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Bracken (*Pteridium aquilinum*)-Induced DNA Adducts in Mouse Tissues Are Different from the Adduct Induced by the Activated Form of the Bracken Carcinogen Ptaquiloside

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Following treatment with bracken fern (*Pteridium aquilinum*) extract and bracken spores a number of DNA adducts were detected by ^{32}P-postlabeling. Three of these adducts have been described previously (Povey *et al., Br. J. Cancer* (1996) 74, 1342–1348) and in this study, using a slightly different protocol, four new adducts, with higher chromatographic mobility, were detected at levels ranging from 50 to 230% of those previously described. When DNA was treated *in vitro* with activated ptaquiloside (APT) and analysed by butanol extraction or nuclease P1 treatment, only one adduct was detected by ^{32}P-postlabeling. This adduct was not present in the DNA from mice treated with bracken fern or spores, suggesting either that bracken contains genotoxins other than ptaquiloside or that the metabolism of ptaquiloside produces genotoxins not reflected by activated ptaquiloside. However, as the ATP-derived adduct has been detected previously in ileal DNA of bracken-fed calves, species-specific differences in the metabolism of bracken genotoxins may exist, thereby leading to differences in their biological outcomes.

Key Words: **bracken fern; ptaquiloside; DNA adducts; ^{32}P-postlabelling.**

Bracken fern (genus *Pteridium*) has been described as one of the most common plants on the planet (Taylor, 1990) and it is the only plant that is known to cause tumours naturally in animals (Shahin *et al.*, 1999). The toxicity and carcinogenicity of bracken fern to domestic and experimental animals has been extensively described (Evans and Mason, 1965; Evans, 1984; IARC, 1986; Pamacku and Price, 1969). This plant contains a number of toxic components, including flavonoid antioxidants such as quercertin and an unstable glycoside, ptaquiloside (PT), which is thought to be the principal carcinogenic compound present in bracken (Hirono *et al.*, 1984a, 1987). Recent work indicates that other toxic compounds related to ptaquiloside are also present in bracken fern (Castillo *et al.*, 1998) but in lower concentrations.

PT is stable at room temperatures but decomposes in aqueous solutions. Under acidic conditions it undergoes aromatisation by elimination of the glucose to give pterosin B, whereas under alkaline conditions it is activated to give a conjugated dienone (activated ptaquiloside: APT) upon the liberation of the sugar group (Fig. 1; Ojika *et al.*, 1987)). APT has been shown to alkylate DNA via a reactive cyclopropyl ring to form a number of different DNA adducts (Ojika *et al.*, 1987, 1989). The main labile adducts occur at the N-3 of adenine, and to a minor extent with N-7 of guanine (Smith *et al.*, 1994b; Kushida *et al.*, 1994). This N3-adenine selectivity has also been observed in anticarcinogenic alkylating agents possessing a cyclopropyl ring intermediate (Hurley *et al.*, 1984; Shalder *et al.*, 1999). Following nuclease P1 enrichment and ^{32}P-postlabelling, a single adduct, as yet uncharacterised, was found in DNA from the ileum of bracken fed calves (Prakash *et al.*, 1996) or APT dosed rats (Shahin *et al.*, 1998), and in DNA treated *in vitro* with APT (Smith *et al.*, 1994b). As ptaquiloside-N3 adenine adducts are labile and depurinate within 24 h (Prakash *et al.*, 1996) they may not be readily detectable in the postlabeling assay. Previously, it has been demonstrated that administration of either extract or spores of bracken fern collected in the UK (*Pteridium aquili-*

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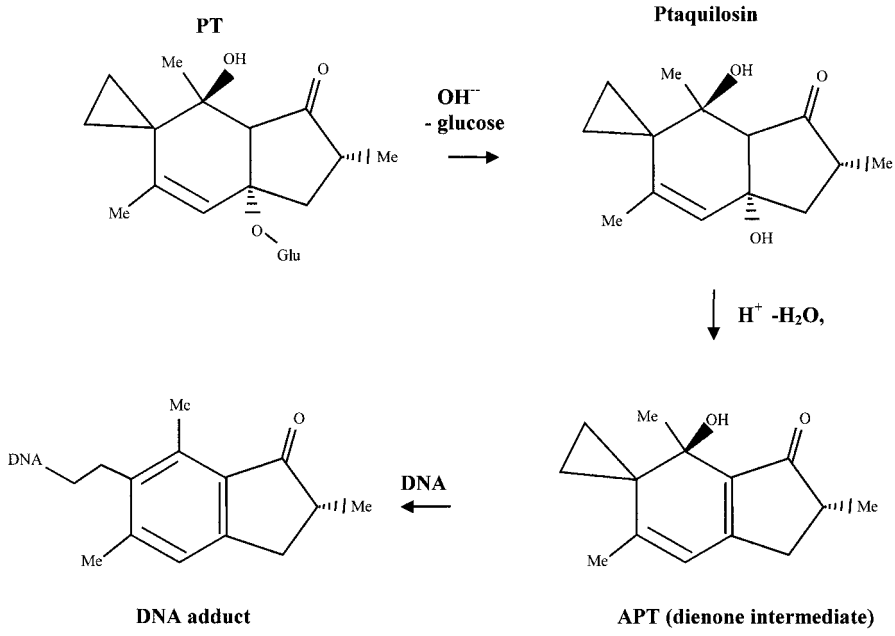

FIG. 1. Structures of ptaquiloside, ptaquilosin, activated ptaquiloside (APT), and its interaction with DNA.

num subsp. *aquilinum*) induced DNA adducts detectable in the upper gastrointestinal tract of BDF1 mice by using ^{32}P-postlabeling after butanol extraction for DNA adducts (Povey *et al.*, 1996). However, the adduct enrichment method used in the former studies (Smith *et al.*, 1994a; Prakash *et al.*, 1996; Shahin *et al.*, 1998) differed from that used in the UK study (Povey *et al.*, 1996) and as these enrichment methods may extract chemically different adducts (Gupta and Early, 1988) it was not immediately apparent whether or not APT-induced DNA adducts were present in the DNA isolated from BDF1 mice.

We thus carried out the present work, using both butanol and nuclease P1 enrichment procedures, to determine whether the uncharacterised adducts found in gastrointestinal tissue from BDF1 mice were the same or different from the one formed by APT *in vitro.*

MATERIALS AND METHODS

^{32}P-γ-ATP (specific activity 7000 Ci/mmol) was purchased from ICN Pharmaceuticals, Inc. (ICN Biomedicals, Kingston-upon-Thames, UK). T_4 polynucleotide kinase (T_4-PNK) and calf spleen phosphodiesterase (CSPE) were both obtained from Boehringer-Mannheim (Germany). Micrococcal nuclease (MN) and alkaline phosphatase were obtained from Sigma (Poole, UK). MN and CSPE were also purchased from Worthington (Lorne Laboratories, Reading, UK). Plastic PEI-cellulose plates were supplied by Schleicher-Schuell (Anderman and Co., Kingston-upon-Thames, UK).

Preparation of Bracken Samples

An extract of fresh bracken fern (*Pteridium aquilinum* subsp. *aquilinum*) collected in Bellech, Anglesey, UK was prepared (Povey *et al.*, 1996). Ptaquiloside was isolated and purified as described elsewhere (Oelrichs *et al.*, 1995) from bracken collected from in Southeast Queensland.

Treatment of Animals

Treatment of animals with bracken fern extracts and spores from the UK has been described previously (Povey *et al.*, 1996) and the DNA obtained in these experiments was used here.

In Vitro Modification of DNA with Activated Ptaquiloside

Ptaquiloside was activated by incubation with NaOH and used *in vitro* to modify calf thymus DNA (Smith *et al.*, 1994a).

Analysis of DNA Adducts

DNA samples from the *in vivo* experiments were extracted using a standard phenol-chloroform method after treatment of the tissue with RNase A and proteinase K (Povey *et al.*, 1996). Both butanol and nuclease P1 enrichment procedures were used to analyse for DNA adducts by ^{32}P-postlabelling.

DNA digestion to nucleotides. 10–25 μg of each DNA sample was digested for 4 h at 37°C using CSPE (0.432 mU/μg DNA) and MN (0.495 U/μg DNA) obtained from Worthington, with 10 mM Tris–HCl, pH 7.4, 5 mM $CaCl_2$ and 1 μM deoxycorfomycin in a final volume of 2.5 μl/μg DNA.

Butanol extraction. Aliquots containing 4 μg of the DNA digest were extracted with 1-butanol (Povey *et al.*, 1996) but using 3 rather than 2 back washes with 1-butanol saturated water.

Nuclease P1 digestion. Aliquots containing 7.89 μg of the initial DNA digest was further incubated with sodium acetate pH 5, zinc chloride, and nuclease P1 at final concentrations of 40 mM, 0.2 mM, and 0.31 μg/μl, respectively. The reaction was allowed to proceed for 30 min at 37°C, then stopped by adding 1 μl of 0.92 mM Tris and the samples were dried *in vacuo.*

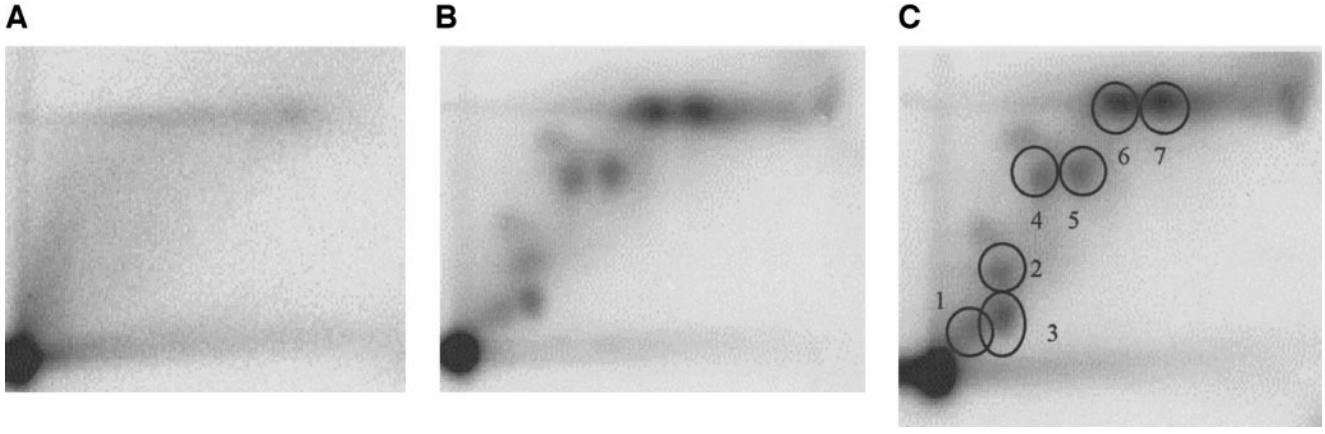

FIG. 2. Phosphorimages of butanol-extractable DNA adducts detected in the upper gastrointestinal tract of mice following treatment with water (A), bracken fern extract (B), and bracken spores (C).

^{32}P-postlabelling and TLC. The adducted nucleotides were ^{32}P-postlabelled for 30 min at 37°C in 30 mM Tris-HCl (pH 9.5) containing 10 mM magnesium chloride, 10 mM DTT, 1 mM spermidine, 3.0 μM ATP (total concentration) using 2.5 units T_4 PNK and 170 μCi ^{32}P-γ-ATP in a total volume of 10 μl. Labelled samples were spotted on PEI-cellulose TLC plates and chromatographed as previously described (Povey *et al.*, 1996) but with the plates only run once in D3 and D4. Solvents used for development of the thin layer plates were as follows: D1, 1.0 M sodium phosphate, pH 6.5; D3, 3.5 M lithium formate, 8.5 M urea, pH 3.5; D4, 1.2 M lithium chloride, 0.5 M Tris, 8.5 M urea, pH 8; and D5, 1.7 M sodium phosphate, pH 6.

Normal nucleotides and adduct quantitation. The plates were dried and exposed to a phosphorimager screen for up to 72 h. The samples were visualised using a Molecular Dynamic Storm 860 image analysis system with ImageQuant software. Adducts were quantified by comparing the signal with that of a known amount of the labelling mix spotted onto a TLC plate and exposed together on the same phosphor screen. To determine the normal levels of nucleotides released, 10 μg aliquots of the same DNA digest were treated with alkaline phosphatase to give nucleosides that were quantified by HPLC (Cooper *et al.*, 1992).

RESULTS

Upper gastrointestinal DNA samples (oesophagus, stomach and ileum) from BDF1 mice treated intragastrically with bracken extract or spores contained several adducts (Figs. 2B and 2C) that were not found in the DNA samples from untreated control animals (Fig. 2A), or in untreated calf thymus DNA used as a negative control (data not shown). The adducts located near to the origin (Nos. 1, 2, and 3 in Fig. 2C) were described previously (Povey *et al.*, 1996). In addition to these adducts, 4 other spots with higher chromatographic mobility were detected (Nos. 4–7 in Fig. 2C). Assuming quantitative recovery, the levels of adducts 4 and 5 were 50 and 90% of adduct 2 respectively whereas levels of adducts 6 and 7 were, respectively, 160 and 230% higher than the level of adduct 2. Adducts recovered using butanol extraction were similar to those recovered using nuclease P1 digestion, except for adduct 1, which was poorly recovered after nuclease P1 digestion (Fig. 3).

Only one adduct was detected in calf thymus DNA reacted *in vitro* with APT when the sample was treated with either nuclease P1 APT (Fig. 4) or extracted with butanol (data not shown). This APT derived adduct had a chromatographic mobility that was similar to adducts 6 and 7 but subsequent cochromatography experiments indicated a chemically different identity for the APT-derived adduct. Hence, we conclude that the APT induced DNA adduct was not present in detectable amounts in the DNA obtained from BDF1 mice treated with bracken fern extract or bracken spores.

DISCUSSION

Previously, the presence of DNA adducts in upper gastrointestinal tissue of BDF1 mice treated with a single dose of bracken extracts or spores has been described (Povey *et al.*, 1996). Using butanol extraction to enrich the adducted nucleotides in these samples, three major (Nos. 1, 2, and 3: Fig. 2C) adducts were found that were not present in untreated animals. These adducts were not characterised, but their chromatographic mobility was similar to adducts formed in the same tissue from mice dosed with synthesised compounds containing a cyclopropyl ring (Povey *et al.*, 1996).

Using a slightly different protocol, not only have the same spots in these samples been detected, but also at least 4 other adducts (Nos. 4, 5, 6, and 7) with higher chromatography mobility (Figs. 2B and 2C) were found that were not present in the control samples (Fig. 2A). Procedures that result in the preferential enrichment of different adduct classes (Reddy and Randerath, 1986; Gupta and Early, 1988) were used to further characterise these adducts. The DNA samples were digested to nucleotides and then either extracted with

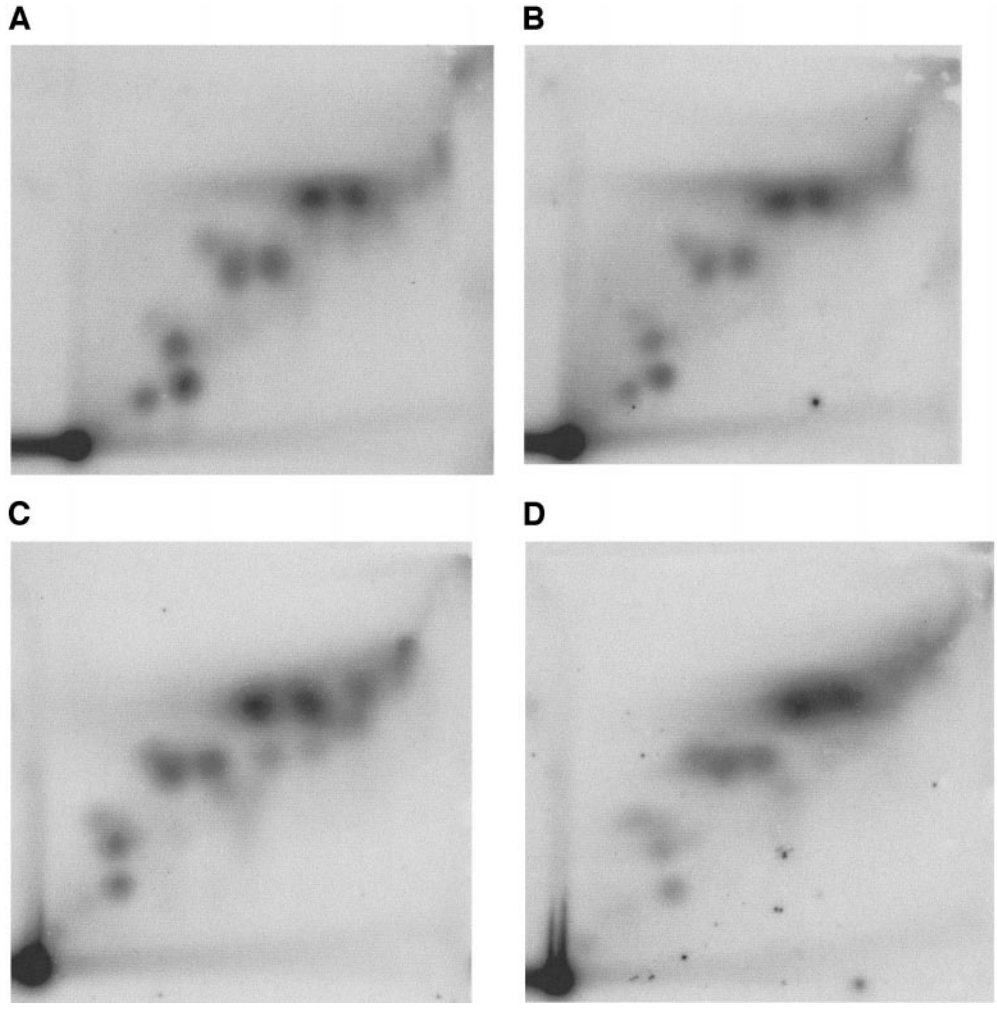

FIG. 3. Phosphorimages of DNA adducts detected in the upper gastrointestinal tract of mice following treatment with bracken fern extract (A, C) or bracken spores (B, D). DNA from treated animals was enzymatically digested to nucleotides and then subjected to butanol extraction (A, B) or further treatment with nuclease P_1 (C, D). The adducts were ^{32}P-postlabelled and chromatographed on PEI-cellulose plates as described in the text.

1-butanol (Gupta, 1985) or treated with nuclease P1 (Reddy and Randerath, 1986). Most of the adducts (Nos. 2–7, Fig. 2C) were readily detected by both techniques indicating that these were not sensitive to nuclease P1 digestion unlike certain adducts, notably those derived from aromatic amines or simple alkylating agents and some cyclic nucleotide adducts (Reddy *et al.*, 1984; Hemmink *et al.*, 1991; Szyfter *et al.*, 1991; Vaca *et al.*, 1992).

In order to know if any of the adducts detected in mouse DNA were identical to that formed by APT *in vitro,* a cochromatography experiment was performed (Fig. 4). This indicated that the principal DNA adduct formed by APT *in vitro* was not found in digests of the upper gastrointestinal tissue DNA of mice treated with bracken fern extracts or spores. The amount of PT in the bracken fern extract and bracken spores used in the mouse experiments was not quantified, but it was previously calculated that approximately 8 mg of ptaquiloside was administered to each animal (Povey *et al.*, 1996) based on a published bracken fern content (Hirono, 1986). However, as PT levels can depend on a number of factors including bracken species, freshness, season and drying conditions (Oelrichs *et al.*, 1995; Smith *et al.*, 1994b), the amount of PT in these samples may have been too low to permit the detection of PT-derived DNA adducts. In a previous work, we (Shahin *et al.*, 1998) could detect in ileum tissue a PT derived DNA adduct from rats dosed intravenously with 3 mg ptaquiloside weekly for 10 weeks. Although, the absence of PT derived DNA adducts may potentially be ascribed to insufficient PT in the initial bracken extract, the presence of DNA adducts as detected by ^{32}P-postlabelling, clearly indicates that the bracken samples did contain other genotoxins: whether these are unrelated to PT, or could arise from the metabolism of PT to forms other than APT remains to be determined. However, as an adduct similar to that arising

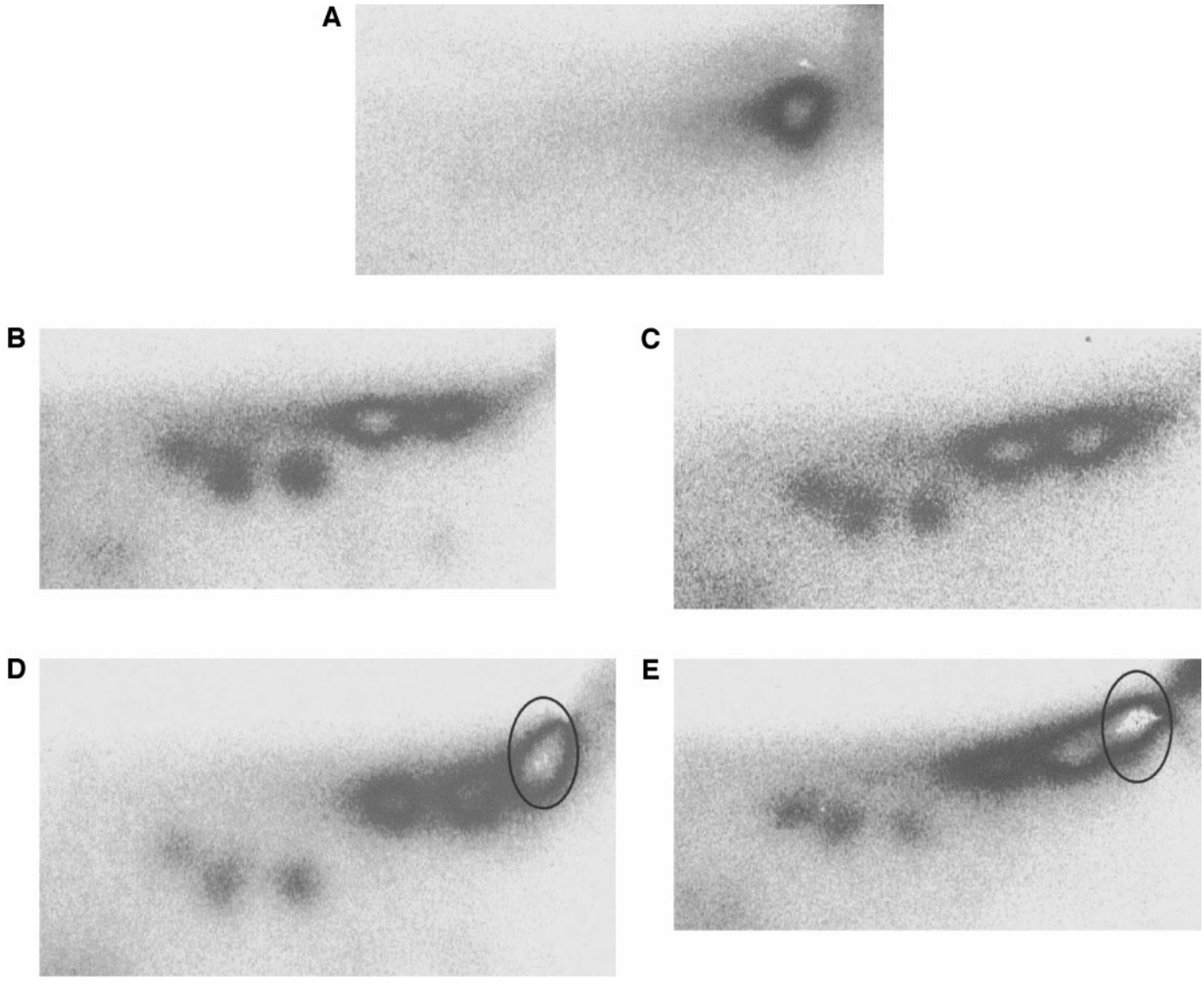

FIG. 4. Phosphorimages of high mobility DNA adducts detected in calf thymus DNA modified *in vitro* with APT (A) and the upper gastrointestinal tract of mice following treatment with bracken fern extract (B) or bracken spores (C). D and E are respectively ^{32}P-postlabelled samples of DNA adducts induced by bracken fern extract (as in B) and bracken spores (as in C) cochromatographed with the adduct from calf thymus DNA modified *in vitro* with APT (as in A). Circles in D and E locate the APT-derived adduct.

from APT-DNA (Prakash *et al.*, 1996), was found in the ileum from a calf fed with bracken fern, species specific differences in PT metabolism may exist. In this regard, it is of interest to note that in calves, the most important site of tumour occurrence is the ileum, followed by urinary bladder and mammary gland whereas in mice, leukaemic, stomach and lung tumours are common but with ileal tumours being a relatively rare occurrence (Evans, 1984; IARC, 1985).

In summary, DNA adducts derived from APT were not detected in tissues from mice treated with *Pteridium aquilinum*. The possibility that this is due to low PT levels in the bracken samples used to treat the mice cannot be ruled out. However as other DNA adducts were detected following treatment with *Pteridium aquilinum* in mice, it is evident that there is more than one genotoxic substance is present in *Pteridium aquilinum*. Whether these genotoxins are related to the new PT-like compounds such as Ptaquiloside Z (Castillo *et al.*, 1998) that are now being identified remains to be determined.

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