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REACTIONS OF THE NITROSO ANALOGUE OF CHLORAMPHENICOL WITH REDUCED GLUTATHIONE

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Abstract—Nitroso-chloramphenicol (NOCAP) was synthetized by reduction of chloramphenicol (CAP) with zinc dust in a modification of the procedure published by Corbett and Chipko. The radioactive derivative was similarly prepared from [dichloroacetamido-1-^{14}C]CAP. NOCAP rapidly reacted with GSH with the formation of hydroxylamino-chloramphenicol (NHOHCAP), D-(−)-*threo*-1-(*p*-hydroxylaminophenyl)-2-dichloroacetamido-1,3-propanediol and glutathione disulfide (GSSG). In addition, a hydrophilic sulfinamide was formed (GSONHCAP), D-(−)-*threo*-1-(*p*-glutathionesulfinamidophenyl)-2-dichloroacetamido-1,3-propanediol. Free amino-chloramphenicol (NH_2CAP), D-(−)-*threo*-1-(*p*-aminophenyl)-2-dichloroacetamido-1,3-propanediol, was not detected. The proportion of NHOHCAP formed increased with increasing GSH concn, at the expense of GSONHCAP. Analysis by stopped-flow spectroscopy revealed formation of a labile adduct in the reaction of NOCAP with GSH (k = 5500 M^{-1} sec^{-1} at 37°, pH 7.4). This reaction was reversible because nearly all NOCAP could be extracted with ether from the labile intermediate. The equilibrium adduct/NOCAP was dependent on GSH concn (K = 4500 M^{-1} at 37°, pH 7.4). The labile intermediate either isomerized to the sulfinamide, GSONHCAP (favoured by decreasing pH at constant GSH), or it was thiolytically cleaved by another GSH molecule to NHOHCAP and GSSG (favoured by increasing GSH at constant pH). At acid pH, GSONHCAP readily hydrolyzed to NH_2CAP and glutathionesulfinic acid. Thus, NOCAP reacts with thiols similar to nitrosobenzene. A scheme is presented for the proposed reaction mechanism. It is concluded that most of the NOCAP, if formed in the intestine or liver, will be rapidly disposed by reactions with GSH. Hence, toxic concns at the sensitive target, i.e. the bone marrow, may usually be prevented.

The use of the antibiotic chloramphenicol has been restricted since its association with bone marrow depression. Two types of bone marrow toxicity have been reported. The more commonly observed one is dose-dependent and usually reversible if chloramphenicol treatment is discontinued. The second type of chloramphenicol-induced bone marrow disease is the rare, but often fatal, aplastic anemia (see Ref. 1 for review). Several groups of investigators have attempted to elucidate the mechanism of this toxicity. There is ample evidence indicating that reversible bone marrow depression by chloramphenicol results from inhibition of mitochondrial protein synthesis [2–8], DNA synthesis [8, 9] or DNA degradation [10]. Moreover, other findings suggest that chloramphenicol may interact with macromolecules of bone marrow cells which results in altered antigenicity and causes thereby an immunological disorder [11–14].

The rare incidence of chloramphenicol-induced aplastic anemia and the lack of adequate experimental animal model systems hitherto prevented the elucidation of the underlying biochemical lesion. It seemed reasonable, however, to suggest that a reactive metabolite of chloramphenicol, which binds to cellular macromolecules, might be responsible for the toxicity. Hence, Pohl and co-workers [15–17] studied the metabolic activation of chloramphenicol and observed covalent binding of chloramphenicol to liver proteins. The reactive metabolite was identified as the oxamyl chloride of chloramphenicol, produced in a cytochrome P-450 dependent reaction. This reactive intermediate seems to be a suicidal product for cytochrome P-450 to which it is covalently bound, especially to the ε-amino groups of lysine [18, 19]. Whereas these findings may reasonably explain the impaired metabolic disposition of certain therapeutic agents during chloramphenicol administration [20], they hardly explain why other chloramphenicol analogues with the intact dichloroacetamido moiety failed to produce aplastic anemia. Thiamphenicol, for instance, readily produces the dose-dependent reversible bone marrow depression but has never been associated with an increased incidence of aplastic anemia, although it is extensively used in Mediterranean countries and the Far East.

This observation suggested to Yunis and co-workers [21, 22] that the *p*-nitro group of chloramphenicol may be somehow causative to aplastic anemia. According to their hypothesis [9], the predisposed host provides the milieu in which the *p*-NO_2 group undergoes metabolic reduction giving rise to reactive intermediates and somehow leading to irreversible stem cell damage. When Corbett and Chipko [23] succeeded in the synthesis of the nitroso and hydroxylamine analogues of chloramphenicol, this hypothesis was experimentally tested. As a result, nitroso-chloramphenicol proved to be considerably more toxic to cultured human bone marrow cells than chloramphenicol. Nitroso-chloramphenicol inhibited irreversibly DNA synthesis and the growth of pluripotential hematopoietic stem cells, whereas the inhibition by chloramphenicol was largely

reversible [9]. However, biochemical formation of nitroso-chloramphenicol has never been reported hitherto. There is only indirect evidence for nitroreduction of chloramphenicol because of the occurrence of the *p*-aminophenyl derivative. Though not exactly characterized, diazotizable material which gave a positive Bratton–Marshal reaction has been reported after administration of chloramphenicol to rats [24]. Though most of this amine has been probably formed by intestinal bacteria [25], chloramphenicol has been shown to be reduced to the arylamine also by different mammalian liver fractions [26] including human liver tissue [27]. The mammalian nitroreductases, however, are inactive in the presence of oxygen and may therefore scarcely contribute to amine formation.

Studying reactions of nitrosoarenes with thiols [28–31], we became interested in this attractive hypothesis that nitroso-chloramphenicol might be the toxic chloramphenicol metabolite responsible for aplastic anemia. Most of the nitrosoarenes tested hitherto rapidly reacted with thiols, especially with the most abundant GSH, and formed hydroxylamines and sulfinamides. These reactions were shown to compete for covalent binding to cellular macromolecules [29, 30], influencing thereby toxicity. Similarly, inhibition of mitochondrial respiration by nitroso-chloramphenicol was prevented by the prior addition of cysteine [32, 10]. Therefore we decided to study whether nitroso-chloramphenicol reacts in a similar way with GSH as other nitrosoarenes. Preliminary results of this study have been presented at the Spring Meeting of the Deutsche Pharmakologische Gesellschaft, Mainz, 1982.

MATERIALS AND METHODS

Chloramphenicol was obtained from Sigma Chemicals (Munich, 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%.

Nitroso-chloramphenicol, D-(−)-*threo*-1-(*p*-nitrosophenyl)-2-dichloroacetamido-1,3-propanediol (NOCAP),* was synthetized by reduction of chloramphenicol with zinc dust and subsequent oxidation with ferric chloride as reported previously by Corbett and Chipko [23]. Following exactly the published procedure gave a greenish product which seemed homogeneous on TLC and exhibited the reported u.v. spectrum. HPLC analysis, however, revealed two different compounds. Figure 1 shows the HPLC chromatogram of such a NOCAP preparation. The u.v. spectra of the two fractionated peaks were virtually identical.

* Abbreviations: NOCAP, D-(−)-*threo*-1-(*p*-nitrosophenyl) - 2 - dichloroacetamido - 1, 3 - propanediol; NHOHCAP, D-(−)-*threo*-1-(*p*-hydroxylaminophenyl)-2-dichloroacetamido-1,3-propanediol; NH_2CAP, D-(−)-*threo*-1-(*p*-aminophenyl)-2-dichloroacetamido-1,3-propanediol; GSONHCAP, D-(−)-*threo*-1-(*p*-glutathionesulfinamidophenyl)-2-dichloroacetamido-1,3-propanediol; GSH, reduced glutathione; GSSG, glutathione disulfide; HPLC, high-performance liquid chromatography.

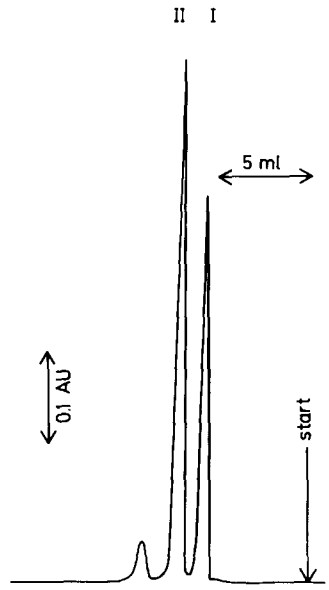

Fig. 1. HPLC analysis of reaction products in the synthesis of NOCAP [μ-Bondapak C_{18}, methanol:water = 50:50 (v/v), flow rate = 2 ml/min].

Large-scale separation of both compounds was performed by column chromatography on Sephadex LH 20 (4 cm i.d. × 200 cm) with water as eluant (Fig. 2). The two separated cuts were lyophilized and seemed to be a homogeneous as checked by HPLC.

For identification, both compounds were oxidized with potassium peroxodisulfate in sulfuric acid. Forty milligrams each of the lyophilized powder of compounds I and II were dissolved in 6 ml of water and mixed with 25 ml of ice-cold peroxodisulfate solution. (This solution was prepared by stirring 6.25 g of solid potassium peroxodisulfate with 6.25 ml of conc. sulfuric acid for 1 hr at 25°. Then, the solution was poured into 500 ml of ice-cold water, adjusted to pH 8.4 with solid potassium carbonate and readjusted to pH 6.4 with acetic acid.) The green colour of both solutions slowly vanished, and after a 6-hr

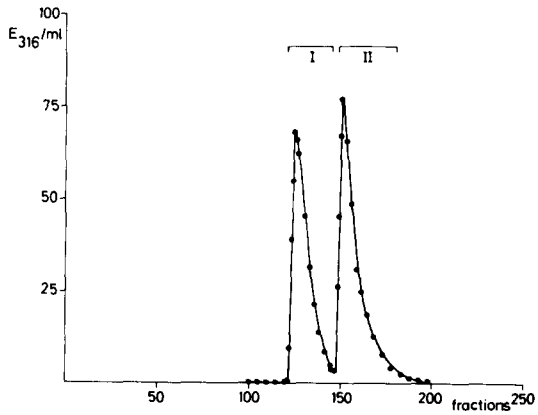

Fig. 2. Chromatographic separation of NOCAP (II) from a dechlorinated product (I) on Sephadex LH 20 (4 cm i.d. × 200 cm) with water as eluant.

reaction nitroso compounds were not detected any longer. Instead, compound II had been oxidized to chloramphenicol (HPLC, u.v., TLC). Oxidized compound I, however, was a more polar product as judged from the chromatographic behaviour (HPLC).

Examination of both compounds by low-resolution mass spectrometry indicated the presence of a molecular ion at *m/z* 273 for compound I and 307 for compound II. The main fragments showed ion peaks at *m/z* 137 and 136 for compound I, and 170 and 137 for compound II. These data pointed to $C_{11}H_{13}ClN_2O_4$ for compound I and $C_{11}H_{12}Cl_2N_2O_4$ for compound II, the latter corresponding to NOCAP.

Proton NMR spectroscopic data as indicated in Table 1 confirmed the identity of compound II with NOCAP and agree well with the data of Corbett and Chipko [23].

Compound I obviously lacks the single dichloroacetamido proton but shows a new signal of two additional protons in a higher field, compatible with a monochloroacetamido structure.

From these data compound I was designated as D-(−)-*threo*-1-(*p*-nitrosophenyl)-2-monochloroacetamido-1,3-propanediol.

Subsequent studies led to an improved synthesis which produced NOCAP at higher yield. To reduce the proportion of the dechlorinated product, the reaction temp. during zinc dust reduction has to be kept below 30° and the total reaction time should not exceed 10 min.

3.23 g of chloramphenicol (10 mmoles) was dissolved in 10 ml of warm ethanol (50°) and mixed with 1 g of ammonium chloride in 30 ml of water which resulted in formation of a white slurry. On vigorous stirring, 2.6 g of zinc dust were added in small portions within 10 min. The temp. was kept constant at 25° in a water bath. After addition of 10 ml of water the suspension was filtered through a Buchner funnel and the filter cake washed with an additional 15 ml of water. The pale yellow filtrate was cooled immediately on ice and mixed with 6.6 g of ferric chloride in 50 ml of ice-cold water. The solution developed a spinach-green colour immediately and, after a 1-min reaction, was extracted 3 times with 50-ml portions of ethyl acetate. After drying over anhydrous sodium sulfate the solvent was evaporated at reduced pressure and the residue dissolved in 10 ml of methanol.

This procedure yielded about 8 mmoles of nitroso compounds (u.v.) with NOCAP > 90%.

For purification, the methanol solution was mixed with 20 ml of water, applied to a column of Sephadex LH 20 (5 cm i.d. × 80 cm) and eluted with water. The pure NOCAP-containing fractions (HPLC) were pooled and lyophilized. The resulting powder was chromatographically pure (HPLC) and yielded 6.5 mmoles of NOCAP. Crystallization studies were unsuccessful and resulted in amorphous precipitates. The u.v. spectra in ethyl ether, ethyl acetate, ethanol and water showed maximal extinction at 316 nm (log $E = 4.11$). For synthesis of the radioactive compound, 48 μmoles chloramphenicol was mixed with about 2 μmoles of [dichloroacetamido-1-^{14}C]chloramphenicol and processed on a correspondingly smaller scale. The final product (27 μmoles) was radiochemically pure (HPLC) and had a sp. act. of 0.54 μCi/μmole.

Hydroxylamino-chloramphenicol (NHOHCAP) was always freshly prepared from NOCAP, because the primary product after zinc dust reduction of chloramphenicol was always contaminated with the dechlorinated compound, and chromatographic purification procedures would result in autoxidation products. NOCAP was reduced by GSH which results in a very rapid adduct formation from which NHOHCAP is easily liberated (see Results). By this procedure formation of a yellow bisazoxy derivative was minimized.

In a typically small-scale preparation 0.1 mmoles of NOCAP in 100 μl of methanol was rapidly mixed with 0.25 mmoles of GSH in 2.5 ml of water adjusted to pH 9 with Tris–potassium phosphate at room temp. After a 5-min reaction, the mixture was 3 times extracted with 8-ml portions of ethyl acetate. The organic solvent was dried over anhydrous sodium sulfate and evaporated under reduced pressure. The resulting pale yellow gum was dissolved in methanol and kept at −50° (yield 0.08 mmoles of NHOHCAP). The u.v. spectrum in methanol showed maximal absorbance at 236 nm (log $E = 4.06$). Aqueous solutions gave positive reaction with pentacyanoamine ferrate and autoxidized quickly under air (3%/min at pH 7.4, 25°). Addition of ferric chloride resulted in rapid formation of NOCAP.

Amino-chloramphenicol (NH_2CAP) was synthetized from NOCAP because the published procedure of chloramphenicol reduction by titanous chloride [33] gave mainly the dechlorinated amino-chloramphenicol. Since NOCAP reduction by titanous chloride resulted also in considerable dechlorination, NH_2CAP was synthetized via glutathione-sulfinam-

Table 1. Proton NMR of chloramphenicol, nitroso-chloramphenicol and compound I in deuterated methanol (ppm, TMS standard)

Chloramphenicol	Compound II = nitroso-chloramphenicol	Compound I
8.18 (d, 2H, aromatic)	7.77 (m, 4H, aromatic)	7.77 (m, 4H, aromatic)
7.64 (d, 2H, aromatic)		
6.23 (s, 1H, dichloroacetamido)	6.23 (s, 1H, dichloroacetamido)	
5.18 (d, 1H, C_1)	5.14 (d, 1H, C_1)	5.12 (m, 1H, C_1)
4.18 (m, 1H, C_2)	4.18 (m, 1H, C_2)	4.18 (m, 1H, C_2)
		3.96 (m, 2H, monochloroacetamido)
3.68 (m, 2H, C_3)	3.65 (m, 2H, C_3)	3.68 (m, 2H, C_3)

idochloramphenicol which is also formed in the reaction of NOCAP with GSH (see Results). To obtain good yields, the pH has to be kept below 7 and the free GSH concn. low. To 1 mmole of NOCAP, dissolved in 100 ml of 0.2 M sodium phosphate, pH 6.6, 2 mmoles of GSH dissolved in the same buffer vol. was added continuously with the aid of a peristaltic pump at room temp. within 10 min. For complete removal of lipophilic products, the mixture was saturated with solid sodium chloride and extracted 3 times with 500-ml portions of ethyl acetate.

On addition of 7 ml of conc. hydrochloric acid to the ice-cold aqueous phase the sulfinamide was completely hydrolyzed within 30 min. After neutralization with solid sodium carbonate, NH_2CAP was extracted 3 times with 500-ml portions of ethyl acetate. After evaporation of the solvent at reduced pressure, the residual gum was dissolved in 10 ml of water and then lyophilized (0.8 mmoles). This product was chromatographically pure (HPLC) and was identical with a specimen kindly provided by Parke & Davis (HPLC, TLC, u.v.).

The u.v. spectrum in water showed maximal absorbance at 236 nm ($\log E = 4.13$). The compound was slowly oxidized by peroxodisulfate with formation of NOCAP and chloramphenicol.

Buffers were prepared as follows: 0.2 M KH_2PO_4 was mixed with 0.2 M Na_2HPO_4 to give the desired pH. NADPH, glutathione reductase, glucose 6-phosphate, glucose-6-phosphate dehydrogenase and glyoxalase I were obtained from Boehringer (Mannheim, F.R.G.) and methylglyoxal from Carl Roth (Karlsruhe, F.R.G.). All other reagents were analytical-grade reagents from Merck (Darmstadt, F.R.G.).

Analytical methods

GSH was determined as *S*-lactyl-glutathione after transformation by glyoxalase I in the presence of methylglyoxal according to Racker [34].

Glutathione disulfide (GSSG) was determined enzymatically by the oxidation of NADPH in the presence of glutathione reductase according to Rall and Lehninger [35].

Glutathionesulfinic acid (GSO_2H) was determined with an amino acid analyzer as already described [28].

TLC. Ethyl acetate extractable reaction products were separated on silica gel plates [silica gel 60 F_{254}, 0.20 mm (Merck)] with chloroform:methanol (90:10 v/v). R_f CAP = 0.64, NOCAP = 0.68, NH_2CAP = 0.44, NHOHCAP = 0.19.

HPLC was performed with a chromatograph ALC/GPC 244 (Waters, Milford, MA) on μ-Bondapak C_{18} (4 mm i.d. × 30 cm; flow rate 2 ml/min; detection at 254 nm). With methanol:water = 50:50 (v/v) NHOHCAP was eluted after 3.4 ml, NH_2CAP after 3.7 ml, chloramphenicol after 6.5 ml and NOCAP after 6.9 ml. With methanol:20 mM sodium phosphate, pH 7.4 = 18:82 (v/v), glutathione-sulfinamidochloramphenicol was eluted after 3.3 ml, NHOHCAP after 5.2 ml and NH_2CAP after 6.2 ml.

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

Rapid changes in extinction of NOCAP in the reactions with GSH were measured in a DW-2 spectrophotometer equipped with an Aminco–Morrow stopped-flow accessory and a DASAR data storage unit (Aminco, Silver Springs, MD).

Radioactivity was measured in Bray's solution with a Packard Tri-Carb 2660 scintillation spectrometer using external standardization. Results have been corrected for recovery of known amounts and background radiation.

RESULTS

Isolation of reaction products

When NOCAP (1 mM) was incubated with GSH (2 mM) in 0.2 M sodium phosphate, pH 7.4, under nitrogen at 25°, all the NOCAP had disappeared within 5 min (HPLC, u.v.). In ethyl acetate extracts only NHOHCAP (0.21 mM) was found, free NH_2CAP was not detected (HPLC, u.v.). Determination of GSH (0.68 mM) and GSSG (0.28 mM) revealed that a major part of glutathione was missing (0.76 mM) corresponding to 0.79 mM of an unidentified reaction product of NOCAP. Hence, formation of a water-soluble adduct was assumed to result from 1 mole of GSH and 1 mole of NOCAP.

Incubation of this water-soluble compound with hydrochloric acid (0.5 M) at room temp. hydrolyzed the adduct completely within 5 min. The cleavage products were 0.79 mM NH_2CAP (HPLC, u.v.) and 0.73 mM glutathionesulfinic acid (amino acid analysis). Hence, formation of a glutathionesulfinamide in the reaction of NOCAP with GSH was assumed.

Kinetics of the reaction of NOCAP with GSH

When [^{14}C]NOCAP reacted with GSH the proportion of the products varied with the concn of GSH. Figure 3 shows the yield of NHOHCAP (HPLC) after reaction of 1 mM NOCAP with GSH at pH 7.4, at 37° under nitrogen which was complete after 5 min. The data fit the slope calculated by the function:

$$\frac{(\text{NHOHCAP formed})}{(\text{NOCAP initially})} = \frac{p\cdot(\text{GSH})}{1 + p\cdot(\text{GSH})}.$$

Residual traces of NOCAP were detected only at 1 mM GSH; at higher GSH concns all the NOCAP

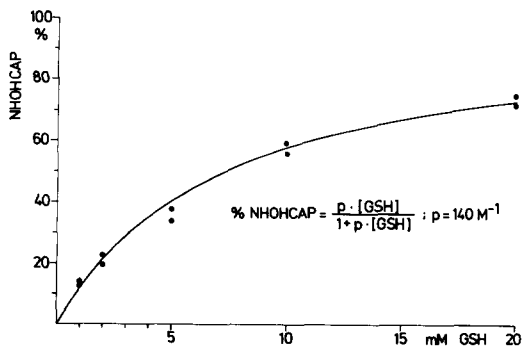

Fig. 3. Formation of NHOHCAP in the reaction of NOCAP (1 mM) with various concns of GSH in 0.2 M sodium phosphate, pH 7.4 (37°, N_2, 5-min reaction).

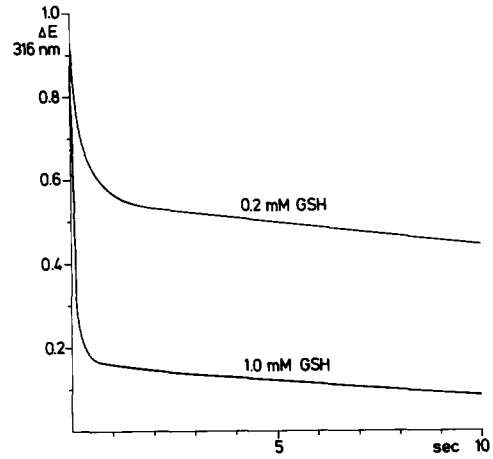

Fig. 4. Decrease in u.v. absorbance of NOCAP (0.08 mM) during the reaction with GSH followed in a stopped-flow spectrophotometer (pH 7.4, 37°).

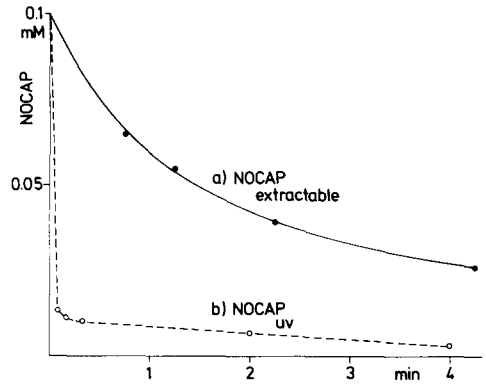

Fig. 5. Comparison of the rates of decrease in NOCAP (0.1 mM) during the reaction with GSH (0.5 mM) at pH 7.4 and 37°. (a) Determined in ether extracts (solid line), or (b) by following the u.v. absorbance of the reaction mixture at a wavelength of 316 nm (broken line).

had disappeared and the glutathionesulfinamide made up the missing radioactive material (HPLC).

To study the reaction rates of NOCAP disappearance in the presence of GSH, the decrease in extinction at 316 nm (the maximum absorbance of NOCAP) was followed in a DW-2 spectrometer equipped with an Aminco–Morrow stopped-flow accessory. As shown in Fig. 4, the kinetics of the decrease in $NOCAP_{UV}$ was biphasic, and the proportion of the rapid reaction depended on the GSH concn. The initial velocity was proportional to the NOCAP and GSH concn indicating a second-order reaction ($k = 5500\ M^{-1}\cdot sec^{-1}$ at pH 7.4, 37°). Variation of the NOCAP and GSH concn, respectively, revealed that the proportion of the rapid decrease in $NOCAP_{UV}$ was proportional to the GSH concn (Table 2). These data pointed to a *reversible* adduct formation of NOCAP with GSH.

To prove this hypothesis, NOCAP disappearance was followed spectroscopically at 316 nm both in ether extracts of incubates and in the reaction mixture directly ($NOCAP_{UV}$). To slow down the reaction rate, NOCAP (0.1 mM) and GSH (0.5 mM) were incubated at 0°. One millilitre of the incubate was extracted with 5 ml of ice-cold ether, and the NOCAP content was determined by u.v. spectroscopy of the extracts. In a parallel experiment the decrease in extinction of the mixture in a cuvette was followed photometrically at a wavelength of 316 nm. As shown in Fig. 5, NOCAP was extracted with ether although it did not exhibit its corresponding absorption at 316 nm in the reaction mixture. These results indicated that formation of the adduct was reversible.

At low GSH concn the adduct was transformed into the sulfinamide. This transformation was observed spectroscopically by rapid repetitive scanning as shown in Fig. 6. After the immediate disappearance of the NOCAP spectrum a transient intermediate appeared which had a maximum in absorbance at around 230 nm.

The maximum difference in absorbance between

Table 2. Reaction rate and equilibrium constants of the reaction of NOCAP with GSH in 0.2 M phosphate, pH 7.4, at 37°

NOCAP (mM)	GSH (mM)	$V_{init.}$ ($\mu M\ sec^{-1}$)	k ($10^3\ M^{-1}\cdot sec^{-1}$)	Q	K ($10^3\ M^{-1}$)
0.01	1.0	58	5.8	0.83	4.59
0.02	1.0	109	5.5	0.82	4.63
0.05	1.0	276	5.5	0.82	4.75
0.10	1.0	533	5.3	0.81	4.64
0.10	0.1	53	5.3	0.25	4.44
0.10	0.2	108	5.4	0.40	4.17
0.10	0.5	290	5.8	0.67	4.69
0.10	2.0	1100	5.5	0.89	4.23
0.10	5.0	n.d.	—	0.95	4.33
0.10	10.0	n.d.	—	0.98	3.94

k, second-order rate constant of the rapid decrease in NOCAP absorption; Q, proportion of the rapid decrease vs total decrease in NOCAP absorption; K, equilibrium constant of the reversible adduct formation: $K = \frac{(NOCAP \infty GSH)}{(NOCAP)\cdot(GSH)}$.

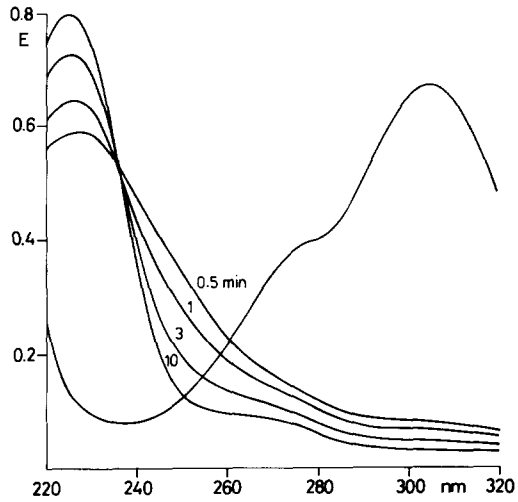

Fig. 6. Rapid repetitive spectrophotometry of a reaction mixture containing NOCAP (0.05 mM) and GSH (0.4 mM) at pH 8.2 and 5°. The reference cuvette contained 0.35 mM GSH. Scans were started at various intervals after addition of GSH (scan speed 10 nm/sec).

the transient adduct and the final sulfinamide was around 255 nm. At this wavelength, the rates of transformation were followed spectroscopically at different pH. Figure 7 shows the reproduction of original test curves. The reaction of NOCAP (0.1 mM) and GSH (0.4 mM) was started at pH 8.2 by the addition of GSH. Twenty seconds later, the pH was rapidly lowered by addition of phosphoric acid or acetic acid which accelerated the disappearance of the transient intermediate. When the pH was lowered to 3 and below, only minimal amounts of NOCAP were extracted with ether indicating that the adduct had been irreversibly transformed into the sulfinamide.

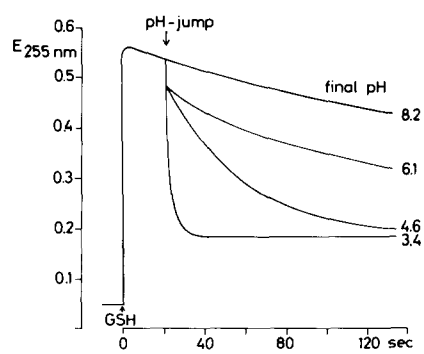

Fig. 7. Velocity of the isomerization of the transient intermediate into GSONHCAP at various pH. Reproduction of original test curves. The sample cuvette contained 0.1 mM NOCAP in 50 mM sodium phosphate, pH 8.2. The reaction was started by addition of 0.4 mM GSH and followed at a wavelength of 255 nm. After a 20-sec reaction, the pH was lowered by acetic acid or phosphoric acid. The reference cuvette contained 0.30 mM GSH.

DISCUSSION

The results indicate that nitroso-chloramphenicol (NOCAP) reacts with GSH very similarly to nitrosobenzene [28]. In a rapid second-order reaction NOCAP combines with GSH and forms a transient intermediate, presumably a semimercaptal, indicated by the loss of u.v. absorption around a wavelength of 300 nm (cf. Fig. 6). The intensive absorption at a low energetic wavelength is characteristic for nitrosoarenes and has been attributed to the N=O bond [36]. This primary reaction, however, seems to be fully reversible since nearly all NOCAP could be extracted with ether from those reaction mixtures at very early states of the reaction (cf. Fig. 5).

As shown in Fig. 4, the u.v. absorption of NOCAP decreased in a biphasic manner. At pH values above 7, both reaction rates differed by nearly two orders of magnitude. The proportion of the rapid reaction on the total decrease in u.v. absorption increased with GSH concn but was independent of the NOCAP concn (see Table 2). Hence, this proportion reflects the equilibrium of adduct formation which was found to be somewhat higher for NOCAP ($K = 4500\ M^{-1}$) than for nitrosobenzene ($K = 2600\ M^{-1}$).

The slow phase of the decrease in u.v. absorption is due to secondary reactions of the transient semimercaptal. As indicated by end-product analysis NOCAP with GSH either forms a sulfinamide or oxidizes GSH with formation of NHOHCAP and a stoichiometric amount of GSSG. This latter reaction is favoured by increasing GSH concns and resembles the thiolytic cleavage of azoester-glutathione adducts by GSH as revealed by Kosower *et al.* [37]. These relationships are illustrated in Fig. 3. At constant pH, only GSH concn determines the pattern of products according to the following equation: NHOHCAP/GSONHCAP = p·(GSH). Interestingly, p was found to be identical for NOCAP and nitrosobenzene. The isomerization of the semimercaptal to the sulfinamide is catalyzed by protons. As shown in Fig. 7, decrease in the pH from 8.2 to 4.6 accelerated the isomerization five-fold. At this pH, hydrolysis of the resulting sulfinamide into glutathionesulfinic acid and NH_2CAP is not observed during this reaction time. As a consequence, at constant GSH concn, formation of the sulfinamide is favoured by lowering the pH. Hence, as described in Materials and Methods, synthesis of the sulfinamide was performed at low steady-state concns of GSH and at pH below neutrality to depress NHOHCAP formation. It should be noted here that, similar to nitrosobenzene, increasing pH markedly increased the velocity of the initial reaction. This is expected, since GS^- rather than GSH is involved in those reactions [38].

Figure 8 schematically summarizes the reactions of NOCAP with GSH. This scheme is essentially analogous to our earlier proposal elaborated for the reactions of nitrosobenzene with GSH [28].

Meanwhile, a variety of other nitrosoarenes have been examined for their ability to react with thiols. Studying reactions of p-nitrosotoluene with GSH, Neumann and co-workers [39, 40] presented evidence that amine can also be formed without con-

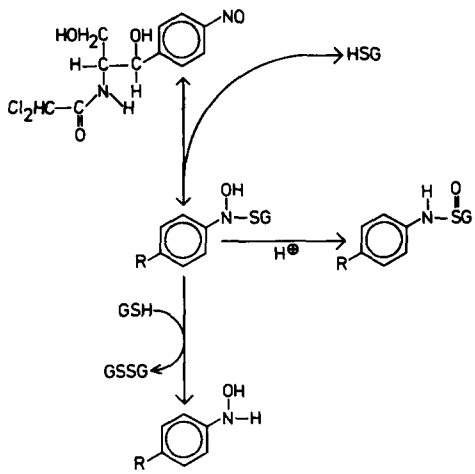

Fig. 8. Tentative scheme of the reactions of NOCAP with GSH.

comitant sulfinic acid production. This slow reaction occurred when all nitrosoarene had disappeared and the reaction mixture was extracted with organic solvents. Since this slow amine liberation needed GSH and produced GSSG, the authors concluded that the primary reaction product, the semimercaptal, adds a second GSH molecule with formation of the full mercaptal. This might be thiolytically cleaved by another two GSH to give finally 2GSSG and the amine. Such a reaction pathway was recently confirmed by Mulder *et al.* [41] for reactions of 2-nitrosofluorene with GSH. These authors succeeded in isolating two adducts by HPLC and identifying them by fast atom bombardment mass spectrometry. One of the compounds exhibited a sulfinamide structure, the other was *N*-(glutathione-*S*-yl)-2-aminofluorene.

Subsequent studies in our laboratory with various *p*-substituted nitrosoarenes and thiols [31] indicated that realization of the three possible reaction pathways presented by Neumann and co-workers depends largely on structure, concn of reactants and pH. Nitrosoarenes with positive σp Hammett constants like nitrosoacetophenone predominantly form the hydroxylamine; *p*-nitroso-*N,N*-dimethylaniline, with its extremely negative σp-value, virtually forms no hydroxylamine and sulfinamide but mainly the amine and GSSG. The bulky *p*-substituent in nitroso-chloramphenicol obviously has no such effects since reaction pathways, reaction rate of semimercaptal formation and equilibrium are nearly identical to nitrosobenzene.

The high reactivity of NOCAP towards GSH obviously raises the question whether NOCAP, if it is formed at all in the intestine or liver, has a chance to reach the sensitive target. In addition to reactions with thiols, NOCAP is also inactivated by reduction. Preliminary experiments with rat liver cytosol and liver alcohol dehydrogenase have shown [42] that NOCAP is rapidly reduced at rates similar to those observed with nitrosobenzene [30]. Experiments are under current progress to investigate whether NOCAP is disposed as effectively as nitrosobenzene [30] in the isolated perfused rat liver.

Even if the first barrier of NOCAP trapping is overcome and NOCAP or NHOHCAP escape from the liver, both compounds can be quickly inactivated in red cells. NHOHCAP forms ferrihemoglobin and is thereby oxidized to NOCAP [42] which is rapidly eliminated in red cells by reaction with intracellular GSH and the reactive SH groups of hemoglobin [42]. These reactions are currently being studied in more detail.

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