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REVIEW

The Identification of Selenium Species in Biological Samples

John S. Edmonds[1]* and Masatoshi Morita[2]
[1]Department of Chemistry, De Montfort University, The Gateway, Leicester, LE1 9BH, UK
[2]National Institute for Environmental Studies, 16-2 Onogawa, Tsukuba, Ibaraki 305-0053, Japan

Selenium is an essential component of glutathione peroxidase and other enzyme systems and is therefore essential for animal metabolism. It is, however, toxic at concentrations little above those required for health. Some plants accumulate selenium from seleniferous soils and constitute a toxic hazard to grazing stock. The more complex organic selenium compounds that occur naturally in plants, animals and microorganisms have been characterized following their isolation or partial isolation, and have often been identified by their similarity to analogous sulphur compounds. The occurrence and identification of selenium compounds in biological samples and methods for their analysis are reviewed.

Keywords: selenium species; biological samples; analysis; essentiality; toxicity

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INTRODUCTION

Selenium was discovered in 1817 by Berzelius and Gahn, who observed the element as a deposit following the oxidation of sulphur dioxide from copper pyrites.[1] It ranks seventieth in abundance among the elements and constitutes approximately 10^{-5}% of the Earth's crust.[2] It is widely distributed in the environment and is mainly found in metal sulphide deposits, mostly of the metals copper, zinc, silver, mercury and lead. Although some selenium minerals, for example Tiemannite and Naumannite, contain up to 24% Se, the element is usually present at very low concentrations compared to sulphur.

In the Periodic Table selenium (atomic number 34, atomic weight 78.96) is located between sulphur and tellurium in Group 16. Six major stable isotopes have been reported and the most abundant in Nature are ^{80}Se (49.6%) and ^{78}Se (23.8%).[3]

Selenium production is usually as a by-product of the processing of other elements, in particular copper, zinc, nickel and silver. In the electrical and electronics industries high purity is essential for its usefulness. It is also used in the chemical, rubber, ceramic and glass industries. The industrial requirement for selenium results in pollution problems because a large part of the total consumption is lost to the environment. Emissions also occur from the burning of fossil fuels, with up to 90% of the selenium present being volatilized.

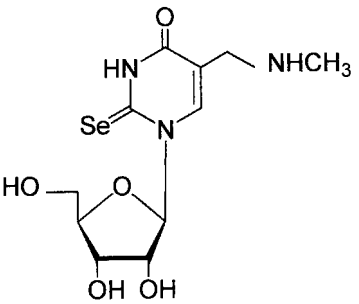
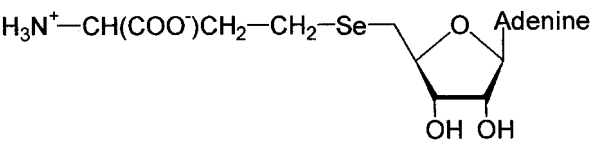
Selenium has a number of oxidation levels and valency states and an extensive organic chemistry in which it forms compounds analogous to those of its congener, sulphur. Both C–Se and Se–Se bonds are, however, less strong than those of their sulphur counterparts. Selenium thus exists in the environment in a number of different chemical species both inorganic and organic, many of which have sulphur-containing analogues see (Table 1).

Selenium has been shown to be essential for animal metabolism and to be toxic at levels little above those required for health. Stock animal diseases caused by both deficiency and excess of selenium are known.[4] The essentiality of selenium results from its presence as a necessary component of glutathione peroxidase and other enzyme systems.[5,6] Selenium has also attracted attention because of its apparent ability, usually when administered as inorganic salts, to ameliorate the toxic effects of heavy metals such as mercury and cadmium.[7,8]

The average crustal abundance of selenium is 0.09 $\mu g\ g^{-1}$;[9,10] in coals concentrations range from 0.47 to 8.1 $\mu g\ g^{-1}$, and in fuel oil from 2.4 to

* Correspondence to: John S. Edmonds, Department of Chemistry, De Montfort University, The Gateway, Leicester LE1 9BH, UK.

Table 1 Selenium species encountered in biological samples

Selenite	SeO_3^-
Selenate	SeO_4^-
Selenocysteine	H_3N^+—CH(COO^-)—CH_2—SeH
Selenocystine	H_3N^+—CH(COO^-)—CH_2—Se—Se—CH_2—CH(COO^-)—NH_3^+
Selenomethionine	H_3N^+—CH(COO^-)—CH_2—CH_2—Se—Me
Dimethyl selenide	Me_2Se
Trimethylselenonium ion	Me_3Se^+
Se-Methylselenocysteine	H_3N^+—CH(COO^-)—CH_2—Se—Me
γ-Glutamyl-*Se*-methylselenocysteine	H_3N^+—CH_2—CH_2—CO—NH—CH(COO^-)—CH_2— Se—Me
Selenocystathionine	H_3N^+—CH(COO^-)—CH_2—CH_2—Se—CH_2—CH(COO^-)—NH_3^+
Selenohomocysteine	H_3N^+—CH(COO^-)—CH_2—CH_2—SeH
Selenocystamine	H_2N—CH_2—CH_2—Se—Se—CH_2—CH_2—NH_2
5-[(Methylamino)methyl]-2-selenouridine	
Se-Adenosylhomocysteine	

7.5 μg g^{-1}.[11] Selenium concentrations in soils can vary widely and values as high as 1200 μg g^{-1} have been reported in seleniferous Irish soils.[12] Levels of 0.02–2.5 μg g^{-1} would appear to be more usual.[13] Robberecht and van Grieken[14] have extensively reviewed the occurrence of selenium in natural waters and report, from a variety of references, levels in river water of 0.016–20 ng l^{-1} (higher levels have been recorded but appear abnormal), in lake water of < 0.1–1.85 ng l^{-1}, and in open ocean seawater of about 0.025–0.2 ng l^{-1}, although higher levels have been recorded in coastal or harbour waters. Selenium concentrations in plants vary enormously. Generally the levels of selenium in grasses and vegetables are only a few micrograms per gram,[12] and growing in non-seleniferous soils throughout the world the selenium content of plants can be as low as 0.01–0.02 μg g^{-1}.[12] On the other hand, plants growing in seleniferous soils can sometimes accumulate surprisingly high concentrations of selenium. Levels of 4–5 μg g^{-1} in forage and crop plants are toxic to stock animals, but apparently the highest level recorded is 18 200 μg g^{-1} selenium in the seeds of the South American nut-bearing tree *Lecythis ollaria*.[12] The concentration of selenium in the edible muscle tissue of farm animals (cattle, pigs, lambs, chickens) appears to be around 0.2 μg g^{-1}, with levels in the liver being rather higher at about 0.5 μg g^{-1}.[15,16] Marine animals have rather higher levels of selenium than do terrestrial animals; concentrations of up to 1 μg g^{-1} in fish muscle appear normal.[17,18] Levels in marine mammals may be much higher than this, with a strong correlation between the accumulated concentrations of selenium and those of mercury.[19] Concentrations in human organs are usually in the range of 0.1–1.0 μg g^{-1}.[20]

2 SELENIUM SPECIES IN BIOTA

Generally the more complex organic selenium compounds that occur naturally in bacteria, plants and animals have been identified following their isolation or partial isolation and have often been characterized, at least in the first instance, by the similarity in their properties to those of analogous sulphur compounds. Sulphur analogues (virtually all naturally occurring selenium compounds are counterparts of well-known and characterized sulphur compounds) have served as makeshift or provisional standards that have allowed a preliminary, usually correct, identification and have provided guidance for the synthesis of the required selenium-containing standards. It is unlikely that analytical methods involving the isolation of compounds of interest will find routine application. Nevertheless where unknown compounds are concerned it is usually necessary to establish the unequivocal identity, which can only be facilitated by their complete isolation. This is the classical approach adopted in natural products chemistry. Standard materials are thereby made available or can become so by synthesis. Routine methods of analysis then become possible.

Although unknown organic selenium compounds have been reported in seawater,[21] it is to selenium compounds in biota rather than other components of the environment that this particularly applies.

Trace methods for the determination of specific selenium species in biological materials, and combination of chromagraphic separation with specific or selective detection methods, have been applied to compounds that are considered important from physiological, nutritional or toxicological aspects. Such compounds include selenoamino acids and simple methylated selenium compounds. Analytical methods for these compounds that can be used on a routine basis are important for the elucidation of biochemical roles and metabolic pathways. The development of analytical methodology is difficult because of the inherent lability of the compounds and problems of achieving the necessary sensitivity of detection.

2.1 Plants

During the 1930s it became apparent that plants that were long suspected of causing certain livestock disorders in western regions of the USA contained selenium.[12] Extensive surveys revealed the relationships between selenium-containing plants, selenium-absorbing capacity, geographical location and geological associations. Certain species were found to be reliable indicators of soil types (seleniferous soils) from which selenium could be accumulated. These plants were designated as primary indicators of seleniferous soils. Some plants accumulate selenium from seleniferous soils, often to toxic levels, but are also found in non-seleniferous regions. Intermittent work through several decades has been directed towards identifying the chemical species of selenium accumulated in such plants. The early recognition that selenium compounds existed in these plants as analogues for well-known and characterized sulphur compounds, particularly amino acids and simple peptides, greatly aided their identification. In several cases identification followed the demonstration that a selenium-containing material had the same chromatographic coordinates as a known sulphur compound.

In 1941, Horn and Jones[22] isolated from the tine-leafed milk-vetch *Astragalus pectinatus* (a selenium-accumulating legume from the central plains and grasslands area of USA), by a series of extractions and precipitations, a crystalline substance containing sulphur and selenium, which was determined by analysis to be a complex of two parts of selenocystathionine and one part of cystathionine. Tentative identification was based largely on elemental analysis and the general amino-acid-like properties of the compounds. In 1960 Trelease *et al.*[23] isolated *Se*-methylselenocysteine from *A. bisulcatus* in almost pure form by ion-exchange and cellulose chromatography. The IR spectrum of the isolated material suggested contamination with *S*-methylcysteine but was very close to that of synthetic *Se*-methylselenocysteine. The R_f values on paper chromatograms (two systems) were identical for synthetic *S*-methylcysteine, for synthetic *Se*-methylselenocysteine, and for the selenium compound isolated from *A. bisulcatus*. Other studies demonstrated the conversion of radio-labelled selenite into *Se*-methylselenocysteine[24] and into a range of selenium-containing amino acids and related compounds, some of which were not identified.[25] Virupaksha and Shrift[26] also demonstrated the biosynthesis of selenocystathionine from a radio-labelled selenate by the prince's plume, *Stanleya pinnata* (Brassicaeae). In all three studies identification, when achieved, was obtained, after preliminary fractionation of extracts by ion-exchange chromatography, by paper chromatography and paper electrophoresis against standards (some of which were the sulphur analogues of the compounds being identified). Radioactive selenium

greatly simplified the location of the selenium-containing compounds on the chromatograms. Peterson and Butler reported their paper and electrophoretic systems for the identification of selenium-containing amino acids as a separate publication.[27] Ion-exchange chromatography, with or without subsequent paper chromatography, was used to investigate the free selenium-containing amino acids in onion after the plant was allowed to absorb radio-labelled selenite.[28] Ion-exchange chromatography (Dowex 50), paper chromatography and paper electrophoresis were used to identify *Se*-methylselenocysteine, selenocystathionine and *Se*-methylselenomethionine in the excised leaves of plant species after they had been exposed to radio-labelled selenite and selenate, and to radio-labelled selenomethionine and methylselenomethionine.[29–31] The distribution of the compounds among the plant species examined reflected their classification as accumulators or non-accumulators of selenium.

A classical natural products chemical approach was taken by Kerdel-Vegas *et al.*,[32] who used dialysis and ion-exchange chromatography to isolate the pharmacologically active compound in the seeds of the South American nut tree *Lecythis ollaria*, and showed it to be selenocystathionine by ^{1}H NMR and IR spectroscopy, elemental analysis, paper chromatography and identification of the products of Raney nickel hydrogenolysis. Peterson and Butler[33] used high-voltage electrophoresis, paper chromatography and identification of the product of hydrogenolysis (by paper chromatography) to identify selenocystathione and indicate an unknown selenium compound in the selenium-accumulating plant *Neptunia amplexicaulis* after seedlings had been allowed to absorb labelled selenite. They also allowed the duckweed *Spirodela oligorrhiza* to absorb labelled selenite, selenate or colloidal selenium and used electrophoresis and paper chromatography to identify chiefly selenomethionine which was released from proteins by enzymic hydrolysis.[34] Thin-layer and paper chromatographies were used to identify selenocysteine and selenomethionine in extracts and enzyme digests of pasture grass that had been exposed to radio-labelled selenite.[35] Demonstration of the presence of dimethyl diselenide in volatile emissions from drying *Astragalus racemosus* was accomplished by the use of gas chromatography (GC).[36] Compounds were collected on activated charcoal and washed off either with water or diethyl ether. The water fractions were examined by ion-exchange chromatography but the two selenium compounds therein could not be identified. Presumably the volatile selenium compounds accounting for the unpleasant odour of *Astragalus* arise from decomposition of selenium-containing amino acids and peptides.

A commercial amino-acid analyser, employing buffered ion-exchange procedures of the type developed by Spackman *et al.*,[37] has been used for the analysis of mixtures containing selenoamino acids.[38,39] The technique successfully separated selenocysteine, selenomethionine, selenocystathionine, *Se*-methylselenocysteine and selenohomocysteine from their sulphur analogues and from other amino acids in a mixture, and was used to characterize the selenoamino acids present in the seeds of certain selenium-accumulating plants. Buffered ion-exchange chromatography on Dowex 1 was also employed to isolate γ-glutamyl-*Se*-methylselenocysteine and *Se*-methylselenocysteine from the seeds and leaves, respectively, of *Astragalus bisulcatus*.[40] The structure of the former was deduced by identification of the products of hydrolysis and by derivatization, and of the latter by comparison with synthetic material. The distribution of selenomethylselenocysteine and other selenoamino acids in species of *Astragalus* was determined using a commercial amino acid analyser, the techniques of Spackman *et al.*[37] and paper electrophoresis.[41]

Ion-exchange chromatography on Dowex 1 followed by paper chromatography was used to purify *Se*-methylselenocysteine from tissues of *Astragalus bisulcatus* that had been subjected to sodium selenate and radio-labelled precursor amino acids as part of an investigation into the biosynthesis of the selenoamino acid.[42] L-Selenocystathionine was isolated from the foliage of *Astragalus pectinatus* by ion-exchange chromatography on Dowex resins.[43] The selenium compound was located in plant material by use of a commercial amino acid analyser and by paper chromatography. Proof of the structure of the isolated material was provided by comparison with synthetic material and by identifying the products of Raney nickel hydrogenolysis. High-voltage paper electrophoresis and paper chromatography were employed to demonstrate the presence of selenocystathionine in extracts of the seleniferous plant *Morinda reticulata*.[44] Confirmation of the presence of selenocystathionine was obtained by subjecting the products of Raney nickel hydrogenolysis to the same electrophoretic and chromatographic techniques. Olson *et al.*[45] employed ion-exchange chromatography (again a commercial amino acid analyser was used) to study selenoamino acids in

pronase hydrolysates of wheat (a non-accumulator plant) protein from plants grown in soil to which [^{75}Se]selenate had been added. Almost half of the selenium in the hydrolysate was accounted for as selenomethionine, and selenocysteine was not detected. The major difference between accumulator and non-accumulator plants appears to be that in the former the selenoamino acids remain largely in the free state, but in the latter they are rapidly and virtually completely incorporated into proteins (hence the use of the hydrolytic enzymes in the study of Olson *et al.*[45] and in the earlier study of Peterson and Butler[25]). The ability to prevent selenoamino acids from being incorporated into proteins may be the reason why selenium-accumulating plants can tolerate seleniferous soils and accumulate considerable quantities of selenium without suffering toxic damage.

Methods used for the determination of selenomethionine have usually relied upon amino-acid analyser columns for the separation of selenomethionine and ^{75}Se radioactivity for its detection. Anaerobic hydrolysis[46,47] or enzymic digestion[48] have been used to obtain selenomethionine as the free amino acid. Derivatization of the selenium has also been employed.[49,50] Selenomethionine is more stable than selenocysteine but not as stable as methionine during acid hydrolysis. HCl (6 M) at 110 ° C for 7 h decomposes selenomethionine but 96% of methionine is unchanged.[51] The loss of selenomethionine can be prevented to some extent, the recovery being improved to 60–70%, by the addition of a reducing agent such as mercaptoethanol or mercaptoethanesulfonic acid.[52]

More recently selenomethionine has been identified in proteolytic enzyme digests of soybean protein by GC–MS after derivatization of the compound following its purification by gel-permeation and thin-layer chromatography.[53] The authors claimed that this study represented the first identification of a selenoamino acid in a plant material when selenium was present at what might be called 'normal' levels (i.e. levels which had not been artificially raised for the purpose of the study).

An indirect method for the determination of selenomethionine in plants and animals has been reported.[54] Material was reacted with cyanogen bromide in the presence of stannous chloride and any protein-bound selenomethionine was converted to CH_3SeCN and was extractable with chloroform. Acid digestion of the extract to form Se(IV) and reaction with 4-nitro-*o*-phenylenediamine reagent gave 5-nitropiazselenol, which was determined by GC with electron capture detection. The authors claimed that losses of selenomethione encountered during the hydrolysis which precedes more direct methods of analysis were avoided by this method. However, theoretically all compounds with a CH_3SeR group can react with CNBr to form RSeCN and thus *Se*-methylselenocysteine could be a major interference in certain circumstances. The detection limit of this method is 6 μg selenomethionine/g of sample, and the recovery is 94.4% for wheat and 84.3% for rice. The precision, estimated as the relative standard deviation for the crop samples, was 12.6–20.0%. The combination of this detection method with ion-exchange HPLC has been examined and a calibration curve obtained over a dynamic range of 30–500 nM selenomethionine.[55]

In recent years mixtures of amino acids have been analysed by reversed-phase liquid chromatography.[56] Presumably this technique will also encompass any selenoamino acids or small peptides that might be included in extracts or digests. For the determination of natural selenium amino acids a sensitive element-specific detector such as ICP–MS in combination with separation by HPLC or amino-acid analyser may ultimately be the method of choice. At present the applications of such techniques have been limited to water and other environmental samples rather than to biological materials.

2.2 Microorganisms

The incorporation of selenoamino acids into proteins in plant materials usually involves the non-specific incorporation of selenomethione. Thus it is usually adequate to identify and estimate the selenomethionine in hydrolysates, and determination of the secondary or tertiary structures of the proteins does not necessarily reveal additional information on selenium speciation. This non-specific incorporation of selenomethionine into proteins also occurs in animals and microorganisms.[57] For example, Hartmanis and Stadtman[58] isolated a selenium-containing enzyme (thiolase) from *Clostridium kluyveri* that was markedly radiolabelled when the bacterium was grown in media containing 1 μM selenite with tracer amounts of [^{75}Se]selenite. The selenium in acid hydrolysates of the radioactive protein was shown to be in the form of selenomethione by co-chromatography with authentic selenomethionine on an amino-acid analyser and on TLC plates in acidic and basic solvents. Also, incubation with *S*-adenosylmethionine synthetase and ATP converted the ^{75}Se-

labelled amino acid to a radioactive basic product indistinguishable from authentic *Se*-adenosylselenomethionine by ion-exchange and thin-layer chromatography. Comparison of peptide maps of those enzymes labelled with ^{35}S or ^{75}Se showed that selenomethione residues were distributed randomly throughout the polypeptide subunits in a pattern resembling the occurrence of sulphur.[59] The non-specific replacement of methionine by small quantities of selenomethionine had no observable effect on the activity of the enzyme.[57]

On the other hand, the highly specific occurrence of selenocysteine in several selenium-dependent enzymes from diverse biological sources, including microorganisms, is well established.[57] Clearly, in such cases, a full definition of the selenium species involved requires elucidation of not only the position of the selenocysteine residue in the peptide chain (secondary structure), but also its location and orientation in the tertiary folded structure of the protein. This is particularly so as the selenocysteine residues are usually directly associated with enzymic function. The identification of selenocysteine as the organoselenium moiety of an enzyme was first achieved by Cone *et al.*,[60] who found it to be present in the selenoprotein component of the clostridial glycine reductase complex. Selenocysteine was isolated as its *Se*-carboxymethyl, *Se*-carboxyethyl, and *Se*-aminoethyl derivatives from trypsin digests of the pure ^{75}Se-labelled protein that had been reduced and treated with the various alkylating agents prior to hydrolysis. The alkylated derivatives of selenocysteine, isolated from the digests, were indistinguishable from the corresponding alkylated derivatives of authentic selenocysteine when chromatographed on a commercial amino-acid analyser and examined by cellulose TLC. Cone *et al.*[60] stress the care necessary (anaerobic conditions etc.) to obtain useful results from this type of study because of the labile nature of the selenium compounds. This careful approach to experimentation has also been advocated by Whanger and Beilstein,[61] and presumably failure to maintain anaerobic conditions resulted in the range of apparent oxidation products that were revealed in the study of Peterson and Butler[25] on digests of selenium-containing proteins of some higher plants. The study of Olson *et al.*[45] on seleniferous wheat would indicate that Peterson and Butler[25] were probably dealing largely, if not solely, with selenocysteine.

A selenium-containing membrane-bound formate dehydrogenase complex from *E. coli* that had been grown under anaerobic conditions was purified and shown to consist of three polypeptides of 110 000, 32 000 and 20 000 Da.[62] Only the polypeptide of 110 000 Da contained significant quantities of selenium. The authors demonstrated that the selenium was likely to have been present covalently bound to carbon. Jones *et al.*[63] reported that the selenium-dependent formate dehydrogenase of *Methanococcus vannielii* contained selenium in the form of selenocysteine. The bacteria were cultured in the presence of [^{75}Se]selenite and the enzyme was purified under strictly anaerobic conditions. It was reduced with borohydride, alkylated with iodoacetamide and subjected to acid hydrolysis which released the radioactive selenoamino acid derivative. Comparison with authentic material by automatic amino-acid analyser[37] and by cellulose TLC identified the selenium compound present in the enzyme as selenocysteine. The authors ensured that no oxidation products (selenones or selenoxides) were detected, by reduction of the derivatives just prior to analysis. Similar techniques (reduction, alkylation, hydrolysis, chromatography by automatic amino-acid analyser) were used to show that selenocysteine was the form of selenium in a selenium-containing hydrogenase from the same organism (*M. vannielii*).[64] However, at least two other bacterial selenoenzymes are known in which selenium is not present as selenocysteine but as an unidentified readily dissociable component. These are nicotinic acid hydroxylase[65] and xanthine dehydrogenase.[66] The nicotinic acid hydroxylase from *Clostridium barkeri* proved extremely sensitive to alkylating agents and was immediately inactivated upon addition of an alkylating agent to a mixture of the enzyme, its cofactors and substrate. When enzyme labelled with ^{75}Se was inactivated by alkylation, all of the ^{75}Se was found in a compound that was easily separated from the protein and had the proportions of a dialkyl selenide.[65,67] No selenocysteine or selenomethionine was detected in the nicotinic acid hydroxylase.

Selenium also occurs in the transfer RNAs (tRNAs) of certain bacteria. In the early 1970s it was reported[68,69] that selenium can be incorporated into the tRNAs of *E. coli*. It was assumed that this represented non-specific substitution of selenium for sulphur in certain modified nucleosides.[70,71] However it was subsequently shown, both for *E. coli* and for some anaerobic bacteria, that selenium occurs as a specific component of a few tRNA species.[57,72] The strong affinity between mercury and selenium enabled Ching and Stadtman[73] to employ organomercury affinity chromatography

procedures to concentrate the selenium-containing tRNA species present in total tRNA populations of *M. vannielii*.[74] Purification of individual seleno-tRNA species was achieved by reversed-phase liquid chromatography. Enzymic digestion of the total ^{75}Se-labelled tRNA population from *E. coli* and HPLC analysis of the resulting nucleoside mixture showed the presence of a single radioactive nucleoside.[75]

The elution position (HPLC) and electronic absorption spectrum of this compound suggested that it might be the selenium analogue of 5-methylaminomethyl-2-thiouridine (already known to be present in *E. coli*). Subsequent synthesis of authentic 5-methylaminomethyl-2-selenouridine and comparison (chromatographic and spectral) with the labelled nucleoside showed that they had indistinguishable properties.[75] This is a further example of similarities between novel selenium and known sulphur analogues leading to the chemical synthesis of the correct selenium-containing standard.

2.3 Animals

The normal presence of selenium at concentrations of about 0.1–1 $\mu g\ g^{-1}$ in a range of human tissues,[20] and the demonstration by Schwarz and Foltz[76] that selenium was essential for the prevention of liver necrosis in rats, led to the identification of selenium as an essential component of glutathione peroxidase.[5,6] This enzyme, found in mammalian and avian blood, protects living cells from oxidative damage by catalysing the decomposition of various harmful peroxides.[77] Flohe *et al.*[6] isolated glutathione peroxidase from bovine blood, obtained it in a crystalline state, and demonstrated that it contained four atoms of selenium per molecule. This value was calculated assuming a molecular weight of 84 000 Da for the native enzyme. Several attempts to determine the chemical species of selenium contained in animal tissues have produced misleading or incomplete results,[78–80] and studies focusing more specifically on glutathione peroxidase have met with a similar fate.[81–83] Forstrom *et al.*[84] were the first to show that selenium in glutathione peroxidase (in this case isolated from rat liver) was present as selenocysteine. They employed techniques similar to those used by Cone *et al.*[60] to demonstrate the presence of selenocysteine in the clostridial glycine reductase complex. Glutathione peroxidase was partially purified (30–50%) from rat livers three days after the rats had been subcutaneously injected with [^{75}Se]selenite. The selenium moiety in the enzyme was identified as selenocysteine by derivatizing the seleno group in the intact protein with either iodoacetate or ethylenemine, hydrolysing the protein with 6 M HCl and co-chromatographing the ^{75} Se-labelled products with authentic standards (anion- and cation-exchange chromatography, gel-permeation chromatography, two-dimensional thin-layer chromatography, and automated amino-acid analysis). Forstrom *et al.*[84] were unsuccessful in their attempts to obtain a mass spectrum of the selenium-containing compound from the enzyme (derivatized selenocysteine) although they obtained mass spectra of *N*-acetyl-*O*-methyl derivatives of the Se-alkylated selenocysteine standards. Cone *et al.*[60] had experienced similar difficulties in their attempts to obtain mass spectra and noted the susceptibility of the selenoether derivatives to oxidation. However Kraus *et al.*[85] were able to obtain satisfactory mass spectra by employing a new derivatization technique based on reaction of the selenocysteine in glutathione peroxidase with Sanger's reagent (1-fluoro-2,4-dinitrobenzene) to form *Se*-(2,4-dinitrophenyl)selenocysteine (*Se*-DNP-selenocysteine). The presence of the DNP moiety made the selenoamino acid easy to isolate from acid hydrolysates and facilitated detection during chromatography (gel-filtration and reversed-phase liquid chromatography). The *Se*-DNP-selenocysteine from the enzyme was identified by mass spectrometry following transformation to the *Se*-methyl-*N*-DNP-selenocysteine methyl ester. This study represented the first identification of a selenoamino acid in a protein by mass spectrometry and, as such, offered a more rigorous proof of identity than provided by co-chromatographic studies.[60,84]

HPLC methods for the estimation of selenocysteine require a selective colorization reaction since it is always a minor amino acid in natural proteins. Selenocysteine reacts with 1-fluoro-2, 4-dinitrobenzene at pH 6 to produce *Se*-DNP-selenocysteine, a yellow derivative ($\lambda_{max} = 335$ nm, $\epsilon_{335nm} = 1.42 \times 10^4\ M^{-1}\ cm^{-1}$). The optical characteristics of *Se*-DNP-selenocysteine are almost the same as those of *S*-DNP-selenocysteine but different from those of most *N*-DNP-amino acids (λ_{max} approximately 360 nm and ϵ_{max} $(1.7–1.8) \times 10^4$ $M^{-1}\ cm^{-1}$).[86] Separation of *Se*-DNP-selenocysteine from DNP derivatives of cysteine, histidine, arginine, lysine and tyrosine can be achieved by reversed-phase HPLC with methanol/ H_2O (30:70) although no data for the application of this method to real samples have yet been reported. There is a

possibility that *Se*-DNP-cysteine will be hidden under the major DNP-amino acid peaks.

The introduction of a fluorescent moiety is another possibility for enhancing analytical sensitivity. Free selenocysteine in plasma or serum was reacted with *N*-(iodoacetylaminoethyl)-5-naphthylamino-1-sulphonic acid (IAEDANS) at pH 6.6, minimizing potential interferences from thiols, to produce derivatives which could be determined by an HPLC system fitted with a fluorescence detector. The detection limit of the method was 0.4 μM.[87] The method was suitable for selenocysteine in plasma or serum but application to other tissues may be limited by high concentrations of glutathione, which might cause chromatographic interference, or by poor recoveries of the selenocysteine–IAEDANS derivatives.

The importance of adequate glutathione peroxidase activity (usually dependent upon levels of available selenium) in mammalian tissues for the maintenance of health has resulted in the development of rapid tests. Such tests are a measure of the quantity of glutathione peroxidase in tissues and are thus, in the strictest sense, estimates of a biologically important selenium species. Tests are based upon measurement of the rate of loss of the fluorescence of NADPH because of its reaction (mediated by glutathione reductase) with oxidized glutathione produced by the reaction of glutathione peroxidase and a synthetic peroxide substrate.[88,89] Both spot tests[89,90] and spectrometric methods[91–93] have been developed. Such techniques, as well as being specific, are often much quicker than determining selenium concentrations.[93]

Molecular biological methods were used to determine the nature of type 1 iodothyronine deiodinase, an enzyme concerned with vertebrate thyroid function, and to show that it contained selenocysteine.[94] A perceived essentiality for selenium in thyroid hormone action led to the recognition of a codon in rat liver nucleic acids known to code for selenocysteine in mammalian and bacterial genes. Enzyme activity was diminished or lost if the relevant nucleic acids were altered to prevent them from specifically coding for selenocysteine in the enzyme.

Several other selenium-containing proteins have been detected in animal tissues.[95–102] Although most seem to contain selenium as selenocysteine, selenomethionine also seems to be involved in some cases.[102] Methods for their estimation will presumably be developed when their structures are elucidated and any functions determined.

Experimental toxicity tests have revealed interactions between selenium and other elements with altered toxicity of both selenium and the element concerned. Elements which have been shown to reduce the toxicity of selenium include arsenic,[103,104] silver,[105] cadmium,[103] copper,[105,106] mercury,[106,107] tungsten,[104] lead,[108] platinum,[108] tin[108] and thallium.[108] Conversely, elements which have had their toxicity reduced by prior or co-administration of selenium include mercury,[109,110] silver,[111] cadmium,[112] lead,[113] platinum,[114,115] tin[116] and thallium.[117] Although such interactions must be based on direct or indirect metabolic transformation of elements concerned, the chemistry is, at best, only partly understood. Attempts have been made to identify mercury–selenium species in dolphin livers. Martoja and Viale[118] considered mercury- and selenium-containing granules therein to consist of mercury(II) selenide, whereas Matsumoto, less specifically, demonstrated the presence of a chemical species of molecular weght less than 1000 and with a selenium/mercury molar ratio of 1:1.[119] Dimethylmercuric selenide was identified in the blood of a mouse that had been administered methylmercury and selenite.[120] The compound was labile and subject to further transformation.

Besides the numerous studies on the form of selenium in animal tissues, there has been a considerable concentration of interest on the form and quantities of selenium in mammalian urine.[121] Selenium species and concentrations in urine are of interest because they may provide information on the selenium status of the body and may offer clues as to essential chemical forms. Byard[122] and Palmer *et al.*[123] independently isolated trimethylselenonium ion from the urine of rats injected with [^{75}Se]selenite. The latter[123] isolated the metabolite by cation-exchange chromatography (AG 50-X8 resin), in the first place by eluting with increasing concentrations of HCl and secondly by buffered ion-exchange chromatography on the same resin employing a pH gradient. Trimethylselenonium ion was finally purified by precipitation as the reineckate salt and was identified by NMR and IR spectroscopy, and co-crystallization and co-chromatography data. Synthetic trimethylselenonium ion served as an authentic standard. Similar methods were used by Byard[122] (ion-exchange chromatography and reineckate precipitation).

Several subsequent studies have involved the administration of radio-labelled selenium compounds to experimental animals, the metabolites being identified by ion-exchange chromatography, paper chromatography and autoradiography.[124–127]

Studies on humans have been made in the same way.[128,129] In all cases trimethylselenonium ion was detectable, but it now seems generally accepted that this ionic species is a major metabolite only when intake of selenium is high.[121,130] Electrothermal atomic absorption spectroscopy (AAS)[131] and molecular neutron activation analysis[132–135] have been used to analyse metabolites of selenium, including trimethylselenonium ion, in human urine following separation by ion-exchange chromatography. Selenite and selenate in human urine have also been analysed by ICP–AES following separation by a resin complexation method.[136] HPLC coupled with ICP–AES by the thermospray system gave enhanced sensitivity for $(CH_3)_3Se^+$, SeO_3^{2-} and SeO_4^{2-} compared with a conventional cross-flow nebulizer. Detection limits based on peak heights ranged from 14 ng for $(CH_3)_3Se^+$ (eluting first) to 54 ng for SeO_4^{2-} (eluting last).[137] An HPLC–thermochemical hydride generation atomic absorption method for the determination of selenocholine and trimethylselenium cations in human urine has been reported.[138] The on-line interface was based on thermospray nebulization of the HPLC methanolic eluent, pyrolysis of the analyte in a methanol–oxygen flame, thermochemical derivatization using excess hydrogen and cool, diffused flame atomization of the product(s) in a quartz cell mounted in the AAS optical beam. The limits of detection were 5 and 7 ng for $(CH_3)_2Se^+CH_2CH_2OH$ and $(CH_3)_3Se^+$, respectively.

ICP–AES was used to measure trimethylselenonium ion in human urine following its precipitation as the reineckate salt, and its subjection to anion-exchange chromatography, thermal decomposition and collection in nitric acid.[139] It is evident that the refractory nature of the trimethylselenonium ion can cause analytical problems, and the use of nitric acid/perchloric acid mixtures to achieve complete conversion of urinary selenium to selenium(IV) has been advocated.[140] The use of HPLC following preconcentration on a cation-exchange column has been used by Shibata *et al.*[121] to examine selenium metabolites in normal human urine. Compounds were separated on a cation-exchange (Nucleosil SA) column using tetramethylammonium chloride (0.2 M) as eluent. Selenium was detected by ICP–AES with the emission line at 196.0 nm being monitored. The detection limit of trimethylselenonium ion was 20 ng Se or 2 μg Se/l of urine, but trimethylselenonium ion was not detected in all the samples examined. An unknown selenium compound was observed which, because of the strong acid used to wash the compounds from the preconcentration column, must have had a similar polarity to that of trimethylselenonium ion.

Shibata *et al.*[121] also used ICP–MS as an element-specific detector for selenium metabolites in human urine following HPLC separation. The low ionization efficiency in the plasma and the low abundance of Se^{82} mean that ICP–MS is not extremely sensitive for selenium detection. Nevertheless the sensitivity of this technique is adequate to enable selenium compounds in urine to be analysed without preconcentration. Capillary zone electrophoresis (CZE) with ICP–MS detection has been used for the identification and determination of selenomethionine and methionine and of selenocystamine and cystamine in milk.[141,142] Recently pneumatically-assisted electrospray tandem MS was used to identify *Se*-adenosylhomocysteine in an extract of selenized yeast.[143] Confirmation of the fragmentation pattern was obtained using the sulphur analogue. The use of LC/MS and LC/MS/MS will be the subject of further study because constant and stable ionization is required for such purposes.

2.4 Determination of volatile selenium compounds

Volatile selenium compounds produced by biomethylation include dimethyl selenide, dimethyl diselenide and dimethylselenone. Mammals, including humans, exposed to a gross excess of selenium develop breath with a smell of garlic[144] which is presumably owing to the exhalation of dimethyl selenide.[145] Volatile selenium compounds have been determined by GC with element-specific detectors. Chau *et al.*[146] used silica furnace AAS as the detection method for GC and demonstrated a lower detection limit of 0.1 ng Se or lower for dimethyl selenide and dimethyl diselenide. The precision of the method was evaluated by running replicate analyses of synthetic air samples spiked with dimethyl selenide and dimethyl diselenide containing 10 and 16 ng Se respectively. The relative standard deviation at these levels waas 80% and 7% respectively. Similarly, use of a gas chromatographic column packed with 20% polymetaphenyl ether on 60% Chromosorb W has been reported.[147] Detection was by AAS with a hot quartz atomizer. Jiang *et al.*[148] modified the method by using graphite furnace AAS for the determination of dimethyl selenide, dimethyl diselenide and dimethylselenone. Specificity was excellent and detection limits with a cryogenic sampling procedure were as low as 0.2 $\mu g\ m^{-3}$ in air. It was noted

Table 2 Certified reference materials that provide a value for total selenium concentration

Supplier	Code no.	Material	Se concentration ($\mu g\ g^{-1}$)
NIST	SRM 1577a	Bovine liver	0.71
	SRM 1566a	Oyster tissue	2.1
	SRM 1567a	Wheat flour	1.1
	SRM 1568a	Rice flour	0.38
	SRM 1515	Apple leaves	0.050
	SRM 1547	Peach leaves	0.120
	SRM 2670	Freeze-dried urine	0.030/0.46
	SRM 1548	Total diet	0.25
	SRM 1549	Non-fat milk powder	0.11
IAEA	MA-B-3/TM	Fish	1.46
	MA-A-10/TM	Copepod homogenate	3.0
	MA-A-2/TM	Fish flesh homogenate	1.7
BCR	CRM 278	Mussel tissue	1.66
	CRM 279	Sea lettuce	0.593
	CRM 281	Rye grass	0.028
NRC	Dolt-1	Dogfish liver	7.34
	Dolm-1	Dogfish muscle	1.62
	LUTS-1	Non-defatted lobster hepatopancreas	0.641
NIES	No. 6	Mussel tissue	1.58
	No. 9	Sargasso	0.05

that the similarity in retention times of diethyl selenide and dimethylselenone made it difficult to distinguish these compounds. In general, the use of quartz capillary columns has advantages over packed columns because of the low adsorption on to the surface of the packing materials. Microwave atomic emission detection has also been employed.[149] The method was specific and was capable of detecting as little as 20 pg of dimethyl selenide.

The analytical study of volatile selenium compounds has been largely restricted to air and water samples, and has not been much directed at biological samples such as body fluids and tissues. The analysis of these materials may well be important to the evaluation of metabolic transformations of the element.

3 CERTIFIED REFERENCE MATERIALS

Certified reference materials derived from biological samples that give values for total selenium concentrations are available from various suppliers (Table 2). No information is available on the chemical forms of selenium in these materials. The stability of the selenium species in these samples is also unknown.

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