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# Aryl Hydrocarbon (Benzo[A]Pyrene) Mono-oxygenase Activity in Human Primary Amnion Cell Cultures

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**Abstract**—*Amnion cells were isolated from placenta at term of healthy women and grown in monolayer cultures. The amnion cells formed dense sheets of cells of epithelial appearance after several days of growth. To evaluate these cells as targets for xenobiotics which have to be metabolically activated we have investigated their level and inducibility of the primary drug-metabolizing enzyme, aryl hydrocarbon (benzo[a]pyrene) mono-oxygenase (AHM). Amnion cells were found to contain very low levels of AHM which were highly inducible by addition of benz[a]anthracence to the growth medium. The inducibility of the enzyme was greatest during the first days of culture and declined by about two-thirds during the 5th and 8th day. After addition of the inducer, AHM activity increased several fold within* 3 hr *and reached a maximum at* 16–24 hr. *The culture age had no significant effect on the time course of induction. Constitutive and benz[a]anthracene-induced AHM were strongly inhibited by* 7,8-*benzoflavone suggesting that amnion cells in culture contain predominantly a cytochrome P-448-dependent mono-oxygenase form. Induced AHM activities in cultured amnion cells from* 13 *healthy donors ranged from* 0.6 *to* 8.0 pmole *of phenolic benzo[a]pyrene products*/min/mg *protein.*

*The results indicate that primary amnion monolayer cultures offer a valuable model to study in human epithelial cells the activation and inactivation of cytotoxic and mutagenic substances which are metabolized by cytochrome P-448-dependent mono-oxygenase(s).*

## INTRODUCTION

THE EXPOSURE to industrial and environmental chemicals poses an ever increasing threat to human health. The great number of chemicals in use or under development precludes an adequate exploration of their potential toxicity in experimental animals. Alternative test systems are presently under study which employ a variety of lower organisms ranging from bacteria to insects [1]. Mammalian cells in culture may offer another possibility for detecting toxic, mutagenic or carcinogenic compounds. In particular, the use of human cells appears to be advantageous since they might reflect more closely the susceptibility of man to the noxious action of chemicals, and may serve as an indicator for the risks of human exposure.

Progress in the development of such a rapid test system has been hampered by the difficulty of obtaining and maintaining suitable human cells in culture. Recently the growth of human placental cells in monolayer cultures has been described [2, 3] suggesting that placenta might be a ready source of human cells for toxicological studies.

A major determinant in the biological effect of chemicals is their metabolic activation and inactivation by a number of enzymes, primarily the microsomal mono-oxygenases. These enzymes are known to convert inert chemicals to reactive intermediates which bind to macromolecules, and may be highly cytotoxic and mutagenic [4–6].

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**Abbreviations:** AHM, aryl hydrocarbon mono-oxygenase: BA. benz[a]anthracene: BP. benzo[a]-pyrene: DMSO. dimethylsulfoxide: 3-OH-BP. 3-hydroxybenzo[a]pyrene: 9-OH-BP. 9-hydroxybenzo[a]-pyrene: PBS. $Ca^{2+}$ and $Mg^{2+}$ free phosphate buffered saline (Dulbecco).

A number of studies have shown that placenta at term contains microsomal mono-oxygenases which are inducible by smoking during late pregnancy [7–10], mediate the binding of benzo[a]pyrene (BP) to DNA [11] and are capable of converting environmental promutagens to their ultimate mutagenic form [12]. Obviously, placental cells in culture will only be as useful in the assessment of activation of chemicals and their biological effects as they retain the mono-oxygenase activity. To our knowledge, cultured placental cells have not been investigated in this respect.

In the following, we describe our studies on the growth of human amnion cells in primary culture and on the presence and inducibility of a representative mono-oxygenase activity, aryl hydrocarbon (benzo[a]pyrene) mono-oxygenase (AHM),* its kinetics of induction, dependency of time in culture and the variability of the induction in cells from 13 individuals.

## MATERIALS AND METHODS

*Materials*

Ham's F10 medium, fetal calf serum, penicillin and streptomycin were purchased from Seromed, Munich, horse serum from Gibco and BP from Roth, Karlsruhe, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and benz[a]anthracene (BA) from Serva, Heidelberg, NADPH from Boehringer, Mannheim, trypsin from Difco and Clophen C from Bayer A.G., Leverkusen. 3-Hydroxybenzo[a]pyrene (3-OH-BP) and 9-hydroxybenzo[a]pyrene (9-OH-BP) were obtained from the Carcinogenesis Standard Reference Compound Bank, NCI, Bethesda, Maryland, U.S.A.

*Preparation of cell cultures*

Amnion membranes were separated from human placentas within 10 hr after normal childbirth and washed thoroughly with sterile phosphate buffered saline (PBS) without $Mg^{2+}$ and $Ca^{2+}$ to remove blood cells and mucous substances. The membranes were pretreated with 0.25% trypsin in PBS at 37°C for 30 min, transfered into 300 ml of a fresh 0.25% trypsin solution and incubated at 37°C under shaking for 3 hr. The membranes were then removed, the cell suspension was filtered through 3-layered gauze, centrifuged at 1900 rev/min for 20 min, and the supernatant was discarded. The membranes were rinsed with 300 ml PBS, and the cells were harvested as described above. The cell sediments were combined and washed twice by centrifugation with complete medium consisting of 2.5% fetal calf serum, 15% horse serum, and 82.5% Ham's F10 medium containing 100 units of penicillin and 100 μg of streptomycin per ml [3]. The pellet was resuspended in 20 ml of complete medium. The yield was about 2–5 $\times 10^8$ cells per membrane from a single donor. The cells were plated in 10 ml or 30 ml medium at a density of $4 \times 10^5$ to $1.2 \times 10^6$/ml in 55 $cm^2$ plastic dishes (Falcon) or 150 $cm^2$ glass Petri-dishes, respectively, and incubated at 37°C in a 5% $CO_2$ : 95% air atmosphere.

*Preparation for enzyme assay*

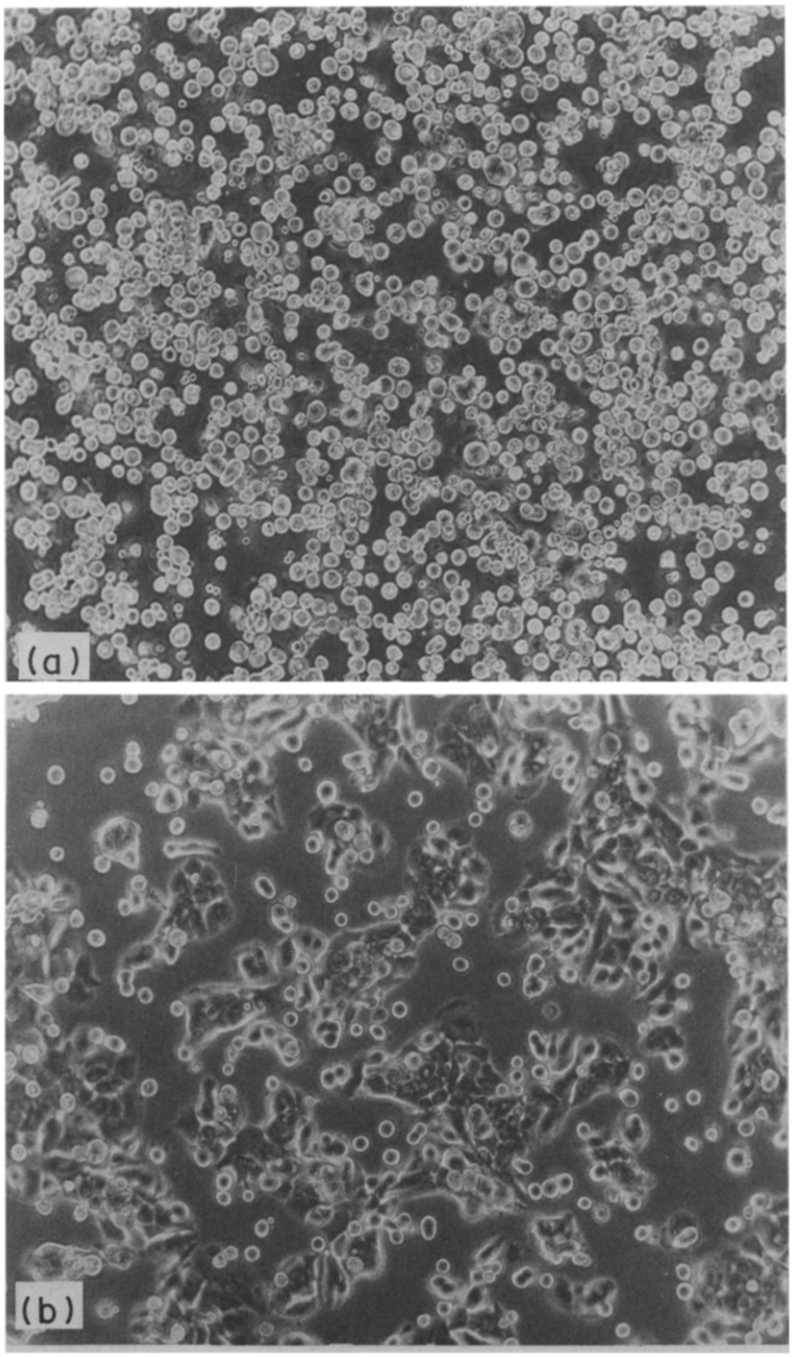
All experiments were performed with primary cultures. The medium was changed 4 days after plating of cells. BA dissolved in dimethylsulfoxide (DMSO) was added to the culture medium to a final DMSO concentration of 0.06–0.07%. The cells were harvested by scraping with a rubber blade after rinsing the cell layer 3 times with cold PBS. They were pelleted by centrifugation at 0°C and stored in liquid nitrogen for later assay.

*Assay of aryl hydrocarbon mono-oxygenase activity*

Cells were homogenized in cold 0.05 M Tris-HCl buffer, pH 7.5, using a glas-glas homogenizer. AHM activity was determined by the method of Nebert and Gelboin [13] with some modifications. The reaction mixture contained in a final volume of 1 ml: 50 μmole of Tris–HCl, pH 7.5, 5 μmole of $MgCl_2$, 0.86 μmole of glucose-6-phosphate, 0.4 units of glucose-6-phosphate dehydrogenase, 0.58 μmole of NADPH, 0.1 μmole of BP in 0.05 ml acetone and cell homogenate (0.1–1.5 mg protein).

The reaction mixture was incubated at 37°C with shaking for 1 hr unless otherwise specified. AHM specific activity is defined as fluorescence equivalent to pmole of 3-OH-BP produced at 37°C/min/mg protein. Protein concentrations were determined by the method of Lowry *et al.* [14] using bovine serum albumin as standard. The amount of DNA was measured by the diphenylamine method with calf thymus DNA as standard [15].

*The mono-oxygenase activity measured by this assay is also known as aryl hydrocarbon hydroxylase, BP-hydroxylase or BP-mono-oxygenase.




*Figs.* 1(*a*) *and* (*b*), *caption on p.* 754.

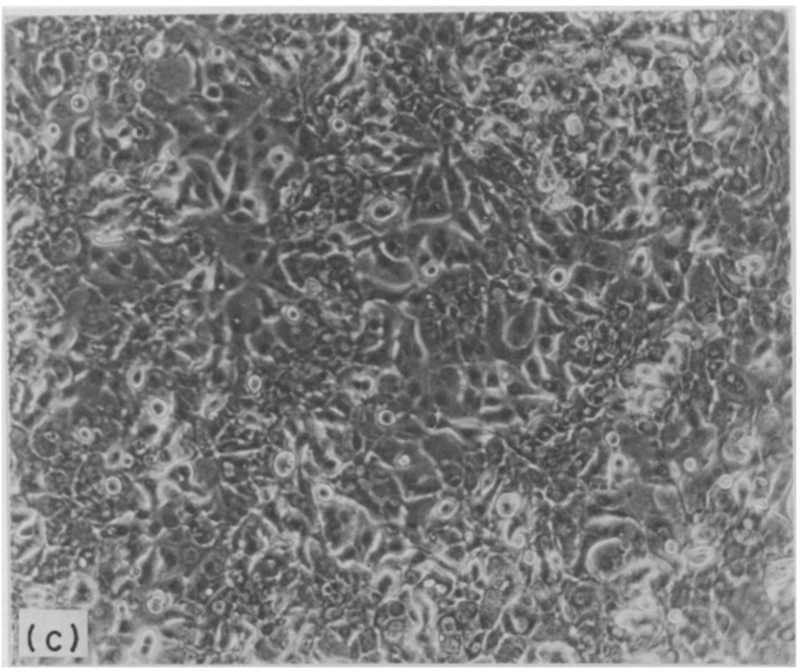



*Fig.* 1. *Amnion cells in primary culture. Amnion cells were isolated and cultured as described in Materials and Methods. Time in culture:* (*a*) 1 day, (*b*) 3 days, (*c*) 5 days. *Magnification* × 125.

## RESULTS

*Growth of human amnion cells in culture*

On the first day after seeding, cells attached loosely to the surface of the culture dishes and retained a spherical shape (Fig. 1a). One or two days later small colonies of cells of epithelial appearance began to grow out (Fig. 1b). Sheets of cells spread over the surface of the dishes within 7–10 days provided the size of the inoculum was at least $10^6$ cells/ml (Fig. 1c). An epithelial-like cell population predominated while spindle- or fibroblast-like cells were rarely observed. The initial plating efficiency of the amnion cells differed significantly between different donors. The dispersion of cells from the amnion membrane required an exposure to trypsin exceeding 3 hr. Possibly, the poor growth of cells from certain donors has to be attributed to an individually greater sensitivity of the cells to the action of trypsin.

*Aryl hydrocarbon mono-oxygenase activity in homogenates of human amnion cells: requirements and assay conditions*

The *in vitro* activity of AHM by amnion cell preparations was linear with respect to the amount of cellular protein in the range of 0.1–1.0 mg/ml (Fig. 2a), and linear with respect to the incubation time up to 1–2 hr (Fig. 2b). Formation of phenolic metabolites increased for at least another hour. AHM activity was dependent on the presence of NADPH, i.e., in the absence of NADPH, enzyme activity was reduced by more than 95% (Fig. 2B). Omission of $MgCl_2$ from the incubation mixture caused a decrease of enzyme activity of less than 20%. A second addition of NADPH (0.58 μmole/ml) after a 90 min incubation period had no stimulatory effect on the enzyme activity. Also, inclusion of a NADPH-generating system had no appreciable influence on either the initial rate of hydroxylation or on the decline of enzyme activity at later incubation times.

*Phenolic products of BP-oxygenation in amnion cell cultures*

As shown in Fig. 3, the fluorescence spectrum of the phenolic BP-metabolites formed in human primary amnion cells differs from that formed by rat liver microsomes. The second major fluorescence peak has a maximum at about 430 nm, i.e., about 20 nm lower than that of 3-OH-BP, but in the same region as 9-OH-BP, suggesting a larger portion of 9-OH-BP in the total fluorescent products. The stability of the fluorescence in alkali argues against the presence of larger amounts of the more labile 1-OH-BP, a moderately fluorescent phenolic product [16], which has been shown to be formed in rodent liver microsomes [17]. There were no apparent differences in the fluorescence spectra of the phenolic products from amnion cells at various times of incubation *in vitro*, with various amounts of protein, at different states of induction, or in cells from different donors (data not shown).

*Induction of aryl hydrocarbon mono-oxygenase in amnion cell cultures*

After addition of BA, enzyme activity increased nearly linearly for about 16–24 hr to reach a plateau level which was maintained for another 24 hr (Fig. 4). Addition of the solvent DMSO did not change the enzyme level over the 48 hr observation period. The kinetics of induction were similar on the 2nd and 5th day in culture (Fig. 4). However, the degree of induction varied considerably with the age of cells in culture. Inducibility was highest from the 2nd to the 5th day and declined thereafter to about 1/3 of the initial level by the 8th day (Fig. 5). The "constitutive" level of enzyme activity also decreased during the first week of culture. The proliferation of cells during this period is indicated by the increase in the amount of DNA (Fig. 5).

The degree of induction was somewhat dependent on the size of the initial inoculum of cells (Fig. 6). Cells plated at greater density, e.g., more than $10^6$ per ml, showed higher induced enzyme levels. The dependency of the mono-oxygenase induction on the concentration of the inducer BA is shown in Fig. 7. Induction occured at concentrations as low as 0.26 μM and reached a maximum above 10 μM.

In a preliminary experiment we tested the effect of BA on the enzyme level in minced placental tissue submerged in growth medium and kept at 37°C for 16 hr. Under these conditions enzyme activity was not detectable either in the absence or in the presence of the inducer (data not shown).

Inhibition of cyclic nucleotide phosphodiesterase has been shown to elevate the mono-oxygenase activity in various cells in culture [18, 19].

Aminophylline at concentrations which stimulated hydroxylase activity in established

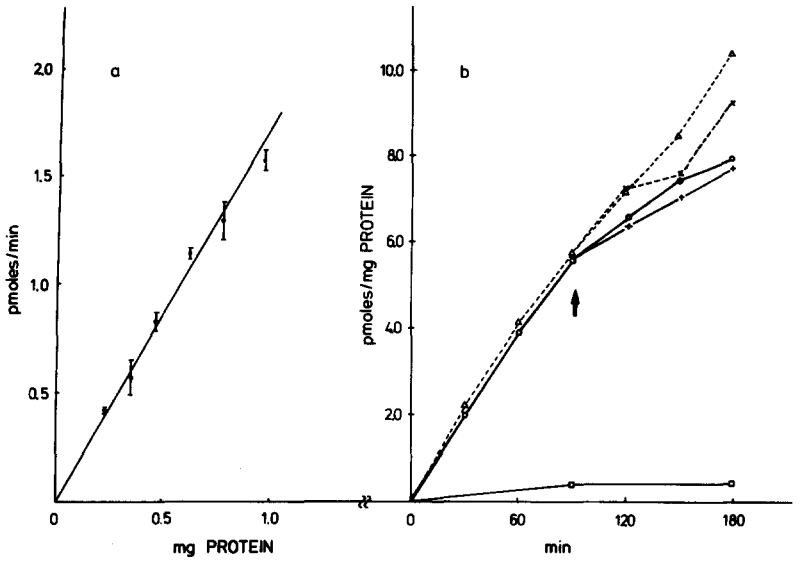



*Fig. 2. Aryl hydrocarbon mono-oygenase activity in human amnion cells as a function of the amount of protein and time of incubation. Cells were cultured* 1–4 days *and treated with BA* (13 μM) *for* 24 hr (*cf. Fig.* 4). *Several cultures were pooled for the determination of enzyme activity. Values represent the mean of duplicate determinations.* (*a*) *Dependency on the amount of cellular protein. Incubation time was* 60 min. (*b*) *Dependency on the time of incubation. Protein concentration was* 0.36 mg/ml. ○—○ *reaction mixture as described in Materials and Methods;* △----△ *without the NADPH-generating system;* +—+ *additional NADPH* (0.58 μmole) *to the reaction mixture containing the NADPH-generating system after* 90 min; ×----× *additional NADPH* (0.58 μmole) *to the reaction mixture lacking the NADPH-generating system after* 90 min; □—□ *reaction mixture without NADPH.*

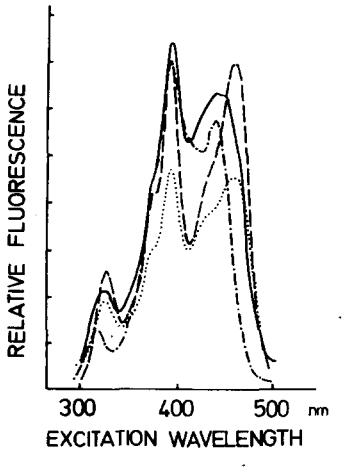



*Fig. 3. Fluorescence spectra of phenolic BP metabolites formed in cultured human amnion cells. Excitation spectra of phenolic metabolites or standard BP-phenols were determined in* 1 N NaOH *at* 522 nm *emission.* (—) *Alkali soluble products formed in amnion cells;* (.....) *in hepatic microsomes of rats pretreated with chlorinated biphenyl mixture, Clophen C:* (----) *of 3-OH-BP and* (-·--·-) *of 9-OH-BP. The two phenols were extracted from acetone:hexane* (1:3, v/v) *into* 1N NaOH. *Other conditions as described in Materials and Methods.*

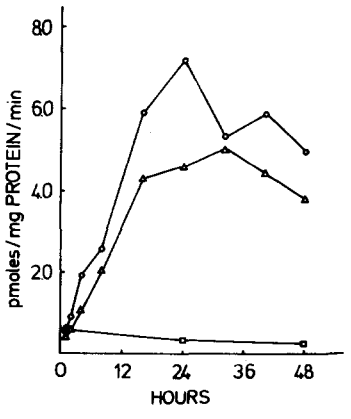



*Fig. 4. Kinetics of aryl hydrocarbon mono-oxygenase induction. Cells were exposed to* 13 μM *BA on the 2nd* (○) *and 5th* (△) *day in culture. Control plates received DMSO* (□) *on the 2nd day. Cells were harvested at times indicated and assayed for enzyme activity. Values represent the mean of duplicate determinations.*

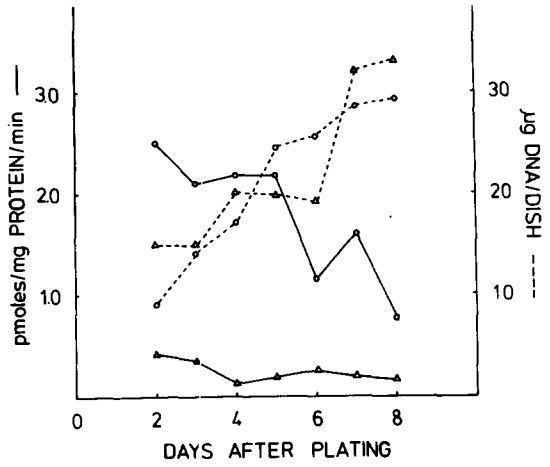



*Fig. 5. Inducibility of aryl hydrocarbon mono-oxygenase activity and DNA content of cell cultures at various times after plating. BA was added to 1–7-day-old cultures; after 24 hr exposure AHM activity and DNA content were determined on the same dish. Controls received DMSO. Values represent the mean of duplicate determinations. (△) DMSO-treated cells; (○) BA-treated cells; (——) AHM activity; (----) DNA content.*

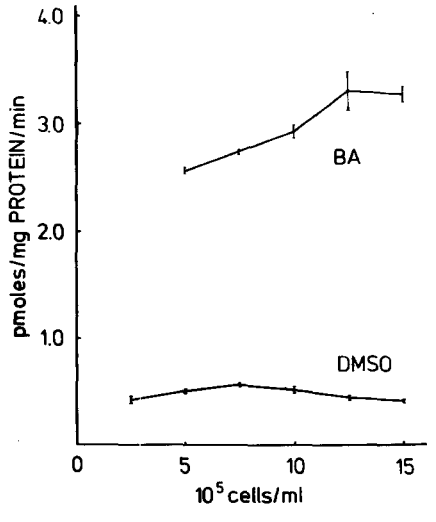



*Fig. 6. Dependency of aryl hydrocarbon mono-oxygenase activity on plating density of amnion cells. The cells were seeded at the population density indicated. They were cultured for 24 hr, exposed to 13 μM BA or DMSO for another 24 hr, and then assayed for enzyme activity. Values give the mean and range of duplicate determinations from one (DMSO-treated) or two (BA-treated) cultures.*

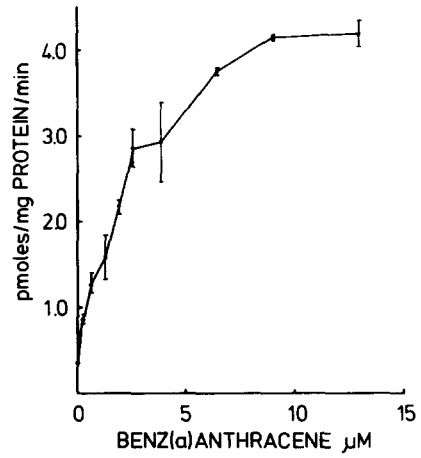



*Fig. 7. Dependency of the induction of aryl hydrocarbon mono-oxygenase on the inducer concentration. BA at concentrations indicated was added to 1-day-old cultures for 24 hr. Values give the mean and range of enzyme activity of two cultures.*

rodent and human lung cells [19] did not enhance enzyme activity in the amnion cells (Table 1). To the contrary, enzyme activity decreased with increasing concentration of aminophylline to about 50% of the control level at 0.7 mM of the inhibitor.

To probe for the nature of the mono-oxygenase in amnion cells we studied the effect of 7,8-benzoflavone, a specific inhibitor of polycyclic hydrocarbon inducible mono-oxygenase [20]. As shown in Table 2, 7,8-benzoflavone inhibited the constitutive and induced mono-oxygenase activity by more than 60% at a concentration of 0.6 mM.

*Variability of aryl hydrocarbon mono-oxygenase activity in amnion cells from different donors*

Figure 8 shows the levels of AHM activity in cultured amnion cells obtained from 13 donors. Basal activities were generally too low to be determined with accuracy. Induced enzyme activities varied widely ranging from 0.6 to 8.0 pmole/min/mg protein. Of the 13 women, only one, donor M, smoked more than 10 cigarettes per day during the last days before childbirth. Although it might be fortuitous, it is striking that the induced enzyme level in amnion cells from this donor is the highest of all donors. AHM activity in replicate cultures from a given donor varied by less than 20%.

## DISCUSSION

The present observations indicate that amnion cells in culture from human term plac-

*Table* 1. *Effect of aminophylline on AHM activity in amnion cells in culture and on the induction by benz(a)anthracene*

| Additions | | AHM activity (pmole/min/mg protein) |
|---|---|---|
| None | — | 0.17 |
| Aminophylline | 0.10 mM | 0.21 |
| Aminophylline | 0.70 mM | 0.19 |
| BA | — | 2.13 |
| BA + aminophylline | 0.10 mM | 1.96 |
| BA + aminophylline | 0.35 mM | 1.52 |
| BA + aminophylline | 0.70 mM | 1.27 |

After 24 hr of culture, the medium was replaced by medium containing variable amounts of aminophylline, BA (13 μM) or both and cultures were incubated for another 48 hr. Values represent the mean of duplicate determinations from 3 culture dishes.

*Table* 2. In vitro *effect of* 7,8-*benzoflavone on AHM activity of human amnion cells*

| Treatment of cultures | 7,8-Benzoflavone | AHM activity (pmole/min/mg protein) | |
|---|---|---|---|
| DMSO | None | 0.22 | (100) |
| DMSO | 0.60 mM | 0.08 | (36) |
| Benz(a)anthracene | None | 6.11 | (100) |
| Benz(a)anthracene | 0.06 MM | 3.39 | (55) |
| Benz(a)anthracene | 0.20 mM | 1.84 | (27) |
| Benz(a)anthracene | 0.60 mM | 0.81 | (13) |

Cells were cultured for 72 hr and exposed to BA for another 24 hr. Control cultures received DMSO. 7,8-Benzoflavone was added in acetone (50 μl) together with the substrate, BP. Numbers in parenthesis give the percentage of the controls, i.e. enzyme activity in the absence of 7,8-benzoflavone. Values represent the mean of duplicate determinations.

enta contain mono-oxygenase activity which is inducible by a polycyclic hydrocarbon. The induction of the enzyme in amnion cells was similar to that in other rodent and human cells in culture with respect to the time course of induction, the dependency of the inducer concentration and the culture age [21–23].

The inhibition of basal as well as induced hydroxylase activity by 7,8-benzoflavone suggests that the mono-oxygenase in amnion cells belongs to the group of cytochrome P-448-dependent enzymes which are found in cultured mammalian cells and in most extrahepatic tissues [24]. In agreement with our findings in cultured placental cells. 7,8-benzoflavone also inhibited BP hydroxylation in homogenates of term placenta obtained from women who smoke [25]. Further proof for the predominance of cytochrone P-448-dependent mono-oxygenase(s) in human amnion cells comes from the fluorescence spectra of phenolic BP metabolites which indicate the formation of large amounts of 9-OH-BP and of only minor quantities of 3-OH-BP. Studies using rodent microsomes have shown that mono-oxygenase forms induced by polycyclic hydrocarbons preferably oxygenate the benzo ring (positions 7–9) of BP in contrast to the constitutive and phenobarbital-induced cytochrome P-450 forms which more strongly attack the pyrene ring of the molecule (positons 1–5) [26, 27]. The predominant oxygenation of BP in the benzo ring by amnion cells and by other cells in culture [19] is of major significance since the oxidative attack on this region of the molecule leads to the

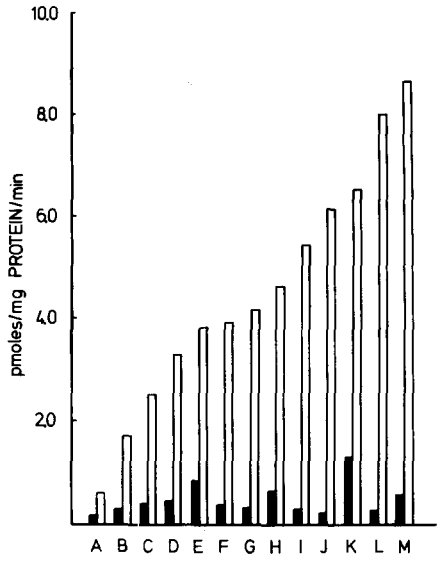



*Fig. 8. Induction of aryl hydrocarbon mono-oxygenase activity in cultured amnion cells from 13 individuals. Cell inocula ranged from $4 \times 10^5$ to $1.2 \times 10^6$ of cells/ml. Cultures were incubated for 24 hr except from donors B and J (48 and 72 hr, respectively) and exposed to 13 μM BA (□) or DMSO (■) for 24 hr. Values represent the mean of duplicate determinations from two to four cultures.*

formation of diol epoxides which are highly mutagenic [28] and possibly the ultimate carcinogenic forms of BP [29, 30].

Generally, cytochrome P-448-dependent mono-oxygenases are known to possess a specific range of substrate/inducers which are typified by planar and highly hydrophobic compounds such as the aromatic polycyclic hydrocarbons. This substrate specificity might limit the usefulness of the human amnion cell cultures as universal target cells for chemical mutagens or carcinogens which have to be activated by microsomal mono-oxygenases. Presently, the cultured amnion cells appear to share this limitation with all established cell lines and primary cells in cultures except short term cultures of hepatocytes [19].

The different levels of mono-oxygenase activity in placental cells from the 13 donors tested may be an expression of the interindividual variability of inducible enzyme activity *in vivo*. Similar differences in hydroxylase activity have been observed in cultured human monocytes [31], lymphocytes [32], pulmonary macrophages [33], skin fibroblasts [34, 35], tracheal organ explants [36], and in liver biopsies [37], and have, to various degrees, been attributed to genetic factors. It is interesting to note that high induced enzyme levels occured in amnion cells from a woman who was a heavy smoker. Relatively high BA-induced enzyme levels were also observed in cultured peripheral lymphocytes of smokers [33]. Presently, we do not know how huch of the variability observed in the amnion cells is due to experimental variation, e.g., in cell isolation, culture conditions and growth rate, or to genetic constitution.

Human fibroblasts which can easily be obtained from skin exhibited considerably lower mono-oxygenase activity than epithelial cells [38, 39] and proved unsuitable for testing premutagens or precarcinogens [40] without an external activation system such as microsomal preparations [41, 42] or "feeder" cells [38]. Although amnion cells in primary culture which consist mainly of epithelial cells by morphological criteria have low constitutive mono-oxygenase level, the induced enzyme attains appreciable levels comparable to these found in fetal liver cells [37] and exceeding those in other human cells in culture [19] except for the choriocarcinoma derived cell line, JEG-3 [22].

Taking into consideration the specificity of mono-oxygenase activity, the amnion cell cultures may represent a useful model to study the effect and the fate of xenobiotics in human epithelial cells, and to follow up their metabolism, binding to macromolecules and removal by repair processes.

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## REFERENCES

1. B. J. Kilbey, J. Legator, W. Nichols and C. Ramel (Editors). *Handbook of Mutagenicity Test Procedures*. Elsevier, Amsterdam (1977).
2. J. Paul, Preparation of primary human amnion cells. In *Cell and Tissue Culture*. (Edited by J. Paul) p. 225. Churchill Livingstone, New York (1975).
3. P. W. Roy, G. E. Ryan and E. D. Bransome, Jr., Primary culture and maintenance of steroid-secreting human placental monolayers. *In Vitro* **12,** 115 (1976).

4. H. V. Gelboin, E. Huberman and L. Sachs, Enzymatic hydroxylation of benzopyrene and its relationship to cytotoxicity. *Proc. nat. Acad. Sci. (Wash)* **64,** 1188 (1969).
5. J. Miller, Carcinogenesis by chemicals: an overview. G.H.A. Clowes memorial lecture. *Cancer Res.* **30,** 559 (1970).
6. H. V. Gelboin and F. J. Wiebel, Studies on the mechanism of aryl hydrocarbon hydroxylase induction and its role in cytotoxicity and tumorigenicity. *Ann. N.Y. Acad. Sci.* **179,** 529 (1971).
7. D. W. Nebert, J. Winkler and H. V. Gelboin, Aryl hydrocarbon hydroxylase activity in human placenta from cigarette smoking and nonsmoking women. *Cancer Res.* **29,** 1763 (1969).
8. R. M. Welch, Y. E. Harrison, A. H. Conney, P. J. Poppers and M. Finster, Cigarette smoking: stimulatory effect on metabolism of 3,4-benzpyrene by enzymes in human placenta. *Science* **160,** 541 (1968).
9. J. Kapitulnik, W. Levin, P. J. Poppers, J. E. Tomaszewski, D. M. Jerina and A. H. Conney, Comparison of the hydroxylation of zoxazolamine and benzo[a]pyrene in human placenta: effect of cigarette smoking. *Clin. Pharmacol. Ther.* **20.** 557 (1976).
10. I. Y. Wang, R. E. Rasmussen, R. Creasey and T. T. Crocker, Metabolites of benzo[a]pyrene produced by placental microsomes from cigarette smokers and nonsmokers. *Life Sci.* **20,** 1265 (1977).
11. D. L. Berry, P. K. Zachariah, T. J. Slaga and M. R. Juchau, Analysis of the biotransformation of benzo[a]pyrene in human fetal and placental tissues with high-pressure liquid chromatography. *Europ. J. Cancer* **13,** 667 (1977).
12. A. H. Jones, A. G. Fantel, R. A. Kocan and M. R. Juchau, Bioactivation of procarcinogens to mutagens in human fetal and placental tissues. *Life Sci.* **21,** 1831 (1977).
13. D. W. Nebert and H. V. Gelboin, Substrate-inducible microsomal aryl hydroxylase in mammalian cell culture. I. Assay and properties of induced enzyme. *J. biol. Chem.* **243,** 6242 (1968a).
14. O. H. Lowry, N. A. Rosebrough, A. L. Farr and R. J. Randall, Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193,** 265 (1951).
15. K. A. Burton, Study of the conditions and mechanisms of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.* **62,** 315 (1956).
16. G. Holder, H. Yagi, W. Levin, A. Y. H. Tu and D. M. Jerina, Metabolism of benzo[a]pyrene. III. An evaluation of the fluorescence assay. *Biochem. biophys. Res. Comm.* **65,** 1363 (1975).
17. R. G. Croy, J. K. Selkirk, R. G. Harvey, J. F. Engel and H. V. Gelboin, Separation of ten benzo[a]pyrene phenols by recycle high pressure liquid chromatography and identification of four phenols as metabolites. *Biochem. Pharmacol.* **25,** 227 (1976).
18. H. Yamasaki, E. Huberman and L. Sachs, Regulation of aryl hydrocarbon (benzo[a]pyrene) hydroxylase activity in mammalian cells: induction of hydroxylase activity by $N^6$, $O^2$-dibutyryl adenosine 3′, 5′monophosphate and aminophylline. *J. biol. Chem.* **250,** 7766 (1975).
19. F. J. Wiebel, S. Brown, H. L. Waters and J. K. Selkirk, Activation of xenobiotics by mono-oxygenases: cultures of mammalian cells as analytical tool. *Arch. Toxicol.* **39,** 133 (1977).
20. F. J. Wiebel, J. C. Leutz, L. Diamond and H. V. Gelboin, Aryl hydrocarbon (benzo[a]pyrene) hydroxylase in microsomes from rat tissues: differential inhibition and stimulation by benzoflavones and organic solvents. *Arch. Biochem.* **144,** 78 (1971).
21. D. W. Nebert and H. V. Gelboin, Substrate-inducible microsomal aryl hydroxylase in mammalian cell culture. II. Cellular responses during enzyme induction. *J. biol. Chem.* **243,** 6250 (1968b).
22. F. J. Wiebel, H. V. Gelboin and H. G. Coon, Regulation of aryl hydrocarbon hydroxylase in intraspecific hydrids of human, mouse and hamster cells. *Proc. nat. Acad. Sci. (Wash.)* **69,** 3580 (1972).
23. J. P. Whitlock, Jr. and H. V. Gelboin, Aryl hydrocarbon (benzo[a]pyrene) hydroxylase induction in rat liver cells in culture. *J. biol. Chem.* **249,** 2616 (1974).

24. F. J. WIEBEL, Activation and detoxification of carcinogens by microsomal mono-oxygenases: modification by benzoflavones and polycyclic aromatic hydrocarbons. In *Modifiers of Chemical Carcinogenesis: An Approach to the Biochemical Mechanism and Cancer Prevention.* (Edited by T. J. Slaga) In press. Raven Press, New York.
25. O. PELKONEN, Differential inhibition of aryl hydrocarbon hydroxylase in human foetal liver, adrenal gland and placenta. *Acta pharmacol. toxicol.* **41,** 306 (1977).
26. G. HOLDER, H. YAGI, P. Dansette, D. M. JERINA, W. LEVIN, A. Y. H. LU and A. H. CONNEY, Effects of inducers and epoxide hydrase on the metabolism of benzo[a]pyrene by liver microsomes and a reconstituted system: analysis by high pressure liquid chromatography. *Proc. nat. Acad. Sci. (Wash.)* **71,** 4356 (1974).
27. W. E. FAHL, C. R. JEFCOATE and C. B. KASPER, Characteristics of benzo[a]pyrene metabolism and cytochrome P-450 heterogeneity in rat liver nuclear envelope and comparison to microsomal membrane. *J. biol. Chem.* **253,** 3106 (1978).
28. E. HUBERMAN, L. SACHS, S. K. YANG and H. V. GELBOIN, Identification of mutagenic metabolites of benzo[a]pyrene in mammalian cells. *Proc. nat. Acad. Sci. (Wash.)* **73,** 607 (1976).
29. T. J. SLAGA, A. VIAJE, D. L. BERRY and W. BRACKEN, Skin tumour initiating ability of benzo[a]pyrene 4,5-7,8-diol-9,10-expoides and 7,8-diol. *Cancer Lett.* **2,** 115 (1976).
30. W. LEVIN, A. W. WOOD, H. YAGI, P. M. DANSETTE, D. M. JERINA and A. H. CONNEY, Carcinogenicity of benzo[a]pyrene 4,5-,7,8- and 9,10-oxides on mouse skin. *Proc. nat. Acad. Sci. (Wash.)* **73,** 243 (1976).
31. R. C. BAST, J. P. WHITLOCK, H. MILLER, H. J. RAPP and H. V. GELBOIN, Aryl hydrocarbon (benz[a]pyrene) hydroxylase in human peripheral blood monocytes. *Nature (Lond.)* **250,** 664 (1974).
32. B. PAIGEN, J. MINOWADA, H. L. GURTOO, K. PAIGEN, N. B. PARKER, E. WARD, N. T. HAYNER, I. D. J. BROSS, F. BOCK and R. VINCENT, Distribution of aryl hydrocarbon hydroxylase inducibility in cultured human lymphocytes. *Cancer Res.* **37,** 1829 (1977).
33. T. L. MCLEMORE, R. R. MARTIN, K. L. TOPPELL, D. L. BUSBEE and E. T. CANTRELL, Comparison of aryl hydrocarbon hydroxylase induction in cultured blood lymphocytes and pulmonary macrophages. *J. clin. Invest.* **60,** 1017 (1977.)
34. W. LEVIN, A. H. CONNEY, A. P. ALVARES, I. MERKATZ and A. KAPPAS, Induction of benzo[a]pyrene hydroxylase in human skin. *Science* **176,** 419 (1972).
35. E. HUBERMAN and L. SACHS, Metabolism of the carcinogenic hydrocarbon benzo[a]pyrene in human fibroblast and epithelial cells. *Int. J. Cancer* **11,** 412 (1973).
36. C. C. HARRIS, H. AATRUP, R. CONNER, L. A. BARRETT, E. M. MCDOWELL and B. F. TRUMP, Interindividual variation in binding of benzo[a]pyrene to DNA in cultured human bronchi. *Science* **194,** 1067 (1976).
37. O. PELKONEN, P. KORHONEN, P. JOUPPILLA and N. KÄRKI, Induction of aryl hydrocarbon hydroxylase in human fetal liver cell and fibroblast cultures by polycyclic hydrocarbons. *Life Sci.* **16,** 1403 (1975).
38. A. E. FREEMAN, R. S. LAKE, H. J. IGEL, L. GERNAND, M. R. PEZZUTTI, J. M. MALONE, C. MARK and W. F. BENEDICT, Heteroploid conversions of human skin cells by methylcholanthrene. *Proc. nat. Acad. Sci. (Wash.)* **74,** 2451 (1977).
39. C. H. FOX, J. K. SELKIRK, F. M. PRICE, R. G. CROYROY, K. K. SANFORD and M. COTTLER-2OX, Metabolism of benzo[a]pyrene by human epithelial cells *in vitro. Cancer Res.* **35,** 3551 (1975).
40. R. H. C SAN and H. F. STICH, DNA repair synthesis of cultured human cells as a rapid bioassay for chemical carcinogens. *Int. J. Cancer* **16,** (1975).
41. B. A. LAISHES and H. F. STICH, Repair synthesis and sedimentation analysis of DNA of human cells exposed to dimethylnitrosamine and activated dimethylnitrosamine.. *Biochem. biophys. Res. Comm.* **52,** 827 (1973).
42. F. E. AHMED, R. W. HART and N. J. LEWIS, Pesticide induced DNA damage and its repair in cultured human cells. *Mutation Res.* **42,** 161 (1977).