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Suicide Inactivation of Rat Liver Cytochrome P-450 by Chloramphenicol *in Vivo* and *in Vitro*

JAMES HALPERT, BIRGITTA NÄSLUND, AND INGVAR BETNÉR

Department of Medical Nutrition, Karolinska Institute, Huddinge Hospital F69, S-141 86 Huddinge, Sweden

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SUMMARY

Intraperitoneal administration of chloramphenicol (100 mg/kg) to phenobarbital-treated rats causes 50% inhibition of liver microsomal 7-ethoxycoumarin and 1,1,2,2 tetrachloroethane metabolism but has no effect on the level of cytochrome P-450 detectable as its carbon monoxide complex or on the NADPH-cytochrome *c* reductase (EC 1.6.2.4) activity. Both the endogenous NADPH oxidase activity and the enzymatic reduction of cytochrome P-450 are inhibited by chloramphenicol treatment, whereas the K_m and K_s for ethoxycoumarin and the cumene hydroperoxide- or iodosobenzene-supported deethylation of ethoxycoumarin are unaffected, suggesting that impaired electron transport to cytochrome P-450 may be the cause of the loss of enzymatic activity. Administration of [^{14}C]chloramphenicol (100 mg/kg) leads to the covalent binding of 0.7 nmole of metabolite(s) per nanomole of the major cytochrome P-450 isozyme. Alkaline hydrolysis of a cytochrome P-450 fraction obtained by chromatography of solubilized ^{14}C-labeled microsomes on octylamino-Sepharose releases oxalic acid and chloramphenicol oxamic acid, whereas enzymatic digestion releases *N*-ε-chloramphenicol oxamyl lysine in addition. These data obtained with radiolabeled chloramphenicol suggest that the same metabolic pathways which lead to the inactivation of cytochrome P-450 *in vitro* are also operative *in vivo*.

INTRODUCTION

The antibiotic chloramphenicol has previously been shown to act *in vitro* as a suicide substrate of the major phenobarbital-induced form of rat liver cytochrome P-450, inactivating the enzyme by virtue of the covalent modification of the protein rather than of the heme moiety (1). The inactivation is accompanied by the covalent binding of 1.5 nmoles of metabolite(s) per nanomole of cytochrome P-450. Approximately 50% of the bound chloramphenicol exists as an adduct of chloramphenicol oxamic acid and the ε-amino group of one or more lysine residues in the cytochrome P-450, whereas the remainder of the bound material is very labile, and appears to be derived from an active metabolite different from the putative acyl chloride which gives rise to the lysine adduct (2–5). *In vitro*, chloramphenicol is a very effective suicide substrate for cytochrome P-450, requiring approximately five turnovers to inactivate the enzyme (2).

In humans, chloramphenicol is known to influence the plasma levels and half-lives of other therapeutic agents (6), and *in vivo* administration of chloramphenicol to experimental animals has been shown to increase barbiturate-induced sleeping time (7–9) and to inhibit various cytochrome P-450-dependent monooxygenase activities assayed *in vitro* (9, 10). Binding of a reactive metabolite of chloramphenicol to cytochrome P-450 *in vivo* has been suggested as the cause of the observed enzyme inhibition (9), and, indeed, covalent binding to rat liver microsomal proteins has been demonstrated 24 hr after administration of radiolabeled chloramphenicol (11). However, recent results suggest that most of the binding observed in the previous studies reflects radiolabel which has become incorporated into various proteins in the form of serine and glycine rather than labeled covalent adducts of chloramphenicol metabolites (12). Furthermore, in none of the previous studies has the precise mechanism of the *in vivo* inhibition of cytochrome P-450 been elucidated, although inhibition of substrate binding to the enzyme has been suggested as a possible explanation (9).

In the present investigation we have sought to examine the nature of the inhibition of cytochrome P-450-dependent monooxygenase activity resulting from the administration of chloramphenicol to phenobarbital-treated rats, and to relate the inhibition to the covalent binding of chloramphenicol metabolites to cytochrome P-450.

EXPERIMENTAL PROCEDURES

Materials. [1,2-^{14}C]1,1,2,2-tetrachloroethane (9.4 mCi/mmole; >99% pure) and [^{14}C]chloramphenicol [(1*R*,2*R*)-(+)1-*p*-nitrophenyl-2-[1,2-^{14}C]dichloroacetamido-1,3-propane-diol] (43.2 mCi/mmole) were purchased from New England Nuclear Corporation (Dreiech, West Germany). The chloramphenicol was found to be radiochem-

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ically pure (>98%) by thin-layer chromatography on silica gel using the solvent systems chloroform/methanol (100:15) and benzene/methanol/acetic acid (45:8:4). Unlabeled chloramphenicol, chloramphenicol base, dilauryl-L-3-phosphatidylcholine, isocitrate, isocitrate dehydrogenase, Lubrol PX, NADP, NADPH, β-naphthoflavone, prolidase, pronase (*Streptomyces griseus* Type XIV), and glucose oxidase (Type V) were purchased from Sigma Chemical Company (St. Louis, Mo.). Catalase, leucine aminopeptidase, and carboxypeptidase A were purchased from Boehringer Mannheim (Mannheim, West Germany). DEAE-Sephacel and Sepharose 4B were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden), and 2′,5′-ADP agarose and 2′-AMP from P-L Biochemicals (Milwaukee, Wisc.). Sodium deoxycholate and sodium cholate were purchased from Merck (Darmstadt, West Germany). The sodium cholate was used without recrystallization. Cumene hydroperoxide, 1,8-diaminooctane, and cyanogen bromide were obtained from Fluka AG (Buchs, Switzerland). Bio-Beads SM-2 were purchased from Bio-Rad Laboratories (Richmond, Calif.). α,α-Dibromotoluene, iodosobenzene diacetate, 7-ethoxycoumarin, and 7-hydroxycoumarin were purchased from Aldrich (Beerse, Belgium).

Chloramphenicol oxamic acid and its methyl ester were synthesized according to the method of Pohl *et al.* (4). *N*-α-*t*-Butyloxycarbonyl-*N*-ε-chloramphenicol oxamyl-L-lysine was synthesized as described previously (2). The *t*-butyloxycarbonyl group was removed by incubation for 15 min at room temperature with 3.5 N HCl in dioxane. Chloramphenicol alcohol was also synthesized as described previously (3).

Treatment of animals and preparation of microsomes. Adult male Sprague-Dawley rats (200 g) were pretreated with phenobarbital or β-naphthoflavone. Phenobarbital treatment consisted of the addition of 0.1% (w/v) sodium phenobarbital to the drinking water for 5 days or of single daily i.p. injections of 80 mg/kg for 3 days. β-Naphthoflavone was injected i.p. once daily for 3 days at a dose of 40 mg/kg in 0.5 ml of corn oil. The animals were fasted for 24 hr after the last phenobarbital treatment, and then four rats per group each received an i.p. injection of chloramphenicol in 0.5 ml of propylene glycol or an injection of vehicle alone. One hour after the chloramphenicol treatment, the animals were killed by a blow to the head and liver microsomes were prepared as described by van der Hoeven and Coon (13). The microsomes were stored at −70° in 10 mM Tris acetate buffer (pH 7.4) containing 20% glycerol and 1 mM EDTA.

Purification of enzymes. NADPH-cytochrome P-450 reductase and the major form of cytochrome P-450 were purified from liver microsomes of phenobarbital-treated rats according to the method of Guengerich and Martin (14), with the following modifications: After sample application the octylamino-Sepharose column was washed with buffer containing 0.5% rather than 0.42% sodium cholate. The cytochrome P-450 was then eluted with buffer containing 0.08% rather than 0.06% Lubrol PX, and 0.40% rather than 0.33% sodium cholate. The reductase was eluted with buffer containing 0.40% rather than 0.35% cholate, and 0.20% rather than 0.15% sodium deoxycholate. The cytochrome P-450 fraction from octylamino-Sepharose was chromatographed on a column (2 × 90 cm) of DEAE-Sephacel. A 1.5-liter linear gradient of 25 mM to 100 mM NaCl in buffer was used to elute the various cytochrome P-450 forms. The altered conditions were necessary to separate the major cytochrome P-450 fraction (B_2) from a later fraction of slightly higher molecular weight (B_3).

The B_2 and B_3 fractions and the reductase used in this study were more than 95% pure as judged by sodium dodecyl sulfate/polyacrylamide gel electrophoresis according to the method of Laemmli (15). The specific content of these cytochrome P-450 preparations was 15 nmoles/mg of protein based on the protein concentration determined by the method of Lowry *et al.* (16), using bovine serum albumin as the standard, whereas the B_1 fraction had a specific content of 11 nmoles/mg and was heterogeneous upon electrophoresis (14). The specific activity of the reductase was 44 μmoles of cytochrome *c* reduced per minute per milligram of protein as assayed in 300 mM potassium phosphate buffer (pH 7.7 at 25°).

Microsomal incubations with chloramphenicol. Unless otherwise noted, microsomal incubations were carried out at 37° using 0.9 mg of microsomal protein, 0.5 mM NADP, 5 mM isocitrate, 15 mM $MgCl_2$, 0.1 mM EDTA, 100 μM [^{14}C]chloramphenicol (added in 10 μl of ethanol), and 0.6 unit of isocitrate dehydrogenase in a volume of 1 ml of 0.05 M Hepes[1] buffer. Incubations were terminated by the addition of 3 ml of methanol. After vortex mixing and centrifugation, the methanol extract was discarded. Three additional methanol washes were performed, after which the protein precipitate was suspended in 0.5 ml of 1 N NaOH and treated at 60° for 60 min. Aliquots of the solubilized protein were taken for liquid scintillation and protein determination (16).

Incubation of a reconstituted system with chloramphenicol. Incubations were carried out for 30 min at 37° using 2 nmoles of cytochrome P-450, 2 units of reductase, 100 μg of dilauryl-L-3-phosphatidylcholine, 200 μg of sodium deoxycholate, 0.05 M Hepes buffer (pH 7.5), 15 mM $MgCl_2$, 0.1 mM EDTA, 0.25 mM chloramphenicol added in 20 μl of absolute ethanol, and 0.4 mM NADPH in a final volume of 2 ml. The NADPH was added in two equal aliquots, one at time zero and one after 15 min. Controls contained NADPH but no chloramphenicol. The samples were dialyzed for 48 hr at 4° against four 1-liter volumes of 10 mM Tris-acetate (pH 7.4) containing 20% glycerol and 0.1 mM EDTA.

Assays of monooxygenase and oxidase activity. Microsomal metabolism of 1,1,2,2-tetrachloroethane was assayed as described previously (17). Microsomal metabolism of 7-ethoxycoumarin was assayed essentially according to the method of Greenlee and Poland (18). The incubation medium contained 0.1 nmole of microsomal cytochrome P-450, 0.5 mM NADP, 5 mM isocitrate, 15 mM $MgCl_2$, 0.1 mM EDTA, 0.3 mM 7-ethoxycoumarin, and 0.6 unit of isocitrate dehydrogenase in a volume of 1 ml of 0.05 M Hepes buffer (pH 7.4). After a 3-min preincubation at 37°, the reaction was started by addition of the isocitrate and allowed to proceed for 10 min. When

[1] The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-pressure liquid chromatography.

the metabolism of ethoxycoumarin was assayed with a reconstituted system, the microsomes were replaced by 0.05 nmole of cytochrome P-450, 0.3 unit of reductase, 30 μg of dilauryl phosphatidylcholine, and 100 μg of sodium deoxycholate, and the NADPH-generating system by 0.2 mM NADPH. After a 3-min preincubation at 37°, the reaction was started by addition of the NADPH and allowed to proceed for 5 min. Incubations were terminated by the addition of 0.1 ml of 2 N HCl; after extraction with chloroform and 30 mM sodium borate, the 7-hydroxycoumarin formed was determined with a Shimadzu RF-510 spectrofluorimeter.

For assay of endogenous microsomal NADPH oxidase activity and steady-state levels of enzymatically reduced cytochrome P-450, microsomes were suspended in 10 mM Tris-acetate buffer (pH 7.4) containing 20% glycerol and 0.1 mM EDTA at a concentration of 0.5 nmole of cytochrome P-450 per milliliter. The NADPH oxidase activity was assayed at 37° with an Aminco DW-2a spectrophotometer by monitoring the decrease in absorbance at 340 nm after addition of NADPH to a final concentration of 0.2 mM. Steady-state levels of the ferrous carbonyl complex of cytochrome P-450 under aerobic conditions were assayed with a Cary 219 spectrophotometer after bubbling with carbon monoxide for 10 min and addition of NADPH to 0.2 mM. Microsomal NADPH oxidase activity in the presence of ethoxycoumarin was monitored at 340 nm as described above, but the microsomes were suspended at a concentration of 0.5 nmole of cytochrome P-450 per milliliter in 1 ml of 0.05 M Hepes buffer containing 15 mM $MgCl_2$ and 0.1 mM EDTA. After a 3-min preincubation at 37°, NADPH was added to a concentration of 0.2 mM, and the reaction was allowed to proceed for 5 min. The incubations were terminated by transfer of the contents of the cuvette to a tube containing 0.1 ml of 2 N HCl, and 7-hydroxycoumarin was determined as described above. NADPH oxidase activity in a reconstituted system in the absence or presence of 0.3 mM ethoxycoumarin was assayed using 0.05 nmole of cytochrome P-450, 0.3 unit of reductase, 30 μg of dilauryl phosphatidylcholine, 100 μg of sodium deoxycholate, 0.2 mM NADPH, 15 mM $MgCl_2$, and 0.1 mM EDTA, in a final volume of 1 ml of Hepes buffer. Incubations were carried out as described for the microsomal system. Under the conditions used, all assays of monooxygenase and oxidase activity were linear with cytochrome P-450 and with time.

Spectral binding studies. Binding studies were carried out at 20° in 50 mM potassium phosphate buffer (pH 7.4) at a microsomal cytochrome P-450 concentration of 1.0 nmole/ml. Ethoxycoumarin was added from a 6 mM or 60 mM solution in dimethyl sulfoxide to give final concentrations ranging from 4 to 240 μM. The amount of dimethyl sulfoxide added never exceeded 15 μl/3-ml sample. A Cary 219 spectrophotometer was used for all measurements.

Kinetics of cytochrome P-450 reduction. The rate of cytochrome P-450 reduction with NADPH under anaerobic conditions in the presence of an enzymatic oxygen-scavenging system was determined essentially according to the method of Peterson *et al.* (19), except that the microsomes were suspended in 50 mM potassium phosphate rather than Hepes buffer. An Aminco DW-2 spectrophotometer equipped with an Aminco-Morrow stopped-flow apparatus was used for these experiments. Measurements were carried out at 25° using 0.5 and 1.0 nmole of cytochrome P-450 per milliliter.

Spin state of cytochrome P-450. The spin state of cytochrome P-450 in liver microsomes from control and chloramphenicol-treated rats was estimated from the difference in absorbance at 648 nm between ferric cytochrome P-450 and its ferrous carbonyl complex (20). Microsomes were suspended at a concentration of 4 nmoles of cytochrome P-450 per milliliter in 50 mM Tris-acetate buffer (pH 7.4) containing 150 mM KCl. Spectra were recorded at 30° using an Aminco DW-2a spectrophotometer.

HPLC of soluble metabolites and amino acid adducts of chloramphenicol. HPLC was performed on a Waters μBondapack C_{18} column (3.9 mm × 30 cm) which was eluted with 25% methanol at a rate of 1.0 ml/min. A Laboratory Data Control instrument equipped with two Constametric II pumps was used. The effluent was monitored continuously at 254 nm, and fractions of 1.0 min were collected and monitored by liquid scintillation counting and by UV spectroscopy. The retention times of the standards used were as follows: chloramphenicol oxamic acid, 2.2 min; chloramphenicol alcohol, 5.7 min; chloramphenicol oxamyl-L-lysine, 6.2 min; and chloramphenicol, 19.3 min.

RESULTS

Effect of in vivo treatment with chloramphenicol on rat liver microsomal enzymes. Previous *in vivo* studies with phenobarbital-treated and untreated mice and with untreated rats have shown significant effects of chloramphenicol on liver microsomal monooxygenase activity at doses of 50–100 mg/kg, using treatment times of 30–60 min (7–10). In the present study, phenobarbital-treated rats received i.p. injections of chloramphenicol (50 or 100 mg/kg), and the animals were killed 60 min later. Initially, the metabolism of 1,1,2,2-tetrachloroethane was monitored. The dose of 50 mg/kg caused a 25% decrease in tetrachloroethane metabolism ($p < 0.05$), whereas the dose of 100 mg/kg caused a 53% inhibition ($p < 0.001$).

The microsomes from the group receiving 100 mg/kg were also examined with respect to cytochrome P-450 content, 7-ethoxycoumarin metabolism, NADPH-cytochrome *c* reductase activity, and NADPH oxidase activity in the absence of substrate. As can be seen in Table 1, there was no effect of chloramphenicol treatment on the level of cytochrome P-450 detectable as its carbon monoxide complex or on the cytochrome *c* reductase activity, as has been shown previously *in vivo* and *in vitro* (1, 9). On the other hand, both the metabolism of 7-ethoxycoumarin and the endogenous NADPH oxidase activity were significantly inhibited by chloramphenicol treatment. Since the above determinations utilizing individual microsomes from the chloramphenicol-treated and control groups clearly showed that inhibition of monooxygenase activity was a specific effect of the chloramphenicol treatment, subsequent experiments aimed at elucidating the precise nature of the inhibition were performed using pooled microsomes from each group.

TABLE 1

Effect of chloramphenicol administration on rat liver microsomal enzymes

Unless otherwise noted, each value represents the mean ± standard deviation of duplicate determinations on individual microsomes from four rats per group

Microsomal constituent[a]	Control	Chloramphenicol[b]
Cytochrome P-450 (nmoles/mg protein)	2.56 ± 0.26	2.55 ± 0.27
NADPH-cytochrome *c* reductase (units/mg)	0.59 ± 0.05	0.67 ± 0.05[c]
NADPH oxidase (nmoles/min/nmole cytochrome P-450)	6.72 ± 1.22	4.85 ± 0.56[c, d]
7-Ethoxycoumarin deethylase (nmoles/min/nmole cytochrome P-450)	2.81 ± 0.10	1.58 ± 0.08[d]
1,1,2,2-Tetrachloroethane metabolism (nmoles/min/nmole cytochrome P-450)	1.84 ± 0.22	0.87 ± 0.11[d, e]

[a] Assays were performed as described under Experimental Procedures.

[b] Chloramphenicol, 100 mg/kg i.p. in 0.5 ml of propylene glycol, 1 hr prior to sacrifice; controls received vehicle only.

[c] Results of duplicate determinations on individual microsomes from three rats per group.

[d] Significantly different ($p < 0.05$) from respective control value; Student's *t*-test.

[e] Results of single determinations on individual microsomes from four rats per group.

Effect of chloramphenicol on kinetics of 7-ethoxycoumarin metabolism and on substrate binding. Three experiments were carried out in which the steady-state kinetics of ethoxycoumarin metabolism was examined at substrate concentrations between 10 and 240 μM. In all cases the V_{max} was found to be 48–50% lower in the microsomes from the chloramphenicol-treated rats, whereas there was no difference in the K_m values (0.15 mM) between the two groups.

In both the control and chloramphenicol-treated microsomes, 7-ethoxycoumarin was found to induce a Type I difference spectrum with a trough at 430 nm and a peak at 410 nm. Double-reciprocal plots revealed no difference in the K_s (15 μM) or in the ΔA_{max} (6 mM^{-1} cm^{-1}) between the two groups.

Effect of chloramphenicol administration on cytochrome P-450 reduction. The chloramphenicol-mediated decrease in endogenous NADPH oxidase activity in the absence of any effect on the level of dithionite-reduced cytochrome P-450 or on the NADPH-cytochrome *c* reductase activity (Table 1) suggested that some modification of the cytochrome P-450 had occurred which inhibited its ability to accept electrons from the reductase. Therefore, the steady-state levels of the ferrous carbonyl complex of cytochrome P-450 under aerobic conditions and the kinetics of cytochrome P-450 reduction under anaerobic conditions were examined using NADPH as the source of reducing equivalents. In the microsomes from the control rats, the level of the ferrous carbonyl complex upon reduction with NADPH was 60% of the level obtained with dithionite, whereas the corresponding value for the microsomes from the treated animals was 42%. Thus the relative amount of enzymatically reducible cytochrome P-450 in the treated group was 30% lower than that in the control group, which agreed well with the 30% loss of endogenous NADPH oxidase activity. To confirm that the decrease in the steady-state level of cytochrome P-450 was due to a slower reduction rather than to an enhanced auto-oxidation (21), the rate of cytochrome P-450 reduction was determined under anaerobic conditions in a stopped-flow spectrophotometer, which yielded initial rates of 18 and 12 min^{-1} for the control and treated groups, respectively.

Effect of chloramphenicol on cumene hydroperoxide- and iodosobenzene-dependent ethoxycoumarin deethylase activity. In order to short-circuit the steps involving an interaction between the cytochrome P-450 and the reductase (22), the metabolism of 7-ethoxycoumarin in the presence of cumene hydroperoxide or iodosobenzene was examined by direct fluorimetry. At room temperature the hydroperoxide-supported reaction was found to be linear with time for up to 25 sec and linear with protein using up to 1.0 nmole of cytochrome P-450 per milliliter. As can be seen in Table 2, there was no difference in the cumene hydroperoxide-supported metabolism of ethoxycoumarin between the chloramphenicol-treated and control groups, whereas the NADPH-dependent activity assayed under the same conditions showed the expected 45% inhibition.

In the presence of 1 mM iodosobenzene diacetate, ethoxycoumarin deethylation was linear for 5 sec using up to 1.0 nmole of cytochrome P-450 per milliliter. Under these conditions the microsomes from both the control and chloramphenicol-treated rats catalyzed the formation of 8 nmoles of hydroxycoumarin per minute per nanomole of cytochrome P-450.

Relationship between loss of NADPH oxidase activity and loss of 7-ethoxycoumarin deethylase activity. The lack of effect of chloramphenicol administration on the apparent binding of or on the cumene hydroperoxide- or

TABLE 2

Effect of chloramphenicol administration on NADPH- and cumene hydroperoxide-supported metabolism of 7-ethoxycoumarin

Results represent the mean ± standard deviation of duplicate determinations on pooled microsomes from three animals per group.

Treatment	7-Hydroxycoumarin formation[a]	
	NADPH-supported[b]	Cumene hydroperoxide-supported[c]
	nmoles/min/nmole cytochrome P-450	
Control	0.450 ± 0.010	0.152 ± 0.006
Chloramphenicol[d]	0.246 ± 0.037	0.153 ± 0.004

[a] Microsomes were incubated at 20° with 300 μM 7-ethoxycoumarin in a fluorimetric cuvette containing 0.05 M Hepes buffer (pH 7.4), 15 mM $MgCl_2$, and 0.1 mM EDTA in a volume of 3.5 ml. After a 3-min preincubation, the reaction was started by the addition of 140 μl of 10 mM NADPH or 35 μl of 100 mM cumene hydroperoxide in absolute ethanol. A Shimadzu RF-510 spectrofluorimeter with a Kipp & Zonen linear recorder was used. The rate of formation of 7-hydroxycoumarin was determined from the linear portion of the curve (excitation 368 nm/emission 450 nm). All reaction rates were linear with cytochrome P-450 under the conditions used.

[b] Cytochrome P-450 0.5 nmole/ml; linear for 5 min.

[c] Cytochrome P-450 1.0 nmole/ml; linear for 25 sec.

[d] Chloramphenicol, 100 mg/kg i.p. in 0.5 ml of propylene glycol, 1 hr prior to sacrifice; the controls received vehicle only.

iodosobenzene-supported metabolism of 7-ethoxycoumarin suggested inhibition of some step intermediate to substrate binding and hydroxylation, and indeed inhibition of the transfer of the first electron from the reductase to the cytochrome P-450 could be directly demonstrated. However, it remained unclear whether the magnitude of the inhibition of the cytochrome P-450 reduction (30%) was sufficient to account for the entire loss of deethylase activity (45%).

To investigate this question further, experiments were also carried out with microsomes pooled from two groups of four phenobarbital-treated rats, each of which had received chloramphenicol (300 mg/kg) or vehicle only. In this case a 51% loss of ethoxycoumarin deethylase activity was observed along with a 40% loss of endogenous NADPH oxidase activity and a 37% decrease in the aerobic steady-state level of enzymatically reducible cytochrome P-450. Furthermore, when NADPH oxidation in the presence of substrate and 7-hydroxycoumarin formation were monitored, the loss of oxidase activity was sufficient to account for the entire loss of monooxygenase activity. Estimation of the spin state of cytochrome P-450 at 30° revealed that approximately 75% was in the high-spin state in the microsomes from both the control and chloramphenicol-treated animals.

Since the microsomes contain several different forms of cytochrome P-450 which might make different relative contributions to the oxidase and monooxygenase activities, four experiments were also carried out with a reconstituted system which was incubated with chloramphenicol at a reductase to cytochrome P-450 ratio of 1 unit/nmole. After removal of all unbound chloramphenicol by dialysis, a saturating amount of reductase was added, and the NADPH oxidase activity in the absence of substrate and the deethylation of 7-ethoxycoumarin were determined. The samples exhibited a 30–50% loss of ethoxycoumarin deethylase activity and a 20–55% loss of NADPH oxidase activity. The loss of oxidase activity amounted to 75–128% of the loss of monooxygenase activity (average 105 ± 20%). In two of the experiments the NADPH oxidase activity in the presence of substrate was also measured, and the ratio of nanomoles of 7-hydroxycoumarin formed to nanomoles of NADPH oxidized was found to be 0.45 for both the treated and control samples.

Covalent binding of chloramphenicol metabolites in vivo. After it was established that a 100 mg/kg dose of chloramphenicol caused approximately 50% inhibition of various monooxygenase activities assayed *in vitro*, the same dose of radiolabeled compound (1 mCi/mmole) was administered to two phenobarbital-treated rats, and microsomes were prepared after 1 hr. The washed microsomes contained 3.3 nmoles of ^{14}C per milligram of protein, which, when expressed on the basis of the cytochrome P-450 content, corresponds to 1.7 nmoles of ^{14}C per nanomole of cytochrome P-450. More than 97% of the radioactivity associated with the microsomes could be precipitated with trichloroacetic acid, suggesting that essentially all of the nonmetabolized chloramphenicol had been removed during preparation of the microsomes. This interpretation was confirmed by a number of criteria, described below.

When the microsomes were treated for 2 hr at room temperature with 1 N NaOH, 70% of the radiolabel was released, in excellent agreement with results obtained with microsomes labeled *in vitro*. This finding indicated that the major part (if not all) of the label associated with the microsomes was externally bound, rather than incorporated into the peptide chain of various proteins in the form of serine or glycine, as is the case with the label associated with serum proteins isolated 24 hr after chloramphenicol administration (12). Upon chromatography on Bio-Rad P-2 (Fig. 1), the material released by the alkaline hydrolysis was found to consist mainly of oxalic acid (*Ve* = 60 ml) and chloramphenicol oxamic acid (*Ve* = 103 ml), also in excellent agreement with results obtained *in vitro*. Furthermore, only a minor amount of radiolabel eluted in the position of dichloroacetic acid (*Ve* = 68 ml), the hydrolysis product of unmetabolized chloramphenicol (3).

The finding that alkaline hydrolysis of the ^{14}C-labeled microsomes released predominantly oxalic and chloramphenicol oxamic acid in approximately equal amounts suggested that the same metabolic pathways previously implicated in the formation of bound adducts of chloramphenicol *in vitro* are also operative *in vivo*. Indeed, chloramphenicol oxamic acid has previously been identified as a urinary metabolite in rats (23), but it was not clear from the study in question whether the acid was formed by a cytochrome P-450-catalyzed dechlorination reaction or by oxidation of chloramphenicol aldehyde, a metabolite produced by the action of glutathione-*S*-transferases (24). To provide further evidence for the cytochrome P-450-dependent oxamyl chloride pathway (2–5) for the formation of chloramphenicol oxamic acid *in vivo*, the ^{14}C-labeled microsomes were digested with

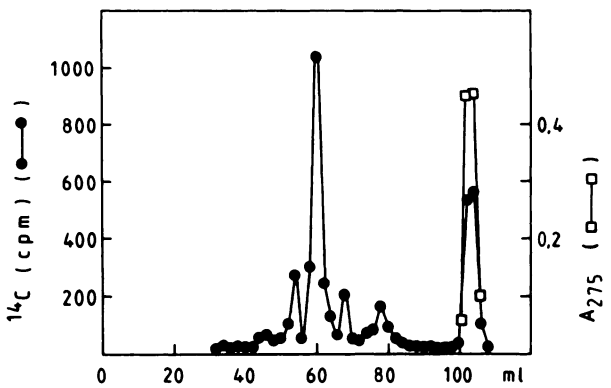

FIG. 1. *Chromatography on a Bio-Rad P-2 column (1 × 150 cm) in 0.05 M N-ethylmorpholine acetate buffer (pH 7.5) of an alkaline hydrolysate of ^{14}C-labeled microsomes*

Two rats each received an i.p. injection of 100 mg/kg [^{14}C]chloramphenicol (1 mCi/mmole) in 0.5 ml of propylene glycol 1 hr prior to sacrifice. Microsomes corresponding to 2 nmoles of cytochrome P-450 were incubated with 0.5 ml of 1 N NaOH in the presence of 250 nmoles of chloramphenicol oxamic acid. After 2 hr at room temperature, 250 μl of 2 N HCl were added, and the microsomal protein was removed by centrifugation. The supernatant was applied to the P-2 column, which was eluted at a rate of 12 ml/hr. Fractions of 2 ml were collected and monitored by liquid scintillation counting (●) and UV spectroscopy (□). The identity of the peak at 103 ml as chloramphenicol oxamic acid was confirmed by HPLC as described under Experimental Procedures.

pronase. Ninety per cent of the radiolabel was solubilized, and this material was examined by chromatography on Bio-Rad P-2. In addition to oxalic acid and chloramphenicol oxamic acid, a peak was observed which coeluted with standard chloramphenicol oxamyl-L-lysine (*Ve* = 120 ml). The identity of the labeled material as the lysine adduct was confirmed by HPLC on C_{18} in 25% methanol, as shown in Fig. 2.

Covalent binding of chloramphenicol metabolites to cytochrome P-450 in vivo. The ^{14}C-labeled microsomes (140 nmoles of cytochrome P-450) were solubilized with 0.6% sodium cholate (14) and chromatographed on a 6-ml column of octylamino-Sepharose. The column was washed with 25 ml of buffer containing 0.5% cholate, and the combined effluents from the sample application and the wash were pooled. The cytochrome P-450 was eluted with 20 ml of buffer containing 0.4% cholate and 0.08% Lubrol. Of the total radioactivity applied, 90% was recovered in the above two fractions, whereas no additional radioactivity could be eluted upon elution of the reductase with buffer containing sodium deoxycholate (14). Of the radioactivity recovered in the sample plus wash, 91% could be precipitated with trichloracetic acid, whereas the corresponding figure for the cytochrome P-450 fraction was >98%. The cytochrome P-450 fraction contained only 30% of the trichloroacetic acid-precipitable radioactivity, and the ratio of nanomoles of ^{14}C to nanomoles of cytochrome P-450 in the P-450 fraction was 0.76.

Treatment of the cytochrome P-450 fraction from octylamino-Sepharose with 1 N NaOH for 2 hr at room temperature released oxalic acid and chloramphenicol oxamic acid, in confirmation of the results obtained with the intact microsomes and in agreement with results obtained with cytochrome P-450 which had been labeled *in vitro*. Furthermore, 35% of the radiolabel in the cytochrome P-450 fraction was stable to hydroxylamine, indicative of a lysine adduct (2, 3). This was confirmed by enzymatic digestion and chromatography on Bio-Rad P-2 and on C_{18}, performed essentially as described for the intact microsomes (Fig. 2), with the exception that the pronase was supplemented with prolidase, leucine aminopeptidase, and carboxypeptidase [each at an enzyme to substrate ratio of 1:20 (w/v)]. Inclusion of these other enzymes was found to be necessary to achieve complete digestion of all small peptides to amino acids.

When the cytochrome P-450 fraction from octylamino-Sepharose was chromatographed on a DEAE-Sephacel column (Fig. 3), approximately equal ratios of $^{14}C/A_{417}$ were found in all four cytochrome P-450 fractions. It should be pointed out, however, that only the B_2 fraction was electrophoretically homogeneous; the A and B_1 fractions contained several other proteins, and the B_3 fraction was partially contaminated with B_2.

To confirm that the B_2 isozyme was inhibited by the covalent binding of chloramphenicol metabolites *in vivo*, the cytochrome was purified from liver microsomes of 10 phenobarbital-treated rats which had received chloramphenicol (300 mg/kg). When assayed toward ethoxycoumarin in a reconstituted system, the B_2 isozyme from the chloramphenicol-treated rats showed only 20% of the activity of a similar preparation from untreated rats.

Comparison of in vivo and in vitro covalent binding of chloramphenicol. Previous *in vitro* studies using intact microsomes, in which covalent binding to different proteins was monitored by gel electrophoresis, indicated that

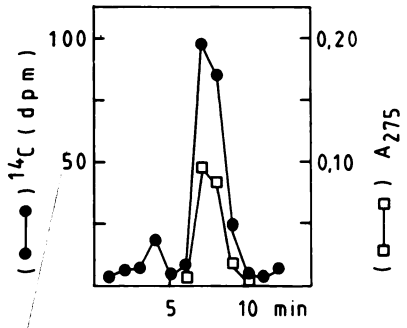

FIG. 2. *HPLC of a ^{14}C adduct derived from a pronase digest of ^{14}C-labeled microsomes*

Microsomes were labeled as described in Fig. 1, and 5 mg of microsomal protein in 0.5 ml of 0.05 M *N*-ethylmorpholine acetate buffer (pH 7.5) containing 0.1% sodium dodecyl sulfate were incubated for 22 hr at 37° with 0.5 mg of pronase. The volume was adjusted to 1 ml, and insoluble material was removed by centrifugation. Standard chloramphenicol oxamyl-L-lysine (250 nmoles) was added, and the sample was chromatographed on Bio-Rad P-2 as described in Fig. 1. The ^{14}C-labeled material which coeluted with the standard (*Ve* = 116 ml) was lyophilized and chromatographed on a Waters μBondapack column as described under Experimental Procedures. Fractions of 1 min were collected and monitored by liquid scintillation counting (●) and by UV spectroscopy (□).

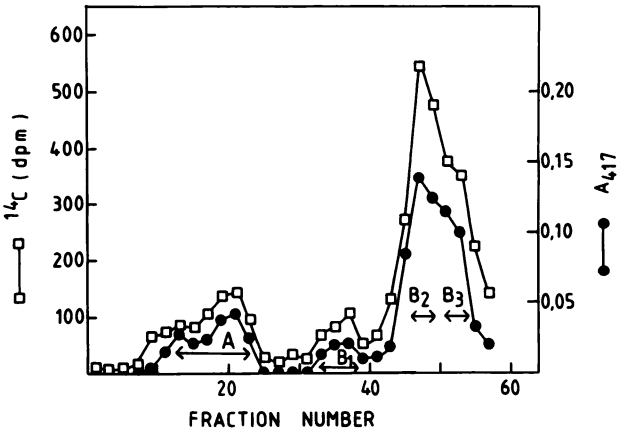

FIG. 3. *Chromatography on a DEAE-Sephacel column (1 × 45 cm) of a cytochrome P-450 fraction obtained by octylamino-Sepharose chromatography of ^{14}C-labeled microsomes*

Microsomes were labeled with chloramphenicol as described in Fig. 1 and solubilized and chromatographed on octylamino-Sepharose as described under Results. After dialysis for 2 hr at 4° against 20% glycerol containing 0.1 mM EDTA, and for 2 hr against 10 mM potassium phosphate buffer (pH 7.7) containing 20% glycerol, 0.1% Lubrol, 0.2% sodium cholate, and 0.1 mM EDTA, the cytochrome P-450 fraction was applied to the DEAE-Sephacel column, which was equilibrated in the same buffer. The column was washed with 30 ml of equilibration buffer and then eluted with a 250-ml linear gradient of 25–100 mM NaCl in buffer. The column was eluted at a rate of 15 ml/hr, and fractions of 3 ml were collected and monitored by liquid scintillation counting (□) and for absorbance at 417 nm (●). Fractions 42–58 were monitored by sodium dodecyl sulfate/polyacrylamide gel electrophoresis to distinguish isozymes B_2 and B_3. The level of radiolabel associated with the homogeneous B_2 isozyme was 0.7 nmole of ^{14}C per nanomole of cytochrome P-450.

>95% of the bound chloramphenicol was associated with proteins in the molecular weight range of cytochrome P-450 (2). However, since the *in vivo* binding in the present study (which indicated considerable labeling of non-cytochrome P-450 proteins) was not examined by gel electrophoresis but by chromatography on octylamino-Sepharose, it was considered important to investigate the *in vitro* binding by the same methodology.

When phenobarbital-induced microsomes were incubated for various times with chloramphenicol, covalent binding was found to reach a plateau at 1.0 nmole of ^{14}C per nanomole of cytochrome P-450 by 60 min. Fifty nanomoles of microsomal cytochrome P-450 were then incubated for 60 min with [^{14}C]chloramphenicol, solubilized with cholate, and chromatographed on a 3-ml column of octylamino-Sepharose. In this way it could be demonstrated that 96% of the protein-bound radiolabel was associated with the cytochrome P-450 fraction, in excellent agreement with the results obtained by gel electrophoresis. When the solubilized microsomes were applied directly to a DEAE-Sephacel column without prior chromatography on octylamino-Sepharose, approximately 1.5 nmoles of ^{14}C per nanomole of cytochrome P-450 were found to be associated with the B_1 and with the combined B_2 and B_3 fractions.

When the individual B_1, B_2, and B_3 forms from phenobarbital-induced microsomes and the B_2 form from microsomes from rats treated with β-naphthoflavone (14) were incubated with chloramphenicol in a reconstituted system, only the phenobarbital-induced B_2 form became covalently labeled. The inability of the β-naphthoflavone-induced form of cytochrome P-450 to form covalent adducts is not surprising, since the corresponding intact microsomes produced only 9% as much covalently bound material as the phenobarbital-induced microsomes when incubated with chloramphenicol for 60 min.

Effect of incubation of a reconstituted system with analogues of chloramphenicol. The functional group in chloramphenicol—the metabolism of which gives rise to intermediates which inactive cytochrome P-450—is the dichloromethyl moiety (2–5). In order to test whether the inhibition caused by chloramphenicol is due to the modification of an important amino acid residue in the cytochrome P-450 or to the introduction of the bulky chloramphenicol oxamyl moiety, experiments were carried out with two other compounds which contain a dihalomethyl moiety, 1,1,2,2-tetrachloroethane and α,α-dibromotoluene. These compounds were incubated with a reconstituted monooxygenase system at a concentration of 0.25 mM, using conditions under which chloramphenicol causes a 30–50% loss of ethoxycoumarin deethylase activity. Tetrachloroethane caused no loss of cytochrome P-450 detectable as its carbon monoxide complex or of monooxygenase activity, consistent with the previous finding that tetrachloroethane metabolism by a reconstituted system causes little covalent binding to cytochrome P-450 (17). On the other hand, in three experiments with α,α-dibromotoluene, a 50–75% loss of cytochrome P-450 and a 70–90% loss of ethoxycoumarin deethylase activity were observed. When the residual ethoxycoumarin deethylase activity was expressed on the basis of the cytochrome P-450 content of the treated samples, a 50% loss of the relative turnover number for ethoxycoumarin could be calculated. Thus it appears that the shorter side chain on α,α-dibromotoluene as compared with that on chloramphenicol allows the active metabolite of the former (presumably benzoyl bromide) to attack both the heme and the protein moiety of cytochrome P-450. The lack of effect of tetrachloroethane suggests that a benzene ring or a similarly hydrophobic group is necessary to direct the active metabolite to functional groups on the enzyme rather than allowing it to be released from the cytochrome P-450. These preliminary experiments suggest that the long side chain in chloramphenicol between the dichloromethyl moiety and the nitrobenzene ring is not required for enzyme inhibition but rather to direct the active metabolite exclusively to the protein rather than to the heme moiety of the cytochrome P-450.

DISCUSSION

The results of the present investigation leave little doubt that the inhibitory effects of chloramphenicol administration *in vivo* on rat liver mixed-function oxidase activity assayed *in vitro* are mediated by the suicide inactivation of cytochrome P-450. Inhibition and covalent modification of the major isozyme of cytochrome P-450 from phenobarbital-treated rats have been demonstrated after chloramphenicol administration *in vivo*, and covalent binding to other cytochrome P-450 isozymes is also indicated. The level of binding (0.7–0.75 nmole of ^{14}C per nanomole of cytochrome P-450) attained with a dose of chloramphenicol which causes approximately 50% inhibition of microsomal 7-ethoxycoumarin or tetrachloroethane metabolism suggests that the stoichiometry of inactivation of 1.5 nmoles of ^{14}C per nanomole of cytochrome P-450 previously shown *in vitro* (1) also applies *in vivo*. Furthermore, examination of the nature of the covalently bound material by alkaline hydrolysis or enzymatic digestion suggests that the same metabolic pathways which give rise to covalently bound adducts of chloramphenicol and cytochrome P-450 *in vitro* are also operative *in vivo*. Thus oxidative dechlorination of chloramphenicol with formation of reactive acyl chlorides (2–5) appears to be an important metabolic pathway *in vivo*. The putative acyl chloride intermediates appear to be less specific for covalent binding to cytochrome P-450 *in vivo* than *in vitro*, since less than one-half of the protein-bound radiolabel is associated with the cytochrome P-450 after *in vivo* administration of [^{14}C]chloramphenicol, whereas after *in vitro* incubation of a reconstituted system or intact microsomes, 95% of the covalent binding is to the cytochrome P-450 (1, 2).

Experiments aimed at elucidating the precise nature of the inhibition of cytochrome P-450-dependent monooxygenase activity by chloramphenicol allow the following conclusions. First, the lack of effect on the K_s and on the cumene hydroperoxide- or iodosobenzene-supported metabolism of 7-ethoxycoumarin suggests that neither substrate binding to the cytochrome P-450 nor substrate hydroxylation and product release are impaired. Second, the inhibition of enzymatic cytochrome P-450 reduction, but not of cytochrome *c* reductase activity caused by chloramphenicol administration *in vivo*, and the inhibi-

tion of endogenous NADPH oxidase activity observed in intact microsomes after *in vivo* administration or in a reconstituted system after incubation with chloramphenicol suggest that some modification of the cytochrome P-450 has taken place which inhibits its ability to accept electrons from the reductase. The loss of oxidase activity appears to be of sufficient magnitude to account for the greater part (if not all) of the loss of monooxygenase activity, and there appears to be little or no effect of chloramphenicol on the efficiency of NADPH utilization by either intact microsomes or a reconstituted system. With regard to the mechanism whereby the covalent binding of chloramphenicol to cytochrome P-450 could inhibit its reduction by the reductase, two attractive hypotheses are alterations in the spin state of the cytochrome P-450 (25) or alterations in the protein-protein interaction between the cytochrome P-450 and the reductase. Spectral estimation of the relative amounts of high-spin cytochrome P-450 (20) in microsomes from control or chloramphenicol-treated rats revealed no significant differences between the two groups. On the other hand, it is of interest that chloramphenicol is activated to a metabolite which binds to a lysine residue in the cytochrome P-450, since complementary charge pairs between cytochrome P-450 and the reductase have been suggested to be necessary for interaction between these two enzymes (26), as has been shown previously for the cytochrome b_5-cytochrome b_5 reductase system (27, 28). Furthermore, membrane charge has been shown to mediate the interaction between cytochrome P-450/$_{LM_2}$ and the reductase in a liposomal system (21), and treatment of cytochrome P-450 with the amino group-specific reagent fluorescamine has been reported to inhibit benzphetamine demethylation without affecting the cytochrome P-450 content or the K_m for the reaction (29). The ability of amino group-specific reagents to mimic the action of chloramphenicol is presently under investigation.

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Send reprint requests to: Dr. James Halpert, Department of Medical Nutrition, Karolinska Institute, Huddinge Hospital F69, S-141 86 Huddinge, Sweden.