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Different Metabolizing Ability of Thiol Reactants in Human and Rat Blood

BIOCHEMICAL AND PHARMACOLOGICAL IMPLICATIONS*

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The effect of oxidants, electrophiles, and NO donors in rat or human erythrocytes was analyzed to investigate the influence of protein sulfhydryl groups on the metabolism of these thiol reactants. Oxidant-evoked alterations in thiolic homeostasis were significantly different in the two models; large amounts of glutathione protein mixed disulfides were produced in rat but not in human erythrocytes by treatment with hydroperoxides or diamide. The disappearance of all forms of glutathione (reduced, disulfide, protein mixed disulfide) was induced by menadione only in human erythrocytes. The treatment of rat red blood cells with electrophiles produced glutathione *S*-conjugates to a much lower extent than in human red blood cells; GSH was only minimally depleted in rat red blood cells. The NO donor *S*-nitrosocysteine induced a rapid transnitrosation reaction with hemoglobin in rat erythrocytes producing high levels of *S*-nitrosohemoglobin; this reaction in human red blood cells was negligible. All drugs were cleared more rapidly in rat than in human erythrocytes. Unlike human Hb, rat hemoglobin contains three families of protein SH groups; one of these located at position β125 is directly implicated in the metabolism of thiol reactants. This is thought to influence significantly the biochemical, pharmacological, and toxicological effects of some drugs.

Glutathione is the major low molecular weight thiol in mammalian cells where it constitutes the most important antioxidant defense. Its action is usually favored by ubiquitous enzymes (*e.g.* glutathione *S*-transferases, glutathione peroxidase). GSH also regenerates other important defensive resources (*e.g.* vitamins E and C) and directly participates in the destruction of reactive oxygen species (1). The alkylation of glutathione by electrophilic reagents and the reduction of chemically reactive oxidant species are biological functions associated with the protection of SH groups of critical cellular macromolecules (2).

The cell concentration of protein SH groups, ranging from 10 to 30 mM, is larger by far than that of GSH (2–10 mM) (3); however, the metabolic role against electrophiles and oxidants is thought to be rather marginal. In fact, the modification of PSH[1] has been usually considered only a potential damaging reaction, even if some authors suggested that chemical alteration of some cysteine residues may have a protective or regulatory role (4, 5).

The participation of PSH in quantitatively important reactions is proportional to their concentration and reactivity. The intrinsic reactivity of PSH is dependent on the pK_a value (the thiolate anion is much more reactive than the undissociated form) and its accessibility (structural and conformational features). In fact, some proteins such as albumin (6) with relatively low pK_a values have an apparent reactivity much lower than that of GSH. It follows from this that PSH may have a wide range of apparent reactivity that spans 4 orders of magnitude (7). This fact has abrogated any efforts to understand the effective defensive contribution of PSH. More recently, the attention on PSH as important modulators of the intracellular redox state has been renewed and emphasized (5, 8, 9), and reports of PSH having reactivity very similar to or greater than that of GSH are increasing.

Recently, the problem has become more interesting because a large number of papers have focused on the importance of the cooperation between GSH and protein SH group, in particular hemoglobin, in nitric oxide transport, and targeting (10, 11). Thus, the role of PSH and GSH as similar cooperating groups in the metabolism of electrophiles, oxidizing agents, and nitric oxide remains an open question.

Our previous studies have demonstrated that rat hemoglobin possesses reacting cysteines located on the β chain at position 125 (12). These cysteinyl residues, given their reactivity and concentration in rat blood, competing and cooperating with GSH, may influence the pharmacokinetics, toxicity, and action of many drugs or their metabolites.

To verify whether and by which mechanism, the reactivity of rat Hb cysteines leads to an unusual metabolism of some thiol reactants, we analyzed the effect of three different drug categories, oxidants, electrophiles, and NO donors on human and rat erythrocytes. Among the oxidants we used were drugs that react with GSH either enzymatically (*via* GSH-peroxidase, *i.e.* hydroperoxydes) or nonenzymatically (diamide, menadione);

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[1] The abbreviation used are: PSH, protein sulfhydryl; BCNU, *N*,*N*′-bis-(2-chloroethyl)-*N*-nitrosourea; CDNB 1-chloro-2, 4-dinitro-benzene; DTNB, 5,5-dithio-bis(2-nitrobenzoic acid); Cys-NO, *S*-nitrosocysteine; EA, ethacrinic acid; GSSP, glutathione-protein mixed disulfhyde; Hb-SH, hemoglobin sulphydryl; GR, glutathione reductase; PBS, phosphate-buffered saline; HPLC, high pressure liquid chromatography; Hct, hematocrit.

similarly, electrophiles that conjugate with GSH by glutathione *S*-transferases catalysis or spontaneously (13) were tested. The membrane-permeable NO donor *S*-nitrosocysteine was also used to evaluate the extent of transnitrosation reactions carried out by PSH.

EXPERIMENTAL PROCEDURES

Chemicals—Glutathione, glutathione disulfide, and glutathione reductase were obtained from Roche Molecular Biochemicals; HPLC grade chemicals were from BDH; BCNU was from Bristol-Myers Squibb (Dublin, Ireland). Diamide (diazenedicarboxylic acid bis(*N*,*N*-dimethylamide)), menadione (2-methyl-1,4-naphthoquinone), *tert*-butyl hydroperoxide, human serum albumin, and all other chemicals of analytical grade were from Sigma.

Blood Collection—Male Wistar rats (2 months old, 300-g body weight) were purchased from Charles River (Como, Italy). Blood was collected from the abdominal aorta under anesthesia with diethylether. Human blood was obtained from healthy volunteers (30–45 years old). For all blood samples K_3EDTA was used as anticoagulant.

Red Blood Cell Treatment—For *in vitro* treatments each blood sample was washed with PBS containing 10 mM glucose and was adjusted to a hematocrit value of 37.5% with the same buffer. Red blood cells samples were placed in plastic tubes, incubated at 37 °C in a thermostatic rotating (100 rev/min) bath, and then exposed to the different substances.

For the *in vivo* treatment BCNU was infused via PE-50 tubing cannulated into the femoral vein, whereas blood was collected from a PE-50 tubing cannulated into the jugular vein. Both tubes were connected to a double valve (model 617, 20 × 20 mm, Danuso Instruments, Milano, Italy). Valves and tubing were implanted 2 days before the experiment, under pentobarbital anesthesia (50 mg/kg body weight).

GSH, GSSG, and GSSP Determinations in Blood Samples—Blood aliquots were deproteinized by the addition of four volumes of 5% trichloroacetic acid; GSH and GSSG were then determined on the clear supernatant. GSH was assayed enzymatically, using CDNB and glutathione *S*-transferases (12); GSSG was assayed enzymatically at 340 nm by the procedure of Klotzsch and Bergmeyer (14).

To determine GSSP concentrations, acid precipitated proteins were washed thoroughly with the precipitating solution until no trace of soluble GSH or GSSG was detected. The pellets were then resuspended and brought to an alkaline pH (pH 7.5–8.0 for samples of rat red blood cells and pH 12.0 for the human erythrocytes). The alkaline pH (12.0) is used in human samples because only under these conditions all mixed disulfides are released; for rat samples slightly alkaline buffers were used (pH 7.5–8.0), but similar results are obtained at pH 12.0. Under these conditions GSH is released via an SH/SS exchange reaction as previously described by Rossi *et al.* (15). The amount of released GSH was assayed enzymatically in the supernatant (see above).

Hemoglobin Preparation—Rat hemoglobin was purified by crystallization from stroma-free hemolysate by the method of Condò *et al.* (16). Human hemoglobin was prepared using the method of Riggs (17). Hemoglobin stocks were prepared and maintained under N_2. The amount of oxidized hemoglobin was checked spectrophotometrically before each experiment on the basis of the 576/541 nm absorbance ratio (18).

Hemoglobin Sulfhydryls Titration and Determination of the Reactivity Constants (k_2)—PSH of hemoglobin were assayed spectrophotometrically with DTNB at 450 nm ($\epsilon_{mM} = 7.0\ mM^{-1}\ cm^{-1}$): hemoglobin was adjusted to pH 7.4 with 0.2 M sodium/potassium phosphate buffer, and then the reaction was started by addition of DTNB (final concentration, 0.2 mM). Fast reacting SH groups were titrated by means of an Applied Photophysics MV 17 stopped flow apparatus; samples of hemoglobin in 0.2 M phosphate buffer, pH 7.4, were rapidly mixed with equal volumes of a solution containing 0.4 mM DTNB in 0.2 M phosphate buffer, pH 7.4. Data were fitted to a single exponential curve for the human protein and a three exponential curve for those of rat using the SigmaPlot, version 2.01 (Jandel Scientific).

Glutathione Reductase Activity—Glutathione reductase activity determination was carried out at room temperature on hemolysate, previously passed through a Sephadex G-25 column, according to standard methods (19).

Nitrosothiols—Cys-NO was freshly prepared by combining equimolar concentrations of 200 mM Cys in 0.75 N HCl and 200 mM potassium nitrite in the presence of 0.1 mM diethylenetriaminepentaacetic acid. After 3–5 min, the mixture was neutralized with 1 M Tris. Cys-NO and nitrosoproteins titration was carried out after decomposition of the S-NO bond with Hg^{2+} and is based on the colorimetric determination of NO_2^- (20).

Glutathione Conjugate and Free Drug Determinations—Glutathione conjugates and free substances were determined on deproteinized samples (final concentration, 5% trichloroacetic acid) by HPLC or by spectrophotometry. Glutathione-CDNB and glutathione-EA conjugates were measured by evaluation of the peak at 340 and 290 nm absorbance, respectively; free CDNB and EA were titrated by end point reactions after the addition of glutathione transferases and an excess of GSH. All other drugs and their adducts with GSH were measured by HPLC. Briefly, deproteinized samples were loaded onto a Sephasil C18 (250 × 4.6 mm) column (Amersham Pharmacia Biotech) and eluted by the application of a linear gradient of methanol: 0–10 min 20% methanol 80% acetate buffer (100 mM, pH 4.5), 10–30 min linear gradient 20–100% methanol.

For HPLC determination of glutathione conjugates of BCNU, a Bio-Rad Biosil NH_2 column (250 × 4.6 mm) was used; deproteinized samples were treated with iodoacetic acid for 45 min (final concentration, 15 mM) at neutral pH and derivatized with an alcoholic 1.5% solution of 2,4-dinitrofluorobenzene (1:1 for 3 h). Samples were eluted by the application of a linear gradient of 0.5 M acetate buffer, pH 4.7, after a 10-min isocratic phase (0–10 min with 20% acetate, 80% methanol; 10–30 min with 20–90% acetate). An Hewlett Packard HPLC Series 1100 equipped with diode array detector was used. All spectrophotometric determinations were carried out with a JASCO UV550 apparatus.

RESULTS

Oxidizing Agents—Treatment of human and rat erythrocytes with oxidizing substances evidenced remarkable differences between the two species; hydrogen peroxide or organic hydroperoxides (*tert*-butyl hydroperoxide) produced a rapid oxidation of GSH to GSSG (Fig. 1), both in human and rat red blood cells. However, rat erythrocytes were also characterized by a subsequent, rapid formation of glutathione-protein mixed disulfides (GSSP). The initial thiolic homeostasis was completely restored within 1 h of drug exposure. Diamide (a specific oxidant for thiol groups) rapidly increased GSSG levels in human red blood cells; conversely, it produced GSSP but not GSSG in rat erythrocytes.

Fig. 1 shows the results of the treatment of rat and human red blood cells with menadione (vitamin K_3). Menadione is a quinone reagent that produces oxidative modifications in biological systems, mainly through redox cycling reactions with oxygen (menadione binds to thiol groups to form a conjugate able to redox cycle like menadione itself) (21). Human red blood cells were essentially characterized by a rapid GSH disappearance with a negligible (if any) production of GSSG and GSSP. In contrast, in rat erytrocytes, menadione immediately produced large amounts of GSSG, which in turn were transformed into GSSP. In both cases, human and rat red blood cells were not able to restore initial levels of GSH. Total glutathione (GSH + GSSG + GSSP) was greatly decreased in human samples, whereas only a slight decrease was found in rat erythrocytes.

Electrophilic Agents—Glutathione alky(ary)lation by electrophilic reagents is considered an important metabolic pathway of many drugs and their intermediates. The formation of these conjugates is a detoxification reaction because the glutathione-electrophile adduct is usually less toxic than the parent molecule; after its formation, the conjugate is exported to the extracellular milieu by specific transporters (22). GSH alky(ary)lation is usually catalyzed by GSH *S*-transferases, a ubiquitous class of isoenzymes, which can be divided into four families (α, μ, π, and τ encoded by different genes) (23).

Rat and human red blood cells were treated with two known substrates of GSH *S*-transferases (CDNB and EA) and with other reagents that conjugate GSH nonenzymatically (*N*-ethylmaleimide) or after decomposition into subproducts (BCNU). After drug addition (1 mM, approximately at a molar ratio of 1:1 with GSH), a rapid increase in intraerythrocytic GSH conjugates (Fig. 2*A*) was detected in all samples, followed by their

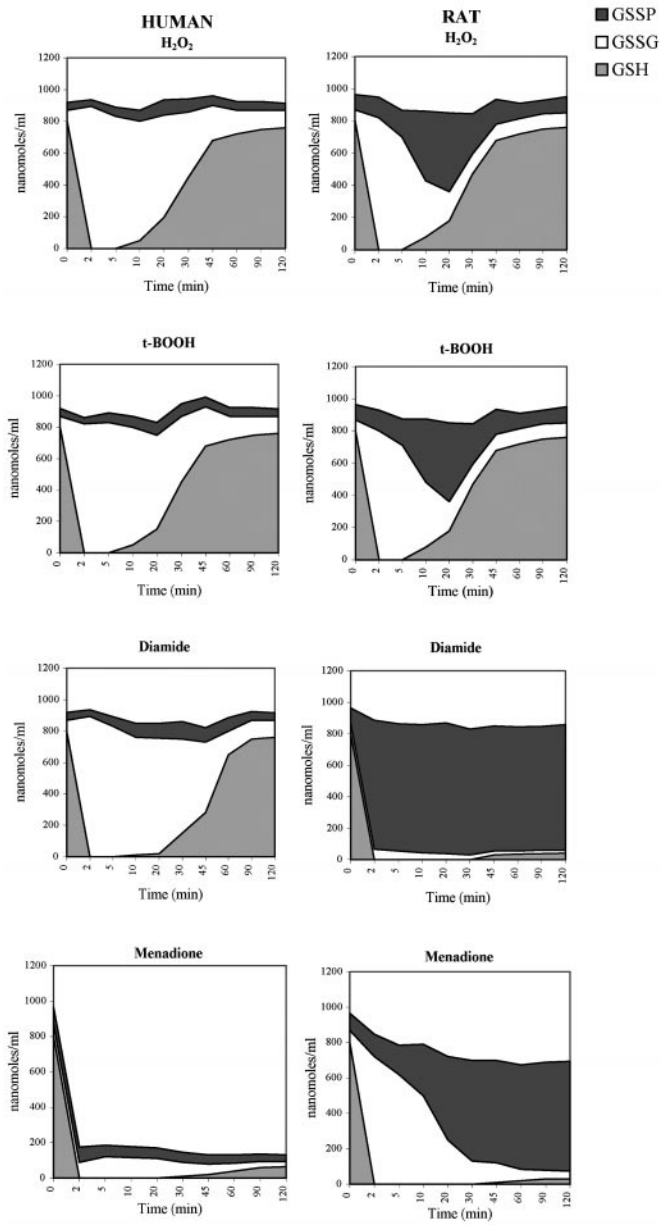

FIG. 1. **Treatment of human and rat red blood cells with oxidants.** Time course of GSH, GSSG, and GSSP in human and rat red blood cells (37.5% Hct in PBS) after treatment with various oxidants (final concentration, 2 mM). At specified times aliquots were withdrawn, and after acid precipitation GSH and GSSG were measured in the clear supernatant; pellets were analyzed for the GSSP content. S.D. values were omitted for clarity. The number of replicate experiments was four. When hydrogen peroxide was used cells were pretreated with 1 mM NaN_3 to inhibit catalase.

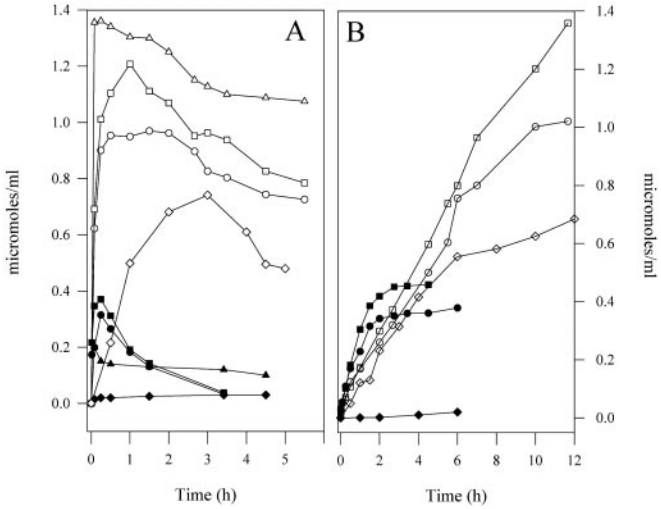

FIG. 2. **Treatment of human and rat red blood cells with electrophilic agents.** Time course of GSH conjugates after treatment of rat (*closed symbols*) or human (*open symbols*) red blood cells (37.5% Hct in PBS) with 1 mM (final concentrations) of CDNB (□, ■), EA (○, ●), *N*-ethylmaleimide (△, ▲), and BCNU (◇, ◆) at 37 °C. *A*, intraerythrocytic levels of conjugates. *B*, extracellular levels of conjugates. At specified times, red blood cells were pelleted by centrifugation, and proteins were removed by acidification and centrifugation. In the supernatant, conjugates were measured by spectrophotometry or HPLC as specified in methods. S.D. values were omitted for clarity. The number of replicate experiments was 3. BCNU does not react directly with thiols, thereby in the plot we refer to the 2-chloroethylisocianate (produced by BCNU decomposition) conjugate with GSH.

TABLE I
GSH concentration in rat or human red blood cells (37.5% Hct in PBS) after 4 h of treatment with various drugs (1 mM, 37 °C)
The number of replicates was three.

	GSH	
	Human	Rat
	nmol/ml	
No treatment	851 ± 84	833 ± 101
CDNB	15 ± 5	684 ± 41
EA	20 ± 5	699 ± 66
NEM	0	721 ± 81
BCNU	44 ± 31	789 ± 54

export to the extracellular medium (Fig. 2*B*). However, remarkable differences were evident between rat and human red blood cells. In particular, in the rat, the peak of intraerythrocytic GSH conjugates was always much lower than that obtained in human samples; similarly, the amount of exported conjugate was considerably lower than in human erythrocytes.

In parallel, GSH was almost totally depleted in human erythrocytes treated with EA, CDNB, BCNU, or *N*-ethylmaleimide, but not in rat red blood cells; a percentage varying from 65 to 95% of initial GSH was still present in rat erythrocytes after 4 h of exposure (Table I).

NO Donors—A large interest in the biochemistry of SH groups has been stimulated by the discovery that they can have an active role in nitric oxide metabolism and targeting through the formation of *S*-nitrosothiols. These molecules have been shown to undergo exchange reactions with low and high molecular weight thiols, reactions of primary importance in the final effect of nitric oxide itself (24).

In Fig. 3, the influence of a treatment of red blood cells with *S*-nitrosocysteine on the levels of intracellular protein *S*-nitrosothiols is shown. *S*-Nitrosocysteine is a compound able to cross plasma membranes rapidly and to exchange NO^+ group with other thiols. The kinetics of these reactions are governed by the reactivity and concentration of various thiol groups. Only slight variations in *S*-nitrosoprotein levels were found when human erythrocytes were used; conversely, under the same conditions rat red blood cells rapidly produced high levels of *S*-nitrosohemoglobin.

Rates of Drug Removal—Among the substances used in the present work, some are known to be exclusively metabolized (at least in red blood cells) through conjugation with SH groups, whereas hydroperoxides (25) and menadione (26) can also react with Fe^{2+} of the Hb heme group. In Table II the $t_{1/2}$ and the time required for complete disappearance of free drugs (we did not consider those metabolized also by heme groups) are reported. Rat red blood cells removed all substances at a significant higher rate; within a few minutes (1–5 min) all substances were in fact under the detection limit, whereas high levels of

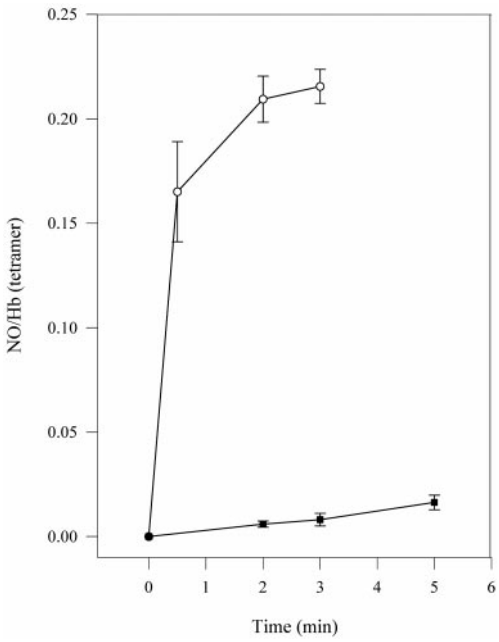

FIG. 3. **Treatment of human and rat red blood cells with Cys-NO.** Rat (○) and human (■) red blood cells (37.5% Hct in PBS) were treated with Cys-NO (final concentration, 1 mM) at 37 °C. At specified times, cells were pelleted by centrifugation and hemolyzed by the addition of 10 volumes of water; the samples were then passed through G25 columns, and nitrosothiols were measured on the protein fraction. The number of replicate experiments was three.

drugs were maintained in human erythrocytes for a long time. BCNU (the rate-limiting step is given by its spontaneous decomposition) was metabolized more slowly by both systems; however, its $t_{1/2}$, as previously reported (27), was half in rat erythrocytes compared with that in human red blood cells.

Hemoglobin SH Groups—The reactivity of various SH groups was tested with the common sulfhydryl titrant 5,5′ dithio-bis-nitrobenzoic acid. Rat hemoglobin (Fig. 4) was characterized by a rapid reaction when compared with other low molecular weight thiols or protein cysteines (human Hb, human serum albumin, GSH, *N*-acetylcysteine); the shape of the titration curve is essentially biphasic, showing an initial burst phase followed by a slower increase in absorbance. The most important feature is that some rat hemoglobin sulfhydryls, located at the β125 position (12), are far more reactive than GSH itself; other cysteines, in positions α13 and β93, are far less reactive. Titration of these cysteines corresponds to the slower phase of the curve. Human Hb possesses only one slow reacting cysteine at position β93 (28). The second order rate constant (k_2) of rat Hb Cys-β125 is about 1 order of magnitude greater than that of GSH and other low molecular weight thiols and 3–4 orders of magnitude greater than the k_2 of other protein cysteinyl residues.

Rat and human red blood cells used in previous experiments (Figs. 2 and 3) were also assayed for Hb-SH content; in Table III data obtained at the end of the experiment (4 h) are reported. Rat Hb-SH β93 and α13, as well as human Hb-SH β93, were modified negligibly by the treatments; in contrast, rat Hb-SH β125 (Table III and Fig. 5) were significantly decreased (the depletion of Cys-β125 is highlighted by the lower absorbance reached by tracings within 1 s). Depletion of rat Hb Cys-β125 elicited by drug addition, ranged from 650 to 850 μM and was consistent with the amount of drug, which was not found as glutathione conjugate. A dose-dependent depletion of Hb Cys-β125 was also induced by the oxidants, diamide, *tert*-butyl hydroperoxide, hydrogen peroxide, and menadione (data not shown).

DISCUSSION

Glutathione is commonly considered to be the first line of defense against oxidizing molecules; it also plays an important role in the detoxification pathways of electrophilic drugs (29). Many models show how GSH depletors may initiate irreversible damage only after a significant decrease (greater than 70–80%) of tissue GSH. The involvement of the SH groups of proteins in GSH-typical reactions has been regarded as marginal, in quantitative terms, and as deleterious to protein structure and function. Even if protein thiols are usually more concentrated than GSH in cellular pools, their reactivity is usually 2–4 orders of magnitude lower; this allows GSH to exhibit its protective role (30). The catalysis of a wide number of conjugation reactions by glutathione transferases provides a further protection of protein SH groups by GSH (2).

The reaction rate of small thiols (*i.e. N*-acetylcysteine, cysteine, or glutathione) with titrating molecules is usually well correlated with the pK_a of the sulfydryl group (which normally lies between pH 8.5 and 9.5). However, for PSH, accessibility as well as the dissociation state of the sulfur must be considered (3). Various protein SH groups have been identified as "fast reacting," for example, SH of cysteine proteases (papain) (31), or SH involved in the catalysis of glycolytic reactions (glyceraldehyde-3-phosphate dehydrogenase) (32); however, their overall quantitative contribution to the metabolism of electrophiles or oxidants is clearly marginal.

Blood is characterized by the presence of high concentrations of Hb (8–10 mM, as monomer), a value significantly larger than GSH (0.8–1 mM). It is well known that human Hb has only one titrable pair of thiols per tetramer (Cys(F9)93β) and that its reactivity is also dependent on the quaternary conformation, being slower in the deoxy state (10). Cys-β93 of human Hb is shielded by a hydrogen bond between Asp-94 and Glu-90 (28) and is also influenced by the vicinity of the terminal carboxyl group.

Rat hemoglobin is known to possess extra reactive SH groups compared with human Hb; cysteines located at β125 and α13 (33) positions were identified as additional potentially reactive cysteinyl groups. Cysteine β125 was shown to have a low pK_a, a high accessibility, and consequently a strong reactivity (12). Moreover, its reactivity is not influenced by allosteric changes in the protein. This low pK is due to a hydrogen bridge between SH of Cys-β125 and Ser-123, where SH is the hydrogen donor.

Comparison (Fig. 4) of the reaction kinetics of various SH groups with DTNB demonstrated how rat Hb is essentially characterized by two different families of thiols. One family has a reaction rate 1 and 3 orders of magnitude greater than small thiols and other protein thiols (*e.g.* human Hb Cys-β93), respectively.

This has proved the existence of thiols on rat Hb possessing an unusually high reactivity. The task is now to verify whether this anomaly can in any way influence the overall metabolism of some agents.

Usually, treatment with oxidizing agents (*e.g.* organic hydroperoxides) leads to the oxidation of GSH into GSSG. After drug reduction, the system recovers the initial levels of GSH. If the oxidative stress is severe, a SH-SS exchange reaction between GSSG and PSH can take place (34, 35) to form protein glutathione mixed disulfides. This reaction is more effective in the presence of highly reactive SH groups (34).

From the data shown in Fig. 1, it can be readily deduced how rat red blood cells, by means of fast reactive Hb thiols, are able to form large amounts of GSSP; in contrast, only GSSG is generated in human erythrocytes. The differences between the two species are more evident when diamide or menadione are used. In the first case, GSSP, but not GSSG, were produced by

TABLE II

Half-life and time required for complete removal of various drugs (diamide, 2 mM; all other drugs, 1 mM; 37 °C) in rat or human red blood cells (37.5% Hct in PBS)

The number of replicate experiments was 4.

	$t_{1/2}$		Time required for complete removal	
	Human	Rat	Human	Rat
Diamide	10 min	<30 s	30 min	<30 s
CDNB	20 min	<30 s	2 h	3 min
EA	25 min	<30 s	2 h	4 min
NEM	1 min	<30 s	2 min	<30 s
BCNU	1 h	30 min	>4 h	3.5 h
Cys-NO	45 min	2 min	2.5 h	5 min

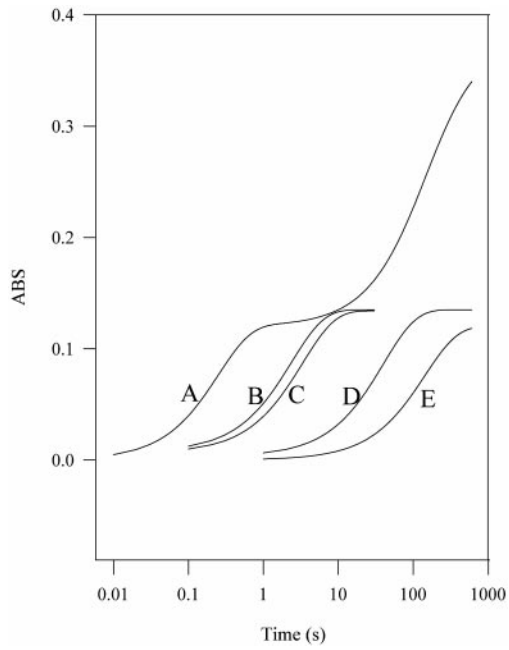

FIG. 4. **Titration of various SH with DTNB.** Thiols (hemoglobins, 10 μM; albumin, 20 μM; GSH and *N*-acetylcysteine, 10 μM) in 0.2 M phosphate buffer, pH 7.4, were titrated with DTNB (final concentration, 0.2 mM) at 25 °C. *A*, rat hemoglobin; *B*, GSH; *C*, *N*-acetylcysteine; *D*, human serum albumin; *E*, human hemoglobin.

rat red blood cells. This was due to the rapid reaction of diamide with Cys-β125 of rat Hb to form an intermediate, which is then cleaved by a molecule of GSH to form mixed disulfide with hemoglobin (36). The irreversible GSSP production in rat red blood cells after diamide treatment is likely to be due to the trapping of all available GSH into GSSP; in fact, the reduction of protein mixed disulfides is catalyzed by thioltransferase, which needs GSH as a cofactor (37).

Menadione-evoked alterations of GSH are rather complex. Because this drug is able both to oxidize and conjugate, the interpretation of its overall action is not easy. The negligible production of GSSG and GSSP in human red blood cells and, in contrast, the generation of both GSSG and GSSP in rat red blood cells, after menadione treatment, can be interpreted in the light of the presence of fast reacting SH in the rat. Menadione is likely transformed into semiquinone by reaction with Fe^{2+} of Hb (26); the semiquinone is probably an electrophile stronger than menadione itself and rapidly conjugates with GSH. This may explain the disappearance of all forms of GSH in human red blood cells (reduced, disulfide, mixed disulfide). On the other hand, rat hemoglobin can intercept the semiquinone by its Cys-β125. The thiolic conjugates of menadione are known to redox cycle (38) (like menadione itself), and this is evident by subsequent oxidation of GSH in rat red blood cells. We can also speculate that the conjugate of menadione with Cys-β125 can redox cycle more efficiently than the GSH menadione adduct.

The conjugation activity of rat Hb toward electrophiles is also demonstrated in Fig. 2; in all time courses of human erythrocytes, a rapid formation of glutathione conjugates is evident, followed by its export to the extracellular milieu. Rat erythrocytes metabolized the drugs by GSH conjugation to a remarkably lower extent; in fact, both intracellular and extracellular levels of conjugates were far lower than that of the corresponding human samples.

In addition, GSH levels were close to 0 in all human red blood cells samples after treatments, whereas in rat erythrocytes most of the GSH was preserved (Table I). Rat erythrocytes usually quickly metabolized all compounds (Table II) unlike human red blood cells (high levels of free drug persisted for minutes to hours). Our data suggest that rat Hb can play a role in the metabolism of such substances by its fast reacting Cys-β125 (Table III and Fig. 5) with a possible influence on the biochemical, pharmacological, and/or toxicological activity of many drugs.

An example confirming this hypothesis can be obtained by data on BCNU (Fig. 6). BCNU is a cytostatic drug that belongs to the group of DNA alkylating and cross-linking agents (39). Additional mechanisms of BCNU action have been proposed (27), notably the inhibition of glutathione reductase. This further mechanism of action is considered to contribute both to the desired effects and the toxicity of the drug (27). Erythrocyte GR activity has been shown to be reduced to 10% of the initial value in patients 10–30 min after BCNU administration (40).

After incubation with BCNU (dose range, 2–200 μM), the residual activity of GR showed little variations in rat red blood cells even when high concentrations of BCNU were used. In contrast, a dramatic decrease was observed in human erythrocytes. The inhibition is due to the binding of the BCNU decomposition product, 2-chloroethylisocianate, to the SH group of the catalytic site of glutathione reductase (27). Rat Hb can intercept this molecule (via Cys-β125), thus protecting GR from inhibition (IC_{50} values were 350 ± 25 and 5.5 ± 2.0 μM for rat and human red blood cells, respectively). Furthermore, the *in vivo* treatment of the rat with BCNU leads to minimal, if any, changes in GR activity. Even if higher doses than that generally used for chemotherapy (which was shown to inhibit GR up to 90% hematic GR) (40) were administered to rats (Fig. 6*B*), no variations in rat hematic GR were found.

Nitric oxide is a signaling molecule that has captured much the attention of researchers. NO has a short half-life *in vivo* and the existence of more stable transport forms of NO has been postulated (41). NO and NO derived species (NO_x) can react with protein and nonprotein thiols forming nitrosothiols. *S*-nitroso derivatives of glutathione, cysteine, hemoglobin, bovine serum albumin, and other protein or nonprotein thiols are potent, fast acting vasodilators as well as strong inhibitors of platelet aggregation (24). Stamler and co-workers (10, 11) reported that Hb can act either as a sink or as a donor of nitric

TABLE III

Levels of Hb SH groups in rat or human red blood cells (37.5% Hct in PBS) after 4 h of treatment with various drugs (1 mM, 37 °C)

The number of replicates was four.

	Hemoglobin SH groups (mM)			
	Human (β93)	Rat (β125)	Rat (α13)	Rat (β93)
No treatment	4332 ± 154	3722 ± 98	4316 ± 121	4451 ± 125
CDNB	4365 ± 95	3065 ± 101[a]	4310 ± 65	4423 ± 69
EA	4299 ± 125	2994 ± 124[a]	4401 ± 34	4401 ± 84
NEM	4321 ± 94	2894 ± 93[a]	4312 ± 94	4382 ± 59
BCNU	4399 ± 84	2994 ± 49[a]	4352 ± 45	4450 ± 61

[a] Statistically significant with respect to control values ($p < 0.001$, Student's t test).

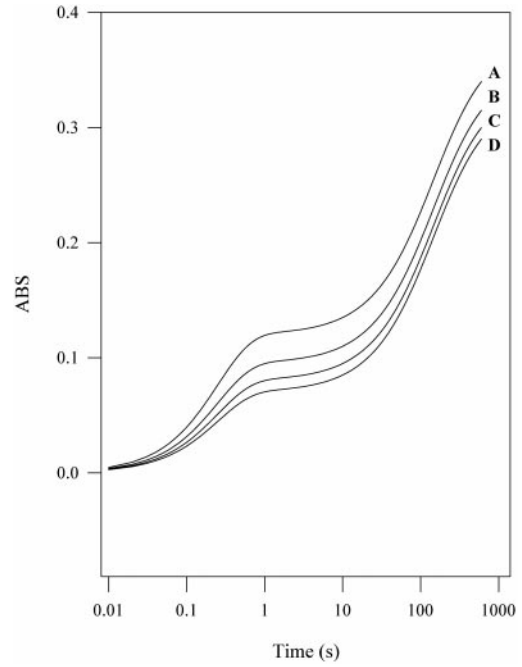

FIG. 5. **Titration of Rat Hb-SH groups after treatment of red blood cells with electrophilic agents.** Rat red blood cells (37.5% Hct in PBS) were treated for 4 h with 1 mM CDNB, EA, or BCNU at 37 °C. Samples were then hemolyzed and passed through G25 columns, and protein content was adjusted to the same final concentration (with phosphate buffer) and titrated with DTNB. *A*, control; *B*, BCNU; *C*, CDNB; *D*, EA.

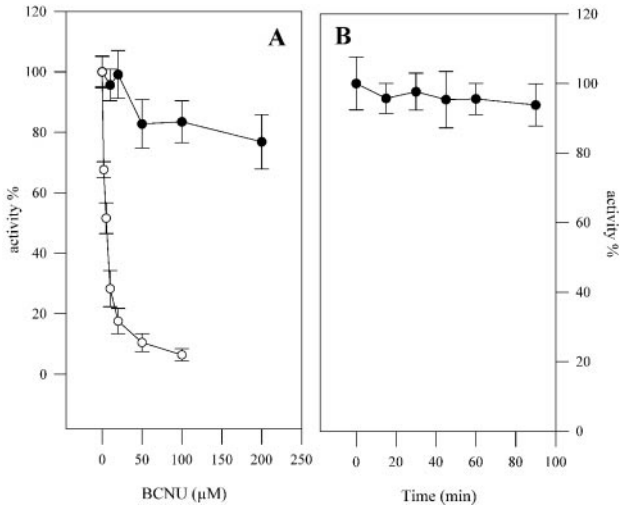

FIG. 6. **Activity of erythrocytic glutathione reductase after treatment with BCNU.** Human (*open symbols*) and rat (*closed symbols*) red blood cells (37.5% Hct) were treated with different concentrations of BCNU (*A*). After 120 min, samples were hemolyzed by the addition of 10 volumes of water and assayed for GR activity. *In vivo* infusion (*B*) of BCNU (200 mg/m^2, 45-min infusion time) through the valve connected with femoral vein. At specified times blood aliquots were withdrawn from the valve connected to the jugular vein, hemolyzed and assayed for GR activity. The number of replicate experiments was three.

oxide depending upon its quaternary state, with a pivotal role in the regulation of blood vessel tone. In these reports they suggest that exchange reactions between low molecular weight nitrosothiols and SH groups of Hb play an important role in mediating nitric oxide effects in the bloodstream. In describing their model, the authors pointed out that Cys-β93 of human Hb is involved in red blood cells nitric oxide handling and that a rapid exchange reaction between *S*-nitrosocysteine and Hb-SH groups takes place. In some experiments however, rat instead of human red blood cells were used. Our data demonstrated that fundamental differences exist among the two systems, with only rat Hb being rapidly nitrosylated. After treatment with *S*-nitrosocysteine (Fig. 3) the intracellular levels of *S*-nitroso hemoglobin rose rapidly in rat red blood cells (0.22 NO mol/mol tetramer of Hb, after 2 min), whereas minimal variation was found in human erythrocytes (0.007 NO mol/mol tetramer of Hb, after 5 min); the use of rat instead of human blood could lead thereby to some misinterpretation of results representing a poor and irrilevant model as to human studies.

In conclusion, data obtained from our work suggest that when the rat is used as an animal model, some attention must be paid to the unusual behavior of its Hb-SH groups. Because of the reactivity of these cysteinyl residues, the rat is an interesting but complicated model in which to study the pharmacological and toxicological action of some drugs. The fact that rat Hb possesses six reactive cysteines/tetramer, makes rat blood *per se* a bad model for some biochemical/physiological interpretations (*i.e.* nitrosothiols action). Furthermore, if we consider that cys β125 is extremely reactive (about 3 orders of magnitude greater than human β93), this makes rat red blood cells able to metabolize some thiol-reacting substances mainly through conjugation/oxidation of its cysteine β125.

Wistar strain male rats were used for all the experiments reported in this work; however, also other strains of rat (*e.g.* Harlan, Sprague-Dawley) behave similarly (not shown). Also rats of different ages and body weights were studied (age, 6–9 months; body weight, 450–550 g), but only slight differences in the metabolism of thiol reactants were found (not shown).

It is well known that some drugs (*i.e.* acetaminophen, ethacrynic acid, and dapsone) (42–44) have different modes of action in rat and man, in terms of dose response curves and toxic limit concentrations; it is thereby possible that as these compounds are able to react with SH groups, such differences can be due (at least in part) to the presence of high reacting thiols on rat Hb. Because the rat is widely used as a model to study the hemotoxicity of xenobiotics, we consider that our results pose an important question and can help to clarify some aspects of metabolism of thiol reactants by rat and human blood.

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