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Biological effects of contaminants: Measurement of DNA adducts in fish by ^{32}P -postlabelling

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Abstract

This document describes in detail the ^{32}P -postlabelling method and its application to fish. Several recent studies have shown that the ^{32}P -postlabelling method can be used to detect and measure the levels of DNA modified by large, hydrophobic aromatic compounds in teleosts. Moreover, the levels of hepatic DNA adducts in wild fish positively correlate with the concentrations of polycyclic aromatic compounds (PACs) present in marine sediments in several cases, and a strong positive correlation has been observed between sediment concentrations of PACs and the prevalence of neoplastic lesions in liver of marine flatfish. Laboratory studies with model PACs and sediment extracts also have shown that the PAC-DNA adducts formed are persistent and have chromatographic characteristics similar to DNA adducts detected in wild fish. These findings suggest that the levels of hepatic DNA adducts found in fish tissues can function as molecular dosimeters of exposure to potentially genotoxic environmental contaminants, such as high molecular weight PACs. The ^{32}P -postlabelling assay has been used as a marker of exposure to potentially genotoxic contaminants in environmental monitoring studies, such as NOAA's National Status and Trends (NS&T) Program and in the Bioeffects Surveys of NOAA's Coastal Ocean Program.

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Keywords: DNA adducts, ^{32}P -postlabelling, polycyclic aromatic compounds.

1 INTRODUCTION

The ^{32}P -postlabelling (PPL) method for the detection of DNA adducts was developed in the laboratory of Dr K. Randerath in the early 1980s (Gupta *et al.*, 1982) and has evolved substantially since then. The ^{32}P -postlabelling technique is currently the most sensitive method for the detection of a wide range of compounds bound to DNA. For hydrophobic aromatic DNA adducts, such as those derived from polycyclic aromatic compounds (PACs), this method can detect one adduct in 10^9 – 10^{10} bases. In addition, the non-specific nature of the ^{32}P -postlabelling assay allows for the detection of a wide range of bulky hydrophobic compounds bound to DNA. This property, coupled with the high sensitivity of the assay, has led to the increased use of the ^{32}P -postlabelling assay in studies assessing the exposure of mammals and fish to environmental genotoxins (Dunn *et al.*, 1987; Varanasi *et al.*, 1989a; Poginsky *et al.*, 1990; Liu *et al.*, 1991; Ray *et al.*, 1991; Stein *et al.*, 1992; van der Oost *et al.*, 1994), to specific genotoxic compounds, such as benzo[a]pyrene (BaP) and 7H-dibenzo[c,g]carbazole (DBC) (Randerath *et al.*, 1984; Schurdak *et al.*, 1987; Varanasi *et al.*, 1989b; Sikka *et al.*, 1991; Stein *et al.*, 1993), and to compounds present in organic-solvent extracts of PAC-contaminated sediments (Stein *et al.*, 1994; Balch *et al.*, 1995).

The use of DNA adduct levels in marine fish shows considerable promise as a biomarker of exposure to hydrophobic pollutants such as those derived from PACs (Varanasi *et al.*, 1989b; Stein *et al.*, 1992; Collier *et al.*, 1993; van der Oost *et al.*, 1994). For example, in a study using the territorially restricted oyster toadfish (*Opsanus tau*), a benthic species, Collier *et al.* (1993) found that hepatic DNA adduct levels in the toadfish were strongly correlated ($p < 0.001$, $r^2 = 0.97$) to sediment levels of creosote-derived PACs. Van der Oost *et al.* (1994) also found a significant relationship between levels of environmental PACs and hepatic DNA adduct levels in eel (*Anguilla anguilla*). In addition, a laboratory study of English sole (*Parophrys vetulus*) exposed to a gradient of contaminated sediments showed that the levels of hepatic DNA adducts increased in both a time- and a dose-dependent manner (French *et al.*, 1996). Furthermore, several studies have shown that an appreciable proportion of the hepatic DNA-formed adducts in fish are highly persistent (Varanasi *et al.*, 1989b; Sikka *et al.*, 1990; Stein *et al.*, 1993, 1994). The demonstration of DNA adduct accumulation and persistence over time and dose suggests that DNA adduct levels can be used as an index of chronic exposure to large hydrophobic compounds. There are several issues, however, that should be considered when using DNA adduct levels in fish as an index of site contamination.

- 1) Adducts formed from PACs are persistent in fish (Sikka *et al.*, 1991; Stein *et al.*, 1993, 1994) and therefore the DNA adduct levels present should be viewed as a 'cumulative index' of current and past exposure. Thus, if a migratory fish has accumulated DNA adducts at a distant site and then migrates, the presence of these accumulated DNA adducts may significantly influence the assessment of contamination present at the capture site. Therefore, the use of another biomarker, such as the levels of fluorescent aromatic compounds in bile, which is a relatively good index of recent exposure to PACs (Collier and Varanasi, 1991; French *et al.*, 1996), could provide information to assess whether DNA adduct levels measured were due to exposure at the capture site or were the result of prior exposure at a remote site.
- 2) Habitat preferences of fish species can significantly influence the extent of exposure. The choice of a target species is dependent on whether the contaminants of interest are associated with the water column or the sediments. The large hydrophobic PAC structures detected by the postlabelling method tend to partition strongly to the sediment fraction and particulates, which is a reflection of their high octanol-to-water partition coefficients (K_{ow})

(Meador *et al.*, 1995). A territorially restricted benthic species, therefore, would be appropriate for evaluating the bioavailability of sediment-associated contaminants, whereas a pelagic species would be more appropriate for measuring exposure to water-column associated contaminants.

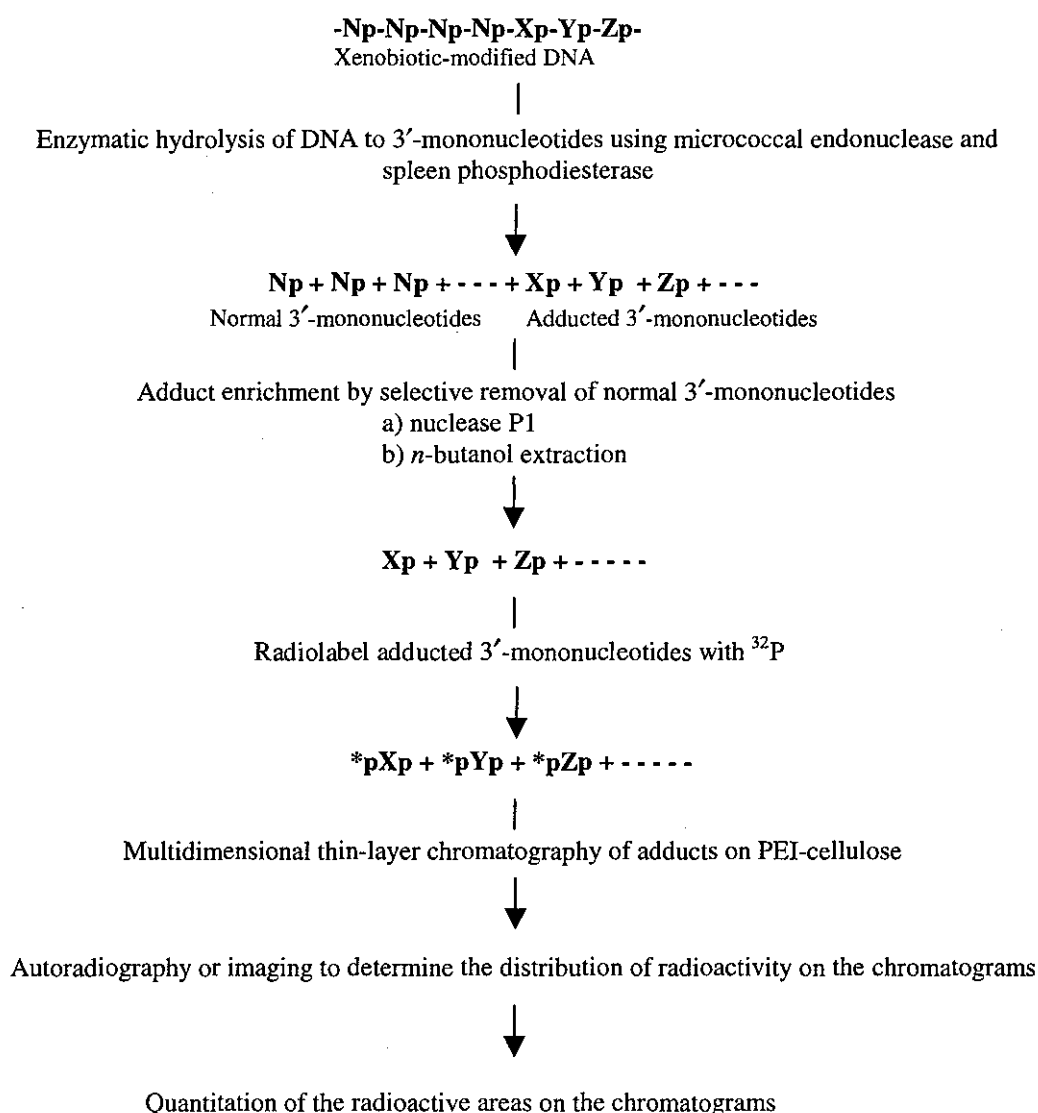
A critical issue in the use of any biomarker is the definition of a baseline value for an animal from a 'reference' site and whether the criteria selected to define a reference site are appropriate. Chemical analysis provides information about the types and levels of contaminants present in sediment but not on their bioavailability. Moreover, because of cost considerations, chemical analysis of sediments is often not done. There are several indicators of pollutant exposure, such as mixed-function oxidase activity (3-OH BaP-hydroxylase, EROD, etc.), levels of CYP1A protein, biliary fluorescent aromatic compounds (FACs), and DNA adduct levels, currently being used. The indications of pollutant exposure obtained from some of these biomarkers can be strongly influenced by factors such as sex, dietary status, and seasonal factors. However, in the fish species that have been extensively investigated by the authors, these factors appear to have, at best, only a minor influence on DNA adduct levels. In the experience of the authors with over 28 marine and freshwater species of teleosts representing 22 genera collected from pristine areas for which we had chemical analyses of sediments, the results show that autoradiograms of ³²P-postlabelled hepatic DNA digests either exhibit no detectable adducts or very faint diagonal radioactive zones. In contrast, fish sampled from PAC-contaminated sites had autoradiograms that clearly indicated PAC exposure. Thus, if current analyses of water and sediment chemistry indicate that no PACs are present at a site and biomarkers show minimal response, then the site is considered a reference site.

The authors have not observed the naturally occurring DNA modifications such as those observed by Randerath and co-workers in mammals (Randerath *et al.*, 1986, 1989; Li *et al.*, 1990; Li and Randerath, 1990). However, these observations do not preclude the possibility that in some fish species naturally occurring DNA modifications may be present in tissues at either appreciable levels or below the current limits of detection. There has been a report suggesting that significant levels of naturally occurring adducts can be present in fish (Kurelec *et al.*, 1989). The criteria used in this study to define pollution levels were based either on benzo[a]pyrene mono-oxygenase activities, which can be rapidly induced by PACs and other compounds, human population densities at sampling sites or the results of chemical analyses of water samples. No sediment contaminant data, which are temporally much less variable than water contaminant levels, were presented. In addition, benzo[a]pyrene mono-oxygenase levels can respond rapidly to changes in contaminant levels and thus historical data may not reflect the current level of contamination. It is difficult to conclude, therefore, without additional analytical and biomarker data on the target fish and sediments, that the DNA modifications observed by Kurelec *et al.* (1989) are unequivocally from naturally occurring compounds and not the result of exposure to contaminants. At present, the findings of Kurelec and co-workers should be considered of interest but inconclusive.

2 METHODOLOGY

The detection of DNA adducts by ^{32}P -postlabelling¹ is a multistep procedure (Figure 1) involving a series of biochemical reactions. Initially, DNA is hydrolyzed enzymatically to 3'-monophosphates. The digest is then enriched in xenobiotic-modified mononucleotides by the selective removal of normal nucleotides. Following the enrichment step, the adducted DNA is enzymatically labelled at the 5'-hydroxyl position with [^{32}P]phosphate to form [5'- ^{32}P]deoxyribonucleoside 3',5'-bisphosphates. Separation of the ^{32}P -labelled adducts is usually accomplished by two-dimensional, thin-layer chromatography (TLC) on polyethyleneimine (PEI)-modified cellulose sheets, however, there is an increasing use of HPLC separation methods. Autoradiography and liquid scintillation counting or imaging analysis (Reichert *et al.*, 1992) are then used to locate and quantitate the radiolabelled adducts on the chromatogram.

Figure 1. Schematic of the ^{32}P -postlabelling assay procedure.



¹For an excellent review of ^{32}P -postlabelling methodology, please see: Phillips, D.H., Castegnaro, M., and Bartsch, H. (Eds.) 1993. Postlabelling methods for detection of DNA adducts. International Agency for Research on Cancer, Lyon, France.

The accurate quantitation of individual adducts is dependent on the specificity and efficiency of the enzymes and the enhancement method used, which can vary substantially for each type of adduct. Optimization of each enzymatic step for specific adducts is required to increase the accuracy of measuring individual adduct levels (Watson, 1987; Gorelick and Wogan, 1989). Currently, the method can be considered only semi-quantitative in organisms exposed to complex mixtures of environmental contaminants that have not been fully characterized. However, because mixtures of chemical contaminants are poorly characterized, the ability of the ^{32}P -postlabelling assay to detect and quantitate a wide range of DNA adducts of unknown structure is a key attribute.

This document describes the procedures and equipment currently used in the authors' laboratories to assay fish DNA by ^{32}P -postlabelling (Reichert and French, 1994). Each section discusses the function and biochemical basis for a particular step, the methodology, and the procedural pitfalls. The samples typically analysed are hepatic tissues from fish; however, the method is applicable to any tissue from which DNA can be extracted.

3 LABORATORY SET-UP

3.1 Facilities

A dedicated laboratory is used for the ^{32}P -postlabelling analysis. This laboratory contains a fume hood, sink, $-20\text{ }^{\circ}\text{C}$ freezer, $-70\text{ }^{\circ}\text{C}$ freezer, and a refrigerator. The fluorescent ceiling lights all have a 400 nm cut-off to reduce photodegradation of samples (i.e., PAC structures such as benzo[a]pyrene-DNA adducts) (Steenwinkel *et al.*, 1993).

3.2 Equipment

Centrifugal vacuum evaporator: A Savant Speed-Vac SVC100H² (Savant Instruments, Farmingdale, NY) equipped with a glass cover is used for evaporating solvents.

Refrigerated microcentrifuge: An Eppendorf Model 5402 refrigerated microcentrifuge is used in postlabelling and DNA extraction procedures.

Tabletop centrifuge: A Sorvall T6000B (DuPont) is used for centrifuging the Plexiglas carousels holding the radioactive postlabelling samples.

A DeSaga TLC spreading device: Desaga 120305 or Whatman 49961-102 is used for making thin-layer chromatographic (TLC) sheets.

A variable speed/pulse kitchen blender is used to mix PEI/cellulose solutions.

A pancake-type radiation monitor (Technical Associates PUG 1 AB, Canoga Park, CA) is used for detecting ^{32}P contamination in the laboratory (see Section 5, Radiation Safety).

² Mention of trade names is for information only and does not constitute an endorsement by the U.S. Department of Commerce.

Autoradiography cassette: Any 14 inch × 17 inch (approximately 35 cm × 43 cm) metal autoradiography cassette will work. The cassettes are lined on one inner side with intensifying screens.

Tissue homogenizers: A Polytron PT 1200C with a 5 mm generator (Brinkmann Instruments, Westbury, NY) or a glass Dounce homogenizer (5 ml to 10 ml size) is used for homogenizing tissue.

Waterbaths: Any standard tabletop waterbath (20–60 °C range) can be used for incubations and enzyme hydrolyses. A separate waterbath is used for radiolabelled samples.

Plexiglas carousels: These carousels are used for holding the microcentrifuge tubes containing radioactive samples during incubations. See Reddy and Blackburn (1990) for design specifications.

Chromatography tanks: Standard glass TLC tanks (inside dimensions 275 mm × 275 mm × 75 mm) can be used; however, if large numbers of samples are processed, then the Plexiglas multisheet holders (see Reddy and Blackburn (1990) for specifications) are preferable.

Spectrophotometer: A UV/VIS spectrophotometer with quartz cuvettes is used for measuring DNA absorbances.

Microcentrifuge tubes: Any high quality microcentrifuge tube (0.5 ml, 1.5 ml, or 1.9 ml) will work. All tubes should be methanol rinsed. Occasionally a residue is present on the tubes that can inhibit enzyme activity.

Freezers: A –70 °C (or colder) freezer is used for the storage of all tissues and purified DNA. A –20 °C freezer may be used for overnight storage of samples but never for extended periods.

Pipettors: Both the Eppendorf and Gilson adjustable micropipettors in sizes of 10 ml, 20 ml, 100 ml, 200 ml, and 1000 ml are used in our laboratory. One set of pipettors is used for radioactive samples and a separate set is used for non-radioactive samples.

Radiation shielding: See Section 5, Radiation Safety.

Liquid scintillation counter: A Packard 1900 TR is used for liquid scintillation counting.

Radioactivity imaging systems: The authors use a Model 425E PhosphoImager (Molecular Dynamics, Sunnyvale, CA) for scanning the radioactive images on the chromatograms.

pH meters: A quality pH meter with a calomel pH electrode is used when preparing buffers containing Tris.

4 CHEMICALS

Carrier-free [³²P]orthophosphate and carrier-free [γ -³²P]ATP (8500–9000 Ci mmol⁻¹) can be obtained from either Amersham (Arlington Heights, IL) or New England Nuclear Research Products (E.I. DuPont, Wilmington, DE). The following chemicals are obtained from Sigma Chemical (St. Louis, MO): Bicine (B-3876), Tris (T-1503), CHES buffer (C-2885), urea (U-1250), spermidine (S-2626), dithiothreitol (D-0632), L-glycerol-3-phosphate (G-7886),

adenosine 5'-diphosphate (A-6521), adenosine 5'-triphosphate (A-6144), 2'-deoxyadenosine 3'-monophosphate (D-3014), sodium pyruvate (P-2256), lithium chloride (L-0505), proteinase K (P-0390), nuclease P1 (N-8630), micrococcal endonuclease (N-3755), calf spleen phosphodiesterase (P-6897), apyrase (A-6132), RNase A (R-4875), RNase T1 (R-8251), RNase T2 (R-3751), polyethyleneimine in 50 % aqueous solution (P-3143), and μ -amylase (A-6255). Cloned T4 polynucleotide kinase (70031) is available from United States Biochemical/Amersham (Cleveland, OH). Cellulose powder (MN-301 is manufactured by Macherey Nagel in Germany) can be obtained from Alltech (Deerfield, IL). Molecular biology grade phenol is available from Boehringer Mannheim (Indianapolis, IN). The DNA adduct postlabelling standard 7R,8S,9S-trihydroxy,10R-(N²-deoxyguanosyl-3'-phosphate)-7,8,9,10-tetrahydrobenzo[a]pyrene (BaPDE-dG-3'p) can be purchased from Midwest Research Institute (Kansas City, MO). All other chemicals used were reagent grade or better.

Enzymes used for the synthesis of [γ -³²P]ATP were as follows: glycerol-3-phosphate dehydrogenase (127124), triosephosphate isomerase (109754), glyceraldehyde-3-P-dehydrogenase (105686), lactate dehydrogenase (127230), 3-phosphoglycerate kinase (108430), and β -NAD (127302); these can be purchased from Boehringer Mannheim (Indianapolis, IN).

Double-distilled, deionized water or high-performance liquid chromatography (HPLC) grade water is used for the preparation of assay reagents. For the preparation of reagents, please see the appropriate sections.

5 RADIATION SAFETY

The ³²P-postlabelling assay uses large amounts of ³²P, which is an energetic beta emitter (1.7 MeV). Therefore, any person using this isotope must receive detailed instruction before handling ³²P. Since an inexperienced person may not realize how easily radioactivity is unintentionally spread, pre-training with a fluorescent solution may be necessary. Thus, a new person should perform laboratory operations using a fluorescent solution (i.e., fluorescein, quinine sulfate) and then turn off the overhead lights and use a black light to reveal handling errors that could result in unwanted spreading of radioactivity (Castegnaro *et al.*, 1993).

Important points to help minimize and monitor ³²P exposure:

- 1) All employees who handle ³²P must wear a thermoluminescent-type finger ring dosimeter on the inside of each hand where the highest potential for radiation exposure exists and a whole body film badge. Rings and badges are monitored monthly.
- 2) All laboratory operations are planned to minimize radioactivity exposure.
- 3) Double latex gloves are worn while handling ³²P and they are frequently checked for radioactivity by passing them under a pancake-type radiation monitor. If the outside gloves require constant changing due to ³²P contamination, sample handling procedures must be reviewed and revised.
- 4) Workers must wear lab coats and use disposable sleeves.
- 5) Laboratory working surfaces must be checked frequently with the radiation monitor when handling ³²P. The monitor probe should be covered with a thin vinyl wrap to prevent contamination of the probe.

- 6) After completion of radioactivity work, the workers must check themselves and their equipment with the radiation monitor. If any radioactivity is detected, they must wash themselves and/or the equipment until free of radioactivity.
- 7) A senior staff member experienced in routinely handling radioactivity checks on the staff to ensure that correct procedures are followed.

5.1 Equipment for Handling ^{32}P

All ^{32}P is handled behind 10-mm to 13-mm Plexiglas shielding. In addition, samples are kept in Plexiglas containers that are at least 13 mm thick. Most of the Plexiglas equipment we use (e.g., carousels for holding the microcentrifuge tubes, shields for the pipettors, and racks for holding the chromatograms during chromatography and drying) is similar to that described by Reddy and Blackburn (1990). This equipment increases sample handling capacity, while substantially lowering radiation exposure risk.

5.2 Radioactive Waste and Disposal

Radioactive waste is temporarily stored in a remote corner of the laboratory in a 13-mm thick Plexiglas box that has a 1.5 mm thick lead foil covering (Josefsen *et al.*, 1993). The ^{32}P waste is then transported to a designated radioactive waste storage site and stored in a 55-gallon steel drum labelled ' ^{32}P only.' Once a drum is filled, it is sealed and dated. After 10 half-lives (143 days), the drum contents are scanned with a survey meter. If there is no survey meter reading, then the barrel contents are disposed of in the regular waste.

See Slobodien (1980), Ballance *et al.* (1984), and Castegnaro *et al.* (1993) for additional information on the safe handling of ^{32}P .

6 POLYETHYLENEIMINE-MODIFIED CELLULOSE SHEETS

Satisfactory chromatography of hydrophobic DNA adducts can be achieved with either commercially available or laboratory-prepared polyethyleneimine-modified cellulose (PEI-cellulose) TLC sheets. The authors have found that the laboratory-prepared PEI-cellulose TLC sheets can give sharper solvent fronts and better resolved spots. In addition, the sheets develop faster and the chromatographic characteristics are more consistent from sheet to sheet as compared to commercial PEI-cellulose sheets. If you plan to do ^{32}P -postlabelling on a large scale, it is cost effective to make your own PEI-cellulose sheets.

6.1 Commercial PEI-Cellulose Sheets

The performance of commercial PEI-cellulose sheets can be improved by soaking the sheets in reagent grade methanol and gently shaking for 2 to 4 minutes to remove impurities from the manufacturing process. After the methanol wash, the sheets are rinsed in distilled water for 10 minutes. The washed sheets are air dried thoroughly and placed in a sealed container for storage; they are stable at $-20\text{ }^{\circ}\text{C}$ for at least two months.

6.2 Preparation of PEI-Cellulose Sheets

The procedure used for the preparation of PEI-cellulose sheets is based on the method of Randerath and Randerath (1964). First, prepare a 0.5 % PEI solution by adding 900 ml of deionized distilled water (ddH₂O) to 10 g of a 50 % PEI solution in a beaker. Adjust the pH to approximately 6 with 6N HCl while constantly stirring. Initially, PEI is a clear, viscous solution at the bottom of the beaker, but as the pH is lowered the PEI will dissolve. After the PEI has dissolved and a pH of 6 has been reached, bring the solution to a final volume of 1 litre. This solution is stable for up to 4 months when stored between 0–4 °C. The retentive characteristics of PEI-modified cellulose sheets can be modified by changing the percentage of PEI present in the solution.

The vinyl sheets used for the backing of the PEI-cellulose sheets must be cleaned before spreading the PEI-cellulose slurry. First, scrub the dull surface of a matte finish vinyl sheet (8.5 inch × 50 inch (21.8 cm × 127 cm), 0.01 inch (0.25 mm) thick, ordered through any commercial plastics dealer) with a commercial cleaner and rinse thoroughly with soft tap water or distilled water. Place the vinyl sheet on a clean glass plate of the same dimensions with the dull side facing up. One of the long edges of the glass plate should barely overhang the countertop edge to allow the TLC spreader to slide smoothly along the vinyl sheet. Dry the vinyl sheet. The TLC spreader is then run along the vinyl sheet to check for smoothness. Any gritty material detected either on top of the vinyl strip or between the vinyl strip and the glass plate is removed.

The PEI-cellulose slurry is prepared by placing 34 g of MN-301³ cellulose in a blender and adding 145 ml of a 0.5 % PEI solution. Stir the solution to wet the cellulose. Pulse the blender a few times and then 'liquefy' on low for 15 seconds. Pour the PEI-cellulose slurry into a vacuum flask and aspirate. Swirl the flask vigorously while aspirating to prevent the PEI-cellulose slurry from foaming. This step may take 10 to 15 minutes because the PEI-cellulose slurry can be slow to degas. Removal of the dissolved gasses is important because it improves adherence of the PEI-cellulose to the vinyl sheets and prevents small bubbles from forming which will leave small holes in the final product.

Set the TLC spreader opening at 0.4 mm. Pour the degassed PEI-cellulose slurry gently into the spreader to avoid generation of bubbles and stir gently with a glass rod to remove any bubbles. Turn the spreader handle and immediately spread the slurry in one slow steady movement along the vinyl strip. At the end of the vinyl strip, turn the spreader handle back to the closed position. The slurry layer is usually thicker at the very ends of the vinyl strip. If pinhead-sized holes are present in the slurry layer, press these dry spots with a fine pointed tool, which will cause the slurry to fill the voids.

Dry the PEI-cellulose sheets overnight and then cut the sheets into 20-cm widths with a paper cutter (do not use sections that were unevenly spread or have holes in them). The sheets can be trimmed to different dimensions to meet other chromatographic needs.

Place the sheets in covered chromatography tanks and develop to the top edge with ddH₂O overnight. Remove the tank cover and continue to develop for 2 to 4 hours more. A faint, yellow, oily band generally develops at the top of the sheet. Trim off the top edge, including the

³ The characteristics of cellulose can change from batch to batch. When Macherey-Nagel replaced their cellulose MN-300 with MN-301, the amount of cellulose used to make a 'durable' sheet increased substantially. Unfortunately, the determination of the proper opening of the spreader and the amount of cellulose to use is a trial-and-error process.

oily band, and the bottom edge of the sheets to obtain 16 cm × 20 cm segments. Air dry the sheets completely (usually 1–2 hours). If they are dried overnight, the chromatographic characteristics may change because of PEI breakdown. *Caution:* If the sheets are placed in the freezer slightly damp, the PEI cellulose may separate from the vinyl backing later during chromatography.

Place the sheets in an airtight container and store at –20 °C. The sheets are stable for 2 to 4 months at –20 °C. *Caution:* Never immerse a dry sheet quickly; instead allow capillary action to wet the sheet, otherwise the PEI-cellulose may separate from the vinyl backing.

7 SYNTHESIS OF $[\gamma\text{-}^{32}\text{P}]\text{ATP}$

The $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ used for labelling DNA adducts can either be purchased or synthesized in the laboratory starting with carrier-free inorganic $[\text{}^{32}\text{P}]\text{phosphate}$ ($^{32}\text{P}_i$) and adenosine diphosphate (ADP).

The procedure for preparing $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ is based on the method of Gupta *et al.* (1982) and Gupta and Randerath (1988) [see also Johnson and Walseth (1979) for the original method]. A synthesis premix containing all of the components for making $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, except the $^{32}\text{P}_i$, can be prepared that is stable for 2 to 3 months at –70 °C or lower. Preparation of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ can then be accomplished by adding the synthesis premix to carrier-free sodium $[\text{}^{32}\text{P}]\text{phosphate}$.

7.1 Preparation and Storage of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ Synthesis Premix

To ensure a high enough $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ specific activity (curies of ^{32}P mmol^{–1} of ATP):

- 1) Soak all glassware, pipette tips, microcentrifuge tubes and any other objects that come in contact with the chemicals and enzymes used to make the $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ synthesis premix in double-distilled, deionized water for several hours to remove non-radioactive phosphates. The presence of non-radioactive phosphate will lower the specific activity of the synthesized $[\gamma\text{-}^{32}\text{P}]\text{ATP}$.
- 2) Keep all components on ice when preparing the ATP synthesis premix and immediately freeze the aliquoted ATP synthesis premix at –70 °C. This precaution will yield a premix that will synthesize $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ with a consistently high level of specific activity.

The following stock solutions are needed for preparation of the ATP synthesis premix:

Enzyme⁴ premix (A)

200 μ l	glycerol-3-phosphate dehydrogenase (2 mg ml ⁻¹)
2 μ l	triosephosphate isomerase (2 mg ml ⁻¹)
40 μ l	glyceraldehyde-3-phosphate dehydrogenase (10 mg ml ⁻¹)
4 μ l	3-phosphoglycerate kinase (10 mg ml ⁻¹)
40 μ l	lactate dehydrogenase (5 mg ml ⁻¹)
284 μ l	total volume

Reagent solution (B)

62.5 μ l	4.4 mg ml ⁻¹ sodium pyruvate
150 μ l	0.1 M dithiothreitol
250 μ l	0.5 M Tris, pH 9.0
125 μ l	2.4 mM l-glycerol-3-phosphate
125 μ l	10 mM b-NAD ⁺
100 μ l	0.3 M MgCl ₂
62.5 μ l	2 mM ADP ⁵ (add last when making solution B)
875 μ l	total volume

Buffer solution (C)

42 μ l	0.1 M dithiothreitol
21 μ l	0.5 M Tris, pH 9.0
375 μ l	ddH ₂ O
438 μ l	total volume

The [γ -³²P]ATP synthesis premix is prepared from the stock solutions as follows:

- 1) Place 30 μ l of enzyme premix A (shake to resuspend the enzymes before taking an aliquot) in a 1.5 ml microcentrifuge tube and centrifuge at 14 000 rpm for 2 minutes. Carefully remove the supernatant with a pipette. The ammonium ions in the supernatant can inhibit the T4-polynucleotide kinase used to phosphorylate the xenobiotic-DNA adducts.
- 2) Add 400 μ l of buffer solution C to the precipitated enzymes in the microcentrifuge tube and mix. Next add a 220 μ l aliquot of the dissolved enzyme mixture to 875 μ l of reagent solution B and vortex.

⁴ These enzymes are shipped individually from the manufacturer as suspensions in ammonium sulfate solution. Before taking an aliquot, the vials containing these enzymes must be shaken to resuspend the enzymes.

⁵ The ADP solution is prepared just before use and kept on ice. The authors order a new package of ADP for each premix preparation. The ADP can decompose with time to give adenosine monophosphate and inorganic phosphate. The inorganic phosphate will be used by the enzyme premix to make non-radiolabelled ATP, which can substantially lower the specific activity of the synthesized [γ -³²P]ATP.

- 3) Aliquot 70 μl of the premix into labelled 0.5 ml microcentrifuge tubes on ice. The premix aliquots are immediately stored at $-70\text{ }^{\circ}\text{C}$.

7.2 [γ - ^{32}P]ATP Synthesis Procedure

Caution: When ordering $^{32}\text{P}_i$ from the manufacturer, request a small delivery volume (100 μl or less), otherwise the synthesized [γ - ^{32}P]ATP may be too dilute for maximum labelling efficiency.

The acid-free/carrier-free $^{32}\text{P}_i$ sometimes arrives contaminated with $^{32}\text{P}_i$ -polyphosphates formed from catenation of $^{32}\text{P}_i$ -monophosphates, which will reduce the quality of the autoradiograms obtained. To eliminate this problem before starting [γ - ^{32}P]ATP synthesis, the $^{32}\text{P}_i$ solution from the supplier is acid-treated with 0.1 volume of 0.1 N hydrochloric acid (HCl) for 0.5 hour at room temperature, followed by addition of 0.055 volume of 0.2 M Tris base. The $^{32}\text{P}_i$ can be ordered in dilute HCl; however, the acid concentration may be variable and this could affect the pH of subsequent enzyme reactions.

To start the [γ - ^{32}P]ATP synthesis, add a 50 μl aliquot of synthesis premix to 5 millicuries (mCi) of carrier-free $^{32}\text{P}_i$ in 100 μl of solution. Vortex and incubate for 1 hour at room temperature (vortex several times during incubation). If less than 5 mCi of $^{32}\text{P}_i$ is used, then the volume of premix to be added is adjusted proportionally. After synthesis, spot a 0.1–0.3 μl aliquot of the reaction mixture on a PEI-cellulose sheet and develop in 1 M lithium chloride solution to check for reaction completion. Dry the chromatogram and autoradiograph. A 10-second exposure at room temperature is usually sufficient. There should be one strong spot due to [γ - ^{32}P]ATP with an R_f of 0.4–0.5 (R_f = distance of the spot from the start/distance of the solvent front from the start) and a faint spot may be present from unreacted $^{32}\text{P}_i$ at an R_f of 0.9 (Figure 2a). This synthesis reaction normally goes to greater than 98 % completion. If the autoradiogram indicates that a substantial amount of $^{32}\text{P}_i$ is still present, then vortex the [γ - ^{32}P]ATP solution and let it stand for an additional 0.5 hour and/or increase the temperature to $37\text{ }^{\circ}\text{C}$ (Talaska *et al.*, 1992). Reanalyse for reaction completion. Usually this reaction will yield [γ - ^{32}P]ATP with a specific activity of 2000–3000 Ci per mmol ATP. The [γ - ^{32}P]ATP should be stored at $-70\text{ }^{\circ}\text{C}$ and should be used within 2 to 3 days for labelling of DNA adduct samples.

7.3 Determination of [γ - ^{32}P]ATP Specific Activity

The specific activity of the [γ - ^{32}P]ATP is determined by labelling a known amount of 2'-deoxyadenosine-3'-monophosphate with [γ - ^{32}P]ATP and separating the products by one-dimensional chromatography. The 3',(^{32}P)5'-deoxyadenosine bisphosphate spot is located by autoradiography and then quantitated by either excising the spot and liquid scintillation spectrometry (LSS) or by image analysis (see Section 13, Imaging and Quantitation).

Specific activity = Ci of ^{32}P associated with the 2'-deoxyadenosine-bisphosphate
spot/mmol of 2'-deoxyadenosine-3' monophosphate labelled

7.4 Assay Protocol

Dissolve a small amount of 2'-deoxyadenosine-3'-monophosphate (Sigma D-3014) in double distilled, deionized water. Determine the concentration at neutral pH by measuring the absorbance at 260 nm and using a molar extinction coefficient of 15 400 litre mole $^{-1}$ for a 1-cm path length (concentration = absorbance/extinction coefficient). Based on the absorbance

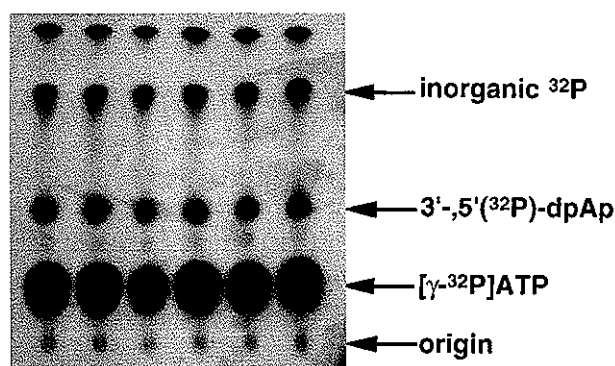
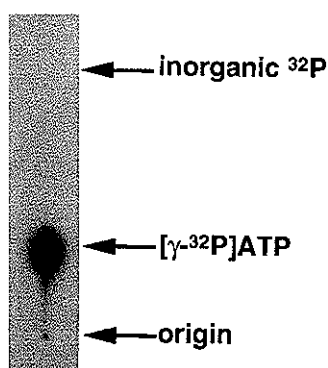
values obtained, dilute the solution to 1×10^{-4} M. Take an aliquot of the dilution and check the absorbance. Aliquot this solution into 0.5 ml microcentrifuge tubes and store at -70°C . This solution is stable for several months at -70°C .

Dilute a 10 μl aliquot of the 1×10^{-4} M 2'-deoxyadenosine-3'-monophosphate solution to 10 ml with ddH₂O to give a final concentration of 1×10^{-7} M. To four replicate 0.5 ml microcentrifuge tubes add the following: 5 μl of 1×10^{-7} M 2'-deoxyadenosine-3'-monophosphate and 5 μl of a [γ -³²P]ATP labelling solution made by combining 3 μl of 33 mCi μl^{-1} [γ -³²P]ATP (each replicate requires 20 mCi of [γ -³²P]ATP); 10 μl of kinase buffer (0.1 M bicine, 0.1 M MgCl₂, 0.1 M dithiothreitol, 10 mM spermidine, pH 9.0); 10 μl of ddH₂O and 1 μl of 30 units μl^{-1} T4-polynucleotide kinase. Mix samples and incubate at 37°C for 40 minutes. Because small volumes ($< 25 \mu\text{l}$) are difficult to mix adequately by vortexing or shaking, one should centrifuge the tubes to bring all liquid to the tube bottom and then mix the samples with a pipettor. After incubation, add 390 μl of H₂O to each replicate tube, vortex and spot 10 μl on a PEI-cellulose sheet. Develop the chromatogram in 0.3 M ammonium sulfate, 10 mM sodium phosphate, pH 7.4. Use 80 % strength for laboratory-prepared sheets. The R_f values are approximately 0.15 for [γ -³²P]ATP, 0.4 for 3',(³²P)5'-deoxyadenosine bisphosphate, and 0.9 for inorganic phosphate (Figure 2b). After chromatography, locate the 3',(³²P)5'-deoxyadenosine bisphosphate spot by autoradiography. Excise the spot and count by LSS. The 3',(³²P)5'-deoxyadenosine can also be quantitated on the chromatogram by imaging methods.

Figure 2. Autoradiograms of one-dimensional chromatograms used in the preparation of [γ -³²P]ATP.

- (a) An aliquot ($\sim 0.1 \mu\text{l}$) of the [γ -³²P]ATP synthesis mixture was eluted with 1 M lithium chloride. The autoradiogram indicates a successful synthesis of [γ -³²P]ATP.

- (b) Aliquots of the specific activity determination digest were eluted in 0.24 M ammonium sulfate and 8 mM sodium phosphate, pH 7.4.



7.5 Sample Calculation for Determining [γ - ^{32}P]ATP Specific Activity

The following information is needed: amount of 2'-deoxyadenosine-3'-monophosphate labelled in moles (i.e., 5×10^{-13} moles), the time at which the 3',(^{32}P)5'-deoxyadenosine bisphosphate spots were counted (i.e., 8/1/96 at 08.00 hours), the average disintegrations per minute (dpm) for the four replicates (i.e., 70 398 dpm), the time the DNA samples were postlabelled (i.e., 8/5/96 at 16.00 hours), the dilution aliquot factor (i.e., 40 if a 10 μl aliquot of a total volume of 400 μl was spotted on a PEI-cellulose sheet), and the conversion factor from dpm to curies (1 curie = 2.22×10^{12} dpm).

$$\text{Specific activity at 16.00 hours on 8/5/96} = (\text{average dpm on 8/1/96}) \times (\text{decay correction}) \times (\text{dilution factor}) / (\text{moles of 2'-deoxyadenosine-3'-monophosphate})$$

$$\text{Decay correction} = N/N_0 = e^{(-0.693T/T_{1/2})}$$

where

T is the time elapsed (decay period),

$T_{1/2}$ is the half-life for the isotope used (14.3 days for ^{32}P),

N is the dpm at time T,

N_0 is the dpm at T_0 .

Specific activity at the time of sample labelling (8/5/96)

$$\begin{aligned} &= (70\,398) \times \{e^{[-0.693 \times 4.33\text{d}/14.3]}\} \times (40) / (5 \times 10^{-13}) \\ &= 4.565 \times 10^{18} \text{ dpm mol}^{-1} \\ &= 2057 \text{ Ci mmol}^{-1} \end{aligned}$$

8 DNA ISOLATION

DNA isolation is a critical step in the ^{32}P -postlabelling method. How the samples are acquired in the field and the care in isolation and handling of the DNA samples can improve the chromatogram quality and lower the background radioactivity on the chromatograms.

Important points to emphasize are:

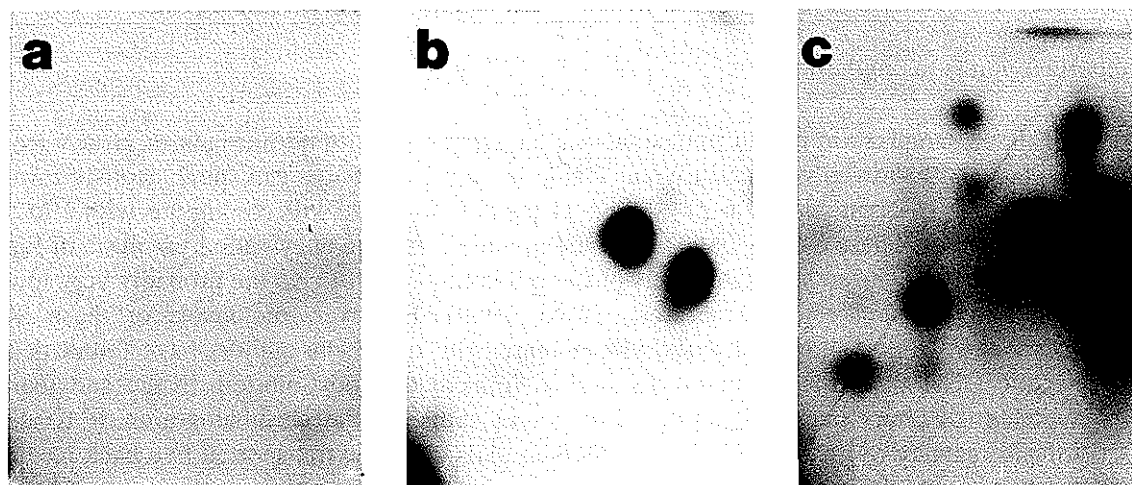
- 1) All tissue samples are frozen immediately after sampling in liquid nitrogen or on dry ice. Gupta (1989) showed with calf thymus DNA that the DNA bases can spontaneously form compounds (S-compounds) at physiological temperature that will co-migrate with PAC-DNA adducts. These S-compounds will be released during the enzymatic hydrolysis of the purified DNA. The steady-state levels of these S-compounds *in vivo* are probably very low owing to efficient repair processes (Gupta, 1989). However, the levels of these S-compounds in post-mortem tissues will increase with time. The authors have found that hepatic DNA from marine mammals packed immediately post-mortem in ice and then sampled 18 hours later will show S-compound spots on the chromatograms. If tissue samples cannot be immediately frozen, then animals could be transported live back to the

laboratory and sampled. An additional benefit when tissue samples are frozen immediately and stored at -70°C or lower is that enzyme-dependent biomarkers can also be evaluated.

- 2) All tissue and purified DNA are stored at -70°C or lower; extended storage (several months) in a -20°C freezer can yield DNA with S-compounds that will be released during the enzymatic hydrolysis of DNA and co-chromatograph and interfere with the quantitation of DNA adducts derived from hydrophobic compounds such as PAHs (Figure 3). After several months at -20°C , the yield of DNA from tissue samples starts to decline and eventually DNA is not recoverable, whereas ^{32}P -postlabelling quality DNA can be extracted successfully from tissues stored at -70°C for at least two years. PAC-DNA adducts appear to be stable in whole tissue for at least two years at -80°C . Ross *et al.* (1995) found that DNA adducts formed from five different PACs were stable for up to three years when the extracted DNA was stored at -80°C . However, it would be desirable to process samples as quickly as possible because some of the possible PAC-DNA adducts present, such as some aromatic amine adducts, may not have long-term stability at -70°C or lower (Gupta, 1996).
- 3) High quality chemicals must be used for the extraction of DNA. The phenol used in the DNA extraction should be a molecular biology grade phenol that has been redistilled and stored under argon. Oxidation of phenol yields quinones that cross-link nucleotides resulting in chromatograms with high backgrounds that can be difficult to quantitate.
- 4) It is important that both RNase A and RNase T1 are used in the RNA removal steps of DNA isolation, otherwise the samples will have substantial RNA contamination which may interfere with subsequent steps in the ^{32}P -postlabelling (PPL) process.

Figure 3. Profiles of ^{32}P -postlabelled 3'-mononucleotides from hepatic tissue from fish showing the chromatographic positions of DNA breakdown products (S-compounds, see Section 8, DNA Isolation). These S-compounds rarely appear in samples that were immediately frozen in liquid nitrogen and then stored at -80°C . These samples were chromatographed on commercial sheets using 1 M sodium phosphate, pH 6.0 for D1, 4.5 M lithium formate, 8.5 M urea, pH 3.5 for D3, and 1.6 M lithium chloride, 0.5 M Tris, 8.5 M urea, pH 8.0 for D4:

- (a) Profile of postlabelled DNA from reference fish hepatic tissue that was immediately frozen in liquid nitrogen and then stored at -80°C .
- (b) and (c) Profiles of DNA S-compound spots that may be observed in *n*-butanol enhanced ^{32}P -postlabelled samples. Profile c has a higher level of S-compounds present and the weaker S-compound spots are showing.



The methods used for DNA extraction from tissues are based on the procedure of Reddy and Randerath (1987), which uses a nuclei precipitation step to minimize RNA contamination. In some species of fish there can be a component present in the cytosolic fraction that will be postlabelled and cause intense streaking on the autoradiograms. The nuclei precipitation step minimizes this problem. The EDTA concentration used in the homogenization step was increased to 100 mM to reduce the activity of endogenous DNAses; however, for the rest of the procedure EDTA concentrations should not exceed 20 mM. A manual procedure for extracting DNA is presented in the following text. This procedure can be adapted for use on commercial DNA extractors.

8.1 Reagents for DNA Isolation

The following is a list of reagents needed for DNA isolation:

- phenol (use a molecular biology grade)
- phenol:chloroform:isoamyl alcohol, 25:24:1, v/v/v (CIP)
- chloroform:isoamyl alcohol, 24:1, v/v (CIA)
- 70 % ethanol (v/v)
- 100 % ethanol (v/v)
- 5 M NaCl
- 1 % SDS/20 mM EDTA (pH 7.4)
- 10 mM Tris/100 mM EDTA, pH 7.4 (ultra TE)
- 2 M Tris, pH 7.4
- 500 mM EDTA, pH 7.4
- 50 mM Tris, pH 7.4
- 10 mM Tris/1 mM EDTA, pH 7.4 (TE)

8.2 Procedure

Keep tissue on ice. Place 125–250 mg of tissue⁶ and 1.7 ml of 10 mM Tris/100 mM EDTA (pH 7.4) either in a glass culture tube (10 mm × 75 mm) and homogenize using a Polytron 1200C at setting 2 for 5–10 seconds or in a 7 ml glass Dounce homogenizer and homogenize using 5–10 slow strokes. *Note: If homogenization is not performed gently, the nuclear membrane may rupture resulting in the loss of DNA during the nuclei precipitation step.*

Transfer the homogenate to a labelled 1.9 ml microcentrifuge tube and centrifuge at 4 °C for 10 minutes at 6000 rpm to pellet the crude nuclei. Decant and discard supernatant. If there is a ring of lipid left on the tube wall, use a cotton swab to remove it.

⁶ The recovery of DNA from fish tissues is dependent on the species and the tissue condition.

Resuspend pellet in 1400 µl of 1 % SDS/20 mM EDTA (pH 7.4) solution and add 8 µl of solution containing 20 µg of heat-treated RNase A, 20 units of RNase T1 and 20 µg of α-amylase and incubate for 30 minutes at 37 °C. The α-amylase is included in the RNase mixture for hydrolysis of glycogen which will interfere with the UV absorbance readings. At the end of the RNase incubation, proteins are digested by the addition of 0.4 mg of proteinase K in 40 µl of 1 M Tris-HCl (pH 7.4) per sample. Incubate samples for 30 minutes at 37 °C with occasional shaking.

Protein is then removed by sequential organic solvent extractions as follows: Add one volume of the appropriate solvent, mix by inverting the tube repeatedly for 1 minute, centrifuge at 14 000 rpm for the recommended time length and remove the organic layer with a pipette. Use the solvent extraction sequence outlined below.

Extraction	Reagent	Centrifugation (min)
1	phenol	7
2	phenol:chloroform:isoamyl alcohol, 25:24:1 (CIP)	5
3	chloroform:isoamyl alcohol, 24:1 (CIA)	3

Transfer the DNA solution (aqueous phase) to a fresh tube after the CIP and CIA extraction steps. Add 0.1 volume of 5 M NaCl to the aqueous phase and invert the tube repeatedly for 30 seconds. Add 1 volume of cold (–20 °C) 100 % ethanol and gently invert the tube repeatedly for 2 minutes to mix. The DNA will precipitate out of solution as a stringy gelatinous clump unless it is sheared. If the DNA is sheared, place the tube in a –20 °C freezer for 20 minutes to overnight. Centrifuge for 5 minutes at 14 000 rpm to pellet the DNA. Wash the DNA pellet with 1 ml of 70 % ethanol by inverting the tube, centrifuging for 5 minutes at 14 000 rpm and decanting the supernatant. Remove any residual 70 % ethanol by centrifuging for a few seconds and then using a pipette. Resuspend the DNA in 35–75 µl of 10 mM Tris/1 mM EDTA, pH 7.4 (TE) buffer, depending on the size of the pellet recovered.

Caution: The DNA precipitate can often be difficult to dissolve. As a result, the DNA can sometimes be a large, clear, colourless aggregate in solution that can be either inadvertently pipetted leading to an abnormally high UV absorbance reading or the aggregate may be missed by the pipette leading to a low UV absorbance value and an erroneous amount of DNA being used for the enzyme hydrolysis step. If this is a persistent problem, then pass the DNA solution through a 26-gauge needle to obtain a homogeneous solution.

Take 5 µl of the DNA solution and dilute to 1 ml with TE and measure the absorbances against a TE blank at 280 nm, 260 nm, and 230 nm. The absorbance ratios of 260/230 and 260/280 should be greater than 2.3 and 1.8, respectively. These ratios will yield information about protein contamination but not RNA contamination. The ³²P-DNA base analysis chromatograms will give an estimate of RNA contamination (Figure 4a).

$$\text{DNA concentration (mg DNA ml}^{-1}\text{)} = (A_{260})(\text{dilution factor})/(22.9 \text{ ml mg}^{-1} \text{ DNA})$$

Example:

dilution factor = $1000\ \mu\text{l final volume} / 5\ \mu\text{l original volume} = 200$

absorbance (A_{260}) = 0.262 OD units

Concentration = $0.262 \times 200 / 22.9 = 2.28\ \text{mg ml}^{-1}$

The DNA concentration obtained by UV measurement at 260 nm is only an estimate and can vary substantially (up to $\pm 50\%$, Gupta (1993)) owing to impurities present and difficulties in accurately pipetting double-stranded DNA solutions. Use the UV-based DNA value to determine the aliquot to take for PPL analysis. Do not assume that you have pipetted an exact amount of DNA. The actual amount of DNA used in the assay will be determined later when the total nucleotides are analysed by postlabelling.

9 ENZYMATIC HYDROLYSIS OF DNA

Successful enzymatic digestion of xenobiotic-modified DNA to 3'-mononucleotides is dependent on several variables. Lower levels of micrococcal nuclease (MN) and spleen phosphodiesterase (SPD) are now being used than were originally recommended (Gupta *et al.*, 1982). Excessive MN levels can lower the recovery of some DNA adducts (Beach and Gupta, 1992). The levels of MN present in the DNA digestion solution should be in the range of 0.15–0.35 $\mu\text{g MN}/\mu\text{g DNA}$. However, SPD concentrations do not appear to have a negative effect on adduct recovery and a concentration of 1 $\mu\text{g SPD}/\mu\text{g DNA}$ works well (Beach and Gupta, 1992). The SPD is relatively unstable and should be discarded 4–5 months after being opened or after the manufacturer's expiration date (Phillips and Castegnaro, 1993). For samples containing DNA adducts derived from PACs, incubation of DNA with MN and SPD for 3–6 hours at 37 °C is sufficient. Overnight digestion of the samples may result in the loss of some aromatic amine adducts and a lower recovery of some PACs (Beach and Gupta, 1992). The choice of MN and SPD concentrations and the length of DNA digestion is adduct-dependent and digestion conditions should be optimized for specific needs. The DNA digest can either be used immediately or stored overnight at –70 °C for processing the next day. In addition to the DNA samples, an aliquot of salmon sperm DNA and a blank containing no DNA are also carried through the postlabelling process. The chromatograms obtained with the high purity salmon sperm DNA should be clear and not show spots. If spots are present on the blank (no DNA) chromatogram, especially when using the butanol enhancement version, they may be arising from impurities on the microfuge tubes used. Rinsing the microfuge tubes with methanol and then water will eliminate this problem.

9.1 Procedure for Digestion of DNA to 3'-Monophosphates

For each sample, place approximately 25 μg of DNA in TE buffer into a 1.5 ml microcentrifuge tube⁷ and bring to a final volume of 15 μl with distilled water. Add 10 μl of the MN/SPD in buffer to each sample.

[Preparation of MN/SPD in buffer: Place an appropriate volume ($10\ \mu\text{l} \times \text{number of samples}$) of SPD suspension in ammonium sulfate ($2\ \mu\text{g SPD}\ \mu\text{l}^{-1}$) in a microcentrifuge tube and centrifuge

⁷ All tubes used in this procedure are rinsed with methanol, then with distilled water and thoroughly dried to remove any residual chemical products that may interfere with enzymatic activities.

at 14 000 rpm for 2 minutes. Remove the supernatant with a pipettor. Then add an appropriate volume ($5\ \mu\text{l} \times \text{number of samples}$) of dialysed MN⁸ ($1\ \mu\text{g MN}\ \mu\text{l}^{-1}\ \text{H}_2\text{O}$) solution and a volume ($5\ \mu\text{l} \times \text{number of samples}$) of succinate buffer (20 mM sodium succinate, 10 mM calcium chloride, pH 6.0), respectively, to the SPD-containing tube.]

Briefly centrifuge the sample tubes to bring the enzyme solution to the tube bottom and then mix with a pipettor. Incubate samples for 3 to 6 hours at 37 °C and vortex occasionally. Remove samples from the waterbath and briefly centrifuge to bring the tube contents to the bottom. A 10 μl aliquot of the enzyme hydrolysate will be taken for the DNA adduct postlabelling assay. In addition, a 5 μl aliquot of each hydrolysate is placed in a 1.5 ml tube along with 495 μl of ddH₂O and vortexed. This sample is used for determination of the total amount of DNA hydrolysed.

Caution: If you change the volumes used in this step, make sure that the calcium ion concentrations present when postlabelling are in the 0.1–1 mM range (Hemminki *et al.*, 1993).

10 DNA ADDUCT ENHANCEMENT PROCEDURES

The xenobiotic-DNA adduct levels in fish are generally below the limits of detection for the standard unenhanced version of the postlabelling assay. Thus, separation of DNA adducts from normal nucleotides is a necessary step in enhancing sensitivity for detection of PAC-caused DNA damage. However, the enhancement methods currently available recover less than 100 % of all DNA adducts present and, in some cases, depending on the method used, specific DNA adducts may not be recovered. If you are analysing for a specific adduct, then the conditions should be optimized for that structure. However, when analysing DNA adducts in fish that have been exposed to complex mixtures of polycyclic aromatic compounds, the method will not label each of the different DNA adducts formed with the same efficiency and therefore the conditions should be optimized for the class of compounds present. It should be recognized that no other method has the extreme sensitivity or range of structures that can be analysed at one time. If the conditions are the same for all samples in a set, then very useful data can be obtained for comparative evaluation of contamination at different sites.

The normal ³²P-postlabelling method can detect DNA adducts in the range of one modification per 10⁶ to 10⁷ nucleotides. However, sensitivity for large, hydrophobic DNA adducts can be substantially improved to detect DNA adducts in the range of one modification per 10⁹ to 10¹⁰ nucleotides with the use of adduct enhancement techniques. The two enhancement methods most commonly used are extraction of the DNA adducts into water-saturated *n*-butanol (Gupta, 1985) and selective enzymatic degradation of normal mononucleotides to nucleosides using nuclease P1 (Reddy and Randerath, 1986). Gupta and Earley (1988) showed for cigarette smoke condensate, a PAC mixture, that nuclease P1 gives results comparable to those of the butanol method. Phillips *et al.* (1990) and Beach and Gupta (1992) also report that the two methods give similar results for PAC mixtures. In general, the less labour-intensive nuclease P1 procedure would be the method of choice for PAC mixtures. However, some aromatic amine adducts (e.g., dG-C8 derivatives of 2-acetylaminofluorene, 2-aminophenanthrene and 4-aminobiphenyl) are substrates for nuclease P1 and may be lost; whereas, the butanol extraction method gives better recoveries for these adducts (Gupta and Earley, 1988; Gallagher *et al.*,

⁸ To prepare dialysed MN, dissolve MN in distilled water to give a concentration of $1\ \mu\text{g}\ \mu\text{l}^{-1}$. Place MN solution in a 10 mm wide dialysis tube and dialyse overnight (4 °C) against distilled water (2000 ml volume).

1989). If the fish being sampled have been exposed to complex PAC mixtures containing an appreciable proportion of aromatic amines, then the samples should be processed by both enhancement procedures to determine which is the better approach.

Samples that have been stored at -20°C for an extended time period or tissue that is partially decomposed because of poor collection procedures may contain S-compounds (see Section 8, DNA Isolation) that will interfere with the detection of DNA adducts when the normal lithium chloride/Tris/urea (LTU) solvent system is used for development in the D4 direction (see Section 12, Chromatography, for a description of solvent systems, and also Figure 3). Figures 3b and 3c show the typical DNA S-compound chromatographic profiles obtained when using the butanol adduct enhancement procedure. The levels of S-compounds present are substantially higher in Figure 3c than in Figure 3b. The isopropanol/4N ammonia solvent system should be used in D4 instead of the LTU system as these S-compounds will migrate with the solvent front and not interfere with the autoradiographic detection of adducts. Another alternative is to use the nuclease P1 method, which removes most, but not all, of these S-compounds before ^{32}P -labelling. However, the problems with DNA S-compounds become non-existent when the samples are handled properly starting with collection.

10.1 Butanol Adduct Enhancement

The butanol adduct extraction procedure is based on the observation that mononucleotides modified with hydrophobic structures will preferentially partition into water-saturated *n*-butanol. The presence of the phase transfer agent, tetrabutylammonium bromide, can enhance the extraction of some structures (Gupta, 1985). This method can process up to 100 mg hydrolysed DNA (Beach and Gupta, 1992). However, the benefits gained by using larger quantities of DNA may be offset by increasing background radioactivity on the chromatograms.

The authors take duplicate samples of a 3'-BaPDE-dG standard through the butanol extraction process as an extraction efficiency standard to assess procedural losses. When using this method for a specific adduct, it would be wise to take a standard of that specific adduct through the butanol method for a recovery estimate.

10.1.1 Procedure

Place an aliquot of the DNA enzyme hydrolysate (up to 40 μg of hydrolysed DNA), 20 μl of 10 mM tetrabutylammonium bromide, 20 μl of 100 mM ammonium formate, pH 3.5, in a 1.5 ml microcentrifuge tube and add sufficient distilled water to bring the volume to 200 μl . The microcentrifuge tubes should be labelled in two places (i.e., top and side) to avoid loss of sample identification from a butanol leak. Extract the enzyme hydrolysate solution twice with 250 μl water-saturated double distilled *n*-butanol by vortexing for 15 seconds and centrifuging for 2 minutes at 14 000 rpm. Transfer the butanol layer containing the DNA adducts (top layer) to a clean 1.5 ml microcentrifuge tube using a pipettor. Do not transfer any water with the butanol phase. Back-extract the butanol fraction with 250 μl of *n*-butanol-saturated water by vortexing for 15 seconds and then centrifuging for 2 minutes at 14 000 rpm. This step removes any normal nucleotides that may have transferred into the butanol phase. For 1–2 μg DNA do one back-extraction, for 3–10 μg DNA do two back-extractions, and for 15–40 μg DNA do three back-extractions. Discard the water extracts (the lower phase). Remove the butanol phase after the last water back-extraction and place in a fresh 0.5 ml microcentrifuge tube. Place samples in a Savant concentrator/evaporator (heater on the unit is set at 45°C) for 30–60 minutes until all the butanol has evaporated. A 1.5 ml microcentrifuge tube is used as a sleeve to hold the 0.5 ml microcentrifuge tubes in the Savant rotor. Add 15 μl ddH₂O and 'flick' the

bottom of the tube vigorously with your finger to dissolve adduct residue. This is an important step because the residue from the butanol fraction coats the walls of the tube rather than concentrating at the bottom (Phillips and Castegnaro, 1993). The samples are now ready for postlabelling.

10.2 Nuclease P1 Enhancement

The nuclease P1 enhancement method is easy to use and based on the observation that chemically modified DNA is resistant to the 3'-phosphatase activity of nuclease P1, whereas normal nucleotides are hydrolysed to nucleosides, which are not substrates for phosphorylation by T4-polynucleotide kinase. However, there are several potential pitfalls. The degree of resistance of DNA adducts to nuclease P1 hydrolysis is dependent on adduct type and the physical size of the adduct (Gupta and Earley, 1988). For some DNA adducts, the percentage of the adducts hydrolysed during nuclease P1 treatment will depend on the amount of nuclease P1 used and how long the samples were hydrolysed. When targeting a specific DNA adduct, adjust the procedure accordingly to maximize recovery.

10.2.1 Procedure

Place 7 μl of a nuclease P1 solution containing 2 μl of 4 μg nuclease P1 μl^{-1} , 0.9 μl of 1 M sodium acetate (pH 5.0), and 4.1 μl of 1 mM zinc chloride at the bottom of a 0.5 ml microcentrifuge tube. Then add 10 μl of DNA enzyme hydrolysate by submerging the pipet tip containing the DNA hydrolysate directly into the nuclease P1 solution, and pumping with the pipettor to mix. This is a critical step because if any of the hydrolysate escapes nuclease P1 digestion then the normal nucleotides will be labelled and the chromatograms will have a high background. Incubate samples for 45 minutes at 37 °C. After incubation, add either 3 μl of 0.5 M CHES buffer (pH 9.6) or 0.5 M Tris base to the hydrolysate, briefly centrifuge the tube contents to the bottom, and mix with a pipettor. The samples are now ready for postlabelling.

11 ³²P-POSTLABELLING OF DNA ADDUCTS AND NUCLEOTIDES

Xenobiotic DNA adducts and DNA nucleotides are postlabelled using T4-polynucleotide kinase (PNK) to enzymatically transfer ³²P from [γ -³²P]ATP to 3'-mononucleotides to form [5'-³²P]deoxyribonucleoside-3',5'-bisphosphates. Important points for successful labelling are that [γ -³²P]ATP is in excess of the DNA adducts present in the sample, sufficient PNK is present to carry out the reaction, and the [γ -³²P]ATP used has a high specific activity. The authors normally use 100 μCi of [γ -³²P]ATP with a specific activity of 2000–3000 Ci per mmol when labelling 10 μg of DNA that has gone through either the butanol or nuclease P1 enhancement procedure. It is important to have the reaction solution concentration of [γ -³²P]ATP > 1 μM to be on a labelling plateau for PACs (Segerbäck and Vodicka, 1993; Hemminki *et al.*, 1993); however, for other types of DNA adducts, the concentration of [γ -³²P]ATP used may need to be higher (Beach and Gupta, 1992).

The sensitivity of DNA adduct detection increases with the specific activity of [γ -³²P]ATP used. However, 100 μCi of [γ -³²P]ATP with a specific activity of 6000 Ci per mmol contains approximately 16 pmol of ([γ -³²P]ATP+ATP) versus 33 pmol when the specific activity is 3000 Ci per mmol (the lower specific activity is due to increased presence of non-radioactive ATP). This means that 200 μCi of [γ -³²P]ATP with a specific activity of 6000 Ci per mmol is needed per sample to have the same starting molar concentration of ATP that 100 μCi of 3000 Ci per

mmol [γ - ^{32}P]ATP would yield. The authors have found that a specific activity for [γ - ^{32}P]ATP in the range of 2000 to 3000 Ci per mmol is satisfactory.

Labelling efficiency is also dependent on the amount of PNK used and the pH of the labelling medium. In a review by Beach and Gupta (1992), they report that the amount of PNK per sample used by various laboratories ranged from 2 to 68 units and was dependent on the compounds that were labelled. Segerbäck and Vodicka (1993) found that a labelling plateau for individual PAHs and PAH mixtures was reached when the PNK concentration in the samples exceeds 0.3 units μl^{-1} . At pH 8.0, PNK has some residual 3'-phosphatase activity which can affect labelling efficiency. However, at higher pH levels (pH > 8.5) this phosphatase activity is almost non-existent. Generally, in most procedures the labelling buffer pH is 9.5 before addition to a sample. However, the actual pH in the sample during labelling is usually lower owing to the buffering capacity of components present in the enzyme hydrolysate. The authors have found that with BaPDE-dG and PAC-DNA adducts, maximum labelling occurred when the actual labelling pH in solution was between 8.4 and 8.8. Labelling efficiency fell off rapidly when the actual labelling pH was above 9.0 (unpublished data). Lee *et al.* (1995) also suggest that a lower labelling pH (< 8.0) would work. However, the level of calcium ions present were in the range where Hemminki *et al.* (1993) reported inhibition of kinase by calcium and this complicates the comparison of the results of Lee *et al.* (1995) with those of others. The authors have used 30 minutes to 45 minutes for labelling time. However, Phillips and Castegnaro (1993) have suggested that 10 minutes may be sufficient for maximal labelling of some types of adducts.

11.1 Procedure for ^{32}P -Postlabelling of DNA Adducts

This procedure is for 1–10 μg DNA samples that have gone through an enhancement step to remove normal nucleotides (i.e., nuclease P1 or butanol extraction). The authors use the Plexiglas carousels described by Reddy and Blackburn (1990) to hold the samples during labelling. The carousels are an efficient means for handling samples and offer excellent radiation protection.

Make sufficient [γ - ^{32}P]ATP labelling solution for the number of samples being processed and then add 10 μl of this solution to each sample. Each 10 μl of [γ - ^{32}P]ATP labelling solution should contain: 100 μCi of 2000 to 3000 Ci per mmol of [γ - ^{32}P]ATP; 10 units of PNK in a 50 % glycerol solution; 5 μl of labelling buffer (0.1 M bicine, 0.1 M MgCl_2 , 0.1 M dithiothreitol, 10 mM spermidine, pH 9.0); additional labelling buffer to bring to a 10 μl volume. This solution is kept on ice until it is added to the samples. Place the samples in the 0.5 ml tubes (cut caps off) and put in a preheated (37 °C) Plexiglas carousel and add 10 μl of [γ - ^{32}P]ATP labelling mix to each tube using a micropipettor. Centrifuge the carousel briefly to concentrate the solutions at the bottom of the microcentrifuge tubes. Mix the contents of each tube using a pipettor and place the carousel in a 37 °C waterbath for 30 minutes. The following step using apyrase is generally omitted when labelling large bulky hydrophobic adducts, such as PAHs (the solvent system used for the D1 chromatography step will remove all of the unreacted [γ - ^{32}P]ATP). (*Optional step*) After incubation with [γ - ^{32}P]ATP labelling mix, add 4 μl of potato apyrase solution (2.5 μg apyrase μl^{-1}) to each sample to degrade any unreacted [γ - ^{32}P]ATP. Centrifuge to concentrate solution at the bottom of the tubes and mix using a pipettor. Incubate solution at 37 °C for 30 minutes. After incubation, centrifuge the carousel briefly to concentrate solution at the bottom of the tubes. After mixing with a pipettor, spot 5–20 μl of each hydrolysate slowly on the origin of a pre-marked PEI-cellulose sheet that has an 11 cm Whatman (CHR 17) filter paper wick stapled to it (Figure 5). Keep the spot as small as possible

by applying the sample slowly to the sheet and allow the PEI-cellulose to draw the moisture from the pipet tip. During chromatography, the spots will expand and the smaller the spot size on application to the PEI-cellulose sheet the smaller and more intense the spot will be on the autoradiogram.

In addition, a small aliquot (<1 µl) is spotted on a separate PEI-cellulose sheet for development in 0.3 M ammonium sulfate and 10 mM sodium phosphate, pH 7.4. This step is necessary to verify that an excess of [γ - 32 P]ATP was present in the sample at the end of the postlabelling. The absence of [γ - 32 P]ATP at reaction completion usually means that the enhancement step (butanol or nuclease P1) was not successful in removing the normal nucleotides which will consume [γ - 32 P]ATP and that 32 P-labelling of samples probably did not go to completion. If an apyrase step is included in the procedure, then take an aliquot of the reaction solution just prior to apyrase addition.

Consult Section 12, for specific instructions on chromatography procedures.

11.2 Procedure for 32 P-Labelling of Nucleotides

Vortex the diluted enzyme hydrolysate (5 µl of each DNA enzyme hydrolysate is diluted to 500 µl with ddH₂O for nucleotide analysis) and place 10 µl in a 0.5 ml microcentrifuge tube for DNA base analysis. Place the tube in a preheated (37 °C) Plexiglas carousel (caps are removed from tubes).

Prepare enough [γ - 32 P]ATP labelling solution for the number of samples being processed and then add 10 µl of this solution to each sample. Each 10 µl of [γ - 32 P]ATP labelling solution will contain: 8 units of PNK in a 50 % glycerol solution; 7.3 µl of labelling buffer (0.1 M bicine, 0.1 M MgCl₂, 0.1 M dithiothreitol, 10 mM spermidine, pH 9.0); 0.5 µCi of [γ - 32 P]ATP⁹; 2.4 µl of non-radioactive ATP solution (0.5 mg ATP ml⁻¹)¹⁰; additional labelling buffer to volume. After addition of the nucleotide labelling solution, briefly centrifuge the carousel to concentrate the solution at the bottom of the tubes and then mix the samples using a pipettor. Incubate samples for 45 minutes at 37 °C.

Take two 5 µl aliquots of the labelling solution for counting later by liquid scintillation spectrometry (LSS) for the determination of the [γ - 32 P]ATP specific activity. In addition, spot two 0.5 µl aliquots of the nucleotide labelling solution on a PEI-cellulose sheet and chromatograph using 1 M LiCl as an eluant. If decomposition of [γ - 32 P]ATP to 32 P_i and ADP has occurred, then two spots will appear on the autoradiogram. The lower spot (R_f 0.4) will be [γ - 32 P]ATP and the upper spot (R_f 0.9) will be 32 P_i. Excise both spots and count them by LSS. The percentage of [γ - 32 P]ATP in the sample is defined as:

$$\% [\gamma\text{-}^{32}\text{P}]\text{ATP} = \{[\gamma\text{-}^{32}\text{P}]\text{ATP dpm}\} / \{[\gamma\text{-}^{32}\text{P}]\text{ATP dpm} + {}^{32}\text{P}_i \text{ dpm}\}$$

⁹ About 50 µCi of [γ - 32 P]ATP will be sufficient for labelling approximately 30–50 samples.

¹⁰ The non-radioactive ATP solution is prepared by dissolving 10 mg of fresh ATP in 20 ml of ddH₂O to give a concentration of 0.907 nmol ATP µl⁻¹ water. It is important that the non-radioactive ATP used to make this solution has not decomposed, otherwise the calculated specific activity will be lower than what is actually present in solution. Also, the amount of radioactive [γ - 32 P]ATP is negligible compared to the amount of non-radioactive ATP used and will not influence the calculation of ATP concentration.

Note: This step for determining the % of [γ - ^{32}P]ATP will not account for the breakdown of non-radioactive ATP.

Specific activity of nucleotide labelling solution =

$$\frac{[(\% \text{ radioactivity due to } [\gamma\text{-}^{32}\text{P}]\text{ATP}) \times (\text{average dpm in a } 5 \mu\text{l} \text{ aliquot of the nucleotide labelling mix})]}{[\text{concentration of ATP in the bases labelling mix aliquot}]}$$

At the end of the incubation, centrifuge the samples briefly to bring contents to the bottom of the tubes. The authors no longer treat the samples with apyrase to remove remaining [γ - ^{32}P]ATP. Mix each sample with a pipettor and spot 10 μl 2 cm above the bottom of a 20-cm high PEI-cellulose sheet and about 2 cm apart. Develop the sheets in 0.3 M ammonium sulfate and 10 mM sodium phosphate, pH 7.4 (dilute the solvent to 80 % strength if you are using laboratory-prepared PEI-cellulose sheets). At the end of the development, dry the sheets and expose the chromatograms to film for 1 hour at room temperature. The sequence of spots from the origin in this solvent system is ATP, dG, dA, dC, dT and the spot at the top is $^{32}\text{P}_i$ (Figure 4b). The approximate R_f values are 0.21, 0.28, 0.49, 0.57, 0.75, and 0.86, respectively (values are for laboratory-prepared PEI-cellulose sheets). Other spots present and adjacent to these four DNA base spots would indicate the presence of RNA contamination (Figure 4a). When apyrase is not used in the nucleotide labelling protocol, the dT spot is generally the best resolved spot (Figure 4b); however, any cleanly separated DNA base spot can be used. Cut out the marked spots using a razor blade and place the piece of PEI-cellulose sheet containing the base spot in a scintillation vial and count. The 5 μl aliquot of the [γ - ^{32}P]ATP nucleotide labelling mix for specific activity determination should be counted at the same time as the nucleotide or calibration spots. This way, all samples are counted at the same time and a decay correction is not required.

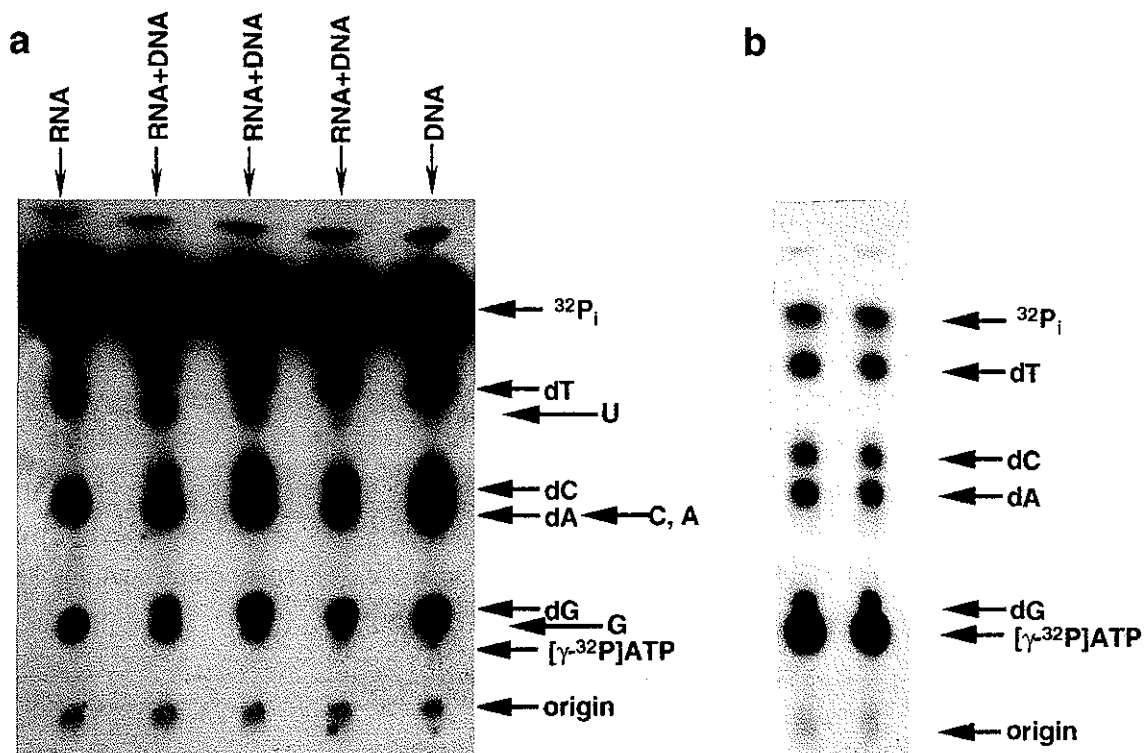
If an imaging system for radioactivity is available, then image the chromatograms and process directly.

Consult Section 13, Imaging and Quantitation, for further information.

12 CHROMATOGRAPHY

The choice of solvent systems for multidimensional chromatography of postlabelled DNA adducts is dependent on the type of adducts to be chromatographed and the removal of interfering ^{32}P -labelled compounds (i.e., normal nucleotides) that can contribute to the radioactive background on the chromatograms. Urea (which reduces the interaction of hydrophobic molecules with the PEI-cellulose sheets), pH, and salt concentrations all influence the rate at which DNA adducts migrate. Increasing the solvent strengths of the salts or urea will generally increase migration. However, as the solubility limits of the solvent components are approached, the risk of these components precipitating out of solution onto the PEI-cellulose sheets increases rapidly. One consequence of this precipitation is the tailing of radioactivity on the chromatograms, which can hamper quantitation. Concentrations of urea greater than 8.5 M should be avoided because chromatography times increase exorbitantly without any appreciable improvement in adduct migration. Generally, the authors dip the edge of the PEI-cellulose sheet in water or the buffer component of the urea/buffer solvents before placing the sheet in the solvent to reduce the problem of supersaturation and precipitation of salts in the solvent front.

Figure 4. (a) Representative autoradiograms showing the chromatographic profiles 3',5'-dpNp and -pNp nucleotides after micrococcal nuclease and spleen phosphodiesterase digestion of DNA and RNA samples and subsequent phosphorylation with polynucleotide kinase and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Excess $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was degraded by apyrase treatment. Samples were chromatographed on polyethyleneimine-cellulose sheets prepared in the laboratory and eluted with 0.24 M ammonium sulfate and 8 mM sodium phosphate, pH 7.4 (b) Representative chromatographic profile of ^{32}P -labelled 3',5'-dpNp nucleotides with the apyrase step omitted.

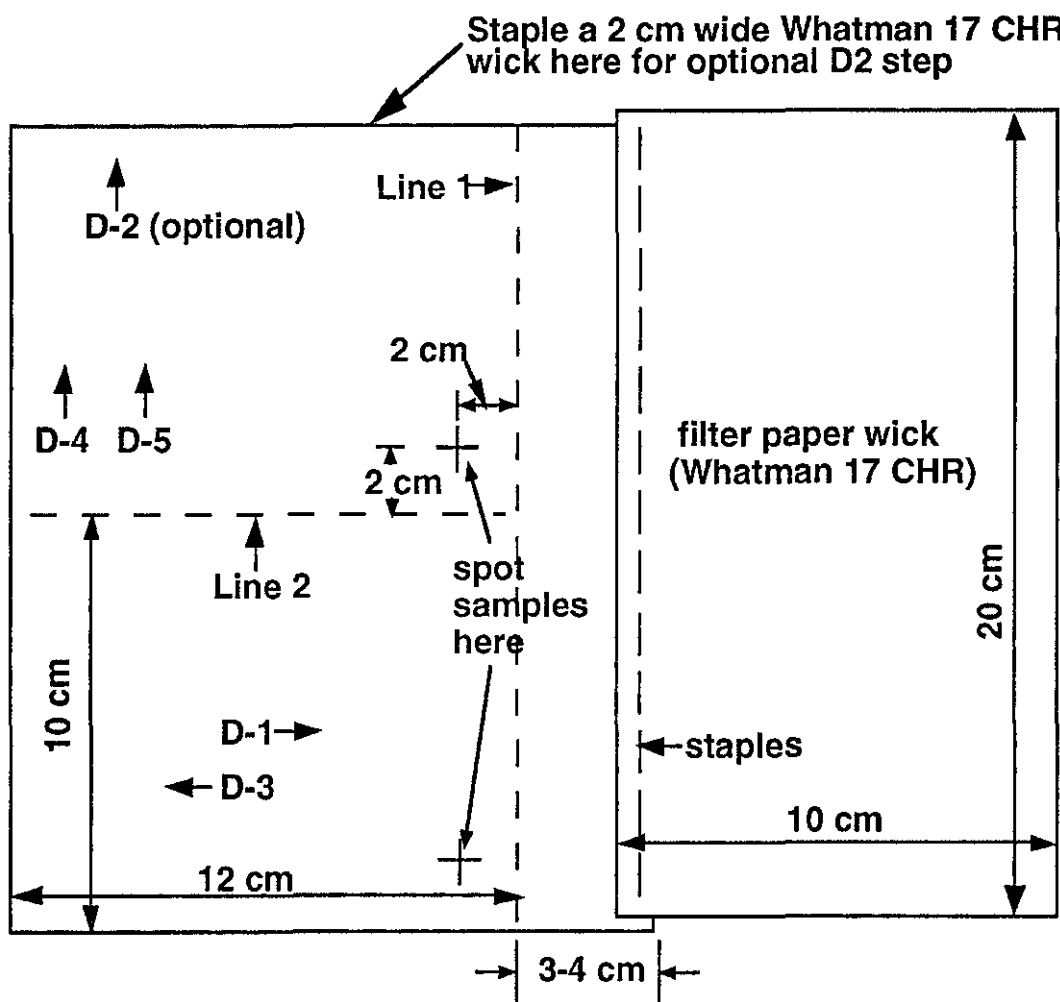


For large hydrophobic structures derived from PAHs (two or more rings), 1 M sodium phosphate, pH 6.0, is generally used for D1 to migrate normal nucleotides and non-specific radioactivity off the PEI-cellulose sheets and onto paper wicks (Reddy, 1993). However, for faster-migrating and less-hydrophobic DNA adducts, the phosphate concentration may have to be raised to 2.3 M sodium phosphate, pH 5.5, to get the DNA adducts to salt out of solution and onto the sheets (Reddy, 1993). The lower pH (5.5) is necessary to minimize the problem of sodium phosphate crystallizing on the PEI-cellulose sheets when using phosphate solutions that are 1.7 M or greater. Also, raising the laboratory temperature and sealing the tanks will help to reduce this problem. However, increasing the ionic strength of the phosphate buffer will also cause some interfering ^{32}P -labelled structures to salt out onto the sheets, and the resulting chromatograms may have a higher background (Beach and Gupta, 1992).

An optional clean-up chromatography step can be done in the D2 direction (Figure 5) using 2.5 M ammonium formate, pH 3.5, although this step is not necessary when chromatographing bulky hydrophobic DNA adducts such as those derived from PAHs (>2 rings). The solvent systems for D3 and D4 are used to migrate and separate the large, hydrophobic DNA adducts.

The most commonly used D3 solvent system for large hydrophobic adducts on commercial sheets is 3.5–4.5 M lithium formate, 8.5 M urea, pH 3.5 (LFU). If the DNA adducts in D3 migrate extremely slowly, then consider 3.5–4.5 M pyridinium formate, 8 M urea, at pH 3.4 (made by neutralizing formic acid with pyridine to the desired pH). The traditional solvents for D4 are 1.6 M lithium chloride, 0.5 M Tris, 8.5 M urea, pH 8.0 (LTU) or 0.8 M sodium phosphate, 0.5 M Tris, 8.5 M urea, pH 8.2 (PTU) for commercial PEI-cellulose sheets. When using laboratory-prepared sheets, dilute the solvents to 80–90 % of the strength used for commercial sheets.

Figure 5. Diagram showing the directions of each of the chromatography steps and the cut lines (---).



The PAC-DNA adducts are generally found within a diagonally shaped region when LFU is used for D3 and LTU is used for D4 (Figure 6b). If the diagonal radioactive zone (DRZ) formed on the chromatogram (as visualized in the autoradiogram) is strong with many intense overlapping spots, then the isopropanol/4 N ammonia system is an excellent alternative for D4, because it will disperse the adducts over a larger area of the chromatogram (Figure 6c).

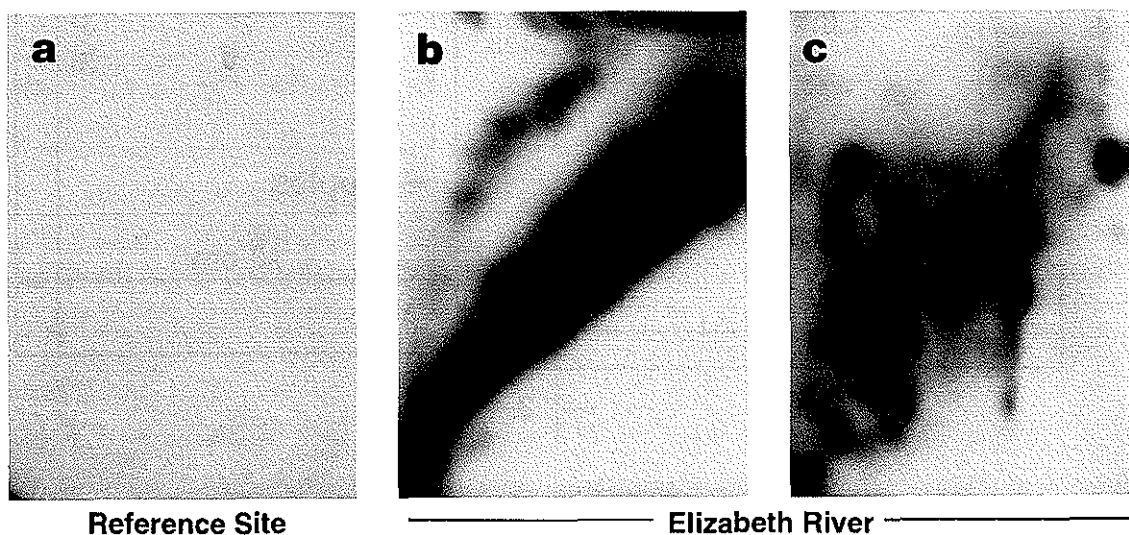
Another promising D4 solvent system is 0.08–0.4 M NH_4OH that gives a zone of adducts more disperse than LTU but less than the isopropanol/ammonia system (Spencer *et al.*, 1993). In addition, this system develops rapidly and chromatography for D4 is complete in approximately 0.5 hours. However, if the DRZ is weak, then the LTU system would be superior because the DNA adducts are compressed into a zone of overlapping spots that can be easily quantitated. If the autoradiograms are hazy, a final development (D5) in 1 M sodium phosphate, pH 6.8, may improve autoradiogram quality by removing some of the non-specific radioactivity from the chromatogram.

12.1 Preparation of Chromatography Solvents

Some of the solvents used for chromatography are based on formic acid or acetic acid. The concentration of these solutions is based on anion concentration, for example, 4 M lithium or ammonium formate, pH 3.5, would have 4 moles per litre of formic acid and sufficient lithium hydroxide (or ammonia) would be added to bring the pH to 3.5. Another important consideration is the type of pH electrode used. With Tris solutions, a calomel electrode is essential because Tris interferes with the standard silver/silver chloride electrode in alkaline media. Also, the pH must be set at the temperature of chromatography because some buffers, such as Tris, have strong temperature-dependent pH profiles. Small changes in pH above 8.0 can have a substantial effect on the migration of some DNA adducts.

Figure 6. Influence of solvent systems on the separation of DNA adducts extracted from hepatic tissues of oyster toadfish captured at a creosote-contaminated site, Elizabeth River, VA. Included is a chromatogram of toadfish hepatic extract from a fish captured at a reference site in Chesapeake Bay, VA. The toadfish from the Elizabeth River site had elevated levels of fluorescent aromatic compounds in bile, which is indicative of PAC exposure. Commercial PEI-cellulose sheets were used.

- (a) and (b) D3: 8.5 M urea, 4.5 M lithium formate, pH 3.5; (c) D3: 8.5 M urea, 4.5 M lithium formate, pH 3.5; D4: isopropanol/4 N ammonia (1:1).
 D4: 8.5 M urea, 1.6 M lithium chloride, 0.5 M Tris, pH 8.0.



12.2 Chromatography of Xenobiotic DNA Adducts

The following protocol is for large hydrophobic DNA adducts. After the spot has been applied, place the sheet in a chromatography tank or a multisheet TLC chamber (see Reddy and Blackburn (1990) for multisheet holder specifications) containing 1.0 M sodium phosphate, pH 6.0, and develop in the D1 direction overnight. The wick will be saturated with the D1 solvent at completion. Remove sheet from chamber and cut the sheet at Line 1 (Figure 5) using a paper cutter. Rinse it in a weak stream of tap water to remove loose specks of PEI-cellulose containing radioactivity. Soak the sheet in either distilled water or soft tap water for 10 minutes in a tank (a 2-gallon aquarium works well with the multisheet holders) to remove the chromatography solvents. Agitate the water with a stirrer. Dry the sheets under a gentle flow of warm air using hair blowers. Be sure that the sheet is completely dry, otherwise solvent migration may not be satisfactory in the next chromatography step.

Dip the bottom edge (approximately 1 cm) of the sheet in 0.45 M lithium formate, pH 3.5, or water, and remove the excess solvent on the bottom edge by touching it with a paper towel. Develop the sheet in the D3 direction (Figure 5) using a lithium formate-urea solvent system (8.5 M urea, 4.5 M lithium formate, pH 3.5, for commercial sheets or 85 % strength for laboratory-prepared sheets). It usually takes 2–3 hours for the solvent front to reach the top of the sheet. *Caution:* The level of the liquid in the chromatography chamber must be below the sample application site on the PEI-cellulose sheets.

Remove the sheet from the TLC chamber and touch the bottom edge to absorbent paper. The PEI-cellulose sheet must be held vertical until the solvent bead has been removed from the bottom of the sheet; holding the sheet in any other position allows the solvent bead to run across the sheet, and a displaced band of radioactivity will appear on the autoradiogram. Also, the solvent bead along the bottom of the sheet must not be allowed to wet the PEI-cellulose side of any sheet in the chromatography tank during removal. Rinse and soak the sheet in the same manner as for D1. Remove the sheet from the tank, cut along Line 2 (Figure 5), and rinse sheet. Dry the sheet as described above.

Dip approximately 1 cm of the bottom edge of the sheet in a 0.5 M Tris, 0.8 M lithium chloride buffer, pH 8.0, and wick the excess solvent with absorbent paper. Develop the sheet in the D4 direction (Figure 5) using 0.5 M Tris, 1.6 M lithium chloride, 8.5 M urea¹¹, pH 8.0 (85 % strength for laboratory-prepared sheets). Remove the sheet and touch the bottom edge to a tissue. Rinse and soak in the same manner as for the D1 step.

Optional clean-up step: Staple a 5 cm filter paper wick (Whatman No. 1) to the PEI-cellulose sheet according to Figure 7. Develop the sheet in the D5 direction (Figure 7) using a 1.0 M sodium phosphate buffer (pH 6.8) overnight. This sodium phosphate development step may improve the autoradiogram by removing some of the residual non-specific radioactivity. After removing the wick, rinse the sheet in the same manner as for D1. Trim the sheet for placement in the cassette and excise the origin while it is still wet (Figure 7). Finally, rinse the sheet gently with tap water to remove any loose PEI-cellulose specks. These specks are caused by the cutting process and can give false images on the autoradiograms. They are usually easy to detect on the autoradiograms because they are intense and have a sharp border. Dry the

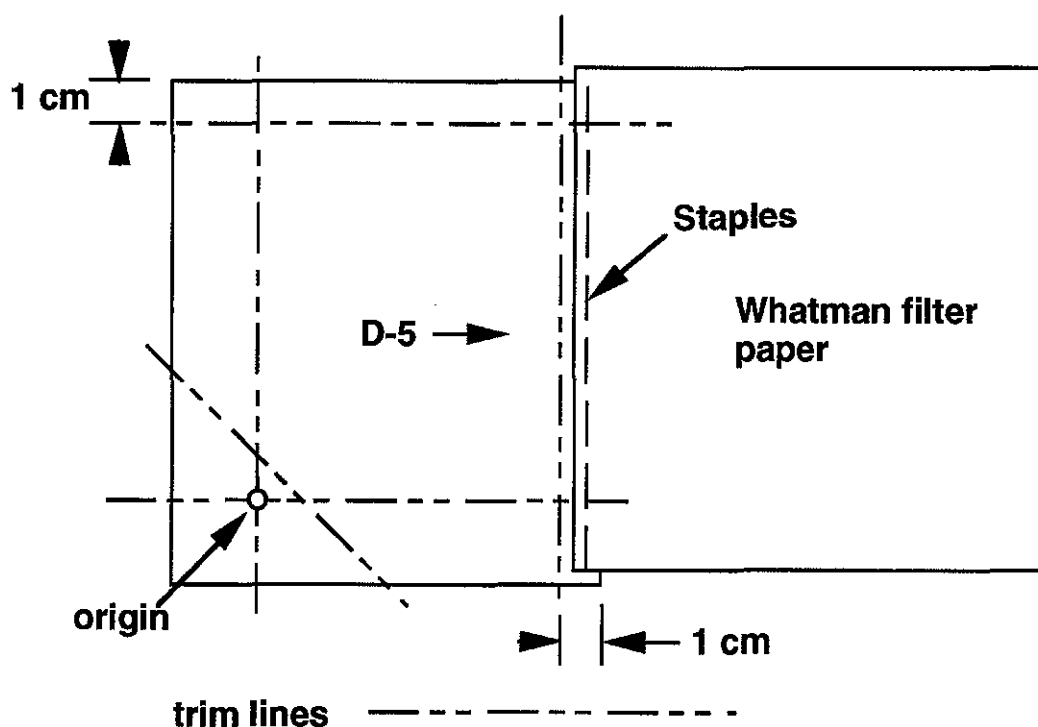
¹¹ When preparing this buffer, be sure to dissolve the urea first and then add the Tris base and lithium chloride and neutralize with hydrochloric acid. If a white precipitate forms, then it may be necessary to start over again with fresh components.

chromatograms and tape to a piece of 14 inch by 17 inch (36 cm × 43 cm) paper. The chromatograms are covered with a thin plastic film and placed in a cassette for autoradiography (see Section 13.1, Autoradiography).

12.3 Alternative D4 Solvent Systems

Isopropanol/4 N ammonia (Figure 6c) and dilute ammonia are excellent alternative D4 solvent systems that will break up the strong DRZs that the LTU solvent system gives and disperse the individual adduct spots over a larger area of the chromatogram (Spencer *et al.*, 1993). The ratio of isopropanol/4 N ammonia (v/v) can vary from 0.8:1 to 2:1; for large hydrophobic adducts on laboratory-prepared sheets use 1.2:1 and for commercial sheets use 1:1. When using the isopropanol/4 N ammonia solvent system for D4, the sheet is placed in 20 mM Tris base solution after the D3 soak step for 3 minutes (this resets the pH of the sheet) and then soaked in water for 5 minutes and dried. A 2 cm filter paper wick (Whatman No. 1) is then stapled to the top edge. A longer wick can be stapled to the top of the PEI-cellulose sheet to increase the distance that the adducts migrate and this can be used as an additional variable along with the isopropanol/4 N ammonia ratio to control adduct migration on the chromatogram. Place the sheet in a TLC tank containing the isopropanol/4 N ammonia, seal the tank with plastic wrap, and develop in the D4 direction. Do not move the tank during development. Development time is usually 2–3 hours. When the solvent has reached the top of the wick, remove the sheet from the tank and let the sheet with the attached wick air dry in a fume hood. Place the dry chromatogram with the attached wick in 1 M sodium phosphate (pH 6.0) for D5. The D5 step is necessary for the isopropanol/4 N ammonia solvent system, otherwise a strong dark band may appear in the middle of the autoradiogram. The dilute ammonia solvent systems also require the Tris base pretreatment and the attachment of a 2 cm wick, as described for the isopropanol/4 N ammonia system. The development time for the dilute ammonia solvent system is 20–40 minutes (Spencer *et al.*, 1993). After D4, develop in D5 direction in the same manner as was done for the isopropanol/4 N ammonia system. Rinse sheet and trim (Figure 7) for autoradiography.

Figure 7. Diagram showing the PEI-cellulose set-up for optional D5. The dashed lines show where the chromatogram is to be trimmed after D5 and before being placed in the autoradiography cassette.



12.4 Chromatography Using HPLC Methodology

An alternative to PEI-cellulose thin-layer chromatography separation of DNA adducts is the use of HPLC with on-line radiometric detection. Pfau *et al.* (1993) and Zeisig and Möller (1995) have shown increased resolution of complex mixtures of DNA adducts formed *in vitro* with bulky hydrophobic compounds. Möller *et al.* (1996) were able to resolve 16 individual peaks or clusters of DNA adducts in lymphocytes from human subjects from the coal-based industrial region of Silesia. Zeisig and Möller (1995) have published the retention times for an extensive number of DNA adducts formed *in vitro* from PACs and their derivatives, which will be useful in the tentative characterization of some DNA adducts. However, absolute structural confirmation of DNA adducts formed in fish from environmental exposure to complex PAC mixtures, based on co-chromatography with standards, by either HPLC or TLC methods is still not possible. Currently, the sensitivity of TLC methods is appreciably greater ($> 10\times$, Pfau *et al.*, 1993) than that of HPLC methods. The lower sensitivity of the HPLC technique may hamper its use when analysing DNA in fish from moderately contaminated sites.

The future for HPLC-based separations of DNA adducts is promising and warrants further development. In addition, the potential exists for using HPLC techniques to automate PPL chromatography steps.

13 IMAGING AND QUANTITATION OF RADIOACTIVITY ON CHROMATOGRAMS

At present, there are several ways to quantitate radioactivity on chromatograms. The traditional 'cut and count method' aligns the autoradiogram with the corresponding chromatogram and the radioactive regions on the chromatogram are outlined with a marking pen. Radioactive regions are then cut from the chromatogram and counted by LSS. Cerenkov counting, which measures the light emitted when a fast-moving charged particle passes through a transparent medium, can also be used in place of LSS for measuring ^{32}P levels but a correction for counting efficiency is necessary. Areas near regions of interest are also excised and counted for background corrections. However, background corrections based on this approach can be subjective and one must exercise careful judgement when the background correction is substantial relative to the spot or region of interest. A superior approach is the use of computer-aided imaging systems that will locate and directly measure the radioactivity on the chromatogram.

13.1 Autoradiography

Autoradiography is used to locate the position of ^{32}P -derived radioactivity on the chromatograms and provides information on the relative intensity of each individual adduct. However, there are restrictions on the interpretation of autoradiograms. First, film has a limited range of response (less than 300) and the range of linear response is less than 100. Multiple exposures are usually required for a complete set of autoradiograms to show both strong and weak regions of radioactivity present on chromatograms. Moreover, the autoradiographic images of faint spots may be only 20–30 % of their expected densities based on the level of radioactivity present, and dark spots will also give an underestimate because of film saturation. Owing to these limitations, caution is required when making assumptions on the relative intensity of faint spots and the levels of background radioactivity present based on a visual inspection of the autoradiogram. The chromatograms can be marked with fluorescent ink (fluorescent ink pens are available from autoradiographic materials suppliers) before autoradiography. The fluorescent image spots will help align the chromatogram with

autoradiograms. Autoradiography of the chromatograms either can be run at room temperature or film sensitivity can be enhanced up to a factor of eighteen with the use of intensifying screens at -80°C (Swanstrom and Shank, 1978).

13.1.1 Procedure

Handle the chromatograms carefully to avoid any flaking or loose particles being generated that are radioactive and could leave confusing images or spots on the autoradiograms. If you do not have fluorescent ink marks to align the chromatogram, then use the solvent front edges on 2D autoradiograms to align the autoradiogram with chromatogram for marking the radioactive regions with a pen.

A map is drawn of the regions that are to be excised and each region is numbered. This will give you a permanent record of the areas quantitated and their respective positions on the autoradiograms. The regions are then excised using a sharp razor blade or scalpel. Each of the excised regions is weighed (this allows a background correction to be made per unit weight of chromatogram) and placed in a scintillation vial for counting.

13.2 Liquid Scintillation Spectrometry (LSS)

Liquid scintillation counting has some restrictions. Samples containing low levels of radioactivity (i.e., faint spots and samples for estimation of background corrections) must be counted for longer time periods than samples containing high radioactivity levels, for adequate counting statistics. Counting statistics are based on total counts measured and not the count rate. A small variation of 3–10 cpm in the estimation of background per cm^2 of surface (easily achievable owing to poor counting statistics for samples with low radioactivity levels) can greatly affect DNA adduct level computations. For single, well-resolved spots, a clean, adjacent area can be used to define background. However, for diagonal radioactive zones, which often have a faint hazy region around them (the haze can be due to DNA adducts), the question of where to sample for background corrections becomes highly subjective. The subjective aspect of defining background from a remote part of the chromatogram points to the need to do the chromatography and measurement of radioactivity carefully.

The liquid scintillation counting efficiency for ^{32}P in weakly quenched conditions is 98–99 %, which means that cpm is essentially the same as dpm.

Correction for background is calculated as follows:

$$\text{Corrected cpm (region of interest)} = \text{cpm (region of interest)} - [\text{wt (mg) of region of interest} \times (\text{background region cpm/wt (mg) of background region})].$$

13.3 Cerenkov Counting

A cheaper alternative to counting samples by LSS is Cerenkov counting in water or with dry samples instead of scintillation cocktail, which eliminates disposal problems associated with some organic-based cocktails. Cerenkov counting efficiencies of 40 % are representative. Because energies involved in Cerenkov radiation are low, one must count samples using tritium detection channels. A correction factor for counting efficiency can be made by counting aliquots of ^{32}P in liquid scintillation cocktail, water, or on a dry sample. Since the counting efficiency in the scintillation cocktail is approximately 99 %, the ratio of cpm-cocktail to cpm-

water or cpm-dry sample will give the appropriate correction factor for converting Cerenkov counts to approximate dpm.

13.4 Imaging Technology

Imaging systems based on multi-wire proportional counting and storage phosphor imaging are currently being used by some laboratories to locate and measure radioactivity associated with the DNA adducts present on the chromatograms. A benefit of using imaging systems is that, unlike radioactivity measurements by liquid scintillation spectrometry, all raw image data for the distribution and intensity of radioactivity on the chromatograms are available for review and reprocessing at any time.

Storage phosphor imaging technology offers high sensitivity (approximately 0.2 nmol adduct mol⁻¹ nucleotides), extremely low counting background, accurate estimation of background radioactivity, and a large linear range of response to radioactivity (10⁵) (Reichert *et al.*, 1992). With the multi-wire proportional counting, the sample image can be monitored constantly during the imaging process. Both imaging systems can handle large numbers of samples. If a decision is made to purchase an imaging system, the following points should be evaluated using your samples: sample throughput, limits of detection, estimating background levels, image quality and cost. In addition, test each machine with a dilution series of ³²P down to 1 dpm and several different chromatograms (i.e., one with a strong DRZ, one with a very weak DRZ, and a control DNA sample).

13.5 Calculations

The ³²P-postlabelling results are usually presented as a ratio of the number of adducts detected divided by the amount of DNA used in the assay (e.g., nmol DNA adducts/mol DNA, amol DNA adducts/μg DNA, fmol DNA adducts/μg DNA). Presenting the data in this fashion avoids the problem of not being able to quantitatively extract DNA from tissues.

It is critical to record all numbers and aliquot factors used in the final calculations. It is also important that your laboratory protocols assure that the necessary information regarding aliquot sizes and the dates when samples are counted is recorded.

13.5.1 Sample calculations

1) Calculation for total DNA adducts measured:

fmol adduct(s) = dpm for DNA adduct spot (or zone) × labelling aliquot correction factor × (1/specific activity of [γ-³²P]ATP).

Where:

dpm for DNA adduct spot (or zone) = 2130,

labelling aliquot correction factor = 2 (15 μ l spotted on chromatography sheet of a 30 μ l ^{32}P -labelled DNA sample),

specific activity of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ = 2000 Ci/mmol = 4400 dpm/fmol.

$$\text{fmol adduct(s)} = 2130 \times 2/4400 = 0.968 \text{ fmol}$$

2) Calculation for total DNA analysed for adducts:

nmol DNA = dpm for DNA base spot \times factor to convert from nmol base to nmol DNA \times spotting aliquot factor \times DNA enzyme hydrolysate dilution correction factor \times (1/specific activity of DNA nucleotides labelling mix $[\gamma\text{-}^{32}\text{P}]\text{ATP}$).

Where:

dpm for DNA nucleotide spot = 24 600 dpm,

factor to convert from nmol base to nmol DNA = 100 % / % of base in DNA; use 4.76 for dG, 3.45 for dT, and 2 for dA+dC,

spotting aliquot factor = 2.4 (10 μ l spotted on chromatography sheet of a 24 μ l ^{32}P -labelled DNA nucleotides sample),

DNA enzyme hydrolysate dilution correction factor = (500 μ l dilution volume/10 μ l taken for nucleotide labelling) \times 2 (to change from 5 μ l of enzyme hydrolysate used for measuring DNA content to 10 μ l of hydrolysate used in the DNA adduct labelling part of the assay) = 100,

specific activity of DNA nucleotides labelling mix $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ = 968 055 dpm/nmol.

$$\text{nmol DNA} = 24\,600 \text{ dpm} \times 4.76 \times 2.4 \times 100 \times 1/(968\,055 \text{ dpm/nmol})$$

$$\text{nmol DNA} = 29 \text{ nmol DNA used in the DNA adduct assay}$$

3) Calculation of DNA damage level:

nmol DNA adducts/mol DNA = amount of DNA adducts measured in a sample/total amount of DNA used in sample.

$$= 0.968 \text{ fmol DNA adducts}/29 \text{ nmol DNA}$$

$$= 968 \text{ amol DNA adducts}/29 \text{ nmol DNA} \times (10^9/10^9)$$

$$\text{nmol DNA adducts/mol DNA} = 33 \text{ nmol DNA adducts/mol DNA}$$

After the calculations have been completed, the results should be reviewed for accuracy. An important check for computational or data entry errors is to compare the final fmols of adducts with the autoradiograms. The results should vary directly with the image density of the region of interest on the autoradiograms, provided that comparable amounts of DNA were assayed.

14 QUALITY ASSURANCE/QUALITY CONTROL

The accuracy of the ^{32}P -postlabelling method in measuring DNA adduct levels is dependent on the efficiencies of enzyme-mediated reactions and radioactivity measurements. The method is semi-quantitative when working with tissue samples from animals exposed to complex PAC mixtures. At present, the necessary standards are not available to optimize the conditions for all adducts present. However, if sample sets are run under uniform conditions, then comparisons between samples collected from different sites can be made. To have increased confidence in the results, appropriate controls must be used to signal the presence of significant problems. The following are quality assurance procedures that the authors have included in the ^{32}P -postlabelling assay:

- 1) Testes DNA from farm-raised Atlantic salmon (*Salmo salar*) is used for measuring the efficiency of DNA hydrolysis and as a sample blank to be carried through the entire postlabelling process. The authors have found that commercial preparations of lyophilized calf thymus, salmon or herring sperm DNA sometimes have appreciable levels of DNA breakdown products (S-compounds) and that it is better to prepare DNA from fresh salmon or herring testes obtained from a source where the probability of PAC exposure is very low.
- 2) The compound 7R,8S,9S-trihydroxy,10R-(N²-deoxyguanosyl-3'-phosphate)-7,8,9,10-tetrahydrobenzo[a]pyrene (BaPDE-dG-3'p) is used as an external standard to monitor the efficiency of enzyme-mediated transfer of the ^{32}P -phosphate from [γ - ^{32}P]ATP to PAC-derived DNA adducts. The concentration of the stock BaPDE-dG-3'p from the manufacturer should be checked by postlabelling the BaPDE-dG-3'p with ^{32}P and using the specific activity of the [γ - ^{32}P]ATP to verify concentration. When used with each set of samples, the radioactivity measurement of the chromatographed postlabelled BaPDE-dG-3'p spot will give information on the efficiency of ^{32}P labelling and can be used as an alternate verification of the specific activity of the [γ - ^{32}P]ATP used in the assay (specific activity of [γ - ^{32}P]ATP = dpm of BaP spot/amount of BaP labelled). In addition, the postlabelled BaPDE-dG-3'-p can be used as a chromatography standard.
- 3) In the butanol adduct enrichment method, an aliquot of BaPDE-dG-3'p is used as an extraction efficiency standard for recovery of PAC-derived DNA adducts. However, when a specific DNA adduct is being targeted, a standard for that compound should be used, if available, to determine extraction efficiency.
- 4) An aliquot of contaminant-modified DNA from fish injected with a PAC-contaminated sediment extract can also be used to monitor inter-assay labelling efficiency of complex mixtures, and as an additional chromatographic standard.
- 5) To assess reproducibility, every tenth tissue sample is analysed in duplicate.

- 6) A 2'-deoxyribonucleotide-3'-monophosphate standard is used to monitor the efficiency of enzyme-dependent labelling of the total normal nucleotides by ^{32}P -phosphate from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$.

15 SOURCES OF ERROR

- 1) Pipetting. The PPL procedure requires numerous pipettings of small volumes. The micropipettors available are excellent at pipetting small volumes. However, the micropipettors must be routinely calibrated. Technicians should evaluate their technique by checking the weight of their aliquots on an analytical balance and practice with both water and the buffer solutions that they will be working with.
- 2) Specific activity of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. The specific activity of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ must be verified for each run to increase comparability between sets. An additional check to verify specific activity is to use a ^{32}P labelling standard, such as BaPDE-dG. Specific activity = dpm of ^{32}P associated with BaPDE-3'-dG spot/moles BaPDE-3'-dG labelled.
- 3) DNA measurement. The amount of DNA used in the assay must be measured by postlabelling. It is our experience and that of others (Gupta, 1993) that DNA concentrations can vary substantially (up to $\pm 50\%$) when measured spectrophotometrically.
- 4) Background corrections. Correction for background levels of ^{32}P on the chromatograms can substantially affect the final calculated values, especially when using the 'cut-and-count' approach (see Section 13.2, above). In the 'cut-and-count' approach, a blank section of the chromatogram is excised and used as an estimate of background. However, the blank areas selected are often not close to nor truly representative of the actual background at the region of interest. Computer-aided image processing gives a better estimate of background, because it is taken at the periphery of the region of interest. The use of an imaging system can substantially reduce the variability in quantitation of chromatograms, especially those with weak DRZs.
- 5) Sample collection and storage. All samples must be frozen immediately when collected with either dry ice or liquid nitrogen. The samples should then be stored at -70°C or lower to prevent the formation of S-compounds that will be released during enzymatic hydrolysis of DNA. If S-compounds form, the chromatograms will have higher background levels and exhibit extraneous spots, which can interfere with quantitation, especially with reference site samples. Samples stored at -20°C for long periods of time slowly deteriorate to the point where DNA is no longer recoverable (6–12 months). See discussion in Section 8, DNA Isolation.

16 INTERPRETATION OF DATA

The results from the ^{32}P -postlabelling assay have been successfully used to rank sites for bioavailable levels of PAC contamination in sediments and as an index of PAC exposure risk for a population of fish (Stein *et al.*, 1992; Collier *et al.*, 1993; van der Oost *et al.*, 1994). Recent findings show that DNA adduct levels are dose and time responsive to PAC concentrations present in sediment and that they are persistent in fish (Sikka *et al.*, 1990; Stein *et al.*, 1993; French *et al.*, 1996) and thus can be used as an index of chronic exposure to bioavailable PACs. Our studies to date suggest that DNA adduct levels are not markedly

dependent on factors such as sex, season, or dietary status, which are factors that can confound the interpretation of other parameters used as biomarkers. However, validation of the DNA adduct biomarker in a species of interest would be prudent to insure against unusual species-specific responses.

16.1 Example of Survey Data

The data in Annex 1 are from a survey of the territorially restricted oyster toadfish (*Opsanus tau*) collected from a reference station in Chesapeake Bay and five stations in the creosote-contaminated Elizabeth River, a tributary to Chesapeake Bay (van Veld *et al.*, 1990; Collier *et al.*, 1993).

The results show a strong correlation between sediment concentrations of polycyclic aromatic compounds (PACs) and the levels of DNA adducts detected in hepatic tissues of the oyster toadfish. Linear regression analysis of the log-transformed average DNA adduct levels at each site versus log-transformed sediment PAC concentrations showed a strong correlation ($r^2 = 0.97$, $p < 0.001$) between DNA adduct levels and sediment PAC levels over a range of 10^5 in PAC concentrations. In addition, the toadfish from PAC-contaminated sites had elevated levels of BaP-like fluorescent aromatic compounds in bile, which is indicative of PAC exposure (Collier *et al.*, 1993). Gas chromatography-mass spectrometry (GC-MS) studies have shown that many of the biliary metabolites observed in PAC-exposed fish arise from PAC metabolism (Krahn *et al.*, 1987). The levels of biliary fluorescent aromatic compounds also correlated with the sediment concentrations of PACs ($r^2 = 0.95$, $p < 0.001$).

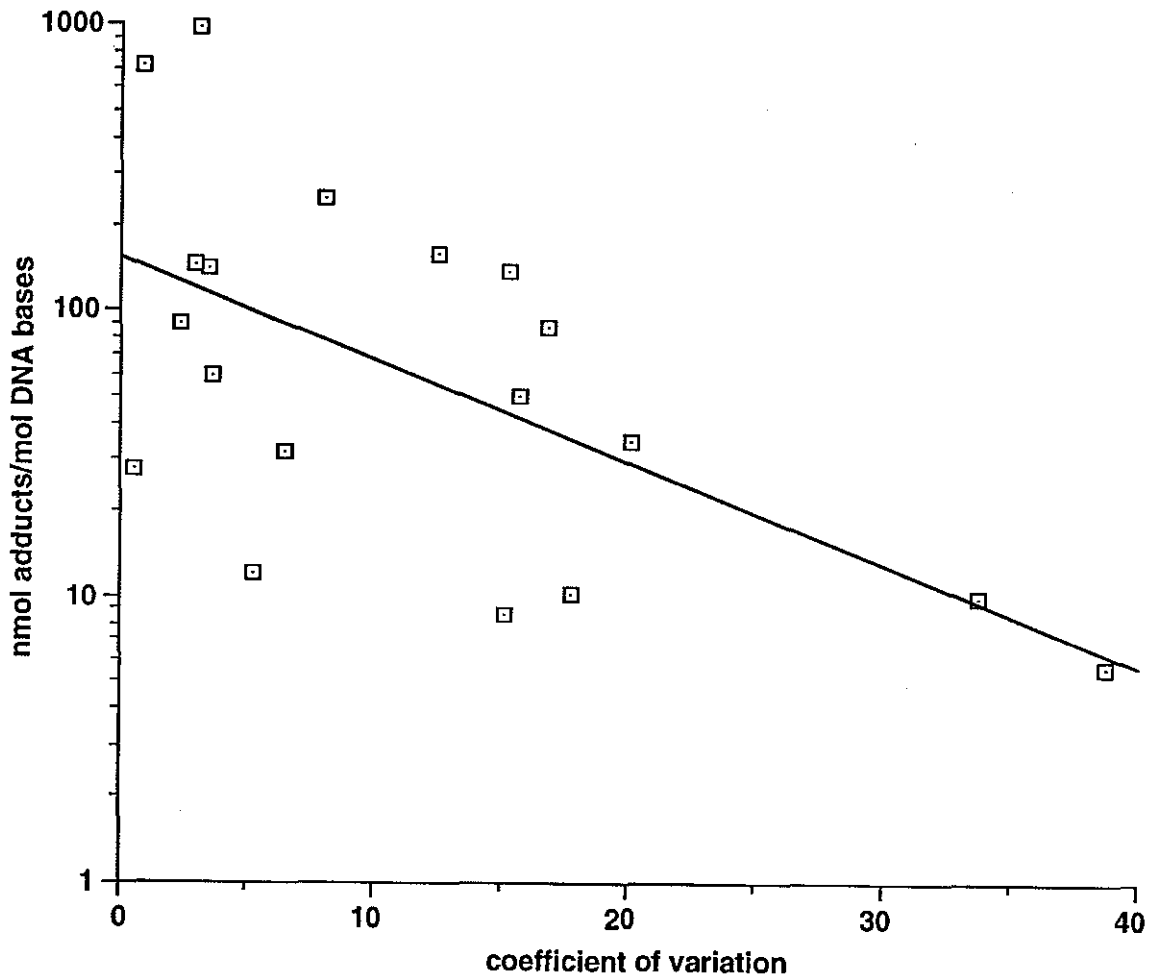
The chromatogram (Figure 6b) of ^{32}P -labelled hepatic DNA digests for fish collected from the Elizabeth River is typical of DNA adduct chromatograms for species exposed to PACs (Varanasi *et al.*, 1989a; Savelle *et al.*, 1989; Stein *et al.*, 1994; French *et al.*, 1996). The DNA chromatogram (Figure 6a) and biliary fluorescent aromatic compound levels for toadfish from the Chesapeake Bay site indicated minimal PAC exposure.

These findings show that DNA adduct levels in a territorially restricted benthic species of fish can correlate strongly with the levels of PACs present in sediments. In addition, the use of another biomarker of PAC exposure, such as biliary fluorescent aromatic compounds, and sediment chemistry can provide additional support for the contention that the DNA adducts arose from PAC exposure.

16.2 Reproducibility and Interlaboratory Comparisons

Reproducibility in the ^{32}P -postlabelling analysis of replicate samples of hepatic DNA from environmentally exposed fish is shown in Figure 8. The average coefficient of variation (CV) was $12 \pm 11\%$ ($n = 19$, range of 1 to 39). In addition, regression analysis of log-transformed levels of DNA adducts vs. CV showed that the CV decreased as the levels of DNA modification increased (1 to 720 nmol adducts/mol DNA bases, $r = 0.607$, $p < 0.01$). The intra-assay and inter-assay CVs for labelling of a BaPDE-3'-dG standard were 7% ($n = 15$) and 15% ($n = 26$), respectively.

Figure 8. Plot of the coefficient of variation (SD/X) versus the average (\bar{X} , $n = 2$) DNA adduct level for replicate samples of hepatic tissue from several species of marine fish. The results show that the CV declines as the DNA adduct levels increase ($r = 0.61$, $p < 0.01$, $n = 19$).



The comparison of PPL results obtained by different laboratories is contingent, primarily, on how each of the enzymatic steps has been optimized and how the distribution of radioactivity and background on the chromatograms was quantitated; there may also be other factors that come into play. At present, there is no widely used standardized procedure for any of these steps. However, there have been several interlaboratory comparisons of standards and human white blood cell samples (Savela *et al.*, 1989; Hemminki *et al.*, 1990; Phillips and Castegnaro, 1993). Savela *et al.* (1989) compared the results obtained by three laboratories assaying the levels of polycyclic aromatic DNA adducts present in the white blood cells from 53 iron foundry workers. They found that the results from the three laboratories were correlated ($p < 0.01$), although the mean adduct values for the foundry workers obtained by the three laboratories were 9.2 ± 23 , 12 ± 10 and 26 ± 43 and the control means from two of the laboratories were 1.7 ± 0.7 and 3.1 ± 1.7 adducts per 10^8 nucleotides. In another study with white blood cells from iron workers, Hemminki *et al.* (1990) found that results between two laboratories were strongly correlated, although the mean adduct values obtained differed by a

factor of 2 to 2.5. The interlaboratory studies (Savelle *et al.*, 1989; Hemminki *et al.*, 1990) show that, in a group of subjects exposed environmentally to PAHs, different laboratories will obtain the same relative rankings by PPL, however, the absolute values obtained by different laboratories may differ appreciably because of differences in protocols and the lack of availability of reference materials. Phillips and Castegnaro (1993) compared the results obtained by fifteen different laboratories sent control DNA, BaP-modified DNA, DNA from the lung of a tobacco smoker, and 2-acetylaminofluorine-modified DNA from mouse liver. The CVs for the results obtained by the different laboratories were in the range of 53 % to 80 % for the nuclease P1 enhancement version and 65 % to 98 % for the butanol version. The high variation in the results obtained is a reflection of the differences in the protocols used by the individual laboratories. The findings also indicated that the levels of DNA modification were under-estimated. Clearly, the availability of DNA from a mammal or fish that has been exposed to PAC-rich sediment extract for broad use as a control material would improve the ability to compare results obtained by different laboratories.

17 FUTURE PROSPECTS

An ultimate goal for using biomarkers, such as DNA adducts, is in improving risk assessment. Recently, a significant association was observed between DNA adduct levels and the prevalence of hepatic pre-neoplastic lesions in English sole (Myers *et al.*, 1996); this has been substantiated in a subsequent study showing that DNA adduct levels are a significant risk factor for certain lesions occurring early in the histogenesis of hepatic neoplasms in feral English sole (M.S. Myers, personal communication). These results suggest that DNA adduct levels, as measured by ^{32}P -postlabelling, have the potential to be molecular dosimeters of exposure to environmental carcinogens and to yield useful information for assessing the risk of neoplasia occurring in a population of wild fish.

In conclusion, DNA adduct levels, as measured by the ^{32}P -postlabelling method, can be used as a biomarker of exposure to PACs and a biomarker of damage. In addition, DNA adducts can potentially yield information that can indicate the class of contaminants involved in the exposure based on DNA adduct profiles. Field studies (Myers *et al.*, 1996) have demonstrated the potential of the method to provide information on the risk for neoplasia from exposure to genotoxic contaminants. There is clearly merit for continued use and evaluation of the DNA adducts biomarker, as measured by the ^{32}P -postlabelling assay, as an important tool in the suite of biomarkers used in environmental assessment.

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ANNEX 1

Data from a survey of territorially-restricted oyster toadfish (*Opsanus tau*) collected from a reference site in Chesapeake Bay and sites in the creosote-contaminated Elizabeth River, a tributary to Chesapeake Bay (Collier *et al.*, 1993). Information is given to show the variation in DNA adduct levels obtained for individual fish captured at the sites and the relationships between DNA adduct levels and concentrations of polycyclic aromatic compounds in sediments.

Station	Site	nmol adducts/ mol nucleotides*	Average	SE	Sediment PACs (g kg ⁻¹ , dry wt)
1	Chesapeake Bay	5, 4, 6, 7, 5	5	1	9
2	Elizabeth River	10, 6, 10, 16, 30	14	4	55
3	Elizabeth River	77, 52, 39, 30, 39	47	8	3 100
4	Elizabeth River	16, 38, 32, 79, 22	37	11	16 000
5	Elizabeth River	148, 187, 94, 179, 98	141	20	96 000
6	Elizabeth River	198, 92, 21, 73, 95	96	30	43 000

*Values are levels of DNA adducts (nmol adducts/mol nucleotides) in individual fish from each site.

ABBREVIATIONS

ADP	adenosine diphosphate	LiCl	lithium chloride
AMP	adenosine monophosphate	LSS	liquid scintillation spectrometry
ATP	adenosine triphosphate	LTU	lithium chloride/Tris/urea solvent system used for developing chromatograms in the D4 direction
BaP	benzo[<i>a</i>]pyrene		
CHES	2[N-cyclohexylamino]ethanesulfonic acid	mCi	millicuries
Ci	curie (1 curie = 2.22×10^{12} dpm)	MN	micrococcal nuclease
CIA	chloroform/isoamyl alcohol, 24:1, v/v	PAC	polycyclic aromatic compound
CIP	chloroform/isoamyl alcohol/phenol, 24:1:25, v/v/v	dpGp	3', 5'-deoxyguanosine bisphosphate
cpm	counts per minute	PEI	polyethyleneimine
D1	direction for solvent development (Figure 5)	PNK	polynucleotide kinase
D2	direction for solvent development (Figure 5)	PPL	³² P-postlabelling
D3	direction for solvent development (Figure 5)	PTU	sodium phosphate/Tris/urea
D4	direction for solvent development (Figure 5)	R _f	distance of the spot from the start/distance of the solvent front from the start
DBC	dibenzo[<i>c,g</i>]carbazole	RNA	ribonucleic acid
ddH ₂ O	deionized distilled water	S-compounds	compounds formed from the spontaneous change of DNA bases
dpm	disintegrations per minute	SDS	sodium dodecyl sulfate
DRZ	diagonal radioactive zone	SPD	spleen phosphodiesterase
EDTA	ethylene diaminetetraacetic acid	TE	10 mM Tris, 1 mM EDTA, pH 7.5
EtOH	ethanol	TLC	thin-layer chromatography
HCl	hydrochloric acid	UV	ultraviolet
HPLC	high performance liquid chromatography	v/v	volume by volume
LFU	lithium formate/urea solvent system used for developing chromatograms in the D3 direction		

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