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GSH: A NEW CANDIDATE NEUROPEPTIDE IN THE CNS

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## ABSTRACT

The physiological significance of glutathione (GSH) in the mammalian central nervous system is still uncertain, although some evidence has indicated that GSH may play an important role in the CNS.

To address the question of whether GSH may be a candidate for a neuropeptide in the CNS, one step is to establish that GSH receptors are present.

In the present study, biotinyl-GSH was synthesized and purified to detect the GSH receptor in the CNS. Histochemical experiments showed that GSH binding sites appeared on the white matter (such as cingulum, dorsal hippocampal commissure, cerebral peduncle, fasciculus retroflexus, mammillothalamic tract etc.) of the rat brain. It thus suggested that the GSH receptors might be on astrocytes or oligodendrocytes. Radioactive receptor assays were performed on cultured astrocytocytes using [ $^{35}\text{S}$ ]GSH. Scatchard analysis revealed two binding sites of  $K_{d1} = 4.67 \pm 0.75$  nM,  $B_{\text{max}2} = 70 \pm 9.2$  fmoles /  $6.4 \times 10^5$  cells (or  $B_{\text{max}1} = 6.6 \times 10^4$  molecules / cell),  $K_{d2} = 35.14 \pm 2.1$  nM,  $B_{\text{max}2} = 260 \pm 12.77$  fmole /  $6.4 \times 10^5$  cell (or  $B_{\text{max}2} = 2.4 \times 10^5$  molecules / cell). The association and dissociation kinetics studies gave a  $K_{+1}$  of  $0.003 \text{ nM}^{-1} \text{ min}^{-1}$ , and a  $K_{-1}$  of  $0.0168 \text{ min}^{-1}$  for site I. These rate constants gave a  $K_{d1}$  of 5.6 nM, consistent with that from Scatchard analysis. Colloidal gold technique and immunofluorescence double staining also showed the GSH binding sites on cultured astrocytes, and suggested that the binding sites might be GSH receptors.

The present study is the first to report the presence of GSH receptors on astrocytes. Based on receptor binding assays and cytochemical experiments, this study not only depicts the biochemical characteristics of GSH receptors in the brain, but also shows the receptor at the cellular level. These results support the view that GSH might be a neuroactively signal substance in the CNS.

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## INTRODUCTION

Glutathione (GSH, reduced form), an acidic tripeptide which has been studied for over a hundred years, is generally considered to be a powerful oxidant playing an important role in peripheral detoxification defense mechanisms (Chance et al, 1979; Mannervik et al, 1989; Ishikawa & Sies, 1989; Sies, 1988; Hinson & Kadlubar, 1988; Chasseaud, 1988). Glutathione is the major low-molecular weight soluble thiol present in mammalian cells. Its function as an antioxidant is linked to the sulfhydryl group. Biologically more important, many GSH-linked reactions are enzyme-catalyzed. The relationship between GSH and some enzymes is illustrated in Fig. 1. GSH, therefore, can be considered as a coenzyme or as an enzyme cofactor.

One important role of GSH in the detoxification of reactive and toxic chemical compounds formed in the metabolism of oxygen is associated with GSH peroxidase, which reduces  $H_2O_2$  and organic hydroperoxides with concomitant formation of glutathione disulfide, GSSG (Mannervik et al, 1988; Ishikawa & Sies, 1989; Chance et al 1979) (Fig. 1). Endogenous oxidative stress is a consequence of aerobic metabolism which in eucaryotes occurs mostly in the mitochondria. About 2% of mitochondrial  $O_2$  consumption generates  $H_2O_2$ .  $H_2O_2$ , if not reduced, can lead to the formation of the very reactive hydroxyl radical and will result in the formation of lipid hydroperoxides that can damage mitochondrial membranes and proteins and their functions. It has been found that mitochondria rely solely on GSH peroxidase to detoxify hydroperoxides (Chance et al, 1979).

To maintain flux through the GSH peroxidase reaction, GSSG must be continuously reduced back to GSH by glutathione reductase at the expense of NADPH. The redox state of cellular glutathione is considered to affect major cellular functions including protein synthesis (Kosower & Kosower, 1978). Glutathione reductase, which catalyzes the ancillary reaction of the glutathione peroxidase system, plays a key role in maintaining GSH essentially completely in its reduced form (Mannervik et al, 1988; Ishikawa & Sies, 1989).

The second major reactivity of GSH involved in detoxification function is the reaction with GSH S-transferases (Sies, 1988; Hinson & Kadlubar, 1988; Chasseaud, 1988). Glutathione S-transferases catalyze the reaction of potentially harmful lipophilic electrophiles with GSH to give a thioether, also



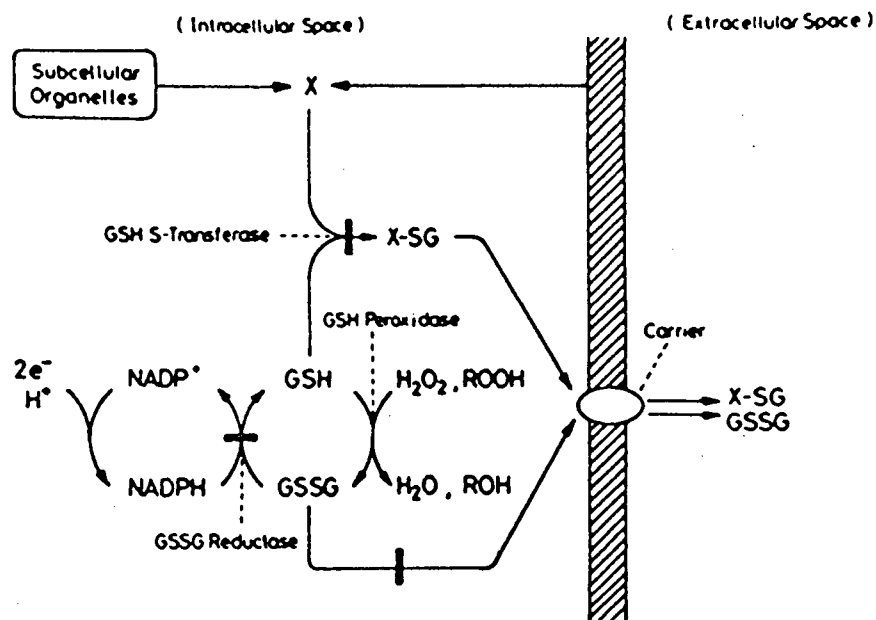


Fig. 1. Illustration of reactions of glutathione S-conjugates in cells. X, electrophile as substrate for glutathione S-transferase; X-SG, glutathione S-conjugates; ROOH, organic hydroperoxides; enzyme reactions or transfer steps inhibited by glutathione S-conjugates are indicated by solid bars. (from H. Sies, 1988).

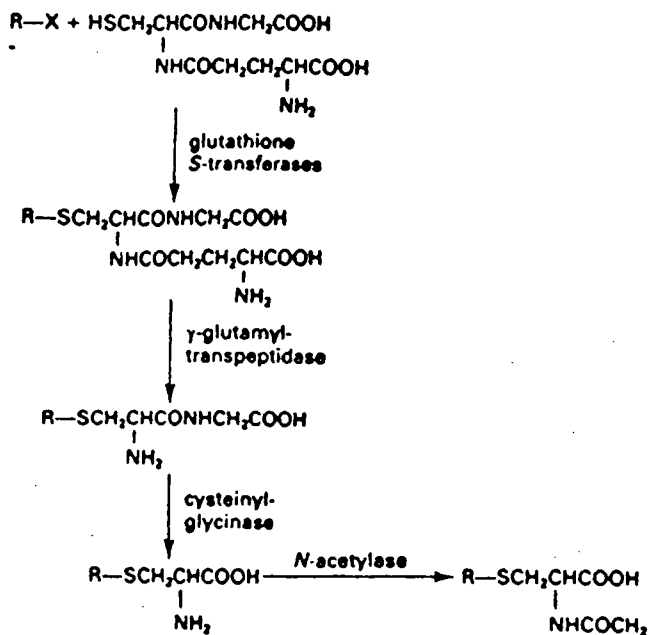


Fig. 2. Mercapturic acid formation through conjugation with GSH (from L.F. Chasseaud, 1988).

called S-conjugate, that is less toxic and more water soluble than the original compound (Fig.1). Glutathione conjugates are thought to be metabolized further by cleavage of the glutamate and glycine residues, followed by acetylation of the resultant free amino group of the cysteinyl residue, to produce the final product, a mercapturic acid ( Boyland & Chasseaud, 1969; Wood, 1970; Chasseaud, 1988) which is then excreted (Fig. 2).

A new development quite unrelated to detoxification, emerged in 1979 when GSH conjugation was shown to participate in the biosynthesis of leukotrienes (Hammarstrom et al, 1979). Leukotrienes are biologically highly active mediators of profound physiological importance (Huber & Kippler, 1988). Enzymatic conjugation of leukotriene A<sub>4</sub> with GSH generates leukotriene C<sub>4</sub> (Hammarstrom et al 1989), which plays a neuroendocrine role in luteinizing hormone secretion (Lindgren et al, 1984; Gerozissis et al, 1987; Samuelsson et al, 1987). Conversion of synthetic leukotriene A<sub>4</sub> into leukotriene C<sub>4</sub> has been shown in human polymorphonuclear leukocytes (Radmark et al, 1980) and platelets (Pace-Asciak et al, 1986), in mouse mastocytoma cells (Dahinden et al, 1985; Soderstrom et al, 1988), and in homogenates of liver and lung from different species (Pace-Asciak et al, 1985; Wu, 1986).

In the eye, a high concentration of GSH has been found in the lens, and the lens epithelium contains a concentration 5 times that of the cortex (Rathbun, 1989). One of the functions ocular GSH appears to be involved in is maintaining the Na<sup>+</sup>-, K<sup>+</sup>-ATPase activity at a normal level in situ ( Rathbun, 1989), and Na<sup>+</sup>-, K<sup>+</sup>-ATPase is quite critical to lens clarity. It has been suggested that GSH either is a cofactor of ionic transport or is directly involved in essential maintenance of Na<sup>+</sup>-, K<sup>+</sup>-ATPase activity.

There is also evidence for another role of GSH in the maintenance of intracellular calcium ion homeostasis (Orrenius et al, 1983). Mammalian cells maintain the ionic calcium concentration in the cytosol ( $\sim 10^{-7}$  M) far below that in the extracellular medium ( $\sim 10^{-3}$  M). To maintain the cytosolic Ca<sup>2+</sup> concentration, the mitochondria and the endoplasmic reticulum represent the predominant sites of Ca<sup>2+</sup> sequestration (Claret-Berthon et al, 1977). The membrane Ca<sup>2+</sup> pump plays a major role in the maintenance of the gradient existing between the extra- and intracellular environments by actively extruding Ca<sup>2+</sup> from the cell (Schatzmann, 1982). GSH was found to protect against the inhibition of Ca<sup>2+</sup> sequestration and exert a stimulatory effect on Ca<sup>2+</sup> transport

in the plasma membrane (Orrenius, 1989). Evidence for an involvement of GSH in the control of  $\text{Ca}^{2+}$  transport through the ATP-dependent translocases in the plasma membrane and endoplasmic reticulum is that the activity of  $\text{Ca}^{2+}$ -ATPase could be restored by treatment of cells with GSH (Orrenius, 1989). However, GSH is also thought to affect non ATP-dependent  $\text{Ca}^{2+}$ -transport control systems: (1) the GSH redox status may control the influx of  $\text{Ca}^{2+}$  through a voltage-dependent plasma membrane channel (Ammon & Mark, 1985), (2) the  $\text{Na}^+/\text{Ca}^{2+}$  exchange system may also be under redox control (Reeves et al, 1986).

Obviously, the concept of GSH function is no longer limited to detoxification of peripheral systems. Actually, research on glutathione has been markedly stimulated by the discovery of the involvement of this tripeptide in a number of important cell functions in addition to its well-characterized role in detoxification reactions. These functions include maintenance of membrane integrity and cytoskeletal organization, regulation of protein conformation and enzymatic activity (Larsson et al, 1983), and augmentation of glucose-induced release of insulin (Meister & Anderson, 1983).

Although the physiological function of GSH in the central nervous system is still uncertain, the presence of large amounts of GSH in the rodent brain (Martin & McIlwain, 1959; Reichelt & Fonnum, 1969) implies that GSH might also play an important role in the CNS. In whole brain, the concentration of total GSH (consisting of both reduced and oxidized forms) is 0.5-3.4 mmol/gm tissue (Orlowski & Karkowsky, 1976), and GSH occurs primarily (97%) in its reduced form. GSH is synthesized rapidly in the brain. Intracisternal administration of [ $^{14}\text{C}$ ]glutamate to cats rapidly labels glutathione in the brainstem, cerebellum, mesodiencephalon, and hippocampus (Berl & Purpura, 1966). In addition to these in vivo studies, in vitro experiments have shown incorporation of [2- $^{14}\text{C}$ ]glycine and DL-[1- $^{14}\text{C}$ ]glutamic acid into glutathione (Takahashi & Akabane, 1961). The first-order rate constant for GSH synthesis in the brain has been calculated as  $0.17 \times 10^3 \text{ min}^{-1}$ . This value is higher than that calculated for human erythrocytes ( $0.12 \times 10^3 \text{ min}^{-1}$ ) and rabbit muscle ( $0.11 \times 10^3 \text{ min}^{-1}$ ), but much lower than that calculated for liver ( $2.9 - 5.8 \times 10^3 \text{ min}^{-1}$ ) and kidney ( $2.4 \times 10^3 \text{ min}^{-1}$ ). These calculations assume a homogeneous pool of both precursors and products (Orlowski & Karkowsky, 1976).

The level of GSH in the brain changes during development. In neonatal cat neocortex, the GSH concentration is about 80% of adult levels. The level markedly increases during the postnatal period and reaches adult levels by the end of the second week. The time course of this increase parallels the morphological development of neocortical elements in the cat (Berl & Purpura, 1963). GSH levels also increase in the hippocampus, brainstem, cerebellum, and mesodiencephalon during the developmental period (Berl & Purpura, 1966).

Recently, Ogita et al (1986) found that GSH exerts a profound suppressive action on the  $\text{Na}^+$ -dependent and -independent binding of glutamic acid. Later, Ogita and Yoneda (1987) reported the possible presence of GSH binding sites in synaptic membranes from rat brain. These results, especially the GSH pattern during development and the binding activity of GSH in the brain suggested that GSH might be a neurally active molecule in the CNS.

To address the question of whether GSH may be a new candidate for a neuropeptide in the CNS, we attempted to determine if there are GSH receptors in the brain.

## EXPERIMENTS AND RESULTS

### 1. The Biotinylated Ligand As A Probe For The Related Receptor

The high affinity ( $K_d = 10^{-15}$  M) of biotin for egg white avidin and streptavidin (Green, 1963; Guesdon et al, 1979) provides an important experimental strategy for the qualitative and quantitative studies of membrane receptors (Newman et al, 1989). In contrast, radio-labeled ligands are usually unsatisfactory in showing cellular resolution in brain sections. For this reason, we synthesized biotinyl-GSH as a probe in the present studies in order to obtain a clearer cellular resolution.

### Materials And Methods

**Materials** GSH and DAB (diaminobenzidine-4HCl) were from Sigma Chemical Comp. (St. Louis, MO), biotinyl- $\epsilon$ -aminocaproic acid-N-hydroxysuccinimide and ABC kit were from Vector Lab. Inc. (Burlingame, CA), and Sephadex G-10 was from Pharmacia LKB Biotechnology (Uppsala, Sweden).

**Synthesis and purification of biotinyl-GSH** 1mg GSH was freshly dissolved in 1 ml 0.15 M borate buffer, pH 8.5. Biotinyl- $\epsilon$ -aminocaproic acid-N-hydroxysuccinimide ester, which was used to introduce the biotin moiety into GSH (Newman et al, 1989), was dissolved in DMF (dimethylformamide). 40  $\mu$ l of biotin reagent (the final molecular ratio of GSH to biotin was 1:4) was quickly added to 1 ml GSH solution. The condensation reaction was allowed to proceed with constant stirring for 1 hr at room temperature. Separation of the reaction products was performed on a Sephadex G-10 column (1.5 x 25 cm) with a Pharmacia UV-1 protein detector at 254 nm. After the reaction mixture was applied to the column, the purification of the reaction products was achieved by isocratic elution with 0.2 N acetic acid at a flow rate of 0.5 ml/min. Fractions were collected with a LKB automatic collector, and then lyophilized. The further purification of the lyophilized sample from the Sephadex-G10 column was performed on a C-18 reversed phase HPLC column with a Waters System 820 with UV detection at 254nm. The elution was completed with a

0-50% or 0-75% gradient of acetonitrile in 0.1% trifluoroacetic acid. Fractions were manually collected and then lyophilized.

Histochemistry Adult male Sprague-Dawley rats were killed by decapitation and the whole brain was removed rapidly. Tissue slices 20-30  $\mu\text{m}$  thick were obtained with a Reichert Histo Cryostat microtome and stored at  $-20^{\circ}\text{C}$  overnight. The ABC (avidin-biotin-complex) method (Hsu et al, 1981) was used for the histochemical localization of GSH binding sites in the brain. Brain sections were fixed with acetone for 45-60 min. After rehydrating through descending ethanol (100% - 30%), the sections were washed with  $\text{H}_2\text{O}$  for 5 min x 3, and incubated with 1.2 mM biotinyl-GSH at  $4^{\circ}\text{C}$  for 1 hr. 0.7%  $\text{H}_2\text{O}_2$  was used to block endogenous peroxidase at room temperature for 15 min and then 1.4%  $\text{H}_2\text{O}_2$  for 3 min. Slides were well washed in  $\text{H}_2\text{O}$  for 15 min x 2, and then incubated with ABC reagent for 60 min at  $4^{\circ}\text{C}$ . After washing with  $\text{H}_2\text{O}$  for 5 min x 2, the sections were stained with 0.08-0.1% DAB in 0.02%  $\text{H}_2\text{O}_2$  (freshly prepared) at room temperature for 15-25 min. Brain sections were washed with  $\text{H}_2\text{O}$  for 5 min x 3, and dehydrated through a graded series of ethanol. After changing two times in xylene, the slides were mounted in DPX for microphotography. Control experiments were performed the same way as described above except for the addition of 100 times unlabeled GSH.

## Results And Discussion

Biotinyl-GSH Two peaks were obtained from Sephadex G-10 separation (Fig. 3). Histochemical experiments showed that only sections incubated with peak II gave a positive color reaction, i.e., peak II eluate contained biotin labeled GSH, while peak I was the unlabeled GSH. Due to the small molecular weights of both GSH and biotin, the separation of Biotinyl-GSH (peak II) from unlabeled GSH (peak I) and free biotin by Sephadex G-10 was not satisfactory (Fig. 3). Chromatography on a C-18 reversed-phase HPLC column, resulted in purified biotinyl-GSH. Fig 4 shows the purification of biotinyl-GSH by reversed-phase HPLC eluted with a 0 - 50% gradient of acetonitrile in 0.1% trifluoroacetic acid. The same result was obtained when eluted with a 0 - 75% gradient of acetonitrile in 0.1% trifluoroacetic acid (data not shown).

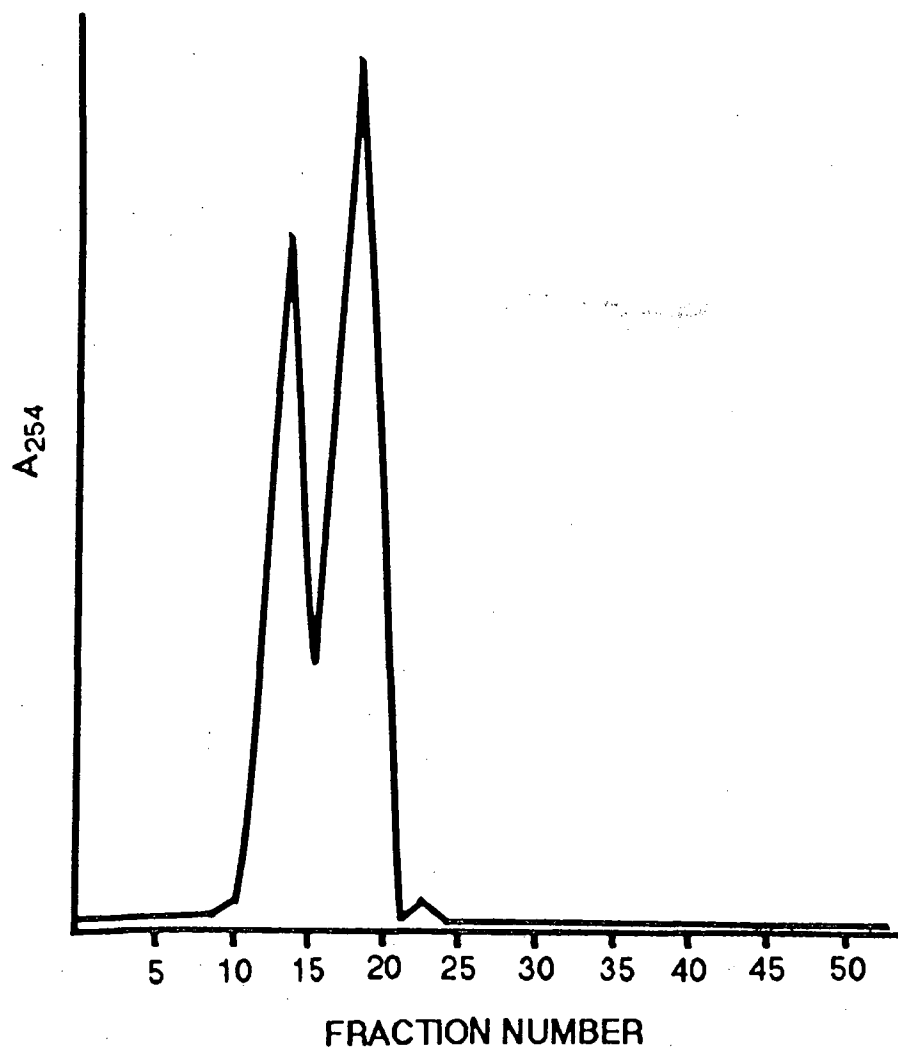


Fig. 3. The elution profile at 254 nm for the crude biotinylated reaction mixture on a Sephadex G-10 column. GSH was reacted with biotinyl-  $\epsilon$ -aminocapric acid-N-hydroxysuccinimide ester for 1 hr. Reaction products were eluted with 0.2 N acetic acid at a flow rate of 0.5 ml / min.

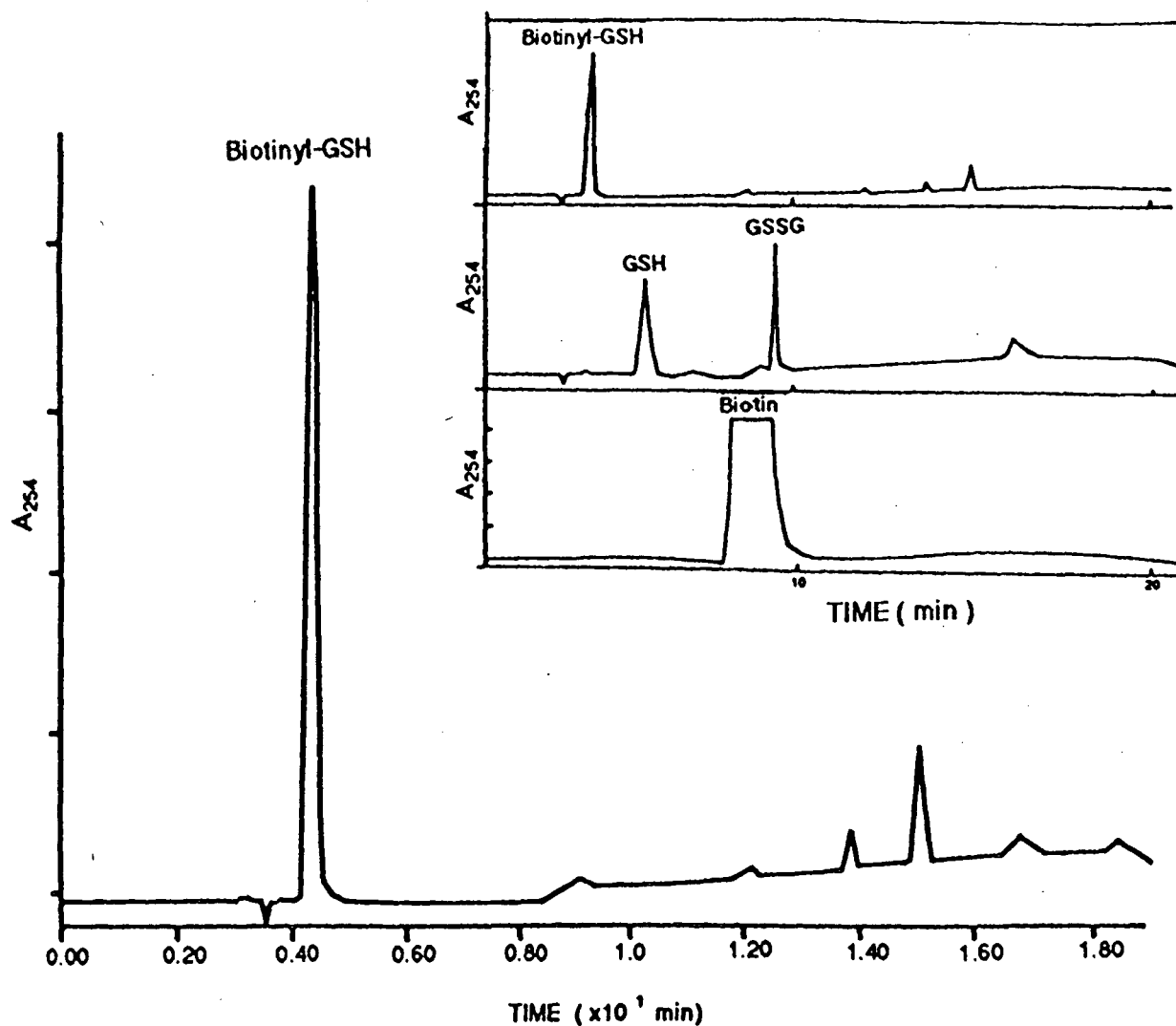


Fig. 4. Purification of biotinyl-GSH by reversed-phase HPLC. Crude biotinyl - GSH from the Sephadex G-10 eluate ( peak 2 ) was applied to a  $C_{18}$  reversed-phase column and eluted with a 0 - 50% gradient of acetonitrile in 0.1% trifluoroacetic acid at 254 nm. The inset shows the elution times of GSH, GSSG, biotinyl-GSH, and biotin.



Histochemistry Figure 5 shows that there are specific binding sites for biotinyl-GSH in the brain while both control groups (GSH only, and Biotinyl-GSH+ 100 x GSH) gave a negative result. Unlike many neuropeptide or neurotransmitter receptors which are usually present on the grey matter such as cortex, hippocampus, and some nuclei of the brain, GSH binding sites appear to be in the white matter. Figure 5A shows that such structures of white matter as cingulum, dorsal hippocampal commissure, cerebral peduncle, brachium superior colliculus, fasciculus retroflexus, habenular commissure, acoustic radiation, brachium superior colliculus, medial lemniscus, optic tract, mamillothalamic tract and external capsule are clearly labeled with the biotin-avidin-enzyme-DAB complex. Control groups gave a negative result (Fig.5B).

Results from further control experiments showed that the labeling on white matter was not non-specific absorption of avidin or DAB itself: (1) The incubation of ABC reagent only (without previous incubation of biotin-GSH) with brain sections gave a negative reaction, and (2) although DAB could be used as a substrate and produce a positive color reaction if the endogenous peroxidase was not blocked by  $H_2O_2$  previously, the DAB staining only appeared in cortex and hippocampus (Fig 5C), a result completely different from the GSH binding.

The specific binding sites of GSH in the brain white matter suggested that there might be GSH receptors on glial cells. However, a number of alternatives to the "GSH receptor hypothesis" could explain the positive binding reaction in the CNS: (1) Biotinyl-GSH might bind to membrane bound enzymes such as GSH-S-transferases and GSH peroxidase, (2) the positive color reaction might reflect the uptake of GSH into astrocytes and not the binding of GSH with its receptors.

To examine the "GSH receptor hypothesis", further experiments were performed.

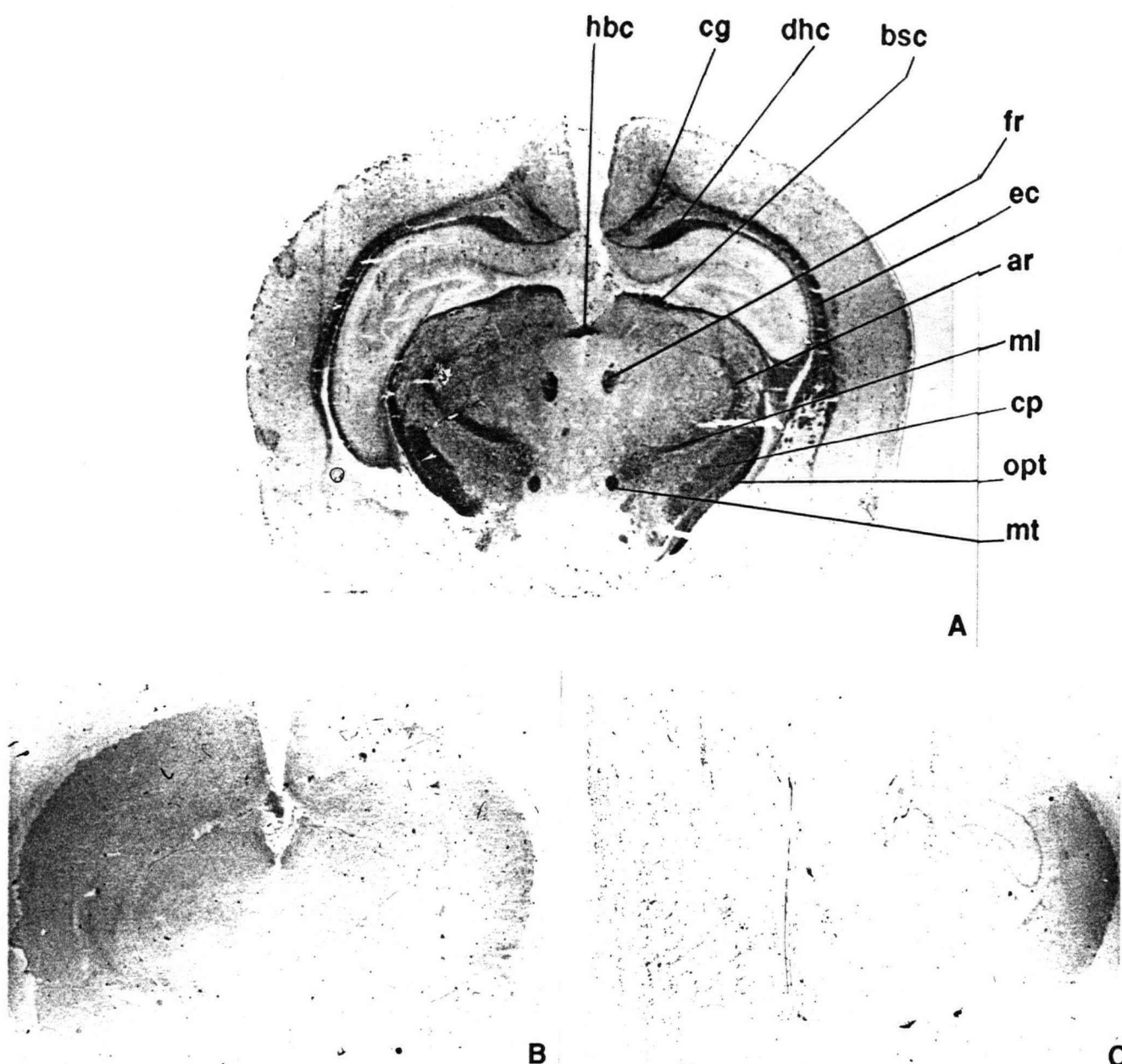


Fig. 5. Biotinyl-GSH binding sites in rat brain. Tissue slices of 20  $\mu\text{m}$  thickness were incubated with biotinyl-GSH, and  $\text{H}_2\text{O}_2$  was used to block the endogenous peroxidase before ABC reagent was applied.

A: Specific binding sites of GSH in the brain (interaural 4.7 mm). ar: acoustic radiation, bsc: brachium superior colliculus, cg: cingulum, dhc: dorsal hippocampal commissure, cp: cerebral peduncle, ec: external capsule, fr: fasciculus retroflexus, hbc: habenular commissure, ml: medial lemniscus, mt: mammillothalamic tract, opt: optic tract.

B: Control of A in the presence of 100 x unlabeled GSH.

C: Nonspecific color reaction caused by endogenous peroxidase. DAB itself, a substrate of endogenous peroxidase, only produced the color reaction in such structures as hippocampus, cortex etc., completely contrary to that of biotinyl-GSH binding in the brain.

## 2. Radioligand Receptor Assay

The major question resulting from the study described above is: Does the positive color reaction reflect a binding of biotinyl-GSH (1) to GSH receptors, (2) to GSH uptake sites, or (3) to membrane bound enzymes in the CNS?

Radioactive receptor assays, which have been widely used for dissecting the characteristics of different receptors of hormones (Rojas & Birnbaumer, 1985; Vale & Shooter, 1985; Teitelbaum et al, 1985), neuropeptides (Hanley et al, 1980; Akil, 1980; Saito et al, 1980; Gammeltoft et al, 1980), and neurotransmitters (Green, 1983; Snyder, 1983; Bockaert & Ebersolt, 1988; Whitker-Azmitia, 1988), are useful to examine this question. If the "receptor hypothesis" is true, the GSH binding sites in the CNS should have such receptor characteristics which are generally regarded as "receptor criteria" (Hughes et al, 1989):

- (1) Finite binding capacity, i.e. a saturable binding of the binding system;
- (2) High affinity ( usually  $K_d < 10^{-8}$  M );
- (3) Ligand specificity;
- (4) Tissue or cellular specificity;
- (5) Correlation with biological response.

Because the previous studies suggested that GSH receptors might exist on glia cells, cultured astrocytes were used to perform the GSH receptor assay.

## Materials And Methods

**Materials** Astrocytes from rat visual cortex were cultured in our laboratory (as described below). Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum were from TFL Media Preparation Service (Vancouver, BC). [ $^{35}$ S]GSH (55.2 Ci/mmol) was purchased from New England Nuclear (Boston, MA). GSH, GSSG, cysteine, S-methyl-GSH, S-hexyl-GSH and other peptides were obtained from Sigma Chemical (St. Louis, MO).

**Cell preparation** For primary cultures, all procedures were done using sterile techniques. Newborn Sprague-Dawley rat pups were decapitated. Visual cortex was rapidly removed from the brain, dissociated, and cultured following the primary culture procedure (McCarthy & De Vellis, 1980). Cells were grown in Falcon dishes (35 x10 and 60 x15 mm) in DMEM containing 10% fetal calf

serum at 37°C in an atmosphere of 95% oxygen and 5% CO<sub>2</sub>. Relative humidity was maintained at 100%.

For binding experiments, cells from the primary cultures were washed with Ca<sup>2+</sup>, Mg<sup>2+</sup>-free phosphate buffered saline (CMF-PBS) (Vale & Shooter, 1985) for 3 min x 2. After centrifuging at 2000 rpm for 4 min, the cells were incubated in 5 ml CMF-PBS containing 1 mM EDTA for 5 min at 37°C. The cells were centrifuged at 2000 rpm for 4 min, and washed with binding buffer (Vale & Shooter, 1985) for 5 min x 2. The cells were then resuspended in an appropriate volume of binding buffer and counted with a hemocytometer. The final cell concentration for binding experiments were 6–6.4 x 10<sup>5</sup> cells/ml.

Receptor Binding [<sup>35</sup>S]GSH binding was determined following the method of whole cell receptor assay (Sutter et al, 1979). Astrocytes (6 - 6.4 x 10<sup>5</sup>/ml) were incubated at 37°C or 4°C for 60 min with 3 nM [<sup>35</sup>S]GSH. The incubation was terminated by a filtration through Whatman GF/B glass filter membranes with the aid of the Millipore Filter (1225 Sampling Manifold). The filter membrane was washed with 10 ml cold CFM-PBS buffer, and the radioactivity trapped on the filter was measured with a liquid scintillation spectrometer (Beckman, LS 6000IC) using 4 ml Formula-963 scintillant (NEN research products) at a counting efficiency of 95% for <sup>35</sup>S. For the Scatchard analysis, cell suspensions were incubated with [<sup>35</sup>S]GSH at various concentration from 0.5-25 nM. Nonspecific binding of [<sup>35</sup>S]GSH was measured in the presence of excess amount of unlabeled GSH at a concentration of 300 μM. Scatchard analysis was performed using a computer (Apple MAC II).

All binding assays were carried out in triplicate per experiment.

## Results And Discussion

Primary cultures Studies on the properties of astrocytes could use cells derived from CNS in one of three ways: (1) cell suspensions derived from perinatal rodents and chicks and maintained in primary culture, (2) bulk separation from mature animals, and (3) perpetual cell lines which exhibit "astrocyte-like" features.

Cells taken directly from the brain tissue and kept in vitro for more than 24 hr are considered to be primary cultures (Fedoroff, 1977), which have been widely

used in studying the properties of astrocytes (Murphy & Pearce, 1987; Walz, 1989; McCarthy et al, 1988). In the present studies primary cultures were utilized to perform receptor assays.

In general, the major proliferative cells in the cerebrum of the new born rat or mouse are astroglial precursor cells (Walz, 1989). Cell suspensions containing viable astroglia may be prepared by dissociating immature brain tissue at a stage when astroglia are mitotic (McCarthy et al, 1988). If the cell suspension is plated at relatively low cell densities, astroglia will overgrow the other cell types present to yield enriched cultures of astroglia (McCarthy et al, 1988). On the other hand, the use of vigorous dissociation procedures on the brain tissue results in the destruction of the majority of the differentiating process-bearing neurons and selective survival of small undifferentiated cells, which are mainly astroglial precursors (Hertz et al, 1985).

By primary culturing, 60-99% of the cells obtained were astrocytes ( see Results and Discussion in part 3). The method used in this study gave a source culture which contained an enriched population of astrocytes > 90% ( McCarthy & De Vellis, 1980). It is thus credible that the receptor assays performed on the primary cultures reflect the receptor characteristics of astrocytes.

Binding assay To examine the "receptor hypothesis", the first attempt was made to determine whether or not the astrocytes demonstrated a specific binding of [ $^{35}$ S]GSH. A significant binding of [ $^{35}$ S]GSH in cultured astrocytes was found in the present study (Fig. 6). The binding was tremendously inhibited by 0.3 mM GSH and independent of the incubation temperature: 97% inhibition at 37°C and 90.9% at 4°C. The specific binding was much higher at 37°C than at 4°C ( *t*-test,  $P < 0.005$ ), while the nonspecific binding, which was found in the presence of an excess amount of non-radioactive GSH, was not affected by the incubation temperature. In the no-cell control group, the [ $^{35}$ S]GSH binding, which was 79% of the nonspecific binding at 37°C and 81% at 4°C, reflected the adsorption of the isotope to glass filters.

The specific binding linearly increased with increasing cell concentration at 37°C and 4°C (Fig.7). Because the specific binding was much higher at 37°C than at 4°C, other experiments including time course, saturation binding, dissociation analysis, and displacer competition etc. were all executed at 37°C.

Fig.8 illustrates the data from experiments in which the time course of the binding was measured at an [ $^{35}$ S]GSH concentration of 25nM, with the cell

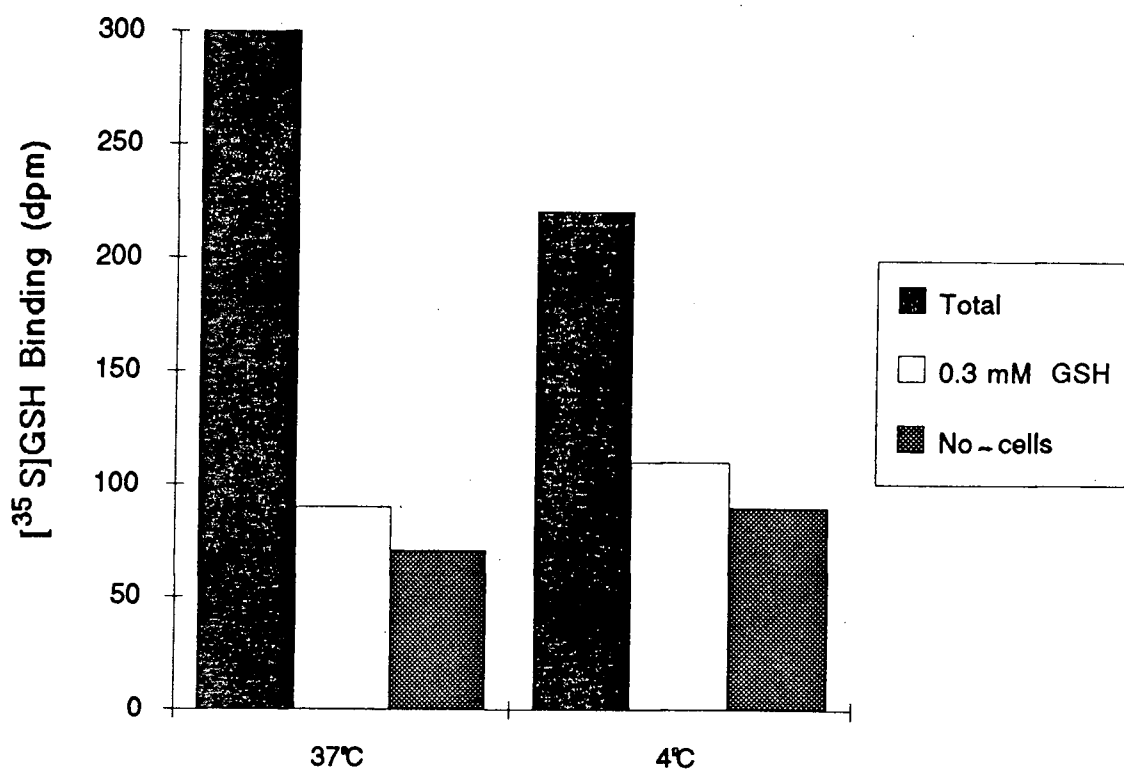


Fig. 6. Effect of excess GSH on [<sup>35</sup>S]GSH binding. Intact cultured astrocytes (  $6 \times 10^5$  /ml) were incubated with 3 nM [<sup>35</sup>S]GSH at 37 °C and 4°C for 60 min in the presence or absence of 0.3 mM unlabeled GSH. For both experiments, triplicate samples were performed.

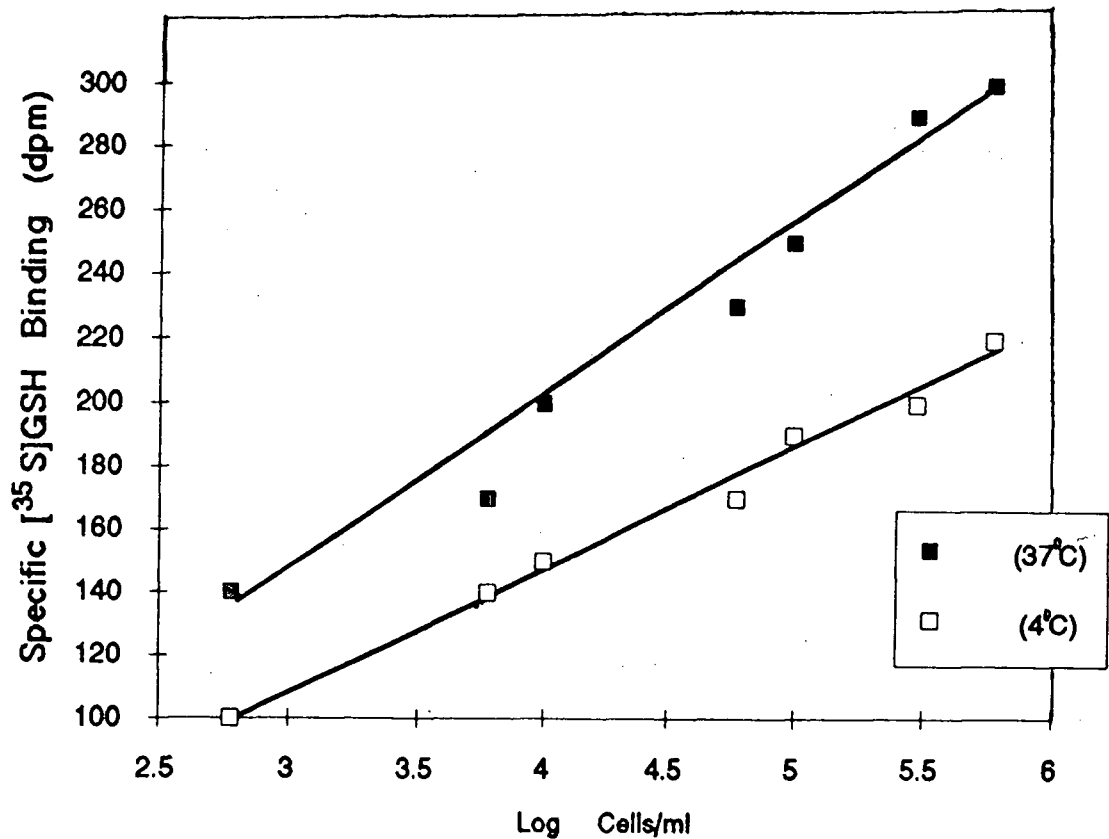


Fig. 7. Effect of cell concentration on [<sup>35</sup>S]GSH binding. Cells in various concentrations ( $6 \times 10^2$  -  $6 \times 10^5$  / ml) were incubated with 3nM [<sup>35</sup>S]GSH at 37°C or 4°C for 60 min. Nonspecific binding was measured in the presence of 300  $\mu$ M unlabeled GSH. Each point was from the triplicate samples of each experiment.

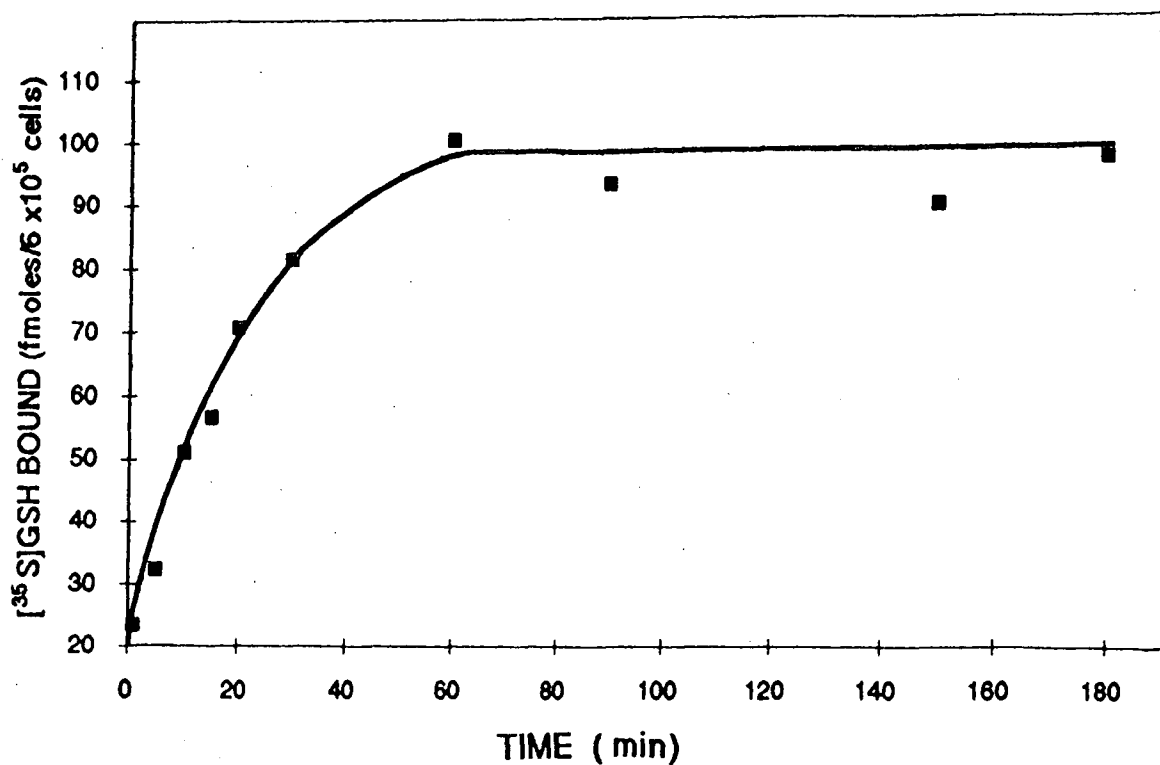


Fig. 8. Time course of [<sup>35</sup>S]GSH to binding sites on astrocytes. Cells ( $6 \times 10^5$  / ml) were incubated with 25 nM [<sup>35</sup>S]GSH at 37°C for various incubation periods indicated. The association of [<sup>35</sup>S]GSH binding was completed after 60 min. The data were corrected for nonspecific binding, and each point was measured in triplicates of 300  $\mu$ l each.



concentration of  $6 \times 10^5$  cells/ml incubated at  $37^\circ\text{C}$ . The association of  $[^{35}\text{S}]\text{GSH}$  binding was completed after 60 min.

Equilibrium binding experiments showed that astrocytes bind  $[^{35}\text{S}]\text{GSH}$  in a saturable manner. The concentration dependence of the binding is shown in Fig.9 over a wide range of  $[^{35}\text{S}]\text{GSH}$  concentrations from 0.5 - 25 nM. The nonspecific binding was determined by adding an excess amount of unlabeled GSH at a concentration of 0.3 mM. Fig.10 depicts the analysis of this binding data according to Scatchard (1949). A non-linear Scatchard plot was obtained that suggested two classes of binding sites on the cultured astrocytes:  $K_{d1} = 4.67 \pm 0.75$  nM,  $B_{\text{max}1} = 70 \pm 9.2$  fmole/  $6.4 \times 10^5$  cells or  $B_{\text{max}1} = 6.6 \times 10^4$  molecules / cell,  $K_{d2} = 35.14 \pm 2.17$  nM,  $B_{\text{max}2} = 260 \pm 12.77$  fmole/  $6.4 \times 10^5$  cells or  $B_{\text{max}2} = 2.4 \times 10^5$  molecules/ cell.

Previous studies have found that the number of different receptors on different cell types varies from  $10^3$  -  $10^5$  or even higher (Gong, 1985). For example, there are  $8 \times 10^4$  EGF receptors per fibroblast cell,  $1.6 \times 10^5$  atropine receptors per smooth muscle cell (Hollenberg & Cuatrecasas, 1975). In the A875 melanoma cell line, NGF receptors are  $7 \times 10^5$  / cell (Fabricant et al, 1977), and in A431 cell line, EGF receptors are  $2 \times 10^6$  / cell (Carpenter, 1987). The GSH receptors shown in the Scatchard analysis are in the range of  $10^4$  -  $10^5$  / cell. The numbers which we report here are in keeping with these previous studies and may imply that GSH receptors serve important physiological functions in astrocytes.

Dissociation and association kinetics For one-site receptor binding, the binding reaches equilibrium following the kinetic equation:



The forward or association ( $K_{+1}$ ) and the reverse or dissociation ( $K_{-1}$ ) rate constants will give the kinetic determination of  $K_d$ , the equilibrium dissociation constant:

$$K_d = \frac{K_{-1}}{K_{+1}} \quad \text{----- [2]}$$

(Bylund, 1980).

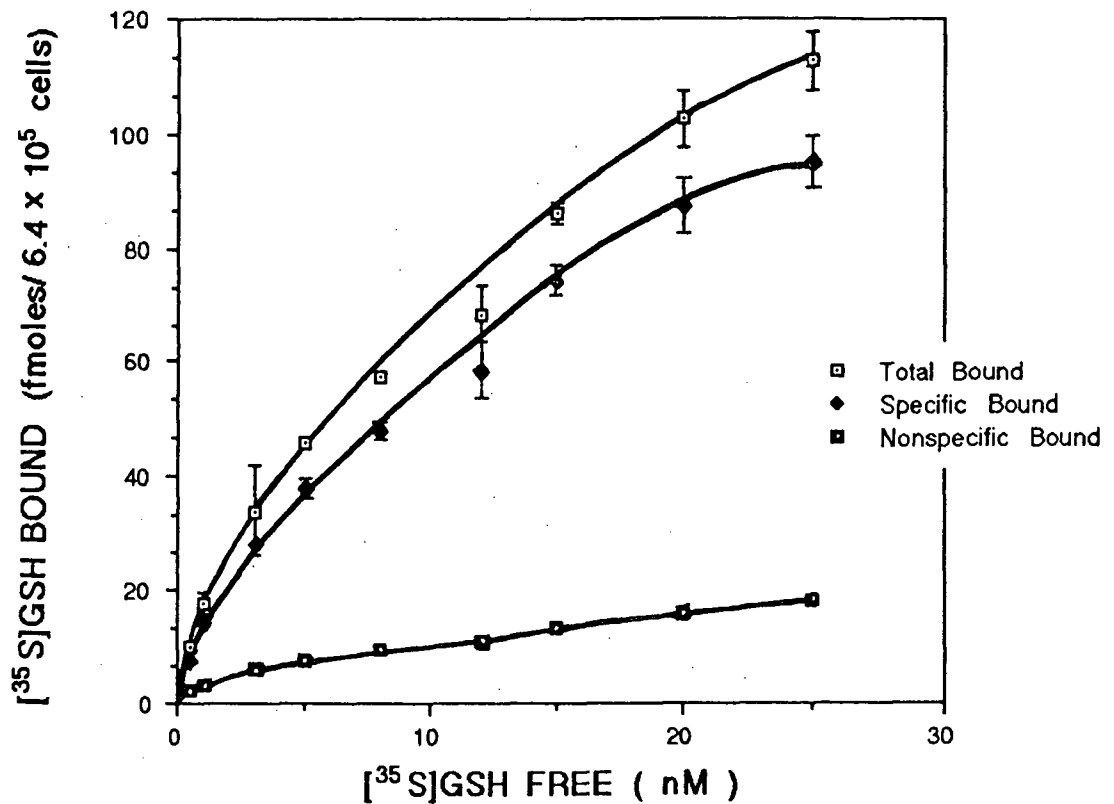


Fig. 9. Binding of  $[^{35}\text{S}]\text{GSH}$  to cultured astrocytes as a function of concentration. Cells ( $6.4 \times 10^5$  / ml) were incubated at  $37^\circ\text{C}$  for 60 min with various concentration (0.5nM to 25nM) of  $[^{35}\text{S}]\text{GSH}$ . Each value of total bound represents the mean  $\pm$  S.E. obtained from 3 separate experiments. Nonspecific binding of  $[^{35}\text{S}]\text{GSH}$  was measured in the presence of 0.3 mM unlabeled GSH.

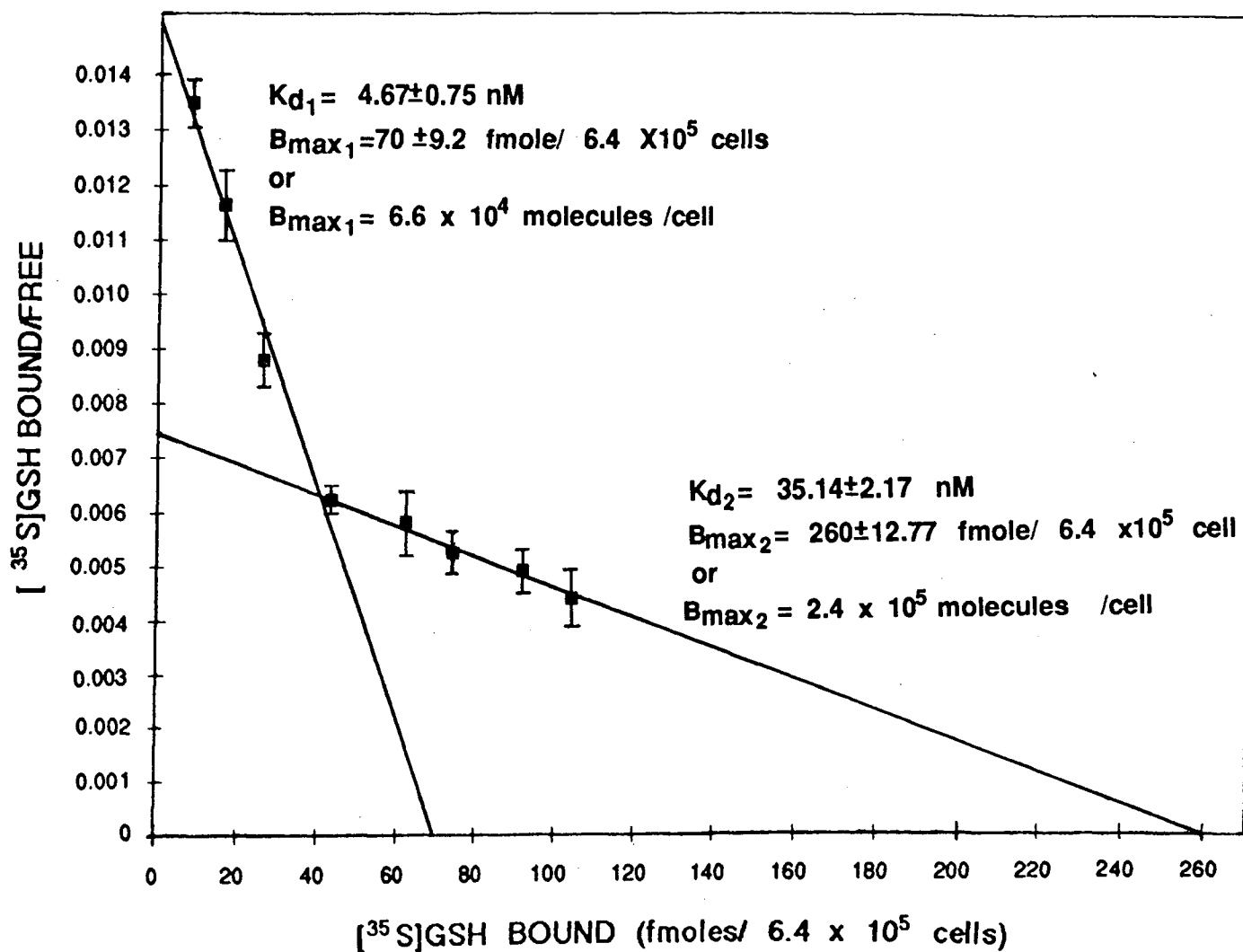


Fig. 10. Scatchard plot analysis of binding equilibrium of [<sup>35</sup>S]GSH to astrocytes. A non-linear plot suggests that there are two classes of binding sites in the cells. Binding constants were calculated as shown. Each point represents the mean  $\pm$  S.E. obtained from 3 separate experiments.

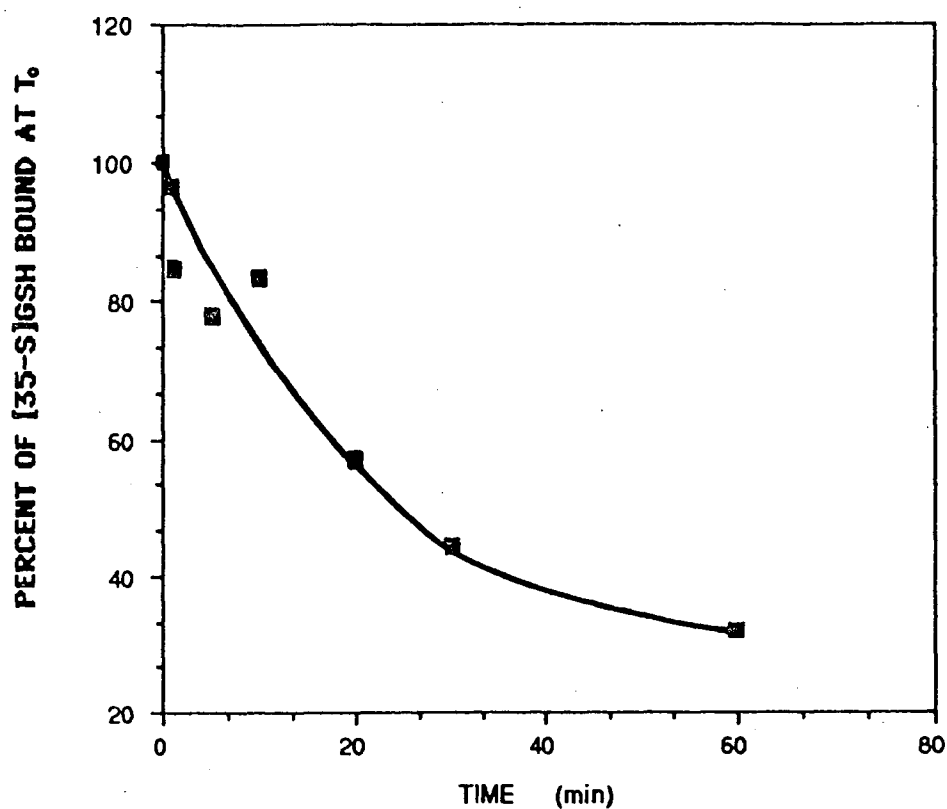


Fig.11. Rate of dissociation of  $[^{35}\text{S}]\text{GSH}$ . Cells were preincubated with 3 nM  $[^{35}\text{S}]\text{GSH}$  for 60 min at 37 °C. The dissociation of  $[^{35}\text{S}]\text{GSH}$  was induced by the addition of 300  $\mu\text{M}$  unlabeled GSH. The specific binding at  $T_0$  and after different times of dissociation was measured in triplicate.

The kinetic rate constants for the dissociation and association of [<sup>35</sup>S]GSH from the higher affinity site I was determined by using [<sup>35</sup>S]GSH concentrations below or at the apparent  $K_D$  of site I. Dissociation was induced by the addition of excess amount of unlabeled GSH. The time course of the dissociation of [<sup>35</sup>S]GSH shown in Fig.11 was obtained under chase condition by the addition of 0.3 mM GSH after pre-equilibrating with 3 nM [<sup>35</sup>S]GSH at 37°C for 60 min. The dissociation rate constant for the reversible binding reaction (equation 1) was determined by a plot of  $\ln(B/B_0)$  vs  $t$  based on the integrated rate equation:

$$\ln(B/B_0) = -K_{-1} \cdot t \quad \text{----- [3]}$$

(Bylund, 1980), where  $B$  is the bound ligand concentration at various dissociation times while  $B_0$  is the bound ligand concentration at dissociation time 0. The graph of this data (Fig.12) gave a slope of  $K_{-1}=0.0168 \text{ min}^{-1}$ .

For the analysis of association data, the pseudo first-order method was used. If the binding reaction is a reversible bimolecular reaction, the integrated rate equation is given by

$$\ln(Be/[Be-Bt]) = (K_{+1}' + K_{-1}) \cdot t = K_{ob} \cdot t \quad \text{----- [4]}$$

(Galper et al, 1977), where  $Be$  and  $Bt$  are the concentrations of bound ligand at equilibrium and at time  $t$ . Therefore, data plotted according to equation 4 should yield a straight line if it is a simple bimolecular reaction (Galper et al, 1977). Fig.13a shows the data from an experiment in which the time course of the association was measured at an [<sup>35</sup>S]GSH concentration of 3 nM. A plot of  $\ln(Be/[Be-Bt])$  vs  $t$  gives a slope of  $K_{ob}=0.008 \text{ min}^{-1}$  ( Fig. 13b). Thus the association rate constant  $K_{+1}$  can be calculated according the equation:

$$K_{ob} - K_{-1} = K_{+1}' = K_{+1} \cdot Lt \quad \text{----- [5]}$$

(Bylund, 1980). Based on equation 5, the present association experiments give a  $K_{+1}$  of  $0.003 \text{ nM}^{-1} \text{ min}^{-1}$ .

Consequently, the dissociation constant of site I for [<sup>35</sup>S]GSH is obtained through these kinetic constants  $K_{-1}$  and  $K_{+1}$  (according to equation 2) as  $K_D = 5.6 \text{ nM}$ , a value consistent with that obtained from Scatchard analysis ( Table 1).

### Dissociation Analysis

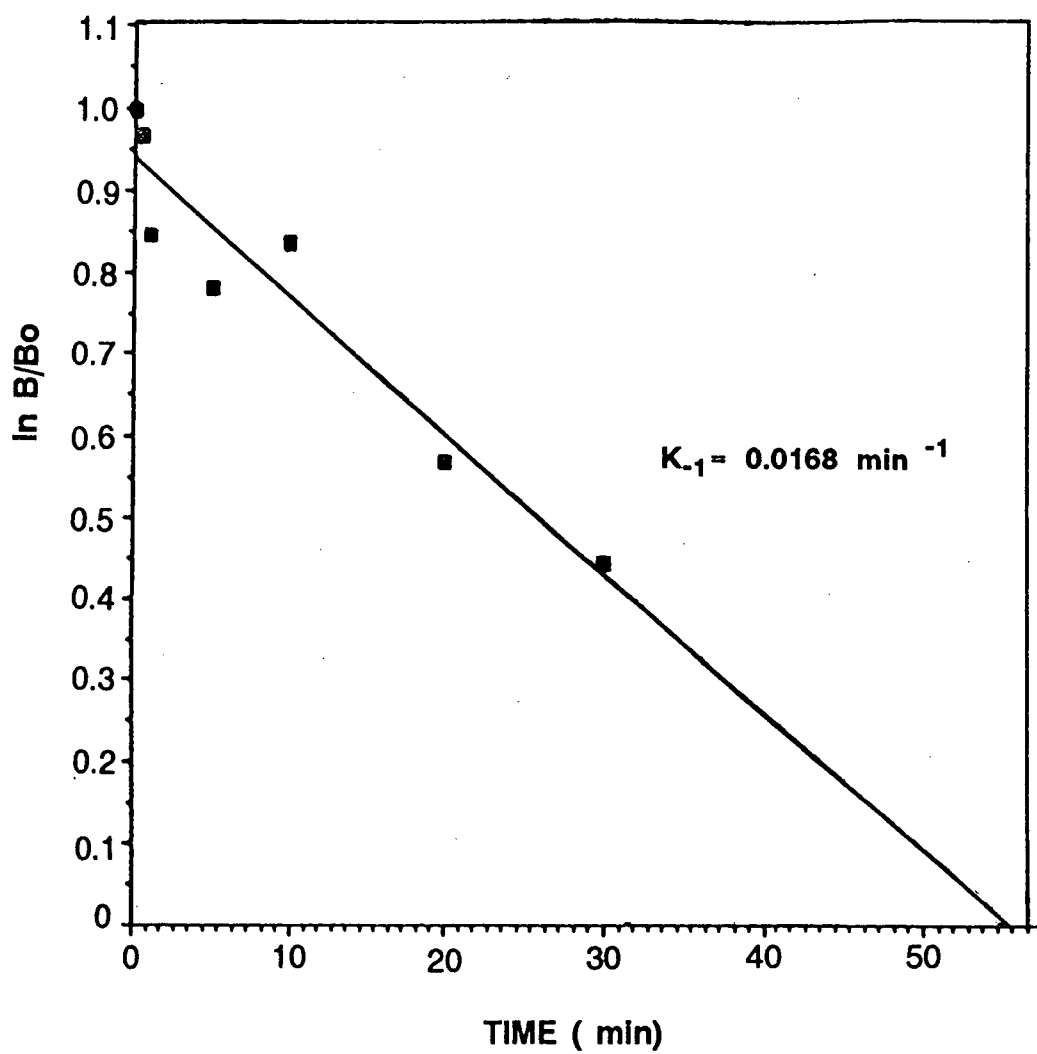


Fig. 12. Site I dissociation rate constant plot analysis. The data were from kinetic experiments of [ $^{35}\text{S}$ ]GSH dissociation.

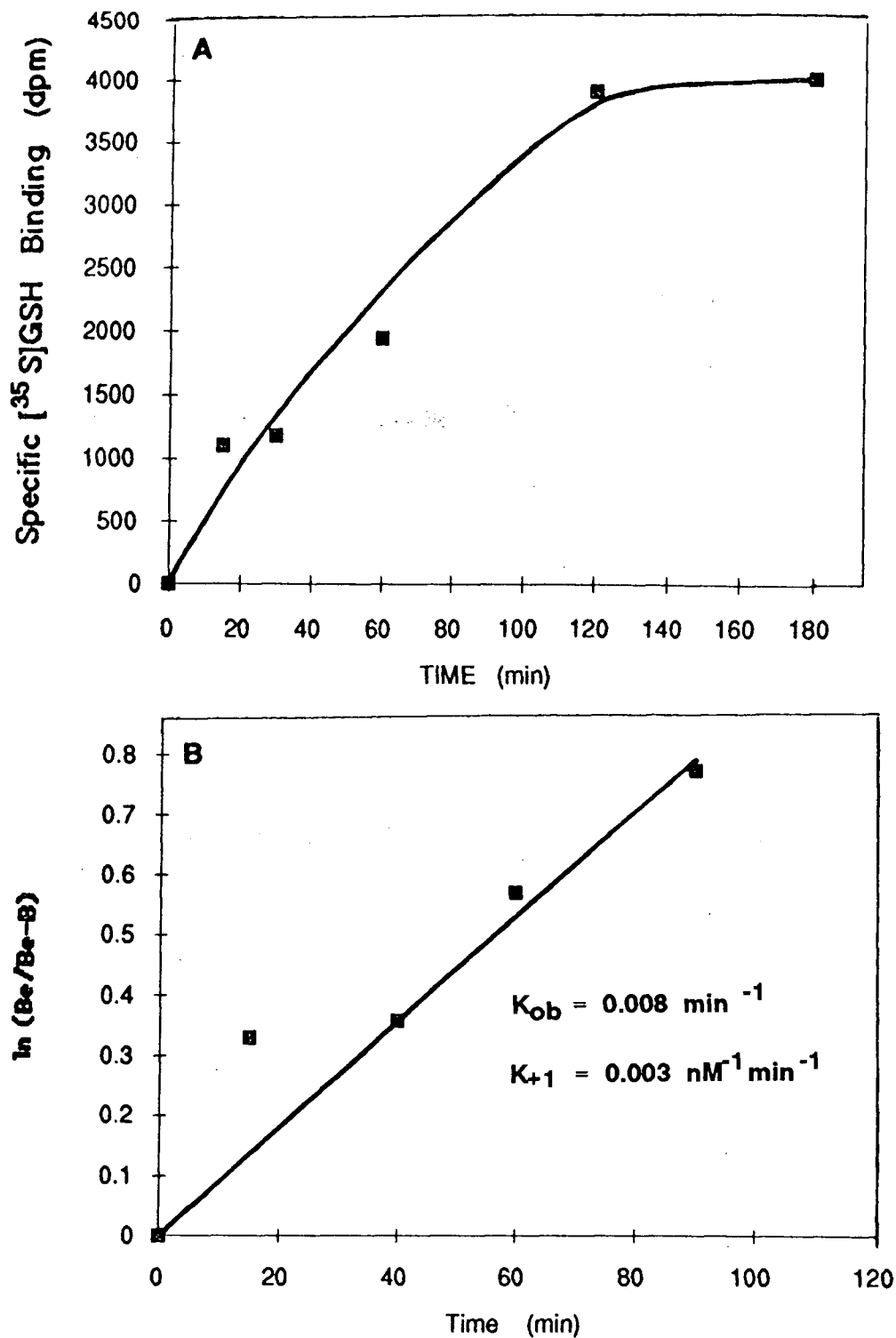


Fig. 13. Site I association rate constant plot analysis. A: Time course of association of  $[^{35}\text{S}]\text{GSH}$  to binding sites on astrocytes. Cells ( $6.4 \times 10^5 / \text{ml}$ ) were incubated with  $3 \text{ nM}$   $[^{35}\text{S}]\text{GSH}$  at  $37^\circ\text{C}$  for various incubation times. B: Site I association rate constant plot according data from A.

Table 1. Kinetic and equilibrium constants for the specific binding of [ $^{35}$ S]GSH to cultured astrocytes.

Binding Constants	Kinetic Analysis	Scatchard Analysis
$K_{-1}$ ( $\text{min}^{-1}$ )	0.0168	
$K_{+1}$ ( $\text{nM}^{-1} \text{min}^{-1}$ )	0.003	
$K_{d1}$ (nM)	5.6	$4.67 \pm 0.75$
$B_{\text{max}1}$ (fmoles/ $6.4 \times 10^5$ cells)		$70 \pm 9.21$
(molecules / cell)		$6.6 \times 10^4$
$K_{d2}$ (nM)		$35.14 \pm 2.17$
$B_{\text{max}2}$ (fmole / $6.4 \times 10^5$ cell)		$260 \pm 12.77$
(molecules / cell)		$2.4 \times 10^5$

Table 2. IC<sub>50</sub> of different displacers for [ $^{35}$ S]GSH binding.

Displacer	IC <sub>50</sub>
GSH	10 nM
GSSG	40 $\mu$ M
S-methyl-GSH	40 $\mu$ M
Cys	1 mM
$\gamma$ -Glu·Glu	1.5 mM
$\gamma$ -D-Glu·Gly	6 mM
$\gamma$ -Glu·Gly	10 mM
S-hexyl-GSH	15 mM



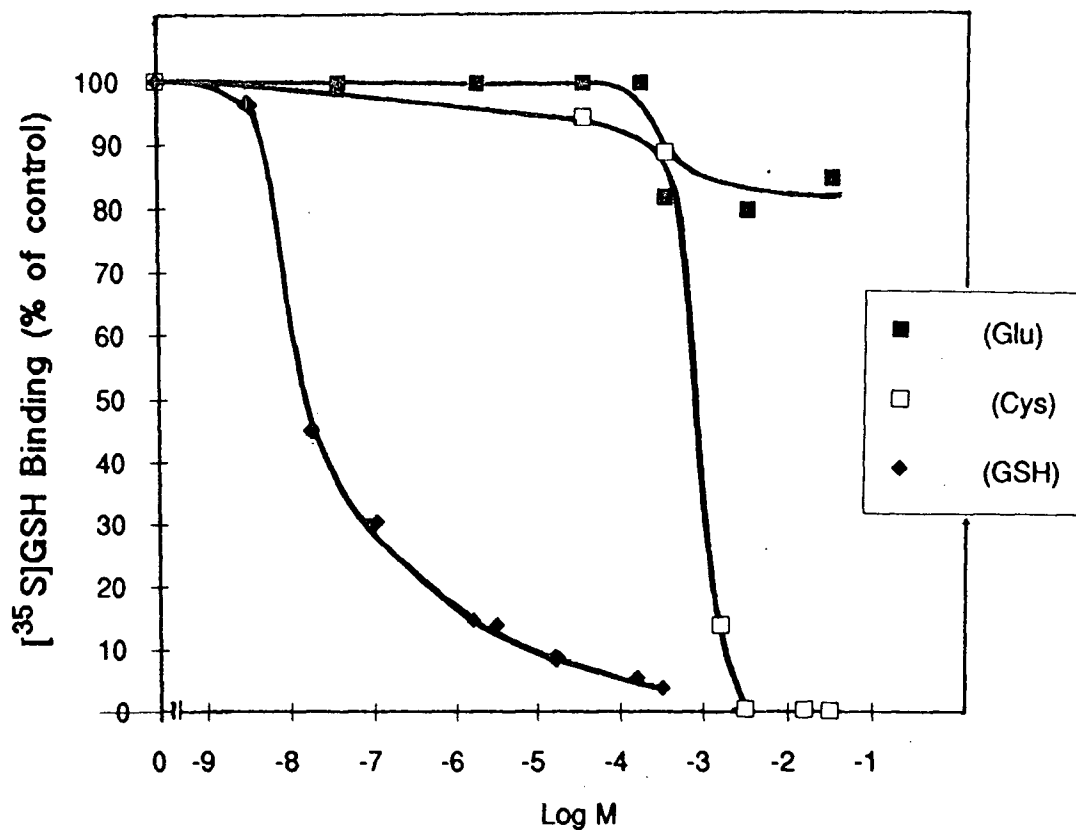


Fig. 14 Comparison of the binding affinity of GSH, Cys, and Glu. The competitive inhibition of [ $^{35}\text{S}$ ]GSH binding was measured by the addition of GSH, Cys, and Glu representively at various concentrations. Cells were incubated with 3nM [ $^{35}\text{S}$ ]GSH at 37°C for 60 min. All experiments were performed in triplicate. IC<sub>50</sub> values: GSH, 10 nM; Cys, 1 mM. Glu had almost no effect on [ $^{35}\text{S}$ ]GSH binding.

Inhibition of [ $^{35}$ S]GSH binding and structure-selectivity Fig.14 shows that GSH elicited a concentration-dependent inhibition of [ $^{35}$ S]GSH binding in the cultured astrocytes. The  $IC_{50}$  of GSH for [ $^{35}$ S]GSH binding is 10 nM. Because cysteine and its analogues have been reported to interact with excitatory amino acid binding sites (Pullan et al, 1987) and GSH contains a cysteine moiety, the relationship between cysteine and GSH receptor was studied by using cysteine as a displacer. A significant inhibition was obtained by the addition of cysteine although its  $IC_{50}$  was much higher than that of GSH (Table 2 and Fig.14). The affinity of cysteine for  $AP_4$  binding sites is 0.34  $\mu$ M, and for NMDA sites is 170  $\mu$ M (Pullan et al, 1987). However, the  $IC_{50}$  of cysteine for the GSH binding sites is 1mM (Fig. 14), suggesting that GSH receptors differ from these excitatory amino acid receptors. Glutamic acid only produced 15% inhibition even with the highest concentration (30 mM) of various concentrations examined (Fig.14). The failure of Glu to inhibit [ $^{35}$ S]GSH binding reflects the specificity of the binding site for its ligand.

The binding was also inhibited by GSSG (oxidized form of glutathione), S-methyl-GSH, and S-hexyl-GSH (Fig. 15). GSSG and S-methyl-GSH have almost the same  $IC_{50}$  (40  $\mu$ M), while S-hexyl-GSH exhibited a much higher  $IC_{50}$  of 15 mM (Table 2). The reason for this might be that the six-carbon side chain connected to GSH obstructs the specific binding of the S-hexyl-GSH molecule to the receptor. The fact that these GSH derivatives, which have no SH group but still maintain in different degrees the binding ability, clearly indicates that the binding in astrocytes is not due to a nonspecific binding of GSH to any membrane protein through an exchange of disulfide bond between GSH and membrane proteins, and also the -SH moiety is not necessarily crucial for the GSH molecule to bind its receptor.

The importance of the  $\gamma$ -Glu structure and its conformation in the ligand was studied. Peptides with the  $\gamma$ -Glu structure in the L-conformation ( $\gamma$ -Glu-Glu,  $\gamma$ -Glu-Gly) and D-conformation ( $\gamma$ -D-Glu-Gly) showed a significant inhibition of [ $^{35}$ S]GSH binding (Fig.16), but obviously they had a much lower affinity to GSH receptors with 2-3 orders of magnitude increase in their  $IC_{50}$ , compared to that of GSH (Table 2). When the  $\gamma$ -Glu structure was substituted by  $\alpha$ -Glu, it not only lost its ability to bind the receptor, but somehow greatly enhanced the [ $^{35}$ S]GSH binding as well (Fig.16). These results suggest that the  $\gamma$ -Glu moiety is of importance, while the cysteine and glycine residues are also essential for the GSH molecule to maintain its binding affinity to the receptor.  $\alpha$ -Glu-Glu,

which increased the [ $^{35}\text{S}$ ]GSH binding in astrocytes, might serve as a synergist of GSH in the binding reaction.



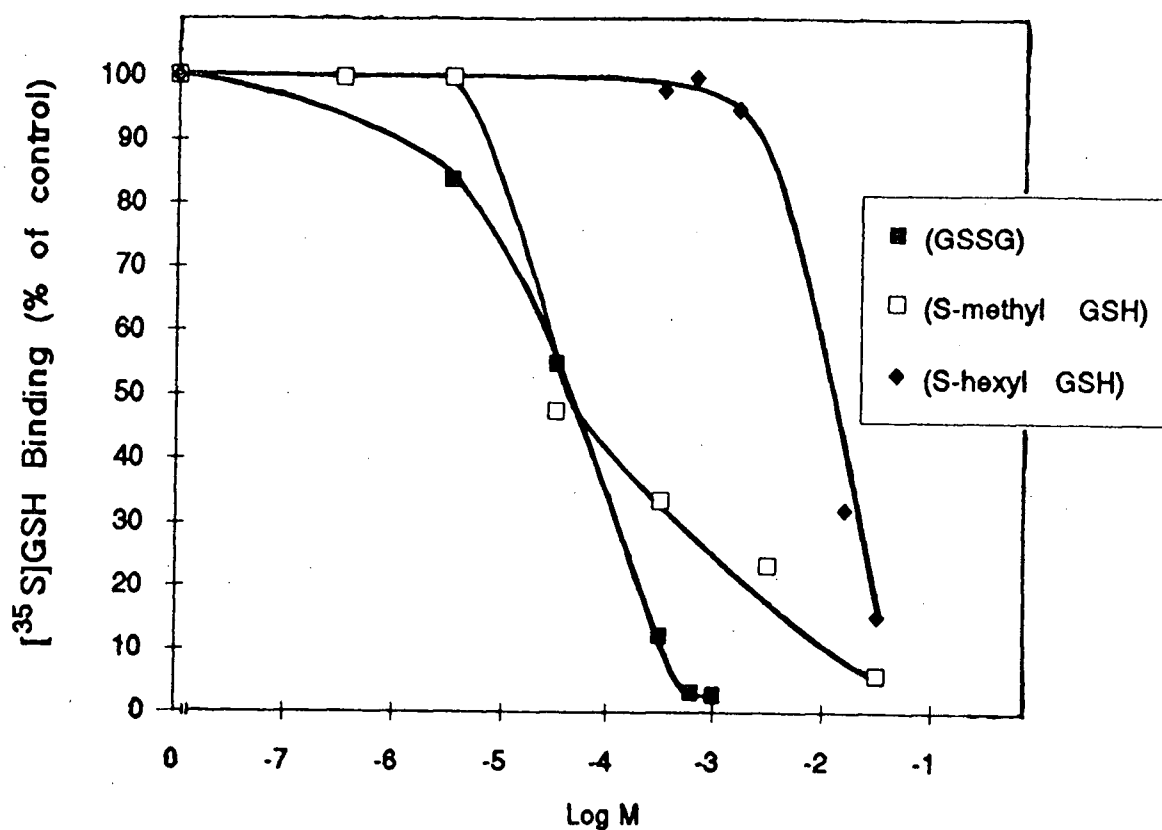


Fig.15. Effect of -SH structure on [ $^{35}\text{S}$ ]GSH binding. Cells were incubated with 3nM [ $^{35}\text{S}$ ]GSH at 37°C for 60 min in the presence of GSSG, S-methyl-GSH, and S-hexyl-GSH at various concentrations. Each experiment was performed in triplicate. IC<sub>50</sub> values: GSSG, 40  $\mu\text{M}$ ; S-methyl-GSH, 40  $\mu\text{M}$ ; S-hexyl-GSH, 15mM.

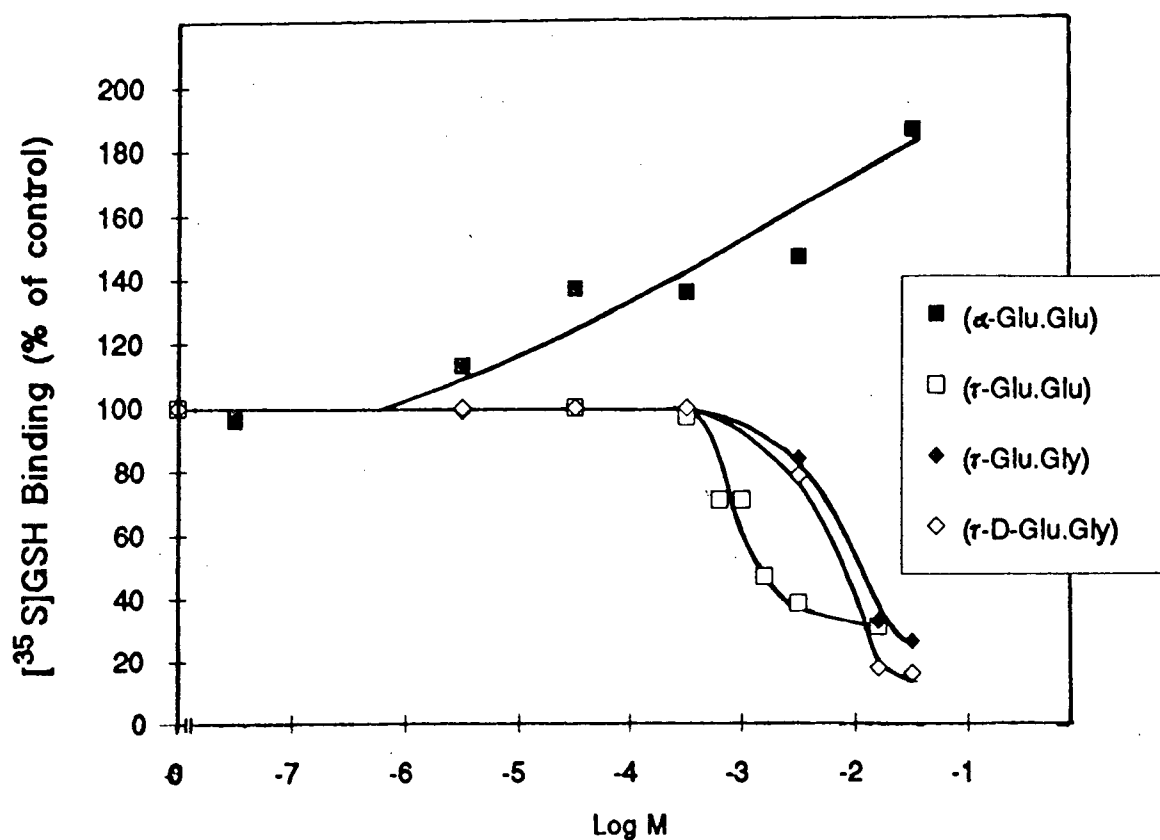


Fig. 16. Effects of  $\gamma$ -Glu and  $\alpha$ -Glu structure on [ $^{35}$ S]GSH binding. Cells were incubated with 3 nM [ $^{35}$ S]GSH at 37°C for 60 min in the presence of  $\gamma$ -Glu.Glu,  $\gamma$ -Glu.Gly,  $\gamma$ -D-Glu.Gly, and  $\alpha$ -Glu.Glu. All experiments were performed in triplicate. IC<sub>50</sub> values:  $\gamma$ -Glu.Glu, 1.5 mM;  $\gamma$ -D-Glu.Gly, 6 mM;  $\gamma$ -Glu.Gly, 10 mM.  $\alpha$ -Glu.Glu did not compete with [ $^{35}$ S]GSH but augmented its binding.

### 3. The Colloidal Gold Technique For The Cellular Localization Of GSH Binding Sites

Although the results from the studies described above suggested that GSH receptors might exist on astrocytes, they did not allow a direct cellular resolution.

If the conclusion based on these previous experiments is correct, i.e. there are GSH receptors on astrocytes, a positive result at the cellular level should be obtained with appropriate cytochemical techniques.

In the present study, the colloidal gold method and fluorescence double staining were used to visualize the GSH receptor at the cellular level.

#### Materials And Methods

Materials Cultured astrocytes were prepared as described above. Streptavidin-gold (AuroProbe) and silver enhancer were from Amersham International Plc (Amersham, UK). Normal goat serum was from Vector Laboratories Inc. (Burlingame, CA). Rabbit immunoglobulins to cow glial fibrillary acidic protein (GFAP) was purchased from Dako Corporation (Santa Barbara, CA). Goat anti-rabbit-Rhodamine was from Cappel/Organon Teknika INC (Turnhout, Belgium).

Colloidal gold method and double staining The colloidal gold method with silver enhancer (Owen et al, 1989) was used in the present study. Cultured cells were washed with CMF-PBS (Vale & Shooter, 1985) for 3 min x 2. Cells were incubated biotinyl-GSH (see " synthesis and purification of biotinyl-GSH " in part 1) for 60 min at room temperature. After the incubation, cells were washed with CMF-PBS for 5 min and with H<sub>2</sub>O for 5 min x 2. Streptavidin-gold ( 1:40 ) was added to cells at room temperature for 40 min. After washing with CMF-PBS for 5 min and with H<sub>2</sub>O for 5 min x 2, cells were fixed with methanol for 30 min at - 20°C, and then washed with H<sub>2</sub>O for 1.5-2 hr. Freshly prepared silver enhancer was added and incubated with cells for 15-25 min at room temperature. After washing with H<sub>2</sub>O for 5 min x3, the cells were mounted with DPX for observation and microphotography. Different control experiments were performed with (1) biotinyl-GSH + GSH ( x 100) + silver, (2) strept-gold + silver, (3) biotinyl-GSH + silver, (4) silver only, and (5) no addition.

A modified double staining method (Owen et al, 1989) was used in order to get a satisfactory result. Cells were fixed with methanol 30 min at  $-20^{\circ}\text{C}$  after washing with CMF-PBS for 3 min x 2. After fixing, cells were washed with  $\text{H}_2\text{O}$  for 1.5 hr. Normal goat serum ( 1:50 ) was added and incubated with cells for 30 min at room temperature. After washing with  $\text{H}_2\text{O}$  for 10 min x 2, the primary antibody, rabbit immunoglobulins to cow GFAP, was added ( 1:100 ) and incubated with cells overnight at  $4^{\circ}\text{C}$ . The cells was washed with  $\text{H}_2\text{O}$  for 5 min x 3, and then the second antibody, goat anti-rabbit IgG-Rhodamine ( 1:100 ), was added. After an incubation of 60 min at room temperature, the cells were washed with  $\text{H}_2\text{O}$  for 10 min x 3. The colloidal gold method (as described above) was then performed. After double staining, the cells were finally mounted in a semipermanent mounting medium special for fluorescent antibody (Rodriguez & Deinhardt, 1960). Phase and immunofluorescence micrographs were recorded with a Zeiss Axiophot microscope.

## Results And Discussion

Colloidal gold decoration Because only one biotin molecule could be conjugated to each GSH molecule, i.e. there is no amplification effect as with the biotinyl-protein / -large peptide, which has much more  $-\text{NH}_2$  groups for biotin to combine with, biotinyl-GSH itself did not give a satisfactory cellular resolution. This problem was solved by taking advantage of the colloidal gold technique.

Colloidal gold -protein complexes have gained wide use as probes in electron and light microscopy procedures ( Roth, 1983; De Mey, 1984).The gold label is a discrete electron-dense, non-fading, red-colored marker capable of strong secondary electron emission, and the signal can be dramatically intensified by reacting with silver (Hacker et al, 1985; Jackson, 1987).

In the present study, the colloidal gold-protein used was streptavidin-gold, which could bind to biotinyl-GSH molecules held by their receptors in the cell membrane. On addition of silver enhancer, precipitation of metallic silver occurs, which enlarges the colloidal gold labels normally visible only at the electron microscope level, yielding high-contrast signals visible by light microscopy. The distribution of GSH receptors on cultured astrocytes was thus visible by labeling cells with biotinyl-GSH. Colloidal gold decoration of biotin-GSH-GSH receptors complex revealed that these receptors were on both

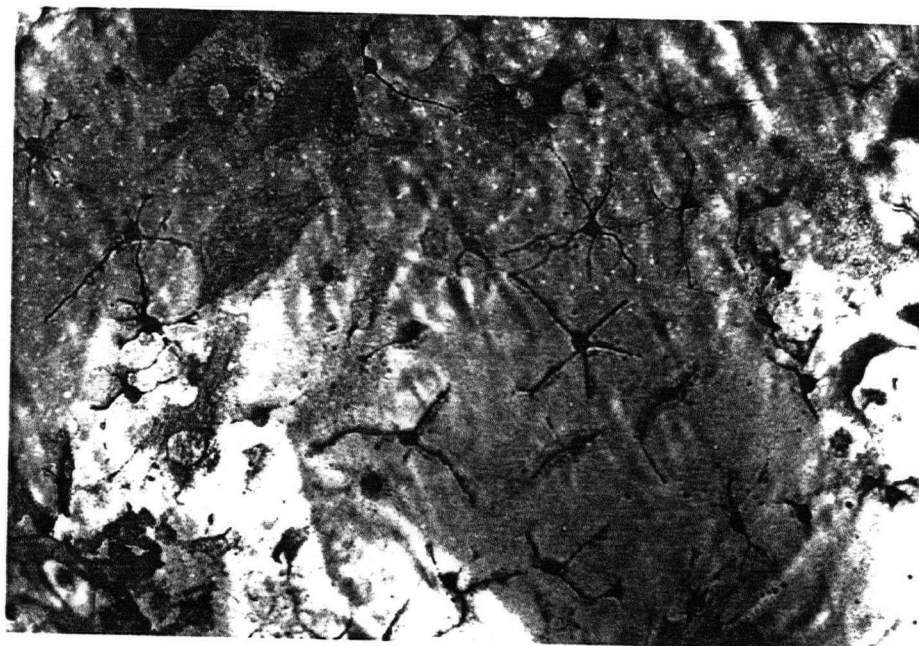


Fig. 17. Distribution of GSH receptors on astrocytes. Both cell bodies and their processes were labeled with biotinyl-GSH which is decorated with colloidal gold.



astrocytic cell bodies and their processes (Fig. 17). This result from the intact cell staining also suggests that GSH receptors exist on the cell membrane of astrocytes. All control groups gave a negative reaction.

GFAP double staining For primary culture, the results at confluence (2-3 weeks in culture) is a population of GFAP-positive cells in a monolayer which may comprise anything from 60-90% of the total number of cells present (Murphy & Pearce, 1987). Chief contaminants are macrophages and fibroblasts, followed by minor contaminants such as capillary endothelial cells, leptomeningeal cells, oligodendrocytes and neurons (usually in that order) (Murphy & Pearce, 1987).

Although the primary cultures used in this study contained an enriched population of astrocytes up to 90% (McCarthy & De Vellis, 1980), it was necessary to identify whether these colloidal gold positive cells, which were labeled with biotinyl-GSH, were wholly astrocytes or included some contaminants such as oligodendrocytes and neurons.

In vitro, the term "astrocytes" is synonymous with a CNS cell that labels with antibodies to glial fibrillary acid protein (GFAP) – the most commonly adopted criterion for positive astrocytes identification, because GFAP positive reaction (GFAP<sup>+</sup>) is restricted in distribution to astroglial cells in the CNS (Bignami & Dahl, 1977). In the present study, Rhodamine-labeled secondary antibody was used to demonstrate the presence of GFAP<sup>+</sup>.

Double staining with both GFAP/Rhodamine and biotinyl-GSH/colloidal gold showed that all cells which were stained by colloidal gold-silver were immunoactive to GFAP, i.e. those cells which possess GSH receptors were astrocytes (Fig. 18). Interestingly, not all the cultured astrocytes have GSH receptors. Fig. 19 shows that many astrocytes which are labeled with Rhodamine (GFAP<sup>+</sup> cells) are not stained with colloidal gold-silver. It seems that only morphologically mature astrocytes possessed GSH receptors and thus are decorated by colloidal gold. But many GFAP<sup>+</sup> cells, which look smaller and have not differentiated their processes, are colloidal gold negative, i.e. they do not have GSH receptors at that ontogenic stage.

The double staining used in the present study also revealed a very interesting phenomenon: in primary cultures, the growth direction of processes of an astrocyte tend towards those of the other astrocytes (Fig. 20).

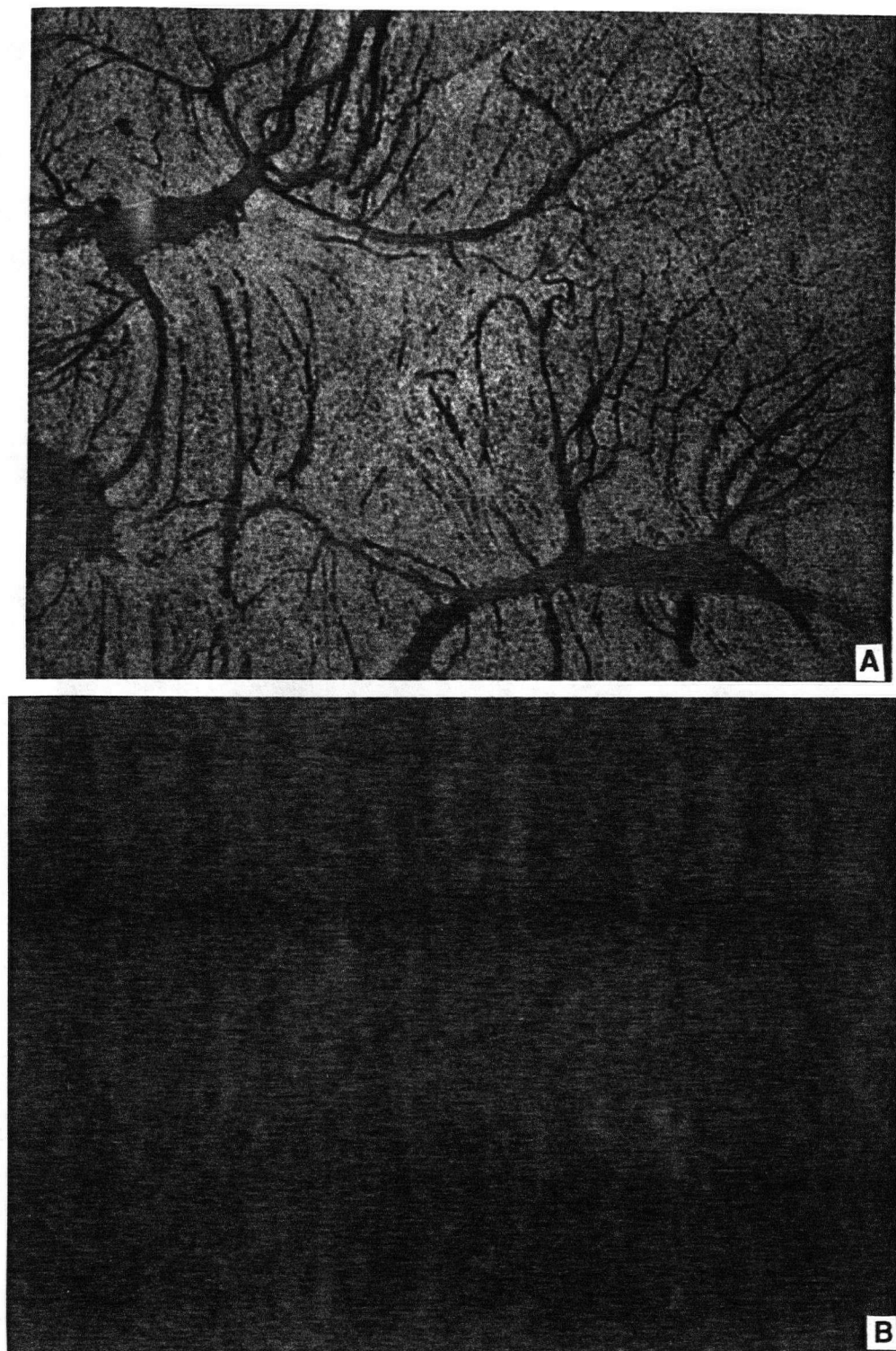


Fig. 18. Distribution of GSH receptors on astrocytes.

A: Colloidal gold decoration of GSH receptor. biotinyl-GSH complex on primary culture cells.

B: Cells in the same field as A were labeled with Rhodamine-antibody. These GFAP<sup>+</sup> cells confirm that GSH receptors exist on astrocytes.

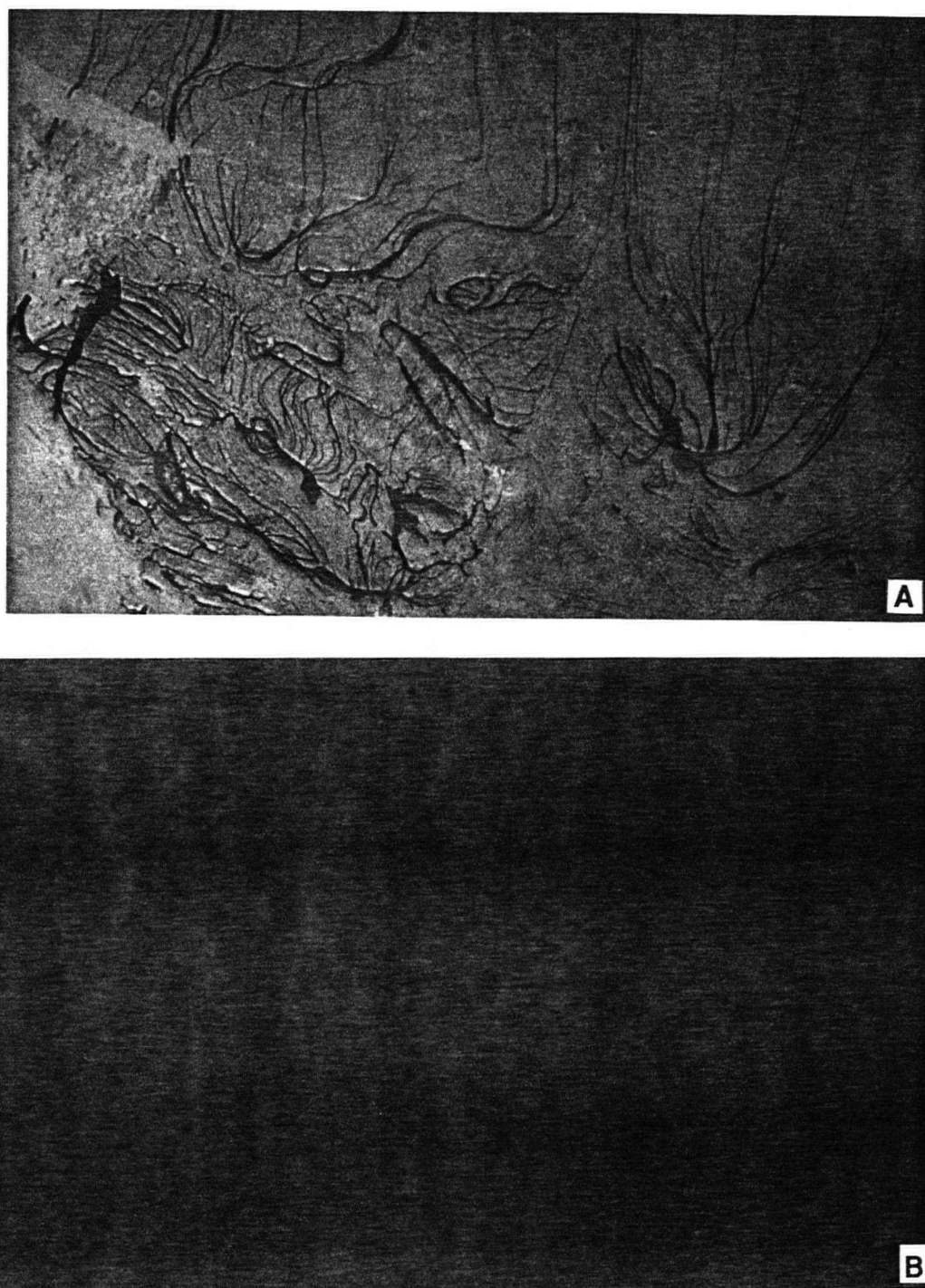


Fig. 19. Distribution of GSH receptors on astrocytes. A: Colloidal gold decoration of GSH receptors. B: Fluorescence double staining of the same field as A. Many GFAP<sup>+</sup> cells, which had no GSH receptors at that ontogenic stage, were Strept-gold<sup>-</sup>.

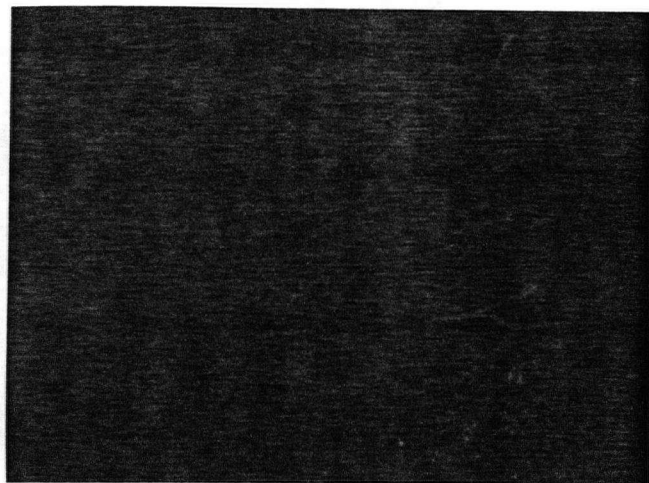
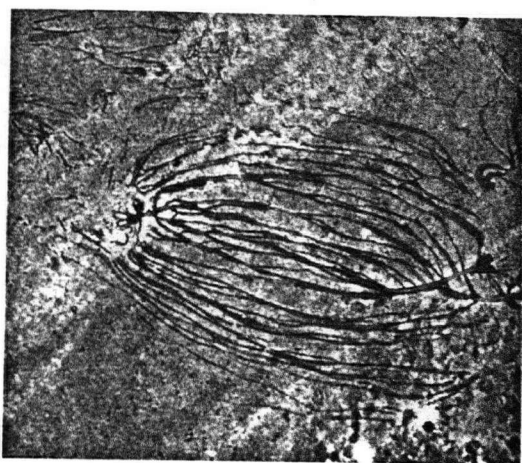


Fig. 20. The growth direction of processes of an astrocyte tends toward that of another. A: Colloidal gold decoration. B: Fluorescence double staining ( the same field as A).

## GENERAL DISCUSSION

High levels of GSH have been found in the brain. A reasonable assumption would be that GSH in the CNS plays the same detoxification function as it does in the peripheral system. But most evidence indicates that detoxification of compounds by their conjugation with GSH occurs primarily in the kidney and liver, while little detoxification seems to take place in the brain (Orlowski & Karkowsky, 1976). In rat brain, little activity of GSH S-alkyltransferase (Johnson, 1966) and GSH S-transferase B (Orlowski & Karkowsky, 1976) has been found. Also, rat cerebrum, cerebellum, and brain stem contain little GSH S-arene oxide transferase activity (Haykawa et al, 1974). Additionally, the decrease in GSH during brain ischemia in vivo was not accompanied by any reciprocal increase in GSSG (Rehncrona et al, 1980). This result thus fails to support the hypothesis that peroxidative damage occurs during or following ischemia, but implies that GSH plays a role other than antioxidant in the brain.

On the other hand, more and more evidence has shown that GSH may be involved in the functional processes of the CNS. The significance of GSH in brain function is suggested by the finding of mental retardation in patients with GSH metabolism problems in the nervous system (Orlowski & Karkowsky, 1976). Patients with GSH synthetase deficiency showed a gradual neurological deterioration of motor functions, retardation of movement, intention tremor and rigidity, and psychomotor retardation apparent in childhood (Jellum et al, 1983). Berl et al (1959) reported focal seizures in cat lesions associated with a significant decrease in GSH. It has also been reported that GSH is virtually absent in the nigra of patients with Parkinson's disease (Perry et al, 1982). Together with the finding of the different pattern of GSH levels in the brain during development (Berl & Purpura, 1963) and binding activity of GSH in the CNS (Ogita & Yoneda, 1987), available evidence suggests that GSH might be a neurally active peptide, acting either as a neuromodulator or neurotransmitter in the brain.

To address the question as to whether GSH is a neuropeptide in the CNS, one key point is to demonstrate whether there are GSH receptors in the CNS.

By focussing on studies of the localization and characterization of GSH receptors in the brain, this thesis reaches a positive conclusion that GSH may indeed be a candidate for a neuropeptide in the CNS. The evidence is summarized as follow: First, GSH binding sites in the brain were clearly revealed by binding of biotinyl-GSH (Fig. 5). The location of these binding sites suggested that GSH receptors might reside on glial cells in the CNS. Further experiments including radioligand receptor assays and cytochemical double staining confirmed that GSH receptors existed on astrocytes.

The binding assays performed on cultured astrocytes showed the GSH binding sites displayed the characteristics of a receptor, such as reversible and saturable binding (Fig. 14 & Fig. 9 -10), high affinity (Table 1), and ligand specificity (Fig.14 -16, Table 2). These results suggest that there are GSH receptors on astrocytes.

Results from the present study are also unlikely to be explained by binding of GSH with the receptors of another ligand in the CNS. Ogita et al ( 1986 ) reported that GSH produced a significant displacement of the  $\text{Na}^+$ -dependent and -independent bindings of  $[^3\text{H}]$ glutamic acid to the membrane preparation from rat brain. There was thus a possibility that the high GSH binding affinity demonstrated in this study might be due to the binding of GSH to Glu receptors on astrocytes. If this were the case, glutamic acid should show a strong displacement of  $[^{35}\text{S}]$ GSH binding. In fact, glutamic acid did not displace  $[^{35}\text{S}]$ GSH binding in astrocytes (Fig. 14). It is thus probable that GSH bind to its own, but not Glu receptors.

A well known function of GSH in peripheral systems is to act as an antioxidant with the association of enzymes (Fig. 1). Is it possible that the specific binding presented in this study reflected the binding of GSH to those enzymes in astrocytes? Results from this study and others also suggest it is unlikely ( because GSH S-transferase and GSH peroxidase are the ones associated directly with GSH in detoxification mechanism, only these two enzymes are discussed here):

- (1) Glutathione S-transferases, a group of dimeric proteins involved in the detoxification of a broad spectrum of xenobiotics, catalyze the conjugation of these compounds with the -SH group of GSH, thereby neutralizing their electrophilic sites and rendering the products more water-soluble for further metabolism and then excretion (Habig et al, 1974; Chasseaud, 1988;

Dekant et al, 1988) (Fig.1). The dissociation constant ( $K_d$ ) of GSH S-transferase A for GSH was found to be around  $7 \mu\text{M}$  with two binding sites each for one of the subunits of the enzyme (Jakobson et al, 1979) although the  $K_m$ s of different GSH S-transferases were reported from 0.2 to 2.0 mM (Habig et al, 1974). GSH peroxidases, which exert their antioxidant function by reducing hydroperoxides and forming glutathione disulfide GSSG, have a  $K_m$  for GSH of 3 mM in pig and 0.7 mM in human (Dolphin, 1989).

But the present study shows that there are two different GSH binding sites in astrocytes with the  $K_{d1}$  of 4.67 nM and  $K_{d2}$  of 35.14 nM, completely different from that of these enzymes (Table 3).

- (2) In the liver, which is GSH S-transferase rich, membrane bound microsomal glutathione transferases were found to be present only on the endoplasmic reticulum and mitochondrial membrane (Morgenstern & Depierre, 1988), and they reacted with oxidants that penetrated the cell or were generated inside the cell (Kosower & Kosower, 1989).

While results from both receptor assays and colloidal gold staining in the present study show that the GSH binding sites are on the cell membrane of astrocytes, it is likely that this binding is not an "enzyme binding", but a receptor binding.

- (3) Even if there is a possibility that some of such enzymes might exist in the cell membrane of astrocytes, the significant displacement of GSH binding by GSSG ( Fig. 13) suggests that it is not due to the binding of GSH to GSH peroxidases which are supposed to bind their substrate GSH to catalyze the formation of GSSG.
- (4) S-hexyl-GSH, a derivative of GSH, has a high affinity to GSH S-transferase and has been used as the " trap ligand " in affinity chromatography for purifying GSH S-transferases (Mannervik & Guthenberg, 1981; Tu et al, 1983; Reddy et al, 1983). If GSH did bind to S-transferases in the cell membrane of astrocytes, the addition of S-hexyl-GSH should easily inhibit the GSH binding. But the present study shows that S-hexyl-GSH is the lowest affinity analogue for the GSH binding sites in astrocytes, with an  $IC_{50}$  6 orders of magnitude higher than that of GSH, 3 orders higher than that of GSSG and S-methyl-GSH (Fig.15, Table 2). It thus reflects the different requirement between an enzyme for its substrates and a receptor for its ligands, and supports the " receptor hypothesis ", i.e. GSH binds to its receptors in the cell membrane of astrocytes.

Table 3. Comparison of kinetic parameters of reactants for GSH.

Reactants	$K_d$ (I)	$K_d$ (II)	$K_m$
GSH receptor	$4.67 \pm 0.75^d$	$35.14 \pm 2.17 \text{ nM}^d$	
S-transferase A*	$7.06 \pm 1.47 \mu\text{M}^a$	$7.06 \pm 1.47 \mu\text{M}^a$	$0.2 \text{ mM}^b$
S-transferase B	—	—	$0.2 \text{ mM}^b$
S-transferase C	—	—	$0.1 \text{ mM}^b$
S-transferase E	—	—	$0.2 \text{ mM}^b$
GSH peroxidase	—	—	$3.0 \text{ mM}^c$

\* Theoretically, in binding kinetics studies, the dissociation constant  $K_d$  is calculated the same way as the  $K_m$  in an enzymatic kinetics study. The difference between  $K_d$  and  $K_m$  for the same enzyme GSH S-transferase A might be caused by different researchers using different measuring methods.

<sup>a</sup> From Jakobson et al ( 1979 ).

<sup>b</sup> From Habig et al ( 1974 ).

<sup>c</sup> From Dolphin (1989).

<sup>d</sup> From the present study.



The result from the binding assay in the present study differs from that reported by Ogita and Yoneda (1987). The present study shows that the  $K_D$ s of GSH binding site I and II on astrocytes are in the nM range (Fig. 10; Table 1), while Ogita and Yoneda reported the  $K_D$ s of GSH binding sites in synaptic membranes in the  $\mu$ M range ( $K_{D1} = 0.76 \mu$ M,  $K_{D2} = 11 \mu$ M). One explanation for such a difference might be that GSH receptors on astrocytes have a different affinity from those in synaptic membranes. It is also possible that the high  $K_D$  values reported by Ogita and Yoneda might reflect the GSH binding characteristics of some enzymes but not the receptors. For the following reasons we are inclined to believe that such is the case:

- (1) The method Ogita and Yoneda used to prepare synaptic membranes produced a mitochondrial fraction (Ogita & Yoneda, 1987; Yong & Snyder, 1973). Since mitochondria in liver and kidney are rich in GSH S-transferases and GSH peroxidases, the membrane preparation used by Ogita and Yoneda might reflect the binding characteristics of these enzymes.
- (2) The  $K_D$  of GSH S-transferase A for GSH is  $7.06 \mu$ M (Jakobson et al, 1979) and the values obtained by Ogita and Yoneda are also in the  $\mu$ M range ( $K_{D1} = 0.76 \mu$ M,  $K_{D2} = 11 \mu$ M).
- (3) Ogita and Yoneda reported that no significant radioactivity was detected in the spots corresponding to the control L-Glu during the ligand incubation period, and thus concluded that the binding was not the association of GSH with its enzymes. But, the ligand they used was glycine-2- $[^3\text{H}]$ GSH, so we do not think the Glu residue could be detected if there is a degradation of GSH catalyzed by enzymes during the binding period.
- (4) Based on the displacement of GSH binding by GSH derivatives such as S-methyl-GSH and S-hexyl-GSH, which do not have the -SH moiety, Ogita and Yoneda suggested that the binding was not the association of GSH with the related enzymes.

Nevertheless, substrates binding to the active sites of enzymes may occur through the formation of specific noncovalent bonds (in some instances, covalent bonds) (Smith et al, 1983), and the binding of enzymes with GSH and its analogues does not depend exclusively on the -SH structure (Fig. 21). In fact, S-hexyl-GSH, which maintains a high affinity to GSH S-transferases, has been successfully used as the "trap ligand" in affinity chromatography for purifying GSH S-transferases (Mannervik & Guthenberg, 1981; Tu et al, 1983; Reddy et al, 1983). The S-hexyl-GSH displacement ( $IC_{50} = 22.5 \mu$ M) reported by Ogita and Yoneda does not

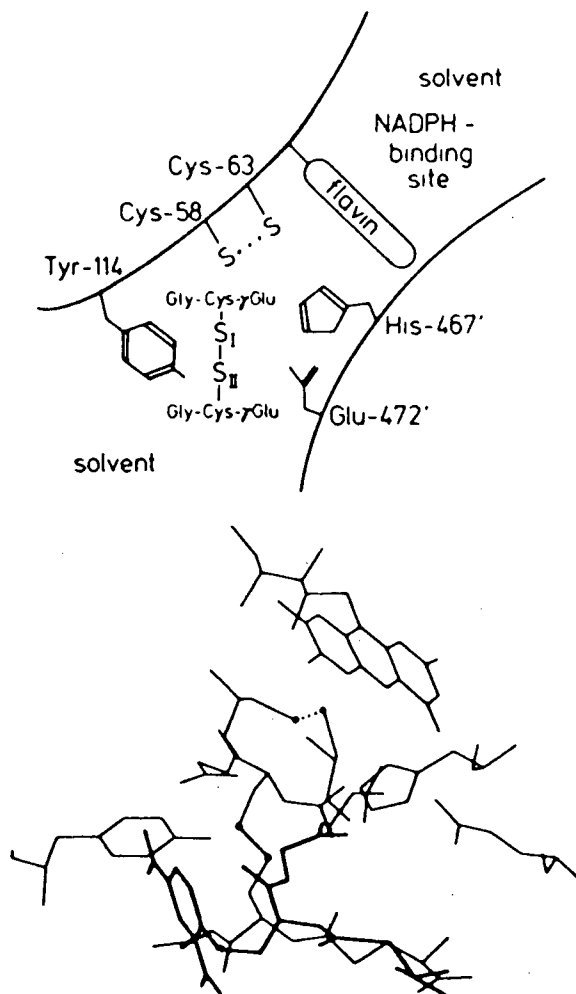


Fig. 21. GSSG binding site of GSSG reductase (top) and the binding of S-(2,4 - dinitrophenyl)glutathione (thick lines) at this site (bottom) (from H. Sies, 1988).

support their conclusion, but implies a binding of GSH to GSH S-transferases in the membrane preparation.

Although the data from the binding assays in the present study demonstrates a saturable and high-affinity GSH binding in astrocytes, an uptake system may also show such characteristics. The synaptic inactivation of several biogenic amine neurotransmitters including dopamine, norepinephrine and serotonin occurs primarily through their reuptake by a sodium-dependent, high-affinity carrier system into the nerve terminals which release the neurotransmitter (Sandberg & Coyle, 1985). The existence of a sodium-dependent, high-affinity choline transport process has also been demonstrated on cholinergic nerve terminals (Kuhar & Murrin, 1978). Ligand-binding techniques showed that these carriers mediating the uptake processes for biogenic amines bound to their competitive inhibitors with high affinity and in a saturable manner (Sandberg & Coyle, 1985; Lee et al, 1982; Javitch et al, 1983). It is thus possible that the high affinity GSH binding in astrocytes is due to similar uptake processes. We do not, however, feel that this is the case for the following reasons:

- (1) Uptake requires energy (Simon & Kuhar, 1976; Kuhar & Murrin, 1978) and is sodium-dependent (Simon & Kuhar, 1976; Pessin & Adler, 1985; Sandberg & Coyle, 1985). Uptake is completely inhibited in  $\text{Na}^+$ -free medium (Pessin & Adler, 1985). In the present study, colloidal-gold staining was performed on fixed cells in a  $\text{Na}^+$ -free medium. The positive colloidal-gold decoration of GSH binding under the conditions of no energy supply and sodium absent suggests that the binding may be receptor binding, but not uptake site binding.
- (2) The equivalent pore radius in different biological membranes ranges between 0.35 - 0.8 nm (DeRobertis et al, 1975), while avidin used in the present study is linked to colloidal gold particles of 5 nm mean diameter (Amersham International plc, Amersham, UK). Even if GSH uptake could take place in astrocytes, the avidin-gold complex can not pass through the cell membrane unless there is a specific transport system (see discussion below).
- (3) There is no doubt that under certain conditions large molecules, such as certain proteins, penetrate the cell because a living cell has transport systems ( active transport, endocytosis, pinocytosis etc.) by which solid or fluid materials are ingested (DeRobertis et al, 1975; Lehninger, 1970). For example, HRP (horseradish peroxidase), with a molecular weight of 40,000,

can be absorbed into neurons by endocytosis or phagocytosis and transported in axons (Kokotas et al, 1978; LeVay & Voigt, 1990). In the present study, however, the colloidal gold staining showed a positive reaction (Fig.18a; 19a; 20a) after the cells were fixed in the first step (Materials and Methods, part 3). Because endocytosis or phagocytosis will not happen in a dead cell, avidin, the tracer molecule used in the present study with a molecular weight of 68,000 (Hofmann & Finn, 1985), can not pass through the cell membrane freely. Thus, if GSH uptake occurred in astrocytes the colloidal gold staining should give a negative result. The positive colloidal gold decoration shown in the present study does not support the "uptake hypothesis", but rather suggests that GSH receptors are membrane receptors on astrocytes.

Implicit in all studies of the putative GSH receptors that bind GSH and meet most criteria for a receptor is the assumption that this binding results in a biological response, i.e., there should be a certain cellular response related to the receptor occupancy. To confirm the GSH receptor serves a physiological role in the CNS, the related physiological and biochemical processes (such as G-protein coupling, activation of second messenger systems, regulation of protein kinases,  $\text{Ca}^{2+}$  release, gene expression, etc.) following the signal-receptor reaction would need to be demonstrated by further studies.

In fact, a growing number of receptors for neuropeptides have been found in astrocytes, including VIP, somatostatin, ACTH,  $\alpha$ - and  $\beta$ -MSH, opioid, substance P, oxytocin, vasopressin, neurokinin, etc. (Wilkin & Cholewinski, 1988; Murphy & Pearce, 1987). Glial cells are now credited with many properties thought some years ago to be exclusively a domain of neurons. For instance, almost every channel found in neurons is known to occur in glial cells including potassium, sodium, chloride, and calcium channels (Walz, 1989). Further evidence is the presence of the release of neuroactive substances by glial cells (Martin et al, 1986; Philibert et al, 1988). In addition, astrocytes appear to be the target of signals released by neurons (Murphy & Pearce, 1987; Kimelberg, 1988). Evidence obtained from studies using techniques such as receptor binding and electrophysiological recording makes it quite clear that glial cells maintained in culture have receptor sites for the majority of CNS neurotransmitters such as noradrenaline, acetylcholine, serotonin, dopamine, histamine, glutamate, and GABA (Murphy & Pearce 1987; Pearce & Murphy 1988; Kettenmann et al, 1988). The present study is the first to report the

presence of GSH receptors on astrocytes. Based on receptor binding assays and cytochemical experiments, this study not only depicts the biochemical characteristics of GSH receptors in the brain, but also demonstrates the receptor at the cellular level. These results suggest that GSH might be a neuroactive signaling substance in the CNS.

According to Schwartz (1985a), the nervous system makes use of two main classes of chemical substances for signaling: (1) small-molecule transmitters and (2) neuroactive peptides. Schwartz (1985a) defined a transmitter as a substance that is released synaptically by one neuron and that affected another cell (neuron or effector organ) in a specific manner. Further, Schwartz (1985a) and Zhou (1988) also suggested that a substance would not be accepted as a transmitter unless the following criteria were met:

- (1) It is synthesized in the neuron from a precursor (that stems from the major cycles of intermediary metabolism) by the biosynthetic enzyme system.
- (2) It is stored in synaptic vesicles in the presynaptic terminal, and released at the synapse through the process of exocytosis.
- (3) After release, it reacts with the postsynaptic receptors thus opening ion channels and producing an EPSP or IPSP in the postsynaptic cell membrane.
- (4) A specific mechanism (diffusion, enzymatic degradation, or reuptake) exists for removing it from the synaptic cleft to terminate synaptic transmission.

Neuropeptides consist of short chains of amino acids. Differing from classical transmitters, which are usually synthesized locally at terminals (Zhou, 1988; Schwartz, 1985a), neuropeptides are synthesized on polyribosomes in the cell body. Some of them are grouped in families, i.e. several different neuropeptides are encoded by a single continuous mRNA, which is translated into one large poly-functional protein precursor (Sossin et al, 1989; Zhou, 1988; Gong, 1985; Schwartz, 1985a). After the post-translational processing, such as specific cleavages, amidation, tyrosine sulfation, phosphorylation, and glycosylation of a precursor, the active neuropeptide is transported to terminals and released from secretory granules (Sossin et al, 1989; Schwartz, 1985a). After release, neuropeptides may diffuse over a relatively long distance before affecting target cells and thus neuropeptide messengers often have more prolonged actions and can function at a distance from release sites (Iversen, 1984; Sossin et al, 1989). Neurotransmitters may represent the predominant mechanism used for the fast point-to-point transmission in the

CNS (Iversen, 1984), while neuropeptide messengers most often act as neuromodulators on target cells (Sossin et al, 1989; Iversen, 1984), affecting RNA and protein synthesis, neurotransmitter release, growth and differentiation, or neuronal metabolism (De Graan et al, 1990).

By comparing the two classes of chemical substances, Schwartz suggested that small-molecule transmitters differed from neuropeptides in the mode of synthesis, as well as in the mode of inactivation after release: Transmitters can be synthesized by enzymes locally at terminals, while peptides are formed only in the cell body (Schwartz, 1985a); neurotransmitter systems have specific degrading or reuptake systems to remove the molecule from the synaptic cleft, but little is currently known about the way neuroactive peptides are removed, and no uptake mechanism for peptides has yet been described (Schwartz, 1985b). Based on these differences and the criteria for classical transmitters, Schwartz thought that no neuropeptide had met all the criteria (Schwartz, 1985a), and suggested that all accepted transmitter substances were those small molecule amines (dopamine, noradrenaline, serotonin, and histamine), acetylcholine, and mono-amino acids (GABA, glycine, and glutamic acid) (Schwartz, 1985a).

In spite of this, much of the present evidence now indicates that neurotransmitters should not be confined to these small-molecule classical transmitters, i.e. a neuropeptide may also be a neurotransmitter. For instance, substance P (SP), a neuropeptide which produces slow EPSP (excitatory postsynaptic potential) in the inferior mesenteric ganglion and dorsal horn neurons, has been suggested as a sensory transmitter in primary afferent neurons (Otsuka & Konishi, 1983) because SP fulfills many of the criteria for transmitter identification: (1) SP is synthesized and stored within central and peripheral neurons (Otsuka & Konishi, 1983; Jessell, 1983), (2) SP is released from nerve terminal in a conventional manner (Jessell, 1983; Otsuka & Konishi, 1983), and (3) SP released from peripheral branches of primary afferents produces a non-cholinergic slow EPSP in the inferior mesenteric ganglia, and SP released from central terminals of certain primary afferent neurons produces an excitatory effect on spinal dorsal-horn neurons (Otsuka & Konishi, 1983). More evidence that neuropeptides can serve as neurotransmitters has been reported: Jan & Jan (1983) demonstrated that a LHRH-like peptide is the transmitter mediating the late slow EPSP in bullfrog sympathetic ganglia.

An emerging view is that the criteria for classical transmitter are no longer sufficient to define neurotransmitters. Neurotransmitters have been found not

only affecting membrane excitability by acting directly on ligand-gated ion channels, but also inducing the activation of adenylyl cyclase (Friele et al 1988; Gilman, 1987) and the breakdown of inositol phospholipid (Nicoletti et al, 1986; Sladeczek et al, 1985) through the coupling of their receptors with G proteins (Gilman, 1987). A neurotransmitter can also affect ion channels indirectly via GTP-binding proteins (Bormann, 1988). Additionally, a classical neurotransmitter may perform a function characteristic of a neuropeptide. Dopamine is considered as a retinal neuromodulator because it does not affect directly the membrane potential of horizontal cells, but activates an intercellular enzyme system, i.e. its principal physiological effect is mediated by an intracellular second messenger (Dowling, 1986).

Because GSH is a peptide, we suggest that it might be a new candidate for a neuroactive peptide. As discussed above, a neuropeptide may act as a modulator or a transmitter, sometimes the same peptide may have roles as a modulator and a transmitter in the same tissue (Lundberg & Hokfelt, 1983; Otsuda & Konishi, 1983; De Graan et al, 1990). Thus, the exact function of GSH, i.e. whether it acts as a neuromodulator (affecting RNA and protein synthesis, neurotransmitter release, etc.) or as a neurotransmitter (affecting ion channels directly or indirectly, or activating the second messenger systems through the coupling of their receptors with G-proteins), requires further study.

The function of mature astrocytes in the CNS and the influence of neurochemicals on these cells has been extremely difficult to define. Only three functions of astrocytes have been firmly established: (1) astrocytes guide both the migration of neurons and the growth of their processes during ontogenesis (Rakic, 1972), (2) astrocytes become phagocytic in response to brain trauma (Bunge et al, 1962), and (3) astrocytes proliferate and hypertrophy to form glial scars (Schultz & Pease, 1959).

Glial functions currently under investigation include (a) influence of the differentiation of neurons in developing tissue (Varon & Somjen, 1979), (b) synthesis, uptake, metabolism and release of neurotransmitters (Kimelberg & Pelton, 1983; Schrier, 1978; Villegas, 1978; Yu & Hertz, 1982), (c) maintenance of the proper ionic environment for neural communication and nutrient factors necessary for neuronal functions (Lindsay, 1979; Varon & Somjen, 1979), and (e) modulation of the extracellular distribution of neurotransmitters and ions by changes in cell shape and volume (Salm et al, 1985).

The importance of the finding of the present study that GSH receptors are present on astrocytes is two fold: It adds another neuropeptide or neurotransmitter to the list of neuroactive signaling substances in the CNS. Further, the biochemical cascades after the GSH-receptor reaction may help to reveal the mechanism of neurochemicals on glial cells and to understand the glial cell function.



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