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Review

# Assay methods and biological roles of labile sulfur in animal tissues

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**Abstract**

Sulfur is a chemically and biologically active element. Sulfur compounds in animal tissues can be present in two forms, namely stable and labile forms. Compounds such as methionine, cysteine, taurine and sulfuric acid are stable sulfur compounds. On the other hand, acid-labile sulfur and sulfane sulfur compounds are labile sulfur compounds. The sulfur atoms of labile sulfur compounds are liberated as inorganic sulfide by acid treatment or reduction. Therefore, the determination of sulfide is the basis for the determination of labile sulfur. Determination of sulfide has been performed by various methods, including spectrophotometry after derivatization, ion chromatography, high-performance liquid chromatography after derivatization, gas chromatography, and potentiometry with a sulfide ion-specific electrode. These methods were originally developed for the determination of sulfide in air and water samples and were then applied to biological samples. The metabolic origin of labile sulfur in animal tissues is cysteine. The pathways of cysteine metabolism leading to the formation of sulfane sulfur are discussed. Finally, reports on the physiological roles and pathological considerations of labile sulfur are reviewed.


*Keywords:* Reviews; Sulphur

## Contents



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## 1. Introduction

Sulfur is a chemically and biologically active element [1–4]. Animal tissues contain various forms of sulfur compounds such as methionine, cysteine, glutathione, sulfate and various intermediates. Methionine and cysteine are sulfur-containing amino acids, and are present in the unbound state and as constituents of proteins and peptides. Glutathione, the most abundant free thiol compound in cells, is a cysteine-containing tripeptide. Sulfate is the major end product of cysteine metabolism and is present in animal tissues as a constituent of sulfated proteoglycans and sulfate esters. Other metabolites of sulfur-containing amino acids are also present in animal tissues. These sulfur compounds participate in various biological processes [1–8].

In animal tissues, the sulfur atom in organic and inorganic compounds can be present in at least five oxidation states: $-2$ (sulfide, organic thiols such as cysteine and glutathione, and most other organosulfur compounds), 0 (elemental sulfur), $+2$ (sulfenic acid), $+4$ (inorganic sulfite, and sulfinic acids such as cysteinesulfinic acid and hypotaurine), and $+6$ (inorganic sulfate, and sulfonic acids such as taurine and cysteic acid). Sulfur atoms in sulfur-containing amino acids are divalent ($-2$) and thus in the most reduced state, and the sulfur atom of sulfate is in the fully oxidized hexavalent ($+6$) state.

Sulfur is taken up in animal tissues mainly in the divalent state as sulfur-containing amino acids, metabolized via the quadrivalent state (sulfinic acids), and finally oxidized to the hexavalent form (sulfuric acid). The sulfur atoms of most sulfur compounds in animal tissues are present as reduced divalent or fully oxidized hexavalent states. Compounds containing such sulfur atoms are stable, and the sulfur atoms in such compounds are not liberated by simple chemical treatment such as acid or dithiothreitol treatment. Such sulfur is designated as stable sulfur.

On the other hand, animal tissues contain a somewhat labile form of sulfur compounds, which can be liberated as hydrogen sulfide ($H_2S$) by simple chemical treatment such as acid or dithiothreitol treatment. Such sulfur compounds include acid-labile sulfur, sulfane sulfur, protein-associated sulfur and

sulfur liberated by dithiothreitol. This review deals with the assay methods for such labile sulfur, mainly in animal tissues, and the roles of these compounds in biological processes.

### 1.1. Definition of labile sulfur species

Various terms have been used to indicate various labile sulfur species in the literature. Clarification of these terms is required.

Acid-labile sulfur is the sulfur which is liberated as $H_2S$ when tissues are treated under acidic conditions. In order to indicate such sulfur species, two terms, namely acid-labile sulfur and acid-labile sulfide, are used. In this review, the term acid-labile sulfur is used to indicate that acid-labile sulfur is a labile sulfur species. It has been established that acid-labile sulfur is contained in iron–sulfur clusters of non-heme iron–sulfur proteins. However, not all acid-labile sulfur is contained in iron–sulfur clusters.

Sulfane sulfur atoms are defined as divalent sulfur atoms bonded only to other sulfur, except that they may bear ionizable hydrogen at some pH values [9]. The following are included in this category: thiosulfate, $S_2O_3^-$; hydrosulfides ($R–SS^-$), which are often called persulfides; thiosulfonates, $R–S_2O_2^-$; polysulfides ($R–S_n–R$), where $n \geq 3$; and polythionates, $^-SO_3–S_n–SO_3^-$, where $n \geq 1$. Elemental sulfur associated with protein is also included in this category. Fig. 1 shows the general structures and examples of biological sulfane sulfur compounds.

Many terms, such as protein-associated sulfur [10], protein-bound sulfur [11], tissue-bound sulfide [12], bound sulfur [13], sulfide liberated by dithiothreitol (or reduction) [12], and non-acid-labile sulfide [12], have been used in the literature. Protein-associated sulfur and protein-bound sulfur appear to exhibit the same behavior as protein-associated elemental sulfur. Sulfide liberated from proteins by dithiothreitol appears to include persulfide and elemental sulfur.

### 1.2. Analytical aspects of labile sulfur species

As stated above, most labile sulfur is liberated as inorganic sulfide. Therefore, the assay method used most often is the determination of inorganic sulfide. In other words, the determination of inorganic sulfide is the basis of labile sulfur determination in animal tissues. In addition, the determination of inorganic sulfide in various samples, including environmental air and water, constitutes the basis of labile sulfur determination of biological samples. Therefore, these methods are included in this review. Inorganic sulfide can be present as $H_2S$, $HS^-$ or $S^{2-}$ in the aqueous phase and as $H_2S$ in the gas phase. In this review, the term sulfide is used in the case of water or tissue samples and $H_2S$ in the case of gas samples.

| General name and structure | | Biological compounds and structure | |
|---|---|---|---|
| Thiosulfate | $\underline{S}SO_3^{2-}$ | Thiosulfate | $\underline{S}SO_3^{2-}$ |
| Persulfide | R - S - $\underline{S}^-$ | Thiocysteine (cysteine persulfide) | Cys - S - $\underline{S}^-$ |
| | | Thiocysteamine (cysteamine persulfide) | $CH_2S - \underline{S}^-$ <br> $CH_2 - NH_2$ |
| | | Glutathione persulfide | GS-$\underline{S}^-$ |
| | | Portein persulfide | Protein - S - $\underline{S}^-$ |
| Thiosulfonate | $RS(O)_2\underline{S}^-$ | Thiotaurine | $CH_2 - SO_2 - \underline{S}^-$ <br> $CH_2 - NH_2$ |
| Polysulfide | R - $\underline{S}_n$ - R (n=3 or greater) | Thiocystine | Cys - S - $\underline{S}$ - S - Cys |
| | | Proteine polysulfide | Protein  |
| Polythionate | $^-O_3S - \underline{S}_n - SO_3^-$ (n=1 or greater) | Tetrathionate | $^-O_3S - \underline{S} - \underline{S} - SO_3^-$ |
| Elemental sulfur ( $\underline{S}^0$ ) |  | Protein associated sulfur | Protein–( $\underline{S}^0$ |

Fig. 1. General structures and examples of biological sulfane sulfur compounds. Sulfane sulfur atoms are underlined.

The determination of sulfide has been performed by many methods, such as spectrophotometry, gas chromatography, ion-specific electrodes, and titration. The determination of elemental sulfur has been performed by methods such as ion chromatography after oxidation of zero-valence sulfur to inorganic sulfate.

### 1.3. Physiological aspects of labile sulfur

Labile sulfur species participate in many physiological processes. As a component of non-heme iron–sulfur proteins, acid-labile sulfur functions in electron transfer in the mitochondrial electron-transfer system and in enzyme activity [3,14–17]. This review does not deal with details of these aspects. Various physiological functions of sulfane sulfur have been reported. The physiological function of sulfide in the nervous system has also been reported.

This review mainly focuses on the functions of labile sulfur species other than acid-labile sulfur, and on sulfide.

### *1.4. Pathological aspects of labile sulfur*

The most important pathological aspect of sulfur species related to labile sulfur is sulfide poisoning. Detoxification of sulfide is an important defense system of animal tissues.

## 2. Analysis of labile sulfur

### *2.1. Spectrophotometric determination of sulfide after derivatization*

#### *2.1.1. Methylene blue method*

The methylene blue method has been widely used since the original description by Fischer [18]. Fogo and Popowsky [19] refined the original method by adapting spectrophotometry to a sensitive determination of $H_2S$ in gases as follows. $H_2S$ is absorbed from gases in an alkaline zinc acetate solution. The precipitate of zinc sulfide formed is dissolved in a hydrochloric acid solution of *p*-aminodimethylaniline (*N*,*N*-dimethyl-*p*-phenylenediamine), and methylene blue is formed within 10 min at 24 °C in the presence of ferric chloride. The blue color of methylene blue is measured at 670 or 650 nm [20]. The color is stable for hours [21]; fading begins after 20 h [19] and it is 95% stable for at least 4 days [20].

The methylene blue method has been applied to the determination of $H_2S$ in air samples in environmental studies [22–24]. In these studies, $H_2S$ was absorbed in an alkaline solution of cadmium hydroxide. The absorption efficiency was studied using an isotope tracer to follow $H_2S$ absorption in the cadmium hydroxide suspension. An impinger with a sintered glass disk was found to have the best absorption of low concentrations of $H_2S$ [25].

Gustafsson [26] studied the application of the methylene blue method for the microdetermination of sulfate in water and biochemical preparations. In this method, sulfate was reduced to $H_2S$ with hydriodic and hypophosphorous acids in 100% yield, and the $H_2S$ formed was precipitated as zinc sulfide before the color reaction [27].

In biological samples, $H_2S$ is determined directly without the absorption step in alkaline solution [20,21,28]. Siegel [20] examined interfering substances and found that 20% inhibition of methylene blue formation was provided by 1 m*M* thiosulfate. Cysteine, glutathione and sulfite strongly inhibited at 10 m*M*, but were relatively innocuous at 1 m*M*. Zinc, cadmium, magnesium and manganese at 10 m*M* concentration did not interfere with the coupling reaction, but copper completely inhibited the reaction because of the low solubility of copper sulfide. The presence of proteins was not inhibitory to the color reaction, and the turbidity produced by the presence of proteins was eliminated by centrifugation [20,28]. In Gilboa-Garber's modification [21], the precipitate of zinc sulfide was collected by centrifugation, dissolved in acidic *N*,*N*-dimethyl-*p*-phenylenediamine solution and the color reaction was performed as in the method of Fogo and Popowsky [19].

The direct methylene blue method has been applied to the determination of biological samples such as the acid-labile sulfur of ferredoxin [29–38], the iron–sulfur protein of mitochondrial complex III [39], enzymes [28,40–42], cellular components [43] and sulfide produced by enzymic reactions [20,44,45].

The methylene blue method has also been applied to the determination of sulfate [46] and total sulfur [47] in biological materials.

A possible problem with the methylene blue method is the presence of interfering colored substances. Sakurai et al. [48] determined acid-labile sulfur and zero-valence (elemental) sulfur in subchloroplast particles from spinach chloroplasts. After color development, the methylene blue formed was separated from interfering colored substances by cellulose phosphate column chromatography [48] in the micromolar range. In this method, elemental sulfur is converted to sulfide by treatment with dithiothreitol, thus permitting the determination of $S^{2-}$ (acid-labile sulfur) + $S^0$ (elemental sulfur). Likewise, Kennedy et al. determined the acid-labile sulfur obtained from beef heart aconitase after conversion to methylene blue, which was separated by Sephadex G-25 column chromatography [42]. They investigated the incorporation of sulfide into an iron–sulfur cluster of aconitase using isotopic sodium sulfide ($Na_2{}^{35}S$). In plant extracts containing chlorophyll and ionic detergents, an ethyl acetate extraction step was used to remove the substances that interfered

with the spectrophotometric assay of methylene blue when detecting acid-labile sulfur and zero-valence sulfur [49]. Thin-layer chromatography and autoradiography of methylene blue were employed as a sensitive method to detect acid-labile sulfur and zero-valence sulfur of $^{35}S$-labeled plant extracts [49].

### 2.1.2. Other derivatization methods

Various methods [50–52] other than the methylene blue method have been developed for derivatization–spectrometric determination of sulfide.

Ellis [50] developed an assay method for sulfite reductase ($H_2S$: $NADP^+$ oxidoreductase, EC 1.8.1.2) from *Escherichia coli*. In this method, the sulfide formed was reacted with excess *N*-ethylmaleimide at pH 8 and the colored adduct formed was measured at 520 nm. The color given by sulfide is much more intense than that given by other sulfur compounds such as cysteine, glutathione and thiosulfate; an absorption of 1.0 is given by 0.26 μmol of sulfide.

Natusch et al. [51] developed a sensitive method for the measurement of atmospheric $H_2S$. In this method, $H_2S$ is extracted from air as $Ag_2S$ by reacting with a $AgNO_3$-impregnated filter. The $Ag_2S$ is dissolved in NaCN solution and the liberated sulfide is reacted with fluorescein mercuric acetate. The fluorescein–sulfide adduct is determined spectrofluorometrically (excitation, 499 nm; emission, 519 nm). The recovery of sulfide from the filter was better than 95%, and this method can measure as low as 5 parts per trillion ($10^{-12}$) of atmospheric $H_2S$.

Balasubramanian and Kumar [52] also developed a sensitive spectrophotometric method for the determination of atmospheric $H_2S$. The $H_2S$ in the air was trapped in zinc acetate–disodium ethylenediaminetetraacetate–sodium hydroxide solution. The fixed sulfide was reacted with iodate, and the ICl formed was stabilized as $ICl^{2-}$ in the presence of excess chloride. The $ICl^{2-}$ was reacted with 2′,7′-dichlorofluorescein to form 2′,7′-dichloro-4′,5′-diiodofluorescein, which was extracted into a mixture of 15% isoamyl acetate in isoamyl alcohol and measured at 535 nm. The method can determine 0.3 μg of $H_2S$.

### 2.1.3. Direct spectrophotometric determination of sulfide

Recently, Guenther et al. [53] developed a direct ultraviolet spectrophotometric method for the determination of total sulfide and iodide in natural waters. At pH near 8, >95% of total sulfide ($H_2S$, $HS^-$ and $S^{2-}$) is present as $HS^-$ [53], which was determined by measuring the absorption from 214 to 300 nm. The $HS^-$ concentration could be determined in samples with low background absorption with a detection limit of <1 μ*M*. The only chemical manipulation required was buffering acidic samples to pH >7 and filtration of particulate-rich samples.

The method of Kovatsis and Tsougas [54] is based on the direct atomic absorption spectroscopy of Pb, in which excess lead acetate is measured after absorption of atmospheric $H_2S$ in a buffered lead acetate solution (pH 5.6).

## 2.2. Liquid chromatographic determination of sulfide without derivatization

### 2.2.1. Ion chromatography of sulfide

Sulfide can be separated from other anions by ion chromatography, but its detection by conductivity is poor. Rocklin et al. [55] determined cyanide, sulfide, iodide and bromide by ion chromatography and electrochemical detection. The detection limits were 2, 30, 10 and 10 ppb, respectively, and sulfide and cyanide could be determined simultaneously.

Goodwin et al. [56] determined trace amounts of sulfide in turbid water by a method with gas dialysis and ion chromatography. They applied this method to the determination of sulfide in brain tissue [57]. Whole rat brains were homogenized in 0.01 *M* sodium hydroxide solution using a Brinkman Polytron homogenizer. For sample preservation, zinc acetate (to ensure preservation of sulfide as insoluble zinc sulfide) and ascorbic acid (to prevent sulfide oxidation during gas dialysis) were added, and sulfide was recovered by a continuous-flow gas dialysis system using a Technicon gas dialysis block and membrane [56]. The stirred sample homogenate stream was reacted with 6 *N* hydrochloric acid and the $H_2S$ released was received in 0.01 *M* sodium hydroxide. Thus, the sulfide determined by this procedure corresponds to acid-labile sulfur as mentioned above. Ion chromatography of sulfide was carried out with a Dionex Model 12 ion chromatograph with a Dionex electrochemical detector. The chromatography eluent was prepared by dissolving 2.473 g of boric acid, 0.8 g of sodium hydroxide and 1 mL of ethylenediamine in 1 L of water. The

detection limit corresponded to 0.02 μg/g brain tissue. The recovery of exogenous sulfide added to the homogenates during gas dialysis was 95–99% and that of the whole procedure was 81.3±1.35%. The brain sulfide level of control rats determined by this method was approximately 1.57 μg/g brain tissue. Forty-five human brainstem controls (36 males, age 52±3.0, and nine females, age 42.2±4.8) were measured for sulfide by this method. Average levels were 0.69±0.12 (SD) μg/g for males and 0.59±0.074 (SD) μg/g for females (Table 1). Brain sulfide levels were compared with subject age, the death-to-autopsy interval, the death-to-refrigeration interval, and the length of storage time in the freezer. Analyses of these comparisons demonstrated no apparent correlation.

Warenycia et al. [58] also applied the method of Goodwin et al. [56] to the measurement of sulfide levels of rat brain regions (brainstem, cerebellum, hippocampus, striatum and cortex) and performed detailed studies. The sulfide level of whole brains was 1.57±0.04 μg/g (mean±SE, $n=16$). Among the brain regions examined, the sulfide level (1.23±0.06 μg/g) of the brainstem was significantly lower than the mean values for the other regions (Table 1). On the other hand, when rats were injected intraperitoneally with 50 mg/kg of sodium hydrogen sulfide (NaHS), the net uptake of sulfide was highest in the brainstem (3.02 μg/g) and was 8–102% higher than that in other brain regions. Under the conditions of the present method, no sulfide was detected with cysteine (4 mg/mL), :methionine (4 mg/mL), taurine (4 mg/mL) and thiosulfate (1.6 mg/mL). However, regional levels of sulfide were correlated with those of methionine and taurine. A higher correlation was demonstrated with taurine ($r=0.968$) than with methionine ($r=0.728$), indicating that endogenous acid-labile sulfur is produced in the metabolism of sulfur-containing amino acids. Examination of the subcellular distribution of sulfide in rat brain revealed that approximately 27% of the endogenous sulfide content of whole rat brain homogenates was found in the fraction enriched in mitochondria, which is known to be rich in acid-labile sulfur.

Warenycia et al. also applied the gas dialysis–ion chromatographic method [56,58] to the determination of sulfide liberated by the dithiothreitol treatment of brain tissue [12]. They found that acid-labile sulfur measured by the conventional gas dialysis–ion chromatographic method accounts for only a proportion of the total sulfide present in brain tissue after poisoning with NaHS. The acid-labile sulfur of the brain tissue of untreated rats was determined to be 1.06 μg/g. When tissue homogenate was treated with 10 m*M* dithiothreitol at 50 °C for 10 min, the sulfide level increased to 1.94±0.24 (a 183% increase) (Table 1). When rats were injected intraperitoneally with 15 or 30 mg/kg NaHS 20 min before sacrifice, the sulfide determined with dithiothreitol treatment increased to 2.83- and 3.79-fold that determined without dithiothreitol treatment. The additional sulfide released by dithiothreitol treatment may indicate that this sulfide exists as persulfide in brain tissues.

$H_2S$ and elemental sulfur were determined by ion chromatography after oxidation to sulfate. Ubuka et al. [59] oxidized $H_2S$ quantitatively to sulfate with hydrogen peroxide and determined the sulfate by ion chromatography. This method was useful for the determination of the sodium sulfide ($Na_2S$) used as a standard in $H_2S$ determination.

$H_2S$ is one of the sulfur species present in volcanic gases and thus analysis of volcanic gases is important in the prevention of $H_2S$ poisoning [60]. $H_2S$ was trapped in sodium hydroxide solution containing cadmium hydroxide. The cadmium sulfide formed was oxidized with hydrogen peroxide to sulfate, which was determined by ion chromatography. Elemental sulfur contained in volcanic gases was oxidized to sulfate by phase-transfer oxidation and the sulfate was determined by ion chromatography.

Fabbri et al. [61] reported a new procedure for the analysis of elemental sulfur in sediments, which was based on the combustion of elemental sulfur to sulfate and ion chromatography.

### *2.3. Liquid chromatographic determination of sulfide after derivatization*

#### *2.3.1. High-performance liquid chromatography (HPLC) of sulfide after conversion to methylene blue*

The HPLC method for the determination of sulfide after conversion to methylene blue is an application of the methylene blue color reaction. Haddad and

Table 1
Concentrations of labile sulfur in mammalian tissues

| Species | Tissue | Treatment | Method | Concentration (*n*) | Ref. |
|---|---|---|---|---|---|
| 1. Rat | Brain | HCl | IC | 1.57 μg/g | [57] |
| 2. Man | | HCl | IC | μg/g | [57] |
| Male | Brainstem | | | 0.69±0.12[a] (36) | |
| Female | Brainstem | | | 0.59±0.074[a] (9) | |
| 3. Rat | | HCl | IC | μg/g | [58] |
| | Whole brain | | | 1.57± 0.04[b] (16) | |
| | Brainstem | | | 1.23±0.06[b] (10) | |
| | Cerebellum | | | 1.58±0.17[b] (10) | |
| | Hippocampus | | | 1.76±0.13[b] (10) | |
| | Striatum | | | 1.85±0.13[b] (10) | |
| | Cortex | | | 1.77±0.08[b] (10) | |
| 4. Rat | Whole brain | HCl | IC | 1.06 μg/g | [12] |
| | Whole brain | DTT | IC | 1.94±0.24[b] μg/g (8) | [12] |
| 5. Bovine | Brain (gray matter) | | MB, HPLC | 166±31.0[a] nmol/g (6) | [63] |
| 6. Mouse | | | MB, HPLC | nmol/g | [64] |
| | Brain | | | 68.9±11.3[a] (25) | |
| | Liver | | | 144.5±12.4[a] (9) | |
| | Kidney | | | 200.1±46.4[a] (9) | |
| 7. Rat | | | MB, HPLC | μg/g | [65] |
| | Hindbrain | | | 1.21±0.05[b] (6) | |
| | Lung | | | 0.54±0.03[b] (6) | |
| | Liver | | | 0.55±0.11[b] (6) | |
| 8. Man | | $H_2SO_4$ | TN, HPLC | μmol/dm$^3$ | [66] |
| Male | RBC | | | 0.165±0.025[a] (5) | |
| Female | RBC | | | 0.151±0.013[a] (5) | |
| 9. Man | | DTT | TN, HPLC | μ*M* | [67] |
| Male | Serum | | | 1.16±0.09[a] (5) | |
| Female | Serum | | | 1.07±0.18[a] (5) | |
| Rabbit | Serum | | | 1.55±0.33[a] (5) | |
| Rat | Serum | | | 1.88±0.65[a] (5) | |
| Guinea pig | Serum | | | 1.65±0.25[a] (5) | |
| Mouse | Serum | | | 1.55±0.86[a] (5) | |
| Bovine | Serum | | | 6.18±0.95[a] (3) | |
| 10. Rat | | $H_2SO_4$ | TN, HPLC | nmol/g | [68] |
| | Liver | | | 25.97±2.96[a] (5) | |
| | Kidney | | | 39.85±7.36[a] (5) | |
| | Heart | | | 129.3±16.5[a] (5) | |
| | Brain | | | 12.51±2.12[a] (5) | |
| | Spleen | | | 6.83±0.63[a] (5) | |
| Rat | | DTT | TN, HPLC | nmol/g | |
| | Liver | | | 66.74±8.89[a] (5) | |
| | Kidney | | | 363.9±104.8[a] (5) | |
| | Heart | | | 128.6±17.8[a] (5) | |
| | Brain | | | 31.05±6.24[a] (5) | |
| | Spleen | | | 40.91±13.81[a] (5) | |
| 11. Man | Serum | DTT | MBB, HPLC | 1.30±0.60[a] μ*M* (5) | [69] |
| 12. Rat | Blood | Alkyl./extract. | GC | <0.01 μg/g | [84] |

Table 1. Continued

| Species | Tissue | Treatment | Method | Concentration (*n*) | Ref. |
|---|---|---|---|---|---|
| 13. Rat | | Alkyl./extract. | GC | nmol/g | [10] |
| | Plasma | | | 0.4 (3) | |
| | RBC | | | 0.2 (3) | |
| | Liver | | | 10.2 (3) | |
| | Kidney | | | 26.8 (3) | |
| | Brain | | | 36.7 (3) | |
| | Heart | | | 35.6 (3) | |
| | Skeletal muscle | | | 6.2 (3) | |
| | Spleen | | | 4.2 (3) | |
| 14. Rat | | $H_3PO_4$ | GC | nmol/g | [59] |
| | Liver | | | 112.2±23.0[a] (7) | |
| | Heart | | | 274.1±34.6[a] (7) | |

Abbreviations: IC, ion chromatography; DTT, dithiothreitol; MB, methylene blue; HPLC, high-performance liquid chromatography; RBC, red blood cell; TN, thionine; MBB, monobromobimane; GC, gas chromatography; *n*, number of animals.

[a] Standard deviation.

[b] Standard error of the mean.

Heckenberg [62] determined trace amounts of sulfide after its reaction with *N*,*N*-dimethyl-*p*-phenylenediamine to form methylene blue, which was determined by reversed-phase HPLC. The detection limit was 0.8 ppb at a signal-to-noise ratio of 3, and the recovery was 98.7% for a 500-ppb standard solution of sulfide.

Savage and Gould [63] determined sulfide in bovine brain tissue and rumen fluid by ion-interaction reversed-phase HPLC (RP-HPLC) after conversion of sulfide to methylene blue. Brain tissue was homogenized in the presence of zinc acetate, and the homogenate containing zinc sulfide was treated with *N*,*N*-dimethyl-*p*-phenylenediamine and ferric chloride to convert sulfide to methylene blue. After centrifugation, the supernatant was subjected to RP-HPLC using toluenesulfonic acid as the ion-interaction reagent. The normal sulfide level in the bovine brain determined by this method was 166±31.0 nmol/g (range 104–191, $n=6$) (Table 1). The formation of methylene blue from 1.0 m*M* glutathione, methionine, cysteine or cystine was less than 1% of sulfur.

Mitchell et al. [64] determined sulfide in tissues of sulfide-treated and control mice according to the method of Savage and Gould [63]. Sulfide levels in the brain, liver and kidney of untreated mice were 68.9±11.3 ($n=25$), 144.5±12.4 ($n=9$) and 200.1±46.4 ($n=9$) nmol/g wet weight (mean±SD), respectively (Table 1). When mice were injected with 60 μg of NaHS, the sulfide levels increased to 108.3±22.0 ($n=28$), 170.6±16.6 ($n=10$) and 327.6±116.1 ($n=10$) nmol/g wet weight (mean±SD), respectively. Although the nature of the sulfide determined in the present experiment was not studied, it is assumed that most of the sulfide determined in the control mice was acid-labile sulfur as mentioned in Section 2.1.1.

Recently, Dorman et al. analyzed sulfide concentrations in normal and $H_2S$-treated rat tissues [65] according to the method of Mitchell et al. [64]. Mean sulfide concentrations in the lung and liver of the control rats were reported to be 0.54±0.03 and 0.55±0.11 μg/g, respectively (Table 1).

### *2.3.2. HPLC of sulfide after conversion to thionine*

Ogasawara et al. [66] determined trace amounts of sulfide in human red blood cells by HPLC with fluorometric detection after precolumn derivatization with *p*-phenylenediamine and iron(III). In this method, sulfide was converted to a fluorescent derivative, thionine, which was chromatographed using a $C_8$ stationary phase with SDS as ion-pairing reagent. A stationary separation was achieved using a mixture of acetonitrile and 50 mmol $dm^{-3}$ sodium phosphate buffer (pH 4.0) (1:1) containing 40 mmol $dm^{-3}$ SDS, and a sharp and symmetrical peak was obtained. Thionine was detected fluorometrically (excitation, 600 nm; emission, 623 nm). Thionine can also be detected in the visible region at 600 nm, but

simultaneous detection of the thionine derivative by fluorescent and visible region detection showed that the former was more sensitive and selective than the latter. Using this method, sulfide ion could be determined in the range from 0.01 to 3.0 μmol $dm^{-3}$ with a relative deviation of 2.54% at 0.02 μmol $dm^{-3}$ and 1.74% at 1.0 μmol $dm^{-3}$. This method was applied to the determination of sulfide in human red blood cells. Sulfide was liberated from washed red blood cells by treatment with dilute sulfuric acid and trapped in 0.1 mmol $dm^{-3}$ sodium hydroxide solution containing 5 mmol $dm^{-3}$ EDTA and 2 mmol $dm^{-3}$ glycerol using a Conway microdiffusion cell. The sulfide concentration in human red blood cells determined by the present method ranged from 0.123 to 0.189 μmol $dm^{-3}$ (packed cells) (average: male, 0.165±0.025, $n=5$; female, 0.151±0.013, $n=5$) (Table 1).

Ogasawara et al. applied the above method [66] to the determination of bound sulfur in serum which was liberated as sulfide by dithiothreitol treatment [67]. An aliquot of serum was treated with 10 m*M* dithiothreitol at 37 °C for 10 min and applied to the gas dialysis system [69] described in Section 2.3.3. $H_2S$ absorbed in 0.1 *M* sodium hydroxide was converted to thionine, which was separated as described above [66]. The standard curve of sulfide in the present method was linear over the range 0.1–10 μ*M* and the minimum detectable level was 250 fmol at a signal-to-noise ratio of 2. Thus, this method is much more sensitive than that of monobromobimane derivatization [69]. The amount of bound sulfur in normal human serum determined by the present method was 1.16±0.09 μ*M* for males ($n=5$) and 1.07±0.18 for females ($n=5$). These values are compatible with that determined by the monobromobimane derivatization method [69]. The mean concentrations in animal sera ranged from 1.55 (rabbit) to 6.18 μ*M* (bovine) (Table 1).

Recovery studies [67] using this method revealed that recoveries of sulfide, cystine trisulfide and human serum albumin treated with $Na_2S$ or elemental sulfur were 98, 96, 97, 102 and 97%, respectively. On the other hand, cysteine, cystine and thiosulfate gave no sulfide.

The nature of the serum-bound form of sulfur determined with the present analytical method has also been studied [67]. These authors found that sulfide was released only when non-deproteinized serum was treated with dithiothreitol; it was different from acid-labile sulfur, and the sulfur species were present in the high-molecular-mass fraction. Upon treatment of serum samples with *N*-ethylmaleimide (NEM), a reagent to combine with free thiol groups ($R–S^-$) and persulfide groups ($R–S–S^-$), the recoveries of sulfide from fresh human serum and from human serum treated with $Na_2S$ were 45 and 24%, respectively, of those without NEM treatment. On the other hand, the recoveries after NEM treatment from cystine trisulfide and human serum albumin treated with elemental sulfur were 91 and 98%, respectively, of those without NEM treatment. These results indicate that dithiothreitol treatment produces sulfide from cysteine trisulfide, serum albumin treated with $Na_2S$ and elemental sulfur, all of which contain sulfane sulfur, and the authors assumed that normal human serum contains three forms of bound sulfur: elemental sulfur bound to hydrophobic sites on protein molecules ($S^0$), reduced sulfur bound to protein as a trisulfide (R–SSS–R), and protein persulfide ($R–SS^-$). The authors suggested that such bound sulfur was in equilibrium with the charged and uncharged state:

$$RS^- + S^0 = RSS^-$$

Further investigations are needed to settle this problem in order to understand the physiological roles of sulfane sulfur.

Using the above method [67], Ogasawara et al. determined labile sulfur released with sulfuric acid (acid-labile sulfur) and that released with dithiothreitol in rat tissues [68] (Table 1). They also reported values of labile sulfur in cell fractions (acid-labile sulfur, and labile sulfur released with dithiothreitol minus acid-labile sulfur) [68].

### *2.3.3. HPLC of sulfide after conversion to the monobromobimane derivative*

Togawa et al. [69] determined sulfide, sulfite and thiosulfate by HPLC after conversion to monobromobimane derivatives. The derivatives were separated on a coupled column chromatograph, with a reversed-phase octadecyl silica column connected to a weakly basic anion-exchange column, by isocratic elution with acetic acid solution (pH 3)–acetonitrile (13:3, v/v) containing 25 m*M* sodium perchlorate.

The monobromobimane derivatives were well separated and detection was performed fluorometrically (excitation at 396 nm, emission at 476 nm).

This method was applied to the determination of bound sulfide in normal human serum. A 500 μL aliquot of serum treated with 10 m*M* DTT at 37 °C for 10 min was applied to a continuous-flow gas dialysis system, which consisted of two pumps and a laboratory made gas dialysis cell, a modification of Goodwin's continuous-flow gas dialysis system [57]. The sample solution was injected into 20% phosphoric acid solution. The $H_2S$ released diffused through a polytetrafluoroethylene membrane and was absorbed in 0.1 *M* sodium hydroxide solution containing 10 m*M* glycerol and 5 m*M* EDTA. A 50 μl portion of the absorbing solution was subjected to monobromobimane derivatization. Recoveries of sulfide added to human serum were 103.2±22.7 (at 1.0 μ*M*) and 99.5±2.8% (at 5 μ*M*). Sulfide released from human serum by dithiothreitol treatment was determined to be 1.30±0.60 μ*M* (mean±SD, $n=5$) (Table 1). Without dithiothreitol treatment, no sulfide was detected under the present conditions, indicating that sulfide was bound to serum proteins, presumably as the persulfide.

Hurse and Abeydeera [70] determined sulfide together with thiosulfate and sulfite in wastewater using monobromobimane derivatization and HPLC.

#### *2.3.4. HPLC of sulfide after labeling with o-phthalaldehyde (OPA)*

Biological thiol compounds are converted into highly fluorescent isoindole derivatives by reaction with OPA and primary amines in aqueous solution and at mildly basic pH, and the derivatives can be separated by reversed-phase HPLC. Nakamura and Tamura [71] applied this method to the analysis of biological thiols such as cysteine, glutathione and coenzyme A using taurine as the primary amine. The detection limits of these thiols were 30, 30, and 100 pmol, respectively. With this method, the relative fluorescence intensity of sulfide was reported to be only 1.23 compared to 100 for glutathione.

Mopper [72] studied OPA derivatization of a number of biological thiols using 2-aminoethanol as the primary amine. The authors reported the chromatography of derivatized sodium sulfides of biothiols such as glutathione, thiolactic acid, 3-mercaptopropionic acid and cysteine. The analytical conditions of sulfide determination were not presented, but this method may be applicable to the determination of $H_2S$.

### *2.4. Gas chromatographic determination of $H_2S$ and labile sulfur*

Gas chromatography with flame ionization or electron capture detection is a sensitive method for the analysis of volatile organic materials. The development of the flame photometric detector (FPD) for gas chromatography by Brody and Chaney [73] enabled the highly sensitive and specific analysis of volatile sulfur and phosphorus compounds. Using FPD or other detection methods, gas chromatographic determination of $H_2S$ was originally developed for atmospheric gas analysis [74–81]. This method was then applied to sulfide analysis of water samples [82,83] and biological samples [10,59,82–91].

#### *2.4.1. Gas chromatographic analysis of sulfur compounds in air samples*

Stevens et al. [74] studied the gas chromatographic analysis of reactive sulfur gases such as sulfur dioxide ($SO_2$) and $H_2S$ in air at the ppb level. These reactive sulfur compounds could not be measured by standard gas chromatographic procedures because of tenacious adsorption on the column walls or solid supports, or irreversible adsorption in the liquid phase. Most metal surfaces were found to react with $SO_2$ and $H_2S$ at levels below 10 ppm, resulting in severe losses. To prevent the occurrence of such interferences, they developed an automated gas chromatographic–FPD sulfur gas analyzer for the measurement of ambient concentrations of $SO_2$ and $H_2S$. In this analyzer they used a Teflon column (0.085 in. I.D.×36 ft) packed with 40:60 mesh Teflon coated with a mixture of polyphenyl ether (five-ring polymer) and orthophosphoric acid, and FPD. Stainless steel tubing, soft glass, and porosilicate glass were all found to exhibit undesirable retention of $SO_2$ at levels below 10 ppm despite vigorous cleaning with solvents and acids. They found that fluorinated ethylene–propylene (FEP Teflon) tubing was satisfactory and adopted this material as standard column material. As the solid support, a variety of packing materials, including glass beads,

firebrick, Chromosorb W and Porapak Q, were found to be unsatisfactory, except for powdered Teflon. Among a number of stationary phase liquids, polyphenyl ether (five-ring polymer) mixed with a small amount of orthophosphoric acid was found to be satisfactory for the separation of $SO_2$ and $H_2S$ and for the prevention of tailing of these sulfur compounds. Chromatography was performed at a flow-rate of 100 mL/min with nitrogen as carrier gas. A FPD was adopted in this analyzer, which was developed by Brody and Chaney [73]. The detector temperature was set at 105±3 °C. Above 130 °C, losses of $SO_2$, $H_2S$ and $CH_3SH$ were found at concentrations below 10 ppm. The losses were attributed to thermal decomposition of these unstable sulfur species. Using this system, $H_2S$, $SO_2$, $CH_3SH$ (methanthiol or methyl mercaptan) and $CH_3SCH_3$ (dimethyl sulfide), at concentrations below 1 ppm, were well separated.

FPD is selective for phosphorus and sulfur [73,74,76,78], and the response to sulfur and phosphorus is of the order of 10 000 times that elicited by hydrocarbons [92]. Phosphorus and sulfur are detected by monitoring narrow band emissions from the simple molecular species HPO and $S_2$ at 526 and 394 nm, respectively, which are generated in the flame. Although the detector response to phosphorus is linear, the response to sulfur compounds containing a single sulfur atom is not linear and is assumed to be proportional to the square of the compound concentration. The relationship between the intensity of the $S_2$ emission and the concentration is given by the equation

$$I_{S_2} = I_0[S]^n$$

where $I_{S_2}$ is the observed intensity of the molecular emission of the $S_2$ species, [S] is the concentration of sulfur atoms and $I_0$ and $n$ are constants under given experimental conditions [78]. The value of $n$ is usually assumed to be 2, but, in general, it is between 1.5 and 2, depending upon the flame conditions. Therefore, the flame conditions should be maintained constant to ensure accurate determination [78]. The $n$ values for $SO_2$, $H_2S$ and $CH_3SH$ in the system of Stevens et al. were close to 2 [74].

Bruner et al. [75] developed an automatic gas chromatographic apparatus with a FPD for monitoring sulfur air pollutants ($SO_2$, $H_2S$, $CH_3SH$ and $CH_3SCH_3$). In their system, the column was of Teflon tubing (3 mm I.D.×1.25 m) packed with graphitized carbon black (surface area, about 90 $m^2/g$; Cabot, Billerica, MA, USA) treated with 0.5% phosphoric acid and 0.3% Dexsil 300 (Ananabs, North Haven, CT, USA). The column used was much shorter than that of Stevens et al. [74]. This allowed a lower working pressure of less than 1 $kg/cm^2$. Sample loops and connecting lines were made entirely of Teflon. Chromatography was performed at a flow-rate of about 100 mL/min with nitrogen as carrier gas and at 40 °C. The detector temperature was kept at 115 °C to prevent losses of reactive sulfur compounds as reported by Stevens et al. [73]. With this system, sharp peaks of $H_2S$ and $SO_2$ were separated.

De Souza et al. [77] analyzed sulfur-containing gases, including $H_2S$ and $SO_2$, by gas–solid chromatography. The column used was acetone-washed Teflon tubing (1/8 in. I.D.×18 in.) packed with 80:100 mesh acetone-washed Porapak QS. A mixture of six sulfur compounds, including $H_2S$ and $SO_2$, was clearly separated in a 7-min temperature program from 30 to 210 °C and detected by FPD. Washing the column with acetone greatly improved the separation of sulfur compounds.

Mangani and Bruner [81] studied the interference in the determination of $H_2S$ in air by gas chromatography with FPD. In this study they used a glass column (4 mm I.D.×1.6 m) packed with Carbopack B coated with 1.2% XE60+0.8% phosphoric acid (Carbopack B HT 100, Supelco). They found that treatment of the Pyrex glass column and gas lines with a stream of dry nitrogen for 2 h at 200 °C makes the glass completely inactive to sulfur compounds. In their system, an ethene concentration of over 10 ppm interfered with $H_2S$ determination in air, but this is unlikely to occur with biological samples.

Stetter et al. [79] developed a system for the electrochemical gas chromatographic detection of $H_2S$ at the ppm and ppb levels. The detector operation is based on measurement of the current when $H_2S$ is electrochemically oxidized at a diffusion electrode. The lower detectable limit was $3\cdot 10^{-12}$ g $H_2S$. They used a FEP Teflon column (1/8 in. O.D.×6 ft) packed with Chromosil 310. Chroma-

tography was performed at a flow-rate of 25.6 mL/min with nitrogen as carrier gas at 40 °C. $H_2S$ was eluted at 1.3 min and $SO_2$ at 2.5 min. The sensitivity for $H_2S$ was approximately 30 times greater than that for $SO_2$. The detector response was proportional to the $H_2S$ concentration and the calibration curve was linear in the range from 25 to 100 ppm. In this system, any gas, such as $N_2$, He or air, which is electro-inactive and does not interfere with the column performance, can be used.

Liu et al. [85] determined sulfur compounds in air at ppb levels by gas chromatography with FPD. They discussed this ''non-absorptive'' system for active sulfur species and the conditions influencing the sensitivity of FPD.

### *2.4.2. Gas chromatographic analysis of sulfur compounds in aqueous samples*

Funazo et al. [93] developed a new derivatizing reagent for anions, including sulfide, for gas chromatographic analysis. They synthesized pentafluorobenzyl *p*-toluenesulfonate and studied its application to the derivatization of anions such as bromide, iodide, cyanide, thiocyanate, nitrate, and sulfide. The derivatives formed were pentafluorobenzyl bromide, iodide, cyanide, thiocyanate, nitrate, and bis(pentafluorobenzyl)sulfide, respectively. The conditions for derivatization and gas chromatography were studied. Although the sensitivity of this method for sulfide was 5 μg/mL and hence it was not practical in the case of small amounts of sulfide [84], it stimulated further studies of the derivatization of sulfide for gas chromatographic analysis.

Cutter and Oatts [83] developed a method for the determination of dissolved sulfide and sedimentary sulfur in aqueous samples which could be used for field studies. The method employed selective generation of $H_2S$, liquid-nitrogen-cooled trapping, subsequent gas chromatographic separation, and photoionization detection.

Ichinose et al. [82] determined dissolved sulfide in anoxic seawaters using a gas chromatograph equipped with FPD.

### *2.4.3. Gas chromatographic determination of sulfide in biological samples*

Kage et al. [84] applied gas chromatography to the analysis of sulfide in blood. They used the ''extractive alkylation'' technique. In this method, derivatization of sulfide to bis(pentafluorobenzyl)sulfide (BPFBS) using pentafluorobenzyl bromide (PFBBr), an alkylating agent, and extraction with ethyl acetate as the solvent and tetradecyldimethylbenzyl ammonium chloride as the phase-transfer catalyst were performed at the same time. A 0.2 g aliquot of a blood sample was mixed with a mixture of alkylation (pH 9.3) and extraction reagents, and stirred vigorously for1 min at room temperature. After centrifugation, the organic phase was analyzed by gas chromatography or gas chromatography–mass spectrometry. Sulfide was stabilized by this procedure and the alkylated sulfide was separated by gas chromatography and detected with $^{63}Ni$ electron capture detection. Confirmation of the alkylated sulfide was performed by mass fragmentography. The detection limit was about 0.01 μg/g. This value is much less than the detection limit of 5 μg/g for the method of Funazo et al. [93], in which sulfide was alkylated with pentafluorobenzyl *p*-toluenesulfonate as mentioned above (Section 2.4.2).

Kage et al. [84] applied their method to the blood of rats exposed to $H_2S$. Varying amounts of $H_2S$, ranging from 0.19 to 0.61 μg/g, were detected and the level determined for the untreated rats was less than 0.01 μg/g (Table 1). Nagata et al. [86] determined sulfide concentrations in postmortem human tissues by this method. Kimura et al. [90] also applied this method for the determination of sulfide in tissues of victims of $H_2S$ poisoning.

Hannestad et al. [10] devised a sensitive method for the determination of elemental sulfur bound to serum albumin and other proteins. In this method, alkylation and extraction were performed in one step. Thus, this method is analogous to the method of Kage et al. [84]. A sample containing protein-bound sulfur was mixed with triphenylphosphine (TPP) in hexane and the mixture was shaken for 60 min at room temperature. The triphenylphosphine sulfide (TPPS) formed was analyzed by a gas chromatograph equipped with FPD. The square root of the detector response (peak height) was proportional to the sulfur content. The effects of pH, TPP concentration and reaction time were examined. TPPS formation from inorganic sulfur bound to serum albumin was 92% and that from inorganic disulfide was 98%. On the other hand, the recovery of TPPS

from inorganic sulfide, thiosulfate, cysteine, cystine, cystine trisulfide (thiocystine), glutathione and glutathione disulfide was negligible or null. Thus, TPPS was not formed from sulfane compounds containing one sulfane sulfur atom and from sulfur compounds without sulfane sulfur. It was reported that thiosulfate reacted quantitatively with TPP in aqueous media [94]. The non-reactivity of thiosulfate and thiocystine with TPP in the present method was explained by the reaction conditions, under which the derivatization was performed in a two-phase system with hexane as the organic phase. Using this method, a fairly high concentration (nmol/g) of protein-associated sulfur was detected in rat tissues: brain, 36.7; heart, 35.6; kidney, 26.8; and liver, 10.2 (Table 1).

Persson et al. [87] determined the sulfide formed by oral bacteria using a gas chromatograph equipped with FPD.

Yaegaki and Sanada [88] determined $H_2S$ and $CH_3SH$ in mouth air from healthy subjects and patients with periodontal disease using a gas chromatograph with FPD. The column used was a Teflon column (1/8 in. I.D.×12 ft) packed with 5% polyphenyl ether–0.05% phosphoric acid on 40:60 mesh Chromosorb T, and 10 mL of mouth air was analyzed.

Christensen and Reineccius [89] determined $H_2S$ and $CH_3SCH_3$ in heated milk using a gas chromatograph with FPD. The column used was a DB-5 (0.32 mm I.D.×30 m), and helium was used as carrier gas. An automatic headspace sampler with a 3 mL sampling loop was used for sample injection.

Furne et al. [91] studied the oxidation of $H_2S$ and $CH_3SH$ by the colonic mucosa of rats. In this study, they determined $H_2S$ using a gas chromatograph equipped with a Teflon column (1/8 in. I.D.×8 ft) packed with Chromosil 330 and a sulfur chemiluminescent detector. Thiosulfate and sulfate, both metabolites of the oxidation of $H_2S$, and $CH_3SH$ were determined by ion chromatography. They found that the colonic mucosa possessed a detoxification system that rapidly oxidized $H_2S$ and $CH_3SH$ to thiosulfate, enabling it to protect the colon from injurious concentrations of these sulfur compounds produced by colonic bacteria.

Ubuka et al. determined $H_2S$ and acid-labile sulfur in rat tissues by gas chromatography and ion chromatography [59]. As for the gas chromatographic analysis of $H_2S$, a gas chromatograph equipped with a Teflon column (3 mm I.D.×6 m) packed with 5% of a five-ringed polyphenyl ether on a Uniport HP 80:100 with FPD was used. Chromatography was performed at 70 °C with nitrogen as carrier gas. The peaks of $H_2S$ and $SO_2$ were sharp and clearly separated at retention times of 3.06 and 3.57 min, respectively. $Na_2S$ was used as the standard material. Standardization of $Na_2S$ was performed by ion chromatography after oxidation to sulfate with hydrogen peroxide. Determination of acid-labile sulfur in rat tissues was performed by direct and indirect methods. In the direct method, tissue homogenate or sample solution placed in a gas equilibration vial was acidified with phosphoric acid, and the headspace gas was subjected to gas chromatography. In the indirect method, tissue homogenate was acidified with phosphoric acid and the $H_2S$ liberated was transferred to a 0.1 *M* sodium hydroxide solution using a gas transfer system with nitrogen as the carrier gas. A 4-mL aliquot of this solution was acidified with phosphoric acid in a vial and the resulting headspace gas was analyzed by gas chromatography as above. Recoveries for these two methods were over 99%. Fresh rat liver and heart tissues were found to contain 112.2±23.0 and 274.1±34.6 nmol/g of acid-labile sulfur, respectively (Table 1). Free $H_2S$ was not detected when gas transfer was performed without acidification with phosphoric acid.

#### *2.4.4. Determination of elemental sulfur in sediments*

Marcó et al. [95] developed a new coupling of organic elemental analysis with flame photometry for the determination of total sulfur at the nanogram level. The method is based on the combustion of solid or liquid samples in oxygen at high temperature, reduction to $SO_2$, and determination of $SO_2$ by gas chromatography with FPD detection.

### *2.5. Determination of sulfide with a sulfide ion-specific electrode*

A sulfide ion-specific electrode has been used for the determination of sulfide in various samples [96–105].

Hseu and Rechnitz [106] prepared a sulfide ion-specific electrode, with which the sulfide ion concentration could be determined by direct potentiometry or potentiometric titration in alkaline solution without interference from other common anions. Using the electrode, Gruenwedel and Patnaik [96] determined the sulfide formed by the pyridoxal-catalyzed elimination from sulfur-containing proteins and amino acids in seafood products. The $H_2S$ released was absorbed in a sulfide antioxidant buffer (50% SAOB, Orion Res.) consisting of 2 *M* potassium hydroxide, 1 *M* salicylic acid and 0.22 *M* ascorbic acid, pH 12.8. Sulfide ions were determined quantitatively using the sulfide ion-specific electrode.

Ehman [97] determined $H_2S$ in air using a sulfide ion-specific electrode after absorption of $H_2S$ in an absorber consisting of 1 *M* sodium hydroxide and 0.1 *M* ascorbic acid. The linearity and accuracy were good for 50–1000 ppb $H_2S$ in air, provided the absorbed sulfide was determined within 2 h.

Cook and Wedding [98,101] followed, using a sulfide ion-specific electrode to monitor the *O*-acetyl serine sulfhydrase (EC 4.2.99.8)-catalyzed reaction between *O*-acetyl serine and sulfide. The enzymic reaction was continuously monitored by measuring the disappearance of sulfide.

McAnalley et al. [99] used a sulfide ion-specific electrode for the determination of sulfide and cyanide in the blood of autopsy cases.

Khan et al. [100] determined sulfide in the rumen of ewes and blood of steers with a sulfide ion-specific electrode.

Lindell et al. [102] studied the determination of sulfide in blood with a sulfide ion-specific electrode after pre-concentration of sulfide in sodium hydroxide solution. Pre-concentration was performed with a wash-bottle reactor assembly, and sulfide trapped in 1 *M* sodium hydroxide was determined with an ion-specific electrode. The detection limit was $3 \cdot 10^{-7}$ *M* (0.01 μg/mL). The method was applied to the determination of the blood sulfide of 12 non-fatal cases of acute $H_2S$ poisoning. The concentration ranged from 1.3 to $18.8 \cdot 10^{-6}$ *M* (0.04–0.6 μg/mL). Jäppinen and Tenhunen [103] also determined sulfide in blood samples of 21 cases of acute $H_2S$ poisoning using a sulfide ion-specific electrode. Recently, Chaturvedi et al. [105] reported sulfide analysis in blood of a case of $H_2S$ poisoning associated with a hazardous material accident. The blood sulfide level was found to be 1.68 μg/mL.

### 2.6. Other methods

#### 2.6.1. Titration method

Smythe [107,108] determined the sulfide formed by incubation of cysteine and cystine with rat liver homogenate. In this method, the enzymic reaction was performed using a Warburg vessel, and the $H_2S$ produced was trapped in cadmium acetate placed in the center inset. The cadmium sulfide formed was treated with excess iodine and acid, and oxidized to free sulfur by iodine. The remaining iodine was determined by titration with sodium thiosulfate solution.

Archer [109] determined sulfate by reduction to $H_2S$, which was titrated with mercuric or cadmium salts using dithizone as indicator.

Puacz and Szahum [104] determined sulfide in human blood using a catalytic method. The method is based on the reaction of iodine with azide ion, which is catalyzed only by compounds that contain divalent sulfur [104]:

$$I_2 + 2N_3^- \xrightarrow{S^{2-}} 3N_2 + 2I^-$$

The unreacted iodine was titrated with sodium arsenite solution using starch as indicator. The iodine consumed in the iodine–azide reaction was directly proportional to the concentration of sulfide in the sample. This method was shown to be useful for sulfide determination in whole blood, with a detection limit of 4 μg $dm^{-3}$. The recovery was close to 100% and the value determined was in good agreement with that determined using a sulfide ion-specific electrode.

#### 2.6.2. Triphenylphosphine sulfide

Kimura and colleagues [33,110] studied the chemical reactivity of acid-labile sulfur of iron–sulfur proteins with tritiated triphenylphosphine. They extracted the reaction product, triphenylphosphine sulfide, with organic solvent, and found that approximately equal amounts of triphenylphosphine sulfide and labile sulfur were recovered from the reaction mixture [33]. The kinetic curves for triphenylphosphine sulfide formation and the loss of

acid-labile sulfur showed a similar pattern with a comparable rate [110].

## 3. Physiological roles of labile sulfur

### *3.1. Metabolism of cysteine and labile sulfur in mammals*

The metabolic origin of labile sulfur is cysteine-sulfur. The metabolism of cysteine in mammalian tissues has been described in reviews [111–113] and reports [114–116] (and references quoted therein). An overview of cysteine metabolism based on these works is described here.

The metabolic reactions of cysteine in mammals may be divided into reactions occurring in the cytoplasm, in mitochondria, or in both. These reactions are summarized in Fig. 2. Cysteine is supplied to the cell by transport or protein catabolism, and utilized for the syntheses of proteins and glutathione. Glutathione biosynthesis occurs in the cytoplasm, and mitochondrial glutathione is transported from the cytoplasm [117].

The catabolism of cysteine occurs via oxidation and non-oxidation pathways. The first reaction of the former pathway is catalyzed by cysteine dioxygenase (EC 1.13.11.20) (reaction 4). The product of this reaction is cysteinesulfinate, which is further metabolized via two reactions: transamination, leading to sulfinopyruvate catalyzed by aspartate aminotransferase (EC 2.6.1.1) (reaction 5), and decarboxylation, leading to hypotaurine catalyzed by cysteinesulfinate decarboxylase (EC 4.1.1.29) (reaction 8). Sulfinopyruvate is non-enzymatically decomposed to pyruvate and sulfite (reaction 6), and the latter is

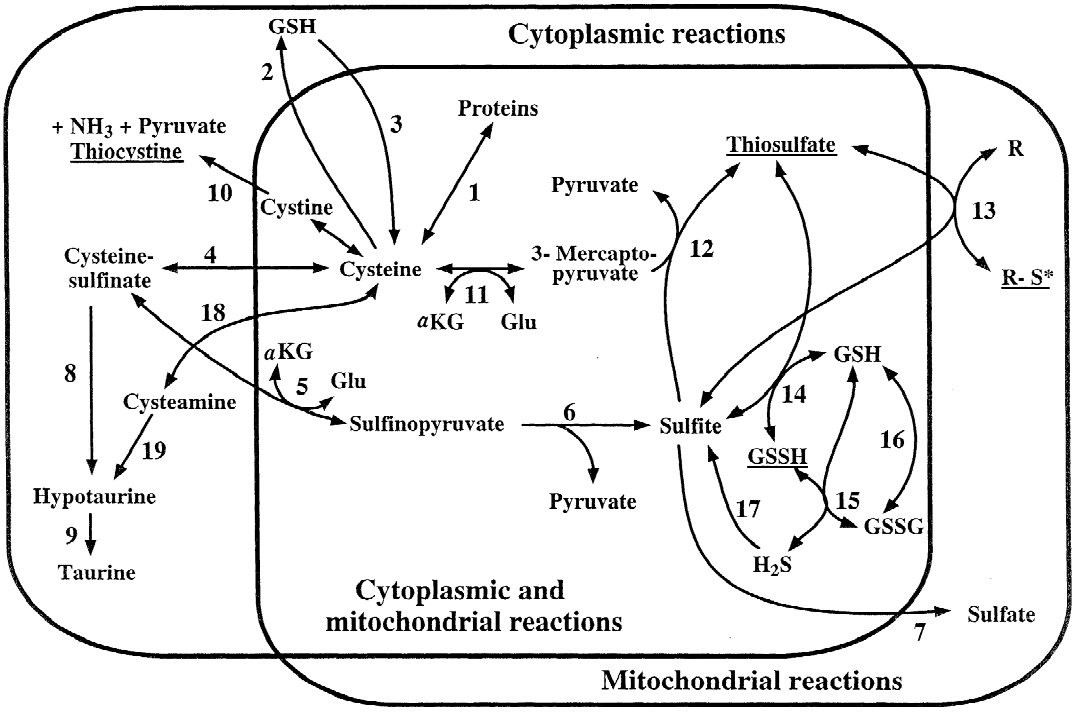


Fig. 2. Mammalian cysteine metabolism. Reactions are catalyzed by: 1, enzymes of protein biosynthesis and catabolism; 2, enzymes of glutathione biosynthesis; 3, enzymes of glutathione catabolism; 4, cysteine dioxygenase; 5, aspartate aminotransferase; 6, non-enzymatic; 7, sulfite oxidase; 8, cysteinesulfinate decarboxylase; 9, ''hypotaurine oxidase'' and/or non-enzymatic; 10, cystathionine γ-lyase; 11, cysteine aminotransferase and/or aspartate aminotransferase; 12, 3-mercaptopyruvate sulfurtransferase; 13, thiosulfate sulfurtransferase; 14, glutathione-dependent thiosulfate reductase; 15, non-enzymatic; 16, glutathione reductase; 17, non-enzymatic; 18, enzymes for the biosynthesis and catabolism of pantetheine and coenzyme A; 19, cysteamine dioxygenase. R: acceptor of the outer atom (S*) of thiosulfate.

oxidized to sulfate by sulfite oxidase (EC 1.8.3.1) (reaction 7). Hypotaurine is oxidized to taurine enzymatically or non-enzymatically. Hypotaurine is also formed via the cysteamine pathway [118]. The formation of sulfate and taurine from cysteine via these pathways appears to be the main metabolic routes for cysteine-sulfur in mammalian tissues.

Non-oxidation pathways of cysteine metabolism involve the cystathionase (cystathionine γ-lyase, EC 4.4.1.1) reaction (reaction 10) and the transamination pathway (3-mercaptopyruvate pathway). The former reaction occurs in the cytoplasm and one of the reaction products is thiocysteine. This compound can combine with cysteine to form thiocystine. It has been shown that enzyme-bound labile sulfur is formed during the cystathionase reaction which can be released as $H_2S$ [111]. Ogasawara et al. [68] reported that the capacity of sulfide production was greatest in the liver cytosol and was well correlated with the distribution of cystathionase in tissues and subcellular fractions.

The first reaction of the transamination pathway is cysteine transamination (reaction 11), occurring both in the cytoplasm and the mitochondria, and the product is 3-mercaptopyruvate. The enzyme catalyzing cysteine transamination, cysteine aminotransferase (EC 2.6.1.3), has not been fully characterized. Ubuka et al. presented evidence that mitochondrial cysteine aminotransferase is identical to aspartate aminotransferase (EC 2.6.1.1) [119]. A cysteine aminotransferase not identical to aspartate aminotransferase was also reported [120]. 3-Mercaptopyruvate is metabolized by 3-mercaptopyruvate sulfurtransferase (EC 2.8.1.2) (reaction 12), and the sulfur atom of this compound is transferred to various acceptor compounds such as sulfite, cyanide, sulfinates and thiols [121]. Among these compounds, sulfite appears to be the main (or one of the main) physiological acceptor(s), and the product is thiosulfate. Another metabolic fate of 3-mercaptopyruvate is reduction by lactate dehydrogenase (EC 1.1.1.27) [111,122] to 3-mercaptolactate, which appears to be the origin of 3-mercaptolactate–cysteine mixed disulfide in human urine [123–128].

The outer sulfur atom of thiosulfate is transferred to various acceptor compounds such as cyanide, sulfite, sulfinates and thiols [121]. The outer sulfur atom of thiosulfate is assumed to be utilized for the formation of iron–sulfur clusters of iron–sulfur proteins [129]. These reactions are catalyzed by thiosulfate sulfurtransferase (rhodanese, EC 2.8.1.1) (reaction 13). Thiosulfate is also a substrate of glutathione-dependent thiosulfate reductase (EC 2.8.1.3) (reaction 14). $H_2S$ is formed in this reaction, and is reported to be oxidized to thiosulfate, and sulfite is assumed to be the intermediate (reaction 17). Sulfite is oxidized to sulfate as mentioned above.

A set of mitochondrial reactions of cysteine metabolism catalyzed by cysteine aminotransferase, mercaptopyruvate sulfurtransferase and thiosulfate sulfurtransferase appears to function for the formation of labile sulfur, including acid-labile sulfur in iron–sulfur clusters. This might be related to the fact that, in eukaryotes, most iron–sulfur proteins are located in mitochondria. Another set of mitochondrial enzyme reactions catalyzed by cysteine aminotransferase, 3-mercaptopyruvate sulfurtransferase, thiosulfate reductase and sulfite oxidase appears to function for sulfate formation. The latter set of reactions may be a system to handle excess thiosulfate to form sulfate. Thus, the transamination pathway of cysteine metabolism appears to be a physiologically important system.

Ogasawara et al. [13] studied the reconstitution of iron–sulfur clusters of spinach ferredoxin with protein-bound sulfur, postulating that it was a possible source of acid-labile sulfur in biological systems.

### *3.2. Physiological roles of labile sulfur and $H_2S$*

Compounds containing labile sulfur exhibit various physiological functions, such as oxidation–reduction, protein structure stabilization, catalytic centers, sensors of iron and oxygen and enzyme regulation.

#### *3.2.1. Iron–sulfur clusters*

Excellent reviews and perspectives on recent developments in iron–sulfur clusters have been published [130,131]. Iron–sulfur clusters are contained in iron–sulfur proteins, which are non-heme iron proteins. Iron–sulfur proteins are ubiquitous in living things, and there are more than 120 distinct classes of proteins and enzymes [131]. They include

rubredoxins, ferredoxins, and enzymes such as succinate dehydrogenase (EC 1.3.99.1), nitrogenase (EC 1.18.6.1), aconitase (EC 4.2.1.3), and ribonucleotide reductase (EC 1.17.4.1). Iron–sulfur clusters are composed of iron atoms and, generally, an equal number of inorganic sulfides with cysteinyl-S iron coordination, as shown in Fig. 3a and b. Most common types of cluster involve [$Fe_2S_2$] (Fig. 3a), [$Fe_3S_4$] and [$Fe_4S_4$] (Fig. 3b). The sulfur atoms of these iron–sulfur clusters are liberated as free sulfide upon treatment of iron–sulfur proteins with acid; thus, they are designated as acid-labile sulfur (also referred to as acid-labile sulfide, as mentioned above). Sulfur atoms are also liberated when proteins are treated with alkaline zinc acetate, which has been applied to the methylene blue method of acid-labile sulfur determination as described above (Section 2.1.1). Among these clusters, rubredoxins, found in bacteria, do not contain acid-labile sulfur, and the iron atom is surrounded by four cysteine residues or sulfur-containing ligands.

The primary function of iron–sulfur clusters lies in mediating one-electron redox processes and, as such, they are integral components of respiratory and photosynthetic electron transfer chains and a host of redox enzymes and proteins involved in carbon, oxygen, hydrogen, sulfur and nitrogen metabolism [131]. In addition to their electron transfer function, iron–sulfur clusters act as catalytic centers and sensors of iron and oxygen [130]. It is considered that iron–sulfur clusters rank with biological prosthetic groups such as hemes and flavins in their pervasive occurrence and multiplicity of function [130].

In relation to the fact that the iron–sulfur cluster is an ancient and ubiquitous form of living matter, the hypothesis of Wächtershäuser is attractive. This proposes that the reaction between FeS and $H_2S$, forming pyrite ($FeS_2$), provided the driving force for the molecular origin of life [132,133]:

$$FeS + H_2S \rightarrow FeS_2 + 2e^- + 2H^+$$

### *3.2.2. Sulfane sulfur*

Sulfane sulfur atoms are defined as divalent sulfur atoms bonded only to other sulfur, except that they may bear ionizable hydrogen at some pH values [9], as mentioned above. Biological compounds containing sulfane sulfur atom(s) are thiosulfate, persulfides, thiosulfonates, polysulfides, polythionates, and elemental sulfur. The structures are shown in Fig. 1. As shown in the figure, the outer sulfur atom(s) of thiosulfate, persulfides and thiosulfonates, the inner sulfur atom(s) of polysulfides and polythionates, and all atoms of elemental sulfur constitute sulfane sulfur. Sulfane sulfur atoms associated with proteins may exist in three forms. One form is a sulfur atom bound to protein as a polysulfide, the second is protein persulfide and the third is protein-associated elemental sulfur. All these forms of sulfane sulfur are rapidly labeled when a compound containing [$^{35}$S]sulfane sulfur is injected into an experimental animal [134], indicating that sulfane sulfur atoms are interchangeable in vivo [135].

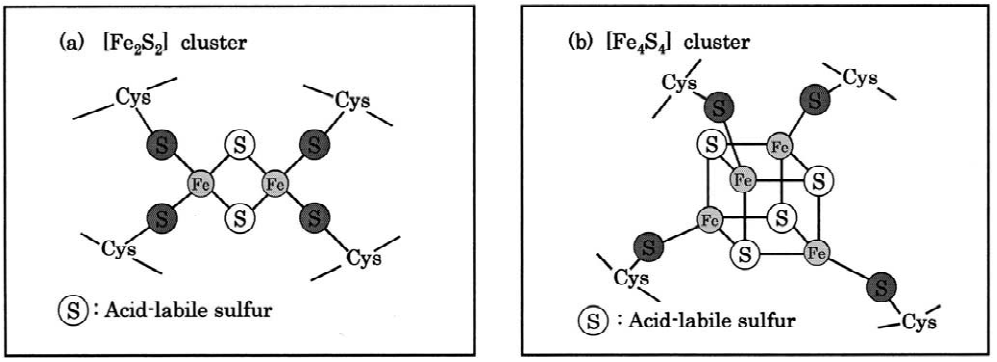


Fig. 3. Structure of iron–sulfur clusters [2Fe–2S] and [4Fe–4S].

Four enzymes are involved in the production or utilization of sulfane sulfur: cystathionase, 3-mercaptopyruvate sulfurtransferase, rhodanese and glutathione-dependent thiosulfate reductase.

The main physiological role of cystathionase is assumed to be the cleavage of cystathionine to form cysteine in the transsulfuration pathway of methionine metabolism. This enzyme also catalyzes the cleavage of cystine to form thiocysteine (cysteine persulfide), which may then combine with cysteine to form thiocystine. Both products of these reactions are sulfane sulfur compounds. Cystathionase appears to be confined to eukaryotic organisms, and intracellularly to the cytosol.

3-Mercaptopyruvate sulfurtransferase transfers the sulfur atom of 3-mercaptopyruvate to cyanide, forming thiocyanate, to thiols, forming the corresponding persulfides, and to sulfite, forming thiosulfate [121]. The role of this enzyme in intracellular cyanide detoxification has been studied [136]. This enzyme is widely distributed in both eukaryotes and prokaryotes. In mammals, the highest activity is found in liver and kidney. Its activity was found in both mitochondria and the cytosol: the ratio in rat liver was found to be 70–80 to 20–30 [137,138]. The distribution in rat tissues and localization in the cytoplasm and mitochondria of this enzyme were confirmed immunohistochemically [139]. The enzyme was purified from rat liver, and it was found that this enzyme was evolutionally related to rhodanese and belongs to the rhodanese family [140].

Rhodanese is widely distributed in the biosphere, and extensive studies of this enzyme have been performed [121,141]. Rhodanese catalyzes the transfer of the outer sulfur atom of thiosulfate to cyanide, forming non-toxic thiocyanate. This reaction is assumed to function for the detoxification of cyanide. However, the role of this enzyme in cyanide detoxification was not assumed to be significant in mammals, because this enzyme is almost exclusively confined to the mitochondrial matrix in rat liver [121,137,138]. Recently, the significance of rhodanese together with mercaptopyruvate sulfurtransferase in the intracellular detoxification of cyanide in liver and kidney was discussed [136]. In frog liver, similar levels of activity of this enzyme were found in both mitochondrial and supernatant fractions [138]. Sulfur donors other than thiosulfate include thiosulfonates, organic and inorganic persulfides and thiocystine. Sulfur acceptors other than cyanide are sulfite, sulfinates and thiols [121]. Thus it seems reasonable to assume that rhodanese functions in the interconversion of sulfane sulfur [135]. Another function of rhodanese appears to be the formation of iron–sulfur clusters [142]. Nishino et al. studied the role of rhodanese in enzyme regulation and found that reversible interconversion between the sulfo (active) and desulfo (inactive) forms of xanthine oxidase occurred in a system containing rhodanese, thiosulfate and sulfhydryl reagent [143].

Glutathione-dependent thiosulfate reductase occurs in both prokaryotes and eukaryotes [137,144–146]. Because of its instability, only the yeast enzyme has been purified extensively [147,148]. The enzyme occurs both in the cytosol and mitochondrial matrix in rat liver [137]. It was shown with yeast enzyme that the reaction proceeded in two steps, and only reaction (A) was enzyme-catalyzed and reaction (B) proceeded non-enzymatically [147,149]:

$$SSO_3^{2-} + GSH \rightleftharpoons SO_3^{2-} + GSSH \quad (A)$$

$$GSSH + GSH \rightleftharpoons H_2S + GSSG \quad (B)$$

Thus, the thiosulfate reductase reaction forms $H_2S$ from the outer sulfur atom of thiosulfate via glutathione persulfide.

With respect to the physiological roles of sulfane sulfur, its participation in detoxification has been reported [135] from studies on the detoxification of cyanide and sulfide, and related enzymes in mammals [121] as stated above.

Hannestad et al. [10] detected significant amounts of protein-associated sulfur in the brain, heart, kidney, liver, skeletal muscle and spleen of rats, but it was negligibly low in blood plasma and erythrocytes. It was suggested that this result did not support the transport function of serum albumin for sulfur.

### *3.2.3. $H_2S$*

The production of sulfide from cysteine in animal tissues was first noted by Fromageot et al. in 1939, who postulated the existence of the enzyme cysteine desulfhydrase [150]. Later studies have shown that sulfide formation from cysteine in animal tissues is

catalyzed by various enzymes, including cystathionase and thiosulfate reductase. Stipanuk and colleagues [44] studied the desulfhydration of cysteine in rat tissues using various assay systems. They found that the liver was most active in sulfide formation, followed by the kidney, and that the desulfhydration was catalyzed by cystathionase, cystathionine β-synthase (EC 4.2.1.22), and cysteine aminotransferase plus 3-mercaptopyruvate sulfurtransferase. It was reported that the cyst(e)ine cleavage reaction catalyzed by cystathionase in a system using isolated rat hepatocytes appeared to account for about 50% of total cyst(e)ine catabolism [151].

Barthoromew et al. [152] studied the oxidation of $Na_2S$ by perfused rat liver, kidney and lung. In liver perfusion experiments, [$^{35}$S]sulfide was oxidized rapidly and almost exclusively to [$^{35}$S]sulfate. The addition of unlabelled thiosulfate inhibited the formation of [$^{35}$S]sulfate and resulted in the release of [$^{35}$S]thiosulfate, suggesting that thiosulfate is an intermediate in sulfide oxidation to sulfate. This sulfide oxidation system was shown to be present in mitochondria and, in the presence of glutathione, thiosulfate was oxidized to sulfate. These results are shown in Fig. 2.

Recently, the possible physiological roles of $H_2S$ have been reported. Abe and Kimura [153] reported the possible roles of $H_2S$ as an endogenous neuromodulator. They showed that $H_2S$ was present at relatively high levels in the brain of rats and that the physiological concentration of $H_2S$ selectively enhanced *N*-acetyl-D-aspartate (NMDA) receptor-mediated responses and facilitated the induction of hippocampal long-term potentiation. Kimura [154] reported that $H_2S$ increased, in primary cultures of brain cells, neuronal and glial cell lines, and Xenopus oocytes, the production of cAMP, which is involved in the phosphorylation of NMDA receptor subunits by protein kinase A. Recently, Eto et al. [155] studied $H_2S$ production in the brain using cystathionine β-synthase (CBS) knock-out mice, and reported observations suggesting that $H_2S$ was produced by CBS in response to neuronal excitation and that $H_2S$ might regulate some aspects of synaptic activity.

Hosoki et al. [156] reported that low concentrations of $H_2S$ greatly enhanced the smooth muscle relaxation induced by NO in the thoracic aorta of the rat. A vasorelaxant effect of $H_2S$ was also reported by Zhao et al. [157]. They showed that $H_2S$ acted as a gaseous $K_{ATP}$ channel opener. Cassanelli and Moulis [158] showed that sulfide could release iron from mammalian ferritins in vitro.

## 4. Pathological aspects of labile sulfur and $H_2S$

### *4.1. $H_2S$ poisoning and detoxification*

Comprehensive and excellent reviews on $H_2S$ toxicity have been published [159,160]. Only some of the toxicological aspects of $H_2S$ are dealt with in this review.

Humans exposed accidentally to $H_2S$ show a variety of symptoms, including dizziness, weakness, nausea, headache, loss of consciousness, confusion and somnolence [161]. A follow-up study of six patients who lost consciousness due to $H_2S$ poisoning revealed that memory and motor function were most affected, and one patient was seriously demented [162]. Repeated exposures of rats to $H_2S$ resulted in an increase in hippocampal electroencephalographic activity [163]. Abnormal growth in developing cerebellar Purkinje cells due to chronic exposure to $H_2S$ during the perinatal period of rats was reported [164].

The primary mechanism of $H_2S$ toxicity has been shown to be the inhibition of cytochrome oxidase in the mitochondrial respiratory chain [165,166]. Dorman et al. reported the relationship between the concentration of $H_2S$ and cytochrome oxidase activity in rat tissues (brain, liver, lung, nasal epithelium) following acute exposure to sublethal concentrations of inhaled $H_2S$ [65]. The results suggested that cytochrome oxidase inhibition was a sensitive biomarker of $H_2S$ exposure in target tissues.

With respect to the detoxification of $H_2S$, Curtis et al. [167] and Bartholomew et al. [152] studied the oxidation of $H_2S$ in rats using [$^{35}$S]sulfide. Whole body autoradiography after intraperitoneal administration of [$^{35}$S]sulfide (5 μmol/200 g body weight) or sodium [$^{35}$S]sulfate (5 μmol/200 g body weight) revealed that the radioactivity was commonly distributed in the gastrointestinal tract and cartilagenous tissues, suggesting that sulfide is oxidized to sulfate

prior to incorporation into mucopolysaccharides. The major portion (up to 94%) of orally or intravenously administered $^{35}S$ was associated with the plasma. Experiments with the perfusion of isolated liver revealed that sodium [$^{35}S$]sulfide was rapidly and almost exclusively oxidized to sulfate. In the isolated perfused kidney system, [$^{35}S$]sulfide was oxidized to [$^{35}S$]sulfate with [$^{35}S$]thiosulfate as a possible intermediate. In the isolated perfused lung system, [$^{35}S$]sulfide was slowly oxidized to [$^{35}S$]thiosulfate. It was shown that the activity of the rapid oxidation of [$^{35}S$]sulfide to [$^{35}S$]sulfate in the liver was present in mitochondria, and, in the presence of glutathione, the thiosulfate was oxidized to sulfate.

Recently, the relationship between $H_2S$ metabolism in the colonic mucosa and ulcerative colitis was studied [91,168,169]. Colonic bacteria liberate a large quantity of $H_2S$ and $CH_3SH$. It was shown that $H_2S$ was metabolized primarily to thiosulfate by rat cecal mucosal homogenate. It was suggested that the mechanism of $H_2S$ detoxification in the cecal mucosa could play a role in colonic diseases such as ulcerative colitis.

### *4.2. Neurological diseases related to labile sulfur*

Friedreich's ataxia is a recessive neurodegenerative disease resulting from insufficient expression of the mitochondrial protein frataxin [170]. In mitochondria, reduced levels of frataxin resulted in the absence of iron–sulfur cluster-dependent enzymes [171]. Puccio et al. [172] reported mouse models for Friedreich ataxia with cardiomyopathy, sensory nerve defect and iron–sulfur enzyme deficiency. Huynen et al. [173] suggested a role for frataxin in the iron–sulfur protein assembly from the phylogenetic distribution of frataxin.

Lin et al. [174] suggested that the 75-kDa iron–sulfur subunit of mitochondrial Complex I was regulated by iron. Complex I activity has been shown to be modified in a number of neurodegenerative diseases, including idiopathic Parkinson's disease [175].

## 5. Concluding remarks

The determination of sulfur compounds is important for the study of their structure, metabolism and function. Because of the high reactivity of sulfur, some sulfur atoms are present in a labile form. These labile sulfur compounds are determined after conversion to sulfide or sulfide derivatives. Many methods have been used for the analysis of labile sulfur, as discussed in this review. However, as shown in Table 1, the values reported are not always in good agreement. The discrepancy among reports seems to be derived from the instability of sulfide, namely its high volatility and great susceptibility to oxidation. Therefore, precautions are necessary to suppress errors arising from the unstable nature of sulfide and labile sulfur compounds. Further studies are needed to develop new, better and stable methods. Sulfur compounds, including labile sulfur, have various physiological and pathological functions. In this respect, further studies on the precise chemical structure of labile sulfur species in animal tissues are important for the elucidation of their physiological roles.

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