Characterization of a Photoproduct of 7,12-Dimethylbenz[*a*]anthracene and its Effects on Chick-Embryo Cells in Culture

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A common impurity of 7,12-dimethylbenz[*a*]anthracene was more effective than 7,12-dimethylbenz[*a*]anthracene in inducing morphological alterations, and in causing an increase in glucose uptake, DNA synthesis and cell number in chick-embryo fibroblasts. Gradual morphological transformation follows the increase in DNA synthesis after 2 days when either primary or secondary cultures are treated with 3 μ g of the compound/ml. The compound, isolated from 7,12-dimethylbenz[*a*]anthracene by alumina column chromatography, was characterized by t.l.c., mass spectroscopy, carbon-hydrogen analysis, u.v. and nuclear-magnetic-resonance spectroscopy and thermal decomposition. It was the photo-oxidation product of 7,12-dimethylbenz[*a*]anthracene, 7,12-epidioxy-7,12-dimethylbenz[*a*]anthracene. It is suggested that some of the biological effects observed after treatment of cultures with 7,12-dimethylbenz[*a*]anthracene may be due in part to the presence of the photo-oxidation product.

Many polycyclic aromatic hydrocarbons have been shown to induce specific alterations in cells in culture (Borenfreund *et al.*, 1966; Chen & Heidelberger, 1969; Grover *et al.*, 1971; Mondal & Heidelberger, 1970; DiPaolo *et al.*, 1972; Rhim *et al.*, 1972; Rhim & Huebner, 1973). Many of these studies have been performed on cell lines where the resulting altered cells (transformed cells) could be cloned and subcultured for further analysis. Except for hamster-embryo cells, which have been studied extensively (Berwald & Sachs, 1965; DiPaolo & Donovan, 1967; DiPaolo *et al.*, 1971; Huberman *et al.*, 1972), little is known about the effect of polycyclic aromatic hydrocarbons on primary or secondary cultures of other animal cells.

Overnight treatment with 100 μ g of 7,12-dimethylbenz[*a*]anthracene/ml caused an increase in DNA synthesis in chick-embryo fibroblasts (Rubin & Koide, 1973). Other polycyclic aromatic hydrocarbon compounds such as benzo[*a*]pyrene and 3-methylcholanthrene were reported to have no appreciable effect (Rubin & Koide, 1973). However, preliminary experiments with commercial preparations of 7,12-dimethylbenz[*a*]anthracene in our laboratory indicated appreciable toxicity even at much lower con-

centrations. After purification by column chromatography and recrystallization the toxicity and general morphological effects of 7,12-dimethylbenz[*a*]anthracene were decreased. A potent impurity in the commercial preparation was thought to be present. The present paper describes (1) the techniques used to purify and identify the impurity and (2) the morphological and biochemical effects of the impurity, as well as of 7,12-dimethylbenz[*a*]anthracene, on chick-embryo cells in culture. An abstract of this work has already appeared (Bissell *et al.*, 1974).

Materials and Methods

Materials

Eggs of C/O- or C/B-type subgroup specific-pathogen-free hens were from H and N, Redmond, WA, U.S.A.; culture plates were from Falcon Plastic, Oxnard, CA, U.S.A.; medium 199 was from Grand Island Biological Co., Grand Island, NY, U.S.A.; calf serum and chick serum were from Microbiological Associates, Bethesda, MD, U.S.A.; tryptose/phosphate broth was from Difco Laboratories, Detroit, MI, U.S.A.; 7,12-dimethylbenz[*a*]anthracene was from Sigma, St. Louis, MO, U.S.A.; [³H]thymidine and 2-deoxy-D-[³H]glucose were from New England Nuclear, Boston, MA, U.S.A. Type-I thin-layer plates (0.1 mm) were from Eastman Kodak, Rochester, NY, U.S.A. Type-II thin-layer plates (0.5 mm) were prepared in this laboratory by using

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silica gel PF 254+366 for preparative t.l.c. from Brinkman Instruments, New York, NY, U.S.A.

Cell culture

Primary cultures were prepared from 10-day-old chick embryos essentially as described by Rein & Rubin (1968) and Bissell *et al.* (1972). Cells were plated at 8.0×10^6 cells per 100mm-diameter tissue-culture plates in medium 199 supplemented with 2% (v/v) tryptose/phosphate broth, 1% (v/v) calf serum and 1% (v/v) chicken serum. The cultures were incubated in an atmosphere of CO₂/air (1:19) at 39°C and received fresh medium on day 3. At 4 days after primary plating, cells were trypsin-treated and replated at 5.0×10^5 cells per 35mm-diameter plate in medium 199 supplemented as above, except that the concentrations of calf serum and glucose were increased to 5% and 11mm respectively. Fungizone (1 µg/ml; E. R. Squibb and Sons, Princeton, NJ, U.S.A.) was included in all culture media. Although some harmful side effects of fungizone at high concentrations have been demonstrated for cells in culture (Dolberg & Bissell, 1974), it has been demonstrated previously that its presence at 1 µg/ml increases the uptake of lipophilic compounds (Van Zutphen *et al.*, 1966).

For studies with virus-transformed cells, one-half of a plate of a single embryo was infected with 0.2ml of Schmidt-Ruppin strain of Rous sarcoma virus (5×10^6 focus-forming units/ml) (Bissell *et al.*, 1972). They were treated as above.

Stock solutions of 7,12-epidioxo-7,12-dimethylbenz[a]anthracene (II) (endoperoxide; preparation is described in the Results section) and purified 7,12-dimethylbenz[a]anthracene (I) were prepared by dissolving the compounds in dimethyl sulphoxide (10 mg/ml). An appropriate sample of each of the stock solutions was then dissolved in 0.5ml of culture medium, and these solutions were added to the cell-culture media. One set of primary cultures was treated with 3 µg of purified endoperoxide/ml [0.3% (v/v) dimethyl sulphoxide] and another set was treated with 10 µg of purified 7,12-dimethylbenz[a]anthracene/ml [0.3% (v/v) dimethyl sulphoxide]. Dimethyl sulphoxide (0.3%) was added to control cultures. Chemically treated cells continued to receive the carcinogens after secondary plating. The control cultures were subdivided further at the time of secondary plating: one half received 3 µg of endoperoxide/ml and the other half continued to receive 0.3% dimethyl sulphoxide. On successive days, cultures were monitored for changes in morphology by using an optical microscope and analysed further in terms of cell number, DNA synthesis and glucose uptake.

Incorporation of [³H]thymidine into trichloroacetic acid-precipitable material and uptake of 2-deoxy-D-[³H]glucose were used as a measure of

DNA synthesis and glucose transport respectively (Dolberg *et al.*, 1975). Protein was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard. Cell number was measured by using a Coulter counter.

Chemical analysis

All melting points were recorded on a Thomas Hoover capillary melting-point apparatus. The u.v. spectra were recorded on a Cary 118 spectrophotometer, the n.m.r. (nuclear-magnetic-resonance) spectra were recorded on a Varian HR 220 MHz spectrometer at 17°C and the mass spectra were obtained on a DuPont 21-491 spectrometer with a 21-094 data system. Other techniques are described in the Results section.

Results

Addition of commercial 7,12-dimethylbenz[a]anthracene (10 µg/ml) to secondary cultures of chick-embryo fibroblasts caused a 50% decrease in cell number in 24h. Preliminary experiments with purified 7,12-dimethylbenz[a]anthracene indicated an appreciable loss of toxicity. We therefore suspected that an impurity in the commercial preparation of 7,12-dimethylbenz[a]anthracene itself, or synergistically with 7,12-dimethylbenz[a]anthracene, was responsible for part of the toxicity observed. To assess accurately the role of 7,12-dimethylbenz[a]anthracene and the impurity on the morphological and biological alterations in chick cells, the compound was isolated and characterized.

Characterization of the compound

When 7,12-dimethylbenz[a]anthracene was chromatographed on type-I thin-layer plates with benzene as a solvent (see the Materials and Methods section), two spots were revealed under a u.v. lamp: one fluorescent spot with an R_F of 0.62, corresponding to the pure compound, and a dark spot with an R_F of 0.45. This indicated that one major impurity was present (quantitative determination of the impurity is described below). To purify 7,12-dimethylbenz[a]anthracene and identify the impurity, the impure compound was dissolved in benzene and chromatographed on a neutral alumina column surrounded with aluminium foil to protect the compounds from light. The impurity remained on the column while pure 7,12-dimethylbenz[a]anthracene was eluted by benzene. After evaporation of the benzene eluate, the residue was recrystallized from a benzene/propan-2-ol mixture giving pale-yellow crystalline flakes of pure 7,12-dimethylbenz[a]anthracene; mp. 122–123°C; λ_{max} (ethanol) 296 (ϵ 72000 litre·mol⁻¹·cm⁻¹), 287 (ϵ 64000 litre·mol⁻¹·cm⁻¹), 276, 264, 398 (weak), 382 (weak), 362 (weak), 236nm; molecular ion (m/e), 256 (parent ion); n.m.r. ([²H]chloroform) (p.p.m.) 3.05 (singlet, 3H), 3.3 (singlet, 3H), 7.6 (multiplet,

5H), 7.8 (multiplet, 1H), 8.1 (doublet, 1H), 8.4 (multiplet, 2H) and 8.5 (multiplet, 1H).

After the 7,12-dimethylbenz[*a*]anthracene had been eluted from the column, the unknown compound was eluted with chloroform. The eluate was evaporated and the residue was recrystallized from benzene/propan-2-ol, giving a colourless powder; m.p. 199–200°C (decomp.); λ_{max} (ethanol) 232 (strong), 298 (weak), 288 (weak), 275 (weak), 267 nm (weak); *m/e* 288 (parent ion) and *m/e* 256 (highest abundance); n.m.r. (^2H)chloroform (p.p.m.) 2.25 (singlet, 3H), 2.70 (singlet, 3H), 7.3 (multiplet, 2H), 7.4 (multiplet, 5H), 7.75 (triplet, 2H) and 8.56 (doublet, 1H).

The above operations were carried out either in the dark or in the absence of direct sunlight. This was particularly necessary during chromatography (Sandin & Fieser, 1940) because 7,12-dimethylbenz[*a*]anthracene can be readily converted into its photo-products in the presence of a room light.

T.l.c. (type-I plates) of 7,12-dimethylbenz[*a*]anthracene and the unknown compound in benzene gave R_F values of 0.62 and 0.45 respectively, whereas t.l.c. in benzene/ethanol (19:1, v/v) gave an R_F of 0.62 for both compounds. The unknown compound was only slightly soluble in hexane and very soluble in chloroform, whereas 7,12-dimethylbenz[*a*]anthracene was very soluble in both solvents. The t.l.c. and solubility properties of the unknown compound indicated that it was a more polar compound than 7,12-dimethylbenz[*a*]anthracene, and the fact that it was colourless was an indication of loss of some aromaticity.

The u.v. spectrum showed loss of the anthracene *p*-bands and a shift of the β -band to 232 nm (ϵ 36000 litre·mol⁻¹·cm⁻¹) (Fig. 1). This type of spectrum was characteristic of a naphthalene-type structure. The mass spectra showed an *m/e* (parent ion) of 288 and an *m/e* of 256 (parent ion) for the unknown compound compared with *m/e* 256 (parent ion) of highest abundance for dimethylbenzanthracene. The difference in the molecular weight was 32, which could have been either CH₃OH or two O atoms. However, the carbon-hydrogen analysis indicated that two O atoms had been incorporated into dimethylbenzanthracene (Calc. for C₂₀H₁₆O₂: C, 83.31; H, 5.59. Found: C, 83.08; H, 5.45%). Lastly, the n.m.r. data (Fig. 2) showed an upfield shift of the two methyl peaks, which was consistent with the loss of some of their aromaticity. Therefore, on the basis of the above work, we concluded that the compound was more polar and less aromatic in character than 7,12-dimethylbenz[*a*]anthracene, had a naphthalene-type structure, a mol.wt. of 288 with the incorporation of two O atoms and a less aromatic environment surrounding the 7- and 12-methyl groups. These characteristics were consistent with those of the endoperoxide of 7,12-dimethylbenz[*a*]anthracene, i.e. 7,12-epidioxy-7,12-dimethylbenz[*a*]anthracene (Cook & Martin, 1940).

To confirm the identification of this compound as the endoperoxide, the thermal decomposition (Southern & Waters, 1960; Wasserman & Scheffer, 1967) of this compound was carried out as follows: 10.6mg of the compound was refluxed for 24h at

Fig. 1. U.v. spectra recorded with a Cary 118 spectrophotometer

—, 7,12-Dimethylbenz[*a*]anthracene (19.8 μM); ----, impurity (33 μM) (both in 95% ethanol). The molar extinction coefficients (litre·mol⁻¹·cm⁻¹) of the indicated peaks are: (1) 232nm, 3.6×10^4 ; (2) 285nm, 6.4×10^4 ; (3) 296nm, 7.2×10^4 .

Fig. 2. *N.m.r. spectra*

The n.m.r. spectra were obtained on a Varian HR 220 MHz spectrometer at 17°C in $[\text{2H}]$ chloroform containing 1% tetramethylsilane with a sweep-width of 2500 Hz and a sweep-time of 500 s. (a) 7,12-Dimethylbenz[a]anthracene (I); (b) 7,12-epidioxy-7,12-dimethylbenz[a]anthracene (II).

140°C in 25 ml of xylene. The reaction was monitored by t.l.c. The reaction mixture was evaporated and the residue chromatographed on a type-II thin-layer plate (see the Materials and Methods section) with benzene as solvent. The various bands were scraped off the plate, extracted in chloroform, filtered and u.v. spectra were recorded. On the basis of the molar extinction coefficients 3.7 mg (46%) of 7,12-dimethylbenz[a]anthracene was generated and 3.2 mg of the endoperoxide was recovered. The formation of 7,12-dimethylbenz[a]anthracene under these conditions confirmed that this unknown com-

pound was indeed the endoperoxide of 7,12-dimethylbenz[a]anthracene.

We then attempted to generate the endoperoxide photochemically in our laboratory. The endoperoxide of 7,12-dimethylbenz[a]anthracene could be synthesized by simple chromatography of 7,12-dimethylbenz[a]anthracene on a neutral alumina column with benzene in the presence of room light.

The amount of endoperoxide in the impure 7,12-dimethylbenz[a]anthracene was then determined as follows: the u.v. spectra of pure and impure compound in ethanol were recorded. The ratios $A_{296}/$

Table 1. *Cellular activity of secondary cultures after 75 h of treatment with the endoperoxide*

DNA synthesis was measured by incorporation of [^3H]thymidine ($2\mu\text{Ci/ml}$ of medium) into trichloroacetic acid-precipitable material (d.p.m./mg of protein). Glucose uptake was measured with 2-deoxy-D- ^3H]glucose ($2\mu\text{Ci/ml}$ of medium; d.p.m./mg of protein). For details see the Materials and Methods section. The percentage relative to control values is given in parentheses.

	DNA synthesis (d.p.m./mg of protein)	Glucose uptake (d.p.m./mg of protein)	$10^{-6} \times$ Cell number (per 35 mm plate)
Control (in 0.3% dimethyl sulphoxide)	24500	6600	1.7
Endoperoxide ($3\mu\text{g/ml}$)	42140 (172%)	10890 (165%)	1.9 (112%)

A_{232} of the pure and the impure compound were 4.73 (1.42/0.30) and 3.82 (1.30/0.34) respectively. The fact that the ratio was smaller for the impure compound indicated that A_{232} was higher, because of the presence of some endoperoxide. The value at 232 nm was then determined for pure 7,12-dimethylbenz[a]anthracene on the basis of the value of 1.30 at 296 nm and the ratio of 4.73 (A_{296} of the endoperoxide is negligible with respect to 7,12-dimethylbenz[a]anthracene). This gave a value for A_{232} of 0.27. Therefore 0.07 ΔA unit was due to the endoperoxide. By using molar extinction coefficients for the endoperoxide at 232 nm and for 7,12-dimethylbenz[a]anthracene at 296 nm, the concentrations for the endoperoxide and 7,12-dimethylbenz[a]anthracene were 1.9 and $18\mu\text{M}$ respectively, indicating that approx. 10% of the commercial 7,12-dimethylbenz[a]anthracene sample was endoperoxide. Chromatography of a small sample of the impure compound on type-II thin-layer plates in benzene resulted in only two bands, which were scraped off, filtered and weighed, yielding 11% of the endoperoxide in the 7,12-dimethylbenz[a]anthracene sample.

The amount of endoperoxide in a 7,12-dimethylbenz[a]anthracene sample, however, will vary depending on the preparation, storage conditions and solvent.

Effects of purified 7,12-dimethylbenz[a]anthracene and its endoperoxide on chick cells

Preliminary testing of 7,12-dimethylbenz[a]anthracene and its endoperoxide on secondary cultures of chick fibroblasts indicated that the latter was more toxic to these cells. In terms of biological and morphological effects $10\mu\text{g}$ of pure 7,12-dimethylbenz[a]anthracene/ml was roughly similar to $3\mu\text{g}$ of the endoperoxide/ml. Treatment of primary chick cultures with $3\mu\text{g}$ of the endoperoxide/ml or $10\mu\text{g}$ of 7,12-dimethylbenz[a]anthracene/ml caused morphological alteration 2 to 3 days after addition of the carcinogen. The elongated cells in the primary control cultures (presumed to be fused fibroblasts or muscle cells) were decreased. The number of vacuoles and the degree of cell 'cross-over', on the other hand, increased. There was also a change in the number of refractile cells. The cells continued to maintain these morphological

alterations through the secondary plating. Cells treated at the time of secondary platings with $3\mu\text{g}$ of the endoperoxide/ml had a morphological similarity to cells transformed with the Schmidt-Ruppin strain of Rous sarcoma virus. Additional changes which are taken as indicative of 'transformation' in culture, such as increased DNA synthesis, glucose uptake and increased cell number after confluency, were also observed after treatment with the endoperoxide (Table 1). These changes were observed after treatment with $10\mu\text{g}$ of 7,12-dimethylbenz[a]anthracene/ml as well, but with less reproducibility than in the case of endoperoxide. However, no RNA-dependent DNA polymerase (reverse transcriptase) activity, which is characteristic of C-type tumour viruses, was found in the medium of the endoperoxide- or 7,12-dimethylbenz[a]anthracene-treated cultures (C. Szabo & M. J. Bissell, unpublished work).

Discussion

The endoperoxide of 7,12-dimethylbenz[a]anthracene is readily produced in the presence of light (Cook & Martin, 1940; Sandin & Fieser, 1940).

The commercial preparations of 7,12-dimethylbenz[a]anthracene may thus contain appreciable amounts of this compound. This photo-oxidation product causes morphological and biochemical changes indicative of 'transformation' in tissue-culture cells. These effects, although produced by both 7,12-dimethylbenz[a]anthracene and the endoperoxide, are more pronounced for the latter, as described in the Results section.

The formation of endoperoxides is a photo-sensitized autoxidation (Corey & Taylor, 1964; Foote *et al.*, 1965; Foote, 1968; Gollnick, 1968) reaction which in reality involves light and singlet O_2 . Other workers (Southern & Waters, 1960; Arbuzov, 1965; Rigaudy & Sparfel, 1972) have shown that endoperoxides under mild conditions lose O_2 with the regeneration of the parent compound or undergo breakage of the O-O bond to form dihydroxy compounds. The loss of O_2 from this compound and other structurally similar polycyclic aromatic hydrocarbons under mild thermal conditions is in the form of singlet O_2 (McKeown & Waters, 1966; Wasserman & Scheffer, 1967).

Singlet O_2 is a very reactive species which can attack purine and pyrimidine bases (Hallett *et al.*, 1970) and protein residues (Spikes & MacKnight, 1970) in the vicinity of its formation and has been postulated to be involved in the carcinogenic activity of these hydrocarbons (Cusachs & Steele, 1967; Kahn & Kasha, 1970; Kearns, 1971). It is therefore possible that some of the biological effects observed after addition of the endoperoxide to cells may be due to the formation of singlet O_2 , followed by nucleophilic attack on cell components leading to genetic or epigenetic changes. On the other hand, breaking of the O-O or C-O bond could lead to metabolic products, i.e. the 7,12-dihydroxy or 7-hydroxy-12-hydroperoxy derivatives, capable of initiating these changes.

Boyland & Sims (1965) studied the metabolism of 7,12-dimethylbenz[*a*]anthracene in liver homogenates and found that the endoperoxide was only formed as a by-product during the isolation and characterization of the metabolites of 7,12-dimethylbenz[*a*]anthracene. However, some of the biological effects observed after treatment of cultures with this compound may be due in part to the presence of its endoperoxide. Secondary chick cells have little or no aryl hydrocarbon hydroxylase (T. Meehan & M. J. Bissell, unpublished work; Nebert & Gelboin, 1968). It has been suggested that polycyclic aromatic hydrocarbons require metabolic activation by this enzyme complex for their carcinogenic activity (Gelboin *et al.*, 1972). Therefore it is possible that the endoperoxide impurity, after conversion into the hydroxyperoxide derivative (Southern & Waters, 1960), can act in place of the hydroxylase for conversion of 7,12-dimethylbenz[*a*]anthracene into more carcinogenic products.

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