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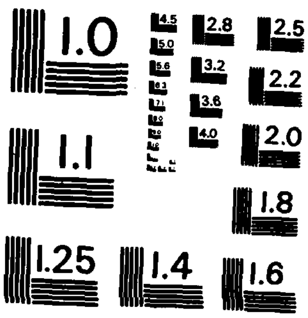

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THE MUTAGENICITY OF SAFROLE IN SALMONELLA *TYPHIMURIUM*, SACCHAROMYCES CEREVISIAE, AND L5178Y MOUSE LYMPHOMA CELLS USING THE FLUCTUATION TEST.

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NOTICES

TECHNICAL REVIEW AND APPROVAL

AFAMRL-TR-82-71

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER

Roger C. Inman

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Safrole mutagenicity was tested using bacteria (Salmonella typhimurium), yeast (Saccharomyces cerevisiae D3) and L5178Y mouse lymphoma cells. The fluctuation method was used in this research and is regarded as 50-100 times more sensitive than conventional methods for detecting mutagenicity. In addition, the effect of the coenzyme for sulfation-3'-phosphoadenosine-5'-phosphosulfate (PAPS)-on mutagenicity was evaluated in the bacteria and yeast. Safrole was mutagenic in *S. typhimurium* strain TA100 at concentrations of 10 and 25 µg/ml. PAPS increased mutation frequency at these safrole concentrations. In yeast these same

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20. ABSTRACT (continued)

concentrations were mutagenic, but the addition of PAPS was without additional effect. Safrole was not mutagenic for mouse L5178Y cells with ouabain as the selective agent.

PREFACE

This research was performed in the Toxicology Branch, Toxic Hazards Division, Aerospace Medical Research Laboratory from December 1979 through September 1981. It was performed in support of Project 6302, "Toxic Hazards of Propellants and Materials"; Task 630201, "Toxicology of Propellants and Materials"; Work Unit 63020104, "Teratogenic Screening of Air Force Chemicals". Drs. To and Rogers were National Research Council research associates. This research was supported by Laboratory Director's Funds.

INTRODUCTION

In standard experiments measuring induced reversion to amino acid prototrophy in auxotrophic strains of bacteria, cells are treated with mutagens and survivors plated on selective agar containing a growth limiting supplement of the required amino acid (Ames et al., 1975). This method works best with higher doses of mutagens, but is rather insensitive with lower doses. Safrole, a hepatocarcinogen, is cytotoxic to Salmonella typhimurium at high doses (To and Andersen, 1981), making evaluation of its mutagenicity difficult in the Ames Salmonella assay. There have been conflicting reports on its mutagenicity in Salmonella. McCann et al. (1975), Dorange et al. (1977), Wislocki et al. (1977), Swanson et al. (1979), and To et al. (1981) reported negative results for Salmonella typhimurium using the method described by Ames et al. (1975). Swanson et al. (1979), however, demonstrated mutagenic activity with 1'-hydroxysafrole in TA100 and with the electrophilic 2',3'-oxides of safrole, 1'-hydroxysafrole, 1'-acetoxysafrole, and 1'-oxosafrole in TA1535. Using a method described by Frantz and Malling (1975), Green and Savage (1978) obtained a 10-fold increase in revertants/10^6 survivors over the control at 0.0025 M/plate in TA1530 and TA1532 using a mouse-liver postmitochondrial fraction for activation. Dorange et al. (1978b) reported mutagenicity with safrole at 0.6 nmoles per plate for strain TA1535 after a 20-min preincubation with liver microsomes and cytosol from 3-methylcholanthrene-treated rats. Dorange et al. (1977, 1978a) also reported mutagenicity in TA1535 and TA100 with 2',3'-oxides of safrole and 1'-hydroxysafrole. To and Andersen (1981) showed a dose-dependent increase in induced mutation frequencies (number of revertants/survivor) in TA100 and TA1535 using a 30-min preincubation with the S9 fraction from Aroclor 1254-induced rats.

Green et al. (1976) have shown that a simplified bacterial fluctuation test is considerably more sensitive than standard plating methods. The modified Luria-Delbruck fluctuation test (Cole, Arlett, and Green, 1976) is regarded as being 50-100 fold more sensitive than these conventional assays. In this investigation, the mutagenicity of safrole in Salmonella typhimurium, Saccharomyces cerevisiae, and L5178Y cells was compared using the fluctuation test. Safrole is oxidized by hepatic microsomes to 1'-hydroxysafrole which can be converted into the DNA binding sulfate ester in the presence of cytosolic sulfotransferases (Wislock et al., 1977). These enzymes require 3'-phosphoadenosine-5'-phosphosulfate (PAPS) as cofactor. Negative results of in vitro mutagenicity assays may be due to lack of the proper coenzymes or soluble enzymes in the activation mixture. This paper describes the effect of PAPS on the mutagenicity of safrole as studied in the fluctuation test with Salmonella and yeast.

MATERIALS AND METHODS

Test strains

Salmonella typhimurium TA100 (histidine auxotroph, R factor strain) was kindly provided by Dr. Bruce Ames (U.C. Berkeley). Saccharomyces cerevisiae D_3 was provided by Dr. F. R. Zimmerman through SRI International (Menlo Park, CA). D_3 is heterozygous for mutation in ADE2 of chromosome XV. Cells homozygous for the ADE2 mutation require adenine and accumulate a red pigment when grown on low-adenine media. Maintenance regimens of Salmonella and Saccharomyces were according to Ames et al. (1975) and Zimmerman (1977).

L5178Y mouse lymphoma cells were originally obtained from Dr. C. F. Arlett, MRC Cell Mutation Unit, Brighton, England. They were routinely screened for PPLO contamination. The cells were grown in suspension culture and cloned in soft agar as described elsewhere (Cole and Arlett, 1976) except that McCoy's 5A medium was used instead of Fischer's medium.

Chemicals

Safrole and 3'-phosphoadenosine-5'-phosphosulfate were from Sigma. Hepatic S9 fractions from Aroclor 1254-induced rats were obtained from Litton Bionetics. For fluctuation tests with Salmonella and yeasts, these working solutions were maintained: Solution A was prepared by dissolving 3.03g Tris-HCl and 0.61g $MgCl_2 \cdot 6H_2O$ in 60 ml distilled water, autoclaving, adjusting pH to 7.5, and adding sterile distilled water to a volume of 100 ml. Solution B was prepared by dissolving sucrose in 70 ml distilled water, autoclaving, and making up to 100 ml by adding sterile distilled water. Solution C was prepared with 20 ml sterile Solution A, 20 ml sterile Solution B, and 10 ml cold sterile distilled water (Rogers, 1978). McCoy's 5A and horse sera were obtained from Gibco-Biocult. Ouabain was obtained from Sigma.

Fluctuation Tests

Salmonella typhimurium

An overnight culture of *S. typhimurium* TA100 in nutrient broth was centrifuged, washed, and resuspended in autoclave-sterilized Davis-Mingioli (DM) salts (Davis and Mingioli, 1950). The following mixture was then prepared: 8 ml of DM salts containing 10^7 bacteria/ml, 67 mg glucose, 12 μg histidine, 6.4 μg biotin, 6 ml S-9, 8 ml Solution C, 12 ml DM salts, 3 ml NADP (5 mg/ml), 3 ml glucose-6-phosphate (8 mg/ml), and 0.4 ml test compound or DMSO control. The mixture was dispensed in 1 ml aliquots to 40 small tubes in a rack. The racks were incubated overnight at 37°C. After incubation, 2 ml DM medium containing 0.4% w/v glucose and 7.5 μg/ml bromocresol purple was added to each tube. Incubation was continued for 3 days. If a mutation occurred in a tube, the revertant growth exhausted the medium, reducing the pH, and causing the medium to change from purple to yellow.

Safrole was tested at 3 concentrations: 1 μg/ml, 10 μg/ml, and 25 μg/ml of mixture. Safrole was toxic at 50 μg/ml. All tests and DMSO controls were run in triplicate. The effect of PAPS on safrole mutagenicity was studied by adding 5 mg PAPS to the above mixture.

Saccharomyces cerevisiae

Saccharomyces cerevisiae D_3 was inoculated in broth containing 1% yeast extract, 2% peptone, and 2% glucose and incubated overnight at 30°C with aeration. This culture was centrifuged and the cells were resuspended at a concentration of 10^6 cells/ml in a synthetic, complete medium containing the following per liter: 6.7 g Difco yeast nitrogen base without amino acids, 5 g glucose, 100 mg adenine sulfate, 10 mg L-arginine-HCl, 10 mg histidine-HCl, 60 mg L-isoleucine, 60 mg L-leucine, 10 mg L-lysine-HCl, 10 mg methionine, 10 mg L-tryptophan, 30 mg L-valine, 10 mg uracil. Then the following mixture was prepared: 8 ml synthetic complete medium containing 10^6 cells/ml, 6 ml S-9, 8 ml solution C, 12 ml synthetic complete medium; 7 ml NADP (5 mg/ml), 3 ml glucose-6-phosphate (8 mg/ml), and 0.4 ml test compound or DMSO solvent control. The mixture was dispensed in 1 ml aliquots to 40 small tubes in a rack. The tubes were incubated overnight in a shaking water bath at 37°C. After incubation, 0.2 ml aliquots were serially diluted to 10^{-5} concention in sterile physiological saline and plated on low adenine (5 mg/liter) synthetic complete medium (Zimmerman, 1977). The plates were incubated for 2 days at 37°C, then for 4 days at 8°C to enhance the detection of red pigmented colonies indicative of adenine-requiring homozygotes. Tubes were scored as positive when red colonies or red sectors were present on the plates and negative when no red colonies were detected. The number of red colonies/plate was not counted.

The standard protocol for the fluctuation test was described by Cole et al. (1976). An exponentially growing culture of L5178Y cells was diluted to 10-25 cells/ml in McCoy's 5A medium + 20% horse serum, 200 μg/ml sodium pyruvate (Sigma) and 100 IU/ml penicillin streptomycin (Sigma). 20 ml aliquots were dispensed into 45 replicate tissue-culture flasks and incubated in a humidified 5% CO_2 incubator. The treatment was applied at 120 hr when the cell density was 25 X 10^4/ml (1 X 10^6 cells/replicate). At this time cells from five of the flasks were counted to determine the exact number of cells per replicate. The flasks were divided into three series of 15 replicates each and were treated as follows: Series A, (control) 0.2 ml ethanol added to each flask; Series B 0.2 ml ethanol containing safrole to give a final concentration of 10 μg/ml was added to each flask; Series C, 0.2 ml ethanol containing safrole 25 μg/ml. The flasks were incubated in a humidified 5% CO_2 incubator for 48 hr. At the end of this period of growth, aliquots (0.1-0.2 ml) were taken from three flasks from each series and counted to determine the mean number of cells per replicate (1-7 X 10^5 cells/ml). Aliquots (0.1 ml) were also withdrawn from three replicates in each series and diluted with 30 ml of non-selective cloning medium to be plated at 15 ml per plate in 9 cm bacterial grade plates (Lux) to determine survival. The contents of every replicate flask were diluted to a total of 60 ml in selective cloning medium containing 1 mM ouabain/ml and plated at 15 ml per plate in four 9 cm dishes. All plates were incubated at 37°C in a humidified 5% CO_2 incubator for 10 days before being scored for colony growth.

RESULTS

The results (Table 1) show three fluctuation tests on Salmonella typhimurium TA100 with safrole, with and without the addition of 3'-phosphoadenosine-5'-phosphosulfate (PAPS). Without PAPS, a significant increase in reversion to histidine prototrophy was not detected at 1 μg/ml, but was detectable at 10 μg and 25 μg/ml. With PAPS, induced mutation was likewise detected at 10 μg and 25 μg/ml but there was a distinct increase in the number of positive tubes.

Table 2 shows the results of three fluctuation tests on Saccharomyces cerevisiae D_3 with safrole with and without the incorporation of PAPS. Induced mitotic recombination was significantly higher than the control at 10 and 25 μg/ml. There was no appreciable difference in the number of positive tubes in the presence of PAPS. The number of tubes positive for mitotic recombination was dose-related.

The results of four experiments with L5178Y cells are shown in Table 3. The data for each experiment were analyzed separately. Only in experiment 1 was the mutation rate greater than control. We conclude that safrole is negative in L5178Y cells in the absence of metabolic activation with this selective system.

TABLE 1. SAFROLE INDUCED MUTATION IN SALMONELLA TYPHIMURIUM TA100 WITH AND WITHOUT COFACTOR PAPS AS SHOWN BY THE FLUCTUATION TEST

CONCENTRATION (μg/mℓ)	NUMBER OF EXPERIMENTS	TOTAL NUMBER OF TUBES	TOTAL NUMBER OF TUBES POSITIVE -PAPS	+PAPS
0	3	120	32 (12,9,11)[a]	35 (12,13,10)
1	3	120	31 (10,10,11)	34 (13,10,11)
10	3	120	54[b] (22,13,14)	73[c] (29,25,19)
25	3	120	70[c] (25,26,19)	103[c] (44,29,30)

[a] Numbers in parenthesis indicate number of positive tubes/40 tubes for each experiment
[b] $P<0.05$ based on the chi-square test (Green et al., 1976)
[c] $P<0.01$ based on the chi-square test (Green et al., 1976)

TABLE 2. SAFROLE INDUCED MIOTIC RECOMBINATION IN SACCHAROMYCES CEREVISIAE D3 WITH AND WITHOUT COFACTOR PAPS AS SHOWN BY THE FLUCTUATION TEST

CONCENTRATION (μg/mℓ)	NUMBER OF EXPERIMENTS	TOTAL NUMBER OF TUBES	TOTAL NUMBER OF TUBES POSITIVE -PAPS	+PAPS
0	3	120	24 (7,7,10)[a]	29 (7,10,12)
1	3	120	28 (8,11,9)	31 (12,7,12)
10	3	120	49[b] (15,18,16)	51[c] (17,20,14)
25	3	120	72[c] (21,26,25)	69[c] (27,19,23)

[a] Numbers in parenthesis indicate number of positive tubes/40 tubes for each experiment
[b] $P<0.05$ based on the chi-square test (Green et al., 1976)
[c] $P<0.01$ based on the chi-square test (Green et al., 1976)

DISCUSSION

The fluctuation test allows the test organism to grow over a long period of time in the presence of a nontoxic concentration of the test agent. The microsomal fluctuation test detects mutagenicity at lower concentrations (10 μg/ml) of safrole in Salmonella typhimurium TA100 than the conventional plate tests (75 μg/ml, see To and Andersen, 1981). In conventional plate tests with an agar overlay, the test agent may diffuse to the bottom layer so that a much higher concentration may be cytotoxic. In addition, the exact concentration of agent is not known due to diffusion.

The addition of PAPS to the fluctuation test in S. typhimurium significantly increased the mutagenicity of safrole at 10 and 25 μg/ml. This suggests that negative results may occur in vitro due to lack of proper micro-environment (i.e. diverse metal ions, various phosphorylated nucleotides, or soluble enzymes). Mutagenicity was also detected at 10 and 25 μg/ml in Saccharomyces cerevisiae D_3. In contrast to its effect in Salmonella, incorporation of PAPS into the fluctuation test did not appreciably increase the mutagenicity of safrole in yeasts. The sulfate ester of safrole may not be as mutagenic for histone-bound DNA. An alternative explanation is that ionized compounds may not pass the intact cell wall of S. cerevisiae as easily as nonionized compounds. The normal lipopolysaccharide barrier coating the surface of bacteria is absent in S. typhimurium TA100 (Ames et al., 1975), facilitating the entry of safrole sulfate ester into the bacterial cell. In the yeast system, the mutagenicity of the sulfate ester may also be reduced by reactions with proteins and other macromolecules before it can bind to yeast DNA.

The sensitivity of the fluctuation test may be enhanced by modifying the incubation procedure and by using postmicrosomal instead of microsomal liver preparations. Kowbel and Nestmann (1981) reported enhanced mutagenicity with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) in Escherichia coli strain WP_2 in the fluctuation test when the incubation temperature was lowered from 37°C to 22°C for 24 hours. They recommend lowering incubation temperature when testing the mutagenicity of relatively volatile or unstable chemicals. Lower temperatures may increase safrole mutagenicity in microbial systems.

Forster et al. (1981) demonstrated increased activation of 2-acetylaminofluorene (2-AAF) to a mutagenic metabolite for S. typhimurium by S105 rat liver postmicrosomal fraction. Like safrole, 2-AAF is converted to a sulfate ester by hepatic cytosolic sulfotransferase activity. They attributed the efficacy of the cytosolic fraction to small inclusions of microsomal proteins necessary for phase I activating reactions. In addition, since high concentrations of microsomal proteins are not present, phase II microsomal detoxifying reactions may have lesser impact on the results. To and Andersen (1981) obtained decreased mutation frequencies in S. typhimurium TA1535 and TA100 with higher concentrations of S9. An investigation of the mutagenicity of safrole using S105 postmicrosomal fraction and PAPS would probably be worthwhile.

The modified Luria-Delbruck fluctuation test is regarded as being 50-100 fold more sensitive than conventional plating techniques (Cole, Arlett and Green, 1976). The experiments reported have given negative results in L5178Y cells suggesting that safrole alone is unable to induce variants resistant to ouabain.

Possible objections to this conclusion are related to the sensitivity of the Oua system. With proven mutagens like ethyl methanesulphonate or methyl methanesulphonate, Oua resistant variants occur with a frequency reduced by a factor of 10 when compared with thioguanine resistance (Cole and Arlett, 1976, 1978). Thus a weak mutagen might prove negative simply as a consequence of the reduced sensitivity of the assay. The negative effect of safrole on this system might be a consequence of either a specific failure to mutate the genes involved at this locus or a weak potential for mutation.

TABLE 3. MUTATION RATES AFTER TREATMENT OF L5178Y MOUSE LYMPHOMA CELLS WITH SAFROLE IN MODIFIED LURIA-DELBRUCK FLUCTUATION TEST

EXPERIMENT NUMBER	CONTROL	TREATMENT 10 μg/ml	25 μg/ml
1	2.3[a]	2.3	9.8[b]
2	3.7	2.9	5.2
3	4.8	6.4	7.0
4	2.4	3.2	4.3

[a] Mutation rate per cell per generation (X 10^{-8}) determined by the method of Cole et al. (1976)
[b] Significant at 0.05 level

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