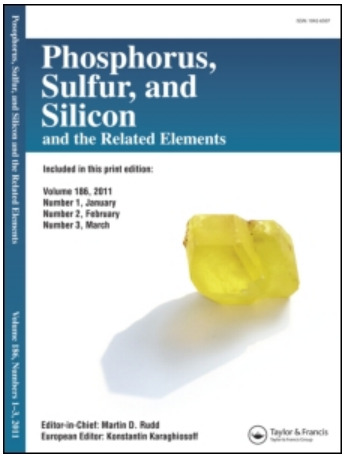

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ASSIMILATION, DISTRIBUTION, AND METABOLISM OF (^{75}Se)-SELENITE, SELENATE, AND SELENOAMINO ACIDS BY *ESCHERICHIA COLI*

Julian E. Spallholz[ab]; John L. Martin[a]; Marlene L. Gerlach[a]
[a] Department of Biochemistry, Colorado State University, Fort Collins, Colorado, USA [b] Laboratory of Experimental Metabolic Diseases, Veterans Administration Hospital, Long Beach, California, USA

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ASSIMILATION, DISTRIBUTION, AND METABOLISM OF (^{75}Se)-SELENITE, SELENATE, AND SELENOAMINO ACIDS BY *ESCHERICHIA COLI*

by

Julian E. Spallholz*, John L. Martin, and Marlene L. Gerlach

Department of Biochemistry, Colorado State University, Fort Collins, Colorado 80521 USA

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ABSTRACT

Assimilation of selenium (Se) by *Escherichia coli* as (^{75}Se)-selenite, selenate, selenomethionine, selenocystine and Se–CH_3-selenocystine revealed that (a) selenoamino acids from a culture media are more completely assimilated than selenite or selenate and (b) that the amount of selenite is assimilated three to four times selenate. Most (>95%) of the Se assimilated by *E. coli* could not be solubilized by sonication and ethanol extraction but much (28% to 70%) of the Se, except Se from selenomethionine, was removed by alkaline dialysis. Se from selenocystine and from Se–CH_3-selenocystine dialyzed from intact cells, whereas Se from selenite and selenate did not. Dialyzable Se is that Se probably present in selenotrisulfide (R–S–Se–S–R) bonds or bound nonspecifically. Analysis of the soluble Se metabolites from selenite, selenate, selenomethionine and selenocystine showed that *E. coli* produces at least one major metabolic product common to all substrates which upon chromatography appeared to be selenocysteic acid. In monogastric animals selenite and selenate Se does not enter the primary protein structure as amino acids yet metabolites of selenite, selenate and selenocystine produced by *E. coli* could enter the primary protein structure of animals in minute amounts.

Introduction

E. coli cultured in media containing selenite assimilates and intracellularly reduces selenium (Se) to its elemental state (Se^0)[1,2,3] or incorporates Se into proteins as selenomethionine and possibly selenocystine.[4,5] Recently, electron microscopy has been used to find the Se^0 localized to the cell membrane and cell wall of *E. coli*.[6] Selenate Se assimilated by *E. coli* has been reported to enter the protein fraction only as selenomethionine.[7] Se from selenomethionine is known only to be directly incorporated into protein of *E. coli*.[8,9] Additionally, Se, at least as selenite, is incorporated into transfer ribonucleic acid (tRNA) of *E. coli* as one or more selenobases.[10]

* Send correspondence to present address:
Laboratory of Experimental Metabolic Diseases
Veterans Administration Hospital
5901 East Seventh Street
Long Beach, California 90801, USA

Most Se however, whether from selenite, selenate or selenomethionine, is predominantly associated with the protein fraction of *E. coli* and it is firmly bound. From the data of Huber *et al.*,[7] 94% of the Se assimilated by *E. coli* as selenate remained bound following sonication and dialysis. Ahluwalia *et al.*[11] reported that 89% of the Se incorporated by *E. coli* as selenite was not extractable with carbon disulfide. For the alga, *P. yezoensis*, Horiguchi *et al*[12] found 90% of its Se content insoluble in hot 70% ethanol.

In animals, Se associated with proteins is also firmly bound. Selenite Se combines with animal and human proteins forming selenotrisulfide (R–S–Se–S–R) bonds which are reducible under various conditions.[13,14,15,16] Unlike *E. coli* and some plants,[17] selenite and selenate Se are not incorporated into the primary structure of proteins of animals as *de novo* selenoamino acids.[13,14]

While considerable information is available on the metabolic fate and biological availability of Se com-

pounds in plants and animals, and studies of the metabolism of selenite, selenate and selonmethionine in strains of *E. coli* have been reported, no systematic investigation of the metabolism of several Se compounds in a single strain of *E. coli* appears available.

The chemical form of Se provided to an organism results in differences in the biological availability and metabolic fates of the Se.[18,19,20] *E. coli* was selected to systematically assess the differences in assimilation of selenite, selenate and selenoamino acids from a defined growth medium with respect to soluble Se metabolites, cellularly bound Se and the bonding of Se as selenotrisulfides (R–S–Se–S–R) or incorporation as amino acids into primary protein structures.

Experimental Section

Materials and Methods

Bacteria: E. coli, wild type, was obtained from the Department of Microbiology, Colorado State University.

Isotopes

(^{75}Se)-selenite, selenate, selenomethionine and selenocystine were obtained from Amersham/Searle Corporation. The specific activities of the isotopes at the time of their use were 43.1, 44.5, 580 and 180 mCi/mmole, respectively. (^{75}Se)–Se–CH_3-selenocysteine was isolated by ion-exchange chromatography from a 70% ethanol extract of *Astragalus osterhouti* which had been grown in the presence of (^{75}Se)-selenite.[21] The specific activity of (^{75}Se)–CH_3-selenocysteine was not determined and was used for the assimilation and dialysis experiments.

Culture Medium

The culture medium was Hank's salt solution (pH 7.2) and dextrose (1.5 g/l). To two liters of culture medium, 20 μCi of either (^{75}Se)-selenite, selenate, selenomethione, or selenocystine was added amounting to 36.7, 35.5, 2.7 and 17.6 μg Se, respectively. An undetermined amount of (^{75}Se)-Se-CH_3-selenocysteine containing 3×10^4 cpm was added. The culture medium and isotopes were autoclaved separately. The isotopes were then added to the culture medium upon cooling.

Growth Conditions

E. coli was cultured in 3:1 Erlenmyer flasks containing 2:1 of media and one of the Se compounds. The flasks were maintained in a 37°C constant temperature water bath for 48–72 h.

Bacterial Isolation

The bacteria were isolated by centrifugation in 250 ml centrifuge bottles at 4°C. The supernatant was decanted and the bacterial pellet was suspended in 150 ml saline and repelleted. Following three washes with saline, the cells were lyophylized. Approximately 125 mg/l (dry weight) of *E. coli* was obtained from each culture

Measurement and Calculation of (^{75}Se)

(^{75}Se) was measured in 16 x 125 mm test tubes using a Baird-Atomic Model 810 B well type gamma ray scintillation detector with an Ortec Model 121 preamplifier. From these data, the amount of (^{75}Se) assimilated by *E. coli* (cpm/mg cells, dry weight basis) was measured and was used to determine the proportion of soluble, bound and nondialyzable content of the bacterial cells.

Extraction of Soluble Se Metabolites

One hundred-fifty mg of lyophylized cells were suspended in 25 ml of 70% ethanol and sonicated (50–60 Hz) for 30 minutes. The sonicated cells were filtered (Whatman #1) and washed with 70% ethanol. The filtrate and washings were concentrated to 1–2 ml and passed through a Millipore filter, 0.45 μ pore size, and counted. The filtrate was evaporated and redissolved in 1.2 ml of 0.2 *N* sodium citrate buffer, pH 2.2 for amino acid analysis.

Dialysis

Whole bacterial cells (2–3 mg) were suspended in 0.25 ml of distilled water (ph 5.8) or distilled water adjusted to pH 12 with NaOH and placed in prewashed 6.35 mm dialysis tubing. (^{75}Se) was then measured. Dialysis was performed against either distilled water (pH 5.8), distilled water adjusted to pH 12 with NaOH or 0.1 *N* NaOH at 22°C. Sonicated cells (50–60 Hz for 30 minutes) were dialyzed against 0.1 *N* NaOH. Nondialyzable (^{75}Se) was periodically determined by placing the dialysis tubing in a 16 x 125 mm test tube and counted as described above.

Amino Acid Analysis

0.1 ± 0.02 ml of the 70% ethanol soluble (^{75}Se) metabolites from 150 mg of bacterial cells was applied from a Bio-Cal Chromatronix injector to an Aminex A-4 column of a modified Beckman Spinco 120-B Amino Acid Analyzer controlled by a Bio-Cal AS 20-1 sequencer. The single column methodology used for the assays is shown in Table I. The colorimeter effluent was collected in 2 ml aliquots using an LKB Ultrarac 7000 fraction collector. Each aliquot was then assayed for (^{75}Se).

TABLE I

Ion Exchange Methodology

Column size	0.9 x 69 cm
Resin type	Aminex A-4 (Bio-Rad)
Resin height	50 cm
Flow rate	60 ml/hr.
First buffer	3.25 (0.2 N)
Second buffer	4.25 (0.2 N)
Third buffer	6.45 (0.9 N)
Buffer change times	78, 115 min.
Column temp.	55° C.

TABLE II

Assimilation of (^{75}Se) Compounds by *E. coli*

Compound	cpm/mg cells (lyophylized)	Relative (^{75}Se) concentration added to culture medium (Selenite Se = 1.00)
(^{75}Se)-selenite	9,600	1.00
(^{75}Se)-selenate	2,800	0.98
(^{75}Se)-selenocystine	24,200	0.50
(^{75}Se)-selenomethionine	24,350	0.07
(^{75}Se)—CH_3-selenocysteine	106[a]	—

[a] Amount approximates total assimilation by *E. coli* from the culture medium.

Results

Assimilation of Se Compounds by E. coli

Cellular assimilation of selenomethionine, selenocystine, and Se—CH_3-selenocysteine by *E. coli* from the culture media was greater than the assimilation of either selenite or selenate even though the quantity of Se as selenite and selenate present in the culture media was greater than that of the selenoamino acids. Amino acid analysis of aliquots of the culture media supernatant using a continuous flow liquid scintillation counter showed that only trace amounts of the selenoamino acids remained in the culture medium following centrifugation. Much more selenite and selenate were detected in the supernatants of their respective growth cultures. Selenite was, however, assimilated by *E. coli* more than selenate by a factor of 3 to 4. The amount of Se assimilated by *E. coli* from selenite, selenate and each of the selenoamino acids is shown in Table II.

Soluble and Insoluble Se in E. coli

After sonication and 70% ethanol extraction, more than 95% of the Se assimilated by *E. coli* as selenite, selenate, selenocystine, selenomethionine or Se—CH_3-selenocysteine (Table II) remained firmly bound and insoluble (Table III). Bacterial cells containing Se from selenomethionine provided the smallest amount of soluble Se (0.5%), whereas cells containing Se from selenate yielded the largest amount of soluble Se (4.3%). Se solubilized from cells grown in the presence of selenocystine, Se—CH_3-selenocysteine and selenite yielded intermediate and similar amounts of soluble Se.

Dialysis of Se from E. coli

Much of the Se assimilated as selenite, selenate, selenocystine, or Se—CH_3-selenocysteine by *E. coli* appears to be bound nonspecifically or bound as "selenotrisulfides" (R—S—Se—S—R) for alkaline dialysis of sonicated cells rapidly removed 30%–55% of the Se content of these cells. Virtually no Se was detectably removed from the bacterial cells cultured with selenomethionine (Figure 1). Alkaline dialysis resulted in the removal of 0 to 11 times more Se from the bacterial cells than could be obtained by sonication and 70% ethanol extraction.

Selenite Se lability from protein by dialysis is pH dependent.[13,14] To ascertain the effects of pH and sonication upon the dialysis of Se from *E. coli*, whole intact cells were sequentially dialyzed against distilled water (pH 12), distilled water (pH 5.8) before and

TABLE III

(^{75}Se) Extracted from *E. coli* by 70% Ethanol

Compound	Total (^{75}Se) Activity (cpm x 10^6)/150 mg	Extracted Activity (cpm)	% of Total Activity
(^{75}Se)-selenite	1.440	33,135	2.4
(^{75}Se)-selenate	0.420	18,230	4.3
(^{75}Se)-selenocystine	3.630	64,400	1.8
(^{75}Se)-selenomethionine	3.653	18,400	0.5
(^{75}Se)—CH_3-selenocysteine	0.016	445	2.8

after sonication and finally against 0.1 *N* NaOH (Figure 2). Dialysis of bacterial cells following this procedure revealed that Se incorporated by *E. coli* either as selenite or selenate was not dialyzable against

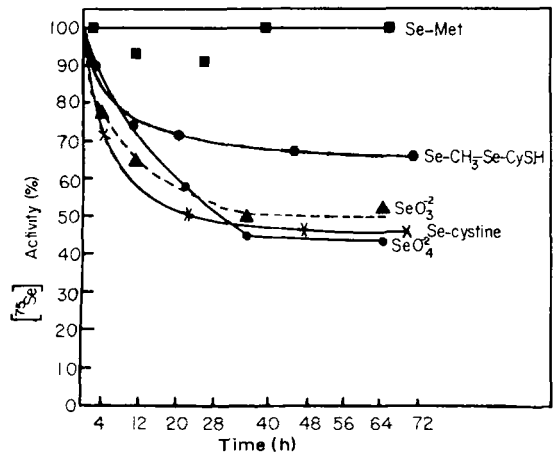

FIGURE 1

Dialysis of sonicated *E. coli* cells. *E. coli* cells were sonicated and dialyzed against 0.1 *N* NaOH. A few mg of cells were placed in 0.25 ml of 0.1 *N* NaOH in 6.35 mm dialysis tubing, dialyzed and counted for (^{75}Se) remaining.

distilled water (pH 12) without first disrupting the integrity of the bacteria either osmotically with distilled water or by sonication. In contrast, Se from the cells incorporating Se–CH_3-selenocysteine could Much of the bound Se from selenocystine was also dialyzable without cellular disruption. Se from selenite, selenate, and selenocystine appeared to dialyze most rapidly against 0.1 *N* NaOH. Over the first 96 h of dialysis, only 3% of the Se from *E. coli* incorporating selenomethionine could be removed by dialysis. Much of the Se not removed by dialysis is probably incorporated into the primary protein as selenoamino acids.

Chromatography of Soluble Se Metabolites

Amino acid chromatograms of the 70% ethanol soluble metabolites from the metabolism of selenite and selenate (Figure 3) and selenocystine and selenomethionine (Figure 4) by *E. coli* revealed striking similarities, as well as differences. Few amino acids or other ninhydrin positive compounds were present in sufficient quantities to be detected with each analysis providing a similar ninhydrin positive chromatogram. The elution time of ^{75}Se and its relative activity, however, served to differentiate the similarities and metabolic differences of selenite, selenate, selenocystine and selenomethione. While it was not possible to identify each of the metabolites observed, three general observations were made. First is the similarity in the amount and number of Se metabolites from selenite and selenate. Second is the striking differences in the number and amounts of Se metabolites from selenocystine. Third is the observation that there is a Se metabolite which is eluted at 28 min, comprises much of the total soluble Se and is common to selenite, selenate, selenocystine and selenomethionine. This metabolite, we believe, is selenocysteic acid. Seleno-

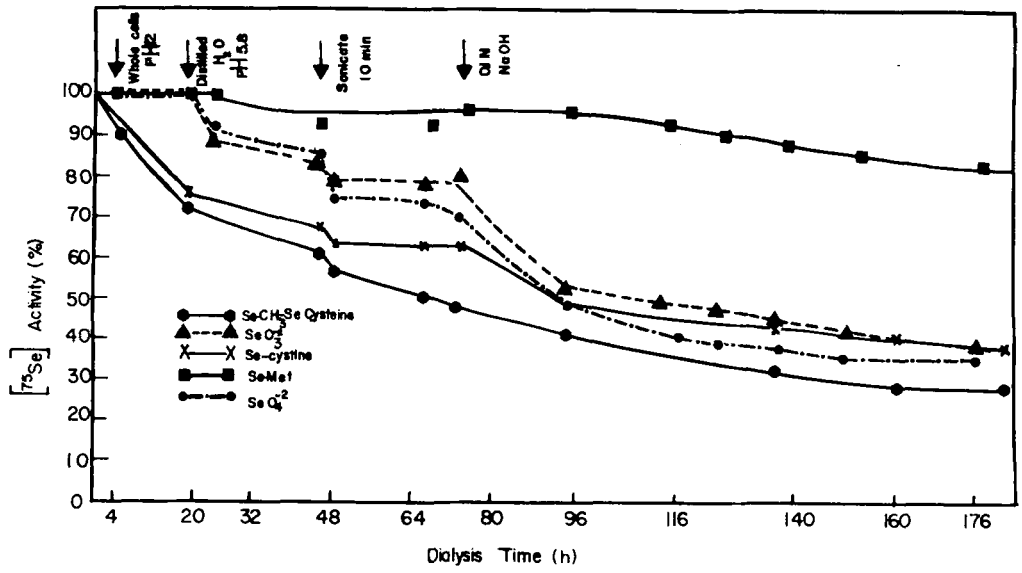

FIGURE 2

Dialysis of intact *E. coli* cells. *E. coli* cells were sequentially dialyzed against distilled water (pH 12), distilled water (pH 5.8) followed by sonication and dialysis against 0.1 *N* NaOH. Dialyzed samples were periodically counted for (^{75}Se) remaining.

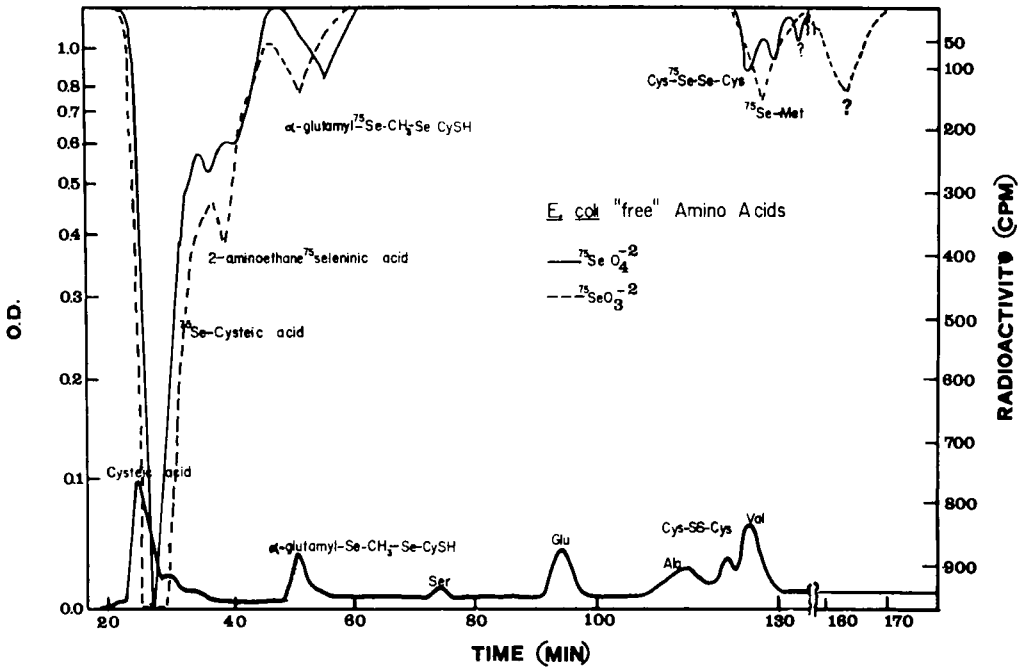

FIGURE 3

Amino acid analysis of 70% ethanol extracts of *E. coli*. Cultured in the presence of (^{75}Se)-selenite or selenate, 150 mg of lyophylized *E. coli* were sonicated in and extracted with 70% ethanol to obtain the soluble metabolites. The samples were prepared as described in the Experimental Section and chromatographed by the methodology of Table I. Aliquots of the effluent were collected and counted for (^{75}Se). Ascending peaks are ninhydrin-positive and descending peaks indicated radioactivity.

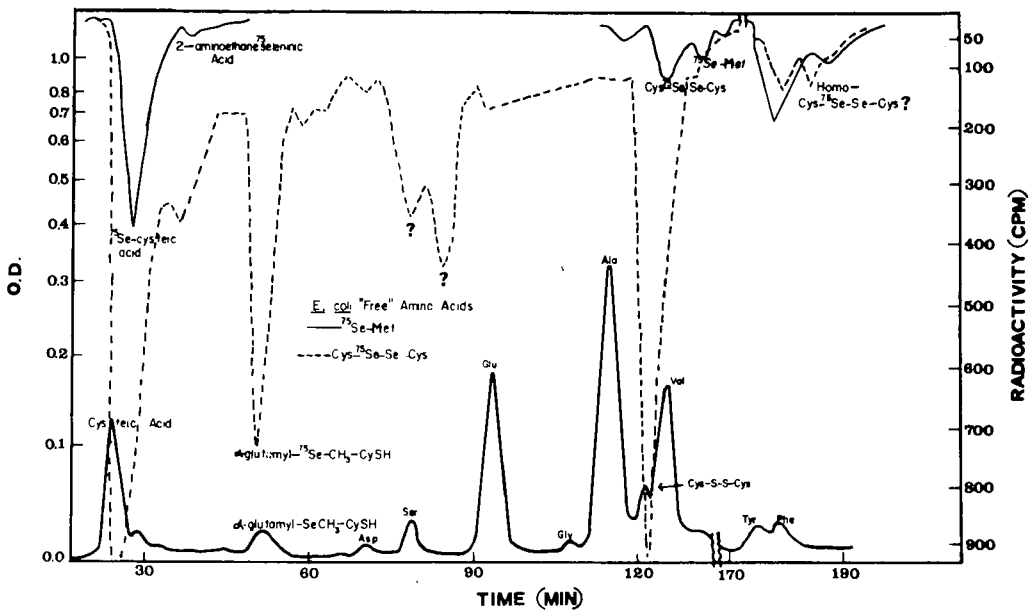

FIGURE 4

Amino acid analysis of 70% ethanol extracts of *E. coli*. Cultured in the presence of (^{75}Se)-selenomethionine or selenocrystine, 150 mg of lyophylized *E. coli* were sonicated in and extracted with 70% ethanol to obtain the soluble (^{75}Se) metabolites. The samples were prepared as described in the Experimental Section and chromatographed by the methodology of Table I. Aliquots of the effluent were collected and counted for (^{75}Se). Ascending peaks are ninhydrin-positive and descending peaks indicated radioactivity.

amino acids elute three to four minutes after their corresponding sulfur analogs and using the methodology of Table I, cysteic acid eluted at 24 min making the elution of this common Se metabolite likely to be selenocysteic acid. Another Se metabolite common to all compounds but occurring in much smaller quantities and eluting at 37 min may be 2-aminoethaneseleninic acid or possibly selenotaurine, the latter being known to be rather unstable.[22] A selenium containing peptide, δ-glutamyl-Se–CH_3-selenocysteine has been reported as a metabolite in some species of the plant genus *Astragulus*[23] eluting at approximately 50 min. Our chromatograms show as Se metabolite from *E. coli* eluting at approximately 50 min, suggesting that this selenopeptide may be a metabolite common to the metabolism of selenite, selenate, and selenocystine. Selenohomocystine appears to be yet another metabolite from the metabolism of the *dl*-isomers of selenomethionine and selenocystine by *E. coli* as a Se metabolite eluted closely to where authentic selenohomocystine eluted using the methodology of Table I.

These Se metabolites which can only be tentatively identified by the elution times of known sulfur analogs and authentic Se compounds occur in addition to the metabolites from selenocystine eluting between 80 and 90 min which also were not identified but eluted where reduced and oxidized selenoglutathione would be expected. Our results agree with previous reports of the synthesis of selenomethionine and possibly selenocystine from selenite by *E. coli*[4,5] and the synthesis of selenomethionine from selenate.[7]

Discussion

Previous reports have noted that selenite, selenate, and selenomethionine incorporated by *E. coli* becomes firmly bound.[7,8,9,11] This study confirms and extends these observations to include selenocystine and Se–CH_3-selenocysteine. In no instance was more than 5% of the Se assimilated as selenite, selenate, selenomethionine, selenocystine or Se–CH_3-selenocystine by *E. coli* solubilized.

Although not soluble, 28-70% of the bound Se, excepting Se from selenomethionine, could be mobilized by sonication and alkaline dialysis. Much of the insoluble bound Se of *E. coli* may be present in protein as reducible selenotrisulfides (RS–Se–SR). Incorporation of Se into proteins as selenotrisulfides has been demonstrated *in vitro*[24,25] and *in vivo* from several protein sources.[13,14,15,16] Reducing agents, denaturants and alkali have been effectively used to mobilize up to 90% of this bound Se.[14] Dialysis of sonicated *E. coli* cells (Figure 1) against 0.1 *N* NaOH produced a fairly rapid release of Se from all assimilated compounds except selenomethionine. The amount of Se initially released provides for an approximation of the maximum percentage of Se which may be bound in the primary protein structure other than as selenotrisulfides and exhaustive dialysis (Figure 2) permits approximation of the minimum amount of Se incorporated as amino acids and tRNA selenobases.[10] With these assumptions, a summary showing an estimate of the distribution of Se in *E. coli* into selenotrisulfides and primary protein structure was made (Table IV). The results show that Se from selenomethionine is mostly incorporated into primary protein and that Se from Se–CH_3-selenocysteine, a nonprotein amino acid, is least incorporated into the primary protein of *E. coli*. The amounts of Se from selenite, selenate and selenocystine appear equally distributed by *E. coli* into the primary protein. It is unlikely, however, that the dialyzable Se from the selenoamino acids is bound like the Se from selenite or selenate, for alkaline dialysis of whole cells mobilized Se from selenocystine and Se–CH_3-selenocysteine but not selenite or selenate. Only upon cellular disruption was dialysis of selenite and selenate Se possible.

TABLE IV

Distribution of Se in *E. coli*

Compound	Soluble Se (%)	Bound Se (%)	% of bound Se (range)[a] Selenotrisulfide or non-specific	% of bound Se (range)[a] [b]Primary Protein
(^{75}Se)-selenite	2.4	97.6	(49)-60.6	(48.6)-37
(^{75}Se)-selenate	4.3	95.7	(53)-60.7	(42.7)-35
(^{75}Se)-selenocystine	1.8	98.2	(52)-61.2	(46.2)-37
(^{75}Se)-selenomethionine	0.5	99.5	(0)-19.5	(99.5)-80
(^{75}Se)–Se–CH_3-selenocysteine	2.8	97.2	(28)-69.2	(69.2)-28

[a] Range estimated from 72-hour ( ) and exhaustive dialysis less soluble fraction.

[b] Amount may include small amounts of Se in tRNA basis[10].

Selenite Se becomes bound as selenotrisulfides, incorporated between inter- and intra-protein cysteine residues upon completion of protein synthesis. The similarities in the amounts of bound and dialyzable selenite and selenate Se and the similarities of their soluble metabolites suggest that selenate is reduced to selenite by *E. coli*. It is unlikely that *E. coli* oxidizes selenite to selenate. Selenite has been observed to be reduced by *E. coli* to Se^0 forming the red allotrope.[1,6] We also observed this reduction as cells grown in the presence of selenite were pink upon pelleting. No Sc^0 formation was observed in cells grown in the presence of selenate, indicating that reduction of selenite by *E. coli* to Se^0 may occur only when the capability of the cell to metabolize selenite is exceeded. Selenate was also assimilated less by *E. coli* than selenite, further indicating a possible rate-limiting reduction of selenate to selenite.

Ion-exchange chromatography of the 70% ethanol extracts of *E. coli* revealed a number of Se metabolites in addition to the previous reports of selenomethionine[4,7] selenocystine.[5] A metabolite comprising most of the soluble Se and common to selenite, selenate, selenomethionine and selenocystine appeared to be selenocysteic acid. The appearance of selenocysteic acid as a soluble metabolite would indicate that small amounts of Se incorporated by *E. coli* follow catabolic pathways which are similar for sulfur. In the gastrointestinal tract of animals and man, *E. coli* could conceivably convert minute amounts of selenite, selenate, and selenoamino acids into other organoselenium compounds which possess either increased or decreased biological activity and availability.

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