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Mutagenesis of the Metabolite of Nonionic Detergents in Water

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SYNOPSIS

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Problem and Research objectives:

Nonionic detergents are used in this country and abroad. The nonionic detergents upon biochemical degradation yield nonylphenol as one of the metabolites. The metabolite is toxic to aquatic animals. The prime objective of this study was to determine if the metabolite is mutagenic.

Methodology

Five tester strains (TA97, TA98, TA100, TA102 and TA104) developed by Ames at the University of California, Berkely, were used to test the hypothesis. The preincubation test procedures were used in this research. Tests were carried with and without S9 activation.

Principal Findings and Significance

The results obtained show that nonylphenol is not a mutagen under the test conditions. However it is toxic at higher concentration (> 100 pg/plate). It may be pointed out that several highly toxic compounds such as DDT and DDE have been proven *non-mutagenic* by Salmonella typhimurium test

(Ames Test). The results indicate that further studies are needed to quantify the toxicity of the compound.

Publication

We will write a technical paper and submit it for publication to the American Water Works Association.

ABSTRACT

Biochemical decomposition of nonionic detergents under aerobic and anaerobic conditions yield a metabolite, 4-nonylphenol. The end use of nonionic detergents in this country is $1^{1}10{,}000$ tons per year. In addition nonylphenol is widely used for manufacturing domestic, agricultural and industrial products. Due to its vast production and consumption nonylphenol is found in European wastewater, sewage sludge and surface water.

Toxicity testing has indicated that nonylphenol is twice more toxic than cadmium.

Studies were conducted using Ames preincubation test procedures to determine the mutagenic activity of nonylphenol. Tester strains used were TA97, TA98, TA100, TA102 and TA104. Both spot test and preincubation tests were done with and without S9 activation.

The results obtained show that nonylphenol is not a mutagenic compound under the test conditions. Dose response studies showed that it was toxic at higher concentration (>100 ug/plate). Nonylphenol should be further tested for its toxicity to Salmonella strains. DDT

and DDE were also not mutagenic as determined by the Ames test.

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INTRODUCTION

Nonionic surfactants [4-alkylphenol polyethoxylates (APE)] are extensively used in the U.S. and other parts of the world. The biodegradation of nonionic surfactants under anaerobic and aerobic conditions yields nonylphenol as a metabolite.

The Ames test is a bacterial test system for detecting chemical mutagens. The test is based on the reversion (back-mutation) of Salmonella mutants.

NONYLPHENOL

Nonylphenol (NP) and nonylphenol-ethyoxylates (NPEO) with one to three oxythylene groups are refactory compounds. Nonionic surfactants of the NPEO type are considered to be the precursors. The nonionic surfactants are manufactured by reacting 4-NP with ethylene oxide. Because NP is made from phenol and tripropylene, various branched chained isomers are also formed in the process. The total consumption of nonionic detergents in the U.S. is about 140,000 metric tons (1). The use of NP in this country is about 70% by surfactant manufactures and 30% by plastic, rubber, fungicide, bactericide and dye industries (2).

NP can also be used as oil additives, synthetic lubricants and pesticides. For example it makes up more than 50% of the aminocarb formation MaticilR, a pesticide used for forest spraying in Canada (4). industry will continue to find more special applications for NP.

A search of the literature* indicates that very limited toxicological health research has been conducted on this chemical. Some preliminary toxicity data on NP were obtained for aquatic organism, rodent and rabbit. The toxicity for aquatic organisms is high. The TL_m96** of NP varies from 130 mg/Z for salmon and 300 mg/1 for shrimp (3). The median effective concentration (EC50) of NP to Daphnia magna is 0.18 mg/Z approximately about one half of cadmium (0.35 mg/1) (4). That means NP is about twice as toxic to D. magna than cadmium. Fish and shrimp are barometers of specific toxicity to humans. In addition, the aquatic organisms- concentrate and accumulate toxic matters and play an important role in food chain magnification. Other toxicity data include an LD50 of 1620 mg/kg in the rat
(oral route) and of 2140 mg/kg in the rabbit (skin application) (5). These results indicate that NP has a moderate acute toxicity in rodents.

NP is a toxic chemical, however, it is not listed among EPA's 129 priority pollutants, primarily because of lack of toxicity data. Consequently, no routine analytical monitoring is made for the presence of this compound in sewage sludge, wastewater effluents and surface waters. NP is not a site specific industrial pollutant, and

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*Databanks searched: Medline, Cancerline, Toxline, Chemical Abstracts and Toxicological databank.

**TL_m96 is the toxic concentration of 50% kill in 96 hours.

it has been detected in domestic wastewater and river water (5). Due to the wide use of this compound, there is a strong possibility of the presence of NP in wastewater and sludge.

THE AMES TEST

There is very limited toxicological health data and there is no reference to the mutagenicity and/or carcinogenicity of NP. It is therefore necessary to test the mutagenicity and/or toxicity of this compound. We started the study of NP with Ames' mutagenicity assay using Salmonella tester strains without and with mammalian microsomal enzyme activation. In 1971, Dr. B.N. Ames and his co-workers (6) of University of California at Berkeley developed a very sensitive bacterial test system for detecting chemical mutagens. The Ames test is based on the back-mutation (reversion) of selected mutants (phenotypes) of Salmonella typhimurium LT2 to wide type (prototype) by muta

gens. Since the development of the Ames test, many specially constructed histidine-dependent mutants of Salmonella have been derived. Strains TA97, TA98, TA100, TA102 and TA104 are recommended by Dr. Ames as standard tester strains. They are often used for general mutagenicity testing. There are many other adjunct strains used for more specific testing.

Over the years, many improvements and modifications have been made to the Ames test to enhance the sensitivities and selectivity of the tester strains in detecting a wide variety of mutagenic com

The incorporation of mammalian microsomal (S9 enzymes) activation and the addition of preincubation period to the standard plate pounds.

incorporation test have made the Ames test far more sensitive for many different categories of chemicals.

The Ames test was first validated in a study of 300 chemicals (7,8). Subsequently the test validation have been made several times. A recent validation was done by Rinkus and Legator (9, 10). Ames and McCann (11) in a discussion of Rinkus and Legator's work, estimated the correlation between the mutagnicity and carcinogenicity of tested chemicals to be 88%. There are test data of Ames technique on more than 5000 chemicals published by Environmental Mutagenic Information Center (EMIC) (17).

METHODOLOGY

MATERIALS

I. The Bacterial Tester Strains

Five standard tester strains obtained from Dr. Ames' lab are TA97, TA98, TA100, TA102, and TA104. Each tester strain contains a different and unique type of mutation in the histidine operon. To increase the sensitivities and selectivities of the tester strain, other types of mutation were also implanted in the tester strain through genetic engineering. A brief discussion of the genetic characteristics of the tester strain, TA97A, TA98, TA100, TA102 and TA104, used in our work is as follows

(1) Histidine mutation of the tester strain is the most important kind of mutation.. It determines the mechanism of the histidine reversion (mutation) in the tester strain. Three strains we used have G-C base pairs at the critical site for reversion.. TA100 derived from TA1535 is a base-pair substitution strain containing 3 G-C base pairs in the hisG46 gene coding for the first enzyme of histidine biosynthesis (6). Both TA98 and TA97 are frameshift-mutation strains. TA98 derived from TA1538 has the risD3052 mutation in the hisD gene coding for histidinol dehydrogenases. The hisD3052 mutation has 8 repetitive C-G residue near the site of a-1 frameshift mutation (12). TA97 is a fairly new frameshift strain. It has an added cytosine resulting in a run of 6 cytosines at the site of the hisD6610 mutation (13). The other two standard tester strains, TA102 and TA104, have ochre mutation. The ochre mutation has A-T base pair at

the critical site for reversion in hisG428 gene. TA102 has ochre mutation in the pAQ1 plasmid (14, 15) while TA104 has the ochre mutation located in the chromosome (16).

(2) Deep rough (rfa) mutation causes partial loss of the lipopalysaccharide barrier that coats the surface of the bacteria and increases permeability to large molecules such as benzo (a) pyrene that do not penetrate the normal cell wall.

(3) The uvrB mutation is a deletion of a gene coding for the DNA excision repair system. For technical reasons, the deletion excising the uvrB gene extends through the biotin-requiring gene. As a consequence, these bacteria also require biotin for growth. All the standard tester strain, except TA102, contain the uvrB mutation. TA102 does not contain the uvrB mutation, because it was constructed primarily for detecting mutagens that require an intact excision repair system.

(4) The R-factor plasmid, pKM101, is the ampicillin resistant plasmid which enhances an error-prone DNA repair system in all five standard tester strains (TA97, TA98, TA100, TA102 and TA104).

(5) The multicopy plasmid, pAQ1, carries the his G428 mutation and a tetracycline resistance gene. The pAQ1 plasmid is only contained in TA102.

Table 1 summarizes the genetic characteristics of the five tester strains used in NP mutagenesis testing.

II. Reagents and Media

The three different media used in the Ames test were minimal glucose agar plate, top agar and nutrient agar. For phenotypes veri

TABLE 1. GENOTYPES OF THE TA STRAINS USED FOR MUTAGENICITY C

NONYLPHENOL

Strain Designation	Histidine Mutation	LBS	uvrB Repair	R-Factor (pKM101)	Mechanism of Reversion
TA97	his D6610	rfa	uvrB	+R	frameshift
TA98	his D3052	rfa	uvrB	+R	frameshift
TA100	his G46	rfa	uvrB	+R	base pair substitution
TA102	his G428 (on pAQ1)	rfa	+	+R	base pair substitution
TA104	his G428 (on chromosome)	rfa	uvrB	+R	base pair substitution

uvrB = deletion through uvrB; + = uvrB intact

fication of the test strains, histidine / biotin plate, ampicillin plate, ampicillin/tetracycline plate, and crystal violet disc were used. Four reagents were required for S9 mix (rat liver microsomal enzymes + cofactors) preparation. The recipies and procedures for preparing these reagents and media are taken from Marron and Ames (17) and can be found in the "Appendix" Section of this report.

III. Mammalian Liver S9

Liver microsomal enzymes (S9) play an important role in chemical mutagens requiring metabolic activation. Its preparation, use, and commercial sources have been described in full detail by Marron and Ames (17). We used commercially prepared S9*.

METHODS

I. Preservation and Storage of the Tester Strains

The tester strains came from Dr. Ames lab. Cultures were embedded in a small sterile disc (1/4" dia.) with soft agar. Upon receipt of the cultures, the organisms-saturated disc was dipped into pre-sterilized Oxide No. 2 Nutrient broth in 13 x_15 mm culture tubes. Three (3) culture tubes were inoculated for each strain. Finally the disc was used to streak an Oxiod No. 2 Nutrient agar plate to check the purity of each strain received. All the inoculated culture tubes (total of 15) were placed in the test tube rack and incubated over

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*Litton Bionetic, Charleston, S.C. and Microbiological Associates, Bethesda, MD).

night in a 37C 'water bath.* To ensure adequate aeration, the culture tubes were shaken at approximately 120 RPM. The freshly' grown cultures are then prepared properly for long term storage without losing tester strains' genetic properties.

The frequently used techniques to preserve bacterial culture; are low temperature freeze (at -80^0C), liquid nitrogen, master plate! and lyophilization. The procedures of each technique are describe; in detail by Maron and Ames (17). The frozen permanent copies of the tester stains stored at -80^0C is the way we stored our tester strains. The frozen permanent copies were made from a fresh overnight Nutrient broth (Oxiod No. 2) culture. To each 1.0 mi o culture was added 0.09 mi of spectro-grade dimethylsulfoxide (DMSO).** Culture and DMSO were combined in a sterilized glass container (test tube, flask, or both according to the number of the permanent copies to be prepared). It was swirled gently until DMS1 was dissolved and then culture + DMSO mixture was distribute aseptically into a sterilized 1.2 mt Cryotube (Nunc). The tube was filled nearly full, allowing for expansion due to subsequent freezing The tube's fullness eliminated the air space at the top of the tub and it helped to minimize the oxidative damage to the cultures. The tube was placed upright in a bed of crushed dry ice until the cultures were frozen and then transferred to a -80°C freezer. The frozen permanent copies were used as inocula to grow cultures for

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*Dubnoff Shaker Incubator, Presifons Scientific

**Fisher Scientific Company

mutagenecity assay. To avoid the damage of culture cells from thawing and re-freezing and the chance to contaminate the culture, the frozen permanent copies were only opened once and discarded. Therefore, at least a dozen of frozen permanent copies were prepared. If more frozen permanent copies are needed, they can always be regenerated from the frozen permanent copies previously set aside.

II. Confirming Genotypes of the Tester Strains

Ge[n]eetic materials of the tester strains have to be intact in order to obtain reliable results for the Ames test. It is absolutely necessary to confirm the genotypes of the tester strains under the (a) immediately after the culture samples have been received in the lab, (b) a new set of frozen permanents or lab prepared, (c) the number of spontaneous revertants per plate falls out of the normal range, and (d) there is a loss of sensitivities to standard mutagens.

Genotype verification include histidine/biotin require(crystal violet sensitivity), uvrB mutation (uv light sensitivity), R-factor (pKM101 plasmid by ampicillin resistant) and pAQ1 plasmid by tetracycline resistant for strain TA102. All the genotype tests for each strain were checked in our study, except for uvrB mutations, due to the lack of a proper uv light source. The tests for genotype confirmation were carried in

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* Bio-Freezer, Forma Scientific

every other mutagenicity assay in our lab. For preparation reagents and media used for the tests, refer to the "Appendix section.

III. The Techniques of the Ames' Mutagenicity Test

The Ames' mutagenecity test can be conducted in various fashion

Spot test is the simplest way for the initial rapid screening of chemical compounds. The plate incorporation test is the standard method. In this test, the mutagen (test compound), bacteria, with or without liver enzymes (S9 mix) were added directly to the top agar. It has been used for validating the test (Ames') using hundreds of chemical: For initial screening of a chemical, testing concentrations over three-log dose range in the absence or the presence of the standard S9 mix are recommended. A positive or questionable result should t confirmed by demonstrating a dose-response relation using a narrower range of concentrations. Compounds that are negative can be retested using the preincubation procedures with or without enzyme activation. Preincubation test has the same test procedures as plate incorporation test, except that a preincubation step precedes the addition top agar. In our testing of the mutagencity of NP, spot test and preincubation test were performed.

a. Spot Test

The spot test has several advantages. A few crystals of a solid chemical or a few ut of a liquid compound can be put directly on the top agar surface, thus saving a great deal of time for preparing solutions of different concentrations for each chemical tested. As the test compound diffuses, a range of concentrations are tested simultaneously.

The spot test can indicate whether the S9 enzyme is required for mutagenesis, and it can also give a preliminary indication of the toxicity of the compound. In the case of a positive result, the spot test identifies the tester strain(s) that should be used for the dose-response relationship. Although the spot test is useful, it is primarily a qualitative test and has its distinct limitation. It can only be used for testing chemicals that are diffusible in the agar, and therefore water-insoluble chemicals, such as NP, are not easily detected by this procedure. Spot test is much less sensitive than. the standard plate incorporation test as only relatively few bacteria on the plate are exposed to the chemical at any particular dose level. Therefore, a negative result in the spot test alone is not a sufficient indication for non-mutagenecity in the Salmonella tester strains.

The spot test for NP (compound of our interest) was carried out in the following manner. One-tenth mt freshly grown culture in Oxiod #2 Nutrient broth was suspended in 0.5 mt of phosphate buffer (0.2 M, pH 7.4), then 2.0 mQ of top agar (0.5 mM Histidine/biotin) in 0.06% agar was added. The contents were vortexed on a mixer* for a few seconds. The mixture (the bacteria + P04 buffer + top agar) was poured on the minimal glucose agar plate. After top agar hardens, 200 ug NP dissolved in methanol was applied in the center of each plate. When NP was dry, the plates were inverted and incubated at

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*Vortex,-Genie, Fisher Scientific Co.

37°C in incubator* for 48 hrs. The inhibition zone and/or increase number of colonies around the tested chemical were recorded after incubation period. Spot test for NP was done for all the standard tester strains (TA97, TA98, TA100, TA102, and TA104).

b. Preincubation Test

The standard plate incorporation test for chemical mutagenicity was first described in 1973 (18, 19, 20). It is the standard method that has been used for validating the Ames test using hundreds of chemicals. Later in 1975 Yahagi (21) found that carcinogenic azo dyes was negative by the original standard plate test, but showed positive result when tested on modified standard plate test. The modification is the preincubation of test mixture before top agar. More and more results have proved that the preincubation modification of the standard plate procedure is more sensitive and selective for some compounds and appears to be at least as good for other compound tested (22, 23, 24). Through our study on NP, the preincubation test was found more suitable.

The increased ability of testing mutagens by preincubation test is attributed to the fact that the test compound, phosphate buffer S9 mix and tester bacteria are incubated at higher concentration in the preincubation assay than in the standard plate test. The procedures of the preincubation discussed below are based on the recommendation of Matsushima et al, (25).

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*Dry Type Bacteriological Incubator, Blue M Electric Co.

Five-tenths ml (0.5 mi) of phosphate buffer (0.2M, pH 7.4) or 0.5 mi of S9 mix, 0.1 ml of bacterial culture and 0.1 mi or less test solution dissolved in appropriate solvent (methanol and DMSO used for NP) were placed in a sterilized 13 x 100 mm capped culture tubes. The culture tubes were then left in an ice bath to prevent the deterioration of S9 enzyme activities. This order of addition of the test compound was essential because it avoids placing the bacteria in direct contact with the undiluted test compound and its solvent. The tube was gently vortexed for a few seconds. Preincubation can be done at 37°C in a water bath with agitation for 20 minutes or at 30°C for 30 minutes. The choice of conditions is purely a matter of convenience. These two sets of conditions give comparable results (25). In our lab, 20 minutes at 37°C and 120 RPM shaking were the conditions used for the preincubation test. When preincubation period was over, 2 ml of top agar (with 0.5 mM histidine/biotin) was added to each tube and mixed well. The contents of the tube were mixed by vortexing the tube for 3 seconds at low speed and then the contents were quickly poured onto a minimal glucose agar plate. To achieve a uniform distribution of the top agar on the surface of the minimal glucose plate, the plate was quickly tilted and rotated (without the cover of the plate) and then the plate was covered. The plate was left on a level surface to harden, (the mixing, pouring and distribution of the contents should take less than 20 seconds, and the plates should be left to harden for several minutes).

It is important to follow these time limits. If the top agar starts to harden in mid-operation a stippled surface will result which makes scoring of revertants difficult. For photosensitive chemicals, the plate should be covered with dark paper. Within an hour the plates should be inverted and incubated in a dark 37°C incubator for 4$hours. First the compound is tested without enzyme activation, if the result is negative, then the compound should be retested with S9 mix for metabolic activation. Both positive (diagnostic mutagens) and negative control (solvent) plates, as well as sterility check for top agar, solvent and S9 mix should also be included in each assay.

In our testing of NP, three experiemental conditions were used:
(a) NP dissolved in methanol, dose of NP varied from 1 pg/plate to 200 ug/plate without liver (S9) activation, (b) NP dissolved in DMSO, NP concentration ranged from 1 pg/plate to 200 vg/plate without S9 activation, (c) NP dissolved in DMSO with S9 activation and only two concentrations (i.e. 0.5 ug/plate and 10 ug/plate) of NP are used. Duplicate plates were poured for each dosage. Researchers have suggested that "the positive control plates should be always included in each run to confirm the reversion properties and specificity of each strain. When S9 is used for metabolic activation, then a diagnostic mutagen which requires the S9 activation should be chosen. Ideally, each tester strain should be checked against its specific diagnostic mutagen." To eliminate the risk of having many kinds of mutagens in the lab, only two

positive controls were used in the course of our study. 4-Nitroquino line-N-oxide (NQO) was used as positive control when S9 mix was not used for activation, while 2-aminofluorene was used when S9 mix was used in the test. These two diagnostic compounds are mutagenic to all five tester strains.

Table 2 lists the constituents contained in each kind of plate.

c. Scoring Revertants and Interpretation of Results

After 48 hours incubation, the revertants on all the plates were counted [m]anually 'or by electronic colony counter.[*] The presence of the background lawn on all the plates should also be confirmed. A bacterial lawn that is thin compared to that on the negative control plates is evidence of bacterial toxicity. Colonies appearing on the plate that has no background lawn are not revertants and should not be scored. These colonies arise from the surviving bacteria that live off the histidine/biotin present in the top agar. The pin-point size colonies on the plate is the indication of massive death of bacterial cells due to bactericidal effect (toxicity) of the test compound.

Each experimental condition was repeated three times. Experimental results are presented as the average of the duplicate plates. A test result is considered as positive if the mutagenic index is two or higher. Mutagenic index is determined as a ratio of the mean of induced revertants to the mean of the spontaneously induced revertants.

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[*]Biotran III, Newbra-unswick Scientific Co.

TABLE 2. CONSTITUTENTS OF THE VARIOUS KINDS OF PLATES USED IN PRE-INCUBATION TEST

Ingredient Plate Type	Culture (0.1ml)	P04/Liver -S9	P04/Liver +S9	Top Agar (2 mt)	Positive Mutagens	Test Compound	Solvent (0.1ml)
Blank	+		+	+	-	-	-
Negative Control	+		+	+	-	-	+
Positive Control	+		+	+	+	+	-
Sterility Check of P04 /S9	-		+	+	-	-	-
Sterility Check of top agar	-		-	+	-	-	-
Mutagen	+		+	+	-	+	+

+ = Ingredient included in the plate.

- = Ingredient omitted from the plate.

RESULTS AND DISCUSSION OF RESULTS

All five tester strains (TA97, TA98, TA100, TA102 and TA104) were monitored for their genotypes except for uvrB mutation. The results of phenotypes met the strains' specification. There was no variation of strain genetic properties. This is not surprising because every precaution was exercised in culture handling and the frozen permanent copies were used only once and discarded.

In the spot test, there was no inhibition zone or increased colony number around the vicinity of test compound. This may be due to the fact that NP did not dissolve in water, therefore, it may not have diffused into the top agar.

In preincubation test, duplicate plates were poured and two or three runs were conducted for each experimental condition. NP does not dissolve in water, but it dissolved very easily in methanol. Testing mutagenicity of NP using methanol as solvent was the first phase of our study. Table 3 shows the results of histidine revertants by NP dissolved in methanol. S9 enzyme activation was not used (¯S9). Results of spontaneous reversing (blank plates), solvent check as well as diagnostic control plates are also illustrated in Table 3. Number of revertants on NP plates was not higher than the blank plates (0.1 mi culture + 0.5 mi phosphate buffer) and the solvent plates (0.1 mi culture, 0.5 mi phosphate + 0.1 mi methanol). Comparing the revertants on the blank plates and or, the solvent plates, the solvent plates had insignificantly less number of re

TABLE 3. REVERSION OF STANDARD TESTER STRAINS BY NP DISSOLVED IN METHANOL WITHOUT S9 ACTIVATION

Strain No. and Run No. / Chemical	TA 97		TA 98		TA 100		TA 102		TA 104	
	1	2	1	2	1	2	1	2	1	2
BLANK[a]	30	10	21	10	75	76	79	130	295	434
MEOH	37	3	17	9	40	72	41	103	104	401
DMSO	54	8	30	14	62	64	144	142	377	426
1 µg NP[b]	34	-[c]	19	-	87	-	159	-	369	-
10 µg NP	2	28	11	14	57	80	131	124	266	518
50 µg NP	1	4	17	8	29	51	30	122	125	180
100 µg NP	7	1	6	7	11	31	36	22	100	94
200 µg NP	26	2	15	1	22	1	3	0	116	1
0.5 µg NQO[d]	199	150	312	377	1183	1661	248	193	1457	1770

a. Blank = Phosphate buffer + culture

b. NP = Nonylphenol

c. - = Not Done

d: NQO = 4-Nitraoquinoline - 1 - oxide

vertants. Perhaps methanol was not one of the preferred solvents for Ames' test. For these reasons, we used the most common solvent DMSO to dissolve NP for the second phase of our study.

The histidine.revertants by NP dissolved in DMSO along with other plate conditions are listed in Table 4. When examining revertants on NP, blank plates and solvent plates, there was no in creased number of revertants on NP plates. Results shown in Table 3 and Table '4 show similar numbers of revertants on NP plates. Conclusions can be made from both sets of experiments that NP did not cause the reversion of the Ames standard tester strains (Salmonella). Hence, NP is not a mutagenic compound by the Ames test under the preincubation condition without S9 activation.

However, there is a common under lying trend in both sets of experiments, that is the number of revertants started to decrease when NP concentration was increased. In both solvent conditions (methanol and DMSO), when 50 mg NP/plate was used, the revertants became less than blank plates and solvent plates. The decreasing of revertants on NP plate became very evident when 200 mg NP was used. Less number of revertants on high concentration NP plates suggested that NP is probably toxic to the Salmonella strains. The toxic effects of NP to the Ames strain are further supported by the pinpoint growth of colonies in the background lawn.

In the third phase of our Ames test, S9 (50 ml enzyme was used to replace phosphate buffer to test whether the enzyme activation will

TABLE 4. REVERSION OF STANDARD TESTER STRAINS BY NONYLPHENOL DISSOLVE DMSO WITH S9 ACTIVATION

Culture No. and Trial No. / Chemical	TA 97		TA 98		TA 100		TA 102		TA 104	
	1	2	1	2	1	2	1	2	1	2
BLANK[a]	19	10	18	16	64	91	71	138	312	470
DMSO	21	15	10	15	56	93	110	159	337	392
1 µg NP[b]	-[c]	18	-	16	-	142	-	138	-	306
10 µg NP	-	7	-	15	57	93	130	49	309	187
50 µg NP	30	2	8	7	34	42	51	21	250	127
100 µg NP	15	1	8	5	38	40	22	12	150	153
200 µg NP	9	1	4	2	30	33	1	2	155	168
0.5 µg NQO[d]	312	256	340	307	1312	1239	211	192	2500	1430

a. Blank = Phosphate buffer + culture

b. NP = Nonylphenol

c: -- = Not done

d. NQOP = 4-Nitroquinoline - 1 - oxide

stimulate the mutagenicity of NP. Concentrations of NP used were 0.5 mg/plate and 10 mg/plate. Low concentration was chosen because NP showed some toxic effect above 50 mg/plate. In our last experimental set up, we assumed that NP may be a weak mutagen and requires enzyme activation. Table 5 summarizes the results for revertants by NP with S9 activation. Number of revertants on NP plates with S9 did not reveal any difference from the blank plates and solvent blank (DMSO). These series of tests show that the NP is nonmutagenic as evaluated by tester strain even with the enzyme activation. 2-Aminofluoene (2-AF) proved to be a weak to strong mutagen to all the five tester strains. It was a weak mutagen to strain TA97 and TA102, a strong mutagen to strain TA98, TA100 and TA104. 2-AF was not mutagen to all the five non-activated strains.

There is no published information of NP in Salmonella studies (Ames test) in the literature, but there are about one hundred references of NP in toxicity studies. This computer search was done at Oak Ridge National Laboratory, Oak Ridge, Tenn. Results of Ames test on NP show that it is toxic rather than mutagenic to standard tester strains (TA97, TA98, WOO, TA102, and TA104).

Our results are comparable to results for NQO (diagnostic mutagens) as obtained by Waleh et. al., (26). In their results, there were 430 revertants/plate and 1606 revertants/plate for TA98 and TA100 respectively when 0.5 mg NQO was used on each plate. Averaging all the 7 runs of 0.5 mg NQO/plate, we had 391 revertants/plate for TA98 and 1605 revertants/plate for TA100.

TABLE 5: REVERSION OF STANDARD TESTER STRAINS BY NONYLPHENOL (IN DMSO) ACTIVATION

Culture No. and Trial No.	TA 97			TA 98			TA 100			TA 102			TA 104		
Chemical	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Blank[a]	11	27	25	22	13	24	101	122	179	95	67	125	485	651	472
DMSO	42	28	40	24	23	18	61	122	173	135	104	204	273	463	569
0.5 µg NP[b]	49	37	46	26	18	19	53	157	189	123	128	209	273	539	635
10 µg NP	37	27	46	24	20	24	53	171	166	146	93	209	254	508	629
0.5 µg NQO[c]	142	145	196	395	407	458	2004	2022	1816	176	199	185	2010	2064	2033
2-AF (+S9)[d]	211	316	293	1564	1287	1834	1008	1133	1156	249	213	268	1617	1296	1630
2-AF (-S9)	16	13	18	37	40	30	125	171	174	98	92	115	438	438	471

a. Blank = Phosphate buffer + culture

b. NP = Nonylphenol

c. -- = Not Done

d. NQO = 4 -Nitroquinoline -1- oxide d. 2-AF = 2-Aminofluorene

Comparing the number of revertants on solvent plates (methanol or DMSO), there is no significant difference. This means methanol (0.1 ml used) is as compatible as DMSO to the Salmonella. The results conclude that 0.1 ml methanol or less can be an acceptable solvent in performing Ames test. This finding is supported by Maron et. al., (27). They tested 14 solvents for compatibility with the Salmonella mutagenicity test; and found ethanol up to 200 ul can be used in the Ames test. Since methanol and ethanol belong to the same homologous series of organic chemicals, it is not surprising that 0.1 ml methanol does not impose a toxic effect on the Ames Salmonella strains.

S9 dose effects on mutagnicity of NP were not carried further because NP did not produce positive results.

In reviewing the number of the spontaneous revertants of the tester strains, except for TA104, the numbers are less than those of Ames' results (17). Nevertheless, Waleh et al. (26), used TA98 and TA100 in their toxicity test, they have 19 spontaneous revertants for strain TA98 and 116 for strain TA100. The average of our total seven runs for spontaneous revertants (Blank plates), had 18 spontaneous revertants for strain TA98 and 101 for TA100. Our results are very similar to the results obtained for TA98 and TA100, tester strains. Although these numbers are somewhat smaller than those found by Ames, they are consistent with our test conditions. Reproducibility of the spontaneous revertants is one of the important criteria while monitoring the stability of the tester strains.

CONCLUSIONS AND RECOMMENDATIONS

In summary it can be said that the test results, as conducted in our laboratory, indicate that 4-nonylphenol is not mutagenic. However, nonylphenol should not be prematurely labeled as a safe com pound. It must be pointed out that the Ames test used in this research is just one test in a battery of tests employed by scientists to evaluate the mutagenicity of compounds. Hence no firm conclusion can be drawn about the mutagenic effect of nonlyphenol. In the past experiments were preformed using Salmonella typhimurium strains to test the Researchers (28, 29) found that DDT and DDE were not mutagenic even on S9 activated strains. In a later study (30) DDE resulted in doubleing the chromosomal aberration. Very often the positive response to an adverse effect resulting from toxicity assay go far beyond negative results. The use of DDT was banned in this country, though it is not mutagenic or teratogenic in all the studies reported.

The studies described in this report indicate that nonylphenol is toxic. Even the earlier studies (3) show that it is toxic to aquatic animals. Such results cause a dilemma to the scientists, and warrant more in-depth studies. When the test compound is strongly toxic and weakly mutagenic or the compound is not mutagenic but only toxic (such as nonylphenol), then toxicity of the compound should be determined quantitatively. The toxicity test protocol has been recently developed by Waleh et. al (26). al (26). Their ingenious assay can be used in conjunction with the

standard Salmonella mutagenicity test. The test is based on the use of two sets of strains that are isogenic with the Ames standard tester strains except for the his (histidine) character. One set of strain is His^- and the other has Tn10 generated His^- mutation. These His- double mutants called "filler cells", are unable to revert to histidine independence and are used to mimic the background lawn present in the standard mutagenicity assay. The killing of Hiscells by the test compound is measured under the conditions that are nearly identical to those of the standard Ames tests. Several of the His^+ cells in the presence of his^- "filler cells" is used to determine the toxicity of the test compound. Hence, toxic effects of NP shown in the Ames test should be tested and confirmed by the toxicity test protocol.

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APPENDIX

RECIPES AND PREPARATIONS OF REAGENTS

AND MEDIA USED IN THE AMES TEST

Recipes for stock solutions and media

Vogel-8onner medium E (SOX)
Use: Minimal agar

Ingredient	**Per liter**
Warm distilled H;O (45°C)	670 ml
Magnesiurn sulfate (MgSO4 7 H;O)	10g
Citric acid monohydrate	I00 g
Potassium phosphate. dibasic (anhydrous) (K:HPO,)	500 g
Sodium ammonium phosphate (NaHNH, P0,-4 H=O)	175 g

Add salts in the order indicated to warm water in a 2-liter beaker or flask placed *sin magnetic stirring hot* plate. Allow each salt to dissolve completely before adding the next. Adjust the volume to 1 liter. Distribute into two 1-Liter glass bottles.. Autoclave, loosely capped. for 20 min at 121 °. When the solutions have cooled. tighten the caps

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0.5 mM histidine/biotin solution
User: Mutageniciry assay (add 10 ml to 100 ml of top agar)

lngredient	**Per 250 ml**
D-Biotin (F.W. 247.3)	30.9 mg
L-Histidine HCl (F.W. 191.7)	24.0 mg
Distilled H2O	250 ad

Dissolve the biotin by heating the water to the boiling point. This can be done in a microwave oven. Sterilize by filtration through a 0.22mm membrane filter or autoclave 20 min at 121°. Store in a glass bottle at 4°.

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*Recipes for stock solutions and media were taken from Maron and Ames paper (17).

Recipes for stock solutions and media*

Top agar
Use: Mutagenicity assay

Ingredient	***Per liter***
Agar	6 g
Sodium chloride (NaCl)	5 g
Distilled H-O	1000 ml

The agar may be dissolved in a steam bath or microwave oven. or by autoclaving briefly. Iviix thoroughly and transfer 100-atl aliquots to 250-ml glass bottles with screw raps. Autoclave for 20 min with loosened caps. Slow exhaust. Cool the agar and tighten caps.

Salt solution (1.65 M KCl + 0.4 M MgC12)
Use: S9 mix for mutagenicity assay

Ingredient	***Per 500*** Ml
Potassium chloride (KCI),	61.5Z
Magnesium chloride (MSC^{1} 2 . 6 H_20)	40.7 g
Distilled H_2O	to final volume of 5.00 Ml

Dissolve ingredients in water. Autoclave for 20 min at 121 Deg. Store in glass bottles in the refrigerator or at room temperature.

0.2 M sodium phosphate buffer. pH 7.4
Use: S9 mix for mutagenicity assay

Ingredient	***per 500 ml***
0.2 M sodium dihydrogen phosphate (NaH_2PO, - H2O) (13.8 g/500 ml)	60 ml
0.2 M disodium hydrogen phosphate (Na_2HPO,) (14.2 g/500 ml)	440 ml

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* These are approximate values. Test the pH. If it is too low. add more 0.2 M disodium hydrogen phosphate to pH 7.4. Sterilize by autoclaving for 20 min at 121°.

0.1 M NADP solution (nicotine adenine dinucleotide phosphate)
Use: S9 for mutagenicity assay

Ingredients	**Per S ml**
NADP (F.W. 765.4)	383 mg
Sterile distilled H,O	5 ml

Add NADP to pre-weighed sterile glass tubes with screw caps. Do not add water. It is convenient to prepare a dozen or more of these dry aliquots at one time. Wrap

Ampicillin solution (8 mg/ml)
Use: Tests of ampicillin resistance
Master plates for R-factor strains

Ingredient	**per 4"PI. 100 m l**
Ampicillin trihydrate	0.8s
Sodium hydroxide (0.02 N)	100 ml

We have never found it necessary to sterilize ampicillin solutions but they can be filtered through a 0.22ml membrane filter. Store in glass bottle at 4°C

.Crystal violet solution (0.1%)
Use: Tests for crystal violet sensitivity (to confirm rfa mutation)

Ingredient	**Per 200 MI**
Crystal violet	0.1g
Distilled H2O	100 all

Store at 4°. in glass bottle with screw cap. Wrap he bottle with metal foil to protect against light.

-Minimal glucose plates
Use: Mutagenecity assay

Ingredient	**per liter**
Agar	*15g*
Distilled H2O	930 ml
SOX VB salts	20 ml
40% glucose	50 ml

Add 15 g of agar to 930 ml of distilled H2O in a 2-liter flask Autoclave for 20 min using slow exhaust. When the solution has cooled slightly. add 20 ml of sterile SOX VB salts and 50 ml of sterile 40% glucose. For mixing. a large magnetic stir bar can be added to the flask before autoclaving. After all the ingredients have been added. the solution should be stirred thoroughly. Pour 30 ml into each petri plate

Note: The SOX VB salts and 40% glucose should be autoclaved separately.

Histidine/biotin plates
Use: Master plates for non R-factor strains
Tests for histidine requirement

Ingredient	**Per liter**
Agar	15g
Distilled H2O	914 ml
SOX VB salts	20 ml
40% glucose	50 ml
Sterile histidine . HCl . H2O (2 g per 400 ml H20)	10 ml
Sterile 0.5 mM biotin	6 ml

Autoclave the agar and water. Add the sterile 40% glucose. 5OX VB salts. and histidine to the hot agar solution. Allow the solution to cool slightly. Add the sterile biotin. Mix. and pour the plates.

Note: A magnetic star bar may be added before autoclaving to facilitate mixing. Autoclave the SOX VB salts. 40% glucose. and histidine solution separately.

Ampicillin plates and ampicillin / tetracycline * plates
Use: Master plates for strains carrying the plasmids pKM101 and pAQ1 + pAQI**
Tests for
Ampicillin / tetracycline resistance

Ingredient	per lliter	Plate concentration
Agar	15g	1.5 %
Distilled H_2O	910 ml	-
SOX VB salts	20 ml	IX
40% glucose	SO ml	2.0%
(Sterile histidine- *H_20)*	10 ml	2.60 mm
Sterile 0·5 mm biotin	·6 ml	3 *mm*
Sterile ampicillin solution (8 mg/ml 0.02 N NaOH)	3.15 ml	25 gg/ml
Sterile tetracycline solution (8 mg/ml 0.02 N *HQ)*	0.25 m	l2 it g/rnl

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Autoclave agar and water for 20 min. Add sterile glucose. SOX VB salts and histidine to the hot solution. Mix. Cool to approximately 50°. Add sterile biotin and ampicillin solutions aseptically *(we* have not found it necessary to sterilize antibiotic solutions but this can be done if necessary, using a *0.2-jum* filter). Tetracycline is added only for use with TA102 which is tetracycline-resistant. It is essential not to exceed or fail below this concentration. See Levin et &L 0982b).

The SOX VB salts and 40% glucose solutions are sterilized separately by autoclav*ing for* 20 min Histadine and biotin solutions can be autoclaved or filter-sterilized .

Plates to be used for tests of tetracycline and/or ampicillin resistance can be stored for approx. 2 months at 4°C. After 2 months they *should be* tested for ampicillin / tetracycline activity with a non R-factor strain such as. Plates should be discarded if the non R-factor strain grows.

Master plates should be prepared within a few days after the agar is poured.

**TA102 master plates should be discarded after 2 weeks. See Master Plates section.

Nutrient agar plates
Use: 1. Tests for genotypes
(a) crystal violet sensitivity *(rfc)*
(b) UV sensitivity *(uvrB)*
2. Tests for viability of bacteria

Ingredient	**Per liter**
Difco bacto nutrient broth *	88
NaCl	5 g
Agar	15 g
Distilled H_20.	1000 ml

Add the ingredients to a 2-liter .flask containing a magnetic sdr bar. Autoclave for 30 nun. slow exhaust. Mix; and pour the plates.

* Oxoid nutrient broth No. 2 can be substituted for Difco bacto nutrient broth in which case 25 g are used and the NaCl is omitted. Since these rich agar plates are used when total growth of bacteria is required and not far mutagcnicity assays it makes no difference what kind of nutrient broth is used. Nutrient broth should not be used for master plated

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LB agar plates
Use: Growing the strains for lyopttiiization

Ingredients	**per liter**
Bacto tryptone	10 g
Bacto yeast extract	5g
NaCl	log
Agar	15g
Distilled H_2O	1000 ml

Dissolve ingredients in'water. Adjust to pH 7.0 with 1 N NaOH (approximately 2 ml/1). Add ingredients to a 2-liter flask containing a magnetic stir bar. Autoclave 30 min at 121°. slow exhaust. Mix, and pour the plates.
