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BACTERIAL MUTAGENICITY OF DITHIOCARBAMATE FUNGICIDE THIRAM

AKTYWNOŚĆ MUTAGENNA TIURAMU W TESTACH BAKTERYJNYCH

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In the present work, within a project of re-evaluation of authorized pesticides coordinated by PZH (National Institute of Hygiene) we aimed at looking for a mechanism of induction of chromosomal aberrations by thiram. We checked its ability to damage bacterial DNA.

INTRODUCTION

Thiram (tetramethyl-bis-thiocarbamyl disulphide) (CAS N° 137-26-8) is an important member of the dithiocarbamic acid derivatives extensively used as fungicide in agriculture as well as in the rubber industry as accelerators and source of sulphur. That is why it is a subject of interest for genotoxic studies for more than twenty years. The main achievement of these studies has been the finding that thiram induces the development of chromosomal aberrations in eukaryotic cells. It was initially shown to occur in murine bone marrow cells [2] and mice sperm [30] and then confirmed by sister-chromatide exchange in human lymphocytes (SCE) [19,20,21], by bone marrow micronucleus test (PCE) [6] and in Chinese Hamster cell lines [17]. In *in vivo* systems thiram was positive in the sex-linked recessive lethal mutations and in the white/white (w/w) somatic mutation and recombination test (SMART) in *Drosophila melanogaster* [4,7].

Thiram is a very reactive compound with a strong metalbinding characteristics and capable to interact with protein SH-group [8]. It has been also shown to induce base substitutions and, at a lower degree, frameshift in several bacterial reversion tests [6, 9, 29] and to activate the excision repair system in *S. typhimurium* [29]. Although the above findings could explain some genotoxic properties of thiram, they are not sufficient to suggest a mechanism of the induction of chromosomal abnormalities caused by this compound.

The main reason of chromosomal abnormalities induced by chemical compounds is a direct interaction of chemicals with DNA resulting in its damage (for review see: [18] and [24]). Looking for the mechanism of thiram's action on chromosomes we checked its ability to damage bacterial DNA using several bacterial strains. *Ames* tester

strain TA1O2, *E. coli* PQ37 which reveals the induction of the SOS response, *E. coli fpg* and *fpg uvr* mutants allowed to check for a possible effect on modification of purines and *E. coli* AB1157 and AB1157 *ada3* which allow to detect DNA alkylation.

MATERIALS AND METHODS

Chemicals

The following chemicals were obtained from the sources listed below: Thiram (CAS No 137-26-8) (purity 99.8%) was a product of „Organica-Azot" Jaworzno, Poland; Tris (hydroxymethyl) - aminomethane (Tris), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), methyl methanesulphonate (MMS), 2-aminoanthracene and mitomycin C (MMC) from Sigma; l-oxide-4-nitroquinoline (NQO) from Fluka AG; aflatoxin Bl (A F B1), O-nitrophenyl-bb-D-galactopyranoside (ONPG) from Calbiochem; p-nitrophenyl disodium phosphate (PNPP) from Merck; Aroclor 1254 from Analabs Inc.; sodium dodecylsulphate (SDS), Bacto trypton and Bacto yeast extract from Difco. All control mutagens and thiram were dissolved in dimethyl sulphoxide (DMSO) - spectrophotometric grade, obtained from Serva. Fresh solution of all chemicals were prepared immediately before use.

Bacterial strains

Strains derived from *E. coli* K-12 were: AB1157, (*F, thr-1, leu-6, proA2, his-4, thi-1, argE, lacY1, galK, ara-14, xyl-5, mtl-1, tsx-33, supE44, rpsL31*); BH2O, as AB1157 but *fpg-l: :Kn*; BH19O, as AB1157 but *fpg-1: :Kn, uvrA: : Tn10* were a gift from *S. Boiteeux* (Inst. Gustave Roussy, Villejiuf, France); PQ37 (*sfiA : : Mudd*) *AP lac/cts, lacU169, mal*$^{+}$, *uvrA, galE, galY, PhoC, rfa, F, thr, leu, his, pyrD, thi, trp: : Muc*$^{+}$, *sr1300: :Tn10, rpoB* used in the SOS Chromotest and AB1157ada3, as AB1157 but *ada3* used for assaying alkylation in the 0^{6}-position of guanine were gifts from *P. Quillardet* and *M. Hofnung* U.B.M.T.G. Institut Pasteur, Paris, 4 France. *S. typhimurim TA102 hisG428 rfa pkM101*, was kindly sent by Prof. *B.N.Ames* (University of California, Berkeley, CA).

Bacterial tests:

Ames test was performed with *S.typhimurium* strain TA102 and liver homogenate fraction (S9) was prepared according to the method of *Maron* and *Ames*, [15].

The induction of the SOS response by thiram was measured in *E.coli* PQ37 strain by means of a *sfi A: :lacZ* operon fusion according to the principle of the SOS Chromotest [24]. Cultivation of bacteria and measurements of bb-galactosidase and alkaline phosphatase activities were performed as described *Quillardet* and *Hofnung* [22]. Bacteria were exposed to thiram for 2 h with vigorous agitation.

Each assay was accompanied by positive controls: 4NQO (20 ng/assay) was used for estimation without metabolic activation, and aflatoxin Bl (20 ng/assay) for estimation with metabolic activation. The S9 mix used in the SOS Chromotest was prepared according to *Quillardet* and *Hofnung* [22].

The units of enzyme activity were calculated using a simplified version of the formula used for calculation of international units [16]:

$$Enzyme\ unit = \frac{1000 \times A_{420}}{t}$$

where:

„A" is the optical density at 420nm

„t" is the time of incubation in the presence of the substrate (ONPG or PNPP) in minutes.

Effect of thiram on survival of *E.coli* AB1157, BH2O *fpg* and BH190 *fpguvrA*. The bacteria were grown at 37°C in LB broth supplemented when required with 40 μg/ml kanamycyn sulphate.

Thiram or MMS were added at the appropriate concentrations and the cell suspension was incubated for 60 min at 37°C with agitation. At the end of incubation the suspension was diluted and placed on nutrient agar. The plates were incubated overnight at 37°C.

The test for alkylation in the 0^6position of guanine was performed by the method described by *Jeggo et. al.*, [11, 12] with modifications described below. All media and salt solutions used in the test were prepared according to *Miller* [16]. The procedure of the test was as follows: the log-phase (A_{600} = 0.2) bacterial cultures were separated from the LB medium by centrifugation. 5 ml of the minimal medium supplemented with M63 salts and thiram (0 - 100 μg/ml) was added to the pellet. Higher doses of thiram were toxic for *E. coli*. In case of metabolic activation the same concentrations of thiram were preincubated for 20 minutes with 100 μl of S9 fraction in 0.5 ml of S9 mix, and, subsequently, were diluted to 5 ml with the minimal medium supplemented with M63 salts.

In both cases, after 30 minutes of incubation, bacterial cultures were centrifuged, washed with 5 ml of M9 salts, and suspended in 5 ml of M9. Samples were diluted accordingly and placed on media selecting for arg^+ revertants. A small amount (1 μg/ml) of arginine was added to the top agar. After 48-60 hours of incubation at 370 C the number of revertants per 10^7 bacterial cells was estimated and compared for AB1157 and AB1157*ada3* trains. MNNG (0.5 μg/ml) was used as a positive control.

RESULTS

In order to reveal possible damages introduced by thiram in DNA we used four bacterial strains. We found no effect of tested fungicide at nontoxic concentrations in any of the above strains. The results ilustrating the lack of ability of thiram to introduce cross-links or oxidative damages *in S. typhimurium* TA102 strain as well as to induce the SOS system in *E.coli* PQ37 are presented in Tabs I and II, respectively. In the latter case the activity of alkaline phosphatase which is syntetized constitutively in the used strain was treated as a control for the overall protein syntesis. Thiram was found to be non deleterious to alkaline phosphatase activity at the tested range of concentration (Table II).

Figs 1A and B show that the sensitivity of bacteria to thiram was not modified in the fpg strain as compared with the wild type. In both strains a similar inhibition of survival was obtained (e.g. about 70% at 420 mM thiram). This was in contrast with the sensitivity to MMS where a clear difference between a survival of the mutant and wild *E. coli* strain was revealed (eg. 1% and 45%, respectively, at 6 mM MMS). Modifications of purines, if they occured, could be excised by UvrABC nucleases [18]. To exclude this possibility we employed in this test additionaly a double mutant fpg uvrA. However, the survival of fpguvrA mutant was not significantly decreased as compared to the fpg mutant or the wild- type strain (Fig. 1A).

The ability of thiram for DNA methylation in the 0^6-guanine position was investigated comparing the mutagenic activity of thiram in the AB1157 strain and

Table I. Mutagenic evaluation of thiram in *Ames* test with *S. typhimurium* strain TA102. Określenie aktywności mutagennej tiuramu testem *Amesa* w szczepie *S. typhimurium* TA102

Compound (μg/plate)	N° of *his*+ revertants/plate ± SD			
	– S9	% ± SD	+ S9	% ± SD
DMSO	460 ± 33	100	630 ± 33	100
2	451 ± 11	98 ± 2	611 ± 130	97 ± 21
5	420 ± 14	91 ± 3	917 ± 70	98 ± 11
10	371 ± 59	81 ± 13	664 ± 94	105 ± 15
25	421 ± 55	92 ± 12	731 ± 92	116 ± 15
50	448 ± 50	97 ± 11	594 ± 123	94 ± 20
100	417 ± 32	91 ± 7	702 ± 99	112 ± 16
150	420 ± 20	91 ± 4	625 ± 60	99 ± 9
250	210 ± 30	46 ± 7	500 ± 30	79 ± 5
mitomycin C	2300 ± 250		NT	
2–aminoanthracene	NT		1150 ± 90	

Revertants expressed as x ± SD, average of 12 plates. 50 μl S9/plate were added
NT – not tested

AB1157*ada3*strain of *E.coli*. The results are presented in Table III. No increase in the number of *arg*$^+$ revertants in the AB1157*ada3* strain in relation to the AB1157 strain was observed, in the presence or absence of the S9 fraction, pointing to inability of thiram and its metabolites to methylate DNA in the 0^6-guanine position. As a positive control MNNG was used, which induced 12 times more *arg*$^+$ revertants in A Bll57*ada3* strain than in AB1157.

DISCUSSION

S. typhimurium TA102 strain enabled the detection of oxidative and cross-linking mutagens [13]. *E. coli* PQ37 was used to reveal the induction of the SOS response, a set of functions specifically induced by a number of types of DNA damage [10, 28]. The strain contained an *uvrA* mutation which abolished excision repair, a process essentially active on lesions affecting DNA conformation [25]. *E. coli fpg* and *fpg uvrA* mutants allowed to check or a possibible effect on modification of purines. The Fpg protein of *E.coli* was initially identified as a DNA glycosylase which excided the imidasole ring-opened form of N-methylguanine residues in DNA (Fapy) [3]. The biological importance of Fapy-DNA glycosylase activities is sugested by the fact that the enzyme has been conserved in both prokaryotes [5] and eukaryotes [14]. The Fpg protein excises imidasole ring-opend purines and nicks DNA at apurinic/apirymidynic sites [4]. *E. coli* AB1157 and AB1157*ada3* non-damaged and damaged function of ada gene, respectively, allow to detect DNA alkylation. This test is based on the ability of bacterial cells to the „adaptation reaction" caused by agents. Low doses of alkylating

Table II. Effect of thiram and its metabolites on the SOS system induction in PQ 37 strain of *E. coli* K–12
Wpływ tiuramu i jego metabolitów na indukcję systemu SOS w szczepie *E. coli* K–12 PQ 37

Compound (nmol / sample)	– S9mix			+ S9mix		
	Uβ	UP	IF	Uβ	UP	IF
DMSO	1.2	19.9	1	0.9	20.8	1
0.002	1.8	21.8	0.9 ± 0.1	0.9	19.8	1.1 ± 0.1
0.02	1.8	22.8	0.8 ± 0.2	0.8	20.3	0.9 ± 0.1
0.1	1.2	19.9	0.8 ± 0.2	0.8	22.7	0.8 ± 0.1
0.2	1.0	18.2	0.8 ± 0.1	0.8	21.6	0.8 ± 0.1
0.3	1.1	21.2	0.7 ± 0.1	0.9	20.9	1.1 ± 0.2
0.4	0.9	17.4	0.9 ± 0.1	0.9	19.6	1.1 ± 0.2
0.6	0.8	16.3	0.8 ± 0.1	0.8	17.6	0.8 ± 0.1
0.8	0.7	14.6	0.8 ± 0.2	0.9	19.6	1.2 ± 0.2
NQO – 105	8.2	19.3	6.7 ± 1.1			NT
AFB1 – 0.064			NT	6.1	19.0	7.1 ± 1.2

Uβ – international units of β–galactosidase activity
UP – international units of alkaline phosphatase activity
IF – induction factor is the ratio of the activities, β–galactosidase/alkaline phosphatase, divided by its value at concentration O of the compound tested.
Each value is an average from 5 independent experiments SD.
NT–not tested.

agents induce the synthesis of 0^6-methylguanine-DNA methyltransferase coded by the *ada* gene [27]. This enzyme transfers alkyl residues from 0^6-guanine onto its own protein repairing in this way the mutagenic damage of DNA and reducing the number of mutations induced by alkylating agents. We found no effect of thiram at nontoxic concentrations in any of the above strains.

The results of this work exclude DNA cross-links, oxidative damage of DNA, opening of imidazole ring in purines and events leading to activation of the SOS system as well as adaptive response as direct reasons of chromosomal abnormalities induced by thiram.

Thiram-induced damages in DNA have been revealed as the activity of the excision repair system in *S. typhimurium* [29]. Such activity can reflect various changes in DNA introduced by the tested agent (for review see: [26]). *Rannug* and *Rannug* [23] have suggested that genotoxicity of thiram could be caused by its ability to induce oxidative damages. Our results do not confirm this suggestion, in agreement with the observation of *Crebelli et al.* [6], who did not identified thiram mutagen of oxidative type. Our results excludes a covalent reaction of thiram with DNA which could be the most serious reason of chromosome aberrations, if occurred. This type of DNA damage

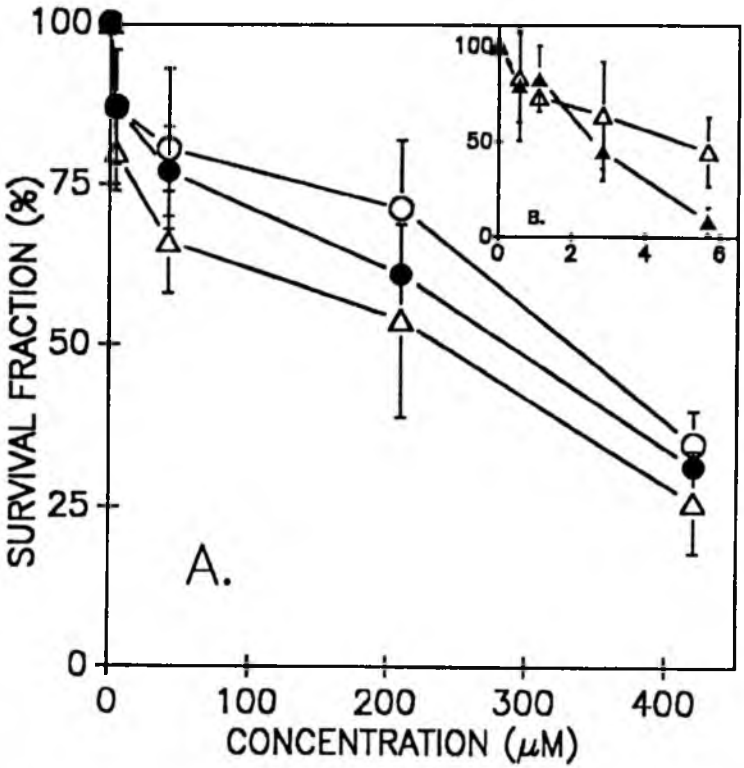

Fig. 1A and B. Effect of thiram and MMS on survival of *E.coli* AB1157, BH20 *fpg* and BH190 *fpguvrA*.
AB1157 with thiram (o — o) and with MMS (△—△); BH20 with thiram (●—●) and with MMS (▲—▲) ; BH190 with thiram (△—△).

would be revealed by two tests performed in this work: that indicating cross-linking mutagenes and the one showing a SOS response.

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Streszczenie

Badając przyczyny indukcji aberracji chromosomowych przez tiuram sprawdzono jego zdolność do wprowadzania uszkodzeń w DNA bakteryjnym. Aktywność mutagenną tiuramu określono stosując: (i) szczep *S. typhimurium* TA102 w teście *Amesa*, (ii) szczep *E.coli* PQ37, który pozwala na wykrycie indukcji systemu naprawy SOS (III) test z mutantami *E. coli BH20 fpg* i *BH190 fpguvrA*, który pozwala na wykrycie modyfikacji puryn, (iv) test ze szczepami *E. coli* AB1157 oraz AB1157*ada3* wykrywającymi alkilację DNA w pozycji 0^6 guaniny.

Stwierdzono, że anomalie chromosomalne indukowane w komórce przez tiuram nie są spowodowane: wprowadzaniem wiązań krzyżowych w DNA, uszkodzeniami oksydacyjnymi oraz indukcją systemu naprawy SOS przez badany związek. Tiuram nie powoduje również otwarcia

Table III. Effect of thiram and its metabolites on arg^+ mutation induction in AB1157 and AB5557*ada3* strains of *E. coli* K–l2
Wpływ tiuramu i jego metabolitów na indukcję mutacji arg^+ w szczepach *E. coli* K–12 AB1157 i AB5557*ada3*

Compound (μg/ml)	N° of arg^+ revertants/10^7 cells ± SD[a]			
	– S9		+ S9[b]	
	AB 1157	AB 1157*ada3*	AB 1157	AB 1157*ada3*
Thiram				
0	0.36 ± 0.12	0.43 ± 0.11	0.16 ± 0.02	0.42 ± 0.12
0.01	0.50 ± 0.13	0.30 ± 0.22	0.57 ± 0.12	0.45 ± 0.13
0.10	0.40 ± 0.11	0.23 ± 0.21	0.10 ± 0.09	0.70 ± 0.13
1.00	0.56 ± 0.14	0.52 ± 0.30	0.39 ± 0.22	0.43 ± 0.11
10.00	2.50 ± 0.32	1.20 ± 0.22	0.71 ± 0.21	0.50 ± 0.14
100.00	2.30 ± 0.25	1.40 ± 0.20	0.65 ± 0.14	0.19 ± 0.01
DMSO-20μl	0.46 ± 0.10	0.86 ± 0.14	0.57 ± 0.13	0.76 ± 0.15
MNNG – 0.5 μg	6.50 ± 1.10	80.50 ± 11	NT	NT

[a] – average of 12 plates
[b] – 100 μl S9 per probe (5ml)
NT–not tested

pierścienia imidazolowego puryn, ani metylacji DNA w pozycji 0^6 guaniny oraz odpowiedzi adaptacyjnej.

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Summary

Looking for a mechanism of induction of chromosomal aberrations by thiram we checked its ability to damage bacterial DNA. Evaluation of the mutagenic effect of thiram was by: (i) the *Salmonella*/mammalian microsome *Ames* test with *S. typhimurium* TA102 strain. (ii) *E. coli* PQ37 strain to reveal an induction of the SOS response, (iii) test with *E. coli* BH20 fpg^- and BH190 fpg^-uvrA^- mutants to check possible effect of modification of purines, (iv) test with *E. coli* AB1157 and AB1157*ada3* to detect DNA alkylation in 0^6-position of guanine. The results of this work exclude DNA cross-links, oxidative damage of DNA, opening of imidazole ring in purines and events leading to activation of the SOS system as well as adaptive response as direct reasons of chromosomal abnormalities induced by thiram.

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Received: 1996.06.12