CHROM. 4402

METABOLISM OF NATURALLY OCCURRING PROPENYLBENZENE DERIVATIVES

I. CHROMATOGRAPHIC SEPARATION OF NINHYDRIN-POSITIVE MATERIALS OF RAT URINE

E. O. OSWALD, L. FISHBEIN AND B. J. CORBETT

National Institute of Environmental Health Sciences, National Institutes of Health, Public Health Service and Department of Health, Education, and Welfare, Research Triangle Park, N. C. 27709 (U.S.A.)

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SUMMARY

Following oral or intraperitoneal administration of myristicin, safrole, isosafrole, asarone (*trans*) or β-asarone (*cis*) to male rats, basic ninhydrin-positive substances were excreted in the urine. These materials were separated and partially characterized by thin-layer chromatography. The same animals when given a control dosage of safflower oil did not excrete these ninhydrin-positive materials.

There is an apparent requirement of a side chain double bond for the production of these products, with greater enhancement by the *trans* isomer than the *cis*. It is suggested that these urinary ninhydrin-positive materials are probably substituted phenylisopropylamines or amphetamines.

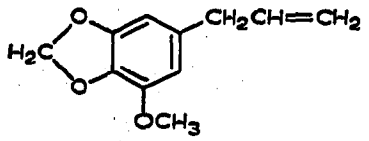
INTRODUCTION

Substituted allyl and propenylbenzene derivatives are widely distributed in nature. The compounds have been isolated and identified in nutmeg[1], parsley[1], parsnip[2], carrots[3], bananas[4], processed tobacco[5] and many other natural oils and flavoring materials with which individuals have contact.

Ingestion of considerable quantities of some of these environmental agents produces significant physiological changes. In the case of nutmeg, myristicin (1-methoxy-2,3-methylenedioxy-5-allyl-benzene) is considered in part to be responsible for a narcotic effect[1]. Depending upon the drug and quantities ingested, these physiological changes[6-8] vary from (a) drop in blood pressure, (b) nausea, and (c) cyanosis to (d) heightening of ego, (e) inability to carry on intellectual processes, (f) insomnia and/or even (g) death. The mechanism by which these materials bring about these responses is unknown.

To account for the observed physiological response, it has been suggested[1] that the substituted benzene derivative may be converted biologically to amphetamines.

The present investigation was undertaken to effect the isolation, separation, and identification of the various urinary metabolites of myristicin (I), safrole (II), isosafrole (III), asarone (IV), β-asarone (V), and dihydrosafrole (VI) following their oral and intraperitoneal administration to male rats, in order to determine if exposure or ingestion of these environmental agents (and other related compounds) would constitute a potential hazard.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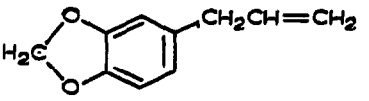
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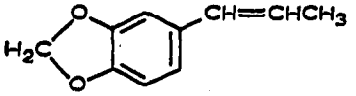
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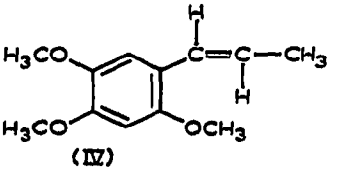
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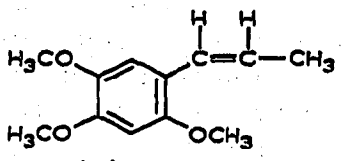
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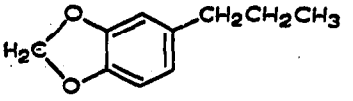
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EXPERIMENTAL

Materials

All of the compounds that were administered to the animals were of 99 % purity or greater as determined by TLC, GLC, IR spectroscopy, and NMR. Myristicin was obtained from parsley seed oil (Fritzsche) by preparative GLC on a 12 ft. × ½ in. 10 % OV-1 column at 215° with a helium flow of 100 ml/min using the Hewlett-Packard 5750B gas chromatograph or on a 10 ft. × ¾ in. 25 % SE-30 column at 235° with a helium flow of 200 ml/min using the Nester Faust 850 Prepkromatic gas chromatograph, followed by column chromatography on silicic acid. Asarone (*trans*) and β-asarone (*cis*) were obtained from an enriched β-asarone mixture (Fritzsche) by preparative GLC, on a 12 ft. × ½ in. 10 % OV-17 column at 250° with a helium flow of 100 ml/min using the Hewlett-Packard 5750B gas chromatograph. The safrole derivatives were commercial preparations (J. T. Baker) which were further purified by silicic acid chromatography. All organic solvents were Baker analyzed reagent grade.

Compound administration, urine collection and extraction

Male Sprague Dawley rats (200–500 g) were given 0.30 ml safflower oil orally or intraperitoneally (i.p.). The animals were then placed in polycarbonate metabolic

cages* which separated the urine from the feces, and the urine was collected for the desired time (1–3 days). Twice each day, urine was removed from the cages and immediately frozen until the time of extraction.

The same animals after the control period were then given 75–300 mg/kg of the desired compound in safflower oil (1:1, w/w). The rats were returned to the metabolic cages and urine was collected for periods up to six days. Pooled urine for each day was kept frozen until extraction. The treated animals after about one week could be re-fed if desired. During the entire urine collection, the rats were housed in a supervised area with free access to food and water.

An aliquot of pooled urine for each control and treated day was extracted as described in Fig. 1. The organic solvent from each of the acidic, neutral, basic and alkaloid fractions was then removed under nitrogen at 30° using a rotary flash evaporator. The final residue was dissolved in spectro-grade chloroform (Matheson, Coleman and Bell) to a final volume so that the ratio of the volume of the final solution to that of the original urine was 1:150. The chloroform solution was then stored in teflon-capped vials in the freezer for later use.

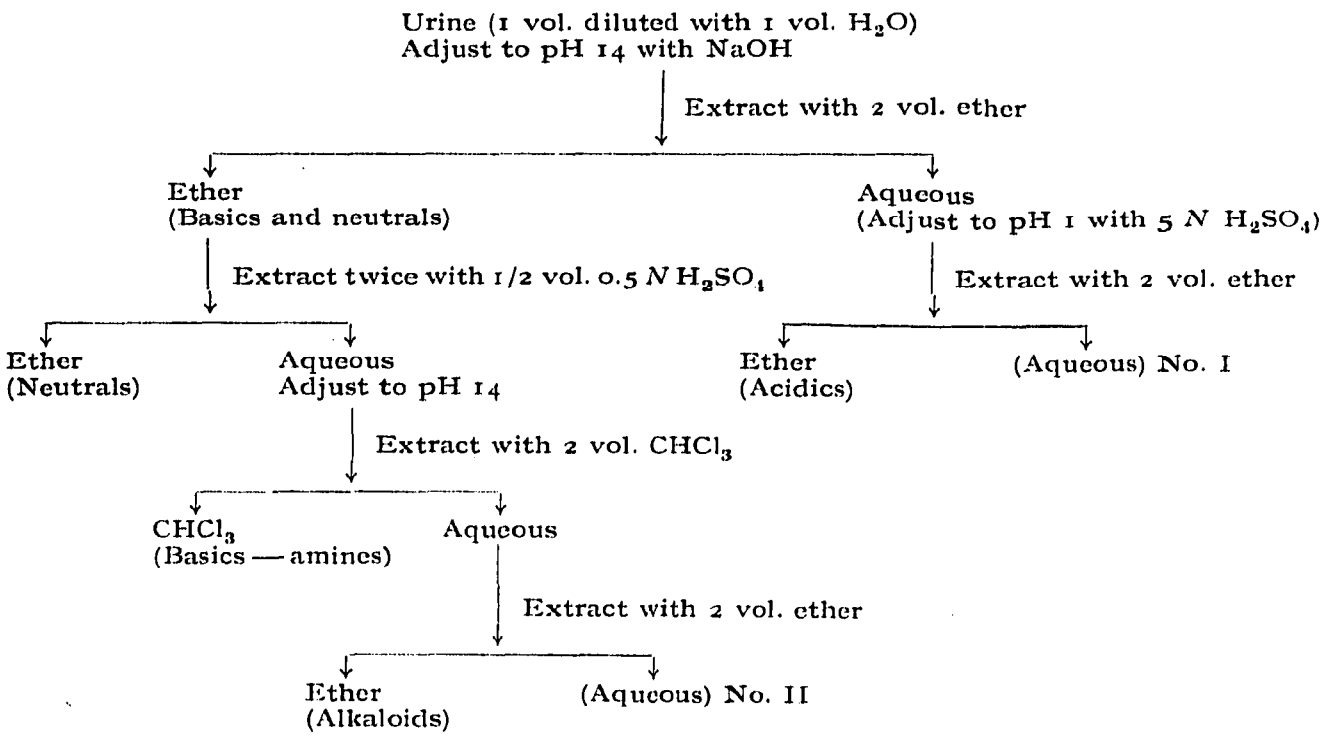

Fig. 1. Extraction of urine.

Thin-layer chromatography

Precoated 250-μ Silica Gel GF plates** were activated at 110° for 10 min. The plates were then scored into lanes and the appropriate amount of sample was applied. In all studies, one to four times as much control sample was applied as treated sample. The plates were then developed 140 mm in lined glass tanks using the desired solvent

* Obtained from Aloe Scientific Co., Chamblee, Ga., U.S.A.
** Obtained from Analtech, Inc., Wilmington, Del., U.S.A.

system. The solvent systems used for the neutral fractions were: (A) benzene, (B) hexane–diethyl ether–acetic acid (90:10:1)[9], and (C) hexane–diethyl ether–methanol–acetic acid (85:20:3:3)[10]. Table I represents the TLC separation obtained for the neutral administered compounds. Using the above three solvent systems, the presence of any of the administered compounds could be detected in the neutral urine fraction very easily.

TABLE I

RELATIVE R_F VALUES OBTAINED ON TLC SEPARATION OF SUBSTITUTED PROPENYL BENZENE DERIVATIVES

Solvent systems: (A) Benzene.
(B) Hexane–diethyl ether–acetic acid (90:10:1).
(C) Hexane–diethyl ether–methanol–acetic acid (85:20:3:3).

Compound	*Solvent system*		
	A	*B*	*C*
Asarone	0.24	0.26	0.58
Myristicin	1.00	1.00	1.00
Safrole	1.27	1.58	1.00

The basic and alkaloid fractions were examined using the following solvent systems: methanol, methanol–water–acetone–acetic acid (200:20:20:10), methanol–water–acetone–pyridine (200:20:20:10), and chloroform–methanol–acetic acid (75:25:5).

After development, the plates were air dried, examined under UV light (2537 and 3660 Å), and then exposed to iodine vapor and/or sprayed with a particular chemical reagent (for the neutral and basic urine fractions, ninhydrin[11], chromotropic acid[12,13], and 2,4-dinitrophenylhydrazine[11] were used).

RESULTS AND DISCUSSION

Myristicin

Following oral or i.p. administration of pure myristicin (75–300 mg/kg) to rats, unmetabolized myristicin is excreted in the urine during the first 17 h after administration. On 10 to 50-fold concentration of tissue extracts, there appears to be no significant accumulation of myristicin in blood, intestine, liver, heart, lung, spleen, and adipose tissue.

In addition to the unmetabolized myristicin excreted in the urine during the first 17 h., two very polar basic ninhydrin-positive materials are present in urine after oral or i.p. administration of myristicin. Semiquantitatively, these basic ninhydrin-positive materials are excreted maximally 24–48 h after administration. Three days after administration of myristicin there is a definite decrease in the excreted ninhydrin-positive materials. By the fourth or fifth day the basic ninhydrin-positive materials are absent. The urine extract of the treated animal by the fifth day is equivalent to that of the control animal, which received none of the test compound. This excretion pattern can be monitored with maximal excretion between 24–48 h and

with the absence of these basic ninhydrin-positive materials from urine by 4–5 days.

The production of these ninhydrin-positive basic materials is in response to administration of myristicin. The same animals when given only safflower oil do not excrete ninhydrin positive materials with an R_F value relative to the standard 3,4-methylenedioxyphenyl-isopropylamine $\geqslant$ 1.0 in the basic urine fraction. It is very probable that these ninhydrin-positive materials are not formed by intestinal flora since the same results were obtained for oral and i.p. administration. A chromatogram of the ninhydrin-positive basic materials after i.p. injection of myristicin is shown in Fig. 2. Both of the basic materials when sprayed with ninhydrin and then heated are dark pink in color. Positive identification of these materials is presently being elucidated.

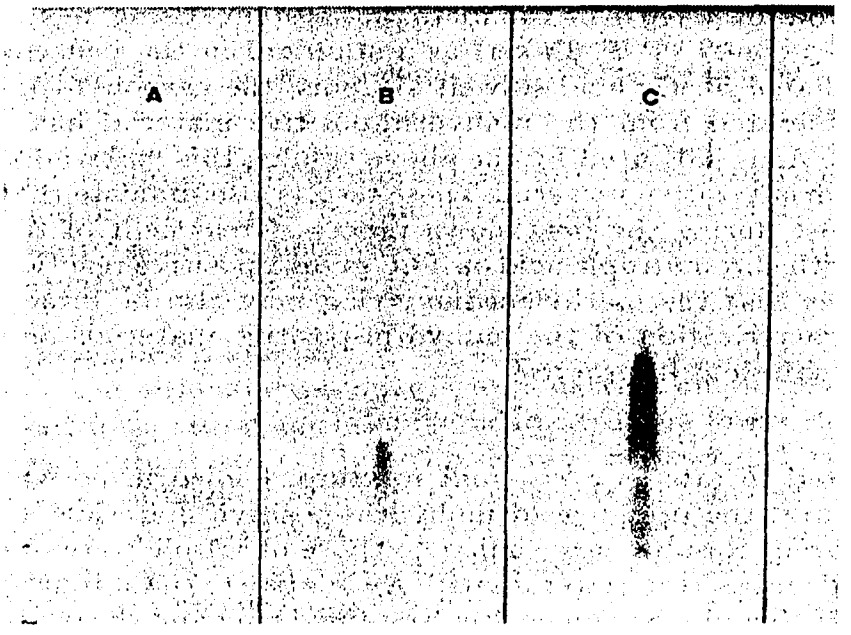

Fig. 2. TLC separation of basic ninhydrin-positive materials. Solvent system: chloroform–methanol–acetic acid (75:25:5). Basic urine fractions: (A) i.p. administered myristicin; (B) orally administered safrole; (C) orally administered asarone (*trans*).

Safrole

Similar experiments to those described for myristicin were also carried out with safrole. Ninhydrin-positive basic materials were also excreted in urine of rats treated with safrole. Maximal excretion of these ninhydrin-positive materials occurred during the 24–48 h period. Four ninhydrin-positive materials were separated using the methanol system. Upon spraying with ninhydrin and heating, the most polar material from safrole (R_F relative to the standard amine of 1.0) was pink while the next less polar material (relative R_F 1.7) was purple. The two remaining less polar ninhydrin-positive material had a relative R_F of 3.9 and 4.5, respectively, in the methanol system. These two much less polar ninhydrin-positive materials (relative R_F 3.9 and 4.5) may be decomposition products, as will be discussed later.

Attempts to isolate and identify the major ninhydrin-positive materials of safrole (relative R_F 1.0) have been unsuccessful to date. Preparative TLC of the safrole basic fraction in the methanol system on either silica gel or kieselgur followed by

elution with methanol and rechromatography in the methanol system destroys the ninhydrin-positive material. Even during storage at 0° under nitrogen there is a decrease in ninhydrin-positive materials in a given basic fraction. With decomposition of the ninhydrin-positive material which contains a nitrogen with at least one hydrogen atom attached, a compound containing a carbonyl group is generated. This carbonyl-containing compound reacts slowly with 2,4-dinitrophenylhydrazine on thin-layer plates, absorbs in the IR region at 1720 cm^{-1}, has no absorption at 3600–3200 cm^{-1}, and is now ninhydrin-negative. The carbonyl-containing compound has been found under all conditions investigated thus far, while analogous treatment of control urine does not yield a carbonyl or ketone material.

The carbonyl-containing material which was generated from the ninhydrin-positive material (relative R_F 1.0) has an R_F relative to the standard amine or most polar ninhydrin spot from safrole of 5.5–6.0 in the methanol system. Using 1-(3,4-methylenedioxyphenyl)-penten-3-one-1) (J. T. Baker) as a standard in the benzene and hexane–diethyl ether–methanol–acetic acid solvent systems, the carbonyl-containing material which was generated from the ninhydrin-positive material has a relative R_F of 1.0 in both cases. As is indicated by the above results, this material is much less polar than the ninhydrin-positive material. These results also indicate that the nitrogen may have been lost during the breakdown process. Treatment of the carbonyl-containing material with chromotropic acid on TLC gives a positive reaction for formaldehyde. This indicates that the methylenedioxy ring may also be intact. Further identification and characterization of the ninhydrin-positive materials and also the ketone materials from safrole is in progress.

Isosafrole

Isosafrole was administered to rats as a *cis–trans* mixture. Pooled urine was extracted as described earlier and the presence of ninhydrin-positive materials in the basic fraction was investigated. Results very similar to those of safrole were obtained for isosafrole. Two ninhydrin-positive materials with R_F relative to the standard amine of 1.0–2.0 were present. With ninhydrin one material stained pink and the other purple. Preparative TLC of the basic urine fractions from isosafrole also gave rise to a 2,4-dinitrophenylhydrazine-positive material (ninhydrin-negative). As discussed earlier, decomposition of the ninhydrin-positive urinary constituent of safrole and isosafrole produces carbonyl-containing materials which are ninhydrin-negative. These carbonyl and ninhydrin-positive materials are presently being identified and characterized.

Dihydrosafrole

Dihydrosafrole was administered orally (75–300 mg/kg) to male rats; pooled urine was extracted and the basic fractions were chromatographed as described earlier. As shown in Table II, there is no detectable quantity of ninhydrin-positive material with a relative $R_F \geqslant 1.0$ as for safrole and isosafrole. The only ninhydrin-positive material that is present in the treated dihydrosafrole urine is a faint purple spot at the origin using the methanol systems. The results for dihydrosafrole urine are equivalent to the control urine and suggest that the production of the ninhydrin-positive material with a relative $R_F \geqslant 1.0$ is dependent upon the presence of an allyl or propenyl double bond in the compound administered. Considering that the double

TABLE II

$R_F \times 140$ VALUES OBTAINED ON TLC SEPARATION OF NINHYDRIN-POSITIVE BASIC MATERIALS

Abbreviations: PSB = pooled safrole basics; PCB = pooled control basics; DHSB = dihydrosafrole basics; 3,4-MDPIA = 3,4-methylenedioxyphenyl-isopropylamine. The color of the ninhydrin-positive spot is given in parentheses.

Fraction	*Solvent system*	
	Methanol	*Chloroform–methanol–acetic acid (75:25:5)*
PSB	12–15 (purple) 32–38 (purple)	58–65 (purple)
PCB	0–10 (pink-purple)	15–20 (pink-purple) 30 (pink
DHSB	0 (purple)	20 (purple) 20–25 (purple)
3,4-MDPIA	10–43 (red-purple)	40–70 (red-purple)

bond is aminated to produce these ninhydrin-positive materials, the propyl derivative could not give rise to ninhydrin-positive materials similar to those of safrole or isosafrole unless there was first a dehydrogenation to produce an allyl or propenyl derivative.

Asarone and β-asarone

Additional experiments were carried out to determine the specificity requirement for a *cis* or *trans* double bond. Asarone (*trans* isomer) or β-asarone (*cis* isomer) was administered orally or i.p. to rats as described earlier. The basic urine fractions were examined by TLC. From earlier experiments with isosafrole, a *cis–trans* mixture, there does not seem to be a great difference in the amount of ninhydrin-positive materials excreted per volume of urine for safrole as compared to the same volume of urine after isosafrole treatment.

All urine extracts were concentrated as described earlier. Equal amounts of the basic fractions from myristicin, safrole, isosafrole, asarone (*trans*) and β-asarone (*cis*)-treated urine were applied to silica gel plates and then developed in the desired solvent system.

In comparison to safrole and myristicin, the asarone (*trans*)-treated urine contained very much more of the ninhydrin-positive material. As discussed earlier for myristicin and safrole, the maximum excretion for the basic ninhydrin-positive materials occurred 24–48 h after administration. For asarone, the *trans* isomer, the majority of the ninhydrin-positive material was excreted during the first 17 h after administration. One day after administration of asarone, very little ninhydrin-positive material with a relative $R_F \geqslant 1.0$ was excreted. In contrast, treatment with β-asarone, the *cis* isomer, produced only a small amount of ninhydrin-positive material in the urine even after two or three days. As is shown in Fig. 2, asarone, the *trans* isomer, produced much more (10 to 50-fold) ninhydrin-positive material than did either the *cis* isomer, safrole, or myristicin.

Using two-dimensional TLC and varying solvent systems, at least three ninhydrin-positive components from the asarone-treated basic urine fraction were separated (Fig. 3). After spraying with ninhydrin and heating, there are two pink spots

and one purple spot. There seem to be also acidic groups present in these materials.

It can be concluded from these preliminary investigations that after treatment of rats either orally or i.p. with myristicin, safrole, isosafrole, asarone (*trans*), or β-asarone (*cis*), basic ninhydrin-positive materials are excreted in the urine. There seems to be a prerequisite of a double bond for the production of these ninhydrin-positive materials. As suggested by unpublished work of BARFKNECHT[8], amphetamine-type materials may be produced in rats after feeding of allylbenzene. More specifically, the *trans* isomer of asarone is a better precursor to the ninhydrin-positive materials than the *cis* isomer. Control rats which did not receive the desired compound do not excrete these ninhydrin-positive materials in urine. These ninhydrin-positive basic materials break down very easily to produce ninhydrin-negative carbonyl-containing compounds.

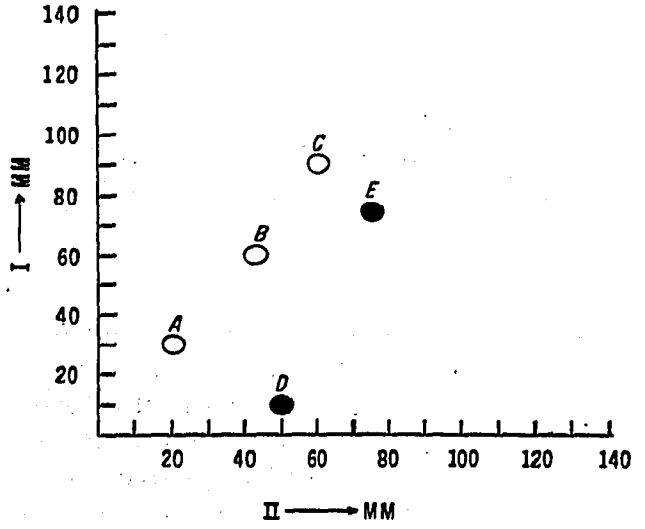

Fig. 3. Two-dimensional TLC separation of the asarone (*trans*) basic urine fraction. Solvent systems: ●, (I) (vertical), methanol–water–acetone–pyridine (200:20:20:10) 140 mm; (II) (horizontal), methanol–water–acetone–acetic acid (200:20:20:10) 140 mm. ○, (I) (vertical), methanol–water–acetone–acetic acid (200:20:20:10) 140 mm. (II) (horizontal), methanol–water–acetone–pyridine (200:20:20:10) 140 mm. Sprayed with ninhydrin. (A) R_F 30↑; 20 (pink); (B) R_F 60↑; 40 (purple); (C) R_F 90↑; 60 (pink); (D) R_F 10↑; 50 (purple); (E) R_F 75↑; 75 (pink).

It is very probable that these ninhydrin-positive materials in urine are phenylisopropylamines or amphetamines which could bring about the psychotropic effect as described earlier for nutmeg and other natural products that contain these constituents. Further identification and characterization of these ninhydrin-positive materials will help to clarify the role of these components in the metabolism of the various substituted benzene derivatives.

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