RODENT ASTROCYTE SUB-TYPES IN VITRO

by

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ABSTRACT

Monolayer cultures of rat cerebellar astrocytes were grown on poly-L-lysine in the presence of foetal calf serum. The non-neuronal cell types in culture were identified using cell-specific antibodies and were found to be primarily astrocytes (83%), the remainder being fibroblasts (5%), endothelial cells (9%) and oligodendrocytes (2%). Astrocytes occurred with either a stellate or non-stellate (epithelioid) morphology however the proportion of stellate cells reached a peak at 5-6 days *in vitro* and thereafter declined whereas the epithelioid form persisted. The two astrocyte types showed differences in expression of a variety of molecular markers including vimentin, glutamine synthetase and the binding sites for the monoclonal antibodies A2B5 and LB1. Autoradiographic studies showed the stellate astrocytes and, to a much lesser extent, the epithelioid astrocytes, able to accumulate GABA The GABA transport system in the epithelioid cells was sensitive to β-alanine and not ACHC; the converse was true for the stellate cells. These specificities were mirrored by the patterns of uptake of the inhibitors. These specificities were not detected *in situ*. Vmax and Km for transport into both cell-types were estimated: the Vmax values were very similar (1.3 and 1.8 nmol/min/mg protein) but there was a difference in Km (6.7μM and 31.2 μM in stellate and epithelioid cells respectively). IC50 values for inhibition of GABA uptake into stellate cells by GABA analogues were estimated; these confirmed the neuron-like specificity of the transport system in these cells. There was no differential accumulation of D-aspartate, glycine or noradrenaline by the cultured astrocytes (D-aspartate was the only one to be significantly transported by any cell). The GABA transport systems in the two astrocyte types appeared to behave differently

towards metabolic inhibitors. Modifications of the culture system and changing the age of animal from which cells were derived were used to "differentiate" the epithelioid astrocytes into the morphologically mature stellate form; this was only minimally successful. Astrocytes having many of the same properties occurred also in cultures from spinal cord and optic nerve. These data are considered with respect to possible astrocyte function and in terms of the differentiation states of astrocytes in culture.

to

HELEN and GAIL

with much love and thanks.

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LIST OF ABBREVIATIONS

The following abbreviations have been used throughout this thesis. In addition to this list, they are defined on their first occurence in the text.

ACHC	cis-1,3-aminocyclohexane carboxylic acid
AOAA	amino-oxyacetic acid
ara C	cytosine arabinoside
BBB	blood-brain barrier
BSA	bovine serum albumin
BSM	Bottenstein-Sato medium
BSS	Earle's balanced salts solution
cAMP	adenosine 3'5'cyclic monophosphate
CNS	central nervous system
d	day(s)
DAB	3,3'diaminobenzidine tetrahydrochloride
DABA	2,4-diaminobutyric acid
DIV	days in vitro
dBcAMP	dibutyryl adenosine-3'5'-cyclic monophosphate
DMEM	Dulbecco's modification of Eagles'medium
dpm	disintegrations per minute
ELISA	enzyme-linked immunosorbent assay
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
GABA	γ-aminobutyric acid; (4-aminobutyric acid)
[GABA]	concentration of 4-aminobutyric acid
GFAP	glial fibrillary acidic protein
GMF	glial maturation factor
h	hour(s)
HRP	horseradish peroxidase
IC50	median effective inhibitory concentration

ICC	immunocytochemical/immunocytochemistry
Ig	immunoglobulin
k	rate constant for an unsaturable transport process
[K]o	concentration of extracellular potassium ion
Km	Michaelis constant; the concentration of substrate eliciting half-maximal transport rate.
KM	Krebs medium (bicarbonate-buffered)
LSC	liquid scintillation counting
mBcAMP	monobutyryl adenosine-3'5'-cyclic monophosphate
min	minute(s)
M_r	relative molecular mass
Na^+K^+ATPase	sodium and potassium-activated adenosine triphosphatase
PAP	peroxidase/anti-peroixidase
PBS	phosphate-buffered saline
PMSF	phenylmethylsulphonyl fluoride
PPO	2,5-diphenyloxazole
rev/min	revolutions per minute
RIA	radioimmunoassay
s	second(s)
SDS	sodium dodecyl sulphate
TBK	tris-buffered Krebs soluition
TEMED	N,N,N'N'-teramethylethylenediamine
THPO	tetrahydroisoxazolo-[4,5c]-pyridin-3-ol
Tris	tris(hydroxymethyl)aminomethane
TRITC	tetramethylrhodamine isothiocyanate
UDR	uptake density ratio
Vmax	maximal transport rate
v	initial transport rate
v/v	volume for volume
w/v	weight for volume

I GENERAL INTRODUCTION

I.A Discovery of astrocytes.

In 1846, when describing the cerebral ventricles and spinal cord, Rudolf Virchow wrote about a substance "of a soft, medullary, fragile nature" which he contrasted with the fibrous connective tissue of the nerves and other organs. He christened this material thus:-

> "This peculiarity of the membrane, namely, that it becomes continuous with interstitial matter, the real cement, which binds the nervous elements together, and that in all its properties it constitutes a tissue different from the other forms of connective tissue, has induced me to give it a new name, that of neuro-glia."

It was also made clear in this early work that Virchow was aware of the possibility of involvement of the neuro-glia (nerve glue) in pathological and physiological conditions of the nervous system:-

> "Experience shows us that this very interstitial matter of the brain and spinal marrow is one of the most frequent seats of morbid change as, for example, fatty-acid degeneration."
>
> (Virchow,1846; quoted in Kuffler & Nicholls, 1976, p256)

It was not until over half a century later that the next major advance in the knowledge of neuroglia was made. In 1913, metallic impregnation staining techniques developed by Ramon y Cajal allowed light microscopic elucidation of morphologies and relative positions of the neuroglial cells within nervous tissues (Cajal,1913; quoted in Peters et al., 1976). Cajal was able to distinguish three classes of glial cells - astrocytes, oligodendrocytes and microglia and further

subdivided the astrocyte class into fibrous and protoplasmic. Further developments in staining technologies and the application of electron microscopy coupled with improved tissue preservation procedures led to the construction, by the 1960s, of a quite detailed picture of the gross and fine structures of a number of glial cell types as well as their anatomical relationships with other nervous system components (see section I.B).

I.B Classification of glial cells.

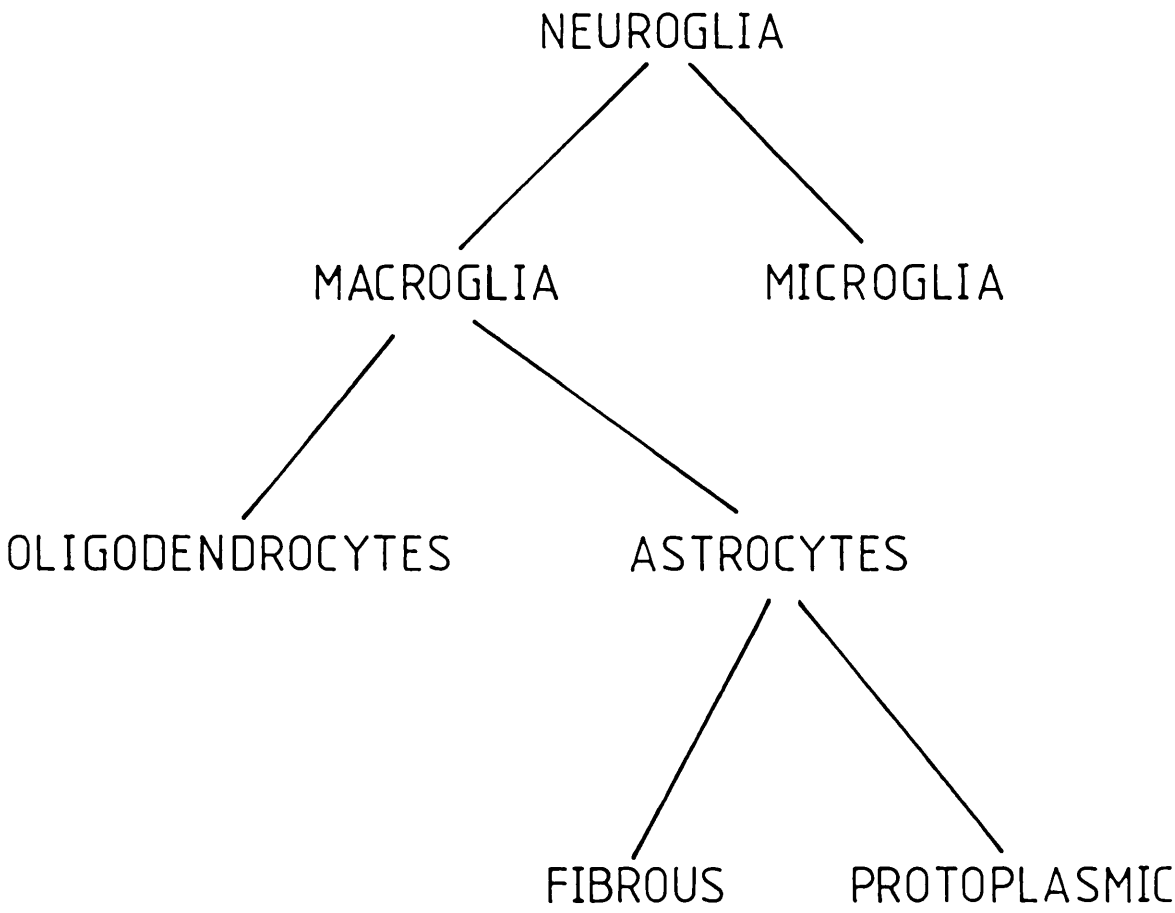
The current classification of the major glial cell types is shown in Fig.1. The structural features of the neuroglia have been comprehensively reviewed by Peters, Palay and Webster (1976) and their observations are summarised in the following sections.

I.B.1 astrocytes

These were identified, using Cajal's gold sublimate method, as star-shaped cells with processes extending into the neuropil. Some of their processes formed end-feet on blood vessels whilst others extended to the surface of the CNS where the end-feet formed the glial limiting membrane or glia limitans. It was appreciated that the astrocytes could be sub-classified into two groups, protoplasmic and fibrous, found predominantly in grey and white matter respectively. These sub-classes were so-called because of the difference in the number of cytoplasmic fibrils seen in the cells.

The fibrous astrocytes are found predominantly or exclusively in the white matter, and are characterised at the electron microscope level as being of a light appearance compared with other cells in the white matter. Throughout the cell body there are many intermediate (8-9nm)

Fig.1 The major glial cell types.



filaments which extend as parallel groups into the processes. The processes from several cells tend to group together to combine nerve fibres into bundles. The end-feet of the processes also form a near-complete layer between the capillary endothelial cells in the white matter and the nerve fibres (this also happens at the glia limitans). Fibrous astrocytes contain few organelles compared with other cell types. Their nuclei are often bean-shaped or irregular and have a deeply-folded membrane. The cytoplasm also contains small, dense glycogen granules which, it is proposed, are localised near neuronal perikarya in areas of high synaptic density. Fibrous astrocytes are physically and chemically connected via gap-junctions with the gap often being occupied by arrays of polygonal subunits (although not all fibrous astrocytes have these arrays). Another type of junction between the cells is the puncta adhaerentia or punctate adhesion.

Protoplasmic astrocytes occur in both white and grey matter areas and also have a light cytoplasm due to the small amount of rough endoplasmic reticulum and free ribosomes. As with the fibrous astrocytes, the protoplasmic variety possess cytoplasmic fibrils. However, there are fewer of these fibrils than in the fibrous type and they occur in bundles rather than filling the cytoplasm. Other features in common with fibrous astrocytes are the formation of end-feet on the capillaries and their contribution to the glia limitans at the surface of the CNS. The nuclei of protoplasmic astrocytes, unlike those of the fibrous type, are round or oval with fewer indentations; the other organelles include microtubules, a centriole and a number of large, elongate mitochondria. The cytoplasm also contains - in common with fibrous astrocytes - glycogen granules. Whereas the processes of the fibrous astrocytes are generally tubular with round or square cross-sections, those of the protoplasmic

astrocytes (especially of the smaller ones) form sheets which often surround synapses. The processes of the protoplasmic astrocytes often follow the outlines of the surrounding tissue but this is not generally true for those of fibrous astrocytes. The processes of the former may be linked by the same types of junctions as found in the fibrous astrocytes i.e. gap junctions and puncta adhaerentia. Even within the class of protoplasmic astrocytes, another sub-grouping has been suggested - the velate protoplasmic astrocyte. This type has been observed only in the cerebellum and is distinguished by having veil-like extensions from the larger processes which pass through the surrounding neuronal material and enclose individual neurons and synaptic complexes.

I.B.2 oligodendrocytes

This glial class was identified and named by del Rio Hortega in 1921 (quoted in Peters et al., 1976) and comprised those cells which had few fine processes (shown by metallic impregnation staining) radiating from spherical or polygonal cell bodies. Oligodendrocytes are found aligned between nerve fibres in white matter (interfascicular oligodendrocytes), in grey matter, (again associated with myelinated fibres) or in grey matter where they are satellite cells closely associated with neurons. At the electron microscopic level, oligodendrocytes are more electron-dense than astrocytes, having a well-developed rough endoplasmic reticulum and many free ribosomes. Their density is also caused by granular material occupying the space between other organelles. The nucleus of the oligodendrocyte can be either round/oval or irregular with clumps of chromatin, and often takes up an eccentric position. Other features in the cytoplasm are a well-developed Golgi apparatus and

many microtubules. In contrast to astrocytes, oligodendrocytes contain few fibrils or glycogen granules (astrocytes have many fibrils but few microtubules). These are some of the structural/ morphological criteria by which astrocytes can be distinguished from oligodendrocytes, others being the cytoplasmic and nuclear densities.

I.B.3. microglia

These cells were finally shown to be different from the oligodendrocytes by the silver carbonate staining method of del Rio Hortega in 1932 (quoted in Peters et al.,1976). The microglia, as the name suggests, are smaller than the astrocytes and oligodendrocytes and are found in both white and grey matter (probably more in grey). They are found throughout the CNS but there is some preferential distribution around blood vessels and neurons. The morphology is of a small cell with wavy projections (often branching to give spines) having little perikaryon and an elongate or triangular nucleus staining very heavily with basic dyes. This description applies, however, only to "resting microglia". Microglia respond in a number of ways when they are "activated" by brain injury and there is still some confusion as to what proportion of activated microglia are endogenous to the brain and what proportion comes in from the blood supply.

I.C Functions of glial cells

Since the discovery of glia as a different cell-type from neurons, many possible functions for these cells have been proposed. The earliest works, generally histological, suggested that glia were simply a structural support for the neuronal elements in the CNS.

However, although this may be one of their functions, much evidence from structural, biochemical, pharmacological and electrophysiological studies has accumulated over the last few decades to support the idea that glia - and particularly astrocytes - have a potentially much wider repertoire of functions than mere physical support.

I.C.1 microglia

Due to the similarity in appearance between the resting microglia in normal brain and the presumed glial precursors in the optic nerves of neonatal rats it has been suggested that, in normal brain, microglia might exist as multipotential neuroglial precursor cells (Vaughn,1969; Vaughn & Peters,1968). However the most-commonly proposed role for the microglia is that of the brain phagocyte. The phagocytic properties of microglia have been observed around nerves during Wallerian degeneration following axonal section (Kerns & Hinsman,1973a,b; Price,1972; Torvik, 1972) and as a result of inflammatory lesions when they proliferate and migrate to the site of injury and, once there, phagocytose the debris. It seems therefore that the major function of the microglia is as a CNS "scavenging dustbin" following injury.

I.C.2 oligodendrocytes

The most prominent and best-documented function of oligodendrocytes appears to be the myelination of axons particularly by the interfascicular oligodendrocytes. The other type of oligodendrocyte - the satellite oligodendrocyte - has been proposed to be involved in neuronal nutrition and it is not yet known whether this cell also has the capacity for myelin production under normal

conditions. The myelinating and nutritional roles of the oligodendrocytes have been proposed for some time now and were suggested partly by anatomical observations. Another function which has been considered recently is interaction with neurotransmitters. They have been shown to take up neurotransmitters (Hökfelt & Ljungdahl,1972a; Messer,1977; Reynolds & Herschkowitz,1984) and to depolarise in response to them (Gilbert et al.,1984). It is also now documented that oligodendrocytes can proliferate in response to brain injury (Ludwin,1984) and when exposed to factors secreted by T-lymphocytes (Merrill et al.,1984) and it has been suggested that they may also have a role to play in facilitating immune responses in the brain (Ting et al.,1981; Wong et al.,1984). These functions have been reported only recently and have been found to be shared by astrocytes (see section I.C.3 for more details)

I.C.3 astrocytes

The astrocyte has been the subject of many studies investigating glial function and there is now evidence to suggest that this cell performs a wide variety of functions other than a structural one. These include involvement in neuronal guidance, extracellular potassium homeostasis, synthesis and release of trophic factors, the blood-brain barrier, repair and regeneration, interaction with neurotransmitters and hormones via receptors or uptake systems and, recently, in CNS immune responses.

I.C.3.a neuronal guidance

During development of the CNS it is often the case that neurons undergo their final mitoses and differentiation at a site other than

where they will lie in the mature brain. They must therefore migrate from their place of development to their final location through a network of other cells. For example, in the cerebellum the stellate, basket and granule neurons originate in the external granule cell layer (a secondary germinal zone) and subsequently migrate inwards to their final locations through the layers of cells which have formed from a primary germinal zone in the roof of the 4th ventricle. It is thought that these migrating neurons are guided on their journey by the radial fibres of the Bergmann glial cells which extend from just below the Purkinje cell layer up to the external granule cell layer (Mugnaini & Forstronen,1967; Rakic,1971b). A similar potential guidance role has also been described in the cerebrum (Rakic,1971a, 1972). It has been shown that the neurons remain attached to the glia throughout their migration (Gona,1978, Rakic & Sidman,1973) even when there are large distances between the various cells (Rakic,1972) and it has been suggested that the guidance by glial cells may help ensure faithful mapping of the ventricular surface on to the cerebral cortex (Rakic, 1978). The mechanism of migration is poorly understood at present, however, it is known that there is a molecular recognition between the neuron and glial cell (Gottlieb & Glaser,1980) and it has been proposed that the migration itself involves a membrane addition mechanism (Bray, 1973; Rakic et al.,1974). The role of astrocytes in neuronal guidance has been confirmed also by many studies using mutant mice with cellular defects (Caviness Jnr. & Rakic,1978; Pinto-Lord et al.,1982; Rakic & Sidman, 1973; Sotelo & Changeux,1974), pharmacologically-lesioned animals (Sotelo & Rio,1980) and by looking at the interactions between neurons and astrocytes in cell cultures (Hatten & Liem,1981; Hatten et al.,1984).

I.C.3.b extracellular potassium homeostasis

One of the consequences of neuronal activity is that the extracellular potassium concentration ([K]o) is elevated from its resting level of around 3mM to somewhere between 10 and 20mM (Futamachi et al.,1974; Orkand et al., 1966; Pedley et al.,1976; Somjen et al.,1976). If this increased level were to remain for some time, the ability of the neuron to function normally would be affected since the propagation of an action potential depends on the ion-distribution across the membrane. It has been proposed by several investigators (Henn et al.,1972; Hertz,1977; Hertz & Schousboe,1975) that an important means of lowering the raised [K]o is by uptake into astrocytes. This may occur by the spatial buffer effect (Orkand et al.,1966; Trachtenberg & Pollen,1969) whereby potassium from a local area of high [K]o passively traverses the glial membrane (which is highly and selectively permeable to potassium ions) with a simultaneous release of potassium from the same cell into a region of normal [K]o. Alternatively there may be an active accumulation of potassium into the astrocyte (Walz et al.,1984). To date, however, there is little evidence to show that these mechanisms would have more than a minor effect in lowering the [K]o from 20mM to 3mM (see Gardner-Medwin,1981 and Varon & Somjen, 1979 and refs therein), and the consensus of opinion is that the neurons themselves play a considerable part in recovering released potassium. Despite this, the glial cells may still have a role to play in the "fine tuning" of the [K]o. Even though the neurons are able to recover a large proportion of the potassium released during activity, this recovery is unlikely to be instantaneous therefore there will be some diffusion away from the axon and into the glia thus creating a localised potassium deficit. Under these

circumstances the potassium-permeable astrocytes may have an important function in balancing potassium levels from area to area by the spatial buffer mechanism described earlier.

I.C.3c The blood-brain barrier

The first evidence for the existence of a blood-brain barrier (BBB) was that of Ehrlich, 1885 (quoted in Dobbing, 1961) who showed that some aniline dyes injected into the bloodstream stained all tissues of the body except the CNS. Since that time this phenomenon has been shown to occur with many other substances and many studies have been concerned with elucidating the nature of the BBB. The two obvious physical barriers between the bloodstream and neurons are the capillary endothelial cells and the perivascular astrocytic end-feet; both of these have, at some time, been proposed as the basis of the BBB. There is now, however, a large amount of evidence supporting the capillary endothelial cells as being the site of the barrier rather then the astrocytes. A major body of evidence against the astrocytic BBB is the many demonstrations that the perivascular endfeet do not form a continuous sheet (Maynard et al.,1957; Patek,1944) and, in support of this, there are now reports showing the impermeability of the endothelial cells and the tight junctions they form with each other (Reese & Karnovsky,1967). What is considered a more likely role for the astrocytes with respect to their relationship to the capillaries is that they regulate the distribution of materials that do cross from the blood into the cerebrospinal fluid (as well, of course, as extracting what they require for their own survival).

I.C.3.d Repair and regeneration

When the brain becomes injured either by physical damage from outside or by cell death from whatever reason, the site of injury becomes enriched in glial material. This "filling in" is usually referred to as scarring and the particular cells involved are termed reactive astrocytes. The properties of reactive astrocytes have been studied and they have been found to differ in several ways from the normal astrocytes. They have larger nuclear diameters (Cavanagh, 1970; Federoff et al.,1984), an increased content of DNA (Lapham & Johnstone,1964), and elevated levels of GFAP (Amaducci et al.,1981) and vimentin (Dahl et al.,1981b, 1982). The origin of these cells was, for many years, obscure: it had been proposed that they arose by amitotic division (Lapham,1962) but it now seems most likely that they originate by hyperplasia and hypertrophy from normal astrocytes (Barrett et al., 1981; Latov et al.,1979) or perhaps from astroblasts (Federoff et al.,1984). The nature of the stimulus that induces the injury-related astrocytosis has not yet been elucidated. The physical damage itself, although it may be the primary stimulus, does not seem to trigger the response directly since it has been shown that reactive gliosis occurs at sites distant from stab wounds (Barrett et al.,1981). Other suggested stimuli include nerve-degeneration, myelin breakdown, ionic imbalance and influx of serum proteins (Barrett et al.,1981; Brotch et al.,1978; Klatzo,1967; Osterberg & Wattenberg, 1962). Recent reports have also shown that astrocytes (and oligodendrocytes) in vitro proliferate in response to factors secreted by cloned, virally-transformed or phytohaemagglutinin-activated T-lymphocytes and B-cells (Merrill et al.,1984). Since T-lymphocytes have been observed in normal white matter as well as in the demyelinated regions found during multiple

sclerosis (Kreth et al.,1982; Traugott et al.,1982), it seems that T-cell secreted factors may also have a role in *in vivo* gliosis. Another potential trigger for astrocytosis may be myelin basic protein since this has been shown to stimulate proliferation of astrocytes (and lymphocytes) *in vitro* (Sheffield & Kim,1977) and this protein is known to be released under demyelinating conditions such as multiple sclerosis (Whitaker,1976). The functions of reactive astrocytes are not yet clearly defined, however, phagocytosis of cellular debris, enhancement of neuronal survival and reconnection, and a physical limitation of the necrotic area are all possibilities. The evidence for phagocytosis is largely circumstantial such as the observation of many cytoplasmic inclusions (Maxwell & Kruger,1965) although these have included lipid droplets and myelin fragments (Vaughn & Pease,1970). It has been reported that following brain injury, and corresponding with the increase in GFAP, there is secretion of neuronotrophic protein around the site of the wound (Nieto-Sampedro et al.,1982) which may be produced by the astrocytes. Another protein produced by the reactive (but not mature, normal) astrocytes is the basement membrane glycoprotein laminin (Liesi et al.,1984b) to which neurons are reported to attach preferentially and on which they display enhanced neurite outgrowth (Baron van Evercooren et al.,1982; Liesi et al.,1984a; Rogers et al.,1983). These observations suggest that rather than filling up space vacated by degenerating neurons, the reactive astrocytes may actively encourage neuronal survival and reconnection. However it has been suggested that, in the mammalian CNS, the glial scarring following injury actually prevents the regeneration and reconnection of neurons (Reier et al.,1983).

I.C.3.e Neuronotrophic factors

That non-neuronal cells might have some trophic influence on neurons was suggested as early as 1928 (Ramon y Cajal, quoted in Müller et al.,1984). During the 1970s, many reports demonstrated that glia from both the PNS and CNS, or factors released from them, could promote neurite outgrowth (Banker,1980; Barbin et al.,1984; Barde et al.,1978; Lindsay et al.,1979; Monard et al.,1973; Muller et al.,1984; Sensenbrenner & Mandel,1974; Whatley & Lim,1981). Most of these factors, when characterised, have been found to be proteins or polypeptides (they are non-dialysable, resistant to extremes of pH and temperature, and resistant to proteases and peptidases). The most celebrated of these is nerve growth factor (NGF) discovered in 1951 (Levi-Montalcini & Hamburger,1951) and thought to be critical in the development and survival of sympathetic and sensory neurons (Levi-Montalcini & Angeletti,1968), involved in the maintenance of neuronal differentiated states (Stoeckel & Thoenen,1975) and may have a chemotactic guiding influence during neuronal migration (Campenot,1977). Recently the actions of trophic agents having a low molecular weight and of a non-protein nature have been reported. One such factor has been shown to be pyruvate (Varon et al.,1984; Selak et al.,1985).

I.C.3.f Accessory cells in the immune system

Since the blood-brain barrier and the lack of lymphatic ducts seems to isolate the brain from many circulating proteins and cells there is much interest in the relationship between the brain and the immune system. In 1981 it was shown that a subpopulation of murine brain cells-probably oligodendrocytes - expressed Ia antigen on the cell

surface (Ting et al.,1981). The authors speculated that these cells may be tissue macrophages (because of the expression of Ia antigen on macrophagous cells in other tissues) and that they may also function as antigen-presenting cells in common with other Ia-bearing accessory cells. It has since been shown that Ia is also expressed on astrocytes and that this is induced by γ-interferon (as is H-2 antigen) (Hirsch et al.,1983; Wong et al.,1984). Recently it was demonstrated that astrocytes do in fact present antigen - in this case myelin basic protein to encephalitogenic T-cells (Fontana et al.,1984) - so it seems that astrocytes are capable of acting as accessory cells in immune responses. This is further supported by the observation that astrocytes stimulated in a number of ways can release an interleukin-1-like substance (Fontana et al.,1982).

I.C.3.g Interactions with neurotransmitters: receptors and uptake.

That neurons communicate with each other by releasing neurotransmitters that bind to specific receptors on a second neuron (within which a number of changes may occur as a consequence of the binding) is well-established. Since the early 1970s a substantial literature has accumulated showing that glial cells (mainly astrocytes) also have the ability to bind and respond to a variety of intercellular signalling molecules. The initial studies showed that in response to stimulation by catecholamines there was an increase in the cytoplasmic concentration of cAMP in glial cells (Clark & Perkins,1971; Gilman & Nirenberg,1970; Newburgh & Rosenberg, 1972; Schimmer,1971), and since then there have been reports showing the presence on glia of receptors (or at least high-affinity, sodium-independent binding sites) for many other neurotransmitters and neuropeptides. These have been reviewed by van Calker &

Hamprecht (1980) and by Henn & Henn (1980).

It has also been shown, unequivocally, that in addition to possessing receptors, astrocytes have the capacity to remove neurotransmitters from the extracellular fluid by selective, sodium-dependent, carrier-mediated active transport systems. One of the earliest reports of glial uptake of neurotransmitters was that of Henn & Hamberger (1971) using bulk-isolated cells. Since that time astrocytic uptake has been shown for almost all the established and some putative neuro-transmitters (with the exception of acetylcholine which is hydrolysed extracellularly by cholinesterases). These include noradrenaline, serotonin, dopamine, GABA, glycine, glutamate, taurine, leucine, lysine, aspartate and β-alanine (Hannuniemi & Oja,1981; Henn & Hamberger,1971; Henn et al.,1974; Kelly & Dick,1975; Schon & Kelly, 1974). The precise role of these uptake systems has not yet been satisfactorily resolved. A frequently suggested reason for their existence is the removal of released neurotransmitter from the perisynaptic area thus quenching synaptic transmission and a recent report has shown directly that the responses of individual neurons to GABA were attenuated by a Na+dependent uptake system (Brown & Scholfield, 1984). However, it has been known for some time that neurons also have Na+-dependent, high-affinity transport systems for transmitters; consequently there is considerable debate over the relative contributions of the glial and neuronal carriers to transmitter uptake. This confusion is not helped by the wide range of values of the kinetic parameters that have been determined for the glial uptake systems (e.g. see Table 12 for GABA, the most studied of the uptake systems). Since many of the transmitters have functions other than intercellular signalling, e.g. protein synthesis, nucleic acid synthesis, energy metabolism etc., it is

possible that the observed uptake systems subserve these processes instead of or as well as the inactivation of neurotransmission. The potential significance of GABA transport by astrocytes will be discussed more fully in Chapter IV.

One of the reasons for the widely-varying values obtained for Vmax and Km of the astrocytic neurotransmitter transport systems is very probably the great range of preparations being used to represent astrocytes. These include cultured cells, freshly-isolated cells, and a number of whole tissue preparations (e.g cortical slices and retinae) taken from a variety of species. The advantages and disadvantages of some of these will be outlined in the following sections.

I.D Methodological approaches

A major obstacle to many attempts to establish, in detail, the properties of individual cell-types in the brain is the great complexity of the CNS. The many types of neurons and glial cells are enmeshed in an intricate network of cellular processes - both glial and neuronal-such that it is virtually impossible to perform studies at a molecular level on a single cell type without having to consider the possible interference from the surrounding cells. Furthermore the more intact the tissue is, the more difficult it will be to determine and control conditions of, for example, oxygen, pH, and metabolite concentrations around the cells of interest, due to diffusion barriers or uptake and metabolism by other cells. In response to these difficulties several attempts have been made to develop experimental systems that will minimise these problems whilst maintaining the normal functions of the cells, but all these procedures have their disadvantages as well as advantages.

The least disruptive of the approaches is to cut slices from the tissue under investigation. These slices have the advantages that all the cells have the same positions and, therefore, anatomical relationship to each other as *in vivo* and that the blood-brain barrier has been removed. Many diffusion barriers remain however (especially in the thicker slices) and there is still the same cellular complexity as found in the whole brain. For these reasons there is a limited range of types of experiment that can be performed successfully on these preparations.

A potentially simpler system can be formed by separating the cells from each other and then fractionating them into groups, each containing a single cell-type. This is frequently done by chopping the tissue into small pieces then breaking the intercellular contacts either by enzymatic digestion (Norton & Poduslo,1970) or by mechanical means such as passing through nylon or steel meshes. These crude cell isolates are subsequently fractionated by centrifugation through sucrose or Ficoll density gradients or under gravity through a BSA gradient (Chao & Rumsby,1977; Farooq & Norton,1978; Nagata et al., 1974; Poduslo & Norton,1975; Wilkin et al., 1976). Preparations of this type have the advantage that all the diffusion barriers have been removed allowing the environment around each cell (or perikaryon) to be precisely controlled. New problems do arise, however, in that a certain amount of mechanical damage is caused during the isolation (this becomes more severe the older the age of animal from which the cells are derived) and the use of proteases during isolation creates the possibility for hydrolysis of cell-surface components. Furthermore the fractionated cell popuations are by no means pure and it may be difficult to determine in many studies what contribution the contaminant perikarya make to

the overall measurements (Cohen et al.,1979; Cohen et al.,1980; Wilkin et al.,1976). In view of these problems the use of bulk-isolated cells is becoming less frequent and many studies now make use of cultured cells.

Cultured cell preparations can be broadly divided into two types cell lines and primary cultures. The use of cell lines is common and they have some advantages over the primary cultures. Cell lines are produced usually by chemical or viral transformation of normal cells from the tissue under study. The essential features of these cells are that they can exhibit differentiated properties and that their tumourous nature makes them "immortal" in that they can be repeatedly subcultured to provide very large numbers of a single cell type. One of the most-commonly used cell lines for glial study is the C6 glioma. This was formed by culturing of N-nitrosomethylurea-induced primary tumours from rat brain and was shown to exhibit some glial characteristics (Benda et al.,1968; Benda et al.,1971). A problem with such tumour-derived cell-lines is their uncertain relationship to normal cells. They have already lost the normal physiological control over division and it has been shown that the expressed phenotype can be different to that displayed <u>in situ</u>. For example the C6 glioma line was considered to be an astrocytic line because of its expression of S100 protein. However GFAP (the most-often used astrocyte marker) was expressed by C6 only under certain conditions of growth (Bissell et al.,1974) and the cells also expressed cyclic nucleotide phosphohydrolase and glycerol-3-phosphate dehydrogenase, thought primarily to be markers for oligodendrocytes (McGinnis & de Vellis,1978; Zanetta et al., 1972). Thus it seemed that the C6 line, and therefore possibly other cell-lines, displayed a mixed astrocyte/ oligodendrocyte phenotype which, as is the case for significantly

impure cell fractions, could lead to difficulties in the interpretation of results. Another problem with cell-lines is that they can be unstable in terms of chromosome number (the diploid lines being the most stable).

The alternative to cell-lines, namely primary cultures, have different benefits and disadvantages. They have the same major technical advantage in that there are few diffusion barriers so their environments can be precisely regulated and manipulated. Another advantage over bulk-isolated cells (and one shared with cell-lines) is that although the cells for culture are often isolated by the same methods, they are generally used some days after dissociation and plating, allowing time for enzymic or mechanical damage to be repaired. They are possibly more representative of the *in vivo* cell because they are not tumour-derived but their non-neoplastic nature means that a new preparation has to be made for each set of experiments. This allows a a "batch variation" to occur which may or may not influence the degree to which different cultures can be compared. A much-discussed topic regarding primary cultures is the degree of differentiation of the cells present. As a result of dissociation then plating, the cells are removed from their normal chemical and cellular environment and therefore from many influences that might control the degree of expression of normal function. It has been observed by many authors that the expression of a variety of characteristics is different *in vitro* compared with *in vivo*. When considering astrocytes the most obvious difference between cells seen *in vitro* and those observed *in vivo* is in the morphologies of the cells. Astrocytes *in situ* as identified by the metallic impregnation staining methods are generally stellate in shape (hence their name). Astrocytes grown in monolayer culture, however,

frequently adopt a polygonal or epithelioid shape unless treated with any of a number of agents e.g. dBcAMP, certain hormones and "factors" or by removing serum from the culture medium (Booher & Sensenbrenner, 1972; Haugen & Laerum,1978; Hertz et al.,1978; Lim & Mitsunobu,1974; Lim et al.,1976,1977; Pettman et al.,1980; Sensenbrenner et al.,1980) Under the latter conditions the cells often extrude processes and the specific activities of certain proteins change such that the cells appear to go through a morphological and biochemical maturation. Occasionally astrocytes *in vitro* develop a stellate morphology without the need for any manipulation of the medium (Eng & DeArmond,1982; Hatten & Liem,1981; Lindsay et al.,1982; Manthorpe et al.,1979; Pruss et al.,1982; Raff et al.,1979; Stallcup et al.,1981; Trimmer et al., 1982), and it seems important to determine what the relationship is between the spontaneously-occurring and the induced stellate astrocytes and between the cultured astrocytes and those *in situ* or *in vivo*. Until this has been done and the degree of differentiation of the cells assessed, the value of the tissue-culture approach in determining the properties of and gaining an insight into the functions of astrocytes *in vivo* will be limited.

I.E Cell-type-specific markers

One way in which different cell types in the CNS and in preparations therefrom can be compared is by the use of a set of reference markers that can identify individual cell types and/or stages of differentiation of any cell type. Over the last two decades such a set of markers has been built up for the major cell types in the CNS.

Due to the complexity of the intact nervous system and the potential

cellular contamination problems with isolated cell preparations (including cultures), some of the most useful markers have been proteins and other molecules against which antibodies have been raised. These have the advantage that they can be used to identify directly the cells expressing the marker molecule whether in situ or in vivo. Many types of molecule (but mostly proteins) have been used for this purpose - cytoskeletal proteins, enzymes, soluble regulatory proteins and a variety of protein, carbohydrate and glycolipid components of the cell membrane. Additionally, the advent of monoclonal antibody technology (Kohler & Milstein, 1975) has allowed the localisation of often-unidentified molecules to particular cell types and these are being increasingly used as cell-type markers (see e.g. Schachner et al., 1982a,b) Some of the well-established markers (detected either biochemically or immunologically) are listed in Table 1 with the cell types with which they are associated. (There are now so many monoclonal antibodies against poorly-characterised or unidentified CNS antigens that these are not listed). It should be noted that the cellular specificities in the table are pertinent only to CNS cells: some of the markers are expressed on different cells in the periphery. Other physiological or pharmacological markers can also be used for comparison of cells in situ with those in vivo; these include neurotransmitter uptake and neurotransmitter-receptor binding studies. The former can give almost the same cellular resolution as the immunological approach if radiolabelled neurotransmitters are used followed by autoradiographic localisation.

I.F The present study

The present work is concerned with the properties of and relationship

between two different forms of astrocyte observed in primary cultures derived from dissociated cerebellum from neonatal rat. As was mentioned in section I.D, two morphological types of astrocyte can be seen in cultures-stellate and epithelioid. In a study of amino-acid neurotransmission Gordon (1982) observed that these two types were able to accumulate 3H γ-aminobutyric acid (GABA, an inhibitory neurotransmitter) to different degrees. This morphological and physiological information suggested that the stellate and polygonal cells may represent differentiated and undifferentiated astrocytes respectively. In the present study several criteria were used to determine whether this was indeed the case. The neurotransmitter transport was investigated in more detail and the properties of the uptake into the stellate cells were compared with uptake into the epithelioid cells. The transport into both cell types was also compared with uptake by cells *in situ*. The different cell types in culture were characterised using some of the molecular markers listed in Table 1 to determine whether there were any antigenic differences between the cells. In addition, antibodies were raised against two neurotransmitter-related enzymes which might be useful as differentiation markers. Finally, interconversion of the two astrocyte forms was investigated using a number of manipulations of the culture conditions.

Table 1 Cell-type-specific markers

marker	cell type	ref
carbonic anhydrase	oligo astro	a,b, c
cyclic nucleotide phosphohydrolases	oligo	d,e,
α,α-enolase (non-neuronal enolase)	astro	f
γ,γ-enolase	neuron	g
galactocerebroside	oligo	h,i j
GFAP	astro	k,h a
glutamine synthetase	astro	l,
glycerol-3-phosphate dehydrogenase	oligo	n
α2-glycoprotein	astro	o,p
myelin basic protein	oligo	q,r
neurofilament protein	neuron	s
S100	astro	t,u v
tetanus toxin	neuron astro	w,x h

a Schachner et al.,1977
b Langley et al.,1980
c Kimelberg et al.,1982
d Nishizawa et al.,1981
e Poduslo & Norton,1972
f Langley et al.,1981
g Marangos et al.,1978
h Raff et al.,1979
i Raff et al.,1978
j Zalc et al.,1981
k Eng & Bigbee,1978
l Norenberg,1979
n Leveille et al.,1980
o Warecka & Bauer,1967
p Langley et al.,1982
q Sternberger et al.,1978a
r Sternberger et al.,1978b
s Anderton et al.,1980
t Matus & Mughal,1975
u Ghandour et al.,1981b
v Ghandour et al.,1981a
w Mirsky et al.,1978
x Dimpfel et al.,1977

II MATERIALS AND METHODS

II.A Rats.

The rats used were of the Sprague-Dawley strain and were obtained from the breeding colony in the Imperial College Biochemistry Department animal unit. The ages of animals used were 2-13 days post-natal for culture and 12 days for slice-uptake experiments. Animals had full access to food and water (in the case of suckling animals this was true for the mothers). The day of birth was designated day 0 and had error limits of $\pm$ 12 h.

II.B Cell Culture.

II.B.1 Preparation of Culture Dishes

Dissociated cells were seeded into 35mm diameter, sterile, polystyrene culture dishes or 24-well multiwell plates each containing a No2-thickness glass coverslip (22x22 mm^2 or 13mm diameter) which had been sterilised with ethanol and flamed dry. Prior to seeding, the dishes and coverslips were coated with filter-sterilised poly-L-lysine (5 μg/ml in H_2O, overnight) to facilitate cell attachment. The poly-L-lysine was removed and replaced with 1ml of DMEM and the dishes returned to the incubator until seeding.

II.B.2 Media

The media used were Dulbecco's Modification of Eagles Medium (DMEM) (Dulbecco & Freeman,1959; Smith et al.,1960) and Bottenstein-Sato Medium (Bottenstein & Sato,1979). DMEM was a commercial preparation to which was added L-glutamine (300 mg/l), gentamicin (100 mg/l), and foetal calf serum (10%). A further addition of KCl (1.864 g/l final concentration) was made when the medium was to be used for neuronal

culturing. Bottenstein-Sato medium was made from a 50:50 mixture of commercial preparations of Ham's F 12 medium (Ham, 1965) and DMEM to which were then added L-glutamine (300 mg/l), gentamicin (100 mg/l), insulin (5 mg/l), progesterone (20 nM), transferrin (100 mg/l), sodium selenite (30 nM) and putrescine (100 μM). This medium did not contain serum of any type. Due to the potential degradation of medium components (especially L-glutamine), complete media were not used later than 1 month after preparation if possible. Whenever this did happen the medium was boosted with L-glutamine as in the original preparation.

II.B.3 Astrocyte culture

(a) Cerebellum

Tissue was dissociated to give a single-cell suspension by a procedure based on a modification (Dutton et al.,1981) of the method of Wilkin et al. (1976). Rats of appropriate age were killed by decapitation. Cerebella were immediately dissected out and cut at 400μm intervals in two mutually-perpendicular directions using a McIlwain chopper (Mickle Laboratories) then transferred to Krebs-bicarbonate solution (121mM NaCl, 4.8mM KCl, 1.2mM KH_2PO_4, 25.5mM $NaHCO_3$, 1.23mM $MgSO_4$, 14.3mM glucose and 0.3% (W/V) BSA, pH 7.4). Cerebella were chopped in groups of four, and transferred to Krebs solution until used (routinely 5 min.). The chopped tissue was centrifuged at 50-100 x g for 15s and the Krebs removed by suction after which the tissue was resuspended in 10ml Krebs containing 0.025% (W/V) bovine pancreatic trypsin (Sigma, Type III) and incubated for 15 min at 37°C with shaking (10ml per 4 cerebella in 25 ml sealed Erlenmeyer flasks). Flask contents were then mixed with an equal volume of Krebs containing 1.28% bovine pancreatic deoxyribonuclease 1, 8.32% soya bean trypsin inhibitor (Sigma, Type

1-S) and an additional 24.8μM $MgSO_4$. Trypsinised tissue was centrifuged at 50-100 x g for 15s , the Krebs removed and replaced with 2ml of Krebs containing 0.008% DNase, 0.052% SBTI and an additional 1.55mM $MgSO_4$. A single-cell suspension was then obtained by triturating the tissue using sterile Pasteur pipettes (250mm long, 1mm nozzle diameter, plugged with cotton-wool) until the fluid appeared milky (15-20 strokes). Any aggregates were allowed to settle over a 10 min period and the milky suspension transferred to 3ml of Krebs containing an additional 1.24mM $MgSO_4$ and 98.4 μM $CaCl_2$. Any remaining cell clumps were again allowed to settle over 10 min and removed by Pasteur pipette before centrifuging the cells at 50-100 x g for 15 min. The pelletted cells were resuspended in 3-5 ml of Dulbecco's Modified Eagle's Medium (DMEM) containing 4500mg/l glucose, no sodium pyruvate, no glutamine and supplemented in the laboratory with 10% foetal calf serum, 2mM L-glutamine and 100 mg/ml gentamicin before counting using an Improved Neubauer haemocytometer. The suspension was adjusted to contain 1 million cells/ml DMEM and the cells seeded at a density of 1 million cells per 35mm pre-prepared culture dish (2.8.1) already containing 1 ml of gassed medium at 37°C. The cells were maintained at 37°C in a water-saturated atmosphere of 9% CO_2 in air.

(b) spinal cord and optic nerve

Due to the smaller amount of tissue available in these cases, a simpler procedure was followed to minimise cell-loss. Rats of 2-4 days old were killed then the tissue dissected out as before (II.B.3a with the exception that optic nerve was chopped only in the direction perpendicular to its long axis) and stored in Earle's balanced salts solution (BSS; 116mM NaCl, 5mM KCl, 0.8mM $MgSO_4$, 1mM NaH_2PO_4, 26.2mM $NaHCO_3$ 1.8mM $CaCl_2$, 5mM D-glucose, 10mg/l phenol red). After chopping, tissue

was immediately incubated in 0.025% trypsin in 5 ml BSS for 15 min at 37°C with shaking. This mixture was then added to 5 ml DMEM containing 10% foetal calf serum and the tissue triturated for about 25 strokes. Dissociated cells were spun down as before, resuspended in fresh DMEM/FCS then plated on poly-L-lysine-coated coverslips in 24well dishes. Initially the cells were plated in a volume of 50 µl then the volume was made up to 1 ml after 1-2 hours; this was done to promote a greater seeding-density which allowed better cell growth. Again, to minimise cell-loss, cells were not counted in this procedure; cells derived from 16 animals were used to seed 12 - 16 wells of a 24-well plate (well-diameter = 16mm).

All manipulations, unless otherwise stated, were performed at room temperature in polystyrene (15ml) or polypropylene (50ml) conical tubes (Falcon). All solutions were sterilised before use by passing through a 0.22µm Millipore filter. Except during centrifugation, tubes were kept in a Hepair laminar flow hood to maintain, as far as possible, aseptic conditions. Before use, Pasteur pipettes and trypsinisation flasks were heat-sterilised (120°C, 1 h) then dried under vacuum.

II.B.4 Neuronal Culture

The procedure for neuronal cell culture was the same as for astrocytes with the following exceptions. The DMEM was supplemented with KCl to a final concentration of 24mM (Lasher & Zagon,1972) and cells were plated at a density of 2.25 million per dish. In addition, cytosine arabinofuranoside (cytosine arabinoside, araC) was added to the culture to a concentration of 10 µM,18 h after plating,in order to arrest the division of astrocytes and other mitotically-competent cells.

II.C Neurotransmitter Transport

II.C.1 Uptake by Cultured Cells

Cells grown as described in II.B.3 were washed with 3x1ml of tris-buffered Krebs solution (128mM NaCl, 1mM $CaCl_2$, 1.2mM $MgSO_4$, 5mM KCl, 5mM Na_2HPO_4, 10mM tris base, buffered to pH 7.4 with HCl) containing 10mM glucose, 0.1% BSA and 10μM amino-oxyacetic acid (TBK). The TBK for washing was maintained at 37°C in a water bath. After the third wash, the TBK was aspirated from the cells and replaced with 900μl of fresh TBK. The cells were preincubated in this for a period of 30 min at 37°C. with gentle shaking. The equipment for this consisted of an orbital shaker (Luckhams) operating at 40 rev/min on top of which was placed a photographic hot plate (Photax) maintained thermostatically at 37 ± 0.5°C.. The thermostat in the hot plate, as purchased, was replaced in the Imperial College electronics workshop in order that this narrow temperature range could be maintained. Temperature was monitored using a thermocouple connected to a digital display unit (Comark) . The thermocouple was inserted through a hole made in the side of a Parafilm-sealed 35mm culture dish containing 1ml of TBK and the unit placed on the rotating hot plate. Temperature was checked at 5 min (approx.) intervals. After the 30 min preincubation, 100μl of ^{3}H-neurotransmitter solution (or mixture of ^{3}H-neurotransmitter and other reagents) in TBK was added to the cells to give the required final concentration at the desired specific radioactivity. In auto-radiographic studies the concentrations ranged from 0.5-21μM with specific radioactivities of 96 mCi/mmol-5μCi/nmol. In kinetic studies the concentrations and specific radioactivities were 0.5-500μM and 0.005-1μCi/nmol respectively. Incubation with the radiolabelled trans-mitter was continued for 5min then the cell-coated coverslips removed

from the dishes with forceps. Excess radiolabel was removed by immersing the coverslips for 2-3s (with gentle circular movement) in 3x250ml volumes of phosphate-buffered saline (PBS; 0.9% NaCl, 0.01M sodium phosphate, pH 7.4). Cells were then processed for autoradiography (II.D) or for protein and radioactivity determinations (II.K and II.L). Control experiments were performed exactly as described above except that the Na^+ ions in the TBK were replaced with choline. The composition of the Na^+-free buffer was 133mM choline chloride, 1 mM $CaCl_2$, 1.2 mM $MgSO_4$, 5 mM K_2HPO_4, 10 mM tris base (buffered to pH 7.4 with HCl), 10 mM glucose, 0.1% BSA and 10uM amino-oxyacetic acid.

II.C.2. Uptake by cerebellar slices.

Neurotransmitter uptake by cerebellar slices was carried out essentially as described by Levi et al. (1982). Rats, 8day old or 13 day old, were killed by decapitation and the cerebella dissected out immediately. 400μm saggital slices were cut using a McIlwain chopper and transferred immediately to a continuously-gassed (95% O_2/5% CO_2 Krebs medium (KM; 118mM NaCl, 4.7mM KCl, 2.5mM $CaCl_2$, 25mM $NaHCO_3$, 1.18mM KH_2PO_4, 1.19mM $MgSO_4$, 11mM glucose, pH 7.4) containing 10μM aminooxyacetic acid. Slices were separated using a dissecting needle and handled using a Pasteur pipette which had been broken just at the beginning of the taper and the broken edges flamed smooth. Slices were then incubated, with gentle shaking, for 1.5h in continuously-gassed KM at 37°C: 8ml KM was used per 6 slices in a 25ml sealed Erlenmeyer flask. Following this preincubation, the slices were transferred to freshly-gassed KM or KM + GABA or KM + inhibitor (as required) in 25ml Erlenmeyer flasks with the same slice:medium ratio as before. Incubation was continued for 30min after which ^{3}H-GABA was added to a final concentration of 0.5 μM, and specific radioactivity of

5 μCi/nmol. Incubation in the presence of radiolabel was continued for 30min under continuous gassing as before. The contents of each flask were next emptied into separate petri dishes then the slices transferred to petri dishes containing 25 ml of freshly-gassed Krebs to remove excess ^{3}H-GABA. Accumulated GABA was fixed in the cells by incubating the slices in 0.1M sodium phosphate buffer (pH 7.4) containing 4% paraformaldehyde/2.5% glutaraldehyde for 30min at room temperature. Fixative was removed by washing the slices with 3x2ml 0.1M cacodylate buffer (pH 7.4) then the tissue postfixed overnight in 1% OsO_4 in cacodylate buffer at 4^{o}C. The OsO_4 was removed and the slices washed with 3x2ml cacodylate buffer before dehydration through a series of alcohols: 10 min in each of 30%, 50%, 70% and 90% (v/v) ethanol followed by 2x15 min in 100% ethanol. Dehydrated slices were embedded in plastic for sectioning. Slices were incubated in propylene oxide for 2 x 10min followed by 30 min successively in 2:1, 1:1 and 1:2 ratios of propylene oxide:resin then overnight in 100% resin. The resin consisted of 26g nonenyl succinic anhydride, 10g cyclohexene dioxide, 8g diglycidyl ether of polypropylene glycol and 0.4g dimethylaminoethanol, well-mixed in that order (Spurr, 1969). Following penetration of the slices by resin, they were embedded in fresh resin which was then cured by baking at 70^{o}C for 10h. 1 μm tissue sections were cut using glass knives on an LKB ultramicrotome, mounted on gelatin/alum-coated microscope slides then processed for autoradiography (II.D).

II.D Autoradiographic Detection of Transported Neurotransmitters in Cells and Slices.

II.D.1 Exposure to photographic emulsion

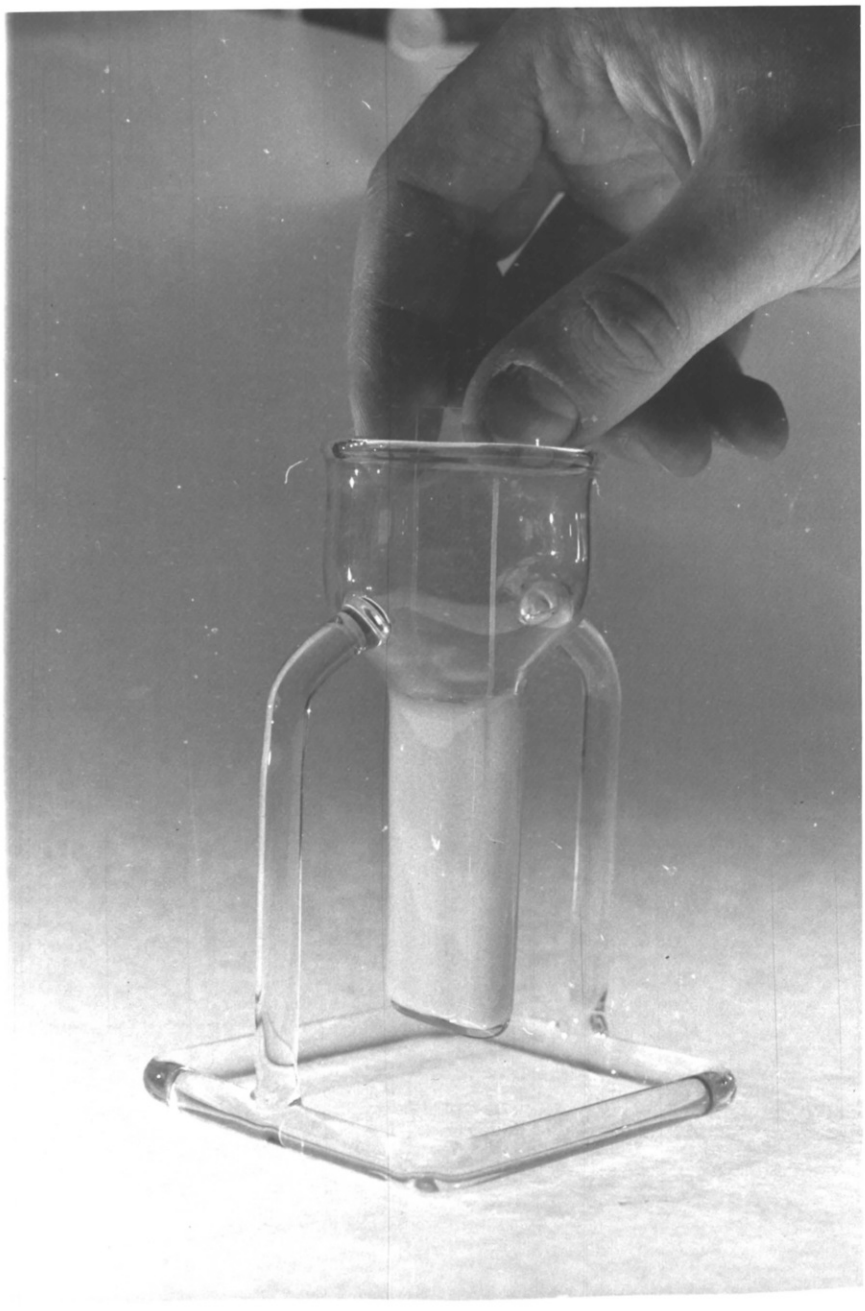
Transported radiolabelled compounds were fixed within the cells by immersing the cells in glutaraldehyde (5% in 0.1M sodium phosphate buffer, pH7.4) for 30 min at room temperature. Cells were next washed with 3x1ml of PBS then allowed to dry overnight under a suitable cover to protect from dust. The dried coverslips were attached, cell-side up, to glass microscope slides using "Loctite Glassbond" adhesive (Loctite U.K. Ltd.) then coated with K5 nuclear emulsion (Ilford) by the dipping technique. 15g of emulsion was melted at 43^{o}C. in the dark and diluted with 22.5ml of water also at 43^{o}C then transferred to a pre-warmed dipping chamber as shown in Fig.2. The diluted emulsion, maintained at 43^{o}C., was stirred with a glass rod (slowly, to avoid air bubbles forming in the mixture) and the slides immersed in and slowly drawn out of the emulsion. Excess emulsion was wiped from the back of each slide with a tissue and the slide left to dry overnight in a vertical position. During the dipping procedure, the emulsion was stirred after every third slide. Dried, coated slides were transferred to a plastic slide-box containing silica gel (as dessicant), the box sealed with sellotape then placed in a light-proof photographic bag for exposure at 4^{o}C. for times ranging between 4d and 6 weeks. Cerebellar slices mounted on glass slides were processed for autoradiography using the same procedure.

II.D.2 Development

After the required exposure time, emulsion coated cells or slices were removed from the light proof bag under safelight (Ilford S902) and

Fig.2 Dipping chamber for preparing emulsion-coated slides.

During dipping, this is immersed to the top of the narrow area in a water bath at 43 ^{o}C.



allowed to equilibrate to the darkroom temperature of 17°C. The autoradiographic image was then developed by immersion of the slide in Kodak D19 developer at 17°C for 3 min without agitation. Development was stopped by immersing the slide in 1% acetic acid (v/v in distilled water) for 1 min (this also prevents swelling and softening of the emulsion). The emulsion was hardened further by standing for 3 min in a solution of 3% chromic potassium sulphate / 6% anhydrous sodium sulphate. After washing for 1 min in distilled water, the autoradiographs were fixed in 25% sodium thiosulphate for 3 min (this solubilises any silver bromide remaining in the emulsion and leaves the emulsion transparent). Finally, the fixed autoradiographs were washed thoroughly in several changes of distilled water then air-dried. All solutions used during the development were at 17°C and all procedures were carried out under safelight illumination.

II.E Staining of autoradiographs

Developed autoradiographs of slices were stained with toluidine blue; those of cultured cells were stained with toluidine blue (in earlier experiments) or with haematoxylin and eosin (later experiments).

II.E.1 Toluidine blue

Cells were stained for 60s with 0.1% toluidine blue in 1% disodium tetraborate (borax, pH 9) then washed to the desired colour-intensity with distilled water. Slice autoradiographs were stained and washed in the same way but using a staining time of 2-2.5 min and with moderate heating of the slide.

II.E.2 Haematoxylin / eosin staining.

(a) preparation of haematoxylin (Mayer's Haemalum)

1g haematoxylin, 50g aluminium potassium sulphate and 0.2g sodium iodate were dissolved in 900 ml distilled water. To this solution was added 50g chloral hydrate and 1g citric acid and the complete solution boiled for 5 min. Once cooled, the volume was made up to 1 litre.

(b) preparation of eosin

0.5g eosin were dissolved in 100ml distilled water then filtered through Whatman No. 1 filter paper.

Before use, both the haematoxylin and eosin solutions were filtered through Whatman No.1 paper.

(c) staining protocol

Autoradiographs were stained for 10 min at room temperature with Mayer's Haemalum then "blued" in running, alkaline tap water for 7 min. (Tap water at Imperial College is pH 8; sodium carbonate solution can be used as an alternative). Staining was checked at this point to ensure the blue nuclear stain was of suitable intensity: the staining was repeated until the required blue colour was achieved. The slide was then stained for 5 min at room temperature with 0.5% eosin followed by destaining and dehydration through 50%, 70%, 90% then 2 changes of absolute alcohol. The slides were left in the 50% and 70% alcohols for only 10-15s (to prevent excessive washing out of the eosin) then 2min in each of the 90% and absolute alcohols. Prior to mounting, cells were soaked in 50:50 ethanol:xylene (2 min) then xylene

(2 min). Finally, the autoradiographs were mounted in D.P.X. mounting medium (Raymond Lamb) and dried for 4 or more hours in a warm (37-40^{o}C) oven.

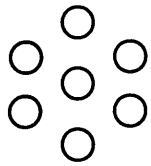
Stained autoradiographs were viewed on a Reichert-Jung Polyvar microscope under bright-field illumination. Photographs were taken using an automatic camera system attached to this microscope.

II.F Immunochemical methods

II.F.1 Ouchterlony double immunodiffusion

(a) preparation of plates

Initial detection of antibody production in immunised animals was done by Ouchterlony double immunodiffusion in agarose gels. 1% agarose gels were made by heating 1g agarose in 100 ml PBS (containing 0.2% NaN_3 as preservative), with stirring, until the agarose had completely dissolved. It was important that the heating was gentle to ensure that the agarose did not char. The solution was allowed to cool to 60^{o}C when it was poured on to detergent-, acetone- and ethanol-cleaned glass plates (15 ml per 100 cm plate area) which had been pre-heated to approximately 40^{o}C. After setting, wells were punched into the gel in the pattern shown below:-



These gels could be stored for several days at 4^{o}C in a sealed, humid box (the plugs were not removed from the gel until immediately before use).

(b) method

After removing the well-plugs from the plates prepared as described in II.F.1a the wells were filled with approximately 15 µl of antigen or antibody diluted in PBS. Diffusion was allowed to proceed for 1-2 h at 37°C, or 10-20 h at room temperature or 24-48 h at 4°C.. Throughout the experiment the gels were kept in sealed, humid boxes. Localisation of precipitin bands was by Coomassie Blue staining (see below).

Following antiserum diffusion, gels were pressed, washed and dried to remove excess protein not in the precipitin bands and to accelerate the staining/destaining process. Wells and troughs were filled with distilled water to prevent cracking and the gel covered with a layer of wet filter paper (Whatman No.1) on top of which were placed several layers of dry filter paper and a weight (approximately 10g/cm^2). After 10 min the top layers of filter paper were replaced with more dry filter paper for a further 10 min. This pressing procedure was repeated a third time after which the gel was washed for 15-20 min in PBS followed by 5 min in distilled water. Pressed, washed gels were dried to a transparent film on the glass plate using a hairdryer, stained for 5 min at room temperature with Coomassie Blue R250 (0.5% in 45% ethanol/10% acetic acid/45% water) then destained for 10 min with two changes of the same solution without the dye. (Care was taken throughout the whole procedure to ensure that the gel did not detach from the plate.) Gels were finally dried again with the hairdryer.

II.F.2 Western Blotting.

Analysis of antiserum cross-reactivity patterns was done by the Western Blotting technique using modifications of the methods of Burnette

(1981) and Towbin et al.(1979). SDS-polyacrylamide gel electrophoresis of the proteins to be probed was carried out as described in section II.G. As a rule the patterns of protein loaded on to the gels were duplicated so that one half of the gel could be used for direct visualisation of Coomassie-stained proteins whilst the other half was used for the blotting procedure. Following electrophoresis, a sheet of nitrocellulose (Bio-Rad), pre-equilibrated for 1 h with transfer buffer (25 mM tris, 190 mM glycine, 20% methanol, pH 8.3) was placed on the gel ensuring that no air bubbles were trapped between them. The gel and nitrocellulose were sandwiched between two "Scotch-Brite" pads (BioRad) and placed in the transfer chamber (Bio-Rad) with the nitrocellulose anodally to the polyacrylamde gel. Transfer was carried out for 14-16 h at 30 V followed by 1-2 h at 60 V after which the transferred proteins were probed with the serum under test. Excess protein binding sites on the nitrocellulose were blocked by incubating the nitrocellulose for 1 h at 37^{o}C in blocking buffer (10 mM tris-HCl, pH 7.4) containing 0.9% NaCl and 10% foetal calf serum. Without prior washing, the nitrocellulose was next exposed to the antibody diluted in blocking buffer for 1.5h at room temperature followed by 2 x 15 min washes with blocking buffer then 2 x 15 min washes in the same buffer without the FCS. Bound antibody was localised using a horseradish peroxidase-conjugated 2nd antibody and 3,3'-diaminobenzidine tetrahydrochloride (DAB) as chromogenic substrate for the peroxidase. 2nd antibody incubations were carried out in the same way as for the first antibody as were the subsequent washes. The blot was then developed as follows. 30 mg DAB were dissolved in 100 ml PBS to which was then added 60 µl of 30% H_2O_2. The nitrocellulose was incubated in this solution until a suitable staining intensity had developed then it was washed thoroughly with PBS then distilled water and finally dried flat

between filter papers. In some cases the reaction product was intensified using nickel sulphate by a simplification of the method of Adams (1981). 25 mg DAB were dissolved in 90 ml PBS and to this was added 10 ml 3% $NiSO_4$ in water and 60 µl of 30% H_2O_2. The nitrocellulose was incubated in this solution, washed and dried as before.

II.G Polyacrylamide gel electropohoresis

Electrophoresis was carried out using the SDS-discontinuous buffer system of Laemmli (1970) using an apparatus of the Studier design (1973) or that made by the Hoeffer Co., USA. Prior to pouring gels, the glass plates were cleaned with detergent, acetone and ethanol. The plates were separated by 0.7mm side spacers and, when the Studier apparatus was used, the spacers were greased with Paraffin Soft White (BDH) and the bottom sealed with a 20% (total monomer) polyacrylamide gel. The gels used were as follows:-

stacking gel 3.5% total monomer in 0.125M tris-HCl/0.1M SDS, pH 6.8

resolving gel 10% total monomer in 0.375M tris-HCl/0.1M SDS, pH 8.8

sealing gel 20% total monomer in 0.025M tris-HCl/0.1% SDS, pH 8.8

Gels were polymerised using 0.075% ammonium persulphate and 0.0375% N,N,N',N'-tetramethylethylenediamine (TEMED). The reservoir (electrode) buffer was 0.025M tris/0.192M glycine /0.1% SDS, pH 8.3. All components were made in concentrated stock solutions. Details of these are given, with recipes for making the various gels, in table 2

Samples for electrophoresis were prepared in 0.0625M tris-HCl, pH 6.8 containing 2% SDS, 5% 2-mercaptoethanol, 10% sucrose and 0.002% bromophenol blue. Molecular weight standards were obtained from Pharmacia

Table 2 Gel recipes for SDS-polyacrylamide gel electrophoresis

component	20% sealing gel	10% resolving gel	3.5% stacking gel
acryl/bisacryl	10ml	13.3ml	2.5ml
stacking buffer	-	-	5.0ml
resolving buffer	1.875ml	4.999ml	-
10% SDS	0.15ml	0.399ml	0.2ml
1.5% amm.persulph.	0.75ml	2.0ml	1.0ml
water	2.22ml	19.266ml	11.3ml
TEMED	15µl	15µl	15µl

stacking buffer stock	0.5M tris-HCl, pH 6.8
resolving buffer stock	3.0M tris-HCl, pH 8.8
reservoir buffer stock	0.25M tris/1.92M glycine/1% SDS pH 8.3
acryl/bisacryl	30% acrylamide/0.8% N,N methylene bisacrylamide

Whilst the resolving gel was polymerising it was overlayed with buffer of the same composition as in the gel (but without the acrylamide or TEMED).

and prepared according to supplied instructions then made up in the sample buffer as already described. Before loading the gel, all samples were heated for 3-5 min in a boiling water bath, allowed to cool then centrifuged at 10,000 x g for 5 min. Enough sample was loaded to give 70-100 µg of a crude protein mixture or 5-10 µg of a purified protein per track.

Following electrophoresis gels were fixed and stained with methanol: water:glacial acetic acid (5:5:2, v/v/v) containing 0.1% Coomassie Brilliant Blue R250. The solution was filtered before use (Whatman No1) and staining performed overnight. Gels were destained with 12.5% propan-2-ol/10% glacial acetic acid in a plastic dish containing foam rubber to which the dye adsorbs. Destained gels were stored wet in 7% glacial acetic acid or dried for 1.5-2h on a Bio-Rad gel drier.

II.H. Preparation of soluble and insoluble brain extracts

These were prepared for use in the testing of antisera by the Western blotting technique. A 10% w/v homogenate (250 µm clearance, 900 rpm, 12 strokes) of adult rat whole brain was made in 0.31M sucrose containing 2mM PMSF and buffered to pH 7.4 with 10mM Tris-HCl. The homogenate was centrifuged at 100,000 x g at 4°C for 1h then the supernatant removed and frozen in 1 ml aliquots. This constituted the soluble fraction. The pellet was resuspended and homogenised (4 strokes, clearance 250µ) in 10mM tris-HCl (pH 7.4)/2mM PMSF to lyse the organelles. After 15 min standing at 4°C the volume was made up to the original homogenate volume and tris/sucrose/PMSF concentrations, then frozen in 1 ml aliquots. This constituted the insoluble fraction.

II.J Immunocytochemistry

II.J.1 Fluorescence Immunocytochemistry

All fluorescence immunocytochemistry (ICC) was carried out by the indirect method using unlabelled primary antibody followed by a secondary antibody covalently-labelled with either fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC). Cells were used either unfixed (surface-labelling) or fixed (internal labelling). They were incubated with primary antibody (45 min incubations), washed thoroughly, incubated with secondary (labelled) antibody (45 min incubations) , washed thoroughly then mounted in PBS/glycerol either with or without a fluorescence anti-fading compound (Citifluor, City University, London). Slides were then viewed on a Reichert Polyvar optical microscope equipped with epifluorescence illumination and appropriate excitation/barrier filters (block G1 for TRITC, block B4 for FITC). One exception to this procedure was in staining for cell components recognised by tetanus toxin; in this case the two antibody steps were preceded by an incubation with tetanus toxin. All antibodies were diluted in and all washes performed with DMEM or PBS containing 10% FCS or 3% BSA with the addition of 0.3% Triton-X100 in the case of fixed material. Actual dilutions of the antibodies used are given in the relevant results chapters.

II.J.2 Peroxidase Immunocytochemistry

Peroxidase ICC was carried out using unlabelled primary antibody followed by either a secondary antibody covalently -labelled with horseradish peroxidase (HRP) or a secondary unlabelled antibody followed by a tertiary antibody-HRP immune complex (the "sandwich" technique of Sternberger et al.,1970). The peroxidase methods were

used only for intracellular staining.

(a) Glutamine synthetase in cultured cells

Cells were fixed for 5 min in 4% paraformaldehyde in PBS then, after thorough washing (3 x 10 min), they were incubated with 10% methanol/3% H_2O_2 in PBS for 10 min. Again, after thorough washing, cells were incubated with 30% normal swine serum for 20 min followed, without washing, by a 1:700 dilution of rabbit anti-glutamine synthetase antiserum (see Chapter V) for 1.5h. With washes between each step, the cells were then exposed to a 1:40 dilution of swine anti-rabbit Ig followed by a 1:70 dilution of rabbit PAP (soluble complex of horseradish peroxidase and rabbit anti-horseradish peroxidase). Following washes with washing buffer (3 x 5 min) and PBS (2 x 5 min) the slides were developed with 3,3'-diaminobenzidine tetrahydrochloride intensified with nickel sulphate as described for Western blots in section II.F.3 The reaction was stopped by thorough washing with PBS and water then the cells dehydratyed through a series of alcohols (10 min in each of 30%, 50%, 70% and 90% ethanol then 2 x 15 min in absolute alcohol) then cleared in xylene (15 min) before mounting in DPX mounting medium (Raymond Lamb).

Unless otherwise stated, all antibodies were diluted with and the cells washed with Dulbecco's modified Eagle Medium (DMEM) or PBS containing 0.3% Triton X-100, 1% normal swine serum and 10% foetal calf serum. All incubations were carried out at room temperature.

(c) Vimentin

Cells were fixed by immersion for 10 min in methanol at -20^{o} C. After

rehydration in washing buffer (10 min) they were stained by the procedure already described for glutamine synthetase staining in cells using a 1:100 dilution of rabbit anti-vimentin as first antibody.

(d) Glial fibrillary acidic protein (GFAP)

Cells were fixed and stained for GFAP as described for vimentin but using a 1:100 dilution of rabbit anti-GFAP as first antibody.

II.K Protein determination

Proteins were determined essentially by the method of Lowry et al. (1951) adapted for automation. BSA standards were made in 0.1M NaOH at concentrations of 5, 10, 15, 20, 25, 50, 100, 150 and 200 ug/ml. The protein contents of cultured cells were measured by solubilising the cell monolayer in 1 ml 0.1M NaOH at 37^{o}C overnight in a humid incubator then taking aliquots of this for protein determinations by the automated Lowry method. Aliquots of this were also used for radioactivity-determinations (see section II.L). All other proteins to be determined were dissolved in 0.1M NaOH.

The sampling and measurement apparatus consisted of a Jencons Autoanalyzer sampler connected to a multichannel peristaltic pump leading into a series of glass coils where the sample and analytical regents were mixed before being fed into a colourimeter set to read absorbance at 547 nm. Absorbance measurements were plotted on a chart recorder from which a standard curve could be drawn and sample values obtained. The minimal concentration of protein which could realistically be measured with this system was approximately 10 - 20 ug/ml; this was sufficient for almost all requirements in this work.

The solutions used in this assay were:-

1 Folin-Ciocalteu Phenol reagent : water 1:2

2 Copper reagent 50 ml 1% $CuSO_4 . 7H_2O$

50 ml 2% Na.K.tartrate

2.5 ml 1M NaOH

made up to 500 ml with water.

3 Alkaline carbonate 10.6% Na_2CO_3 in 1M NaOH, dilute 1 in 5 with water.

II.L Radioactivity determinations

Radioactivity (tritium) was determined in a Packard TriCarb Liquid Scintillation Spectrometer preprogrammed to convert counts to disintegrations per minute using the sample channels ratio method. The scintillation fluid was:-

toluene : triton X-100 2:1

2,5-diphenyloxazole (PPO) 4 g/l final concentration

0.5 ml aliquots of sample in 0.1 M NaOH (see II.M) were neutralised with HCl then mixed with 4 ml scintillation fluid. These were counted for 10 min after allowing time for any chemiluminescence to subside. Efficiency of counting was generally around 30%.

III CHARACTERISATION OF PRIMARY CULTURES OF CEREBELLAR ASTROCYTES: INFLUENCE OF CULTURE CONDITIONS

III.A. Introduction.

One of the attractive features of tissue culture in the study of cellular activity/function is that the the possibility exists for manipulation of the conditions under which the cells are grown such that there is an enrichment of cells exhibiting the function to be studied, or that the cells may be induced to express a particular activity of interest. That primary cultures derived from rodent brain can contain astrocytes showing two different morphologies has been shown by several workers (Lasher,1974; Manthorpe et.al.,1979; Stieg et.al.,1980), however, little work has been done to characterise further these two cell types. The major aim of the present work has been to compare and contrast some of the properties of these cell types with a view to establishing their relationship to astrocytes *in vivo* and assessing their potential as *in vitro* models for mature astrocytes. An important part of this work is to determine the behaviour of these cells in culture under a variety of conditions and to attempt to enrich for one or other of the two astrocyte types previously observed. This work is dealt with in the present chapter.

III.B. Development of cells *in vitro* under astrocyte-enriching conditions.

III.B.1 Morphological development *in vitro* of cells derived from 8-day old rat cerebellum.

In choosing the starting material for tissue-culture, many factors must be taken into consideration; this usually entails making a compromise between selecting tissue from which a large number of cells may be obtained - in effect older tissue - and selecting tissue in which the cell-cell

adhesions are weak enough to allow dissociation without permanent harm. For these reasons the cultures used initially were derived from 8-day old rat cerebellum (Woodhams et.al.,1981)

Cells were dissociated by trituration in the presence of trypsin, suspended in DMEM/FCS then seeded onto poly-L-lysine coated coverslips at a density of 1 million cells per 35mm diameter dish. Cells were grown at 37°C and dishes were taken at intervals to study the morphological development as determined by immunofluorescent labelling of GFAP (glial fibrillary acidic protein, a 10nm intermediate-filament protein which has been shown to be expressed in the CNS exclusively by astrocytes and is probably, therefore, one of the best criteria for identifying cells as astrocytes.

Cultured cells were taken at daily intervals, fixed and permeabilised by incubation in methanol or 95% ethanol/5% acetic acid at -18°C for 10 min. Fixed cells were treated with a rabbit antibody (A10) raised against human GFAP (Woodhams et al.,1980); the antibody was diluted 1:50 in PBS/1% BSA/0.3% triton-X100, incubations were for 45 min. The secondary antibody was TRITC-conjugated swine anti-rabbit Ig used in the same way. Fluorescently-labelled cells were mounted in 90% glycerol/10% PBS (v/v) then viewed and photographed on a Reichert Polyvar microscope using rhodamine optics and an automatic camera system. The developmental time-course is shown photographically in Fig.3.

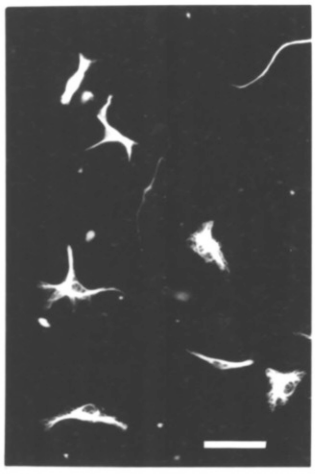
When plated, all cells were spherical, then over the first day took up characteristic shapes. The time course shows that at 2 DIV (days in vitro) most of the cells expressing GFAP were of a polygonal form and occasionally there were cells extending very fine $GFAP^+$ processes. From 2 DIV to 5 DIV the morphologies of the cells developed such that two different types could be recognised by the end of this period and almost

Fig.3 Fluorescent micrographs of 2-11 DIV cerebellar astrocytes labelled with anti-GFAP.

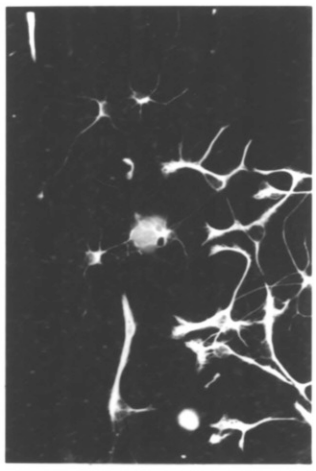
Astrocytes from 8 day old rat cerebella were labelled at various stages in culture with rabbit anti-GFAP / TRITC swine anti-rabbit Ig. Antibodies were used at 1:50; cells were pre-fixed in methanol at $-18^{o}C$.

The figures illustrate the development of the two forms of astrocyte (stellate and epithelioid) from shortly after plating until confluence and show the appearance then disappearance of the stellate morphology.

Scale bar = 50um



2 DIV



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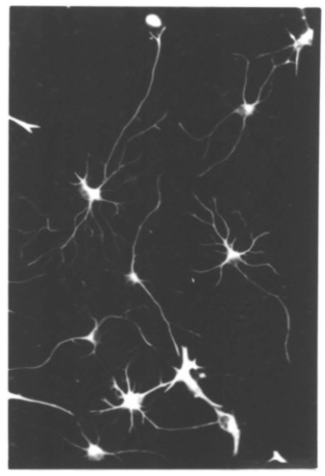
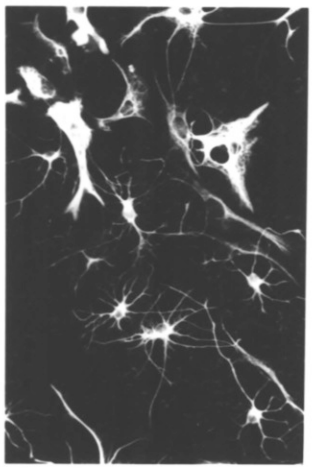
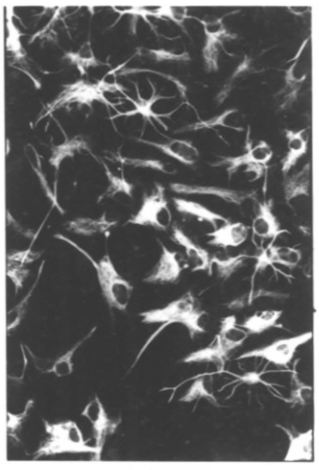


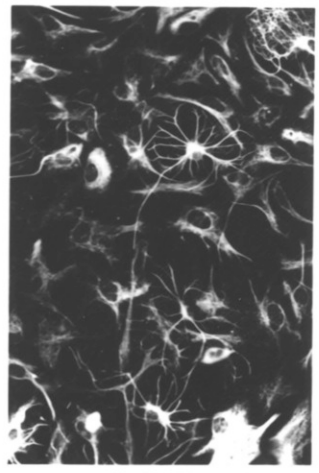
4 DIV



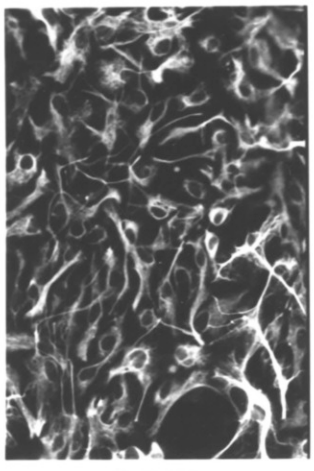
5 DIV



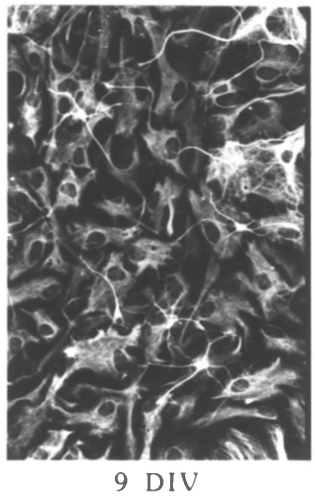
6 DIV



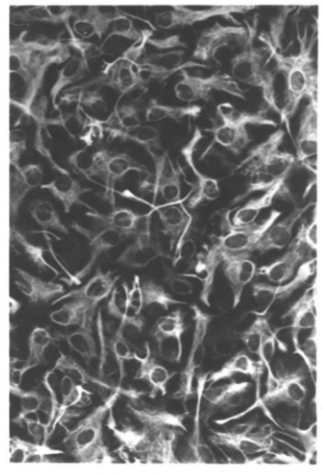
7 DIV



8 DIV



9 DIV



11 DIV

all the cells expressed GFAP. One population of cells extruded several radial processes, had a small perikaryon containing an eccentric nucleus and were labelled intensely with the anti-GFAP antibody. These I have termed stellate or S-type astrocytes. Another population of cells developed a more epithelioid shape with few processes (any processes that did develop being much thicker than those in S-type astrocytes), a large central nucleus and an apparently less intense staining with the anti-GFAP antibody which bound to many fine fibrils in the cell body. These I have termed epithelioid or type-E astrocytes.

Between 5 DIV and 12 DIV there was a general reduction in the proportion of stellate astrocytes in the culture so that at 12 DIV about the time when the cultures reached confluence - almost all the cells present were of the epithelioid variety. The reason for this happening has not been fully investigated, however, there is some evidence to suggest that the type-S cells are changing morphology into that resembling type-E cells. The alternative explanation, that type-S cells die and their places then occupied by type-E cells, does not seem likely since there is no evidence to show the dying stellate cells or gaps where they have left the plate. In addition, the GFAP labelling during the transition period 5 DIV - 12 DIV shows many cells with morphologies intermediate between the stellate and epithelioid astrocytes. Finally, a cinemicrographic study of the development of such cultures has shown the ruffling of membrane and the drawing-in of processes of the S-type cell after contact with E-type cells suggesting that this interaction may trigger the shape transition (Wilkin et.al.1983).

III.B.2 Morphometry of 5 DIV cerebellar astrocytes.

Due to the highly irregular shapes of the cells (especially the stellate cells) it was difficult to make any quantitative comparisons between the two types (e.g. surface area, perimeter, diameter). One measurement that could be made was that of the nuclear area. Photographic negatives of cells stained with antibodies against GFAP or vimentin (another intermediate filament protein) were projected on to a digitising surface and the outlines of the nuclei followed by a cursor. The output from the pad and cursor were fed to a computer which subsequently calculated the nuclear areas. This was done for the stellate and epithelioid astrocytes and the results are shown in Fig.4.

These results confirm that the two morphologically-distinct astrocyte types are also significantly different with respect to their nuclear areas. The stellate cells have a nuclear area of 101 $\pm$ 28 μm^2 and the epithelioid cells' nuclei an area of 280 $\pm$ 75 μm^2 (means $\pm$ S.D.) A Student's t-test analysis shows the difference between the two populations to be highly significant ($P < 0.001$). This comparison is interesting in that it may help to give an idea as to the types of astrocyte represented by the two cultured forms. Federoff et.al. (1984) made similar measurements (diameter rather than area) on normal and cAMP-treated astrocytes in culture and on normal and reactive astrocytes *in vivo*. These results are summarised below after recalculation of nuclear area from diameter, making the assumption that the nuclei are circular:-

normal, *in vitro*	86.6 $\pm$ 0.05 μm^2
normal, *in situ*	86.9 $\pm$ 0.01 μm^2
reactive, *in situ*	229.7 $\pm$ 0.04 μm^2
dBcAMP-treated, *in vitro*	160.8 $\pm$ 0.02 μm^2

Comparison of these figures with those obtained in the present work indicate that the stellate astrocytes are similar in nuclear area to both

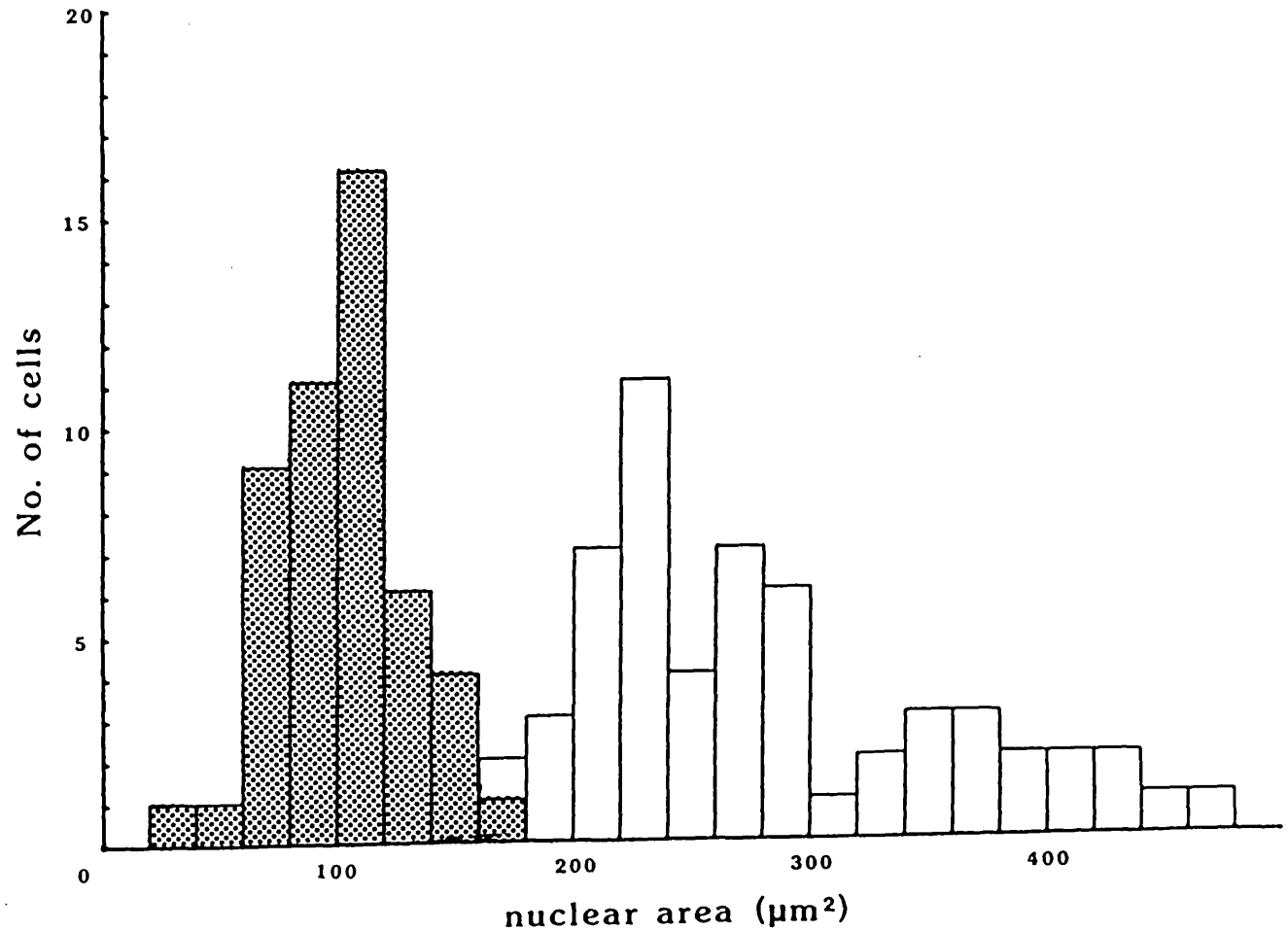
Fig.4 Nuclear areas of stellate and epithelioid astrocytes.

Photographic negatives of 5 DIV cerebellar astrocytes stained with anti-GFAP or anti-vimentin antibodies (fluorescence or peroxidase) were projected onto an electronic digitising surface linked to a computer (Hewlett-Packard). The nuclear perimeters were traced using a hand-held cursor also linked to the computer which then calculated nuclear areas from the perimeter data. Calculated areas were plotted as a histogram. This shows a clear distinction between the stellate and epithelioid astrocytes on the basis of nuclear area, the stellate cells having a significantly smaller mean nuclear area than the epithelioid cells.

stellate cells - dotted bars

epithelioid cells - blank bars

Fig.4



normal cultured astrocytes and normal astrocytes in vivo. The epithelioid astrocytes, on the other hand, exhibit nuclear areas in and beyond the range observed by Federoff et al for dBcAMPtreated cells. These authors considered that the upwards shift in nuclear area following treatment with dBcAMP suggested that these cells were more representative of reactive astrocytes since the latter also showed a larger nuclear area than normal astrocytes both *in vitro* and *in vivo*. By the same argument it might be that in the cultures used in the present work, the epithelioid cells represent the reactive type whilst the stellate ones are normal.

III.B.3 Influence of age of source animal on astrocyte sub-types *in vitro*.

If the development of astrocytes *in vitro* follows the same or a similar pattern to those *in vivo* then it may be possible to enrich a culture by altering the age of animal from which the cells are initially derived i.e. so that the cell-type required can be removed from the animal at the time approaching the height of the proliferative capacity of that cell.

In order to investigate this, cultures were grown as in II.B.1 using cells from cerebella of animals ranging from 1 to 13 days old. At 5-6 DIV they were labelled with the anti-GFAP antiserum and the numbers of stellate and non-stellate astrocytes counted (5-6 DIV was chosen because this gave the largest number of stellate cells in the initial studies on 8-day old animals and would give a better base for comparing changes in numbers as the age of source animal was varied). Fig.5 shows (●) the total number of astrocytes per field and (O) the number of stellate astrocytes per field as a percentage of the total astrocytes per field.

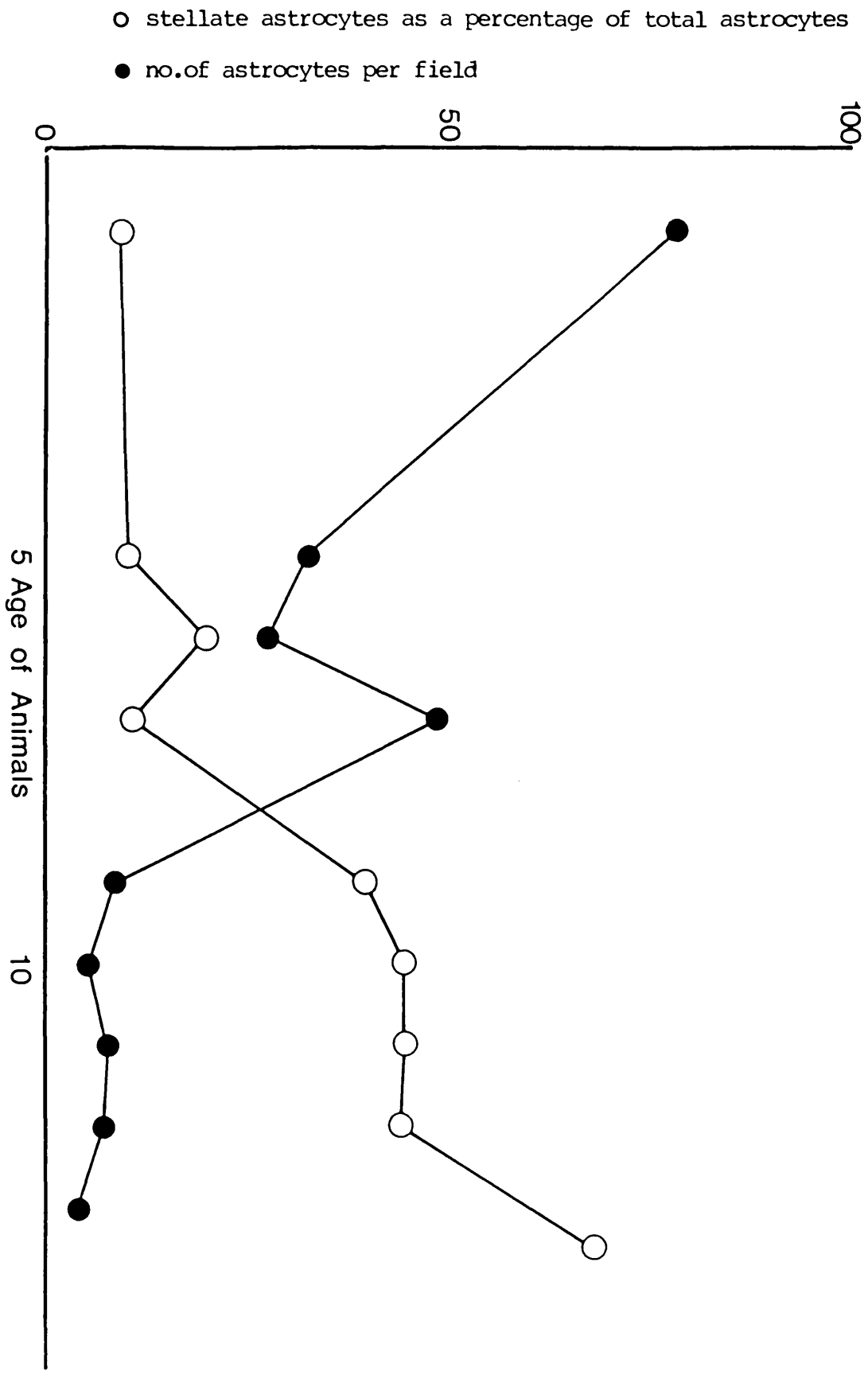
Fig.5 Influence of age of source animal on astrocyte forms in culture.

● no. of $GFAP^+$ cells per field

○ no. of stellate $GFAP^+$ cells per field as a percentage of total GFAP+ cells per field.

Field size = $280 \times 10^3 \ \mu m^2$

Cells were dissociated from animals of 1-13 days (abscissa) and grown to 6DIV when they were labelled with anti-GFAP antiserum. Each point represents the mean ± SEM from at least 18 fields. Mean no. of fields counted was 38. The no. of cells counted at each age ranged from 1402 (at 1DIV) to 160 (13DIV) with the mean being 671


o stellate astrocytes as a percentage of total astrocytes
● no.of astrocytes per field
0
50
100
5 Age of Animals
10

This figure indicates (●) that the older the source animal used, the lower the number of cells which remain in culture at 6 DIV. This may be due either to a reduced plating efficiency or to a decreased survival in culture, however a contributing factor may be an increased inaccuracy in the initial number of cells seeded. This might come about because the concentration of cells in the post-dissociation suspension may be over-estimated due to confusion with cellular debris which is present in greater amounts after dissociation of older tissue. Determination of cell viability using Fluorescein Diacetate / Ethidium Bromide method would overcome this. This figure also shows (O) however that as the age of the source animal was increased there was an increase in the proportion of all surviving astrocytes showing the stellate morphology. Posible reasons for this are that there is preferential attachment and/or survival of the stellate cell precursors following dissociation of older tissue or that the potentially-stellate astrocytes have been dissociated at a more favourable point in their developmental pathway so that they have been able to proliferate in culture whereas the potentially epithelioid astrocytes were not isolated at such an opportune moment.

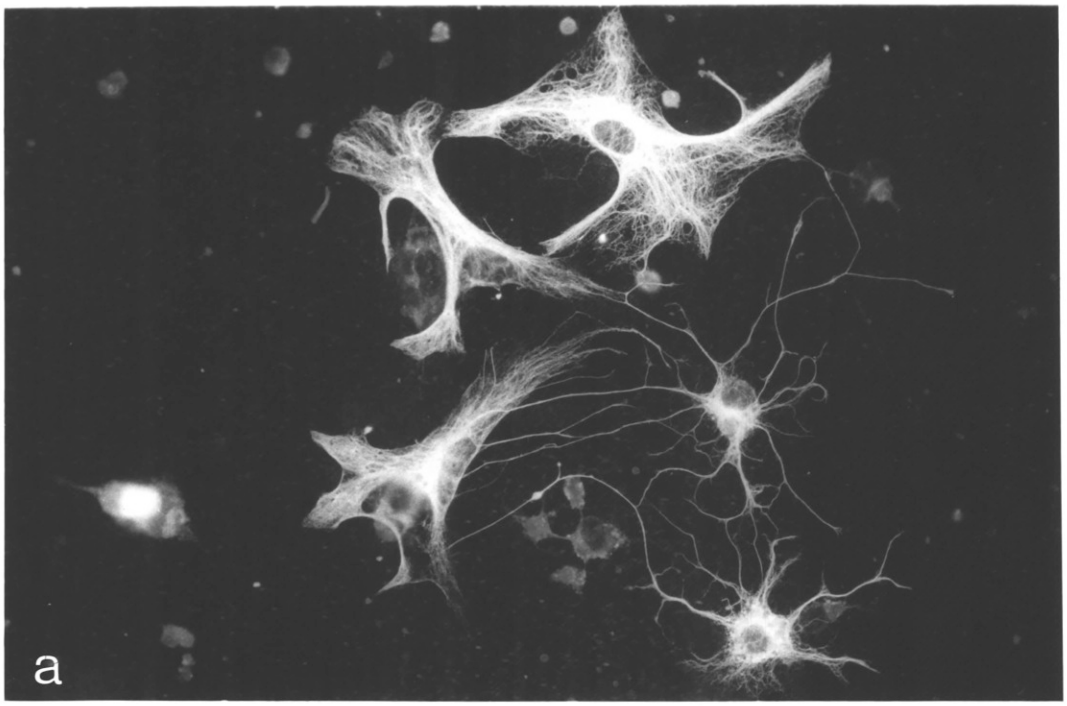
Another feature of the cells grown from older animals is that their morphologies (or at least their GFAP distributions) were different from those seen in cells grown from younger animals. This is illustrated in Fig.6 where stellate astrocytes at 6 DIV grown from 8-day and 12-day old rats are shown for comparison. It is clear that although the cells from 12-day animals still possess a radial symmetry, they do not possess the distinct, fine processes that were the striking features of the stellate cells grown from 8-day old animals, however they equally do not fit into the epithelioid or E-type classification defined earlier.

From this study it seems that increasing the age of the source animal progressively selects for the stellate or S-type astrocytes, however,

Fig.6 Fluorescent micrographs of 5 DIV cerebellar astrocytes from 8 and 12 day old animals.

Cerebellar astrocytes from 8 and 12 day old rats were labelled at 5 DIV with rabbit anti-GFAP / TRITC swine anti-rabbit Ig. Antibodies were used at 1:50; cells were pre-fixed in methanol at $-18^{o}C$. The photographs show that the same general forms of cell are present in the cultures from both (a) 8 and (b) 12 day old rats although the stellate cells tend to have thicker processes in the cultures from 12 day old animals.

scale ▬▬▬ = 50μm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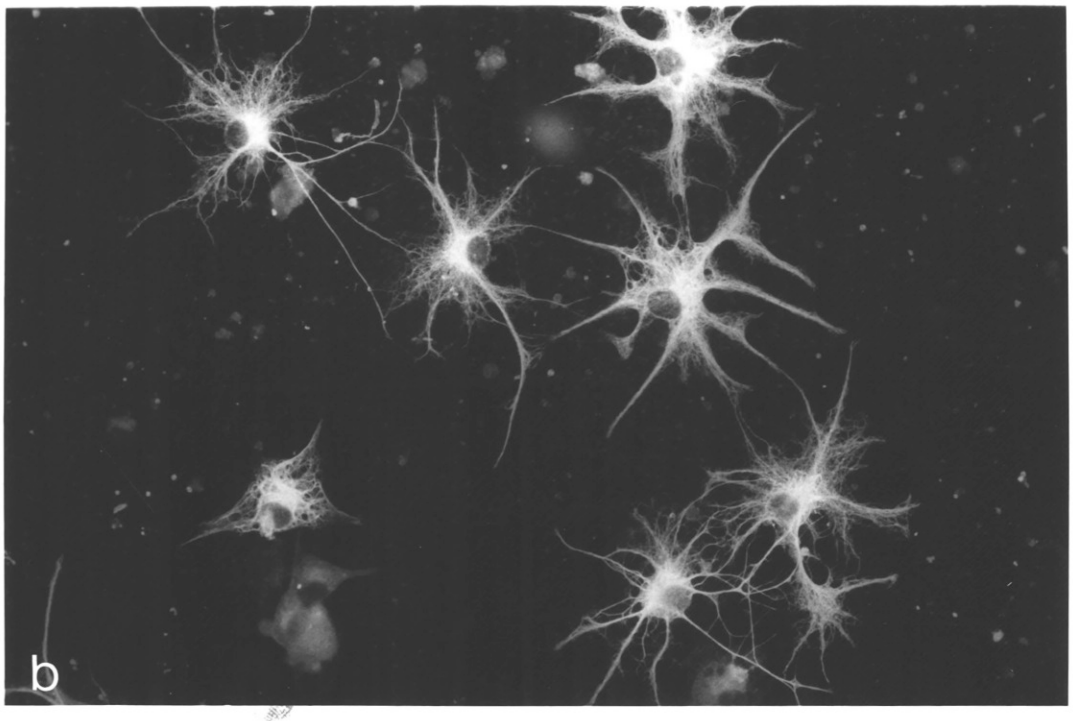

b

there are drawbacks to the application of this procedure for that purpose. Firstly, as shown in Fig.6 although the stellate astrocytes from 8-day and 12-day rats do share some morphological characteristics, they are obviously not identical (although they may be a homologous cell-type). Secondly, even if it could be established that, for all useful purposes, these cells can be considered identical, the yield is so small that it would greatly increase the difficulty in using cells derived in this manner as a routine preparation and would require the use of a much larger number of animals per experiment.

For these reasons many of the experiments in this work were carried out on the cultures derived from 8-day old animals which, at 6 DIV, allowed the comparison of some of the properties of both the E- and S-type astrocytes.

III.B.4 Cellular composition of 5 DIV cultures derived from 8-day rat cerebellum.

Despite the fact that about 80-90% of the non-neuronal cells in cultures prepared as described above are astrocytes, it is important and useful to be aware of the presence of other cell-types and to be able to recognise them routinely with some accuracy without time-consuming identification procedures. To this end the 5 DIV cultures from 8-day rat cerebellum were investigated with a number of antibodies recognising specific cell-types.

The most common contaminating mammalian cells in many culture systems are fibroblasts and endothelial cells; additional contaminating cells in nervous system astrocyte cultures are oligodendrocytes and neurons. It has been documented that these cells possess molecules on their surfaces that enable them to be distinguished from each other. Thus astrocytes are identified by the anti-GFAP antibody, A 10; neurons by their ability to

bind tetanus-toxin, fibroblasts by the MRC OX 7 monoclonal antibody against Thy 1.1, endothelial cells (as well as a few others) by serum obtained from patients suffering from Chagas' disease and oligodendrocytes by antibodies against galactocerebroside (a major glycolipid of myelin). These antibodies, the principal cell-types recognised by them, the antigens to which they bind and the appropriate references are outlined in Table 3.

To elucidate the composition of the cerebellar cultures, cells from 8-day animals were cultured for 5 days, then fixed (if required) and treated with antibody/toxin and appropriate fluorochrome-conjugate. All the primary reagents except anti-GFAP and Chagas'serum were directed against surface-antigens therefore no prior fixation or permeabilisation steps were needed. For staining with anti-GFAP and Chagas' serum, permeabilisation and physical fixation were employed; post-fixation was used for all other reagents. The fixation procedures, antibody dilutions and incubation times for each staining method are outlined in Table 4. Photographs of cells labelled with these reagents are shown in Fig.7. Table 5 shows the number of cells identified by these reagents as a proportion of all cells present (obtained under phase-contrast optics), each figure corresponding to at least 20 fields on 2 coverslips.

Table 5 clearly demonstrates that under the conditions used here, the cultures are heavily enriched in astrocytes which constitute about 83% of the non-neuronal cells. The major contaminating cells are the endothelial cells which constitute about 9% of the cells whilst the fibroblasts and oligodendrocytes form approximately 8%. Neuronal cell bodies are not included in these figures since their numbers varied widely between cultures, ranging from less than 5% to as much as 30-40% of the total cells. In addition, the neuronal perikarya often detached from the

Table 3. Molecular markers for identifying cell types in culture

cell type	antibody		antigen	reference
astrocytes	A-10 anti-GFAP	(P)	glial fibrillary acidic protein	1
neurons	tetanus toxin	-	GD1b, GT1	2
oligodendrocytes	anti-Gal C	(P)	galactocerebroside	3
fibroblasts	MRC OX-7	(M)	Thy-1.1	4
endothelial cells	Chagas serum	(P)	unknown	5

1 Bignami et al,.,1972
2 Dimpfel et al.,1977
3 Ranscht et al.,1982
4 Stern et al.,1973
Mason & Williams,1979
5 Woodhams et al.,1980

Table .4 Protocols for immunofluorescent staining of cells in culture

primary reagent	dilution / concentration	pre-fixation	2nd antibody	3rd antibody	post-fixation
anti-GFAP	1:50	methanol, -18°C 10 min	TRITC Sw @ Rb	-	none
tetanus toxin	10 ug/ml	none	H @ tet	FITC Hu @ H	3.5% paraformaldehyde, 5min
anti Gal-C	1:250	none	FITC G @ M	-	3.5% paraformaldehyde, 5 min
anti-Thy-1	supernatant	none	FITC G @ M	-	3.5% paraformaldehyde, 5 min
Chagas serum	1:30	5% acetic acid in ethanol,-18°C	FITC Sh @ Hu	-	none

After the final washes, cells were mounted in 'Citifluor' mounting medium.

All antibody dilutions were in DMEM + 10% foetal calf serum.

Incubation times for all antibodies was 45 min.

TRITC and FITC indicate rhodamine and fluorescein conjugates respectively.

Sw @ Rb swine anti-rabbit Ig; H @ tet horse anti-tetanus toxoid; G @ M goat anti-mouse Ig

Sh @ Hu sheep anti-human Ig; Hu @ H human anti-horse Ig.

Fig.7 Fluorescent micrographs of astrocyte cultures labelled with cell type specific markers.

5 DIV cerebellar astrocyte cultures from 8 day old rats were labelled with:-

(a) anti-GFAP

(b) tetanus toxin

(c) anti-galactocerebroside

(d) anti-Thy.1

(e) Chagas serum

and the appropriate fluorochrome-labelled 2nd antibodies. The antibodies were used as described in Table 4 as are the fixation details.

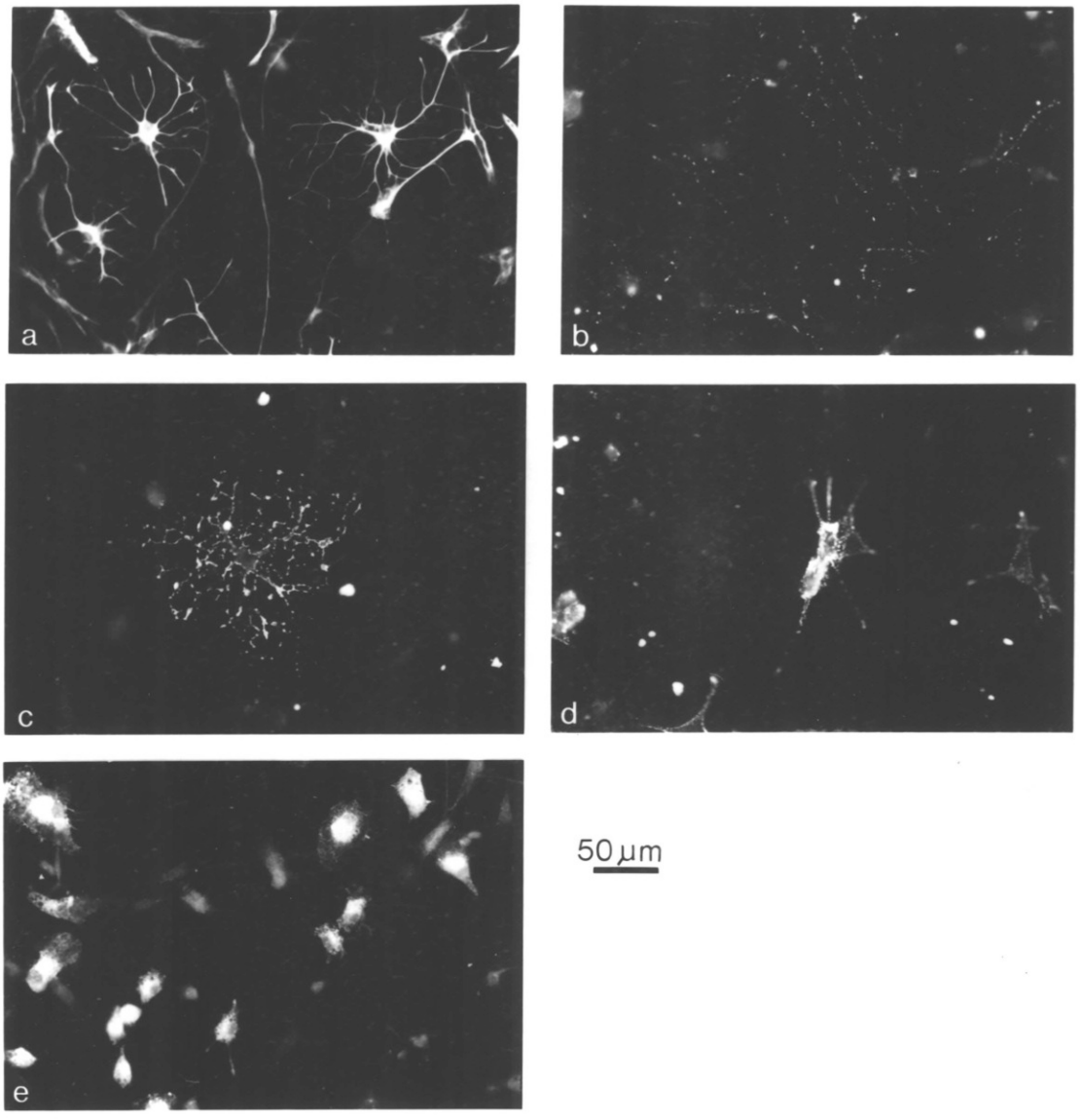

a
b
c
d
e
50µm

Table 5. Proportions of non-neuronal cells in 5 DIV cerebellar astrocyte cultures

cell type	antibody	% of total cells
astrocytes	anti-GFAP	82.8 ± 4.5 %
oligodendrocytes	anti-galactocerebroside	1.5 ± 0.7 %
fibroblasts	Thy-1	4.8 ± 1.5 %
endothelial cells	Chagas serum	9.4 ± 1.9 %

plate leaving the remnants of neuronal processes detectable by tetanus toxin (see Fig.7b)

III.C. Antibodies showing differential labelling of the stellate and epithelioid astrocytes in culture.

In addition to antibodies that recognise different cell types in the astrocyte cultures, two other antibodies were shown to label to different degrees the stellate and epithelioid astrocyte sub-types. These were a polyclonal antibody against vimentin and a monoclonal antibody designated LB1.

III.C.1 Anti-vimentin.

The anti-vimentin antibody recognised the 57,000 dalton intermediate filament protein found in many cell types, especially mesenchymal cells (Franke et al.,1978). It has been suggested that vimentin can be expressed by most cells in culture (Franke et al.,1979a) and that it may be associated with cell growth in vitro (Franke et al.,1979b). Of particular relevance to the present work is that vimentin has also been shown to be expressed in immature glia (Dahl et al.,1981a), cerebellar Bergmann glia, the major processes of fibrous astrocytes (but not fine fibrils) and reactive astrocytes (Dahl et al.,1981b). Since the stellate astrocytes and epithelioid astrocytes in culture display different growth characteristics and because it was hypothesised that the stellate cells may be more differentiated than the epithelioid ones, vimentin expression was potentially a good basis for discrimination between these two forms of astrocyte.

III.C.I.a. Labelling of cells with anti-vimentin antibody.

Astrocyte cultures from 8 day old rat cerebellum were grown on cover-slips as described in section II.B. At 5 DIV the cells were fixed and permeabilised by immersion in methanol at -18^{o}C for 10 min. They were then labelled with rabbit anti-vimentin antibody followed by TRITC-conjugated swine antri-rabbit Ig using the conditions described in Table 4 for anti-GFAP staining. After the final washes the cells were mounted in Citifluor mountant and viewed under rhodamine optics. A fluorescent micrograph from this experiment is shown in Fig.8.

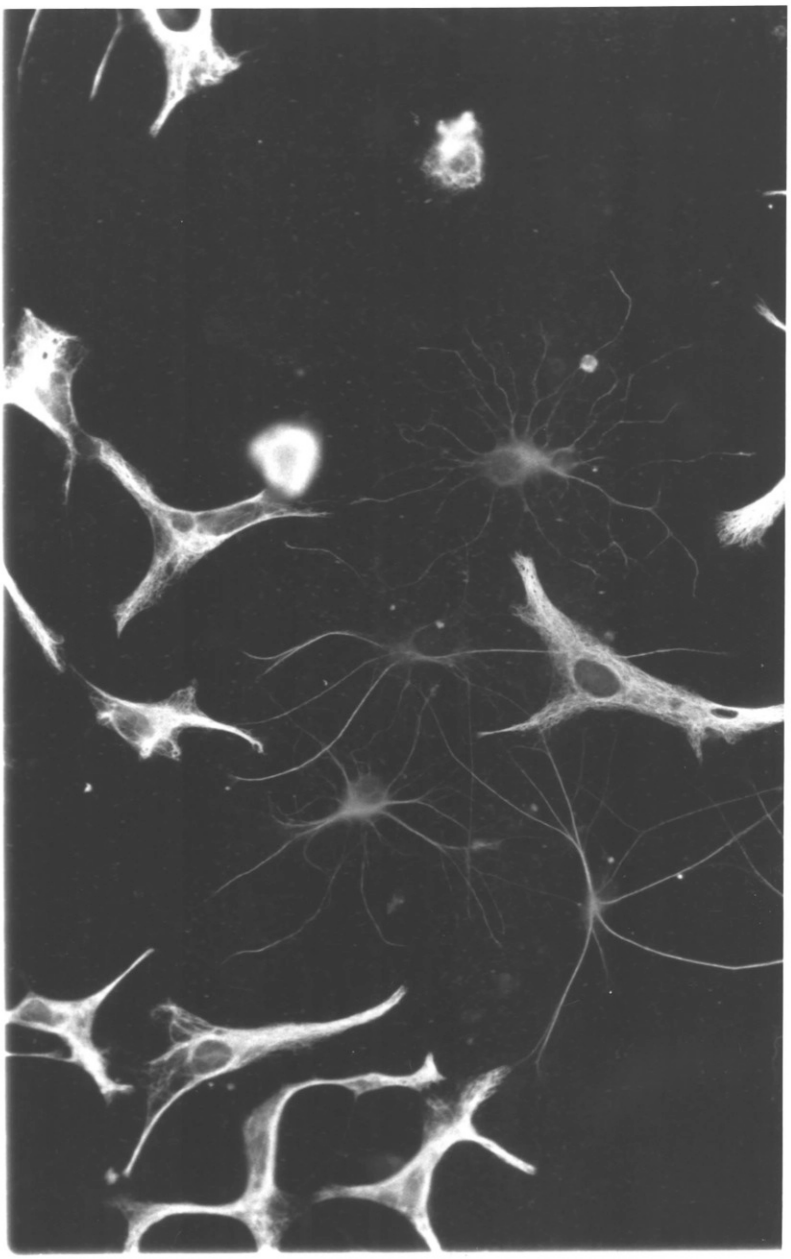
From this figure it appears that the stellate astrocytes contain considerably less vimentin than do the epithelioid cells. Although the possibility exists that this apparent difference may have arisen from different distributions of the protein in the two cell types, this seems unlikely because this differential staining pattern was not seen when using the anti-GFAP antibody in combination with the same 2nd antibody (if anything the stellate cells were more intensely labelled with anti-GFAP than were the epithelioid cells). A similar, but less pronounced, difference in the level of labelling in the two astrocyte types was also seen when vimentin was labelled using the peroxidase method (not shown). This staining pattern was also observed by Yen & Fields (1981).

This result lends weight to the idea that the stellate astrocytes are a more differentiated form of astrocyte than the epithelioid type. This is not proved conclusively, however, because the result is also consistent with the epithelioid cells' being reactive astrocytes, Bergmann glia or white matter astrocytes (Dahl et al.,1981; Yen & Fields,1981). Chiu et al. (1981) suggested, additionally, that the relative amounts of GFAP and vimentin in normal mature astrocytes may vary from region to region.

Fig.8 Fluorescent micrograph of 5 DIV cerebellar astrocytes labelled with anti-vimentin antibody.

5 DIV cerebellar astrocytes from 8 day old rats were labelled with rabbit anti-vimentin / TRITC swine anti-rabbit Ig. Antibodies were used at 1:50; cells were pre-fixed in methanol at -18^{o}C. The figure shows a markedly less-intense staining of the stellate astrocytes than the epithelioid astrocytes with the vimentin antibody (compare with the GFAP staining patterns shown in Figs 3 and 6).

Scale ▬▬▬ = 50μm



III.C.2. LB1 monoclonal antibody.

This antibody was raised by Dr J.Cohen (MRC Neuroimmunology Project, University College, London) who kindly donated clones to this laboratory. It was raised by injecting whole cells from 10 DIV cultures of mouse spinal cord into BALB/c mice from which spleen cells were subsequently fused with NS1 myeloma cells. The hybridomas produced by this procedure were cloned by classical methods and screened for cell-surface binding properties by fluorescence immunocytochemistry (personal communication; Wilkin & Cohen in preparation). One clone, designated LB1, produced an antibody which looked useful for discriminating between the two astrocyte forms in culture.

III.C.2.a. Double labelling of cells with LB1 and GFAP.

Astrocyte cultures from 8 day old rat cerebellum were grown on coverslips as described in section II.B. At 5 DIV the cells were incubated for 45 min, without prior fixation, with LB1 hybridoma supernatant diluted 1:5 with DMEM + 10% FCS. Excess LB1 was removed by washing and the cells subsequently incubated for 45 min with FITC-conjugated goat anti-mouse Ig. Following washing, the cells were fixed for 5 min at room temperature with 4% paraformaldehyde in PBS then washed with 3 x 1ml of PBS over 10 min. For GFAP staining the cells were next permeabilised and fixed for 10 min in 5% glacial acetic acid in absolute ethanol at -18°C. After thorough washing with PBS the cells were incubated with anti-GFAP then TRITC swine anti-rabbit Ig using the conditions described in Table 4 (but omitting the methanol fixation step). Following washing, cells were mounted in Citifluor mountant then viewed under rhodamine (GFAP) or fluorescein (LB1) optics. Fluorescent micrographs obtained under these conditions are shown in Fig.9.

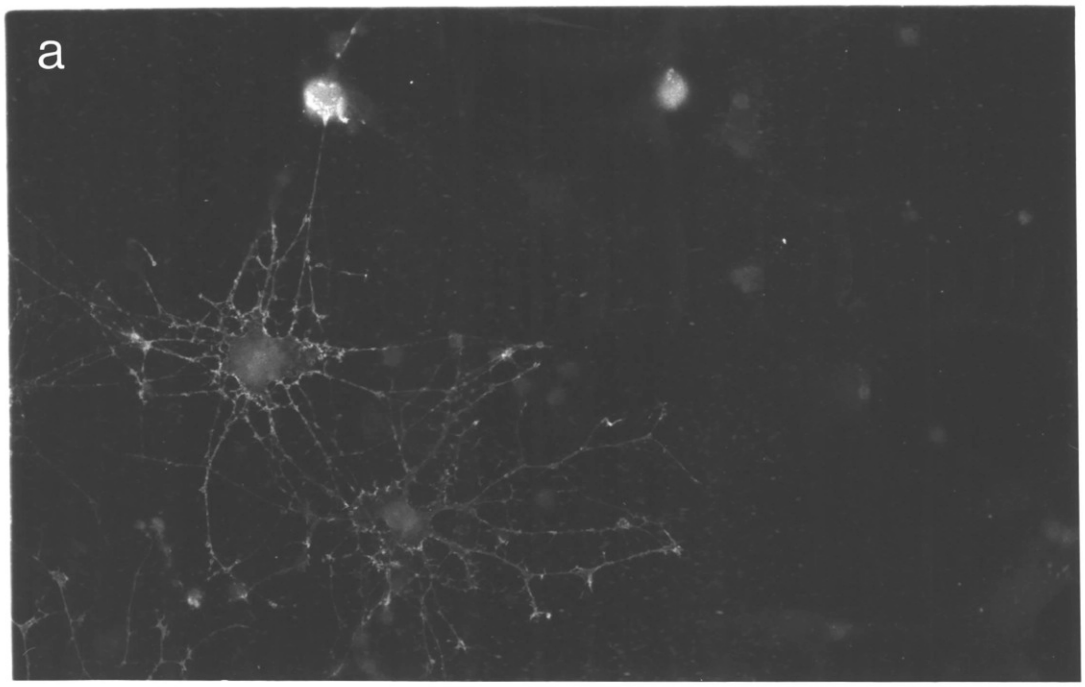
Fig.9 Fluorescent micrographs of 5 DIV astrocytes labelled with LB1 and anti-GFAP

5 DIV astrocytes from 8 day rat cerebellum were labelled successively with mouse monoclonal antibody LB1 / FITC goat anti-mouse Ig then rabbit anti-GFAP / TRITC swine anti-rabbit Ig. Antibodies were used at 1:50. Before GFAP labelling, cells were fixed with 4% paraformaldehyde for 5 min at room temperature then 5% glacial acetic acid in absolute ethanol for 10 min at -18°C.

(a) Fluorescein optics showing LB1 labelling of two stellate cells.

(b) Same field under rhodamine optics showing GFAP labelling of non-stellate cells in addition to the stellate cells labelled by LB1 in (a).

Scale = 50μm


a

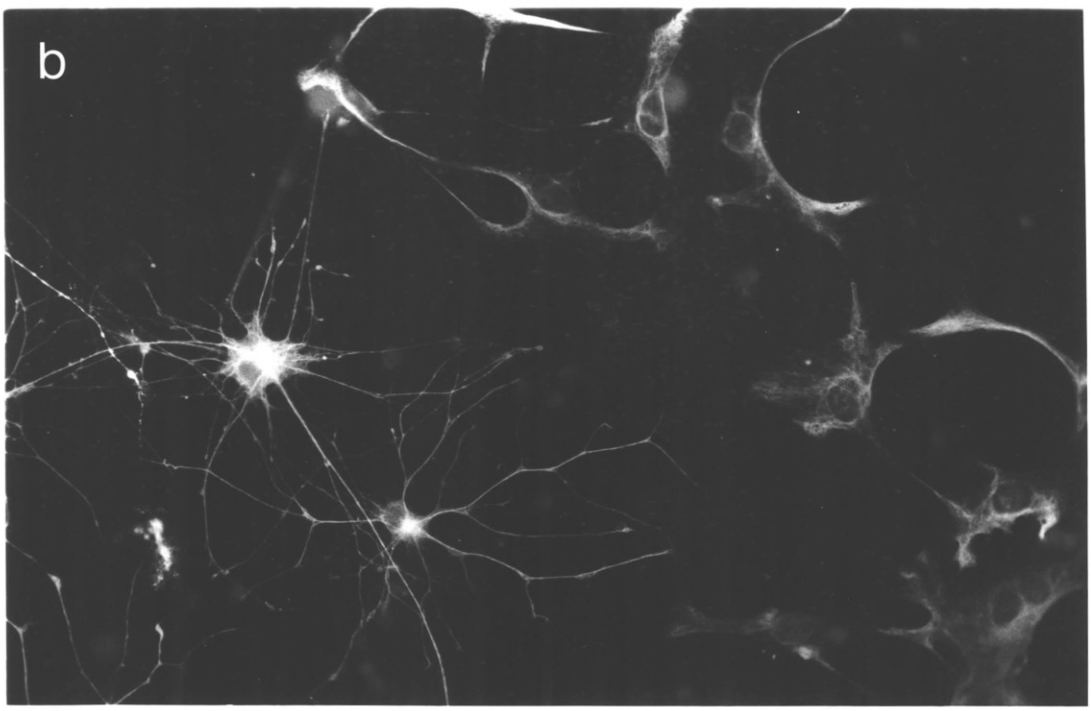

b

Fig.9 shows that the anti-GFAP antibody labelled both the stellate and the epithelioid astrocytes (b) but that LB1 labelled only the stellate cells (a). This was a potentially useful situation in that expression of the antigen recognised by LB1 (Ag-LB1) on only the stellate astrocytes could provide a means towards separation of this cell type from the epithelioid cells. Work by others in this laboratory have shown that Ag-LB1 is expressed by stellate astrocytes (or their precursors) from 1 DIV until 8-10 DIV and I have determined that Ag-LB1 is still present on cell surfaces immediately after isolation of the cells by the usual method (section II.B). This prompted an initial attempt at immunoaffinity plating of cells expressing Ag-LB1 onto LB1-coated culture dishes. This was unsuccessful, however, there remain many possibilities for using LB1 for cell purification and further studies are required. (An interesting limitation to this approach may have been shown in that I was unable to grow, in culture, cells which had been labelled with LB1/FITC goat anti-mouse Ig immediately after isolation despite their remaining viable up to at least 3 DIV as determined by exclusion of ethidium bromide.

III.D Attempted enrichment for stellate astrocytes by chemical manipulation of the culture medium.

It was of interest to be able to enrich the cultures for stellate astrocytes for two reasons. The first is that although the properties of epithelioid astrocytes could be investigated essentially in the absence of other cell-types by using cultures at later stages, this was not true for the stellate cells which were always observed in the presence of epithelioid astrocytes at a ratio of 60:30:10 epithelioid : stellate : others in the best cases. The second is that the stellate morphology of the type-S astrocyte was very similar to the morphologies observed *in situ* by the metal-impregnation techniques (and from which the term

astrocyte - "star cell" was derived) and so it was a possibility that this form would represent more closely the mature, differentiated astrocyte than would the epithelioid cells or many of the other models reported in the literature If this were the case then a purified population of these cells may prove to be a very useful in vitro model for differentiated astrocytes which would be amenable to extensive experimentation.

In this section are described experiments designed to enrich the cultures for stellate astrocytes using two general approaches - (a) to retard the more quickly dividing cells such that the stellate cells would survive and proliferate in preference to others and (b) to convert the apparently less differentiated type-E astrocytes to the more differentiated type-S cells. For approach (a) cytosine arabinoside was used; for approach (b) three manipulations were investigated. These were (i) treatment of cells with dibutyryl cyclic AMP, (ii) treatment of cells with a glial maturation factor and (iii) growing the cells in a chemically-defined, serum-free culture medium.

III.D.1 Treatment with cytosine arabinoside

In cultures grown under conditions selecting for neurons (section II.B.4), it was observed that these conditions were also able to select for stellate astrocytes over the epithelioid type (Levi et al.,1983). If this effect were due solely to the action of cytosine arabinoside (ara C) directly on the astrocytes and not as a result of enhanced neuronal survival (or other factors e.g. high [KCl]), and it could be combined with conditions unfavourable to neuronal survival, then this might provide a means for selecting for stellate astrocytes over both epithelioid astrocytes and neurons.

Astrocyte cultures were prepared from cerebella from 8-day old rats as

before then experimental dishes given a 24h pulse of ara C (50 µl dose, final concentration = 10 µM) over 1-2 DIV, 2-3 DIV or 3-4 DIV. The ara C-containing medium was replaced with fresh medium and the cells grown to 5 DIV when they were stained immunofluorescently with antiGFAP anti-serum. Cells were then counted and the number of stellate $GFAP^+$ cells expressed as a percentage of total GFAP+ cells. These results are presented in Table 6.

The counts show that independent of the time of addition of ara C, there is an approximately 50% reduction in the total number of astrocytes (GFAP+ cells) present at 5 DIV compared to the control values, however it is also clear that there has been only a modest increase in the "purity" of the culture with respect to the stellate cells. (Although there was approximately a 40-50% increase in the proportion of stellate out of total astrocytes, this still represented only a small fraction of the total). It was interesting to note that the ara C effect on the stellate astrocytes was most prominent over 1-2 DIV and virtually absent over 3-4 DIV. This suggested that these cells or their precursors go through perhaps a single division soon after being plated and, thereafter, remain mitotically quiescent. This confirms the results of Wilkin et al.(1983) who showed a maximum incorporation of ^{3}H-thymidine into stellate cell nuclei when this was added over 1-2 DIV.

III.D.2 Treatment of cultures with dibutyryl cyclic AMP

There is considerable evidence showing the ability of cAMP (or its cell-permeant monobutyryl and dibutyryl derivatives - mBcAMP and dBcAMP) to alter the morphology and biochemistry of cells in culture (Hsie & Puck, 1971; Johnson et al.,1971; Lim et al.,1973; Shapiro, 1973) These changes are usually from a less-differentiated to an apparently more-

Table 6. Effect of ara C on astrocyte numbers in culture

period with ara c treatment	total GFAP$^+$ cells per field (mean ± S.D)	stellate GFAP$^+$ cells per field (mean ± SD)	stellate as % of total
no treatment	49.7 ± 12.4	7.7 ± 3.5	15.5 %
1-2 DIV	26.6 ± 6.9	3.3 ± 2.3	12.4 %
2-3 DIV	19.8 ± 7.1	5.2 ± 3.1	26.3 %
3-4 DIV	26.8 ± 6.6	6.5 ± 3.7	24.3 %

Cerebellar astrocyte cultures were grown to 6 DIV then stained with anti-GFAP antibody. Experimental dishes were treated with 24h pulses of ara C (10μM final concentration) over the periods indicated. Counts were made of stellate and non-stellate GFAP$^+$ cells. The results are from a single experiment. Each figure was obtained by counting 500-1,000 cells from 20 fields on 3 coverslips. Figures are mean no. of cells per field ± S.D.

100

differentiated state. Of interest to this work are the demonstrations by several authors of the morphological transformation of cultured glial cells, or their precursors, following treatment with dBcAMP (Lim et al.,1973; Shapiro,1973). These observations suggested that it may be possible to convert type E to type S astrocytes by a similar treatment if the latter were a more-differentiated stage of the former.

Astrocyte cultures were prepared as before and allowed to grow to 6DIV. At this stage dBcAMP was added to a final concentration of 1mM. After 2h some cultures were stained immunofluorescently with anti-GFAP, others were given a second dose of dBcAMP after 24h and stained with anti-GFAP after a further 2h. Cells were then counted and the number of stellate cells expressed as a proportion of the total number of astrocytes. Fluorescent micrographs of the treated cells are shown in Fig.10 and the cell counts are given in Table 7. In addition to labelling with GFAP, the ability of the cells to accumulate the neurotransmitter γ-aminobutyric acid (GABA) was investigated since this function is known to be exhibited by mature astrocytes <u>in vivo</u> (see Chapter IV for a detailed investigation of this property and Chapter II for the methodology). The results from this study were expressed as the number of stellate, GABA-transporting cells as a percentage of total cells present and are given in Table 7. Autoradiographs are shown in Fig 11.

The figures in Table 7 showing the relative proportions of epithelioid and stellate $GFAP^+$ cells (astrocytes) indicate that a 2h exposure to dBcAMP had a marked effect, increasing the fraction of stellate astrocytes from 15% in the absence of treatment to 41% after treatment. This suggests a significant progress towards purifying the stellate astrocytes. However the dBcAMP effect was not permanent, the cultures having returned to their original appearance after about 6-8h. Further treatment 24h later was not very effective in altering the stellate/ epithelioid ratio (only a 4%

Fig.10 **Fluorescent micrograph of dBcAMP-treated cells labelled with anti-GFAP antibody.**

5 DIV cerebellar astrocytes from 8 day old rats were treated for 2h with 1mM dBcAMP then stained with rabbit anti-GFAP / TRITC swine anti-rabbit Ig. Antibodies were used at 1:50; cells were pre-fixed in methanol at -18^{o}C. Two cells at top right show normal stellate astrocyte characteristics. The two next to these are more elongate but with long processes; these are putative dBcAMP-induced "stellate astrocytes".

Scale ▬▬▬ = 50μm

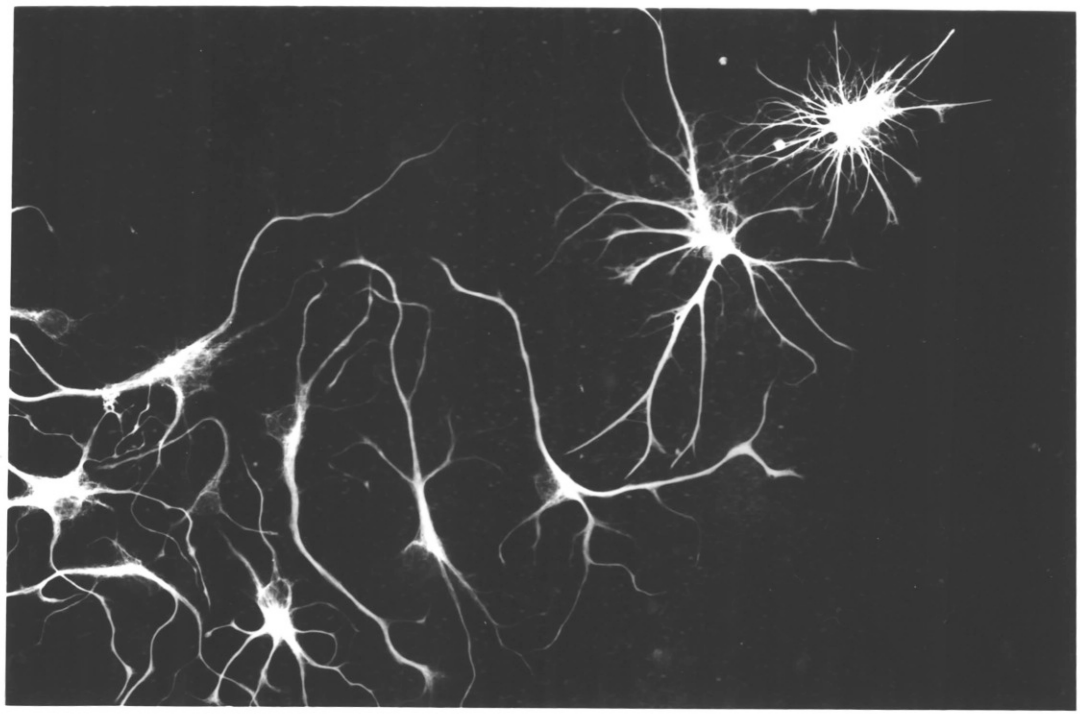


Table 7. Effect of dBcAMP on cellular composition of cultures

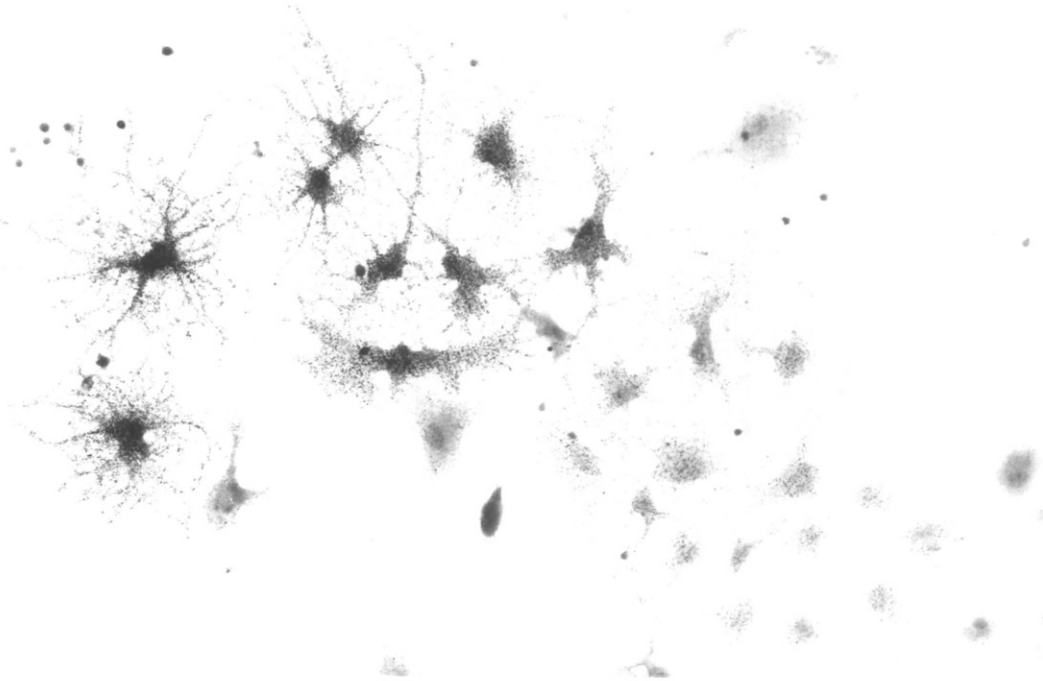
	GFAP immunofluorescence			GABA autoradiography		
	a	b		c	d	
treatment	total GFAP$^+$ cells	stellate GFAP$^+$ cells	b/a %	total cells	GABA-transporting stellate cells	d/c %
control	26.2 ± 12.8	3.8 ± 2.1	14.3 %	45.0 ± 21.0	4.6 ± 2.1	10.1 %
2h dBcAMP	43.0 ± 17.6	17.6 ± 5.8	40.9 %	56.0 ± 29.0	3.6 ± 2.9	6.6 %
24h + 2h	50.0 ± 19.3	9.1 ± 5.2	18.2 %	37.6 ± 19.3	3.8 ± 4.6	10.2 %

Astrocyte cultures from 8-day rat cerebellum were grown for 6 days in DMEM + 10% FCS. At 6 DIV, dBcAMP was added to a final concentration of 1mM with some plates receiving a second dose 24h later. 2h after addition of dBcAMP cultures were (i) stained with anti-GFAP antiserum or (ii) exposed to 3H-GABA (1µM, 5 min) then processed for autoradiography. Counts were made of total GFAP+ cells and stellate GFAP+ cells (from the GFAP-stained slides) and total cells and GABA-accumulating sellate cells (from the autoradiographs). For each treatment counts were made from 20 fields on 2 or 3 coverslips and the total no. of cells counted was between 500 and 1100. Figures in the table are mean no. of cells per field ± S.D.

Fig.11 Light microscopic autoradiograph of dBcAMP-treated cells following ^{3}H-GABA uptake.

5 DIV cerebellar astrocytes from 8 day old rats were treated for 2h with 1mM dBcAMP then exposed to ^{3}H-GABA (1μM, 5μCi/nmol) for 5 min. Cells were fixed with glutaraldehyde, exposed to photographic emulsion for 14d then developed and stained with haematoxylin and eosin. The figure shows process-bearing cells (top left) displaying heavy labelling with GABA and other cells (bottom right) with a much less-intense accumulation of GABA. The former are characteristic of the stellate cells normally seen in untreated cultures; the latter are presumably dBcAMP-transformed epithelioid cells (which in the absence of dBcAMP display a non-process-bearing morphology).

Scale ▬▬ = 50μm



increase towards the stellate morphology) suggesting a refractoriness of the dBcAMP response.

An important feature of the dBcAMP response was that although there was an increase in the number of cells extending processes there appeared to be now two populations of process-bearing cells. One, presumably, was the original stellate astrocyte population and the other the morphologically-transformed epithelioid astrocytes. The latter appeared to be only partially differentiated in that they had fewer processes than the type-S astrocytes and these often appeared thicker than in the type-S cells.

Further evidence for the only partially-differentiated nature of the dBcAMP-transformed cells came from the GABA-accumulation experiments. As described more-fully in Chapter IV the type-S astrocytes were able to transport GABA to a much greater extent than the type-E cells. A complete transformation of E to S -type astrocytes would require an alteration in the GABA uptake properties as well as a morphological change. When dBcAMP-treated cells were exposed to GABA there appeared to be little alteration in the proportion of cells which were stellate and accumulated GABA to a significant degree when compared with untreated cultures. The results in Table 7 show that 6.5% of all cells in treated cultures were stellate, GABA-accumulating cells compared with 10.1% in untreated cultures. Again there was no change after a second dose of dBcAMP 24h after the first. Longer-term treatments with dBcAMP also did not change the proportion of GABAaccumulating stellate cells.

It seems, therefore, that although dBcAMP may effect a partial differentiation of astrocytes in vitro it will not induce a complete transformation.

III.D.3 Treatment ofcultures with glial maturation factor

It was shown in 1973 (Lim et al.,1973)) that a macromolecular factor in extracts of adult rat brain was capable of transforming (morphologically) dissociated embryonic rat brain cells in monolayer culture. A similar activity was found also in extracts from porcine (Lim & Mitsunobu,1975) and bovine (Kato et al.,1981) brains. These activities are associated with proteins of M_r = 13,000 daltons for bovine GMF (Lim & Miller,1984) and M_r = 40,000 and 200,000 daltons for porcine GMF (Kato et al.,1979). These factors have been shown to induce both biochemical and morphological maturation of astrocytes (astroblasts) in culture therefore it seemed possible that GMF would be able to trigger differentiation of the epithelioid astrocytes into the stellate form in the cultures used in the present work (which have many similarities to those used by the investigators studying GMF action).

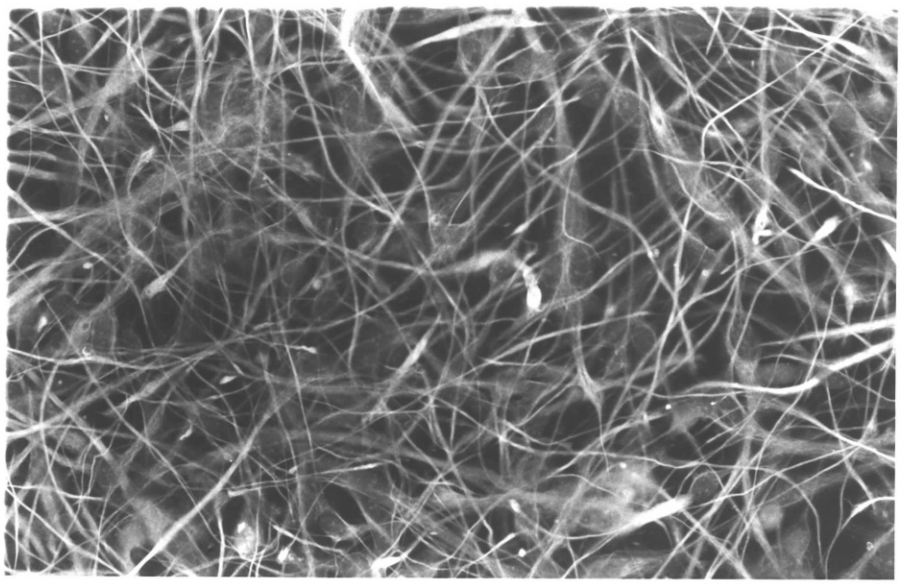
Astrocyte primary cultures from 8-day old rat cerebellum were prepared as before and grown to 13 DIV. At 13 DIV the medium was removed and replaced with fresh medium containing 0.003mg (equivalent to 0.076 units) of GMF from bovine brain (lot 82:66 + 67 ; a kind gift of Dr Ramon Lim, Dept. of Neurology, University of Iowa, USA). This replacement of medium with fresh medium containing the same amount of GMF was repeated at 15, 18 and 20 DIV. In control cultures the medium was replaced with fresh medium containing no GMF but an equivalent amount of the buffer in whch the GMF was dissolved (0.15M NaCl/0.02M tris-HCl, pH 7.4). At 21 DIV cells were immunofluorescently stained with anti-GFAP antibodies. Photographs of control and treated cultures stained with anti-GFAP are shown in Fig.12. GABA uptake studies were also carried out (see section II.C and Chapter IV) and the resultant autoradiographs are shown in Fig.13.

Fig.12 **Fluorescent micrograph of confluent cerebellar astrocytes treated with glial maturation factor and labelled with anti-GFAP antibody.**

13 DIV (confluent) cerebellar astrocytes from 8 day old rats were treated for 7d with (a) glial maturation factor (GMF, 3ug per 2ml DMEM in a 35mm culture dish) or (b) 2ml fresh DMEM. Cells were then labelled with rabbit anti-GFAP / TRITC swine anti-rabbit Ig. Control dishes (b) showed individual cells with few, if any, processes. GMF-treated cells (a) showed a fibrous network of GMF-transformed cells with the individual cells being difficult to distinguish.

Scale ▬▬ = 70 μm

a



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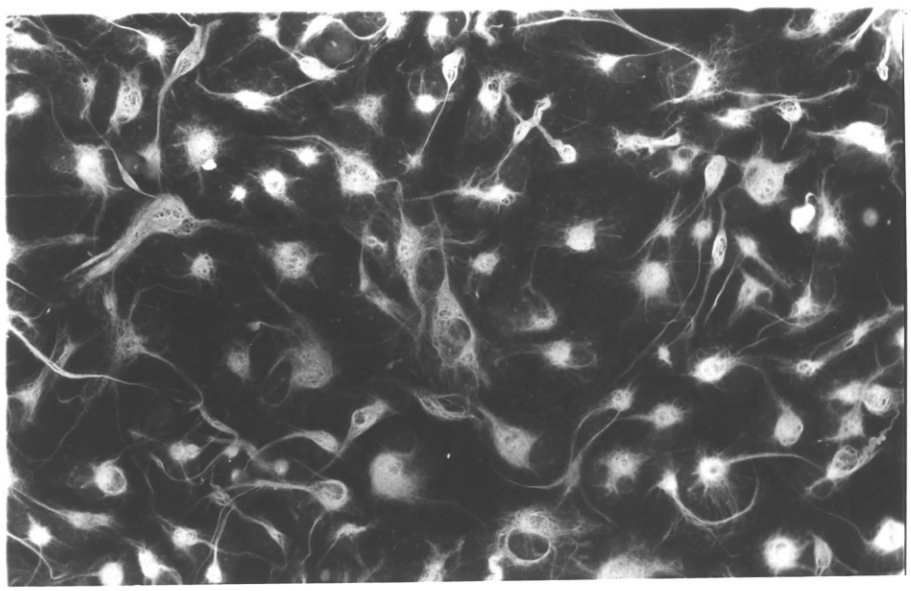
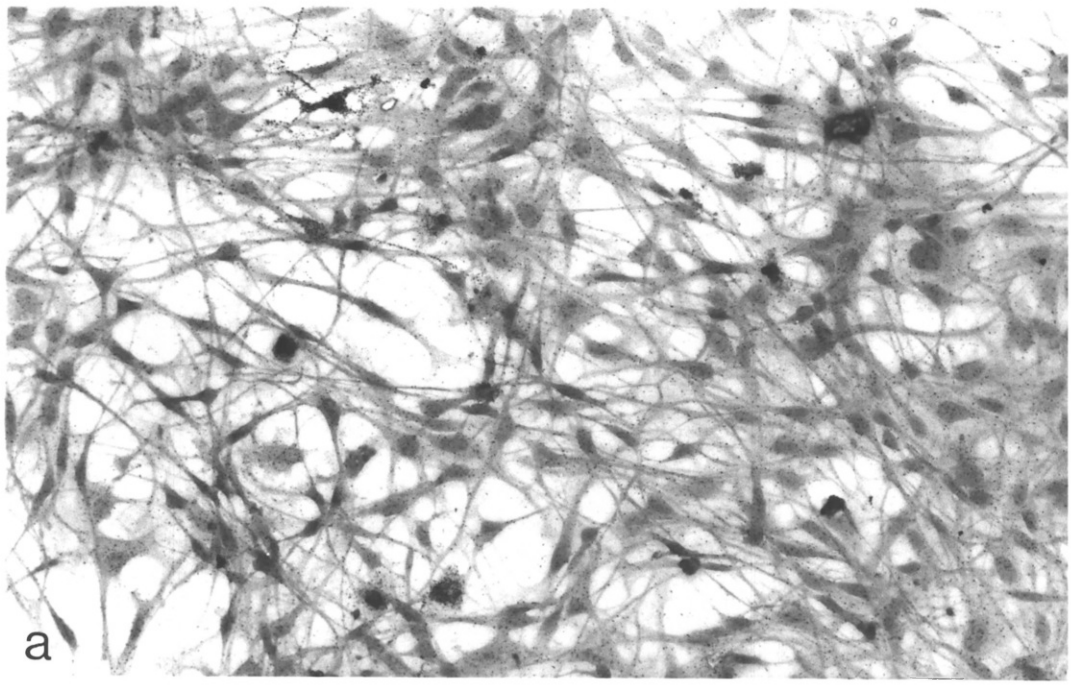


Fig.13 Autoradiograph of confluent cerebellar astrocytes treated with glial maturation factor then exposed to ^{3}H-GABA.

13 DIV (confluent) cerebellar astrocytes from 8 day old rats were treated for 7d with (a) glial maturation factor (GMF, 3ug per 2ml DMEM in a 35mm culture dish) or (b) 2ml fresh DMEM. Cells were then incubated with ^{3}H-GABA (1μM, 5μCi/nmol) for 5 min, fixed with glutaraldehyde, exposed to photographic emulsion for 14d then developed and stained with haematoxylin and eosin. A marked morphological difference can be seen between the treated (a) and untreated (b) cells but there was no obvious change in the amount of GABA accumulated.

Scale ▬▬ = 50μm


a

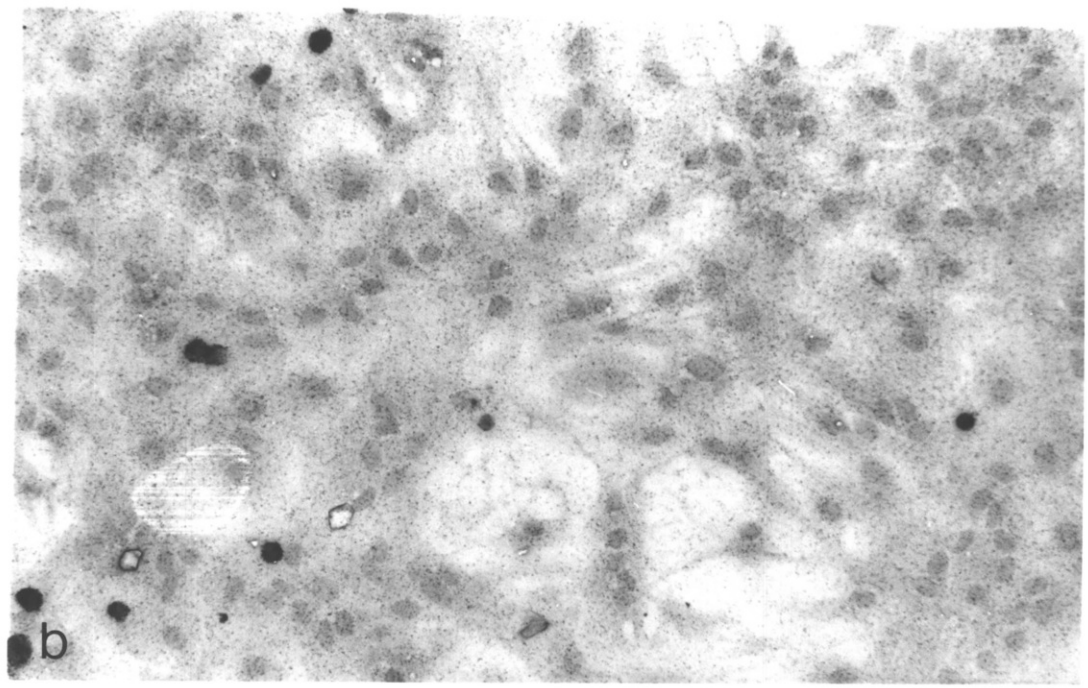

b

Fig.12 shows that there has been a marked morphological transformation following treatment of cells with GMF. The appearance (as demonstrated by GFAP staining) before treatment was of distinct individual cells with, generally, very fine, wispy processes no longer than the width of the cell-body similar to the 12 DIV cultures and 5 DIV E-type cells observed before. After treatment this had changed to a cellular network of $GFAP^+$ cells with ramifying processes considerably longer than the cell bodies (although the latter were often difficult to detect amongst the processes and it was difficult to trace the origins of most of the processes). It should be noted that despite the marked change in morphology, very few, if any, adopted the stellate shape observed at 5DIV in untreated cultures. The transformed morphology also did not resemble that seen after dBcAMP treatment. From the autoradiographs (Fig 13) following exposure to GABA it is clear that this function has not apparently been affected by treatment with GMF; the treated cells all show the same low level of accumulation as was observed in untreated cells.

The morphological effect was similar to that observed on foetal rat glioblasts in response to bovine GMF (Lim & O'Connell,1982) and also to the effects of GMF from rat and pig brains (Lim et al.,1973; Lim & Mitsunobu; 1975). Lim & O'Connel (1982) also pointed out an intensification of GFAP-immunostaining in the processes and end-feet of the GMF-transformed glioblasts; this was also observed in the present work.

From these results it appears that GMF does have a significant "maturation" activity on the morphology of epithelioid astrocytes *in vitro*, however, this influence does not extend to all the functional activities of the cell as demonstrated by the inability of this factor to stimulate GABA transport in the morphologically-differentiated cells. For this reason, as was the case with dBcAMP, treatment with GMF is not a simple means of obtaining homogeneous cultures of type-S astrocytes.

III.D.4 Growth of cells in serum-free, chemically-defined medium

For many years a common constituent of tissue-culture media has been mammalian serum of some type e.g. foetal calf serum, newborn calf serum, horse serum etc.) There have been moves throughout this period, however, to discontinue the use of serum and replace it with defined chemical supplements for at least two reasons. Firstly, one of the aims of tissue culture is to provide a controlled, extensively manipulable experimental system; this can never be achieved whilst the "unknown" FCS remains a requirement for successful culture. Secondly, there have been reports indicating that serum may retard the differentiation of cells (Moonen et al.,1985; Seeds et al.,1970) and it was this point that suggested that removal of serum from the medium might allow the full differentiation of the cultured astrocytes.

Astrocyte cultures from 8-day old rat cerebellum were prepared as before and grown to 6 DIV and 32DIV in DMEM + 10% foetal calf serum (FCS). At 6 DIV the medium was removed and replaced with chemically-defined, serum-free Bottenstein-Sato Medium (BSM). After 4h and 28h in the BSM cells were immunofluorescently stained with antiGFAP antibody and investigated for their GABA transport capability by autoradiography. Cells were counted to ascertain the number of stellate astrocytes as a proportion of total cells stained with antiGFAP, and the number of stellate, GABA-accumulating cells as a percentage of total cells. Cell counts are given in Table 8.

These figures indicate that removal of serum from the culture medium will not effect any enrichment of the culture for stellate astrocytes (identified by GFAP immunostaining) or for GABA-transporting stellate cells.

Table 8. Effect of BSM on cellular composition of cultures

	GFAP immunofluorescence			GABA autoradiography		
	a	b		c	d	
treatment	total GFAP+ cells	stellate GFAP+ cells	b/a%	total cells	GABA-transporting stellate cells	d/c%
control	26.2 ± 12.8	3.8 ± 2.1	14.3 %	45.0 ± 21.0	4.6 ± 2.1	10.1 %
6h in B-S	69.0 ± 30.0	5.5 ± 3.4	8.0 %	55.4 ± 19.6	4.0 ± 2.7	7.2 %
24h + 6h in B-S	44.2 ± 15.2	6.4 ± 2.8	14.4 %	41.5 ± 22.9	3.6 ± 3.4	8.7 %

Astrocyte cultures from 8 day old rat cerebellum were grown for 6 days in DMEM + 10% FCS then transferred to Bottenstein-Sato (B-S) medium. After 6h and 30h in B-S medium, cultures were (i) stained with anti-GFAP antiserum or (ii) exposed to ^{3}H-GABA (1μM, 5 min) then processed for autoradiography. Counts were made of total GFAP+ cells and stellate GFAP+ cells (from the GFAP-stained slides) and total cells and GABA-accumulating sellate cells (from the autoradiographs). For each treatment counts were made from 20 fields on 2 or 3 coverslips and the total no. of cells counted was between 500 and 1100. Figures in the table are mean no. of cells per field ± S.D.

III.D. Summary

It is clear, then, that in cultures derived from neonatal rat cerebellum there develop two morphologically-distinct astrocyte sub-types stellate (S-type) and epithelioid (E-type). The stellate cells first appeared at approximately 2-3 DIV, reached a peak (in terms of proportion of all astrocytes present) at 5-7 DIV and thereafter declined so that by 12-15 DIV few, if any, remained. The epithelioid cells were apparent as early as 1 DIV and continued to increase in number until confluence was reached.

From their morphologies, from their degree of expression of vimentin, and from their GABA-transporting capabilities (see Chapter IV) it appeared that the stellate form represented a more highly differentiated astrocyte than did the epithelioid type. It should be noted that it is the combination of these properties rather than the possession of any single one that suggests the more-differentiated nature of the stellate cells. Using a variety of potential "differentiating" agents, however, little success was achieved towards changing the epithelioid cells into the stellate type. Whenever this was achieved to any degree, the effect was generally incomplete and reversible. This suggests, perhaps, that there may not be a simple relationship between the two cell types and could indicate that they are, in fact, different classes of astrocyte (although it could equally well reflect a lack of the correct differentiation signal or combination of signals under the conditions used). The relationships between the two forms of astrocyte and between the astrocytes *in vitro* and *in vivo* are now being addressed using combinations of antibodies to identify more thoroughly the astrocyte types in culture and also as cytotoxic agents (in combination with complement) to remove specific populations from the cultures. Work from this laboratory

(Johnstone et al.,1985; Wilkin & Cohen, in preparation) and from other groups (Levi et al., personal communication; Raff et al., 1983) supports the idea that the stellate and epithelioid astrocytes in these cultures are different cell types.

IV TRANSPORT OF NEUROTRANSMITTERS BY CULTURED ASTROCYTES

IV.A. Introduction

In Chapter III it was apparent that in primary cultures of dissociated rat cerebellum, spinal cord and optic nerve, the astrocyte population (defined by the expression of GFAP) was morphologically heterogeneous. At least two forms were observed: one had a small perikaryon and many fine, radial processes whereas the other was polygonal or elongate (epithelioid) with a large perikaryon and few (if any) thick processes. Although the stellate shape for astrocytes was expected from the classical studies of Cajal and others using metallic impregnation staining methods, the epithelioid form did not fit easily into the established classification of astrocyte types (see Fig.1)

One hypothesis that might explain this is that the morphologically-distinct populations of astrocytes observed in culture represent not two discrete astrocyte types but different stages of differentiation of the same cell, the stellate cells being the more differentiated of the two types. A way in which this hypothesis could be tested would be to look for a difference in expression by the cells of differentiated astrocyte properties. The most commonly used astrocyte marker is glial fibrillary acidic protein (GFAP), however, expression of this marker has already been shown (Chapter III) not to distinguish between the two cultured astrocyte types since it was expressed by both stellate and epithelioid forms. Another property exhibited by astrocytes <u>in vivo</u> is the ability to transport neurotransmitters from the extracellular fluid, across the cell membrane to the inside of the cell. Initial studies by Gordon (1982) showed that the two astrocyte forms in culture could indeed be distinguished by their differing abilities to take up γ-aminobutyric acid (GABA) from the external medium.

In this chapter are presented the results of experiments to investi-

gate further the abilities of cultured astrocytes to transport neurotransmitters (especially GABA) and to determine some biochemical and kinetic properties of the transport processes. These properties were then used as the basis for comparison of the stellate and epithelioid astrocytes with other cultured astrocyte systems and with astrocytes *in vivo*.

IV.B. Autoradiographic localisation of transported neurotransmitters in cultured cerebellar astrocytes

IV.B.1. Localisation of transported GABA

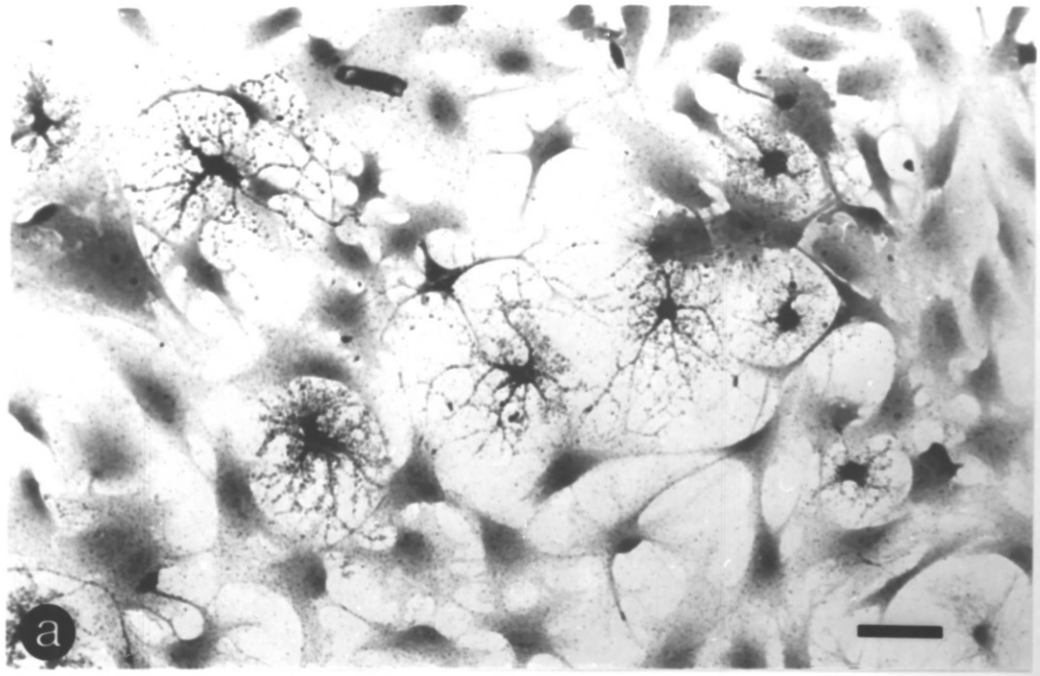
Localisation of transported ^{3}H-GABA in cultured cerebellar astrocytes at 5 DIV and 12 DIV was carried out as described in sections II.C & D. The cells were exposed to ^{3}H-GABA (1μM, 5μCi/nmol) for 5 min at 37^{o}C then fixed with 5% glutaraldehyde for 30 min at room temperature after which they were processed for autoradiography, stained and mounted. Uptake was carried out in Tris-buffered Krebs solution, pH 7.4 (TBK) containing 138 mM Na^{+} and in a sodium-free medium (5 DIV only). Autoradiographs from the uptake in the presence and the absence of sodium are shown in Figs 14 and 15 (5 DIV and 12 DIV respectively).

Comparison of Figs 14a with 14b show that under the conditions employed here, almost all the ^{3}H-GABA localised autoradiographically was transported in a Na^{+}-dependent manner since no silver grains were observed over any cells when sodium was omitted from the buffer. At 5 DIV in the presence of sodium (Fig.14a) these experiments show ^{3}H-GABA accumulation by two populations of cells. One type was very heavily labelled with silver grains, had a small cell body and many radial processes with both the cell body and the processes being labelled. In contrast, a second population was much less intensely labelled, had a

Fig.14 Light microscopic autoradiograph of 5 DIV cerebellar astrocytes following uptake of ^{3}H-GABA.

5 DIV cerebellar astrocytes from 8 day old rats were incubated with ^{3}H-GABA (1μM, 5μCi/nmol) for 5 min in (a) a medium containing 128mM Na^{+} or (b) a medium in which the Na^{+} had been replaced with choline. Cells were fixed with glutaraldehyde, exposed to photographic emulsion for 14d then developed and stained with toluidine blue. In the presence of Na^{+} (a), process-bearing (stellate) cells were heavily labelled with GABA and non-process-bearing (epithelioid) cells were poorly labelled. In the absence of Na^{+} (b) no silver grains were seen over any cell type indicating inhibition of uptake.

Scale bar = 50μm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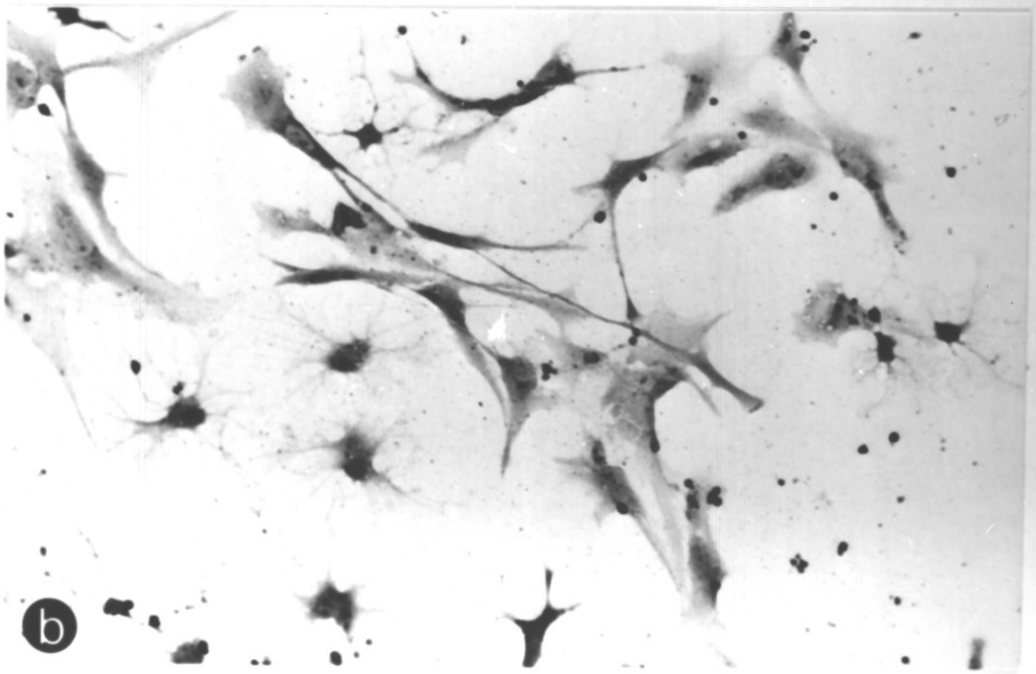
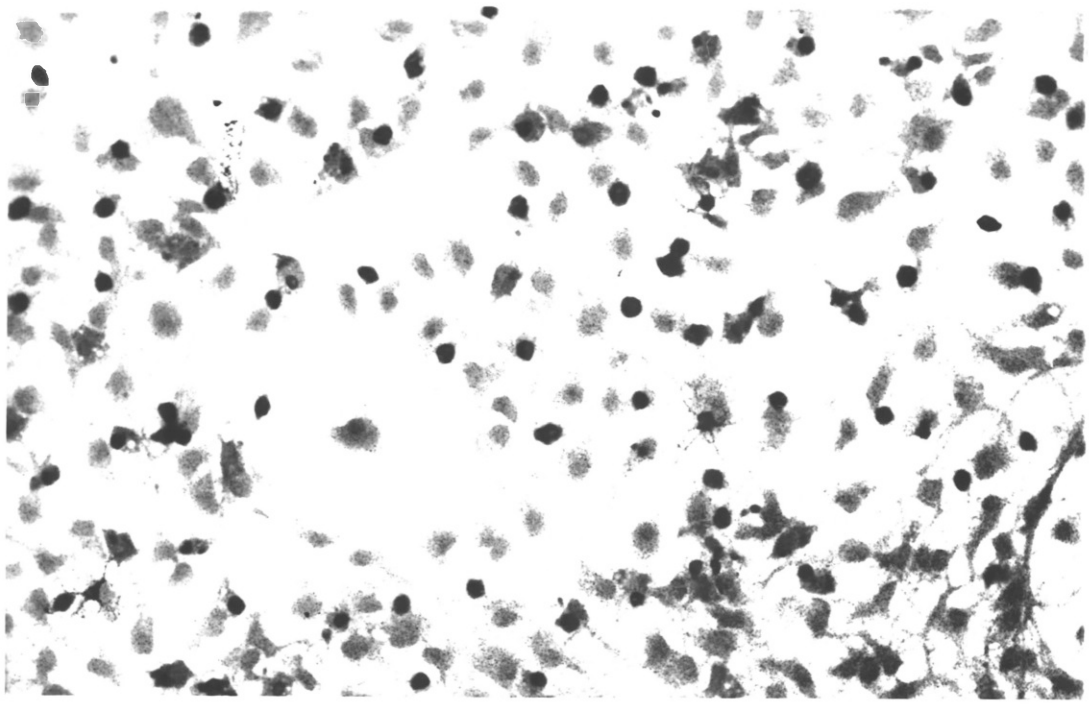

b

Fig.15 Light microscopic autoradiograph of 12 DIV cerebellar astrocytes following uptake of ^{3}H-GABA.

5 DIV cerebellar astrocytes from 8 day old rats were incubated with ^{3}H-GABA (1μM, 5μCi/nmol) for 5 min in a medium containing 128mM Na^{+}. Cells were fixed with glutaraldehyde, exposed to photographic emulsion for 14d then developed and stained with toluidine blue. The figure shows that almost all cells were of the non-process-bearing type and that they showed a low degree of GABA accumulation.

Scale ▬▬ = 50 μm



considerably larger cell body and possessed no fine processes. The heavily-labelled stellate cells constituted about 15-30% of the total cell number and frequently occurred in groups of 5-10 cells in isolated patches. The less heavily labelled non-stellate cells made up most of the remainder of the culture with only a few, if any, cells showing no labelling at all.

At 12 DIV there were many fewer of the stellate cells showing heavy labelling with GABA (<1% of the total astrocytes) with almost all cells being of the non-stellate or epithelioid type and showing a similar degree of labelling as they did at 5 DIV. At 12 DIV the cultures had reached confluence.

Occasionally at 5 DIV and very infrequently at 12 DIV, small round or ovoid cells with one or a few long thin processes were seen to be very intensely labelled with ^{3}H-GABA. Transport into these cells was again Na^{+}-dependent in that such forms were never seen to be labelled in the absence of Na^{+}. Immunofluorescent labelling studies (Chapter III) performed on parallel cultures allowed the identification of the GABA-transporting cells with some certainty as follows:

stellate, GABA-transporting cells	-	astrocytes
epithelioid, GABA-transporting cells	-	astrocytes
ovoid, GABA-transporting cells	-	neurons

IV.B.2. Efficiency of fixation of transported neurotransmitters by glutaraldehyde.

In order to determine the proportion of transported ^{3}H-GABA and (other transmitters) retained in the cells by the glutaraldehyde fixation step, the amount of isotope in the cells after fixation was counted by liquid scintillation counting (LSC) and compared with the amount present

Table 9 Efficiency of fixation of neurotransmitters and analogues in cultured cells by glutaraldehyde.

ACHC		27 %
β-alanine		28 %
GABA	(5 DIV)	32 %
	(12 DIV)	36 %
glycine		25 %
noradrenaline		31 %

Uptake of radiolabelled compounds was carried out over 5 min. The radioactivity in the cells was determined either immediately or after fixation in 5% glutaraldehyde for 30 min. The radioactivity in fixed cells was expressed as a percentage of that measured in unfixed cells. The values shown are from triplicate measurements from single experiments except for the case of GABA where values are from triplicates from three experiments.

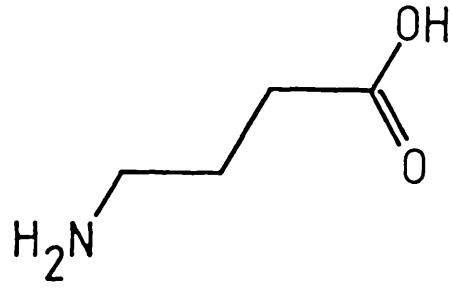
immediately after stopping the uptake process. The procedure followed was the same as for IV.A.1 except that the cells were prepared for LSC and protein determinations rather than for autoradiography. The fixation efficiency was measured at 5 DIV and 12 DIV for ^{3}H-GABA and at 5 DIV for ^{3}H-ACHC, ^{3}Hß-alanine, ^{3}H-noradrenaline and ^{3}H-glycine. The results were expressed as amount of transmitter retained as a percentage of total amount transported and are shown in table 9. The results show that between 25% and 36% of the accumulated transmitter is fixed within the cells using the conditions of 5% glutaraldehyde for 30 min at room temperature. The efficiency for ^{3}H-GABA fixation was similar in astrocytes of 5 DIV and 12 DIV. This represents a significant loss of labelled compound - a point that should be kept in mind since the loss may not be equal from all cell types (although the similarity between the 5 DIV and 12 DIV data suggests this is not the case).

IV.B.3. Effect of structural analogues of GABA on ^{3}H-GABA transport.

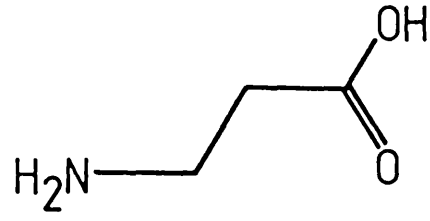
Several structural analogues of GABA have been reported to inhibit transport of ^{3}H-GABA by astrocytes, neurons or both: some of these were tested in the culture system used in the present work. Those tested were cis-1,3-aminocyclohexane carboxylic acid (ACHC), 2,4-diaminobutyric acid (DABA), β-alanine, nipecotic acid (piperidine-3-carboxylic acid), cis-4-hydroxynipecotic acid, and 4,5,6,7-tetrahydro-isoxazolo-[4,5c]-pyridin-3-ol (THPO). The structures of these compounds are shown in Fig 16.

Uptake experiments were carried out as in section IV.A.1 with the additional step of adding a small volume of inhibitor solution immediately before the ^{3}H-GABA to give a final concentration of 1mM. The ^{3}H-GABA was used at 1µM with a specific radioactivity of 5µCi/nmol. The

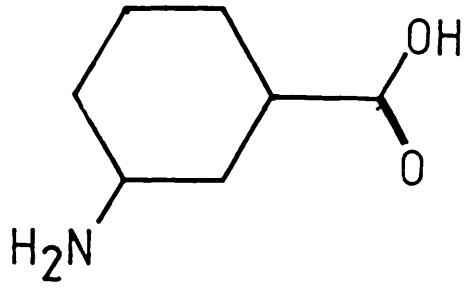
Fig.16 Structures of GABA analogues used as inhibitors of uptake.



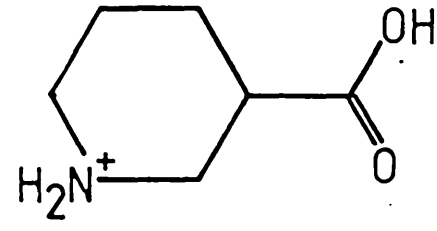
GABA
(γ-aminobutyric acid)



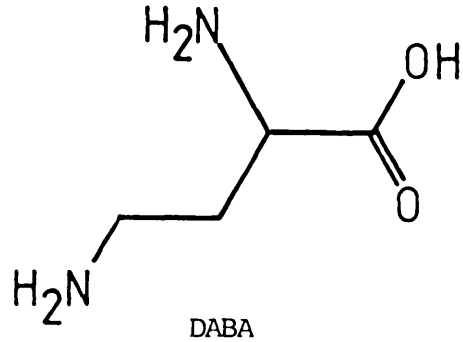
β-alanine



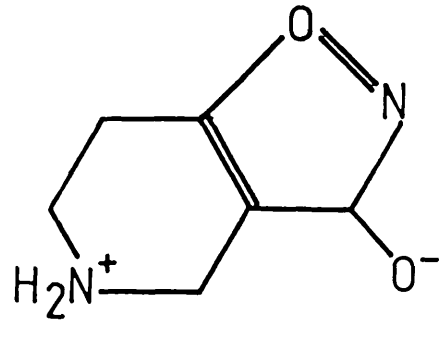
ACHC
(cis-1,3-aminocyclohexane-
carboxylic acid)



nipecotic acid
(piperidine-3-
carboxylic acid)



DABA
2,4-diaminobutyric acid



THPO
(4,5,6,7- tetrahydroisoxazolo-
(4,5c)-piperidin-3-ol)

final volume in each dish was 1ml. The cultures were used at 5 DIV. Autoradiographs were prepared as in section II.D. and the results are shown in Fig 17.

In the case where no inhibitor was present (see Fig.14) the pattern of ^{3}H-GABA accumulation was the same as described before, namely heavy labelling of the stellate cells with markedly less labelling of the epithelioid cells. In the presence of the GABA analogues a range of inhibition was observed. In the cases of nipecotic acid, cis-4-hydroxynipecotic acid and THPO there was almost total inhibition of uptake into both stellate and non-stellate cells. In the presence of ACHC there was a very marked (but not complete) inhibition of uptake by the stellate cells; The effect, if any, on the epithelioid cells was difficult to assess (but see section IV.E.2). β-alanine was without apparent effect whereas DABA had an effect somewhere between those of ACHC and β-alanine (closer to ACHC).

IV.B.4. Transport of ^{3}H-ACHC and ^{3}H-β-alanine by cultured cerebellar astrocytes

In order to determine whether uptake inhibitors were alternative substrates for the glial GABA transporter(s) and in an attempt to characterise further any selectivity of these compounds for the stellate or epithelioid cells, the uptake of two of the inhibitors ACHC and β-alanine - was investigated. The experiments were performed exactly as for ^{3}H-GABA transport using the ^{3}H-ACHC and ^{3}H-βalanine at 20μM and specific radioactivity of 96 mCi/mmol. (The changes from the usual concentrations and specific activities occurred because the ^{3}H-ACHC was supplied at 96 mCi/mmol and the specific activities and concentrations of the other isotopes were changed to this level). The resultant

Fig.17 Light microscopic autoradiograph of 5 DIV cerebellar astrocytes following exposure to ^{3}H-GABA in the presence of GABA-analogues.

5 DIV cerebellar astrocytes from 8 day old rats were incubated with ^{3}H-GABA (1μM, 5μCi/nmol) for 5 min, in the presence of:-

(a) cis-1,3-aminocyclohexane carboxylic acid

(b) β-alanine

(c) nipecotic acid

(d) 2,4-diaminobutyric acid

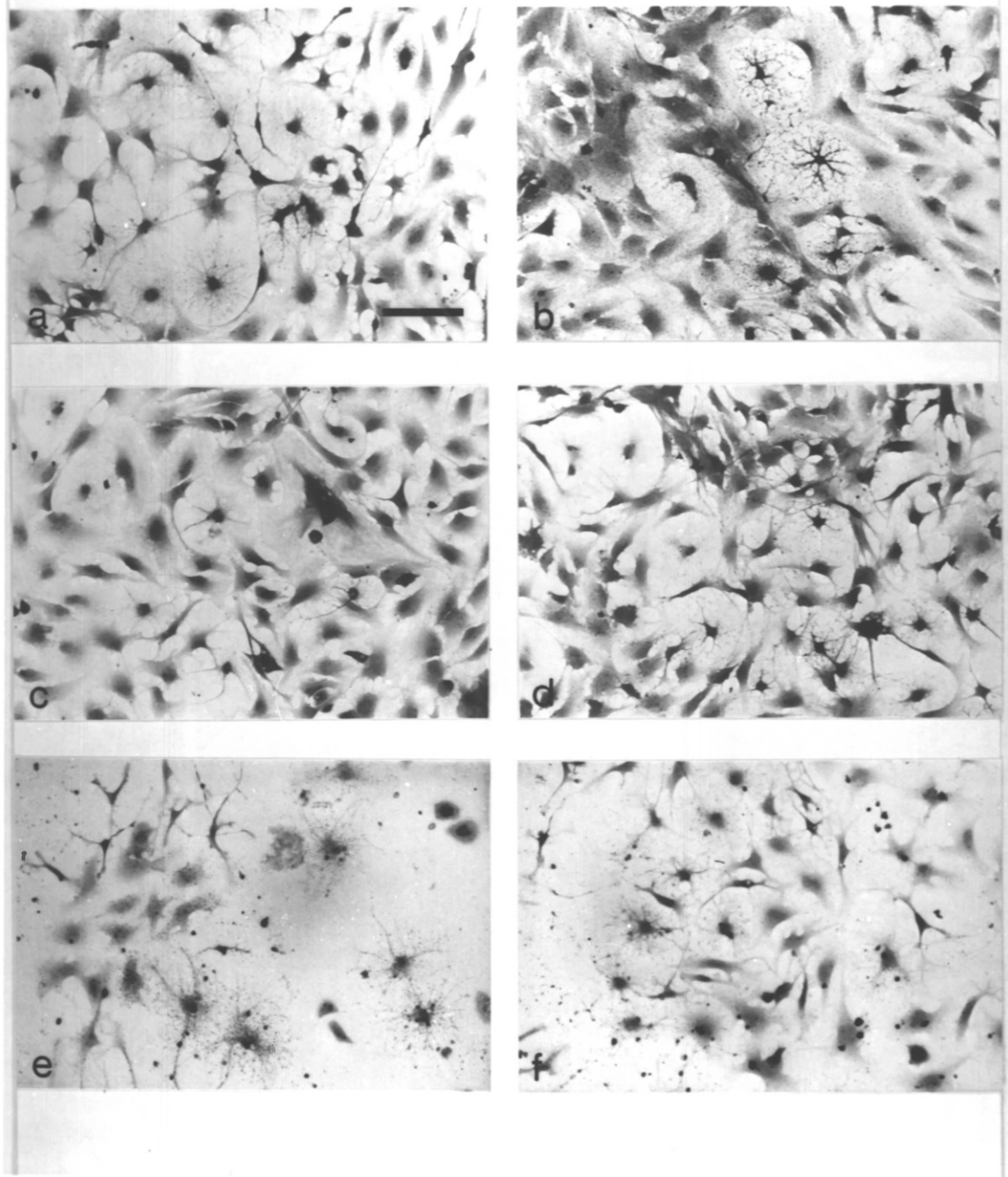
(e) 4,5,6,7-tetrahydroisoxazolo-[4,5,c]-pyridin-3-ol (THPO)

(f) cis-4-hydroxynipecotic acid

Cells were fixed with glutaraldehyde, exposed to photographic emulsion for 14d then developed and stained with haematoxylin and eosin.

Compared with the case when no inhibitor was present (Fig.17a), ACHC (a), nipecotic acid (c), DABA (d) and cis-4-hydroxynipecotic acid (f) were very effective inhibitors of GABA uptake. THPO (e) was moderately effective but β-alanine had no obvious inhibitory action.

Scale bar = 100μm


a
b
c
d
e
f

autoradiographs are shown in Fig.18. Efficiency of fixation of ^{3}H-ACHC and ^{3}H-β-alanine were determined; these values are shown in Table 9 and indicate a similar level of retention of GABA, ACHC and β-alanine. The same experiment was performed on a neuron-enriched culture (Fig.19); this showed the same labelling pattern as in the astrocyte culture and shows more clearly the labelling of neurons by β-alanine.

The autoradiographs show a clear difference in the distribution of the two inhibitors. ^{3}H-ACHC was accumulated to a moderate degree by the stellate cells but not by the epithelioid ones whereas β-alanine was transported very well by the epithelioid astrocytes but poorly by those showing the stellate morphology. Uptake into neuronal cells indicates that ^{3}H-ACHC is accumulated by only a small proportion of neurons however β-alanine seems to be taken up by almost all cells.

IV.B.6. Effects of metabolic inhibitors on ^{3}H-GABA transport

A number of inhibitors of cellular metabolism were tested for their effects on GABA transport in 5 DIV and 12 DIV astrocytes. The procedures followed for these experiments were the same as used for kinetic studies (section II.C) with the modification that the inhibitor under test was added to the incubation mixture at a specified time before the ^{3}H-GABA. The inhibitors used were ouabain, rotenone, NaCN, and NaF; their sites of action are shown in Fig.20. The final concentrations, times of addition prior to ^{3}H-GABA and the results are given in Table 10. The latter are expressed as nmol GABA accumulated per min per mg cell protein in the presence of inhibitor as a percentage of the same parameter measured in the absence of inhibitor.

These figures show that the transport systems at both ages in culture

Fig.18 Light microscopic autoradiographs of 5 DIV cerebellar astrocyte-enriched cultures following exposure to ^{3}H-ACHC and ^{3}H-β-alanine.

5 DIV cerebellar astrocyte-enriched cultures from 8 day old rats were incubated with ^{3}H-GABA, ^{3}H-ACHC or ^{3}H-β-alanine (21μM, 96mCi/mmol) for 5 min, fixed with glutaraldehyde, exposed to photographic emulsion for 3 weeks then developed and stained with haematoxylin and eosin.

GABA labelled the stellate astrocytes heavily (arrowheads in (a)) with the epithelioid cells labelled less heavily (arrows in (a)). ^{3}H-ACHC labelled stellate astrocytes most heavily (b) with only few grains seen over epithelioid cells. β-alanine (c) labelled epithelioid astrocytes moderately/heavily with little labelling of the stellate cells.

Scale bar = 50μm

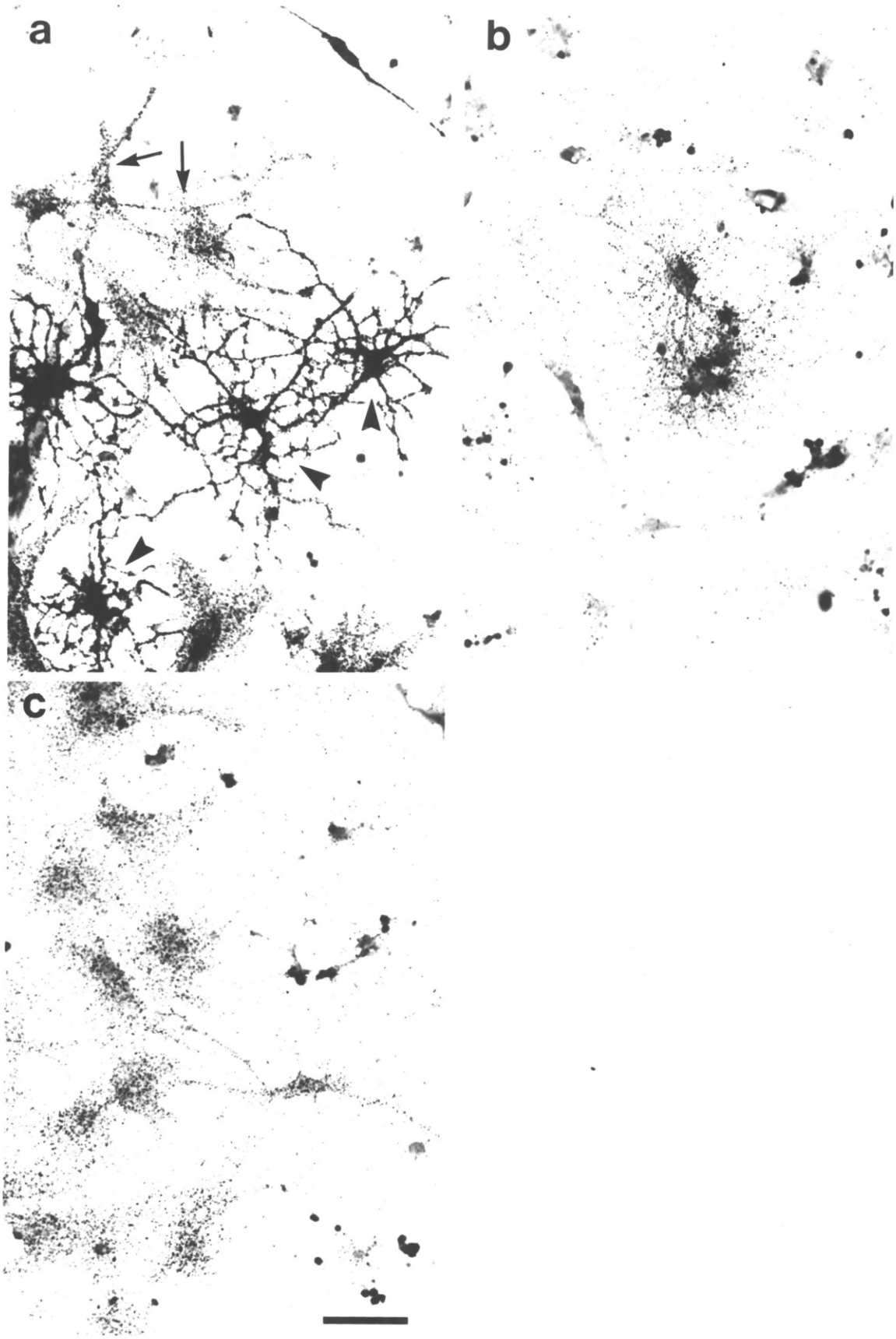

a
b
c

Fig.19 Light microscopic autoradiographs of 5 DIV cerebellar neuron-enriched cultures following exposure to ^{3}H-ACHC and ^{3}H-β-alanine.

5 DIV cerebellar neuron-enriched cultures from 8 day old rats were incubated with ^{3}H-GABA, ^{3}H-ACHC or ^{3}H-β-alanine (21μM, 96mCi/mmol) for 5 min, fixed with glutaraldehyde, exposed to photographic emulsion for 3 weeks then developed and stained with haematoxylin and eosin.

In these cultures, ^{3}H-β-alanine labelled many neuronal processes and perikarya (b); ^{3}H-ACHC labelled a smaller proportion of the neuronal processes and perikarya (c). The astrocytes in these cultures had the same labelling pattern as they showed in astrocyte-enriched cultures.

Scale bar = 50μm

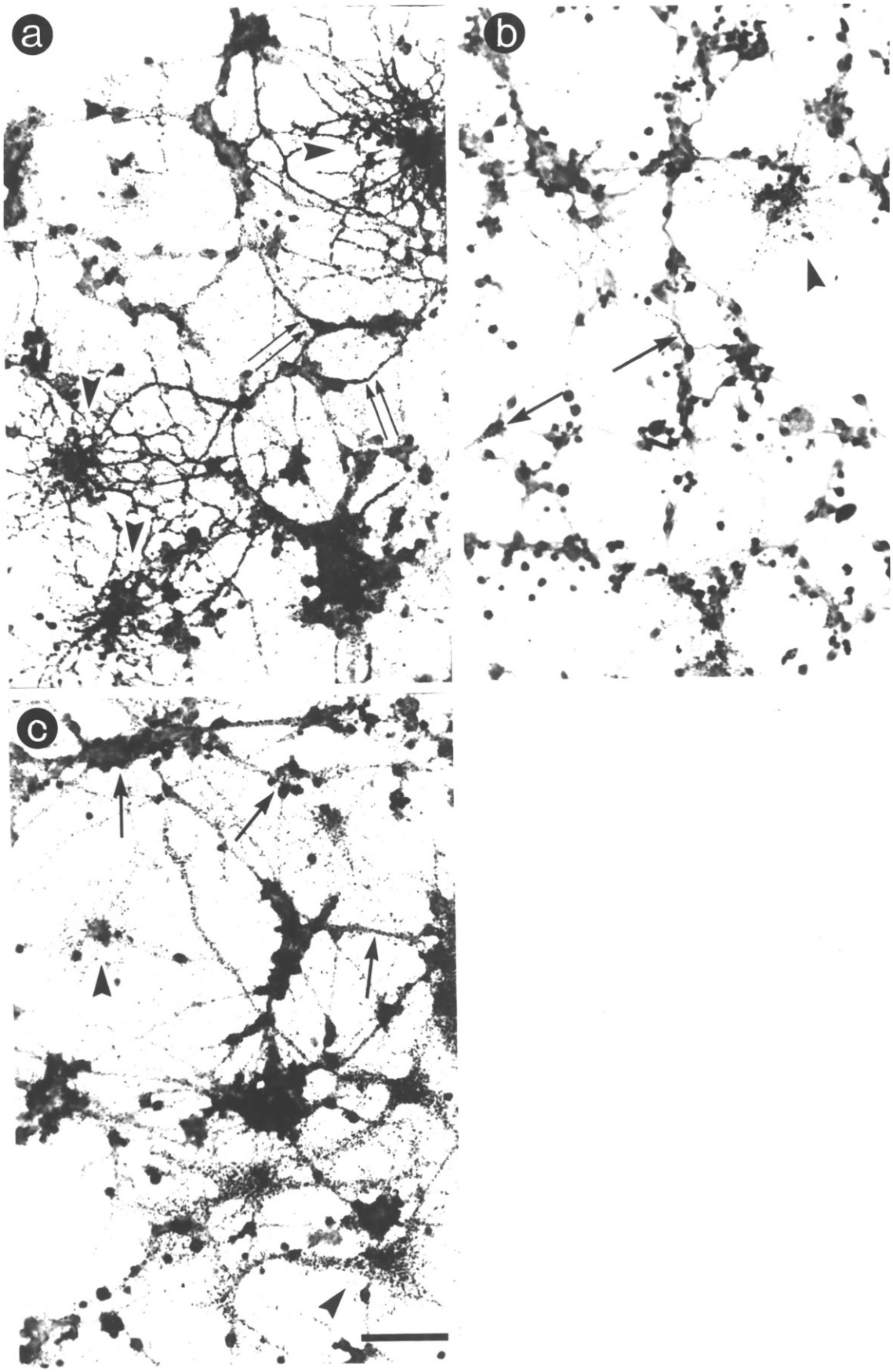

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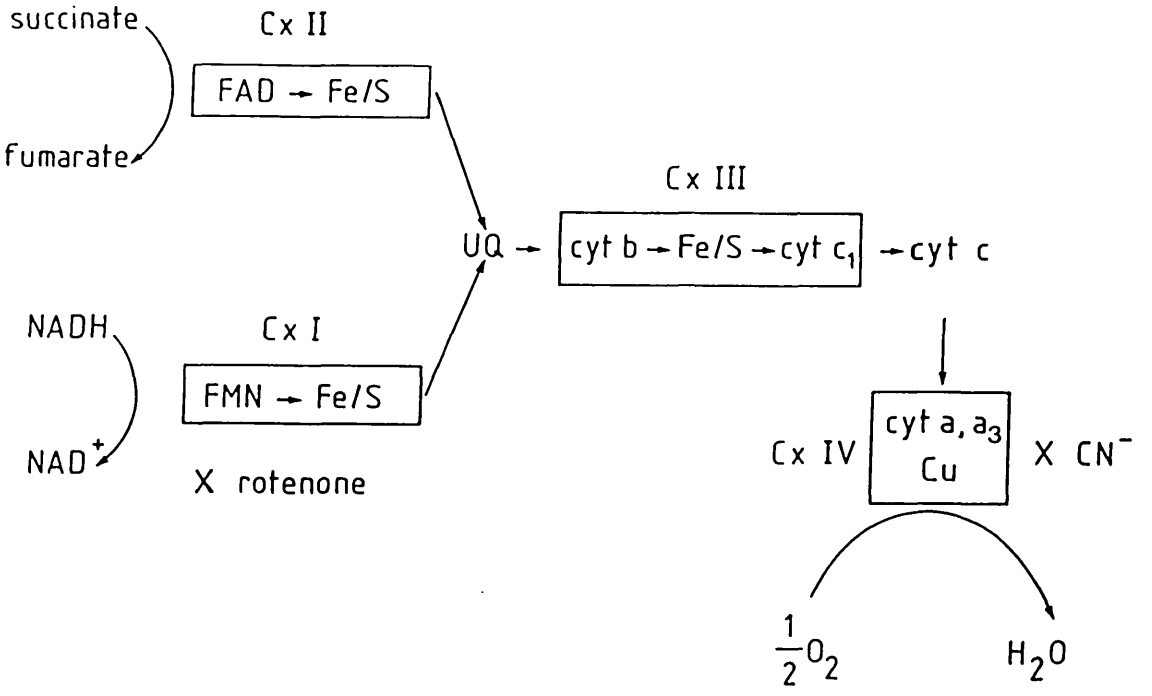
Fig.20 Sites of action of metabolic inhibitors



ouabain is an inhibitor of Na^+ pumps

NaF is an inhibitor of the glycolytic pathway

Table 10. Effects of metabolic inhibitors on ^{3}H-GABA uptake by cultured astrocytes.

INHIBITOR	CONCENTRATION	UPTAKE AS % OF CONTROL VALUE	
		5 DIV	12 DIV
rotenone	1μM	102% (NS)	88% (NS)
ouabain	400μM	73% (****)	44% (**)
NaF	20μM	79% (****)	21% (***)
NaCN	1mM	107% (*)	97% (NS)

*	$0.05 < P < 0.1$
**	$P = 0.01$
***	$P = 0.002$
****	$P = 0.001$
(NS)	

Inhibitors were added to the cells 15 min before the ^{3}H-GABA except in the case of ouabain which was added 30 min before ^{3}H-GABA.

^{3}H-GABA was used at 1uM, 5uCi/nmol. Uptake was over 5 min, 37°C

The values are from triplicate measurements from 2 or 3 experiments. P values were established using a Students' t-test

are sensitive to ouabain and fluoride with the 12 DIV cells being affected to a greater extent than those at 5 DIV. At the concentrations used in these experiments neither rotenone nor cyanide caused significant inhibition of uptake. Cyanide, surprisingly, caused a small but reproducible stimulation of the uptake by 5 DIV cells. The significance of these findings will be discussed at the end of this chapter.

The effects of these inhibitors were also investigated autoradiographically to determine whether there was differential inhibition of uptake into the different cell types. The effects of the inhibitors as shown in Table 10 were not obvious in the autoradiographic study (Figs 21 and 22); possible reasons for this will be considered in the discussion at the end of this chapter.

IV.B.7. Transport of neurotransmitters other than GABA: ^{3}H-glycine, ^{3}H-noradrenaline and ^{3}H-D-aspartate.

Previous sections in this chapter have shown ^{3}H-GABA to be transported by two morphologically-different populations of astrocytes <u>in vitro</u>, the rate of transport being different in the two populations. In order to ascertain whether this uptake is specific for GABA i.e. that it is not a general "amino acid" transport or a general uptake system for all neurotransmitters, the ability of the cells to accumulate a number of other transmitters was studied. These were glycine (an inhibitory amino acid transmitter), D-aspartate (a non-metabolised analogue of the excitatory transmitter glutamate) and noradrenaline (a non-amino acid transmitter). Uptake and autoradiography were carried out using the same methods as for ^{3}H-GABA uptake. ^{3}H-glycine, ^{3}H-Daspartate and ^{3}H-noradrenaline were used at a concentration of 0.5 μM with specific radioactivities of 5 μCi/nmol in each case. The resultant

Fig.21 Light microscopic autoradiographs of 5 DIV cerebellar astrocytes following exposure to ^{3}H-GABA in the presence of metabolic inhibitors.

5 DIV cerebellar astrocytes from 8 day old rats were incubated with ^{3}H-GABA (1μM, 5μCi/nmol) for 5 min in the presence of:-

(a) no inhibitor

(b) rotenone

(c) ouabain

(d) NaCN

(e) NaF

Cells were fixed with glutaraldehyde, exposed to photographic emulsion for 14d, then developed and stained with haematoxylin and eosin. None of the inhibitors (b-e) appeared to have an inhibitory effect on the stellate cells when compared with (a). There was the suggestion of some inhibition into the epithelioid cells by NaF (e) but not by the other compounds.

Scale bar = 50μm

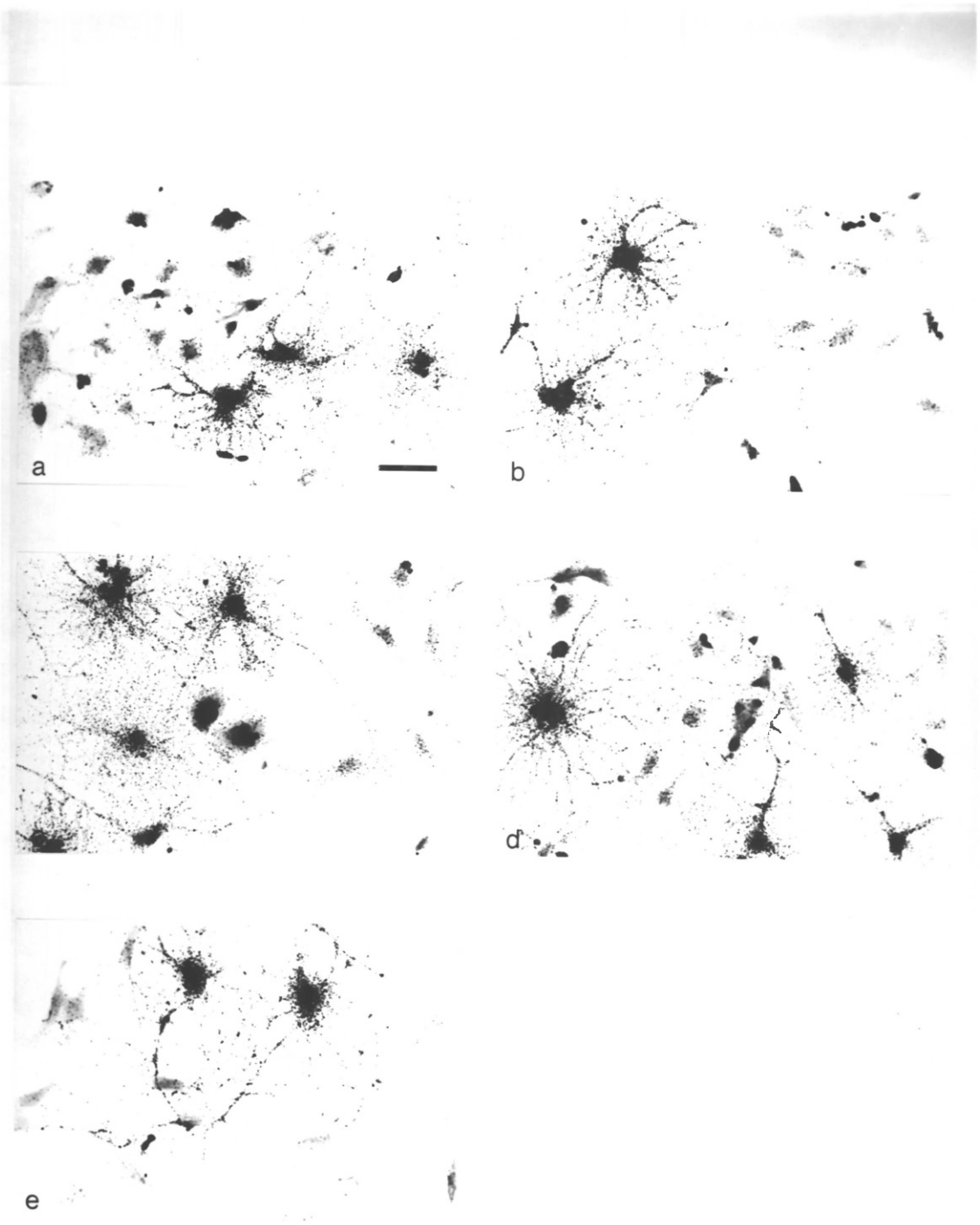

a
b
d
e

Fig.22 Light microscopic autoradiographs of 12 DIV cerebellar astrocytes following exposure to ^{3}H-GABA in the presence of metabolic inhibitors.

12 DIV cerebellar astrocytes from 8 day old rats were incubated with ^{3}H-GABA (1μM, 5μCi/nmol) for 5 min in the presence of:-

(a) no inhibitor

(b) rotenone

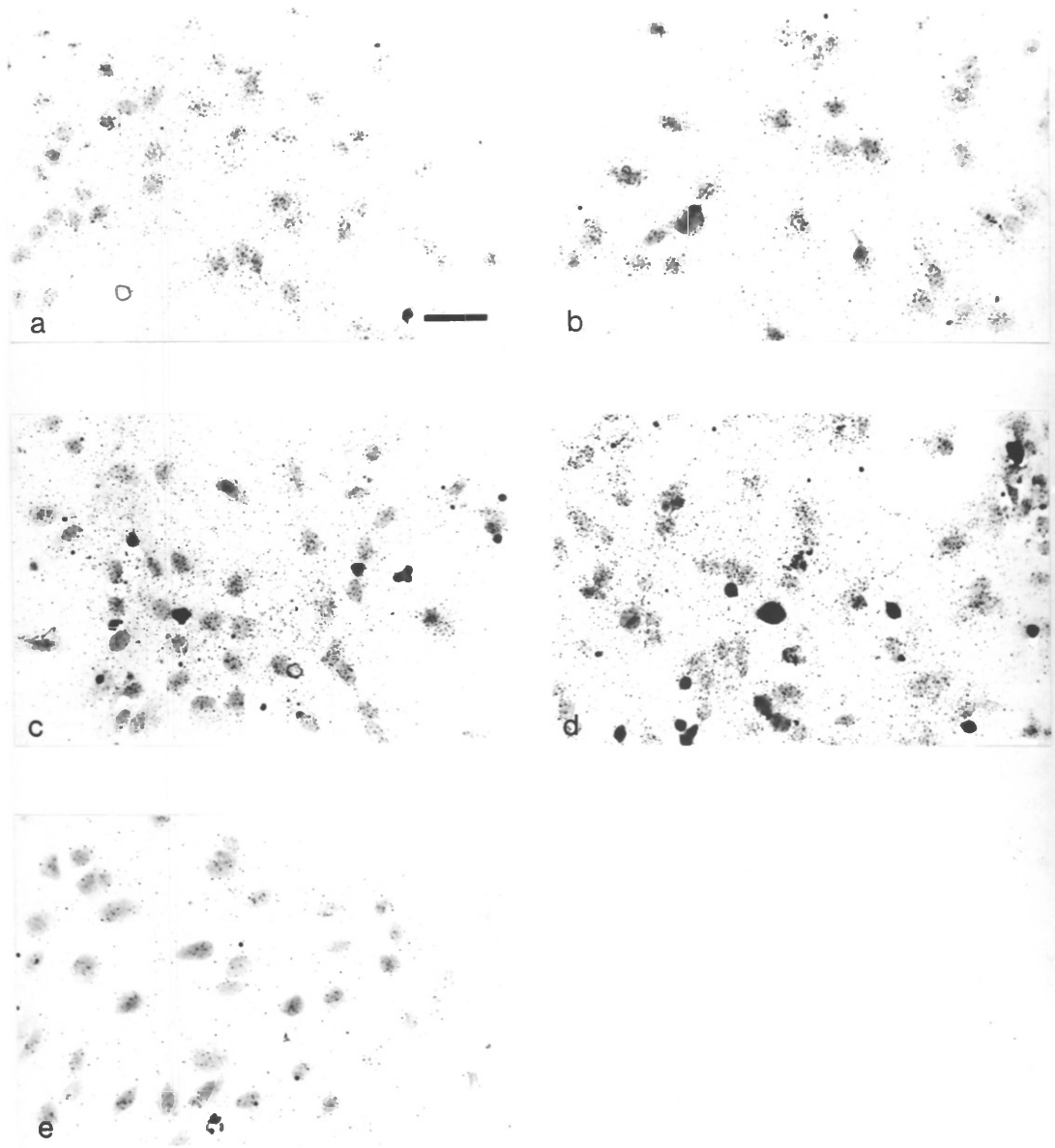
(c) ouabain

(d) NaCN

(e) NaF

Cells were fixed with glutaraldehyde, exposed to photographic emulsion for 14d then developed and stained with haematoxylin and eosin. Compared with the control (a) only NaF showed a noticeable inhibition of uptake.

Scale bar = 50μm


a
b
c
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e

autoradiographs are shown in Fig.23. Efficiencies of fixation were also measured and are shown in Table 9..

The autoradiographs show that the uptake patterns for these three compounds are different to that for GABA. ^{3}H-D-aspartate was accumulated by both the stellate and epithelioid cells to apparently the same extent - similar to or greater than ^{3}H-GABA transport by the stellate cells. ^{3}H-glycine was transported sparingly by both stellate and non-stellate cells. Noradrenaline appeared to be transported by none of the cells under the present conditions. The three transmitters were fixed in the cells by glutaraldehyde to about the same extent (although the actual amounts fixed were different being least for noradrenaline and greatest for D-aspartate). These experiments demonstrate that the ^{3}H-GABA transport systems in cultured astrocytes are specific for that compound rather than being carriers with a broader specificity, e.g. for a wide range of amino acids or for a range of neurotransmitters.

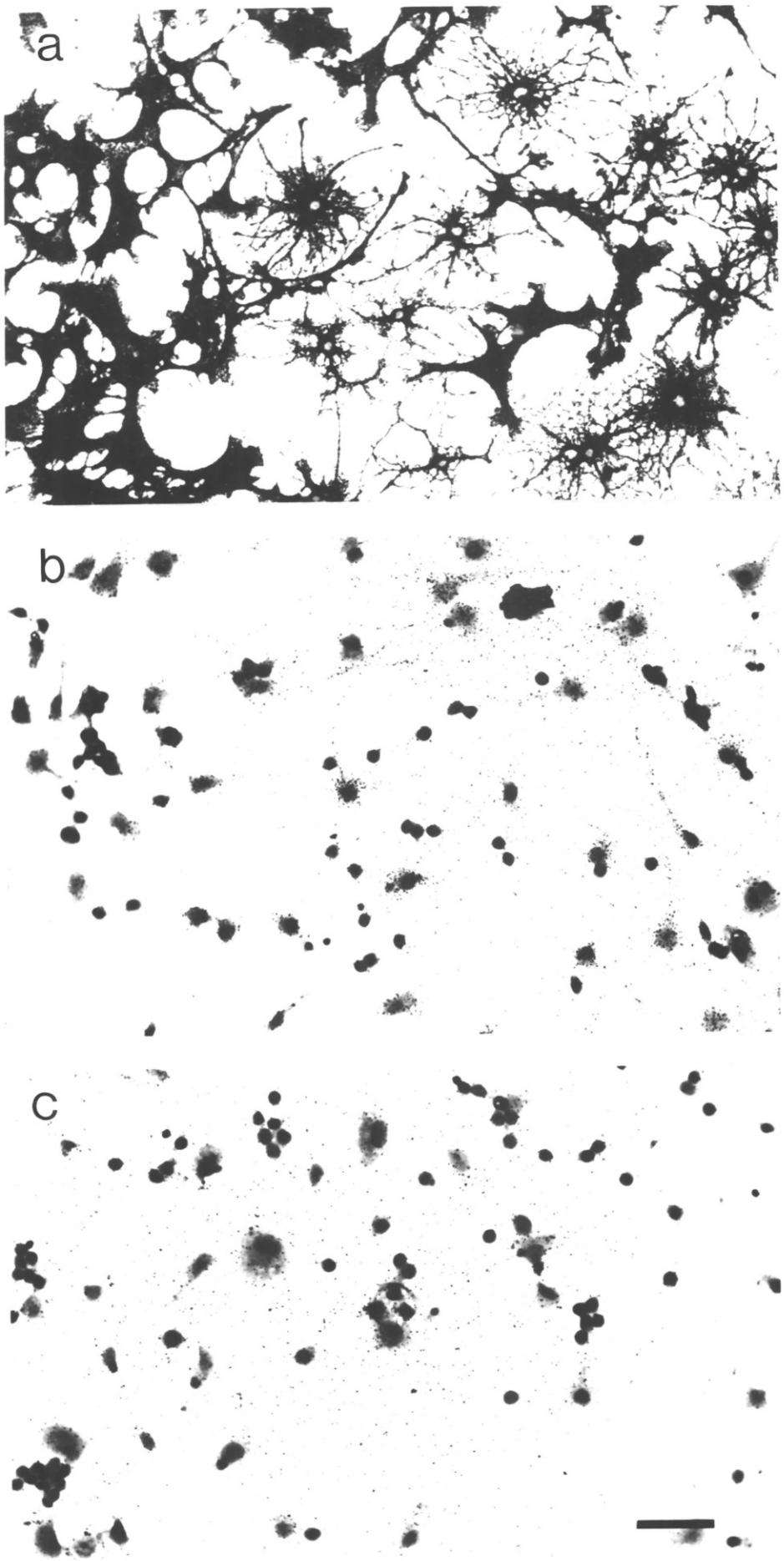
IV.C. Autoradiographic localisation of transported neurotransmitters in astrocytes cultured from spinal cord and optic nerve.

It has been demonstrated in foregoing sections that astrocytes in primary cultures derived from rat cerebellum have the ability to transport various neurotransmitters (or their analogues) in particular patterns. It would seem interesting and important to establish whether these properties are peculiar to astrocytes from the cerebellum or whether they are shared by astrocytes from other brain regions, and whether there is any relationship between astrocytic neurotransmitter uptake and the particular transmitters functioning in any given brain area. To this end the ability of astrocytes from optic nerve and spinal cord to transport ^{3}H-GABA and ^{3}H-glycine were carried out.

Fig.23 Light microscopic autoradiographs of 5 DIV cerebellar astrocytes following exposure to ^{3}H-D-aspartate, ^{3}H-glycine and ^{3}H-noradrenaline.

5 DIV cerebellar astrocytes from 8 day old rats were incubated with ^{3}H-D-aspartate, ^{3}H-glycine or ^{3}H-noradrenaline (1μM, 5μCi/nmol) for 5 min, fixed with glutaraldehyde, exposed to photographic emulsion for 4 weeks then developed and stained with haematoxylin and eosin. (a) ^{3}H-D-aspartate labelled both stellate and epithelioid astrocytes heavily. (b) ^{3}H-glycine labelled both stellate and epithelioid astrocytes only sparingly. (c) ^{3}H-noradrenaline appeared not to label any of the cells specifically.

Scale bar = 50μm


a
b
c

IV.C.1. Transport of ^{3}H-GABA in spinal cord astrocytes and effects of inhibitors.

Uptake and autoradiography were carried out as described for cerebellar astrocytes (sections II.C and II.D). Experiments were done in the presence and absence of Na^{+} and cultures were used at 4 DIV. Inhibitors (ACHC and β-alanine), when included, were used at 1 mM; ^{3}H-GABA was used at 0.5 μM with specific radioactivity of 5μCi/nmol. Exposure time was 14 d and the autoradiographs are shown in Fig.24.

Fig.24 shows that, as was observed for cerebellar cultures, astrocytes from spinal cord occurred in at least two morphologically-distinct types having the ability to transport GABA and that the rate of transport was greater in those cells showing a stellate morphology. This uptake was abolished by the replacement of Na^{+} by choline in the incubation medium. When the inhibitor-sensitivity of the uptake was investigated it was found that in comparison to the control, ACHC reduced but did not abolish the uptake by stellate astrocytes but had little effect on the non-stellate forms. In contrast, β-alanine was particularly effective as an inhibitor of transport into the nonstellate cells but not into the stellate astrocytes.

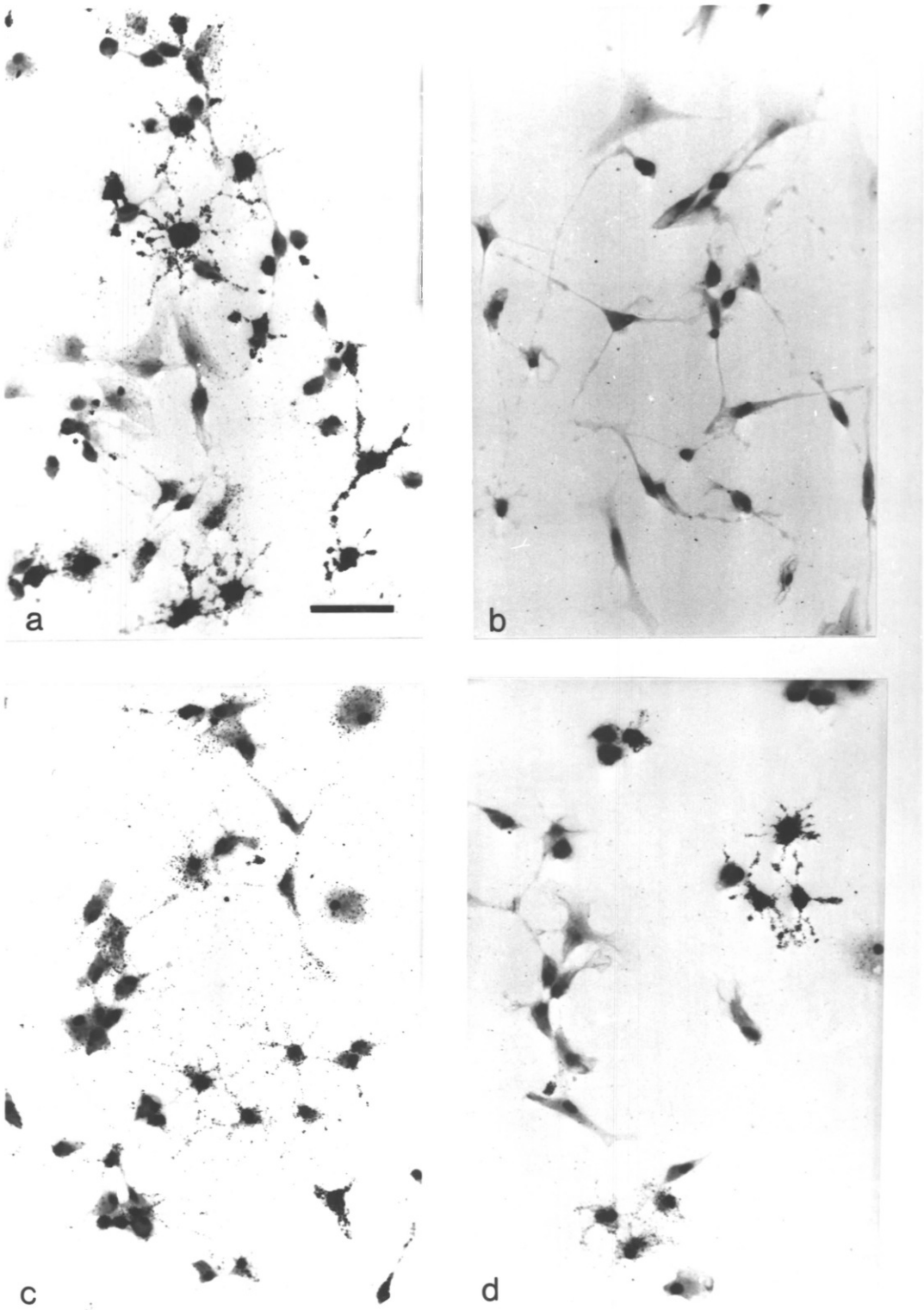
IV.C.2. Transport of ^{3}H-glycine by spinal cord astrocytes.

Glycine is the major inhibitory neurotransmitter in the spinal cord. If astrocytic neurotransmitter uptake plays an important role in the inactivation of released neurotransmitter then it might be expected that cultured spinal cord astrocytes would show significant uptake of ^{3}H-glycine. This was investigated using the methods of section II.C.1.

Fig.24 Light microscopic autoradiographs of 3 DIV spinal cord astrocytes following exposure to ^{3}H-GABA in the presence of GABA-analogues.

3 DIV spinal cord astrocytes from 2 day old rats were incubated for 5 min with ^{3}H-GABA (1μM, 5μCi/nmol) in the presence (a) and absence (b) of Na^{+} and in the presence of (c) 1mM ACHC and (d) 1mM β-alanine. Cells were fixed with glutaraldehyde, exposed to photographic emulsion for 14d then developed and stained with haematoxylin and eosin. ^{3}H-GABA was taken up strongly by process-bearing cells and weakly by epithelioid cells (a), both uptakes being inhibitable by the removal of Na^{+} from the incubation medium (b). Comparison of (c) with (a) shows significant inhibition by ACHC of uptake into the process-bearing cells but not into the epithelioid cells. Comparison of (d) with (a) shows virtually complete inhibition by β-alanine of uptake into epithelioid cells but a much lesser effect on stellate cells.

Scale bar = 50μm


a
b
c
d

Glycine was used at a concentration of 0.5 μM with a specific radioactivity of 5μCi/nmol. Cells were used at 4 DIV and uptake was carried out in the presence and in the absence of sodium. Exposure was for 14d and the resultant autoradiographs are shown in Fig.25. Fig.25 shows that 4 DIV spinal cord astrocytes exhibit a low level of glycine uptake into both stellate and epithelioid forms with the uptake by stellate cells being higher than that into epithelioid. The rate of uptake into the stellate cells was not, however, nearly as great as the rate of GABA transport by the same cells under the same conditions. Similar to GABA transport, glycine transport by spinal cord astrocytes was highly sensitive to removal of sodium from the incubation medium.

IV.C.3. Transport of ^{3}H-GABA by optic nerve astrocytes and effects of inhibitors.

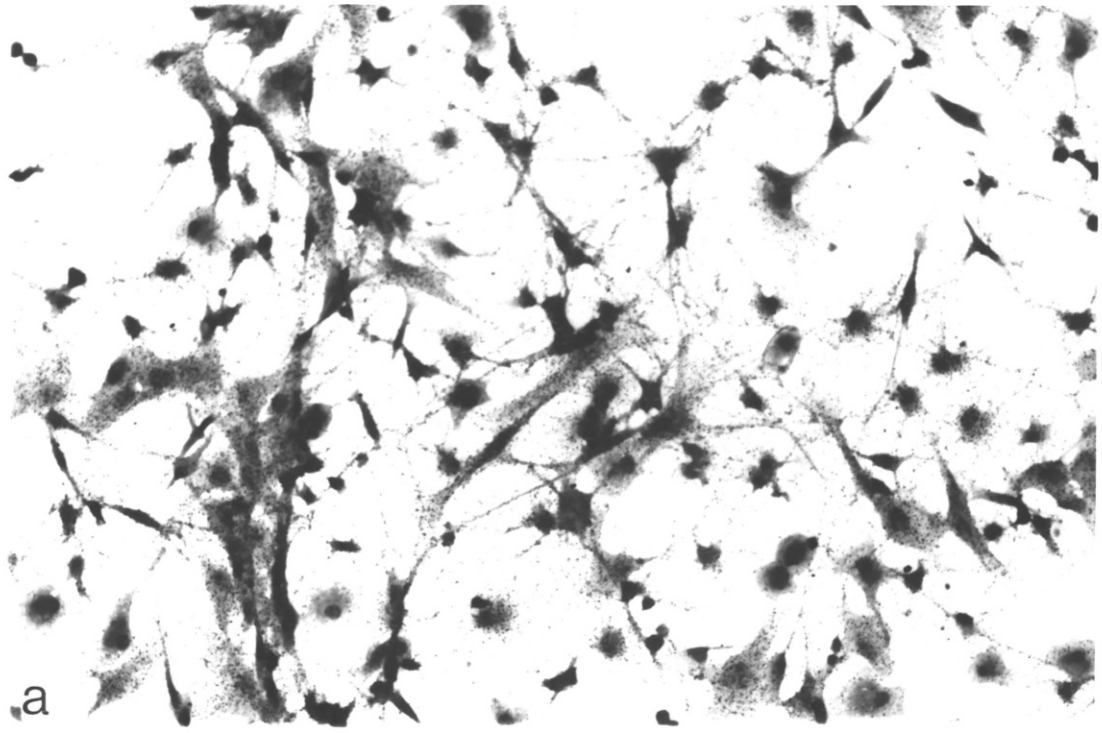
The optic nerves project from the retinae to the lateral geniculate nuclei without any intervening synapses. It might be expected, therefore, that astrocytes cultured from the optic nerve would not have an uptake system for any particular neurotransmitters if such a function were solely to serve as a mechanism for inactivation of neural signals (if a transport system were present one might expect it to be for glutamate which is the transmitter used by the retinal ganglion cells). An initial study of this was carried out by looking at ^{3}H-GABA uptake by 5 DIV astrocytes cultured from rat optic nerve. The methods used were as described in section II.C. ^{3}H-GABA was used at a concentration of 0.5μM with specific radioactivity of 5μCi/nmol. Uptake was performed in the presence and in the absence of sodium. Exposure was for 14d and the resultant autoradiographs are shown in Fig.26.

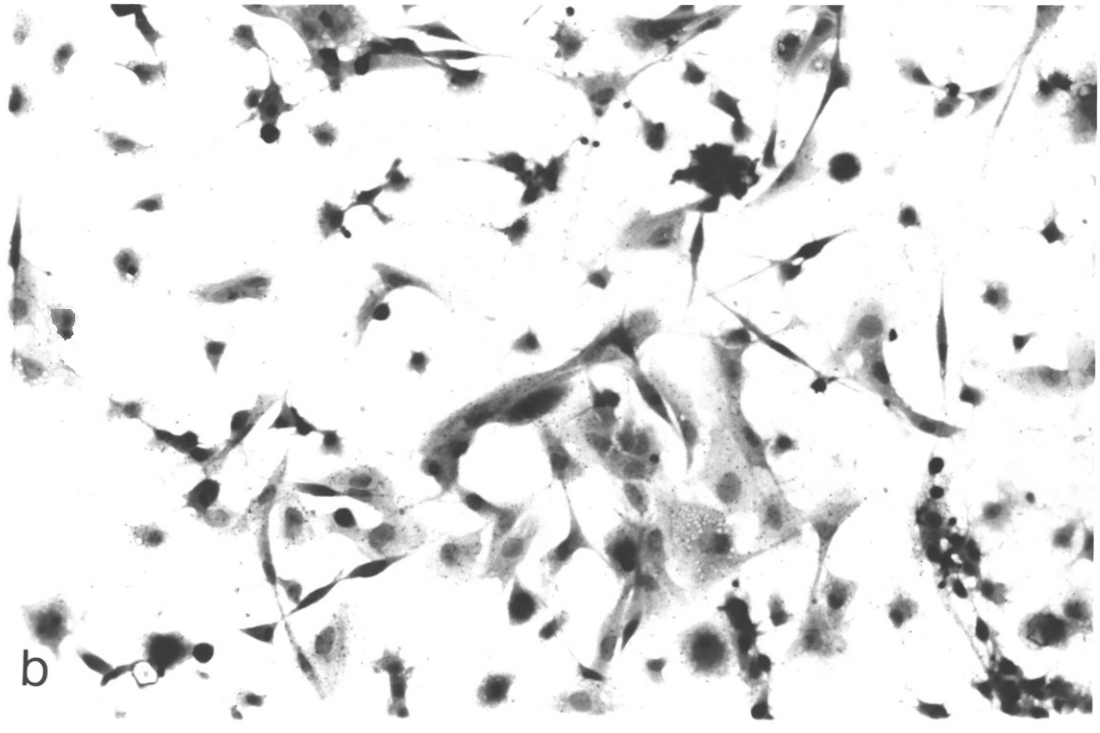
As was the case for both cerebellum and spinal cord, there were two

Fig.25 Light microscopic autoradiographs of 3 DIV spinal cord astrocytes following exposure to ^{3}H-glycine.

3 DIV spinal cord astrocytes from 2 day old rats were incubated with ^{3}H-glycine (1μM, 5μCi/nmol) for 5 min, fixed with glutaraldehyde, exposed to photographic emulsion for 15 days then developed and stained with haematoxylin and eosin. Two astrocyte forms were seen to take up glycine very poorly (a) and any uptake was inhibited by the removal of Na^{+} from the incubation medium (b).

Scale ▬▬ = 50μm


a


b

morphological types of cell transporting ^{3}H-GABA in the optic nerve cultures: a stellate form transporting GABA to a significant extent and an epithelioid form transporting GABA to a much lesser degree. The uptake into both of these cell types was abolished by replacement of sodium by choline in the incubation medium. There was an obvious difference between the effects of the inhibitors ACHC and β-alanine. ACHC markedly reduced the uptake by stellate optic nerve astrocytes but had little apparent effect on the epithelioid cells. In contrast, β-alanine inhibited almost totally the uptake of GABA by the epithelioid cells but had little apparent effect on the transport by the stellate astrocytes. The astrocytic nature of the spinal cord and optic nerve cells was shown by labelling them with an antibody against GFAP (Fig.27, and see Chapter II).

IV.D. Uptake of ^{3}H-GABA by isolated cerebellar slices and effects of inhibitors.

In order to correlate the findings from the ^{3}H-GABA transport studies in culture to the <u>in vivo</u> situation, the uptake of GABA and its inhibitor-sensitivity by cerebellar slices was studied autoradiographically as described in section II.C.2. 12 day old rat tissue was used in this study because the cells in this tissue would be of approximately the same age as those used in the culture studies (cells from 8 day old animals used at 5 DIV).

Tissue was incubated for 30 min with 0.5μM GABA with or without inhibitor (1mM) then fixed, dehydrated and plastic-embedded. 1μm sections were cut and used for autoradiography. Autoradiographs were developed after 6 weeks and stained with toluidine blue; these are shown in Fig.28. In the absence of inhibitor, ^{3}H-GABA was accumulated by cells

Fig.26 Light microscopic autoradiographs of 3 DIV optic nerve astrocytes following exposure to ^{3}H-GABA in the presence of GABA-analogues.

3 DIV optic nerve astrocytes from 2 day old rats were incubated for 5 min with ^{3}H-GABA (1μM, 5μCi/nmol) in the presence (a) and absence (b) of Na^{+} and in the presence of (c) 1mM ACHC and (d) 1mM -alanine. Cells were fixed with glutaraldehyde, exposed to photographic emulsion for 14d then developed and stained with haematoxylin and eosin. ^{3}H-GABA was taken up strongly by process-bearing cells and weakly by epithelioid cells (a), both uptakes being inhibitable by the removal of Na^{+} from the incubation medium (b). Comparison of (c) with (a) shows strong inhibition by ACHC of uptake into the process-bearing cells but not into the epithelioid cells. Comparison of (d) with (a) shows strong inhibition by -alanine of uptake into epithelioid cells but a much lesser effect on stellate cells.

Scale bar = 50μm

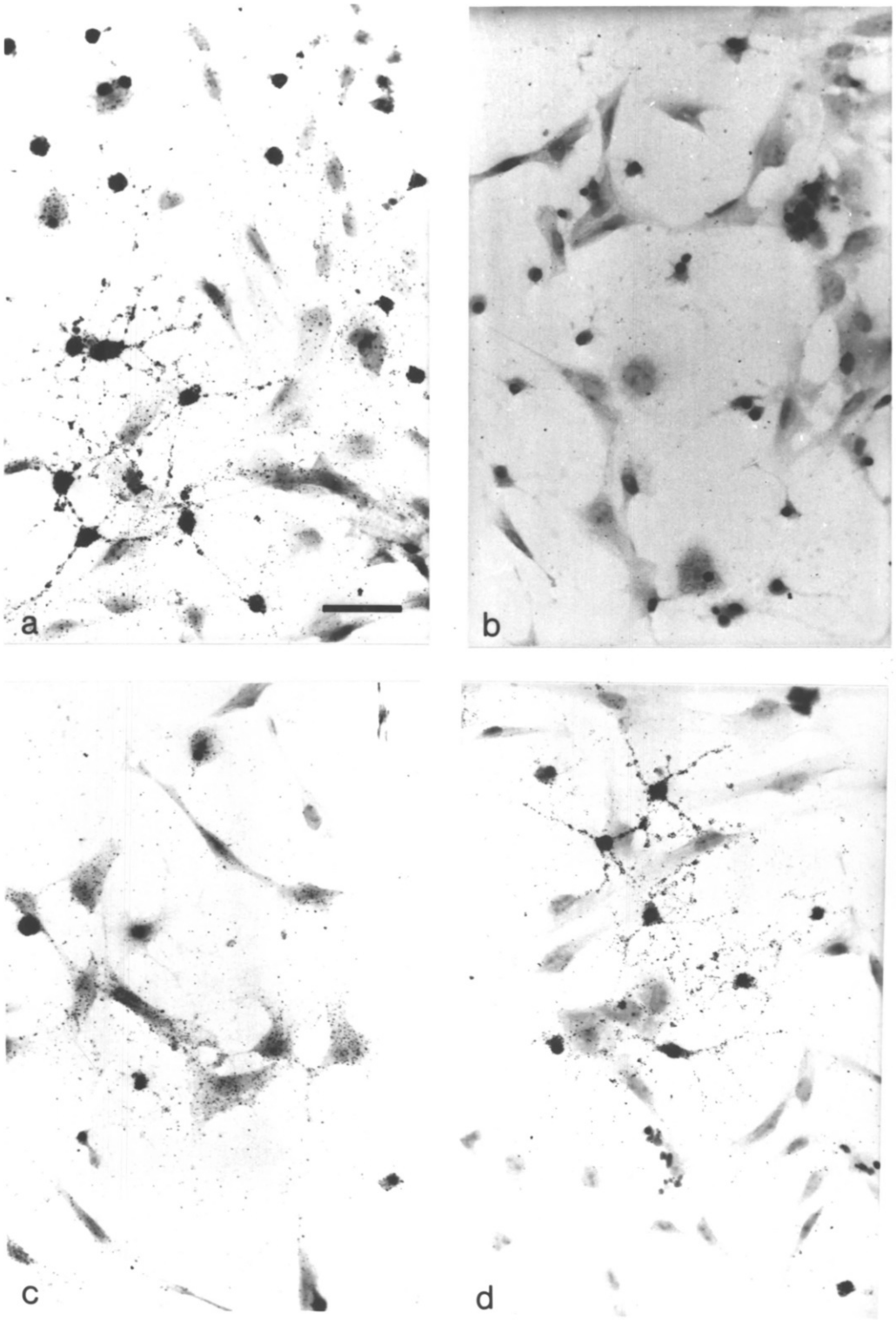
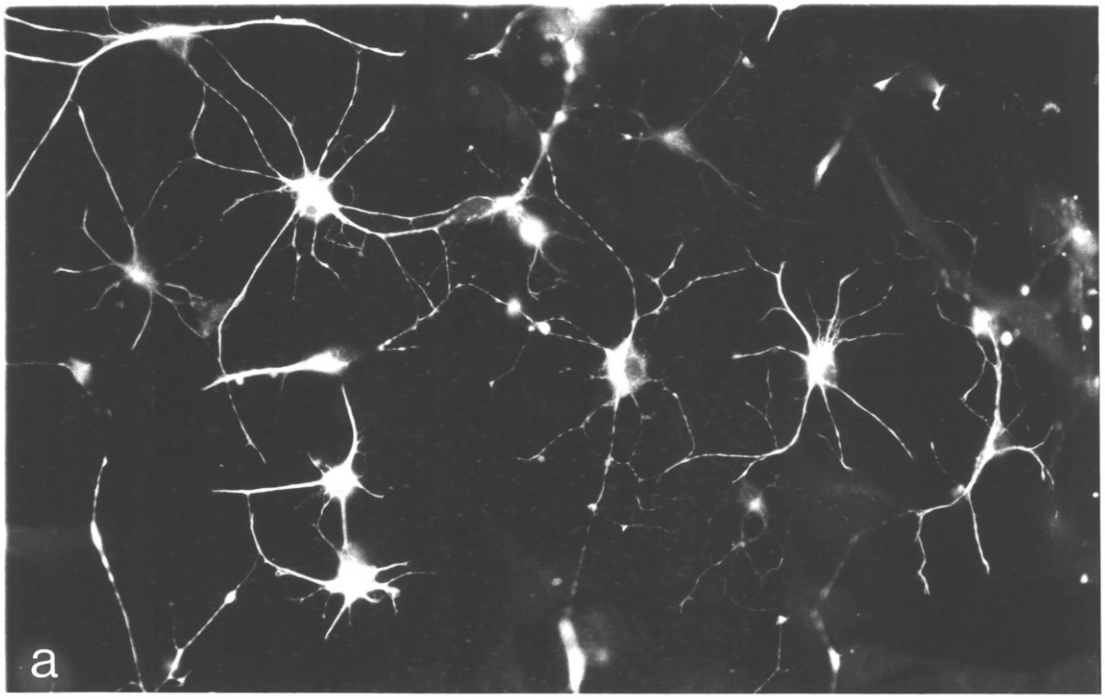
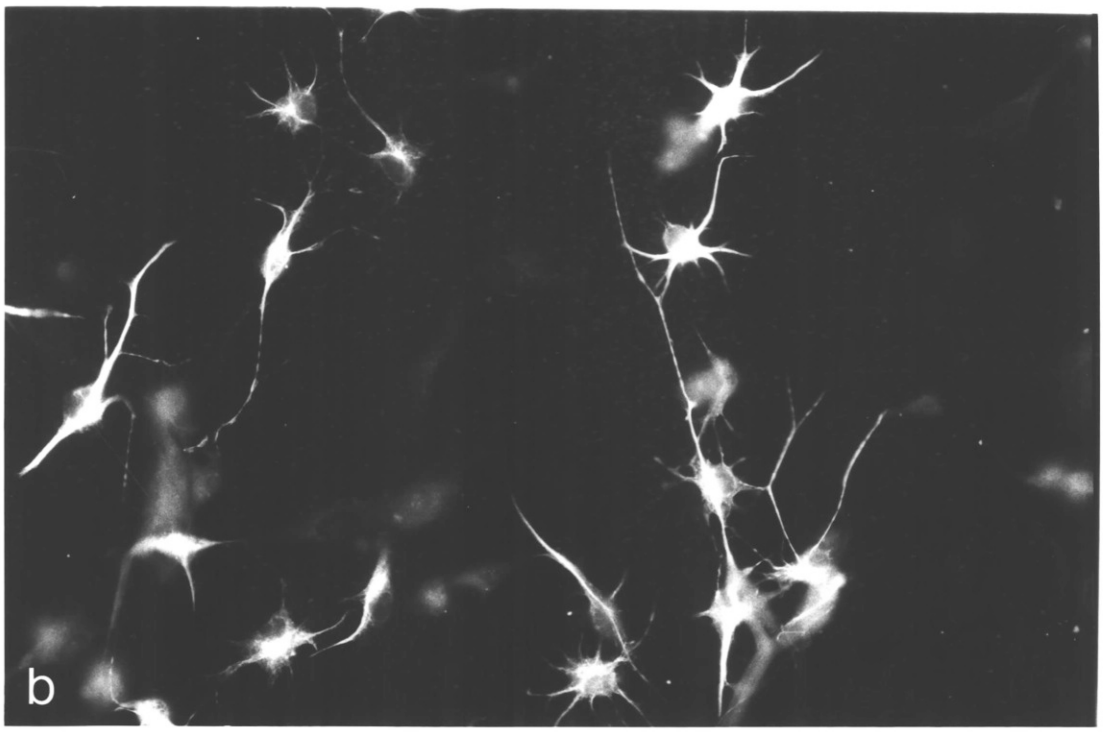

a
b
c
d

Fig.27 Fluorescent micrographs of 3 DIV astrocytes from 2 day rat optic nerve and spinal cord labelled with anti-GFAP.

3 DIV astrocyte cultures from 2 day old rat optic nerve (a) and spinal cord (b) were labelled with rabbit anti-GFAP / TRITC swine anti-rabbit Ig . Antibodies were used at 1:50; cells were pre-fixed in methanol at -18 C for 10 min. These micrographs show that the cell types which were shown to accumulate 3H-GABA in optic nerve and spinal cord cultures (Figs 26 and 24 respectively) can be identified as astrocytes by the anti-GFAP antiserum.

Scale ▬▬▬ = 50μm


a


b

in all layers of the cerebellum:Bergmann glial processes, cells around the base of the Purkinje cells, cells of the internal granule cell layer and cells in the white matter. There was also considerable uptake by cells in the external granule cell layer which is still present at this young age. In the cases where inhibitors were present, The results were different from those obtained in cultures. Neither ACHC nor -alanine appeared to have a marked effect on the accumulation pattern. Interpretation of the results from these experiments was difficult however because there was a significant variation in labelling intensity between different areas of the sections. The presence of excess unlabelled GABA totally abolished the uptake of ^{3}H-GABA by the cells in all layers of the slice.

IV.E. Quantitative pharmacology of ^{3}H-GABA transport in cerebellar astrocyte cultures.

In section IV.B.3 it was shown that a variety of structural analogues of GABA could influence the function of the GABA transport system(s) in primary cultures of cerebellar astrocytes. From the autoradiographic data it was possible to obtain only a crude estimate of the extent to which these compounds affected the transport. In the present section is described work carried out to determine the IC50 value - that concentration of inhibitor which reduces uptake to 50% of that measured in the absence of inhibitor - for four of the inhibitors tested previously. The compounds tested were ACHC, β-alanine, nipecotic acid and DABA. These values would allow a more accurate comparison of the (relative) potencies of these GABA-analogues as inhibitors of astrocytic ^{3}H-GABA transport.

Fig.28 Light microscopic autoradiographs of the pattern of ^{3}H-GABA accumulation in rat cerebellar slices in the presence and absence of GABA-analogues.

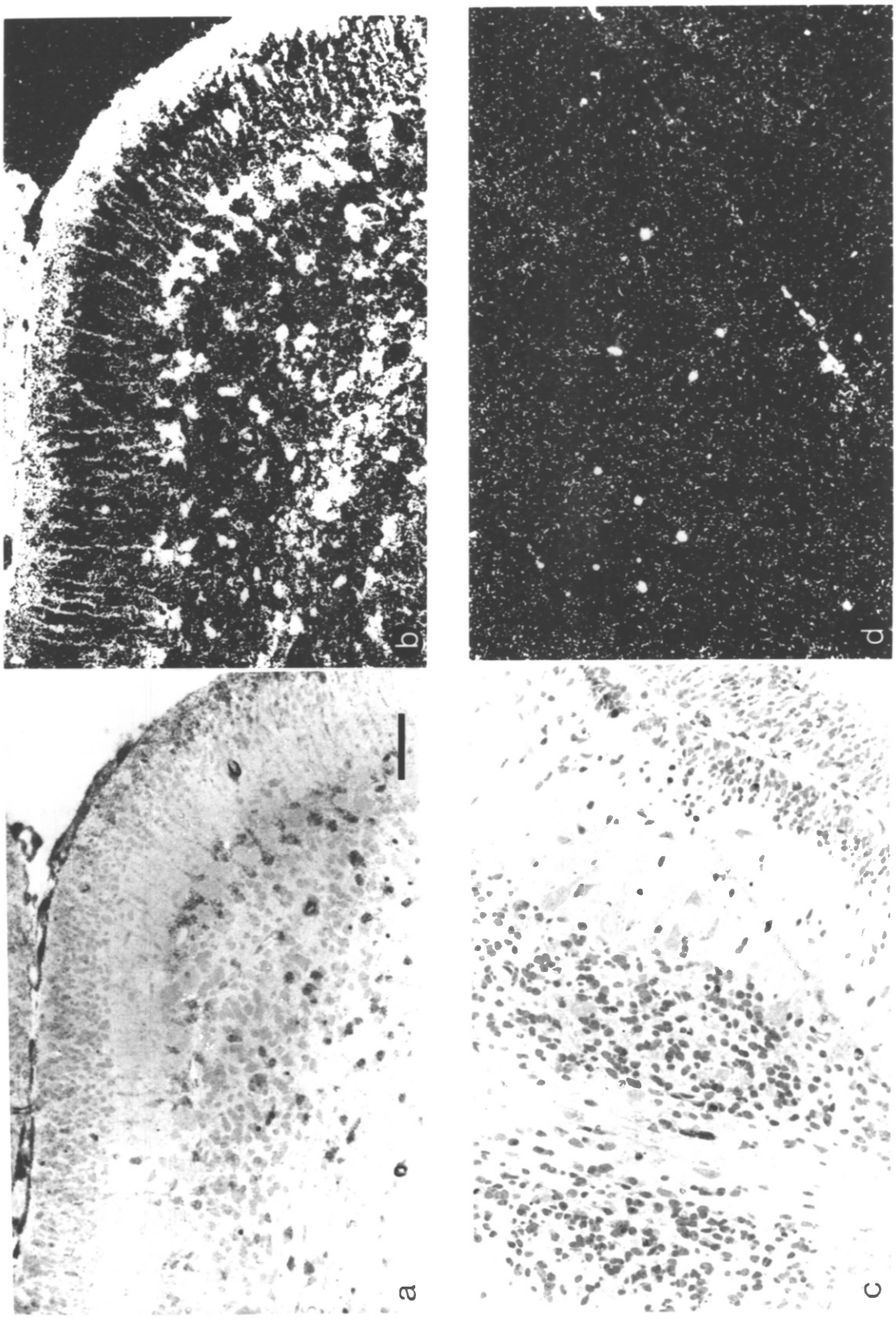
400µm cerebellar slices fron 12 day old rats were incubated for 30 min in the presence of ^{3}H-GABA (0.5µM, 5µCi/nmol) or 3H-GABA + 1mM unlabelled GABA (a,b), 1mM β-alanine (c,d), or 1mM ACHC (g,h). Slices were fixed with a glutaraldehyde / paraformaldehyde mixture and post-fixed in OsO4. 1µm plastic sections were used to produce autoradiographs (8 weeks exposure time) which were counterstained with toluidine blue.

(a), (c), (e), and (g) - bright-field illumination

(b), (d), (f), and (h) - dark-field illumination

Accumulation of silver grains are shown in the outer layers of the external granule cell layer, over the Bergmann glial perikarya and radial processes, over probable astrocytes in the internal granule cell layer and over the developing white matter (a) and (b). Unlabelled GABA was shown to inhibit totally the uptake into all layers (c) and (d) but no inhibition was seen in the presence of either β-alanine or ACHC (e,f,g and h).

Scale bar = 50µm


b
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a
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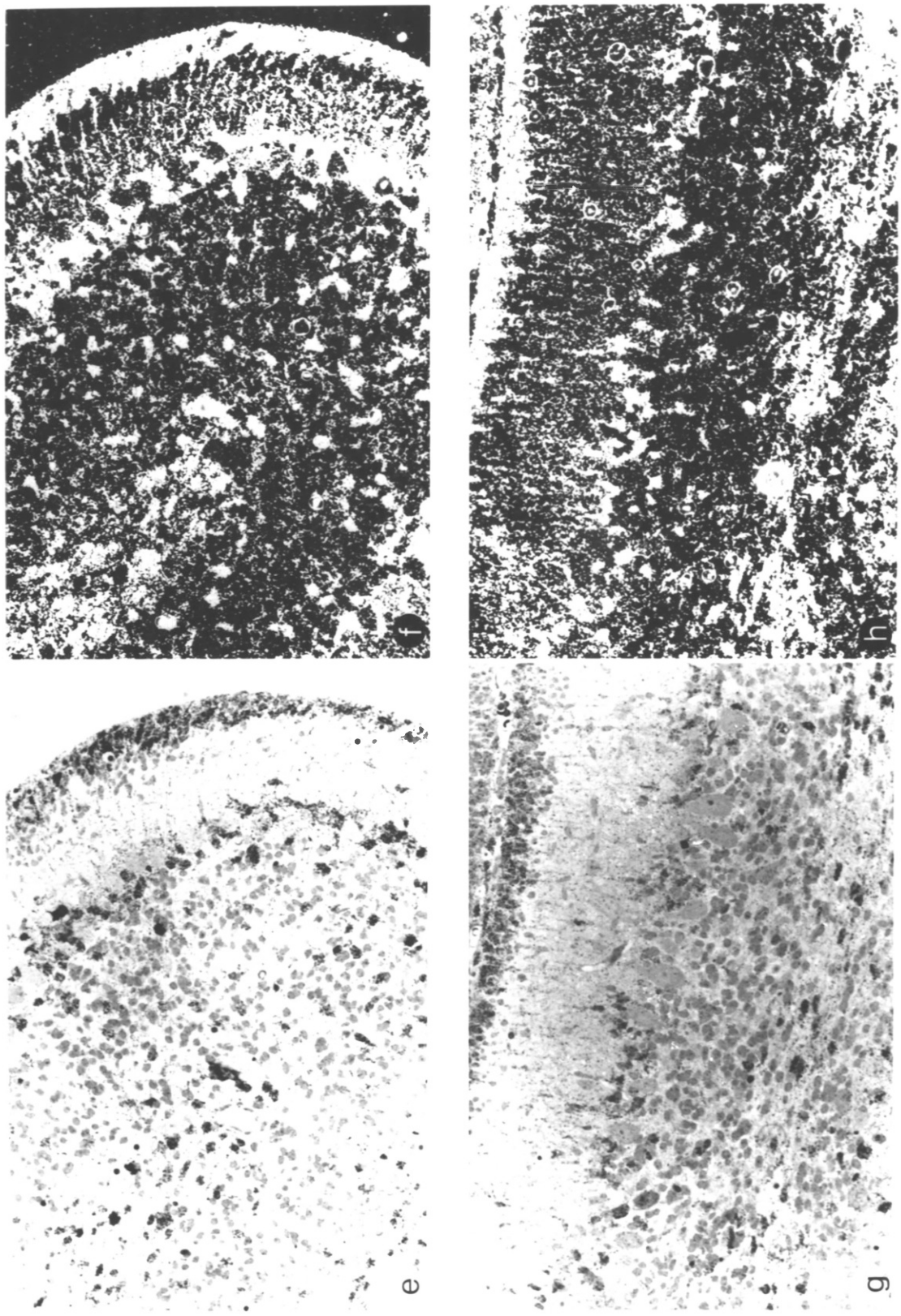

f
h
e
g

IV.E.1. ^{3}H-GABA transport by cultured cerebellar astrocytes: determination of IC50 values for four inhibitors of transport.

^{3}H-GABA transport was carried out as described in section II.C. After washing and preincubating the cells in tris-buffered Krebs solution (TBK) at 37^{o}C, inhibitor was added to a final concentration ranging from 0.1μM to 10mM. Immediately after addition of inhibitor, ^{3}H-GABA was added to a final concentration of 0.5μM with specific radioactivity of 5μCi/nmol. The cells were washed after 5 min then processed for determinations of protein and radioactivity. The accumulation of ^{3}H-GABA in each dish was calculated as dpm/μg cell protein and the results for each inhibitor concentration expressed as:-

$$\frac{\text{dpm/μg protein in presence of inhibitor}}{\text{dpm/μg protein in absence of inhibitor}} \times 100\%$$

These values were then plotted against log [inhibitor] to give a straight line graph from which the IC50 was obtained by interpolation. These graphs are shown in Fig.29. The measured IC50 values of nipecotic acid, 6μM; ACHC, 19μM, DABA, 292 μM and β-alanine, 403μM, when placed in rank order, confirm the rank order of potencies of these inhibitors as perceived from the autoradiographs of Fig.17. As mentioned in that section, this order of potency is unexpected in the light of the previously published reports. This point will be discussed at the end of this chapter. Another interesting feature of the IC50 data is that the graphs of the ACHC data and DABA data have very similar gradients but different from those of the nipecotic acid and β-alanine graphs which are themselves similar to each other. This may suggest that ACHC and DABA inhibit ^{3}H-GABA transport into cultured cerebellar astrocytes by the same mechanism but that this mechanism is

Fig.29 Plots to determine IC50 values for four inhibitors of GABA transport into astrocytes.

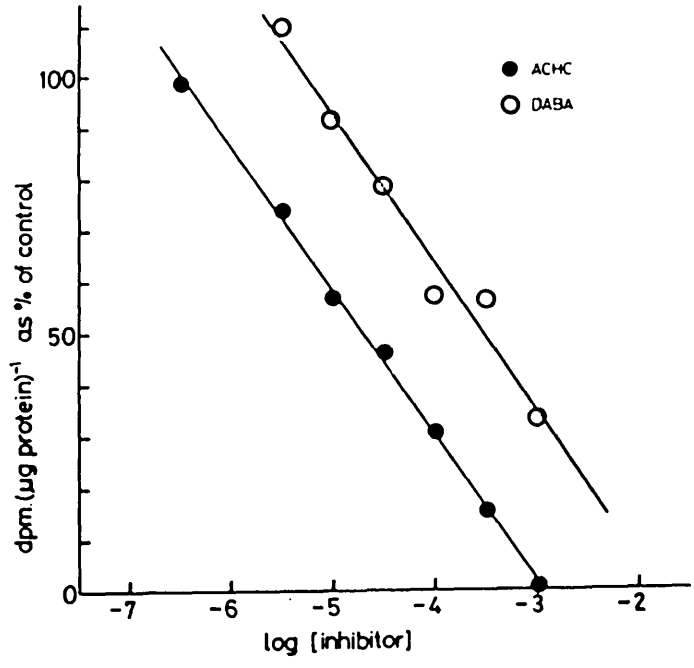
6 DIV astrocytes from 8 day old rat cerebellum were exposed to ^{3}H-GABA (0.5μM, 5μCi/nmol) for 5 min in the presence of a range of inhibitor concentrations. The amount of GABA accumulated (measured by liquid scintillation counting) was expressed as dpm/μg cell protein and this value plotted against log[inhibitor]. Control incubations were carried out in the absence of inhibitor at 37°C and 0°C. Amino-oxyacetic acid (10μM) was present in all incubations to inhibit GABA transaminase.

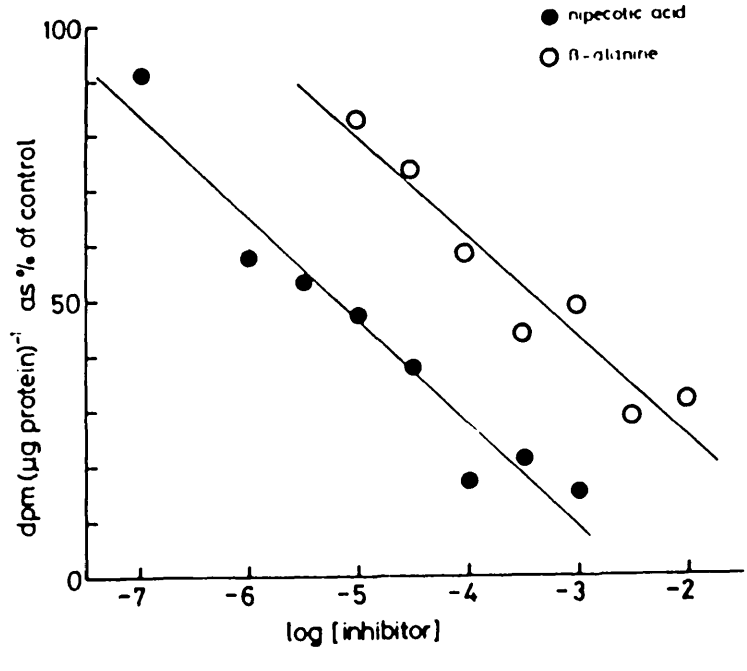
(a) ACHC ● DABA ○ (b) nipecotic acid ● β-alanine ○

Each point represents the mean from at least 3 experiments each done in triplicate. Maximum error around each point is 12%

The IC50s calculated from these plots are:-

nipecotic acid	6μM
ACHC	19μM
DABA	292μM
β-alanine	403μM


ACHC
DABA
100
50
0
-7
-6
-5
-4
-3
-2
dpm (µg protein)$^{-1}$ as % of control
log [inhibitor]


nipecotic acid
β-alanine
100
50
0
-7
-6
-5
-4
-3
-2
dpm (µg protein)$^{-1}$ as % of control
log [inhibitor]

different in some way to that mediating the inhibition by nipecotic acid and β-alanine.

IV.E.2. Differential effects of GABA-analogues on ^{3}H-GABA transport by stellate and epithelioid astrocytes.

In section IV.E.1 it was assumed, from the autoradiographs in Fig.14 that the uptake of ^{3}H-GABA by the non-stellate cells in culture was so small that it would not contribute significantly to the total ^{3}H-GABA accumulation by these cultures. Thus the IC50 data obtained would largely reflect the behaviour of the stellate astrocytes. It is obvious however, that the non-stellate cells do transport a small amount of GABA and, furthermore, that this is inhibited to some extent by some of the GABA structural analogues (c.f. Figs.14a and 17c). Due to the great differences in the actual amounts of GABA accumulated by the two cell types (reflected in the different densities of silver grains over the cells) it was difficult to determine by eye from the autoradiographs whether the stellate cells were affected to the same extent as the non-stellate cells by any of the inhibitors. In an attempt to overcome this problem, use was made of a photometer system which could give a measure of the relative grain densities over different cells. The system used was the Leitz MPV.

Using this system the densities of silver grains over a number of cells of each type was measured as a percentage of the maximum grain density found on the particular autoradiograph studied. These values were then used to calculate an uptake density ratio (UDR):-

$$UDR = \frac{\text{density over stellate cells}}{\text{density over epithelioid cells}}$$

A comparison was then made between the UDRs obtained in the absence of inhibitor and those found when inhibitor was present. The absolute values of these ratios are probably of little use but the direction of change of these values between the case with inhibitor present and inhibitor absent could give some indication of how the inhibitor is affecting the two cell types. Thus, when such comparisons are made, an increase in the value of the ratio when inhibitor was present would suggest that the inhibitor was having a greater effect on the non-stellate cells than on the stellate population whereas a decrease in ratio might indicate the converse. The results from such a study based on the autoradiographs obtained as described in section IV.B.1 and IV.B.3. are presented in Table 11.

These figures show the following directional changes in the UDRs:-

ACHC	▼▼
β-alanine	▲
nipecotic acid	▼▼▼
DABA	▼▼

In all cases except that of β-alanine there was a decrease in ratios indicating a greater effect of the inhibitors on the stellate than the epithelioid cells. The increase in UDR between β-alanine and control suggests that this particular inhibitor has a more pronounced effect on the non-stellate cells than on the stellate variety. This is also shown clearly by the unmanipulated figures for the percentage grain densities (Table 11) over the two cell types. In the case of the epithelioid cells, the reduction in density in the presence of β-alanine compared with control is greater than that observed with any other inhibitor. In contrast, β-alanine treatment of the stellate cells caused a smaller reduction of uptake than did any other inhibitor

Table 11. Stellate/Epithelioid GABA Uptake Density Ratios Using Uptake Inhibitors

condition	density over stellate cells	density over epithelioid cells	stellate epithelioid
control	79.5 ± 11.7	5.8 ± 3.5	13.7
β-alanine	61.0 ± 14.1	2.7 ± 1.5	22.6
(±)ACHC	20.5 ± 15.1	3.9 ± 3.0	5.3
DABA	11.4 ± 7.6	3.0 ± 0.9	3.8
nipecotic acid	5.0 ± 3.1	4.6 ± 2.2	1.1

A Leitz MPV photometer system was used to estimate the grain densities over stellate and epithelioid astrocytes from autoradiographs following GABA uptake in the presence and absence of GABA analogues. [GABA] = 1μM; [inhibitor] = 1mM The figures in the table are from a single experiment and show grain density per unit area as a percentage of the maximum found on that coverslip (mean ± S.D.; 20 fields, 3 coverslips).

tested.

These observations confirm the idea already suggested by the results from IV.B.4 (uptake of ^{3}H-ACHC and ^{3}H-β-alanine) that there may be more than one type of carrier for the transport of ^{3}H-GABA into cultured astrocytes. One will transport ACHC but not β-alanine and will transport GABA in an ACHC-sensitive manner; this carrier is found predominantly in the stellate astrocytes. The second carrier type will transport β-alanine but not ACHC and will transport GABA in a β-alanine-sensitive manner; this system is located primarily in the epithelioid astrocytes.

IV.F. Kinetics of ^{3}H-GABA transport by cultured cerebellar astrocytes.

One of the major roles proposed for astrocytes in the CNS is the inactivation of intercellular signals by the uptake of the neurotransmitters involved in this process. It is well-known, however, that neurons possess high-affinity transport systems for the transmitters (in fact this was often used as one of the criteria in determining neurotransmitter identity) so it is unclear to what extent astrocytes may be important in this function. The relative contributions of astrocytes and neurons to total transmitter uptake (and thus to the inactivation of synaptic signals) will depend on several factors including the proximity of astrocytic membrane to the synapse, the ease with which transmitter is able to diffuse from the synaptic cleft and the relative values of the kinetic parameters - Km and Vmax - of the neuronal and astrocytic transport systems. The first two points will be important in determining the concentration of transmitter at the astrocyte membrane; the third will describe how the carriers will behave kinetically (i.e. how fast they will transport transmitter) when faced

with the transmitter at any particular concentration. In this section is described work done to determine the kinetic parameters of the GABA transport systems in cultured cerebellar astrocytes at 5 DIV and 12 DIV.

IV.F.1. Determination of kinetic parameters for GABA transport by cultured cerebellar astrocytes.

Km and Vmax for cultured cerebellar astrocytes were determined for cells of 5 DIV and 12 DIV. Uptake was carried out as described in section II.C. Cells were washed and preincubated in TBK, or TBK in which the sodium had been replaced with choline, at 37°C. They were then exposed for 5 min to ^{3}H-GABA at concentrations between 0.5 and 500μM with specific radioactivities between 0.005 and 1μCi/nmol. Excess ^{3}H-GABA was washed off and the cells processed for determination of protein and radioactivity. Transport rates were expressed as nmol ^{3}H-GABA accumulated per mg cell protein per minute (nmol/mg/ min). The saturable, sodium-dependent component of the uptake was plotted as a graph of [GABA]/uptake rate against [GABA] (S/V against S, a Hanes plot) from which the values of Km and Vmax were determined. The sodium-dependent transport used for this plot was obtained by (i) subtracting the graph of Na^{+}-free transport from total transport in the presence of sodium then (ii) subtracting the extrapolated linegraph for unsaturable transport from the graph of total Na^{+}-dependent transport. The graphs obtained by these procedures are shown in Figs 30, 31 and 32.

Fig.30a shows the total transport (●) and Na^{+}-independent transport (○) of GABA into 5 DIV astrocytes. This demonstrates that at low GABA concentrations (<10μM) a large proportion of the accumulation of GABA by the cultures is by a Na^{+}-dependent mechanism, the Na^{+}-independent uptake

Fig.30 Kinetics of GABA transport in 5 DIV astrocytes: variation of Na^+-dependent and Na^+-independent GABA uptake with varying [GABA]

5 DIV astrocytes from 8 day old rat cerebellum were exposed for 5 min to ^{3}H-GABA over a range of concentrations in the presence and absence of Na^+. Radioactivity and protein determinations were made and uptake was calculated as nmol GABA accumulated/mg cell protein/min. These values were plotted against [GABA].

(a) (●) total uptake in the presence of Na^+

(O) total uptake in the absence of Na^+

(O) was subtracted from (●) to give (b)

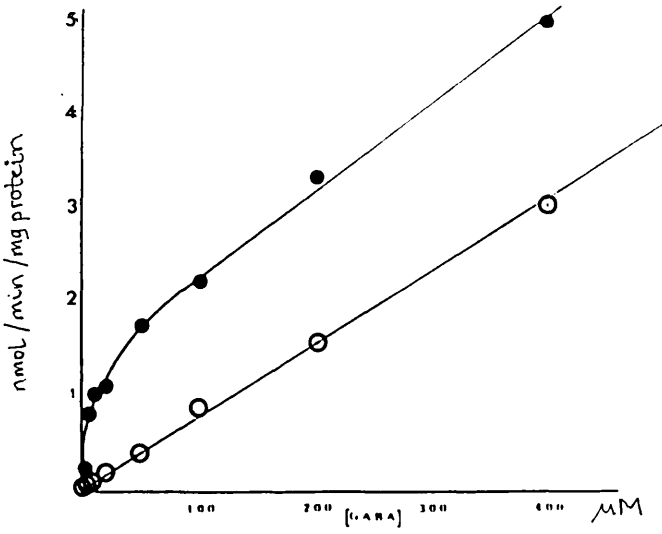
(b) Na^+-dependent uptake

The unsaturable component in (b) was subtracted from (b) to give (c)

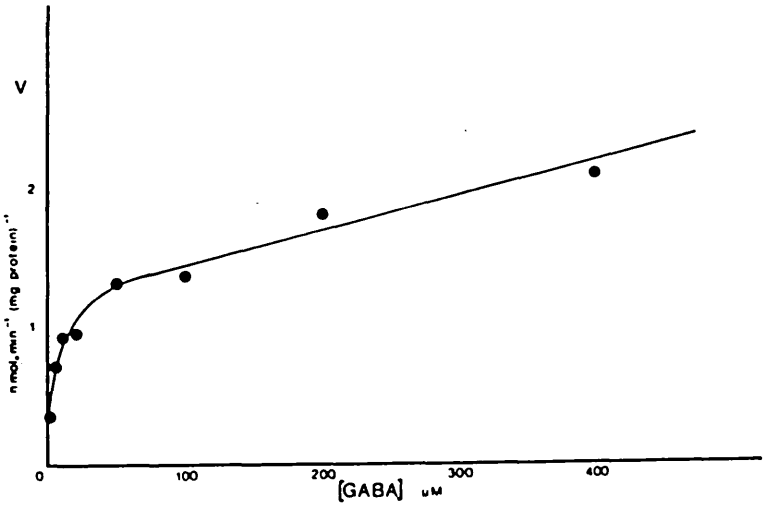
(c) saturable, Na^+-dependent uptake

10μM amino-oxyacetic acid was present in all incubations to inhibit GABA transminase. The points in (a) are the means from 3 different experiments, each done in triplicate. (b) and (c) were formed by subtracting the graphs rather than individual points.

a


nmol/min/mg protein
5
4
3
2
1
100
200
[GABA]
300
400
μM

b


V
nmol.min⁻¹ (mg protein)⁻¹
2
1
0
100
200
300
400
[GABA] μM

c

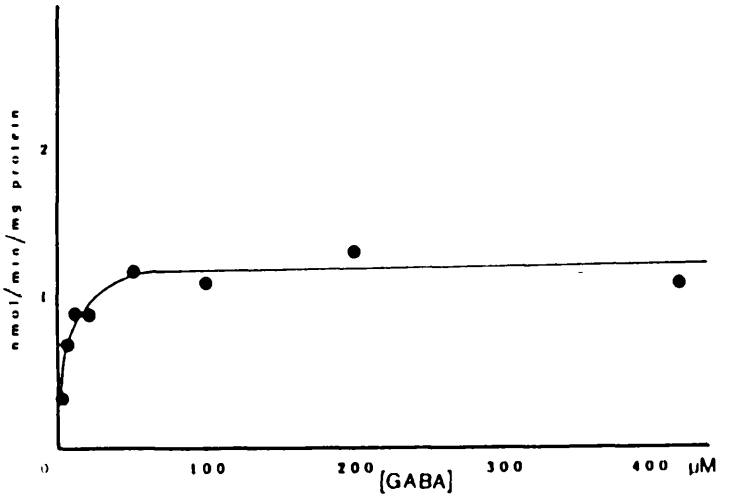

nmol/min/mg protein
2
1
0
100
200
300
400
[GABA]
μM

Fig.31 Kinetics of GABA transport in 12 DIV astrocytes: variation of Na^+-dependent and Na^+-independent GABA uptake with varying [GABA]

12 DIV astrocytes from 8 day old rat cerebellum were exposed for 5 min to ^{3}H-GABA over a range of concentrations in the presence and absence of Na^+. Radioactivity and protein determinations were made and uptake was calculated as nmol GABA accumulated/mg cell protein/min. These values were plotted against [GABA].

(a) (O) total uptake in the presence of Na^+

(●) total uptake in the absence of Na^+

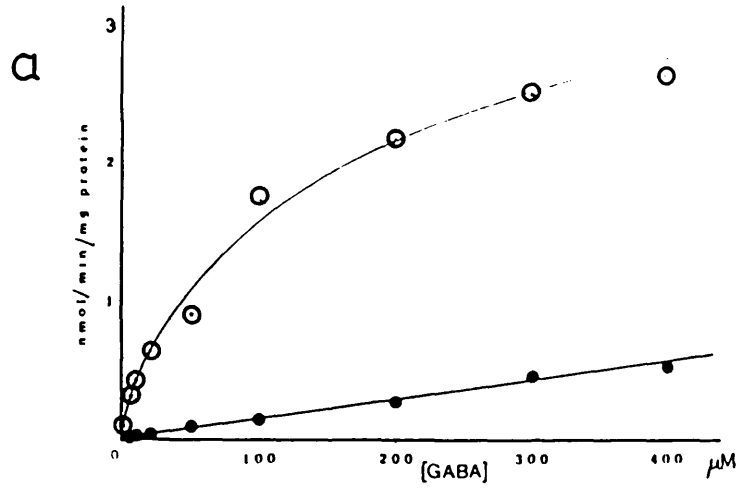
(●) was subtracted from (O) to give (b)

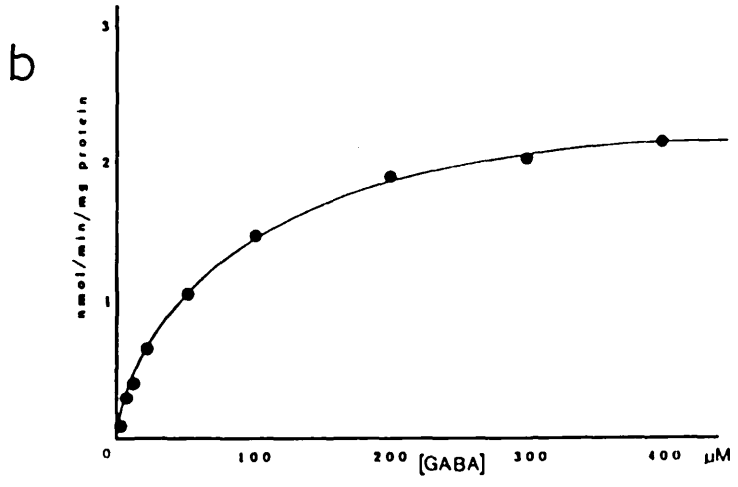
(b) Na^+-dependent uptake

The unsaturable component in (b) was subtracted from (b) to give (c)

(c) saturable, Na^+-dependent uptake

10μM amino-oxyacetic acid was present in all incubations to inhibit GABA transminase. The points in (a) are the means from 3 different experiments, each done in triplicate. (b) and (c) were formed by subtracting the graphs rather than individual points.


a
3
2
1
0
nmol/min/mg protein
100
200
[GABA]
300
400
μM


b
3
2
1
0
nmol/min/mg protein
100
200
[GABA]
300
400
μM

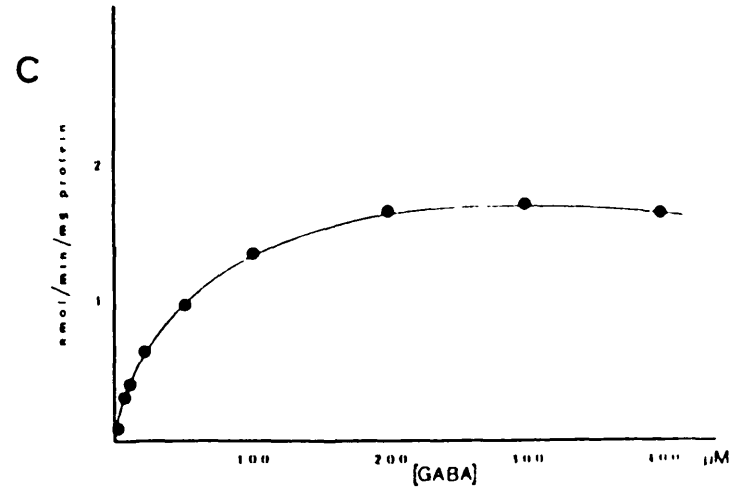

c
2
1
nmol/min/mg protein
100
200
[GABA]
μM

Fig.32 **Hanes plots for determination of kinetic parameters for GABA transport in 5 DIV and 12 DIV astrocyte cultures.**

The values for saturable, Na^+-dependent GABA transport in 5 and 12 DIV astrocyte cultures were obtained from Fig.30(c) and 31(c) respectively. Various values for substrate concentration (S) were divided by the velocity of uptake at that concentration (V) and the quotients (S/V) plotted against (S).

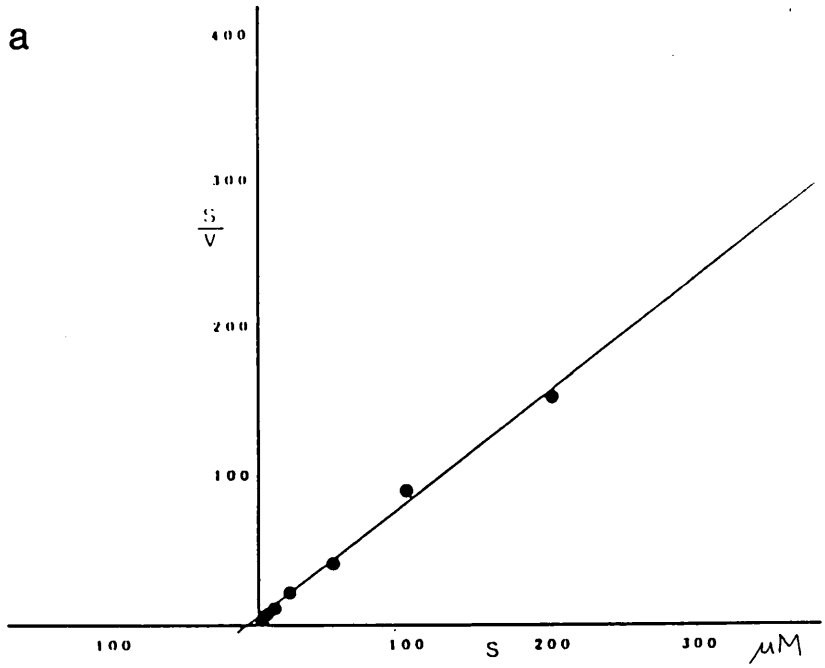
(a) 5 DIV

(b) 12 DIV

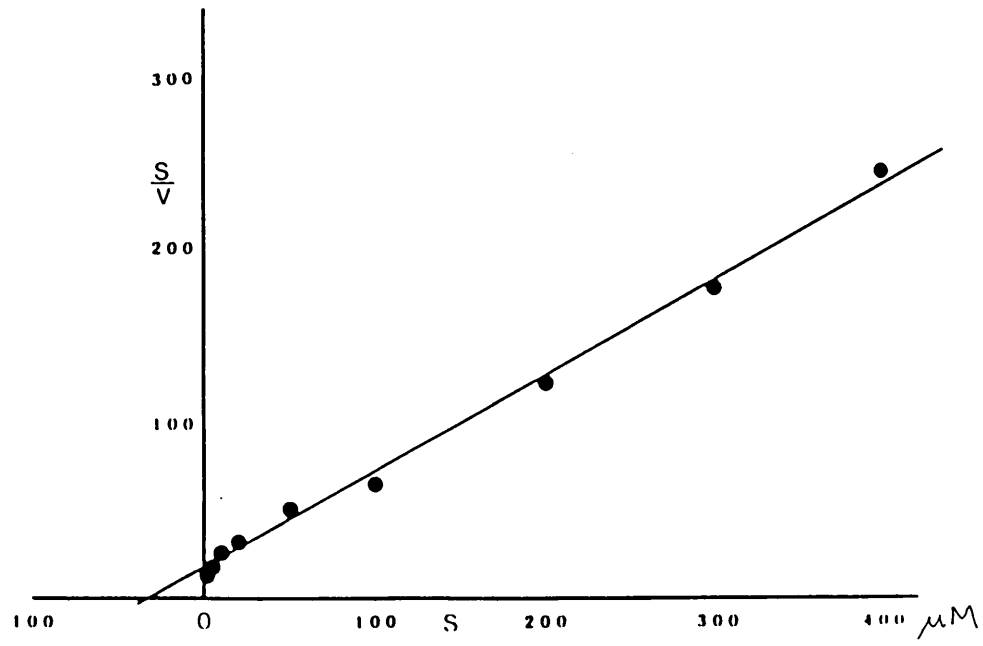
Km and Vmax estimated from these plots are:-

	Km	Vmax
5 DIV	6.7 μM	1.3 nmol/mg protein/min
12 DIV	31.2 μM	1.8 nmol/mg protein/min

a


400
300
S/V
200
100
100
100
S
200
300
μM

b


300
S/V
200
100
100
0
100
S
200
300
400
μM

constituting only 10-20% of the total. At higher concentrations (>200uM) the Na^+-independent uptake contributes more than half of the total transport. When the Na^+-independent component is subtracted from the total, the resultant plot suggests a transport system whose kinetics obey an equation of the following form:-

$$v = \frac{S.Vmax}{Km + S} + k.S$$

where S = concentration of substrate

Vmax = maximum transport velocity

Km = the "Michaelis" constant (a combination of individual rate constants and equivalent to the substrate concentration giving half-maximal velocity).

v = initial velocity at a given substrate concentration.

k = rate constant for an unsaturable transport system.

In order to predict the rate of transport at a given substrate concentration it is necessary to know the values of Km and Vmax. These were obtained graphically from the present data using a linearising transformation of the Micahaelis-Menten equation:

$$S/V = (1/Vmax).S + Km/Vmax$$

Thus a plot of S/Vmax against S will give a straight line graph with gradient 1/Vmax and intercept on the y-axis of Km/Vmax. The intercept on the x-axis is -Km.

Fig.32a shows such a plot for GABA uptake by 5DIV astrocyte cultures. This gives a linear relationship between S/Vmax and S from which the following values were estimated:-

Km = 6.7μM

Vmax = 1.3 nmol/mg/min

The uptake data for 12 DIV astrocyte cultures were analysed in the same manner (Figs.31 and 32b). Again the Na^+-independent fraction of total transport is lower at low substrate concentrations than at higher although at all concentrations it is lower at 12 DIV than at 5 DIV. The saturable, Na^+-dependent uptake was calculated then transformed to the linear plot from which the following kinetic parameters were calculated:-

Km = 31.2μM

Vmax = 1.8 nmol/mg/min

IV.G. Summary and discussion.

As stated at the beginning of this chapter, initial studies by Gordon (1982) showed that cerebellar astrocytes in primary culture showed two distinct morphologies - stellate and epithelioid. These two types also showed different rates of accumulation of GABA, the stellate cells exhibiting a much faster uptake rate than the epithelioid type. The shapes of these cells suggested that one form, the stellate, might represent a differentiated form of astrocyte whereas the other, epithelioid, form could be either a less differentiated astrocyte or, alternatively, a different astrocyte type. Similarly, the extent to which GABA was accumulated by these cells may also be an indication of their differentiated state.

That glial cells are able to transport neurotransmitters was first reported by Henn & Hamberger, 1971 who showed concentrative uptake of serotonin, noradrenaline, dopamine and GABA by bulk-isolated glia. Since then, the list of transported transmitters has steadily increased and now includes, in addition to the four above, taurine (Hosli &

Hosli,1980), glutamate (Henn et al.1974), glycine, leucine, and aspartate (Hannuniemi & Oja 1981).

In the study of Henn & Hamberger (1971), GABA was the most rapidly transported (by glia) of the transmitters tested, and it has been shown repeatedly to be accumulated by differentiated astrocytes in vivo (Halasz et al.,1979; Kelly & Dick,1975; Neal & Iversen,1972). Furthermore, Wolff et al. (1979) showed GABA to be accumulated by only the most differentiated of the glial cells in the embryonic rat neocortex. Thus it seemed that GABA uptake could be used as an astrocyte differentiation marker. With these points in mind the properties of the GABA transport systems in the two types of cultured astrocytes were investigated with a view to comparing them with the carriers found in vivo/in situ. Such comparisons could possibly give some further indication of the relative differentiated states of the stellate and epithelioid cultured astrocytes.

In the culture system used in the present work, autoradiographic studies showed that ^{3}H-GABA was indeed transported by astrocytes in culture but that the stellate astrocytes accumulated the transmitter at a faster rate than did the epithelioid cells. That this was not due simply to the different glial cells' geometries was shown by the equal distribution between the cell types of ^{3}H-D-aspartate and by unequal distribution of ^{3}H-GABA in cells whose morphologies had been altered using dBcAMP. These observations would support the idea that the stellate cells were more differentiated than the epithelioid ones, however it is possible that the uptake capacity of the former had been artificially enhanced by some aspect of the culture conditions. If this were the case it may be that it is the lower-affinity uptake (seen in the epithelioid cells) that is the true physiological uptake observed

by others in more intact tissue preparations such as ganglia, cortical prisms and slices (Bowery et al.,1979; Schon & Kelly,1974b, Snodgrass & Iversen, 1973)

Which, if any, of the *in vitro* transport systems reflected most closely the physiological carrier was investigated using a variety of structural analogues of GABA known to interfere with the transport systems for this transmitter in both neurons and glia (Bowery et al., 1976; Kelly & Dick, 1975; Krogsgaard-Larsen & Johnston,1975; Schon & Kelly,1974b,1975; Schousboe et al.,1978; Autoradiographs from these studies showed an interesting but unexpected pattern of inhibition of GABA uptake. Of the inhibitors tested it was predicted that β-alanine would be very effective since it has been shown to act preferentially on glial rather than neuronal GABA transport and also to be accumulated selectively by glial cells in several studies (Hosli & Hosli,1980; Iversen & Johnston,1971). ACHC, on the other hand, was reported to be specifically neuronal (Bowery et al., 1976) and was expected to have little effect on astrocytes. The study undertaken in the present work, however, showed precisely the opposite pattern in the stellate astrocytes. GABA uptake by these cells was inhibited very markedly by the allegedly neuron-specific inhibitor ACHC, whereas there was no obvious inhibition by the glial-specific compound β-alanine (although the uptake into the astrocytes was so intense that in an autoradiograph a small inhibition may not have produced a perceptible reduction in silver grains). 2,4-diaminobutyric acid (DABA), supposedly a neuron-selective agent was moderately effective in reducing uptake into the stellate astrocytes whereas (±)nipecotic acid, reported to inhibit uptake into both neurons and glia (Balcar et al., 1979; Schousboe et al.,1978) appeared the most inhibitory. These autoradiographic studies were complemented with and confirmed by

biochemical studies in which the IC50 values for the inhibitors were determined. The IC50 values obtained were nipecotic acid, 6μM; ACHC, 19μM; DABA, 209μM and β-alanine, 403μM.

These values are markedly different from those reported by other groups. In the present study ACHC was shown autoradiographically and by its IC50 value to be a potent inhibitor of GABA uptake by the stellate astrocytes. However, Bowery et al.(1976) found that ACHC at a concentration of 1mM had no effect on GABA uptake by rat retina or sympathetic ganglia (in which most of the GABA transport is in to glial cells (Neal & Iversen,1972, Young et al.,1973)). In contrast, uptake by rat cortical slices and frog retinae (predominantly into neurons (Iversen & Bloom,1972; Voaden et al.,1974)) was inhibited to 50% of control values by 62μM and 960μM ACHC respectively. Other studies by Currie & Dutton (1980) and Cohen et.al. (1980) also demonstrated effective ACHC-inhibition of GABA-uptake into neurons but only weak effects on glial cells. β-alanine was shown autoradiographically in several studies to be transported selectively into glial cells by a GABA-inhibitable process (Hosli & Hosli,1980; Kelly & Dick,1975; Schon & Kelly,1975) and numerous reports have described the inhibition of glial GABA transport by β-alanine (Bowery et al.,1979; Cohen et al.,1980; Currie & Dutton,1980; Hosli & Hosli,1980). In contrast, the autoradiographic data from the present study showedβ-alanine to be a poor inhibitor of GABA uptake by the stellate astrocytes, this being confirmed by the determination of an IC50 of 403μM. It should be noted that two reports also showed β-alanine to be a weak inhibitor of GABA uptake into cultured astrocytes (Schousboe et al.,1978) or to be poorly transported (Cummins et al., 1982). Nipecotic acid has been found to inhibit strongly the uptake of GABA by both glia (Schousboe et al.,1978) and neurons (Balcar et al.,1979; Bowery et al., 1976). The present

study confirms the effect on glial transport in that autoradiography following GABA uptake by astrocytes in the presence of 1mM nipecotic acid showed an absence of silver grains over all cells. In addition the measured IC50 value of 6μM revealed nipecotic acid to be the most potent of the inhibitors tested. DABA, initially thought to be specific for neuronal GABA transport systems (Balcar et al., 1979; Kelly & Dick,1975; Schon & Kelly,1974b; Weitsch-Dick et al., 1978) was moderately effective at inhibiting GABA transport into the cultured stellate astrocytes, having an IC50 value of 209μM. There have, however, been reports of strong glial as well as neuronal inhibition by DABA (Bowery et al.,1976; Hösli & Hösli,1980) with a quoted IC50 value as low as 58μM (Cohen et al.,1980).

Effects of the inhibitors on the epithelioid astrocytes were more difficult to determine since any inhibition had to be measured against a rather low level of transport even in the absence of inhibitor. Two efforts were made to overcome this problem. Firstly an attempt to quantitate the autoradiographic data was made using a photometer system. The results from this suggested that β-alanine alone, of the inhibitors tested, had more effect on the epithelioid astrocytes than on uptake by the stellate cells and that its effect on the epithelioid cells was greater than the effects of the other inhibitors on the same cells. The second approach was to investigate the transport of the inhibitors themselves. In these autoradiographic studies, ^{3}H-ACHC and ^{3}H-β-alanine were shown to have different distributions between the two astrocyte types. ACHC was transported to a small degree by the stellate cells but not at all by the epithelioid cells; in contrast βalanine was taken up by the epithelioid cells to a moderate degree whilst there was less intense labelling of the stellate cells.

In cultures grown under conditions such that neurons survived and

extended processes, autoradiographic studies showed that the GABA transport system(s) in the neurons behaved similarly to that in the stellate astrocytes, namely a strong inhibition by ACHC but not by β-alanine, and an ability to transport ACHC (although β-alanine was also transported by the neurons (see Hitzemann & Loh, 1978).

A brief summary of the autoradiographic data from cerebellar cultures allows the conclusion that, in vitro, two forms of astrocyte can exist both of which are able to accumulate GABA from the surrounding medium and that the rates of transport and the pharmacological properties of these uptake systems are different in the two cell types. The epithelioid astrocytes take up GABA poorly at low external concentrations (<1μM) and show patterns of sensitivity to GABA analogues similar to the "classical" glial transport system. The stellate cells however show a much more rapid accumulation of GABA at low concentrations and show patterns of inhibitor sensitivity more closely resembling the classical neuronal transport system and the neuronal carrier observed in the present studies.

When the autoradiographic studies were extended to other areas of the CNS - optic nerve and spinal cord - the same general characteristics of GABA transport were observed. Firstly, cultures from both areas showed differential GABA uptake by two morphologically distinct astrocyte types. A stellate form was particularly effective at accumulating exogenous ^{3}H-GABA whereas the epithelioid form was much less so. This was particularly apparent in spinal cord cultures. Secondly, uptake into all cells was abolished by the removal of Na^{+} from the medium and its replacement with choline. The effects of the GABA analogues ACHC and β-alanine on GABA transport by these two cultures were especially interesting in that they showed clearly defined selectivity between the

two astrocyte forms. In spinal cord cultures ACHC caused a noticeable decrease in the density of grains over the stellate cells but little effect on the epithelioid cells (in fact there may even be an increased labelling). β-alanine in these cultures abolished uptake into epithelioid cells but not stellate cells on which its effect appeared less than that of ACHC. This cellular specificity was even clearer in the optic nerve astrocytes. ACHC inhibited almost completely the uptake by stellate cells but had little effect on the epithelioid type, whereas β-alanine had minimal effect on stellate cells but caused a complete loss of uptake by the epithelioid astrocytes.

These observations are consistent with those obtained from cerebellar cultures with regards to inhibition of ^{3}H-GABA uptake and accumulation of ^{3}H-inhibitor compounds. The conclusion that may be drawn from these is that in cultures from three CNS regions there exist, in each culture, three sodium-dependent GABA transport systems:-

α - found primarily in stellate astrocytes, inhibited strongly by ACHC and poorly by β-alanine, and which can use ACHC as an alternative substrate in preference to β-alanine.

β - found primarily in epithelioid astrocytes, inhibited strongly by β-alanine and poorly by ACHC, and which can use β-alanine as an alternative substrate in preference to ACHC.

γ - found in presumed GABA-ergic neurons and having similar behaviour with respect to inhibitors as stellate astrocytes.

As the cultures aged from 5 DIV to 11 or 12 DIV the proportion of stellate astrocytes out of total astrocytes progressively decreased so that at 12 DIV only an occasional stellate astrocyte was seen. During this process there was a parallel loss of the neuron-like GABA trans-

porting activity previously seen in the stellate astrocytes so that all astrocytes remaining exhibited a similar rate of transport to that observed in the epithelioid astrocytes at 5-7 DIV. In view of this the 12 DIV cultures were used as pure epithelioid astrocyte cultures for kinetic and metabolic inhibitor studies of GABA transport. The 5 DIV cultures were used to glean some information about the kinetics and metabolic requirements of transport into the stellate cells. Although the 5 DIV cultures were far from pure stellate cultures, measurements made using the Leitz photometer system indicated that uptake into the stellate cells was at least 14x that into the epithelioid cells on a "grains per unit area" basis. This meant that at a composition of 70% epithelioid:30% stellate, the contribution of each cell type to the total GABA uptake would be stellate, 86%, epithelioid, 14% therefore any data from kinetic/inhibitor experiments would be more representative of stellate cell uptake than of epithelioid cell uptake. Probably the major effect of the epithelioid "contamination" in the 5 DIV cultures would be to give an erroneously low measurement for transport rates and thus for Vmax. This is because although the epithelioid cells were contributing a small amount to the total uptake, they might contribute very significantly to the protein determinations in each sample. Since uptake rates were expressed per mg cell protein it is clear that for 5 DIV cultures these rates would be higher if the cultures were truly pure stellate astrocytes. Nevertheless, in the absence of such a pure culture, kinetic studies and studies on the effects of metabolic inhibitors were performed on the mixed, 5 DIV cultures and the results compared with the 12 DIV epithelioid cultures.

When the metabolic requirements for GABA transport were studied there were again differences between the stellate and the epithelioid cells. Neither rotenone nor cyanide caused inhibition of GABA accumulation by

either of the astrocyte types, suggesting that a negligible proportion of the energy for this process is derived directly from oxidative phosphorylation. This is in agreement with the results of Balcar et al.(1979) Schrier & Thompson (1974) and Cohen et al. (1980), (who used 2,4-dinitrophenol as an uncoupler of oxidative phosphorylation). Consistent with the insensitivity of uptake to the blocking of oxidative phosphorylation was the marked inhibition of uptake into epithelioid cells by fluoride (NaF), an inhibitor of glycolysis, suggesting that the glycolytic pathway provides a large proportion of the required energy for the uptake. The reasons for the relatively small effect of NaF on transport by the stellate cells (a decrease of 25% of the control value, a proportion of which may be due to the contaminating epithelioid cells) is not clear (but see below).

That external sodium is required for GABA transport has been shown both autoradiographically and kinetically, and the need for the maintenance of a sodium gradient across the cell membrane is supported by the inhibition by ouabain of uptake into both cell types. Again the inhibitory effect was considerably greater in the epithelioid than the stellate cells. One reason for this may be that there was a larger Na^+-independent component to the total GABA uptake by stellate than non-stellate astrocytes (shown in the kinetic studies), therefore treatments affecting the transmembrane sodium gradient would have a lesser inhibitory effect on the total uptake in the former. An alternative explanation is that the permeabilities to sodium of the membranes of the two cell types may differ from each other. Kuffler et al.(1966) showed that the membrane potential of glia from Necturus (mudpuppy) was very close to the potassium equilibrium potential and suggested that for this to be the case the sodium permeability must be very low. Similarly, Moonen & Nelson (1978) showed a near-Nernstian

slope for the membrane potential versus log $[K^+]$ relationship in rat astroblast cultures. Also in these cells the mean membrane potential did not change when the sodium content of the bathing saline was reduced to 20% of its usual concentration. It is therefore possible that the stellate astrocytes have Na^+-permeabilities similar to those described in the aforementioned reports, whilst the epithelioid cells are more similar to most other cells, having an appreciable Na^+-permeability. The consequence of this would be a prolonged maintenance of the transmembrane Na+-gradient in the stellate cells even after inhibition of the Na^+K^+ATPase, so that compounds such as ouabain would have only a small effect on GABA transport in the short term. In epithelioid cells the Na^+-gradient would collapse more quickly with parallel loss of GABA transporting ability.

A third possible reason for the differential effect of ouabain on the two astrocyte types is that these cells might possess different ATP-ases which vary in sensitivity to ouabain. Marks & Seeds (1978) observed a heterogeneous interaction between ouabain and ATPase in mouse brain. The heterogeneity was shown to be due to two molecular forms of the enzyme (Sweadner, 1979). One form existed predominantly in the neuronal fraction whereas the other occurred primarily in glial fractions, these two forms having high and low affinities, respectively, for ouabain. This was confirmed recently by Atterwill et al. (1984) who showed that the Na^+K^+ATPase of cultured astrocytes was predominantly (73% of total activity) of the low-affinity type, whilst this form constituted only 55% of total Na^+K^+ATPase in neuronal cultures.

As well as measuring the amounts of GABA accumulated by the cultured cells in the presence of metabolic inhibitors, some cultures from the same experiments were used for autoradiography. These studies did not show, however, the effects of the metabolic inhibitors suggesting that

this technique is not sensitive to anything but the largest changes in uptake. One exception to this was in the detection of uptake into neurons. In the 5 DIV cultures a number of neurons were very heavily labelled with ^{3}H-GABA; such figures were not seen when either fluoride or ouabain were present indicating a much higher sensitivity of the neuronal transport system to these inhibitors in comparison with the astrocyte carrier (neuronal cells recognised by morphology were shown to be present in these cultures by phase contrast microscopy). An alternative explanation would be that the astrocytic transport system is so much more active than the neuronal one that equal inhibition of both carriers would be apparent in the neurons but not the astrocytes. This seems unlikely, since if the autoradiographs following incubation with ^{3}H-GABA are developed after a variety of exposure times, the neurons are generally the first cells to appear heavily labelled, followed by the stellate astrocytes then the epithelioid astrocytes.

When kinetic studies were carried out on the 5DIV and 12DIV (stellate and epithelioid) cultures it was found that the two astrocyte types had very similar Vmax values for GABA transport (1.3 and 1.8 nmol/ min/mg protein respectively). There was, however, a 4.5-fold difference in the K_m values — 6.7 μM for the stellate astrocytes and 31.2 μM for the epithelioid cells . This meant that at the low concentrations of GABA used in the autoradiographic studies (<1μM) most of the ^{3}H-GABA should have been taken up by the stellate cells rather than the epithelioid astrocytes; this was observed to be the case. There have been many studies aimed at determining the kinetic parameters of GABA transport in a variety of cell and tissue preparations. In Table 12 are listed the kinetic parameters for GABA transport in several experimental models for both glia and neurons (taken from Schousboe, 1981) against which the values obtained in the present work will be compared later in this

Table 12. Kinetic constants for high-affinity uptake of GABA into a variety of cell and tissue preparations.

Tissue preparation	Km (uM)	Vmax (nmol/min/mg protein)
Adult brain slices	11-31	0.36-1.76
Neonatal brain slices	5-43	0.05
Synaptosomes	13	2.2
"	4	1.1
" "	0.42	-
"	4	1.3
Cerebellar glomeruli	9.6	1.6
"	15	-
"	10	1.5
Dorsal spinal roots	24	0.07
Ventral spinal roots	33	0.05
Bulk-prepared neurons	0.72	-
Cultured cerebellar neurons	0.33	0.21-0.84
NB41 neuroblastoma cells	0.15	0.0021
Bulk-prepared glial cells	0.27	-
"	0.6	-
Superior cervical ganglia	7	0.002
Sensory ganglia	10	0.02
"	9.7	0.03
Cultured cerebellar glial cells	0.29	0.0005-0.002
C6 glioma cells	32	0.023
"	0.22	0.0014
"	50	-
Cultured astrocytes mouse brain	40	0.35
"	45	0.40

discussion.

The fundamental question is whether or not these cells exhibit *in vitro* the same GABA-transporting properties as they do *in vivo*. The approach taken to elucidate this was to study, autoradiographically, the effects of ACHC and β-alanine on ^{3}H-GABA transport by freshly-cut slices of cerebellum. The results from these experiments were unclear in that although there was considerable uptake of ^{3}H-GABA into glia in all layers of the cerebellar cortex, there was no obvious inhibition of uptake by either ACHC or β-alanine. The reasons for this remain unresolved. One difficulty with this technique is that there was noticeable variation in labelling intensity across individual sections, possibly due to unevenness in the cut tissue. However it seems unlikely that this would mask any considerable inhibition of uptake. The problem does not seem to be one of simple diffusion barriers between the medium and the cells of the tissue since (a) the ^{3}H-GABA was able to reach and be taken up by the tissue and (b) this uptake was totally inhibited by the excess unlabelled GABA. In terms of the chemistry of the molecules, they all have similar pKa values for the ionisable amino and carboxyl groups and so are likely to have similar ratios of ionised to unionised states; this suggests that there would be no particular disadvantage to ACHC or β-alanine compared with GABA for diffusion through a charged environment (although the physical size of ACHC may cause it to be retarded slightly more than the others). Lack of apparent inhibition of uptake due to emulsion saturation by overloaded cells was also ruled out by developing the autoradiographs at a variety of intervals after exposure; this failed at all times tested to show any differences in labelling between the different cell types (the shortest exposure time was 7 days after which very few silver grains were seen). A reduction in inhibitor concentration (and thus in its

effect) by uptake and metabolism is unlikely since both ACHC and β-alanine have been reported to be minimally metabolised in experiments of this nature (Neal & Bowery,1977; Schon & Kelly,1975); in addition, AOAA was present throughout the experiment to block GABA-T for which β-alanine is a substrate. In the absence of a suitable explanation for the lack of effects of the inhibitors tested in this experiment, the tentative conclusion must be drawn that the astrocytes *in vitro* differ from those *in situ* with regard to GABA transport under the conditions used in this study.

A striking difference between uptake by cultures and uptake in slices was that not only was there no obvious effect in slices of established uptake inhibitors, but that although in cultures there appeared two astrocyte types having differing GABA transport rates, this difference was not observed *in situ*. Astrocytes in all layers of the cerebellar cortex molecular layer, Purkinje cell layer, granule cell layer and white matter - were labelled to a similar extent. This may have been a result of the extended uptake period used in the slice experiments compared with the culture studies (30 min and 5 min respectively) which might saturate both astrocyte types to a similar level. This might also account for the inability to detect, autoradiographically, differences between cells after even the shortest exposure periods. An alternative to this is that only one of the ^{3}H-GABA transport systems observed in culture is functional in slices.

Despite the uncertainties regarding the extent to which the cultured astrocytes faithfully represent those *in situ* (uncertainties which were not resolved by the slice experiments in this study), it seems unlikely that the cells *in vitro* express a property totally alien to astrocytes. In other words, even if the properties observed *in vitro*

are not always apparent *in vivo*, it is probable that they are displayed at some point throughout the life of the cell and possible that the tissue culture conditions are able to unmask important properties which might remain hidden using other experimental methods. In view of this it is interesting to consider the potential physiological significance of the astrocytic GABA transport observed *in vitro*.

The most obvious roles for GABA uptake by any cells in the CNS are (a) to inactivate signalling at GABAergic synapses and (b) to contain GABA within a defined region of the brain. GABA transport is a function shared by both neurons and glial cells *in vitro* and *in vivo* therefore the relative contributions of neuronal and glial transport to the total uptake must be assessed. As mentioned earlier, this will depend on the topography of the synaptic area, ease of neurotransmitter diffusion and the kinetic parameters of the neuronal and glial uptake processes.

Considering firstly Vmax values, comparison with Table 13 shows the values obtained in the present study of 1.8 nmol/min/mg protein and 1.3 nmol/min/mg protein for the cultured stellate and epithelioid astrocytes, respectively, to be at least an order of magnitude greater than values quoted for other astrocyte models, with the data obtained by Schousboe et al.(1977) and Hertz et al.(1978) with cultured astrocytes from mouse brain being the closest. The values obtained from the present work are the same as or very close to those obtained by others from synaptosomes (Hitzemann & Loh,1978; Levi & Raiteri, 1973) or cerebellar glomeruli (Wilkin et al.,1974; Wilson et al.,1976). In terms of Km values, 31.2μM obtained with epithelioid astrocytes is comparable with values measured in a variety of glial preparations, notably other cell preparations such as C6 glioma (Bowery & Brown, 1972; Schrier & Thompson,1974) and cultured astrocytes from mouse brain (Schousboe et al.,1977b; Hertz et al., 1978), but differs markedly from other glial

models, especially bulkprepared glia (Henn, 1976; Henn & Hamberger, 1971).

With reference to their potential capacities for transport, it would appear that both the stellate and epithelioid astrocytes under study would, at high enough GABA concentrations, show similar uptake rates (i.e they have similar Vmax values) to many of the neuronal transport systems presented in Table 12 The Vmax of the transport systems may not therefore be a determining factor as regards the relative importance of glia and neurons in total GABA uptake. On the other hand Km values may be important. From Table 13 it would appear that an average Km value for neuronal uptake is approximately 8μM (using the values for synaptosomes and cerebellar glomeruli). This is very close to the value of 6.7μM obtained for the cultured stellate astrocytes, so it might be expected that stellate astrocytes and neurons, having similar Km and Vmax values would, at any given [GABA] take up approximately equal amounts of this transmitter. In contrast, the epithelioid astrocytes have a markedly higher Km value than do neurons, therefore at low [GABA] they would take up only a fraction of the amount of this transmitter taken up by neurons. To date it has not been possible to measure directly the concentration of GABA in the synaptic area during transmission, however, Desarmenien et al.(1980) found the threshold [GABA] for response in dorsal root ganglion neurons to be 2-20μM, with a mean of 9μM. When the mean value is used to calculate GABA transport rates into neurons, stellate astrocytes and epithelioid astrocytes, the following rates are obtained:-

	Km	Vmax (nmol/min/mg)	uptake rate (nmol/min/mg)
neurons	8μM	1.54	0.86
stellate astrocytes	6.7μM	1.3	0.78
epithelioid astrocytes	31.2μM	1.8	0.44

Therefore, under conditions where all cell types are subjected to the same (estimated) physiological concentration of GABA, the epithelioid cells can, at best, transport GABA at only about half the rate of the neurons or the stellate astrocytes. Since the neuron will be closer to the released GABA than would the astrocytes, it would be expected to experience a higher [GABA] than the latter so it is probable that maximum uptake of GABA would be into neurons followed by stellate astrocytes then epithelioid astrocytes.

It seems, therefore, on the basis of the kinetic data, that the stellate astrocytes would be capable of contributing significantly to the inactivation of GABAergic neurotransmission by removing GABA from the perisynaptic area. A feature of the stellate-cell (or aa) GABA transport system is that it was lost as the cultures aged (along with a probable change in cell shape). If this observation in the culture system is fairly representative of the <u>in vivo</u> situation then it would be interesting to speculate on the reasons why such an intense GABA-transporting ability should be required at the early stages of development but disappears at later stages. This, again, leads to the question of what the prime purpose of GABA transport really is.

At the developmental stage under consideration in this work (approximately 6-12 DIV) synaptogenesis in the cerebellum is only at an early stage (Jacobson, 1978), so it seems most unlikely that such an intense GABA transporting capabilty would be required to inactivate GABA which

had been synaptically released during the normal course of neurotransmission. The use of GABA as a metabolic fuel, via the GABA shunt, might be considered (it was very well taken up by cells in the external granule cell layer in 12d cerebellar slices, Fig.28). However it has been calculated that GABA contributes to only about 8% of the flux through the tricarboxylic acid cycle (Balazs et al.,(1970). A further possible role for a highly-active, high-affinity glial GABA transport system at this stage of development might be to limit any potential effect of "stray" GABA.

During synaptogenesis it is possible that GABAergic neurons would have developed the capacity to synthesise GABA and to release it even before the synapse has been formed. This would allow some GABA to "escape" into non-synaptic extracellular fluid where it could interact with any cells it encounters. It has been shown that the stimulation of postsynaptic receptors by neurotransmitters can alter the biochemistry of the postsynaptic cell e.g. the stimulation of tyrosine hydroxylase activity in the adrenal medulla or superior cervical ganglion (Zigmond,1985). Additionally, modulation of receptor number has been demonstrated in a number of situations (Epstein & Grundfest, 1970; Katz,1957; Sarne,1976). It can be imagined, therefore, that if stray transmitter should come into contact with the wrong cells or with any cells at the wrong time, then the functions of those cells might be regulated to a degree or in a manner inappropriate to their role in CNS function. If, however, glial cells are able to remove the transmitter efficiently from the extracellular fluid then such false regulatory signals would be abolished or, at least, minimised. Thus the glial cells might form "regulatory domains" allowing particular cells to be included under the regulatory influence of a set of developing neurons and others to be excluded. That this type of effect may operate during

development has been suggested recently by Madtes Jnr. & Redburn (1985). They showed that as a consequence of inhibition of GABA uptake in the immature retina, receptor binding increased four-fold (Madtes & Redburn,1983a) and that this could be mimicked by the GABA receptor agonist THIP (4,5,6,7tetrahydro-[5,4c]pyridin-3-ol) ((Madtes & Redburn,1983b).

V GLUTAMINE SYNTHETASE AND GABA TRANSAMINASE IN CULTURED ASTROCYTES: AN IMMUNOCYTOCHEMICAL STUDY

V.A Introduction.

In Chapter III it was shown that in primary cultures from rat cerebellum there develop two distinct populations of astrocyte (based on morphology) one being stellate and the other epithelioid in shape. In chapter IV it was shown that these types differed not only in their shapes but also in their abilities to transport neurotransmitters and neurotransmitter analogues - especially GABA. These observations promoted the suggestion that the stellate cells were more differentiated than the epithelioid ones and that they might provide a useful *in vitro* model for the mature astrocyte. In view of the GABA transport capacity observed in the cultured astrocytes and of the potential usefulness of enzymes as indicators of the differentiation state of the cells it was of interest to look at some of the enzymes involved in the GABA synthetic and degradative processes.

The metabolism of GABA is part of a complex of relationships involving intra- and intercellular compartmentalisation of reactions. This metabolic network is shown diagrammatically in Fig.33. Five enzymes are involved in this scheme:

1. glutamic acid decarboxylase (E.C. 4.1.1.15) which converts glutamic acid to GABA.

2. 4-aminobutyrate:2-oxoglutarate aminotransferase (GABA transaminase, GABA-T, E.C. 2.6.1.19) which converts GABA to succinic semialdehyde.

3. succinic semialdehyde dehydrogenase (E.C. 1.2.1.24) which converts succinic semialdehyde to succinic acid.

4. glutaminase (E.C. 3.5.1.2) which converts glutamine to glutamic acid.

5. glutamine synthetase (E.C. 6.3.1.2) which converts glutamic acid to glutamine.

The enzymes chosen for study in the present work were glutamine synthetase and GABA-T.

V.B. Glutamine synthetase (GS)

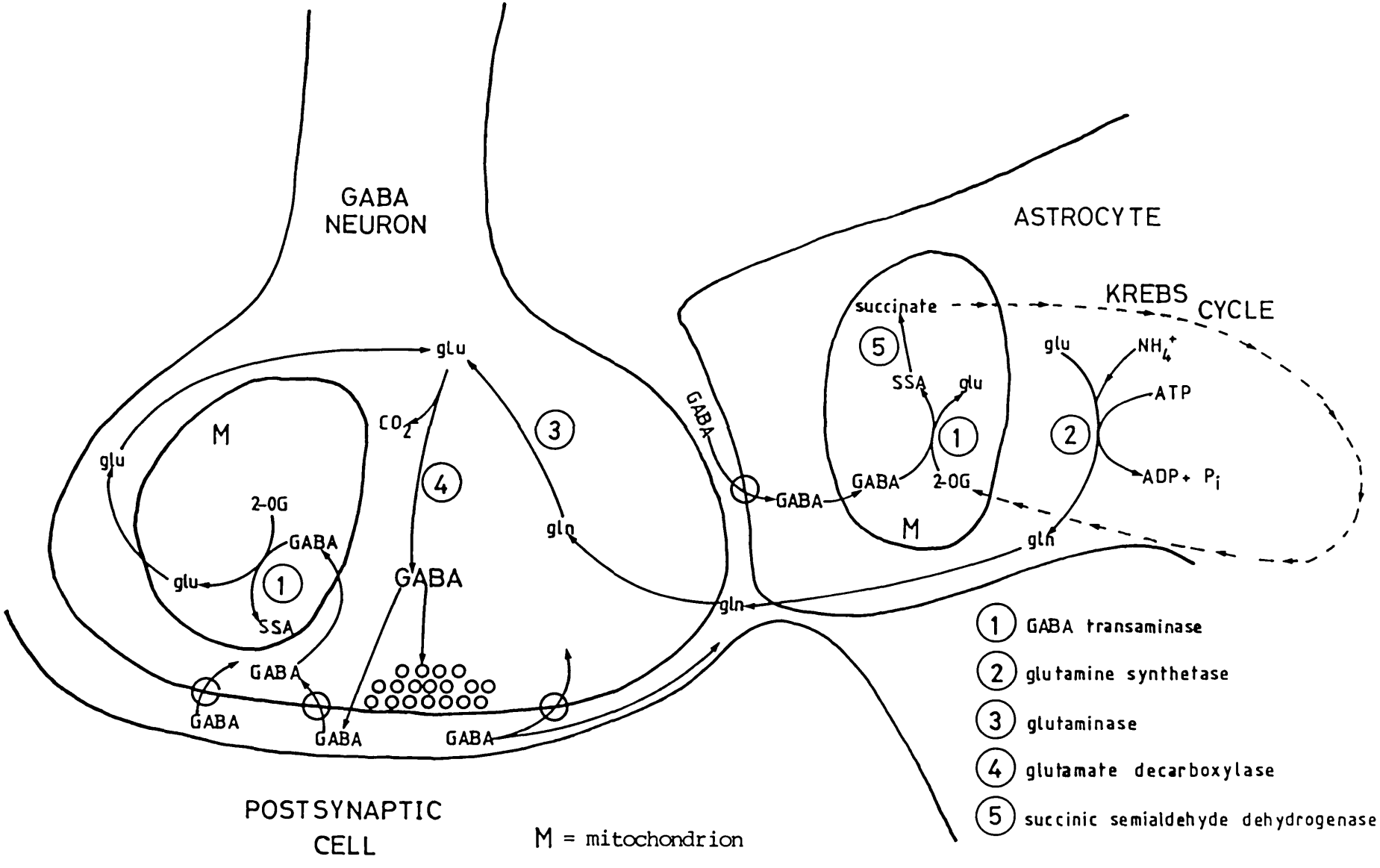
Glutamine synthetase (L-glutamate:ammonia ligase (ADP-forming), E.C. 6.3.1.2) catalyses the reaction:-

$$ATP + L\text{-glutamate} + NH_3 \rightleftharpoons ADP + Pi + L\text{-glutamine}$$

The enzyme has a number of proposed functions in the brain including ammonia detoxification, and as a central enzyme in the glutamine - glutamate cycle which is involved with recycling of the neurotransmitters glutamate and GABA and in the energy metabolism of the cell. In mammals the enzyme has a wide distribution; the greatest activity is found in the liver (which may be expected due to the importance of this organ in detoxification processes) however the next most active tissue is the brain which shows about 50% of the activity seen in liver (Dixon & Webb, 1979).

The localisation of glutamine synthetase in the nervous system has been studied extensively in the rat and to some extent in other species using immunological methods (Norenberg, 1979). where the staining pattern was almost identical to immunoreactive GFAP and to that observed with the metal impregnation staining methods designed to identify astrocytes. These findings suggested that GS was located exclusively in the astrocytic glial cells. This is supported by

Fig.33 Compartmentalisation of glutamine, glutamate and GABA metabolism



other studies showing GS in the cerebellar Bergmann glia (Norenberg, 1979), the Muller cells of the retina (Linser & Moscona, 1979; Riepe & Norenberg, 1977) cerebellar white matter (Martinez-Hernandez et.al., 1977) as well as the demonstration of astrocytic GS in an ultrastructural study (Norenberg & Martinez-Hernandez, 1979). Using isolated cells, the specific activity of glutamine synthetase has been shown to be at least five times greater in astrocytes than in neurons (Patel et.al., 1982) and its presence at high levels has also been demonstrated in Muller cells from turtle (Sarthy & Lam, 1978). Tissue culture studies have also confirmed the presence of GS in astrocytes using both enzymatic and immunocytochemical techniques Hallermayer et.al., 1981; Juurlink et.al., 1981; Schousboe, Svenneby & Hertz, 1974). It has further been shown immunocytochemically that astrocytes but not oligodendrocytes express GS in vitro (Raff et.al., 1983).

Another aspect of GS in astrocytes is the observation that its expression can be induced by hydrocortisone in embryonic chick retinal glia both in ovo and in vitro (Linser & Moscona, 1979; Norenberg et.al., 1980). as well as in monolayer primary cultures derived from newborn mouse cerebrum (Juurlink et.al., 1981)

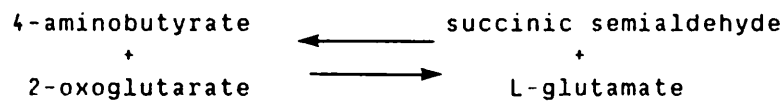
The many demonstrations of the astrocytic localisation of GS promoted the idea of using this enzyme as an astrocyte marker. This is given added weight by investigations showing the presence of GS in cells which do not express GFAP but which are considered to be glial. For example the GFAP staining patterns in rat, human and chick CNS are very similar to each other. However in chick Bergmann glia, which stain strongly for GS (Norenberg, 1985), GFAP-immunoreactivity was not found (Dahl & Bignami, 1973). Furthermore, lower species such as goldfish and toad do not possess astrocytes but do have "ependymoglial" cells

whose perikarya reside at the ventricular zone and whose processes project to the pial surface. These cells stain positively for GS but not for GFAP (Norenberg, 1985).

Developmental studies on GS expression suggest that GS appears at earlier stages than does GFAP (Bignami & Dahl, 1973,1974; Norenberg, 1985) and therefore GS may be one of the earliest markers we have at present for identifying glioblasts which have entered the differentiation pathway towards astrocytes. For this reason it would be useful to be able to detect the expression of GS in cultured cells and to have some means of determining the amount of the enzyme present at various stages in culture. The production of reagent antibodies against GS would serve both of these purposes since they can be used immunocytochemically to study the cellular distribution of the enzyme, and can also be used quantitatively as the basis of an ELISA or RIA for determining the amount of enzyme present.

V.C. GABA Transaminase

GABA-transaminase (GABA-T; 4-aminobutryrate:2-oxoglutarate aminotransferase, E.C. 2.6.1.19) catalyses the reaction:



This activity has been identified in the brains of a variety of mammals including pig (Bloch-Tardy et.al., 1974), rat (Maitre et.al., 1974), mouse (Schousboe et.al., 1973), human (Jeremiah & Povey, 1981), monkey (Salvador & Albers, 1959) cow (Baxter & Roberts, 1958) and rabbit (John & Fowler, 1976) as well as from non-mammalian tissue such as turtle retina (Sarthy & Lam, 1978) and Pseudomonas (Scott & Jakoby, 1959). As can be seen from Fig.33 one of the established roles of

GABA-T is the degradation of GABA released during neurotransmission and subsequently accumulated by neurons and/or glial cells in the vicinity of the synapse. This allows the amino group to be recycled to glutamate, glutamine and GABA for reusage in neurotransmission or more mainstream metabolism.

GABA-T has been localised in the brain using both histochemical and immunohistochemical methods with many studies focussing on the cerebellum which utilises GABA extensively as a transmitter. Histochemical studies based on the formazan-dye method (Van Gelder, 1965) have localised GABA-T activity to Purkinje cells, granule cells, basket cells, stellate cells, as well as Bergmann glia (Galustyan & Prianishnikov, 1978; Hyde & Robinson, 1976a,b; Martinez-Hernandez et.al. 1982). Immunohistochemical studies have also shown immunoreactive GABA-T in Purkinje cells, granule cells, basket cells, Golgi neurons, Bergmann glia and radial fibres, granule layer astrocytes and white matter astrocytes (Cavallotti et.al., 1983; Chan-Palay et.al., 1979). It is clear, therefore, that both neurons and glial cells, at least in the cerebellum, possess the GABA-T protein.

Developmental studies on the rat showed that the levels of GABA-T activity in cerebral hemispheres from young postnatal animals (3 days) were only around 14% of the adult level and that this initial low level increased steadily to reach adult levels at between 14 and 21 days postnatal. The expression of GABA-T in cultured brain cells (of special relevance to this work) has been reported not to follow this pattern. Astroglial cultures derived from rat cerebrum showed an initially low activity which increased only very slowly over the first 14 days in culture to a level only 25% of that in the whole cerebral hemisphere (Hansson & Sellstrom, 1983). A similar study on astro-

glial cultures from neonatal mouse also reported a rise in GABA-T levels between 1 and 3 weeks in culture but again there was a failure to reach adult levels - only 33% of the adult activity was measured (Schousboe, Hertz & Svenneby, 1977). In contrast to this, Sellstrom et.al. (1975) showed that using freshly-isolated (bulk-prepared) neuronal perikarya, synaptosomes and glial cells, the highest activity of GABA-T was found in the glial-cell fraction, the specific activity being similar to brain homogenate.

In view of the reported increases, over the first 3 weeks post partum, in GABA-T activity associated with the astrocyte compartment but not observed in cultured astrocytes, it would seem that the degree of expression of GABA-T might be a rather strict marker for the complete differentiation of astrocytes and, therefore, particularly useful in tissue culture studies. This should not be confused with a marker specific for astrocytes but, since both glutamine synthetase and GFAP can be detected in astrocytes in vivo and in vitro before they are fully differentiated, GABA-T expression may be useful as a measure of the degree of differentiation along the astrocyte lineage. In other words GS and GFAP can be used as qualitative differentiation markers whereas GABA-T can be used as a quantitative marker.

For the reasons described above it would be useful to have a means of detecting GABA-T in cultured cells and also a means of estimating the amount present. This is particularly true also in view of the marked GABA-transporting ability of the stellate but not the epithelioid astrocytes observed in cultures (Chapters III and IV). As already discussed in these chapters, it was hypothesised that, in view of their morphologies and their GABA transport properties, the stellate astrocytes were more differentiated than the epithelioid type. The levels of expression of GABA-T, a function presumably related to the

GABA transport capacity, may be a further means of determining whether this is the case.

For the same reasons of applications as described in V.A.1 for glutamine synthetase coupled with the ease of use over other means, an immunological route was taken for the detection and estimation of GABA-T in cultures.

V.D. Production of anti-glutamine synthetase antiserum.

V.D.1. Glutamine synthetase protein.

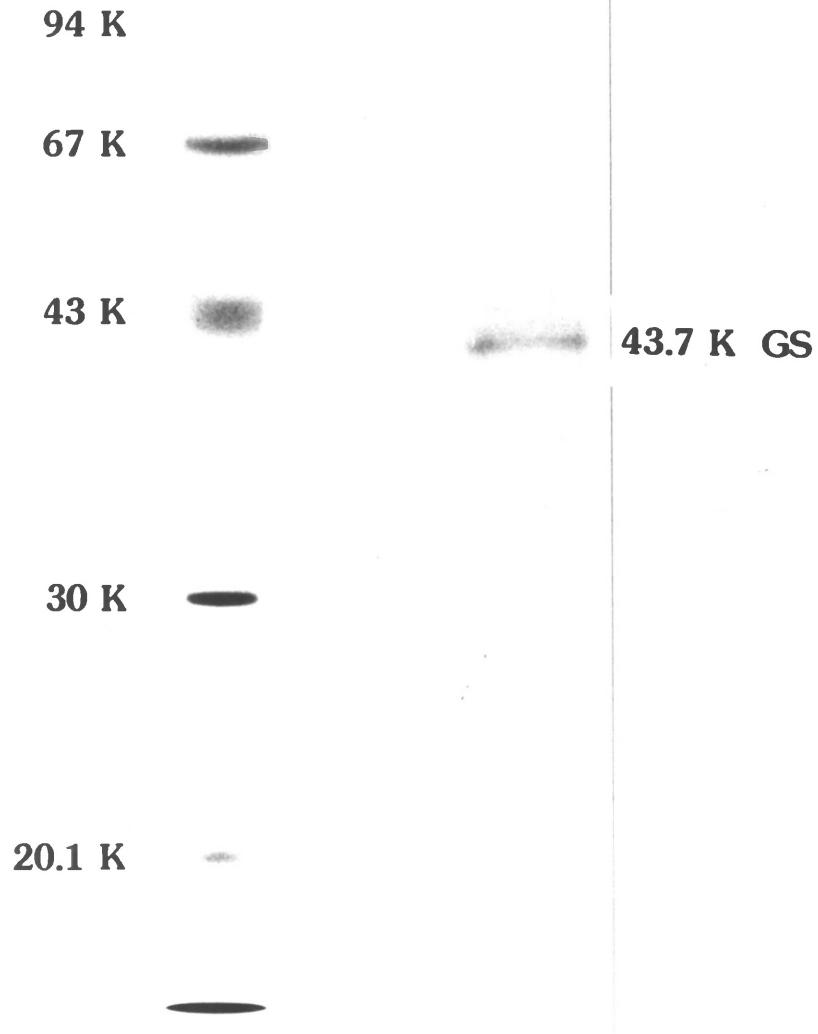
The protein used for immunisation was L-glutamate:ammonia ligase (ADP-forming) (EC 6.3.1.2.) from ovine brain and was obtained from Sigma Chemical Company (Type III, lot 85C-85 III). It was supplied as a lyophilised powder containing 5% protein and 95% buffer salts as potassium phosphate, sodium citrate and magnesium acetate. A stock solution of 0.97 mg/ml was made in 0.2M sodium phosphate buffer, pH 7.4. SDS-polyacrylamide gel electrophoresis (II.G) showed a single band of M_r = 46,000 (Fig.34)

V.D.2. Immunisation schedule.

Before immunisation 30-35 ml of blood was taken from each of two rabbits and used to prepare pre-immune sera (see V.B.3). Each animal was immunised by subcutaneous injection at four sites (on the back) of a total of 0.1mg of protein mixed with the same volume of Freund's Incomplete Adjuvant (0.5ml enzyme solution + 0.5ml adjuvant). This procedure was repeated after two weeks and then one week after the second injection blood was taken to check for a response. A further series of injections was made 10 months later. In this series one

Fig. 36 SDS-polyacrylamide gel electrophoretogram of glutamine synthetase used for antiserum production.

7-10 μg of glutamine synthetase and approximately the same amount of each of the molecular weight standards were run on a 10% resolving gel with 3.5% stacking gel under reducing/dissociating conditions. A plot of log(molecular weight) against Rf for the standards gave a curve from which the molecular weight of the glutamine synthetase protein was estimated to be 43,700 Daltons.


94 K
67 K
43 K
30 K
20.1 K
43.7 K GS

injection of 0.2mg then three of 97µg were done as above at weekly intervals.

V.D.3. Bleeding of animals and serum preparation.

15-20ml of blood were taken from each animal via an ear vein into glass tubes. These samples were heated for 1h at 37°C during which the blood clotted. The clot was then loosened and the tubes transferred to a fridge overnight when the serum was expressed by contraction of the clot. The serum was separated from the clot by decanting into a 50ml polypropylene Falcon tube and any remaining red blood cells removed by centrifugation at 2600 x g for 15 min. Finally, complement was inactivated by heating at 56°C for 30 min. Sera prepared in this way were stored frozen at -18°C.

V.D.4. Characterisation of antisera.

V.D.4.a. Ouchterlony double immunodiffusion.

Immune responses to the injected glutamine synthetase were initially screened for by Ouchterlony double immunodiffusion in agarose gels. The centre wells contained either preimmune or immune serum and the peripheral wells rat brain extract from adult or baby (7 days) rats or with the protein solution used for immunisation (each well held 9-13µl of sample). After 18h at room temperature the plates were washed and stained with Coomassie Brilliant Blue R250; the staining patterns are shown in Fig.35. These showed a definite response to the injected protein, and cross-reactivity with components in adult brain extract, although it was difficult to detect any cross-reactivity with neonatal brain extract components. Neither of the preimmune sera showed signs of reacting with any of the potential antigens.

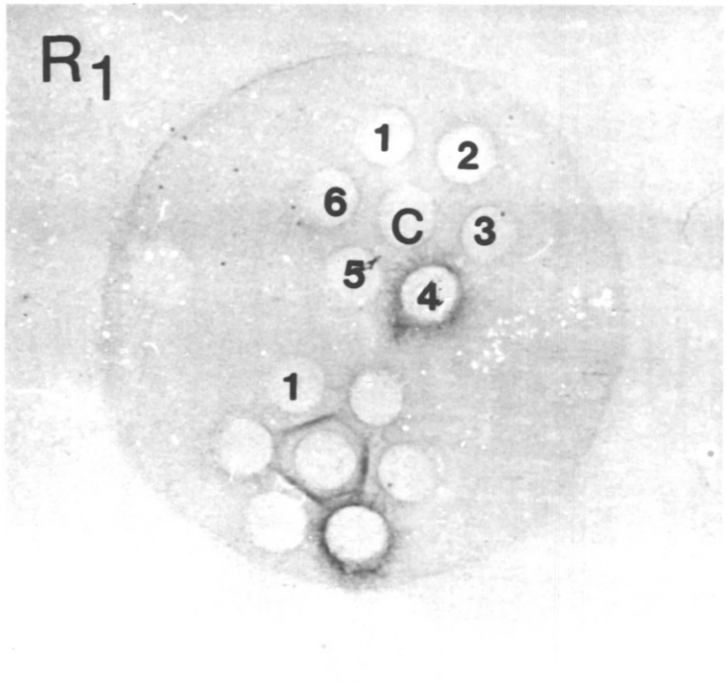
Fig.35 Ouchterlony double immunodiffusion with serum from rabbits immunised with glutamine synthetase.

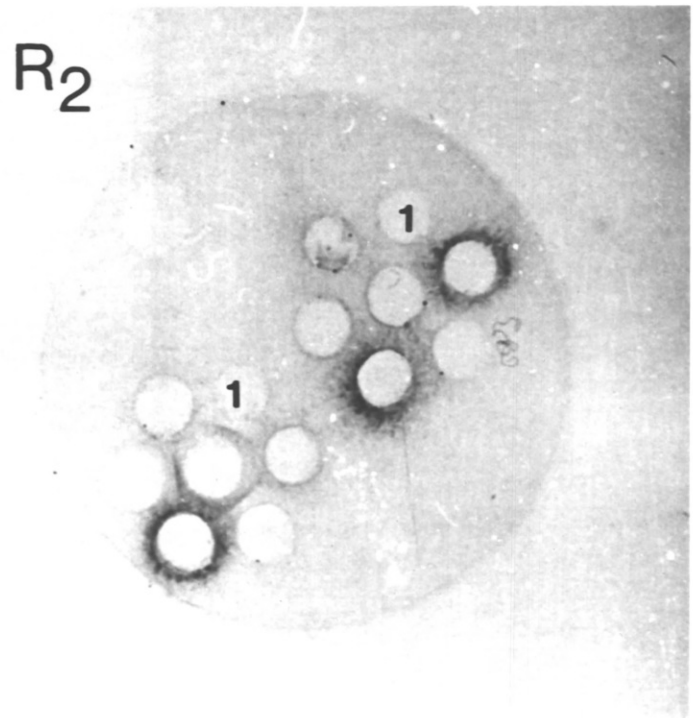
Sera from two rabbits immunised with glutamine synthetase (GS) as described in section V.D were screened for immune response against the injected material and brain extracts by Ouchterlony double immunodiffusion.

Wells are numbered 1-6 in a clockwise direction.
For rabbits 1 and 2, the centre wells contained pre-immune serum or immune serum (top and bottom patterns respectively on each plate). The contents of the other wells were as follows:-

1 sheep glutamine synthetase (the injected material)
2 adult rat brain extract
3 sheep glutamine synthetase
4 adult rat brain extract
5 sheep glutamine synthetase
6 7 day old rat brain extract

Gels were 1% agarose in barbital buffer (0.1M, pH 8.6) containing 0.1% Triton X-100. Diffusion was over 18h at room temperature. Gels were stained with Coomassie blue R250.


R1
1
2
6
C
3
5
4
1


R2
1
1

V.D.4.b. Western blotting.

The antisera were analysed by the Western blotting technique (Towbin et.al., 1979) to investigate whether any proteins in rat brain other than glutamine synthetase would cross-react with the anti-GS antisera. Insoluble brain extract (see II.H) was electrophoresed, 100ul per track, under reducing conditions (mercaptoethanol) in an SDS-discontinuous buffer system (Laemmli, 1970, see II.G) on a 10% resolving gel with 3.5% stacking gel. Separated proteins were electrophoretically transferred to nitrocellulose (NC) for 16h at 30V then 1.5h at 60V in tris/glycine/methanol buffer. Excess protein-binding sites were blocked with 10% foetal calf serum in tris-buffered saline (1h at 37^{o}C). Individual lanes were next incubated with dilutions of antiserum (from 1 in 500 to 1 in 16000) in blocking buffer for 1.5h at room temperature. Excess antiserum was removed by washing the NC for 1h with blocking buffer (2 x 15 min) and tris-buffered saline (TBS, 2 x 15 min) then the NC incubated with a 1 in 200 dilution of horseradish peroxidase-conjugated swine anti-rabbit antiserum for 1.5h at room temperature. Excess antibody was removed by washing as before and the NC developed using 3,3'diaminobenzidine tetrahydrochloride with nickel enhancement as described for immunocytochemistry (II.J). The blot was finally washed with PBS then distilled water and dried flat between filter papers. The results of this procedure for the GS I antiserum are shown in Fig.36.

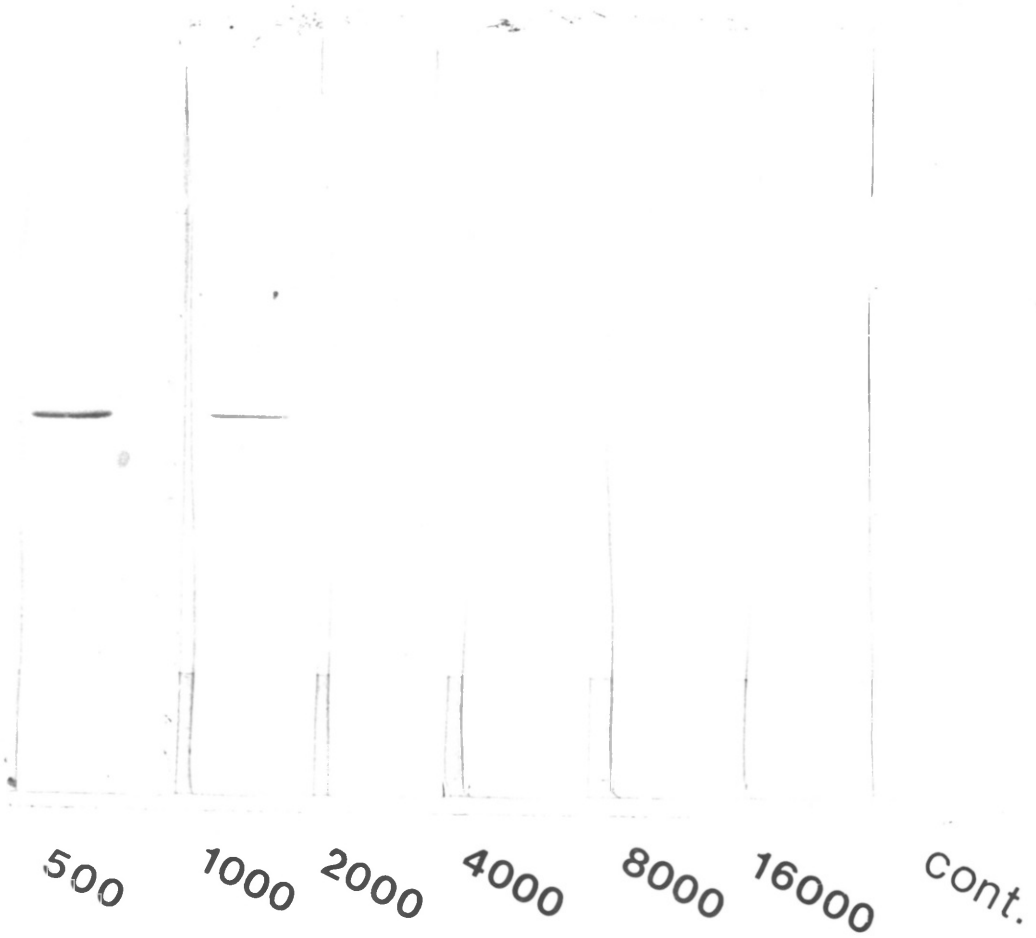
The blot (Fig.36) shows a strongly stained band at M_r=45,000 corresponding to glutamine synthetase (M_r of injected material from sheep brain was approximately 46,000) and a much less intense band of a lower M_r. With dilution of antiserum from 1:500 down to 1:16000 the staining of the weak band was no longer apparent by 1:1000 whilst the

Fig.34 **Western blot analysis of anti-glutamine synthetase antiserum.**

Soluble brain extract was electrophoresed under reducing/ dissociating conditions (10% gel) then electrophoretically transferred to nitrocellulose. The separated proteins were probed with dilutions of anti-glutamine synthetase from 1 in 500 to 1 in 16,000 and any binding localised by the PAP/nickel sulphate method. In control experiments the anti-glutamine synthetase antiserum was omitted from the 1st incubation.

500, 1000,.....16000 = anti-GS dilutions of 1 in 500 - 1 in 16,000.

Cont. = control


500
1000
2000
4000
8000
16000
cont.

GS band was still clearly visible. In view of this the serum was used for immunocytochemistry at a dilution of 1:700.

V.C. Immunocytochemical localisation of glutamine synthetase in cultured astrocytes: induction by hydrocortisone.

Cerebellar astrocytes were grown from 8 day old rats as described previously (II.B). At 4 DIV, filter-sterilised hydrocortisone-21-hemisuccinate (Sigma) in DMEM was added to the culture dish to give a final concentration of 10μM or 20μM. Control dishes were given an equal volume (20 or 40μl) of DMEM. 72h after addition of the hydrocortisone the cells were stained using the glutamine synthetase antiserum by the PAP/nickel sulphate method as described in II.J.2. Controls for the immunocytochemistry were carried out using normal rabbit serum as the first layer antibody (this was used at the same dilution as the immune serum). The results from this staining are shown in Fig.37.

Fig.37c shows that using a non-immune serum the only staining observed was a slight nuclear staining which could not be confused with the staining patterns observed with the immune serum. In the absence of hydrocortisone there was a clear difference in staining between the stellate and nonstellate cells in culture. The non-stellate cells were only just visible whereas some (but not all) of the stellate cells were very intensely labelled with reaction product indicating the presence of GS in the cell bodies and processes. Following a 72h treatment with 10μM hydrocortisone the stellate cells displayed the same intense labelling as before, however the non-stellate cells now

Fig.37 **Glutamine synthetase in cultured astrocytes: induction by hydrocortisone.**

7 DIV cerebellar astrocytes from 8 day old rats were stained with anti-glutamine synthetase antiserum using the PAP/nickel sulphate method for localisation. Some cultures were grown between 4 DIV and 7 DIV in the presence of 10μM hydrocortisone.

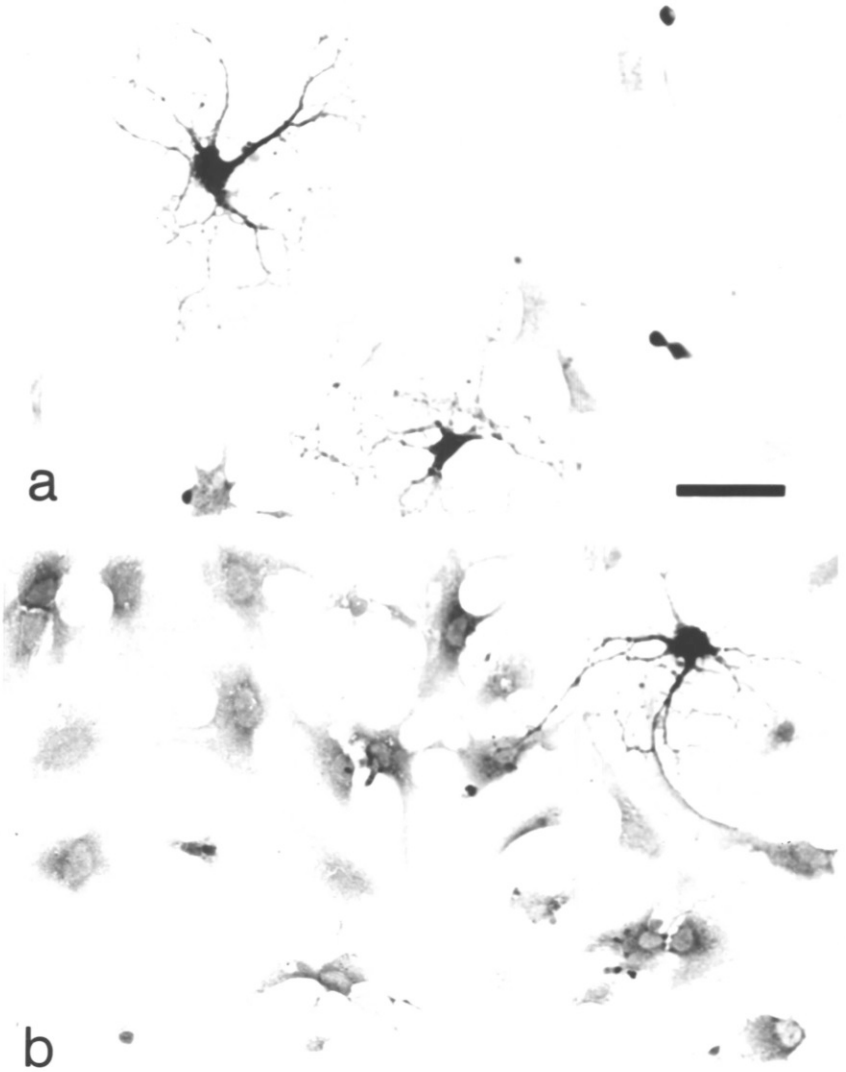
Cells were pre-fixed and permeabilised with paraformaldehyde and methanol. Anti-GS was used at 1 in 700. In controls for the staining procedure, the anti-GS antiserum was replaced with normal rabbit serum.

(a) untreated cells

(b) hydrocortisone-treated cells

(c) control (normal rabbit serum as 1st antibody)

Scale bar = 50μm



c

appeared more heavily labelled than in the absence of hydrocortisone indicating an induction of the enzyme by the hormone. There was no apparent difference between the cultures treated with 10μM hydrocortisone and those treated with 20μM.

From this study it appears that GS is expressed in both the stellate and the epithelioid astrocytes in culture (although at only just-detectable levels in the latter). The induction of the enzyme in the epithelioid astrocytes by hydrocortisone treatment confirms the findings of Juurlink et.al. (1981) and of Hallermayer et.al. (1981). These studies showed induction of GS (detected by either biochemical assay or by immunofluorescence) in response to hydrocortisone or dexamethasone. The present work also showed that, at least in the rat, the hormonal stimulus is sufficient for the induction to occur. This was not found to be the case in embryonic chick retina where the enzyme was shown to be present and inducible in organ cultures, in vivo and in retinotypic cell culture but not in monolayer culture (Linser & Moscona, 1979) implying that a degree of cell-cell interaction may be required in this case.

In terms of cellular differentiation, the heavier staining of the stellate astrocytes suggests that these cells are more differentiated than the epithelioid type since the levels of GS (which is exclusively astrocytic) are low at birth and increase to approximately 80% of the adult level over the first two weeks of life (Ozand & Stevenson, 1979; Bayer & McMurray, 1967). On the other hand it is impossible to tell whether the astrocyte subtypes represent different developmental stages of the same cell or whether they are different types of astrocyte - one (stellate) with constitutive expression of GS, the other (epithelioid) with hormonally-regulated GS. (The inducibility of GS in the stellate cells was not detected in the present study).

V.F. Production of anti-GABA transaminase (GABA-T) antiserum

The production of antiserum against GABA-T was carried out using the same general procedures as described for production of anti-GS antiserum. The antigen used was GABA-T isolated from rabbit brain and was a generous gift from Dr Leslie Fowler, School of Pharmacy, University of London. An SDS electrophoretogram of the injected material is shown in Fig.38.

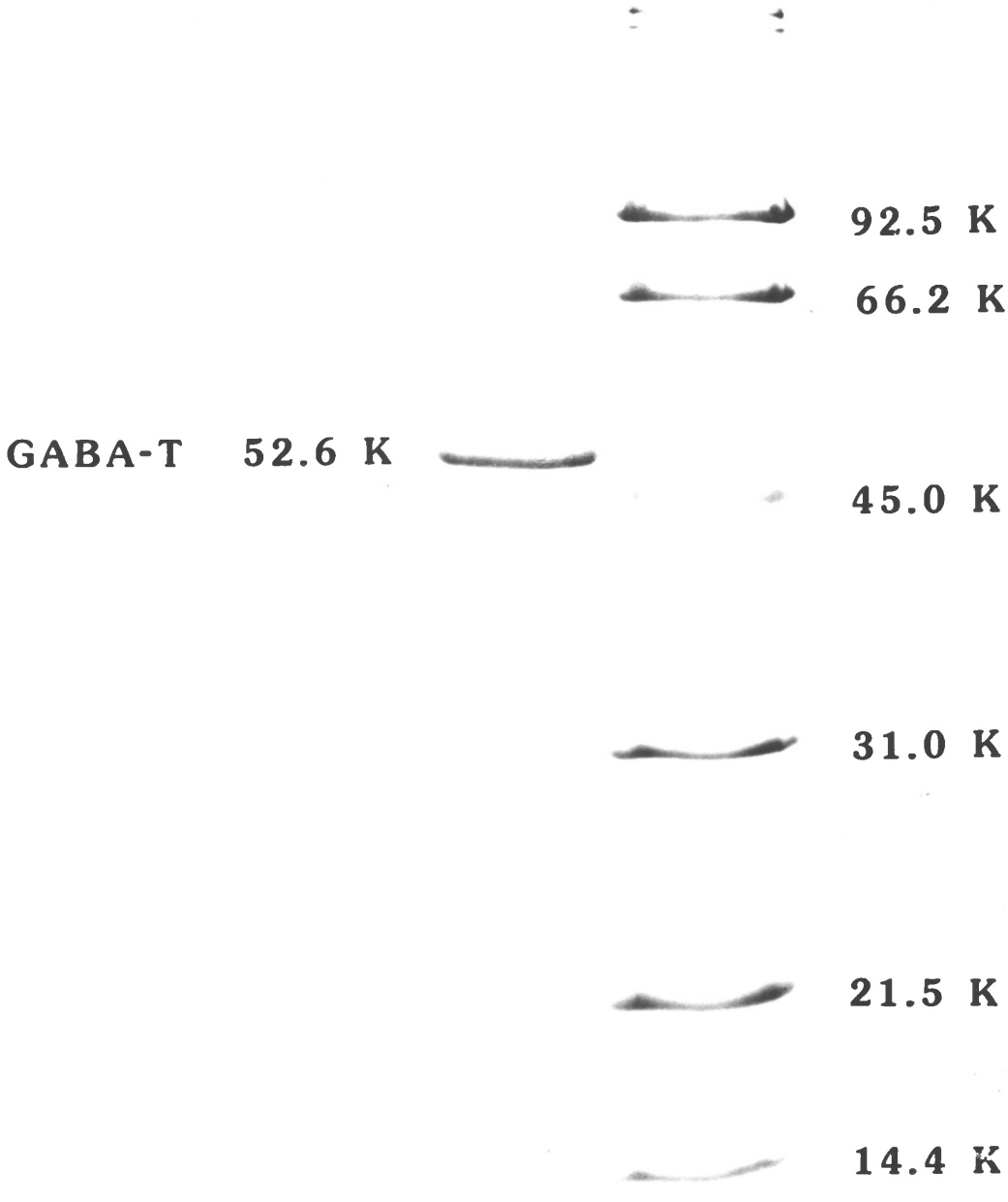
Initially the protein was injected into rabbits using the same schedule as described for glutamine synthetase. This procedure gave no detectable response on Ouchterlony gels. It was thought that this might have been due to the rabbits' being tolerant to the injected protein (since the latter had been obtained from rabbit) so a second attempt was made using guinea pigs.

A 50:50 mixture of Freund's Complete Adjuvant : GABA-T solution containing 0.3 mg of protein was injected subcutaneously into each of four animals at three sites on the back. This was repeated after 4 weeks with 0.42 mg of protein (using Incomplete Adjuvant this time) and after a further five weeks with 0.47 mg of protein in Incomplete Adjuvant. Antisera were analysed by immunodiffusion and Western blotting as before. The Ouchterlony gels (Fig.39) indicated that there had indeed been a response to the injected GABA-T, however the Western blot (Fig.40) showed cross-reactivity with a number of bands in a crude brain extract which could not be removed by dilution of the antiserum. This meant that the antiserum could not be used for immunocytochemistry without further purification by, for example, absorp-

Fig.38 **SDS-polyacrylamide gel electrophoretogram of GABA transaminase used for antiserum production.**

GABA transaminase and molecular weight standards were run under reducing/dissociating conditions in the laboratory of Dr Leslie Fowler, The London School of Pharmacy.

A plot of log(molecular weight) against Rf for the standards gave a curve from which the moleculkar weight of the GABA-T protein was estimated to be 52,600 Daltons.


92.5 K
66.2 K
GABA-T 52.6 K
45.0 K
31.0 K
21.5 K
14.4 K

tion or immunoaffinity purification. This has not yet been undertaken.

Fig.39 **Ouchterlony double immunodiffusion with serum from rabbits immunised with GABA transaminase.**

Sera from two rabbits immunised with GABA transaminase (GABA-T) as described in section V.F were screened for immune response against the injected material and brain extracts by Ouchterlony double immunodiffusion.

Wells are numbered 1-6 from the top in a clockwise direction (as in (a)) and the plates are shown as pairs corresponding to guinea pigs I (a,b), II (c,d,) and III (e,f). The centre wells contained either 1mg/ml GABA-T (a,c and e) or neat antiserum (b,d and f). Numbered wells contained:-

a,c and e	b, d and f
1. neat antiserum	GABA-T, 1 mg/ml
2. 1 in 2 dilution of antiserum	0.5 "
3. 1 in 4 " " "	0.25 "
4. 1 in 8 " " "	0.12 "
5. 1 in 16 " " "	0.06 "
6. 1 in 32 " " "	0.03 "

Gels were 1% agarose in PBS containing 0.1% Triton X-100. Diffusion was over 20h at room temperature. Gels were stained with Coomassie blue R250.

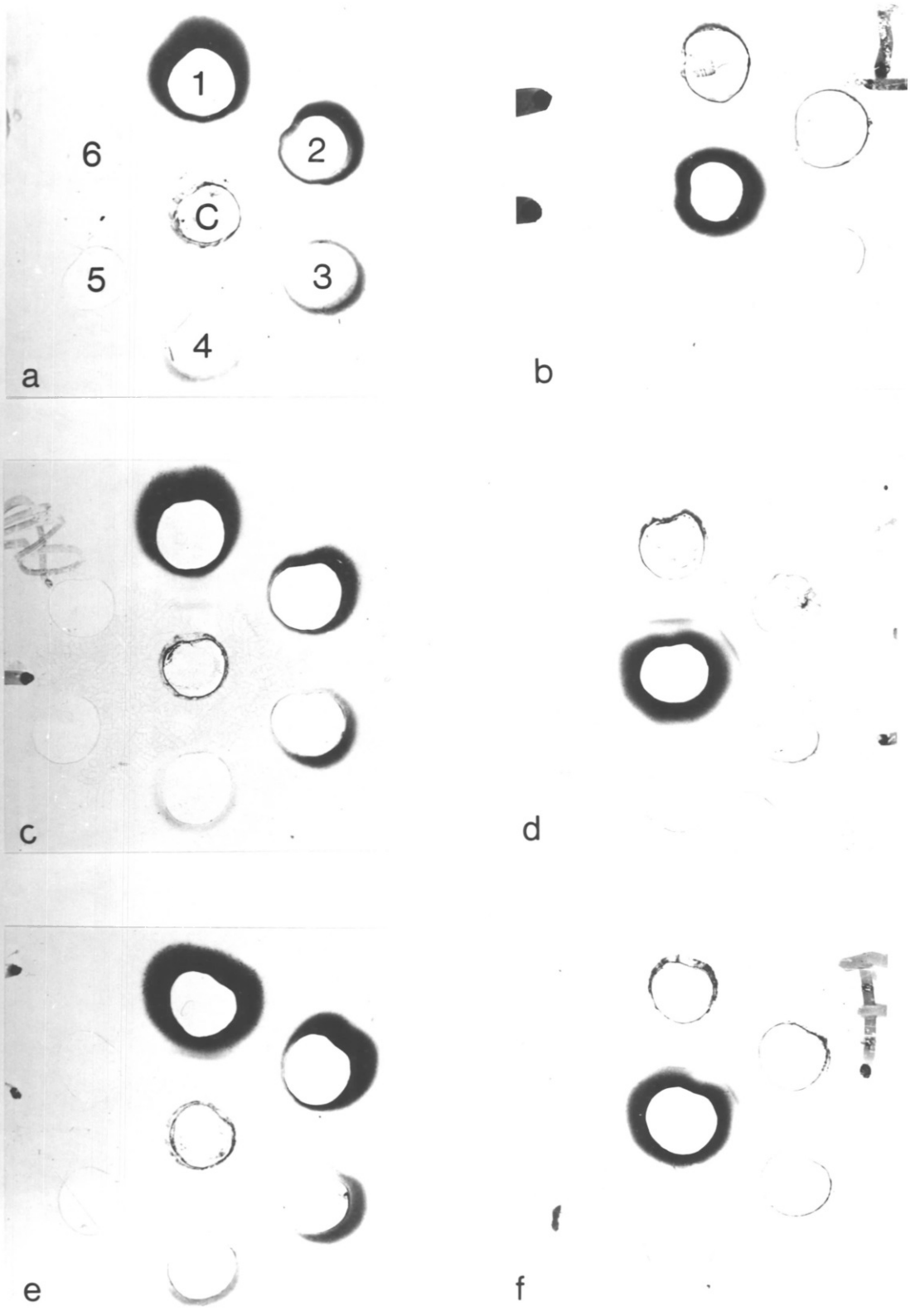
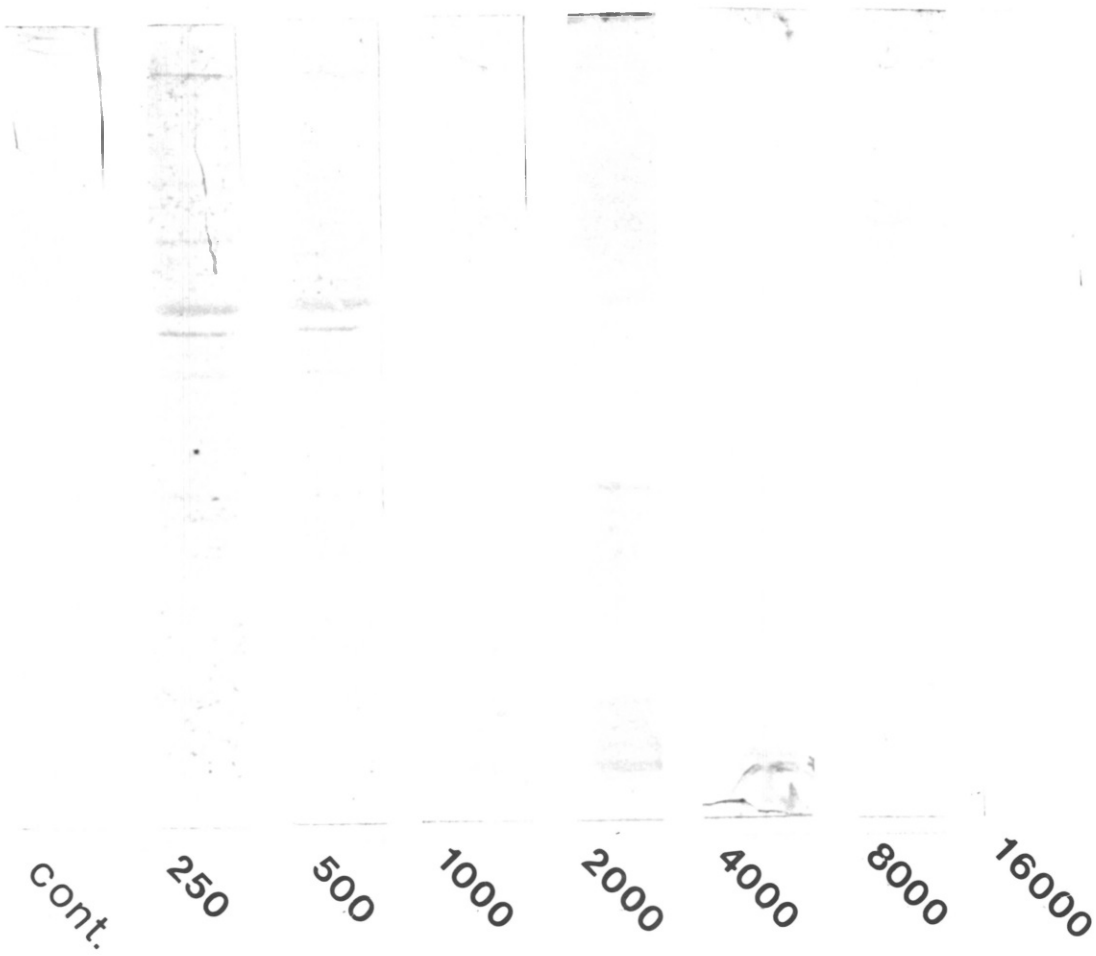

1
2
3
4
5
6
C
a
b
c
d
e
f

Fig.40 Western blot analysis of anti-GABA transaminase antiserum.

Soluble brain extract was electrophoresed under reducing/ dissociating conditions (10% gel) then electrophoretically transferred to nitrocellulose. The separated proteins were probed with dilutions of anti-GABA transaminase from 1 in 250 to 1 in 16,000 and any binding localised by the PAP/nickel sulphate method. In control experiments the anti-GABA transaminase antiserum was omitted from the 1st incubation

250, 500,.....16000 = anti-GS dilutions of 1 in 250 - 1 in 16,000.

Cont. = control


cont.
250
500
1000
2000
4000
8000
16000

VI GENERAL SUMMARY AND DISCUSSION

In primary cultures of non-neuronal cells from the neonatal rat cerebellum, the major cell type present after 3-4 days in vitro (DIV) was the astrocyte, defined by the expression of glial fibrillary acidic protein (GFAP). Astrocytes in such cultures occurred in two broad morphological classes. One can be described as polygonal or elongate or epithelioid in shape, with a large nucleus and was often apparent after 1 DIV but certainly after 2 DIV. The other astrocyte sub-type can be described as process-bearing or stellate, with a smaller nucleus than the epithelioid cells, and became apparent by 2-3 DIV when labelled with anti-GFAP antiserum. As a proportion of the total astrocyte population, the stellate cells on reached a peak at around 5-6 DIV when they constituted approximately 15% of GFAP$^+$ cells (on occasions this figure was as high as 29%). As the cultures aged the number of cells showing the stellate morphology decreased until, by about 12 DIV, almost all cells had no fine processes. The events underlying these changes in culture composition may include cell death, however, several observations by myself and more detailed studies by others in this laboratory suggest that the stellate cells themselves undergo a shape change. It was noticeable that during the period between 5 DIV and 12 DIV, many cells labelled with the antiGFAP antiserum showed morphologies intermediate between those of the stellate and epithelioid astrocytes: either their processes had retracted or their perikarya extended (or both) indicating a transition between one morphology and the other. That this was the case was supported by the patterns of expression of a cell-surface antigen recognised by the monoclonal antibody A2B5 (Eisenbarth et al.,1979). Such labelling studies showed that at 5 DIV A2B5 labelled most (but not all) stellate astrocytes but not the epithelioid ones. As the culture aged the cells expressing A2B5 binding sites progressively showed a non-stellate morphology, one possible explanation for this

being that the stellate, GFAP$^+$, A2B5$^+$ cells had changed shape whilst retaining the two antigens (Johnstone et al.,1985). These observations have been confirmed recently by Wilkin & Cohen (in preparation) using a new monoclonal antibody LB1 (see Chapter 3). The stimulus for the change in shape has not been unequivocally identified, however there is some evidence to suggest that contact with other cells might be involved. It became apparent that when cells were plated at lower densities than usual or when mitotic inhibitors such as cytosine arabinoside were used (as in neuronal cultures) the stellate astrocyte form persisted to a later stage in culture indicating that a morphological change was not a strictly pre-programmed event but required some external signal related to the other cells in culture. This could be either some soluble factor secreted by other cells or cell-cell contact, both of which would be increased by increased cell density. That cell-cell contact might be important was indicated by a cinemicrographic study from this laboratory showing the withdrawal of processes of a stellate cell following contact with a non-stellate cell (Wilkin et al.,1983)

As well as the difference in shapes of the astrocyte subtypes, they varied markedly in their rates of transport of the amino acid neurotransmitter γ-aminobutyric acid (GABA). Autoradiographic studies showed that the stellate cells accumulated this transmitter at a faster rate than did the epithelioid cells but this was not simply a consequence of the different geometries of the two cell types. The autoradiographic data were confirmed by determining the kinetic parameters of the transport at 5 DIV (mixed stellate/epithelioid) and 12 DIV (epithelioid). These studies showed the uptake systems in the two cell types to have similar Vmax values but different Km values, the stellate cells having the lower Km. The Km and Vmax of the

stellate cell transport system compared favourably with those reported for neuronal uptake systems thereby supporting the idea that removal of transmitter from the synaptic area by astrocytes may be important in the inactivation of signalling in GABAergic systems. The substrate-specificities of the GABA transport systems were also found to differ between the two cell types. The uptake of GABA by the stellate cells was inhibited strongly by cis-1,3-aminocyclohexane carboxylic acid (ACHC) but poorly by β-alanine. Conversely, GABA uptake by the epithelioid cells was inhibited more strongly by β-alanine than by ACHC. These specificities were supported by studies on the uptake of radiolabelled versions of the inhibitors: whereas ACHC was transported well by the stellate astrocytes but not the epithelioid cells, β-alanine was taken up to a greater extent by the epithelioid cells than the stellate type. These observations indicated that the epithelioid cell uptake was similar to the "classical" astrocytic GABA uptake but that the transport system exhibited by the stellate cells had more similarities with that usually described as neuronal. The differences in GABA transport rates (studied autoradiographically) observed in the cultured astrocytes were not seen with D-aspartate (an analogue of the neurotransmitter glutamate) which was accumulated rapidly by both astrocyte types, or with glycine or noradrenaline which were taken up very slowly, if at all.

The two astrocyte morphologies and the associated GABA transport properties were not confined to cultures from the cerebellum; cultures from optic nerve and spinal cord also contained both process-bearing and elongate or epithelioid astrocytes. These additionally showed similar GABA transport proerties to the cerebellar cells in that the stellate cells from both regions were capable of accumulating

GABA much more rapidly than the non-stellate cells (autoradiographic study). Furthermore, the patterns of inhibition of GABA uptake by GABA analogues mimicked those observed in cerebellar cultures.

The most obvious roles for GABA uptake by CNS cells is the inactivation of GABA released during neurotransmission. That stellate astrocytes might fulfil this role was supported by the rapid uptake of GABA by these cells in cultures derived from cerebellum (where GABA is a prominent transmitter). The evidence presented here from optic nerve cultures suggests, however, that this may not be the sole purpose for astrocytic GABA transport since GABA is not known to be a transmitter in that region. Similarly, the apparent lack of a rapid transport system for glycine in cultures derived from spinal cord (where glycine is a major transmitter) indicates that in this region also there is a poor correlation between the predominant transmitter and the astrocytic transport systems. It is possible, therefore, that under some circumstances (including those in culture) a proportion of astrocytes do in fact use GABA as an energy source. It may be that although only approximately 8% of the flux through the tricarboxylic acid (TCA) cycle is provided by GABA, this represents a large amount of the energy metabolism in a population of astrocytes and only a small fraction from other cells. It would be interesting, therefore, to compare the fluxes (originating in GABA) through the TCA cycle in the two astrocyte types.

An alternative, as suggested previously, is that the stellate astrocytes are a "developmental stage". This may allow some flexibility in properties so that a mismatch between astrocytic uptake systems and the prevalent transmitter in any region may not be of great importance. Some light may be thrown on this by investigating the correl-

ations between the major regional transmitters and the uptake by astrocytes from that region derived from older or, if possible, adult animals

Concerning the topic of differentiation, the hypothesis was that, on the basis of morphology and on GABA transport, the stellate cells represented differentiated astrocytes whereas the epithelioid cells were undifferentiated or dedifferentiated astrocytes. If this were the case, however, the transformation from the un- or dedifferentiated state to the differentiated state was not simple to effect in that two well-known differentiating agents -glial maturation factor and dibutyryl cAMP were unable to bring about this change (they did have an effect on the morphology of the epithelioid cells but the GABA uptake was apparently unaltered, at least in the short term). Two other properties of these cells supported the idea of the stellate cells' being more differentiated than the epithelioid cells. These were the apparently higher level of expression of glutamine synthetase and the lower level of expression of vimentin in the stellate cells compared with the epithelioid. In the latter, however, glutamine synthetase could be induced by hydrocortisone - a documented property of astrocytes. Also vimentin may be expressed *in vitro* by a wider variety of cells than *in vivo* so that the results from both the glutamine synthetase and vimentin studies do not allow the unequivocal assignment of relative differentiation states to the stellate and non-stellate astrocytes. One criterion suggesting that the stellate cells are not highly differentiated astrocytes is the presence in them of a neuron-like specificity of the GABA transport: this has not been observed in mature astrocytes *in vivo* or *in situ*.

It appears therefore that the stellate cells exhibit an unusual combination of glial properties (GFAP, glutamine synthetase, vimentin) and

neuronal properties (ACHC-inhibitable/β-alanine-insensitive GABA transport, possibly tetanus-toxin binding (Johnstone et al.,1985), binding sites for the monoclonal antibody A2B5 (Johnstone et al.,1985) and L81 (Wilkin & Cohen, in preparation). The doubt as to the astrocytic or neuronal nature of the stellate astrocytes has been partially answered by Raff et al.(1983a), but it would be interesting and useful to investigate other potential distinguishing characteristics (e.g S100 protein, isozymes, electrophysiological properties). Furthermore, once these studies have been carried out on cells *in vitro*, similar investigations must be done *in situ* to identify if, where and when these cells occur in the animal.

Much work towards identifying the two astrocyte types *in situ* has been reported by Raff and colleagues (Raff et al.,1983a,b; Miller & Raff, 1984; Raff et al.,1984; Temple & Raff,1985) using antibodies to determine antigenic phenotypes of cells in culture and in the brain. They first reported the presence in culture of stellate and non-stellate astrocytes and designated them type 2 and type 1 respectively (Raff et al.,1983a). These cells were derived primarily from optic nerve but were also seen in cultures from cerebellum and corpus callosum. In these cultures, type 2 astrocytes expressed GFAP and had binding sites for tetanus toxin and the monoclonal antibody A2B5 ($GFAP^+$,$tetanus^+$, $A2B5^+$) whereas the type 1 astrocytes were $GFAP^+$,$tetanus^-$,$A2B5^-$. The type 2 astrocytes were quickly overgrown in culture by the type 1 cells but, when cell division was arrested, the type 2 antigenic phenotype persisted although their morphology had changed to one resembling a type 1 astrocyte.

These properties reported by Raff et al. are the same as those shown by the astrocytes studied in the present work (with perhaps some doubt

over the tetanus-positivity) therefore it seems safe to assume that the stellate or type-S cerebellar astrocytes are equivalent to type 2 optic nerve astrocytes and similarly with epithelioid or type-E and type 1. The observation by Raff et al.(1983a) that a high proportion of astrocytes cultured from white matter displayed a type 2 phenotype, whereas astrocytes cultured from grey matter were predominantly type 1, led them to suggest that type 1 might represent protoplasmic astrocytes and type 2 fibrous astrocytes. They recognised, however, that this simple scheme could not explain all their observations since optic nerve cultures and freshly-isolated optic nerve suspensions contained many type 1 cells. Interestingly, the type 1 phenotype was apparently also shared by reactive astrocytes following corpus callosum lesions (which is consistent with the observations in the present study on nuclear sizes and vimentin expression) and suggests a potential origin for the reactive cells.

One of the most interesting points in this paper (Raff et al. 1983a), and which was developed in subsequent publications (Raff et al.,1983b) was that the type 2 astrocytes developed from $GFAP^-$,$A2B5^+$ precursor cells which could also, under different conditions, give rise to oligodendrocytes ($GFAP^-$,galactocerebroside$^+$,$A2B5^{\pm}$). It was further shown that there was some plasticity in the choice of differentiation pathway for these cells in that the pathway could be switched at any time during the first 2 days of culture (Raff et al.,1984, Temple & Raff,1985). A similar study on cerebellar cultures suggests that type 2 astrocytes are formed from $GFAP^-$,$LB1^+$ precursors (Wilkin, personal communication). It has also been observed that the neuron-like GABA transport system of the type 2 astrocytes is apparent before the expression of GFAP in these cells, so it would be interesting to investigate the GABA transport characteristics of precursor cells which had

progressed along the oligodendrocyte pathway.

With respect to whether the cultured cells truly represent cells *in vivo*, Miller et al.,(1984) investigated antigenic phenotypes *in situ* and found that in *adult* mouse optic nerve both type 1 and type 2 phenotypes were present. Moreover, the type 2 astrocytes *in situ* appeared to follow a similar developmental pathway as their *in vitro* counterparts ($GFAP^-, A2B5^+$ to $GFAP^+, A2B5^+$) although the time-scales *in vitro* and *in situ* may be different.

It seems certain, therefore, that type 1 and type 2 astrocytes *in vitro* are representative of cell types found *in situ* and that they are not simply different differentiation states of the one cell but two co-existing astrocyte types, at least in optic nerve. Studies from this laboratory (including the present work) indicate that type 1 and type 2 astrocytes from other regions (e.g.spinal cord, cerebellum) have similar properties to their optic nerve counterparts, and therefore it seems likely that all brain regions will have these two astrocyte types, perhaps in variable proportions. It will now be important to establish some of the functional properties of type 1 and type 2 astrocytes in order that some idea of their contributions to CNS function can be assessed. Such studies will also be required before an assessment can be made regarding the extent to which the two astrocyte sub-types *in vitro* are a good model for their *in vitro* counterparts. The neurotransmitter transport studies (Johnstone et al., 1985; Levi et al.,1983; Wilkin et al.,1983 and the present study) have made a start in this direction; other clues may be gained from studies of the distributions of these two astrocyte types in both time (development) and space (different regions) and perhaps from comparisons between different animals. Additionally, many of the functions already proposed for astrocytes (section I.C.3) should now be

thoroughly investigated from a different viewpoint - that of being aware of the potentially different behaviours of different astrocyte types rather than expecting uniform responses.

As a final note I would like to return to a question and answer proposed by Raff et al. (1983a). They raised the problem of the identification of cells, pointing out that type 2 astrocytes shared as many properties with neurons as with type 1 astrocytes but gave various reasons for accepting the non-neuronal nature of the type 2 cells. It remains true, however, on the basis of the information available that there are rather few points of similarity between type 1 and type 2 astrocytes. They differ in morphology, surface antigenic properties, neurotransmitter transport, growth characteristics and even in their immediate precursor cells. In fact the single major point of similarity is the expression of GFAP and it is perhaps questionable whether these two rather different cells should be given the same name. There is a requirement, therefore, for yet more markers that can identify particular cell types <u>in vitro</u> and <u>in vivo</u> and which can be used to classify the various nervous system cells into highly-defined groupings; the use of cell-surface-directed monoclonal antibodies still appears to provide a very useful tool in such studies. These antibodies, either singly or in combinations, can be used to trace the development and distribution of perhaps very small groups of cells and they may also be used as a means of enriching or depleting nervous system cultures of precisely-defined cells with a view to understanding the interactions between different cell types in terms of both the mechanisms and the consequences of these interactions.

To conclude, the information obtained in the present study both in isolation but also, importantly, when viewed in the light of recent

publications, indicates that the initial hypothesis - that the stellate cells represented differentiated astrocytes and the epithelioid cells a less-differentiated form - should not be accepted. Both cell types displayed some astrocytic characteristics, however they equally displayed properties which might be considered unexpected for a mature astrocyte. For example the stellate cells expressed higher levels of glutamine synthetase than the epithelioid type and apparently lesser amounts of vimentin, both of which would indicate a more differentiated astrocyte. On the other hand although both cell-types had the ability to transport GABA, it was the epithelioid rather than stellate form which showed glial-like substrate-specificity of this transport system. The initial studies on antigenic phenotype and development also strongly indicate that these are two distinct cell types. It is now time to investigate what the functions of these two astrocyte types may be, whether they act in isolation from each other or whether there is an over-all role for astrocytes which are shared by these two types.

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PUBLICATIONS

Some of the work presented in this thesis has already appeared in the following publications:-

S.R.Johnstone, G.Levi, G.P.Wilkin, A.Schneider, M.T.Ciotti; Dev.Brain Res. in press.

G.Levi, G.P.Wilkin, M.T.Ciotti, S.R.Johnstone; Dev.Brain Res. 10 227-241 (1983). Enrichment of differentiated, stellate astrocytes in cerebellar interneuron cultures as studied by GFAP immunofluorescence and autoradiographic uptake patterns with ^{3}H-D-aspartate and ^{3}H-GABA.

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APPENDIX

Sources of chemicals:-

ACHC	Dr Norman Bowery, St Thomas' Hospital
Citifluor mounting medium	City University, London
DMEM	GIBCO
D19 developer	Kodak
DPX mounting medium	Raymond A.Lamb
diaminobenzidine $(HCl)_4$	Aldrich Chemicals
eosin	Raymond A Lamb
foetal calf serum	Flow Laboratories
gentamicin	Flow Laboratories
glutaraldehyde	Agar Aids
Ham's F12 medium	Flow Laboratories
haematoxylin	Raymond A Lamb
molecular weight markers	Pharmacia
nipecotic acid	Dr Norman Bowery, St Thomas' Hospital
cis-4 hydroxy nipecotic acid	Dr P. Krogsgaard-Larsen
nitrocellulose sheets	BioRad
osmium tetroxide	Agar Aids
Spurr's resin kit	Agar Aids
toluidine blue	Raymond A Lamb
tetanus toxin	Dr R.O.Thompson

Antibodies

All fluorescein-, rhodamine- and peroxidase-conjugated antibodies were obtained from Wellcome or Dako.
Anti-GFAP and Chagas serum were from Graham Wilkin (Imperial College) LB1 monoclonal supernatant was from a clone donated by Dr J Cohen,Unversity College,London. Anti-galactocerebroside was from Dr B Ranscht,

Other reagents were of analytical grade from Sigma, Fisons or BDH. Solvents were from Fisons or Burroughs.