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FORMATION AND TRANSPORT OF XENOBIOTIC GLUTATHIONE-*S*-CONJUGATES IN RED CELLS

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Abstract—*In vitro* studies with freshly drawn human erythrocytes showed 4-dimethylaminophenol, a cyanide antidote, to be rapidly metabolized with the formation of a transient *S*,*S*-(2-dimethylamino-5-hydroxy-1,3-phenylene)bis-glutathione conjugate and a stable *S*,*S*,*S*-(2-dimethylamino-5-hydroxy-1,3,4-phenylene)tris–glutathione conjugate. The stable tri-glutathionyl derivative was actively transported across the red cell membrane with an apparent $V_{max} = 1$ nmol/min/ml red cell suspension (15 g hemoglobin/100 ml) and $K_m = 0.5$ mM. The transport system was strictly unidirectional, inhibited completely by sodium fluoride and reduced to one-fifth by lowering the temperature from 37 to 22°. Similarly *S*-(2,4-dinitrophenyl)-glutathione, the glutathione-*S*-transferase mediated glutathione-*S*-conjugate with 1-chloro-2,4-dinitrobenzene, was unidirectionally transported, a process which was inhibited by sodium fluoride. Kinetic analysis revealed two different transport processes: $V_{max} = 0.9$ nmol/min/ml, $K_m = 1.4$ μM and $V_{max} = 4.5$ nmol/min/ml, $K_m = 700$ μM. Mutual inhibition of the low affinity transport system was found for both glutathione-*S*-conjugates. The apparent energies of activation for all these transport processes and for GSSG were identical (70 kJ/mol) suggesting at least one common carrier for the excretion of the three glutathione-*S*-conjugates.

Little attention has been paid to the capacity of red cells to metabolize xenobiotics, although these abundant cells are equipped with a variety of enzymes that are required for the biotransformation of foreign compounds. Thus, oxyhemoglobin with its intracellular concentration of 25 mM iron acts like a monooxygenase and has been shown to *C*-oxygenate aniline [1] and to *N*-oxygenate 4-chloroaniline [2]. Even more impressive is the peroxidatic activity of oxyhemoglobin, especially in its reactions with phenylhydroxylamines and aminophenols.

Reactions of aminophenols in red cells have been studied intensively with 4-dimethylaminophenol (DMAP)* which catalyzes ferrihemoglobin formation *in vitro* and *in vivo* at high rates. DMAP, therefore, has been proposed for the treatment of cyanide poisoning [3–6]. The reaction mechanism of ferrihemoglobin formation was studied with purified human hemoglobin. DMAP, like other aminophenols [7, 8], catalytically transfers electrons from ferrohemoglobin to oxygen. Thereby DMAP is oxidized to the phenoxyl radical and *N*,*N*-dimethylquinonimine [9]. Both species are reduced by ferrohemoglobin with the formation of ferrihemoglobin. The catalytic ferrihemoglobin formation is terminated by side reactions, i.e. quick binding of oxidized DMAP to the reactive SH groups in hemoglobin and thioether formation with reduced glutathione.

Formation of glutathione-*S*-conjugates with DMAP was shown to occur *in vitro* in red cells [10], and *in vivo* in dogs [11] and in man [12, 13]. Kinetic studies on thioether formation of DMAP in the presence of purified dog hemoglobin and glutathione in a reducing system showed that initially *S*,*S*-(2-dimethylamino-5-hydroxy-1,3-phenylene)-bis-glutathione (di(GS)-DMAP) is formed [14]. This thioether is highly autoxidizable and adds another thiol giving the stable *S*,*S*,*S*-(2-dimethylamino-5-hydroxy-1,3,4-phenylene)tris–glutathione (tri(GS)-DMAP).

Despite the chemical stability of tri(GS)-DMAP in red cells this thioether disappeared from the erythrocytes of a dog *in vivo* with an apparent half life of about 1 hr. Concomitantly, pre-mercapturic acids of DMAP appeared in the urine, mostly as the tricysteinyl derivative [11]. Essentially the same behaviour was also observed in man [12]. Since the faint hemolysis which has been observed after i.v. injection of DMAP in man [13] could not explain reasonably the disappearance of tri(GS)-DMAP from erythrocytes, a transport mechanism for this bulky, polar molecule had to be assumed. The present study was undertaken to investigate the formation and transport of glutathione-*S*-conjugates of DMAP in human red cells *in vitro* by a newly developed h.p.l.c. method. Additionally we compared the fate of another electrophilic compound, 1-chloro-2,4-dinitrobenzene (CDNB), which is an established substrate for glutathione-*S*-transferase.

MATERIALS AND METHODS

Red cells were obtained from freshly drawn heparinized human blood, washed five times with five vol. of Ringer-phosphate solution, pH 7.4, containing 10 mM glucose, and suspended to a final content of 15 g Hb/100 ml.

* Abbreviations: DMAP, 4-dimethylaminophenol; di(GS)-DMAP, *S*,*S*-(2-dimethylamino-5-hydroxy-1,3-phenylene)bis-glutathione; tri(GS)-DMAP, *S*,*S*,*S*-(2-dimethylamino-5-hydroxy-1, 3, 4-phenylene)tris–glutathione; CDNB, 1-chloro-2,4-dinitrobenzene; GS-DNP, *S*-(2,4-dinitrophenyl)-glutathione; Hb, hemoglobin.

DMAP hydrochloride and the radioactive compound, 4-dimethylamino[U-^{14}C]phenol, sp. act. 9.5 μCi/μmol, were synthesized by Farbwerke Hoechst (Frankfurt, F.R.G.).

[^{14}C]-Di(GS)-DMAP and [^{14}C]-tri(GS)-DMAP were prepared from DMAP and GSH as described elsewhere [11] and had a sp. act. of 78 μCi/mmol. The radiochemical purity was greater than 98% (h.p.l.c.).

CDNB was from Aldrich Chemie (Steinheim, F.R.G.). Equine liver glutathione-*S*-transferase, 65 U/mg protein, was obtained from Sigma Chemie (München, F.R.G.), GS-DNP was synthesized enzymatically from GSH and CDNB in the presence of glutathione-*S*-transferase according to Kondo *et al.* [15]. The thioether was purified by h.p.l.c. and determined at 334 nm using a molar extinction coefficient of 9600/cm.

Silicon oil (AR 200) was obtained from Wacker Chemie (München, F.R.G.). All other reagents (purest grade available) were purchased from Boehringer (Mannheim, F.R.G.) and Merck (Darmstadt, F.R.G.).

Ferrihemoglobin and the total hemoglobin content of the red cell suspensions were measured by the method reported by Kiese [16]. Extracellular hemoglobin in the red cell supernatant was determined as carbon monoxide derivative at 419 nm according to Antonini and Brunori [17]. GSH and GSSG were determined enzymatically [8].

For h.p.l.c. analysis of DMAP- and CDNB-thioethers red cells were separated from the extracellular fluid by centrifugation through a silicon oil layer as described earlier [18]. The extracellular fluid was deproteinized by 0.6 M trichloroacetic acid (TCA, v/v). Total thioethers were determined after deproteinization of the sample with 4 vol. of 1 M $HClO_4$ or 0.6 M TCA, respectively. For complete recovery of GS-DNP, the precipitates were extracted additionally twice with 1 M $HClO_4$. In that way the yield of GS-DNP was increased from 70 [19] to 98%.

For calculation of the intracellular thioether concentration, the intracellular volume of distribution was assumed to be 0.36 ml [20] at an extracellular volume of 0.50 ml/ml red cell suspension ([^{14}C]-inulin distribution) [18]. H.p.l.c. was performed with a chromatograph SP-4/M 600 (Gynkotek, München, F.R.G.) on μ-Bondapak C-18 (0.4 × 30 cm; Waters, Milford, MA, U.S.A.). Di(GS)-DMAP and tri(GS)-DMAP were separated with methanol: 20 mM lithium-citrate, pH 2.2 (5:95, v/v, flow rate 1.5 ml/min, detection at 320 nm). Under these conditions the DMAP thioethers were eluted after 4.8 min (tri(GS)-DMAP) and 6.5 min (di(GS)-DMAP), respectively.

The separated thioethers were quantitated by measuring their radioactivity in Bray's solution with a LKB Rackbeta 1217 liquid scintillation counter. Results were corrected for recovery and background radiation.

GS-DNP was determined by h.p.l.c. on μ-Bondapak C-18 with methanol: 10 mM phosphoric acid (50:50, v/v, flow rate 2 ml/min, detection at 334 nm, R_t = 4.9 min). Quantitation was performed by comparing the peak areas with authentic standards.

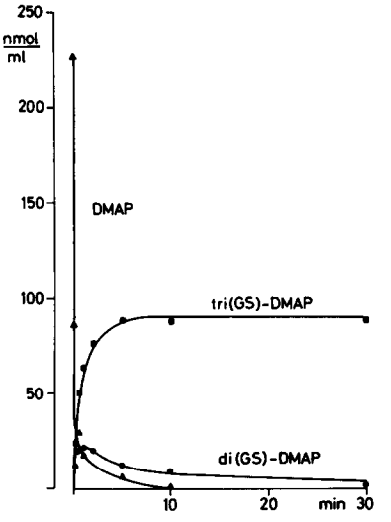

Fig. 1. Kinetics of 4-dimethylaminophenol (DMAP) decrease and thioether formation in human red cells. [^{14}C]DMAP (0.23 mM) was incubated in a suspension of human red cells (15 g Hb/100 ml Ringer-phosphate solution, containing 10 mM glucose, pH 7.4, 37°).

RESULTS

When radioactive DMAP (0.23 mM) was added to a suspension of human red cells (15 g hemoglobin/100 ml, in Ringer-phosphate-solution containing 10 mM glucose, pH 7.4, 37°), more than 90% of DMAP had disappeared within 1 min and the content of GSH had decreased by 0.31 μmol/ml. About half the radioactive material was bound covalently to hemoglobin, the remaining radioactivity was found in two polar metabolites. Analysis of these metabolites by h.p.l.c. indicated formation of two glutathione conjugates, a transient di(GS)-DMAP and the stable tri(GS)-DMAP. The kinetics of these rapid reactions are shown in Fig. 1.

To study the fate of tri(GS)-DMAP we incubated red cells under identical conditions for longer periods and observed a time-dependent increase of tri(GS)-DMAP in the extracellular fluid. After 3 hr incubation half the tri(GS)-DMAP was found in the supernatant (Fig. 2). Because hemolysis was insig-

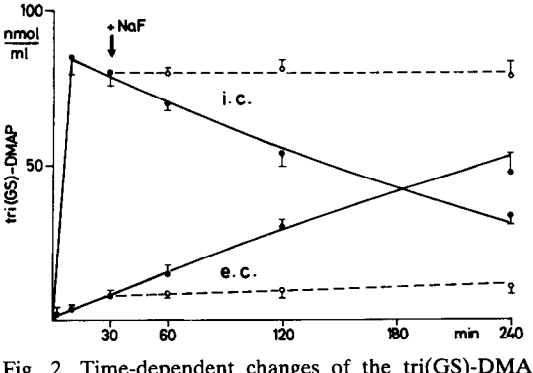

Fig. 2. Time-dependent changes of the tri(GS)-DMAP distribution in the intracellular (i.c.) and extracellular (e.c.) compartment of red cell suspensions, incubated with DMAP (same conditions as in Fig. 1). Broken lines show the influence of 10 mM NaF, added after 30 min. (Means of three experiments ± S.E.M.).

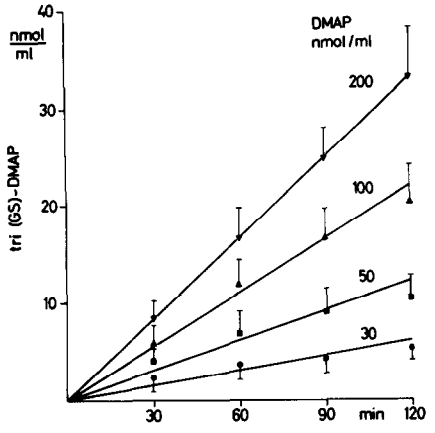

Fig. 3. Concentration dependence of tri(GS)-DMAP transport. Red cells were incubated with various concentrations of DMAP (30–200 μM) under the same conditions as described in Fig. 1. (Means of four experiments ± S.E.M.).

nificant during the incubation period (1.2%), we assumed some active process to transport this bulky molecule (MW = 1053) out of red cells. Actually, tri(GS)-DMAP remained in the intracellular compartment when the energy supply was inhibited by NaF (10 mM). The transport was unidirectional. When radioactive tri(GS)-DMAP (0.1 mM) was added to a suspension of red cells (15 g Hb/100 ml), only 3.5% of the radioactivity was incorporated within 4 hr.

The temperature dependence of the tri(GS)-DMAP transport across the cell membrane was studied between 22 and 37°. Increasing the temperature by 10° accelerated the transport 2.5 fold. It should be noted that tri(GS)-DMAP formation was complete within 5 min at all temperatures tested.

To evaluate the kinetic parameters of the tri(GS)-DMAP transport we incubated red cells with various concentrations of DMAP. The used concentration range was rather narrow, because of (a) the limited detection sensitivity and (b) the limited GSH content of red cells. In addition, high concentrations of DMAP might result in membrane damage and in disturbed energy supply which is consumed for

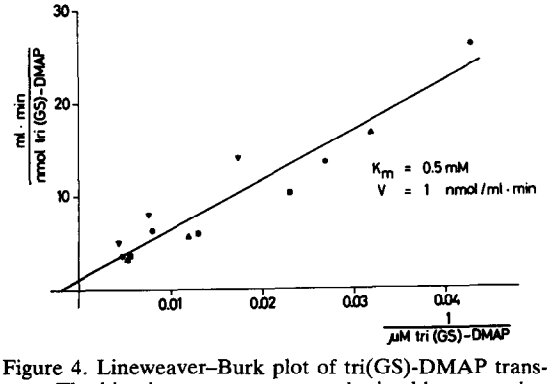

Figure 4. Lineweaver–Burk plot of tri(GS)-DMAP transport. The kinetic parameters were obtained by regression analysis of the transport rates (shown in Fig. 3) at respective intracellular tri(GS)-DMAP concentrations. Data points were obtained from experiments with four different red cell preparations.

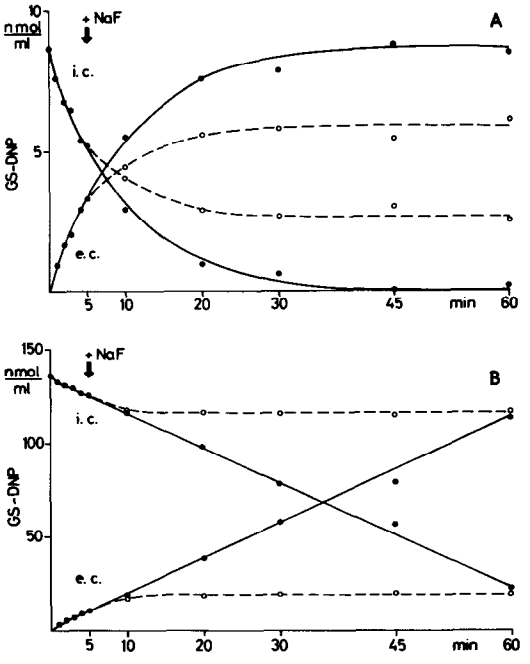

Fig. 5. Time-dependent changes of the GS-DNP distribution in the intracellular (i.c.) and extracellular (e.c.) compartment of red cell suspensions (cf. Fig. 1), incubated with CDNB (panel A: 10 nmol/ml; panel B: 150 nmol/ml). Broken lines show the influence of 10 mM NaF, added after 5 min.

methemoglobin reduction. Figure 3 shows that tri(GS)-DMAP transport was linear for at least 1 hr. The kinetic parameters of the tri(GS)-DMAP transport were obtained by regression analysis of the transport rates at various intracellular concentrations of tri(GS)-DMAP (see Methods) according to Lineweaver–Burk (Fig. 4).

In addition to tri(GS)-DMAP, the thioether from CDNB and GSH, catalyzed by glutathione-S-transferase, showed similar transport characteristics. At surplus GSH, CDNB in red cells is quantitatively transformed into the thioether S-(2,4-dinitrophenyl)-glutathione (GS-DNP), more than 90% within 1 min.

Figure 5 shows the time-dependent changes of GS-DNP distribution at 10 and 150 nmol CDNB per ml red cell suspension. Addition of NaF (10 mM) inhibited the transport of GS-DNP completely. The delayed inhibitory effect of NaF was not observed at higher NaF concentrations, e.g. 100 mM. The transport was strictly unidirectional and showed the same temperature dependence as tri(GS)-DMAP. Omission of calcium ions and the presence of EDTA (1 mM) was without effect on GS-DNP transport.

In contrast to tri(GS)-DMAP, apparently two systems are involved in the transport of GS-DNP. Covering a range from 10 to 1000 nmol CDNB/ml red cells, we observed transport rates of GS-DNP which did not follow a Michaelis–Menten kinetics. The Lineweaver–Burk plot (Fig. 6) clearly shows deviation of the data points from a straight line. The kinetic parameters shown in Fig. 6 resulted from iterative nonlinear curve fitting on the assumption of two different transport systems, one of which having a remarkably high affinity.

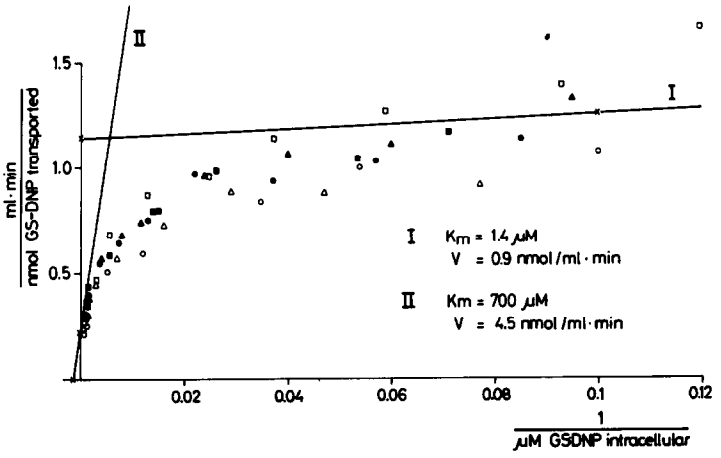

Fig. 6. Lineweaver–Burk plot of *S*-(2,4-dinitrophenyl)-glutathione (GS-DNP) transport. Red cells (15 g Hb/100 ml) were incubated with various concentrations of 1-chloro-2,4-dinitrobenzene (CDNB, 10–1000 μM) at 37°. The kinetic parameters were calculated on the assumption of two different transport systems. (The different symbols indicate experiments with six different red cell preparations.)

Mutual inhibition of the transport was found for both thioethers. In the presence of surplus DMAP (DMAP 0.2, CDNB 0.04 μmol/ml) GS-DNP transport was inhibited by about 20%, in the presence of surplus CDNB (CDNB 0.5, DMAP 0.2 μmol/ml) tri(GS)-DMAP transport was inhibited by about 60%.

DISCUSSION

The energy-dependent transport system for GSSG was first reported by Srivastava and Beutler [21] using erythrocytes exposed prior to oxidative stress. The movement of GSSG was shown to be strictly unidirectional proceeding even against a concentration gradient. Pre-incubation of red cells in a glucose-free medium or the presence of 0.1 M NaF resulted in a cessation of GSSG transport [21]. Using inside-out vesicles from human red cells Kondo *et al.* [22] detected two ATP-dependent transport systems for GSSG, $K_m = 0.1$ and 7.3 mM, $V = 20$ and 210 nmol/ml/hr, respectively. In addition to the demonstrated capacity of red cells to transport GSSG, it has been shown that other tissues, such as lens [23], liver [24] and heart [25] can also extrude GSSG. Moreover, it has been reported that glutathione-*S*-conjugates formed in the isolated perfused liver were excreted preferentially into bile [26], and competition has been observed between GSSG and GS-DNP for the transport across the liver canalicular membrane [27]. Such a glutathione-*S*-conjugate transport was also detected in red cells by Board [19], who reported GS-DNP transport at a rate of 7.6 nmol/ml/min packed red cells, whereas Srivastava and co-workers [28] found a transport rate of 2.3 nmol/ml/min. In addition, GS-DNP was shown by Kondo *et al.* [15] to inhibit competitively the high-K_m process of GSSG transport but not significantly the low-K_m process in inside-out vesicles from human red cells. These authors reported on a single GS-DNP transport system with an apparent K_m value of 0.94 mM.

In contrast to Kondo *et al.* [15] who investigated the GS-DNP transport only in the range from 0.2 to 2 mM GS-DNP, we found two GS-DNP transport processes in intact red cells of humans. The high-affinity process has an extremely low K_m value of 1.4 μM and $V = 0.9$ nmol/ml/min. Obviously, the high affinity process is easily overlooked if the transport is studied at concentrations above 0.1 mM where the low affinity process with its five-fold higher capacity is operating ($K_m = 0.7$ mM, $V = 4.5$ nmol/ml/min). For maximal transport of GS-DNP in packed cells a value of 9 nmol/ml/min is calculated which agrees with the data given by Board [19]. This transport rate exceeds the maximum reported rate of GSSG transport [21] by a factor of ten. Similarly, the cardiac transport of GS-DNP was five-fold higher than the GSSG transport in isolated perfused rat heart [25]. At present we do not know whether the high affinity transport system for GS-DNP is identical with that for GSSG as observed in inside-out vesicles of human red cells [22]. Interestingly, the affinity of the low-K_m system for the 'unphysiological' GS-DNP is by two orders of magnitude higher than for GSSG.

Mutual inhibition of the transport of glutathione-*S*-conjugates was found between GS-DNP and tri(GS)-DMAP, the stable thioether produced by DMAP in red cells [10]. The transport of GS-DNP (40 μM) was inhibited by tri(GS)-DMAP (80 μM) by some 20% and a similar inhibition was found for the tri(GS)-DMAP transport. Tri(GS)-DMAP transport was not inhibited by 10 μM GS-DNP but reduced to 40% in the presence of 500 μM GS-DNP. These data suggest that tri(GS)-DMAP competes with the low affinity transport system of GS-DNP.

The transport of GS-DNP and tri(GS)-DMAP was strictly unidirectional, completely inhibited by NaF and had an identical energy of activation as shown in the Arrhenius-plot (Fig. 7). There was no difference in the temperature dependence between the high and low affinity transport of GS-DNP, the transport of tri(GS)-DMAP and GSSG (calculated from the data published by Srivastava and Beutler [21]). the values for the energy of activation (70 kJ/mol) were far beyond that for simple diffusion processes.

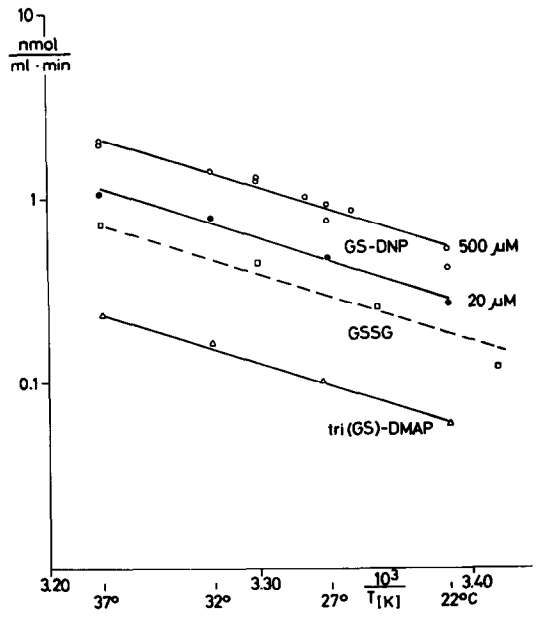

Fig. 7. Arrhenius plot of the transport of tri(GS)-DMAP, GS-DNP and GSSG across the red cell membranes. Red cell suspensions (cf. Fig. 1) were incubated at various temperatures with DMAP (0.23 mM) and with CDNB (20 and 500 μM). Data for GSSG transport (broken line) were calculated from [21].

The existence of at least two different transport systems for glutathione-*S*-conjugates enables erythrocytes to eliminate not only unphysiologically high amounts of GSSG but also glutathione-*S*-conjugates which might be formed during exposure to various electrophilic compounds. These findings underline the physiological significance of red cells in the detoxification of xenobiotics.

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