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Treatment of YAC128 mice and their wild-type littermates with cystamine does not lead to its accumulation in plasma or brain: implications for the treatment of Huntington disease

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Abstract

Cystamine is beneficial to Huntington disease (HD) transgenic mice. To elucidate the mechanism, cystamine metabolites were determined in brain and plasma of cystamine-treated mice. A major route for cystamine metabolism is thought to be: cystamine → cysteamine → hypotaurine → taurine. Here we describe an HPLC system with coulometric detection that can rapidly measure underivatized cystamine, cysteamine and hypotaurine, as well as cysteine and glutathione in the same deproteinized tissue sample. A method is also described for the coulometric estimation of taurine as its isoindole-sulfonate derivative. Using this new methodology we showed that cystamine and cysteamine are undetectable (≤ 0.2 nmol/100 mg protein) in the brains of 3-month-old HD transgenic (YAC128) mice (or their wild-type littermates) treated daily for 2 weeks with cystamine (225 mg/kg) in their drinking water. No significant changes were observed in brain glutathione and taurine but significant increases were observed in brain cysteine. Cystamine and cysteamine were not detected in the plasma of YAC128 mice treated daily with cystamine between the ages of 4 and 12 or 7 and 12 months. These findings suggest that cystamine is not directly involved in mitigating HD but that increased brain cysteine or uncharacterized sulfur metabolites may be responsible.

Keywords: cystamine, cysteamine, cysteine, glutathione, Huntington disease, transglutaminase.

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At least nine inherited neurodegenerative diseases are caused by a CAG expansion in the coding region of the affected gene (e.g. Maryuma *et al.* 2002; Taylor *et al.* 2002; Zoghbi and Orr 2002). The most prevalent of these is Huntington disease (HD), which affects approximately 1 in 10 000 individuals in the USA. The genes responsible for the disorders appear to be unrelated except for the presence of a CAG expansion. In each of these disorders, the mutated protein containing an expanded polyglutamine (Q_n) domain appears to be normally expressed but this expression eventually leads to accumulation of abnormal protein deposits in affected brain. The Q_n expansion confers a pathological gain of function although, in some cases, a pathological loss of function may also occur. In general, the size of the CAG/Q repeats correlates with the severity of disease progression as well as age of onset of symptoms.

Several authors have suggested that the reciprocal relationship between length of the Q_n domain and age of onset of symptoms in the Q_n expansion diseases may be due, in part,

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Abbreviations used: GSH, glutathione; HD, Huntington disease; MPA, metaphosphoric acid; PBS, phosphate-buffered saline; Q_n, polyglutamine; TGase, transglutaminase.

to additional substrate sites for transglutaminases (TGases) (e.g. Kahlem *et al.* 1996, 1998; Cooper *et al.* 1997, 2002; Igarashi *et al.* 1998; Karpuj *et al.* 1999; Iuchi *et al.* 2003). TGases belong to a family of enzymes which catalyses: (i) cross-linking between a protein/polypeptide Q residue (acyl donor; amine acceptor) and K residue (acyl acceptor; amine donor) with concomitant formation of a proteolysis-resistant N^{ε}-(γ-L-glutamyl)-L-lysine isopeptide bridge and (ii) polyamination of susceptible proteins (e.g. Folk 1983; Lorand and Conrad 1984; Aeschlimann and Thomazy 2000). It was proposed that the length-dependent formation of cross-links among mutant proteins by TGases contributes to the formation of the abnormal protein aggregates associated with HD brain (Green 1993; Cooper *et al.* 1997, 2002). At least three TGases are known to be present in brain (TGase isoforms 1, 2 and 3) (Kim *et al.* 1999). In support of the TGase hypothesis, Q_n domains, especially those of pathological length, are excellent TGase 2 substrates *in vitro* (Kahlem *et al.* 1996, 1998; Cooper *et al.* 1997; Gentile *et al.* 1998; Lesort *et al.* 1999). Two groups have shown that the proteinaceous deposits in HD brain are immunohistochemically positive for N^{ε}-(γ-L-glutamyl)-L-lysine cross-links (Dedeoglu *et al.* 2002; Zainelli *et al.* 2003, 2004). Moreover, TGase 2 expression has been shown to increase the aggregation of Q_n-containing proteins in a cell model system (de Cristofaro *et al.* 1999). Finally, cross-linking involving Q_n domains has the potential to obstruct the proteasome machinery (Cooper *et al.* 2002). Evidence that this may occur in a model of the Q_n-expansion disease, spinobulbar muscular atrophy, has been presented by Mandrusiak *et al.* (2003).

Protein aggregates in HD brain have been reported to be detrimental (Bates 2003), neutral (Saudou *et al.* 1998) and even beneficial (Kuemmerle *et al.* 1999; Arrasate *et al.* 2004). Moreover, it was recently reported that the aggregate number in the striatum does not correlate with disease progression in the R6/1 mouse model of HD (Bailey and Johnson 2005). While the weight of evidence now suggests that the visible macroaggregates *per se* are not toxic, the toxicity status of aggregate precursors such as protein oligomers is unclear (Arrasate *et al.* 2004). Furthermore, the contribution of TGases to formation of aggregates is complicated by the fact that aggregation may involve both TGase-catalysed covalent bond formation and non-covalent interactions (Lai *et al.* 2004). In this regard, Mastroberardino *et al.* (2002) showed that R6/1 mice lacking TGase 2 exhibit significantly more brain aggregates than R6/1 TGase 2+/+ mice, despite a 90% decrease in brain N^{ε}-(γ-L-glutamyl)-L-lysine linkages.

In HD, total TGase activity is increased in brain homogenates (Karpuj *et al.* 1999; Lesort *et al.* 1999). Lesort *et al.* (1999) reported a stage-dependent increase in TGase 2 message and protein in HD brain. More recently, Zainelli *et al.* (2005) reported a large increase in TGase 2 mRNA in HD cortex and striatum relative to controls. An influx of intracellular Ca^{2+} together with higher inherent TGase activity, more favorable substrate and perhaps lowered GTP levels (GTP is an inhibitor of the transamidating activity of TGase 2) led Lesort *et al.* (2000) to suggest that TGase activity is increased in HD brain *in vivo*. Evidence in favor of this conclusion is the finding that free N^{ε}-(γ-L-glutamyl)-L-lysine is increased three- and ~8–10-fold in the CSF and brains of patients with HD, respectively (Jeitner *et al.* 2001; Dedeoglu *et al.* 2002). The possibility that TGase activity is detrimental in HD brain is indicated by the work of Mastroberardino *et al.* (2002), who reported a reduction in neuronal cell death, improved behavior and prolonged survival in R6/1 mice lacking TGase 2.

Cystamine is an *in vitro* TGase inhibitor (Lorand and Conrad 1984) which is currently being investigated as a possible therapeutic agent for treating patients with HD. Cystamine participates in disulfide exchange reactions (Duffel *et al.* 1987) and, as TGases contain critical cysteine sites (Folk 1983), disulfide exchange could theoretically inactivate these enzymes. Kahlem *et al.* (1998) showed that the *in vitro* covalent polymerization of mutant huntingtin (the affected protein in HD) catalysed by TGase 2 is inhibited by cystamine. Other studies demonstrated that cystamine treatment of COS-7 or neuroblastoma SK-N-BE cells reduced the aggregation of Q_n-containing proteins with a concomitant reduction in cell death and proteasome dysfunction (Igarashi *et al.* 1998; de Cristofaro *et al.* 1999). Based on these previous findings, two groups investigated the effects of cystamine treatment in the R6/2 mouse model of HD. Both groups reported improvement in life expectancy, behavior and body mass in the cystamine-treated R6/2 mice (Karpuj *et al.* 2002; Dedeoglu *et al.* 2002).

Although the studies of Karpuj *et al.* (2002) and Dedeoglu *et al.* (2002) are consistent with a role for TGase in HD pathogenesis, other explanations for the beneficial effects of cystamine have been considered. Lesort *et al.* (2003) showed that cystamine inhibits recombinant caspase 3 activity *in vitro* in a concentration-dependent manner. As caspases are involved in programmed cell death and have been implicated in CAG-expansion diseases (e.g. Ona *et al.* 1999; Chen *et al.* 2000; Wellington *et al.* 2002; Shoesmith Berke *et al.* 2004), reduction in caspase activity might account for some of the beneficial effects of cystamine. Lesort *et al.* (2003) also found that cystamine promotes the formation of the antioxidant glutathione (GSH) in a cell model. Increased GSH levels may be beneficial because HD brain is under oxidative stress (e.g. Browne and Beal 2004). The reduced form of cystamine (i.e. cysteamine) is a potent antioxidant (e.g. Duffel *et al.* 1987). Thus, the protective effect of cyst(e)amine in HD mice may theoretically be due to increased antioxidant capacity. Finally, treatment of R6/2 mice with cystamine has been shown to increase the

expression of heat shock proteins that can assist in proper folding or degradation of proteins containing expanded Q_n domains (Karpuj *et al.* 2002).

In order to determine the mechanism(s) whereby cystamine is protective and to design the best treatment regimens involving cystamine for patients with HD, it is important to understand its metabolic fate. HPLC methods with coulometric detection are available for the measurement of sulfhydryl-containing compounds and disulfides (e.g. Melnyk *et al.* 1999; Shirin *et al.* 2001). However, such methods do not include cystamine or its reduced form cysteamine. We have adapted the HPLC-CoulArray (coulometric) detection procedure of Shirin *et al.* (2001) to quantitate cystamine and cysteamine in deproteinized brain and plasma samples. The procedure, which does not require prior derivatization, has the added advantage that it can be used to rapidly determine cysteine, GSH and hypotaurine simultaneously in the same sample preparations. In addition, we were able to measure the potential cyst(e)amine metabolite taurine by HPLC/coulometric detection of its isoindole-sulfonate derivative. Here we report on the levels of GSH, cysteine, cyst(e)amine and its metabolites hypotaurine and taurine in the plasma and brains of: (i) untreated YAC128 mice, (ii) untreated littermate control mice, (iii) cysteamine-treated YAC128 mice and (iv) cysteamine-treated littermate control mice. We also report on the stability of cyst(e)amine in plasma, liver, kidney and brain homogenates of control mice. The implications of our findings for understanding the mechanisms contributing to the beneficial effects of cystamine in HD mice are discussed.

Materials and methods

Materials

Cysteamine.HCl, cystamine.2HCl, L-cysteine.HCl, GSH, diethylenetriaminepenta-acetic acid, taurine, hypotaurine, GSH, uric acid, ascorbic acid, *o*-phthaldialdehyde, octane sulfonic acid and metaphosphoric acid (MPA) were obtained from Sigma Chemical Company (St Louis, MO, USA). OmniSol^R solvents (acetonitrile and *N,N*-dimethylformamide) were HPLC grade and obtained from EM Science (Gibbstown, NJ, USA).

Mice

In one set of experiments, 3-month-old YAC128 transgenic mice (Slow *et al.* 2003) of both sexes and their wild-type littermates were maintained under a 12-h dark/light cycle with free access to food and water. Half of the animals of each genotype were treated with cystamine (Sigma Chemical Company) in autoclaved drinking water at a concentration of 900 mg/L to yield an approximate dose of 225 mg/kg/day as described by Dedeoglu *et al.* (2002). The remainder of the animals received autoclaved drinking water alone. Water volumes were monitored to ensure consumption of the cystamine-treated water. [To ensure that cystamine is stable in the drinking water, aliquots of a solution of cystamine in autoclaved water (900 mg/L) were incubated at 25 or 37°C for 5 days and then analysed by the HPLC coulometric procedure described below. The cystamine in the autoclaved water was stable under these conditions (~100% recovery) and no trace of redox-active breakdown products could be detected.] After 2 weeks of cystamine treatment, the mice were killed by asphyxiation with carbon dioxide. Brains were removed, immediately placed in 1.5-mL Eppendorf tubes and snap frozen in isopentane on dry ice. The brains were then maintained at −80°C before assay for cystamine and other metabolites of interest. In another set of experiments, 4-month-old mice of both sexes were used. Plasma was obtained from four groups: (i) untreated YAC128 mice; (ii) cystamine-treated YAC128 mice; (iii) untreated wild-type littermate controls and (iv) cystamine-treated wild-type littermate controls. Treatment was with cystamine in the drinking water (225 mg/kg/day) for 8 months beginning at 4 months of age. As noted above, YAC128 mice at 4 months of age are mildly symptomatic. In a third set of experiments, fully symptomatic 7-month-old mice of both sexes were used. Plasma was obtained from four groups: (i) untreated YAC128 mice; (ii) cystamine-treated YAC128 mice; (iii) untreated wild-type littermate controls and (iv) cystamine-treated wild-type littermate controls. Treatment was with cystamine in the drinking water (225 mg/kg/day) for 5 months. At the time of killing, blood was drawn from the heart into a syringe rinsed with a solution of heparin. The blood was transferred to tubes containing EDTA (1.6 mg/mL blood) and inverted a couple of times. The blood was then centrifuged and the plasma was removed and frozen at −80°C until analysed for cystamine and related metabolites. The mice were housed at the University of British Columbia and all experiments described herein were approved by the University of British Columbia Institutional Animal Care and Use Committee (A03-0312). Frozen specimens of brain and plasma were shipped on dry ice to the laboratories of JTP and AJLC where biochemical analyses were performed.

In a fourth experiment, the endogenous levels of sulfur-containing compounds and the stability of added cystamine and cysteamine were determined in homogenates of tissues and plasma obtained from adult wild-type C57BL/6N (control) male mice [redacted](Harlan Sprague Dawley, Indianapolis, IN, USA) housed at the Burke Medical Research Institute (White Plains, NY, USA). The mice were fed *ad libitum* and had full access to water. The mice were killed by decapitation. Plasma was obtained from collected neck blood. Brain, liver and kidney were quickly frozen in liquid nitrogen and stored at −80°C until analysed.

Preparation of mouse tissues for metabolite analysis

Ten volumes of ice-cold 5% (w/v) MPA containing 5 mM diethylenetriaminepenta-acetic acid were added to samples of frozen (−80°C) mouse tissues (50–75 mg) or plasma, which were then homogenized in a Dounce homogenizer. After incubation for 10 min on ice, the samples were centrifuged at 0°C for 5 min at 13 000 *g* in a microfuge to sediment coagulated protein. Precipitates were dissolved in 0.1 N NaOH and protein was quantitated by a spectrophotometric method using bicinchoninic acid reagent (Pierce Chemical Co., Rockford, IL, USA). In many cases, supernatant fractions were analysed immediately after removal of denatured protein for compounds of interest by using HPLC separation (see below). However, this was not possible in those experiments requiring consecutive analyses of cysteamine and cystamine in tissue homogenates at time-points separated by less

than 12 min (i.e. the time for a complete HPLC run plus column re-equilibration; see below). For these experiments, the tissue homogenate was deproteinized with 5% (w/v) MPA, stored briefly on ice, centrifuged and then frozen at −80°C until analysis could be completed. Control experiments showed that such storage in 5% (w/v) MPA had no effect on the recovery of cystamine and cysteamine.

Determinations of redox-active sulfur-containing compounds by HPLC and coulometric detection

Concentrations of the redox-active sulfur-containing compounds cysteine, cysteamine, cystamine, hypotaurine and GSH were measured without prior derivatization by using a Liquid Chromatograph (Perkin-Elmer) equipped with an eight-channel coulometric array (CoulArray) detector (ESA, Inc., Chelmsford, MA, USA) (Lakritz *et al.* 1997). The procedure also quantitates the redox-active compounds ascorbic acid and uric acid that do not contain sulfur.

The supernatant fractions from the MPA homogenates were injected directly onto a Bio-Sil ODS-5S C18 column (5-μm particle size, 4.0 × 250 mm; Bio-Rad, Life Science Research Group, Hercules, CA, USA) and eluted with a mobile phase consisting of 50 mM NaH_2PO_4, 0.05 mM octane sulfonic acid, 1% (v/v) acetonitrile and 0.5% *N,N*-dimethylformamide (v/v) (pH 2.52) at a flow rate of 1 mL/min. PEEK™ (polyetheretherketone) tubing was used throughout the HPLC system and a 0.2-μ PEEK™ filter was placed pre- and post-column to protect both column and flow cells, respectively, from any particulate matter. A Rheodyne injection valve with a 5-μL sample loop was used to manually introduce samples. The eight-channel CoulArray detectors were set at 250, 400, 450, 500, 550, 600, 650 and 700 mV, respectively.

Measurements were also made of the sulfur-containing amino acid taurine. This sulfur-containing amino acid is not amenable to direct quantitation by coulometric analysis. To measure taurine, a redox active derivative was employed. After deproteinization of the tissue sample with MPA, the supernatant fraction was analysed immediately for taurine by using a modification of the method of Rowley *et al.* (1995). An aliquot of the supernatant fluid (50 μL) was mixed with 200 μL 0.5 M sodium borate buffer (pH 10.0) and 2 μL of working *o*-phthaldialdehyde reagent [4.4 mg *o*-phthaldialdehyde dissolved in 0.1 mL of 1 M sodium sulfite to which was added 100 μL of absolute ethanol and 1.8 mL of 0.5 M sodium borate buffer (pH 10.0)] was added. Derivatized samples were injected onto an α-Chrom™ C18 column (5 μ, 3 × 150 mm; Upchurch Scientific, Inc., Oak Harbor, WA, USA) and eluted with a mobile phase of 100 mM NaH_2PO_4 and 14% (v/v) methanol (pH 4.52) at a flow rate of 0.8 mL/min. PEEK™ tubing was used throughout the HPLC system and a 0.2-μ PEEK™ filter was placed pre- and post-column to protect both column and flow cells, respectively, from any particulate matter. The eight-channel CoulArray detectors were set at 300, 350, 400, 450, 500, 550, 600 and 700 mV, respectively. The isoindole-sulfonate derivative of taurine eluted at 3 min. The total run time was 6 min and the next sample could be injected immediately.

Peak areas were analysed using software from ESA, Inc. Concentrations of each metabolite were obtained from appropriate standard curves and are reported as nmol/mg protein.

Determination of cystamine and cysteamine stability in mouse tissue homogenates

The stabilities of cystamine and cysteamine were determined in brain, liver and kidney homogenates obtained from an adult C57BL/6N (wild-type control) male mouse. The tissues were disrupted at 0°C in a 10-fold excess of 100 mM potassium phosphate buffer (pH 7.4) by means of a Dounce homogenizer. After freeze–thawing, the homogenates were spiked with stock solutions containing either 1 μmol/mL cystamine or 1 μmol/mL cysteamine to yield a final concentration of ~250 nmol/mL (experiment 1). Experiment 2 was identical to experiment 1 except that tissues from a different C57BL/6N control mouse were used, the concentration of added cysteamine was ~500 nmol/mL in the tissue homogenate and the tissues were homogenized in a 10-fold excess of phosphate-buffered saline (PBS). The homogenates were then incubated at 37°C for various lengths of time. Measurements of endogenous cystamine and cysteamine were made before the addition of either compound. At intervals, 50-μL aliquots were withdrawn and added to 200 μL of 6.25% (w/v) MPA to precipitate proteins. Thus, the final concentration at the time zero time-point in the denatured samples was 50 nmol/mL for both cystamine and cysteamine (experiment 1) and 50 and 100 nmol/mL, respectively, for experiment 2. Denatured protein was removed by centrifugation at 0°C for 5 min at 13 000 *g*. Simultaneous measurements of both cystamine and cysteamine levels were made regardless of whether cystamine or cysteamine was added to the homogenate. Detection of cystamine and cysteamine was by HPLC with coulometric detection as outlined above.

Determination of cystamine and cysteamine stability in mouse plasma

Plasma samples from an adult C57BL/6N male mouse were diluted 10-fold in PBS. This diluted plasma (450 μL) was spiked with 12 μL of 10 μmol/mL cystamine (final concentration of cystamine, 246 nmol/mL) and incubated at 37°C. At intervals beginning immediately after the addition of cystamine, 50-μL spiked plasma samples were removed and treated with 150 μL of water and 50 μL of 25% (w/v) MPA. Thus, the time zero deproteinized sample had a concentration of 49.3 nmol/mL of cystamine. In another experiment, 6 μL of 20 μmol/mL cysteamine was added to serum from an adult C57BL/6N male mouse diluted 10-fold in PBS (final concentration of cysteamine, 250 nmol/mL). At intervals beginning immediately after addition of cysteamine, 50-μL spiked plasma samples were removed and treated with 150 μL of water and 50 μL of 25% (w/v) MPA. Thus, the time zero deproteinized sample had a concentration of 50 nmol/mL of cysteamine. After removal of denatured protein by centrifugation the samples were stored at −80°C until analysed by coulometric detection.

Statistical analysis

For determinations where $n \geq 3$, the mean ± SEM is reported. Regression analyses were carried out with Sigma Plot 2000 software. After statistical analysis, a p-value of ≤ 0.05 was considered significant. As noted in the Results, analyses of 28 brains [eight from cystamine-treated YAC128 mice, seven from cystamine-treated wild-type littermates (controls), six from untreated YAC128 mice and seven from untreated controls] for redox-active compounds showed that 23 yielded relatively tight

values of taurine (range 368–543 nmol/mg of protein). The other five brains yielded remarkably low values (56–79 nmol/mg of protein). In order to reduce the influence of the five 'anomalous' outliers, the statistical differences between cystamine-treated and untreated mice or between YAC128 mice and their control littermates were assessed by performing two-way ANOVA on normal score ranks of the individual variables measured (taurine, ascorbate, GSH and cysteine) using Proc Rank and Proc GLM (SAS software version 9; SAS Institute, Cary, NC, USA). The ANOVA was also performed after omitting data sets from the five 'anomalous' mice or by including an indicator variable to identify them. There were two such mice in the cystamine-treated controls and one in each of the three treatment–genotype combination groups. This relative balance across the four groups reduced the effects of the outliers.

Results

Cystamine and cysteamine quantitation by HPLC coupled to coulometric detection

Aliquots of stock solutions (1 μmol/mL) of cystamine, cysteamine, cysteine, GSH, ascorbic acid and uric acid were prepared in deionized water and frozen at −80°C. A working standard mixture of the above compounds [20 or 25 nmol/mL in 5% (w/v) MPA] was prepared fresh before each analysis. Figure 1 shows that the peaks associated with cysteamine and cystamine in this reference mixture are well separated from each other by the HPLC procedure and from other potentially interfering redox-active compounds. Moreover, the analysis is rapid. A single run is accomplished in less than 8 min. Recharging the column with the mobile phase requires only an additional 4 min. Working standards for both cysteamine and cystamine were prepared in the range 5–50 nmol/mL. The linear response range for coulometric detection of these compounds is 0.2–200 nmol/mL. Ascorbic acid was included in the standard reference mixture because it is generally present in biological samples at relatively high (mM) concentrations and generates strong signals along with the signals obtained from the redox-active sulfur-containing compounds. Ascorbic acid is, however, sensitive to rapid decomposition if samples are not carefully processed. Thus, ascorbic acid can be used for quality control of the deproteinized tissues. Uric acid is present at a relatively high concentration in plasma. As uric acid generates a strong redox signal it can also be used for quality control of deproteinized plasma samples. However, uric acid levels are very low in rat (Yamamoto and Zhu 1998) and human (Mirecki *et al.* 2004) brain. The concentration of uric acid in rat brain is ~50 pmol/mg of protein (Yamamoto and Zhu 1998) or < 0.1% of the level of GSH and ascorbic acid in normal rodent brain (cf. Table 1). Interestingly, the level of uric acid in autopsied human brain is approximately eight times higher than in biopsied human brain (Mirecki *et al.* 2004), suggesting time-dependent formation of uric acid in autolysing human brain tissue. This finding suggests that, for mouse brain, a coulometric HPLC profile of a good quality sample will contain a large ascorbic acid peak and negligible uric acid. Glutathione disulfide can also be analysed by the present procedure (elution time, ~5.5 min) but was not included in the reference mixture because tissue levels are generally low (usually ≤ 1% of GSH levels). The potential cystamine metabolite hypotaurine is also detected by this procedure and elutes at ~2.1 min but was not included in the standard mixture.

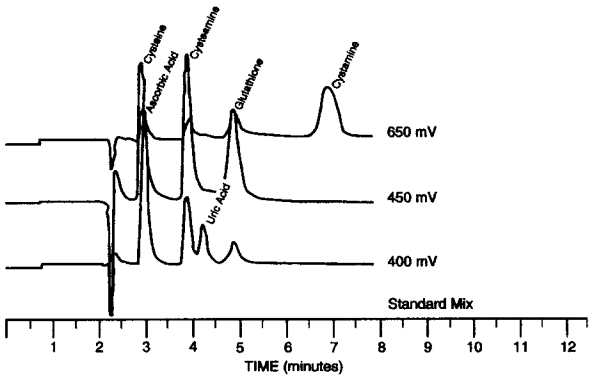

Fig. 1 Rapid separation and quantitation of cysteine, cystamine, cysteamine, glutathione (GSH), ascorbic acid and uric acid by HPLC coupled to CoulArray (coulometric) detection. A standard mixture containing 25 nmol/mL of each compound indicated (uric acid, 10 nmol/mL) was injected onto the HPLC and the peak responses were monitored over eight channels (three channels shown here). Accordingly, ascorbic acid and uric acid exhibit peak oxidation patterns at 400 mV that subside after 450 mV; the thiol-containing compounds cysteine, cysteamine and glutathione (GSH) begin oxidation at 400 mV and exhibit maxima at 450 mV. By contrast, the disulfide-containing compound cystamine begins oxidizing at 600 mV (data not shown) and maximizes at 650 mV. Full-scale deflection is 5 μC. See Materials and methods.

Cystamine, cysteamine and hypotaurine cannot be detected in the brains of cystamine-treated YAC128 mice or their cystamine-treated wild-type littermates

No cystamine, cysteamine or hypotaurine [a metabolite of cyst(e)amine] could be detected (Table 1) when deproteinized brain samples from 3-month-old: (i) untreated YAC128 mice, (ii) cystamine-treated YAC128 mice, (iii) untreated wild-type littermate controls and (iv) cystamine-treated wild-type littermate controls were analysed by HPLC with coulometric detection. The accurate detection limit for all three compounds is ~2 nmol/100 mg of protein. To ensure that MPA treatment does not interfere with the detection of cystamine or cysteamine, deproteinized brain samples from cystamine-treated YAC128 mice were spiked with authentic cystamine or cysteamine and checked for recovery. When 5 μL of cysteamine (50 nmol/mL) and 5 μL of cystamine (50 nmol/mL) were added to 100 μL of MPA-deproteinized

Table 1 Levels of taurine, ascorbic acid, glutathione (GSH) and cysteine (nmol/mg protein) in brain tissues of untreated YAC128 mice, untreated wild-type littermates, cystamine-treated YAC128 mice and cystamine-treated wild-type littermates

Group (sex ratio)	Taurine	Ascorbic acid	GSH	Cysteine
Untreated YAC128 mice (4F, 2M)	362 ± 64 [*52.3* (397–473)]	83.9 ± 3.8 (66.1–92.2)	71.9 ± 4.6 (51.9–81.6)	7.08 ± 1.88 [*15.3* (3.01–9.64)]
Untreated littermate controls (5F, 2M)	370 ± 57 [*61.4* (314–543)]	81.2 ± 3.2 (73.4–87.1)	70.5 ± 2.7 (57.4–78.5)	6.61 ± 1.58 [*15.1* (2.26–7.43)]
Cystamine-treated YAC128 mice (3F, 5M)	411 ± 51 [*74.9* (368–508)]	90.9 ± 3.1 (74.2–100)	72.7 ± 3.2 (56.2–82.3)	10.9 ± 2.1[a] (6.68–17.2)
Cystamine-treated littermate controls (4F, 3M)	311 ± 52 [*56.1, 79.0* (356–481)]	84.6 ± 4.3 (64.7–98.0)	68.2 ± 3.0 (59.2–77.6)	10.6 ± 1.8[a] (7.44–20.9)

Three-month-old mice were either treated with cystamine in their drinking water for 2 weeks or given plain drinking water (untreated groups) (see Materials and methods). Cystamine, cysteamine and hypotaurine could not be detected (≤ 0.2 nmol/100 mg of protein) in the brains of any of the four groups of mice. The range of values (excluding outliers) for each metabolite is shown in parenthesis; individual outliers are shown in italics. [a]ANOVA showed no significant effect of genotype on cysteine levels but a highly significant effect of cystamine treatment on cysteine levels with $p = 0.003$. [The outliers were not discarded in this statistical analysis.] See Materials and methods and Results for more details of the analysis.

brain samples from wild-type mice, the recoveries were ~98, 95 and ~97, 96%, respectively ($n = 2$). Representative HPLC profiles of a brain sample from a cystamine-treated YAC128 littermate control in the absence and presence of added standards are shown in Figs 2(a–c). [In principle, an appropriate control is to spike brain (or other tissue) homogenate with cystamine and cysteamine before addition of deproteinizing agent. However, we found that these compounds are metabolized rapidly in mouse tissue homogenates prepared in phosphate buffer or PBS (see below). Therefore, we deemed it more appropriate to homogenize the frozen tissue directly into deproteinizing agent before spiking with standards.]

Glutathione and taurine levels are not altered but cysteine levels are elevated in the brains of cystamine-treated YAC128 mice and their cystamine-treated control littermates

Levels of brain cysteine were significantly higher in the cystamine-treated mice than in the untreated mice ($p = 0.003$), with no significant differences between YAC128 mice and their control littermates and no significant interactions between the treatment and genotype factors. [The results were similar if data for the five mice with low taurine values were omitted from the analysis ($p < 0.001$) or if an adjustable variable was included in the model ($p = 0.002$).] Ascorbate levels were slightly higher for cystamine-treated compared with untreated mice and for YAC128 mice vs. control littermates but the p-values were not significant ($p = 0.065$ and 0.066, respectively). The ascorbate results were marginally significant, however, if data from the five mice with low taurine values were excluded or an adjustment variable was included. The p-values were 0.011 for 'treatment' and 0.052 for 'genotype' if an indicator variable was used and 0.027 and 0.015, respectively, if values from the five outliers were omitted. As ascorbate levels were not the focus of the present work, possible changes of ascorbate levels in the cystamine-treated mice were not further investigated here. There were no significant differences based on treatment or genotype for brain taurine or GSH, whether or not data from the five taurine outliers were excluded.

Cystamine and cysteamine cannot be detected in the plasma of cystamine-treated YAC128 mice or their cystamine-treated control littermates

As was noted with the deproteinized brain samples, neither cystamine nor cysteamine (≤ 0.2 nmol/mL) could be detected in deproteinized plasma samples of the two groups of cystamine-treated littermate control mice or two groups of cystamine-treated YAC128 mice. To ensure that MPA treatment does not interfere with the detection of cystamine and cysteamine, MPA-deproteinized plasma samples from cystamine-treated YAC128 mice were spiked with authentic cystamine and cysteamine as described above for the deproteinized brain samples. As was noted for the MPA-deproteinized brain samples, recovery of cystamine and cysteamine was essentially quantitative (≥ 96%).

Cystamine added to mouse tissue homogenates is rapidly metabolized

As cystamine was not detected in the brains (≤ 0.2 nmol/100 mg of protein) or plasma (≤ 0.2 nmol/mL) of cystamine-treated YAC128 mice (or their wild-type littermate controls), the possibility was considered that the compound is rapidly metabolized. Evidence in support of this conclusion was

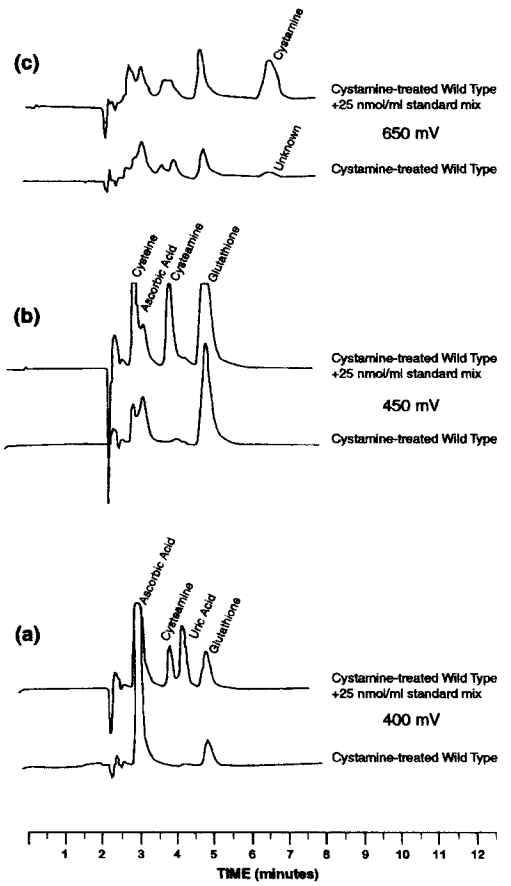

Fig. 2 HPLC profiles of deproteinized brain samples obtained from cystamine-treated wild-type littermates of YAC128 mice. Comparison of 5% (w/v) metaphosphoric acid supernatant fractions of brain homogenates in the absence and presence of a 25 nmol/mL standard mixture of cysteine, ascorbic acid, cysteamine, uric acid, glutathione (GSH) and cystamine. (a–c) Measurements at 400, 450 and 650 mV, respectively. As shown for the standards in Fig. 1, the thiol-containing compounds cysteine, cysteamine and GSH begin oxidation at 400 mV and exhibit peak maxima at 450 mV. By contrast, the disulfide-containing compound cystamine begins oxidizing at 600 mV (data not shown) and its peak maximizes at 650 mV. Note the lack of a uric acid peak in the deproteinized unspiked brain samples. The concentration of uric acid in rat brain has been reported to be ~0.05 nmol/mg of protein. Assuming that the concentration is similar in mouse brain, this level of uric acid (< 0.1% that of GSH and ascorbic acid) will not show above the baseline when the *Y*-axis is set to show on-scale peaks of ascorbate and GSH. A peak labeled 'unknown' in (c) elutes at ~0.5 min before cystamine and appears to be a mixed disulfide based on its pattern of oxidation. The unknown peak appeared in chromatographs of brain samples from both untreated and cystamine-treated YAC128 mice and their littermate controls and did not show differences in its area of integration among the different groups. Full-scale deflection is 5 μC.

obtained when tissue homogenates [1/10 dilution (w/v) in 100 mM potassium phosphate buffer, pH 7.4] of brain, liver and kidney obtained from a single adult C57BL/6N (wild-type control) male mouse were spiked with cystamine or cysteamine (final concentration, ~250 nmol/mL) and incubated for various lengths of time at 37°C (Figs 3a and b) (experiment 1). In each tissue homogenate, > 88% of the added cystamine was metabolized within 15 min (Fig. 3a) with concomitant formation of 55, 18 and 48% cysteamine in brain, liver and kidney homogenates, respectively (Fig. 3b). When the tissue homogenates were spiked with cysteamine, a trace of cystamine (≤ 2% of the cysteamine added) was detected at time zero (Fig. 3c). [Note the difference in scale between Figs 3a and b.] The minute amount of cystamine in tissues spiked with cysteamine at time zero is presumably a minor contaminant arising from air oxidation of cysteamine or rapid, transient (but very minor) *in situ* oxidation within the tissue homogenates. As with the cystamine added directly to the homogenates (Fig. 3a), this cysteamine-derived cystamine (< 1 nmol/mL) disappeared rapidly in the tissue homogenates (Fig. 3c). No evidence was found that cysteamine can give rise to cystamine on prolonged incubation in the tissue homogenates.

The experiment shown in Fig. 3 was repeated except that the tissues were homogenized in PBS, cystamine was added to a final concentration of ~500 nmol/mL and extra sampling times (from 0 to 5 min) were included (experiment 2). Essentially similar results were obtained with experiment 2 to those obtained with experiment 1 shown in Fig. 3. Note that in experiment 1, time-points were chosen such that consecutive deproteinized samples could be analysed immediately by HPLC without the need for storage. In experiment 2, where samples were deproteinized at 30-s intervals for the first few minutes after addition of cystamine (or cysteamine), immediate analysis was not possible and the deproteinized tissue samples were stored at −80°C until analysed. As we were able to obtain more data points at earlier time-points in experiment 2 we were able to define the *in vitro* rate of loss of cystamine more accurately. For all three tissue homogenates, ~95% of the added cystamine was metabolized within 5 min.

Cysteamine added to mouse tissue homogenates is metabolized more slowly than cysteamine

One possibility for the extremely rapid removal of cystamine from the tissues is facile reduction to cysteamine. We found that cystamine was indeed converted to cysteamine but the maximal conversion in the brain, kidney and liver homogenates in experiment 1 was ~55, 50 and 45%, respectively (Fig. 3b). In experiment 2, the maximal conversions were ~17, 20 and 40%, respectively (data not shown). In both experiments, maximal concentrations of cysteamine in brain and kidney homogenates were attained by about 10 min after spiking with cystamine, whereas in the liver homogenates maximal cysteamine concentration was attained by about

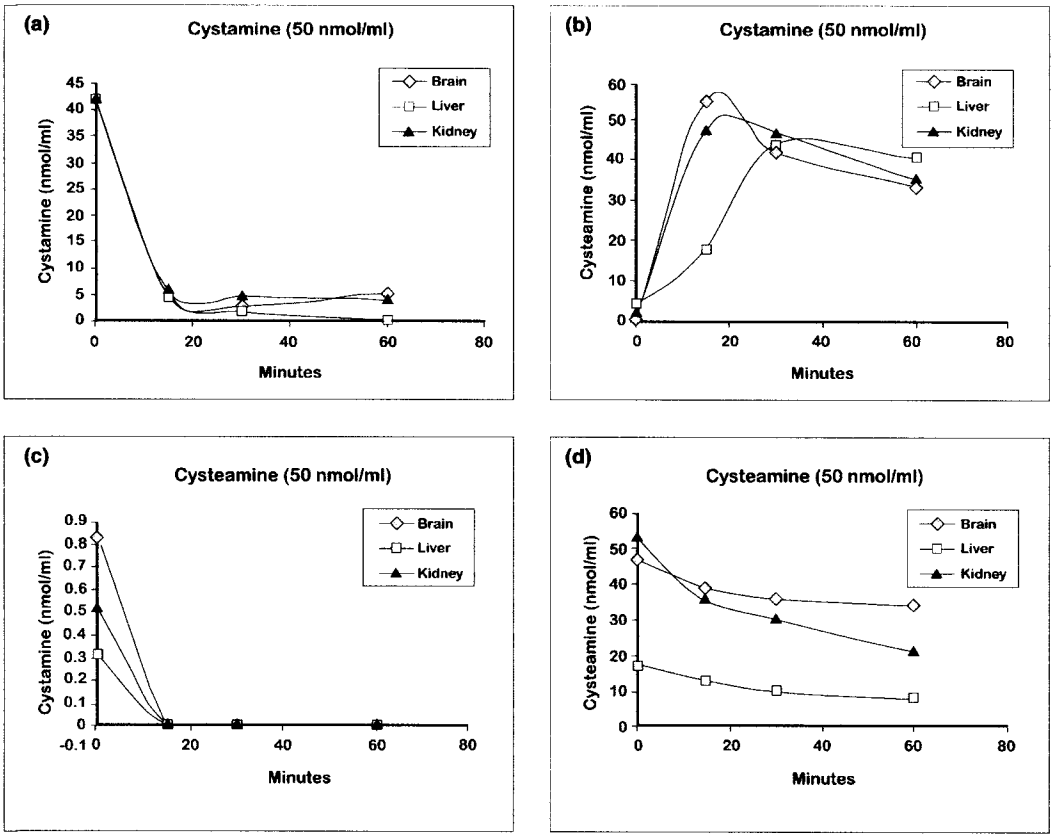

Fig. 3 Rapid metabolism of cystamine and cysteamine in homogenates of tissues from C57BL/6N (wild-type control) mice. Aliquots of tissue homogenates (95 μL) diluted 10-fold (w/v) in 100 mM potassium phosphate buffer (pH 7.4) were spiked with either 5 μL of 1 mmol/L cystamine (a and b) or 5 μl of 1 mmol/l cysteamine (c and d) to yield the final concentration of 250 nmol/mL and incubated at 37°C for the times shown. After deproteinization, 100% recovery at the time zero time-points would be 50 nmol/mL for cystamine (or cysteamine). At the times shown, the deproteinized samples were simultaneously analysed for cystamine and cysteamine. A trace of cystamine was observed at time zero in the tissue homogenates spiked with cysteamine. This cystamine rapidly disappeared. The data shown in each of the panels are from a single experiment with tissue homogenates obtained from a single mouse. The findings in this experiment were similar to those found for tissues obtained from another wild-type control mouse spiked with cystamine or cysteamine (see Results).

40 min. It is interesting that, in both experiments, maximal levels of cysteamine in the brain and kidney homogenates were attained at a time when almost all the cystamine had been removed, suggesting direct reductive cleavage of cystamine to cysteamine. On the other hand, there was a considerable lag in maximal cysteamine formation in the liver homogenate after addition of cystamine, suggesting that for this tissue most of the cysteamine arose indirectly from cystamine.

As cysteamine derived from cystamine declined somewhat slowly in the tissue homogenates, cysteamine added directly should likewise decline slowly and with similar kinetics. This was found to be the case (experiment 1; compare Fig. 3b with 3d). In experiment 2, the kinetics of the disappearance of cysteamine also followed a similar pattern whether the cysteamine was derived from cystamine or added directly to the homogenate. Interestingly, in experiment 1, after adding cysteamine to the brain and kidney homogenates, the 'zero' time-point for cysteamine concentration was close to the expected value but this was not the case for the liver homogenate (Fig. 3d). In experiment 2, where we were able to sample at 30-s intervals, the time zero level of cysteamine was also low. However, we noted a rapid climb from the 'zero' time-point after addition of cysteamine to a maximum at about 1–2 min followed by a slower decline. Thus, it appears that for liver homogenates, cysteamine may be reacting extremely rapidly with another biomolecule only to be released as the tissue is incubated.

Cystamine is rapidly removed from mouse plasma

When cystamine was added to mouse plasma diluted 1 : 10 with PBS, it was rapidly removed in a process that exhibits triphasic kinetics (Fig. 4a). A large percentage of the

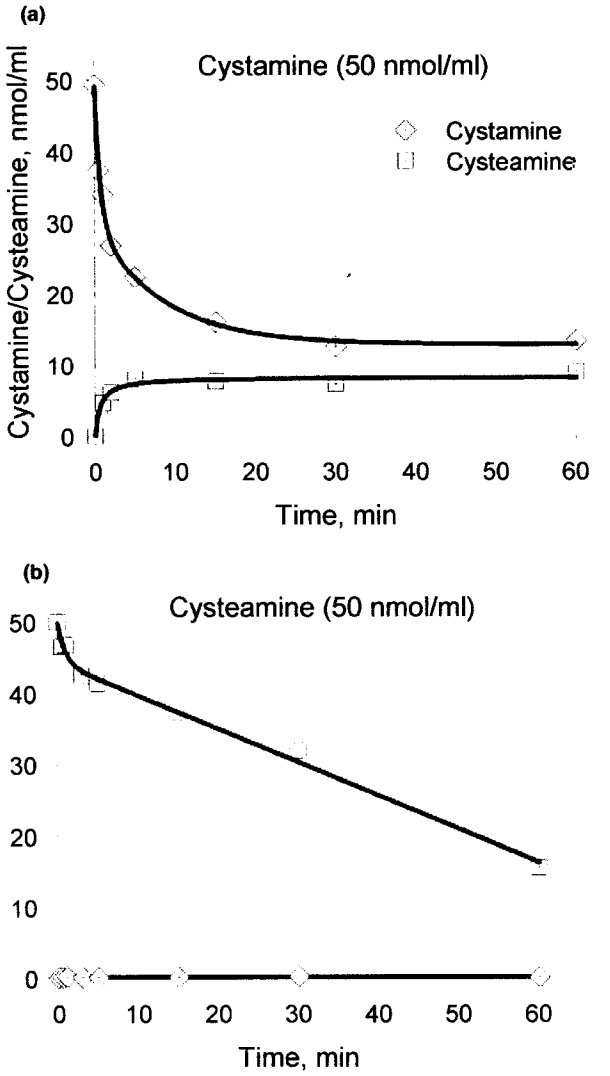

Fig. 4 Disappearance of (a) cystamine and (b) cysteamine added to mouse plasma diluted 10-fold in phosphate-buffered saline (PBS). (a) An aliquot of plasma from a single mouse diluted 10-fold in PBS was spiked with a solution of cystamine so that the final concentration was 246 nmol/mL. (b) An aliquot of diluted plasma was spiked with cysteamine so that the final concentration was 250 nmol/mL. After dilution into deproteinizing agent, the final concentration of both cysteamine and cystamine was ~50 nmol/mL at time zero. The spiked plasma samples were incubated at 37°C. At the times shown aliquots were withdrawn and stored at −80°C until analysed for cysteamine and cystamine (see Materials and methods). In (a), the disappearance of cystamine fits a triphasic process [$Y = Y_0 + a^*e^{(-b^*x)} + c^*e^{(-d^*x)}$; Y_0 = 12.8, a = 19.2, b = 1.4, c = 17.0, d = 0.12, r^2 = 0.997], whereas the appearance of cysteamine is best described as monophasic [$Y = (a^*x)/(b + x)$; a = 8.21, b = 0.62, r^2 = 0.963]. In (b), the disappearance of cysteamine fits a biphasic process [$Y = Y_0 + a^*e^{(-b^*x)} + c^*x$; Y_0 = 44.3641, a = 5.45, b = 1.04, c = −0.47, r^2 = 0.994].

cystamine was removed within about 1 min. After about 20 min only about 30% of the added cystamine was still present in the plasma. The rapid disappearance of cystamine was accompanied by the rapid appearance of cysteamine but the maximal conversion of cystamine to cysteamine (taking into account the fact that reduction of cystamine produces two equivalents of cysteamine) was only about 8–9%. On the other hand, when cysteamine was added to the diluted plasma sample it disappeared in a biphasic manner (Fig. 4b). There was a rapid loss of about 10–15% of the added cysteamine in the first few minutes followed by a slower exponential loss. No cystamine (< 1 nmol/mL) could be detected in this experiment. The half-life for cysteamine loss from mouse plasma in this experiment was about 45 min. Previously, Hsuing *et al.* (1978) reported the half-life of cysteamine added to rat plasma to be about 1.7 h.

The concentration of free GSH in plasma is relatively low (e.g. Meister 1989). Therefore, this compound was not used for quality control in the spiked plasma samples. On the other hand, ascorbic acid is present in the diluted plasma sample at a relatively high concentration (final concentration in the deproteinized, diluted plasma samples, ~4 nmol/mL). However, ascorbic acid is extremely sensitive to air oxidation at pH values near neutral (e.g. Cooper *et al.* 1980). Therefore, as expected, the ascorbic acid peak completely disappeared from the 1 : 10 diluted plasma samples within minutes. On the other hand, uric acid is relatively stable and remained constant in the two diluted plasma samples shown in Figs 4(a and b) even over a period of 1 h at 37°C (final concentration in the deproteinized, diluted plasma ~1.2 nmol/mL).

Discussion

Development of a CoulArray (coulometric) system for the analysis of sulfur-containing compounds

Although, as noted in the Introduction, HPLC methods coupled to redox detection of small molecular weight sulfur-containing biomolecules have been published previously, the current procedure is the first to permit simultaneous measurement of cystamine and cysteamine in biological samples without prior derivatization. The procedure is rapid (five determinations can be performed per hour), relatively simple and requires minimal sample preparation. The method has the added advantage of also providing levels of GSH, cysteine and ascorbic acid (and, where present in sufficient quantities, uric acid, glutathione disulfide and hypotaurine). In addition, the system was adapted so that the sulfur-containing metabolite taurine can be rapidly quantitated in biological samples as its isoindole-sulfonate derivative. The procedures should be of general interest to biochemists studying sulfur metabolism.

To our knowledge, cystamine concentrations in tissues have not been published. On the other hand, tissue levels of cysteamine have been reported but the values in the literature vary enormously. For example, the rodent kidney has been reported to contain 0.17, 7.9, 10 and 15 nmol cysteamine/g wet weight (Huxtable and Bressler 1976; Ziegler *et al.* 1983; Garcia *et al.* 1988; Pitari *et al.* 2000, respectively). The values for rodent liver range from 0.21, 1, 8.5, 24 to 267 nmol/g wet weight (Huxtable and Bressler 1976; Kelley *et al.* 1967; Ziegler *et al.* 1983; Garcia *et al.* 1988; Pitari *et al.* 2000, respectively). Ricci *et al.* (1983) reported a concentration of cysteamine in bovine kidney, liver, heart and brain of 23, 20, 1.7 and 1.6 nmol/g wet weight, respectively. In contrast, Ida *et al.* (1984) reported values of cyst(e)amine (i.e. cysteamine plus cystamine) in rat kidney, heart and liver of 150, 124 and 3.5 pmol/g wet weight; cysteamine was not detectable in rat brain. We determined that ≤0.2 nmol of cysteamine/100 mg of protein (or ≤0.2 nmol/g wet weight) was present in normal mouse brain, liver or kidney. Our values are consistent with the lower values reported in the literature. Part of the variation in the reported values may be due to species differences. However, Garcia *et al.* (1988) noted that cysteamine values in rat liver and kidney increased significantly with post-mortem time. Thus, post-mortem interval may also be a factor in the variability of reported cysteamine values. Our measurements are likely to be accurate for rat tissues based on the following criteria. As noted above, the CoulArray system directly detects cysteamine without prior derivatization. Moreover, the voltage required to oxidize cysteamine provides an additional 'fingerprint' which can be used to verify the presence of cysteamine. In addition, separation of cyst(e)amine using reverse phase HPLC eliminates cross-contamination measurements of other compounds with free sulfhydryl moieties. The cyst(e)amine fingerprints can be seen in brain homogenates spiked with cysteamine (Fig. 1). The low level of cysteamine in mouse brain (present work) is consistent with the low levels of radioactivity in mouse brain detected autoradiographically after systemic administration of [^{35}S]cysteamine (Nelson and Ullberg 1960). In summary, although the literature values vary considerably, the most realistic values for levels of cysteamine in normal rodent tissues are on the extremely low side.

Several studies have employed cystamine in cell culture studies (e.g. Lesort *et al.* 2003; Fox *et al.* 2004). In these studies relatively low levels of cystamine were used in the medium (0.2 and 0.05 mM, respectively) compared with the 8 mM present in the drinking water of the mice used in the present study. Although the present HPLC procedures were developed to determine levels of cystamine (and its metabolites) in mouse tissues, the procedure should be suitable for measuring sulfur-containing metabolites in cells exposed to cystamine in culture.

Pharmacological and metabolic interconversions of cyst(e)amine in mice

Using our newly developed HPLC–coulometric detection system we showed that the levels of cystamine and cysteamine are below the accurate detection limit (≤ 0.2 nmol/100 mg of protein) in the brains of 3-month-old YAC128 mice (and their wild-type control littermates) provided for 2 weeks with drinking water containing pharmacological doses of cystamine. In addition, we showed that the levels of cystamine and cysteamine are below the accurate detection limit (< 0.2 nmol/mL) in the plasma of YAC128 mice (and their wild-type littermates) provided with pharmacological doses of cystamine in their drinking water for 8 months beginning at the age of 4 months or in plasma from YAC128 mice (and their wild-type littermates) provided for 5 months with pharmacological doses of cystamine beginning at the age of 7 months. This inability to detect cyst(e)amine in plasma and brain may result in part from rapid metabolism. Indeed, we found that cystamine was rapidly (in minutes) metabolized in homogenates of mouse brain, liver and kidney (Fig. 3; Results). By contrast, the addition of cysteamine to tissue homogenates did not result in appreciable conversion to cystamine. Instead, cysteamine was metabolized to additional compounds (Fig. 3).

The rapid disappearance of cystamine in plasma (Fig. 4) is consistent with the known chemical properties of this disulfide. Wilson *et al.* (1980) demonstrated that reduction of cystamine by thiols is a very facile reaction (~10^3/M/s). Thus, we hypothesize that cystamine is rapidly reduced by thiol-bearing compounds (RSH) in plasma (reaction 1, Fig. 5). This reduction would be accompanied by the concomitant oxidation of the thiol to a mixed disulfide and the release of cysteamine (reaction 1, Fig. 5). The resulting mixed disulfide would then be reduced by a second thiol species (R′SH) to release the remaining cysteamine (reaction 2, Fig. 5). The plasma thiol-bearing entities responsible for reactions 1 and 2 (Fig. 5) have not yet been identified but are currently under investigation in our laboratories. However, plasma albumin is likely to participate in reaction 1 as it is present at high concentrations (60 mg/dL) and has several available thiol groups. Moreover, Sengupta *et al.* (2001) have described an analogous reaction, the formation of mixed disulfides of plasma albumin and homocysteine from homocystine. The reduction of the RS-cysteamine mixed disulfide could be mediated by plasma GSH, cysteine or albumin (reaction 2, Fig. 5).

Cysteamine is also short-lived in plasma (Fig. 4). This finding is in accord with the low reducing potential of plasma, which has only ~10 μM GSH versus the mM amounts found in most cells (Cooper *et al.* 1980; Griffith 1981; Cooper 1998; Dringen 2000). Values of plasma cysteamine of up to 40 μM have been reported in subjects receiving cysteamine for the treatment of cystinosis (Jonas and Schneider 1981, 1982). However, the samples in these

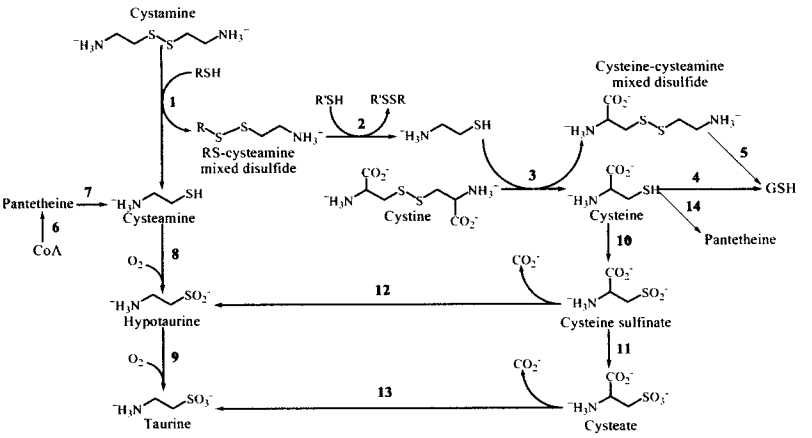

Fig. 5 Pharmacological and metabolic interconversions of cystamine. Reactions 1–3 depict thiol-disulfide interchanges that reduce cystamine to cysteamine and cystine to cysteine. This cysteine can then be incorporated into glutathione (GSH) (reaction 4) by the combined actions of glutamate-cysteine ligase and GSH synthetase. The reduction of cystine by cysteamine is accompanied by oxidation of cysteamine to cysteine-cysteamine mixed disulfide (reaction 3) which, in turn, can be reduced to cysteine and also utilized for GSH synthesis (reaction 5). Cysteamine is metabolically derived from CoA (reaction 6) and released from pantetheine by pantetheinase (reaction 7). Cysteamine dioxygenase oxidizes cysteamine to hypotaurine (reaction 8) which is further oxidized to taurine by hypotaurine dehydrogenase (reaction 9). Cysteine dioxygenase catalyses the oxidation of cysteine to cysteine sulfinate (reaction 10). The latter undergoes a second oxidation to yield cysteate (reaction 11). Cysteine sulfinate can be decarboxylated by cysteine sulfinate decarboxylate to give taurine (reaction 12). Cysteate can also be decarboxylated to taurine (reaction 13). Finally, cysteine can be incorporated into pantetheine (reaction 14) as well as GSH. Note that, although cysteamine is formally decarboxylated cysteine, cysteamine does not arise *in vivo* from the decarboxylation of this amino acid. Note also that taurine may arise directly from cysteamine (reactions 8 and 9). This pathway occurs in the liver. In the brain, taurine predominantly arises from cysteine (reactions 8–13). (Adapted and expanded from Griffith 1987 and Huxtable 1989.)

studies were reduced before analysis and therefore reflect the total plasma content of cysteamine rather than the concentration of reduced cysteamine *per se*. Cysteamine is thought to reduce plasma cystine to cysteine while being oxidized to cysteine-cysteamine mixed disulfide (reaction 3, Fig. 5) (Meier and Issels 1992; Jókay *et al.* 1998). In the brain, cysteine is used for GSH (reaction 4, Fig. 5) and protein (Dringen 2000) synthesis. Cysteine-cysteamine mixed disulfide resembles lysine and enters cells via amino acid transporters (Pisoni *et al.* 1985, 1995; Meier and Issels 1992). Cellular GSH would then reduce the cysteine-cysteamine mixed disulfide to cysteine and cysteamine by reactions analogous to reactions 1 and 2 (substitute cysteine-cysteamine mixed disulfide for cystamine and GSH for RSH and R'SH in Fig. 5) (Meier and Issels 1992). The released cysteine would then be available for GSH and protein synthesis (reaction 4, Fig. 5). Thus, cyst(e)amine can promote GSH synthesis by supplying cysteine and this has been validated in numerous *in vivo* experiments (Meier and Issels 1992; Jeitner and Lawrence 2001). The administration of cystamine to animals, however, does not promote a significant increase in the GSH content of the brain (Fig. 3 and Fox *et al.* 2004). There are at least two possible explanations for this finding. GSH synthesis begins with the formation of γ-glutamylcysteine and is catalysed by glutamate-cysteine ligase (γ-glutamylcysteine) synthetase (Meister 1989; Griffith 1999; Dringen 2000). The brain content of glutamate-cysteine ligase is low (Liu 2002; Liu and Choi 2000) and is thought to account, in part, for the very slow GSH turnover time in this tissue (~60–80 h) (Douglas and Mortensen 1956; Chang *et al.* 1997). It is possible that glutamate-cysteine ligase in the brain is saturated at very low concentrations of cysteine. This explanation, however, cannot account for the observation that GSH content can be increased by other agents that increase cysteine levels in the brain (Dringen 2000). Cystamine is a potent inhibitor of glutamate-cysteine ligase (Griffith *et al.* 1977). If cystamine was able to enter cells intact then it might be able to inhibit glutamate-cysteine ligase and thereby prevent the utilization of cysteine for GSH synthesis. Cystamine is similar in structure to polyamines and is an effective inhibitor of cellular polyamine uptake (Wyatt *et al.* 1989; Hoet *et al.* 1993, 1994, 1995). Thus, cystamine could enter cells and inhibit glutamate-cysteine ligase if it escapes reduction by intracellular thiols. This is possible as glutamate-cysteine ligase is situated at the plasmalemma in close proximity to the sites of entry for cystamine. In addition, the redox environment of degenerating neurons is likely to be more oxidizing and less likely to reduce cystamine (Bogdanov *et al.* 2000, 2001; Mecocci *et al.* 1993, 1994; Polidori *et al.* 1999; Klivenyi *et al.* 2000; Schulz *et al.* 2000).

As noted above, the influx of cysteine-cysteamine mixed disulfide is expected to result in an accumulation of cysteine. This expectation is supported by our present findings (Table 1). Cysteamine would also be released by the intracellular reduction of cysteine-cysteamine mixed disulfide (discussed above). No appreciable amounts of cysteamine, however, were detected in the brains of the cystamine-treated mice (Fig. 1). Cysteamine normally arises as a metabolite during the catabolism of CoA (Abiko 1975) (Fig. 5). This cofactor is converted to pantetheine which, in turn is hydrolysed to cysteamine and pantothenic acid (Pitari *et al.* 2000). Cysteamine can then be oxidized to hypotaurine or taurine through the actions of cysteamine dioxygenase and hypotaurine dehydrogenase, respectively (reactions 8 and 9, Fig. 5). Although cystamine acts to deliver cysteamine to the brain (Golubentsev and Titov 1973; Titov *et al.* 1974; Widmann *et al.* 1988), we were unable to detect hypotaurine in the brains of cystamine-treated YAC128 mice (and their littermates) and taurine levels were unchanged (Table 1). These observations suggest that oxidation to hypotaurine and taurine is probably not a major pathway for the catabolism of cysteamine in the brain. Taurine is thought to arise in the brain via cysteine (reactions 9–13, Fig. 5) (Huxtable 1989). However, despite the fact that cysteine is elevated in the brains of the cystamine-treated mice, taurine levels were not elevated. This may be due, in part, to the 50-fold greater pool size of taurine relative to cysteine in the normal mouse brain (Table 1).

Mechanisms for the therapeutic benefit of cystamine

The lack of detectable cysteamine in the brains of animals treated with cystamine suggests a rapid utilization of this aminothiol by pathways other than those shown in reactions 8 and 9 (Fig. 5). One possibility involves the action of TGases. TGase activity is significantly increased in both HD and animal models of this disease (Karpuj *et al.* 1999, 2002; Lesort *et al.* 1999; Jeitner *et al.* 2001; Dedeoglu *et al.* 2002; Zainelli *et al.* 2003). Jeitner *et al.* (2005) have demonstrated that TGase 2 catalyses the covalent attachment of cysteamine to a suitable protein/peptide substrate, resulting in the formation of a γ-glutamylcysteamine moiety attached to a modified Q residue. This alteration would result in an increase in the thiol content of the modified protein and thereby offer greater antioxidant protection in the immediate vicinity of the peptide. Free γ-glutamylcysteamine is also a potent antioxidant. Thus, the formation of γ-glutamylcysteamine may significantly augment the cellular antioxidant defenses. However, it remains to be determined whether such a mechanism contributes to the neuroprotection afforded by cyst(e)amine. Determining the basis of this neuroprotection is obviously important. The protection is likely to involve metabolites of cyst(e)amine and is the topic of active investigation in our laboratories.

The increase in brain cysteine content is the most profound biochemical effect of cystamine administration reported thus far (Table 1). While the present work was in progress, Fox *et al.* (2004) reported that brain GSH levels were unaltered whereas brain cysteine levels were greatly elevated in R6/2 HD transgenic mice administered cystamine i.p. (112 mg/kg/day) for 4 weeks beginning at 23 days of age. These authors, however, did not report on the levels of cystamine and cysteamine (or of their metabolites hypotaurine and taurine) in the brains of the cystamine-treated mice. Despite the fact that we used a different HD mouse model (YAC128 vs. R6/2), different routes of administration (drinking water vs. i.p.) and different analytical techniques, we have robustly confirmed the findings of Fox *et al.* (2004) that short-term (weeks) cystamine administration to mice does not result in an appreciable increase of brain GSH but leads to a significant increase of brain cysteine. Our results extend the current understanding of sulfur metabolism in brains of cystamine-treated mice by providing a method for the simultaneous estimation of cysteamine and cystamine in biological samples in addition to those of cysteine, GSH, hypotaurine and taurine.

As discussed earlier, cysteine is unlikely to contribute to the therapeutic effects by promoting the formation of GSH, hypotaurine or taurine. Cysteine also does not serve as a significant substrate for TGase 2 (unpublished observations). Although elevated cysteine does not promote increased GSH synthesis, this molecule is also a good antioxidant (Winterbourn 1985; Winterbourn and Metodiewa 1999; Peskin and Winterbourn 2001). As oxidative processes figure prominently in the neurodegeneration, cysteine may be acting to slow oxidative damage in the brain (Fox *et al.* 2004).

Summary

As noted in the Introduction, cystamine participates in rapid disulfide interchange reactions with protein cysteine residues (Duffel *et al.* 1987). Such a disulfide interchange reaction is likely to account for the inactivation of glutamate-cysteine ligase (Griffith *et al.* 1977), guanylate cyclase (Brandwein *et al.* 1981), placental GSH *S*-transferase (Nishihara *et al.* 1991) and caspases (Lesort *et al.* 2003) by cystamine *in vitro* and *in situ* in 5H-SY5Y cells in culture (Lesort *et al.* 2003). Our findings, however, suggest that the levels of cystamine in cystamine-treated mice are too low to significantly affect intracellular enzymes in brain that require a critical cysteine residue for activity. The findings seemingly rule out the possibility that cystamine treatment is beneficial in HD mice by inhibiting caspases. We have verified the previous findings of Fox *et al.* (2004) that short-term (weeks) cystamine treatment results in no change in mouse brain GSH but increased brain cysteine. Whether this increase in brain cysteine is protective in HD remains to be determined. It is probable that cyst(e)amine metabolism is complex. The ability of cystamine to form mixed disulfides, γ-glutamylcysteamine and other, as yet unidentified, metabolites is

currently under investigation. It is anticipated that a more complete knowledge of the metabolism of this small, but fascinating, molecule will shed light on mechanisms contributing to the protective effect of cyst(e)amine in HD, whether or not TGases are involved and may suggest improved regimens for the treatment of HD.

Acknowledgements

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References

Abiko Y. (1975) Metabolism of coenzyme A, in *Metabolism of Sulfur Compounds* (Greenberg D. M., ed.), pp. 1–25. Academic Press, New York.

Aeschlimann D. and Thomazy V. (2000) Protein crosslinking in assembly and remodelling of extracellular matrices: the role of transglutaminases. *Connect. Tissue Res.* **41**, 1–27.

Arrasate M., Mitra S., Schweitzer E. S., Segal M. R. and Finkbeiner S. (2004) Inclusion body formation reduces levels of mutant huntingtin and the risk of neuronal death. *Nature* **431**, 805–810.

Bailey C. D. C. and Johnson G. V. W. (2005) Tissue transglutaminase contributes to disease progression in the R6/2 Huntington's disease mouse model via aggregate-independent mechanisms. *J. Neurochem.* **92**, 83–92.

Bates G. (2003) Huntingtin aggregation and toxicity in Huntington's disease. *Lancet* **361**, 1642–1644.

Bogdanov M., Brown R. H., Matson W., Smart R., Hayden D., O'Donnell H., Flint B. M. and Cudkowicz M. (2000) Increased oxidative damage to DNA in ALS patients. *Free Rad. Biol. Med.* **29**, 652–658.

Bogdanov M. B., Andreassen O. A., Dedeoglu A., Ferrante R. J. and Beal M. F. (2001) Increased oxidative damage to DNA in a transgenic mouse model of Huntington's disease. *J. Neurochem.* **79**, 1246–1249.

Brandwein H. J., Lewicki J. A. and Murad F. (1981) Reversible inactivation of guanylate cyclase by mixed disulfide formation. *J. Biol. Chem.* **256**, 2958–2962.

Browne S. E. and Beal M. F. (2004) The energetics of Huntington's disease. *Neurochem. Res.* **29**, 531–546.

Chang M. L., Klaidman L. K. and Adams J. D. Jr (1997) The effects of oxidative stress on *in vivo* brain GSH turnover in young and mature mice. *Mol. Chem. Neuropathol.* **30**, 187–197.

Chen M., Ona V. O., Li M. *et al.* (2000) Minocycline inhibits caspase-1 and caspase-3 expression and delays mortality in a transgenic mouse model of Huntington disease. *Nat. Med.* **6**, 797–801.

Cooper A. J. L. (1998) Role of astrocytes in maintaining cerebral glutathione homeostasis and in protecting the brain against xenobiotics and oxidative stress, in *The Role of Glutathione in the Nervous System* (Shaw C. A., ed.), pp. 91–115. Taylor & Francis, Washington, DC.

Cooper A. J. L., Pulsinelli W. A. and Duffy T. E. (1980) Glutathione and ascorbate during ischemia and post-ischemic reperfusion. *J. Neurochem.* **35**, 1242–1245.

Cooper A. J. L., Sheu K.-F. R., Burke J. R., Onodera O., Strittmatter W. J., Roses A. D. and Blass J. P. (1997) Polyglutamine domains are substrates of tissue transglutaminase. Does transglutaminase play a role in expanded CAG/poly-Q neurodegenerative diseases? *J. Neurochem.* **69**, 431–434.

Cooper A. J. L., Jeitner T. M., Gentile V. and Blass J. B. (2002) Cross linking of polyglutamine domains catalyzed by tissue transglutaminase is greatly favored with pathological length repeats: Does transglutaminase activity play a role in $(CAG)_n/Q_n$-expansion diseases? *Neurochem. Int.* **40**, 53–67.

de Cristofaro T., Affaitati A., Cariello L., Avvedimento E. V. and Varrone S. (1999) The length of polyglutamine tract, its level of expression, the rate of degradation, and transglutaminase activity influence the formation of intracellular aggregates. *Biochem. Biophys. Res. Commun.* **260**, 150–158.

Dedeoglu A. D., Kubilus J. K., Jeitner T. M. *et al.* (2002) Therapeutic effects of the transglutaminase inhibitor, cystamine, in a murine model of Huntington's disease. *J. Neurosci.* **22**, 8942–8950.

Douglas G. W. and Mortensen R. A. (1956) The rate of metabolism of brain and liver glutathione in the rat studied with C^{14}-glycine. *J. Biol. Chem.* **222**, 581–585.

Dringen R. (2000) Metabolism and functions of glutathione in brain. *Prog. Neurobiol.* **62**, 649–671.

Duffel M. W., Logan D. J. and Ziegler D. M. (1987) Cysteamine and cystamine. *Meth. Enzymol.* **143**, 149–154.

Folk J. E. (1983) Mechanism and basis for specificity of transglutaminase-catalyzed ε-(γ-glutamyl) lysine bond formation. *Adv. Enzymol.* **54**, 1–56.

Fox J. H., Barber D. S., Singh B. *et al.* (2004) Cystamine increases L-cysteine levels in Huntington's disease mouse brain and in a PC12 model of polyglutamine aggregation. *J. Neurochem.* **91**, 413–422.

Garcia R. A., Hirschberger L. L. and Stipanuk M. H. (1988) Measurement of cyst(e)amine in physiological samples by high performance liquid chromatography. *Anal. Biochem.* **170**, 432–440.

Gentile V., Sepe C., Calvani M., Melone M. A. B., Cotrufo R., Cooper A. J. L., Blass J. P. and Peluso G. (1998) Tissue transglutaminase-catalyzed formation of high-molecular-weight aggregates *in vitro* is favored with long polyglutamine domains: a possible mechanism contributing to CAG-triplet diseases. *Arch. Biochem. Biophys.* **352**, 314–321.

Golubentsev D. A. and Titov A. V. (1973) Chemical transformations of cystamine in mouse tissues. *Voprosy Med. Khimii* **19**, 177–181. (In Russian.)

Green H. (1993) Human genetic diseases due to codon reiteration: relationship to an evolutionary mechanism. *Cell* **74**, 955–956.

Griffith O. W. (1981) The role of glutathione turnover in the apparent renal secretion of cystine. *J. Biol. Chem.* **256**, 12 263–12 268.

Griffith O. W. (1987) Mammalian sulfur amino acid metabolism: An overview. *Meth. Enzymol.* **143**, 366–376.

Griffith O. W. (1999) Biologic and pharmacologic regulation of mammalian glutathione synthesis. *Free Rad. Biol. Med.* **27**, 922–935.

Griffith O. W., Larsson A. and Meister A. (1977) Inhibition of γ-glutamylcysteine synthetase by cystamine: An approach to the therapy of 5-oxoprolinuria (pyroglutamic aciduria). *Biochem. Biophys. Res. Commun.* **79**, 919–925.

Hoet P. H., Dinsdale D., Lewis C. P., Verbeken E. K., Lauweryns J. M. and Nemery B. (1993) Kinetics and cellular localisation of putrescine uptake in human lung tissue. *Thorax* **48**, 1235–1241.

Hoet P. H., Lewis C. P., Demedts M. and Nemery B. (1994) Putrescine and paraquat uptake in human lung slices and isolated type II pneumocytes. *Biochem. Pharmacol.* **48**, 517–524.

Hoet P. H., Lewis C. P., Dinsdale D., Demedts M. and Nemery B. (1995) Putrescine uptake in hamster lung slices and primary cultures of type II pneumocytes. *Am. J. Physiol.* **269**, L681–L689.

Hsuing M., Yeo Y. Y., Itiaba K. and Crawhall J. C. (1978) Cysteamine, penicillamine, glutathione, and their derivatives analyzed by automated ion exchange column chromatography. *Biochem. Med.* **19**, 305–317.

Huxtable R. J. (1989) Taurine in the central nervous system and the mammalian actions of taurine. *Prog. Neurobiol.* **32**, 471–533.

Huxtable R. J. and Bressler R. (1976) The metabolism of cysteamine to taurine, in *Taurine* (Huxtable R. J. and Barbeau A., eds), pp. 45–57. Raven Press, New York.

Ida S., Tanaka Y., Ohkuma S. and Kuriyama K. (1984) Determination of cystamine by high-performance liquid chromatography. *Anal. Biochem.* **136**, 352–356.

Igarashi S., Koide R., Shimohata T. *et al.* (1998) Suppression of aggregate formation and apoptosis by transglutaminase inhibitors in cells expressing truncated DRPLA protein with an expanded polyglutamine stretch. *Nat. Genet.* **18**, 111–117.

Iuchi S., Hoffner G., Verbeke P., Djian P. and Green H. (2003) Oligomeric and polymeric aggregates formed by proteins containing expanded polyglutamine. *Proc. Natl Acad. Sci. USA* **100**, 2409–2414.

Jeitner T. M. and Lawrence D. A. (2001) Mechanisms for the cytotoxicity of cysteamine. *Toxicol. Sci.* **63**, 57–64.

Jeitner T. M., Bogdanov M. B., Matson W. R. *et al.* (2001) N^{ε}-(γ-L-Glutamyl)-L-lysine (GGEL) is increased in the cerebrospinal fluid of patients with Huntington's disease. *J. Neurochem.* **79**, 1109–1112.

Jeitner T. M., Delikatney E. J., Ahlqvist J., Capper H. and Cooper A. J. L. (2005) Mechanism for the inhibition of transglutaminase 2 by cystamine. *Biochem. Pharmacol.* in press.

Jókay I., Kelemenics K., Gyuris A. and Minárovits J. (1998) S-Methylthio-cysteine and cystamine are potent stimulators of thiol production and glutathione synthesis. *Life Sci.* **62**, PL27–33.

Jonas A. J. and Schneider J. A. (1981) A simple, rapid assay for cysteamine and other thiols. *Anal. Biochem.* **114**, 429–432.

Jonas A. J. and Schneider J. A. (1982) Plasma cysteamine concentrations in children treated for cystinosis. *J. Pediatr.* **100**, 321–323.

Kahlem P., Terré C., Green H. and Djian P. (1996) Peptides containing glutamine repeats are substrates for transglutaminase-catalyzed cross-linking: relevance to diseases of the nervous system. *Proc. Natl Acad. Sci. USA* **93**, 14 580–14 585.

Kahlem P., Green H. and Djian P. (1998) Transglutaminase action imitates Huntington's disease: Selective polymerization of huntingtin containing polyglutamine. *Mol. Cell* **1**, 595–601.

Karpuj M. V., Garren H., Slunt H., Price D. L., Gusella J., Becher M. W. and Steinman L. (1999) Transglutaminase aggregates huntingtin into nonamyloidogenic polymers, and its enzymatic activity increases in Huntington's disease nuclei. *Proc. Natl Acad. Sci. USA* **96**, 7388–7393.

Karpuj M. V., Becher M. W., Springer J. E., Chabas D., Youssef S., Pedotti R., Mitchell D. and Steinman L. (2002) Prolonged survival and decreased abnormal movements in transgenic model of Huntington disease, with administration of the transglutaminase inhibitor cystamine. *Nat. Med.* **8**, 143–149.

Kelley J. J., Herrington K. A., Ward S. P., Meister A. and Friedman O. M. (1967) Studies on latent derivatives of aminoethanethiols as potentially selective cryoprotectants. II. *In vivo* distribution of cysteamine liberated in rat tissues. *Cancer Res.* **27**, 137–142.

Kim S.-Y., Grant P., Lee J.-H., Pant H. C. and Steinert P. M. (1999) Differential expression of multiple transglutaminases in human brain. Increased expression and cross-linking by transglutaminases 1 and 2 in AD. *J. Biol. Chem.* **274**, 30 715–30 721.

Klivenyi P., Andreassen O. A., Ferrante R. J., Dedeoglu A., Mueller G., Lancelot E., Bogdanov M., Andersen J. K., Jiang D. and Beal M. F. (2000) Mice deficient in cellular glutathione peroxidase show increased vulnerability to malonate, 3-nitropropionic acid, and 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine. *J. Neurosci.* **20**, 1–7.

Kuemmerle S., Gutekanst C.-A., Klein A. M., Li X.-J., Li S. H., Beal M. F., Hersch S. M. and Ferrante R. J. (1999) Huntingtin aggregates may not predict neuronal death in Huntington's disease. *Ann. Neurol.* **46**, 842–849.

Lai T.-S., Tucker T., Burke J. R., Strittmatter W. J. and Greenberg C. S. (2004) Effect of tissue transglutaminase on the solubility of proteins containing expanded polyglutamine repeats. *J. Neurochem.* **88**, 1253–1260.

Lakritz J., Plopper C. G. and Buckpitt A. R. (1997) Validated high-performance liquid chromatography-coulometric method for determination of glutathione and glutathione disulfide in small tissue samples. *Anal. Biochem.* **247**, 63–68.

Lesort M., Chun W., Johnson G. V. W. and Ferrante R. J. (1999) Tissue transglutaminase is increased in Huntington's disease brain. *J. Neurochem.* **73**, 2018–2027.

Lesort M., Tucholski J., Zhang J. and Johnson G. V. W. (2000) Impaired mitochondrial function results in increased tissue transglutaminase activity *in situ*. *J. Neurochem.* **75**, 1951–1961.

Lesort M., Lee M., Tucholski J. and Johnson G. V. W. (2003) Cystamine inhibits caspase activity. Implication for the treatment of polyglutamine disorders. *J. Biol. Chem.* **278**, 3825–3830.

Liu R. and Choi J. (2000) Age-associated decline in γ-glutamylcysteine synthetase gene expression in rats. *Free Rad. Biol. Med.* **28**, 566–574.

Lorand L. and Conrad S. M. (1984) Transglutaminases. *Mol. Cell Biochem.* **58**, 9–35.

Mandrusiak L. M., Beitel L. K., Wang X., Scanlon T. C., Chevalier-Larsen E., Merry D. E. and Trifiro M. A. (2003) Transglutaminase potentiates ligand-dependent proteasome dysfunction induced by polyglutamine-expanded androgen receptor. *Hum. Mol. Genet.* **12**, 1497–1506.

Maryuma H., Izumi Y., Morino H., Oda M., Toji H., Nakamura S. and Kawakami H. (2002) Differences in disease-free survival curve and regional distribution according to subtype of spinocerebellar ataxia: a study of 1,286 Japanese patients. *Am. J. Med. Genet.* **114**, 578–583.

Mastroberardino P. G., Iannocola C., Nardacci R., Bernassola F., De Lurenzi V., Melino G., Pavone F., Oliverio S., Fesus L. and Piacentini M. (2002) 'Tissue' transglutaminase ablation reduces neuronal death and prolongs survival in a mouse model of Huntington's disease. *Cell Death Differ.* **9**, 873–880.

Mecocci P., MacGarvey U., Kaufman A. E., Koontz D., Shoffner J. M., Wallace D. C. and Beal M. F. (1993) Oxidative damage to mitochondrial DNA shows marked age-dependent increases in human brain. *Ann. Neurol.* **34**, 609–616.

Mecocci P., MacGarvey U. and Beal M. F. (1994) Oxidative damage to mitochondrial DNA is increased in Alzheimer's disease. *Ann. Neurol.* **36**, 747–751.

Meier T. and Issels R. D. (1992) Promotion of cyst(e)ine uptake. *Meth. Enzymol.* **252**, 103–112.

Meister A. (1989) Metabolism and function of glutathione, in *Glutathione: Chemical, Biochemical and Medical Aspects — Part A.* (Dophin D., Poulson R. and Avramović O., eds), pp. 367–474. John Wiley and Sons, New York.

Melnyk S., Pogribna M., Progribny I., Hine R. J. and James S. J. (1999) A new HPLC method for the simultaneous determination of oxidized and reduced plasma aminothiols using coulometric detection. *J. Nutr. Biochem.* **10**, 490–497.

Mirecki A., FitzMaurice P., Ang L. *et al.* (2004) Brain antioxidant systems in human methamphetamin users. *J. Neurochem.* **89**, 1396–1408.

Nelson A. and Ullberg S. (1960) Distribution of S^{35} in mice after injection of S35-cysteamine: An autoradiographic investigation. *Acta Radiol.* **53**, 305–313.

Nishihara T., Maeda H., Okamoto K., Oshida T., Mizoguchi T. and Terada T. (1991) Inactivation of human placenta glutathione *S*-transferase by SH/SS exchange reactions with biological disulfides. *Biochem. Biophys. Res. Commun.* **174**, 580–585.

Ona V. O., Li M., Vonsatell J.-P. *et al.* (1999) Inhibition of caspase-1 slows disease progression in a mouse model of Huntington's disease. *Nature* **399**, 263–267.

Peskin A. V. and Winterbourn C. C. (2001) Kinetics of the reactions of hypochlorous acid and amino acid chloramines with thiols, methionine, and ascorbate. *Free Rad. Biol. Med.* **30**, 572–579.

Pisoni R. L., Thoene J. G. and Christensen H. N. (1985) Detection and characterization of carrier-mediated cationic amino acid transport in lysosomes of normal and cystinotic human fibroblasts. Role in therapeutic cystine removal? *J. Biol. Chem.* **260**, 4791–4798.

Pisoni R. L., Park G. Y., Velilla V. Q. and Thoene J. G. (1995) Detection and characterization of a transport system mediating cysteamine entry into human fibroblast lysosomes. Specificity for aminoethylthiol and aminoethylsulfide derivatives. *J. Biol. Chem.* **270**, 1179–1184.

Pitari G., Malergue F., Martin F., Philippe J. M., Massucci M. T., Chabret C., Maras B., Dupré S., Naquet P. and Galland F. (2000) Pantetheinase activity of membrane-bound Vanin-1: lack of free cysteamine in tissues of Vanin-1 deficient mice. *FEBS Lett.* **483**, 149–154.

Polidori M. C., Mecocci P., Browne S. E., Senin U. and Beal M. F. (1999) Oxidative damage to mitochondrial DNA in Huntington's disease parietal cortex. *Neurosci. Lett.* **272**, 53–56.

Ricci G., Nardini M., Chiaraluce R., Duprè S. and Cavallini D. (1983) Detection and determination of cysteamine at the nanomole level. *J. Appl. Biochem.* **5**, 320–329.

Rowley H. L., Martin K. F. and Marsden C. A. (1995) Determination of *in vivo* amino acid neurotransmitters by high-performance liquid chromatography with *o*-phthalaldehyde-sulphite derivatisation. *J. Neurosci. Meth.* **57**, 93–99.

Saudou F., Finkbeiner S., Devys D. and Greenberg M. E. (1998) Huntingtin acts in the nucleus to induce apoptosis but death does not correlate with the formation of intranuclear inclusions. *Cell* **95**, 55–66.

Schulz J. B., Dehmer T., Schols L. *et al.* (2000) Oxidative stress in patients with Friedreich ataxia. [See comment.] *Neurology* **55**, 1719–1721.

Sengupta S., Chen H., Togawa T., DiBello P. M., Majors A. K., Budy B., Ketterer M. E. and Jacobsen D. W. (2001) Albumin thiolate anion is an intermediate in the formation of albumin-S-S-homocysteine. *J. Biol. Chem.* **276**, 30 111–30 117.

Shirin H., Pinto J. T., Kawabata Y., Soh J.-W., Delohery T., Moss S. F., Murty V., Rivlin R. S., Holt P. R. and Weinstein I. B. (2001) Antiproliferative effects of *S*-allylmercaptocysteine on colon cancer cells when tested alone or in combination with sulindac sulfide. *Cancer Res.* **61**, 725–731.

Shoesmith Berke S. J., Flores Smith F. A., Brunt E. R., Ellerby L. M. and Paulson H. L. (2004) Caspase-mediated proteolysis of the polyglutamine disease protein ataxin-3. *J. Neurochem.* **89**, 908–918.

Slow E. J., van Raamsdonk J., Rogers D. *et al.* (2003) Selective striatal neuronal loss in a YAC128 mouse model of Huntington disease. *Hum. Mol. Genet.* **12**, 1555–1567.

Taylor J. P., Hardy J. and Fischbeck K. H. (2002) Toxic proteins in neurodegenerative diseases. *Science* **296**, 1991–1995.

Titov A. V., Golubentsev D. A. and Mikhailova E. G. (1974) Specific differences in cystamine metabolism in mice and rats. *Radiobiologiia* **14**, 907–909. (In Russian.)

Wellington C. L., Ellerby L. M., Gutekunst C. A. *et al.* (2002) Caspase cleavage of mutant huntingtin precedes neurodegeneration in Huntington's disease. *J. Neurosci.* **22**, 7862–7872.

Widmann R., Maas D. and Sperk G. (1988) Effect of local injection of cysteamine and cystamine on somatostatin and neuropeptide Y levels in the rat striatum. *J. Neurochem.* **50**, 1682–1686.

Wilson J. M., Wu D., Molixw-DeGrood R. and Hupe D. J. (1980) A spectrophotometric method for studying the rates of reaction of disulfides with protein thiol groups applied to bovine serum albumin. *J. Am. Chem. Soc.* **120**, 359–363.

Winterbourn C. C. (1985) Comparative reactivities of various biological compounds with myeloperoxidase-hydrogen peroxide-chloride, and similarity of the oxidant to hypochlorite. *Biochim. Biophys. Acta* **840**, 204–210.

Winterbourn C. C. and Metodiewa D. (1999) Reactivity of biologically important thiol compounds with superoxide and hydrogen peroxide. *Free Rad. Biol. Med.* **27**, 322–328.

Wyatt I., Moore R. B. and Smith L. L. (1989) Competition for polyamine uptake into rat lung slices by WR2721 and analogues. *Int. J. Rad. Biol.* **55**, 463–472.

Yamamoto B. K. and Zhu W. (1998) The effects of methamphetamine on the production of free radicals and oxidative stress. *J. Pharmacol. Exp. Ther.* **287**, 107–114.

Zainelli G., Ross C. A., Troncos J. C. and Muma N. A. (2003) Transglutaminase-catalyzed cross-links in intranuclear inclusions in Huntington disease. *J. Neuropath. Exp. Neurol.* **62**, 14–24.

Zainelli G., Ross C. A., Troncoso J. C., Fitzgerald J. K. and Muma N. A. (2004) Calmodulin regulates transglutaminase 2 cross-linking in huntingtin. *J. Neurosci.* **24**, 1954–1961.

Zainelli G. M., Dudek N. L., Ross C. A., Kim S.-Y. and Muma N. (2005) Mutant huntingtin protein: a substrate for transglutaminase 1, 2 and 3. *J. Neuropath. Exp. Neurol.* **64**, 58–65.

Ziegler D. M., Poulsen L. L. and York B. M. (1983) Role of flavin-containing monooxygenases in maintaining cellular thiol: disulfide balance, in *Function of Glutathione: Biochemical, Physiological, Toxicological and Clinical Aspects*, pp. 297–305. Raven Press, New York.

Zoghbi H. Y. and Orr H. T. (2002) Glutamine repeats and neurodegeneration. *Annu. Rev. Neurosci.* **23**, 217–247.

