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# FORMATION AND DISPOSITION OF NITROSOCHLORAMPHENICOL IN RAT LIVER

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**Abstract**—It has been suggested that in the chloramphenicol-induced aplastic anemia nitroso-chloramphenicol may be involved as a toxic intermediate. We found that aminochloramphenicol, which reportedly is formed from chloramphenicol by intestinal bacteria, is N-oxygenated by liver microsomes of untreated rats with apparent $K_m$ = 0.4 mM and $V_{max}$ = 0.28 nmole/min/mg protein. These values are in close agreement with those reported for aniline N-oxygenation. Reductive reactions, however, eliminate the N-oxygenation products at markedly higher rates. As judged from hemoglobin-free single-pass liver perfusion experiments, N-hydroxy-chloramphenicol is reduced at rates faster than 300 nmole/min/g liver wet, and nitrosochloramphenicol is eliminated at rates faster than 1.5 μmole/min/g liver. At least two NADPH- and two NADH-dependent cytosolic enzymes are responsible for nitroso-chloramphenicol reduction. Determination of the kinetic parameters of these enzymes by stop-flow analysis revealed the contribution of enzymes, one of it being alcohol dehydrogenase, with Michaelis constants in the micromolar range. Despite this high reducing capacity, about 10% of nitroso-chloramphenicol reacted with GSH under formation of glutathionesulfinamidochloramphenicol and GSSG released from the liver into bile and venous effluent. At high nitrosochloramphenicol load these reactions led to glutathione depletion of the liver, caused membrane damage, and impaired bile production. At low nitrosochloramphenicol load, i.e. below 0.5 μmole/min/g, no relevant nitroso-chloramphenicol passed the liver. These data together with the previously reported reactions of nitrosochloramphenicol within human blood suggest that nitrosochloramphenicol, if formed at all in the intestine or liver, is rather unlikely to be transferred to the critical target.

The use of the broad spectrum antibiotic chloramphenicol (CAP)† has been restricted after its association with bone marrow depression. The more common type of bone marrow toxicity is a dose-dependent and usually reversible suppression of mainly the erythroid elements probably caused by the inhibition of mitochondrial protein synthesis. Contrasting with this type of injury, CAP induces occasionally aplastic anemia (approximately 1 in 20,000 patients). The underlying biochemical lesion responsible for this often fatal effect is still obscure, and adequate animal models are lacking. Since thiamphenicol, the *p*-methylsulfonyl analogue of CAP has never been associated with aplastic anemia, Yunis and co-workers have been suggesting that the *p*-nitro group of CAP may be involved in the development of aplastic anemia. These authors elaborated the working hypothesis that CAP causes bone marrow aplasia in the genetically predisposed host who provides the milieu for the reduction of the *p*-nitro group to toxic intermediates somehow leading to stem cell damage [for review see ref. 1]. When Corbett and Chipko [2] succeeded in the synthesis of a variety of CAP reduction products, this hypothesis was experimentally tested.

Using several lymphocytic precursor cell assays, Pazdernik and Corbett [3] demonstrated that NOCAP was the most active of the CAP reduction products tested in suppressing lymphocyte action in mice. In addition, NOCAP proved to be considerably more toxic to cultured human bone marrow cells than CAP, and inhibited irreversibly DNA synthesis as well as the growth of pluripotential hematopoietic stem cells, but the inhibition by CAP was largely reversible [4]. NOCAP inhibited mitochondrial respiration and proton translocation [5] and mediated DNA damage at micromolar concentrations at which CAP was without effect [6]. Pohl *et al.* [7] investigated the influence of CAP and several of its known or potential metabolites on DNA synthesis of rat and human bone marrow cells, and also found NOCAP to be the most potent inhibitor of DNA synthesis; moreover, 500-times more [$^{14}$C]-labelled NOCAP was irreversibly bound to viable bone marrow cells than [$^{14}$C]-labelled CAP. From all these data, it seems reasonable to suspect NOCAP as the most probable candidate from all the CAP metabolites tested hitherto being responsible for the bone marrow cell injury, irrespective of the molecular mechanism involved.

Although biochemical formation of NOCAP was not reported, there is indirect evidence for nitro reduction of CAP because of the occurrence of diazotizable material in urine which leads to a positive Bratton–Marshal reaction [8]. Though most of this amine has been probably formed by intestinal bacteria [9], CAP was shown to be reduced to the arylamine also by different mammalian liver fractions [10] including human liver tissue [4]. The mammalian nitro reductases, however, are inactive in the presence of oxygen [11] and may therefore scarcely

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† For abbreviations and formula cf. Fig. 6.

contribute to marked amine formation. On the other hand, NOCAP can be formed conceivably also by oxidation of the known arylamine metabolite by various tissue microsomal oxidases.

In the present study, we investigated the formation of NOCAP from $NH_2CAP$ in rat liver microsomes and the disposition of NOCAP in the isolated perfused rat liver and in subcellular rat liver fractions. As a result, we found that $NH_2CAP$ is N-oxygenated by rat liver microsomes. The reductive reactions, however, eliminate NHOHCAP and especially NOCAP at high rates. Hence, the liver does not allow the passage of relevant NOCAP concentrations, because NOCAP is metabolized by enzymic reduction and conjugation with GSH [12]. These data, together with the previously reported reactions of NOCAP within human blood [13], suggest that NOCAP, if formed at all in the gut or liver, is rather unlikely to be transferred unchanged to the critical target.

## MATERIALS AND METHODS

Nitroso-chloramphenicol (NOCAP), D(−)-threo-1-(*p*-nitrosophenyl)-2-dichloroacetamido-1,3-propanediol, and the radioactive compound- (dichloro-acetamido-1-[$^{14}$C])- were synthesized as described previously [12].

Hydroxylamino-chloramphenicol (NHOHCAP), D(−)-threo-1-(*p*-hydroxylaminophenyl)-2-dichloroacetamido-1,3-propanediol, and amino-chloramphenicol ($NH_2CAP$), D(−)-threo-1-(*p*-aminophenyl)-2-dichloroacetamido-1,3-propanediol were prepared as described [12].

*N*-Acetylamino-chloramphenicol (NAcCAP), D-(−)-threo-1-(*p*-*N*-acetylaminophenyl)-2-dichloroacetamido-1,3-propanediol, was prepared by acetylation of $NH_2CAP$. Sixty μmole of $NH_2CAP$ dissolved in 5.4 ml ethyl acetate reacted with 0.6 ml acetanhydride for 2 hr at room temperature. The solvent and acetanhydride were removed by repetitive rotary evaporation and the residue was dissolved in methanol. Small impurities were removed by preparative HPLC. After lyophilization 42 μmole NAcCAP (amorphous powder) were obtained. The proton NMR in deuterated methanol proved the structure (ppm, TMS): 7.4 (m, 4H, phenyl); 6.27 (s, 1H, dichloroacetamido); 4.95 (d, 1H, C-1), 4.01 (m, 1H, C-2); 3.62 (dd, 2H, C-3); 2.09 (s, 3H, acetylamino).

Glutathionesulfinamido-chloramphenicol (GSONHCAP), D(−)-threo-1-(*p*-glutathionesulfinamidophenyl)-2-dichloroacetamido-1,3-propanediol was prepared from NOCAP by reaction with GSH [12]. To 5.4 ml of 3 mM [$^{14}$C]-NOCAP (0.54 μCi/μmole) in 0.2 M sodium phosphate, pH 6.6, 0.6 ml of 60 mM GSH were added in three portions within 1 min. After 5 min reaction at 37°, the incubate was adjusted with tri-potassium phosphate to pH 7.4 and extracted three times with 10-ml portions of ethyl acetate to remove lipid-soluble by-products (mainly NHOHCAP). The aqueous phase contained GSONHCAP (radiochemical purity > 97% (HPLC), 80% yield), was divided in small portions, and kept at −20°. Within 1 month, 5% of the compound had been hydrolyzed with formation of free $NH_2CAP$ and glutathionesulfinic acid.

### *Animals*

Unstarved male Sprague–Dawley rats, weighing 190–220 g and fed with Altromin® with free access to food and water, were anaesthetized by i.p. injection of 50 mg/kg pentobarbital sodium (Nembutal®). Liver perfusion experiments were performed between 10 hr and 11 hr a.m. to reduce variance in thiol content of livers due to circadian rhythm [14].

### *Liver perfusion*

The procedure for removing the liver from the animal and the perfusion apparatus, suitable for both non-recirculating and circulating organ perfusion, have been described earlier [15]. The perfusion medium was a modified Krebs–Henseleit [16] solution which contained 110 mM NaCl, 5.9 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM $NaH_2PO_4$, 6 mM $Na_2SO_4$, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$ and 10 mM glucose, equilibrated with $O_2/CO_2$ (95:5, v/v). The mean liver flow rate was about 4 ml/min/g liver at 37°. The mean liver weight was about 10 g wet wt. The bile was collected at 3 cm negative pressure by a tube tied into the common bile duct. First, the liver was perfused substrate-free with the recirculating buffer for 10 min and for an additional 15 min by open flow. Because NOCAP is poorly water-soluble, the substrate for once-through perfusion was dissolved in the perfusion buffer which had to pass the oxygenator for 3 min before entering the liver.

### *Preparation of rat liver microsomes*

Male Sprague–Dawley rats (220–250 g) were kept at Altromin® and water *ad libitum*. The animals were fasted for 24 hr prior to use. Usually, five rats were decapitated, the livers removed immediately, freed from the larger vessels and connective tissue, chopped into small pieces and forced through a 1 mm mesh screen as described by Jagow *et al.* [17]. The resulting pulp was weighed, suspended in two-fold volume (w/w) of 0.15 M KCl containing 10 mM Tris–HCl, pH 7.4, and 0.1 mM EDTA, and stirred for 30 min on a magnetic stirrer. The homogenate was centrifuged at 9000 *g* for 30 min to remove undisrupted cells, nuclei, and mitochondria. The supernatant was carefully decanted and further centrifuged at 100,000 *g* for 60 min. The microsomes containing pellet was suspended in fresh buffered KCl and centrifuged at 100,000 *g* for 60 min. After a third washing the microsomes were suspended in 0.1 M Tris–HCl, pH 7.4, containing 5 mM $MgCl_2$ and 1 mM EDTA. The total procedure was run between 0 and 4°.

The protein concentration in the final suspension was measured by the biuret method, cytochromes P-450 and *b* 5 were determined according to Omura and Sato [18]. Usually, 8.7 ± 2.3 mg microsomal protein was obtained from one g of liver (wet wt) (N = 9). The cytochrome P-450 content was 1.2 ± 0.3 nmole/mg protein and the cytochrome *b* 5 content was 0.6 ± 0.1 nmole/mg protein.

Postmicrosomal supernatant was prepared from

rat liver homogenates after centrifugation at 100,000 *g* for 1 hr. Homogenates were prepared from chopped liver pieces in 5 vol. of 100 mM Tris–HCl, pH 7.4, containing 1 mM EDTA by homogenisation in an Ultra-Turrax® (4 × 30 sec with 1 min interval, at 0°). The supernatant was dialyzed against 2 × 100 vol. of the same buffer overnight.

*Microsomal incubations*

Incubations were carried out in 50 ml round flasks with glass stoppers in a gyrotory water bath at 37°. The reaction mixture (3.5 ml) consisted of 0.1 M Tris–HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA, 5 mM glucose-6-phosphate, 1 mM NADPH, 2 mM 5′-AMP, 1.4 U/ml glucose-6-phosphate dehydrogenase and 5 mg microsomal protein/ml. After 5 min preincubation the substrate dissolved in 20 μl of methanol was added.

*Sample preparation*

One ml of a microsomal sample was saturated with solid NaCl, mixed with 2–5 nmole of the radioactive tracer, and extracted with 2 ml of ice-cold ethyl acetate. In the experiments with $NH_2CAP$ as substrate, unreacted $NH_2CAP$ was removed from the organic phase by extraction with 0.05 M $H_2SO_4$ containing 15 μmole hexacyanoferrate (III) to convert all NHOHCAP to NOCAP. The ethyl acetate phase was evaporated by a stream of nitrogen, the residue was dissolved in 0.1 ml of methanol and kept at −20° until HPLC analysis.

*Analytical methods*

Autoxidation of NHOHCAP during liver perfusion, which would lead to erroneous results in NOCAP determination, was largely prevented by connecting the effluent tube of the caval perfusate to a through-flow cell in a PMQ II photometer (Carl Zeiss, Oberkochen) equipped with a Servogor® (BBC-Goerz) recorder. The lag phase between the caval vein and the cuvette was less than 10 sec. The determination of NOCAP at 316 nm ($\varepsilon$ mM = 14.3) was not disturbed by other compounds, e.g. 4,4′-bisazoxy-chloramphenicol which was not existent to any significant extent.

NHOHCAP and $NH_2CAP$ were determined by HPLC. The perfusate was frozen immediately after sampling at −40°, and thawed immediately before injection into the apparatus. HPLC was performed on μBondapak $C_{18}$ (4 mm i.d. × 30 cm) with methanol:20 mM sodium phosphate, pH 7.4 (10:90, v/v, flow rate 2 ml/min) in an ALC/GPC 244 chromatograph (Waters Milford, MA) with detection at 254 nm and peak integration by a Data Modul®. Calibration was performed with authentic standards. GSONHCAP was eluted after 3.7 ml, NHOHCAP after 6.4 ml, and $NH_2CAP$ after 7.8 ml. With methanol:20 mM sodium phosphate, pH 7.4 (25:75) NOCAP was eluted after 17.2 ml, NAcCAP after 7.2 ml, $NH_2CAP$ after 5.2 ml and $NH_2CAP$-glucuronide after 3.3 ml. GSONHCAP in liver perfusates was additionally determined by isotope dilution analysis. The pooled caval perfusate after NOCAP-perfusion was mixed with 0.1 μmole of authentic [$^{14}$C]-GSONHCAP and extracted three times with an equal volume of ethyl acetate. The aqueous phase was lyophilized, dissolved in a small volume of water and chromatographed on Sephadex G 10 (4 cm i.d. × 40 cm) with 10 mM ammonium acetate, pH 7.4. The radioactive fractions were pooled and applied to column chromatography on $DE_{52}$-cellulose (2.5 cm i.d. × 12 cm) equilibrated with 10 mM ammonium acetate, pH 7.4. GSONHCAP was eluted from the column with a linear salt gradient (200 ml 10 mM ammonium acetate, pH 7.4, to 200 ml 250 mM ammonium acetate, pH 7.4). The radioactive fractions were pooled, lyophilized and dissolved in 0.5 ml of water. HPLC revealed one single peak after 3.7 ml. When 0.1 ml of the sample was hydrolyzed with 5 μl of conc. hydrochloric acid, all the radioactivity was found in $NH_2CAP$ (HPLC). $GSO_2H$, liberated by the acid hydrolysis was determined on an amino acid analyzer [19]. Its amount agreed with $NH_2CAP$ within 5%. From the radioactivity of $NH_2CAP$ and the total amount of $NH_2CAP$ determined by HPLC, the amount of GSONHCAP in the perfusate was calculated. Conjugates of $NH_2CAP$ and NAcCAP were identified as glucuronides because they were completely hydrolyzed to the corresponding aglycons (HPLC) upon treatment with β-glucuronidase (helix pomatia, 10.000 Fishman units/ml, pH 5, 37°, 16 hr). Addition of 20 mM D-saccharic acid-1.4-lactone inhibited the hydrolysis completely. Since the glucuronide of CAP, which was proven to be a 3-glucosiduronate [20], exhibited the same hydrolytic behaviour, we assume by analogy that the corresponding glucuronides of $NH_2CAP$ and NAcCAP were also 3-glucosiduronates.

Nitrosoarene reduction kinetics in postmicrosomal supernatant or in presence of horse liver ADH was carried out in an Aminco DW 2A dual-wavelength spectrophotometer equipped with an Aminco-Morrow stop-flow accessory. Initial velocities of the reactions, stored in an Aminco-DASAR digital unit, were determined in at least triplicate measurements agreeing within 5 per cent. Kinetic parameters were calculated from regression lines of Lineweaver–Burk plots which were carried out by programs designed for a Hewlett Packard 97 pocket computer.

Glutathione in caval perfusate and bile was determined as the sum of GSH and GSSG equivalents by the colorimetric micromethod of Owens and Belcher [21] modified by Oshino and Chance [22]. Prior to the assay the perfusate was extracted immediately after sampling with a 5-fold vol. of ethyl acetate to remove reactive xenobiotics. GSH, GSSG and glutathione sulfinic acid in perchloric acid extracts of the livers (5 vol/g wet wt) were determined as described elsewhere [19]. Lactate, pyruvate and LDH were determined after ethyl acetate extraction of the caval perfusate by standard methods [23]. Oxygen consumption was recorded with a Clark-type electrode inserted in the caval perfusate line.

Radioactivity was measured in Bray's solution in an LKB Wallac 1217 liquid scintillation spectrometer. Radioactive material bound to liver proteins was determined after three washings each with 0.5 M perchloric acid and methanol and combustion in a Packard Sample Oxidizer® using Carbosorb® as trapping agent for $^{14}CO_2$ and Permafluor® scintillation cocktail (Packard). All

results have been corrected for background radiation and recovery using external standard.

## RESULTS

### (1) *Single-pass perfusion of isolated rat livers with nitrosochloramphenicol*

*Biotransformation products.* Single-pass perfusion was chosen to avoid the consequences of autoxidation of the expected reduction product, NHOHCAP. This compound autoxidized under pure oxygen with an apparent half-life of 70 min at pH 7.4 and 25°. Figure 1 shows the averaged data of three experiments during 30 min perfusion with 0.46 mM prehepatic NOCAP, followed by a 15 min washout period. As seen from the upper panel, at steady state more than 90% of NOCAP was metabolized, and 0.32 mM NHOHCAP and 0.08 mM $NH_2CAP$ had been formed. In addition, 0.027 mM GSONHCAP was found in the pooled perfusate between min 38 and 43. Hence, virtually all NOCAP metabolized can be attributed to these three metabolites. As shown in the lower panel of Fig. 1, oxygen consumption was not significantly altered by NOCAP, and the lactate/pyruvate ratio remained almost constant. The sum of lactate and pyruvate tended to decrease slightly during NOCAP perfusion (data not shown).

*Glutathione status.* During NOCAP perfusion the glutathione status of the liver was altered. As shown in the upper panel of Fig. 2, the glutathione release (GSH + 2GSSG) into the venous effluent was increased from 10 to 30 nmole/min/g. This effect,

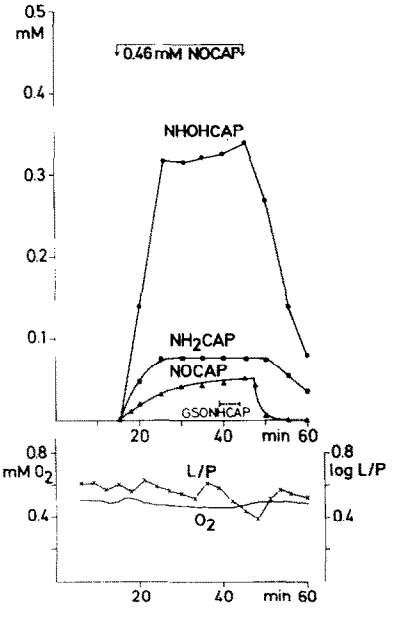


Fig. 1. Concentrations of NOCAP and its metabolites and the metabolic response of rat liver during hemoglobin-free single-pass perfusion. Upper panel: post-hepatic NOCAP, NHOHCAP, $NH_2CAP$ and GSONHCAP during perfusion with 0.46 mM NOCAP (means of three expts). Lower panel: post-hepatic oxygen concentration and the lactate/pyruvate ratio (log scale).

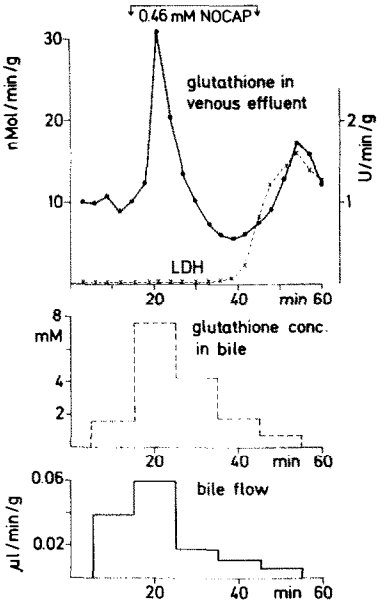


Fig. 2. Glutathione release in venous effluent and bile during hemoglobin-free single-pass perfusion of rat liver with 0.46 mM NOCAP (means of three expts).

however, was only observed at the beginning of NOCAP-perfusion; at the later stage, the glutathione release decreased even to subnormal values. The later re-increase of the glutathione release is attributed to membrane damage, as indicated by the simultaneous LDH release. Such an initial transient increase in glutathione release was also observed in the bile (mid panel of Fig. 2). During the first 10-min-period of NOCAP perfusion the glutathione concentration in the bile increased from 1.6 to 7.6 mM but decreased steadily thereafter reaching subnormal values at the end of the perfusion (means of three expts). Concomitantly with the glutathione concentration, the bile flow rate was slightly stimulated by NOCAP only at the beginning of the perfusion. After 60 min the bile flow rate decreased to 1/10th of the initial rate (lower panel of Fig. 2). In contrast, control livers without NOCAP showed a 20% reduction in bile flow rate after the 60 min perfusion period [24].

At 0.19 mM prehepatic NOCAP, 0.095 mM NHOHCAP, 0.075 mM $NH_2CAP$ and 0.017 mM GSONHCAP had been formed under steady state conditions. Only traces of NOCAP appeared in the venous effluent. The enhanced glutathione release into the venous effluent and the bile decreased later, and the bile flow was less inhibited.

The glutathione content of the livers after NOCAP perfusion was diminished depending on the total load with NOCAP (Table 1). The amount of glutathione released with the bile and the venous effluent accounted only for a small part, the major part of the missing glutathione was found in GSONHCAP. Because this compound is quite labile and readily hydrolyzes at acidic or alkaline pH [12], we examined its stability during recirculating liver perfusion with 50 µM [$^{14}$C]GSONHCAP. After 80 min perfusion,

Table 1. Liver glutathione after hemoglobin-free single-pass perfusion of rat livers with nitrosochloramphenicol (NOCAP)

| NOCAP perfused | Glutathione in the liver | Glutathione in GSONHCAP | Glutathione in ven. effl. | Glutathione in the bile |
|---|---|---|---|---|
| 47* | 2.59 | 2.20 | 0.72 | 0.055 |
| 48 | 2.35 | 2.05 | 0.75 | 0.085 |
| 47 | 2.59 | 2.25 | 0.45 | 0.025 |
| 30 | 3.25 | 2.10 | 0.60 | 0.015 |
| 21 | 4.21 | 1.29 | 0.54 | 0.024 |
| 0 | 6.36 | 0 | 0.35 | 0.020 |
| 0 | 6.54 | 0 | 0.34 | 0.027 |
| 0 | 5.54 | 0 | 0.34 | 0.053 |
| 0 | 5.97 | 0 | 0.27 | 0.061 |

* Mole per g liver wet wt; 15 min single-pass equilibration period followed by 30 min NOCAP once-through perfusion and 15 min washout period. Controls were perfused for 60 min without substrate.

virtually all [$^{14}$C]GSONHCAP was recovered unchanged in the perfusate. Despite of its high molecular weight, GSONHCAP was not excreted by the bile, nor was any radioactivity detected in the bile. Thus, GSONHCAP is sufficiently stable during liver perfusion.

(2) *Reduction of NOCAP by postmicrosomal supernatant of rat liver*

Since the kinetic constants of NOCAP reduction could not be determined in the isolated perfused liver, attempts were made to estimate the reducing capacity of the soluble fraction of the liver. When 50 $\mu$M NOCAP was incubated with 100 $\mu$M NADH in the presence of postmicrosomal supernatant (1 mg protein/ml), 38 $\mu$M NHOHCAP was found after 1 min reaction (isotope dilution analysis).

Similarly, 100 $\mu$M NOCAP was completely reduced by 5 $\mu$M NADPH in the presence of 0.5 mg of postmicrosomal protein and a NADPH regenerating system (10 mM glucose-6-phosphate and 7 U glucose-6-phosphate-dehydrogenase in 100 mM Tris–HCl, pH 7.4, containing 1 mM EDTA).

For kinetic analysis of NOCAP reduction, disappearance of NOCAP and NAD(P)H was determined by a stop-flow method at 345 nm wavelength in presence of 0.5 mg/ml of postmicrosomal protein. The stop-flow method was indispensable because of the high rate of spontaneous reduction of NOCAP by NAD(P)H in the absence of postmicrosomal supernatant. The second order rate constant of this latter reaction was 260 $M^{-1}sec^{-1}$ at pH 7.4 and 37°. In addition, NOCAP disappeared in the presence of carefully dialyzed postmicrosomal supernatant even in the absence of any additional nucleotide. This reaction was attributed to the addition of NOCAP to protein sulfhydryl groups [25]. For calculation, the enzyme-catalyzed reduction rates were corrected correspondingly. Figure 3 shows the influence of 50 $\mu$M NAD(P)H on enzymic NOCAP reduction of various substrate concentrations. Since aromatic C-nitroso compounds are known to be substrates for liver ADH (EC 1.1.1.1) [26, 27], NOCAP reduction by NADH was also followed in the presence of 0.5 mM 4-methylpyrazole, a powerful inhibitor of liver ADH [28]. The difference between the reduction rates in the absence or presence of 4-methylpyrazole was attributed to ADH-dependent NOCAP reduction and is shown by the dashed line in Fig. 3. Attempts to evaluate the kinetic parameters from Lineweaver–Burk plots were only successful in case of the NADH-dependent NOCAP reduction in the presence of 4-methylpyrazole ($r^2 = 0.95$). The other slopes systematically deviated from straight lines indicating the existence of several enzymes with different kinetic parameters.

Assuming two different enzymes which catalyze NOCAP reduction by NADH (one of which being ADH), and two different enzymes for NOCAP reduction by NADPH, the data fitted satisfactorily the calculated slopes generated by a special computer program, which was designed for Scatchard analysis by Dr. R. Elbers at this institute. The generated curves fit satisfactorily the data points shown in Fig. 1. The corresponding kinetic parameters are given in Table 2. The $K_m$-values for NAD(P)H at 50 $\mu$M NOCAP were calculated from straight regression

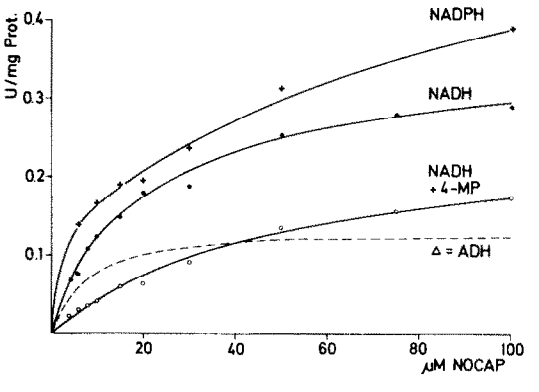


Fig. 3. Influence of NAD(P)H on enzymic NOCAP reduction at various substrate concentrations. NOCAP reduction (U/mg = $\mu$mole/min/mg protein) by NAD(P)H (50 $\mu$M) in the presence of post-microsomal supernatant of rat livers was followed by stop-flow spectroscopic analysis (see Methods). The difference between the reduction rates by NADH in the absence and presence of 4-methylpyrazole (4-MP; 0.5 mM) was attributed to ADH activity (dashed line).

Table 2. Kinetic parameters for nitrosochloramphenicol (NOCAP) reduction in the postmicrosomal supernatant of rat liver

| Condition | $K_m$ ($\mu$M NOCAP) | $V_{max}$ (nmole/min/mg protein) |
|---|---|---|
| NADH (50 $\mu$M) | 8 (1.2)* | 130 (140) |
| | 40 (30) | 250 (270) |
| NADH (50 $\mu$M) + 4-methylpyrazole (0.5 mM) | 50 (40) | 260 (300) |
| NADPH (50 $\mu$M) | 1 | 130 |
| | 100 | 550 |

The apparent $K_m$ and $V_{max}$ values were calculated from computer generated Scatchard plots assuming two enzymes each for NADH- and NADPH-dependent NOCAP reduction. Photometric determination of initial rates of NOCAP and NAD(P)H disappearance was achieved by a stop-flow method. All experiments were carried out in 0.1 M Tris–HCl, containing 1 mM EDTA, pH 7.4, at 37° in the presence of 0.5 mg post-microsomal protein/ml.

* Data given in brackets show the kinetic parameters for nitrosobenzene reduction under identical experimental conditions.

lines ($r^2 = 0.98$) of Lineweaver–Burk plots giving 13 $\mu$M for NADH and 9 $\mu$M for NADPH. Hence, the various enzymes with different $K_m$-values for NOCAP had very similar affinities for both reduced nucleotides. The existence of at least two NADH- and two NADPH-dependent enzymes in liver cytosol was confirmed by HPGPC separation of post-microsomal supernatant. Figure 4 shows a separation of 3 mg cytosolic protein. The enzyme activity in 0.2 ml fractions was determined at 20 $\mu$M NOCAP and 20 $\mu$M NAD(P)H. Complete inhibition of the enzyme activity by 50 $\mu$M 4-methylpyrazole was attributed to ADH activity. In contrast to the NADH-dependent enzymes, the NADPH-dependent enzymes were poorly separated. Therefore, kinetic analysis of this material seemed not to be feasible. It should be noted that the fractions containing NOCAP-reducing activity did also reduce nitrosobenzene, 4-nitrosophenetol and 4-nitroso-*N,N*-dimethylaniline (data not shown). As can be seen from the data in Table 2, the kinetic parameters of the NADH-dependent enzymes are very similar for NOCAP and nitrosobenzene in the case of the non-ADH enzyme. The affinity of ADH, however, is significantly lower for NOCAP than for nitrosobenzene. This difference is even more pronounced in the case of crystalline horse liver ADH. As shown in Table 3, the $K_m$-values for a variety of 4-substituted nitrosobenzenes with small-sized substituents do not markedly differ from nitrosobenzene. The affinity for NOCAP, however, is lower by two orders of magnitude. This large difference might be attributed to steric hindrance of the bulky residue in NOCAP. This effect would also explain the marked species difference of the enzyme affinities ($K_m$NOCAP/nitrosobenzene: rat = 7, horse = 90).

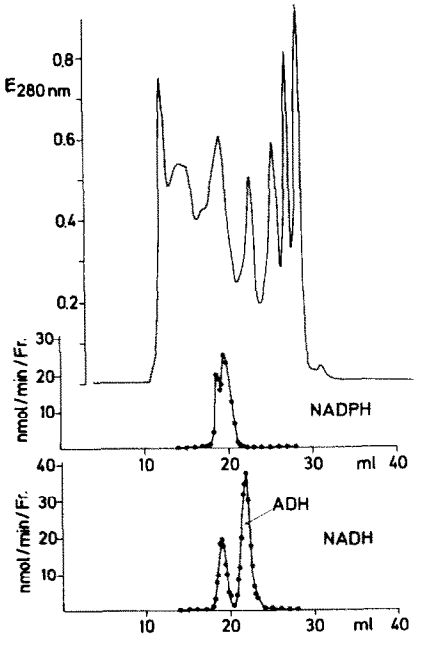


Fig. 4. Separation of NOCAP reducing enzymes in post-microsomal supernatant of rat livers by high performance gel permeation chromatography. Postmicrosomal proteins (3 mg in 0.2 ml) were chromatographed on Ultropac® TSK-G 3000 SW (LKB; 7.5 mm i.d. × 600 mm) with 50 mM triethanolamine-HCl, pH 7.2, at 1 ml/min flow rate. In 0.2 ml fractions NOCAP (20 $\mu$M) reducing activity was followed in the presence of 20 $\mu$M NADPH (mid panel) or NADH, respectively (lower panel). Complete inhibition by 4-methylpyrazole was attributed to ADH activity.

### (3) *Reduction of NHOHCAP by rat liver fractions*

In contrast to NOCAP, NHOHCAP was slowly reduced by cytosolic enzymes. At 10 $\mu$M NHOHCAP, $NH_2$CAP was produced at a rate of about 2 pmole per min per mg protein in the presence of 1 mM NADH or NADPH, respectively ($N_2$, 37°).

Rat liver microsomes from untreated animals reduced NHOHCAP much faster than cytosolic enzymes. As shown in Table 4, 10 $\mu$M [$^{14}$C] NHOHCAP was completely metabolized within 10 min in the presence of 1 mM NADPH and microsomal protein (5 mg/ml) when oxygen was excluded (maximal rate > 200 pmole/min/mg). The main product was $NH_2$CAP, besides some polar metabolites (presumably a glucuronide of $NH_2$CAP; see below). Covalent binding to microsomal protein was insignificant. Under air, NHOHCAP reduction was slower (about 50 pmole per min per mg), the amount of $NH_2$CAP was less, and the proportion bound covalently to proteins was higher than under nitrogen. When microsomes were omitted from the system, NHOHCAP did not decrease within 30 min.

The aerobic biotransformation of NHOHCAP by rat liver microsomes showed no saturation kinetics between 10 and 50 $\mu$M. NHOHCAP apparently

Table 3. Kinetic parameters for nitrosoarene reduction by NADH in the presence of crystalline horse liver alcohol dehydrogenase

| Nitrosoarene | $K_m$ NOAr ($\mu$M) | $K_m$ NADH ($\mu$M) | $V_{max}$ ($\mu$mole/min/mg) |
|---|---|---|---|
| Nitrosobenzene | 1.8 | 10.4 | 119 |
| 4-Chloronitrosobenzene | 1.1 | 10.1 | 118 |
| 4-Nitrosophenetole | 0.9 | 11.3 | 106 |
| 4-Nitroso-*N,N*-dimethylaniline | 3.1 | 10.2 | 111 |
| Nitrosochloramphenicol | 165 | 9.5 | 100 |

The apparent $K_m$ and $V_{max}$ values were calculated from regression lines ($r^2 = 0.99$) of Lineweaver–Burk plots (at seven concentrations within 20-fold range) after correction of the data for the non-enzymic nitrosoarene reduction. Photometric determination of initial rates of nitrosoarene and NADH disappearance was achieved by a stop-flow method. All experiments were carried out in 0.1 M Tris–HCl, containing 1 mM EDTA, pH 7.4 at 37° in presence of 0.05 mg horse liver alcohol dehydrogenase.

decreased in a pseudo-first order kinetics with a half-life of approx. 30 min (pH 7.4, 37°, air) at all concentrations tested.

(4) *Biotransformation of* $NH_2CAP$ *during recirculating perfusion of rat liver*

During recirculating perfusion with 5 $\mu$mole $NH_2CAP$ (110 ml perfusate, 9 g liver wet wt), $NH_2CAP$ disappeared with an apparent half-life of about 30 min. After 90 min perfusion, 0.065 $\mu$mole $NH_2CAP$, 1.2 $\mu$mole $NH_2CAP$-glucuronide and 1.1 $\mu$mole NAcCAP were found in the perfusate. About 1.5 $\mu$mole $NH_2CAP$ glucuronide and traces of free $NH_2CAP$ and NAcCAP were excreted with the bile.

When the liver was perfused in a similar manner with 5 $\mu$mole NAcCAP, about 10% disappeared from the perfusate within 90 min, 0.05 $\mu$mole NAcCAP and 0.07 $\mu$mole NAcCAP-glucuronide were excreted with the bile. Free or conjugated $NH_2CAP$ were not detected.

(5) N-*oxygenation of* $NH_2CAP$ *by rat liver microsomes*

N-oxygenation of $NH_2CAP$ in the presence of microsomal protein (5 mg/ml) and 1 mM NADPH in a regenerating system proceeded linearly during the first 10 min at $NH_2CAP$ concentrations below 1 mM. Figure 5 shows the Lineweaver–Burk plot of data obtained from the initial rates of NHOHCAP formation at various $NH_2CAP$ concentrations (means of three different microsomal preparations). The apparent $K_m$ was 0.4 mM $NH_2CAP$ and the $V_{max}$ 0.28 nmole per min per mg protein. This activity was measured with fresh preparations (24 hr after removal of the livers). Microsomes tested after 48 hr showed only 2/3 of that activity. In the absence of microsomes no N-oxygenation products were detected. Obviously, the observed rates do not reflect true formation rates, since about 20% of the NHOHCAP formed is reduced back to $NH_2CAP$ within 10 min.

## DISCUSSION

The high capacity of the liver to reduce nitrosoarenes has been previously shown for nitrosobenzene [27] and for 4-nitrosophenetol [24]. The experiments presented here extend these investigations to nitrosochloramphenicol which is eliminated at rates faster than 1.5 $\mu$mole/min/g liver. This high efficiency of the liver allows a nearly quantitative elimination of NOCAP during single passage through the liver. The high reductive capacity of the liver is due to various cytosolic enzymes with remarkable kinetic constants. From Fig. 3 and the data in Table 2 it is evident that at least two reductases using NADH and NADPH, respectively, are effective even at micromolar concentrations according to their low Michaelis constants both for NAD(P)H and NOCAP. Assuming 82 mg cytosolic protein/g liver wet wt [29], each enzyme system can reduce up to 10 $\mu$mole NOCAP/min/g liver at NOCAP concentrations of 20 $\mu$M. At higher NOCAP concentrations, the reduction is

Table 4. Influence of oxygen on the biotransformation of hydroxylaminochloramphenicol (NHOHCAP) by rat liver microsomes

| | Air | | Nitrogen | |
|---|---|---|---|---|
| | 10 min | 30 min | 10 min | 30 min |
| NHOCAP + NOCAP ($\mu$M) | 7.0 | 3.6 | 0 | 0 |
| $NH_2CAP$ ($\mu$M) | 1.6 | 2.8 | 6.8 | 6.3 |
| Acid soluble metabolites ($\mu$M) | 1.0 | 2.5 | 2.2 | 3.0 |
| Protein bound metabolites ($\mu$M) | 0.4 | 0.8 | 0.08 | 0.08 |

[$^{14}$C]-NHOHCAP (10 $\mu$M) was incubated with rat liver microsomes (5 mg/ml) in the presence of 1 mM NADPH in a regenerating system at pH 7.4, 37°. NHOHCAP was determined as NOCAP after oxidation with $K_3Fe(CN)_6$ (means of two expts).

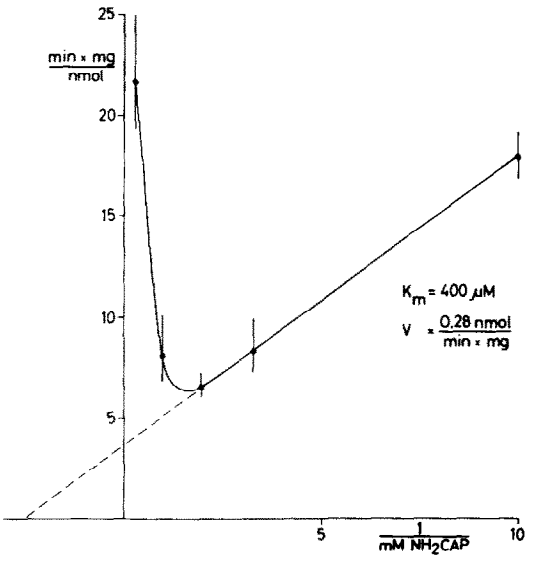


Fig. 5. Lineweaver–Burk plot of data obtained from the initial rates of NHOHCAP formation by rat liver microsomes at various $NH_2CAP$ concentrations (means of three different microsomal preparations).

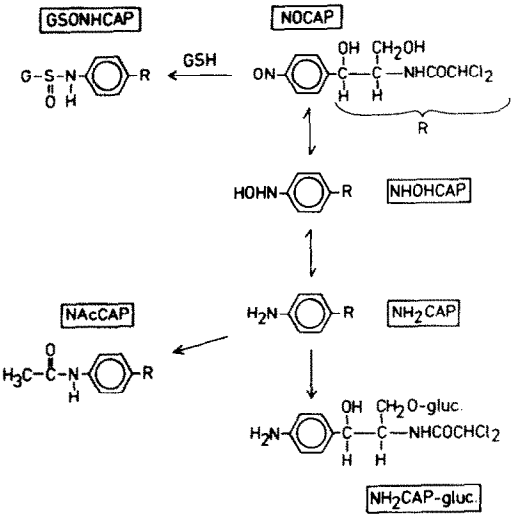


Fig. 6. Scheme of the metabolic routes of chloramphenicol derivatives in rat liver.

augmented by at least two further enzymes, one of them being alcohol dehydrogenase. The occurrence of multiple cytosolic enzymes with C-nitroso-reductase activities have been previously described by others [30]. With this equipment liver cells are protected to some degree from the otherwise deleterious action of NOCAP. NOCAP rapidly reacts with thiol groups of cellular constituents, especially with GSH [12]. The significance of this reaction in the perfused liver can be deduced from Figs 1 and 2. About 10% of NOCAP reacted with GSH under formation of the sulfinamide, GSONHCAP. In addition, NOCAP is reduced non-enzymatically by GSH [12] with formation of NHOHCAP and GSSG, the latter being partly excreted by the bile and venous effluent. These reactions lead to glutathione depletion of the liver (Table 1). Other cytotoxic reactions, like covalent binding to cellular constituents may impair bile production (Fig. 2) and cause membrane damage as signalled by increased LDH release.

The primary reduction product, NHOHCAP, is further reduced to the amine, $NH_2CAP$. This reduction seems to function less effectively than NOCAP reduction and is saturated at lower concentrations as indicated by the dose-dependence of the relative amounts of both reduction products: at 0.46 mM NOCAP the ratio of NHOHCAP/$NH_2CAP$ was 4, at 0.19 mM NOCAP, this ratio decreased to unity. Nevertheless, more than 300 nmole NHOHCAP were reduced per min/g liver during the single passage. This value is similar to the apparent $V_{max}$ of 600 nmole/min/g of reduced phenylhydroxylamine observed under identical conditions [27]. The enzymes responsible for NHOHCAP reduction seem to be located mainly in the microsomal fraction. It should be noted that these metabolic flow profiles were obtained under the steady state conditions of single pass perfusion. Actually, NHOHCAP is concomitantly re-oxidized to NOCAP (e.g. autoxidation), enzymatically reduced to the arylamine, and regenerated by N-oxygenation. Likewise, $NH_2CAP$ is also a transient which is partly N-oxygenated, conjugated with glucuronic acid (acid stable $C_3$-O-conjugate, no N-conjugate) and N-acetylated. These latter reactions proceed rather slowly, and the products were detected only during recirculating perfusion. A scheme of the proposed metabolic pathways is shown in Fig. 6.

As to our knowledge, N-oxygenation of $NH_2CAP$ has not been reported hitherto. Under our conditions $NH_2CAP$ was N-oxygenated by rat liver microsomes with an apparent $K_m$ of 0.4 mM $NH_2CAP$ and a $V_{max}$ of 0.28 nmole min/mg protein. These values are in close agreement with those of aniline N-oxygenation as reported by Smith and Gorrod [31]. These authors found a $K_m$ of 0.4 mM and a $V_{max}$ of 0.17 nmole/min/mg microsomal protein. Similar data were also published by Blaauboer *et al.* [32] for aniline N-oxygenation in isolated rat liver cells. This metabolic potential enables the liver to produce N-oxygenated CAP species from $NH_2CAP$, which might be provided by the intestinal flora. Studies in progress should clarify whether N-oxygenated CAP metabolites, especially NHOHCAP, are released by the liver either following reductive metabolism of CAP (e.g. under reduced oxygen tension) or by oxidative metabolism of $NH_2CAP$.

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