Cytogenetic Effects of the Insecticide Methamidophos in Mouse Bone Marrow and Cultured Mouse Spleen Cells*

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Dedicated to Professor Erich Hecker on the occasion of his 60th birthday

Insecticides, Methamidophos, Cytogenetic Effect

The cytogenetic effect of the insecticide methamidophos (0,S-dimethylphosphoroamidothiolate) was studied in mouse bone marrow and mouse spleen cells in culture. *In vivo* the ability of methamidophos to induce micronuclei and sisterchromatid exchange in mouse bone marrow was investigated. *In vitro* mouse spleen cells in culture were used to assess the ability of the insecticide to induce chromosomal aberrations and sister chromatid exchange.

Three different routes of application for the pure insecticide were tested so as to cover the different possibilities for human exposure to the insecticide. Intraperitoneal, oral and dermal treatment with methamidophos caused toxicity to marrow as indicated by a significant increase in the percentage of polychromatic erythrocytes (PEs) over that of the control.

Methamidophos showed mutagenic potential as evidenced by a positive response in the micronucleus and chromosomal aberrations assays. Thus, single and multiple i.p. injections at 6 and 4.5 mg methamidophos/kg body wt., oral administration of the insecticide for 14 consecutive days at a dietary level of 100 ppm and multiple dermal treatments (total 4) with 24 mg/kg body wt. induced a statistically significant increase in the frequency of PEs with micronuclei in mouse bone marrow. Moreover, the tested concentrations of methamidophos as low as 0.25 μg/ml induced a high percentage of metaphases with chromosomal aberrations in cultured mouse spleen cells.

Methamidophos is a weak inducer of SCEs in mouse bone marrow and cultured mouse spleen cells.

The organophosphorous insecticide and acaricide methamidophos (0,S-dimethyl phosphoroamidothiolate, marketed under the name monitor or tamaron is a chemically simple compound with a broad spectrum of insecticidal activity [1].

In Egypt methamidophos is widely used to control pests which attack different crops *e.g.* cotton, tomato, potato broad bean, and soyabean (Program of pest control, Ministry of Agriculture, A.R.E, 1972/1973–1980/1981).

Interest in the toxicology and pharmacology of methamidophos was stimulated following the observation that a combination of methamidophos and gusathion caused serious illness of 3000 agricultural workers in Egypt in 1976 with a fatality rate of 1% [2]. The toxic effect of the pure insecticide in mice was found to be more pronounced when the insecticide was ingested than when applied dermally or intraperitoneally, and caused a progressive inhibition of both erythrocyte and plasma cholinesterase activity [3].

In the present investigation the cytogenetic effect of the insecticide was studied *in vivo* and *in vitro*. *In vivo* the ability of methamidophos to induce micronuclei and sister chromatid exchange in mouse bone marrow was investigated. *In vitro* mouse spleen cells in culture were used to assess the ability of the insecticide to induce chromosomal aberrations and sister chromatid exchange.

Materials and Methods

In vivo studies

The micronucleus test

Random-bred white Swiss mice aged 6–8 weeks were used. The source of mice is the Egyptian Organization for Biological and Vaccine Production-Agouza. The mice used for any one experiment were selected from mice of similar age (±1 week) and weight (±2 gm). Both male and female mice were

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used, since, differences in sensitivity among animals of different age or sex were not demonstrable [4].

Methamidophos used in this study was synthesized by the Laboratory of Organic Chemistry, National Research Centre and kindly given by Prof. Dr. S. M. A. D. Zayed, Head of the Laboratory.

Three different routes of application for the pure insecticide were tested so as to cover the different possibilities for human exposure to the insecticide.

a) Intraperitoneal treatment

Each dose was dissolved in 0.1 dimethyl sulphoxide (DMSO). The doses were 4–5 and 6 mg methamidophos/kg body weight. Mice were injected once with 6 mg methamidophos/kg body wt. and killed one, two and seven days after injection. For repeated treatment, injections were performed twice weekly with 4.5 mg methamidophos/kg body weight. Mice were killed 24 h after the last treatment. A group of mice received multiple injections (total of 3) was left to recover for 14 days. Control animals received 0.1 ml dimethyl sulphoxide only.

b) Oral treatment

Mice received standard rat chow (commercial name: Nahi) containing two levels of the insecticide, 50 and 100 ppm. Dietary concentrations of the insecticide were prepared by thoroughly mixing the calculated amount of the insecticide in 5 ml pure acetone (Spectroscopic grade) with the chow. Acetone was allowed to evaporate at room temperature for 24 h before the diet was consumed. Samples were taken: one day, 7 days and 14 days after the treatment. A group of mice treated for 14 days was left to recover for two weeks.

c) Dermal treatment

Two doses of the insecticide 12,24 mg/kg body weight were applied as a solution in 0.1 ml DMSO on a preshaved part of the back of the animal (about 2 cm^2). Applications were performed twice weekly over a period of two weeks. Mice were killed 24 h after the last treatment. Controls received the solvent only. A group of animals received multiple treatments (total 4) was left to recover for another 2 weeks.

Animals were housed in cages and received rat chow (commercial name, Nahi). Food consumption and mortality were observed in all treatment groups, growth of mice was evaluated on the basis of rate of increase in body weight gain and was determined at 0 and sampling times.

The bone marrow from both femora was flushed into a test tube containing calf serum (3 ml) and then centrifuged at 1500 rpm. From the pellet, smears were made on slides and the air-dried preparations were stained by the May-Grünwald-Giemsa method [5]. For each mouse, 2 or 3 slides were prepared. Micronuclei were counted in polychromatic erythrocytes only. Micronuclei were identified as dark-blue-staining bodies in the cytoplasm of polychromatic erythrocytes (PEs). The results are expressed as the average number of micronucleated PEs/100 PEs (%). About 6000 nucleated cells including PEs were analysed per animal. Since the insecticide response did not indicate large variations between individual animals or sexes, data from animals within a particular dose group were pooled (compare [6]).

The ratio of PEs to nucleated cells was determined and expressed as the percentage of PEs/100 nucleated cells. Doses that induced a statistically significant increase in the percentage of PEs over that of the control were considered to cause marrow toxicity (compare [7–10]).

Statistical evaluation

The significance of the difference between experimental and control data was calculated using differences between 2 proportions [11] for % PEs/nucleated cells and the tables of Kastenbaum and Bowman [12] for polychromatic erythrocytes with micronuclei. The calculations were made using a computer program in the Institute of Statistical Studies and Research, Cairo University.

Sister chromatid exchange

The experimental procedure was conducted in accordance with the protocol of Allen [13] with some modifications.

Random-bred, male, Swiss mice, aged 3–4 months and weighing 30–40 gm, were used. The used doses were: 4,6 and 8 mg methamidophos/kg body weight.

BrdU (Sigma) tablets weighing approximately 55 ± 2 mg were placed subcutaneously. Mice were injected intraperitoneally with the insecticide solution in distilled water 8 h following BrdU treatment. Control mice were injected with distilled water. Mice

were injected with colchicine (0.6 mg/kg body wt.) 2 h prior to killing.

The bone marrow from both femora was flushed into a test tube containing phosphate buffered saline, 0.075 M KCl was added. The cells were fixed in methanol: acetic acid (3:1). The cell suspension was dropped on dry slides which were stained with Hoechst 33258 dye then rinsed in distilled water and subjected to U.V. light and incubated for one hour in $2 \times$ SSC buffer at 60 °C. Staining followed in 7% Giemsa dye (Gurr improved R66 bio/medical specialities, Box 1687, Santa Monica, Calif. 90406 USA) in 0.06 M phosphate buffer pH 6.8.

Only cells with well spread chromosomes were selected for scoring, 40 metaphase spreads per animal were examined microscopically for SCEs, 200 metaphase spreads were scored from each treatment and 120 metaphase spreads from the controls.

Evaluation of the differences in mean SCE frequencies between treated and control groups was made using the t-test.

In vitro studies

Cell culture

The spleen was washed with RPMI medium cut in small pieces, and the cells were centrifuged at 1000 rpm for 10 minutes. Spleen cells at a concentration of 2×10^5 were cultured in plastic tissue culture dishes (falcon) containing, RPMI 1640 medium plus 10% faetal calf serum, antibiotics (100 units/ml of penicillin and 100 µg/ml of streptomycin) and 2 µg/ml concanavalin A, and were maintained in 5% CO_2 incubator at 37 °C.

Toxicity

Toxicity was measured as the loss in growth potential of the cells induced by 4-h exposure to the test article followed by a 24-h expression period in growth medium [14].

Spleen cells, 16 h old, were exposed to a wide range of methamidophos concentrations ranging from 10^{-7} M to 10^{-3} M for 4 h. After that the medium was removed, then the cells were left to grow in fresh medium for another 20 h. The number of viable cells was estimated using Trypan Blue (a vital stain) and Neubauer Chamber. The percentage of dead cells was determined from three separate experiments for each concentration of the insecticide.

Chromosomal aberrations

Spleen cells were exposed to four different concentrations of the insecticide for 4 h. The used concentrations were: 0.25, 0.50, 1.00 and 2.00 µg/ml. Colchicine was added to the cell culture 2 h prior to harvesting. The cell pellet was suspended in 0.075 M KCl at 37 °C for 20 minutes. The cells were fixed in methanol acetic acid (3:1), dropped onto dry slides, air-dried and stained by Giemsa in phosphate buffer (pH 6.8). Three separate experiments were conducted using each concentration of the insecticide.

Sister-chromatid exchange

BrdU was added at the time of culturing the cells. The cell cultures were treated with the insecticidal solution for the last 4 h. The used doses were: 0.25, 0.50, 1.00 and 2.00 µg methamidophos/ml. Control cultures were treated with distilled water. Two hours before cell harvesting, colchicine was added. Hypotonic treatment, fixation of the cells and chromosome preparations were made by the standard air-drying technique previously described.

A separate experiment was conducted using mitomycin C, as a positive control, dissolved in distilled water at a final concentration of 0.15 µM.

Two separate experiments were conducted using each dose. Twenty-five cells per culture were analyzed for SCE's/cell (50 cells per dose).

Results

In vivo studies

The micronucleus test

a) Intraperitoneal treatment

The percentage of PEs increased significantly one and two days after injection of mice at 6 mg methamidophos/kg body wt. then returned to the normal value 7 days after cessation of the treatment (Table I).

Multiple treatments (total of 3 injections) at 4.5 mg methamidophos/kg body wt. for each injection over a period of 2 weeks caused a significant increase in the percentage of PEs over the period of treatment indicating marrow toxicity. When the treated mice were left to recover for two weeks the percentage of PEs was found to be normal (Table II).

Signs of acute toxicity which consisted of tremors, abnormal irregular movements and reduction of

Table I. Percentage of polychromatic erythrocytes (PEs) and PEs with micronuclei in mouse bone marrow after single intraperitoneal injection at 6 mg/kg body wt. of methamidophos in DMSO.

Treatment	Number of mice	Number of counted nucleated cells + PEs	Maximal and minimal number of PEs/animal	PEs %	PEs with micronuclei %
1 day					
Control[△]	2	12605	511 505	8.1	0.79
Methamidophos	3	18735	716 705	11.4**	1.9*
2 days					
Control	2	12138	493 491	8.1	0.81
Methamidophos	3	19336	881 834	13.2**	2.1**
7 days					
Control	2	12238	497 488	8.1	0.91
Methamidophos	3	18009	490 469	7.9	1.04

△ Control animals received 0.1 ml DMSO.
* Significant at 0.05 level.
** Significant at 0.01 level.

Table II. Percentage of polychromatic erythrocytes (PEs) and PEs with micronuclei in mouse bone marrow after repeated intraperitoneal injection at 4.5 mg/kg body wt. of methamidophos in DMSO.

Treatment	Number injections	Mice killed after (h)	Number of mice	Number of counted nucleated cells + PEs	Maximal and minimal number of PEs/animal	PEs %	PEs with micronuclei %
Control[△]	Single injection	24	2	12247	515 505	8.3	0.98
Methamidophos			3	18505	694 631	10.7**	1.5
Control	Total of 2 injections	24	2	12089	529 489	8.4	0.88
Methamidophos			3	18273	772 758	12.6**	1.74*
Control	Total of 3 injections	24	2	12093	525 521	8.7	0.76
Methamidophos			3	18084	900 835	14.4**	1.73*
Control	Total of 3 injections	14 days	2	12092	496 491	8.2	0.91
Methamidophos			3	18774	555 486	8.4	1.08

△ Control animals received 0.1 ml DMSO.
* Significant at 0.05 level.
** Significant at 0.01 level.

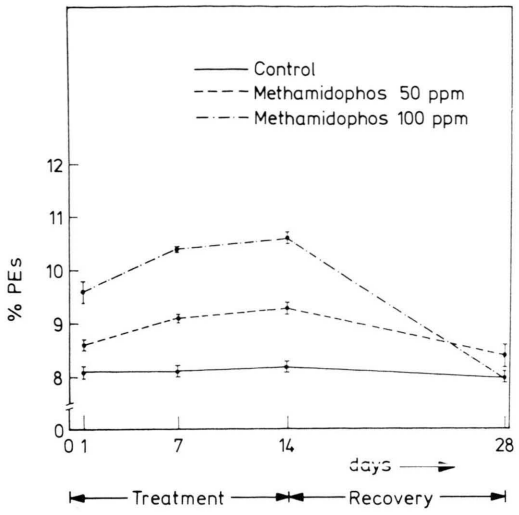

Fig. 1. Percentage of (PEs) in mouse bone marrow after oral treatment with various concentrations of methamidophos.

body wt. were observed following the treatment with the higher dose and the second injection with the lower dose and no mortality was observed.

A significant increase in the frequency of PEs with micronuclei was observed one and two days after the treatment (Table I). Repeated treatment (total of 3 injections) at 4.5 methamidophos/kg body wt. affected a significant increase in the frequency of micronucleated PEs over that of the control (Table II).

b) Oral treatment

Administration of methamidophos for 14 consecutive days at dietary levels of 50 and 100 ppm affected a significant increase in the percentage of PEs over that of the control. However, the percentage of PEs attained the normal value two weeks after cessation of the treatment (Fig. 1).

Groups of mice receiving dietary levels of 50 and 100 ppm methamidophos for two weeks showed decrease in initial body wt. and no mortality was observed.

A dietary level of 100 ppm of the insecticide caused increase in the percentage of PEs with micronuclei after treatment for 7 and 14 days, such percentage was found to be statistically significant (Table III).

c) Dermal treatment

Treatment of mice with 12 and 24 mg methamidophos/kg body wt. caused a significant increase in the percentage of PEs over that of the control 7 and 14 days after the treatment. When the treated mice were left to recover for two weeks, the percentage of PEs was found to be normal (Fig. 2).

Table III. Percentage of polychromatic erythrocytes (PEs) with micronuclei in mouse bone marrow after oral treatment with various concentrations of methamidophos.

Period of treatment	Period of recovery (days)	Number of mice	Number of PEs	Micronucleated PEs No.	PEs with micronuclei [%]
24 hours	00				
Control		2	991	8	0.81
50 ppm		2	1102	14	1.3
100 ppm		3	1707	25	1.5
7 days	00				
Control		2	968	8	0.83
50 ppm		2	1193	16	1.34
100 ppm		3	1957	36	1.84*
14 days	00				
Control		2	1003	9	0.89
50 ppm		2	1224	21	1.7
100 ppm		3	1898	40	2.11*
14 days	14				
Control		2	961	9	0.94
50 ppm		2	1039	11	1.1
100 ppm		3	1453	17	1.2

* Significant at 0.05 level.

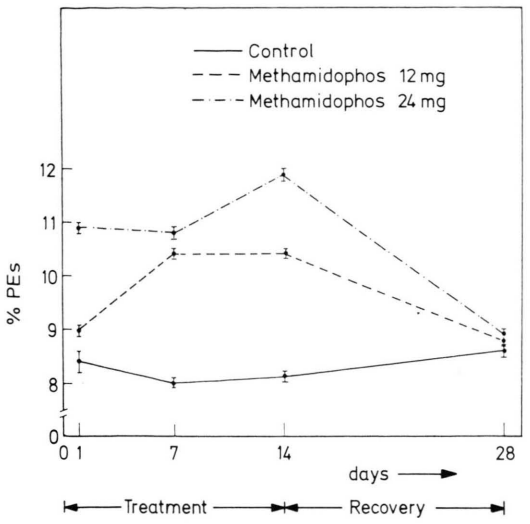

Fig. 2. Percentage of (PEs) in mouse bone marrow after dermal treatment with various concentrations of methamidophos.

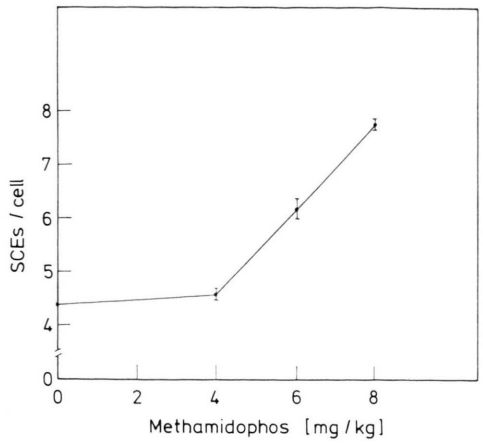

Fig. 3. Frequency of sister chromatid exchanges in mouse bone marrow cells after i.p. injection with different concentrations of methamidophos.

The high dose of the insecticide caused a decrease in the body weight gain after two weeks as compared with the controls.

Multiple treatments (total 4 over a period of 2 weeks), with 24 mg methamidophos/kg body wt. caused a significant increase in the percentage of micronucleated PEs (1.77 ± 0.13)* over that of the control (0.82 ± 0.03). The frequency of micronucleated PEs returned to the normal value two weeks after cessation of the treatment.

Sister chromatid exchange

A slight but significant increase (at 0.01 level) in the frequency of SCE's was noted at the doses 6 and 8 mg methamidophos/kg body wt. (Fig. 3).

In vitro studies

Toxicity

Methamidophos at a concentration of 10^{-6} M affected a slight decrease in cell survival. A linear increase in the mean percentage of dead cells was observed as the concentration of the insecticide increased (Fig. 4).

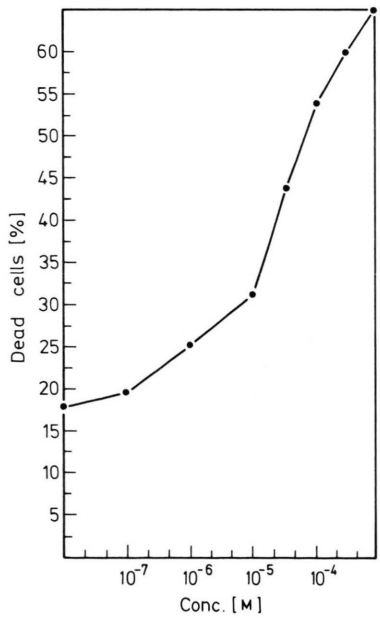

Fig. 4. Percentage of dead cells in cultured mouse spleen cells treated with different concentrations of methamidophos for 4 h and 20 h in fresh medium.

Chromosomal aberrations

The mean percentage of chromosomal aberrations induced after treatment of cultured mouse spleen cells with methamidophos increased progressively as the concentration of the insecticide increased

* Significant at 0.05 level.

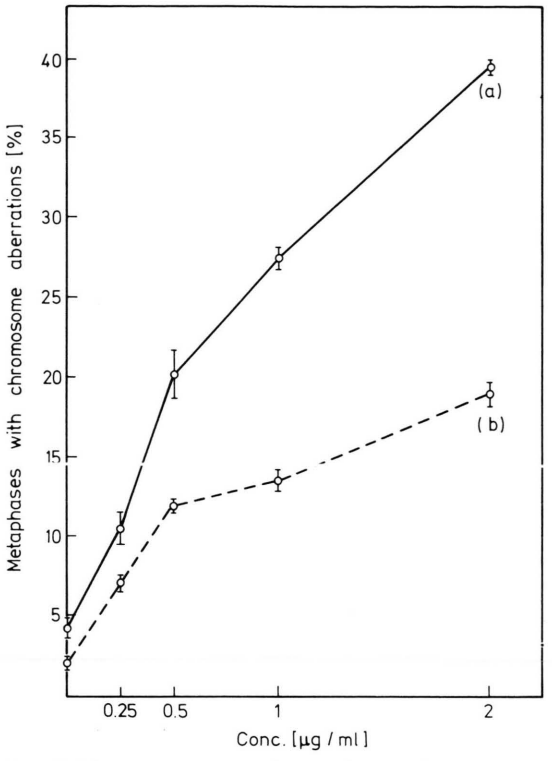

Fig. 5. Mean percentage of metaphases with chromosome aberrations in cultured mouse spleen cells after treatment with different concentrations of methamidophos: (a) with gaps; (b) without gaps.

(Fig. 5). All the tested concentrations of the insecticide resulted in a highly significant percentage of cells with chromosomal aberrations. The percentage of aberrations without chromosome and chromatid gaps was found also to be statistically significant (Fig. 5).

When cultured mouse spleen cells were treated for 4 h with 2 µg methamidophos/ml, then left to grow in fresh medium for another 20 h, the percentage of chromosomal aberrations though decreased markedly yet, it was still statistically significant (17.1 ± 0.8* compared with 4.9 ± 0.11 for the control cell culture).

Table IV and Fig. 6 and 7 represent the different types of chromosomal aberrations in cultured mouse spleen cells treated with different concentrations of methamidophos.

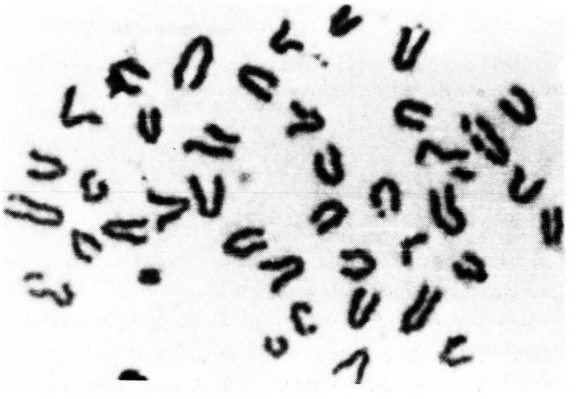

Fig. 6. Metaphase spread from cultured spleen mouse cells treated with 0.5 µg methamidophos for 4 h, showing chromatid fragments and chromatid gaps.

* Significant at 0.01 level.

Table IV. Mean percentage of metaphases with different types of chromosomal aberrations in cultured mouse spleen cells treated with different concentrations of the insecticide methamidophos.

Conc. [µg/ml]	Metaphase No.	% Metaphases with different types of chromosomal aberrations: Frag.	Chromat. gap	Chrom. gap	Frag. chromat. or chrom. gap	Chrom. gap + chromat. gap	Tetraploid	Ring chrom.	Mean % metaph. with chrom. aberr.
2	524	12.6	10.5	6.1	4.4	4.2	1.1	0.76	39.7*
1	511	9.6	5.1	8.2	2.2	0.78	0.9	0.78	27.6*
0.5	527	8.5	3.8	3.6	2.2	0.95	0.6	0.4	20.11*
0.25	581	4.9	2.2	1.03	0.86	0.17	0.5	0.7	10.5*
Control	1029	1.4	0.7	0.97	0.39	0.29	0.09	0.29	4.08
Mitomycin C (positive control)	582	5.8	2.7	1.2	0.86	0.52	0.69	0.52	12.4*

* Significant at 0.01 level.

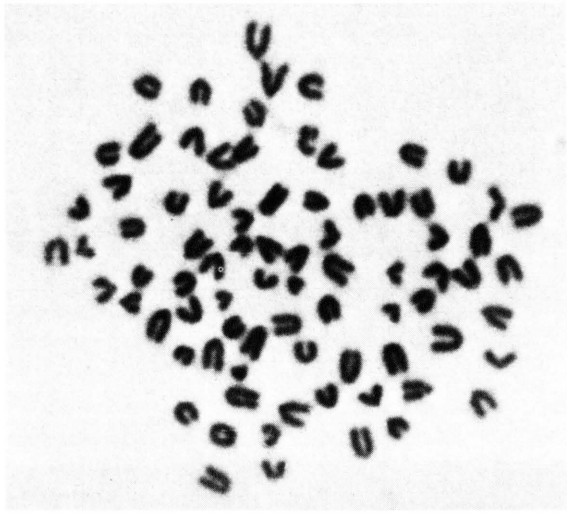

Fig. 7. A tetraploid metaphase from cultured mouse spleen cells treated with 2 μg methamidophos/ml.

Sister chromatid exchange

The tested concentrations of the insecticide affected a significant increase in the frequency of SCE's in cultured mouse spleen cells (Fig. 8). The increase was however, lower than that induced by the positive control mitomycin C. The frequency of SCE's in mouse spleen cell cultures treated with 0.15 μM mitomycin C reached 12.4 ± 0.1* per cell.

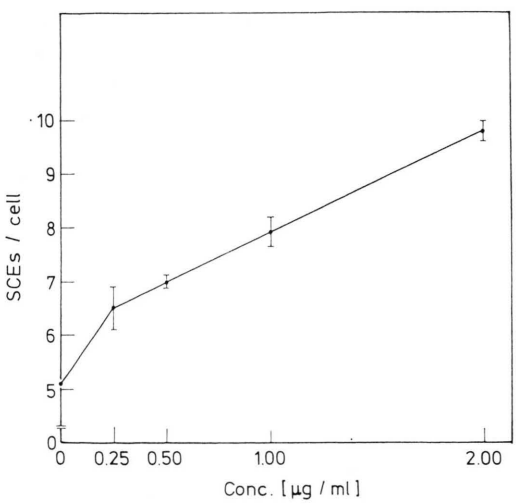

Fig. 8. Frequency of sister chromatid exchanges in cultured mouse spleen cells after treatment with different concentrations of methamidophos.

* Significant at 0.01 level.

Discussion

The three routes of application for the insecticide caused toxicity to marrow indicated as significant increase in the percentage of polychromatic erythrocytes over that of the control. Bone marrow depression was caused also by other chemicals including mutagenic and carcinogenic agents. Thus methylmethanesulfonate, ethylmethanesulfonate and colchicine produced marrow depression and micronuclei in bone marrow of CD mice [6]. Moreover the carcinogens, dimethylnitrosamine, acetylaminofluorene, aflatoxin B, and 3-methylcholanthrene caused marrow toxicity in mouse and hamster bone marrow. In this respect it is worth mentioning that the different routes of treatment with: the organophosphorous insecticides dursban and gardona, the pyrethroid insecticide cypermethrin and the botanical insecticide rotenone, caused marrow toxicity indicated as significant increase in the percentage of PEs in mouse bone-marrow [8–10].

Micronuclei are believed to be formed from chromosomes or chromosome fragments left behind during anaphase and can be scored during interphase because they persist [15]. The micronuclei are found in a variety of different bone-marrow cells of animals treated with chemical mutagens: myeloblasts, myelocytes, erythrocytes etc. The majority, however is observed in polychromatic erythrocytes [16].

Evans [17] has recommended the micronucleus test in polychromatic erythrocytes as a method for detecting chemical mutagens in animal test systems.

A total of three injections though effected increase in the frequency of PEs with micronuclei yet, the increase was nearly the same as that induced after double injection. The presented results are in line with those of Salamone *et al.* [18] who, suggested that multiple (3 or more) treatments are non-additive and may depress the PEs frequency.

Only the higher dietary level of methamidophos (100 ppm) affected a significant increase in the percentage of PEs with micronuclei over that of the control after treatment for 7 days. Such increase in the frequency of micronucleated PEs become higher when mice received the insecticide for further 7 days. The results obtained are in line with those of Seiler [19] who found that the number of micronucleated erythrocytes showed a dose-dependent increase when mice were treated orally by (gavage) with MBC (a benzimidazole derivative).

SCE's are currently recognized as being sensitive indicators of agents which damage mammalian DNA [20, 21] and their formation has been compared with that of mutations [22]. Various authors have suggested that sister chromatid exchange (SCE) analysis using the 5-bromodeoxyuridine probe offers a cytogenetic technique for determining the potential genetic hazards of chemicals in the environment [21, 23–27]. The *in vitro* technique is much more sensetive to detect genotoxic damage than the *in vivo* technique. Although *in vivo* testing is less sensitive, it may be more useful in prediciting possible health risks since *in vivo* studies more closely resemble human exposure [28].

The higher tested doses of methamidophos (6 and 8 mg/kg body weight) increased the frequency of SCE's to a small but statistically significant extent. In this respect it may be mentioned that, Latt *et al.* [29] reported that small, but statistically significant increases in SCE frequencies, may not be indicative of a meaningful biological effect, but may reflect inherent variability in the system used.

The ability of methamidophos to induce chromosomal aberrations and SCE's *in vitro* was tested in a primary culture of mouse spleen cells. The method used in our laboratory for culturing mouse spleen cells and described here provides a reproducible means for culturing the spleen cells and yields sufficient numbers of metaphases for scoring both chromosomal aberrations and SCE's.

Analysis of chromosomal aberrations has been the most commonly used method of detecting DNA damage in mammalian cells [30, 31].

The chromosomal aberrations induced by methamidophos are structural including fragments, gaps, few ring chromosomes and numerical in the form of tetraploid cells. These aberrations were observed after treatment of cultured mouse spleen cells with all the used concentrations of methamidophos. Richardson *et al.* [32] reported that, when assessing the data derived from *in vitro* cytogenetic assays it is usual to regard gaps as a separate category of damage and to then accord all other types of damage a greater but equivalent weighting.

The percentage of chromosomal aberrations induced by the different concentrations of methamidophos was statistically significant after excluding the metaphases with chromosome and chromatid gaps.

In the present study, mitomycin C, was included essentially as a positive control. It has been established that mitomycin C is mutagenic [22, 33, 34] and clastogenic [35–37]. Mitomycin C is one of the most powerful inducers of sister-chromatid exchanges [25]. The induction of SCE's by mitomycin C may reflect the action of basis cellular DNA repair processes. It is known to alkylate DNA [38].

The induced increase in the SCE's frequency after treatment the spleen cell cultures with the tested concentrations of methamidophos was dose-dependent and was much lower than that produced by the positive control mitomycin C.

Methamidophos was found to be a weak chemical mutagen in cultured red muntjac diploid cells using assays for chromosome aberrations, sister chromatid exchanges and cell cycle delay [39]. Methamidophos induced no increase of SCE and induced cell cycle delay in Chinese hamster V79 cells after 27 h of treatment [40].

However, methamidophos showed mutagenic potential in our studies. In one study, treatment of both seeds and roots of *Vicia faba* induced a statistically significant percentage of abnormal mitoses in root meristems [41]. Spraying *Vicia faba* plants with a solution of pure methamidophos (500 ppm) caused a significant percentage of abnormal pollen mother cells [42]. In this study, single and multiple i.p. injections at 6 and 4.5 mg/kg body weight, oral administration for 14 consecutive days at dietary level of 100 ppm and multiple dermal treatments (total 4) with 24 mg/kg body wt. induced a statistically significant increase in the frequency of PEs with micronuclei in mouse bone marrow. Moreover, the tested concentrations of methamidophos as low as 0.25 μg/ml induced a high percentage of metaphases with chromosomal aberrations in cultured mouse spleen cells.

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