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Authors

Bartholomew, James C.
Salmon, Andrew G.
Gamper, Howard B.
et al.

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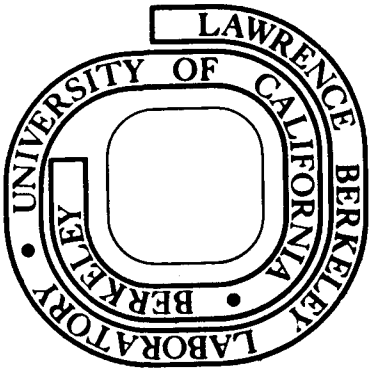

BENZO[a]PYRENE EFFECTS ON MOUSE EPITHELIAL CELLS IN CULTURE

I. Cytoxicity and Induction of Aryl Hydrocarbon Hydroxylase

JAMES C. BARTHOLOMEW, ANDREW G. SALMON, HOWARD B. GAMPER, and MELVIN CALVIN

Laboratory of Chemical Biodynamics, Lawrence Berkeley Laboratory,
University of California, Berkeley, California 94720 USA

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SUMMARY

The effect of benzo[a]pyrene on the growth in culture of five mouse epithelial cell strains was examined. These epithelial cells are highly sensitive to the cytotoxic action of benzo[a]pyrene. In addition, the level of one of the benzo[a]pyrene metabolizing enzymes, aryl hydrocarbon hydroxylase, is low, but highly inducible by the carcinogen. As the sensitivity of a cell strain to the cytotoxic action of benzo[a]pyrene decreased, the inducibility of the hydroxylase also decreased. However, a strong correlation could not be found between cytotoxicity and the level of uninduced or induced hydroxylase when the values from different cell strains were compared. Furthermore, 7,8-benzoflavone, an inhibitor of the hydroxylase, inhibited the cytotoxic action of benzo[a]pyrene. These experiments suggest that the hydroxylase is important in determining the sensitivity of epithelial cells to the cytotoxic action of benzo[a]pyrene, but other factors may also modulate this sensitivity.

INTRODUCTION

It has been estimated that 90% of all cancers in man are caused by environmental chemicals [1]. Of these malignancies, most arise in epithelial tissue. However, most studies of the malignant transformation of cells in culture have utilized either fibroblastic cell lines or early passage embryo cells. The inability to study the effects of oncogenic agents on more highly differentiated epithelial cell strains in culture has been due to the difficulty of separating and culturing epithelial cells from the more rapidly proliferating fibroblastic cells found in the primary cultures of embryos. Recently, Owens [2] has described a technique for selectively culturing epithelial cells from different tissues. We have been studying the effect of benzo[a]pyrene(BaP), a common environmental carcinogen, on the epithelial cells obtained by Owens. The aim of this research is to characterize the action of chemical carcinogens on more highly differentiated cells than the whole embryo fibroblasts commonly used.

Carcinogenic polycyclic aromatic hydrocarbons like BaP have been shown to be cytotoxic to mammalian cells [3-8]. Gelboin et al. [4] have reported that the susceptibility of cells grown in culture to the cytotoxic action of BaP is related to the level of one of the enzymes which metabolizes the hydrocarbons, aryl hydrocarbon hydroxylase. The hydroxylase has also been implicated in the malignant transformation of mammalian cells. Kinoshita and Gelboin [9] have shown that 7,8-benzoflavone, a potent inhibitor of the hydroxylase, inhibits the binding of polycyclic aromatic hydrocarbons to biological macromolecules such as DNA, RNA, and protein, and also inhibits the formation of tumors in mice treated with 7,12-dimetylbenz[a]anthracene. Since the hydroxylase has been implicated in both the toxic and carcinogenic

effects of these hydrocarbons, it was originally thought that cytotoxicity was a manifestation of the carcinogenicity of these compounds. These two processes seem to be distinct, however, since it has been shown by Huberman and Sachs [3] that normal cells resistant to the cytotoxic action of BaP can still be transformed by that compound. In addition, there are a number of reports that concentrations of hydrocarbon in the medium which are maximal for cytotoxic effects of cells, are still submaximal for the transformation of these cells [3,10]. This suggests that the relationship between cytotoxicity and transformation is a complex function of a cell's ability to metabolize the hydrocarbon to cytotoxic and/or carcinogenic intermediates, and the turnover of these derivatives once formed.

MATERIALS AND METHODS

Cell Culture Techniques. The cells used in this study are described in Table 1. The method of isolation and the characterization of the epithelial cells has been described by Owens [2]. All cells were carried in 100 mm plastic dishes (Falcon) and incubated in a CO_2 incubator at 37°. Epithelial cells were grown in Vogt and Dulbecco's modification of Eagle's medium [11], containing 10% fetal calf serum (GIBCO) and 10 µg/ml insulin. The fibroblastic cells were maintained in the same medium without insulin. All cell lines were transferred twice weekly, by removal from the dishes with 0.05% trypsin (Difco, 1:250) in 25 mM Tris buffer, pH 7.4, containing 140 mM NaCl, 5 mM KCl, and 0.7 mM Na_2HPO_4 (isotonic Tris buffer). The cloning experiments were carried out by seeding 100 cells on a 100 mm dish in growth medium and incubating the cells until colonies were formed. The colonies were isolated and carried as described above.

The toxicity studies were carried out by plating 5 x 10^4 cells to be tested in each 35 mm plastic dish. The concentration of carcinogen to be tested was added to four dishes 24 h after seeding the cells. The BaP used in all these experiments was purified prior to use by neutral alumina column chromatography using benzene as a solvent followed by recrystallization from benzene/isopropanol. The carcinogen was added in DMSO which had a final concentration of 0.5% in the growth medium. Control dishes minus carcinogen were 0.5% in DMSO. Three days after adding the carcinogen the surviving cells were washed 2 times with isotonic Tris buffer and removed from the dishes by treatment with 2.0 ml 0.01% trypsin in isotonic Tris buffer for 20 min at 37°. The number of cells per dish was determined by counting an aliquot of the trypsin solution in a Coulter counter Model Fn.

Aryl Hydrocarbon Hydroxylase Induction and Assay. Cells were seeded onto 100 mm plates at a density such that they would continue to grow logarithmically throughout the induction period. This density varied with cell line but was usually in the range 1 to 5 x 10^5 cells per plate. Twenty-four hours later BaP was added as a solution in DMSO, the final concentration of DMSO in the experimental and control plates was 0.1%. Each BaP concentration point was set up in 5 plates. After 24 h the cells were removed from the plates with 0.1 mg/ml EDTA in isotonic Tris buffer, washed once with the same buffer by centrifugation, and resuspended in 50 ml Tris buffer, pH 7.5, containing 0.25 M sucrose. The yield from each set of 5 plates was combined in a final volume of 0.5 ml Tris-sucrose buffer. The cells were disrupted by sonication for 3 min at a power of 75 watts in the cooled horn (0°) of a Heat Systems Ultrasonic, Inc. cell disrupter.

Each sample was enclosed in an airtight plastic tube, thus avoiding aerosol problems with carcinogen-containing material. Protein concentration of the cell sonicate was measured by the method of Lowry et al. [12] using bovine serum albumin as a standard. Before taking a sample for protein estimation or enzyme assay, the solution was mixed by vortexing to ensure homogeneity.

The standard assay method used was that described by Nebert and Gelboin [13], with the addition of NADH as well as NADPH as suggested by Nebert and Gielen [14]. A typical assay tube contained the following in a total volume of 1.0 ml: between 20 and 100 µl of total cell sonicate; 360 µM NADPH; 390 µM NADH; 600 µg/ml crystalline bovine serum albumin; and 3 mM $MgCl_2$, in 50 mM Tris buffer, pH 7.5. The samples were pre-warmed to 37°, and the reaction was started by addition of 10 µl of a BaP solution in DMSO to give a final BaP concentration of 80 µM. Incubation was for 30 min with shaking, after which the reaction was stopped by the addition of 1 ml of acetone. Blanks were treated as experimentals except that BaP was not added until after the addition of acetone. After all reactions were stopped by th acetone addition, 3.25 ml of hexane was added to each sample, which was agitated with a vortex mixer, then allowed to stand for 10 min. Two ml of the hexane layer was added to 4 ml of 1 N NaOH, mixed, and allowed to stand for 10 min before measuring the fluorescence of the NaOH layer. The fluorimeter (Hitachi-Perkin Elmer MPF2A) was used with excitation and emission slits giving 10 mm bandwidth. The excitation monochrometer was set at 393 nm and emission measured at 520 nm. Fluorescence was measured relative to a standard of 10^{-6} M quinine sulfate in 1 N H_2SO_4, and this intensity converted to concentrations of 3-hydroxybenzo[a]pyrene or equivalent products by means of a previously determined

standard curve. The standard curve was based on a solution of pure 3-hydroxybenzo[a]pyrene in 1 N NaOH, this material being a gift from Dr. H. V. Gelboin. The reaction was linear with respect to time and amount of cellular protein. Specific activities were expressed as pmoles of 3-hydroxybenzo[a]pyrene produced per mg protein during the 30 min incubation period.

RESULTS

Benzo[a]pyrene Cytotoxicity to Low Passage Mouse Epithelial and Fibroblastic Cells

Fig. 1 compares the effect of different concentrations of BaP on the growth of two cell strains in culture. The epithelial cells (NMuLi) were isolated from mouse liver and were passaged 8 times before testing. The whole embryo fibroblasts [Mm 15 WE(A)] were passaged 5 times before testing. As the carcinogen concentration was increased a decrease in the number of cells relative to the control was observed. The concentration of hydrocarbon at which this cytotoxic effect is first observed differed for the two cell systems tested. To compare the sensitivity of a number of cell strains of epithelial and fibroblastic origin, we determined the concentration of carcinogen required to give half-maximal cytotoxicity on each of the strains listed in Table 1. These values are given in column 2 of Table 2 and indicate that the epithelial strains are more sensitive to the carcinogen than the fibroblastic strains. There is a broad distribution of sensitivity to the hydrocarbon. For example, the two mouse ovary strains derived from the same strain of mice vary considerably in their sensitivity to BaP.

A second parameter that can be used to compare the sensitivity of cells to BaP is maximum cytotoxicity. The maximum cytotoxicity values for the cell systems tested are reported in column 3 of Table 2. Again, there is a diverse range of values; however, there is a greater maximum cytotoxic effect on the epithelial cells than on the fibroblasts.

In heterogeneous systems such as those seen in the culturing of whole embryo cells or even cell lines, the maximum sensitivity of a strain can be a function of the ratio of sensitive to resistant cells in the population. If the population does contain resistant cells, or if resistant cells can be induced by BaP, then the population surviving the initial carcinogen treatment should be enriched in resistant cells. To determine if the population was being enriched with resistant cells by BaP treatment, the various epithelial strains were seeded at 1×10^5 cells per 60 mm dish and after 24 h BaP dissolved in DMSO was added to a final concentration of 20 µg/ml of medium. The cells were incubated for 3 days in the carcinogen and the surviving cells on the dishes were washed 3 times with isotonic Tris buffer and overlayed with fresh medium not containing carcinogen. The surviving cells were left to grow to their staturation density. These cells were tested for their sensitivity to BaP in the normal cytotoxicity assay. The concentration of carcinogen required to give half-maximal cytotoxicity and the maximum cytotoxicity for each of the treated strains is reported in the bottom of Table 2. The growth of the two mouse ovary strains surviving the initial treatment with high concentrations of carcinogen is just as sensitive to further BaP treatment as the parent strains. This suggests that their maximum cytotoxicity values reflect a maximum effect on the growth of a homogeneous population, and that pretreatment with BaP

does not enrich for cells resistant to BaP cytotoxicity. The mouse liver cells and the mammary cells surviving the high concentration of BaP all showed an enrichment for resistance to further BaP treatment indicating that the liver and mammary cell strains either contain a population of cells resistant to BaP, or that the BaP treatment induced the formation of resistant cells. We compared the sensitive NMuLi and the resistant NMuLi-BaP and found that this difference in sensitivity to the carcinogen is not simply due to differences in growth potential of the two types of cells since both NMuLi and NMuLi-BaP cells have a doubling time in the absence of carcinogen of 20 $\pm$ 3 h when grown as described in MATERIALS AND METHODS. Furthermore, the resistant and sensitive cells appear to accumulate BaP to the same extent and at roughly the same rates [15]. This suggests that the different sensitivities are due to the processing of BaP once it has entered the cell or the ability of the cells to repair the cytotoxic damage.

Comparison of Low and High Passage Mouse Epithelial Cells

It has been reported that the prolonged cultivation of fibroblasts results in the loss of sensitivity to the cytotoxic action of BaP [16]. We have carried NMuLi in culture for approximately 500 population doublings. During this time we observed no degeneration in the growth rate that is characteristic of fibroblasts when they become established cell lines. The concentration of BaP required to inhibit growth of the high passage cells by one-half was 0.04 µg/ml or a fourfold increase in comparison to the value obtained with low passage cells (Table 2). The maximum cytotoxicity of BaP on NMuLi decreased from 98% after 8 passages or 16 population doublings to 80% after 500 population doublings. As seen in Fig. 2,

these high passage cells have become morphologically heterogeneous with an increase in fibroblastic cells. When low and high passage NMuLi cells were cloned and the clones compared for their sensitivity to BaP, the clones from low passage cells were all as sensitive to the carcinogen as the parent, but the clones from high passage NMuLi showed a considerable variation in sensitivity to the carcinogen. Some of these clones were as sensitive as low passage cells. The ability to clone these high passage cells allowed us to recover a population of cells highly sensitive to BaP.

Induction of Aryl Hydrocarbon Hydroxylase

Numerous workers [4,7,17] have shown that the sensitivity of cells to cytotoxicity of BaP is related to the ability of the cells to metabolize the hydrocarbon to more oxidized derivatives. The metabolic conversion is carried out by an enzyme induced by the hydrocarbon called aryl hydrocarbon hydroxylase [18]. Gelboin, Huberman and Sachs [4] reported that there is a correlation between the level of the enzyme and the susceptibility of the cells to cytotoxicity.

To test whether this correlation is found in our systems, we have assayed the level of hydroxylase after 24 h induction with various concentrations of BaP. Fig. 3 compares the induction by BaP of the enzyme in NMuLi epithelial cells with the induction in Mm 15 WE(A) fibroblasts. Hydroxylase activity is significantly induced in NMuLi after 24 h of treatment with concentrations of BaP as low as 0.1 μg/ml. The enzyme in Mm 15 WE(A) embryo cells is induced at levels of BaP starting at 0.05 μg/ml. These fibroblasts are not only induced maximally to a higher specific activity than the epithelial cells, but also this maximum induction occurs at a lower concentration of BaP (Fig. 3). At concentrations of carcinogen

above 0.50 µg/ml the induction of the fibroblasts falls off until at 10 µg/ml the specific activity is only half that of the epithelial cells. A similar decrease in specific activity of the hydroxylase at high carcinogen concentrations was seen by Nebert and Gelboin [19] using benz[a]anthracene or 7,12-dimethylbenz[a]anthracene to induce secondary hamster embryo cells. The degree of induction for the Mm 15 WE(A) is only fivefold in comparison to a 220-fold induction of NMuLi. Thus, the epithelial strain which is more sensitive to BaP cytotoxicity has a lower basal and induced hydroxylase activity than the fibroblast, but a much higher degree of induction.

Table 3 lists the results of our induction studies on the various epithelial and fibroblastic cells. There is no simple relationship between the level of uninduced or induced enzyme and cell type. In general, the degree of induction for the epithelial cells is higher than for the fibroblastic cells.

As described above, epithelial cells can be rescued from a 3-day treatment with high concentrations of BaP. We tested the cells which survived this treatment for their uninduced and induced hydroxylase activities. These experiments were done after the rescued cells had been cultured free of carcinogen for a number of generations. The data in the bottom of Table 3 indicate that when the pretreatment with BaP resulted in the enrichment for resistant cells (see Table 2), these populations have similar uninduced but lower induced levels of the hydroxylase than the parent. The concentration of carcinogen required for maximal induction in these resistant cells is also lower. However, when the BaP pretreatment did not alter the sensitivity of cells to BaP cytotoxicity, there was little change in the induction parameters. Thus, it appears that the resistance to the

cytotoxicity is paralleled by a decreased inducibility of the hydroxylase. However, the specific activity of the hydroxylase in these resistant cells is often still higher than that seen in some of the sensitive epithelial cells. It is unlikely that these resistant cells are selections of fibroblast cells in the original culture since they continue to grow with the clonal morphology characteristic of epithelial cells.

The loss of inducibility of the hydroxylase is also observed as the population becomes more resistant to BaP by continued passage. The NMuLi cells which after 58 passages showed a fourfold increase in the amount of carcinogen required to give half-maximal cytotoxicity also had a maximally induced specific activity of only one-fourth that seen for the low passage cells.

Inhibition of Cytotoxicity by 7,8-Benzoflavone

Gelboin et al. [20] have shown that 7,8-benzoflavone inhibits the aryl hydrocarbon hydroxylase. DiPaolo et al. [8] have used this inhibitor to demonstrate the role of the hydroxylase in cytotoxicity of BaP to Syrian hamster cells. We tested the effect of 7,8-benzoflavone on the cytotoxicity of BaP to NMuLi. 7,8-Benzoflavone at a concentration that by itself did not affect the growth of NMuLi inhibited the cytotoxicity of BaP to a level one-fifth of that seen with BaP alone. This suggests that the function of the aryl hydrocarbon hydroxylase is required for the cytotoxicity with BaP.

DISCUSSION

One of the difficulties of extrapolating information gained in the study of transformation of cells grown in culture to the onset of malignancies in the whole animal is that until recently cells in culture were

either undifferentiated embryo cells, cell lines of fibroblastic nature, or malignant cells. It is clear that in vivo most malignancies arise in highly differentiated epithelial cells [1]. Therefore, it seems likely that the cellular functions necessary for the oncogenic action of viruses, chemicals, and radiation would more likely be found in such epithelial cells. We have described in this report the characterization of the response of a number of epithelial cell strains obtained from Robert Owens [2] to the chemical carcinogen, BaP. A detailed investigation of one mouse liver strain (NMuLi) indicated that unlike fibroblasts the sensitivity to cytotoxicity by BaP is only slightly affected by long term culturing. By cloning this strain we can maintain a population highly sensitive to BaP cytotoxicity. We have selected for variants of NMuLi, Mm 15 Li, and Mm 5 MT/c1, which are resistant to the cytotoxicity of BaP. These variants have identical clonal morphology and growth properties as their parents and do not give rise to malignant tumors when tested in newborn isogeneic mice (unpublished results).

The level of aryl hydrocarbon hydroxylase in most of these uninduced epithelial cell strains is very low and showed good induction after 24 h in the presence of BaP. This induction is dependent on the concentration of BaP in the medium, and is significant at levels as low as 0.1 μg BaP/ml (4×10^{-7} M). The maximum induction seen with these strains was 220 fold for NMuLi. This value compares favorably with an approximately 30 fold induction seen by Whitlock and Gelboin [21] using benz[a]anthracene on Buffalo rat liver epithelial cells. The maximum induced specific activity of hydroxylase was 261 pmoles 3 HO-BaP/mg protein/30 min for Mm 160V as compared with values of greater than 6,000 reported for mouse livers induced in vivo with 3-methylcholanthrene [5].

The coefficients of linear correlation between parameters describing the cytotoxic effect (level of carcinogen to give half-maximal cytotoxicity and maximum cytotoxicity) and those describing enzyme activity (basal activity, induced activity, and fold induction), show a moderate positive correlation between enzyme inducibility and cytotoxicity. These correlation coefficients increase if the correlation is calculated over a more closely related group of cell strains (e.g., epithelial cells only, or NMuLi derivatives only). This confirms the correlation observed by Gelboin et al. [4], and suggests that sources of the remaining variation are other characteristics of the individual strains, which remain unidentified. For example, transport and further metabolism of the products must play a role in determining the response of a cell to the carcinogen.

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Table 1. Cell strains tested

Cell strain	Tissue of origin	Passage level at time of testing***	Laboratory of origin
Epithelial			
Mm 140V	Ovary of a HRS/J Mouse	9	Owen, NBRL*
Mm 160V	Ovary of a HRS/J Mouse	10	" "
NMuLi	Liver of a Namru Mouse	8	" "
Mm 15Li	Liver of a HRS/J Mouse	9	" "
Mm 5 MT/c1	Mammary Carcinoma of a C3H Mouse	23	" "
Fibroblastic			
Mm 15 SP+Th	Spleen and Thumus of a HRS/J Mouse	5	" "
Mm 15 WE(A)	Whole Embryo of HRS/J Mouse	5	" "
3T3 c1 4A	Whole Embryo of a Swiss Mouse	-	Holley, Salk Inst.**
SV3T3 c1 56	SV40 Virus Transformed Swiss 3T3	-	Dulbecco, " "
Balb 3T3 c1 A31	Whole Embryo of a Balb/c Mouse	-	Smith, NBRL

*Naval Biomedical Research Laboratory, Oakland, California.

**The Salk Institute, San Diego, California.

***Each passage represents approximately one population doubling.

Table 2. Benzo[a]pyrene toxicity of different cells

Cell strain	µg/ml BaP* in Growth Medium for Half-Maximal Cytotoxicity	Maximum Cytotoxicity (%)
Epithelial		
Mm 140V	0.22	78
Mm 160V	0.04	82
NMuLi	0.01	98
Mm 15 Li	0.07	81
Mm 5 MT/c1	0.01	46
Fibroblastic		
Mm 15 WE(A)	0.02	22
3T3 c1 4A	0.50	42
SV3T3 c1 56	No effect	0
Balb 3T3 c1 A31	No effect	0
Epithelial - recovered from treatment with 20 µg/ml BaP* (see text)		
Mm 140V-BaP	0.13	82
Mm 160V-BaP	0.04	80
NMuLi-BaP	Slight toxicity	36
Mm 15 Li-BaP	No effect	0
Mm 5 MT/c1-BaP	No effect	0

The maximum cytotoxicity is the highest % decrease in cell number relative to a control seen after 3 days' treatment with carcinogen. The half-maximum cytotoxicity values are the concentration of carcinogen required to give 50% of the maximum cytotoxicity in this 3-day assay.

*BaP refers to benzo[a]pyrene.

Table 3. Induction of aryl hydrocarbon hydroxylase in different cell systems

Cell Strain	Specific Activity pmoles 3 HO-BaP/mg Protein/30 min Basal	Induced*	Degree of Induction
Epithelial			
Mm 140V	1.6	17.0 (10)	10
Mm 160V	15.0	261.0 (10)	17
NMuLi	0.2	43.0 (1)	220
Mm 15 Li	0.6	18.0 (10)	30
Mm 5 MT/cl	1.3	118.0 (10)	90
Fibroblastic			
Mm 15 WE(A)	24.0	125.0 (0.5)	5
3T3 cl 4A	5.8	61.0 (10)	10
SV3T3 cl 56	0.4	13.0 (1)	32
Balb 3T3 cl A31	3.2	4.6 (0.001)	1
Epithelials - recovered from treatment with 20 g/ml BaP (see text)			
Mm 140V-BaP	1.1	8.0 (5)	7
Mm 160V-BaP	12.4	99.0 (5)	8
NMuLi-BaP	2.2	30.0 (0.5)	14
Mm 15 Li-BaP	0.4	6.0 (0.5)	15
Mm 5 MT/cl-BaP	0	8.0 (10)	-

The specific activity of the hydroxylase was determined on the whole cell sonicate after 24 h of induction with benzo[a]pyrene [3].

*The numbers in parentheses are the concentrations in μg/ml of benzo[a]-pyrene at which maximal induction occurred.

FIGURE CAPTIONS

Fig. 1. Abscissa: Benzo[a]pyrene concentration in µg/ml of growth medium; Ordinate: % cells relative to control remaining after 3 days' treatment with the carcinogen. (o——o) Mm 15 WE(A); whole embryo fibroblasts. (●——●) NMuLi; mouse liver epithelials. Cytotoxocity of benzo[a]pyrene to low passage mouse cells.

Fig. 2. (A) NMuLi passage level 58. (B) NMuLi passage level 8. Accumulation of cells with fibroblastic morphology after long term culturing of NMuLi.

Fig. 3. Abscissa: Benzo[a]pyrene concentration in µg/ml of growth medium; Ordinate: specific activity of aryl hydrocarbon hydroxylase (pmoles 3 HO-BaP/mg Protein/30 min). (o——o) Mm 15 WE(A); whole embryo fibroblasts. (●——●) NMuLi; mouse liver epithelials. Induction of aryl hydrocarbon hydroxylase by benzo[a]pyrene in low passage mouse cells.

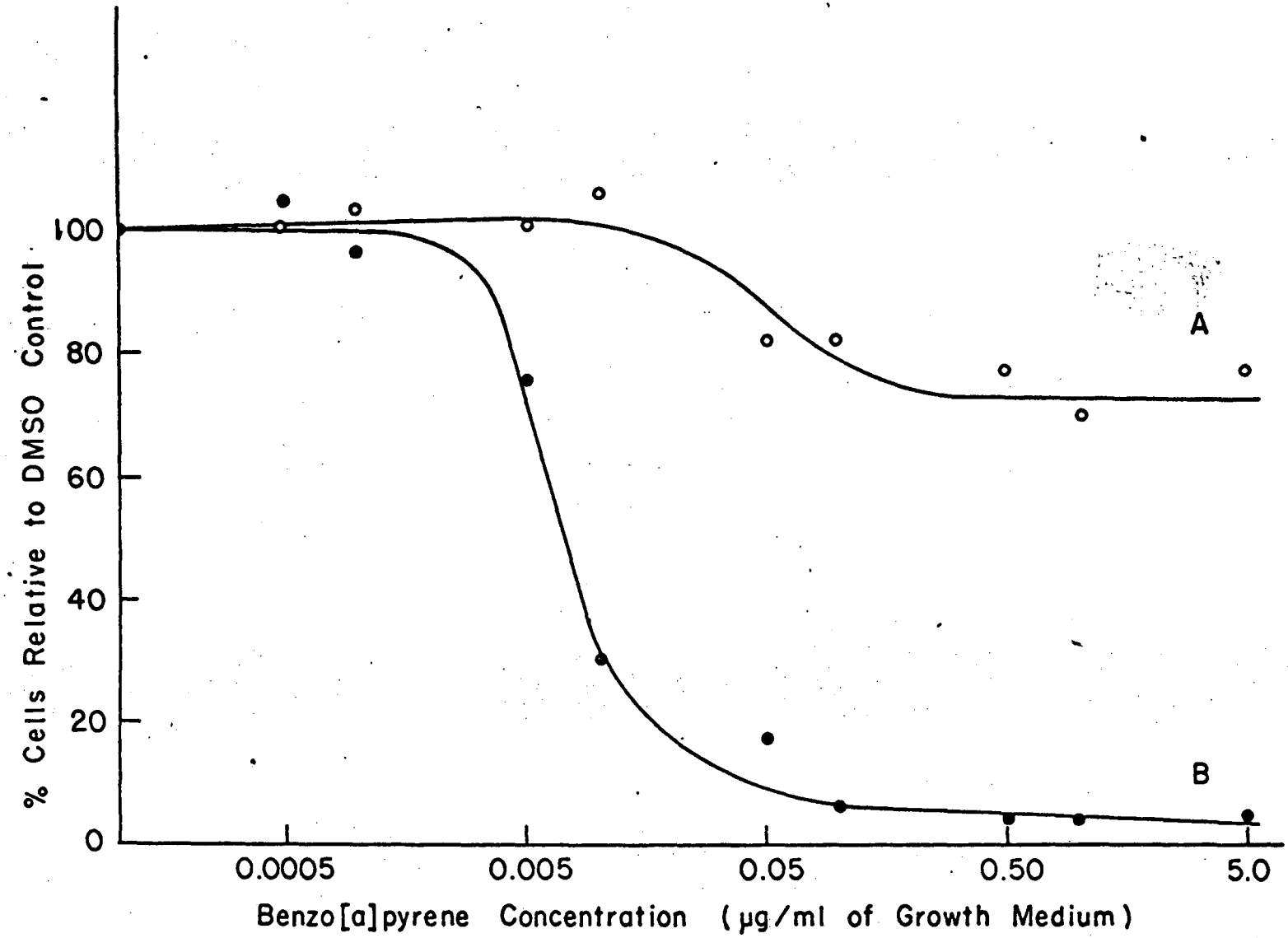

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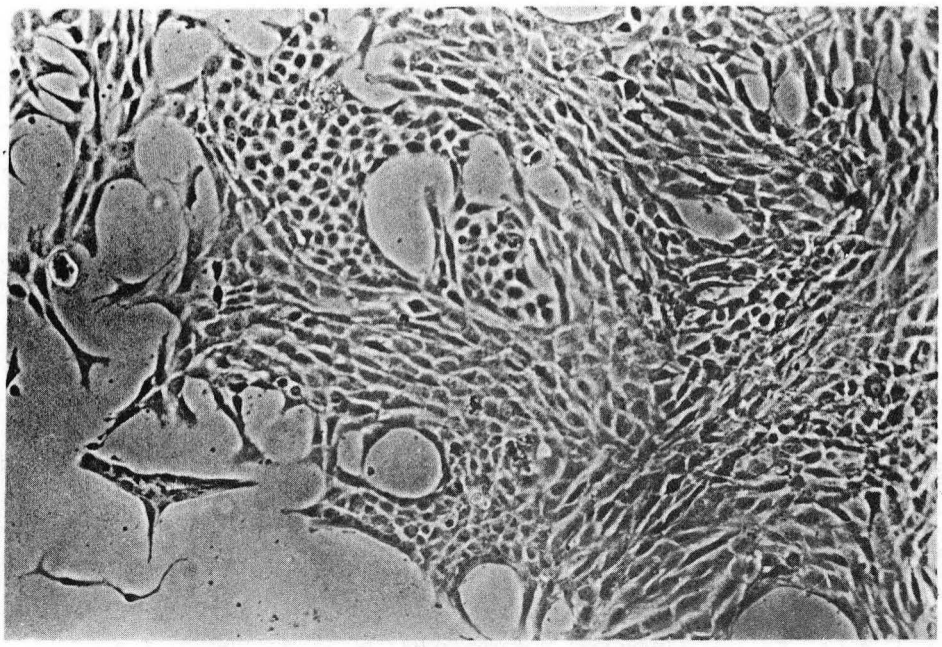
Fig. 1.



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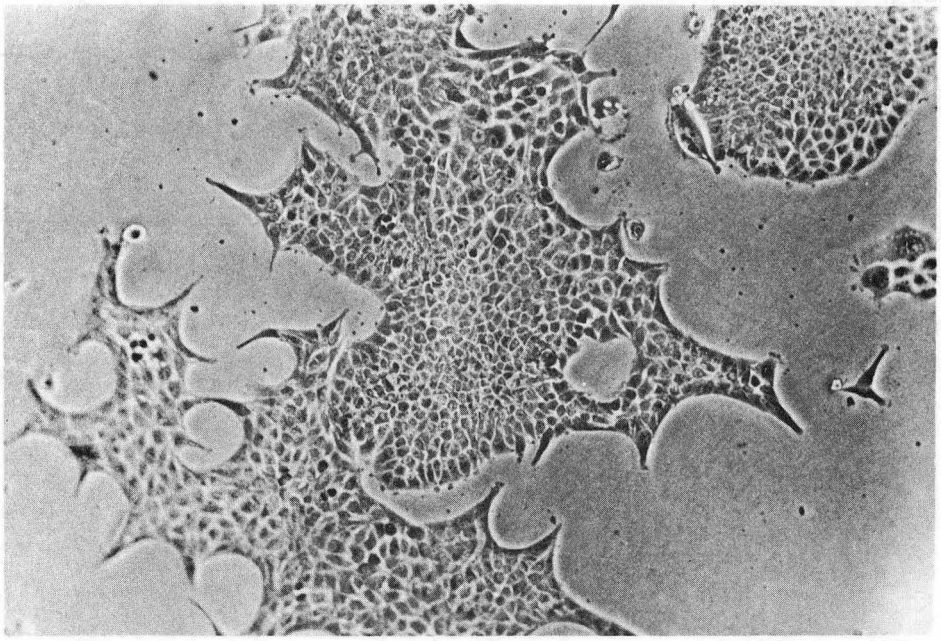

XBB 745-3353

(B)

Fig. 2.

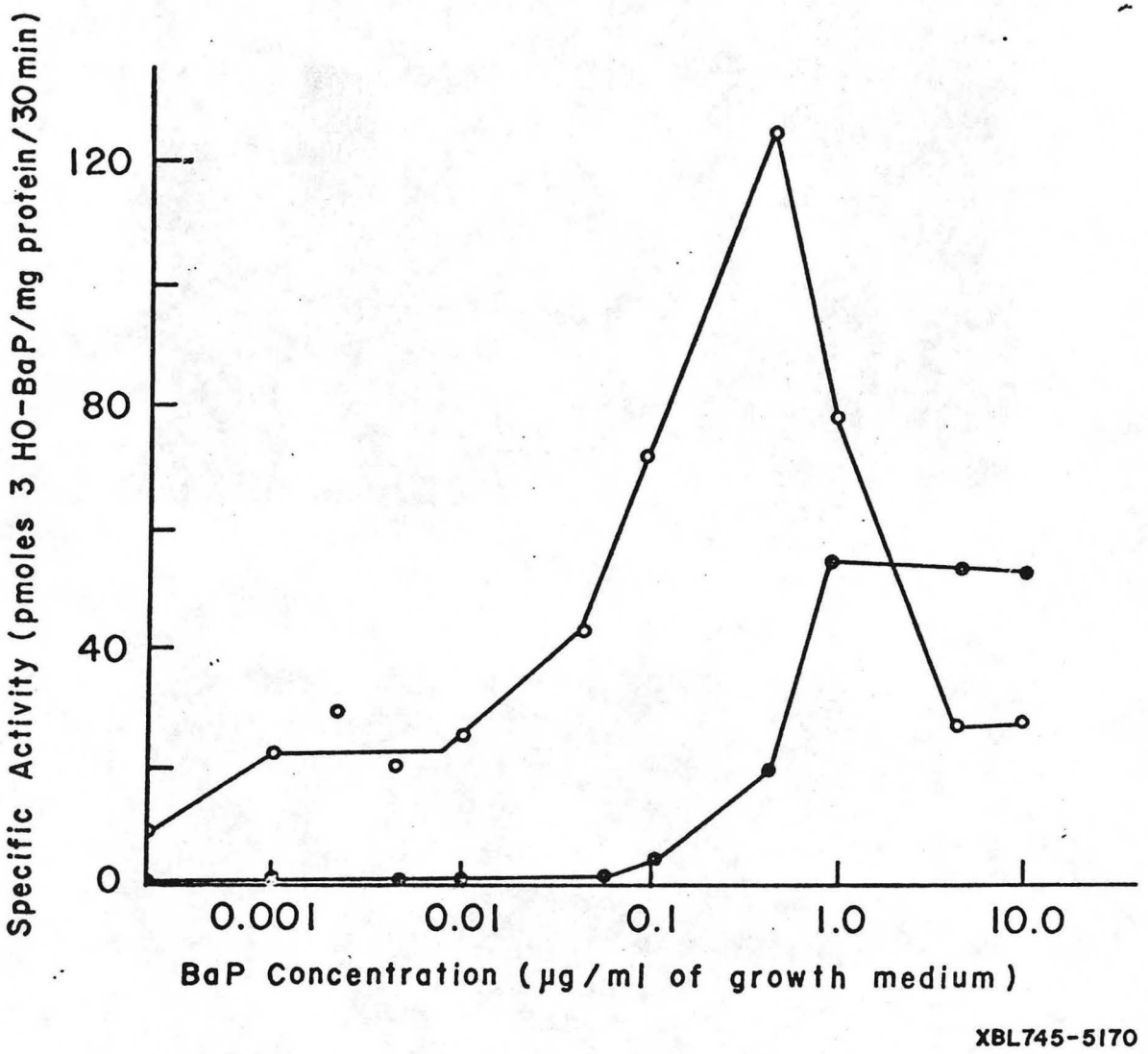
Specific Activity (pmoles 3 HO-BaP/mg protein/30 min)
120
80
40
0
0.001
0.01
0.1
1.0
10.0
BaP Concentration (µg/ml of growth medium)
XBL745-5170

Fig. 3.

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