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# $^{32}$P-Postlabeling Determination of DNA Adducts in the Earthworm *Lumbricus terrestris* Exposed to PAH-Contaminated Soils

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The importance of the search for reliable biomarkers of DNA damage in environmental health assessment is well recognized by the scientific community and regulatory agencies (IARC 1988; Fowle and Sexton 1992). Among the major biomarkers of DNA damage is the measurement of DNA adducts in target cells or tissues. Up to now, DNA adduct determinations have been directed mostly toward human exposure to toxic substances from the workplace and environment (Hemminki et al. 1988; Talaska et al. 1992). Moreover, techniques for measuring DNA adducts, and in particular the $^{32}$P-postlabelling technique, presented also the possibility of determining DNA adduct levels in endogenous animal populations exposed to polluted environments as early warning monitors of ecotoxicity (Dunn et al. 1987; Varanasi et al. 1989; Liu et al. 1991).

Soil contamination is becoming a major environmental issue. Therefore, numerous contaminated sites must now be remediated to protect human health and to permit new uses of these sites as agricultural, residential, or industrial areas. Fulfillment of this task requires standardized and sensitive bioassays to carry out site evaluations and to establish scientifically defensible soil quality criteria. To that effect, the earthworm appears to be one of the best organisms for use in soil toxicity evaluation (Bouché 1992). Earthworms are probably the most relevant soil species, representing 60 to 80% of the total animal biomass in soil (Rida 1994). Present soil bioassays focus mostly on plant species with end points like seed germination, root elongation, seedling growth and seedling emergence, and on acute toxicity evaluation (re: LC 50) on the earthworm *Eisenia fetida* (OECD 1984; Keddy et al. 1992). As yet, a standardized soil invertebrate test for teratogenic or mutagenic end points has not been developed (Sheppard et al. 1992). In this paper, we report the feasibility of DNA adduct determination by $^{32}$P-postlabelling in the earthworm *Lumbricus terrestris* as a way to detect the presence of genotoxic substances in soils.

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## MATERIALS AND METHODS

Contaminated soils were collected from two sites in the province of Québec, Canada. Site 1, located in Québec City, was used by a coal gasification plant in operation from 1907 to 1973. During this period, various chemical wastes were buried on site (e.g. oil, wood chips impregnated with naphtalene, coke, sulfides, coal dust, and coal tar). An earlier study of this site showed it to be heavily contaminated with polyaromatic hydrocarbons (PAH) (data not shown; Hydro-Québec, Internal Report 1989). The maximum concentration of total PAHs was about 3500 ppm, with 209 ppm of benzo(a)pyrene (BaP) and 11 ppm of benzo(b,k)fluoranthene. Site 2, located near Montréal, was the site of illegal dumping of different chemical wastes. The soil from this site has very high levels of total PAHs (up to 6000 ppm) and numerous other chemicals, such as phenol and chlorobenzene, (data not shown; Ministère de l'Environnement du Québec, Internal Report, 1991).

*Lumbricus terrestris* were purchased from a local fish bait supplier (Les Appâts du Nord, Black Lake, Québec); these earthworms were collected in southern Québec and Ontario. For evaluation of site 1, control earthworms were kept in the soil provided by the supplier. Four to five earthworms were put in a one-liter plastic box containing approximately 500 g of a single sample of contaminated soil. After 2 or 3 wk of exposure, the earthworms were removed, washed in distilled water, dried, and rapidly frozen in liquid nitrogen. For site 2, the high concentrations of toxic substances in the soil sample caused the death of earthworms after only 24 to 48 hr of exposure. Therefore, to detect a genotoxic response without an acute toxicity effect, this soil sample was diluted at 10% with a preparation of artificial soil made of sand (70%), kaolinite (20%), and sphagnum peat (10%) (Greene et al. 1989). Earthworm exposure was done as described above except the duration of the test was limited to 1 wk. For this site, control earthworms were kept in the artificial soil preparation for the same period. Thereafter, the worms were handled the same way as for those of site 1. Before being analyzed, all the earthworms were kept frozen at -85°C.

For the DNA extraction, a procedure developed for the earthworm species *Eisenia fetida fetida* was used (El Adlouni et al., 1994). Essentially, the earthworms were crushed in liquid nitrogen, and 2 to 3 g of the crushed tissue powder were incubated at 65°C for 60 min in SET buffer (100 mM NaCl; 20 mM EDTA; 50 mM Tris base, pH 8.0) with 20% SDS. Fourteen milliunits (mU) of proteinase K (Sigma Chemical Co. St-Louis, MO; EC 3.4.21.14) were added and the tubes were incubated at 37°C for another 2 hr. Proteins were precipitated by adding potassium acetate (6M; pH 5). The samples were kept on ice for 30 min before being centrifuged for 10 min at 10,000 x g and 4°C. The samples were thereafter submitted to partitions in phenol, phenol:chloroform:isoamyl alcohol (25:24:1, v:v:v) and chloroform:isoamyl alcohol (24:1, v:v) (CSH 1989). DNA was precipitated in cold ethanol, spooled out, centrifuged for 10 min, and rinsed twice with 70% ethanol. To eliminate RNA, 1 unit (U) of RNAse A (Sigma

Chemical Co., EC 3.4.27.5) and 5 U of RNAse T1 (Sigma Chemical Co., EC 3.1.27.3) were added to 150 µg of nucleic acids and incubated at 37°C for 1 hr. The extraction procedure was repeated to eliminate proteins from the samples. DNA was then dried under vacuum and stored at -85°C until utilization. The DNA concentration was evaluated spectrophotometrically considering that 50 µg/mL of double stranded DNA should give an OD value of 1.0 at 260 nm.

In this study, we used the nuclease P1 modification of the $^{32}$P-postlabelling method (Reddy and Randerath 1986). DNA samples (10 µg) were digested (4 hr) with 0.5 U of micrococcal nuclease (grade IV; Sigma Chemical Co., EC 3.1.31.1) and 4 mU of spleen phosphodiesterase (Boehringer Mannheim; EC 3.1.16.1). After another digestion (45 min) with 5 U of nuclease P1 (Boehringer Mannheim; EC 3.1.30.1), the samples were $^{32}$P-labelled by incubation with 100 µCi of carrier free γ-[32P]-ATP (3000 Ci/mM; New England Nuclear) and 5 U of T4 polynucleotide kinase (Pharmacia, Piscataway NJ; EC 2.7.1.78) at 37°C for 45 min. The reaction was terminated by the addition of 0.016 U of potato apyrase (grade I; Sigma Chemical Co.; EC 3.6.1.5) to convert unused γ-[32P]-ATP to inorganic phosphate and AMP. These procedures were designed to yield biphosphate adducts and normal nucleosides. Resolution of $^{32}$P-labelled adducts was carried out on polyethyleneimine(PEI)-cellulose plates (Macherey-Nagel, Germany) for the selective detection of aromatic nucleotide adducts using the following solvent mixtures: 1st dimension (D1): 2,3 M sodium phosphate pH 5.7; 2nd dimension (D2): 5.3 M lithium formate and 8.5 M urea, pH 3,5; 3rd dimension (D3): 0.7 M sodium phosphate and 7 M urea, pH 6.4; 4th dimension (D4): 1.7 M sodium phosphate, pH 6.0 (IARC 1993). Eight to 10 samples were first spotted at the bottom of a 20 x 20 cm plate then run in solvent D1 for approximately 16 hr. After autoradiography, the initial spots containing the hydrophobic adducts were cut out of the plate. The samples were transferred between two magnets at the bottom left of a new 18 x 13 cm plate. After chromatography in solvent D2 (3-5 hr), the top 2 cm of the plates was cut out and the plates thereafter were resolved perpendicularly to the previous migration in solvent D3 (2-3 hr). Finally, the plates were run for 2-3 hr in solvent D4, in the same direction as the 3rd dimension. After each migration, the plates were washed twice in distilled water and dried. Radioactive adduct spots identified by autoradiography were excised from the chromatograms and the $^{32}$P levels were determined by Cerenkov counting. Knowing the specific activity of the ATP and taking into account the $^{32}$P decay, the total levels of adducts were calculated by adding the radioactivity of the individual spots on the chromatograms. The results are given in relative adduct levels (adducts/$10^9$ normal nucleotides), considering that 1 µg of DNA is equal to 3.03 pmoles of nucleotides.

## RESULTS AND DISCUSSION

Figure 1 shows the DNA adduct patterns of earthworms exposed for 1 to 3 wk to PAH - contaminated soils. We noted the presence of spots on the

horizontal axis of the chromatograms, which were present in some instances in blank runs in which DNA, DNA hydrolyzing, or phosphorylating enzymes were omitted (Fig 1a). These spots were not quantitated. Our results indicate that, with the exception of these spots, all DNA adducts analyzed in this study derived from earthworms. Figures 1c and 1d show the chromatograms of *Lumbricus terrestris* DNA after 2 and 3 wk of exposure of the earthworms to the soil of site 1. The numerous adduct spots and the diffuse diagonal zone of radioactivity shown on these chromatograms are characteristic of an exposure to complex mixtures of DNA reacting substances. Adduct levels (Table 1) show a gradual increase from $16.8/10^9$ nucleotides in the control worm taken from the supplier stock and not exposed to contaminated soil (Fig. 1b), to $57.7/10^9$ nucleotides after 3 wk of exposure to soil 1 (Fig. 1d). Previous analyses of soil samples from site 1 showed elevated PAH concentrations with the presence of some highly carcinogenic congeners such as benzo(a)pyrene. Because no other DNA reacting substances are expected on this site and since the solvent mixtures used for chromatography are suitable for bulky non-polar aromatic adducts resolution, the diagonal zone and discrete spots most probably represent adducts from PAH metabolites. The formation of adducts in *Lumbricus terrestris* earthworms exposed to PAH-contaminated soil suggests that this species may have some enzymatic ability to form oxidized PAH metabolites (Sternensen 1992).

Figures 1e and f illustrate the results of earthworm exposure to the soil from site 2. As mentioned, the acute toxicity of this soil was very high; consequently, this sample was diluted (10%) in an artificial soil preparation. After 1 wk in the artificial soil sample, only a low amount of adducts ($3.9/10^9$ nucleotides) was detected in the control earthworm (Table 1; chromatogram not shown). However, after 1 wk of exposure to the contaminated soil sample from site 2, adduct levels of 56.1 and 69.1 adducts/$10^9$ nucleotides were measured in duplicate earthworms. Qualitatively, the pattern of adducts in those two earthworms appears the same. The small difference in adducts levels (<25%, Table 1) may be more a reflection of the variation from one individual to another since both earthworms come from the same experimental container (see also the areas limited by a dotted line on fig. 1e and 1f). Variations much larger are often seen and expected (Phillips and Castegnaro 1993).

To our knowledge, these results represent the first DNA adduct determinations in earthworms exposed to contaminated soils. Although this study was aimed primarily to assess the feasibility of the $^{32}$P-postlabelling method to earthworms, it is shown that one can at least qualitatively evaluate the genotoxic potential of PAH-contaminated soils. However, irregardless of the nature of the contaminants in the soils under study, earthworms exposed to these contaminated soils exhibited a 3.5 to 15 fold increase in DNA adducts over non-exposed (control) animals. Even if $^{32}$P-postlabelling appears to be one of the best techniques to estimate the totality of non-polar aromatic DNA adducts caused by complex mixtures of genotoxic substances found in the environment (IARC 1993),

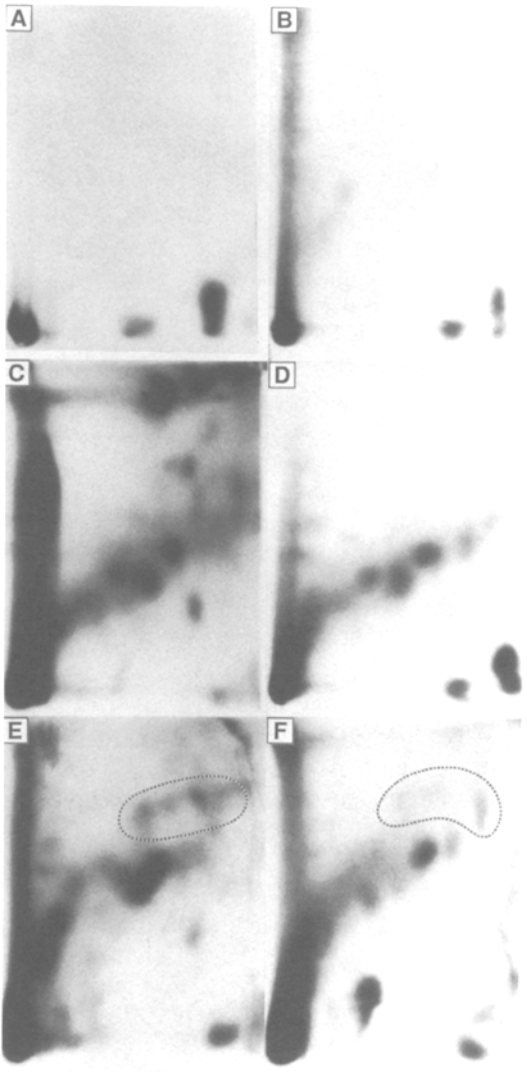


Figure 1. Autoradiograms of the DNA adduct patterns from *Lumbricus terrestris* earthworms exposed to PAH - contaminated soils: a) reaction mixture without DNA (blank); b) control earthworm from the supplier; c) 2-wk exposure to site 1; d) 3-wk exposure to site 1; e) and f) 1-wk exposure to a preparation of 10% contaminated soil from site 2 and 90% artificial soil. The origin of the PEI-cellulose chromatograms is at the lower left corner of each plate. All the autoradiograms were developed for 48 hr except plate d) which was developed for 24 hr. Each $^{32}$P-postlabelling bioassay involved a single DNA extraction from a single earthworm.

Table 1. DNA adduct levels in *Lumbricus terrestris* exposed to contaminated soils

| Figure | Site | Exposure conditions | Adducts/$10^9$ nucleotides |
|---|---|---|---|
| 1b | 1* | Control | 16.8 |
| 1c | 1 | 2 wk | 36.5 |
| 1d | 1 | 3 wk | 57.7 |
| — | 2** | Control | 3.9 |
| 1e | 2 | 1 wk | 56.1 |
| 1f | 2 | 1 wk | 69.1 |

* Québec City, Québec, Canada; coal gasification plant (tested as collected)

** Site near Montréal, Québec, Canada; illegal dump (tested as a 10% mixture with an artificial soil)

it is difficult at this time to evaluate the extent of the relation between adduct concentrations in earthworms and chemical loads. Nonetheless, there appears to be a time-dependent increase in adduct level in earthworms exposed to the soil of site 1. Further experiments will be necessary to properly address this question.

As mentioned, earthworms are currently used in soil toxicity evaluations; a standardized acute toxicity test already exists (OCDE 1984). The development of new toxicological parameters in earthworms (Venables et al. 1992) will facilitate future soil toxicity assessment and the establishment of soil quality criteria. To that effect, DNA adduct determinations could be an interesting parameter to add to a battery of tests that include the current LC50 earthworm test. Because no lethality was observed in our bioassays, the $^{32}$P-postlabelling technique seems however much more sensitive than LC50 determinations for assessing toxicity in earthworms. Consequently, in the case of genotoxic pollutants that are not acutely toxic to earthworms, DNA adduct determinations could provide relevant risk assessment information complementing acute toxicity tests that otherwise would have been completely negative.

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