Biochemical Pharmacology, 1966, Vol. 15, pp. 1507–1521. Pergamon Press Ltd., Printed in Great Britain.

THE ABSORPTION, EXCRETION, AND METABOLISM OF CHLORPHENESIN CARBAMATE IN THE DOG

DONALD R. BUHLER,* HARRY HARPOOTLIAN and R. L. JOHNSTON

Research Laboratories, The Upjohn Company, Kalamazoo, Mich., U.S.A.

(*Received* 15 *October* 1965; *accepted* 28 *March* 1966)

Abstract—The absorption, excretion, and fate of orally administered chlorphenesin carbamate-^{3}H have been studied in the dog. The drug was well absorbed, with about 67–79 per cent of a single oral dose of 100 mg/kg appearing in the urine within 48 hr and only 4–12 per cent in the feces.

Approximately 81 per cent of the urine radioactivity was present in neutral metabolites or their conjugates while 19 per cent of the radioactivity appeared as free or conjugated acidic metabolites. The major urinary metabolite in the dog, accounting for 59 per cent of the radioactivity in 0–12 hr and 32 per cent of the radioactivity in 12–48 hr, was a glucuronide of chlorphenesin carbamate. An additional 25 per cent of the radioactivity in urine was accounted for as the free drug or its sulfate conjugate. The major acidic metabolites in dog urine were *p*-chlorophenol, *p*-chlorophenoxylactic acid, and *p*-chlorophenoxyacetic acid. Several additional minor acidic and neutral metabolites also were isolated and characterized.

CHLORPHENESIN carbamate (3-[*p*-chlorophenoxy]-1,2-propanediol-1-carbamate)† is a new skeletal muscle relaxant recently described by Matthews *et al.*[1] Studies of the metabolism of chlorphenesin carbamate indicate that a substantial portion of the administered drug is oxidized to a number of acidic metabolites by the rat.[2] In the human, however, the drug undergoes little oxidation and is excreted mainly as a glucuronide conjugate.[2, 3]

The related drug mephenesin (3-[*o*-methylphenoxy]-1,2-propanediol) is rapidly oxidized *in vivo* and excreted primarily as β-(*o*-methylphenoxy)-lactic acid.[4–8] When the terminal carbon group of mephenesin is protected by formation of the carbamate ester, the resulting drug mephenesin carbamate shows an increased efficiency, apparently due to its decreased rate of oxidation and metabolic inactivation.[9–11] Hence, the principal urinary metabolite of mephenesin carbamate in dogs is a conjugate of the unmodified drug.[12] Studies with another related drug methocarbamol in dogs confirmed that the carbamate ester group is not hydrolyzed *in vivo*, since the drug is excreted primarily as a glucuronide conjugate.[13] The carbamate groups of other drugs also were found to be quite stable *in vivo*.[14, 15]

In view of the observed differences in the stability of the carbamate ester group of chlorphenesin carbamate in the rat[2] and human,[2, 3] it was of interest to investigate its fate in a third test species. Therefore, a detailed investigation of the absorption, excretion, and metabolism of tritium-labeled chlorphenesin carbamate has now been carried out in the dog.

* Present address: Biology Department, Pacific Northwest Laboratory, Batelle Memorial Institute, Richland, Washington.

† Registered by The Uphohn Co. under the trade name Maolate.

METHODS

Chlorphenesin carbamate-^3H. Tritium-labeled chlorphenesin carbamate was prepared by gaseous exchange procedure and thoroughly purified.* The resulting product (4·5 μc/mg) was radiochemically and chemically pure as evidenced by paper chromatography, infrared and ultraviolet spectra, elemental analysis, and melting point.

Radioactivity measurement. All counting was performed on a Tri-Carb 314X or 314EX-2A liquid scintillation spectrometer (Packard Instrument Co., La Grange, Ill.) at settings suitable for measurement of tritium. Aliquots of urine, water, or organic solvents were counted in 10 ml of diotol scintillator [toluene:dioxane: methanol (350:350:210 by volume) containing 73 g naphthalene, 4·6 g 2,5-diphenyloxazole (PPO), and 0·080 g 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) per liter]. The tritiated water content of urine samples was determined by counting aliquots of water obtained by lyophilization of urine. Dried blood and fecal samples were burned in an atmosphere of oxygen,[16] and the resulting water was trapped and counted. All samples were recounted after addition of tritium-labeled toluene internal standard and the results then expressed as disintegrations per minute (dis/min).

Animal studies. Three female beagle dogs (9·90, 13·55, and 9·80 kg) were fasted overnight; each animal then was given orally one gelatin capsule containing 1·00 g and $1{\cdot}73 \times 10^8$ dis/min of tritium-labeled chlorphenesin carbamate. Blood samples were taken from the jugular vein of the unanesthetized dogs at 0·5, 1, 2, 4, 6, 8, 12, and 24 hr and the blood immediately frozen for eventual lyophilization. Urine was removed from each dog by catheterization after 2, 4, 8, 12, and 24 hr respectively. The animals were then placed in individual metabolism cages and urine collected at 24-hr intervals over 8 days. Feces were collected at 24-hr intervals, homogenized with water, and aliquots lyophilized to dryness.

In order to examine the effect of sex upon the metabolism of chlorphenesin carbamate and to obtain sufficient quantities of metabolites for identification purposes, two male (9·4 and 9·6 kg) and two female (9·4 and 10·4 kg) beagle dogs were each given capsules containing 1·0 g nonradioactive chlorphenesin carbamate every 24 hr for 5 days. The urine was collected from each dog over a 6-day period and pooled according to sex. The pooled male and female dog urines were then hydrolyzed and extracted as described below.

Extraction of metabolites. Pooled urine samples were adjusted to pH 2 with glacial acetic acid and extracted 5 times with 1/3 volume chloroform to remove the nonconjugated metabolites. This extract was in turn fractionated into a *nonconjugated acidic* fraction and a *nonconjugated neutral* fraction by basic washing of the chloroform extract and subsequent re-extraction of the acidified basic wash. The urine residue then was adjusted to pH 5 and incubated under toluene with a β-glucuronidase preparation (Ketodase; Warner-Chilcott Co., Morris Plains, N.J.) at 37° for 120 hr. The incubation mixture was again adjusted to pH 2, extracted exhaustively with chloroform, and the chloroform extract separated into *glucuronide acidic* and *glucuronide neutral* subfractions. Finally, the urine residue from the above extraction was readjusted to pH 5 and incubated under toluene at 37° for 120 hr with a combination sulfatase and β-glucuronidase preparation (Glusulase; Endo Laboratories, Inc., Richmond Hill, N.Y.). The digest was adjusted to pH 2, extracted five times with 1/3 volume chloro-

* R. C. Thomas, D. R. Buhler and G. J. Ikeda, in preparation.

form, and the chloroform extract separated into *sulfate acidic* and *sulfate neutral* fractions. All chloroform extracts were concentrated *in vacuo* with a minimum of heating and with cooling of the condensing flasks in dry ice–methanol in order to minimize the loss of volatile metabolites. The urine hydrolysis and extraction procedure is shown diagrammatically in Fig. 1.

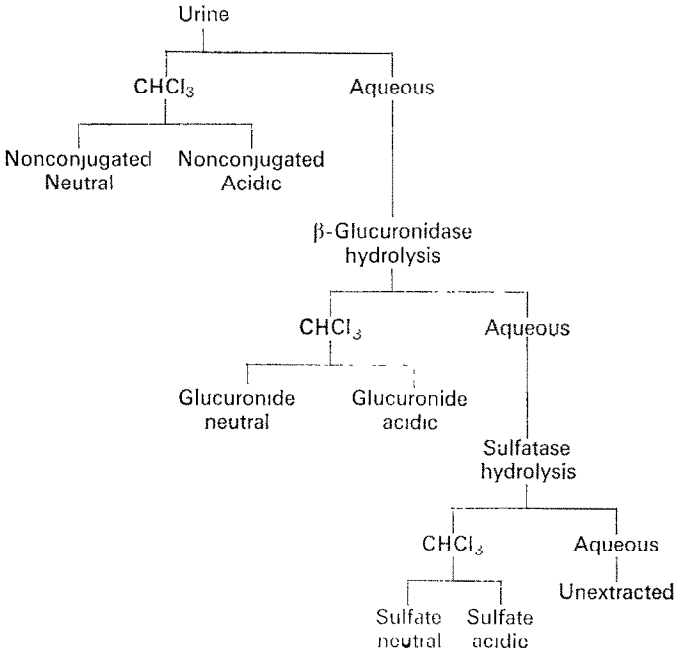

FIG. 1. Urine hydrolysis and extraction flow chart.

Chromatography. Paper chromatography of neutral metabolites was performed in the Bush B-5 system, benzene:methanol:water (10:5:5)[17] or the FBW system, paper impregnated with formamide, mobile-phase butyl acetate:formamide:water (100:5:5).[18] Acidic metabolites were chromatographed in isopropanol:aqueous ammonia:water (8:1:1) (IAW); benzene:propionic acid:water (2:2:1) (BPW); chloroform:methanol:formic acid:water (1000:100:4:96) (CMF); and benzene:acetic acid:water (1:1:2) (BZAW). Dried paper chromatograms were examined under u.v. light. Carbamates were detected with the sodium hypochlorite spray,[2] acids were visualized by spraying with 0·1% bromphenol blue indicator, and phenolic compounds were detected with diazotized sulfanilic acid.

Dried blood samples were leached overnight with 90% aqueous methanol, filtered, the filtrates evaporated to dryness *in vacuo*, and aliquots chromatographed on paper in the IAW system. The dried radioactive chromatograms were cut into sequential 1-cm portions, the strips transferred to individual counting vials containing 1 ml methanolic Hyamine hydroxide (Packard Instrument Co., La Grange, Ill.), and the vials allowed to stand at 25° for 16 hr. Diotol (10 ml) was added to each vial and the vials then counted in the usual manner.

Thin-layer chromatography (TLC) of neutral metabolites was performed on alumina GF (Brinkmann Instrument Co., Great Neck, N.Y.) and developed in ethyl acetate: cyclohexane (1:3). Acidic metabolites were fractionated on silica gel plates eluted with BPW, CMF, or methanol:chloroform (1:4). Radioactive zones were located by transferring sequential 1-cm portions along one edge of a streaked plate to individual counting vials and counting in the usual manner, with 10 ml diotol containing 0·3 ml water. Neutral compounds were eluted from silica gel with methanol and the evaporated extract leached with methylene chloride. Acids were eluted with 0·1 N hydrochloric acid which was then extracted with methylene chloride.

Neutral metabolites were separated on a column 4·8 × 69 cm made from 384 g of acid-washed Celite 545 which had been previously mixed with 346 ml of 70% methanol saturated with cyclohexane. Elution was initiated with the organic phase of cyclohexane:methanol:water (20:7:3) and continued with cyclohexane:benzene:methanol: water (10:10:7:3), benzene:methanol:water (20:7:3) and benzene:ethyl acetate: methanol:water (19:1:7:3). Flow rate was maintained at 80 ml/hr, and 20-ml fractions were collected.

Certain neutral fractions were further purified by chromatography on small Florisil columns eluted with acetone–isooctane mixtures of increasing polarity.

Acidic metabolites were separated on a silica gel column (3·7 × 92 cm) containing 500 g silica gel mixed with 500 ml 0·05 M citrate buffer, pH 3·42. Elution was initiated with chloroform saturated with buffer and continued with 0·5% ethanol plus 99·5% chloroform saturated with buffer. Twenty-ml fractions were collected at a flow rate of 60 ml/hr.

RESULTS

The recovery of radioactivity in the urine and feces of dogs after an oral dose of tritium-labeled chlorphenesin carbamate is summarized in Table 1. The data demonstrate that the orally administered drug was well absorbed and then excreted primarily

TABLE 1. EXCRETION OF RADIOACTIVITY FROM DOGS AFTER ORAL ADMINISTRATION OF TRITIUM-LABELED CHLORPHENESIN CARBAMATE

Time (hr)	% Dose in urine			% Dose in feces			% Dose as THO in urine		
	Dog A	Dog B	Dog C	Dog A	Dog B	Dog C	Dog A	Dog B	Dog C
0–2	6·65	7·80	5·22	†	†	†	0·0116	0·0370	0·0247
2–4	12·3	5·66	17·5	†	†	†	0·0162	0·0059	0·0270
4–8	11·2	25·43	22·9	†	†	†	0·0444	0·0379	0·0223
8–12	8·50	13·65	12·95	†	†	†	0·0480	0·0414	0·0386
12–24	9·80	19·5	0·32*	10·5	4·41	3·11	0·0496	0·103	0·0120*
24–48	18·6	6·88	16·4	1·62	1·43	1·01	0·682	0·405	1·360
48–72	1·90	1·28	1·42	†	†	0·37	0·129	0·192	0·0842
72–96	2·20	1·79	2·12	†	0	†	0·275	0·244	0·250
96–120	1·18	1·68	0·99	0·23	0·81	4·16	0·169	0·317	0·266
120–144	0·26	0·51	0·40	0·16	0·047	0·47	0·121	0·175	0·175
144–168	0·25	0·49	0·15	0	0·041	0·21	0·257	0·202	0·142
168–192	0·11	0·31	0·12	0	0·028	0	0·127	0·133	0·220
Total	72·95	84·95	80·49	12·51	6·77	9·33	1·93	1·90	2·62

* Lost part. † No excretion.

via the urine. Approximately 50 to 70 per cent of the dose was eliminated during the initial 24 hr, and the total recovery of radioactivity in both urine and feces after 8 days ranged between 85·5 per cent and 91·7 per cent of the dose. Examination of the urine from dogs A, B, and C showed that 1·93, 1·90, and 2·62 per cent of the dose, respectively, was excreted into the urine as tritiated water. After 5 days the bulk of the radioactivity in urine was present as tritiated water (Table 1). Excretion at the time, therefore, largely reflected the biological half-life of tritiated water rather than continued excretion of drug.

Peak blood radioactivity levels occurred 2–3 hr after oral administration of tritiated chlorphenesin carbamate (Fig. 2). Radioactivity decreased initially with a half-life

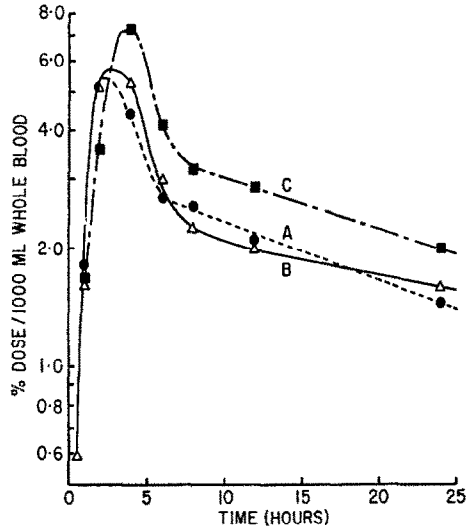

FIG. 2. Semilogarithmic plot of blood radioactivity in dogs that received an oral dose of chlorphenesin carbamate-^{3}H.

of about 2·5 hr, but a second slower process soon predominated with a half-life of about 25 hr. Scans of blood extract chromatograms indicated that the free drug disappeared from the blood within about 2 hr. Chlorphenesin carbamate O-glucuronide (IAW R_f 0·35) was present in maximal concentration in blood at 4 hr and thereafter decreased with a half-life of 2·7 hr. Chromatography showed that the subsequent longer rate process with a half-life of 25 hr represented mainly the disappearance of chlorphenesin carbamate metabolites from the blood.

Urine was extracted as previously described, and the distribution of radioactivity is presented in Table 2. The dog excreted initially a major portion of orally administered chlorphenesin carbamate into the urine as neutral metabolites. A substantial increase in the excretion of acidic metabolites was observed in subsequent urine samples along with a marked decrease in the proportion of conjugated metabolites.

Chlorphenesin carbamate (metabolite N-1) was the only radioactive material present in concentrations sufficient for detection by paper chromatography in the 0 to 12-hr glucuronide and sulfate neutral extract fractions and in all 12 to 48-hr neutral extract fractions. No trace of the parent drug chlorphenesin was found in any neutral fraction.

The glucuronide and sulfate neutral fractions from the 0 to 12-hr and 12 to 48-hr urine collections were recrystallized twice from methylene chloride (charcoal treatment) to yield crystalline products. The melting points, elemental analyses, infrared spectra,* and nuclear magnetic resonance† (NMR) spectra confirmed their identity with authentic chlorphenesin carbamate.

TABLE 2. DISTRIBUTION OF URINE RADIOACTIVITY FROM DOGS RECEIVING TRITIUM-LABELED CHLORPHENESIN CARBAMATE

Fraction	% Total urine radioactivity	
	0–12 hr*	12–48 hr†
Nonconjugated neutral extract	0·89	15·0
Nonconjugated acid extract	2·32	17·2
Glucuronide neutral extract	58·9	31·8
Glucuronide acid extract	2·32	2·47
Sulfate neutral extract	24·4	12·1
Sulfate acid extract	2·44	10·8
Unextracted	5·89	4·48
Total	97·2	93·9
Neutral total	84·2	58·9
Acidic total	7·08	30·5

* Total urine radioactivity $2{\cdot}46 \times 10^8$ dis/min, 47·5 per cent of the dose.
† Total urine radioactivity $1{\cdot}77 \times 10^8$ dis/min, 34·2 per cent of the dose.

TABLE 3. PROPERTIES OF CHLORPHENESIN CARBAMATE RECOVERED FROM URINE EXTRACTS OF DOGS RECEIVING TRITIUM-LABELED CHLORPHENESIN CARBAMATE

Fraction	Weight of isolate* (mg)	Melting point	$[\alpha]_D$†	Specific activity (dpm × 10^7/mmole)	Elemental analyses‡			
					C	H	N	Cl
0- 12-hr Nonconjugated neutral§	6,380	88–89°	0°		49·07	5·16	5·74	14·87
0- 12-hr Glucuronide neutral	605	88–90°	−6°	4·01	48·47	5·22	6·15	14·28
0- 12-hr Sulfate neutral	164	86–87°	−7°	3·90	49·20	5·02		14·30
12- 48-hr Nonconjugated neutral	26	87–88°	0°	4·00	48·76	5·11		
12- 48-hr Glucuronide neutral	139	87–88°	0°	3·93	48·89	4·83		
12- 48-hr Sulfate neutral	19	84–85°	0°	4·20	48·16	4·88		

* All isolates exhibited an infrared spectrum identical with that of authentic chlorphenesin carbamate.
† Carried out in chloroform at a concentration of about 0·70 g/100 ml.
‡ Theoretical for $C_{10}H_{12}ClNO_4$: C, 48·89; H, 4·92; N, 5·70; Cl, 14·43.
§ This fraction was diluted with nonradioactive carrier as described in the text.

The 12 to 48-hr nonconjugated neutral fraction was chromatographed on a small Florisil column. The major radioactive zone eluted from the column with acetone was purified and identified as chlorphenesin carbamate, as previously described (Table 3).

* KBr pellet i.r. spectra were obtained in a Perkin-Elmer model 421 apparatus.
† Determined in $CDCl_3$ in a Varian model A-60 apparatus. The authors are indebted to G. Slomp and F. MacKellar, Physical and Analytical Chemistry Research, The Upjohn Co., for these analyses.

A minor radioactive zone eluted from the column with acetone:isooctane (1:4) contained a minor neutral metabolite (designated metabolite N-4) with chromatographic properties as outlined in Table 4.

TABLE 4. CHROMATOGRAPHIC PROPERTIES AND CARBAMATE REACTION OF NEUTRAL URINARY METABOLITES FROM DOGS RECEIVING TRITIUM-LABELED CHLORPHENESIN CARBAMATE

Metabolite	Probable identity	R_f Value in solvent*		u.v.	Carbamate test
		A	B		
N-1	Chlorphenesin carbamate	0·45	0·55	+	+
N-2		0·08	0·08	±	+
N-3		0·40	0·41	±	−
N-4		0·40	0·85	+	−

* The solvent systems employed were: A, FBW; and B, Bush B-5.

In addition to chlorphenesin carbamate and metabolite N-4, the 0 to 12-hr nonconjugated neutral fraction contained two additional minor neutral components, metabolite N-2 and metabolite N-3 (Table 4). To provide sufficient material for identification of the minor neutral compounds, the radioactive 0 to 12-hr nonconjugated neutral extract (121 mg, $2{\cdot}19 \times 10^6$ dis/min) was pooled with the neutral extracts from a study with nonradioactive chlorphenesin carbamate (no differences had been observed in the chromatography patterns from male and female dogs). The combined neutral extract was fractionated on a Celite column and the major radioactive eluate purified and characterized as chlorphenesin carbamate (Table 3). The remaining column fractions were pooled (2·43 g, $7{\cdot}98 \times 10^5$ dis/min) and chromatographed on a small Florisil column. A radioactive material eluted from the column with acetone:isooctane (1:4) and (3:7) gave 295 mg material containing $7{\cdot}34 \times 10^4$ dpm. This neutral metabolite proved volatile and exhibited chromatographic properties similar to those of metabolite N-4 (Table 4). Chromatography of an aliquot of the impure material on silica gel GF developed with ethyl acetate:cyclohexane (1:3) gave a u.v.-absorbing radioactive zone with an R_f of 0·21. Upon elution of the zone with acetone, a glassy material was recovered which exhibited an i.r. spectrum with λ_{max} at 3380, 1735, 1590, 1270, 1140, 1096 and 825 cm^{-1}. Attempts to crystallize or characterize this material further were unsuccessful, but the data suggest that metabolite N-4 could be a compound such as 1-(*p*-chlorophenoxy)-3-hydroxy-2-propanone.

Sufficient quantities of metabolites N-2 and N-3 could not be recovered from the remaining column fractions to attempt further characterization. The lack of suitable reference standards has precluded more definitive identification of these minor neutral metabolites.

Examination of the 0 to 12-hr acidic extract fractions by paper chromatography showed four major acidic metabolites which have been designated metabolites A-2, A-4, A-6 and A-10. The chromatographic properties of these major metabolites and those of a number of minor metabolites are summarized in Table 5. The nonconjugated acidic extract contained primarily metabolite A-4 and a smaller amount of metabolite A-10. The major component of the glucuronide acidic extract fraction was metabolite

TABLE 5. CHROMATOGRAPHIC PROPERTIES, COLOR REACTIONS AND RELATIVE DISTRIBUTION OF 0 TO 12-HR URINARY METABOLITES FROM DOGS RECEIVING TRITIUM-LABELED CHLORPHENESIN CARBAMATE

Metabolite	Fraction number*	Probable identity	% of Total acid radioactivity	R_f Value in solvent†					Color reactions‡		
				A	B	C	D	u.v.	A	B	C
A-2	30–43	*p*-Chlorophenol	29·2	0·81	0·77	0·87	0·90	+	−	red-orange	
A-4	60–105	*p*-Chlorophenoxyacetic acid (m.p. 156–157°)	12·4	0·51	0·75	0·88	0·81	+	+		
A-5	120–130		1·18	0·82			0·56	+	±		
A-6(N-1)§	138–162	Chlorphenesin carbamate (m.p. 88–94°)		0·75	0·66	0·76		+	−	yellow	+
A-10	165–192	*p*-Chlorophenoxylactic acid (m.p. 137–137·5°)	45·8	0·55	0·50	0·81	0·48	+	+		
A-11	253–262		1·74	0·50	0·80		0·48	+	+		
A-12	253–262			0·70	0·90		0·81	+	±	yellow	
A-13	345–382	(m.p. 194–195°)	8·69	0·59			0·11	+	−	orange	

* A 3·6 × 92 cm column was prepared from 500 g silica gel mixed with 250 ml 0·05 M citrate buffer, pH 3·42, and eluted with chloroform (fractions 1–109), 99·5% chloroform + 0·5% ethanol (fractions 110–213), 99% chloroform + 1% ethanol (fractions 214–278), and 97·5% chloroform + 2·5% ethanol (fractions 279–382). Twenty-ml fractions were collected at a flow rate of 60 ml/hr.

† The solvent systems were: A, IAW; B, BPW; C, CMF; and D, BZAW.

‡ Spray reagents were: A, bromphenol blue indicator; B, diazotized sulfanilic acid; and C, sodium hypochlorite reagent.

§ Identified as chlorphenesin carbamate contamination, and therefore not listed in acid radioactivity.

A-6 with smaller quantities of metabolites A-2, A-4 and A-10. The sulfate acidic fraction contained principally metabolite A-2 with smaller quantities of metabolites A-6 and A-10.

In order to provide sufficient quantities of acidic metabolites for isolation and characterization purposes, the 0 to 12-hr nonconjugated acidic, glucuronide acidic, and sulfate acidic extracts were pooled and an aliquot (339 mg, $1{\cdot}14 \times 10^7$ dis/min) combined with the total acidic extract (2·56 g) from the study with nonradioactive chlorphenesin carbamate. The resulting fraction then was chromatographed on a silica gel column. The paper chromatographic properties, spray reaction, order of elution, relative distribution, and probable identification of the acidic metabolites are summarized in Table 5.

Metabolite A-2 was found to be very volatile and was easily lost unless special precautions were observed during isolation. Paper chromatography of the combined metabolite A-2 fractions demonstrated that this metabolite was phenolic and that it resembled authentic *p*-chlorophenol in its chromatographic properties. Vapor-phase chromatography* of metabolite A-2 and reference *p*-chlorophenol indicated that both compounds had identical retention times (8·2 min) and that a mixture of the two gave one symmetrical peak. Evaporation of an aliquot of metabolite A-2 as a film on the surface of a sodium chloride block (other techniques led to the complete loss of metabolite) gave an infrared spectrum which was identical with that of authentic *p*-chlorophenol.

The major portion of the metabolite A-2 fraction was condensed with an excess of chloroacetic acid (Distillation Products, Inc., Rochester, N.Y.) under basic conditions to give *p*-chlorophenoxyacetic acid. The crude reaction product was recrystallized from methylene chloride and then from ethylene chloride to give 43 mg crystals, m.p. 155·5–156·5°. The m.p. of authentic *p*-chlorophenoxyacetic acid was 157–158°.[2] The i.r. spectrum of this material was identical with that of reference *p*-chlorophenoxyacetic acid. *Anal.* Calcd. for $C_8H_7O_3Cl$: C, 51·49; H, 3·78. found: C, 50·82; H, 3·99. These data identify metabolite A-2 as *p*-chlorophenol.

The combined column fractions containing the acid, metabolite A-4, were recrystallized from methylene chloride and then from ethylene dichloride to yield 91 mg crystals, m.p. 156–157°. The i.r. spectrum of the purified metabolite was identical with that for authentic *p*-chlorophenoxyacetic acid. *Anal.* Calcd. for $C_8H_7O_3Cl$: C, 51·49; H, 3·78. Found: C, 51·08; H, 3·99.

Paper chromatography of the metabolite A-6 peak showed a single radioactive component which did not react with bromphenol blue or with diazotized sulfanilic acid but which gave a positive test for the carbamate group. The material exhibited chromatographic properties identical with those of authentic chlorphenesin carbamate, including identical mobilities (31·5 cm) in the Bush B-5 system. The crude metabolite A-6 was purified by chromatography on a small Florisil column. After elution with acetone, the crude metabolite was crystallized from methylene chloride to give 5 mg product melting at 88–94°. A portion of the impure material was streaked at the origin of a 10×20 cm alumina GF plate adjacent to a spot of authentic chlorphenesin

* Carried out on an F and M model 609 flame ionization gas chromatograph with a quarter-inch coiled glass column containing 2·6% Carbowax 20M on 100–200 mesh Gas Chrom Z. Column temperature 140°, injection port temperature 300°, and detector temperature 290°. The authors are indebted to R. E. Nygren, Physical and Analytical Chemistry Research, The Upjohn Co., for these analyses.

carbamate. The plate was developed in methanol:chloroform (1:4). The metabolite A-6 peak (R_f 0·82) had the same mobility as the reference standard. The i.r. spectrum of the eluted metabolite was found to be identical with that of authentic chlorphenesin carbamate, confirming the identification of metabolite A-6 with chlorphenesin carbamate. The presence of this neutral metabolite in the acid extracts probably resulted from an incomplete extraction of the very large quantities of neutral metabolite present during the initial fractionation procedure. Alternatively, presence of this neutral compound could reflect cleavage of an acid-labile acidic conjugate of chlorphenesin carbamate during the isolation process.

The major acidic metabolite of dog, metabolite A-10, was an acid that chromatographed in a manner similar to that of *p*-chlorophenoxylactic acid. The fraction was twice recrystallized from methylene chloride to yield 47 mg crystals, m.p. 137–137·5° (the melting point of authentic *dl-p*-chlorophenoxylactic acid is 137·5–138°)[2] and $[\alpha]_D = 0°$ (C = 0·58, methanol). The i.r. spectrum of the purified metabolite was identical with that of authentic *p*-chlorophenoxylactic acid. *Anal.* Calcd. for $C_9H_9O_4Cl$: C, 49·90; H, 4·18. Found: C, 49·80 H, 4·75.

Metabolites A-11 and A-12 were eluted from the column together in approximately equal concentration. Metabolite A-11 was an acid that exhibited chromatographic properties very similar to those of authentic *p*-chlorophenoxyaceturic acid.* Metabolite A-12 was a phenolic acid which did not appear identical with any of the available reference standards. The small quantities of both metabolites prevented further characterization.

Crude metabolite A-13 (242 mg, 6·04 × 10^5 dis/min) was recrystallized twice from acetone–Skellysolve B to yield crystals, m.p. 187–188°. The purified material proved to be nonradioactive, and i.r. analysis indicated that the crystalline product was identical with hippuric acid. The filtrates from the above crystallizations containing all the initial radioactivity were streaked at the origin of two 20-cm^2 silica gel plates and developed in BPW. The u.v. absorbing radioactive zones (R_f 0·43) were separated from large bands of residual hippuric acid (R_f 0·25). Radioactive areas were scraped from the plates, eluted with 0·1 N HCl and metabolite A-13 recovered after ethyl acetate extraction. The crude metabolite was recrystallized from acetone–Skellysolve B to yield 11 mg crystals, m.p. 194·5°. The i.r. spectrum showed λ_{max} at 3330, 1580, 1485, 1230, 1085, 1005 and 820 cm^{-1}. The identity of this metabolite has not been established.

To examine the nature of the acidic metabolites excreted at a later time, the nonconjugated acidic, glucuronide acidic, and sulfate acidic extracts from the 12 to 48-hr urine were combined (712 mg, 2·46 × 10^7 dis/min) and the pooled fraction separated on a silica gel column as previously described. In Table 6 are summarized the chromatographic properties, order of elution, probable identification, and per cent distribution of the various acidic metabolites excreted during this period.

An aliquot of the chloroform solution containing metabolite A-2 from the 12 to 48-hr acid extracts exhibited vapor-phase and paper chromatographic properties and an i.r. spectrum (salt block) identical with those of authentic *p*-chlorophenol.

* Prepared by condensation of *p*-chlorophenoxyacetyl chloride with glycine under alkaline conditions. The crude product was twice recrystallized from aqueous methanol to yield crystals, m.p. 144–5°. *Anal.* Calcd. for $C_{10}H_{10}ClNO_4$: C, 49·29; H, 4·14; N, 5·75; Cl, 14·55. Found: C, 49·29; H, 4·14; N, 5·90; Cl, 14·76.

TABLE 6. CHROMATOGRAPHIC PROPERTIES, COLOR REACTIONS, AND RELATIVE DISTRIBUTION OF 12 TO 48-HR URINARY METABOLITES FROM DOGS RECEIVING TRITIUM-LABELED CHLORPHENESIN CARBAMATE

Metabolite	Fraction number*	Probable identity	% of Total acid radioactivity	R_f Value in solvent†		u.v.	Color reactions‡	
				A	B		A	B
A-1	16–20		4·07	0·77	0·87	+	–	yel-orange
A-2	22–25	*p*-Chlorophenol	35·9	0·78	0·91	+	–	red-orange
A-3	44–54		1·10	0·71	0·91	+	+	yel-orange
A-4	56–75	*p*-Chlorophenoxyacetic acid (m.p. 153–154°)	13·6	0·51	0·87	+	+	
A-7	84–95	(m.p. 93–94°)	1·91	0·66	0·80	+	+	red-orange
A-8	84–95	(m.p. 119–121°)	3·84	0·74	0·90	+	+	yel-orange
A-9	96–113	(m.p. 100–105°)	3·71	0·48	0·82	+	+	
A-10	96–113	*p*-Chlorophenoxylactic acid (m.p. 135–6°)	32·4	0·46	0·77	+	+	
A-11	282–300		1·46	0·63	0·52	+	–	orange
A-12	282–300			0·82	0·90	+	+	yel-orange
A-13	325–340		2·03	0·66	0·88	+	+	yel-orange

* A 3·0 × 60 cm column was prepared from 200 g silica gel mixed with 100 ml 0·1 M citrate buffer, pH 3·1, and eluted with chloroform (fractions 1–121), 99·5% chloroform + 0·5% ethanol (fractions 122–209), 99% chloroform + 1% ethanol (fractions 210–270), and 97·5% chloroform + 2·5% ethanol (fractions 271–373). Twelve-ml fractions were collected at a flow rate of 55 ml/hr.

† The solvent systems were: A, IAW; and B, CMF.

‡ Spray reagents were: A, bromphenol blue indicator; and B, diazotized sulfanilic acid.

Condensation of a portion of metabolite A-2 with chloroacetic acid gave 5 mg crystals, m.p. 156–157°, which exhibited chromatographic properties and i.r. spectrum identical with those of authentic *p*-chlorophenoxyacetic acid. The specific activity of the purified derivative was $3{\cdot}15 \times 10^7$ dis/min/m-mole.

Attempts to recrystallize metabolite A-4 failed. Therefore, an aliquot of the crude metabolite was purified via TLC on a 20-cm² silica gel plate developed in BPW. The eluted radioactive zone (R_f 0·80) still containing impurities was rechromatographed on a 10 × 20 cm silica gel plate in the same solvent system. The radioactive zone (R_f 0·67) was eluted to give 6 mg crystals, m.p. 153–154°, which showed chromatographic properties and i.r. spectrum identical with those of the reference *p*-chlorophenoxyacetic acid. The specific activity of the isolated material was $3{\cdot}64 \times 10^7$ dis/min/m-mole. *Anal.* Calcd. for $C_8H_7O_3Cl$: C, 51·49; H, 3·78. Found: C, 52·15; H, 3·99.

The pooled fraction containing metabolites A-7 and A-8 also contained a small amount of metabolite A-10. The crude fraction (52 mg, $1{\cdot}25 \times 10^6$ dis/min) was streaked at the origin of a 20-cm² silica gel plate which then was developed in the BPW system. The radioactive bands of metabolite A-7 (R_f 0·40) and A-8 (R_f 0·69) were well separated from the small metabolite A-10 zone (R_f 0·16). The two u.v.-absorbing bands containing metabolites A-7 and A-8 were scraped from the plate and eluted. Crude metabolite A-7 was recrystallized from methylene chloride to yield 3 mg needles, m.p. 93–94°. The i.r. spectrum of this metabolite exhibited λ_{max} at 3540, 2600, 1740, 1600, 1585, 1495, 1245, 1170, 1125, 1045 and 850 cm^{-1}; its identity has not been determined.

Metabolite A-8 was recrystallized from acetone–Skellysolve B to give 10 mg crystals, m.p. 119–121°. The i.r. spectrum of the purified metabolite showed λ_{max} at 3360, 2700, 2620, 1615, 1603, 1495, 1235, 1180, 1112, 803 and 770 cm^{-1}. This metabolite has not been characterized further.

Chromatography of the major fraction containing metabolite A-10 showed evidence for a second minor component, metabolite A-9. Attempts to isolate metabolite A-10 by crystallization were unsuccessful; therefore, the crude fraction (99 mg, $7{\cdot}84 \times 10^6$ dis/min) was streaked at the origin of two 20-cm² silica gel plates and chromatographed in BPW. The major radioactive zones (R_f 0·37) were scraped from the plates, eluted with acid, and the acid extracted with chloroform. The recovered metabolite A-10 was recrystallized twice from methylene chloride to give 22 mg crystals, m.p. 135–136°, which possessed the same i.r. spectrum and chromatographic properties as those of reference *p*-chlorophenoxylactic acid. The specific activity of this material was $3{\cdot}71 \times 10^7$ dis/min/m-mole. *Anal.* Calcd. for $C_9H_9O_4Cl$: C, 49·90; H, 4·18. Found: C, 48·85; H, 3·93.

The u.v.-absorbing band containing metabolite A-9 (R_f 0·57) was eluted and crystallized from methylene chloride to give crystals, m.p. 100–105°. The i.r. spectrum of this acidic metabolite showed λ_{max} at 3390, 2480, 1725, 1675, 1630, 1550, 1495, 1245, 1215, 1175, 1095, 1060 and 820 cm^{-1}. The i.r. spectrum of metabolite A-9 proved identical with that of metabolite J previously isolated from the rat.[2] Metabolite A-9 has not been identified.

Attempts to characterize further other acidic metabolites were unsuccessful because of the low concentrations of radioactive compounds in the various pooled fractions and the absence of appropriate reference standards.

DISCUSSION

Orally administered chlorphenesin carbamate was quickly absorbed by the dog, with peak blood levels appearing 2–3 hr after ingestion. The drug was readily eliminated from the body as was shown by the initial rapid decrease in blood radioactivity ($t_{\frac{1}{2}} = 2{\cdot}5$ hr) and the high excretion of radioactivity during the first 24 hr. After 8 days, approximately 70–80 per cent of the dose was eliminated into the urine and 6–12 per cent into the feces. The ratio of urinary to fecal excretion and the total recoveries of radioactivity from the dogs are quite comparable to those observed previously in the rat.[2]

The principal urinary metabolite of chlorphenesin carbamate in the dog was the glucuronide conjugate of the drug (Fig. 3). This metabolite is assumed to be chlorphenesin carbamate O-glucuronide, since the O-glucuronide was the only glucuronidate of chlorphenesin carbamate detected previously in the rat and human.[3] The O-glucuronide and sulfate conjugates of chlorphenesin carbamate accounted for the majority of the drug excreted in urine during the initial 12 hr after dosage with the tritiated drug (Table 2). A considerably smaller portion (7 per cent) of the urinary radioactivity was excreted in this period as acidic materials. A significant reduction in the concentration of neutral metabolites occurred in the 12 to 48-hr urine sample, with a concomitant increase in the concentrations of acidic products. In addition, the recovery of conjugated drug decreased in these later urine samples, whereas the concentrations of nonconjugated drug increased significantly.

Optically active chlorphenesin carbamate was isolated after enzymatic cleavage of the O-glucuronide and sulfate conjugates in the 0 to 12-hr urine sample, while chlorphenesin carbamate isolated from the 12 to 48-hr urine extracts failed to show such optical activity. The presence of optically active drug in the urine after administration of a racemic mixture suggests either the preferential conjugation of one isomer of chlorphenesin carbamate or an enrichment in one isomer through selective absorption, localization, hydrolysis, or oxidation of the second isomer. This stereochemical disparity subsequently disappeared, since both isomers were found in equal concentration in later urine samples. An analogous situation has been observed previously in the rat.[2, 5] Such stereochemical specificity in metabolism and excretion also has been reported with ethambutal,[19] nirvanol,[20] and noradrenaline.[21]

In addition to chlorphenesin carbamate and several unidentified neutral compounds, the dog excreted substantial quantities of a variety of acidic metabolites (Fig. 3). The majority of these products are probably formed after hydrolysis of the carbamate ester group. Such oxidations must take place very rapidly, since no trace of the initial hydrolysis product chlorphenesin was detected in the urine. Although cleavage of the carbamate group of chlorphenesin carbamate occurs to an appreciable extent in the dog and the rat,[2] other carbamate-containing drugs such as mephenesin carbamate,[12] methocarbimol,[13] meprobamate,[14] and mebutamate[15] appear to be quite stable *in vivo*.

p-Chlorophenoxylactic acid is apparently formed via oxidation of the terminal hydroxyl group of the initial hydrolysis product chlorphenesin. This acid then is probably decarboxylated to produce *p*-chlorophenoxyacetic acid. The formation of *p*-chlorophenol apparently does not proceed via *p*-chlorophenoxyacetic acid, since this acid is recovered unaltered in the urine of rabbits,[22] and 2,4-dichlorophenoxyacetic acid is excreted unchanged by sheep.[23] The *p*-chlorophenol may be produced by direct

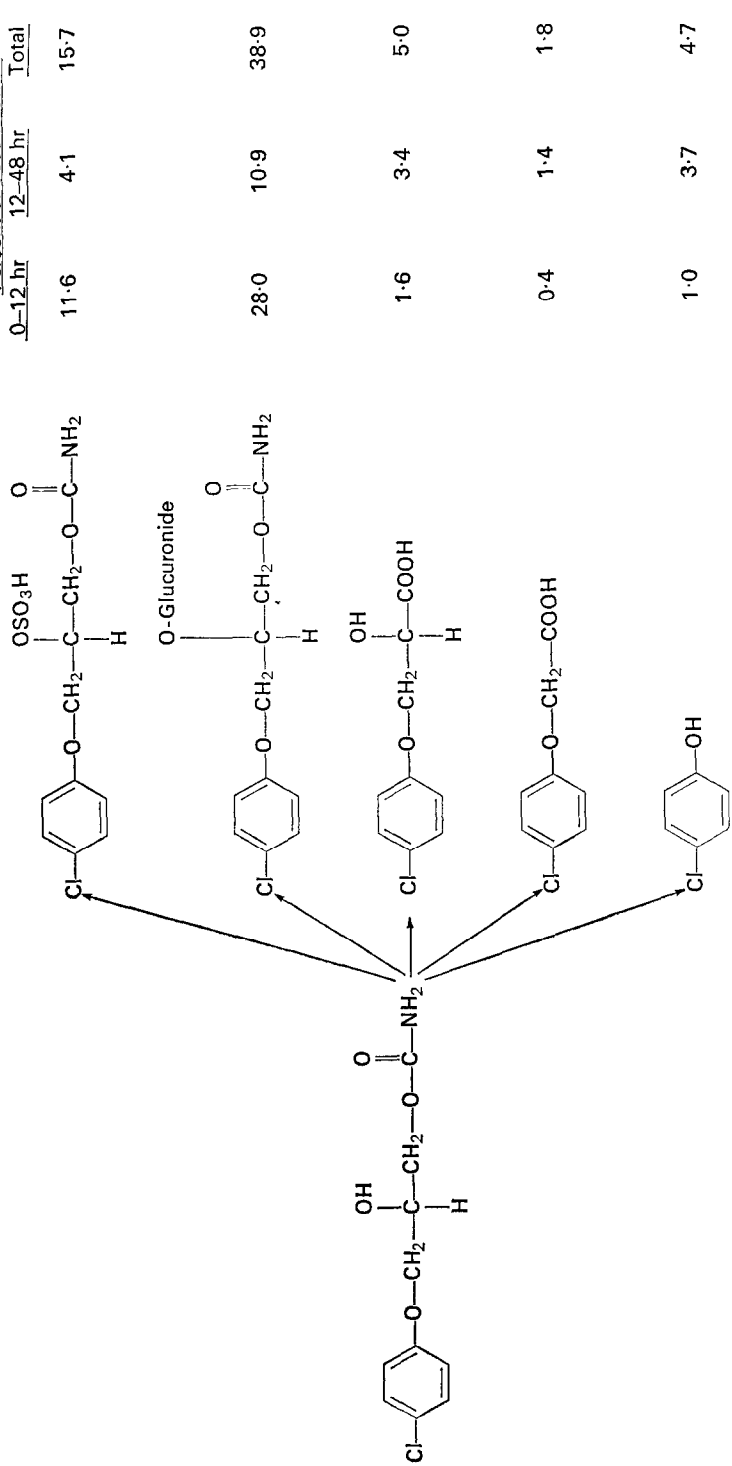

FIG. 3. Pathways for metabolism of chlorphenesin carbamate in the dog.

cleavage[24] of chlorphenesin carbamate or chlorphenesin. The origin of the majority of the minor acidic metabolites is unknown, but these compounds may represent phenolic, aceturic, or mercapturic derivatives of the major urinary metabolites.

Acknowledgement—The authors would like to acknowledge the technical assistance of Mr. F. LaPlante and Mr. R. S. Schwikert and the cooperation of Physical and Analytical Chemistry Research, The Upjohn Co., for the many analyses necessitated by this study.

REFERENCES

1. R. J. MATTHEWS, J. P. DAVANZO, R. J. COLLINS and M. J. VANDERBROOK, *Archs int. Pharmacodyn. Thér.* **143**, 574 (1963).
2. D. R. BUHLER, *J. Pharmac. exp. Ther.* **145**, 232 (1964).
3. D. R. BUHLER, *Biochem. Pharmac.* **14**, 371 (1965).
4. R. F. RILEY and F. M. BERGER, *Archs. biochem.* **20**, 159 (1949).
5. E. L. GRAVES, T. J. ELLIOTT and W. BRADLEY, *Nature, Lond.* **162**, 257 (1948).
6. R. F. RILEY, *J. Pharmac. exp. Ther.* **99**, 329 (1950).
7. R. F. RILEY, *J. Am. chem. Soc.* **72**, 5712 (1950).
8. B. J. LUDWIG, H. LUTS and W. A. WEST, *J. Am. chem. Soc.* **77**, 5751 (1955).
9. J. B. WYNGAARDEN, L. A. WOODS and M. H. SEEVERS, *Proc. Soc. exp. Biol.* **66**, 256 (1947).
10. J. L. MORRISON, P. SAPERSTEIN, H. A. WALKER and A. P. RICHARDSON, *Proc. Soc. exp. Biol.* **68** 339 (1948).
11. E. TITUS, S. ULICK and A. P. RICHARDSON, *J. Pharmac. exp. Ther.* **93**, 129 (1948).
12. A. P. RICHARDSON, P. S. JONES and H. A. WALKER, *Fedn. Proc.* **12**, 361 (1953).
13. A. D. CAMPBELL, F. K. COLES, L. L. EUBANK and E. G. HUF, *J. Pharmac. exp. Ther.* **131**, 18 (1961).
14. S. S. WALKENSTEIN, C. M. KNEBEL, J. A. MACMULLEN and J. SEIFTER, *J. Pharmac. exp. Ther.* **123**, 254 (1958).
15. J. F. DOUGLAS, B. J. LUDWIG, T. GINSBERG and F. M. BERGER, *J. Pharmac. exp. Ther.* **136**, 5 (1962).
16. R. G. KELLY, E. A. PEETS, S. GORDON and D. A. BUYSKE, *Analyt. Biochem.* **2**, 267 (1961).
17. I. E. BUSH, *Biochem. J.* **50**, 370 (1952).
18. V. R. MATTOX and M. L. LEWBART, *Archs Biochem. Biophys.* **76**, 362 (1958).
19. E. A. PEETS and D. A. BUYSKE, *Biochem. Pharmac.* **13**, 1403 (1964).
20. T. C. BUTLER and W. J. WADDELL, *J. Pharmac. exp. Ther.* **110**, 120 (1954).
21. F. P. LUDUENA, E. ANANENKO, O. H. SIEGMUND and L. C. MILLER, *J. Pharmac. exp. Ther.* **95**, 155 (1949).
22. S. LEVEY and H. B. LEWIS, *J. biol. Chem.* **168**, 213 (1947).
23. D. E. CLARK, J. E. YOUNG, R. L. YOUNGER, L. M. HUNT and J. K. MCLARAN, *J. Agric. Fd. Chem.* **12**, 43 (1964).
24. J. AXELROD, *Biochem. J.* **63**, 634 (1956).