Mutagenic evaluation of trichlorfon using different assay methods with *Salmonella typhimurium*

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Evaluation of the mutagenicity of trichlorfon pesticide was carried out with strains TA1535, TA100, TA97, TA98 and TA104 of *Salmonella typhimurium* by means of several assay methods: (i) spot test; (ii) standard plate incorporation test; (iii) plate incorporation test with preincubation; (iv) fluctuation test and (v) fluctuation test with preincubation, with and without post-mitochondrial liver fraction (S9) from Wistar rats pretreated with phenobarbital and 5,6-benzoflavone as a metabolic activation system. Trichlorfon induced base-pair substitution mutations, and its mutagenic activity was decreased by the addition of S9 mix. The fluctuation test and fluctuation test with preincubation were the most sensitive assay methods for detecting the mutagenicity of trichlorfon.

Introduction

Trichlorfon was introduced as a commercial chemical in 1952. It is made by the reaction of dimethyl hydrogen phosphite with trichloroacetaldehyde (Worthing, 1979). Its use as an insecticide in agricultural applications is a potential source of exposure, both of workers and of the general population.

Results of mutagenicity with bacteria have not been consistent; the differences reported are probably due to differences in methodology. Trichlorfon was negative in spot tests with *Salmonella typhimurium* strains TA1535, TA1536, TA1537 and TA1538 at 2000 µg/plate (Carere *et al.*, 1978a). Others have reported mutagenicity in *S.typhimurium* (Byeon *et al.*, 1976; Kawachi *et al.*, 1980; Shirasu *et al.*, 1982). Although Shirasu *et al.* (1977) reported that trichlorfon did not cause mutations in *S.typhimurium* strains TA1535, TA1536, TA1537 and TA1538 in the absence of metabolic activation, several authors (Wildemaue *et al.*, 1983), using variants of the Ames test, observed a direct mutagenic effect. Hanna and Dyer (1975) found a mutagenic effect in *S.typhimurium* TA1535 only after incubating the tester plates for 72 h, rather than for 48 h. Carere *et al.* (1978b) found that after incubation in liquid culture, trichlorfon caused mutations in *S.typhimurium* TA1535 in the absence of metabolic activation. Batzinger and Bueding (1977), using a preincubation assay, found trichlorfon to be directly mutagenic to *S.typhimurium* TA98 and TA100. Braun *et al.* (1982) observed no mutagenic effect with the plate incorporation test, but after preincubation trichlorfon appeared to cause mutation in *S.typhimurium* TA100 without metabolic activation. Byeon *et al.* (1976) reported a weak direct mutagenic effect in *S.typhimurium* TA100 and Waters *et al.* (1982) found a direct mutagenic effect on one of the Ames strains using the plate incorporation assay. Moriya *et al.* (1983) established the mutagenic potency of

trichlorfon in 0.0067 revertants/nmol for the TA100 strain without metabolic activation.

In general, induction of base-pair substitution or mutation was observed and exogenous metabolic activation was not required. This fact is consistent with the activity of an alkylating agent (IARC, 1983).

In the presence of metabolic activation trichlorfon was observed to cause mutation in *S.typhimurium* TA100 (Poole *et al.*, 1977; Batzinger and Bueding, 1977; Simmon, 1979; Braun *et al.*, 1982; Waters *et al.*, 1983) and, according to Braun *et al.* (1982), also in TA98. However, Braun (1982) reported that microsomal enzymes decreased the genotoxic activity of trichlorfon. Panda and Sharma (1980) obtained a negative result for trichlorfon with a host-mediated assay on *S.typhimurium*, but Batzinger and Bueding (1977) observed trichlorfon to cause mutation in *S.typhimurium* TA98 and TA100 in a host-mediated assay.

In this study a combination of different assays using *S.typhimurium* was applied to provide additional data on the genotoxicity of trichlorfon. The strain TA104, which detects mutagens damaging A-T base pairs, was also tested.

Materials and methods

Chemicals

Trichlorfon [dimethyl(2,2,2-trichloro-1-hydroxyethyl)phosphonate] (purity 97%) was supplied by ERT (Explosivos de Río Tinto, España). This compound was dissolved in dimethyl sulphoxide (DMSO), supplied by Merck.

Positive control chemicals were sodium azide, 9-aminoacridine and 4-nitroquinoline-N-oxide, from Fluka; methylglyoxal, from Aldrich Chemical Co.; and 2-aminoanthracene, from Sigma Chemical Co.

NADP, D-glucose-6-phosphate and phenobarbitone sodium salt (PB) were supplied by Fluka, and 5,6-benzoflavone (β -naphthoflavone) was supplied by Aldrich Chemical Co., Inc.

All other chemicals were of laboratory reagent grade.

Bacterial strains

S.typhimurium strains TA1535, TA97, TA98, TA100 and TA104 were kindly supplied by Professor B.N.Ames, University of California, Berkeley, CA, USA. Their genotypes have been described previously (Levin *et al.*, 1982; Maron and Ames, 1983).

Their genetic markers and other characteristics, such as response to positive controls and the number of spontaneous revertants, were routinely verified as described by Maron and Ames (1983).

Metabolic activation system

The supernatant of the post-mitochondrial liver fraction (S9) from Wistar male rats weighing ~200 g was used as the metabolic activation system.

For induction we used a combined injection of PB and β -naphthoflavone (Matsushima *et al.*, 1976). The rats were injected i.p. with PB (30 mg/kg), PB (60 mg/kg), PB (60 mg/kg) and β -naphthoflavone (80 mg/kg), and PB (60 mg/kg), 4, 3, 2 and 1 day(s), respectively, before they were killed.

Table I. Mutagenicity of trichlorfon in the spot test

S9 mix	<i>S.typhimurium</i> strains				
	TA100	TA97	TA98	TA104	TA1535
Without	—	—	—	?	—
With	—	—	—	?	—

— = Non-mutagenic.

? = The induced revertant colonies appeared only in one of three plates.

Table II. Mutagenicity of trichlorfon in the Ames test^a

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		mg/plate	TA100		TA104 ^b		TA98		TA97		TA1535		
			<i>x</i> ± SD	MI	<i>x</i> ± SD	MI	<i>x</i> ± SD	MI	<i>x</i> ± SD	MI	<i>x</i> ± SD	MI	
Assay I													
Without S9 mix	DMSO	94 ± 14.1			513 ± 14.4		11 ± 4.9						
	0.1	102	0.1		499 ± 26.6		10 ± 2.1						
	1	77 ± 2.1			684 ± 32.6	0.3	9 ± 2.1						
	5	107	0.1		1165 ± 76.2	1.2	13 ± 5.0	0.2					
	25				436 ± 3.5								
	SR	91 ± 24.7			537 ± 35.1		11 ± 0.6						
	SM	900	8.8		9000	15.8	45	3.1					
	With S9 mix	DMSO	84 ± 7.8			418 ± 20.0		18 ± 3.5					
0.1	90 ± 19.8	0.1		424 ± 108.2		12 ± 4.6							
1	112 ± 15.5	0.3		603 ± 55.1	0.4	15 ± 3.2							
5	123 ± 18.6	0.4		915 ± 67.7	1.2	13 ± 2.1							
25				591 ± 61.0	0.4	24 ± 5.6							
SR	95 ± 25.5			416 ± 22.1		15 ± 3.8							
SM	425	3.6		1640	2.9	1000	65.5						
Assay II													
Without S9 mix	DMSO	140 ± 1.0			577 ± 29.0		17 ± 0.6		97 ± 2.4		40 ± 3.5		
	5	327 ± 10.2	1.3		1142 ± 39.9	1.0	26 ± 3.5	0.6	91 ± 12.5		53 ± 11.8	0.2	
	7.5	259 ± 13.6	0.8		1043 ± 34.0	0.9	21 ± 2.1	0.3	131 ± 3.0	0.3			
	10	290 ± 12.1	1.0		1093 ± 33.5	1.0	18 ± 7.5		130 ± 22.2	0.3	60 ± 2.6	0.4	
	12.5	355 ± 26.6	1.4		1077 ± 18.5	0.9	22 ± 6.1	0.4	37 ± 13.1		54 ± 9.3	0.3	
	15										50 ± 10.1	0.2	
	20										29 ± 6.3		
	25	209 ± 103.6	0.5		621 ± 105.9		5 ± 3.2		3		36 ± 24.7		
	SR	148 ± 7.0			538 ± 50.2		14 ± 0.6		99 ± 9.8		51 ± 4.5		
	SM	689	3.77		7000	11.9	136 ± 1.4	8.0	700	6.0	3254	63	
	With S9 mix	DMSO	135 ± 13.4			495 ± 20.1		20 ± 2.1		101 ± 8.7		39 ± 1.0	
		5	286 ± 26.3	1.1		869 ± 59.9	0.6	17 ± 6.6		91 ± 10.7		44 ± 4.1	0.1
		7.5	234 ± 24.5	0.8		904 ± 48.6	0.7	32 ± 5.9	0.5	103 ± 3.0			
		10	227 ± 13.7	0.7		757 ± 141.6	0.4	24 ± 7.8	0.2	116 ± 22.6	0.1	45 ± 5.5	0.1
12.5		259 ± 10.3	0.9		1187 ± 83.3	1.2	13 ± 3.5		66 ± 24.0		46 ± 3.5	0.1	
15											35 ± 4.5		
20											23 ± 2.8		
25		159 ± 26.5	0.2		745 ± 30.3	0.4	9 ± 2.1		32 ± 17.5		24 ± 5.6		
SR		131 ± 9.5			581 ± 46.7		23 ± 6.6		110 ± 4.9		60 ± 7.2		
SM		500	2.8		2000	2.6	800	33.9	1000	8.2	863	13.7	

^a*x* ± SD, mean of revertants of triplicate plates ± standard deviation; MI, mutation index; SR, spontaneous revertants; DMSO, dimethylsulphoxide; SM, standard mutagen.

^bStatistical analysis applied to assay I with TA104: without S9 mix: ANOVA *P* < 0.01; regression analysis *P* < 0.01; slope = 0.130; with S9 mix: ANOVA *P* < 0.01; regression analysis *P* < 0.025; slope = 0.097.

The liver S9 fraction was prepared according to the method described by Maron and Ames (1983). The protein concentration was 74.5 mg/ml as determined by the procedure of Lowry *et al.* (1951). The NADPH-cytochrome-c-reductase activity, measured according to the method of Masters (1967), was 2.3×10^{-7} mol/min/mg protein.

Immediately before the mutagenicity tests S9 mix was prepared, i.e. S9 with NADP and glucose-6-phosphate cofactors.

S9 mix was prepared as follows: S9 (4%) with 4 mM NADP and 5 mM glucose-6-phosphate cofactors, for plate incorporation tests, according to Maron and Ames (1983). S9 mix for fluctuation tests contained 700 µl S9 fraction/ml, 0.32 mmol MgCl₂, 33.1 µmol glucose-6-phosphate, 26.79 µmol NADP and 5.13 mmol Sørensen phosphate buffer, pH 7.4 (Herrera, 1989).

Mutagenicity assays with bacteria

Testing for mutagenicity was carried out using several assay methods: spot test, standard plate incorporation assay, plate incorporation assay with preincubation, fluctuation test and fluctuation test with preincubation.

The overnight cultures of the tester strains were prepared by inoculation from the stock cultures into Difco Bacto nutrient broth and incubated for 14 h at 37°C with shaking.

Fig. 1. Ames test with TA104 (Assay I)

Fig. 2. Ames test with preincubation and TA104 (Assay I)

Spot test. This assay was carried out according to the method described by Ames *et al.* (1975). TA1535, TA100, TA97, TA98 and TA104 strains were used. Trichlorfon was dissolved in DMSO and applied at doses of 500 and 5000 µg/plate. All experiments were performed in triplicate. Results were expressed qualitatively.

Standard plate incorporation assay. This assay was carried out as described previously (Ames *et al.*, 1975; Maron and Ames, 1983). To 2 ml of top agar supplemented with 0.2 ml of a 0.5 mM histidine/biotin solution were added 100 µl of the trichlorfon solution, 100 µl of an overnight culture of the tester strain (TA100, TA104, TA98, TA97 or TA1535) and, when the experiment was performed using a metabolic activation system, 500 µl of S9 mix. After shaking, the mixture was spread onto a minimal glucose agar plate. Plates were placed in a 37°C incubator for at least 48 h after which revertants were counted. All experiments were carried out in triplicate, and results were expressed as the average number of revertants per plate. For each manipulation, spontaneous reversion and mutation in response to DMSO and positive response to the standard mutagen controls were included to ensure the validity of the test.

The positive controls used per plate were (with corresponding strains in

Table III. Mutagenicity of trichlorfon in the Ames test with preincubation^a

		Mg/plate	TA100 ^b		TA104 ^c		TA1535	
			<i>x</i> ± SD	MI	<i>x</i> ± SD	MI	<i>x</i> ± SD	MI
Assay I								
Without S9 mix	DMSO	108 ± 3.5			647 ± 31.0		27 ± 7.8	
	5	230 ± 24.2	0.8		1000	0.5	45	0.5
	10	243 ± 32.9	0.9		1317 ± 68.5	1.0	28 ± 10.4	
	12.5	259 ± 26.9	1.0		750 ± 223.2	0.2	31 ± 3.2	0.1
	15	157 ± 40.3	0.3		761 ± 107.1	0.2	19 ± 2.5	
	20	66 ± 8.0			178 ± 173.2		8 ± 0.7	
	25	28 ± 8.6			5 ± 2.1		7 ± 2.1	
	SR	155 ± 5.1			637		37 ± 6.0	
	SM	903	5.1		3000	3.7	3500	93.9
With S9 mix	DMSO	101 ± 13.6			532 ± 23.4		22 ± 2.0	
	5	287 ± 22.5	1.1		853 ± 158.8	0.5	18 ± 0.6	
	10	311 ± 15.1	1.2		1023 ± 61.8	0.8	19 ± 4.5	
	12.5	342 ± 27.6	1.4		417 ± 117.2		15 ± 2.6	
	15	351 ± 42.4	1.5		214 ± 105.3		12 ± 7.1	
	20	245 ± 113.4	0.8		25 ± 10.4		10 ± 4.2	
	25	28 ± 36.5			45 ± 49.4		3 ± 2.8	
	SR	170 ± 13.2			648 ± 43.4		46 ± 4.7	
	SM	822	4.2		2256	2.7	900	19.1
Assay II								
Without S9 mix	DMSO	130			442 ± 31.8			
	1	156 ± 13.6	0.2		667 ± 56.9	0.5		
	2.5	201 ± 19.8	0.5		905 ± 38.3	1.1		
	5	255 ± 3.5	0.9		1365 ± 362.9	2.1 ^d		
	12.5	283 ± 80.3	1.1		1344 ± 82.6	2.1 ^d		
	25	7 ± 4.2			0			
	SR	137 ± 7.1			447 ± 30.9			
	SM	554	3.1		3200	6.2		
With S9 mix	DMSO	134 ± 9.9			427 ± 35.4			
	1	206 ± 2.1	0.4		541 ± 42.0	0.3		
	2.5	263 ± 14.5	0.8		977 ± 18.9	1.3		
	5	349 ± 19.2	1.3		1237 ± 198.8	2.0 ^d		
	12.5	445 ± 66.2	1.9		1736 ± 266.3	3.2 ^d		
	25	2 ± 1.5			0			
	SR	164 ± 9.2			412 ± 39.8			
	SM	1236	6.7		2224	4.4		

^a*x* ± SD, mean of revertants of triplicate plates ± standard deviation; IM, mutation index; SR, spontaneous revertants; DMSO, dimethylsulphoxide; SM, standard mutagen.

^bStatistical analysis applied to assay II with TA100: without S9 mix: ANOVA *P* < 0.01; regression analysis *P* < 0.05; slope = 0.012; with S9 mix: ANOVA *P* < 0.01; regression analysis *P* < 0.025; slope = 0.023.

^cStatistical analysis applied to assay II with TA104: without S9 mix: ANOVA *P* < 0.01; regression analysis *P* < 0.025; slope = 0.067; with S9 mix: ANOVA *P* < 0.01; regression analysis *P* < 0.05; slope = 0.049.

^dMutagenic concentration.

parentheses) 1.5 µg sodium azide (TA1535 and TA100), 100 µg 9-aminoacridine (TA97), 0.5 µg 4-nitroquinoline-*N*-oxide (TA98) and 50 µg methylglyoxal (TA104). When assays were performed in the presence of S9 mix, 15 µg of 2-aminoanthracene was used for all strains.

The concentration range for trichlorfon was selected taking into account the spot test results.

In order to determine the existence of a mutagenic effect caused by trichlorfon and to quantify it, two methods were used: (i) calculation of the mutation index and (ii) a statistical method based on least-squares linear regression (Barrueco and de la Peña, 1988, 1989; Herrera and Laborda, 1988).

A concentration of the test compound is considered mutagenic when the mutation index is at least 2.

We can only calculate the mutagenic effect using statistical methods when the analysis of regression is significant, then the slope will be a measure of the mutagenic effect. When the analysis of regression is significant it means that there is a dose-response relationship and we can calculate the mutagenic potency, expressed as the number of induced revertants/nmol of product taking the number of revertants from the top level of the linear part of the dose-response curve.

Plate incorporation assay with preincubation. The preincubation procedure was carried out as described by Yahagi *et al.* (1975). To sterile tubes, placed in an ice bath, were added 500 µl of S9 mix (or phosphate buffer, pH 7.4), 100 µl of an overnight culture of the tester strain (TA100, TA104 or TA1535) and 100 µl of the trichlorfon solution. Tubes were placed in a shaking bath at 37°C for 20 min. After incubation, top agar was added to the tubes and the procedure from then on is the same as that used in the standard incorporation assay.

Fluctuation test. This assay was carried out as described by Green *et al.* (1976, 1977). Liquid medium was Davis Mingioli salts supplemented with 40% (w/v) glucose, 0.57 mM histidine and 0.0245 mM biotin. To liquid medium (99 ml) was added 20 µl of an overnight culture of the tester strain TA100 and 1 ml of trichlorfon solution. The mixture was dispensed in 2 ml aliquots into each well of a 48-well sterile tray. The trays were incubated at 37°C. After 2 days of incubation, bromocresol purple (5 µg/ml) was added. The trays were incubated for a further 24 h. Positive wells containing prototrophic mutants turn yellow, whereas negative wells remain purple. For each manipulation, spontaneous reversion and mutation in response to DMSO and positive response to the standard mutagen were included to ensure the validity of the test. The positive control used was 0.01 µg/ml sodium azide. The strain and the concentration range for trichlorfon were selected taking account of the plate incorporation test results.

The statistical significance of the results was assessed by the χ^2 test (Green *et al.*, 1976). Results of different experiments with trichlorfon were combined for statistical analysis.

Fluctuation test with preincubation. This assay was carried out using an adapted procedure from Levin *et al.* (1981) (Herrera, 1989). Reaction tubes contained the following: 100 µl of a log-phase TA100 bacterial culture, 2 ml of liquid medium, 25 µl of the trichlorfon solution and 2.85 ml of S9 mix when it was used. These tubes were incubated in a shaking bath at 37°C for 20 min. A 1 ml sample was added to 99 ml of liquid medium and the mixture was dispensed in 2 ml aliquots into each well of a 48-well sterile tray and the test proceeded as the fluctuation test until the end. The positive controls used were 0.3 µg/ml sodium azide and 2 µg/ml 2-aminoanthracene for assays performed in the absence or presence of S9 mix, respectively.

Results

The number of spontaneous revertants observed for each strain was within the range previously established in our laboratory. Similarly, the response of the positive controls was as expected.

Spot test results are shown in Table I. These indicated that trichlorfon was not mutagenic either in the presence or the absence of S9 mix for TA100, TA97, TA98 and TA1535 strains. The results with strain TA104 were inconclusive, because the induced revertant colonies appeared only in one of the three plates. Furthermore, the spot test results indicated low toxicity of trichlorfon as judged by the absence of an inhibition zone around the spot at a dose of 500 µg/plate; 5000 µg/plate dose was toxic.

In the plate incorporation assay this compound was usually tested at a range of 0.1–25 mg/plate using at least three concentrations in a preliminary assay and at least four concentrations of trichlorfon in a second assay in order to establish a dose-response relationship. Similar results were obtained in

either the absence or the presence of S9 mix with all strains. According to the mutation index, trichlorfon did not show mutagenic activity. Nevertheless, trichlorfon increased significantly the number of revertants in TA104 although the mutation index did not reach 2. The maximum mutation index obtained for TA104 was 1.2, and for TA100 strain, 1.4 (Table II and Figure 1).

In the plate incorporation test with preincubation trichlorfon was tested with strains TA100 and TA104. In this test TA1535 was also included because it had been reported that trichlorfon shows activity in this strain in the Ames test with preincubation. Trichlorfon showed weak mutagenic activity with strain TA104 both in the presence and the absence of S9 mix (Table III and

Fig. 3. Ames test with preincubation and TA100 (Assay II)

Table IV. Fluctuation test TA100 strain.

a: preliminary experiments—effect of trichlorfon on bacteria growth

Trichlorfon (µg/ml)	Viable bacterium/ml
DMSO	4.71×10^8
50	5.45×10^8
500	4.05×10^8
1250	2.66×10^8
2500	7.00×10^7
5000	7.00×10^7
Control	7.22×10^8

b: mutagenicity assay

Trichlorfon (µg/ml)	No. of experiments	Total wells	No. of turbid wells		χ^2	Significance	Q ^a
			control	treated			
2.5	1	48	15	42	31.00	$P \ll 0.001$	M4
5	1	48	15	48	50.28	$P \ll 0.001$	M4
10	2	96	25	55	19.28	$P < 0.001$	M3
50	1	48	15	32	12.05	$P < 0.001$	M3
100	1	48	15	37	20.30	$P < 0.001$	M3
250	2	96	25	50	13.67	$P < 0.001$	M3
500	2	96	10	0	11.16	$P < 0.001$	T
1250	1	48	10	0	11.16	$P < 0.001$	T
2500	1	48	10	0	11.16	$P < 0.001$	T
5000	1	48	10	0	11.16	$P < 0.001$	T
Sodium azide (0.01 µg/ml)	2	96	25	75	52.17	$P \ll 0.001$	M4

^aQ, quality; M3, mutagenic; M4, highly mutagenic; T, toxic effect.

Figure 2), its mutagenic potency being established at 0.048 revertants/nmol. With strain TA100 trichlorfon increased significantly the number of revertants per plate although the mutation index did not reach 2 (Table III and Figure 3).

In the fluctuation test with TA100 trichlorfon showed mutagenic activity in the concentration range 2.5–250 µg/ml (Table IV).

In the fluctuation test with preincubation with strain TA100 trichlorfon showed mutagenic activity mainly in the absence of S9 mix in the concentration range 5–2500 µg/ml. The mutagenic activity of trichlorfon was not enhanced by addition of the S9 mix (Table V).

Discussion

The results of the spot test suggested trichlorfon was of low toxicity and that it was not mutagenic. In the case of TA104 these results were inconclusive. These results confirm those obtained by Carere *et al.* (1978a) with TA1535.

The standard plate incorporation assay confirmed the results obtained in the spot test. Similar results were obtained in both the absence and presence of S9 mix. As regards strain TA104, although trichlorfon was negative according to the mutation index, it produced a significant dose–response relationship indicating the existence of a possible mutagenic effect. In the case of TA100 our results agree with those of Moriya *et al.* (1983) which showed a small increase in the number of revertants due to trichlorfon but they disagree with us in the criterion used to decide whether a result is positive or negative. We apply a 'modified 2-fold' rule and Moriya *et al.* (1983) considered a positive result when the number of revertants was greater than that of spontaneous

revertants +100. Thus, Moriya *et al.* (1983) established the mutagenic potency of trichlorfon as 0.0067 revertants/nmol. Although we did not consider trichlorfon as a mutagen to TA100 in this assay, we calculated the mutagenic potency in order to compare our data with those of Moriya *et al.* (1983). Thus, the mutagenic potency was established as 0.0098 revertants/nmol, in good agreement with the value obtained by Moriya *et al.* (1983). In both studies the maximum number of revertants was ~300 at a concentration of 5000 µg/plate.

The plate incorporation test with preincubation was more sensitive than the Ames test in detecting the mutagenic activity of trichlorfon with TA104 either in the absence or presence of S9 mix. The mutagenic potency was 0.048 revertants/nmol. In TA100, trichlorfon was negative according to the mutation index but the dose–response relationship observed was significant, indicating the existence of a possible mutagenic activity.

According to these results and those obtained by other authors (Wildemaue *et al.*, 1983), it appears that variants of the Ames test must be used in order to detect the mutagenicity of trichlorfon.

The use of the fluctuation test allowed us to detect the mutagenicity of trichlorfon with TA100 and showed that preincubation with S9 mix decreased the mutagenic activity of trichlorfon.

Clearly, liquid medium assays are the most sensitive tests to detect the mutagenicity of trichlorfon.

According to Carere *et al.* (1978a,b) the negative result obtained with *S.typhimurium* TA1535 using the standard plate protocol was changed to a positive result if the trichlorfon solution is stored for 12 h before application to the cells for 1 h at 12 mg/ml. Mutagenicity following storage was also noted by Hanna and Dyes (1975).

Table V. Fluctuation test with preincubation strain TA100: summary of results of trichlorfon in the absence or presence of S9 mix

Trichlorfon ($\mu\text{g/ml}$)	No. of experiments	Total wells	No. of turbid wells		χ^2	Significance	Q ^a
			control	treated			
Without S9 mix							
1.25	1	48	12	16	0.81	$P > 0.05$	NM
2.50	2	96	12	20	2.4	$P > 0.05$	NM
5	1	48	0	17	20.65	$P < 0.001$	M3
12.5	2	96	12	38	18.28	$P < 0.001$	M3
25	1	48	0	10	11.16	$P < 0.001$	M3
50	2	96	12	32	11.79	$P < 0.001$	M3
250	2	96	12	30	9.87	$P < 0.01$	M2
500	2	96	12	32	11.16	$P < 0.001$	M3
2500	2	96	12	42	23.19	$P < 0.001$	M3
5000	1	48	12	15	0.46	$P > 0.05$	NM
Sodium azide	2	96	12	33	12.8	$P < 0.001$	M3
With S9 mix							
1.25	1	48	9	14	1.43	$P > 0.05$	NM
2.50	2	96	18	22	0.50	$P > 0.05$	NM
5	1	48	9	17	3.37	$P > 0.05$	NM
12.5	1	48	9	8	0.07	$P > 0.05$	NM
25	1	48	9	13	0.94	$P > 0.05$	NM
50	2	96	18	23	0.77	$P > 0.05$	NM
250	2	96	18	39	11.00	$P > 0.01$	M3
500	2	96	18	28	2.86	$P > 0.05$	NM
2500	2	96	18	19	0.03	$P > 0.05$	NM
5000	1	48	9	10	0.06	$P > 0.05$	NM
2-Aminoanthracene	2	96	18	53	27.37	$P < 0.001$	M3

^aQ, quality; NM, non-mutagenic; M2, weakly mutagenic; M3, mutagenic.

In the fluctuation test trichlorfon was in a liquid medium for at least 72 h. This fact and the greater sensitivity of this assay compared with the Ames test allowed the detection of the mutagenic effect of trichlorfon at low concentrations. Our results agree with Carere's in that degradation products such as dichlorvos may be present and must be considered in all studies with trichlorfon.

The fact that the mutagenicity of trichlorfon or its degradation products does not require metabolic activation (Batzinger and Bueding, 1977; Kawachi *et al.*, 1980) is consistent with our results obtained in the fluctuation test with preincubation. According to this, trichlorfon behaves as an alkylating agent.

In conclusion, the results obtained using different assay methods with several strains of *S.typhimurium* showed that trichlorfon induced base-pair substitution mutations and that exogenous metabolic activation was not required. Furthermore, the use of different assay methods allowed us to obtain more information about the behaviour of trichlorfon.

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References

- Ames, B.N., McCann, J. and Yamasaki, N. (1975) Methods for detecting carcinogens and mutagens with the *Salmonella/mammalian-microsome* mutagenicity test. *Mutat. Res.*, **31**, 347–364.
- Barrueco, C. and de la Peña, E. (1988) Mutagenic evaluation of pesticides, captan, folpet, captafol, dichlofluanid and related compounds with the mutants TA102 and TA104 of *Salmonella typhimurium*. *Mutagenesis*, **3**, 33–38.
- Barrueco, C. and de la Peña, E. (1989) Métodos utilizados para interpretar los resultados del ensayo *Salmonella/microsoma*. *Rev. Toxicol.*, **4**, 121–135.
- Batzinger, R.P. and Bueding, E. (1977) Mutagenic activities in vitro and in vivo of five antischistosomal compounds. *J. Pharmacol. Exp. Ther.*, **200**, 1.
- Braun, R., Schöneich, L., Weissflog, L. and Dedek, W. (1982) Activity of organophosphorus insecticides in bacterial tests for mutagenicity and DNA repair-direct alkylation vs. metabolic activation and breakdown. I. Butonate, vinylbutonate, dichlorvos, dimethyl dichlorvos and dimethyl vinylbutonate. *Chem.-Biol. Interactions*, **39**, 339.
- Byeon, W.H., Hyun, H.H. and Lee, S.Y. (1976) Mutagenicity of pesticides in the *Salmonella/microsome* system. *Korean J. Microbiol.*, **14**, 128–134.
- Carere, A., Ortali, V.A., Cardamone, G., Torracca, A.M. and Raschetti, R. (1978a) Microbiological mutagenicity studies of pesticides *in vitro*. *Mutat. Res.*, **57**, 277–286.
- Carere, A., Ortali, V.A., Cardamone, G. and Morpurgo, G. (1978b) Mutagenicity of dichlorvos and other structurally related pesticides in *Salmonella* and streptomycetes. *Chem.-Biol. Interactions*, **22**, 297.
- Green, M.H.L., Muriel, W.J. and Bridges, B.A. (1976) Use of a simplified fluctuation test to detect low levels of mutagens. *Mutat. Res.*, **38**, 33–42.
- Green, M.H.L., Bridges, B.A., Rogers, A.M., Horspool, G., Muriel, W.J., Bridges, J.W. and Fry, J.R. (1977) Mutagen screening by a simplified bacterial fluctuation test: use of microsomal preparations and whole liver cells for metabolic activation. *Mutat. Res.*, **48**, 287–294.
- Hanna, P.J. and Dyes, K.F. (1975) Mutagenicity of organophosphorus compounds in bacteria and *Drosophila*. *Mutat. Res.*, **28**, 405.
- Herrera, A. (1989) Estudio genotóxico de piretroides utilizando mutantes de *Salmonella typhimurium*. Tesis Doctoral. Ediciones de la Universidad Autónoma de Madrid.
- Herrera, A. and Laborda, E. (1988) Mutagenic activity of synthetic pyrethroids in *Salmonella typhimurium*. *Mutagenesis*, **3**, 509–514.
- IARC (1983) *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Miscellaneous Pesticides*. IARC, Lyon, Vol. 30, pp. 206–207.
- Kawachi, T., Yahagi, T., Kada, T., Tazima, Y., Ishidate, M., Sasaki, M. and Sugiyama, T. (1980) Cooperative programme on short-term assays for carcinogenicity in Japan. In Montesano, R., Bartsch, H. and Tomatis, L. (eds), *Molecular and Cellular Aspects of Carcinogen Screening Tests*. IARC Scientific Publication no. 27. IARC, Lyon, pp. 323–330.
- Levin, D.E. (1982) A new *Salmonella* tester strain (TA102) with A-T base pairs at the site of mutation detects oxidative mutagens. *Proc. Natl. Acad. Sci. USA*, **79**, 7445–7449.
- Levin, D.E., Blunt, E.L. and Levin, R.E. (1981) Modified fluctuation test for the direct detection of mutagens in food with *Salmonella typhimurium* TA98. *Mutat. Res.*, **85**, 309–321.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275.
- Maron, D.M. and Ames, B.N. (1983) Revised methods for the *Salmonella* mutagenicity test. *Mutat. Res.*, **113**, 173–215.
- Masters, B.S.S. (1967) The preparation and properties of microsomal NADPH-cyt-c-reductase from pig liver. *Methods Enzymol.*, **10**, 565–573.
- Matshushima, T., Sawamura, M., Hara, K. and Sugimura, T. (1976) A safe substitute for polychlorinated biphenyls as an inducer of metabolic activation system. In de Serres, F.J., Fouts, J.R., Bend, J.R. and Philpot, R.M. (eds), *In Vitro Metabolic Activation in Mutagenesis Testing*. Elsevier/North Holland Biomedical Press, Amsterdam, pp. 85–88.
- Moriya, M., Ohta, T., Watanabe, K., Miyazawa, T., Kato, K. and Shirasu, Y. (1983) Further mutagenicity studies on pesticides in bacterial reversion assay systems. *Mutat. Res.*, **116**, 185–216.
- Panda, B.B. and Sharma, C.B.S.R. (1980) Cytogenetic hazards from agricultural chemicals. 3. Monitoring the cytogenetic activity of trichlorfon and dichlorvos in *Hordeum vulgare*. *Mutat. Res.*, **78**, 341.
- Poole, D.C., Simmon, V.F. and Newell, G.W. (1977) *In vitro* mutagenic activity of fourteen pesticides. *Toxicol. Appl. Pharmacol.*, **41**, 196.
- Shirasu, Y., Moriya, M., Kato, K.K., Lienard, A.F., Tezuka, H. and Teramoto, S. (1977) Mutagenicity screening on pesticides and modification products: a basis of carcinogenicity evaluation. *Cold Spring Harbor Corp. Cell Proliferation*, **4**, 267.
- Shirasu, Y., Moriya, H., Tezuka, H., Teramoto, S., Ohta, T. and Inoue, T. (1982) Mutagenicity screening studies on pesticides. In Sugimura, T., Kondo, S. and Takebe, H. (eds), *Environmental Mutagens and Carcinogens*. University of Tokyo Press, Tokyo, pp. 331–335.
- Simmon, V.F. (1979) *In vitro* microbiological mutagenicity and unscheduled DNA synthesis studies of eighteen pesticides. US Environmental Protection Agency, Research Triangle Park, NC 27711. No. EPA-600/1-79-041.
- Waters, M.D., Sandhu, S.S., Simmon, V.F., Mortelmans, K.E., Mitchell, A.D., Jorgenson, T.A., Jones, D.C.L., Valencia, R. and Garrett, N.E. (1982) Study of pesticide genotoxicity. In Fleck, A. and Hollaender, A. (eds) *Genetic Toxicology: An agricultural perspective*. Plenum Press, New York, pp. 275–326.
- Wildemaue, C., Lontie, J.F., Schooes, L. and Larebeke, N. (1983) The mutagenicity in procaryotes of insecticides, acaricides and nematocides. *Res. Rev.*, **89**, 128–177.
- Worthing, C.R. (ed.) (1979) *The Pesticide Manual. A World Compendium*, 6th edn. British Crop Protection Council, Croydon, UK, p. 531.
- Yahagi, T., Degawa, M., Seino, Y., Matsushima, T., Nagao, M., Sugimura, T. and Hashimoto, Y. (1975) Mutagenicity of carcinogenic azo dyes and their derivatives. *Cancer Lett.*, **1**, 91–96.

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