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Metabolism of selenite labelled with enriched stable isotope in the bloodstream

K.T. Suzuki*, M. Itoh

Faculty of Pharmaceutical Sciences, Chiba University, 1–33 Yayoi, Inage, Chiba 263, Japan

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Abstract

The metabolism of selenium (Se) in the bloodstream of rats was studied using HPLC–ICP-MS with an enriched Se stable isotope, and the results were used as Se-specific indicators for Se nutritional status. Concentration of endogenous Se in plasma depended on dietary Se, while changes in concentrations and distributions of exogenous Se revealed its metabolic pathway. Namely, selenite was taken up by red blood cells and reduced to selenide, and then reappeared in plasma in a form bound selectively to albumin within 10 min, disappeared from plasma again within 30 min after injection. Then, the concentration of labelled Se started to increase slowly as selenoprotein P and extracellular glutathione peroxidase, and attained a maximum level at about 6 h after injection. The isotope ratio of endogenous to exogenous Se concentrations in plasma after 48 h post-injection was proposed to represent the Se-specific indicator in plasma reflecting the nutritional status of Se.

Keywords: Enriched stable isotope; Selenium; Selenite; Selenoprotein P; Albumin

1. Introduction

Selenium (Se) is an essential element having a very narrow range of dietary level for the necessary amount, and both inorganic and organic Se in nutrients are assumed to be metabolized through the key intermediate, selenide [1–3]. Inorganic Se, selenite and selenate are reduced to selenide by glutathione (GSH) in the body owing to the intrinsic low ionization potential of Se, and then utilized to produce Se-containing proteins (selenoproteins) through selenocysteine or excreted after being methylated. Thus, Se is chemically a unique element being present in both inorganic and organic forms with a variety of oxidative states from +6 to −2. In addition, Se alters its localization in the body depending on its chemical forms. Although Se administered in a form of selenite or selenate is known to appear in the bloodstream in quite different chemical forms as mentioned above, details of metabolic changes in the chemical forms of Se have been studied only to a limited extent owing to the absence of appropriate analytical methods.

Chemical forms of metals among biological constituents can be estimated efficiently by detecting metals in the eluate of various columns by HPLC and liquid chromatography with the use of an atomic absorption spectrometer (AAS) [4,5], atomic emission spectrometer (AES) by excitation with inductively coupled argon plasma (ICP) (ICP-AES) [6,7], and mass spectrometer (MS) by ionization with ICP (ICP-MS) [8–11]. Our recent application of HPLC–

*Corresponding author.

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ICP-MS further demonstrated that endogenous and exogenous (labelled with an enriched stable isotope) metals in different chemical forms can be specified simultaneously by a single experiment [9,10]. Simultaneous speciation of both endogenous and exogenous metals can be performed only by utilizing MS as a detector of separation tools such as HPLC. Thus, HPLC–ICP-MS can be effectively used as a powerful way of specifying metabolites, and also for revealing their metabolic fate and interaction with internal pools.

One additional aim in the present study was to develop sensitive and selective biological indicators that would reflect the nutritional status of respective trace metals, and also would be required to diagnose a status of 'deficient to excessive' for essential trace metals in the body. Recently we successfully applied the HPLC–ICP-MS method with enriched stable isotopes and demonstrated that the nutritional status of Se in the body can be diagnosed by its distribution in biological fluids [10]. By this application, we showed that the nutritional status of Se in rats by the feeding of diets of differing Se contents is reflected in Se-containing biological constituents in body fluids (blood and urine) and organs. The present study was performed to reveal the detailed metabolic process of Se in the bloodstream, and to find and to propose specific and selective indicators in plasma for the nutritional status of Se based on the speciation study. The isotope ratio of exogenous to endogenous Se was proposed as an indicator that can be used without referring to the reference material or value, and certainly is applicable as a better indicator than our previous one in urine [10].

2. Experimental

2.1. Reagents

Sodium selenite (99%) was purchased from Aldrich (Milwaukee, WI, USA). Concentrated HNO_3 and $HClO_4$ (analytical grade), and standard solution of Se (1000 μg Se/ml) were from Wako (Osaka, Japan). Sodium selenite enriched with ^{82}Se was prepared by oxidation of the enriched metallic ^{82}Se (97.02%, Oak Ridge National Laboratory, Oak Ridge, TN, USA). Oxidation of metal ^{82}Se was achieved by dissolving it in concentrated metal-free nitric acid and subsequent neutralization with 1 *M* NaOH.

2.2. Animals and treatment

Male Wistar rats were purchased at 4 weeks of age from a breeder (Clea Japan, Tokyo, Japan). After feeding a normal diet (CE-2, Clea Japan) and tap water ad libitum for 1 week, three groups of rats were fed a Se-deficient (<0.03 μg Se/g diet, Oriental Yeast, Tokyo, Japan), Se-adequate (0.2 μg Se/g diet prepared by addition of sodium selenite to the Se-deficient diet) or Se-excessive (2.0 μg Se/g diet) diet and deionized water ad libitum for 3 weeks. The composition of the Se-deficient diet has been reported elsewhere [9]. ^{82}Se-enriched sodium selenite was singly injected intravenously at a dose of 25 μg ^{82}Se/kg body weight under pentobarbital anesthesia. Blood was collected with heparin under light ether anesthesia at various time points after injection or without heparin at 48 h post-injection. The blood specimen was immediately centrifuged at 8000 *g* for 1 min to separate plasma or sera.

2.3. Analysis of distributions and concentrations of Se

A 0.1-ml aliquot of plasma was applied to a size-exclusion column (Asahipak GS-520, 500×7.6 mm, Showa Denko, Tokyo), and the column was eluted with 50 m*M* Tris–HCl buffer (pH 7.4) at a flow-rate of 1.0 ml/min on HPLC (LC-10AD, Shimadzu, Kyoto, Japan). The elute was introduced directly into the nebulizer tube of ICP-MS (PMS 2000, Yokogawa Analytical Systems, Musashino, Japan) to monitor Se (*m/z* 78 and 82) and sulfur (S, *m/z* 34) concentrations continuously. The analytical conditions of ICP–MS were reported in the previous paper [9]. Results for S and endogenous Se (*m/z* 78) are presented as direct counts from ICP–MS, while results of the exogenous Se (*m/z* 82) are presented as calculated counts.

A 0.5-ml aliquot of plasma or serum was wet-digested with an acid mixture of HNO_3 and $HClO_4$ (4:1, v/v) and diluted to 5 ml with purified water. Concentrations of Se in the samples were analyzed by detection at *m/z* 78 and 82 using ICP-MS with calibration carried out by the standard addition

Table 1
Concentrations of endogenous Se in plasma

Group	Endogenous se (ng Se/ml plasma)	Number of samples (*n*)
Deficient	118±3*	13
Adequate	362±9	12
Excessive	419±15*	13

Rats were fed a Se-deficient, -adequate or -excessive diet for 3 weeks, and then plasma was obtained from the rats. Values are means±S.E.M. *$P<0.05$, compared with the value of the adequate group

method. Concentrations of endogenous Se were calculated from counts of m/z 78 and those of exogenous Se were calculated by the following formula:

(counts of m/z 82 originated from exogenous Se)
= (measured counts of m/z 82)
− (measured counts of m/z 78)
× (ratio of counts of m/z 82/78 in standard solution of natural abundance Se)

2.4. Statistics

Student's t-test was utilized to evaluate differences between means.

3. Results

3.1. Concentrations of the endogenous and exogenous Se in plasma reflect the nutritional status

Concentrations of endogenous Se were detected by natural abundance ^{78}Se, while concentrations of exogenous Se were estimated by enriched (97.02%) ^{82}Se after subtracting the ^{82}Se concentration in the natural abundance Se from the experimental ^{82}Se concentration. Concentrations of the endogenous Se in plasma reflected the nutritional status that was produced by feeding diets of differing Se contents as demonstrated in Table 1. The concentration in the Se-deficient group was decreased to 1/3 of the Se-adequate group. On the other hand, the concentration of the Se-excessive group was increased only 16% over the adequate group after 3 weeks of feeding despite the fact that 10 times less and more the concentrations of Se were contained in the deficient and excessive groups, respectively.

Although the same dose of labelled selenite was loaded to the three groups of rats, concentrations of labelled Se in serum at 48 h post-injection were dependent on the nutritional status (Table 2). That is, exogenous Se concentrations in plasma increased with decreasing Se content in the diet.

3.2. Speciation of exogenous Se in plasma; uptake and reduction in RBC, and binding to albumin

Distributions of endogenous and exogenous Se among constituents in plasma were determined by separation on a size-exclusion column and monitoring Se concentrations at m/z 78 and 82, respectively, using ICP-MS. Simultaneous detection of endogenous and exogenous Se revealed that concentrations and distributions as well, of endogenous Se were not altered by the injection of the labelled selenite, indicating that the present dose was sufficiently small so as not to disturb the internal pool. However, dramatic changes were shown in both concentrations (Fig. 1) and distributions (Fig. 2) of labelled Se. (Only the typical profile was shown in Fig. 2).

Table 2
Concentrations of endogenous and exogenous Se in serum at 48 h post-injection of selenite

Group	Concentration (ng Se/ml serum)		Ratio endogenous Se/exogenous Se
	Endogenous Se	Exogenous Se	
Deficient	166±15**	56.4±3.3*	3.0±0.4**
Adequate	384±10	42.3±0.8	9.1±0.3
Excessive	443±46	12.5±1.6**	36.2±1.8**

Rats were fed a Se-deficient, -adequate or -excessive diet for 3 weeks, and then labeled (^{82}Se-enriched) selenite was injected intravenously and serum was obtained from the rats 48 hr later. Values are means±S.E.M. (n=3~4). *$P<0.05$, **$P<0.0001$, compared with the corresponding values of the adequate group

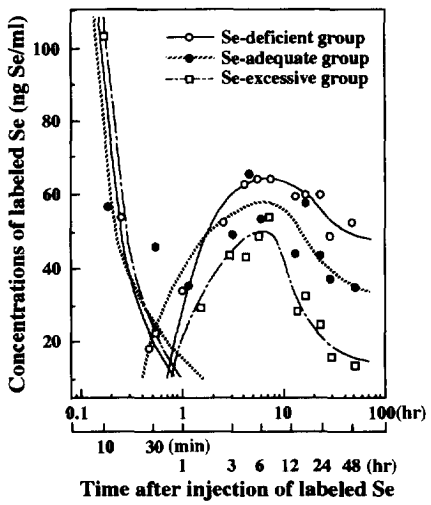

Fig. 1. Time-dependent changes in concentrations of labelled Se in plasma after a single intravenous injection of ^{82}Se-enriched selenite into rats, maintained for three weeks on three diets of different Se content. A single intravenous dose of ^{82}Se-enriched selenite (25 μg ^{82}Se/kg body weight) was injected into rats that had been maintained for three weeks on three different diets of Se content, and the concentrations of ^{82}Se in plasma were plotted against time after the injection. Se-deficient group (○), Se-adequate group (●) and Se-excessive group (□).

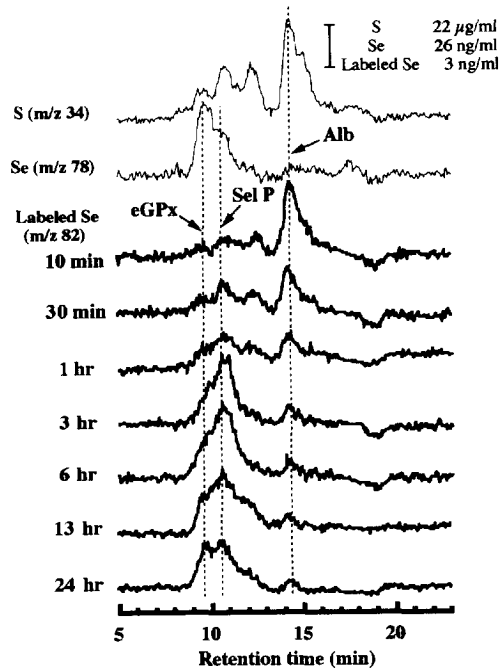

Fig. 2. Time-dependent changes in the distributions of labelled Se in plasma after a single intravenous injection of ^{82}Se-enriched selenite into rats maintained for three weeks on a Se-adequate diet. Selenite enriched with ^{82}Se was singly injected intravenously into rats of the Se-adequate group, and blood specimens were obtained 10, 30 min, 1, 3, 6, 13 and 24 h after the injection. Distributions of exogenous Se were determined on an Asahipak GS 520 column. Distributions of sulfur (S) (*m/z* 34) and endogenous Se (*m/z* 78) were determined at the same time as references. The vertical bar indicates the detection level for the three elements: albumin (Alb), extracellular glutathione peroxidase (eGPx), and selenoprotein P (Sel P).

Labelled Se injected as selenite disappeared from plasma within 30 min (Fig. 1), and this Se was shown not to be present as selenite even at only 10 min post-injection. The labelled Se disappearing rapidly from plasma within 30 min after the injection was designated to be bound to albumin from the retention time on the present column (Fig. 2) separating plasma proteins largely into albumin and globulins [12]. The in vitro mixing of selenite with plasma demonstrated that Se did not bind to albumin, selenite being eluted as the anion without being bound to any plasma constituents on a size-exclusion column by HPLC (Fig. 3a). However, incubation of selenite in plasma in the presence of GSH (2 m*M*) at 37°C for 10 min (data not shown), or in heparinized whole blood in the absence of glutathione, produced a Se peak at the retention time corresponding to albumin (Fig. 3b, 10 min). As is evident in Fig. 3b, selenite was taken up by RBCs within 1 min under the latter conditions. Further, in the absence of albumin (heparinized blood of analbuminemic rats) [12], it was shown that the labelled Se incorporated into RBCs was not effluxed from RBCs (data not shown), confirming earlier suggestions by Gasiewics and Smith [13].

3.3. Speciation of exogenous Se in plasma; extracellular glutathione peroxidase and selenoprotein P

The labelled Se disappearing from plasma and reappearing in plasma within 10 min after the injection disappeared again within 30 min (Fig. 1). However, after 30 min post-injection of ^{82}Se-enriched selenite, concentrations of labelled Se in plasma started to increase with time, and new peak

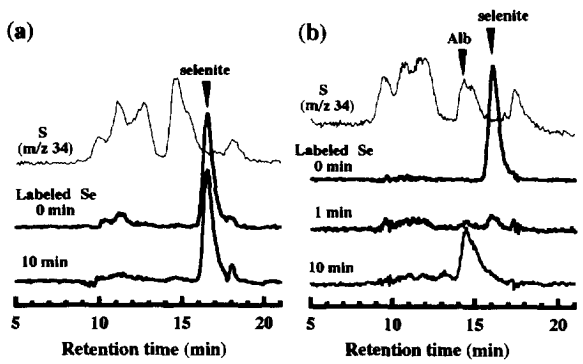

Fig. 3. Distributions of labelled Se in plasma (a) and whole blood (b) after incubation with ^{82}Se-enriched selenite on a size-exclusion column by HPLC–ICP-MS. Selenite enriched with ^{82}Se was incubated in plasma (a) for 0 and 10 min, and in whole blood (b) for 0, 1 and 10 min at 37°C, and the distributions of labelled Se in plasma were determined on an Asahipak GS 520 column. Distributions of sulfur (S) (m/z 34) were determined at the same time as the references.

concentrations were attained at about 6 h after the injection in the three dietary groups (Fig. 1). The distribution profiles of ^{82}Se in plasma after 30 min post-injection indicated that the labelled Se peak at the retention time of albumin decreased with time and, instead, two new labelled Se peaks increased with time at retention times of 9.6 and 10.5 min (Fig. 2). Retention times of these two labelled Se peaks corresponded to the two endogenous Se peaks in the same plasma. When distributions of Se were first demonstrated by HPLC–ICP-MS, we tentatively designated the two Se peaks in plasma as selenoprotein P (Sel P) and extracellular glutathione peroxidase (eGPx) in the order of elution from the column [9]. However, this assignment was reversed here, based on the observation that the slower eluting Se peak was produced by the interaction with heparin and that this peak in serum was eluted more slowly at 12.0 min as a sharper peak without heparin (Fig. 4), which corresponded to the heparin-binding property of Sel P [14]. Therefore, the two Se peaks increasing in plasma at 9.6 and 10.5 min with time after 30 min post-injection in Fig. 2 were designated as eGPx and Sel P, respectively, indicating that selenide transferred from albumin in plasma to the liver was incorporated into the two selenoproteins and reappeared in plasma after 30 min post-injection. Fig. 2 revealed that the labelled Se transferred to the liver was incorporated faster and at a greater amount into Sel P than into eGPx. These observations confirm previous suggestions by Burk and his coworkers [15,16].

3.4. Isotope ratio of endogenous to exogenous Se concentration as a specific and sensitive indicator for nutritional status

The difference in concentrations of labelled Se in plasma of the three dietary groups became more evident with time later than 6 h (Fig. 1). To investigate the cause of this difference, distributions of labelled Se were compared between the deficient dietary group (the slowest rate in disappearance of

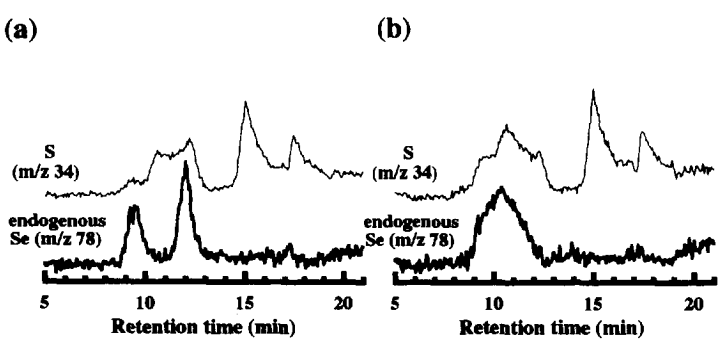

Fig. 4. Effect of heparin on the distribution profile of the two major Se-containing peaks in the bloodstream on a size-exclusion column by HPLC–ICP-MS. Two distinct Se peaks were observed at 9.5 and 12.0 min on an Asahipak GS 520 column by HPLC–ICP-MS in the serum of the male Wistar rat of 15 weeks of age (a), while an addition of 10% heparin into the serum (serum/heparin solution = 9:1, v/v) caused a shift of the slower eluting peak (b). The faster and slower eluting Se peaks were designated as extracellular glutathione peroxidase (eGPx) and selenoprotein P (SelP), respectively.

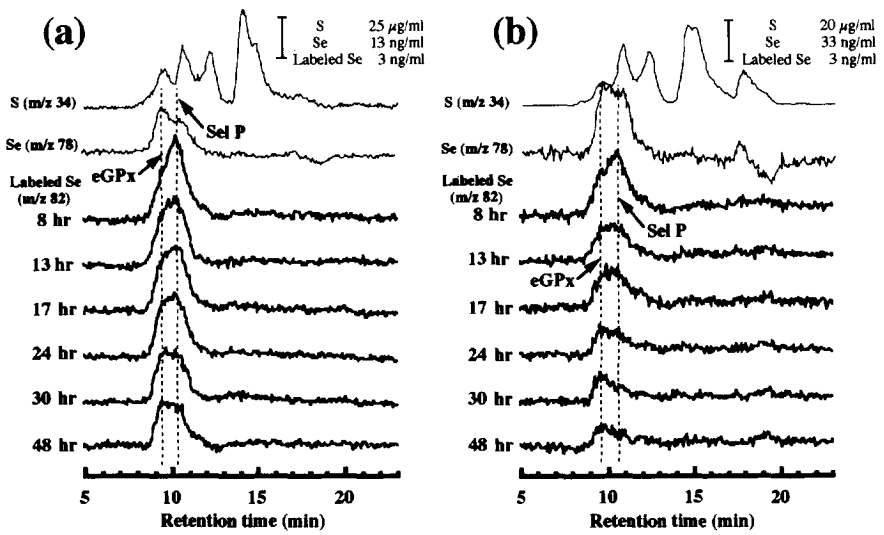

Fig. 5. Time-dependent changes in distributions of labelled Se in plasma at the disappearance phase after a single intravenous injection of ^{82}Se-enriched selenite into rats of the Se-deficient (a) and Se-excessive rats (b) on an Asahipak GS 520 column by HPLC–ICP-MS. For details, see the legend to Fig. 3.

labelled Se) and the excessive dietary group (the fastest rate in disappearance of labelled Se) (Fig. 5). In the disappearance step, labelled Se incorporated into Sel P disappeared rapidly as demonstrated in the two dietary groups in Fig. 5. Further, the distribution profiles in Fig. 5 indicated that the labelled Se incorporated into Sel P disappeared more rapidly in the excessive group than in the deficient group. These changes in distribution profiles of labelled Se were shown as the change in concentrations of the labelled Se in serum at 48 h post-injection (Table 2).

4. Discussion

Separation by HPLC and on-line detection with an element-specific detector have been demonstrated to give more valuable information than a simple measurement of element concentrations. ICP–MS was used as an element-specific detector, and this method was used to analyze Se in the body in the present study. ICP-MS is currently the most sensitive means of detecting concentrations of metals and, in a previous study, HPLC–ICP-MS was effectively applied for the first time to specify endogenous Se in plasma [9]. In addition, ICP-MS can be applied to analyzed stable isotopes, and makes it possible to use enriched stable isotopes as labels to trace the metabolic fate of elements [10,11]. As a result, the application of HPLC–ICP-MS with enriched stable isotopes to Se in the present study has successfully demonstrated that the method can be used efficiently to specify endogenous Se by detecting the natural abundance ^{78}Se, and at the same time, to specify exogenous Se by detecting enriched ^{82}Se.

4.1. Reduction of Se in RBCs

The present HPLC–ICP-MS study with ^{82}Se-enriched selenite revealed the metabolic pathway of Se administered as selenite into the bloodstream, and it was found to be mostly transferred to RBCs. Extremely rapid and complete uptake of selenite by RBCs occurred at first within several min, and selenite was assumed to be reduced to selenide in RBCs. Selenide produced in RBCs by reduction with GSH was effluxed into plasma (only when albumin was present), and sequestered selectively by albumin (Fig. 2). Direct transfer of selenide to the liver without being sequestered by albumin was not possible because selenide was not effluxed from RBCs in the absence of albumin.

These observations indicate that Se alters its localization by changing chemical forms. Namely, Se in a form of selenite is taken up efficiently by RBCs and reduced possibly to selenide, and then the selenide is effluxed and bound selectively to albumin in plasma.

4.2. Mechanisms of the transfer of Se to and from albumin

Selenide was assumed to be produced in RBCs, and it was demonstrated to be effluxed into plasma and bound selectively to albumin. However, the mechanism to explain the transfer of selenide based on chemical reactions is not known. It seems reasonable to assume that there is a formation of (albumin)–S–Se–(selenide) bridge between selenide and albumin. This chemical reaction has to be verified together with the reasonable mechanism for the selective binding of selenide to albumin. Further, although the transfer of selenide bound to albumin to the liver observed in the present study (Fig. 2) was already proposed by Sandholm [17], its mechanism is also not known yet. In addition, there was one more observation that still requires an explanation. Namely, selenide is sequestered only by about 5% of the albumin existing in plasma (data not shown). The rest of the albumin was silent to this sequestration reaction.

4.3. Nutritional status and metabolic pool

A large amount of exogenous Se was shown to disturb the endogenous metabolism of Se, as demonstrated by the administration of ^{82}Se-enriched selenite at a single dose of 50 μg Se/rat [10]. However, a smaller dose of 25 μg Se/kg body weight in the present study did not disturb endogenous Se, and no appreciable changes in concentrations and distributions of endogenous Se were observed. The nutritional effect of Se content in diet was reflected by the endogenous Se concentration in plasma or serum of rats in the three dietary groups (Tables 1 and 2). The nutritional effect was also observed in the metabolism of exogenous Se (Fig. 5), and labelled Se in Sel P was shown to respond more sensitively than in eGPx (Fig. 2 and Fig. 5), indicating that the metabolic turnover of Sel P is faster than that of eGPx.

4.4. Isotope ratio of endogenous to exogenous Se concentration as a specific and sensitive indicator for nutritional status

As the concentration of the endogenous Se in plasma was shown not to be affected by a small amount of exogenous Se despite reflecting the nutritional status, it can be used as a reference value before or without injection of labelled Se. Further, the concentration of the exogenous Se in plasma at a later time after the injection, reflecting the disappearance rate of Sel P, was also shown to sensitively reflect the nutritional status (Fig. 1, Table 2). The rapid metabolism of Sel P in the excessive dietary group must be related to its, as yet, unknown biological roles. These results also suggest that the ratio of concentrations of exogenous Se to endogenous Se after 48 h post-injection of the enriched selenite represents a very sensitive and accurate indicator of the nutritional status of Se. On this assumption, the ratios of concentrations of endogenous to exogenous Se in serum at 48 h post-injection of labelled selenite were calculated from the data shown in the left columns of Table 2. As this new indicator is based on the ratio between the two Se isotopes, no reference materials are required to obtain absolute values with accuracy and precision.

4.5. Metabolic characteristics of selenoprotein P and scheme for proposed metabolism of selenite in bloodstream

Although the mechanisms for the uptake of selenide bound to albumin by the liver and the incorporation of the labelled Se into eGPx and Sel P at the different rate were not studied in the present study, it has been proposed that selenide is incorporated into proteins through selenophosphate and selenocysteinyl tRNA [18,19]. The present results confirmed the observation using radio-labelled selenite by Burk and colleagues [15] that Sel P responds more sensitively to exogenous Se supplied in a form of selenite than eGPx. This fast response of Sel P may be related to its biological role. A schematic

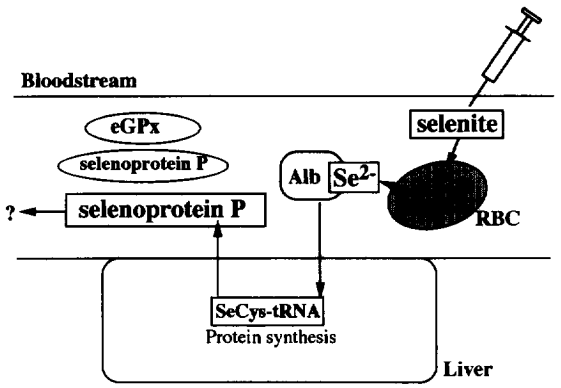

Fig. 6. Proposed scheme for the metabolic fate of Se injected into bloodstream in the form of selenite. Se-containing compounds originating from exogenous and endogenous Se are represented in rectangular and oval shapes, respectively. Se injected in a form of selenite is incorporated into RBCs, reduced to selenide or its equivalent, and then effluxed to plasma in the presence of albumin and sequestered selectively by albumin. Selenide bound to albumin is transferred to the liver and is then incorporated into selenoproteins such as selenoprotein P (Sel P) and extracellular glutathione peroxidase (eGPx) through selenocysteine (SeCys). The two selenoproteins are excreted into plasma, Sel P being the major one and disappearing from plasma faster.

diagram of the proposed metabolism of selenite in the bloodstream is shown in Fig. 6.

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References

[1] H.E. Ganther, Biochemistry, 10 (1971) 4089–4098.
[2] H.S. Hsieh and H.E. Ganther, Biochemistry, 14 (1975) 1632–1636.
[3] N. Esaki, T. Nakamura, H. Tanaka and K. Soda, J. Biol. Chem., 257 (1982) 4386–4391.
[4] K.T. Suzuki, Anal. Biochem., 102 (1980) 31–34.
[5] C.D. Klaassen and L.D. Lehman-McKeeman, Methods Enzymol., 205 (1991) 190–197.
[6] H. Sunaga, E. Kobayashi, N. Shimojo and K.T. Suzuki, Anal. Biochem., 160 (1987) 160–168.
[7] K.T. Suzuki, Methods Enzymol., 205 (1991) 198–205.
[8] L. Stuhne-Sekalec, S.X. Xu, J.G. Parkes, N.F. Olivieri and D.M. Templeton, Anal. Biochem., 205 (1992) 278–284.
[9] K.T. Suzuki, M. Itoh and M. Ohmichi, J. Chromatogr. B, 666 (1995) 13–19.
[10] K.T. Suzuki, M. Itoh and M. Ohmichi, Toxicology, 103 (1995) 157–165.
[11] K.T. Suzuki, S. Yoneda, M. Itoh and M. Ohmichi, J. Chromatogr. B, 670 (1995) 63–71.
[12] K.T. Suzuki and A. Karasawa, Arch. Biochem. Biophys., 278 (1990) 120–124.
[13] T.A. Gasiewicz and J.C. Smith, Chem.-Biol. Interact., 21 (1978) 299–313.
[14] J.T. Deagen, J.A. Butler, B.A. Zachara and P.D. Whanger, Anal. Biochem., 208 (1993) 176–181.
[15] R.F. Burk, K.E. Hill, R. Read and T. Bellew, Am. J. Physiol., 261 (1991) E26–E30.
[16] R.F. Burk, K.E. Hill, J.A. Awad, J.D. Morrow, T. Kato, K.A. Cockell and P.R. Lyons, Hepatology, 21 (1995) 561–569.
[17] M. Sandholm, Acta Pharmacol. Toxicol., 33 (1973) 1–5.
[18] R.F. Burk and K.E. Hill, Annu. Rev. Nutr., 13 (1993) 65–81.
[19] Z. Veres, I.Y. Kim, T.D. Scholz and T.C. Stadtman, J. Biol. Chem., 269 (1994) 10597–10603.