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SELENITE BIOTRANSFORMATION TO VOLATILE METABOLITES IN AN ISOLATED HEPATOCYTE MODEL SYSTEM

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Abstract—The biotransformation of selenite to dimethylselenide was studied in an oxygenated hepatocyte model system. The concentrations of selenite used were 20–100 μM. A lag period of one hour or more, during which no net formation of selenide could be detected characterized the system. The maximal rate of volatilization was recorded during the second hour and was 0.13 nmoles/10^6 cells/min with 50 μM selenite. The rate then declined and volatilization eventually ceased. Two-thirds of the added amount of Se was lost within 4 hr. Oxidation of glutathione (GSH) by cumene hydroperoxide delayed volatilization. An inhibitor of gluconeogenesis, *p*-tert-butylbenzoic acid (3 μM) prevented volatilization. There were indications that GSSG reductase dependent metabolism was the only major metabolic pathway in hepatocytes under the conditions studied. During the lag period Se accumulated in cells, but was subsequently partially released during volatilization. The accumulation of Se was paralleled by an increase in oxygen uptake. The above mentioned inhibitors of volatilization prolonged the phase of accumulation. With 50 μM selenite the rate of accumulation was 0.06 nmoles/10^6 cells/min and maximally 30–35% of the added dose was retained in the cells. The results are compatible with the assumption that Se mainly accumulated as Se–glutathione complexes. The possibility that such complexes autooxidized and entered futile redox cycles during the lag period is discussed.

Selenite reacts chemically with sulfhydryl compounds. The reaction with glutathione (GSH) is the first step in a metabolic pathway in the liver which leads to the formation of volatile metabolites. The first stable intermediate, GSSeSG, is a substrate for GSSG reductase [1] and is reduced to GSSeH at the expense of NADPH. GSSeH can be further reduced to selenide by the same enzyme [2]. Selenide is methylated under physiological conditions but may evaporate at low pH [3]. *In vivo* formed dimethylselenide is excreted through the lungs and thought to be responsible for the "garlic breath" of selenite poisoned animals [3]. A trimethyl selenonium ion is also formed *in vivo* and excreted in urine [4, 5].

The volatilization of Se in this coupled reductive-methylating pathway has mainly been characterized in homogenized liver or liver fractions under anaerobic conditions as air strongly inhibits dimethylselenide formation [6]. The mechanism behind this inhibition has not been studied directly, but it is known since many years that selenite in small amounts catalyzes GSH oxidation [7]. It was suggested that GSSeSG may autooxidize to GSSG and selenite [7]. An alternative is that selenide autooxidizes to elemental selenium which reacts with GSH (to form GSSeH) [8].

In a previous work we have studied some aspects of selenite toxicity in isolated hepatocytes under aerobic conditions [9]. We found several indications that cellular lysis mediated by selenite was related to selenite metabolism. Thus, methionine which stimulates dimethylselenide formation [6], potentiated selenite induced cellular lysis. It was also found that cells isolated from starved animals were more sensitive than control hepatocytes, and the addition of glucose or alanine prevented lysis [9]. These results suggested a critical role for the regeneration of NADPH in this type of toxicity. Stoichiometric calculations, however, showed that merely a 6 valence reduction of selenite to selenide, could not possibly explain the drop in NADPH levels and subsequent lysis, as, for example, hydroperoxide metabolism may consume more NADPH without damaging cells [10–13]. In order to further characterize deleterious effects of selenite in isolated liver cells this work was extended to involve volatilization studies. In this paper we present data which show that selenium can be rapidly metabolized in an oxygenated isolated hepatocyte system. However, volatilization was proceeded by a long lag period, and it is suggested that the rate of autooxidation of Se metabolites was high during this period.

MATERIALS AND METHODS

Male Sprague–Dawley rats were used as liver donor animals. Their weight was 180–240 g and they had free access to standard laboratory food and water. Collagenase (Grade II) was purchased from Boehringer Mannheim (F.R.G.), selenite ($Na_2SeO_3 \times 5H_2O$) from Merck (F.R.G.). The radioactive isotopes $^{75}SeO_3^{2-}$ (2–20 mCi/mg Se) and L-(methyl-^{14}C)-methionine were delivered from Amersham (U.K.). One microcurie of $^{75}SeO_3^{2-}$ was used per 20 ml of incubation medium. This isotope

was chromatographed [14] and found to contain 10–20% impurities. However, only marginal effects could be related to the impurities.

Hepatocytes were isolated by the collagenase perfusion technique and cell vialibility was determined [15]. Freshly prepared cells were suspended in Krebs–Henseleit buffer (pH 7.4) supplemented with 25 mM *N*-2-hydroxyethylpiperazine-*N'*-ethane sulphonic acid (HEPES). Methionine (0.5 mM) was also included as this amino acid stimulated volatilization. The final incubation volume was 20 ml with a cell concentration of 2.0–2.5 × 10^6/ml. The suspended cells were incubated at 37° in rotating round-bottom 50 ml flasks and carbogen gas (93.5% O_2 and 6.5% CO_2) was supplied to the surface [15]. Gas leaving the flasks was usually let out into the atmosphere (into ventilated hoods). Up to ten flasks were incubated simultaneously.

Volatilization was measured as the disappearance of ^{75}Se from the total incubate. Aliquots (0.5 ml) were withdrawn and usually put directly into counting vials. The vials were sealed immediately and the radioactivity counted in an automatic gamma-counter (LKB). Some samples, kept on ice, were also bubbled with N_2 for 30 min but only small (less than 5%) effects of the bubbling was noticed. In some short term experiments with a single incubation flask, the leaving gas was passed through 8 N HNO_3 [6]. Only dimethylselenide will be trapped under these conditions [6] and it was found that more than 90% of the evaporated ^{75}Se was recovered in the HNO_3 solution. When L-(methyl-^{14}C)-methionine was used ^{14}C-label followed the ^{75}Se-label and a constant stoichiometric relationship of 2.11 ± 0.15 S.D. (after adjustment for quenching) was found in the HNO_3 solution. These results are in agreement with those presented in ref. 6 as identification of the volatile metabolite as dimethylselenide.

Accumulation of Se was measured in cell pellets from aliquots of incubate (1.0 ml) centrifuged at 500 *g* for 1 min. GSH was assayed in the acid soluble fraction of the cell pellet. The method used [16] is specific for thiols and an oxidation of GSH could thus be detected as a decrease in GSH concentration. Oxygen consumption was measured polarographically with a Clark type electrode at 37°. Measurements were performed without and with antimycin A (25 μM) to inhibit mitochondrial oxygen uptake.

Results presented in this paper have been taken from typical experiments. All experiments were repeated at least three times with different cell batches and with purified or commercial radioactive isotope.

RESULTS

Volatilization of ^{75}Se could be effectively studied in a relatively narrow concentration range. As previously reported for homogenized liver [6] 100 μM partially inhibited Se volatilization in isolated hepatocytes (Fig. 1). At lower concentrations the fraction that was volatilized progressively decreased, and with 20 μM the volatilization was insignificant (Fig. 5). Fifty micromolar selenite was used in most experiments since a substantial fraction (more than 50%) was volatilized with this concentration, and the cell lysing effects could easily be controlled.

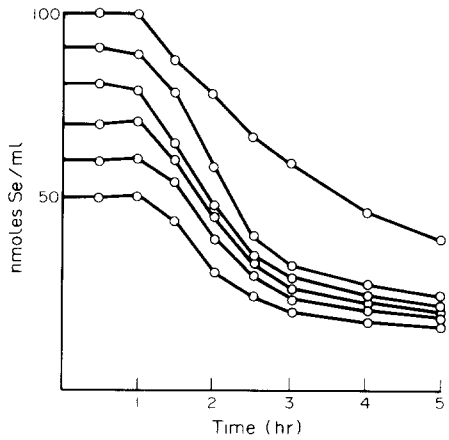

Fig. 1. Volatilization of Se, added as selenite, by isolated hepatocytes. Suspensions of isolated hepatocytes (2.2 × 10^6 cells/ml) were incubated with selenite (50–100 μM). Volatilization was measured as the disappearance of ^{75}Se from the total incubate.

As also can be seen from Fig. 1, a lag phase of about 1 hr preceded volatilization. Then there was a second phase with a rapidly increasing rate of volatilization and a third phase with a decreasing rate of volatilization which approached zero at 5 hr. With 30 μM selenite the lag period had a duration of 2 hr (Fig. 5), and, as previously reported, a similar lag period could also be seen when measuring loss of selenite (50 and 25 μM) from the medium [9]. The maximal rate of volatilization with 50 μM selenite in a series of eight experiments (volatilization was measured every 30 min) was 0.13 (± 0.02) nmoles/10^6 cells/min. Control experiments (see Materials and Methods) indicated that dimethylselenide was the major if not the only metabolite volatilized under these conditions. In some experiments incubations were run for 18 hr, but no further loss of ^{75}Se occurred after 5 hr.

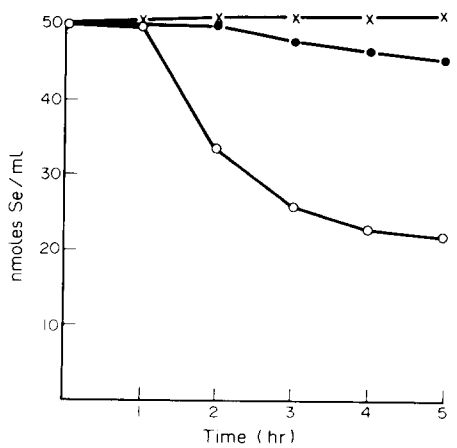

Fig. 2. Se volatilization; effect of cell lysis induced by chlorpromazine. Suspensions of isolated hepatocytes (2.3 × 10^6 cells/ml) were incubated with selenite (50 μM). Chlorpromazine (0.4 mM) was added at 0 min (×—×) or at 60 min (●—●) and cellular lysis was complete within 5–10 min. Chlorpromazine was not included in (○—○). The disappearance of radiolabel was measured at times indicated.

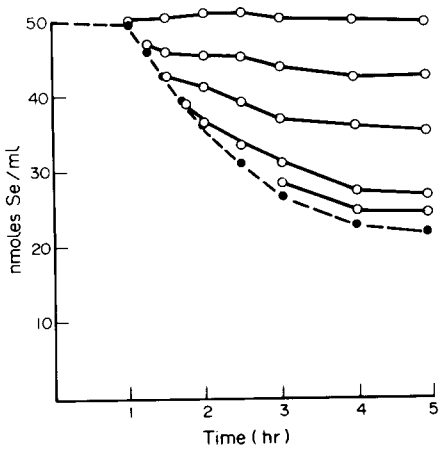

Fig. 3. Se volatilization; effect of perchloric acid addition. Six incubation flasks were used. The final concentration of selenite was 50 μM and the incubate contained 2.2×10^6 cells/ml. The reaction was stopped by the addition of perchloric acid (0.5 ml, 30%) to five of the flasks at 60, 75, 90, 105 and 180 min and the incubation was then continued. One flask (●– – –●) was incubated without perchloric acid. Aliquots were withdrawn at times indicated and the disappearance of radiolabel determined.

Cells, lysed by chlorpromazine treatment [17] at the start of the incubation did not volatilize Se and only a small amount was volatilized when cells were lysed at 1 hr (Fig. 2). However, when cells were lysed after 2 hr the inhibitory effect on volatilization was insignificant (not shown). When perchloric acid was used to lyse cells at different time points the lag phase remained. When the incubation was continued after perchloric acid addition there was a further loss of Se (Fig. 3) which might be due to a delay in volatilization of dimethylselenide as previously reported in liver tissue and attributed to physical factors [18]. In this experiment it is also possible that the acidic condition promoted evaporation of small amounts of hydrogen selenide. In any event it appears that 80% of the metabolites that eventually evaporated, were reduced to selenide already 45 min after the end of the lag period, and thus that selenite reduction to selenide occurred at a higher rate than that given above.

Table 1 summarizes experiments which were basically performed as in Figs. 1 and 2, but in which cold and radiolabelled selenite were added separately and at different time points. Experiment 1 shows that radiolabelled selenite, added at 60 and 90 min, rapidly evaporated without an apparent lag phase and indicates that selenite freely passed the plasma membrane. Additions at 0 and 120 min resulted in a slower rate of volatilization which is in accordance with the results presented in Figs. 1–3. Experiment 2 shows that the cells retained the capacity to convert selenite to volatile metabolites also during the fourth hour. It also shows that addition of cold selenite at 120 min did not promote volatilization of radiolabelled Se added at 0 min, indicating that this dose no longer was available for volatilization but converted to stable water soluble or protein bound metabolites. Experiment 3 shows that preincubated cells exhibited the same lag period as did nonpreincubated cells.

Selenium transiently accumulated in the hepatocytes (Fig. 4) and this process started without an apparent lag phase. With 50 μM selenite a peak was reached at 1.5 hr. During the first hour 3.7 nmoles accumulated per 10^6 cells. Then a rapid release of Se was observed, which coincided with the rapid phase of volatilization. In the concentration range of 50–90 μM the accumulation rate was always slower than the rate of release, and this rate was always slower than the maximal apparent rate of volatilization (Fig. 1). At 100 μM the release appeared to be inhibited, as well as volatilization. The fraction associated with the cells at 1.5 hr increased with increasing selenite concentration up to a level of about 30% bound. Some Se was retained in the cells even after 5 hr

Table 1. Characterization of the lag period and metabolism of selenite by adding cold and radioactive selenite separately and at different time points

Experiment	Time of ^{75}Se addition (min)	Time of sampling (min)	Rate of volatilization (cpm/ml/min)
1. Cold Se added at 0 min	0	0 + 30	43
	60	60 + 90	576
	90	90 + 120	1866
	120	120 + 150	103
2. Cold Se added at 0 and 120 min	120	120 + 150	31
	120	180 + 210	413
	0	120 + 150	220
	0	180 + 210	67
3. Cold Se added at 60 min	90	60 + 90	53
	60	120 + 150	481

Incubations were started at 0 min. Unlabelled (cold) selenite (50 μM) was added at 0 min (exp. 1 and 2) or at 60 min (exp. 3). A second dose of cold selenite (50 μM) was added at 120 min in exp. 2. A single addition of ^{75}Se labelled selenite (70 nM) was made at times indicated in the first column. Two samples were withdrawn from each incubate at times depicted in the second column. The difference in counts between the two samples is expressed as the rate of volatilization (cpm/ml/min) in the third column.

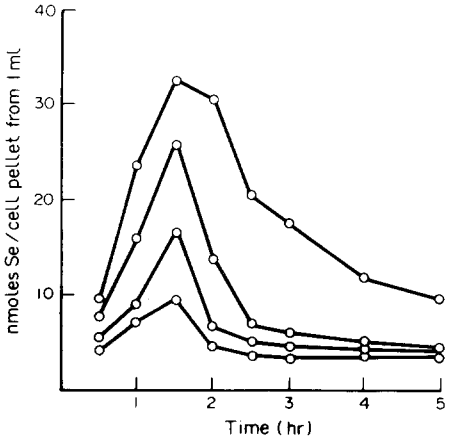

Fig. 4. Se accumulation by isolated hepatocytes. Selenite (50, 70, 90 or 100 μM) was added to four suspensions of isolated hepatocytes (2.2 × 10⁶ cells/ml). Aliquots were withdrawn and immediately centrifuged. Radioactivity was measured in pelleted cells.

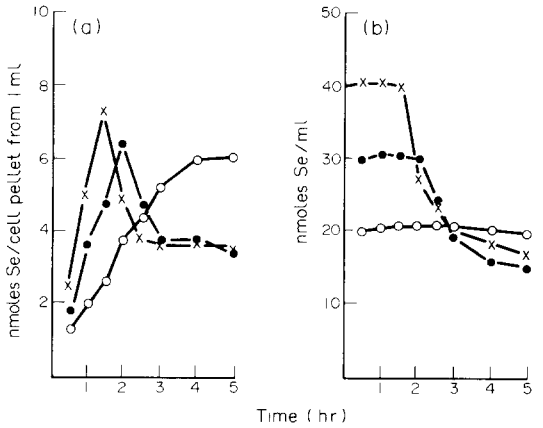

Fig. 5. Se accumulation (a) and volatilization (b) as a function of selenite concentration. Selenite, 20 (○—○), 30 (●—●) and 40 (×—×) μM was added to isolated hepatocytes (2.2 × 10⁶ cells/ml). Volatilization was measured in aliquots of the total incubate, and accumulation in pelleted cells.

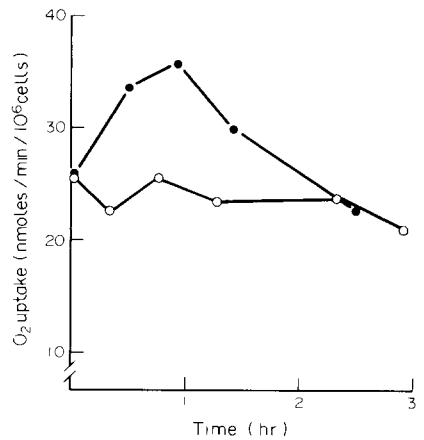

Fig. 6. Effect of selenite on oxygen uptake in isolated hepatocytes. Suspensions of isolated hepatocytes (2.0 × 10⁶ cells/ml) were incubated without (○—○) or with selenite (50 μM; ●—●). Aliquots (2 ml) were withdrawn at times indicated and the incubation continued in a thermostated cell fitted with an oxygen electrode.

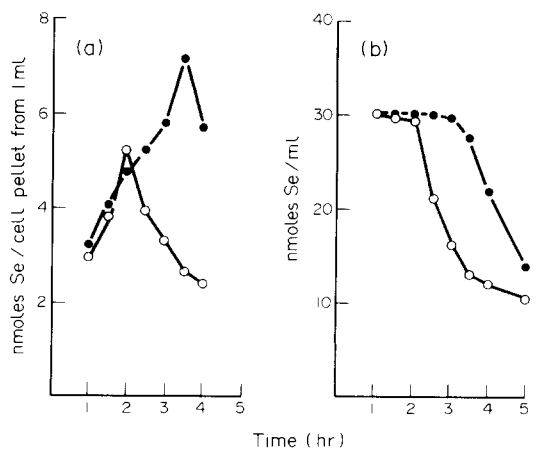

Fig. 7. Se accumulation (a) and volatilization (b); effect of cumene hydroperoxide. Selenite (30 μM) was added to suspensions of isolated hepatocytes (2.2 × 10⁶ cells/ml). Cumene hydroperoxide was added at 60 min and the concentration was 225 μM (●—●).

incubation. Figure 5 shows that, with 20 μM, when usually no volatilization was detected, the accumulation continued with an unchanged rate for 3 hr. At the end, about 30% of the added dose was trapped in the cells. With 30 μM the accumulation stopped at 2 hr and at the same time volatilization started. With 40 μM the turning point was at 1.5 hr.

The accumulation of Se in the cells was paralleled by an increase in oxygen consumption (Fig. 6). A maximal oxygen uptake was seen at 60 min. It then declined during the volatilization phase until control uptake rates were recorded. The pattern of changes in oxygen uptake was the same when antimycin A (25 μM) was used to inhibit mitochondrial oxygen uptake.

Cumene hydroperoxide, at a concentration of 225 μM, readily oxidizes GSH without damaging hepatocytes [15]. This concentration did not affect the rate, or the extent of metabolism, but markedly delayed volatilization and prolonged the phase of Se

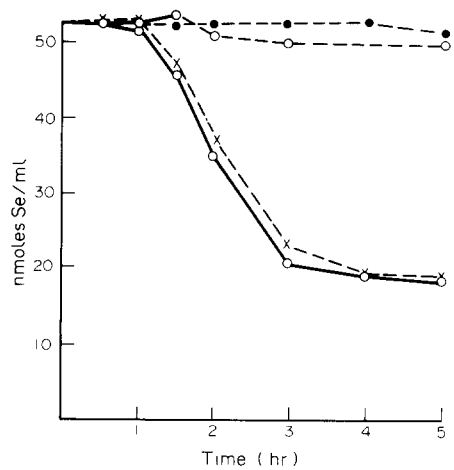

Fig. 8. Se volatilization; effect of *p*-tert-butylbenzoic acid. Selenite (50 μM) was added to suspensions of isolated hepatocytes (2.0 × 10⁶ cells/ml). *p*-Tert-butylbenzoic acid was added in dimethylsulfoxide (20 μl) and the concentrations were: 5 μM: (●- - -●); 3 μM: (○- - -○); 2 μM: (×- - -×) and zero: (○—○).

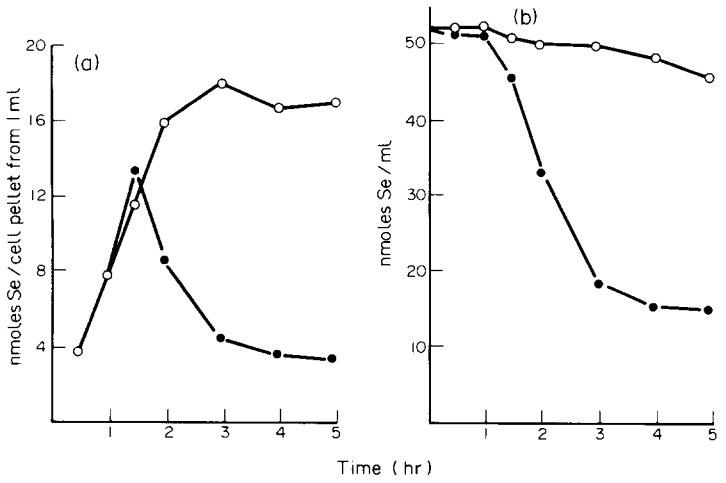

Fig. 9. Se accumulation (a) and volatilization (b); effect of *p*-tert-butylbenzoic acid and glucose. Selenite (50 μM) was added to suspensions of isolated hepatocytes (2.3×10^6 cells/ml). *p*-Tert-butylbenzoic acid (10 μM) was included in both incubates, and glucose (40 mM) in one (●—●).

accumulation in the cells (Fig. 7). *p*-Tert-butylbenzoic acid, an inhibitor of gluconeogenesis [19], inhibited volatilization more or less completely at 3 μM when 2×10^6 cells/ml was used (Fig. 8). This is a lower concentration than that used in ref. 19 to inhibit gluconeogenesis. However, the concentration needed to inhibit volatilization increased with a higher cell concentration. Glucose effectively counteracted the inhibition by *p*-tert-butylbenzoic acid when included in the medium (Fig. 9). Glucose also decreased the amount of Se that accumulated in the cells. *p*-Tert-butylbenzoic acid alone prolonged the accumulation phase until a level of about 17 nmoles/ml was reached, which corresponds to 34% of the total amount of Se added to the system. Glucose induced a release of Se from the cells, similar to that seen in non-inhibited hepatocytes.

Figure 10 shows the effect of selenite and *p*-tert-butylbenzoic acid on intracellular GSH levels. As previously shown [9, 20] 50 μM selenite only marginally decreased GSH concentration while 70 μM had a more pronounced effect. When *p*-tert-butylbenzoic acid was included in the incubate a more marked decrease in GSH levels was seen (Fig. 10). Also this effect of *p*-tert-butylbenzoic acid was counteracted by glucose (not shown). *p*-Tert-butylbenzoic acid alone had no significant effect on GSH levels during the first 1.5 hr (not shown).

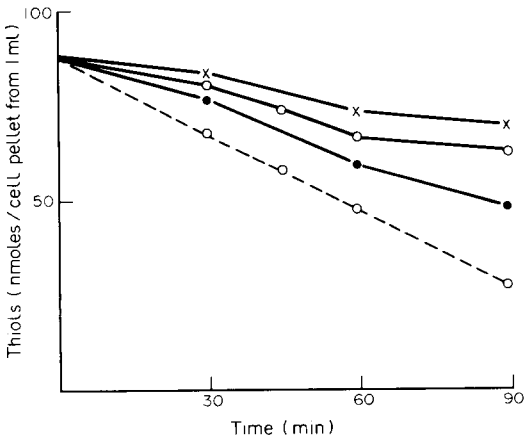

Fig. 10. GSH levels, effect of selenite and *p*-tert-butylbenzoic acid. Control incubation: (×—×), 50 μM selenite: (○—○), 70 μM selenite: (●—●) and 50 μM selenite plus 10 μM *p*-tert-butylbenzoic acid: (○- - -○). The suspensions contained 2.5×10^6 cells.

DISCUSSION

This study shows that intact hepatocytes in an oxygen rich atmosphere convert selenite to dimethylselenide after a distinct lag period. The maximal rate of selenide formation, as calculated from Fig. 3, was 0.22 nmoles/min/10^6 cells which roughly corresponds to 25 nmoles/min/g liver. Available data in the literature indicate that rat liver homogenates volatilize Se at a rate of 22 nmoles/min/g liver under anaerobic conditions [21]. In comparison with mouse liver homogenates, in which the reaction has been characterized in more detail, there are other similarities such as the stimulating effect of methionine, the inhibition of the reaction at 100 μM selenite and a lack of complete volatilization of 50 μM selenite [6]. It can thus be concluded that, with the exception the longer lag period, the reaction in isolated hepatocytes exhibited prominent similarities to that in homogenized liver in spite of the aerobic condition.

The effect of inhibitors used in this study further support an involvement of GSSG reductase in selenite metabolism. GSSG generated through cumene hydroperoxide metabolism might have acted as a competitive inhibitor of GSSeSG and GSSeH reduction. It is of interest to note that neither the rate, nor the extent of metabolism was altered by the addition of hydroperoxide; this indicates that biotransformation through other metabolic pathways than the GSSG reductase dependent one was insignificant or occurred at a comparatively slow rate. *p*-Tert-butylbenzoic acid, which inhibits fatty acid synthesis and gluconeogenesis, possibly through

CoA sequestration [19] may have impaired NADPH regeneration. Support for this interpretation are the findings that glucose counteracted the inhibition and that *p*-tert-butylbenzoic acid potentiated the loss of GSH in selenite exposed cells, and thus probably shifted the GSH/GSSG ratio.

As much as 30–34% of the available Se accumulated in the cells. This implies that Se was concentrated several times in the cellular compartment as 10^6 cells can be expected to have a volume of less than 0.01 ml [15]. The accumulation curve attenuated at this level under quite different incubation conditions (cf. Figs. 4, 5 and 9), indicating that the maximal concentrating capacity had been reached with the amount of cells used. In erythrocytes incubated in buffered saline more than 90% accumulated but the cell volume used in those experiments seems to have been much higher [22]. The uptake of selenite in erythrocytes was related to interactions with GSH [22] and similar mechanisms may have operated in the hepatocytes as indicated by the decrease of GSH levels (Fig. 10). Se–glutathione complexes can be expected to be less permeable than selenite over the plasma membrane so the formation of such metabolites may explain the generation of a concentration gradient.

The length of the lag period prior to volatilization always correlated with the length of the accumulation phase so that the release coincided with the phase of rapid volatilization. This pattern strongly suggests that one or several intermediary metabolite(s) accumulated in the cells during the lag period. The transient nature of the accumulation and the inhibition studies are also compatible with the assumption that these metabolites were mainly Se–glutathione complexes. It is not likely that major amounts of selenide accumulated in the cells during the lag period as this metabolite would have been volatilized upon perchloric acid addition (c.f. Fig. 3).

The reason why postulated intermediary metabolites accumulated prior to further metabolism is not fully understood. However, data calculated from Figs. 5 and 7 suggest that an intracellular level of about 2.5 nmoles Se/10^6 cells had to be established before volatilization could start. With an intracellular GSH concentration of about 35 nmoles/10^6 cells, this signifies a Se/GSH ratio of approximately 0.07 in the cells. Dilworth and Bandurski have studied the reaction between selenite and GSH in the presence of oxygen and described a transition from catalytic oxidation of GSH to reduction of selenite (to elemental Se) at a ratio of 0.04 [23]. Considering, for example, possible protein binding in the hepatocyte system these ratios can be regarded as compatible. It is thus possible that the peak in the oxygen uptake curve indicates the same type of transition and that the transition in our system triggered volatilization. The finding that much more than 2.5 nmoles Se/10^6 accumulated with the higher concentrations of selenite does not contradict this assumption. Volatilization might have been delayed at higher concentrations, implying that considerable amounts of dimethylselenide was retained in the cells during 60–120 min. It is also possible that the enzymatic conversion to dimethylselenide was temporarily inhibited, by selenite catalysed GSH oxidation [6].

Even though no net formation of selenide could be detected during the lag phase, results in earlier publications show that there was an increased turnover in the GSH redox system during the first hour. Thus, there was a decrease in the NADPH/$NADP^+$ ratio [9, 17], and a complete oxidation of GSH provided GSSG reductase was partially inhibited [9]. These findings taken together with the accelerated oxygen uptake suggest that during the lag period autooxidation of Se metabolites counteracted the GSSG reductase catalyzed reduction of selenite.

A futile metabolism of selenite in redox cycles may have biological implications. It can explain a high consumption of NADPH and thus also the cellular lysis mentioned in the introduction. However, redox cycles may generate reactive oxygen species [24] and we have recently found that selenite, in the presence of GSH, is a very potent inhibitor of the mirosomal calcium pump [25]. This enzyme is often implicated in cell damaging reactions and seems to be sensitive to oxidative stress [26], so it is possible that selenite toxicity also involves oxygen activation.

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