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Chemical Transformations of Selenium in Living Organisms. Improved Forms of Selenium for Cancer Prevention

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Abstract: Compounds having cancer-preventing activity are developed during the metabolism of selenium in plants and animals. Monomethylated forms of selenium appear to be one class of chemopreventive metabolites. Synthetic organoselenium compounds have been used to explore determinants of activity and differentiation from other biological effects of selenium. Triphenyl selenonium chloride, a new type of chemopreventive selenium compound, has been synthesized in radioactive form for use as a tracer to facilitate studies of its mode of action.

INTRODUCTION

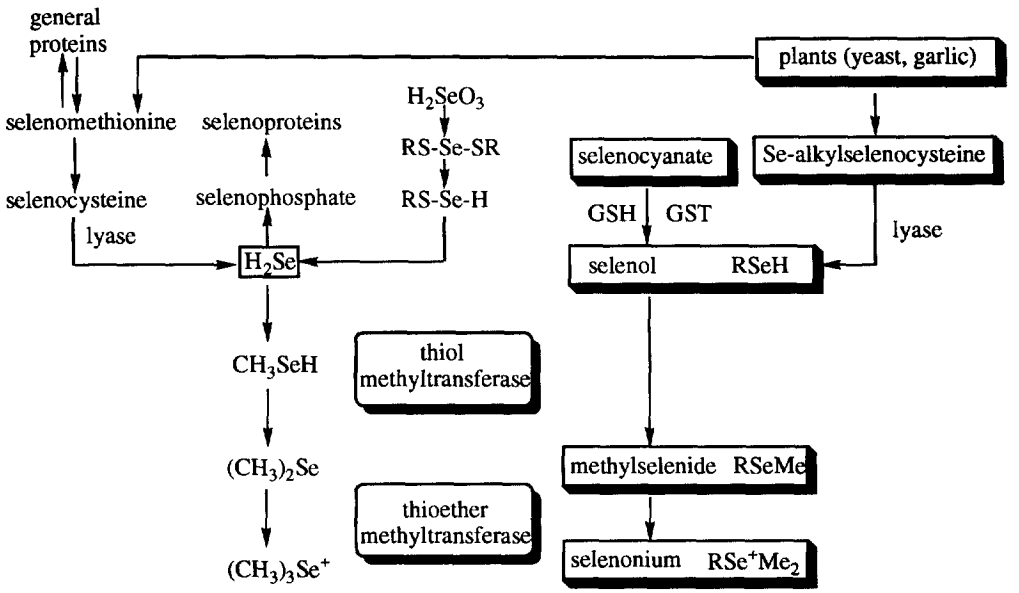
Selenium has been shown to prevent cancer in studies with experimental animals[1] and with humans[2]. The objectives of this article are to provide a perspective on the chemical fate of selenium in living organisms, and the origins of chemopreventive compounds when selenium is metabolized in plants and animals.

In animals, the activities of selenium as an essential nutrient, cancer-preventing agent, and toxicant, are developed as the dietary selenium level is increased over an approximately 100-fold range. As a point of reference, the nutritional requirement for selenium in animals is comparable to that for iodine, and the toxicity of inorganic sodium selenite is comparable to that of sodium arsenite. The nutritional requirement for selenium, like iodine, can be met by providing simple inorganic salts, and both selenium and iodine are metabolized in animals to their active organic forms.

Following the discovery in 1957 that selenium was an essential trace element for animals, considerable effort was made by Schwarz and others to isolate and identify a low molecular weight form of selenium ("Factor 3") that would be the putative active form[3]. Hundreds of organoselenium compounds were synthesized and fed to animals for asssay of biological activity in the prevention of selenium deficiency. These studies constitute a rich source of information on the relative bioavailability of selenium in different chemical forms, reflecting the ease with which selenium can be released from diverse chemical structures. However, this approach failed to identify any selenium compound that was more than a few-fold more active than inorganic selenium salts. Beginning with the discovery that selenium was an essential component of glutathione peroxidase[4], all the known functions of selenium as an essential nutrient in animals and certain microorganisms have been associated with selenoproteins. Usually these selenoproteins contain selenocysteine at the active site of an enzyme. There are elaborate mechanisms to ensure the specific incorporation of selenium into selenoproteins; assimilatory

activation of inorganic selenide to a selenophosphate[5] is followed by transfer of the selenium to a three-carbon intermediate at the level of transfer RNA to form selenocysteine[6].

Two kinds of evidence suggest that selenium's anticarcinogenic action may not involve its usual roles as an essential nutrient: (1) Se-dependent enzyme activities are already at a maximum at levels of selenium below its effective anticarcinogenic level; (2) forms of selenium that lack nutritional activity (unavailable for synthesis of Se-dependent enzymes) show good cancer preventing activity. If low molecular weight forms of selenium are involved in its anticarcinogenic activity, what are the forms and how are they produced? Scheme 1 summarizes known pathways of selenium metabolism that are discussed in regard to origins of chemopreventive activity.

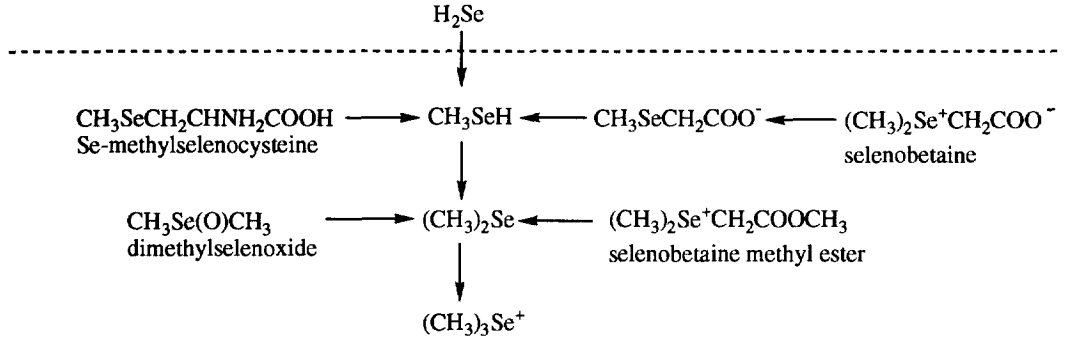


Scheme 1. Selenium metabolism, emphasizing reactions for generating possible chemopreventive metabolites.

Biosynthesis of methylated selenium compounds. Methylation is an important pathway of selenium metabolism. Methylated selenoaminoacids are formed in plants[7]. Animals also synthesize methylated selenides, as summarized in Scheme 1 and reviewed elsewhere in more detail[7, 8]. Hydrogen selenide is the common intermediate in both the assimilatory pathway for synthesis of selenoproteins, and for the synthesis of methylated selenium excretory products. For inorganic selenite, reduction occurs by reaction with the major cellular thiol (glutathione) and certain dithiol proteins[9, 10, 11]. Hydrogen selenide also is formed through the action of a lyase on selenocysteine[12]. Selenomethionine can be converted to hydrogen selenide via selenocystathione and selenocysteine[13]. Methylation of the inorganic selenide by thiol methyltransferase[10, 14] forms methyl selenol and dimethyl selenide, and further methylation by thioether methyltransferase[15, 16] forms trimethylselenonium ion. These methyltransferases play a major role in sulfur, selenium, and tellurium metabolism.

SELENIUM METABOLISM AND CHEMOPREVENTIVE ACTIVITY

Collaborative studies were begun with Dr. Clement Ip and Dr. Henry Thompson using animal models of mammary cancer to explore the basis for the anticarcinogenic action of selenium. We began our studies on the premise that (1) metabolites of sodium selenite were responsible for its anticarcinogenic activity, and (2) the quantitative output of such metabolites would increase as the dose of selenite was increased to the chemopreventive range. We sought compounds that would deliver chemopreventive activity but have low toxicity. Although there is considerable interest in the metabolites formed in the course of selenite metabolism via GSSeSG to hydrogen selenide, these also may be associated with toxic effects[17]. Methylation is the best known fate of selenium and the fully methylated metabolites are regarded as detoxified forms of selenium. Dr. Foster in this laboratory had shown that when animals were given methylated selenium compounds that enter the metabolic pathway beyond the inorganic pool, methylated metabolites were formed in large amounts[18, 19]. We chose such compounds (Scheme 2) for initial studies of cancer prevention activity.



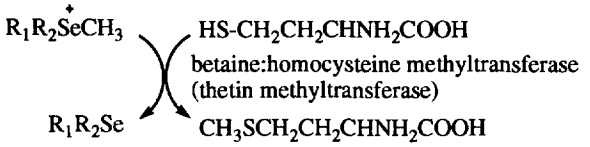
Scheme 2. Entry of methylated forms of selenium below the inorganic pool (indicated by dashed line).

The generation of a monomethylated form of selenium was a prominent feature of selenium compounds having good anticarcinogenic activity. Se-methylselenocysteine was about 650-fold more active than its sulfur analog[20], and a monomethylated form of selenium was the major excreted metabolite[21]. Its metabolism is discussed later in more detail. Selenobetaine and selenobetaine methyl ester had good anticarcinogenic activity[22], but dimethyl selenoxide and trimethylselenonium had little or no activity[22,24]. Even though half or more of the administered selenium was excreted as dimethyl selenide or trimethylselenonium ion with all four of these compounds, chemopreventive activity was markedly different, so that activity did not correlate with the excretion of the distal metabolites[21]. The metabolite profile also provided clear evidence that all of the methylated selenium compounds underwent partial demethylation, so that even di- and tri-methylated precursors formed inorganic and mono-methylated products (the biochemical basis for demethylation is discussed below). The amount of inorganic selenium produced by demethylation of the active methylated selenium compounds was not correlated with their relative anticarcinogenic activity. Taken with other studies, these results indicate that formation of inorganic selenium is not essential for expression of anticarcinogenic activity, although it provides bioavailable

selenium for synthesis of selenoproteins. Clearly, the animal has extensive capabilities for interconverting these forms of selenium.

Demethylation of methylselenonium compounds

It was clear that demethylation of selenium occurred in animals, but the biochemical basis for such reactions had not been established. It was shown[23] that a homocysteine-dependent methyltransferase activity was present in liver that demethylates selenobetaines and trimethylselenonium:



When tested at near-optimal substrate concentrations, selenobetaine, selenobetaine methyl ester, and sulfobetaine gave much higher rates compared to betaine, the "physiological" substrate. Selenonium compounds were more active than their sulfonium analogues. Trimethylselenonium ion gave the highest rate of all the compounds tested. These results establish a biochemical basis for selenium demethylation, a metabolic process largely ignored in many discussions of selenium metabolism. This demethylation reaction probably competes with the production of sulfonium and selenonium derivatives by the recently discovered thioether methyltransferase[15,16], so that the steady-state level of such compounds in tissues that contain both enzymes (liver) will reflect the interplay of both enzyme activities. This is an important concept in view of the hypothesis that certain methylselenonium compounds generated by the thioether methyltransferase reaction may be mediators of selenium's anticarcinogenic action.

Anticarcinogenic activity and metabolism of Se-methylselenocysteine

One of the best chemopreventive forms of selenium in our studies was Se-methylselenocysteine[24]. It is a naturally-occurring form of selenium, and is a major constituent of plants grown on selenium-rich media[24]. This amino acid does not get incorporated into proteins, in contrast to selenomethionine, thus minimizing the possibility for excessive accumulation in tissues. As a monomethylated form of selenium, the metabolic point of entry is below the level of inorganic selenide. The metabolism of Se-methylselenocysteine, as described previously, gave monomethylated selenium as the major excretory metabolite. There was also extensive conversion to inorganic selenium, and this result was corroborated by the high bioavailability observed in other studies[20]. Because monomethylated selenium is the major excretory product, it seemed likely that direct scission of the Me-Se moiety from the amino acid would be catalyzed by an enzyme such as a lyase[19].

Cysteine conjugate β-lyase. Several pyridoxal phosphate-dependent enzymes that catalyze cleavage of the C-S bond of cysteine conjugates to form the thiol, pyruvic acid, and ammonia have been described, and have received considerable attention because of their importance to sulfur toxicology and metabolism[25]. The enzyme activity is predominantly located in liver and kidney, and in intestinal contents (almost all in association with

microorganisms). S-aryl-L-cysteine conjugates (having the sulfur attached directly to an aromatic ring) appear to be the best substrates for the tissue β-lyases of mammals as well as intestinal flora[25]. The microbial β-lyase has a broader substrate specificity and acts on S-alkyl as well as S-aryl-cysteine derivatives. Gut flora are exposed to high amounts of various cysteine conjugates present in diets, such as cysteine conjugates in kale or *Allium* species. It is apparent that intestinal flora may be involved in the metabolism of dietary Se-alkylselenocysteine derivatives. Recently it has been shown that cysteine conjugate β-lyase of kidney origin will cleave the C-Se bond to release alkyl- or aryl-selenols from alkyl- or aryl-selenocysteine derivatives[26]. Good activity was observed for the lower alkyl series of selenocysteine conjugates, whereas the coresponding sulfur analogues were inactive. For some selenocysteine conjugates, fairly rapid non-β-lyase scission was observed that may involve a β-elimination mechanism[27].

We propose that the thiols or selenols released by cysteine conjugate ß-lyase will be methylated by thiol methyltransfersase, and further methylated by the thioether methyltransferase to give the dimethylselenonium derivative[15, 16]. Se-glucuronidation also may occur, as observed with other organoselenium compounds[28]. Besides the action of cysteine-conjugate β-lyase on the selenocysteine conjugates, N-acetylation is likely to be a competing reaction, since this is a well-established activity for formation of mercapturic acids[29]. The relative activity of various Se-alkyl selenocysteine derivatives with respect to N-acetylation vs. scission to release the selenol may vary, and may be a factor to consider in designing anticarcinogenic forms of such compounds. Oxidation by monooxygenases to a selenoxide (see below) also may be a factor in regard to selenocysteine conjugate metabolism, favoring selenenic acid elimination[27].

Oxidation of selenoethers by microsomal monooxygenases

Dimethyl selenide is an excellent substrate for microsomal flavin monooxygenases, even at sub-micromolar concentrations[30]. The reaction is easily monitored by the oxidation of NADPH using purified pig liver enzyme. The selenoxide product undergoes rapid reduction back to the selenoether, and this facile redox cycling may be important in regard to some of the biological activities of selenium. A number of synthetic selenoethers also were shown to be oxidized to selenoxides[31]; cytochrome P_{450}-catalyzed oxidation was significant for some of the organoselenium compounds. Selenium analogs of sulfur aminoacids such as S-alkylcysteine derivatives and methionine that are substrates for certain flavin monoxygenase isozymes[32,33] also are likely to undergo oxidation *in vivo*. Because the selenoxide products undergo rapid reduction back to the selenoether, it is possible that those selenoethers that can undergo facile methylation will eventually be methylated to the selenonium derivative due to the sustained action of the thioether methyltransferase.

Anticarcinogenic activity and metabolism of selenocyanates

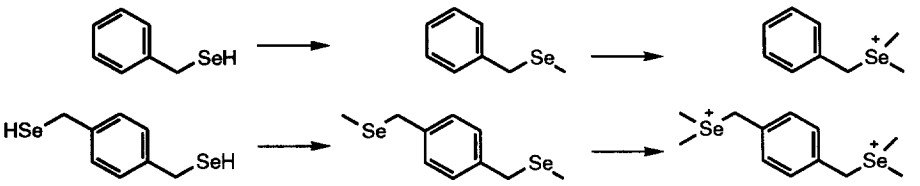
Benzylselenocyanate and various isomers of xylyl-bis(selenocyanate) were shown to be active in chemoprevention[34,35]. When fed to animals, the xylyl derivatives were relatively less toxic in relation to chemopreventive activity, due to a lower absorption from the intestinal tract[36]. A series of alkylselenocyanates evaluated for their ability to block the initiation phase (administered only at the time of carcinogen administration) showed increasing activity with increasing chain length up to five carbons[37]. The anticarcinogenic activity[38] and metabolism[39] of potassium selenocyanate also has been reported. In contrast to the relative inertness of

thiocyanate, which is excreted as an end product of sulfur metabolism in urine, potassium selenocyanate was efficiently catabolized and had similar bioavailability to other inorganic forms of selenium[40]. The cyanide moiety is converted to thiocyanate, as shown by labeling studies[39]. For organic selenocyanates, it is likely that scission of the Se-CN bond involves glutathione and is catalyzed by glutathione transferases, since the analogous organic thiocyanates are known to be substrates for this enzyme[41]:

$$RSeCN + GSH \longrightarrow RSeSG + HCN \quad (1)$$

$$RSeSG + GSH \longrightarrow RSeH + GSSG \quad (2)$$

The metabolism of benzyl selenocyanate to benzyl selenol and the disposition of the benzyl moiety has been described[42]. We suggest that further metabolism of the selenol intermediates formed from benzyl and xylylselenocyanates would occur by thioether methyltransferases, to give the mono- or bis- methyl selenides and dimethylselenonium derivatives:

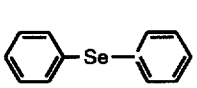


In a study of the metabolism of methylselenocyanate, about 40% of the dose was excreted as dimethyl selenide plus trimethylselenonium[43]; double-labeled studies showed retention of methyl by selenium in the products[43].

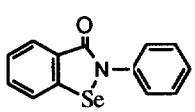
Anticarcinogenic activity and metabolism of phenyl selenides and triphenylselenonium chloride

The possible importance of lipophilic character for anticarcinogenic activity was suggested by the studies with benzyl or xylene-type selenocyanates, as well as aliphatic selenocyanates (RSeCN). A drawback of these types of selenocyanates is the facile scission of Se from the organic moiety. The bioavailability of methyl selenocyanate and benzyl selenocyanate is comparable to selenite, and 1,4-xylyl-bis(selenocyanate) also had substantial bioavailability, as measured by the restoration of glutathione peroxidase[38].

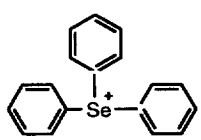
In order to retain lipophilic character but reduce the bioavailability of the Se, we turned to aromatic organoselenium compounds where Se is bonded directly to an unsubstituted benzene ring. Such compounds, and the phenyl selenide drug, Ebselen, have very low toxicity and bioavailability[44, 45]. These characteristics likely are explained by the inherent chemical and metabolic stability of the Se in such compounds, involving sp^2 bonding and delocalization of electrons of Se into the aromatic ring. Any biological activity of such compounds is more likely to be associated with the intrinsic molecule, rather than selenium released from the structure.



diphenyl selenide



Ebselen



triphenylselenonium

Of particular interest is the triphenylselenonium ion, since it has three benzene rings attached to selenium, but has a permanent positive charge due to the onium center, conferring solubility in water. Thus, triphenylselenonium ion is an amphiphilic or lipophilic cation, a class of compound having antitumor activity[46].

Triphenylselenonium (fed to animals as the chloride salt) proved to have very low toxicity and good efficacy at 10-30 parts/10^6 in the diet, giving the best ratio of efficacy to toxicity for any selenium compound tested to date[47]. Tissue selenium levels were increased only slightly by feeding a chemopreventive level of triphenylselenonium, in contrast to most forms of selenium used in chemoprevention[47]. This is a very favorable property, alongwith water solubility and lack of odor, for any agent being considered for use in cancer prevention. Very low toxicity but good cytostatic activity also was observed when triphenylselenonium chloride was added to cultured mammary tumor cells[48]. Cytostasis was associated with decreased cell proliferation and delayed cell cycle progression. Effects of triphenylselenonium on cellular metabolism (increased rate of glucose consumption and lactic acid production) were observed; this apparent enhancement of glycolytic metabolism may be a compensatory effect resulting from decreased mitochondrial energy production. Lipophilic cations are known to be accumulated in mitochondria because of the negatively charged mitochondria matrix, thus one possible site of action for triphenylselenonium chloride is mitochondria[48]. The activity of triarylselenonium compounds establishes a new class of chemoprevention compounds, and directs attention to anticarcinogenic selenium compounds having lipophilic character along with cationic properties, or the potential for generating such types of compounds when metabolized in animals.

Synthesis of [^{75}Se]triphenylselenonium derivatives. Little is known about the tissue distribution and metabolism of triphenylselenonium ion. To facilitate such studies, we have synthesized the radioactive compound by a series of reactions starting with commercially-available radioactive selenious acid. The method involves the classic sequence of converting the element to potassium selenocyanate, which is then reacted with diazotized aniline to form phenyl selenocyanate. Along with the selenocyanate, radioactivity was recovered in diphenyl selenide (relative yield of products 2:1, respectively). The structure of both products was confirmed by mass spectrometry. After converting the phenylselenocyanate to diphenyl selenide by reaction with phenyl lithium, the diphenyl selenide was converted to the dichloride and subjected to Friedel-Crafts reaction to form the triphenylselenonium product. This was adsorbed onto a weak-cation ion exchanger, and washed to remove impurities. Because of the dual retention mechanisms involvng hydrophobic interactions as well as electrostatic interactions, the triphenylselenonium remains bound to the ion exchanger during washing with 90% methanol (as well as 0.5 N perchloric acid), but is eluted by a combination of 50% methanol and 0.5 N perchloric acid, and crystallizes in this solvent in the cold as the perchlorate salt. Although the optimal conditions have not been worked out and the yield was low, the product was very pure. HPLC showed a single radioactive and ultraviolet peak having a spectrum and retention time (12.3 min) identical with that of standard triphenylselenonium chloride, using a perchlorate-perchloric acid eluting buffer (Figs. 1, 2). The UV maxima for triphenylselenonium perchlorate (266 and 272 nm) are at slighly lower wavelengths compared to triphenylsulfonium perchlorate (267 and 275 nm)[49], but otherwise the spectra are very similar. In a separate study comparing the triphenyl derivatives of the Group VI elements, the retention times increased in the order triphenylsulfonium (9.8 min), triphenylselenonium (11.4 min), triphenyltelluronium (17.2 min) using the polymer-based PRP-1 column. Using the same elution solvent with a C18 silica reversed phase column (TSK phenyl), the elution order was reversed.

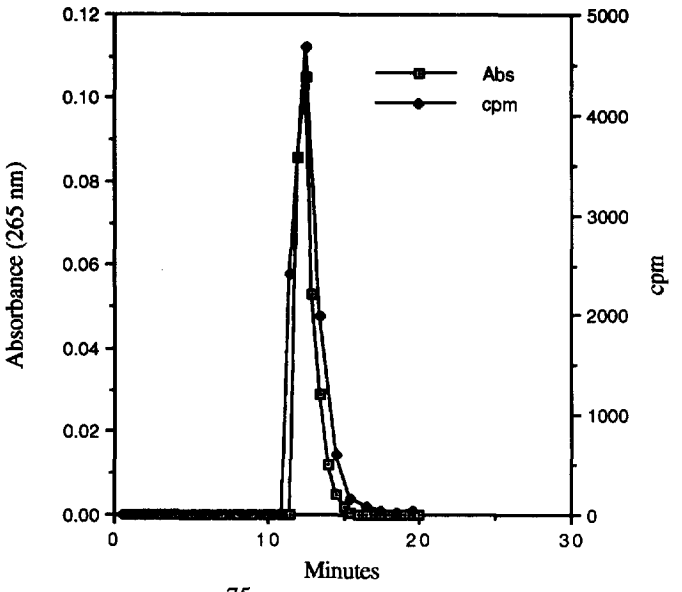


Fig. 1. Reversed phase HPLC of [^{75}Se]triphenylselenonium perchlorate. Sample: 40 μL [^{75}Se]$(C_6H_5)_3Se^+ClO_4^-$. Retention time: 12.3 min.

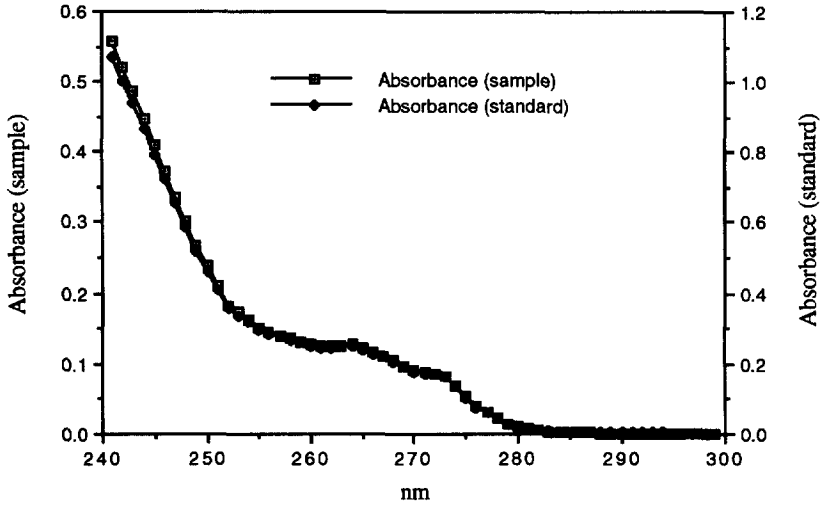


Fig. 2 HPLC diode array spectra of triphenylselenonium derivatives. Standard: 40 μL 1 mM $(C_6H_5)_3Se^+Cl^-$. Sample: 40 μL [^{75}Se]$(C_6H_5)_3Se^+ClO_4^-$. Retention times: 12.3 min.

DISCUSSION

Evidence has been summarized that supports a biosynthetic origin of active chemopreventive selenium metabolites involving the attachment of suitable carbon chains to a selenium atom (X):

$$X \rightarrow R_1X \longrightarrow R_1R_2X \longrightarrow R_1R_2R_3X^+$$

The monomethylated form of selenium appears to be a critical metabolite formed by metabolism of inorganic selenium, or formed from precursors such as Se-methylselenocysteine. The monomethylated form appears to lack some of the adverse toxic effects associated with inorganic forms of selenium and hydrogen selenide (genotoxicity); one possible mechanism of action may be induction of apoptosis in cancer cells[50].

If alternative types of carbon chains (such as allyl) are available, more active metabolites might be formed. Plants such as *Allium* species can transfer allyl groups to sulfur, and possibly selenium. The C6 product formed by transfer of two allyl groups to sulfur can undergo methylation when metabolized in the animal to give a C7 onium product[16]. The point is that the potential activity of selenium can be enhanced in the course of being metabolized in plants, especially in those species that have specialized alkyl-group transfer capabilities. Furthermore, the higher chemopreventive activity of selenium compounds compared to sulfur analogs could involve superiority in *generating* the alkylated derivatives (greater nucleophilic character and greater availability of its electrons for alkylation, especially in forming the onium center). This factor would be in addition to any differences due to the elements once they are incorporated into a given chemical structure. The greater chemopreventive activity of garlic grown on selenium as compared to regular garlic has been demonstrated[51]. Thus, natural products formed from selenium in plants ultimately can give rise to more active chemopreventive metabolites in animals, as compared to the chemopreventive products formed in animals from inorganic selenium.

Triphenylselenonium chloride and related phenyl selenide derivatives represent novel organoselenium chemopreventive compounds with useful properties. They have greater metabolic stability because selenium is bonded directly to an unsubstituted benzene ring. Their mechanisms of action remain to be established.

ACKNOWLEDGEMENTS

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EXPERIMENTAL

Synthesis of [^{75}Se]triphenylselenonium chloride. The starting material was [^{75}Se]H_2SeO_3 obtained from the University of Missouri Research Reactor Facility, Columbia, Missouri. An aqueous solution containing 182 μCi of radioactivity plus 1 mmol (0.110 g) of carrier SeO_2 was treated with ascorbic acid to reduce the selenious acid to elemental Se. The aqueous phase was removed and the pellet of Se converted to KSeCN with 1 mmol KCN plus 1 drop of conc. ammonium hydroxide in 1 mL of water at 50°. Aniline (1 mmol) was diazotized by the slow addition of $NaNO_2$ to an aqueous HCl solution at 4°. The pH was adjusted to 4-5 by the addition of 1 M sodium acetate and the solution of KSeCN added to the chilled solution of diazotized aniline over a 30 min period. The organic products were extracted into dichloromethane and dried under nitrogen. The oil was taken

up in 2:1 heptane:dichloromethane and chromatographed on a silica gel column using the same solvent. The first fraction collected yielded a colorless oil, identified as diphenylselenide by HPLC/UV diode array and mass spectroscopy. A second fraction (yellow, unidentified) was eluted followed by a third fraction identified as phenylselenocyanate on the basis of HPLC/diode array and mass spectroscopy. The phenylselenocyanate was reacted with phenyllithium in THF for 30 min at 0° under N_2, quenched with water, and extracted with dichloromethane, then purified by silica gel chromatography to give diphenyl selenide. The two diphenylselenide portions were combined and converted to the dichloride using nitric acid followed by HCl. The reaction mixture was then diluted with water and the suspended yellow solids extracted into chloroform and evaporated to dryness under nitrogen. A solution of the diphenylselenide dichloride in benzene was converted to triphenylselenonium chloride by the Friedel-Crafts reaction with excess $AlCl_3$ in five portions, at low temperature (about 8°). After 0.5 h a small piece of ice was added to the deep red solution, after which the triphenylselenonium chloride product (13 μCi, 7 % yield) was obtained by extraction with water.

Purification of [^{75}Se]triphenylselenonium by ion exchange chromatography. Adsorption onto a weak cation exchange column (Amberlite CG-50, H^+ form) followed by elution with aqueous methanol containing perchloric acid gave [^{75}Se]triphenylselenonium perchlorate, which crystallized as fine needles in the cold. Procedure: The aqueous solution of radioactive triphenyl selenonium chloride was adsorbed onto the column (previously washed with methanol and equilibrated with water). After sample application, the column was washed with water to remove a small amount of radioactive impurity, followed by aqueous methanol (to 90% methanol). After equilibrating the column with 50% aqueous methanol, elution was begun using 50% methanol containing 0.5 N perchloric acid. Fractions were collected and assayed for radioactivity. A broad peak containing 85% of the applied radioactivity was eluted, and these fractions were cooled to -20°. The crystalline product was collected and dissolved in a small volume of methanol for subsequent assay of purity by HPLC.

HPLC analysis. For analysis of triphenylselenonium chloride and related compounds, a polymer-based reversed phase column (Hamilton PRP-1, 1 x 10 cm, fitted with a guard column) was operated at 25°, using isocratic elution (1 mL/min) with methanol:water (65:35) containing 5 mM $NaClO_4$ plus 5 mM $HClO_4$, pH 2.5. A photodiode array detector (Waters model 991) was used to monitor the ultraviolet spectra of eluted compounds. For analysis of crystalline [^{75}Se]triphenylselenonium perchlorate, a fraction collector was used to collect 1 min fractions for direct assay of ^{75}Se by gamma ray scintillation counting.

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