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REVIEW

BIOSYNTHESIS AND BIOLOGICAL PROPERTIES OF COMPOUNDS CONTAINING HIGHLY REACTIVE, REDUCED SULFANE SULFUR

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The covalent modifications of sulfhydryl groups (–SH) may occur through oxidation to mixed disulfides (S-thiolation), S-nitrosylation, as well as persulfide and trisulfide formation. The latter possibilities of –SH group modification connected with compounds containing sulfur called sulfane sulfur are described in this paper. Sulfane sulfur compounds contain a labile, highly reactive sulfur atom at a reduced oxidation state with a valence of 0 or –1, covalently bound to another sulfur atom. These compounds include persulfides, polysulfides, polythionates, thiosulfate, elemental sulfur and disulfides, which enable tautomerization to thiosulfoxides. Sulfane sulfur compounds are formed in the anaerobic cysteine sulfur metabolism with the participation of such enzymes as cystathionase (CST), 3-mercaptopyruvate sulfurtransferase (MpST) and rhodanese (thiosulfate: cyanide sulfurtransferase). Compounds containing sulfane sulfur participate in cell regulation processes through activation or inactivation of some enzymes. Other important roles of sulfane sulfur compounds are their antioxidative properties, significance in the processes of carcinogenesis, participation in the tRNA sulfuration as well as an influence on the activity of immune cells. To recognize completely the biological role of compounds with sulfane sulfur it is necessary to have sensitive methods of quantitative determination, so a review of these methods is presented in this paper. Moreover, biosynthetic pathways and biological properties of these compounds have been discussed.

Key words: *sulfane sulfur, persulfides, polysulfides, polythionates, thiosulfoxides*

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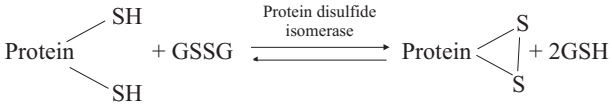
Introduction

Sulfhydryl groups –SH are most reactive in protein molecules (enzymatic and receptor), and the possibility of their chemical modification has an important regulative role. It may occur through covalent protein modifications such as oxidation to mixed disulfides (S-thiolation), S-nitrosylation, as well as persulfide and trisulfide formation. The S-thiolation process is connected with the participation of thioltransferases and involves low molecular disulfides (e.g. oxidized glutathione GSSG) and protein sulfhydryl groups, which can affect the structure and function of proteins, and enzyme activity [12]. This process can also occur under the influence of compounds oxidizing glutathione in the cells (e.g. diamide) [26]. Not all –SH groups are thiolated to the same extent, and since thiolate ions react faster than non-dissociated –SH groups, the specificity of above reaction depends on pK of sulfhydryl group [15]. Protein monothiol (i.e. albumine) reactions with glutathione disulfide (GSSG) result in the formation of mixed disulfides.

$$\text{Protein-SH} + \text{GSSG} \longrightarrow \text{Protein-SS-G} + \text{GSH}$$

In case of S-thiolation of protein dithiols, the reaction may result in the formation of intristic disulfide bridges [33].



At physiological GSSG concentration in cells S-thiolation is a reversible process and is recognized as one of the mechanisms of posttranslational protein modification related to covalent modification of –SH groups [15]. Moreover, this reaction is believed to be a regulative mechanism as well as a mean of protection from nonreversible oxidation of –SH groups [34, 35, 54].

The other covalent modification of –SH groups is S-nitrosylation of cysteine residues in proteins, or low molecular thiols in NO-dependent process. It is believed that this reaction assures NO molecule stabilization and is a prompt and direct transduction signal [63]. The formation of S-nitrosoproteins may occur in a S-transnitrosylation reaction by heterolytic release of nitrosonium cation (NO^+) and then by its transport to the following thiolate ion [6, 62]. Thus, the S-transnitrosylation process is a series of reactions where nitrosonium cation (NO^+) is passed to following thiols until the critical S-nitrosylation reaction.

$$\begin{array}{ccc} R_2SH & & R_1SH \\ \downarrow\uparrow & & \downarrow\uparrow \end{array}$$

$$R_1\,S\text{-}NO + R_2\,S^- \longrightarrow R_1\,S^- + R_2\,S\text{-}NO$$

It has been recently found that S-nitrosylation may be a result of a direct NO reaction with –SH groups in proteins with intermediate radical formation [25].

$$\text{R-SH} + {}^{\bullet}\text{NO} \rightleftharpoons \text{R-S-N}^{\bullet}\text{-O-H}$$

$$\text{R-S-N}^{\bullet}\text{-O-H} + O_2 \longrightarrow \text{R-S-N=O} + O_2^{\bullet-}$$

S-nitrosylation of proteins, which can change protein conformation, leads to the activation or inactivation of enzymes or receptor proteins. These reactions are recently more and more often being compared to phosphorylation reactions [41, 62, 63]. S-nitrosylation is not only a temporary and reversible modification of –SH groups but it may be the preliminary step leading to disulfide formation in proteins. This may occur both by homolytic and heterolytic dissociation of S-nitrosothiols (SNT) [6].

$$\text{R-S-N=O} \xrightarrow{H^+} [\,RS^+\text{=N-O}^-\,] \xrightarrow{RS^-} \text{RSSR} + \text{HNO}$$

$$\text{R-S-N=O} \xrightarrow{Cu^+} RS^{\bullet} + {}^{\bullet}\text{NO}$$

$$2RS^{\bullet} \longrightarrow \text{RSSR}$$

Therefore, if the formation of S-nitrosothiols is only a kind of temporary and reversible regulation, subsequent formation of disulfides in the same process will be more permanent [6, 32]. S-nitrosylation of proteins may indirectly result in the formation of disulfides both with vicinal –SH groups and the formation of mixed disulfides. There are numerous examples of the regulatory function of protein S-nitrosylation [32, 62].

In this paper the authors decided to focus on the possibilities of a covalent modification of –SH groups with compounds containing highly reactive sulfane sulfur and all their regulatory implications. Biotransformations of sulfur-containing amino acids

leading to the formation of sulfane sulfur compounds in the cells and the characteristics of these compounds have been reviewed. Furthermore, the possibilities of modulation of sulfane sulfur level using different systems generating this reactive form of sulfur have been discussed. Some pathological states related to disorders in sulfane sulfur biosynthesis as well as methods of their quantitative determination have also been described.

Anaerobic metabolism of cysteine and methionine leading to sulfane sulfur formation

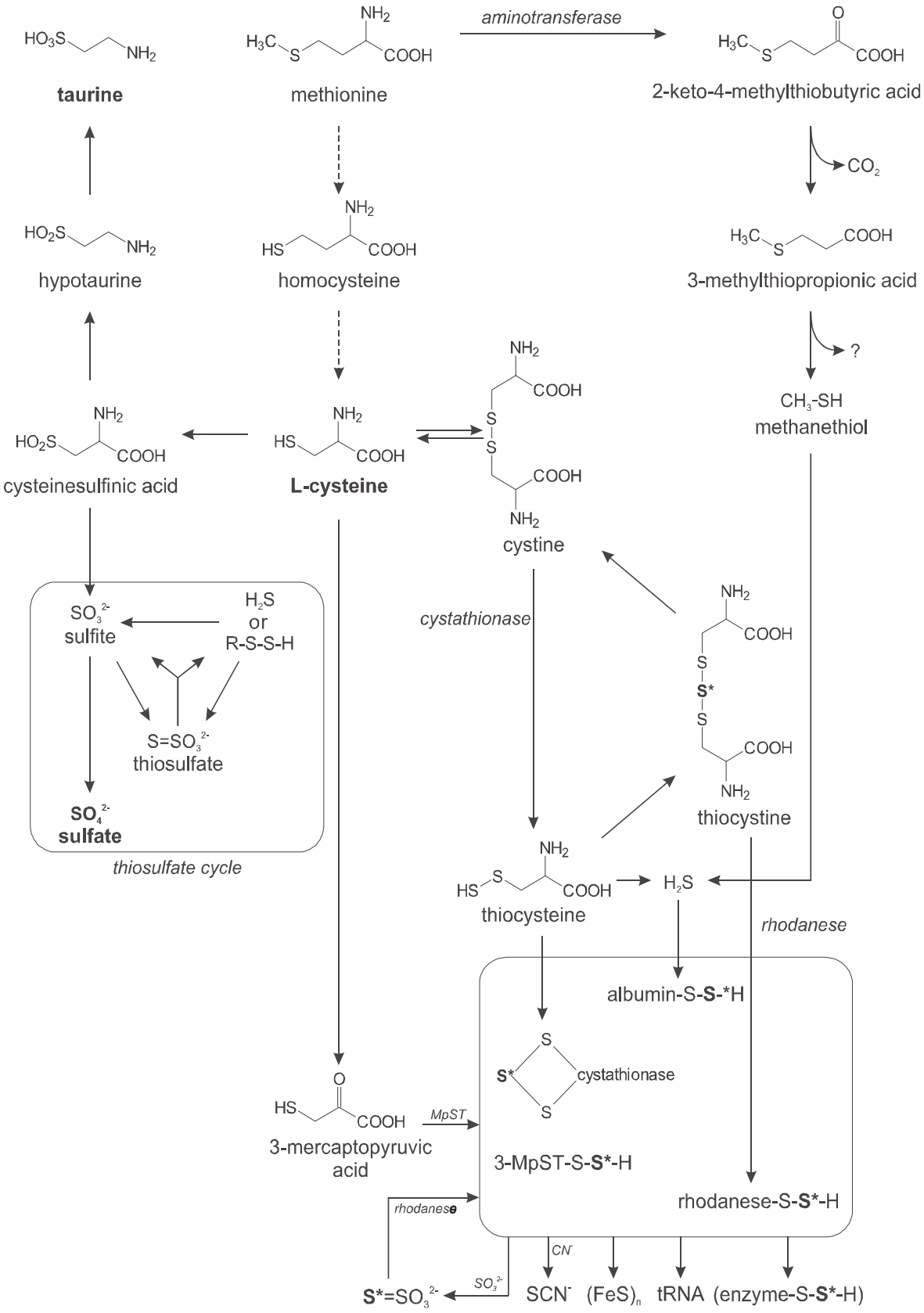
Cysteine catabolism follows two pathways. The first one, called cysteinesulfinic acid dependent, consists in the changes in aerobic environment with formation of such metabolites as sulfates and taurine (Scheme 1). The second pathway, called cysteinesulfinic acid non-dependent, consists in anaerobic changes producing highly metabolically reactive reduced sulfur called sulfane sulfur, which has been reported to have an important biological function [73, 75, 82]. Compounds containing reactive sulfane sulfur are endogenous metabolites, which are produced in mammalian cells during cysteine desulfuration process in reactions catalyzed by such enzymes as cystathionase (CST), 3-mercaptopyruvate sulfurtransferase (MpST), cysteine transaminase and rodanese (thiosulfate: cyanide sulfurtransferase) [17]. The anaerobic pathway of cysteine conversion can be initiated by cysteine aminotransferase catalyzing cysteine transamination to 3-mercaptopyruvate acid, the donor of sulfur transported by MpST to different nucleofilic acceptors such as CN^- and SO_3^{2-}. MpST can also transport sulfur from 3-mercaptopyruvate to sulfhydryl groups of proteins with persulfide formation. Another pathway of cysteine desulfuration is associated with its spontaneous oxidation to disulfide (cystine), which is a substrate for CST and can be converted into sulfane sulfur-containing cysteine hydropersulfide, called also thiocysteine (Cys-S-SH). This unstable compound is transformed into the stable trisulfide-thiocystine (Cys-S-S-S-Cys), rodanese substrate (Scheme 1) [67]. This means that the enzymes taking part in the formation of sulfane sulfur-containing compounds may include cysteine transaminase (indirectly), and MpST and CST (directly). CST and MpST can, beside their ability to take part in sulfane sulfur formation, also transport sulfane sulfur. However, the function of rodanese is mainly to transport reactive sulfur from anionic donors (thiosulfate, persulfides, polisulfides, polythionates) to thiophilic acceptor (cyanide, sulfate (IV)) with the formation of thiocyanate and thiosulfate, respectively, and to iron-sulfur proteins.

Plasma albumins also belong to proteins transporting sulfane sulfur in the form of persulfides [72, 76]. The presence of sulfane sulfur in organs such as cardiac muscle or spleen, with no or trace enzymatic activity related to sulfane sulfur biosynthesis proves the importance of the role of albumin in sulfane sulfur transportation [46]. This means that plasma albumin in blood circulation may be a specific sulfane sulfur carrier from liver and kidney to other tissues.

In the process of cysteine desulfuration by CST, and in catabolism of other sulfur-containing amino acid, i.e. methionine (Scheme 1), hydrosulfide ions are produced, which can react with protein disulfide groups to form protein persulfides containing sulfane sulfur [13, 73]. Hydrosulfuric acid formation from methionine has not been studied in detail yet. It is known that methionine can be transformed directly in transamination reaction or indirectly to 5'-methylthioadenosine and to 2-keto-4-methylthiobutyric acid (Scheme 1) [7, 8, 56]. Research on rat liver has shown that 2-keto-4-methylthiobutyric acid is decarboxylated to 3-methylthiopropionate and then to methanethiol (CH_3SH) [9, 64, 65, 74]. This compound is known as a precursor of hydrosulfuric acid produced in mammalian cells from methionine [65]. Methanethiol can also be formed directly from methionine in a reaction catalyzed by CST (Scheme 1) [73]. Both methanethiol and its disulfides were recognized as natural components of human breath and urine [14, 39, 86].

Characteristic properties of compounds with sulfane sulfur

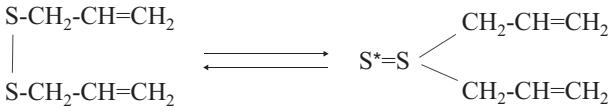
Sulfane sulfur compounds contain a labile, highly reactive sulfur atom in a reduced oxidation state with a valence of 0 or –1, covalently bound to another sulfur atom. Sulfur with such features easily leaves the compound structure and can be transferred to such acceptors as sulfates (IV) SO_3^{2-}, sulfenic acid (R-SO-H) or cyanide (CN^-). Because of the latter acceptor, such sulfur is also often called cyanolysable sulfur. Examples of the compounds from physiological sulfane sulfur pool include per-



Scheme 1. The biodegradation of sulfur amino acid to sulfane sulfur metabolites. (S* – sulfane sulfur)

sulfides (R-S-S-H), polysulfides (R-S_n-R where n > 3) and polythionates (^-O_3S-S_n-SO_3^-). The outer sulfur atom of thiosulfate ($S{=}SO_3^{2-}$) and elemental sulfur (S_8) also have sulfane sulfur properties [45, 73, 75, 82].

Another group of combinations related to sulfane sulfur includes disulfides containing double bond, carbonyl or enol group (R-S-S-CH_2-CH= =CH-R; R-S-S-CH_2-CO-CO_2H and R-S-S-CH_2- -COH or R-S-S-CH_2-CH=CH-OH) in its molecule, which enables tautomerization to sulfane sulfur-containing thiosulfoxides. In this way, thiosulfoxide tautomers of e.g. allyl disulfides from garlic can be a source of labile and reactive sulfur [73].



A similar mechanism occurs in disulfides containing carbonyl or enol group whose presence, as in case of non-saturated allyl radical, enables vicinal C-S bounds "labilisation" and promotes disulfides tautomerization to thiosulfoxides [73].

Sulfane sulfur participation in cell regulatory processes

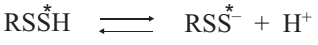
Sulfane sulfur plays an important role in cyanide detoxication processes and in iron-sulfur protein formation, and may have regulatory function in the cells [11, 46, 52, 68, 73]. Regulatory properties of sulfane sulfur-containing compounds are related to the possibility of covalent modification of –SH groups of receptor and enzymatic proteins with persulfide and trisulfide formation, which can directly influence their biological activity [73]. Thus, the activation of some and the inactivation of other enzymes can be caused by sulfur transferases and sulfane sulfur. Among enzymes whose activity is increased under sulfane sulfur influence are oxidoreductases containing iron or molybdenum atom such as: xanthine oxidase [37], aldehyde oxidase [10], malate dehydrogenase [2]. Also 5-aminolevulinate synthetase is activated by cystine incubated together with CST [58, 84]. Furthermore, such disulfides as cystamine or 2-mercaptoethanol disulfide stimulate the activity of fructose-1,6-bisphosphatase [51]. In case of the last disulfide oxidation to dialdehyde is necessary for sulfur "labilisation". Compounds with sulfane sulfur can inhibit the activity of some enzymes, as it was observed for serine dehydratase [29]. The inhibition of this enzyme activity is observed during incubation with cystine and CST, or with elemental sulfur. Enzymes inactivated by sulfane sulfur also include: 3-hydroxybutyrate dehydrogenase, alcohol dehydrogenase [48], adenylate kinase [16, 57], tyrosine aminotransferase [28], ornithine decarboxylase [42]. The inhibition of these enzymes takes place due to hydrodisulfide formation and is reversible under thiol influence (i.e. GSH or dithiotreitol) [73].

What is more, both *in vitro* inhibition and activation of enzymes by systems able to produce and transport sulfane sulfur occur at a very low and narrow concentration range [72, 73]. Thus, the participation of sulfane sulfur-containing compounds enables another –SH group covalent modification, which is an alternative to S-thiolation or S-nitrosylation reactions.

Antioxidative properties of sulfane sulfur-containing compounds

Compounds containing sulfane sulfur are not only active in the regulation of protein activity, but also exhibit antioxidant and protective properties. This means that they are able both to scavenge free radicals and to increase the activity of such antioxidative enzymes as: glutathione peroxidase, glutathione reductase, superoxide dismutase [22, 47, 59].

Perthiols can, therefore, scavenge free radicals by either hydrogen- and electron-transfer processes, depending on the nature of the free radical species [20, 21]. At physiological pH, the position of the perthiol acid-base equilibrium shifts towards the deprotonated perthiolate anion compared to the corresponding ionization of thiol.



Persulfides (RSSH), compared to the sulfhydryl group (–SH), are more effective hydrogen donors, and as persulfide ions (RSS^-) – electron donors, which makes them efficient scavengers.

Thiols scavenge highly damaging hydroxyl radicals ($^{\bullet}OH$) with the formation of thiyl radicals ($RS^{\bullet}$), whereas perthiols (RSSH) and perthiolate anions (RSS^-) with perthiyl radicals ($RSS^{\bullet}$) formation. The latter ($RSS^{\bullet}$) are more stable and, in consequence, less reactive and less toxic than thiyl radical $RS^{\bullet}$ [20].

$$RSH + {}^{\cdot}OH \longrightarrow RS^{\cdot} + H_2O$$

$$RSS^{*}H + {}^{\cdot}OH \longrightarrow RSS^{*\cdot} + H_2O$$

$$RSS^{-} + {}^{\cdot}OH \longrightarrow RSS^{*\cdot} + OH^{-}$$

Allyl disulfide, which occurs in garlic extracts, inhibits lipid peroxidation [22]. All this permits to regard compounds with reactive sulfane sulfur as an important element of cell antioxidation system [15, 21].

Significance of compounds containing sulfane sulfur in processes of carcinogenesis

A characteristic feature of neoplastic cells is a total lack of CST activity, while the activity of cysteine aminotransferase, MpST, and rodanese is residual only [79]. As a result, biosynthesis and transport of compounds from the sulfane sulfur pool does not occur in these cells. Toohey suggests that uncontrolled proliferation of neoplastic cells is a result of sulfane sulfur deficiency and overactivity of those enzymes, which would be inhibited in normal cells by this active form of sulfur [73]. The remission of transplanted tumor in mice [4, 5] and inhibition of tumor induction by carcinogens under the influence of different sulfane sulfur precursors [36, 69] confirms the above hypothesis.

At present reports on beneficial, antiproliferative action of compounds with sulfane sulfur and its precursors on neoplastic cells are becoming more frequent. Allyl disulfide, present in garlic extracts, amounts to ca. 60% of total sulfur in oil received from the bulb of this plant, inhibits neoplastic growth in animals and decreases damage caused by exposure to gamma radiation [1, 18, 23, 60]. Furthermore, an epidemiological study suggests that garlic-rich diet reduces the risk and incidence of neoplastic disease [19, 85]. Allyl disulfide effectively inhibits *in vitro* proliferation of a number of neoplastic cells [30, 49, 66].

On the other hand, it was confirmed that *in vitro* proliferation of malignant lymphoma cells is completely dependent on the presence of sulfane sulfur, and that those cells, to proliferate normally, require a source of this sulfur. A stimulating effect of sulfane sulfur was also observed in case of normal bone marrow cells [72].

All attempts to induce the activity of enzymes associated with sulfane sulfur generation by the introduction of adequate substrates into neoplastic cells failed [78, 83]. Thus, further research should focus on direct introduction of exact systems generating sulfane sulfur to neoplastic cells.

At the same time it was confirmed that MpST and CST activity in mouse liver with Ehrlich ascites tumor cells (EATC) is significantly lowered as compared to livers of healthy animals [83]. This means that the development of cancer causes a radical decrease in the activity of enzymes involved in sulfane sulfur generation in liver. It was also reported that such cysteine precursors as thiazolidine derivatives, i.e. 2-methyl-thiazolidine-2,4-dicarboxylic acid, increase the MpST and CST activity in mouse livers with tumors much more efficiently than sulfur-containing amino acids (cysteine, methionine) [83]. This suggests the possibility of correction of disordered metabolism related to sulfane sulfur generation in liver of animals with cancer.

The possibility of –SH group level modulation, and activity of enzymes related to anaerobic cysteine transformation by NO donors and by NO synthase inhibitors is also interesting [63].

Other biological properties of compounds with sulfane sulfur

Sulfane sulfur also takes part in the process of tRNA sulfuration, which is a posttrancriptional tRNA modification. It was reported that sulfur atom is transferred on tRNA by MpST from 3 mercaptopyruvate, the product of cysteine transamination [3, 73, 80, 81]. Although mercaptopyruvate does not contain sulfane sulfur according to its classical definition, the presence of carbonyl group increases sufficiently its reactivity in comparison to cysteine sulfur. The possibility of oxidation to respective disulfide and tautomerization to sulfoxide cannot be excluded, either. Thiopyrimidines and methylthiopurines are normal tRNA components, and they play a major role in the translation process [3]. Thus, it can be said that anaerobic transformation of cysteine supplies sulfane sulfur compounds necessary for posttranscriptional tRNA sulfuration.

The influence of sulfane sulfur compounds on the activity of cells involved in the immune system was also shown [72, 73]. 2-Mercaptoethanol or 1-thioglycerol are in wide use in *in vitro* immune cell cultures because of a large proliferation stimulation capacity. This may be a result of reducing properties of these thiols, but may be also a conse-

quence of their oxidation to disulfides containing carbonyl groups and the following tautomerization to compounds containing sulfane sulfur. Immunothiol (diethyldithiocarbamate), used in AIDS treatment, has immune-enhancing properties in humans and mice [31, 50, 55]. This compound is metabolized to carbon disulfide, which is next transformed to a compound containing reactive sulfane sulfur.

The compound containing sulfane sulfur, cystine trisulfide (CysSSSCys), and elemental sulfur catalyse the non-enzymic reduction of cytochrome C by GSH [38, 53].

The authors have also recently observed a significant decrease in sulfane sulfur level in plasma of the patients with chronic renal insufficiency in comparison to healthy people's plasma. The process of hemodialysis caused a further drop in the plasma level of this reactive form of sulfur [77].

Relations between granulations rich in reduced sulfur of the periventricular glial cells in the brains of mice and rats and transformations related to sulfane sulfur remain unknown as yet [61].

Sulfane sulfur-generating systems

Toohey draws attention to the problem that compounds containing sulfane sulfur cannot be applied in biological systems because they are quickly degraded at physiological pH and "they don't have time" to be effective [73]. This is why Toohey suggests different sulfane sulfur-generating systems, e.g. the abovementioned disulfides with allyl, carbonyl or enol group [72, 73]. Pyridoxal phosphate and cysteine can constitute another system of this kind. Pyridoxal catalyzes a non-enzymatic reaction of cystine -elimination, with the formation of cysteine hydropersulfide containing sulfane sulfur (RSSH). Cystamine may be another sulfane sulfur-generating system in cell culture. Its oxidation by diamine oxidase present in plasma results in aldehyde formation, whose isomer contains sulfane sulfur [24, 73]. A substrate of alcohol dehydrogenases, 2-mercaptoethanol disulfide, also undergoes a transformation into aldehyde disulfide. The isomer of this compound contains sulfane sulfur. Sulfide-treated proteins (such as serum albumin or egg globulin) are stable and non-toxic sources of sulfane sulfur [13, 72]. On the other hand, sulfane sulfur-generating systems applied in concentration higher than optimal can be toxic and can contribute to the so-called giant cell creation [72, 73].

Methods of sulfane sulfur assay

To describe completely the biological role of sulfane sulfur compounds, it is necessary to possess methods sensitive enough to assay its level in biological samples. As mentioned above, compounds with sulfane sulfur react easily with cyanide forming thiocyanate that reacts with Fe^{3+} ions to give a red complex.

The most popular spectrophotometric assay of sulfane sulfur level, first described by Wood [82], is based on this reaction. Unfortunately, this method and its modifications have limited sensitivity and specificity, and, therefore, cannot be applied for quantification of trace amounts of sulfane sulfur in biological samples.

This is why a search for a new method allowing precise sulfane sulfur determination has been going on in recent years. A gas chromatography assay of protein-associated sulfur has been described by Sörbo et. al. [27]. This method, however precise, cannot be applied to determine non-protein sulfane sulfur forms. Westley and Westley, in turn, developed a polarographic method based on the conversion of sulfane sulfur (called by them cyanide-reactive sulfur) to thiocyanate by using rodanese as a catalyst, with cyanide acting as an acceptor and glutathione as a cofactor [75]. This method is more sensitive than the colorimetric one, however it is also quite time-consuming and demanding due to a complicated detection system.

Looking for new determination methods of compounds with sulfane sulfur, Ogasawara et al. discovered that sulfur bound to normal human serum can be released as sulfide by reduction in the presense of excess of dithiothreitol (DTT) [44, 46]. Released sulfide can be converted in reaction with p-phenylenediamine and Fe^{3+} ions into fluorescent derivative, thionine, which can be determined by HPLC with fluorometric detection in combination with flow gas dialysis [43, 70]. Ogasawara called sulfur released by dithiothreitol reduction "bound sulfur". This method is highly sensitive and specific but thiosulfonates containing sulfane sulfur

and thiosulfate cannot be determined, because they could not be reduced by dithiothreitol [44]. According to Ogasawara "bound sulfur" is only a fraction of sulfane sulfur. He introduced the term "acid-labile sulfur", to describe sulfur released, in H_2S form, from iron-sulfur proteins following the addition of hydrochloric acid. This type of sulfur was located mostly in mitochondrial fraction, in contrast to "bound sulfur" found in cytosolic fraction [45]. These observations are in agreement with earlier data on iron-sulfur cluster location in mitochondrial fraction [40].

Using the properties of released sulfane sulfur in the form of H_2S by dithiothreitol reduction, Toohey determined sulfane sulfur level by diffusion assay [71]. Released sulfide diffuses in the form of H_2S in a hermetic diffusion vessel and is trapped in an adjoining vessel containing 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and a yellow product is determined spectrophotometrically.

Every method of sulfane sulfur assay corresponds to a different type of compounds containing this reactive form of sulfur. The term "sulfane sulfur" refers to the whole pool of compounds containing reduced atom of sulfur covalently bound with other sulfur atom, whereas "protein-associated sulfur", "bound sulfur" and "acid-labile sulfur" refer to particular compounds with sulfane sulfur and should not be used interchangeably.

Summary

Sulfane sulfur is generated in a number of the described metabolic pathways (Scheme 1). Protein carriers, which stabilize and transport sulfane sulfur, are widespread. Compounds containing sulfane sulfur efficiently regulate the *in vitro* activity of many enzymes and have antioxidative properties. Furthermore, a connection exists between disturbed anaerobic sulfur metabolism and neoplastic processes, virus infections and immunodeficiency. All these reasons suggest that sulfane sulfur can exert regulative function in the cells through –SH group modification, and its high activity and short half-life endows it with features of a regulator with high operating precision.

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