Levels and ^{75}Se-Labeling of Specific Proteins as a Consequence of Dietary Selenium Concentration in Mice and Rats (43171)

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Abstract. **Selenium-labeled proteins (SLP) distinct from glutathione peroxidase (GSH-PX) recently have been purified and partially characterized. Antisera to two SLP, a 56-kDa and a 14-kDa protein, were generated in rabbits and used to examine expression of these proteins as a consequence of dietary selenium concentration (0.02, 0.2, 2.0 ppm) in mice and rats. Additionally, the kinetics of ^{75}Se labeling in plasma, liver, kidney, and mammary gland were examined over a 40-hr time period as a function of dietary selenium concentration. A plasma 57-kDa protein was labeled by 30 min after ^{75}Se injection and reached maximum labeling by 4 hr. The cellular 56-kDa and 14-kDa proteins, as well as GSH-Px, labeled progressively over 40 hr starting between 1 and 4 hr after injection. In general, the 56-kDa and GSH-Px followed similar labeling patterns, whereas the 14-kDa protein was labeled less and was not labeled in discernible quantities until 40 hr. The extent of labeling of all proteins was inversely proportional to the dietary selenium concentration and was probably a reflection of different endogenous selenium body pools. The most important observation was generated by the immunoblot data. The amount of 56-kDa and 14-kDa proteins as detected and measured on immunoblots was not a function of dietary selenium concentration. This result suggests that the synthesis and maintenance of the 56-kDa and 14-kDa proteins are not selenium dependent, a characteristic which distinguishes the two proteins from GSH-Px. The single exception to the above results was the 40% decrease of liver 14-kDa protein concentration in carcinogen-treated rats fed 2.0 ppm of selenium. An organic selenium compound, selenobetaine, did not lead to a decrease under similar conditions. In 15 rat mammary tumors induced by 7,12-dimethylbenzanthracene and analyzed on immunoblots, the SLP-56 was undetected in 5 cases and appeared as two bands (56,000 Da, 50,000 Da) in 10 cases. This latter result raises the possibility that the expression of SLP-56 may be altered in mammary tumors as compared with normal mammary gland.** [P.S.E.B.M. 1991, Vol 196]

Numerous experiments have documented the efficacy of selenium as a potent chemopreventive agent for epithelial tumors induced by chemical carcinogens (reviewed in 1, 2). In mice and rats, selenium (generally administered as Na_2SeO_3) reduces the incidence of 7,12-dimethylbenzanthracene (DMBA)-induced mammary adenocarcinomas by 50% when fed in the diet at 2–3 ppm (3, 4). The biochemical basis underlying the chemopreventive effects of selenium is not understood. At nutritional levels, selenium is essential for the enzyme glutathione peroxidase (GSH-Px), which catalyzes the reduction of hydrogen peroxide and organic hydroperoxides (5). However, several experiments have ruled out a function for enhanced GSH-Px activity in selenium-mediated inhibition of mammary tumorigenesis (6–9).

Numerous investigators have documented the presence of selenium-labeled proteins other than GSH-Px

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(10–19). These proteins have been identified using column chromatography or electrophoretic methods; however, only a few have been purified and characterized by other methods. Yang *et al.* (16) have purified a 57-kDa protein (also known as selenoprotein P) from rat plasma and developed antibodies specific for the protein. The protein has been proposed to function as a selenium-transport protein (20) and to protect against oxidant stress (21). Bansal *et al.* (22) have purified proteins of 56 kDa and 14 kDa from mouse liver and developed antisera specific for each protein. The antiserum to the liver 56 kDa did not recognize the plasma 57 kDa in mice or rats (22, 23). Both the 14- and 56-kDa proteins have been further characterized. The 14-kDa protein was specific to liver and was identified as mouse liver fatty acid-binding protein (FABP) for which selenium is a ligand (24). The 56 kDa has been sequenced at the nucleotide and amino acid levels and is a unique protein (25). The nature of selenium binding to each of the proteins is unknown; however, a TGA codon specific for selenocysteine was not present in either sequence (24, 25).

Experiments in cell culture using mammary epithelial cells demonstrated that the amount of ^{75}Se labeling of the 56-kDa protein was proportional to the amount of selenium in the growth medium and inversely proportional to the proliferative phase of the cell population (26, 27). The cell culture experiments led us to propose that the 56-kDa protein (as distinct from GSH-Px) was involved in the growth inhibitory effects of selenite. In this article, we report on the tissue levels and ^{75}Se labeling of the 56-kDa and 14-kDa proteins as a function of dietary selenium concentration. We term these proteins selenium-labeled proteins (SLP) to convey the fact that they avidly bind selenium (retain the label after electrophoresis). GSH-Px is a classical selenoprotein with selenium present as selenocysteine. It is a selenium-labeled protein whose mechanism of selenium incorporation is well established. For simplicity's sake, we refer to the 56 kDa, 14 kDa, and GSH-Px proteins as selenium-labeled proteins. The term is not meant to convey any statement regarding the specific nature of selenium binding to the protein.

Materials and Methods

Diet. Mice and rats were maintained on a basal A1N-76 purified diet but using a mineral mixture without selenium (9). This basal diet contained approximately 0.02–0.03 ppm of selenium (4, 9). Additional selenium was added as sodium selenite (0.1–2.0 ppm) or selenobetaine (2.0 ppm) (9). In mice, the animals were fed the control diets from the time of weanling and the specific selenium diets started at 8 weeks of age. In rats, the specific selenium diets were started at approximately 6 weeks and continued until they were sacrificed. DMBA (10.0 mg) was given at approximately 8 weeks of age (9).

^{75}Se Injections. Mice were given a single intraperitoneal injection of 0.25 mCi of ^{75}Se as selenious acid (sp act = 179.3 mCi/mg). At different time periods thereafter, the animals were killed and their tissues collected and processed as described below.

Preparation of Plasma and Tissues Samples for Immunoblot/Polyacrylamide Electrophoresis. The animals were anesthetized with ether and exanguinated by cardiac puncture. Blood was collected using EDTA as an anticoagulant. The plasma was separated from the cellular components by centrifugation. The required tissues (300–400 mg) were removed rapidly from each mouse/rat, cut into pieces, and washed in ice-cold 0.9% NaCl solution containing di-NaEDTA (1 m*M*), dithiothreitol (1 m*M*), Aprotinin (0.5 μg/ml), and phenylmethylsulfonyl fluoride (1 m*M*) to inhibit protease activity. After washing, the tissues were blotted dry and homogenized. A 10% homogenate of each tissue sample was made using 20 m*M* Tris (pH 7.4) containing di-NaEDTA (1 m*M*), dithiothreitol (1 m*M*), Aprotinin (0.5 μg/ml), and phenylmethylsulfonyl fluoride (1 m*M*) to inhibit protease activity.

Sodium Dodecyl Sulfate-Polyacrylamide Electrophoresis (SDS-PAGE). Discontinuous SDS-PAGE was performed as described by Laemmli (28) with minor modifications. Fifteen percent slab gels (1.5 mm) were allowed to polymerize overnight. To prevent damage due to free radicals and oxidants trapped in the gel matrix, 0.1 m*M* sodium thioglycolate was added to the running buffer in the upper reservoir. One-hundred-microgram protein samples were applied in each well as described previously (22). Following electrophoresis at a constant current of 30 mA/gel, the gels were stained with Coomassie brilliant blue R250, dried, and exposed to Kodak AR film.

Immunoblot Assay. The Western immunoblot assay was performed as described previously (22). Briefly, 25- to 100-μg protein samples were run on 15% SDS-PAGE gels, as explained above. The gels were then placed on a nitrocellulose membrane (Millipore) and the proteins were transferred in the presence of Tris-glycine-methanol buffer for 1 hr using a Hoefer Scientific Instrument apparatus at full voltage at 4°C. The membrane was then washed in Tris-buffered saline (TBS) for 20 min to remove glycine and placed in blocking buffer (TBS with 5% nonfat milk) for 20 min.

Following incubation in blocking buffer, the membrane was incubated with primary antibody diluted in blocking buffer (1/200) at room temperature for 60 min, followed by consecutive 10-min washes in TBS, TBS containing 0.1% NP-40 and TBS, respectively. The membrane was incubated in ^{125}I-labeled conjugated protein A (1 μCi/lane) diluted in blocking buffer for 60 min at room temperature. The membrane was

then washed in TBS as described above and air dried for 1 hr. The membrane was then exposed to Kodak AR film and developed.

Autoradiographs of immunoblots and also of ^{75}Se SDS-PAGE gels were scanned on a Helena Labs Quick-Scan densitometer (Beaumont, TX). The ^{75}Se signal and the ^{125}I signal were converted to optical density units in order to determine the relative ^{75}Se-labeling patterns or protein concentration, respectively. The ^{75}Se-labeling patterns were interpreted as qualitative assays and were not construed as quantitative determinations of selenium since the body pools of selenium were different in the groups fed the different selenium diets. Multiple gels were run at one time to control for sample variation and inter-gel signal variation on the autoradiographs.

Protein concentration was determined according to the microassay method of Bradford (29).

Results

Labeling of SLP in Mouse Organs. Experiment 1 determined the labeling kinetics of SLP-57 in plasma of female mice fed deficient (0.02 ppm), sufficient (0.2 ppm), and supplemental (2.0 ppm) selenium diets. A pilot experiment which examined labeling of plasma proteins at 4, 12, 24, and 40 hr indicated that maximum labeling of plasma SLP-57 occurred by 4 hr. Based on the findings of the pilot experiment, the plasma from 10-week-old female mice (n=2–3) fed deficient, sufficient, or supplemental selenium diets for 4 weeks was examined at 10, 30, 60, and 240 min after intraperitoneal injection of 250 μCi of ^{75}Se. The results are shown in Figure 1. In mice fed the 0.02-ppm selenium diet, the uptake of ^{75}Se in SLP-57 was detectable by 30 min and increased progressively over the 4-hr period. The sample variation was 1–5% between triplicates. The uptake of ^{75}Se in SLP-57 in plasma from mice fed the sufficient (not shown in Fig. 1) and supplemental diets (Fig. 1) was 90% and 39%, respectively, that observed in plasma from mice fed the deficient diet.

Experiment 2 determined the labeling kinetics for SLP in organs from mice fed a low selenium diet. Figure 2 illustrates the ^{75}Se binding to the three SLP in liver, kidney, and mammary gland from virgin mice fed a 0.02-ppm selenium diet for 8 weeks from 6 to 14 weeks of age. The organs were collected at 1, 4, and 40 hr after injection of 250 μCi of ^{75}Se. Panel A shows the labeling kinetics of SLP-56. Interestingly, the protein in the mammary gland was labeled to a greater extent than in liver or kidney. Labeling was detectable by 1 hr, increased sharply at 4 hr, and then decreased in rate over the last 36 hr. Panel B shows the labeling kinetics of SLP-26. Liver showed the greatest labeling, kidney significantly high labeling, whereas the mammary gland only demonstrated significant labeling at the 40-hr time point. Panel C shows the labeling kinetics of SLP-14.

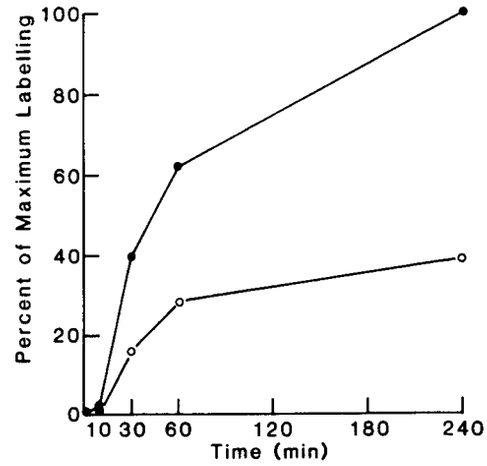

Figure 1. Illustrates the mean percentage of ^{75}Se labeling of plasma 57-kDa protein in female mice. Maximum labeling (100%) was considered the value observed at 240 min. Samples were done in triplicate and the variation was 1–5% of the mean. Mice were fed 0.02 ppm of selenium (●——●) or 2.0 ppm of selenium (○——○). The mice fed the 0.2-ppm selenium diet were not graphed, but the values were 90% of the values seen with the 0.02-ppm selenium diet. Labeling of 57 kDa in plasma in mice fed the high selenium diet was 39% of that in the low selenium diet.

The protein was labeled less than SLP-26 and SLP-56 in all three organs. The labeling was very rapid in liver (1 hr), slightly delayed in kidney (4 hr), but observed only at 40 hr in the mammary gland. The results of these experiments established that 40 hr was a good time point to examine labeling of these proteins.

Experiment 3 determined the effects of dietary selenium concentration on ^{75}Se labeling of SLP. Figure 3 illustrates the ^{75}Se binding of the three SLP as a function of dietary selenium. The mice were separate from Experiment 2 but followed the same experimental design. In each organ, the labeling of each protein was highly dependent upon the dietary selenium level. Generally, only SLP-26 was significantly labeled by 40 hr when the mice were fed 2 ppm of selenium. In the three organs, the extent of labeling in the mice fed the low selenium diet was greater than in mice fed the other two selenium diets.

The 40-hr values for the mice fed 0.02 ppm of selenium from this experiment agreed with the 40-hr values of the previous experiment (Fig. 2) in eight of nine cases. Thus, for SLP-56, the extent of labeling in mammary gland was greater than in liver and kidney. For SLP-14, liver = kidney $\gg$ mammary gland. For SLP-26, liver > kidney. The only discordant value was for mammary gland, where in the prior experiment, the labeling of the SLP-26 protein in mammary gland was significantly lower than liver. In Experiment 3, the labeling was similar between the two organs. The discrepancy may reflect the known cellular heterogeneity of the mammary gland or, perhaps, circadian rhythms of hormones which affect mammary gland function.

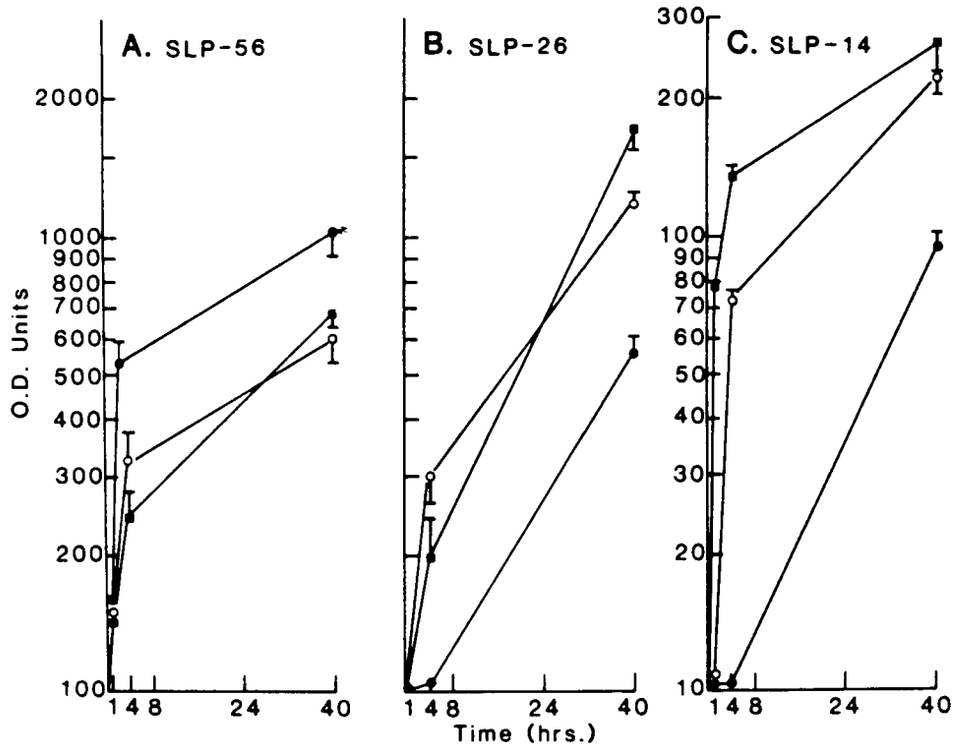

Figure 2. Illustrates the ^{75}Se labeling of SLP-56, SLP-26 (GSH-Px), and SLP-14 over 40 hr in liver (■——■), kidney (○——○), and mammary gland (●——●) of female mice fed the 0.02-ppm selenium diet. The time periods analyzed were 1, 4, and 40 hr after ^{75}Se injection. Each time point was done in triplicate. The values are the mean optical density units ± SE. It was of interest that the labeling of SLP-56 was significantly higher in mammary gland than liver and kidney, whereas the labeling of SLP-14 was significantly lower in mammary gland. Also the labeling of 14,000 Da was lower than that of the other two proteins.

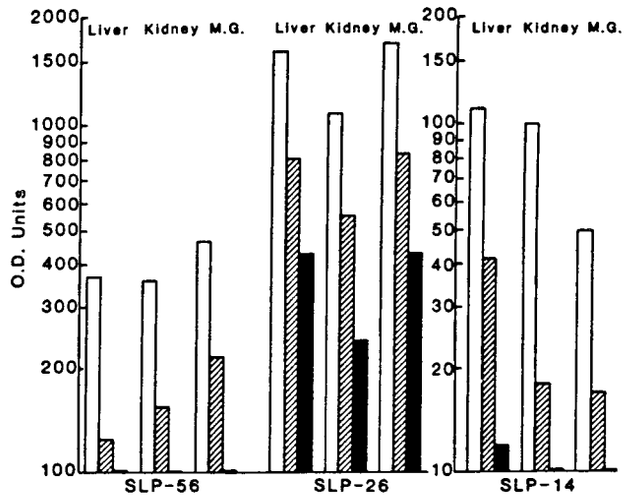

Figure 3. Illustrates the ^{75}Se labeling after 40 hr of the SLP in liver kidney and mammary gland (MG) of female mice fed the three selenium diets (□, 0.02 ppm; ▨, 0.2 ppm; ■, 2.0 ppm). Three mice were assayed for each group. The values are the mean of the optical density units with a variation of 3–8%. Labeling was inversely proportional to the dietary selenium concentration. Although it was easy to label GSH-Px at 2.0 ppm of selenium, the other proteins were difficult to label at this dietary selenium level. The optical density units for SLP-56 and SLP-14 (kidney, mammary gland) at 2.0 ppm of selenium were drawn at the minimum values of the ordinate; however, the actual values were lower.

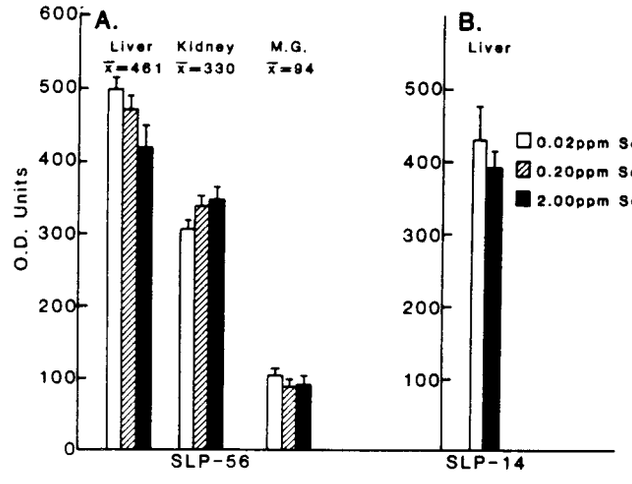

Figure 4. Illustrates the result from the immunoblotting of the SLP-56 in the three organs and of SLP-14 in liver. The same samples were used as in Figure 3. The optical density units represent the mean ± SE. There were no significant differences in the amount of SLP-56 protein in liver, kidney, or mammary gland in mice fed the three diets or of SLP-14 in liver.

Figure 4 illustrates the data from the immunoblotting of the SLP-56 in the three organs and of the SLP-14 in liver. The levels of the SLP-56 did not change significantly over the three dietary selenium concentra-

tions. One possible exception was the level of SLP-56 in liver where the protein concentration showed a decreasing linear trend with selenium dose, however, the probability was >0.05. For SLP-14, the levels were not different between the 0.02- and 2.0-ppm selenium groups (Fig. 4B).

Labeling of SLP in Rat Organs. In the experiments with virgin female Sprague-Dawley rats, the rats were maintained on one of three diets (0.03, 0.1, and 2.0 ppm selenium) for 2 weeks before receiving 10 mg of DMBA, then the animals were killed at 2, 6, or 24 weeks thereafter. The 24-week group animals bore mammary adenocarcinomas which were also collected and assayed. In the 2-week group, the rats received 300 μCi of ^{75}Se (sp act, 61.5 mCi/mg) 24 hr before sacrifice.

Figure 5A illustrates the ^{75}Se labeling of the SLP in liver and mamamry gland. For SLP-56 in mammary gland, there was a clear linear trend in ^{75}Se labeling which was inversely proportional to dietary selenium concentration. These same results were observed with SLP-26 in liver. For the SLP-56 in liver, only a low level of labeling was observed and a trend could not be discerned. For SLP-26 in mammary gland, and SLP-14 in liver and mammary gland, detectable labeling was extremely low; therefore, the data could not be plotted reliably.

Figure 5B illustrates the immunoblotting of the SLP-56 and SLP-14 in the liver and mammary gland. In liver, SLP-56 did not vary with dietary dose, although in mammary gland, protein was inversely proportional to dietary selenium concentration. However, it is important to note that the amount of SLP-56 detected in the rat mammary gland was only 10% of the amount detected in rat liver, thus the significance of this difference is debatable. At the 2-week time period, the amount of liver SLP-14 was significantly less ($P < 0.05$) in the high selenium diet than in either of the other two diets.

Figure 6A shows the immunoblotting of the SLP-56 and SLP-14 in rat liver at the 6-week period. The mammary gland SLP-56 levels were not plotted since the levels remained 10% of liver values. In this group, the only consistent result was the decreased amount of SLP-14 in the high selenium diet (65% of control diet level). Figure 6B shows the levels of the same two proteins at 24 weeks post-DMBA treatment, but in this group, the rats fed 2.0 ppm of selenium as selenobetaine were also compared with the control and 2.0-ppm selenium (as selenite) diets. Whereas the SLP-56 levels did not vary in the three dietary selenium groups, the level of SLP-14 was less in the 2-ppm selenium (selenite) group (60% of control) but not in the 2.0-ppm selenobetaine group. Rats fed selenobetaine methyl ester, selenomethionine, and Ebselen did not exhibit a decrease in either SLP-56 or SLP-14 (data not plotted). Similarly, at the 6-week time period, rats fed selenobetaine did not show a significant change in the levels of SLP-56 (data not plotted).

Two noticeable differences occurred in the immunologic detection of SLP-56 in mammary tumors compared with mammary gland. First, of the 15 tumors examined from the six dietary selenium groups, 5 showed undetectable levels of the SLP-56 protein. Second, in the other 10 tumors, the SLP-56 appeared as

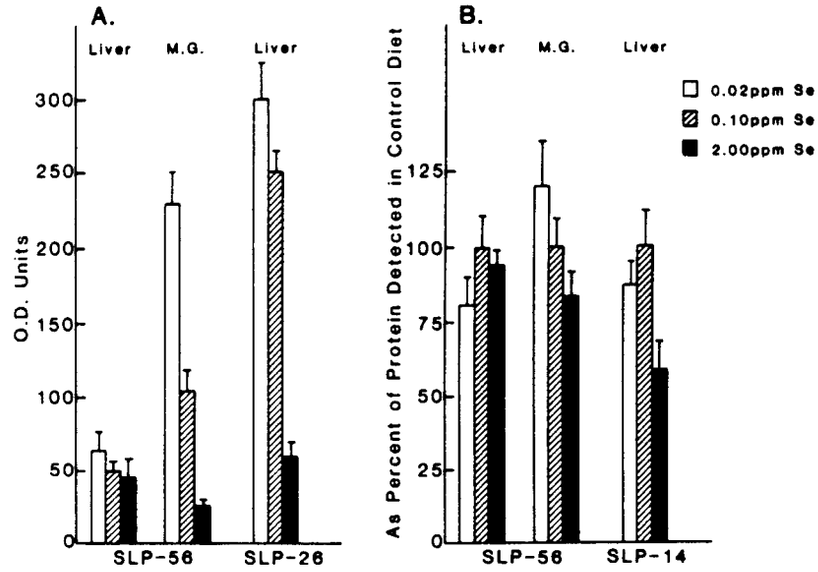

Figure 5. Illustrates the ^{75}Se labeling and levels of protein in the rat mammary gland fed three levels of selenium. The optical density units represented the mean ± SE. (A) ^{75}Se labeling. For mammary gland SLP-56 and liver SLP-26, there was a definite inverse correlation between ^{75}Se labeling and dietary selenium concentration. The labeling of liver SLP-56 was low and did not show a trend. (B) Immunoblot ($n = 5$). As with the mouse experiments, there was not a significant difference in concentration of liver and mammary gland SLP-56; however, the concentration of SLP-14 was significantly lower ($P < 0.05$) in livers of rats fed 2.0 ppm of selenium compared with normal selenium diets.

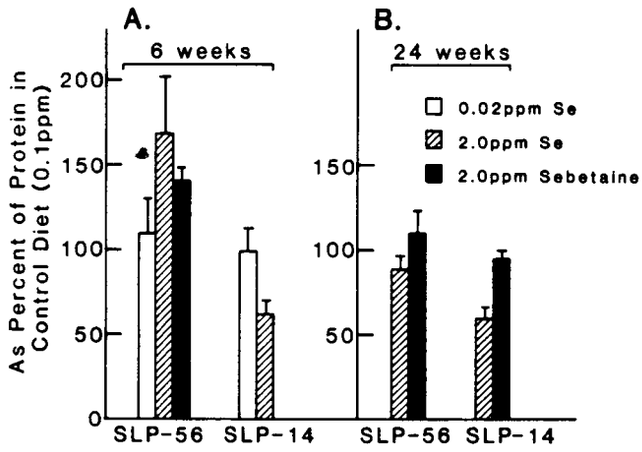

Figure 6. Illustrates the results of immunoblots from liver cytosols of rats fed different selenium diets ($n = 5$). The optical density values of the control diet (0.1 ppm) was considered as unity (100%) and the optical density values of the other groups were then plotted as percentage of values in the control diet (0.1 ppm of selenium). Note that the levels of liver SLP-56 at both 6 and 24 weeks were the same whether the rats were fed selenium as selenite or selenobetaine. The levels of liver SLP-14 were significantly less ($P < 0.05$) in the rats fed 2.0 ppm of selenite at 6 and 24 weeks after DMBA feeding, but selenobetaine had no effect at 24 weeks.

two protein bands, 56,000 Da, and 50,000 Da which was not observed on the mammary gland tissue immunoblots.

Discussion

The study of selenium-labeled proteins other than GSH-Px is still in a preliminary stage. Several laboratories have documented the existence of multiple selenium-labeled proteins in a variety of organs (10–23); however, there are only a few published reports on the kinetics of ^{75}Se labeling of these proteins (17, 30, 31) and only two which have examined concentration (16, 31). The existing literature presents the following picture about the SLP's other than GSH-Px. First, such proteins are present in most all organs of the body and number anywhere from 3 to 12. Second, in rats fed a diet with inadequate selenium (Torula yeast diet), the brain, reproductive organs, and endocrine organs preferentially sequester selenium into proteins other than GSH-Px, which suggests that these proteins have important functions (18). Previous experiments (12, 32) have suggested the importance of proteins other than GSH-Px in understanding some of the biologic effects of selenium. Third, Calvin *et al.* (30), on the basis of ^{75}Se-labeling kinetics of proteins, suggested that some of the larger SLP's (54 kDa and 47 kDa) might be precursors to the 15- to 17-kDa SLP of testis. The 15- to 17-kDa SLP is essential for viability of rat sperm (30). Fourth, the kinetics of ^{75}Se labeling of proteins indicated that the plasma SLP-57 was labeled by 1 hr, whereas other proteins such as the 65 kDa in liver kidney, the 45 kDa in kidney, and the 17 kDa in testes were significantly labeled at later time periods, from 12 hr (liver) to 72 hr (testes). The extent of labeling was dependent upon the dietary selenium concentration and, in all cases, inhibited by cycloheximide (17).

The results from the experiments reported in this article confirm and extend the information on these selenium-labeled proteins in three areas. First, the kinetics of the ^{75}Se labeling of the SLP in plasma and organs were similar to those reported by other groups (17, 30). The ^{75}Se labeling of plasma SLP-57 was very rapid in both deficient and adequate selenium mice and occurred within 30 min. The labeling maximized between 1 and 4 hr and remained high for 40 hr. This is a pattern very similar to that reported by Evenson and Sunde (17). Similarly, in liver, kidney, and mammary gland, the ^{75}Se labeling of SLP-56, SLP-26 (GSH-Px), and SLP-14 was initially low and increased over a 40-hr period. The ^{75}Se labeling of SLP-14 in mammary gland was very slow compared with the labeling of the other two proteins. This same pattern was reported by Evenson and Sunde (17). It is tempting to propose that the large SLP's (i.e., 56 kDa) serve as precursors or transfer selenium to SLP-14 (30). Indeed, a transport function has been proposed for the plasma 57 kDa (20, 21). However, it should be noted that a precursor-product relationship between the 56- and 14-kDa proteins is not evident (22–24). Additionally, the plasma 57 kDa and cellular 56 kDa are not immunologically related (22, 23). At this time, the data support only a difference in labeling kinetics, but do not rule in or out a role of the larger proteins as selenium-transfer proteins.

Second, the immunoblot data indicate that the SLP-56 and SLP-14 differ from GSH-Px in a fundamental manner. Unlike GSH-Px, the two proteins are not induced by or dependent upon dietary selenium concentration. This property is fundamentally different from GSH-Px, where biologic activity (33), protein concentration (32), and mRNA levels (34) are dependent upon dietary selenium concentration. The lack of selenium dependency was observed over a ×100 range of selenium concentration (0.02–2.0 ppm) for both SLP-56 and SLP-14. Interestingly, in the pilot experiment, animals fed the 2.0-ppm selenium diet did not show a significant increase in labeling for SLP-26 over a 40-hr time period. This is consistent with the fact that GSH-Px incorporates selenium as selenocysteine and the level of GSH-Px is maximum at low selenium levels (0.1 ppm) (35). The different nature of SLP-56 and SLP-14 is substantiated further by the absence of the selenocysteine codon, TGA (36), in the coding portion of the DNA sequence. Liver SLP-14 has recently been identified as fatty acid-binding protein (24) which does not have an in-frame TGA. Similarly, an in-frame TGA is not present in the DNA which codes for mouse liver SLP-56 (25). These data lend support to the proposition

that SLP-56 and SLP-14 are fundamentally different from GSH-Px regarding the incorporation and/or binding of selenium to the proteins.

Finally, the experiments in DMBA-treated rats showed very similar patterns as in mice. Of particular interest was the observation that the concentration of SLP-14 was 40% less in rats fed the 2.0-ppm selenium diet. This was observed in all three groups (2-, 6-, and 24-week post-DMBA). This is interesting in view of the fact that liver FABP is hypothesized to be a growth regulatory molecule in rat liver (37). Thus, it is conceivable that selenium binding to this protein alters its properties. At first glance, it is disconcerting that a decrease in SLP-14 was not observed in mouse liver examined from the animals fed 2.0 ppm of selenium. The main difference between the mouse and rat experiments was that the latter were fed DMBA. It is established that liver FABP binds chemical carcinogens avidly for a prolonged time period (37), thus it is possible that the FABP which bound both ligands was less stable than its unbound counterpart. It should be noted that the SLP-14 could only be assayed in liver since the mammary gland protein is unrelated immunologically to the liver protein (23).

The most important result from these experiments is the demonstration that the proteins SLP-56 and SLP-14, although avidly selenyated, are not selenium-dependent proteins. These proteins are viewed as representing a distinct class of proteins from the prototypic selenoprotein, GSH-Px. It is our working hypothesis that selenium acts to modulate the function of these proteins. Whether these proteins are important in the chemopreventive property of selenite remains an unanswered, but interesting, question.

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