Binding of Diethylstilboestrol to Deoxyribonucleic Acid by Rat Liver Microsomal Fractions *in vitro* and in Mouse Foetal Cells in Culture

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Diethylstilboestrol, a synthetic and carcinogenic hormone, binds to DNA as a result of incubation with a liver microsomal preparation *in vitro* and on incubation with primary mouse foetal cells in culture. Enzymic digestion of DNA samples thus prepared gives several covalent deoxyribonucleoside–diethylstilboestrol products from the microsomal system. One of these is produced in small but significant yield in the tissue-culture system.

Diethylstilboestrol has been known for many years to be capable of inducing tumours in experimental animals (Fels, 1950; Kirkman & Bacon, 1950). More recently it has been linked to cases of cancer of the vagina in humans (Herbst *et al.*, 1971). Although this compound can be bonded covalently to DNA by photochemical or oxidative means (Blackburn *et al.*, 1974), in the Ames *Salmonella*/microsome test (McCann *et al.*, 1975) its bacterial toxicity (McCann & Ames, 1976) precludes the application of the test as an indicator of DNA damage. We therefore thought it desirable to evaluate its binding to DNA by using microsomal fractions and in tissue culture, since considerable progress has resulted from the use of these systems for the binding of carcinogens, particularly polycyclic aromatic hydrocarbons, to DNA (Gelboin, 1969; Baird *et al.*, 1975; Thompson *et al.*, 1976).

Materials and Methods

[G-³H]Diethylstilboestrol was purchased from The Radiochemical Centre, Amersham, Bucks., U.K., with a specific radioactivity of 10 Ci/mmol. It was used without dilution. Native calf thymus DNA was type V from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.

Preparation of microsomal fractions

Male CB hooded rats (approx. 150 g) were given injections intraperitoneally with a suspension of 3-methylcholanthrene (3 mg) in arachis oil (0.5 ml); 1 day later the animals were deprived of food for a 12 h period, decapitated, and their livers removed. Microsomal fractions were prepared from the livers of three animals (Kinoshita *et al.*, 1973),

washed by resuspension on 0.25 M-sucrose and re-centrifugation at 10⁵ g for 1 h, and finally suspended at 20–40 mg of protein/ml in 0.25 M-sucrose. Batches (1 ml) were stored at –196°C. Protein was assayed by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. The RNA content, determined spectrophotometrically (assuming $E_{260} = 1$ for 50 µg of RNA/ml), was 5–7 mg/ml.

Binding of diethylstilboestrol to DNA in the presence of microsomal fractions

Diethylstilboestrol (0.5 mCi, 0.05 µmol) was incubated at 37°C for 30 min in 5.0 ml of aqueous suspension containing 1 mM-NADPH, 0.1 mM-EDTA, 3 mM-MgCl₂ and 0.1 M-potassium phosphate buffer, pH 7.5, with 10 mg of calf thymus DNA, 4 mg of microsomal protein and 5% (v/v) acetone. The DNA was recovered by adding sodium dodecyl sulphate to the mixture (final concn. 1%), followed by 2.5 ml of phenol reagent (Kirby, 1965). The DNA was then precipitated with ethanol, purified by re-precipitation four times, extraction with ethanol and with diethyl ether, and dried under N₂.

Degradation and analysis of the DNA

The DNA obtained above was assayed by dissolution in water (2.0 ml) and partially degraded to oligonucleotides by the addition of deoxyribonuclease I and MgCl₂ (King *et al.*, 1975). From the E_{260} and measured radioactivity, the DNA was shown to have a specific radioactivity of 26 000 d.p.m./mg, corresponding to a gross binding of 0.4 µmol of diethylstilboestrol/mol of DNA P. The DNA was degraded to deoxyribonucleosides by incubation with deoxyribonuclease I (Sigma), snake-venom diesterase (Sigma) and alkaline phosphatase (Sigma) as previously described (King *et al.*, 1975).

Duplicate samples of this mixture of deoxyribonucleosides were fractionated by gradient elution [500ml of methanol/water (3:7, v/v) to 500ml of methanol] through a column (80cm $\times$ 1.5cm) of Sephadex LH-20 standing in methanol/water (3:7, v/v). Samples (approx. 5ml) were collected and their E_{260} and radioactivity were measured. Whole-volume samples were counted for radioactivity in Triton X-100 phosphor (10ml) made up from 1,4-bis-(5-phenyloxazol-2-yl)benzene (POPOP) (3.5g), 2,5-diphenyloxazole (PPO) (70g), Triton X-100 (4 litres) and toluene (6 litres).

Binding of diethylstilboestrol to DNA of primary mouse-embryo cells in tissue culture

Primary mouse-embryo cells were obtained by trypsin treatment of 12–18-day C57SB mouse embryos and were grown in plastic culture flasks (175cm²; Nunclon, Nunc, Denmark) in Eagle's minimal essential medium (catalogue no. F14; Grand Island Biological Co., Grand Island, NY, U.S.A.) supplemented with 10% of foetal calf serum (Flow Laboratories Ltd., Irvine, Scotland, U.K.). The cells were grown to confluence in ten bottles, each containing 50ml of this medium, during 48h and then treated with [G-³H]diethylstilboestrol (10 μ mol, 0.1mCi per bottle) for 48h. The cells were harvested by using EDTA (Baird & Brookes, 1973), and the DNA was isolated by the method of Kirby (1965) as modified by Duncan *et al.* (1969).

A total of 1.4mg of DNA was recovered with a specific radioactivity of 18000d.p.m./mg of DNA, equivalent to a gross binding of 0.3 μ mol of diethylstilboestrol/mol of DNA P. Subsequent degradation of this DNA (as described above) to deoxyribonucleosides was followed by chromatographic analysis on Sephadex LH-20.

Results

The extent of binding of diethylstilboestrol (I) to calf thymus DNA as a result of incubation with a microsomal preparation was determined under the strictly controlled conditions hitherto used for polycyclic aromatic hydrocarbons and their metabolites (Thompson *et al.*, 1976) and found to be 0.4 μ mol of diethylstilboestrol/mol of DNA P, uncorrected for any metabolic loss of ³H.

The DNA was digested enzymically to give a mixture of deoxyribonucleosides, which on standard chromatographic analysis revealed that an excellent degradation of the DNA had occurred. The four deoxyribonucleosides, identified by u.v. absorption, were eluted characteristically at 95–100ml (dC), 110ml (dT), 130ml (dG) and 160ml (dA).

The pattern of radioactivity from the [³H]diethylstilboestrol showed the resolution of at least eight bound species, which were eluted later than the

unmodified deoxyribonucleosides (Fig. 1), in a similar fashion to that for aromatic hydrocarbon-deoxynucleoside products. In addition, two major peaks were resolved that were eluted ahead of the four nucleosides and probably corresponded to undegraded oligodeoxynucleotides.

The extent of gross binding of the synthetic hormone to DNA in mouse foetal cells in culture amounted to 0.3 μ mol of diethylstilboestrol/mol of DNA P. This DNA was totally degraded to deoxyribonucleosides, as revealed by the u.v. trace of the column eluate. The bulk of the ³H label is accounted for by the large amount of material eluted between 50 and 80ml, corresponding to oligonucleotides and nucleotides [and any protein that survived the Kirby (1965) DNA isolation procedure], and by ³H incorporated into the purine and pyrimidine deoxyribonucleosides, which were eluted between 95 and 160ml (Fig. 1). A small amount of radioactivity, corresponding to some 5% of the total, was eluted at approx. 550ml at the same methanol concentration as a major peak in the microsomal binding experiment.

Discussion

The above data show that diethylstilboestrol is covalently bound to DNA as a result of incubation with a rat liver microsomal system. Protein- and RNA-bound impurities would not contribute significantly to the measured DNA-bound radioactivity because of the addition of excess of DNA and the elution of any protein-bound hormone in the void volume. The association of some radioactivity with material of high molecular weight suggests a limited resistance to enzymic hydrolysis of the DNA in regions of hormone binding. Among the degradation products, some eight low-molecular-weight bound species are resolved (Fig. 1). These are not likely to be metabolites of diethylstilboestrol occluded in the DNA, since the purification sequence was designed to extract such species. Moreover, they are eluted at a methanol concentration characteristic for polyhydroxylated aromatic hydrocarbon-deoxyribonucleoside products (Thompson *et al.*, 1976).

Fig. 1. Elution profile from Sephadex LH-20 of enzyme digests of DNA isolated after incubation of diethylstilboestrol (I) with primary mouse foetal cells in culture (—) and with calf thymus DNA in the presence of a rat liver microsomal preparation (.....)

The void volume of the column was 45 ml. Other experimental details are described in the text.

The metabolism of diethylstilboestrol *in vivo* involves epoxidation of the ethylenic double bond and hydroxylation of the phenolic rings (Metzler, 1975). These features are also characteristic of the metabolism of polycyclic aromatic hydrocarbons and are associated with binding such materials to DNA. It is thus reasonable to expect that microsomal incubation should lead, as shown, to the binding of diethylstilboestrol to DNA.

The extent of such binding for this hormone is only one-tenth that for hydroxylic metabolites of benzo[*a*]pyrene of low carcinogenicity and two orders of magnitude lower than that for benzo[*a*]pyrene itself (Thompson *et al.*, 1976). It therefore seems possible that diethylstilboestrol would exhibit little if any toxicity were it not that hormone-target tissue shows remarkable receptor properties (Jensen *et al.*, 1972) and specific transport of the hormones into the nucleus (Jensen & DeSombre, 1973).

The gross binding of diethylstilboestrol to DNA in primary mouse foetal embryo cells in culture is 0.3 $\mu\text{mol/mol}$ of phosphate. Gauged in terms of the amount of hormone metabolized, that gives a binding index which is greater than that for the weak carcinogens benz[*a*]anthracene and dibenz[*a,c*]anthracene (Brookes *et al.*, 1974).

It is possibly misleading. Enzymic degradation (Fig. 1) shows that most of the radioactivity incorporated either is eluted with high-molecular-weight materials, resistant to enzymic digestion, or is incorporated into purine and pyrimidine deoxyribonucleosides, as has been observed previously for binding [^3H]7-methylbenz[*a*]anthracene to DNA (Baird & Brookes, 1973). There is a small but significant amount of radioactivity that is eluted in the region characteristic of deoxyribonucleoside conjugates with polyhydroxylated aromatic hydrocarbons, and is moreover coincident with a major

product of the microsomal binding system. We believe that this is a diethylstilboestrol-deoxyribonucleoside product, albeit formed in very low yield.

These data show that diethylstilboestrol, a known carcinogen that cannot be thoroughly tested by the Ames *Salmonella*/microsome system (McCann & Ames, 1976), is capable of damaging DNA as a result of covalent binding to it through metabolic processes.

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