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COVALENT MODIFICATION OF LYSINE DURING THE SUICIDE INACTIVATION OF RAT LIVER CYTOCHROME P-450 BY CHLORAMPHENICOL

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Abstract—During the metabolism of [^{14}C]chloramphenicol by a reconstituted monooxygenase system or by intact liver microsomes from phenobarbital-treated rats, a ^{14}C-containing metabolite covalently bonded to cytochrome P-450, and the enzyme was irreversibly inhibited. In both systems, approximately 95 per cent of the ^{14}C that was covalently bound to protein was associated with cytochrome P-450. In the presence of 0.5% sodium dodecylsulfate, half of the ^{14}C that was bound to the proteins of the reconstituted system was dissociated by treatment with 1 N hydroxylamine (pH 7.5) or by mild alkaline hydrolysis (pH 10.5). Most of the hydroxylamine-labile protein-bound material was readily degraded to oxalate under the conditions used to digest the P-450 with proteolytic enzymes. The radiolabeled material stable to hydroxylamine was isolated as an amino acid adduct from pronase digests of the ^{14}C-labeled proteins of a reconstituted system or of the ^{14}C-labeled microsomal proteins. This adduct was identified as *N*-ε-chloramphenicol oxamyl lysine based on co-chromatography with the synthetic compound and on the release of free lysine plus chloramphenicol oxamic acid upon alkaline hydrolysis of the ^{14}C product isolated from digests of the microsomal protein. Evidence is presented that modification of lysine is at least partially responsible for the suicide inactivation of cytochrome P-450 by chloramphenicol.

The antibiotic chloramphenicol has been shown recently to be an irreversible inhibitor of cytochrome P-450 dependent metabolism in intact rat liver microsomes [1] and in a reconstituted monooxygenase system [2]. This irreversible inhibition may be the mechanism whereby chloramphenicol potentiates the action of certain therapeutic agents that are normally metabolized by liver microsomal enzymes [3]. In the reconstituted system, the inactivation of cytochrome P-450 is accompanied by the covalent binding of 1.5 nmoles ^{14}C from [1,2-^{14}C]chloramphenicol per nmole P-450, but not by a decrease in heme or in cytochrome P-450 detectable as the carbon monoxide complex [2]. Inhibition of liver microsomal mixed-function oxidase activity with no apparent decrease in the cytochrome P-450 level has been shown previously by Adams *et al.* [4] upon *in vivo* administration of chloramphenicol to mice. These findings suggested that chloramphenicol might be inactivating the cytochrome P-450 by modifying important amino acid residues rather than by altering the heme moiety. The active intermediate of chloramphenicol responsible for the covalent binding observed *in vitro* using intact microsomes has been postulated to be an oxamyl chloride. Such an intermediate might be expected to acylate amino, hydroxyl, or sulfhydryl groups in proteins [5, 6]. The present investigation has focused on the nature of the amino acid adducts formed during the covalent binding of activated chloramphenicol to cytochrome P-450 in both a reconstituted system and in intact liver microsomes from phenobarbital-treated rats.

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MATERIALS AND METHODS

Materials. [^{14}C]Chloramphenicol [(1R,2R)-(+)1*p*-nitro - phenyl - 2 - [1,2 - ^{14}C]dichloroacetamido-Ĭ,3-propane-diol], 43.2 mCi/mmole, was purchased from the New England Nuclear Corp., Boston, MA. The compound was found to be radiochemically pure (>98 per cent) 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), thermolysin, glucose-6-phosphate dehydrogenase, NADP, and NADPH were from the Sigma Chemical Co., St. Louis, MO. α-Chymotrypsin was purchased from Worthington, and leucine aminopeptidase and glucose-6-phosphate from Boehringer-Mannheim. Methyl oxalyl chloride was purchased from the Aldrich Chemical Co., Milwaukee, WI. *N*-α-t-Butyloxycarbonyl-L-lysine was purchased from Vega. Chloramphenicol oxamic acid and its methyl ester were synthesized according to Pohl *et al.* [6].

Synthesis of N-α-*t-butyloxycarbonyl*-N-ε-*chloramphenicol oxamyl*-L-*lysine.* Chloramphenicol acid methyl ester (1.1 mmole) and *N*-α-t-butyloxycarbonyl-L-lysine (1.2 mmole) were dissolved in 7 ml of methanol. Sodium methoxide (0.6 mmole) was added from a 1 M solution in methanol, and the mixture was allowed to react for 2 hr at room tem-

perature. An additional equal aliquot of sodium methoxide was then added, and the reaction was allowed to proceed for another 2 hr. The reaction mixture was rotary evaporated to dryness and dissolved in 2 ml of methanol. Aliquots of 400 μl were applied to each of five Whatman PK1F silica gel plates, which were developed in $CHCl_3$–MeOH (75:25) and scanned under a u.v. light. In this system, chloramphenicol acid methyl ester has an R_f of 0.9, and chloramphenicol acid an R_f of 0.25, whereas *N*-α-t-Boc-lysine does not migrate. In addition to the ester and the acid, a broad zone with an R_f of about 0.5 was observed, which was scraped from the plates and eluted with absolute ethanol (10 ml/plate). The product was rotary evaporated to dryness, dissolved in 1 ml of methanol, and re-chromatographed on silica gel in $CHCl_3$–MeOH (100:10) to remove remaining traces of chloramphenicol acid methyl ester. The purified product was eluted with absolute ethanol and rotary evaporated to given an amorphous solid, 0.47 mmole (42 per cent yield). The identity of the compound was confirmed by the following criteria: (1) n.m.r. (^1H, CD_3OD, TMS internal standard, δ) 8.17 (d, J = 9 Hz, 2H, aromatic), 7.61 (d, J = 10 Hz, 2H, aromatic), 5.13 [d, J = 4 Hz, 1H, —CH(OH)—], 3.8 to 4.2 [m, 2H, —CH(CH_2OH)— and —CH(COOH)—], 3.69 (m, 2H, —CH_2OH), 3.21 (m, 2H, —NH—CH_2—CH_2—), 1.2 to 1.8 (m, 6H, —CH_2—CH_2—CH_2—CH_2—), 1.43 [s, 9H, (CH_3—)$_3$]; (2) n.m.r. (^{13}C, CD_3OD, TMS internal standard, ppm) 178.7 (—COOH), 161.5, 161.3 (—CO—CO—), 157.7 (—NH—CO—O—), 151.6, 148.7, 128.3, 124.2 (aromatic), 80.2 [(CH_3)$_3$—C—O—], 71.5, 62.4, 49.3 [—CH(OH)——CH(CH_2OH)—], 58.4 [—CH(COOH)—], 40.4, 33.6, 29.8, 24.0 [—NH—(CH_2)$_4$—], 28.7 [(CH_3—)$_3$]; and (3) acid hydrolysis (6 N HCl, 24 hr, 110°, μmoles product/μmole hydrolysate) chloramphenicol base = 0.99, oxalic acid = 1.02, lysine = 0.99. Chloramphenicol base and oxalic acid were identified by chromatography on Sephadex G-10 and quantitated by u.v. and titration with permanganate respectively. Lysine was determined by amino acid analysis.

Preparation of microsomes. Adult male Sprague–Dawley rats (100–200 g) were given phenobarbital (0.1% in their drinking water) for 5 days prior to their being killed. 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′ Sepharose [10]. Both enzyme preparations were ≥95 per cent pure as judged by sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis, performed by the method of Laemmli [11] using 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 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 cytochrome P-450, 4 units reductase, 60 μg dilauryl L-3 phosphatidylcholine, 200 μg 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 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 4 × 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 cytochrome P-450) was suspended in 50 μl *N*-ethyl morpholine acetate buffer (pH 7.5), containing 0.1% SDS, and the suspension was incubated at 37° with 20 μg pronase (enzyme: P-450 ratio ≃ 1:10, w/w) until solubilized (*ca.* 4 hr). Leucine aminopeptidase (10 μg) was then added, and the digest was allowed to proceed overnight.

Microsomal incubations with chloramphenicol. These were carried out using 6 mg 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 absolute ethanol, and 2 units 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. The precipitate was suspended in 2 ml H_2O and re-precipitated with 6 ml of ethanol. This procedure was repeated two more times. To obtain sufficient protein-bound material for characterization, 700 mg of microsomal protein was incubated as described above, using six centrifuge bottles containing 40 ml of incubation mixture each. For these experiments [^{14}C]chloramphenicol with a specific activity of 0.3 mCi/mmole was used, and the incubation time was increased to 60 min.

Isolation of a chloramphenicol–amino acid adduct from ^{14}C-labeled microsomal protein. Ethanol-washed microsomes corresponding to 700 mg of microsomal protein were suspended in 50 ml of 0.05 M *N*-ethyl morpholine acetate buffer (pH 7.5) containing 0.1% SDS and were incubated with stirring for 24 hr at 37° with 70 mg of pronase. After centrifugation, the supernatant fraction was lyophilized, dissolved in 10 ml buffer, and chromatographed on a column (2.5 × 90 cm) of Sephadex

* HEPES = 4-(2-hydroxyethyl-1-piperazine-ethanesulphonic acid.

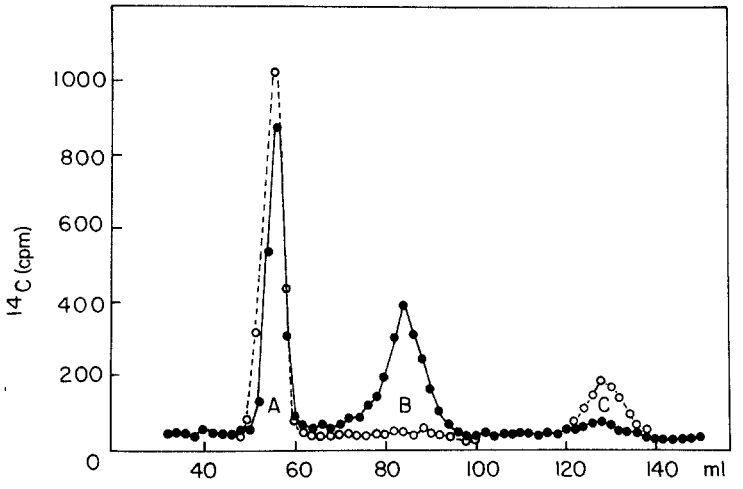

Fig. 1. Gel filtration on a Sephadex G-10 column (0.9 × 150 cm) in 0.05 M *N*-ethyl morpholine acetate buffer (pH 7.5) of a pronase and leucine aminopeptidase digest of the ^{14}C-labeled proteins of a reconstituted system after incubation with [^{14}C]chloramphenicol, as described in Materials and Methods. One 5-μl aliquot of the digest was diluted to 0.5 ml and chromatographed directly on the column (●——●). An equal aliquot was dried, dissolved in 20 μl 1 N NaOH, and incubated for 2 hr at room temperature. After addition of 20 μl of 1 N HCl, the sample was diluted to 0.5 ml with buffer and chromatographed on the column (○---○). The column was operated at 12 ml/hr, and fractions of 2 ml were collected and analyzed for radioactivity. The Sephadex G-10 was washed with 1 M pyridine prior to use. Unmodified chloramphenicol elutes at 295 ml on the same column under the same conditions.

G-10. The column was operated at 20 ml/hr, and fractions of 5 ml were collected. Two radioactive peaks were obtained, the first (V_e = 225 ml) corresponding to the material in peak G-10 A (Fig. 1), and the second (V_e = 365 ml) corresponding to the material in peak G-10 B. The second peak was pooled, lyophilized, and chromatographed on a column (1.5 × 100 cm) of Bio-Rad P-2. The column was operated at 8 ml/hr, and fractions of 2 ml were collected. A single radioactive peak was obtained (V_e = 168 ml), the center cut fractions of which (40 nmoles) were pooled, lyophilized, and chromatographed on a Beckman 121 amino acid analyzer using the sodium citrate buffer system. The material eluting between 97 and 100 min was pooled, lyophilized, and extracted with absolute ethanol (2 ml). The ethanol extract containing the purified amino acid adduct was dried under nitrogen.

RESULTS

Enzymatic hydrolysis of the ^{14}C-labeled proteins of a reconstituted system. As seen in Fig. 1, an overnight pronase plus leucine aminopeptidase digest of the ^{14}C-labeled proteins of the reconstituted system after incubation with [^{14}C]chloramphenicol could be resolved into two major peaks by chromatography

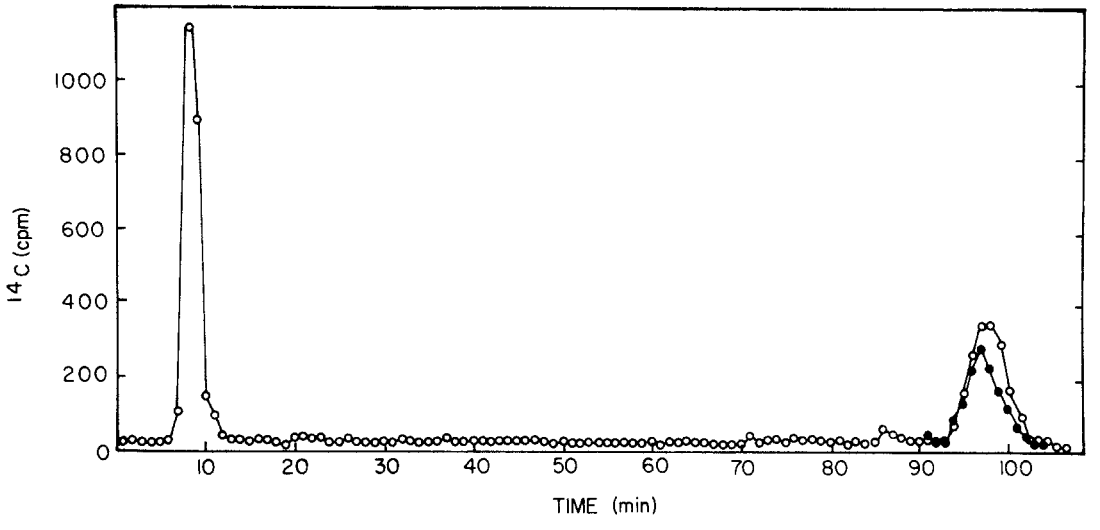

Fig. 2. Ion-exchange chromatography on a Beckman 121 amino acid analyzer of a pronase and leucine aminopeptidase digest of the ^{14}C-labeled proteins of a reconstituted system (○——○) and of an adduct isolated from a pronase digest of ^{14}C-labeled microsomal protein (●——●). Experimental details are given in Materials and Methods.

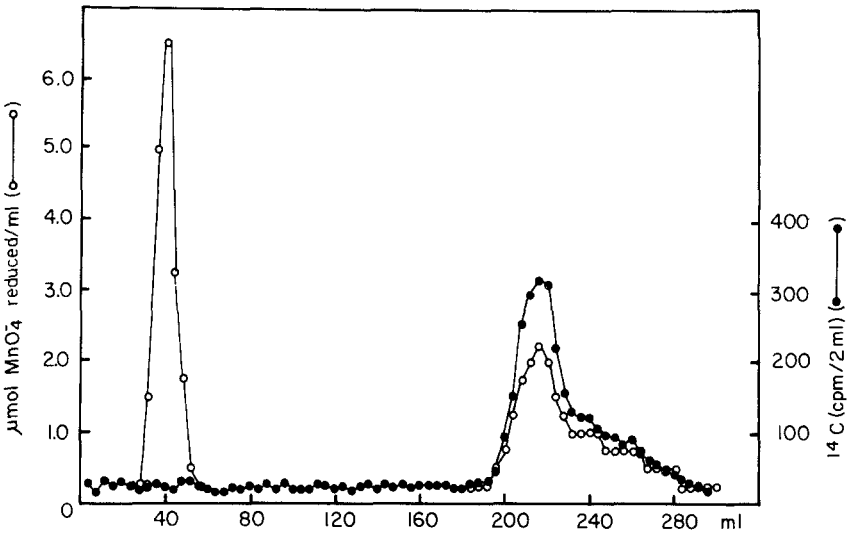

Fig. 3. Ion-exchange chromatography on a Dowex 1X8 column (1 × 15 cm) in 0.08 M sodium nitrate–0.02 M boric acid (pH 7.1), of peak G-10 A (Fig. 1) together with 5 mg each of glyoxyalate and oxalate. The standards were dissolved in 1 ml of the buffer, and the pH was adjusted to 7.1 with 1 N NaOH. The column was operated at 80 ml/hr, and fractions of 4 ml were collected. Two-ml aliquots were monitored for radioactivity, and 1-ml aliquots for glyoxyalate (peak 1) and oxalate (peak 2) by permanganate oxidation. The elution positions of the standards were established by chromatographing them individually.

on Sephadex G-10. Peak G-10 B (V_e = 84 ml) eluted between phenylalanine (V_e = 76 ml) and tyrosine (V_e = 92 ml), an elution position consistent with an amino acid adduct of chloramphenicol. In contrast, the elution position of peak G-10 A (V_e = 58 ml) was not consistent with such an adduct. In similar fashion, the digest could be resolved into two radiolabeled peaks by ion-exchange chromatography on a Beckman 121 amino acid analyzer (Fig. 2). The first peak (R_t = 8 min) was completely excluded by the column, whereas the second peak (R_t = 98 min) eluted after phenylalanine. This second peak was shown to be derived from peak G-10 B by chromatography of the individual G-10 peaks.

Identification of peak G-10 A as oxalate. Peak G-10 A was identified as oxalate by co-chromatography with standard oxalate in the following systems: Sephadex G-10 and G-25 in 0.05 M *N*-ethyl morpholine acetate (pH 7.5), Dowex 1 in 0.08 M sodium nitrate–0.02 M boric acid, pH 7.1 (Fig. 3), and thin-layer chromatography on cellulose in the solvent system formic acid–ethyl acetate–H_2O (1:3:1) (R_f = 0.60). The oxalate appeared to be a non-enzymatic degradation product of the protein-bound ^{14}C-material, since it could be formed in the absence of proteolytic enzymes under the conditions of the digestion. Oxalate, however, could not be produced under such conditions from chloramphenicol acid, or from any other metabolites found in the supernatant fraction of a microsomal incubation with [^{14}C]chloramphenicol. Treatment of the ^{14}C-labeled reconstituted-system protein in the presence of 0.5% SDS with 1 N hydroxylamine (pH 7.5) or with dilute base (pH 10.5) for 18 Hr at room temperature removed 50 per cent of the radioactivity. No peak G-10 A could be found in an enzymatic digest of the hydroxylamine-treated protein, indicating that the oxalate was derived from a hydroxylamine-sensitive adduct (Fig. 4).

Heterogeneity of peak G-10 B. When chromatographed on the amino acid analyzer, peak G-10 B yielded two components, a minor one (R_t = 8 min) corresponding to 20 per cent of the radioactivity

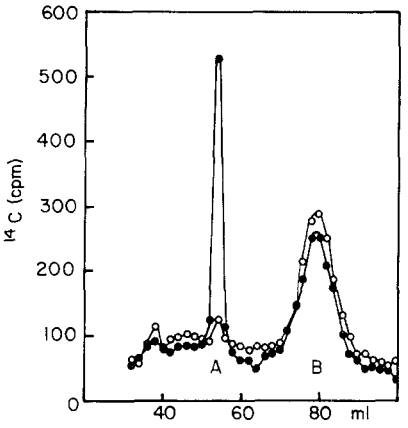

Fig. 4. Chromatography on a Sephadex G-10 column (0.9 × 150 cm) in 0.05 M *N*-ethyl morpholine acetate buffer (pH 7.5) of a pronase and leucine aminopepetidase digest of control (●——●) and hydroxylamine-treated (○——○) ^{14}C-labeled proteins of a reconstituted system after incubation with [^{14}C]chloramphenicol, as described in Materials and Methods. The treatment with hydroxylamine (1 N) was for 18 hr at room temperature and pH 7.5 in the presence of 0.5% SDS. Prior to digestion of the protein with proteolytic enzymes as described in Materials and Methods, the samples were washed twice with absolute ethanol to remove any radioactivity released from the protein during the hydroxylamine treatment. The Sephadex column was operated at 12 ml/hr, and fractions of 2 ml were collected.

recovered, and a major one (R_t = 98 min) corresponding to 80 per cent (not shown). Upon treatment of the pronase digest of the ^{14}C reconstituted-system protein with 1 N hydroxylamine, the amount of radioactivity eluting in peak G-10 B could be reduced by 20 per cent. At the same time a new peak appeared at V_e = 66 ml which accounted for the radioactivity lost from peak B. When chromatographed on the amino acid analyzer, the material in peak G-10B from the hydroxylamine-treated digest yielded only a single peak (R_t = 98 min). Thus, the material in peak G-10 B from the non-treated protein or digest appeared to consist of two chemically distinct adducts, one of which (20 per cent of the material) was readily cleaved by hydroxylamine and appeared to be unstable under the conditions of the amino acid analysis. On the other hand, the adduct eluting at 98 min on the amino acid analyzer (80 per cent of the material) was stable to hydroxylamine. In accordance with the above findings, treatment of the pronase digest with 5 mM dithiothreitol for 18 hr at room temperature and pH 7.5 or with performic acid reduced the amount of material eluting as G-10 B by 20 per cent.

Thin-layer chromatography of the digest of the ^{14}C*-labeled reconstituted system protein.* The recoveries of radioactivity from the chromatography of the enzymatic digest of the reconstituted-system protein on Sephadex G-10 and on the amino acid analyzer were 70 and 85 per cent respectively. To ensure that no minor component had been lost during the column chromatography, the digest was also examined by thin-layer chromatography on silica gel in isopropanol–H_2O (70:30) and on cellulose in *n*-butanol–pyridine–acetic acid–H_2O (15:10:3:12). In each case, approximately 90 per cent of the radioactivity applied to the plate was recovered and was found to be distributed among three components, each of which could be accounted for by the material identified by column chromatography.

Identification of the major adduct as N-ε*-chloramphenicol oxamyl lysine.* Peak G-10 B of Fig. 1 could be completely hydrolyzed by treatment with 1 N NaOH for 2 hr at room temperature, most of the radioactivity appearing as chloramphenicol acid

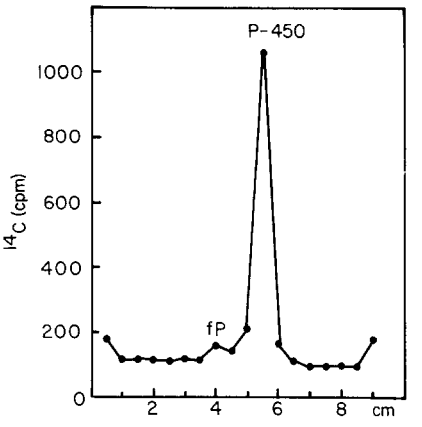

Fig. 5. SDS–polyacrylamide gel electrophoresis of ^{14}C-labeled microsomal protein incubated with [^{14}C]chloramphenicol and washed with ethanol essentially as described in Materials and Methods except that the incubation volume was scaled down 20-fold, and 0.05 mM substrate (43.2 mCi/mmole) was used. Electrophoresis was carried out using 7.5% gel slabs (0.75 mm × 10 cm) prepared according to Laemmli [11] and using double-strength cathode buffer as described by Guengerich [12]. The gel was stained, dried, and cut into 0.5 cm strips, which were assayed for radioactivity by combustion in a Packard 306 sample oxidizer, followed by liquid scintillation counting.

(peak C in Fig. 1). The identity of peak C as chloramphenicol acid was confirmed by co-chromatography with the standard acid on the same Sephadex G-10 column, by thin-layer chromatography on silica gel in methanol (R_f = 0.69), and by DEAE-cellulose thin-layer chromatography in 0.05 M *N*-ethyl morpholine acetate, pH 7.5 (R_f = 0.42).

The conversion of most of peak G-10 B to chloramphenicol acid by the treatment with NaOH, together with the resistance to cleavage by hydroxylamine of that component of peak G-10 B that eluted at 98 min on the amino acid analyzer, suggested that the latter represented an amino acid adduct of chloramphenicol acid containing an amide linkage. To isolate a sufficient quantity of this adduct to permit

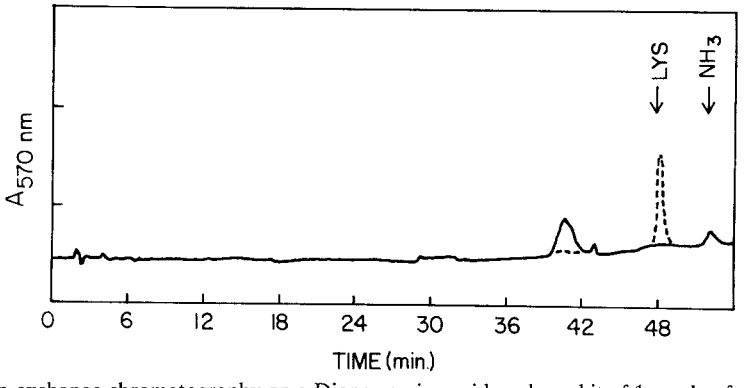

Fig. 6. Ion-exchange chromatography on a Dionex amino acid analyzer kit of 1 nmole of unhydrolyzed (——) and acid or base hydrolyzed (– – –) ^{14}C-labeled amino acid adduct of chloramphenicol isolated from a pronase digest of ^{14}C-labeled microsomal protein, as described in Materials and Methods. The base hydrolysis was accomplished by treatment with 1 N NaOH for 2 hr at room temperature, and the acid hydrolysis with 6 N HCl for 24 hr at 110° *in vacuo*.

identification, a large scale incubation was carried out with [^{14}C]chloramphenicol, using intact rat liver microsomes as described in Materials and Methods. This approach seemed justified since (1) SDS–polyacrylamide gel electrophoresis of the ^{14}C-labeled microsomal proteins revealed that *ca.* 95 per cent of the radioactivity was associated with the major cytochrome P-450 band (Fig. 5), as had been shown previously with the reconstituted system [2]; (2) pronase digestion of the ^{14}C microsomal protein yielded an amino acid adduct of chloramphenicol having the same elution position on Sephadex G-10, on Bio-Rad P-2, and on the amino acid analyzer (Fig. 2), as the adduct from the digest of the reconstituted system.

By consecutive chromatography on Sephadex G-10, Bio-Rad P-2, and the amino acid analyzer, the ^{14}C-containing adduct from microsomes could be isolated free of all amino acids and small peptides (Fig. 6). Upon acid or base hydrolysis, a nearly stoichiometric amount of free lysine was released. These data suggested that the radiolabeled material, derived from the pronase digest of microsomal protein labeled with [^{14}C]chloramphenicol, that eluted at 98 min on the amino acid analyzer represented a lysine adduct of chloramphenicol acid containing an amide linkage between the chloramphenicol acid moiety and the ε-amino group of lysine (Fig. 7). By inference, the corresponding material derived from the digest of the reconstituted system protein should represent the same adduct.

To confirm the structure of the adduct, *N*-α-t-butyloxycarbonyl-*N*-ε-chloramphenicol oxamyl-L-lysine was synthesized as described in Materials and Methods. After removal of the t-butyloxycarbonyl group by incubation for 15 min with 1 N HCl in glacial acetic acid or with 3.5 N HCl in dioxane, the synthetic compound yielded a single ninhydrin-positive component upon chromatography on the Beckman 121 amino acid analyzer. The ninhydrin-positive material from the synthetic adduct and the ^{14}C from the adduct derived from pronase digests of the ^{14}C-labeled reconstituted-system protein co-chromatographed exactly on Sephadex G-10 (not shown) and on the amino acid analyzer (Fig. 8).

Role of modification of lysine in the suicide inactivation of cytochrome P-450 by chloramphenicol. Previous studies indicated that 15–20 per cent of the ^{14}C which becomes covalently bound to the cyto-

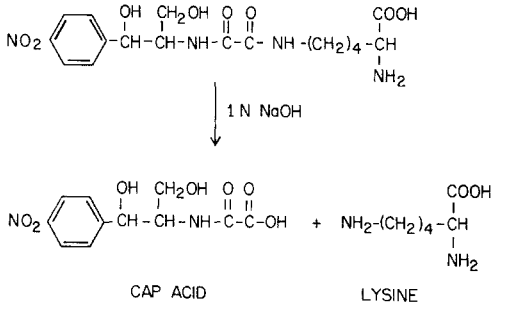

Fig. 7. Proposed structure of the hydroxylamine-stable amino acid adduct of chloramphenicol (CAP = chloramphenicol).

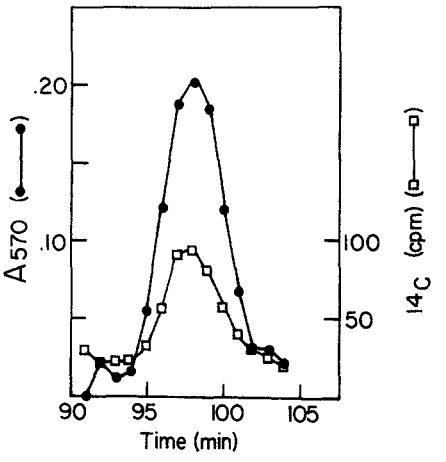

Fig. 8. Ion-exchange chromatography on a Beckman 121 amino acid analyzer of the hydroxylamine-stable adduct from a pronase and leucine aminopeptidase digest of the ^{14}C-labeled proteins of a reconstituted system together with synthetic *N*-ε-chloramphenicol oxamyl-L-lysine. One-minute fractions were collected, aliquots of which were analyzed by ninhydrin (●——●) and by liquid scintillation counting (□——□).

chrome P-450 of a reconstituted system during the metabolism of [^{14}C]chloramphenicol could be removed by treatment with dithiothreitol with no restoration of enzymatic activity [2]. In agreement with this result, treatment of the intact ^{14}C-labeled protein with hydroxylamine *in the absence of SDS* was found to remove 16 per cent of the label, but no reactivation of the ethoxycoumarin deethylase activity of the cytochrome P-450 was observed. After the treatment with hydroxylamine or dithiothreitol in the absence of SDS, the stoichiometry of inactivation was approximately 1.2 nmoles ^{14}C/nmole P-450, of which approximately 0.8 nmole/nmole P-450 appeared to be represented by *N*-ε-chloramphenicol oxamyl-L-lysine. On this basis, it would seem that modification of lysine was responsible for most of the observed inactivation of the cytochrome P-450 by chloramphenicol, but that formation of the as yet unidentified, less stable adducts may also contribute to the loss of monooxygenase activity.

Effectiveness of chloramphenicol as a suicide substrate of cytochrome P-450. Demonstration of the formation of *N*-ε-chloramphenicol oxamyl lysine as the major protein-bound adduct of chloramphenicol is consistent with the proposal by Pohl and co-workers [5, 6] that the protein-bound material and chloramphenicol acid are derived from a common intermediate. This is also supported by the finding that inclusion of free cysteine during microsomal incubations with chloramphenicol led to the formation of a cysteine adduct at the expense of the formation of chloramphenicol acid (data not shown). By comparing the amount of free chloramphenicol acid formed with the amount of *N*-ε-chloramphenicol oxamyl lysine, it should be possible to judge the effectiveness of chloramphenicol as a suicide substrate. In the microsomal system, approximately 4 nmoles chloramphenicol acid was formed per nmole protein-bound *N*-ε-chloramphenicol lysine,

whereas the corresponding value for the reconstituted system was 8 nmoles/nmole. Since formation of approximately 0.8 nmole of the lysine adduct/nmole P-450 was accompanied by complete inactivation of the P-450, it would appear that on the order of five turnovers of the substrate are sufficient to inactivate the enzyme.

DISCUSSION

The data presented in this report indicate a heterogeneous distribution of the ^{14}C-material that was covalently bound to the cytochrome P-450 of a reconstituted system during the metabolism of [^{14}C]chloramphenicol. In the presence of 0.5% SDS, approximately 50 per cent of the protein-bound ^{14}C could be readily removed with neutral hydroxylamine or by mild alkaline hydrolysis. This radioactivity appeared to be distributed between two classes of amino acid adducts, the major one of which was degraded to oxalate under the conditions of the enzymatic digestion of the protein. The other class of hydroxylamine-sensitive adduct was stable to such conditions, but not to the conditions of chromatography on the amino acid analyzer or to treatment with dithiothreitol or performic acid, suggesting a cysteine adduct.

The hydroxylamine-stable material from pronase digests of the ^{14}C-labeled protein from a microsomal or reconstituted system was identified as *N*-ε-chloramphenicol oxamyl lysine based on co-chromatography with the synthetic compound and on the release of chloramphenicol acid plus lysine upon base hydrolysis. Based on the stoichiometry of the inactivation, formation of the lysine derivative appears to have been at least partially responsible for the suicide inactivation of cytochrome P-450 by chloramphenicol. The structure of the lysine adduct is consistent with the proposal by Pohl and co-workers [5, 6] that the protein-bound material and chloramphenicol acid are derived from a common intermediate, probably chloramphenicol oxamyl chloride. On the other hand, it is unclear how a protein-bound derivative of such an intermediate would give rise to oxalate at neutral pH, since this would require hydrolysis of two linkages.

Two observations support the hypothesis that chloramphenicol is a very effective suicide substrate for cytochrome P-450. First, approximately five turnovers of the substrate appear to have been sufficient to inactivate the enzyme. Second, in both a microsomal and reconstituted system, the only significant covalent binding of chloramphenicol was to the cytochrome P-450 itself. If a similar situation exists *in vivo*, then it appears unlikely that the active species of chloramphenicol, which is responsible for the inactivation of cytochrome P-450, would be formed in sufficient amounts to cause any of the other adverse effects of chloramphenicol.

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