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Endogenous production of hydrogen sulfide in mammals

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Summary. Hydrogen sulfide is one of three gases involved in biological functions and synthesized *in vivo*. Like NO and CO, it seems to act as a neuromodulator: it modulates NMDA glutamate receptor function. CBS seems to be the only source of hydrogen sulfide in the brain, whereas the liver synthesizes hydrogen sulfide via cystathionase. In the heart, the third pathway for the hydrogen sulfide synthesis, the 3-mercaptopyruvate pathway is used. Only two diseases characterized by alterations of hydrogen sulfide metabolism have been described: decreased hydrogen sulfide synthesis in the brains of Alzheimer's disease patients and increased hydrogen sulfide synthesis due to the overexpression of CBS in Down syndrome patients.

Keywords: Cystathionase – Cystathionine beta synthase – Cystathionine gamma lyase – Down syndrome – Hydrogen sulfide – H2S – Mammals – Mercaptopyruvate sulfurtransferase

Hydrogen sulfide (H2S) was traditionally considered to be a toxic gas. It is also, however, generated endogenously during cysteine metabolism. Relatively high endogenous levels of hydrogen sulfide have been measured in the brains of rats, humans and cattle (Goodwin et al., 1989; Warenycia et al., 1989; Savage and Gould, 1990), suggesting that hydrogen sulfide plays a physiological role. Indeed, hydrogen sulfide has many physiological functions and is now considered to be an atypical neural messenger (Baranano et al., 2001). Hydrogen sulfide is generated by various enzymatic and non-enzymatic steps in the brain and in other tissues. Many enzymes are involved in the catabolism of hydrogen sulfide. Finally, some enzymatic defects have provided clues on the mechanism of hydrogen sulfide production and about its physiological action.

Three enzymes are able to produce hydrogen sulfide

The desulfhydration of cysteine (or cystine) is catalyzed by several enzymes present in mammalian tissues (Stipanuk and Beck, 1982). Some of these enzymes are produced both in mitochondria and in the cytosol e.g. cysteine aminotransferase and 3-mercaptosulfurtransferase (MSPT; 2.8.1.2) whereas others -e.g. cystathionase (cystathionine gamma lyase; EC 4.4.1.1) and cystathionine beta synthase (CBS; EC 4.2.1.22) -are only produced in the cytosol. The respective amount of hydrogen sulfide produced in mitochondria and the cytosol depends on the species and organ considered as described below. Cytosolic and mitochondrial cysteine aminotransferase are identical to cytosolic (Akagi, 1982) and mitochondrial (Ubuka et al., 1978) aspartate aminotransferase, respectively (EC 2.6.1.1 and EC 2.6.1.3, respectively). One pathway of cysteine metabolism (see Fig. 1) involves its oxidation to cysteinesulfinate. Cysteinesulfinate is then either decarboxylated in cytosol to yield hypotaurine, which is further oxidized to taurine, (Fig. 1) or transaminated in mitochondria to yield pyruvate and sulfite (Stipanuk, 1986) (Fig. 2). There appears to be a relationship between hepatic cysteinesulfinate decarboxylase activity and the ability to synthesize taurine. This supports the consensus that the cysteinesulfinate pathway is the major route of taurine synthesis in animal tissues. The most marked difference between rat and cat hepatocytes incubated with L-cysteine or with cysteinesulfinate is that hepatocytes from rats convert cysteinesulfinate to taurine rapidly, whereas hepatocytes from cats do not convert cysteinesulfinate to hypotaurine or taurine (De La Rosa et al., 1987). Most of the exogenous cysteinesulfinate present in rat hepatocytes is decarboxylated, but this is not the case in cat hepatocytes. These results are in agreement with the difference in levels of hepatic cysteinesulfinate decarboxylase (CSAD; EC 4.1.1.29) activity in the two species: 200-to

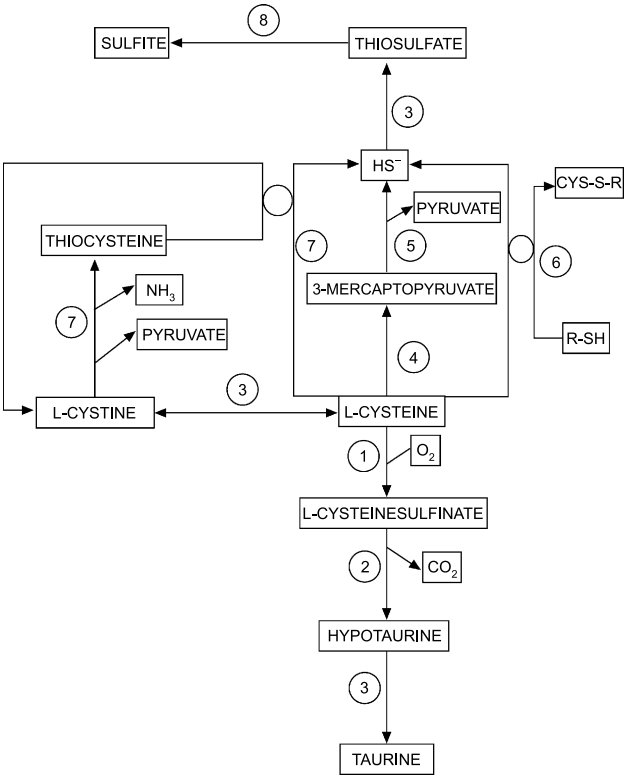

Fig. 1. Hydrogen sulfide production in the cytosol. *1*, Cysteine dioxygenase [EC 1.13.11.20]; *2*, cysteinesulfinate decarboxylase [EC 4.1.1.29]; *3*, non enzymatic reaction; *4*, aspartate aminotransferase (cytosolic) [EC 2.6.1.1]; *5*, 3-mercaptopyruvate sulfurtransferase [EC 2.8.1.2]; *6*, cystathionine beta synthase [EC 4.2.1.22]; *7*, cystathionase [EC 4.4.4.1]; *8*, thiosulfate reductase [EC 2.8.1.3]

300-fold higher in rats than in cats (De La Rosa et al., 1987). CSAD is the only enzyme of the cysteine catabolic pathway that is repressed by a protein-rich diet; in rats, this regulation occurs at the mRNA level (Jerkins et al., 1998). The physiological importance of CSAD regulation by dietary protein is not understood, but may be related to the partioning of cysteinesulfinate between sulfate and taurine (Bagley and Stipanuk, 1995). In rat enterocytes, cyst(e)ine is metabolized to pyruvate and inorganic sulfur, but not to taurine (Coloso and Stipanuk, 1989). About half of the cyst(e)ine is oxidized to cysteinesulfinate, which is then transaminated in mitochondria to the putative intermediate, sulfinylpyruvate, which decomposes to yield sulfite and pyruvate (Fig. 2). Catabolism of the remaining of cyst(e)ine in enterocytes appears to involve the release of sulfur from cyst(e)ine prior to its oxidation (Coloso and Stipanuk, 1989). The incubation of rat hepatocytes with D, L-propargylglycine, an irreversible cystathionase inhibitor, decreases the production of ^{14}C-labeled CO_2 from 1-^{14}C cysteine by about 50% and markedly decreases the production of ammonia and urea; cysteinesulfinate catabolism is not affected (Drake et al., 1987). These data suggest that a substantial proportion of cysteine is catabolized by cysteinesulfinate-independent pathways in rat hepatocytes and that the cleavage of cyst(e)ine by cystathionase is an important physiological pathway for cysteine catabolism in the rat liver.

Cystathionase, a pyridoxal 5′-phosphate-dependent enzyme, catalyzes the desulfhydration of cystine (Fig. 1). Cystathionase catalyzes a β-disulfide elimination reaction that results in the production of pyruvate, NH_4^+ and thiocysteine. Thiocysteine may react with cysteine or other thiols to form hydrogen sulfide (Stipanuk and Beck, 1982; Yamanashi and Tuboi, 1981). D, L-propargylglycine is a specific covalent inhibitor of the cysteine desulfhydration, reaction catalyzed by cystathionase. This inhibitor considerably reduces hydrogen sulfide production in the rat liver and slightly reduces hydrogen sulfide production in the rat kidney (Stipanuk and Beck, 1982). This suggests that a larger proportion of the hydrogen sulfide production is catalyzed by cystathionase in the liver than in the kidney. However, the effect of treating tissue homogenates with S-adenosylmethionine, a specific activator of CBS, did not suggest that CBS plays a greater relative role in the catalysis of cysteine desulfhydration in the kidney than in the liver (Stipanuk and Beck, 1982). The apparent Km of rat liver cystathionase for L-cystine has been estimated to be about 0.03–0.07 mM compared to 0.8–3.5 mM for L-cystathionine and 15–20 mM for L-homoserine, the natural substrates of cystathionase. Thus, cystine should compete favorably with other substrates for the enzyme (Stipanuk, 1986). Cystathionase activity is about 10-times higher in the rat liver than in the liver of full-term human infants and over four times higher than in the adult human liver (Zlotkin and Anderson, 1982; Stipanuk, 1979). Cystathionase activity is lower in guinea pig tissues than in rat tissues: five-fold lower in the liver and 18-fold lower in the kidney (Wrobel et al., 1997). Isolated renal cortical tubules contain active cystathionase, which is consistent with the metabolism of cystine by a cysteinesulfinate-independent desulfhydration pathway in which the β-cleavage of cystine is catalyzed by cystathionase (Stipanuk, 1990). The relative flux through the β-cleavage reaction catalyzed by cystathionase and through the cysteinesulfinate pathway is similar to that observed for another type of epithelial cell, the rat enterocyte (Coloso and Stipanuk, 1989). The human fetal liver lacks cystathionase activity (Sturman, 1970), as well as immunoreactive protein (Gaull et al., 1972). In contrast, cystathionase mRNA can be detected from the 19th gestational week onwards and the mRNA levels are similar to

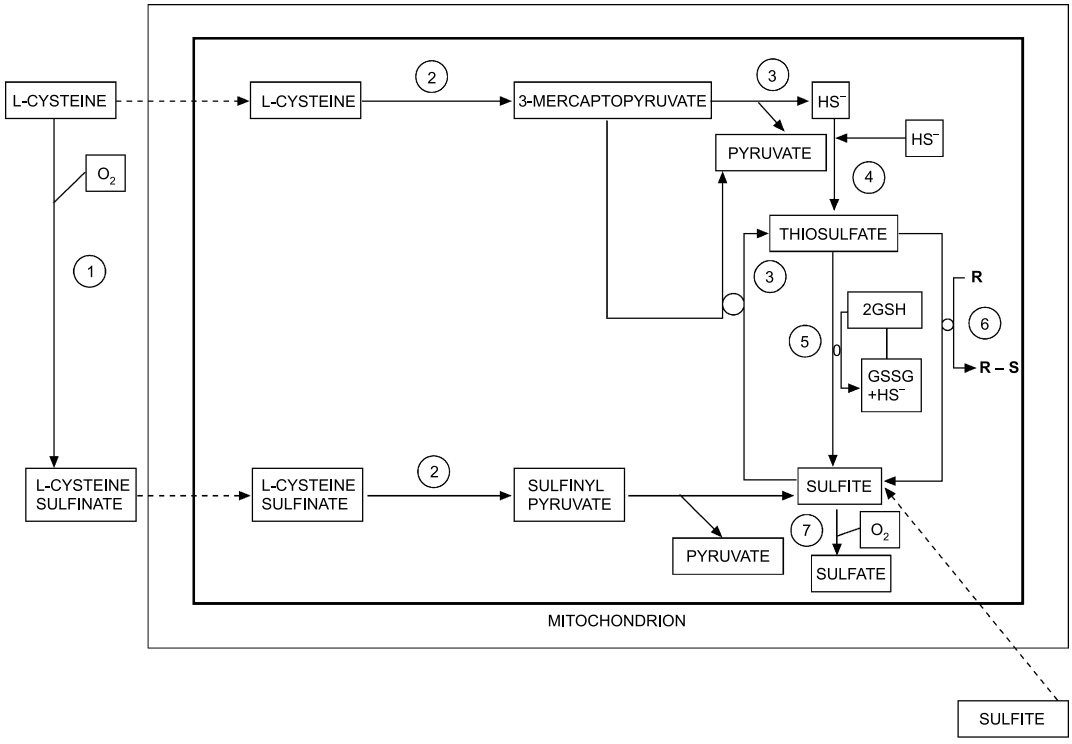

Fig. 2. Hydrogen sulfide and Other sulfur compounds production in mitochondria. *1*, Cysteine dioxygenase [EC 1.13.11.20]; *2*, aspartate aminotransferase (mitochondria) [EC 2.6.1.3]; *3*, 3-mercaptopyruvate sulfurtransferase [EC 2.8.1.2]; *4*, non enzymatic reaction; *5*, thiosulfate reductase [EC 2.8.1.3]; *6*, thiosulfate sulfurtransferase [EC 2.8.1.11]; *7*, sulfite oxidase [EC 1.8.2.1]

those of adult liver samples (Levonen et al., 2000). The most plausible explanation for this discrepancy is the post-transcriptional regulation of cystathionase gene expression. In the rat liver, the activity is low during fetal development, but increases rapidly during the last three days of gestation (Heinonen, 1973). The subcellular distribution of cystathionase has been studied in the rat liver and kidney (Ogasawara et al., 1994): cystathionase activity was mainly detected in the cytosolic fractions in the both tissues. Cystathionase activity is significantly lower in the liver of 24-month-old mice. As rats mature, total cystathionase activity in the liver increases, peaking at 24 months of age and then decreasing to the same level found in five-week-old rats (Nakata et al., 1996). Finally, diabetes increases the concentration of cystathionase in the rat liver (Hargrove et al., 1989). A very low level of activity has been described in the rat brain compared to in other tissues (Ogasawara et al., 1994; Finkelstein, 1990) (see Table 1). Cystathionase activity has been measured in various regions of the developing rat brain. Cystathionase activity increases during development, reaching the adult level in postnatal week 2. However, increases enzyme activity clearly increases less in the cerebellum (about 1.8-fold) than in the other regions (about 4-fold). The cystathionase content in various regions of the 3-week-old rat brain estimated by immunoblotting is consistent with the enzyme activity; the enzyme level is lower in the cerebellum than in the other regions (Awata et al., 1995). Small amounts of cystathionase mRNA have been detected in the brain (Erickson et al., 1990). In contrast to the liver and kidney, hydrogen sulfide production in brain seems to be unrelated to cystathionase activity. Cystathionase inhibitors, D,L-propargylglycine and β-cyano-L-alanine, do not suppress the production of hydrogen sulfide in the brain (Abe, 1996) although they effectively suppress hydrogen sulfide production in the liver and kidney (Stipanuk and Beck, 1982).

Cystathionine beta synthase (CBS), a pyridoxal-5′-phosphate-dependent heme protein, can synthesize hydrogen sulfide from L-cysteine (Stipanuk and Beck, 1982; Griffith, 1987; Swaroop et al., 1992) (see Fig. 1). Hydrogen sulfide is formed by the substitution of the thiol group of L-cysteine with a variety of thiol compounds to form the corresponding thioether. However, CBS appears to have a much higher Km for L-cysteine (36 mM) than for its natural substrates, L-serine (2–8 mM) and L-homocysteine (0.1–9 mM) (Stipanuk, 1986). CBS is mainly located in the cytosol. CBS is activated approximately

Table 1. Relative activities of hydrogen sulfide producing enzymes in different tissues and species

	Cystathionine beta-synthase	Cystathionase			3-Mercaptopyruvate sulfurtransferase		
	Rat	Rat		Guinea pig	Rat		Guinea pig
	Rat Finkelstein (1990)	Ogasawara et al. (1994)	Wrobel et al. (1997)	Wrobel et al. (1997)	Wrobel et al. (1997)	Ogasawara et al. (1994)	Wrobel et al. (1997)
Liver	100	100	100	100	100	100	100
Kidney	45	48	34	10	147	90	41
Heart	–	0.5	–	–	–	20	–
Brain	20	1.1	0.3	0.9	6.5	8.2	3.8
Spleen	2	undetectable	–	–	–	14	–
Small intestine	17	–	–	–	–	–	–
Pancreas	90	–	–	–	–	–	–
Adipose	21	–	–	–	–	–	–

two-fold by the allosteric regulator, S-adenosylmethionine (SAM). Binding of NO or CO to the heme pocket of CBS appears to modulate its catalytic activity, suggesting that the heme of CBS serves a regulatory role. The binding affinities of CBS for NO and CO are quite different. The enzyme binds NO about 200-fold less tightly than it does CO (Taoka and Banerjee, 2001). The binding of NO is accompanied by the inhibition of enzyme activity, whereas CO is a reversible and competitive inhibitor with respect to homocysteine (Taoka et al., 1999). This indicates that CO and homocysteine bind in a mutually exclusive manner. In physiological conditions, the intracellular concentration of NO is unlikely to be sufficiently high to affect CBS activity. In contrast, CBS displays a high affinity for CO, which raises the question as to whether CBS is a CO receptor in human cells. CBS activity has been measured in various regions of the developing rat brain. CBS activity gradually increases during development at almost the same rate in each region, until the adult level is reached at week 4 (about 4-fold increase) (Awata et al., 1995). The level of CBS gene expression was studied during early human embryogenesis by *in situ* hybridization and in fetal and adult tissues by northern-blot analysis. CBS is continuously produced at an especially high level in the neural and cardiac systems (Quere et al., 1999). Studies on CBS knock-out mice (Eto et al., 2002a) showed that CBS is involved in the production of hydrogen sulfide in the brain. Endogenous hydrogen sulfide could not be detected in CBS knock-out mice and intermediate levels were detected in heterozygous mice (Eto et al., 2002a). These observations are in agreement with the inhibition of *in vitro* hydrogen sulfide production from L-cysteine by brain homogenate in the presence of CBS inhibitors, such as hydroxylamine and aminooxyacetate (Abe and Kimura, 1996).

3-Mercaptopyruvate sulfurtransferase (MST; EC 2.8.1.2) activity can be detected in mitochondria (Koj et al., 1975; Ubuka et al., 1977) and cytosolic fractions of rat liver and kidney (Nagahara et al., 1998; Ogasawara et al., 1994). MST, a zinc-dependent enzyme, is predominantly localized in proximal tubular epithelium in the kidney, pericentral hepatocytes in the liver, cardiac cells in the heart and neuroglial cells in the brain (Nagahara et al., 1998). In mitochondria, MST can produce hydrogen sulfide from 3-mercaptopyruvate or transfers its sulfur to sulfite, which then forms thiosulfate (see Fig. 2). In the cytosol, the thiocysteine formed by cystathionase can act as an acceptor of the sulfur transferred from 3-mercaptopyruvate by MST (see Fig. 1). In patients suffering from an MST defect (β-mercaptolactate-cysteine disulfiduria; see also below) lactate dehydrogenase transforms, the uncatabolized 3-mercaptopyruvate to 3-mercaptolactate, which can form a mixed disulfide with cysteine (Crawhall et al., 1973). One patient was found to excrete approximately 1.2 mmoles of the mixed disulfide per day. This increased to 11 mmoles per day when he was given 67 mmoles of oral cysteine. Thus, about 15% of the catabolized cysteine enters the 3-mercaptopyruvate pathway. The mixed disulfide is excreted in small amounts in normal human urine (Ubuka et al., 1968). The increased excretion after cysteine load indicates that the MST-catalyzed reaction is the rate-limiting step in this pathway. MST activity has been measured in various rat and guinea pig tissues (Table 1). MST activity is sixty times lower in the guinea pig liver than in the rat liver (Wrobel, 1997). Aminoguanidine, an inhibitor of NO synthase, considerably decreases cystathionase activity in the stem and in cortex of the rat brain and leads to a simultaneous increase in MST activity. These results confirm the relationship between intercellular levels of NO and sulfur metabolism (Sokolowska et al., 1999).

Endogenous reduced sulfur, with a reduced oxidation state and a valence of 0 or 1, can be produced via the desulfuration of cyst(e)ine by the three enzymes listed above. The sulfur from the sulfide released from cyst(e)ine via desulfuration pathways *in vivo* may be incorporated into some pools of active reduced sulfur (sulfane sulfur). This active reduced sulfur has a relatively long half-life prior to its oxidation to sulfate (Stipanuk, 1986). Sulfane refers to all compounds containing sulfur-bonded sulfur. In many sulfane compounds, the sulfur is labile, readily coming out of the structure. The released sulfur then reacts with cyanide ion, and is deposited as elemental sulfur or is reduced to hydrogen sulfide by reducing agents such as dithiothreitol (Tooney, 1989). Sulfane sulfur appears to be the physiological source of the sulfur in iron sulfur cluster enzymes (Tooney, 1989; Ogasawara et al., 1995). Endogenous levels of bound sulfur and acid-labile sulfur can be determined experimentally by acidification of the samples (acid-labile sulfur) or by incubation with dithiothreitol before acidification (total sulfur = the sum of bound sulfur and acid-labile sulfur). The amount of sulfur released in various rat tissues is shown in Table 2. Bound sulfur is localized in the cytosol, whereas acid-labile sulfur is mainly localized in mitochondria. All tissues studied, except the lungs and muscles, contain acid-labile sulfur. The acid-labile sulfur/total sulfur ratio is almost 1 in the heart, 0.4 in the liver and brain and about 0.15 in the spleen and kidney (Table 2). The acid-labile sulfur concentrations are similar

Table 2. Releasable sulfur in rat tissues [nmol/g of wet tissue; mean ± SD (number of measurements)]

	Acid-labile sulfur	Total sulfur	References
Liver	28.5 ± 3.5 (5)	–	Nagata et al. (1990)
	26.0 ± 3.0 (5)	66.7 ± 8.9 (5)	Ogasawara et al. (1994)
	112.2 ± 23 (7)	–	Ubuka et al. (2001)
Brain	–	57.1 ± 20.0 (8)	Warenycia et al. (1990)
	46.2 ± 4.7 (16)	–	Warenycia et al. (1989)
	12.5 ± 2.1 (5)	31.1 ± 6.2 (5)	Ogasawara et al. (1994)
Kidney	35.0 ± 6.8 (5)	–	Nagata et al. (1990)
	39.9 ± 7.4 (5)	363.9 ± 104.8 (5)	Ogasawara et al. (1994)
Heart	129.3 ± 16.5 (5)	128.6 ± 17.8 (5)	Ogasawara et al. (1994)
	274.1 ± 34.6 (7)	–	Ubuka et al. (2001)
Spleen	6.8 ± 0.6 (5)	40.9 ± 13.8 (5)	Ogasawara et al. (1994)
Lung	0	–	Nagata et al. (1990)
Muscles	0	–	Nagata et al. (1990)

Table 3. Relative ⟨⟨Total sulfur⟩⟩ production capacity from cysteine in rat tissues (from Ogasawara et al., 1995)

	Control	With propargyl glycine	With aspartate
Liver	100	23	95
Kidney	50	6	47
Heart	4	4	3
Brain	5	5	3

in various regions of the rat brain (Warencyia, 1989). The capacity for sulfide production from cysteine in rat tissues and subcellular liver fractions was studied as means of determining the enzymatic pathway responsible for this production (Table 3). The higher sulfide production capacity of the liver and kidney is markedly inhibited by propargylglycine, a specific inhibitor of cystathionase, whereas L-aspartate, an inhibitor of the MST pathway, significantly inhibits sulfide production from L-cysteine in the heart but not in the liver or kidney (Ogasawara et al., 1994). Thus, sulfide production is mainly due to cystathionase rather than MST in the whole body (Ogasawara et al., 1994). The amount of sulfide produced in various human tissues *in vivo* is unknown. Recently, the amount of hydrogen sulfide in the frontal cortex was determined post mortem in thirteen Alzheimer's disease patients and six age-matched normal subjects. Endogenous hydrogen sulfide levels were significantly lower (0.22 ± 0.07 nmol/mg protein) in patients than in controls (0.49 ± 0.07 nmol/mg protein) (Eto et al., 2002b). This could be related to decreased amounts of the natural activator of CBS, S-adenosylmethionine (SAM), in the brain of Alzheimer's disease patients (Morrison et al., 1996).

Catabolism of hydrogen sulfide

The catabolism of hydrogen sulfide has been studied by the organ perfusion method. Rat blood containing sodium ^{35}S-sulfide was perfused through isolated rat lungs, kidney or liver (Bartholomew et al., 1980). The rate and the extent of sulfide oxidation varied from one organ to another. In isolated perfused lung, ^{35}S-sulfide was slowly oxidized to ^{35}S-thiosulfate and only small amounts of ^{35}S-sulfate were detectable, possibly due to the absence of sulfite oxidase. In the isolated perfused kidney system, ^{35}S-sulfide was oxidized to ^{35}S-sulfate possibly via ^{35}S-thiosulfate. In liver perfusion experiments, ^{35}S-sulfide was oxidized almost exclusively to ^{35}S-sulfate. The addition of unlabeled thiosulfate inhibited the formation of ^{35}S-sulfate and caused the release of ^{35}S-thiosulfate from the isolated liver. This suggests that thiosulfate is an intermediate in sulfide oxidation to sulfate. The pathways by which hydrogen sulfide is converted to sulfate (Figs. 1 and 2) have also been elucidated (Ubuka et al., 1990, 1992; Huang et al., 1998). The oxidation of hydrogen sulfide to thiosulfate in the rat liver is catalyzed by heme compounds (Sorbo, 1958), metal-protein complexes and ferritin (Ubuka et al., 1992). One molecule of thiosulfate is formed from two molecules of hydrogen sulfide. Two enzymes can act on thiosulfate: thiosulfate sulfurtransferase and thiosulfate reductase. A large amount of thiosulfate sulfurtransferase is present in the livers of most mammals; less than half as much is found in the kidneys and

even less is found in all other organs. The activity is confined to the mitochondrial matrix (Westley, 1980). The distribution of thiosulfate reductase in mammalian tissues differs from that of thiosulfate sulfurtransferase. The liver and kidney contain approximately equal amounts, and the brain, heart, intestine and testis all have substantial reductase contents (one quarter to one-third of the specific activity of the liver). The cytosol contains about one fifth of the amount of this reductase compared to the mitochondrial matrix (Westley, 1980). Thiosulfate reductase catalyzes the reaction of thiosulfate with reduced glutathione. ($S_2O_3^{=} + 2\,GSH \rightarrow GSSG + SO_3^{=} + HS^- + H^+$).

Oxidized glutathione is reduced by the mitochondrial glutathione reductase. The metabolic pathway involved in the conversion of cysteine to sulfate in intact cells has been shown to be glutathione-dependent (Huang et al., 1998). The addition of L-cysteine does not lead to the hepatocyte-catalyzed formation of sulfate if hepatocyte glutathione is depleted beforehand. Furthermore, sulfate formation does not recover in glutathione-depleted hepatocytes if glutathione synthesis is prevented by a glutathione synthesis inhibitor (buthionine sulfoximine, an inhibitor of the gamma-glutamyl-cysteine synthetase). Thiosulfate formation is, however, markedly enhanced in glutathione-depleted hepatocytes. These results suggest that thiosulfate is an intermediate in the formation of inorganic sulfate from L-cysteine and that glutathione is required for the conversion of thiosulfate to inorganic sulfate. These results also suggest that thiosulfate sulfurtransferase is poorly efficient in the catabolism of thiosulfate, the main pathway being the thiosulfate reductase pathway. Sulfite oxidase (EC 1.8.2.1) is a soluble protein found in the intermembrane space of mitochondria (Cohen et al., 1972). This location means that it has ready access to its reducing substrate, sulfite, which can diffuse across mitochondrial membranes, and to its physiological acceptor, cytochrome c. Sulfite oxidase, a molybdenum containing enzyme, forms sulfate from sulfite ($SO_3^{=} + O_2 \rightarrow SO_4^{=}$).

The secretion of thiosulfate in urine seems to be the best biological indicator of acute or chronic hydrogen sulfide intoxication (Kangas and Savolainen, 1987; Beauchamp et al., 1984). Many methods have been used to determine thiosulfate in urine (Table 4). The amount of thiosulfate in urine (about 30 µmoles per day in adults) is very low compared to the amount of sulfate excreted (about 5 mmoles per day in adults). As thiosulfate is derived from sulfur-containing amino acids, the effect of a large, protein load (70 g) was determined in volunteers.

Table 4. Thiosulfate urinary excretion in humans (mean ± SD; number of subjects between brackets)

References	Technique used	µmol/day	µmol/mmol creatinine
Sorbo (1978)	A	31.7 ± 12.8 [31]	–
Belardinelli (2001)	A	–	2.6 ± 1.9 [17]
Kamoun (2003)	A	–	2.7 ± 2.0 [41]
Kangas (1987)	B	–	2.9 ± 2.5 [29]

A, Cyanolysis procedure in combination with ion exchange techniques and colorimetric determination of thiocyanate formed
B, Formation of a bromobimane complex and assay after liquid chromatography

Urine samples collected two hours after the load showed no marked or consistent alterations in the thiosulfate/creatinine concentration ratio (Reynolds and Harkness, 1991). The duration of the urinary sampling was very short, only two hours after the protein load which may explain the lack of effect on urinary thiosulfate excretion.

Physiological action of hydrogen sulfide

Hydrogen sulfide was first shown to have a physiological function in 1996 (Abe and Kimura, 1996). After this article, many authors published articles on the physiological action of hydrogen sulfide and some recent reviews have been published on the ability of hydrogen sulfide to act as a neuromodulator (Kimura, 2002; Wang, 2002). Indeed, high concentrations of hydrogen sulfide inhibit synaptic transmission in the hippocampus. Physiological concentrations of hydrogen sulfide facilitate the induction of hippocampal long-term potentiation. Hydrogen sulfide enhances NMDA receptor-mediated responses. These observations suggested that endogenous hydrogen sulfide functions as a neuromodulator in the brain, in the same way as other gaseous neuromodulators, (e.g. nitric oxide and carbon monoxide) (Baranano et al., 2001). Shortly afterwards, it was also demonstrated that a low concentration of hydrogen sulfide enhances the smooth muscle relaxation effect of nitric oxide by up to 13-fold. Other thiols, including endogenous substances such as cysteine and glutathione, do not relax smooth muscle by themselves and do not have a synergistic effect with nitric oxide. These observations suggest that the synergistic relaxation effect with nitric oxide on smooth muscle is specific to hydrogen sulfide (Hosoki et al., 1997). The hydrogen sulfide-induced activation of NMDA receptors is related to cAMP production (Kimura, 2000). Indeed, NMDA receptor subunits are directly phosphorylated at specific sites by protein kinase A (PKA); the NMDA R1

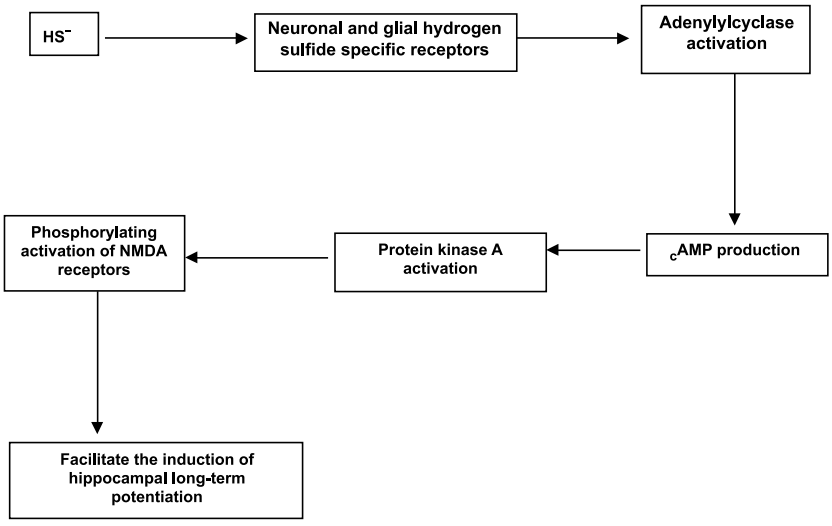

Fig. 3. Mechanism of HS^- action on neuronal cells

subunit contains a consensus PKA phosphorylation site (Leonard and Hell, 1997). The increased activity of adenylyl cyclase and PKA enhances NMDA currents in the neostriatum (Colwell and Levine, 1995), indicating that the NMDA receptor may be modulated through the cAMP cascade. Physiological concentrations of hydrogen sulfide increase the production of cAMP in primary cultures of brain cells, neuronal and glial cell lines (Kimura, 2000). The NMDA receptors expressed on *Xenopus* oocyte membranes are modulated by hydrogen sulfide and this modulation is specifically inhibited by an adenylylcyclase-specific inhibitor. Thus, unlike other gaseous neurotransmitters/modulators, such as nitric oxide and carbon monoxide, which activate guanylyl cyclase and increase the production of cGMP, hydrogen sulfide has a unique signal transduction pathway (Kimura, 2000). Figure 3 summarizes the mechanism of HS^- action in neuronal cells. *In vitro* studies using the sodium salt of hydrogen sulfide, NaHS, which generates the gas once in solution, demonstrated that hydrogen sulfide has a neuroendocrine effect. NaHS inhibits the release of KCl-stimulated corticotropin-releasing hormone from hypothalamic explants in a concentration-dependent manner. The effect is significant at 1 mM (Dello Russo et al., 2000; Navarra et al., 2000).

Hydrogen sulfide induces a concentration-dependent relaxation of phenylephrine-precontracted rat aortic tissues. Again, this vasorelaxant effect of hydrogen sulfide is not mediated by the cGMP pathway (Zhao et al., 2001). KCa and Kv channels are not involved in hydrogen sulfide-induced vasorelaxation. Hydrogen sulfide has a direct effect on KATP channel currents and membrane potential in vascular smooth muscle cells. KATP channel blockers inhibit the hypotensive effect of an intravenous bolus injection of hydrogen sulfide. When the specific irreversible cystathionase inhibitor, D, L-propargylglycine, is used *in vitro*, hydrogen sulfide production is completely abolished in arteries (mesenteric artery, tail artery, pulmonary artery and aorta) (Zhao et al., 2001). This indicates that the generation of hydrogen sulfide from vascular tissues is due to the specific catalytic activity of cystathionase. The same isoform of the cystathionase gene is expressed in rat vascular tissues and in the liver. Cystathionase mRNA can clearly be identified in the smooth muscle layer of the artery wall, but not in the endothelial layer (Zhao et al., 2001). Unlike NO, which can be produced by both endothelial cells and smooth muscle cells, hydrogen sulfide is only generated from smooth muscle cells. The effect of NO on the endogenous production of hydrogen sulfide was examined by incubating homogenized rat vascular tissues with different concentrations of a NO donor. Hydrogen sulfide production was upregulated by the NO donor in a concentration-dependent manner. The transcriptional level of cystathionase increased significantly in cultured vascular smooth muscle cells in the presence of a NO donor (Zhao et al., 2001). NO appears to be a physiological modulator of the endogenous production of hydrogen sulfide in smooth muscle cells by increasing cystathionase expression and stimulating cystathionase activity (Zhao et al., 2001).

Cystathionase cannot be detected in the brain by northern blot analysis. Cystathionase inhibitors, such as D, L-propargylglycine and β-cyano L-alanine, a competitive inhibitor, do not suppress the production of hydrogen sulfide in the brain (Abe and Kimura, 1996), although these inhibitors suppress hydrogen sulfide production effectively in the liver and kidneys (Stipanuk, 1982). In

contrast, hydrogen sulfide production from L-cysteine in brains homogenates is suppressed by CBS inhibitors, such as hydroxylamine and aminooxyacetate, and is increased by the CBS activator, S-adenosylmethionine (Abe and Kimura, 1996). Endogenous hydrogen sulfide could not be detected in the brains of two-week-old CBS knock-out mice (Eto et al., 2002a). The hydrogen sulfide level in heterozygous mice (0.76 ± 0.04 nmoles/mg protein) was less than half of that in wild-type mice (1.60 ± 0.32 nmoles/mg protein) (Eto et al., 2002a). These observations clearly show that CBS produces the endogenous hydrogen sulfide in the brain.

CBS contains a consensus sequence that is conserved in calmodulin-binding proteins (Rhoads and Friedberg, 1997). *In vitro* purified CBS produces hydrogen sulfide 3.5-times faster in the presence of Ca^{+} and calmodulin than in their absence. In the presence of its activator, S-adenosylmethionine, CBS activity which is enhanced by Ca^{2+} and calmodulin, is potentiated. Calmodulin inhibitors prevent hydrogen sulfide production (Eto et al., 2002a). These observations confirm that the production of hydrogen sulfide by CBS is regulated by Ca^{2+} and calmodulin. A 19-amino acid sequence of the C-terminal domain of CBS suppresses CBS activity in the absence of Ca^{2+} and calmodulin. When calmodulin binds to this sequence, CBS is activated. A model for the regulation of CBS by S-adenosylmethionine has been proposed, in which the C-terminal domain of CBS bends over and covers its own catalytic domain, suppressing CBS activity. When S-adenosylmethionine binds to the regulatory domain of CBS, a conformational change occurs that frees the catalytic domain, and CBS becomes active (Rhoads and Friedberg, 1997). A similar mechanism has been proposed for the regulation of CBS by Ca^{2+} and calmodulin. In the absence of Ca^{2+} and calmodulin, the C-terminal domain may cover the catalytic domain, keeping CBS activity at a basal level. When Ca^{2+} and calmodulin bind to the 19-amino acid sequence, the catalytic domain is exposed by the opening of the C-terminal domain and CBS becomes active (Eto et al., 2002a). A novel mechanism for the regulation of CBS activity has been described recently (Eto and Kimura, 2002c). Sodium nitroprusside, an agent that modifies cysteine residues, enhances CBS activity. Site-directed mutagenesis of cysteine residues from CBS led to the identification of four cysteine residues that are involved in the regulation of the basal CBS activity.

The production of hydrogen sulfide by brain cell suspensions is greatly enhanced by L-glutamate, NMDA or AMPA in the presence of Ca^{2+} (Eto et al., 2002a). Thus,

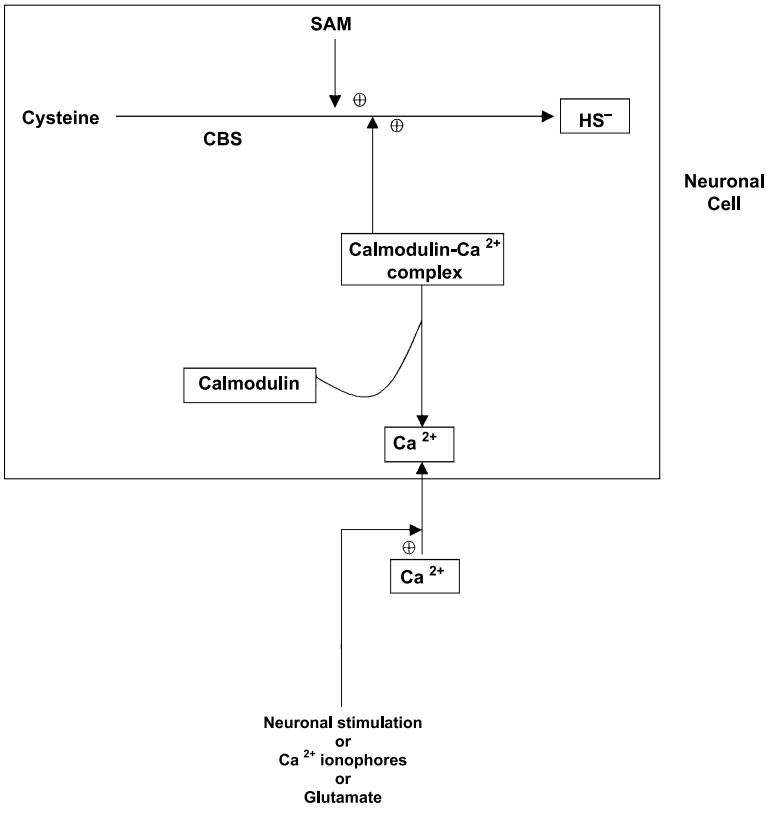

Fig. 4. Control of HS^{-} biosynthesis in neuronal cells (*SAM*, S-adenosylmethionine; *CBS*, cystathionine beta synthase)

hydrogen sulfide is produced when Ca^{2+} enters cells by the activation of at least two classes of ionotropic glutamate receptor. When the neuronal membrane is depolarized, voltage-activated Ca^{2+} channels are opened and Ca^{2+} enters cells. Ca^{2+} ionophores also potentiate hydrogen sulfide production. L-glutamate (100 μM) or electrical stimulation of cerebral cortex slices increases hydrogen sulfide production (Eto et al., 2002a). Figure 4 summarizes the control of CBS activity in neuronal cells. In addition to the fast regulation exerced by the Ca^{2+}/calmodulin-mediated pathway, a slower form of the regulation of hydrogen sulfide production by testosterone and SAM has recently been described (Eto and Kimura, 2002d). The brains of female mice contain less hydrogen sulfide than do those of male mice. The administration of single testosterone dose to female mice increases the endogenous hydrogen sulfide and SAM concentrations to levels similar to those found in male mice. In contrast, castration of male mice decreases the levels of testosterone, hydrogen sulfide and SAM in the brain. Administration of SAM once a day for 3 days increases the concentration of hydrogen sulfide in the brain without significantly changing the testosterone levels. These observations suggest that testosterone regulates the brain hydrogen sulfide level by controlling the level of SAM.

Genetic alterations of hydrogen sulfide metabolism

β-Mercaptolactate-cysteine disulfiduria (Crawhall et al., 1973) is an inherited disease caused by an error in L-cysteine metabolism due to the lack of 3-mercaptopyruvate sulfurtransferase (MST). Patients with this disorder excrete more than 250 μmoles of 3-mercaptolactate-cysteine disulfide in their urine per day. This disulfide is normally excreted in small amounts (Ubuka et al., 1968). The results of the cysteine loading test in the MST-deficient patients revealed that less than 15% of cysteine is normally catabolized by the MST pathway (see above) (Crawhall et al., 1973). Urinary thiosulfate excretion has not been measured in β-mercaptolactate disulfiduria in patients with classical homocystinuria (CBS defect) or in those with cystathioninuria (cystathionase defect).

Inherited sulfite oxidase deficiency (see Fig. 2) leads to the decreased urinary excretion of sulfate and to the increased excretion of thiosulfate, sulfite and S-sulfocysteine, the product of cysteine sulfitation by sulfite. Less than 5% of the total sulfur excreted in the urine was in the form of inorganic sulfate in the first patient described (normal 75 to 90%) (Irrevere et al., 1967). S-sulfocysteine excretion accounted for 13 to 20% of the total sulfur excretion in the patient (compared to 0.1% in controls). The sulfite excreted corresponded to 11% of the total sulfur excreted (0.08% in controls). The thiosulfate excreted corresponded to 16% of the total sulfur excreted (normal children, up to 2%) and the amount of taurine excreted was slightly elevated. Thus, inherited sulfite oxidase deficiency results in abnormal hydrogen sulfide catabolism, but not in abnormal hydrogen sulfide synthesis.

Cystathionine beta synthase (CBS) is encoded by a gene on chromosome 21 ($21q^{22\text{-}3}$). The enzymatic activity of CBS is approximately 150% higher in fibroblasts from Down syndrome patients than in those from normal individuals (Chadefaux et al., 1985). The *in vivo* increase in CBS activity induces a decrease in the amount of CBS substrate (homocysteine) in the plasma of Down syndrome patients (Chadefaux et al., 1988). Down syndrome patients suffer from progressive mental retardation: individuals are born with normal intelligence, which starts to decline linearly within the first year. This phenomenon is compatible with a metabolic intoxication, as observed in phenylketonuria patients. It has been hypothesized that hydrogen sulfide is the toxic compound involved (Kamoun, 2001). Hydrogen sulfide is overproduced in Down syndrome patients: 2.3 times more thiosulfate is excreted in the urine of Down syndrome patients than in that of controls (Belardinelli et al., 2001; Kamoun et al., 2003). Sulfhemoglobin production requires hydrogen sulfide and methemoglobin. The concentration of sulfhemoglobin in erythrocytes has also been used to assess hydrogen sulfide overproduction in Down syndrome: sulfhemoglobin levels are significantly higher in Down syndrome patients than in controls ($p < 0.001$) (Kamoun et al., 2003). Clinical and biological observations have established a relationship between Down syndrome and chronic hydrogen sulfide poisoning [see review (Kamoun, 2001)]. Nevertheless, the role of hydrogen sulfide overproduction in mental retardation remains to be established.

Conclusion

Hydrogen sulfide is endogenously generated from cysteine by three enzymes. 3-mercaptopyruvate sulfurtransferase is the only enzyme that is efficient both in mitochondria and in the cytosol. Cystathionine beta synthase and cystathionase are cytosolic enzymes. In the rat liver, kidney, enterocytes and vascular smooth muscle cells, hydrogen sulfide is mainly synthesized by cystathionase, whereas it is mainly synthesized by cystathionine beta synthase in the brain and by 3-mercaptopyruvate

sulfurtransferase in heart tissues (partially). Hydrogen sulfide production differs considerably between the various mammalian species studied. Hydrogen sulfide is mainly catabolized in mitochondria by thiosulfate reductase and the sulfite formed is oxidized to sulfate by sulfite oxidase. Normally, about 31 μmoles of thiosulfate are excreted in the urine per day and this seems to be the best indicator of hydrogen sulfide biosynthesis. Hydrogen sulfide functions as a neuromodulator in the brain: enhancing NMDA receptor-mediated responses, facilitating the induction of hippocampal long-term potentiation, inhibiting synaptic transmission in the hippocampus. Hydrogen sulfide also has a direct vasorelaxant effect on KATP channel and affects the membrane potential of vascular smooth muscle cells. The hydrogen sulfide concentration is abnormally low in the brains of Alzheimer's disease patients. The overproduction of endogenous hydrogen sulfide described in Down syndrome patients is probably related to the overexpression of the CBS gene which is located on chromosome 21.

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