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REACTIONS OF NITROSOCHLORAMPHENICOL IN BLOOD

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Abstract—It has been suggested that nitrosochloramphenicol (NOCAP), a possible metabolite of chloramphenicol (CAP), may be involved in CAP-induced aplastic anemia. We found that NOCAP was rapidly eliminated from human blood *in vitro* (more than 90% in less than 15 sec). Analysis of the different reactions showed that 5% of NOCAP was covalently bound to plasma proteins, mainly to albumin, the remainder being metabolized in red cells. The most important reaction in red cells was the very rapid adduct formation with GSH ($k = 5{,}500\ M^{-1}s^{-1}$), yielding presumably a semimercaptal which either isomerized to a sulfinamide (GSONHCAP, $k = 0.05\ s^{-1}$) or was thiolytically cleaved by another GSH molecule with formation of the hydroxylamine (NHOHCAP) and GSSG ($k = 7.1\ M^{-1}s^{-1}$). Another important elimination reaction was the covalent binding of NOCAP to the SH groups of hemoglobin ($k = 5\ M^{-1}s^{-1}$), also yielding a sulfinamide. Besides these reactions with thiols, NOCAP was enzymatically reduced to NHOHCAP in the presence of NADPH (K_m NADPH = 10^{-5} M; K_m NOCAP = 10^{-4} M; $V_{max} = 2\ \mu$mole/min per ml). This reaction was only effective at NOCAP concentrations below 10^{-4} M, probably because of limited NADPH-regeneration. Further reduction of NHOHCAP to NH_2CAP was a slow process which did not exceed 0.5 nmole/min per ml. NH_2CAP was mainly formed from GSONHCAP, a reaction which depended on NADPH and the presence of hemolysate, indicating an enzymatic reaction. In contrast to smaller nitrosoarenes, NOCAP was a poor ligand for ferrohemoglobin (probably due to steric hindrance by its bulky molecule) and was therefore much more exposed to biotransformation. NOCAP and NHOHCAP formed ferrihemoglobin at a rate 5000 times slower than did phenylhydroxylamine. In contrast to NOCAP, NHOHCAP penetrated slowly the red cell membrane ($t_{\frac{1}{2}}$ about 5 min), and its disposition in blood was quite ineffective. From these data, it seems likely that most of the NOCAP formed by microorganisms in the intestine or produced in the liver, will be degraded in blood before it can reach the bone marrow.

Administration of the broad spectrum antibiotic chloramphenicol (CAP)* may cause aplastic anemia in humans. This uncommon, but often fatal complication has restricted the clinical use of this antibiotic. The underlying biochemical lesion responsible for this effect is still obscure, and adequate animal models are lacking. Since thiamphenicol, which has been extensively used in Mediterranean countries and the Far East, has never been associated with fatal aplastic anemia, Yunis and co-workers have suggested that the *para*-nitro group of CAP may be somehow involved in the development of aplastic anemia (see Ref. 1 for review). It has been shown that the *para*-nitro group of CAP is reduced *in vivo* [2], probably by intestinal bacteria [3], but also by the liver [4, 5]. Recently, CAP has been shown to be reduced also by human liver extracts, giving aminochloramphenicol (NH_2CAP) [6]. Even if the significance of this bioreduction of CAP is uncertain, Yunis *et al.* presented evidence that nitrosochloramphenicol (NOCAP) is considerably more toxic to cultured human bone marrow cells than CAP [7]. NOCAP inhibited mitochondrial respiration [8] and proton translocation [9] even at micromolar concentrations, whereas CAP, at these low concentrations, had no effect. These observations have led to the hypothesis that NOCAP may play an important role in CAP-induced aplastic anemia.

Studying reactions of nitrosoarenes in biological systems [10–14], we have found that NOCAP rapidly reacts with reduced glutathione (GSH) with formation of a sulfinamide and hydroxylamino-chloramphenicol (NHOHCAP) [15]. Preliminary results have shown that reactions of NOCAP with thiols are responsible also for the rapid elimination of NOCAP in the liver [16] and red cells [17]. Since NOCAP, formed in the gut or liver, will at first be in contact with blood before reaching its critical target (the bone marrow), it seemed rational to study more intensively the fate of NOCAP in human blood *in vitro*. Hence, we analyzed the reactions of NOCAP with hemoglobin, red cells and plasma proteins, which have been reported to be covalently labelled by [^{14}C]-CAP [18]. As a result, we found that NOCAP is effectively eliminated from the blood within a few seconds, and it seems rather unlikely that NOCAP itself reaches the critical target.

MATERIALS AND METHODS

Chloramphenicol was obtained from Sigma Chemicals (München, F.R.G.), the radioactive preparation, D-(-)-*threo*-[dichloroacetamido-1-^{14}C]-chloramphenicol from Amersham Buchler GmbH (Braunschweig, F.R.G.). The sp. act. was 14.9 mCi/mmole and the radiochemical purity > 98%.

NOCAP, NHOHCAP, NH_2CAP, and the radioactive derivatives were prepared as described

* For abbreviations and formula, c.f. Fig. 12.

previously [15]. For GSONHCAP-synthesis, NOCAP (0.05 mmole) in 0.1 ml methanol and GSH (0.05 mmole) in 0.1 ml water adjusted to pH 6.6 with tri-potassium phosphate were added to 0.9 ml of 0.2 M sodium phosphate, pH 6.6, with the aid of two motor-driven Hamilton syringes within 15 min. The reaction mixture was kept at 37° for additional 10 min. The pH was adjusted to 7.4 and the mixture extracted three times with 5 ml portions of ethyl acetate for complete removal of lipophilic material. The product (65% yield) was pure as judged by HPLC and released equal amounts of glutathione sulfinic acid, and NH_2CAP upon acid hydrolysis. The solution stored at −20° was stable for two weeks. Glutathionesulfinanilide was prepared as already described [11].

Inulin-[carboxyl-^{14}C], sp. act. 2.4 mCi/g, was obtained from NEN Chemicals (Dreieich, F.R.G.), *p*-chloromercuribenzoate from Serva (Heidelberg, F.R.G.), and *N*-ethylmaleimide and crystallized human serum albumin (globulin-free) from Sigma Chemicals. NADH, NADPH, GSH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and catalase were obtained from Boehringer (Mannheim, F.R.G.), all other reagents were analytical grade chemicals from Merck (Darmstadt, F.R.G.). Silicon oil (Merck Art. No. 7742) was mixed with 4% CCl_4 (v/v) to increase its density.

Purified human hemoglobin and hemolysate were prepared as described elsewhere [11]. Hemoglobin with its SH groups blocked was obtained by addition of the two-fold stoichiometric amount of *N*-ethylmaleimide or *p*-chloromercuribenzoate followed by dialysis. Deoxyhemoglobin was prepared by repetitive evacuation and flushing with nitrogen (>99.99% pure, Linde, Höllriegelskreuth, F.R.G.).

The NADPH-regenerating system consisted of 10 mM glucose-6-phosphate, 0.1 mg glucose-6-phosphate dehydrogenase/ml (14U), and various amounts of NADPH in 0.1 M Tris–HCl, pH 7.4, containing 5 mM $MgCl_2$ and 1 mM EDTA.

Analytical methods. Total hemoglobin and ferrihemoglobin were measured by the method reported by Kiese [19]. Glutathione sulfinic acid, cysteic acid and cystine were determined with an amino acid analyzer as already described [10]. SH groups in proteins were titrated according to Boyer [20].

Unless stated otherwise, NOCAP, NHOHCAP, NH_2CAP and bisazoxy-CAP were determined by HPLC after extraction of the samples with 5 vol of ice-cold ethyl acetate. Addition of saturating NaCl improved the yield >95%. The organic solvent was dried over anhydrous sodium sulfate and kept at −70° until analysis.

HPLC was performed immediately afterwards with a chromatograph ALC/GPC 244 (Waters, Milford, MA) on μ-Bondapak C_{18} (4 mm i.d. × 30 cm; flow rate 1.5 ml/min; simultaneous detection at 254 and 280 nm wavelength). With methanol–water 50:50 (v/v) NOCAP was eluted after 6.75 ml and bisazoxy-CAP after 7.8 ml. With methanol – 20 mM sodium phosphate, pH 7.4, 18:82 (v/v), GSONHCAP was eluted after 3.6 ml, NHOHCAP after 5.4 ml, and NH_2CAP after 6.3 ml.

The concentrations of glutathione sulfinamides were determined as the corresponding amines after hydrolysis of the sample in 1 M hydrochloric acid at room temperature for 10 min. Then the samples were readjusted to pH 7, saturated with NaCl and extracted with 5 vol of ether. The ether extracts were determined spectroscopically (aniline log $E_{238\ nm}$ = 4.03; NH_2CAP log $E_{236\ nm}$ = 4.13). The concentration of NH_2CAP was additionally checked by HPLC.

Separation of plasma proteins was performed on Ultropak® TSK-G 3000 SW (LKB, Sweden), 7.5 mm i.d. × 600 mm, with 50 mM triethanolamine–HCl, pH 7.2, at a flow rate of 1 ml/min.

Red cells were separated from the extracellular fluid by centrifugation through a silicon oil layer. Typically, 0.5 ml of red cell suspension was layered on 0.5 ml silicon oil in 1.5 ml centrifugation tubes (Eppendorff) and centrifuged for 10 sec at 12,000 rpm in an Eppendorff 3200 centrifuge (Netheler und Hinz, Hamburg, F.R.G.).

U.v. spectra were recorded on a Cary 219 spectrophotometer (Varian, Palo Alto, CA), equipped with

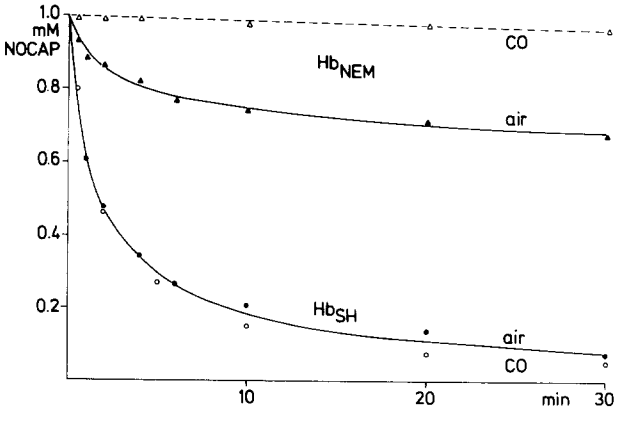

Fig. 1. Binding of NOCAP to human hemoglobin. NOCAP (1 mM) was incubated with purified human hemoglobin (3 mM Fe), either native (HbSH) or with the SH groups blocked by *N*-ethylmaleimide (HbNEM), in 0.1 M Tris–HCl, pH 7.4, at 37°. Reactions were carried our either under air or an atmosphere of carbon monoxide (means of 2 experiments).

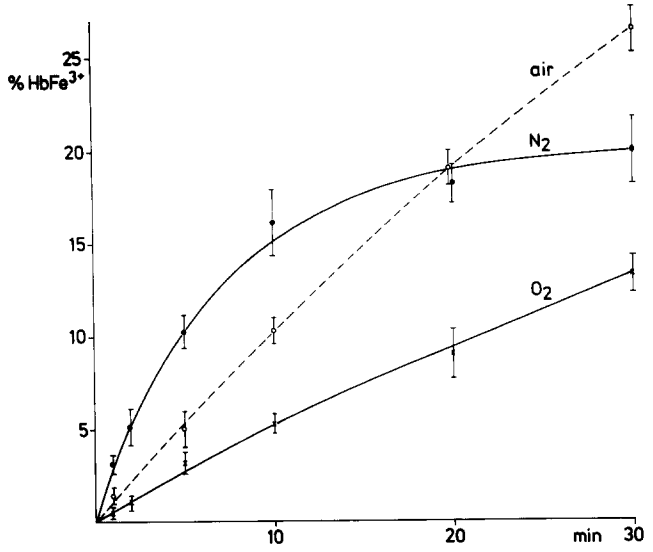

Fig. 2. Formation of ferrihemoglobin by NOCAP. NOCAP (1 mM) reacted with purified human hemoglobin (3 mM Fe, with the SH groups blocked by *p*-chloromercuribenzoate) in 0.1 M Tris–HCl, pH 7.4, at 37° under air, one atmosphere of oxygen or nitrogen (means of 3 experiments ± S.E.M.).

a thermostat. Radioactivity was measured in Bray's solution by a LKB Wallac 1217 RackBeta Liquid Scintillation Counter using external standardization. Results have been corrected for recovery and background radiation.

RESULTS

Reactions of NOCAP with hemoglobin. When NOCAP was incubated with purified human hemoglobin, the yield of NOCAP in ethyl acetate extracts quickly decreased. As shown in Fig. 1, pretreatment of hemoglobin with *N*-ethylmaleimide diminished the binding of NOCAP to hemoglobin; this binding was further reduced in the presence of carbon monoxide. Therefore, two different binding sites for NOCAP were assumed: the reactive SH groups, and the divalent iron in hemoglobin.

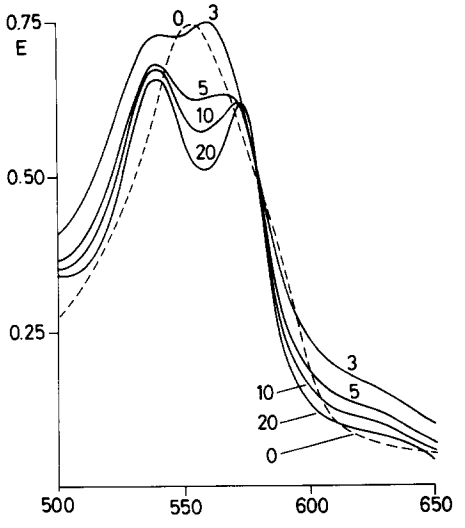

Fig. 3. Ligation of NOCAP with deoxyhemoglobin and the kinetics of displacement by oxygen. Purified human hemoglobin (0.06 mM Fe with the SH groups blocked by *p*-chloromercuribenzoate) in 0.1 M Tris–HCl, pH 7.4 at room temperature reacted with NOCAP (1 mM). After 3 min the concentration of nitrosoarene-hemoglobin was maximal. Then the solution was purged with pure oxygen and the kinetics of oxyhemoglobin formation was followed at the time intervals indicated (min).

In another experiment [^{14}C]NOCAP (1 mM) reacted with carbonyl-hemoglobin (3 mM) at pH 7.4, 37° for 1 hr. Subsequent SH group titration revealed that 0.9 mM of the reactive SH group had been blocked. When this hemoglobin was chromatographed on Sephadex G-75 fine, all radioactivity was found in the hemoglobin containing fractions. By treatment of these pooled fractions with 1 M hydrochloric acid (0°, 24 hr) 80% of the radioactive material was split off from hemoglobin and was identified as NH_2CAP (HPLC, u.v.). Complete hydrolysis of this hemoglobin (6 M HCl, 110°, 24 hr) released 1.2 cysteic acid and 4.8 half-cystine per tetramer. Hence, 0.9 mM of the reactive SH groups from 3 mM hemoglobin had been oxidized by 1 mM NOCAP. These data suggest that NOCAP had formed a sulfinamide with the cysteine residues of hemoglobin.

To study the rate of reaction of NOCAP with the SH groups of hemoglobin, the rate of NOCAP-disappearance was followed directly at 316 nm, the wavelength of maximal absorbance of NOCAP (0.2 cm light path). In the presence of carbonyl-hemoglobin (0.1–0.4 mM), NOCAP (0.05–0.2 mM) disappearance followed second order kinetics, $k_2 = 5\ M^{-1}s^{-1}$, pH 7.4 and 37°. The same constant was calculated from the initial rate of NOCAP disappearance in ethyl acetate extracts, when NOCAP 0.2 mM) had reacted with carbonylhemoglobin (1.2 mM).

Since carbon monoxide prevented the binding of NOCAP to hemoglobin and NOCAP was not bound to ferrihemoglobin provided the SH groups had been

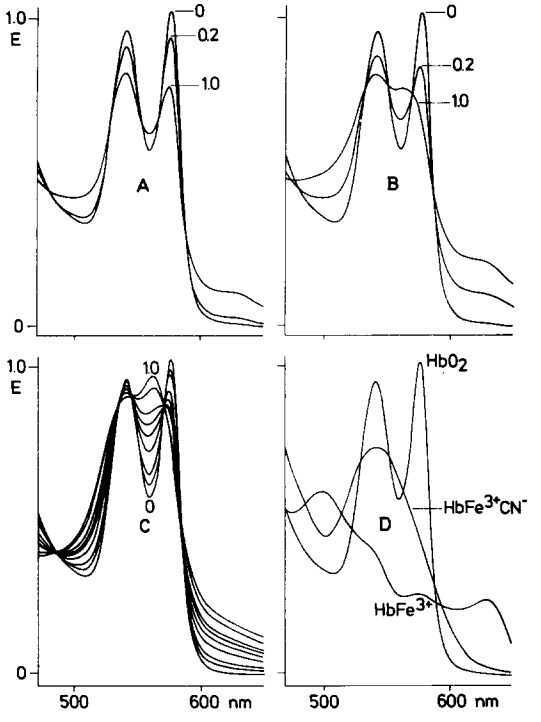

Fig. 4. Electronic spectra of hemoglobin derivatives. Purified human hemoglobin (0.075 mM with the SH groups blocked) in 0.1 M Tris–HCl, pH 7.4 at room temperature under air. (A) After 5 min reaction in the presence of 0.2 and 1.0 mM NOCAP. (B) After 5 min reaction in the presence of 0.5 M $MgCl_2$ and 0.2 and 1.0 mM NOCAP. (C) After 5 min reaction in the presence of nitrosobenzene 5, 10, 20, 40, 60, 80, 200 and 1000 μM, from bottom to top. (D) Oxyhemoglobin, methemoglobin, and cyanomethemoglobin.

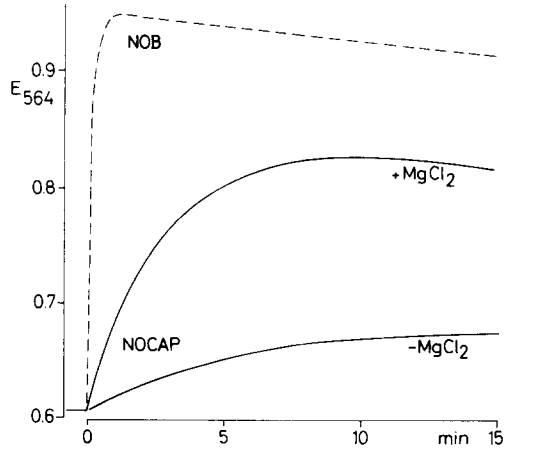

Fig. 5. Rates of nitrosoarene-hemoglobin formation. Purified human hemoglobin (0.07 mM Fe with the SH groups blocked) in 0.1 M Tris–HCl, pH 7.4 containing 10 mM cyanide reacted with NOCAP (1 mM) in the absence or presence of 0.5 M $MgCl_2$. For comparison, ligation with nitrosobenzene (1 mM) in the absence of $MgCl_2$ is also shown. Nitrosoarene-hemoglobin formation was followed at 564 nm, the isosbestic point for oxyhemoglobin and cyanomethemoglobin.

blocked, we assumed that NOCAP is a ligand for the heme iron like other nitrosoarenes [21]. However, NOCAP produced hardly any ligand spectrum immediately after addition to oxyhemoglobin. The small spectral changes which were observed after longer reaction times rather pointed to ferrihemoglobin formation. As shown in Fig. 2, NOCAP produced significant amounts of ferrihemoglobin with the highest initial rate in the absence of oxygen. When deoxyhemoglobin was mixed with NOCAP, however, spectral changes were observed as typically seen with other nitrosoarenes, i.e. an increase in absorbance at about 600 and 560 nm. The maximal absorbance at 560 nm was reached only after some minutes, thereafter ferrihemoglobin formation led to a decrease in absorbance at that wavelength. Interestingly, oxygen and even carbon monoxide displaced NOCAP slowly from the divalent iron. Such an experiment is shown in Fig. 3. NOCAP (1 mM) was mixed with deoxyhemoglobin and the spectrum recorded after 3 min reaction. Then oxygen was bubbled for 30 sec and spectra were recorded at the various time intervals indicated. The resulting spectrum after 20 min reaction pointed to a mixture of oxyhemoglobin, ferrihemoglobin, and nitrosoarene-hemoglobin. At this experimental stage we concluded, that steric hindrance of the bulky NOCAP was responsible for the slow association and dissociation rates. When we reexamined the reaction of oxyhemoglobin with higher concentrations of NOCAP, we observed nitrosoarene-hemoglobin formation after some min. As shown in Fig. 4 (panel A), the increase in absorbance at 560 nm after 5 min reaction points to a small fraction of nitrosoarene-hemoglobin besides the obvious changes due to ferrihemoglobin formation (e.g. the shoulder at 630 nm). The presence of $MgCl_2$ which favours dimerization of human oxyhemoglobin [22] markedly enhanced nitrosoarene-hemoglobin formation (panel B). Nevertheless, the affinity of NOCAP to oxyhemoglobin was considerably weaker than that of nitrosobenzene, with which half-saturation was obtained at 0.02 mM free nitrosobenzene (c.f. panel C). To examine the kinetics of ligation of NOCAP without interference of the simultaneously formed ferrihemoglobin, we followed the changes in absorbance at 564 nm in the presence of cyanide. At this wavelength, the extinctions of oxyhemoglobin and cyanomethemoglobin are isosbestic (c.f. Fig. 4, panel D). As shown in Fig. 5, ligation of NOCAP to oxyhemoglobin was very sluggish as compared to the reaction of nitrosobenzene. In the presence of $MgCl_2$, the rate and yield of nitrosoarene-hemoglobin formation was considerably increased. When oxyhemoglobin (3 mM), pretreated with *N*-ethylmaleimide, reacted with NOCAP (1 mM) at 37°, the increase in absorbance at 564 nm (measured in cuvettes of 0.1 cm light path) corresponded to roughly 0.25 mM nitrosoarene-hemoglobin formed within 10 min. This value agrees with the bound proportion of NOCAP in the experiment depicted in Fig. 1.

Reactions of NHOHCAP with hemoglobin. In contrast to the analogous phenylhydroxylamine, NHOHCAP slowly produced ferrihemoglobin (Fig. 6). Since the initial rates of ferrihemoglobin form-

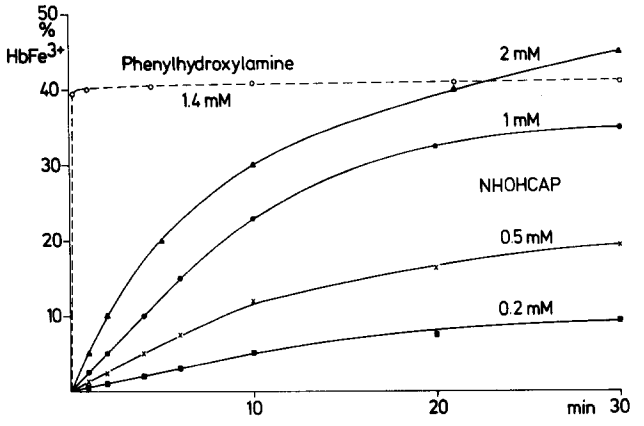

Fig. 6. Ferrihemoglobin formation by NHOHCAP and phenylhydroxylamine. Purified human hemoglobin (3 mM Fe) reacted with various amounts of hydroxylamines in 0.1 M Tris–HCl, pH 7.4, at 37° under air (means of 2 experiments).

ation were directly proportional to the NHOHCAP concentrations, a second order reaction was assumed. Catalase (13.000 U/ml) diminished the rate of ferrihemoglobin formation by about 10%, which excludes any significant role of hydrogen peroxide in ferrihemoglobin formation by NHOHCAP. During ferrihemoglobin formation NHOHCAP is oxidized. As seen from Fig. 7(A), only part of NHOHCAP was recovered as NOCAP since the latter was bound to the SH groups of hemoglobin and liganded to the heme iron (see above). Hence, with native hemoglobin maximal NOCAP concentration was only one fourth the initial NHOHCAP concentration. AzoxyCAP formation in hemoglobin solution was less than 3%. The kinetics of autooxidation of NOCAP is shown in Fig. 7(B). This reaction rate was only one third the rate in the presence of oxyhemoglobin. The reaction products observed were NOCAP and AzoxyCAP. The formation rate of the latter was highest at the 10th min, when the product of the NOCAP and NHOHCAP concentration was maximal.

Metabolism of NOCAP in red cells. Washed human red cells (10 g Hb/100 ml) were incubated with [^{14}C]NOCAP (1 mM) in Ringer–phosphate solution containing glucose (10 mM) at pH 7.4 and 37° under air. Samples were extracted with ethyl acetate after 0.5 and 3 hr and assayed for NOCAP, NHOHCAP, NH_2CAP and AzoxyCAP. The extracted incubates (4 ml) were hemolyzed by ultrasonic treatment and chromatographed on Sephadex LH-20 (2.5 cm i.d. × 90 cm) with ammonium acetate (10 mM, pH 7.0). The hemoglobin containing fractions were treated with 1 M hydrochloric acid at 0° overnight and assayed for liberated NH_2CAP. The second radioactive peak eluted from

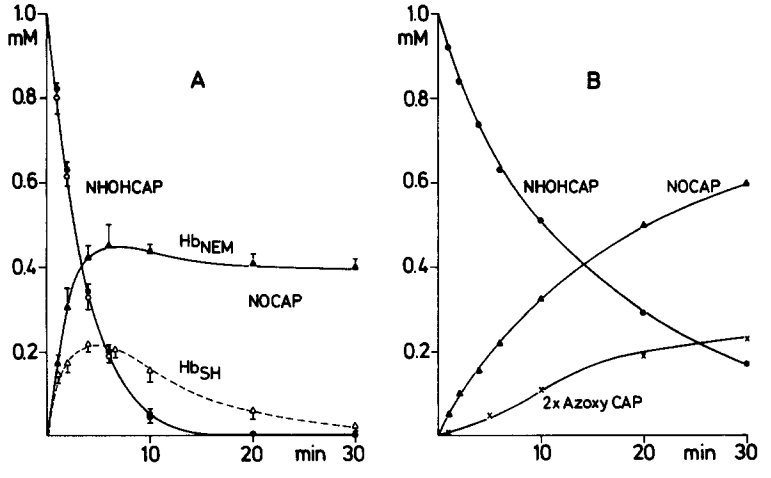

Fig. 7. Oxidation of NHOHCAP in the presence of oxyhemoglobin (panel A) or in 0.1 M Tris–HCl, pH 7.4, only (panel B). NHOHCAP (1 mM) reacted with purified human hemoglobin (3 mM Fe) either native (HbSH) or with the SH groups blocked by *N*-ethylmaleimide (HbNEM) at 37° under air. Only part of NHOHCAP was recovered as NOCAP in the presence of hemoglobin where azoxyCAP formation was minimal (means of 3 experiments ± S.E.M.).

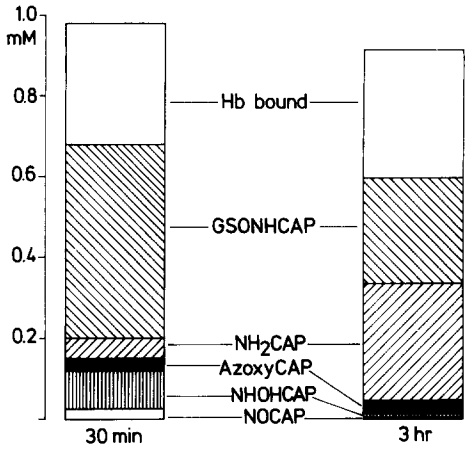

Fig. 8. Pattern of metabolites formed in the reaction of NOCAP with red cells. NOCAP (1 mM) reacted with washed human red cells (10 g Hb/100 ml) in Ringer–phosphate solution containing 10 mM glucose, pH 7.4, at 37° under air for 30 and 180 min, respectively (means of 2 experiments).

Table 1. Liberation of amine from glutathionesulfinamides in the presence of hemolysate

Incubation mixture	Amine liberated (nmole/ml per min)
1 mM GSONHCAP	
Buffer	0.05
Buffer + 1.0 mM NADH	0.1
Buffer + 1.0 mM NADPH	0.1
Hemolysate	0.8
Hemolysate + 0.001 mM NADPH	1.7
Hemolysate + 0.01 mM NADPH	2.6
Hemolysate + 0.1 mM NADPH	2.8
Hemolysate + 1.0 mM NADPH	2.2
1 mM Glutathionesulfinanilide	
Hemolysate + 1.0 mM NADPH	36.4

The glutathionesulfinamides (1 mM) were incubated with freshly prepared, dialyzed hemolysate of washed human red cells (10 g Hb/100 ml) and incubated at pH 7.4 and 37° under air in an NADPH-regenerating system (see Methods).

the column (low-molecular fraction) was further chromatographed on DE_{52}-cellulose (2 cm i.d. × 10 cm) equilibrated with the ammonium acetate buffer. Using a linear salt gradient (10–250 mM ammonium acetate, pH 7.0, 200 ml each), 92% of the applied radioactivity was eluted in a single peak.

After hydrolysis in 1 M hydrochloric acid at 0° for 30 min, equal amounts of NH_2CAP (HPLC, u.v.) and glutathione sulfinic acid were liberated. The pattern of metabolites are shown in Fig. 8. Obviously, the glutathionesulfinamide was decomposed in the red cells during the 3 hr incubation period giving free NH_2CAP. Since the glutathionesulfinamide penetrated the red cell membrane very slowly (about 5% per hr) and was found to be stable in buffer solutions at pH 7.4, it seemed of interest to study the fate of the glutathionesulfinamide in human hemolysate. As shown in Table 1, NH_2CAP liberation from the glutathionesulfinamide was increased in the presence of hemolysate and NADPH. This catalytic activity, however, was considerably weaker with the sulfinamide from NOCAP than from nitrosobenzene. Nevertheless, the observed rate of NH_2CAP liberation from the sulfinamide (2–3 nmole/min per ml of hemolysate,

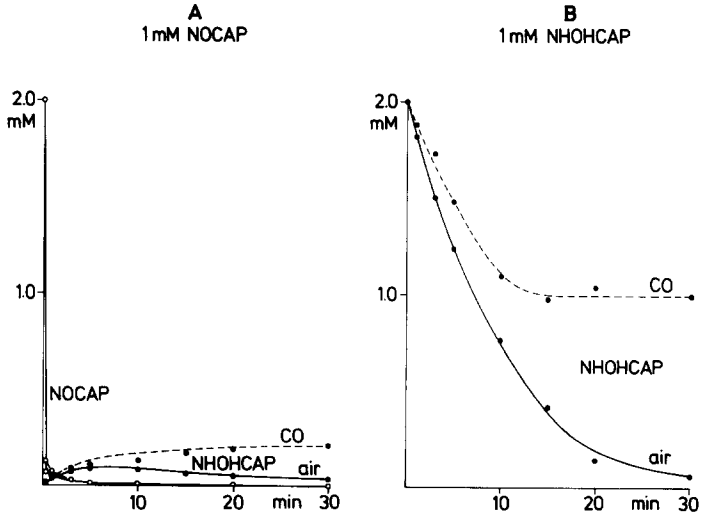

Fig. 9. Distribution of NOCAP and NHOHCAP between red cells and extracellular fluid. NOCAP and NHOHCAP, respectively, (1 μmole/ml, each) were mixed with red cells (15 g Hb/100 ml) in Ringer–phosphate solution containing 10 mM glucose, pH 7.4, at 37°. Panel A shows the rapid trapping of NOCAP by red cells and the slow release of NHOHCAP. Panel B shows the slow equilibrium formation of NHOHCAP between the two compartments. Under air, NHOHCAP was partly autoxidized and the NOCAP formed was trapped by red cells.

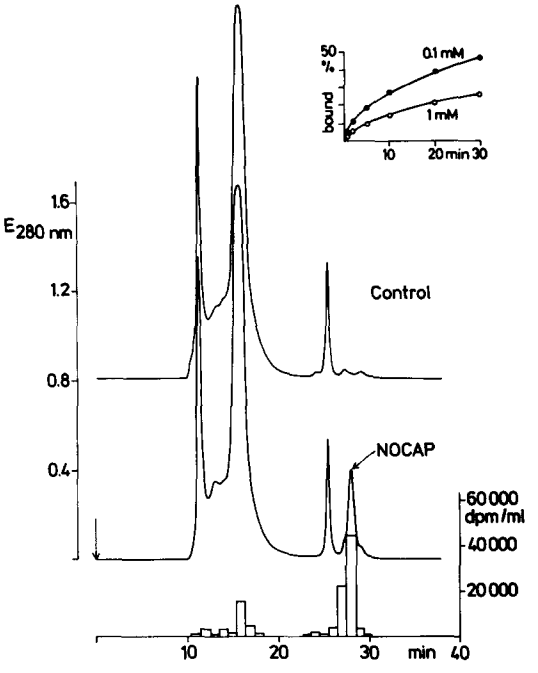

Fig. 10. Covalent binding of NOCAP to plasma proteins. NOCAP [^{14}C] (1 mM) was incubated with human plasma at pH 7.4, 37° for 30 min. The HPLC chromatograms show control plasma on top, the plasma incubated with NOCAP in the middle and the radioactivity in 1 ml-fractions on the bottom. The insert shows the kinetics of covalent binding of [^{14}C]NOCAP to plasma proteins.

containing 10 g Hb/100 ml) was sufficient to explain the decomposition rate of the glutathionesulfinamide in red cells (220 nmole/150 min/ml) as shown in Fig. 8. In addition, NH_2CAP was formed from NHOHCAP in the red cells. NHOHCAP (1 mM) was reduced in dialyzed hemolysate (10 g Hb/100 ml), in the presence of NADPH (0.1 mM) and a regenerating system under carbon monoxide, at a rate of 0.5 nmole/min/ml. For comparison, phenylhydroxylamine was reduced at a rate of 15 nmole/min/ml of hemolysate.

To study the enzymatic reduction of NOCAP into NHOHCAP, NOCAP and NADPH at various concentrations were incubated in the presence of dialyzed hemolysate and a NADPH-regenerating system under carbon monoxide. NHOHCAP was determined in ethyl acetate extracts by HPLC. After correction of the initial rates of NHOHCAP formation for the non-enzymatic reduction [16], Lineweaver–Burk plots were constructed giving the following kinetic constants (pH 7.4, 37°): K_m NADPH = 10^{-5}M (at 0.2 mM NOCAP), K_m NOCAP = 10^{-4} (at 10 μM NADPH), V_{max} = 2 μmol/min/ml hemolysate (15 g Hb/100 ml).

Penetration of NOCAP and NHOHCAP into red cells. To study the permeability of the red cell membrane for the chloramphenicol derivatives, red cells were mixed with NOCAP or NHOHCAP and rapidly separated from the extracellular fluid by centrifugation through a silicon oil layer. As shown in Fig. 9, NOCAP penetrated the red cell membrane about two orders of magnitude faster than NHOHCAP. Since the extracellular volume represented 50% of the total ([^{14}C] inuline distribution), an initial concentration of 2 mM in the extracellular space was calculated. Experiments performed under carbon monoxide to avoid autoxidation of NHOHCAP revealed that diffusion equilibrium was obtained only after 10 min. Under air, NHOHCAP steadily decreased in the extracellular fluid due to formation of the more diffusible NOCAP. NOCAP reduction within red cells with formation of NHOHCAP was small even in the presence of carbon monoxide. Under air, the NHOHCAP liberated at early times, progressively decreased at later times.

Reactions of NOCAP with plasma proteins. When [^{14}C]NOCAP (1 mM) was mixed with human plasma from heparinized blood at 37°, covalent binding occurred to various plasma proteins as shown in the chromatogram in Fig. 10. After 2 min reaction covalently bound material was found only in the albumin fraction (R_t = 16 min), after 30 min reaction radioactivity was also detected in higher molecular weight proteins as shown in Fig. 10 (R_t = 12 and 14 min). As shown in the insert of Fig. 10, the proportion of bound NOCAP was higher with 0.1 mM than with 1 mM NOCAP which points to saturation of the binding sites at mM NOCAP concentrations. When NOCAP (0.1 to 0.01 mM) reacted with human serum albumin (40 mg/ml) containing 0.145 mM reactive SH groups as determined by titration, NOCAP decreased biphasically when followed by direct photometry at 316 nm wavelength. From the initial reaction rates a second order rate constant of 70 $M^{-1}s^{-1}$ was calculated (pH 7.4, 37°). The proportion of the rapid decrease vs total decrease in NOCAP absorption was about 0.3 as observed also with GSH [15]. Since at that early reaction stage more than 92% of NOCAP was extractable with ethyl acetate, reversible formation of an adduct, presumably a semimercaptal, was suggested which isomerizes then to a sulfinamide.

Reactions of NOCAP with whole blood. When [^{14}C] NOCAP was mixed with freshly drawn heparinized human blood, the concentration of NOCAP in the plasma quickly decreased. Separation of cells and plasma was achieved by the silicon oil method; the extracellular volume was 50% of the total, the hemoglobin content 16g Hb/100 ml. NOCAP and its metabolites in the plasma were extracted twice with 10 vol of ethyl acetate, which lead to recoveries of the metabolites >95%. As shown in Fig. 11, 95% of NOCAP was eliminated from the plasma within 2 min, and about 5% was bound irreversibly to plasma proteins. NHOHCAP which was formed within red cells appeared only temporarily in the plasma, and then decreased as NH_2CAP appeared.

When NHOHCAP (1 mM) was mixed with whole blood, half the calculated initial concentration was found in the plasma after 5 min showing similar kinetics as observed with red cells suspended in buffer (c.f. Fig. 9). The maximal NOCAP concentration in the plasma was 0.06 mM after 2 min declining to 0.02 mM after 30 min.

DISCUSSION

Our experiments show that NOCAP is rapidly eliminated from the blood, mainly due to its fast

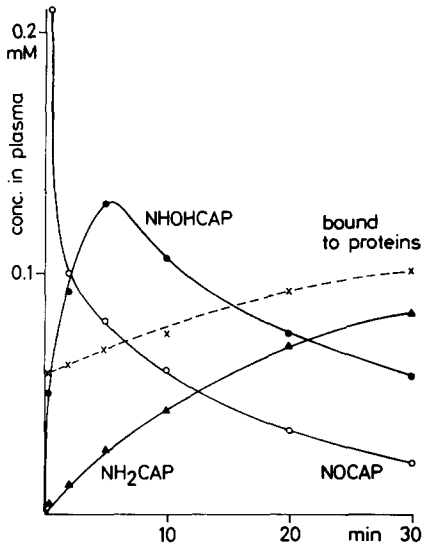

Fig. 11. Elimination of NOCAP from the extracellular fluid in whole blood. NOCAP (1 μmole) was mixed with 1 ml of heparinized human blood (pH 7.4, 37° under air). NOCAP and metabolites were determined in the plasma after separation from the cells by centrifugation through a silicon oil layer. The calculated initial concentration of NOCAP in the plasma was 2 mM.

reaction with GSH [15] in erythrocytes. This fast reaction essentially protects plasma proteins from covalent binding by NOCAP. Nevertheless, at 1 mM NOCAP, about 5% was irreversibly bound to plasma proteins, which contain 0.4 mM SH groups [23]. The main target for irreversible binding appears to be albumin which rapidly forms an adduct with NOCAP. This reaction was about 15-times faster than with the SH groups in hemoglobin. In addition, high molecular weight proteins, presumably immunoglobulins [24] were labelled by NOCAP. This reaction occurred slowly and to a small extent only.

Within red cells, the most important reaction is the very rapid adduct formation with reduced glutathione, followed by binding of NOCAP to the SH groups of hemoglobin, and enzymatic reduction by NADPH. In contrast to nitrosobenzene and a variety of other nitrosoarenes, NOCAP reacts very slowly with the divalent iron of hemoglobin; indeed, it was rather difficult to prove whether such a ligation occurred. Whereas nitrosobenzene produced nitrosoarene-hemoglobin with half-saturation at 4 μM in the absence of air [21] and at about 20 μM in its presence (this paper, [26, 27]), NOCAP (1 mM) produced only about 20% nitrosoarene-hemoglobin under air and this reaction was very sluggish (Fig. 5). Similarly, the dissociation of the nitrosoarene-hemoglobin-complex was tardy as shown in the experiment depicted in Fig. 3. We assume that steric hindrance of the bulky NOCAP molecule to enter the heme crevice may be the reason for the low affinity. Similar observations have been previously reported for nitrosobenzene substituted at the *ortho* position with bulky residues like *tert*-butyl [21]. Steric hindrance may be also the cause for the slow ferrihemoglobin formation by NHOHCAP. Indeed, the second order rate constant of this reaction ($0.4\ M^{-1}s^{-1}$) was only 1/5000 of that observed with phenylhydroxylamine [28].

This low affinity of NOCAP for the hemoglobin iron may have important consequences for the further fate of NOCAP in red cells and may explain its unusually rapid breakdown ($t_{\frac{1}{2}} = 0.5$ min). In contrast, nitrosobenzene is almost completely liganded

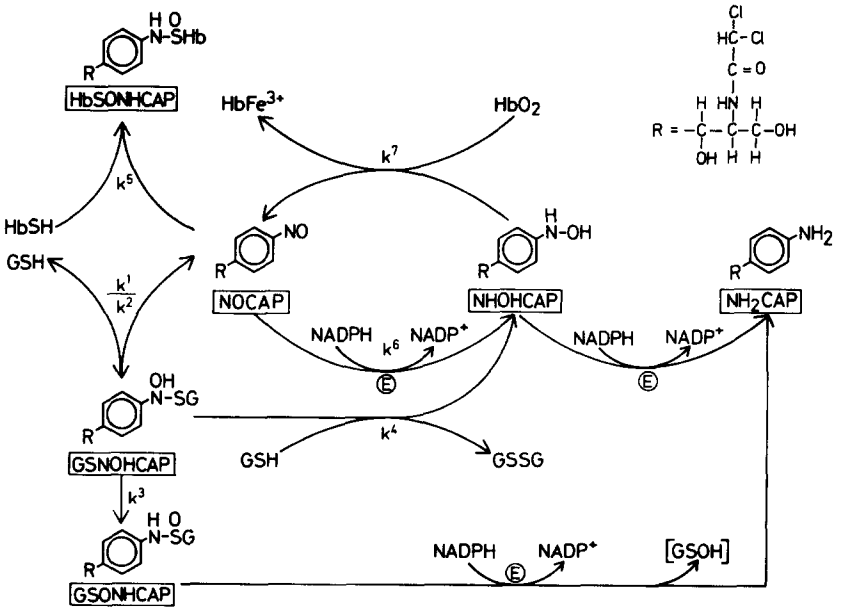

Fig. 12. Scheme of the reactions of NOCAP in red cells. The reaction rate constants (k) are listed in Table 2. Ⓔ indicates enzymatic reactions.

Table 2. Kinetic constants of the various reactions of NOCAP in red cells

(1) NOCAP + GSH ⟶ GSNOHCAP	$k_2^1 = 5{,}500\ M^{-1}s^{-1}$
(2) GSNOHCAP ⟶ NOCAP + GSH	$k_1^2 = 1.22\ s^{-1}$
(3) GSNOHCAP ⟶ GSONHCAP	$k_1^3 = 0.05\ s^{-1}$
(4) GSNOHCAP + GSH ⟶ NHOHCAP + GSSG	$k_2^4 = 7.1\ M^{-1}s^{-1}$
(5) NOCAP + HbSH ⟶ HbSONHCAP	$k_2^5 = 5\ M^{-1}s^{-1}$
(6) *NOCAP + NADPH ⟶ NHOHCAP + NADP	$k_2^6 = 260\ M^{-1}s^{-1}$
(7) NHOHCAP + HbO_2 ⟶ NOCAP + $HbFe^{3+}$	$k_2^7 = 0.4\ M^{-1}S^{-1}$

* In addition NOCAP is reduced enzymatically: K_m NADPH = 10^{-5} M, K_m NOCAP = 10^{-4} M, V_{max} = 2 μmole/min/ml hemolysate (15 g Hb/100 ml).
All constants were determined at pH 7.4 and 37°.

to hemoglobin in red cells (>99%) and decays with an apparent half-life of about 0.5 hr [29, 11]. Analysis of the reaction product of NOCAP with hemoglobin points to sulfinamide formation with the SH groups of hemoglobin, as previously suggested in the case of nitrosobenzene [11]. This sulfinamide formation deserves some comment.

During the reaction of NOCAP with GSH, reversible formation of a semimercaptal was observed ($k_2 = 5500\ M^{-1}s^{-1}$) which isomerizes readily to the sulfinamide ($k_1 = 0.05\ s^{-1}$). These consecutive reactions differ greatly in rate so that the semimercaptal transiently accumulates. From this semimercaptal NOCAP is extractable with organic solvents because the backward reaction is favoured by disturbance of the equilibrium. Hence, NOCAP which has already disappeared as revealed by direct spectroscopy of the solution is still extractable with organic solvents [15]. In the reaction of hemoglobin with NOCAP, however, no difference in the rates of disappearance obtained by the two methods was observed. In this case, the rate of the *intermolecular* adduct formation ($k_2 = 5\ M^{-1}s^{-1}$) is obviously too slow (as compared with the *intramolecular* isomerization) to allow any significant accumulation of the transient semimercaptal. The very slow rate of adduct formation with the SH groups of hemoglobin may be also explained by steric hindrance.

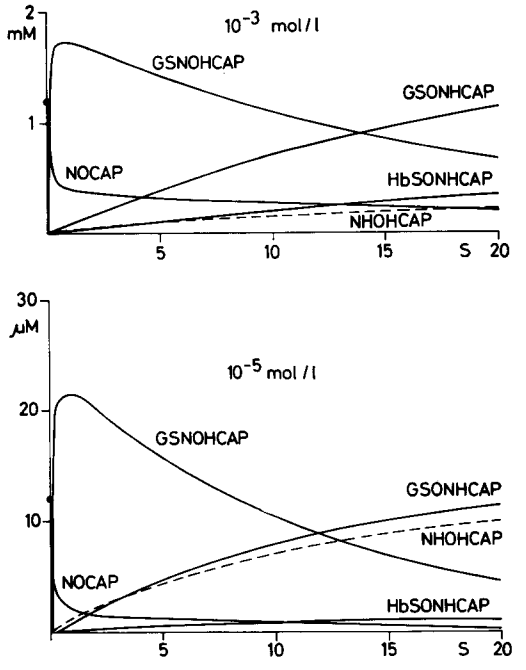

Fig. 13. Computer-generated reaction curves of NOCAP in red cell suspension using the kinetic constants listed in Table 2 and the enzymatic parameters of NOCAP-reduction given in "results". The curves were calculated at 0.02 sec intervals where actual reaction rates approach the initial ones. The following assumptions were made: NADPH-regeneration = 80 nmole/min/ml, red cell suspension (15 g Hb/100 ml) with a hematocrit = 50% and an intracellular reaction volume of 38%; no influence of non-covalent protein binding, immediate diffusion of NOCAP through the red cell membrane, no diffusion of NHOHCAP into plasma and no autoxidation during the first 20 sec. The concentrations given are intracellular ones, where initial concentrations were: GSH = 2.9 mM, HbSH = 12.3 mM, NADPH = 40 μM.

The most important elimination reaction of NOCAP in red cells is formation of the glutathionesulfinamide. This product which is rather stable at neutral pH, was slowly decomposed in red cells with formation of NH_2CAP. The reaction was catalyzed by hemolysate in the presence of NADPH which indicates an enzymatic reaction. Such a reaction has been previously described for glutathionesulfinanilide [11]. Interestingly, the latter substrate was metabolized 20-times faster than the NOCAP-analogue which points to steric hindrance also of the enzymatic reaction. At last, NOCAP is enzymatically reduced to NHOHCAP in red cells. This reaction was only examined with NADPH, which is known to be used by the NADPH-dependent "methemoglobin reductase" which catalyses also nitrosobenzene reduction [30, 31]. Although the capacity of the enzymatic reduction is rather high (2 μmole/min/ml), this reaction seems of significance only at low NOCAP concentrations, since maximal NADP-reduction in red cells does not exceed 80 nmol/min per ml blood [32–35], so that NOCAP-reduction at high concentrations is limited by the poor NADPH-supply. As the oxidation of NHOHCAP to NOCAP catalyzed by hemoglobin is also a rather slow process (initial rate 60 nmole/min per ml of blood at 0.1 mM NHOHCAP) a significant proportion of NHOHCAP was found at the early reaction stage. In contrast, the proportion of phenylhydroxylamine present in red cells has been calculated to be in the order of 10^{-4} that of total nitrosobenzene [11].

Besides NH_2CAP-formation from the sulfinamide,

NH_2CAP was also produced by an enzymatic reduction of NHOHCAP. This reaction was rather slow, however, and formed only 0.8 nmole/min per ml blood which is 30-times slower than the reduction of phenylhydroxylamine. Hence, NH_2CAP-formation by this route is thought to be rather unimportant, which is underlined by the experiment shown in Fig. 8, where NH_2CAP was mainly formed from the sulfinamide.

The scheme in Fig. 12 summarizes the various reactions of NOCAP in erythrocytes, while the kinetic constants of these reactions are listed in Table 2. With these data it was possible to calculate reaction curves for various NOCAP concentrations. Such a computerized simulation is presented in Fig. 13 and shows the importance of the semimercaptal formation of NOCAP with reduced glutathione which allows the very rapid sequestration of NOCAP from the blood and diminishes covalent binding of this reactive intermediate to plasma proteins.

From these data is seems very unlikely that NOCAP, which has been formed by microorganisms in the gut or produced in the liver, can be transported unchanged by the blood to bone marrow to any significant extent. This assumption does not hold for NHOHCAP, because this hydrophilic hydroxylamine does not penetrate the red cell membrane quickly enough to be removed from the blood before entering the bone marrow. In addition, NHOHCAP is formed within red cells in significant amounts at low NOCAP concentrations, where the enzymatic reduction is not limited by the NADPH-supply (c.f. Fig. 13). This NHOHCAP is either autoxidized within red cells or crosses the red cell membrane and reaches the plasma. Hence, the effects of NHOHCAP on bone marrow cells should be studied.

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