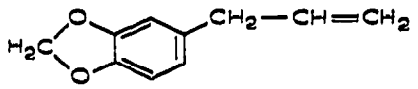
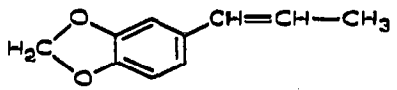
Thin-layer chromatography of rat bile and urine following intravenous administration of safrole, isosafrole, and dihydrosafrole

In a previous paper[1] we reported on the elimination of pesticidal synergists (piperonyl butoxide and tropital) and their metabolites in rat bile and urine, resulting from single intravenous administrations of the above compounds.

The purpose of this investigation was to continue the thin-layer chromatographic elaboration of rat bile and urine following the single intravenous administration of three basic methylenedioxyphenyl derivatives, *viz.*, safrole (I), isosafrole (II), and dihydrosafrole (III)*.


I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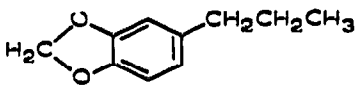
III

Safrole, the principal component of oil of sassafras[2], has until recently, been widely used in foods chiefly as a flavoring agent in root beer[3]. Both safrole and isosafrole are employed in the manufacture of heliotropin (piperonal) and are active synergists for pyrethrum and Sevin[4] (1-naphthyl methyl carbamate) as well as being inhibitors of the hydroxylation of naphthalene in houseflies[5].

Safrole has been shown to be a relatively weak hepatic carcinogen when fed to rats for chronic periods[6,7] while dihydrosafrole produced benign and malignant esophageal tumors during similar feeding studies with rats[8] and fatty degeneration of the liver when fed to mice[9]. Although liver changes in rats resulting from chronic feeding of isosafrole, safrole and dihydrosafrole were of the same general type (including hepatic cell enlargement, cystic necrosis and bile duct proliferation), the magnitude of the liver changes was much greater with safrole than with the other two compounds[8].

Experimental

Preparation of the plates

Silica Gel Df-5 chromatoplates were prepared as previously described[1].

Solvent systems

(A) Toluene–acetic acid–water (10:10:1)
(B) Ethyl acetate–acetic acid–methanol (70:10:20)
(C) *n*-Butanol–acetic acid–water (10:1:1)
(D) Acetone–benzene (1:39).

Detecting reagents

(1) Chromogenic agents: (a) Conc. sulfuric acid–*n*-butanol (15:85)[10]; (b) Chromotropic acid reagent[11].

(2) Radiation sources: (a) U.V. 3660Å—Mineralight, Blak-Ray Model UVL-22**; (b) U.V. 2537Å—Mineralight, Model UVS-11**.

* 3,4-Methylenedioxyallylbenzene, 3,4-methylenedioxypropenylbenzene and 3,4-methylenedioxypropylbenzene, respectively. All obtained from J. T. Baker Chemical Co., Phillipsburg, N.J. (U.S.A.).

** Obtained from Allied Impex Corp., New York, N.Y.

TABLE I

SUMMARY OF RAT BILE R_F (× 100) DIFFERENCES ON SILICA GEL DF-5 RESULTING FROM SINGLE INTRAVENOUS ADMINISTRATION OF SAFROLE, ISOSAFROLE AND DIHYDROSAFROLE

Detectors: (1) 2537Å U.V.; (2) sulfuric acid–butanol, 3660Å; (3) sulfuric acid–butanol, visible light; (4) chromotropic acid, 3660Å; (5) chromotropic acid, visible light.
Colors: B = blue; Bl = black; Bn = brown; G = green; Gr = grey; O = orange; Ob = obscured by other 2537Å absorbing spots; Q = quench; R = red; V = violet; W = white; Y = yellow; (—) = not detected.

Developer	*Safrole*						*Isosafrole*						*Dihydrosafrole*					
	R_F	*Detector and color*					R_F	*Detector and color*					R_F	*Detector and color*				
		1	*2*	*3*	*4*	*5*		*1*	*2*	*3*	*4*	*5*		*1*	*2*	*3*	*4*	*5*
Toluene–acetic acid–water (10:10:1)	0	Ob	Q	G	Q	Bn-G	0	Ob	Q	G	Q	G	0	Ob	Q	G	Q	G
	8	—	V	V	Q	V												
	20	Q	Q	V	Q	V	20	Q	—	Bn	—	Bn						
	23	Q	R-V	Y-Bn	R-V	Y-Bn	23	Q	R-V	Y-Bn	R-V	Y-Bn						
	28	Ob	—	—	—	V	28	Ob	—	—	—	V	28	Ob	—	—	—	V
	36	B	—	—	—	—	36	B	—	—	—	—						
	38	—	—	—	—	V	38	—	—	—	—	V						
	48*	—	W	Y-O	W	Y-O	45*	—	W	Y-O	W	Y-O	45*	—	W	Y-O	W	Y-O
	55	Q	Q	Gr	Q	Gr	55	Q	—	Gr	—	Gr	55	—	—	Gr	—	Gr
	58	—	W	—	W	—												
	72	Ob	—	Gr	—	Bl	72	Ob	—	Gr	—	Bl	72	Ob	—	Gr	—	Bl
Ethyl acetate–acetic acid–methanol (70:10:20)	0	Ob	Q	Bn	Q	Bn	0	Ob	Q	Bl	Q	Bl	0	Ob	Q	Bl	Q	Bl
	25	—	V	G-Bn	V	Gr-Bn	25	—	V	Bn	V	Gr	25	—	V	Bn	V	Gr
	38	Q	R-V	Gr	R-V	Gr	38	Q	R-V	Gr	R-V	Gr						
	60	Ob	—	Gr	—	Gr	60	Ob	—	Gr	—	Gr	60	Ob	—	Gr	—	Gr
	70	Ob	—	Gr	—	Gr	70	Ob	—	Gr	—	Gr	70	—	—	Gr	—	Gr
	75*	—	W	Y-O	W	Y-O	75*	—	W	Y-O	W	Y-O	75*	—	W	Y-O	W	Y-O
	81	B	—	—	—	V	81	B	—	—	—	V						
n-Butanol–acetic acid–water (10:1:1)	0	Ob	Q	Bl	Q	Bl	0	Ob	—	G	—	G	0	Ob	—	G	—	Gr
	5	Q	Q	Gr	—	Gr	5	Q	Q	Gr	—	Gr	5	Q	Q	Gr	—	Gr
	13	—	Q	Bl	Q	Bl												
	18	—	Q	G-Bl	Q	Bn												
	22	Ob	—	V	—	V												
	28	Ob	V	V	V	V	28	Ob	—	Bn	—	Gr						
	47	Ob	—	Gr	—	Gr												
	56	—	—	V	—	V							56	Ob	—	Bn	—	Bn
	68	B	—	—	—	—	68	B	—	—	—	—						
	75*	—	W	Y-O	W	Y-O	75*	—	W	Y-O	W	Y-O	75*	—	W	Y-O	W	Y-O
	78	Ob	—	Gr	—	Gr	78	Ob	—	Gr	—	Gr	78	Ob	—	Gr	—	Gr

* Quenching spot.

Bile and urine sampling

Single intravenous injections of 0.04 ml each of safrole, isosafrole, and dihydrosafrole were given to adult male rats of the Sprague-Dawley strain, averaging 350 g in weight. Bile samples were collected by fistula and urine samples by cannulation from each rat. Details on the handling of the animals, anesthesia, surgery and sample collection and timing have previously been described[12].

At least three urine samples were collected, one before intravenous injection, a second at an appropriate interval after injection and a final sample at the termination of the bile collection. All samples were kept frozen until ready for analysis.

Photography of chromatoplates

The thin-layers plates were photographed in color and black and white using equipment and procedures previously described[1].

Procedure

Time study of metabolite formation. Twenty microliters of all bile and urine samples were applied as half-inch streaks* on Silica Gel Df-5 plates. One microliter of standard RBY dye** was applied to each plate as a reference marker. The plates were developed in ethyl acetate–acetic acid–water (10:10:1), the solvent removed with a hot air dryer and each plate photographed under U.V. at 2537Å. The plates were then treated with the chromotropic acid reagent. After spraying, all plates were heated in a circulating air oven at 120° until color development was maximum (10–20 min). The plates were then photographed under U.V. at 3660Å and visible light. R_F values and spot colors were taken directly from the photographs because many of the colors were unstable on the silica gel plates.

Metabolite characterization. Bile and urine samples exhibiting optimum concentration of metabolites were pooled and applied as a streak (60 μl/1.25 in.) on Silica Gel Df-5 plates and were compared with controls taken before injection. After solvent development, they were first photographed under U.V. at 2537Å. One half of each streak*** was sprayed with sulfuric acid–butanol reagent and the other half with the chromotropic acid reagent, then the spots developed at 120° as described above.

Results and discussion

Chromatographic differences in bile and urine samples resulting from intravenous administration of safrole, isosafrole and dihydrosafrole are summarized in Tables I and II, respectively. Tables I and II list the R_F values of each component and data regarding its characterization (means of detection, color, etc.).

It is apparent from the data shown in Table I that the presence of an unsaturated side chain, as in safrole and isosafrole, results in a larger number of metabolites in the bile than in the case of dihydrosafrole where the side chain is saturated. For safrole and isosafrole there exists the possibility of degradation at the double bond of the aliphatic side chain and/or at the methylenedioxy ring leading to the type of products that are diagrammed in Fig. 1.

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* Bile and urine samples were applied with a RADIN-PELIDS thin-layer sample streaker obtained from Applied Science Laboratories, State College, Pa., U.S.A.

** Obtained from Camag, Muttenz, Switzerland.

*** Two thin-layer glass plates were used to cover the areas that were not to receive the spray.

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TABLE II

SUMMARY OF RAT URINE R_F (× 100) DIFFERENCES ON SILICA GEL DF-5 RESULTING FROM SINGLE INTRAVENOUS ADMINISTRATION OF SAFROLE, ISOSAFROLE AND DIHYDROSAFROLE

Detectors: (1) = 2537Å; (2) = sulfuric acid–butanol, 3660Å; (3) = sulfuric acid–butanol, visible light; (4) = chromotropic acid, 3660Å; (5) = chromotropic acid, visible light.
Colors: B = blue; Bl = black; Bn = brown; G = green; Gr = grey; O = orange; Ob = obscured by other 2537Å absorbing spots; Q = quench; V = violet; W = white; Y = yellow; (—) = not detected.

Developer	*Safrole*						*Isosafrole*						*Dihydrosafrole*					
	R_F	*Detector and color*					R_F	*Detector and color*					R_F	*Detector and color*				
		1	*2*	*3*	*4*	*5*		*1*	*2*	*3*	*4*	*5*		*1*	*2*	*3*	*4*	*5*
Toluene–acetic acid–water (10:10:1)	8	B	Q	Bn	Q	G	7	Ob	Q	Gr	Q	Gr	7	Ob	Q	Gr	Q	Gr
	20	Q	Q	V	Q	V	20	Q	Q	V	Q	V	20	Q	Q	Gr	Q	Gr
	23	Ob	—	Gr	—	Gr												
							40	Ob	Q	V	Q	V	40	Ob	—	Gr	—	Bl
							42	Ob	—	—	—	Bl						
							45	Ob	—	Gr	—	Bn						
	59	Q	Q	Y-G	Q	Y-G							60	Q	Q	Y-G	Q	Y-G
	72	Q	B-W	Gr	B-W	Gr												
							75	B	—	—	—	—						
													79	—	B-W	Gr	B-W	Gr
Ethyl acetate–acetic acid–methanol (70:10:20)	5–32	—	Q	Bn	Q	Bn												
							10	—	—	Bn	—	Bn	10	—	—	Bn	—	Bn
							28	Q	—	—	—	—						
													30	Ob	—	Gr	—	Gr
							57	Ob	Q	V	Q	V						
	59	Q	Q	V	Q	V												
							63	Ob	—	—	—	Gr						
							72	—	—	—	—	Bl-V						
	79	Ob	—	—	—	Bl	79	Q	—	—	—	V	79	Q	—	—	—	Bl
n-Butanol–acetic acid–water (10:10:1)	5–30	—	—	—	Q	Bn												
							10	Q	—	—	—	—						
							38	B	—	—	—	V						
	58	Q	—	—	Q	V	58	Q	—	—	Q	V	58	Q	—	—	Q	V
							68	Q	—	—	—	Gr						
	72	Q	—	—	Q	Y-G	72	B	—	—	—	Bn						
	80	Ob	—	—	B	Gr	80	Q	—	—	—	Gr						

Although the products depicted in Fig. 1 have not been unequivocally identified in this study, previous bile experiments with piperonal and piperonylic acid have indicated steps 2 → 3 of Fig. 1 and will be reported separately.

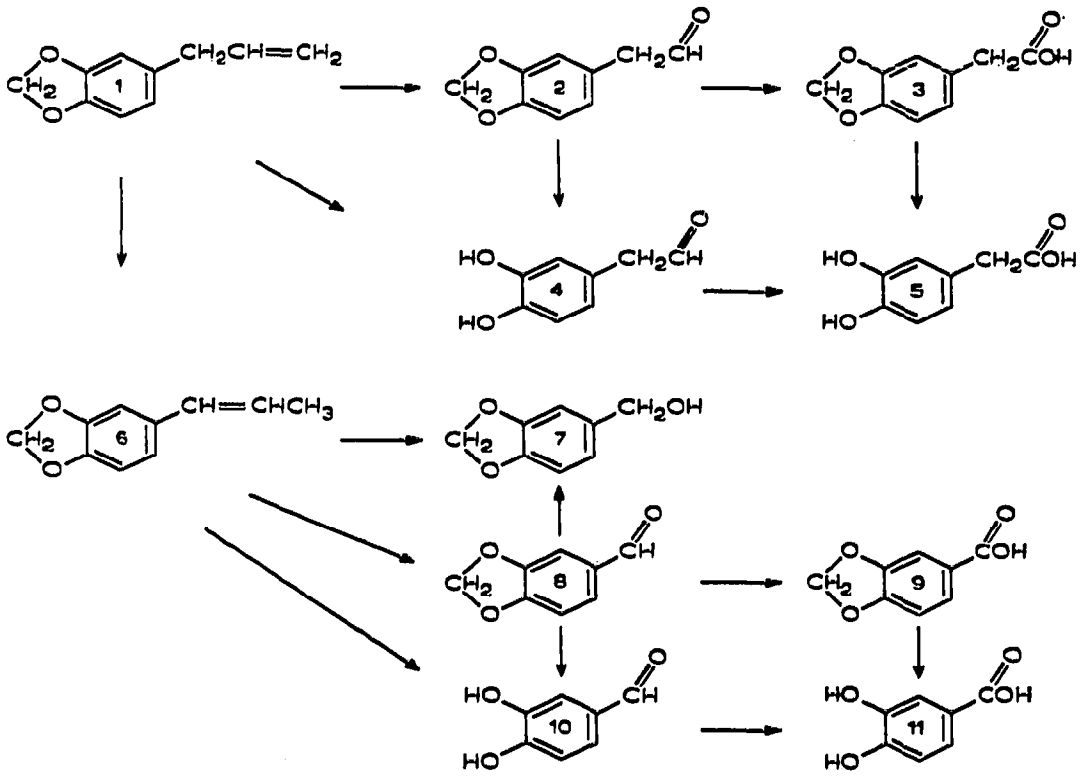
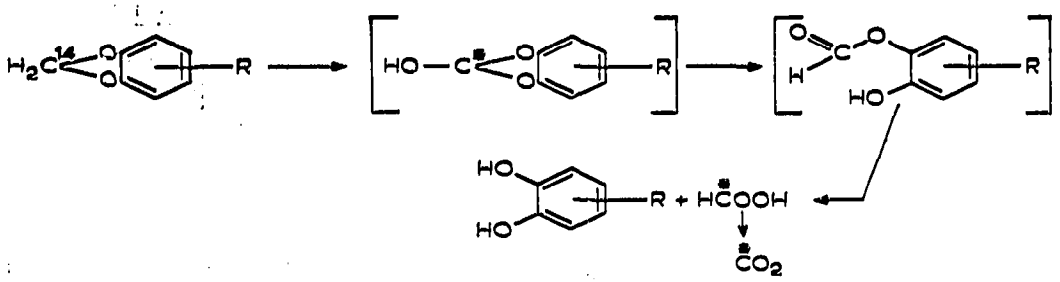


Fig. 1. Possible routes of metabolism for safrole and isosafrole. 1 = Safrole; 2 = 3,4-methylenedioxyphenylacetaldehyde; 3 = 3,4-methylenedioxyphenylacetic acid; 4 = 3,4-dihydroxyphenylacetaldehyde; 5 = 3,4-dihydroxyphenylacetic acid; 6 = isosafrole; 7 = piperonyl alcohol; 8 = piperonal; 9 = piperonylic acid; 10 = 3,4-dihydroxybenzaldehyde; 11 = 3,4-dihydroxybenzoic acid.

It is of interest to note the similarity (R_F and color) for the bile metabolites of safrole and isosafrole utilizing toluene–acetic acid–water and ethyl acetate–acetic acid–methanol developers (Table I).

CASIDA and co-workers[13] utilized methylene-^{14}C-safrole and dihydrosafrole to elaborate the metabolism in relation to synergistic action. It was found that the methylene-^{14}C group is hydroxylated as shown below:



When urine chromatograms from safrole and isosafrole were compared with those from piperonal and piperonylic acid injected rats, a similarity of grey bands occurring at R_F 0.68 for the isosafrole and piperonylic acid urines was noted.

WILLIAMS[14] has reported the isolation of piperonylic acid conjugates (in the urine) such as the ester glucuronide and a glycine conjugate after the administration of safrole and isosafrole to dogs.

Fig. 2 depicts the relationship of bile components with time after intravenous administration of safrole (the $R_F \times 100$ values shown correspond to those in Table I found utilizing *n*-butanol–acetic acid–water (10:10:1) developer). Graph (A) indicates the metabolites that occur within a few minutes after injection. Graphs (B) and (C) indicate those that begin to appear 70–90 min and 130 min, respectively, after injection.

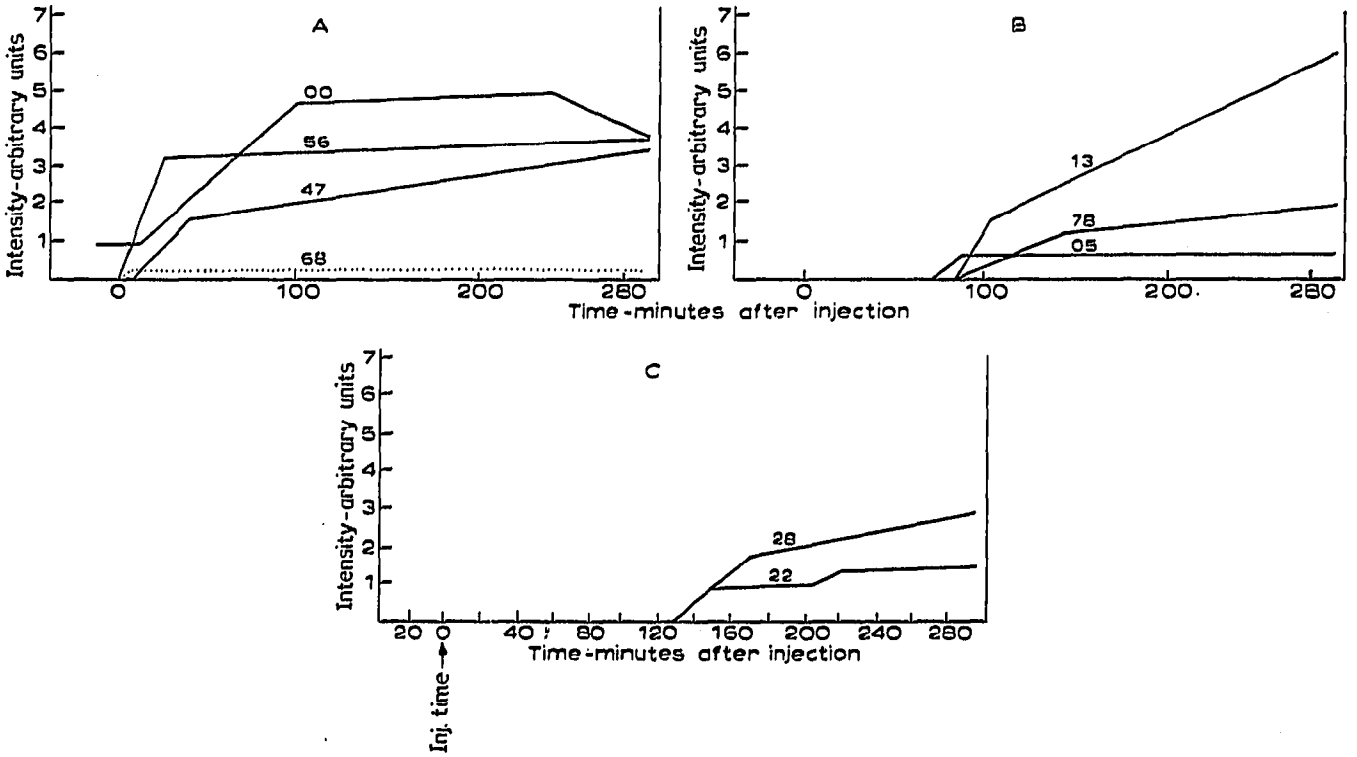


Fig. 2. Relationship of spot intensity with time of various metabolites occurring in rat bile after intravenous administration of safrole. The data shown were taken from thin-layer plates developed with *n*-butanol–acetic acid–water (10:1:1). Concentrations were estimated visually as degrees of intensity of absorbance or fluorescence. Portion (A) of figure indicates metabolites that occur within a few minutes after injection. Portions (B) and (C) indicate those that appear 70–90 min and 130 min after injection, respectively. Dotted curve (R_F 68; portion A) was viewed under U.V. at 2537Å; all other components were plotted after spraying the plate with chromotropic acid.

tion. The bile acid, cholic acid (R_F 75) (Table I) showed no discernable change in concentration before or after safrole injection and hence was not included in Fig. 2. It is of interest to note that in analogous studies with tropital and piperonyl butoxide[1] the cholic acid concentration was shown to rise steadily throughout the length of the experiment in tropital injected animals whereas the cholic acid level remained constant in the case of piperonyl butoxide treated animals.

Conclusions

The methylenedioxyphenyl derivatives studied in these experiments were found to be altered chemically with the following apparent order of metabolism: safrole > isosafrole > dihydrosafrole. The elimination of products occurred largely in the bile after intravenous injection.

The rate of elimination of metabolites resulting from the administration of safrole does not reach a rapid peak with rapid decline thereafter, but similarly to the earlier experiments with piperonyl butoxide and tropital, suggests slow prolonged elimination of the metabolites in the bile. As suggested earlier[15], inhibition of certain detoxification mechanisms and delayed elimination from the body of pesticides and other chemicals could constitute a hazard to the health of man exposed to these compounds.

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1 L. FISHBEIN, J. FAWKES, H. L. FALK AND S. THOMPSON, *J. Chromatog.*, 27 (1967) 153.
2 F. B. POWER AND C. KLEBER, *Pharm. Rev.*, 14 (1896) 101.
3 M. B. JACOBS, *Am. Perfumer. Aromat.*, 71 (1958) 57.
4 S. KUWATSUKA AND J. E. CASIDA, *J. Agr. Food Chem.*, 13 (1965) 528.
5 W. W. PHILLEO, R. D. SCHONBROD AND L. C. TERRIERE, *J. Agr. Food Chem.*, 13 (1965) 113.
6 E. L. LONG AND A. A. NELSON, *Federation Proc.*, 20 (1961) 287.
7 E. L. LONG, A. A. NELSON, O. G. FITZHUGH AND W. H. HANSON, *Arch. Pathol.*, 75 (1963) 595.
8 E. C. HAGAN, P. M. JENNER, W. I. JONES, O. G. FITZHUGH, E. L. LONG, J. W. BROUWER AND W. K. WEBB, *Toxicol. Appl. Pharmacol.*, 7 (1965) 18.
9 F. VON GRAEVENITZ, *Arch. Exptl. Pathol. Pharmakol.*, 104 (1924) 298.
10 W. L. ANTHONY AND W. T. BEHER, *J. Chromatog.*, 13 (1964) 567.
11 M. BEROZA, *J. Agr. Food Chem.*, 11 (1963) 51.
12 P. KOTIN, H. L. FALK AND R. BUSSER, *J. Natl. Cancer Inst.*, 23 (1959) 541.
13 J. E. CASIDA, J. L. ENGEL, E. G. ESSAC, F. X. KAMIENSKI AND J. KUWATSUKA, *Science*, 153 (1966) 1130.
14 R. T. WILLIAMS, *Detoxification Mechanisms*, 2nd Ed., Wiley, New York, 1959, p. 371.
15 H. L. FALK, S. THOMPSON AND P. KOTIN, *Arch. Environ. Health*, 10 (1965) 847.

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