Metabolism of the Acaricide Chemical, Fenazaflor (5,6-Dichloro-1-phenoxycarbonyl-2-trifluoromethylbenzimidazole), and Related 2-Trifluoromethylbenzimidazoles in Certain Mammals, Insects, and Plants

D. M. Bowker[1] and John E. Casida

Mouse liver and mouse plasma hydrolyze fenazaflor (5,6-dichloro-1-phenoxycarbonyl-2-trifluoromethylbenzimidazole) to 5,6-dichloro-2-trifluoromethylbenzimidazole (5,6-Cl_2-TFB). Microsomal mixed-function oxidases of mouse liver hydroxylate the benzene moiety of 4,5-dichloro-2-trifluoromethylbenzimidazole (4,5-Cl_2-TFB) and 5,6-Cl_2-TFB. Rats or mice treated orally with fenazaflor or 5,6-Cl_2-TFB excrete the following products in urine: 5,6-Cl_2-TFB; 5,6-Cl_2-TFB N-glucuronide (major product in mice); conjugates of 4-hydroxy-5,6-Cl_2-TFB, 4,7-dihydroxy-5,6-Cl_2-TFB, and 6-chloro-5-hydroxy-TFB (major products in rats). Certain conjugates chromatograph in the manner anticipated for riboside and glucoside derivatives. Urine of rats treated orally with 4,5-Cl_2-TFB contains conjugates of 6-hydroxy-4,5-Cl_2-TFB (major), 7-hydroxy-4,5-Cl_2-TFB (minor), and 5-hydroxy-4,6-Cl_2-TFB (minor). Houseflies excrete 4,5-Cl_2-TFB and 5,6-Cl_2-TFB as the N-glucoside and, possibly, the N-glucoside-6-phosphate derivatives. Fenazaflor is more persistent than 5,6-Cl_2-TFB on bean foliage but, in bean plants, each compound converts to products retained in the acetone-insoluble residue. Apple foliage and fruit treated with fenazaflor contain 5,6-Cl_2-TFB, N- and O-conjugates of 4-hydroxy-5,6-Cl_2-TFB (possibly the glucosides), an unidentified trifluoromethylbenzimidazole occurring only on the surface, and unextractable derivatives formed from the absorbed material.

The biological activity of benzimidazoles varies dramatically, in type and potency, with the substituents involved. Herbicidal and insecticidal activities occur with 2-trifluoromethylbenzimidazole (TFB) derivatives containing strongly electronegative substituents on the benzene moiety and either an acidic N—H group or appropriate replacement groupings on the imidazole hydrogen (Burton et al., 1965, 1968). The mode of action of TFB derivatives appears to be related to uncoupling of oxidative phosphorylation in mammals (Beechey, 1966; Buchel et al., 1965a; Burton et al., 1965; Ilivicky and Casida, 1969; Jones and Watson, 1965, 1967; Weinbach and Garbus, 1966; Wilson and Chance, 1966; Wilson and Merz, 1967), and in insects (Ilivicky et al., 1967; Ilivicky and Casida, 1969; Williamson and Metcalf, 1967), and to inhibition of photosynthesis reactions in plants (Buchel et al., 1965b, 1966).

Fenazaflor (5,6-dichloro-1-phenoxycarbonyl-2-trifluoromethylbenzimidazole, formerly known as fenoflurazole and NC 5016) is a nonsystemic acaricide chemical with outstanding residual activity in controlling all stages of phytophagous mites, including the eggs, and for the control of strains resistant to organophosphates (Saggers and Clark, 1967). Although its water solubility is low (less than 1 p.p.m.), fenazaflor hydrolyzes fairly readily in solution to 5,6-dichloro-2-trifluoromethylbenzimidazole (5,6-Cl_2-TFB), phenol, and carbon dioxide. Knowledge of the metabolic fate of fenazaflor and related TFB derivatives is of importance in determining safe and effective conditions for the use of this new group of pesticide chemicals.

The available information on metabolism of compounds related to fenazaflor and TFB derivatives concerns benzimidazole (Kapoor and Waygood, 1965a, b), 5,6-Cl_2-TFB and 4,5-dichloro-2-trifluoromethylbenzimidazole (4,5-Cl_2-TFB)

Division of Entomology, University of California, Berkeley, Calif. 94720

[1] Present address, Plant Protection, Ltd., Jealott's Hill Research Station, Berkshire, England.

(Flockhart et al., 1968), imidazole, and intermediates in histidine degradation (Karjala et al., 1956; Schayer, 1956; Williams, 1959). Benzimidazole incorporates into the "nucleic acid" fraction when incubated with the detached, first leaves of Khapli wheat, this fraction yielding benzimidazole nucleoside on acid hydrolysis. Wheat embryo enzymes convert benzimidazole to the corresponding mononucleotide and catalyze the substitution of benzimidazole for the nicotinamide moiety of nicotinamide–adenine dinucleotide. In rats and rabbits, 4,5-Cl_2-TFB and 5,6-Cl_2-TFB are hydroxylated on the nonchlorinated position(s) of the benzene moiety, and conjugated at the resulting hydroxyl group(s) and at the nitrogen. When administered to rabbits, imidazole excretes, in large part, without chemical change. In contrast, 4-imidazolylacetic acid, from histidine metabolism, excretes as the N-methyl and N-riboside derivatives; however, it is not known whether the riboside formation is a special reaction of 4-imidazolylacetic acid or is an extension of normal nucleoside formation.

The present study concerns the metabolic fate, in certain mammals, insects, and plants, of fenazaflor, 5,6-Cl_2-TFB, and a number of other chlorinated TFB derivatives. As a result, sufficient data exist to postulate tentative metabolic pathways for conjugation reactions occurring on the imidazole moiety and for hydroxylation reactions occurring on the benzene moiety, based on radiotracer studies and thin-layer cochromatography of metabolites or their derivatives.

MATERIALS AND METHODS

Chemicals. The 42 TFB derivatives studied are listed in Table I. The compounds indicated by Roman numerals, without a letter, were provided by E. F. Edson, Chesterford Park Research Station, Fisons, Ltd. (Cambridge Division), Saffron Walden, England. Those designated by a numeral and a letter were prepared in these laboratories, as described below. The abbreviations used in the text and tables are given in Table I. In accordance with this system of abbreviations, 5,6-dichloro-4,7-dimethoxy-2-trifluoromethylbenzim-

Table I. Designations, Composition, and Chromatographic Characteristics of the 2-Trifluoromethylbenzimidazoles Studied
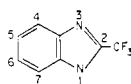
Designation	Substituents and Their Positions[a]					TLC Systems Used and Respective R_f Values				
	N	4	5	6	7	A	B	C	D	E
Unsubstituted and Monochloro Derivatives										
I	H	H	H	H	H		0.66			0.82
II	H	H	Cl	H	H		0.66			0.71
IIa	Me	H	Cl	H	H	0.29, 0.34	0.66			
III	H	OH	Cl	H	H		0.66			
IIIa	Me	OMe	Cl	H	H	0.36, 0.42				
IV	H	H	Cl	OH	H	0.02	0.66		0.49	
IVa	Me	H	Cl	OMe	H	0.25, 0.33				
4,5-Dichloro Derivatives										
V	H	Cl	Cl	H	H		0.66		0.64	0.30
Va	Me	Cl	Cl	H	H	0.39, 0.45	0.66			
VI	glr	Cl	Cl	H	H	0.00	0.16			
VIa	mglr	Cl	Cl	H	H		0.60			
VII	H	Cl	Cl	OH	H	0.02	0.66	0.13	0.35	
VIIa	Me	Cl	Cl	OH	H		0.66	0.27		
VIIb	H	Cl	Cl	Ac	H		0.66	0.32		
VIIc	Me	Cl	Cl	OMe	H	0.27, 0.35	0.66			
VIId	Me	Cl	Cl	Ac	H		0.66	0.61, 0.50		
VIII	H	Cl	Cl	H	OH	0.02	0.66	0.22	0.49	
VIIIa	Me	Cl	Cl	H	OH		0.66	0.36		
VIIIb	H	Cl	Cl	H	Ac		0.66	0.34		
VIIIc	Me	Cl	Cl	H	OMe	0.36, 0.45	0.66			
VIIId	Me	Cl	Cl	H	Ac		0.66	0.65, 0.51		
5,6-Dichloro Derivatives										
IX	H	H	Cl	Cl	H	0.13	0.66	0.50	0.68	0.44
X	Phc	H	Cl	Cl	H	0.59	0.66			
XI	Me	H	Cl	Cl	H	0.40	0.66	0.83		
XIa	rib	H	Cl	Cl	H		0.61			
XII	gls	H	Cl	Cl	H		0.55			
XIII	glr	H	Cl	Cl	H		0.16			
XIIIa	mglr	H	Cl	Cl	H		0.60			
XIV	H	OH	Cl	Cl	H	0.02	0.66	0.23	0.48	
XIVa	Me	OH	Cl	Cl	H	0.06	0.66	0.41		
XV	H	OMe	Cl	Cl	H	0.15	0.66	0.60		
XIVb	H	Ac	Cl	Cl	H		0.66	0.37		
XIVc	Me	OMe	Cl	Cl	H	0.42, 0.49	0.66			
XIVd	Me	Ac	Cl	Cl	H		0.66	0.62, 0.69		
XVI	H	OMe	Cl	Cl	OMe	0.09	0.66	0.54		
XVIa	Me	OMe	Cl	Cl	OMe	0.39	0.66			
XVII	H	(=O)	Cl	Cl	(=O)	0.01	0.47		0.28	
XVIIa	Me	(=O)	Cl	Cl	(=O)	0.38				
Other Di-, Tri-, and Tetrachloro Derivatives										
XVIII	H	Cl	OH	Cl	H	0.02	0.66		0.57	
XVIIIa	Me	Cl	OMe	Cl	H	0.33, 0.38	0.66			
XIX	H	Cl	Cl	Cl	H		0.66			0.16
XX	H	Cl	Cl	Cl	Cl		0.66			0.04

a Ac = acetyl; Cl = chlorine; glr = β-D-glucopyranosiduronic acid or β-D-glucuronide; gls = β-D-glucopyranoside or β-D-glucoside; OH = hydroxyl; Me = methyl; MeO = methoxyl; (=O) represents carbonyl function of a quinone; mglr = methyl ester of β-D-glucopyranosiduronic acid or β-D-glucuronide methyl ester; Phc = phenoxycarbonyl; rib = β-D-ribofuranoside or β-D-riboside; TFB = 2-trifluoromethylbenzimidazole.

idazole (XVI) is written as 5,6-Cl$_2$-4,7-OMe$_2$-TFB, and 4,5-dichloro-2-trifluoromethylbenzimidazole N-glucuronide (VI) abbreviates to N-glr-4,5-Cl$_2$-TFB.

Three trifluoromethyl-C^{14}-benzimidazoles were provided by Fisons, Ltd.: fenazaflor-C^{14} (1.8 mCi. per mmole); 5,6-Cl$_2$-TFB-C^{14} (0.9 mCi. per mmole); and 4,5-Cl$_2$-TFB-C^{14} (0.9 mCi. per mmole). Partial to complete chlorination of 5,6-Cl$_2$-TFB-C^{14}, by the procedure of Buchel et al. (1965a), was used to prepare 4,5,6-Cl$_3$-TFB-C^{14} (XIX) and 4,5,6,7-Cl$_4$-TFB-C^{14} (XX) (0.9 mCi. per mmole, each); the products were isolated by preparative-scale thin-layer chromatography (see below), using dichloromethane with three separate developments, in sequence and in the same direction, followed by recovery and rechromatography of the individual chlorinated derivatives using a dichloromethane-chloroform-ethyl acetate-concentrated ammonium hydroxide mixture (120:40:10:1). The radiochemical purity of each labeled compound was greater than 99%.

Several compounds were subjected to methylation, acetylation, or both. Methylation, of both the imidazole nitrogen and the phenolic oxygen, was quantitative on reacting the appropriate benzimidazole (10 mg.) with excess diazomethane [20 mg. in 2 ml. of ethereal-alcoholic solution, prepared according to DeBoer and Backer (1954)] for 12 hours at 25 °C. The reaction was rapid, the bubbling due to nitrogen evolution occurring for only a few minutes, and the yellow color of

excess diazomethane persisted for a relatively long period. After evaporation to dryness, crystalline products were obtained. Acetylation of the phenolic oxygen but, in no case, of the imidazole nitrogen was accomplished in almost quantitative yields by reaction of the hydroxybenzimidazoles (10 mg.) with acetic anhydride (1 ml. containing 20 mg. of concentrated sulfuric acid) for 30 minutes at 50°C., with shaking. After the reaction mixture was cooled, ice and water were added to a volume of 4 ml., and the pH was adjusted to 6.0 with solid sodium bicarbonate, allowing adequate time for complete hydrolysis of the acetic anhydride. The organo-soluble products were recovered by extraction with 6 ml. of chloroform, and the chloroform was dried (sodium sulfate) and evaporated. Methylation of the acetoxybenzimidazoles (as described above) gave the N-methylacetoxy derivatives. The acetoxy group of the N-methylacetoxy compounds was completely hydrolyzed, to the corresponding N-methylhydroxy compounds, on heating at 100°C. with 0.5 ml. of $12N$ hydrochloric acid in an open beaker until a dry residue was obtained, requiring approximately 15 minutes. The crystalline solids were then methylated to obtain the N-methyl-O-methyl derivatives with R_f values identical to those formed on direct methylation of the original hydroxybenzimidazoles.

On methylation, each symmetrical benzimidazole gave one product and each unsymmetrical benzimidazole gave two products as a result of the tautomeric nature of the imidazole nucleus (Forsyth and Pyman, 1925; Hofmann, 1953). The ratio of the N-methyl isomers formed on methylation varied with the ring substituents and reaction conditions, in accordance with the findings of Forsyth and Pyman (1925). The fact that two isomers are formed on methylation and appear in a nearly constant ratio was found useful in comparison of authentic compounds with metabolites, by forming the N-methyl derivatives of the latter and determining their approximate isomer ratio. There were two exceptions to this relationship. Methylation of 5,6-Cl₂-4,7-(=O)-TFB (XVII) gave one major product, probably the N-methyl derivative; however, several minor, unidentified products were also formed, possibly resulting from the instability of the quinone. Unfortunately, the chromatographic methods used were inadequate to resolve the two isomers of the three unsymmetrical N-methylhydroxybenzimidazoles (VIIa, VIIIa, and XIVa).

As expected from the work on 1,2-glycols by Malaprade (1928) and Buchanan *et al.* (1950), each of the N-glucoside, N-glucuronide, and N-riboside derivatives (VI, IXa, XII, XIII, and certain C¹⁴-labeled metabolites) was attacked on treatment with 6 mg. of potassium metaperiodate in 1.0 ml. of aqueous solution. The R_f values of the oxidized glycosides, as authentic compounds or C¹⁴-labeled metabolites, using TLC-system B, differed significantly from those of the parent compounds before oxidation.

To prepare 5,6-Cl₂-TFB N-riboside (IXa), 5,6-Cl₂-TFB (IX) was converted to the silver salt by reaction with less than an equivalent amount of silver nitrate in 60% ethanol, filtering, washing the silver salt with ethanol, and drying the salt in vacuo in the dark (calculated: C = 26.6%, H = 0.56%, Ag = 29.8%, N = 7.7%; found: C = 26.8%, H = 0.71%, Ag = 30.9%, N = 7.7%). 2,3,5-Tri-O-benzoyl-D-ribosyl chloride (2 mmoles, prepared according to Yung and Fox, 1963) was added to a suspension of the silver salt (1.1 mmoles) in refluxing xylene (25 ml.) and, after completion of the addition, the reflux was continued for one hour, the reaction mixture was cooled and evaporated under reduced pressure to a sirup, and methanol was added to obtain a crystalline material.

Recrystallization from methanol gave 1-(tri-O-benzoyl-β-D-ribofuranosyl)-5,6-Cl₂-TFB (m.p. 156°C.) (75% yield) (calculated: C = 58.4%, H = 3.3%, Cl = 10.1%, N = 4.0%; found: C = 58.3%, H = 3.5%, Cl = 10.2%, N = 3.9%). The benzoyl ester (0.25 gram) suspended in anhydrous methanol (30 ml.) was hydrolyzed by saturating the mixture with dry ammonia gas at 0°C., holding for 4 days at 0°C., evaporating the clear solution to a low volume, removing the benzoic acid by steam distillation, extracting the nondistillable fraction with ether, evaporating the ether, and recrystallizing the product twice from benzene (m.p. 119–20°C.) (30% yield) (calculated: C = 40.3%, H = 2.9% Cl = 18.3%, N = 7.2%; found: C = 40.8%, H = 2.7%, Cl = 18.1%, N = 7.2%).

Thin-Layer Chromatography (TLC). Preparative-scale TLC on silica gel, for preliminary cleanup and resolution of metabolites, utilized SilicAR TLC-7GF (Mallinckrodt Chemical Co., St. Louis, Mo.), in a thickness of 1 mm. on 20×20 cm. glass plates. Better resolution was obtained with silica gel F_{254} precoated chromatoplates (Merck AG, Darmstadt, Germany); accordingly, all R_f values and quantitative data on authentic compounds and/or metabolites were determined on the precoated plates. The solvent systems used are shown in Table II. Radioactive products were detected by radioautography with Kodak Medical No-screen x-ray film and unlabeled compounds were detected by their quenching of gel fluorescence when viewed under short-wavelength ultraviolet light. Radioactive products were essentially quantitatively recovered from the TLC plates by scraping the radioactive gel region free from the glass support and extracting the scrapings twice with methanol.

Fenazaflor-C¹⁴ is converted partially to 5,6-Cl₂-TFB-C¹⁴, when exposed for a few minutes under ambient laboratory conditions as a spot on the precoated plates; however, it is not extensively degraded during the period of chromatographic development with TLC-system A. The extent of conversion to 5,6-Cl₂-TFB, as a function of time on the plate before the solvent came in contact with the spot for development, is: 1 minute = 2%, 5 minutes = 26%, 10 minutes = 41%, 30 minutes = 88%, 60 minutes = 97%. This degradation rate is essentially the same for pure fenazaflor as for fenazaflor recovered, as surface residues, from apple leaves and apple fruit. When appropriate, corrections were made for this breakdown in the determination of fenazaflor levels.

Enzyme Incubation Studies. In the fenazaflor-C¹⁴ hydrolysis studies, 100 μl. of mouse plasma or 20% mouse liver homogenate and 1.0 μl. of benzene containing 5 μg. of fenazaflor-C¹⁴ were added, in sequence, to 1.0 ml. of 0.05M phosphate solution (pH 7.0). After incubation for various periods of time at 37°C., the reaction mixtures were immediately diluted with 2 ml. of water and extracted twice with 3 ml. of benzene, and the combined benzene extracts were concentrated by evaporation to approximately 2 ml. The labeled products in the resulting benzene phase were methylated by addition of 1.0 ml. of ether containing 10 mg. of diazomethane; after 2 minutes, the excess diazomethane was destroyed by adding 20 mg. of glacial acetic acid and the solvent was removed by evaporation. The products were separated by TLC system A, using a 2-hour interval between spotting and solvent development, to allow complete degradation of fenazaflor-C¹⁴ to 5,6-Cl₂-TFB-C¹⁴. In this procedure, fenazaflor is determined as 5,6-Cl₂-TFB (resulting from breakdown on the TLC plate, 100% recovery) and 5,6-Cl₂-TFB is determined as N-Me-5,6-Cl₂-TFB (formed on methylation before spotting on the TLC plate, 91% recovery). The ratio of fenazaflor-C¹⁴ to 5,6-Cl₂-TFB-C¹⁴, under the various incubation conditions, was used as an

Table II. Components and Characteristics of Thin-Layer Chromatographic Systems Utilizing Silica Gel F_{254} Precoated Chromatoplates

System	Development Solvent	Characteristics
A	Dichloromethane	HO-TFB derivatives and N-conjugates of TFB remain at or near origin; products of higher R_f and adequately resolved are Cl-TFB, MeO-TFB, N-Me-TFB, N-Me-OH-TFB, and N-Me-MeO-TFB derivatives.
B	2-Methyl-1-propanol saturated with water	Introduction of —OH into TFB derivatives does not greatly alter R_f; all Cl-TFB derivatives are of similar R_f; N-conjugates of TFB are well resolved.
C	Benzene-acetone (10:1)	Within series of —OH derivatives of 4,5-Cl_2-TFB or 5,6-Cl_2-TFB, resolution is adequate for HO-TFB, N-Me-HO-TFB, MeO-TFB, Ac-TFB, and N-Me-Ac-TFB derivatives.
D	Ethyl acetate–benzene–acetone (20:10:1)	Introduction of —OH into TFB derivatives reduces R_f value, but OH-TFB derivatives are not at or near the origin.
E	Dichloromethane-chloroform–ethyl acetate-concentrated aqueous ammonium hydroxide (120:40:10:1)	Separates Cl-TFB derivatives on basis of pK_a.

index of the catalytic activity of the mouse tissue preparations. In separate experiments, the reaction mixtures were preincubated for 5 minutes in the presence of dichlorvos or carbaryl $(10^{-5}M)$.

The microsome fraction or the microsome-plus-soluble fraction, equivalent to 400 mg. of mouse liver, was incubated for 2 hours at 37°C., with 20 μg. of 4,5-Cl_2TFB-C^{14} or 5,6-Cl_2-TFB-C^{14}, in 2.0 ml. of $0.25M$ sucrose–$0.05M$ phosphate (pH 7.5), in the presence and absence of 3 mg. of reduced nicotinamide–adenine dinucleotide phosphate (NADPH), using the general procedure of Oonnithan and Casida (1968). The labeled substrates were added, in 20 μl. of ethanol, just before the incubation period. After incubation, each reaction mixture was acidified with hydrochloric acid to pH 1 and extracted three times with 5-ml. portions of ether, and the ether was concentrated and applied to the precoated TLC plates, as a band rather than as a spot, for chromatography by TLC system D. Quantitative data were determined for the radiocarbon content of water-soluble products, unmetabolized compound, and the various ether-soluble metabolites.

Treatment of Mammals, Plants, and Insects. The organisms studied were male albino mice (20 grams, Swiss-Webster strain, Bioscience Animal Laboratories, Oakland, Calif.), male albino rats (160 to 180 grams, Sprague-Dawley strain, Bioscience Animal Laboratories, Oakland, Calif.), adult female houseflies (*Musca domestica* L., SCR-susceptible strain, 4 days after emergence), 11-day-old bean plants (Contender variety, trifoliate stage, 5-gram average fresh weight), and a mature apple tree (Golden Delicious variety, 0.33-gram average fresh weight of leaf at the time of treatment and 0.45-gram after 30 days, and 25-gram average fresh weight of fruit at the time of treatment and 31- to 32-gram at the termination of the experiment, 20 or 30 days after treatment).

Fenazaflor-C^{14}, 4,5-Cl_2-TFB-C^{14}, 5,6-Cl_2-TFB-C^{14}, 4,5,6-Cl_3-TFB-C^{14}, and 4,5,6,7-Cl_4-TFB-C^{14}, in dimethyl sulfoxide (DMSO) solution, were administered orally by stomach tube to the mammals at a dose of 1.5 mg. per kg.; the volume of DMSO used was 50 μl. for each mouse and 0.5 ml. for each rat. Immediately after treatment, the mammals were placed individually in metabolism chambers designed for the separate collection of urine and feces, and held there for 72 hours while receiving normal laboratory rations and water

ad libitum. In the 48-hour metabolism study with mice, expired $C^{14}O_2$ and other volatile products were trapped by absorption in monoethanolamine–methyl Cellosolve mixture (2 to 1) (Jeffay and Alvarez, 1961). Houseflies were injected with 0.8 μl. of acetone-water mixture (1 to 2) containing 0.15 μg. of 4,5-Cl_2-TFB-C^{14} or 5,6-Cl_2-TFB-C^{14} in solution. Groups of 25 flies were held in small glass vials for 24 hours after treatment, while receiving sugar and water, and then removed to obtain the excreta deposited on the glass surface. The test compounds did not result in symptoms of poisoning in the mammals, but an average mortality of 30% occurred in the housefly studies.

Foliage treatment of beans involved individual application of fenazaflor-C^{14} (14 μg.) or 5,6-Cl_2-TFB-C^{14} (10 μg.) dissolved in 20 μl. of ethanol, as uniformly as possible, to the upper surface of the two primary leaves. Immediately after this treatment, the plants were placed outside in an unshaded, unprotected area on the Berkeley campus. The studies took place in July 1967. The sunlight was almost continuous through the daylight hours, and there was no rain; temperature ranged from 50° to 60°F. at night to 70° to 85°F. in the daytime. These treatments resulted in some phytotoxicity, as evidenced by small necrotic areas on the treated but not the untreated foliage. Alternatively, the labeled compounds were injected into the stem by the procedure of Abdel-Wahab *et al.* (1966), using 20 μl. per plant of the DMSO solution containing 40 μg. of fenazaflor-C^{14} or 20 μg. of 5,6-Cl_2-TFB-C^{14}. Following injection, the plants were held in a greenhouse on the Berkeley campus. Both fenazaflor and 5,6-Cl_2-TFB stunted the plant growth and caused a twisting of the stem above the site of injection.

Leaves and fruit on an apple tree, located in an unshaded, unprotected part of an orchard in Albany, Calif., were treated (July 1968) in the following manner. Each of six apple fruit was painted, as uniformly as possible over the greater part of the available surface, with 250 μl. of an acetone solution of fenazaflor-C^{14}. Five leaves, attached a few centimeters above or below untreated fruit, were painted in a similar manner with 100 μl. of acetone containing fenazaflor-C^{14} in solution; the associated fruit was enclosed in an open-ended, clear plastic bag to avoid airborne or surface contamination of the untreated fruit by radiocarbon from the treated leaves. Sufficient fenazaflor-C^{14} was applied to give a

final concentration, based on the estimated initial fresh weight of the treated plant materials, of 10 p.p.m. in the fruit and 250 p.p.m. in the leaves. Treated fruit were removed for analyses 20 and 30 days after treatment, and treated leaves 30 days after treatment. Protected fruit, not treated with fenazaflor-C^{14} but associated with groups of treated leaves, were also removed 30 days after treatment. Rain did not fall during the experimental period. The treatment chemicals and procedure did not adversely affect the apple fruit or leaves.

Extraction and Analysis of Metabolites from in Vivo Studies. Mouse urine (5 ml.) and rat urine (up to 30 ml.), each including a small volume of water rinse of the metabolism chamber, were evaporated to dryness at 30°C. or, preferably, were lyophilized to dryness. The residue from evaporation of mouse urine was dissolved in 0.5 ml. of water before spotting on TLC plates. For preliminary cleanup of rat urine metabolites, the residue from evaporation was extracted with 50-, 20-, and 10-ml. portions of acetonitrile-water mixture (50 to 1), sequentially. The acetonitrile-insoluble residue was evaporated to remove trace amounts of acetonitrile, dissolved in 10 ml. of $0.1N$ hydrochloric acid, and extracted twice with 20-ml. portions of ether, to yield an ether phase and an acidic aqueous phase (which was discarded). The acetonitrile and ether extracts were combined for TLC analysis. Feces (20 grams) from rats were homogenized in 500 ml. of water, in a Waring Blendor, and the homogenate was extracted twice with 500-ml. portions of ether; the ether phase was dried (sodium sulfate) and evaporated to obtain the products for TLC analysis. Excreta from houseflies was suspended, with mixing, in 5 ml. of water and the water was extracted twice with 5-ml. portions of ether. The ether phase was dried (sodium sulfate) and the water phase was concentrated to about 0.5 ml. for separate TLC analyses.

Bean leaves, from topical treatment, were cut at the petiole and, in groups of two leaves, were rinsed three times with separate 10-ml. portions of acetone. The acetone was evaporated for TLC analysis. Each whole bean plant, which had been injected with the labeled compound, was macerated three times with separate 20-ml. portions of acetone to yield, after filtration, an acetone-soluble fraction and a fibrous residue fraction. Interfering pigments in the acetone extract were removed, prior to TLC analysis and without loss of radiocarbon, by passing the concentrated extract through a column of Sephadex LH-20 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) packed in acetone. The radiocarbon content of the acetone-insoluble fraction was determined by combustion.

Individual apple fruit or groups of five apple leaves were placed in 80 ml. of benzene and washed, by occasional swirling of the benzene, for 15 minutes, and the washed material and container were rinsed with 40 ml. of benzene. The combined washings were dried (sodium sulfate), evaporated to small volume, and analyzed by TLC. At times, the washings and/ or the benzene-washed apples were held in the frozen state for several days prior to analysis. After washing, each benzene-washed apple, or group of five leaves, was macerated in a Waring Blendor with 300 ml. of water and 500 ml. of ether, the macerate was filtered through Celite (5 grams, analytical Filter-Aid, Johns-Manville), and the pulp remaining on the filter was washed with 100 ml. of water followed by 200 ml. of ether. The ether and water phases of the filtrate were both analyzed by TLC, and the residue on the filter was used for radiocarbon analysis after combustion. Radiocarbon components in the benzene wash were designated as surface residues, and those in the ether and aqueous extracts as absorbed or internal residues.

Table III. Effect of Time and Inhibitors on Cleavage of Fenazaflor to 5,6-Cl$_2$-TFB by Mouse Plasma and Liver Homogenate

| Incubation Time, Min. | Inhibitor, $10^{-5}M$ | % Radiocarbon, in 5,6-Cl$_2$-TFB-C^{14} Produced from 5 μg. Fenazaflor-C^{14} | | |
		No tissue	Plasma, 100 μl.	20% liver homog., 100 μl.
0	None	2	3	17
5	None	3	22	22
30	None	8	36	21
30	Dichlorvos		30	29
30	Carbaryl		33	38

The metabolites in rat urine (acetonitrile-ether fraction), rat feces (ether fraction), and the absorbed material in apple fruit and leaves (separate ether and water phases) were further cleaned up by application of the extract, as an 18-cm. band along the origin, to preparative-scale TLC plates, development with TLC system B, radioautography, scraping the bands of silica gel containing radioactive material free from the glass support, and thoroughly mixing the dry powder by stirring. Dry aliquots (10 weight %) of the silica gel from each band were mixed, to reconstitute the metabolites in their original proportion, and the resulting mixture was extracted with methanol to effect almost quantitative recovery of the metabolite mixture free from many of the interfering materials originally present. After evaporation, the methanol extract was analyzed by TLC system B, using spots rather than bands of the material on precoated plates. The remaining silica gel from each band was extracted with methanol and the individual metabolites so obtained were purified for characterization studies.

Except as noted above, the metabolite extracts were analyzed by direct application to precoated TLC plates without preliminary cleanup. In each case, whether or not a preliminary cleanup was used, the recovery of the radiocarbon transferred to the precoated TLC plate was greater than 95%. Preparative-scale TLC separations (see above) were used, in all cases, to obtain the amounts of individual metabolites needed for characterization experiments; in such cases, 18-cm. bands were applied to the chromatoplates.

All radioactive measurements were made by scintillation counting with a Packard Tri-Carb Model 3003 or Beckman Model LS-150 liquid scintillation spectrometer, using 15 ml. of 0.55% 2,5-diphenyloxazole in toluene-methyl Cellosolve solution (2 to 1) as the scintillation mixture. The samples for counting consisted of organic extracts (after removal of the solvent from the scintillation vial by evaporation), 50-μl. portions of aqueous extracts, 2-ml. aliquots of carbon dioxide-trapping mixture from combustion analyses (Krishna and Casida, 1966), or radioactive regions of silica gel scraped from TLC plates. Corrections for quenching were made, when appropriate.

Results were calculated as percentages of the administered dose in all studies except those involving apple fruit and foliage, when the results were calculated as parts per million of compound based on the initial fresh weight of the plant material. Three to five replicate experiments were made and the quantitative data obtained for the replicates were averaged.

RESULTS

Hydrolysis of Fenazaflor and Hydroxylation of 4,5-Cl$_2$-TFB and 5,6-Cl$_2$-TFB by Mouse Liver Enzymes. Fenazaflor

Table IV. Effect of NADPH on Products Formed in the Incubation of 4,5-Cl$_2$-TFB and 5,6-Cl$_2$-TFB with Microsome or Microsome-Plus-Soluble Fraction of Mouse Liver Homogenate

Products	$R_f{}^a$	% Radiocarbon Recovered, in Products Obtained with Indicated Incubation Mixture			
		Microsomes only	Microsomes + NADPH	Microsomes + soluble	Microsomes + soluble + NADPH
4,5-Dichloro-2-trifluoromethylbenzimidazole					
Ether-soluble					
Unidentified	0.00	0.3	2.2	0.8	2.5
Unidentified	0.04	0.0	0.0	0.0	1.3
Unidentified	0.11	0.0	1.9	0.0	3.1
4,5-Cl$_2$-6-OH-TFB, 4,5-Cl$_2$-7-OH-TFB, and 4,6-Cl$_2$-5-OH-TFB	0.17	0.0	4.8	0.0	13.0
Unidentified	0.29	0.0	0.0	0.0	1.6
4,5-Cl$_2$-TFB	0.59	99.7	90.6	96.8	68.3
Water-soluble		0.0	0.5	2.4	10.2
5,6-Dichloro-2-trifluoromethylbenzimidazole					
Ether-soluble					
Unidentified	0.00	0.5	1.8	4.7	6.2
4-OH-5,6-Cl$_2$-TFB	0.28	0.0	4.3	0.0	7.8
Unidentified	0.39	0.0	0.7	0.0	1.7
5,6-Cl$_2$-TFB	0.66	99.5	91.8	93.8	80.5
Water-soluble		0.0	1.4	1.5	3.8

^{a} TLC system D. R_f values differ from those in Table I because products were developed as bands rather than as spots.

cleavage to 5,6-Cl$_2$-TFB is catalyzed by mouse plasma and mouse liver homogenate, but the catalytic activity is not reduced by preincubation of either plasma or liver homogenate with 10^{-5} M dichlorvos or carbaryl (Table III). The low rate of cleavage of fenazaflor to 5,6-Cl$_2$-TFB is possibly related either to the resistance of the carbamate ester groups to enzymatic attack or to the limited solubility of fenazaflor when used an an enzyme substrate.

The liver microsome in vitro system, in the presence but not absence of NADPH, converts 4,5-Cl$_2$-TFB and 5,6-Cl$_2$-TFB to several ether-soluble and water-soluble products (Table IV). The activity of the microsome-NADPH system is enhanced by the addition of the soluble fraction. The R_f values, in TLC system D, for the major ether-soluble metabolite(s) of each of 4,5-Cl$_2$-TFB and 5,6-Cl$_2$-TFB suggest that they are Cl-OH-TFB derivatives. Methylation of the major ether-soluble metabolite(s) of 4,5-Cl$_2$-TFB yields five resolvable products which co-chromatograph, in TLC system A, with the two isomers of each of N-Me-4,5-Cl$_2$-6-OMe-TFB (VIIc), N-Me-4,5-Cl$_2$-7-OMe-TFB (VIIIc), and N-Me-4,6-Cl$_2$-5-MeO-TFB (XVIIIa). Methylation of the major ether-soluble metabolite of 5,6-Cl$_2$-TFB yields only two products which cochromatograph, in TLC system A, with the two isomers of N-Me-4-MeO-5,6-Cl$_2$-TFB (XIVc). Hydroxylation by microsomal mixed-function oxidases converts 4,5-Cl$_2$-TFB to almost equal amounts of 4,5-Cl$_2$-6-OH-TFB (VII) and 4,5-Cl$_2$-7-OH-TFB (VIII), plus a small amount of 4,6-Cl$_2$-5-OH-TFB (XVIII); with 5,6-Cl$_2$-TFB, only the 4-position is attacked to yield 4-OH-5,6-Cl$_2$-TFB (XIV). The chemical nature of the other ether-soluble and water-soluble metabolites is not known.

Fate of Radiocarbon from Trifluoromethyl-C^{14}-Labeled Fenazaflor, 5,6-Cl$_2$-TFB, and Related Compounds in Rats and Mice. The pattern of radiocarbon excretion in urine and in feces, from both mice and rats, is very similar for fenazaflor-C^{14} and 5,6-Cl$_2$-TFB-C^{14} given orally (Table V). Very little radiocarbon is excreted after the initial 48-hour period and, 72-hours after administration, the products in urine account for 46 to 57% and in feces for 10 to 14% of the oral dose. Products volatilized (as C^{14}O$_2$ or other volatile compounds) from treated mice over a 48-hour period account for only 0.2% of the radiocarbon from fenazaflor-C^{14} and 0.1% from 5,6-Cl$_2$-TFB-C^{14}. The excretion patterns for 4,5-Cl$_2$-TFB-C^{14}, 4,5,6-Cl$_3$-TFB-C^{14}, and 4,5,6,7-Cl$_4$-TFB-C^{14} are given in Table VI. Even though a portion of the results are with rats and the remainder are with mice, the data indicate that the results are more compound-dependent than species-dependent, because the excretion patterns of fenazaflor and 5,6-Cl$_2$-TFB do not vary greatly with species. The extent of

Table V. Excretion of Radiocarbon in Urine and Feces of Male Mice and Rats Receiving 1.5 Mg. per Kg. of Fenazaflor-C^{14} or 5,6-Cl$_2$-TFB-C^{14}, Orally

Source of Excreted Radioactivity	Interval after Treatment, Hr.	Radiocarbon Recovered, %			
		Mouse		Rat	
		Fenazaflor	5,6-Cl$_2$-TFB	Fenazaflor	5,6-Cl$_2$-TFB
Urine	0–12	34.1	34.0	36.4	17.7
	12–24	14.9	7.3	8.1	16.7
	24–48	7.6	10.8	3.0	10.3
	48–72	0.7	1.6	1.3	0.9
	0–72	57.3	53.7	48.8	45.6
Feces	0–72	14.0	12.0	13.3	10.0
Urine plus feces	0–72	71.3	65.7	62.1	55.6

Table VI. Excretion of Radiocarbon in Urine and Feces of Male Mice or Rats 72 Hours after Receiving 1.5 Mg. per Kg. of 4,5-Cl$_2$-TFB-C^{14}, 4,5,6-Cl$_3$-TFB-C^{14}, and 4,5,6,7-Cl$_4$-TFB-C^{14}, Orally

Source of Excreted Radioactivity	Radiocarbon Recovered, %		
	4,5-Cl$_2$-TFB, rat	4,5,6-Cl$_3$-TFB, mouse	4,5,6,7-Cl$_4$-TFB, mouse
Urine	60	53	4
Feces	13	16	10
Urine plus feces	73	69	14

Table VII. Metabolites Present in Urine of Male Mice and Rats 72 Hours after Receiving 1.5 Mg. per Kg. of Fenazaflor-C^{14} or 5,6-Cl_2-TFB-C^{14}, Orally

Metabolite Description			Radiocarbon Recovered, %			
			Mouse		Rat	
Designation	Tentative identification	R_f [a]	Fenazaflor	5,6-Cl_2-TFB	Fenazaflor	5,6-Cl_2-TFB
M1 }	Conjugate(s) of 5-Cl-6-OH-TFB	{ 0.05	< 0.1	< 0.1	1.8	0.7
M2 }	(plus unidentified product?)	{ 0.10	< 0.1	< 0.1	4.2	1.2
M3	N-glr-5,6-Cl_2-TFB	0.16	43.0	40.8	6.4	4.3
M4	Conjugate(s)	0.34	< 0.1	< 0.1	3.9	4.8
M5	Conjugates of 5-Cl-6-OH-TFB, 4-OH-5,6-Cl_2-TFB, and 4,7-OH_2-5,6-Cl_2-TFB	0.46	5.5	3.7	24.6	17.7
M6	Conjugate(s) of hydroxy derivative(s) of 5,6-Cl_2-TFB	0.54	3.9	2.5	4.0	4.5
M7	Conjugate(s) of hydroxy derivative(s) of 5,6-Cl_2-TFB	0.61	3.5	4.1	2.2	10.2
M8	5,6-Cl_2-TFB plus two minor hydroxy derivatives of 5,6-Cl_2-TFB	0.66	1.4	2.6	1.7	2.2
	Total		57.3	53.7	48.8	45.6

[a] TLC-system B.

excretion, in the 72-hour period, does not depend on the position or degree of chlorination, except with 4,5,6,7-Cl_4-TFB, where very little of the radiocarbon appears in urine. Although tissue analyses are lacking, it seems reasonable to assume that the radiocarbon not accounted for after 72 hours is in the animal bodies (Flockhart *et al.*, 1968). The chemical nature of fragments remaining in the body still needs to be determined.

Identity of Metabolites Excreted from Rats and Mice Receiving Fenazaflor or 5,6-Cl_2-TFB, Orally. Fenazaflor-C^{14} and 5,6-Cl_2-TFB-C^{14} both yield the same eight products (or mixtures of products), in almost the same proportion, in the urine of rats (Table VII, Figure 1). Five of these products (M_3, M_5, M_6, M_7, and M_8), but not those designated as M_1, M_2, and M_4, are also present in the urine of mice and, in the manner found with the rats, the products and their ratios are similar in the case of fenazaflor-C^{14} and 5,6-Cl_2-TFB-C^{14}.
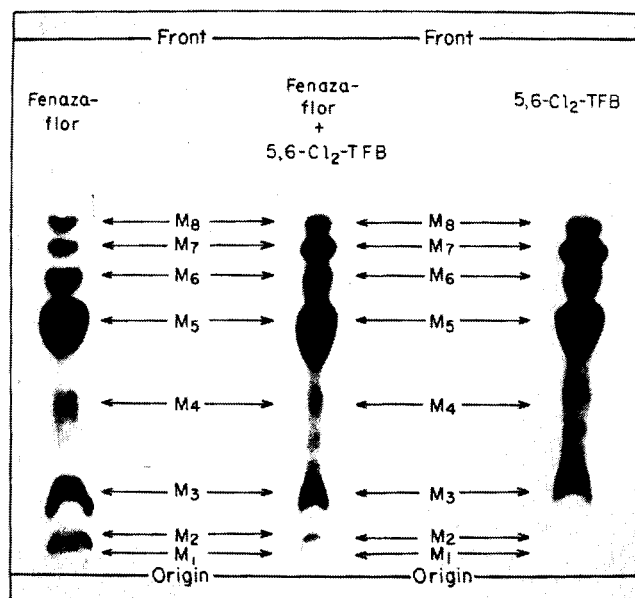

Figure 1. TLC radioautogram of radioactive metabolites in urine of rats receiving fenazaflor-C^{14} or 5,6-Cl_2-TFB-C^{14} and cochromatography of their metabolites

TLC system B

The justification for assigning the same identities to products from fenazaflor-C^{14} and 5,6-Cl_2-TFB-C^{14} in both mice and rats is based not only on their respective R_f values but also on cochromatography studies with the various possible combinations of metabolites from the two species (Figure 1). Although studies on individual metabolites were made with the products from each of mice and rats and from each of fenazaflor-C^{14} and 5,6-Cl_2-TFB-C^{14}, the products are considered irrespective of their source because the evidence for their identity is the same in each case.

Metabolites M_1 and M_2, which are poorly resolved in TLC system B, are hydrolyzed as a mixture in acid to a material(s) with a R_f value, in TLC system D, intermediate between the origin and that of 5,6-Cl_2-TFB. Methylation of the hydrolyzate yields two products cochromatographing, in TLC system A, with the two products (IVa) formed on methylation of 5-Cl-6-OH-TFB, indicating that one or both of M_1 and M_2 are conjugates of 5-Cl-6-OH-TFB (IV). There is limited evidence that both O- and N-conjugates of 5-Cl-6-OH-TFB are present in the M_1 and M_2 region, based on an analytical approach, described below, which was used to study the polar metabolite(s) in apples. Metabolite M_4 is converted, on acid hydrolysis, to a product(s) with a R_f value of 0.66 in TLC system B; this suggests that M_4 is a conjugate, but the nature of the TFB moiety and of the conjugating moiety is not known.

M_3, the predominant metabolite in mouse urine but not in rat urine, cochromatographs with N-glr-5,6-Cl_2-TFB (XIII) in TLC system B, is converted by methylation to a derivative which cochromatographs with N-mglr-5,6-Cl_2-TFB (XIIIa) in TLC system B, and is hydrolyzed in acid to 5,6-Cl_2-TFB (IX) as determined with TLC systems B and E, in two dimensions, along with a minor product having R_f = 0.00 in TLC system B. Methylation of the acid hydrolyzate yields a single product, which cochromatographs with N-Me-5,6-Cl_2-TFB (XI) in TLC system A. Potassium metaperiodate converts product M_3 to a derivative having R_f = 0.66 in TLC system B. These studies establish the identity of product M_3 as N-glr-5,6-Cl_2-TFB (XIII).

Product M_5, the predominant metabolite in rat urine but not in mouse urine, is a single spot in TLC system B, and yields a single spot on hydrolysis (R_f = 0.00, TLC system A; R_f = 0.66, TLC system B; R_f = 0.49, TLC system D); these properties suggest that M_5 is a conjugate(s) of a Cl-OH-TFB

derivative but not a dechlorinated product without a hydroxy group. On methylation of the hydrolyzed material, five products are obtained (R_f = 0.25, 0.33, 0.39, 0.42, and 0.49, TLC system A), establishing that M$_5$ is a mixture of conjugates of at least three different Cl-OH-TFB derivatives. Critical intercomparisons of R_f values in TLC system A established the following identities for the methylated products: IVa = N-Me-5-Cl-6-OMe-TFB (R_f = 0.25, 0.33); XIVc = N-Me-4-OMe-5,6-Cl$_2$-TFB (R_f = 0.42, 0.49); XVIa = N-Me-4,7-OMe$_2$-5,6-Cl$_2$-TFB (R_f = 0.39). Thus, three Cl-OH-TFB derivatives appear as conjugates in M$_5$: 5-Cl-6-OH-TFB (IV), 4-OH-5,6-Cl$_2$-TFB (XIV), and 4,7-OH$_2$-5,6-Cl$_2$-TFB. The site of conjugation and the conjugating moiety are not known.

The R_f values for products M$_6$ and M$_7$, in TLC system B, are identical to those for N-gls-5,6-Cl$_2$-TFB (XII) and N-rib-5,6-Cl$_2$-TFB (IXa), respectively; in addition, product M$_7$ is converted, by metaperiodate, to a product having R_f 0.66 in TLC system B. Acid hydrolysis converts products M$_6$ and M$_7$ to derivatives with R_f values, in TLC system D, intermediate between the origin and that for 5,6-Cl$_2$-TFB, suggesting that the TFB moieties of the conjugates are hydroxy derivatives of 5,6-Cl$_2$-TFB. It is possible that M$_6$ is a riboside(s) and M$_7$ is a glucoside(s) of a Cl-OH-TFB derivative(s) because TLC system B differentiates the type of conjugate but not the presence of hydroxy groups in TFB derivatives. The major component of M$_8$ cochromatographs, in two dimensions using TLC systems B and F, with 5,6-Cl$_2$-TFB (IX) and, after methylation, the derivative cochromatographs with N-Me-5,6-Cl$_2$-TFB (XI) in TLC system A. Thus, the major component of product M$_8$ is 5,6-Cl$_2$-TFB. Product M$_8$ also contains two other compounds remaining at or near the origin in TLC system A, a chromatographic behavior which suggests that they are hydroxy derivatives of 5,6-Cl$_2$-TFB.

Ether extraction of feces, obtained during a 72-hour period following administration of 5,6-Cl$_2$-TFB-C^{14} to rats, results in recovery of a material which chromatographs, using TLC system B (both before and after methylation of the extract), as a single product having the same R_f value as that of 5,6-Cl$_2$-TFB-C^{14}, before methylation, and of N-Me-5,6-Cl$_2$-TFB-C^{14} (XI), after methylation. This characterization of the product excreted in feces as 5,6-Cl$_2$-TFB is tentative only, because it is possible that interfering material might not have been adequately removed prior to chromatographic analysis.

Identity of Metabolites Excreted from Rats and Mice Receiving 4,5-Cl$_2$-TFB, 4,5,6-Cl$_3$-TFB, and 4,5,6,7-Cl$_4$-TFB, Orally. 4,5-Cl$_2$-TFB-C^{14} gives four products (M$_9$, M$_{10}$, M$_{11}$, and M$_{12}$, as resolved in TLC system B) in rat urine, with M$_{10}$ clearly predominating (Table VIII). Product M$_9$ cochromatographs in TLC system B with N-glr-4,5-Cl$_2$-TFB

(VI), before methylation, but is of lower R_f value than N-mglr-4,5-Cl$_2$-TFB (VIa), after methylation. Cleavage of M$_9$ with acid yields a single product with a R_f value between the origin and that of 4,5-Cl$_2$-TFB in TLC system D and, on methylation, the two derivatives cochromatograph, in TLC system A, with the isomers of N-Me-4,5-Cl$_2$-6-OMe-TFB (VIIc), indicating that M$_9$ is a conjugate(s) of 4,5-Cl$_2$-6-OH-TFB (VII). Metabolite M$_{11}$ also is a conjugate(s) of 4,5-Cl$_2$-6-OH-TFB (VII), because the products obtained on hydrolysis and methylation are the same as those indicated for M$_9$. On the same basis, M$_{12}$ is converted on methylation to the isomers of N-Me-4,5-Cl$_2$-6-OMe-TFB (VIIc) and so this metabolite probably is 4,5-Cl$_2$-6-OH-TFB (VII) in an unconjugated form.

The major metabolite, M$_{10}$, for 4,5-Cl$_2$-TFB is converted by acid hydrolysis to a new compound having R_f = 0.00, TLC system A, R_f = 0.66, TLC system B, and R_f = approximately 0.35, TLC system D. These properties establish that metabolite M$_{10}$ is a conjugate and that it is not a conjugate of 4,5-Cl$_2$-TFB, being a Cl-OH-TFB derivative(s), instead. Methylation of the hydrolysis product gives two major derivatives, cochromatographing, in TLC system A, with the two isomers of N-Me-4,5-Cl$_2$-6-OMe-TFB (VIIc) but not with the two isomers of N-Me-4,5-Cl$_2$-7-OMe-TFB (VIIIc); thus, the major portion of this metabolite has a hydroxy group in the 6- but not in the 7-position. Direct comparison of the major portion of the hydrolyzed metabolite with authentic, unlabeled 4,5-Cl$_2$-6-OH-TFB (VII) reveals that both have identical R_f values when subjected to the following sequential reactions and the following TLC systems: acetylation to 4,5-Cl$_2$-6-Ac-TFB (VIIb), TLC system C; methylation to N-Me-4,5-Cl$_2$-6-Ac-TFB (VIId), TLC system C; acid hydrolysis to N-Me-4,5-Cl$_2$-6-OH-TFB (VIIa), TLC system C; methylation to N-Me-4,5-Cl$_2$-6-OMe-TFB (VIIc), TLC system A. Thus, the TFB moiety of the major portion of metabolite M$_{10}$ is 4,5-Cl$_2$-6-OH-TFB (VII). The site of conjugation and the nature of the conjugating moiety are not known.

Minor products (accounting for approximately 10% of the total) also appear in the metabolite M$_{10}$ region, but they are not evident until the product is hydrolyzed, methylated, and chromatographed in TLC system A. This procedure gives five derivatives (R_f = 0.27, 0.33, 0.35, 0.38, and 0.45), the major two of which (R_f = 0.27 and 0.35) result from methylation of 4,5-Cl$_2$-6-OH-TFB. The remaining derivatives are consistent with the assumption that methylation also yields N-Me-4,5-Cl$_2$-7-OMe-TFB (VIIIc) (R_f = 0.36 and 0.45) and N-Me-4,6-Cl$_2$-5-OMe-TFB (XVIIIa) (R_f = 0.33 and 0.38). A less likely alternative for the R_f = 0.35 and 0.45 derivatives is that they are the isomers of N-Me-4-OMe-5-Cl-TFB (IIIa) formed on methylation of 4-OH-5-Cl-TFB (III). The simplest

interpretation of this complex picture is that product M_{10} contains conjugates of 4,5-Cl_2-7-OH-TFB and 4,6-Cl_2-5-OH-TFB, in addition to the major conjugates of 4,5-Cl_2-6-OH-TFB. The nature of the conjugating moieties is not known.

Very little information is available on the nature of the products in urine following oral administration of 4,5,6-Cl_3-TFB-C^{14} and 4,5,6,7-Cl_4-TFB-C^{14} to mice. 4,5,6-Cl_3-TFB gives four products having R_f values of 0.16, 0.55, 0.60, and 0.66 in TLC system B, the one having R_f = 0.55 predominating, whereas 4,5,6,7-Cl_4-TFB gives three products having R_f values = 0.16, 0.55, and 0.66 in TLC system B, the one having R_f = 0.55 predominating.

Housefly Metabolism of 4,5-Cl_2-TFB and 5,6-Cl_2-TFB. When 4,5-Cl_2-TFB-C^{14} and 5,6-Cl_2-TFB-C^{14} are injected into houseflies, each is excreted as metabolites to the extent of 20 to 30% within 24 hours. With each compound, two metabolites (R_f = 0.02 and 0.55, TLC system B) are present in the excreta, the compound of lower R_f value being predominant. The ether extract contains only products having R_f = 0.55, while the products with R_f = 0.02 and a trace amount of those with R_f = 0.55 appear in the aqueous phase. An extremely small amount of the original 4,5-Cl_2-TFB or 5,6-Cl_2-TFB is excreted without metabolism or is present as a result of leakage from the injected flies. Acid hydrolysis converts each of the 5,6-Cl_2-TFB metabolites to 5,6-Cl_2-TFB, as determined with TLC systems A and B, before methylation, and with TLC system A, after methylation. On a similar basis, acid hydrolysis converts each of the 4,5-Cl_2-TFB metabolites to 4,5-Cl_2-TFB, based on the use of TLC system B for the metabolite having R_f = 0.55, without methylation, and of TLC system A for the metabolite with R_f = 0.02 after methylation. Thus, each metabolite is a N-conjugate of the administered benzimidazole. Metaperiodate converts the metabolites having R_f = 0.55, TLC system B, to products with R_f values identical to the free TFB derivatives in this solvent system, in the manner anticipated for N-glucosides, N-glucuronides, and N-ribosides. The metabolite with R_f = 0.55 of 5,6-Cl_2-TFB cochromatographs, in TLC system B, with N-gls-5,6-Cl_2-TFB (XII) but not with N-glr-5,6-Cl_2-TFB (XIII) or with N-rib-5,6-Cl_2-TFB (IXa). Although an authentic sample of N-gls-4,5-Cl_2-TFB was not available, the metabolite of 4,5-Cl_2-TFB with R_f = 0.55 was found to cochromatograph, in TLC system B, with N-gls-5,6-Cl_2-TFB (XII); the solvent in TLC system B does not differentiate on the basis of the position of chlorination. These findings, for the metabolites with R_f = 0.55, establish that N-gls-5,6-Cl_2-TFB is excreted following injection of 5,6-Cl_2-TFB and indicate that the N-glucoside also is formed from 4,5-Cl_2-TFB. The more polar N-conjugate (R_f = 0.02) of 5,6-Cl_2-TFB is not the N-glucuronide (XIII) or N-riboside (IXa) and that from 4,5-Cl_2-TFB is not the N-glucuronide (VI). It is possible that the metabolites with R_f = 0.02 are the N-glucoside-6-phosphate derivatives of 4,5-Cl_2-TFB and 5,6-Cl_2-TFB, based on analogy with the studies of Heenan and Smith (1967) which established that glucoside-6-phosphate conjugates accompany glucoside conjugates in the excreta of houseflies treated with phenols.

Plant Metabolism of Fenazaflor-C^{14} and 5,6-Cl_2-TFB-C^{14}. When individually applied to bean foliage, fenazaflor-C^{14} is more persistent than 5,6-Cl_2-TFB-C^{14}. The recoveries of administered radiocarbon, on washing the leaves with acetone, are: fenazaflor-C^{14}, 0 days = 98%, 1 day = 95%, 2 days = 72%, 3 days = 65%, 13 days = 49%; 4,5-Cl_2-TFB-C^{14}, 0 day = 94%, 1 day = 40%, 2 days = 29%, 7 days = 6%. The material recovered 13 days after fenazaflor-C^{14} application (49% of original dose) consists essentially of 99% pure

fenazaflor-C^{14} or 5,6-Cl_2-TFB-C^{14}, as the case may be, based on chromatography, in sequence, with TLC system A immediately after spotting, TLC system A after time for fenazaflor-C^{14} cleavage on the plate, and TLC system E. In addition, the use of TLC system E reveals a trace of material having the R_f value of 5-Cl-TFB (II). It is not known whether this dechlorination product is an impurity in the original labeled material, as a phenoxycarbonyl derivative, or results from photodecomposition.

The radiocarbon from each of fenazaflor-C^{14} and 5,6-Cl_2-TFB-C^{14} is translocated into both the veins and the intraveinal areas of bean foliage within 6 days after injection into the stem, as revealed by radioautography of the treated plants. Acetone extraction of the plants, immediately after injection of fenazaflor-C^{14} or 5,6-Cl_2-TFB-C^{14}, gives almost quantitative radiocarbon recovery in the acetone and chromatography by TLC system A reveals no degradation products, with the exception of some breakdown of fenazaflor to 5,6-Cl_2-TFB during chromatography. Six days after treatment the extract contains only 5,6-Cl_2-TFB (as such or resulting from fenazaflor breakdown in the plants or on the plate) based on TLC system A. Twenty-eight days after injection, the recovery values and their standard deviations relative to administered radiocarbon are as follows: fenazaflor-C^{14}, acetone-soluble 14.8 ± 5.1%, acetone-insoluble residue 5.4 ± 2.7%, total 20.2 ± 7.2%; 5,6-Cl_2-TFB-C^{14}, acetone-soluble 26.5 ± 1.0%, acetone-insoluble residue 8.8 = 4.7%, total 35.3 ± 4.5%. More than 99% of the labeled material, in the acetone-soluble fraction from both series of plants at 28 days, is 5,6-Cl_2-TFB as determined with TLC system A, both before and after methylation. A minor product is also present which, after methylation, does not move from the origin in TLC system A. Based on a separate study, the described extraction procedure is known to give complete recovery of N-gls-5,6-Cl_2-TFB (XII), without decomposition, when this material is added to bean plants immediately before extraction. These findings establish that 5,6-Cl_2-TFB persists in the bean plants in an unconjugated form. The nature of the labeled materials present in the acetone-insoluble residue is not known.

The results of the studies on fenazaflor-C^{14} breakdown on apple fruit and foliage are given in Table IX. Washing of the fruit and leaves with benzene, immediately after treatment, results in almost quantitative recovery of fenazaflor-C^{14}, using TLC systems A and B, in sequence. Twenty or 30 days after treatment with fenazaflor-C^{14}, apple fruit and leaves contain the same four products, as resolved in TLC systems A and B; they are, based on the identification procedure given below: fenazaflor, 5,6-Cl_2-TFB, an unknown, and N-gls-4-OH-5,6-Cl_2-TFB combined with an equal amount of 4-O-gls-5,6-Cl_2-TFB. Fenazaflor chromatographs in TLC system A with the anticipated R_f value, and hydrolyzes on standing on the plates used in TLC-system A to 5,6-Cl_2-TFB, and the hydrolysis product methylates to give a product with the R_f value on N-Me-5,6-Cl_2-TFB (XI) in TLC system A. Identification of 5,6-Cl_2-TFB in the surface and absorbed material is based on chromatography before and after preparing the N-methyl derivative (see above). The unknown, of R_f = 0.00 in TLC system A and of R_f = 0.33 in TLC system B, remains unidentified.

An elaborate procedure is required for tentative characterization of the polar fenazaflor metabolite in apple fruit and foliage. Metaperiodate treatment as well as acid hydrolysis converts the polar metabolite to a product with R_f = 0.66 in TLC system B; this evidence suggests that the polar metabolite is a conjugate of 5,6-Cl_2-TFB or of a Cl-OH-TFB derivative. Chromatography with TLC system B fails to

Sample	Application Site	Application Dosage, p.p.m.	Interval, Days, after Application	Surface Fenazaflor	Surface 5,6-Cl$_2$-TFB	Surface Unknown	Surface Conj. of 4-OH-5,6-Cl$_2$-TFB[a]	Interior 5,6-Cl$_2$-TFB[b]	Interior Conj. of 4-OH-5,6-Cl$_2$-TFB[a]	Interior Unextractable
Apples	Apples	10	0	8.90	0.60	0.00	0.00	—	—	—
Apples	Apples	10	20	2.26		0.23	0.00	0.43	1.50	1.14
Apples	Apples	10	30	1.39	0.32	0.19	0.17	0.34	1.94	0.73
Apples[c]	Leaves	250	30	<0.001				<0.025		< 0.004
Leaves	Leaves	250	0	197.7	19.6	0.0	0.0	—	—	—
Leaves	Leaves	250	30	22.1	5.3	1.6	0.0	11.1	19.2	16.8

[a] Consists of both N- and O-conjugate, each possibly as β-D-glucoside.
[b] Contains small amount of fenazaflor, but method of analysis did not allow differentiation from 5,6-Cl$_2$-TFB.
[c] Associated with, but protected from, leaves on same branch treated with fenazaflor-C[14].

differentiate the polar metabolite from N-gls-5,6-Cl$_2$-TFB (XII), each having a R_f value = 0.55, but the R_f value is lower for the polar metabolite than for N-gls-5,6-Cl$_2$-TFB in ethyl acetate (R_f values being 0.07 and 0.19, respectively) and in ethyl acetate–methanol mixture (10-to-1) (R_f values being 0.44 and 0.57, respectively). The acid-hydrolyzed product remains at the origin in TLC system A, suggesting that the ring is hyroxylated. Methylation converts the acid-cleavage product to two materials (R_f = 0.42 and 0.49, TLC system A). the R_f of the lower spot approximating that of N-Me-5,6-Cl$_2$-TFB (XI) and both spots cochromatographing with the two isomers of N-Me-4-OMe-5,6-Cl$_2$-TFB (XIVc). These findings tentatively identify the TFB moiety of the conjugate as 4-OH-5,6-Cl$_2$-TFB (XIV) but do not establish the site of conjugation or identify the conjugating moiety. Methylation of the conjugate does not shift the R_f value from 0.00, in TLC system A, but hydrolysis of the methylated conjugate gives two major and one minor product (R_f = 0.06, 0.15, and 0.10, respectively, TLC system A). One methyl derivative released on acid hydrolysis cochromatographs, in TLC system A, with 4-OMe-5,6-Cl$_2$-TFB (XV) (R_f = 0.15) and the other major derivative with N-Me-4-OH-5,6-Cl$_2$-TFB (XIVa) (R_f = 0.06); it is possible that the minor R_f = 0.10 derivative is one of the isomers of N-Me-4-OH-5,6-Cl$_2$-TFB which was not detected in the preparation of an unlabeled sample of this material. After first introducing a methyl group in the conjugate form, additional methylation of the hydrolysis product results in derivatives which cochromatograph, in TLC system A, with the isomers of N-Me-4-OMe-5,6-Cl$_2$-TFB (XIVc). Methylation of the conjugate occurs at both the O- and the N-position, indicating that a mixture of conjugates is involved: The N-conjugate is methylated to the 4-methoxy derivative and cleaved to 4-OMe-5,6-Cl$_2$-TFB, while the O-conjugate is methylated at the N-position and cleaved to N-Me-4-OH-5,6-Cl$_2$-TFB. Thus, the polar metabolite is a mixture of the N-conjugate of 4-OH-5,6-Cl$_2$-TFB and the O-conjugate of 4-OH-5,6-Cl$_2$-TFB, each possibly as the β-D-glucoside derivative.

Some information is available on the distribution of fenazaflor metabolites after treatment of apple leaves and foliage (Table IX). Treatment of apple leaves does not result in translocation of labeled compounds into nearby fruit. The surface material, removed by benzene washing, consists of fenazaflor, 5,6-Cl$_2$-TFB, the unknown, and, in the apple fruit after 30 days only, the conjugates of 4-OH-5,6-Cl$_2$-TFB. The internal material, in leaves and fruit, consists of only the conjugates in the aqueous fraction, whereas both the conjugates and 5,6-Cl$_2$-TFB (or fenazaflor hydrolyzed to this compound during workup) appear in the ether fraction. Some of the material inside the apples and leaves appears in a form not extracted from the residue by the procedure used.

DISCUSSION

Tentative metabolic pathways in various organisms for fenazaflor and 5,6-Cl$_2$-TFB are given in Figure 2 and for 4,5-Cl$_2$-TFB in Figure 3.

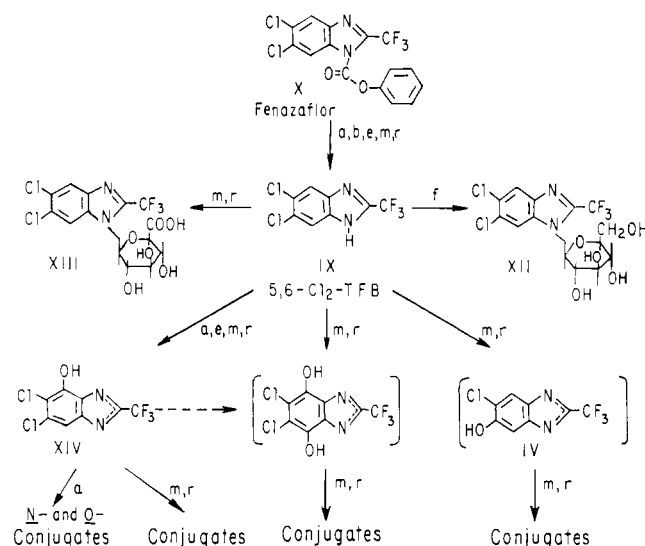
Figure 2. Tentative metabolic pathways for fenazaflor and 5,6-Cl$_2$-TFB

a. Apples
b. Beans
e. Enzymatic in vitro systems
f. Houseflies
m. Mice
r. Rats

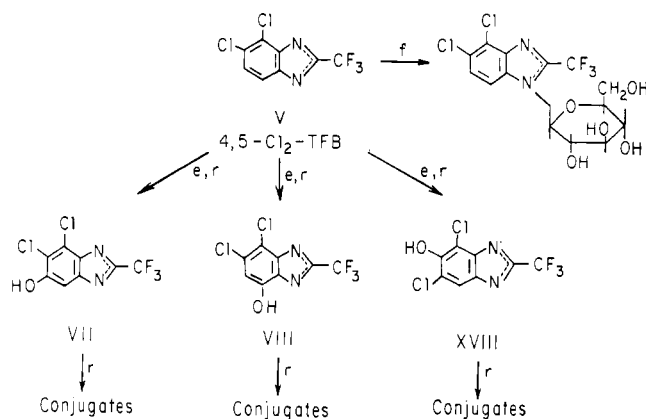Roman numerals refer to compound designations in text and Table I

Figure 3. Tentative metabolic pathways for 4,5-Cl$_2$-TFB
e. Enzymatic in vitro systems
f. Houseflies
r. Rats
Roman numerals refer to compound designations in text and Table I

Fenazaflor is hydrolyzed readily to 5,6-Cl$_2$-TFB under each biological situation investigated. Enzymes or other constituents in mouse liver and mouse plasma catalyze this conversion, but the catalytic effect is not highly sensitive to inhibition by carbaryl and dichlorvos. [The present studies did not consider the metabolic fate of phenol released on cleavage of fenazaflor, because this material and its further metabolites are normal constituents of human urine and enter known metabolic pathways in insects and plants (Parke, 1968; Williams, 1959).] The further reactions of 5,6-Cl$_2$-TFB result in metabolites which, for the most part, are conjugates and not free benzimidazoles. These conjugates include the N-glucuronide, and products chromatographing in the manner anticipated for glucoside and riboside conjugates. The benzene moiety of 5,6-Cl$_2$-TFB is hydroxylated to yield three different hydroxy derivatives and there are indications that the liver microsome–NADPH system is responsible for the hydroxylation. The hydroxybenzimidazoles do not persist as such but rather are conjugated, based on the ones studied. In mammals and plants, the metabolism of fenazaflor involves similar pathways, of hydrolysis, hydroxylation of the benzene moiety, and conjugation.

4,5-Dichloro-2-trifluoromethylbenzimidazole is metabolized, in rats and houseflies, by pathways similar to those involved in the metabolism of the 5,6-isomer and, as with 5,6-Cl$_2$-TFB, the liver microsome–NADPH system is probably responsible for the hydroxylation reactions.

The metabolites tentatively appear to have structures consistent with previous studies on detoxication mechanisms. There is much previous indication of the formation of N-glucosides in plants (Casida and Lykken, 1969) but not in insects or, particularly, in mammals. N-Riboside formation is not inconsistent with previous studies, because histidine converts to the N-riboside of 4-imidazolylacetic acid (Schayer, 1956; Williams, 1959), and the purine bases, which have some structural similarity to the benzimidazoles, convert to ribonucleic acids via N-riboside intermediates. There is some precedent, with related compounds, for suggesting (Kapoor and Waygood, 1965a; Williams, 1959) but no evidence for concluding that the N-ribosides of the trifluoromethylbenzimidazoles serve as intermediates for incorporation of the benzimidazole moiety into tissue constituents. There is precedent for glucoside-6-phosphate conjugation of phenols in houseflies (Heenan and Smith, 1967) but these are O- rather than N-conjugates. The sites of hydroxylation on the benzene moiety are not unusual and there is precedent for both the hydroxyl replacement of chlorine and the hydroxylation-induced migration of chlorine (Guroff et al., 1967).

Conjugation appears to occur slowly in beans and apples, but fast in houseflies; it is possible that this phenomenon is related to the low toxicity of certain benzimidazoles to houseflies (Ilivicky and Casida, 1969) and to their high herbicidal activity (Burton et al., 1965) because the biological activity, in both cases, is probably dependent on the free N-H grouping. Trifluoromethylbenzimidazoles readily bind to plasma albumin (Beechey, 1966; Ilivicky et al., 1967; Ilivicky and Casida, 1969; Weinbach and Garbus, 1966) and probably to other proteins as well; such binding possibly reduces the ease of detoxicating these compounds in the body and minimizes their rate of excretion.

The TFB ring structure is not degraded in any of the metabolizing reactions studied. The resistance of the TFB ring to metabolic decomposition possibly is conferred by the tri-

fluoromethyl group because the imidazole ring of related natural products undergoes metabolic fission or modification (Williams, 1959). Certain products persist in the body with each of the di-, tri-, and tetrachloro-2-trifluoromethylbenzimidazoles studied, but little is known about their identity or the products excreted in the feces. Data given in this paper lay the background for, but do not provide a complete knowledge of, the metabolic fate of trifluoromethylbenzimidazoles in mammals, insects, and plants.

LITERATURE CITED

Abdel-Wahab, A. M., Kuhr, R. J., Casida, J. E., J. AGR. FOOD CHEM. 14, 290 (1966).
Beechey, R. B., Biochem. J. 98, 284 (1966).
Buchanan, J. G., Dekker, C. A., Long, A. G., J. Chem. Soc. 1950, 3162.
Buchel, K. H., Draber, W., Trebst, A., Pistorius, E., Z. Naturforsch. 21b, 243 (1966).
Buchel, K. H., Korte, F., Beechey, R. B., Angew. Chem. Intern. Ed. 4, 788 (1965a).
Buchel, K. H., Korte, F., Trebst, A., Pistorius, E., Angew. Chem. Intern. Ed. 4, 789 (1965b).
Burton, D. E., Lambie, A. J., Ludgate, J. C. L., Newbold, G. T., Percival, A., Saggers, D. T., Nature 208, 1166 (1965).
Burton, D. E., Lambie, A. J., Newbold, G. T., Percival, A., Senciall, I. R., Brit. Patent 1,104,936 (March 6, 1968).
Casida, J. E., Lykken, L., Ann. Rev. Plant Physiol., in press (1969).
DeBoer, T. J., Backer, H. J., Rec. Trav. Chim. Pays-Bas 73, 229 (1954).
Flockhart, I. R., Smith, R. L., Williams, R. T., Biochem. J. 110, (3), 32P (1968).
Forsyth, W. G., Pyman, F. L., J. Chem. Soc. 127, 573 (1925).
Guroff, G., Daly, J. W., Jerina, D. M., Renson, J., Witkop, B., Udenfriend, S., Science 157, 1524 (1967).
Heenan, M. P., Smith, J. N., Life Sciences 6, 1753 (1967).
Hofmann, K., "Imidazole and Its Derivatives," pp. 247–324, Interscience, New York, 1953.
Ilivicky, J., Casida, J. E., Biochem. Pharmacol., in press (1969).
Ilivicky, J., Chefurka, W., Casida, J. E., J. Econ. Entomol. 60, 1404 (1967).
Jeffay, H., Alvarez, J., Anal. Chem. 33, 612 (1961).
Jones, O. T. G., Watson, W. A., Biochem. J. 102, 564 (1967).
Jones, O. T. G., Watson, W. A., Nature 208, 1169 (1965).
Kapoor, M., Waygood, E. R., Can. J. Biochem. 43, 153 (1965a).
Kapoor, M., Waygood, E. R., Can. J. Biochem. 43, 165 (1965b).
Karjala, S. A., Turnquest, B., 3rd, Schayer, R. W., J. Biol. Chem. 219, 9 (1956).
Krishna, J. G., Casida, J. E., J. AGR. FOOD CHEM. 14, 98 (1966).
Malaprade, M. L., Bull. Soc. Chim. France (iv) 43, 683 (1928).
Oonnithan, E. S., Casida, J. E., J. AGR. FOOD CHEM. 16, 28 (1968).
Parke, D. V., "Biochemistry of Foreign Compounds," pp. 146–8, Pergamon Press, New York, 1968.
Saggers, D. T., Clark, M. L., Nature 215, 275 (1967).
Schayer, R. W., Brit. J. Pharmacol. 11, 472 (1956).
Weinbach, E. C., Garbus, J. J. Biol. Chem. 241, 3708 (1966).
Williams, R. T., "Detoxication Mechanisms," pp. 133–5, 278–9, 549, 577–8, Wiley, New York, 1959.
Williamson, R. L., Metcalf, R. L., Science 158, 1694 (1967).
Wilson, D. F., Chance, B., Biochem. Biophys. Res. Comm. 23, 751 (1966).
Wilson, D. F., Merz, R. D., Arch. Biochem. Biophys. 119, 470 (1967).
Yung, N., Fox, J. J., Methods Carbohydrate Chem. 2, 108 (1963).