TRANSPORT OF GLUTATHIONE *S*-CONJUGATE FROM HUMAN ERYTHROCYTES

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1. Introduction

Reduced glutathione (GSH) is a major product of erythrocyte metabolism and is vital for protection of red cells from oxidative damage. GSH is synthesized in erythrocytes from its constituent amino acids and has a turnover half-life of ~4–5 days [1]. The mechanism of GSH degradation in erythrocytes has been the subject of some dispute. Some reports [2–4] have suggested that GSH is broken down by γ-glutamyl transpeptidase. However, while this may be the case in other tissues, more recent investigations have clearly demonstrated that erythrocytes are devoid of γ-glutamyl transpeptidase [5,6]. Recently, evidence has been presented suggesting that, although GSH is not transported from erythrocytes, its turnover is due to the active transport of oxidized glutathione (GSSG) [7].

The glutathione *S*-transferases catalyze the formation of glutathione conjugates from GSH and a wide variety of electrophilic metabolites and zenobiotics [8]. A single form of glutathione *S*-transferase has been shown to be present in erythrocytes [9,10] and this indicates their capacity to form glutathione conjugates. It is generally considered that the first step in the metabolism and degradation of glutathione conjugates is the cleavage of the γ-glutamyl cysteine peptide bond by the action of γ-glutamyl transpeptidase [8]. Since this enzyme is not present in erythrocytes, glutathione conjugates would be expected to either accummulate, or to be transported from the cell.

The experiments reported here demonstrate the transport of a glutathione *S*-conjugate, (*S*-(2,4-dinitrophenyl)glutathione) out of erythrocytes. The formation of glutathione conjugates by the action of glutathione *S*-transferase, and their transport out of the cell, may constitute a significant component of the turnover and metabolism of erythrocyte glutathione.

2. Materials and methods

Blood samples were collected from normal blood donors onto heparin, as an anticoagulant, washed twice in cold isotonic saline and filtered through cotton wool to remove leucocytes and platelets. The conversion of erythrocyte GSH to the conjugate *S*-(2,4-dinitrophenyl)glutathione, (GS–DNP) was carried out by exposure of washed erythrocytes to 4 vol. isotonic saline containing 1.2 mM 1-chloro-2,4-dinitrobenzene for 15 min at 37°C. The cells were then washed twice in 5 vol. ice cold saline and suspended in a buffer containing 1% bovine serum albumin, 8 mM glucose, 62 mM NaCl, 40 mM NaH_2PO_4/$Na_2H\,PO_4$, 35 mM Na-TES [*N*-Tris (hydroxymethyl) methyl-2-aminoethane sulphonic acid] (pH 7.4) (buffer A) [7]. Erythrocytes treated in this manner were used immediately in transport experiments, and GSH and GSSG determinations [11] indicated that ~70% of the initial GSH was converted to the glutathione-*S*-conjugate.

Transport of GS–DNP was evaluated by two different methods:

Method 1: Intracellular glutathione was biosynthetically labelled using [^{3}H]glycine (New England Nuclear) and the specific activity determined by the procedure in [7]. After labelling, the GSH was converted to GS–DNP and the cells suspended in buffer A at ~25% and incubated at 37°C. Aliquots were taken at timed intervals and the supernatants obtained by centrifugation. Supernatant (200 μl) was diluted with 2 ml water containing 0.5 mM glycine and 0.5 mM GSSG, and washed through a 5 × 60 mm column of Dowex 1 (formate form) with 4 × 1 ml pulses of water. The [^{3}H]GS–DNP and GSSG carrier were then eluted with 2 × 1 ml

pulses of 4 N formic acid followed by 1 ml water. The eluate was collected and the [^{3}H]GS–DNP quantitated by liquid scintilation counting.

Method 2: Erythrocytes pre-treated with 1-chloro-2,4-dinitrobenzene were suspended in buffer A at ~25% and incubated at 37°C. Aliquots were withdrawn at timed intervals and the supernatants collected by centrifugation. GS–DNP concentrations were determined spectrophotometrically in the supernatant at 340 nm using a mM extinction coefficient of 9.6 [12,13].

GSH, GSSG and ATP concentrations were determined by the methods in [11].

3. Results

As shown in fig.1 the treatment of erythrocytes with 1-chloro-2,4-dinitrobenzene results in the rapid efflux of ^{3}H-labelled glutathione conjugate. The rate of this transport was linear for at least 2 h. Untreated erythrocytes showed a much lower transport rate, which may represent either the transport of GSSG or endogenously formed glutathione *S*-conjugates. Over the period studied, there was <1% haemolysis in both the treated and untreated cells. Similar results were obtained by measuring the appearance of GS–DNP in the supernatant spectrophotometrically.

Because of the necessity to determine the specific activity of [^{3}H]glutathione in each sample of erythrocytes, and the increased number of manipulations required, evaluation of the transport rate by the radiometric method proved to be considerably more time consuming and less accurate that the spectrophotometric method. Consequently, most studies were carried out by method 2. The rate of GS–DNP transport from erythrocytes obtained from normal blood donors determined spectrophotometrically over a 2 h incubation period is given in table 1. These data show that after treatment with 1-chloro-2,4-dinitrobenzene the level of GSH decreases and does not regenerate during the course of the experiment.

Table 1
Transport of *S*-(2,4-dinitrophenyl)-glutathione from normal human erythrocytes

No. individuals	Initial GSH (μM/ml RBC)	GSH after 2,4-DNB (μM/ml RBC)	GSH after transport (μM/ml RBC)	Conjugate transport (nM . ml RBC^{-1} . h^{-1})
10	2.54 ± 0.266	0.72 ± 0.477	0.53 ± 0.202	496.2 ± 51.26

All data expressed as mean ± SD

To determine the effect of temperature on the transport rate, 1-chloro-2,4-dinitrobenzene treated cells were incubated in buffer A at different temperatures. After 2 h the GS–DNP concentration in the supernatant was determined. The data presented in fig.2 show that transport of GS–DNP is slow at low temperatures and increases rapidly at >23°C.

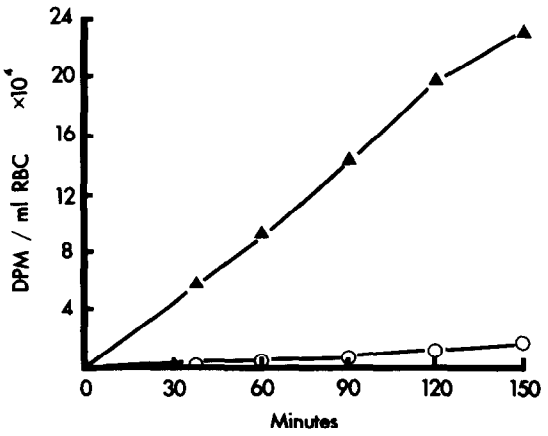

Fig.1. Transport of [^{14}C]glutathione out of erythrocytes: (▲) erythrocytes treated with 1-chloro-2,4-dinitrobenzene; (○) untreated erythrocytes.

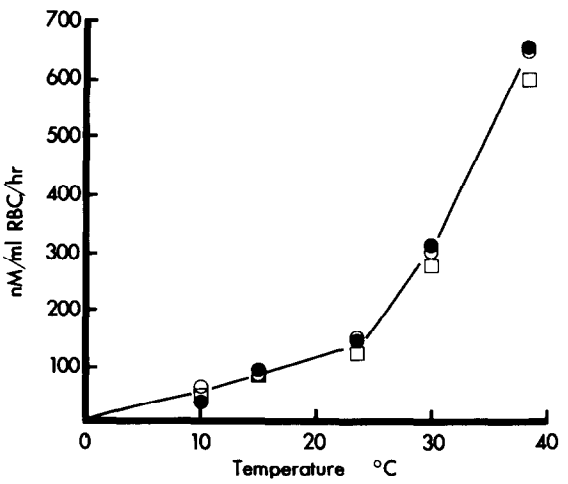

Fig.2. Transport of *S*-(2,4-dinitrophenyl)-glutathione from erythrocytes at different temperatures. Erythrocytes were obtained from 3 normal blood donors (●, ○, □).

Table 2
The effect of ATP depletion and fluoride on the transport of *S*-(2,4-dinitrophenyl)-glutathione from human erythrocytes

	ATP (μM/ml RBC)	Conjugate transport (nM . ml^{-1} RBC . h^{-1})
Fresh samples[a]	1.4 ± 0.15	457 ± 51.1
ATP-depleted[a]	0.4 ± 0.14	148 ± 48.5
ATP-regenerated[a]	2.2 ± 0.27	419 ± 54.6
NaF[b], 2 mM		103

[a] Mean 6 obs. ± SD; [b] Mean of 2 obs.

The effect of ATP depletion was studied by preincubating erythrocytes for 8 h in buffer A without glucose. Over this period ATP was significantly depleted. These cells showed a marked reduction in GS–DNP transport rate (table 2). Aliquots of these ATP depleted cells were incubated in buffer A with 10 mM inosine and 1 mM adenine for 2 h to regenerate ATP. Transport experiments with these cells showed that regeneration of high ATP concentrations restored the transport of GS–DNP (table 2).

The effect of fluoride in the buffer A incubation medium is also shown in table 2. Transport was substantially inhibited by the inclusion of 2 mM NaF.

4. Discussion

These data show that the glutathione conjugate *S*-(2,4-dinitrophenyl)glutathione (GS–DNP) formed by glutathione *S*-transferase, when erythrocytes are exposed to 1-chloro-2,4-dinitrobenzene, is transported from the cell. The infusion of 1-chloro-2,4-dinitrobenzene into a perfused rat liver had been shown [13] to result in the appearance of GS–DNP in the bilary compartment, but no evidence was presented to indicate whether this transposition of glutathione conjugate was due to diffusion or some metabolically-dependent route.

Oxidized glutathione (GSSG) was shown [14] to be actively transported from erythrocytes while the red cell membrane appeared to be impermeable to GSH. The transport system described here is sensitive to changes in temperature, ATP concentration and is inhibited by fluoride. These properties suggest that that transport of glutathione conjugates from erythrocytes is metabolically dependent, and is probably an active process. These characteristics are the same as those observed during GSSG transport [14] and suggest that both GSSG and glutathione conjugates may well be transported from erythrocytes via the same mechanism.

The rate of GS–DNP efflux measured here was found to be ~10-fold, the maximum reported rate of GSSG transport [14]. This suggests that, if both GSSG and glutathione conjugates are transported via the same mechanism, the system may be primarily involved in glutathione conjugate transport and transports GSSG only as a secondary process, since most GSSG should normally be reduced to GSH by glutathione reductase [6]. The finding that glutathione conjugates are transported from erythrocytes supports the concept that glutathione turnover in erythrocytes results from its efflux, however, it remains to be clearly demonstrated what proportion of this efflux is in the form of the disulphide GSSG, or glutathione conjugates as suggested by these data.

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