Tissue and Subcellular Distribution of Bound and Acid-Labile Sulfur, and the Enzymic Capacity for Sulfide Production in the Rat

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The tissue and subcellular distribution in liver and kidney of bound and acid-labile sulfur as well as the enzyme capacity for sulfide production related to the desulfuration pathway were determined in rats. Bound sulfur was widely distributed in tissues and highest in kidney, whereas acid-labile sulfur was highest in heart. Bound sulfur was found primarily in the cytosolic fraction in the form of high molecular weight material in liver, and as both high and low molecular weight material in kidney. Acid-labile sulfur was located in the mitochondrial fraction. Sulfide production capacity from cysteine was greatest in liver cytosol. This capacity was well correlated with the distribution of γ-cystathionase in tissues and subcellular fractions.

Keywords bound sulfur; desulfuration pathway; distribution; acid-labile sulfur; γ-cystathionase; 3-mercaptopyruvate sulfurtransferase

Endogenous reduced sulfur, having a reduced oxidation state with a valence of 0 or −1, can be produced via desulfuration of cysteine by several enzymes present in mammalian tissues; these enzymes include γ-cystathionase (CST, E.C.4.4.1.1.) and cysteine aminotransferase (E.C. 2.6.1.3.) in conjuction with 3-mercaptopyruvate sulfurtransferase (3-MST, E.C. 2.8.1.2.), as illustrated in Fig. 1.

Stipanuk has suggested that sulfide released from cysteine via desulfuration pathways in vivo is followed by incorporation of the sulfur into some pools of active reduced sulfur (sulfane sulfur), which has a relatively long half-life prior to its oxidation to sulfate.1)

On the other hand, Westley et al. have introduced the concept of a metabolic pool of sulfane sulfur for biosynthetic purposes, e.g., to form iron-sulfur protein and for detoxication, e.g., of cyanide.2−5) Sulfane sulfur is defined as divalent sulfur bonded only to other sulfur atoms. Furthermore, Toohey has reviewed the topic and stated that sulfane sulfur has effects in biological systems and suggested that it has a natural regulatory function.6)

Although recent evidence indicating the existence of a reduced sulfur pool has been reported, little quantitative or qualitative data have been produced so far and in sufficient characterization of it as a biological constituent has been provided. Recently, we found that sulfide was released from human serum following reduction with excess thiols.7) Further, we determined sulfur in various mammalian serum samples using a fluorometric high performance liquid chromatographic (HPLC) method combined with flow gas dialysis; this reduction-labile sulfur has been referred to as “bound sulfur.”8) Figure 2 shows

\[
\text{cysteine} \xrightarrow{\text{cysteine}} \text{thiocysteine} + \text{pyruvate} + \text{NH}_4^+ \\
(1) \xrightarrow{\text{amino acid}} \xrightarrow{\text{3-mercaptopyruvate}} S^0 \\
(2) \xrightarrow{\text{keto acid}} \xrightarrow{\text{cysteine}} \\
(3) \xrightarrow{\text{pyruvate}} \xrightarrow{S^0} \xrightarrow{(HS^-)}
\]

Fig. 1. Pathways of Reduced Sulfur Production from Cysteine in Mammalian Tissues

Enzyme: (1) cysteine aminotransferase; (2) CST; (3) 3-MST.

cysteine \xrightarrow{\text{cysteine}} thiocysteine + pyruvate + NH₄⁺

low molecule
\[ R - S - N - R (s > 2) \quad R - S - S - R \quad O_3S - S - SO_3^- (s > 2) \]
poly sulfide persulfide polythionate

high molecule (protein-associated species)
\[
\text{protein} \xrightarrow{S} \text{protein-S-S}^- \xrightarrow{\text{protein}} \text{protein-S}^\circ
\]
sulfur bound to protein as a polysulfide protein persulfide elemental sulfur bound to protein

Fig. 2. Various Types of Biological Components Containing Bound Sulfur

*: bound sulfur atom.

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the compounds thought to involve bound sulfur atoms. This sulfur belongs to sulfane pool components. Although all of these components have been considered to be involved in biological systems, our knowledge of bound sulfur compounds has been limited by lack of quantitative information. The level of bound sulfur that we found in mammalian serum was low, and the bound sulfur compounds exist in high molecular weight forms as a protein-associated species (Fig. 2), as reported previously by us.8)

The purpose of the present study is to measure the endogenous levels of bound sulfur and acid-labile sulfur in rat tissues, and their distribution in subcellular fractions of rat kidney and liver, using an analytical method established by us5); acid-labile sulfur means sulfur that is released to form H₂S, from the iron-sulfur cluster proteins in which bound sulfur appears to be the physiological source of the sulfur, by treatment with acid. Moreover, we examined the form of bound sulfur in the kidney and liver cytosolic fractions by means of gel chromatography. We also confirmed the location of 3-MST and CST activities, and the distribution of the enzymic capacity for sulfide production in rat tissues and subcellular fractions under more-physiological assay conditions, to investigate the relationship between endogenous sulfur and the sulfur-producing enzymes involved in the cysteine desulfuration pathway.

MATERIALS AND METHODS

Reagents Standard solutions of analytes and reagents for bound and acid-labile sulfur determination were prepared according to our previous report.7) Triton-X was from Nakarai Tesque, Inc. (Kyoto, Japan). The compounds β-NADPH, β-NADH and glucose-6-phosphate(G-6-P) were purchased from Oriental Yeast Co. (Osaka, Japan). Cytochrome c was obtained from Sigma (St. Louis, MO). Other reagents used were of analytical grade. Molecular weight marker proteins (thryoglobin, ferritin, bovine serum albumin, ribonuclease) were obtained from Pharmacia LKB Biotechnology Inc. (Uppsala, Sweden).

Animals Male Wistar rats weighing between 230 and 280 g were used for all experiments. Rats were housed in stainless-steel cages in an air-conditioned room at 23 °C with a 12-h light/dark cycle. All animals had access ad libitum to water and commercial laboratory chow CE-2 (CLEA Japan Inc., Tokyo) for at least one week before the start of the experiments.

Tissue Preparations Rats were exsanguinated under light ether anesthesia, and individual tissues were removed immediately and washed in phosphate buffered saline (pH 7.4).

For the tissue distribution study, tissue samples (0.5—1.0 g) were homogenized in 4 volumes of 20 mm potassium phosphate buffer (pH 7.4) using a Potter-Elvahjem homogenizer with a Teflon pestle to form a 20% (w/v) homogenate. To 5.0 ml of iced homogenate, 0.05 ml of 20% (w/v) Triton X-100 was added. The mixture was centrifuged at 4 °C for 20 min, and the supernatant was retained as the sample material.

Subcellular Fractionation For the subcellular distribution study, liver and kidney were immediately minced and homogenized in ice-cold 0.25 M sucrose solution with five strokes of a Potter-Elvahjem homogenizer. Subcellular components were separated by the procedure of Hogeboom.6)

The nuclear, mitochondrial and microsomal fractions were each suspended in an appropriate amount of 10 mm phosphate buffer, pH 7.4, to give a concentration of 10% (w/v). These suspensions were sonicated for three 15-s intervals with a Sonifier (Branson Instruments, Inc., Stamford, Conn.) with cooling in an ice bath during sonication. The final supernatant (12000 g) was used for the measurement of sulfur and enzyme activity.

Gel Chromatography of Cytosol Fractions The gel chromatographic conditions were as follows: gel bed, Superose 6 (Pharmacia LKB, 30 cm x 1 cm i.d.); eluent, 50 mm phosphate buffer (pH 7.4) containing 0.15 M NaCl; flow rate, 0.4 ml/min; fraction volume, 1 ml/tube. Cytosol fractions (0.2 ml) from liver and kidney were filtered using a 0.22 µm membrane (Millipore, Japan) and then chromatographed on a Superose 6 column that had been pre-equilibrated at 4 °C with eluent buffer. Eluent fractions were collected in 10 ml tubes. This separation procedure was repeated six times and each fraction (total 6 ml/tube) was concentrated to 1.2 ml for the assays of bound and acid-labile sulfur.

Determination of Bound and Acid-Labile Sulfur The bound and acid-labile sulfur content were differentially determined fluorometrically by an HPLC method based on the formation of thioine in combination with gas dialysis, as reported previously.8) Each sample was diluted with 50 mm borate buffer (pH 8.0) prior to measurement. For the determination of total sulfur released as sulfide, a 500 µl portion of sample and 500 µl of 20 mm dithiothreitol (DTT) solution were placed in a snap-cap tube. After incubation at 37 °C for 15 min, the reaction mixture was subjected to flow gas dialysis followed by derivatization and chromatographic separation. The determination of acid-labile sulfur was similar except that the DTT reduction procedure was performed before gas dialysis treatment. The subsequent procedure was carried out exactly according to the previous method.8) From the total releasable sulfur values obtained, the amount of acid-labile sulfur, calculated by analyzing parallel samples, was subtracted. Net values of the bound sulfur content were thus achieved.

Assay of Capacity for Sulfide Production The formation of sulfide from cysteine by rat tissues and subcellular fractions was measured as follows. A volume of 0.9 ml phosphate buffered saline (PBS, pH 7.4) containing 2 mm cysteine was added to 0.1 ml of a 10% (w/v) tissue homogenate or subcellular fraction (from liver) in 10 mm phosphate buffer (pH 7.4). After incubation for 60 min at 37 °C, the total sulfide produced was determined by the method described above. Sample blanks were measured for all samples in a similar manner except for cysteine addition. The incubation conditions for cysteine desulfuration assay were fixed at pH 7.4 (PBS) without any addition of co-factor to be as close to physiological conditions as possible. All determinations were made in triplicate.
Assay of Enzyme Activity  CST was assayed by the method of Greenberg\(^{10}\) using homoserine as a substrate. 3-MST was determined as previously described.\(^{11}\) Glutamate dehydrogenase (GDH, E.C. 1.4.1.2) was assayed as described by Arnold.\(^{12}\) NADPH cytochrome c reductase (E.C. 1.6.2.4) was assayed as described by Strobel.\(^{13}\) G-6-P dehydrogenase (G6PDH, E.C. 1.1.1.49) was assayed by the method of Kornberg and Horecker.\(^{14}\) The protein concentration in all tissues and subcellular preparations was determined by the method of Lowry et al.\(^{15}\) using bovine serum albumin as a standard.

## RESULTS

### Tissue Distribution

The distribution of bound and acid-labile sulfur and the activities of enzymes involved in the desulfuration pathway of each tissue are shown in Table I. A significant tissue difference in the levels of bound and acid-labile sulfur was observed. The highest total releasable sulfur content was found in kidney, and about 90% of the sulfur was of the bound type. Total releasable sulfur content in liver, brain and spleen was relatively similar, and 60–80% of the total sulfur was present as bound sulfur. Releasable sulfur in heart was completely of the acid-labile type and its content was markedly higher than in other tissues. The tissue distribution of 3-MST and CST activities related to reduced sulfur production in rat revealed that the highest activities were present in liver and kidney. Slight CST activity was detected in other tissues.

### Subcellular Distribution

The subcellular distributions of the bound and acid-labile sulfur and the 3-MST and CST activities in rat liver and kidney are summarized in Tables II and III. The contamination from other subcellular fractions was estimated by using marker enzymes for each fraction. The recovery of each fraction was relatively good, but the separation of the mitochondrial and microsomal fractions was not homogeneous. An

### Table I. Tissue Distribution of the Bound and Acid-Labile Sulfur and the Enzyme Activities Related to the Desulfuration Pathway in Rats

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Total (nmol/g)</th>
<th>Bound(^{b}) (nmol/g)</th>
<th>Acid-labile (nmol/g)</th>
<th>3-MST(^{a}) (U/g)</th>
<th>CST(^{a}) (U/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>66.74 ± 8.89</td>
<td>40.76 ± 11.42</td>
<td>25.97 ± 2.96</td>
<td>472 ± 55.6</td>
<td>8.03 ± 2.43</td>
</tr>
<tr>
<td>Kidney</td>
<td>363.9 ± 104.8</td>
<td>324.1 ± 100.7</td>
<td>39.85 ± 7.36</td>
<td>425 ± 80.7</td>
<td>3.85 ± 0.40</td>
</tr>
<tr>
<td>Heart</td>
<td>128.6 ± 17.8</td>
<td>Not detected</td>
<td>129.3 ± 16.5</td>
<td>95.7 ± 17.3</td>
<td>0.04 ± 0.02</td>
</tr>
<tr>
<td>Brain</td>
<td>31.05 ± 6.24</td>
<td>18.53 ± 8.09</td>
<td>12.51 ± 2.12</td>
<td>38.7 ± 5.01</td>
<td>0.09 ± 0.03</td>
</tr>
<tr>
<td>Spleen</td>
<td>40.91 ± 13.81</td>
<td>34.06 ± 14.21</td>
<td>6.830 ± 0.63</td>
<td>64.8 ± 6.43</td>
<td>Not detected</td>
</tr>
</tbody>
</table>

\(\text{a) All values represent the means±S.D. for triplicate determinations on tissues from five male rats.}\)
\(\text{b) Bound sulfur=total sulfur-acid-labile sulfur.}\)

### Table II. Subcellular Distribution of Bound and Acid-Labile Sulfur and the Enzyme Activities Related to the Desulfuration Pathway in Rat Liver

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (mg/g)</th>
<th>Total (nmol/g)</th>
<th>Bound(^{b}) (nmol/g)</th>
<th>Acid-labile (nmol/g)</th>
<th>3-MST(^{a}) (U/g)</th>
<th>CST(^{a}) (U/g)</th>
<th>Marker enzyme activity (U/g)(^{b})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole extracts</td>
<td>133.15 ± 14.22</td>
<td>41.82 ± 11.65</td>
<td>43.57 ± 12.12</td>
<td>188.70 ± 34.70</td>
<td>5.33 ± 1.20</td>
<td>103.20 ± 19.33</td>
<td>GDH(^{d}) 6.74 ± 0.80 1.03 ± 0.29</td>
</tr>
<tr>
<td>Nuclei</td>
<td>9.87 ± 1.50</td>
<td>2.21 ± 1.41</td>
<td>6.52 ± 0.71</td>
<td>17.20 ± 2.76</td>
<td>0.04 ± 0.05</td>
<td>13.87 ± 3.27</td>
<td>Cyt. Red.(^{d}) 0.20 ± 0.07 0.01 ± 0.01</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>34.02 ± 3.98</td>
<td>1.08 ± 1.35</td>
<td>22.14 ± 0.62</td>
<td>116.60 ± 8.55</td>
<td>0.14 ± 0.11</td>
<td>64.82 ± 23.37</td>
<td>G6PDH(^{d}) 0.80 ± 0.29 0.08 ± 0.05</td>
</tr>
<tr>
<td>Microsomes</td>
<td>36.12 ± 8.26</td>
<td>1.70 ± 1.98</td>
<td>8.78 ± 2.94</td>
<td>6.50 ± 2.45</td>
<td>0.40 ± 0.21</td>
<td>20.90 ± 4.08</td>
<td>Glucose-6-phosphate dehydrogenase.</td>
</tr>
<tr>
<td>Cytosol</td>
<td>58.07 ± 8.09</td>
<td>27.65 ± 11.71</td>
<td>7.88 ± 1.67</td>
<td>77.20 ± 11.90</td>
<td>1.41 ± 0.25</td>
<td>3.09 ± 0.74</td>
<td>GDH(^{d}) 1.38 ± 0.76 0.77 ± 0.44</td>
</tr>
</tbody>
</table>

\(\text{a) All values represent the means±S.D. (per gram wet weight tissue) for triplicate determinations on tissues from four male rats.}\)
\(\text{b) Unit=μmol product/min.}\)

### Table III. Subcellular Distribution of Bound and Acid-Labile Sulfur and Enzyme Activities Related to the Desulfuration Pathway in Rat Kidney

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (mg/g)</th>
<th>Total (nmol/g)</th>
<th>Bound(^{b}) (nmol/g)</th>
<th>Acid-labile (nmol/g)</th>
<th>3-MST(^{a}) (U/g)</th>
<th>CST(^{a}) (U/g)</th>
<th>Marker enzyme activity (U/g)(^{b})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole extracts</td>
<td>138.82 ± 10.47</td>
<td>337.42 ± 25.50</td>
<td>84.35 ± 6.31</td>
<td>255.80 ± 44.00</td>
<td>2.79 ± 0.58</td>
<td>57.95 ± 5.47</td>
<td>GDH(^{d}) 2.29 ± 0.28 1.52 ± 0.14</td>
</tr>
<tr>
<td>Nuclei</td>
<td>10.00 ± 1.71</td>
<td>25.61 ± 2.21</td>
<td>13.22 ± 0.46</td>
<td>16.00 ± 3.57</td>
<td>0.04 ± 0.01</td>
<td>5.57 ± 0.45</td>
<td>Cyt. Red.(^{d}) 0.21 ± 0.03 0.03 ± 0.01</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>31.07 ± 3.88</td>
<td>52.38 ± 8.16</td>
<td>35.77 ± 9.68</td>
<td>86.20 ± 18.10</td>
<td>0.18 ± 0.04</td>
<td>31.45 ± 3.53</td>
<td>G6PDH(^{d}) 0.58 ± 0.13 0.20 ± 0.02</td>
</tr>
<tr>
<td>Microsomes</td>
<td>44.50 ± 3.43</td>
<td>31.88 ± 2.20</td>
<td>8.81 ± 1.69</td>
<td>3.94 ± 1.19</td>
<td>0.12 ± 0.01</td>
<td>12.82 ± 1.05</td>
<td>Glucose-6-phosphate dehydrogenase.</td>
</tr>
<tr>
<td>Cytosol</td>
<td>62.12 ± 8.26</td>
<td>172.02 ± 12.87</td>
<td>16.78 ± 1.26</td>
<td>186.00 ± 30.00</td>
<td>0.65 ± 0.11</td>
<td>2.62 ± 0.54</td>
<td>GDH(^{d}) 1.38 ± 0.14 0.38 ± 0.14</td>
</tr>
</tbody>
</table>

\(\text{a) All values represent the means±S.D. (per gram wet weight tissue) for triplicate determinations on tissues from four male rats.}\)
\(\text{b) Unit=μmol product/min.}\)
\(\text{c) GDH: glutamate dehydrogenase.}\)
\(\text{d) Cyt. Red.: NADPH cytochrome c reductase.}\)
\(\text{e) G6PDH: glucose-6-phosphate dehydrogenase.}\)
Fig. 3. This Figure Depicts a Plot of the Relative Content and Specific Activity versus Protein (%)

Liver homogenate was fractionated and the sulfur species, enzymes and protein were assayed as described in the Materials and Methods section. Results are presented as means ± S.D. (n=4). Protein (%) is the percentage recovery of protein from the liver whole extracts. The relative content and specific activity are calculated as the content and specific activity in the fraction divided by those in the liver whole extracts. A, acid-labile sulfur; B, bound sulfur; C, CST; D, 3-MST; E, cytochrome c reductase; F, GDH; G, G6PDH. Fractions: ☐ nucleus; ☐ mitochondrial; ☐ microsomal; ☐ -cytosolic.

An even more clear picture was obtained after graphical presentation of the distribution pattern of the investigated components and enzymes in terms of their relative values (Figs. 3 and 4). Approximately 42—45% of the releasable sulfur content in whole liver and kidney extracts was found in the enriched fraction in cytosol: the mitochondrial fraction contained 21—27% of the total releasable sulfur contents (Tables II and III). Acid-labile sulfur was present
Fig. 4. This Figure Depicts a Plot of the Relative Content and Specific Activity versus Protein (%)

Kidney homogenate was fractionated and the sulfur species, enzymes and protein was assayed as described in the Materials and Methods section. Results are presented as means ± S.D. (n=4). Protein (%) is the percentage recovery of protein from the kidney whole extracts. The relative contents and specific activity are calculated as the content and specific activity in the fraction divided by those in the kidney whole extracts. A, acid-soluble sulfur; B, bound sulfur; C, CST; D, 3-MST; E, cytochrome c reductase; F, GDH; G, G6PDH. Fractions: □, nuclei; □, mitochondrial; □, microsomal; □, cytosolic.

mainly in the mitochondrial fraction, whereas, most of the bound sulfur was located in the cytosolic fractions. On the other hand, about 60% of the 3-MST activity in the rat liver whole extracts was located in the mitochondrial fraction and 40% was found in the cytosolic fraction; in the case of rat kidney, 70% of the 3-MST activity in the
Fig. 5. Elution Profiles of Bound Sulfur in Rat Liver (A) and Kidney (B) Cytosolic Fractions on Superose 6 Column Gel Chromatography
Chromatographic conditions are described in the text. Each profile was produced from data obtained in triplicate experiments. —○—, bound sulfur. BSA: bovine serum albumin, RNase: ribonuclease.

Fig. 6. Sulfide Production Capacity from Cysteine in Various Rat Tissues
Experimental details are given in the text. Activity is expressed as total sulfide production in 1 g tissue. Results are presented as means ± S.D. for three rats.

Whole extracts was contained in the cytosolic fraction. CST activity was located mainly in the cytosolic fractions in both tissues.

Molecular Form of Bound Sulfur Components We further studied the molecular form of the bound sulfur components contained in the cytosolic fractions from liver and kidney, by means of Superose 6 column chromatography. As shown in Fig. 5A, components containing bound sulfur from liver cytosol were eluted in the high molecular weight fractions ranging from about 10 to 600 kDa. On the other hand, the bound sulfur components from kidney cytosol were detected in both the high molecular weight fractions and also in the low molecular weight fractions (Fig. 5B).

Capacity for Sulfide Production The distribution of the capacity for sulfide production from cysteine in the rat tissues and subcellular fractions from liver are shown in Figs. 5 and 6, respectively. Sulfide production was determined as the increased amounts of total releasable

Fig. 7. Sulfide Production Capacity from Cysteine in Subcellular Fractions of Rat Liver
Experimental details are given in the text. Results are expressed as relative capacity (%), means ± S.D. (n = 3) vs. total sulfide production in 1 g of liver homogenate (capacity: 22.1 ± 2.2 nmol/g/min). N: nuclei, Mic: microsome, Mit: mitochondria, Cyt: cytosol.

Table IV. Effect of Propargylglycine and Aspartate on the Sulfide Production Capacity from Cysteine in Rat Tissues

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Released sulfide (nmol/g/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Liver</td>
<td>38.0</td>
</tr>
<tr>
<td>Kidney</td>
<td>18.9</td>
</tr>
<tr>
<td>Brain</td>
<td>1.78</td>
</tr>
<tr>
<td>Heart</td>
<td>1.52</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.89</td>
</tr>
</tbody>
</table>

a) Average of three rats.
sulfur. As shown in Figs. 6 and 7, sulfide production was greatest in the liver and cytosolic fractions, which corresponded to the distribution of CST activities (see Tables I and II). Although no large differences in releasable sulfur between liver and other tissues (except kidney) were observed, a significant difference in sulfide production capacity was found.

Sulfide production from cysteine by rat liver and kidney extracts was markedly inhibited by the presence of propargylglycine, a suicide inactivator of CST, and slightly inhibited by aspartate, a competitive inhibitor of the 3-MST pathway including cysteine aminotransferase (Table IV).

**DISCUSSION**

In our previous studies, we suggested that reduction-labile sulfur species commonly exist in mammalian serum. This sulfur species is referred to as "bound sulfur," which is defined as divalent sulfur that is easily liberated as sulfide by reduction with excess thiols. Thus, bound sulfur has been presumed to be a normal constituent in vivo, but the exact details are not known. To characterize the bound sulfur in biological systems, it is necessary to study the distribution of bound sulfur and related enzymes and the state of bound sulfur in mammalian tissues and subcellular fractions. This study provides fundamental information about the production and physiological role of bound sulfur.

Recently, Stipanuk et al. suggested that the desulfuration pathway may be an important route for cysteine catabolism, and sulfide released from cysteine via the desulfuration pathway in vivo is followed by incorporation of the sulfur into a variety of bound forms. Therefore, it was presumed that some reduced sulfur is generated as a normal metabolite of cysteine. However, there was no good evidence for the existence of endogenous reduced sulfur.

In the present study, we applied a differential determination method for various sulfur species to normal rat tissues and subcellular fractions. As shown in Table I, the existence of acid-labile and bound sulfur was confirmed; however, free sulfide ion was not detected in all tissue samples following deproteinization (data not shown). It is likely that this releasable sulfur is widely distributed in mammalian tissues. The highest level of bound sulfur was found in kidney (Table I) and it was shown that bound sulfur was localized primarily in the cytosolic fraction (Figs. 3 and 4). In contrast, acid-labile sulfur was mainly concentrated in the mitochondrial fraction, as shown in Figs. 3 and 4. This is in agreement with the fact previously elucidated that iron-sulfur clusters are concentrated in the mitochondrial fraction. The acid-labile sulfur in the heart is relatively high compared with other tissues, along with the cytochrome components of the respiratory chain. It may be possible that the bound sulfur produced or transported in heart is rapidly utilized for biosynthesis of iron-sulfur clusters in mitochondria.

The form of bound sulfur in mammals has remained unknown until now. From the results of the gel chromatographic separation of the rat liver cytosolic fractions (Fig. 5), bound sulfur components are present in high molecular weight fractions. On the other hand, both high molecular and low molecular bound sulfur, probably non-protein components, were found in the kidney cytosolic fraction. These results suggest that bound sulfur is present in a wide variety of forms in mammalian tissues.

Our assay results for the sulfide production capacity of each tissue support the assumption described by Stipanuk that sulfide is released from cysteine via the desulfuration pathway (mainly dependent on CST) and this is followed by incorporation of the sulfur into some pools of reduced sulfur. The higher sulfide production capacity in liver and kidney is markedly inhibited by propargylglycine, which is a specific inhibitor for CST, whereas using l-aspartate as an inhibitor for the 3-MST pathway showed significant inhibition of sulfide production from cysteine in the heart, but not in liver and kidney (Table IV). Thus, the sulfide production capacity in the whole body seems mainly due to CST rather than 3-MST. These results agree with a previous report.

Furthermore, the rate of sulfide production was much slower when the increased free sulfide content was assayed in the deproteinized reaction mixture after incubation with cysteine. However, a higher production capacity was observed by determination of total releasable sulfur with DTT reduction. This means that much of the sulfur released from cysteine does not exist in its free form (sulfide ion) in tissue or cell extracts, but as the bound form. Our assay for the sulfide production capacity in vitro is more precise than previous methods because the increased amounts of both free sulfide and bound sulfur were measured as total sulfide in the present study. Although the sulfide production activity from cysteine is localized in the liver and kidney cytosol fractions, there are significant amounts of acid-labile or bound sulfur in other tissues. In addition, total releasable sulfur in kidney is about five times greater than that in liver, whereas the sulfide production capacity of kidney is less than half of that of liver. It is presumed that the transport mechanism for endogenous sulfur is present in vivo. That is, the sulfide produced mainly in liver cytosol is rapidly converted to bound sulfur, and then transferred to other tissues or intercellular fractions by some transport mechanism. The sulfur transported to mitochondria may be incorporated into iron-sulfur clusters by the action of sulfurtransferases, e.g. rhodanese. The transport mechanism for bound sulfur (or sulfide) is unclear so far, however, Westley et al. proposed that serum albumin might act as a specific carrier of elemental sulfur formed in liver for transport to other tissues.

On the other hand, bound sulfur retained in the cytosolic fraction is not only nonspecifically bound to protein for storage, but is also significantly incorporated into cytosolic enzymes: xanthine oxidase, aldehyde oxidase and tyrosine aminotransferase. A number of enzymes in biological systems are activated or inactivated by reduced sulfur through a mechanism which involves the incorporation of a sulfur atom.

The wide distribution of bound sulfur and sulfurtransferases may indicate the existence of other unknown
functional proteins which are regulated by reversible incorporation. Further investigation is necessary to identify the high and low molecular weight components containing bound sulfur and to elucidate the transport of bound sulfur in vivo.

Since it is assumed that bound sulfur is produced from cysteine in vivo, it is important to make correlations with the cysteine and glutathione levels in tissues in any further study of bound sulfur metabolism.

Acknowledgement We are grateful to Asahi Chemical Industry, Tokyo, Japan for the donation of pyruvate oxidase.

REFERENCES