ENVIRONMENTAL BIOTECHNOLOGY 9 (2) 2013, 65-71; doi:10.14799/ebms193

Assessment of mutagenic activity of methyl- and phenylphenanthrenes based on *Salmonella* test and micronucleus test

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Key words: Ames test, *in vitro*, micronucleus test, mutagenicity, phenanthrenes

Received in February 2013. Published in April 2014.

ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are widely spread environmental pollutants mainly originating from anthropogenic sources such as fossil fuel combustion, industries, and others. Although a large body of literature exists on the toxicity and carcinogenicity of PAHs, primarily benzo[*a*]pyrene, toxicity data for phenanthrene deriveratives are very limited. The main aim of the experiment was to investigate if there exists correlation between molecular structure and mutagenic activity of four phenanthrene derivatives: 1-methylphenanthrene, 4-methylphenanthrene, 1-phenylphenanthrene, and 4-phenylphenanthrene. An Ames assay using two strains of histidine dependent *Salmonella* Typhimurium (TA98 and TA100) was conducted to assess the mutagenic activity of studied compounds both in the presence (+S9) and in the absence (-S9) of an exogenous source of metabolic activation. The compounds were also tested in an *in vitro* chromosome aberration assay in which V-79 cells were exposed to the phenanthrene derivatives investigated both in the presence and in the absence of metabolic activation. The phenylphenanthrenes showed no mutagenic effect. These compounds occasionally induced significant decrease in the number of revertants in the Ames test. The greatest mutagenic effects were observed for 1-methylphenanthrene after metabolic activation (+S9). In the micronucleus test the greatest mutagenic effect was observed for 4-methylphenanthrene also in the presence of metabolic activation system. The results obtained are comparable to those reported earlier for the methylphenanthrenes.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are widely spread environmental pollutants mainly originating from anthropogenic sources such as fossil fuel combustion, industries, and others, however, vehicular traffic has been one of the major sources of air pollution (Gaffney and Marley 2009; Ravindra et al. 2008). PAHs which are released into the atmosphere are redistributed between the gaseous and particulate phases. Low-molecular-weight (LMW) PAHs tend to be more concentrated in the vapour phase, while those with higher molecular weight (HMW) are often associated with particulates (Bidleman 1988; Harner and Bidleman 1998; Pankow 1998). Dry and wet deposition and volatilization from the water and soil are the main processes that contribute to air/water and air/soil exchange of PAHs (Godish 1991). These molecules are predominantly found in the environment surrounding urban zones with high

vehicular density and/or presence of industrialized areas (Godish 1991; Macdonald et al. 2005).

PAH toxicity ranges from non-toxic through extremely toxic to humans. Many PAHs are mutagenic (Mielzynska et al. 2006), carcinogenic (Bostrom et al. 2002), and/or teratogenic (Singh et al. 2008).

Phenanthrene and its derivatives can be derived from coal tar. Phenanthrene is ubiquitous in the environment as a product of incomplete combustion of fossil fuels and wood (Wrobel and Reinhardt 2003). It appears in the exhaust gas produced in diesel engines (Levsen 1988) and has been identified in ambient air, surface and drinking water, and in foods (IARC 1983; U.S. EPA 1988). Based on data from animal bioassays, U.S. EPA (1987, 1993) has placed phenanthrene in weight-of-evidence group D, not classifiable as to human carcinogenicity. The compound accumulates in the tissues and is toxic to the aquatic form of liverwort *Riccia fluitans* (Burrit 2008).

Data regarding the gastrointestinal or pulmonary absorption of phenanthrene in humans are not available. However, data from structurally related PAHs suggest that phenanthrene would be absorbed readily from the gastrointestinal tract and lungs. In general, PAHs are highly lipid-soluble and can pass across epithelial membranes (U.S. EPA 1987). Although many PAHs accumulate in body fat, Bock and Dao (1961) found little phenanthrene in the perirenal and mammary fat of rats administered the compound by gavage 24 hours earlier. Phenanthrene itself is one of the PAH model molecules because of its low toxicity, as well as its structural and physical similarity to other PAHs (Dabestani and Ivanov 1999).

Among others, phenanthrenes and their derivatives are the most common PAHs widely distributed in the whole environment. Although an extensive body of literature exists regarding the toxicity and carcinogenicity of PAHs, primarily benzo[*a*]pyrene, toxicity data for phenanthrene and their deriveratives are very limited. Phenanthrene is a relatively good substrate for cytochrome P-450 monooxygenases (Nordqvist et al. 1981). Studies in the early 1980-ties have shown that for different strains of *Salmonella* Thyphimurium (TA97, TA98, TA100) phenanthrene, as well as its metabolites, was not mutagenic (Sakai et al. 1985). Mutagenic activity of some methyl derivatives of phenanthrene has been previously reported (LaVoie et al. 1981). In 1953, attempts were made to study 2-phenylphenanthrene derivatives, which were characterized by a slight activity or did not show any biological activity. Even in small quantities these compounds occurring together with other fuel components, can cause mutagenic or carcinogenic effects (Levsen 1988). The carbon skeleton of the phenanthrene molecule is an excellent model for the study of correlations of PAH structure and substitution pattern with its genotoxic activity (Łuczyński et al. 2005).

In this paper, from the large group of methyl- and phenylphenanthenes, four compounds were selected because it is likely that the structure of their molecules promotes their conversion by organisms to mutagenic active metabolites.

MATERIAL AND METHODS

Chemistry

1-methylphenanthrene, 4-methylphenanthrene, 1-phenylphenanthrene and 4-phenylphenanthrene were synthesized because they were not available commercially. All chemicals were synthesized at the Faculty of Chemistry in Jagiellonian University by the research group of Chemistry of Carbocyclic Compounds. Purity of samples was confirmed by NMR spectroscopy and was greater then 95%. Gas chromatography (GC-FID) analysis showed no impurities. Yield of isolated phenananthrene derivatives was within range of 81-95%. Chemical structure of these compounds is in Figure 1.

Synthesis scheme for methyl-, and phenylphenanthrene is shown in Figure 2.

The methyl and phenylphenanthrenes were obtained according to modified synthesis published by Haworth (1932) except for the first two steps leading to naphthylbutyric acids (Figure 2 steps **4** and **5**) which remained unmodified. Compared to the original procedure significant improvements were achieved at the cyclisation step. Heating of **4** and **5** in anhydrous methanesulfonic acid led directly to the production of phenanthrenones **6** and **7** with yields exceeding 60%. This

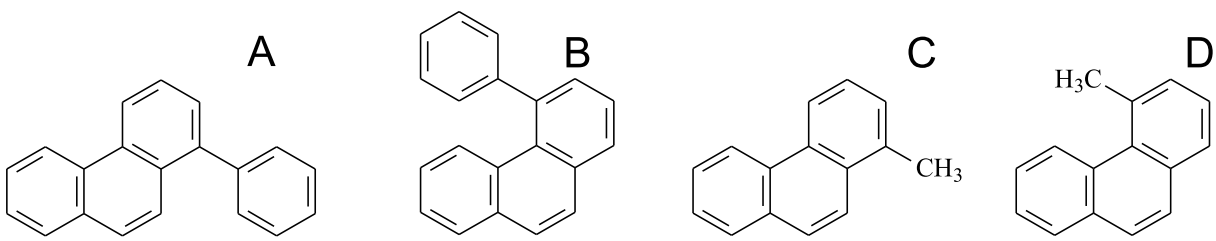

Figure 1. The structure of tested chemicals: A, 1-phenylphenanthrene; B, 4-phenylphenanthrene; C, 1-methylphenanthrene; D, 4-methylphenanthrene.

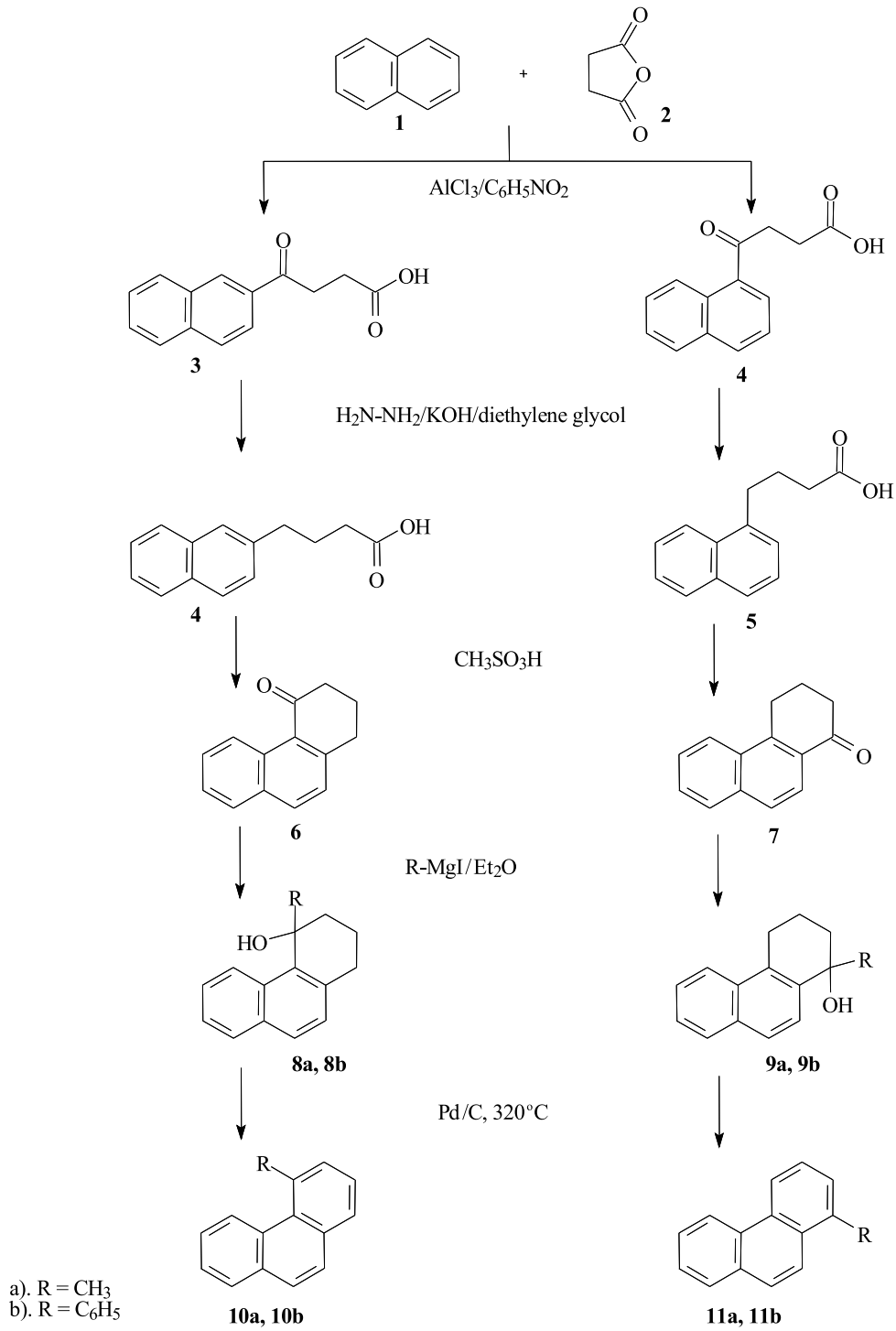

Figure 2. Scheme of synthesis of methyl- and phenylphenanthrene (Rospondek et al. 2009).

procedure allow to avoid the conversion of the naphthylbutyric acids to appropriate acid chlorides and further cyclisation in presence of $SnCl_4$. Reaction of phenylmagnesium or methylmagnesium iodides with 1,2,3,4-tetrahydrophenanthrenones **6** and **7** resulted in formation of phenanthrols **8** and **9**. Heating of isolated phenanthrols with 10% palladium on charcoal under slow stream of argon caused their simultaneous dehydration and aromatization to methyl- and phenylphenanthrenes **10** and **11**. Melting points and spectroscopic data of isolated methyl- and phenylphenanthrenes were identical with those previously obtained (Rospondek et al. 2009).

The *Salmonella* test

Histidine dependent strains TA98 and TA100 of *Salmonella* Typhimurium have been purchased from TRINOVA Biochem GmbH Germany.

The Ames mutagenicity test was conducted with two bacterial strains of *S*. Typhimurium (strains TA98 and TA100) with and without metabolic activation (±S9 mix). The S9 microsome fraction is induced with Aroclor 1254 (Trinova Biochem GmbH Germany) and is able to stimulate eukaryotic processes that cannot be conduced by microorganisms themselves. The strains were cultured as described by Maron and Ames (1983) and Mortelmans and Zeiger (2000). Confirmation of the genotypes of the tester strains was routinely carried out, including crystal violet, UV and ampicillin sensitivities. The number of spontaneous revertants, induced in the negative controls (solvent, S9) and following exposure to such diagnostic mutagens as NQNO ($10\mu g \cdot plate^{-1}$) and B[*a*]P ($10\mu g \cdot plate^{-1}$) were measured in each experiment. In these studies, the tested compound was dissolved in 80μL of DMSO and added to 2.5mL molten top agar (at 42°C) with 18h of nutrient broth culture of appropriate strain of *S*. Typhimurium and 0.5mL of S9 mix. The final mixture was poured onto minimal glucose agar plates. Mutations from histidine-dependent to histidine-independent bacteria were assessed 48h after plating by counting the colonies of bacteria in the Petri dishes. The compounds were assayed in triplicate at each dose level based on the solubility of the compounds.

In vitro micronucleus test (MN)

Cell line V-79 was purchased from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Germany.

In vitro micronucleus test was conducted with V-79 cell line according to ISO 21427-2:2006. It was performed on 4-well plates (1mL of medium) and consisted of four stages. Test conditions for all steps were the same: temperature 37°C, humidity 95% and 5% carbon dioxide atmosphere. For all the steps culture medium consisted of 90% of MEM (Minimal Essential Medium), 10% of FBS (Fetal Bovine Serum) and 1% of antibiotics solution (Amphotericin B, Streptomycin, Penicillin) (Rudnicka et al. 2012).

The pre-incubation of cell cultures was carried out with use of minimal essential medium supplemented with 10% of fetal bovine serum, 1% antibiotics. At the beginning of the test, cell density was $5\text{-}8 \cdot 10^4$ cells per well. Incubation was carried out for 48 hours. Next stage was divided into two variants. In the variant without metabolic fraction (-S9) the cell cultures after pre-incubation were covered with fresh culture medium supplemented with appropriate concentrations of test substances, based on the toxicity according to OECD guideline for testing chemicals (maximal volume of tested chemical if dissolved in DMSO should be 1% of volume of the well), with negative and positive controls. Then the cells were incubated for 24 hours. The second variant was preceded by incubation of the test substances, with negative and positive controls, with addition of S9 (+S9) (in this variant FBS should not be added to culture medium) and the appropriate volume of culture medium for 1 hour. After incubation, the solutions obtained were filtered on sterile syringe filters (0.22μm). Cell cultures were covered with solutions of test substances. Incubation was performed for 24h. After incubation with the respective chemicals culture medium was removed from the solutions of test substances. Then the cultures were covered with fresh medium supplemented with cytochalasin B at a concentration of $3\mu g \cdot mL^{-1}$. Cells were then incubated for 24h. At the end of the test, the Giemza staining protocol was employed. Thereafter probes were closed with use of DPX mounting medium and counted with use of light microscope (Fenech 2006; Fenech et al. 1999; Phelps et al. 2003).

Statistical data analysis

The method of least squares regression analysis and analysis of variance were used. Doses of test compounds were treated as an independent variable, and the number of revertants induced in strains of *S*. Typhimurium (TA98 and TA100) as the dependent variable. The number of revertants in the corresponding negative control was treated as a response to zero dose. In the event of a toxic effect or appearance of the same response with increasing doses, the analysis was performed again with the rejection of the results for the highest doses. In order to compare the mutagenic potency of the tested compounds their mutagenic rates (MR) were calculated. MR is a relative measure of the mutagenic potency and is defined as the ratio of the number of revertants observed at a given dose to the average number of revertants observed in the negative control. A positive mutagenic response is indicated when MR values are ≥2.

For the *in vitro* micronucleus test data from the experiments were analyzed using Student t-test, considering number of micronuclei observed in 1000 cells at each concentration level, including the control. A type I error rate of 0.05 was used in the statistical analyses. A positive effect was declared for a chemical when *p*-value was less than or equal to 0.05.

RESULTS

Salmonella test

None of the four studied compounds showed mutagenic activity in the absence of a metabolic activation (-S9).

For *S.* Typhimurium strain TA98, only 1-methylphenanthrene showed a distinct mutagenic effect in the presence of S9. The highest activity appeared at a concentration of 50μg·plate^{-1} and was associated with MR value of 11.25. The number of revertants at the 200μg·plate^{-1} dose level was significantly lower, but there was still a significant mutagenic effect observed (MR=8.44) (Figure 3). 4-methylphenanthrene showed weak toxic effect for strain TA98 (Figure 4).

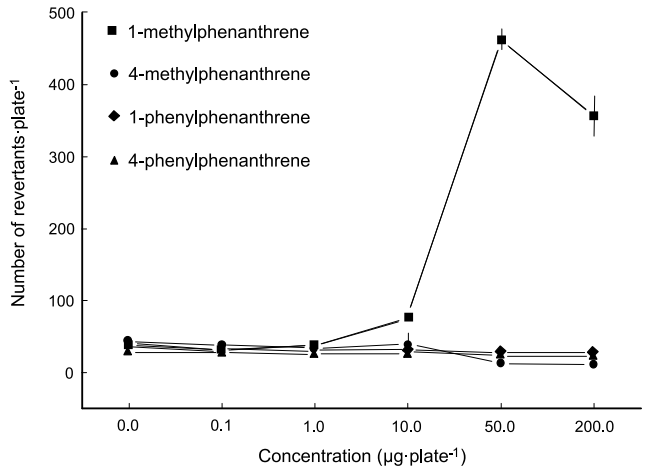

Figure 3. Number of revertants caused by phenanthrene derivatives and their metabolites (+S9) in the *Salmonella* Typhimurium strain TA98, mean values±0.95 confidence interval.

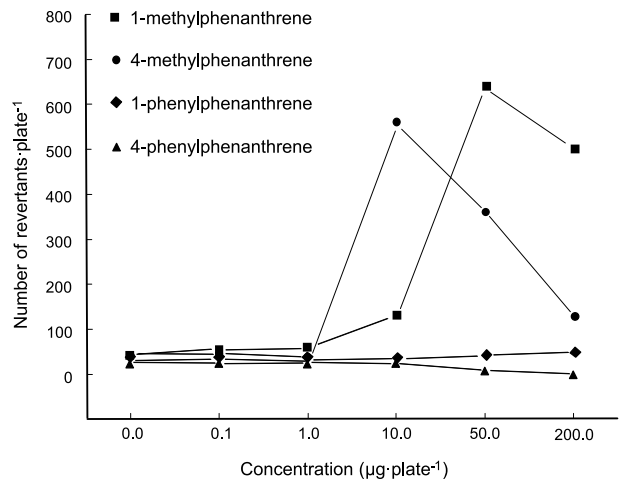

Figure 4. Number of revertants caused by phenanthrene derivatives and their metabolites (+S9) in the *Salmonella* Typhimurium strain TA100, mean values±0.95 confidence interval.

In the case of strain TA100, a mutagenic effect occurred in the variant of the enzyme activation. 1-methylphenanthrene induced higher number of revertants (Figure 4) what indicated mutagenic activity of this compound. The highest effect was caused by the dose of 50μg·plate^{-1} (MR=4.13). The highest number of revertants for 4-methylphenanthrene appeared at the concentration of 10μg·plate^{-1} (MR=3.56).

In vitro micronucleus test

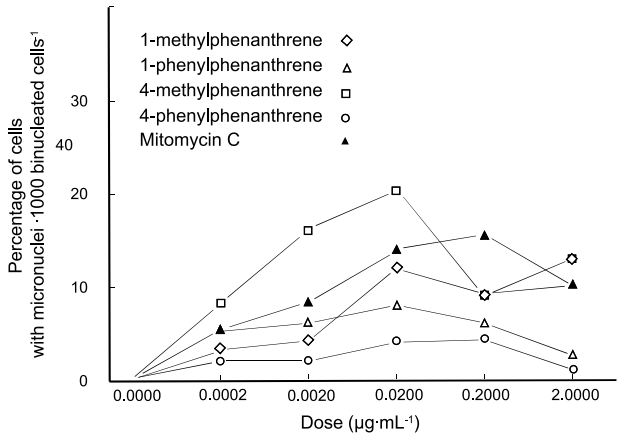

Figure 5. Percentage of micronuclei among 1000 binucleated cells induced by phenanthrene derivatives in V-79 cells (-S9), mean values±0.95 confidence interval (spontaneous micronulei 0.3%).

All examined methyl- and phenylphenanthrenes showed evidence of mutagenic effects at all concentrations (Figure 5, 6). These chemicals induced significant increase in number of

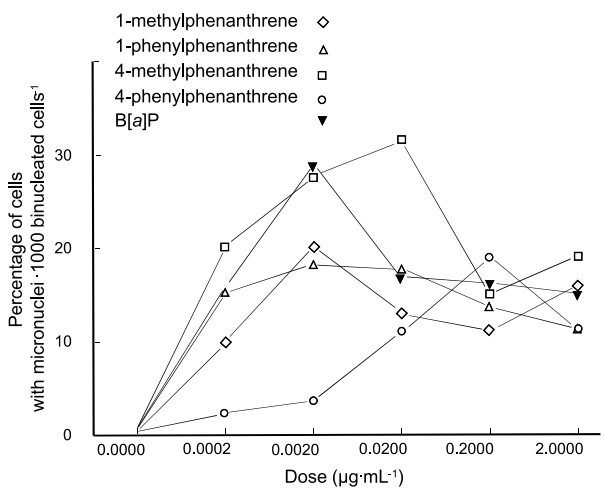

Figure 6. Percentage of micronuclei among 1000 binucleated cells induced by phenanthrene derivatives in V-79 cells (+S9), mean values±0.95 confidence interval (spontaneous micronulei 0.3%).

micronuclei in V-79 cells, but the effect was more pronounced with S9 metabolic activation. The greatest effect was observed for metabolites of 4-methylphenanthrene. These compounds at a dose of 0.02μg·mL^{-1} induced the maximum number, 317 cells with micronuclei per 1000 binucleated cells (MN/1000BC). At the same dose, metabolites of 1-phenylphenanthrene caused 178 MN/1000 BC. The weaker response at the same dose appeared for metabolites of 1-methylphenanthrene (120 MN/1000BC) and 4-phenylphenanthrene (115 MN/1000 BC), but there was still a mutagenic effect observed.

DISCUSSION

The main aim of the experiment was the examination of potential mutagenic activity of four phenanthrene derivatives and their metabolites. In their pure form, PAHs, like phenanthrene, cannot be considered as strong carcinogens (Korytko et al. 2000), but they can become highly mutagenic as a result of their metabolism, especially by P-450 enzyme systems (Ioannides 2007).

The mutagenicity and *in vitro* metabolism of selected methylphenanthrenes have been previously evaluated. The monomethyl isomers which were mutagenic toward *S.* Typhimurium were 1- and 9-methylphenanthrenes. Among the disubstituted phenanthrenes assayed for mutagenicity, 1,4-dimethylphenanthrene was active in the presence of metabolic activation (LaVoie et al. 1981). In our studies we confirmed the mutagenic activity of 1-methylphenanthrene in the *Salmonella* test, which was observed for both tested strains: TA98 and TA100 after metabolic activation (+S9). This result indicates that metabolites of this compound can cause both frameshift and base-pair substitution point mutation. Metabolites of 4-methylphenanthrene showed mutagenic effects only for *S*. Typhimurium strain TA100. In the case of this chemical the mutagenic effect appeared at a lower concentration compared to 1-methylphenanthrene, what may suggest greater mutagenic activity of 4-methylphenanthrene. This may result from the position of the methyl group, located in position 4 (i.e., close to "bay-region"). In this position, the methyl substituent can form a "fjord-region", which has a higher affinity to DNA and is able to create DNA-adducts (LaVoie et al. 1981).

Phenylphenanthrenes and their metabolites were not mutagenic to both *Salmonella* strains. This was probably due to the fact that the test compounds did not form DNA adducts and did not lead to the formation of mutations. Mutagenicity potential of 1-methylphenanthrene is associated with inhibition of 9,10-dihydrodiol formation. LaVoie et al. (1981) studies of the methylphenanthrenes metabolism confirm that for the non-mutagenic derivatives, the of 9,10-dihydrodiol is a major metabolite. *In vivo* and *in vitro* studies indicate that metabolism of phenantrene proceeds via epoxidation at the 1-2, 3-4, and 9-10 carbons, with dihydrodiols as primary metabolites of which the 9,10-dihydrodiol is the major component. The 9-10-, 1,2-, and 3,4-dihydrodiols of phenanthrene were identified unaltered or as glucuronic acid conjugates in the urine of rats and rabbits that received intraperitoneal injections of phenanthrene (Boyland and Sims 1962; Boyland and Wolf 1950). Also the glucuronic acid conjugates of 1-, 2-, 3-, and 4-hydroxyphenanthrene, 1,2-dihydroxyphenanthrene, and 3,4-dihydroxyphenanthrene were identified (Boyland and Sims 1962). *In vitro* studies with guinea pig, rat, and mouse liver preparations also identified the 9-10-, 1,2-, and 3,4-dihydrodiols. Further oxidative metabolism to the 9,10-oxide and 1,2-diol-3,4-epoxide of phenanthrene has also been reported (Chaturapit and Holder 1978; Nordqvist et al. 1981; Sims 1970).

The results of the micronucleus test showed that metabolites of 4-methylphenanthrene caused formation of more micronuclei then 1-methylphenanthrene. These results indicate that 4-methylphenanthrene may be considered a stronger mutagen relative to 1-methylphenanthrene, a result trends to echo that of the *Salmonella* test. An increased frequency of micronuclei formation in the case of 4-methylphenanthrene may result from the position of methyl substituent which is close to the "bay region". It can be concluded that the presence of such a substituent in the "bay region" increases the mutagenic activity of phenanthrene derivatives. Methyl group in position 1 does not produce the same strong effect as for 4-methylphenanthrene. This results from the fact that the methyl group is a small substituent which does not prevent the access of metabolic enzymes to the "K-region". In contrast, greater mutagenic effect of phenyl derivatives was observed for 1-phenylphenanthrene than that for 4-phenylphenanthrene. The reason for theses results could be due to the difference in the location of the phenyl substituent. For 1-phenylphenanthrene it is close to the "K-region" what may result in the formation of diolepoxides.

Parent phenanthrene has a reduced mutagenic effect on bacteria and mammalian cells relative to 1- and 4-methylphenanthrenes (Wood et al. 1979). This difference in the type of interaction may result from differences in the molecular structure of the studied compounds. Substituents in the molecule may influence interactions between the molecule and the genetic material.

To our knowledge, there are no reports concerning particular metabolic pathways of 1-phenylphenanthrene and 4-phenylphenanthrene, so further experiments on this subject would be of interest.

ACKNOWLEDGEMENTS

This work was supported by Ministry of Science and Higher Education: project N N305 323136 and 0536/R/TO2/ 2007/03.

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