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Solvent-mutagen interactions in the *Salmonella*/microsome assay

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Abstract

The *Salmonella*/microsome assay is the most used test for assessing the mutagenic potential of chemicals and/or their metabolites. A modified *Salmonella* assay has also been employed to evaluate antimutagenic activities of natural and synthetic substances. Although several solvents used for testing water insoluble compounds are regarded as compatible with the assay, so far their effects on the mutagenic response have not been systematically studied. This study was conducted to investigate the effects of dimethylsulfoxide (DMSO), ethanol (EtOH) and acetone (ACE) on the *Salmonella*/microsome assay sensitivity to detect a set of mutagens generally used as positive controls. Non-toxic doses of solvents (0, 12.5, 25, 50 and 100 µl/plate) were tested against non-toxic doses of mutagens with a sensitive tester strain without (TA100: SA; TA98: NPD and 2-NF; TA97a: 4-NQNO) and with (TA98:AFB1, B[a]P and 2-AF; TA1535: CP) a rat liver S9 mixture. Results showed that the solvents did not alter the effects of direct acting mutagens except for an EtOH-induced nearly 30% enhancement of SA mutagenicity. Nonetheless, EtOH, ACE and to a lesser extent DMSO attenuated the effects of most tested promutagens. The foregoing findings suggested that the solvents impaired the conversion of promutagens into their active metabolites by the rat liver S9. The mode by which solvents interfered with the extrinsic metabolic activation system, however, remains unclear.

Keywords: Antimutagenic agents; *Salmonella typhimurium*; ethanol; acetone; dimethylsulfoxide.

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Introduction

The *Salmonella*/microsome assay (Ames test)is a sensitive, fast and inexpensive mutagenicity test and the most used assay to screen chemicals and/or their metabolites for genotoxic potential.[1]To investigate the antimutagenicity of natural and synthetic substances researchers often use a modified version of the assay protocol.[2]

Dimethyl sulfoxide (DMSO) has been the solvent of choice to test water insoluble or poorly soluble compounds in the Ames assay.[2],[3]Ethanol (EtOH), acetone (ACE), and ten other solvents which are non-mutagenic and not too toxic to *S. typhimurium* tester strains, have been used as well.[3]The effects of these solvents on the assay sensitivity to detect mutagens, however, remains largely unclear.

Some studies suggest that mutagenicity test results may depend on the solvent. For instance, DMSO reacts with trichloroacetic acid to form a short-lived mutagenic derivative which is detected by the Ames test.[4]Solutions of p-phenylenediamine in DMSO – after standing at room temperature for 4 h prior to testing – produced marked positive responses whereas compound aqueous solutions were negative in the assay.[5] Similarly, hexachloroacetone in DMSO was mutagenic whereas it was non-mutagenic when ACE was the solvent.[6]Another study employing the Ames test found that using different amounts of DMSO or ACE to dissolve 2-aminoanthracene (2-AA) gave rise to varying mutagenic responses. Amount of solvent-related differences in the mutagenic response, however, were unclear with benzo[*a*]pyrene (B[*a*]P).[7] In the SOS chromotest, responses to α,β unsaturated ketones and aldehydes dissolved in EtOH were more potent than

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those obtained when test compounds were dissolved in DMSO.[8],[9]A study by Quintero et al. reported that solvents (acetone, dichoromethane and methanol) used to dissolve vitamin E increase its antigenotoxic activity against mitomycin C and 4-nitroquinoline-1-oxide in the SOS chromotest.[10]

This study investigated the effects of different amounts of DMSO, EtOH and ACE in the Ames test response to compounds with a variety of physico-chemical properties and mutagenic potencies.

Material and methods

Chemicals and culture media

Sodium azide - SA (99%) and 2-nitrofluorene - 2-NF (98%) were from Aldrich Chemical Co. 4-nitro-*o*-phenylenediamine - NPD (>97%) was from Fluka (Milwaukee, WI, USA). 4-nitroquinoline-N-oxide - 4-NQNO (98%), 2-aminofluorene - 2-AF (≥98%), and 2-aminoanthracene - 2AA (96%), benzo-[*a*]-pyrene - B[*a*]P (≥96%), cyclophosphamide monohydrate - CP (>97%), aflatoxin B1 - AFB_1 (≥98%) were from Sigma Chemical Co (St. Louis, MO, USA). Dimethylsulfoxide UVASOL spectrophotometric grade – DMSO (99.8%), and acetone analytical grade - ACE (99.98%) were from Merck KGaA (Darmstadt, Germany), while ethanol - EtOH (99.8%) was from Vetec® (Rio de Janeiro, RJ, Brazil).

Nutrient Broth N° 2 was from Oxoid (Hampshire, UK) and Bacto Agar was from Becton, Dickinson and Co (Maryland, USA). D-(+)-glucose was from Vetec® while all other top agar and minimal glucose medium ingredients were from Merck. Culture media and solutions were prepared as described by Maron and Ames.[11]

Metabolic activation system (S9 mixture)

Lyophilized Sprague-Dawley male rat liver post-mithocondrial (S9) fraction induced by Aroclor 1254 (Moltox®, Molecular Toxicology Inc, Boone, NC, USA) was used. β-nicotinamide adenine dinucleotide phosphate-sodium salt (NADP) and β-D-glucose-6-phosphate (monosodium salt) were from Sigma Chemical. Sodium phosphate was from Isofar®(Rio de Janeiro, RJ, Brazil) while disodium phosphate, magnesium chloride hexahydrate and potassium chloride were from Merck. The S9 mixture (10% of S9 fraction) was prepared for use as reported by Gomes-Carneiro et al.[12]

Bacterial tester strains

The *S.typhimurium* strains TA100, TA98, TA97a and TA1535 were kindly supplied by Dr Bruce N. Ames (University of California, Berkeley, USA). In all cases, an inoculum (200 µl) of a thawed permanent culture was added to 20 ml of Oxoid Nutrient Broth N° 2 and incubated at 37°C with shaking until a concentration of approximately 1-2 x 10^9 bacteria per milliliter was obtained. In each experiment, the concentration of viable cells in the culture medium was determined by counting the bacteria colonies (colony-forming units) formed on Oxoid nutrient broth agar plates.

Selection of doses of solvents and mutagens

Preliminary assays were conducted to evaluate the toxicity of solvents (0, 12.5, 25, 50, 100, 250 and 500 µl/plate) and mutagens to tester strains.[12]

SA and CP were dissolved in ultrapure water (Milli-Q®) while 4-NQNO, 2-NF, NPD, B[*a*]P, AFB_1, 2-AA and 2-AF were dissolved in DMSO. Tester strain and positive control (mutagens) were as follows TA100 (SA), TA97a (4-NQNO), TA98 (NPD, 2-NF, B[*a*]P, AFB_1, 2-AA and 2-AF), and TA1535 (CP).

Test on the interaction of solvents with mutagens

Solvent effects on the assay (plate incorporation procedure) response to mutagens (positive controls) were tested as described elsewhere.[13]Briefly, 100 µl of a grown culture, different volumes of the solvent (0, 12.5, 25, 50 or 100 µl), 50 µl of the mutagen solution in DMSO or water and 500 µl of sodium-phosphate buffer pH 7.4 or the S9 mix, were added to 2 ml of molten top agar. This mixture was poured onto minimal glucose plates that were then incubated in the dark at 37°C for 72 h and scored for revertant *his*$^+$ bacteria colonies. Each determination was made in 3-4 replicates. The background reversion rate was determined for each tester strain, in the absence of solvents and mutagens, in every experiment.

A solvent-mutagen interaction response was defined as any statistically detected variation (increase or decrease) in the average number of revertants compared to the average number of revertants in control plates (solvent dose 0). To check reproducibility and consistency of findings, each experiment was repeated at least once.

Effects of solvents and solvent-mutagen combinations on bacteria viability

A bacteria viability test was conducted using an overnight grown culture (16 h - 2-3x10^9 bacteria/ml) that was diluted (1:10^6) in liquid Oxoid medium. Molten top agar was mixed with 100µl of the diluted culture, and with the solvent alone (12.5-500µl) or solvent-mutagen combinations (12.5-100µl) in the presence of S9 mixture. Treated bacterial cultures were then plated on the Oxoid medium agar. After plate incubation for 48-72 h at 37 °C, colony-forming units were scored using a colony counter.

Statistical analysis

Data (number of *his*$^+$ revertants)were evaluated by one-way ANOVA and Dunnett's test (GraphPad Prism5) and the significance level was $p< 0.05$.

Results

Toxicity of solvents and positive control mutagens to tester strains

Mutagen doses used in the study were within the linear part of the dose-response curve (obtained in previous studies) and were non-toxic to the tester strain. Non-toxic doses caused no discernible reduction in the number of *his*$^+$colonies and no alteration of the "background lawn". The non-toxic dose intervals (µg/plate) were as follows: SA (TA100) 0.2-2.0, NPD (TA98) 1.0-5.0, 2-NF (TA98) 1.0-4.0, 4-NQNO (TA97a) 0.25-2.0, AFB_1 (TA98 +S9) 0.05-0.25, B[*a*]P (TA98 +S9) 1.0-3.0, 2-AA (TA98 +S9) 0.25-2.0, 2-AF (TA98 +S9) 0.5-3.0, and CP (TA1535 +S9) 500-5000 (data not shown). The (non-toxic) doses of mutagens selected to be tested against the solvents were: SA: 0.25, 0.5 and 1.0; NPD: 2.5 and 5.0; 2-NF: 0.5, 1.0 and 2.5; 4-NQNO: 0.5 and 1.0; AFB_1: 0.06 and 0.15; B[*a*]P: 1.5, 2,5 and 5.0; 2-AA: 0.5 and 1.0; 2-AF: 2.5 and 5.0; CP: 500 and 1000.

Solvent (0, 12.5, 25, 50, 100, 250, 500 µl/plate) toxicity to tester strains was tested as well. The highest dose (500 µl/plate) of the solvents was toxic to all tester strains. At doses ≤ 250µl/plate no solvent-related toxicity was noted [Table 1]. Taking into account these findings, we selected 100 µl/plate as the upper limit of the solvent dose-range tested against non-toxic doses of mutagens.

Dimethylsulfoxide (DMSO)

DMSO up to 100 µl/plate did not alter the response to any direct acting mutagen (SA, NPD, 2-NF, or 4-NQNO) in the Ames test (data not shown). Nonetheless, the assay response to the promutagen AFB_1 was attenuated by DMSO in a fairly dose dependent manner [Figure 1]. The mutagenic response to AFB_1 0.06 and 0.15 µg/plate in the presence of DMSO 100 µl was 50.5 and 57.8%, respectively, the response in control plates. DMSO 12.5 and 25 µl/plate, but not at higher doses (50 and 100 µl/plate), attenuated the response to 2-AA [Figure 1] and [Table 2]. In plates treated with DMSO 12.5 and 25 µl/plate, the average number of revertants induced by 2-AA 0.5 µg/plate declined to 52.1 and 66.1% the average number of revertants in control plates, respectively. Similarly, solvent low dose-only effects were also noted when DMSO was tested against a higher dose of 2-AA (1.0 µg/plate) [Figure 1] and [Table 2]. These findings indicated that DMSO attenuated the mutagenic effect of AFB_1 and 2-AA, promutagens activated by the S9 mixture. The inhibitory effect of DMSO on AA-induced mutagenesis, however, was non-linear and observed only at the lowest doses tested. The response to promutagens B[*a*]P, 2-AF and CP was not altered by DMSO (data not shown).

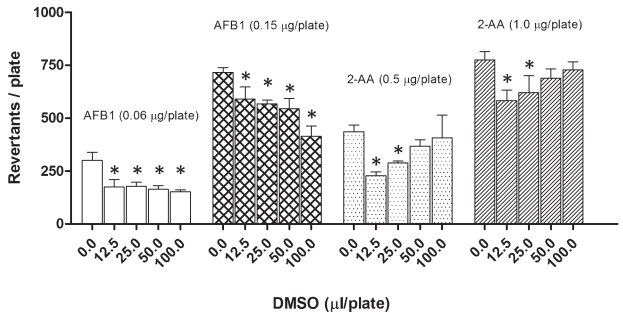


Figure 1. Effect of dimethylsulfoxide (DMSO) on the number of *his*$^+$ revertants induced by promutagens in the *Salmonella*/microsome assay. Values are Means ± SD of three to four plates of one representative experiment. The mean number of spontaneous revertants (TA98) obtained in each assay (48, 60, 37, and 58) was subtracted. *: Different (ANOVA and Dunnett's test; $P< 0.05$) from DMSO dose 0. AFB_1: aflatoxin B1. 2AA: 2-aminanthracene. 2-AF, B[a]P and CP were not affected.

Table 1. Toxicity of ethanol (EtOH), acetone (ACE) and dimethylsulfoxide (DMSO) to *S.typhimurium* tester strains TA97a, TA98, TA100 and TA1535 without (-) and with (+) addition of S9 mixture and effects of solvents on the viability of TA98 strain

Doses (µl/ plate)		TA98/-S9	TA100/-S9	TA97a/-S9	TA1535/+S9	TA98/+S9	
		N°. of revertants (Mean ± SD)	N°. of revertants (Mean ± SD)	N°. of revertants (Mean ± SD)	N°. of revertants (Mean ± SD)	Nº. of revertants (Mean ± SD)	Nº. of viable colonies (x 10^7)" (Mean ± SD)
EtOH	500	18 ± 8 T	43 ± 37 T	62 ± 6 T	11 ± 11 T	2 ± 2 T	0 ± 1 *
	250	29 ± 5	116 ± 12	120 ± 12	23 ± 1	38 ± 9	40 ± 5 *
	100	34 ± 6	135 ± 13	125 ± 8	17 ± 1	46 ± 6	146 ± 28 *
	50	37 ± 6	133 ± 6	145 ± 3	19 ± 3	49 ± 6	260 ± 36
	25	45 ± 4	134 ± 12	158 ± 10	15 ± 3	53 ± 6	238 ± 39
	12.5	38 ± 2	128 ± 2	154 ± 7	14 ± 3	48 ± 4	232 ± 34
	0	35 ± 7	166 ± 17	142 ± 19	20 ± 2	48 ± 4	242 ± 11
	PC	381 ± 39	814 ± 58	803 ± 84	218 ± 5	425 ± 39	-
ACE	500	10 ± 9 T	31 ± 46 T	32 ± 15 T	12 ± 2	34 ± 7	20 ± 6 *
	250	37 ± 7	127 ± 11	124 ± 28	16 ± 3	44 ± 4	191 ± 38
	100	36 ± 6	152 ± 19	126 ± 5	17 ± 5	50 ± 8	219 ± 18
	50	34 ± 1	149 ± 11	151 ± 57	15 ± 5	50 ± 2	234 ± 2
	25	34 ± 6	136 ± 11	154 ± 13	17 ± 4	43 ± 5	254 ± 40
	12.5	39 ± 6	165 ± 15	148 ± 8	13 ± 3	54 ± 13	229 ± 4
	0	35 ± 7	166 ± 17	142 ± 19	20 ± 2	47 ± 5	222 ± 11
	PC	381 ± 39	814 ± 58	803 ± 84	218 ± 5	576 ± 33	-
DMSO	500	26 ± 3	145 ± 8	124 ± 12	16 ± 1	30 ± 3	66 ± 61 *
	250	32 ± 8	127 ± 4	124 ± 11	19 ± 3	40 ± 3	173 ± 38
	100	31 ± 7	137 ± 16	142 ± 7	16 ± 2	42 ± 8	180 ± 34
	50	38 ± 8	140 ± 17	162 ± 9	14 ± 3	43 ± 6	177 ± 65
	25	35 ± 8	153 ± 4	148 ± 23	17 ± 3	47 ± 6	129 ± 16
	12.5	34 ± 3	141 ± 20	153 ± 12	18 ± 3	47 ± 1	222 ± 24
	0	35 ± 7	166 ± 17	142 ± 19	16 ± 2	47 ± 5	222 ± 11
	PC	381 ± 39	814 ± 58	803 ± 84	213 ± 6	576 ± 33	-

Values are Mean ± SD of three plates. Dose 0 – spontaneous reversion; PC – positive control mutagen for TA98/-S9, 2NF (1µg/plate); TA100/-S9, SA (1µg/plate); TA97a/-S9, 4-NQNO (1µg/plate); for TA98/+S9, 2-AA (1µg/plate); TA1535/+S9, 2-AA (2µg/plate). SA: sodium azide, 2-NF: 2-nitrofluorene, 4-NQNO: 4-nitroquinoline-N-oxide, 2AA: 2-aminoanthracene. T: Toxicity apparent as a change of the auxotrophic background lawn. Statistical analysis was performed by comparing the number of viable colonies in solvent treated Oxoid plates and in untreated plates (Dose 0 = controls). *: Differs from negative control (dose 0) plates ($p < 0.05$).

Table 2. Solvent-mutagen interactions in the *Salmonella typhimurium* / microsome assay

Mutagen (µg/plate)	Tester strain	Solvent		
		DMSO	EtOH	ACE
Direct (- S9):				
SA (0.25)	TA100	no	↑ [23%]	-
SA (0.5)	TA100	no	↑ [31%]	no
SA (1.0)	TA100	no	no	no
NPD (2.5)	TA98	no	no	no
NPD (5.0)	TA98	no	no	no
2-NF (0.5)	TA98	no	no	no
2-NF (1.0)	TA98	no	no	no
2-NF (2.5)	TA98	no	no	no
4-NQNO (0.5)	TA97a	no	no	no
4-NQNO (1.0)	TA97a	no	no	no
Indirect (+ S9):				
AFB1 (0.06)	TA98	↓ [50%]	↓ [36%]	↓ [43%]
AFB1 (0.15)	TA98	↓ [42%]	↓ [36%]	↓ [51%]
B[a]P (1.5)	TA98	-	↓ [27%]	↓ [35%]
B[a]P (2.5)	TA98	no	↓ [31%]	↓ [34%]
B[a]P (5.0)	TA98	no	-	-
2-AA (0.5)	TA98	↓ [48%]	↓ [27%]	↓ [39%]
2-AA (1.0)	TA98	↓ [25%]	no	↓ [37%]
2-AF (2.5)	TA98	no	no	no
2-AF (5.0)	TA98	no	no	no
CP (500)	TA1535	no	no	no
CP (1000)	TA1535	no	no	no

(-) not tested; no: no interaction. ↑: increase in the number of *his*$^+$ colonies. ↓: reduction in the number of *his*$^+$ colonies. In brackets are the maximum increment or decrement expressed as % of the number of *his*$^+$ revertants recorded in solvent control plates (solvent dose 0 = 100%). SA: sodium azide; NPD: nitro-o-phenylenediamine; 2-NF: 2-nitrofluorene; 4-NQNO: 4-nitroquinoline-N-oxide; AFB1: aflatoxin B1; B[*a*]P: benzo-[*a*]-pyrene; 2AA: 2-aminoanthracene; 2-AF: 2-aminofluorene; CP: cyclophosphamide.

Ethanol (EtOH)

Except for SA, effects of direct-acting mutagens were unaffected by EtOH. ETOH (100 µl/plate) enhanced the assay response to the two lowest (0.25 and 0.5 µg/plate) of SA in 23 and 31%, respectively [Figure 2] and [Table 2]. The effects of the highest doses of SA (1.0 and 2.5 µg/plate) remained unaltered. The effects of three promutagens (AFB_1, B[*a*]P, 2-AA) were attenuated by ETOH at doses as high as 50 and 100µl/plate [Figure 3]. The mutagenic response to 2-AF and CP, however, were unaltered by addition of ETOH (data not shown).

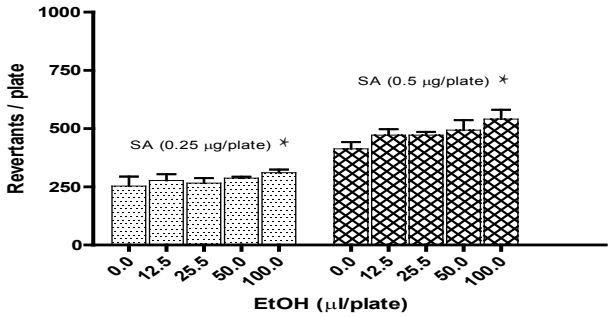


Figure 2. Effect of ethanol (EtOH) on the number of *his*$^+$ revertants induced by the direct acting mutagen sodium azide (SA) in the *Salmonella*/microsome assay. Values are Means ± SD of three to four plates of one representative experiment. The mean number of spontaneous revertants (TA100) obtained in each assay (131, 148) was subtracted. *: Different (ANOVA and Dunnett's test; P< 0.05) from EtOH dose 0.

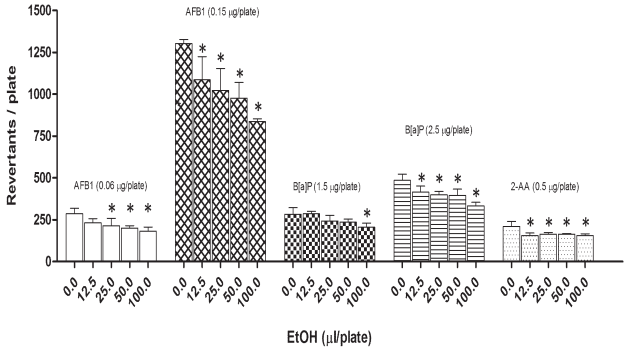


Figure 3. Effect of ethanol (EtOH) on the number of *his*$^+$ revertants induced by pro-mutagens in the *Salmonella*/microsome assay. Values are Means ± SD of three to four plates of one representative experiment. The mean number of spontaneous revertants (TA98) obtained in each assay (48, 50, 48, 51 and 47) was subtracted. *: Different (ANOVA and Dunnett's test; P< 0.05) from EtOH dose 0. AFB_1: aflatoxin B1. B[a]P: benzo-[a]-pyrene. 2AA: 2-aminoanthracene. 2-AF and CP were not affected.

Acetone (ACE)

ACE did not alter the mutagenic responses to direct-acting mutagens and to promutagens 2-AF and CP (data not shown). The effects of AFB_1, B[*a*]P, and 2-AA, however, were attenuated by ACE. ACE 100µl/plate reduced the response to AFB_1, B[*a*]P, and 2-AA to 49 to 69% the response observed in the solvent-untreated plates (100%) [Figure 4] and [Table 2].

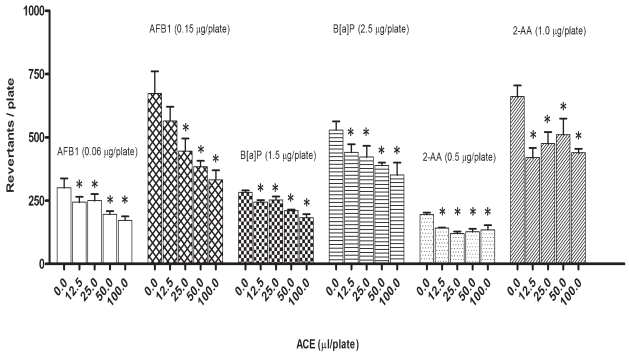


Figure 4. Effect of acetone (ACE) on the number of *his*$^+$ revertants induced by pro-mutagens in the *Salmonella*/microsome assay. Values are Means ± SD of three to four plates of one representative experiment. The mean number of spontaneous revertants (TA98) obtained in each assay (48, 50, 53, 55, 47 and 47) was subtracted. *: Different (ANOVA and Dunnett's test; P< 0.05) from ACE dose 0. AFB_1: aflatoxin B1. B[a]P: benzo-[a]-pyrene. 2AA: 2-aminoanthracene. 2-AF and CP were not affected.

Confirmation of solvents' antimutagenic effects

Reductions in the number of revertants can arise from a bactericidal effect and we need to exclude this possibility before concluding that the solvent exerted an anti-mutagenic effect.

In a preliminary dose-range finding assay, solvent toxicity was assessed as treatment-related reductions in the number of revertants per plate and/or appearance of an auxotrophic "background lawn".[11]A great number of tiny colonies of *his*$^-$ bacteria, the growth of which was promoted by trace amounts of histidine due to massive bacterial deaths, forms a"background lawn". Detection of changes of "background lawn" appearance, however, depends on evaluators' subjective judgment and grading and thus they are not direct and quantitative measurements of bacteria viability. To confirm (assays against AFB_1, B[*a*]P, and 2-AA with TA98) that the decrement in the number of revertants was an antimutagenic effect of the solvent and did not arise from a decreased bacteria survival caused by solvents and/or solvent-mutagen combinations, we assessed their effects on bacteria viability. In other words, we conducted bacteria viability tests to clarify whether solvent- or solvent-mutagen combination-caused deaths of auxotrophic *his*$^-$ bacteria could explain the decreases in the yield of prototrophic *his*$^+$ colonies.

The column on the right of Table 1 shows the effects of solvents on bacteria viability. A reduction of cell viability noted with ETOH 100 µl/plate was the only case in which a solvent toxicity to TA98 occurred in the dose-range tested for anti mutagenicity (12.5-100 µl/plate). Solvent-mutagen combinations with EtOH 100 µl/plate, however, caused no decline of TA98 viability (data not shown). Overall, these findings suggested that, except for ETOH 100 µl/plate, in all other cases the decline in the number of revertants was in fact an antimutagenic effect of the solvent.

Discussion

This study showed that,except for a nearly 30% enhancement of the mutagenic effect of SA by EtOH, assay response to direct-acting mutagens was not altered by the solvent [Table 2]. EtOH, ACE, and to a lesser extent DMSO, however, attenuated the effects of promutagens [Table 2]. The magnitude of the solvent-caused depression of assay response to promutagens was in most cases dose-dependent and more pronounced at the highest dose.

The fact that EtOH, ACE and DMSO attenuated pro mutagen effects while effects of direct-acting mutagens were unaltered indicates that the solvents may have impaired the conversion of promutagens into their active metabolites. This interpretation is consistent with some previous findings. Mori et al.[14] described that the number of TA100 revertants induced by N-nitrosodialkylamines (NNDAs) declined after addition of DMSO, EtOH (95%), ACE, dimethyl formamide or acetonitrile to the incubation mixture (pre-incubation method). Recoveries of unmetabolized NNDAs from pre-incubation mixtures and agar plates with solvents and S9 were higher than recoveries with S9 and without solvents, thereby suggesting that the solvent blocked the activation of NNDAs by rat liver S9. Mutagenic NNDAs undergo hydroxylation by CYP2E1 to form DNA adducts, and EtOH, ACE, DMSO and dimethyl formamide are substrates for CYP2E1. A competitive enzyme inhibition is, therefore, a plausible explanation for solvent-caused impairment of NNDAs activation.[14] Similarly, the solvent-produced attenuation of the assay response to promutagens in this study may have resulted from an inhibition of enzymes that mediate their conversion into active metabolites.

This interpretation is also consistent with data showing that solvents inhibit CYP oxidative reactions in *in vitro*

test systems. When methanol, acetonitrile or DMSO (0.1, 1 and 2% v/v) were added to human hepatocyte cultures, DMSO decreased the activities of CYP2E1, 2C9, 2C19 and/or 3A4, while methanol inhibited CYP2C9 and 2E1, and acetonitrile did not produce any noticeably change of CYP activities.[15] In solvent-treated human liver microsomes, DMSO (0.2% v/v) inhibited CYP2C8/9, 2C19, 2E1 and 3A4-catalyzed reactions, while methanol (≤1% v/v) reduced activities of CYP2C8/9 and 2E1, and acetonitrile (up to 1%) did not affect any CYP activity.[16] Busby et al.[17] treated human B lymphoblastoid cell lines (stably expressing CYP1A1, 1A2, 2A6, 3A4, 2B6, 2C9, 2C19 and 2D6) and baculovirus-insect cell (expressing CYP2C8) systems with methanol, EtOH, DMSO or acetonitrile. Methanol 1%v/v inhibited CYP1A, 2B6, 2C9 and 2D6 activities by 12-26%, EtOH 0.1% decreased CYP1A1, 2B6 and 2C19 activities by 20 to 30%, DMSO 0.1% reduced CYP3A4, 2C19 and 2D6 by 15-25% and acetonitrile 1% depressed CYP1A1 and 2B6 by 40-60% and inhibited 2A6, 3A4, 2C19 and 2D6 by 10-20%.[17] Hickman et al. reported inhibitory effects of DMSO and ACE (at 1% v/v) on CYP-mediated reactions in human liver microsomes and showed that DMSO depressed CYP2E1, and to a lesser degree 2C19, 1A2 and 2C9, while ACE strongly inhibited 2E1, and to varying degrees 2C19, 2A6 and some other forms.[18]

Solvent-caused stimulation of CYP activities (yet rarer than inhibition) was also described. ACE (1%) produced a two-fold increase in CYP1A2-mediated activity (i.e., caffeine N3 demethylation).[18] Additional examples of CYP stimulation are enhancements of aldrin epoxidation and alprazolam hydroxylation by EtOH and ACE, respectively.[19],[20]Acetonitrile and ACE (at 2-4% final concentrations) stimulated NADPH-dependent tolbutamide hydroxylation by nearly two- to three-fold in human liver microsomes and CYP2C9-reconstituded systems.[21]

These studies indicated that EtOH, ACE, DMSO and other solvents, at low concentrations (<1%v/v), inhibit or stimulate catalytic activities of a diversity of CYP forms in *in vitro* test systems. Our results are consistent with this notion because nontoxic doses of EtOH, ACE and DMSO attenuated the effects of promutagens converted into active metabolites by CYP-mediated reactions. The hypothesis that solvents inhibit assay response to promutagens by acting at a stage other than that of metabolic activation is unlikely, because the solvents did not inhibit any direct-acting mutagen in a similar manner.

The metabolic activation of most promutagens/carcinogens in humans and rodents is catalyzed by a limited group of CYP forms/subfamilies such as CYP1A1, 1A2,1B1, 2A (2A6 in humans), 2B (2B6 in humans), 2C (2C9 in humans), 2E1, and 3A (3A4 in humans).[22],[23] The oxidation of AFB_1 to its mutagenic metabolite AFB_1 8,9-epoxide in humans is mediated predominantly by CYP3A4, and to a lesser degree by CYP1A1 and 1A2. [23]Some studies suggested that CYP2E1 and/or 2A6 also mediate the activation of AFB1.[24] The activation of polycyclic aromatic hydrocarbons (e.g. B[*a*]P) and aromatic amines (e.g. 2-AF and 2-AA) is catalyzed by CYP1A1 and 1A2 forms.[22], [23]EtOH, ACE and DMSO, therefore, inhibit CYP forms (1A1, 1A2, 2E1, 3A) that take part in the activation of promutagens used in this study (AFB_1, B[*a*]P, 2AA and CP).

Depending on which CYP forms metabolize the test substance, and on the solvent and its amount added to the plate, assay sensitivity to detect promutagens can be reduced by 27-35% (B[*a*]P) and 25-48% (2-AA) or nearly 50% (AFB_1) [Table 2]. The influence of the solvent on the outcome of *Salmonella*/microsome assay with a substance for which there is no previous data, however, is difficult to anticipate, avoid and/or control. Taking into account only the promutagens tested in this study, DMSO seems to be the most suitable solvent for the assay because it interferes less than EtOH and ACE with the mutagenic response. Nonetheless, similarly to ETOH and ACE, DMSO also markedly depressed the mutagenic effect of AFB_1 in the assay. Along the same line, unexpected low dose/concentration *in vivo* and *in vitro* (retinal cell line) toxicity of DMSO was also reported.[25]

If we know which CYP forms play a major role in the metabolism of the test compound, it is possible to test the effects of the solvent on the activity of these enzymes. If not, it should be borne in mind that solvents may reduce *Salmonella*/microsome assay sensitivity to detect weak mutagens that require metabolic activation. At any rate, the amount of solvent added to the assay mixture should always be the minimum amount required to dissolve the test compound. Current Bacterial Reverse Mutation Test protocols (e.g., OECD 471) recommend that, if organic solvents are necessary to dilute water insoluble or poorly soluble chemicals, they should be compatible with the survival of bacteria and the S9 activity. According to OECD 471, organic solvents compatible with the Ames test are cited in Maron et al. [3]This article, however, did not provide any information on the impact of solvents

on the assay sensitivity to detect mutagens or on the rat liver S9 activity. Findings presented here, therefore, add to these guidelines by indicating that DMSO, EtOH and ACE, depending on their concentrations, may decrease assay sensitivity to detect promutagens.

Furthermore, whenever a modified assay protocol is used for anti mutagenicity tests, control plates should be included to detect effects of the solvent upon the yield of revertants by the mutagen against which the substance (or plant extract) is tested. Possible antimutagenic effects of the solvent have been overlooked in tests on the antimutagenic activity of chemicals and plant extracts. Water insoluble compounds require the use of solvents to be tested in *in vitro* assays and, in the case of plant extracts, it should also be taken into consideration that they at times contain a certain amount of the solvent (methanol, ethanol, acetone and others) used in the extraction procedure. Results from this study suggested that solvents used to dissolve test compounds might have contributed to some of antimutagenic effects reported in the literature.

In conclusion, the solvents evaluated in this study did not alter the effects of direct-acting mutagens in the *Salmonella*/microsome assay, except for a nearly 30% enhancement of sodium azide effects by ethanol. Ethanol, acetone and DMSO, however, decreased the number of *his*$^+$ revertants induced by most of tested promutagens. Based on data provided here and on results of published studies showing that solvents inhibit CYP-catalyzed reactions, a hypothesis was advanced that solvents even at low and nontoxic doses may impair the metabolic activation of promutagens in the *Salmonella*/microsome assay.

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Conflict of Interest

None Declared

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