Short Communications

Evidence for the Binding of the Carcinogen 3-Methylcholanthrene to Both the Purine and the Pyrimidine Bases of Hamster Fibroblast Deoxyribonucleic Acid

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The binding of [^3H]3-methylcholanthrene to the DNA of hamster fibroblasts was studied by using chemical methods for DNA degradation. DNA depurinated by mild acid hydrolysis released approximately half of the radioactivity at the same rate as the purine bases, but the resulting apurinic acid still contained radioactive carcinogen.

It has now been established that the carcinogenic aromatic hydrocarbons can covalently bind to DNA (Gelboin, 1969; Kuroki & Heidelberger, 1971). This binding is thought to be preceded by metabolic activation of the hydrocarbons to give active electrophiles, which react with tissue constituents (Miller, 1970). Although the interaction of the carcinogen with DNA may be of importance in initiating the carcinogenic response (Brookes & Lawley, 1964), attempts to study the chemical nature of this interaction have been unsuccessful, because of the low degree of binding and the difficulty in obtaining sufficient DNA (Brookes & Heidelberger, 1969). We have studied the binding of 3-methylcholanthrene to hamster fibroblast DNA by first selectively removing, by dilute acid hydrolysis, the purine bases from DNA isolated from treated cells, and then degrading the resulting apurinic acid with alkali to yield pyrimidine 'runs'. Hamster fibroblasts were chosen for this work because they are transformable by 3-methylcholanthrene (Berwald & Sachs, 1965), and because preliminary experiments showed them to be a good source of DNA containing bound carcinogen.

Experimental

Primary cultures of hamster embryo cells were initiated and grown as previously described (Jones *et al.*, 1972). Generally labelled [^3H]3-methylcholanthrene was specially prepared by the New England Nuclear Corp. (Boston, Mass., U.S.A.) and was purified before use by t.l.c. on silica gel G with benzene as solvent. The carcinogen was dissolved in acetone and diluted with tissue-culture medium to give a final concentration of $2\mu\text{Ci/ml}$ ($0.24\mu\text{g/ml}$). The concentration of acetone in the medium was 0.2%

(v/v) and this was shown to have no adverse effect on cellular growth. Cultures were always treated with this medium 24 h after being seeded as secondary cultures, and treatment was continued for a further 24 h.

The cells were harvested with 0.1% (w/v) EDTA made up in phosphate-buffered saline (Dulbecco & Vogt, 1954) and the DNA was extracted by a modification of the method of Chiarugi (1969). This modification consisted of a digestion of the crude deoxyribonucleoprotein with $50\mu\text{g}$ of self-digested Pronase/ml for 30 min at 37°C immediately after treatment with ribonuclease. The purified DNA was dissolved in water and dialysed for 24 h against three changes of deionized water at 4°C before depurination. To ensure that the carcinogen required metabolic activation before binding to DNA, a zero-time experiment was also conducted, in which the labelled carcinogen was added to the cells immediately after they were harvested. Only minimal amounts of radioactivity were then detected in the extracted DNA.

The DNA was depurinated by the method of Tamm *et al.* (1952). The course of depurination was followed by carrying out the reaction inside a dialysis bag and observing the appearance of both 260 nm-absorbing material and radioactivity in the diffusate.

DNA obtained from the extraction of 60×10^6 treated cells was mixed with 2.5 mg of carrier DNA isolated by similar procedures from hamster liver. The volume was 1.5 ml, and 0.2 M-HCl was slowly added to adjust the pH to 1.6, yielding a heavy precipitate. The mixture was made up to 2 ml with 25 mM-HCl at pH 1.6, transferred to a dialysis bag and dialysed against 15 ml of 25 mM-HCl at pH 1.6 for 48 h at 37°C . Samples of the diffusate were taken at various intervals for absorbance and radioactivity

Fig. 1. Release of radioactivity during the depurination of DNA containing [^3H]3-methylcholanthrene

A 2 ml portion of a solution (pH 1.6) of DNA containing radioactive carcinogen was placed in a dialysis bag and dialysed against 15 ml of 25 mM-HCl at pH 1.6 at 37°C. At the indicated times samples of the diffusate were taken for E_{260} (●) and radioactivity (○) determinations.

determinations. Radioactivity was determined on 0.5 ml samples, which were neutralized with NaOH and their radioactivities counted with Bray's (1960) solution at an efficiency of 40%.

Results and discussion

Fig. 1 depicts a result that was typical of three separate experiments conducted on different DNA samples. The courses of release of radioactivity and purine bases were identical. The total recovery of radioactivity varied from 50 to 75%, and approximately half of the recovered radioactivity was retained in the bag whereas the other half was found in the diffusate at equilibrium. To ensure that the depurination gave rise to only adenine and guanine and also that the released radioactivity represented the carcinogen and was not due to hydrogen exchange with the bases, the diffusate was concentrated in a rotary evaporator and the concentrate applied to a thin-layer cellulose plate. This was developed with butan-1-ol saturated with water in an NH_3 atmosphere. Two u.v.-absorbing bands corresponding to the purines migrated under these conditions and all the applied radioactivity was confined to the origin. Since the released radioactivity could represent unchanged carcinogen or a phenolic derivative, the solubility of the radioactivity in organic solvents

was examined. It was found that only 10% of the radioactivity was extracted by an equal volume of ether under acid conditions.

The fate of the radioactivity remaining in association with high-molecular-weight material was then examined by column chromatography. One-third of a sample of carcinogen-containing DNA (0.5 ml) was applied to a 60 cm × 1 cm column of Sephadex G-50 (medium grade). The column was run in 20 mM-sodium citrate buffer, pH 5.0, at 30 ml/h, and 2 ml fractions were collected. After the absorbancies at 260 nm had been recorded, the complete fractions were each evaporated to a volume of 1 ml in scintillation vials and then their radioactivities were counted after the addition of 10 ml of Insta-Gel (Packard Instruments, Downers Grove, Ill., U.S.A.) to each. Efficiency was determined to be 41% by automatic external standardization.

The remaining two-thirds of the same sample of DNA (1 ml) was then depurinated by adjusting the pH to 1.6 with 0.2 M-HCl and incubating for 24 h at 37°C. One-half of this reaction mixture was then applied to the same column described above, and the pH of the remaining mixture was adjusted to 14 with KOH. These alkaline conditions further degrade apurinic acid (Shapiro & Chargaff, 1964), and after 5 h at 37°C the reaction mixture was analysed on the column.

Results of these experiments are given in Fig. 2. Before depurination the radioactivity is associated with high-molecular-weight DNA (Fig. 2a). After depurination (Fig. 2b) two peaks of absorbance at 260 nm are found; these correspond to apurinic acid and released purines. One-half of the applied radioactivity is still associated with apurinic acid, but the radioactivity released with the purine bases (Fig. 1) is not recovered from the column. The reason for this is not apparent, but a possibility is strong binding of the aromatic carcinogen nucleus to the polysaccharide column. The non-recovery of nucleosides containing bound dimethylbenz[*a*]anthracene from Celite columns was observed by Brookes & Heidelberger (1969). Fig. 2(c) shows two peaks of absorbance corresponding to pyrimidine isostichs and purine bases, and the radioactivity is distributed over a wide region. Thus the radioactivity is still firmly attached to short pyrimidine 'runs' and pyrimidine nucleotides.

The investigation of the nature of carcinogen binding to DNA has been complicated by the fact that the carcinogen renders the DNA resistant to complete enzymic hydrolysis to the level of mono-nucleotides (Brookes & Heidelberger, 1969). HClO_4 hydrolysis of DNA containing bound carcinogen was also unsuccessful owing to degradation of the carcinogen. The relatively mild chemical methods used in this study may therefore be useful in the identification of the carcinogen moiety and the type

Fig. 2. Analysis of the degradation products of DNA containing $[^3\text{H}]3\text{-methylcholanthrene}$ by column chromatography

Material to be analysed was chromatographed on a column (60cm $\times$ 1cm) of Sephadex G-50 (medium grade). The absorbancies at 260nm (1cm cell) (●) and radioactivities (○) of the 2ml fractions collected are shown. (a) Profiles before depurination; (b) profiles after depurination; (c) profiles after depurination followed by alkaline hydrolysis of the resulting apurinic acid.

of linkage involved, and the evidence suggests binding to both the purine and the pyrimidine bases. Support for this suggestion comes from the work of Dipple & Slade (1971), who have identified the binding sites

of 7-bromomethylbenz[*a*]anthracene as being the amino groups of the DNA bases. The radioactivity released on depurination would therefore represent purine bases containing the carcinogen bound to

amino functions, whereas that remaining on the apurinic acid would represent carcinogen bound to the amino function of cytosine.

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