



UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

APR 18 1989

OFFICE OF
PESTICIDES AND TOXIC SUBSTANCES

MEMORANDUM

SUBJECT: ASANA (Esfenvalerate) - Minutes of Meeting of
January 13, 1989 - EPA Registration Nos. 352-496
and 352-515

TOX Chem No.: ~~ATA~~ 268 J
Project No.: 9-0801A
Record Nos.: 239684,
239685

FROM: William B. Greear, M.P.H. *William B. Greear 4/10/89*
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TO: Adam Heyward/George LaRocca, PM Team 15
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THRU: Marion P. Copley, D.V.M., Section Head *Marion Copley 4/18/89*
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and

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Toxicology Branch I - Insecticide, Rodenticide Support
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Under the cover letter dated February 1, 1989, D.A. Wustman of Dupont has submitted the minutes of a meeting generated from a meeting that was held on January 13, 1989 to discuss miscellaneous registration issues.

A correction should be noted on page 2 of the minutes of the meeting. The OPP RfD Committee has not yet convened to discuss whether the ADI of 0.045 mg/kg/day, derived from the 20-month mouse oncogenicity study, should be divided by a factor of 4. The reason 4 may be used is because the mouse study was conducted with the racemic technical which contains 4 isomers in equivalent proportions, of which only the **SS**-isomer is insecticidally active; however, the new technical is almost exclusively composed of the **SS**-isomer. A decision on whether the factor 4 should be used to decrease the ADI will be held in abeyance pending the submission of a number of reprints on the activity and relationship of the 4 isomers of fenvalents.

MEMORANDUM OF DISCUSSION

Time: 10:00-12:00 noon, January 13, 1989

Place: EPA Offices, Arlington. VA

Present: From EPA Adam Heyward Registration
J. W. Hauswirth Toxicology
J. H. Olney Dietary Exposure
H. Fonouni Dietary Exposure
S. L. Stanton Dietary Exposure

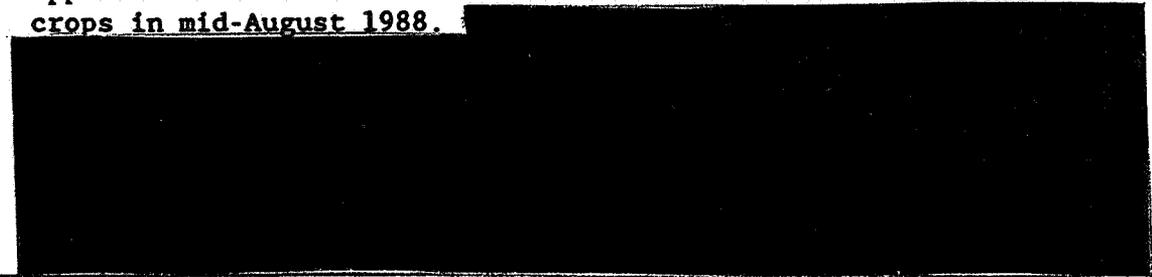
From DuPont N. L. Redfearn Registration
D. A. Wustner Registration
P. W. Lee Environmental Studies

A general introduction was presented to acquaint all participants with the planned agenda and the objectives of the meeting (DAW). As previously committed, DuPont is now in a position to replace DuPont PYDRIN 2.4 EC (containing fenvalerate) for all uses in food crops with the single isomer product, DuPont ASANA XL 0.66 EC (containing esfenvalerate). To do this, ASANA XL must have a use label that includes all of the present PYDRIN 2.4 EC uses. With PYDRIN out of food use, DuPont wished to address the pending uses for fenvalerate and new uses for (es)fenvalerate, the magnitude of the tolerances, and the tolerance assessment. The visuals used are included as attachment I.

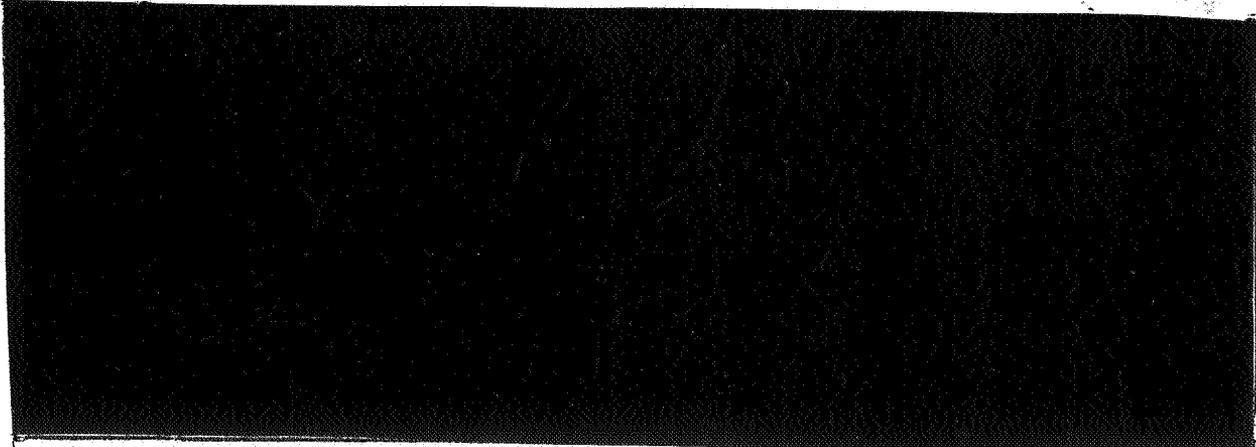
Subsequent discussions mainly addressed the two major concerns of the Agency--(1) the nature and magnitude of the residue from ASANA versus PYDRIN on crops, and secondary residues in livestock and milk from ASANA uses; and (2) the determination of an appropriate ADI for ASANA from the current chronic toxicology data base. The various issues that were discussed will be treated separately in this memorandum.

Issue: DuPont ASANA XL 0.66 EC Use Label.

ASANA XL is presently labeled for use on a subset of PYDRIN labeled crops. Before DuPont will remove Pydrin from agricultural use, all of the present PYDRIN uses must be transferred to the ASANA label--the "parity" label. DuPont applied for a label amendment to ASANA for all the additional crops in mid-August 1988.



COMMERCIAL/FINANCIAL INFORMATION IS NOT INCLUDED



Cancellation of PYDRIN 2.4 EC will have two other beneficial effects. First the reduced use rates, by elimination of the insecticidally inactive isomers, reduces environmental contamination. Second, It will not be necessary to reregister food uses of PYDRIN under the new amended FIFRA minimizing cost to both the Agency, and DuPont.

Issue: Acceptable Daily Intake.

The OPP ADI committee met on November 15, 1988 to consider establishing a new ADI for fenvalerate of 0.05 mg/kg/day based on a NOEL of 200 ppm (5.3 mg/kg/day) for esfenvalerate in a recently completed one-year dog feeding study. Reevaluation of all of the chronic data caused the committee to propose an ADI of 0.045 mg/kg/day based on the NOEL from a mouse oncogenicity study of fenvalerate, with a 100-fold safety factor. The committee then divided this number by 4 in that the technical used was racemic--i.e. composed of the four isomers of fenvalerate while the chronic toxicity of fenvalerate is attributed to the SS-isomer of fenvalerate. Thus, the mice were challenged with racemic fenvalerate, only approximately one-fourth of which was the neuro-toxic SS-isomer.

There are two technical bases supporting the use of the NOEL from the one-year dog study in determining the ADI for esfenvalerate. These are: the definition of the residue, and how it is measured; and the causes of the toxicological effects that determined the NOEL's in the two chronic studies.

The definition of the residue: The dose is compared with the sum of the tolerances--or the anticipated residues--multiplied by appropriate food factors to determine acceptability of dietary exposure. Presently, the toxic residue from either fenvalerate, or esfenvalerate uses, is defined as parent only, and the approved enforcement methods evaluate the sum of all four isomers. The ADI, the tolerances, and the enforcement methods must be chemically consistent. Thus, unless the tolerances are based and enforced on only the single SS-isomer, the ADI should continue to be based on all four isomers. It should be emphasized that the analytical method which is stereospecific to the SS-isomer of fenvalerate--esfenvalerate--using chiral HPLC is

extremely difficult and time-consuming. The method requires equipment that is not readily available in most residue laboratories and is not rugged enough to be used as a routine enforcement method.

To further support the definition of the residue as the sum of the isomers, some isomerization of field residues has been detected. Under weakly basic conditions, the cyanohydrin carbon is easily racemized. All of the data that are available are consistent with a interconversion of the SS- and SR-isomers. This is observed in the present GC method as an increase in the size of the first peak (containing the SR- and RS-diastereomers) and a concomitant reduction of the second peak (containing the SS- and RR- diastereomers). This isomerism is not common nor has it been seen to go to completion, however, it does complicate the definition of an ASANA residue.

Toxicological effects: The low dose effects generally seen in chronic and subchronic studies with (es)fenvalerate are either microgranulomas in liver, spleen and lymph nodes, or neurological effects. The SS-isomer of fenvalerate is a sodium channel agonist. This activity is completely stereospecific to the SS-isomer and is responsible for the activity of (es)fenvalerate on nerve membranes. Microgranuloma formation in mice is also a stereospecific effect, however this effect is only caused by the RS-isomer. Work by Sumitomo scientists in Japan has unequivocally demonstrated that the microgranulomas are a consequence of the accumulation of cholesteryl (2R-2-(4-chlorophenyl)isovalerate --that is the cholesterol ester of the R-isomer of the fenvalerate acid--in the affected tissues. The formation of this ester conjugated by metabolism, and the formation of the microgranulomas, are completely specific to the RS-isomer of fenvalerate. Microgranuloma formation was not observed in a companion study using the resolved SS-isomer of fenvalerate as the test material. An interim report through 26 weeks of this Japanese work can be found in Tab 44 of Volume IV of the original ASANA petition--EPA Accession No. 254115. Copies of two reprints that report the work through 52-weeks are included as Attachments II and III. These studies also provide chronic toxicity data (1-year mouse feeding studies) that directly compare the SS-isomer, the SS- SR-isomer pair, and racemic fenvalerate as test materials.

The technical fenvalerate that was used in the mouse feeding study proposed for use to determine the ADI contained about 25% of the RS-isomer, the isomer that causes microgranuloma formation. In contrast, the ASANA technical typically contains around 5 % of the RS-isomer. Therefore one would calculate that approximately five-times as much ASANA would have to be consumed by the mice to reach the equivalent NOEL with regard to the RS-isomer which cause the microgranulomas. This further suggests an ADI of 0.23mg/kg/day (150 ppm . 0.15 / 100 = 0.23) if extrapolation is done from the mouse NOEL. The fenvalerate mouse study is not appropriate to use in the ASANA tolerance assessment. The low dose effects in the dog feeding study with ASANA are neurological, and microgranuloma formation was not

observed. Thus, the one-year dog feeding study is the most appropriate to address the chronic toxicological end-point for ASANA.

Considering that esfenvalerate has already largely displaced fenvalerate for use in the US, and when fenvalerate (PYDRIN) is out of the channels of trade, all of the subsequent use will be SS-isomer, the one-year dog feeding study using esfenvalerate technical as the test material is the most appropriate study for use in determining the ADI.

Issue: Tolerance Assessment.

It appears that the tolerance assessments performed for DuPont and that performed by the Agency reach similar conclusions. It was noted that different ADI values were used, the reasons for which are discussed above.

Issue: Residue chemistry--General.

From the viewpoint of performance, the active ingredient in PYDRIN is the SS-isomer, i.e. esfenvalerate, the ai in ASANA. All of the studies using racemic fenvalerate that support the registration of PYDRIN also support the registration of ASANA since the test material always contained the active isomer as well as the other three, inactive isomers. At low concentrations, the isomers appear to act, and are acted upon independently. Thus, the SS-isomer alone will perform, degrade, etc. in an identical manner to the SS-isomer in the presence of similar concentrations of the other three isomers. The fate of ASANA will be identical to the fate of the SS-isomer as part of the residues from PYDRIN. Moreover, a number of studies have compared the behavior of isomer preparations to the behavior of the racemic--several of these studies will be discussed below and have previously been submitted to the Agency. These studies all show that different isomer preparations behave no differently than the racemic. Residues from ASANA will be quantitatively less than those from PYDRIN because of the reduced use rate. Qualitatively ASANA residues will be the same.

Issue: Magnitude of the ASANA residues.

The present tolerances for fenvalerate were established based on a PYDRIN use. Given the definition of the composition of the products, and the definition of the residue, the tolerances will be quite large for an exclusive ASANA use. The Agency has very little field residue data comparing PYDRIN to ASANA. DuPont has about 40 additional field studies that report residues from PYDRIN and ASANA side-by-side on a variety of crops. These data will be submitted to the Agency. Because there continues to be some PYDRIN in channels of trade, the tolerances probably cannot be changed for some time but progress towards resolution of this question can be made.

Issue: Chemical Nature of the Residue on Plants.

Several plant metabolism studies have been conducted with racemic fenvalerate to determine the fate of fenvalerate on crop plants, and to define the chemical nature of the residue. These studies have been summarized in "Residue Chemistry and Metabolism of SD43775 (Shell Summary, 1985)", MRID No. 151250, and are also summarized in the table below. In short, little absorption or translocation of the ¹⁴C-fenvalerate was evident. In addition to undegraded fenvalerate (ca. 80 % of extractable), the decarboxylated fenvalerate (SD54597, a photoproduct) was the major degradation product detected on plant surfaces. There were several minor products (from the acid and alcohol moieties) resulting from the cleavage of the parent ester; however, all were present in very small amounts--less than 5 % of the applied radioactivity.

Test plant	Wheat(1)		Cotton(2)		Tomato(3)	
Test location	Outdoor (CA)		Outdoor (CA)		Outdoor (CA)	
Time (weeks)	10		4		7	
14-C Position	Acid	Alcohol	Acid	Alcohol	Acid	Alcohol
Solvent Ext.	36	27	53	56	79	84
As SD43775	30	22	35	40	66	66
SD54597	<1	<1	8	7	4	6
Minor	<5	<5	10	9	9	12
Solid Resid.	8	8	2	4	3	4
Percent Appl.	44	35	55	60	82	86

Test plant	Soybean (leaf)(4)		Soybean (pod)(4)		Bean(5)
Test Location	Outdoor (CA)		Outdoor (CA)		Greenhouse
Time (weeks)	5		5		8.6
14-C Position	Acid	Alcohol	Acid	Alcohol	Cyano
Solvent Ext.	47	66	83	79	59
As SD43775	37	55	79	76	43
SD54597	6	6	<1	<1	1
Minor	4	5	4	3	15
Solid Resid.	4	6	1	2	8
Percent Appl.	51	72	84	81	87

(1) RIR-22-001-85, no MRID or Accession No's. Reviewed by RCB in a review dated May 3, 1985 in connection with PP#4F3003/4H5419 and PP#4F3021 transmitted to Shell by letter dated May 10, 1985.

(2) TIR-22-110-76, MRID No. 71632

(3) TIR-22-104-79, MRID No. 35651

(4) TIR-22-111-79, MRID No. 100104

(5) Pesticide Science. 5, 215-23, 1980., MRID No. 141454.

There has been a considerable toxicology, environmental chemistry, residue and secondary residue data base developed on the photoproduct. The results of these studies have convinced the Agency that the photoproduct, SD54597, is not of toxicological concern and is not a necessary part of the tolerance expression.

Issue: Stereoselectivity of Plant Metabolism of Racemic and the SS-Isomer Fenvalerate.

Reference: Mikami, N., N. Wakabayashi, H. Yamada, and J. Miyamoto. 1985. The metabolism of fenvalerate in plants: The conjugation of the acid moiety. *Pesticide Science*. 16, 46-58. Attachment IV.

The paper referenced above directly compares the metabolism of racemic fenvalerate with the SS-isomer on cabbage plants grown and treated under laboratory conditions. The data clearly show that there were no significant qualitative or quantitative differences between the metabolism on cabbage of the racemic or the SS-isomer. The undegraded parent remained the major radioactive component recovered from the leaf surfaces. Since this study was conducted in the greenhouse, the decarboxylated photoproduct was observed as a minor metabolite. Ester cleavage products, free and conjugated, were observed from both the acid and alcohol portions of the ester. The only degradation product to exceed 5 % of the applied radioactivity was the conjugated fenvalerate acid. Differences in the relative magnitude of metabolites in this study relative to those discussed above are most likely attributable to the greenhouse conditions leading to lower rates of photolysis.

Together, the plant metabolism studies with fenvalerate or the SS-isomer demonstrate (1) that the bulk of the residue remaining after a (es)fenvalerate treatment is parent, (2) that the residue remains on the plant surface where applied, (3) that the only significant outdoor degradation product is a photolysis product that is not of toxicological significance, and (4) that other potential degradation products are in amounts less than 5 % of applied. It is important to emphasize that virtually all of the degradation of fenvalerate occurs on the outside of the plant, and that the major pathway outdoors is photolysis. Photolytic reactions, such as the loss of carbon dioxide from fenvalerate, would be expected to be unaffected by optical isomerism, thus, the single SS-isomer will degrade photolytically in an identical manner and rate to the racemic mixture.

Issue: Chemical Nature of the Radioactivity in Fat of Rats.

Four rat metabolism studies have been conducted to demonstrate the overall pharmacokinetic characteristics of racemic fenvalerate and an SS-isomer enriched preparation of fenvalerate SD92459. These four studies have MRID No's of 85749, 99109,

144166, and 144165. The results were consistent and demonstrated that there were no apparent differences in metabolism, or tissue accumulation of racemic fenvalerate, or the SS-isomer enriched fenvalerate (ca. 45 % SS-isomer), with one exception. The reviewer noted that there was a discrepancy in the proportion of parent in the fat of the rats. In earlier rat metabolism studies, undegraded parent accounted for greater than 90 % of the radioactivity in the fat. In these studies, only about 55 % of the fat residues were identified as parent for the racemic, and 15 % in the SS-isomer enriched studies.

Further characterization of the rat body fat was conducted and the results were submitted to the agency on March 15, 1985. These results were reported in MO-RIR-22-005-85 which is included as Attachment V. Also included as Attachment VI is the Agency's response and review of the study (dated August 6, 1985). An EPA MRID or Accession No. is not available. The submission was in connection with EPA File Symbol 201-URI, which became 352-LNE and then DuPont ASANA Insecticide 1.9 EC EPA Registration No. 352-502.

The results of the reanalysis of the rat body fat from the four studies showed approximately 99% of the ¹⁴C-residues in the body fat could be recovered by hexane-acetone (3:1) homogenization and extraction. Greater than 95 % of the organic-extractible residues were characterized as the undegraded parent. Significant levels of water-soluble or tissue-bound and other minor organic-extractible metabolites were not detected. There were no differences evident in the overall residue distribution pattern between the sex of the test animals, label position, or the isomeric composition of the test compound. The differences between original metabolism studies, and these studies questioned by the original EPA reviewer were attributed to incomplete extraction. Upon reviewing the supplementary report, the Agency agreed.

Issue: Chemical Nature of the Secondary Residues in Animal Tissues and Milk From SS-Isomer Residues in Animal Feed.

A summary of the many secondary residue studies that have been submitted for fenvalerate is available as "Summary: Residue of Pydrin Insecticide in Milk, Meat and Eggs." Shell Chemical Company, 1983., MRID No. 132554. There have been three 28-day feeding studies performed on lactating cows using ¹⁴C-fenvalerate. Feeding levels were 79, 10.9, and 0.15 ppm radiolabeled material in the cow diet. The magnitude of the radiocarbon residue was investigated in milk and tissues, and the magnitude of the parent residues were determined in the milk in all three studies. In the 79 ppm study the chemical nature of the residue was extensively investigated in various tissues. The data show that within analytical the error of the determinations, virtually all of the residue in the milk was parent. Material balance indicates that the concentration of metabolite(s) is negligible.

It has been demonstrated in animal and plant studies that, excepting the mode of action of the SS-isomer on nerve membranes, and the chronic effect of the cholesteryl ester metabolite from the RS-isomer, the SS-isomer (ASANA) behaves no differently at residue levels than the racemic (PYDRIN). The SS-isomer was approximately 25 % of the radioactive material used in the ruminant studies briefly reviewed above. There was parent, and no metabolites, found in milk as a result of feeding high levels of the racemic fenvalerate. Therefore it is most likely that there will be parent, and no metabolites found from feeding the SS-isomer of fenvalerate. It should be emphasized that the residue levels on animal feeds will be smaller from the field use of the lower use rate SS-isomer, and consequently, the secondary residues in meat, milk, and eggs will be correspondingly smaller.

ATTACHMENT I

AGENDA

(ES)FENYALERATE-- PYDRIN ASANA

1/13/89

DU PONT PYDRIN 2.4 EC

DU PONT ASANA XL 0.66 EC

NEW USES

TOLERANCE ASSESSMENT

DAW 1/12/89

PYDRIN / ASANA

- PYDRIN--**
- o First registered in 1977
 - o Pyrethroid insecticide--fenvalerate.
 - o Racemic mixture of 4 optical isomers.
 - o Demonstrated early-on that one isomer is active.
 - o Presently registered for use on many crops.
 - o Field use rate in the 0.1-0.2 lb. ai/a range.

- ASANA--**
- o First registered in 1986.
 - o Pyrethroid insecticide--esfenvalerate.
 - o Single active optical isomer of fenvalerate.
 - o Presently registered on a subset of PYDRIN crops.
 - o Field use rate in the 0.03- 0.05 lb. ai/a range.

DAW 1/12/89

OBJECTIVES

- 1. PARITY LABELING FOR ASANA XL SO THAT DU PONT CAN EXIT THE PYDRIN BUSINESS, AND THAT THE FOOD USES OF PYDRIN 2.4 EC CAN BE CANCELED.**
- 2. A FORWARD PLAN TO DEAL WITH THE RESIDUE QUESTIONS, OUTSTANDING PETITIONS, AND NEW USES FOR ASANA XL.**

DAW 1/12/89

Asana XL

Label Parity Amendment

Apply: 8/17/88
No new tolerances

Add:	Sugarcane	Blueberry
	Sunflower	Caneberry
	Stone fruit	Carrots
	Pears	Collards
	Almonds	Okra
	Filberts	Radish
	Ornamentals	Turnip

Revise: Directions for use

DAW 1/12/89

New Uses Applied

<u>CROP</u>	<u>PP No.</u> (FAP No.)	<u>SUBMITTED</u>
Grapes	2F2657 2H5340	3/82
Forage	2F2746	8/82
Sorghum, Alfalfa	4F3003 4H5415	11/83 (alfalfa withdrawn 4/85)
Range, Pasture	4F3004	11/83
Wheat, Barley	4F3021 4H5423	1/84
Celery	4F3023	1/84
Brussels S.	4F3030	1/84
Sugarbeets	4F3120 4H5437	7/84
Spinach	5F3172	11/84
Head Lettuce	2F2599	11/81 (withdrawn 1/85)
Citrus	4F3022 4H5424	1/84 (withdrawn 5/85)

(ES)FENVALERATE

TOLERANCE ASSESSMENT

Analysis performed by Technical Assessment Systems Inc.

- o All residue data used has been submitted to EPA
- o ADI 0.050 mg/kg body wt/day
- o ADI based on chronic dog using esfenvalerat

SUMMARY (U. S. Pop)

Analysis	Total Exposure	
	MG/KG	% ADI
EXISTING TOLERANCES	0.0196	39
PROPOSED TOLERANCES (exist + proposed + livestock)	0.0474	95
ANTICIPATED RESIDUES (as above, fenvalerate data)	0.0058	12
ANTICIPATED RESIDUES (as above, esfenvalerate "data")	0.0026	5

DAW 1/12/89

PROPOSED TOLERANCES VS ANTICIPATED RESIDUES

	TOLERANCE (PPM)	ANTICIPATED RESIDUE	
		PYDRIN	ASANA
SORGHUM	8	1.25	0.55
WHEAT	1	0.22	0.10
BARLEY	5	1.35	0.60
SPINACH	30	17.33	7.63

ANTICIPATED DIETARY INTAKE FOR POULTRY

COMMODITY	ANTIC. RES. IN FEED	(ppm)	
		PROP. DIET	ANTIC, RES.
SORGHUM	1.25	0.60	0.750
BARLEY	1.35	0.50	<u>0.675</u>

MAXIMUM TOTAL ANTICIPATED DIETARY INTAKE --- 1.425

(Using tolerances the maximum would be --7.3)

PYDRIN / ASANA RESIDUE STUDIES

- o Performed from 1983-1986
- o Three have been submitted to the agency as part of the ASANA registration.
- o The ASANA / PYDRIN residue ratio of 0.44 was derived from these three studies.
- o 73 separate locations.
- o 44 were performed with PYDRIN and ASANA in side-by-side experiments.
- o 29 are ASANA alone.
- o 25 crops are represented (14 reg., 6 pend, and 5 other)
- o ASANA / PYDRIN residue ratio is 0.35 using data from above where residues were greater than 0.1 ppm.

DAW 1/12/89

ATTACHMENT II

Differential Metabolism of Fenvalerate and Granuloma Formation

I. Identification of a Cholesterol Ester Derived from a Specific Chiral Isomer of Fenvalerate

HIDEO KANEKO, MASATOSHI MATSUO, AND JUNSHI MIYAMOTO

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Reprinted from TOXICOLOGY AND APPLIED PHARMACOLOGY, Volume 83, No. 1, March 30, 1986
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Received April 24, 1985; accepted October 7, 1985

Differential Metabolism of Fenvalerate and Granuloma Formation. I. Identification of a Cholesterol Ester Derived from a Specific Chiral Isomer of Fenvalerate. KANEKO, H., MATSUO, M., AND MIYAMOTO, J. (1986). *Toxicol. Appl. Pharmacol.* 83, 148-156. On a single po administration of the four chiral isomers of fenvalerate ([*RS*]- α -cyano-3-phenoxybenzyl [*RS*]-2-(4-chlorophenyl)isovalerate) to rats and mice at 2.5 mg/kg, the [*2R, α S*]-isomer showed relatively higher residues in all analyzed tissues as compared with the other three isomers. Similarly, this isomer showed higher tissue concentrations than other isomers when mice were fed a diet containing 500 ppm of the [*2S, α S*]-, [*2R, α S*]-, and [*2R, α R*]-isomers for 2 weeks. The [*2R, α S*]-isomer produced a lipophilic metabolite in all the examined tissues on the basis of thin-layer chromatography analysis, but not for the other isomers. The amounts of lipophilic metabolite differed among tissues, being higher in adrenal, liver, and mesenteric lymph nodes following feeding to mice at 500 ppm of the [*2R, α S*]-isomer for 2 weeks. However, the amount did not increase proportionally with time and apparently reached a plateau within a rather short time. This metabolite was identified as cholesteryl [*2R*]-2-(4-chlorophenyl)isovalerate ([*2R*]-CPIA-cholesterol ester) on the basis of spectroanalysis and chromatographic behavior after purification on silica gel, Florisil, thin-layer, and high-pressure liquid chromatography. The presence of the same metabolite also was indicated in rat tissues. The CPIA-cholesterol ester was rapidly formed and found in all the analyzed tissues of mice 1 hr after a single po administration of the [*2R, α S*]-isomer. © 1986 Academic Press, Inc.

It will be reported in a subsequent paper of this series that fenvalerate, a photostable pyrethroid insecticide ([*R,S*]- α -cyano-3-phenoxybenzyl [*RS*]-2-(4-chlorophenyl)isovalerate; Sumicidin, Pydrin), caused microgranulomatous lesions in adrenal glands, mesenteric lymph nodes, liver, and/or spleen of rats and mice, and that of the four chiral isomers, one specific isomer, the [*2R, α S*]-isomer, produced such histological changes (Okuno *et al.*, 1986). With respect to the metabolic fate of fenvalerate, the racemic mixture and the [*2S, α S*]-isomer, which is insecticidally most active, were extensively studied in rats,

mice, and dogs by using three radioactive preparations labeled with ^{14}C in the acid and alcohol moieties as well as the cyano group (Ohkawa *et al.*, 1979; Kaneko *et al.*, 1981, 1984). The major metabolic reactions were oxidation at the 2- and 3-positions of the acid moiety, and at 2'- and 4'-positions of the alcohol moiety, ester cleavage, and conjugation reaction of the resultant carboxylic acids and phenols with glucuronic acid, sulfuric acid, and amino acids (glycine and taurine), as well as conversion of the cyano group to thiocyanate and CO_2 . No noteworthy retention of the radiocarbon from either acid or alcohol moiety

0041-008X/86 \$3.00

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was observed, but the cyano carbon was retained to a certain extent mainly in skin and hair.

Thus, although it appears that there is no significant difference in general metabolism among the four fenvalerate isomers, a more detailed comparative metabolism study of the four isomers was undertaken in rats and mice with acid-labeled preparations in order to find out possible differences in bioretention of metabolites, which may be associated with the difference in capability of inducing granuloma formation.

METHODS

Spectroscopy

Proton magnetic resonance (PMR) spectra were determined in deuteriochloroform with tetramethylsilane as an internal standard on a Hitachi R-40 spectrometer at 90 MHz. Electron impact mass spectra (MS) were obtained with a Shimadzu gas chromatograph-mass spectrometer Model 6020 with direct insertion method at 70 eV. The polarity of samples in chloroform was measured with a Perkin-Elmer Model 241 polarimeter at 23°C.

Chemicals

Fenvalerate labeled uniformly with ^{14}C in the chlorophenyl ring [chlorophenyl- ^{14}C] was synthesized (Kanamaru *et al.*, 1981). The specific activity and the radiochemical purity were 53.2 mCi/mmol and more than 99%, respectively. To obtain each of the [chlorophenyl- ^{14}C] [2*S*,*aS*] (abbreviated as A α), [2*S*,*aR*](A β), [2*R*,*aS*](B α), and [2*R*,*aR*](B β)-fenvalerate isomers (Fig. 1), racemic [chlorophenyl- ^{14}C]fenvalerate was subjected to high-pressure liquid chromatography (HPLC) under the following conditions: Jasco FLA-A20 (Japan Spectroscopic Co., Ltd.); detector, UVIDEC 100-II (254 nm); recorder, UN-

ICORDER U-228 (Japan Spectroscopic Co., Ltd.); column, SUMIPAX OA-2000 (2 × (5 μm , 8 mm i.d. × 250 mm) plus 10 μm , 8 mm i.d. × 500 mm) (Sumitomo Chemical Analysis Service); solvent, *n*-hexane:1,2-dichloroethane:isopropyl alcohol (500:30:1) or *n*-hexane:isopropyl alcohol (500:30); flow rate, 3 or 4 ml/min (Chapman, 1983). About 10 mg of [chlorophenyl- ^{14}C]fenvalerate in 50 μl chloroform was injected. The chromatographic eluting pattern of the four isomers is shown in Fig. 2. The optical purities of ^{14}C -labeled preparations obtained were 97.7, >99, >99, and 96.6% for the A α -, A β -, B α -, and B β -isomers, respectively, determined as described above except for column and flow rate; column, OA-2000 (5 μm , 4 mm i.d. × 250 mm) and flow rate, 1 ml/min. Fenvalerate labeled with ^{14}C in the carbonyl group (^{14}CO ; 24.5 mCi/mmol) of the acid moiety was also synthesized in this laboratory (Kanamaru *et al.*, 1981).

Nonradioactive A α - and A β -isomers were synthesized in this laboratory (Aketa *et al.*, 1978), and the B α - and B β -isomers were purified from the B-isomers (B α /B β , 1:1) by silica gel column chromatography with *n*-hexane:ethylacetate (10:1 or 20:1) as the eluting solvent. The chemical and optical purities of these samples were determined by HPLC to be more than 97%.

The authentic standard cholesteryl [2*R*]-2-(4-chlorophenyl)isovalerate ([2*R*]-CPIA-cholesterol ester) was synthesized as follows: [2*R*]-2-(4-chlorophenyl)isovaleric acid chloride was condensed with equimolar cholesterol in benzene and pyridine to yield the corresponding cholesterol ester. The crude CPIA-cholesterol ester was purified on silica gel column chromatography with the solvent *n*-hexane:diethyl ether (9:1, v/v). Recrystallization of the cholesterol ester from *n*-hexane yielded white crystals with a melting point of 134 to 138°C. [2*S*] and [2*R*]-CPIA-cholesterol esters were synthesized in a similar manner. PMR spectra including assignment of proton signals are shown in Fig. 3. The weak molecular ion peak (M) was observed at m/z 580, together with M + 2 at m/z 582, as shown in Fig. 4. The base peak at m/z 368 shows the cholesteryl moiety (Brooks *et al.*, 1968; Fears *et al.*, 1982). Other CPIA-cholesterol esters showed the identical spectra as [2*R*]-CPIA-cholesterol ester. The optical rotations of three CPIA-cholesterol esters are shown in Table 1.

Thin-Layer Chromatography

Precoated silica gel 60F254 chromatoplates (20 × 20 cm, 0.25 mm layer thickness, E. Merck) and reverse phase RP-8F254S chromatoplates (10 × 10 cm, 0.25 mm layer thickness, E. Merck) were used for both analytical and preparative purposes. For identification of metabolites, one- and two-dimensional thin-layer chromatography (TLC) was carried out: (A, B) in two-dimensional TLC means that A is solvent for the first direction and B for the second direction. The standards used in this study were prepared similarly to those in the previous studies and

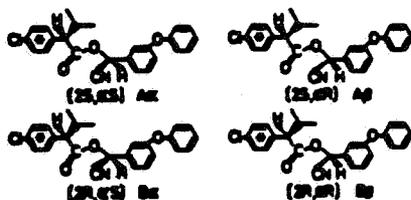


FIG. 1. The four chiral isomers of fenvalerate. ^{14}C -labeled carbon.

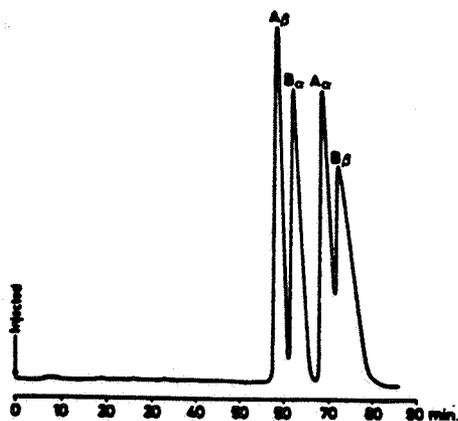


FIG. 2. The chromatogram of the four fenvalerate isomers on the preparative HPLC. Column: Sumipex OA2000 solvent; *n*-hexane:1,2-dichloroethane:isopropyl alcohol (500:30:1) flow rate; 4 ml/min. Monitored at 254 nm.

their abbreviations are as follows: CPIA, 2-(4-chlorophenyl)isovaleric acid; 3-OH-CPIA, 2-(4-chlorophenyl)-3-hydroxymethyl-butanoic acid; 2,3-OH-CPIA, 2-(4-chlorophenyl)-2-hydroxy-3-hydroxymethyl-butanoic acid; Cl-Bacid, 2-(4-chlorophenyl)-3-methyl-2-butenoic acid; Cl-Bacid anhydride, 2-(4-chlorophenyl)-3-methyl-2-butenedioic acid anhydride, 4'-OH-fenvalerate, α -cyano-3-(4'-hydroxyphenoxy)benzyl 2-(4-chlorophenyl)isovalerate (Ohkawa *et al.*, 1979, Kaneko *et al.*, 1981). The R_f values for fenvalerate metabolites were reported previously (Ohkawa *et al.*, 1979). The following solvent systems were used: (A) *n*-hexane:toluene:acetic acid (3:15:2, two developments); (B) petroleum ether:diethyl ether (7:3); (C) petroleum ether:diethyl ether:acetic acid (90:10:1); (D) carbon tetrachloride; (E) tetraline:*n*-hexane (1:3); (F)

cyclohexane:benzene (4:1); (G) *n*-hexane:diethyl ether (19:1); (H) tetraline:*n*-hexane (1:1), (I) acetic acid; (J) methyl ethyl ketone:methanol (7:3); and (K) benzene saturated with formic acid:diethyl ether (10:3, two developments). The radioactive spots were localized by radioautography, and the authentic standards on TLC plates were visualized under uv light (254 nm). CPIA-cholesterol ester on TLC plates was detected by spraying with Zatkis reagent ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ dissolved in water, acetic acid, and sulfuric acid) and then heating.

Column Chromatography

Silica gel column chromatography (Kiesel Gel 60, 70-230 mesh, E. Merck) was carried out with the following solvent systems; (a) *n*-hexane:diethyl ether (9:1); (B) *n*-hexane:acetone (4:1); (C) benzene; and (D) petroleum ether:diethyl ether (19:1). Florial column chromatography (Florial PR, Wako Pure Chemical Industries, Ltd., Japan) was performed by using *n*-hexane:diethyl ether (19:1).

High-Pressure Liquid Chromatography

Five systems were used for preparative and analytical purposes: (A) Lichrosorb RP-18 (5 μm ; 4 mm i.d. \times 25 cm, E. Merck) with acetonitrile:isopropyl alcohol (3:7), flow rate, 0.8 ml/min; (B) same column with acetonitrile:isopropyl alcohol:*n*-hexane (3:2:2), flow rate, 0.8 ml/min; (C) Lichrosorb SI-100 (10 μm , 4 mm i.d. \times 25 cm, E. Merck) with *n*-hexane:diethyl ether (199:1), flow rate, 1 ml/min; (D) I-15 ODS (15 μm , 20 mm i.d. \times 250 mm, Yamamura Chemicals Co., Japan) with acetonitrile:chloroform:isopropyl alcohol (1:1:1) (Perkins *et al.*, 1981), flow rate, 3 ml/min; (E) same column with acetonitrile:chloroform:methanol (1:1:1), flow rate, 3 ml/min.

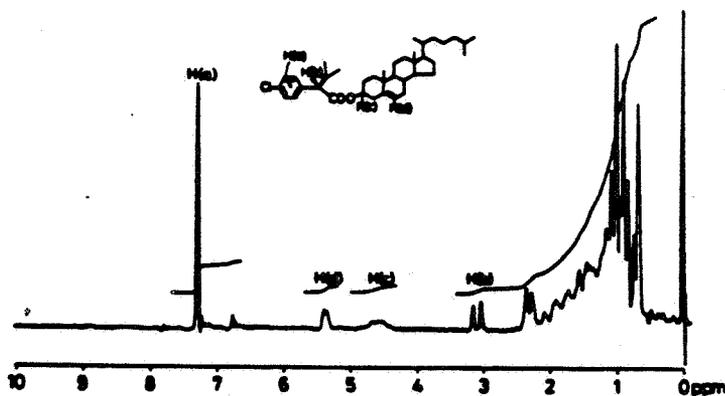


FIG. 3. PMR spectra of authentic standard [2R]-CPIA-cholesterol ester.

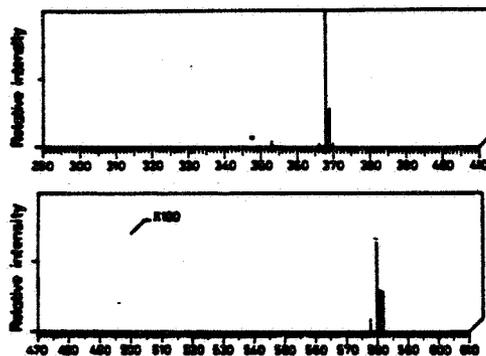


FIG. 4. MS of authentic standard [2R]-CPIA-cholesterol ester.

Animals and Treatments

Male *ddY* mice and male Sprague-Dawley rats were purchased from Shizuoka Agricultural Cooperative Association for Experimental Animals (Shizuoka, Japan). Animals were allowed to acclimatize for at least 7 days prior to use. All experiments were carried out in a temperature- and humidity-controlled room ($25 \pm 2^\circ\text{C}$, $60 \pm 10\%$, respectively) with a 12-hr light/dark cycle. Animals were supplied with a diet (CE-2, Clea Japan Inc., Osaka, Japan) and water *ad libitum*.

Each of the ^{14}C -labeled preparations was suspended in 10% Tween 80 aqueous solution, and four mice (30 to 35 g) and two rats (250 for 270 g) were treated with a single po dose of each of these labeled preparations at 2.5 mg/kg. The treated animals were housed in all-glass metabolism cages (Metabolica-CO2, Sugiyamagen Iriki, Japan) for 6 days after administration. Blood was collected under light diethyl ether anesthesia 6 days after administration and then several tissues were excised to determine the radioactivity by combustion and liquid scintillation counting (LSC).

Male *ddY* mice (30 to 35 g) were housed in suspended stainless-steel cages with wire mesh in the bottom and fed a diet containing 500 ppm [*chlorophenyl*- ^{14}C]A α -, B α -, or B β -isomers (1.6 mCi/mmol) for 1 and 2 weeks. They were then killed, and tissues were excised. The diets were prepared by adding a corn oil solution of each labeled compound to the pulverized rat chow (CE-2) and mixing for 30 min. The rate of addition of corn oil was 2% on the basis of weight. Homogeneity and content of each ^{14}C -labeled compound in the diet were determined by a combination of combustion analysis and LSC of 3 to 6 samples. Tissues were pooled and extracted with the following organic solvents to get sufficient radioactivity to analyze the metabolites in each tissue by TLC. Tissues, except adrenal glands and mesenteric lymph nodes, were homogenized in methanol with a Polytron (Kinematica, Switzerland) and then centrifuged at 3000 rpm. The following extraction

procedure was twice performed: the precipitate was re-suspended with ethyl acetate:methanol (2:1), shaken for 10 min, and centrifuged at 3000 rpm. Adrenals and lymph nodes were homogenized with ethyl acetate in a Potter-Elvehjem homogenizer with a Teflon pestle, and the homogenate was filtered through paper. The supernatant fractions, filtrates, and precipitates were each radioassayed for determination of total radioactivity in the tissues. The extracts were subjected to TLC analysis in solvent systems A and C, and amounts of metabolites on TLC plates were quantified by radioanalysis.

Furthermore, seven male *ddY* mice (30 to 35 g) were treated with a single po dose of [*chlorophenyl*- ^{14}C]Ba (2.3 mCi/mmol) suspended in 10% Tween 80 solution at about 70 mg/kg which is roughly equivalent to 500 ppm. Tissues were excised at appropriate intervals to determine the concentrations of the metabolites by TLC analysis as described above.

Separation and Identification of Metabolites

To purify and identify persistent tissue metabolites, rats and mice were fed diets containing fenvalerate and/or the B-isomer (1:1 mixture of the B α - and B β -isomers) as follows: Animal care and preparation of diets were the same as those described in the 500-ppm feeding study.

Rats. One hundred female Sprague-Dawley rats (7 weeks old, Charles River, Japan) were fed 1500 ppm [^{14}C]fenvalerate (2.0 mCi/mmol) for 2 weeks and then killed to remove liver, adrenal glands, ovaries, spleen, mesenteric lymph nodes, and kidneys. These tissues were homogenized with methanol, ethyl acetate, or chloroform:methanol (2:1) in a Waring blender, and centrifuged at 3000 rpm. The precipitate was homogenized similarly twice. The supernatant fractions of each tissue after centrifugation were concentrated, and the metabolites were partitioned between *n*-hexane and acetonitrile. The ^{14}C -labeled metabolites present in the *n*-hexane layer of the tissues were purified successively by silica gel column chromatography (solvent systems B, C, and D), Florisil column chromatography (*n*-hexane:diethyl ether, 19:1), and preparative TLC (solvent systems B and I). The pu-

TABLE I
OPTICAL ROTATION OF THREE AUTHENTIC
CPIA-CHOLESTEROL ESTERS

Compound	$[\alpha]_D^{25}$
[2R]-CPIA-cholesterol ester	-37.7
[2RS]-CPIA-cholesterol ester	-17.7
[2S]-CPIA-cholesterol ester	+3.1

Note. All compounds were dissolved in chloroform at the concentrations of 6.4 to 7.7 mg/ml.

rified metabolites from tissues were subjected to MS analysis, two-dimensional TLC cochromatography (solvent systems (D, E); (D, F); and (F, H)) and/or to analytical HPLC (systems A, B, and C). They were also hydrolyzed in a solution of 10% NaOH methanol solution overnight and analyzed by TLC with solvent systems A and K after adjustment of the pH to 2 and extraction with a solution of diethyl ether and ethanol (2:1).

Mice. The livers of 125 male *ddY* mice (7 weeks old, Shizuoka Agricultural Cooperative Association for Experimental Animals, Japan) treated with 4000 ppm B-isomer for 2 weeks and 1500 ppm [*chlorophenyl*-¹⁴C]-fenvalerate (0.5 mCi/mmol) for the following 1 week were excised and extracted with chloroform:methanol (2:1), and the extracts were partitioned between *n*-hexane and acetonitrile. The ¹⁴C-labeled metabolites in the *n*-hexane layer were purified by silica gel (solvent A) and Florisil column chromatography (*n*-hexane:diethyl ether, 19:1), followed by TLC (solvent C) and preparative HPLC (systems D and E). The purified metabolites were subjected to spectroanalysis.

Radioassay and Radioautography

The content of radiocarbon in the organosoluble fractions and in the urine was determined with a Packard

Model 460CD liquid scintillation spectrometer. An aliquot (0.5 ml) was added to a low-potassium glass vial containing 10 ml of a toluene scintillation fluid (7 g of 2,5-diphenyl-oxazole and 1 g of 1,4-bis(2-methylstyryl)benzene in 1 liter of a 1:1 mixture of toluene and ethylene glycol monoethyl ether). For determination of ¹⁴C in tissues and unextractable fecal residues, samples were air dried and then about 200 mg of each sample was oxidized in a Packard Model 306 sample oxidizer prior to liquid scintillation counting. A 9:15 mixture of Oxisorb-CO₂ and Oxiprep-2 (New England Nuclear) was used for combustion analysis. Quantification of radioactive metabolites on TLC plates was carried out by scraping appropriate gel regions into scintillation vials and counting, as described above.

RESULTS

¹⁴C-Tissue Residue

Table 2 shows ¹⁴C-tissue residues 6 days after a single administration of ¹⁴C-chiral isomers to rats and mice. The A α -, A β -, and B β -isomers showed very low residues in all the

TABLE 2
¹⁴C-TISSUE RESIDUES AT 6 DAYS AFTER A SINGLE po ADMINISTRATION OF [*chlorophenyl*-¹⁴C]A α -, A β -, B α -, OR B β -ISOMER TO MALE *ddY* MICE AND SPRAGUE-DAWLEY RATS AT 2.5 mg/kg

Tissue	Rat				Mouse			
	A α	A β	B α	B β	A α	A β	B α	B β
	ng equivalents/g wet tissue							
Adrenals	12 \pm 6 ^a	14 \pm 2	371 \pm 67	23 \pm 4	38 ^b	66	597	53
Blood	— ^c	—	—	—	7 \pm 0	13 \pm 0	19 \pm 0	3 \pm 0
Fat	511 \pm 26	326 \pm 73	304 \pm 44	756 \pm 10	431 \pm 129	496 \pm 48	117 \pm 9	314 \pm 40
Heart	2 \pm 0	2 \pm 1	40 \pm 19	4 \pm 2	<3 ^d	<3	62 \pm 25	<3
Kidneys	9 \pm 1	6 \pm 4	25 \pm 7	7 \pm 3	3 \pm 3	7 \pm 2	43 \pm 11	4 \pm 1
Liver	22 \pm 1	20 \pm 1	72 \pm 28	19 \pm 12	13 \pm 7	21 \pm 4	369 \pm 32	22 \pm 23
Lungs	3 \pm 0	3 \pm 1	25 \pm 7	5 \pm 7	<3	<3	45 \pm 19	<3
Mesenteric lymph nodes	45 \pm 11	68 \pm 12	318 \pm 9	94 \pm 51	59 \pm 50	47 \pm 17	205 \pm 154	33 \pm 25
Spleen	3 \pm 1	2 \pm 0	62 \pm 11	4 \pm 1	<3	<3	92 \pm 11	<3
Testes	<1	<1	4 \pm 1	<1	<3	<3	26 \pm 6	<3

Note. Four mice and two rats were killed 6 days after a single po administration of each of the ¹⁴C-labeled chiral isomers. The data represent mean values of four mice and two rats.

^a Results expressed as $\bar{x} \pm$ SD of four samples.

^b Analyzed together.

^c Not measured.

^d Below detection limits.

tissues analyzed, although the radiocarbon in the fat was relatively high. On the other hand, the $B\alpha$ -isomer showed a quite different pattern, with relatively higher residues in the analyzed tissues of both animals, particularly in the adrenal glands, liver, mesenteric lymph nodes, and spleen. However, the fat did not contain the highest amount of the radiocarbon among the analyzed tissues.

To demonstrate the difference in the tissue residues more clearly, ^{14}C concentrations in mouse tissues were measured after male mice had been kept on a diet containing 500 ppm [*chlorophenyl- ^{14}C*] $B\alpha$ -isomer for 1 and 2 weeks. The $A\alpha$ - and $B\beta$ -isomers were used as references and the same dosage was given to mice. The maximally tolerated dosage of the $A\alpha$ -isomer was 500 ppm, and the $B\alpha$ -isomer at this dose is reported to cause granulomatous changes in mice (Okuno *et al.*, 1986). Table 3 gives the results. As indicated by ^{14}C tissue residues on single po doses, the $B\alpha$ -isomer also showed higher ^{14}C concentrations in all the tissues analyzed, particularly in the adrenals, liver, and mesenteric lymph nodes, than the $A\alpha$ - and $B\beta$ -isomers. TLC analysis showed a lipophilic metabolite, less polar than fenvalerate (e.g., 0.55 and 0.18 in solvent system C for a lipophilic metabolite and fenvalerate, respectively), was present in all the tissues of mice treated only with $B\alpha$ -isomer. This metabolite was found as a predominant metabolite in all the analyzed tissues of mice treated with only $B\alpha$ -isomer (Table 3), together with the parent compound and CPIA and 3-OH-CPIA as minor metabolites. The adrenal glands showed the highest concentration of this metabolite, followed by liver and mesenteric lymph nodes. On the other hand, amounts in the brain, blood, and testes were smaller than those in other tissues. Amounts in most of the tissues were not in parallel with feeding periods, although the amounts increased slightly after 2 weeks. The amount of a lipophilic metabolite in the tissues tends to reach a plateau easily. On the other hand, no detectable lipophilic metabolite was found in any tissues of mice treated with the $A\alpha$ -isomer.

The metabolite was not formed from the $B\beta$ -isomer in any tissues of mice except in the liver which contained trace amounts (less than one-five hundredth of $B\alpha$). Tissues of mice treated with the $A\alpha$ - and $B\beta$ -isomers contained unchanged parent compound, CPIA, and 3-OH-CPIA as major components.

Identification of a Lipophilic Metabolite in Rat and Mouse Tissues

Rats. The ^{14}C distribution in *n*-hexane and acetonitrile layers after extraction of the tissues was variable. Of ^{14}C in adrenal, ovary, and spleen, more than 70% was found in the *n*-hexane layer, whereas ^{14}C in liver, kidneys, and mesenteric lymph nodes was partitioned into the *n*-hexane layer to the extent of 30 to 50%. The TLC analysis (solvent system A or C) of the *n*-hexane layer of these tissues revealed the presence of the same lipophilic metabolite as that described above. On Florisil column chromatography, the bulk of *n*-hexane-soluble ^{14}C in the liver was found in the cholesterol ester fraction (Carroll *et al.*, 1961). This finding implies that the metabolite appeared to be a cholesteryl ester conjugated with the acid moiety of fenvalerate. The metabolite from the liver was not purified in sufficient quantity to confirm the chemical structure by PMR. Thus, the following procedures were used to identify it.

(A) Release of [^{14}C]CPIA by hydrolysis of this metabolite with 10% NaOH methanol solution as evidenced by TLC analysis in solvents A and K.

(B) Identify the ^{14}C -lipophilic metabolite with the authentic standard CPIA-cholesterol ester by chromatography, in the following manner:

(a) TLC: Two-dimensional TLC (silica gel); (1) (D, E), (2) (D, F), and (3) (F, H). One-dimensional TLC (reverse phase); (1) I and (2) J. *R_f* values of CPIA-cholesterol ester were as follows: 0.32, 0.41, 0.32, 0.73, 0.64, and 0.64 for solvents D, E, F, H, I, and J, respectively.

(b) HPLC: The retention times were as follows; 8.1, 6.1, and 5.2 min for HPLC systems A, B, and C, respectively.

(c) MS: The ^{14}C metabolite from livers gave the same spectra as those of the authentic standard, the base peak being m/z 368.

TABLE 3

¹⁴C AND A LIPOPHILIC METABOLITE CONCENTRATION IN THE TISSUES OF MICE FED DIETS CONTAINING 500 ppm [chlorophenyl-¹⁴C]A α -, B α -, OR B β -ISOMER FOR 1 AND 2 WEEKS

Tissue	Weeks	A α	B α	B β
µg equivalents/g wet tissue				
Adrenals	1	19.0 (ND) ^a	128.9 (112.8)	26.1 (ND) ^b
	2	10.4 (ND)	173.2 (128.7)	21.1 (ND)
Blood	1	4.7 (ND)	5.1 (0.4)	3.5 (ND)
	2	4.1 (ND)	5.6 (2.9)	3.0 (ND)
Brain	1	— ^c	1.2 (ND)	0.7 (ND)
	2	—	1.4 (0.8)	0.9 (ND)
Heart	1	1.9 (ND)	10.7 (7.8)	1.9 (ND)
	2	1.7 (ND)	15.1 (11.4)	2.3 (ND)
Kidneys	1	8.3 (ND)	24.0 (9.7)	8.5 (ND)
	2	10.3 (ND)	21.5 (6.7)	8.8 (ND)
Liver	1	14.4 (ND)	92.4 (42.4)	12.9 (0.1)
	2	12.6 (ND)	105.0 (73.4)	12.8 (ND)
Lungs	1	2.3 (ND)	16.9 (8.8)	4.0 (ND)
	2	1.8 (ND)	31.2 (20.0)	5.1 (ND)
Mesenteric lymph nodes	1	12.3 (ND)	73.4 (57.8)	9.4 (ND)
	2	7.5 (ND)	86.2 (64.0)	12.1 (ND)
Spleen	1	1.9 (ND)	14.3 (12.3)	0.8 (ND)
	2	0.7 (ND)	20.6 (15.0)	1.0 (ND)
Testes	1	—	3.3 (1.8)	3.6 (ND)
	2	—	6.2 (4.8)	1.4 (ND)

Note. Seven to ten male *ddY* mice were fed diets containing 500 ppm [chlorophenyl-¹⁴C]A α -, B α -, or B β -isomer for 1 and 2 weeks. Each tissue removed after death was extracted with organic solvents and subjected to radioanalysis and TLC analysis in solvents A and C. The *R_f* values for a lipophilic metabolite were 0.55 and 0.91 for the former and latter solvent systems, respectively. Quantification of radioactive metabolites on TLC plates was carried out by a combination of scraping the appropriate silica gel region and LSC.

^a The figures in the parentheses show amounts of a lipophilic metabolite.

^b ND—below detection limit.

^c Not analyzed.

Based on the above findings, the ¹⁴C-metabolite in the hexane layer of the liver was identified as CPIA-cholesterol ester. The metabolite in the hexane layer from the adrenal, ovary, kidneys, spleen, and mesenteric lymph nodes showed the same retention time by HPLC as those of the authentic CPIA-cholesterol ester.

Mice. PMR were measured at each step of the purification procedure to confirm purifi-

cation. The samples contained triglycerides and cholesterol esters after purification on silica gel column chromatography, but only cholesterol esters after Florisil column chromatography on the basis of PMR. The crude cholesterol ester fraction (261 mg) was subjected to preparative HPLC (systems D and E) giving 31 mg of the purified metabolite. This metabolite was subjected to PMR and MS. These spectra agreed with those of the

authentic standard (cf. Figs. 3 and 4). The optical rotation of the purified metabolite was $[\alpha]_D^{25} = -38.5$. From these findings, this hepatic metabolite was identified as [2*R*]-CPIA-cholesterol ester. Based on the TLC cochromatography in solvent systems A and C, a lipophilic metabolite detected in tissues of mice treated with 500 ppm β -isomer was demonstrated to be the CPIA-cholesterol ester.

CPIA-Cholesterol Ester Amounts

To have information on organ specificity of the presence of the CPIA-cholesterol ester, male *ddY* mice were given a single po dose of [*chlorophenyl*- ^{14}C] β -isomer at 70 mg/kg, and several tissues were analyzed at appropriate intervals. CPIA-cholesterol ester was found in the intestines, mesenteric lymph nodes, blood, and kidneys 30 min after administration, but not in the liver (Table 4). However, this metabolite was detected in all the analyzed tissues after 1 hr. The amount of this ester was relatively high in the adrenals, liver, and mesenteric lymph nodes. The liver tended to contain the highest amount.

DISCUSSION

Only the β -isomer of fenvalerate yielded a persistent metabolite in tissues of rats and mice. This metabolite was identified as [2*R*]-CPIA-cholesterol ester, which was actually retained in large amounts particularly in adrenals, spleen, mesenteric lymph nodes, and liver. CPIA-cholesterol ester was rapidly formed and detected in all the analyzed tissues within 1 hr after a single po dose of the β -isomer. So far, there have been a few reports on conjugation of xenobiotics with cholesterol. These are carboxylic acid and ester derivatives such as cycloprate (Schooley and Quistad, 1982), methoprene (Quistad *et al.*, 1976), BRL 24139 (Fears *et al.*, 1982) and chlorambucil (Gunnarsson *et al.*, 1984). However, the critical difference in conjugation reactions with cholesterol among chiral isomers as revealed in the present paper has not been reported.

Interestingly only the β -isomer gave [2*R*]-CPIA-cholesterol ester, although the β - and β -isomers will yield the same [2*R*]-CPIA which is the acid component of [2*R*]-CPIA-cholesterol ester. This fact implies that CPIA-cholesterol ester appears not to be produced

TABLE 4
CONCENTRATIONS OF CPIA-CHOLESTEROL ESTER IN MOUSE TISSUES AFTER A SINGLE po DOSE OF THE FENVALERATE β -ISOMER AT 70 mg/kg

Tissue	0.5	1.0	1.5	2.0	3.0	6.0	12.0	24.0
	µg equivalents/g wet tissue							
Adrenals	— ^a	—	—	—	8.2	12.9	22.0	15.8
Blood	0.2	0.5	0.6	ND ^b	0.4	0.2	0.9	0.6
Brain	—	—	—	—	ND	ND	ND	ND
Intestines	2.7	4.6	4.8	6.0	—	—	—	—
Kidneys	0.4	3.2	6.6	5.0	2.7	1.3	3.3	ND
Liver	ND	1.3	3.9	4.6	7.5	12.4	14.3	17.8
Mesenteric lymph nodes	2.7	9.2	12.7	21.4	24.4	20.2	29.5	15.3
Spleen	ND	3.5	4.5	5.7	3.4	2.5	3.2	2.0

Note. Seven male *ddY* mice were killed at appropriate intervals, and tissues were excised. Each type of the individual tissues from the seven mice was combined and homogenized with organic solvents. Concentrations of CPIA-cholesterol ester were determined in the same manner to those described in the footnote of Table 3.

^a Not analyzed.

^b Below detection limit.

via CPIA-CoA derivative mediated by acyl-CoA:cholesterol *O*-acyltransferase (ACAT).

To date, conjugation reactions have generally been considered to be associated with rapid excretion and detoxication (Paulson, 1982). In fact, most of the classical conjugation reactions of xenobiotics with glucuronic acid, sulfuric acid, glutathione, and amino acids, for example, lead to rapid elimination of the aglycones from the animal body. On the contrary, the lipophilic conjugates such as cholesterol esters, triglycerides, and fatty acid esters are in general excreted slowly and are rather persistent in tissues (Hutson, 1982). Thus, the toxicological significance of lipophilic conjugates will be duly considered. In this connection, it is noteworthy that only the *B α* -isomer which produced microgranulomatous lesions in rats and mice is capable of forming CPIA-cholesterol ester. The possible relationship between granuloma formation and CPIA-cholesterol ester is discussed in an accompanying paper (Okuno *et al.*, 1986).

ACKNOWLEDGMENTS

The authors thank Messrs. T. Izumi and Y. Ueda, and Miss Y. Nakade for their skilled technical assistance in carrying out this work.

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ATTACHMENT III

Differential Metabolism of Fenvalerate and Granuloma Formation

II. Toxicological Significance of a Lipophilic Conjugate from Fenvalerate

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Reprinted from **TOXICOLOGY AND APPLIED PHARMACOLOGY**, Volume 83, No. 1, March 30, 1986
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Differential Metabolism of Fenvalerate and Granuloma Formation. II. Toxicological Significance of a Lipophilic Conjugate from Fenvalerate. OKUNO, Y., SEKI, T., ITO, S., KANEKO, H., WATANABE, T., YAMADA, T., AND MIYAMOTO, J. (1986). *Toxicol. Appl. Pharmacol.* 83, 157-169. Male mice of the *ddY* strain were fed a diet containing the [2*S*, α *S*]-, [2*S*, α *R**S*]-, [2*R*, α *S*]-, and [2*R*, α *R*]-isomers of fenvalerate. Microgranulomatous changes were observed only in mice treated with the [2*R*, α *S*]-isomer at 125 and 1000 ppm for 1, 2, or 3 months. In contrast, the changes did not occur in mice treated with the [2*R*, α *R*]-isomer under the same conditions. Feeding of the [2*S*, α *S*]- and [2*S*, α *R**S*]-isomers for 1 year did not cause the microgranulomatous changes at 500 or 1000 ppm. To clarify the causative agent of granuloma formation, cholesterol ester of 2-(4-chlorophenyl)isovaleric acid (CPIA), a lipophilic conjugate from the [2*R*, α *S*]-isomer of fenvalerate, was injected iv into *ddY* mice. Microgranulomatous changes were observed in the liver of mice treated with the [2*R*]-, [2*S*]-, or [2*R**S*]-CPIA-cholesterol ester 1 week after a single treatment of 1, 10, or 100 mg/kg, as well as in liver of mice treated with a single dose of 10 or 30 mg/kg of the [2*R*]-CPIA-cholesterol ester and kept up to 26 weeks afterward. Histochemistry and microscopic autoradiography of the liver of mice demonstrated the presence of tritium derived from ³H-labeled [2*R*]-2-(4-chlorophenyl)isovalerate and cholesterol. Histochemistry also was positive for cholesterol ester in livers of mice treated with the [2*R*, α *S*]-isomer of fenvalerate. These results lend support for the hypothesis that CPIA-cholesterol ester is the causative agent of microgranulomatous changes induced by fenvalerate. © 1986 Academic Press, Inc.

With respect to the subchronic and chronic toxicities of fenvalerate (Sumicidin, Pydrin, [2*R**S*]- α -cyano-3-phenoxybenzyl[2*R**S*]-2-(4-chlorophenyl)isovalerate), a synthetic pyrethroid, several studies have reported that chronic dietary administration of fenvalerate did not produce a carcinogenic response to mice and rats (Anonymous, 1981; Parker *et al.*, 1983a, 1984). However, in a 2-year study of mice, treatment-related, nonneoplastic pathological changes, which were diagnosed as multifocal microgranulomas, were observed in lymph nodes, liver, and spleen of male mice treated with 50, 250, and 1250 ppm or of female mice treated with 250 and 1250 ppm

(Parker *et al.*, 1983a). The microgranulomatous changes were also observed in lymph nodes, liver, spleen, and adrenal glands of male and female Wistar rats treated with 500 and 1500 ppm in a life span study (Anonymous, 1981). Parker *et al.* (1983b) also reported hepatic microgranulomas in a 6-month feeding study of fenvalerate in Beagle dogs. It was considered important to examine the changes in more detail and to clarify the mechanism of development of the hepatic lesion in order to assess the toxicological significance. Therefore, metabolism studies have been reported in the first paper of this series in relation to granuloma formation (Kaneko *et al.*, 1986).

The present study is intended to demonstrate that a lipophilic conjugate from a chiral isomer of fenvalerate is the causative agent of granuloma formation, taking the results of metabolism studies into consideration.

METHODS

Animals. Male *ddY* mice that were 4 weeks of age were purchased from Shizuoka Agricultural Cooperative Association for Experimental Animals (Shizuoka, Japan). They were housed five mice per cage in polycarbonate cages, at the bottom of which were shavings, and they were given food (CE-2 Type, Clea Japan Inc., Osaka, Japan) and tap water *ad libitum*. The animals used for the feeding study with fenvalerate isomers and a 6-month study with 2-(4-chlorophenyl)isovaleric acid (CPIA)-cholesterol ester were held in rooms in a specific pathogen-free experimental unit and maintained at a temperature of 21 to 26°C with

a relative humidity of 40 to 70%. Other animals were held in rooms of a conventional unit controlled at a temperature of approximately 25°C, and a relative humidity of approximately 60%. The animals were acclimatized for 1 week before dosing.

Test materials and diets. The [2*S*,*aS*] (designated as A α), [2*S*,*aRS*](A), [2*R*,*aS*](B α) and [2*R*,*aR*](B β)-isomers were prepared in the authors' laboratory. The A α -isomer (chemical purity, 100.0%; optical purity, 99.6%) was prepared from the A-isomer (chemical purity, 95.2%; optical purity, 98.3%) which was synthesized by condensation of [2*S*]-2-(4-chlorophenyl)isovaleric acid chloride with α -cyano-3-phenoxybenzyl alcohol. Three lots of the B α - and the B β -isomers were purified from the [2*R*,*aRS*]-isomer synthesized analogously to the A-isomer by silica gel column chromatography with *n*-hexane:ethyl acetate (20:1) as an eluting solvent, as described by Kaneko *et al.* (1986). The chemical and optical purities of the B α -isomer were 97.1 to 99.1% and 97.3 to 98.6%, respectively. Those of the B β -isomer were 99.3 to 99.7% and 99.3 to 100.0%, respectively. Technical (racemic) fenvalerate (chemical purity 96.1%) was manufactured by Sumitomo Chemical

TABLE I

INCIDENCE OF FENVALERATE-INDUCED GRANULOMATOUS CHANGES SUCH AS MICROGRANULOMAS AND GIANT CELL INFILTRATION IN LIVER, SPLEEN, AND LYMPH NODES OF MICE TREATED WITH FENVALERATE ISOMERS^a

Chemical	Dosage (ppm)	Feeding period (weeks)					
		4	8	13	26	39	52
Control I	0	— ^b	—	0%	0% ^c	0% ^d	0% ^{e,f}
A α	500	—	—	0% ^g	0%	0%	0% ^{e,h}
A	500	—	—	0%	0%	0% ⁱ	0% ^{e,i}
	1000	—	—	0% ^g	0% ^{e,h}	0% ^{e,j}	0% ^{e,k}
Racemic	500	—	—	100% ^g	100% ^g	100% ^g	95% ^{e,l}
Control II	0	0%	0%	0%	—	—	—
B α	125	0% ^c	70% ^g	60% ^g	—	—	—
	1000	100% ^g	100% ^g	100% ^g	—	—	—
B β	125	0%	0%	0%	—	—	—
	1000	0%	0%	0%	—	—	—

^a Unless otherwise stated, the number of animals examined was 10.

^b No scheduled termination.

^c Very weak granulomatous changes were observed in the liver and mesenteric lymph nodes of a few animals. However, the changes were judged not to be related to the chemical because fenvalerate had generally induced the granulomatous formation in both the liver and mesenteric lymph nodes in a former study (Parker *et al.*, 1983a).

^d Granulomatous changes similar to the fenvalerate-induced changes were observed in the liver and lymph nodes of one control animal.

^e N = 20.

^f N = 9.

^g N = 11.

^h N = 8.

ⁱ N = 15.

^j N = 6.

^k N = 12.

^l p < 0.01.

Company, Ltd. These fenvalerate isomers were dissolved in an appropriate volume of corn oil, and then the solutions were mixed with basal diet (CE-2 type) and given to the mice. The final concentration of corn oil was adjusted to 2% in the diet for both treated and control groups. Cholesteryl [2*R*]-, [2*S*]-, and [2*RS*]-2-(4-chlorophenyl)-isovalerate (abbreviated as [2*R*]-, [2*S*]-, and [2*RS*]-CPIA-cholesterol) were synthesized by the method reported by Kaneko *et al.* (1986). The chemical purities of [2*R*]-, [2*S*]-, and [2*RS*]-CPIA-cholesterol were 97.0, 96.8, and 93.8%, respectively. The [2*RS*]-CPIA-cholesterol labeled with ³H at the chlorophenyl ring with the specific activity of 460 μCi/mg was synthesized by condensation of ³H-[2*RS*]-CPIA acid chloride with cholesterol. Then ³H-[2*R*]-CPIA-cholesterol was separated by high-pressure liquid chromatography (HPLC; column: SUMIPAX OA-2000, 2 × 5 μm, 8 mm i.d. × 250 mm; solvent: *n*-hexane). To prepare the suspension for iv administration, 1.5 g of the [2*R*]-, [2*S*]-, or [2*RS*]-CPIA-cholesterol was mixed overnight with 70 g of glass beads, 3 g of Tween 80, and 30 ml of distilled water in a Sandgrinder (Igarashi Kikai Seizo Co., Ltd., Tokyo, Japan). The beads were removed by

centrifugation (1900g, 30 min; stock solution). The mean particle diameter of CPIA-cholesterol was 0.2 μm after the centrifugation. The stock solution was diluted with a 10% Tween 80 aqueous solution before dosing. The radioactive CPIA-cholesterol was suspended in a 10% Tween 80 aqueous solution with a mortar and pestle.

Feeding of fenvalerate isomers. Groups of 50 male mice were fed diets containing corn oil only, 500 and 1000 ppm Aα-isomer, 500, 1000, and 2000 ppm A-isomer, or 500 ppm technical (racemic) fenvalerate for up to 52 weeks. Ten males of each group were killed at 13, 26, or 39 weeks and 20 males of each group were killed at the termination of feeding. In a separate experiment, additional groups of 30 males each were fed diets containing corn oil only, 125 or 1000 ppm Bα-isomer, or 125 or 1000 ppm Bβ-isomer. Ten males of each group were killed at 4, 8, and 13 weeks after dosing.

Intravenous injection of CPIA-cholesterol. Groups of five mice were treated with single iv injections of [2*R*]-, [2*S*]-, or [2*RS*]-CPIA-cholesterol through the tail vein at dosages of 1, 10, and 100 mg/kg. The administration volume was 8 ml/kg body wt. The mice were killed 1 week after in-

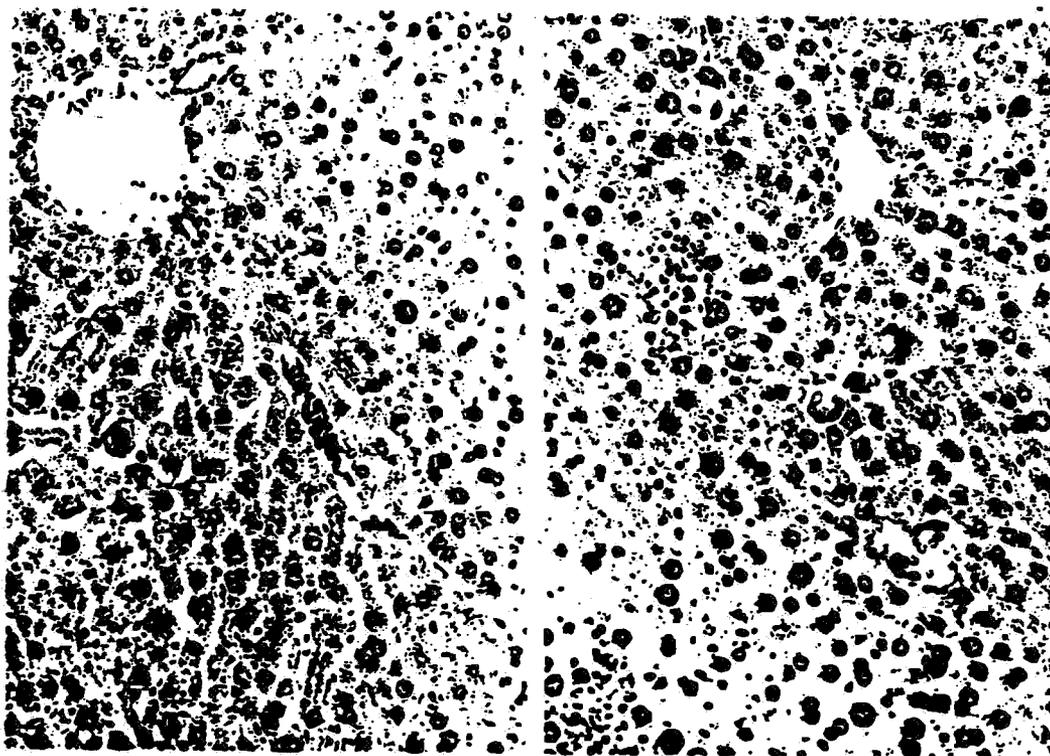


FIG. 1. Granuloma formation in mouse liver as related to chirality of fenvalerate (HE, ×155). Left, the liver after 13-week feeding of the [2*S*,α*S*]-isomer of fenvalerate at 500 ppm. No granulomatous changes are observed. Right, the liver after 13-week feeding of the [2*R*,α*S*]-isomer of fenvalerate at 1000 ppm. Microgranuloma and giant cell infiltration (Langhans' type cells) are noticed.

jection. In another trial, groups of 25 mice were similarly treated with iv [2R]-CPIA-cholesterol doses at 10 and 30 mg/kg. The administration volume was 3.5 ml/kg body wt in this case and the mice had been fed only the basal diet (CE-2 Type) for 26 weeks. Five mice of each group were killed at 1, 4, and 8 weeks after the injection. The surviving animals were finally killed after 26 weeks. In both experiments, the control animals were treated with the vehicle only.

Intravenous injection of ^3H -[2R]-CPIA-cholesterol. The suspension of radioactive CPIA-cholesterol in 10% Tween 80 aqueous solution was given intravenously to mice at the rate of 400 μCi /mouse or 0.87 mg/mouse (approximately 30 mg/kg), and microscopic autoradiography was carried out with liver from mice killed 1 and 5 weeks thereafter.

Observation items. Observation for mortality was made at least once a day during the experimental period of all studies.

Histopathological examination. At every scheduled interval, the mice treated with fenvalerate isomers or unlabeled CPIA-cholesterol were necropsied, and liver, spleen,

adrenal glands, and mandibular and mesenteric lymph nodes were dissected out. Any other tissues indicating gross abnormality were also dissected out. All these tissues were fixed in neutral buffered Formalin. The fixed tissues were processed and embedded in paraffin. Sections (5 to 10 μm) were stained with hematoxylin and eosin (HE) and examined under a light microscope. Liver and mesenteric lymph nodes of mice from all the groups in the feeding study of fenvalerate isomers killed at 4 and 13 weeks, liver and mesenteric lymph nodes from control and 500 ppm technical fenvalerate groups killed at 52 weeks, as well as livers of mice from the 8- and 26-week groups treated with 30 mg/kg [2R]-CPIA-cholesterol, were also subjected to transmission electron microscopy. Samples of liver and lymph nodes were fixed in 2.5% phosphate-buffered glutaraldehyde (pH 7.4), washed in the buffer alone, postfixed in 1% phosphate-buffered osmium tetroxide, and then embedded in epoxy resin (WE812, Wako Pure Chemical, Osaka, Japan). Thin sections (600–700 \AA) were stained with lead citrate and uranyl acetate and examined with a Hitachi H-300 electron microscope (Hitachi Ltd., Tokyo, Japan) at 75 kV.

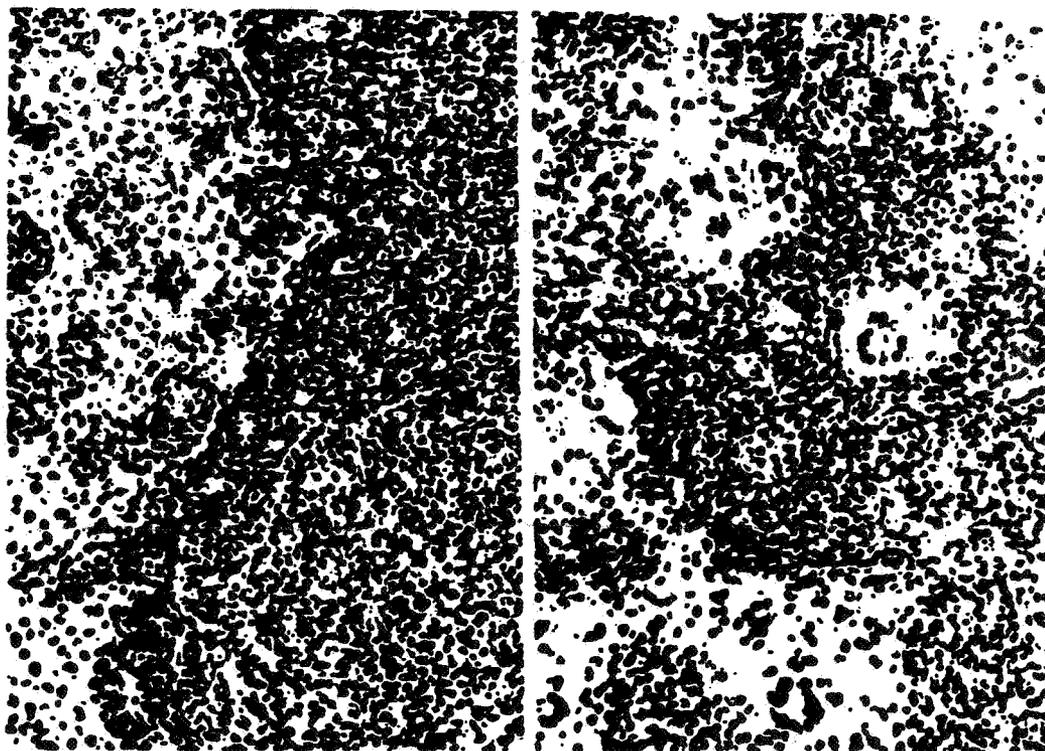


FIG. 2. Granuloma formation in mouse mesenteric lymph nodes as related to chirality of fenvalerate (HE, $\times 155$). Left, the lymph node after 13-week feeding of the [2S, α S]-isomer of fenvalerate at 500 ppm. No granulomatous changes are observed. Right, the lymph node after 13-week feeding of the [2R, α S]-isomer of fenvalerate at 1000 ppm. Both giant cells of Langhans' and foreign body types are seen.



FIG. 3. A giant cell in microgranulomatous foci of mouse liver ($\times 10,000$). Liver of a male mouse fed the [2*R*, α S]-isomer of fenvalerate at 1000 ppm for 4 weeks. Crystalline rods or needles (arrows) are seen.

Microscopic autoradiography: A dipping technique similar to that described by Bogoroch (1972) was applied as follows. Frozen blocks of liver of mice treated with the tritium-labeled CPIA-cholesterol at about 30 mg/kg and killed after 1 and 5 weeks were fixed in 10% phosphate-buffered Formalin and sliced at 8 μ m. The sections were mounted onto glass slides and then coated with Sakura NR-M2 emulsion (Konishiroku Photo. Industries, Tokyo, Japan). The sensitizer was exposed at 4°C overnight and developed. The tissues were then stained with HE and examined under a light microscope.

Histochemistry of cholesterol ester. To demonstrate the presence of CPIA-cholesterol in the phagocytes, histochemical staining of cholesterol ester was performed on the same liver used for microscopic autoradiography. The staining was carried out by Schultz's method (Schultz, 1924). Frozen blocks of liver fixed in 10% phosphate-buffered Formalin were sliced at 10 to 20 μ m, placed in ferric ammonium sulfate solution for 48 to 72 hr at 37°C, and rinsed briefly in distilled water. One drop of freshly prepared acetic-sulfuric acid solution (1:1) was then placed on the sliced sections, which were examined under a light microscope within a few min. To demonstrate identity of crystalline rods in the cells of microgranulomatous foci induced by fenvalerate, the same histochemical staining of cholesterol ester was performed in livers of male *ddY*

mice fed diets containing 1000 ppm $B\alpha$ - and $B\beta$ -isomers for 8 and 13 weeks.

Statistical analysis. The statistical significance of differences in incidence of pathological findings was tested with the Fischer's exact test. Differences were considered significant when $p < 0.05$.

RESULTS

Mortality Rate

Almost all the mice fed 1000 ppm of the $A\alpha$ -isomer and 2000 ppm of the A-isomer developed severe hyperexcitability and tremor which are characteristic of the compounds; they were dead within 2 weeks. There was no mortality in groups exposed to racemic fenvalerate or the $B\alpha$ - and $B\beta$ -isomers. A higher mortality was observed at 100 mg/kg for both [2*R*]- and [2*RS*]-CPIA-cholesterol (2/5 and 3/5, respectively). No mice died in the group given [2*S*]-CPIA-cholesterol. All the treated



FIG. 4. Crystalline rod (arrow) in giant cells of mouse lymph node ($\times 10,000$). Mesenteric lymph node of a male mouse fed the [2*R*, α S]-isomer of fenvalerate at 1000 ppm for 4 weeks.



FIG. 5. Crystalline rod (arrow) and lysosomes (arrowheads) in giant cells of mouse lymph node ($\times 15,000$). Mesenteric lymph node of male mice fed technical fenvalerate at 500 ppm for 52 weeks.

animals in the second trail with [2*R*]-CPIA-cholesterol survived for 26 weeks, and one control mouse died after 100 days.

Microgranulomatous Changes Produced by Fenvalerate Isomers

Light Microscopy

Liver, lymph nodes, and spleen. Table 1 shows the results of histopathological examination of surviving mice treated with fenvalerate isomers. Under the present conditions, the A α -, A-, and B β -isomers were not found to cause granuloma formation, whereas typical changes were observed in the B α -treated groups as shown in Figs. 1 and 2. The histopathological changes observed in liver, spleen, and lymph nodes in B α -treated groups were identical to those in racemic fenvalerate-treated groups. The granulomatous changes

consisted of microgranulomas and giant cell infiltration. The granulomatous changes were mainly observed in the medullary cord of the lymph nodes, in the white pulp of spleen, and in the periportal area of the hepatic lobules. Hepatic microgranulomatous foci were less frequently present in the midzonal and centrilobular areas. There were no remarkable changes in the tissues surrounding the microgranulomatous foci.

A treatment-related change was observed in the livers of 500 ppm A α and 1000 ppm A groups; namely, a decreased incidence of hepatocellular cytoplasmic vacuolation was noticed in the groups. The incidences in control, 500 ppm A α , and 1000 ppm A groups were 9/10, 2/9, and 3/9 at 13 weeks; 10/10, 4/10, and 1/8 at 26 weeks; 10/10, 4/10, and 4/6 at 39 weeks; and 14/20, 4/11, and 5/12 at 52 weeks, respectively. Hepatocellular adenoma was observed in one control mouse killed at 52 weeks, and one mouse found dead at A α 500 ppm had leukemia.

TABLE 2

FORMATION OF MICROGRANULOMATOUS CHANGES IN LIVERS OF MALE MICE TREATED WITH [2*R*]-, [2*S*]-, AND [2*RS*]-CPIA-CHOLESTEROL BY A SINGLE *iv* INJECTION^{a,b}

Chemical	Dosage (mg/kg)	Microgranuloma	Giant cell infiltration
Control	0	-	\pm^c
[2 <i>R</i>]	1	+	-
	10	+	\pm
	100 ^d	++	++
[2 <i>S</i>]	1	\pm	-
	10	+	\pm
	100	++	+++
[2 <i>RS</i>]	1	+	\pm
	10	++	++
	100 ^e	++	+++

^a -, negative; \pm , trace; +, slight; ++, moderate; +++, severe.

^b Unless otherwise stated, the number of animals examined was 5.

^c Solitary focus of giant cell was observed in a few mice.

^d *N* = 3.

^e *N* = 2.

TABLE 3
FORMATION OF MICROGRANULOMATOUS CHANGES IN LIVERS OF MALE MICE TREATED WITH [2R]-CPIA-CHOLESTEROL BY A SINGLE iv INJECTION^a

Dosage (mg/kg)	Finding	Week after injection			
		1 ^b	4 ^b	8 ^b	26 ^c
0	Microgranuloma	-	-	-	-
	Giant cell infiltration	± ^d	-	-	-
10	Microgranuloma	+	+	+	-
	Giant cell infiltration	-	-	+	+
30	Microgranuloma	++	++	+	-
	Giant cell infiltration	+	++	+++	++

^a The symbols are the same as in Table 2.

^b Five animals per group were examined at each stage.

^c Nine control animals and 10 each of the treated groups were examined at 26 weeks.

^d Solitary focus of giant cell was observed in one mouse.

Adrenals and other tissues. No microgranulomatous changes were observed in adrenal glands or any other tissues of mice in any groups. The incidence of neoplastic changes was very low with the exception of lung adenoma. Lung adenomas were observed in several animals. However, there was no difference in incidence of this common spontaneous tumor between control and treated groups.

Electron Microscopy

Liver and lymph nodes were examined by transmission electron microscopy. The results were as follows. The ultrastructure of granulomatous foci was similar in $B\alpha$ - and racemic fenvalerate-treated groups. There were many activated macrophages and giant cells which phagocytosed needle- or rod-shaped crystals as shown in Figs. 3 and 4. The crystals were more often found in the 13-week groups than in the 4-week groups. The crystals were also observed in liver cells of mice fed 1000 ppm of the $B\alpha$ -isomer for 13 weeks. Such crystalline inclusions were never observed in the tissues of mice in the control, $A\alpha$ -isomer-, and A -isomer-treated groups. The phagocytes often formed numerous pseudopods loosely intertwined with those of adjacent macrophages,

so that the cells were not interlocked by the pseudopods. Heterochromatin was often margined along the nuclear membrane. Lipid droplets were sporadically found in the cytoplasm of these phagocytes. There were no remarkable changes in the tissues surrounding the phagocytes. The ultrastructural findings of liver and lymph node in the 52-week group of the racemic fenvalerate were basically the same as those in 4- and 13-week groups. However, the phagocytes often had a large number of lysosomes within the cytoplasm, as shown in Fig. 5. The lysosomes sometimes contained the crystalline rods.

Microgranulomatous Changes Produced by CPIA-Cholesterol

Liver. The results of histopathological examination of mice treated with CPIA-cholesterol are shown in Table 2. The microgranulomas or compact accumulation of mononuclear phagocytes, and formation of multinucleated giant cell (giant cell infiltration) in livers of mice treated with [2R]-, [2S]-, and [2RS]-CPIA-cholesterol were essentially identical to those observed in fenvalerate-treated mice. The changes in the [2RS]-CPIA-

cholesterol group were more prominent than in the [2*R*]- or [2*S*]-CPIA-cholesterol group. For all three compounds the changes were dose related. Table 3 shows grades for microgranulomatous changes in the liver of mice treated with a single iv dose of [2*R*]-CPIA-cholesterol and kept for a 6-month observation period. The granulomatous changes were observed at all the periods. Giant cell infiltration was clearly observed at later stages, while microgranulomas were clearly present at the early stages, as shown in Fig. 6. At 26 weeks, microgranulomas were no longer observed in the treated mice. These changes were mainly noticed in the midzonal and periportal areas and were localized in sinusoidal spaces in affected animals. Electron microscopic examination was carried out on liver of mice in the 30 mg/

kg group killed at 8 and 26 weeks. Giant cells and activated macrophages had phagocytosed numerous crystalline rods. The rods were similar in appearance to those observed in fenvalerate-treated mice. The phagocytes had numerous pseudopods and an abundant cytoplasm laden with a large number of organelles. In the livers of the 26-week group, the phagocytes often had a large number of lysosomes within the cytoplasm, and the lysosomes sometimes contained crystalline rods, as shown in Fig. 7.

Spleen and lymph nodes. The formation of microgranulomatous changes was not as clear in spleen and lymph nodes. Namely, giant cell infiltration of spleen was noticed in two mice treated with 100 mg/kg of the [2*RS*]-CPIA-cholesterol as well as in one mouse from the

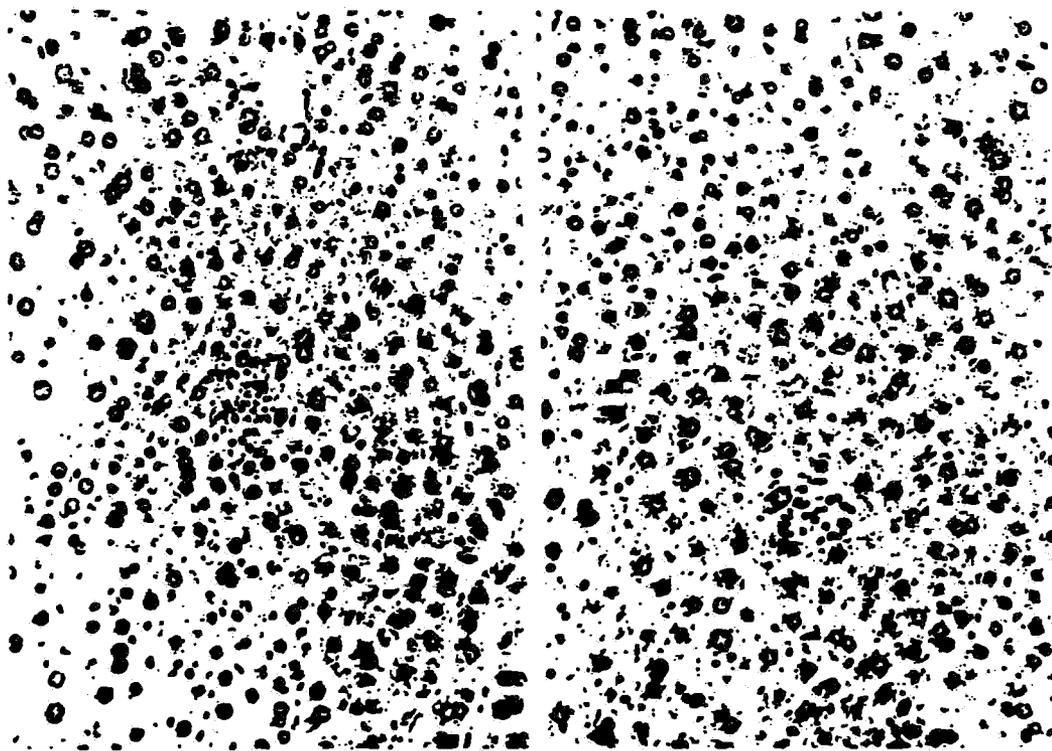


FIG. 6. Microgranulomatous changes in mouse liver (HE, $\times 155$). Left, microgranuloma in liver of a male mouse killed 1 week after a single iv injection of 30 mg/kg of [2*R*]-CPIA-cholesterol. Right, giant cell infiltration in liver of a male mouse killed 26 weeks after the same treatment. Two giant cells of foreign body type are seen.

8-week group treated with 30 mg/kg of [2R]-CPIA-cholesterol. Giant cell infiltration of mesenteric and mandibular lymph nodes was observed in one mouse treated with 100 mg/kg of [2RS]-CPIA-cholesterol.

Histochemistry and Microscopic Autoradiography

Figure 8 shows the results of microscopic autoradiography of the liver obtained from a mouse treated with ^3H -labeled [2R]-CPIA-cholesterol. The ^3H label was clearly localized in giant cells and Kupffer cells in both the 1- and 5-week groups. Giant cell infiltration was more frequently observed in the 5-week group than in the 1-week group. Figure 9 shows the histochemistry results of cholesterol ester in mouse liver obtained from the 5-week group treated with the labeled compound (left). The mouse liver obtained after feeding of 1000 ppm Ba -isomer for 8 weeks was also stained (right). The positive color was observed most frequently in giant cells of livers from mice given CPIA-cholesterol and the Ba -isomer. The results were also positive for liver of mice given 1000 ppm of the Ba -isomer for 13 weeks.

DISCUSSION

The histopathological examinations as reported herein demonstrated that the Ba -isomer of fenvalerate is the causative component for granulomatous changes. Electron microscopic examination of liver and lymph nodes demonstrated the presence of crystalline inclusions in the cytoplasm of the phagocytes in microgranuloma foci in both Ba -isomer- and racemic fenvalerate-treated groups. Such crystals were never observed in either liver or lymph nodes of mice treated with three other isomers. It was shown in the preceding paper (Kaneko *et al.*, 1986) that several tissues, including liver, spleen, and lymph nodes, from mice fed the Ba -isomer contained CPIA-cholesterol. These observations suggest the caus-

ative relationship between the granuloma formation and CPIA-cholesterol. Moreover, it is known that cholesterol crystals cause granulomatous changes (Hiraide *et al.*, 1982; McAllister and Ferrans, 1983; Perzin, 1983; Manz, 1983). Bayliss (1976) reported that granulomas were found in the subcutaneous tissues of rats treated sc with crystalline cholesterol. Therefore, it can be presumed that the CPIA-cholesterol derived from fenvalerate caused the granulomatous changes. This presumption is supported by the fact that microgranulomatous changes were found in the present studies in all the groups treated with the synthesized [2R]-CPIA-cholesterol. Electron microscopy examination of livers of mice treated with the [2R]-CPIA-cholesterol demonstrated the presence of crystalline rods similar to those observed in liver of mice treated with fenvalerate.

The results of histochemistry and micro-



FIG. 7. Crystalline rods in a giant cell of mouse liver ($\times 10,000$), 26 weeks after a single iv injection of 30 mg/kg of [2R]-CPIA-cholesterol. Crystalline rods (arrows) are seen inside lysosomes (arrowheads).

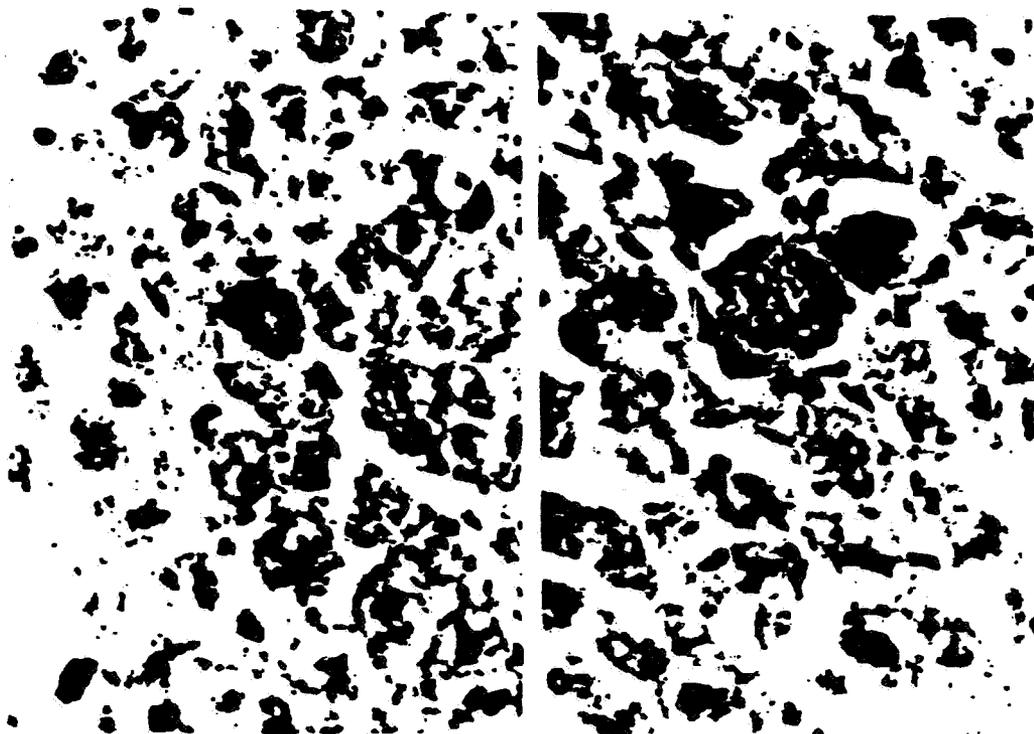


FIG. 8. Microscopic autoradiography of mouse liver treated with tritium-labeled CPIA-cholesterol ($\times 464$). 1 week (left) or 5 weeks (right) after a single iv injection of about 30 mg/kg of ^3H -[2R]-CPIA-cholesterol. ^3H label is localized in Kupffer and giant cells.

scopic autoradiography clearly demonstrated that [2R]-CPIA-cholesterol was phagocytosed by phagocytic cells in the microgranulomatous foci in mouse liver. Moreover, the histochemical staining of cholesterol ester in liver of mice fed the $B\alpha$ -isomer of fenvalerate developed positive coloring in the giant cells.

It is concluded that the causative agent of the microgranulomatous changes induced by fenvalerate is CPIA-cholesterol which is produced in several tissues of rats and mice from the fenvalerate $B\alpha$ -isomer (Kaneko *et al.*, 1986). As described in Table 2, not only [2R]-CPIA-cholesterol but also the [2S] analog which is the component acid moiety of fenvalerate $A\alpha$ - and $A\beta$ -isomers was capable of inducing granulomatous changes. It has been shown that the $A\alpha$ - and $A\beta$ -isomers (more ex-

actly the A -isomer or a mixture of $A\alpha$ and $A\beta$) never produce such histological changes, which is, apparently, a discrepancy from the results shown in Table 2. However, if the absence of *in vivo* biosynthesis of [2S]-CPIA-cholesterol, as demonstrated earlier (Kaneko *et al.*, 1986), is taken into account, then the apparent discrepancy can be resolved.

Clinical observation of mice treated with fenvalerate isomers reveals that remarkable differences exist in the toxicological properties among the chiral isomers of fenvalerate. The most potent isomer in producing neurotoxic signs and death was the $A\alpha$ -isomer. In contrast, the $B\alpha$ - and $B\beta$ -isomers caused neither neurotoxic signs nor death. These data agree with those reported by Nakayama *et al.* (1978) in which the $A\alpha$ -isomer had the highest po-



FIG. 9. Histochemistry of cholesterol ester in mouse liver ($\times 619$), 5 weeks after a single iv injection of about 30 mg/kg of ^3H -[2R]-CPIA-cholesterol (left); and after 8-week feeding of the [2R, α S]-isomer of fenvalerate at 1000 ppm (right). Positive coloring (arrows) is observed in giant cells in both livers.

tency in terms of both insecticidal activity and acute toxicity in mammals while the B-isomers had little effect on insects or mammals. In mice treated with CPIA-cholesterol, there was a significant difference in mortality among [2R]-, [2S]-, and [2RS]-CPIA-cholesterol-treated groups. The mortality of [2R]- and [2RS]-CPIA-cholesterol-treated groups was higher than that of [2S]-CPIA-cholesterol-treated group. Although the mechanism of lethality is known, the [2R]-CPIA-cholesterol seems to be more toxic with respect to the lethal action.

The microgranulomatous changes were observed in lymph nodes, liver, and spleen of fenvalerate-treated mice as already described. However, in mice injected with CPIA-cholesterol, the changes were not clearly seen in lymph nodes and spleen. The difference in the

organ specificity of granulomatous changes between fenvalerate and CPIA-cholesterol may be due to the difference in administration route and/or due to endogenous or exogenous CPIA-cholesterol.

Kaneko *et al.* (1986) reported the *in vivo* retention of CPIA-cholesterol esters by various tissues such as adrenal glands, heart, kidneys, lymph node, spleen, and liver in mice. However, fenvalerate was not reported to cause the microgranulomatous changes in such tissues as heart, kidneys, and adrenal glands in mice (Parker, 1983a). The apparent contradiction may result from the following differences among organs. The content of CPIA-cholesterol in liver and lymph nodes where the granulomas were observed was generally higher than in other organs except for the adrenal

glands (Kaneko *et al.*, 1986). There might be a threshold value for the formation of microgranulomatous changes. However, the content of CPIA-cholesterol in adrenal glands of mice was even higher than in liver and lymph nodes (Kaneko *et al.*, 1986). The following consideration could be made with respect to the absence of granulomatous changes. It is reported that the adrenal glands contain large amounts of cholesterol which is mostly esterified and present in large cytoplasmic droplets of steroid-forming cells (Myant, 1981). Although the localization of CPIA-cholesterol in the organs has not been clarified, it can be speculated that CPIA-cholesterol is contained in the lipid droplet because of its physicochemical properties. If the ester is present in the lipid droplet and is not released from the steroid-forming cells, macrophages cannot phagocytose the ester, so that no granulomatous changes would be expected.

The process of microgranuloma formation by CPIA-cholesterol, shown in Table 3 and Fig. 6, was clearer than those by fenvalerate. The process is considered to be as follows: First, the CPIA-cholesterol is phagocytosed into mononuclear phagocytes or Kupffer cells. Second, the cells mature and form an aggregation, called a microgranuloma. Then, the cells fuse with one another to build multinucleated giant cells.

The CPIA-cholesterol ester is an example of a lipophilic conjugate in addition to triglyceride and fatty acid conjugates (Hutson, 1982). Although the formation of these lipophilic conjugates has been reported earlier (Leighty *et al.*, 1976, 1980; Crayford and Hutson, 1980; Fears *et al.*, 1978, 1982; Quistad *et al.*, 1976, 1978; Schooley and Quistad, 1982; Gunnarsson *et al.*, 1984), the toxicological significance of lipophilic conjugates has not been elucidated. The present report demonstrates that CPIA-cholesterol is a causative agent of granuloma formation in animals. This is the first example of a lipophilic conjugate whose toxicological significance has been reported.

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ATTACHMENT IV

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The Metabolism of Fenvalerate in Plants: The Conjugation of the Acid Moiety

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(Revised manuscript received 25 July 1984)

The metabolism of the pyrethroid insecticide fenvalerate [(*RS*)- α -cyano-3-phenoxybenzyl (*RS*)-2-(4-chlorophenyl)-3-methylbutyrate] (I), and of its most insecticidal ($\alpha S, 2S$) isomer (II), has been examined in cabbage plants grown and treated under laboratory conditions with [^{14}C]chlorophenyl- and [*ring*- ^{13}C]benzyl-labelled preparations of the two compounds. Both insecticides disappeared from the treated leaves with similar half-lives of approximately 12-14 days; they underwent ester cleavage to a significant extent, together with some hydroxylation at the 2- or 4-position of the phenoxy ring, and hydrolysis of the nitrile group to amide and carboxyl groups. Most of the carboxylic acids and phenols thus produced occurred as glycoside conjugates. In separate experiments, the uptake and metabolism of 2-(4-chlorophenyl)-3-methylbutyric acid (X), the acidic half of the molecule, were examined in the laboratory, using abscised leaves of kidney bean, cabbage, cotton, cucumber and tomato plants. The acid X was found to be readily converted, mainly into glucose and 6-*O*-malonylglucose esters in kidney bean, cabbage and cucumber plants, into glucosylxylose, sophorose and gentiobiose esters in cotton, and into two types of triglucose esters with differing isomerism in tomato. One of the acetyl derivatives of the trisaccharide conjugates was identical with the synthetic deca-acetyl derivative of the [1 \rightarrow 6]-triglucose ester.

1. Introduction

Fenvalerate [(*RS*)- α -cyano-3-phenoxybenzyl (*RS*)-2-(4-chlorophenyl)-3-methylbutyrate] ('Sumicidin') is one of the most potent pyrethroid insecticides, controlling various insect pests on cotton plants and other crops.^{1,2} The metabolism of fenvalerate (I) in bean plants has already been reported³ and it was shown that a large number of metabolites were formed, mainly as a result of hydrolysis at the ester bond and/or hydroxylation of the alcohol moiety. Major metabolites were 3-phenoxybenzoic acid (VI) and its hydroxy derivatives, and 2-(4-chlorophenyl)-3-methylbutyric acid (X). Both of these metabolites occurred also as glycoside conjugates in plants. In a previous publication,⁴ it has been shown that VI was rapidly converted in abscised leaves of various plants into more polar products by esterification with glucose, glucosylxylose, gentiobiose, cellobiose, malonylglucose and two types of triglucose with differing isomerism. Further, More *et al.*⁵ suggested the presence of glucosylarabinose ester of VI in cotton, vine and other plants.

Fenvalerate has two chiral centres: at C-2 of the acid moiety and at the α -C of the alcohol moiety. Among the four isomers, the most active possesses the absolute configuration of ($\alpha S, 2S$). In the present paper the metabolic fate of fenvalerate and the ($\alpha S, 2S$) isomer in cabbage, is reported, together with results of the subsequent work on the fate of X in abscised leaves of cabbage, tomato, bean, cucumber and cotton plants.

2.1. Radiolabelled

The following optically pure [chlorophenyl-compounds; a phenyl nucleus Sumitomo Ch [chlorophenyl-mical purities

2.2. Unlabelled

The unlabelled procedures for reported elsewhere synthesised by spectrometric

2.3. Chromatography

Precoated silica were used for thin-layer chromatography

- A, hexane
- B, benzene
- C, hexane
- D, ethyl acetate

Esters with skeleton

I	(<i>RS</i>)
II	(<i>S</i>)- <i>c</i>
III	α -C ₂
IV	α -C ₁
V	3-(4)

3-Phenoxybenzoic acid

VI	3-P
VII	3-(2)
VIII	3-(4)
IX	3-P

4-Chlorophenyl

X	2-(4)
---	-------

Glycoside derivatives

XI	1-(4)
XII	1-(4)
XIII	1-(4)
XIV	1-(4)
XV	1-(4)
XVI	1-(4)
XVII	1-(4)
	ac

2. Experimental methods

2.1. Radiolabelled compounds

The following ^{14}C preparations were used: fenvalerate (I) and its ($\alpha S, 2S$) isomer (II), >99% optically pure, both labelled uniformly either in the chlorophenyl ring, referred to as the [chlorophenyl- ^{14}C] compounds, or in the ring of the benzyl group, referred to as the [benzyl- ^{14}C] compounds; and (RS)-2-(4-chlorophenyl)-3-methylbutyric acid (X) labelled uniformly in the phenyl nucleus. These labelled compounds were prepared in Takarazuka Research Center, Sumitomo Chemical Co. Ltd.^{6,7} The specific activity was 15.6 Ci mol^{-1} for [^{14}C]X and the [chlorophenyl- ^{14}C] compounds, and 20.8 Ci mol^{-1} for the [benzyl- ^{14}C] compounds. The radiochemical purities of all samples, as determined by thin-layer chromatography (t.l.c.) were >99.5%.

2.2. Unlabelled reference compounds

The unlabelled compounds listed in Table 1 were prepared for reference purposes. The procedures for the synthesis of I-X, and their R_f values by t.l.c. in various solvent systems, are reported elsewhere.⁶⁻¹⁰ The acetyl derivatives of the mono-, di- and tri-saccharides of X were synthesised by the same procedures used for those of VI.⁴ The R_f values by t.l.c., along with the spectrometric properties are summarised in Table 2.

2.3. Chromatography

Precoated silica gel 60F-254 chromatoplates (20×20 cm, 0.25 mm layer thickness, E. Merck) were used for analytical and preparative purposes. The solvent systems used to develop thin-layer chromatograms were (by volume):

- A, hexane + toluene + acetic acid (3+15+2);
- B, benzene (saturated with formic acid) + diethyl ether (10+3);
- C, hexane + diethyl ether (20+1);
- D, ethyl acetate + ethanol + water (4+2+1);

Table 1. Fenvalerate and its derivatives

<i>Esters with skeleton intact and a decarboxylated derivative</i>	
I	(RS)- α -Cyano-3-phenoxybenzyl (RS)-2-(4-chlorophenyl)-3-methylbutyrate
II	(S)- α -Cyano-3-phenoxybenzyl (S)-2-(4-chlorophenyl)-3-methylbutyrate
III	α -Carbamoyl-3-phenoxybenzyl 2-(4-chlorophenyl)-3-methylbutyrate
IV	α -Carboxy-3-phenoxybenzyl 2-(4-chlorophenyl)-3-methylbutyrate
V	3-(4-Chlorophenyl)-4-methyl-2-(3-phenoxyphenyl)pentanenitrile
<i>3-Phenoxybenzyl derivatives</i>	
VI	3-Phenoxybenzoic acid
VII	3-(2-Hydroxyphenoxy)benzoic acid
VIII	3-(4-Hydroxyphenoxy)benzoic acid
IX	3-Phenoxybenzyl alcohol
<i>4-Chlorophenyl derivative</i>	
X	2-(4-Chlorophenyl)-3-methylbutyric acid
<i>Glycoside derivatives of X</i>	
XI	1-[2-(4-Chlorophenyl)-3-methylbutyryl]- β -D-glucopyranose tetra-acetate
XII	1-[2-(4-Chlorophenyl)-3-methylbutyryl]- β -D-sophorose hepta-acetate
XIII	1-[2-(4-Chlorophenyl)-3-methylbutyryl]- β -D-laminarabinose hepta-acetate
XIV	1-[2-(4-Chlorophenyl)-3-methylbutyryl]- β -D-cellobiose hepta-acetate
XV	1-[2-(4-Chlorophenyl)-3-methylbutyryl]- β -D-gentiobiose hepta-acetate
XVI	1-[2-(4-Chlorophenyl)-3-methylbutyryl]-2,3,4-tri-O-acetyl-6-O-(2,3,4-tri-O-acetyl- β -D-xylopyranosyl)- β -D-glucopyranose
XVII	1-[2-(4-Chlorophenyl)-3-methylbutyryl]-O-2,3,4,6-tetra-O-acetyl- β -D-glucopyranosyl-(1-6)-O-2,3,4-tri-O-acetyl- β -glucopyranosyl-(1-6)-O-2,3,4-tri-O-acetyl- β -glucopyranosyl-(1-6)-O-2,3,4-tri-O-acetyl- β -D-glucopyranose

Table 2. Analytical data for 2-(4-chlorophenyl)-3-methylbutyric acid (X) and its glycoside acetyl derivatives

Compound	R_f values ^a		$[\alpha]_D^{25}$ at 25°C (chloroform)	¹ H-N.m.r. data (ppm) (deuteriochloroform solution)
	E	F		
X	0.05	0.10	0° (C5.0)	11.0 (1H, 6s), 7.31 (4H, s), 3.15 (1H, d, J=10 Hz), 2.3 (1H, m), 1.06 (1H, d, J=7 Hz), 0.70 (1H, d, J=7 Hz)
XI	0.05	0.5 ^b	-17.4° (C8.6)	7.30 (4H, s), 5.75 (1H, d, J=9 Hz), 5.0-5.3 (3H, m), 3.7-4.5 (3H, m), 3.20 (1H, d, J=10 Hz), 2.4 (1H, m), 2.0-2.1 (12H, 4xs), 1.05 (3H, dd, J=7 Hz), 0.7 (3H, dd, J=7 Hz)
XII	0.29	0.4 ^b	-11.2° (C7.3)	7.35 (4H, s), 5.65 (1H, d, J=9 Hz), 5.53 (1H, d, J=9 Hz), 3.4-5.5 (12H, m), 3.25 (1H, d, J=10 Hz), 2.4 (1H, m), 2.0-2.1 (21H, 7xs), 1.07 (3H, dd, J=7 Hz), 0.75 (3H, dd, J=7 Hz)
XIII	0.29	0.49	-35.1° (C7.3)	7.30 (4H, s), 5.63 (1H, d, J=9 Hz), 5.53 (1H, d, J=9 Hz), 3.16 (1H, d, J=10 Hz), 2.4 (1H, m), 1.95-2.10 (21H, 7xs), 1.0 (3H, dd, J=7 Hz), 0.7 (3H, dd, J=7 Hz)
XIV	0.33	0.53	-30.7° (C5.5)	7.30 (4H, s), 5.68 (1H, d, J=9 Hz), 5.53 (1H, d, J=9 Hz), 3.5-5.3 (15H, m), 3.15 (1H, d, J=10 Hz), 2.45 (1H, m), 2.0-2.1 (21H, 7xs), 1.0 (3H, dd, J=7 Hz), 0.7 (3H, dd, J=7 Hz)
XV	0.31	0.51	-25.0° (C4.6)	7.35 (4H, s), 5.76 (1H, d, J=9 Hz), 5.65 (1H, d, J=9 Hz), 3.5-5.5 (15H, m), 3.23 (1H, d, J=10 Hz), 2.4 (1H, m), 2.0-2.15 (21H, 7xs), 1.05 (3H, dd, J=7 Hz), 0.75 (3H, dd, J=7 Hz)
XVI	0.41	0.51	-2.5° (C4.0)	7.40 (4H, s), 5.57 (1H, d, J=9 Hz), 5.53 (1H, d, J=9 Hz), 3.5-5.4 (14H, m), 3.2 (1H, d, J=10 Hz), 2.4 (1H, m), 2.0-2.1 (18H, 6xs), 1.0 (3H, dd, J=7 Hz), 0.7 (3H, dd, J=7 Hz)
XVII	0.23	0.45	-5.3° (C7.4)	7.35 (4H, s), 5.70 (3H, 3xddd, J=9 Hz), 3.4-5.5 (22H, m), 3.2 (1H, d, J=10 Hz), 2.4 (1H, m), 1.95-2.15 (30H, 10xs), 1.06 (3H, dd, J=7 Hz), 0.7 (3H, dd, J=7 Hz)

^aSolvent systems: E, chloroform-ethyl acetate (2-1 by volume); F, benzene+ethyl acetate (1-2 by volume).

E, chloroform+ethyl acetate (2+1);

F, benzene-ethyl acetate (1+2).

The solvent systems used for two-dimensional development are illustrated, for example, as follows: (A × 2, B) indicates development in the first direction twice with solvent system A, and in the second direction with solvent system B. The radioactive areas on chromatograms were detected by autoradiography, and unlabelled standard chemicals were detected under ultraviolet light.

High-performance liquid chromatography (h.p.l.c.) was performed with a Hitachi Model 635 using a stainless steel column (20 × 0.4 mm i.d.) packed with Hypersil ODS, and eluted with water+acetonitrile (1-4, by volume) at a flow rate of 1.3 ml min⁻¹. Component elution was monitored with a multi-wavelength detector (230 nm) and a Berthold radioactivity monitor 1B 503 linked in series.

2.4. Spectroscopy

Proton nuclear magnetic resonance (n.m.r.) and ¹³C-n.m.r. were determined, in either deuteriochloroform or tetradeuteromethanol, with Hitachi model R-40 and R-900 spectrometers at 90 MHz, respectively. Infrared (i.r.) spectra were recorded on a Hitachi model 385 spectrometer, as potassium bromide disks or as a liquid thin film on sodium chloride crystals. Optical rotations were measured with a Perkin-Elmer polarimeter at 25°C in chloroform (1-dm cell). Gas chromatography-mass spectrometry (g.c./m.s.) analysis was carried out on Finnigan model 4000 gas chromatograph-mass spectrometer equipped with model 6111 data system. The column conditions were: column, 5% PEG-20M on 80-100 mesh GasChrom Q (50 × 0.2 cm i.d. glass column); oven temperature, 110°C; injector temperature, 140°C; separator temperature, 250°C; carrier gas, helium at 20 ml min⁻¹. The ionisation energy and trap current were 20 eV and 150 μA, respectively. Field desorption mass (f.d.m.s.) spectra were recorded on a Hitachi M-80 spectrometer equipped with model M-003 data system.

2.5. Radioassay

Liquid scintilla reported previ

2.6. Plant treat

2.6.1. Treatme

Cabbages (*Bra* 25(±2)°C befo I or II was ev seedlings at th microsyringe. 7 them into the cut into small acetone + water the residuum and then evap small volume c to t.l.c.

2.6.2. Treatme

In addition to daylight and te Giza): kidney Kurumeochiai) removed as rec Groups of thre an aqueous sol They were ma described abo

Separate ex aqueous soluti between dieth the mass of co aqueous fracti acetate to extr was concentra and by h.p.l.c yellow syrup. and Sephadex system D and

2.7. Characte

Metabolites w enzymic hydro two labelled p from the hydro

2.7.1. Hydroly

The conjugate in an acetate Sigma Ltd) o

2.5. Radioassay

Liquid scintillation counting (l.s.c.), combustion analysis and autoradiography were performed as reported previously.¹¹

2.6. Plant treatment and extraction

2.6.1. Treatment with [¹⁴C]fenvalerate (I) and its optical isomer (II)

Cabbages (*Brassica oleracea*, var *capitata* cv. Shikidori) were allowed to grow in a greenhouse at 25(±2)°C before or after treatment. Either a [*chlorophenyl*-¹⁴C] or a [*benzyl*-¹⁴C] preparation of I or II was evenly applied to the upper surface of two fifth-sixth leaves of the 4-week old seedlings at the rate of 20 µg in 200 µl acetone per leaf (approximate area 25 cm²) by a microsyringe. The treated plants were sampled by cutting them off at ground level and separating them into the treated leaves and the non-treated shoot portions. Each portion of the plant was cut into small pieces and homogenised in a Waring blender for 10 min with 10 ml of acetone + water (1+1 by volume) per gram of the plant material. The mixture was filtered and the residuum was re-extracted twice in the same way. The combined filtrate was radioassayed and then evaporated to dryness on a rotary evaporator at 40°C. The residue was redissolved in a small volume of acetone + water (1+1 by volume), and an aliquot of this solution was subjected to t.l.c.

2.6.2. Treatment with [¹⁴C]-2-(4-chlorophenyl)-3-methylbutyric acid (X)

In addition to cabbages, the following four species of plants, grown in the greenhouse with daylight and temperature at 25(±2)°C, were used in the experiments: cotton (*Gossypium* spp. cv. Giza); kidney bean (*Phaseolus vulgaris* L. cv. Nagauzura); cucumber (*Cucumis sativus* L., cv. Kurumeochiai); tomato (*Lycopersicon esculentum* Mill. cv. Fukuju No. 2). Mature leaves were removed as required. The petiole was immersed in water and cut an angle of 45° using a scalpel. Groups of three abscised leaves were immediately transferred to a 100-ml glass flask containing an aqueous solution (100 ml) of [¹⁴C]X (1 µg ml⁻¹), so that the cut ends dipped into the solution. They were maintained in the greenhouse before sampling. The treated leaves were extracted as described above in section 2.6.1.

Separate experiments were carried out using 200 abscised leaves that were supplied with an aqueous solution (4 litres) of [¹⁴C]X (100 µg ml⁻¹) for up to 5 days. The extracts were partitioned between diethyl ether (200 ml) and distilled water (300 ml) to separate the ¹⁴C-metabolites from the mass of coloured plant materials. Approximately 90% of the radiocarbon remained in the aqueous fraction. After acidification to pH 3, the aqueous layer was partitioned with ethyl acetate to extract the starting material and malonylglucoside conjugates. The ethyl acetate layer was concentrated, and the metabolites were purified by preparative t.l.c. in solvent system D, and by h.p.l.c. The remaining aqueous fraction was neutralised at 0-5°C and lyophilised to give a yellow syrup. Some metabolites were purified by column chromatography on a Sephadex G-25 and Sephadex G-50 column (3 cm i.d. × 80 cm), and by repeated preparative t.l.c. in solvent system D and in chloroform + methanol (1+1 by volume), and by h.p.l.c.

2.7. Characterization of metabolites

Metabolites were identified by t.l.c. cochromatography with authentic samples, before or after enzymic hydrolysis, methylation or acetylation. Direct comparison by t.l.c. of metabolites from two labelled preparations of I or II was used to distinguish products retaining the ester linkage from the hydrolysis products.

2.7.1. Hydrolysis with enzymes

The conjugated metabolites of I, II (>5000 disintegrations min⁻¹) and X (1-2 mg) were dissolved in an acetate buffer (1 ml) at pH 4.5, and incubated with β-glucosidase (2 mg, almond, from Sigma Ltd) or cellulase (2 mg, *Aspergillus niger*, from Sigma Ltd) at 37°C for 12 h. The enzymic

cleavage was complete under these conditions because no further cleavage occurred on either longer incubation or reincubation with enzymes.

2.7.2. Analysis of the aglycone moiety

The aglycone released on treatment with enzyme was extracted with ethyl acetate (1 ml×3) for radioassay by l.s.c. and analysis by t.l.c.

2.7.3. Analysis of the sugar moiety

The aqueous fraction, containing sugars released on treatment with cellulase, was analysed directly by t.l.c. in butan-1-ol + propan-2-ol + water (130+47+23 by volume), or in butan-1-ol + acetone + water (4+5+1 by volume). Sugars on t.l.c. plates were detected by spraying with either silver nitrate solution (50 g litre⁻¹), or a solution of 2,3,5-triphenyltetrazolium chloride (20 g litre⁻¹) in 1M-methanolic sodium hydroxide solution, followed by heating to 110°C for 5 min. The *R_f* values of the various saccharides were reported previously.¹² In parallel, a certain volume of the aqueous layer was subjected to colorimetric analysis at 620 nm by the anthrone-sulphuric acid method,¹³ to determine the glucose content.

2.7.4. Acetylation

Samples were treated with acetic anhydride + pyridine (2+1 by volume) at 80°C for 1 h. The reagents were then removed under vacuum and the products were analysed directly by t.l.c.

2.7.5. Methylation

Samples were methylated with an ethereal solution of diazomethane for 1 h. The products were subjected to analysis by g.c./m.s.

3. Results

3.1. Metabolism of fenvalerate (I) and its (*αS*,2*S*) isomer (II) in cabbages

After foliar application of ¹⁴C-labelled I or II to cabbages, the recovery of total radiocarbon gradually decreased. After 48 days, approximately 36–39% of the applied radiocarbon was recovered from plants treated with [¹⁴C]II. Most of the recovered radiocarbon resided in the treated leaves, with less than 3% of the dose in other parts of the plants. Analysis by t.l.c. showed that no parent compound was found in the shoots, except in the treated leaves.

Fenvalerate (I) and its isomer (II) disappeared from the treated leaves with initial half-lives of 14 and 12 days, respectively. The concentrations of I and II after 48 days were 0.63 and 0.56 μg g⁻¹, respectively. There seemed to be no difference in the rates of metabolism of I and II in cabbages.

A number of metabolites were present in the plant extracts, in free and conjugated forms. More than nine free products were detected by t.l.c. on silica gel plates in solvent system A. Each product, separated by preparative t.l.c., was identified by two dimensional cochromatography with the synthesised compounds, in the following solvent systems: I, III, IV and VI–X (A, B); II and V (A, C×6).

The conjugated metabolites, located at the origin of the t.l.c. plates in solvent system A, were scraped off, eluted with methanol + water (1+1 by volume) and then developed again by t.l.c. in solvent system D. At least four products were detected with the [*chlorophenyl*-¹⁴C] label, and seven products with the [*benzyl*-¹⁴C] label. The individual conjugates were subjected to β-glucosidase and/or cellulase hydrolysis, and the released aglycones were identified as described above. The minor conjugates were readily cleaved by β-glucosidase, whereas the major conjugates were resistant to β-glucosidase hydrolysis but completely cleaved by cellulase.

The amounts of I, II and their metabolites in cabbages are summarised in Table 3. The (*αR*,2*S*) isomer of II was hardly found in the treated leaves, suggesting that no *αR/αS* epimerisation occurred in plants. The major metabolites of I and II were ester cleavage products, such as 3-phenoxybenzoic acid (VI) and 2-(4-chlorophenyl)-3-methylbutyric acid (X), which

Table 3. Amount

Sample
Treated leaves
Extract ¹⁴ C
Unchanged
III
IV (free)
(conj)
V
VI (free)
(conj)
VII (free)
(conj)
VIII (free)
(conj)
IX (free)
(conj)
X (free)
(conj)
Others
Bound ¹⁴ C
Untreated shoot
Total ¹⁴ C

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Table 3. Amounts of fenvalerate (I), the (α S,2S)-fenvalerate isomer (II), and their metabolites in cabbages, 24 and 48 days after foliar application of 14 C-labelled preparations

Sample	14 C (as % of the applied dose)							
	(α RS,2RS)-Fenvalerate				(α S,2S)-Fenvalerate isomer			
	[Chlorophenyl- 14 C]		[Benzyl- 14 C]		[Chlorophenyl- 14 C]		[Benzyl- 14 C]	
	24 days	48 days	24 days	48 days	24 days	48 days	24 days	48 days
Treated leaves	56.8	33.3	52.6	37.5	56.4	32.7	48.9	31.5
Extract 14 C	54.6	32.2	45.9	31.8	52.7	28.3	45.0	30.3
Unchanged I or II	40.7	18.5	36.2	19.9	37.5	17.3	31.5	17.1
III	1.1	1.3	0.8	1.6	0.8	1.0	0.7	1.4
IV (free)	0.5	0.7	0.3	0.6	0.1	<0.1	0.3	0.1
(conjugated)	0.7	0.7	0.2	0.4	0.2	0.3	0.5	0.3
V	1.8	2.2	1.9	2.1	0.6	0.1	0.9	0.5
VI (free)	—	—	0.1	0.1	—	—	0.1	<0.1
(conjugated)	—	—	3.2	3.1	—	—	4.5	4.0
VII (free)	—	—	<0.1	<0.1	—	—	0.7	0.5
(conjugated)	—	—	0.3	0.2	—	—	0.1	0.3
VIII (free)	—	—	<0.1	<0.1	—	—	<0.1	0.3
(conjugated)	—	—	1.1	1.1	—	—	2.2	1.9
IX (free)	—	—	<0.1	0.1	—	—	0.2	<0.1
(conjugated)	—	—	0.1	0.1	—	—	1.0	<0.1
X (free)	0.1	0.3	—	—	1.1	1.9	—	—
(conjugated)	7.1	4.7	—	—	8.5	5.4	—	—
Others	2.6	3.8	1.7	2.5	3.9	2.3	2.3	3.9
Bound 14 C	2.2	1.1	6.7	5.7	3.7	4.4	3.9	1.2
Untreated shoots	1.4	3.0	0.6	1.0	0.9	0.5	1.2	0.8
Total 14 C	58.2	36.3	53.2	38.5	57.3	33.2	50.1	32.3

occurred mainly as glycoside conjugates. It appears that there was no significant difference between the amounts of metabolites formed from I and II.

3.2. Uptake and metabolism of [14 C]X in abscised leaves

As the glycoside conjugates of X were major plant metabolites of I and II, separate studies of the metabolism of X in abscised leaves were undertaken in order to obtain more information on the nature of the conjugates formed.

3.2.1. Comparative metabolism of [14 C]X in abscised leaves of various plants

The abscised leaves of cabbages were treated with an aqueous solution of X ($1 \mu\text{g ml}^{-1}$) for up to 3 days. Uptake occurred readily, and analysis by t.l.c. showed that one major product (designated as C6), together with small amounts of unchanged X and two minor products (C2, C5), were present in the plant extracts (Figure 1). The parallel experiments were also carried out with abscised leaves of tomato, cucumber, bean and cotton plants. The metabolic profiles of X in cabbages were very similar to those in bean and cucumber plants, but different from those in tomato and cotton plants. Relatively large amounts of C5 were found in tomato and cotton plants. In addition, C3 was found only in cotton, and C7 and C8 only in tomato. In contrast, C6 was hardly detected in extracts of cotton and tomato leaves.

3.2.2. Identification of metabolites

Some components (approximately 3–15 mg) were isolated from a total of 200 abscised leaves of tomato, cotton and cabbages treated with an aqueous solution of [14 C]X ($100 \mu\text{g ml}^{-1}$) for 5 days. Each of the purified samples was quantitatively hydrolysed by cellulase, and the released aglycone and sugar moieties were identified by thin-layer cochromatography. Furthermore, the [14 C]aglycone and sugar moieties were quantitatively analysed by l.s.c. and colorimetric methods.

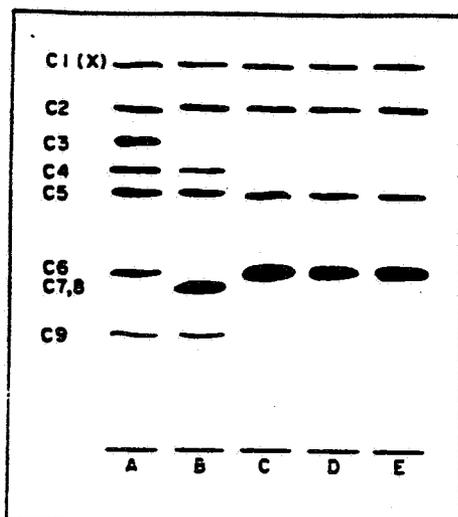


Figure 1. Autoradiogram of a thin-layer chromatographic plate, showing separation of the metabolites of 2-(4-chlorophenyl)-3-methylbutyric acid (X) in abscised leaves of various plants: (A) cotton, (B) tomato, (C) cabbage, (D) kidney bean and (E) cucumber. The developing solvent was ethyl acetate + ethanol + water (4+2+1 by volume).

respectively, and the molar ratios of glucose to the aglycone were determined. The results are summarised in Table 4.

Component C1 was identified as unchanged X by thin-layer cochromatography in solvent systems (A, B).

Component C2 was identified as 1-[2-(4-chlorophenyl)-3-methylbutyryl]- β -D-glucopyranose based on the information described below. The component released an equimolar mixture of X and glucose, and upon acetylation gave one product of low polarity by t.l.c., which had identical specific optical rotation ($[\alpha]_D -16.8^\circ$) and R_f values with those of XI.

Component C3 released unchanged X, glucose and xylose. Although the molar ratios of the sugars to X were not determined, acetylation gave one less polar product with a molecular ion of $m/e=759$, suggesting the presence of equal moles of X, glucose and xylose in the molecule. As the specific optical rotation ($[\alpha]_D -1.4^\circ$) and the R_f values of the corresponding acetate were very close to those of XVI, component C3 was identified as 1-[2-(4-chlorophenyl)-3-methylbutyryl]-6-O-(β -D-xylopyranosyl)- β -D-glucopyranose.

Each component of C4 and C5 released two molar equivalents of glucose to one of X, and upon acetylation gave less polar products with the same molecular ion of $m/e=835$. It is likely, therefore, that these products are diglucose conjugates with different glucosidic linkages. The

Table 4. Analytical data for glycoside conjugates of 2-(4-chlorophenyl)-3-methylbutyric acid (X)

Metabolite	H.p.l.c. retention time ^a (min)	Cellulase treatment			Molar ratio (glucose/aglycone)	Acetyl derivative (m/e by f.d.m.s.)
		Aglycone	Sugar(s)	Fatty acid		
C1	43.5	X	Glucose	ND	1.0	543
C3	—	X	Glucose-xylose	ND	—	759
C4	20.5	X	Glucose	ND	2.1	835
C5	25.8	X	Glucose	ND	2.0	835
C6	2.8	X	Glucose	Malonic acid	1.2	—
C7	1.9	X	Glucose	ND	3.1	1119
C8	8.6	X	Glucose	ND	3.0	1119
C9	10.7	X	Glucose	Malonic acid	2.1	—

^a Retention time of X=4.3 min.
ND=not detected.

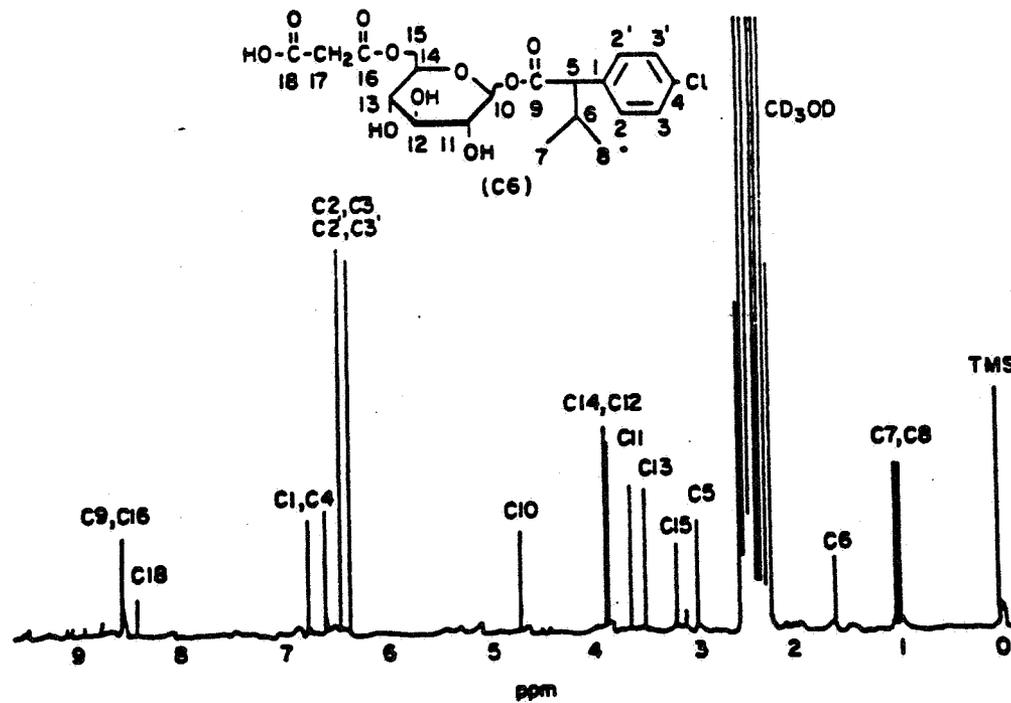
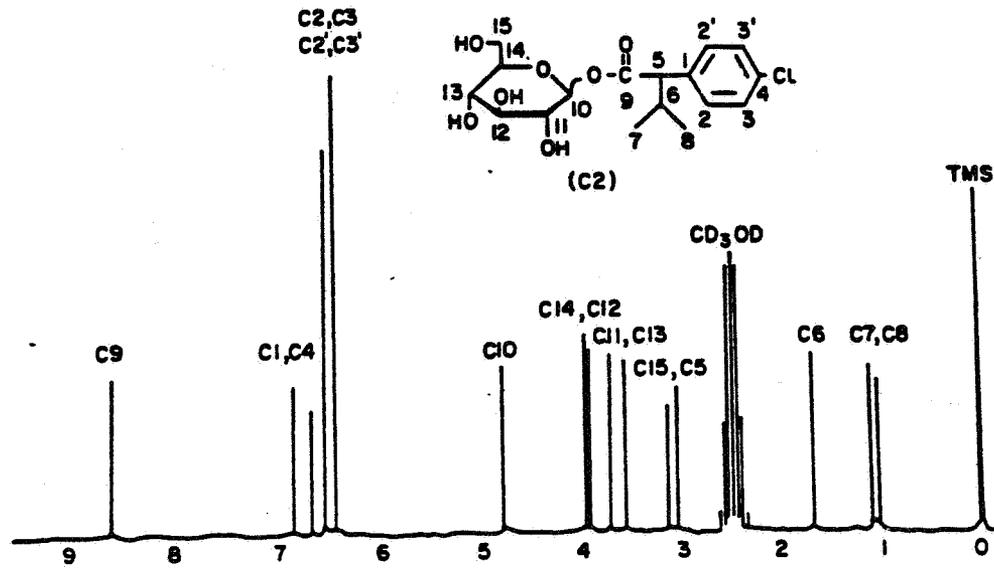


Figure 2. ^{13}C -N.M.R. spectra of metabolites C2 and C6 extracted from cabbage leaf. TMS=tetramethylsilane.

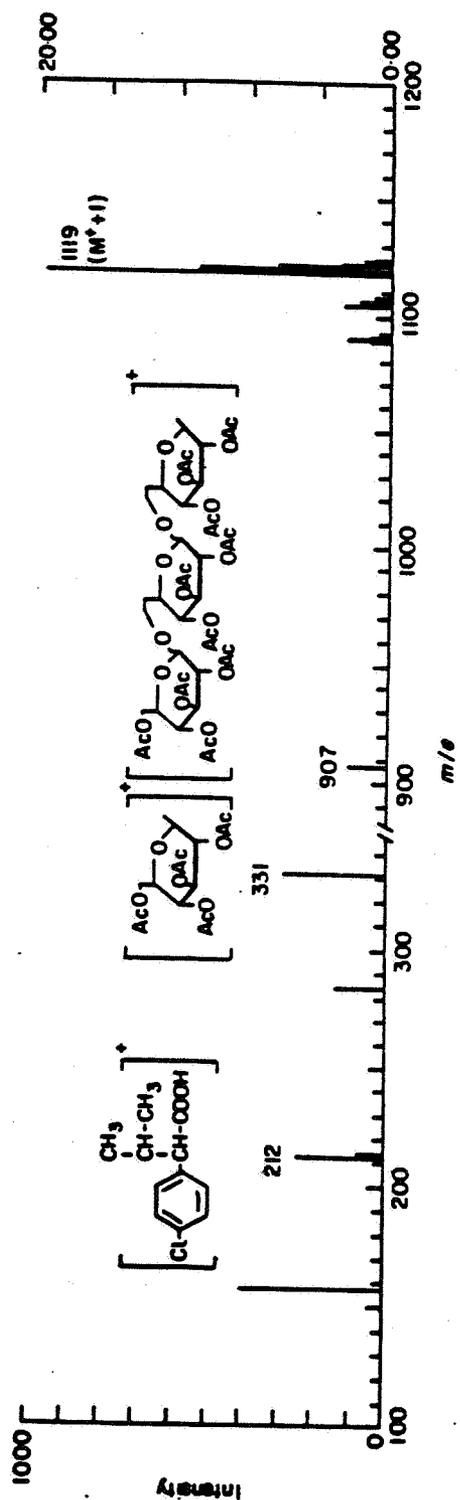


Figure 3. Field desorption mass spectra of deca-acetyl derivatives of metabolites C7 and C8 extracted from immature leaf.

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Component Malonic acid spectrometry C6 was labile spectra show the carboxyl derivative of determined 1 Figure 2, the from the carb 10⁻⁶ to 63.6; signal due to Based upon 6-O-malonyl

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Table 5. Uptake

Sample
¹⁴ C in plants
Extracts
C1 (X)
C2
C3
C4
C5
C6
C7
C8
C9
Others
Bound reau
¹⁴ C in water
Total ¹⁴ C

acetyl derivative of C4 had an R_f value that was very close to those of XII or XIII in solvent system F. However, the specific optical rotation ($[\alpha]_D -12^\circ$) was in good agreement with that of XII and apparently different from that of XIII. On the other hand, the R_f value of the C5 acetyl derivative was close to those of XIII, XIV or XV in solvent system F. However, the specific optical rotation ($[\alpha]_D -23.8^\circ$) was in good accord with that of XV, but different from that of the laminarabinose (XIII) or cellobiose (XIV) ester. Thus, components C4 and C5 were considered to be 1-[2-(4-chlorophenyl)-3-methylbutyryl]-2-*O*-(β -D-glucopyranosyl)- β -D-glucopyranose (a sophorose conjugate) and 1-[2-(4-chlorophenyl)-3-methylbutyryl]-6-*O*-(β -D-glucopyranosyl)- β -D-glucopyranose (a gentiobiose conjugate), respectively.

Component C6 released unchanged I, glucose and malonic acid in molar ratios of 1.0:1.2:0.8. Malonic acid was identified by g.c./m.s. and estimated by selected ion monitoring mass spectrometry at $m/e=101$ and 132, according to the methods reported previously.⁴ Component C6 was labile and was converted into C2 to a limited extent on t.l.c. in solvent system D. The spectra showed the strong absorption bands at 2500-3000, 1600 and 1390 cm^{-1} corresponding to the carboxyl and carboxylate groups. This evidence suggests that C6 is a malonate hemi-ester derivative of C2. The substitution position of the glucose moiety with malonic acid was determined by comparison of the ^{13}C -n.m.r. spectra in tetradeuteromethanol. As shown in Figure 2, the spectrum of C6 differed from that of C2 primarily by the additional signals derived from the carbonyl carbons (188.1, 190.7 parts 10^{-6}), and the lower shift of the signal (from 61.4 parts 10^{-6} to 63.6 parts 10^{-6}) originating from the C-6 position of the glucose moiety. It appears that the signal due to the methylene carbon of malonic acid overlaps with that of tetradeuteromethanol. Based upon these results, component C6 was identified as 1-[2-(4-chlorophenyl)-3-methylbutyryl]-6-*O*-malonyl- β -D-glucopyranose.

Although C7 and C8 appeared to be a single product on t.l.c. in solvent system D, they were separated from each other by h.p.l.c. (Table 4). Components C7 and C8 released three molar equivalents of glucose to one of X, and upon acetylation gave less polar products with respective R_f values of 0.23 and 0.20 on t.l.c. in solvent system E; both of these products showed the molecular ion of $m/e=1119$ (M^++1) by f.d.m.s. (Figure 3). Therefore, they were isomers of a triglucose ester of X, with different substitution positions for the glucose moiety. As the deca-acetyl derivative of C7 was identical to XVII on t.l.c. in solvent systems E and F, component C7 was tentatively identified as 1-[2-(4-chlorophenyl)-3-methylbutyryl]-*O*- β -D-

Table 5. Uptake and metabolism of 2-(4-chlorophenyl)-3-methylbutyric acid (X) in abscised leaves of various plants over a 24- or 72-h period

Sample	^{14}C (as % of the applied dose)									
	Bean		Cabbage		Cotton		Cucumber		Tomato	
	24 h	72 h	24 h	72 h	24 h	72 h	24 h	72 h	24 h	72 h
^{14}C in plants	12.3	58.2	49.6	70.0	32.6	69.8	6.9	12.8	26.8	67.8
Extracts	0.7	54.8	44.8	63.7	29.6	64.9	6.1	11.7	24.7	62.4
C1 (X)	2.5	0.5	3.3	4.8	11.0	3.3	2.7	2.1	1.4	3.3
C2	0.5	3.2	2.2	3.4	3.2	1.9	0.2	0.5	1.2	1.3
C3	—	—	—	—	8.0	29.9	—	—	—	—
C4	—	—	—	—	3.7	12.7	—	—	0.3	1.4
C5	0.2	1.3	<0.1	<0.1	2.4	12.1	0.1	0.4	0.8	2.3
C6	6.2	46.0	35.9	52.0	0.2	0.9	3.0	8.3	—	—
C7	—	—	—	—	—	—	—	—	8.9	25.1
C8	—	—	—	—	—	—	—	—	9.8	22.0
C9	0.1	<0.1	<0.1	<6	<0.1	<0.1	<0.1	0.1	0.4	2.7
Others	1.2	3.8	3.4	3.5	4.8	4.1	0.1	0.3	2.3	7.0
Bound residue	1.6	3.4	4.8	6.3	3.0	4.9	0.8	1.1	1.7	2.7
^{14}C in water	85.6	27.2	31.8	13.4	54.9	23.5	91.4	77.4	62.6	25.0
Total ^{14}C	97.9	85.4	81.4	83.4	87.5	93.3	98.3	90.2	87.4	92.8

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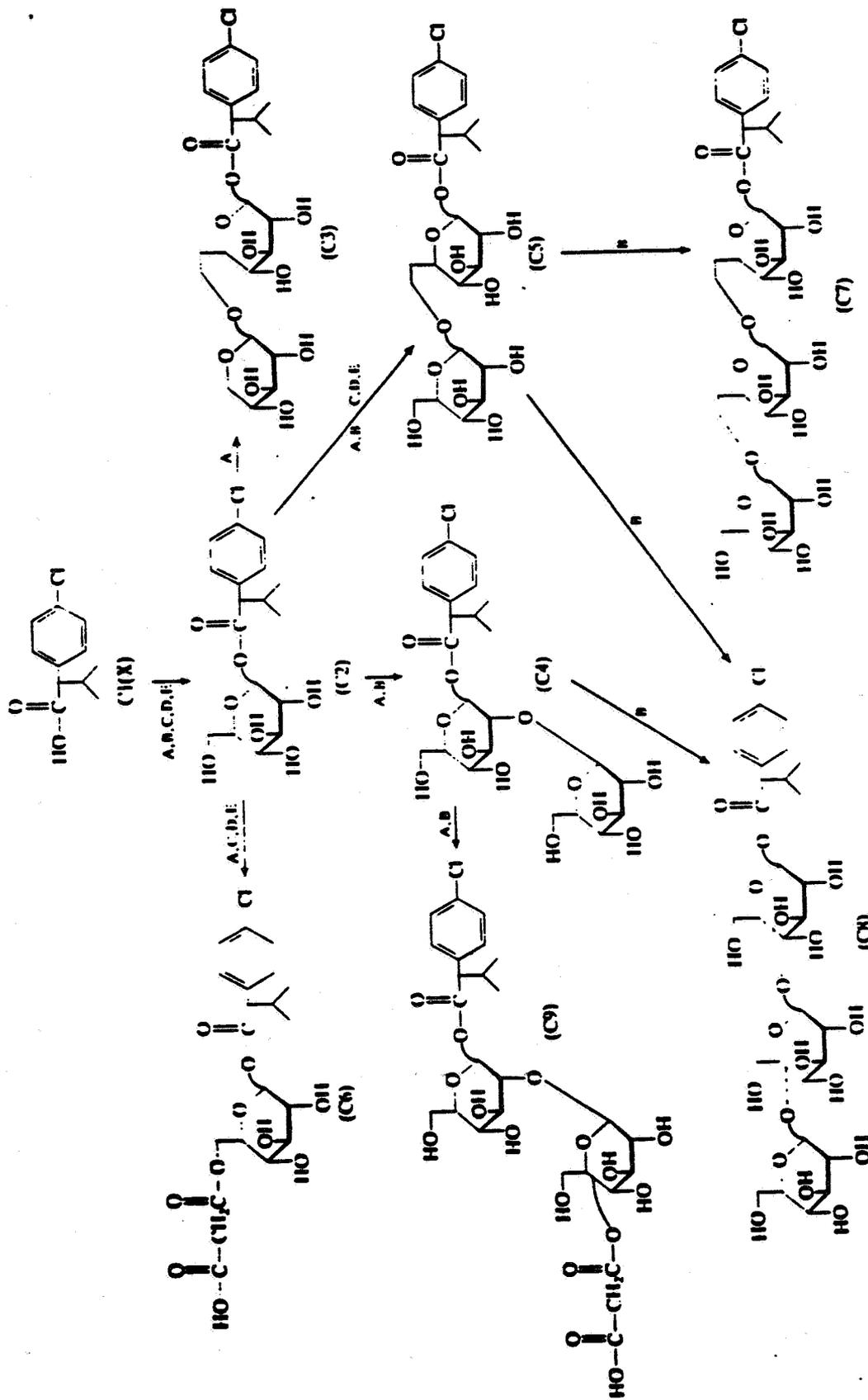


Figure 4. Proposed metabolic pathways for 2-(4-chlorophenyl)-3-methylbutyric acid (X) in various plants: (A) coffee, (B) tomato, (C) cabbage, (D) kidney bean seed (E) cucumber.

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4. Mikami.

glucopyranosyl-(1→6)-O-β-D-glucopyranosyl-(1→6)-O-β-D-glucopyranose. Further characterisation of C8 has yet to be carried out.

Component C9 released X, glucose and malonic acid in molar ratios of 1.0:2.1:0.9. As only limited conversion to C4 occurred on t.l.c. in solvent system D, C9 was considered to be the malonate hemi-ester derivative of C4. The substitution position of malonic acid with sophorose remains unknown.

The amounts of these conjugated metabolites in various plants are summarised in Table 5.

4. Discussion

After foliar treatment, fenvalerate and its (α S,2S) isomer similarly disappeared from the treated leaves of cabbages, with half-lives of 12–14 days under laboratory conditions. Both insecticides underwent hydroxylation at the 2- or 4-position of the phenoxy group, hydration of the nitrile group to the amide group with subsequent hydrolysis to the carboxyl group, and cleavage of the ester linkage, as in the case of metabolism in bean plants.³ It appears that the rate and route of metabolism of I are not affected significantly by the chirality at C-2 of the acid moiety and the α -C atoms of the alcohol moiety of the molecule.

The major metabolic route in cabbage and bean plants was by cleavage of the ester linkage, leading to the formation of 3-phenoxybenzoic acid (VI) and 2-(4-chlorophenyl)-3-methylbutyric acid (X). The resultant carboxylic acids occurred in plants, mainly as two types of glycoside conjugates. The acid (X) was found to be further metabolised, mainly to its malonylglucoside, together with small amounts of glucose and the gentiobiose ester in abscised leaves of cabbages. It appears that these glycoside conjugates also occurred in intact cabbages to which I had been applied as a starting material: this evidence was based on the comparison of the R_f values on t.l.c. The malonylglucoside was resistant to β -glucosidase hydrolysis but was completely cleaved by cellulase, whereas glucose and the gentiobiose conjugates were readily hydrolysed by both enzymes.

As shown in Figure 4, the metabolic pathways for X were somewhat plant-specific. The malonylglucoside was predominant in bean, cabbage and cucumber plants, whereas the two types of triglucose esters with different isomerism, and the glucosylxylose, sophorose and gentiobiose esters were predominant in tomato and cotton plants, respectively. The glucosylxylose ester was found only in cotton, and the triglucose esters only in tomato. Although one of the triglucosides was not identical with the synthetic [1→6] triglucoside derivative, it could be formed by the stepwise addition of the glucose unit to the sophorose (C4) or gentiobiose (C5) ester. Such a hypothesis is supported by several authors.^{14–15} It seems possible that introduction of the pentose unit or malonic acid, at the primary alcohol of the glucose moiety, interrupts the build-up of a longer sugar side-chain.¹⁶ The structures of these components were closely analogous to those found for the metabolites of VI.⁴

Based upon the available information, the terminal residues of I and II in plants are likely to be the unchanged parent compound, together with glycoside conjugates of VI or X. These metabolites in plants were analogous to those in mammals,⁹ except for the nature of the conjugating moieties. It has been reported that upon oral administration to rats, the glucoside conjugates of VI, IX and X were absorbed, mainly as their corresponding aglycones, after cleavage of the glycoside linkage by gut microflora, and were then completely eliminated as a mixture of metabolites very similar to those derived from VI, IX and X, respectively.^{20,21} Conjugation with glucose significantly lowered the acute oral toxicity of VI, IX and X in mice.²²

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A Sensitive of N₂N'-Bi

Weiling Hsu

Schwarz Research
Institute of Cereals

(Revised manuscript)

A sensitive
semi-quantitative
method for the
determination of
this metabolite
Using thin layer
chromatography

The antitumor
activity of the
compound against
Xanthomonas
N,N'-bis(1,3,4-oxadiazol-5-yl)ethane-2-thione
The antitumor
activity of the
1,5-dithioether
126 times stronger
leaf blight disease
5-10 times lower

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ATTACHMENT V

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Pages 61 through 68 are not included in this copy.

The material not included contains the following type of information:

- Identity of product inert ingredients
 - Identity of product impurities
 - Description of the product manufacturing process
 - Description of product quality control procedures
 - Identity of the source of product ingredients
 - Sales or other commercial/financial information
 - A draft product label
 - The product confidential statement of formula
 - Information about a pending registration action
 - FIFRA registration data
 - The document is a duplicate of page(s) _____
 - The document is not responsive to the request
-

The information not included is generally considered confidential by product registrants. If you have any questions, please contact the individual who prepared the response to your request.

ATTACHMENT VI



UNITED STATES ENVIRONMENTAL PROTECTION AGENCY

WASHINGTON, D.C. 20460

OFFICE OF PESTICIDES AND TOXIC SUBSTANCES

RECEIVED
AUG 8 1985
SHELL OIL COMPANY
WASHINGTON, D.C. OFFICE

AUG 6 1985

Shell Development Co.
Modesto
AUG 15 1985
(DAW)
511285-A1

Dr. E. L. Hobson
Shell Oil Company
Suite 200
1025 Connecticut Ave., NW.
Washington, DC 20036

Dear Dr. Hobson:

Subject: SS Pydrin® Insecticide 1.9 EC
EPA File Symbol 201-URI
Your Letter Dated March 15, 1985

This is in response to your letter of the above date, transmitting residue information in support of the above product. After reviewing the additional residue information, the Agency has concluded that the deficiencies cited in our letter of December 13, 1984, have been resolved. Please refer to our letter dated October 17, 1984, for other data requirements and/or deficiencies.

The toxicology data package is still under review. When completed, we will send you the results. As you requested, enclosed is a copy of our residue chemistry scientific review for your information.

Sincerely yours,

George T. LaRocca
Product Manager (15)
Insecticide-Rodenticide Branch
Registration Division (TS-767)

Enclosure

Page 71 is not included in this copy.

Pages _____ through _____ are not included in this copy.

The material not included contains the following type of information:

- Identity of product inert ingredients
 - Identity of product impurities
 - Description of the product manufacturing process
 - Description of product quality control procedures
 - Identity of the source of product ingredients
 - Sales or other commercial/financial information
 - A draft product label
 - The product confidential statement of formula
 - Information about a pending registration action
 - FIFRA registration data
 - The document is a duplicate of page(s) _____
 - The document is not responsive to the request
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The information not included is generally considered confidential by product registrants. If you have any questions, please contact the individual who prepared the response to your request.

fenvalerate, only ca 15% of the fat residues was determined to be the parent. No additional identification of residues or explanation of discrepancies has been furnished. This will be required.

Shell's Response

Shell has submitted a report, MO-RIR-22-005-85, entitled "Characterization of ^{14}C -Residues in the Body Fat of Rats Following a Single Oral Dose of ^{14}C -SD 43775 and ^{14}C -SD 92459". Incomplete extraction was perceived to be the cause of these discrepancies.

RCE's Comment

This report is presumably the written form of what was presented on this issue in a meeting held earlier this year (memo of Conference, 3/14/85). SD 43775 denotes the racemic mixture and SD 92459 refers to the SS isomer-enriched fenvalerate.

In this report, a mixture of hexane and acetone (3:1) instead of just hexane was used for extraction. A larger sample size was also employed (5 grams of composite body fat tissues rather than 1 gram of inguinal fat). The hexane-acetone extracts were combined, suspended in 0.1 N citric acid buffer (pH 3) and the ^{14}C -residues were partitioned into chloroform. Greater than 99% of the activity in the fat tissues was extracted into the chloroform while the aqueous phase and the insoluble solid tissues contained negligible levels of radioactivity. The chloroform extracts after concentration were further partitioned against 1:1 hexane-acetonitrile to remove lipophilic coextractives. The acetonitrile phase which contained the ^{14}C -residues was concentrated. The residue thus obtained was placed on a silica gel plate and developed first with hexane followed by 25:25:1 hexane-acetone-acetic acid and 75:25:1 toluene-ether-acetic acid. Autoradiograms showed greater than 95% of the recovered activity was undegraded parent from all four treatment groups. The identity of fenvalerate was confirmed by GC.

We conclude undegraded parent is the residue of concern in the body fat of rats regardless of the isomeric ratio in the fenvalerate administered. The results agree with those from previous metabolism studies. This deficiency is resolved.