

Mutagenicity of rubber additives and curing fumes

Results from five short-term bioassays

by Maria Donner, PhD,¹ Kirsti Husgafvel-Pursiainen, MSc,¹ Dag Jenssen, PhD,² Agneta Rannug, MSc²

DONNER M, HUSGAFVEL-PURSIAINEN K, JENSSEN D, RANNUG A. Mutagenicity of rubber additives and curing fumes: Results from five short-term bioassays. *Scand work j environ health* 9 (1983): suppl 2, 27-37. The mutagenicity of rubber additives and curing fumes from some rubber formulations were studied in five short-term tests: point mutations in Chinese hamster V79 cells, the induction of sister chromatid exchanges in Chinese hamster ovary cells, micronuclei in erythrocytes of Chinese hamster bone marrow, sex-linked recessive lethal mutations in *Drosophila melanogaster*, and reverse mutations in the *Salmonella*/microsome assay. When 46 rubber additives were tested with *Salmonella*, 15 compounds were mutagenic, 16 were negative, and 15 were negative with no observed toxicity. Only one negative response was obtained for the nine tested samples of curing fumes. Thiuram and dithiocarbamate additives and some curing fumes with mutagenic responses in *Salmonella* were the primary objects of study in the other four test systems. Although the results of these four tests did not completely parallel the *Salmonella* results, they indicate that the individual compounds and the curing fumes from different types of rubber formulations may involve genotoxic risks.

Key terms: chloroprene, dithiocarbamates, *Drosophila*, genotoxicity, micronuclei, point mutations, *Salmonella*, sister chromatid exchange, thiurams.

It has been known for some time that mutagenic and potentially carcinogenic chemicals are used in the rubber industry. A review by the International Agency for Research on Cancer (10) reported that there is sufficient evidence to associate some job categories with an increased risk of cancer.

The processing of rubber involves different combinations of several hundred additives. Although the toxicology of these chemicals is still poorly known, studies on carcinogenicity in animals have shown that both monomers (contained as residues in the polymers) and additives constitute a carcinogenic hazard. Possibly the action of some carcinogens can also be enhanced

by simultaneous exposure to agents that potentiate their action (23).

The primary aim of the Swedish-Finnish collaborative investigation on genotoxic health risks in the work environment of the rubber industry was to identify categories of jobs in which exposure to mutagenic and potentially carcinogenic compounds occur. Short-term bioassays for mutagenicity in vitro and in vivo were applied so that mutagens could be detected from the extensive number of compounds used as additives in the composition of rubber. The compounds were selected for testing on the basis of the toxicologic data collected by Holmberg & Sjöström in 1977 (8) on chemicals used in the Swedish rubber industry. Mutagenicity tests were also applied to curing fumes produced in laboratory model systems.

The *Salmonella*/microsome mutagenicity test was carried out with 46 compounds from the following four main classes of additives: curing agents, accelerators, antioxidants, and retarders. The

¹ Institute of Occupational Health, Helsinki, Finland.

² Division of Toxicological Genetics, Wallenberg Laboratory, University of Stockholm, Stockholm, Sweden.

Reprint requests to: Ms A Rannug, Wallenberg Laboratory, University of Stockholm, S-106 91 Stockholm, Sweden.

Table 1. Frequencies of sex-linked recessive lethals per X chromosome tested in *Drosophila* exposed to tetramethylthiuram monosulfide (TMTM), zinc diethyldithiocarbamate, or zinc dimethyldithiocarbamate (ziram) dissolved in ethanol + sucrose.

Compound	Days after treatment		
	0-3	3-6	6-9
Ethanol + sucrose ^a	4/2,370 (0.17 %)	1/2,253 (0.04 %)	2/2,534 (0.08 %)
TMTM			
0.04 mg/ml	0/ 646	1/ 603	0/ 707
0.4 mg/ml	0/ 690	2/ 495	0/ 489
4.0 mg/ml	1/ 484 ^b	1/ 393 ^c	—
	1/1,820 (0.05 %)	4/1,491 (0.27 %)	0/1,196
Ziram			
0.05 mg/ml	0/ 653	1/ 541	0/ 567
0.25 mg/ml	3/ 540	5/ 620	0/ 455
0.5 mg/ml	5/ 938*	6/1,066	1/ 655
	8/2,131 (0.38 %)	12/2,227** (0.54 %)	1/1,687 (0.06 %)
Zinc diethyl- dithiocarbamate			
0.05 mg/ml	2/1,023	0/ 772	—
0.25 mg/ml	1/ 729	0/ 776	—
0.5 mg/ml	0/ 689	0/ 791	—
	3/2,441 (0.12 %)	0/2,279	—

^a Solvent control A.

^b A cluster of three lethals excluded.

^c A cluster of ten lethals excluded.

* 0.01 < p ≤ 0.05, ** 0.001 < p ≤ 0.01, Fisher's exact probability test.

Table 2. Frequencies of sex-linked recessive lethals per X chromosome tested in *Drosophila* exposed to tetramethylthiuram disulfide (TMTD) or tetraethylthiuram disulfide (TETD) dissolved in ethanol + sucrose.

Compound	Days after treatment	
	0-6	6-9
Ethanol + sucrose ^a	4/2,854 (0.14 %)	2/2,376 (0.08 %)
TMTD		
1 mg/ml	9/ 834	1/ 585
2 mg/ml	2/ 496	1/ 498
3 mg/ml	3/ 615	0/ 507
4 mg/ml	11/ 571	0/ 693
5 mg/ml	1/ 441	1/ 526
	26/2,957*** (0.88 %)	3/2,809 (0.11 %)
TETD		
3.7 mg/ml	1/ 431	1/ 420
5.0 mg/ml	2/ 563	0/ 449
6.2 mg/ml	0/ 424	0/ 337
8.6 mg/ml	2/ 810	0/ 544
12.3 mg/ml	1/ 612	1/ 683
	6/2,840 (0.21 %)	1/2,733 (0.04 %)

^a Solvent control B.

*** p ≤ 0.001, Fisher's exact probability test.

results of these tests have been presented in detail elsewhere (6, 23). In addition many different curing fumes from rubber materials that represented the major types of rubber formulation were tested in the *Salmonella* test (4, 5, 24).

On the basis of results of the *Salmonella* tests, other screening tests were used to

study both additives and curing fumes. Specific attention was paid to thiuram and dithiocarbamate additives because of the positive results obtained in the *Salmonella* tests and because these chemicals are widely used. The test systems used were (i) the sex-linked recessive lethal test with *Drosophila melanogaster*, (ii) the induction of sister chromatid exchanges in Chinese hamster ovary cells, (iii) the induction of point mutations in Chinese hamster V79 cells, and (iv) the induction of micronuclei in the bone marrow erythrocytes of Chinese hamsters.

This paper provides the detailed results from these four test systems (tables 1-9) and a qualitative comparison of the data from all five test systems, including the results of the *Salmonella* tests (tables 10 & 11).

Materials

The additives and rubber mixtures tested in this study (see tables 10 and 11 for their names and abbreviations) were provided by Swedish and Finnish rubber factories. All the additives were of technical purity. The rubber mixtures consisted primarily

Table 3. Frequencies of sex-linked recessive lethals per X chromosome tested in *Drosophila* exposed to condensed vulcanization fumes from chloroprene rubber (CR), ethylene propylene rubber (EPDM), styrene butadiene rubber (SBR), or chlorosulfonated polyethylene rubber (CSM). The fumes were dissolved in ethanol + sucrose.

Rubber sample	Days after treatment		
	0-3	3-6	6-9
Ethanol + sucrose ^a	4/2,370 (0.17 %)	1/2,253 (0.04 %)	2/2,534 (0.08 %)
CR			
0.1 %	5/ 794 (0.63)	1/ 280 (0.36)	1/ 415 (0.24)
1.0 %	3/ 625 (0.48)	0/ 356	3/ 591 (0.51)
	8/1,419* (0.56 %)	1/ 636 (0.16 %)	4/1,006 (0.40 %)
EPDM			
1.0 %	2/2,412 (0.08)	1/2,253 (0.04)	0/1,538
10.0 %	0/2,214	0/2,144	0/1,978
	2/2,626 (0.08 %)	1/4,379 (0.02 %)	0/3,516
SBR			
1.0 %	2/2,137 (0.09)	1/2,026 (0.05)	0/1,921
10.0 %	1/2,025 (0.05)	2/2,133 (0.09)	1/1,998 (0.05)
	3/4,162 (0.07 %)	3/4,159 (0.07 %)	1/3,919 (0.03 %)
CSM			
1.0 %	1/2,048 (0.05)	0/2,131	0/1,733
10.0 %	0/1,793	2/1,805 ^b (0.11)	1/1,885 (0.05)
	1/3,841 (0.03 %)	2/3,936 (0.05 %)	1/3,618 (0.03 %)

^a Solvent control A.

^b A cluster of three lethals excluded.

* 0.01 < p ≤ 0.05, Fisher's exact probability test.

(approximately 90 %) of polymers, carbon black, and mineral oils. Curing agents, accelerators, activators, antidetergents, and retarders were present in the mixtures as additives.

Fifty grams of rubber material was heated in a Pyrex reaction flask to temperatures that corresponded to industrial curing operations (150–220° C). The vapors formed by this thermal treatment were collected on a water-cooled condenser that reached down into the flask. The condenser was removed every 5 min and washed in a tube containing 10 ml of acetone. A total sampling period of 30 min was used for each condensate.

Methods

Sex-linked recessive lethals in Drosophila

The sex-linked recessive lethal test was carried out according to the standard pro-

cedure (30). Males, 2–3 d old, of the “white” strain were starved for 3–5 h and then exposed for 24 h by the adult feeding method. The compounds were suspended in 0.5 ml of 94 % ethanol, and 4.5 ml of 1 % sucrose was added (controls A and B: ethanol + sucrose). The solution was sucked into Kleenex[®] tissue paper. The virgin females of the *y Basc mal* strain used in the P matings were ≤ 2 d old when mated. Only complete lethals (vials with no P-type males) were scored as sex-linked recessive lethals in the F₂ generation.

The concentrations tested were chosen on the basis of approximate lethal concentration values (data not shown in the present communication). None of the compounds yielded concentration-dependent responses, but all the compounds were lethal to some extent. This phenomenon was considered to mean that the compounds were taken up by the flies; the concentrations tested for mutagenicity were well below the approximate median lethal concentration values.

Table 4. Number of sister chromatid exchanges (SCE) in Chinese hamster ovary cells treated with tetramethylthiuram monosulfide (TMTM), tetramethylthiuram disulfide (TMTD), or tetraethylthiuram disulfide (TETD) dissolved in acetone. (S9 - = S9 not present, S9 + = S9 present)

Compound	SCE per cell		Number of cells analyzed
	Mean	SE	
Acetone ^a			
S9 -	9.06	0.19	296
S9 +	9.23	0.18	326
TMTM			
10 ⁻⁷ M			
S9 -	10.23	0.41**	60
S9 +	9.56	0.44	57
10 ⁻⁶ M			
S9 -	10.89	0.38***	90
S9 +	9.77	0.38	90
5 × 10 ⁻⁶ M			
S9 -	9.43	0.46	60
S9 +	9.80	0.41	60
2 × 10 ⁻⁵ M			
S9 -		toxic	-
S9 +		toxic	-
TMTD			
10 ⁻⁷ M			
S9 -	8.35	0.55	46
S9 +	9.08	0.38	60
10 ⁻⁶ M			
S9 -	9.60	0.42	84
S9 +	9.57	0.36	90
5 × 10 ⁻⁶ M			
S9 -		toxic	-
S9 +	9.37	0.36	60
10 ⁻⁵ M			
S9 -		toxic	-
S9 +	9.57	0.50	60
TETD			
10 ⁻⁶ M			
S9 -	9.33	0.36	78
S9 +	8.99	0.29	120
5 × 10 ⁻⁶ M			
S9 -		toxic	-
S9 +	10.12	0.39*	60
10 ⁻⁵ M			
S9 -		toxic	-
S9 +	11.62	0.49***	60
10 ⁻⁴ M			
S9 -		toxic	-
S9 +		toxic	-
CP ^b (10 ⁻⁵ M)			
S9 -	9.18	0.21	232
S9 +	24.62	0.46***	215

^a Solvent control.

^b Positive control, cyclophosphamide (CP).

* 0.01 < p ≤ 0.05, ** 0.001 ≤ p < 0.01, *** p ≤ 0.001, Student's t-test.

Table 5. Number of sister chromatid exchanges (SCE) in Chinese hamster ovary cells treated with the curing fumes of chloroprene rubber (CR). (S9 - = S9 not present, S9 + = S9 present).

Rubber sample	SCE per cell		Number of cells analyzed
	Mean	SE	
Acetone ^a			
S9 -	9.06	0.19	296
S9 +	9.23	0.18	326
CR			
0.001 %			
S9 -	10.43	0.60*	30
S9 +	10.65	0.43**	60
0.01 %			
S9 -	9.30	0.38	60
S9 +	9.68	0.45	60
0.1 %			
S9 -	11.28	0.50***	60
S9 +	10.70	0.53**	30
0.2 %			
S9 -	9.53	0.35	60
S9 +	11.32	0.53***	60
0.4 %			
S9 -	11.25	0.42***	60
S9 +	12.42	0.47***	60
0.8 %			
S9 -	14.35	0.46***	60
S9 +	14.95	0.57***	60
1.2 %			
S9 -		toxic	-
CP ^b (10 ⁻⁵ M)			
S9 -	9.18	0.21	232
S9 +	24.62	0.46***	215

^a Solvent control.

^b Positive control, cyclophosphamide (CP).

* 0.01 < p ≤ 0.05, ** 0.001 < p ≤ 0.01, *** p ≤ 0.001, Student's t-test.

and 10 % heat-inactivated fetal calf serum (Flow Laboratories). The incubation took place at 37° C in 5 % carbon dioxide in air. The medium for selection was prepared with 6-thioguanine (5 µg/ml) (Sigma), which was five times the concentration needed to kill cells with active hypoxanthine guanine phosphoribosyl transferase (HGPRT⁺) in the actual serum batch used. Vulcanization fume condensates were dissolved in Hanks' balanced salt solution (HBSS).

The S9 mix was prepared from male rats pretreated with Arochlor 1254. It contained 0.3 ml of S9, 1.5 µmol of nicotinamide adenine dinucleotide phosphate (NADP) and 15 µmol of glucose-6-phosphate (G6P) per milliliter of HBSS. The concentration of magnesium in the S9 mix was 0.8 mM (12, 13).

V79 cells seeded the day before were treated in HBSS with or without S9 mix. After treatment three plates per group were seeded for toxicity, and two Falcon

Point mutations in V79 cells

The screening for mutagenicity and toxicity was performed according to the optimal protocol described by van Zeeland & Simons (28). The cells were cultured in Eagle's Minimal Essential Medium supplemented with 1.8 mM L-glutamine, 90 units/ml of penicillin, 90 µg/ml of streptomycin,

vessels (75 cm²) were seeded with 2×10^5 cells for 7 d of expression. The cells were reseeded once during the expression time. After the expression six plates per group with 10^5 cells per plate were seeded for selection with 6-thioguanine, and three plates were seeded for cloning efficiency. Both the plates for survival and those for mutagenicity were fixed, coded, and scored for colonies 7 d after incubation. The mutation frequencies shown in the tables were corrected according to the cloning efficiency of each group.

Sister chromatid exchanges in Chinese hamster ovary cells

The induction of sister chromatid exchanges in Chinese hamster ovary cells was studied according to standard methodology (16). Each chemical dissolved in acetone was tested in at least four concentrations, three of which were in the sub-toxic range. All the treatments were performed both with and without a metabolic

activation system of rat liver microsomes (S9) from Clophen-induced rats. Cyclophosphamide (Lääke, Turku, Finland) was used as a positive control agent requiring metabolic activation. The period of treatment was 60 min. The cells were washed thoroughly after the treatment and grown in the presence of bromodeoxyuridine (Calbiochem, La Jolla, California) for 25 h. Mitotic cells were subsequently collected, fixed, and stained for the sister chromatid exchange analysis as described earlier (16, 17). The statistical analysis of the results was done with the Student's t-test.

Micronucleus test

The Chinese hamsters used in the study were males and females (weight 23–36 g) bred in the laboratory of the Institute of Occupational Health. This outbred colony originates from Shell Research Centre (Sittingbourne, Kent, United Kingdom). Each of the chemicals tested was dissolved in olive oil and administered by a single intraperitoneal injection at the doses

Table 6. Survival and frequency of 6-thioguanine-resistant mutants of Chinese hamster V79 cells treated with tetramethylthiuram monosulfide (TMTM), tetramethylthiuram disulfide (TMTD), or zinc dimethyldithiocarbamate (ziram). The cells were treated for 4 h without S9 mix or with different amounts of S9 in the mix.

Compound	Survival (%)				Number of 6TG ^r mutants per 10 ⁵ cells			
	0 % S9	2 % S9	10 % S9	20 % S9	0 % S9	2 % S9	10 % S9	20 % S9
TMTM								
0 µg/ml	100	100	100	100	0.4	0.9	0.2	0.7
0.2 µg/ml	98	..	..	..	0.2	..	..	..
0.3 µg/ml	70	..	..	..	0.4	..	..	..
0.4 µg/ml	19	104	81	..	0.3	0.4	1.5	..
0.8 µg/ml	..	123	94	97	..	0.7	0.8	0.5
1.6 µg/ml	..	56	..	119	..	0.8	..	0.7
3.2 µg/ml	..	..	..	104	..	..	..	0.8
TMTD								
0 µg/ml	100	100	100	100	0.7	1.2	0.9	1.2
0.2 µg/ml	13	112	..	..	0.2	2.0	..	..
0.25 µg/ml	3	..	95	..	0.2	..	..	..
0.5 µg/ml	2	..	92	..	0.2	..	0.7	..
0.8 µg/ml	..	31	..	109	..	0.7	..	0.2
1.6 µg/ml	..	..	..	116	..	..	..	0.8
Ziram								
0 µg/ml	100	100	..	100	0.7	0.2	..	0.2
0.01 µg/ml	94	..	..	..	0.2	..	..	..
0.02 µg/ml	14	121	..	114	0.5	0.7	..	0.2
0.04 µg/ml	..	97	..	109	..	0.8	..	0.4
0.08 µg/ml	..	..	..	95	..	..	..	0.5
EMS ^a (500 µg/ml)	93	..	..	..	11.8***	..	..	..
DMN ^a (740 µg/ml)	..	..	73	95	..	..	22.9***	20.0***

^a Positive controls, ethylmethanesulfonate (EMS) and dimethylnitrosamine (DMN).

*** $p \leq 0.001$, Student's t-test.

Table 7. Survival and frequency of 6-thioquanine-resistant Chinese hamster V79 cells treated with N-phenyl-N'-isopropyl-p-phenylenediamine (IPPD), N,N'-diphenyl-p-phenylenediamine (DPPD), dimethyldithiocarbamic acid (DTCA), N,N'-diphenylguanidine (DPG), or 2-mercaptobenzothiazole (MBT) for 4 h with (+) and without (−) S9 mix (10% S9).

Compound	Survival (%)		Number of 6TGr mutants/10 ⁵	
	− S9	+ S9	− S9	+ S9
IPPD				
0 µg/ml	100	100	0.4	0.9
0.8 µg/ml	91	125	..	..
4 µg/ml	44	89	..	..
5 µg/ml	28	..	0.4	..
10 µg/ml	24	..	0.4	..
20 µg/ml	23	72	..	0.2
40 µg/ml	..	19	..	0.3
DPPD				
15 µg/ml	58	..	0.8	..
20 µg/ml	..	84	..	0.4
30 µg/ml	58	..	3.1**	..
40 µg/ml	..	75	..	0.7
60 µg/ml	59	..	0.8	..
90 µg/ml	57	..	2.5*	..
DTCA				
0 µg/ml	100	..	0.8	..
0.05 µg/ml	47	..	0.5	..
0.10 µg/ml	25	..	0.3	..
0.15 µg/ml	12	..	0.3	..
0.30 µg/ml	4	..	0.7	..
DPG				
100 µg/ml	95	..	0.3	..
200 µg/ml	89	..	1.5	..
500 µg/ml	94	..	0.9	..
MBT				
0 µg/ml	100	..	1.4	..
50 µg/ml	107	..	0.1	..
100 µg/ml	88	..	2.5	..
300 µg/ml	97	..	1.4	..

* 0.01 < p ≤ 0.05, ** 0.001 < p ≤ 0.01, Student's t-test.

shown in table 9. Control animals were given olive oil alone (10 ml/kg body weight). Each group consisted of three to five animals, except the control group, which had ten animals.

The Chinese hamsters were decapitated 30 h after treatment. Slides for the micronucleus test were prepared from their bone marrow as described by Schmid (25) with minor modifications (20). The frequency of micronucleated cells was studied in 2,000 polychromatic erythrocytes and 2,000 normochromatic erythrocytes per animal whenever possible. The ratio of polychromatic to normochromatic erythrocytes was determined simultaneously with the analysis of micronucleated cells. The chi-square test was used for the statistical evaluation of the results.

Results

Sex-linked recessive lethals

Additives. The results from the recessive lethal tests with *Drosophila* are given in tables 1 and 2. Of the five accelerators tested, two, TMTD and ziram, were found positive. The mutagenicity was restricted to certain germ cell stages. TMTD and TETD were investigated with a rough brooding scheme. All postmeiotic germ cells (0–6 d) were examined as one brood, and the premeiotic (6–9 d) as another. In a smaller experiment (data not shown) TMTD induced recessive lethals both in sperm/spermatids (2/553, control 1/597) and in the spermatids/spermatocytes (4/425, control 0/538). For the other compounds a differentiation was made between the germ cell stages.

The mutagenic action of both TMTD and ziram may be of an indirect type, as the effect was manifested especially in the metabolically active spermatid/spermatocyte stages (3–6 d). No mutations were found in the premeiotic stages (6–9 d); this finding was possibly due to a delayed toxic effect that caused the death of the mutated cells.

Curing fumes. The results of the sex-linked recessive lethal tests on curing fumes are presented in table 3. Only the fumes derived from chloroprene rubber produced a statistically significant effect in these tests. The data indicate that the chloroprene rubber sample acted as a direct mutagen, as its effect was noted only in the late germ cell stages where either no or very limited metabolic activation takes place.

Sister chromatid exchanges in Chinese hamster ovary cells

Additives. TMTM, TMTD, and TETD were tested for the induction of sister chromatid exchanges in Chinese hamster ovary cells. The results are given in table 4. All of the thiurams were toxic, even in low concentrations, although the incorporation of a metabolic activation system decreased the toxicity of the compounds. TMTM and TETD gave weak positive responses, whereas TMTD was negative. TMTM was

Table 8. Survival and frequency of 6-thioquinane-resistant Chinese hamster V79 cells treated with condensed fumes from different vulcanization processes with chloroprene rubber (CR), styrene butadiene rubber (SBR), or ethylene butadiene rubber (EPDM). Different amounts of the condensates were dissolved in acetone and added to the treatment medium. Treatment was maintained for 4 h, and, in the case of CR rubber, different amounts of S9 were added to the medium.

Rubber sample	Survival (%)			Number of 6TG ^r mutants per 10 ⁵ cells		
	0 % S9	2 % S9	20 % S9	0 % S9	2 % S9	20 % S9
CR						
0 %	100	..	100	0.2	..	1.0
0.13 %	62	76	..	0.2	0.2	..
0.25 %	50	70	96	0.4	0.4	0.3
0.5 %	..	..	50	..	..	0.4
SBR						
0 %	100	..	..	0.4	..	..
0.05 %	78	..	..	0.8	..	..
0.13 %	97	..	..	0.3	..	..
0.25 %	84	..	..	0.9	..	..
0.5 %	32	..	..	0.7	..	..
EMS ^a (2mM)	87	..	..	16.0***	..	..
EPDM						
0	.. b	..	..	0.6	..	..
0.06 % ^c	.. b	..	..	0.7	..	..
0.13 % ^d	.. b	..	..	0.7	..	..
0.13 % ^c	.. b	..	..	0.3	..	..
0.25 % ^d	.. b	..	..	1.6	..	..
0.25 % ^c	.. b	..	..	0.6	..	..
0.50 % ^d	.. b	..	..	0.4	..	..
EMS ^a (2mM)	.. b	..	..	8.5***	..	..

^a Positive control, ethylmethanesulfonate (EMS).

^b Data lost.

^c Sample number II.

^d Sample number I.

*** $p < 0.001$, Students t-test.

Table 9. Frequency of micronucleated polychromatic and normochromatic erythrocytes and the ratio of polychromatic to normochromatic erythrocytes in the bone marrow of Chinese hamsters 30 h after treatment with tetramethylthiuram monosulfide (TMTM), tetramethylthiuram disulfide (TMTD), or tetraethylthiuram disulfide (TETD) dissolved in olive oil. Number of cells analyzed is given in parentheses.

Compound	Animals		Polychromatic cells with micronuclei			Normochromatic cells with micronuclei			Polychromatic : normochromatic	
	Male	Female	%	SE	(n)	%	SE	(n)	Ratio	SD
Olive oil ^a	4	6	0.16	0.03	(16,000)	0.13	0.08	(16,500)	0.69	0.30
TMTM (mg/kg body weight)										
100	2	3	0.16	0.04	(7,000)	0.13	0.07	(8,000)	0.40	0.10
250	2	0	0.39	0.11	(2,500)*	0.18	0.11	(4,000)	0.12	0.01
500	2	0	0.33	0.08	(3,500)	0.18	0.04	(4,000)	0.32	0.27
TMTD (μg/kg body weight)										
100	2	2	0.23	0.03	(7,000)	0.13	0.04	(7,500)	0.44	0.14
200	1	2	0.20	0.03	(5,000)	0.13	0.10	(6,000)	0.37	0.20
500	1	2	0.26	0.03	(4,500)	0.20	0.10	(5,000)	0.33	0.10
TETD (μg/kg body weight)										
100	2	3	0.13	0.04	(8,500)	0.11	0.07	(9,500)	0.55	0.14
250	2	3	0.25	0.06	(9,000)	0.16	0.07	(10,000)	0.48	0.14
500	1	2	0.16	0.05	(5,500)	0.08	0.03	(6,000)	0.70	0.24

^a Solvent control.

* $0.01 < p \leq 0.05$, chi-square-test.

found to be active without metabolic activation and TETD with metabolic activation only. However, as none of the compounds was able to double the frequency of sister chromatid exchanges in the Chinese hamster ovary cells, the com-

pounds cannot be considered potent inducers of sister chromatid exchanges.

Curing fumes. The results of the sister chromatid exchange analysis of Chinese

Table 10. Comparison of the results of different mutagenicity tests on additives.

	Test system ^a				
	<i>Salmo-</i> <i>nella</i>	V79	Chinese hamster ovary cells	<i>Droso-</i> <i>phila</i>	Micro- nucleus
Curing agents					
Dicumylperoxide	(-)	..	..	..	
2,5-Di-methyl-2,5-di(t-butylperoxi) hexane	(-)	..	..	..	..
Dipentamethylenethiuram tetrasulfide (PTT)	-	..	..	..	..
Dipentamethylenethiuram hexasulfide (PTH)	-	..	..	..	..
4,4-Dithiodimorpholine	+	..	..	..	..
Accelerators					
N,N'-diphenylguanidine (DPG)	-	-	..	..	..
1,3-Diphenylthiocarbamide	-	..	..	..	..
0-Totylbiquanide	(-)	..	..	..	..
N,N'-di-o-totylguanidine (DoTG)	(-)	..	..	..	..
1,3-Diethyl-2-thiocarbamide (DETU)	(-)	..	..	..	..
Zinc-2-mercaptobenzothiazole (Zn-MBT)	(-)	..	..	..	..
2-Mercaptobenzothiazole (MBT)	(-)	-	..	..	..
2,2-Dibenzothiazyl disulfide (MBTS)	(-)	..	..	..	..
Tetramethylthiocarbamide	(-)	..	..	..	..
Hexamethylenetetramine	(-)	..	..	..	..
N-cyclohexyl-2-benzothiazylsulfenamide (CBS)	(-)	..	..	..	..
Tetramethylthiuram disulfide (TMTD)	+	-	-	+	-
Tetraethylthiuram disulfide (TETD)	+	..	+	-	-
Tetramethylthiuram monosulfide (TMTM)	+	-	+	-	+
Zinc dimethyldithiocarbamate (ziram)	+	-	..	+	..
Copper dimethyldithiocarbamate	+	..	..	..	..
Zinc diethyldithiocarbamate	+	..	..	-	..
Cadmium diethyldithiocarbamate	+	..	..	..	..
Tellurium diethyldithiocarbamate	-	..	..	..	..
Zinc dibutyldithiocarbamate	-	..	..	..	..
Nickel dibutyldithiocarbamate	-	..	..	..	..
Zinc ethylphenyldithiocarbamate	+	..	..	..	..
Piperidine pentamethylenedithiocarbamate	-	..	..	..	..
N,N'-dimethyldiphenylthiuram disulfide	(-)	..	..	..	..
Dimethyldithiocarbamate Na + salt (DTCA)	+	-	..	..	..
Antioxidants					
6-Ethoxy-1,2-dihydro-2,2,4-trimethyl quinoline (EDT)	+	..	..	..	..
Polymer. 2,2,4-trimethyl-1,2-dihydro quinoline	(-)	..	..	..	..
N,N'-diphenyl-p-phenylenediamine (DPPD)	+	+	..	..	..
N-phenyl-N'-isopropyl-p-phenylenediamine (IPPD)	-	-	..	..	..
N-(1,3-dimethylbutyl)-N'-phenyl-PPD (6PPD)	-	..	..	..	..
N,N'-dicyclohexyl-PPD	+	..	..	..	..
N,N'-bis (1,4-dimethylpentyl)-PPD	-	..	..	..	..
2,6-Di-t-butyl-1-hydroxy-4-toluene (BHT)	(-)	..	..	..	..
4,4'-Diaminodiphenylmethane	+	..	..	..	..
2-Mercaptobenzimidazole	-	..	..	..	..
Aldol- α -naphthylamine	(-)	..	..	..	..
Fenyl- α -naphthylamine	-	..	..	..	..
Fenyl- β -naphthylamine	-	..	..	..	..
Retarders					
N-nitrosodiphenylamine	-	..	..	..	..
B-(cyclohexylthio)-phthalimide	-	..	..	..	..
N-methyl-N-4-dinitrosoaniline	+	..	..	..	..

^a + = statistically significant mutagenic response; - = statistically nonsignificant mutagenic response; (-) = statistically nonsignificant mutagenic response, toxic concentration not reached; .. = not tested.

hamster ovary cells treated with curing fumes from a chloroprene rubber sample is given in table 5. A dose-related increase in sister chromatid exchanges was noted both with and without metabolic activation. The increase was more pronounced when a metabolic activation system was used.

Point mutations in V79 cells

Additives. Six accelerators (TMTM, TMTD, ziram, DTCA, DPG & MBT) were tested with the V79/HGPRT⁺ system together with two antioxidants, DPPD and IPPD, and the results are shown in tables 6 and 7.

The accelerators of the thiuram or dithio-

Table 11. Comparison of the results of different mutagenicity tests on curing fumes.

Polymer	Test system ^a			
	<i>Salmo-nella</i>	V79	Chinese hamster ovary cells	<i>Droso-phila</i>
Natural rubber (NR)	—	..	..	..
Styrene butadiene rubber (SBR)	+	—	..	—
Butadiene acrylonitrile rubber (NBR)	+	..	..	..
Chloroprene rubber (CR)	+	—	+	+
Ethylene propylene rubber (EPDM)	+	..	..	—
Butyl rubber (IIR)	+	..	..	..
Chlorosulfonated polyethylene rubber (CSM)	+	..	..	—
Fluor rubber (FPM)	+	..	..	..
Epichlorohydrin rubber (CO)	+	..	..	..

^a + = statistically significant mutagenic response; — = statistically nonsignificant mutagenic response; (—) = statistically nonsignificant mutagenic response, toxic concentration not reached; .. = not tested.

carbamic type (TMTM, TMTD, ziram & DTCA) were all negative in this test system. Also negative were the other two accelerators (DPG & MBT). Because neither DPG nor MBT reduced survival even at high concentrations, it is possible that they did not pass into the cells.

IPPD was toxic but not mutagenic to the V79 cells. DPPD, an indirect mutagen in the *Salmonella* test, produced mutants in this test system without the addition of S9 mix. The addition of S9 mix abolished the mutagenic effects of DPPD.

Curing fumes. The results of the test of three curing fumes are given in table 8. Negative results were obtained with all three samples, both with and without metabolic activation.

Micronuclei

Additives. Table 9 shows the results of tests on the induction of micronucleated erythrocytes in bone marrow of Chinese hamsters by three thiurams (TMTM, TMTD & TETD). In a comparison with a control group of ten animals, a statistically significant increase in micronucleated polychromatic erythrocytes was detected only in hamsters treated with 250 mg of TMTM/kg of body weight. None of the treatments caused an increase in normochromatic cells with micronuclei. Almost any dose of the three thiurams decreased the ratio of polychromatic erythrocytes to normochromatic ones, an occurrence which indicates that the chemicals were cytotoxic. The decreased proportion of polychromatic erythrocytes probably re-

flected mitotic delay or death of the maturing cells due to the treatments.

Discussion

The results of the eucaryotic mutagenicity tests presented in this paper do not completely confirm the positive mutagenic responses observed with *Salmonella*. This differential sensitivity is to be expected, and it is mainly due to the particular physiological, genetic, and toxicokinetic features of the in vitro and in vivo short-term tests used. The enzymatic pathways involved in the activation/inactivation of indirect mutagens are highly important in determining the ultimate mutagenic action (2, 11). The positive results with an in vivo test system such as *Drosophila* deserve specific emphasis. *Drosophila* has a well-characterized xenobiotic metabolizing system (1), which particularly applies to the germ cells (29). Furthermore the specificity of *Drosophila* (ie, the lack of false positive results) is high (2).

Some other investigations have also shown that thiurams and dithiocarbamates damage deoxyribonucleic acid (DNA). Direct mutagenicity has been found in short-term test systems for mutagenicity in bacteria and yeast (6, 18, 31, 32), as well as in the silk worm (14). Both chromosome damage (14, 15, 21, 22, 26) and aneuploidi (27) have been reported.

These results are difficult to explain on the basis of binding to DNA, because none of these additives can, from a chemical point of view, be classified as alkylating, a fact confirmed by tests for the alkylating activity of thiuram and dithiocarbamate

derivatives performed with 4-(p-nitrobenzyl)-pyridine and deoxyguanosine as nucleophiles (7).

Two properties of the derivatives of dialkylthiuramic acid render them biologically very active. These are (i) their ability to form chelates with metals and (ii) their binding to SH groups and the subsequent formation of mixed disulfides (19). Many fundamentally important cellular functions (such as respiration, cell division, and protection against endogenous and exogenous oxidants) may be disturbed through enzyme inhibitions (3). A hypothesis explaining the mutagenic action of dialkylthiuramic acid derivatives via indirect function on enzymes involved in the protection against oxygen radicals has been presented recently by our group on the basis of experiments on *Salmonella* (23).

Except for the analysis of sister chromatid exchanges in Chinese hamster ovary cells, all the mutagenicity tests used in this study are recommended for the prediction of carcinogenicity (9). Our investigations of the genotoxic-associated risks with the work environment in the rubber industry indicate the possibility of genotoxic risks caused by individual additives, their reactive combinations, or fumes from complex rubber formulation.

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