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Evaluation of Mutagenic Activities of Endosulfan, Phosalone, Malathion, and Permethrin, Before and After Metabolic Activation, in the Ames *Salmonella* Test

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Wide spread use of insecticides in agriculture for many years can lead to their contamination in the food chain and the environment (Manske and Johnson 1977; Anon 1973). In recent years, it is reported that mutagenic activation or inactivation of the ingested chemicals can occur through various metabolic processes in animal body (Lu et al. 1972; Prins 1978). In such transformation of the chemicals, liver microsomal enzymes and intestinal microflora play major roles (Prins 1978).

The work reported here evaluates the mutagenic activities of commonly used insecticides - endosulfan (organochlorine), phosalone and malathion (organophosphorus) and permethrin (pyrethroid), before and after activation with cecal microbial extract or with liver post-mitochondrial fraction (S9-fraction) of rat, in Ames test with Salmonella typhimurium tester strains TA 97a, TA 98 and TA 100. As far as we are aware, no study has yet addressed whether the insecticides mentioned above can be mutagenic following their activation by mammalian cecal microorganisms.

MATERIALS AND METHODS

Salmonella typhimurium strains TA 100 (sensitive to base pair substitution mutagens), TA 98 and TA 97a (sensitive to frame shift mutagens) were kindly provided by Prof. B.N. Ames, University of California, Berkeley, USA. Recently developed strain TA 97a is reported to be more sensitive than strain TA 98 (Levin et al. 1982).

Nicotinamide adenine dinucleotide phosphate (NADP), glucose-6-phosphate (G-6-P), l-histidine, biotin, rutin, benzo(α)pyrene and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. USA; and Aroclor 1254 from Monsanto, St. Louis, USA. Endosulfan [α,β-1,2,3,4,7,7-hexachlorobicyclo-2,2,1-heptene-2-biosoxymethylon -5,6-sulphite] and Malathion [0,0-dimethyl-S-(1,2-dicarbethoxy-ethyl)-dithiophosphate] were obtained from Excel Industries, Bombay, India. Phosalone [0,0-diethyl-S-(6-chloro-2-oxobenzoxa-zolin-3-yl)methyl phosphorodithioate] and permethrin [3-phenoxy-

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benzyl-cis-trans-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylate] were acquired from Volhro Ltd., Hyderabad, India, and Alkali Chemical Corporation, Calcutta, India, respectively. The concentration of the insecticide expressed in the experiments is based upon its purity (around 90-94%). Solutions of endosulfan, phosalone and malathion were prepared in DMSO and that of permethrin in absolute alcohol. The concentrations of these solvents in experiments were maintained at or less than 2 per cent (v/v). DMSO or ethyl alcohol at the concentration of 2 per cent (v/v) did not exhibit mutagenic activities in Ames assay with the three tester strains.

Non-toxic dose (a concentration at maximum level that did not affect the cell growth) and 90% toxic dose (a concentration that inhibited the cell growth by 90%) for each of the four insecticides were determined by measuring the growth (18 hr, 37°C) of S.typhimurium tester strains - TA 97a, TA 98 and TA 100. The non-toxic doses of endosulfan, phosalone, malathion and permethrin for the tester strains were 41, 42, 33 and 39 mg/L, respectively, and the 90% toxic doses were 3256, 2100, 1650, 2730 mg/L, respectively (results not included).

Cell free extract of microorganisms located in the rat cecum was prepared by the procedure of Brown and Dietrich (1979). The procedure involved removal of rat cecal contents (about 4.5 gm), sonication for the disruption of cells (MSE ultrasound disintegrator, Model - 100 W1, 5 ultrasound bursts each of 30 sec with 60 sec gap at 0-4°C), centrifugation (13000 x g, 20 min, 4°C) and collection of the supernatant termed as 'cecal cell-free extract' (CCE). Freshly prepared CCE was filter sterilised before use. That CCE was metabolically active was ensured by assaying the mutagenicity of rutin, with and without CCE activation, using TA 98 strain. Mutagenicity of rutin (around 200 revertants/100 µg/-plate) was discernible exclusively after the activation with CCE (Brown and Dietrich 1979; Pamuken et al. 1980; Tamura et al. 1980).

Aroclor 1254 (200 mg/ml DMSO) induced rat (male Wistar strain, Aroclor administration intraperitoneal, 500 mg kg body wt.) liver S9-factor was prepared according to Ames et al. (1975) and stored at -80°C until use. S9-mix (containing per ml, S9-factor, 0.4 ml; NADP, 4 mM; G-6-P, 5 mM; Na_2HPO_4, 100 mM; $MgCl_2$, 8 mM and KCl, 33 mM) was prepared freshly before use (Ames et al. 1975). The metabolic activity of S9-mixture was determined by assessing the mutagenicity of benzo(α)pyrene with TA 100 strain. The compound showed mutagenic activity following the activation (around 900 revertants/µg/plate) (Ames et al. 1975).

For the assessment of mutagenicity of endosulfan, phosalone, malathion and permethrin at 90% toxic doses (3256, 2100, 1650, 2730 mg/L, respectively) (Maron and Ames 1983) cells of the tester strains grown in nutrient broth (0.1 ml, 1 x 10^8 cells/ml) were incubated for 3 hr at 37°C in a system containing minimal medium (2 ml), insecticide in solvent (0.2 ml) liver-S9-mix (0.1 ml) or CCE (0.4 ml) whenever required, centrifuged, washed, resuspended in minimal medium(0.1 ml) and used immediately in the mutagenicity

assay as described below. In the control groups, the cells were treated with solvents - DMSO or ethanol (2% v/v).

The Salmonella typhimurium mutagenicity assay with tester strains TA 97a, TA 98, TA 100 was conducted according to Ames et al. (1975).

Cells grown in nutrient broth for 16-18 hr (0.1 ml, 1 x 10^8 cells-/ml), insecticide at non-toxic concentration (0.1 ml), CCE (0.4 ml) or liver S9-mixture (0.4 ml) were added to 2 ml of molten (40-42°C) soft agar butt (agar, 0.6%; NaCl, 0.5%; histidine, 0.05 mM; biotin, 0.05 mM and glucose, 4% for TA 98, TA 100 and 0.4% for TA 97a) and spread on the surface of the pre-set agar base (20 ml, minimal medium with 1.5% agar) in petri plate. The plates were incubated at 37°C for 48 hr and histidine revertant colonies were counted.

The cells of the tester strains were pretreated with the insecticide at 90% toxic dose or with the solvents -DMSO or ethyl alcohol as described earlier. The pretreated cells in suspension (0.1 ml, 1 x 10^7 cells) were added to molten soft agar butt (2 ml) and spread on the surface of the pre-set agar base in petri plate as described above. The plates were incubated (37°C, 48 hr) before scoring for the revertant colonies.

The solvents DMSO or ethyl alcohol (2% v/v) or the tester strain cells pretreated with these solvents did not show change in the patterns of spontaneous revertants of the strains.

RESULTS AND DISCUSSION

Synthetic insecticides can enter animal body through their contamination in foods and environment (Manske and Johnson 1977; Anon 1973). The microflora of the intestine and the microsomal enzymes of the liver can transform and/or breakdown such chemicals by enzymic hydrolysis, reduction, degradation and various other reactions (Lu et al. 1974; Prins 1978). It is shown - that metabolism of the insecticides of pyrethroid group involves mainly hydrolysis of ester bond by liver microsomal esterases and various c-hydroxylations probably by cytochrome P-450 dependant monooxygenases (Hutson 1979); - that hydrolysis of malathion, an organophosphorous insecticide, in mammals leads to the formation of 'malathion acid', a triester of phosphoric acid having alkylating properties (Wild 1975); - that the cleavage of malathion by microbial phosphatase action in rumen produces dimethyl phosphate and O,O'-dimethyl phosphorothioate (Prins 1978; James et al. 1975); - that metabolism of endosulfan in rat, cattle and sheep generates endosulfan diol, endosulfan α-hydroxy ether, endosulfan sulfate and endosulfan lactone (Dorough et al. 1978; Beck et al. 1966; Gorbach et al. 1968). Thus, the ingested insecticides or their transformed forms and breakdown products may have adverse genetic effects on human beings.

The mutagenic potentials of the four synthetic insecticides - endosulfan, phosalone, malathion and permethrin - at non-toxic and 90% toxic doses, in the presence and in the absence of rat-liver post-mitochondrial fraction (S9-fraction) or rat-cecal microbial extract, were systematically examined in Ames Salmonella Assay system with three tester strains TA 97a, TA 98 and TA 100. The non-toxic and the toxic concentrations of endosulfan, phosalone, malathion and permethrin were 41, 42, 33, 39 mg/L, and 3256, 2100, 1650, 2730 mg/L, respectively. The testing for the mutagenicity of the insecticides at non-toxic doses was carried out by plate--incorporation-assay, whereas, the testing at 90% toxic doses was performed by using the pretreated (for 3 hr) cells in the assay.

The results from Table 1, 2 and 3, do not diaplay mutagenic activities of endosulfan, phosalone, malathion and permethrin at the respective non-toxic and toxic doses, either before or after the activation with rat-liver S9-fraction with three S.typhimurium tester strains. These findings confirm the reported non-mutagenicity of the four insecticides with the test assays using S.typhimurium strains TA 98, TA 100, TA 1535, TA 1537 and TA 1538; Escherichia coli WP$_2$ and Bacillus subtilis (Shirasu et al. 1976; Moriya et al. 1983; Wildemauwe et al. 1983). However, other reports have ascribed genotoxic properties to endosulfan and malathion. These insecticides induce chromosomal breaks in human beings (Yoder et al. 1973; Van Bao et al. 1974); significant increase in the frequency of micronuclei in mice (Sylianco 1978; Usha Rani et al. 1980; Dulout et al. 1982) and clastogenic effects in several cultured cell lines (Nicholas et al. 1979; Chen et al. 1981; Yadav et al. 1982). In addition, endosulfan causes recessive lethals and sex chromosomal losses in Drosophila melanogaster (Velazquez et al. 1984); reverse mutation, mitotic gene conversion and increase in the frequency of abberant colonies in Saccharomyces cerevisiae (Yadav et al. 1982) and chromosomal damage in barley root-tip cells (Grover and Tyagi 1980).

The results from Tables 1, 2, 3 demonstrate non-mutagenicity of endosulfan, phosalone, malathion and permethrin (at non-toxic and 90% toxic doses) following their activation with rat-cecal microbial extract in Ames assay system with S.typhimurium tester strains TA 97a, TA 98, TA 100. This would imply that the transformations or the metabolites of the four insecticides arising through the action of intestinal microflora may not be genotoxic. As far as we are aware no laboratory has yet reported these findings.

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Table 1. Results of mutagenicity test using S.typhimurium strain TA 97a on the synthetic insecticides - endosulfan, phosalone, malathion and permethrin

Insecticide	Concentration (mg/L)	Concentration (M)	Without CCE or S9 (Number of revertants per plate)	With CCE (Number of revertants per plate)	With S9 (Number of revertants per plate)
SR	-	-	94 ± 2.00	107 ± 1.82	105 ± 3.40
Endosulfan	41	1×10^{-4}	97 ± 1.82	119 ± 2.91	117 ± 2.90
	3256	8×10^{-3}	94 ± 1.71	110 ± 1.53	111 ± 3.10
Phosalone	42	1×10^{-4}	102 ± 3.10	117 ± 1.08	119 ± 3.10
	2100	5×10^{-3}	98 ± 2.91	118 ± 2.90	116 ± 2.90
Malathion	33	1×10^{-4}	93 ± 2.38	109 ± 1.24	107 ± 1.93
	1650	5×10^{-3}	106 ± 2.34	120 ± 2.21	121 ± 2.25
Permethrin	39	1×10^{-4}	98 ± 2.13	108 ± 1.85	110 ± 1.36
	2730	7×10^{-3}	99 ± 1.25	120 ± 2.19	118 ± 1.19

SR = spontaneous revertants; CCE = rat cecal cell free extract.
S9 = rat liver post-mitochondrial fraction (S9-fraction).

Controls with DMSO or ethyl alcohol [2% (v/v) per plate] did not alter the pattern of spontaneous revertants of the strain TA 97a.

Each value is an average of six replicates of seven independent experiments ± standard error of the mean.

Table 2. Results of mutagenicity test using S.typhimurium strain TA 98 on the synthetic insecticides - endosulfan, phosalone, malathion and permethrin

Insecticide	Concentration (mg/L)	Concentration (M)	Without CCE or S9	With CCE	With S9
			(Number of revertants per plate)		
SR	-	-	7 ± 0.82	15 ± 0.94	13 ± 1.01
Endosulfan	41	1×10^{-4}	8 ± 0.91	17 ± 1.01	14 ± 0.51
	3256	8×10^{-3}	10 ± 1.12	16 ± 0.75	15 ± 0.70
Phosalone	42	1×10^{-4}	12 ± 0.95	18 ± 1.00	16 ± 1.21
	2100	5×10^{-3}	11 ± 0.75	19 ± 0.82	17 ± 0.70
Malathion	33	1×10^{-4}	9 ± 0.91	15 ± 0.72	14 ± 0.91
	1650	5×10^{-3}	11 ± 1.01	13 ± 0.56	16 ± 1.98
Permethrin	39	1×10^{-4}	9 ± 0.91	14 ± 0.72	15 ± 2.10
	2730	7×10^{-3}	12 ± 1.02	17 ± 0.62	17 ± 0.85

SR = spontaneous revertants; CCE = rat cecal cell free extract.
S9 = rat liver post-mitochondrial fraction (S9-fraction).

Controls with DMSO or ethyl alcohol [2% (v/v) per plate] did not alter the pattern of spontaneous revertants of the strain TA 98.

Each value is an average of six replicates of seven independent experiments ± standard error of the mean.

Table 3. Results of mutagenicity test using S.typhimurium strain TA 100 on the synthetic insecticides - endosulfan, phosalone, malathion and permethrin

Insecticide	Concentration		Without CCE or S9	With CCE	With S9
	(mg/L)	(M)	(Number of revertants per plate)		
SR	-	-	112 ± 1.68	130 ± 2.89	128 ± 5.71
Endosulfan	41	1×10^{-4}	114 ± 1.24	133 ± 3.10	129 ± 3.81
	3256	8×10^{-3}	117 ± 2.10	134 ± 1.39	133 ± 2.25
Phosalone	42	1×10^{-4}	109 ± 1.69	127 ± 1.28	124 ± 3.3
	2100	5×10^{-3}	114 ± 1.75	131 ± 2.14	130 ± 2.91
Malathion	33	1×10^{-4}	111 ± 3.10	130 ± 3.75	128 ± 2.00
	1650	5×10^{-3}	118 ± 3.20	132 ± 2.13	137 ± 1.37
Permethrin	39	1×10^{-4}	112 ± 1.50	128 ± 1.95	126 ± 1.36
	2730	7×10^{-3}	110 ± 1.34	125 ± 2.12	122 ± 1.32

SR = spontaneous revertants; CCE = rat cecal cell free extract.
S9 = rat liver post-mitochondrial fraction (S9-fraction).

Controls with DMSO or ethyl alcohol [2% (v/v) per plate] did not alter the pattern of spontaneous revertants of the strain TA 100.

Each value is an average of six replicates of seven independent experiments ± standard error of the mean.

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