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Selenium Incorporation into Selenoproteins in the Se-Adequate and Se-Deficient Rat[1,2] (42651)

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Abstract. To rapidly discriminate between selenoproteins and Se-binding proteins, SDS + 2-mercaptoethanol treatment and then gradient SDS–slab gel electrophoresis was used to remove loosely bound Se from proteins while separating protein subunits according to molecular weight. This technique was used to study the nature and time course of ^{75}Se incorporation into selenoproteins. Male weanling rats were fed either a Se-adequate (0.35 ppm Se) or a Se-deficient (<0.02 ppm Se) diet for 20–41 days, injected iv with 50 μCi [^{75}Se]selenite (100 μCi/μg Se), and sacrificed 1, 3, 24, or 72 hr after ^{75}Se injection. At 1 and 3 hr, a 55-kDa plasma ^{75}Se protein contained the most ^{75}Se of any ^{75}Se protein observed in any tissue in either Se-adequate or Se-deficient rats. At 24 and 72 hr, a 23-kDa ^{75}Se protein (glutathione peroxidase subunit) in liver cytosol was the second-most labeled ^{75}Se protein observed in Se-adequate rats. The second-most labeled ^{75}Se protein in Se-deficient rats was a 17-kDa ^{75}Se protein in testes at 24 and 72 hr. ^{75}Se proteins of 10, 14, 45, and 65 kDa as well as lesser amounts of other ^{75}Se proteins were also detected. In a separate experiment, cycloheximide pretreatment eliminated ^{75}Se labeling of any of the ^{75}Se proteins, demonstrating that protein synthesis is required for Se incorporation. The rise and fall of various ^{75}Se proteins with time suggests that these selenoproteins may be important in the flux of Se between tissues.

Seven bacterial selenoenzymes as well as several additional forms of glutathione peroxidase have been reported (1, 2) since Rotruck and co-workers (3) discovered that glutathione peroxidase was a selenium-containing enzyme. Mammalian glutathione peroxidase (GSH-Px) activity is catalyzed by several different enzymes including the 80-kDa glutathione peroxidase (GSH:H_2O_2 oxidoreductase, EC 1.11.1.9) discovered by Mills (4), the non-Se-dependent glutathione-S-transferase that has peroxidase activity (5), and a reportedly distinct Se-dependent phospholipid hydroperoxide glutathione peroxidase in pig heart (6). In addition to these selenoenzymes, a number of other mammalian selenoproteins have been identified or suggested (2). Using column chromatography, Hawkes and co-workers (7) were able to partially resolve up to 23 selenoproteins in addition to GSH-Px. Thus it is becoming apparent that Se-containing proteins are not exceedingly rare.

In order to better resolve and quantitate the various selenoproteins present in animal tissues, we have developed a technique using pretreatment of samples with SDS + 2-mercaptoethanol and conventional SDS–slab gel electrophoresis (SDS–PAGE), followed by fixation, gel slicing, and ^{75}Se counting, that can monitor the labeling of selenoproteins in various tissues of rats administered ^{75}Se. The present study was designed to use this technique to investigate the time course of labeling of ^{75}Se proteins in both Se-adequate and Se-deficient rats. We further used inhibition of protein synthesis by cycloheximide treatment to determine whether the identified ^{75}Se proteins were selenoproteins that required protein synthesis for Se incorporation. We have used the term "selenoprotein" to describe proteins that contain Se which is not removed by the SDS–PAGE procedure, and used the term "Se-binding protein" to

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[2] A preliminary report of these experiments was presented at the meetings of the Federation of American Societies for Experimental Biology: Evenson JK, Sunde RA. Fed Proc **45**:371, 1986.

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refer to proteins that bind Se which is removed by this procedure.[3]

Materials and Methods. *Rats and diets.* Male weanling 21-day-old rats (Holtzman Co., Madison, WI) were housed individually in hanging wire mesh cages in an environmentally controlled room. Diet and deionized water were provided *ad libitum.* In Experiment 1, rats were fed a Se-adequate stock diet (Rodent Blox 8604, Wayne Pet Food Division, Chicago, IL) that contained 0.347 ± 0.006 ppm Se as determined by fluorometric analysis (8) in our laboratory. In Experiments 2 and 3, rats were fed a torula yeast-based diet as described by Schwarz (9) and modified by Hafeman and Hoekstra (10). It was composed of 30% torula yeast (Rhinelander Paper Co., Rhinelander, WI), 59% sucrose, 5% lard, 5% mineral mix,[4] 0.9% vitamin mix[5] and 0.1% choline chloride. To prevent liver necrosis, the diet was supplemented with 0.4% D,L-methionine (U.S. Biochemical Corp., Cleveland, OH) and 100 IU/kg of all *rac*-α-tocopheryl acetate (Sigma Chemical Co., St. Louis, MO) at the expense of sucrose. The basal diet contained <0.02 ppm Se (0.018 ± 0.002 ppm) by fluorometric analysis (8).

Experiment 1. To study Se metabolism in the Se-adequate rat, weanling rats were fed the Se-adequate stock diet for 20 days prior to treatment. The ^{75}Se incorporation patterns in the tissues of these Se-adequate rates were qualitatively identical to the patterns we observed in other experiments with rats fed a Se-adequate (+0.2 ppm Se as selenite) torula yeast-based diet.[6] Rats (171–209 g) were anesthetized with ether and injected with 50 μCi ^{75}Se per rat as sodium [^{75}Se]selenite (453 mCi/mg Se, ICN Radiochemicals, Irvine, CA) into the right femoral vein. The total injected Se was adjusted to 0.5 μg Se/rat with unlabeled sodium selenite (Sigma). At 1, 3, 24, and 72 hr postinjection, rats were reanesthetized, blood was drawn via heart puncture with a heparinized syringe, and the liver was perfused *in situ* with ice-cold 0.15 *M* KCl to remove erythrocytes. Kidneys, heart, testes, and epididymides were removed and placed on ice. Plasma was separated from red blood cells (RBCs) by centrifugation (2000*g*, 15 min) and the packed RBCs were restored to the original volume with saline phosphate buffer, pH 7.4.

The left liver lobe and the kidney were each fractionated by homogenizing a portion in 9 vol of 0.25 *M* sucrose containing 0.25 m*M* EDTA, pH 7.4, with a Potter–Elvehjem homogenizer. The homogenate was centrifuged (800*g*, 10 min) and the supernatant was then centifuged (14,000*g*, 10 min) to obtain the mitochondrial pellet. The pellet was homogenized for 30 sec (Brinkmann Polytron PT 1035 with PTA-205 generator, Brinkmann Instrument Co., Westbury, NY) in 5 ml of 10 m*M* Tris, 1% SDS, 10 m*M* 2-mercaptoethanol, pH 7.4 (Tris/SDS), and centrifuged (105,000*g*, 60 min) to obtain a mitochrondrial extract. The supernatant from the 14,000*g* centrifugation was centrifuged (105,000*g*, 60 min) to obtain the cytosolic fraction. The pellet from this centrifugation was homogenized using the Polytron homogenizer in 5 ml Tris/SDS buffer, and centrifuged (105,000*g*, 60 min) to obtain a microsomal extract. Heart, testes and epidi-

[3] Metalloproteins contain integral, stoichiometric quantities of the metal (29). As shown in Fig. 8, cycloheximde treatment blocks the ^{75}Se incorporation into the proteins identified by SDS–PAGE, suggesting that these proteins contain integral, stoichiometric quantities of Se and thus can be called selenoproteins. The "Se-binding proteins" would include proteins which bind Se nonspecifically as well as any selenoproteins that bind a stoichiometric quantity of Se which is released by SDS–PAGE.

[4] Mineral mix (g/kg mix): $CaCo_3$, 526.76; $MgCO_3$, 25.0; $MgSO_4 \cdot 7H_2O$, 32.76; NaCl, 69.0; KCl, 108.0; KH_2PO_4, 212.0; ferric ammonium citrate-green, 20.5; KI, 0.08; $MnSO_4 \cdot H_2O$, 3.33; NaF, 1.00; $AlNH_4(SO_4)_2 \cdot 12H_2O$, 0.16; $CuSo_4 \cdot 5H_2O$, 0.90; $CrCl_3 \cdot 6H_2O$, 0.51.

[5] Vitamin premix (mg/kg diet): glucose monohydrate, 8797.5; thiamin · HCl, 4.0; riboflavin, 2.5; pyridoxine · HCl, 2.0; calcium-D-pantothenate, 20; niacin, 100; menadione, 1.0; folic acid, 2.0; d-biotin, 1.0; vitamin B_{12} (0.1% triturate), 10.0; retinyl acetate and ergocalciferol (500,000 IU vitamin A/g and 50,000 IU ergocalciferol/g), 40.0; retinyl palmitate (250,000 IU vitamin A/g), 20 (providing a total of 25,000 IU vitamin A/kg diet and 2000 IU vitamin D/kg diet).

[6] Evenson JK, Sunde RA. Time course of selenium labeling of selenoproteins in Se-adequate and high Se rats. Fed Proc **46**:907, 1987.

dymides were each homogenized for 45 sec in 9 vol of Tris/SDS buffer using the Polytron homogenizer, and centrifuged (105,000*g*, 60 min) to obtain a tissue extract. All procedures were carried out at 4°C.

Experiment 2. To study Se metabolism in the Se-deficient rat, rats were fed the basal torula yeast-based diet for 41 days. The rats were Se-deficient as shown by an average liver GSH-Px activity of 0.016 EU/mg protein as compared to 0.594 EU/mg protein for the Se-adequate rats used in Experiment 1. Se-deficient rats (weighing 250–255 g) were administered ^{75}Se as described for Experiment 1, and tissue samples were prepared using identical procedures.

Experiment 3. To determine the importance of protein synthesis on the ^{75}Se labeling of selenoproteins, rats were pretreated with cycloheximide to block protein synthesis. Rats were fed either the Se-deficient torula yeast-based diet or that diet supplemented with 0.2 ppm Se as Na_2SeO_3 (Sigma). After 64 days, the rats (357–380 g) were injected ip with 5 mg/kg cycloheximide as described previously (11), and then administered 50 μCi ^{75}Se 30 min after the cycloheximide injection. Rats were killed 1, 3, and 6 hr post ^{75}Se administration. Rats killed 3 hr after ^{75}Se administration received a booster cycloheximide injection (1.5 mg/kg) 1.5 hr after the ^{75}Se injection; rats killed 6 hr post ^{75}Se administration received booster cycloheximide injections 1.5 and 3.5 hr after the ^{75}Se injection, as described previously (11).

Analyses. The GSH-Px activity of the various fractions was determined using the coupled assay procedure with H_2O_2 (12). Protein was determined as described by Lowry (13). ^{75}Se was measured using a gamma counter (MiniGamma 1275, LKB Instrument Co., Gaithersburg, MD) with an efficiency of 25%. To quantitate ^{75}Se labeling of proteins, proteins were separated according to subunit molecular weight using SDS gradient polyacrylamide slab gel electrophoresis using a slabgel apparatus (SE600 Hoefer, San Francisco, CA), and power supply (Hoefer PS2500). Samples (1500 μg protein) from individual rats were mixed with sample buffer (50 m*M* Tris, 1% SDS, 2% 2-mercaptoethanol), heated in a boiling water bath for 15 min, and then were electrophoresed using 3 mm SDS gels (14 × 16 cm; 0.1% SDS, pH 8.8; 7.5 to 20% acrylamide gradient; with 4.5% acrylamide stacking gel, pH 6.8) prepared according to the method of Laemmli (14). Each sample was loaded in a 1-cm-wide well using 30 mA per gel and then run at 60 mA per gel. In each experiment, the samples for each timepoint for a given tissue were electrophoresed on the same gel so that the ^{75}Se incorporation into a specific selenoprotein could be compared directly. Myofibrilar proteins from bovine muscle were used as molecular weight standards. Gels were fixed and stained for 12–14 hr in aqueous 50% (v/v) methanol, 10% (v/v) acetic acid, and 0.25% (w/v) Coomassie brilliant blue R and destained for 6 hr (aqueous 50% (v/v) methanol and 10% (v/v) acetic acid), followed by further destaining for 48 hr (aqueous 7.5% (v/v) acetic acid and 5% (v/v) methanol). ^{75}Se distribution was quantitated after fixing by cutting out each lane and slicing the lane into 2-mm slices which were counted individually for 3 min. The counts were corrected for decay, and the data were plotted for each sample without background subtraction to indicate the "signal-to-noise" ratio of the profile. Profiles for different time points were offset using an in-house basic program, and the composite figure for each tissue for a four-rat block was plotted. All chemicals and diet components were obtained from Sigma unless otherwise noted.

Results. To directly compare the ability of the SDS–PAGE with that of gel filtration chromatography to detect ^{75}Se incorporation into selenoproteins, liver cytosol from a Se-deficient rat was subjected to both procedures as described in Fig. 1. Sephadex G-150 chromatography (Fig. 1A) showed four major ^{75}Se peaks corresponding to >200, 80–95, 20–30, and <5 kDa, and containing 6, 20, 12, and 53%, respectively, of the applied ^{75}Se. The SDS–PAGE (Fig. 1B) resulted in two major ^{75}Se-labeled proteins of 65 and 23 kDa (GSH-Px), which contained 17 and 6%, respectively, of the applied ^{75}Se. When the pooled fractions eluting at the position of GSH-Px (42–55) were concentrated and then subjected to the SDS–PAGE, the resulting profile (data not shown) revealed that the 80- to 95-kDa ^{75}Se peak contained the 65-kDa, the 23-kDa, and several of the minor ^{75}Se

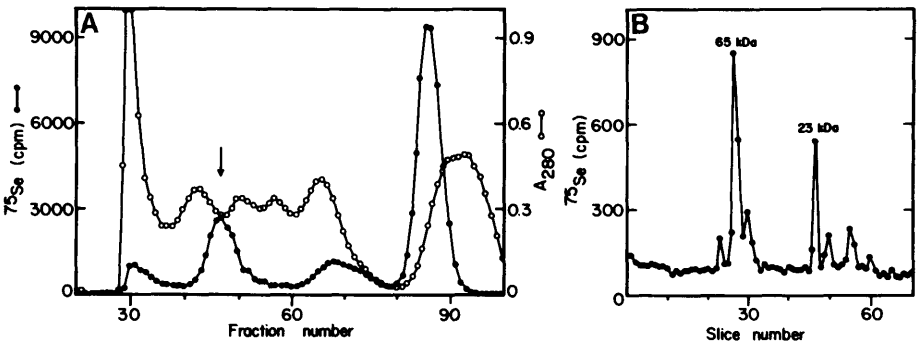

FIG. 1. Analysis of ^{75}Se labeling of proteins in rat liver cytosol using gel filtration chromatography (A) or SDS–PAGE (B). A 330-g Se-deficient rat was injected with 50 μCi [^{75}Se]selenite (1.3 μg Se) and killed 3 hr later, and liver cytosol was prepared as described under Materials and Methods. (A) For gel filtration chromatography, 10 ml (656,000 cpm) was applied to a Sephadex G-150 column (2.6 × 96 cm) and chromatographed as described previously (11). For each 5-ml fraction, 1 ml was counted using a Packard Model 5630 gamma counter (60% efficiency), and the resulting ^{75}Se profile and A_{280} profile are shown. The arrow indicates the elution position of GSH-Px. (B) For SDS–PAGE, 200 μl (13,200 cpm) of the same cytosol was analyzed as described for Fig. 2. Gel slices were counted using the Packard Model 5630 gamma counter, and the ^{75}Se profile resulting from the SDS–PAGE is plotted.

proteins detected using the SDS–PAGE procedure alone. We have previously shown that cycloheximide pretreatment blocks ^{75}Se incorporation into the 80- to 95-kDa ^{75}Se peak but does not block labeling of the >200-kDa, 20- to 30-kDa, and <5-kDa ^{75}Se peaks in the G-150 profile (11). This preliminary experiment thus demonstrates that SDS–PAGE is readily able to distinguish ^{75}Se-labeled selenoproteins from proteins that bind ^{75}Se, and that SDS-PAGE can separate ^{75}Se-labeled proteins (subunits) that are not separated using gel filtration chromatography.

When the Se-adequate rats were injected iv with a tracer dose of [^{75}Se]selenite, over 40% of the ^{75}Se was present in the liver 1 hr after ^{75}Se administration (Table I). The percentage of the injected ^{75}Se found in the liver declined to 12% of the dose by 72 hr postinjection, but the liver remained the tissue with the highest amount of ^{75}Se at all times in the Se-adequate rat. Blood, kidney, heart, and epididymides ^{75}Se increased from 1 to 3 hr, and then decreased by 24 hr, whereas testes ^{75}Se in Se-adequate rats increased from 0.17 to 1.38% of the dose by 24 hr postinjection (Table I). In Se-deficient rats, the liver contained 70% of the injected ^{75}Se 1 hr after ^{75}Se administration, and liver ^{75}Se then declined to 17% by 72 hr postinjection. Kidney ^{75}Se increased 2.4-fold and testes ^{75}Se increased 108-fold in the Se-deficient rats over the course of the experiment. Cycloheximide pretreatment (Table I) lessened the decline in liver ^{75}Se in both Se-adequate and Se-deficient rats, and it blocked the increase in blood ^{75}Se (from 1 to 3 hr) and in testes ^{75}Se that was observed in untreated rats.

In plasma of Se-adequate and Se-deficient rats (Fig. 2), a 55-kDa protein was the sole ^{75}Se protein observed at 1 and 3 hr. This protein was labeled with fourfold more ^{75}Se in deficient rats as compared to Se-adequate rats, and at 3 hr this selenoprotein contained more ^{75}Se than any other ^{75}Se protein in either Se-adequate or Se-deficient rats. At 24 and 72 hr postinjection, the ^{75}Se labeling of this protein was reduced, but labeling of GSH-Px (23-kDa subunit) increased concomitantly at 24 and 72 hr in both Se-adequate and Se-deficient plasma. Quantitatively similar patterns of labeling occurred in liver microsomes (not shown) and heart (Fig. 6). Table II shows the cpm ^{75}Se applied to the gel and the percentage of applied ^{75}Se recovered in the gel for each sample.

In liver cytosol (Fig. 3), GSH-Px (23 kDa) was the major ^{75}Se-labeled species at all times in the Se-adequate rats; labeling at 24 and 72 hr was only twice that observed at 1 hr. In the

TABLE I. TISSUE DISTRIBUTION OF ^{75}Se

	1 hr		3 hr		6 hr		24 hr		72 hr	
Tissue	+Se	−Se	+Se	−Se	+Se	−Se	+Se	−Se	+Se	−Se
Se-adequate and Se-deficient rats[a,b]										
Blood[c]	12.5	26.4	17.5	38.0			5.3	16.5	7.0	11.7
Liver	43.9	70.3	30.1	36.0			11.3	14.4	11.6	16.6
Kidney	4.9	5.6	6.6	6.4			4.5	8.0	2.3	13.4
Heart	0.2	0.4	0.4	1.0			0.3	0.4	0.2	0.4
Testis	0.2	0.6	0.3	2.2			1.4	16.7	1.3	18.3
Epididy.	0.1	0.1	0.1	0.0			0.1	0.3	0.1	0.3
Muscle[d]	6.3	9.8	6.9[e]	11.0			8.4	15.7	7.7	11.3
Total	68.1	113.2	61.9	94.6			31.3	72.0	30.2	72.0
Cycloheximide-treated rats[f]										
Blood[c]	13.3	12.8	9.3	12.3	10.4	13.8				
Liver	53.3	54.7	49.1	77.6	18.8	56.5				
Kidney	4.0	5.2	8.0	7.8	9.9	11.0				
Heart	0.2	0.3	0.2	0.2	0.2	0.3				
Testis	0.4	0.6	0.4	0.4	0.3	0.4				
Epididy.	0.1	0.1	0.1	0.1	0.2	0.2				
Muscle[d]	8.6	8.3	6.9	7.7	8.0	8.0				
Total	79.9	82.0	74.0	106.1	47.8	90.2				

[a] Percentage of administered dose (50 μCi/rat).

[b] Se-adequate rats (Experiment 1, 0.35 ppm dietary Se) and Se-deficient rats (Experiment 2, <0.02 ppm dietary Se) are identified as +Se or −Se, respectively. The data at each time point are for the one rat that provided the samples for the SDS–gel electrophoresis analyses.

[c] Calculation based on a total whole blood weight of 8% of body weight and a blood density of 1.05 g/ml.

[d] Calculation based on a total muscle weight of 40% of body weight.

[e] Sample lost; value estimated from Experiment 3.

[f] Se-adequate (Experiment 3, 0.2 ppm dietary Se as sodium selenite) and Se-deficient rats (<0.02 ppm dietary Se) were pretreated with cycloheximide (5 mg/kg body weight) 30 min prior to ^{75}Se injection; rats killed 3 and 6 hr after ^{75}Se injection were given booster cycloheximide injections (1.5 mg/kg body weight) as described in the text.

Se-adequate rat, liver cytosolic GSH-Px contained more ^{75}Se than any other ^{75}Se protein in any tissue except the plasma ^{75}Se protein. Distinctly labeled ^{75}Se proteins of 14, 55, and 65 kDa with low levels of ^{75}Se were observed in addition to GSH-Px in the Se-adequate liver cytosol. The 20-kDa ^{75}Se protein (adjacent to the 23-kDa GSH-Px subunit) most likely is a smaller molecular weight form of GSH-Px, as shown by immunoblotting and Coomassie blue staining of purified GSH-Px (15). In the Se-deficient liver cytosol, a moderately labeled ^{75}Se protein of 65 kDa was observed at 3, 24, and 72 hr; GSH-Px (23 and 20 kDa) and the 55-kDa ^{75}Se protein were labeled to a lesser extent. In liver mitochondrial extract (Fig. 4), a 65-kDa ^{75}Se protein at 1 hr and a 23-kDa ^{75}Se-protein at 3 hr were the major ^{75}Se proteins in the Se-adequate rat. The magnitude of labeling of these ^{75}Se proteins was higher in Se-deficient liver mitochondrial extract, and a number of lesser labeled ^{75}Se proteins were observed. Similar labeling was observed in liver microsomal extract (now shown) from Se-adequate and Se-deficient rats.

In Se-adequate kidney cytosol (Fig. 5), GSH-Px as well as a 10 and a 65-kDa ^{75}Se protein progressively increased in magnitude from 1 to 72 hr. A similar labeling pattern with larger amounts of ^{75}Se was observed in the Se-deficient kidney cytosol, including a 45-kDa ^{75}Se protein. In kidney mitochondrial extract (not shown), GSH-Px was the

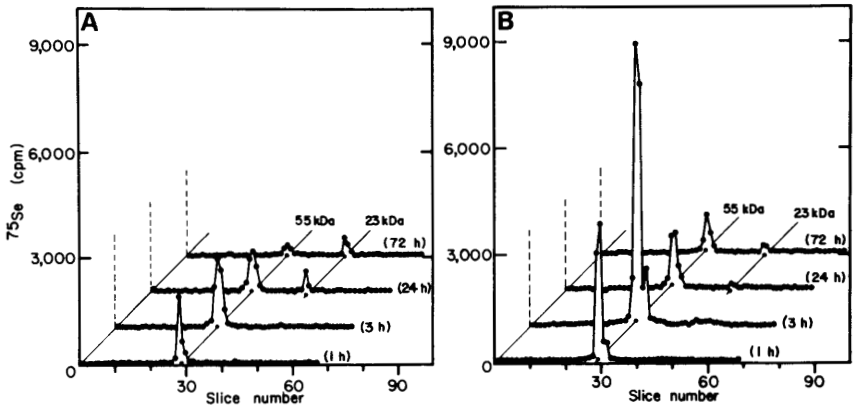

FIG. 2. Incorporation of ^{75}Se into plasma proteins. Se-adequate (A) and Se-deficient (B) rats were injected iv with 50 μCi [^{75}Se]selenite (0.5 μg Se), and killed 1, 3, 24, or 72 hr after injection. Each profile is from one rat. Plasma samples (1500 μg protein) were separated using gradient SDS/PAGE. The counts per minute (cpm) of ^{75}Se in each sample applied to the gel are indicated in Table II. Sample lanes in each gel were cut into 2-mm slices and counted. The cpm for each slice were plotted to show the ^{75}Se incorporation into the various ^{75}Se proteins of different subunit molecular weights. Slice 1 contains polypeptides of the highest molecular weight. Plots for 3, 24, and 72 hr were staggered, and diagonal lines were drawn through the major ^{75}Se proteins to show the change in ^{75}Se incorporation in these species with time.

major ^{75}Se protein at all times in the Se-adequate and Se-deficient rats; other small ^{75}Se proteins were more distinct in Se-deficient than in Se-adequate kidney mitochondrial extracts. A multitude of ^{75}Se proteins was observed in Se-adequate kidney microsomal extract (not shown), but GSH-Px was the sole moderately labeled ^{75}Se protein in Se-deficient kidney microsomal extract.

In Se-adequate and Se-deficient hearts (Fig. 6) there was a progressive increase from 1 to 72 hr in ^{75}Se labeling of GSH-Px (23 kDa in Se-adequate and 20 kDa in Se-deficient heart). A prominent 65-kDa ^{75}Se protein was also observed 1 to 24 hr after ^{75}Se injection. In testes (Fig. 7), the only distinct ^{75}Se protein in the Se-adequate rat was a 17-kDa ^{75}Se protein observed at 72 hr. This ^{75}Se protein

TABLE II. ^{75}Se APPLIED TO AND RECOVERED IN THE GEL[a]

Tissue	Se status[b]	1 hr	3 hr	24 hr	72 hr
		(cpm)			
Plasma	+Se	7,980 (41)	12,700 (48)	6,510 (67)	3,560 (55)
	−Se	10,500 (85)	20,700 (100)	7,050 (71)	4,640 (58)
Liver cytosol	+Se	17,900 (16)	10,500 (19)	5,100 (53)	3,810 (59)
	−Se	10,800 (16)	4,810 (38)	3,120 (47)	2,460 (51)
Liver mito.	+Se	7,100 (44)	5,040 (43)	2,580 (68)	1,830 (40)
	−Se	9,870 (24)	7,310 (44)	2,460 (45)	2,220 (43)
Kidney cytosol	+Se	7,750 (15)	11,300 (17)	5,720 (41)	3,160 (61)
	−Se	6,560 (31)	5,420 (41)	4,700 (51)	5,630 (49)
Heart extract	+Se	870 (68)	1,430 (65)	1,070 (105)	752 (96)
	−Se	1,070 (35)	1,900 (45)	1,080 (47)	1,010 (35)
Testis extract	+Se	699 (13)	1,050 (41)	2,390 (45)	2,670 (56)
	−Se	850 (58)	2,890 (49)	16,800 (50)	27,300 (54)

[a] Counts per minute (cpm) ^{75}Se above background applied to the gel for each time point shown in Figs. 2–7. The percentage of the applied ^{75}Se recovered in the slices is given in parentheses.

[b] Se-adequate rats and Se-deficient rats are identified as +Se and −Se, respectively.

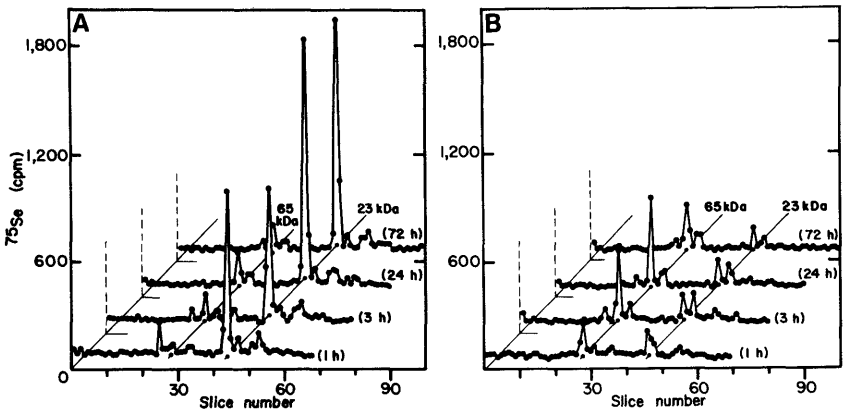

FIG. 3. Incorporation of ^{75}Se into proteins in liver cytosol. Liver cytosol from Se-adequate (A) and Se-deficient (B) rats, injected with [^{75}Se]selenite 1, 3, 24, or 72 hr prior to sacrifice, was analyzed as described for Fig. 2.

had the second largest level of ^{75}Se labeling of all tissues examined in Se-deficient rats.

Cycloheximide pretreatment. The above results indicate that a number of proteins were labeled by administration of ^{75}Se, but the nature of this labeling was unclear. Cycloheximide pretreatment, which has been shown to block Zn-induced synthesis of metallothionein (16) and ^{75}Se incorporation into GSH-Px (11), completely blocked incorporation of ^{75}Se into any of the selenoproteins at 1, 3, or 6 hr postinjection in both ^{75}Se-adequate and ^{75}Se-deficient rats (Fig. 8). No ^{75}Se proteins were detected using this SDS–PAGE procedure in spite of the high levels of ^{75}Se present in the tissues of the cycloheximide-treated rats (Table I), showing that protein synthesis is required for ^{75}Se incorporation into these selenoproteins.

Discussion. These experiments have examined the ^{75}Se incorporation of ^{75}Se into selenoproteins in various tissues of Se-adequate and Se-deficient rats using SDS–PAGE. This technique separates the subunits of proteins according to size, and permits the quantitation of ^{75}Se incorporation by count-

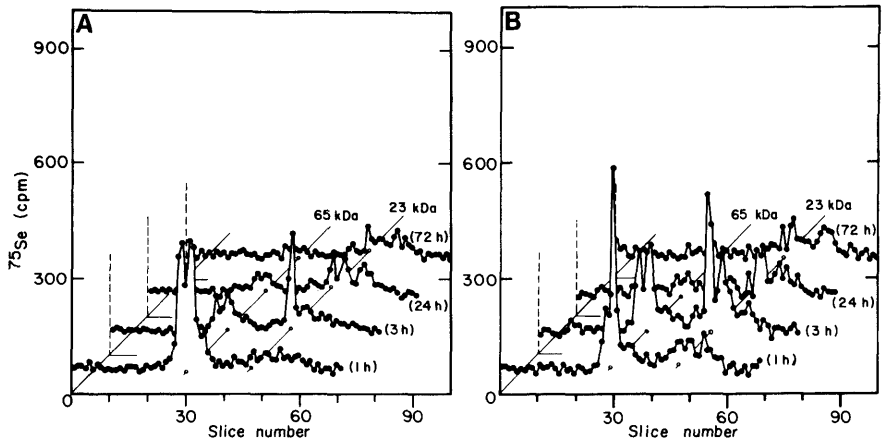

FIG. 4. Incorporation of ^{75}Se into mitochondrial proteins of liver. Liver mitochrondria from Se-adequate (A) and Se-deficient (B) rats, injected with [^{75}Se]selenite 1, 3, 24, or 72 hr prior to sacrifice, were homogenized in Tris/SDS and then centrifuged (105,000*g*, 60 min) to obtain a mitochondrial extract that was analyzed as described for Fig. 2.

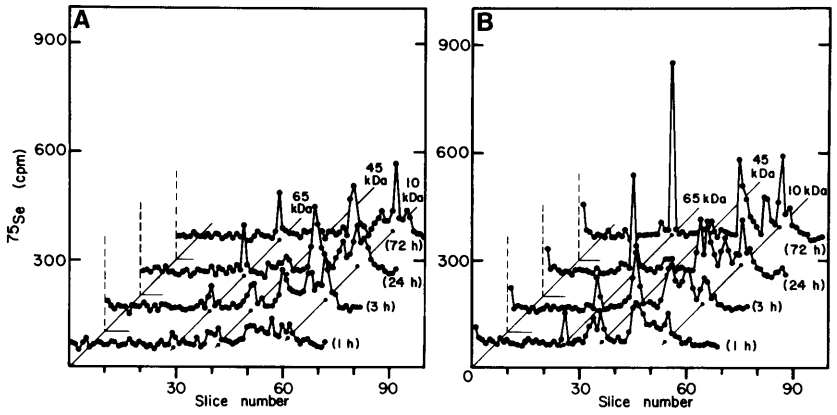

FIG. 5. Incorporation of ^{75}Se into proteins in kidney cytosol. Kidney cytosol from Se-adequate (A) and Se-deficient (B) rats, injected with [^{75}Se]selenite 1, 3, 24, or 72 hr prior to sacrifice, was analyzed as described for Fig. 2.

ing of slices of the fixed protein bands (Fig. 1). A 55-kDa plasma protein subunit contained the highest level of ^{75}Se of any single ^{75}Se protein in both Se-adequate and Se-deficient rats 3 hr after administration of 50 μCi of [^{75}Se]selenite. The 55-kDa protein in Se-deficient rat plasma at 3 hr contained four times the ^{75}Se as did the 55-kDa protein in Se-adequate rat plasma. This protein is most likely the large subunit of the 80-kDa plasma selenoprotein first reported by Herrman (17), and later further characterized by Burk and Gregory (18) and Motzenbocker and Tappel (19). The use of SDS–PAGE permits clear discrimination between the 55-kDa plasma protein and the 23-kDa subunit of plasma GSH-Px (Fig. 2).

The second-highest labeling of a single ^{75}Se protein in Se-adequate rat tissue was GSH-Px in rat liver cytosol 24 to 72 hr after ^{75}Se injection. Immunoblotting of this protein band using IgG prepared against purified rat liver GSH-Px has shown that this protein subunit is GSH-Px (15). Coomassie blue staining, immunoblotting (15), and ^{75}Se incorporation (Fig. 3) all show a doublet of GSH-Px subunits, indicating that two slightly different molecular weight forms of

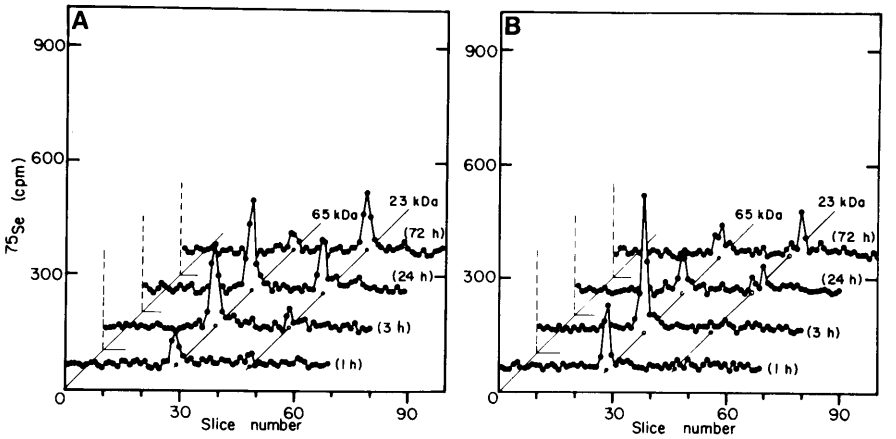

FIG. 6. Incorporation of ^{75}Se into heart proteins. Hearts from Se-adequate (A) and Se-deficient (B) rats, injected with [^{75}Se]selenite 1, 3, 24, or 72 hr prior to sacrifice, were homogenized in Tris/SDS and then centrifuged (105,000*g*, 60 min) to obtain an extract that was analyzed as described for Fig. 2.

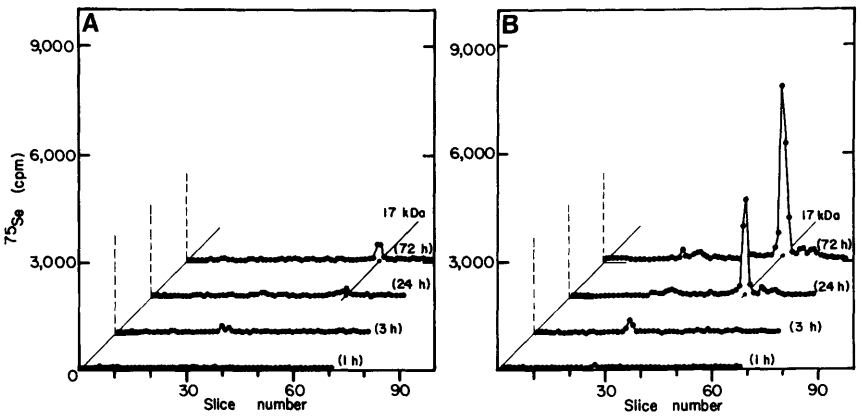

FIG. 7. Incorporation of ^{75}Se into testes proteins. Testes from Se-adequate (A) and Se-deficient (B) rats, injected with [^{75}Se]selenite 1, 3, 24, or 72 hr prior to sacrifice, were homogenized in Tris/SDS and then centrifuged (105,000*g*, 60 min) to obtain a supernatant that was analyzed as described for Fig. 2.

GSH-Px occur. The 20-kDa form may be the result of post-translational processing of the larger molecular weight form *in vivo*, or it may have resulted from proteolytic cleavage during tissue preparation. GSH-Px was labeled with ^{75}Se to a lesser extent in Se-adequate kidney and heart than in liver. In Se-deficient rats, the ^{75}Se-labeling of GSH-Px in liver, kidney, and heart was also reduced as compared to Se-adequate rats. In these studies, we have not attempted to distinguish between the classical GSH-Px subunit and the recently discovered phospholipid hydroperoxide GSH-Px, also with a reported molecular weight of approximately 23 kDa (6).

The second-most labeled ^{75}Se protein in Se-deficient rat tissue was a 17-kDa protein in testes 24 and 72 hr but not 1 or 3 hr after ^{75}Se administration. The lack of substantial labeling of a 17-kDa protein in any other tissue at any time point indicates that synthesis of this protein occurs specifically in the testes. The level of ^{75}Se incorporation in the Se-adequate rat testes was 10% of that observed in the Se-deficient rat, most likely due to the dilution of ^{75}Se by endogenous Se in the Se-adequate rat. This 17-kDa protein would appear to be the 17- to 20-kDa selenoprotein discovered in rat sperm (20) and bovine sperm (21), and this protein may account for the sixfold increase in testes Se in the rat at sexual maturity (22). The origin of the ^{75}Se that was incorporated into this 17-kDa protein has not been established, but the timing and quantity of ^{75}Se necessary to label this protein would suggest that the plasma 55-kDa ^{75}Se protein may be the source of the ^{75}Se. This idea is further supported by the low level of ^{75}Se in testes (Table I) when cycloheximide pretreatment also blocked the synthesis of the plasma 55-kDa protein. This hypothesis was previously suggested by Motzenbocker and Tappel (19), and the low levels of a 55-kDa ^{75}Se protein in liver microsomes and cytosol observed in our experiments are consistent with this hypothesis. These experiments thus suggest that metabolism of these selenoproteins is an important aspect of Se flux between tissues.

A previously unreported 65-kDa selenoprotein was observed in these studies in liver, kidney, heart, and testes, but the protein is otherwise completely uncharacterized. A number of other ^{75}Se-labeled proteins, containing lesser amounts of ^{75}Se, were also observed, especially in Se-deficient liver microsomal and mitochondrial extracts, and in Se-adequate kidney microsomal extract. These results clearly suggest that a number of other selenoproteins are present in mammalian cells in addition to GSH-Px, the 17-kDa sperm selenoprotein, and the 55-kDa plasma selenoprotein.

We began using SDS–PAGE to study selenoproteins because we thought that it might electrophoretically remove Se from Se-binding proteins so that we could resolve selenoproteins, such as GSH-Px, from pro-

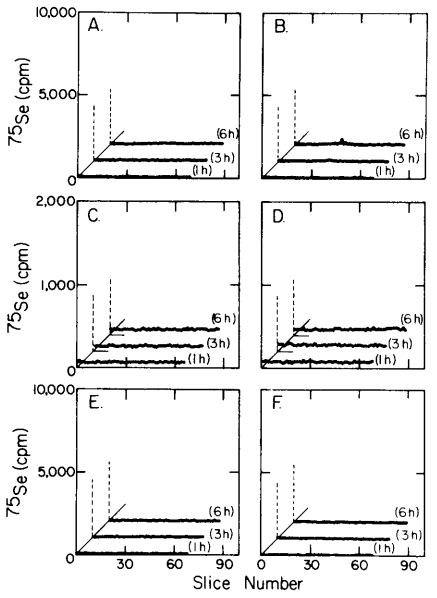

FIG. 8. Cyclohexamide inhibition of ^{75}Se incorporation into protein in plasma, liver cytosol, and testes supernatant. Rats were injected ip with cycloheximide (5 mg/kg rat weight) 30 min prior to [^{75}Se] selenite injection, as described in the text, and killed 1, 3, or 6 hr after ^{75}Se administration. Plasma (A, B), liver cytosol (C, D), and testes supernatant (E, F) were analyzed as described for Figs. 2, 3, and 7, respectively. (A, C, and E) Se-adequate tissues, (B, D, and F) Se-deficient tissues. The profiles were plotted using the same axis scales as were used for the non-cycloheximide-treated tissues (Figs. 2, 3, and 7). The samples applied to the gel had cpm of ^{75}Se with ranges of 3940–7750, 4,800–14,700, and 334–628 cpm for the plasma, liver cytosol, and testes extract samples, respectively.

teins that bind Se but which can be dissociated by SDS + 2-mercaptoethanol and separated by electrophoresis. The multitude of selenoproteins observed in Experiment 1 and 2, however, initially suggested that SDS–PAGE may not have removed the bound Se from all the binding proteins. The use of cycloheximide as a protein synthesis inhibitor (Experiment 3), however, clearly demonstrated that protein synthesis was required for the labeling of the detected ^{75}Se proteins, just as shown previously for GSH-Px (11). This indicates that the detected ^{75}Se was incorporated into these selenoproteins rather than just bound to ^{75}Se-binding proteins. The form of Se in all of these selenoproteins is not known, but because protein synthesis is required, the Se in these species may be present as selenocysteine just as in GSH-Px. These selenoproteins would thus be prime candidates for the reservoir of selenocysteine that Hawkes and co-workers (7) found in rats administered ^{75}Se.

Previous chromatographic analyses of tissues for selenoproteins have detected a number of different ^{75}Se-labeled proteins (7, 11, 23). These chromatographic procedures, however, were not able to distinguish selenoproteins containing the selenocysteine moiety, such as GSH-Px, from proteins that bind ^{75}Se in a different manner. In a preliminary experiment (Fig. 1), Sephadex G-150 chromatography suggested that three or more ^{75}Se-labeled proteins may have been present in Se-deficient liver cytosol in addition to the low-molecular-weight (<5 kDa) species, whereas SDS–PAGE detected only two major selenoproteins, and further analysis revealed that both of these selenoproteins were contained in the same 80- to 95-kDa ^{75}Se peak in the G-150 chromatographic profile. Previous chromatographic analysis (11) of Se-adequate liver cytosol 1 and 3 hr after ^{75}Se administration indicated that GSH-Px contained only 4 and 9%, respectively, of the ^{75}Se present in the profile, whereas SDS–PAGE showed clearly that GSH-Px was the major cytosolic ^{75}Se protein in Se-adequate rat liver at these and later times (Fig. 3). Sephadex G-150 chromatography of liver cytosol (11) from cycloheximide-treated rats showed apparent ^{75}Se-labeled proteins with molecular weights of >200 and 20–30 kDa, whereas SDS–PAGE (Fig. 8) detected no selenoproteins. Thus SDS–PAGE is a useful technique that can distinguish selenoproteins like GSH-Px from proteins that bind Se in a more labile manner.

We have reported that inorganic forms of Se are more readily incorporated into GSH-Px than was Se from selenocysteine (23), and we have recently shown that the carbon skeleton of the Se–Cys moiety of GSH-Px was derived from serine (24). Hawkes *et al.* (25), however, reported that Se incorporation was mediated by a selenocysteine tRNA. The implied conflict between these results has seemingly been resolved by researchers who

determined the nucleotide sequence for GSH-Px (26) and for bacterial formate dehydrogenase (27). Both groups have shown that the nonsense codon UGA codes for selenocysteine in these two selenoenzymes. Two naturally occurring serine tRNAs that are specific for UGA have been identified in mammalian cells (28). These tRNAs are unique because the serine is further phosphorylated by a kinase while the serine is esterified to the tRNA. We have suggested (24) that an additional metabolic step, replacing phosphate with -SeH, may be the mechanism for synthesis of selenocysteine. Thus Se incorporation into GSH-Px may be directed by a tRNA that has its serine phosphorylated before the phosphoserine is converted to selenocysteine, either on the tRNA or after incorporation into the protein. The selenoproteins observed in this study are likely to be formed by the same cotranslational mechanism, since protein synthesis is necessary for ^{75}Se labeling of these selenoproteins as well as GSH-Px.

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1. Stadtman TC. Specific occurrence of selenium in enzymes and amino acid tRNAs. FASEB J. **1**:375–379, 1987.
2. Sunde RA. The biochemistry of selenoproteins. J Amer Oil Chem Soc **61**:1891–1900, 1984.
3. Rotruck JT, Pope AL, Ganther HE, Swanson AB, Hafeman DG, Hoekstra WG. Selenium: Biochemical role as a component of glutathione peroxidase. Science **179**:588–590, 1973.
4. Mills GC. Hemoglobin catabolism. I. Glutathione peroxidase, an erythrocyte enzyme which protects hemoglobin from oxidative breakdown. J Biol Chem **229**:189–197, 1957.
5. Lawrence RA, Burk RF. Glutathione peroxidase activity in selenium deficient rat liver. Biochem Biophys Res Commun **71**:952–958, 1976.
6. Ursini F, Maiorino M, Gregolin C. The selenoenzyme phospholipid hydroperoxide glutathione peroxidase. Biochim Biophys Acta **839**:62–70, 1985.
7. Hawkes WC, Wilhelmsen EC, Tappel AL. Abundance and tissue distribution of selenocysteine-containing proteins in the rat. J Inorg Biochem **23**:77–92, 1985.
8. Oh SH, Ganther HE, Hoekstra WG. Selenium as a component of glutathione peroxidase isolated from ovine erythrocytes. Biochemistry **13**:1825–1829, 1974.
9. Schwarz K. Development and status of experimental work on factor 3-Selenium. Fed Proc **20**:666–673, 1961.
10. Hafeman DG, Hoekstra WG. Protection against carbon tetrachloride-induced lipid peroxidation in the rat by dietary vitamin E, selenium and methionine as measured by ethane evolution. J Nutr **107**:656–665, 1977.
11. Sunde RA, Hoekstra WG. Incorporation of selenium into liver glutathione peroxidase in the Se-adequate and Se-deficient rat. Proc Soc Exp Biol Med **165**:291–297, 1980.
12. Lawrence RA, Sunde RA, Schwartz GL, Hoekstra WG. Glutathione peroxidase activity in rat lens and other tissues in relation to dietary selenium intake. Exp Eye Res **18**:563–569, 1974.
13. Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem **193**:265–275, 1951.
14. Laemmli UK. Cleavage of structual proteins during the assembly of the head of bacteriophage T_4. Nature (London) **227**:680–685, 1970.
15. Knight SAB, Sunde RA. The effect of progressive selenium deficiency on anti-glutathione peroxidase-antibody reactive protein in rat liver. J Nutr **117**:732–738, 1987.
16. Richards MP, Cousins RJ. Mammalian Zn homeostasis: Requirement for RNA and metallothionein synthesis. Biochem Biophys Res Commun **64**:1215–1223, 1975.
17. Herrman JL. The properties of a rat serum protein labelled by the injection of sodium selenite. Biochim Biophys Acta **500**:61–70, 1977.
18. Burk RF, Gregory PE. Some characteristics of ^{75}Se-P, a selenoprotein found in rat liver and plasma and comparison of it with selenoglutathione peroxidase. Arch Biochem Biophys **213**:73–80, 1982.
19. Motzenbocker MA, Tappel AL. A selenocysteine-containing selenium-transport protein in rat plasma. Biochim Biophys Acta **719**:147–153, 1982.
20. Calvin HI. Selective incorporation of selenium-75 into a polypeptide of the rat sperm tail. J Exp Zool **204**:445–452, 1978.
21. Pallini V, Bacci E. Bull sperm selenium is bound to a structural protein in mitochondria. J Submicrosc Cytol **11**:165–170, 1979.
22. Behne D, Duk M, Elger W. Selenium content and glutathione peroxidase activity in the testis of the maturing rat. J Nutr **116**:1442–1447, 1986.
23. Sunde RA, Hoekstra WG. Incorporation of selenium from selenite and selenocystine into glutathione peroxidase in the isolated perfused rat liver. Biochem Biophys Res Commun **93**:1181–1188, 1980.

24. Sunde RA, Evenson JK. Serine incorporation into the selenocysteine moiety of glutathione peroxidase. J Biol Chem **262**:933–937, 1987.
25. Hawkes WC, Lyons DE, Tappel AL. Identification of a selenocysteine-specific aminoacyl transfer RNA from rat liver. Biochim Biophys Acta **699**:183–191, 1982.
26. Chambers I, Frampton J, Goldfarb P, Affara N, McBain W, Harrison PR. The structure of the mouse glutathione peroxidase gene: The selenocysteine in the active site is encoded by the 'termination' codon, TGA. EMBO J **5**:1221–1227, 1986.
27. Zinoni F, Birkmann A, Stadtman TC, Bock A. Nucleotide sequence and expression of the selenocysteine-containing polypeptide of formate dehydrogenase (formate-hydrogen-lyase-linked) from *Escherichia coli.* Proc Natl Acad Sci USA **83**:4650–4654, 1986.
28. Hatfield D. Suppression of termination codons in higher eukaryotes. Trends Biochem Sci **10**:201–204, 1985.
29. Vallee BL, Wacker, WEC. Metalloproteins. In: Neurath, H, Ed. The Proteins. New York, Academic Press, Vol 5:pp25–60, 1970.

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