acid levels, expressed as $\mu\text{mol/g}$ wet tissue, in extracts of spinal cord from rats of different ages

Amino acid	Postnatal Age		
	1 day	10 days	13 weeks
Taurine	9.97 \pm 0.95	4.90 \pm 0.42	1.30 \pm 0.05
Aspartate	1.22 \pm 0.08	1.63 \pm 0.10	1.83 \pm 0.11
Serine	0.72 \pm 0.08	0.77 \pm 0.13	0.46 \pm 0.12
Glutamine	5.82 \pm 0.09	4.76 \pm 0.51	2.34 \pm 0.39
Proline	0.56 \pm 0.10	0.39 \pm 0.05	0.09 \pm 0.03
Glutamate	4.52 \pm 0.51	3.06 \pm 0.18	3.67 \pm 0.31
Glycine	3.98 \pm 0.52	3.22 \pm 0.33	3.22 \pm 0.17
α -Alanine	2.73 \pm 0.97	1.93 \pm 0.48	0.45 \pm 0.22
Valine	0.31 \pm 0.11	0.15 \pm 0.03	0.07 \pm 0.01
Cystathionine	0.31 \pm 0.02	0.34 \pm 0.05	0.30 \pm 0.02
Isoleucine	0.18 \pm 0.03	0.14 \pm 0.01	0.15 \pm 0.05
Leucine	0.27 \pm 0.03	0.19 \pm 0.03	0.11 \pm 0.03
Tyrosine	0.28 \pm 0.03	0.37 \pm 0.05	0.08 \pm 0.01
Phenylalanine	0.10 \pm 0.01	0.10 \pm 0.02	0.03
GABA	1.15 \pm 0.20	1.59 \pm 0.23	0.80 \pm 0.10
Lysine	0.73 \pm 0.11	0.65 \pm 0.03	0.88 \pm 0.07
Histidine	0.61 \pm 0.13	0.39 \pm 0.05	0.08 \pm 0.01

Values are means \pm S.E.M. of 3 - 5 determinations, unless individual values are given. Because of the lack of separation between the serine and glutamine peaks in the system used, the values given for serine are only approximate.

no significant change. In contrast to findings for rat brain however, the spinal levels of glutamate, glutamine and GABA decreased.

Turning to brain, a comparison of the regional levels in 10-day-old rats (Table 3.1) with those reported by KANDERA, LEVI & LAJTHA (1968) for adult rats (Data reproduced in Table 3.1a), shows that for all brain regions there is a developmental decrease in the levels of taurine, valine, cystathionine, isoleucine, leucine, tyrosine, phenylalanine, lysine and histidine and an increase in the levels of aspartate and glutamate. The level of GABA increased in all regions except the midbrain, whereas that of α -alanine decreased in all regions except the pons-medulla. The glycine level decreased in all brain regions except the pons-medulla where the level increased. There appeared to be little change in the serine level in any brain region, in agreement with studies on whole brain development (BAYER & McMURRAY, 1967; AGRAWAL, DAVIS & HIMWICH, 1966).

The levels of α -alanine showed greater regional variation in 10-day-old rats than in adults. The midbrain level of α -alanine in 10-day-old rats is very high compared with adult levels and varied over a wide range in the five animals examined. The level of GABA in the midbrain in 10-day-old rats is higher than in the other CNS regions, as is the case for adult rats (KANDERA, LEVI & LAJTHA, 1968).

A decreasing rostrocaudal gradient was very apparent for taurine in the 10-day-old rats, with levels ranging from 14.7 $\mu\text{mol/g}$ in the cerebral cortex to 4.9 $\mu\text{mol/g}$ in the cord. A similar pattern occurs in adult rats where levels decrease from 7.6 $\mu\text{mol/g}$ in the cerebral

cortex to 2.0 $\mu\text{mol/g}$ in the pons-medulla (KANDERA, LEVI & LAJTHA, 1968) and 1.3 $\mu\text{mol/g}$ in spinal cord. The decrease in taurine levels in all regions is very marked and suggests that taurine may play an important role during the development of the CNS (AGRAWAL, DAVISON & KACZMAREK, 1971; KACZMAREK, AGRAWAL & DAVISON, 1971).

Serine hydroxymethyltransferase activity. The regional distribution of serine hydroxymethyltransferase activity in extracts of CNS tissue from rats of different postnatal ages (1-day, 10-day and 13-week) is shown in Table 3.3. There is a decrease in activity of the enzyme from newborn to adult rats. The highest levels of activity were found in extracts of the cerebellum of the 10-day-old rats. The effects of a number of inhibitors on this activity (Table 3.4) were similar, but not identical to those observed on the hydroxymethyltransferase activity in extracts of adult spinal cord (Table 2.4).

Glycine:2-oxoglutarate aminotransferase activity. The data obtained for the regional distribution of glycine:2-oxoglutarate activity (Table 3.5) suggest a slightly greater activity in 10-day-old than 1-day-old animals. Activities measured in four brain regions in adult rats have shown that levels in cerebral grey matter and the midbrain are the same. Slightly higher levels are found in the corpus callosum and the lowest levels are in the spinal cord (BENUCK, STERN & LAJTHA, 1972).

4. DISCUSSION

From a regional comparison of glycine levels in 10-day-old rats, it is apparent that the increasing rostrocaudal gradient so prominent in adults (Table 2.1) is established in young rats at least as early as ten days

Table 3.3. Regional distribution of serine hydroxymethyltransferase activity in extracts of rat CNS

Region	Serine hydroxymethyltransferase activity in extracts from					
	1-day-old rats		10-day-old rats		13-week-old rats (from table 2.1)	
Cerebral cortex	6.03 ± 0.45	0.128 ± 0.009	4.15 ± 0.38	0.076 ± 0.006	2.36 ± 0.19	0.037 ± 0.004
Cerebellum	5.79 ± 0.45	0.129 ± 0.009	8.45 ± 0.38	0.163 ± 0.034	2.42 ± 0.18	0.040 ± 0.005
Midbrain	3.81 ± 0.23	0.087 ± 0.005	2.40 ± 0.44	0.047 ± 0.009	2.32 ± 0.37	0.034 ± 0.005
Pons-medulla	4.21 ± 0.48	0.095 ± 0.011	3.75 ± 0.48	0.073 ± 0.009	2.64 ± 0.32	0.039 ± 0.006
Spinal cord	4.79 ± 0.45	0.124 ± 0.012	3.97 ± 0.56	0.089 ± 0.013	2.53 ± 0.23	0.043 ± 0.004

For each age group the left column is activity expressed as μmol of formaldehyde produced/h/g wet tissue and the right column is activity expressed /mg of protein. Values are means \pm S.E.M. from four separate experiments.

Table 3.4. Inhibitors of serine hydroxymethyltransferase activity in extracts of 10-day-old rat cerebellum

Inhibitor 0.5 mM	% Inhibition
Aminooxyacetic acid	95.8 \pm 0.1
Thiosemicarbazide	61.5 \pm 0.1
O-Glycyl-L-serine	48.9 \pm 0.3
L-Serine-O-phosphate	25.9 \pm 0.2
DL-Serine-O-sulphate	21.3 \pm 0.3
L-Threonine	23.7 \pm 0.5
Aminopterin	23.3 \pm 0.3
Strychnine	16.2 \pm 0.2
β -Phenylserine	16.1 \pm 0.3
Kojic acid	11.8 \pm 0.2
DL-2-Methylserine	9.5 \pm 0.2
<u>No significant inhibition:</u> DL-C-allylglycine, 5 aminotetrazole	

Values are expressed as % inhibition and are means \pm S.E.M. of four determinations. Inhibitors were tested in the standard assay.

Table 3.5. Regional distribution of glycine:2-oxoglutarate aminotransferase activity in extracts of rat CNS

Region	Glycine:2-oxoglutarate aminotransferase activity in extracts from	
	1-day-old rats	10-day-old rats
Cerebral cortex	3.72 ± 0.78	5.69 ± 0.46
Cerebellum	4.26 ± 0.90	5.82 ± 0.48
Midbrain	5.34 ± 0.96	5.24 ± 0.63
Pons-medulla	5.88 ± 1.02	6.64 ± 1.00
Spinal cord	4.92 ± 0.48	6.00 ± 1.06
		0.104 ± 0.008
		0.112 ± 0.009
		0.101 ± 0.012
		0.129 ± 0.019
		0.132 ± 0.023

For each age group, the left column is activity expressed as μmol of glycine produced/h/g wet tissue and the right column is activity expressed /mg of protein. Values are means \pm S.E.M. from four separate experiments.

after birth. The present study shows that the overall developmental changes in amino acids in the different brain regions are consistent with developmental changes of total brain amino acids (HIMWICH & AGRAWAL, 1969; AGRAWAL, DAVIS & HIMWICH, 1966; BAYER & McMURRAY, 1967; DAVIS & HIMWICH, 1973) with the exception of glycine in the pons-medulla which is lower in 10-day-old rats than in the adults. In the spinal cord a somewhat different developmental pattern from that of higher brain regions is observed in that only aspartate, and not glutamate or GABA, increased during development. The levels of isoleucine, cystathionine and lysine showed no change. Glycine showed no significant decrease, while the level of all other amino acids decreased.

There is no correlation of either serine hydroxymethyltransferase activity or glycine:2-oxoglutarate aminotransferase activity with glycine levels in the CNS of 10-day-old rats. There is however, a general decrease in the measured activity of the hydroxymethyltransferase with development, which is in contrast to many other enzymes which increase in activity as the brain matures. This finding is in agreement with that of BRIDGERS (1968), who found that the activity was highest in new-born mouse brain and then declined to reach a steady level between two and three weeks postnatal age. BRIDGERS (1968) suggested that hydroxymethyltransferase activity essentially paralleled the growth rate of neonatal mouse brain. The present results in rat CNS, especially in the cerebellum, support this suggestion.

A growth 'spurt' or period in which there is a marked increase in the rate of gain of weight, occurs in the ontogenetic development of mammalian brain (DAVISON & DOBBING, 1968). In the rat this growth peak is maximal at 11 days after birth and is due mainly to cerebellar development. There is a twenty-fold growth in the cerebellum between 1-21 days (measured as increase in area of saggittal sections), primarily due to development of the cortex (ALTMAN, 1969). The addition of new cells accelerates rapidly in the first week of postnatal

life and it remains at a high level until about the end of the second week (ALTMAN, 1966). However, the rate of growth of other regions declines from birth. Estimates of cell proliferation, as measured by in vitro DNA synthesis have been reported by SUNG (1968). The following activities (μmol [2- ^{14}C] thymidine incorp. into DNA/g tissue/h) were recorded in six regions; cerebral cortex (1.27), cerebellum (23.9), midbrain (0.78), pons-medulla (1.09), olfactory bulb (2.50) and hypothalamus (0.74). Further results show that the activity remains high in the cerebellum until 6-10 days after birth before decreasing, whereas activity in the cerebral cortex decreases from birth.

Ornithine decarboxylase is an enzyme involved in the synthesis of spermine and spermidine, polyamines which probably have an important function during cell growth. The levels of this enzyme in the rat cerebrum, cerebellum and brainstem have been shown to parallel those periods of maximal cell proliferation (ANDERSON & SCHANBERG, 1972). The level in the cerebellum increases from birth to a maximum at ten days then decreases, whereas activity in the cerebrum decreases from birth almost to the adult level by the twelfth postnatal day. This pattern is much the same for serine hydroxymethyltransferase (Table 3.3). If serine hydroxymethyltransferase levels in cerebrum and cerebellum at three stages of development, 1-day, 10-days and 13 weeks postnatal age are compared with the levels of ornithine decarboxylase measured in the same regions at 1-day, 10-days and 12 or 15-days (when adult values are reached), a correlation coefficient of 0.96 is obtained. Therefore the results reported here suggest that hydroxymethyltransferase levels correlate with the growth rate of neonatal rat brain. It appears that the major role of this enzyme, during development at least, is in the formation of 1-carbon units from the α -carbon of glycine and the β -carbon of serine, which are used for the biosynthesis of thymine and various purines, precursors of DNA and RNA.

IV. POSTNATAL CHANGES IN THE HIGH AFFINITY UPTAKE
OF GABA AND GLYCINE IN THE
RAT CENTRAL NERVOUS SYSTEM

1. INTRODUCTION

Carrier-mediated, structurally specific uptake systems for glycine and GABA are considered to be extremely important in the maintenance of low extracellular levels of these amino acids, both as a means of terminating transmitter action and of providing transmitter for re-use (CURTIS & JOHNSTON, 1974). There is no evidence to indicate that glycine or GABA is inactivated enzymically in the extracellular synaptic environment. The observed inhibition of glycine and GABA uptake by p-chloromercuriphenylsulphonate (BALCAR & JOHNSTON, 1973) would explain the effect of this mercurial in vivo in potentiating and prolonging the depressant actions of GABA and glycine on the firing of feline spinal interneurons when administered microelectrophoretically (CURTIS, DUGGAN & JOHNSTON, 1970).

Much recent neurochemical research has been concerned with studying the specificity, kinetics, sensitivity to inhibitors, regional distribution and association with subcellular particles of the uptake processes for particular amino acids using tissue slices, homogenates, subcellular fractions and cultured glia and neurons (COHEN & LAJTHA, 1972; SNYDER, LOGAN, BENNETT & ARREGUI, 1973). Structurally specific uptake systems fall into two groups on the basis of their kinetic parameters, subcellular and regional distributions. The first group is characterized by K_m s higher than 5×10^{-4} M (SMITH, 1967; BLASBERG, 1968). The second have K_m s lower than 5×10^{-5} M and are referred to as 'high affinity' uptake systems. Those high affinity uptake systems which are sodium-dependent have been found to be specific for the amino acids considered most likely to be transmitters, GABA, glycine, taurine and L-glutamate/L-aspartate and appear to be associated with distinct populations

of isolated nerve terminals (synaptosomes). This latter observation is based on evidence from autoradiographic (BLOOM & IVERSEN, 1971; HÖKFELT & LJUNGDAHL, 1971; MATUS & DENNISON, 1971) and subcellular fractionation studies (IVERSEN & SNYDER, 1968; IVERSEN & JOHNSTON, 1971; WOFSEY, KUHAR & SNYDER, 1971; ARREGUI, LOGAN, BENNETT & SNYDER, 1972) and suggests that these uptake systems are intimately involved in CNS synaptic function by providing a supply of transmitter for re-use. In fact, sodium-dependent, high affinity uptake into synaptosomes has been proposed as a means of identifying amino acid transmitters (BENNETT, LOGAN & SNYDER, 1972; SNYDER, YOUNG, BENNETT & MULDER, 1973; BENNETT, MULDER & SNYDER, 1974).

Although synaptosomal uptake appears to be very important, glial uptake of neurotransmitter candidates has been demonstrated (HENN & HAMBURGER, 1971; LJUNGDAHL & HÖKFELT, 1973a, b; SCHRIER & THOMPSON, 1974). A possible role of central nervous system glial cells could be to ensure more rapid or efficient removal of neuronally-released transmitter. It is becoming increasingly apparent that sodium-dependent high affinity uptake systems for amino acids are not necessarily solely related to synaptic transmission mediated by these amino acids, since a high affinity sodium-dependent GABA uptake has been observed in sympathetic ganglia (BOWERY & BROWN, 1972), rat dorsal root ganglia and autonomic ganglia (YOUNG, BROWN, KELLY & SCHON, 1973; SCHON & KELLY, 1974a, 1974b) and in dorsal and ventral roots (DAVIES & JOHNSTON, 1974), sites where GABA seems to be most unlikely to be important as a transmitter. Accumulation in rat sensory ganglia and autonomic ganglia was exclusively localized within satellite glial cells. However, the rate of uptake of GABA as measured

by V_{\max} is very low in roots and ganglia and indicates the presence of comparatively few uptake sites. Furthermore this high affinity uptake seems to differ with respect to inhibitors and substrate specificity from that high affinity uptake observed in the CNS (SCHON & KELLY, 1974b).

IVERSEN & BLOOM (1972) have studied the uptake of [^3H]GABA and [^3H]glycine into slices and homogenates of rat brain and cord by electron microscopic autoradiography. Using substrate concentrations suitable for examining high affinity uptake they have demonstrated a predominant localization of [^3H]GABA and [^3H]glycine over nerve terminals of cortex and spinal cord respectively.

The work described in this chapter was undertaken to determine if high affinity uptake systems for glycine and GABA existed in slices prepared from tissue of young animals, and if so, to determine changes with respect to kinetic characteristics, sodium-dependence and substrate specificity. Experimental conditions chosen for uptake studies were very similar to those which have been used to demonstrate high affinity glycine and GABA uptake in adult CNS tissue slices (IVERSEN & JOHNSTON, 1971; JOHNSTON & IVERSEN, 1971).

2. METHODS AND MATERIALS

Glycine and GABA uptake by tissue slices. The procedures used for measuring the uptake of glycine into spinal cord slices and of GABA into cerebral cortex slices were essentially similar to those described previously (JOHNSTON & IVERSEN, 1971; BEART, JOHNSTON & UHR, 1972). Wistar rats of 1-day, 10-days and 13-weeks postnatal age were used and it was necessary to pool tissue from more than one animal as follows: (a) 1-day-old rats, six to nine animals per experiment, and (b) 10-day-old rats, three to

six animals per experiment. Tissue was chopped into mini-slices (0.1 x 0.1 x 1-2 mm) using a McIlwain tissue chopper and immediately suspended in cold buffer (1 ml/100 mg tissue). 100 μ l aliquots (10 mg wet weight of tissue) were dispensed into incubation bottles (Kimax 15105-L, 30 ml capacity) containing 10 ml of oxygenated buffer. The bottles were preincubated for 15 min at 25°C or 37°C (details are given in the tables of results) in a shaking water bath, before the addition of radioactive substrate (100 μ l) and incubation continued for a further 10 min. All determinations, including blanks (i.e. not incubated) were done in quadruplicate. Slices were collected by vacuum filtration on filter paper circles (Whatman No.1, 23 mm diam). Bottles and filters were washed with 10 ml of 154 mM sodium chloride. Papers were transferred to scintillation vials and extracted with 1 ml of water for one hour, then 10 ml dioxan-based scintillator (0.5% (w/v) 2,5-diphenyloxazole and 10% (w/v) naphthalene in dioxan) was added and radioactivity measured in a scintillation counter (Beckman LS-100). CPM were converted to DPM by means of an external standard (see Appendix 1).

For kinetic analysis of uptake, 100 μ l of appropriate concentrations of non-radioactive substrate were added at the same time as the radioactive substrate. Blanks were carried out at several different substrate concentrations over the range used. The results of these experiments were analysed by the methods of CLELAND (1963) using his Fortran programmes and an IBM 360/50 computer as described previously (BEART, JOHNSTON & UHR, 1972). In this procedure the fit of the data is made to the rate equation in its hyperbolic form and the kinetic constants determined ($v = VA/(K + A)$; where v = uptake velocity, V = maximal velocity of uptake (V_{\max}), A = substrate concentration and K = Michaelis constant (K_m)).

Potential uptake inhibitors were preincubated with the slices for 15 min before the addition of radioactive substrate. Using a PDP-8L computer the mean blank was subtracted from the four individual determinations for each drug or amino acid analogue and a mean \pm S.E.M. calculated. These were compared with the mean control value (no added inhibitor) to yield a percentage inhibition of uptake.

Uptake buffer. The Krebs Ringer phosphate buffer solution used for uptake consisted of 118.5 mM NaCl, 4.75 mM KCl, 1.77 mM CaCl₂, 1.18 mM MgSO₄ and 5.8 mM D-glucose in 15.4 mM sodium phosphate buffer, pH 7.4. Buffer, kept cold on ice, was oxygenated for at least 20 min before being dispensed into incubation bottles. For analysis of the dependence of uptake of glycine and GABA on external sodium ion concentration, the sodium phosphate buffer was replaced with tris-HCl buffer, and varying proportions of the sodium chloride were replaced by choline chloride.

Bicuculline and strychnine injections. These alkaloids were injected intraperitoneally into young rats as 0.5 - 5 mM solutions of their hydrochlorides. Control animals received 0.9% (w/v) saline.

Metabolism. Under the conditions used, with GABA uptake measured after 10 min at 25°C and glycine uptake after 10 min at 37°C, essentially all accumulated radioactivity in the slices remained as unchanged GABA or glycine (JOHNSTON & IVERSEN, 1971; IVERSEN & JOHNSTON, 1971).

Chemicals. [2,3-³H] GABA, specific activity 2 Ci/mmol, and [2-³H]glycine, 10.2 Ci/mmol, were purchased from New England Nuclear, Boston. The following compounds were gifts: Amiloride (A.W. Cuthbert, Cambridge), N-methyl-GABA and N,N-dimethyl-GABA (D. Butler, Canberra). The following compounds were prepared by published procedures (see BEART

& JOHNSTON, 1973; BEART, JOHNSTON & UHR, 1972): trans-4-aminocrotonic acid, 4-amino-tetrolic acid, 4-aminovaleric acid and 2-hydroxy-GABA. Hydrazinoacetic acid was prepared by the procedure of CARMI, POLLAK & YELLIN (1960). Other drugs and amino acids were purchased from commercial suppliers.

3. RESULTS

Kinetics of GABA uptake into slices of cerebral cortex. Both apparent kinetic parameters for GABA uptake into slices of cerebral cortex changed during development (Table 4.1). There was a 20-fold increase in the maximal velocity of uptake (V_{\max}) and a four-fold increase in K_m from 1-day-old to adult rats.

Kinetics of glycine uptake into slices of spinal cord. The changes in the apparent kinetic parameters for the uptake of glycine into spinal cord slices (Table 4.2) were less marked than those for GABA uptake. There was a 5-fold increase in V_{\max} from 1-day-old to adult rats. For the K_m there was a significant increase from 1-day to 10-day-old animals, but no significant change from 10-day to adult animals.

Sodium dependence of glycine and GABA uptake. The changes during development in dependence of uptake on the external sodium ion concentration were more marked with GABA (Fig. 4.1) than with glycine (Fig. 4.2). At only one measured sodium-ion concentration (46 mM) did the uptake of glycine differ significantly between the 1-day-old and adult rat, whereas the uptake of GABA differed at three sodium ion concentrations (23, 46 and 72 mM). At all ages studied the uptake of both glycine and GABA showed an absolute dependence on added sodium ions in the incubation medium, in that measured uptake in the absence of external sodium did not differ significantly from zero time blank values.

Inhibition of GABA uptake. Of the structural analogues of GABA that inhibit GABA uptake (Table 4.3), several were more potent inhibitors of uptake in the tissue from young animals than from the adults; N,N-dimethyl-GABA, N-methyl-GABA 4-amino-valeric acid (4-methyl-GABA) and glycine. The reverse was true for a number of drugs (Table 4.3) :

Table 4.1. Apparent kinetic parameters for high affinity GABA uptake into slices of cerebral cortex from rats of different ages

Postnatal Age	K_m (μM)	V_{max} ($\mu\text{mol}/\text{min}/\text{g}$)
1 day	5 ± 1	0.005 ± 0.0002
10 days	5 ± 1	0.020 ± 0.001
Adult*	24 ± 1	0.129 ± 0.006

Initial velocities of GABA uptake were measured at 25°C with 8 substrate concentrations (10-167 μM). Slices were preincubated for 15 min in the medium before the addition of radioactive substrate, and uptake measured after a further 10 min incubation. The values are weighted means \pm S.E.M. of 2 experiments, each of which involved quadruplicate determinations of uptake at the various substrate concentrations. The results of the individual experiments were analysed by the method of CLELAND (1963) to yield mean values \pm S.E.M. of the apparent kinetic parameters.

*Values for adults from IVERSEN AND JOHNSTON (1971). Similar values have been obtained in this laboratory under experimental conditions which were the same as used in the IVERSEN & JOHNSTON (1971) study.

Table 4.2. Apparent kinetic parameters for high affinity glycine uptake into slices of spinal cord from rats of different ages

Postnatal Age	K_m (μM)	V_{max} ($\mu\text{mol}/\text{min}/\text{g}$)
1 day	15 \pm 2	0.037 \pm 0.003
10 days	50 \pm 4	0.142 \pm 0.008
Adult*	36 \pm 9	0.21 \pm 0.05

Initial velocities of glycine uptake were measured at 37°C with 5-6 substrate concentrations (5-50 μM). Details as for Table 4.1.

*Values for adults from JOHNSTON & IVERSEN (1971).

Fig.4.1. Analysis of the dependence on external sodium ion concentration of [³H] GABA (0.0125 μM) uptake into slices of cerebral cortex from 5-hour-old (closed circles), 10-day-old (closed triangles) and adult rats (open circles). Uptake was measured in medium in which the normal sodium phosphate buffer was replaced by Tris-HCl buffer and in which varying proportions of the sodium chloride were replaced by choline chloride. The points are means ± S.E.M. of four experimental values, and uptake is expressed as a percentage of the uptake observed at the 'normal' sodium ion concentration.

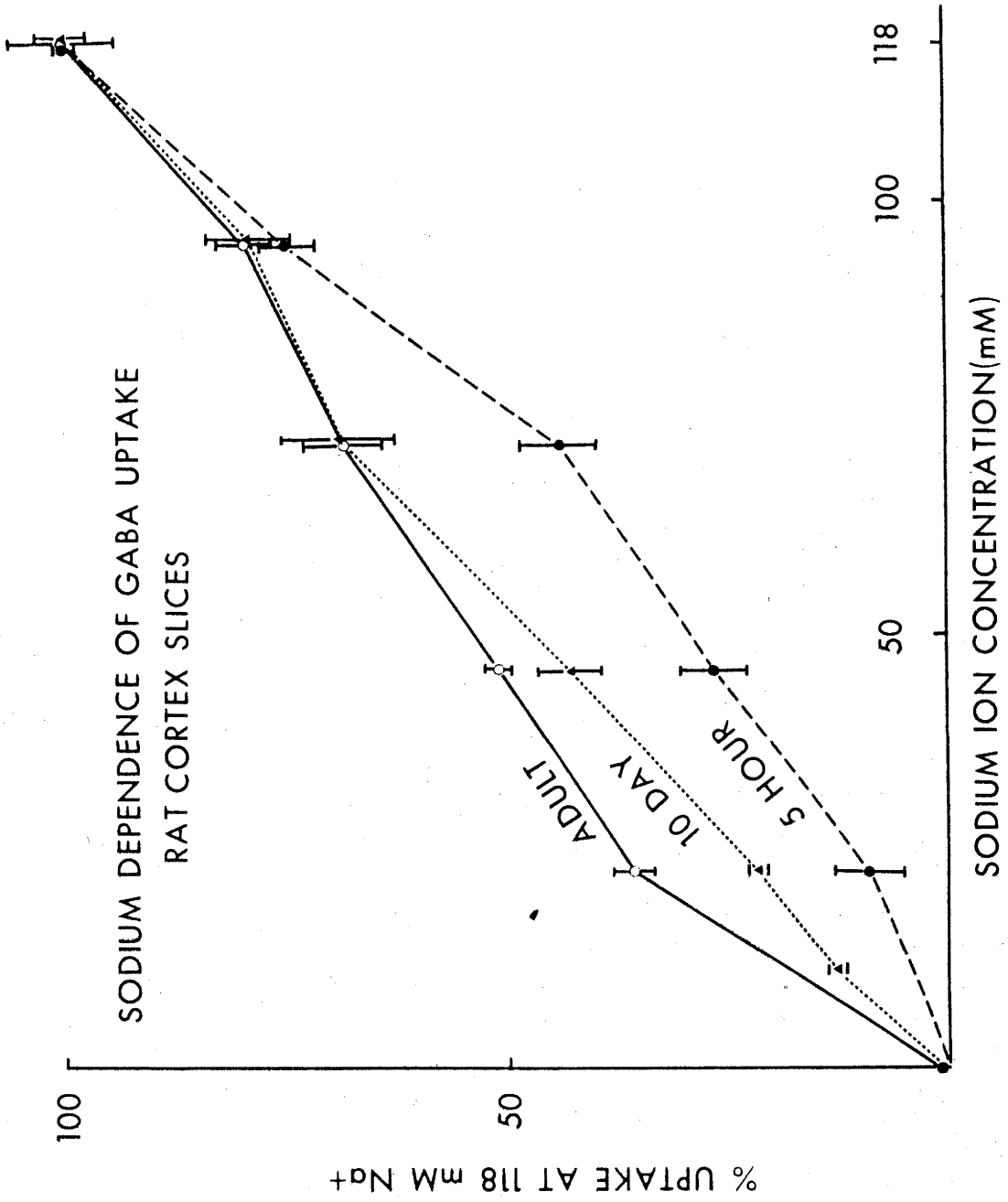


Fig.4.2. Analysis of the dependence on external sodium ion concentration of [³H]glycine (0.57 μM) uptake into slices of spinal cord from 1-day-old (closed circles), 10-day-old (closed triangles) and adult rats (open circles). Details as for Fig. 4.1.

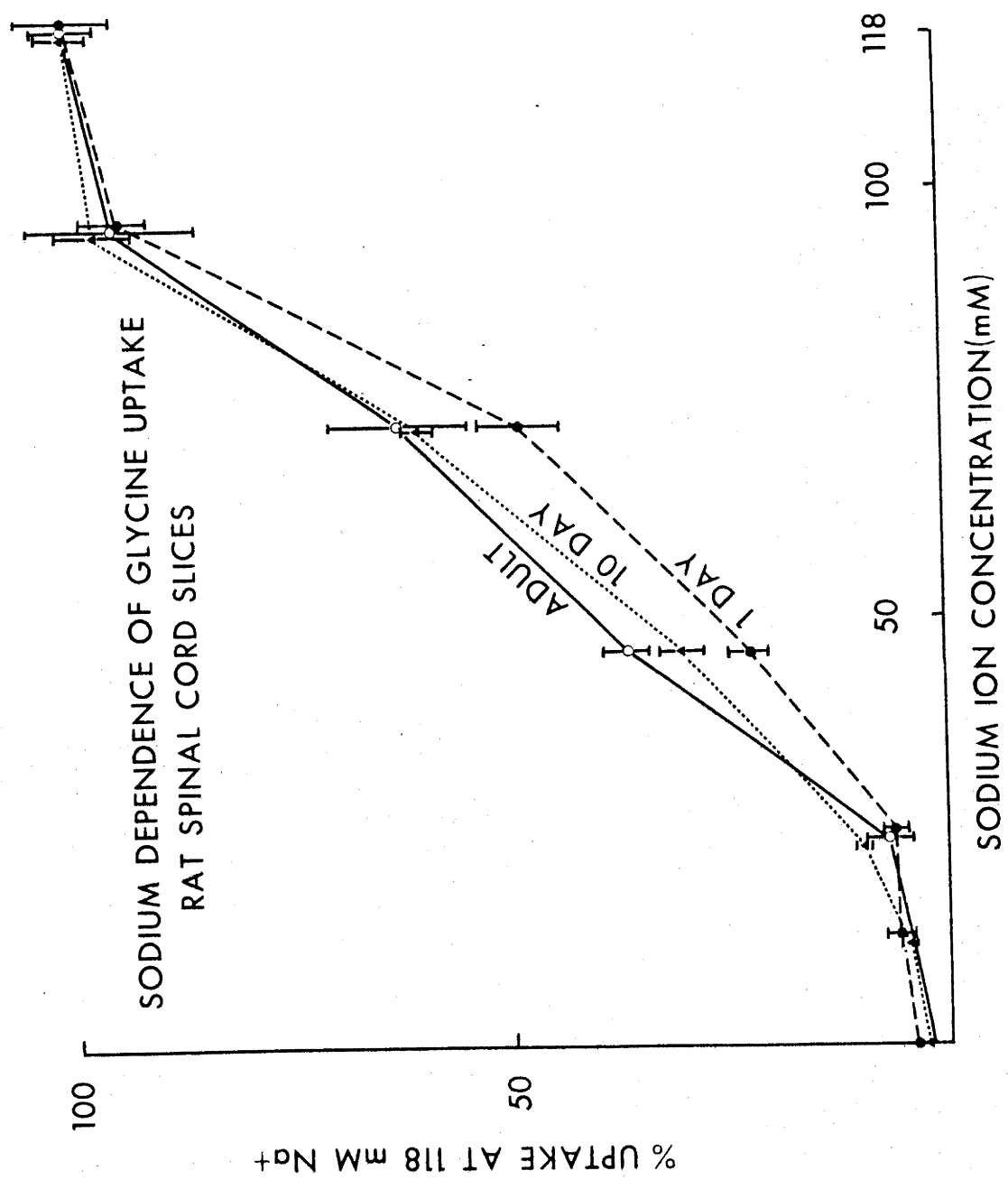


Table 4.3. Inhibition of high affinity GABA uptake into cerebral cortex slices from rats of different ages

Inhibitor	% Inhibition of control uptake at postnatal age:		
	1 day	10 days	adult
(a) amino acids (0.5 mM)			
trans-4-aminocrotonic acid	110 ⁺ 1	97 ⁺ 1	96 ⁺ 1
4-aminovaleric acid	103 ⁺ 3	91 ⁺ 1	82 ⁺ 1
2-hydroxy-GABA	103 ⁺ 1	88 ⁺ 1	90 ⁺ 1
N-methyl-GABA	89 ⁺ 4	73 ⁺ 1	54 ⁺ 1
N,N-dimethyl-GABA	68 ⁺ 2	54 ⁺ 2	29 ⁺ 2
4-aminotetrolic acid	63 ⁺ 1	57 ⁺ 1	55 ⁺ 7
L-2,4-diaminobutyric acid	53 ⁺ 6	50 ⁺ 1	47 ⁺ 1
glycine	27 ⁺ 3	11 ⁺ 5	n.s.
(b) drugs (0.1 mM)			
p-chloromercuriphenylsulphonate	95 ⁺ 1	93 ⁺ 1	92 ⁺ 2
chlorpromazine	94 ⁺ 1	n.t.	89 ⁺ 1
juglone	87 ⁺ 1	n.t.	93 ⁺ 5
protoveratrine A	40 ⁺ 1	35 ⁺ 11	77 ⁺ 4
protoveratrine B	35 ⁺ 1	38 ⁺ 7	70 ⁺ 9
dipyridamole	35 ⁺ 9	19 ⁺ 3	19 ⁺ 1
ouabain	37 ⁺ 8	62 ⁺ 1	67 ⁺ 1
strophanthidin	30 ⁺ 5	55 ⁺ 1	44 ⁺ 2
amiloride	n.s.	26 ⁺ 6	28 ⁺ 5
atractyloside	n.s.	n.s.	n.s.

The tissue slices were pre-incubated with the inhibitors at the concentrations shown for 15 min at 25°C, [³H]GABA (0.0125 μM) added and uptake measured after 10 min. Values are means ± S.E.M. of results from one or more quadruplicate experiments.

n.t. = not tested

n.s. = not significant

ouabain, protoveratrine A and B were more potent inhibitors of GABA uptake in adult tissue than in tissue from 1-day-old animals.

Inhibition of glycine uptake. L- α -alanine, D- α -alanine, β -alanine and GABA inhibited glycine uptake to a greater extent in tissue from young animals than in that from adults, while the reverse was true for ouabain and the related aglycone, strophanthidin (Table 4.4).

Convulsant effects of bicuculline and strychnine. The alkaloids bicuculline and strychnine were significantly more potent convulsants of 1 - 10-day-old rats than of adult rats (Table 4.5). These results are similar to those of more extensive studies reported by PYLKKO & WOODBURY (1961) and VERNADAKIS & WOODBURY (1969) on the susceptibility to strychnine of rats ranging from 1 to 36 days old. In the 1- and 2-day-old rats, generalized convulsions could not be elicited; at these ages the alkaloids produced body tremors, often accompanied by tail extension and sideways twisting of the body or hind limbs (see also PYLKKO & WOODBURY, 1961). At 10 days of age the alkaloids produced seizures comparable in type with those observed in adult animals. No gross differences were observed in the nature of the convulsions induced in the immature animals by strychnine or by bicuculline.

4. DISCUSSION

Using slices of adult rat CNS, the high affinity uptake of glycine is observed only in tissue prepared from the spinal cord and pons-medulla, regions where glycine is likely to be a transmitter, whereas the low affinity uptake is found in slices prepared from all areas of the CNS (JOHNSTON & IVERSEN, 1971; NEAL, 1971). A high affinity

Table 4.4. Inhibition of high affinity glycine uptake into spinal cord slices from rats of different ages

Inhibitor	%Inhibition of control uptake at postnatal age:		
	1 day	10 days	adult
(a) amino acids (1 mM)			
hydrazinoacetic acid	79 ± 5	71 ± 1	93 ± 1
L-α-alanine	67 ± 1	43 ± 1	8 ± 3
D-α-alanine	57 ± 6	34 ± 3	27 ± 7
β-alanine	40 ± 2	20 ± 4	17 ± 9
taurine	40 ± 8	20 ± 2	30 ± 4
GABA	22 ± 3	12 ± 3	n.s.
2-amino-isobutyric acid	11 ± 3	9 ± 1	13 ± 6
allylglycine	n.s.	n.s.	22 ± 5
L-serine	n.s.	n.s.	n.s.
(b) drugs (0.1 mM)			
juglone	103 ± 1	96 ± 1	100 ± 1
p-chloromercuriphenylsulphonate	99 ± 2	86 ± 1	100 ± 3
chlorpromazine	98 ± 2	89 ± 1	99 ± 2
protoveratrine A	n.t.	81 ± 1	94 ± 1
ouabain	77 ± 4	79 ± 1	97 ± 1
strophanthidin	26 ± 12	25 ± 11	79 ± 2
dipyridamole	58 ± 3	53 ± 4	56 ± 2
pyridoxamine	20 ± 6	21 ± 5	n.s.
amiloride	20 ± 3	13 ± 4	n.s.
atractyloside	n.t.	21 ± 9	8 ± 1

The tissue slices were pre-incubated with the inhibitors at the concentrations shown for 15 min at 37°C, [³H]glycine (0.57 μM) added and uptake measured after 10 min. Values are means ± S.E.M. of results from one or more quadruplicate experiments.

n.t. = not tested

n.s. = not significant

Table 4.5. Convulsant doses of bicuculline and strychnine in rats of different ages

Postnatal Age	CD ₅₀ (μmol/kg i.p.)	
	Bicuculline	Strychnine
1 day	1.4 ± 0.7	1.8 ± 0.4
2 days	2.6 ± 0.2	2.2 ± 0.2
10 days	1.2 ± 0.4	2.8 ± 0.05
Adult	12.1 ± 0.6 [§]	6 ^{§§}

For each age group, 3-4 different doses of the alkaloids were injected intraperitoneally to each of 4 animals, and convulsions scored if occurring within 5 min. The percentage convulsing at each dose was plotted on a probability scale against the log of the dose to obtain an estimate of the CD₅₀.

§ JOHNSTON & MITCHELL (1971).

§§ PYLKKO & WOODBURY (1961).

uptake for glycine, strictly dependent on sodium, has been demonstrated in synaptosomal preparations of spinal cord (BENNETT, LOGAN & SNYDER, 1972) but not of cerebral cortex (LOGAN & SNYDER, 1972). These results suggest that within the CNS, the sodium-dependent high affinity transport of glycine is associated with its transmitter function.

A high affinity uptake for GABA is present in slices of all regions of the CNS (IVERSEN & JOHNSTON, 1971): on the basis of maximal rates of uptake measured in vitro, results are consistent with the view that GABA is a major inhibitory transmitter in supraspinal regions, but of less importance in spinal inhibitory mechanisms. For GABA uptake into cortex and glycine uptake into cord, experiments (JOHNSTON & IVERSEN, 1971; IVERSEN & JOHNSTON, 1971) have shown that more than two-thirds of the uptake sites in slices of intact tissue are still present in homogenates and that radioactivity accumulated by homogenates is associated with osmotically sensitive particles with sedimentation characteristics of synaptosomes. Autoradiographic evidence for a predominantly synaptic-terminal localization of accumulated glycine and GABA in tissue slices has been discussed (Section IV.1). A recently described low affinity transport for GABA in CNS tissue slices and homogenates (LEVI & RAITERI, 1973; BOND, 1973) has yet to be studied extensively.

The high affinity uptake systems for GABA and glycine found in CNS tissue slices from adult rats are present in tissue from 1- and 10-day-old animals. The high affinity uptake of GABA has been investigated previously in slices of immature rat brain by LEVI & RAITERI (1973), who also demonstrated the existence of a low affinity system. LEVI & RAITERI (1973) found a slightly higher K_m

for GABA uptake into newborn brain slices (43 μM) than into adult brain slices (31 μM), whereas the present investigation suggests that the K_m for high affinity GABA uptake increases during post-natal development. For both GABA and glycine the maximal velocity of uptake increases with development suggesting possibly an increased density of transport sites. For glycine uptake into spinal cord slices, the developmental change in K_m was less marked than that for GABA uptake in the cortex. From 10-day-old to adult rats, there was no significant difference in the K_m . Relative constancy of K_m and increases in the maximal velocity of uptake during development have been reported for a number of substrates including noradrenaline in rat brain (COYLE & AXELROD, 1971) and -aminoisobutyric acid, L-leucine, D-glutamate and L-lysine in chick brain (LEVI, 1972).

Recent work on developmental changes for glycine uptake into rat cerebral synaptosomal fractions (PETERSON & RAGHUPATHY, 1973) suggests that accumulation proceeds by two independent systems, one totally dependent on external sodium, the other independent of sodium. The sodium-dependent system was more active in fractions from newborn and adult rats than in fractions from 12 - 17-day-old animals; in these, the sodium-independent system was more active and its contribution to total uptake declined with development. This uptake demonstrated high affinity kinetics and was characterized by a decline in V_{max} and increase in K_m with development from 12 - 17-day-old to adult animals. The function and subcellular site(s) of this sodium-independent accumulation is at present unknown. BENNETT, LOGAN & SNYDER (1973) have also reported Na-dependent and independent glycine uptake into rat cerebral synaptosomes, although they find a somewhat different developmental pattern.

A substrate for a particular transport system will effectively inhibit the observed uptake of another

substrate by this transport system when present in sufficient molar excess to overcome differences in affinity of the two substrates for the transport system: thus, putative substrates can be screened rapidly and an assessment made of the possible substrate specificity. In adult rats the high affinity uptake systems for glycine and GABA are essentially independent. In the 1- and 10-day-old rats, however, there is a small but significant inhibition by GABA of the uptake of glycine and vice versa, suggesting that in these animals the transport systems may be less specific than in the adults. There are some other changes with development in the susceptibility of the two systems to inhibition by amino acid analogues which also indicate increased specificity of the transport carrier with development. Other authors have noted changes in the apparent substrate specificity with development of other amino acid transport systems (PICCOLI, GRYBAUM & LAJTHA, 1971; LEVI, 1972).

Inhibition of GABA and glycine uptake by drugs interfering with sodium ion channels is generally more apparent in adult tissue than in that from immature animals. The veratrine alkaloids, protoveratrine A and B, apparently interfere with sodium ion movements by stabilizing the sodium-ion-selective channels once they have been formed (BAKER, 1968), but act at different sites to ouabain and do not affect the sodium/potassium dependent ATPase (YOSHIDA, KANIIE & NAMBA, 1963). It has been proposed that protoveratrine may exert its effect by blocking sodium-dependent binding of GABA to the membrane, a process that may occur before membrane transfer (GOTTESFELD & ELLIOT, 1971). Strophanthin, an aglycone structurally related to ouabain blocks active sodium transport in a variety of preparations (HOROWICZ & GERBER, 1965). Amiloride, a potent inhibitor

of sodium transport in toad bladder (CUTHBERT & WONG, 1972) was only a weak inhibitor of GABA and glycine uptake in tissue from young and adult rats.

Slight differences in the dependence on the external sodium ion concentrations are observed for GABA and glycine uptake into tissue prepared from animals of different post-natal ages. In common with other high affinity amino acid transport systems in adults (BENNETT, LOGAN & SNYDER, 1972), the systems for GABA and glycine showed an absolute dependence of sodium ions at all ages examined. Plotting the rate of uptake of glycine into spinal cord slices of young and adult tissue versus sodium ion concentration (Fig. 4.2) revealed sigmoidal curves reminiscent of allosteric interactions found in certain enzyme systems. Similar results have been reported for glycine uptake into spinal cord homogenates (BENNETT, LOGAN & SNYDER, 1973) and suggest possible allosteric interactions between sodium ions and the glycine transport system. A sigmoidal curve has been observed for the sodium-dependence of GABA uptake by cerebral cortical synaptosomes (MARTIN & SMITH, 1972). The sodium requirement of L-glutamate uptake in homogenates and slices of cortex, however, appears to be less complex (BENNETT, LOGAN & SNYDER, 1973; BALCAR & JOHNSTON, 1972a).

In the cat CNS, bicuculline and strychnine have been shown to be relatively specific antagonists of the post-synaptic actions of 'GABA-like' and 'glycine-like' amino acids respectively (CURTIS, HÖSLI, JOHNSTON & JOHNSTON, 1968; CURTIS, DUGGAN, FELIX & JOHNSTON, 1971). In the rat cord this is also the case. In the rat cortex, bicuculline antagonized the actions of the depressant amino acids while strychnine had no specific antagonistic effect, possibly

reflecting the lower proportion of neurons relatively sensitive to glycine in the cortex than in the cord (BISCOE, DUGGAN & LODGE, 1972). The convulsions produced in 1-, 2- and 10-day-old rats by these antagonists suggests that at these ages the depressant amino acids, glycine and GABA are being used as transmitters. However, maturational changes in the blood-brain barrier to these alkaloids (PYLKKO & WOODBURY, 1961; VERNADAKIS & WOODBURY, 1969) and the hepatic drug-metabolizing system (DAVIS & YEH, 1969) could influence the amount of these convulsants reaching post-synaptic receptor sites. Thus it is not possible to deduce from the present CD_{50} measurements the degree to which glycine and GABA are being used as inhibitory transmitters.

Although recent work has shown that for GABA and L-glutamate at least, a high affinity uptake exists in peripheral nervous system tissue where on current evidence they are unlikely to have a transmitter function, most experimental findings considered above suggest that within the central nervous system the association of sodium dependent high affinity uptake systems and the probable transmitter function of certain amino acids is good. Thus it is tempting to speculate that the existence of sodium dependent, high affinity uptake systems for GABA and glycine in 1-day-old rats is indicative of them functioning as transmitters at this stage. However, it is possible that the development of uptake systems precedes transmitter function; developing synapses may accumulate amino acids before they are morphologically (ADINOLFI, 1971) or physiologically mature, or newly-differentiated glial cells insulating developing synapses may possess the ability to transport amino acids from the extra-cellular environment. In this regard it is interesting to note that membrane-receptor sensitivity appears to precede development of functional synapses, since micro-electrophoretically applied GABA and noradrenaline can inhibit the spontaneous discharges of immature Purkinje cells in 1-day rat cerebellum (WOODWARD, HOFFER & LAPHAM, 1969). Alternatively, transmitter function and high affinity

uptake into terminals may develop simultaneously, since there is some evidence to suggest that mechanisms responsible for sodium-dependent, high affinity transport of putative amino acid transmitters may be involved in their release from presynaptic terminals (e.g. LEVI & RAITERI, 1974). Obviously further studies are required before definite conclusions can be drawn as to the association of the presence of high affinity uptake systems for amino acids in immature animals and their function as transmitters. Additional experiments could include -

1. A comparison of glycine uptake in the spinal cord and cerebral cortex of immature animals, in view of the finding (JOHNSTON & IVERSEN, 1971) that the high affinity uptake is only present in those regions where glycine is likely to be a transmitter.
2. An extensive study of GABA uptake in the cerebellum at a large number of stages during growth and maturation. The cerebellum may be the best brain region in which to attempt to relate developmental changes in neurochemical correlates of synaptically-active amino acids to changes in the number of synaptic junctions, since the developing cerebellum has been studied extensively with respect to cellular and morphological changes (e.g. ALTMAN, 1966, 1969) and estimates have been made of developing synaptic junction numbers (WOODWARD, HOFFER, SIGGINS & BLOOM, 1971).
3. Electron-microscope autoradiographic studies of uptake of putative amino acids in slices and homogenates of immature tissue, under conditions of labelling in which high affinity uptake predominates. Such studies of [^3H] GABA uptake in slices of rat cerebellum may be particularly worthwhile, in view of the well known inhibitions mediated by GABA in the adult cerebellum (CURTIS & JOHNSTON, 1974).

As discussed above, the problem with approaches (2) and (3) is that the terminals appearing in morphological studies of

immature tissue may not be synaptically active. A combined morphological and electrophysiological approach could help to determine at what stage of ultrastructural development a synapse becomes functional, although the problems of electrophysiological recording in immature brain (WOODWARD, HOFFER & LAPHAM, 1969) may render such an approach impractical.

In addition to the existence of sodium dependent, high affinity uptake systems for glycine and GABA, it has been shown that compounds which antagonise the postsynaptic actions of these amino acids cause convulsions in 1 and 2-day-old rats. In the following chapter the ability of immature rat CNS tissue to release these amino acids in vitro under conditions of stimulation which may approximate a model of in vivo synaptic release is examined.

V. POSTNATAL CHANGES IN THE POTASSIUM-STIMULATED,
CALCIUM-DEPENDENT RELEASE OF RADIOACTIVE GABA AND GLYCINE
FROM SLICES OF RAT CENTRAL NERVOUS TISSUE

1. INTRODUCTION

An important criterion for the establishment of a neurotransmitter role for a substance is that it should be released from nerve terminals by presynaptic impulses. A rigorous experimental proof of release is very difficult for central synaptic transmitters and results of most investigations on endogenous amino acid efflux from the CNS have provided, at most, indirect evidence of synaptic release of putative amino acid transmitters by impulses in particular pathways.

The release of GABA from the mammalian cortex has been studied in vivo, in response to stimulating various pathways (JASPER & KOYAMA, 1969; OBATA & TAKEDA, 1969; IVERSEN, MITCHELL & SRINIVASAN, 1971). There is now substantial evidence that GABA is released by neural activity, especially in the cerebral cortex and Deiter's nucleus. It has proved difficult to demonstrate changes in the release of glycine from the spinal cord after nerve stimulation, although JORDAN & WEBSTER (1971) did observe increased efflux of radioactive glycine from cat spinal cord perfused in vivo as a result of stimulating various nerves, provided p-hydroxymercuribenzoate was present in the superfusate, presumably to block re-uptake (NEAL & PICKLES, 1969). APRISON (1970) has observed release of radioactive glycine from hemisected toad spinal cord as the result of dorsal-root stimulation.

The calcium-dependent release of radioactive amino acids from preloaded slices and synaptosome preparations of adult CNS tissue in response to electrical stimulation or high potassium concentration has been studied extensively as a model for transmitter release (e.g. MACHIYAMA, BALÁZS & RICHTER, 1967; SRINIVASAN, NEAL & MITCHELL, 1969; KATZ,

CHASE & KOPIN, 1969; HOPKIN & NEAL, 1971; HAMMERSTAD, MURRAY & CUTLER, 1971; ARNFRED & HERTZ, 1971; MULDER & SNYDER, 1974). This type of experiment serves to eliminate some of the difficulties encountered in studying amino acid release in vivo, including the problem of collection of sufficient amounts of endogenous amino acid to quantify, as well as the inability to collect directly at the site of release. However the ability to stimulate particular neuronal pathways is lost; furthermore, care must be exercised in equating release of preloaded exogenous amino acid with release of endogenous compound from a transmitter pool, because of possible metabolic compartmentation (BERL & CLARKE, 1969; VAN DEN BERG, 1973).

In this chapter a simple system, made from readily available apparatus, has been used to study potassium-stimulated release of amino acids from 'mini-slices' (0.1 x 0.1 x 1-2 mm) of CNS tissue. 'Mini-slices' have commonly been used for studying those high affinity uptake systems which appear to be associated with transmitter pools of amino acids, but previous release studies have usually been carried out with relatively large slices (e.g. 0.5 x 1 x 1 mm cerebral slices; MULDER & SNYDER, 1974; 1 mm transverse discs of spinal cord; HOPKIN & NEAL, 1971). The apparent kinetics of amino acid uptake into rat brain slices can be influenced by the size of the slices, with 'low affinity' uptake predominating over 'high affinity' uptake in thicker slices (LEVI & RAITERI, 1973). In the case of glycine, 'low affinity' uptake is much less specific than the 'high affinity' uptake and may serve a general metabolic function (JOHNSTON & IVERSEN, 1971). 'Mini-slices' were used in order to minimise uptake of radioactivity by 'low affinity' systems.

This apparatus was used to examine whether a potassium-stimulated, calcium-dependent release could be observed from slices of cerebral cortex and spinal cord prepared from rats of 1 day and 10 days postnatal age.

2. METHODS AND MATERIALS

Preparation of tissue slices. Wistar rats of 1 day, 10 days or 13 weeks postnatal age were killed by decapitation and the brains or spinal cords rapidly removed. The cerebral hemispheres were separated from the brainstem and midbrain (diencephalon and mesencephalon) and, in the case of adult rats, dissected free of underlying white matter. The spinal cords were freed from spinal roots and the dura removed. The tissue was chopped into 'mini-slices' (approximately 1.0 x 0.1 x 0.1 mm) using a McIlwain tissue chopper and suspended in cold buffer (1 ml per 100mg tissue). Aliquots of 500 μ l were distributed to incubation bottles containing 5 ml of cold oxygenated buffer.

Incubation of slices. Slices were preincubated with shaking at 37°C for 10 min before the addition of amino-oxyacetic acid (2×10^{-5} M final concentration) and [U-¹⁴C] GABA, (0.5 μ Ci), [2-³H] glycine or [1-¹⁴C] 2-aminoisobutyric acid (1.0 μ Ci). Amino-oxyacetic acid was added to inhibit the catabolism of GABA (COLLINS, 1973). The slices were incubated for a further 20 min, then collected by vacuum filtration on glass fibre filter discs (Whatman GF/A, 2.5 cm diameter) and washed with 10 ml of warm (37°C) buffer.

Perfusion of slices. The filter discs were transferred rapidly to filter holders (Swinnex 25, Millipore Corp.) and perfusion commenced. The apparatus (Fig. 5.1) consisted of up to 7 Swinnex filter holders held by spring tool clips on a 37°C water bath. A multichannel peristaltic pump (Buchler Dekastaltic) was used to pump oxygenated

Krebs Ringer buffer solution through a heating coil contained in the bath and into the adapters at a rate of 0.5 ml/min. Fractions (2.8 min) were collected using an automatic fraction collector (Isco, model 328), modified to collect up to 7 fractions at once directly into scintillation vials (fractions were changed as each row of vials moved sideways). Slices were perfused with normal buffer for different times before switching to high K-buffer for 10 min. Perfusion was continued with normal buffer, then the system was pumped dry and the filter discs with tissue were removed and transferred to scintillation vials containing 1 ml of water.

In initial experiments on GABA release, slices were perfused with normal buffer for 40 min before the 10 min high-potassium stimulation. However, this initial perfusion time was reduced to 25 min for adult and 10-day cortex slices, and to 30 min for 1-day cortex slices, since it was observed that pre-stimulation spontaneous efflux reached a constant level within these times. Unlike GABA release from cortex, the resting release of glycine from spinal cord slices did not attain a constant basal level. To determine what effect this had on the measured release parameter (peak percentage increase over immediate pre-stimulation spontaneous efflux) the experiments were repeated using longer initial perfusion times. The calculated potassium-stimulated release of glycine was not significantly different in experiments involving 25 min, 35 min and 40 min initial perfusion times. Thus 30 min perfusion was routinely used. A 25 min pre-stimulation perfusion was used for 2-aminoisobutyric acid.

Perfusion buffer. The 'control' Krebs Ringer buffer solution used for uptake and release consisted of 118.5 mM NaCl, 4.75 mM KCl, 1.77 mM CaCl₂, 1.18 mM MgSO₄, and 5.8 mM D-glucose

Fig. 5.1. Apparatus used to perfuse 'mini-slices' of nervous tissue. The slices were retained on glass-fibre filter discs in Swinnex filter holders. Up to seven such holders were used in parallel.

TISSUE SLICES ON
GLASS FIBRE FILTER

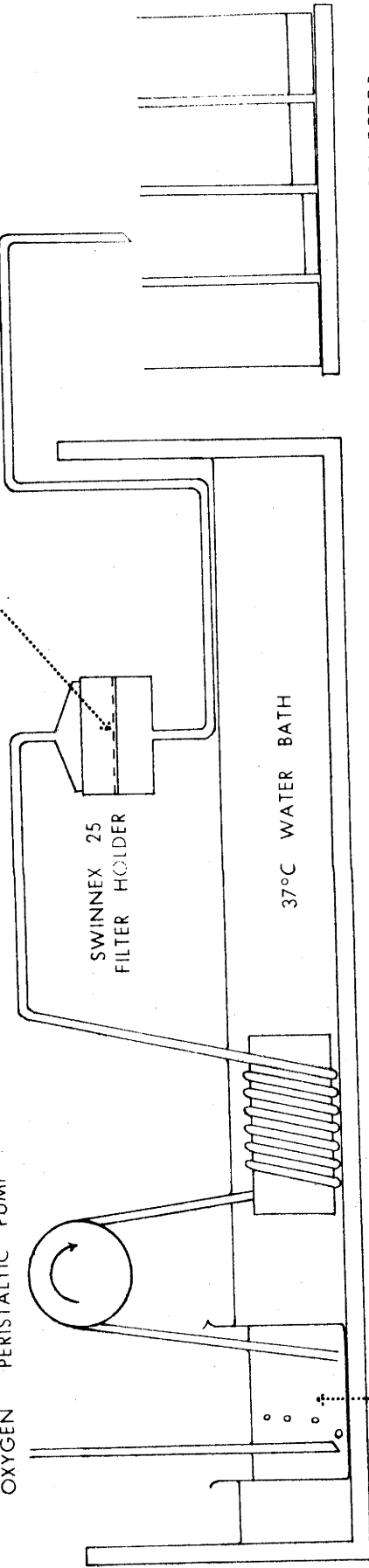
OXYGEN PERISTALTIC PUMP

SWINNEX 25
FILTER HOLDER

37°C WATER BATH

FRACTION COLLECTOR

KREBS RINGER
SOLUTION



in 15.4 mM sodium phosphate buffer, pH 7.4, oxygenated before incubation of slices and during the course of release experiments. Calcium-free buffer was normal buffer with CaCl_2 omitted. For high K-buffer, KCl was increased from 4.75 to 44.75 mM. Low sodium buffer had the NaCl replaced with choline chloride (118.5 mM) reducing the sodium ion concentration to approximately 26 mM.

Chemicals. Chemicals were purchased from the following sources; amino-oxyacetic acid (Eastman); $[2\text{-}^3\text{H}]\text{glycine}$, 10.2Ci/mmol (New England Nuclear, Boston); $[\text{U-}^{14}\text{C}]\text{GABA}$, 228 mCi/mmol and $[1\text{-}^{14}\text{C}]\text{aminoisobutyric acid}$, 53.8 mCi/mmol (Radiochemical Centre, Amersham); Teric N8 detergent (Imperial Chemical Industries, Australia) and 2,5-diphenyloxazole (Merck).

Radioactivity in the aqueous samples was determined by liquid scintillation counting after the addition of 9 ml of scintillator. This scintillator contained 0.5% (w/v) of 2,5-diphenyloxazole in a 2:1 mixture of xylene:Teric N8 detergent.

Metabolism. The extent of metabolism of radioactive GABA and glycine occurring during the course of perfusion experiments was examined. For analysis of radioactivity retained in the tissue at the end of perfusion, tissue slices on filter discs were homogenized in 0.45 N perchloric acid or 0.5 N trichloroacetic acid and supernatants collected by centrifugation. Perchloric acid extracts were neutralized with potassium hydroxide and insoluble potassium perchlorate removed by centrifugation, while trichloroacetic acid was removed from supernatants by ether extraction. For analysis of released radioactivity, efflux fractions were pooled and concentrated in vacuo. Acidified samples of tissue radioactivity and released radioactivity were applied to

small columns of Dowex 50W x 4 (200-400 mesh), washed with 0.01 N hydrochloric acid and water, then eluted with 2 N ammonia solution. Aliquots of the wash were counted to determine radioactivity in acidic metabolites, while the alkaline eluent was evaporated to dryness in vacuo at 37°C, resuspended in a small volume of water and analysed by paper electrophoresis or thin-layer chromatography on micro-crystalline cellulose (Avicel). In other experiments, tissue radioactivity was analysed directly without prior column fractionation. Electrophoresis buffer was 2% pyridine, 1% acetic acid, pH 5.2. The solvent for chromatography of glycine was butanol:acetone:diethylamine:water (20:20:3:10) which clearly separated glycine and serine, and for GABA, butanol:acetic acid:water (4:1:1).

3. RESULTS

Release of radioactive amino acids from slices of adult rat cerebral cortex

GABA. As previously observed using larger slices (SRINIVASAN, NEAL & MITCHELL, 1969; MULDER & SNYDER, 1974) of adult rat cerebral cortex, increasing the potassium ion concentration in the perfusing buffer led to a markedly enhanced release of radioactive GABA, and this K-stimulated release was almost completely abolished if the perfusing buffer contained no added calcium ions (Fig.5.2). The results of individual experiments can be expressed in terms of the peak percentage increase over the immediate pre-stimulation spontaneous release of radioactivity, as described by MULDER & SNYDER (1974); this data is presented in Table 5.1.

Lowering the sodium ion content of the perfusing medium from 146 mM to 26 mM had the effect of slightly increasing the spontaneous release of radioactivity and greatly increasing the K-stimulated release (Fig.5.2, Table 5.1).

Fig. 5.2. Release of [U-¹⁴C]GABA and [1-¹⁴C]2-aminoisobutyric acid from slices of adult rat cerebral cortex. The slices (50 mg) were preloaded by incubation in buffer at 37°C for 20 min with either 0.5 µCi of GABA or 1.0 µCi of 2-aminoisobutyric acid, collected by filtration on a glass-fibre filter disc, washed with warm buffer, transferred to Swinnex filter holder and perfused at 37°C with Krebs Ringer phosphate buffer. Each point represents the mean ± S.E.M. of 3-4 experiments, of the radioactivity in fractions of the perfusate collected over 2.8 min periods. The 'control' curve represents perfusion with buffer containing 1.77 mM CaCl₂, while the curve labelled 'no added calcium' represents experiments in which the calcium chloride was omitted from the perfusion buffer. The hatched bar represents perfusion with buffer containing 44.75 mM KCl for the 10 min period indicated (normal KCl is 4.75 mM).

**[¹⁴C]GABA RELEASE
ADULT RAT CORTEX SLICES**

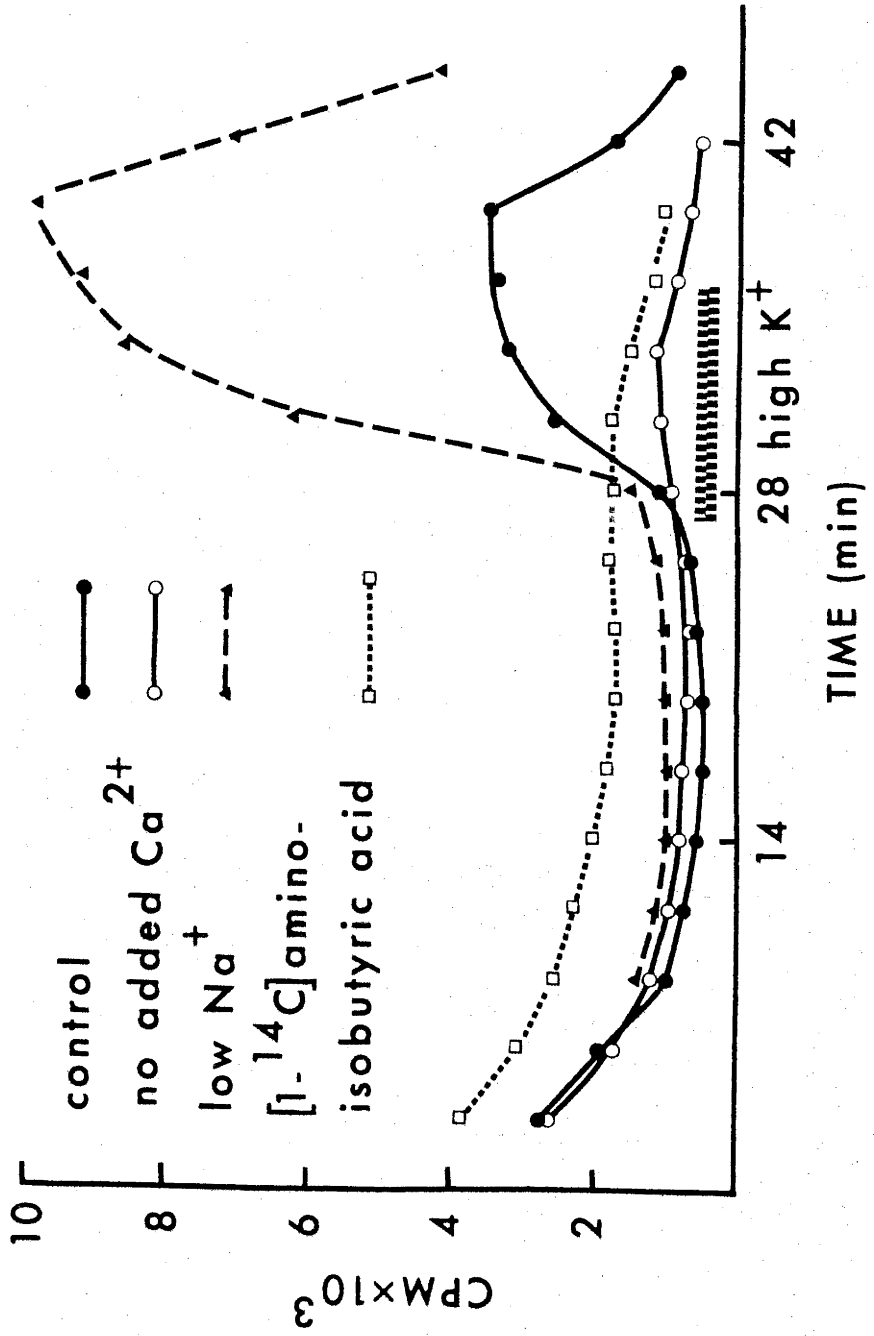


Table 5.1. Potassium-stimulated release of radioactive amino acids from adult rat CNS tissue slices

	K-Stimulated release	
	+ Calcium	- Calcium
<u>(a) Cerebral cortex slices</u>		
GABA	505 \pm 54 (6)	63 \pm 11 (4)
(Low Na)*	763 \pm 69 (5)	-
(Low Na)**	1006 \pm 141 (5)	-
2-Aminoisobutyric acid	8 \pm 1 (5)	-
Glycine	34 \pm 4 (4)	21 \pm 3 (4)
<u>(b) Spinal cord slices</u>		
GABA	368 \pm 75 (3)	61 \pm 15 (5)
2-Aminoisobutyric acid	-1 \pm 2 (5)	-
Glycine	63 \pm 4 (5)	13 \pm 3 (4)

K-Stimulated release is expressed as the peak percentage increase in release over immediate pre-stimulation spontaneous release. Values are means \pm S.E.M. for the number of experiments shown in brackets.

* Expressed as increase over low Na resting release

** Expressed as increase over normal Na resting release

2-Aminoisobutyric acid. Increasing the potassium ion concentration in the perfusing buffer did not lead to any marked increase in the release of radioactivity from slices preloaded with [1-¹⁴C]2-aminoisobutyric acid (Fig. 5.2, Table 5.1).

Glycine. There was a significant increase in the rate of release of radioactivity from adult cerebral cortex slices in response to perfusion with high K-buffer, and this K-stimulated release was in part dependent on calcium ions in the perfusing buffer (Table 5.1).

Release of radioactive amino acids from slices of adult rat spinal cord

GABA. There was an appreciable calcium-dependent, K-stimulated release of radioactive GABA from slices of adult rat spinal cord (Table 5.1). This stimulated release was not significantly different from that observed from slices of adult rat cerebral cortex.

2-Aminoisobutyric acid. Radioactive 2-aminoisobutyric acid was not released by high K-buffer (Table 5.1).

Glycine. The calcium-dependent, K-stimulated release of glycine from slices of adult spinal cord was approximately twice that observed from slices of adult cerebral cortex (Table 5.1).

Postnatal changes in the stimulated release of radioactive GABA from slices of cerebral cortex. Calcium-dependent, K-stimulated release of radioactive GABA was apparent with slices of cerebral cortex from 1-day and 10-day-old animals (Fig.5.3, Table 5.2). With increasing age there appeared to be an increase in the amount of radioactive release on K-stimulation.

Fig. 5.3. Release of [U-¹⁴C] GABA from slices of cerebral cortex from animals of 1 day and 10 days postnatal age. Other details as for Fig. 5.2.

[U-¹⁴C]GABA RELEASE FROM SLICES OF CEREBRAL CORTEX

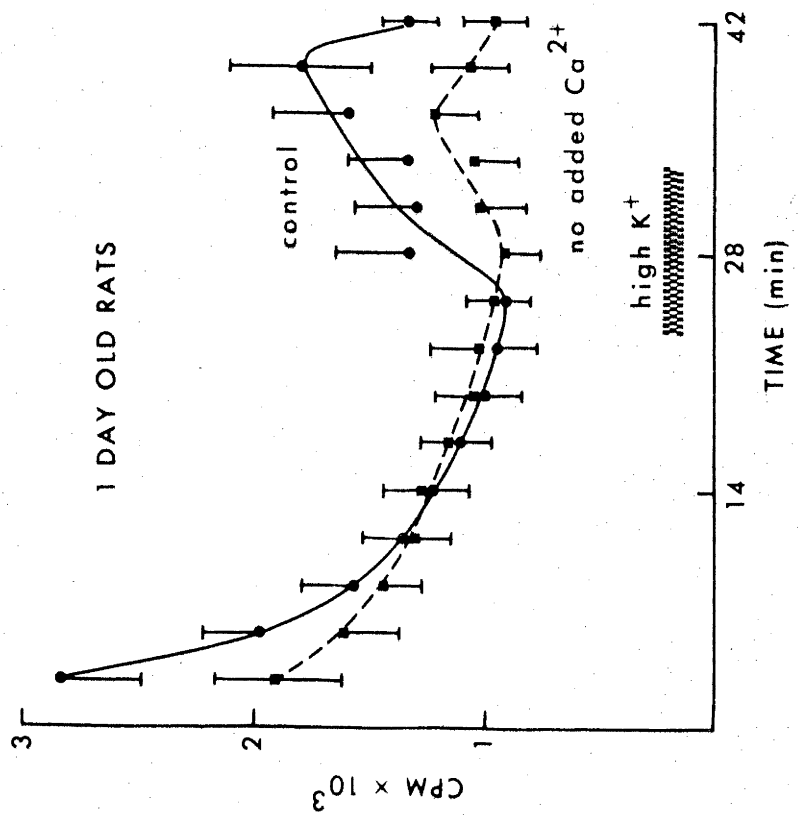
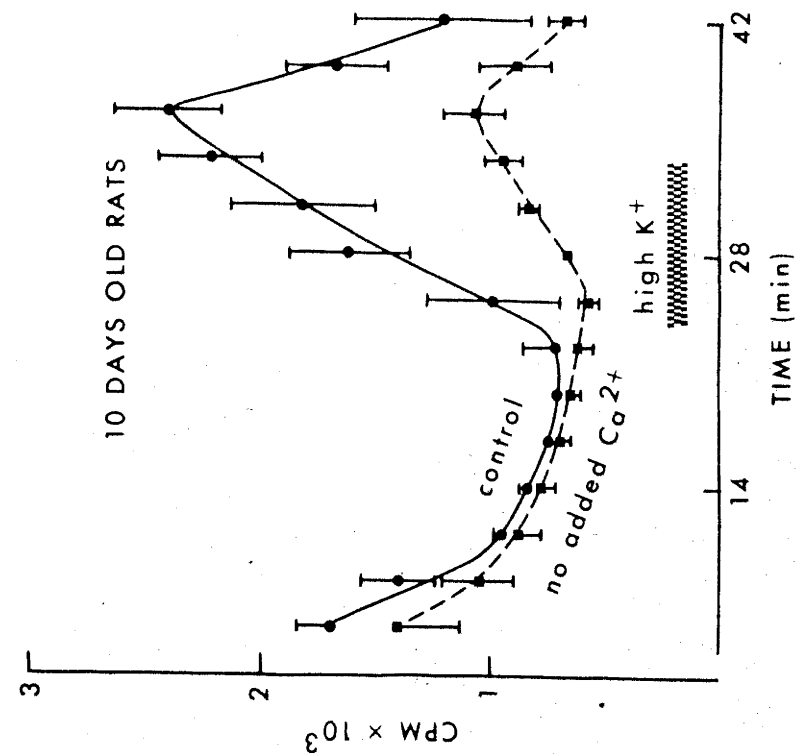


Table 5.2. Postnatal changes in potassium-stimulated release of radioactive GABA from slices of cerebral cortex

Postnatal age of animals	K-Stimulated release	
	+ Calcium	- Calcium
1 Day	104 \pm 17 (7)	27 \pm 3 (3)
10 Days	249 \pm 29 (5)	76 \pm 9 (6)
Adult (13-week)	505 \pm 54 (6)	63 \pm 11 (4)

K-Stimulated release is expressed as the peak percentage increase in release over immediate pre-stimulation spontaneous release. Initial perfusion time for adult and 10-day cortex slices was 25 min and for 1-day slices, 30 min. Values given are means \pm S.E.M. of the number of experiments shown in brackets.

Postnatal changes in the stimulated release of radioactive glycine from slices of spinal cord. Little difference could be discerned in the calcium-dependent, K-stimulated release of radioactive glycine from cord slices prepared from 1-day, 10-day and 13-week-old animals (Fig.5.4, Table 5.3).

Radioactivity retained in the tissue after perfusion. The percentage of the total accumulated radioactivity retained in the tissue slices at the conclusion of each set of perfusion experiments is given in Table 5.4. In all experiments using radioactive GABA, more than 80% of the radioactivity remained in the tissue slices: this retained radioactivity did not represent 'non-releasable' GABA since a second high K-stimulus (10 min) could release as much radioactivity from slices of adult cerebral cortex as did the first stimulus (Fig.5.5). It is apparent that the changes observed in K-stimulated GABA release with development are not due to differences in the amount of accumulated radioactive GABA present in the tissue at the onset of the K-stimulus. In general the α -amino acids, 2-aminoisobutyric acid and glycine, were retained less efficiently than was GABA.

Metabolism

GABA. The extent of metabolism of GABA released from 1-day-old rat cortex slices, before, during and after high-potassium stimulation was examined. Radioactivity released from slices of 1-day rat cortex during a 60 min perfusion was collected in three fractions which were released over 15 min periods before, during and after a 10 min high-potassium stimulus. Radioactivity was analysed

Fig. 5.4. Release of [2-³H] glycine from slices of spinal cord from animals of 1 day and 10 days postnatal age. Other details as for Fig. 5.2 except 1 μ Ci of radioactivity was used.

[2-³H]GLYCINE RELEASE FROM SLICES OF SPINAL CORD

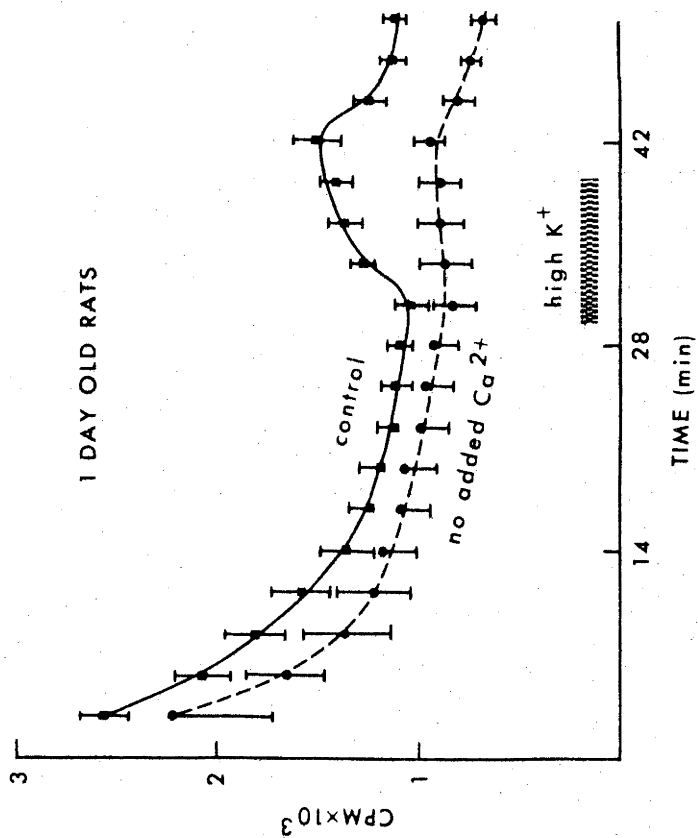
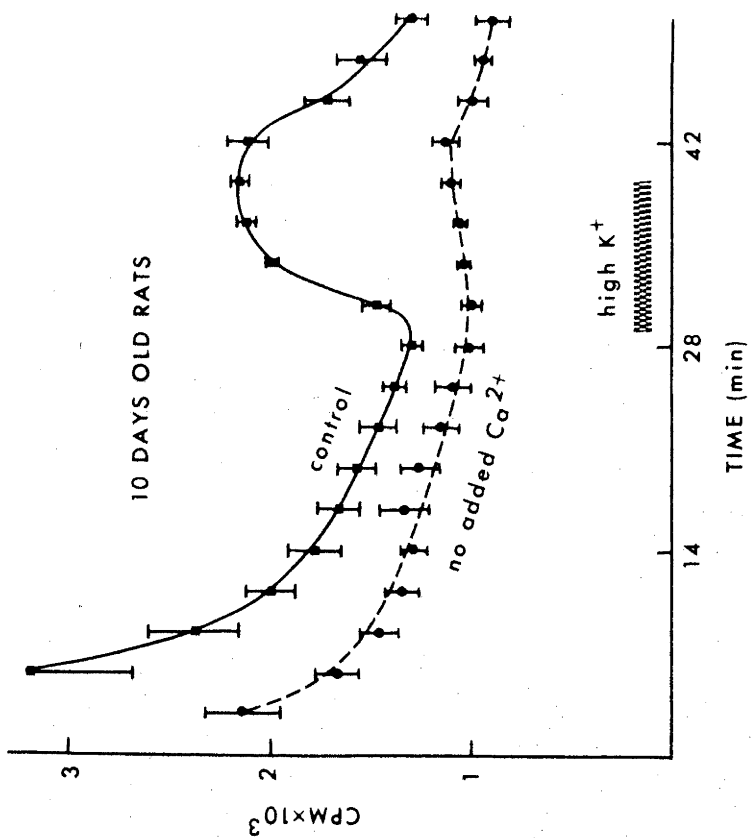


Table 5.3. Postnatal changes in potassium-stimulated release of radioactive glycine from slices of spinal cord

Postnatal age of animals	K-Stimulated release	
	+ Calcium	- Calcium
1 Day	52 \pm 7 (4)	19 \pm 6 (3)
10 Days	72 \pm 5 (5)	18 \pm 3 (4)
Adult (13-weeks)	63 \pm 4 (5)	13 \pm 3 (4)

K-Stimulated release is expressed as the peak percentage increase in release over immediate pre-stimulation spontaneous release. Values given are means \pm S.E.M. of the number of experiments shown in brackets. Initial perfusion time with normal buffer before switching to high K was 30 min.

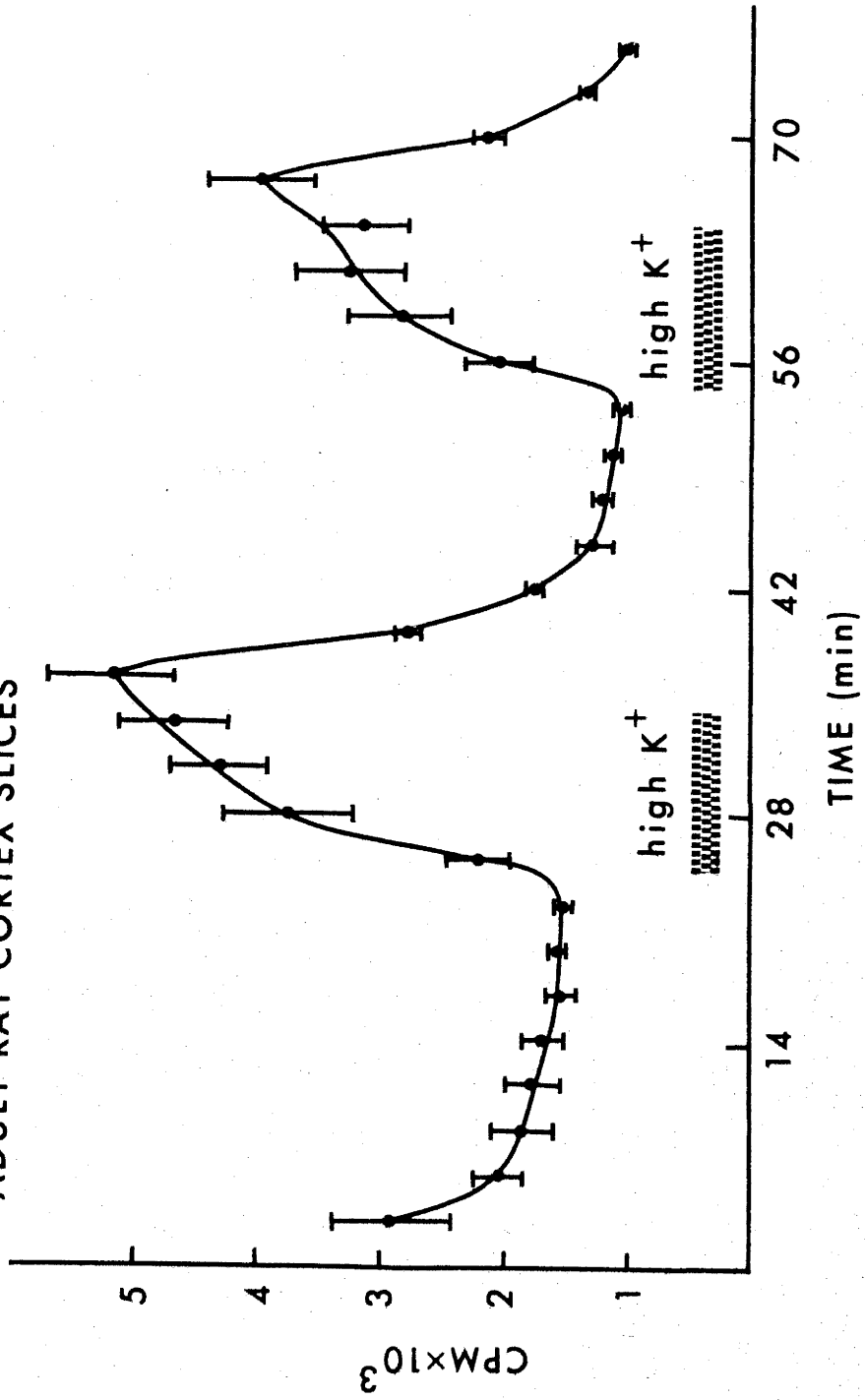
Table 5.4. Percentage of accumulated radioactivity retained in tissue at end of perfusion

Rat age	Total Perfusion Time (min)	Cortex		Spinal cord	
		+ Ca	- Ca	+ Ca	- Ca
(a) <u>GABA</u>					
1 Day	55	85 \pm 2 (5)	87 \pm 1 (3)		
10 Days	50	90 \pm 4 (5)	95 \pm 1 (6)		
13 Weeks	50	94 \pm 0.5 (4)	96 \pm 1 (2)	81 \pm 1 (3)	87 \pm 1 (5)
(b) <u>2-Aminoisobutyric acid</u>					
13 Weeks	50	47 \pm 2 (5)		38 \pm 2 (5)	
(c) <u>Glycine</u>					
1 Day	65			68 \pm 1 (4)	68 \pm 4 (4)
10 Days	65			67 \pm 3 (5)	80 \pm 1 (5)
13 Weeks	65	56 \pm 2 (4)	56 \pm 2 (4)	53 \pm 3 (5)	56 \pm 2 (4)

The amount of accumulated radioactivity retained in tissue slices at the end of each set of experiments is given, along with total perfusion times. Values given are means \pm S.E.M. of the number of experiments shown in brackets.

Fig. 5.5. Release of [U-¹⁴C]GABA from slices of adult rat cortex in response to two high-potassium stimuli, each separated by 20 min perfusion with normal buffer. Each point represents the mean \pm S.E.M. of 3 separate perfusion experiments. Other details as for Fig. 5.2.

**[¹⁴C]GABA RELEASE
ADULT RAT CORTEX SLICES**



by Dowex 50W fractionation and thin-layer chromatography. In the released fractions, 75%, 71% and 71% of the radioactivity remained as unchanged GABA respectively, while 76% of the acid-soluble radioactivity remaining in the slices at the end of the perfusion was unchanged GABA (values are the means of two experiments).

In one experiment, with adult rat cortex slices, the peak of radioactivity released during high-potassium stimulation was analysed. 74% of the released radioactivity was found as unchanged GABA, while GABA accounted for 76% of the acid-soluble radioactivity retained in the slices at the end of the 75 min perfusion.

Glycine. Radioactivity released from pre-labelled slices of 1-day-old rat spinal cord was pooled in three fractions collected over 15 min periods before, during and after 10 min high-potassium stimulus. Of the radioactivity released before, during and after the stimulus, 78%, 74% and 63% remained as unchanged glycine, while 89% of the acid-soluble radioactivity retained by the tissue after a 60 min perfusion was unchanged glycine (values are the means of two experiments).

80% of the acid-soluble radioactivity retained by adult rat cortex slices after 65 min perfusion ran as a single spot on thin-layer chromatography in a position equivalent to glycine.

4. DISCUSSION

A potassium-stimulated release of radioactive GABA and glycine has been demonstrated from slices of cerebral cortex and spinal cord respectively, from rats of 1 day, 10 days and 13 weeks postnatal age. This stimulated release was largely calcium-dependent, as is common to neurosecretory processes (RUBIN, 1970; SIMPSON, 1968).

The initiating factor in release of transmitters from terminals appears to be depolarization of the terminal rather than ionic fluxes which produce the depolarization (HUBBARD, 1973). The high potassium concentration used in these experiments has been shown to depolarize neurones in brain slices (HILLMAN & McILWAIN, 1961). A survey of studies on release of amino acids suggests that it is easier to demonstrate calcium dependence of release mechanisms when tissue is stimulated with high K rather than with electrical impulses (see e.g. MULDER & SNYDER, 1974). Such an observation has been made for neurohumoral release mechanisms (RUBIN, personal communication cited by SRINIVASAN, NEAL & MITCHELL, 1969), and may be explained by the fact that potassium ions appear to have a potentiating action on transmitter release in addition to their depolarizing action (at least at cholinergic synapses; PARSONS, HOFFMAN & FEIGEN, 1965; GAGE & QUASTEL, 1965). Such an action may result from potassium mobilizing more quanta into the readily-releasable store (PARSONS *et al.*, 1965).

A substantial increase in the K-stimulated release of radioactive GABA from slices of adult rat cerebral cortex was observed when the sodium concentration in the perfusing medium was lowered to 26 mM. Similar observations have been made for catecholamine release from the adrenal medulla (DOUGLAS, 1966), acetylcholine release at the neuromuscular

junction (GAGE & QUASTEL, 1966), noradrenaline release from the spleen (KIRPEKAR & WAKADE, 1968) and the release of vasopressin and oxytocin from the neurohypophysis (DOUGLAS & POISNER, 1964). A possible explanation for the observed increase in GABA release could be that it is the result of inhibition of a sodium-dependent re-uptake mechanism (BENNETT, LOGAN & SNYDER, 1972; SNYDER, YOUNG, BENNETT & MULDER, 1973; see also previous chapter, Section IV.3). The perfusion system used should ensure relatively rapid removal of buffer surrounding the tissue slices, although it is possible that equilibration of perfusing medium with extracellular spaces in the tissue may be slow enough to allow some re-uptake. Another possible explanation involves a sodium-calcium antagonism such as has been observed at the neuromuscular junction (GAGE & QUASTEL, 1966; BIRKS, BURSTYN & FIRTH, 1968). The inward movement of calcium is likely to be a critical step in the process of 'stimulus-secretion coupling' (RUBIN, 1970). Calcium has been proposed to interact with a receptor on the presynaptic membrane which influences transmitter release; sodium may compete with calcium by binding to the receptor or may reduce the ability of calcium to bind to the receptor. In the squid axon, it has been shown that calcium influx is increased 5-40 times when external sodium is removed (BAKER, BLAUSTEIN, HODGKIN & STEINHARDT, 1969). An examination of the effect of polyvalent cations on the stimulus-coupled secretion of radioactive GABA from rat brain synaptosomes (LEVY, HAYCOCK & COTMAN, 1974) suggests that the system is 'strikingly similar' to the release of acetylcholine from the neuromuscular junction.

For GABA release from cortex slices, there is an increase in the measured release parameter with development,

with significant K-stimulated, calcium-dependent release being measured in tissue from 1-day rats. It is possible that this increase reflects the development of inhibitory synapses in the cerebral cortex. The development of synaptic junctions has been observed in the parietal cortex molecular layer of rats 12 days to 12 weeks old (AGHAJANIAN & BLOOM, 1967). Few synaptic junctions were evident on postnatal day 12 or 13; however, deeper cortical layers showed earlier development than the outermost layers (EAYRS & GOODHEAD, 1959).

Preliminary observations have been made on synaptic development in rat spinal cord (MAY & BISCOE, 1973). The appearance of synapses has been observed at foetal days 14-18 (gestation period, 21 days); the calcium-dependent, K-stimulated release of glycine from cord slices of 1-day-old animals may represent neurotransmitter release from inhibitory synapses. The phenomenon of potassium-stimulated, calcium-dependent release of amino acids from brain slices may be considered only as an approximate model for the release of transmitters from presynaptic terminals however, since the cellular origin of the released radioactivity is uncertain (CURTIS & JOHNSTON, 1974). Although several studies have shown a presynaptic location of accumulated radioactive GABA and glycine (IVERSEN & BLOOM, 1972; MATUS AND DENNISON, 1972), it is clear that glial elements are also involved (HÖKFELT & LJUNGDAHL, 1972; LJUNGDAHL & HÖKFELT, 1973a, b). Some experimental findings pertinent to this problem have been discussed in the previous chapter (Section IV.1). Radioactive GABA accumulated by rat sensory or superior cervical ganglia becomes exclusively localized in glial cells (GOTTESFELD, KELLY & SCHON, 1973; YOUNG, BROWN, KELLY & SCHON, 1973) from whence it can be released by high potassium-stimulation in a calcium-dependent manner

(BOWERY & BROWN, 1972; ROBERTS, 1974; MINCHIN & IVERSEN, 1974). Subject to these unresolved difficulties, the present results demonstrating potassium-stimulated, calcium-dependent release of radioactive GABA and glycine from tissue slices are consistent with these inhibitory amino acids functioning as transmitters at least as early as 1 day after birth.

The release of radioactive glycine by potassium from slices of adult cerebral cortex is interesting in view of the relative lack of evidence for glycine acting as a synaptic transmitter in this region of the brain (CURTIS & JOHNSTON, 1974). 'High affinity' uptake of glycine into slices of adult cerebral cortex cannot be demonstrated (JOHNSTON & IVERSEN, 1971) although a 'high affinity', sodium-independent uptake is present in synaptosomes prepared from the same tissue (PETERSON & RAGHUPATHY, 1973). Most of the glycine uptake into slices of cerebral cortex appears to be mediated by the 'low affinity', 'small neutral' amino acid system which also transports α -alanine and aminoisobutyric acid (SMITH, 1967; BLASBERG, 1968). It does appear from the present release experiments that glycine enters, more efficiently than does 2-aminoisobutyric acid, a pool from which it can be released by potassium stimulation. This pool may represent the glycine taken up by the 'high affinity' system which cannot be detected in conventional uptake experiments on slices of cerebral cortex.

These experiments were carried out with a simple apparatus that has proved convenient and reliable for studying the release of radioactive amino acids from mini-slices of nervous tissue. A similar system may be useful for studying the effects of centrally-active drugs on transmitter release and for distinguishing between compounds

that influence amino acid transport by inhibiting uptake, potentiating release, or sharing the transport carrier.

Obviously, a rigorous study of development of amino acid transmitter function in the CNS must include a combined neurochemical, neurophysiological, neuropharmacological, anatomical and autoradiographic approach. Work presented in the previous three chapters represents the application of some current neurochemical ideas and techniques which have been helpful in establishing transmitter roles for GABA and glycine in the adult CNS, to the problem of the time during development when these compounds are first used as transmitters in young animals. The data on (1) postnatal levels of glycine and GABA (2) the presence and characteristics of high affinity uptake systems specific for these amino acids in CNS slices (3) the susceptibility of young animals to convulsions caused by the glycine and GABA antagonists, strychnine and bicuculline, and (4) the observation of potassium-stimulated calcium-dependent release from CNS slices provide circumstantial evidence that these compounds are functioning as transmitters at least as soon as 1 day after birth.

VI. THE EFFECT OF SOME DRUGS ON AMINO ACID LEVELS
IN 10-DAY RAT SPINAL CORD

1. INTRODUCTION

A large number of studies have examined the levels of mammalian brain amino acids after administration of convulsive drugs. Attempts have been made to correlate CNS hyperexcitability with increases in levels of those amino acids which excite central neurons when applied microelectrophoretically or with decreases in levels of those amino acids with inhibitory effects. The discovery of compounds which can profoundly raise or lower GABA levels has aided in an understanding of GABA metabolism and physiology (e.g. WOOD & ABRAHAMS, 1971; KURIYAMA, ROBERTS & RUBINSTEIN, 1966).

In the present investigation, a number of drugs were tested in 10-day-old rats with the main aim of finding compounds which could alter glycine levels in the spinal cord and thus aid in studies of glycine metabolism. 10-day-old rats were used for two reasons: (1) the less developed blood-brain barrier system (JOHNSTON, 1973) and, (2) the relative ease of injection and removal of spinal cord. Changes observed in spinal cord levels of amino acids from drug administration were compared with changes in brain levels observed in other studies.

2. METHODS AND MATERIALS

Drugs were administered to unanaesthetized 10-day-old rats, weighing between 16 and 22 g, by intraperitoneal injection of aqueous solutions. Control animals received 0.9% (w/v) saline and volumes of solutions administered were less than 1% of body weight. After the stated times (see Table 6.1) the rats were decapitated and spinal cords removed and weighed. The procedure for preparation of extracts for amino acid analysis has been described (Section III.2).

Extracts were analysed on a Beckman amino acid analyser using a modified system (JAMES, unpublished results) which allows GABA to be simultaneously analysed with aspartate, glutamate, glycine and alanine.

Drugs were purchased from the following sources: amino-oxyacetic acid (Eastman); cycloserine (Nutritional Biochemicals); thiosemicarbazide (B.D.H.); β -phenyllactate and oxythiamine chloride hydrochloride (Sigma); methionine sulphoximine (Calibiochem) and Nikethamide (Ciba).

3. RESULTS AND DISCUSSION

Results for seven drugs are presented in Table 6.1.

Amino-oxyacetic acid. After 3 h, 50 mg/kg amino-oxyacetic acid almost doubled GABA levels and reduced the levels of glutamate and aspartate in 10-day rat spinal cord. This dose of amino-oxyacetic acid, administered to adult rats 2 h before decapitation, was shown to cause a large accumulation of GABA and a decrease in the levels of glutamate, aspartate and alanine in the brain (YOSHINO & ELLIOT, 1970). The large increase in GABA can be explained on the basis of inhibition of GABA:2-oxoglutarate aminotransferase (COLLINS, 1973); increased levels of inhibitory synaptic transmitter may explain the anticonvulsant properties of amino-oxyacetic acid (DE VANZO, GREIG & CRONIN, 1961; KURIYAMA, ROBERTS & RUBINSTEIN, 1966; TAPIA, DE LA MORA & MASSIEU, 1969). It appears that amino-oxyacetic acid also affects the activity of pyruvate-glutamate and oxaloacetate-glutamate transaminases, although we did not observe any significant change in alanine levels in 10-day rat spinal cord. Amino-oxyacetic acid has been shown to inhibit serine hydroxymethyltransferase (Section II.3) and glycine:2-oxoglutarate

Table 6.1. Effect of various drugs on amino acid levels in 10-day rat spinal cord

Drug	Amino-oxycetic acid	Cycloserine	Thio-semicarbazide	β -Phenyl-lactate	Oxythiamine	Methionine sulphoximine	Nikethamide
Drug dose mg/kg	50	50	50	50	500	20	50
mmol/kg	0.56	0.49	0.55	0.30	1.48	0.11	0.28
Time killed (min)	185 \pm 3	149 \pm 5	147 \pm 3	163 \pm 3	117 \pm 12	237 \pm 2	204 \pm 3
No. of animals	5	4	4	5	3	3	3
Taurine		+24 (4)					
Aspartic acid	-69 (5)		-23 (1)				
Serine/Threonine							
Glutamine			+24 (2)				
Glutamic acid	-21 (4)						
Glycine	-17 (1)	+22 (4)					
Alanine							
Valine	+46 (1)			+28 (1)			
Methionine							
Isoleucine							
Leucine							
Tyrosine	+20 (1)						
Phenylalanine	+33 (1)		-42 (3)				
GABA	+89 (5)	+13 (2)	-46 (5)				

Numbers given represent the percentage increase (+) or decrease (-) in amino acid levels when compared with levels from control (saline injected) animals. Six animals were used to determine control levels for the first four drugs listed, while the last group of three drugs listed were tested in an experiment in which four controls were used. The numbers in brackets give the level of significance (student t-test) of changes observed after drug administration, according to: (1) $p < 0.05$ (2) $p < 0.025$ (3) $p < 0.02$ (4) $p < 0.005$ (5) $p < 0.001$. A dash shows that a particular amino acid was not measured; otherwise, no significant change is represented by a blank space.

aminotransferase (JOHNSTON & VITALI, 1969) in vitro. A slight decrease in glycine level ($p < 0.05$) was observed in this experiment.

Cycloserine. Cycloserine (50 mg/kg) had the effect of increasing the levels of glycine and taurine by about 20%, with a smaller, less significant increase in GABA. No tremors or convulsions were observed. Cycloserine inhibits serine hydroxymethyltransferase activity in vitro (Section II.3) as well as other pyridoxal-dependent enzymes, because of its ability to bind strongly to pyridoxal phosphate.

Thiosemicarbazide. $2\frac{1}{2}$ h after the administration of 50 mg/kg (0.55 mmole/kg) of this compound there was a 50% decrease in GABA levels, with a less significant decrease in phenylalanine and increase in glutamine. Slight tremors were evident after $1\frac{1}{2}$ - 2 h. No significant effect was observed on glycine levels. WOOD & ABRAHAMS (1971) have examined the effects of various hydrazines on GABA levels and metabolism in chick brain. 30 min after injection of chickens with 2.5 mmol/kg of thiosemicarbazide, the GABA level had dropped by 25%; very similar effects were observed with other hydrazides.

DL- β -Phenyllactate. This compound failed to produce any significant changes in 10-day spinal cord amino acid levels at the dose given. It has been reported to be a specific inhibitor of rat liver glycollate oxidase (RICHARDSON, 1973) and was tested in view of the possible role of glycollate oxidase in glycine synthesis in the CNS (CURTIS & JOHNSTON, 1974; see Fig. 2.1).

Oxythiamine. An analogue of thiamine, this compound inhibits the formation of the coenzyme, thiamine pyrophosphate. Chronic administration results in symptoms of thiamine

deficiency and eventual death. This substance was tested because thiamine-deficient animals have increased levels of glyoxylate (LIANG, 1962; BUCKLE, 1963). Elevated levels are probably caused by inhibition of the thiamine pyrophosphate-dependent, pyruvate dehydrogenase and α -oxoglutarate dehydrogenase enzyme complexes, which catalyse the condensation of glyoxylate with pyruvate and α -oxoglutarate respectively (KUBASIK, RICHERT, BLOOM, HSU & WESTERFELD, 1973; SCHLOSSBERG, BLOOM, RICHERT & WESTERFELD, 1970). A single administration of this drug had no effect on amino acid levels.

Methionine sulphoximine. No changes in amino acid levels were observed with the low dose used in this experiment, although all animals displayed slight and spasmodic tremors. This compound produces 'running fits' and grand-mal seizures in dogs, 16-18 h after i/p administration of 10 mg/kg (BROWN & STONE, 1973). A dose of 2 mmol/kg produced convulsions in 10-day-old rats in 4¹/₂ - 5 h (JOHNSTON, 1973).

Nikethamide. This drug which has been used as an analeptic and as a respiratory and cardiovascular stimulant has been shown to cause convulsions in high doses (80 mg/kg) (ECKENHOFF & HAFKENSCHIEL, 1947). Nikethamide (50 mg/kg) showed no effect after 3 h although all animals showed slight tremors before being killed.

None of these drugs produced any large change in glycine levels. Cycloserine increased glycine by 22%, approximately 2 h after administration (50 mg/kg). Amino-oxyacetic acid and thiosemicarbazide were observed to have drastic effects on GABA levels, similar to their effects in brain. It is suggested that attempts to relate the onset of experimentally-induced convulsions with changes in brain GABA levels should include measurement of spinal cord levels.

PART TWO: D- AND L-ASPARTATE

VII. THE UPTAKE AND RELEASE OF D- AND L-ASPARTATE
BY RAT BRAIN SLICES

1. INTRODUCTION

The four most abundant amino acids in the adult mammalian brain are glutamic acid, glutamine, GABA and aspartic acid. Both L-glutamate and L-aspartate are likely to be major excitatory CNS transmitters. The central levels of L-aspartate vary from region to region, but only within the feline spinal cord have levels been correlated with a possible function as a transmitter of excitatory interneurons (DAVIDOFF, GRAHAM, SHANK, WERMAN & APRISON, 1967).

L-glutamate, more concentrated in the dorsal than in ventral roots of feline spinal cord, has been proposed as the excitatory transmitter released by the terminals of primary afferent fibres (GRAHAM, SHANK, WERMAN & APRISON, 1967). In other areas no obvious correlation exists between the levels of L-glutamate, activities of its various metabolising enzymes and suspected transmitter function (JOHNSON, 1972a, b).

Both L-glutamate and L-aspartate excite feline central neurons by a reversible depolarization accompanied by an increase in membrane conductance (CURTIS, PHILLIS & WATKINS, 1960; KRNJEVIĆ & SCHWARTZ, 1967). The corresponding D-isomers are only slightly less potent excitants when tested by microelectrophoresis (CURTIS & WATKINS, 1963; CRAWFORD & CURTIS, 1964).

In a study on the high affinity uptake of L-aspartate and L-glutamate in slices of rat cerebral cortex and feline spinal cord, BALCAR & JOHNSTON (1972a, b) showed D-aspartate to be a strong inhibitor of L-glutamate and L-aspartate uptake; in fact, in rat cortex, it was a stronger inhibitor of L-glutamate uptake than was L-aspartate. D-glutamate, however, was weaker than the corresponding L-isomer in inhibiting L-glutamate or L-aspartate accumulation. This work

suggested that D-aspartate inhibition of L-glutamate uptake was consistent with linear competitive inhibition, but did not indicate whether D-aspartate was accumulated by the slices (i.e. whether it was a substrate for the dicarboxylic amino acid transport 'carrier').

Work reported in this chapter describes the preparation of radioactive D-aspartate and its use to determine whether D-aspartate was actively accumulated by slices, and furthermore, to examine kinetics, substrate specificity and subcellular distribution of any such uptake.

2. METHODS AND MATERIALS

Preparation of [^{14}C]D-aspartic acid. [$4\text{-}^{14}\text{C}$]D-aspartic acid was prepared from [$4\text{-}^{14}\text{C}$]DL-aspartic acid using an acetone powder extract of Clostridium welchii (containing L-aspartate 4-carboxy-lyase activity), under conditions similar to those described by MARGOLIS & LAJTHA (1968). Reaction mixtures, in Kontes flasks (25 ml), contained 10.8 μmoles of [$4\text{-}^{14}\text{C}$]DL-aspartate acid (0.05 mCi), 120 μmoles of sodium pyruvate, 150 mg of Cl.welchii acetone powder and 2 μmole of L-aspartic acid in a total volume of 5.2 ml of 0.2 M sodium acetate buffer, pH 4.9. To collect $^{14}\text{CO}_2$ evolved, a rolled piece of Whatman 3MM paper (4 x 2 cm), which had been soaked in 3 N potassium hydroxide and dessicated over potassium hydroxide pellets, was placed in a small plastic cup hanging from the rubber stopper. Reactions were incubated at 37°C in a shaking water bath for 6 1/2 h, then a further 2 μmoles of L-aspartate and 30 mg of Cl. welchii extract in 0.2 M acetate buffer was added and incubation continued for a further 3 h. Reactions were stopped with 1 ml of 4.5 M perchloric acid and incubated for a further hour before removal of potassium hydroxide papers for scintillation counting. Efficiency of $^{14}\text{CO}_2$ capture by the papers was measured

simultaneously using flasks containing $\text{NaH}^{14}\text{CO}_3$. The perchloric acid precipitated protein was removed by centrifugation and the supernatant neutralized with potassium hydroxide. Potassium perchlorate was removed by centrifugation and the supernatant concentrated in vacuo to approximately 3 ml. This was loaded onto a small column of Dowex 1 x 8 (acetate form; 200-400 mesh, 5 ml of resin, I.D. of column = 7 mm). Columns were washed with 45 ml of water, 20 ml of 0.1 N acetic acid, 50 ml of 0.4 N acetic acid and then with 2 N acetic acid. The peak of radioactivity was eluted with 0.4 N acetic acid; these fractions were pooled, reduced to dryness in vacuo several times to remove acetic acid and then aliquots were subject to a second incubation with Cl. welchii extract to check on the completeness of L-aspartate digestion. Less than 3% of added radioactivity was released as $^{14}\text{CO}_2$.

Aspartic acid uptake by tissue slices. The procedures used for measuring the uptake of $[4\text{-}^{14}\text{C}]$ D-aspartic acid and $[U\text{-}^3\text{H}]$ L-aspartic acid were essentially the same as those described in a previous chapter (Section IV.2). All experiments, unless otherwise stated, were carried out at 25°C. For kinetic analysis, initial velocities of aspartate accumulation at ten concentrations of substrate in the range of 9-400 μM were determined. Cold substrate (100 μl) was added at the same time as the radioactive substrate (100 μl). Potential inhibitors (10^{-4} M) were preincubated with the slices for 15 min before the addition of radioactivity. All experiments, including the blank (not incubated) were done in quadruplicate. Slices, on filter paper discs, were extracted with water for 1 h at room temperature, then 10 ml of scintillator (either 0.5% PPO and 10% naphthalene in dioxan or 0.3% PPO in a 1:3 mixture of Triton X-114:xylene) added and radioactivity measured in a scintillation counter (Beckman LS-100). Counts per minute (CPM) were converted to disintegrations per minute (DPM) by means of an external standard.

Density gradient fractionation of accumulated radioactivity.

The procedure used was similar to that described by ARREGUI, LOGAN, BENNETT & SNYDER (1972). All operations were performed at 0-4°C. The cerebral hemispheres of 13-week-old rats were separated from the midbrain, cerebellum and pons-medulla, dissected free of underlying white matter and homogenized in 20:1 (v/w) 0.32 M sucrose by 12 up-and-down strokes of a motor-driven perspex pestle (rotating at 870 rev./min, clearance 0.25 mm difference in diameter) in a stainless-steel homogenizer. The homogenate was centrifuged at 1000 x g. for 10 min to remove nuclei and cell debris and 1.5 ml aliquots of the supernatant were added to Kontes flasks (25 ml) containing 15 ml of oxygenated Krebs Ringer phosphate buffer, pH 7.4. After preincubation at 37°C for 5 min in a shaking water bath, radioactive amino acids (1 µCi of [¹⁴C]D-aspartate and 1.5 µCi of [³H]L-aspartate) were added and incubation continued for 5 min. Contents of flasks were cooled on ice and transferred to 40 ml centrifuge tubes over a layer of 10 ml of ice-cold 0.32 M sucrose. Tubes were centrifuged at 27,000 x g. for 5 min to form a crude mitochondrial pellet. The pellet was rinsed with 10 ml of cold sucrose and thoroughly suspended in 3 ml of 0.32 M sucrose using a pasteur pipette and vortex mixer, taking care to eliminate particle aggregates. In one experiment, the pellet was osmotically shocked by resuspension in water instead of 0.32 M sucrose. The suspension was layered on a continuous sucrose density gradient formed by linear dilution of 13 ml of 1.5 M sucrose with 13 ml of 0.5 M sucrose. Gradients were prepared at room temperature and left in the cold room (4°C) for 4-5 h. These were centrifuged at 53,000 x g. for 1 h with a SW 25.1 head in an ultracentrifuge (Spinco, model L). Fractions were obtained

by piercing the bottoms of the tubes and collecting 20-drop fractions directly into scintillation vials. [^3H] and [^{14}C] radioactivity was determined after the addition of 9 ml of Bray's scintillator. Results were corrected for spillover of [^{14}C] CPM into the [^3H] channel.

Release of aspartic acid from slices. The apparatus and procedure for release studies has been described previously (Section V.2). Adult rat cortex slices were preloaded with [^{14}C]D- or [^3H]L-aspartic acid for 20 min, then filtered onto glass fibre filter discs and transferred to the perfusion apparatus. Initial perfusion time before perfusion with high potassium buffer was 25 min. 10 min perfusion with high potassium was followed by a return to normal buffer for 25 min. Fractions of superfusate were collected every 2.8 min directly into scintillation vials and radioactivity determined after the addition of 9 ml of xylene:Teric N8 scintillator (see Section V.2).

Metabolism. Rat brain cortex slices (100 mg) were incubated in 10 ml of oxygenated Krebs ringer phosphate buffer at 37°C in a shaking water bath for 1 h, in the presence of [^3H]L-aspartate or [^{14}C]D-aspartate. The slices were collected by centrifugation and the extracts concentrated to dryness in vacuo. Extracts were resuspended in 200 μl of water and 50 μl aliquots subject

to electrophoresis on paper strips in 2% pyridine, 1% acetic acid, pH 5.2 at 300 V for 2¹/₂ h. Strips were cut into 1 cm pieces, placed in scintillation vials with 1 ml water for 1 h, then scintillator added and radioactivity determined.

The radioactivity retained in cerebral cortical slices at the end of perfusion experiments was examined. Tissue was homogenized in 5 ml of 0.45 N perchloric acid in a glass-teflon homogenizer and the supernatant collected by centrifugation. The extract was neutralized with potassium hydroxide, the potassium perchlorate removed by centrifugation and the supernatant concentrated in vacuo. The extract (acidified to pH 1-3) was applied to a small column of Dowex 50W x 4 (200-400 mesh), washed with 0.01 N HCl and water, then eluted with 2N ammonia. A 1 ml aliquot of the wash was counted to determine radioactivity in acid metabolites, while the 2 N ammonia eluent was evaporated to dryness in vacuo (several times to remove ammonia), resuspended in a small volume of water and subjected to electrophoresis on paper strips under the conditions described above.

Chemicals. [4-¹⁴C]DL-aspartic acid (4.64 mCi/mmol) was purchased from I.C.N., Cleveland, [U-³H]L-aspartic acid (2.2 Ci/mmol) from New England Nuclear, Boston, and [¹⁴C] sodium bicarbonate (42.4 mCi/mmol) from the Radiochemical Centre, Amersham. Other chemicals were purchased from the following sources; L-aspartic acid, sodium pyruvate and L-glutamate decarboxylase (crude acetone powder from Clostridium welchii, containing L-aspartate β -decarboxylase activity) (Sigma); D-glutamic acid, threo- β -hydroxy-DL-aspartic acid and L-cysteinesulphinatate (Calbiochem); D-aspartic acid (Nutritional Biochemicals); monosodium L-glutamate (B.D.H.); L-cysteic acid (Light and Co.); amino-oxyacetic acid (Eastman); and sucrose (Mann).

N-methyl-L-aspartic acid and N-methyl-D-aspartic acid were prepared by Dr J.C. Watkins (WATKINS, 1962).

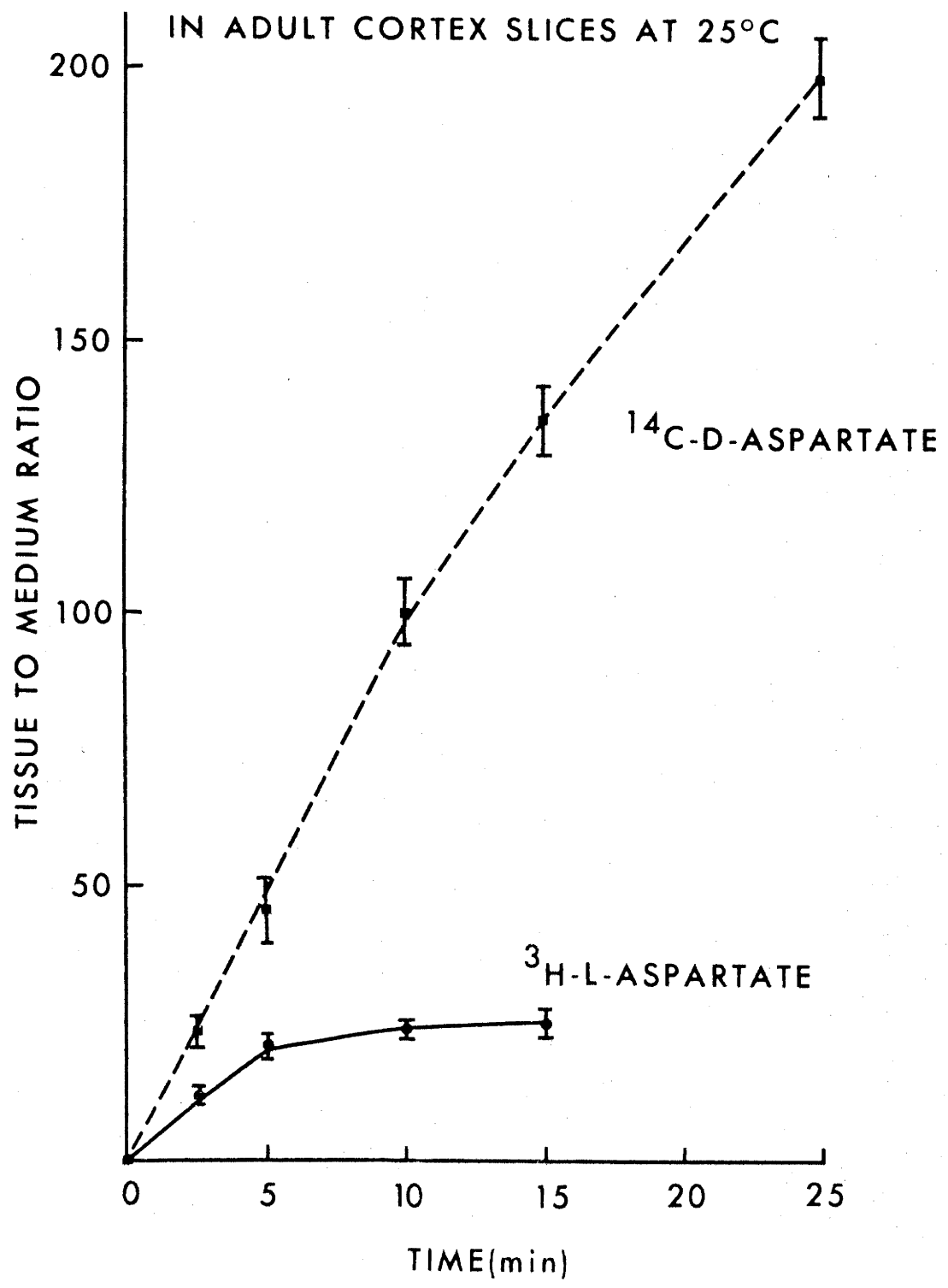
3. RESULTS

Time course of D- and L-aspartate uptake. Adult rat brain cortex slices were preincubated at 25°C for 5 min, radioactivity added and incubation continued for different times before the slices were filtered and washed with saline. The time course of uptake of [4-¹⁴C]D-aspartate and [U-³H]L-aspartate is illustrated in Fig. 7.1. The specific activity of the [¹⁴C]D-aspartate used was 4.6 mCi/mmol; approximately 0.1 µCi of radioactivity was used per incubation vial, giving a final concentration of 2 µM in the 10 ml of incubation medium. The specific activity of the L-aspartate was 2.2 Ci/mmol; approximately 0.22 µCi was used per bottle, giving 0.01 µM. Assuming the endogenous concentration of L-aspartate in the rat cortex to be 2 µmol/g wet weight and that 50% of this is released on preparation of slices (BALCAR & JOHNSTON, 1975), the contribution to the external L-aspartate concentration from endogenous L-aspartate is 1 µM. With these concentrations of D- and L-aspartic acid it can be seen that D-aspartic acid is accumulated to a greater extent than the L-isomer, with uptake linear for a longer period of time. In most subsequent experiments, unless otherwise stated, L-aspartic acid uptake was measured over 5 min and D-aspartate over 10 min.

Kinetics of D- and L-aspartate uptake. The initial rates of accumulation of D- and L-aspartic acid were measured in adult rat brain cortex slices over a range of aspartic acid concentrations from 9 to 400 µM. For L-aspartic acid uptake, analysis of the data by the method of CLELAND (1963) revealed a 'high affinity' system with a K_m of 15.6 ± 2.1 µM and a

Fig. 7.1. Time course of accumulation of radioactive [^{14}C]D-aspartate and [^3H]L-aspartate in slices of adult rat cerebral cortex. Slices were preincubated at 25°C for 5 min, radioactivity added and incubation continued for different times before slices were filtered and washed with saline. The tissue to medium ratio is the CPM accumulated per ml of tissue water/CPM per ml of incubation medium; in this case the parameter was calculated assuming the tissue to be 100% water.

TIME COURSE OF ASPARTATE UPTAKE
IN ADULT CORTEX SLICES AT 25°C



V_m of 0.10 ± 0.007 $\mu\text{mol}/\text{min}/\text{g}$ wet weight, and a 'low affinity' uptake with a K_m of 125 ± 14 μM and a V_m of 0.25 ± 0.01 $\mu\text{mol}/\text{min}/\text{g}$ wet weight. Analysis of D-aspartate uptake revealed only a 'high affinity' system of lower K_m to that for L-aspartate, i.e., $K_m = 7.5 \pm 1.3$ μM , and with a higher maximal velocity of uptake of 0.22 ± 0.01 $\mu\text{mol}/\text{min}/\text{g}$ (Fig. 7.2).

Sodium-dependence of D-aspartic acid uptake. In common with high affinity uptake of the transmitter candidates L-aspartate, L-glutamate, GABA, proline and glycine (in the spinal cord), the uptake of D-aspartate was completely dependent on the presence of sodium. The absence of sodium resulted in radioactive accumulation not significantly different from zero time blank values (Fig. 7.3). In another experiment, the 118.1 mM sodium chloride was replaced with lithium chloride or choline chloride and the ability of lithium to support uptake in the absence of sodium was examined (Table 7.1). Uptake of D-aspartate in the presence of lithium was about 4% of that in the presence of sodium, and was 4-5 times more than the value for L-glutamate uptake. GABA uptake was not significantly different from the CPM accumulated in the absence of sodium.

Inhibitors. Several amino acids and analogues were tested at 10^{-4} M concentration against D- and L-aspartate uptake (Table 7.2). The pattern of inhibition of the D- and L-isomers was similar. D-glutamate showed significant inhibition of D-aspartate uptake ($27 \pm 5\%$) and no significant inhibition of L-aspartate uptake, while D-aspartate was a better inhibitor of L-aspartic acid uptake than was the L-isomer itself. D-glutamate was less effective in inhibiting either D- or L-aspartate than was L-glutamate.

Fig. 7.2. Kinetic analysis of data for the uptake of D- and L-aspartate in slices of adult rat cerebral cortex. The slices were preincubated for 5 min at 25°C before addition of radioactive substrate and incubation continued for a further 5 min for L-aspartate or 10 min for D-aspartate. Each point represents the mean \pm S.E.M. of 3-4 experimental values. Each set of points represents a separate experiment. The open circles are derived from measurements of initial velocities of L-aspartate accumulation over a range of external L-aspartate concentrations from 9-81 μ M, the closed circles from L-aspartate accumulation over the concentration range 81-400 μ M, while the black squares are from measurements of D-aspartate accumulation over the concentration range 9-81 μ M. The lines represent Lineweaver-Burk plots of the computed best fit of the individual experimental values obtained in each experiment, to the hyperbola $v = VA/(K + A)$ (CLELAND, 1963), for the range of substrate concentrations given. The units of uptake velocity, v , are μ mol/min/g wet weight and those of A , the external substrate concentration, are μ M.

KINETIC ANALYSIS OF THE UPTAKE
OF ASPARTATE IN SLICES OF
RAT CEREBRAL CORTEX

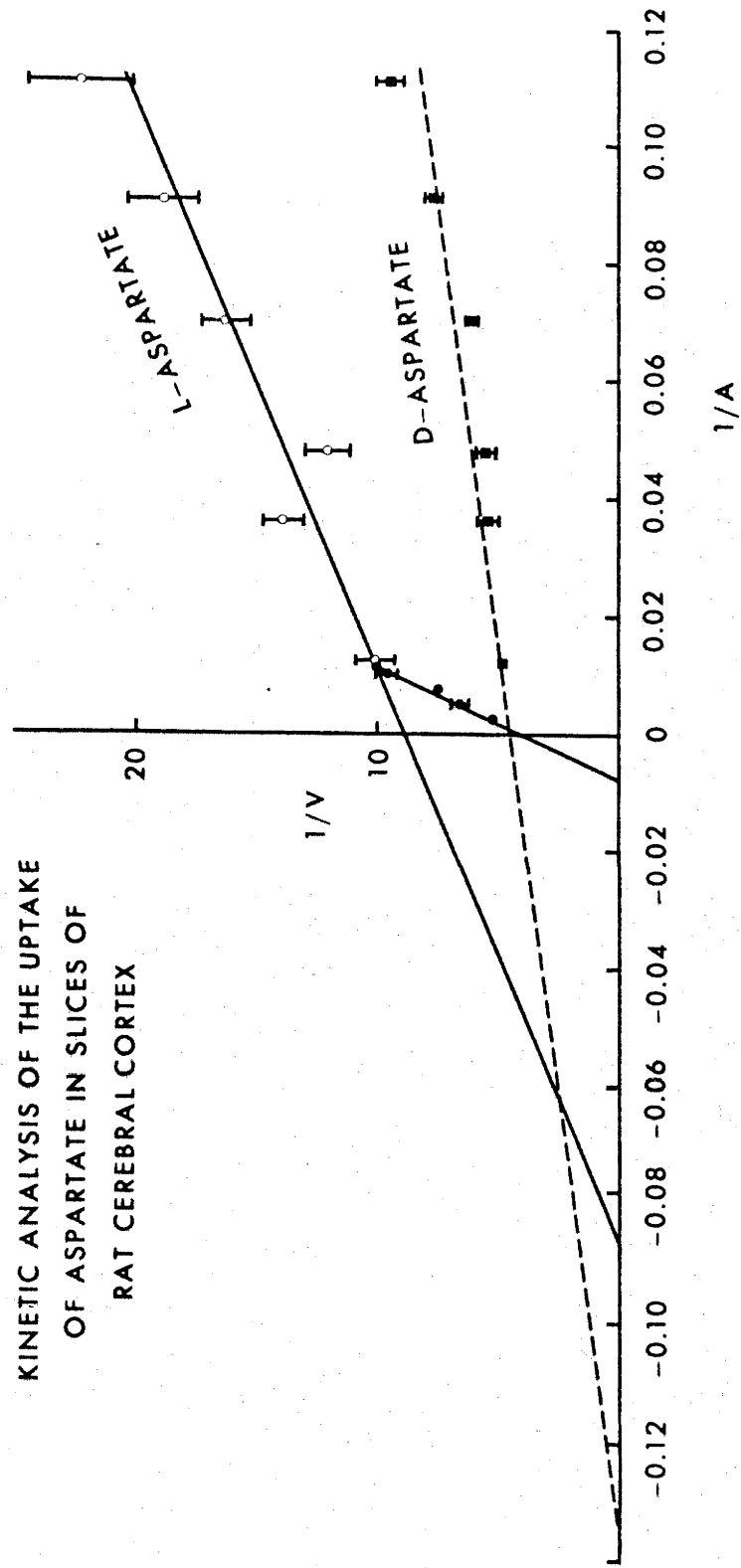


Fig. 7.3. Analysis of the dependence on external sodium ion concentration of [¹⁴C]D-aspartate (2 μM) uptake into adult rat cerebral cortex slices. Slices were preincubated at 25°C for 15 min before the addition of radioactive substrate and uptake measured after 10 min. Uptake was measured in medium in which the normal sodium phosphate buffer was replaced with tris-HCl buffer and in which varying proportions of the sodium chloride were replaced with choline chloride. The points are means ± S.E.M. of 4 determinations and uptake is expressed as the percentage of uptake observed at 118.1 mM sodium.

SODIUM DEPENDENCE OF D-ASPARTATE UPTAKE
ADULT RAT CORTEX SLICES

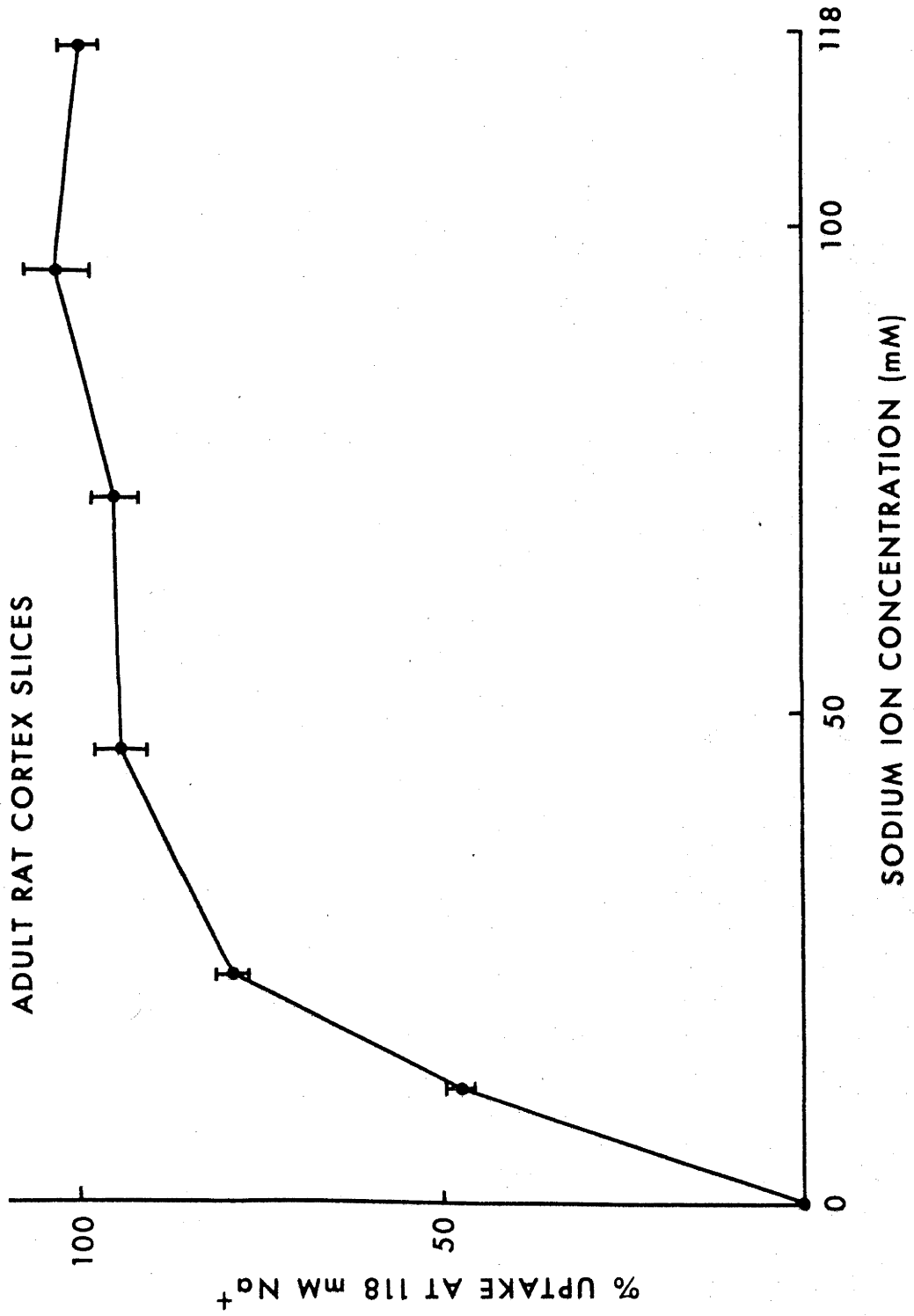


Table 7.1. Effect of replacement of sodium with lithium on amino acid accumulation in rat brain slices

Amino acid	CPM accumulated from incubation medium					$\frac{\text{TLi-Tc}}{\text{TNa-Tc}}$
	TC	TNa	TLi	TC(blank)		
L-glutamate	192 ± 10	7,388 ± 57	252 ± 13	109 ± 9	0.8 (3.5)	
D-aspartate	292 ± 18	26,071 ± 1,038	1,275 ± 159	287 ± 6	3.8 (26.3)*	
GABA	203 ± 10	7,434 ± 24	183 ± 8	126 ± 6	-0.3 (-0.3)	

Values represent the means ± S.E.M. of four determinations of radioactivity accumulated by rat brain cortex slices (10 mg) over either a 10 min incubation period for D-aspartate and GABA or an 8 min period for L-glutamate, at 25°C. Incubation buffer was normal Krebs ringer tris-HCl buffer, pH 7.4, in which the sodium chloride (118.1 mM) was replaced by either lithium chloride or choline chloride.

TC = buffer with choline chloride

TNa = buffer with sodium chloride

TLi = buffer with lithium chloride

Values in brackets refer to the equivalent parameter (TLi-TS/TNa-TS; TS = tris-HCl buffer with sucrose, not choline as used here) calculated from the results of PETERSON & RAGHUPATHY (1974) on amino acid accumulation in rat cerebral cortical synaptosomes.

* This value is for L-aspartate accumulation, not D-aspartate as used here.

Added CPM per 10 ml of incubation buffer were, L-glutamate 79,550; D-aspartate 172,690; GABA 51,900.

Table 7.2. Inhibition of the high affinity L-aspartate and D-aspartate uptake into adult cat cortex slices

Inhibitor (10^{-4} M)	% Inhibition of control uptake	
	L-aspartic acid	D-aspartic acid
Threo- β -hydroxy-DL-aspartate	101 \pm 1	89 \pm 2
L-cysteinesulphinate	87 \pm 1	95 \pm 1
L-cysteate	89 \pm 1	90 \pm 1
L-aspartate	78 \pm 2	83 \pm 4
D-aspartate	89 \pm 1	82 \pm 1
L-glutamate	74 \pm 2	66 \pm 1
D-glutamate	n.s.	27 \pm 5
N-methyl-L-aspartate	n.s.	n.s.
N-methyl-D-aspartate	n.s.	n.s.

The tissue slices were preincubated with the amino acids at the concentration shown for 15 min at 25°C, [3 H]L-aspartate (0.02 μ M) and [14 C]D-aspartate (2.0 μ M) added and uptake measured after 10 min. Values are means \pm S.E.M. of results from quadruplicate experiments. In double-label scintillation counting, [3 H] CPM were corrected for spillover from the [14 C] channel.

Subcellular distribution of accumulated radioactivity.

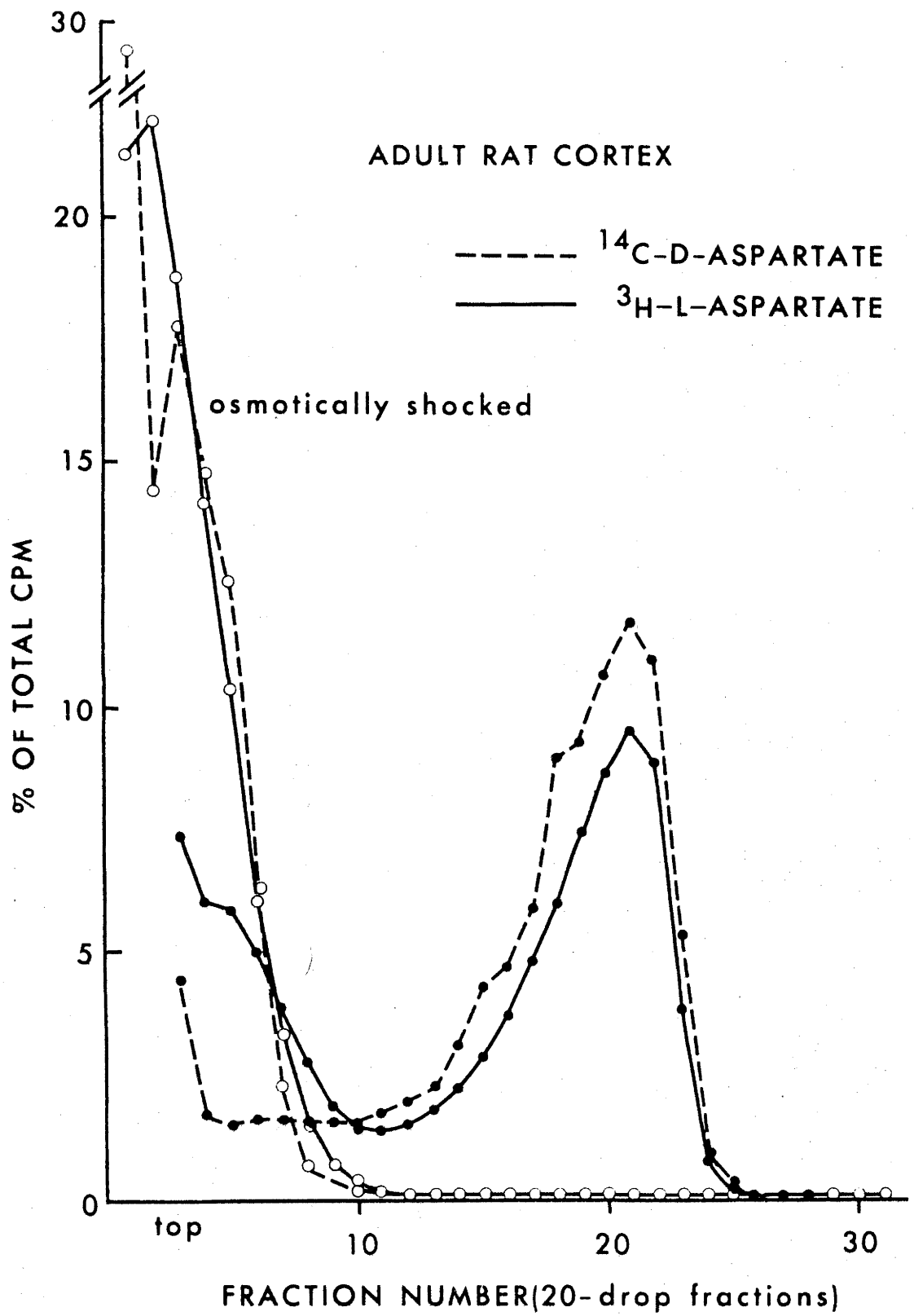
Homogenates of rat cerebral cortex were incubated with [³H]L-aspartate and [¹⁴C]D-aspartate, the crude mitochondrial fraction separated by centrifugation and subjected to density gradient fractionation on a sucrose gradient. Both D- and L-aspartate appear to be accumulated into the same subcellular particles, since peaks of radioactivity were not separable on sucrose density gradients (Fig. 7.4). Both isomers were accumulated into osmotically-sensitive particles, since suspension of the crude mitochondrial pellet in water instead of 0.32 M sucrose, prior to applying it to the top of the gradient, resulted in release of radioactivity into the medium. In repeated experiments it was observed that there was always less of the total radioactivity from D-aspartate at the top of the gradient (i.e. radioactivity released from particles in the crude mitochondrial pellet during suspension and layering on the gradient) than there was of the L-aspartate radioactivity. To examine further the efflux of aspartate from sites of accumulation, release of D- and L-aspartate was followed, using apparatus previously described (Section V.2) for studying the release of GABA and glycine.

Release of D- and L-aspartic acid. Cerebral cortical slices were preloaded with [4-¹⁴C]D-aspartate and [U-³H]L-aspartate at 37°C. Slices were perfused with normal buffer for 25 min before switching to high potassium buffer for 10 min.

In two experiments (slices prelabelled either in the presence or absence of 2×10^{-5} M amino-oxyacetic acid) D-aspartate was released with very little release of L-aspartate.

Increasing the initial perfusion time to 40 min before the potassium-stimulation did result in release of L-aspartate; this however, was still much less than that observed for

Fig. 7.4. Subcellular distribution of radioactive [^3H]L-aspartate and [^{14}C]D-aspartate accumulated by homogenates of adult rat cerebral cortex. Homogenates were incubated with radioactive D- and L-aspartate for 5 min, the crude mitochondrial fractions separated by centrifugation and subjected to density gradient fractionation on sucrose gradients formed by linear dilution of 13 ml of 1.5 M sucrose with 13 ml of 0.5 M sucrose. Gradients were centrifuged at 53,000 x g. for 60 min in a swinging-bucket rotor. The closed circles are an experiment in which the crude mitochondrial pellet was suspended in 0.32 M sucrose prior to density gradient fractionation. The open circles are an experiment in which the crude mitochondrial pellet was osmotically shocked by resuspending in water instead of 0.32 M sucrose, before being layered onto the sucrose gradient.



D-aspartate (Table 7.3). It was also apparent from these perfusion experiments that [^{14}C]D-aspartate radioactivity was retained to a much greater extent than [^3H]L-aspartate radioactivity (Table 7.3). To determine the reason for lack of release of L-aspartate the extent of metabolism was examined under the same conditions.

Metabolism. Under the conditions of the uptake experiments, with 5 min incubation for L-aspartate, less than 10% was metabolised (BALCAR & JOHNSTON, 1972a). Analysis of radioactivity accumulated by rat brain cortex slices (100 mg) after 1 h incubation at 37°C with [^3H]L-aspartate or [^{14}C]D-aspartate in 10 ml of Krebs ringer phosphate buffer showed that of the CPM recovered from electrophoresis strips, more than 97% of D-aspartate radioactivity ran as aspartate (two experiments), while only 66-72% of radioactivity from L-aspartate ran as aspartate (two experiments). Under conditions of the perfusion experiments, with 20 min preloading of tissue with radioactive compound and subsequent perfusion for 50-65 min, an examination of radioactivity retained in the slices at the end of perfusion showed that between 46% and 71% of the L-aspartate was metabolised. There was less than 3% metabolism of the D-aspartate.

Kinetics of inhibition by the opposite isomer. Data from two separate experiments, in which the kinetics of L-aspartate accumulation were examined in the presence of 13.3 and 26.7 μM D-aspartate, were fitted to the equation for linear competitive inhibition (CLELAND, 1963; BEART, JOHNSTON & UHR, 1972). The following results were obtained (weighted mean \pm S.E. of the weighted mean, for values \pm S.E. of the kinetic constants from the two experiments; see Appendix 2):
 $K_m = 17.3 \pm 1.8 \mu\text{M}$; $V_m = 0.10 \pm 0.006 \mu\text{mol}/\text{min}/\text{g wet weight}$; $K_{is} = 13.0 \pm 0.9 \mu\text{M}$. Data from three experiments,

Table 7.3. Release of radioactive aspartate from rat cerebral cortex slices

Amino acid	Initial perfusion			Potassium-stimulated release		Percent accumulated CPM retained by tissue
	time (min)	+Ca ²⁺	-Ca ²⁺	+Ca ²⁺	-Ca ²⁺	
D-aspartate	25	217 ± 41 (4)	49 ± 14 (3)			93 ± 0.5
	25*	220 ± 85 (4)	-			91 ± 1.4
L-aspartate	25	5 ± 3 (4)	-			55 ± 1.4
	25*	10 ± 7 (4)	-			56 ± 1.9
	40	31 ± 6 (4)	-			59 ± 0.2

Release is calculated as the peak percentage increase in release over immediate pre-stimulation spontaneous efflux. Values given are means ± S.E.M. of the number of experiments shown in brackets. The right-hand column shows the percentage of accumulated radioactivity retained by the slices at the end of perfusion, and are for the experiments performed in the presence of calcium.

*These values are for an experiment in which the slices were preloaded in the absence of amino-oxyacetic acid. In other experiments, slices were preloaded in the presence of amino-oxyacetic acid (2×10^{-5} M).

in which the kinetics of D-aspartate accumulation were examined in the presence of several concentrations of L-aspartate were variable, possibly because of metabolism of the L-aspartate occurring over the time of preincubation and then incubation with radioactive D-aspartate.

4. DISCUSSION

The uptake of D-aspartate was linear for at least 10 min, whereas that of L-aspartate levelled off after 5 min. This was presumably due to metabolism and release of metabolites into the medium. Release experiments showed that D-aspartate radioactivity was retained to a much greater extent than radioactivity from L-aspartate.

Lineweaver-Burk plots of the dependence of aspartate uptake on the external concentration of aspartate showed two kinetically distinct systems for L-aspartate accumulation. This has been shown to be the case for L-glutamate, L-aspartate and glycine uptake in cat spinal cord slices (BALCAR & JOHNSTON, 1973). The K_m for the high affinity uptake into rat brain slices ($16 \pm 2 \mu\text{M}$) was very similar to that measured in cat cord slices ($13 \pm 4 \mu\text{M}$). The K_m for low affinity uptake in rat brain slices was $125 \pm 14 \mu\text{M}$, and $185 \pm 143 \mu\text{M}$ for cat spinal cord slices. D-aspartate accumulation measured over the range 10-400 μM showed only a high affinity uptake, with a K_m somewhat lower than that for L-aspartate and with a maximal velocity of accumulation approximately twice that for the L-isomer. This difference may explain why D-aspartate is a slightly less effective neuronal excitant than L-aspartate, when tested by microelectrophoresis.

The specificity of a transport system, presumably determined by the attachment of the transport substrate to

the binding site of the carrier, is usually characterized by competitive inhibition between analagous substrates. The presence of competitive inhibition between D- and L-aspartate, which are both transported, suggests that they use the same transport system. A more rigid proof stipulates that the following requirements must be fulfilled if two substrates (A and B) use the same transport system (HEINZ, 1967):

1. The inhibitory constant (K_i) of A with respect to B must be the same as the K_m of A.
2. The inhibitory constant of B with respect to A must be the same as the K_m of B.
3. A competing third substrate must have the same inhibitory constant with respect to either A or B.

Results obtained here satisfy one of the first two requirements and while K_{is} values for a third substrate have not been calculated against D- or L-aspartate uptake, the observed inhibitions of uptake of the two isomers by fixed concentrations of a number of inhibitors are similar.

The pattern of dependence of rat cerebral cortex high affinity D-aspartate uptake on external sodium ion concentration was very similar to that observed for L-glutamate measured under the same conditions (BALCAR & JOHNSTON, 1972a). In the presence of approximately $1 \mu\text{M}$ D-aspartate, half-maximal uptake was calculated to occur at an external sodium ion concentration of $14 \pm 2 \text{ mM}$, which is the same as that for L-glutamate uptake ($13 \pm 5 \text{ mM}$; external L-glutamate concentration approximately $3.6 \mu\text{M}$). A comparison with the sodium-dependence curves for GABA and glycine high affinity uptake (Figs 4.1 and 4.2), measured under the same conditions, reveals very different patterns,

with glycine showing a sigmoidal-shaped curve and GABA being much more sensitive to changes in external sodium concentration. The sodium-dependence curves for high affinity L-glutamate and glycine uptake into synaptosomes (BENNETT, LOGAN & SNYDER, 1973) are exactly the same as have been obtained with slices.

MARTIN & SMITH (1972) have reported a sigmoidal sodium-dependence curve for GABA uptake into synaptosomes, although in the present results on slices, this is only apparent in tissue from immature rats. The fact that the uptake of amino acids which have excitatory effects on neurons is less sensitive to reduction in external sodium ion concentration and can function maximally at sodium ion concentrations above about 30 mM, may be relevant to the need for uptake to function efficiently during extracellular decreases in sodium ion concentration resulting from inwardly-directed sodium ion fluxes occurring during post-synaptic depolarization (BALCAR & JOHNSTON, 1972a). Postsynaptic hyperpolarization by GABA or glycine does not involve sodium ion movements (CURTIS & JOHNSTON, 1974) and uptake processes necessary for termination of inhibitory transmitter action are not required to function efficiently at low sodium ion concentrations.

The effects of lithium on the uptake of glutamate, aspartate and GABA are qualitatively similar to those obtained by PETERSON & RAGHUPATHY (1974) on uptake into synaptosomal fractions. Quantitative differences are probably explained by the use of slices instead of synaptosomes and the presence, in the medium used in the current experiments, of calcium, magnesium and potassium; PETERSON & RAGHUPATHY reported that the presence of magnesium lowered the ratio of Li/Na-supported uptake. From these

experiments it would seem that the mechanism of glutamate and aspartate uptake may not be quite the same, although their sodium-dependence curves are very similar. It would appear that lithium supports D-aspartate uptake to a similar extent as it does L-aspartate.

The uptake of D- and L-aspartate was inhibited by structurally-related amino acids bearing one amino and two carboxyl or equivalent groups. Substituents on the amino group abolished inhibition. As discussed previously (BALCAR & JOHNSTON, 1973), the high affinity uptake and postsynaptic action of aspartate (and glutamate) are different processes, since the strong excitants, N-methyl-L-aspartate and N-methyl-D-aspartate had no influence on uptake of the natural transmitters. D-aspartate appeared to be slightly more effective in inhibiting L-aspartate uptake than the L-isomer. D-aspartate was also more effective than L-aspartate in inhibiting L-glutamate uptake (BALCAR & JOHNSTON 1972a, b). D-glutamate was weaker than L-glutamate in inhibiting uptake of L-glutamate (BALCAR & JOHNSTON, 1972a, b), L-aspartate and D-aspartate. Briefly, the order of potency against uptake of L-aspartate, D-aspartate and L-glutamate is D-aspartate, L-aspartate, L-glutamate and D-glutamate, with the latter being substantially weaker than the first three. With respect to excitation by these compounds when tested by micro-electrophoresis, both L-glutamate and L-aspartate had similar potencies when tested on spinal interneurons and cortical neurons; the corresponding D-isomers were only slightly less effective, with the D-aspartate being slightly more potent than D-glutamate on cortical neurons (CRAWFORD & CURTIS, 1964; CURTIS & WATKINS, 1963): on cortical neurons, KRNJEVIĆ & PHILLIS (1963) have found D-glutamate

to be consistently less effective than the L-form, while D-aspartate is not very different in potency from L-aspartate.

D-aspartate and L-aspartate appear to be taken up into the same osmotically-sensitive subcellular particles of crude cortical homogenates. Potassium-stimulation caused the release of both isomers, although the extent of release of the L-isomer was very low, presumably because of extensive metabolism occurring during the course of the experiments.

From experiments described in this chapter it appears that D-aspartate is actively accumulated by rat CNS tissue slices, shares the same transport system as L-aspartate, is accumulated at the same subcellular sites as L-aspartate, is absolutely dependent on the presence of sodium for uptake to occur and shows the same sodium-dependence pattern as L-glutamate. It is released from tissue slices in response to potassium depolarization, provided calcium is present in the perfusing buffer. In fact, radioactive D-aspartate may prove very useful in examining the neurotransmitter role of the naturally-occurring L-aspartate, its main advantage being that it does not undergo rapid metabolism. Studies on the autoradiographic localization of radioactive D-aspartate may help in determining sites of high affinity uptake of L-glutamate and L-aspartate. Such information could be of great value in identifying those neurons using these compounds as excitatory transmitters. To date no electron-microscopic autoradiographic studies on the localization of sites of high affinity uptake of the dicarboxylic excitatory transmitters have been reported (IVERSEN & SCHON, 1973).

VIII. D-ASPARTATE OXIDASE ACTIVITY IN EXTRACTS OF
MAMMALIAN CENTRAL NERVOUS TISSUE

1. INTRODUCTION

The well known D-amino acid oxidase (D-amino-acid: oxygen oxidoreductase (deaminating), EC 1.4.3.3), a flavoprotein occurring especially in kidneys, oxidises many D-amino acids, but has no action on the dicarboxylic acids, D-aspartate and D-glutamate (DIXON & KLEPPE, 1965). These two amino acids, however, are oxidised by kidney extracts; the enzyme responsible for these oxidations is D-aspartate oxidase (D-aspartate:oxygen oxidoreductase (deaminating), EC 1.4.3.1) (DIXON & KENWORTHY, 1967).

D-amino acid oxidase activity has been described in extracts of mammalian central nervous tissue (NIEMS, ZIEVERINK & SMILACK, 1966; GOLDSTEIN, 1966). DeMARCHI & JOHNSTON (1969) have measured this activity in different regions of the mammalian CNS, particularly the spinal cord, because of its possible involvement with glycine metabolism.

Investigations on the extent of metabolism of L- and D-aspartate accumulated by tissue slices of rat brain, carried out concurrently with uptake studies on these compounds, prompted further work to see if the mammalian brain contained D-aspartate oxidase, particularly since DeMARCHI & JOHNSTON (1969) observed significant uptake of oxygen by sheep cerebellum extract in the presence of 60 mM D-aspartate. During the course of these investigations YUSKO & NIEMS (1973) presented evidence for the broad distribution of D-aspartate oxidase activity in hog brain and noted that the activity was also detected in cat brain.

2. METHODS AND MATERIALS

Enzyme assays. The activity of D-aspartate oxidase was measured by either of two assays. The first, used in initial studies with the cat and rabbit kidney enzyme, was performed

essentially by the method of DIXON (1970), using ferricyanide as acceptor and following its reduction with a recording spectrophotometer (Varian Techtron, model 635) at 420 nm. Reaction mixtures contained in a Thunberg cuvette (1 cm light path) consisted of 6.67 mM D-aspartate, 83 mM potassium phosphate buffer, pH 7.6, 16.7 μ M FAD, 0.76 mM potassium ferricyanide and 0.1 ml of enzyme extract in a final volume of 3.0 ml. Initially the ferricyanide was contained in the hollow stopper. After evacuation of the cuvette using a water pump and a 15 minute preincubation period, the reaction was initiated by inverting the cuvette and mixing the ferricyanide with the other reactants. The assay was performed at 30°C.

The second method involved the determination of the product 2-oxo-acid, oxaloacetate in the case of D-aspartate, as the 2,4-dinitrophenylhydrazone (NIEMS, DeLUCA & HELLERMAN, 1965). Assays were performed open to the air at 37°C in a shaking water bath. Reaction mixtures consisted of 76 mM sodium pyrophosphate buffer, pH 8.5, 10 μ M FAD, 40 μ g catalase and 20 mM D-aspartate (or other substrate), and up to 0.3 ml of extract in a final volume of 1.0 ml. The enzyme was pre-incubated with FAD for 15 min before initiation of the reaction by addition of substrate. Reactions were stopped with 0.2 ml of 50% trichloroacetic acid and centrifuged. Product oxo-acids were determined on 1.0 ml aliquots of the supernatants by addition of 0.1 ml of 0.2% 2,4-dinitrophenylhydrazine in 2 N HCl, incubation at 37°C for 30 min, then reading absorption at 416 nm (KATSUKI, YOSHIDA, TANEGASHIMA & TANAKA, 1971) in a micro-sample spectrophotometer, (Gilford, model 300-N) 10 min after the addition of 0.5 ml of 5 N NaOH. D-amino acid oxidase was assayed in a similar way, using 20 mM D-alanine as substrate. Substrate blanks were run as controls.

Preparation of tissue extracts. The enzymes were measured in water extracts of acetone powders. The tissue was homogenized in 10-20 volumes of acetone at approximately -10°C in a Waring blender. The homogenate was centrifuged at 1000 g for 3 min at 0°C and the supernatant decanted. The sediment was washed twice with 5 volumes of acetone. The powder was air dried while kept cold on dry ice, then desiccated in vacuo over NaOH pellets and paraffin wax shavings (McILWAIN & RODNIGHT, 1962).

Water extraction involved stirring with 50 volumes of ice-cold water for 30 min, then centrifugation to remove undissolved material. Ammonium sulphate was added to the supernatant to give the desired saturation and stirred for 20 min at 0°C before collecting the precipitate by centrifugation at 27000 g for 20 min.

Protein assays. Protein concentrations in the tissue extracts were measured by the method of LOWRY, ROSEBROUGH, FARR & RANDALL (1951).

Chemicals. Reagents were purchased from the following sources: D-aspartate (Nutritional Biochemicals); GABA, L-glutamine, L-aspartate, catalase (partially purified, 1500 units/mg), malonic acid and hog-brain acetone powder (Sigma); D- α -alanine, D-serine (Fluka); Glycine (Mann); D-asparagine and D-glutamine (Cyclo Chemicals); FAD, FMN, L- α -alanine and D-glutamate (Calbiochem); 2,4-dinitrophenylhydrazine, L-glutamate, sodium benzoate, malic acid, D-tartaric acid and sodium barbitone (BDH); and potassium ferricyanide (May & Baker). The N-substituted aspartic acid derivatives were prepared by Dr J.C. Watkins (WATKINS, 1962).

3. RESULTS

Using the ferricyanide assay, no D-aspartate oxidase activity was observed in water extracts of acetone powders of cat and rat CNS tissue, although activity was readily observable in cat and rabbit kidney extracts. With the 2,4-dinitrophenylhydrazine assay, however, activity which was linear with respect to time and amount of added protein (Fig.8.1) was found in ammonium sulphate fractions of water extracts of acetone powders prepared from cat, rat, hog and sheep brain. Using crude water or buffer homogenates of CNS tissue it was not possible to unequivocally demonstrate the existence of enzyme activity because of high background colour formed as a result of endogenous oxo-acids.

Sensitivity of the assay procedures. A cat kidney extract was assayed using both assay procedures. Under the assay conditions described in the methods, and with amounts of extract chosen to give accurate measure of initial velocities in each of the two assays, it was found that the ferricyanide assay, using $K_3Fe(CN)_6$ as electron acceptor, gave an optical density change at 420 nm of 0.030 per min per mg of protein whereas the 2,4-dinitrophenylhydrazine assay, in which molecular O_2 acted as an electron acceptor, gave an optical density change at 416 nm of 0.464 per min per mg of protein (taking 1.0 ml of the 1.2 ml assay supernatant). Presumably the sensitivity of the ferricyanide assay could have been increased by increasing the pH (7.6 to 8.5), the substrate concentration (6.67 to 20.0 mM) and the temperature ($30^\circ C$ to $37^\circ C$), but under the conditions used, the 2,4-dinitrophenylhydrazine assay was some 15 times as sensitive.

D-aspartate oxidase activity in brain extracts. Table 8.1 shows activities of D-aspartate oxidase in extracts from various brain regions of cat, hog, rat and sheep brain.

Fig. 8.1.

A. 2,4-Dinitrophenylhydrazine assay. Linearity of oxo-acid formation from D-aspartate with respect to time and amount of added protein using a 0-35% ammonium sulphate fraction of water extract cat brain acetone powder.

B. Ferricyanide assay. Linearity of ferricyanide reduction (and D-aspartate oxidation) with respect to amount of added protein using a 0-35% ammonium sulphate fraction of a water extract of cat kidney acetone powder. This assay was a continuous assay and initial velocities were determined from the slope of the spectrophotometer trace after the reaction was initiated by the addition of ferricyanide.

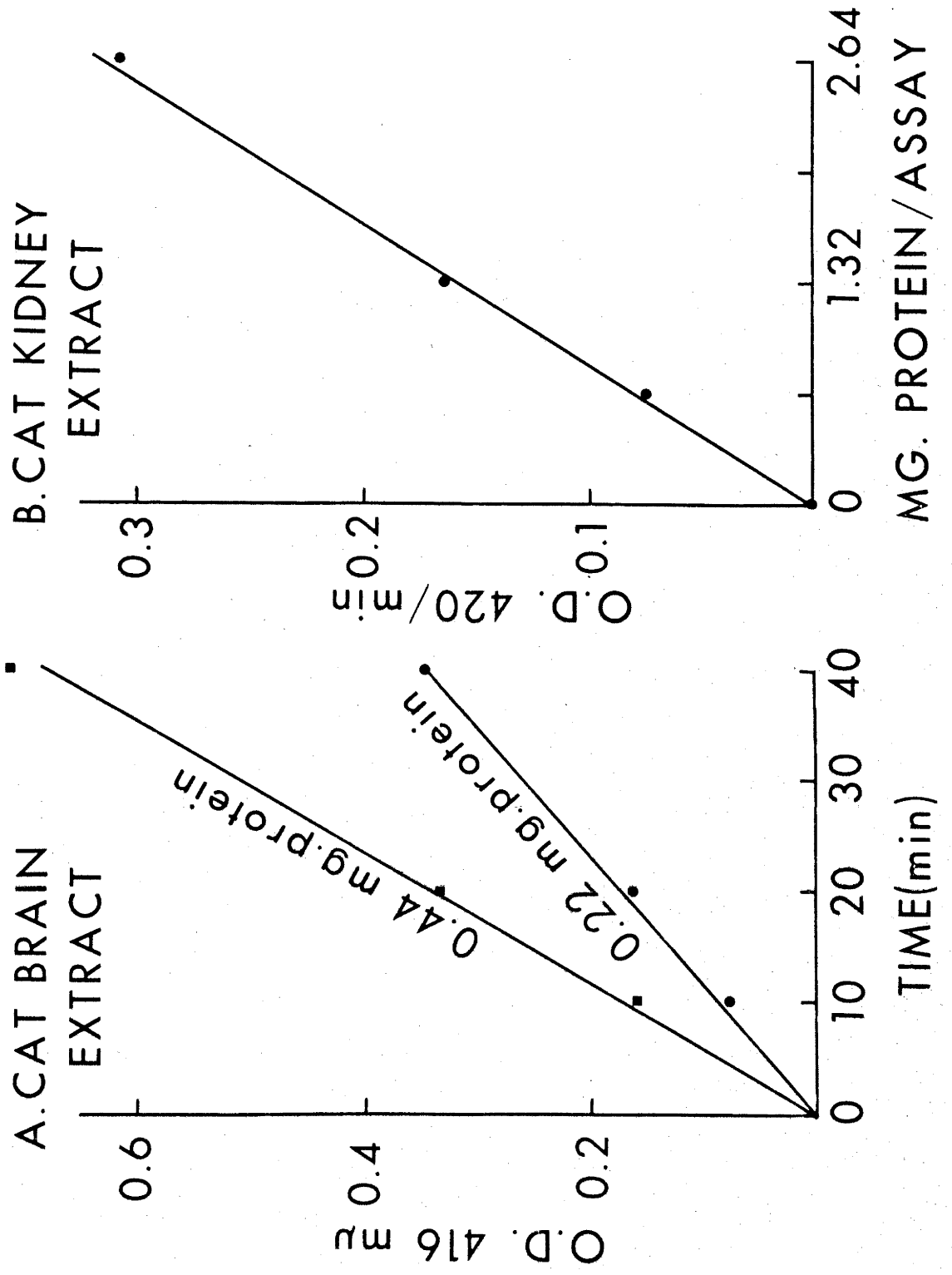


Table 8.1. Enzymic activities in CNS and kidney tissue:
2,4-dinitrophenylhydrazine assay

Tissue	Ammonium Sulphate Fraction	Enzymic Activity (nmol of oxo-acid produced/min/mg protein)	
		D-Aspartate oxidase	D-Amino Acid oxidase
Cat forebrain*	0-50%	2.89	
	0-35%	7.31	
	35-100%	< 0.09	
Cat cerebellum	0-35%	3.44	
	35-100%	0.11	
Cat spinal cord	0-35%	2.31	
Hog brain	0-35%	1.08	1.61
	35-50%	3.36	0.77
	50-65%	0.53	0.02
	65-100%	<0.03	<0.01
Rat brain	0-35%	1.67	
	35-55%	0.40	
Sheep brain stem	0-50%	2.89	
Rabbit kidney**	0-35%	410	
Cat kidney**	0-35%	270	
	35-100%	0	
Cat kidney	0-35%	115	

Each value represents a single determination of the initial rate of substrate oxidation, calculated from time-course experiments.

* Includes hemispheres, midbrain, thalamus and hypothalamus.

** These results are calculated from those obtained in the ferricyanide assay, multiplied by a factor of 15 (see text) to give values which can be compared with results obtained in the 2,4-dinitrophenylhydrazine assay.

These extracts were ammonium sulphate fractions of water extracts of acetone powders. Activities obtained from similar extracts of rabbit and cat kidney are given for comparison. It is apparent that the activity of brain D-aspartate oxidase is much lower than that of renal tissue, which accounts for the activity not being observed in CNS tissue using the ferricyanide assay.

Little reliance should be placed on the regional differences observed for the enzymic activity in cat CNS because of possible differential water extraction and ammonium sulphate precipitation of the enzyme from acetone powders. Using water extracts of different regions YUSKO & NIEMS (1973) found a relatively uniform distribution of the enzyme in hog brain, in contrast to D-amino acid oxidase activity, which is very much higher in cerebellum and pons-medulla than in other regions (DeMARCHI & JOHNSTON, 1969).

Ammonium sulphate fractionation of the hog brain acetone powder extract (Table 8.1) showed that the fraction with the highest specific activity for the oxidation of D-aspartate was different from the fraction with highest activity for the oxidation of D-alanine. This suggests that the two activities are due to two different enzymes, as shown conclusively for the kidney (STILL & SPERLING, 1950; STILL, BUELL, KNOX & GREEN, 1949; KREBS, 1935).

Substrate specificity of assayed activity. The apparent activity of D-aspartate oxidase from cat brain with various substrates is shown in Table 8.2. This experiment was performed in the presence of 2mM sodium benzoate, an inhibitor of D-amino acid oxidase, to prevent this enzyme contributing to oxo-acid oxidation products (KLEIN & KAMIN, 1941; FRISELL, LOWE & HELLERMAN, 1956). Sodium benzoate was shown

Table 8.2. Substrate specificity of D-aspartate oxidase from cat brain and kidney: 2,4-dinitrophenylhydrazine assay

Substrate	Relative Activity	
	Brain	Kidney
D-aspartate	100	100
N-Methyl-D-Aspartate	94	93
N-Ethyl-D-Aspartate	58	49
N-Propyl-D-Aspartate	31	39
N-Butyl-D-Aspartate	41	44
D-Glutamate	6	6
L-Glutamate)		
L-Aspartate)		
N-Methyl-L-Aspartate)		
D-Glutamine)		
L-Glutamine)		
D-Serine)	No significant activity was observed with these compounds	
L-Serine)		
D-Asparagine)		
L-Alanine)		
Glycine)		
GABA)		

The above compounds were tested at 20 mM final concentration in the standard assay system. Each value represents a single determination from 50 min incubations for the brain enzyme and duplicate determinations from 10 min incubations for the kidney enzyme. The extracts used were a 0-35% ammonium sulphate fraction of a water extract of cat cortex acetone powder and a 0-50% ammonium sulphate fraction of a water extract of cat kidney acetone powder. Sodium benzoate (2 mM) was present to inhibit D-amino acid oxidase activity in these extracts. For the brain extract, no significant activity was observed with D-alanine under these conditions. For the kidney extract, relative activity with D-alanine was 16% (corrected for the different colour value of its oxo-acid product). Increasing the sodium benzoate to 10 mM reduced this to 6% without affecting the other values.

to have no effect on the activity of D-aspartate oxidase using D-aspartate as substrate. All compounds were tested at 20 mM final concentration, and activities are expressed relative to D-aspartate as 100. Standard curves of colour development for the 2,4-dinitrophenylhydrazones of 2-oxoglutarate and pyruvate, the products of oxidation of D-glutamate and D-alanine respectively, were determined (Fig.8.2) to check that observed lack of activity was not due to lack of colour development in the standard oxo-acid assay. It is apparent that D-aspartate and its N-substituted derivatives are the only amino acids tested which are oxidised to a significant extent by this enzyme, with activity generally decreasing with increasing size of the N-substituent. The cat kidney D-aspartate oxidase activity, assayed by the 2,4-dinitrophenylhydrazine method (Table 8.2), shows a very similar substrate specificity.

The substrate specificity of the cat kidney D-aspartate oxidase assayed by the ferricyanide method is given (Table 8.3). Benzoate is not needed to inhibit D-amino acid oxidase in this assay because D-amino acid oxidase does not use ferricyanide as an electron acceptor (DIXON & KLEPPE, 1965). The values obtained for the relative rates for different substrates are not directly comparable with those values obtained in the 2,4-dinitrophenylhydrazine assay (Table 8.2) due to differences in substrate concentration, pH, acceptor and temperature in the two assays. The obvious difference for D-glutamate can be explained by the fact that the value of K_m for this substrate depends very much on the acceptor used (DIXON & KENWORTHY, 1967). From the kinetic data of DIXON & KENWORTHY (1967) the rate of oxidation of D-glutamate (relative to D-aspartate as 100) using ferricyanide as acceptor can be calculated as 20.6 at

Fig. 8.2. Colour development of oxo-acids in the standard 2,4-dinitrophenylhydrazine assay described in methods. Linearity of colour formation with amount of added oxo-acid is shown for oxaloacetate, 2-oxoglutarate and pyruvate, the oxidative deamination products of D-aspartate, D-glutamate and D-alanine respectively.

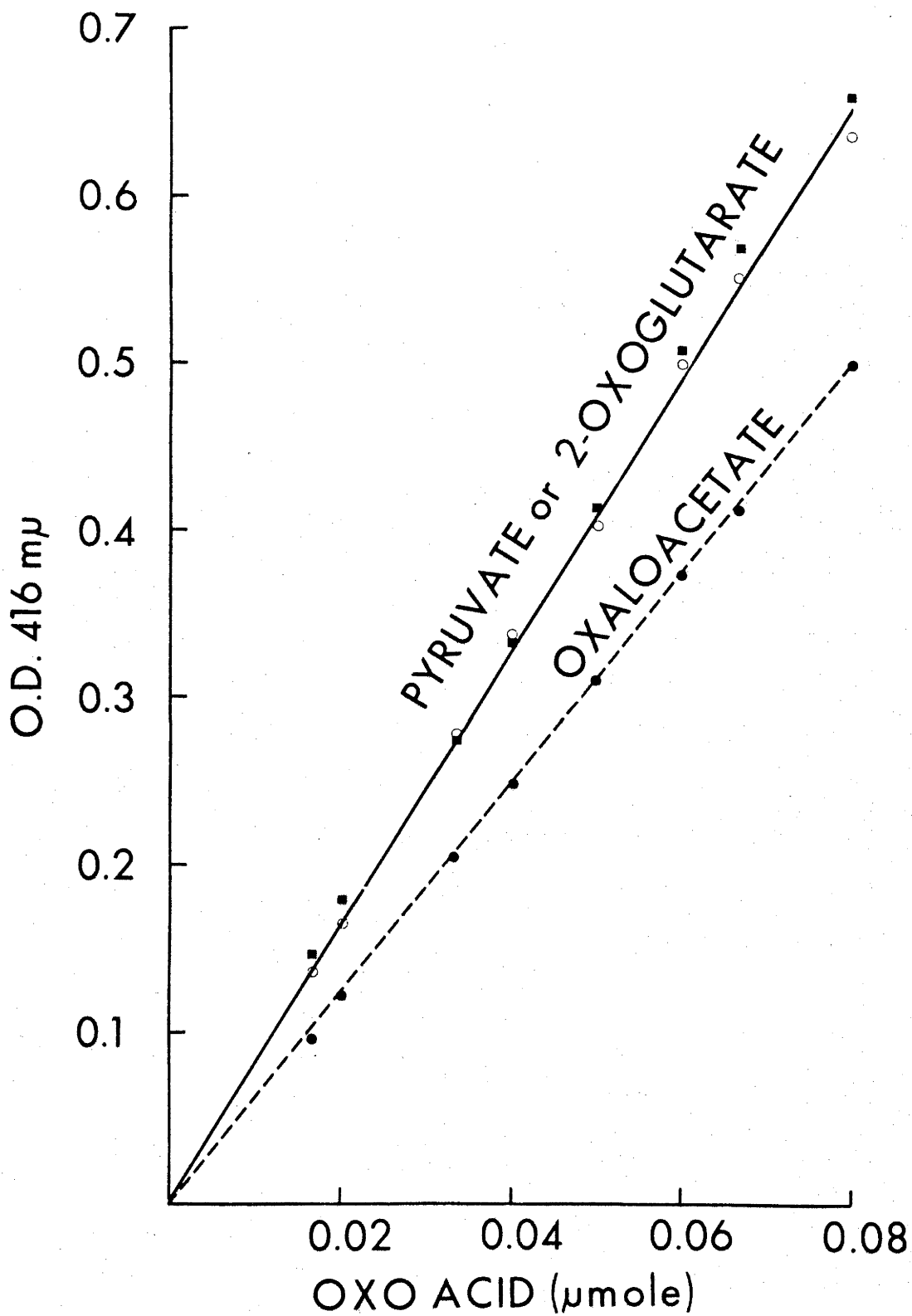


Table 8.3. Substrate specificity of D-aspartate oxidase from cat kidney: ferricyanide assay

Substrate	Relative Activity
D-Aspartate	100
D-Glutamate	42
N-Methyl-D-Aspartate	38
N-Propyl-D-Aspartate	6
D-Serine	2
D-Alanine	2
L-Alanine)	No significant activity was observed with these compounds
L-Serine)	
Glycine)	
GABA)	
L-Glutamine)	
D-Glutamine)	
D-Asparagine)	
L-Aspartate)	
L-Glutamate)	

The above compounds were tested at 6.7 mM in the standard ferricyanide assay. The enzyme was a 0-35% ammonium sulphate fraction of an acetone powder extract of cat kidney. Each value represents the mean of duplicate determinations of the initial velocity of ferricyanide reduction.

6.67 mM substrates and 40.5 at 20 mM substrates, whereas the rate with O_2 as acceptor is 8.4 at 6.67 mM substrate and 11.2 at 20 mM substrates, i.e. there is much less oxidation of D-glutamate with O_2 as acceptor than with ferricyanide as acceptor. Using a cat kidney extract, activity was measured by the ferricyanide assay at 6 concentrations of D-aspartate in the range of 6.7-50 mM, and for 6 concentrations of D-glutamate in the range 8.3-83 mM. At pH 7.6 and $30^\circ C$, approximate K_m and V_{max} values for D-aspartate were 35 mM and 0.41 O.D.₄₂₀/min/mg respectively, whereas for D-glutamate the corresponding values were 231 mM and 0.67 O.D.₄₂₀/min/mg. Values for D-glutamate are very approximate because the range of substrate concentrations used was well below the actual K_m , but results are in general, comparable with those of DIXON & KENWORTHY (1967) in that with ferricyanide as acceptor the K_m for D-glutamate is much higher than for D-aspartate and under saturating conditions for both substrates, D-glutamate is oxidized faster than D-aspartate.

Cofactor requirement. D-aspartate oxidase from cat cortex and cat cerebellum exhibited less than 20% of maximal activity when FAD was not included in the assay. Addition of FMN did not restore activity (Table 8.4).

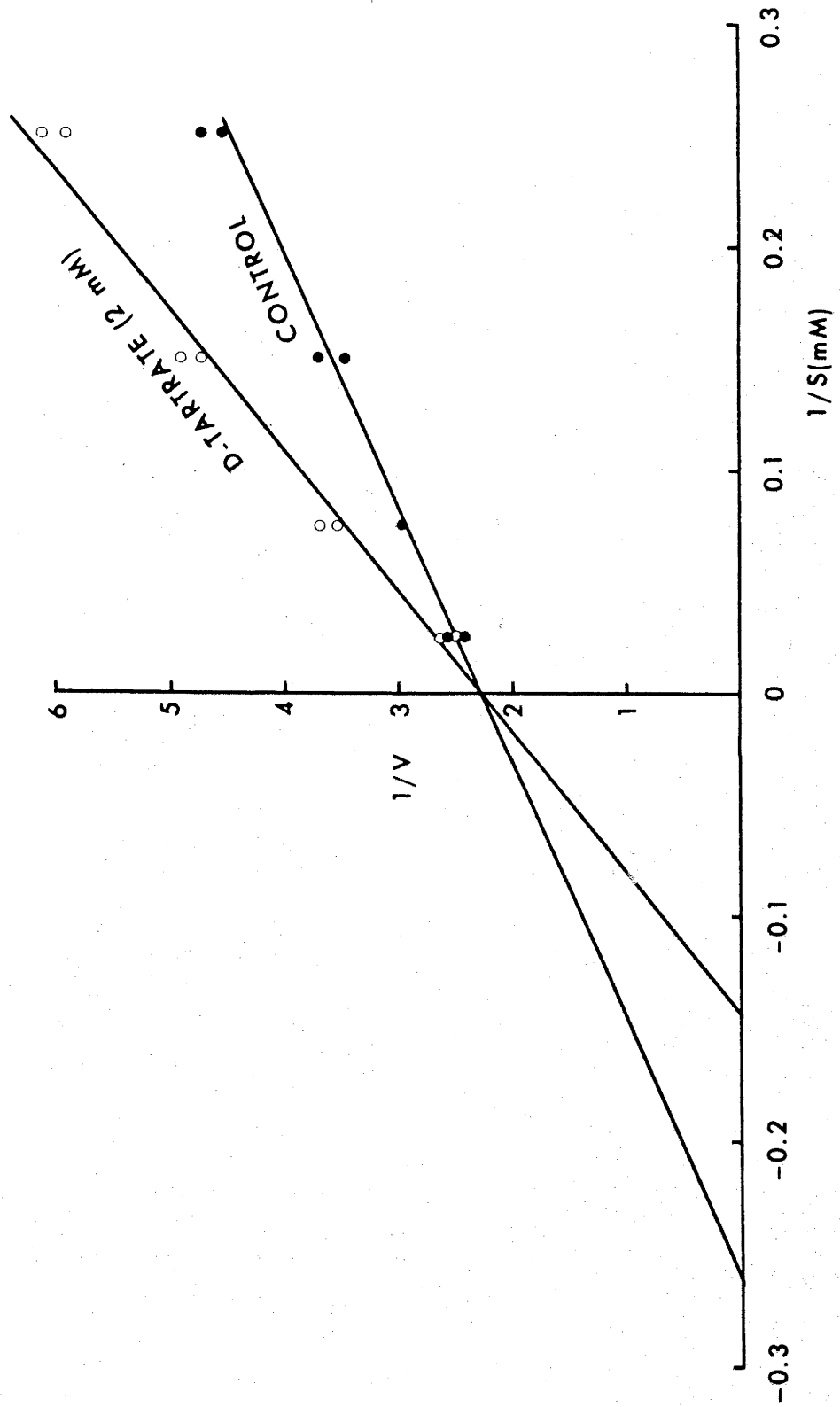
Inhibitors of D-aspartate oxidase activity. Sodium benzoate, a relatively potent inhibitor of D-amino acid oxidase activity from extracts of human cerebellum and hog kidney (NIEMS, ZIEVERINK & SMILACK, 1966) was without effect on cat brain D-aspartate oxidase activity (Table 8.4). Sodium barbitone at 10 mM final concentration also failed to inhibit D-aspartate oxidase activity; GOLDSTEIN (1966) reported that sodium pentobarbitone was a weak competitive inhibitor of sheep cerebellum D-amino acid oxidase activity. D-tartrate (2mM) (Fig.8.3) competitively inhibited sheep brain-stem

Table 8.4. Inhibitors and cofactor requirements of
cat brain D-aspartate oxidase:
2,4-dinitrophenylhydrazine assay

Assay Conditions	Oxidase Activity	
	Cortex	Cerebellum
Complete	100	100
-FAD	11	21
-FAD, +FMN (10 μ M)	24	30
-FAD, +FMN (25 μ M)	26	-
Sodium Benzoate (1 mM)	99	95
Sodium D-Malate (10 mM)	79	73
Sodium Barbitone (10 mM)	98	-

Activities are expressed as percentage of the activity observed with the complete assay system. Each value represents the mean of two determinations from 45 min incubations. The extracts used were 0-35% ammonium sulphate fractions of water extracts of cat brain acetone powders.

Fig. 8.3. Lineweaver-Burk plots of the activity of D-aspartate oxidase with concentrations of D-aspartate in the range 4-40 mM, in the presence and absence of 2 mM D-tartrate. Each point represents a single determination and results were fitted to the equation for linear competitive inhibition by the method of CLELAND (1963) to yield mean values \pm S.E.M. of the apparent kinetic parameters. The extract was a 0-50% ammonium sulphate fraction of a water extract of sheep brain stem acetone powder. Units of v are optical density change at 416 nm/45 min/0.2 ml of extract. The K_m for D-aspartate was calculated as 3.8 ± 0.2 mM and V_{max} as 2.77 ± 0.07 nmol/min/mg protein. K_{is} for D-tartrate was 2.43 ± 0.31 mM.



D-aspartate oxidase activity when tested against varying concentrations of D-aspartate in the standard, 2,4-dinitrophenylhydrazine assay. D-tartrate, malonate and DL-malate, when tested at 20 mM final concentration in the standard assay against the hog brain enzyme gave inhibitions in the range 66-83%. The kidney enzyme was quite strongly inhibited by dicarboxylic acids (DIXON & KENWORTHY, 1967) as was the hog brain enzyme by sodium mesotartrate (YUSKO & NIEMS, 1973).

Kinetics of D-aspartate oxidase activity. Using ammonium sulphate fractions of brain and kidney extracts, initial velocity of oxo-acid formation was measured for a range of concentrations of D-aspartate from 2 - 40 mM (Table 8.5, Fig. 8.4). When assayed under identical conditions, the K_m 's for D-aspartate are very similar for the kidney and brain enzymic activities. The V_{max} parameters, although not directly comparable because of possible differential water extraction and to the fact that different ammonium sulphate cuts were used, show that kidney has much more maximum activity than brain, although the affinity for D-aspartate, as evidenced by the K_m value, is similar. This is consistent with the same or at least a very similar enzyme being present in brain and kidney. An attempt was made to measure velocities for a range of D-glutamate concentrations from 10 to 100 mM, but activities were too low to give accurately measurable colour. At 20 mM D-glutamate both the hog brain and cat kidney enzyme had approximately 6% of the activity observed with 20 mM D-aspartate, while at near saturating concentrations of both substrates the activity with D-glutamate was of the order of 10% of the activity with D-aspartate.

Table 8.5. Apparent kinetic constants of D-aspartate oxidase from brain and kidney acetone powder extracts: 2,4-dinitrophenylhydrazine assay

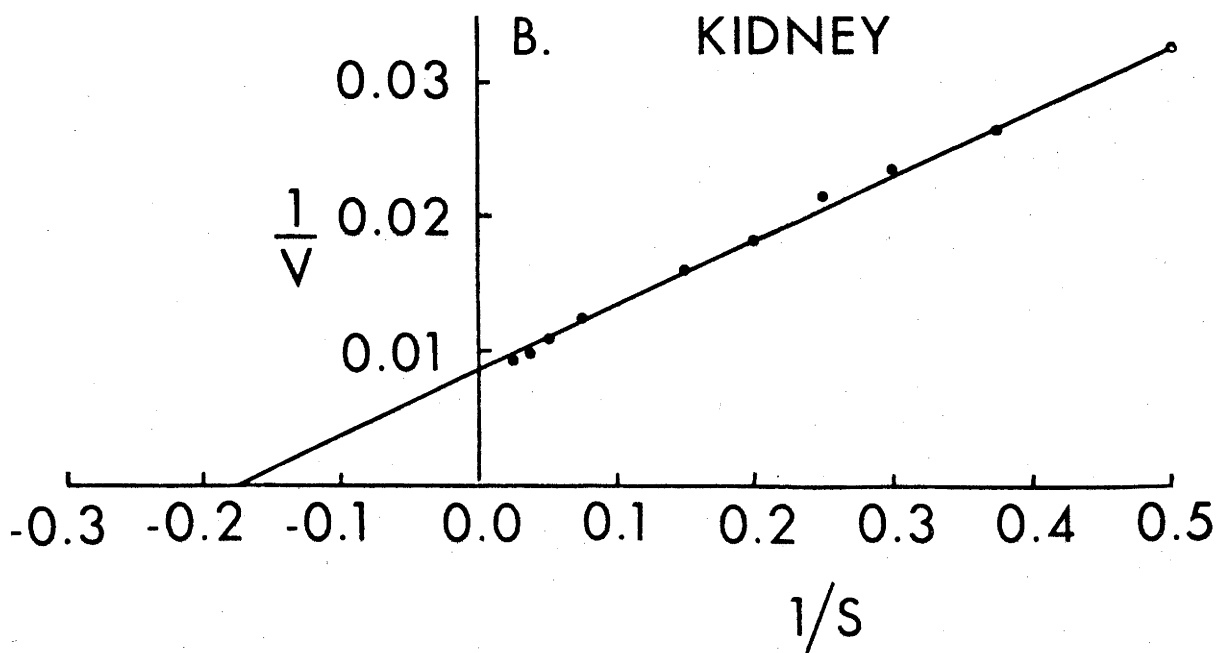
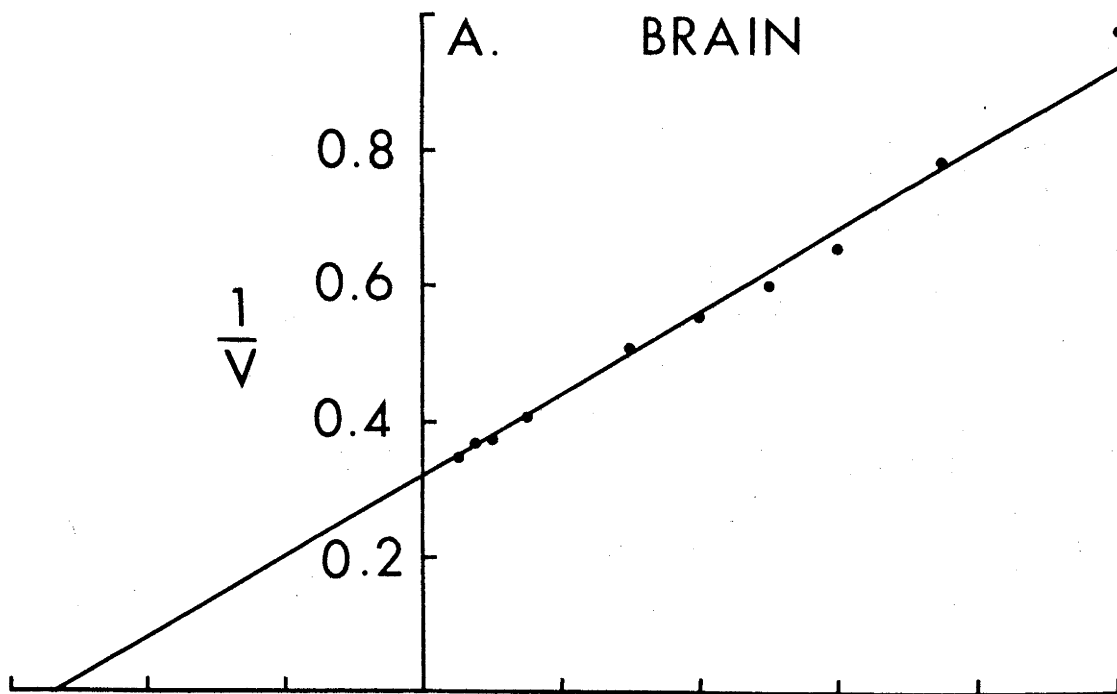
Tissue	Ammonium Sulphate Fraction	K_m (mM)	V_{max} (nmol/min/mg protein)
Sheep brain stem	0-50%	3.8 ± 0.2	2.77 ± 0.07
Cat brain	0-50%	5.8 ± 0.2	3.74 ± 0.05
Hog brain	35-50%	3.7 ± 0.1	3.18 ± 0.04
Cat kidney	0-50%	6.5 ± 0.3	124.0 ± 1.9

Initial velocities of D-aspartate oxidation by the acetone powder extracts were determined for a range of D-aspartate concentrations from 2-40 mM. The results were analysed by the method of CLELAND (1963) to yield mean values \pm S.E.M. of the apparent kinetic parameters. V_{max} values are not directly comparable due to possible differential water extraction of acetone powders and different ammonium sulphate cuts.

Fig. 8.4. Lineweaver-Burk plots of the activity of D-aspartate oxidase with concentrations of D-aspartate in the range 2-40 mM. Each point represents the mean of duplicate determinations and results were analysed by the method of CLELAND (1963) to yield mean values \pm S.E.M. of the apparent kinetic parameters (see Table 8.5).

A: The extract was a 35-50% ammonium sulphate fraction of a water extract of hog brain acetone powder.

B: The extract was a 0-50% ammonium sulphate fraction of a water extract of cat kidney acetone powder.



4. DISCUSSION

An enzymic activity capable of oxidizing D-aspartate has been demonstrated in water extracts of acetone powders prepared from cat forebrain, cerebellum and spinal cord, rat brain, hog brain and sheep brain stem. Activities obtained for brain D-aspartate oxidase activity were in the range 1-7 nmole/min/mg protein for the partially purified extracts used, a figure which is reasonably similar to the activities observed by YUSKO & NIEMS (1973) who obtained values in the range 11-200 nmole/min/g wet weight of tissue for water homogenates. Like D-amino acid oxidase, the D-aspartate oxidase activity of brain is much less than that in kidney. In agreement with YUSKO & NIEMS (1973) this enzyme appears to be broadly distributed within the CNS and appears to be a separate enzyme from D-amino acid oxidase.

The results of studies involving the specificity of the enzyme towards substrates, inhibitors and activating cofactors suggest that the brain enzyme is the same, or at least very similar to the D-aspartate oxidase (D-aspartate: oxygen oxidoreductase (deaminating), EC 1.4.3.1) of kidney which was first studied by STILL, BUELL, KNOX & GREEN (1949). Crude extracts (ammonium sulphate fractions of water extracts of acetone powders) displayed little activity without added FAD; FMN could not replace FAD. Those amino acids susceptible to oxidation by D-amino acid oxidase were not oxidised by the D-aspartate oxidase. As previously observed for the kidney D-aspartate oxidase activity, dicarboxylic acids competitively inhibited this enzymic activity in brain extracts, while sodium benzoate and sodium barbitone, inhibitors of D-amino acid oxidase, were without effect. With oxygen as electron acceptor, the enzyme oxidized D-aspartate much more rapidly than D-glutamate,

and displayed quite high activities with N-substituted derivatives of D-aspartate as substrates.

The enzymic activity observed with N-substituted derivatives of D-aspartate is consistent with the mechanism of enzyme-substrate interaction proposed by DIXON & KENWORTHY (1967). The active centre of D-aspartate oxidase is postulated to contain groups which combine with the two carboxyl groups of the substrate (or dicarboxylic acid inhibitor), giving two links which hold the substrate molecule to the enzyme. When the D-dicarboxylic amino acid substrate is in position on the active site the amino group is directed away from the enzyme surface, whereas in the L-configuration, the inwardly directed amino group is sufficient to overcome the attraction of the two carboxyl groups for the enzyme. It appears that a substituent on an amino group in the D-configuration does not drastically affect enzyme substrate binding and oxidative deamination. Further, the competitive inhibition of the enzyme by the dicarboxylic acids D-tartrate, malonate and malate is consistent with the ability of these dicarboxylic acids to compete with D-aspartate for the active centre.

The regional distribution of the D-aspartate oxidase activity within the CNS differed from that of the D-amino acid oxidase. An attempt to measure the subcellular distribution of the enzyme in the rat CNS was unsuccessful because of the low activity of the enzyme. Measurements of subcellular distribution in kidney may prove easier, because of the greater activity in this organ. The cellular distribution of the enzyme in brain and kidney tissue also remains unknown; in fact, the function of enzymes in these tissues which oxidise the D-isomers of many amino acids remains enigmatic.

IX. D-ASPARTATE IN THE CENTRAL NERVOUS SYSTEM:
PRESENCE OR ABSENCE?

1. INTRODUCTION

In the previous two chapters a number of observations have been made about the D-isomers of the dicarboxylic acids, aspartate and glutamate.

1. D-aspartate and D-glutamate excite cat spinal neurons when administered microelectrophoretically, being only slightly less potent than the corresponding L-isomers (CURTIS & WATKINS, 1960).
2. D-aspartate is a good inhibitor of [³H]L-aspartate and [³H]L-glutamate high affinity uptake into cat spinal cord slices (BALCAR & JOHNSTON, 1973) and of [³H]L-glutamate high affinity uptake into rat cerebral cortex slices (BALCAR & JOHNSTON, 1972a, 1972b). In fact, D-aspartate is a more powerful inhibitor of [³H]L-glutamate uptake than either D- or L-glutamate.
3. In rat brain, a similar high affinity uptake system to that described for L-glutamate (BALCAR & JOHNSTON, 1972a) exists for D-aspartate (Section VII). Much of this uptake appears to take place into osmotically-sensitive particles which band in the same position in sucrose density gradients as those particles which accumulate L-aspartate. D-aspartate is a more potent inhibitor of [³H]L-aspartate uptake than is the L-isomer.
4. D-aspartate oxidase, an enzyme specific for the oxidation of D-dicarboxylic acids and more active on D-aspartate than D-glutamate (at least under in vitro conditions), appears to be broadly distributed within the mammalian CNS (Section VIII).

Furthermore, it was noted that there is a discrepancy in two studies reporting the level of aspartate in six regions of the feline spinal cord. Using the ninhydrin colour reaction, JOHNSTON (1968) obtained values which, in five of the six regions, were between 44% and 96% higher than those obtained using a fluorometric enzymic method specific for the L-isomer (GRAHAM, SHANK, WERMAN & APRISON, 1967). A comparison of other amino acids measured, including glutamate, glutamine and glycine showed either no significant difference or ninhydrin values slightly lower than enzymic method values. In view of the foregoing observations, an investigation was undertaken to see whether the CNS contained any D-aspartate.

For many years it was believed that only L-amino acids occurred in nature and thus the D-isomers were categorized as 'unnatural isomers'. The occurrence of D-amino acids in a variety of microorganisms in both the free state and in peptide linkage has now been demonstrated (see MEISTER, 1965). Of a number of invertebrate species shown to contain D-amino acids, the earthworm Lumbricus terrestris has been shown to contain free D-serine, the milkweed bug Oncopeltus fasciatus free D-alanine, while the luciferin of fireflies contains a D-cysteine moiety (MEISTER, 1965). There is no conclusive evidence, however, for the occurrence of D-amino acids in mammals. It has been claimed that tumour proteins contained D-amino acids (KOGEL & ERXLEBEN, 1939), but these results have not been verified. KUHN (1958) isolated small amounts of D-leucine from horse hair protein hydrolysates; however this was probably due to racemization occurring during hydrolysis (CORRIGAN, 1969).

A large amount of work has been devoted to the resolution and analysis of the optical isomers of amino acids. These methods have been reviewed by GREENSTEIN (1961). His extensive review covered selective crystallization of free amino acids or salts of amino acids and derivatives, biological methods utilizing optically-specific enzymes for free or appropriately substituted amino acids and paper or column chromatographic approaches. Recently, much work has developed methods suitable for automated column chromatography, either by synthesis of suitable diastereoisomeric derivatives of amino acids, or by synthesis of optically-active stationary phases. L-leucyl dipeptides of 21 racemic amino acids have been synthesized and the L-L and L-D dipeptides clearly separated by ion exchange chromatography (MANNING & MOORE, 1968). Gas-liquid chromatography has been used to successfully separate diastereoisomeric derivatives of seven racemic amino acids (POLLOCH & KAWAUCHI, 1968). The free amino acids, isoleucine, proline and allothreonine have been separated into isomers on an asymmetric complex-forming polystyrene resin (ROGOZKIN, DAVANKOV, YAMSKOV & KABANOV, 1972).

The first approach made in determining if D-aspartate was present in CNS extracts was to use specific enzymes. An experiment, using the standard ferricyanide assay for D-aspartate oxidase (Section VIII), was performed to see whether an acid-soluble CNS extract could support reduction of ferricyanide in the presence of kidney D-aspartate oxidase. However, the K_m of the kidney extract oxidase activity was too high for this approach to be sufficiently sensitive.

By using enzymes specific for one isomer it was thought that a possible approach would be to remove L-aspartate

from a brain extract by exhaustive digestion with the enzyme, then analyse the reaction supernatant by an amino acid analyser to see if the ninhydrin-positive aspartate peak was completely removed. To this end the enzymes L-aspartate β -decarboxylase (L-aspartate 4-carboxy-lyase) and L-amino acid oxidase were used. L-aspartate β -decarboxylase has been used previously to prepare [^{14}C]D-aspartate from [^{14}C]DL-aspartate (MARGOLIS & LAJTHA, 1968; see Section VII.2), while procedures describing the use of L-amino acid oxidase for small scale preparation of D-isomers of a number of amino acids have been well documented (PARIKH, GREENSTEIN, WINITZ & BIRNBAUM, 1958).

Because of difficulties experienced with this approach, L-leucyl dipeptides of amino acids in CNS extracts were synthesised and subjected to column chromatography on an amino acid analyser. This involved synthesis of L-leucine-N-carboxyanhydride and its subsequent reaction with CNS amino acid extracts under carefully controlled conditions (MANNING & MOORE, 1968). The use of thin-layer chromatography for separation of diastereoisomeric peptides was investigated.

2. METHODS AND MATERIALS

Incubation of a synthetic amino acid mixture and cat brain and spinal cord extracts with a *Clostridium welchii* extract.

Reaction mixtures, in a final volume of 2.6 ml, consisted of 0.5 ml of 0.2 M sodium acetate buffer, pH 4.9, 0.6 ml of 100 mM sodium pyruvate in sodium acetate buffer, 0.05 ml of [^3H]L-aspartate (0.1 μCi), 75 mg of *Cl.welchii* acetone powder (containing L-aspartate β -decarboxylase) and 1.4 ml of one of the following amino acid extracts: (1) a synthetic mixture of 14 naturally-occurring amino acids (0.93 μmol of each); (2) neutralized perchloric acid extract of

cat cortex; (3) neutralized perchloric extract of cat cord. Reaction mixtures, contained in Kontex flasks (25 ml), were stoppered and incubated for $10\frac{1}{2}$ h at 37°C in a shaking water bath. Enzyme blanks (complete reaction mix minus enzyme extract) for each extract and a substrate blank (to check on amino acid contamination from the acetone powder) were run concurrently. [^3H]L-aspartic acid was included to follow the extent of decarboxylation to L-alanine. Pyruvate was added to reaction mixtures as the presence of α -oxo-acids has been shown to stimulate L-aspartate β -decarboxylase (MEISTER, SOBER & TICE, 1951).

Reactions were stopped by the addition of 6.1 ml of absolute ethanol (to give a final ethanol concentration of 70%) and centrifuged to remove protein. Samples were evaporated to dryness in vacuo (temperature $< 37^{\circ}\text{C}$) and resuspended in 4 ml of 0.02 N hydrochloric acid. To check on the extent of reaction 1 ml of each sample was brought to pH 7-8 and loaded onto small columns of Dowex 1 x 8 (acetate form; 200-400 mesh; 5 ml of resin contained in glass columns of 7 mm I.D.). Columns were eluted with water (40 ml) and 0.02 N acetic acid (10 ml), then with 2N acetic acid (40 ml). L- α -alanine came through in the water wash while L-aspartate was eluted with the 2 N acetic acid. The remainder of the samples from the enzymic digestion were used for amino acid analysis.

Extraction of L-aspartate decarboxylase activity from Cl. welchii acetone powder. Previous work on the preparation of radioactive D-aspartate from [^{14}C]DL-aspartate (Section VII.2) showed that brief stirring of the acetone powder with 0.2 M sodium acetate buffer, pH 4.9 failed to extract L-aspartate β -decarboxylase activity. For this reason the acetone powder was weighed out and added directly to the incubation mixtures. To eliminate the amino acid contamination

present in these crude powders, attempts were made to purify the decarboxylase activity. 350 mg of acetone powder was suspended in 7 ml of 0.17 M sodium chloride and dialysed against 0.17 M sodium chloride and 1 mM EDTA in the cold (4°C). Dialysis with stirring was continued against several changes of the salt solution for approximately 20 h, then against sodium acetate buffer, pH 4.9, for a further 4 h. The suspension was then centrifuged and the supernatant incubated with a synthetic amino acid mixture and with extracts of cat cortex and cord for 21¹/₂ h. Reaction mixtures were the same as described above, with the exception that the amount of pyruvate added per incubation flask was reduced from 60 µmol to 5 µmol. Reactions were stopped and processed as described above.

Incubation of a synthetic amino acid mixture with L-amino acid oxidase from rattlesnake (*Crotalus adamanteus*) venom.

The procedure used was a modification of the method described by PARIKH, GREENSTEIN, WINITZ & BIRNBAUM (1958). Crude *Crotalus adamanteus* venom extract (Sigma Type I) was dissolved in 0.2 M potassium phosphate buffer, pH 7.2 and dialysed for 7-8 h at 4°C in water then potassium phosphate buffer. Catalase was also dialysed under the same conditions. Extensive dialysis was carried out to remove free amino acids contained in these extracts. To each of three Kontes flasks (25 ml), was added 1.5 ml of 0.2 M potassium phosphate buffer, pH 7.2 and 0.1 ml of catalase (1 mg/ml). To one flask was added 1.0 ml of L-amino acid oxidase (5 mg/ml) and 2 ml of a synthetic mixture containing 2 µmol each of 18 amino acids (see Table 9.2). To the second was added 2 ml of the amino acid mixture and 1.0 ml of buffer (i.e. no L-amino acid oxidase), while the third received 1.0 ml of L-amino acid oxidase extract and 2 ml of water

(i.e. no amino acids). Reaction bottles were gassed with oxygen, stoppered and incubated at 37°C in a shaking water bath for 20 h. Reactions were stopped with 10.4 ml of absolute ethanol (to give a final concentration of 70% ethanol) and left for 1 h. Protein was sedimented by centrifugation (12,000 x g for 10 min), the supernatants concentrated to dryness in vacuo and resuspended in 5 ml of water.

2.5 ml aliquots were acidified and loaded onto small columns of Dowex 50W x 4 (200-400 mesh; 11 mm I.D., 2.5 cm high) (LAZARUS, 1973). Columns were washed with 45 ml of 0.01 N hydrochloric acid, 5 ml of water and eluted with approximately 30 ml of 2N ammonia. These eluates were evaporated to dryness several times to remove ammonia, resuspended in 2 ml of 0.01 N hydrochloric acid and used for amino acid analysis.

Synthesis of L-leucine-N-carboxyanhydride. L-leucine-N-carboxyanhydride (L-4-isobutyloxazolidine 2:5-dione) was prepared by a modification of a published procedure (COLEMAN, 1950). L-leucine (11.6 g) was suspended in dry dioxan (200 ml; purified by filtration through 100 g aluminium oxide and freshly distilled from sodium shavings) at 40°C. With vigorous stirring, a solution of phosgene (12.5% in toluene, 100 ml) was added dropwise over a 2 h period. Then a stream of air was passed rapidly through the reaction mixture (kept at 40°C) for 16 h to remove excess phosgene. All exhaust gases from the reaction mixture were passed through five bubblers of 20% sodium hydroxide. The mixture was filtered and the filtrate concentrated in vacuo. The product crystallized almost immediately and was recrystallized from ether-petroleum ether to give small white flakes.

The reaction yielded 5.3 g of the product which was identified as being the required material on the basis of its solubility in petroleum ether, insolubility in ether, its reaction with water to give L-leucine and its ability to react with amino acids (as evidenced by thin-layer chromatography).

Preparation of acidic amino acid extracts of CNS tissue. Rat cord, rat brain, cat cord and cat brain tissue samples were homogenized with 10 volumes of 0.6 N perchloric acid. After standing 30 min on ice, the extracts were centrifuged (17,000 x g for 10 min), supernatants decanted and the precipitates washed with 10 ml of 0.6 N perchloric acid. Following recentrifugation, supernatants and washings were combined and neutralized with potassium hydroxide. Insoluble potassium perchlorate was removed by centrifugation, the supernatants evaporated to dryness in vacuo (temperature < 37°C), and resuspended in 6 ml of water. After a recentrifugation to remove remaining potassium perchlorate, 3 ml of each sample (pH 7-8) was loaded onto Dowex 1 x 8 columns (acetate form; 200-400 mesh; 5 mm I.D., 3.5 ml of resin) and washed with 37 ml of water. Acidic amino acids were eluted with 30 ml of 2 N acetic acid. The acid eluates were evaporated to dryness in vacuo, suspended in a small volume of water and re-evaporated to dryness, then suspended in 4 ml of sodium borate buffer (pH 10.2 at 25°C). 2 ml of these samples were used for derivitization, while the rest was used for thin-layer chromatography, to check the presence of acidic amino acids in the CNS extracts.

Reaction of L-leucine-N-carboxyanhydride with optical isomers of amino acids and with CNS tissue amino acid extracts.

The procedure used was that described by MANNING & MOORE (1968). 2 ml of 10 mM L-aspartate, D-aspartate, L-glutamate and D-glutamate in borate buffer (13.9 g of boric acid to

480 ml with freshly boiled distilled water, pH adjusted to 10.2 at room temperature with 5 N sodium hydroxide and made up to 500 ml) were added to separate graduated glass centrifuge tubes. Tubes containing 2 ml of extract of cat cortex, cat cord, rat brain and rat cord in borate buffer were also prepared. Amounts of L-leucine-N-carboxyanhydride, chosen to give approximately 5-fold molar excess over amino acid (assuming 20 $\mu\text{mol/g}$ wet weight of acidic amino acids in tissue extracts) were weighed out in small glass vials. The coupling reactions were performed in the cold room (4°C) and tubes were cooled on ice before derivitization. The tubes were vigorously agitated on a vortex mixer at maximum setting and the L-leucine-N-carboxyanhydride quickly added to the swirling solution. The agitation was continued for two minutes, briefly removing the tubes from the mixer approximately every 30 sec to ensure complete solution of the N-carboxyanhydride. The reaction was terminated by adding 0.8 ml of 1 N hydrochloric acid to bring the pH to 1-3. At this stage the peptide solution was filtered to remove slight cloudiness and stored in the freezer until chromatographic analysis was carried out.

Thin-layer chromatography of peptide solutions. Derivatized solutions were spotted and run on the following plates: (1) pre-coated silica-gel plates (Merck), 5 x 20 or 20 x 20 cm plates, 0.25 mm thick; (2) microcrystalline cellulose (Avicel). 100 g of Avicel were suspended in 430 ml of water with a Waring blender and spread on 20 x 20 cm glass plates, 0.25 mm thick. Plates were allowed to dry for 24 h before use.

The following solvent systems were used:

- (a) absolute ethanol:water (60:40);
- (b) n-propanol:25% ammonia (67:33);
- (c) butanol:acetic acid:water (4:1:1).

L-leucine, L-aspartate and L-glutamate were run with samples of the dipeptides. Plates were developed with ninhydrin spray (0.2% w/v in acetone).

Column chromatography of peptide solutions. Dipeptide derivatives were separated by Mr L.B. James on a Technicon Auto-Analyzer modified for amino acid analysis (JAMES, 1972). A 23 cm glass column (0.9 cm I.D.) containing Beckman PA-35 resin (height of bed = 14 cm) was used. Eluting buffer was 0.2 M sodium citrate, pH 3.25.

Chemicals. [U-³H]L-aspartic acid was purchased from New England Nuclear, Boston. L-glutamate decarboxylase (Type III, crude acetone powder from Clostridium welchii) and L-amino acid oxidase (Type I, crude Crotalus adamanteus rattlesnake venom extract) were purchased from Sigma. Catalase was from Nutritional Biochemicals. Other chemicals were obtained from the following sources: L-leucine, GABA and L-methionine (Calbiochem); L-serine, β -alanine and DL-threonine (Fluka); aspartic acid, L- α -alanine, L-proline (Sigma); glutamic acid, taurine, L-valine and phosgene (B.D.H.); asparagine (Cyclo); glycine (Mann); L-phenylalanine (Koch-Light) and L-lysine (Merck).

3. RESULTS

Incubation of cat brain and spinal cord extracts with Cl. welchii acetone powder (containing L-aspartate β -decarboxylase). Incubation of amino acids with Cl. welchii acetone powder resulted in almost complete destruction of [³H]L-aspartate (Table 9.1). However, amino acid analysis of the substrate blank incubation (complete incubation mix minus added amino acids) revealed the presence of extensive amino acid contamination from the acetone powder. The chromatogram of acidic and neutral amino acid analysis

Table 9.1. Incubation of amino acid mixtures with crude acetone powder from Clostridium welchii

Sample	Acetone powder	CPM in 2 N acetic acid wash	% of CPM left in [³ H]L-asp.
Synthetic amino acid mix (1)	+	261	2.5
	-	8805	
Synthetic amino acid mix (2)	+	1583	17.7
	-	8759	
Cat cortex extract	+	401	4.2
	-	8489	
Cat cord extract	+	425	4.2
	-	9096	
Substrate 'blank'	+		3.9

A synthetic amino acid mixture, or cat CNS extracts were incubated with acetone powder from Clostridium welchii (containing L-aspartate β -decarboxylase activity) under conditions described in 'Methods and Materials' (Section VII.2). [2-³H]L-aspartate was added to follow the breakdown of aspartic acid. '+' and '-' indicate incubation of the amino acids in the presence or absence of the acetone powder, respectively. Reaction supernatants were applied to columns of Dowex 1 x 8 (acetate form; 200-400 mesh). Radioactive L- α -alanine, the breakdown product of aspartate decarboxylation, came through in the water wash and substrate L-aspartate in the 2 N acetic acid wash. The Substrate 'blank' had [³H]L-aspartate but no other added amino acids.

showed 22 major and eight to nine minor ninhydrin positive peaks. In the amino acid digestion experiments the L-alanine peaks were greatly increased, while glutamate and aspartate peaks were reduced in comparison to enzyme-blank controls. However, interpretation of analyses was rendered virtually impossible by exogenous peaks from the Cl. welchii extract. Furthermore, the presence of bacterial amino acids precluded any conclusions as to the presence or absence of D-aspartate. Therefore an attempt was made to extract the L-aspartate β -decarboxylase activity from the acetone powder and to dialyse away amino acids and other low-molecular weight components.

Attempts to extract activity into buffer by extensive dialysis were unsuccessful. The supernatant from an acetone powder suspension which was dialysed in buffer for 24 h with stirring was inactive in decarboxylating [³H]L-aspartate. Failure to observe activity could have been due to the following factors: (1) loss of pyridoxal 5'-phosphate from the enzyme during dialysis, with resultant lack of activity of the apoenzyme in the incubation mixture which did not include pyridoxal 5'-phosphate; (2) loss of activity over the extended dialysis period; (3) failure of the sodium acetate buffer to extract the activity from the acetone powder.

It was evident from the work of NISHIMURA, MANNING & MEISTER (1962) that lack of activity was not due to the first point. Working on purified L-aspartate β -decarboxylase from lyophilized Cl. welchii, all attempts to remove enzyme bound vitamin B₆ by extensive dialysis or enzyme precipitation were unsuccessful. The only way to reduce the concentration of the prosthetic group was by U.V. irradiation. Added pyridoxal phosphate stimulates the enzyme, and it appears that this can act both as a prosthetic group and as a less

tightly bound cofactor. Furthermore, added α -oxo acids produce a stimulation equivalent to that observed by pyridoxal phosphate. Only a slight further stimulation occurs when both are added. The second possibility, i.e. inactivation over the long dialysis period, cannot be discounted, although in the purification procedure used by NISHIMURA, MANNING & MEISTER (1962) an 18 h dialysis was used. If the third possibility was the case, then the aspartate β -decarboxylase activity would have been removed from the dialysed acetone powder suspension by centrifugation; this would explain why the supernatant used lacked activity. Interestingly, several studies (EPPS, 1945; GALE, 1945; GALE, 1957) have shown that of a number of bacterial decarboxylase enzymes, the glutamate decarboxylase was often inactivated by preparation of acetone powders. The stability of aspartate β -decarboxylase to acetone powder preparation has not been reported, but all purifications of this enzyme from bacteria have commenced with either freeze-dried material (NISHIMURA, MANNING & MEISTER, 1962; MEISTER, SOBER & TICE, 1951) or sonically-disrupted frozen cell paste (TATE, NOVOGRODSKY, SODA, MILES & MEISTER, 1970; MILES & SPARROW, 1970).

Incubation of a synthetic amino acid mixture with L-amino acid oxidase from rattlesnake (*Crotalus adamanteus*) venom.

PARIKH, GREENSTEIN, WINITZ & BIRNBAUM (1958) reported amounts of crude *Crotalus adamanteus* venom suitable for preparing optically-pure D-amino acids from racemic mixtures. Using an excess amount of venom, oxidation of dicarboxylic acids, L-aspartate and L-glutamate was negligible (Table 9.2).

Results of GREENSTEIN, BIRNBAUM & OTEY (1953) on the rate of oxidation of various amino acids by rattlesnake venom are given for comparison. A report (KEARNEY & SINGER, 1949)

Table 9.2. Oxidation of amino acids by L-amino acid oxidase from rattlesnake (Crotalus adamanteus) venom

Amino acid	Percent amino acid oxidized	Oxidation by L-amino acid oxidase **
Methionine	99.2	243
Leucine	99.0	225
Phenylalanine	98.7	185
Valine	99.4	9
Cystine	82.5	63
Alanine	41.1	0.9
Lysine	n.d.	0.4
Glutamic acid	6.8	0.2
Proline	1.2	0.0
Aspartic acid	0.0	0.1
Threonine	0.0	0.0
β -alanine	0.0	n.d.
Glycine	0.0	n.d.
Serine*	32.0	0.0
Glutamine*	-	n.d.

Values are expressed as percent amino acid oxidized during 20 h incubation of 2 μ moles each of 18 amino acids with 5 mg of L-amino acid oxidase from Crotalus adamanteus venom. There was no contribution to amino acids from the enzyme extract.

* Serine and glutamine peaks not separated on the amino acid analyser.

** Data from GREENSTEIN, BIRNBAUM & OTEY (1953). Rates are in terms of μ moles of oxygen consumed/hour/mg of protein. n.d. = not determined.

which showed L-amino acid oxidase to be largely inhibited by inorganic phosphate was subsequently found. Thus the phosphate buffer used here was a poor choice; the tris-HCl buffer of PARIKH et al. (1958) was not used because it was thought that the tris carried through in the column desalting procedure would interfere with the amino acid analyses. The use of another buffer and the elimination of inorganic phosphate may allow this approach to be utilized successfully for determining the presence or absence of D-isomers of amino acids. Using tris buffer under the same incubation conditions, acidic amino acids were separated from a reaction supernatant and analysed. However, there appeared to be little oxidation of glutamate and aspartate in the 20 h incubation period.

Separation of diastereoisomeric dipeptide derivatives of amino acids in cat and rat CNS extracts. Thin-layer chromatography of CNS extracts (before dipeptide derivitization) in butanol:acetic acid:water (4:1:1) showed the presence of major spots of glutamate and aspartate, and several other spots which were not directly identified. Chromatography of derivatized samples on silica-gel plates in butanol:acetic acid:water resulted in good separation of diastereoisomers, with clear sharp spots and reproducible Rf's (Fig. 9.1). None of the other solvents was as effective, while chromatography on Avicel plates resulted in smearing and diffuse overlapping spots. From TLC analysis it was apparent that neither in cat or rat tissue samples was there a spot corresponding to authentic L-leucyl-D-aspartate. In all samples spots corresponding to the L-leucyl-L-aspartate and L-leucyl-L-glutamate were present, while in cat cortex and cord extracts spots running in an equivalent position to standard L-leucyl-D-glutamate were apparent. To check whether this was the L-leucyl-dipeptide of D-glutamate,

Fig. 9.1. Thin layer chromatography of amino acid samples, before and after derivitization with L-leucine N-carboxyanhydride. 20 x 20 cm precoated silica gel plates (Merck), layer thickness 0.25 mm, were used. The solvent system was butanol:acetic acid:water (4:1:1). Plates were run for 3.5 h at room temperature and spots developed with ninhydrin spray (0.2% w/v in acetone).

A. Aspartate, glutamate and rat and cat CNS extracts before derivitization.

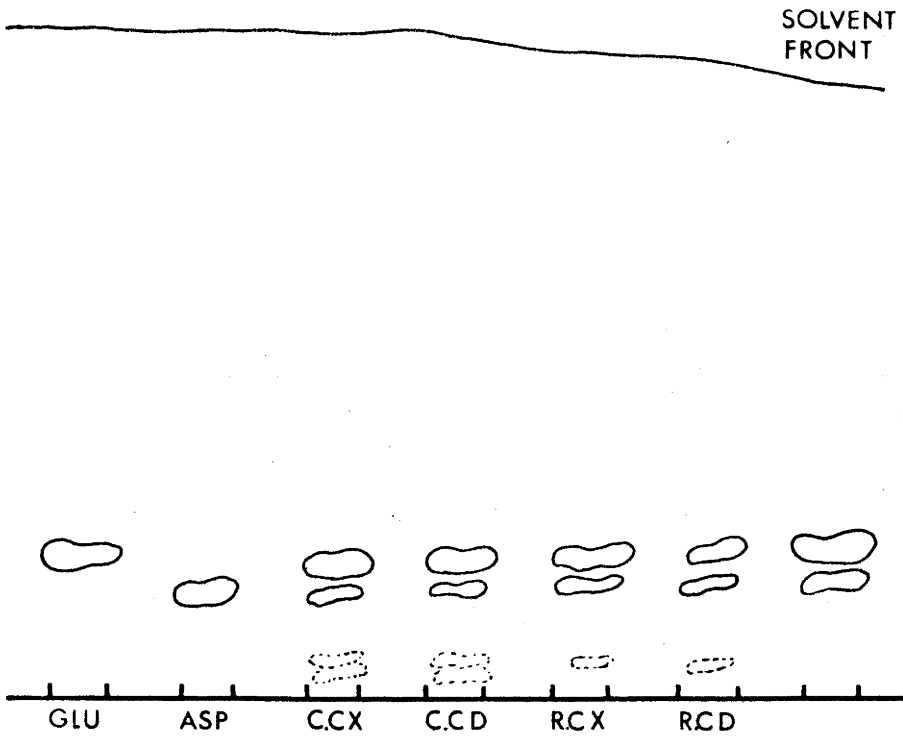
B. D- and L-aspartate, D- and L-glutamate and rat and cat CNS extracts after derivitization with L-leucine N-carboxyanhydride. Free aspartate, glutamate and L-leucine were also run on the same plate.

The following abbreviations are used:

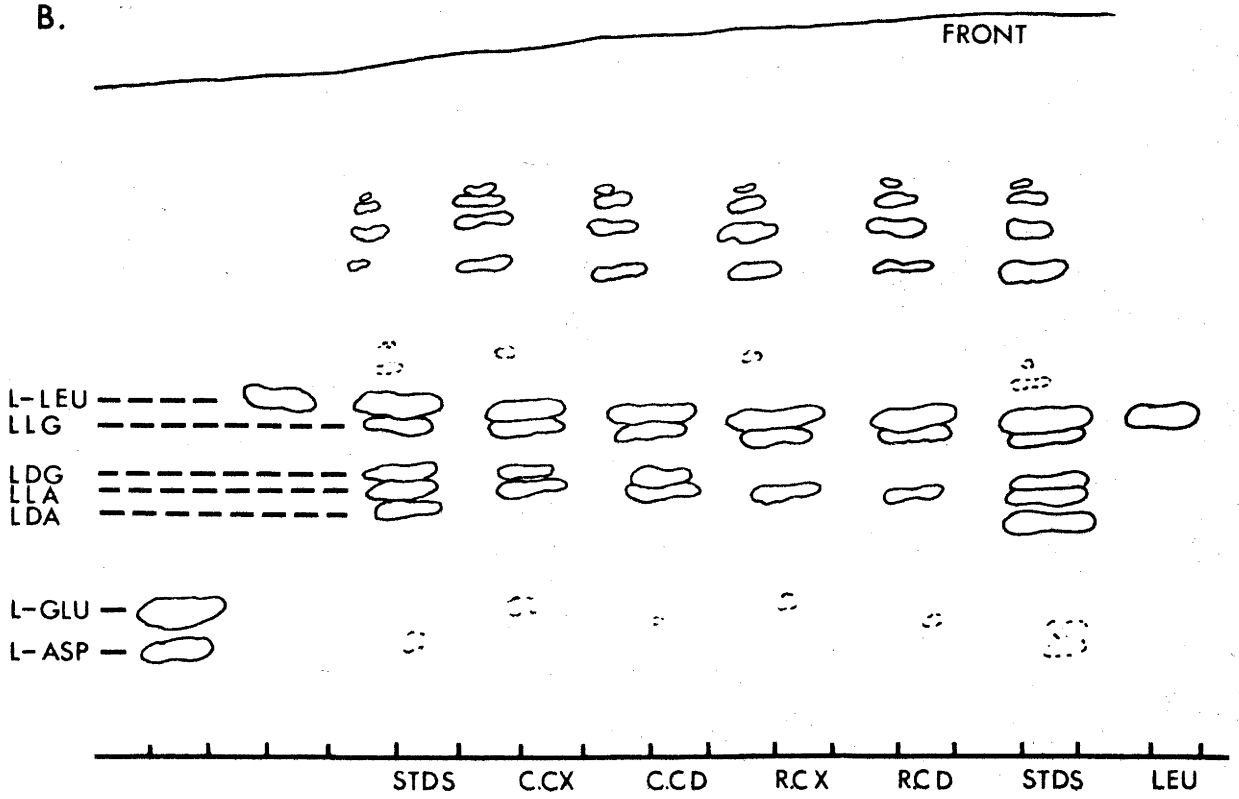
LLG = L-leucyl-L-glutamate
LDG = L-leucyl-D-glutamate
LLA = L-leucyl-L-aspartate
LDA = L-leucyl-D-aspartate

C.CX = cat cerebral cortex extract
C.CD = cat spinal cord extract
R.CX = rat cerebral cortex extract
R.CD = rat spinal cord extract
STDS = derivatized standards

A.



B.



samples were run on an amino acid analyser. Spots travelling ahead of L-leucine on TLC were presumed to be tri- and higher peptides, although not positively identified.

Column chromatography of derivatized standards on the amino acid analyser resulted in the following order of elution (elution times are given in brackets): L-leucyl-D-aspartate (89 min); L-leucyl-L-aspartate (102 min); L-leucyl-D-glutamate (125 min); L-leucine (140 min) and L-leucyl-L-glutamate (158 min). Chromatographic analysis of derivatized samples of brain and spinal cord extracts from cat and rat did not reveal the presence of any peak running in an equivalent position to L-leucyl-D-aspartate. With the amounts of samples analysed, it was calculated that if D-aspartate was present, it would be so at a level of less than 1% of L-aspartate. In all extracts there was a small peak running in a position equivalent to the L-leucyl-D-glutamate standard; this was observed on TLC of cat cortex and cord extracts. The amount was between 4-6% of L-leucyl-L-glutamate for cat brain, rat brain and cord extracts and about 19% for the cat cord sample (assuming equal colour development values with ninhydrin for the two derivatives). This minor peak however, was not further identified and is quite possibly a derivative of some other amino acid in the CNS extracts.

4. DISCUSSION

It can be concluded from these results that the brain of cat and rat does not contain D-aspartate, at least not in amounts in excess of 1% of the level of the L-isomer. A peak, about 4-6% of the L-glutamate peak, ran in an equivalent position to the L-leucyl-D-glutamate standard in two chromatographic systems. It could represent a

derivative of some other amino acid, possibly that of an unidentified amino acid which elutes just before aspartate in routine analyses (JAMES, 1972) of rat and cat brain extracts. This peak was first noticed by JOHNSTON (unpublished results) and was seen in extracts of 10-day-old rat CNS (Section III).

The use of isomer-specific enzymes, coupled with automated column-chromatographic amino acid analysis, should be a feasible method of determining optical configurations of amino acid samples, provided enzyme preparations free of contaminating amino acids can be prepared or obtained. This was not the case in the present investigation.

The formation of diastereoisomeric dipeptide derivatives of amino acids by reaction with L-leucine N-carboxyanhydride, and subsequent separation by column chromatography, seems to be a convenient method of determining the optical configuration of an amino acid sample, provided a supply of L-leucine N-carboxyanhydride can be either obtained commercially or synthesized. The synthesis described here was relatively straightforward, although great care is necessary in the handling of the phosgene and in isolating the reaction in a fume-cupboard. The separation of such derivatives by TLC should be useful in checking the optical purity of radiolabelled compounds.

APPENDIX 1

All scintillation counting was done on a Beckman LS-100, which uses an External Standard-Channels Ratio operation as a means of correcting for counting efficiency variations caused by sample quenching. CPM were converted to DPM by means of this External Standard Ratio. Known amounts of [^3H] or [^{14}C] radioactivity were added to a number of scintillation vials containing appropriate scintillator solution and a quenched series made by adding different volumes of chloroform. Samples were counted and the External Standard Ratio plotted versus counting efficiency (CPM obtained \times 100/known DPM added). The External Standard channel window was set to obtain a linear relationship between the External Standard Ratio and efficiency; for each scintillator used the appropriate constants for the experimentally-determined straight lines were calculated for both isotopes and used in computer programmes written to convert CPM to DPM.

APPENDIX 2

Where appropriate, weighted averages of the values \pm S.E.M. from analysis of individual experiments were calculated using the following equations (MORRISON & JAMES, 1965):

$$\text{Weighted means of } x \text{ values} = \frac{\sum w_i x_i}{\sum w_i}$$

$$\text{and S.E.M. of weighted mean value} = \frac{1}{\sqrt{\sum w_i}}$$

$$\text{where } w = \frac{1}{[\text{S.E.M.}(x_i)]^2}$$

REFERENCES

- AGHAJANIAN G.K. & BLOOM F.E. (1967) Brain Res. 6, 716-727.
- AGRAWAL H.C., DAVIS J.M. & HIMWICH W.A. (1966) J. Neurochem. 13, 607-615.
- AGRAWAL H.C., DAVISON A.N. & KACZMAREK L.K. (1971) Biochem. J. 122, 759-763.
- ANDERSON T.R. & SCHANBERG S.M. (1972) J. Neurochem. 19, 1471-1481.
- APRISON M.H. (1970) Pharmacologist 12, 128.
- APRISON M.H., DAVIDOFF R.A. & WERMAN R. (1970) In Handbook of Neurochemistry Vol.3 (LAJTHA A., ed.) pp.381-397. Plenum Press, New York.
- APRISON M.H., SHANK R.P., DAVIDOFF R.A. & WERMAN R. (1968) Life Sci., Oxford 7, 583-590.
- APRISON M.H. & WERMAN R. (1965) Life Sci., Oxford 4, 2075-2083.
- ARNFRED T. & HERTZ L. (1971) J. Neurochem. 18, 259-265.
- ARREGUI A., LOGAN W.J., BENNETT J.P. Jr & SNYDER S.H. (1972) Proc. natn. acad. Sci. U.S.A. 69, 3485-3489.
- BAKER P.F., BLAUSTEIN M.P., HODGKIN A.L. & STEINHARDT R.A. (1969) J. Physiol., Lond. 200, 431-458.
- BALCAR V.J. & JOHNSTON G.A.R. (1972a) J. Neurochem. 19, 2657-2666.
- BALCAR V.J. & JOHNSTON G.A.R. (1972b) J. Neurobiol. 3, 295-301.
- BALCAR V.J. & JOHNSTON G.A.R. (1973) J. Neurochem. 20, 529-539.
- BALCAR V.J. & JOHNSTON G.A.R. (1975) Brain Res. 83, 173-175.
- BAYER S.M. & McMURRAY W.C. (1967) J. Neurochem. 14, 695-706.
- BEART P.M. & JOHNSTON G.A.R. (1973) J. Neurochem. 20, 319-324.
- BEART P.M., JOHNSTON G.A.R. & UHR M.L. (1972) J. Neurochem. 19, 1855-1861.

- BENNETT J.P. Jr, LOGAN W.J. & SNYDER S.H. (1972) Science, N.Y. 178, 997-999.
- BENNETT J.P. Jr, LOGAN W.J. & SNYDER S.H. (1973) J. Neurochem. 21, 1533-1550.
- BENNETT J.P. Jr, MULDER A.H. & SNYDER S.H. (1974) Life Sci., Oxford 15, 1045-1056.
- BENUCK M, STERN F. & LAJTHA A. (1972) J. Neurochem. 19, 949-957.
- BERL S. & CLARKE D.D. (1969) In Handbook of Neurochemistry Vol. 7 (LAJTHA A., ed.) pp. 447-472. Plenum Press, New York.
- BIRKS R.I., BURSTYN P.G.R. & FIRTH D.R. (1968) J. gen. Physiol. 52, 887-907.
- BISCOE T.J., DUGGAN A.W. & LODGE D. (1972) Comp. gen. Pharmac. 3, 423-433.
- BLASBERG R.G. (1968) Prog. Brain Res. 29, 245-256.
- BLOOM F.E. & AGHAJANIAN G.K. (1966) Science, N.Y. 154, 1575-1577.
- BLOOM F.E. & IVERSEN L.L. (1971) Nature, Lond. 239, 628-630.
- BOND P.A. (1973) J. Neurochem. 20, 511-518.
- BOWERY N.G. & BROWN D.A. (1972) Nature, Lond. 238, 89-91.
- BRIDGERS W.F. (1968) J. Neurochem. 15, 1325-1328.
- BRODERICK D.S., CANDLAND K.L., NORTH J.A. & MANGUM J.H. (1972) Archs biochem. Biophys. 148, 196-198.
- BROWN D.J. & STONE W.E. (1973) J. Neurochem. 20, 1461-1467.
- BUCKLE R.M. (1963) Clin. Sci. 25, 207-217.
- CARMI A., POLLACK G. & YELLIN H. (1960) J. org. Chem. 25, 44-46.
- CLELAND W.W. (1963) Nature, Lond. 198, 463-365.
- COHEN S.R. & LAJTHA A. (1972) In Handbook of Neurochemistry Vol. 7 (LAJTHA A., ed.) pp. 543-572. Plenum Press, New York.

- COLEMAN D. (1950) J. Chem. Soc. 1950, 3222-3229.
- COLLINS G.S. (1973) Biochem. Pharmac. 22, 101-111.
- CORRIGAN J.J. (1969) Science, N.Y. 164, 142-149.
- COYLE J.T. & AXELROD J. (1971) J. Neurochem. 18, 2061-2075.
- CRAWFORD J.M. & CURTIS D.R. (1964) Br. J. Pharmac. 23, 313-329.
- CURTIS D.R., DUGGAN A.W., FELIX D. & JOHNSTON G.A.R. (1971)
Brain Res. 32, 69-96.
- CURTIS D.R., DUGGAN A.W. & JOHNSTON G.A.R. (1970) Expl
Brain Res. 10, 447-462.
- CURTIS D.R., HÖSLI L., JOHNSTON G.A.R. & JOHNSTON I.H.
(1968) Expl Brain Res. 5, 235-258.
- CURTIS D.R. & JOHNSTON G.A.R. (1970) In Handbook of
Neurochemistry Vol.4 (LAJTHA A., ed.) pp.115-134.
Plenum Press, New York.
- CURTIS D.R. & JOHNSTON G.A.R. (1974) Ergebn. Physiol. 69,
97-188.
- CURTIS D.R., PHILLIS J.W. & WATKINS J.C. (1960) J. Physiol.,
Lond. 150, 656-682.
- CURTIS D.R. & WATKINS J.C. (1960) J. Neurochem. 6, 117-141.
- CURTIS D.R. & WATKINS J.C. (1961) Nature, Lond. 191, 1010-1011.
- CURTIS D.R. & WATKINS J.C. (1963) J. Physiol., Lond. 166,
1-14.
- CUTHBERT A.W. & WONG P.V.D. (1972) Molec. Pharmac. 8, 222-229.
- DALY E.C. & APRISON M.H. (1974) J. Neurochem. 22, 877-885.
- DAVIDOFF R.A., GRAHAM L.T., SHANK R.P., WERMAN R. & APRISON
M.H. (1966) Physiologist, 9, 165.
- DAVIDOFF R.A., GRAHAM L.T., SHANK R.P., WERMAN R. & APRISON
M.H. (1967) J. Neurochem. 14, 1025-1031.
- DAVIDOFF R.A., SHANK R.P., GRAHAM L.T., APRISON M.H. &
WERMAN R. (1967) Nature, Lond. 214, 680-681.
- DAVIES J. & JOHNSTON G.A.R. (1974) J. Neurochem. 22, 931-935.
- DAVIS J.M. & HIMWICH W.A. (1973) In Biochemistry of the
Developing Brain Vol.1 (HIMWICH W.A., ed.) pp.55-99.
Marcel Dekker, New York.

- DAVIS W.M. & YEH J.Z. (1969) Experientia 25, 1291-1293.
- DE MARCHI W.J. & JOHNSTON G.A.R. (1969) J. Neurochem. 16, 355-361.
- DE VANZO J.P., GREIG M.E. & CRONIN M.A. (1961) Am. J. Physiol. 201, 833-837.
- DIXON M. (1970) In Methods in Enzymology Vol.XVII, Part A, (COLOWICK S.P. & KAPLAN N.O., eds) pp.713-718. Academic Press, New York/London.
- DIXON M. & KENWORTHY P. (1967) Biochim. biophys. Acta. 146, 54-76.
- DIXON M. & KLEPPE K. (1965) Biochim. biophys. Acta. 96, 368-382.
- DODGSON K.S., LLOYD A.G. & TUDBALL N. (1961) Biochem. J. 79, 111-117.
- DOUGLAS W.W. (1966) Pharmac. Rev. 18, 471-480.
- DOUGLAS W.W. & POISNER A.M. (1964) J. Physiol., Lond. 172, 19-30.
- EAYRS J.T. & GOODHEAD B. (1959) J. Anat., Lond. 93, 385-402.
- ECCLES J.C., FATT P. & KOKETSU K. (1954) J. Physiol., Lond. 126, 524-562.
- ECKENHOFF J.E. & HAFKENSCHIEL J.H. (1947) J. Pharmac. exp. Ther. 91, 362-369.
- EPPE H.M.R. (1945) Biochem. J. 39, 42-46.
- FRISELL W.R., LOWE H.J. & HELLERMAN L. (1956) J. biol. Chem. 223, 75-83.
- GAGE P.W. & QUASTEL D.M.J. (1965) Nature, Lond. 206, 625-626.
- GAGE P.W. & QUASTEL D.M.J. (1966) J. Physiol., Lond. 185, 95-123.
- GALE E.F. (1945) Biochem. J. 39, 46-52.
- GALE E.F. (1957) In Methods of Biochemical Analysis Vol.4 (GLICK D., ed.) pp.285-306. Interscience, New York.
- GOLDSTEIN D.B. (1966) J. Neurochem. 13, 1011-1016.

- GORNALL A.G., BARDAWILL J. & DAVID M.M. (1949) J. biol. Chem. 177, 751-766.
- GOTTESFELD Z. & ELLIOT K.A.C. (1971) J. Neurochem. 18, 683-690.
- GOTTESFELD Z., KELLY J.S. & SCHON F. (1973) Br. J. Pharmac. 47, 640P.
- GRAHAM L.T. Jr & APRISON M.H. (1969) J. Neurochem. 16, 559-566.
- GRAHAM L.T. Jr, SHANK R.P., WERMAN R. & APRISON M.H. (1967) J. Neurochem. 14, 465-472.
- GREENSTEIN J.P., BIRNBAUM S.C. & OTEY C. (1953) J. biol. Chem. 204, 307-321.
- HAMMERSTAD J.P., MURRAY J.E. & CUTLER R.W.P. (1971) Brain Res. 35, 357-367.
- HATEFI Y., TALBERT P.T., OSBORN M.J. & HUENNEKENS F.M. (1960) In Biochemical Preparations Vol.7 (LARDY H.A., ed.) pp.89-92. Wiley, New York.
- HEINZ E. (1967) Ann. Rev. Physiol. 29, 21-58.
- HENN F.A. & HAMBURGER A. (1971) Proc. natn. Acad. Sci. U.S.A. 68, 2686-2690.
- HILLMAN H.H. & McILWAIN H. (1961) J. Physiol., Lond. 157, 263-278.
- HIMWICH W.A. & AGRAWAL H.C. (1969) In Handbook of Neurochemistry Vol.1 (LAJTHA A., ed.) pp.33-52. Plenum Press, New York.
- HÖKFELT T. (1970) Brain Res. 22, 147-151.
- HÖKFELT T. & LJUNGDAHL A. (1971) Brain Res. 32, 189-194.
- HÖKFELT T. & LJUNGDAHL A. (1972) Expl Brain Res. 14, 354-362.
- HOPKIN J. & NEAL M.J. (1971) Br. J. Pharmac. 42, 215-223.
- HOROWICZ P. & GERBER C.J. (1965) J. gen. Physiol. 48, 489-514.
- HUBBARD J.I. (1973) Physiol. Rev. 53, 674-723.
- IVERSEN L.L. & BLOOM F.E. (1972) Brain Res. 41, 131-143.
- IVERSEN L.L. & JOHNSTON G.A.R. (1971) J. Neurochem. 18, 1939-1950.

- IVERSEN L.L., MITCHELL J.F. & SRINIVASAN V. (1971)
J. Physiol., Lond. 212, 519-534.
- IVERSEN L.L. & SCHON F.E. (1973) In New Concepts in Neurotransmitter Regulation (MANDELL A.J., ed.) pp. 153-193.
Plenum Press, New York/London.
- IVERSEN L.L. & SNYDER S.H. (1968) Nature, Lond. 220, 796-798.
- JAMES L.B. (1972) Lab. Pract. Sept., 639-642.
- JASPER H.H. & KOYAMA I. (1969) Can. J. Physiol. Pharmac.
47, 889-905.
- JOHNSON J.L. (1972a) Brain Res. 37, 1-19.
- JOHNSON J.L. (1972b) Brain Res. 45, 205-215.
- JOHNSTON G.A.R. (1968) J. Neurochem. 15, 1013-1017.
- JOHNSTON G.A.R. (1973) Biochem. Pharmac. 22, 137-139.
- JOHNSTON G.A.R. & IVERSEN L.L. (1971) J. Neurochem. 18,
1951-1961.
- JOHNSTON G.A.R. & IVERSEN L.L. (1971) J. Neurochem. 18,
1951-1961.
- JOHNSTON G.A.R. & MITCHELL J.F. (1971) J. Neurochem. 18,
2441-2446.
- JOHNSTON G.A.R. & VITALI M.V. (1969) Brain Res. 12, 471-472.
- JOHNSTON G.A.R., VITALI M.V. & ALEXANDER H.M. (1970)
Brain Res. 20, 361-367.
- JORDAN C.C. & WEBSTER R.A. (1971) Br. J. Pharmac. 43, 441P.
- KACZMARECK L.K., AGRAWAL H.C. & DAVISON A.N. (1971) In
Inherited Disorders of Sulphur Metabolism Proceedings
for the 8th Symposium of the Society for the Study of
Inborn Errors of Metabolism (CARSON N.A.J. & RAINER
D.N., eds) pp. 63-69. Churchill Livingstone, Edinburgh.
- KANDERA J., LEVI G. & LAJTHA A. (1968) Archs biochem.
Biophys. 126, 249-260.
- KATSUKI H., YOSHIDA T., TANEGASHIMA G. & TANAKA S. (1971)
Analyt. Biochem. 43, 349-356.
- KATZ R.I., CHASE T.N. & KOPIN I.J. (1969) J. Neurochem. 16,
961-967.

- KEARNEY L.B. & SINGER T.P. (1949) Archs biochem. Biophys.
21, 242-245.
- KIRPEKAR S.M. & WAKADE A.R. (1968) J. Physiol., Lond. 194,
595-608.
- KLEIN J.R. & KAMIN H. (1941) J. biol. Chem. 138, 507-512.
- KOGL F. & ERXLEBEN H. (1939) Hoppe-Seyler's Z. physiol. Chem.
258, 57-95.
- KREBS M.A. (1935) Biochem. J. 29, 1620-1644.
- KRNJEVIĆ K. & PHILLIS J.W. (1963) J. Physiol., Lond. 166,
KUBASIK N.P., RICHERT D.A., BLOOM R.J., HSU R.Y. & WESTERFELD
W.W. (1973) Biochem. 11, 2225-2229.
- KUHN W. (1958) Advan. Enzymol. 20, 1-29.
- KURIYAMA K., ROBERTS E. & RUBINSTEIN M.K. (1966) Biochem.
Pharmac. 15, 221-236.
- KURTZ D.J., LEVY H. & KANFER J.N. (1972) J. Nutr. 102, 291-298.
- LIANG C.C. (1962) Biochem. J. 82, 429-434.
- LAZARUS W. (1973) J. Chromatogr. 87, 169-178.
- LEVI G. (1972) Archs biochem. Biophys. 151, 8-21.
- LEVI G. & RAITERI M. (1973) Life Sci., Oxford 12, 81-88.
- LEVY W.B., HAYCOCK J.W. & COTMAN C.W. (1974) Molec. Pharmac.
10, 438-499.
- LJUNGDAHL A. & HÖKFELT T. (1973) Brain Res. 62, 587-595.
- LJUNGDAHL A. & HÖKFELT T. (1973) Histochemie 33, 277-280.
- LOGAN W.J. & SNYDER S.H. (1972) Brain. Res. 42, 413-431.
- LOWRY O.H., ROSEBROUGH N.H., FARR A.L. & RANDALL R.J.
(1951) J. biol. Chem. 193, 265-273.
- McBRIDE W.J., DALY E. & APRISON M.H. (1974) J. Neurobiol.
4, 557-566.
- MACHIYAMA Y., BALÁZS R. & RICHTER D. (1967) J. Neurochem.
14, 591-594.

- McILWAIN H. & RODNIGHT R. (1962) Practical Neurochemistry
Churchill, London, pp.221-224.
- McLENNAN M. (1970) Synaptic Transmission (2nd ed.) pp.78-105.
W.B. Saunders, Philadelphia.
- MANNING J.M. & MOORE S. (1968) J. biol. Chem. 243, 5591-5597.
- MARGOLIS R.K. & LAJTHA A. (1968) Biochim. biophys. Acta 163,
374-385.
- MARTIN D.L. & SMITH A.A. (1972) J. Neurochem. 19, 841-855.
- MATUS A.T. & DENNISON M.E. (1972) J. Neurocytol. 1, 27-34.
- MAY M.K. & BISCOE T.J. (1973) Brain Res. 53, 181-186.
- MEISTER A., SOBER A. & TICE S.V. (1951) J. biol. Chem. 189,
577-590.
- MILES E.W. & SPARROW L.G. (1970) In Methods in Enzymology
Vol.XVII, Part A (TABOR H. & TABOR C.W., eds) pp.689-693.
Academic Press, New York/London.
- MINCHIN M.C.W. & IVERSEN L.L. (1974) J. Neurochem. 23, 533-540.
- MORRISON J.F. & JAMES E. (1965) Biochem. J. 97, 37-52.
- MULDER A.H. & SNYDER S.H. (1974) Brain Res. 76, 297-308.
- NAKANO Y., FUJIOKA M. & WADA H. (1968) Biochim. biophys.
Acta 159, 19-26.
- NEAL M.J. (1971) J. Physiol., Lond. 215, 103-117.
- NEAL M.J. & PICKLES H. (1969) Nature, Lond. 223, 679-680.
- NIEMS A.H., DeLUCA D.G. & HELLERMAN I. (1965) Biochem.
5, 203-213.
- NIEMS A.H., ZIEVERINK W.D. & SMILACK J.D. (1966) J. Neurochem.
13, 163-168.
- NISHIMURA J.S., MANNING J.M. & MEISTER A. (1962) Biochem.
1, 442-447.
- OBATA K. & TAKEDA K. (1969) J. Neurochem. 16, 1043-1047.
- PARIKH J.R., GREENSTEIN J.P., WINITZ M. & BIRNBAUM S.M.
(1958) J. Am. Chem. soc. 80, 953-958.
- PARSONS R.L., HOFFMAN W.W. & FEIGEN G.A. (1965) Nature,
Lond. 208, 590-591.

- PETERSON N.A. & RAGHUPATHY E. (1973) J. Neurochem. 21, 97-110.
- PETERSON N.A. & RAGHUPATHY E. (1974) Biochem. Pharmac. 23, 2491-2494.
- PICCOLI F., GRYNBAUM A. & LAJTHA A. (1971) J. Neurochem. 18, 1135-1148.
- POLLOCK G.E. & KAWAUCHI A.H. (1968) Analyt. Chem. 40, 1356-1358.
- PYLKKO O.O. & WOODBURY D.M. (1961) J. Pharmac. exp. Ther. 131, 185-190.
- RICHARDSON K.E. (1973) Toxicol. Appl. Pharmac. 24, 530-538.
- ROBERTS, P.J. (1974) Brain Res. 74, 327-332.
- ROGOZKIN S.V., DAVANKOV V.A., YAMSKOV I.A. & KABANOV V.P. (1972) J. Gen. Chem., U.S.S.R. 42, 1605-1607.
- RUBIN P. (1970) Pharmac. Rev. 22, 389-428.
- SCHLOSSBERG M.A., BLOOM R.J., RICHERT D.A. & WESTERFELD W.W. (1970) Biochem. 9, 1148-1153.
- SCHON F. & KELLY J.S. (1974a) Brain Res. 66, 275-288.
- SCHON F. & KELLY J.S. (1974b) Brain Res. 66, 289-300.
- SCHRIER B.K. & THOMPSON E.J. (1974) J. biol. Chem. 249, 1769-1780.
- SHANK R.P. & APRISON M.H. (1970) J. Neurochem. 17, 1461-1475.
- SHANK R.P., APRISON M.H. & BAXTER C.F. (1973) Brain Res. 52, 301-308.
- SHAW R.K. & HEINE J.D. (1965) J. Neurochem. 12, 151-155.
- SIMPSON L.L. (1968) J. Pharm. Pharmac. 20, 889-910.
- SMITH S.E. (1967) J. Neurochem. 14, 291-300.
- SNYDER S.H., LOGAN W.J., BENNETT J.P. Jr & ARREGUI A. (1973) Neurosci. Res. 5, 131-157.
- SNYDER S.H., YOUNG A.B., BENNETT J.P. Jr & MULDER A.H. (1973) Fedn Proc. Fedn Am. Socs exp. Biol. 32, 2039-2047.
- SPACKMAN D.H., STEIN W.H. & MOORE S. (1958) Analyt. Chem. 30, 1190-1206.

- SRINIVASAN V., NEAL M.J. & MITCHELL J.F. (1969) J. Neurochem. 16, 1235-1244.
- STILL J.L., BUELL M.B., KNOX W.E. & GREEN D.E. (1949) J. biol. Chem. 179, 831-837.
- STILL J.L. & SPERLING E. (1950) J. biol. Chem. 182, 585-589.
- SUNG S.C. (1969) Can. J. Biochem. 47, 47-50.
- TAPIA R., DE LA MORA M. & MASSIEU G.H. (1969) Ann. N.Y. Acad. Sci. 166, 251-266.
- TATE S.S., NOVOGRODSKY A., SODA K., MILES E.W. & MEISTER A. (1970) In Methods in Enzymology Vol. XVII, Part A (TABOR H. & TABOR C.W., eds) pp.681-688. Academic Press, New York/London.
- TAYLOR R.T. & WEISSBACH H. (1965) Analyt. Biochem. 13, 80-84.
- TIMIRAS P.S. & VERNADAKIS A. & SHERWOOD N. (1968) In Biology of Gestation Vol.2 (ASSALI N.S., ed.) pp.261-319. Academic Press, New York.
- UHR M.L. & SNEDDON M.K. (1971) FEBS Lett. 17, 137-140.
- UHR M.L. & SNEDDON M.K. (1972) J. Neurochem. 19, 1495-1500.
- VAN DEN BERG C.J. (1973) In Metabolic Compartmentation in the Brain (BALÁZS R. & CREMER J.E., eds) pp.129-136. McMillan, London.
- VERNADAKIS A. & WOODBURY D.M. (1969) Epilepsia 10, 163-178.
- WALSH D.A. & SALLACK H.J. (1966) J. biol. Chem. 241, 4068-4076.
- WATKINS J.C. (1962) J. med. Chem. 5, 1187-1199.
- WOFSEY A.R., KUCHAR M.J. & SNYDER S.H. (1971) Proc. natn. Acad. Sci. U.S.A. 68, 1102-1106.
- WOOD J.D. & ABRAHAMS D.E. (1971) J. Neurochem. 18, 1017-1025.
- WOODWARD D.J., HOFFER B.J., SIGGINS G.R. & BLOOM F.E. (1971) Brain Res. 34, 73-97.
- YOSHIDA H., KANIIKE K. & NAMBA J. (1963) Nature, Lond. 198, 191-192.
- YOSHINO Y. & ELLIOT K.A.C. (1970) Can. J. Biochem. 48, 228-235.

- YOUNG A.C., BROWN D.A., KELLY J.S. & SCHON F. (1973)
Brain Res. 63, 479-486.
- YUSKO S.C. & NIEMS A.H. (1973) J. Neurochem. 21, 1037-1039.

ADDITIONAL REFERENCES

- ADINOLFI A.M. (1971) In Brain Development and Behaviour
(STERMAN M.B., MCGINTY D.J. & ADINOLFI A.M., eds)
pp. 73-89. Academic Press, New York/London.
- ALTMAN J. (1966) J. comp. Neurol. 128, 431-474.
- ALTMAN J. (1969) J. Comp. Neurol. 136, 269-294.
- BAKER P.F. (1968) Brit. med. Bull. 24, 179-182.
- DAVISON A.N. & DOBBING J. (1968) In Applied Neurochemistry
(DAVISON A.N. & DOBBING J., eds) pp. 253-286. Blackwell,
Oxford/Edinburgh.
- DOBBING J. (1968) In Applied Neurochemistry (DAVISON A.N.
& DOBBING J., eds) pp. 287-316. Blackwell,
Oxford/Edinburgh.
- GREENSTEIN J.P. (1961) In Chemistry of the Amino acids
Vol. I, pp. 715-760: Wiley, New York/London.
- MEISTER A. (1965) In Biochemistry of the Amino acids
Vol. I, pp. 113-118. Academic Press, New York/London.
- RASSIN D.K. & GAULL G.E. (1975) J. Neurochem. 24, 669-678.
- WOODWARD D.J., HOFFER B.J. & LAPHAM L.W. (1969) In
Neurobiology of Cerebellar Evolution and Development
Proceedings of the 1st International Symposium of the
Institute for Biomedical Research (LLINÁS R. ed.)
pp. 725-741. A.M.A. Education and Research Foundation
Chicago.