Biochemical Pharmacology, Vol. 26, pp. 1481–1487. Pergamon Press, 1977. Printed in Great Britain.

# THE METABOLISM OF BENZO(a)PYRENE, 7,8-DIHYDRO-7,8-DIHYDROXYBENZO(a)PYRENE AND 9,10-DIHYDRO-9,10-DIHYDROXYBENZO(a)PYRENE BY SHORT-TERM ORGAN CULTURES OF HAMSTER LUNG

GERALD M. COHEN and BRIAN P. MOORE
Department of Biochemistry, University of Surrey, Guildford, GU2 5XH, Surrey, England

(*Received* 10 *November* 1976; *accepted* 4 *February* 1977)

**Abstract**—The metabolism of benzo(a)pyrene and two of its metabolites 7,8-dihydro-7,8-dihydroxybenzo(a)pyrene (7,8-dihydrodiol) and 9,10-dihydro-9,10-dihydroxybenzo(a)pyrene (9,10-dihydrodiol) to both ethyl acetate-soluble and water-soluble metabolites has been studied using short-term organ cultures of hamster lung. Benzo(a)pyrene is metabolised to ethyl acetate-soluble metabolites which co-chromatograph with 9,10-dihydrodiol, 7,8-dihydrodiol and benzo(a)pyren-3-yl hydrogen sulphate but little or no 3-hydroxybenzo(a)pyrene and 4,5-dihydro-4,5-dihydroxybenzo(a)pyrene (4,5-dihydrodiol) are detected. After culture with benzo(a)pyrene, the amount of 9,10-dihydrodiol in the medium is 9-fold greater than the amount of 7,8-dihydrodiol. Benzo(a)pyrene is also metabolised by short-term organ cultures of hamster lung to water-soluble metabolites, which on hydrolysis with β-glucuronidase yield metabolites co-chromatographing with 3-hydroxybenzo(a)pyrene, quinones, 4,5-dihydrodiol and 7,8-dihydrodiol. However little or no 9,10-dihydrodiol is detected. Both 7,8- and 9,10-dihydrodiols are metabolised by cultures of hamster lung to an ethyl acetate-soluble metabolite which co-chromatographs and has similar fluorescence excitation and emission spectra to 7,8,9,10-tetrahydro-7,8,9,10-tetrahydroxybenzo(a)pyrene (7,8,9,10-tetrahydrotetrol). More 7,8,9,10-tetrahydrotetrol is formed from 7,8- than 9,10-dihydrodiol. A major route for metabolism of 7,8-dihydrodiol is conversion into water-soluble metabolites, which on hydrolysis with β-glucuronidase yield an ethyl acetate-soluble metabolite co-chromatographing with 7,8-dihydrodiol. However only small amounts of water-soluble metabolites are observed after short-term organ culture with 9,10-dihydrodiol. The amount of covalent binding after short-term organ culture with 7,8-dihydrodiol was greater than that with 9,10-dihydrodiol and benzo(a)pyrene. This was in agreement with the many observations showing the high biological activity of the further metabolite of 7,8-dihydrodiol, i.e. 7,8-dihydro-7,8-dihydroxybenzo(a)pyrene 9,10-oxide. These results however, also raise the possibility of a role for 9,10-dihydrodiol as a precursor of active metabolites.

The widespread occurrence of carcinogenic polycyclic aromatic hydrocarbons, such as benzo(a)pyrene, as environmental pollutants and constituents of cigarette smoke may be a contributory factor to the increasing number of deaths from lung cancer observed in many countries [1]. Most chemical carcinogens have to be metabolically activated to reactive electrophiles in order to initiate tumour formation [2]. The toxicity, mutagenicity and carcinogenicity of polycyclic aromatic hydrocarbons is also believed to require metabolic activation to reactive intermediates which can then bind covalently with cellular macromolecules [2–5]. Polycyclic aromatic hydrocarbons are metabolised both to organic-soluble metabolites (such as epoxides, dihydrodiols, diol-epoxides, quinones and phenols and their sulphate esters) and to water-soluble conjugates [3, 4, 6, 7]. Many of these metabolites arise from epoxide intermediates which may: (i) re-arrange spontaneously to form the phenol; (ii) be converted into the dihydrodiol by the microsomal enzyme epoxide hydratase; (iii) conjugate with glutathione; (iv) react with a cellular nucleophile in what is believed to be the toxic reaction [3, 4]. K-region epoxides have shown to be mutagenic and to induce malignant transformations of cells in culture [8, 9]. However, in *in vivo* studies the K-region epoxides are less carcinogenic than the parent compound [3, 10] and in cells treated with polycyclic aromatic hydrocarbons, the metabolite which reacted with the DNA was not the K-region epoxide [11].

More recently, using Syrian hamster embryo cells and rat liver microsomes, Sims and co-workers have shown the further metabolism of dihydrodiols such as 7,8-dihydro-7,8-dihydroxybenzo(a)pyrene (7,8-dihydrodiol) and 8,9-dihydro-8,9-dihydroxybenz(a)anthracene to their respective diol-epoxides 7,8-dihydro-7,8-dihydroxybenzo(a)pyrene 9,10-oxide and 8,9-dihydro-8,9-dihydroxybenz(a)anthracene 10,11-oxide [6, 12]. They have also shown that it is this type of diol-epoxide which reacts with DNA of Syrian hamster embryo cells treated with the parent hydrocarbon [6, 12]. These diol-epoxides, but not the parent dihydrodiols, do not require metabolic activation in order to be potent mutagens to both *S. typhimurium* strain TA 100 and Chinese hamster V 79 cells [13–16]. The diol-epoxides have also been implicated in the metabolic activation of benzo(a)pyrene in human bronchial mucosa and mouse skin [17, 18]. All this data strongly implicates 7,8-dihydro-7,8-dihydroxyben-

zo(a)pyrene 9,10-oxide as the major proximate carcinogen derived from benzo(a)pyrene.

Here we report the metabolism of 9,10-dihydro-9,10-dihydroxybenzo(a)pyrene (9,10-dihydrodiol), benzo(a)pyrene and 7,8-dihydrodiol by short-term organ cultures of hamster lung to both ethyl acetate-soluble and water-soluble metabolites. 7,8- and 9,10-Dihydrodiols are further metabolised to 7,8,9,10-tetrahydro-7,8,9,10-tetrahydroxybenzo(a)pyrene (7,8,9,10-tetrahydrotetrol). Significant amounts of the 7,8-dihydrodiol but not of 9,10-dihydrodiol are converted into water-soluble conjugates.

## MATERIALS AND METHODS

### *Materials*

G-[$^3$H]Benzo(a)pyrene (sp. act. 5 Ci/m-mole and 25 Ci/m-mole) was obtained from the Radiochemical Centre, Amersham, Bucks, England and purified according to the method of De Pierre *et al.* [19]. Benzo(a)pyrene was obtained from Aldrich Chemicals and added to the labelled benzo(a)pyrene to the appropriate concentration. β-Glucuronidase (Type H-1, Helix Pomatia which also contains sulphatase) was obtained from Sigma Chemical Co. Leibovitz L-15 medium containing 2 mM L-glutamine and foetal calf serum were obtained from Biocult Laboratories Ltd., Paisley, Scotland and Flow Laboratories, Irvine, Scotland respectively. Thin layer chromatography (t.l.c.) plates used were either pre-coated glass or aluminium with silica gel (0.25 mm thickness) both obtained from Merck, Darmstadt, Germany. The labelled reference compounds 4,5-, 7,8- and 9,10-dihydrodiols and 3-hydroxybenzo(a)pyrene were prepared essentially as described by Sims and their identity was confirmed by comparison of their u.v. spectra with those reported in the literature [20]. 7,8- and 9,10-Dihydrodiols were incubated with liver microsomes as described by Booth and Sims and metabolites isolated with identical u.v. spectra to the proposed 7,8,9,10-tetrahydrotetrol and 9,10-dihydroxybenzo(a)pyrene [21].

### *Preparation of* [$^3$H]*7,8- and* [$^3$H]*9,10-dihydrodiols*

[$^3$H]7,8- and [$^3$H]9,10-Dihydrodiols were prepared essentially as described for [$^3$H]3-hydroxybenzo(a)-pyrene [7] with minor modifications as follows. After the initial t.l.c. in a mixture of benzene–ethanol 9:1 (v/v), the bands co-chromatographing with reference 7,8- and 9,10-dihydrodiols were scraped off, extracted with ethyl acetate, and further purified by rechromatographing twice more in benzene–ethanol (9:1, v/v). The identity of the final products was confirmed by their u.v. spectra, fluorescent characteristics and mobility when chromatographed in benzene–ethanol (9:1, v/v and 19:1, v/v). When chromatographed in either of these solvent systems, the metabolites gave one major fluorescent and radioactive band which contained >97 per cent of the radioactivity.

### *Short-term organ cultures of lung*

Male Syrian golden hamsters DSN (100–140 g) were obtained from D. Roberts, Basingstoke, England, and maintained on Sterolit bedding (Englehard Corp., U.S.A.). Hamster lung samples (200 ± 10 mg) were cultured in short-term organ culture for 16–18 hr at 37° in 10 ml of Leibovitz L15 medium with 2 mM L-glutamine containing 10% foetal calf serum, penicillin 100 U ml$^{-1}$ and streptomycin 100 μg ml$^{-1}$ [22]. [$^3$H]Benzo(a)pyrene or [$^3$H]7,8- or [$^3$H]9,10-dihydrodiol was added to a final concentration of 1 μM.

After culture, examination of haematoxylin and eosin sections showed that the lungs retained normal histological architecture except for very slight changes in the nuclear staining indicating early signs of autolysis. Tissue samples were separated from the medium at the end of the incubation period and stored at −20° until required for covalent binding studies. Radioactive metabolites in the medium were examined immediately after culture.

### *Determination of metabolism of* [$^3$H]*benzo(a)pyrene and* [$^3$H]*7,8- and* [$^3$H]*9,10-dihydrodiols by short-term organ culture*

*Ethyl acetate-soluble metabolites.* Ethyl acetate-soluble metabolites were extracted by shaking the medium for a minimum of 40 sec with 2 × 1 vol. of ethyl acetate and separating the aqueous and ethyl acetate phases. Control extractions, with medium incubated for 16–18 hr with no tissue, extracted essentially all the radioactivity from the medium. The pooled ethyl acetate extracts were dried with sodium sulphate and examined by t.l.c. in solvent systems of either benzene–ethanol (9:1, v/v) (A) or benzene–ethanol (4:1, v/v) (B) or chloroform–methanol (4:1, v/v) (C) [20, 21]. The fluorescent products were located by inspection of the wet plates in u.v. light (254 nm) and the radioactivity quantified as previously described [22].

*Water-soluble metabolites.* After the initial ethyl acetate extractions, the medium remaining was extracted once more with ethyl acetate immediately prior to enzymic incubations. Samples taken from this extraction showed that only very low amounts of radioactivity were being released into the organic phase. The medium was then divided into two and incubated in stoppered tubes with either equal volumes of β-glucuronidase (10 mg/ml) in 0.1 M acetate buffer pH 5.0 or buffer alone. The tubes were flushed with nitrogen, stoppered and incubated overnight in a shaking water bath at 37°. The enzymic hydrolysates were then extracted with ethyl acetate and the the organic-soluble metabolites examined by t.l.c. in either solvent systems A, B or C.

*Covalent binding.* The method used was essentially that of Siekevitz [23] with modifications as follows. Lung samples were homogenised in 0.9% saline (approximately 3 ml) and an equal volume of 1 M perchloric acid added. The precipitated protein was sedimented by centrifugation and the precipitate washed consecutively with 2 × 3 ml 0.5 M perchloric acid, 2 × 5 ml 96% ethanol, 2 × 5 ml of diethyl ether–ethanol–chloroform (2:2:1) and 4 × 5 ml acetone. The final acetone wash was checked for the presence of any radioactivity and in all cases, the counts were the same as background.

The final precipitates were dissolved in 0.5–1.0 ml soluene 350 (Packard Instrument Co). and radioactivity counted in a scintillant containing 0.5% w/v PPO (2,5-diphenyloxazole) and 0.02% w/v dimethyl POPOP [1,4 bis[2-(4-methyl-5-phenyloxazolyl)]benzene] in toluene. Quenching was determined by internal standardisation using [$^3$H]toluene.

Table 1. Distribution of radioactivity of benzo(a)pyrene and metabolites after short-term organ culture

| Compound (1 μM) | Percentage radioactivity, expressed as a fraction of initial radioactivity | | | |
|---|---|---|---|---|
| | Remaining in tissue after culture | Ethyl acetate extractable after culture | Remaining in medium after ethyl acetate extraction | Unchanged starting compound in medium |
| Benzo(a)pyrene | 34.0 ± 2.1 | 45.8 ± 1.9 | 20.0 ± 2.0 | 38.5 ± 1.7 |
| 7,8-Dihydrodiol | 52.4 ± 2.3 | 21.3 ± 0.8 | 26.3 ± 1.9 | 16.1 ± 0.8 |
| 9,10-Dihydrodiol | 41.3 ± 1.7 | 49.9 ± 1.3 | 8.8 ± 1.2 | 48.1 ± 1.2 |

All results are expressed as mean ± S.E.M. of 4 determinations.

[$^3$H]Benzo(a)pyrene, [$^3$H]7,8-dihydro-7,8-dihyroxybenzo(a)pyrene (7,8-dihydrodiol) or [$^3$H]9,10-dihydro-9,10-dihydroxybenzo(a)pyrene (9,10-dihydrodiol) were incubated for 16 hr with hamster lung (200 mg). The medium was extracted with 2 × 1 vol. ethyl acetate, and the organic-soluble extracts separated by t.l.c. in benzene–ethanol (9:1 or 4:1, v/v) for quantitative determination of the unchanged starting compound.

## RESULTS

### *Metabolism of* [$^3$H]*benzo(a)pyrene by short-term organ cultures of hamster lung*

*Ethyl acetate-soluble metabolites.* After short-term organ culture of hamster lung, benzo(a)pyrene was converted to ethyl acetate-soluble metabolites and to more polar metabolites which remained in the culture medium after ethyl acetate extraction (Table 1). When the ethyl acetate-soluble extracts were concentrated and chromatographed in solvent system (A), radioactive bands which co-chromatographed with 9,10- and 7,8-dihydrodiols were observed (Fig. 1). Little or no 4,5-dihydrodiol and 3-hydroxybenzo(a)pyrene were observed (Fig. 1). Some of the radioactivity, which migrates just beyond the origin, is associated with benzo(a)pyren-3-yl hydrogen sulphate [7]. The amount of this metabolite is dependent on the size of lung used for culture (unpublished observations). With the larger portions of lung used in earlier studies, this metabolite was the major ethyl acetate-soluble metabolite after lung culture. In this study 9,10-dihydrodiol was the major ethyl acetate-soluble metabolite formed (Fig. 1). The ratio of 9,10- to 7,8-dihydrodiol formation was 8.6:1. The rates of production of 9,10- and 7,8-dihydrodiols were 2.24 ± 0.20 and 0.26 ± 0.01 pmoles $g^{-1}$ $min^{-1}$ respectively (Mean ± S.E. $n = 4$).

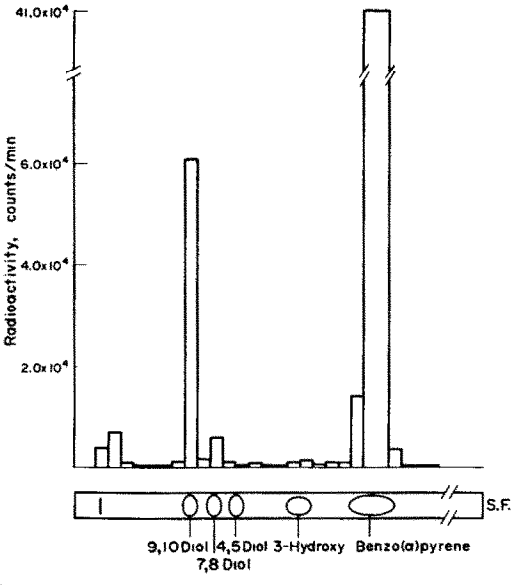


Fig. 1. Ethyl acetate-soluble metabolites from the culture medium after 16 hr short-term organ culture of hamster-lung (200 mg) with [$^3$H]benzo(a)pyrene (1 μM). The radioactive products were separated by t.l.c. in a mixture of benzene–ethanol (9:1, v/v). Part of the material migrating just beyond the origin co-chromatographed with benzo(a)-pyren-3-yl hydrogen sulphate. S.F. indicates solvent front. The abbreviations used in the figure are 9,10-dihydro-9,10-dihydroxybenzo(a)pyrene (9,10-Diol), 7,8-dihydro-7,8-dihydroxybenzo(a)pyrene (7,8-Diol), 4,5-dihydro 4,5-dihydroxybenzo(a)pyrene (4,5-Diol) and 3-hydroxybenzo(a)pyrene (3-hydroxy).

*Water-soluble metabolites.* In order to determine the nature of some of the radioactivity remaining in the medium after extraction with ethyl acetate, the medium was subjected to enzymic hydrolysis with β-glucuronidase. The hydrolysate was extracted with ethyl acetate and the extract was chromatographed in solvent system (A). Radioactive bands which co-chromatographed with 7,8-dihydrodiol, 3-hydroxybenzo(a)pyrene, quinones (migrating just beyond 3-hydroxybenzo(a)pyrene) and benzo(a)pyrene were observed (Fig. 2). In some experiments a radioactive band which co-chromatographed with 4,5-dihydrodiol was also observed. In all experiments, little or no radioactivity, which co-chromatographed with 9,10-dihydrodiol was observed (Fig. 2). Most of the ethyl acetate-soluble radioactivity, which was released following enzyme hydrolysis, co-chromatographed with 3-hydroxybenzo(a)pyrene (Fig. 2).

### *Metabolism of 7,8-dihydrodiol*

*Ethyl acetate-soluble metabolites.* [$^3$H]7,8-Dihydrodiol was metabolised by short-term organ culture of hamster lung to both ethyl acetate-soluble products and to water-soluble conjugates. When the ethyl acetate-soluble radioactivity was examined using either solvent systems (B) or (C), the material associated with most of the radioactivity co-chromatographed with, and had similar fluorescence excitation and emission spectra to those of, reference 7,8-dihydrodiol (Fig. 3). A major metabolite with $R_f = 0.14$ (relative to benzo(a)pyrene) in solvent system (B) was also

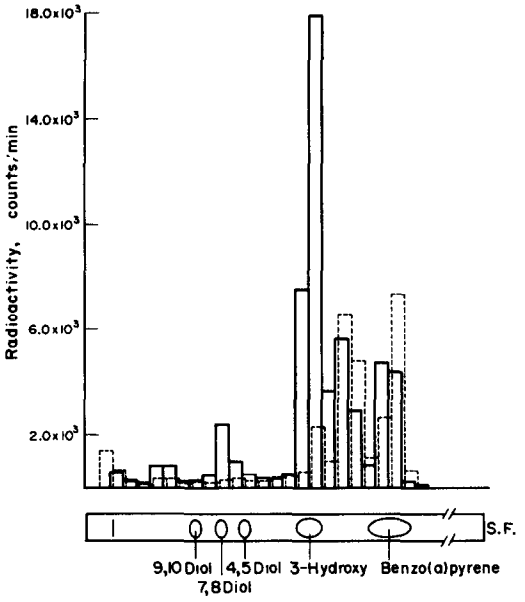


Fig. 2. Enzymic hydrolyses of water-soluble metabolites from benzo(a)pyrene. Water-soluble metabolites from the culture medium, after 16 hr short-term organ culture of hamster lung (200 mg) with [$^3$H]benzo(a)pyrene (1 $\mu$M), were hydrolysed with either $\beta$-glucuronidase (——) or buffer alone (---) and extracted with ethyl acetate. The ethyl acetate-soluble radioactivity was separated by t.l.c. in a mixture of benzene–ethanol (9:1, v/v). S.F. indicates solvent front. The abbreviations used are the same as those in the legend to Fig. 1.

observed (Fig. 3). This major metabolite co-chromatographed with, and had similar fluorescence excitation and emission spectra to, reference 7,8,9,10-tetrahydrotetrol in solvent systems (B) and (C), (Fig. 4). The rate of formation of the 7,8,9,10-tetrahydrotetrol was $0.91 \pm 0.31$ pmoles $g^{-1}$ $min^{-1}$ (mean $\pm$ S.E. $n = 4$). After short-term organ culture of hamster lung with 7,8-dihydrodiol, a significantly smaller percentage of unchanged parent compound remained in the medium than in corresponding experiments with either benzo(a)pyrene or 9,10-dihydrodiol (Table 1).

*Water-soluble metabolites.* When the water-soluble metabolites of the short-term organ culture of 7,8-dihydrodiol were hydrolysed with $\beta$-glucuronidase, the greater part of the radioactivity (up to 91%) was released as ethyl acetate-soluble radioactivity. However this figure could not be accurately obtained in all experiments because of gelling problems on extraction of the enzymic hydrolysate with ethyl acetate. Control experiments, with buffer only, released little or no radioactivity. When the ethyl acetate-soluble radioactivity of the enzyme hydrolysate was examined by t.l.c. using solvent system (B), the major radioactive band co-chromatographed with 7,8-dihydrodiol (Fig. 5). Thus the majority of the water-soluble metabolites from 7,8-dihydrodiol were present as either glucuronide or sulphate conjugates and only small amounts were present as glutathione or other conjugates of 7,8-dihydrodiol or 7,8,9,10-tetrahydrotetrol.

*Metabolism of 9,10-dihydrodiol*

*Ethyl acetate-soluble metabolites.* A smaller percentage of radioactivity remained in the lung after culture with 9,10-dihydrodiol than with 7,8-dihydrodiol (Table 1). The rate of metabolism of [$^3$H]9,10-dihydrodiol, by short-term organ culture of hamster lung, was much slower than that of either benzo(a)pyrene or 7,8-dihydrodiol. When the ethyl acetate-soluble radioactivity was examined using either solvent systems (B) or (C), the material associated with most of the radioactivity co-chromatographed with, and had similar fluorescence excitation and emission spectra to, reference 9,10-dihydrodiol (Table 1). Small amounts of a metabolite, which co-chromatographed and had similar fluorescence excitation and emission spectra to the standard 7,8,9,10-tetrahydrotetrol but not with 9,10-dihydroxybenzo(a)pyrene, were observed (Fig. 4). The small amounts of tetrahydrotetrol detected prevented its accurate quantitative determination.

*Water-soluble metabolites.* Very small amounts of water-soluble metabolites were detected following lung culture with 9,10-dihydrodiol (Table 1). Enzymic hydrolysis of the aqueous phase with $\beta$-glucuronidase released very small amounts of ethyl acetate-soluble, radioactive material which on t.l.c. gave no major radioactive bands.

*Covalent binding*

The values for covalent binding were obtained by incubation of the [$^3$H]metabolite or [$^3$H]benzo(a)pyrene with tissue (Table 2). Whilst more 7,8-dihydrodiol is bound than either benzo(a)pyrene or 9,10-dihydrodiol, significant amounts of the 9,10-dihydrodiol were also bound. In preliminary experiments with [$^3$H]4,5-dihydrodiol and [$^3$H]3-hydroxybenzo(a)pyrene the amount of covalently bound material was less than that found with either [$^3$H]7,8- or [$^3$H]9,10-dihydrodiol.

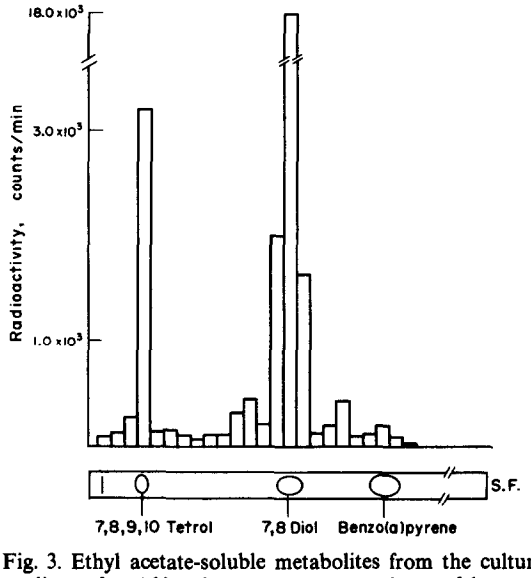


Fig. 3. Ethyl acetate-soluble metabolites from the culture medium after 16 hr short term organ culture of hamster lung (200 mg) with [$^3$H]7,8-dihydro-7,8-dihydroxybenzo(a)pyrene (1 $\mu$M-7,8-Diol). The radioactive products were separated by t.l.c. in a mixture of benzene–ethanol (4:1, v/v). The major metabolite formed co-chromatographed with 7,8,9,10-tetrahydrotetrol of benzo(a)pyrene (7,8,9,10-tetrol). S.F. indicates solvent front.

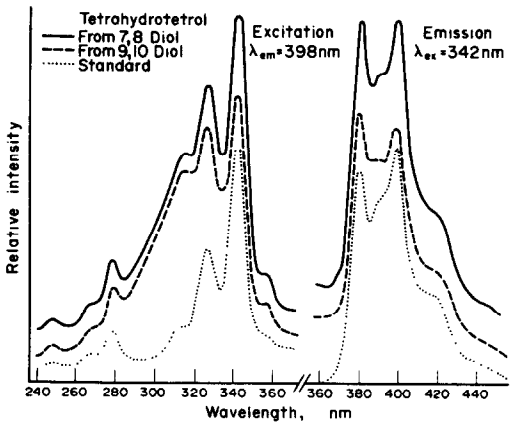


Fig. 4. Fluorescence spectra of 7,8,9,10-tetrahydrotetrol of benzo(a)pyrene. Uncorrected fluorescence excitation and emission spectra of standard 7,8,9,10-tetrahydrotetrol of benzo(a)pyrene (· · · · ·) and the suspected tetrahydrotetrols, obtained from incubation of 7,8-dihydro-7,8-dihydroxybenzo(a)pyrene (7,8-Diol) or 9,10-dihydro-9,10-dihydroxybenzo(a)pyrene (9,10-Diol) with short-term organ culture of hamster lung, were measured in 95% ethanol.

## DISCUSSION

Benzo(a)pyrene was metabolised by short-term organ culture of hamster lung to (i) ethyl acetate-soluble metabolites which co-chromatographed with 9,10- and 7,8-dihydrodiols and benzo(a)pyren-3-yl hydrogen sulphate but as reported in earlier studies [7, 22] little or no 4,5-dihydrodiol and 3-hydroxybenzo(a)pyrene were formed (Fig. 1) and (ii) to water-soluble metabolites which on enzyme hydrolysis released metabolites co-chromatographing with 3-hydroxybenzo(a)pyrene, 7,8- and 4,5-dihydrodiols but not with 9,10-dihydrodiol (Fig. 2). Thus the low amounts

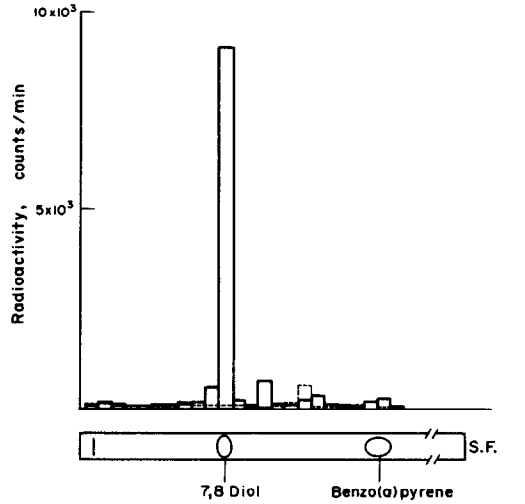


Fig. 5. Enzymic hydrolysis of water-soluble metabolites from 7,8-dihydro-7,8-dihydroxybenzo(a)pyrene (7,8-Diol). Water-soluble metabolites from the culture medium, after 16 hr short-term organ culture of hamster lung (200 mg) with 7,8-dihydro-7,8-dihydroxybenzo(a)pyrene (1 μM), were hydrolysed with either β-glucuronidase (——) or buffer alone (---), extracted with ethyl acetate and the organic-soluble extracts separated by t.l.c. in a mixture of benzene–ethanol (4:1, v/v). S.F. indicates solvent front.

of 3-hydroxybenzo(a)pyrene and 4,5-dihydrodiol detected after the initial culture were due most probably to their relatively rapid conjugation to water-soluble metabolites or possibly to their binding to tissue macromolecules. All the metabolites reported in this study, with the exception of benzo(a)pyren-3-yl-hydrogen sulphate, have previously been identified as metabolites following incubation of benzo(a)pyrene with rat lung microsomes [24].

9,10-Dihydrodiol was metabolised relatively slowly when incubated with hamster lung. Most of the radioactivity remained as unchanged 9,10-dihydrodiol and very small amounts of water-soluble metabolites were obtained, whereas 7,8-dihydrodiol was metabolised more quickly, primarily to a water-soluble conjugate (Table 1 and Fig. 5). These results are in agreement with the relative rates of glucuronide conjugation of these metabolites using a rat liver microsomal system fortified with UDP-glucuronic acid [25]. The very low conversion of 9,10-dihydrodiol to water-soluble metabolites may explain why it is the major ethyl acetate-soluble metabolite obtained from benzo(a)pyrene (Fig. 1) in the present study.

7,8- and 9,10-Dihydrodiols were both metabolised, presumably via their diol-epoxide intermediates, i.e. 7,8-dihydro-7,8-dihydroxybenzo(a)pyrene 9,10-oxide and 9,10-dihydro-9,10-dihydroxybenzo(a)pyrene 7,8-oxide respectively, to a product with similar fluorescent excitation and emission spectra to 7,8,9,10-tetrahydrotetrol (Fig. 4).

Many workers have suggested that it is the diol-epoxide from 7,8-dihydrodiol which is the ultimate carcinogen from benzo(a)pyrene. This was based on: (i) the original observation of Borgen *et al.* [26], that 7,8-dihydrodiol when activated with microsomes was bound to DNA to a 15-fold greater extent than benzo(a)pyrene; (ii) the exceptional mutagenicity of this dio-epoxide in several test systems [13–16]; and (iii) its identification as the metabolite bound to DNA both in cultured cells [6, 27] and in mouse skin and human bronchial mucosa [17, 18].

After short-term organ culture with 7,8-dihydrodiol, the radioactivity remaining associated with the lung was greater than with either 9,10-dihydrodiol or benzo(a)pyrene (Table 1). This may be connected with the reported greater biological activity of 7,8-dihydro-

Table 2. Covalent binding of benzo(a)pyrene and its metabolites in short-term organ cultures of hamster lung

| Metabolite or benzo(a)pyrene | Covalent binding pmoles bound/g lung |
|---|---|
| Benzo(a)pyrene | 1200 ± 123 (4) |
| 9,10-Dihydrodiol | 2018 ± 286 (4) |
| 7,8-Dihydrodiol | 2796 ± 179 (4) |

The covalent binding was determined after 16 hr organ culture of hamster lung (200 mg) with 1 μM of either [$^3$H]benzo(a)pyrene or 7,8-dihydro-7,8-dihydroxybenzo(a)pyrene (7,8-dihydrodiol) or 9,10-dihydro-9,10-dihydroxybenzo(a)pyrene (9,10-dihydrodiol) at 37°. Results at 37° represent mean ± S.E.M. of 4 determinations. The amount of covalent binding observed in controls incubated at 4° to minimise metabolism was always <672 pmoles bound/g lung.

diol but such interpretation is fraught with difficulty as the nature of most of the radioactivity remaining in the lung was not determined in the present study. The results reported here also show that after culture with 7,8-dihydrodiol the covalent binding is greater than after culture with either 9,10-dihydrodiol or benzo(a)pyrene (Table 2). Similar results have been obtained in preliminary experiments using short-term organ cultures of rat lung. The higher covalent binding from 7,8-dihydrodiol was consistent with the higher amounts of 7,8,9,10-tetrahydrotetrol formed from 7,8-dihydrodiol. Thus, whilst these results are consistent with the observations suggesting that the diol-epoxide derived from 7,8-dihydrodiol may be the most reactive metabolite from benzo(a)pyrene, they raise the possibility that the diol-epoxide from 9,10-dihydrodiol may also be of importance. This is based on the above observations and also on the findings in this study that: (i) 9,10-dihydrodiol was the major ethyl acetate-soluble metabolite formed from benzo(a)pyrene; (ii) it was conjugated very slowly; and (iii) the only detectable route of metabolism was via the 9,10-dihydro-9,10-dihydroxybenzo(a)pyrene 7,8-oxide to the 7,8,9,10-tetrahydrotetrol as no 9,10-dihydroxybenzo(a)pyrene was detected. In contrast to this, when 9,10-dihydrodiol was incubated with rat liver microsomes, the major metabolite was 9,10-dihydroxybenzo(a)pyrene although some 7,8,9,10-tetrahydrotetrol was also formed [21]. In preliminary experiments with short-term organ cultures of both human and rat lung with 9,10-dihydrodiol significant amounts of unidentified water-soluble metabolites were obtained in contrast to the very small amounts obtained from hamster lung. The further metabolism of 7,8-dihydrodiol to 7,8,9,10-tetrahydrotetrol in this study is in agreement with its metabolism with rat liver microsomes [21].

The possible different routes of 9,10-dihydrodiol metabolism, depending on the metabolising system, may have important implications in assessing the biological activity of the metabolites in many of the test systems used. Both the initial observation of Borgen *et al.* showing the higher binding of 7,8-dihydrodiol [26] and some mutagenicity tests [13] have used a microsomal activating system which converts 9,10-dihydrodiol to a catechol rather than to the tetrahydrotetrol. Thus these studies may have underestimated the potential biological activity of 9,10-dihydrodiol. The hypothesis of possible activity of metabolites derived from 9,10-dihydrodiol also receives some support from the findings that the fluorescent benzo(a)pyrene-DNA product in mouse skin had identical fluorescent properties to that of salmon sperm DNA reacted with either 7,8-dihydro-7,8-dihydroxybenzo(a)pyrene 9,10-oxide or 9,10-dihydro-9,10-dihydroxybenzo(a)pyrene 7,8-oxide [17]. When incubated with rat liver microsomes fortified with the soluble liver fraction and GSH, similar amounts of glutathione conjugates were obtained from the 7,8- and 9,10-dihydrodiols, indicating similar amounts of diol-epoxides from both substrates [21]. The evidence presently available which might argue against this hypothesis are: (i) hydrolysates of DNA from embryo cells treated with [$^3$H]9,10-dihydrodiol did not appear to contain radioactive hydrocarbon-deoxyribonucleoside products [6]; (ii) 7,8-dihydrodiol in a cell-mediated mutagenic assay gave a very marked increase in mutagenicity whereas 9,10-dihydrodiol even at higher concentrations gave only a slight increase in mutation frequency [14]; and (iii) when applied to mouse skin 7,8-dihydrodiol but not 9,10-dihydrodiol gave rise to hydrocarbon-nucleoside products [18]. However, these criticisms are only valid if both the cell systems used in these studies do not artificially favour formation of catechol and conjugates from 9,10-dihydrodiol and also that the 9,10-dihydrodiol when applied to mouse skin can penetrate into the cell. Thus, whilst most of the data strongly implicate a major role for the 7,8-dihydrodiol as the precursor of a highly reactive diol-epoxide of benzo(a)pyrene, the possible role of other metabolites, e.g. 9,10-dihydrodiol, in the biological activity of benzo(a)pyrene, must also be considered. It is thus preferable to assess the potential activity of metabolites using a system with both metabolic activating and deactivating enzymes. This system should wherever possible utilise a tissue in which these compounds are biologically active and thus simulate the *in vivo* situation.

*Acknowledgements*—We wish to thank Dr. J. W. Bridges for valuable discussions and criticisms of the manuscript and Ms S. Haws for excellent technical assistance. This work was supported in part by grants from the Medical Research Council and the Cancer Research Campaign.

## REFERENCES

1. Natn. Acad. Sci. *Biological Effects of Atmospheric Pollutants, Particulate Polycyclic Organic Matter*, Washington D.C. (1972).
2. E. C. Miller and J. A. Miller, *Pharmac. Rev.* **18**, 805 (1966).
3. P. Sims and P. L. Grover, *Adv. Cancer Res.* **20**, 165 (1974).
4. D. M. Jerina and J. W. Daly, *Science, N.Y.* **185**, 573 (1974).
5. C. Heidelberger, *Ann. Rev. Biochem.* **44**, 79 (1975).
6. P. Sims, P. L. Grover, A. Swaisland, K. Pal and A. Hewer, *Nature, Lond.* **252**, 326 (1974).
7. G. M. Cohen, S. M. Haws, B. P. Moore and J. W. Bridges, *Biochem. Pharmac.* **25**, 2561 (1976).
8. E. Huberman, L. Aspiras, C. Heidelberger, P. L. Grover and P. Sims, *Proc. natn Acad. Sci. U.S.A.* **68**, 3195 (1971).
9. P. L. Grover, P. Sims, E. Huberman, H. Marquardt, T. Kuroki and C. Heidelberger, *Proc. natn. Acad. Sci. U.S.A.* **68**, 1098 (1971).
10. W. Levin, A. W. Wood, H. Yagi, P. M. Dansette, D. M. Jerina and A. H. Conney, *Proc. natn. Acad. Sci. U.S.A.* **73**, 243 (1976).
11. W. M. Baird, A. Dipple, P. L. Grover, P. Sims and P. Brookes, *Cancer Res.* **33**, 2386 (1973).
12. A. J. Swaisland, A. Hewer, K. Pal, G. R. Keysell, J. Booth, P. L. Grover and P. Sims, *FEBS Lett.* **47**, 34 (1974).
13. C. Malaveille, H. Bartsch, P. L. Grover and P. Sims, *Biochem. biophys. Res. Commun.* **66**, 693 (1975).
14. E. Huberman, L. Sachs, S. K. Yang and H. V. Gelboin, *Proc. natn. Acad. Sci. U.S.A.* **73**, 607 (1976).
15. P. G. Wislocki, A. W. Wood, R. L. Chang, W. Levin, H. Yagi, O. Hernandez, D. M. Jerina and A. H. Conney, *Biochem. biophys. Res. Commun.* **68**, 1006 (1976).
16. R. F. Newbold and P. Brookes, *Nature, Lond.* **261**, 52 (1976).

17. P. Daudel, M. Duquesne, P. Vigny, P. L. Grover and P. Sims, *FEBS Lett.* **57,** 250 (1975).
18. P. L. Grover, A. Hewer, K. Pal and P. Sims, *Int. J. Cancer* **18,** 1 (1976).
19. J. W. De Pierre, M. S. Moron, K. A. M. Johannesen and L. Ernster, *Analyt. Biochem.* **63,** 470 (1975).
20. P. Sims, *Biochem. Pharmac.* **19,** 795 (1970).
21. J. Booth and P. Sims, *Biochem. Pharmac.* **25,** 979 (1976).
22. G. M. Cohen and B. P. Moore, *Biochem. Pharmac.* **25,** 1623 (1976).
23. P. Siekevitz, *J. biol. Chem.* **195,** 549 (1952).
24. P. L. Grover, A. Hewer and P. Sims, *Biochem. Pharmac.* **23,** 323 (1974).
25. N. Nemoto and H. V. Gelboin, *Biochem. Pharmac.* **25,** 1221 (1976).
26. A. Borgen, H. Darvey, N. Castagnoli, T. T. Crocker, R. E. Rasmussen and I. Y. Yang, *J. med. Chem.* **16,** 502 (1973).
27. H. W. S. King, M. H. Thompson, E. M. Tarmy, P. Brookes and R. G. Harvey, *Chem.-Biol. Interact.* **13,** 349 (1976).