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Genotoxicity testing of the herbicide Roundup and its active ingredient glyphosate isopropylamine using the mouse bone marrow micronucleus test, Salmonella mutagenicity test, and Allium anaphase-telophase test

J. Rank, A.-G. Jensen, B. Skov, L.H. Pedersen and K. Jensen

Department of Environment, Technology and Social Studies, Roskilde University, Denmark

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Summary

The genotoxic potential of the herbicide Roundup and its active agent, glyphosate isopropylamine salt, was studied in three different assays. No clastogenic effects were found in the mouse bone marrow micronuleus test for either of the two agents. In the Salmonella assay only Roundup was tested. It showed a weak mutagenic effect for the concentrations 360 μg/plate in TA98 (without S9) and 720 μg/plate in TA100 (with S9). These concentrations are close to the toxic level. The anaphase-telophase Allium test showed no effect for the glyphosate isopropylamine salt, but a significant increase in chromosome aberrations appeared after treatment with Roundup at concentrations of 1.44 and 2.88 mg/l when calculated as glyphosate isopropylamine. The most frequent aberrations observed could be characterized as disturbances of the spindle.

Roundup is a relatively new herbicide first marketed in the USA in 1974. The active agent in Roundup is glyphosate (*N*-phosphonomethyl glycine), which is considered almost non-toxic in mammals (LD_{50}, rat oral = 4.3 g/kg) (Atkinson, 1985). The herbicide Roundup is commonly used in agriculture, forestry and nurseries all over the world and is expected to be used even more in the future, when utility plants with resistance to glyphosate are introduced on the market. Spraying with Roundup on glyphosate-resistant crops will make it possible to eliminate all other unwanted plants on the field.

The genotoxicity of Roundup and glyphosate has been investigated in different assays (Seiler, 1977; Vigfusson and Vyse, 1980; Li and Long, 1988; Njagi and Gopolan, 1981; De Marco et al., 1992). The results from these studies are conflicting, and none of the investigators have tested both Roundup and glyphosate in the same assay.

In the present study we have examined the effect of the formulated commercial Roundup

Correspondence: Jette Rank, Department of Environment, Technology and Social Studies, Roskilde University, Box 260, DK-4000 Roskilde, Denmark.
Tel. +4546757711; Fax. +4546754403.

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and the active agent, glyphosate isopropylamine salt, in the mouse bone marrow micronucleus test, the Salmonella mutagenicity test using strains TA98 and TA100, and in the Allium anaphase-telophase test.

Materials and methods

Organisms

NMRI-Bom mice, weighing 30–35 g and 11 weeks of age, were obtained from Bom-mice (Denmark). *Allium cepa* onion bulbs, 15–22 cm in diameter, without any treatment for growth restriction, were obtained from Dähnfeldt, Denmark. The Salmonella tester strains TA98 and TA100, originally obtained from B.N. Ames, University of California, CA, USA, were kindly supplied by the Institute of Toxicology, the National Food Agency, Denmark.

Chemicals

S9 mix induced with Aroclor was obtained from Bio-test Laboratories (Denmark); D-biotin from Merck (Germany); L-histidine from BDH Chemicals (UK); Oxoid nutrient broth from Oxoid Ltd. (UK); bacto agar from Difco Co. (USA); glyphosate isopropylamine salt (CAS No. 38541-94-0) was a mixture of glyphosate and isopropylamine (1:1) obtained from Dr. S. Ehrenstorfer (Germany); and Roundup, containing 480 g glyphosate isopropylamine salt per liter, was from Monsanto (USA). Other chemicals were of analytical grade.

Mutagenicity assays

Mice bone marrow micronucleus assay. The micronucleus test followed the procedure described by Schmid (1975). Fetal calf serum was used to flush out the cells from the bone marrow, which after centrifugation were smeared and stained with May–Grünwald and Giemsa solutions.

The concentrations of glyphosate isopropylamine salt were 100, 150, and 200 mg/kg body weight and those of Roundup were 133 and 200 mg/kg body weight, calculated as glyphosate isopropylamine for the purpose of an easier comparison of the two agents. Methyl methanesulfonate (MMS), 50 mg/kg body weight, was used as positive control and 0.9% NaCl in a volume corresponding to 10 ml/kg body weight was used as negative control.

The intervals from intraperitoneal injection of the test chemicals to the harvest of blood cells from bone marrow were 24 and 48 h for glyphosate isopropylamine salt and 24 h for Roundup. Groups of 7–10 mice (half of them female and half male) were used per concentration. The counting procedure was done according to Hart and Engberg-Pedersen (1983). Per animal 1000 polychromatic erythrocytes (PCE) were examined to determine the frequency of micronucleated PCE (MNPCE). The first 200 cells counted included both normo- and polychromatic erythrocytes and were used for the calculation of PCE as percent of both classes of cells.

Salmonella plate incorporation assay. The test was performed according to Ames et al. (1975) and Maron and Ames (1983), using two of the recommended *Salmonella typhimurium* tester strains: TA98 and TA100. The mutagenicity of Roundup was tested with and without Aroclor induced S9 mix with three plates per dose in two experiments. Positive controls were 10 μg 4-nitro-*o*-phenylenediamine (NPD), 0.5 μg MMS, 1.0 μg benzo[*a*]pyrene (B(a)P), 0.1 μg 2-nitrofluorene, and 0.4 μg sodium azide per plate.

Allium anaphase-telophase assay. The test was performed according to Fiskesjö (1985) and the modifications proposed by Rank and Nielsen (1993).

In one experiment the Allium root cells were exposed for 24 h to 720, 1440, and 2880 μg glyphosate isopropylamine salt per liter, and in another experiment the onion cells were exposed to three concentrations of Roundup, corresponding to the concentrations of glyphosate isopropylamine used in the first experiment. Tap water was used as control and five onions were exposed per concentration. Root tips were prepared for the microscopic examination by squashing and staining in 2% orcein (Fiskesjö, 1985).

Mitotic index was calculated from the observation of mitoses in 400 cells per onion. The chro-

MONGLY00409563

TABLE 1

MICRONUCLEUS TEST WITH GLYPHOSATE ISOPROPYLAMINE SALT AND ROUNDUP

Agent	Interval (h)	Dose (mg/kg)	Mice n (♂, ♀)	% MNPCE (mean ± SD)	% PCE (mean ± SD)
NaCl (0.9%)	24		10 (5, 5)	0.27 ± 0.11	53 ± 11
MMS	24	50	9 (4, 5)	2.53 ± 0.59	47 ± 12
Glyphosate isopropylamine	24	100	10 (5, 5)	0.20 ± 0.13	50 ± 10
		150	10 (5, 5)	0.21 ± 0.13	56 ± 10
		200	10 (5, 5)	0.25 ± 0.10	47 ± 14
	48	150	9 (5, 4)	0.13 ± 0.09	51 ± 11
		200	10 (5, 5)	0.12 ± 0.09	51 ± 6
Roundup	24	133 [a]	8 (4, 4)	0.28 ± 0.13	41 ± 16
		200 [a]	7 (3, 4)	0.24 ± 0.17	36 ± 9 *

[a] Calculated as glyphosate isopropylamine salt.
* $P < 0.01$ in Student's t-test.

mosome aberrations were scored in 100 normal anaphase and early telophase cells per onion (Rank and Nielsen, 1993). The aberrations scored were: bridges, fragments, vagrants (e.g., lagging whole chromosomes), and other aberrations (e.g., c-mitotic, multipolar, or polyploid anaphase).

Toxicity test

The general toxicity of both agents was tested using *Allium cepa* onion bulbs. The onions were allowed to produce roots in test solutions of different concentrations, six onions per concentration, and tap water as control. Every day the

TABLE 2

SALMONELLA MUTAGENICITY TEST WITH ROUNDUP

Agent	Concentration (μg/plate)	Revertants/plate (mean ± SD)			
		TA98		TA100	
		−S9	+S9	−S9	+S9
Control	–	24 ± 7	26 ± 3	97 ± 1 [c]	109 ± 10
Roundup [a]	360	99 ± 25 [c]*	24 ± 1	97 ± 7	28 ± 19
	720	4 ± 4	16 ± 6	33 ± 23	350 ± 114 *
	1081	2 ± 4	11 ± 3	30 ± 7	104 ± 9
	1440	0	39 ± 30 [c]	38 ± 10	87 ± 21
Nitrofluorene	0.1	38 ± 1	–	–	–
Sodium azide	0.4	–	–	212 ± 6 [c]	–
Benzo[*a*]pyrene	1.0	–	113 ± 6	–	441 [b]
Control	–	19 ± 1 [d]	25 ± 9	95 ± 4	111 ± 16
Roundup [a]	180	16 ± 1 [d]	24 ± 1	120 ± 29 [d]	53 ± 3
	360	34 ± 8 [d]*	22 ± 4	107 ± 23 [d]	151 ± 3
NPD	10	292 ± 25 [d]		474 ± 25 [d]	
MMS	0.1	24 ± 7 [d]		1117 ± 14	
Benzo[*a*]pyrene	1.0	16 ± 1 [d]	105 [b]	111 ± 10 [d]	230 [b]

[a] Calculated as glyphosate isopropylamine salt.
[b] 1 plate, [c] 2 plates, [d] 4 plates.
* $P < 0.05$ in Student's t-test.

MONGLY00409564

onions were given fresh test solutions. On day 2 the onion with the poorest growth for each concentration was removed and on day 5 the experiment was terminated by measuring the length of the root bundles as described by Fiskesjö (1985). For each concentration the average root length was calculated as percent of the control. The growth inhibition values, EC_{50}, were interpolated from a plot of root length % against the log concentrations. The plots were made by the SYGRAPH computer program using the DWLS smoothing with a tension of 0.1.

Statistics

Statistical analyses were performed using the χ^2-test for the incidence of micronuclei and chromosome aberrations and Student's *t*-test for the other results.

Results

The results from the micronucleus test are shown in Table 1. As no significant difference between male and female mice was found, we have combined the results from the two sexes. The incidence of MNPCEs was not increased by glyphosate isopropylamine salt or Roundup. At the highest concentration of Roundup, 200 mg/kg body weight of glyphosate isopropylamine, a statistically significant decrease in the number of PCEs was found. This indicates that the formulated glyphosate product, Roundup, is more toxic than the glyphosate isopropylamine salt itself.

In Table 2 the results from the Salmonella mutagenicity test are shown. A slight but significant number of revertants was observed at concentrations of 360 μg/plate for TA98 (without S9) and 720 μg/plate for TA100 (with S9). The results indicate that Roundup, at concentrations close to the level of toxicity, can induce point mutations.

In Table 3 the results from the Allium test are shown. At the two highest concentrations of Roundup there was a statistically significant increase in chromosome aberrations, whereas no increase in aberrations was found for the glyphosate isopropylamine salt exposed cells. When the four classes of aberrations induced by Roundup are compared to those induced by the positive control, MMS, it can be seen that the two patterns are different. MMS as a well known clastogen agent induces bridges and fragments, as

TABLE 3

GLYPHOSATE AND ROUNDUP IN THE ALLIUM TEST

Agent	Concentration (μg/l)	Mitotic index	Chromosome aberrations per 500 cells					
			bridges	fragments	vagrants	other aberr.	total	% CA ± SD
Control		21.8 ± 4.3	7	4	3	1	15	3.0 ± 1.8
Glyphosate	720	21.8 ± 4.1	9	4	1	5	19	3.8 ± 1.8
isopropylamine	1440	24.6 ± 5.5	12	6	5	0	23	4.6 ± 1.5
	2880	25.2 ± 5.2	7	3	3	0	13	2.6 ± 2.1
MMS	10	23.4 ± 3.4	23	14	8	0	45	9.0 ± 2.6
Control		32.0 ± 3.6	10	1	2	1	14	2.8 ± 0.4
Roundup [a]	720	26.2 ± 5.4	6	1	3	15	25	5.0 ± 3.3
	1440	28.2 ± 6.6	13	8	7	31	59	11.8 ± 6.6 **
	2880	24.2 ± 6.7	5	12	1	10	28	5.6 ± 2.8 *
MMS	10	26.6 ± 3.9	28	17	10	3	58	11.6 ± 2.7

For each concentration five onions were exposed and 100 anaphase/early telophase cells were analyzed per onion.

[a] Calculated as glyphosate isopropylamine.

* $P < 0.05$, ** $P < 0.005$ in χ^2 test.

MONGLY00409565

opposed to Roundup, for which most aberrations are in the category 'other aberrations'. Most of these aberrations could be described as weak spindle disturbances, as shown in Fig. 1 (A,B,C).

A simple positive relationship between frequency and dose was not found, as a smaller

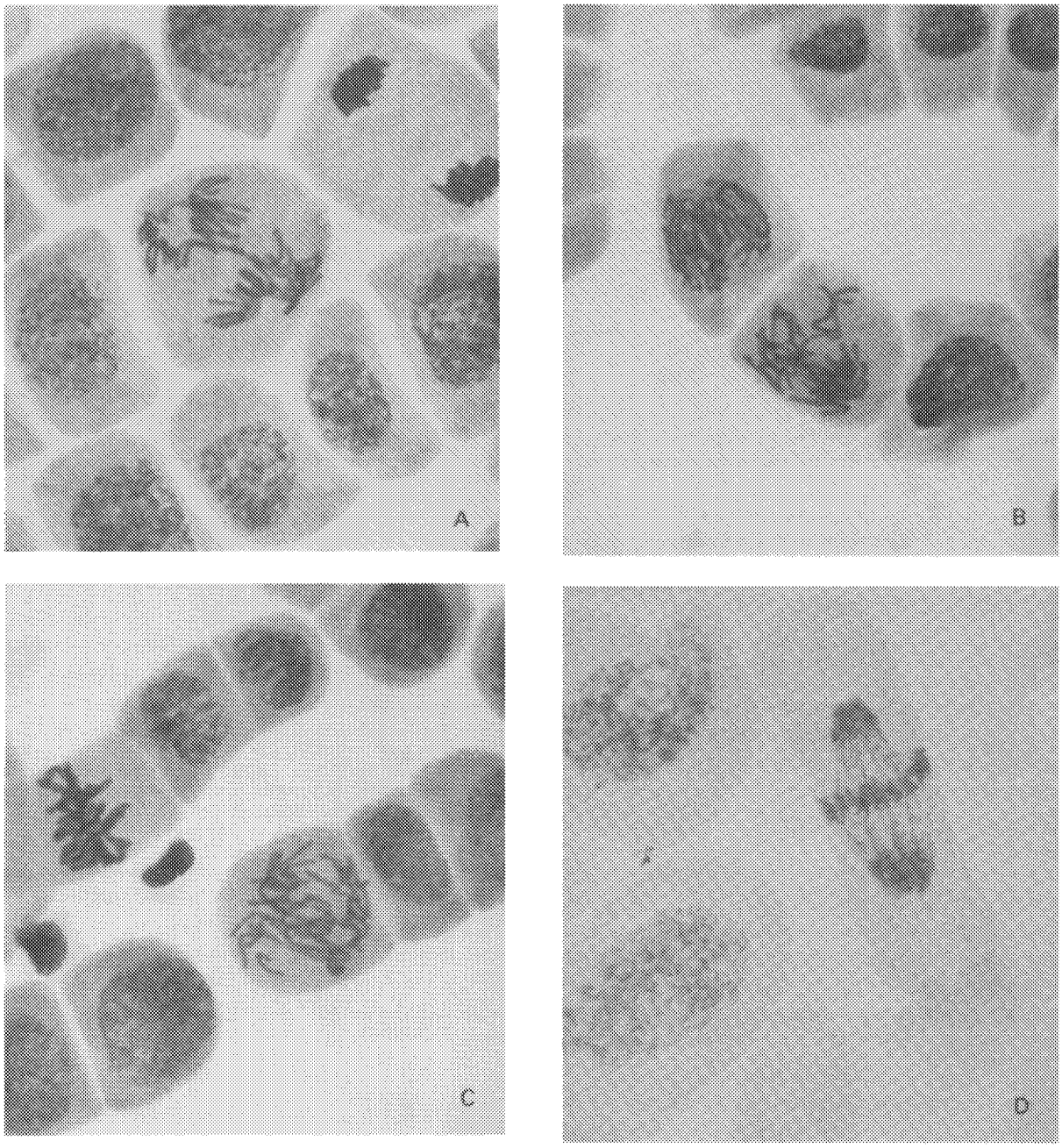

Fig. 1. *Allium cepa* root cells after 24 h treatment with Roundup. A, B, C: 1.44 mg glyphosate isopropylamine per liter, weak disturbances in the anaphase with disoriented chromosomes and tendency to diffuse polarity. D: 2.88 mg glyphosate isopropylamine per liter, a special disturbance with some of the chromosomes trapped in the middle of the spindle.

MONGLY00409566

number of aberrations were observed for the highest dose of Roundup (2880 μg/l) than for the next highest dose (1440 μg/l). However, this could be due to the toxic effect of Roundup, as there is a significant decrease in the mitotic index for the highest concentration of Roundup.

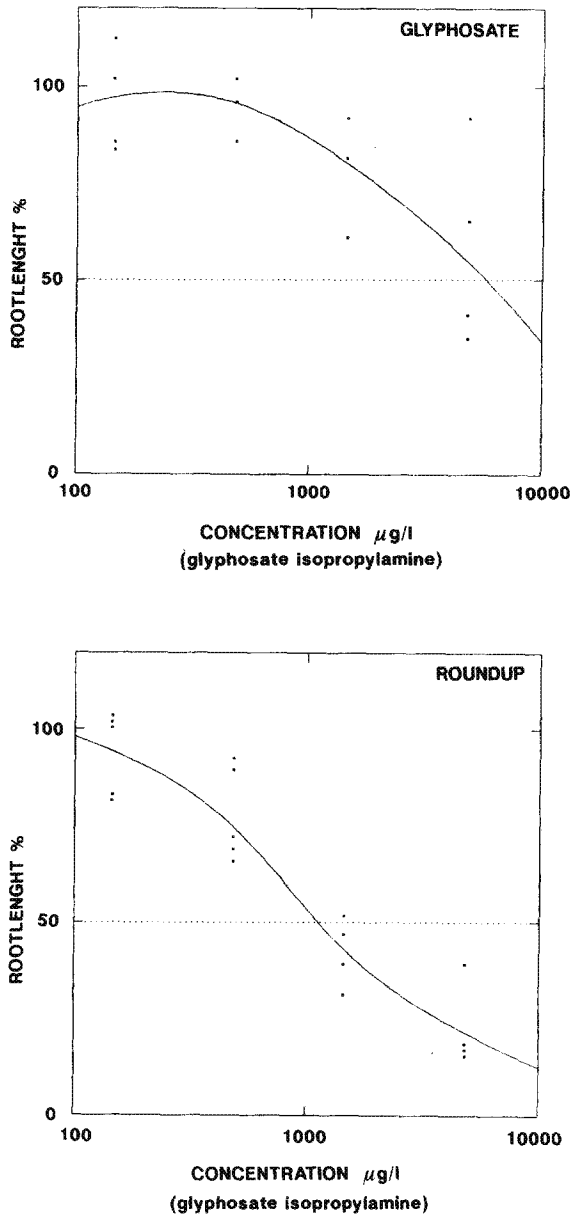

Fig. 2. Toxicity of glyphosate isopropylamine salt (top) and Roundup (bottom), calculated as glyphosate isopropylamine salt and measured as growth inhibition of the onion root bundles.

The toxicity of the two agents is shown in Fig. 2. The EC_{50} values are 5.5 mg/l for glyphosate isopropylamine salt and 1.2 mg/l for glyphosate isopropylamine in Roundup. The results show that the formulated product, Roundup, is about 5 times more toxic to the Allium root cells than the glyphosate isopropylamine salt.

Discussion

The concentrations used in the mouse bone marrow micronucleus assay were chosen at the highest possible level, close to the LD_{50} value for glyphosate (130 mg/kg, mouse, i.p.) reported by Olorunsogo and Bababunmi (1980). However, neither glyphosate isopropylamine salt nor Roundup showed clastogenicity in the test. This is in accordance with a study of De Marco et al. (1992), where no statistically significant increase in the incidence of micronucleus was found in *Vicia faba* root cells after treatment with glyphosate (21% in Solado trading formulation), and also in accordance with the micronucleus study done by Li and Long (1988), where glyphosate (*N*-phosphonomethyl glycine) was tested in the rat bone marrow cytogenicity assay. No statistically significant increases were found in either chromosomal aberrations or achromatic lesions observed after 6, 12 and 24 h exposure to a concentration of 1000 mg/kg body weight, i.p., which is a rather high concentration compared with the LD_{50} value (235 mg/kg, rat, i.p.) found by Olorunsogo and Bababunmi (1980).

Li and Long (1988) also tested glyphosate in the *Salmonella typhimurium* and *Escherichia coli* reversion assay, the *Bacillus subtilus* recombination assay, Chinese hamster ovary cell gene mutation assay and the rat primary hepatocyte/DNA repair assay; they did not observe any genotoxic activity in the assays performed. The strains used in the Salmonella assay were TA1535, TA100, TA1537, TA1538 and TA98, and toxicity was first seen at 5000 μg/plate. When we compare the toxicity level from this study with the results from our own study, it is clear that the toxicity of glyphosate in Roundup is much higher than for glyphosate alone. The toxicity of glyphosate iso-

MONGLY00409567

propylamine in Roundup showed up already at a concentration of 360 μg/plate, which equals 266 μg/plate of glyphosate.

In another study (Seiler, 1977) glyphosate was tested, together with other nitrogenous pesticides, in the Salmonella mutagenicity test after reaction with sodium nitrite in acidic solution. Glyphosate was the only agent out of six amides, for which an extract of the reaction mixture produced weak but significant mutagen activity in *Salmonella typhimurium* hisG46. As no *N*-nitroso derivatives were detected in the reaction extract the mutagenicity could be due to either glyphosate or some unknown reaction compounds.

A positive effect of Roundup in the SCE assay was reported by Vigfusson and Vyse (1980). Although the authors describe the effect as weak compared with the stronger response of other pesticides, Roundup at a concentration of 6.5×10^{-4} M showed an increase in the number of SCEs close to that induced by EMS at a concentration of 10^{-4} M. No dose related effect was shown for the three concentrations of Roundup, where the highest dose, 6.5×10^{-2} M, inhibited cell growth completely.

A genotoxicity test was also done in the *Vicia faba* assay (Njagi and Gopolan, 1981). No chromosome aberrations were found, but it was observed that Roundup (120 ppm) induced pycnotic nuclei and premature chromosome condensation in meristem root cells after 120 min exposure, indicating some effects on the chromosomes. Popov and Popov (1987) found that Roundup, at a concentration of 1%, suppressed the despiralization chromosomal process during the reconstruction of the interphase nucleus. They also found injuries of chromosomal migration during anaphase.

The present study shows that Roundup, which is a mixture of several agents, and among these 360 g glyphosate per liter, can induce weak mutations in *Salmonella typhimurium* TA98 and TA100, and can induce chromosome aberrations in *Allium cepa* meristem root cells at concentrations close to the level of toxicity. As no genotoxicity was found for glyphosate isopropylamine the effect must be due to some other ingredients in Roundup. Menkes et al. (1991) reported that the formulation of Roundup contains 15% polyoxyethylene surfactants, with an oral LD_{50} value in the range of 1000–4000 mg/kg, which could explain the higher toxicity of Roundup compared with glyphosate. To our knowledge there are no published genotoxcity data on these surfactants.

The conclusion of the present study is that we find it insufficient only to test the active agent in a formulated product. Because of the possibility of additive and synergistic effects it is just as important to investigate the complex mixture as well as the other ingredients of the product.

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MONGLY00409568

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MONGLY00409569