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Benzo(*a*)pyrene Metabolism in Bovine Aortic Endothelial and Bovine Lung Fibroblast-like Cell Cultures[1]

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ABSTRACT

The metabolism of [^{3}H]benzo(*a*)pyrene ([^{3}H]BP) in bovine aortic endothelial and bovine lung fibroblast-like cells *in vitro* was investigated. Both cell types metabolized BP to organic solvent-extractable and water-soluble metabolites. The major organic solvent-extractable metabolites were 9-hydroxybenzo(*a*)pyrene and 3-hydroxybenzo(*a*)pyrene; 7,8-dihydro-7,8-dihydroxybenzo(*a*)pyrene, 9,10-dihydro-9,10-dihydroxybenzo(*a*)pyrene, and BP quinones were also formed. No glucuronide or sulfate conjugates of BP metabolites were detected. When exposed to [^{3}H]-3-hydroxybenzo(*a*)pyrene, both cell types metabolized this phenol to water-soluble derivatives, probably through oxidation rather than conjugation of the molecule.

These results demonstrate that endothelial cells metabolize BP to a proximate carcinogenic derivative, the 7,8-dihydrodiol. Thus, efforts to predict the biological effects of hydrocarbons on an organism must take into account possible metabolic activation by endothelial cells as well as by other target tissues. The formation of unconjugated, phenolic hydrocarbon derivatives by bovine cells suggests their use as a model system for studying the contribution of phenols to the induction of biological effects by hydrocarbons.

INTRODUCTION

BP,[5] a widespread environmental contaminant, is a potent carcinogen in experimental animals and can induce transformation and mutation of cells in culture (see chapters in Refs. 20, 22, and 28). The hydrocarbon metabolic pathways, both those leading to the induction of biological effects and those leading to detoxification and removal, have been studied in cells in culture and in microsomal systems (14–16, 20, 28, 38). Cells with detectable metabolic activity first oxidize the hydrocarbon to primary metabolites such as dihydrodiols, phenols, or quinones and then convert these into water-soluble metabolites, either through further oxidation or through conjugation to substrates such as glucuronic acid, sulfate, or glutathione (1, 4, 5, 12, 14, 33, 38–40). Some primary metabolites, such as the 7,8-diol, may be further metabolized to reactive intermediates that bind covalently to cellular nucleic acids and proteins (11, 19, 37, 41, 42). The relative proportions of the various hydrocarbon metabolism pathways may determine whether the hydrocarbon will be detoxified or will induce damage (20, 22, 28). Thus, it is essential to understand how cells from different tissues metabolize polycyclic aromatic hydrocarbons.

The endothelium, which forms the luminal surfaces of the vascular and lymphatic systems, is particularly germane to studies of chemical carcinogenesis. It is the target tissue in humans for carcinogens such as vinyl chloride (17), and its unique and strategic location dictates that carcinogens passing to and from the blood stream will traverse endothelial cells. Juchau *et al.* (29, 30) have shown that polycyclic hydrocarbons are metabolized by aortic tissue of several species, and they have postulated that polycyclic hydrocarbons may have a role in the genesis of atherosclerotic lesions (29). If endothelial cells activate compounds to proximate or ultimate carcinogens, they could serve both as target cells and as a source of activated metabolites that pass into the blood stream to other tissues. Recent improvements in the cultivation of endothelial cells provide an opportunity to study endothelial cell functions *in vitro* (21). In this study, the metabolism of BP by a cloned strain of bovine fetal vascular endothelial cells (31, 35) was investigated to determine if endothelial cells can metabolize a carcinogenic hydrocarbon and if any metabolites formed are proximate carcinogenic forms. To compare BP metabolism in endothelial cells to that in another type of bovine cell and in cells of another species, BP metabolism was also studied in a line of bovine fetal lung fibroblast-like[6] cells and in hamster embryo cells.

MATERIALS AND METHODS

Radiochemicals. [G-^{3}H]BP (specific radioactivity, 16 to 19 Ci/mmol; Amersham/Searle Corporation, Arlington Heights, Ill.) was diluted with unlabeled BP to a specific activity of 5 Ci/mmol prior to use. [G-^{3}H]-3-OH-BP (specific radioactivity, 0.146 Ci/mmol) was provided by the Cancer Research Program, Division of Cancer Cause and Prevention, National Cancer Institute, Bethesda, Md.

Cell Cultures. Bovine endothelial cells were isolated from the thoracic aorta of a fetal calf, as described previously (31); a cloned strain, BFA-1c, with a proliferative life span *in vitro* of 80 CPDL (35) was used.

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[5] The abbreviations used are: BP, benzo(*a*)pyrene; 7,8-diol, 7,8-dihydro-7,8-dihydroxybenzo(*a*)pyrene; 3-OH-BP, 3-hydroxybenzo(*a*)pyrene; CPDL, cumulative population doubling level; HPLC, high-pressure liquid chromatography; 9,10-diol, 9,10-dihydro-9,10-dihydroxybenzo(*a*)pyrene; 9-OH-BP, 9-hydroxybenzo(*a*)pyrene; BP-7,8-diol-9,10-epoxide, 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene.

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[6] The term "fibroblast-like" (TCA Manual *4*: 779, 1978) is used to indicate the morphology of the majority of the cells in the lung cell cultures: bi-polar cells which grew in parallel arrays and multiple layers. However, these uncloned cultures derived from lung fragments showed heterogeneity of cell type, especially at early passages, and included some epithelial-like cells. The possibility that these included some endothelial cells must be considered.

Bovine fetal lung cell cultures were prepared from a fetal calf by removing aseptically a lobe of the lung and mincing the tissue with scissors into approximately 1-cu mm pieces. These explants were placed into a 75-sq cm tissue culture flask in 3 ml Ham's F-12 medium (23) plus 20% fetal bovine serum and allowed to attach for 24 hr. Cultures were fed weekly. Fibroblast-like cells migrated from the explants, and the cultures were confluent in approximately 3 weeks. These cultures were then subcultivated, and their CPDL was determined every 14 days by methods described previously (35). The line used for these studies, BFL-25, has a proliferative life span *in vitro* of 60 CPDL. All serially cultivated cultures were monitored for *Mycoplasma* infection weekly and found to be free of contamination (35).

Second-passage Syrian hamster embryo cells were prepared and grown as described previously (6).

Hydrocarbon Metabolism. Confluent cultures in 25-sq cm culture vessels containing 10 ml medium were exposed to [^{3}H]BP (0.1 nmol/ml) or [^{3}H]-3-OH-BP (0.5 nmol/ml). Hydrocarbons were added in dimethyl sulfoxide; the final concentration of dimethyl sulfoxide was 0.1% or less. After a 24-hr exposure, samples of medium were removed and extracted with chloroform:methanol:water, as described previously (4, 6). This 2-step procedure, which involves extraction first with chloroform:methanol:water as a single phase followed by the addition of chloroform and water to give 2 phases, one of chloroform and one of methanol:water, allows complete extraction into the chloroform of primary oxidation products of BP (dihydrodiols, quinones, and phenols) as well as unchanged BP (6). BP conjugates and secondary oxidation products such as tetrols remain in the water:methanol phase. Aliquots from both aqueous and organic phases were removed to determine the radioactivity present, and the chloroform phases were analyzed by HPLC. To detect sulfate or glucuronic acid conjugates, some samples were incubated for 2 hr at 37° prior to organic solvent extraction with 2000 Fishman units of β-glucuronidase from bovine liver or 11 units of arylsulfatase from *Patella vulgata*, as described previously (3–5).

Samples were chromatographed at 30° on a 3.2-mm × 25-cm Lichrosorb RP18 (10 μm) reverse-phase column (Altex Scientific Inc., Berkeley, Calif.) on an Altex Model 312 HPLC chromatograph, eluted at 1 ml/min for 40 min with a linear methanol:water gradient from 11:9 to 17:3, followed by elution for 10 min at a methanol:water ratio of 17:3 (4). One hundred fractions (0.5 ml) were collected, and radioactivity in each was measured by liquid scintillation counting. Authentic samples of BP metabolites were provided by the Cancer Research Program, Division of Cancer Cause and Prevention, National Cancer Institute, Bethesda, Md., and their elution positions were determined by monitoring UV absorption at 254 nm.

A blank flask without cells containing [^{3}H]BP or [^{3}H]-3-OH-BP and medium was incubated with each experiment. The BP or 3-OH-BP present at the end of the experiment was determined as described above, and experimental results were corrected for this blank value.

To detect highly polar BP oxidation products such as tetrols, some samples were extracted with ethyl acetate:acetone:water, and the ethyl acetate phases were analyzed by HPLC (4). This solvent extracts secondary oxidation products such as tetrols (44, 45) as well as primary oxidation products and unchanged BP (39). This extraction procedure was used only for the analysis of secondary oxidation products of BP because conjugates such as BP glucuronides partition between the 2 phases depending upon the pH of the solution (4), and a small amount of BP may become trapped in the partially denatured protein of the aqueous phase due to the initial 2-phase extraction. Ethyl acetate extracts were chromatographed at 30° on a 4.6-mm × 25-cm Spherisorb-ODS (5 μm) reverse-phase column eluted with linear methanol:water gradients from 1:1 to 7:3 for 50 min and 7:3 to 9:1 for 10 min and then eluted at 9:1 for 10 min. One hundred forty 0.5-ml fractions were collected. Markers of BP tetrols were prepared from *anti*- and *syn*-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene [National Cancer Institute Chemical Repository Nos. 137 and 152 (8, 44, 45).] provided by the Cancer Research Program of the National Cancer Institute, and the elution positions of these compounds were determined by monitoring UV absorption at 254 nm.

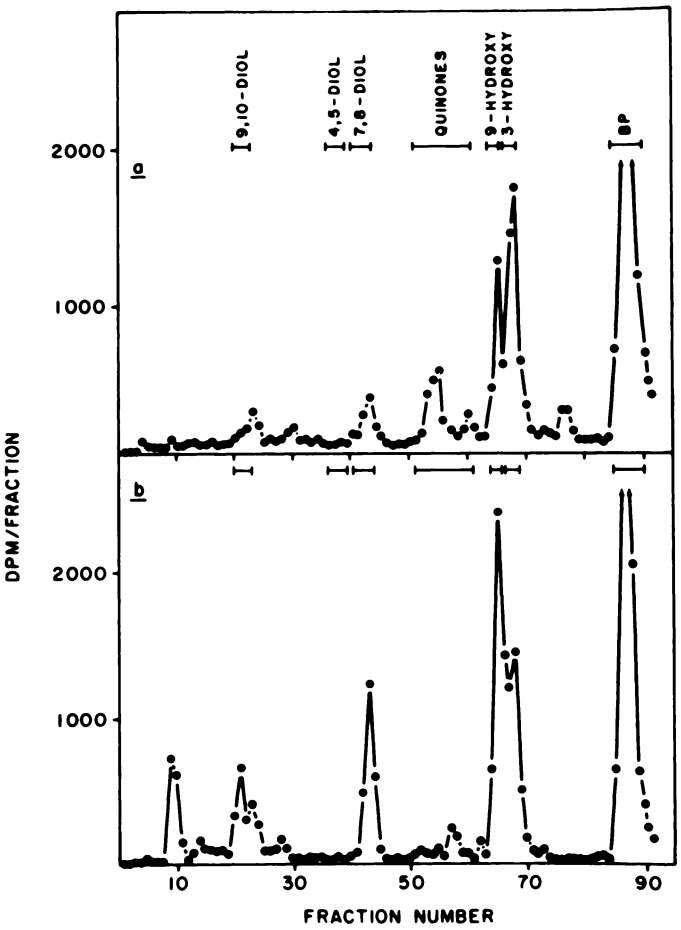

Chart 1. HPLC elution profiles of chloroform-extractable metabolites formed by [^{3}H]BP-treated bovine fetal endothelial cells and lung fibroblast-like cells. Cultures were exposed to 0.1 nmol [^{3}H]BP per ml medium for 24 hr. Samples of medium were extracted with chloroform:methanol:water, and the chloroform-extractable metabolites were analyzed by HPLC on a 3.2-mm × 25-cm Lichrosorb RP18 column eluted with a methanol:water gradient, as described in "Materials and Methods." One hundred 0.5-ml fractions were collected, and the radioactivity in each was determined by liquid scintillation counting. Elution positions of BP metabolite markers are shown at the *top*; data are expressed as dpm/0.5-ml fraction. *a*, endothelial cells (CPDL 51), 76% of the total radioactivity in the medium was chloroform extractable; *b*, lung fibroblast-like cells (CPDL 20), 50% of the total radioactivity in the medium was chloroform extractable. *4,5-diol*, 4,5-dihydro-4,5-dihydroxybenzo(*a*)pyrene.

RESULTS

HPLC elution profiles of the chloroform-extractable metabolites from cultures of bovine aortic endothelial cells and bovine lung fibroblast-like cells that were exposed to [^{3}H]BP (0.1 nmol/ml medium) for 24 hr are shown in Chart 1. Both cell types metabolized BP to the 9,10-diol, 7,8-diol, quinones, 9-phenol, and 3-phenol. In Table 1, the metabolites in each of these peaks are expressed as the percentage of the total metabolites formed and are compared with values obtained in another cell system, hamster embryo cells (5). In both bovine cell types, the major dihydrodiol formed was the 7,8-diol, in contrast to hamster embryo cells in which it was the 9,10-diol. The phenols, 9-OH-BP and 3-OH-BP, were also major metabolites in medium from the bovine cells, whereas almost no free phenols were present in hamster cell medium. The bovine lung fibroblast-like cells metabolized more BP per cell than did the bovine endothelial cells, and both bovine cell types metabolized less than did the hamster embryo cells.

In all 3 cell types, more than one-half of the metabolites could not be extracted into chloroform (Table 1). To determine if these non-chloroform-extractable metabolites resulted from conjugation of a hydrocarbon oxidation product to glucuronic acid or to sulfate, medium samples were treated with either β-glucuronidase from bovine liver to cleave glucuronide conjugates or arylsulfatase from *P. vulgata* to cleave sulfate conjugates, and the amount of chloroform-extractable material was determined (Table 2). In hamster embryo cells, some of the major metabolites of BP have been shown to be phenol-glucuronides (5); treatment of medium from hamster cells with β-glucuronidase reduced the percentage of non-chloroform-extractable metabolites by one-half (Table 2). In contrast, neither β-glucuronidase nor arylsulfatase treatment altered the percentage of non-chloroform-extractable metabolites from either type of bovine cell, suggesting that the BP metabolites formed in bovine cells are not conjugated with either of these substrates.

Of the metabolites remaining in the water phases after chloroform extraction, 45% of those from the endothelial cell medium and 43% of those from the fibroblast-like cell medium could be extracted into ethyl acetate. To determine if these ethyl acetate-extractable metabolites resulted from further oxidation of primary hydrocarbon oxidation products, samples of

Table 1

BP metabolism in bovine cells

Cultures of bovine endothelial cells (CPDL 51) and bovine lung fibroblast-like cells (CPDL 20) were exposed to 0.1 nmol [^{3}H]BP per ml medium (10 ml total) for 24 hr; second-passage hamster embryo cells were exposed to 0.5 nmol [^{3}H]BP per ml medium. The metabolites in samples of medium were extracted with chloroform:methanol:water, and the chloroform phase was analyzed by HPLC as described in Chart 1. The percentage of the BP (added to each culture) that was metabolized was: endothelial, 44.3; lung, 71.6; and hamster, 77.8. The average number of cells in each culture vessel was: endothelial, 2.2 × 10^6; lung, 1.1 × 10^6; and hamster, 3.4 × 10^6.

Metabolite	% of the total metabolites formed		
	Bovine endothelial	Bovine fibroblast-like	Hamster embryo
9,10-Diol	1.8 ± 0.2[a]	2.7 ± 0.5	8.4 ± 0.5
7,8-Diol	2.2 ± 0.1	3.3 ± 0.2	1.1 ± 0.1
Quinones	5.5 ± 0.4	1.5 ± 0.2	1.1 ± 0.1
9-OH-BP	5.8 ± 0.3	6.0 ± 0.1	2.0 ± 0.2
3-OH-BP	10.2 ± 0.6	4.3 ± 0.2	0.2 ± 0.1
Not chloroform extractable	56.5 ± 3.0	71.7 ± 1.9	69.0 ± 3.0

[a] Mean ± S.D. of samples from at least 3 culture vessels.

Table 2

Effects of β-glucuronidase and arylsulfatase treatment on BP metabolites

Cultures of bovine endothelial cells (CPDL 68), bovine lung fibroblast-like cells (CPDL 16), and second-passage hamster embryo cells were exposed to [^{3}H]BP as in Table 1. Samples of this medium were treated with β-glucuronidase or arylsulfatase prior to chloroform:methanol:water extraction, as described in "Materials and Methods." After incubation of [^{3}H]BP in culture medium without cells for 24 hr, less than 2% of the radioactivity remained in the methanol:water phase. The results are representative of those obtained in 2 to 3 experiments. The values for hamster embryo cells are the average of values obtained previously (5).

Cells	% of material not extractable into chloroform		
	Control	β-Glucuronidase	Arylsulfatase
Bovine endothelial	20.7 ± 1.2[a]	24.5 ± 1.8	22.5 ± 0.7
Bovine lung fibroblast-like	73.7 ± 1.5	71.6 ± 0.4	71.5 ± 0.8
Hamster	67.0	30.0	68.0

[a] Mean ± S.D. of 3 or more medium samples from a single culture vessel.

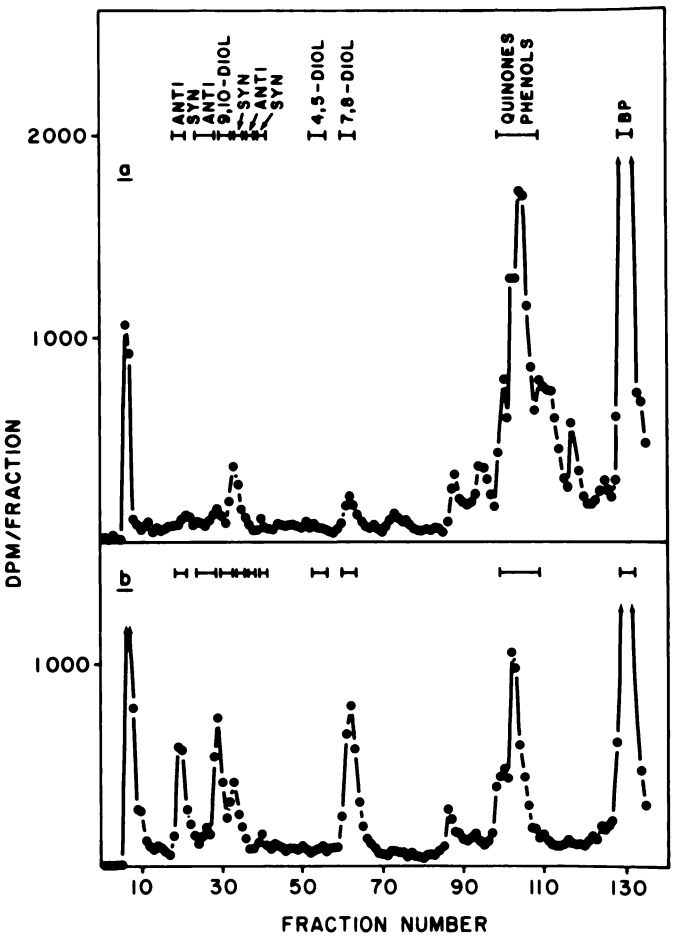

Chart 2. HPLC elution profiles of ethyl acetate-extractable metabolites formed by [^{3}H]BP-treated bovine fetal endothelial cells and bovine fetal lung fibroblast-like cells. Cultures were treated as described in Chart 1, and samples of medium were extracted with ethyl acetate, as described in "Materials and Methods." HPLC was carried out with a 4.6-mm × 25-cm Spherisorb-ODS (5 μm) reverse-phase column eluted with methanol:water, as described in "Materials and Methods." Elution positions of BP metabolite markers are shown at the *top*; data are expressed as dpm/0.5-ml fraction. *anti* and *syn* refer to the hydrolysis products of the corresponding BP-7,8-diol-9,10-epoxides. *a*, endothelial cells (CPDL 50), 89% of the total radioactivity in the medium was ethyl acetate extractable; *b*, lung fibroblast-like cells (CPDL 20), 70% of the total radioactivity in the medium was ethyl acetate extractable. *4,5-diol*, 4,5-dihydro-4,5-dihydroxybenzo(*a*)-pyrene.

medium were extracted with ethyl acetate, and the organic solvent phases were analyzed by HPLC using a gradient capable of resolving various BP tetrols (Chart 2). In both bovine cell types, several peaks chromatographed at the same position as BP tetrols, and some even more polar peaks were also recovered. The identities of these polar metabolites have not been established.

The BFA-1c clone of bovine endothelial cells used in these studies has a finite life span in culture of 80 CPDL (35). To determine if BP metabolism changes with increasing age of the culture, BP metabolism in cells at middle passage (CPDL 51) and late passage (CPDL 79) were compared (Chart 3). HPLC profiles of the metabolites formed in these cells show that the chloroform-extractable metabolites found in the late-passage cells were similar to those in the middle-passage cells. The major diol formed in both was the 7,8-diol. Both 9-OH-BP and

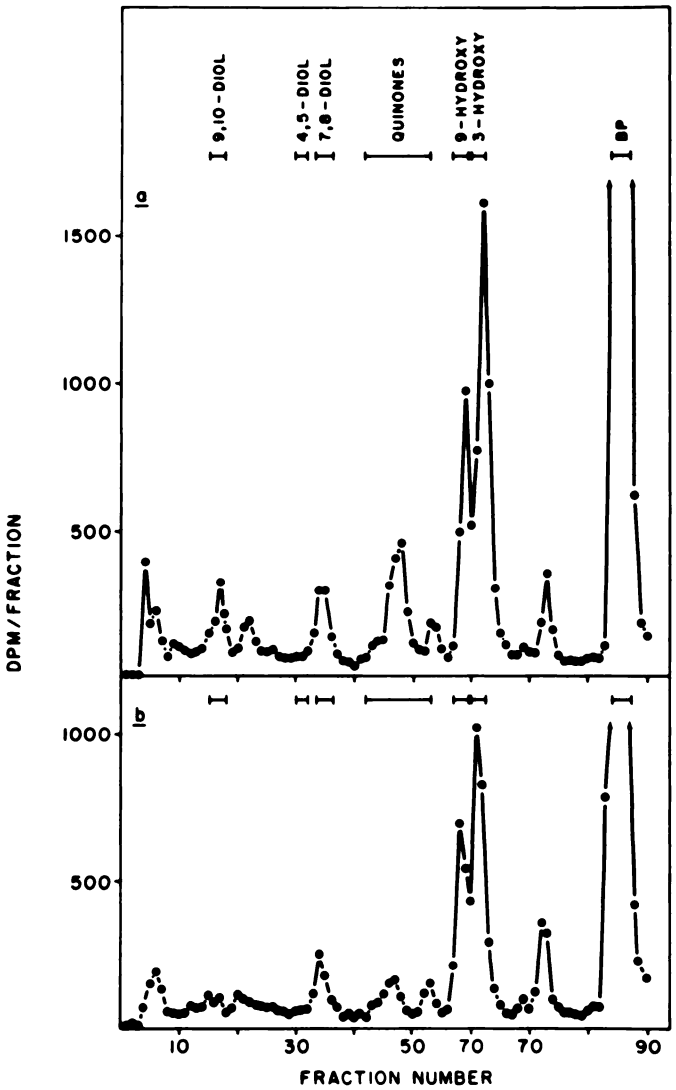

Chart 3. HPLC elution profiles of the chloroform-extractable metabolites formed by CPDL 51 (*a*) and CPDL 79 (*b*) [^{3}H]BP-treated bovine fetal endothelial cells. Cultures were treated, and HPLC was done as in Chart 1. Elution positions of BP metabolite markers are shown at the *top*; data are expressed as dpm/0.5-ml fraction. *a*, endothelial cells (CPDL 51), 76% of the total radioactivity in the medium was chloroform extractable; *b*, endothelial cells (CPDL 79), 93% of the total radioactivity in the medium was chloroform extractable. *4,5-diol*, 4,5-dihydro-4,5-dihydroxybenzo(*a*)pyrene.

Table 3

Metabolism of [^{3}H]-3-OH-BP by bovine cells

Cultures of bovine endothelial cells (CPDL 50) and bovine lung fibroblast-like cells (CPDL 16) were exposed to 0.5 nmol [^{3}H]-3-OH-BP per ml medium for 24 hr. Samples of medium were treated with β-glucuronidase, treated with arylsulfatase, or left untreated as control and were extracted with chloroform:methanol: water, as described in "Materials and Methods." The ethyl acetate extraction was carried out as described in "Materials and Methods." After incubation in medium with no cells for 24 hr, 90% of the [^{3}H]-3-OH-BP was extractable by either chloroform or ethyl acetate. The results are representative of those obtained in 2 to 3 experiments.

Treatment		% of radioactivity extractable by organic solvent	
Enzyme	Organic solvent	Bovine endothelial cells	Bovine lung fibroblast-like cells
None	Chloroform	67.7 ± 1.9[a]	26.5 ± 9.7
β-Glucuronidase	Chloroform	72.1 ± 4.2	29.7 ± 1.9
Arylsulfatase	Chloroform	64.9 ± 2.0	27.6 ± 1.5
None	Ethyl acetate	89.7 ± 0.2	87.7 ± 0.1

[a] Mean ± S.D. of 3 or more medium samples from a single culture vessel.

3-OH-BP were formed in late-passage cells, and these free phenols represented a slightly higher proportion of the total metabolites formed than they did in middle-passage cells (Chart 3). The total amount of BP metabolized by CPDL 79 cultures was less than CPDL 51 cultures, but the CPDL 79 cultures metabolized as much BP per cell as did the CPDL 51 cultures. Thus, BP metabolism in bovine endothelial cells remains essentially the same over the *in vitro* life span of these cells.

To determine if BP phenols are oxidized further by these cells rather than conjugated, the metabolism of 3-OH-BP was examined in both bovine cell types. After a 24-hr exposure to [^{3}H]-3-OH-BP (0.5 nmol/ml), the bovine lung fibroblast-like cells converted more than 70% and the bovine endothelial cells converted 30% of the 3-OH-BP to metabolites that could not be extracted into chloroform (Table 3). The amount of chloroform-extractable material was not increased by treatment of the medium samples from these cultures with β-glucuronidase or arylsulfatase. However, when samples of media were extracted with ethyl acetate, which extracts secondary oxidation products as well as the primary oxidation products extracted by chloroform, more than 87% of the radioactivity in the bovine lung fibroblast-like cell medium and 89% of that in the bovine endothelial cell medium was extracted. Thus, a major portion of the 3-OH-BP in each culture was converted to a form (5) that was ethyl acetate extractable but not chloroform extractable. Secondary oxidation products such as tetrols or triols display this type of extraction specificity; conjugates such as glucuronides and glutathiones do not.

DISCUSSION

Moore and Schwartz (34) reported that early-passage bovine fibroblast-like cells metabolize BP to water-soluble metabolites, but the metabolic pathways were not characterized. DNA isolated from [^{3}H]BP-treated bovine bronchial explants was found to contain adducts formed by reaction of a BP-7,8-diol-9,10-epoxide with deoxyguanosine (27). Elucidating the pathways of hydrocarbon metabolism in bovine cells is important because, although cattle may be resistant to the induction of tumors by BP or 3-methylcholanthrene (13), cattle are a major source of human food, and hydrocarbon metabolites in bovine

tissues could be ingested by humans. The availability of well-characterized bovine endothelial cell clones permitted us to elucidate hydrocarbon metabolism in this cell type and compare it with that in bovine fibroblast-like cells and in cells of another species.

Both bovine endothelial and fibroblast-like cells metabolized BP to the 9,10-diol and the 7,8-diol. Unlike hamster embryo cells in which the 9,10-diol is the major diol formed, slightly more 7,8-diol than 9,10-diol was formed in both bovine cell types. Similar amounts of these diols are formed in human cell lines such as WI-38 (7) and in explant cultures from several human tissues (2, 18, 24, 25). In numerous biological systems, the oxidation of the 7,8-diol to a 7,8-diol-9,10-epoxide results in one of the most toxic, mutagenic, transforming, and tumor-inducing metabolites of BP (reviewed in Refs. 20, 22, and 28). Thus, the formation by bovine cells of the 7,8-diol and metabolites that cochromatograph with those formed by hydrolysis of BP-7,8-diol-9,10-epoxide suggests that these cells may be susceptible to several hydrocarbon-induced biological effects. Indeed, preliminary studies indicate that some bovine cells are susceptible to BP-induced toxicity; additional studies will determine if these cells can be transformed by BP.

The bovine cells differ from cells of other species in that they form large amounts of free phenols. These phenols are the major organic solvent-extractable metabolites in both cell types, with the endothelial cells forming more 3-OH-BP than 9-OH-BP and the lung fibroblast-like cells forming more 9-OH-BP than 3-OH-BP. The phenols are found mainly as glucuronic acid conjugates in medium from rodent embryo cell cultures and as sulfate conjugates in some tissues (4, 12). High concentrations of BP phenols are toxic to cells (26, 32, 43); however, in cells capable of conjugating these compounds, toxicity is inversely related to cell density (5). The conjugation of phenols in many cell types with high hydrocarbon-oxidizing activity has complicated efforts to determine how phenols induce toxicity and if they are capable of inducing other biological effects upon further oxidation. The bovine cell systems should permit these questions to be answered because, although BP phenols are metabolized by these cells, this metabolism does not involve formation of glucuronic acid or sulfate conjugates. It is unlikely that the metabolites found in this study represent BP-phenol-glutathione conjugates. Although hydrocarbon-glutathione conjugates are formed from hydrocarbon epoxides by purified enzyme preparations (36) and although unidentified hydrocarbon-glutathione conjugates are formed in some cells *in vitro* (24), such conjugates cannot be extracted from culture medium by organic solvents such as chloroform or ethyl acetate (9, 10).

We have not detected any major differences between the pathways of BP metabolism in bovine endothelial and bovine lung fibroblast-like cells. Both cell types are capable of metabolizing BP to a proximate carcinogenic form, the 7,8-diol. The fact that this diol is formed by endothelial cells and could pass from them into the blood stream suggests that hydrocarbon metabolites could pose a danger both to endothelial tissue and to other, distant tissues. The possibility that endothelial cells in various organs could form and release similar hydrocarbon metabolites emphasizes the importance of determining not only how hydrocarbons are metabolized in target tissue but also how proximate carcinogens formed in other tissues could interact with target tissues.

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