Metabolism of *m*-Tolyl N-Methylcarbamate(Tsumacide®) in Rats, Houseflies and Bean Plants

Hideo OHKAWA, Reiko YOSHIHARA, Toshiko KOHARA
and Junshi MIYAMOTO

*Research Department, Pesticides Division, Sumitomo Chemical Co., Ltd.,
Takarazuka, Hyogo 665*

Received December 13, 1973

The life of *m*-tolyl N-methylcarbamate (Tsumacide®) in rat body is very short. Depending on the sex, from 78 to 96.6% of an administered dose of ¹⁴C-Tsumacide is eliminated in the urine by 24 hr after treatment. In rats, Tsumacide is mainly metabolized by oxidative reactions at *m*-methyl group and 4-position of the aromatic ring. Hydrolysis of the ester linkage occurs only to a minor extent. The resulting oxidation products and phenols are partly conjugated as sulfate and glucuronides. The major metabolic pathways are identical in male and female rats.

From 78.7 to 85.8% of the radiocarbon is rapidly lost from the bean plants treated with ¹⁴C-Tsumacide and -Meobal by 24 hr after treatment. Approximately 70% of the dose is also lost within 24 hr after topical application of ¹⁴C-Tsumacide to houseflies. Oxidation of *m*-methyl group, N-methyl group and the aromatic ring at 4-position occurs with Tsumacide in houseflies and bean plants. Meobal undergoes hydroxylation at 4-methyl group and N-methyl group in bean plants. The oxidation products are conjugated as glucuronide and glucoside in houseflies and as glucosides in bean plants.

Tsumacide metabolites formed by oxidation are less active than the original compound in anticholinesterase activity.

m-Tolyl N-methylcarbamate (Tsumacide®) is useful for the control of various species of plant hoppers and leaf hoppers on rice and being widely used in Japan. It is important to clarify the metabolic fate of this compound in plants as well as in insects and mammals.

The present knowledge shows that substituted phenyl N-methylcarbamates are usually metabolized by oxidative reaction in mammals, insects and plants.^{1~4)} In most cases, the oxidative reaction is catalyzed by mixed function oxidases in mammals and insects. Although the site of metabolic attack in the carbamates varies with the nature of the molecules, similar oxidative reactions are known to occur in plants and animals. Hydrolysis of the carbamate linkage also is another important metabolic reaction, usually leading to phenolic compounds.⁵⁾ The resulting oxidation products and phenols are further conjugated to water-soluble products.

This paper deals with the elucidation of metabolic pathways of Tsumacide when it is incubated with mixed function oxidases of mammalian livers, administered to male and female rats and houseflies and applied to bean foliage. Also, the metabolism of 3,4-xylyl N-methylcarbamate (Meobal®) in bean plants is compared with that of Tsumacide.

MATERIALS AND METHODS

Chemicals. Two methylcarbamates labeled with ¹⁴C were prepared in this laboratory; 3-methyl-labeled *m*-tolyl N-methylcarbamate (Tsumacide, 5.38 mCi/mmole, 23.4 mCi), 4-methyl-labeled 3,4-xylyl N-methylcarbamate (Meobal, 1.52 mCi/mmole, 1.5 mCi).⁶⁾ The radiochemical purity of each labeled preparation was more than 99% and no radioactive impurities were detected by thin-layer chromatography (tlc). The following carbamates were prepared by the reaction of corresponding phenols with methyl isocyanate in anhydrous ether containing triethylamine for 16 hr at 25°C. The desired product was isolated by filtering,

evaporating the filtrate, and crystallized from isopropylether. The liquid product was purified by passing through florisil column. The products were: 3-hydroxymethylphenyl N-methylcarbamate (*m*-CH₂OH-T; calcd. for C₉H₁₁O₃N·1/5H₂O: C, 58.49; H, 6.11; N, 7.58; found C, 58.04; H, 5.89; N, 7.42%); 3-carboxyphenyl N-methylcarbamate (*m*-COOH-T; mp 173°C; calcd. for C₉H₉O₄N: C, 55.38; H, 4.65; N, 7.18; found C, 55.39; H, 4.54; N, 7.20%). Similar procedures were used to prepare 3-methyl-4-(N-methylcarbamoyloxy)phenyl N-methylcarbamate (1,4-bis-carbamate; mp 142°C; calcd. for C₁₁H₁₄O₄N₂: C, 55.45; H, 5.92; N, 11.76; found C, 55.33; H, 5.89; N, 11.75%) and 3-methyl-5-(N-methylcarbamoyloxy)phenyl N-methylcarbamate (1,5-bis-carbamate; mp 153°C; calcd. for C₁₁H₁₄O₄N₂: C, 55.45; H, 5.92; N, 11.76; found C, 55.57; H, 5.91; N, 11.82%). Two N-hydroxymethylcarbamates were prepared from phenylcarbamates by the reaction with paraformaldehyde in tetrahydrofuran according to the procedure reported by Fahmy and Fukuto.⁷⁾ The products were recrystallized from ether-benzene: *m*-tolyl N-hydroxymethylcarbamate (N-CH₂OH-T; mp 110°C; calcd. for C₉H₁₁O₃N: C, 59.66; H, 6.12; N, 7.73; found C, 59.79; H, 6.13; N, 7.73%) and 3,4-xylyl N-hydroxymethylcarbamate (N-CH₂OH-M; mp 122°C; calcd. for C₁₀H₁₃O₃N: C, 61.53; H, 6.71; N, 7.17; found C, 61.46; H, 6.73; N, 7.18%). The preparation of 3-methyl-4-hydroxymethylphenyl N-methylcarbamate (4-CH₂OH-M) was reported previously.⁸⁾ The following compounds were purchased: *m*-hydroxybenzoic acid and methylhydroquinone from Tokyo Kasei Kogyo, Tokyo, Japan; 3-hydroxybenzyl alcohol from Aldrich Chemical Company, Milwaukee, Wis., U.S.A.; reduced nicotinamide adenine dinucleotide phosphate (NADPH) from Oriental Yeast Co., Osaka, Japan. Aryl sulfatase (limpets), β -glucuronidase (bovine liver) and β -glucosidase (almonds) were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.

Analysis and identification of metabolites. Reaction products and organosoluble metabolites were resolved by tlc using silica gel 60F₂₅₄ precoated plate with a gel thickness of 0.25 mm (E. Merck, Darmstadt, W. Germany). *R_f* values and solvent systems used are given in Table I. Two-dimensional tlc was used for identification of metabolites. The neutral solvent system (*n*-hexane-ether, 1:5) was used in the first direction and the second direction was with the acidic solvent system (benzene saturated with formic acid-ether mixture, 2:1). Unlabeled methylcarbamates and phenolic products were detected by spraying phosphomolybdic acid (10% w/v in ethanol) and heating for 10 min at 110°C.⁹⁾ Ninhydrin was also used as a chromogenic reagent to detect methylcarbamates.¹⁰⁾ N-Hydroxymethylcarbamates were visualized with chromatropic acid.¹¹⁾ Radioactive materials were detected

TABLE I. *R_f* VALUES OF TSUMACIDE, MEOBAL AND THEIR METABOLITES ON THIN-LAYER CHROMATOGRAPHY

Compound	<i>R_f</i> in solvent system			
	I	II	III	IV
Tsumacide	0.45	0.39		
<i>m</i> -CH ₂ OH-T	0.10	0.09		
<i>m</i> -COOH-T	0.07	0.18		
N-CH ₂ OH-T	0.17	0.16		
<i>m</i> -Cresol	0.58	0.60		
3-OH-Benzyl alcohol			0.26	0.16
<i>m</i> -OH-Benzoic acid			0.30	0.22
Methylhydroquinone			0.44	0.30
Meobal	0.56	0.42		
4-CH ₂ OH-M	0.16	0.09		
N-CH ₂ OH-M	0.27	0.15		

Solvent system I=*n*-hexane-ether (1:5), II=benzene saturated with formic acid-ether (2:1), III=*n*-hexane-ether (1:8), IV=benzene-ethyl acetate (1:1).

by a radio thin-layer chromatogram scanner (Aloka TLC 201) and by radioautography. For quantitation, radioactive areas observed on the radioautograms were scraped from the plate into scintillation vials for determination of radiocarbon with a liquid scintillation spectrometer. One of Tsumacide metabolites, 3-methyl-4-hydroxyphenyl N-methylcarbamate (4-OH-T), was treated with methyl isocyanate in anhydrous ether containing triethylamine to form 1,4-bis-carbamate, which was identified by cochromatography with the authentic compound, using the following solvent systems: isopropylether-ethyl acetate (1:1), *R_f*=0.22; benzene-ethanol (7:1), *R_f*=0.31. The structure of all prepared compounds and some of metabolites was also confirmed by elemental analysis, infrared and NMR spectra.

Radioanalysis and radioautography. For direct radiocarbon assay with a liquid scintillation spectrometer, the following scintillation solutions were used; (A) 2.7 g of PPO in 1 liter of toluene, (B) 15 g of PPO and 300 g of naphthalene in 3 liters of dioxane. Radiocarbon in feces, plant extracts and pulp was determined after combustion by using an oxidizer (Tri-carb automatic sample oxidizer, Packard). About 100 to 200 mg of the dried sample was used for a typical combustion analysis.

Industrial X-ray film (Fuji X-ray film 150) was used for radioautography. The developed tlc was placed under film and held at 4 to 8°C for several days depending on the radioactivity. The harvested plants were pressed between sheets of filter paper with gently heated iron and then exposed to the X-ray film. Radioautogram of whole body of rats was taken ac-

cording to Ullberg's technique with certain changes as noted.¹²⁾ At the stated periods, the rats were anesthetized with ether and then immersed into a mixture of acetone and dry ice. Sagittal sections (20 μ) of the whole body were cut by a cryostat (Bright OT/SEH, England). The sections attached to tapes were dried at -15°C and exposed to X-ray film.

Enzyme assay. Mammalian livers were homogenized at 20% (w/v) in phosphate buffer, 0.1 M, pH 7.4, and the homogenates were centrifuged at 20,000 g for 20 min at 2°C and the supernatant fractions obtained were centrifuged at 105,000 g for 60 min to get the soluble fraction and the microsome pellet. The microsome pellet was washed once by resuspension in the same buffer and recentrifuged to obtain the microsome fraction. The microsome fraction equivalent to 400 mg of the liver was incubated in air for 4 hr at 37°C with 1 μ mole of ^{14}C -Tsumacide in 2 ml of 0.1 M phosphate, pH 7.4, in the presence or absence of 5 mg of NADPH. After incubation, 1 g of ammonium sulfate was added to each reaction mixture which was then extracted twice with 10 ml of ether-ethanol mixture (2:1). The radioactive compounds were analyzed by tlc after concentration of the organic extract.

Anticholinesterase activity of the carbamates was assayed as follows. Rabbit blood was heparinized and centrifuged at 1,000 g for 5 min to separate plasma from blood cells, which were washed three times with 0.9% saline. The washed blood cells were hemolyzed by addition of three times volume of distilled water. Fly heads (Takatsuki strain) were homogenized at 5% (w/v) in M/15 phosphate, pH 6.8, and the homogenate was centrifuged at 1,000 g for 10 min to remove the debris. Each chemical was previously placed in the test tube by evaporation of its acetone solution and preincubated with 0.5 ml of the enzyme solution and 1.2 ml of M/15 phosphate, pH 6.8, at 37°C for 10 min. The incubation mixture was further incubated at 37°C for 20 min following addition of 0.3 ml of acetylcholine solution (4 mg of acetylcholine chloride/ml). After reaction, the remained acetylcholine was colorimetrically determined by Hestrin's method.¹³⁾

Metabolism in rats. Male and female rats (SD strain, 7 weeks old) weighing approximately 200 g were used for all experiments. ^{14}C -Tsumacide was suspended in 10% Tween 80 at the concentration of 30%. The rats were orally treated by a stomach tube with 100 mg of ^{14}C -Tsumacide per kg of body weight (LD_{50} to male rats, 580 mg/kg). The treated animals were housed in a metabolism cage and urine and feces were collected every 24 hr. Expired radioactive carbon dioxide was trapped as reported previously.¹⁴⁾ The excreted urine was first extracted with ether.

The remaining aqueous layer was acidified to pH 2 with hydrochloric acid and then extracted three times with 2 volumes of ether. The combined ether layers were dried over anhydrous sodium sulfate (ether soluble). Radioactive metabolites in ether extract were directly analyzed by tlc after evaporation of the solvent. The aqueous phase was neutralized with 5% sodium hydroxide and lyophilized. The residue was extracted with methanol (water soluble). The methanol extract was directly added to a column of Amberlite XAD-2 for desalting as described by Yang *et al.*¹⁵⁾ The metabolites were removed by elution with methanol. A part of the residue remaining after evaporation of methanol was subjected to acid hydrolysis which was carried out at 100°C for 1 hr in 1 N hydrochloric acid. The reaction solution was extracted with ether. The ether extract was assayed for radioactivity and analyzed by tlc. The water soluble metabolites were also incubated with β -glucuronidase (2 mg) or aryl sulfatase (5 mg) in 4 ml of 0.2 M acetate buffer, pH 5.0, at 37°C for 16 hr. The liberated aglycones were extracted with ether and analyzed by tlc.

Metabolism in houseflies. ^{14}C -Tsumacide in acetone was applied topically to female houseflies (Takatsuki strain) at a dose of 1 μg per fly in 1 μl of acetone (LD_{50} , 4.8 $\mu\text{g}/\text{fly}$). Usually, 400 flies were treated in each experiment. The treated flies were held at room temperature for 24 hr while receiving sugar and water in glass beakers covered with gauze. After 24 hr, the treated flies were anesthetized with carbon dioxide and then rinsed with acetone. The beaker containing excreta was also washed with acetone. Both acetone solutions were combined to form wash-excreta fraction. The washed flies were then homogenized in 0.1 N hydrochloric acid and the homogenate was extracted three times with equal volume of ether. The ether extracts were combined, dried and concentrated (acid-ether extract). The aqueous layer was neutralized with 10% sodium hydroxide and lyophilized. The residue was extracted with methanol (methanol extract). The wash-excreta fraction and the acid-ether extract were directly subjected to tlc analysis. The methanol extract was treated with 1 N hydrochloric acid and β -glucuronidase as with the water-soluble metabolites from rats. The methanol extract was also incubated with β -glucosidase (3 mg) in 4 ml of 0.2 M acetate buffer, pH 4.5, at 37°C for 6 hr. In each case, the radioactive aglycones were extracted with ether. The ether extract was concentrated and analyzed by tlc.

Metabolism in bean plants. Each of the two radioactive carbamates was individually applied to the upper surface of one primary leaf of 12 days old bean seedlings (*Phaseolus vulgaris*). Tsumacide and

Meobal, 0.5 μ mole equivalent to approximately 0.2 μ Ci, were each applied to the leaf in 50 μ l of methanol solution by microsyringe. The treated plants were maintained in the green house and provided water daily. At predetermined intervals, two plants were removed from the green house and analyzed in the following manner. The treated leaves were excised at the base of the stem and rinsed with ether to remove surface residue (surface wash). The whole plants including the washed leaves were homogenized with three 100 ml portions of ether and then with three 100 ml portions of methanol. The combined ether extracts and methanol extracts were each dried over anhydrous sodium sulfate, concentrated to a known volume and appropriate aliquots were taken for combustion analysis to determine radioactivity. The remaining colorless pulp was dried in a vacuum desiccator and retained for combustion analysis. The surface wash and the ether extract were directly analyzed by tlc. β -Glucosidase hydrolysis was carried out on the methanol extract. The reaction solution was extracted with ether and the ether soluble components were analyzed by tlc.

RESULTS

Metabolism of Tsumacide in rats

Male and female rats were orally treated with 100 mg of Tsumacide per kg of body weight. Several minutes after treatment, both male and female rats produced symptoms of poisoning

and they were fully recovered approximately a few hours later. Distribution of radioactivity in tissues was determined by whole body radioautography. After 30 min, high radioactivity was detected in brawn fat and saliva gland of male rats which were showing symptoms of poisoning. Thereafter, radioactivity was found in all tissues observed, especially higher activity being in liver, kidney, stomach and intestines 3 hr after treatment. There was no detectable 14 C residues in the rat body 24 hr after dosing. No significant differences in distribution of radiocarbon was apparent between male and female rats (Fig. 1).

When administered orally to both male and female rats, Tsumacide was excreted from the body very rapidly (Fig. 2). In male rats, 96.6% of the dose was eliminated in the urine and 0.1% in the feces within 24 hr after treatment. During 4 days, 99.3% of radiocarbon was recovered in the urine (98.7%) and in the feces (0.6%). Elimination of Tsumacide was rather slow in female rats. By 4 days after treatment, 83.4% of the dose was eliminated from female rats; 78.3% in the urine and 5.1% in the feces. No radioactive carbon dioxide was detected in the air expired from male and female rats.

FIG. 1. Radioautograms Showing Distribution of Radiocarbon in the Rat Treated with 14 C-Tsumacide.

A=30 min after treatment, B=3 hr after treatment.

FIG. 2. Excretion of Radiocarbon from Male and Female Rats Treated Orally with 100 mg of ^{14}C -Tsumacide per kg.

Excreted urine during 2 days was pooled and submitted to acid-ether extraction. As shown in Table II, in male rats, 66.1% of the urinary metabolites was obtained in the ether phase and 33.9% was in the aqueous phase. The ether extract was directly analyzed by tlc and metabolites were identified by cochromatography with authentic compounds. Only trace of intact Tsumacide was found in the ether extract from male and female rats. The main metabolite in the ether extract proved to be *m*-COOH-T, which accounted for 59.3% and 59.7% of the excreted radioactivity from male and female rats, respectively. The following metabolites were also identified; *m*-CH₂OH-T, 4-OH-T, *m*-hydroxybenzoic acid and *m*-cresol. Analysis of the ether-soluble metabolites indicates that the same metabolites in the same proportions were present in the urine from male and female rats. Thus, the major metabolic pathways seem to be identical in both male and female rats.

The water-soluble metabolites were submitted to acid hydrolysis and enzyme hydrolysis. Over 50% of the water-soluble metabolites from male rats was cleaved by acid hydrolysis and transferred to ether phase. The following products were found; *m*-CH₂OH-T, *m*-COOH-T, 4-OH-T, *m*-cresol, *m*-hydroxybenzyl alcohol and methylhydroquinone. The

TABLE II. METABOLITES OF ^{14}C -TSUMACIDE FOLLOWING ORAL ADMINISTRATION TO MALE AND FEMALE RATS
Tsumacide administered at 100 mg/kg in Tween 80.

Products	Radiocarbon recovered as indicated products, %	
	Male	Female
Ether-soluble		
Tsumacide	0.3	0.3
<i>m</i> -CH ₂ OH-T	1.1	0.2
<i>m</i> -COOH-T	59.3	59.7
4-OH-T	0.1	0.2
<i>m</i> -OH-Benzoic acid	1.2	2.2
<i>m</i> -Cresol	0.1	
Unknowns	4.0	7.2
Total	66.1	69.8
Water-soluble		
<i>m</i> -CH ₂ OH-T	} Glucuronide	4.0
<i>m</i> -COOH-T		
4-OH-T		
<i>m</i> -Cresyl sulfate	1.6	
4-OH-T Deriv.	13.6	
3-OH-Benzyl alcohol Deriv.	<0.1	
Methylhydroquinone Deriv.	<0.1	
Unknowns	14.7	
Total	33.9	30.2

main component proved to be 4-OH-T, which was led to 1,4-bis-carbamate by the reaction with methyl isocyanate in the presence of triethylamine and then identified by cochromatography with the authentic compound. By β -glucuronidase hydrolysis, 12% was cleaved to yield ether-soluble products (aglycones) which consisted of *m*-CH₂OH-T, *m*-COOH-T and 4-OH-T. This finding indicates that the water phase contains conjugates which are possibly β -glucuronides. *m*-Cresol was liberated on incubation with aryl sulfatase, indicating that it is present as a conjugate and probably *m*-cresyl sulfate. Although a part of 4-OH-T present in water phase seems to be conjugated as glucuronide, the compound was not liberated by aryl sulfatase hydrolysis. Thus, most of the 4-OH-T may be present as another type of conjugate or as some complex with excreta.

Metabolism in vitro by mixed function oxidases

When ^{14}C -Tsumacide was incubated with each microsome fraction from rabbit, rat and mouse livers in the presence of NADPH, metabolites found were common to three animal species. Two major metabolites were identified as $m\text{-CH}_2\text{OH-T}$ (main) and 4-OH-T (Table III). Among three animal species, the

TABLE III. METABOLITES OF ^{14}C -TSUMACIDE FOLLOWING INCUBATION WITH MIXED FUNCTION OXIDASES FROM MOUSE, RAT AND RABBIT LIVERS

The incubation mixture consisted of 1 μmole of ^{14}C -Tsumacide, microsomes equivalent to 400 mg of the liver and 5.5 μmole of NADPH in 2.5 ml of 0.1 M phosphate buffer, pH 7.4, was incubated at 37°C for 4 hr.

Product	Radiocarbon recovered as indicated products, %		
	Mouse	Rat	Rabbit
$m\text{-CH}_2\text{OH-T}$	18.4	11.1	39.4
4-OH-T	11.7	4.0	11.6
$m\text{-Cresol}$	2.3	1.3	0.6
Unknowns	1.7	0.8	5.3

enzyme from rabbit liver was most active in hydroxylating Tsumacide at m -methyl group. It seems that the initial attack involves hydroxylation at m -methyl group and 4-position of the ring in the metabolism of Tsumacide by mammals.

Metabolism of Tsumacide in houseflies

When ^{14}C -Tsumacide was applied topically to the houseflies, about 70% of the dose was lost within 24 hr after application. Approximately 30% of the dose was recovered in the following fractions; 1.4% in wash-excreta fraction, 15.8% in the acid-ether extract of the flies and 11.6% in the methanol extract of the flies (Table IV). Intact Tsumacide was detected in both wash-excreta fraction and acid-ether extract of the flies in a small amount. The following oxidation products were obtained in the wash-excreta fraction; $\text{N-CH}_2\text{OH-T}$, $m\text{-CH}_2\text{OH-T}$, $m\text{-COOH-T}$ and 4-OH-T . So, they appear to be excreted from the flies

following absorption and metabolism. Major metabolites internally obtained were $m\text{-CH}_2\text{OH-T}$ and $m\text{-COOH-T}$. Also, $\text{N-CH}_2\text{OH-T}$ and 4-OH-T were found in relatively small amounts. Methanol soluble products were almost quantitatively hydrolyzed with 1N hydrochloric acid to give the following ether-soluble products; $m\text{-CH}_2\text{OH-T}$, $m\text{-COOH-T}$ (major) and 4-OH-T . The main product, $m\text{-COOH-T}$, was also obtained by β -glucuronidase and β -glucosidase hydrolysis. Thus, $m\text{-COOH-T}$ seems to be present as conjugates such as β -D-glucuronide and β -D-glucoside. These findings indicate that Tsumacide is mainly metabolized by oxidation at m -methyl group, N-methyl group and 4-position of the ring and a part of the oxidation products is conjugated as glucuronide and glucoside.

TABLE IV. METABOLITES OF ^{14}C -TSUMACIDE FOLLOWING TOPICAL APPLICATION TO HOUSEFLIES

Tsumacide administered at 1 $\mu\text{g/fly}$ in acetone.

Product	Radiocarbon recovered as indicated products, %		
	Wash-excreta fraction	Acid-ether extract	Acid hydrolyzate of methanol extr.
Tsumacide	0.3	2.5	
$\text{N-CH}_2\text{OH-T}$	0.1	0.5	
$m\text{-CH}_2\text{OH-T}$	0.4	6.4	0.6
$m\text{-COOH-T}$	0.3	4.9	7.0
4-OH-T	0.1	0.5	2.2
$m\text{-Cresol}$		0.1	
Unknowns	0.2	0.9	1.8
Total	1.4	15.8	11.6

Metabolism of Tsumacide and Meobal in bean plants

Topical application of both Tsumacide and Meobal to bean plants resulted in a rapid loss of radiocarbon from the leaf surface as shown in Table V. The total recovery of the radiocarbon applied was only 14.2% with Tsumacide and 20.3% with Meobal at one day after application and then gradually reduced to 2.7% and 5.7% over several days, respectively. The radiocarbon in organosoluble fractions and

TABLE V. FRACTIONATION OF RADIOCARBON FROM ^{14}C -TSUMACIDE AND -MEOBAL AT DIFFERENT TIME INTERVALS AFTER TOPICAL APPLICATION TO BEAN PLANTS

Fraction	Radiocarbon recovered at various days after treatment, %					
	1	3	6	7	9	12
Tsumacide						
Surface wash	4.8	1.8	0.3		0.2	
Ether extract	8.3	2.7	0.8		0.4	
Methanol extract	0.5	0.8	0.4		0.9	
Pulp	0.6	0.8	1.3		1.2	
Total	14.2	6.1	2.8		2.7	
Meobal						
Surface wash	15.6	6.3		1.5		0.5
Ether extract	3.6	4.5		1.0		0.6
Methanol extract	0.8	1.6		1.8		3.0
Pulp	0.3	1.6		2.2		1.6
Total	20.3	14.0		6.5		5.7

TABLE VI. METABOLITES OF ^{14}C -TSUMACIDE AND -MEOBAL FOLLOWING TOPICAL APPLICATION TO BEAN PLANTS

Product	Radiocarbon recovered as indicated products, %		
	Surface wash	Ether extract	β -Glucosidase hydrolyzate of methanol extr.
Tsumacide	6.8	12.2	
N-CH ₂ OH-T	<0.1		0.4
<i>m</i> -CH ₂ OH-T	0.1		1.8
4-OH-T	<0.1		0.1
Unknowns	0.1		0.3
Total	7.0	12.2	2.6
Meobal	21.3	9.7	
N-CH ₂ OH-M			0.5
4-CH ₂ OH-M			6.6
Unknowns	2.6		0.1
Total	23.9	9.7	7.2

unextractable residue increased but in very small amounts. Radioautographic studies showed that the radiocarbon was mostly located on the leaves treated with the carbamates over a period of several days. These findings indicated that both carbamates applied to the bean foliage are rapidly lost from the surface and very slowly absorbed into the plants.

The surface-wash fractions, ether extracts and methanol extracts obtained during the course of the experiment showed in Table V were each combined and radioactive products

were analyzed by tlc. The data in Table VI show that Tsumacide and Meobal are the principal component in the acetone washing of the surface and the ether extract, although several metabolites are present in the surface wash to minor extents. The following metabolites are identified; N-CH₂OH-T, *m*-CH₂OH-T and 4-OH-T. Since three oxidative metabolites were obtained in the acetone washing of the treated leaves, Tsumacide appears to be metabolized on the leaf surface, probably by photochemical reaction. Meobal also gave some metabolites in the surface wash to a minor extent but their structures remain unknown. Methanol soluble products from both Tsumacide and Meobal were cleaved, almost quantitatively, by the action of β -glucosidase to yield ether-soluble products (aglycones). The aglycones derived from Tsumacide are as follows; N-CH₂OH-T, *m*-CH₂OH-T (major) and 4-OH-T. Meobal yielded the following aglycones; N-CH₂OH-M and 4-CH₂OH-M (major). Therefore, they appear to be present as β -D-glucosides in bean plants. The chemical nature of the metabolites in the unextractable residue (pulp) remains unknown. Hydrolysis of the carbamate esters do not appear to take place in the metabolism of Tsumacide and Meobal in bean plants. No carboxy derivatives of *m*-CH₂OH-T and 4-CH₂OH-M

were detected. Thus, the oxidation of methyl groups seems to stop at the hydroxymethyl stage.

Anticholinesterase activity of Tsumacide metabolites and their related compounds

The biological activity of the Tsumacide metabolites was estimated by assaying the inhibition of rabbit blood and fly head cholinesterases (Table VII). Anticholinesterase ac-

TABLE VII. INHIBITION OF CHOLINESTERASES FROM RABBIT BLOOD CELLS AND HOUSEFLY HEAD BY TSUMACIDE METABOLITES AND RELATED COMPOUNDS

Compound	Rabbit blood ChE I_{50} ($M \times 10^5$)	Fly head ChE I_{50} ($M \times 10^5$)
Tsumacide	3.3	1.40
N-CH ₂ OH-T	50.0	> 300
<i>m</i> -CH ₂ OH-T	12.0	3.8
<i>m</i> -COOH-T	> 300	190
5-OH-T	15.0	0.98
1,5-Bis-carbamate	8.5	0.51
1,4-Bis-carbamate	1.1	1.1

tivity of three metabolites, N-CH₂OH-T, *m*-CH₂OH-T and *m*-COOH-T, toward both cholinesterases was less than that of the parent compound but *m*-CH₂OH-T could not be considered inactive to fly head cholinesterase. The molar I_{50} values for this compound is 3.8×10^{-5} (fly head ChE). On the other hand, 5-OH-T analogous to the metabolite, 4-OH-T, and 1,5-bis-carbamate were highly active in the inhibition of fly head cholinesterase but not so active to rabbit blood cholinesterase. 1,4-Bis-carbamate was an active inhibitor of both rabbit blood and fly head cholinesterases. Thus, Tsumacide derivatives which have a substituent at 5-position of the ring appear to be specific inhibitors of the fly head cholinesterase.

DISCUSSION

Tentative metabolic pathways for Tsumacide are given in Fig. 3. The life of Tsumacide in the rat body was very short. From 78 to 96.6% of an administered dose was eliminated,

primarily in the urine, by 24 hr after treatment. No residual radioactivity was detected in certain tissues of the male and female rats, although the rate of elimination was rather slow in the female rats. In the metabolism of Tsumacide by rats, the initial metabolic attack involved hydroxylation at *m*-methyl group and 4-position of the aromatic ring. This reaction was mediated by the mixed function oxidases from the livers of three animal species. The produced *m*-CH₂OH-T was further oxidized to *m*-COOH-T in rats. Hydrolysis of the carbamate esters occurred only to a minor extent to form the corresponding phenols. The oxidation products and phenols were subsequently conjugated and excreted as sulfate and glucuronides. The female rats metabolized and excreted Tsumacide in a manner quite similar to that of the male rats. The sites of the initial metabolic attack in Tsumacide molecule appear to be the same in rabbits, rats and mice, although the enzyme activity of the mixed function oxidases varied with the mammalian species.

Most of the radiocarbon was rapidly lost from the houseflies treated with ¹⁴C-Tsumacide and from the bean plants treated with ¹⁴C-Tsumacide and -Meobal. Both carbamates may be easily removed from the treated surface by wind, volatilization and decomposition to more volatile compounds. In houseflies, Tsumacide underwent oxidation at *m*-methyl group, N-methyl group and 4-position of the aromatic ring. One of the major products, *m*-COOH-T, subsequently formed conjugates cleavable with β -glucuronidase and β -glucosidase. However, the cleavage of insect conjugates by β -glucuronidase must be carefully interpreted as pointed out by Binning *et al.*²²⁾ In the metabolism of Tsumacide and Meobal by bean plants, the major attack involved hydroxylation at one of the methyl substituents and N-methyl group. The oxidation of the methyl substituent mostly stopped at the hydroxymethyl stage in bean plants. Ring hydroxylation also took place with Tsumacide at the 4-position. The hydroxylated products were subsequently conjugated and persisted as

FIG. 3. Proposed Metabolic Pathways for Tsumacide in Rats, Houseflies and Bean Plants.

R=Rat, F=Housefly, P=Bean plant

glucosides in bean plants. In general, Tsumacide is rapidly metabolized by oxidative reactions in rats, houseflies and bean plants. Hydrolysis of the carbamate esters is not major pathway. The metabolites formed are very similar to those formed in rats, houseflies and bean plants.

The results obtained from Tsumacide and Meobal are consistent with previous studies on the metabolism of related phenyl methylcarbamate. Metabolism of Meobal has been investigated in rats.⁸⁾ When orally administered, more than 90% of the radiocarbon was eliminated in the urine by 24 hr after treatment. Major metabolites found in the urine were the 4-carboxy derivatives. Therefore, the oxidation of the 4-methyl group seems to be one of the principal metabolic pathway. In the metabolism of the two major Landrin constituents (3,4,5- and 2,3,5-trimethylphenyl N-methylcarbamates) by bean plants, mice and houseflies, oxidation of one of the ring-methyl groups proved to be the major metabolic attack.⁹⁾ Carbanolate (2-chloro-4,5-dimethyl-

phenyl N-methylcarbamate) is closely related structurally to Meobal and the Landrin isomers. The major pathway of Carbanolate in the plant was oxidative but hydrolysis was an important degradation mechanism in rats.^{16,17)}

Although metabolism of carbamate ester generally leads to non-toxic products, there are a number of examples where toxic metabolites are produced. Therefore, the toxicological importance of the oxidative metabolites of Tsumacide was estimated. Three metabolites of Tsumacide were of low anticholinesterase activity toward both rabbit blood and fly head cholinesterases, while Tsumacide derivatives having a substituent at the 5-position of the ring were specific inhibitors of fly head cholinesterase. This finding is consistent with previous studies indicating that di-meta-(3,4)-substituted compounds are highly active against insect acetylcholinesterase.¹⁸⁾ On the other hand, it was reported that Tsumacide showed no teratogenicity when it was given to pregnant SD-JCL rats from day 8 to 15 of gestation at levels of 80 to 4,000 ppm (about 6 and 280 mg/

kg/day, respectively).¹⁹⁾ Also, the no-effect level of Tsumacide proved to be 600 ppm (36.9 mg/kg/day) in the diet after 90 consecutive days feeding to rats.²⁰⁾ The results of residue analysis showed that rice grains contain 0.02 to 0.067 ppm of Tsumacide under regular spraying programme in Japan. Metabolism studies also supported that the residue level of Tsumacide in crops could be extremely low. Thus, as evidenced with Meobal,²¹⁾ Tsumacide can be used without any potential danger of residual toxicity to humans.

Acknowledgement. The authors thank Mr. K. Kamoshita and Mr. M. Sasaki for synthesizing the intermediates of carbamates, and Miss H. Kawai for the technical assistance. The authors wish to express their thanks to Sumitomo Chemical Co., Ltd., for permission to publish this paper.

REFERENCES

- 1) T. R. Fukuto, *Drug Metab. Revs.*, **1**, 117 (1972).
- 2) J. B. Knaak, *Bull. Wld. Hlth Org.*, **44**, 121 (1971).
- 3) H. W. Dorough, *J. Agr. Food Chem.*, **18**, 1015 (1970).
- 4) R. J. Kuhr, *ibid.*, **18**, 1023 (1970).
- 5) J. E. Casida and K. B. Augustinsson, *Biochem. Biophys. Acta*, **36**, 411 (1959).
- 6) M. Hazue, Unpublished.
- 7) M. A. H. Fahmy and T. R. Fukuto, *J. Agr. Food Chem.*, **20**, 169 (1972).
- 8) J. Miyamoto, K. Yamamoto and T. Matsumoto, *Agr. Biol. Chem.*, **33**, 1060 (1969).
- 9) M. Slade and J. E. Casida, *J. Agr. Food Chem.*, **18**, 467 (1970).
- 10) R. L. Metcalf, T. R. Fukuto, C. Collins, K. Borch, S. A. El-Aziz, R. Munoz and C. C. Cassil, *ibid.*, **16**, 300 (1968).
- 11) M. Beroza, *ibid.*, **11**, 51 (1963).
- 12) S. Ullberg, *Proc. Second U.N. Intern. Confer. Peaceful Uses At. Energy*, **24**, 248 (1958).
- 13) S. Hestrin, *J. Biol. Chem.*, **180**, 249 (1949).
- 14) J. Miyamoto, Y. Sato, K. Yamamoto, M. Endo and S. Suzuki, *Agr. Biol. Chem.*, **32**, 628 (1968).
- 15) R. S. H. Yang and C. F. Wilkinson, *Pest. Biol. Physiol.*, **1**, 327 (1971).
- 16) R. L. Baron and J. D. Doherty, *J. Agr. Food Chem.*, **15**, 830 (1967).
- 17) A. R. Friedman and A. J. Lemin, *ibid.*, **15**, 642 (1967).
- 18) R. L. Metcalf, *Bull. Wld. Hlth Org.*, **44**, 43 (1971).
- 19) T. Yamamoto, M. Kimura, H. Katayama and C. Saito, Unpublished.
- 20) M. Yasuda, *Botyu-kagaku*, **37**, 161 (1972).
- 21) J. Miyamoto and K. Fukunaga, "Pesticide Chemistry," Vol. 4, ed. by A. S. Tahori, Gordon and Breach Science Publishers, 1972, pp. 319~324.
- 22) A. Binning, F. J. Darby, M. P. Heenan and J. N. Smith, *Biochem. J.*, **103**, 42 (1967).