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PENETRATION OF 2,4,6-TRINITROBENZENESULFONATE INTO HUMAN ERYTHROCYTES

CONSEQUENCES FOR STUDIES ON PHOSPHOLIPID ASYMMETRY

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Summary

The glutathione content of human erythrocytes rapidly diminishes when cells are exposed to 2,4,6-trinitrobenzenesulfonate (20 μmol/l cells) at 37°C. Even at 0°C a slow decrease in glutathione content is observed. The uptake of trinitrobenzenesulfonate by the cells is retarded by inhibitors of the inorganic anion exchange system, indicating that trinitrobenzenesulfonate enters the cells by this pathway.

The disappearance of glutathione most probably results from the reaction:

2 GSH + trinitrobenzenesulfonate → GSSG + aminodinitrobenzenesulfonate

The reaction of trinitrobenzenesulfonate with glutathione occurs prior to its covalent binding to amino groups of hemoglobin which makes this reaction a more sensitive method of detection of penetration of trinitrobenzenesulfonate into erythrocytes. Results of studies on the asymmetric distribution of phospholipids using trinitrobenzenesulfonate as the only probe should be reconsidered in the light of these new data.

Introduction

The distribution of phospholipids between the two lipid layers of biological membranes has been studied using amino reagents, phospholipases and phos-

Abbreviations: DNDS, 4,4'-dinitro-2,2'-stilbenesulfonate; DIDS, 4,4'-diisothiocyano-2,2'-stilbenedisulfonate.

pholipid exchange proteins [1]. These probes only react with phospholipids of the outer layer as they do not penetrate into the cell. Penetration enables the reagent to attack the cytoplasmic surface of the cell membrane [2]. In almost all cells macromolecular probes such as enzymes and exchange proteins can be considered impermeable with high certainty. In the case of low molecular weight probes, however, the possibility of their uptake into a cell must be checked under the conditions of the particular experiment. The amino reagent, 2,4,6-trinitrobenzenesulfonate, has been used in a number of investigations to demonstrate an asymmetric arrangement of phosphatidylethanolamine and phosphatidylserine in erythrocytes [2]. This evidence can be confirmed by studies with phospholipases [3]. For other membranes, an asymmetric distribution of amino phospholipids has been claimed on the basis of studies using trinitrobenzenesulfonate [4—7].

In the case of erythrocytes, trinitrobenzenesulfonate has been regarded as highly impermeable whenever no reaction of the reagent with amino groups of hemoglobin could be detected [8—12]. It is known, however, that trinitrobenzenesulfonate can also react with SH groups [13—15]. The reaction with SH groups can probably occur even faster than that with amino groups [13—16]. Since erythrocytes contain a large reservoir of SH groups in their intracellular and membrane proteins and in the tripeptide GSH, the absence of binding of trinitrobenzenesulfonate to amino groups of hemoglobin might be due not so much to its low permeability, but to its preferential binding to SH groups, which would thus act as a sink for the probe.

In the present study we present evidence that this is in fact the case and that penetration of trinitrobenzenesulfonate into erythrocytes can easily be detected by the disappearance of SH groups of GSH.

Methods

Determination of the disappearance of GSH upon treatment of erythrocytes with trinitrobenzenesulfonate

Erythrocytes from freshly collected, heparinized human blood were washed three times with isotonic NaCl. 1 vol. of the cells was then suspended in 10 vol. of a medium containing (concentrations in mmol/l): KCl (90), NaCl (45), Na_2HPO_4/NaH_2PO_4 (10) and sucrose (44) (medium A). Subsequently, 2,4,6-trinitrobenzenesulfonate (Sigma; 2 mmol/l) was added. The cells were then incubated at different pH values and temperatures for various time periods, as indicated in Results and Discussion. After the incubation, the cells were washed three times with medium A at 0° C and GSH content determined [17].

Determination of the uptake of trinitrobenzenesulfonate by erythrocytes

4 g of packed erythrocytes were suspended in 6 g of medium A, containing 0.2 mmol/l trinitrobenzenesulfonate (pH 8.0, 20°C). After various time intervals, 1.5 ml samples were centrifuged and 0.8 ml of the supernatant mixed with 0.02 ml of $HClO_4$ (60%, w/w). After a 15 min incubation the sample was centrifuged. 0.7 ml of the supernatant was mixed with 0.3 ml medium A and 0.08 ml 10 N NaOH, and the absorbance read at 412 nm as a measure of the concentration of trinitrobenzenesulfonate.

Results and Discussion

Disappearance of GSH in erythrocytes treated with trinitrobenzenesulfonate

Incubation of erythrocytes in 10 vol. of medium A containing 2 mmol/l trinitrobenzenesulfonate at pH 8.0 and 37°C leads to a decrease of GSH to 40% of its original concentration within 15 min (Fig. 1). The rate of decrease of GSH diminishes with decreasing temperature. Even at 0°C, however, a slow disappearance of GSH is still observed. Spontaneous oxidation of GSH can be excluded under our experimental conditions, since in the absence of trinitrobenzenesulfonate GSH does not decrease. Thus, it seems most likely that the observed disappearance is linked to a penetration of trinitrobenzenesulfonate into the cells.

Further experiments were performed to substantiate this concept. Trinitrobenzenesulfonate has been supposed to penetrate the erythrocyte membrane via the inorganic anion-exchange system and via the lipid phase [18]. We therefore studied the effect of inhibitors of the inorganic anion exchange system on the disappearance of GSH in erythrocytes incubated with trinitrobenzenesulfonate. As could be shown, DNDS, a reversible inhibitor [19], and DIDS, an irreversible covalent inhibitor [19], at concentrations known to completely block inorganic anion exchange [19] markedly retarded the effect of trinitrobenzenesulfonate (Fig. 2). The effect of DIDS was more pronounced than that of DNDS. An increase in the concentration of DIDS did not enhance the inhibition further. The residual uptake of trinitrobenzenesulfonate after blockage of the inorganic anion pathway is due to diffusion via the lipid phase (see below).

Since the rate of reaction of trinitrobenzenesulfonate with amino groups is known to increase with pH, studies on the arrangement of proteins and phospholipids in the membrane using trinitrobenzenesulfonate have usually been

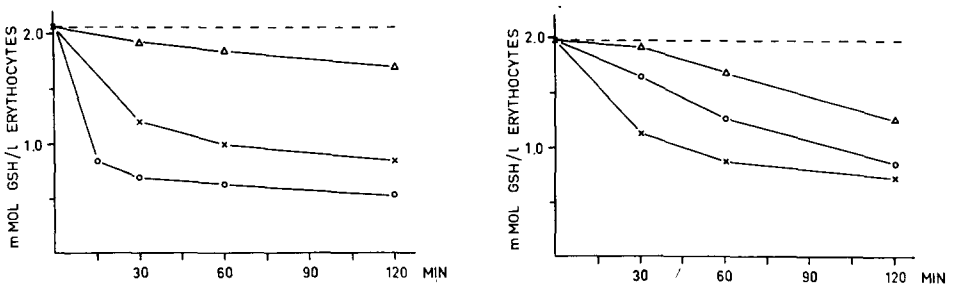

Fig. 1. Temperature dependency of the disappearance of GSH during the incubation with 2,4,6-trinitrobenzenesulfonate. 1 vol. of erythrocytes were incubated at 0°C (△), 20°C (X) and 37°C (○) with 10 vol. medium A containing 2 mmol/l trinitrobenzenesulfonate (pH 8.0). After varying times of incubation, erythrocytes were isolated by centrifugation, washed three times with medium A (0°C) and free GSH quantified.

Fig. 2. Effect of inhibitors of the inorganic anion-exchange system on the disappearance of GSH of erythrocytes during an incubation with 2,4,6-trinitrobenzenesulfonate. Erythrocytes were exposed with trinitrobenzenesulfonate (pH 8.0, 20°C) in the absence (X) or the presence (○) of DNDS (1 mmol/l). Alternatively, to study the effect of the irreversible inhibitor, DIDS, cells were pretreated with 20 μmol/l DIDS for 30 min (37°C, pH 8.0), centrifuged and exposed to trinitrobenzenesulfonate (△). After different time periods of incubation at 20°C, intracellular free GSH was quantified.

performed at alkaline pH values [2,4—12]. It seemed of interest therefore to check the pH dependency of the disappearance of GSH in erythrocytes incubated with trinitrobenzenesulfonate. As shown in Fig. 3, the rate of disappearance of free GSH is accelerated at increasing pH. This pH dependency parallels the pH dependency of the reaction of trinitrobenzenesulfonate with SH groups [13].

Direct measurements of the uptake of trinitrobenzenesulfonate

Further studies served to obtain direct data on the rate of uptake of trinitrobenzenesulfonate by erythrocytes. To this end we measured the disappearance of trinitrobenzenesulfonate from the external medium. As shown in Fig. 4A, penetration of trinitrobenzenesulfonate into erythrocytes decreases with increasing pH. This pH-dependency parallels the pH-dependency — in intact erythrocytes — of the exchange of monovalent inorganic anions [20]. Since the rate of disappearance of GSH of erythrocytes incubated with trinitrobenzenesulfonate increases under these conditions (Fig. 3), we conclude that the rate of reaction of trinitrobenzenesulfonate with GSH and not its penetration into the cells is the rate-limiting step.

In analogy to the transfer of other anions passing the membrane via the inorganic anion-exchange system [21], the uptake of trinitrobenzenesulfonate is inhibited by a pretreatment of the cells with this amino reagent (Fig. 4B). Trinitrobenzenesulfonate thus inhibits its own uptake. Further evidence that trinitrobenzenesulfonate passes the membrane via the inorganic anion pathway comes from the strong inhibition of its uptake by the specific inhibitor of this pathway, DIDS (Fig. 4B). This observation excludes the possibility that mere binding of trinitrobenzenesulfonate to the cell surface is reponsible for the disappearance of the reagent from the medium. Such a possibility becomes even more unlikely in view of our finding that the fractional uptake of the reagent into the cells is not greatly affected by a 10-fold increase of its concentration (data not shown) and in view of results [22] showing that the number of superficial groups likely to react with trinitrobenzenesulfonate

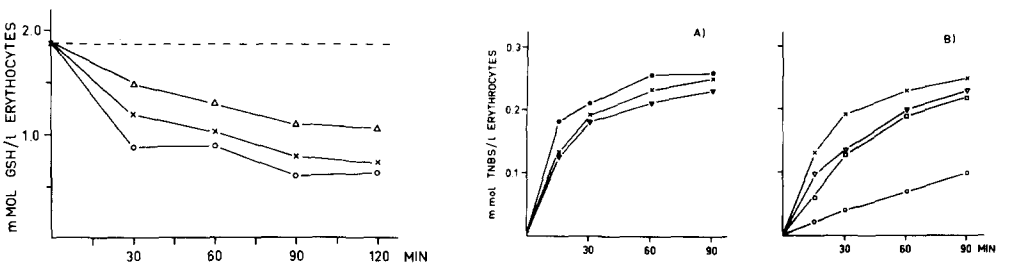

Fig. 3. pH dependency of the disappearance of GSH of erythrocytes upon incubation with 2,4,6-trinitrobenzenesulfonate. Erythrocytes were treated (20°C) with trinitrobenzenesulfonate in medium A of different pH values. After various times intracellular GSH was quantified. △, pH 7.4; X, pH 8.0; ○, pH 8.5.

Fig. 4. Uptake of 2,4,6-trinitrobenzenesulfonate (TNBS) by erythrocytes. A, Uptake of trinitrobenzenesulfonate by erythrocytes as measured at pH 7.4 (●), 8.0 (X) and 8.5 (▲). B, Fresh erythrocytes (X) and cells pretreated with 0.2 mmol/l (□) or 2 mmol/l (△) trinitrobenzenesulfonate (15 min, pH 8.0, 20°C) and cells pretreated with DIDS (○) (0.02 mmol/l, 30 min, pH 8.0, 37°C) were washed three times and packed by centrifugation. Subsequently, uptake of trinitrobenzenesulfonate by the cells was measured.

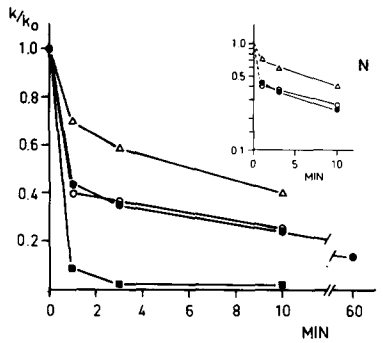

Fig. 5. Time course of the inhibition of the anion-exchange system in human erythrocytes by trinitrobenzenesulfonate. Cells were loaded for 45 min with oxalate at pH 8.0, 37°C in a medium containing (concentrations in mmol/l) KCl (84), NaCl (42), Na_2HPO_4/NaH_2PO_4 (9), sucrose (41), disodium oxalate (7.5); hematocrit 3%. Subsequently, the hematocrit was raised to 10% by removing some of the medium and the cells loaded with [^{14}C]oxalate. After 45 min incubation the temperature of the suspension was lowered to 20°C and aliquots of a 0.11 M stock solution of 2,4,6-trinitrobenzenesulfonate or 1-fluoro-2,4-dinitrobenzene were added to appropriate aliquots of the suspension. The reaction was allowed to proceed for 1,3 or 10 min, after which it was stopped by addition of 0.5 ml of a 90 mmol/l solution of glycylglycine, pH 8.0. The suspensions were then centrifuged, and the cells washed once in medium A at pH 8.0, 0°C. Back-exchange of [^{14}C]oxalate into tracer-free incubation media was measured at 10°C and evaluated as described elsewhere [27]. k/k_0, ratio of rate coefficient of tracer efflux for test versus control cells. Trinitrobenzenesulfonate: ●, 2 mmol/l; ○, 0.6 mmol/l; △, 0.2 mmol/l. Fluorodinitrobenzene: ■, 2 mmol/l. Insert, semi-logarithmic plot of the data.

is much lower than the amount of reagent taken up by the cells in our experiments.

In order to obtain more precise information on the extent to which trinitrobenzenesulfonate inhibits its own uptake, we studied the time course of the inactivation of the self-exchange of oxalate, a well-established substrate of the anion-exchange system, at different concentrations of trinitrobenzenesulfonate. As becomes evident from Fig. 5, the reagent inhibits anion exchange with a peculiar biphasic time-dependence not observed for fluorodinitrobenzene. A fraction of the transport is inhibited almost instantaneously. The size of this fraction saturates with increasing concentration of trinitrobenzenesulfonate and reaches its maximum at about 0.6 mmol/l (hematocrit 10%). This maximum depends on the temperature at which the cells are exposed to the inhibitor (data not shown).

The further inhibition of oxalate self-exchange develops much more slowly, with an apparent first-order rate coefficient of about 0.07 min^{-1} ($t_{1/2}$ = 10 min). Provided that these data are applicable to the inhibition of the penetration of trinitrobenzenesulfonate, one may conclude that the uptake of the reagent, as measured at 0.2 mmol/l for about 10 min is only 60—70% of the true, uninhibited flux. For defining the true 'permeability' of trinitrobenzenesulfonate, fluxes have to be corrected for this inhibition. An evaluation of the data in Fig. 4 under this aspect is given in Table I. As is evident, trinitrobenzenesulfonate penetrates the erythrocyte membrane via the inorganic anion-exchange system at a rate about 10—15 times higher than that of the movements of inorganic phosphate [23] or *N*-(4-azido-2-nitrophenyl)-2-aminoethylsulfonate [24]. After blockage of the anion transport system by DIDS,

TABLE I

TRANSMEMBRANE FLUX AND PERMEABILITY OF 2,4,6-TRINITROBENZENESULFONATE IN HUMAN ERYTHROCYTES, AS COMPARED TO OTHER ANIONS PENETRATING VIA THE ANION-EXCHANGE SYSTEM

	Flux (μmol/ml per h)	Apparent [e] permeability (cm/s)
Trinitrobenzenesulfonate (total) [a]	0.87	$8.7 \cdot 10^{-8}$
Trinitrobenzenesulfonate (DIDS-insensitive) [b]	0.07	$\leq 0.7 \cdot 10^{-8}$
N-(4-azido-2-nitrophenyl)-2-aminoethylsulfonate [c]	0.04	—
Orthophosphate [d]	0.024	—

[a] This study, pH 8, 20°C, C_{ex} = 0.2 mmol/l, probably hetero-exchange against chloride.
[b] This study, pH 8, 20°C, C_{ex} = 0.2 mmol/l, cells pretreated with DIDS (20 μmol/l, pH 8, 30 min, 37°C, hematocrit 10%).
[c] Calculated from the J_{max} values for hetero-exchange efflux given by Knauf et al. [24] for C = 0.2 mmol/l, using a K_m value of 2 mmol/l and assuming a Q_{10} value of 5.
[d] From Deuticke [23]. Data for equilibrium exchange, pH 8, 20°C, C_{ex} = 1 mmol/l.
[e] Calculated from the flux and the initial transmembrane gradient of trinitrobenzenesulfonate.

a residual permeability of trinitrobenzenesulfonate, probably that of the lipid bilayer, can be demonstrated, about one order of magnitude smaller.

Mechanism of reaction of trinitrobenzenesulfonate with GSH

Trinitrobenzenesulfonate can react with SH-groups of GSH by two mechanisms [14]: The first involves an addition mechanism leading to the formation of *S*-trinitrophenyl groups. In the second one, which occurs in the presence of an excess of SH groups, SH groups are oxidized by trinitrobenzenesulfonate. In the course of this process trinitrobenzenesulfonate is reduced to 2- or 5-aminodinitrophenylsulfonate. The first mechanism seems unlikely to be responsible for the disappearance of GSH under our conditions, since *S*-trinitrophenyl groups are known to hydrolyse spontaneously at alkaline pH under formation of trinitrophenol and restoration of SH groups after removal of trinitrobenzenesulfonate [13—15]. Removal of trinitrobenzenesulfonate is part of our experimental procedure in order to be able to quantify GSH (see Methods). Moreover, the persistence of *S*-trinitrophenyl groups would have been observed in the cell extracts due to their yellow color [13—16].

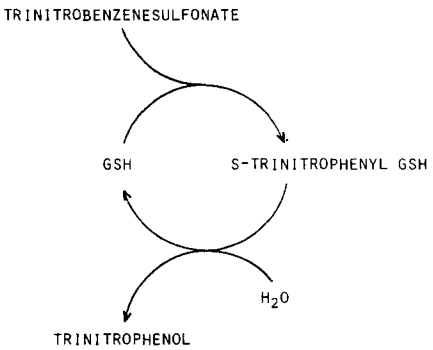

Fig. 6. Reversible binding of trinitrobenzenesulfonate by GSH.

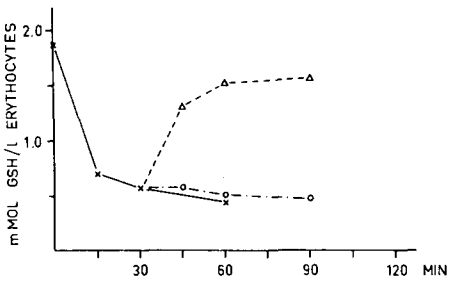

Fig. 7. Restoration of GSH by energy-dependent processes after a pretreatment of erythrocytes with 2,4,6-trinitrobenzenesulfonate. Erythrocytes were treated (37°C, pH = 8.0) with trinitrobenzenesulfonate (X). After 30 min, glycylglycine (5 mmol/l), which blocks trinitrobenzenesulfonate was added to one aliquot (○), and to another were added (△) glycylglycine and adenosine (5 mmol/l). After various times of incubation (37°C, pH 8.0) free GSH was quantified.

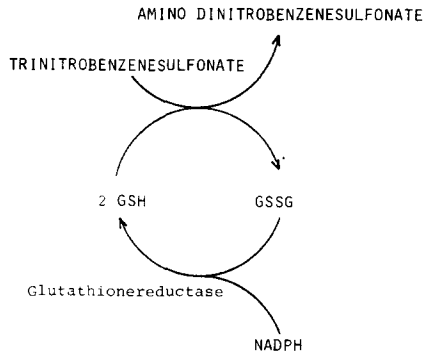

Fig. 8. Oxidation of GSH by trinitrobenzenesulfonate followed by its reduction by metabolic processes.

Nevertheless, trinitrophenol is formed in erythrocytes during exposure to trinitrobenzenesulfonate. Evidence for this process comes from our observation that a yellow compound having the spectral properties of trinitrophenol is released from the cells into the medium under these conditions. From the amount of trinitrophenol released during the first 30 min of incubation, it can be calculated that almost 50% of the trinitrobenzenesulfonate taken up by the cells is transformed into trinitrophenol. In this calculation it has been taken into account that trinitrophenol is distributed between cells and medium at a ratio of about 5 : 1 (data not shown), probably due to binding to hemoglobin. As an explanation for the formation of trinitrophenol we propose a sequence of reactions involving a recycling of GSH between a trinitrophenylated and a 'free' state (Fig. 6).

Calculations from data in Figs. 1 and 4 show that the 50% of the trinitrobenzenesulfonate which is taken up by the cells, but not consumed in this reaction, is just sufficient to oxidize GSH to GSSG. Evidence for such an oxidation comes from the observation that GSH that has reacted with trinitrobenzenesulfonate can be reduced by energy-dependent processes. In the experiment shown in Fig. 7, a treatment of erythrocytes with trinitrobenzenesulfonate decreased GSH to 30% of its original value. Subsequent blockage of the residual trinitrobenzenesulfonate by glycylglycine did not reverse this effect. Addition of adenosine after glycylglycine, however, caused a rapid increase of GSH. An interpretation of these findings is given in Fig. 8. Stimulation of the hexose monophosphate pathway by adenosine improves regeneration of NADPH consumed in reductive processes and thus enhances reduction, by glutathione reductase, of GSSG to GSH. In other experiments, addition of adenosine during trinitrobenzenesulfonate treatment retarded the decrease of GSH, probably as a consequence of a continuous energy-dependent reduction of GSH oxidized by trinitrobenzenesulfonate (data not shown). These results make it rather unlikely that a trinitrobenzenesulfonate-induced increase of membrane permeability to glutathione and a subsequent leakage of GSH

(and GSSG) from the cells is responsible for the disappearance of GSH in the presence of the reagent.

From all these results it can be concluded that trinitrobenzenesulfonate penetrates into the cells, and that part of it is consumed to oxidize GSH to GSSG, while the rest is converted into trinitrophenol.

Oxidation of membrane SH groups by trinitrobenzenesulfonate

A number of agents have previously been shown to oxidize SH groups of GSH as well as of membrane proteins [25,26]. In view of its now established ability to oxidize GSH, trinitrobenzenesulfonate may be expected to oxidize SH groups of membrane proteins, too. As shown in Table II, membrane SH groups of erythrocytes, in fact, decrease in cells incubated with trinitrobenzenesulfonate, most probably due to oxidation. However, as compared to other oxidizing agents, e.g. diamide, trinitrobenzenesulfonate is much less potent [25,26]. The finding of a reaction of trinitrobenzenesulfonate with protein SH groups demonstrates that this reagent cannot be used as a specific reagent to modify membrane amino groups. In studies showing inhibition of enzyme and transport proteins by this reagent, cross-linking of SH groups should be considered as the underlying mechanism apart from the involvement of amino groups.

General considerations

Penetration of trinitrobenzenesulfonate into human erythrocytes has been checked previously [8—12] by its reaction with amino groups of hemoglobin. We were not able to obtain evidence for penetration at 20°C and pH 8.0 during the first hour of incubation, even after blockage of GSH by SH reagents [10]. This result was in agreement with the lack of reaction of trinitrobenzenesulfonate found by others under more extreme conditions such as pH 8.5, 23°C, and higher amounts of trinitrobenzenesulfonate per cell [11,12]. The present study, however, clearly demonstrates penetration of trinitrobenzenesulfonate, as detected by the oxidation of GSH, into the cell even under rather mild conditions (20°C, pH 7.4). Nevertheless, a reaction of trinitrobenzenesulfonate with cytoplasmic amino groups in fresh erythrocytes does not take

TABLE II

DECREASE OF MEMBRANE SH GROUPS AFTER AN INCUBATION OF ERYTHROCYTES WITH 2,4,6-TRINITROBENZENESULFONATE

Erythrocytes were incubated in medium A containing differing concentrations of trinitrobenzenesulfonate (pH 8.0, 37°C, 1 h). After three washings (0°C), ghosts were prepared from the cells and membrane SH groups quantified [26].

Concentration of trinitrobenzenesulfonate (mmol/l)	SH groups remaining (nmol/mg protein (%))
0	90 (100)
2	73 (81)
5	67 (74)
10	65 (72)

place as long as GSH is available [8—12]. This is particularly true for metabolizing cells.

According to our results two processes consuming trinitrobenzenesulfonate keep the concentration of the agent at very low levels as long as free GSH is present (Figs. 6 and 8). A significant reaction of trinitrobenzenesulfonate with amino groups of the inner lipid layer of the membrane is thus prevented. The general agreement between studies with trinitrobenzenesulfonate and phospholipases in human erythrocytes [2,3] is thus more or less fortuitous, due to the high GSH content of the cells.

In conclusion, the claim that trinitrobenzenesulfonate does not penetrate into a certain type of cell cannot be based on the argument that it does not react with cytoplasmic amino groups of hemoglobin of human erythrocytes. The present study offers a simple sensitive method to detect penetration of trinitrobenzenesulfonate into cells by its reaction with intracellular non-protein SH groups.

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