Fate of 6-Chloropicolinic Acid following Oral Administration in Rats

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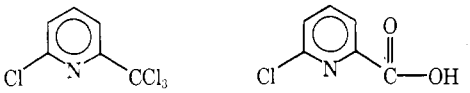
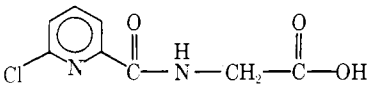
Carboxyl-labeled 6-[^{14}C]chloropicolinic acid (6-[^{14}C]CPA) was administered to three male and three female rats as a single oral dose of approximately 10 mg/kg. Forty-eight hours after administration, 97.64 ± 3.43% of the total dose of ^{14}C activity had been eliminated in the urine, with 1.48 ± 0.47 and 1.30 ± 0.07% eliminated in the feces and air, respectively. Less than 0.3% of the administered ^{14}C remained in liver, kidneys, and skinned carcasses 48 hr following administration. Thin-layer chromatography and mass spectrometry verified that 6-[^{14}C]CPA and its glycine conjugate, *N*-(6-chloropicolinoyl)glycine, accounted for ^{14}C eliminated in the urine during the first 8 hr following administration. The half-life for elimination of 6-[^{14}C]CPA from the body was calculated to be 2.4 hr, and the half-life for clearance of ^{14}C from blood plasma was only 1.1 hr. Thus, it is concluded that following oral administration to rats 6-CPA is partially conjugated with glycine and both the parent compound and its glycine conjugate are rapidly eliminated from the body.

2-Chloro-6-(trichloromethyl)pyridine (CTP) is the active ingredient of N-SERVE (Registered Trademark of The Dow Chemical Co., Midland, Mich. 48640) nutrient stabilizer, a product applied to the soil to inhibit the nitrification of ammonia by soil microorganisms (Goring, 1962). The metabolism of CTP has been studied in a dog (Redemann *et al.*, 1966) which was fed [^{14}C]CTP (labeled in the trichloromethyl carbon) in its daily diet. About 80% of the ingested ^{14}C appeared in the urine, and most of the urinary ^{14}C was accounted for as *N*-(6-chloropicolinoyl)-glycine (N-6-CPG). The conversion of [^{14}C]CTP to N-6-CPG was also demonstrated in two male rats (Redemann and Clark, 1967) in which most of the urinary ^{14}C was ascribed to 6-chloropicolinic acid (6-CPA) with a smaller amount being due to N-6-CPG. Since CTP is converted by soil bacteria and by plants (Redemann *et al.*, 1965) to 6-CPA, the absorption, distribution, and excretion of 6-CPA were evaluated in rats to provide data for assessing the safety of this compound.



CTP

6-CPA



N-6-CPG

EXPERIMENTAL SECTION

Materials. *Reagents.* The carboxyl-labeled 6-[^{14}C]CPA was supplied by R. Meikle, Dow Chemical U. S. A., Walnut Creek, Calif. The compound had a specific activity of 0.94 mCi/mmol, and the radiochemical purity as established by thin-layer chromatography in three different solvent systems was greater than 98%. All thin-layer chromatography was carried out on 0.25-mm precoated silica gel plates. The solvent systems were (a) ethyl acetate–methanol–acetic acid (8:1:1), (b) chloroform–methanol–25% NH_4OH (4:5:2), and (c) dioxane–acetic acid–water (90:6:4).

Both unlabeled 6-CPA (purity greater than 99%) and N-6-CPG were obtained from the Ag-Organics Department, Dow Chemical U. S. A., Midland, Mich. Aquasol Universal L.S.C. cocktail was used as purchased from New England Nuclear. (Aquasol is a trademark of New England Nuclear, Boston, Mass.) 3-Methyl-1-*p*-tolyltriazene was obtained from Eastman Organic Chemicals (No. P9973).

Apparatus. The metabolism experiment was conducted in glass metabolism cages designed for the separate collection of urine, feces, and expired air. The urine traps were immersed in a Dry Ice bath throughout each collection interval. Room air was pulled through the cages at a flow rate of 500–600 ml/min. The air exiting each cage was bubbled through 140 ml of 5 *M* 2-aminoethanol in 2-methoxyethanol, and subsequently through 40 ml of the same solution to trap expired CO_2. The cages were maintained in an air-conditioned room at a temperature of 24°, and with a light–dark cycle of 12 hr on–12 hr off.

All radioactive counting was done with a Nuclear-Chicago Mark II liquid scintillation system utilizing external standard ratios to determine quench correction. The validity of the quench correction curve was spot-checked with various samples by using [^{14}C]toluene as an internal standard.

Mass spectra were obtained with a CEC 21-490 single-focusing mass spectrometer using the direct probe inlet. The probe temperature was 120°, electron energy was 70 eV, and ion source pressure about 1×10^{-6} Torr. Oxidation of biological samples to CO_2 was carried out in a Beckman Biological Material Oxidizer.

Methods. *Metabolism and Balance Study.* For the metabolism and balance study, three male and three female 42-day old Sprague-Dawley (Spartan strain) rats were acclimated to the metabolism cages for 4 days prior to dosing. They were supplied with food and water *ad libitum* throughout the experiment. Prior to dosing the rats, unlabeled 6-CPA dissolved in pH 7.3 phosphate buffer was added to the 6-[^{14}C]CPA to obtain a dosing solution at a concentration of 2 mg of 6-CPA/ml and a specific activity of 4 μCi/mg. Appropriate volumes of this solution were administered to the rats using a syringe fitted to a feeding needle to obtain dose levels of about 10 mg of 6-CPA/kg body weight. Aliquots of the dosing solution measured in the same syringe used to feed the animals were counted directly to ascertain the quantity of ^{14}C administered. Body weights, milligrams of 6-CPA administered, and doses in milligrams of 6-CPA/kilogram are summarized in Table I, part A.

After initiation of the experiment, urine and fecal samples were collected at 8- and 24-hr intervals, respectively, and stored frozen until analyzed. The solutions used to trap expired CO_2 were collected every 8 hr and stored in a refrigerator. Forty-eight hours after dosing, the rats were sacrificed by decapitation. The carcasses were skinned and the liver, kidneys, and a sample of perirenal fat were removed from each animal. The carcasses, skins, livers, kidneys, and fat samples were stored frozen. Each metab-

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Table I. Body Weights and Dose Levels for Two Groups of Rats Given a Single Oral Dose of 6-[^{14}C]Chloropicolinic Acid

Rat no.	Sex	Wt, g	6-CPA, mg	mg of 6-CPA/kg
	(A) Metabolism and Balance Study[a]			
1	M	241	2.50	10.4
2	M	250	2.50	10.0
3	M	238	2.50	10.5
4	F	177	2.00	11.3
5	F	161	2.00	12.4
6	F	178	2.00	11.2
	(B) Blood Plasma Levels[a]			
1	M	205	2.00	9.76
2	M	235	2.00	8.51
3	M	210	2.00	9.52
4	F	149	1.40	9.40
5	F	152	1.40	9.21
6	F	147	1.40	9.52

[a] The rats were deprived of solid food for 12 hr prior to dosing, and were administered the dose at the beginning of the normal light cycle.

olism cage was rinsed with water which was saved for ^{14}C assay.

Aqueous homogenates of the carcasses, livers, kidneys, and feces were prepared and aliquots of the homogenates containing 150 mg of sample were oxidized in the Biological Material Oxidizer. The resulting CO_2 was trapped in 2-aminoethanol for subsequent liquid scintillation counting. Samples of skin (including hair) and perirenal fat were oxidized directly. Aliquots of each urine sample (250 μl) were counted directly in a solution of 1 ml of water plus 15 ml of Aquasol. One-milliliter aliquots of the expired CO_2 traps were counted in 15 ml of a solution of 4 g of PPO and 0.7 g of POPOP per l. of 2-methoxyethanol–toluene (1:1). The lower limit of detection for these samples was estimated as 0.005, 0.01, and 0.04% of the administered ^{14}C for tissue samples, CO_2, and urine, respectively.

A 20-μl aliquot of a representative urine sample was spotted on each of three uv-activated silica gel thin-layer plates and cochromatographed with 30 μg each of standard 6-CPA and N-6-CPG in the three solvent systems described previously. After visually locating the positions of 6-CPA and N-6-CPG under uv, the plates were scanned for radioactivity with a thin-layer plate scanner equipped with a pen recorder. In each chromatogram, the radioactive spots coincided with the position of the 6-CPA and N-6-CPG standards. Subsequently, all the urine samples from each rat were cochromatographed with 6-CPA and N-6-CPG in one solvent system (dioxane–acetic acid–water) and the radioactive regions were measured either with the thin-layer plate scanner or by scraping the plates into liquid scintillation vials and counting in a thixotropic gel suspension.

Since the only two ^{14}C-labeled compounds detected in the urine by thin-layer chromatography appeared to be 6-CPA and N-6-CPG, the 0–8-hr urine samples from each rat were pooled to form a composite sample containing about 80% of the total ^{14}C activity excreted *via* the urine. A portion of this sample was adjusted to pH 1 with H_3PO_4, saturated with NaCl, and extracted twice with ethyl ether. The ether extract was then concentrated, streaked on a preparative-scale thin-layer silica gel plate, and developed in the same solvent system described previously. The zones corresponding to the location of standard 6-CPA and N-6-CPG were scraped from the plate, eluted with methanol, and filtered, and the methanol evaporated. The residues were taken up in several milliliters of toluene containing 10 mg of 3-methyl-1-*p*-tolyltriazene and heated, and the toluene was evaporated (White *et al.*, 1968). The resulting derivatives were then subjected to mass spectral analysis utilizing the direct probe inlet.

Blood Plasma Levels. Another group of six rats (three of each sex) were used for the determination of blood plasma levels. They were administered a dose of 6-[^{14}C]CPA similar to that described previously and maintained in the same experimental apparatus. Body weights and dose levels for this group of rats are summarized in Table I, part B. Whole blood samples were obtained in heparinized capillary tubes by orbital sinus puncture at intervals of 1, 2, 4, 8, 12, 16, 24, 36, and 48 hr after dosing. The tubes were immediately sealed at one end with clay and centrifuged in a hematocrit centrifuge. They were then broken close to the plasma–cell interface and the plasma was drained into preweighed liquid scintillation vials. Radioactivity in the plasma samples was determined after the addition of 1.5 ml of water and 15 ml of Aquasol to each vial.

RESULTS AND DISCUSSION

A tabulation of the radioactivity recovered after 48 hr from the excreta and tissues of each rat expressed as per cent of ^{14}C administered is presented in Table II. Within 48 hr an average of 97.64 ± 3.43% (± standard deviation) was excreted *via* the urine, while only 1.48 ± 0.47 and 1.30 ± 0.07% was excreted *via* the feces and expired air, respectively. The livers and kidneys contained only 0.04 ± 0.01 and 0.02 ± 0.01%, respectively, and the carcasses accounted for 0.21 ± 0.12%.

Since almost all of the ^{14}C activity was excreted *via* the urine, the logarithm of the per cent ^{14}C eliminated by this route during successive 8-hr intervals was plotted as a function of time following administration as shown in Figure 1. Urinary excretion of ^{14}C apparently followed first-order kinetics during the 24 hr immediately following administration. Therefore, the data set for this interval was analyzed by linear regression to obtain the solid line plotted in Figure 1. The first-order rate constant for urinary excretion of ^{14}C during this interval determined by the slope of the line is 0.28 hr^{-1} (95% confidence limits,

Table II. Per Cent of ^{14}C Activity Recovered from the Excreta and Tissues of Rats 48 hr after a Single Oral Dose of 6-[^{14}C]Chloropicolinic Acid

Sample	% ^{14}C recovered for animal no.						Mean ± SD[a]
	1	2	3	4	5	6	
Urine	94.99	97.95	92.31	99.09	101.72	99.76	97.64 ± 3.43
Feces	1.46	2.20	1.81	0.87	1.27	1.27	1.48 ± 0.47
Air	1.37	1.30	1.30	1.26	1.20	1.39	1.30 ± 0.07
Carcass	0.38	0.34	0.21	0.11	0.10	0.14	0.21 ± 0.12
Skin	0.08	0.03	0.15	0.08	0.14	0.07	0.09 ± 0.05
Liver	0.05	0.04	0.04	0.04	0.03	0.03	0.04 ± 0.01
Kidney	0.02	0.01	0.02	0.01	0.03	0.02	0.02 ± 0.01
Perirenal fat	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	
Total[b]	98.35	101.87	95.84	101.46	104.49	102.68	100.78 ± 3.14

[a] SD = standard deviation. [b] Cage washings represented less than 1% of the dose and are not included in this table.

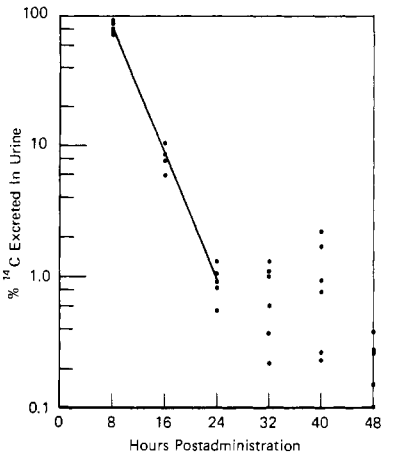

Figure 1. Per cent of administered ^{14}C excreted in the urine of rats during successive 8-hr intervals following a single oral dose of 6-[^{14}C]chloropicolinic acid. (Rat no. 6 did not urinate during the first 8-hr interval. Therefore, the data for this rat were not plotted.)

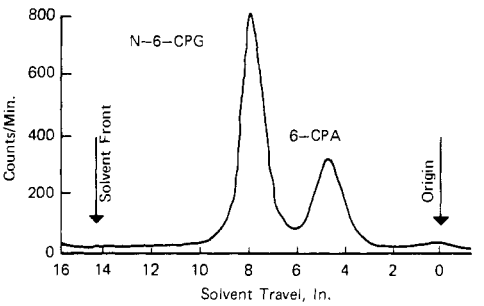

Figure 2. Typical radiochromatogram scan of urine collected from a rat during the first 8 hr following a single oral dose of 6-[^{14}C]chloropicolinic acid.

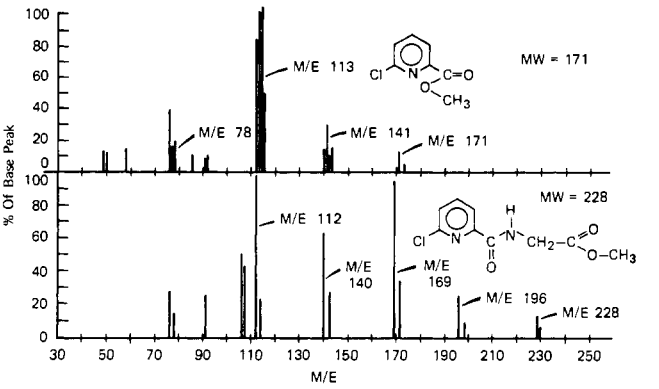

Figure 3. Normalized mass spectra of the methyl ester of urinary 6-CPA (top) and of the methyl ester of urinary N-6-CPG (bottom).

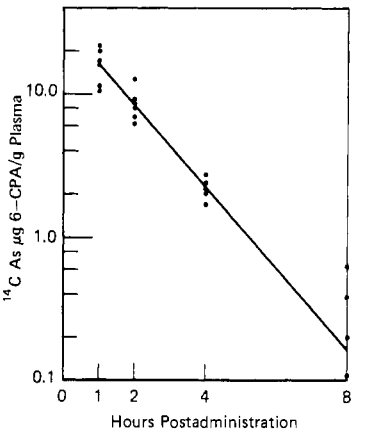

Figure 4. ^{14}C in blood plasma plotted as micrograms of 6-CPA/gram of plasma in rats following a single oral dose of 6-[^{14}C]chloropicolinic acid.

±0.02). The half-life, $t_{1/2}$, corresponding to this value is 2.4 hr. Since urinary excretion during the first 24 hr accounted for an average of 95.64 ± 3.92% of the administered dose, it is concluded that the half-life for excretion by this route closely approximates the half-life for all the elimination of 6-[^{14}C]CPA from the body during this time interval. This rapid elimination of ingested 6-[^{14}C]CPA from the body of the rat is supported by the very small fractions of the administered dose of ^{14}C remaining in the body 48 hr after dosing, as shown in Table II.

Thin-layer chromatograms of urine eliminated by each rat during the first 8 hr revealed only two radioactive peaks, which were located in the same positions as the cochromatographed standards of 6-CPA and N-6-CPG. A typical radiochromatogram scan of one of these plates is shown in Figure 2. The relative areas under each radioactive peak were approximated by cutting and weighing the paper, and it was determined that an average of about 30% of the urinary ^{14}C activity during the first 8-hr interval was due to 6-CPA and the remainder was due to N-6-CPG. This proportion was confirmed by radioactivity measurements of the thin-layer plate scrapings from subsequent urine samples in which 30 ± 3% of the ^{14}C in each 8-hr interval was excreted as 6-CPA and 62 ± 8% was due to N-6-CPG. The relative amounts of these two compounds in the urine remained constant throughout the experiment.

Verification of the structure of these excretion products was provided by mass spectrometry of their methyl ester derivatives. Figure 3 shows normalized mass spectra of the methyl ester of urinary 6-CPA (top) and of the methyl ester of urinary N-6-CPG (bottom). The mass spectrum of the methyl ester of 6-CPA isolated from urine was virtually identical with that prepared from a standard sample, and showed a moleculer ion at m/e 171 with major fragments at m/e 141, 113, and 78. The methyl ester of N-6-CPG isolated from urine exhibited a molecular ion at m/e 228, and major fragments at m/e 196, 169, 140, and 112, as did the methyl ester prepared from the standard sample of N-6-CPG.

Counting of the blood plasma samples revealed that only those samples collected during the first 8 hr following administration contained measurable quantities of radioactivity. The amount of ^{14}C activity present in each of these samples was calculated as micrograms of 6-CPA/gram of plasma, and the data are presented in the semilogarithmic plot shown in Figure 4. Regression analysis of these data points resulted in the solid line shown in Figure 4 and a first-order rate constant for the clearance of ^{14}C activity from blood plasma of 0.64 hr^{-1} (95% confidence limits, ±0.09) and a $t_{1/2}$ of 1.1 hr. Since the analysis of ^{14}C activity in plasma as early as 1 hr following administration failed to provide points on the ascending portion of the plasma concentration curve, it was concluded that 6-CPA is very rapidly absorbed following oral administration.

None of the data determined in this experiment indicated a significant difference in the metabolism of 6-[^{14}C]CPA between male and female rats. Thus, it is concluded that following oral administration to rats 6-CPA is

partially conjugated with glycine and both the parent compound and its glycine conjugate are rapidly eliminated from the body.

LITERATURE CITED
Goring, C. A. I., *Soil Sci.* **93**, 211 (1962).
Redemann, C. T., Clark, H. W., Jr., *J. Agr. Food Chem.* **15**, 1127 (1967).
Redemann, C. T., Martin, R. T., Wien, J. D., Widofsky, J. G., *J. Agr. Food Chem.* **13**, 518 (1965).
Redemann, C. T., Williams, E. A., Clark, H. W., Jr., Kaku, J. J., *J. Agr. Food Chem.* **14**, 530 (1966).
White, E. H., Baum, A. A., Eitel, D. E., *Org. Syn.* **48**, 102 (1968).

Received for review September 14, 1973. Accepted May 15, 1974. This paper was presented at the Fall Meeting of the American Society for Pharmacology and Experimental Therapeutics, East Lansing, Mich., Aug 19–23, 1973.

Comparative Toxicity of Acetylcholine Mustard (Methyl-2-acetoxyethyl-2′-chloroethylamine) in the Mouse and American Cockroach

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The toxicity of acetylcholine mustard has been investigated in the mouse and the American cockroach. This compound, resembling acetylcholine when dissolved in water, was expected to have high toxicity in the mouse and low toxicity in the insect. In contrast to acetylcholine, high toxicity was obtained in the cockroach with an LD_{50} approximating that of nicotine (base). Toxicity depended on the route of drug administration and the use of nonaqueous solvents. This procedure maintained the tertiary lipophilic nature of the pure compound and it may be an important factor in allowing the substance to reach a tissue target site. High toxicity was obtained in the mouse; toxicity was related to route of administration of the compound. The aziridinium ion of acetylcholine mustard was more toxic in the mouse than the parent tertiary compound. Symptoms of toxicity and the lack of cholinergic stimulation are discussed for both species of experimental animal. The mechanism of action of acetylcholine mustard in the mouse and insect is unknown.

Hanby and Rydon (1947) first synthesized methyl-2-acetoxyethyl-2′-chloroethylamine subsequently termed acetylcholine mustard (AChM) by Hudgins and Stubbins (1971). The toxicity of AChM in mice was investigated by Anslow *et al.* (1947) with a reported LD_{50} of 36.5 mg/kg following intravenous injection in propylene glycol. More recent work of Hudgins and Stubbins (1972), Hirst and Jackson (1972), and Robinson *et al.* (1974) has revealed muscarinic activity of the aziridinium ion of AChM which is formed in aqueous medium. Nicotinic activity of this ion was greater than acetylcholine (ACh), when tested on the frog rectus abdominis muscle and chick biventer cervicus muscle (Jackson, 1972). Although suggested by Hudgins and Stubbins (1972) and Robinson *et al.* (1974), the alkylation of receptor anions was not an obvious property of the aziridinium ion (Hirst and Jackson, 1972). Thus it appears unlikely that cytotoxicity is the prime mode of action of AChM in animals.

The preliminary data of Clement *et al.* (1973) showed that toxicity of AChM in the American Cockroach, *Periplaneta americana*, depended on injection or topical application of AChM as the tertiary base. Injection of AChM in nonaqueous media enhanced the toxicity of AChM. It is not clear from the results of Anslow *et al.* (1947) whether the toxicity of AChM in mice was dependent on the use of propylene glycol as the solvent system or indeed if the aziridinium ion was the toxic component. This problem has been investigated in the mouse and data are given for the actions of AChM in the cockroach in which ACh has no known toxicity (Colhoun, 1963).

MATERIALS AND METHODS

Male mice weighing 25–30 g obtained from Bio-Breeding Laboratories, Ottawa, Ontario, were used for toxicity studies. The mice supplied with water and Purina pellets were acclimated in the animal rearing laboratories for 48 hr before use in the toxicological experiments. The test substances, dissolved in physiological saline, were injected intraperitoneally (IP) or intravenously (IV) into the tail vein. Eight to ten mice were used for each experiment. Individual mice were marked and those given the same dose of drug were held together in a colony cage until the termination of the experiment. Respiratory arrest was the criterion for death in the mouse.

Male American cockroaches supplied by the Department of Zoology, or by the Research Institute, Canada Department of Agriculture, University of Western Ontario, London, Canada, were used for toxicity studies on a species of insect. The cockroaches were acclimated in the laboratory for 24 hr within glass jars containing water and Purina chow. A layer of vaseline was smeared along the top of the jar to prevent escape of the cockroaches. Similar jars were used for the insects following exposure to various drugs. Ten cockroaches were used for each experiment and all experiments were replicated at least three times. Compounds, dissolved in water or in an organic solvent, were applied topically to the dorsal abdomen of the cockroach. Acetone was used as a water-free solvent by drying it with a molecular sieve, Type 4A, B.D.H. Some of these compounds were injected intraabdominally (IAb), with a number 30 needle, between the fourth and fifth ventral abdominal sclerites. The needle was inserted at a shallow angle and allowed to penetrate approximately 0.25 in. All drugs were injected in a fluid volume of 2 μl. For control purposes the solvents were injected or applied topically in the same fluid volume. In the American cockroach the inability to stand was termed knockdown. Prolongation of knockdown to 48 hr was recorded as death. LD_{50} estimations were made according to Miller and Tainter (1944).

Precursors, metabolites, and congeners of AChM are listed below. They were prepared according to the method

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