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Bacterial Reverse Mutation (*Ames*) Test with Potassium Nitrate and 2-Methylpentane

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We investigated the genotoxicities or mutagenicities of two chemicals (potassium nitrate and 2-methylpentane) that have limited toxicological data in spite of their common usage based on *Ames* reverse mutation test. In this test, treatment of two chemicals at each five dose did not induce mutagenicity in *Salmonella typhimurium* TA98, TA100, TA1535, TA 1537, and in *Escherichia coli* WP2uvrA with and without metabolic activation. These results indicate that two chemicals do not have mutagenic potentials under the conditions of our study.

Key words: *Ames*, potassium nitrate, reverse mutation, 2-methylpentane

1. Introduction

Despite the necessity of hazard assessment of many chemicals which are exposed to workers more frequently with the development of the chemical industries, there is no sufficient and definite information.

The bacterial reverse mutation (*Ames*) test of 2 chemicals, potassium nitrate (CAS No. 7757-79-1) and 2-methylpentane (CAS No. 107-83-5) was performed because the information of hazard assessment was needed. Moreover the toxicological information of these two chemicals will be used for workers' rights to know and to prepare or update the Material Safety Data Sheet (MSDS) of these chemicals.

It was reported that cyanosis among infants who drink well water is a frequently encountered clinical manifestation of nitrate toxicity. Ingestion of large quantities may cause violent gastroenteritis. Prolonged exposure to small amounts may produce anemia, methemoglobinemia, nephritis[1].

The toxic dose varies greatly; from 15 to 30 g KNO_3 may prove fatal but much larger doses have been taken without serious effects[2]. And the lethal oral dose of potassium nitrate for an adult has been estimated to be between 4 and 30 g (about 40 to 300 mg NO^{3-}/kg)[3].

It was reported that one patient died of cardiac arrest with methemoglobin concentration of 65%. A 22 yr old woman developed an intense cyanosis without respiratory distress or heart attack; it was a case of toxic methemoglobinemia[4].

The general population will be exposed to 2-methylpentane in ambient air, especially in areas of high traffic and at filling stations. Exposure via inhalation and dermal contact may also result from the use of glues and other products in which 2-methylpentane is contained as a solvent[5]. NIOSH (NOES Survey 1981-1983) has statistically estimated that 42 workers are potentially exposed to 2-methylpentane in the USA[6].

The gasoline vapor that workers are exposed to at bulk terminals and marine loading facilities contained an average of 3.5 and 4.9% 2-methylpentane by weight. Emissions will occur from the evaporation of petroleum during transfer, transport and storage of petroleum products and evaporation of gasoline from carburetors,

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gas tanks, and during refueling of motor vehicles[7].

2-Methylpentane emissions would therefore occur as a result of natural oil seepages or gas vents and emissions from vegetation. Forest fires are another emission source[8].

2-Methylpentane causes narcosis in mice at 30,000 ppm in 30-60 minutes; it is not converted in the body into metabolites that cause peripheral neuropathy. No listed effects of short-term or long-term exposure but inhalation of high concentrations can cause CNS depression[9].

It was evaluated that potassium nitrate and 2-methylpentane are non-mutagenic by bacterial reverse mutagenic assays with *Salmonella typhimurium* and *E. coli* with or without metabolic activation, but all of them are non-GLP tests[10], and the available genotoxicity data on these two chemicals are still controversial. Thus it was evaluated to determine the genotoxicity or mutagenicity with *Ames* test.

2. Materials and methods

2.1. Chemicals and bacterial strains

The potassium nitrate (Sigma-Aldrich, ≥99%, MO, USA, Lot No MKBC7980) was dissolved in distilled water. The 2-methylpentane (Sigma-Aldrich, ≥99%, MO, USA, Lot No. 95797LJ) dissolved in vehicles (Acetone, 99.8%, Merck, Darmstadt, Germany, Lot No. K38502214812) was added directly to the test systems and/or diluted prior to treatment.

Sodium azide (WAKO, 98%, Osaka, Japan, Lot No. KSJ6151), 9-aminoacridine (9AA) (WAKO, 98%, Lot No. 03024JR), 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide (AF-2) (WAKO, 98%, Lot No. WAP0369) and 2-aminoanthracene (2AA) (WAKO, 95%, Lot No. KSF1039) were used as positive controls. Dimethyl sulfoxide (Sigma-Aldrich, 99.5%, MO, USA, Lot No. 108K0185) was used as a solvent.

Five strains used in this study include four strains of *S. typhimurium* (TA100 Lot No. 4475D, TA1535 Lot No. 4487D, TA98 Lot No. 4486D and TA1537 Lot No. 4455D, Moltox, NC, USA) and one strain of *E. coli* (WP2uvrA, Moltox, NC, USA, Lot No. 4473D) that have been shown to be reliable and reproducibly responsive between laboratories. An appropriate minimal agar (e.g. containing Vogel-Bonner minimal medium E and glucose, Junsei, Tokyo, Japan, Lot No. 0A1198) and an overlay agar containing histidine and biotin or tryptophan (Bacto-agar, BD, NJ, USA, Lot No. 7144748), to allow for a few cell divisions, are used (No. 2 Nutrient Broth, Oxoid, Cambridge, UK, Lot No. 612715; Shaker bath, Precision, VA, USA, Model 50, 180 rpm).

2.2. Mutagenicity assay

The most commonly used metabolic activation system is a cofactor supplemented post-mitochondrial fraction (S9, Moltox, NC, USA, Lot No. 2553) prepared from the livers of rodents treated with enzyme-inducing agents such as Aroclor 1254 or a combination of phenobarbitone and β-naphthoflavone.

For the preincubation method the test substance/test solution is preincubated with the test strain (containing approximately 10^8 viable cells) and sterile buffer or the metabolic activation system (0.5 ml) usually longer than 20 min at 30~37°C prior to mixing with the overlay agar and pouring onto the surface of a minimal agar plate.

For an adequate estimate of variation, triplicate plating was used at each dose level. All plates in a given assay were incubated at 37°C for 48 hours. After the incubation period, the number of reverting colonies per plate is counted.

2.3. Evaluation and Analysis of Results

The number of reverting colonies per plate was determined (Bio-multiscanner, BMS-400 system, Toyo Sokki, Kanagawa, Japan). The mutant frequency was expressed as the quotient of the number of reverting colonies over the number of colonies in the negative control.

A mutagenic potential of a test item was assumed if the mutant frequency is 2.0 or higher. A possible mutagenic potential was assumed if the quotient ranges between 1.7 and 1.9 in combination with a dose effect relationship. No mutagenic potential was assumed if all

quotients range between 1.0 (and lower) to 1.6.

3. Results

The treatment of two chemicals at each five dose did not induce mutagenicity in *Salmonella typhimurium* TA98, TA100, TA1535, TA 1537 and in *Escherichia coli* WP2uvrA with and without metabolic activation. These results indicate that two chemicals do not have mutagenic potentials under the conditions examined in each study.

All *Salmonella* strains are histidine-, the used *E. coli* strain tryptophan- dependent. Revertants are identified as colonies that grow in low levels of histidine or tryp-

Table 1. Reverse mutation (*Ames*) assay using *Salmonella typhimurium* and *E. coli* treated with potassium nitrate without (upper panel) and with metabolic activation (middle panel), respectively. The lower panel is positive control (without and with metabolic activation)

With/Without S9-mix	Conc. of test material	Number of reverse mutation (colony number/plate)				
		Base-pair substitution type			Frameshift type	
		TA100	TA1535	WP2uvrA	TA98	TA1537
S9 Mix(-)	0	109 110 128 (116)	5 6 7 (6)	47 50 34 (44)	43 36 48 (42)	2 8 9 (6)
	312.5	104 110 117 (110)	9 7 9 (8)	40 39 57 (45)	36 37 40 (38)	9 12 7 (9)
	625	126 121 100 (116)	7 8 13 (9)	48 36 46 (43)	48 44 46 (46)	8 8 7 (8)
	1,250	124 121 133 (126)	7 4 8 (6)	48 39 36 (41)	40 41 35 (39)	6 6 12 (8)
	2,500	125 118 122 (122)	6 8 8 (7)	42 45 48 (45)	35 45 36 (39)	9 6 9 (8)
	5,000	107 110 114 (110)	11 10 5 (9)	45 34 49 (43)	37 41 41 (40)	6 13 10 (10)
S9 Mix(+)	0	153 130 119 (134)	11 10 11 (11)	38 47 63 (49)	46 55 37 (46)	18 12 11 (14)
	312.5	123 125 123 (124)	10 13 15 (13)	44 30 61 (45)	56 50 53 (53)	11 10 8 (10)
	625	135 123 121 (126)	10 9 13 (11)	50 40 49 (46)	52 58 43 (51)	15 13 15 (14)
	1,250	115 94 90 (100)	9 9 10 (9)	50 54 50 (51)	50 47 45 (47)	8 11 12 (10)
	2,500	119 130 123 (124)	17 11 9 (12)	55 48 40 (48)	40 64 46 (50)	15 15 12 (14)
	5,000	111 136 108 (118)	10 8 11 (10)	48 46 49 (48)	63 53 55 (57)	17 12 16 (15)
+Control Without S9 Mix	Compound	AF-2	NaN3	AF-2	AF-2	9-AA
	Conc. (μg/plate)	0.01	0.5	0.01	0.1	80
	Colony number/plate	344 340 366 (350)	480 476 477 (478)	428 425 430 (428)	292 283 290 (288)	952 997 943 (964)
+Control With S9 Mix	Compound	2-AA	2-AA	2-AA	2-AA	2-AA
	Conc. (μg/plate)	1.0	2.0	10	0.5	2.0
	Colony number/plate	680 676 670 (675)	156 160 163 (160)	392 390 388 (390)	436 440 439 (438)	96 111 106 (104)

( ): mean value

tophan. Frameshift and base-pair substitution defects are represented to identify both types. Additional genetic markers (e.g., his, uvr, rfa, bio, pKM101, pAQ1) serve to make the strains more sensitive to certain types of mutagens.

Table 1 shows the results of the reverse mutation (*Ames*) assay using *Salmonella typhimurium* and *E. coli* treated with potassium nitrate without and with metabolic activation, and Table 2 shows the results of the same assay with 2-methylpentane.

Table 2. Reverse mutation (*Ames*) assay using *Salmonella typhimurium* and *E. coli* treated with 2-methylpentane without (upper panel) and with metabolic activation (middle panel), respectively. The lower panel is positive control (without and with metabolic activation).

With/Without S9-mix		Conc. of test material (μg/plate)	Number of reverse mutation (colony number/plate)				
			Base-pair substitution type			Frameshift type	
			TA100	TA1535	WP2uvrA	TA98	TA1537
S9 Mix(-)		0	100 120 115 (112)	10 5 5 (7)	36 40 30 (35)	15 15 18 (16)	8 6 12 (9)
		312.5	108 106 126 (113)	7 5 7 (6)	33 27 34 (31)	13 16 13 (14)	9 10 10 (10)
		625	87 79 86 (84)	5 7 8 (7)	35 29 25 (30)	15 14 15 (15)	4 7 4 (5)
		1,250	72 72 79 (74)	4 11 9 (8)	26 22 20 (23)	17 19 12 (16)	2 3 3 (3)
		2,500	94 74 80 (83)	6 8 5 (6)	28 26 31 (28)	14 15 11 (13)	8 9 4 (7)
		5,000	83 87 65 (78)	4 8 12 (8)	31 19 30 (27)	14 14 13 (14)	6 5 6 (6)
S9 Mix(+)		0	120 125 125 (123)	5 14 7 (9)	51 55 38 (48)	22 17 23 (21)	12 6 8 (9)
		312.5	141 126 110 (126)	4 8 12 (8)	55 54 52 (54)	24 20 11 (18)	9 13 8 (10)
		625	136 113 137 (129)	11 8 2 (7)	44 66 51 (54)	20 14 26 (20)	7 12 12 (10)
		1,250	118 102 87 (102)	8 8 12 (9)	43 39 20 (34)	22 16 20 (19)	8 11 10 (10)
		2,500	90 87 91 (89)	9 7 5 (7)	31 29 39 (33)	11 24 17 (17)	6 11 10 (9)
		5,000	112 99 101 (104)	10 10 9 (10)	34 15 30 (26)	18 28 14 (20)	11 5 3 (6)
+Control	Without S9 Mix	Compound	AF-2	NaN3	AF-2	AF-2	9-AA
		Conc. (μg/plate)	0.01	0.5	0.01	0.1	80
		Colony number/ plate	432 430 437 (433)	321 311 325 (319)	150 161 158 (156)	375 377 359 (370)	456 480 442 (459)
	With S9 Mix	Compound	2-AA	2-AA	2-AA	2-AA	2-AA
		Conc. (μg/plate)	1.0	2.0	10	0.5	2.0
		Colony number/ plate	692 683 681 (685)	143 148 151 (147)	448 460 455 (454)	81 102 96 (93)	108 120 113 (114)

( ): mean value

4. Discussion

It was reported that the cDNA microarray technology was used to determine the impact of 2,4-D and nitrate in an in vitro model of exposure. The affected genes indicate that HepG2 cells respond to environmental, low-level exposure and produce a cellular response that is associated with alterations in the expression of many genes. The affected genes were characterized as stress response; cell cycle control, immunological and DNA repair genes[11]. The Unscheduled DNA synthesis (UDS) was determined in leukocytes of 10 human subjects after the consumption of meals containing varying amount of nitrate, nitrites or nitrosamines. In 6/10 subjects, UDS was significantly increased but no correlation was found with dietary nitrate, nitrite and nitrosamine levels or with blood nitrosamine levels[12].

The majority of the studies revealed no correlation, or in some cases a negative correlation, between nitrate intake and gastric cancer. A study of 556 grinders occupationally exposed from 1958 to 1976 to cutting fluids containing nitrite and amines, did not reveal an increased risk of cancer[13]. A causative connection between nitrate/nitrite and cancer through the formation of N-nitroso compounds is suspected. The role of nitrate and nitrite in the etiology of cancer in humans, especially gastric cancer, is addressed in numerous studies[14].

A survey of a high volume service station in eastern, whose site represented a maximum exposure to gasoline vapors, found that all 15 personal-long term samples contained 2-methylpentane; the 3 samples that were quantifiable ranged from 0.1 to 0.3 ppm[15]. Air concentrations (95% confidence limits) of 2-methylpentane measured by eight petroleum companies for service station attendants (49 measurements), transport drivers (49 measurements), and outside operators (56 measurements) were 2.01 (1.55-2.47), 1.69 (0.80-2.57), and 0.61 (0.37-0.84) mg/m^3, respectively[16].

The *Ames* test is a biological assay to assess the mutagenic potential of chemical compounds. A positive test indicates that the chemicals might act as a carcinogen (although a number of false-positives and false-negatives are known). All observed responses are verified in repeat tests. If no increase in mutant colonies is seen after testing several strains under several different culture conditions, the test chemical is considered to be nonmutagenic in the *Ames* test[17]. As cancer is often linked to DNA damage, the test also serves as a quick assay to estimate the carcinogenic potential of a compound since it is difficult to ascertain whether standard carcinogen assays on rodents were successful. The procedure is described in a series of papers from the early 1970s by Bruce Ames and his group at the University of California, Berkeley[18]. In addition, many studies have been performed to determine the sensitivity and correlation of the *Ames* test with animal carcinogenicity studies. It has indeed been established that there is a high predictability of a positive mutagenic response in the *Ames* test for rodent carcinogenicity, ranging from 90% to 77%, the primary differences being the chemical composition of the compiled databases[19]. The Acetone(99.8%, CAS No. 67-64-1) which was used as an vehicle were evaluated as negative in *Ames* test[20].

Despite the results of this test, it can affect by inducing inhalation, skin or eye contact, ingestion, and have affected central nervous system as a target organ. We suggest that future studies should be directed toward chronic inhalation, carcinogenic tests. The methods of assessment have been developed to provide indirect evidence of potential DNA damage or protective activities; for example, antioxidant effects can be monitored by measuring the lipid peroxidation and the formation of oxidized macromolecules. Further, enzyme measurements may provide information on processes that are associated with DNA protection[21]. The major advantage of methods that detect primary DNA damage is that no cell divisions are required. These methods have been largely replaced with single-cell gel electrophoresis (SCGE, and also called “Comet”) assays.

Based on this study, we suggest that future studies with cellular and molecular level such as Fragment Length Analysis using Repair Enzymes (FLARE) assay, comet assay with repair enzyme as Fpg, Endo III, and real time RT-PCR, and other developing tools. These

tools with many other chemicals would be also useful as a biomarker for chemical risk assessment.

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References

1. Gosselin RE, Smith RP, Hodge HC. *Clinical Toxicology of Commercial Products*. 5th ed. **1984**, p. II-124, Williams and Wilkins, Baltimore, U.S.A.
2. Reynolds JEF, Prasad AB. *Martindale-The Extra Pharmacopoeia*. 28th ed. **1982**, p. 1746, The Pharmaceutical Press, London, U.K.
3. International Program on Chemical Safety (IPCS). CEC; *International Chemical Safety Card on Potassium nitrate.* **2001.**
4. International Uniform Chemical Information Database (IUCLID). European Chemicals Bureau; Potassium nitrate (7757-79-1). **2006.**
5. Wallace LA, Pellizzari ED, Hartwell T, Rosenzweig R, Erickson M, Sparacino C, Zelon H. Personal exposure to volatile organic compounds. I: Direct measurement in breathing zone air drinking water, food, and exhaled breath. *Environ Res*, **1984**, 35, 293-319.
6. National Occupational Exposure Survey (NOES), Atlanta (USA): National Institute for Occupational Safety and Health (USA). **1989**.
7. Halder CA, Van Gorp GS, Hatoum NS, Warne TM. Gasoline vapor exposure. Part 1. *Am Ind Hyg Assoc J*, **1986**, 47, 164-72.
8. Bonsang B, Kanakidou M, Lambert G, Monfrey P. The marine source of C2-C6 aliphatic hydrocarbons. *J Atmos Chem*, **1988**, 6, 3-20.
9. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Some Organic Solvents, Resin Monomers and Related Compounds, Pigments and Occupational Exposures in Paint Manufacture and Painting, Volume 47, **1989**, International Agency for Research on Cancer, Lyon. France.
10. Fujita H, Nakano M, Sasaki M. Mutagenicity test of food additives with *Salmonella typhimurium* TA97 and TA102. *Kenkyu nenpo-TOKYO-toritsu eisei kenkynsho*, **1988**, 39, 343-50.
11. Bharadwaj L, Dhami K, Schneberger D, Stevens M, Renaud C, Ali A. Altered gene expression in human hepatoma HepG2 cells exposed to low-level 2,4-dichlorophenoxyacetic acid and potassium nitrate. *Toxicol In Vitro*, **2005**, 19(5), 603-19.
12. International Uniform Chemical Information Database (IUCLID). European Chemicals Bureau; Sodium nitrate, containing in the dry state more than 16.3 per cent by weight of nitrogen (7631-99-4). **2006**.
13. World Health Organization (WHO). "*WHO Food Additives Series*" 35 (844): Nitrite.
14. International Program on Chemical Safety (IPCS). 1996. *Poisons Information Monograph G016: Nitrates and nitrites*. **2006**.
15. Kearney CA, Dunham DB. Gasoline vapor exposures at a high volume service station. *Am Ind Hyg Assoc J*, **1986**, 47, 535-9.
16. Rappaport SM, Selvin S. Exposure hydrocarbon components of gasoline in the petroleum industry. *Appl Ind Hyg*, **1987**, 2, 148-54.
17. Mortelmans K, Zeiger E. The *Ames Salmonella*/microsome mutagenicity assay. *Mutat Res*, **2000**, 455(1-2), 29-60.
18. Ames BN, McCann J, Yamasaki E. Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. *Mutat Res*, **1975**, 31, 347-364.
19. Zeiger E. Identification of rodent carcinogens and noncarcinogens using genetic toxicity tests: premises, promises, and performance. *Regul Toxicol Pharmacol*, **1998**, 28, 85-95.
20. International Uniform Chemical Information Database (IUCLID). European Chemicals Bureau; Acetone (67-64-1). **2000.**
21. Knasmller S, Nersesyan A, Misk M, Gerner C, Mikulits W, Ehrlich V, Hoelzl C, Szakmary A, Wagner KH. Use of conventional and -omics based methods for health claims of dietary antioxidants: a critical overview. *Br J Nutr*, **2008**, 99(E Suppl 1), ES3-52.