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IN VITRO HEMOLYSIS OF RAT ERYTHROCYTES BY SELENIUM COMPOUNDS*

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Abstract—Rat erythrocytes were incubated *in vitro* with various selenium compounds at 37°. Hemolysis occurred with some selenium compounds but not with corresponding sulfur analogues. Selenite induced more rapid loss of intracellular glutathione (GSH) than did selenocystine but was less hemolytic. Cystine caused neither loss of intracellular GSH nor hemolysis. Addition of GSH to the incubation medium enhanced hemolysis by selenite and selenium dioxide but inhibited hemolysis by selenocystine. Inclusion of glucose in the incubation medium also inhibited selenocystine-induced lysis of erythrocytes from both selenium-supplemented rats and selenium-deficient rats. The results suggest a relationship between the oxidation of intracellular GSH and the hemolysis by selenocystine, selenite and selenium dioxide.

Selenium is an element of considerable controversy. It is a nutritionally essential element [1] but is also known to be highly toxic [2]. It was suggested by an early study to be carcinogenic [3], but increasing evidence has indicated that it may have an anticarcinogenic function [4, 5]. Selenium toxicity in animals is well known and anemia is a common manifestation of selenosis in all species [2]. In humans, however, selenium poisoning is rare and mostly accidental in relation to occupation [6]. A valuable experience of observed selenium toxicity in humans occurred from the treatment of leukemic patients with selenocystine which reduced leukocyte counts but resulted in severe toxicity symptoms [7].

The mechanism(s) of selenium toxicity has been investigated to a limited extent. The *in vitro* experiments of Wright [8, 9] indicated a general inhibition of dehydrogenases and urease by sodium selenite or selenate but no impairment of cytochrome-indophenoloxidase, catalase or liver arginase. The results suggested that oxidation of sulfhydryl groups essential to redox processes was the possible biochemical site of selenium toxicity. Recently, Young *et al.* [10] reported that selenite caused lysis of sheep erythrocytes *in vitro*, and intracellular glutathione (GSH) was implicated in the lytic process.

Although various selenium compounds have been shown to have different toxicities [4, 11], studies concerning the actions of organic selenium compounds at toxic levels, to our knowledge, have not been available. The present study investigates the hemolytic effects of various selenium compounds with emphasis on an organic selenium compound, selenocystine. This is considered to be of importance because selenium occurring naturally in food is predominantly organic rather than inorganic [12].

MATERIALS AND METHODS

A total of forty Sprague–Dawley rats was used in the present experiments. Twenty rats were fed a commercial chow, which contains approximately 0.26 ppm selenium [13]. Unless otherwise specified, blood samples, from these twenty animals were used in the experiments described below. To investigate the effect of dietary selenium on the lysis of rat erythrocytes induced by selenocystine, ten rats were fed a selenium-deficient diet [14] and another ten rats were fed the same diet supplemented with 0.5 ppm selenium as sodium selenite. Blood samples were taken from ether-anesthetized rats by cardiac puncture or by retroorbital plexus puncture with a citrated (3.8% sodium citrate) syringe or a capillary tube.

Materials. Selenocystine, selenomethionine, selenium dioxide (SeO_2), sodium selenite (Na_2SeO_3) and 5,5′-dithiobis(2-nitrobenzoate) (DTNB) were obtained from the Sigma Chemical Co., St. Louis, MO. Sodium selenate (Na_2SeO_4) was obtained from Alpha Products, Denvers, MA. Metaphosphoric acid was purchased from Matheson, Coleman & Bell, Norwood, OH.

Lysis experiment. Blood samples were washed three times in 5 vol. saline-phosphate buffer (pH 7.4) [15], and the cells were suspended in buffer to make 30% (v/v) suspension (hematocrit approx. 13.5%); a sample (0.1 ml) of the erythrocyte suspension was delivered to a test tube and 2.9 ml of an appropriate incubation medium was then added to make a 1% (v/v) suspension (hematocrit approx. 0.45%), which was then incubated at 37° with gentle agitation every 2 hr. Centrifugation was carried out at 2000 rpm for 2 min. Hemoglobin released to supernatant fractions was measured at 415 nm, and percent hemolysis was calculated as described by Draper and Csallany [15]. All of the solutions added to erythrocytes were prepared in saline-phosphate buffer and were maintained at pH 7.4 with care to maintain isotonicity.

Determination of GSH. GSH determinations were

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performed by the method of Beutler *et al.* [16] with some modification. During the experiments, erythrocytes were quickly centrifuged and washed twice with buffer. To the intact cells 3 ml of a metaphosphate–EDTA–NaCl solution was added. After standing 5 min the mixture was filtered and 2.0 ml of the filtrate was mixed with 1.5 ml of 0.5 M Na_2HPO_4 buffer (pH 9.0) and with 0.5 ml of DTNB (40 mg DTNB in 1% trisodium citrate solution). Absorbance at 412 nm was read within 5 min. The sensitivity of GSH assay was approximately 2.0 mg/100 ml of blood.

Enzyme assay. Glutathione peroxidase activities of erythrocytes and liver of selenium-deficient and selenium-supplemented rats were assayed by a modified method of Paglia and Valentine [17] with 0.25 mM H_2O_2 as substrate. Assays were carried out in 100 mM phosphate buffer (pH 7.0) containing 2.0 mM GSH, 0.2 mM NADPH, 1.0 units/ml glutathione reductase, 1.0 mM NaN_3 and 3.0 mM EDTA. Glutathione peroxidase activity in the liver was assayed in a 20% (w/v) homogenate after centrifugation at 48,000 *g* for 40 min. Liver protein in the supernatant fraction was assayed by the method of Lowry *et al.* as modified by Schacterle and Pollack [18]. Erythrocyte glutathione peroxidase was assayed in the hemolysate by adding 0.1 ml of 30% erythrocyte suspension to 2.9 ml of 20 mM phosphate buffer containing 0.6 mM EDTA. Hemoglobin was measured at 540 nm with a hemoglobin standard supplied in a commercial kit (No. 525, Sigma Chemical Co.).

RESULTS

Figure 1 demonstrates that selenocystine, selenium dioxide and sodium selenite caused lysis of rat erythrocytes *in vitro*, whereas selenomethionine and sodium selenate had no hemolytic effect. Furthermore, selenocystine was found to be more strongly hemolytic than either selenite or selenium dioxide. High concentrations of sodium selenite and selenium dioxide *per se* apparently did not contribute to erythrocyte lysis since no hemolysis was induced by the same concentration (10.0 mM) of sodium selenate (Fig. 1) or sodium sulfite (data not shown). Figure 2 shows that lysis of erythrocytes induced by selenocystine was preceded by the loss of intracellular GSH. Cystine at the same concentration (2.5 mM) caused neither loss of intracellular GSH nor hemolysis. Although data are not shown, selenomethionine and sodium selenate were also found to have no effect on levels of intracellular GSH. Figure 2 also shows that selenite at 2.5 mM produced an almost complete loss of intracellular GSH in the erythrocyte within 10 min but induced no detectable hemolysis within 6 hr. The present study provides no explanation for the delay between the disappearance of intracellular GSH and hemolysis by selenite. However, the rapid loss of intracellular GSH caused by selenite incubation is not likely to have been due to the reaction between GSH and remaining selenite outside the cell during the GSH assays since identical results were obtained from cells washed up to four times with buffer. From our results, rat erythrocytes appear to be more resistant to lysis by selenite than sheep erythrocytes are since 1.0 mM selenite induces about 50% lysis of normal sheep erythrocytes within 4 hr [10] and 10.0 mM selenite caused only 36.5% lysis of rat erythrocytes within 12 hr (Fig. 1).

The hemolytic effects of selenite and selenium dioxide were greatly enhanced when GSH was included in the incubation medium (Fig. 3). In the presence of 1.0 mM extracellular GSH, cell lysis

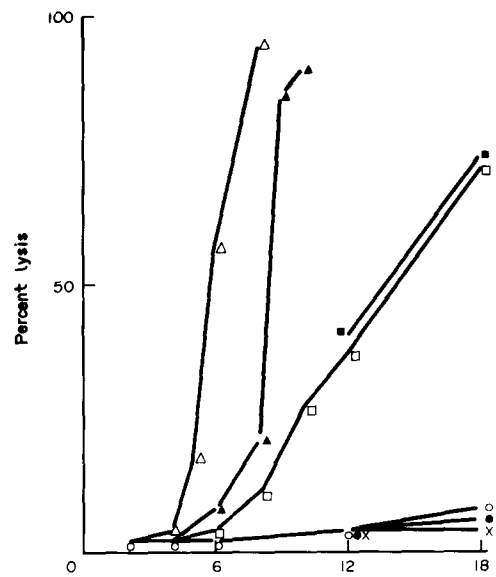

Fig. 1. Lysis of rat erythrocytes (1% suspension, v/v) induced by various selenium compounds. Key: (○) saline-phosphate buffer (●) 10.0 mM Na_2SeO_4; (×) 10.0 mM selenomethionine; (■) 10.0 mM SeO_2; (□) 10.0 mM Na_2SeO_3; (△) 5.0 mM selenocystine; and (▲) 2.5 mM selenocystine. Cells lysed by H_2O equalled 100% lysis.

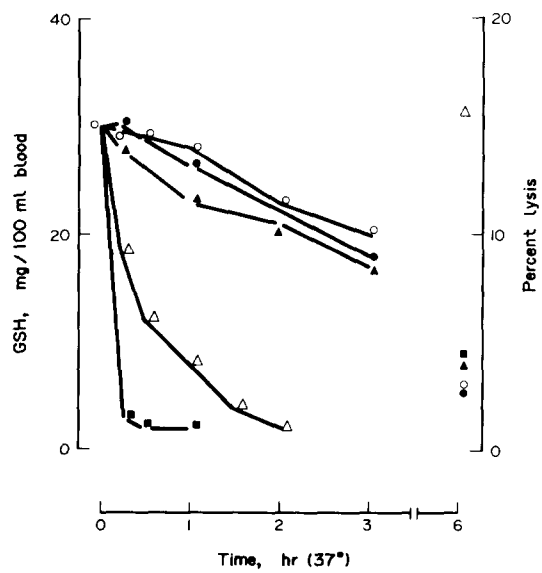

Fig. 2. Percent hemolysis and intracellular GSH concentrations in rat erythrocytes (1% suspension, v/v) incubated with selenite, selenocystine and cystine. Key: (○) saline-phosphate buffer; (△) 2.5 mM selenocystine; (▲) 2.5 mM selenocystine plus 10 mM glucose in the medium; (●) 2.5 mM cystine; and (■) 2.5 mM selenite. Percent lysis of erythrocytes was measured at 6 hr only.

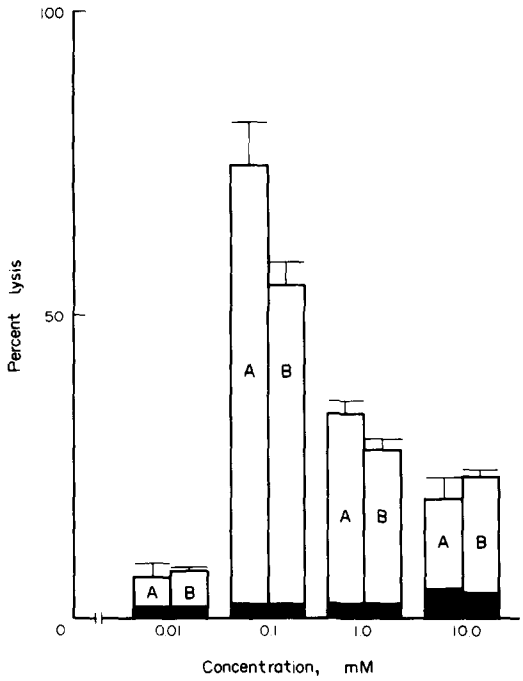

Fig. 3. Lysis of rat erythrocytes (1% suspension, v/v) induced by selenite (A) and selenium dioxide (B) in the presence of 1.0 mM extracellular GSH. Incubations were for 6 hr at 37°. Each value represents the mean ± S.E.M. of three animals. The dark-shaded areas represent percent lysis by selenite (A) or by selenium dioxide (B) in the absence of extracellular GSH. Cell lysis in buffer control is less than 4%.

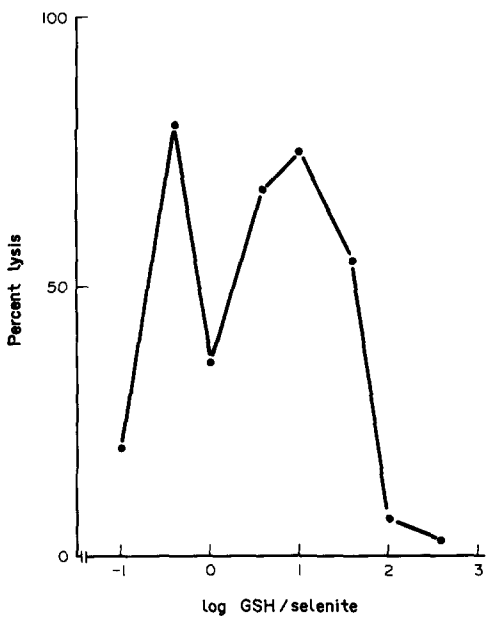

Fig. 4. Percent lysis of rat erythrocytes (1% suspension, v/v) as a function of the logarithm of the ratio of the concentration of extracellular GSH to that of selenite. Selenite was added to cell suspensions to final concentrations of 0.01, 0.1, 1.0 and 10.0 mM respectively. Glutathione was then added to each suspension to a final concentration of 1.0 and 4.0 mM. Incubations were for 6 hr at 37°.

Table 1. Effect of extracellular GSH on selenocystine-induced lysis of rat erythrocytes*

Additions	% Lysis†
Saline-phosphate buffer, pH 7.4	3.2 ± 0.4
Selenocystine (5.0 mM)	57.2 ± 4.9
Selenocystine (5.0 mM) + GSH (1.0 mM)	27.4 ± 2.6
Selenocystine (5.0 mM) + GSH (2.0 mM)	2.7 ± 0.2

* Erythrocytes (1% suspension, v/v) were incubated at 37° for 6 hr.

† Percent lysis was calculated using water-lysed cells as 100%. Each value represents the mean ± S.E.M. of three animals.

reached about 80% as the selenite concentration increased from 0.01 to 0.1 mM. Lysis of cells then decreased as the selenite concentration was increased. The results indicate a relationship between hemolysis and the ratio of GSH to selenite or to selenium dioxide. Using two levels of GSH (1.0 and 4.0 mM) and four levels of selenite (0.01, 0.1, 1.0 and 10.0 mM), cell lysis as a function of the ratio of extracellular GSH to selenite was investigated. Figure 4, expressed as log GSH/selenite, confirms the findings of Young *et al.* [10] with sheep erythrocytes that there are at least two separate components of selenite-induced hemolysis. One occurred at the lower GSH/selenite ratio and the other occurred at the higher ratios.

In contrast to selenite-induced hemolysis, added extracellular GSH had an inhibitory effect on selenocystine-induced hemolysis, and the inhibitory effect increased as the concentration of extracellular GSH increased (Table 1). Addition of 10.0 mM glucose to the incubation medium also inhibited selenocystine-induced hemolysis and the loss of intracellular GSH (Fig. 2). No elemental selenium was found following cell lysis induced by selenite and selenocystine, although selenocystine in saline-phosphate buffer (pH 7.4) was relatively unstable at room temperature and formed elemental selenium upon prolonged standing. The stability of the selenocystine solutions decreased as the concentrations increased. A 10 mM concentration of selenocystine in buffer formed elemental selenium after approximately 12 hr at room temperature but no elemental selenium was formed in 2.0 mM selenocystine for up to 2 days.

Table 2 shows that the percentages of inhibition of selenocystine-induced lysis of erythrocytes from selenium-deficient and selenium-supplemented rats were equal when 10.0 mM glucose was included in the incubation medium. No significant differences were found in selenocystine-induced hemolysis in the absence of glucose between cells from selenium-deficient rats and cells from selenium-supplemented rats (Student's t-test, $P > 0.05$). Small amounts of glutathione peroxidase activity were detected in the erythrocytes from selenium-deficient rats even though the liver had no detectable glutathione peroxidase activity.

Table 2. Effect of dietary selenium on hemolysis by selenocystine (2.0 mM) in the presence and absence of glucose in the incubation medium*

Dietary treatment	Glutathione peroxidase†		% Lysis	
	Liver (units/mg protein)	Erythrocyte (units/mg Hb)	−Glucose	+Glucose
−Se	ND	0.04 ± 0.01	56.7 ± 3.0	3.5 ± 0.2
+Se (0.5 ppm)	1.64 ± 0.27	0.67 ± 0.09	62.3 ± 5.4	4.4 ± 0.4

* Erythrocyte samples were taken from 14- to 16-week-old rats receiving the respective dietary treatment. Lysis of erythrocytes (1% suspension, v/v) was carried out at 37° for 9 hr. Each value is the mean ± S.E.M. of three animals.

† A unit is 1 μmole NADPH oxidized/min. Glutathione peroxidase activity in liver from selenium-deficient animals was not detectable (ND).

DISCUSSION

The present results demonstrate that selenocystine is more hemolytic than selenite and selenium dioxide to rat erythrocytes *in vitro*. Selenate and selenomethionine had no hemolytic effects under the present experimental conditions. Hemolysis by selenocystine and selenite was associated with loss of intracellular GSH. Our results on the lytic mechanism of selenite and selenium dioxide support the suggestion of Young *et al.*, who used sheep erythrocytes [10], that intermediates such as selenotrisulfides (GS-Se-SG) and selenopersulfides (GS-SeH) produced from the reaction of selenite with GSH may be responsible for the erythrocyte lysis by selenite. The reaction of selenite with thiols was originally proposed in 1941 by Painter [19] and was later extensively investigated by Ganther [20–22]. The reactions, which occur spontaneously and are independent of oxygen, are:

$$4\ GSH + SeO_2 \rightarrow GS\text{-}Se\text{-}SG + GSSG + 2\ H_2O$$
$$4\ GSH + SeO_3^{2-} \rightarrow$$
$$GS\text{-}Se\text{-}SG + GSSG + 2\ OH^- + H_2O$$

The reactions form moderately stable derivatives having an enhanced absorption in the 260–380 nm region [20]. Selenotrisulfides are relatively stable but can be reduced to selenopersulfides (highly reactive nucleophiles) either by excess thiol [23, 24] or by glutathione reductase [22]. Both selenotrisulfides and selenopersulfides undergo further transformation leading to the formation of elemental selenium.

Selenate and selenomethionine apparently do not react with GSH to form selenotrisulfides and selenopersulfides since no enhanced absorption (determined at 340 nm) or formation of elemental selenium was observed (data not shown). Selenate and selenomethionine also did not cause loss of intracellular GSH in rat erythrocytes. These observations may explain the lack of hemolytic effects of selenate and selenomethionine on rat erythrocytes. There may be other factors that affect hemolytic effects of various selenium compounds. For example, selenomethionine has been reported to be actively transported across cell membranes, whereas selenite and selenocystine are not activity transported [25]. It has also been suggested that selenate is reduced to selenite in normal cellular metabolism [26]. It may be that reduction of selenate to selenite does not occur or proceeds less effectively in rat erythrocytes since selenate does not induce lysis of rat erythrocytes. It is interesting to note that selenite–GSH mixtures are among the most mutagenic substances tested in a DNA repair assay and, in contrast, selenocystine causes much less DNA damage in the presence or absence of added GSH [27].

Three observations suggest that selenocystine induces erythrocyte lysis by oxidizing intracellular GSH. First, selenocystine caused loss of intracellular GSH and hemolysis. Second, addition of glucose to erythrocytes inhibited loss of intracellular GSH and hemolysis by selenocystine. Extensive evidence suggests that glucose protects the erythrocyte from oxidative damage through its ability to generate intracellular GSH by the combined actions of glucose-6-phosphate dehydrogenase and glutathione reductase. Our results indicate that the inhibitory effect of glucose on selenocystine-induced lysis of erythrocytes is independent of the levels of glutathione peroxidase in the erythrocyte (Table 2). Third, in contrast to selenite-induced hemolysis, addition of GSH to erythrocytes inhibited hemolysis by selenocystine. The inhibitory effect of added GSH on selenocystine-induced hemolysis may result from either the reduction of selenocystine to selenocysteine by GSH or the protection of membrane sulfhydryl groups by GSH, or both. Dickson and Tappel [28] have reported that the reduction of selenocystine by GSH to selenocysteine occurs very rapidly, and they suggest that the ratio of GSH to selenocystine may be a critical factor in tissue toxicity to selenium.

The present results suggest that selenocystine, selenite and selenium dioxide cause erythrocyte lysis because of their oxidation of intracellular GSH. Selenocystine is believed to be reduced by intracellular GSH to selenocysteine, with the loss of intracellular GSH resulting in hemolysis by selenocystine. In contrast, erythrocyte lysis by selenite and selenium dioxide may be mediated by one or more intermediates (selenotrisulfides and selenopersulfides) produced from the reaction of intracellular GSH with selenite or with selenium dioxide. Selenate and selenomethionine, being incapable or less capable of oxidizing or otherwise reacting with GSH, are not hemolytic to rat erythrocytes. In his conclusion, Ganther [20] indicated that the reaction of selenite with thiols provides a plausible means of incorporating inorganic selenite into a stable organic

moiety. Diplock [29] also concluded that, although there may be other reactions in which selenium, at toxic levels, is involved, the reaction of selenite with thiols holds the key to the biochemistry of selenium toxicity. Since the present study deals with only one thiol, GSH, which contributes almost all of the non-protein thiols to erythrocytes [16, 30] and is essential to erythrocyte integrity, it is not known whether the oxidation of other thiols (such as protein thiols) by selenite, selenium dioxide or selenocystine also contributes to lysis of rat erythrocytes.

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