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Mutagenicity of *trans*-Anethole, Estragole, Eugenol, and Safrole in the Ames *Salmonella typhimurium* Assay

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Safrole (4-allyl-1,2-methylenedioxybenzene, estragole (4-allyl-1-methoxybenzene), eugenol (4-allyl-1-hydroxy-2-methoxybenzene), and trans-anethole (4-propenyl-1-methoxybenzene) are structurally related, naturally occurring plant constituents which are hepatotoxic to humans and other animals (Figure 1). Both safrole and estragole are weak hepatocarcinogens when fed to adult rats and mice (MILLER & MILLER 1977), but safrole has a greater hepatocarcinogenic activity when injected into preweanling mice. Safrole is still used as a food additive in some countries and as a synergist in pesticide formulations while eugenol and trans-anethole are widely used flavoring agents (FURIA & BELLANCO 1971; GUENTHER & ALTHAUSEN 1949).

In general, a good correlation exists between carcinogenic activities of chemicals and their mutagenic activities in Salmonella typhimurium (MCCANN et al. 1975; Sugimura et al. 1976). Nevertheless, there have been conflicting reports on the mutagenicity of safrole. MCCANN et al. (1975), DORANGE et al. (1977), WISLOCK et al. (1977), and SWANSON et al. (1979) reported negative results for S. typhimurium strains TA98, TA100, TA1535, TA1537, and TA1538. In contrast, GREEN & SAVAGE (1978) and DORANGE et al. (1978) reported mutagenicity with safrole for strains TA1530, TA1532, and TA1535.

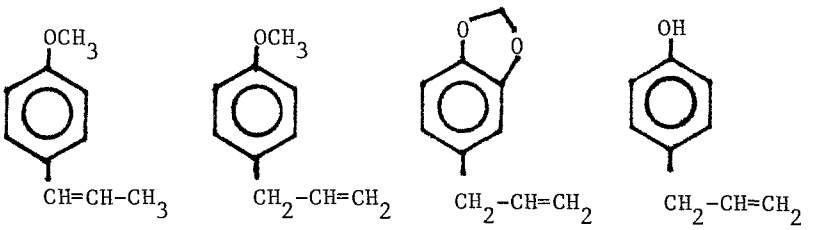

Fig. 1 The structural formulae of safrole and analogues.

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Negative results in S. typhimurium may possibly be due to lack of the appropriate cofactors in the activation system. We have re-investigated the mutagenic activities of safrole, estragole, anethole, and eugenol in S. typhimurium. This report presents a study on the mutagenicity of safrole and related compounds using the method of AMES et al. (1975) as well as the effect of the incorporation of 3'-phosphoadenosine-5'-phosphosulfate.

MATERIALS AND METHODS

Chemicals: trans-Anethole, estragole, eugenol, and safrole were purchased from Aldrich Chemical Company (Milwaukee, WI).

Metabolic activation mixture: Hepatic S-9 fractions from Aroclor 1254-induced rat were obtained from Litton Bionetics (Baltimore, MD). S-9 mix was prepared according to AMES et al. (1975) with 0.1 mL of S-9 fraction/mL mix, $MgCl_2$ (8μM), KCl (33μM), glucose-6-phosphate (5μM), NADP (4μM), and sodium phosphate, pH 7.4 (100μM).

Bacteria: Cultures of S. typhimurium TA1535, TA100 (for detection of base-pair substitution) and TA1537, TA1538, and TA98 (for detection of frameshift mutations) were kindly provided by Dr. Bruce Ames of the University of California at Berkeley. The general procedures for maintenance of stock cultures were those described by AMES et al. (1975).

Toxicity tests: Ethanolic solutions of the test compounds were prepared in the following concentrations: 100, 30, 10, 1, 0.3, and 0.1 mg/mL. Then, 0.1 mL of an overnight bacterial liquid culture (adjusted to 70% transmission), 0.1 mL test chemical, and 0.5 mL of S-9 mix were added (in order) to a tube of 2 mL molten top agar at 45 C. This mixture was quickly swirled and poured onto minimal glucose medium plates. After 48 h at 37 C, the plates were examined for the presence of a background lawn. Absence or reduction of the background lawn to discrete colonies was interpreted as a toxic response.

Mutagenicity assays: Plate incorporation tests with and without 0.5 mL S-9 mix were done according to the method of AMES et al. (1975), at the following concentrations in all 5 tester strains: X, 0.3X, 0.1X, 0.02X, 0.004X, and 0.0001X where X is one-half (1/2) the highest nontoxic concentration. In strain TA1535, a modification of the Ames test was also performed by the addition of 5 mg of 3'-phosphoadenosine-5'-phosphosulfate (PAPS) to the S-9 mix.

RESULTS AND DISCUSSION

The lowest, overtly toxic concentrations for trans-anethole, estragole, and safrole were 1 mg/plate, while eugenol was toxic at 3 mg/plate in all 5 strains.

Although occasional instances of statistical significant differences were seen in the direct bacterial and microsomal mutagenicity assays (see Tables 1 and 2), these differences are small and the biological significance uncertain. GABRIDGE & LEGATOR (1969) stated that a 10-fold increase in mutagenicity is required to classify a compound as mutagenic. If this criterion is applied, none of the above compounds would be classified as mutagens.

The lack of mutagenicity of safrole in our tests is in agreement with other studies using the Ames method (MCCANN et al. 1975; DORANGE et al. 1977; WISLOCK et al. 1977; SWANSON et al. 1979). Our results differ from reports of mutagenicity of safrole at 0.6 nM/plate (DORANGE et al. 1978) and at 0.0025 M/plate (GREEN & SAVAGE 1978). DORANGE et al. (1978) used a 20 min preincubation with liver microsomes and cytosol from 3-methylcholanthrene-treated rats. SWANSON et al. (1979) were unable to confirm the results of DORANGE et al. (1978) using 0.6 nM to 30 µM safrole with a liver S13 fraction from 3-methylcholanthrene-treated rats even with a 20 min preincubation. GREEN & SAVAGE (1978) obtained a mutagenic response with 0.0025 M safrole in TA1530 and TA1532, both in a host-mediated system and in a system using a mouse-liver postmitochondrial fraction for activation. In view of differences in bacterial test strains and activating systems, comparison of our findings with those of DORANGE et al. (1978) or GREEN & SAVAGE (1978) is difficult.

Aside from differences in bacterial test strains and activating systems, the major difference among these reports is in the methods of mutagenicity assay in Salmonella. Ames' method does not permit calculations of mutation frequencies while FRANTZ & MALLING'S (1975) quantitative microsomal assay does. GREEN & SAVAGE (1978) used FRANTZ & MALLING'S (1975) method in the microsomal assay, and mutagenicity was measured in terms of reversion/10^6 survivors. Induced mutation frequencies with safrole in S. typhimurium support the GREEN & SAVAGE (1978) results when we used a similar method (TO & ANDERSEN 1981).

The addition of 3'-phosphadenosine-5'-phosphosulfate (PAPS) to the microsomal assay markedly increased the mutagenicity for all test compounds in TA1535 (Table 3). The ultimate carcinogenic forms of many chemical carcinogens are strong electrophiles (MILLER 1970). These strong electrophiles react with nucleophilic DNA and other cellular macromolecules. Consequently, DNA alterations have received considerable attention as a molecular basis of chemical carcinogenesis. BORCHERT et al. (1973) demonstrated that safrole metabolized into 1'-hydroxysafrole which is more carcinogenic than the parent compound. This hydroxylated metabolite is reported to be mutagenic in TA100 (SWANSON et al. 1979), but not in TA1535 (WISLOCKI et al. 1977). 1'-Hydroxy-safrole can be further metabolized to electrophilic epoxide derivatives by hepatic

TABLE 1. Effect of Compounds on *Salmonella* in Direct Bacterial Assay

Compound	Concentration (μg/plate)	REVERTANTS PER PLATE *SALMONELLA* STRAINS TA98	TA1537	TA1538	TA100	TA1535
Ethanol (Control)		17,24,26	1,7,11	8,11,13	42,56,85	9,13,15
Anethole	0.05	21,26,26	3,9,11	6,9,15	72,81,87	9,15,15
	0.20	20,51,55	9,12,17	10,12,14	62,69,74	17,24,26
	1.0	21,30,36	6,6,10	5,9,15	64,65,66	15,16,20
	5.0	8,20,20	6,6,7	6,9,14	65,66,87	18,18,25
	15.0	2,6,8	8,13,16	4,4,7	63,64,79	16,24,25
	50.0	12,20,24	7,9,9	9,11,16	53,72,86	14,21,24
Estragole	0.05	19,19,21	6,7,11	8,12,13	57,67,69	20,21,21
	0.20	20,26,32	4,5,6	6,7,11	56,57,77	11,12,15
	1.0	25,26,29	3,4,4	6,9,9	48,60,87	16,19,24*
	5.0	25,31,37	5,7,7	7,8,11	51,68,85	17,19,31*
	15.0	17,24,26	5,5,10	7,7,13	30,39,53	15,19,37
	50.0	19,33,37	5,5,13	9,11,13	44,48,50	18,19,27*
Eugenol	0.50	15,19,29	4,7,8	9,9,18	62,63,90	19,19,19*
	2.00	18,27,38	4,8,9	4,8,12	65,85,86	12,18,19
	10.0	14,19,30	4,5,7	7,10,15	63,75,77	12,12,18
	50.0	7,8,19	3,6,12	9,13,16	69,74,79	14,18,25
	150.0	18,36,51	5,7,9	7,7,8	30,50,54	8,16,24
	500.0	26,32,38*	3,9,10	7,7,12	73,92,97	7,12,19
Safrole	0.05	18,25,36	4,9,10	5,6,11	54,55,68	8,17,18
	0.20	16,30,37	5,7,10	4,11,13	53,61,79	16,21,31
	1.0	20,25,32	6,7,12	6,8,8	65,67,98	9,14,17
	5.0	14,14,27	5,6,7	5,13,15	63,65,67	12,14,18
	15.0	13,17,30	4,8,9	5,5,13	77,87,94	8,17,18
	50.0	29,31,38*	4,5,9	8,8,11	64,73,89	19,21,21
2-Aminoanthracene	10.0	21,25,37	4,4,13	8,8,11	76,88,92	16,21,31

* $P < 0.05$ using the Mann-Whitney U test.

TABLE 2. Effect of Compounds on *Salmonella* in Microsomal Assay

Compound	Concentration (µg/plate)	REVERTANTS PER PLATE SALMONELLA STRAINS TA98	TA1537	TA1538	TA100	TA1535
Ethanol		33,38,39	5,12,14	17,20,25	65,86,99	20,24,39
Anethole	0.05	13,24,45	5,7,10	12,20,22	69,93,105	14,18,27
	0.20	19,25,30	6,7,7	21,21,25	65,69,110	7,14,30
	1.0	14,14,19	12,16,23	24,27,30	69,81,81	16,24,31
	5.0	18,19,25	5,9,11	12,14,24	76,80,96	16,16,18
	15.0	19,29,29	4,5,9	11,13,15	87,91,102	8,12,14
	50.0	28,30,36	2,3,5	18,19,23	81,88,90	17,24,25
Estragole	0.05	14,27,29	10,12,13	16,23,24	75,81,81	8,15,19
	0.20	17,27,39	7,7,16	16,21,22	78,88,98	12,20,50
	1.0	14,15,21	3,4,7	11,14,15	78,81,90	13,14,26
	5.0	21,29,41	2,7,7	19,25,26	67,77,79	9,13,15
	15.0	16,18,25	8,10,10	12,22,26	49,54,57	13,17,20
	50.0	20,20,33	12,14,18	18,18,22	66,72,88	12,21,27
Eugenol	0.50	17,24,30	7,8,18	20,24,26	87,96,122	14,19,20
	2.0	19,24,37	10,12,18	20,21,24	105,109,117	20,25,38
	10.0	18,32,43	15,19,24*	13,15,20	67,78,81	19,21,26
	50.0	28,29,30	17,18,24*	14,28,37	101,113,124*	18,20,43
	150.0	18,29,33	14,15,19*	19,19,27	65,84,88	17,19,60
	500.0	26,29,42	18,20,20*	14,21,26	92,117,117	15,15,21
Safrole	0.05	12,27,38	14,14,21	13,20,27	61,92,124	17,19,21
	0.20	18,30,40	9,10,16	11,17,18	80,89,105	15,20,21
	1.0	30,33,33	10,12,20	18,29,36	102,120,128*	9,17,25
	5.0	29,37,55	9,13,15	20,21,27	68,89,100	7,14,24
	15.0	29,30,31	10,13,21	15,15,17	116,136,146*	9,17,25
	50.0	31,39,48	11,11,19	7,15,17	98,108,132	7,14,24
2-Aminoanthracene	10.0	405,396,385*	319,404,332*	167,151,202*	109,364,418*	241,243,278*

* $P < 0.05$ using the Mann-Whitney U test.

TABLE 3. Effect of Cofactor PAPS on the Mutagenicity of Compounds on Salmonella Typhimurium TA1535

COMPOUND	CONCENTRATION (µg/plate)	REVERTANTS PER PLATE - S-9	+ S-9
Ethanol		7,7,13	6,15,19
Anethole	0.05	8,17,19	43,55,78*
	0.20	14,14,21	53,73,77*
	1.0	13,13,14*	101,124,174*
	5.0	14,17,17*	101,165,184*
	15.0	13,15,20*	91,121,127*
	50.0	7,8,18	15,26,69*
Estragole	0.05	12,12,14	18,29,38
	0.20	12,13,16	19,33,36*
	1.0	7,9,15	62,84,123*
	5.0	12,18,28	62,88,91*
	15.0	5,6,12	38,44,65*
	50.0	13,18,22	28,32,33*
Eugenol	0.50	12,13,18	32,45,67*
	2.0	4,5,12	41,63,111*
	10.0	2,6,8	32,64,77*
	50.0	3,4,7	25,28,31*
	150.0	7,9,12	41,42,42*
	500.0	9,12,13	13,31,37
Safrole	0.05	13,13,15	14,20,21
	0.2	9,14,16	73,87,122*
	1.0	5,7,18	52,78,116*
	5.0	9,17,28	32,53,62*
	15.0	9,16,17	20,24,27*
	50.0	12,20,20	20,27,28*
2-Aminoanthracene	10.0	10,12,13	333,339,352*

* $P < 0.05$ using the Mann-Whitney U test.

microsomes or to its sulfuric acid ester by hepatic cytosol (WISLOCKI et al. 1976). SWANSON et al. (1979) showed marked increases in revertants in S. typhimurium TA1535 with 2',3'-oxides of 1'-hydroxysafrole, safrole, 1'-acetoxy-safrole, 1'-oxosafrole, eugenol, estragole, and 1'-hydroxy-estragole.

The increase of revertant colonies as a result of incorporating PAPS into the microsomal, but not in the direct bacterial (Table 3) assay, suggests that safrole and related compounds maybe converted to DNA binding sulfuric acid esters under these conditions. It is crucial to incorporate proper cofactors (metal ions, phosphorylated nucleotides) and soluble enzymes in in vitro mutagenicity screens to optimize the mutagenic response.

Even with PAPS in the microsomal assay, there was no consistent dose-dependence of the mutagenic response. This may be an artifact since mutation was not reported in terms of revertants/survivor (induced mutation frequency) but only as number of revertants/plate.

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