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PIOTR JADCZYK*

METHODS OF EXAMINATION OF GENOTOXICITY OF ATMOSPHERIC POLLUTANTS

Current physicochemical monitoring of atmospheric pollutants fully reflects their biological activity. This should be supplemented with evaluation of mutagenicity and carcinogenicity of those pollutants by means of biotests. Test selection should take into consideration the sensitivity of test organisms to genotoxic activity of atmospheric pollutants, conformity of the results with those of other biotests, common use of environmental mutagenesis and carcinogenesis in the tests with special consideration of atmospheric pollutants, possibility of examining the activity of atmospheric pollutants independent of atmospheric conditions, short duration of the test cycle, relatively low cost, reducing the suffering of laboratory animals to minimum. Those criteria are best fulfilled by the following tests on microorganisms and tissue cultures: the Ames test with *Salmonella typhimurium*, sister chromatide exchange induction and canceration in cells of human respiratory systems and identification of DNA adducts in human organisms.

1. INTRODUCTION

An important element of integrated environmental monitoring is the monitoring of atmospheric pollutants of genotoxic activity. It includes two complementary groups of research methods: physicochemical monitoring and biological monitoring. Physicochemical monitoring provides more and more precise information on concentration of selected pollutants. It, however, does not provide information about the effect of those pollutants on organisms living in the polluted environment. Such information can be provided only by biological monitoring.

Studies concerning mutagenicity and carcinogenicity of atmospheric pollutants, particularly those adsorbed on airborne particulate, have been conducted since invention and dissemination of mutagenicity tests, i.e., the second half of the 1970's [13], [65]. So far they have been conducted primarily in cities, i.e., in areas of relatively high atmospheric air pollution and at the same time the greatest concentration of population for which those pollutants constitute health hazards [13], [23], [74], [85].

* Institute of Environment Protection Engineering, Wrocław University of Technology, Wybrzeże Wyspiańskiego 27, 50-370 Wrocław, Poland.

Such studies have been conducted primarily in countries from West Europe [4], [13], [74], North America [23], [89], [93] and Japan [17], [48], [85]. In Poland, the study on mutagenic properties of air particulate pollutants has been conducted thus far in Upper Silesia [55]–[61], [64], [83], Warsaw [38]–[40] and Wrocław [1], [30]–[36], [87], [94]. Interest in mutagenic and carcinogenic atmospheric pollutants is gradually increasing. Authors of the majority of works confine themselves, however, to the study of a relatively small number of samples and concentrate their attention on identification of groups of mutagenic and carcinogenic pollutants and identification of the other types of mutations caused by atmospheric pollutants. There are not so many works presenting results of the studies [31], [49], [74], [75], [88], [94].

An extensive literature dedicated to mutagenic and carcinogenic atmospheric pollutants provides basis for indication of directions of studies essential to proper selection of methods, which in the future will be in routine use in evaluation of mutagenic and carcinogenic activity of atmospheric pollutants in integrated environmental monitoring. This is the goal of this paper.

2. SAMPLE COLLECTION

Most often mutagenic and carcinogenic activity of pollutants adsorbed on airborne particulate is studied, omitting volatile fractions of pollutants. For examination of genotoxic activity airborne particulate samples are collected on glass filters [50], [74], [89]. They keep particles of particulate matter of sizes smaller than the ones kept on teflon filters and fibreglass coated teflon filters. Glass filters are also capable of retaining volatile hydrocarbons [69].

To sample organic pollutants adsorbed on the smallest granules of airborne particulates, some researchers use Amberlite XAD-2 resin [36], [53], [67], [91], less frequently polyurethane foam [87] or wood coal [18]. The materials are usually placed behind the filters or they are used as an exclusive means of sample acquisition. The results of comparative studies indicate differentiation of mutagenic activity of pollutants adsorbed on fine and coarse fractions of airborne particulates and they also indicate interactions between those two groups of pollutants [87]. Therefore, monitoring of atmospheric pollutants displaying mutagenic and carcinogenic activity should not be confined to coarse fractions of airborne particulates.

3. SAMPLE EXTRACTION

Filters with airborne particulates are most often extracted in Soxhlet apparatus with organic solvents such as benzene [17], [57], [92], dichloromethane [1], [4], [23], [68], [93], methanol [2], [11], [46], acetone [74], toluene [74], cyclohexane [46], [68]. There are also employed other extraction methods, e.g., with use of ultrasonic bath

[73]. Dichloromethane is advantageous due to its low boiling point, which causes that relatively few of the compounds under study volatilise during extraction.

The extraction times are different, e.g., 20 minutes when using ultrasounds [73], 6–48 hours in Soxhlet apparatus [2], [12], [17], [18], [68]. There is, however, lack of data about the influence of extraction time on the effectiveness of the extraction of mutagenic and carcinogenic compounds.

Solvents used for examination of mutagens adsorbed on airborne particulate cannot always be introduced to the tests as they are toxic to the organisms tested. On that occasion they are evaporated in vacuum evaporator, then the sample is solubilized in another solvent, which can be introduced to the test without affecting its result. Most often it is DMSO [17], [22], [57], [68], [89], rarely ethanol [13].

To determine precisely the groups of chemical compounds responsible for mutagenic activity, the extract obtained undergoes fractionation. A method that is often used is sequential elution solvent chromatography (SESC) [13], [21], [48], [55]–[58], [60], [61], [92], [93].

4. PHYSICOCHEMICAL ANALYSIS

Organic extracts of atmospheric pollutants and their fractions undergo physicochemical analysis with liquid chromatography (HPLC) [74], capillary gas chromatography [46], [60], [93] or gas chromatography coupled with mass spectrometry [4], [93]. As regards the accuracy of ion identification (many atmospheric pollutants are of polar character), the most recommendable seems to be liquid chromatography.

The presence of many organic compounds on particulates makes this analysis more difficult. Moreover, compounds effecting mutagenic and carcinogenic activity of extracts constitute only their insignificant part and are masked by compounds present in greater quantities. Analysing several hundreds of organic pollutants in atmosphere would be impossible. A routine analysis is confined to the analysis of PAHs from EPA list or to benzo(a)pyrene only (the Journal of Laws 55/98, item 335). This is, however, a simplification which omits many other mutagenic compounds.

5. CRITERIA OF BIOTESTS SELECTION

In research on mutagenicity and carcinogenicity of atmospheric pollutants there have been used so far many biotests on microorganisms, tissue cultures and higher organisms. Routine biomonitoring of mutagenic and carcinogenic pollutants should be based, at the most, on a few carefully selected biotests. The criteria of selection should be as follows:

- sensitivity of the test organisms to genotoxic activity of atmospheric pollutants,
- conformity with results of other biotests,

- common use in studying environmental mutagenicity and carcinogenicity with particular consideration given to atmospheric pollutants,
- the possibility of studying the activity of atmospheric pollutants independently of atmospheric conditions,
- a short examination cycle,
- a relatively low cost,
- limiting the suffering of laboratory animals to minimum.

The number of short-term tests enabling evaluation of mutagenic activity of samples have already exceeded 100 [29]; new biotests are still being introduced. To enable a comparison of salubriousness of atmospheric air in various places and periods of year, scientists working in different laboratories should not only agree to the use of a few common tests but also to unification of the sampling procedures and sample preparation, performance of biological tests and methods of presenting and interpreting the results. This should enable in the future collection of comparable data of mutagenic and carcinogenic activity of atmospheric pollutants in different places of the world and of different degree of atmospheric contamination. The comparison of mutagenic and carcinogenic activities of atmospheric pollutants in areas of different degree of urbanisation and industrialisation should make it possible to adopt values analogous to allowable concentration limits.

6. EVALUATION OF USEFULNESS OF DIFFERENT BIOTESTS FOR ROUTINE MONITORING OF GENOTOXIC ATMOSPHERIC POLLUTANTS

Human DNA-adduct examination. The method enables thorough evaluation of the influence of environmental mutagens and carcinogens in people *in vivo*. By adduct is meant a mutagen connected with a chemical bond with DNA nucleotide. In quantitative analysis, use is often made of chromatographic methods of mixture separation, and then of radiometric, immunological and spectrophotometric methods of adduct detection. With this method it is possible to confirm directly the cause–effect connections between exposure to selected pollutants and tumour-related diseases [72]. It is, however, more difficult to indicate the route of carcinogen invasion to organism, many of them being present both in atmosphere and in drinking water and food. Also, a significant role play exposure to occupational hazards and tobacco smoking. Additional difficulty is due to population migration. The handicaps of this method can, however, be compensated for by simultaneous genotoxic activity examination of pollutants extracted from individual environmental media. Adduct examination in human organisms is significantly limited due to poor accessibility of cancerous tissues. A direct study is possible on organs removed during surgical intervention. This significantly reduces the quantity of material for assay. Therefore, a substitute material is

often used for assaying the presence of DNA adducts in urine or lymphocyte of peripheral blood, and not in tissues suffering from tumour disease.

The heretofore studies on formation of DNA adducts by atmospheric pollutants, such as PAHs, indicate that frequency of their formation is greater in winter than in summer [5], [24]. PAHs adducts constitute about half of the adducts formed by atmospheric pollutants, more frequently they are formed in inhabitants of industrial areas than agricultural ones [86]. The number of aromatic adducts in blood corpuscles of Upper Silesia inhabitants indicated correlation with the number of chromosomal mutations caused by those pollutants in sister chromatide exchange test and chromosomal aberration test [6].

Rodent tests *in vivo*. Rodent tests *in vivo* seem at first glance to be most suitable for examination of environmental mutagenesis and carcinogenesis with regard to their genetic similarity to a human being, and what follows, a significant reliability of results and ease of relating them to human organisms. The manner of dosing laboratory animals with samples enables studying different issues, e.g., variations in seasonal and spatial mutagenic and carcinogenic activities of atmospheric pollutants, relation of their activity to atmospheric conditions and emission source. They, however, take a long time, and hence are costly. A long examination cycle is of less significance in studying a new medicine or pesticide before their introduction to the market. In environmental studies, e.g., of atmospheric pollutants, a short examination cycle is important, as it enables obtaining the results quickly. Experiments in which formation of mutagens or tumours is induced in rodents are connected with inflicting pain on laboratory animals. Similar results can be obtained in a shorter time and without inflicting pain on laboratory animals, by conducting a study on tissue cultures. Therefore, tests on rodents are used sporadically in examination of mutagenic and carcinogenic activity of atmospheric pollutants. Rodents were used, among other things, for examination of carcinogenesis of diesel engine emission with the method of skin test [9] and micronuclei formation in mice into which particulate extract of atmospheric pollutants was injected [55].

SMART test on fruit fly, *Drosophila melanogaster*. The test enables studying somatic mutation and recombination on fruit flies, *Drosophila melanogaster*, which are manifested in deformation of hairs on their wings. This test has already been adapted to study mutagenicity of tar substances contained in airborne particulates [38]–[40]. Its value lies in that with costs comparable to those of bacterial tests it enables evaluation of mutagenic activity of the substance on eucaryotic organisms, it is also relatively easy to perform and does not require growing of tissue cultures. However, it requires a relatively large quantity of test sample, which is administered to the fly larvae as food, this being an unnatural way in the case of atmospheric pollution. Microscopy analysis of the changed hairs is very laborious, and hence, time-consuming. Due to those reasons it is not in frequent use.

The study on mutagenicity of airborne particulates in Warsaw demonstrated that greater concentration of airborne particulates in winter than in summer entails greater

mutagenicity given per 1 m^3 of air. Mutagenicity of airborne particulates in the city centre is greater than in the suburbs [39].

Tissue culture assay. In the tissue culture assay, there was studied the influence of atmospheric pollutants on formation of micronuclei [7], frequency of sister chromatide exchange [3], [6], [10], [11], [21], [22], [37], [68], [76], [77], [80], [81], frequency of chromosomal aberrations [3], [6], [21], [22], [37], [58], aneuploids [59], production of mutation in the gene responsible for synthesis of thymine kinase [23] and cell transformation [9], [16], [78], [79], [82].

The main advantage of all those tests is that they use mammalian cell cultures, very often human. The study cycle is in this case shorter than in the case of *in vivo* tests on rodents. The experiments are not connected with infliction of harm on laboratory animals. Sample proportioning for the tissue tests enables such preparation of samples as to make it possible, on the basis of the results obtained, to answer questions concerning seasonal and spatial differentiation of mutagenic and carcinogenic activity of atmospheric pollutants and relation of this activity to atmospheric conditions, distance from the emission source, etc.

Most of the biotests on tissue cultures are sensitive to activity of mutagens and carcinogens polluting the atmosphere. Results obtained from different biotests are mutually consistent. This is proved not only by the positive results obtained by authors studying different particulate samples with different methods, also with tests on microorganisms, plants and fruit fly, but also by the results of comparative examination on the same samples whose mutagenic activities have been evaluated with different biotests. For example, a sister chromatide exchange test displayed conformity of results with the Ames test [3], [68], chromosome aberration test [3], [22] and micronucleus assay [3]. So far most reports have concerned successful use of sister chromatide exchange test for evaluation of mutagenicity of atmospheric pollutants. Thus this test can be recommended for use in a set of routine tests which will be applied in monitoring mutagenic atmospheric pollutants. This set should also include biotests enabling direct evaluation of carcinogenic activity of those pollutants. The other biotests are less commonly used. Some of them such as micronucleus assay display, moreover, less sensitivity to activity of mutagenes present in atmosphere.

For evaluation of mutagenic and carcinogenic activity of atmospheric pollutants there have been used different tissues: egg cells of Chinese hamster [11], [68], embryo cells of rats, mice and hamsters [9], [16], [19], Chinese hamster lung cells V79 [10], [61], and also human cells: lymphocytes [3], [6], [21], [22], [37], [76], [77], [80], [81], bronchial epithelium [26], [27]. Atmospheric pollutants get to the human organism via respiratory route. Therefore, for evaluation of their mutagenic and carcinogenic activity most suitable seem to be cells of respiratory system, especially lungs and bronchi. Human cells better reflect human health hazards than cells of other mammals.

***In situ* plants assay.** For *in situ* plants assay of mutagenic activity of atmospheric pollutants, examination has been performed of micronuclei induction and mutations

of the gene encoding colour of clones' stamens of *Tradescantia paludosa* BNL 4430 [25], [42], [43] as well as examination of the formation of lethal mutation of pollen of plants from the area under study [51], [52], [62].

The unquestionable advantages of both tests are the possibility of evaluating *in situ* mutagenic activity of atmospheric pollutants and a relatively low cost. The results of comparative examinations with the use of tests on *Tradescantia* and other plant tests demonstrated that it is a perfect plant for the monitoring of mutagenicity of atmospheric pollutants [20]. Plant tests have, however, many limitations. Probably this is the reason why they do not find a broader use. On the basis of their results it is possible to draw conclusions with greater precision about the effect of pollutants on local flora than about human health. Many limitations are connected with the life cycle of plants. Formation of lethal mutation in pollen and mutation of the gene encoding colour of stamens can be studied only in the period of flowering. *Tradescantia* is a thermophilous plant, its exposition to atmospheric conditions in the moderate climate region is not possible in winter. Both tests are thus not suitable for examination of seasonal changes of mutagenicity of atmospheric pollutants. The time-consuming examination cycle and necessity of permanent exposition to activity of mutagenic factors prevent use of those tests for examination of, e.g., relation of mutagenic activity of atmospheric pollutants and frequently changing atmospheric conditions. Those tests enable only evaluation of spatial differentiation of mutagenicity, e.g., in relation to the distance from point emission sources, e.g., close landfill sites and incinerators [43], [45], parking garages [41], nuclear power plants [62]. The result obtained will however be a mean value of this activity for a longer period of time. The one on *Tradescantia* can also be performed under *in vitro* conditions. In this case, a greater sample selection is possible, and what follows, use of the test for examination of issues for which it is not suitable under *in situ* conditions [30], [44].

Tests on microorganisms. For evaluation of potential mutagenic activity of atmospheric pollutants there have been used so far several bacterial tests, of which the most widespread one is the Ames test [1], [2], [13], [17], [57], [94]. The test is characterised by high sensitivity and ability to predict carcinogenicity of mutagenic compounds [29]. Its results display considerable conformity with those of other tests, including studies of mutagenicity of atmospheric particulate pollutants. WALKER et al. [89] established correlation between results of studies on air mutagenicity with the Ames test and morbidity and mortality rates of rats due to pulmonary carcinomas. For these reasons, the Institute of Environmental Health in Stockholm recommends inclusion of the Ames test in the set of routine examinations of monitoring environmental pollutants [84].

In the Ames test, use is made of mutants of *Salmonella typhimurium* incapable of production of histidine. This genetic defect can get repaired under influence of the mutagen tested as a result of reverse point mutation frame-shift or base-pair substitution. For examination of mutagenicity of atmospheric pollutants the following strains

are most often used: TA 98 enabling detection of frame-shift mutation and TA 100 enabling detection of base-pair substitution [1], [2], [57]. Depending on the composition of the pollutants being examined, greater sensitivity to their mutagenic activity displays one or the other strain. In the preliminary phase of the studies, it is advisable to use both strains in order to check their sensitivity to activity of mutagenes of locally predominant atmospheric pollutants. Afterwards, there can be used only the strain of greater sensitivity.

The Ames test, similarly to other bacterial tests, enables examination of activity of direct mutagens and indirect mutagens called also promutagens. The latter do not display direct mutagenic activity. As the result of biochemical change in vertebrate organism, catalysed primarily by liver enzymes, they undergo changes to the forms capable of reacting with DNA. To simulate biochemical conditions of mammal organism in the bacterial test, there is used a rat liver fraction S-9 [47].

Genetic modifications of strains TA 98 and TA 100, consisting in deleting or multiplying the gene responsible for encoding nitroreductase and O-acetyltransferase, enable use of the Ames test for evaluation of the share of nitro-, amine- and hydroxyloamine-derivatives of PAHs in mutagenicity of multicompound mixtures which are organic extracts of atmospheric pollutants. This also enables a more thorough evaluation of those derivatives in the form of single compounds [8], [14], [28], [54], [66], [70], [71]. Use of the strain YG 1024 of enhanced O-acetyltransferase activity enabled determination of the fact that 3-nitro-7H-benz(d,e)anthracene-7-one extracted from exhaust gas and atmospheric pollutants is more mutagenic than 1,8-dinitropyrene having been considered earlier the most active direct mutagen present in exhaust gas and atmospheric airborne particulates, the result being confirmed by micronuclei test [15].

The results of studies obtained so far indicate significant sensitivity of the Ames test to activity of mutagenes present in the atmosphere [1], [2], [16], [57], [74], hence there is no need in this case for preincubation, although authors of the test envisage such possibility [47]. Therefore, only few researchers decide to use such a version of the test [17].

Other bacterial tests are far less frequently used for evaluation of mutagenic activity of atmospheric pollutants. The positive results obtained not only indicate their sensitivity to activity of mutagens polluting the atmosphere, but they also confirm the positive results obtained in the Ames test, being the most commonly used. In the test on strain of *Salmonella typhimurium* TM 677, there was proved a forward point mutation, caused by atmospheric pollutants, manifesting itself in that the mutants become insensitive to toxic activity of 8-azaguanine [23]. They also cause mutations disturbing the process of DNA replication, and thus activating the SOS system. This is indicated by the results of chromotest on the strain of *Escherichia coli* K12 PQ37 [11], [63] and umu test on the strain of *Salmonella typhimurium* TA1535/pSK1002 [90].

From among eucaryotic microorganisms for evaluation of mutagenicity of atmospheric pollutants there have been used yeast strains (*Saccharomyces cerevisiae* D7 and XV185-

14C) [35], [74]. Pollutants adsorbed on atmospheric airborne particulates sampled in Italy induced sporadic mutations in mitochondrial DNA, mitotic gene conversion and mutation reversion [74]. Yeast assay displayed complementarity to the Ames test providing positive results towards non-mutagenic samples in the Ames test. Particulate samples collected in Wrocław were clearly mutagenic in the Ames test and did not cause reverse mutations in yeast assay [35]. Yeast assay is, therefore, suitable only for confirming negative results obtained sometimes in examination of mutagenicity of atmospheric pollutants.

7. CONCLUSIONS

Supplementing the currently used physicochemical monitoring of atmospheric pollutants with a set of biotests being carried out in order to evaluate their actual mutagenic and carcinogenic activity will enable a more precise evaluation of human health hazards caused by those pollutants. Learning about spatial differentiation of genotoxic activity of atmospheric pollutants will make it possible to concentrate activities of prophylaxis and early tumour detection in areas of greatest environmental hazards. The set of those biotests should include induction of point mutation reversion in *Salmonella typhimurium* in the Ames test, induction of sister chromatide exchange and transformation in cells of human respiratory systems and identification of DNA adducts in humans.

Based on the study the following conclusions hold true:

1. In the atmosphere, there are many mutagenic and carcinogenic pollutants of anthropogenic and natural origin. Most of them are organic compounds adsorbed on airborne particulates.

2. Many of the pollutants react in atmosphere with other compounds. In human organism, they undergo enzymatic change. Products of the change can be more or less biologically active. In the multicompound mixtures, mutagens and carcinogens display mutually synergistic and antagonistic action. Total activity of the mixture is not the sum of activities of the compounds.

3. Currently used physicochemical monitoring does not entirely reflect biological activity of atmospheric pollutants. Therefore, it should be supplemented with evaluation of mutagenic and carcinogenic activity of atmospheric pollutants with biotests.

4. When selecting biotests for routine monitoring of mutagenic and carcinogenic activity of atmospheric pollutants it is required considering the following criteria: sensitivity of the experimental organisms to genotoxic activity of atmospheric pollutants, conformity of the results with those of other tests, common use in studying atmospheric pollutants, the possibility of examining the activity of atmospheric pollutants independently of atmospheric conditions, a short time of study cycle, relatively low cost, reducing the suffering of laboratory animals to the minimum.

5. Those criteria are fulfilled best by the tests on microorganisms and tissue cultures. Biomonitoring of mutagenic and carcinogenic pollutants should include induc-

tion of reversion of point mutation in *Salmonella typhimurium* in the Ames test, induction of sister chromatide exchange and canceration in cells of human respiratory systems and identification of DNA adducts in human organisms.

6. Routine use of biotests for monitoring of atmospheric pollutants should be preceded by a standardisation of sample collection and preparation, biotest procedure and presentation and interpretation of results.

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METODY BADANIA GENOTOKSYCZNOŚCI ZANIECZYSZCZEŃ ATMOSFERY

Stosowany obecnie monitoring fizykochemicznych zanieczyszczeń atmosfery nie odzwierciedla w pełni ich aktywności biologicznej. Powinien on być uzupełniony oceną mutagenności i kancerogenności tych zanieczyszczeń za pomocą biotestów. Wybierając biotesty, należy wziąć pod uwagę: wrażliwość testowanych organizmów na genotoksyczne działanie atmosfery, zgodność otrzymanych wyników z wynikami innych biotestów, powszechność stosowania testów w badaniu mutagenezy i kancerogenezy środowiskowej ze szczególnym uwzględnieniem zanieczyszczeń atmosfery, możliwość badania aktywności zanieczyszczeń atmosfery niezależnie od warunków atmosferycznych, krótkotrwałość cyklu badawczego, relatywnie niski koszt oraz ograniczenie do niezbędnego minimum cierpienia zwierząt laboratoryjnych. Kryteria te w największym stopniu spełniają testy na mikroorganizmach i kulturach tkankowych: test Amesa na *Salmonella typhimurium*, indukowanie wymiany chromatyd siostrzanych i transformacji nowotworowej w komórkach narządów oddechowych człowieka oraz identyfikacja adduktów DNA u ludzi.