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HAEMOLYSIS OF NORMAL AND GLUTATHIONE-DEFICIENT SHEEP ERYTHROCYTES BY SELENITE AND TELLURITE

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Abstract—Both selenite and tellurite caused lysis of normal sheep erythrocytes *in vitro*. GSH-deficient sheep erythrocytes were considerably more resistant to haemolysis than normal cells. This effect was independent of the biochemical lesion responsible for GSH-deficiency (amino acid transport lesion or γ-glutamyl cysteine synthetase deficiency). These and other observations directly implicate intracellular GSH in the lytic mechanism. Selenite and tellurite-induced haemolysis therefore provides a simple method for detecting GSH-deficient cells. The lytic effect of selenite may explain some of the symptoms associated with selenium poisoning.

Two types of inherited erythrocyte GSH†-deficiency can be found in sheep. The first (referred to as lesion 1 in this paper) results from an impaired transport system for certain amino acids including cysteine, a precursor of GSH [1–3]. The second type of deficiency (lesion 2) is the result of a diminished maximum activity of γ-glutamyl cysteine synthetase, the first enzyme of GSH biosynthesis [4, 5]. Selective breeding experiments have produced sheep which manifest both types of deficiency. Such animals with lesions 1 + 2 have very low concentrations of erythrocyte GSH (< 15 per cent of normal) [6].

Before selenium was shown to be an essential trace element [7] and an integral part of glutathione peroxidase [8] it was known to be highly toxic both in man and sheep [9]. One symptom of selenium poisoning is anaemia. Tellurite (TeO_3^{2-}), which is closely related to selenite (SeO_3^{2-}), has been shown to cause lysis of human erythrocytes *in vitro* [10] and intracellular GSH appeared to be involved in the lytic process [11]. The aim of the present study was to investigate this phenomenon further by comparing the effects of selenite and tellurite on normal and GSH-deficient sheep erythrocytes. A preliminary report of some of these findings has already been published [12].

MATERIALS AND METHODS

Sheep of the 4 phenotypes were identified as described previously [6]. Blood was collected by jugular venepuncture into heparinized tubes. All animals were maintained at Babraham under standard husbandry conditions and blood samples were generally used within 5 hr of bleeding.

Materials. K_2TeO_3 and 4-acetamido-4′-isothiocyanato-stilbene-2,2′-disulphonic acid (SITS) were obtained from BDH Chemicals Ltd., Poole, U.K. K_2SeO_3 was purchased from Hopkin & Williams Ltd., Chadwell Heath, U.K. Diazenedicarboxylic acid-bis-(*N*,*N*-dimethylamide) (diamide) and 5,5′-dithiobis-(2-nitrobenzoate) (DTNB) were obtained from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, U.K. $Na_2[^{35}SO_4]$ was purchased from the Radiochemical Centre, Amersham, U.K.

Lysis experiments. Erythrocytes were washed 3 times with 20 vol. of a medium containing 140 mM NaCl, 5 mM KCl, 20 mM Tris–HCl (pH 7.2 at 37°), 2 mM $MgCl_2$ and 0.1 mM EDTA. The buffy coat was discarded.

Cell lysis was measured at 37° by mixing 0.6 ml of prewarmed washed erythrocytes (haematocrit approx. 1 per cent) with an equal volume of prewarmed medium containing the appropriate additives. Incubations were stopped at predetermined time intervals by rapidly centrifuging the cells using an Eppendorf 3200 microcentrifuge (15,000 *g*, 20 sec). The degree of cell lysis was determined by measuring the extinction of the supernatant at 540 nm and relating this value to the extinction obtained when complete lysis was achieved by osmotic shock or addition of Triton X-100.

GSH estimations. Erythrocyte GSH was assayed using the nonspecific thiol reagent DTNB [13]. Previous studies have shown > 92 per cent of the DTNB-reactive nonprotein thiol in sheep erythrocytes is GSH [14].

In one experiment, erythrocyte GSH was converted to GSSG by incubating cells at a haematocrit of 10 per cent for 1 hr at 0° in normal incubation medium containing 1.5 mM diamide [15]. Excess diamide was removed by washing the cells 4 times with 20 vol. ice-cold medium.

SO_4^{2-} *uptake.* The incubation medium used in this series of experiments contained 20 mM sodium phosphate (pH 7.2) in place of Tris–HCl. Uptake of 1 mM $Na_2[^{35}SO_4]$ at 37° was measured by centrifugation of cells through *n*-dibutyl phthalate as described previously [16]. Erythrocytes were treated with SITS by

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† Abbreviations: GSH, reduced glutathione; GSSG, oxidized glutathione.

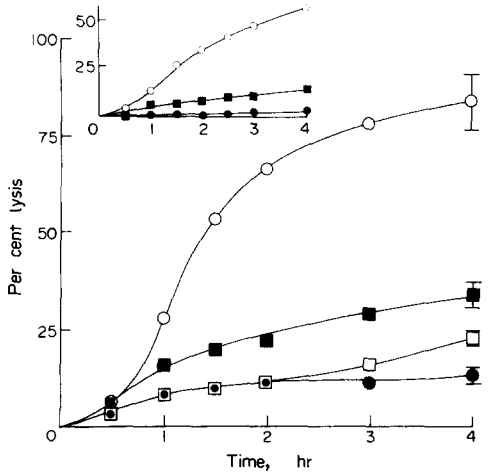

Fig. 1. Effect of tellurite and selenite on normal and GSH-deficient sheep erythrocytes. Erythrocytes were incubated with 1 mM tellurite at 37° as described in the text. The curves are the means ± S.E.M. for 3 animals of each type. ○, Normal cells; ■, cells with lesion; □, cells with lesion 2; ●, cells with lesion 1 + 2. The inset diagram shows the result of a corresponding exeriment where cells from 3 sheep (one high-GSH type, one with lesion 1 and one with lesions 1 + 2) were incubated with 1 mM selenite. Maximum lysis in the absence of added tellurite or selenite was 2 per cent.

incubation of cells with 0.2 mM SITS for 2 hr at 4° and a haematocrit of 2 per cent. Excess inhibitor was removed by washing the cells in ice-cold incubation medium.

RESULTS

Figure 1 shows that addition of 1 mM tellurite to normal (high-GSH type) sheep erythrocytes at 37° resulted in almost complete haemolysis within 4 hr. Similar results were found with human cells (data not shown). In contrast, tellurite had much less effect on GSH-deficient sheep erythrocytes, with the rate

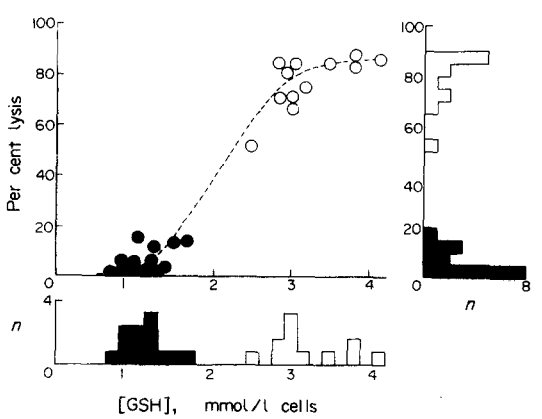

Fig. 2. Relationship between tellurite-induced haemolysis and intracellular GSH concentration. Cells from 14 high-GSH type sheep (○, open squares) and 12 animals with lesion 1 (●, hatched squares) were incubated with 1 mM tellurite for 4 hr at 37°. See text for other details. *n*, No. of animals.

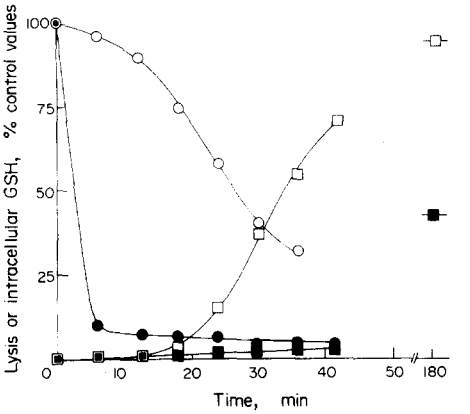

Fig. 3. Effect of selenite and tellurite on the intracellular GSH concentration of normal sheep erythrocytes. Cells were incubated with 1 mM selenite or tellurite at 37°. Per cent lysis was determined as described in the text. Residual cell pellets were rapidly washed free of excess tellurite (selenite and the intracellular GSH concentration of the remaining intact cells measured using DTNB as chromogen. ○, □, Intracellular GSH and per cent lysis in the presence of tellurite, respectively; ●, ■ intracellular GSH and per cent lysis in the presence of selenite, respectively.

of haemolysis proportional to the intracellular GSH-concentration (high GSH > lesion 2 > lesion 1 > lesion 1 + 2; 2.98 ± 0.28, 1.56 ± 0.08, 1.21 ± 0.33 and 0.82 ± 0.19 mmol GSH/l cells, respectively (mean ± S.E.M. [3]). The inset diagram in Fig. 1 further demonstrates that incubation of normal sheep erythrocytes with 1 mM selenite also induced lysis, but the rate of haemolysis was slower than that with tellurite (50 per cent after 4 hr). In agreement with the tellurite results, selenite had much less effect on GSH-deficient cells (high GSH > lesion 1 > lesion 1 + 2). Other experiments established that 1–2 mM was the optimal haemolytic concentration of tellurite or selenite for normal sheep erythrocytes, but that 0.1 mM tellurite was still capable of substantial cell lysis (70 per cent after 3 hr). Haemolysis by both tellurite and selenite was not associated with methaemoglobin formation.

Additional evidence that tellurite-induced lysis is related to the intracellular GSH concentration is presented in Fig. 2 where the effects of 1 mM tellurite on cells from 14 high-GSH type sheep and 12 animals with lesion 1 are compared. Normal cells lysed considerably more readily than GSH-deficient erythrocytes, with no overlap between the two groups (50–85 per cent lysis and 0–25 per cent lysis, respectively after 4 hr).

Further experiments were carried out to investigate the mechanism(s) responsible for tellurite and selenite-induced cell lysis. Figure 3 demonstrates that tellurite lysis of normal cells was preceded by a decrease in intracellular GSH concentration. This lysis was also associated with the formation of elemental tellurium (dark grey precipitate). Other experiments found that no detectable tellurium was formed in cells with lesions 1 + 2. Selenite caused a much more rapid and complete loss of intracellular GSH from normal cells, but the onset of haemolysis was considerably slower (see also Fig. 3) and was

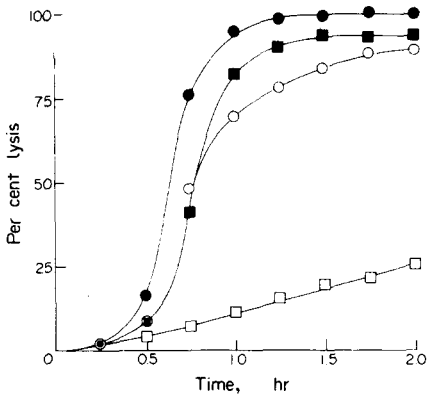

Fig. 4. Effects of glucose on tellurite-induced lysis of normal and GSH-deficient erythrocytes. Normal sheep erythrocytes (○, ●) and cells from an animal with lesions 1 + 2 (□, ■) were incubated with 1 mM tellurite in the presence (closed symbols) or absence (open symbols) of 5 mM glucose.

not associated with selenium formation (red precipitate). The relatively slow decrease in intracellular GSH concentration induced by tellurite was not a consequence of slower reaction between GSH and tellurite compared with selenite, since incubation of 1 mM GSH with 1 mM tellurite in normal medium at room temperature resulted in a 92 per cent decrease in GSH concentration within 2 min. It is therefore probable that tellurite enters erythrocytes at a slower rate than selenite.

Oxidation of intracellular GSH with diamide of pretreatment of cells with the anion transport inhibitor SITS abolished tellurite (1 mM) induced haemolysis of high-GSH cells. The former result provides additional evidence that cellular thiol groups are important for lysis while the latter experiment suggests that tellurite (and presumably also selenite) enters cells via the inorganic anion transport mechanism [17]. Control experiments established that normal cells and GSH-deficient erythrocytes (lesions 1 + 2) transported SO_4^{2-} at the same rate (influx 0.100 ± 0.003 and 0.099 ± 0.003 mmol/l cells in 30 min, respectively, at 1 mM extracellular SO_4^{2-}. Uptake by SITS-treated cells was < 10 per cent of control values. From this it is assumed that lesions 1 and 2 do not affect selenite or tellurite permeability, though this was not tested for directly.

Tellurite-induced haemolysis of GSH-deficient cells (lesions 1 + 2) was increased 8-fold when 5 mM glucose was included in the incubation medium (Fig. 4). The lysis was associated with tellurium formation. Glucose had a much smaller effect on the rate of haemolysis of normal cells and no increased tellurium production was observed. Finally, incubation of cells with lesions 1 + 2 in the presence of added extracellular GSH resulted in rapid lysis when either tellurite or selenite were added (Fig. 5). Maximum haemolysis occurred at GSH/tellurite (selenite) ratios of 5–10. However, the shape of the selenite curve suggested that there were two separate components of selenite-induced haemolysis: one occurring at high GSH/selenite ratios and comparable to that seen with tellurite, and another occurring at lower GSH/selenite ratios. The latter component was not observed with tellurite. Incubation of 0° abolished lysis at low GSH/selenite ratios. Haemolysis at high GSH/tellurite and GSH/selenite ratios was associated with visible metal formation, suggesting that the two compounds undergo similar reactions in the presence of excess GSH.

DISCUSSION

The present results demonstrate that both selenite and tellurite cause erythrocyte lysis *in vitro*. Four observations directly implicate intracellular GSH in the lytic process. First, GSH-deficient cells were considerably more resistant to lysis than normal cells, an effect which was independent of the lesion responsible for GSH-deficiency. Second, lysis by both tellurite and selenite was preceded by a drop in intracellular GSH concentration. Third, the thiol-oxidizing agent, diamide, inhibited tellurite-induced lysis. Finally, addition of extracellular GSH to

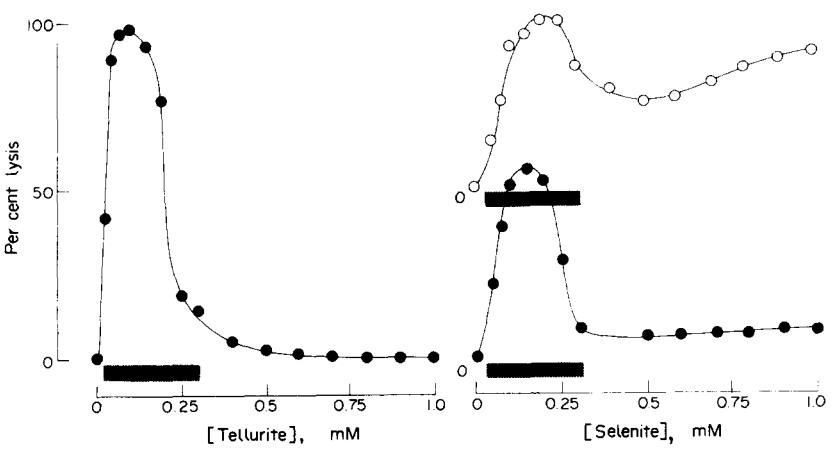

Fig. 5. Effects of extracellular GSH on lysis of GSH-deficient erythrocytes by selenite and tellurite. Erythrocytes from an animal with lesions 1 + 2 were incubated in medium containing GSH (1 mM) and varying concentrations of selenite or tellurite. ○, Selenite (3.5 min at 37°); ●, selenite (41 min at 1°). Incubations in the presence of tellurite were for 10 min at 37°. The hatched areas indicate selenium and tellurium formation. Maximum lysis in the absence of added tellurite (selenite) or GSH was 2 per cent.

GSH-deficient erythrocytes resulted in rapid lysis when tellurite (or selenite) were also present.

An important intermediate in the series of reactions between selenite and GSH is 2-seleno-1,3-diglutathione, GS–Se–SG [18]:

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

GS–Se–SG is relatively stable but is rapidly reduced in the presence of excess thiol to glutathione selenopersulphide (GS–SeH), a highly reactive nucleophile which undergoes further transformations leading to the eventual formation of elemental selenium [19–22]. GS–SeH can also be formed from GS–Se–SG enzymically by glutathione reductase [20]. Our findings suggest that erythrocyte lysis by selenite (and tellurite) may be mediated by one or more of these intermediates. However, the situation is complex. For example, the experiments employing extracellular GSH to lyse GSH-deficient erythrocytes indicate that there are at least two separate components of selenite-induced lysis; one occurring under conditions where GS–Se–SG is relatively stable, and the other occurring at GSH/selenite ratios where GS–Se–SG is rapidly reduced. Haemolysis of cells by reaction of selenite with intracellular GSH is likely to occur by the former mechanism whereas tellurite-induced cell lysis only occurs at high GSH/tellurite ratios. The ability of glucose to enhance tellurite lysis of GSH-deficient erythrocytes is presumably due to increased reduction of otherwise stable GSH-tellurium complexes.

In the present series of experiments we have made use of GSH-deficient sheep erythrocytes. Inherited erythrocyte GSH-deficiency also occurs in man and is often associated with the occurrence of haemolytic anaemia, especially noticeable after exposure to oxidant drugs (see Ref. [23] for a recent review). One implication of the present results is that tellurite or selenite could be used as a simple means of detecting such GSH-deficient erythrocytes. The lytic action of selenite may also contribute to the haematological symptoms associated with selenium poisoning [9].

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