IN VITRO TRACER STUDY OF A CHEMICAL CARCINOGEN

A Thesis

Submitted to

The Division of Biological Sciences

Emporia State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Noboru Nakamichi

August 1980

AN ABSTRACT OF THE THESIS OF

Noboru Nakamichi for the Master of Science

in Biology presented on August 5, 1980

Title: In Vitro Tracer Study of a Chemical Carcinogen

Abstract approved: Gaylen Rempel for H. M. LeFever

A tracer study of a chemical carcinogen, benzo(a)pyrene, was conducted using mouse embryo cells in culture. In addition, this study challenged the credibility of autoradiography techniques to visualize the chemical carcinogen in the cell.

Autoradiographic results showed that benzo(a)pyrene was accumulated in cytoplasm and the nucleus at maximum levels after 24 hours of incubation. Chromosome spreads from benzo(a)pyrene treated cells were prepared to investigate the possibility of benzo(a)pyrene-chromosome interaction. Autoradiographic results indicated that benzo(a)pyrene molecules reacted with chromosomes.

Hayden Mayfield for H. M. LeFever
Approved for Major Department

Jared E. Hurst
Approved for Graduate Council

ACKNOWLEDGEMENT

I would like to express my deepest appreciation to Dr. Michael LeFever for his guidance and constructive help during this research. Furthermore, I would like to thank him for his patience and continuous help during the time required for the completion of the investigation.

Sincere appreciation is also extended to my committee members, including Dr. Gaylen Neufeld and Dr. Helen McElree.

Deep appreciation goes to Mr. Mel Fuqua, my friend and colleague who was of great help in the preparation of this thesis.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

All living organisms, including human beings, are exposed to both external and internal environmental factors (see diagram on page 3). A close study of these environmental factors in relationship to cancer led investigators to conclude that many human cancers have chemicals as their causative agents (Boyland and Sims, 1965; Van Cantfort et al., 1975; Hecht et al., 1974; Henderson et al., 1975). Although the existence of cancer has been known for many years, intensive investigations into its origins were not conducted until the 19th century.

A Japanese research group performed one of the initial studies in cancer research. They repeatedly applied coal tar to rabbit ears for 100-580 days and a consequent development of skin cancer was observed (Yamagiwa and Ichikawa, 1918).

This study stimulated many investigators to search for cancer causing chemicals. Among the cancer causing chemicals found, benzo(a)pyrene was isolated as well as with other chemical constituents from coal tar (Cook et al., 1932, 1933). Benzo(a)pyrene has also been isolated from numerous materials found in environment (Table 1; Hirono et al., 1970, 1972, 1973; Pamukcu et al., 1968; Falk et al., 1958; Evans and Mason, 1965).

Recent investigations have classified chemical carcinogens by chemical structures such as, a) carcinogenic aromatic hydrocarbons (see diagram on page 3), b) carcinogenic aromatic amines, c) carcinogenic compounds (amino azo dyes, nitroso compounds, alkylating agents), and d) inorganic carcinogens (Weisburger, 1977).

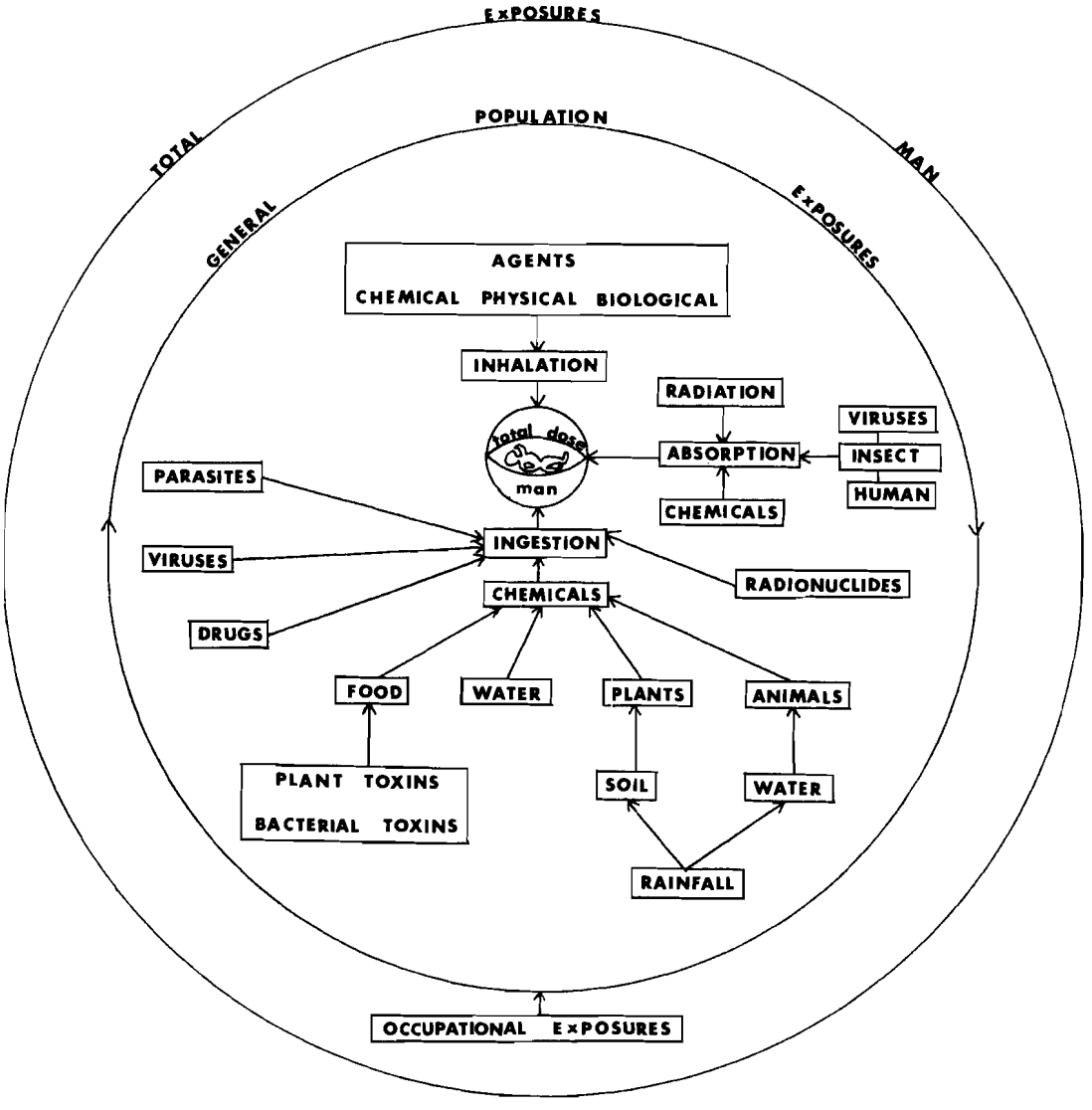
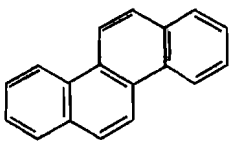


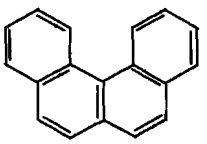
Figure 1. Human exposure to hazardous environmental agents. (Kraybill, 1977)

Disputed
(33% or less)*



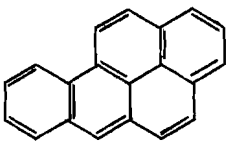
Chrysene

Moderate
(33 - 66%)

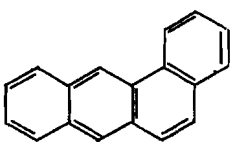


Benzo(c)phenanthrene

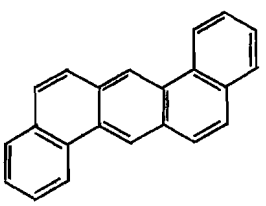
High activity
(66% or higher)



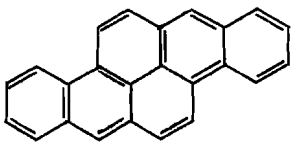
Benzo(a)pyrene



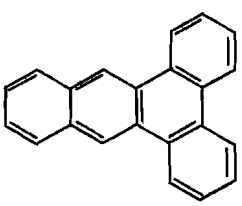
Benz(a)anthracene



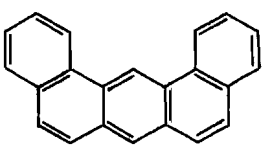
Dibenz(a,h)anthracene



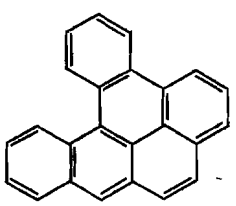
Dibenzo(a,h)pyrene



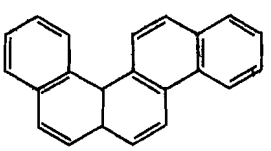
Dibenz(a,c)anthracene(1,2:3,4-Dibenzonthracene)



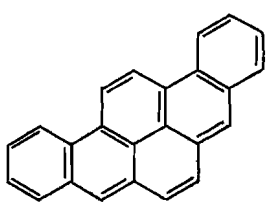
Dibenz-(a,j)anthracene



Dibenzo(a,l)pyrene



Benzo(c)chrysene-(1,2:5,6-dibenzphenanthrene)



Dibenzo(a,i)pyrene

Table 1. Polycyclic aromatic hydrocarbons in food.

	Benzo(a) pyrene (ug/Kg)	Benz(a) anthracene (ug/Kg)	
Charcoal broiled meat	8	4.5	Lijinsky and Shubik, 1964
Smoked fish	12-37	91-189	Masuda and Kuratune, 1971
Singed sheep head:			
Coal, raw	21	15	Thorsteinsson and
Coal, cooked	9	5	Thordarson, 1968
Singed sea bird:			
Coal, raw	99	69	
Coal, cooked	96	50	
Smoked food:			
Mutton	1.3	--	Bailey and Dungal,
Trout	2.1	--	1958
Cod	0.5	--	
Red Fish	0.3	--	
Barbecued ribs	10.5	3.6	Lijinsky and Shubik, 1965
Coffee	4	--	Kuratsune and Hueper, 1960

Researchers' attention up to this point was devoted to isolating chemical carcinogens from our environment and to characterizing the chemical carcinogens. Some researchers, Allison and Mallucci (1964); Shires et al. (1966); Brooks and Lawley, (1964); Wang et al. (1972), started to investigate the mechanisms involved in chemical carcinogenesis.

Allison and Mallucci (1964) utilized fluorescence microscopy, phase contrast microscopy and autoradiography techniques to locate carcinogenic hydrocarbons in mammalian cell cultures. These investigators concluded that the hydrocarbon carcinogens were not concentrated in either mitochondria or the nuclei. They were, however, found to be within lysosomes. Allison and Paton (1965), Allison and Dingle (1966), and Allison (1968) centered their studies on lysosomes and their relationship to cancer.

A study similar to the one mentioned above was carried out two years later by Shires and Richter (1966). These workers exposed mammalian cells in vitro to carcinogenic hydrocarbons for periods ranging from 12 hours to 4 days. The treatment was terminated at different intervals for microscopic observations. The researchers were unable to detect any incorporation of benzo(a)pyrene into the nuclei or chromosomes during a complete generation cycle.

On the other hand, Brooks and Lawley (1964) tried to show the binding of carcinogenic hydrocarbons to the nucleic acids in mouse skin. They did so by applying the hydrocarbon carcinogens to shaved regions on the backs of mice. The mice were sacrificed at various times, and the treated areas of skin were removed and used for deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) isolation. This study indicated that

DNA reacts with the hydrocarbon carcinogens tested. Experimental results did not indicate how the hydrocarbon carcinogens are bound to the nucleic acids.

Lerman (1960) investigated the way in which polynuclear hydrocarbons react with DNA. He used mutagenic acridine dye derivatives for his study. This investigation showed that the dyes are intercalating into the DNA helix through extension of DNA backbone. This information enhanced the idea that polycyclic aromatic hydrocarbons require structural planality for the carcinogenic potency. A similar mechanism of the intercalation reaction between hydrocarbon carcinogens and DNA was proposed by Liquori (1962). Liquori's findings were based upon the increased solubility of benzo(a)pyrene in DNA aqueous solutions. This is significant because benzo(a)pyrene is virtually insoluble in water.

Boyland and Green (1964) expressed a slightly different opinion. They showed that carcinogenic hydrocarbon molecules react with DNA by slipping into the sites where the DNA is already stretched. Such stretching of DNA can only occur during DNA replication or RNA transcription. An expanded idea was postulated by Arcos and Argus (1968). They indicated that the solubilization of carcinogenic hydrocarbons by DNA can be accomplished by either intercalation or through external binding mechanisms (Figure 2.).

Van Duuren et al. (1969) suggested a reconsideration of the intercalation mechanism. This was proposed because a) solubilization of detergent micelles of sodium lauryl sulfate and aqueous DNA solution caused the same results which were found with hydrocarbon carcinogens

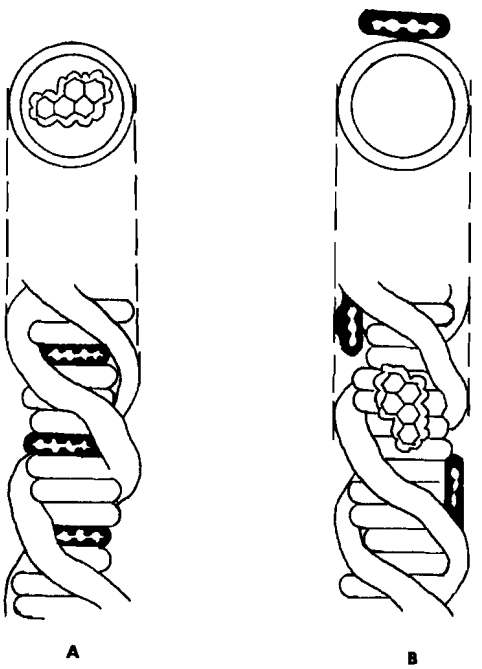


Figure 2. Noncovalent interactions of polycyclic aromatic hydrocarbons (illustrated with 3,4-benzpyrene) with DNA. A. Intercalation into DNA double helix. B. External binding to DNA. (From Arcos and Argus, 1968.)

and aqueous DNA solution, and b) potent carcinogens such as 7,12-dimethylbenz(a)anthracene, and 3-methylcholanthrene, are thicker than the distance between adjacent base pairs in DNA.

Because previous investigations had been unable to prove whether or not particular parent carcinogenic hydrocarbon molecules react with DNA, research interest was directed to the isolation and characterization of intermediate metabolites. Benzo(a)pyrene-4,5-oxide (K-region epoxide) proved to be one of the highly reactive metabolites of benzo(a)pyrene (Wang et al., 1972; Selkirk et al., 1971). Another metabolite, 7,8-dihydro-7,8-dihydroxybenzo(a)pyrene-9,10-oxide has been investigated for its metabolism and mutagenicity. It was confirmed to be carcinogenic (Neubold and Brooks, 1976; Sims et al., 1974).

Thus far, accumulated data favor the theory of hydrocarbon carcinogens binding to DNA. On the other hand, the significance of lysosomes in regard to carcinogenesis cannot be ignored. Both Allison and Mallucci (1964) and Shires and Richter (1966) utilized fluorescence and phase microscopies and concluded that lysosomes were somehow involved. In addition to optical systems, Allison and Mallucci (1964) used autoradiography techniques for localization of hydrocarbon carcinogens in mammalian cell cultures. They concluded that the amount present, if any, in cellular organelles other than lysosomes is too small to detect by these methods.

It was the purpose of this investigation to challenge the credibility of autoradiographic techniques as an indicator of benzo(a)pyrene-DNA incorporation, and to consider the problems that were encountered with this technique.

MATERIALS AND METHODS

Experimental animals

Heterogenic white mice were obtained from the Emporia State University research animal facility and from the Mid-Continent Research Company (Kansas City, Kansas). They were maintained in the animal facility at Emporia State University.

Chemicals

Benzo(a)pyrene (B(a)P), obtained from Sigma Chemical Company, was first dissolved in acetone (10 mg/ml) and then dispersed into complete growth medium with a Pyrex disposable syringe to give a concentration of 100 ug/ml of medium (Berwald and Sachs, 1965). This B(a)P suspension was used as stock solution from which dilutions were made. Dilutions were 0.1 ug, 10 ug and 50 ug B(a)P per milliliter of medium. The stock solution was stored at 0°C. The desired concentrations of B(a)P solution were applied to cells in culture.

Tissue culture medium

Dulbecco's modified Eagle's medium containing 4.5 g/l glucose was purchased from GIBCO. The growth medium was supplemented with 10% fetal bovine serum (GIBCO) and 0.5 ml combiotics. This complete growth medium was used to grow and maintain the cells *in vitro*. GKN (calcium and magnesium free saline) was used for cell harvest and cell transfer with 0.25% trypsin and trypsin-versene (0.002%-0.05%) solutions, respectively.

Stock combiotic solutions were prepared by combing penicillin "G" sodium (1650 U/mg) and streptomycin sulfate (740 mg/gm) in 1X Hank's BSS. Stock solutions were sterilized using Millipore filters. One milliliter aliquots were prepared and stored frozen until needed. Cell

harvesting solutions were prepared by adding 0.5 ml of the combiotic to every 100 ml of GKN. Likewise, cell maintenance solutions were prepared by adding 0.5 ml of combiotic stock solution to every 100 ml of growth medium.

Fetal bovine serum, which was mycoplasma tested and virus screened, was obtained from GIBCO and used throughout this study.

Carbon dioxide gas, prepared by mixing calcium carbonate and sulfuric acid, was used to maintain the desired pH of the medium.

Glassware

Glassware was cleaned by soaking in an acid bath (1:7 mixture of concentrated hydrochloric acid and concentrated sulfuric acid) overnight, followed by washing with Alconox solution and repetitive rinsing with tap water. All glassware was rinsed with deionized distilled water after being washed.

Microscope coverslips (22 X 50 mm) were used to make cell growing coverslips (22 X 8 mm). These were immersed in an Alconox solution and rinsed with running tap water for 10-13 hours. They were rinsed individually 10 times in three consecutive deionized distilled water baths and one 100% ethanol bath, then stored in an absolute ethanol bath until use.

Cell culture

Minced whole embryos of heterogenic white mice served as the source of normal cells. Pregnant mice (15-18 days in term) were sacrificed by cervical dislocation. Embryos were then removed aseptically from the mouse and transferred to a petri dish containing GKN-combiotics mixture. Embryos were minced and placed into a trypsinizing flask. The tissue

chunks were subjected to gentle stirring while being trypsinized at room temperature for 30 minutes. Trypsinized tissue was filtered through four layers of cheese cloth. The filtrate was washed with one change of GKN and one change of complete growth medium. Cell populations were adjusted with complete growth medium to a concentration of 3×10^6 cells/ml. Three milliliter aliquots were planted in 25 cm^2 polystyrene culture flasks and incubated at 37°C for one to three days (primary cell culture). Primary cell cultures were treated with trypsin-versene solution for two minutes, washed, centrifuged and resuspended to give a concentration of 6×10^5 cells/ml. They were inoculated in test tubes containing coverslips (7 X 22 mm) for 24 hours at 37°C (secondary culture) prior to usage in the studies. Secondary cell cultures were treated with either a colloidal suspension of B(a)P (10 ug/ml) and ^{3}H-B(a)P (10 uCi/ml), or ^{3}H-B(a)P (10 uCi/ml) alone.

Autoradiography

Tritium-labeled B(a)P treated cells growing on the coverslips were removed from the test tubes, rinsed with two changes of GKN solution and fixed in an absolute methanol bath for five minutes. The coverslips were mounted on a slide with the cell side up, using Pro-texx mounting medium. The slides were dried overnight on a heating plate at 37°C.

Ilford emulsion (L-4, Polysciences, Inc.) was melted in a 40°C water bath. Ten milliliters of melted emulsion were diluted with an equal volume of deionized distilled water, and any bubbles resulting in the mixing operation were removed through a layer of cheese cloth. The slides were dipped into the diluted emulsion and immediately removed. Residual emulsion was cleaned from the back of the slides, and the

slides held in a vertical position for 15 minutes to allow the emulsion to dry. Slides were sealed in a light tight slide box by wrapping the box with aluminum foil. The slides were exposed for 10 days at 0°C.

The exposed slides were developed in Dektol (D-17) for two minutes, rinsed in a deionized distilled water stop bath, and fixed in acid fixer for three minutes. After washing for 15 minutes in slowly running tap water, the slides were dried and stained for four minutes in 0.05% basic fuchsin solution. The slides were dehydrated in a series of ethanol baths and immersed in a mixture of cedar wood oil and absolute ethanol (1 : 1) for one hour followed by infiltration of a Pro-texx mounting medium and xylene mixture (1 : 1) for one hour.

The slides were examined for the appearance of silver grains (black dots) located within the cells, thereby indicating B(a)P uptake by the cell.

Cell viability test

The toxic effect of B(a)P on cultured cells was examined by adding 5 ml of B(a)P at different concentrations. Concentrations used ranged from 0.1 to 50 ug B(a)P per milliliter of growth medium. At regular intervals, B(a)P-growth medium mixtures were collected from culture flasks and centrifuged for five minutes at 800 r.p.m. The supernatant was siphoned off and discarded. Pellets were resuspended with 1 ml growth medium, and 0.1 ml of erythrosin (0.4%) was added and stained for two minutes. Dead cells, staining pink, were counted on a hemocytometer. Numbers of dead cells were plotted against the durations of B(a)P treatment.

Inoculation of B(a)P treated cells into animals

Cells treated with B(a)P were harvested with a trypsin-versene solution for three minutes. Cell harvest was made at preselected intervals of time. The cell population was adjusted to 10 X 10^6 cells/ml with complete growth medium. From this solution, 0.2 ml were injected into a group of five male mice. Injections were made subcutaneously on the left thigh (modified from Evans et al., 1957). Inoculation sites were checked every 14 days for tumor development until termination of the experiment.

Histological study

Twenty-eight days after inoculation of B(a)P treated cells, mice which developed tumors were sacrificed for histological studies. The tumor tissue was excised and fixed overnight in Bouin's fixative. Several solution changes of 70% ethanol saturated with lithium carbonate were made to eliminate any fixative in the tissue. Dehydration of fixed tissue was accomplished in a series of ethanol baths of one hour each. The tissue was then immersed for one hour in a mixture of absolute ethanol and xylene (1 : 1). It was soaked in a xylene bath overnight and then transferred to a mixture of xylene and paraffin (56° tissuemat) for 15 minutes. Infiltration of hot tissuemat was carried out in four changes of tissuemat solution. Tissue was then embedded in a plastic ice cube tray with hot tissuemat and cooled to room temperature. The blocks were sectioned on the microtome at a thickness of seven microns. The sections were stained by standard hematoxylin and eosin procedures. The slides were then carefully examined for histological abnormalities.

Chromosome preparation

Cells in culture were mitotically blocked by lyophilized colcemid, 0.06 ug/ml, (GIBCO) for two hours. Harvesting of cells was accomplished by trypsinization and centrifuging for five minutes at 800 r.p.m. The pellet was resuspended in 1 ml of complete growth medium with a Pasteur pipette. Hypotonic solution (3 ml deionized distilled water) was added to this suspension for seven minutes at room temperature. One milliliter of freshly prepared methanol-acetic acid (3 : 1) fixative was introduced to the hypotonic solution for 10 minutes. The suspension was centrifuged and fixed by repeated dispersion at 10 minute intervals in three changes of 3:1 methanol-acetic acid fixative. During these fixation periods, microscope slides were washed in an Alconox solution and stored in deionized distilled water at 4°C. After the third fixation, the pellet was resuspended in 0.3-1 ml (depending on size of the pellet) fixative solution. Several drops of the final cell suspension were applied to the slides. The backs of the slides were wiped dry with tissue paper. A film of water on the upper surface of the slides was left to facilitate spreading of the cells. The slides were air-dried and stained for 15 minutes in modified Giemsa stain. Modification was made according to Gude, et al., (1955). For the autoradiographic study, the slides were stained for 15 minutes after the completion of photographic processes.

RESULTS

Cell viability test

The toxic effect of B(a)P was tested for nine days. Results are summarized in Figure 3. From the data, linear regression curves were prepared for each concentration. The slope of the untreated cell line is 4.8. The slopes of the B(a)P treated cell lines are 6.5 for 0.1 ug B(a)P treated cells, 7.0 for 10 ug B(a)P treated cells, and 7.6 for 50 ug B(a)P treated cells. A statistical analysis did not indicate a significant difference between the normal cell line and the B(a)P treated cell lines. Re-examination of the data used in Figure 3 will help in understanding effect of treatment on cell viability for specific periods of time. In Figure 4, one and three day incubation periods showed a smaller rate of increase (slope for one day: 0.07; and slope for three days: 0.16) which implies that the B(a)P concentration did not have a significant effect in terms of cell killing ability. The dead cell numbers in five day B(a)P treated cultures presented about 2.8-fold increase from that of three day B(a)P treated cultures. A statistical analysis showed a significant difference indicating that the critical factor in cell killing ability of B(a)P is dependent upon the exposure period.

Cell morphology in culture

Mouse embryo cells in culture were observed under the light microscope for their morphology. Embryonic cells were round immediately after implantation into culture flasks. Approximately three hours incubation at 37°C after initial cell plantation, some cells started to establish their natural morphological appearance (elongation of cytoplasm). The

elongation process was an indication of cell attachment on the surface of culture flasks. Owens et al. (1974) reported that in earlier days of incubation, two cell types, epithelial cells and fibroblast cells, can be identified. Epithelial cells from monolayered sheets which are attached to each other (Figures 5 and 9). Vasiliev and Gelhand (1977) reported that fibroblast cells form several morphological varieties in culture; monolayers of mutually oriented cells which are not firmly attached to each other (Figures 6 and 8), multilayered sheets (Figure 7), or spherical aggregates (Figure 7 lower right and upper left darkly stained aggregates). Fibroblasts and/or fibroblast-like cells became predominant throughout the entire cell population when cells were cultured for over 30 days (Figures 10, 11 and 12). These fibroblast cells are characterized by elongated or stellate cytoplasmic structures (Figures 10, 11 and 12).

Mouse embryo cells were treated with 10 ug B(a)P for predesignated exposure periods to observe an effect on cell morphology. Some B(a)P treated cells showed an abnormal spindle shape while the other cells retained their normal morphological appearance (Figures 13, 15 and 16). In Figures 15 and 16, some cells were found to have vacuoles within their cytoplasm. Many of these also showed attenuated cytoplasms (Figure 16).

Autoradiography

Cultured cells were incubated with B(a)P for several different types of treatment. One treatment consisted of incubating cultured cells with B(a)P at 10 ug/ml and ^{3}H-B(a)P at 10 uCi/ml until the termination of experiment (full-term treatment). Other cells were partially exposed to B(a)P at the same concentration and radioactivity level. The exposure

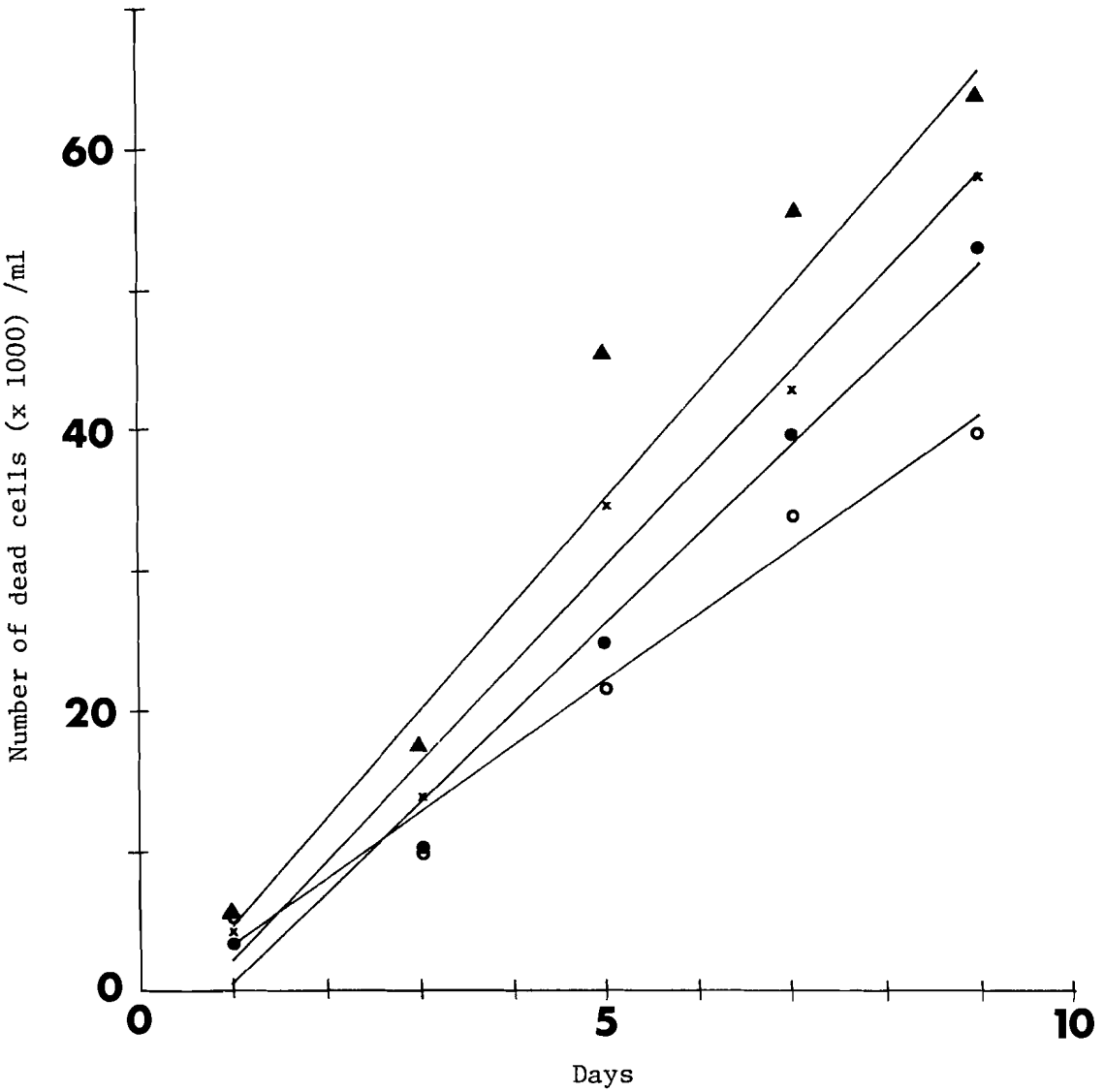


Figure 3. Toxic effect of B(a)P on cultured mouse embryo cells. Untreated cells; 0, Cells treated with 0.1 ug B(a)P; ●, Cells treated with 10 ug B(a)P; X, Cells treated with 50 ug B(a)P; ▲.

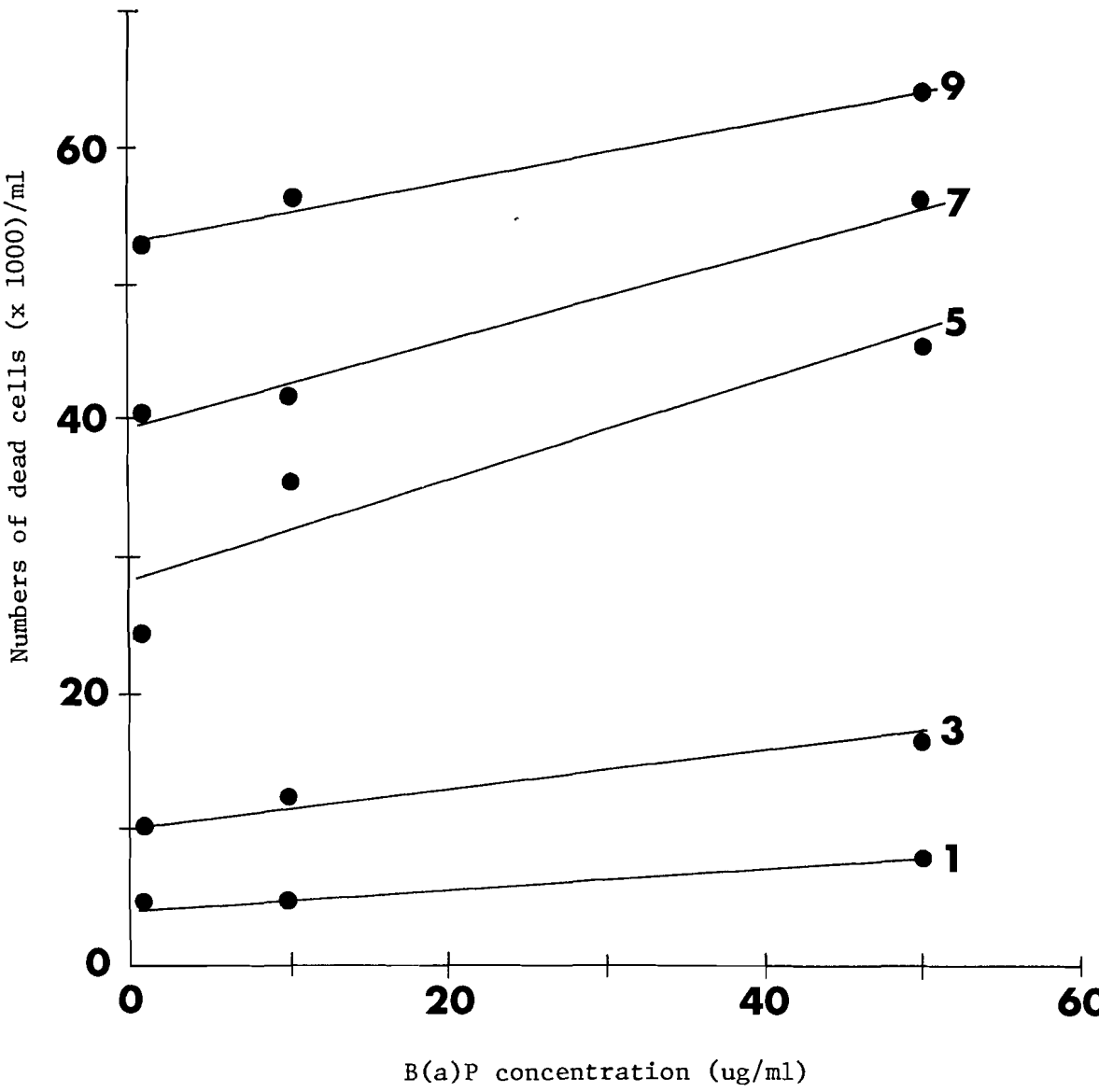
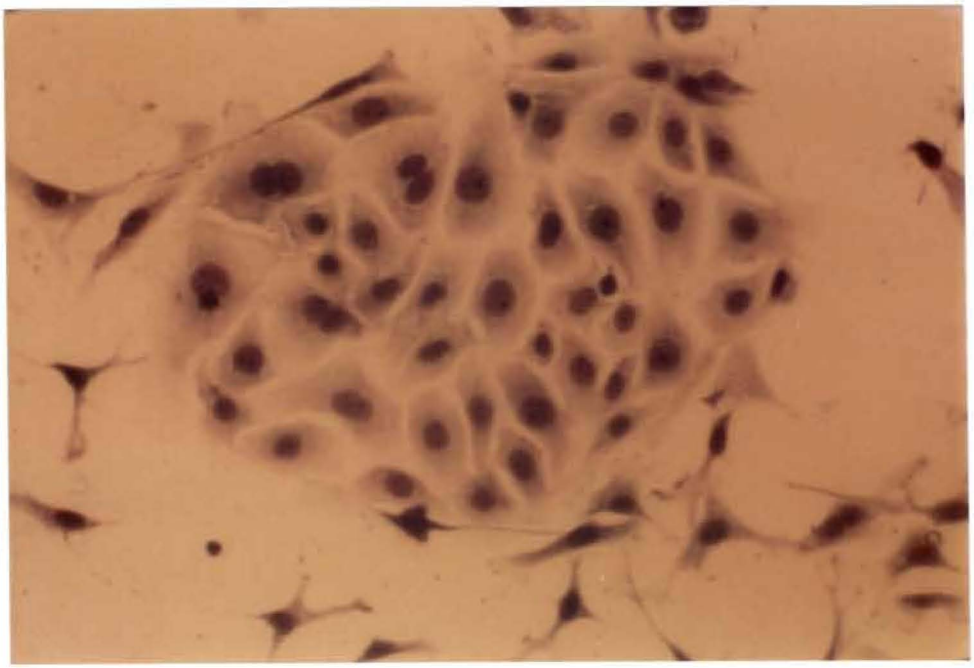


Figure 4. Toxicity effect of B(a)P concentration. The numbers 1, 3, 5, 7 and 9 indicate the days of B(a)P treatment.

Figure 5. Two day old normal mouse embryo cells in culture. Epithelial cells in the central area of fibroblast cells surrounding the epithelial cells are shown here. May-Grunwald and Giemsa stain (100 X).

Figure 6. Four day old normal mouse embryo cells in culture. Large nucleated epithelial-like cell and fibroblast cells are shown here. May-Grunwald and Giemsa stain (400 X).



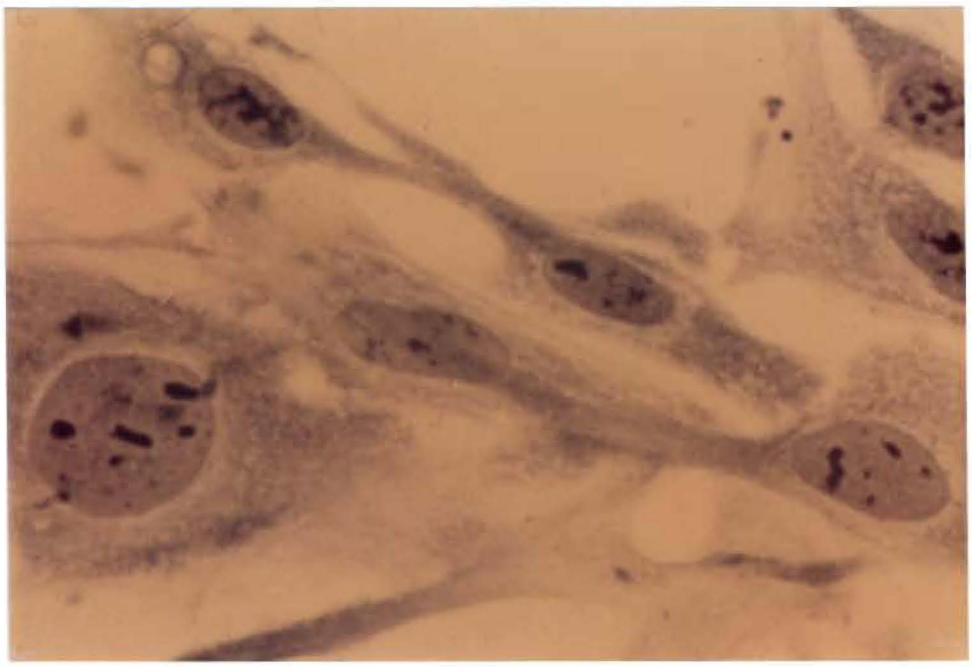


Figure 7. Six day old normal mouse embryo cells in culture. Multilayered fibroblast cells and two heavily stained aggregates are shown here. May-Grunwald and Giemsa stain (100 X).

Figure 8. Eight day old normal mouse embryo cells in culture. Fibroblast cells formed a monolayer sheet. May-Grunwald and Giemsa stain (100 X).

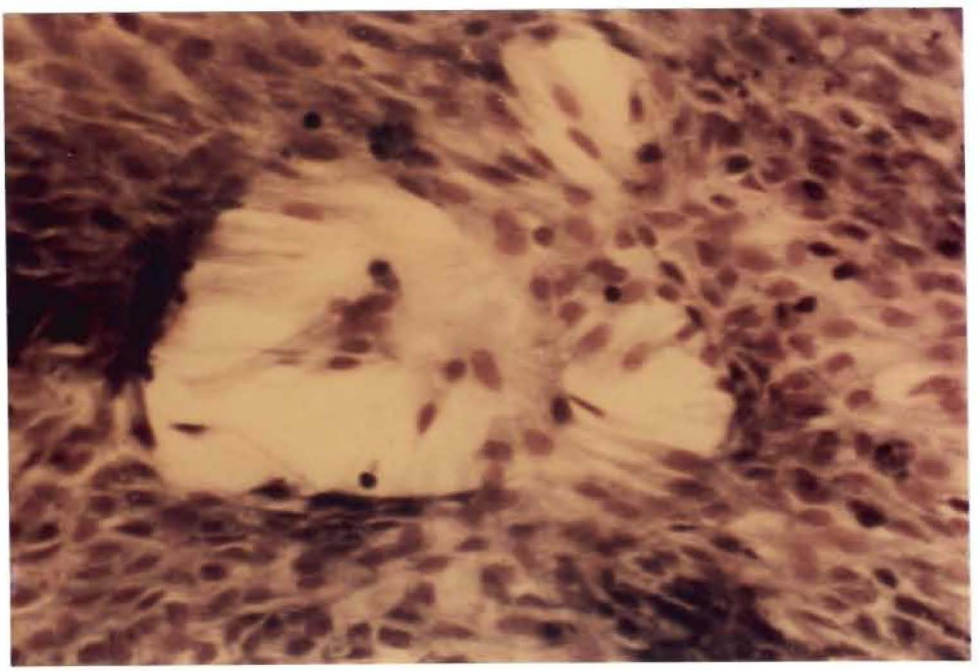
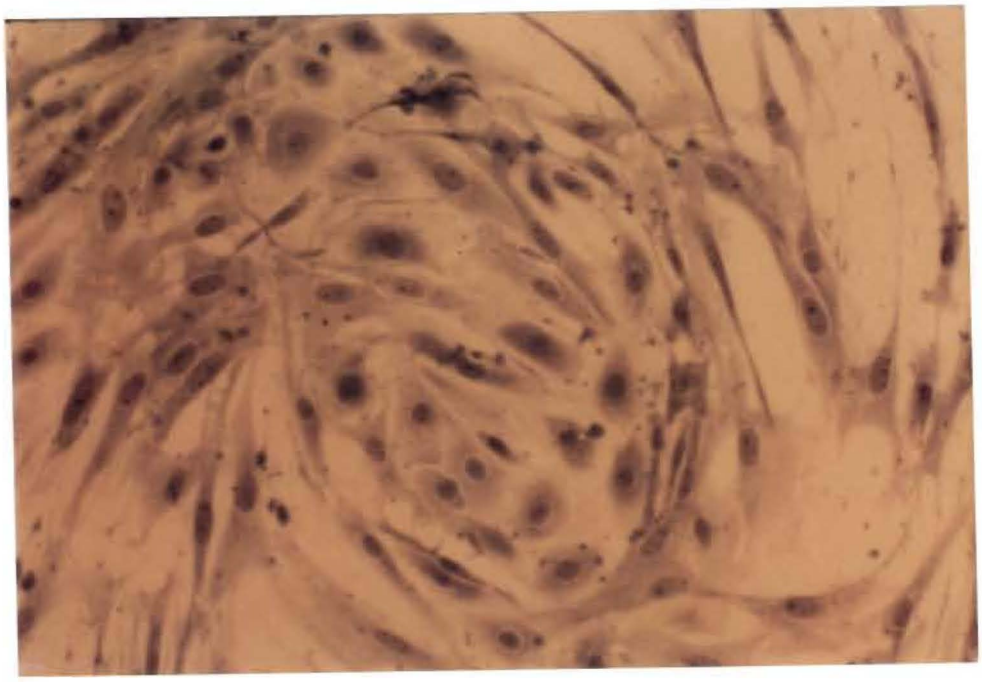


Figure 9. Thirteen day old normal mouse embryo cells in culture. Epithelial cells in the central and upper left areas are surrounded by well elongated fibroblast cells. May-Grunwald and Giemsa stain (100 X).

Figure 10. Thirty day old normal mouse embryo cells in culture. May-Grunwald and Giemsa stain (100 X).



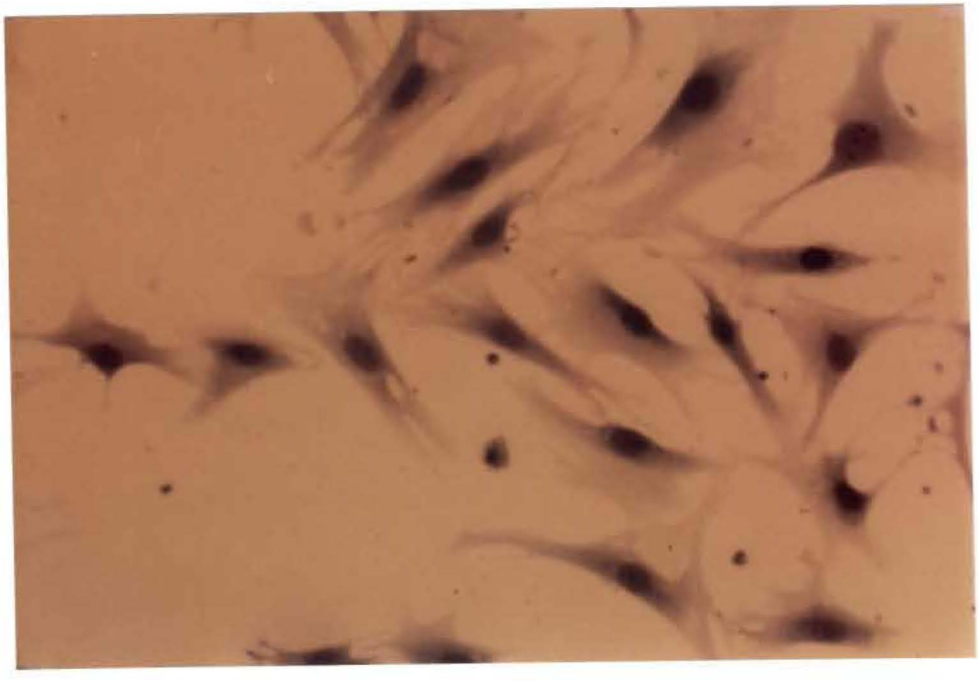
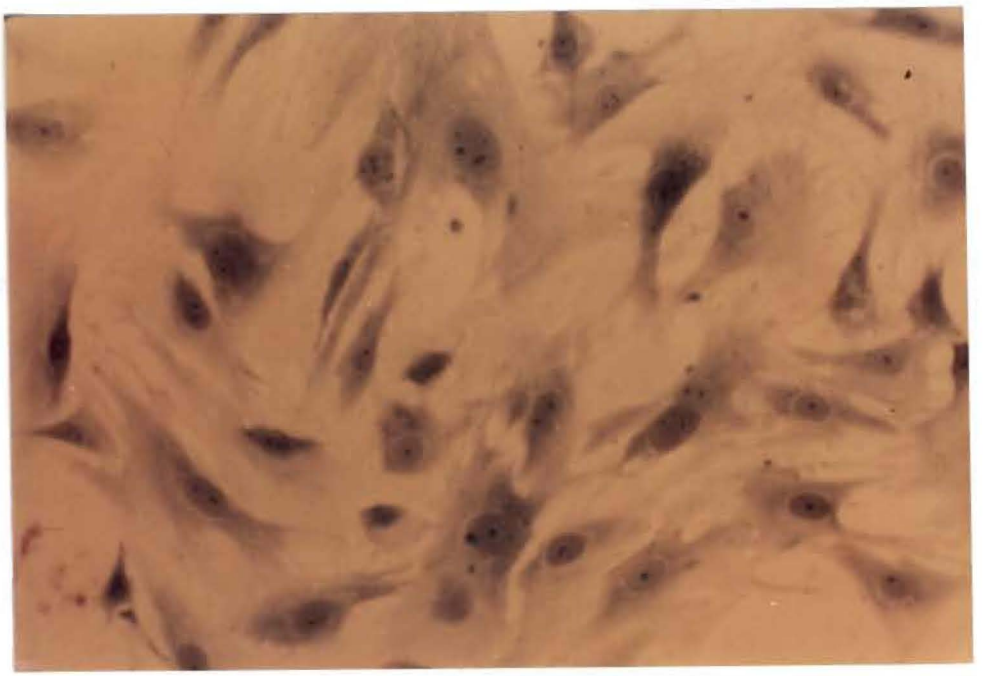


Figure 11. Thirty five day old normal mouse embryo cells in culture. Cell orientation is obscure and vacuoles are seen. May-Grunwald and Giemsa stain (100 X).

Figure 12. Forty day old normal mouse embryo cells in culture. Cell orientation is obscure and vacuoles are present. May-Grunwald and Giemsa stain (100 X).



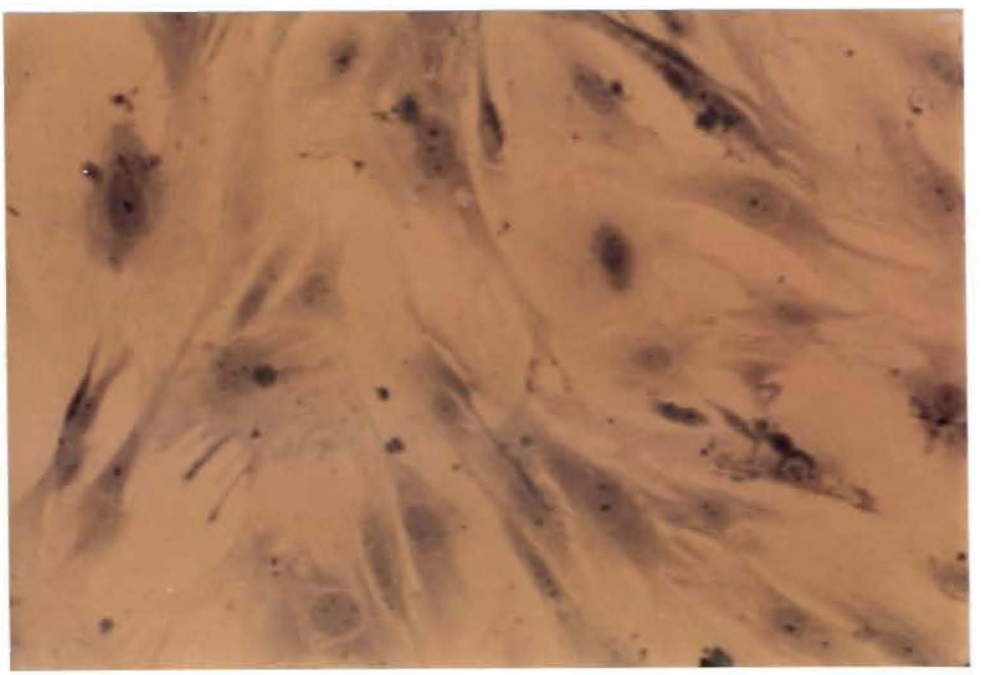
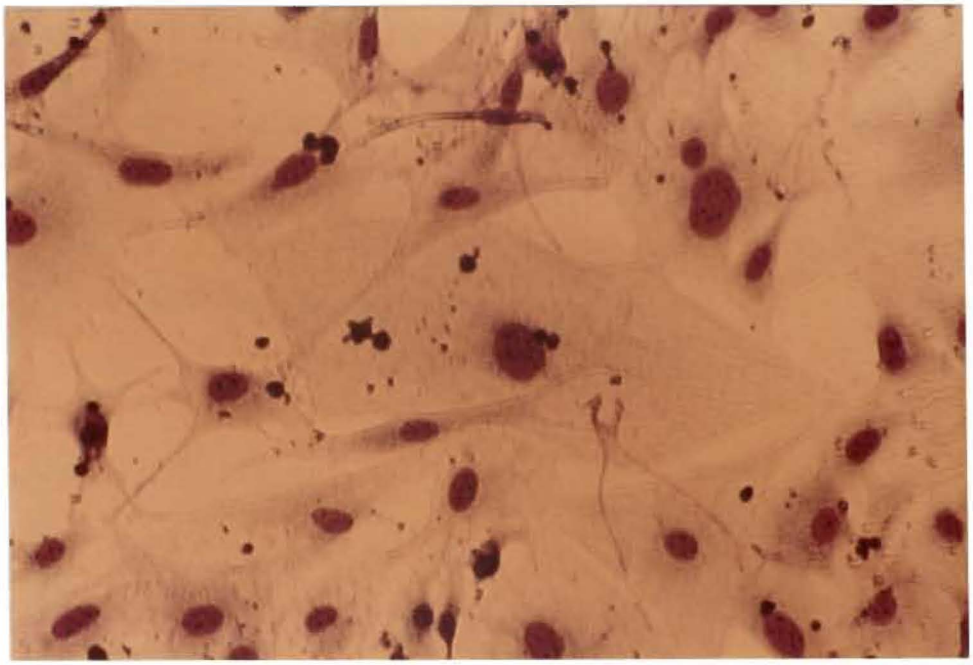


Figure 13. Mouse embryo cells incubated with 10 ug B(a)P for 1 day. May-Grunwald stain (100 X).

Figure 14. Mouse embryo cells incubated with 10 ug B(a)P for 2 days. May-Grunwald stain (100 X).



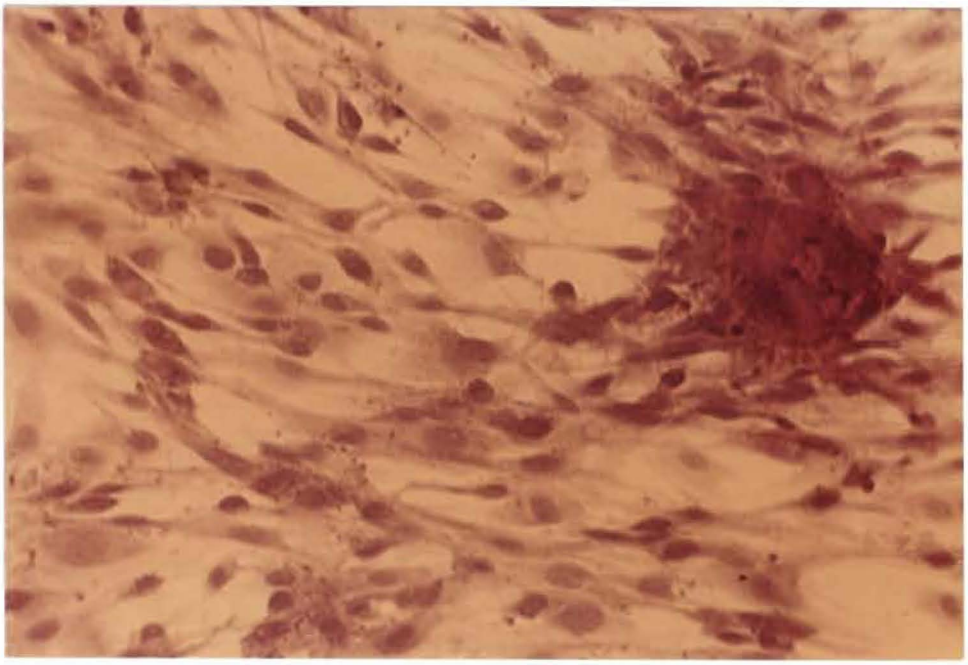
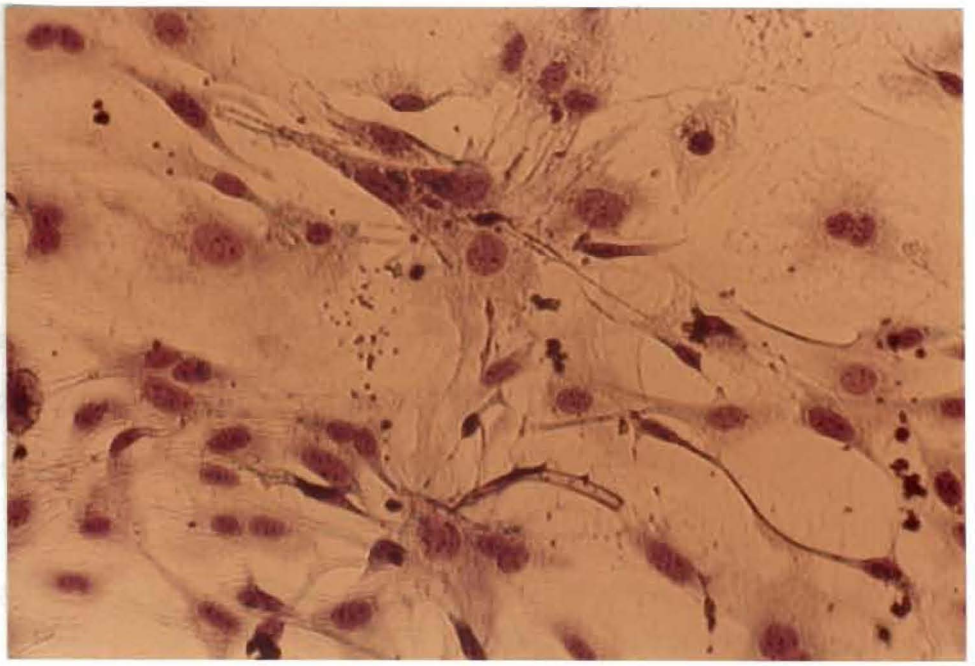
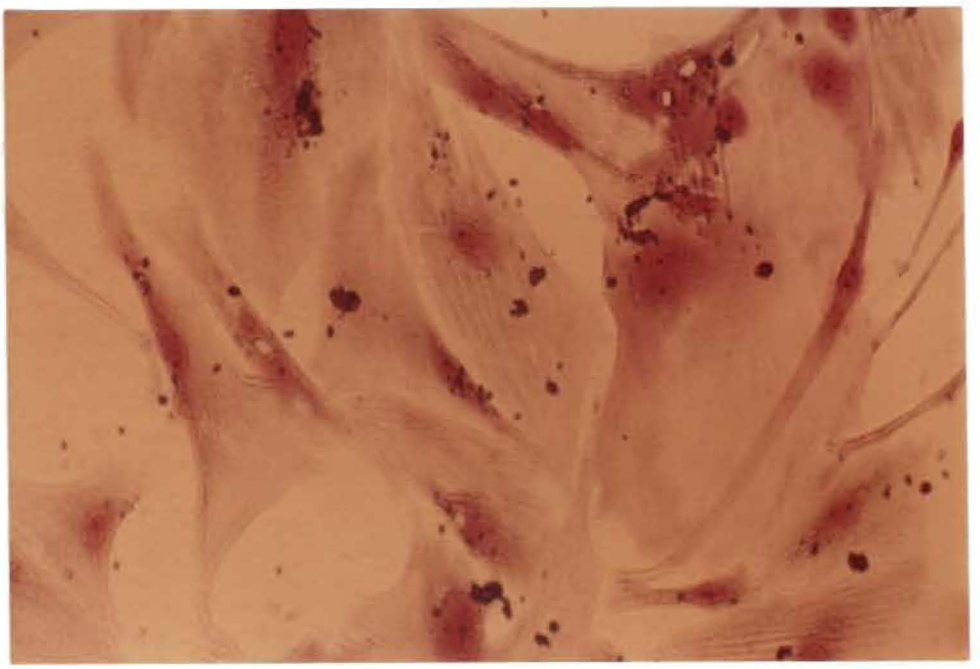


Figure 15. Mouse embryo cells treated with 10 ug B(a)P for 6 days. May-Grunwald stain (100 X).

Figure 16. Mouse embryo cells treated with 10 ug B(a)P for 9 days. Basic Fuchsin stain (100 X).





periods were 24 hours and four days.

Figures 18 through 23 represent the full-term treatment, and events observed for the first 17 hours are summarized in Figure 17. After only 1.5 hours exposure to ^{3}H-B(a)P, B(a)P accumulation in the nuclei was very low. Its cytoplasmic concentration was also minimal (Figure 18). ^{3}H-B(a)P uptake by the cells increased rather sharply after 3.5 hours of incubation (Figures 19, 20 and 21). Long term incubation (six and 20 days) showed a concentration of B(a)P in the cytoplasm while no nuclear concentration of B(a)P was observed (Figures 22 and 23).

In other treatments, cultured cells were partially exposed to B(a)P at the same concentration. Radioactivity levels were as in the full-term treatment. The cells were exposed to B(a)P for one and four days. At the end of the exposure period, the complete growth medium was replaced for further incubation. Figures 24 through 27 indicate some of the results of 24 hour treated cells. Overall results are summarized in Figure 24. Nuclear accumulation of ^{3}H-B(a)P was maximum at 24 hours and decreased drastically when fresh complete growth medium was introduced and incubation was continued (Figures 24, 27 and 28). The nuclear accumulation of B(a)P exceeded that of cytoplasm only at 24 hours incubation (Figure 24). Cytoplasmic accumulation of ^{3}H-B(a)P was at its highest peak on the 6th day of incubation and followed the same pattern of decrease as the nuclear accumulation curve. Extrapolation of the two curves suggests disappearance of B(a)P from the cells occurs between the 24th and 26th day. Some cells, as exemplified in Figure 28, lost nuclear B(a)P and most of the cytoplasmic B(a)P at

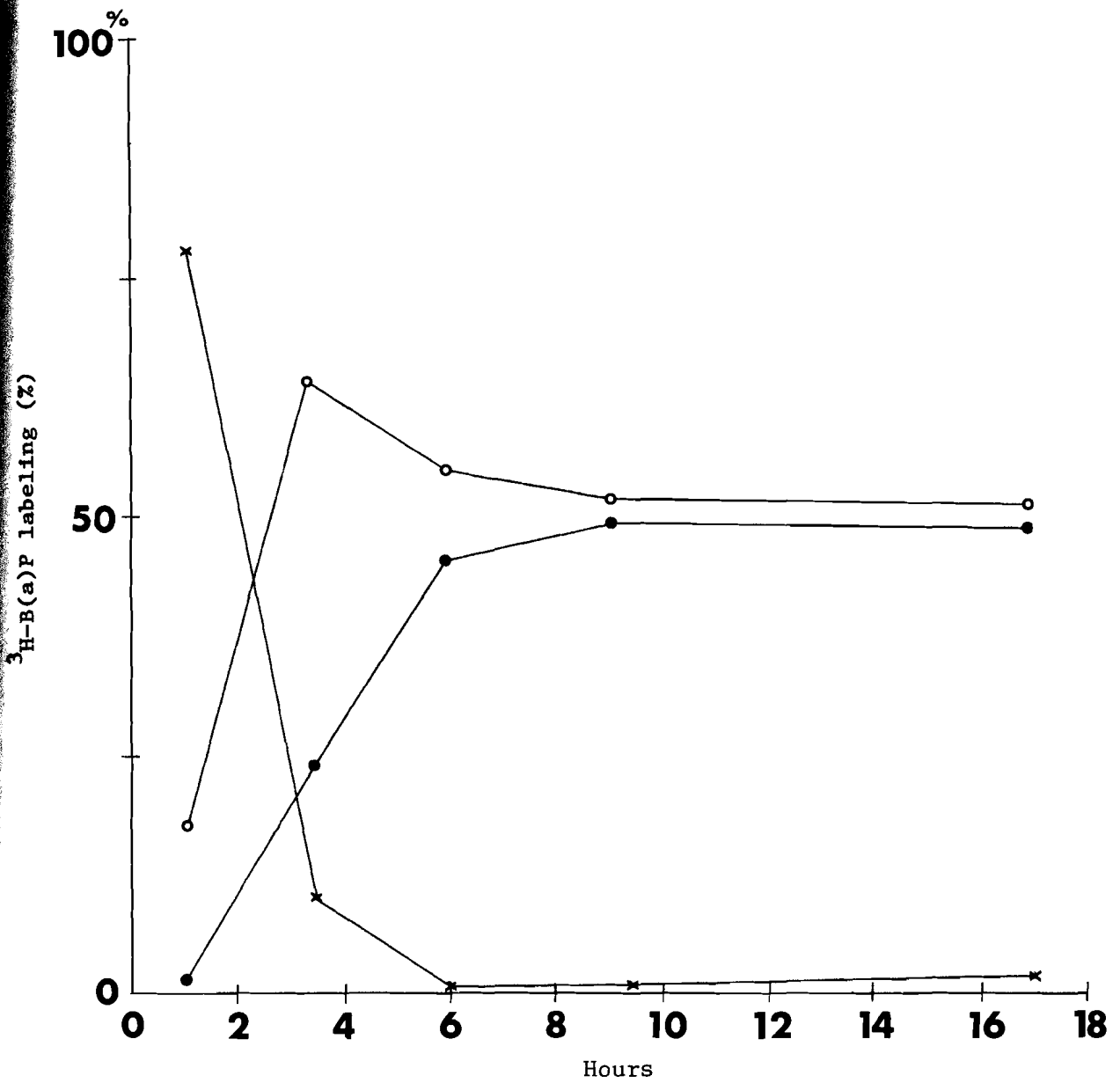


Figure 17. Time-course of ^{3}H-B(a)P accumulation in the cells. Cells in autoradiograms were counted for nuclear accumulation (●), cytoplasmic accumulation (0), and no accumulation (X).

21 days of incubation in the growth medium.

Figures 29 through 32 represent the results of four day treated cells. Four (no additional incubation in growth medium) and 11 days (additional six day incubation in growth medium) cells show nuclear and cytoplasmic accumulation of B(a)P but not as intense as seen in the one day treated cells (Figures 29 and 30). Fifteen day incubated cells had some B(a)P molecules in the cytoplasm. The cells incubated for 27 days had no retention of B(a)P in any part of the cells (Figure 32).

Chromosome study

Figures 33 through 35 are the chromosome spreads from ^{3}H-B(a)P treated cells. Silver grains (black dots) were seen on or near the chromosomes in three autoradiograms (Figures 33, 34 and 35). Autoradiograms of chromosomes prepared from one, two and three day treated cells with ^{3}H-B(a)P (10 uCi/ml) were counted for use in labeling analysis. Chromosome spreads from one and two day treated cells produced 93.6% and 92.0% labeled chromosome spreads respectively. Total chromosome spreads examined were 94 and 100. Cells treated for three days gave 30.4% labeled chromosome spreads.

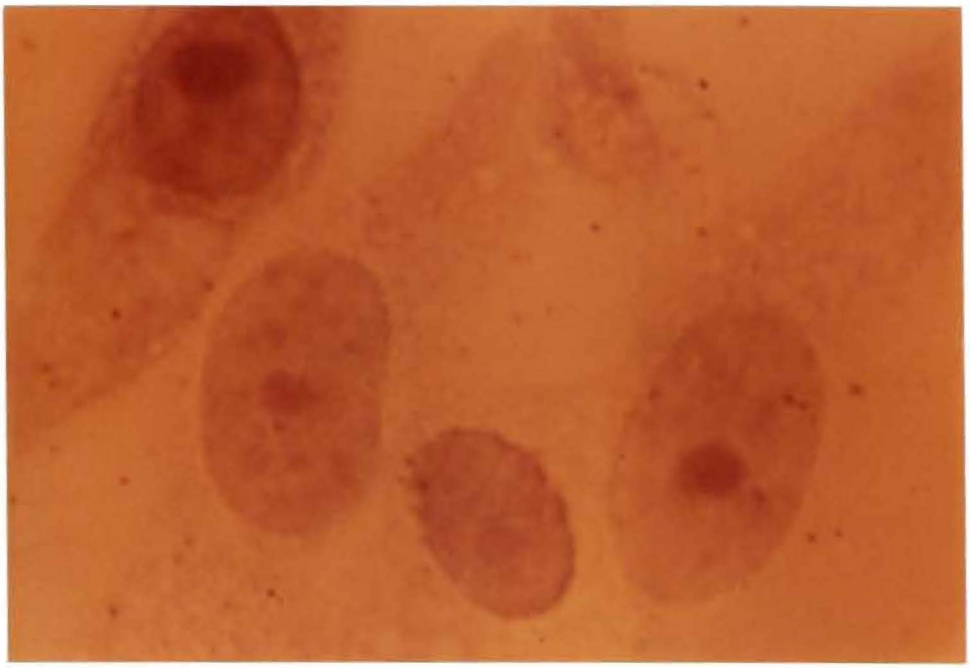
Figures 34 and 35 showed chromosome breakages.

Induction of abnormalities and its histology

B(a)P treated cells were injected into adult mice. The mouse in Figure 36 was a normal male mouse. The mouse in Figure 37 exemplifies the abnormal genital inflammation characterized by many of the mice injected with the B(a)P treated cells. From the 26 injected mice, 31% developed a genital inflammation. In contrast, only 3.8% of the control mice exhibited genital abnormalities. The controls consisted of three groups of mice. One group of 32 mice received no injections. Another

Figure 18. Autoradiogram. Mouse embryo cells in culture were incubated for 1.5 hours with 10 ug B(a)P(10 uCi). Only a few radioactive molecules are present. Hematoxylin and eosin stain (100 X).

Figure 19. Autoradiogram. Mouse embryo cells in culture were incubated for 3.5 hours with 10 ug B(a)P(10 uCi). Nuclear and cytoplasmic accumulation of B(a)P is seen. Hematoxylin and eosin stain (100 X).



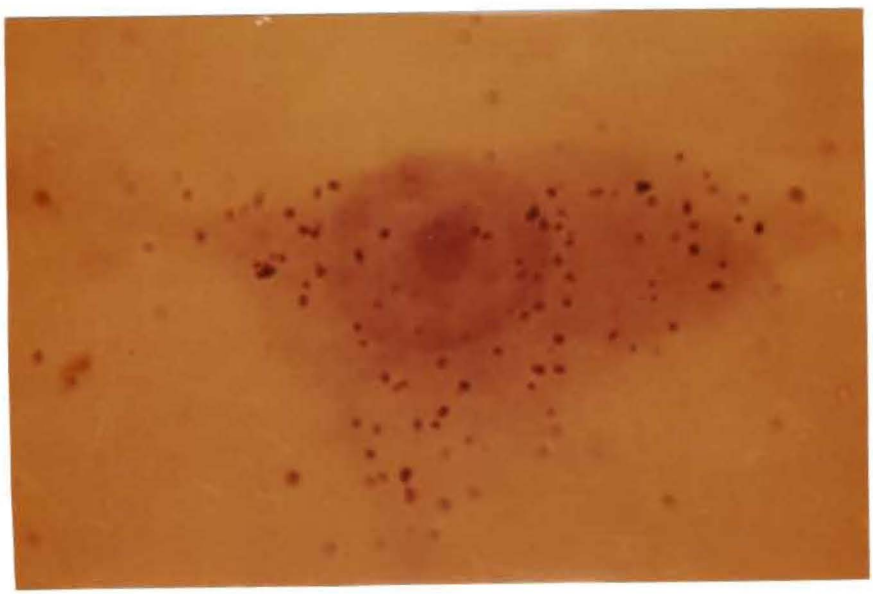
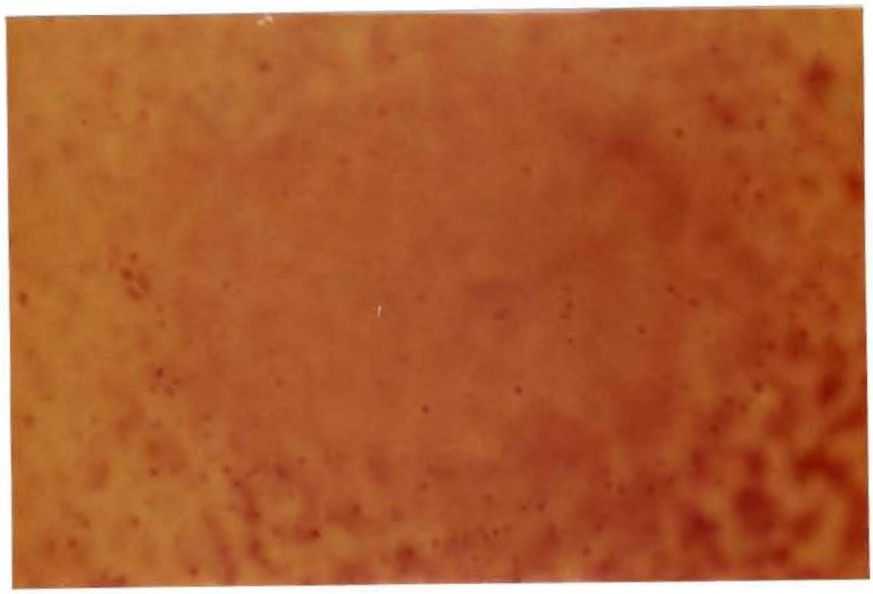


Figure 20. Autoradiogram. Mouse embryo cells in culture were incubated for 17 hours with 10 ug B(a)P(10 uCi). Basic Fuchsin stain (1000 X).

Figure 21. Autoradiogram. Mouse embryo cells in culture were incubated for 24 hours with 10 ug B(a)P(10 uCi). Basic Fuchsin stain (1000 X).



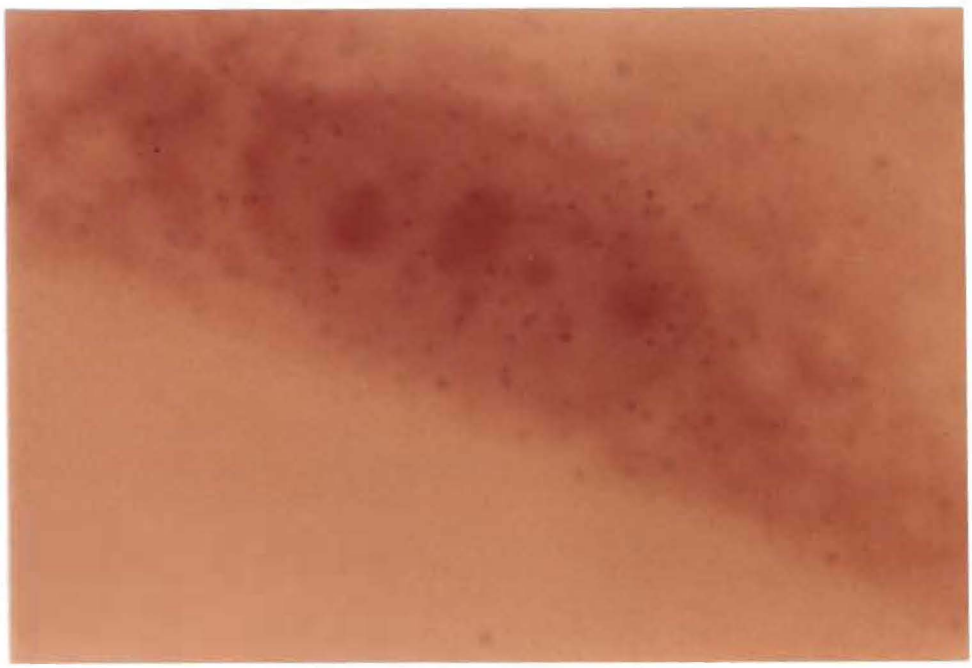
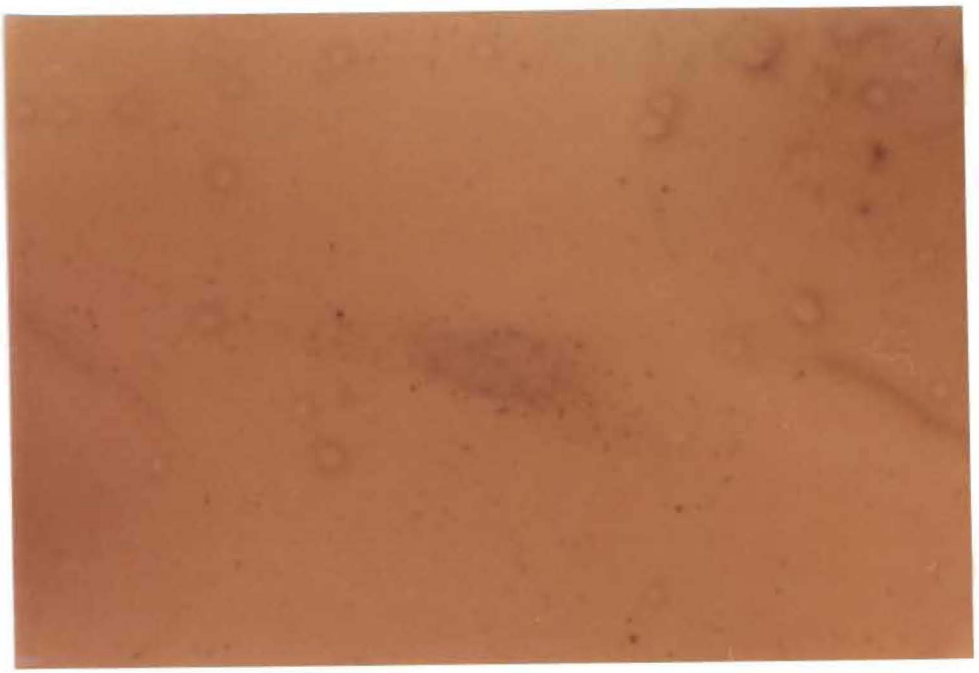
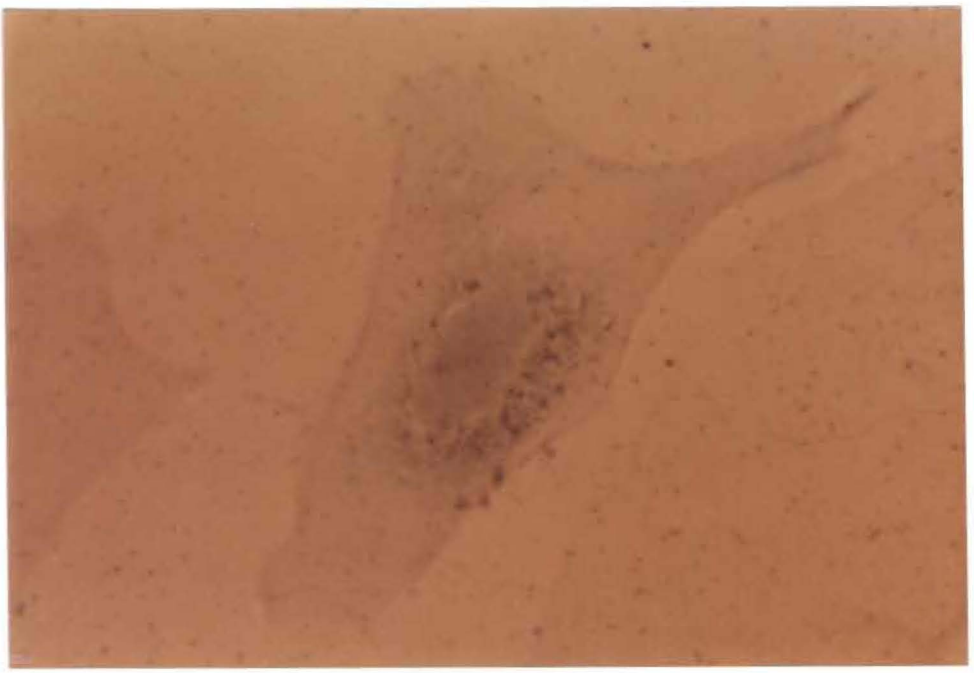


Figure 22. Autoradiogram. Mouse embryo cells in culture were incubated for six days with 10 ug B(a)P(10 uCi). Hematoxylin and eosin stain (100 X).

Figure 23. Autoradiogram. Mouse embryo cells in culture were incubated for 20 days with 10 ug B(a)P(10 uCi). A heavy accumulation of B(a)P in cytoplasm is seen here. Hematoxylin and eosin stain (400 X).





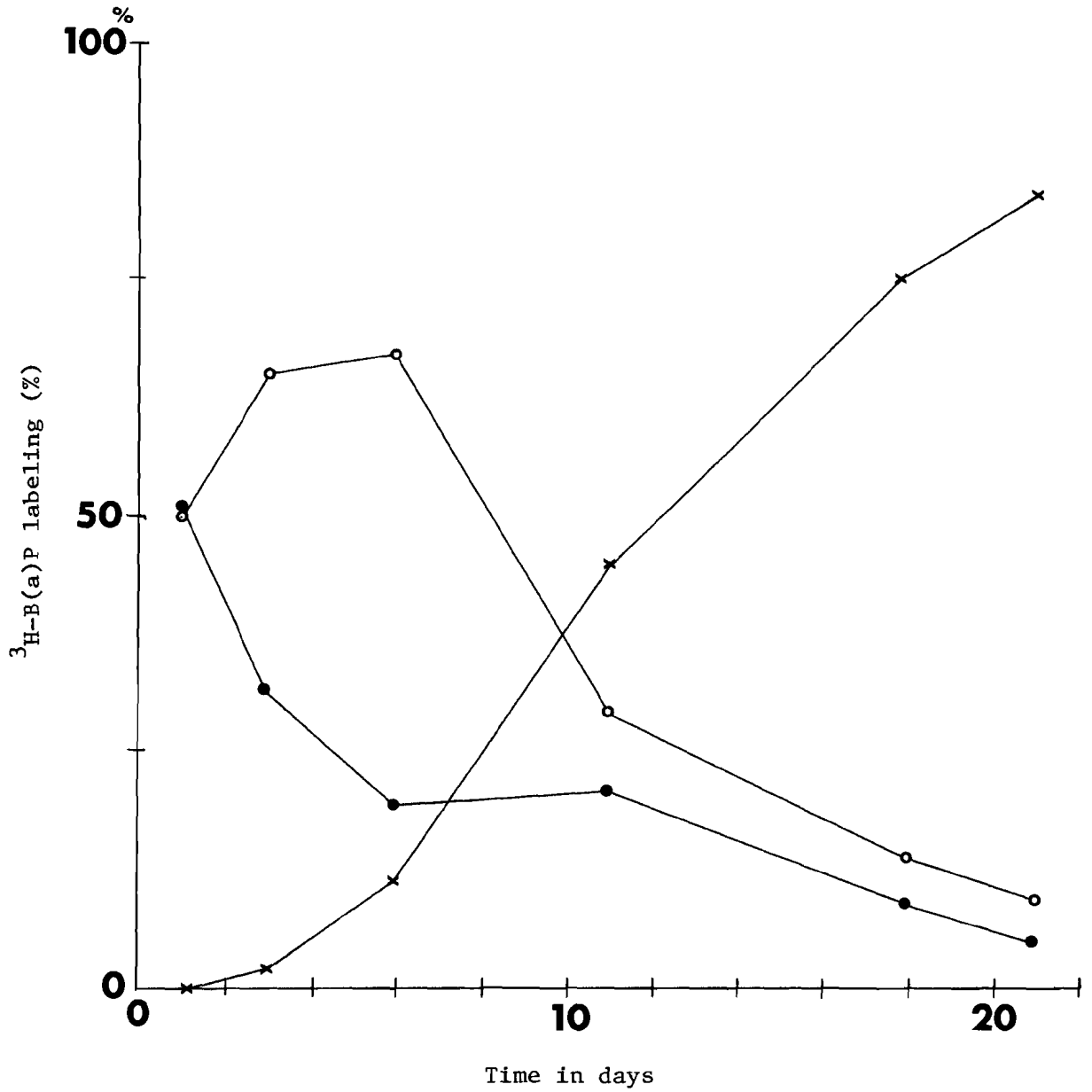
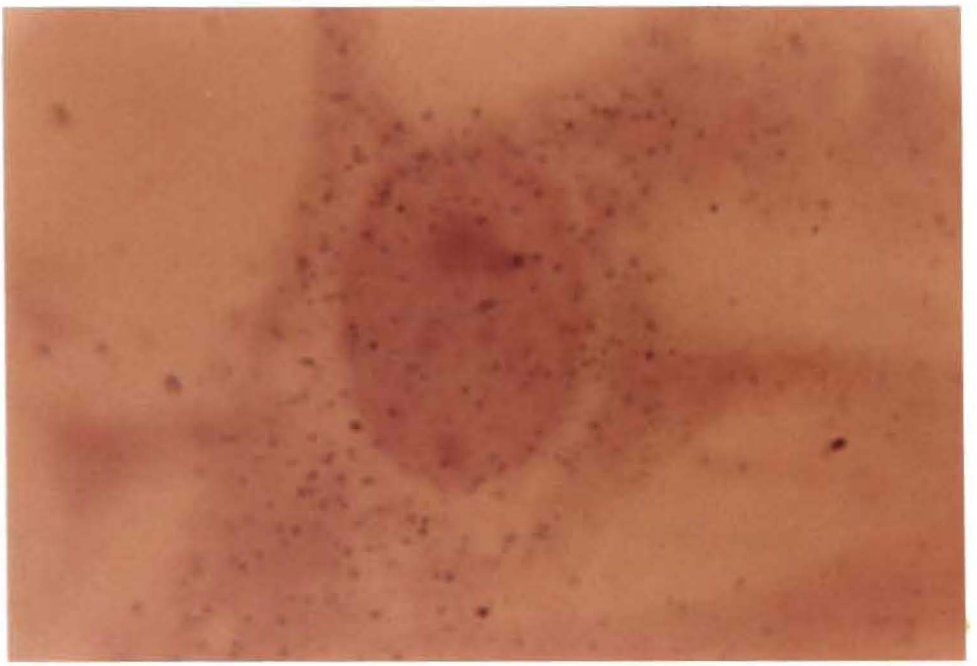


Figure 24. Time-course of ^{3}H-B(a)P accumulation in the cells. ^{3}H-B(a)P was replaced with normal growth medium after 24 hours incubation. Cells in autoradiograms were counted for nuclear accumulation (●), cytoplasmic accumulation (O), and no accumulation (X).

Figure 25. Autoradiogram. Mouse embryo cells in culture were incubated for one day with 10 ug B(a)P(10 uCi). Nuclear and cytoplasmic accumulation of B(a)P is seen. Basic Fuchsin stain (1000 X).

Figure 26. Autoradiogram. Mouse embryo cells in culture were incubated for one day with 10 ug B(a)P(10 uCi). The B(a)P was then removed. The cells were incubated an additional 3 days in complete growth medium. Basic Fuchsin stain (1000 X).



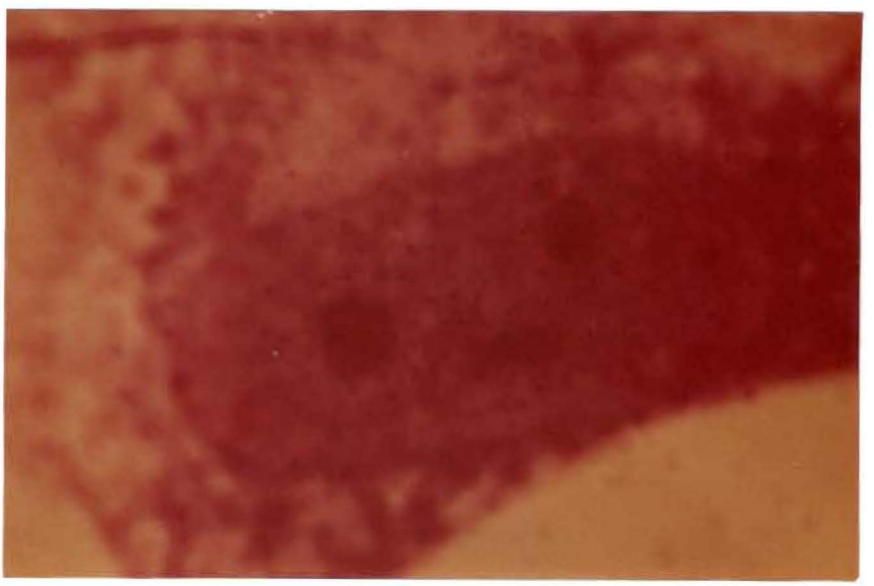
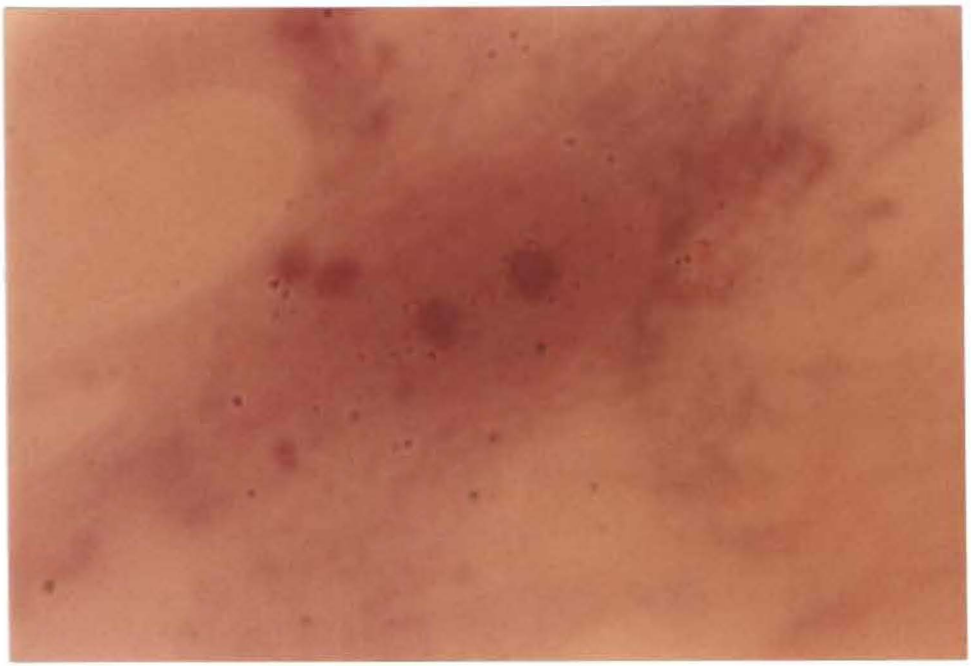


Figure 27. Autoradiogram. Mouse embryo cells in culture were incubated for one day with 10 ug B(a)P(10 uCi). The B(a)P was then removed. The cells were incubated an additional 11 days in complete growth medium. Basic Fuchsin stain (1000 X).

Figure 28. Autoradiogram. Mouse embryo cells in culture were incubated for one day with 10 ug B(a)P(10 uCi). The B(a)P mixture was then removed. The cells were incubated an additional 21 days in complete growth medium. Basic Fuchsin stain (1000 X).



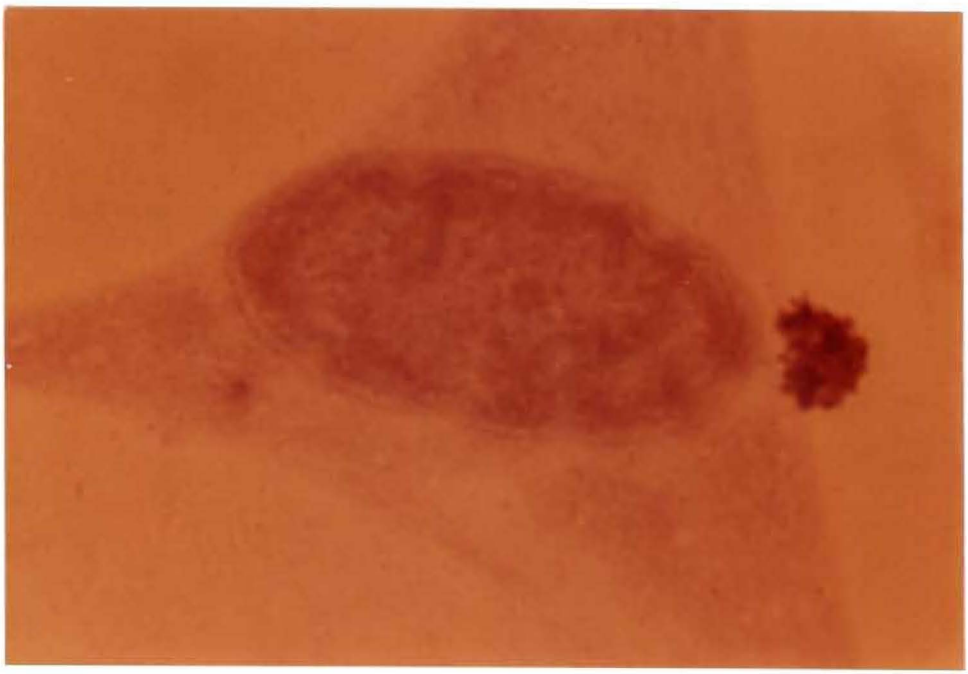
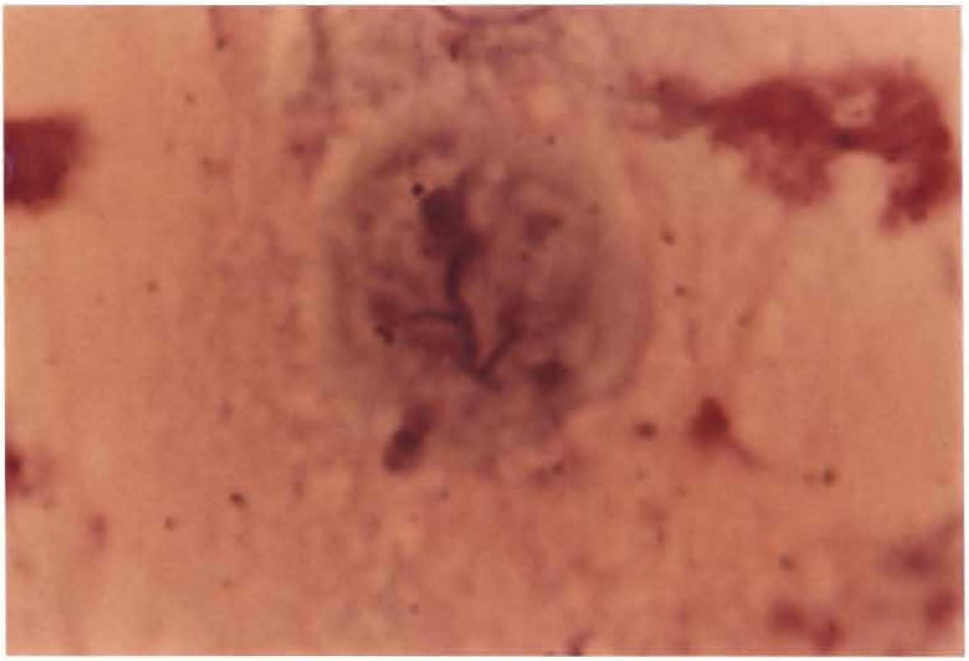


Figure 29. Autoradiogram. Mouse embryo cells in culture were incubated for four days with 10 ug B(a)P(10 uCi). Negri body stain (1000 X).

Figure 30. Autoradiogram. Mouse embryo cells in culture were incubated for four days with 10 ug B(a)P(10 uCi). The B(a)P mixture was then removed. The cells were incubated an additional six days in complete growth medium. Some silver grains are seen. Basic Fuchsin stain (1000 X).



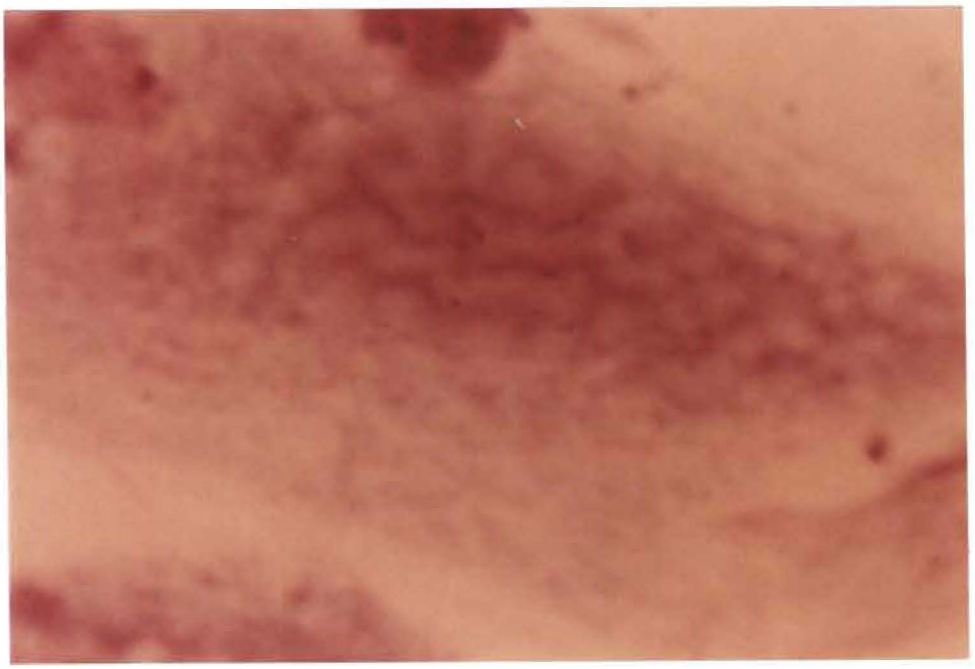
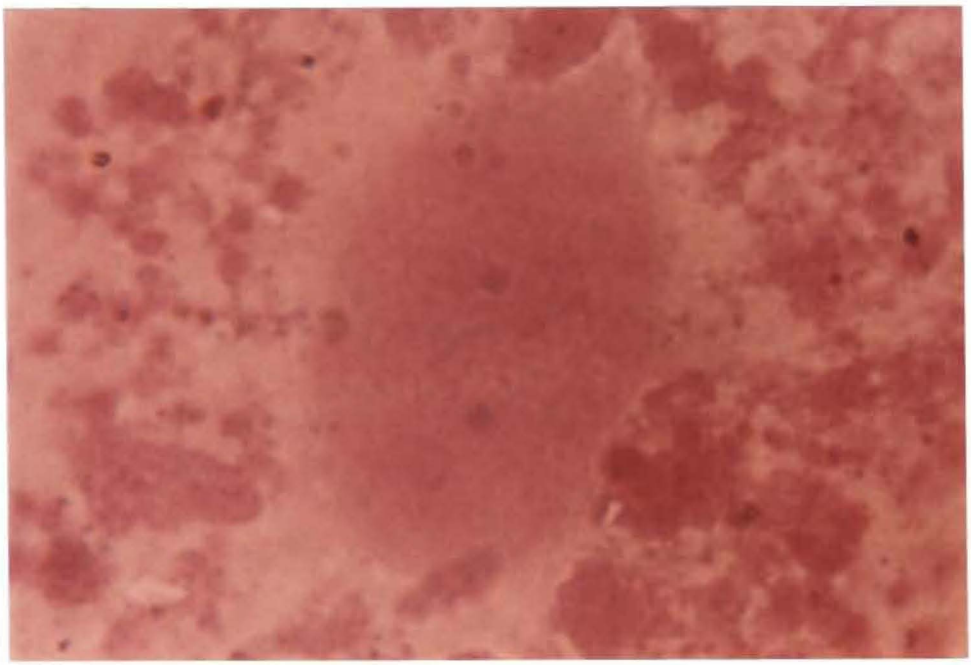


Figure 31. Autoradiogram. Mouse embryo cells in culture were incubated for four days with 10 ug B(a)P(10 uCi). The B(a)P was then removed. The cells were incubated an additional 11 days in complete growth medium. Some silver grains were seen in the cytoplasm. Basic Fuchsin stain (1000 X).

Figure 32. Autoradiogram. Mouse embryo cells in culture were incubated for four days with 10 ug B(a)P(10 uCi). The B(a)P mixture was then removed. The cells were incubated an additional 23 days in complete growth medium. Basic Fuchsin stain (1000 X).



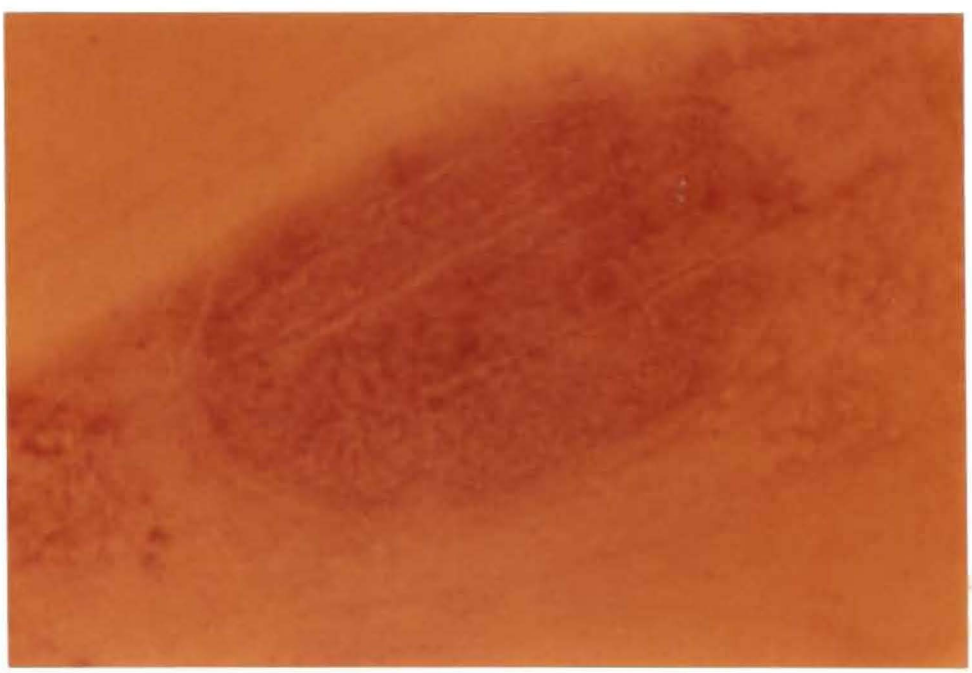
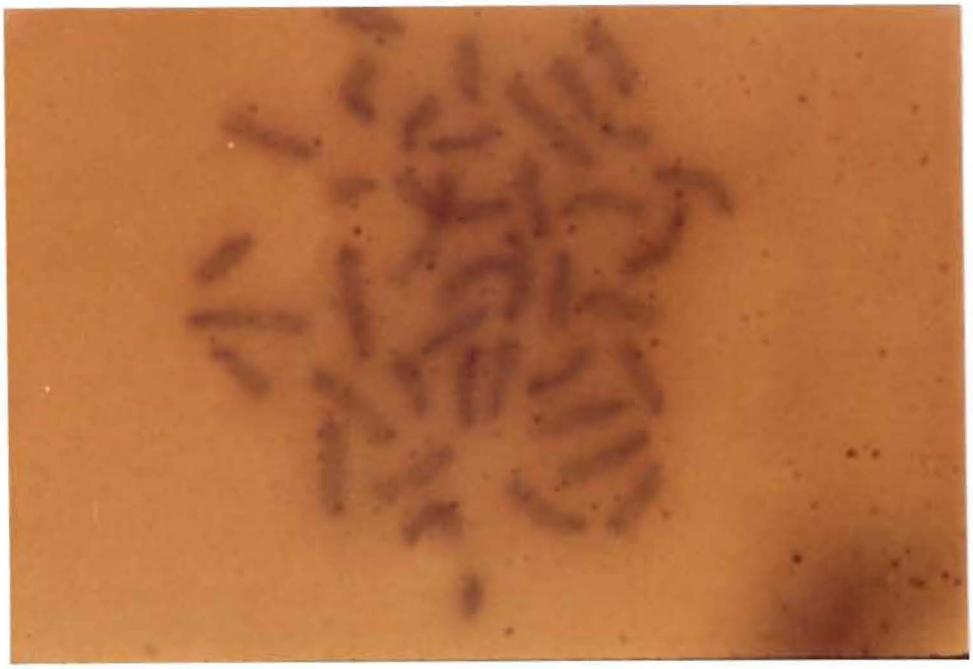


Figure 33. Autoradiogram of chromosomes. Mouse embryo cells in culture were incubated for one day with 0.05 ug B(a)P(10 uCi). Giemsa stain (1000 X).

Figure 34. Autoradiogram of chromosomes. Mouse embryo cells in culture were incubated for two days with 0.05 ug B(a)P(10 uCi). Giemsa stain (1000 X).



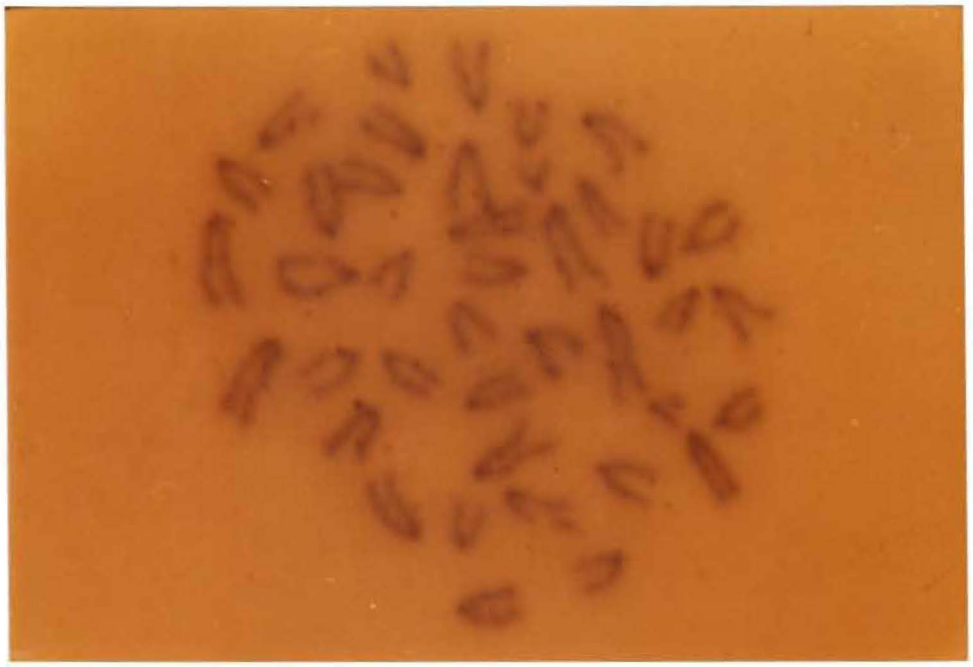
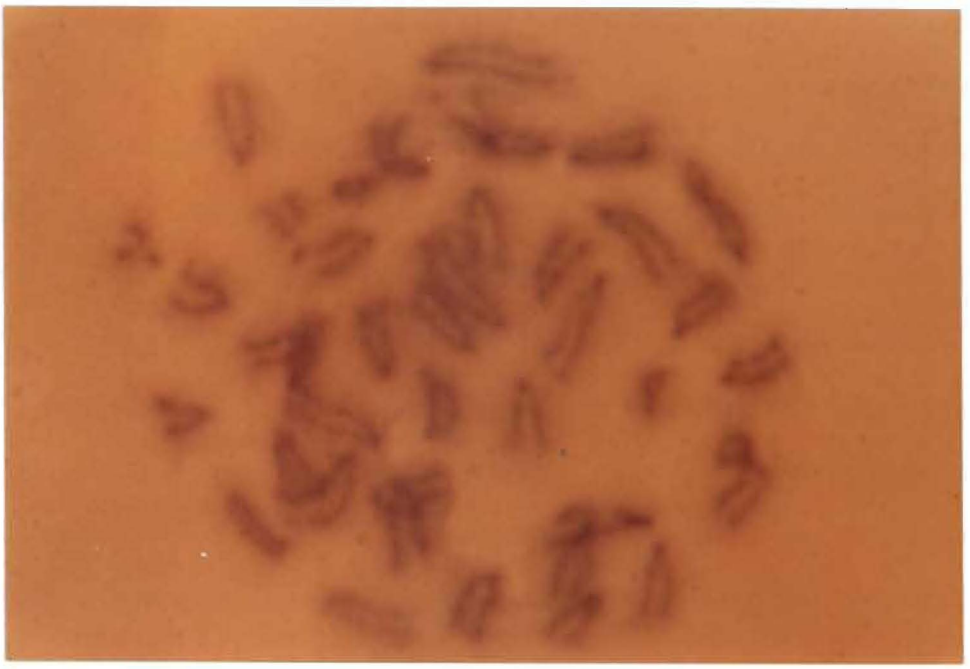


Figure 35. Autoradiogram of chromosomes. Mouse embryo cells in culture were incubated for three days with 0.05 ug B(a)P(10 uCi). Giemsa stain (1000 X).

Figure 36. Normal male mouse.



group of 10 mice were injected with normal untreated cells. The last group of 10 mice was inoculated with growth medium that was used to maintain the cells (Table 2). The effect of B(a)P treated cells in the mice is summarized on Table 2. The mice inoculated with the cells of four days B(a)P preincubation resulted in a 25-50% incidence of abnormal development. Of these abnormal mice, two mice developed a small nodule (ca. 4-5 mm in diameter) on the peritoneum (Figure 38). This nodule was processed for histological study. Microscopic examination revealed that the nodule was the undescended testis and showed hyperplasia of Leydig cells (Figure 43). Atrophy of seminiferous tubules (Figure 39) is clearly recognized when they are compared with those of normal tissue (Figures 42 and 43). Seminiferous tubules from abnormal tissue did not show spermatogenesis (Figure 43). The ductus epididymis (Figure 41), however, showed normal morphological appearance but clumps of spermatozoa were not seen in the ductuli. Hyperplasia of connective tissue surrounding ductus epididymis was observed as well. The mice inoculated with 10 day B(a)P treated cells had a higher incidence (80%) of abnormal development. Long term observation (146 days) did not reveal the development of any abnormality although the inoculated cells were treated for one day with B(a)P.

Table 2. Effect of B(a)P treated cells in adult mice

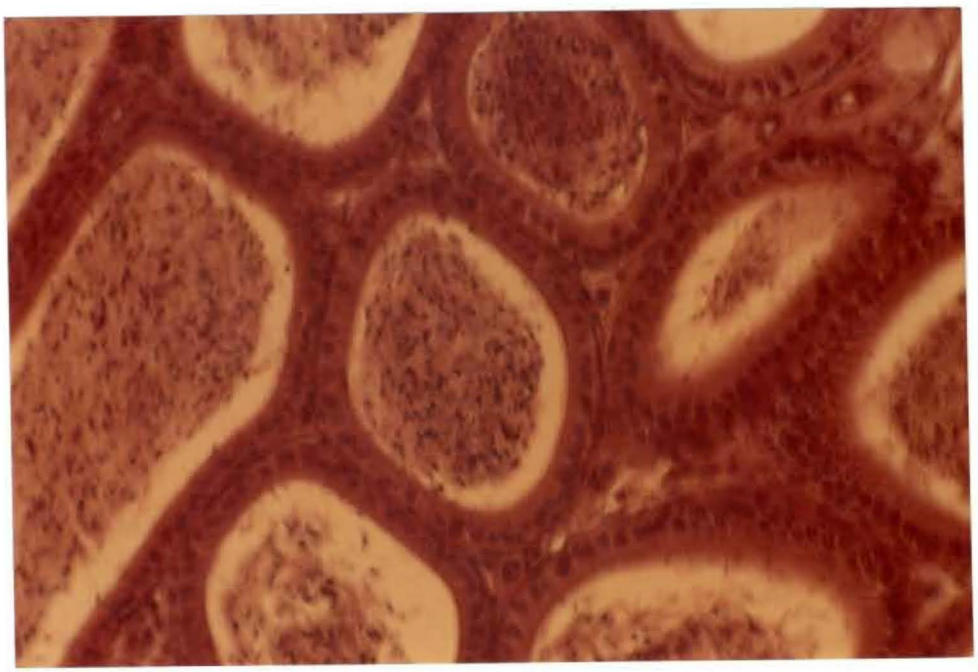
Days of incubation with B(a)P	Days of incubation after B(a)P removed	Number of cells inoculated (x 10^6)	Days in culture	Days after inoculation	Abnormalities
4	0	10	47	62	2/4
4	4[a]	10	43	68	1/4
4	6	10	41	70	1/4
10	0	10	43	70	4/5
1	0	1	6	146	0/5
1	0	10	6	146	0/4
Controls					
Untreated Mouse		--	--	---	1/32
Normal cells		10	11	146	1/10
Growth medium only (from a)			4	70	0/10

Figure 37. Mouse with genital abnormality.

Figure 38. Mouse with a nodule on peritoneum.

Figure 40. Transverse section of ductus epididymis from normal mouse testis. Hematoxylin and eosin stain (100 X).

Figure 41. Transverse section of ductus epididymis from undescended testis. Clumps of spermatozoa are not seen in the ductuli. Spaces among the ductuli are abnormally large. Hematoxylin and eosin stain (100 X).



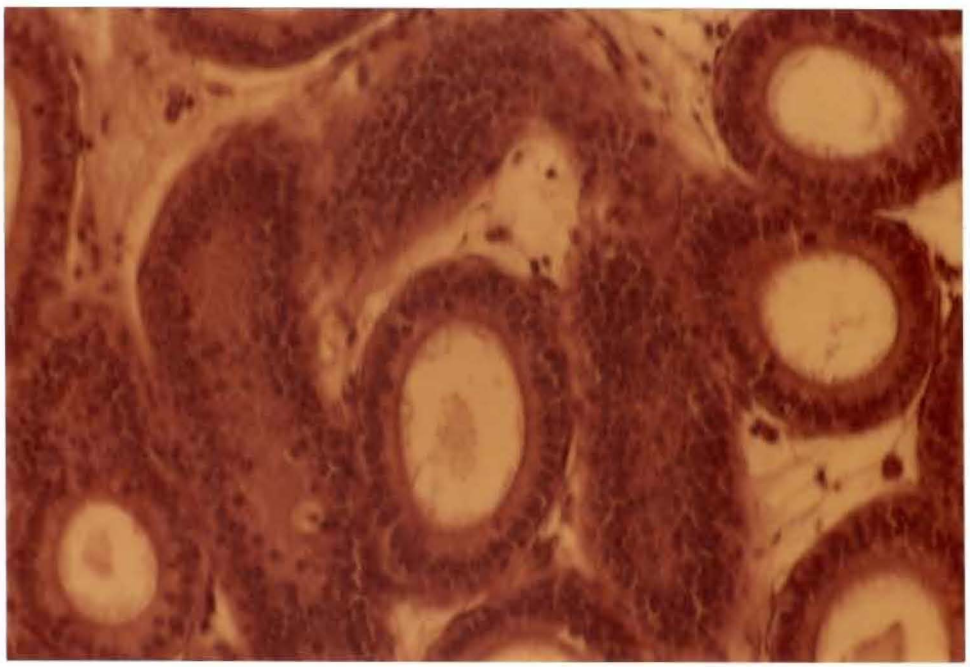
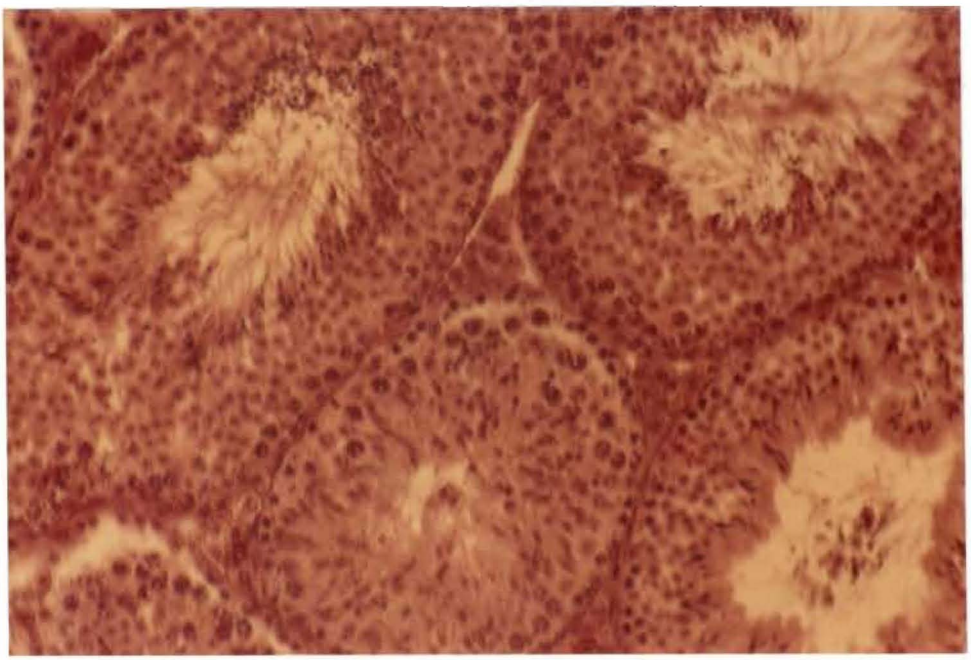
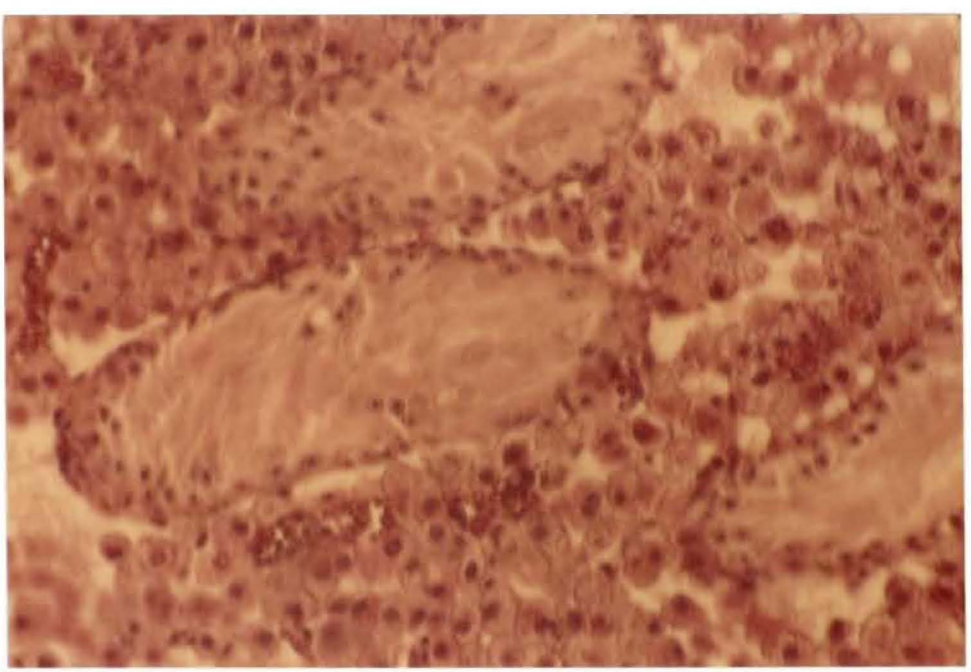


Figure 42. Transverse section of seminiferous tubules from normal mouse testis. Seminiferous tubules are separated by a slight amount of interstitial connective tissue containing some Leydig cells. Hematoxylin and eosin stain (100 X).

Figure 43. Transverse sections of seminiferous tubules from undescended testis. Atrophy of seminiferous tubules is seen. Hyperplasia of Leydig cells is recognized. Hematoxylin and eosin stain (100 X).





DISCUSSION

Cook et al. (1932, 1933) isolated carcinogenic hydrocarbons from coal tar pitch. The melting point (m.p.) of one of the hydrocarbons was determined to be 175.5-176.5°C. This particular hydrocarbon species was shown to be pure benzo(a)pyrene (B(a)P) by comparison with the synthesized B(a)P. B(a)P has alternative names such as 1,2-benzpyrene and 3,4-benzpyrene. It has the molecular weight of 252.30; its structure is shown in diagram on page 3.

B(a)P and other closely related carcinogenic hydrocarbons (see diagram on page 3) are being used to determine a mechanism of carcinogenesis in several research systems. B(a)P is known to be one of the chemically inert carcinogens. To be activated, B(a)P must first be taken up by the cells. Thus, invasion into the cell is the first step in the mechanism. B(a)P particles in the growth medium are taken into the cells by endocytosis (Allison and Mallucci, 1964). Engulfed B(a)P particles then require enzymatic activations to acquire cytotoxic, mutagenic (Huberman et al., 1971, 1976; Newbold and Brooks, 1976), and carcinogenic abilities in the cells (Wood et al., 1976; Wislocki et al., 1976).

The greatly increased number of dead cells in cultures which had been treated with B(a)P for five days suggested that enzymatic activations of ingested B(a)P might have occurred during a one to three day incubation period. As a result, B(a)P acquired cytotoxic potency to the cells (Figure 4). Figures 17 and 24 indicate that the cytoplasmic accumulation had maximum values between 3.5 hours and six days. This suggested the possibility that B(a)P metabolism takes place between these periods. A reliable explanation for the cytotoxic ability of B(a)P was

made by several workers (Nebert and Gelboin, 1968a, 1968b; Kasper, 1971; Khandwala and Kasper, 1973). There exists an enzyme, aryl hydrocarbon hydroxylase, that functions in the metabolism of B(a)P. The metabolite is 3-hydroxybenzopyrene which also possesses cytotoxic potency to the cell. Benzo(a)pyrene-hydroxylase was shown to be present at low concentrations in the nuclear membrane. The existence of inducible B(a)P-hydroxylase in a microsomal preparation was reported as well. The major difference between nuclear and microsomal membranes is its specificity. Nuclear membranes produce hydrocarbon hydroxylase specifically by the stimulation of hydrocarbon carcinogens while microsomal membranes produce hydrocarbon hydroxylase in response to the addition of hydrocarbon carcinogens or phenobarbital.

In addition to enzymatic activation in the cytoplasm, B(a)P molecules may be accumulated in one or some of the cellular organelles. To determine the ultimate B(a)P accumulation site in the cell, it is important to define the "target organelle(s)" for hydrocarbon carcinogens. Several techniques were developed for this purpose, and an autoradiography technique was the one utilized in this study. Autoradiography techniques in the biomedical field are a powerful tool for localization and visualization of desired chemicals in the cell or the tissues. Autoradiograms obtained in this study indicated that the cytoplasmic accumulation of B(a)P occurred as early as 1.5 hours after the initial application and lasted about 14 days (Figure 17 and 24). The silver grains seen in Figures 19, 20 and 27 indicate that B(a)P molecules may be encapsulated as a phagosome, perhaps independently from lysosomes, or fused to lysosomes where enzymatic alterations as

well as detoxification reactions take place. Along with the cytoplasmic accumulation a maximum nuclear accumulation (52% labeling) was observed in one day (Figure 24). Although nuclear accumulation of B(a)P was shown, it does not clearly indicate the incorporation of B(a)P into genetic material (DNA). To answer this question, chromosome spreads combined with autoradiography were prepared (Figures 33, 34 and 35). These figures indicate that an interaction of B(a)P and chromosomes might have occurred in the nuclei. This result is supported by the work of Brooks and Lawley (1964) who isolated DNA and RNA from mouse skin to which radioactive carcinogenic hydrocarbons were previously applied. One of their results is shown in Figure 44 which demonstrates that the maximum binding of B(a)P to proteins occurred about 24 hours after application of B(a)P. This result suggests the possibility that B(a)P molecules bind to DNA.

Figures 34 and 35 demonstrated chromosome breakages. A generally accepted explanation for chromosome aberrations is that a direct chemical or physical attack on DNA has occurred. However, Allison and Patton (1965) proposed another possible mechanism which could cause chromosome breakages. The idea was that damage to lysosomes can cause chromosomal damage. It was demonstrated by De Duve and his colleagues that lysosomes contain hydrolytic enzymes, acid phosphatase, acid DNase, acid RNase, and acid protease (reviewed by Allison and Mallucci, 1964). Among these lysosomal enzymes, DNase can break the DNA, which is the backbone of the uncoiled interphase chromatid. Deoxyribonuclease must therefore enter into the nucleus for chromosome breakages to occur. Widnell and Tata (1964) reported a result which supports DNase migration:

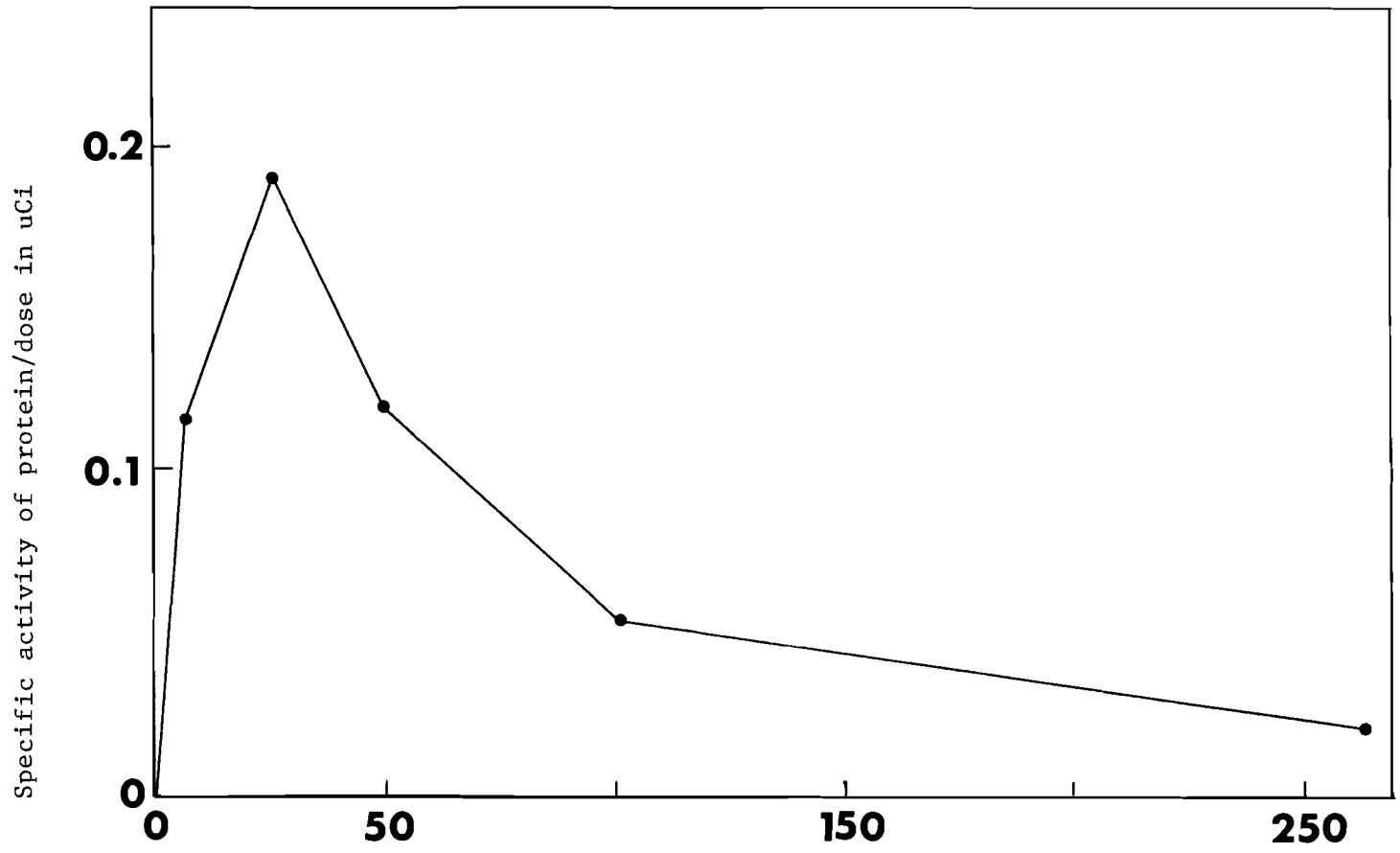


Figure 44. Time-course of the binding of (^{3}H) labelled hydrocarbons to total proteins of mouse skin. Results are expressed as specific radioactivity of protein (uCi/g) divided by the dose (uCi/mouse) given at zero time, plotted against time. ●, 3:4-benzpyrene. (Partially reproduced from Brookes and Lawley, 1964.)

thus DNA-dependent RNA polymerase activity in isolated nuclei is blocked by addition of DNase. Newton et al. (1962) also reported that DNA breakdown occurred shortly after infection with herpes virus. This report implies that lysosomal damage by herpes virus induced a liberation of DNase which in turn caused DNA breakage.

The studies conducted so far do not give any indication concerning mechanisms by which B(a)P binds to the nucleic acids. Many investigators worked to clarify the steps involved as B(a)P migrates from the cytoplasm to the nucleus. B(a)P is enzymatically converted to at least four phenols, three dihydrodiols, and three quinones and epoxides (reviewed by Yung et al., 1976). The active B(a)P metabolites which are converted by microsomal hydroxylase within the cells possess a relatively high tendency to react with the nucleophilic sites of the macromolecules. Thus, the B(a)P metabolite has a rare chance of reacting with nuclear DNA (Alexandrov and Thompson, 1977). This report implies that further metabolic activation of B(a)P metabolites by the other enzymes of microsomes or the nuclear envelope becomes necessary (Rogan et al., 1976). A similar interpretation of a two-step activation mechanism was reported by several workers (Sims et al., 1974; Weinstein et al., 1976; Spelsberg et al., 1977). Diol-epoxide intermediates (7,8-dihydro-7,8-dihydroxybenz(a)pyrene and 7,8-dihydro-7,8-dihydroxybenzo(a)pyrene-9,10-oxide) were reported to be the metabolites responsible for DNA-hydrocarbon binding (Brooks et al., 1975; Sims et al., 1974). Brooks et al. (1975) also indicated that the purine moieties of DNA react with diol-epoxide intermediates. A specific isomer of a diol-epoxide derivative of B(a)P, ($\pm$)-7B, 8a-dihydroxy-9a, 10a-7,8,9,10-tetra-hydrobenzo(a)pyrene, also

appeared to be a B(a)P metabolite in terms of covalent binding to nucleic acids (Weinstein et al., 1976). Another isomeric form of a diol epoxide derivative of B(a)P, 7a ,8B-dihydroxy-9B,10B-epoxy-7,8,9, 10-tetrahydrobenzo(a)pyrene, was proved to be a highly mutagenic intermediate of B(a)P metabolism (Wood et al., 1976; Huberman et al., 1976). This metabolite is suspected to be the ultimate carcinogenic species in B(a)P carcinogenesis. The reactions mentioned above are the events occurring as B(a)P molecules move into the nucleus of the cell. It is important to see the relationship between the duration of B(a)P treatment and cell transformation. To determine the tumorigenicity of B(a)P in the cells in vitro, suspected cells must be injected into a host animal to observe the behavior of the cells (Fedoroff, 1967). In this experiment, several conditions for B(a)P treatments were designed and inoculation of mice with B(a)P treated cells was carried out. The results are summarized in Table 3. Two mice developed palpable tumors (Figure 38) while the remainder showed genital abnormality (Figure 37). The mice inoculated with four day B(a)P treated cells developed 25-50% abnormality development. Some four day B(a)P treated cells were incubated additional days in a normal growth medium before inoculation. Abnormal development was not affected significantly by the additional incubation period. This is because during a four day incubation period, most of the cells may be transformed. This idea can be supported by the work of Berwald and Sachs (1965). They reported that two days of incubation with B(a)P is necessary for cell transformation. Ten day B(a)P incubation created an 80% rate of abnormal development. Higher genital development in 10 day B(a)P treated cells was possibly due to

the increased probability of cell transformation. Berwald and Sachs (1965) reported that the normal mouse cells in vitro showed spontaneous transformation sometime after 45 to 80 days in the culture. As can be seen on Table 3, the cells used for the inoculations were cultured from 41 to 47 days which is in the range of Berwald's figures. Thus, this spontaneous transformation factor may be involved in the results shown on Table 3. The mice inoculated with one day B(a)P treated cells developed no abnormalities (Table 3). One reason for the absence of abnormal development may be related to the age of the cells in culture, the cells used were cultured for six days, while the abnormality-producing cells were cultured from 41 to 47 days.

A tumor development in host mice by the inoculation of suspected cells must be further examined by histological means. Figures 40 through 43 are transverse sections of the ductus epididymis and the seminiferous tubules from abnormal and normal mouse testis. Figures 40 and 42 from the control mouse show a normal histological appearance. The ductuli are well packed and contain aggregates of spermatozoa. In contrast, Figures 41 and 43 show atrophy of ducts of the epididymis resulting in the formation of large interstitial spaces. Closer observations revealed a deterioration of the sterocilia as well. Figures 40 and 42 show the normal condition in which seminiferous tubules contain newly formed spermatozoa, primary spermatocytes and secondary spermatocytes. Figures 41 and 43 show atrophy of seminiferous tubules with a few supporting cells (Sertoli) located near the basement membrane. An abnormal increase in the number of interstitial Leydig cells was also seen. Occasionally, binucleated Leydig cells were observed. Detailed observation of the excised nodule revealed it to be histologically abnormal. It is, however,

difficult to determine if the abnormal nodule was caused by the direct action of B(a)P treated cells.

Allison and Mallucci (1964) reported that autoradiographic techniques failed to visualize accumulation of chemical carcinogens in the nucleus. Through this study, it was demonstrated that autoradiographic techniques could show the existence of B(a)P in the nucleus. Furthermore, autoradiograms of chromosomes gave an indication that interaction between B(a)P and chromosomes may have occurred.

SUMMARY

1). Benzo(a)pyrene accumulation in the cell occurred after 24 hours of incubation period.

2). Autoradiograms of chromosomes prepared from benzo(a)pyrene treated cells indicated that benzo(a)pyrene reacted with chromosomes.

3). Autoradiographic techniques were shown to have the capacity to localize the sites of benzo(a)pyrene accumulation in the cell.

4). Cytotoxicity studies of benzo(a)pyrene showed a time-dependent effect on the cultured mouse embryo cells.

5). A tumor induction study indicated that tumor inducibility in the host animal seems to be affected by the length of cell culture period.

LITERATURE CITED

LITERATURE CITED

Allison, A. C. and L. Mallucci. 1964. Uptake of hydrocarbon carcinogens by lysosomes. Nature. 203:1024-1027.

Allison, A. C. and G. R. Paton. 1965. Chromosome damage in human diploid cells following activation of lysosomal enzymes. Nature. 209:1170-1173.

Allison, A. C. and J. T. Dingle. 1966. Role of lysosomes in adrenal necrosis caused by dimethylbenzanthracene. Nature. 209:303-304.

Allison, A. C. 1968. Lysosomes in relation to cancer induction and treatment. Europ. J. Cancer. 3:481-490.

Arcos, C. J. and F. M. Argus. 1968. Molecular geometry and carcinogenic activity. pp. 348-435. Advances in Cancer Research. Vol. 11. Academic Press, Inc.

Alexandrov, K. and H. M. Thompson. 1977. Influence of inducers and inhibitors of mixed-function oxidase on benzo(a)pyrene binding to the DNA of rat nuclei. Cancer Res. 37:1443-1449.

Bailey, J. E. and N. Dungal. 1958. Polycyclic hydrocarbons in icelandic smoked food. Brit. J. Cancer. 12:348-350.

Berwald, Y. and L. Sachs. 1965. In vitro transformation of normal cells to tumor cells by carcinogenic hydrocarbons. J. Natl. Cancer Inst. 35(4):641-661.

Boyland, E. and B. Green. 1964. On the reported sedimentation of polycyclic hydrocarbons from aqueous solutions of DNA. J. Mol. Biol. 9:589-597.

Boyland, E. and P. Sims. 1965. Induction of adrenal damage and cancer with metabolites of 7,12-dimethylbenz(a)anthracene. Nature. 207:816-817.

Brookes, P. and P. D. Lawley. 1964. Evidence for the binding of polynuclear aromatic hydrocarbons to the nucleic acids of mouse skin: Relation between carcinogenic power of hydrocarbons and their binding to deoxyribonucleic acid. Nature. 202:781-784.

Brookes, P., P. Jones and J. Amos. 1975. The nature of the deoxyribonucleosides involved in the binding of carcinogenic hydrocarbons to the DNA of mouse embryo cells. Int. J. Cancer. 15:912-917.

Cook. J. W., C. Hewett and L. Hieger. 1932. Coal tar constituents and cancer. Nature. 130:926.

Cook, J. W. 1933. The isolation of a cancer producing hydrocarbon from coal tar. Chem. Society J. 398:395-405.

Dipple, A. 1976. Chemical Carcinogens. ACS Monograph. pp. 245-314.

Evans, V. J., N. M. Hawkins, B. B. Westfall and W. R. Earle. 1957. Studies on culture lines derived from mouse liver parenchymatous cells grown in long term tissue culture. Cancer Res. 18:261-273.

Evans, I. A. and J. Mason. 1965. Carcinogenic activity of bracken. Nature. 208:913-914.

Falk, H. L., A. Miller and P. Kotin. 1958. Elution of 3,4-benzpyrene and related hydrocarbons from soots by plasma proteins. Science. 127:474-475.

Fedoroff, S. 1967. Proposed usage of animal tissue culture terms. J. Natl. Cancer Inst. 38(4):607-611.

Gude, W. D., A. C. Upton and T. T. Odell. 1955. Giemsa staining of autoradiograms prepared with stripping film. Stain. Tech. 30:161-162.

Hecht, S. S., R. M. Ornaf and D. Hoffman. 1974. Chemical studies on tobacco smoke, XXXIII. N'-nitrosonornicotine in tobacco: Analysis of possible contributing factors and biologic implications. J. Natl. Cancer Inst. 54(5):1234-1244.

Henderson, E. B., J. R. Gordon, H. Menck, J. Soohoo, P. S. Martin and C. M. Pike. 1975. Original contributions: Lung cancer and air pollution in South central Los Angeles county. American J. of Epidemiology. 101(6):477-488.

Hirono, I., C. Shibuya, K. Fushimi and M. Haga. 1970. Studies on carcinogenic properties of bracken, Pteridium aquilium. J. Natl. Cancer Inst. 45(1):179-188.

Hirono, I., C. Shibuya, M. Shimizu and K. Fushimi. 1972. Carcinogenic activity of processed bracken used as human food. J. Natl. Cancer Inst. 48(4):1245-1249.

Hirono, I., K. Fushimi, H. Mori, T. Miwa and M. Haga. 1973. Comparative study of carcinogenic activity in each part of bracken. J. Natl. Cancer Inst. 50(5):1367-1371.

Haberman, E., L. Aspiras, C. Heidelberger, P. L. Grover and P. Sims. 1971. Mutagenicity to mammalian cells of epoxides and other derivatives of polycyclic hydrocarbons. Natl. Acad. Sci. USA. Proceeding. 68(12):3195-3199.

Kasper, C. B. 1971. Biochemical distinctions between the nuclear and microsomal membranes from rat hepatocytes. J. Biol. Chem. 246(3):577-581.

Khandwala, A. S. and C. B. Kasper. 1973. Preferential induction of aryl hydroxylase activity in rat liver nuclear envelope by 3-methylcholanthrene. Biochem. Biophys. Res. Commun. 54(4):1241-1246.

Kuratsune, M. and W. C. Hueper. 1960. Polycyclic hydrocarbons in roasted coffee. J. Natl. Cancer Inst. 24(2):463-469.

Kraybill, H. F. 1977. Advances in Modern Toxicology. Vol. 3. pp. 27-29. Hemisphere Publishing Co., Washington, D. C.

Lerman, L. S. 1960. Structural considerations in the interaction of DNA and acridines. J. Mol. Biol. 3:18-30.

Lijinsky, W. and P. Shubik. 1964. Benzo(a)pyrene and other polynuclear hydrocarbons in charcoal-broiled meat. Science. 145:53-55.

_____. 1965. Polynuclear hydrocarbon carcinogens in cooked meat and smoked food. Indus. Med. Surg. 34:152-154.

Liquori, A. M. 1962. Interaction between DNA and polycyclic aromatic hydrocarbons. J. Mol. Biol. 5:521-526.

Masuda, Y. and M. Kuratsune. 1971. Polycyclic aromatic hydrocarbons in smoked fish "Katsuobushi". Gann. 62:27-30.

Nebert, D. W. and H. V. Gelboin. 1968a. Substrate-inducible microsomal aryl hydroxylase in mammalian cell culture. I. Assay and properties of induced enzyme. J. Biol. Chem. 243(23):6242-6249.

_____. 1968b. Substrate-inducible microsomal aryl hydroxylase in mammalian cell culture. II. Cellular responses during enzyme induction. J. Biol. Chem. 243(23):6250-6251.

Newton, A., P. P. Dendy, C. L. Smith and P. Wildy. 1962. A pool size problem associated with the use of tritiated thymidine. Nature. 194:886-887.

Newbold, R. F. and P. Brooks. 1976. Exceptional mutagenicity of a benzo(a)pyrene diol epoxide in cultured mammalian cells. Nature. 261:52-54.

Owens, R. B., H. S. Smith and A. J. Hackett. 1974. Epithelial cell cultures from normal glandular tissue of mice. J. Natl. Cancer Inst. 53(1):261-269.

Owens, R. B. 1974. Brief communication: Glandular epithelial cells from mice: A method for selective cultivation. J. Natl. Cancer Inst. 52(4):1375-1378.

Pamukcu, A. M. and M. J. Price. 1969. Induction of intestinal and urinary bladder cancer in rats by feeding bracken fern (Pteris aquilina). J. Natl. Cancer Inst. 43(1):275-281.

Rogan, E. G., P. Mailander and E. Cavalieri. 1976. Metabolic activation of aromatic hydrocarbons in purified rat liver nuclei: Induction of enzyme activities and binding to DNA with and without monooxygenase-catalyzed formation of active oxygen. Natl. Acad. Sci. USA. Proceeding. 73(2):457-461.

Selkirk, J. K., E. Huberman and C. Heidelberger. 1971. An epoxide is an intermediate in the microsomal metabolism of the chemical carcinogen, dibenz(a,h)anthracene. Biochem. Biophys. Res. Commun. 43(5):1010-1016.

Shires, T. K. and K. M. Richter. 1966. The distribution of 3,4-benz(a)pyrene in replicating mammalian cells in vitro. Exp. Cell Res. 44:617-620.

Sims, P., P. L. Grover, A. Swaisland, K. Pal and A. Hewer. 1974. Metabolic activation of benzo(a)pyrene proceeds by a diol-epoxide. Nature. 252:326-328.

Spelsberg, T. C., T. H. Zytkovicz and H. L. Moses. 1977. Effects of metabolism on the binding of polycyclic hydrocarbons to nuclear subfractions of cultured AKR mouse embryo cells. Cancer Res. 37:1490-1496.

Thorsteinsson, T. and G. Thordarson. 1968. Polycyclic hydrocarbons in singed food in ice land. Cancer. 21:390-392.

Van Cantfort, J. and J. Gielen. 1975. Organ specificity of aryl hydrocarbon hydroxylase induction by cigarette smoke in rats and mice. Biochem. Pharmacol. 24:1253-1256.

Van Duuren, B. L., B. M. Goldschmidt and H. H. Seltzman. 1969. The interaction of mutagenic and carcinogenic agents with nucleic acids. Ann. N. Y. Acad. Sci. 153:744-757.

Vasiliev, J. M. and I. M. Gelhand. 1977. Mechanisms of Morphogenesis in cell cultures. International Review of Cytology. Vol. 50. pp. 159-163. Academic Press, Inc.

Wang, I. Y., R. E. Rasmussen and T. T. Crocker. 1972. Isolation and characterization of an active DNA-binding of benzo(a)pyrene from hamster liver microsomal incubation system. Biochem. Biophys. Res. Commun. 49(4):1142-1149.

Weinstein, B. I., A. M. Jeffery, K. W. Jennette, S. H. Blobstein, R. G. Harvey, C. Harris, H. Autrup, H. Kasai and K. Nakanishi. 1976. Benzo(a)pyrene diol epoxides as intermediates in nucleic acid binding in vitro and in vivo. Science. 193:592-595.

Weisburger, E. K. 1977. Cancer-causing chemicals. Chemistry. 50(1): 42-48.

Widnell, C. C. and J. R. Tata. 1964. Evidence for two DNA-dependent RNA polymerase activities in isolated rat-liner nuclei. Biochem. J. 92:313-315.

Wislocki, P. G., A. W. Wood, R. L. Chang, W. Levin, H. Yagi, O. Hernandez, P. M. Dansette, D. M. Jerina and A. H. Conney. 1976. Mutagenicity and cytotoxicity of benzo(a)pyrene arene oxides, phenols, quinones, and dihydrodiols in bacterial and mammalian cells. Cancer Res. 36:3350-3357.

Wood, A. W., P. G. Wislocki, R. L. Chang, W. Levin, Y. Anthony, H. Lu, H. Yagi, O. Hernandez, D. M. Jerina and A. H. Conney. 1976. Mutagenicity and cytotoxicity of benzo(a)pyrene benzo-ring epoxides. Cancer Res. 36(9):3358-3366.

Yamagiwa, K. and K. Ichikawa. 1918. Experimental study of the pathogenesis of carcinoma. J. Cancer Res. 3(1):1-29.

Yung, S. K., D. W. McCourt, P. P. Roller and H. V. Gelboin. 1976. Enzymatic conversion of benzo(a)pyrene leading predominantly to the diol-epoxide r-7,t-8-dihydroxy-t-9,10-oxy-7,8,9,10-tetrahydrobenzo(a)pyrene through a single enantiomer of r-7, t-8-dihydroxy-7,8-dihydro-benzo(a)pyrene. Natl. Acad. Sci. USA. Proceeding. 73(8):2594-2598.