Biochemical Pharmacology, Vol. 27, pp. 491-496. Pergamon Press, 1978. Printed in Great Britain.

INVESTIGATION OF THE MECHANISM OF THE METABOLIC ACTIVATION OF CHLORAMPHENICOL BY RAT LIVER MICROSOMES

IDENTIFICATION OF A NEW METABOLITE

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(*Received* 15 *February* 1977; *accepted* 6 *May* 1977)

Abstract—A new metabolite of chloramphenicol (CAP) has been isolated from incubation mixtures containing CAP and liver microsomes from phenobarbital-pretreated rats. This compound has been identified as the oxamic acid derivative of CAP by reverse isotope dilution, high pressure liquid chromatography, and isobutane chemical ionization mass spectroscopy. It has also been identified as a product of the chemical hydrolysis of radiolabeled microsomal protein. These results support the proposal that CAP is metabolically activated by liver microsomes to an oxamyl chloride reactive intermediate which either hydrolyzes to the oxamic acid or acylates protein.

Previous studies with the antibiotic chloramphenicol (CAP) revealed that this compound is bound irreversibly to tissue proteins when it is either incubated with liver microsomes of rat or is given to rats [1, 2]. A recent study on the mechanism of the binding of CAP to liver microsomes *in vitro* using specifically labeled ^{14}C and ^{3}H derivatives of CAP [3, 4] established that intact CAP is activated by a cytochrome P 450 monooxygenase, which is induced by phenobarbital. Moreover, the C—H bond of the dichloromethyl carbon of CAP appeared to be the position of activation. These findings suggested to us that CAP is hydroxylated to yield a hydroxydichloroacetamido intermediate which spontaneously eliminates hydrochloric acid to produce an oxamyl chloride (Fig. 1). This reactive compound would be expected to acylate microsomal protein [1–4].

If the oxamyl chloride, compound 3, were an electrophilic intermediate in the bioactivation of CAP, it seemed reasonable that this compound would react not only with tissue macromolecules such as protein, to form compound 5, but also with water to yield the hydrolyzed product, CAP oxamic acid, compound 4. In order to test this possibility, we have studied the biotransformation of radiolabeled CAP by liver microsomes of rat. In addition, we have investigated the composition of radiolabeled products that were isolated from the chemical hydrolyzate of radiolabeled microsomal protein.

MATERIALS AND METHODS

Materials

Chemicals. [^{14}C]chloramphenicol [(1R,2R)-(+)-1-*p*-nitrophenyl-2-[1,2-^{14}C]dichloroacetamido-1,3-propanediol], 4.87 mCi/m-mole, was purchased from New England Nuclear, Boston, MA. [1-^{3}H]chloramphenicol [(1R,2R)-1-*p*-nitrophenyl-2-dichloroacetamido-1,3-[1-^{3}H]propanediol], 23.4 mCi/m-mole, was synthesized previously [4]. These compounds were found to be radiochemically pure (99 per cent) by thin-layer chromatography (t.l.c.) with solvent systems 1–3 (refer to Thin-Layer chromatography in Methods). NADP, NADH, glucose 6-phosphate, nicotinamide, chloramphenicol and chloramphenicol base [(1R,2R)-(−)-*p*-nitrophenyl-2-amino-1,3-propanediol] were purchased from Sigma Biochemicals, Saint Louis, MO. Glucose 6-phosphate dehydrogenase was purchased from CalBiochem, La Jolla, CA. Methyl oxalyl chloride and Diazald were purchased from Aldrich, Milwaukee, WI. Scintillation fluid, which consists of a mixture of 0.4% BBOT [2,5-bis (5-tert-butyl-benzoxyzoyl) thiopene, 0.8% naphthalene, and 40% methylcellulose in toluene], was purchased from Yorktown Research, Ensack, NJ.

Analytical equipment. Melting points were determined on a Fisher–Johns melting point apparatus and are uncorrected. High pressure liquid chromatography (h.p.l.c.) was performed with a Spectra-Physics model 3500 instrument. Radioactivity was measured by scintillation counting with a Tri-Carb model 3375 spectrophotometer, using 15 ml of scintillation mixture. ^{1}H n.m.r. spectra tetramethylsilane internal standard) were obtained with a Varian A-60 nuclear magnetic spectrophotometer and u.v. spectra with a Cary 15 spectrophotometer. Chemical ionization mass spectrometry (c.i.m.s.) was performed by direct insertion probe on a VG Micro Mass 16F at an accelerating voltage of 4 kV, an electron energy of 100 eV, an ionizing current of 200 μA, and an ion source pressure of approximately 0.3 Torr (isobutane reactant gas). Chemical analysis was performed at the microanalytical laboratory of the National Institute of Arthritis, Metabolic and Digestive Diseases, Bethesda, MD.

Methods

Thin-layer chromatography. Thin-layer chromatography was performed using Eastman 6060 or Analtec GF Silica gel plates. The chromatograms were usually developed 15 cm with one of the following

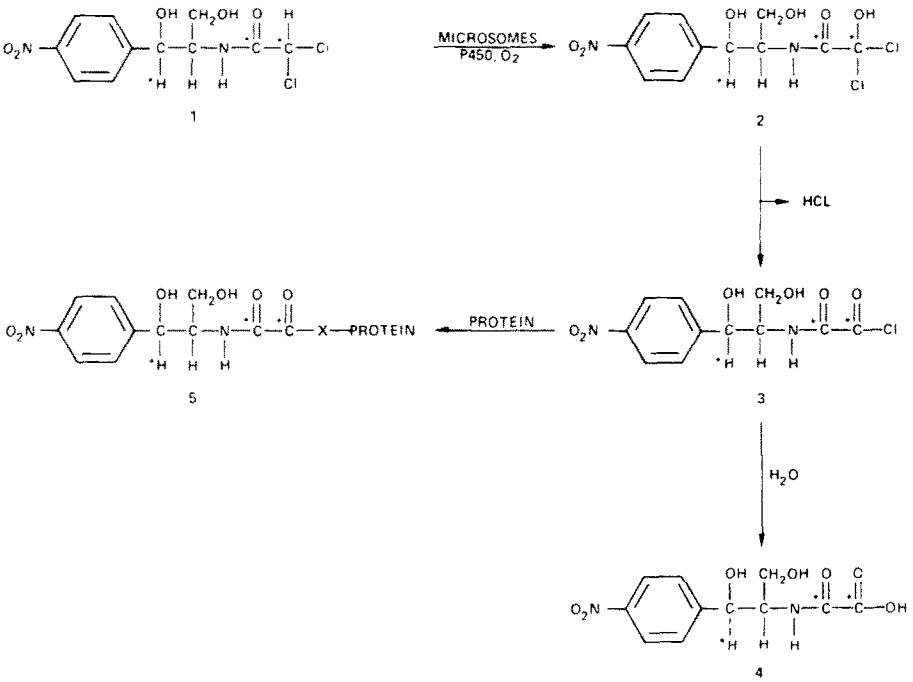

Fig. 1. Possible mechanism for the metabolic activation of chloramphenicol *in vitro* by liver microsomes from phenobarbital-pretreated rats. The asterisks represent the position of ^{14}C and ^{3}H labels. The X represents an O, S or N atom of an amino acid in the protein.

four solvent systems: solvent system 1, chloroform–methanol (100:15); 2, benzene–methanol–acetic acid (45:8:4); 3, isopropanol–ammonium hydroxide–water (85:5:15); and 4, propanol–H_2O (70:30). Compounds were detected on t.l.c. plates by visualization under a short wave u.v. lamp. Radioactive fractions were typically detected by autoradiography with X-ray film, after exposure for 7 days.

Synthesis of methyl (1*R*,2*R*)-1-p-*nitrophenyl*-1,3-*propanediol*-2-*oxamate* (*CAP methyl oxamate*). CAP base (10 g, 47.2 m-moles) and a sodium bicarbonate solution (7.9 g, 94.4 m-moles in 60 ml water) were stirred with ethyl acetate (200 ml) and cooled to 0° in an ice bath. Methyl oxalyl chloride (11.5 g, 94.4 m-moles) was then added dropwise (2 min) to the reaction suspension, which was magnetically stirred for an additional 40 min at 0°. The reaction mixture was transferred to a separatory funnel, and the lower aqueous phase was extracted with ethyl acetate (25 ml). The ethyl acetate extract was then combined with the upper ethyl acetate phase, and the combined extracts were washed first with 1 N hydrochloric acid (25 ml, three times), then sodium bicarbonate solution (25 ml, three times and finally with water (25 ml, twice). The ethyl acetate extract was dried ($MgSO_4$) and evaporated under vacuum to give a pale yellow viscous oil, which was crystallized from water to yield white needle crystals, 5.6 g (40 per cent), m.p. 142–46°. Recrystallization from water yielded white needle crystals, m.p. 146–48°. The identity of the compound was confirmed by the following criteria: (1) n.m.r. (60 MHz, DMSO-d_6, TMS internal standard) δ (ppm) 8.20 (*m*, 2 H, aromatic), 7.64 (*m*, 2 H aromatic), 5.07 (*m*, 1 H, benzylic), 4.08 (*m*, 1 H, methine), 3.76 (*s*, 3 H, methyl ester), 3.50 (*m*, 2 H, methylene); (2) u.v. (H_2O), 277 nm (ϵ = 9395); (3) c.i.m.s. (source and probe temperature 210 and 120° respectively), ions with m/e $\geq$ 120 and relative intensity $\geq$ 10 per cent of base peak were as follows: m/e (relative intensity) 299 (MH^+, 80), 281 (63), 251 (33), 223 (38), 221 (25), 180 (40), 122 (100); and (4) calculated analysis for $C_{12}H_{14}O_7N_2$: C, 48.33; H, 4.73; N, 9.39. Found: C, 47.80; H, 4.97; N, 9.50.

Synthesis of (1*R*,2*R*)-1-*nitrophenyl*-1,3-*propanediol*-2-*oxamic acid* (*CAP oxamic acid*). CAP methyl oxamate (4 g, 13.4 m-moles) was dissolved in 1 N sodium hydroxide (30 ml) and stirred at room temperature for 30 min. The reaction solution was then extracted with ethyl acetate (30 ml), acidified with 6 N hydrochloric acid (8 ml) and extracted with ethyl acetate (40 ml, five times). The combined ethyl acetate extracts of the acidic aqueous solution were washed with water (10 ml, twice), dried ($MgSO_4$) and evaporated under vacuum to yield a pale yellow solid, which was washed with benzene and air dried to give a white solid 3.05 g (80 per cent, t.l.c. pure), m.p. 125–26° (gas liberated upon melting). The product was recrystallized from a mixture of ethanol and ethylene dichloride to yield white needle crystals, m.p. 127–28° (gas liberated upon melting). The identity of the product was confirmed by the following criteria: (1) n.m.r. (60 MHz, DMSO-d_6, TMS internal standard) δ (ppm) 8.27 (*m*, 2 H, aromatic), 7.68 (*m*, 2 H, aromatic), 5.13 (*m*, 1 H, benzylic), 4.07 (*m*, 1 H, methine), 3.58 (*m*, 2 H, methylene); (2) u.v. (H_2O) 277 nm (ϵ = 9431); (3) c.i.m.s. (source and probe temperature 180 and 160° respectively) ions with m/e $\geq$ 120 and relative intensity $\geq$ 10 per cent of base peak were as follows: m/e (relative intensity) 223 ($MH^+ - 62$, 100), 207 (12), 193 (26), 175 (14), 163 (35), 122 (12); and (4) calculated analysis for $C_{11}H_{12}O_7N_2$: C, 46.50; H, 4.22; N, 9.87. Found: C, 46.14; H, 4.09; N, 9.93.

The structure of CAP oxamic acid was further confirmed by chemical degradation and synthesis.

Hydrolysis of CAP oxamic acid. CAP oxamic acid (100 mg) was refluxed in 1 N hydrochloric acid for 1 hr. The reaction mixture was extracted with ethyl acetate, then basified with 1 N sodium hydroxide and re-extracted with ethyl acetate. The ethyl acetate extract of the alkaline solution was dried ($MgSO_4$) and evaporated under vacuum to give a pale yellow solid which was recrystallized from water to yield CAP base: pale yellow plate crystals, 10 mg, m.p. 160–62° (lit. [5] 160–62°).

Methylation of CAP oxamic acid with diazomethane. CAP oxamic acid (500 mg) was methylated with diazomethane which was generated from Diazald following the directions given on the reagent bottle. The resulting ethyl ether solution was evaporated to dryness to give a pale yellow solid, which was recrystallized from ethylene dichloride to yield white crystals, 222 mg (42 per cent), m.p. 142–45° (nearly t.l.c. pure). The n.m.r. and isobutane c.i.m.s. analyses of the product were consistent with CAP methyl oxamate.

Preparation of microsomes. Male Sprague–Dawley rats (160–200 g) obtained from Hormone Assay Laboratories (Chicago, IL) were used in the experiments. Animals were allowed free access to water and food (Purina Lab Rat Chow). Rats were pretreated with phenobarbital (80 mg/kg, in saline, i.p.) 72, 48 and 24 hr before the experiment. The rats were killed by decapitation and their livers were removed immediately and washed in 0.9% saline. The livers of three rats were combined, homogenized in 3 vol. of 0.25 M sucrose and the microsomes were isolated by the calcium aggregation method [6, 7]. The microsomal pellet was resuspended in 1.15% KCl–0.02 M Tris–HCl, pH 7.4 (Tris–KCl) to a concentration of microsomal protein of 2 mg/0.8 ml. Protein concentration was determined by the method of Lowry *et al.* [8].

Incubation of CAP in vitro. The incubations were conducted using the same basic procedure that was previously described [1, 2, 4]. Each incubation vial contained 2 mg of microsomal protein, 0.1 mM NADH, 2.0 mM nicotinamide, 0.2 mM NADP, 2.0 mM glucose 6-phosphate, and 1 unit of glucose 6-phosphate dehydrogenase in a total volume of 1 ml of Tris–KCl buffer (pH 7.4). The mixture was preincubated at 37° for 1 min and the reaction was started by the addition of a mixture of [^{14}C]CAP (5.2 dis./min/pmole) and [1-^{3}H]CAP (25.9 dis./min/pmole) in 10 μl dimethylformamide to give a final concentration of CAP of 0.1 mM. The incubation was performed at 37° in air for 20 min and the reaction was stopped by the addition of absolute ethanol (3 ml).

Biotransformation of CAP in vitro. To obtain a sufficient amount of metabolite for characterization, 100 incubations were conducted with microsomes from phenobarbital-pretreated rats as described in the previous section. After addition of ethanol the reaction mixtures were centrifuged and the ethanol supernatants were combined and evaporated under vacuum. The residue was dissolved in water (15 ml, approximately pH 6) and extracted with ethyl acetate (30 ml, five times). The extracted aqueous phase was acidified with concentrated hydrochloric acid (2 ml) and extracted with ethyl acetate (30 ml, five times). The combined ethyl acetate extract was filtered through magnesium sulfate, evaporated under vacuum to give an oily residue, which was dissolved in methanol (100 μl) and chromatographed on an Analtec Silica gel GF, 1 mm, t.l.c. plate. The plate was developed with solvent system 1, air dried and re-developed in the same dimension with system 4. A fraction corresponding to authentic CAP oxamic acid (dark blue band under u.v.) was scraped from the t.l.c. plate and eluted with water (3 ml). The aqueous extract was acidified with 6 N hydrochloric acid (1 ml), and extracted with ethyl acetate (10 ml, four times). The ethyl acetate extract was then evaporated under vacuum to give a small amount of pale yellow oil, which was further characterized by the following three criteria: (1) reverse isotope dilution with authentic CAP oxamic acid, using ethanol: ethylene dichloride as the recrystallization solvent, (2) high pressure liquid chromatography, and (3) isobutane c.i.m.s. after derivatization with diazomethane.

Chemical hydrolysis of radiolabeled microsomal protein. The radiolabeled microsomal protein from the above 100 incubation mixtures containing [^{14}C]- and [1-^{3}H]CAP was collected as a pellet after the reactions were stopped with ethanol (3 ml) and the reaction mixtures centrifuged. The protein pellets were washed with methanol–ether (3:1, 5 ml) ten times, as described in the previous investigations on covalent binding [1, 4]. After aspiration of the final wash, which contained virtually no radioactivity, the protein was dissolved in 1 ml of 1 N sodium hydroxide. The covalently bound radioactivity was determined by counting an aliquot (0.5 ml) of the alkaline solution [1, 4]. Protein was determined by the method of Lowry *et al.* [8].

Acid hydrolysis: a sample (10 mg) of washed microsomal protein was hydrolyzed with 6 N hydrochloric acid (5 ml) at 110 ± 2°, under an atmosphere of nitrogen, for 25.5 hr. The reaction mixture was extracted with ethyl acetate (5 ml, five times), basified with 10 N sodium hydroxide (5 ml) and extracted with ethyl acetate (12 ml, four times). The radiochemical composition of the ethyl acetate extracts was investigated by scintillation counting, reverse isotope dilution and comparative t.l.c. with synthetic standards.

Base hydrolysis: another sample (10 mg) of washed microsomal protein was hydrolyzed in 1 N sodium hydroxide at room temperature under an atmosphere of nitrogen for 17 hr. The reaction mixture was acidified with concentrated hydrochloric acid, and extracted with ethyl acetate (5 ml, five times). The radiochemical composition of the extract was investigated by scintillation counting and comparative t.l.c. with synthetic standards.

RESULTS

Identification of CAP oxamic acid as a metabolite of CAP in vitro *by reverse isotope dilution, h.p.l.c., and isobutane c.i.m.s.*

A radioactive fraction, with the solubility properties of a carboxylic acid, was isolated by extraction and preparative t.l.c. from the incubation mixture of [^{14}C]- and [1-^{3}H]CAP with microsomes from phenobarbital-pretreated rats. Recrystallization of this fraction to constant specific activity with authentic CAP

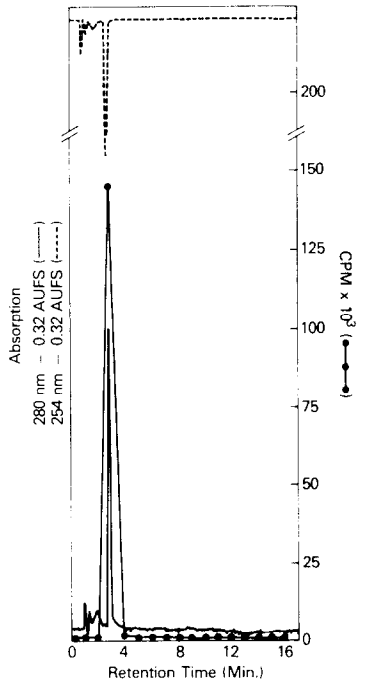

Fig. 2. High pressure liquid chromatography of a metabolite of chloramphenicol (CAP) isolated by extraction and preparative t.l.c. from the incubation mixture of [^{14}C]- and [1-^{3}H]CAP with liver microsomes from phenobarbital-pretreated rats. The isolated metabolic fraction was dissolved in water and chromatographed on an ODS Spherisorb, 3 mm × 250 mm, 5-μm column with an eluant of 0.02 M phosphate buffer, pH 4.0, and a flow rate of 1 ml/min. The instrument was equipped with a dual wave length u.v. detector, 280 and 254 nm; 1-ml samples were collected for 30 min, and aliquots were taken for scintillation counting. Authentic CAP oxamic acid had the same retention volume and u.v. properties as the metabolite.

oxamic acid (Fig. 1, compound 4) revealed that the sample contained [^{14}C] and [^{3}H] labels in a ratio of 1:1; calculations based on the specific activity indicated that nearly 100 per cent of the radioactivity in the fraction was associated with CAP oxamic acid. High pressure liquid chromatography of the fraction indicated that the radioactivity in the sample was associated with a component that had a relative u.v. absorption at 280–254 nm of 2.51 (Fig. 2). The retention volume and u.v. properties of this component were identical to authentic CAP oxamic acid. The eluted radioactive fraction (Fig. 2) was isolated from the aqueous solution, methylated with diazomethane and analyzed by isobutane c.i.m.s. (Fig. 3). The c.i.m.s. of the metabolite derivative (Fig. 3b) was nearly identical to the methyl ester of CAP oxamic acid (Fig. 3a), with an ion at m/e 299, corresponding to the molecular ion (MH^+), and fragment ions at m/e 281 ($MH^+ - 18$, H_2O), 251, 223, 221, 180 and 122.

When a mixture of [^{14}C]- and [1-^{3}H]CAP was incubated with microsomes from untreated rats, no radioactive fraction corresponding to CAP oxamic acid could be detected in the reaction mixtures by t.l.c.; nor was there any covalent binding of the radioactivity to protein.

Identification of oxalic acid, CAP base and CAP oxamic acid as products of the chemical hydrolysis of radiolabeled microsomal protein

Microsomal protein was isolated from the incubation mixture of [^{14}C]- and [1-^{3}H]CAP with microsomes from phenobarbital-pretreated rats and was found to contain both [^{14}C] and [^{3}H] labels irreversibly bound in a ratio of 1:1. After the protein was treated with 6 N hydrochloric acid for 25.5 hr at 110°, nearly all of the bound radiolabel was extracted from acidic and alkaline solutions with ethyl acetate. The extract of the acidic solution contained only [^{14}C] label. At least 65 per cent of this fraction was identified as [^{14}C]-oxalic acid by reverse isotope dilution with authentic oxalic acid. In contrast, the extract of alkaline solution contained only [^{3}H] label. Approximately 70 per cent of the fraction was identified as [1-^{3}H]CAP base by comparative t.l.c. with authentic CAP base, using solvent system 2, $R_f = 0.19$ and solvent system 3, $R_f = 0.58$.

Another sample of microsomal protein was treated with 1 N sodium hydroxide for 17 hr at room temperature. The ethyl acetate extract of the acidified reaction mixture contained approximately 50 per cent of the [^{3}H] and 70 per cent of the [^{14}C] label which was bound originally to protein. Nearly 80 per cent

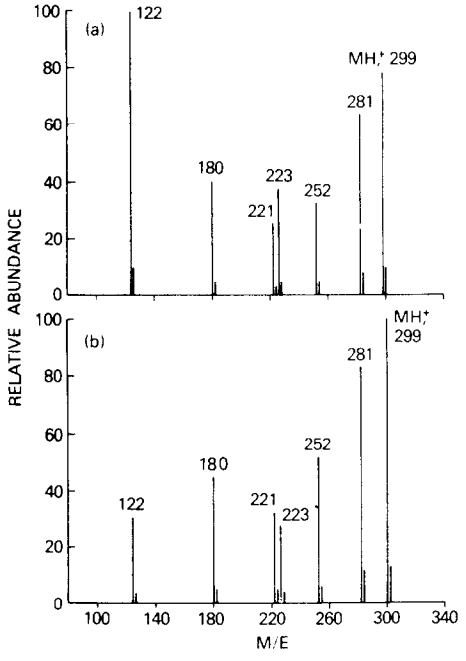

Fig. 3. Isobutane chemical ionization mass spectra (c.i.m.s.): (a) authentic methyl ester of CAP oxamic acid, (b) metabolite of CAP *in vitro* purified by h.p.l.c. and derivatized with diazomethane. Ions with an m/e of ⩾120 and a relative intensity of ⩾10 per cent of the base peak are reported. The c.i.m.s. of the authentic ester was performed at a source temperature of 210° and a probe temperature of 120°; the metabolite was analyzed at 230 and 60° respectively. The metabolite was quantitatively extracted with ethyl acetate from the phosphate buffer eluant of h.p.l.c. after the solution was acidified with 3 N hydrochloric acid. The ethyl acetate extract was then evaporated under vacuum and the residue was derivatized with diazomethane.

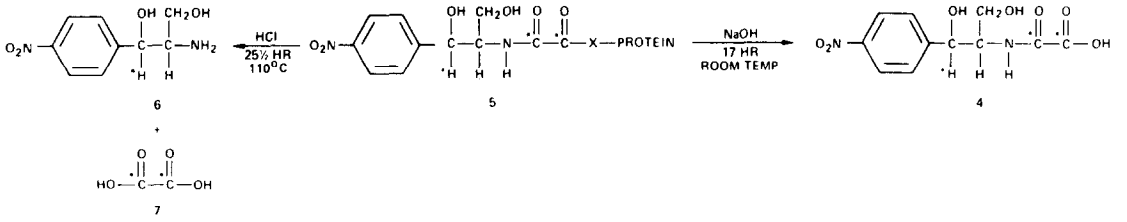

Fig. 4. Products isolated from the chemical hydrolysis of [^{14}C]- and [^{3}H]-labeled microsomal protein. Compound 5 is the proposed covalently bound product of the metabolism of CAP by liver microsomes from phenobarbital-pretreated rats. The X represents an O, S or N atom of an amino acid in the protein. The asterisks represent the positions of [^{14}C] and [^{3}H] labels. The procedures used for the hydrolysis and identification of products are represented in Methods.

of the extract was identified as [^{14}C]- and [^{3}H]-labeled CAP oxamic acid by comparative t.l.c. with authentic compound on Analtec silica gel 250-μm plates, using solvent system 3, $R_f = 0.51$, and solvent system 4, $R_f = 0.64$.

DISCUSSION

In this investigation, a new metabolite of CAP was isolated from the incubation mixture of [^{14}C]- and [1-^{3}H]CAP with microsomes from phenobarbital-pretreated rats. This compound was identified as CAP oxamic acid by reverse isotope dilution and h.p.l.c. (see Fig. 2) with authentic CAP oxamic acid. This assignment was confirmed by the isobutane c.i.m.s. of the methylated derivative of the metabolite (see Fig. 3). Derivatization of the metabolite with diazomethane was performed prior to c.i.m.s., since it was found that the spectrum of authentic CAP oxamic acid did not contain a characteristic protonated molecular ion, MH^+; instead, this compound underwent thermolysis upon analysis to yield ions of lower m/e. Moreover, authentic CAP oxamic acid was not appreciably volatile and therefore was relatively insensitive to detection. The more volatile and stable methyl ester of CAP oxamic acid, however, was readily detected and yielded a characteristic protonated molecular ion at m/e 299 (see Fig. 3a). The presence of this ion in the c.i.m.s. of the derivatized metabolite (see Fig. 3b) was consistent with the metabolite being CAP oxamic acid. The differences observed in the relative abundance of ions in the c.i.m.s. of authentic CAP methyl oxamate (see Fig. 3a) and the methylated metabolite (Fig. 3b) were probably due to the differences in source and probe temperature which were used for analyses (see Fig. 3); small changes in these temperatures were found to effect the c.i.m.s. of authentic CAP methyl oxamate.

The isolation and identification of CAP oxamic acid, as a metabolite of CAP, support the proposed pathway for the bioactivation of CAP *in vitro* by liver microsomes from phenobarbital-pretreated rats [1–4] (see Fig. 1). In this mechanism, the reactive oxamyl chloride, compound 3, either acylates protein to form compound 5, or is hydrolyzed by water to CAP oxamic acid, compound 4. Therefore, a correlation should exist between the level of covalent binding and the amount of CAP oxamic acid formed. Indeed, when CAP was incubated with microsomes from control rats, no binding was observed and CAP oxamic acid was not detected by t.l.c. analysis of the reaction mixture.

The formation of the oxamyl chloride is also suggested by the products isolated from chemical hydrolyzates of radiolabeled microsomal protein. The oxamyl chloride would be expected to bind covalently to microsomal protein by forming ester, thioester or amide bonds to amino acids in protein (Fig. 4). When the radiolabeled protein is hydrolyzed exhaustively in hydrochloric acid, [1-^{3}H]CAP base, compound 6, and [^{14}C]-oxalic acid, compound 7, would be expected to be formed. In fact, these products not only were isolated and identified by t.l.c. and isotope dilution analysis after this chemical treatment was performed but actually accounted for the majority of the radiolabel which was bound initially to the microsomal protein.

The hydrolysis of the radiolabeled protein under mild alkaline conditions would be expected to hydrolyze selectively ester and thioester bonds. If this occurred, CAP oxamic acid which was covalently linked to protein through an ester or a thioester bond would be released (see Fig. 4). When this experiment was conducted, the amount of [^{14}C]- and [^{3}H]-labeled CAP oxamic acid released accounted for approximately 50 per cent of the radioactivity that was extracted from the protein. Enzymatic hydrolysis of the radiolabeled microsomal protein with Pronase, and separation of the solubilized radiolabeled products on a Sephadex G-10 column, revealed that free CAP oxamic acid was not present in the hydrolyzate.* This observation confirms the idea that CAP oxamic acid is covalently bonded to microsomal protein through an ester, thioester or amide bond (see Figs. 1 and 4) and not merely entrapped by microsomal protein.

The results presented in this study are consistent with the findings of previous investigations [1–4] and, therefore, support the proposal that CAP is metabolically activated by liver microsomes of phenobarbital-pretreated rats by an oxidative dechlorination mechanism (see Fig. 1). The relevance of this pathway of biotransformation to the pharmacologic and toxicologic properties of CAP is currently being investigated.

Acknowledgements—We wish to thank Dr. James R. Gillette for the critical reviewing of this manuscript and Miss E. Boykins for valuable technical assistance. Lance R. Pohl was supported by NIGMS Fellowship 5 F22 GM 02365-02.

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