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# The dynamics of cysteine, glutathione and their disulphides in astrocyte culture medium

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**Glutathione (GSH) plays an important neuroprotective role, and its synthesis depends on the amount of available cysteine (CSH) in the cells. Various kinds of evidence suggest that astrocytes can provide CSH or GSH to neurons, but the delivery mechanism of the thiol-compounds has not been elucidated. In this study, the dynamics of CSH, GSH and their disulphides in astrocyte culture medium were investigated by following the time-course of concentration changes and by computer simulation and curve fitting to experimental data using a mathematical model. The model consists of seven reactions and three transports, which are grouped into four categories: autoxidation of thiols into disulphides, thiol–disulphide exchange and reactions of thiols with medium components, as well as the cellular influx and efflux of thiols and disulphides. The obtained results are interpreted that cystine (CSSC) after entering astrocyte is reduced to CSH, most of which is released to medium and autoxidized to CSSC. The efflux of GSH was estimated to be considerably slower than that of CSH, and most of the excreted GSH is converted to cysteine–glutathione disulphide principally through the thiol–disulphide exchange. The results seem to indicate that astrocytes provide neurons mainly with CSH, rather than GSH, as the antioxidant material for neuroprotection.**

*Keywords*: astrocyte/cysteine/glutathione/high-performance liquid chromatography/mathematical model.

*Abbreviations*: CSH, cysteine; CSSC, cystine; CSSG, cysteine–glutathione disulphide; DMEM, Dulbecco's modified Eagle medium; FDNB, 1-fluoro-2,4-dinitrobenzene; γ-GT, γ-glutamyl transpeptidase; GSH, glutathione; GSSG, glutathione disulphide; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline.

Glutathione (GSH) is an essential cellular antioxidant. GSH reacts non-enzymatically with free radicals and acts as the electron donor in the reduction of peroxides catalysed by glutathione peroxidase (*1*). In the brain of animals, a relatively large amount of $O_2$ is consumed (*2*), which leads to continuous production of reactive oxygen species including peroxides. It has been thought that GSH plays a key role also for the defence of brain against oxidative stress (*3*), and actually, GSH depletion causes a serious disorder in central and peripheral nervous systems (*4*).

GSH is a tripeptide composed of glutamate, cysteine (CSH) and glycine. CSH is a rate-limiting precursor for GSH synthesis, and the cellular GSH level is significantly affected by the supply of CSH (*5*). Neurons require CSH for GSH synthesis. When cultured separately from astrocytes, neurons decrease CSH and GSH contents, and therefore the provision of thiols, such as CSH or GSH, from astrocytes has been considered necessary to maintain GSH levels in neurons (*6*). However, it has not been still cleared by what pathways CSH or GSH are provided to neurons. Because CSH is highly reactive, it is difficult to speculate its dynamics in cell culturing medium. Dringen *et al.* (*6*) have reported that GSH released from astrocytes is possibly converted to CysGly by the action of an ectoenzyme, γ-glutamyl transpeptidase (γ-GT) and that the dipeptide, CysGly, is hydrolyzed and used as the GSH precursor by neurons. However, there is no evidence for the existence of CysGly in cerebrospinal fluid and astrocytes culturing medium. Wang and Cynader (*7*) have proposed that GSH released from astrocytes reacts with extracellular cystine (CSSC), producing CSH in the medium. This CSH is thought to be provided to neurons as a GSH precursor. On the other hand, Guebel and Torres (*8*) concluded from theoretical inspection using a model that CSH is directly effluxed from astrocytes and is provided to neurons, because the uptake and reduction of CSSC in astrocytes are greater than their CSH consumption.

In this study, as a first step to understand the dynamics of CSH, GSH and their disulphides between astrocytes and neurons, we have attempted to evaluate their reactions quantitatively in the astrocyte culturing medium. We have constructed a mathematical model describing chemical reactions and transports of thiols and disulphide in the cell culturing medium, and the kinetic parameters were optimized to fit the experimental data. The results indicate that astrocytes export CSH faster than GSH by one order of magnitude, suggesting that astrocytes could predominantly provide CSH for neurons.

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## Materials and Methods

### *Materials*

Dulbecco's modified Eagle medium (DMEM) was obtained from Invitrogen. Foetal bovine serum (FBS) was from Thermo Fisher Scientific Inc. Cysteine–glutathione disulphide (CSSG) was obtained from Toronto Research Chemicals (North York, Canada) to be used as high-performance liquid chromatography (HPLC) standard.

### *Cell culture*

Astrocytes were prepared and cultured as described previously (*9*). The cultures were grown to confluency in DMEM containing 10% FBS under 5% $CO_2$ at 37°C. The culture medium was changed every 5 days, and the cells were used after 10 days of culture.

### *Time-course experiments*

To follow the autoxidation of thiols in phosphate-buffered saline (PBS) or DMEM without the cells, each concentration of CSH or GSH was solved in 200 µl PBS or 250 µl CSSC-free DMEM, and the solution was incubated 37°C. The medium used in this work contained 10% FBS. At given intervals, portions of the solution were treated for HPLC analysis, as described below. Each experiment was performed in duplicate.

For experiments using cell culture, astrocytes were plated on 60-mm diameter dishes and were incubated for 3 days to become confluent. On the day before the experiment, the medium was changed. Then the cells were washed once with PBS containing 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 5.6 mM glucose and were washed once with DMEM containing 50 µM CSSC. The reaction was started by giving the cells 5 ml of DMEM containing 50 µM CSSC at 37°C. At given intervals, 250 µl of portions were sampled and treated for HPLC analysis, as described below. To examine the influence of reagents affecting the CSSC influx, the cells were washed once with PBS containing 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 5.6 mM glucose and were washed with DMEM containing 50 µM CSSC. Then the cells were incubated at 37°C with 5 ml of DMEM containing 50 µM CSSC and 2.5 mM glutamate or 1 µM $CdCl_2$. The experiment was conducted in triplicate.

### *Derivatization for HPLC of thiols and disulphides*

Before analysis of thiols and disulphides by HPLC, free thiol groups in the samples were blocked with iodoacetic acid and were then reacted with 1-fluoro-2,4-dinitrobenzene (FDNB) to produce *N*-dinitrophenyl derivatives. The samples in PBS were treated according to Wang and Cynader (*7*). To analyse the samples in DMEM, 12.5 µl of 70% perchloric acid was added to each of 250 µl solution. The precipitated protein was removed by centrifugation at 10,000 *g* for 10 min at 0–4°C. The supernatant (200 µl) was treated immediately with 25 µl of a 100 mM fresh iodoacetic acid solution and then neutralized with ~0.035 g of $NaHCO_3$. After incubation for 30 min at room temperature, 225 µl of 1% (v/v) FDNB in ethanol was added and the mixture was stored in the dark at 4°C for 24 h or longer. Thereafter, 25 µl of 1 M lysine was added to eliminate the unreacted FDNB and the samples were stored at 4°C for 12 days or less until analysis by HPLC.

### *HPLC analysis*

HPLC was performed using Shimadzu LC-10Ai system at room temperature. The Inertsil $NH_2$ column, with a particle size of 5 µm and dimensions of 4.0 × 250 mm, was obtained from GC Science (Tokyo). Solvent A was 80% methanol in water, and Solvent B was 0.8 M sodium acetate in 64% methanol (*10*). The stored samples were centrifuged at 10,000 *g* for 20 min at 2°C, and 20 µl each of the supernatant was applied. The mobile phase was maintained at 80% A/20% B for 7.5 min, followed by a gradient elution to 1% A/99% B for 15 min, and was held for 5 min at 1% A/99% B. Finally, elution was held at 80% A/20% B for 5.5 min. The flow rate was 1.0 ml $min^{-1}$, and the elution was monitored at 365 nm. The standard mixtures containing 20 µM CSH, GSH, CSSC, GSSG and CSSG were applied three times, and the averages of peak areas were calculated.

### *Analysis based on the mathematical model*

A metabolic simulator, GEPASI 3 (*11*), was used for simulation and curve fitting using the mathematical model. The chemical and

**Table I. Reactions and transports of thiol and disulphide compounds considered for the astrocyte culturing medium.**

| Reaction | Stoichiometry | Rate formula ($M\,s^{-1}$) | Rate constant |
|---|---|---|---|
| CSH autoxidation in PBS, pH 7.4 | 2CSH → CSSC | $k_C$ (PBS) [CSH] | $(5.7 \pm 0.7) \times 10^{-5}$ |
| GSH autoxidation in PBS, pH 7.4 | 2GSH → GSSG | $k_G$ (PBS) [GSH] | $(3.8 \pm 0.7) \times 10^{-5}$ |
| Reaction 1: CSH autoxidation | 2CSH → CSSC | $k_C$ [CSH] | $(3.6 \pm 0.1) \times 10^{-5}$ |
| Reaction 2: GSH autoxidation | 2GSH → GSSG | $k_G$ [GSH] | $(2.2 \pm 0.3) \times 10^{-5}$ |
| Reaction 3: Mixed autoxidation | CSH + GSH → CSSG | $k_{CG}$ [CSH] [GSH] | $(9.7 \pm 0.7) \times 10^{-1}$ |
| Reaction 4: CSH with medium | CSH→ | $k_{M\text{-}C}$ [CSH] | $(5.3 \pm 0.9) \times 10^{-5}$ |
| Reaction 5: GSH with medium | GSH→ | $k_{M\text{-}G}$ [GSH] | $(4.6 \pm 0.2) \times 10^{-5}$ |
| Reaction 6: GSH-CSSC exchange | GSH + CSSC ↔ CSH + CSSG | $k_1$ [GSH] [CSSC] − $k_2$ [CSH] [CSSG] | $k_1 = 10$, $k_2 = 3.2$ (Ref. *12*) |
| Reaction 7: GSH-CSSG exchange | GSH + CSSG ↔ CSH + GSSG | $k_3$ [GSH] [CSSG] − $k_4$ [CSH] [GSSG] | $k_3 = 9.0$, $k_4 = 7.5$ (Ref. *12*) |
| Transport 1: CSSC influx | Astrocyte + CSSC → Astrocyte | $k_{A\text{-}CC}$ [A] [CSSC] | $(6.6 \pm 0.5) \times 10^{-5}$ |
| Transport 2: CSH efflux | Astrocyte → Astrocyte + CSH | $k_{A\text{-}C}$ [A] | $(4.0 \pm 0.4) \times 10^{-9}$ |
| Transport 3: GSH efflux | Astrocyte → Astrocyte + GSH | $k_{A\text{-}G}$ [A] | $(2.7 \pm 0.5) \times 10^{-10}$ |

The square brackets show molar concentrations of the compounds, except that [A], the concentration of astrocyte, was expressed in mg cell protein/ml. The units of rate constants are: $s^{-1}$ for $k_C$ (PBS), $k_G$ (PBS), $k_C$, $k_G$, $k_{M\text{-}C}$, and $k_{M\text{-}G}$; $M^{-1}s^{-1}$ for $k_{CG}$, $k_1$, $k_2$, $k_3$ and $k_4$; (mg cell protein/ml)$^{-1}s^{-1}$ for $k_{A\text{-}CC}$; M (mg cell protein/ml)$^{-1}s^{-1}$ for $k_{A\text{-}C}$ and $k_{A\text{-}G}$.

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transport reactions considered and their rate equations are shown in Table I. The unknown parameter values were estimated by the least-square method using the simplex method, and the uniqueness of the fitted values was confirmed by starting from several different initial values.

## Results

In culture media, thiols are susceptible to various reactions depending on conditions. Among them, the following reactions are thought to be principal: autoxidation of thiols by dissolved $O_2$, reactions between thiols and culture medium components, and thiol–disulphide exchange reaction. As for these reactions, kinetic properties of the exchange reactions under physiological conditions have been investigated in detail by Jocelyn (*12*), but those of other two reactions are still not well established. Therefore, prior to assay and numerical analyses on cell culturing medium, we tried to find rate equations and constants for the reaction taking place in the cell-free medium.

### *Autoxidation of CSH and GSH in PBS*

Figure 1A and B show the time course of CSH and GSH autoxidation in PBS at pH 7.4 and 37°C under air, respectively. As the concentrations of CSH or GSH decreased, formation of stoichiometric amounts (1/2 mol) of disulphides, CSSC or glutathione disulphide (GSSG), was observed. The formation of oxidized products other than disulphides was not examined in this study. It has been reported that thiols are oxidized by $O_2$ only to the disulphides at neutral pH (*13*, *14*) and that the more oxidized products such as sulphinic and sulphonic acids are formed appreciably only at alkaline pH (*15*). In any case, if such reactions take place in the present experiments, they will be taken as a part of Reactions 4 and 5 in the mathematical model described below (Table I). As shown in Fig. 1A, the observed time course of CSH oxidation was well fitted by assuming the first-order kinetics. The obtained values for the rate constants were $6.2 \times 10^{-5}$ and $5.2 \times 10^{-5}\,s^{-1}$ at initial CSH concentrations of 50 and 100 µM, respectively, and the average value of $5.7 \times 10^{-5}\,s^{-1}$ was used for simulation (Table I, CSH autoxidation in PBS). With GSH of concentrations higher than 100 µM, a lag phase was observed in the product (GSSG) formation (data not shown), but at concentrations <50 µM, no such lag was observed, as shown in Fig. 1B. As shown in the figure, the reactions were fitted well by assuming the simple first-order kinetics. The obtained values for rate constants were $4.3 \times 10^{-5}$ and $3.3 \times 10^{-5}\,s^{-1}$ at initial GSH concentration of 25 and 50 µM, respectively, and the average value of $3.8 \times 10^{-5}\,s^{-1}$ was used for simulation (Table I, GSH autoxidation in PBS).

The $pK_a$ values of CSH and GSH thiols are 8.3 and 8.7, respectively, at 25°C. In PBS at pH 7.4, ~11 and 5% of CSH and GSH are calculated to be present in the thiolate form. In the present experiments, though the temperature was somewhat higher (37°C), the thiols can be ionized more or less to similar extents. In general, the thiols are autoxidized faster at higher pH, but their pH dependencies are reported to be rather complex (*15*).

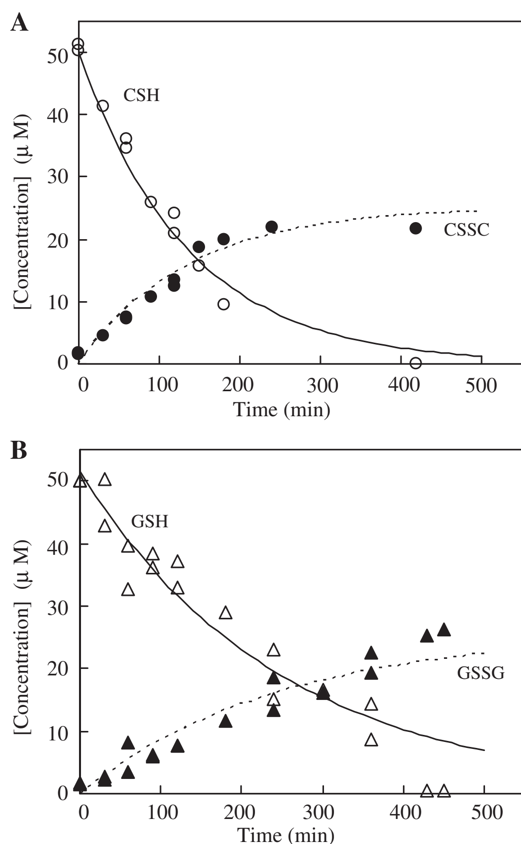


**Fig. 1 The time course of concentration changes of thiols (CSH and GSH) and disulphides (CSSC and GSSG) during autoxidation in PBS at pH 7.4.** About 50 µM each of (A) CSH and (B) GSH were incubated in PBS at 37°C. Symbols and lines show the experimental and theoretical values, respectively. The theoretical values were obtained by least-square curve fitting using GEPASI 3 with $k_C$ (PBS) or $k_G$ (PBS) as a variable parameter.

### *Reactions of CSH and GSH in DMEM*

Figure 2A and B show the time course of CSH and GSH concentration changes in CSSC-free DMEM in the absence of the cells. Unlike the reactions in PBS (Fig. 1), the formation of CSSC or GSSG was significantly less than that expected from the stoichiometry. After 8–9 h, CSSC and GSSG concentrations reached only ~50% of the stoichiometric values. It is thought that CSH and GSH also undergo other reactions, such as those with culture medium components, and first-order reactions for the disappearance of CSH and GSH were added to the scheme (Reactions 4 and 5 in Table I). As shown in Fig. 2, the reaction time courses were fitted well to those calculated based on Reactions 1 and 4 for CSH, or Reactions 2 and 5 for GSH by adjusting the respective two parameter values. The values of the rate constants for autoxidation in the medium, $k_C$ and $k_G$, were somewhat smaller than those in PBS. The values of the rate constants, $k_{M\text{-}C}$ and $k_{M\text{-}G}$, were comparable with those of $k_C$ and $k_G$, which indicates that other reactions are not negligible in considering the thiol dynamics in the culture medium.



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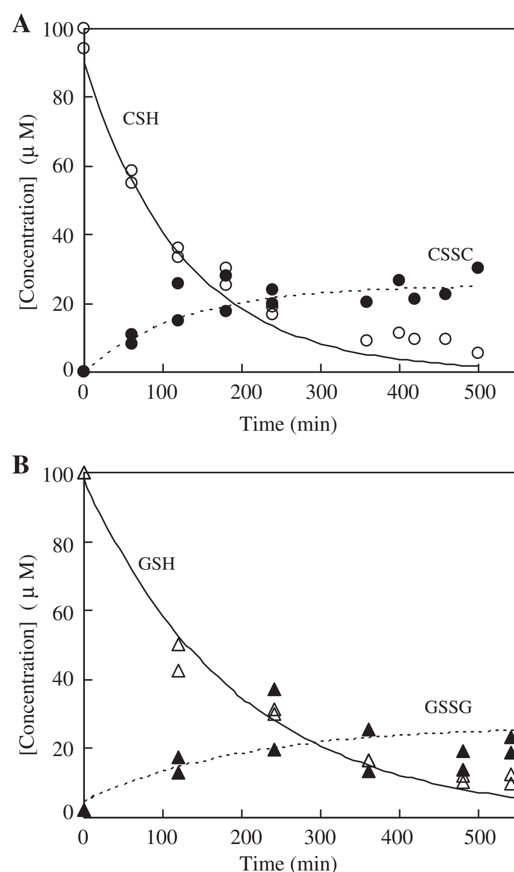


**Fig. 2 The time course of concentration changes of thiols (CSH and GSH) and disulphides (CSSC and GSSG) during autoxidation in CSSC-free DMEM.** About 100 μM each of (A) CSH and (B) GSH were incubated in the medium at 37°C. Symbols and lines show the experimental and theoretical values, respectively. The theoretical values were obtained by curve fitting with $k_C$ and $k_{M\text{-}C}$ or $k_G$ and $k_{M\text{-}G}$ as variable parameters.

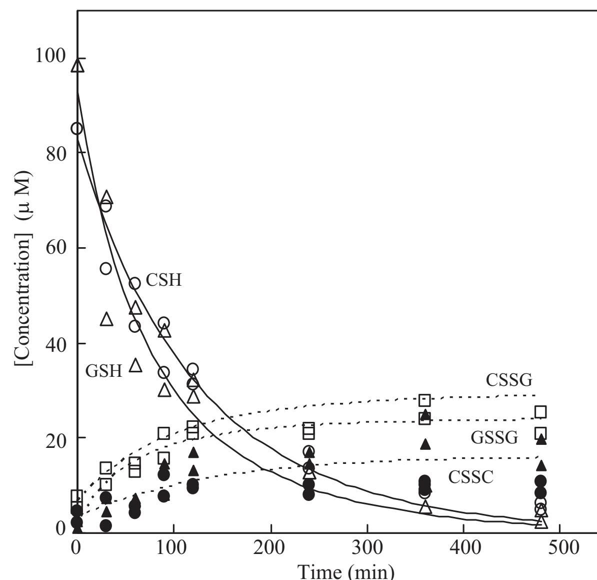


**Fig. 3 The time course of concentration changes of thiols (CSH and GSH) and disulphides (CSSC, GSSG and CSSG) after mixing CSH and GSH in CSSC-free DMEM.** About 100 μM each of CSH and GSH were incubated in the medium at 37°C. Symbols and lines show the experimental and theoretical values, respectively. The theoretical values were obtained by curve fitting with $k_{CG}$ as a variable parameter.

### Mixed autoxidation between CSH and GSH in DMEM

When both CSH and GSH are present in the medium, a mixed disulphide, CSSG is possibly formed as one of the autoxidation products, in addition to CSSC and GSSG. To assess its formation, we next examined the reaction between CSH and GSH in CSSC-free DMEM, and the results are shown in Fig. 3. As the reaction proceeded, a considerable amount of CSSG formation was observed in addition to CSSC and GSSG. CSSG can be derived from the mixed autoxidation between CSH and GSH, as well as from the thiol–disulphide exchange, and Reactions 3, 6 and 7 were added to the reaction model (Table I). The rate of mixed autoxidation (Reaction 3) was assumed to be proportional to the concentrations of CSH and GSH. We refer to the rate constants as $k_1$ and $k_2$ for GSH–CSSC exchange, and $k_3$ and $k_4$ for GSH–CSSG exchange (Table I) according to the notation by Jocelyn (*12*). In this study, the rate constant values obtained at pH 7.4 and 37°C (*12*) were used for the calculation. The rate constant for the mixed autoxidation ($k_{CG}$) was unknown, and its value was estimated by least-square curve fitting to the observed time course (Fig. 3) on the basis of Reactions 1–7 (Table I). As seen in Fig. 3, the fitting was sufficiently good, and a statistically significant value for $k_{CG}$ was obtained (Table I).

### Analysis of thiol and disulphide concentration changes in astrocyte culturing medium

The astrocytes were incubated in the culture medium containing 50 μM CSSC, and concentration changes of the thiols and disulphides were measured. The time course was followed up to 72 h, and the results are shown in Fig. 4. The cells remained alive during the period. As shown in Fig. 4, the CSSC concentration decreased to less than half of the original during the incubation. The CSH concentration reached a steady-state at 5 μM after a few hours. The CSSG concentration increased gradually up to 5 μM, while the amounts of GSSG and GSH were mostly less than the detectable level (1.5 and 0.5 μM, respectively). To explain the results, it seemed necessary to include the following three transport processes: CSSC influx into the cell (Transport 1 in Table I), CSH efflux from the cell (Transport 2) and GSH efflux from the cell (Transport 3). As shown in Table I, the cells were regarded as a reactant. It was assumed that the rate of Transport 1 is proportional to the concentrations of CSSC and the cell but that the rates of Transports 2 and 3 are proportional only to that of cell. To test the applicability of the model, we fitted Reactions 1–7 and Transports 1–3 (Table I) to the observed data (Fig. 4) with the rate constants for Transports 1–3 ($k_{A\text{-}CC}$, $k_{A\text{-}C}$ and $k_{A\text{-}G}$, respectively) as variable parameters. In the present study, ~4.0 μM of CSH was observed at the beginning of the reaction. It seems that a portion of CSSC had reacted with the medium components to form CSH during the storage (at 4°C for 3 days). In the curve fitting, the initial concentrations of CSH and



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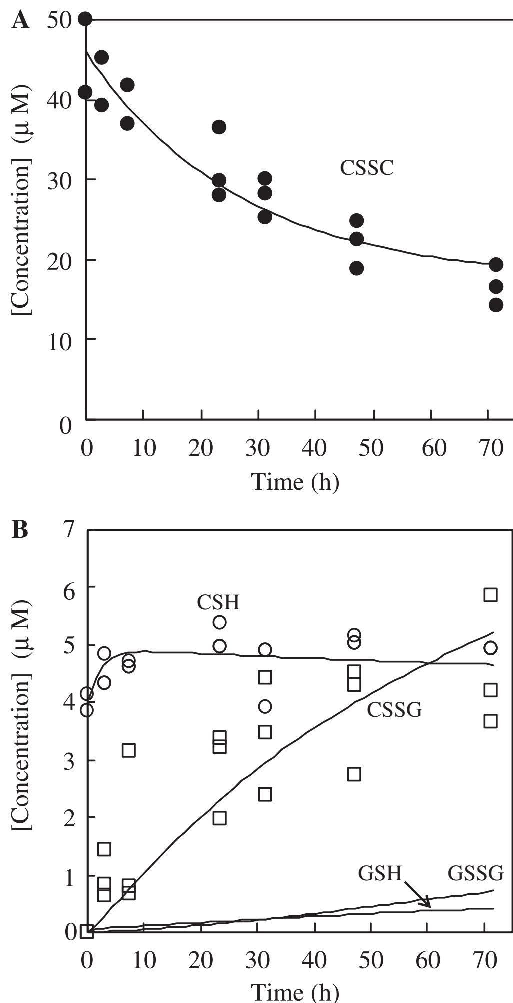


**Fig. 4 The time course of concentration changes of thiols and disulphides in astrocyte culturing medium.** The cells were incubated with 50 μM CSSC in DMEM at 37°C in a humidified atmosphere of 5% $CO_2$ and 95% air. CSSC concentration change (A) and CSH, CSSG, GSH, and GSSG concentration changes (B) in the same experiments are shown in different scales. Symbols and lines show the experimental and theoretical values, respectively. The amounts of GSH and GSSG were less than the detectable levels, and only the calculated values are shown.

CSSC were set variable in the range of ±10% in addition to the kinetic parameters.

### *Effects of reagents affecting the CSSC transporter*

Glutamate and $Cd^{2+}$ alter the activity of CSSC transporter, system $x_c^-$ (*9*). To test the involvement of the transporter in the influx of CSSC, we examined the effect of glutamate and $Cd^{2+}$ on the rate constants for the influx and efflux. A set of three experiments were performed using nearly equal amounts of cells. The rate constants for the influx and efflux could be estimated with statistically significant accuracy by applying the model, and their values are compared in Fig. 5. After 3 days of incubation, no significant changes in the cellular protein mass were observed in both presence and absence of the reagents. Glutamate, which competitively inhibits CSSC influx by system $x_c^-$, significantly decreased the CSSC influx rate constant ($k_{A\text{-}CC}$). On the other hand, $Cd^{2+}$, which induces system $x_c^-$ activity, slightly increased the $k_{A\text{-}CC}$ value. Glutamate decreased considerably the efflux rate of GSH ($k_{A\text{-}G}$) but did not alter the CSH efflux rate ($k_{A\text{-}C}$). $Cd^{2+}$ caused no appreciable changes in both $k_{A\text{-}C}$ and $k_{A\text{-}G}$.

### *Dynamics of the thiols and disulphides in the cell culturing medium*

To view the dynamics of thiols and disulphides in astrocyte culturing medium, we computed the flux rates of the individual reactions and transports as functions of time on the basis of the model using the obtained parameter values (Table I). The results are shown in Fig. 6. Since the cell amount was presumed to be unchanged throughout the incubation period, the efflux rates for Transports 2 and 3 were constant. The efflux rate of CSH was an order of magnitude larger than that of GSH. The CSSC uptake rate by the cell (Transport 1) decreased only slowly, because astrocytes constitutively excreted CSH into the medium, and most of them were autoxidized to CSSC. When the stoichiometry (CSH/CSSC = 2) is taken into account, the rate of CSH efflux was of similar magnitude to that of CSSC influx. It is thus thought that the incorporated CSSC is reduced to CSH, most of which is then excreted to medium. Up to 60 h, CSSG was produced principally through Reaction 6 (GSH–CSSC exchange), but the reverse of Reaction 7 (CSH–GSSG exchange) subsequently increased to become comparable with Reaction 6. The mixed autoxidation of CSH and GSH forming CSSG (Reaction 3) was relatively slow, and therefore its contribution to the CSSG formation is regarded as small. On the whole, the reaction sequence forms a cycle involving CSH and CSSC, starting with the influx of CSSC into the cell, intracellular reduction to CSH, efflux of CSH into the medium and autoxidation to CSSC. In addition to this, a portion of extracellular CSH branches to undergo Reaction 4, because the first-order rate constants, $k_C$ and $k_{M\text{-}C}$, are of similar magnitudes (Table I).

## Discussion

GSH is the major cellular antioxidant, acting to protect neurons against oxidative stress. Neural GSH maintenance has been thought to require CSH (*16*). Thiols are metabolized by various enzymes in cells. In addition, outside of cells they pass into the various non-enzymatic processes. When material exchanges between neurons and astrocytes are investigated, the reactions occurring in the extracellular fluid have to be considered. Our final aim is to better understand the dynamics of CSH-related compounds between neurons and astrocytes with the aid of mathematical models. As a first step, in this study, we tried to find a minimum model for describing the reactions of CSH, GSH and their disulphides occurring in the astrocyte culturing medium. Among the possible reactions, autoxidation of thiols (Reactions 1–3 in Table I) and thiol–disulphide exchanges (Reactions 6 and 7) are considered as principal and were included in the

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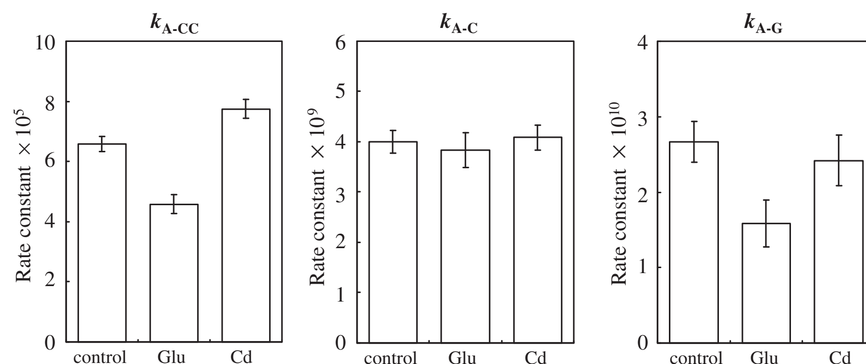


**Fig. 5 The effects of glutamate and $Cd^{2+}$ on the rate constants for CSSC influx ($k_{A\text{-}CC}$), CSH efflux ($k_{A\text{-}C}$) and GSH efflux ($k_{A\text{-}G}$).** The astrocytes were incubated with 50 μM CSSC in DMEM in the presence or absence of 2.5 mM glutamate (Glu) or 1 μM $CdCl_2$ (Cd) for 72 h. The rate constants for cellular influx and efflux were estimated by curve fitting of the mathematical model (Table I). The units of rate constants are: (mg cell protein/ml) $^{-1}s^{-1}$ for $k_{A\text{-}CC}$; M (mg cell protein/ml) $^{-1}$ $s^{-1}$ for $k_{A\text{-}C}$ and $k_{A\text{-}G}$.

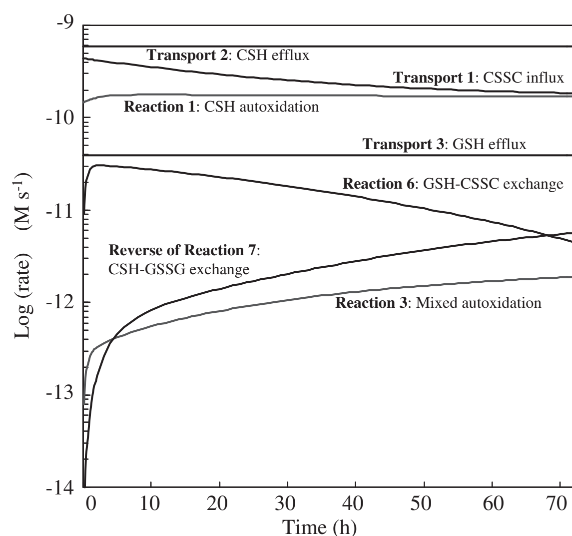


**Fig. 6 Time course of the reaction and transport rates calculated on the basis of the model using the estimated rate constant values (Table I).** The initial CSSC concentration was set at 50 μM. Reaction and transport numbers, shown in Table I, are indicated.

model. Furthermore, Reactions 4 and 5 were introduced to explain the stoichiometry of the thiol oxidation in the culture medium. Although Reactions 4 and 5 may include various types of reactions, the most possible candidates are those with bovine serum albumin, which is contained in FBS supplement, and this is discussed below in more detail. Among the cellular transport reactions of CSH-related compounds, the uptake of CSSC and release of CSH and GSH are thought to be predominant and necessary in the present system (Transports 1–3). The transport reactions may be kinetically more complex, but, for the sake of simplicity, the reaction rates were assumed to be constant or to follow the first-order kinetics. Possible errors arising from the assumptions are also discussed blow. The present results showed that, in spite of the simplifications and approximations, the model can be fitted to the experimental results quantitatively satisfactorily and can explain the effects of CSSC transport modifiers (glutamate and $Cd^{2+}$). The results seem to indicate that the present model provides enough accuracy for our purpose and can serve as a basis for more extended analyses.

The autoxidation of CSH or GSH was found to be of first-order with respect to its concentration, though the stoichiometry of the reaction is 2 mol of thiol to 1 mol of disulphide. It is likely that multiple sequential steps are involved in the reaction and that the rate-limiting step is of first-order. Actually, for the autoxidation of thiols, the following reaction sequence is proposed, as discussed by Schöneich (*17*).

where RSH, RSSR and RS· stand for thiol, disulphide and thiyl radical, respectively. Although atmospheric $O_2$ is involved in Step 1, the reaction is apparently of first-order with respect to RSH, because $O_2$ concentration is regarded as constant. When Step 2 is much faster than Step 1, the rate of overall reaction is governed by Step 1 and shows the first-order kinetics. Nevertheless, when the reaction was started from a high concentration (≥100 μM) of GSH in PBS, a lag phase was observed in the product (GSSG) formation. In the framework of reaction scheme shown above, this may be interpreted as lowering the rate of Step 2 under those conditions. However, it is unlikely as considered from the nature of free radical reaction. More possibly, more number of steps may be involved in the reaction sequence, as proposed by Bagiyan *et al.* (*15*), and the rate-limiting step may vary with conditions.

When CSH or GSH was incubated in the culture medium, unlike in PBS, the disulphide formation was considerably less than that expected from the stoichiometry. Apparently, the thiols seem also to be involved in other reactions in addition to autoxidation, and in the model, they are represented by first-order reactions (Reactions 4 and 5 in Table I). As to the nature of these reactions, the most likely are reactions with a CSH residue of albumin. The culture medium used in the present work was supplemented with 10% FBS, and the medium contains ~60 μM bovine serum albumin. Albumin contains a CSH residue which is reactive at physiological pH (*18*). Some portion of the residue is

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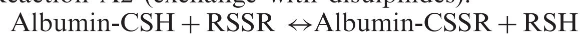
present as mixed disulphide with CSH or GSH (*18*), and the following reactions can take place with thiols and disulphides (*19*, *20*).

Reaction A1 (mixed autoxidation with thiols):

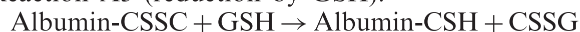
Albumin-CSH + RSH $\rightarrow$ Albumin-CSSR

Reaction A2 (exchange with disulphides):



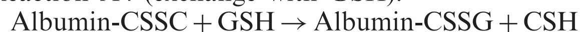
Albumin-CSH + RSSR $\leftrightarrow$ Albumin-CSSR + RSH

Reaction A3 (reduction by GSH):



Albumin-CSSC + GSH $\rightarrow$ Albumin-CSH + CSSG

Reaction A4 (exchange with GSH):



Albumin-CSSC + GSH $\rightarrow$ Albumin-CSSG + CSH

where RSH, RSSR and CSSR stand for thiol, disulphide and mixed disulphide of CSH and RSH, respectively. In the present experiments, only free thiols and disulphides in the medium were quantified. Reaction A1 is included in Reactions 4 and 5 of the present model (Table I). In Reaction A2, RSSR in the medium is reduced to RSH, and, in the analysis of this study, the reaction should cause apparent decreases in the rate constants for autoxidation, $k_C$ and $k_G$. In fact, the observed $k_C$ and $k_G$ values were ~40% smaller than of those of $k_C$ (PBS) and $k_G$ (PBS), respectively (Table I). This may be, at least partly, due to Reaction A2 taking place in the culture medium. Reactions A3 and A4 are estimated as negligible in the present experiments, since neither CSSG nor CSH was observed in the experiment of GSH autoxidation in the culture medium without cells. The nature of the reaction between GSH and medium components (Reaction 5) is not clear. It is possible, by analogy with Reaction A1 above, that GSH undergoes mixed autoxidation to form Albumin-CSSG.

The mixed disulphide, CSSG, is produced through the following three reactions: mixed autoxidation of CSH and GSH (Reaction 3), and exchange reactions between GSH and CSSC (Reaction 6) and also between CSH and GSSG (the reverse of Reaction 7). As seen from the results of simulation (Fig. 6), the rate for the mixed autoxidation of CSH and GSH is relatively slow. At the initial stage of reaction, GSH–CSSC exchange is predominant in the CSSG formation, but the contribution of CSH–GSSG exchange gradually increases with the decrease in CSSC concentration and the increase in GSSG concentration (Figs 4 and 6). Since the concentrations of GSH and GSSG in the medium were very low (Fig. 4), the excreted GSH is thought to be mostly converted to CSSG.

From the results of the present study, it is inferred that CSH is produced by the reduction of CSSC imported into astrocytes and is mostly released from the cells. Its efflux is estimated to be much faster than that of GSH. This is in accord with the idea that neurons maintain their GSH level by taking up CSH provided by astrocytes, as proposed previously (*16*). It is considered that most of GSH released to the medium reacts with CSSC to produce a mixed disulphide CSSG. Thus formed CSSG was accumulated even in the presence of astrocytes, indicating that the activity of γ-GT to break down of GSH, GSSG, S-conjugates of GSH (*21*), and probably CSSG is low in astrocytes, as reported by Stastný *et al.* (*22*). Since the activity of γ-GT is high in choroid plexus (*23*) and brain vasculature (*24*), CSSG may be decomposed there.

Astrocytic CSSC influx is mediated by system $x_c^-$ and is inhibited by glutamate. In the present study, the rate constant for CSSC influx was decreased by the addition of glutamate. The GSH efflux rate was also decreased, indicating that GSH synthesis is influenced by CSSC influx. In mammalian, Nrf2 binds to antioxidant response elements and regulates the genes involved in protecting cells from oxidative damage (*25*). In rat kidney cells, $Cd^{2+}$-induced oxidative stress regulates Nrf2 activation (*26*). The treatment with $Cd^{2+}$ increased the rate constant of CSSC influx, indicating that GSH production was increased to cope with $Cd^{2+}$-induced oxidative stress in the cells. On the other hand, the rate of CSH efflux was unchanged under the treatment of these agents. CSH is transported mainly by system ASC in a variety of cells. System ASC mediates both inward and outward flows of neutral amino acids such as CSH, alanine, serine and glutamine (*27–29*). The amino acids other than CSH, which are present abundantly outside of the cells, inhibit competitively the influx of CSH (*cis*-inhibition) and stimulate the efflux of CSH (*trans*-stimulation) (*5*). System ASC thus seems to be unaffected by the treatment of glutamate or $Cd^{2+}$.

In our model, it is approximated that the efflux rates of CSH and GSH are constant throughout the experiment (Transports 2 and 3). As for astrocytes, the apparent $K_m$ of GSH efflux is considerably higher than the cellular GSH concentration (*9*), while the $K_m$ of CSH efflux by system ASC is similar to or slightly higher than the cellular CSH concentration (~0.1 mM) (*16*, *27*, *28*). Thus the GSH and CSH efflux rates are significantly influenced by their intracellular concentrations. GSH concentration in astrocyte is altered considerably in the early stage (~12 h) of glutamate or $Cd^{2+}$ addition (*30*, *31*). The CSH concentration is thought to change in parallel with that of GSH, because CSH is the rate-limiting substrate for GSH synthesis (*5*), and also because the CSH/CSSC redox state is nearly equilibrated with the GSH/GSSG couple by the action of enzymes (*32*). Therefore, the efflux rates of CSH and GSH possibly alter with the changes in CSH and GSH concentrations at the early stage of the reactions in the presence of glutamate or $Cd^{2+}$, and accordingly it may have brought some errors to the results of simulation. In the present study, however, the model gave reasonable results on the effects of glutamate and $Cd^{2+}$, as described above. This seems to imply that CSH and GSH concentrations are nearly at steady-state and remain almost constant during most of the experimental periods.

*In vivo*, $O_2$ concentrations are considerably lower than those of solutions under air and culture condition. For a model study on the dynamics of autoxidizable compounds, it would be necessary to take into account the effects of $O_2$ concentration on the individual reactions. As a future problem, it would be of importance and interest to improve the model to include the $O_2$ concentration as well as those of intracellular metabolites, as noted above.

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The metabolism and interaction of thiols and disulphides are too complicated to comprehend the whole circulation system ranging from intracellular to extracellular fluid. Then employment of a mathematical model will help much to interpret the material-flow quantitatively. Moreover, if the model is further extended to include the neuronal influx and efflux of materials, it will become a useful tool for elucidation of interactions between neurons and astrocytes.

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**Conflict of interest**
None declared.

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