Metabolism of *p*-Chlorophenyl *N*-Methylcarbamate in the Chicken

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Leghorn hens were given a single oral dose of either *p*-chlorophenyl-^{14}C(U) *N*-methylcarbamate (ring label) or *p*-chlorophenyl *N*-methylcarbamate ^{14}C (carbonyl label). When the carbonyl-labeled compound was administered, 69.5% of the carbon-14 was expired during the 48-hr collection period, whereas no expiratory carbon-14 was detected when the ring-labeled compound was given. Total carbon-14 excreted in the urine accounted for 95% of the activity given as the ring-labeled compound and 14% of the activity given as the carbonyl-labeled compound; the feces was a minor route of excretion in both cases (less than 2%). Carbon-14 remaining in the hens 48 hr after dosing accounted for 0.7 and 7.5% of the activity given as the ring-labeled and carbonyl-labeled compounds, respectively. Eggs collected for 8 days after dosing contained 0.27% of the carbon-14 given as a single oral dose of the ring-labeled compound. Urinary metabolites were identified as *p*-chlorophenyl sulfate (I), *p*-chlorophenyl glucuronide (II), and *p*-chlorophenol (III). The feces contained I, II, III, and trace amounts of the parent compound.

Earlier reports have indicated that *p*-chlorophenyl *N*-methylcarbamate had slight herbicidal activity (Herrett and Berthold, 1965) and was a weak acetylcholinesterase inhibitor (Kolbezen *et al.*, 1954). Dawson (1969) reported that a combination of chloropropham [isopropyl *N*-(3-chlorophenyl)carbamate] and *p*-chlorophenyl *N*-methylcarbamate controlled dodder twice as long as did chloropropham alone. A recent report (Technical Service Bulletin 105-F-1, 1970) indicates that *p*-chlorophenyl *N*-methylcarbamate is a competitive inhibitor of hydrolytic enzyme systems which degrade carbanilates, carbamates, thiocarbamates, acetamides, and anilides. Thus *p*-chlorophenyl *N*-methylcarbamate may be an effective synergist in pesticide formulations. One prerequisite for the safe usage of any biologically active compound in the environment is complete knowledge of its metabolic fate in animals. This study was initiated to determine the metabolic fate of *p*-chlorophenyl *N*-methylcarbamate in the chicken.

EXPERIMENTAL

Radio-Labeled *p*-Chlorophenyl *N*-Methylcarbamate. The purity of *p*-chlorophenyl-^{14}C(U) *N*-methylcarbamate, and *p*-chlorophenyl *N*-methylcarbamate-^{14}C was greater than 99%, as determined by comparing their chromatographic behavior with that of authentic *p*-chlorophenyl *N*-methylcarbamate on silica gel thin-layer plates developed in hexane ether (2:1, v/v). To verify further their purity, both the ring-labeled and the carbonyl-labeled compounds were acetylated by the method of Sullivan *et al.* (1967); in both cases the product migrated as a single radioactive component in the gas chromatograph. The infrared spectra of the acetylated products were identical with that of *p*-chlorophenyl *N*-acetyl-*N*-methylcarbamate.

Synthesis of Chemicals. Potassium *p*-chlorophenyl sulfate was prepared by a slight modification of the method of Feigenbaum and Neuberg (1941). A solution of 10 g of *p*-chlorophenol in 60 ml of dimethylaniline was cooled in an ice bath, and 7 ml of freshly distilled chlorosulfonic acid was slowly added with constant stirring. The solution was made to pH 8 by the addition of KOH (1:1, w/v); the solid which formed was removed by filtration and washed with diethyl ether. The product was recrystallized twice from hot ethanol.

Methyl (*p*-chlorophenyl tri-*o*-acetyl-β-glucopyranosid)uronate was prepared by the method used by Robbins *et al.* (1969) for the synthesis of methyl (4-benzothienyl tri-*o*-acetyl-β-glucopyranosid)uronate. For the synthesis of *p*-chlorophenyl glucuronide, 2 g of methyl (*p*-chlorophenyl tri-*o*-acetyl-β-glucopyranosid)uronate was dissolved in 40 ml of boiling ethanol, 2 ml of 1 *N* KOH was added, and the solution was heated to 65° C for 1 hr. The solution was then cooled and the white precipitate which formed was removed by filtration and washed repeatedly with cold ethanol. The product was recrystallized twice from hot ethanol.

The *p*-chlorophenol-^{14}C(U) was prepared by basic hydrolysis of *p*-chlorophenyl-^{14}C(U) *N*-methylcarbamate (0.1 *N* NaOH for 20 hr at room temperature). The solution containing the hydrolysis product was then made to pH 6 with HCl and extracted three times with an equal volume of CCl_4. The hydrolysis product was identified as *p*-chlorophenol by comparison of its infrared spectrum with that of an authentic sample. For the synthesis of *p*-chlorophenyl-^{14}C(U)-2-cyclopentyl acetate, 1 mg of *p*-chlorophenol-^{14}C(U), 0.5 ml of benzene, 0.2 ml of pyridine, and 0.2 ml of 2-cyclopentylacetyl chloride were mixed in a tightly stoppered vial and allowed to react at room temperature for 2 days. The product was purified using a Barber-Colman series 5000 gas chromatograph equipped with a hydrogen flame detector and a radioactivity monitoring system (column, 6 ft, 5 mm i.d.; support, Chromosorb G, 80–100 mesh; liquid phase, 3% neopentyl glycol succinate; helium flow rate, 70 ml per min; injection port temperature, 350° C; column temperature, 170° C). A capillary tube was used for trapping the product from the gas chromatograph. The product was identified as *p*-chlorophenyl-2-cyclopentyl acetate by inspection of its infrared spectrum and mass spectrum (*m/e* parent ion, 238, 1 chlorine; phenol, 128, 1 chlorine; cyclopentyl acetate, 111, no chlorine).

The method of Sullivan *et al.* (1967) was used to synthesize *p*-chlorophenyl *N*-acetyl-*N*-methylcarbamate and *p*-chlorophenyl acetate.

Treatment of Birds. Mature white Leghorn hens were surgically modified to facilitate separate collection of feces and urine (Paulson, 1969). In experiment I, three hens were given a single oral dose of *p*-chlorophenyl-^{14}C(U) *N*-methylcarbamate (ring label) and three hens were given *p*-chlorophenyl *N*-methylcarbamate-^{14}C (carbonyl label). The dose was

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Table I. Cumulative Elimination of Carbon-14 in the Feces, Urine and Expiratory Gases from the Chicken after a Single Oral Dose of Radio-Labeled *p*-Chlorophenyl *N*-Methylcarbamate

Time Hours	*p*-Chlorophenyl-^{14}C(U) *N*-Methylcarbamate			*p*-Chlorophenyl *N*-Methylcarbamate-^{14}C		
	Feces % dose	Urine % dose	Expiratory gases % dose	Feces % dose	Urine % dose	Expiratory gases % dose
6	1.2 ± 0.7[a]	80.7 ± 11.1	ND[b]	0.7 ± 0.3	7.5 ± 1.0	53.0 ± 5.2
12	1.5 ± 0.7	92.0 ± 1.0	ND	1.0 ± 0.4	10.0 ± 0.9	64.7 ± 3.9
24	1.7 ± 0.7	94.3 ± 1.8	ND	1.1 ± 0.4	12.3 ± 1.0	68.2 ± 3.8
36	1.8 ± 0.8	94.8 ± 1.7	ND	1.3 ± 0.5	13.3 ± 1.0	69.1 ± 3.7
48	1.9 ± 0.8	95.0 ± 1.8	ND	1.4 ± 0.5	14.0 ± 1.0	69.5 ± 3.7

[a] Average ± standard error. [b] None detected.

dissolved in polyethylene glycol 400 and was administered in a gelatin capsule. The dose contained from 5 to 10 μCi of carbon-14 and was made to supply 10 mg of *p*-chlorophenyl *N*-methylcarbamate per kg of body weight by the addition of an appropriate amount of the unlabeled compound. Feces, urine, and expiratory gases were collected (Paulson, 1969) at 6, 12, 24, 36, and 48 hr after the dose was given; the hens were sacrificed after 48 hr, and tissues were removed. The expiratory gases, urine, feces, and tissues were analyzed for carbon-14, as previously described (Paulson and Feil, 1969).

In experiment II, four surgically modified hens were given a single oral dose of *p*-chlorophenyl-^{14}C(U) *N*-methylcarbamate at the rate of 100 mg per kg of body weight (30–40 μCi carbon-14). Urine and feces were collected for 6 hr after the dose was given.

In experiment III, three laying white Leghorn hens were given a single oral dose of *p*-chlorophenyl-^{14}C(U) *N*-methylcarbamate. The dose contained 10 mg per kg of body weight (23–25 μCi carbon-14). Eggs were collected for 8 days, and the yolk and white were separated, freeze-dried, and analyzed for carbon-14 as previously described (Bakke *et al.*, 1967).

In all experiments the hens had free access to water and a commercial 16%-protein laying mash for several weeks prior to and throughout the experimental period.

Purification of Metabolites. The urine, collected from 0 to 6 hr after the dose was given, was extracted three times with an equal volume of diethyl ether (Figure 1). After ether extraction, the aqueous phase was concentrated to a small volume and applied to a 2.5- × 60-cm Sephadex G-10 column poured in water. The radio-labeled metabolites were eluted with water (flow rate, approximately 0.5 ml per min), and the effluent was monitored with a model 2002 Packard liquid scintillation spectrometer equipped with a continuous flow cell, rate meter, and strip chart recorder. Fraction B (Figure 1) from the Sephadex G-10 column was concentrated to a small volume and applied to a 1- × 60-cm Sephadex LH-20 column poured in water, and the radioactivity was eluted with water (flow rate, approximately 0.2 ml per min). The single radioactive fraction from the Sephadex LH-20 water column was evaporated to a small volume, applied to a 1- × 60-cm Cellex-GE column poured in water, and eluted with 1% KCl (flow rate, approximately 0.2 ml per min). The single radioactive component eluted from the Cellex-GE column was evaporated to dryness; the radioactivity in the residue was extracted with methanol and concentrated to a small volume and applied to a 1- × 60-cm Sephadex LH-20 column poured in methanol. The radiolabeled metabolite was eluted with methanol (flow rate, approximately 0.2 ml per min). Fraction C from the Sephadex G-10 column was concentrated to a small volume and a tenfold molar excess of KBr was added as previously described (Paulson *et al.*, 1970); the sample was then evaporated to dryness, and the radioactivity was extracted from the residue with methanol. The methanol solution was concentrated to a small volume, applied to a Sephadex LH-20 column poured in methanol, and chromatographed as described for metabolite B. Each column effluent containing a purified metabolite was concentrated to a small volume on a flash evaporator for characterization studies.

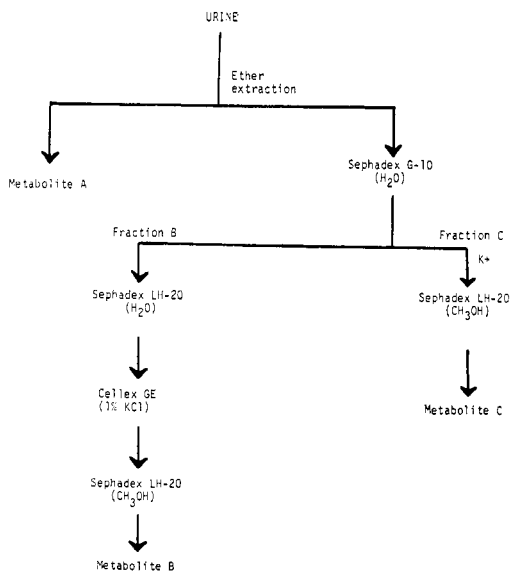

Figure 1. Separation and purification of metabolites of *p*-chlorophenyl *N*-methylcarbamate in chicken urine

The ether-soluble metabolite A (Figure 1) was maintained at 0° C and concentrated to a small volume under vacuum. The concentrate was mixed with benzene, pyridine, and 2-cyclopentylacetyl chloride, and allowed to react at room temperature for 1 to 2 days; the product which formed was purified by gas chromatography as previously described for the synthesis and purification of *p*-chlorophenyl-2-cyclopentyl acetate.

The feces collected from 0 to 6 hr in experiment II were mixed with an equal amount of water, homogenized, and extracted three times with an equal volume of benzene. The aqueous phase was then evaporated to dryness, and the residue was extracted with methanol as previously described (Paulson *et al.*, 1970). The radioactive metabolites in the benzene and methanol extracts were purified and identified as outlined for urinary metabolites.

Enzymatic Hydrolysis of Metabolites. A tube containing from 2 to 3 mg of metabolite, 1 ml of enzyme solution (12,000

Table II. Carbon-14 in Tissues from the Chicken 48 Hr after a Single Oral Dose of Radio-Labeled *p*-Chlorophenyl *N*-Methylcarbamate

Tissue	*p*-Chlorophenyl-^{14}C(U) *N*-Methylcarbamate		*p*-Chlorophenyl *N*-Methylcarbamate-^{14}C	
	% dose/g dry matter	% dose in total fraction	% dose/g dry matter	% dose in total fraction
Liver	0.006 ± 0.002[a]	0.068 ± 0.002	0.144 ± 0.005	0.955 ± 0.090
Blood	0.004 ± 0.002	0.034 ± 0.022	0.081 ± 0.016	0.448 ± 0.052
Kidney	0.003 ± 0.001	0.010 ± 0.001	0.062 ± 0.011	0.107 ± 0.021
Lung	0.003 ± 0.001	0.004 ± 0.001	0.053 ± 0.010	0.082 ± 0.012
Proventriculus	0.001 ± 0.000	0.002 ± 0.001	0.023 ± 0.001	0.032 ± 0.001
Gizzard	0.002 ± 0.001	0.011 ± 0.003	0.022 ± 0.006	0.128 ± 0.027
Intestine	0.001 ± 0.000	0.012 ± 0.001	0.023 ± 0.002	0.161 ± 0.017
Intestinal contents	0.005 ± 0.002	0.022 ± 0.001	0.034 ± 0.002	0.173 ± 0.005
Heart	0.001 ± 0.000	0.002 ± 0.001	0.022 ± 0.004	0.368 ± 0.005
Carcass	0.001 ± 0.000	0.537 ± 0.062	0.012 ± 0.002	5.403 ± 0.625
Total		0.703 ± 0.066		7.530 ± 0.762

[a] Mean ± standard error.

Fishman units of β-glucuronidase and 10,000 Whitehead units of aryl sulfatase, *Helix pomatia*, Calbiochem), and 1 ml of 0.1 *M* sodium acetate buffer (pH 4.5) was incubated at 39° C for 4 hr. The radio-labeled hydrolysis product was then extracted from the mixture with an equal volume of benzene and characterized by infrared spectrometry. Direct acetylation of metabolites was carried out as previously described (Paulson and Portnoy, 1970).

Instrumental Analysis. Infrared spectra were taken with a model 337 Perkin-Elmer grating infrared spectrometer, using the micro KBr technique. Mass spectra were taken with a Varian M-66 mass spectrometer equipped with a V5500 control console.

RESULTS AND DISCUSSION

In the present study there was no apparent toxicity, due to the single oral dose of *p*-chlorophenyl *N*-methylcarbamate (10 and 100 mg per kg of body weight), as evaluated by outward appearance and gross postmortem examination of the hens.

Radioactivity from the ring-labeled *p*-chlorophenyl *N*-methylcarbamate was rapidly excreted in the urine during the first 6 hr after the dose was given (Table I); during subsequent collection periods there were significant, but progressively smaller, amounts in the urine. The cumulative urinary excretion of carbon-14 from the ring-labeled compound was 95% of the dose, whereas only 14% of the carbon-14 given as the carbonyl-labeled compound appeared in that fraction.

Table III. Carbon-14 in the Egg after a Single Oral Dose of *p*-Chlorophenyl-^{14}C(U) *N*-Methylcarbamate

Day	Yolk % of dose in total fraction	White % of dose in total fraction
1	0.0240 ± 0.0039[a]	0.0346 ± 0.0131
2	0.0404 ± 0.0104	0.0024 ± 0.0004
3	0.0380 ± 0.0055	0.0012 ± 0.0004
4	0.0454 ± 0.0118	0.0004 ± 0.0000
5	0.0423 ± 0.0098	0.0007 ± 0.0001
6	0.0264 ± 0.0030	0.0006 ± 0.0001
7	0.0081 ± 0.0013	0.0005 ± 0.0003
8	0.0015 ± 0.0006	0.0004 ± 0.0001
Total	0.2261	0.0408

[a] Mean ± standard error.

Approximately 70% of the carbon-14 given as the carbonyl-labeled compound appeared in the expiratory gases during the 48-hr collection period (Table I), whereas no radioactivity from the ring-labeled compound was detected in that fraction. The feces were a minor route of excretion of carbon-14 given as both the ring-labeled and the carbonyl-labeled compound.

Less than 1% of the carbon-14 given as the ring-labeled compound remained in body tissues 48 hr after the dose was given (Table II); the specific activity was highest in the liver, blood, lung, and kidney. There was approximately ten times as much of the carbon-14 from the carbonyl-labeled compound retained in the tissues as from the ring-labeled compound; these data suggested that some of the carbonyl carbon was cleaved from the molecule and subsequently incorporated into other compounds *via* CO_2 fixation.

Less than 0.3% of the carbon-14 given as the ring-labeled compound was present in eggs collected for 8 days after dosing (Table III). The highest specific activity in the egg white was on the first day after the dose was given, whereas the highest specific activity in the yolk was on the fourth day. The activity in both the white and yolk declined to trace amounts 8 days after the dose was given. No attempts were made to characterize the nature of the carbon-14-containing compound(s) in body tissues and eggs.

One radioactive compound (metabolite A) was removed from the urine by ether extraction (Figure 1); preliminary studies demonstrated that it cochromatographed with *p*-chlorophenol in a variety of tlc and glc systems. The structure of metabolite A was verified by preparing its 2-cyclopentyl acetate derivative; the product was purified by glc and identified as *p*-chlorophenyl-2-cyclopentyl acetate by comparison of its infrared spectrum with that of an authentic sample.

The radioactivity remaining in the aqueous phase after ether extraction was separated into two fractions when chromatographed on a Sephadex G-10 column (Figure 1); final purification of the two polar radio-labeled metabolites was accomplished by chromatographing on Cellex GE and/or Sephadex LH-20 columns. Metabolites B and C were both readily hydrolyzed enzymatically (a mixture of β-glucuronidase and aryl sulfatase); the aromatic hydrolysis product from both metabolites was identified as *p*-chlorophenol by comparison of its infrared spectrum with that of an authentic sample. Metabolite C was converted to *p*-chlorophenyl acetate by the method of Paulson and Portnoy (1970). Proof of structure of metabolites B and C was by synthesis; metabolite B was identified as *p*-chlorophenyl glucuronide and me-

Table IV. The Effect of Dose Rate on the Distribution of Metabolites of *p*-Chlorophenyl *N*-Methylcarbamate in Chicken Urine[a]

Metabolite	Dose: 10 mg/kg body weight, % of total ^{14}C in urine	Dose: 100 mg/kg body weight, % of total ^{14}C in urine
p-Chlorophenol	4.5	4.6
p-Chlorophenyl glucuronide	3.1	29.1
p-Chlorophenyl sulfate	92.4	66.3

[a] Urine was collected for 6 hr after dosing with chlorophenyl-^{14}C(U) *N*-methylcarbamate.

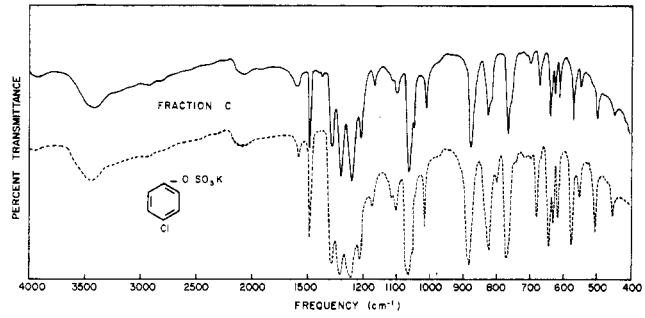

Figure 3. Infrared spectra of metabolite C and authentic *p*-chlorophenyl sulfate

tabolite C was identified as *p*-chlorophenyl sulfate by comparison of their infrared spectra with those of authentic samples (Figures 2 and 3). The percent distribution of the three metabolites in the urine from birds dosed with 10 and 100 mg per kg of body weight is shown in Table IV. In both cases *p*-chlorophenyl sulfate was the predominant metabolite; however, when the birds were given the larger dose, the proportion of the urinary metabolites accounted for as *p*-chlorophenyl glucuronide increased. The size of the dose had very little effect on the percent of the activity in the urine accounted for as *p*-chlorophenol.

An interesting observation made during these studies was that three different salt forms of metabolite C could be separated on a Sephadex LH-20 column developed with methanol (separation of salt forms not shown in Figure 1). The infrared spectra of the three different salt forms of *p*-chlorophenyl sulfate were very similar to those shown in Figure 3; however, most of the absorption bands from 600 to 1300 cm^{-1} were shifted slightly, and there were more distinct differences in the 2500 to 3500 cm^{-1} region. Separation of different salt forms of 1-naphthyl sulfate and other metabolites of carbaryl on Sephadex LH-20 columns developed with methanol has been reported (Paulson *et al.*, 1970). In the present study, the different salt forms of *p*-chlorophenyl sulfate were readily converted to a single salt form by mass action when mixed with an excess of an appropriate inorganic salt in water, as previously described (Paulson *et al.*, 1970).

Six percent of the radio-labeled material in the 0 to 6-hr feces from hens in experiment II was identified as *p*-chlorophenol and 9% was identified as the parent compound, *p*-chlorophenyl *N*-methylcarbamate. The phenol was identified by the procedures outlined for that metabolite in chicken urine. The parent compound was identified by preparing the acetyl derivative, purifying the product by glc, and comparing the infrared spectrum of the purified acetyl derivative

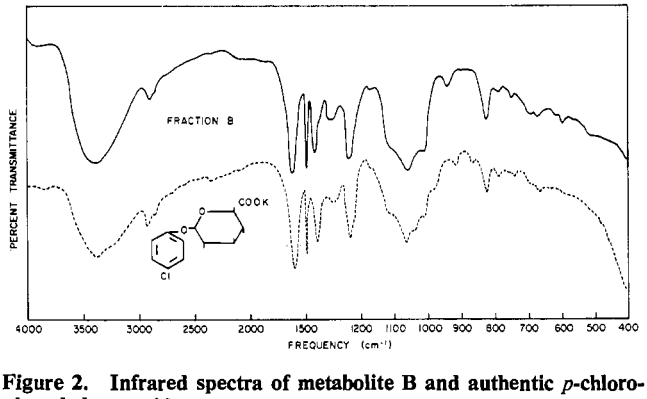

Figure 2. Infrared spectra of metabolite B and authentic *p*-chlorophenyl glucuronide

with that of an authentic sample of *p*-chlorophenyl *N*-acetyl-*N*-methylcarbamate. The metabolites in the feces, soluble in both water and methanol, were not isolated in pure form; however, based on cochromatography and similar infrared spectra, 29% of the activity was tentatively identified as *p*-chlorophenyl sulfate and 18% as *p*-chlorophenyl glucuronide. The nature of the remaining radio-labeled compound(s) in the feces was not determined.

These studies have demonstrated that *p*-chlorophenyl *N*-methylcarbamate and/or its hydrolysis product were very efficiently absorbed from the gastrointestinal tract of the chicken. At least 80 to 95% of the metabolism of the parent compound involved cleavage of the carbamate side chain; most of the hydrolytic product, *p*-chlorophenol, was conjugated with sulfate or glucuronic acid and eliminated in the urine. Less than 1% of the radioactivity remained in the tissues of hens 48 hr after dosing with *p*-chlorophenyl-^{14}C(U) *N*-methylcarbamate, and less than 0.3% of a similar dose appeared in the eggs collected for 8 days after administration. The nature of the radio-labeled compound(s) in the tissues and eggs was not determined in this study; however, it is an area of research worthy of further investigation.

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