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Further Studies of the Suicide Inactivation of Purified Rat Liver Cytochrome P-450 by Chloramphenicol

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SUMMARY

The kinetics and reversibility of the suicide inactivation of rat liver cytochrome P-450 by chloramphenicol have been investigated with the use of a reconstituted monooxygenase system purified from liver microsomes of phenobarbital-treated rats. At a ratio of 1 unit of NADPH-cytochrome P-450 reductase per nanomole of cytochrome P-450 and a chloramphenicol concentration of 1 mM, the $t_{1/2}$ for the inactivation of cytochrome P-450 is less than 2 min. The inactivated cytochrome regains some of its activity upon incubation at 25° or 37°, and experiments with [^{14}C]chloramphenicol show that this partial reactivation is accompanied by the release of some of the ^{14}C originally bound covalently to the cytochrome P-450. Previous work has shown that the ^{14}C-labeled material spontaneously released from ^{14}C-labeled cytochrome P-450 is in the form of oxalic acid, and that the latter is derived from a hydroxylamine-labile adduct of chloramphenicol and cytochrome P-450 [*Biochem. Pharmacol.* 30:875–881 (1981)]. In the present investigation the ^{14}C-labeled material released by hydroxylamine was identified as the hydroxamic acid of oxalic acid. Trapping experiments with the amino acid cysteine suggest that the adduct, the spontaneous degradation of which appears to be involved in the reactivation of cytochrome P-450, contains an ester rather than a thioester linkage between cytochrome P-450 and a metabolite of chloramphenicol. However, this metabolite may not be identical with chloramphenicol oxamyl chloride, which was the active metabolite implicated in the formation of the 50% covalently bound material which was stable to hydroxylamine treatment.

INTRODUCTION

The antibiotic chloramphenicol has recently been shown to act 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, 2). Approximately 50% of the covalently-bound metabolite(s) was found to exist in the form of *N*-ε-chloramphenicol oxamyl-L-lysine (2), apparently formed by the reaction of the active metabolite chloramphenicol oxamyl chloride (3, 4) with the ε-amino group of one or more lysine residues in cytochrome P-450. Modification of lysine was shown to play a major role in the loss of cytochrome P-450-dependent monooxygenase activity caused by chloramphenicol.

The remaining 50% of the covalently bound chloramphenicol appeared to reside in an ester or a thioester linkage with the cytochrome P-450, since this material could be readily cleaved from the protein with neutral hydroxylamine (2). However upon hydrolysis, most of this component of the covalently bound chloramphenicol was released as oxalic acid rather than chloramphenicol oxamic acid, the expected product if the hydroxylamine-labile material were derived from chloramphenicol oxamyl chloride (2–4). The stoichiometry of the inactivation of the cytochrome P-450 suggested that the hydroxylamine-labile adducts might play a role in the loss of monooxygenase activity. The present investigation has focused on the nature of the hydroxylamine-labile adducts formed during the suicide inactivation of purified rat liver cytochrome P-450 by chloramphenicol and on the role of such adduct formation in the loss of enzymatic activity.

MATERIALS AND METHODS

Materials. [^{14}C]chloramphenicol [(1R,2R)-(+)-1-*p*-nitrophenyl-2-[1,2-^{14}C]dichloroacetamido-1,3-propanediol], 43.2 mCi/mmole, was purchased from New England Nuclear Corporation (Boston, Mass.). The compound was found to be radiochemically 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, pronase (*Streptomyces griseus* protease, Type VI), glucose 6-phosphate dehydrogenase, NADP, and

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NADPH were purchased from Sigma Chemical Company (St. Louis, Mo.); leucine aminopeptidase and glucose 6-phosphate were obtained from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). Methyl oxalyl chloride, *N*-hydroxysuccinimide, and *N,N'*-dicyclohexyl carbodiimide were purchased from Aldrich Chemical Company (Milwaukee, Wisc.). *N*-α-*t*-butyloxycarbonyl-L-lysine was purchased from Vega-Fox (Tucson, Ariz.). 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*-butyloxylcarbonyl group was removed by incubation for 15 min at room temperature with 3.5 N HCl in dioxane.

Synthesis of N-ε-oxalyl-L-lysine. *N*-α-*t*-butyloxylcarbonyl-L-lysine (1.0 mmole), methyl oxalyl chloride (1.8 mmoles), and sodium methoxide (2 mmoles) were incubated in 5 ml of methanol for 30 min at room temperature. The incubation mixture was rotary-evaporated to dryness and dissolved in 3 ml of distilled water, and the pH was adjusted to 5 with glacial acetic acid (20 μl). The sample was extracted with three washes of 10 ml of ethyl acetate, and the combined extracts were washed with two 1-ml portions of 0.1 N acetic acid. Thin-layer chromatography on silica gel in methanol followed by spraying with ninhydrin confirmed that the unreacted *N*-α-butyloxycarbonyl lysine had been removed by this procedure.

The ethyl acetate extract was rotary-evaporated to dryness and treated for 30 min at room temperature with 4 ml of 3.5 N HCl in dioxane to remove the *t*-butyloxycarbonyl group. After removal of the dioxane, 2 ml of distilled water were added and the sample was washed with two 10-ml portions of ethyl acetate. The water phase containing the *N*-ε-methyl oxalyl-L-lysine was examined by DEAE-cellulose thin-layer chromatography in 0.05 M *N*-ethylmorpholine acetate buffer (pH 7.5), which revealed a single ninhydrin-positive spot with an R_F of 1. After incubation for 30 min at room temperature with 1 N NaOH to remove the methyl group, a ninhydrin-positive spot with an R_F of 0.4 on DEAE-cellulose appeared. The structure of this product as *N-E*-oxalyl lysine was confirmed by acid hydrolysis followed by amino acid analysis and titration with permanganate which indicated a concentration of 0.98 μmole of oxalic acid per micromole of lysine. From comparison of the ninhydrin color value before and after hydrolysis, the color factor of the *N-E*-oxalyl lysine was determined to be 90% that of free lysine, which agrees well with various reports in the literature that the extra amino group in lysine makes about a 10% contribution to the total ninhydrin color.

Synthesis of chloramphenicol alcohol. Chloramphenicol alcohol was synthesized from chloramphenicol base and glycolic acid essentially according to the method of Bald *et al.* (5) for the preparation of the monoiodo analogue of chloramphenicol from chloramphenicol base and iodoacetic acid. The structure of the synthetic alcohol was confirmed by gas chromatography-mass spectrometry using a Ribermag R10-10B mass spectrometer equipped with a Serie 31 gas chromatograph and an RDS GC/MS data system. The trimethylsilyl derivative was prepared by treating 40 nmoles of sample with 50 μl of TRISIL (Pierce) for 15 min at 70°. The sample was injected onto a 6 foot × 2 mm glass column packed with 3% OV-17. Helium was employed as the carrier gas at a flow rate of 20 ml/min. The column was operated at 200° for 1 min and then programmed to 280° at 10°/min. The injector and interface temperatures were 300°. The mass spectrometer was operated at 70 eV and 200 μamp. The retention time of the derivatized sample under these conditions was 9.42 min. The mass spectrum was characterized by a base peak at 262 atomic mass units and fragments at 471, 383, 334, 246, 225, and 208 atomic mass units, in excellent agreement with the results of Martin *et al.* (6).

Preparation of microsomes. Adult male Sprague-Dawley rats (100–200 g) were given phenobarbital (0.1% in the drinking water) for 5 days prior to sacrifice. Microsomes were prepared as described by Neal (7), with the final centrifugation performed at 105,000 × *g* for 60 min. Microsomes were stored frozen at −70° in 10 mM Tris-acetate (pH 7.4) containing 20% glycerol and 1 mM EDTA.

Preparation of enzymes. The major form of cytochrome P-450 from liver microsomes of phenobarbital-treated rats was purified as described by Guengerich (8). The method involves chromatography of cholate-lysed microsomes on octylamino-Sepharose 4B (9) in the cold followed by chromatography on DEAE-cellulose at room temperature. The NADPH-cytochrome P-450 reductase-containing-fractions from the octylamino-Sepharose column were further purified by affinity chromatography on ADP 2′,5′ agarose (10). Both enzyme preparations were ≥95% pure as judged by SDS[1]-polyacrylamide gel electrophoresis, performed by the method of Laemmli (11), using a double-strength cathode buffer as described by Guengerich (12). The specific content of the cytochrome P-450 preparation used in this investigation was 15 nmoles/mg of protein based on the protein concentration determined by the method of Lowry *et al.* (13) using bovine serum albumin as standard.

Incubations of the reconstituted system with chloramphenicol. Incubations were carried out for 30 min at 37° using 4 nmoles of cytochrome P-450, 4 units of reductase, 60 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, 0.05 mM [^{14}C]chloramphenicol (43.2 mCi/mmole) added in 40 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 zero time and one after 15 min.

The samples were dialyzed for 48 hr at 4° against four 1-liter portions of 50 mM Tris-acetate (pH 7.4) containing 20% glycerol and 0.1 mM EDTA, and then for 24 hr against distilled water. The protein, which precipitated during the dialysis against distilled water, was collected by centrifugation.

Enzymatic hydrolysis of the ^{14}C-labeled reconstituted system protein. The dialyzed protein (4 nmoles of cytochrome P-450) was suspended in 50 μl of *N*-ethylmorpho-

[1] The abbreviations used are: SDS, sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

line acetate buffer (pH 7.5) containing 0.1% SDS and incubated at 37° with 20 μg of pronase [enzyme/P-450 ratio 1:10 (w/w)] until solubilized (approximately 4 hr). Leucine aminopeptidase (10 μg) was then added, and the digest was allowed to proceed overnight.

Microsomal incubations with chloramphenicol. Microsomal incubations with chloramphenicol were carried out using 6 mg of microsomal protein, 0.2 mM NADP, 2 mM glucose 6-phosphate, 0.1 mM [^{14}C]chloramphenicol (1.2 mCi/mmole) added in 10 μl of absolute ethanol, and 2 units of glucose 6-phosphate dehydrogenase in 2 ml 0.05 M Hepes buffer (pH 7.5) containing 15 mM $MgCl_2$ and 0.1 mM EDTA. The incubation was allowed to proceed for 30 min at 37° and was stopped by addition of 6 ml of absolute ethanol. The reaction mixture was centrifuged and the supernatant was rotary-evaporated to dryness.

High-performance liquid chromatography of a neutral metabolite of chloramphenicol. A large-scale incubation of a reconstituted system containing 25 nmoles of P-450 was carried out with chloramphenicol, and the incubation mixture was extracted three times with 25-ml portions of ethyl acetate. The extract was rotary-evaporated to dryness and dissolved in 50 μl of methanol. One-half was then injected onto a Zorbax ODS column (4.6 mm × 25 cm) which was eluted with 25% methanol at a flow rate of 1 ml/min. Fractions of 1 ml were collected and monitored for radioactivity. In addition to chloramphenicol (R_t = 25 min), a ^{14}C-containing peak at 6 min was detected, which was collected and lyophilized.

RESULTS

Kinetics and reversibility of suicide inactivation of cytochrome P-450 by chloramphenicol. As shown in Fig. 1, when a reconstituted system containing 1 unit of reductase per nanomole of cytochrome P-450 was incubated with chloramphenicol, a rapid inactivation of the 7-ethoxycoumarin deethylase activity of cytochrome P-450 was observed. At chloramphenicol concentrations of 0.1 mM and 1 mM, the $t_{1/2}$ values for the inactivation of the cytochrome P-450 were 5 min and <2 min, respectively. However, after approximately 5 min, the inactivation appeared to cease, and a slow reactivation of the enzyme was subsequently observed. NADPH oxidation was also found to level off at 5 min, and it thus appeared that the inactivation of the P-450 had ceased owing to a lack of NADPH for the further metabolism of chloramphenicol. To test this hypothesis an experiment was carried out with 1 mM chloramphenicol but at a reductase/P-450 ratio of 0.1:1. Under such conditions the NADPH was consumed much more slowly, and the inactivation of the P-450 was linear for at least 20 min (Fig. 1).

Previous studies had shown that a portion of the ^{14}C which becomes covalently bound to the cytochrome P-450 of a reconstituted system during the metabolism of [^{14}C]chloramphenicol is released spontaneously as [^{14}C] oxalic acid upon incubation of the protein at 37° in *N*-ethylmorpholine acetate buffer (pH 7.4) (2). In the present investigation, overnight incubation of the ^{14}C-labeled protein in 50 mM Tris-acetate buffer (pH 7.4) containing 20% glycerol was found to release 10% or 25% of the

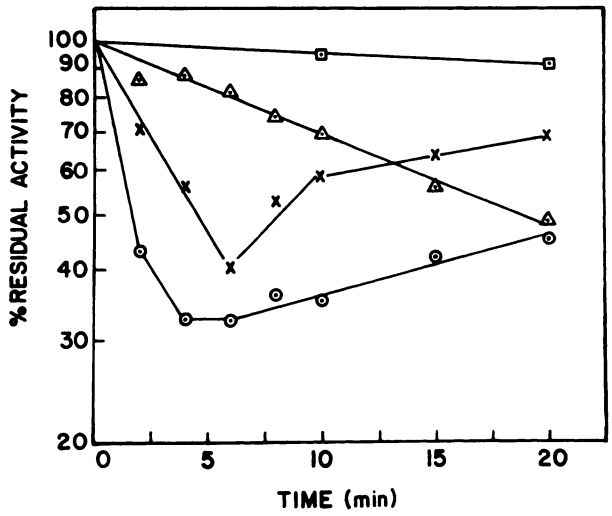

FIG. 1. *Loss of 7-ethoxycoumarin deethylase activity of the cytochrome P-450 of a reconstituted system during incubation with chloramphenicol*

In all experiments the reconstituted system contained 1 nmole of cytochrome P-450, 15 μg of dilauryl L-3-phosphatidyl choline, 50 μg of sodium deoxycholate, 15 mM $MgCl_2$, and 0.1 mM EDTA in a final volume of 0.5 ml of 0.05 M Hepes buffer (pH 7.5). Other components of the incubation mixtures were as follows: ⊡, 1 unit of reductase, 1 mM chloramphenicol *or* 1 unit of reductase, 0.2 mM NADPH; ⊙, 1 unit of reductase, 1 mM chloramphenicol, 0.2 mM NADPH; ×, 1 unit of reductase, 0.1 mM chloramphenicol, 0.2 mM NADPH; △, 0.1 unit of reductase, 1 mM chloramphenicol, 0.2 mM NADPH. Incubations were performed at 37°, and at the times indicated 50-μl aliquots corresponding to 0.1 nmole of cytochrome P-450 were taken and added immediately to 660 μl of ice-cold 0.075 M Hepes buffer. The samples were kept on ice until the final aliquot had been taken, at which time all samples were assayed for ethoxycoumarin deethylase activity in the presence of a saturating amount of the reductase as described previously (1). The presence of up to 0.05 mM chloramphenicol during the ethoxycoumarin assays did not affect the linearity of product formation with time or cytochrome P-450 concentration. However, since chloramphenicol causes competitive inhibition as well as inactivation of the cytochrome P-450 (1, 14), the absolute values of the ethoxycoumarin deethylase activity at time 0 are not the same for all sets of samples. To simplify the data presentation, activities are therefore expressed as a percentage of the value at 0 min for each set of data.

radioactivity at 25° or 37°, respectively. The release of ^{14}C upon incubation at 37° was accompanied by a reactivation of the enzyme relative to untreated samples incubated at 37°, but it was not possible to calculate the precise stoichiometry of the reactivation. In this context it should be noted that reactivation of cytochrome P-450 in intact rat liver microsomes incubated with chloramphenicol has been inferred from the unusual substrate dependence of *o*-nitroanisol metabolism after the chloramphenicol treatment (14).

Nature of hydroxylamine-labile adducts. Previous results had indicated that the [^{14}C]oxalic acid which is released from the labeled cytochrome P-450 during digestion with proteolytic enzymes was derived from a hydroxylamine-labile adduct (2). Evidence was also obtained for the presence of two chemically distinct classes of hydroxylamine-labile adduct, a major one representing approximately 40% of the total radioactivity bound to the P-450, and a minor one representing approximately

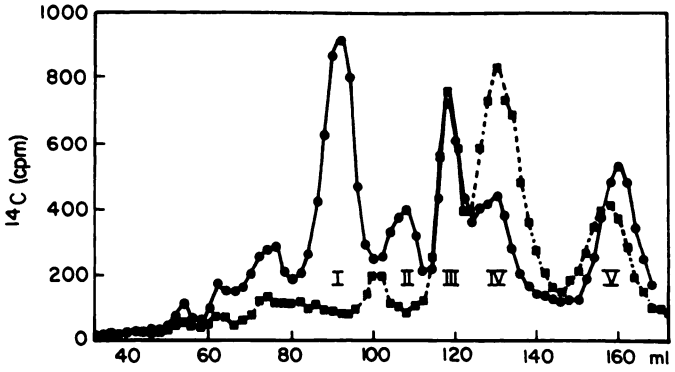

FIG. 2. *Gel filtration on a Sephadex G-10 column (0.9 × 150 cm) in 0.05 M N-ethylmorpholine acetate buffer (pH 7.5) of the supernatant fraction from microsomal incubations with [^{14}C]chloramphenicol in the absence (■- - -■) or presence (●——●) of 5 mM cysteine as described under Materials and Methods*

The column was operated at 12 ml/hr, and fractions of 2 ml were collected and analyzed for radioactivity. The column was washed with 1 M pyridine prior to use. Unmodified chloramphenicol elutes at 295 ml on the same column under the same conditions.

10% of the covalently bound label. The major labile adduct was entirely degraded to oxalic acid under the conditions used to digest the labeled P-450 with proteolytic enzymes, whereas the minor adduct could be isolated as such at neutral but not at acidic pH. The chemical properties of the minor adduct were suggestive of a cysteine adduct.

To prepare a cysteine adduct of chloramphenicol, trapping experiments were carried out by including 5 mM cysteine in the incubation medium during microsomal incubations with chloramphenicol. As shown in Fig. 2, in the absence of cysteine three metabolites are obtained: Peak III, which corresponds to the known microsomal metabolite dichloroacetic acid (15); Peak IV, which corresponds to chloramphenicol oxamic acid (3, 4); and Peak V, which is as yet unidentified. Peak V requires the presence of NADPH and appears to have its dichloroacetyl moiety intact, since it can be hydrolyzed to dichloroacetic acid upon treatment with 1 N NaOH for 2 hr at room temperature. In the presence of cysteine, two new peaks (Peaks I and II) appear, whereas the amount of chloramphenicol acid is decreased substantially. Peak I elutes in the same position as the amino acid adducts of chloramphenicol and cytochrome P-450 isolated from pronase digests of the ^{14}C-labeled protein (2). Peak I was found to consist of two chemically distinct classes of adducts, one of which was stable to hydroxylamine and to chromatography on a Beckman amino acid analyzer, suggesting derivatization of the α-amino group. The other class of cysteine adduct was cleaved by hydroxylamine and was not retained by the amino acid analyzer, suggesting that it had been hydrolyzed at the low pH (2.2) used to dissolve the sample. The hydroxylamine-labile cysteine adduct had an R_F of 0.95 upon thin-layer chromatography on cellulose in 1-butanol/pyridine/acetic acid/water (15:10:3:12). With respect to chemical stability and chromatographic behavior, the labile cysteine adduct exhibited properties identical with those of the minor hydroxylamine-labile adduct isolated from the digest of the ^{14}C-labeled cytochrome P-450 (2).

The appearance of the cysteine adducts in Peak I at the expense of chloramphenicol oxamic acid is consistent with the acyl chloride intermediate of Pohl *et al.* (3) (Fig. 3), as is the structure of the lysine adduct *N*-ε-chloramphenicol oxamyl-L-lysine (Fig. 4), previously shown to account for approximately 50% of the chloramphenicol covalently bound to the P-450 (2). However, it was unclear how such an adduct of chloramphenicol acid would give rise to oxalic acid at neutral pH, since this would require the hydrolysis of two linkages.

Model studies with derivatives of chloramphenicol oxamic acid. In order to investigate whether the ^{14}C oxalic acid derived from the ^{14}C-labeled cytochrome P-450 could have resulted from the degradation of a protein-bound derivative of chloramphenicol oxamic acid, the products of the alkaline hydrolysis of chloramphenicol acid methyl ester and of *N*-ε-chloramphenicol oxamyl-L-lysine were examined. In neither case was any evidence obtained for the cleavage of more than one of the linkages susceptible to hydrolysis. Thus the methyl ester of chloramphenicol acid yielded only the free acid but no chloramphenicol base upon treatment with 1 N NaOH for 2 hr at room temperature. When the lysine derivative of chloramphenicol acid was treated in a similar fashion, amino acid analysis revealed that 60% of the ninhydrin-positive material was released as lysine and 40% as *N*-ε-oxalyl lysine (Fig. 5), whereas chromatography on Sephadex G-10 revealed that 60% of the UV-

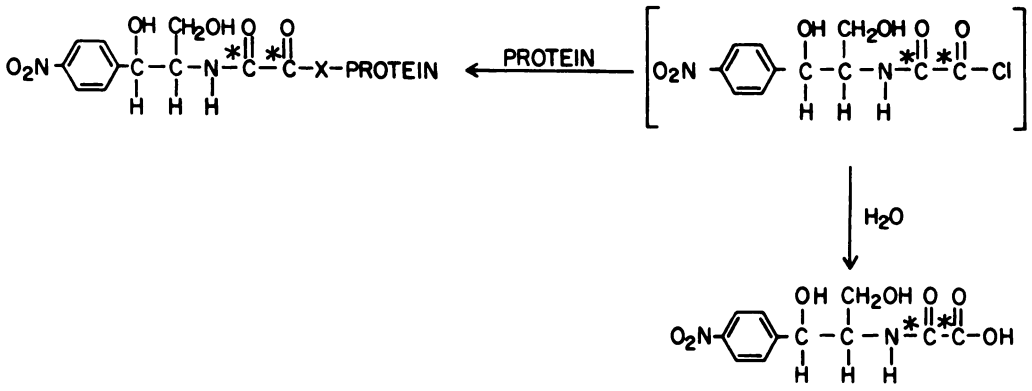

FIG. 3. *Structure and reactions of the putative active intermediate of chloramphenicol produced by the cytochrome P-450-dependent monooxygenase system as proposed by Pohl et al. (3, 4)*

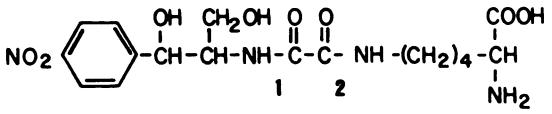

FIG. 4. *Structure of N-ε-chloramphenicol oxamyl-L-lysine showing the two amide linkages*

Hydrolysis of Linkage 1 yields chloramphenicol base and *N*-ε-oxalyl lysine, whereas hydrolysis of Linkage 2 yields chloramphenicol oxamic acid and lysine.

absorbing material was released as chloramphenicol acid and 40% as chloramphenicol base. All of these results are consistent with the hypothesis that the negative charge introduced upon hydrolysis of either the amide linkage in chloramphenicol or the linkage between the terminal carboxyl group of chloramphenicol acid and a hydroxyl or amino group inhibits the hydrolysis of the second susceptible linkage. Thus it does not appear likely that a protein-bound derivative of chloramphenicol oxamic acid would degrade spontaneously to oxalic acid at neutral pH.

The nature of the ^{14}C-labeled material released from the labeled P-450 by hydroxylamine-treatment was also examined. As seen in Fig. 6, this material was distinctly different from the hydroxamic acid of chloramphenicol acid formed upon incubation of chloramphenicol acid methyl ester with hydroxylamine. The ^{14}C-labeled material was subsequently identified as the hydroxamic acid of oxalic acid by chromatography on Dowex 1 (Fig. 7). This finding is consistent with the spontaneous release of oxalic acid from the protein but again would appear difficult to reconcile with a protein-bound derivative of chloramphenicol oxamic acid.

Evidence for the cytochrome P-450 dependent formation of a neutral polar metabolite of chloramphenicol. Since the model studies suggested that the release of oxalic acid or its hydroxamic acid upon hydrolysis or hydroxylaminolysis of the ^{14}C-labeled P-450 was not due to the inherent instability of the amide linkage in the chloramphenicol moiety, the possibility was considered that some other metabolic alteration in addition to dechlorination of the chloramphenicol had taken place. For example, if the same substrate molecule were to undergo sequential metabolism of both the amide nitrogen atom and the dichloromethyl moiety then a rather labile derivative capable of covalently binding to the P-450 might result. In such a case one might expect to find evidence of a polar metabolite with its dichloroacetyl moiety intact, representing those substrate molecules which had undergone only one cycle of metabolism.

To test the above hypothesis, a neutral ethyl acetate extraction of incubations of the reconstituted system with chloramphenicol was performed. In the presence but not the absence of NADPH, a metabolite was formed which was isolated by reverse-phase liquid chromatography on Zorbax in 25% methanol as described under Materials and Methods. Under the conditions used, this metabolite had a retention time of 6 min, which can be compared with that of chloramphenicol acid (R_t = 3 min) and chloramphenicol alcohol (R_t = 7 min). This metabolite was found to have its dichloroacetyl moiety intact, but was not identical with Peak V in Fig. 2. Rather, when chromatographed on G-10, the neutral metabolite exhibited multiple peaks eluting between Peaks IV and V. The neutral metabolite was found to react with both the amino acid cysteine, giving rise to a peak in the same position as Peak II in Fig. 2, and with 2,4-dinitrophenylhydrazine. However, after treatment with sodium borohydride for 90 min at room temperature, reactivity toward both cysteine and dinitrophenylhydrazine was lost. Attempts to identify the structure of this metabolite by gas chromatography-mass spectrometry have so far been unsuccessful. However, high-performance liquid chromatography of a methanol extract of incubations of the reconstituted system with chloramphenicol suggests that the neutral metabolite and chloramphenicol oxamic acid are formed in approximately equal amounts.

DISCUSSION

Evidence is presented in this paper that three chemically different classes of adducts are formed between active metabolites of chloramphenicol and the cytochrome P-450 of a reconstituted monooxygenase system. Approximately 50% of the covalently bound chloramphenicol has previously been shown to exist in the form of *N*-ε-chloramphenicol oxamyl lysine, which is stable to hydroxylamine (2). The remainder of the covalently bound material can be readily cleaved from the protein

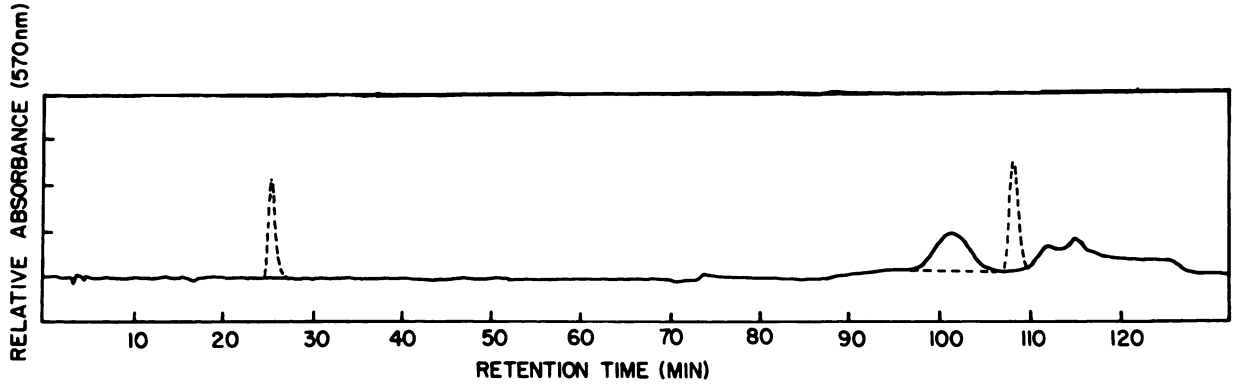

FIG. 5. *Ion-exchange chromatography on a Beckman 121 amino acid analyzer of synthetic N-ε-chloramphenicol oxamyl-L-lysine before (——) and after (- - -) treatment with 1 N NaOH for 2 hr at room temperature*

The peak at 108 min in the alkaline hydrolysate corresponds to lysine. The peak at 26 min was identified as *N-E*-oxalyl lysine by comparison with a standard synthesized as described under Materials and Methods.

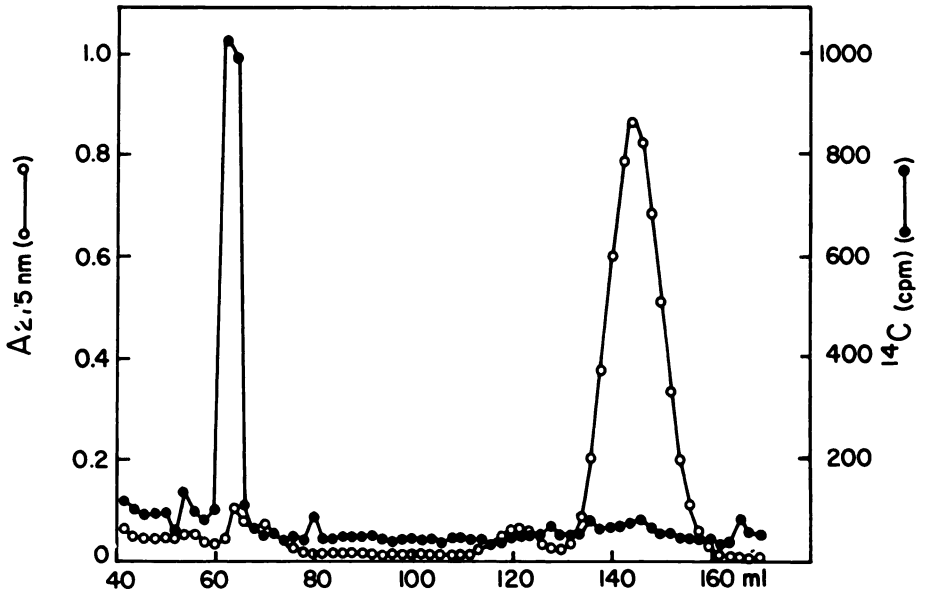

FIG. 6. *Gel filtration on a Sephadex G-10 column of the supernatant fraction from a mixture of 10 nmoles of ^{14}C-labeled cytochrome P-450 and 1 μmole of chloramphenicol oxamic acid methyl ester after treatment with hydroxylamine*

The protein was labeled by incubation with [^{14}C]chloramphenicol as described under Materials and Methods. Noncovalently bound material was removed by dialysis also as described under Materials and Methods. The protein and the synthetic ester were suspended in 0.4 ml of 1 N hydroxylamine (pH 7.5) containing 0.5% SDS. After incubation for 5 hr at room temperature, 1 ml of absolute ethanol was added to precipitate the protein. The ethanol extract was evaporated under nitrogen, and the sample was dissolved in 1 ml of *N*-ethylmorpholine acetate buffer. Chromatography on Sephadex G-10 was performed as described in the legend to Fig. 2. Under these conditions the unmodified ester elutes at 120 ml. The synthetic compound was detected by its absorbance at 275 nm (○——○), and the ^{14}C-labeled compound by liquid scintillation counting (●——●).

as 2-hydroxyoxamic acid upon treatment with neutral hydroxylamine. Approximately 20% of the hydroxylamine-labile material exhibits chemical and chromatographic properties similar to those of a cysteine adduct formed during microsomal incubations with chloramphenicol carried out in the presence of free cysteine. The remainder of the hydroxylamine-labile material cannot be isolated as an amino acid adduct, but is readily degraded to oxalic acid upon incubation of the protein at 37° and at neutral pH. The release of oxalic acid is accompanied by partial reactivation of the enzymatic activity of the cytochrome P-450. This is consistent with the previous conclusion based on the stoichiometry of the inactivation that formation of a labile as well as of the lysine adduct is somehow involved in the loss of monooxygenase activity (2).

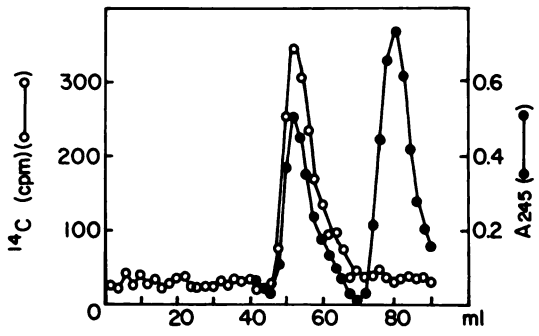

FIG. 7. *Ion-exchange chromatography on a Dowex 1X8 column (1 × 15 cm) in 0.08 M sodium nitrate-0.02 M boric acid (pH 7.1) of the ^{14}C-labeled material released upon hydroxylamine treatment of ^{14}C-labeled cytochrome P-450 together with the hydroxamic acid of oxalic acid (Peak 1) and the 2-oxime of glyoxylic acid (Peak 2)*

The column was operated at 70 ml/hr, and fractions of 2 ml were collected and monitored by liquid scintillation counting (○——○) or UV spectroscopy (●——●). The methyl ester of 2-hydroxyoxamic acid was synthesized by allowing 1 mmole of hydroxylamine to react with 2 mmoles of methyl oxalyl chloride in 2 ml of 1 M sodium biocarbonate. After 10 min at room temperature, the pH was raised to 13 by addition of 5 N NaOH, and the sample was incubated for 3 hr at room temperature to remove the methyl group. The oxime of glyoxylic acid was prepared by incubating 25 μmoles of glyoxylic acid with 100 μmoles of hydroxylamine for 1 hr at pH 7.

All of the structural data on the different adducts are consistent with the proposal of Pohl *et al.* (3, 4) that the covalently bound material is derived from an acyl chloride intermediate formed during the cytochrome P-450-dependent oxidative dechlorination of chloramphenicol. Moreover, the structure of the lysine adduct suggests that this intermediate is chloramphenicol oxamyl chloride. However, the release of oxalic acid rather than chloramphenicol oxamic acid upon hydrolysis and of the hydroxamic acid of oxalic acid rather than of chloramphenicol oxamic acid upon hydroxylaminolysis of the ^{14}C-labeled protein would appear not to be consistent with such an intermediate, since this would require the cleavage of two linkages. This conclusion is supported by model studies with *N*-ε-chloramphenicoloxamyl-L-lysine and chloramphenicol oxamic acid methyl ester. Thus neither compound is hydrolyzed to oxalic acid, even upon treatment with 1 N NaOH, suggesting that the negative charge introduced upon hydrolysis of one of the susceptible linkages inhibits the hydrolysis of the second. Moreover, upon treatment with hydroxylamine, chloramphenicol acid methyl ester retains the aromatic ring, demonstrating that the amide linkage in the chloramphenicol

moiety is intact. Evidence is presented for the formation of a previously unreported neutral metabolite of chloramphenicol produced by the cytochrome P-450-dependent monooxygenase system. This metabolite, which is formed in amounts approximately equal to those of the known metabolite chloramphenicol oxamic acid (3, 4), exhibits reactivity toward both cysteine and 2,4-dinitrophenylhydrazine. However, after treatment with sodium borohydride such reactivity is lost. Although such data would be consistent with chloramphenicol aldehyde (6), the neutral polar metabolite has its dichloroacetyl moiety intact, and after reduction it does not co-chromatograph with chloramphenicol alcohol, the reduction product of the aldehyde (6). Whether further metabolism of this neutral metabolite is responsible for the formation of the hydroxylamine-labile adducts of cytochrome P-450 and chloramphenicol remains to be elucidated.

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