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Tellurite-induced damage of the erythrocyte membrane. Manifestations and mechanisms

B. Deuticke, P. Lütkemeier and B. Poser

Institut für Physiologie, Medizinische Fakultät der RWTH Aachen, Aachen (Germany)

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Chemical and biophysical mechanisms underlying the thiol-dependent lytic action of tellurite (and selenite) on human erythrocytes were investigated using native and GSH-depleted cells. Exposure of GSH-depleted cells to tellurite alone produces oxidative cross-linking of membrane thiols paralleled by a moderate membrane leakiness comparable in its extent to that induced by other SH-oxidizing agents (diamide, periodate). Exposure to tellurite in presence of endogenous or exogenous GSH produces marked leakiness which stems from the formation of aqueous leaks permeant to ions and nonelectrolytes and sensitive to inhibition by phloretin. Apparent pore radii, derived from exclusion limits for polar non-electrolytes, range from 0.3 to at least 1.3 nm. Leak size increases with increasing exposure time and concentration of the modifier. Leak formation is paralleled by membrane rigidification based on the cross-linking of spectrin. Thiol-dependent leak formation by tellurite in GSH-depleted cells can be sustained not only by exogenous GSH but also by other thiols. Progress of leak formation by tellurite/thiol can not be reliably quenched by procedures such as removal of tellurite from the medium, inhibition of anion transport via band-3 protein, washing of the cells or low temperature. The reaction can, however, be terminated, even in the presence of tellurite, by addition of *N*-ethylmaleimide, presumably due to the blockage of thiols or thiol-analogous tellurium compounds. *N*-ethylmaleimide even brings about a partial reversal of leakiness, suggesting the contribution of a reversible and an irreversible component of tellurite damage. Membrane perturbation by tellurite/thiol involves the formation of a membrane permeant tellurium species, possibly HTe^-, which is likely to induce progressive damage of membrane proteins by a redox shuttle going along with a formation of elemental tellurium and its reduction by thiols.

Introduction

Data scattered over the literature for the last two decades indicate that tellurite (TeO_3^{2-}) and selenite (SeO_3^{2-}) induce alterations of the red cell membrane going along with cell swelling, hemolysis and shedding of cell fragments [1–7]. Tellurite has even been envisaged, in view of this volume-expanding effect, as a potential antisickling agent [5]. In view of the established toxicity of tellurium compounds for eucaryotic [8,9] and procaryotic [10] organisms studies on the mechanisms underlying its membrane perturbing effects are of biomedical and toxicological interest. A crucial role of cellular GSH in membrane damage induced by tellurite was observed in the original studies. GSH-deficient cells and cells depleted of endogenous GSH by oxidation or alkylation are essentially insensitive to the lytic action of tellurite [1,3,6]. This was taken to indicate a causal role of products of the reaction between tellurite (selenite) and thiols.

In order to characterize the formation and the properties of membrane defects induced by tellurite in greater detail, we took advantage of our former studies of red cell-membrane leaks induced by SH-oxidizing or radical-forming agents [11–14]. Strategies developed for the characterisation of these leaks have now proven useful for the study of the kinetics of leak formation by tellurite, and the analysis of leak sizes (and numbers). Evidence for a redox-cycling of tellurium, sustained by thiols, could be obtained.

Preliminary results were reported elsewhere (Deuticke, B. (1988) Biannual Meeting of the European Association for Red Blood Cell Research, Cadarache, France).

Correspondence to: B. Deuticke, Institut für Physiologie, Medizinische Fakultät der RWTH Aachen, Pauwelsstr. 30, W-5100 Aachen, Germany.

Abbreviations: CDNB, chlorodinitrobenzene; DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid; NEM, *N*-ethylmaleimide; r_{SE}, Stokes-Einstein radius.

Materials and Methods

Materials

Human blood from healthy donors, obtained from the local blood bank, was anticoaculated with citrate and stored up to 5 days at 4°C in a conventional storage medium. Erythrocytes were isolated by centrifugation (5 min, $6000 \times g$), plasma and buffy coat removed and the cells washed three times with isotonic saline at room temperature.

Potassium tellurite (K_2TeO_3) was from Fluka, Neu Ulm, chlorodinitrobenzene (CDNB) from Sigma, 4,4'-diisothiocyanostilbene-2,2'-disulfonate (DIDS) from Calbiochem, Dextran 1 (FP1) and Dextran 4 from Serva, Heidelberg.

Methods

Depletion of GSH by treatment with chlorodinitrobenzene. Cells were depleted of endogenous GSH by reaction with CDNB [15]. Briefly, cells were suspended in 10 vols. of phosphate buffered medium A (concentrations in mM): KCl (90), NaCl (40), NaH_2PO_4/Na_2HPO_4 (12.5) (pH 7.4). 37°C. CDNB was added, dissolved in a small volume of dimethylformamide, to a final concentration of 0.3 mM. After 30 min incubation at 37°C, the cells were spun down and washed three times in an excess of medium A.

Characterisation of leak permeabilities induced by tellurite. K^+-leakage from tellurite-damaged cells was followed continuously, at 37°C, by a K^+-selective glass-electrode (Tacussel X 110, Solea-Villeurbane, France) combined with a double-compartment reference electrode (K 701 Radiometer). 0.3 ml native or CDNB-treated cells were rapidly mixed into a reaction vessel containing isotonic choline chloride, dextran 4 (100 mg/ml final concentration), KCl (0.3) and K_2TeO_3 at the concentration desired. pH was adjusted to 7.4 by tetraethylammonium hydroxide. In experiments on cells depleted of endogenous GSH by CDNB treatment, GSH was added as the neutralized tetraethylammonium salt. For details of the measurements and evaluations see Ref. 16.

Leak permeability to salts was derived from half times of colloid-osmotic hemolysis in isotonic NaCl, following principles and using procedures described elsewhere [12,14,16]. Lysis of cells suspended (Hct 5%) in phosphate-buffered (2.5 mM) isotonic NaCl (pH 7.4) was followed in the presence of tellurite (native cells) or tellurite plus thiol (CDNB-pretreated cells).

Leak permeabilities to a model nonelectrolyte (erythritol), were measured as described in Ref. 11. Native or CDNB-pretreated, GSH-depleted cells were first preloaded with [^{14}C]-erythritol (Hct 30%) in phosphate-buffered saline containing 300 mg/ml dextran 8 as a protectant against colloid-osmotic lysis and then modified by either tellurite or tellurite plus thiol, respectively. Fluxes were initiated by suspending the cells in tracer-free media without tellurite. In experiments in which the action of tellurite on native cells was to be interrupted by blockage of thiols (see Results), NEM was added to the tellurite-containing suspension (Hct 25%) at a concentration of 4 mM unless indicated otherwise. After 10 min at 37°C (unless indicated otherwise) the suspensions were cooled to 0°C, centrifuged and the cells immediately used for efflux measurements.

Leak sizes were determined in cells in which the action of tellurite had been interrupted by addition of NEM, by applying the equilibrium procedure described in former work [14,17] using the following compounds: mannitol, M_r 180, r_{SE} 0.36 nm; sucrose, M_r 340, r_{SE} 0.46 nm; dextran 1, mean r_{SE} 0.75 nm; dextran 4, mean r_{SE} about 1.5 nm; dextran 8, mean r_{SE} about 2.1 nm. Radii were adopted from Ref. 18. Osmolarities of these solutions were determined by freeze point depression osmometry using a Gonotec Osmomat 030. The radius of a nonelectrolyte providing 50% protection against lysis after this long incubation period was obtained by interpolation and used as an indicator of the 'apparent pore radius'.

Membrane rigidification. The deformability of normal and modified cells as a measure of mechanical properties of the membrane was assessed by quantifying the mean elongation of erythrocytes subjected to viscometric shearing by laser ectacytometry. Measurements were carried out in the prototype of a simple new apparatus (Rheodyne, Myrenne, Roetgen, Germany). Experimental details were as described previously [19].

Further analytical procedures. Membrane SH-groups were determined in ghosts prepared from modified cells following the procedure described in Ref. 13. SDS-PAGE of the membrane polypeptide patterns was carried out as described in Ref. 20.

Results

Leak formation by tellurite in native cells

As in previous studies of others [1–4] on leakiness induced by tellurite (and selenite), we used colloid-osmotic lysis in the presence of tellurite as a primary indicator (Fig. 1). The time course of colloid-osmotic lysis is, however, usually slower than that of the formation of the ion leaks responsible for the slow net uptake of salt and water into the cell leading to colloid-osmotic swelling and lysis (see e.g. Ref. 21). This is also true for human erythrocytes exposed to tellurite. As evident from Fig. 2A, K^+ permeates the membranes of such cells at rates exceeding markedly those of lysis. Both K^+ leakiness and lysis already occur at very low tellurite levels ($\leq 5\ \mu M$) and increase with increasing levels of tellurite. Selenite tested under

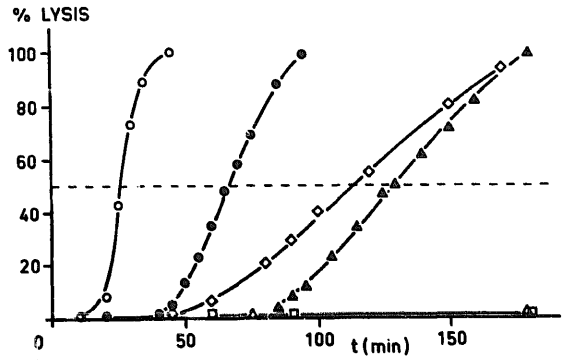

Fig. 1. Time course of lysis of erythrocytes in the presence of tellurite. Cells were exposed to the agent and lysis measured as described in Materials and Methods. ○, Tellurite 0.5 mM; ●, tellurite 0.1 mM; ▲, tellurite 0.01 mM; ◇, tellurite 0.5 mM in the presence of DIDS 300 μM; □, cells pretreated with CDNB 0.3 mM, 37°C, 30 min, then tellurite 0.5 mM; △ selenite 2.5 mM.

the same conditions did not cause lysis for periods up to 4 h at concentrations ≤ 2.5 mM (Fig. 1).

Tellurite-induced leakiness is not restricted to small ions but also concerns larger hydrophilic nonelectrolytes. This was demonstrated in tellurite pretreated cells, as described in Materials and Methods. The rapidly increasing leak permeability of such cells to erythritol is shown in Fig. 2B. Rates of K^+-release and of lysis were also studied in tellurite-pretreated cells after resuspension in tellurite-free media. These rates are generally higher than rates obtained for the same concentration of tellurite in the presence of the modifier. k_{lysis} (at 37°C) in presence of 0.5 mM tellurite, for instance, is about 0.033 min^{-1}, ($t_{1/2}$ = 30 min, Fig. 1), while k_{lysis} (at 0°C) in cells only pretreated with tellurite at 37°C is about 0.045 min^{-1} after 5 min exposure to tellurite, 0.27 min^{-1} after 10 min and 0.4 min^{-1} ($t_{1/2}$ = 2.5 min) after 15 min pretreatment.

TABLE I

Progress of membrane damage after pulse treatment of erythrocytes with 0.5 mM tellurite (5 min, 37°C) and subsequent washing of the cells

Cells were treated with tellurite, washed in saline media containing dextran 4 as a protectant against lysis and suspended for measuring rates of lysis in saline media containing 40 mM raffinose, either immediately or after an interposed incubation in the washing medium at 37°C.

Lysis phase	Rate of lysis (10^{-2} min^{-1})
Started immediately	1.5
Started after 30 min at 37°C	3.7
60 min at 37°C	5.0
90 min at 37°C	6.7
Started after 90 min at 0°C	1.6

These observations suggested, that formation of leaks, not just fluxes through the leaks, might proceed in tellurite-pretreated cells even after removal of tellurite from the suspension medium. As shown in Table I, this is in fact the case. Cells pretreated with tellurite for 5 min and then washed and incubated for 0–90 min at 37°C under conditions protecting them against lysis lyse increasingly fast during a subsequent incubation in non-protecting medium. After incubation at 0°C this effect was not observed.

Steps preceding tellurite-induced leak formation in native cells

Tellurite-induced leak formation in native cells is assumed to require entry of the anion into the cell and its reaction with GSH [1,3,7]. The relevance of the first step could be confirmed by demonstrating inhibition of leak formation in cells pretreated with the potent inhibitor of anion transport in erythrocytes, DIDS (Fig. 1). Surprisingly, however, the action of tellurite was not

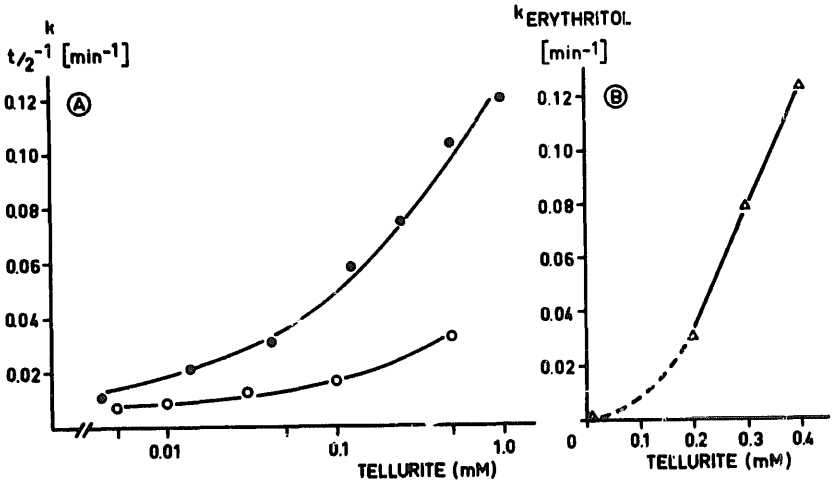

Fig. 2. Development of membrane leakiness in erythrocytes exposed to tellurite under various conditions. (A) Rates of loss of K^+ (●) and of colloid-osmotic lysis (○) in the presence of tellurite. Cells exposed to the agent as described in the Methods, at 37°C. (B) Rates of erythritol leak fluxes at 0°C after 10 min pretreatment of cells with tellurite at 37°C (Hct 30%), measured as described in Materials and Methods.

TABLE II

Lack of a simple relationship between decrease of GSH concentration and rate of lysis of erythrocytes exposed to tellurite

For experimental details see Materials and Methods.

Tellurite (mM)	Decrease of cellular GSH at 50% lysis (μmol/ml cells)	Time required for 50% lysis (min)
0.5	−1.75	25
0.025	−0.31	60
0.005	−0.11	91

completely suppressed even under conditions of DIDS treatment known to go along with complete inhibition of anion exchange. This finding either indicates additional DIDS-insensitive pathways providing for a slow uptake of tellurite or extracellular mechanisms of its activation even in the absence of exogenous thiols.

Reaction of tellurite with intracellular GSH, suppressible by DIDS treatment, could be demonstrated directly. In the presence of 0.5 mM tellurite, cellular GSH (normal content 2.7 μmol/ml cells) disappeared at a rate of 0.06 μmol/ml per min. Pretreatment of the cells with DIDS (1 μmol/ml cells, 20 min) fully prevented this disappearance for at least one hour. The amount of GSH consumed at 50% lysis is, however, not the same at each concentration of tellurite (Table II), as might be expected in the case of a direct relationship between consumption of GSH and lysis. Higher levels of tellurite, inducing 50% lysis after short time, produce a more marked depletion of GSH than low levels. The consumption of GSH thus depends on the level of tellurite but the extent of lysis is not related to the consumption of GSH. On the other hand, the extent of leakiness induced by a given concentration of tellurite is an almost linear function of the level of cellular GSH (Fig. 3). This could be demonstrated by lowering GSH levels to various extents by a CDNB pretreatment of the cells.

Leak formation by tellurite in cells depleted of endogenous GSH

Effects in the absence of exogenous thiols. While a crucial role of GSH for tellurite-induced leak formation is unquestionable, it must also be considered that tellurite may react directly with membrane protein thiols and thereby induce leakiness in analogy to other SH oxidizing and SH modifying agents [11,13]. Such a direct effect would seem likely in view of the chemical similarity of tellurite with selenite, for which cross-linking and other reactions with proteins are well established [22].

To study this problem red cells were depleted of endogenous GSH by CDNB (0.3–0.5 mM) [15], washed,

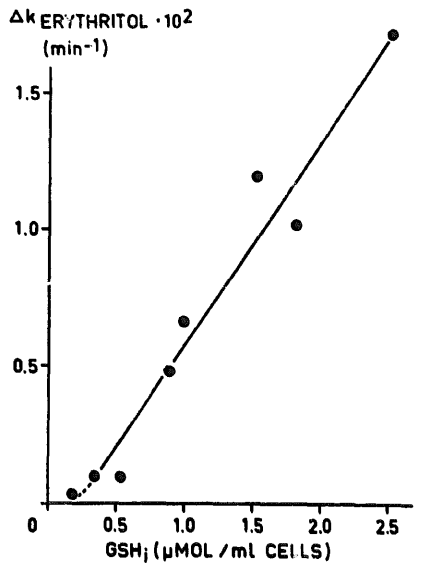

Fig. 3. Linear dependence of leak formation by tellurite on the initial intracellular level of GSH. Levels of GSH were decreased by incubating the cells with various concentrations of CDNB prior to their exposure to tellurite (1.2 mM) for 10 min. Effect of tellurite stopped by NEM. Leak formation characterized by the tellurite-induced enhancement of erythritol flux rates (Δk) measured at 0°C.

and treated with tellurite (0.5 or 1.0 mM, pH 7.4), for 60 or 120 min. Tellurite induced the disappearance of up to 40% of the membrane SH groups within that period (from 102 to 61 nmol/mg protein for the most extensive treatment (1 mM, 120 min)). Moreover, this tellurite treatment made the cells less deformable as indicated by a diminished elongation under viscometric shear stress at shear rates up to 10.5 Pa (Fig. 4). Selenite had the same effect, though to a somewhat

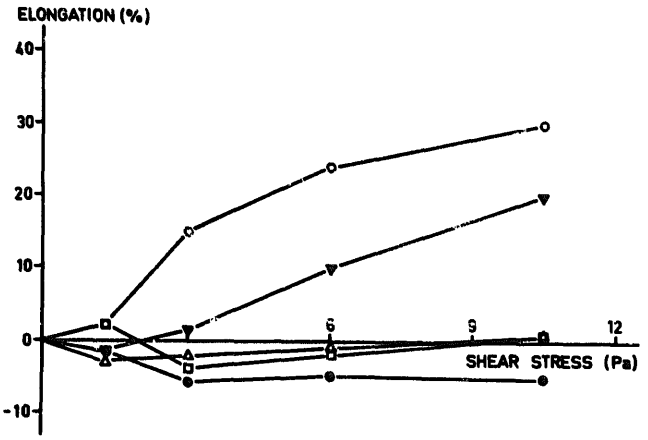

Fig. 4. Influence of treatment of erythrocytes with tellurite or selenite on cell deformability under various conditions. Native or CDNB-pretreated cells (see Materials and Methods) were exposed to tellurite or selenite at 37°C as indicated below. After washing (three times in medium B without erythritol) cell deformability was measured by laser ektacytometry as described in Materials and Methods and Ref. 19. ○, Controls; ●, native cells, tellurite 0.5 mM, 40 min; △, CDNB-treated, tellurite 1 mM, 60 min; ▼ CDNB-treated, selenite 1 mM, 60 min; □, CDNB-treated, GSH 2 mM plus tellurite 0.25 mM, 40 min.

lesser extent. Such 'rigidification' is likely to result from alterations or crosslinking of the membrane skeletal proteins due to reversible formation of intermolecular disulfide bonds (cf. Ref. 23). This is indicated by the reversibility of the rigidification upon subsequent reductive treatment with dithioerythritol. SDS-PAGE of ghosts treated under the same conditions with tellurite revealed reversible formation of high molecular weight material, derived from spectrin. (Fig. 5). Densitometric evaluation of scanned gels (n = 3) revealed the reversible disappearance, in the presence of tellurite, of 20–30% of the material from the spectrin region. Treatment of isolated spectrin with tellurite induced disappearance of SH-groups (data not shown).

Besides increasing membrane rigidity, tellurite by itself also produces membrane leak permeability, though to a much lesser extent than in combination with thiols. On the other hand, for the same extent of SH group modification, tellurite treatment of GSH-depleted cells induces about the same leakiness as other mild oxidants, e.g., diamide or periodate at 0°C (Table III). Independent of its destructive effect in presence

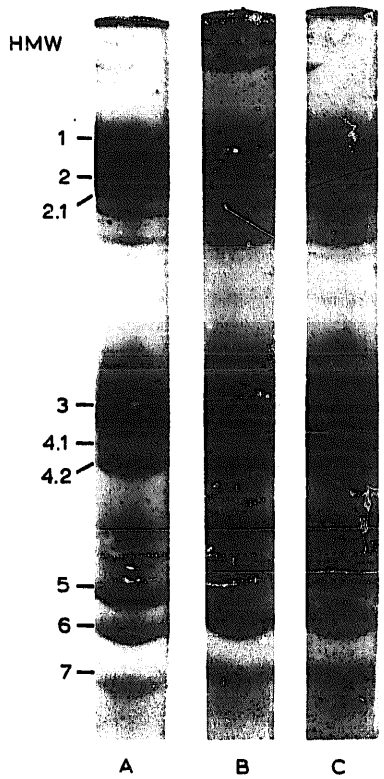

Fig. 5. Reversible formation, by tellurite, of cross-linked high-molecular-weight protein material (HMW) in erythrocyte ghosts. White ghosts prepared in 0.5 mM phosphate buffer were treated with 0.5 mM tellurite for 60 min at 37°C and washed. A portion was subsequently exposed to dithioerythritol (10 mM) for 60 min at 37°C and washed again. The membranes were solubilized and subjected to SDS gel electrophoresis as described earlier [20]. A, Control; B, tellurite; C, tellurite, than dithioerythritol. Enumeration of bands according to Ref. 35.

TABLE III

Comparison of tellurite with two mild SH oxidizing agents in their effects on membrane SH groups and leak permeabilities (Δk, at 0°C) for small solutes

Experimental details for tellurite as described in Materials and Methods, for periodate and diamide-treated cells as described in Refs. 11 and 13. Fluxes of erythritol in presence of 10 μM cytochalasin B, of chloride in DIDS (10 μM)-pretreated cells.

	ΔSH (%)	$\Delta k_{Erythritol}$ (10^{-3} min^{-1})	Δk_{Cl} (10^{-3} min^{-1})
Tellurite 1 mM, 60 min (CDNB-pretreated cells)	−30	3.5	10.0
Periodate 5 mM, 15 min, pH 7.4	−30	7.5	10.0
Diamide 5 mM, 15 min, pH 8	−27	3.0	11.9

of soluble thiols, tellurite is thus a membrane modifying, disulfide-forming reagent like the other agents mentioned.

Effects in the presence of added thiols. In native cells treated with tellurite, the aggressive species arising from its reaction with GSH is formed intracellularly. This is, however, not a prerequisite for membrane attack. Cation leakiness and subsequent lysis also occur when CDNB-pretreated erythrocytes are exposed to external impermeable GSH and tellurite. (Fig. 6).

At a given combination of the two agents, e.g., 2 mM GSH and 0.25 mM tellurite, loss of K^+ is regularly about 3-times faster than lysis ($k_{K^+} \sim 0.3$ min^{-1}, k_{lysis} approx. 0.1 min^{-1}). This is the same ratio as observed with native cells, containing about 2 mM GSH, in presence of 0.25 mM tellurite. Fig. 6 also demonstrates a marked dependence of the membrane perturbing effect of tellurite plus external GSH on the concentration ratio of the two components. The effect is maximal at a ratio GSH/tellurite of about 8–12. It decreases when this crucial value is exceeded or undercut. Similar findings were previously reported in genetically GSH deficient sheep erythrocytes by Young et al. [3]. An analogous maximum was observed by Ganther [24] for the in vitro rates of reaction between thiols and selenite.

These data raise the question whether membrane attack induced by tellurite in presence of an exogenous impermeable thiol occurs extra- or intracellularly, i.e., whether the aggressive species formed extracellulary has to enter the cell. Evidence for the latter assumption comes from two findings: (1) Lysis by external GSH plus tellurite is significantly (about 50%) suppressed by DIDS (data not shown). (2) Marked rigidification, which requires modification of the membrane skeleton, occurs in presence of external thiol and tellurite, exceeding the extent observed with tellurite alone (Fig. 4).

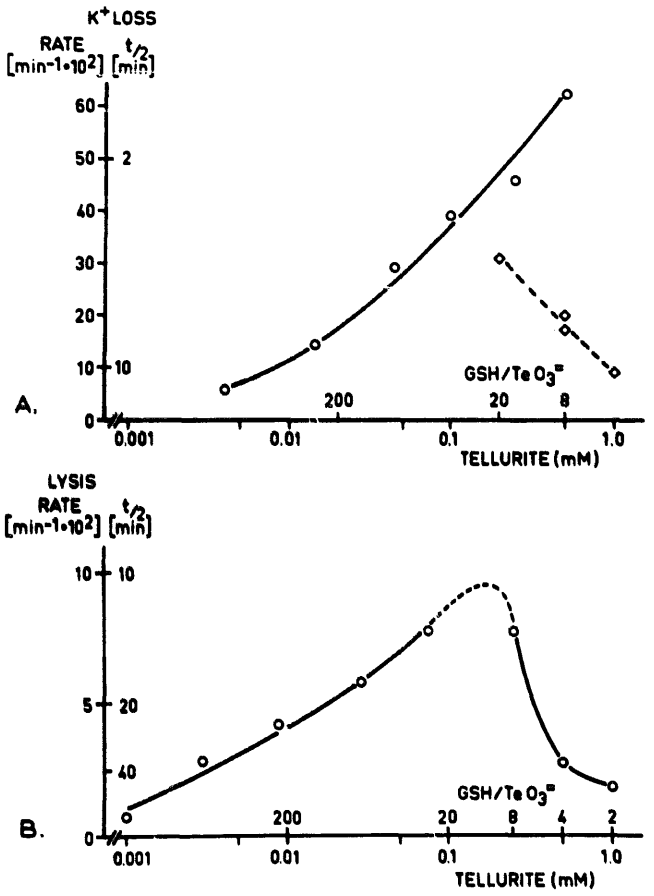

Fig. 6. Biphasic dependence, on the thiol/tellurite concentration ratio, of leak formation mediated by extracellular thiol and tellurite. (A) K^+ release from cells pretreated with CDNB and exposed to tellurite in the presence of external GSH (4 mM, ○——○; 2 mM, ◇— — —◇). (B) Colloid-osmotic lysis of cells pretreated with CDNB and exposed to tellurite in the presence of 2 mM external GSH. For further details see Materials and Methods.

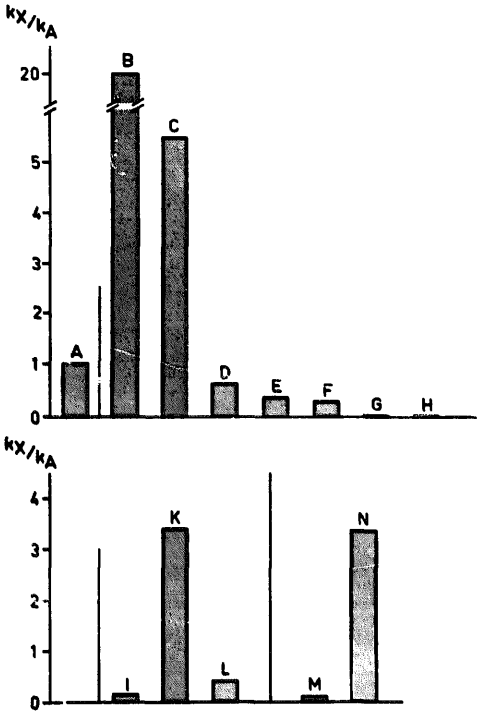

Fig. 7. Comparative evaluation of thiols with respect to their capacity for promoting lysis by, tellurite (A–H) or selenite (I–N). Cells were depleted of endogenous GSH by pretreatment with CDNB and exposed to tellurite (0.5 mM) or selenite (0.5 or 1 mM) in the presence of the various thiols. Rates of induced colloid-osmotic lysis (k_x) are normalized to the rate in the presence of GSH and tellurite (k_A). (Upper panel) All thiols at 2 mM: A, GSH; B, thioglycol (mercaptoethanol); C, cysteine; D, mercaptoethanesulfonate; E, dithioerythritol; F, cysteamine; G, thioglycolate; H, mercaptosuccinate. (Lower panel) I–L, Selenite 0.5 mM (I, GSH 1 mM; K, thioglycol 1 mM; L, dithioerythritol 2 mM. M, N, Selenite 1 mM (M, GSH 2 mM; N, thioglycol 2 mM). k = rate of lysis $(t_{1/2})^{-1}$.

GSH can be replaced in its lysis-promoting action in CDNB pretreated cells by other thiols (Fig. 7). At a ratio thiol:tellurite of 4, most thiols are less effective than GSH. Only cysteine and thioglycol (mercaptoethanol) are much more effective, inducing lysis in presence of tellurite within a few minutes. There is no obvious relationship between the effectivity of the various thiols and their charge or permeability: Both, thioglycol and thioglycolate are readily permeable, mercaptoethanesulfonate is a slowly permeating anion, cysteamine a rapidly permeating weak base, GSH is impermeant. Selenite, which has little lytic effect in combination with GSH (compare columns I and A), has a pronounced effect in combination whith thioglycol (columns K and N).

The product of the reaction between tellurite and GSH which induces membrane damage is not stable. A time-dependent decrease in the level of aggressiveness could be demonstrated by experiments in which the reactants (GSH and tellurite) were incubated with each other for varying periods of time before addition of the cells. The mixture became less aggressive with time (Fig. 8), the half-time of lysis increased considerably.

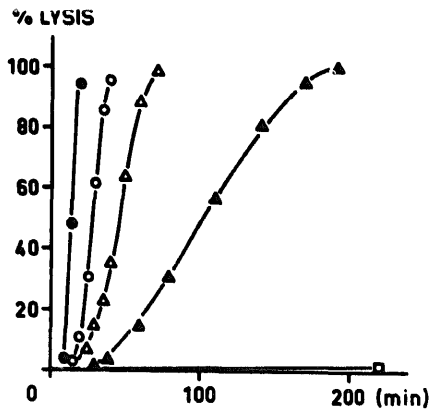

Fig. 8. Changes in the extent of lytic efficiency of tellurite/GSH mixtures in dependence of the time passed after mixing the two components prior to addition of CDNB-treated cells. (A) Cells added at 0 min (●), 10 min (○), 20 min (△) or 40 min (▲) after mixing GSH (2 mM) with tellurite (0.25 mM) in phosphate-buffered saline. □, Controls, not exposed.

Interruption and reversibility of the effect of tellurite. The process of leak formation by tellurite, as quantified by an increasing rate of lysis or of leak fluxes, cannot be interrupted by transferring the cells into a new, tellurite-free medium, even after only a short (5 min) exposure. This is true for native cells exposed to tellurite (Table I), as well as for CDNB-treated cells exposed to exogenous, impermeable GSH plus tellurite (data not shown). In native cells the progress of leak formation can be interrupted by cooling the cells to 0–4°C (cf., Table I). Rewarming, however, leads to a renewed progress of leak formation (data not shown).

In view of the crucial role of soluble thiols for the membrane damage by tellurite the blockage of such groups might be expected not only to interrupt, but even to terminate the chain of events. Indeed, *N*-ethylmaleimide (5 mM) added to suspensions of native cells 10 min after tellurite (0.25 mM) increases the half-time of colloid-osmotic lysis 10-fold from 40 to 410 min. This suppressive effect becomes less pronounced when the latency time between addition of tellurite and of *N*-ethyl maleimide is increased, although the agent was always added before the cells began to lyse. This has to be expected, since the leaks already formed when leak formation is interrupted will still account for a progress of colloid-osmotic lysis. Iodoacetamide is much less effective than *N*-ethylmaleimide. Addition of CDNB also retards lysis, providing evidence, in view of its specific, enzyme-catalysed reaction with GSH [15], that at least part of the protection stems from blockage of GSH.

Termination of leak formation by addition of *N*-ethylmaleimide can be demonstrated more directly by measuring leak permeabilities. When native cells are loaded with labelled erythritol and treated with tellurite (0.6 mM) for 10 min at 37°C immediately prior to measuring erythritol efflux at 0°C, a very substantial increase of permeability becomes evident (Δk 0.0493 min^{-1}). When NEM is added to the tellurite-containing suspension for 5 min at 37°C prior to the flux measurement at 0°C, the measured increase of permeability is much lower ($\Delta k = 0.0106$ min^{-1}). This effect must stem from a partial reversal of the tellurite-induced leakiness upon addition of NEM. This became evident in experiments in which NEM was added to tellurite-treated cells at the start and during the course of erythritol efflux. As evident from Fig. 9, the slope of the efflux kinetics decreases immediately after addition of NEM. The possible mechanisms of such a reversal is presently not clear.

NEM also interrupted leak formation induced in CDNB-pretreated cells by exogenous GSH plus tellurite and even stopped the spontaneous progress, at 37°C, of this leakiness after removal of the extracellular agents by washing at 0°C. Since no intracellular GSH is available under these conditions, some NEM-reactive, probably non-thiol reaction product of extracellular GSH and tellurite must be claimed to cause leak formation under these conditions.

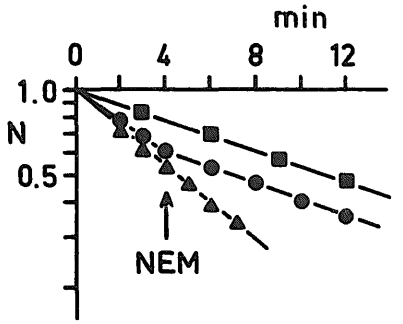

Fig. 9. Time course of [^{14}C]erythritol efflux from tellurite-treated cells under various conditions. Cells were exposed to tellurite (0.3 mM, 10 min) while being loaded with labelled erythritol, separated from the loading medium at 0°C and immediately resuspended for efflux measurement in tellurite-free medium B at 35°C. ■, NEM present from beginning ($k = 0.0648$ min^{-1}), ▲, no NEM ($k = 0.1378$ min^{-1}); ●, NEM (8 mM) after 4 min ($k_{0-4\ min} = 0.1174$ min^{-1}, $k_{4-12\ min} = 0.0630$ min^{-1}). Addition of NEM after 4 min lowers the flux rate to that of cells in the presence of NEM from the beginning. $N = 1 - (cpm_t / cpm_\infty)$.

Progress of damage by tellurite in native cells was not interrupted by even extensive washing of the cells (Table I). This may at least partly be due to the fact, that tellurite can not quantitatively be removed from the cells even under the conditions of thorough washing. CDNB-treated cells exposed to tellurite (1.2 mM) for 10 min and than washed three times at 37°C in an excess of tellurite-free medium become leaky upon subsequent addition of permeant but not impermeant thiols. This indicates that tellurite could not be removed by the washing procedure and thus reacted with the thiols. The reasons for this strong 'binding' of tellurite remain to be clarified.

Quenching of tellurite-induced leak formation by NEM provides a means to establish characteristics of the defect not accessible to determination otherwise. Thus, we could establish the apparent activation energy (E_a) of the diffusion of erythritol through the tellurite-induced leak to be about 20 kJ/mol. This corresponds to E_a for diffusion in bulk water and clearly points to the involvement of an aqueous 'hole'. In line with this concept, phloretin, which has recently been shown [25] to be an inhibitor of aqueous leaks induced in the erythrocyte membrane by various types of modification, inhibited the leak fluxes of erythritol up to about 50–60% (data not shown). The sizes of these putative holes could be determined as described in the following section.

Size of the tellurite induced leak

The best available method to assign apparent radii to 'holes' induced by membrane damage on the basis

of exclusion limits requires a 24–28-h incubation of the cells in the leaky state at 0°C [14,17]. This 'equilibrium method' could be used in tellurite-modified cells after quenching the reaction by NEM. Since colloid-osmotic lysis is an all-or-none phenomenon with respect to the single cell, the curves shown in Fig. 10 demonstrate a considerable heterogeneity in the distribution of the pore sizes over the cell population. From the interpolated molecular weights of – virtual – solutes required for protection of 50% of the cells against lysis, apparent mean radii can be obtained for holes induced by tellurite. At 0.5 mM tellurite mean values for these radii amount to 0.61 ± 0.15 nm ($n = 5$) after 10 min exposure and increase to 0.98 ± 0.3 nm ($n = 6$) after 16 min.

The increases in erythritol permeability resulting from the formation of holes with these apparent radii (r_{50}) roughly correspond (Fig. 11) to the increase in pore area (πr_{50}^2), predicted from the Renkin-Pappenheimer model of restricted diffusion [26]. It would, thus, appear that the apparent number of holes per cell does not change very much with increasing leak permeability, at least in the range of low leak permeabilities. Calculated numbers of pores per cell are very small (≤ 1 per cell) as in other cases of induced leakiness [17], indicating a dynamic nature of the structural defects forming the 'holes'.

Discussion

This paper reports on the long established but causally not yet well defined effect of tellurite on biological membranes. The following major information could be obtained:

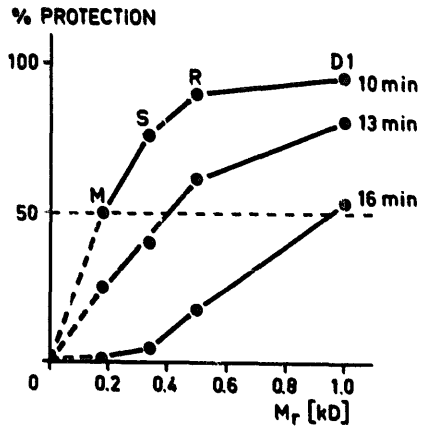

Fig. 10. Protective effect of polar nonelectrolytes varying in size, against the colloid-osmotic lysis induced in native cells by pretreatment with 0.6 mM tellurite for the time periods indicated. Damage by tellurite was stopped by addition of NEM (4 mM) and the extent of colloid-osmotic lysis determined as described in Materials and Methods. 'Protection' defined as percentage of non-lysed cells after 26 h incubation in the respective media. Data from a characteristic experiment out of a series of six with comparable results. M = mannitol, S = sucrose, R = raffinose, D1 = dextran 1.

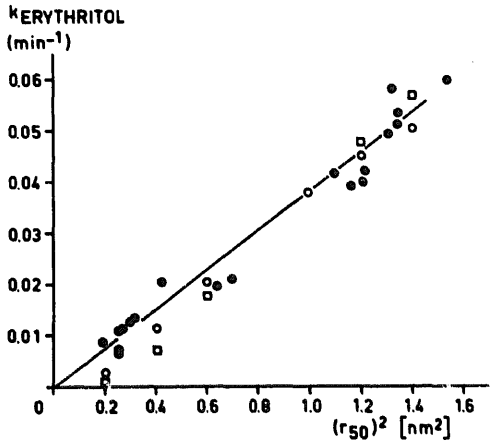

Fig. 11. Linear relationship between leak permeabilities k to erythritol induced by treatment of native cells with tellurite (0.6 mM) for various time periods, and the square of the apparent radii of the leaks determined for the same cells according to the procedure shown in Fig. 10. Closed symbols: Experimental values. Open symbols: Permeability changes predicted from the model of restricted diffusion [26], using two different numbers for the radius of erythritol (□, 0.28 nm; ○, 0.35 nm). Predicted permeabilities normalized to the measured permeability value for $r_{50} = 1$ nm. Note the fairly close correspondence between measured and predicted permeability changes.

(1) Membrane damage by tellurite has at least two components. A minor direct and reversible effect probably arises from the modification of membrane SH-groups and is comparable to the action of diamide or periodate. The major, partly irreversible effect requires the reaction of tellurite with soluble thiols in either the intra- or the extracellular space. While cellular GSH is the natural reactant for tellurite, the tellurite-induced lowering of its concentration is not the crucial event responsible for membrane damage. The progress of irreversible membrane damage by tellurite can not be terminated by washing the cells. It can be stopped, and even be partly reverted, however, by an alkylating agent like *N*-ethylmaleimide. This is mainly, but probably not exclusively, due to blockage of thiols reacting with tellurite. The time-course of the development of an aggressive species from tellurite and thiols seems to depend on chemical properties of the thiol not related to their permeability.

(2) Membrane damage includes increasing leakiness to ions and non-electrolytes as well as membrane rigidification.

(3) The leaks can be described in terms of aqueous 'holes' to which mean radii can be assigned by means of the colloid-osmotic nature of the lysis. The increase of leakiness with increasing extent of treatment with tellurite can be accounted for by an increase of the apparent radius of the defects which are present at a very low number per cell (< 1), indicating a fluctuating nature.

The weak 'direct' action of tellurite most likely derives from the primary formation of tellurotrisulfides subsequently converted to stable disulfides. The formation of corresponding disulfide bonds and protein cross-linking have been reported for selenite [22,24,27,28]. The strong similarity between SeO_3^{2-} and TeO_3^{2-} in their chemical reactivity certainly permits this argument by analogy. The cross-linking effect of tellurite even seems to be more pronounced than that of selenite in the erythrocyte membrane as can be concluded from the much lower rigidifying effect of selenite in cells free of soluble thiols (Fig. 4). Since tellurite is probably taken up less readily by cells [3] than the highly permeable selenite [29], the difference in reactivity is even more pronounced than indicated by the differences in rigidification.

Inter- and intra-monomeric cross-linking of membrane proteins, particularly of spectrin, by tellurite also provides a further piece of evidence for our claim [30] that a considerable fraction of SH-groups in the erythrocyte membrane proteins must be located pairwise in close (< 0.4 nm) vicinity to each other.

The indirect effect of tellurite, which requires soluble thiols in the intra- or the extracellular compartment, is much stronger than the direct one. It is at least partly irreversible and can be terminated, once initated, only by a thiol-blocking agent. A reaction mechanism which may in part explain the events can be derived from data for selenium chemistry [22,24,27,28]. These events, thought to occur in native cells exposed to tellurite, are shown in Fig. 12. Reaction between GSH and tellurite leads to the formation, via a tellurotrisulfide (GSTeSG), of the telluropersulfide of GSH ($GSTe^-$), which probably rapidly decomposes to form the highly reactive hydrogen telluride anion (HTe^-, pK' approx. 2.6) [28]. Besides its possible but yet undefined reaction with membrane constituents, HTe^- is oxidized by free oxygen to elemental tellurium which is also formed by decomposition of the (mixed) tellurotrisulfides of soluble thiols and proteins. Since elemental Te may again react with GSH to form the

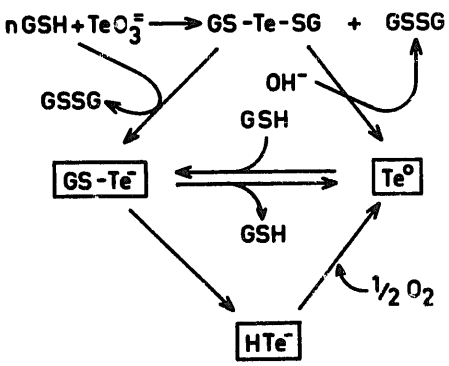

Fig. 12. Provisional model of conversions of tellurite occurring in cell suspensions exposed to tellurite in the presence of GSH.

persulfide, the recycling of tellurium could sustain formation of HTe^- at the expense of available thiols.

A further feature of tellurite-promoted membrane damage also points to a role of HTe^-. As shown above, the combination of extracellular GSH and tellurite produces leakiness, although GSH is impermeant. Since neither of the above mentioned GSH-derived intermediates is likely to permeate the erythrocyte membrane, hydrogen telluride may be the most likely candidate for importing the damage into the cell interior and promoting leak formation. The incomplete inhibition of this effect by DIDS (see above) is in line with this interpretation, since HTe^- probably penetrates the erythrocyte membrane to some extent by non-ionic diffusion or as a lipophilic anion. HTe^- may be assumed to be a labile intermediate of the reaction between TeO_3^{2-} and GSH, which leads to the final formation of elemental Te^0 indicated by the slow formation of a brownish precipitate in mixtures of the two. This conversion also accounts for the time-dependent decrease of the toxic effect after premixing of the components (Fig. 8).

Under physiological conditions GSH and cysteine are likely to be the 'activators' of tellurite. GSH will probably be more important than cysteine in view of its higher concentration in cells which more than compensates for its lower reactivity compared to cysteine (Fig. 7).

Selenide formation from selenite in presence of GSH has been shown to be stimulated (catalyzed) by glutathione reductase [28]. An analogous type of enzymatic stimulation may also contribute to the membrane damage caused by tellurite in native cells. Pretreatment of erythrocytes with the inhibitor of GSH reductase, 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) inhibits colloid-osmotic lysis in presence of 0.5 mM tellurite by about 30% (data not shown).

In terms of apparent size, the leaks produced by tellurite/thiol resemble the numerous types of membrane leaks which we have studied in recent years [11–17,19,21]. There is obviously a considerable heterogeneity in the cell population with respect to leak sizes. Nevertheless, it is probably permitted to speculate that in their structural basis the leaks produced by tellurite-derived reactive species are not intrinsically different from leaks produced by oxygen-derived reactive species (oxy-radicals). This view is also supported by the partial inhibition of the tellurite-induced leak by phloretin, which has recently been shown [25] to inhibit many types of leak fluxes following covalent membrane modification.

The primary sites of membrane attack by the tellurite-derived reactive species are proteins. The evidence is as follows:

(1) The pattern of membrane lipid fatty acids, in particular the fractional content of unsaturated fatty

acids, is not changed after the treatment (Heller K. and Deuticke B., unpublished observations), excluding a role for lipid peroxidation.

(2) The extent of decrease of membrane protein SH-groups is probably much higher in the presence of tellurite plus GSH than after tellurite alone *. Membrane protein SH-modification, in particular cross-linking and oxidation are known from other studies [11–14] to be a major basis for the formation of membrane leaks.

(3) Membrane rigidification, known to be the consequence of covalent modification or even intermolecular cross-linking of membrane proteins [31] accompanies leak formation.

The type of the protein modification involved remains to be defined. Binding of tellurium to the membrane in the course of the reaction of telluride with functional groups of the proteins may induce structural alterations leading to leakiness of the lipid/protein mosaic forming the natural membrane barrier. Membrane modification by tellurite goes along with irremovable binding, to the membrane, of elemental tellurium, as indicated by the finding of a black material adsorbed to ghosts prepared from erythrocytes exposed to tellurite in the presence of thiols (see also Ref. 3). This is, however, not merely a 'passive' deposit, since incubation of open ghost membranes in solutions containing Te^0, formed by reaction of tellurite with GSH in cell-free solution, does not lead to substantial binding of such black material on the membrane (Deuticke, B. unpublished observations, see also Ref. 22). Deposition of membrane-associated Te^0 has also been observed in microorganisms [10].

Which proteins have to be modified to produce leakiness of the erythrocyte membrane is not yet finally clear. Recent studies [36–38] have provided further evidence for the claim [11,12] of a crucial involvement of membrane skeletal proteins in leak formation after chemical or enzymatic membrane modification. Similar leak formation was not observed in membranes devoid of the major skeletal proteins.

Acknowledgements

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* Precise determinations of SH contents in membranes from cells damaged by tellurite plus thiol are impeded by the irreversible deposition of brownish-black material, probably elemental Te, on the membrane.

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