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CYANURIC CHLORIDE HAS NO GENOTOXIC AND MUTAGENIC PROPERTIES IN BACTERIA AND BONE MARROW CELLS

KALINA WYSZYŃSKA[1], BARBARA PRZYBOJEWSKA[1], EWA SPIECHOWICZ[1], WIESŁAWA CHWIAŁKOWSKA-LIRO[1], ELŻBIETA DZIUBAŁTOWSKA[1] and KONRAD RYDZYŃSKI[2]

[1]Department of Carcinogenicity, Teratogenicity and Mutagenicity
[2]Department of Toxicology Evaluation
The Nofer Institute of Occupational Medicine
Lodz, Poland

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Abstract. Three short-term tests were used; Salmonella/microsome assay, *in vivo* sister chromatid exchange assay (SCE) and micronucleus assay to evaluate mutagenic and genotoxic properties of 2,4,6-trichlorotriazine; cyanuric chloride. Mutagenicity assays were carried out using the standard top agar overplay plate assay decribed by Maron and Ames (9). Tester strains TA97a, TA98, TA100 and TA102 were used. Compound was dissolved in 0.1 ml of DMSO and doses of 1, 10, 100 and 500 μg/plate were tested in the absence and in the presence of the S9-mix. From the results obtained it appeared that incubation of the test substance with the bacteria did not increase the number of His+ revertants with any of the strains of *S. typhimurium*, either in the absence or in the presence of the S9-mix. At the high dose level used i.e. 100 and 500 μg/plate, the test substance appeared to be slightly toxic for strain TA 97a (in the absence of the S9-mix), as was seen from a diminished number of revertant colonies. The SCE test was performed according to the GENE-TOX programme. No significant increase was noted in the incidence of SCE in the groups treated with all tested doses of cyanuric chloride. Thus, in this test cyanuric chloride did not induce chromosomal damage resulting in SCE formation in bone marrow cells of mice. The micronucleus assay *in vivo* was performed on mice bone marrow cells. The incidence of micronucleated polychromatic erythrocytes after administration of all doses of cyanuric chloride used were not statistically different ($p > 0.05$) as compared to negative controls. However, the compound caused significant decrease of polychromatic erythrocytes to normochromatic erythrocytes ratio in the bone marrow at 30, 48 and 72 hours after administration of both tested doses of cyanuric chloride. Negative results in Ames test and lack of induction of sister chromatid exchange and micronuclei in bone marrow cells of mice indicate that cyanuric chloride is not an effective mutagenic and genotoxic agents.

Address reprint requests to Prof. K. Rydzyński, Department of Toxicity Evaluation, The Nofer Institute of Occupational Medicine, P.O.Box 199, 90-950 Lodz, Poland.

INTRODUCTION

Cyanuric chloride (2,4,6-trichlorotriazine; CAS No. 198-77-0) is used predominantly as an intermediate in the production of dye and herbicydes. Cyanuric chloride has strong irritant properties to the eyes, skin and respiratory tract (5, 13, 15 and our unpublished data). In the literature there are no information concerning genotoxic properties of this compound. However, our earlier studies indicated that some of the reactive dyes based on the cyanuric chloride have genotoxic properties. It was found that Reactive Brown 9 was positive in Ames test and micronucleus test, but negative in SCE assay; Reactive Black 8 was positive in SCE test (4).

The aim of this study was to evaluate mutagenic and genotoxic properties of cyanuric chloride. For this purpose, three short-term tests were used; Salmonella/microsome assay, *in vivo* sister chromatid exchange assay (SCE) and micronucleus assay. All tests were performed according to standardized protocols based on OECD and EU guideliness (6, 10, 14).

MATERIALS AND METHODS

Cyanuric chloride (2,4,6-trichlorotriazine; Degussa) was used throughout the study. The purity of the compound was > 95%.

Salmonella/microsome assay

The mutagenicity assay was carried out according to the plate-incorporation method with the histidine requiring *S. typhimurium* mutants TA 97a, TA 98, TA 100 and TA 102 as indicator strains. Tests were performed both in the absence and in the presence of 10% S9 fraction prepared from Wistar Imp: DAK rats liver induced with Aroclor 1254. The S9 used contained 4.61 nmol of cytochrome P−450. The cytochrome P−450 content was determined by the method of Omura and Sato (11). This assay has been described in detail elsewhere (1, 7, 9).

Briefly, to 2 ml molten top agar (containing 0.6% Difco agar, 0.5% NaCl and 0.05 mM L-histidine HCl/0.05 mM biotin), maintained at 45°C, were added in this sequence: 0.1 ml of the appropriate solution of the test substance; 0.1 ml of about 10 h grown culture of the appropriate tester strain and 0.5 ml S9-mix, if any. The ingredients were thoroughly mixed and mix was immediately poured onto minimal glucose agar plates (1.5% Bacto-Difco agar in Vogel and Bonner medium E with 2% glucose). After incubation for two days at 37°C, the background lawn of bacterial growth was examined and the His^+ revertants were counted using a Biotran II colony counter; New Brunswick Sci. Co, N.Y.

A preliminary test with 6 concentrations of the test substance and the vehicle, DMSO as negative control was carried out to assess the toxicity of the test substance for *S. typhimurium* strains on TA98. For this test appropriate dilutions of the test substance in DMSO, containing 0; 0.5, 1; 10; 100; 1000 and 5000 μg/0.1 ml were used.

The results, summarized in Table 1, show that the test substance was toxic for TA 97a at a dose level of 1000 μg/plate and above. No toxicity was observed at any of the lower dose levels used. In view of these observations 500 μg/plate was chosen as the highest dose level for the mutagenicity assay, both in the absence and in the presence of the S9-mix.

Table 1. Results of the toxicity test with cyanuric chloride in *S. typhimurium* TA 98

Amount of test substance per plate (μg)	without S9-mix		with S9/mix	
	background growth[1]	number of revertants	background growth[1]	number of revertants
0.0	ng	27	ng	44
0.5	ng	29	ng	34
1.0	ng	24	ng	40
10.0	ng	22	ng	45
100.0	ng	24	ng	41
1000.0	0	–	0	–
5000.0	0	–	0	–

[1]Abbreviations used:
ng: normal background lawn of bacterial growth
0: no background lawn of bacterial growth

For the main study 4 concentrations of cyanuric chloride; 1; 10; 100 and 500 μg/0.1 ml were used. The repeat experiment was carried out with the same dose levels. Each culture was examined for the number of spontaneous revertants, histidine requirement and sensitivity to ampicilin, crystal violet and UV radiation. The reference mutagens used as positive controls were:

— sodium azide: 1.5 μg per plate with the strains TA 100, in the absence of the S9-mix 4-nitro-o-phenylenediamine 3.0 μg per plate with the strains TA 98, in the absence of the S9-mix

— 4-nitroquinoline-N-oxide 0.5 μg per plate with strain TA 97a and TA 102 in the absence of the S9-mix

— 2-aminofluorene 5.0 μg per plate with strains TA 97a, TA 100 and TA 102 in the presence of the S9-mix

— benzo(a)pyrene: 5.0 μg per plate with strain TA 98 in the presence of the S9-mix

A positive response in the assay system is taken to be a two-fold or greater increase in the mean number of revertant colonies appearing in the test plates above the background spontaneous reversion rate observed with the vehicle, together with evidence of a dose-response.

In vivo sister chromatid exchange assay (SCE)

The SCE test was performed according to the GENE-TOX programme (8). In brief, assay was carried out on 9 week-old male mice, Balb/C strain with mean b.w. of 25 g, from Animal Hausbandry at the Nofer Institute of Occupational Medicine in Lodz. The animals were fed with standard diet and water was given *ad libitum*. Cyanuric chloride diluted in DMSO was injected intraperitoneally to five male mice per group at doses of 8, 16, 24 and 32 mg/kg.b.w. at the volume of 0.3 ml/animal. The additional groups of mice received only DMSO (negative control; n = 5)) or cyclophosphamide (positive control; n = 4) One hour prior to injection of the test chemical animals were lightly anesthetized and tablets of 5-bromodeoxyuridine subcutaneously implanted. Twenty-one hours after administration of the compound

bone marrow cells mitosis was inhibited by i.p. administration of 3.33 mg/kg. b.w. colchicine. Two hours later animals were sacrificed by cervical dislocation; both femures of each mouse were isolated and bone marrow was collected by flushing the femurs with Hank's solution. After the hypotonic treatment with 0.075 M KCl the cells were fixed using methanol: glacial acetic acid (3:1) and then dropped onto chilled slides. The differential staining of sister chromatids was carried out using the method of Antoshina and Porjadkova (2). To determine SCE frequency, 50 second-generation metaphase cells from each mouse were analyzed. The results were statistically evaluated by one-way variance analysis with Scheffe's test for multiple comparisons.

Micronucleus assay

The micronucleus assay *in vivo* on mice bone marrow cells was conducted according to the method described in detail by Przybojewska (12). Cyanuric chloride was dissolved in corn oil and injected intraperitoneally to adolescent male and female Balb/C outbred mice. Compound was tested at two dose levels; at 80% and at 40% of LD_{50} value. Each dose was divided into two equal parts and given at 24 hours intervals. Two-stage experimental model was used. At first cyanuric chloride was injected to male mice at dose equal to 80% of LD_{50} and bone marrow cells collected 30, 48 and 72 hours afterwards. At the second stage, compound was given to male mice at dose equal to 80% and 40% of LD_{50} and to female mice at dose of 80% of LD_{50} and bone marrow sampled at time as above. The preparation of bone marrow and staining were carried out according to the procedure described earlier (12). Mitomycin C (Sigma) dissolved in distilled water to a total dose of 2.5 mg/kg.b.w. was given i.p. in the same manner as tested compound and served as the positive control. The negative control groups for cyanuric chloride and Mitomycin C received corn oil and distilled water, respectively. For each animal 1000 polychromatic erythrocytes (PCE) were analysed for the presence of micronucleated PCEs (MN PCE). Additionally, the ratio of PCEs to normochromatic erythrocytes was determined by counting the number of PCEs among 200 normochromatic erythrocytes (NCE). The obtained frequency of MN PCEs was verified with a one-way analysis of variance. The compound was considered to be positive when a statistically significant and reproducible increase in the number of MN PCEs were obtained for at least one experimental point.

RESULTS

Salmonella/microsome assay

The results of the Ames test are summarized in Table 2. From the results obtained in both experiments it appeared that incubation of the test substance with the bacteria did not increase the number of His^+ revertants with any of the strains of *S. typhimurium*, either in the absence or in the presence of the S9-mix.

At the highest dose level used i.e. 100 and 500 μg/plate, the test substance appeared to be slightly toxic for strain TA 97a (in the absence of the S9-mix), as was seen from a diminished number of revertant colonies (Table 1).

Table 2. Results of the Salmonella/microsome mutagenicity test with cyanuric chloride

Dose µg/pl	TA 97a				TA 98				TA100				TA 102			
	−S9		+S9		−S9		+S9		−S9		+S9		−S9		+S9	
Spontaneous revertants	103	112	114	122	117	128	27	27	34	44	29	37	131	117	148	122	269	257	283	334	286	361
	94	108	139	120	116	149	24	24	31	44	30	30	134	104	124	122	236	246	251	378	292	296
	105	107	105	131	112	127	31	26		30	31	33	113	112	141	133						
0																						
mean		111			125			28			34		119		132			257			325	
SD		13			11			4			6		12		11			17			39	
Solvent control	85	125	136	122	124	140	26	29	21	31	32	32	120	132	124	140	269	279	283	311	322	330
	109	85	103	101	103	84	20	27	19	38	33	34	131	123	110	133	179	230	255	322	317	310
DMSO 100µl													133	109	120	121	276			273		
mean		107			112			24			33		125		125			267			312	
SD		21			20			4			3		9		11			19			19	
1	107	94	118	108	112	103	24	20	25	40	48	29	119	107	108	120	283	231	245	304	296	292
	104			107	93		28			29			124	115	114	120	227	244	217	381	295	314
mean	106			105				24			37		116		116			234			314	
SD	10			7				3			9		7		6			11			34	
10	115	114	112	90	124	126	22	24	29	45	28	35	114	112	138	137	196	222	259	361	325	366
	110	92	79	128	92		26			23			146	112	154	140	266	236	270	361	310	283
mean		104			112			25			33		121		142			242			334	
SD		15			19			3			10		17		8			29			34	
100	115	79	90	134	120	139	24	18	26	41	27	40	123	122	100	140	279	230	297	305	340	359
	116	94	86	136	104	110	23	21	23	26	35		114	113	108	110	265	260	255	341	340	335
mean		97			124			23			34		118		115			264			337	
SD		15			15			3			7		5		18			23			18	
500	81	98	87	126	170	160	29	28	16	36	28	28	132	120	180	198	246	234	259	336	365	345
	113						15			39			117	116	171	212	183	227		365	392	352
mean		95			152			22			33		121		190			230			359	
SD		14			23			8			6		7		18			29			20	
Positive controls																						
mean		389			597			311			280		951		798			570			607	
SD		41			65			28			47		50		64			21			58	

The positive controls used in the present assay gave the expected strong increase in the number og His^+ revertants, both in the absence and in the presence of the S9-mix.

In vivo sister chromatid exchange assay

The individual animal and group mean SCE frequencies for animal treated with cyanuric chloride are shown in Table 3. Among animals in the same groups no significant variation was observed. DMSO control showed an average of 4.97 + 2.45 SCE/cell. No significant increase was noted in the incidence of SCE in the groups treated with all tested doses of cyanuric chloride. Thus, in this test cyanuric chloride did not induce chromosomal damage resulting in SCE formation in bone marrow cells of mice. Animals treated with cyclophosphamide were all positive; significant increase of SCE/cell was noted ($p < 0.025$).

Table 3. Sister chromatid exchange frequency in bone marrow cells of mice following *in vivo* exposure to cyanuric chloride

Dose (mg/kg)	No. of animals	SCE/cell + SD	Mean SCE/cell ± SD
Negative Control[a]	5	4.20 + 2.36	
		5.20 + 2.78	
		5.32 + 2.74	4.97 ± 2.45
		4.98 + 2.06	
		5.16 + 2.15	
8	5	5.06 + 2.88	
		5.20 + 3.32	
		4.36 + 1.67	4.89 ± 2.55
		5.20 + 2.51	
		4.64 + 2.00	
16	5	5.00 + 2.22	
		5.36 + 2.16	
		5.76 + 2.34	5.33 ± 2.33
		5.20 + 2.59	
		5.32 + 2.31	
24	5	5.84 + 2.84	
		5.64 + 2.89	
		5.32 + 1.63	5.56 ± 2.37
		5.28 + 2.03	
		5.72 + 2.23	
32	5	5.08 + 2.07	
		5.64 + 2.27	
		5.40 + 2.20	5.36 ± 2.33
		5.16 + 2.51	
		5.50 + 2.60	
Positive Control[b]	4	16.44 + 4.40	
		15.88 + 4.26	
		16.16 + 4.75	16.20 ± 4.18*
		16.32 + 4.20	

[a] – DMSO
[b] – Cyclophosphamide, 15 mg/kg b.w.
* – Different from negative control ($p < 0.025$).

Micronucleus assay

The results obtained in the micronucleus assay are summarized in Table 4. The incidence of MN PCEs after administration of all doses of cyanuric chloride used were not statistically different ($p > 0.05$) as compared to negative controls. However, the compound caused significant decrease of PCEs NCEs ratio in the bone marrow noted 30, 48 and 72 hours after administration of both tested doses of cyanuric chloride.

Table 4. The frequency of micronuclei in bone marrow polychromatic erythrocytes of Balb/C mice and the ratio of polychromatic to normochromatic erythrocytes.

Compound	Total dose mg/kg	Number of polychromatic erythrocytes with micronuclei (% ± SD)			Ratio of polychromatic to normochromatic erythrocytes		
		Bone marrow cells					
		30 h	48 h	72 h	30 h	48 h	72 h
		Males					
Negative control[a]	–		0.15 + 0.10			1.47	
Cyanuric chloride	3.05	0.10 ± 0.05	0.30 ± 0.00	0.17 ± 0.07	0.56*	0.55*	0.44*
	6.10	0.16 ± 0.05	0.17 ± 0.07	0.17 ± 0.04	0.43*	0.62*	n.t.
Negative control[b]	–		0.20 + 0.08			1.63	
Positive control[c]	2.50	3.47 ± 0.20*	3.42 ± 0.68*	0.80 ± 0.45*	0.51*	0.20*	0.09*
		Females					
Negative control[a]	–		0.17 ± 0.07			1.69	
Cyanuric chloride	6.10	0.17 ± 0.07	0.17 ± 0.07	0.12 ± 0.03	1.02*	1.02*	0.84*

[a] – corn oil (0.5 ml/30 g)
[b] – sterile water
[c] – Mitomycin C (Sigma Co., U.K.)
* – difference statistically significant ($p < 0.05$)

DISCUSSION

In view of the fact that mutagenic and potential carcinogenic agents may induce different types of genetic reaction, testing of chemicals by several methods increased the probability of detection of genotoxic properties (3, 7). In our studies we used three assays based on the evaluation of various biological end-points regarding mechanism of their occurrence.

From the above findings it is concluded that cyanuric chloride did not show mutagenic activity in *Salmonella typhimurium* TA 97a, TA 98, TA 100 or TA 102, either in the absence or in the presence of the S9-mix, under the conditions employed in this evaluation. At the highest dose level used, namely 100 and 500 µg/0.1 ml, the test substance appeared to be slightly toxic for strain TA 97a (in the absence of the S9-mix).

No differences in SCE frequency, as compared with the level observed in the controls were found after administration of 4 doses of cyanuric chloride. Thus, in this assay cyanuric chloride did not induce chromosomal damage resulting in SCE formation in mice bone marrow cells.

Cyanuric chloride revealed no genotoxic activity when tested for the incidence of micronuclei in the bone marrow polychromatic erythrocytes. However, significant decrease of PCEs to NCEs ratio in the bone marrow may indicate the cytostatic properties of cyanuric chloride.

It was found that some of the reactive dyes based on the cyanuric chloride, namely Reactive Brown 9 and Reactive Black 8 have genotoxic properties (4). Negative results in Ames test and a lack of induction of sister chromatid exchange and micronuclei in bone marrow cells of mice obtained in this study indicate that cyanuric chloride by itself is not an effective mutagenic and genotoxic agents.

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