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METABOLISM AND MACROMOLECULAR BINDING OF BENZO(A)PYRENE BY TRANSFORMABLE AND NON-TRANSFORMABLE HUMAN SKIN FIBROBLAST CELLS

The Ohio State University

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METABOLISM AND MACROMOLECULAR BINDING OF BENZO(a)PYRENE
BY TRANSFORMABLE AND NON-TRANSFORMABLE HUMAN SKIN FIBROBLAST CELLS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
Raman Tejwani, M.S.

* * * * *

The Ohio State University
1980

Reading Committee:
Dr. George E. Milo
Dr. Ronald W. Trewyn
Dr. Thomas E. Webb

Approved By

George E. Milo
Adviser
Department of
Physiological Chemistry
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Raman Tejwani
1980
Dedicated To My Parents
I would like to thank Dr. Milo for his constant guidance and supervision throughout this work. I am grateful to Dr. Nesnow at Research Triangle Park, North Carolina, Dr. Jeffrey at Columbia University, New York and Dr. Trewyn for assisting me with the HPLC analysis. I am also thankful to Inga, for helping with the chick skin organ culture system, Jim for helping with the gel electrophoresis and Aline for typing the manuscript. I sincerely appreciate the cooperation and support of my husband, Gopi and my son, Samir, without which this work could not have been completed.
VITA

March 11, 1950 ........ Born - New Delhi, India

1969 ............... B.S. (Chemistry), Delhi University, New Delhi

1972 ............... M.S. (Biochemistry), All India Institute of Medical Sciences, New Delhi

1973-1974 ........ Research Assistant, St. Louis University, St. Louis, Missouri

1974-1975 ........ Research Assistant, Roche Institute of Molecular Biology, Nutley, New Jersey

1976-1977 ........ Teaching Associate, Department of Physiological Chemistry, The Ohio State University, Columbus, Ohio

1977-1980 ........ Research Associate, Department of Physiological Chemistry, The Ohio State University, Columbus, Ohio

PUBLICATIONS


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FIELDS OF STUDY

Major Field: Biochemistry

Studies in Metabolism and Macromolecular Binding of Benzo(a)pyrene.
Professor George E. Milo
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ABBREVIATIONS

BP ................ Benzo(a)pyrene
MCA .............. 3-Methylcholanthrene
DMBA ............. 7,12-Dimethylbenzanthracene
TDMDA ........... 1,2,3,4-Tetrahydro dimethylbenzanthracene
DBA ............. Dibenzanthracene
AAF .............. 2-Acetyl aminofluorene
4-NQO .......... 4-Nitroquinoline-1-oxide
MNNG ............ N-Methyl-N'-Nitro-N-nitrosoguanidine
BPDE ............ Benzo(a)pyrene-7,8 diol-9,10-epoxide
PNH ............. Polynuclear hydrocarbon
HNF ............. Human neonatal foreskin
FBS ............. Fetal bovine serum
PDL ............. Population doubling
LP ............... Low passage
HP ............... High passage
MFO ............. Mixed function oxidase
AHH ............. Aryl hydrocarbon hydroxylase
BHT ............. Butylated hydroxytoluene
HPLC ........... High pressure liquid chromatography
VLDL ........... Very low density lipoprotein
LDL ............. Low density lipoprotein
HDL ............. High density lipoprotein
HRP/H2O2 ....... Horseradish peroxidase/hydrogen peroxide
CHAPTER I

INTRODUCTION

Polynuclear hydrocarbons (PNH) like benzo(a)pyrene (BP) are environmental pollutants which exert their carcinogenic effect by covalent binding to cellular macromolecules like DNA, RNA and protein. This binding is preceded by conversion of these proximate carcinogens to highly reactive metabolites by the microsomal mixed-function oxidases (MFO's) (49,86,115). The PNH's are initially oxygenated by this enzyme system, and subsequent metabolism of the oxygenated products involves either hydration of the epoxide intermediates to dihydrodiols, further oxygenation of the latter to diol-epoxides by the MFO's or conjugation of the oxygenated intermediates to water-soluble glutathione, glucuronide or sulfate conjugates (32,125,126,157,187).

Certain cellular proteins have been shown to be primary targets of chemical carcinogens in vivo and in culture. Thus, during transformation to malignancy in rat liver by aminoazodyes and 2-acetylaminofluorene (162-165) and in mouse skin as well as in cells in culture by PNH's (95,169), metabolites of the carcinogens are covalently bound to mainly very few protein species with a high degree of specificity, in proportion to their carcinogenic activities. The carcinogen-protein complexes isolated from the cytosol are relatively
basic proteins and belong to the electrophoretic class of proteins termed "h".

Miller first reported fluorescent protein bound derivatives of the PNH, BP in the skin of mice treated topically with the carcinogen (113). Kuroki and Heidelberger later observed binding of the PNH's BP, 3-methylcholanthrene (MCA), 7,12-dimethylbenzanthracene (DMBA) and dibenzanthracene (DBA), to the proteins of cultured embryonic cells from C3H mice and hamsters and cells derived from adult mouse prostates (94).

The azodye hepatocarcinogens have been shown to bind covalently to three rat liver proteins that have sedimentation coefficients of 4.7S, 3.5S and 1.7S. The 4.7S target protein also called the slow "h2-5S azo protein" is associated with the major fraction of the bound azodye and has been purified (165). This protein derives from an unknown target protein which has been detected immunologically in the cytosol of normal rat and mouse liver (150). The 3.5S protein, referred to as ligandin has been purified and characterized by Ketterer et al (84) and Morey and Litwack (123). In the rat, ligandin is found in the liver, kidney, intestinal mucosa, lungs, skin and gonads (46,85). It is particularly rich in the liver, where it comprises 4% of the protein of the soluble supernatant and is induced by substances like phenobarbital which also induce cytochrome P-450 (46). Ligandin has a wide range of binding affinities towards carcinogens and their metabolites, anionic steroid metabolites, bilirubin, heme and a number of exogenous anions (100). Singer and Litwack have observed binding of metabolites of MCA to rat liver ligandin (160). The rat ligandin has been shown to be
identical to glutathione-S-transferase B which is involved in the detoxification of various foreign compounds by converting them to glutathione conjugates (55).

Even though the two hepatocarcinogens 2-acetyl aminofluorene (AAF) and 3'-methyl-4-dimethylaminoazobenzene, and the two skin carcinogens MCA and DBA interact with target proteins of closely similar charge (h2 or h2-like), the principal conjugates are of considerably different molecular weights: aminoazo dye, 60,000-80,000, AAF, 150,000 and PNH, 40,000 (162). This diversity indicates that the target proteins of the three carcinogens are different at least in molecular size or if identical are altered in different ways by the three carcinogens. This raises the possibility that carcinogens may have specific protein receptors. It has been postulated that such a receptor may facilitate transport of the activated metabolites of the carcinogen to specific nuclear sites or may function in a detoxification role to facilitate excretion of the carcinogen (123).

Mainigi and Sorof have reported that the azodye-protein conjugate exists in two forms (107). In one form, the two moieties appear to be non-covalently held in vivo but after protein denaturation the complex is maintained covalently. The dual existence of the complex indicates that the carcinogen in the native complex is in an activated state capable of yielding a reactive electrophile. In agreement with this, the activated carcinogen, styrene epoxide (91,175) rapidly, reversibly and non-covalently complexes with horse liver alcohol dehydrogenase in its hydrophobic pocket (40) while also reacting covalently at a slow rate.
It has been suggested (107) that the nature of the carcinogen-protein linkage may determine the potential significance of the complex in carcinogenesis. If the complex is bound covalently, the carcinogenesis may result in part from an alteration of the target protein brought about by the carcinogen. However, non-covalent association would suggest that the protein might protect the activated carcinogen in a hydrophobic environment removed from cellular nucleophiles, and then transport it to the nucleus to interact with critical macromolecules. Bakay et al have thus reported the presence of small amounts of nuclear h$_2$-5S azoprotein complex in liver of rats fed with the carcinogen (7).

Recently, the hepatocarcinogen Aflatoxin-B$_1$ has been shown to bind to multiple proteins present in the cytoplasmic fraction of rat liver (106). The ability of the metabolites of Aflatoxin-B$_1$ to interact extensively with DNA, RNA and a wide variety of proteins suggests that the carcinogen interacts non-specifically with liver macromolecules and the unusual potency of Aflatoxin-B$_1$ may result from a capacity to inflict multiple molecular insults on target cells.

Three carcinogen-protein conjugates have been identified in mammary glands of mice treated with MCA (35). The principal conjugate has a molecular weight of 83,000 but has not been further characterized. Also, sucrose gradient sedimentation studies by Toft and Spelsberg (173) have shown that MCA is bound to a protein fraction in rat lung, not present in the liver. The lung carcinogen-protein conjugate has a sedimentation coefficient of 7S at low ionic strength which changes to
5S in presence of 0.3M KCl, a behavior similar to that observed with the steroid receptor proteins (131). This raises the possibility that cellular uptake of the PNH may be followed by binding to a specific site on a cytosol receptor protein. This inducer receptor complex may then be activated to a larger molecular weight size and/or to an altered molecular configuration and may interact with one or more nuclear sites.

Recent reports have also indicated binding of PNH's to plasma proteins, particularly the plasma lipoproteins, by a hydrophobic interaction (110,161). Thus, using gel filtration, Skalsky and co-workers have observed binding of BP, DMBA and MCA to rat serum proteins, preferentially to the lipoprotein fraction (161). Recently McKenzie et al have observed binding of BP to a low molecular weight protein (plasma fraction 1) present in the human plasma of smokers and non-smokers (110). The protein which has a molecular weight of 15,000 and a high specificity of binding for BP has been identified as a lipoprotein (D. Busbee, personal communication). These workers have also detected the plasma fraction 1 in cytoplasmic fractions prepared from cultured human lymphocytes but have shown it to be absent in homogenates of excised lung tissue (110).

The lipoproteins originally discovered by Macheboeuf in 1929, represent macromolecular complexes of specific lipid and protein components. Lipoproteins are important cellular constituents occurring both in the cell membrane and in the mitochondria and are also involved in transporting a large quantity of lipid material in the aqueous environment of the blood. Because of their high lipid content, the
Lipoproteins are of lower density than other proteins in the circulation. This property makes possible the isolation of lipoproteins by ultracentrifugation floatation in salt solutions. The human plasma lipoproteins have been separated into four major classes, which include the chylomicrons (d<0.94), very low density lipoproteins (VLDL, d=0.94-1.006), low density lipoproteins (LDL₁ and LDL₂, d=1.006-1.073) and high density lipoproteins (HDL₂ and HDL₃, d=1.073-1.21). The lipoproteins consist of a lipid core of non-polar triacylglycerol and cholesteryl ester, surrounded by more polar phospholipids, cholesterol and apoproteins which can solubilize the particle in the aqueous plasma around it. In the larger lipoproteins, chylomicrons and VLDL, the predominant lipid is triacylglycerol whereas the major lipids in HDL and LDL are cholesterol and phospholipid.

In the body, VLDL secreted by the liver undergoes stepwise delipidation and is converted to LDL, which functions to transport cholesterol into peripheral cells. HDL on the other hand has been postulated to play a major role in the transport of cholesterol from the peripheral cells to the liver, for removal from the body. Low density lipoproteins have also been implicated in the transport of cholesterol into cultured cells. Thus, cultured human skin fibroblasts grown in the standard growth medium containing lipoproteins have very low sterol synthesis activity (4,5) and obtain their cholesterol mainly from LDL (22). On the other hand, if the cells are grown in lipid deficient medium, enzymes involved in sterol synthesis are induced and the cells are able to synthesize cholesterol (20).
Studies of Brown and Goldstein (21) and others (27,92,132) have indicated that LDL binds to specific high affinity receptor sites on the human skin fibroblast cell surface, is internalized and hydrolyzed in lysosomes to yield amino acids and free cholesterol, which inhibits the synthesis of cholesterol and LDL receptor sites, and stimulates cholesterol ester formation in the cell. The presence of the LDL receptor sites has also been confirmed by Kruth and Vaughan (93), who have quantitated LDL binding in individual cultured human fibroblasts by indirect immunofluorescence. In human fibroblast and endothelial cells in culture, HDL has been shown to competitively inhibit LDL binding (116,166).

Thus, in cultured human fibroblast cells, hydrophobic interaction of PNH's with LDL may be followed by binding to specific LDL receptor sites on the cell surface and eventual uptake into the cell. Subsequent metabolism of the lipoproteins by specialized group of enzymes may then provide a site of activation for attached procarcinogen, in close proximity to the nucleus.

MATERIALS AND METHODS
Establishing Primary Fibroblast Cell Cultures:

Human neonatal foreskin (HNF) tissue samples were collected in the operating room in sterile glass vials containing 5 ml of sterile 1X Minimal Essential medium in Earle's balanced salt solution supplemented with 25mM Hepes buffer (Grand Island Biological Co., Grand Island, New York) to which were added 50μg/ml gentocin (Schering Veterinary Surgical Corp., Kenilworth, New Jersey), 8.8% sodium bicarbonate (NaHCO₃ was
sterilized by filtration through a 0.22 micron pore diameter Nalgene filter (Nalge Co., Rochester, New York) and 5% fetal bovine serum (Reheis Chemical Co., Phoenix, Arizona). The sterile NaHCO₃ solution was added to the medium until the color was orange red (pH 7.2). Tissue samples were processed within 24 hours of collection. All tissue processing and cell maintenance was carried out under sterile conditions in a vertical laminar air flow hood (Bellco Glass Co., Vineland, New Jersey).

Five ml of Detroit 550 special medium [1X MEM-Hepes buffer, 50μg/ml gentocin, 2mM glutamine, 0.1mM non-essential amino acids, 1mM sodium pyruvate (Microbiological Associates, Inc., Walkersville, Maryland), 8.8% NaHCO₃, pH 7.2] without fetal bovine serum (FBS) was placed in each of the four wells of a plastic Linbro tissue culture plate (Linbro Scientific Co., Inc., Hamden, Connecticut). Using two sterile disposable scalpels, the tissue was cut into three or four pieces, 8 to 10 mm in size. The tissue pieces were rinsed by swirling in the medium, then transferred and rinsed in the remaining three wells. In the fourth well, the tissue was minced to smaller pieces, 2mm. in diameter. The medium was removed from the fourth well using a plastic pipet and 5 ml of 1% collagenase (Worthington Biochemical corp., Freehold, New Jersey) was added to the minced tissue (1g of collagenase dissolved in 100ml of MEM-Hepes buffer was centrifuged at 12,000xg for 10 minutes and was sterilized by filtration through 0.45 micron and 0.22 micron Nalgene filters. 5 ml volumes were dispersed in sterile test tubes and stored at -19°C). This was then transferred to a preincubated 75 sq.cm. Lux flask
(Lux Scientific Corp., Newbury Park, CA.) containing 15ml complete medium (Detroit 550 special medium containing 20% FBS) and incubated at 37°C overnight. Following the incubation, the collagenase and tissue were centrifuged at 650xg in an IEC centrifuge (Model CRU-5000) for 7 minutes in sterile centrifuge tubes. The supernatant was decanted and the tissue and cells were washed twice by resuspension in complete growth medium. The final pellet was suspended in 5 ml of complete growth medium and then transferred with a plastic pipet to a preincubated 75 sq.cm. flask containing 10ml of complete medium. Twenty four to 48 hours after seeding, the medium was decanted and the cell sheet was rinsed three times with 10ml of Detroit 550 Special medium without FBS (incomplete medium) to remove cell debris and unattached cells. The cell sheet was refed with 15 to 20 ml of growth medium containing 20% FBS and the flask was incubated at 37°C in an atmosphere of 5% CO₂ - 95% air until confluency was reached.

Cell Maintenance

Cell cultures derived from HNF tissue were grown to confluency in growth medium supplemented with 10% or 20% (v/v) FBS. The medium was decanted and the cell sheets were rinsed once with 10ml of incomplete medium. The cells were removed from the 75 sq. cm. flasks by trypsinisation with one ml of 0.1% trypsin (Worthington Biochemical Corp.) and detachment of the cell monolayer was observed microscopically. 1% trypsin was prepared in MEM-Hepes buffer, pH 7.2 and was sterilized by filtration through 0.45 micron and 0.22 micron Nalgene filters. One ml volumes were added to sterile test tubes which were stored at -19°C.
One ml frozen trypsin was thawed immediately prior to use and was
diluted with 9 ml incomplete medium to give a final concentration of
0.1%. The trypsin was immediately neutralized with 10ml of incomplete
medium and the cell clumps were dispersed with a 10ml plastic pipet.
The appropriate number of detached cells were seeded into preincubated
75 sq.cm. flasks containing 15 to 20 ml of complete growth medium. The
flasks were continued to be incubated at 37°C and were refed with
complete growth medium at three day intervals, until the cell sheets
were confluent.

Cell Treatment: \([G^{3}\text{H}]\text{BP}(16-40 \text{ Ci/mmole, } 1\text{mCi/ml})\) was purchased from
Amersham Searle (Arlington Heights, IL.) and was diluted with
appropriate amounts of unlabeled BP(1 mg/ml) under red or gold light.

HNF cells (population doubling 5 to 28) growing on 75 sq.cm. flasks
were trypsinised and seeded at 5000 cells per sq.cm. For dual labeling
experiments, each flask was treated with 0.05 \(\mu\text{M L-(4,5-}\text{3}\text{H})\) leucine,
immediately after seeding. After 48 to 72 hours, when the cells were 40
to 60% confluent, the growth medium was removed and replaced with medium
containing 13\(\mu\text{M}[G^{3}\text{H}]\text{BP}\) for 12 to 15 hours. For dual labeling
experiments, the preconfluent cells prelabeled with L-(4,5-\(\text{3}\text{H})\) leucine
were treated with 2\(\mu\text{M of }[7,10-^{14}\text{C}]\text{BP}\) for 12 hours.

Preparation of a Crude \([G^{3}\text{H}]\text{BP-Cytoplasmic Protein Complex}: \) All
procedures were carried out at 4°C under gold light (F40G0 bulb, G.E.).
After carcinogen treatment, the cell sheets were rinsed with 10ml of
incomplete medium and the cells were harvested by scraping with a rubber
The cell suspension was centrifuged at 650xg for 7 minutes. The cell pellet was washed twice by resuspension in 10ml of cold Dulbecco's phosphate buffered saline, (0.01M Na₂HPO₄, 0.01M KH₂PO₄, 0.15 M NaCl, 3mM KCl, 1mM CaCl₂, 0.5mM MgCl₂·6H₂O), pH 7.4. The final cell pellet was suspended in 2 ml of 0.02M Na phosphate - 0.03M Tris-HCl-2.5mM Na₂EDTA - 0.5mM DTT, pH 7.5 and was homogenized in a 5ml prechilled stainless steel homogenizer (5X10⁻⁴ to 1.7X10⁻³ inch clearance) using a teflon pestle connected to a Tri-R stirrer. The homogenate was centrifuged at 1800xg in an IEC model B-20 centrifuge for 25 minutes to remove cell debris, nuclei and whole cells. Non-specifically bound [G⁻³H]BP was removed from the 1800xg crude cytoplasmic fraction by an adaptation of Korenman's charcoal procedure (90) described by Chamness and McGuire (28). 2.5g/liter of Norit A and 25 mg/liter of Dextran-T70 were suspended in 0.02M Na phosphate - 0.03M Tris-HCl-2.5mM Na₂EDTA - 0.5 mM DTT buffer (pH 7.5). 5ml of the suspension was centrifuged at 12,000xg for 10 minutes and the supernatant was discarded. 2.0ml of the 1800xg cytoplasmic fraction was added to the pellet. After gently stirring at 4°C for 10 to 15 minutes, the suspension was centrifuged at 12,000xg and the supernatant was carefully removed. Aliquots of the supernatant were placed in scintillation vials containing 10ml of Instagel liquid scintillation cocktail (Packard Instruments Co., Inc., Downers Grove, IL.) and the radioactivity was assayed in a Packard Tri-Carb liquid scintillation counter at a tritium counting efficiency of 38%.
Effect of pH on Binding of $[^3H]BP$ to the Cytoplasmic Protein

Complex: Two 75 sq. cm. flasks of HNF cells (PDL5) were split at 5000 cells per sq. cm. and when preconfluent were incubated with 13μM $[^3H]BP$ for 12 hours. Cells from each flask were harvested and homogenized in 2ml of 0.05 M Tris-maleate buffer (pH 5.5 to 8.5) containing 0.5mM DTT.

The 1800xg cytoplasmic fractions were prepared from each homogenate and were treated with dextran-coated charcoal at 4°C for 15 minutes. The suspensions were centrifuged at 12,000xg, aliquots removed from the supernatants and the radioactivity assayed.

Preparation of 100,000g $[^3H]BP$-Cytoplasmic Protein Complex. All procedures were carried out at 4°C under red or gold light. After carcinogen treatment, the medium was decanted and the cell sheet was rinsed once with 10ml of incomplete medium. The cells were harvested by scraping with a rubber policeman and centrifuged at 650xg for 7 minutes. The cell pellet was washed twice by resuspension in 10ml of cold Dulbecco's phosphate buffered saline, suspended in 2 ml of 0.02M Na phosphate - 0.03M Tris-HCl-2.5mM Na2EDTA-0.5mM DTT (pH 7.5) and homogenized in a prechilled homogeniser. The homogenate was centrifuged at 105,000xg (40,000 rpm) for 1 hour in an L2-65B or L8-55 Beckman Ultracentrifuge using a Ti50 or a Ti75 rotor. After centrifugation, the supernatant was carefully removed and treated with dextran-coated charcoal to remove non-specifically bound $[^3H]BP$. After gently stirring at 4°C for 10 to 15 minutes, the suspension was centrifuged at
12,000xg for 10 minutes and the supernatant was removed. Aliquots of the supernatant were placed in scintillation vials and the radioactivity was assayed.

**Sephadex G-200 Column Chromatography**

For column chromatography, Sephadex G-200 (dry particle diameter 40-120μm, Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) was suspended in a large excess of double distilled water and was allowed to swell for 72 hours at room temperature. The water overlay was then decanted and replaced with an appropriate volume of 0.02M Na phosphate - 0.03M Tris-HCl - 2.5 mM Na$_2$EDTA - 0.5mM DTT buffer (pH 7.5) containing 0.2% sodium azide. Immediately prior to use, part of the buffer was removed to form a fairly thick slurry which was deaerated. It was then slowly poured along the wall of a 0.9x60 cm chromatographic column with a gel and eluant reservoir attached to it. The gel was allowed to settle under gravity and was equilibrated with two bed volumes of the buffer.

The column was standardized with cytochrome C (12,500), chymotrypsinogen A (25,000), hen egg albumin (45,000), bovine serum albumin (67,000) and blue dextran (200,000) (Boehringer Mannheim, Indianapolis, ID, and Pharmacia Chemicals, Inc., Piscataway, N.J.). 5mg of the standards were dissolved in 0.5ml of 0.02M Na phosphate - 0.03M Tris-HCl - 2.5mM Na$_2$EDTA - 0.5mM DTT buffer (pH 7.5) and were applied on an equilibrated Sephadex G-200 column (0.9x58 cm.). Elution of 0.5ml fractions was carried out with the above buffer at a flow rate of 10 to 15ml per hour. The absorbance of each fraction was determined at 280nm in a PMQII spectrophotometer (Carl Zeiss, Oberkochen, West Germany).
The 1800xg or 100,000xg $[^{3}H]$BP-cytoplasmic protein complex isolated from HNF cells (population doubling 5 and 25) was then applied on the Sephadex G-200 column and eluted with 0.02M Na phosphate-0.03M Tris-HCl-2.5mM Na$_2$EDTA-0.5mM DTT buffer (pH 7.5). In specific experiments, Triton X-100 (0.1-1.0%) or ascorbate (1mM) were added to the elution buffer. Five hundred microliter fractions were collected at a flow rate of 10 to 15ml per hour and aliquots were removed to assay the radioactivity in each fraction.

**Organic Extraction of the $[^{3}H]$BP-Cytoplasmic Protein Complex:**

The fractions eluting with the low molecular weight peak or the high molecular weight peak were pooled and extracted with 3 volumes of ethyl acetate. The organic and aqueous phases were separated and aliquots were removed to determine the radioactivity associated with the organic soluble and water soluble fractions.

**Determination of Stability of the $[^{3}H]$BP-Protein Complex**

Low passage cells were seeded at 5000 cells per sq. cm. into 150 sq. cm. tissue culture dishes (Falcon Plastics, Oxnard, CA) and when preconfluent, were harvested. A 100,000xg cytoplasmic fraction was prepared in 2ml of 0.02M Na phosphate-0.03M Tris-HCl-2.5mM Na$_2$EDTA-0.5mM DTT (pH 7.5). 0.9ml of the cell free cytoplasmic preparation was incubated with 0.30μM of $[^{3}H]$BP (18 Ci/m mole) for ten minutes at 25° with constant stirring. The $[^{3}H]$BP-cytoplasmic protein complex was partitioned with dextran-coated charcoal as described earlier in this section and aliquots were removed to determine the radioactivity. The cytoplasmic preparation was then dialyzed against 100ml of the 0.02M
Na phosphate-0.03M Tris-HCl - 2.5mM Na₂EDTA - 0.5mM DTT buffer (pH 7.5) at 4°C for one hour. The dialysate was partitioned with dextran-coated charcoal and aliquots were removed before and after the dextran-coated charcoal treatment to assay the radioactivity.

**Nature of [G⁻³H]BP-Denatured Cytoplasmic Protein Complex:**

LP cells were seeded at 5000 cells per sq. cm. into 150 sq. cm. tissue culture dishes and when preconfluent, were harvested and a 100,000xg cytoplasmic fraction was prepared. It was denatured in a boiling water bath for 30 minutes and then incubated with 0.10µM [G⁻³H]BP (18 Ci/mmole) for 5 to 10 minutes at 25°, with constant stirring. The [G⁻³H]BP-cytoplasmic protein complex was partitioned with dextran-coated charcoal and applied on a Sephadex G-200 column. Elution of 0.5ml fractions was carried out at a flow rate of 12ml per hour. An aliquot of each fraction was removed and the radioactivity was assayed.

**Isolation of Lipoproteins by Ultracentrifugation**

Low passage (PDL 4) HNF cells grown on 150 sq.cm. tissue culture dishes were seeded at 5000 cells per sq. cm. Forty eight to 72 hours after seeding, the cell sheets were treated with 0.105µM [G⁻³H]BP (19Ci/mmole) for 12 hours. The cells were harvested and a 100,000xg [G⁻³H]BP-cytoplasmic protein complex was prepared in 2ml of 0.01M Tris-maleate buffer (pH 7.5) containing 1mM Na₂EDTA and 1mM DTT. After treatment with dextran coated charcoal, the [G⁻³H]BP-cytoplasmic fraction was layered over a potassium bromide gradient, to determine the radiolabel associated with the various lipoprotein classes.
Tris-maleate buffer was adjusted to densities of 1.006, 1.073, 1.125, 1.25 and 1.279 with solid potassium bromide. The gradient was prepared in an SW 41 rotor tube, by layering 2.2ml of the buffers in an order of decreasing density. After centrifugation at 200,000xg (40,000 rpm) in a Beckman Model L8-55 Ultracentrifuge for 24 hours, the various fractions were carefully removed with a pasteur pipette. Aliquots from each fraction were placed in scintillation vials containing 10ml of Instagel and the radioactivity was determined in a Beckman Model LS-9000 liquid scintillation counter with a tritium counting efficiency of 50%.

Preparation of Lipoprotein Deficient Serum

Lipoprotein deficient serum (LDS) was prepared by the method of Kruth et al. (92). Fetal bovine serum was centrifuged at 200,000xg (40,000 rpm) in a Beckman Model L8-55 Ultracentrifuge at 4°C for 48 hours, after adjustment of the density to 1.25 with potassium bromide. The top lipoprotein layer was removed and the serum was dialyzed at 4°C for 48 hours against four changes of 0.85% saline. The lipoprotein deficient serum was sterilized by filtration through 0.45 micron and 0.22 micron Millipore filters and stored at 4°C.

Uptake of \([\text{G}^3\text{H}]\text{BP}\) by Low Passage Cells Grown in LDS

HNF cells (PDL2), grown on 150 sq. cm. Falcon tissue culture dishes were passaged as described earlier in this section. The control cell cultures were maintained in complete growth medium supplemented with 10% FBS while the experimental cell cultures (from the same primary) were grown in complete medium supplemented with 10% LDS. 48 to 72 hours post-seeding, the cells were treated with 0.105µM \([\text{G}^3\text{H}]\text{BP}\)
(19Ci/m mole, 1mCi/ml) for 12 hours and a 100,000xg cytoplasmic fraction was prepared in 0.01M Tris-maleate-1mM Na₂EDTA - 1m M DTT buffer (pH 7.5) as described earlier. Aliquots were removed from the cytoplasmic fraction after dextran coated charcoal treatment and the radioactivity was assayed.

RESULTS

To determine the effect of pH on binding of [G⁻³H]BP to the cytoplasmic protein complex, cells preincubated with [G⁻³H]BP for 12 hours were harvested and a crude 1800xg cytoplasmic fraction was prepared in Tris-maleate buffer (pH 5.5 to 8.5). A significant portion of the radiolabel was found to be associated with the cytoplasmic protein complex at pH 7.5. When this 1800xg cytoplasmic fraction was isolated and chromatographed on a Sephadex G-200 column, a major portion of BP was associated with a protein complex of molecular weight 200,000, eluting at the void volume and a minor portion of the BP was associated with a protein complex of molecular weight 12,500 (Figure 1). Also, the ratio of area under the low molecular weight peak (peak II) to the high molecular peak (Peak I) was 0.038. When the 1800xg cytoplasmic protein complex isolated from LP cells was chromatographed on a Sephadex G-200 column in presence of 0.1% Triton X-100, there was no significant effect on the distribution of the radiolabel between the two peaks and the ratio of area under peak II to peak I was 0.034.

A 100,000xg cytoplasmic fraction isolated from [G⁻³H]BP treated LP cells (≤ PDL6) was also chromatographed on a Sephadex G-200 column. A major portion of the radiolabel co-chromatographed with the low
Figure 1: Sephadex G-200 chromatography of a 1800g [G-³H]BP-cytoplasmic protein complex isolated from low passage cells. PDL 5 HNF cells treated with [G-³H]BP for 12 hours were harvested and an 1800xg cytoplasmic fraction was prepared. 200,000 cpm were applied to a Sephadex G-200 column (58x0.9cm) and were eluted with 0.02M Na phosphate - 0.03M Tris-HCl - 2.5mM Na₂EDTA - 0.5mM DTT buffer (pH 7.5). 0.5ml fractions were collected and the radioactivity was assayed. Blue Dextran - 200,000; Aldolase - 158,000; Bovine Serum Albumin - 67,000; Hen Egg Albumin - 45,000; Chymotrypsinogen - 25,000 and Cytochrome C - 12,500 served as molecular weight standards.
Figure 1.
molecular weight protein and the ratio of under peak II to peak I was 6.5 (Figure 2). When Sephadex G-200 chromatographic analysis of the \([G^-\text{3H}]\text{BP}-\text{cytoplasmic protein complex}\) from LP cells was carried out in the presence of 0.1 to 1% Triton X-100 or 1mM ascorbate the distribution of the radiolabel between the low molecular weight peak and the high molecular weight was altered. There was an increase in the radiolabel associated with peak I and the ratio of area under peak II to peak I was 0.8 to 1.0 (Fig. 3).

To determine the Sephadex G-200 profile of the \([G^-\text{3H}]\text{BP}\) protein complex present in HP cells (>PDL20), a 100,000xg cytoplasmic fraction was prepared and chromatographed. In contrast to the LP cells, in HP cells a major portion of the BP was associated with a protein complex of molecular weight 200,000 and the ratio of area under the high molecular weight peak to the low molecular weight peak was 1.8 (Fig. 4). Also, when HNF cells prelabeled with L-[4,5-\text{3H}]leucine were labeled with \([7,10-\text{14C}]\text{BP}\), a major portion of the BP in the LP and HP cells co-chromatographed with cytoplasmic protein complexes of molecular weight 12,500 and 200,000.

When the \([G^-\text{3H}]\text{BP}-\text{cytoplasmic protein complexes}\) prepared from LP and HP cells were chromatographed on Sephadex G-200 columns and peaks I and II were partitioned with ethyl acetate, a major portion (90%) of the radiolabel under both the peaks was associated with the organic phase. Also, when \([G^-\text{3H}]\text{BP}\) was incubated with a cell free cytoplasmic fraction prepared from LP cells and the \([G^-\text{3H}]\text{BP-protein complex}\) was dialyzed at 4°C, within one hour greater than 96% of the radiolabel was
Figure 2. Sephadex G-200 chromatography of a 100,000g [G-3H]BP-cytoplasmic protein complex isolated from low passage cells. A 100,000g [G-3H]BP-cytoplasmic protein complex prepared from PDL 6 cells was chromatographed on a Sephadex G-200 column as described under Figure 1.
Figure 2: Chromatographic profile of various proteins.

- **Blue Dextran**
- **Aldolase**
- **Hen Egg Albumin**
- **Chymotrypsinogen**
- **Cytochrome C**

Log MW vs. Fraction No. and CPM.
Figure 3. Effect of ascorbate on the Sephadex G-200 chromatographic profile of a 100,000g [G-3H]BP-cytoplasmic protein complex isolated from low passage cells. A 100,000g cytoplasmic fraction prepared from PDL 6 cells was applied on a Sephadex G-200 column and eluted with 0.02M Na phosphate - 0.03M Tris-HCl - 2.5mM Na2EDTA-0.5mMDTT buffer (pH 7.5) containing 1mM ascorbate, as described under Figure 1.
Figure 3.
Figure 4. Sephadex G-200 chromatography of a 100,000g [G-3H]BP-cytoplasmic protein complex isolated from high passage cells. A 100,000g [G-3H]BP-cytoplasmic protein complex prepared from PDL 27 cells was chromatographed on a Sephadex G-200 column as described under Figure 1.
Blue Dextran

Aldolase

BSA

Hen Egg Albumin

Chymotrypsinogen

Cytochrome C

Figure 4.

Log MW

CPM

Fraction No.
dissociated from the protein complex. When the dialysate was partitioned with dextran-coated charcoal, only 1% of the \([G^3H]BP\) remained associated with the cytoplasmic fraction.

Sephadex G-200 chromatographic analysis of the \([G^3H]BP\)-protein complex, prepared by incubating \([G^3H]BP\) with a heat denatured 100,000xg cytoplasmic fraction from LP cells indicated greater than 90% of the radiolabel associated with the low molecular weight peak. But, when the peak II fractions were partitioned with ethyl acetate, a major portion (99%) of the radiolabel was associated with the aqueous phase.

To investigate the possibility of association of \([G^3H]BP\) with a lipoprotein complex in the cytoplasm, a 100,000xg cytoplasmic fraction prepared from carcinogen treated LP cells, was adjusted to a density of 1.25 (with solid potassium bromide) and centrifuged for 24 hours. When the top lipoprotein fraction was removed and assayed for radioactivity, 20 to 30% of the radiolabel was found to be associated with it.

To determine the specific classes of lipoproteins to which the BP was bound, a \([G^3H]BP\)-cytoplasmic fraction prepared from LP cells was centrifuged through a sequential potassium bromide gradient (density 1.006-1.279). The very low density, low density and high density lipoprotein fractions were then removed. Although the radiolabel was associated with all the lipoprotein classes, a major portion (30 to 40%) of the \([G^3H]BP\) was associated with the low density lipoprotein fraction corresponding to a density of 1.073 (Expts 1 and 2, Table).

In experiment 3, the \([G^3H]BP\)-cytoplasmic fraction was stored at 4°C overnight, before centrifugation through the potassium bromide gradient,
which resulted in a change in distribution of the radiolabel among the lipoprotein fractions. An increase in the radiolabel associated with the very low density lipoprotein fraction (density, 1.006) was accompanied by a decrease in the radiolabel associated with the low density lipoprotein fraction (Table 1), and 28-29% of the radiolabel was associated with both these fractions.

**TABLE 1**

Distribution of BP Radioactivity in Various Lipoprotein Classes

<table>
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<th>VLDL</th>
<th>LDL</th>
<th>HDL$_2$</th>
<th>HDL$_3$</th>
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<td>33.1</td>
<td>25.3</td>
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<tr>
<td>Experiment 1</td>
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</tr>
<tr>
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<tr>
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<td>d = 1.21</td>
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</table>

Low passage HNF cells from the same tissue sample were grown and passaged in Detroit 550 special medium supplemented with 10% LDS or 10% FBS to compare BP uptake in absence and presence of lipoprotein transport into these cells. When [$\text{G}^{-3}\text{H}$]BP-protein complexes prepared from the cytoplasm of these populations were assayed for radioactivity, no significant differences were observed in the radiolabel associated with the two fractions. Also, the uptake of BP by the cells in absence of lipoproteins was 72% of that in presence of lipoproteins.
DISCUSSION

Previous work from our laboratory has shown that significant uptake of BP in the cytoplasm of HNF cells in culture is observed at 12 hours; under these conditions, BP has been shown to bind to a cytoplasmic protein complex (39). Subsequently, optimum accumulation in the nucleus is observed at 24 hours. Although BP has been shown to induce DNA damage in logarithmically growing low passage (<PDL6) and high passage (>PDL20) cells (118), only the low passage cells can be neoplastically transformed by the PNH (39).

Preliminary studies to determine the nature of association of BP with the protein complex were done with a crude 1800xg cytoplasmic preparation. The binding of [G-3H]BP to the cytoplasmic fraction was maximum at pH 7.5. Also, the [G-3H]BP-cytoplasmic protein complex isolated from LP cells co-chromatographed mainly with a protein of molecular weight 200,000 on a Sephadex G-200 column. Since the crude 1800xg cytoplasmic fraction contains various organelles in addition to the soluble proteins, all the later studies were carried out with a 100,000xg cytoplasmic preparation.

Labeling of LP and HP cells with L-[4,5-3H]leucine before treatment with [7,10-14C]BP confirmed the binding of the PNH to a cytoplasmic protein complex. It was of interest to compare the nature of association of BP with the cytoplasmic protein complex in the transformable (low passage) and non-transformable (high passage) HNF cells in culture. Sephadex G-200 chromatographic separation of the cytoplasmic protein complexes from LP and HP cells indicated the
distribution of BP between protein complexes of molecular weights 12,500 and 200,000. In low passage cells, the amount of BP associated with the low molecular weight protein complex was 4 to 7 times that associated with the high molecular weight protein complex. However, in high passage cells, the amount of BP associated with the high molecular weight protein complex was 0.8 to 1.8 times that associated with the low molecular weight protein complex. A similar distribution of radiolabel was also obtained when the [G\(^3\)H]BP-protein complex, prepared by incubating [G\(^3\)H]BP with a cell free cytoplasmic fraction, was analyzed by Sephadex G-200 chromatography.

The [G\(^3\)H]BP associated with the two cytoplasmic protein complexes isolated from the LP and HP cells was extractable with ethyl acetate. Also, the total [G\(^3\)H]BP-cytoplasmic protein complex prepared from LP cells completely dissociated during dialysis. This indicated association of the PNH with the cytoplasmic protein complex by a non-covalent linkage. The non-covalent association of BP with the total cytoplasmic protein complex suggests that this complex may function as a carrier to transport BP to specific nuclear sites. But, on the other hand, when [G\(^3\)H]BP total cytoplasmic protein complex prepared from LP cells was incubated with isolated nuclei, in presence of an NADPH regenerating system, there was no radiolabel associated with the nuclear fraction. Since this translocation of the [G\(^3\)H]BP-cytoplasmic complex into isolated nuclei may require the presence of the microsomal AHH activity or other extrinsic factors, further studies need to be done to determine the role of the BP binding
protein. Also, since BP is bound to different cytoplasmic protein complexes in low and high passage cells, the transport of BP into the nucleus of these cells may involve an activation of the BP-cytoplasmic protein complex, similar to that observed with the steroids. Only binding of BP to the lower molecular weight protein complex (the predominant complex in the transformable cells) may result in an activation of the complex. Therefore, the accessibility of BP metabolites to specific nuclear binding sites in low and high passage cells may be different and may account for the susceptibility or refractoriness to BP-induced carcinogenesis in human fibroblast cells in vitro.

Although in low passage cells, a major portion of the BP was associated with the low molecular weight protein complex, some inter-individual variation was observed. Thus, in the LP cells derived from some tissue samples, BP was associated mainly with the high molecular weight protein complex and the Sephadex G-200 profile was similar to that observed with the HP cells.

Sephadex G-200 has been known to have an affinity for hydrophobic compounds like PNH's. Thus, in most of our experiments only 20% to 30% of the original radioactivity applied to the column was recovered. In presence of Triton X-100 and ascorbate, a 2 to 3 fold increase in recovery was accompanied by an increase in the radiolabel associated with the high molecular weight peak. This was probably due to solubilization of the high-molecular weight [G-3H]BP-cytoplasmic protein complex that was adsorbed to the Sephadex G-200. It was
interesting to observe that the \([G^{-3}H]BP\)-cytoplasmic protein complex derived from LP cells was more stable in presence of EDTA. Also, overnight storage at 4°C altered the Sephadex G-200 profile of the protein complex and resulted in an increase in the radiolabel associated with the high molecular weight peak. When heat denatured cytoplasmic protein complex prepared from LP cells was reacted with \([G^{-3}H]BP\), the PNH was associated with the cytoplasmic fraction by a covalent link. Thus, heat denaturation resulted in an aggregation of the protein complex. These properties are characteristic of a lipoprotein complex.

Serum lipoproteins have been shown to be unstable during isolation and storage. Prolonged storage at 4°C results in denaturation of the LDL fraction and is also accompanied by a change in the floatation pattern. These effects have been attributed to autoxidation of the lipids, which is minimized in presence of EDTA. This chelating agent complexes copper, which catalyzes autoxidation of both fatty acids and cholesterol. Also heat denaturation has been shown to result in partial aggregation of LDL.

Preliminary evidence from ultracentrifugation experiments also indicated that the 100,000xg cytoplasmic protein complex, to which BP was bound, was composed of various lipoprotein classes and a significant portion of the radiolabel was associated with the low density lipoprotein fraction. McKenzie et al have also observed binding of BP to a low molecular weight lipoprotein present in human plasma and in the cytoplasmic fraction of cultured human lymphocytes (110). It has been previously reported that when cultured human skin fibroblasts are grown
in complete medium containing lipoprotein (present in the serum), the LDL functions to transport cholesterol into these cells and is taken up by binding to high affinity receptors on the cell surface \((21,22)\). Our preliminary experiments suggested that the LDL present in the cytoplasmic fraction of LP cells to which BP was bound, was not derived from the fetal bovine serum in which the HNF cells were grown, since there was no significant reduction in the uptake of BP by cells grown in absence of lipoproteins. Thus, a major fraction of \([\text{G}^{-3}\text{H}]\text{BP}\) taken up by the LP cells was bound to lipoproteins already present in the cytoplasmic fraction.

In conclusion, previous studies by McKenzie et al and the present study have demonstrated binding of the PNH, benzo(a)pyrene to a lipoprotein fraction present in human plasma, lymphocytes and skin fibroblasts. In the human skin fibroblast cells in culture, BP is bound to the lipoprotein fraction by a non-covalent association. It has also been demonstrated that LDL in the circulation is degraded in various extrahepatic tissues, after being taken up by binding to specific receptors on the cell surface.

These observations suggest a possible mechanism of uptake of chemicals carcinogens like BP by various human tissues in vivo. Thus, on exposure to chemical carcinogens like BP, the PNH may accumulate in the blood and bind to the low-density lipoprotein fraction. This LDL, in non-covalent association with the BP, may then be taken up from the circulation by lymphocytes, fibroblasts and other extrahepatic tissues, by binding to specific cell surface receptors. Once in the cytoplasm, the BP-lipoprotein complex may eventually be transported to the nucleus.
CHAPTER 2
INTRODUCTION

The discovery of the carcinogenic effects of polynuclear hydrocarbons (PNH) was made about 200 years ago by a British physician Percival Pott (140), who attributed the high incidence of scrotal cancer among the chimney sweeps to their continual contact with coal tar. In 1915, Yamagiwa and Ichikawa first produced malignant tumors on the ears of rabbits by repeated applications of coal tar (186) and this represented the first example of experimental chemical carcinogenesis. In the mid 1930's Cook and co-workers isolated a few milligrams of benzo(a)pyrene (BP) from several tons of coal tar (34).

BP is a universal air pollutant and about 2000 tons of it are released into the environment of the United States each year from sources such as heat and power generation, refuse burning, industrial processes and transportation systems (33). BP is also a major carcinogen found in tobacco smoke, the inhalation of which is associated with an increased risk of bronchogenic carcinoma (33). The PNH can be either inhaled directly or more likely in combination with a carrier, like industrial soot, airborne particulates and particulates in tobacco smoke.
The covalent binding of a PNH (dibenzanthracene) to the DNA of mouse skin was first observed by Heidelberger and Davenport (64). Later work by Brookes and Lawley showed that the amount of hydrocarbon bound to the DNA of mouse skin was related to its carcinogenic activity as measured by Iball's index (19). Grover and Sims (53) and Gelboin (50), were the first to show increased binding of PNH's to DNA in presence of metabolizing enzymes from rat liver microsomal fractions. These early observations gave rise to the concept that metabolic activation of PNH's was necessary before they could interact with DNA.

The initial biological receptors for the PNH's have been identified as a group of enzymes known collectively as the cytochrome P-450 mediated monooxygenases (mixed function oxidases, MFO's) (49,86,115) which are involved in the detoxification of PNH's as well as in the formation of metabolites which are more cytotoxic and carcinogenic than the parent compound. MFO's are part of the endoplasmic reticulum and consist of two protein components - cytochrome P-450 and NADPH-cytochrome P-450 reductase and a lipid fraction - phosphatidylcholine. Cytochrome P-450 is the substrate and oxygen binding site of the enzyme, whereas the reductase serves as an electron carrier shuttling electrons from NADPH to cytochrome P-450.

Recent studies have shown the presence of six different forms of cytochrome P-450 in phenobarbital and 3-methylcholanthrene induced rat liver. One of these forms, the cytochrome P-448 is induced by PNH's in many mammalian systems, both in vivo and in culture. The specific enzyme system which utilizes cytochrome P-448 and metabolizes the PNH's
is referred to as aryl hydrocarbon hydroxylase (AHH). AHH activity has been identified in several adult human tissues including liver, lung, placenta, foreskin and breast (29,51,136,141). The enzyme activity has also been observed in human fetal liver (136), adrenal gland (136) and aortic smooth muscle (12) and human neonatal foreskin (1,118).

Several workers have also reported the existence of inducible AHH activity associated with purified nuclear envelope (43,78,139,176,177). Thus, incubation of BP with isolated rat liver or lung nuclei, in presence of NADPH, results in covalent binding of active metabolites of the PNH to DNA, RNA, histones and non-histone proteins (138). Also, Bresnick and coworkers have shown the presence of a part of the intracellular hepatic cytochrome P-448 in isolated nuclei, by a combined histochemical - immunological technique (18). It has been suggested, that due to its intracellular location, the microsomal AHH activity may play only a detoxification function and activation of PNH's like BP by the nuclear AHH activity may be followed by their binding to nuclear macromolecules.

The initial oxygenation of BP by the MFO results in the formation of 4,5-, 7,8- and 9,10- epoxides (62,146). The most stable of these, the 4,5- epoxide has been isolated under special conditions (151). The formation of the three epoxides has been confirmed by the presence of the corresponding dihydrodiols. These are formed by the action of the microsomal epoxide hydratase (EH) on the epoxide intermediates (52,65,179,189). Also, 1,2-epoxy-3,3,3-trichloropropane (TCPO), an EH inhibitor has been shown to inhibit the formation of the three
dihydrodiols (152) which can be overcome by readdition of the partially purified enzyme (EH) (65). All the three dihydrodiols formed enzymatically from BP have been shown to be trans isomers (189). Recently, all three trans-diols were found to be optically pure (-) enantiomers (187). Borgen et al have shown that when the dihydrodiols derived from BP are further metabolized by hamster liver microsomial preparations in the presence of DNA, the covalent binding of the 7,8-diol to DNA is 10 fold higher than BP itself (16). This observation has been confirmed using rat liver microsomal preparation. The further metabolism of the 7,8-diol followed by DNA binding is also observed in cultured hamster embryo cells (158), in cultured human bronchial mucosa (54) and in mouse skin in vivo (54).

Sims et al then showed that the hydrolysis products of DNA that were allowed to react with the 7,8-diol-9,10-epoxide (anti) of BP coeluted from a Sephadex LH-20 column with the hydrolysis products of DNA isolated from hamster embryo cells treated with the parent hydrocarbon (158). The 7,8-diol-9,10-epoxide (anti) of BP has also been shown to be the major reactive metabolite bound to DNA in cultured human bronchus, trachea, esophagus, colon, lung and skin (2,3,6,45,58,59).

Based on the above experimental evidence the following pathway of BP metabolism has been postulated. BP is oxygenated by the MFO to a 7,8 epoxide which is acted upon by EH to form a (-) trans 7,8-diol. This is further oxygenated, predominantly at the 9,10 double bond, trans to the 7-hydroxyl group, to form a 7β, 8α-dihydroxy-9α,10α-epoxy-7,8,9,10-
tetrahydrobenzo(a)pyrene (BPDE-I) and to a smaller extent at the 9,10 double bond, cis to the 7-hydroxyl to form a 7β, 8α-dihydroxy-9β, 10β-epoxy 7,8,9,10-tetrahydrobenzo(a)pyrene (BPDE-II). The diol epoxides are rapidly hydrolyzed in an aqueous medium to a pair of tetrols. BPDE-I is hydrolyzed trans-stereoselectively at the C-10 position to form a major 7,10/8,9-tetrol and a minor 7/8,9,10 tetrol while BPDE-II is hydrolyzed cis-stereoselectively at the C-10 position to form a major 7,9,10/8 tetrol and a minor 7,9/8,10 tetrol.

BP has been shown to be also metabolized to a 2,3-epoxide intermediate which rearranges to the 3-phenol (77). The 7- and 9-phenols have been reported to be formed by rearrangement of the 7,8- and 9,10-epoxides (65,179) and the other phenols may be direct hydroxylation products of the MFO's. The conversion of BP to a 6-phenol followed by formation of 1,6- 3,6-, and 6,12- quinones has also been reported (97,101). The primary metabolites of BP have been shown to be inactivated, mainly in the liver by conjugation with polar groups like glutathione, glucuronic acid and sulfate. These detoxification pathways are catalyzed by glutathione-S-transferase, uridine phosphate glucuronyl transferase and the sulfotransferase. At least seven forms of the glutathione-S-BP-4,5-epoxide-transferase have been purified to homogeneity (125). Five of these forms have been isolated from rat liver and two from the human liver (125). Each of these purified forms has a unique specificity towards BP-4,5-epoxide. A uridine diphosphate glucuronyl transferase has been identified in rat liver microsomes and has been shown to conjugate UDP-glucuronic acid with BP diols, epoxides
and phenols to form the respective glucuronides (126). These
detoxification products have also been detected in urine and bile
(44,180). The sulfotransferase enzyme activity has been detected in
lung organ cultures from rats, hamsters and humans. This soluble enzyme
has been found to catalyze the formation of sulfate conjugates with BP
3- and 9-phenols (32,127) and the 1-, 7- and 6- phenols as well as the
9,10-, 4,5- and 7,8-diols (128). The specificity and the amount of each
enzyme present in a given tissue of an individual may effect the
efficiency of the detoxification of the arene oxides of PHN's and thus
may play an important role in the removal of carcinogenic intermediates.
In contrast, recent studies of Kinoshita and Gelboin have suggested that
glucuronide conjugates of BP may be converted by β-glucuronidase, an
enzyme widely distributed in the animal tissues, to active intermediates
of the PNH that may be carcinogenic (87). This event may occur at
intracellular and organ sites distal to the initial sites of oxygenation
and conjugation.

MATERIALS AND METHODS
Preparation of Cytoplasmic and Nuclear Fractions

HNF cells between PDL 5 and 25, grown on 75 sq. cm. flasks were
serially passaged as described in Section B of Chapter I. After 48 to
72 hours, the cell sheets were refed with complete medium supplemented
with 0.072-26μM [G-3H]BP (27 Ci/mole) for 12 to 24 hours and the
nuclear and cytoplasmic fractions were prepared.

The nuclear fraction was prepared by a modification of the
procedure of Chauveau et al (130). Twenty four hours after carcinogen
treatment, the medium was decanted and the cell sheets were rinsed with 10ml of incomplete medium. All subsequent procedures were carried out at 4°C. The cells were harvested by scraping with a rubber policeman and centrifuged at 650xg for 10 minutes. The cell pellet was washed twice by resuspension in 10ml of incomplete medium and a small aliquot was removed for determining the cell number. The final cell pellet was suspended in 1ml of 10mM Tris maleate - 1mM DTT- 3mM Ca(Ac)2-2mM Mg(Ac)2 buffer, pH 7.5 and homogenised in a 5ml prechilled stainless steel homogeniser with a Teflon pestle attached to a Tri-R stirrer. 6.5 ml of 2.0 M sucrose in 10mM Tris maleate - 1mM DTT - 3mM Ca(Ac)2-2mM Mg(Ac)2 buffer (pH 7.5) was added to the homogenate, which was then layered over 5 ml of 2 M sucrose in an SW 41 Ti rotor tube. The nuclear fraction was centrifuged at 40,000xg (15,000 rpm) for 1 hour in a Beckman Model L2-65B Ultracentrifuge. The nuclear pellet was suspended in 0.5 ml to 1.0 ml of 10mM Tris maleate-1mM DTT - 3mM Ca(Ac)2-2mM Mg(Ac)2 buffer (pH 7.5) and a small aliquot was removed to determine the nuclear count in a Spencer Improved Neubauer hemocytometer. Contrast-interference Nomarski microscopic examination of the nuclear suspension indicated a 30-40% recovery of nuclei. The cytoplasmic fraction was prepared as described under Section B of Chapter I.

**Organic Extraction of [G-3H]BP Metabolites**

Extraction of either the BP-cytoplasmic protein complex or the nuclear fraction was completed with 3 volumes of ethyl acetate in the presence of 0.8 mg/ml butylated hydroxytoluene (BHT); the organic phase was passed over anhydrous sodium sulfate, filtered, dried under argon
and dissolved in 0.5 ml of acetone/methanol (2:1 v/v). Aliquots were removed for counting and the remaining sample was dried under argon and stored at -90°C. The sample was reconstituted with methanol-acetone-DMSO (2:1:1), nonradioactive BP metabolite standards added, and the extract chromatographed on a Dupont Instruments Model 848 High Pressure Liquid Chromatograph with 4 mm x 30 cm μ-Bondapak C18 column (Waters Associates) using an isocratic elution solvent of methanol-water-ethyl ether (66.3:30.4:3.3) at a flow rate of 1.4 ml/min. The effluent was monitored by uv spectrometry to identify metabolites, which were quantitated by collecting appropriate fractions of the effluent for liquid scintillation analysis. Six second fractions were collected for 11-12 minutes, then 12 second fractions were collected for 8-9 minutes and lastly, 60 second fractions were collected until the completion of the chromatographic run. Typical retention times in minutes for each metabolite were: BP-9,10-diol, 3.6; BP-4,5-diol, 5.5; BP-11,12-diol, 5.8; BP-7,8-diol, 6.5; BP-1,6-quinone, 8.6; BP-11,12-quinone, 9.1; BP-3,6-quinone, 9.6; BP-6,12-quinone, 11.0; BP-9-phenol, 16.5; BP-3-phenol, 19.1; BP, 37.5. The overall recovery of radioactivity from the column was greater than 90%.

Pulse Label Experiments

Generally labeled [G-3H]BP (37 ci/m mole) was diluted with unlabeled BP (50μg/ml) to a specific activity of 19 Ci/m mole. All BP dilutions were carried out under gold light (F40G0 bulb, G.E.) in an atmosphere of argon and the stock solution was stored at -20°C. The [G-3H]BP sample was about 95% pure as determined by HPLC.
HNF cells between PDL 5 to 24, grown on 150 sq. cm. tissue culture dishes (Falcon Plastics, Oxnard, CA) were passaged as described in Section B of Chapter I. When preconfluent, the cells were incubated with 0.105\mu M [G-3H]BP (19Ci/mmole, 1mCi/ml) for 24 hours.

A 100,000xg cytoplasmic fraction was prepared in 2 ml of 0.02M Na phosphate - 0.03M Tris HCl - 2.5mM Na₂EDTA - 0.5 mM DTT (pH 7.5), as described in Section B of Chapter I. A nuclear fraction was prepared as described in Section B of this Chapter and suspended in 1ml of 10mM Tris maleate - 1mM DTT-3mM Ca(Ac)₂ -2mM Mg (Ac)₂ buffer (pH 7.5). The extracellular medium sample was removed from one 150 sq. cm. tissue culture dish, 24 hours after carcinogen treatment.

**Pulse Chase Experiments**

HNF cells between PDL 5 and 25, grown on 150 sq. cm. tissue culture dishes were passaged as described in Section B of Chapter I and seeded at 5000 cells per sq. cm. Five tissue culture dishes were used for each of the time points studies. Forty eight hours after seeding, the cells were incubated with 0.105\mu M [G-3H]BP (19Ci/mmole, 1mCi/ml) in 20 ml of complete growth medium. Twelve hours after treatment, the medium was removed and the cell sheets were rinsed six times with 5 to 10ml of incomplete medium (growth medium without FBS). The cells were then refed with 20 ml of growth medium supplemented with 13\mu M unlabeled BP (1mg/ml).

At 24 hours, 48 hours, 72 hours, and 96 hours following initiation of [G-3H]BP treatment, cells from five 150 sq. cm. tissue culture dishes were harvested by scraping with a rubber policeman. The
cytoplasmic and nuclear fractions were prepared as described in Section B of Chapters I and II. The extracellular medium samples from one 150 sq. cm. tissue culture dish were also removed at these time intervals.

**Extraction of \([G-^3H]BP\) Metabolites:**

The extracellular medium, cytoplasmic and nuclear fractions were extracted with 3 volumes of ethyl acetate, the organic and aqueous phases separated and aliquots removed to assay the radioactivity. The organic phase was passed over anhydrous sodium sulfate, filtered, dried under argon and stored at -20°C.

**Comparison of \([G-3H]BP\) Metabolism in Randomly Proliferating and Synchronized Preconfluent Cell Cultures and in Confluent Cell Cultures.**

Low passage cells (<PDL 6) were seeded at 5000 cells per sq. cm. in 25 sq. cm. flasks, preincubated at 37°C with 5ml of Detroit 550 special medium or non-proliferating Dulbecco's modified Eagle's medium supplemented with 10% FBS. Twenty four hours (or 168 hours in case of confluent cell cultures) after seeding, the growth medium was removed and the cells were refed with 3.3 ml of complete growth medium containing 0.105μM \([G-^3H]BP\) (19Ci/m mole, 1μCi/ml). The medium was removed after 24 hours and partitioned with 3 volumes of ethyl acetate as described earlier in this section. The non-covalently bound hydrocarbon and its metabolites were then separated by High Pressure Liquid Chromatography (HPLC).
High Pressure Liquid Chromatography Analysis

The samples were dissolved in 0.4ml methanol (Spectrar grade, Mallinckrodt, Inc., St. Louis, MO) and centrifuged at 12,000xg for 2 minutes (Eppendorf Model 5412 microcentrifuge) to remove particulate matter. Twenty microliter samples were analyzed by HPLC with a Beckman Model 322MP Chromatograph fitted with a 150 x 4.6 m.m. Ultrasphere-ODS column (Beckman Instruments, Inc., Irvine, CA). Elution was initiated with 85% methanol. After 0.5 minutes, the methanol concentration was increased to 100% over a period of 1.5 minutes. The flow rate was maintained at 1 ml/minute. Fractions (0.2 ml) were collected directly in minivials and 2 ml of Instagel scintillation cocktail (Packard Instruments Co., Downers Grove, IL) was added. The radioactivity was assayed in a Beckman LS-9000 liquid scintillation counter at a tritium counting efficiency of 50%. Authentic BP metabolite standards were detected by UV absorbance at 254 nm.

RESULTS

HPLC Analysis of Cytoplasmic and Nuclear Metabolites

Low passage and high passage human fibroblast cells were treated with [G-3H]BP for 12 hours and the cytoplasmic fractions (100,000xg) were prepared in 2ml of Tris-phosphate-EDTA-DTT buffer (pH 7.5). The total cytoplasmic protein complexes isolated from the LP and HP cells were extracted with 3 volumes of ethyl acetate and the non-covalently bound hydrocarbon and its metabolites were co-chromatographed on a reverse phase column by HPLC with authentic reference standards. In the metabolite profiles of BP treated low passage cells (Figure 5),
Figure 5. HPLC analysis of BP metabolites associated with the cytoplasmic protein complex isolated from LP and HP cells. PDL 5 cells (bottom panel) and PDL 25 cells (top panel) were treated with [G-3H]BP for 12 hours and the organic extract of the [G-3H]BP-total cytoplasmic protein complex was analyzed by HPLC in presence of non-radioactive BP metabolite standards.
Figure 5.
unmetabolized BP was the only radiolabeled fraction. Similar results were obtained with BP radioactivity isolated from the total cytoplasmic protein complex from high passage cells, except that a small peak of radioactivity eluted prior to the BP-9,10 diol (Figure 5).

To determine the nuclear metabolites, low passage and high passage human fibroblast cells were treated with \([\text{G}^{-3}\text{H}]\)BP for 24 hours and the nuclear fractions were prepared in 1 ml of Tris-maleate - Ca(Ac)\(_2\)-Mg(AC)\(_2\)-DTT buffer (pH 7.5). After extraction with three volumes of ethyl acetate, the radioactivity associated with the organic phases was chromatographed as described above (Figure 6). The nuclei from high passage cells had radioactivity which co-chromatographed with BP-1,6, 3,6 and 6,12 quinones and BP-9-phenol; 89% of the counts were associated with BP. However, the radioactivity isolated from \([\text{G}^{-3}\text{H}]\)BP treated low passage cells co-chromatographed with BP-9-phenol and 90% of the counts eluted with BP. The unknown peaks at 2.4 and 4.5 minutes represent void volume radioactivity (pre BP-9,10 diol) and an unidentified metabolite respectively. Co-chromatography with BP-11,12-diol and BP-11,12- quinone indicated that the 4.5 minute peak represented neither of these metabolites.

24 Hour Pulse Label Experiment

The distribution of \([\text{G}^{-3}\text{H}]\)BP and metabolites was determined in the cytoplasmic, nuclear and extracellular medium fractions of low passage and high passage HNF cells, derived from the same tissue sample. The cells were treated with \([\text{G}^{-3}\text{H}]\)BP for 24 hours and the cytoplasmic
Figure 6. HPLC analysis of BP metabolites associated with the nuclear fractions isolated from LP and HP cells. PDL 5 cells (bottom panel) and PDL 25 cells (top panel) were treated with [G-3H]BP for 24 hours and the organic extract of the nuclear fraction was analyzed by HPLC in presence of non-radioactive BP metabolite standards.
Figure 6.
and nuclear fractions were prepared. The cytoplasmic fraction was used before treatment with dextran-coated charcoal. The extracellular medium sample was also removed at this time point.

The cytoplasmic, nuclear and medium fractions were partitioned with ethyl acetate and the organic-soluble and water-soluble fractions were separated. As seen in Table 2, organic solvent extractable metabolites accounted for a major portion of the radiolabel associated with the nuclear, cytoplasmic and extracellular medium fractions of the LP and HP

**TABLE 2**

**Distribution of BP Radioactivity**

*in Transformable and Non-transformable Cells*

<table>
<thead>
<tr>
<th></th>
<th>Transformable</th>
<th>Non-Transformable</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nucleus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Organic solvent-soluble</td>
<td>15.9</td>
<td>2.7</td>
</tr>
<tr>
<td>Water-soluble</td>
<td>0.7</td>
<td>0.1</td>
</tr>
<tr>
<td><strong>Cytoplasm</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Organic solvent-soluble</td>
<td>2.1</td>
<td>3.1</td>
</tr>
<tr>
<td>Water-soluble</td>
<td>0.3</td>
<td>0.1</td>
</tr>
<tr>
<td><strong>Extracellular Medium</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Organic solvent-soluble</td>
<td>433.5</td>
<td>545.4</td>
</tr>
<tr>
<td>Water-soluble</td>
<td>42.3</td>
<td>46.7</td>
</tr>
</tbody>
</table>

*p moles in each fraction were calculated by direct determination of radioactivity and the data was normalized to 10^6 cells.*
cells. Also, it was interesting to observe that the uptake of BP by the nuclear fraction of LP cells was six times higher than that by HP cells.

The non-covalently bound PNH and its metabolites were separated by HPLC and identified by co-chromatography with authentic reference standards (Figures 7,8). In all the fractions isolated from LP and HP cells, the various BP metabolites (BP-tetrols, diols, quinones and phenols) accounted for less than 10% of the total radioactivity eluting from the column; between 85 to 93% of the radiolabel was associated with the parent BP.

Although there were no significant BP-tetrols, diols and quinones formed in the nuclear fractions isolated from LP and HP cells (Table 3), a small amount of the radiolabel in these fractions was associated with the BP-phenol peak and an unknown peak eluting at 4.6 minutes. When the extracellular medium obtained from LP and HP cells was analyzed by HPLC, a minor portion of the radiolabel co-chromatographed with the BP phenol peak and the unknown peak (Table 3). In addition, the extracellular medium from HP cells had a small fraction of the radiolabel associated with the BP-tetrols. The cytoplasmic fraction from LP cells had 2 to 3 times more radiolabel associated with BP-tetrols, diols and the unknown peak compared to the high passage cells (Table 3). The major metabolite observed in the latter was BP-phenol.
Figure 7. HPLC analysis of BP metabolites associated with the nuclear, cytoplasmic and extracellular medium fractions of LP cells, 24 hours after carcinogen treatment. PDL 6 cells were treated with 0.1μM [G-3H]BP for 24 hours and the nuclear, cytoplasmic and extracellular medium fractions were prepared. Ethyl acetate extractable radioactivity from each of the fractions was analyzed by HPLC.
Figure 7.
Figure 8. HPLC analysis of BP metabolites associated with the nuclear, cytoplasmic and extracellular medium fractions of HP cells, 24 hours after carcinogen treatment. PDL 25 cells were treated with 0.1μM [G-3H]BP for 24 hours and the nuclear, cytoplasmic and extracellular medium fractions were prepared. Ethyl acetate extractable radioactivity from each of the fractions was analyzed by HPLC.
Figure 8.
TABLE 3

Distribution of BP and Metabolites in the Nucleus, Cytoplasm and Extracellular Medium of Transformable and Non-transformable Cells

<table>
<thead>
<tr>
<th></th>
<th>Extracellular</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nucleus</td>
<td>Cytoplasm</td>
<td>Medium</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LP</td>
<td>HP</td>
<td>LP</td>
<td>HP</td>
</tr>
<tr>
<td>Tetrols</td>
<td>0.37</td>
<td>0.50</td>
<td>1.20</td>
<td>0.66</td>
</tr>
<tr>
<td>Diols</td>
<td>0.69</td>
<td>0.56</td>
<td>1.91</td>
<td>0.70</td>
</tr>
<tr>
<td>Unknown</td>
<td>2.20</td>
<td>1.35</td>
<td>2.32</td>
<td>0.82</td>
</tr>
<tr>
<td>Phenols</td>
<td>1.50</td>
<td>1.06</td>
<td>3.26</td>
<td>2.60</td>
</tr>
<tr>
<td>Quinones</td>
<td>0.43</td>
<td>0.47</td>
<td>0.28</td>
<td>0.45</td>
</tr>
<tr>
<td>BP</td>
<td>89</td>
<td>90</td>
<td>85</td>
<td>89</td>
</tr>
</tbody>
</table>

The metabolism of $[G-3^H]BP$ was also studied in preconfluent randomly proliferating and synchronized cell cultures and in confluent cell cultures of LP human fibroblast cells. The cells were treated with $[G-3^H]BP$ for 24 hours and the non-covalently bound hydrocarbon and its metabolites were separated by HPLC. There was no significant metabolism (<7%) occurring in both the randomly proliferating and synchronised cell cultures and greater than 90% of the radiolabel was associated with the parent BP (Table 4). In contrast, there was a dramatic increase in the production of BP metabolites (41%) in the confluent cell cultures and
only 51% of the total radioactivity co-chromatographed with the parent BP (Figure 9). There was about a 7 to 10 fold increase in the formation of BP-diols and BP-tetrols and about a 2 to 3 fold increase in the radiolabel associated with the BP-quinone and the unknown peak (Table 4).

TABLE 4

Distribution of BP and Metabolites in the Extracellular Medium of LP Cells

<table>
<thead>
<tr>
<th>Percentage of Total Radioactivity</th>
<th>Proliferating</th>
<th>Confluent</th>
<th>Synchronized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetrols</td>
<td>2.2</td>
<td>24.9</td>
<td>2.4</td>
</tr>
<tr>
<td>Diols</td>
<td>1.1</td>
<td>7.4</td>
<td>0.8</td>
</tr>
<tr>
<td>Unknown</td>
<td>1.8</td>
<td>4.9</td>
<td>2.4</td>
</tr>
<tr>
<td>Phenols</td>
<td>0.9</td>
<td>2.1</td>
<td>1.6</td>
</tr>
<tr>
<td>Quinones</td>
<td>0.3</td>
<td>1.3</td>
<td>0.4</td>
</tr>
<tr>
<td>BP</td>
<td>91.8</td>
<td>51.3</td>
<td>90.5</td>
</tr>
</tbody>
</table>

Pulse Chase Experiments

The distribution of [G-3H]BP was determined in the extracellular medium, nuclear and cytoplasmic fractions of low passage and high passage cells, at specific time intervals following initiation of treatment. Cells were treated with 0.105 μM [G-3H]BP for 12 hours, after which time the experimental medium was replaced with growth medium
Figure 9. HPLC analysis of BP metabolites in the extracellular medium of confluent low passage cells. Confluent PDL 6 cells were treated with 0.1μM [G-3H]BP for 24 hours and the organic soluble radioactivity associated with the extracellular medium fraction was analyzed by HPLC.
containing 13 μM unlabeled BP. Samples were removed at 24, 48, 72 and 96 hours following the initiation of [G-3H]BP treatment.

Influx of [G-3H]BP into the cytoplasmic fraction of HNF cells, 24 hours after carcinogen treatment was followed subsequently by an optimum appearance of the parent radiolabeled BP in the nuclear fraction at 48 hours (Table 5). Between 24 and 48 hours a large efflux of bound [G-3H]BP from the cell into the extracellular experimental medium was observed. This release of radiolabeled BP then decreased as a function of time from 48 to 96 hours.

The nuclear, cytoplasmic and medium fractions from treated cells were partitioned with ethyl acetate and the organic and water-soluble fractions separated. A major portion of [G-3H]BP (>89%) was associated with the organic phase and water-soluble metabolites constituted a minor portion (<11%) of radioactivity in all the fractions of both low passage and high passage cells (Table 5).

At 24 and 48 hours after initiation of [G-3H]BP treatment, the nuclear fraction from LP and HP cells contained 4 to 7% of the various BP metabolites and there were no significant differences in the HPLC profiles of these cells (Figures 10 and 11). While the HPLC profile of [G-3H]BP metabolites from the nuclear fraction of LP cells, 72 hours after the initiation of PNH treatment, contained maximum amount of radiolabeled metabolites (15%), the nuclear fraction from HP cells had only 6% of the total radiolabel associated with the various metabolites (Figure 12). Also, the nuclear fraction from the LP cells had approximately four times more radiolabel chromatographing with BP-tetrols, twelve times more radiolabel chromatographing with BP-diols
<table>
<thead>
<tr>
<th>TABLE 5</th>
</tr>
</thead>
</table>

Distribution of BP in Low Passage (LP) and High Passage (HP) Cells

<table>
<thead>
<tr>
<th>pmoles BP/10^6 Cells^b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>24 Hours</td>
</tr>
<tr>
<td>LP</td>
</tr>
<tr>
<td>48 Hours</td>
</tr>
<tr>
<td>72 Hours</td>
</tr>
<tr>
<td>96 Hours</td>
</tr>
</tbody>
</table>

**Nucleus**

| organic-soluble | 1.96(94)^b | 2.24(94) | 3.96(95) | 2.79(98) | 2.40(96) | 1.60(94) | 1.28(96) | 1.44(99) |
| water-soluble    | 0.13       | 0.14     | 0.23     | 0.05     | 0.10     | 0.10     | 0.05     | 0.01     |

**Cytoplasm**

| organic-soluble | 0.25(93) | 0.42(91) | 0.18(90) | 0.24(96) | 0.11(92) | 0.23(89) | 0.11(92) | 0.17(90) |
| water-soluble   | 0.02     | 0.04     | 0.02     | 0.01     | 0.01     | 0.03     | 0.01     | 0.02     |

**Extracellular medium**

| organic-soluble | 101.65(96) | 114.58(98) | 74.84(96) | 118.91(96) | 87.59(96) | 94.77(97) | 78.89(95) | 81.45(91) |
| water-soluble   | 4.47      | 2.90      | 1.72      | 5.23      | 3.42      | 2.56      | 3.79      | 7.66      |

a) pmoles in each fraction were calculated by direct determination of radioactivity and data was normalized to 10^6 cells.

b) The numbers in parenthesis correspond to percentage of counts in the organic solvent soluble fractions.
Figure 10. HPLC analysis of [G-3H]BP metabolites in the nuclear fractions of LP and HP cells 24 hours after initiation of carcinogen treatment. PDL 3 (top panel) and PDL 25 (bottom panel) HNF cells, seeded at 2,000 cells per sq.cm. were treated with 0.105μM [G-3H]BP for 12 hours. The cell sheets were rinsed and treated with 13μM unlabeled BP for 12 hours. BP and the organic soluble metabolites in the nuclear fractions were analyzed by HPLC.
Figure 10.
Figure 11. HPLC analysis of \([G-^3H]BP\) metabolites in the nuclear fractions of LP and HP cells 48 hours after initiation of carcinogen treatment. PDL 6 (top panel) and PDL 28 (bottom panel) HNF cells, treated with \([G-^3H]BP\) for 12 hours were subsequently incubated with unlabeled BP for 36 hours. BP and the organic soluble metabolites in the nuclear fractions were analyzed by HPLC.
Figure 11.

PERCENT OF TOTAL RADIOACTIVITY

RETENTION TIME (minutes)
Figure 12. HPLC analysis of [G-3H]BP metabolites in the nuclear fractions of LP and HP cells 72 hours after initiation of carcinogen treatment. PDL 6 (top panel) and PDL 28 (bottom panel) HNF cells, treated with [G-3H]BP for 12 hours were subsequently incubated with unlabeled BP for 60 hours. BP and the organic soluble metabolites in the nuclear fractions were analyzed by HPLC.
Figure 12.
Figure 13. HPLC analysis of $[G-^{3}H]BP$ metabolites in the nuclear fractions of LP and HP cells 96 hours after initiation of carcinogen treatment. PDL 6 (top panel) and PDL 28 (bottom panel) HNF cells, treated with $[G-^{3}H]BP$ for 12 hours were subsequently incubated with unlabeled BP for 84 hours. BP and the organic soluble metabolites in the nuclear fractions were analyzed by HPLC.
Figure 13.
and two times more radiolabel associated with the BP-phenols, compared to the nuclear fraction from HP cells, at this time point (Table 6). At 96 hours after initiation of [G-3H]BP treatment, 11% and 6% of the total radioactivity co-chromatographed with the various metabolites in the LP and HP cells (Figure 13). There was also a four fold increase in the amount of tetrols and diols formed in the nuclear fraction of LP cells (Table 6).

**TABLE 6**
Distribution of BP and Metabolites in the Nucleus

<table>
<thead>
<tr>
<th></th>
<th>24 Hours</th>
<th>48 Hours</th>
<th>72 Hours</th>
<th>96 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LP</td>
<td>HP</td>
<td>LP</td>
<td>HP</td>
</tr>
<tr>
<td>Tetrol</td>
<td>1.2</td>
<td>1.2</td>
<td>0.6</td>
<td>1.0</td>
</tr>
<tr>
<td>Diol</td>
<td>0.9</td>
<td>0.7</td>
<td>0.6</td>
<td>0.8</td>
</tr>
<tr>
<td>Unknown</td>
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<td>77.0</td>
<td>88.9</td>
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a) An aliquot of the ethyl acetate extract of the nuclear fraction was analyzed by HPLC and the percentage of total radioactivity recovered from the column which eluted with unmetabolized BP and with each of the standard metabolite peaks was determined.

b) BP-4,5 quinone, BP-7,8 quinone and 6-hydroxymethyl BP coelute at this position.
In the HPLC metabolite profiles of the cytoplasmic preparations, the distribution of oxygenated metabolites was altered compared to the nuclear distribution over the 96 hour time period. At 24 hours, between 8 to 10% of metabolism was observed (Figure 14). There was a significant increase in the production of BP-quinones and BP-phenols in the cytoplasmic fraction from LP cells (Table 7). The cytoplasmic fraction prepared from LP and HP cells, 48 hours after initiation of [G-3H]BP treatment had 9 to 14% of radiolabel co-chromatographing with the BP metabolites (Figure 15). There was a 2 to 5 fold increase in the radiolabel associated with BP-tetrols, phenols and the unknown peak (Table 7). At the 72 hour time point, less than 10% BP metabolites were formed (Figure 16). There was a 2 to 3 fold increase in the BP-diols and BP-quinones formed in the LP cells and a significant increase in the radiolabel associated with the unknown peak, at this time point (Table 7). The cytoplasmic fraction from LP and HP cells had 8 to 13% BP metabolites formed (Figure 17) at the 96 hour time point. Although the cytoplasmic fraction from HP cells had a two fold increase in the radiolabel co-chromatographing with BP-tetrols, quinones, phenols and the unknown peak, there was a slight increase in diol production in the cytoplasmic fraction of the LP cells at this time point (Table 8).
Figure 14. HPLC analysis of [G-3H]BP metabolites in the cytoplasmic fractions of LP and HP cells 24 hours after initiation of carcinogen treatment. PDL 6 (top panel) and PDL 28 (bottom panel) HNF cells, treated with [G-3H]BP for 12 hours, were subsequently incubated with unlabeled BP for 12 hours. BP and the organic soluble metabolites in the cytoplasmic fractions were analyzed by HPLC.
Figure 14.

PERCENT OF TOTAL RADIOACTIVITY

RETENTION TIME (minutes)
Figure 15. HPLC analysis of \([G-^{3}H]BP\) metabolites in the cytoplasmic fractions of LP and HP cells 48 hours after initiation of carcinogen treatment. PDL 6 (top panel) and PDL 28 (bottom panel) HNF cells, treated with \([G-^{3}H]BP\) for 12 hours were subsequently incubated with unlabeled BP for 36 hours. BP and the organic soluble metabolites in the cytoplasmic fractions were analyzed by HPLC.
Figure 15.
Figure 16. HPLC analysis of [G-3H]BP metabolites in the cytoplasmic fractions of LP and HP cells 72 hours after initiation of carcinogen treatment. PDL 6 (top panel) and PDL 28 (bottom panel) HNF cells, treated with [G-3H]BP for 12 hours, were subsequently incubated with unlabeled BP for 60 hours. BP and the organic soluble metabolites were analyzed by HPLC.
Figure 16.

RETENTION TIME (minutes)

PERCENT OF TOTAL RADIOACTIVITY

B(a)P Phenols
B(a)P Diols
B(a)P Tetrols

B(a)P Quinones

B(a)P

Phenols
Diols
Tetrols
Quinones
Figure 17. HPLC analysis of [G-\textsuperscript{3}H]BP metabolites in the cytoplasmic fractions of LP and HP cells 96 hours after initiation of carcinogen treatment. PDL 6 (top panel) and PDL 28 (bottom panel) HNF cells, treated with [G-\textsuperscript{3}H]BP for 12 hours were subsequently treated with unlabeled BP for 84 hours. BP and the organic soluble metabolites were analyzed by HPLC.
Figure 17.

PERCENT OF TOTAL RADIOACTIVITY

RETENTION TIME (minutes)

B(a)P Phenols
B(a)P Diols
B(a)P Tetrois
B(a)P Quinones

B(a)P Phenols
B(a)P Diols
B(a)P Tetrois
B(a)P Quinones
TABLE 7

Distribution of BP and Metabolites in the Cytoplasm

<table>
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<th>24 Hours</th>
<th>48 Hours</th>
<th>72 Hours</th>
<th>96 Hours</th>
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<td>80.7</td>
<td>84.7</td>
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</table>

* See legends a and b under Table 6

There were no significant metabolites found in the extracellular medium of LP or HP cell cultures and greater than 90% of the radiolabel was associated with [G-3H]BP (Table 8).
### TABLE 8

Distribution of BP and Metabolites in the Extracellular Medium

<table>
<thead>
<tr>
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<th>24 Hours</th>
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<th></th>
<th>72 Hours</th>
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*See legends a and b under Table 6

### DISCUSSION

A variety of human as well as other mammalian cell types and many other lower organisms are able to metabolize PNH's like BP to dihydrodiols, epoxides, quinones, phenols and water-soluble conjugates (2,3,6,125,126,127). This metabolism is primarily an enzymatic oxidation catalyzed by the microsomal and/or nuclear MFO enzyme complex and is necessary to activate these PNH's to active proximate carcinogens (49,86,115,43,78,139,176,177).

The various metabolites of BP can be separated and identified by HPLC analysis, which is a rapid, efficient, reproducible and sensitive method. High pressure is used to obtain rapid analysis through long, narrow columns containing small size particles. Selkirk and co-workers
first reported separation of various metabolites of BP by this method (151).

Ekelman and Milo have reported neoplastic transformation of normal human skin fibroblast cells in culture, by BP (39). Studies of Milo et al have also indicated that although BP can induce DNA damage in logarithmically growing LP (<PDL 6) and HP (>PDL 10) cells (118), only the LP cells can be neoplastically transformed by the carcinogen (119).

Cell proliferation has been shown to be required for the fixation of the carcinogenic event. In our laboratory, neoplastic transformation of normal human fibroblasts by a variety of chemical carcinogens like BP is observed only when the cells are in an enhanced proliferative state (119,120). Thus, confluent, non-proliferating human skin fibroblasts cannot be transformed, even if the cells are allowed to divide immediately after carcinogen treatment.

Although cell proliferation is required for chemical carcinogen induced neoplastic transformation, most studies of BP activation make use of non-proliferating cell cultures (6,145,188). Thus, non-proliferating human skin fibroblast cell cultures, established by seeding cells at a low density in nutrient deficient medium, have been reported to yield ten times more oxygenated BP metabolites compared to the proliferating cultures (145). The significance of these oxygenated metabolites to carcinogenesis is unknown. Milo et al have studied the subcellular localization of BP in HNF cell monolayers by autoradiographic analysis (118) and have demonstrated that BP is first concentrated at the cell, then distributed randomly throughout the cytoplasm and finally concentrated at the nuclear membrane or in the
nucleus (118). Later studies by Ekelman and Milo have also demonstrated significant uptake of BP in the cytoplasm of HNF cells in culture at 12 hours; under these conditions BP has been shown to bind to a cytoplasmic protein complex (39). Subsequently, optimum accumulation in the nucleus is observed at 24 hours (39).

In our present study, HPLC analysis of the BP radioactivity separated from the BP-total cytoplasmic protein complex of LP and HP cells indicated that the major fraction was the parent BP. This was in contrast to other studies in which active metabolites of various carcinogens were shown to bind to cytoplasmic protein complexes in rat liver (107). It was also interesting to observe that unmetabolized [G-^H]BP made up the major fraction of the PNH associated with the nuclei from LP and HP cells. Also, there were only minor differences in the metabolites non-covalently bound to DNA in these transformable and non-transformable cells.

The metabolism of BP in the cytoplasmic, nuclear and extracellular medium fractions was examined in randomly proliferating HNF cells, under conditions of optimum PNH accumulation in the nucleus. After 24 hours of incubation with LP and HP cells, BP was metabolized primarily to organic-soluble metabolites, with only relatively small amounts (<10%) of water-soluble metabolites formed. When these organic-soluble and water-soluble metabolites were analyzed by HPLC, a low level of BP metabolism was observed and parent BP accounted for about 90% of the counts, in all the fractions of the transformable and non-transformable cell populations.
It was previously reported from our laboratory that LP cells contained measurable amounts of inducible microsomal AHH activity, with an optimum induction at 24 hours after carcinogen treatment (118). But as the HNF cells were passaged a decreased microsomal AHH inducibility was observed (118). In our present studies, 24 hours after BP treatment, there was no significant difference in the metabolism of BP in the LP and HP cells, although there was a significant increase the uptake of BP by the nuclear fraction of LP cells.

Since randomly proliferating human cell populations have an extremely efficient "error-free" DNA repair system and 90% of the DNA damage induced by a carcinogen can be repaired within hours (117), these cell populations can not be transformed in vitro. But neoplastic transformation is observed when the normal human cell populations are blocked in G1/S interphase of the cell cycle (synchronized), then released and treated with the carcinogen during the S phase of the cell cycle (119).

When BP metabolites in the extracellular medium of the randomly proliferating and synchronized LP cell populations were analyzed by HPLC, no significant metabolism was observed. Also, a major portion of the radiolabel co-chromatographed with the BP, even under conditions in which neoplastic transformation of LP cells is observed. In contrast, when non-proliferating LP cell cultures were treated with BP for 24 hours, there was a seven to ten fold increase in the amount of BP-diols and BP-tetrols formed and about a seven fold increase in the total BP metabolism observed in these cells, compared to randomly proliferating
LP cells. However, as previously reported from our laboratory, these non-proliferating HNF cells do not transport BP into the nucleus.

Thus, in conclusion, LP synchronized cells, which can be neoplastically transformed by BP in vitro, take up the carcinogen and transport it into the nucleus as the parent compound. In contrast, LP confluent cells which do not transport BP into the nucleus show a significant increase in BP metabolism. These observations suggest that the microsomal MFO activity may play only a detoxification function in these cells and activation of PNH's like BP by the nuclear MFO activity may be a more significant event prior to interaction with cellular macromolecules.

We also investigated the metabolism of BP in transformable and non-transformable human foreskin fibroblasts, 24 to 96 hours after carcinogen treatment. Cells exposed to a low concentration of [G-3H]BP (0.105 μM) for 12 hours were subsequently treated with a high concentration (13μM) of unlabeled BP for up to 96 hours to avoid a passive release of the [G-3H]BP into the extracellular medium. The optimum uptake of [G-3H]BP into the cytoplasm and localization into the nuclear fractions was observed at 24 hours and 48 hours after initiation of treatment. There was a greater uptake of [G-3H]BP by the nuclei of transformable cells, while efflux of the PNH into the extracellular medium of non-transformable cells was higher. Specifically, the uptake of [G-3H]BP by the nuclei from LP cells was 1.5 fold greater than uptake by the HP cells. Moreover, 2 to 3 times more unmetabolized [G-3H]BP was associated with the cytoplasm and
extracellular medium of HP cells. These differences, although minor, may play a significant role in the ability of the low passage human skin fibroblasts to be transformed in vitro.

Also, 72 hours and 96 hours after initiation of [G-3H]BP treatment, we observed an increase in the level of BP-diols and tetrols in the nuclear fraction of low passage cells. At this time point there was a decrease in the release of [G-3H]BP into the extracellular medium of these cells. These data indicated a low level of metabolism taking place in the nucleus of the low passage cells, possibly at the chromatin level.

There was no significant difference in the cytoplasmic and extracellular medium metabolites produced by low and high passage cells at any of the time points studied. This further suggested little role for the microsomal MFO activity in formation of active metabolites of [G-3H]BP in these cells.

Activation of BP to a diol-epoxide is generally considered to be required for DNA binding leading to carcinogenesis (2,3,6,45,58,59). Since the amount of tetrols and diols in the transformable human skin fibroblasts is very low, formation of the diol-epoxide may not be necessary for macromolecular binding and subsequent carcinogenesis in these cells. Rogan and co-workers have observed horseradish peroxidase/hydrogen peroxide (HRP/H_2O_2) catalyzed covalent binding of BP to calf thymus DNA, and postulate that it occurs by one electron oxidation with the formation of a radical cation intermediate (144). In view of the strong electron donating properties of polycyclic
hydrocarbons and the existence in vivo of several oxidants in the form of metal ion containing enzymes, one electron oxidation of the hydrocarbon to reactive radical cation intermediates capable of binding to cellular nucleophiles may constitute the critical first step in BP carcinogenesis in the low passage cells.
CHAPTER 3
INTRODUCTION

Several studies have documented that the BP-7,8-dihydrodiol-9,10-epoxide (BPDE) is the major metabolic intermediate involved in the covalent binding of the PNH BP to nucleic acids (2,3,6,45,58,59,158). Two steroisomers of BPDE have been synthesized. In isomer I, the 7-hydroxyl and the 9,10-epoxide groups are on the opposite sides of the plane of the ring while in isomer II, they are on the same side (184,185). Each stereoisomer has two enantiomers (75) and although in the 7R enantiomer, the 7-hydroxyl faces upwards with respect to the plane of the ring system, it faces downward in the 7S enantiomer. Theoretically, each enantiomer could also form two different guanine adducts, resulting from a 9,10 cis or trans addition of the guanine and thus allow both BPDE-I and BPDE-II to generate four different isomers of a BPDE-guanine adduct. The BPDE isomers I and II have been shown to be formed in vivo (170,190).

Several research groups have investigated the structures of the nucleic acid adducts formed when benzo(a)pyrene is incubated with mammalian tissues in culture. Thus, Weinstein et al have found that when BP is incubated with cultured bovine bronchial mucosa, one of the major adducts formed with RNA is identical to the product formed in the chemical reaction of BP-diol-epoxide-I (anti) with poly G, as shown by
an examination of the ribonucleoside derivatives obtained by hydrolysis of the polymers (181). Jeffrey et al have deduced from circular dichroism and NMR spectra that the adduct is formed between the 10 position of the diol-epoxide and the 2-amino group of guanine and is generated by 9,10 trans addition of guanine to the 7R enantiomer of BPDE-I (74). Meehan et al (111) have also reported that 92% of the reaction of the anti diol-epoxide with DNA involves covalent interaction of the diol-epoxide with deoxyguanosine, but minor adducts are also observed with deoxyadenosine and deoxycytidine. The major deoxyguanosine adduct has been shown to be formed by covalent bonding between the 10-position of the diol epoxide and the 2-amino group of guanine. A similar DNA adduct has also been observed on incubation of human and bovine bronchial explant tissue with BP (75).

In certain rodent cell cultures and in mouse skin exposed to BP, the DNA appears to contain adducts derived from both the 7R and 7S enantiomers and from both 9,10 trans and cis additions to the guanine residues (72,122). Each type of modification may result in subtle changes in the DNA conformation which could be of biological significance. Although guanine residues are the major nucleic acid targets, both BPDE-I and II can also react with adenine and cytosine residues in vitro and perhaps in vivo. In recent studies by Kakefuda and Yamamoto (79), BPDE modified superhelical double stranded circular SV-40 or plasmid Col El DNA was degraded with endonuclease S1, which cleaves single stranded and not double stranded regions in DNA. Although the carcinogen modification of G residues was about ten fold greater than A residues, the S1 nuclease preferentially excised the
modified A residues, which indicated a greater localized denaturation of the helix by these residues. Since BPDE modification of A residues produces a marked destabilization of the A-T base pairs, determination of the structure and conformation of the adduct may be of significance. Recent studies have also suggested that BPDE may react with the N-7 position of guanine but this adduct is readily lost from the DNA due to labilization of the glycoside bond followed by depurination (133). Depurination can lead to spontaneous or enzymatic chain scissions which could have major biological consequences.

Recent evidence obtained from fluorescence quenching (142) and optically detected magnetic resonance technique (96) has suggested that, in the BPDE-DNA adduct formed in vitro, the pyrene like chromophore of BPDE is not intercalated between the DNA base pairs but is located on the outside of the DNA helix, in the minor groove. Relatively little information is available on the actual distribution of the hydrocarbon bound to the DNA. Zeiger et al have isolated the mouse satellite and main band skin epidermal DNA after topical application of 7,12-DMBA to mouse skin and have shown that the amount of hydrocarbon bound per milligram of mainband or satellite DNA is similar (191). Meunier and Chauveau have also studied the microsome mediated binding of BP to calf thymus DNA and have found a higher level of binding of the PNH to the satellite DNA as compared to the mainband DNA (112). Zytkovicz and co-workers have reported that in mouse embryo cells treated with BP, the PNH is preferentially bound to a particular subnuclear fraction isolated by sucrose density gradient centrifugation of the sheared nuclei (192). The fraction has been shown to be more transcriptionally active than other fractions and has a higher RNA and non-histone protein content.
The accessibility of DNA in chromatin can determine the distribution of binding of a carcinogen. Studies of a variety of cell types indicate that chromatin has a repetitive subunit structure consisting of a string of beads or nucleosomes, of complexed DNA and histones, with connecting interbead or spacer regions of DNA (134). Jahn and Litman have demonstrated that when calf thymus DNA is incubated with BP in the presence of rat liver microsomal fraction and digested with specific nucleases, the PNH is mainly bound to the spacer region and the outermost portions of the nucleosome core (73).

Recently, Whitlock has reported the interaction of BPDE-I with HeLa chromatin core particles (183), which contain a 140 base pair segment of DNA, two molecules each of histones H2A, H2B, H3, H4 and about 0.4 mg of nonhistone protein per mg of DNA. Core particles were allowed to react with $^{14}$C-BPDE for increasing times and the modified histone and non-histone proteins were purified and analyzed by polyacrylamide gel electrophoresis and fluorography. The results indicated that, early in the reaction, BPDE bound predominantly to a single non-histone protein (M.W. 40,000). Later in the reaction BPDE bound to other non-histone proteins, followed by binding to the histones. The H2A and H2B histones were modified by BPDE to a greater extent than histone H3, while histone H4 was modified only slightly.

The rapidly labeled non-histone protein has been shown to be present in very small amounts in the core particle and is modified by BPDE to a high specific activity. When core particles are incubated with BPDE in presence of an excess of BP or the tetrol, 7,8,9,10-tetrahydro-tetrahydroxy benz(a) pyrene, the binding of the BPDE
to the rapidly labeled protein is reduced, suggesting that the unreactive PNH's compete with BPDE for the binding site. The role that such a protein may play in carcinogenesis is not known. It has been postulated (183) that interactions between BPDE and the histones may reflect a tendency of many non-polar compounds, such as PNH's, to preferentially interact non-covalently with hydrophobic regions of the histone proteins, with the result that, over a short term, less of the carcinogen may be available to interact with DNA. On the other hand, the effective lifetime of a potentially reactive molecule may be substantially longer in a hydrophobic environment than in an aqueous medium and the non-covalent interaction of a foreign compound with chromosomal proteins could conceivably prolong its lifetime, enough to actually increase the probability of its ultimate reaction with DNA.

Thus, chemical carcinogens like BP are metabolically activated to electrophilic intermediates that covalently bind to nucleophilic regions in nucleic acids. The change in the nucleic acid conformation resulting from this modification may be related to mechanisms by which DNA repair enzymes recognize and excise damaged regions of DNA. Studies with AAF (187) have indicated that the PNH adducts on the C-8 position of guanine are repaired more efficiently than those on the N-2 position since only the former are associated with a gross change in nucleic acid conformation. It is thus possible that the potency and tissue specificity of a carcinogen relate not only to its extent of cellular uptake, activation and DNA binding, but also the ability to bind to DNA with a conformation that minimizes recognition by DNA excision repair mechanism and yet cause functional impairment of the DNA.
MATERIALS AND METHODS

Preparation of Basic Nuclear Proteins

Low passage (transformable) cells were seeded at 5000 cells per sq. cm. into eight 150 sq. cm. tissue culture dishes and when preconfluent were incubated with 0.112 μM \( \text{[G}^3\text{H}]\)BP for 24 hours. The cells were harvested and nuclei were prepared as described under Section B of Chapter 2. The nuclear pellet was suspended in 0.25 N HCl at 4°C and the acid extraction of the histone and non-histone basic proteins was continued for three hours by gentle stirring of the suspension at 4°C. The suspension was centrifuged at 10,000xg for 20 minutes and the supernatant was dialyzed overnight against 20% sucrose - 0.9M acetic acid. The isolated histone and non-histone basic proteins were then separated by polyacrylamide gel electrophoresis.

Preparation of Polyacrylamide Gels

The entire procedure was performed at room temperature. The following solutions were used to make up the gels: A) 60% acrylamide (w/v) and 0.4% bis-acrylamide w/v (electrophoresis purity grade; Bio-Rad, Richmond, CA) were made up in double distilled water and were stored at 4°C in the dark B) 43.2% glacial acetic acid, v/v, and 4% TEMED, N, N, N', N'-tetramethylene diamine (Eastman Kodak Co., Rochester, N.Y.) C) 4.0M urea (Bio-Rad) and 0.2% ammonium persulfate. Solutions B and C were made up immediately prior to use.

In a 50ml flask, two parts of solution A and five parts of solution C were combined and aspirator drawn suction was applied to the contents of the flask for at least one minute to deaerate the acrylamide solution. One part of the solution B was then added to initiate
polymerization. Immediately after mixing, acid washed and dried electrophoresis tubes (placed in the polymerization rack) were filled up to 100mm of their length. Double distilled water was gently layered over the gel surface by a 3ml syringe with a small gauge needle fitted on it. The gels were allowed to polymerize for at least three hours at room temperature, prior to pre-electrophoresis.

Pre-Electrophoresis

The tubes were transferred to the upper section of the electrophoresis chamber, which was then inserted into the lower chamber filled with 500 ml of electrophoresis buffer (0.9N acetic acid). The water layer over the gels was removed and was replaced with the electrophoresis buffer. The upper section of the electrophoresis chamber was filled with 500ml of electrophoresis buffer and the chamber was leveled. Pre-electrophoresis was carried out at 1mA/tube, overnight.

Electrophoresis

The electrophoresis buffer from the top and bottom sections of the electrophoresis chamber was discarded. The buffer over the gel surface was removed and was replaced with 0.08 ml of the nuclear protein sample (50μg protein) in 20% sucrose and 0.9N acetic acid. Five microliters of 1% methylene blue was also added to one tube as a marker. All the tubes were then filled with the electrophoresis buffer, without disturbing the sample layer. The tubes were transferred to the upper section of the electrophoresis chamber, which was then inserted into the buffer filled lower chamber. Electrophoresis buffer was added to the upper section of the electrophoresis chamber and the chamber was leveled.
Electrophoresis of the histone and non-histone basic proteins was carried out for one hour at 1mA/tube and then for three hours at 2mA/tube, using a current regulated DC power supply unit (Buchler Instruments, Fort Lee, N.J.). The electrophoresis buffer and gels were cooled during the run by circulating water (room temperature) through the water jacket of the lower chamber. After completion of the electrophoresis, the gels were loosened from the side of the tubes by a 12ml syringe, with a small gauge needle attached to it, and were expelled into test tubes, for staining.

The gels were stained for three hours with 0.1% Amido-Black containing 40% ethanol and 7% acetic acid. Destaining of the gels was carried out in repeated changes of 7% acetic acid and 40% ethanol for 24 hours. The gels were stored in 7% acetic acid and 40% ethanol, in the dark.

The gels were sliced into 1mm. pieces using a Joyce Loebel Gel Slicer (Vickers Instruments, Inc., Woburn, Mass.). Each piece was placed in a scintillation vial containing 4ml of Instagel liquid scintillation cocktail and the radioactivity was assayed in an LS-9000 liquid scintillation counter, at a $^3$H counting efficiency of 50%.

Analysis of [G-3H]BP-DNA Adducts

HNF cells (PDL 1), growing on three 75 sq. cm. tissue culture flasks, were trypsinized and pooled, before seeding at 5000 cells per sq.cm. into 150 sq. cm. tissue culture dishes. The cells were passaged as described under Section B of Chapter I. Sixty tissue culture dishes of preconfluent HNF cells between PDL 6 to 25 (derived from the same tissue sample) were incubated with 0.112$\mu$M [G-3H]BP (19 Ci/m mole) for
24 hours. The cell sheets were harvested and the nuclear fraction was prepared as described under Section B of Chapter 2. The nuclear pellet, suspended in 0.5 ml of 0.01M Tris-HCl - 0.001M Na₂EDTA buffer (pH 7.5) containing 0.1% SDS was immediately frozen under liquid nitrogen and stored at -20°C.

The nuclear suspension was diluted to 2 to 5 ml with 10mM Tris-HCl, pH 7.0 containing 0.15M NaCl and 1% SDS and was extracted several times (3 to 6) with water saturated, redistilled phenol containing 0.1% SDS and 0.1% 8-hydroxyquinoline. The DNA was then precipitated by addition of 0.2M sodium acetate, pH 5.0 and two volumes of ethanol at -25°C overnight. The DNA was redissolved in water and was precipitated with ethanol in presence of sodium acetate, until at least 95% of the radioactivity was precipitable. The DNA (<1mg/ml) was dissolved in 0.01M Tris-HCl-0.1M NaCl - 0.005M MgCl₂ (pH 7.9) and was treated consecutively with 200 units DNase I for 4 hours, 5 units of alkaline phosphatase for 2 hours, 2 units of phosphodiesterase I for 4 hours, 2 units of phosphodiesterase II for 4 hours and 5 units of alkaline phosphatase for 4 hours.

The resulting deoxynucleosides were then chromatographed on a Sephadex LH-20 column. After application of the sample, the column was washed with water to remove unmodified deoxyribonucleosides. The modified adducts were eluted with 80% methanol and the solvent was evaporated under reduced pressure. The sample was dissolved in 10µl of methanol containing unlabeled BPDE-DNA adducts and was analyzed by HPLC. The sample was chromatographed on a Dupont Instruments Model 830 or 850 High Pressure Liquid Chromatograph fitted with a C₁₈ µ-Bondapak column.
(Waters Associates) using a concave gradient number 2 from 30 to 60% methanol in water at 50° and a flow rate of 1ml/minute (850) or at 750 psi (830). Samples were collected and the radioactivity was assayed.

RESULTS AND DISCUSSION

Recent studies have strongly implicated that, in vivo, the major metabolic intermediate of the chemical carcinogen BP is the diol-epoxide, which is enzymatically converted to two stereoisomeric forms, 7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (BPDE-I or "anti" form) and 7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzopyrene (BPDE-II or "syn" form). Both isomers have also been chemically synthesized as mixtures of (±) racemic enantiomers (184). These isomers have been found to form covalent adducts, preferentially with the guanine residues of nucleic acids, in vitro (181). The major in vivo adduct on incubation of human and bovine bronchial explant tissues with BP has been shown to be generated by 9,10 trans addition of guanine (at the N-2 group) to the 7R enantiomer of BPDE-I (75). Although a single BP deoxyribonucleoside adduct is formed in the bronchial tissue, multiple DNA adducts can occur in other tissues and species. Thus, Shinohara et al and Ivanovic et al have found both BPDE-I and BPDE-II deoxyribonucleoside adducts in BHK cells, mouse-embryo fibroblasts and hamster embryo cells (72,157).

The active metabolites of BP have also been shown to form covalent adducts with adenine and cytosine residues in vivo (72,75) and in vitro (76), although to a much smaller extent. Jeffrey and co-workers have identified four BPDE-adenosine adducts, formed by reacting poly A with (±) BPDE in vitro (76). These adducts have been shown to be formed by
cis- and trans- addition of the N-6 amino group of adenine to the 10 position of both enantiomers of BPDE, as determined by NMR, mass and CD spectral analysis. Although modification of the N-2 amino group of guanine occurs preferentially with 7R-BPDE, the N-6 amino group of adenine reacts preferentially with the 7S enantiomer (76). It is interesting to observe that the N-6 position of adenine is located in the major groove of the DNA while the N-2 position of guanine is located in the minor groove. Recent reports have also suggested that the conformation of DNA at sites of BPDE modification of adenine residues differs from the conformation at sites of guanine modification (80). Further studies are required to determine conformational differences associated with the various DNA adducts and their relevance to DNA repair, mutagenesis and carcinogenesis. Studies of Koreeda et al and Gamper et al have shown that in addition to the reaction with nucleic acid bases, BPDE can also modify phosphate residues of the nucleic acid backbone (48,89). Such a modification that may result in breaks in the nucleic acid backbone could be of major biological significance.

In the present study, LP and HP human skin fibroblast cells were treated with [G-3H]BP and the cellular DNA was isolated and digested to release the modified deoxyribonucleosides. The latter were separated by Sephadex LH-20 chromatography and then analyzed by HPLC using appropriate markers synthesized in vitro. Preliminary experiments indicated that although a major portion of the radiolabel in LP cells co-chromatographed with 7R BPDEI-dG and two unknown peaks, a small amount of radiolabel was also associated with the 7S-BPDEI-dG and BPDEII-dG adducts (Figure 18). Very little radioactivity coeluted with
Figure 18. HPLC profile of DNA adducts formed by low passage HNF cells incubated with [G-3H]BP for 24 hours. PDL 6 cells were treated with [G-3H]BP for 24 hours and the DNA was isolated and enzymatically digested. The modified deoxynucleosides were isolated by Sephadex LH-20 chromatography and co-chromatographed on HPLC with authentic reference standards.
Figure 18. The diagram shows a distribution of CPM (counts per minute) across fraction numbers. Peaks are labeled for 7S-BPDE I-dG, 7R-BPDE I-dG, BPDE II-dG, Tetrol, and dA-BPDE.
the adenosine adducts. The DNA isolated from HP cells was modified to a much less extent by BP and small amounts of 7R-BPDE I-dG and 7S-BPDEI-dG adducts were formed (Figure 19). These minor differences may play a significant role in the ability of only the LP cells to be neoplastically transformed in vitro by BP.

It has become increasingly evident that the complexity of PNH carcinogenesis can best be understood in terms of multiple mechanisms of activation, producing different ultimate metabolites. The predominance of one mechanism over the other may change depending on the nature of the activating enzymes present in the target organs of the various animal tissues. Since the cytochrome P-450 is a one electron acceptor (137), and has peroxidase activity (57), it may also activate some PNH's by one electron oxidation. Recent studies of Cavalieri and co-workers have indicated that in the rat mammary glands the carcinogenicity of BP may not be understood in term of an ultimate diol-epoxide intermediate, since the presumed ultimate carcinogenic metabolite BP-7,8-dihydrodiol, in contrast to the parent BP, is not tumorigenic in this system (25). The only PNH's that are carcinogenic in mammary glands are those with generally low ionizing potentials. This indicates involvement of radical cations in tumor initiation, since the ease of formation of these intermediates is dependent on the ionization potential of the PNH. Also, compounds like 5-methylchrysene and dibenz(a,h)anthracene, which have relatively high ionization potential, and can not be activated by one electron oxidation, are inactive in the mammary glands. These studies thus suggest that one electron oxidation of PNH's to radical cations may also be an important mechanism of activation leading to
Figure 19. HPLC profile of DNA adducts formed by high passage HNF cells incubated with [G-^3H]BP for 24 hours. PDL 25 cells were treated with [G-^3H]BP for 24 hours and the DNA adducts were analyzed by HPLC as described under Figure 18.
Figure 19

CPM

FRACTION NUMBER

17 34 51 68 85

Tetrol

7S-BPDEI-dG

7R-BPDEI-dG

BPDE II-dG

da-BPDE

16 32 48 64 80
carcinogenesis. Removal of one electron from an aromatic \( \pi \) system can generate a radical cation in which the charge distribution varies with the structure of the hydrocarbon and the position(s) of highest charge density is most susceptible to nucleophilic attack.

Rogan and co-workers also studied the mechanism of \( \text{HRP}/\text{H}_2\text{O}_2 \) mediated binding of PNH's to DNA using BP labeled with \(^{14}\text{C}\) and \(^3\text{H}\) at specific known positions (144). A 94% loss of tritium label from bound \([6-3\text{H},^{14}\text{C}]\text{BP}\) indicated binding of BP to DNA mainly at the C-6 position, which is the most likely position to react by one electron oxidation. When possible metabolites of \(^{14}\text{C}-\text{BP}\) were analyzed by HPLC, the only significant radioactivity observed corresponded to the parent BP. Since no BP-6-phenol was found to be bound to DNA, these studies indicated that the activation of BP by \( \text{HRP}/\text{H}_2\text{O}_2 \) occurred by one electron oxidation, with the formation of a radical cation intermediate, and not by formation of a diol-epoxide or a 6-oxybenzopyrene radical (formed from BP-6-phenol).

Despite localized changes in DNA structure and the covalent attachment of the bulky BP group to the 2-amino group of guanine, DNA modified by reaction with BPDE has been shown to be reassociated with histones to form a chromatin-like nucleosome structure (182). On the other hand, completely denatured DNA cannot function in the reconstitution of chromatin. These results suggest that although DNA must have a structure which is predominantly double stranded, localized distortions in base pairing do not interfere with the DNA-histone associations involved in the nucleosome formation. On the other hand, modification of DNA by BPDE has been shown to result in inhibition of
chain elongation, but not chain initiation, during transcription (182). This impairment in chain elongation might reflect conformational distortions at sites of BPDE modification and/or interference with the base pairing capacity of the modified guanine residues in the DNA template.

The in vitro studies with BPDE modified intact chromatin (182) or core particles (183) have indicated association of the carcinogen with both the spacer and the nucleosome regions of DNA, as defined by susceptibility to single strand specific S1-nuclease. In the present study, when the basic nuclear proteins isolated from BP treated low passage HNF cells were separated by polyacrylamide gel electrophoresis, a major portion of the radiolabel was associated with a non-histone acid extractable nuclear protein (Figure 20).

As previously reported from our laboratory, uptake of BP by HNF cells in culture is followed by its binding to a cytoplasmic protein complex and transport to the nucleus within 24 hours (39). Also, as described under Chapter 2, when HNF cells (LP and HP) are treated with BP for 24 hours and the metabolites are analyzed by HPLC, a major portion of the PNH (90%) bound to the cytoplasmic protein complex and transported to the nucleus is unmetabolized BP. Preliminary evidence for the presence of the 7R BPDEI-dG adduct in LP cells and to a lesser extent in the HP cells, 24 hours after carcinogen treatment and the binding of BP to a non-histone nuclear protein at this time point, indicates a low level activation of BP taking place at the level of chromatin.
Figure 20. Polyacrylamide gel electrophoresis of histone and non-histone nuclear proteins.
CHAPTER 4
INTRODUCTION

The study of the chemical carcinogen induced transformation of normal human cells into neoplastic cells \textit{in vitro} can be very useful for identifying environmental agents that may be responsible for causing cancer in humans. Berwald and Sachs were the first to demonstrate chemical carcinogen induced neoplastic transformation of cells \textit{in vitro} (10,11). After treating secondary cultures of Syrian hamster embryo cells with MCA or BP, they obtained cells capable of producing primarily fibrosarcomas, when injected into hamsters. The transformation of these cells was quantitated by the number of colonies exhibiting an altered morphology following carcinogen treatment. This dose dependent morphological transformation could be induced only by known chemical carcinogens and not by structurally related non-carcinogens.

Neoplastic transformation of fibroblast cells by chemical carcinogens has also been reported with Chinese hamster lung cells, rat embryo cells, guinea pig fetal cells, BHK cells, mouse 3T3 cell lines and a mouse C3H/10T1/2 cell line (17,42,81,88,108,121,148). However, since 80% of all human cancers are of epithelial origin, systems employing epithelial cells are also important in understanding human malignancies. Recently, neoplastic transformation by chemical carcinogens of epithelial cells derived from various tissues have also been reported (23,41,129).
The encounter of the cells with a chemical carcinogen results in modification of the DNA and almost every base in the macromolecule has been reported as a site of reaction with some carcinogen. The amino groups of guanine, adenine and cytosine are particularly susceptible to reaction with PNH's (36) and the C-8 sites of adenine and guanine are reactive with AAF (114). The ring nitrogens are sites of alkylation and the oxygens in the macromolecule have also been reported as reactive (159). Once bound to the DNA bases, these compounds introduce a distortion in the double helix which acts as a stimulus for DNA repair. There is evidence that these carcinogens may remain associated with DNA for relatively long periods and may even remain bound when the DNA replicates.

The predominant lesion formed by ultraviolet (UV) light in DNA is the pyrimidine dimer (154) but additional damage occurs through the formation of DNA - protein crosslinks (172). The greater portion of DNA damage produced by ionizing radiation has been attributed to the production (in oxygenated medium) of hydroxy radicals which interact with DNA to cause strand breaks (26). It has been recently suggested that active oxygen radicals may also be generated during metabolism of PNH's like BP (26). Thus, the repair of this type of damage is of significance, since production of active metabolites leading to DNA damage can be initiated by several environmental agents.

As in bacteria, several DNA repair activities have been shown in mammalian cells. These include rejoining of strand breaks (109,98), excision repair (102,104,143,155,167), replacement of altered bases
in DNA (99) and photoreactivation (83,168). All these repair activities have been associated with an "error free" DNA repair which is initiated immediately following damage and presumably before replication of the DNA (45,188). An "error prone" DNA repair may also take place following semi-conservative replication of the damaged DNA in mammalian cells (31,37,103). This post-replication repair mechanism is of significance since it is during this stage that mutation, and in multicellular organisms, tumorogenesis are initiated.

Following treatment of cultured cells with a carcinogen, at least one cell division is required for the fixation of the transforming event, and several cell divisions are required for the expression of the transformed phenotype. Borek and Sachs (13-15) have demonstrated that the morphologically transformed state of Syrian hamster embryo cells treated with X-rays is lost if the cells are maintained for two days in a confluent state, in which cell division does not occur. However, if the cells are allowed to undergo two cell divisions after treatment, the X-ray induced transformed state is "fixed". Kakunaga has studied the role of cell division in malignant transformation of mouse 3T3 cells treated with 3-MCA and 4-nitroquinoline-1-oxide (NQO) (80,81). His results clearly demonstrate that one cell division is needed for fixation and several additional cell divisions are required for expression of the transformed state.

Neoplastic transformation in vitro of normal human diploid fibroblast cells has also been achieved. Recently, Kakunaga has reported transformation of cells from a skin biopsy taken from the lip
of an adult female by 4-NQO and MNNG (82). Milo and DiPaolo have also reported transformation of human foreskin cell populations by the carcinogens propane sultone, B-propiolactone, Aflatoxin-B1, MNNG, ethylmethane-sulfonate and 4-NQO (119). In both studies, the neoplastically transformed cells have been shown to produce tumors when injected into athymic nude mice.

In our laboratory, we have defined several indices of human fibroblast cell transformation in response to a variety of chemical carcinogens. These include morphological changes, extended lifetime, growth in culture conditions toxic for untreated normal cells, increase in lectin agglutinability and alteration in the cellular prostaglandin levels. The anchorage-independent growth of transformed cells in soft agar has been the most consistent and reliable indicator of tumor production in athymic nude mice and on the chick embryonic skin organ culture system.

Previous reports from our laboratory have indicated that the PNH benzo(a)pyrene is taken up by human skin fibroblast cells in culture and is initially bound to a cytoplasmic protein complex before being transported to the nucleus (39). DMBA, on the other hand, is randomly dispersed throughout the cell and is not bound to the cytoplasmic protein complex (39). These differences may explain the induction of neoplastic transformation in the normal human skin fibroblast cells by BP and not by DMBA (Milo et al., unpublished data). PNH's like BP and DMBA require metabolic activation to bay region diol-epoxides before binding to cellular macromolecules like DNA (2,3,6,71,124). The bay
region diol-epoxides of BP and DMBA have been shown to be much more mutagenic and carcinogenic than the parent compounds (16,71,124,188). In contrast, other studies have demonstrated that substitution of methyl and fluoro groups in the bay region of these PNH's does not abolish their carcinogenic activity (60,156). Recent reports have also shown that 1,2-dihydro-DMBA and 1,2,3,4-tetrahydro-DMBA, analogs of DMBA that are partially or completely reduced in the bay region are highly mutagenic in the Ames assay (70).

It was thus, of interest, to test BP-7,8-dihydriodiol-9,10-epoxide-I (BPDE-I), the bay region diol epoxide and 1,2,3,4-tetrahydro-DMBA (TD MBA), the DMBA analog completely reduced in the bay region, for their ability to induce neoplastic transformation in low passage HNF cells in culture.

MATERIALS AND METHODS

Cell Synchronization

Actively growing preconfluent HNF cell monolayers between PDL 4 to 6 were trypsinized and reseeded at 5000 cells per sq. cm. into 75 sq. cm. flasks preincubated at 37°C with non-proliferating Dulbecco's Modified Eagle's medium (Biolabs Inc., Northbrook, IL.) supplemented with 50 μg/ml gentocin, 1mM sodium pyruvate, 10% dialyzed FBS (FBS was dialyzed against three changes of the above medium over a period of 24 hours, sterilized by filtration through 0.2 micron Nalgene filters and the pH was adjusted to 7.2 with 8.8% NaHCO3). The flasks were incubated at 37°C in a 95% air 5% CO2 atmosphere.

Twenty four hours after seeding, the non-proliferative medium was removed and the cells were refed with complete growth medium.
supplemented with 0.5U insulin (23.6U/mg, Sigma Chemical Co., St. Louis, MO) per ml of medium (A stock solution of 100 units/ml of insulin was prepared, immediately prior to use, by dissolving 4.24 mg in 10 ml of sterilized water, in presence of four drops of glacial acetic acid. It was sterilized by filtration through 0.4 micron and 0.22 micron Millipore filters and stored at 4°C).

Thirty four hours after seeding, the flasks were treated with 0.5μg/ml (ED25 dose) or 1μg ml (ED50 dose) of TDMBA, in acetone. Monolayers to which an equal volume of acetone was added served as controls. The synchronized monolayers were treated with BPDE-I, by the procedure of Heflich et al. (63). Thirty six hours after seeding, the complete growth medium was decanted and the flasks were refed with incomplete medium (Detroit 550 special medium without FBS) supplemented with 0.5 units/ml insulin. The flasks were treated with 0.057μM (ED25 dose) or 0.114 μM (ED50 dose) of BPDE-I in acetone. Three hours after treatment, the incomplete growth medium containing BPDE-I was removed and the cell monolayers were refed with complete growth medium supplemented with 0.5U/ml insulin.

Forty eight hours after seeding, the BPDE-I and TDMBA treated and control cell monolayers were trypsinized and reseeded at 5000 cells per sq. cm. (a one-to-two split) into 75 sq. cm. flasks containing 15ml of selection medium [Detroit 550 special medium supplemented with 2 ml of essential vitamins (M.S. Bioproducts, Walkersville, MD) per 100 ml of medium, 8 ml of non-essential amino acids per 100 ml of medium and 20% FBS]. Four to five days after reseeding, when the cells were
preconfluent, the monolayers were trypsinized and the cells reseeded at 2000 cells per sq. cm. (a one-to-ten split) in the selection medium. The control and transformed cell cultures were passaged and maintained in the selection medium till they were put into soft agar.

Growth of Morphologically Transformed Cells in Soft Agar

Bacto-agar (Difco Labs, Detroit, Michigan) used for making base plates was washed by mixing with distilled water, permitting the agar to settle out of the suspension, aspirating the water overlay and resuspending the settled agar until the water over the agar was clear. The 4% agar and 0.6% agar: double distilled water solutions were then sterilized by autoclaving for 15 minutes.

Immediately prior to use the 4% agar was heated to a liquid state and kept in a 45°C water bath. 2X RPMI 1629 medium (Grand Island Biological Co., Grand Island, New York) containing 2mM sodium pyruvate, 0.2 mM non essential amino acids, 4mM glutamine, 0.1mg/ml gentocin and 8.8% NaHCO3 (pH 7.0) was supplemented with 2 ml each of essential vitamins and essential amino acids per 100 ml of medium and 40% FBS, and was kept in a 45°C water bath. Equal volumes of the two solutions were mixed and five ml of this medium: agar mixture was placed in each well of four-well Falcon (60mm. diameter) plates. The plates were incubated at 37°C in a 95% air-5% CO2 atmosphere and used within a week of preparation.

To prepare the seeding agar overlay, 0.6% agar solution was heated to a liquid state and kept in a 45°C water bath. To 2X of Dulbecco's LoCal medium [prepared by dilution of 5X LoCal medium (Bio Labs Inc.)
with sterile double distilled water] were added 2 mM sodium pyruvate, 0.2 mM non-essential amino acids, 4 mM glutamine, 0.1 mg/ml gentocin and the pH was adjusted to 7.2 with 8.8% NaHCO₃. This medium was then supplemented with 2 ml each of essential vitamins and essential amino acids per 100 ml medium and 40% FBS and kept in a 45° water bath. Preconfluent flasks of passage 20 cells (Carcinogen treated or control) were trypsinized and then neutralized with 2X LoCal complete medium. One ml of the single cell suspension was added to a flask preincubated with 3 ml of Dulbecco's LoCal complete medium and 4 ml of 0.6% agar. Two ml of this seeding agar (50,000 cells) was layered gently in each well of a Falcon four well plate, over the solidified 2% agar: 1629 medium base. The plates were continued to be incubated at 37°C in a 95% air 5% CO₂ atmosphere for about three weeks. The wells were refed each week with 0.5 ml of 1X Lo Cal complete medium.

After three weeks the large, well defined boluses from three wells were transferred with a 23 gauge needle and syringe to a 75 sq. cm. flask preincubated with 15 ml of selection medium supplemented with 20% FBS. When big clones had formed, the flask was trypsinized and reseeded into a 75 sq. cm. flask. The cells were passaged and maintained in the selection medium till they were seeded on chick skins.

**Invasiveness of Anchorage Independent Cells**

The carcinogen treated cell population at PDL 36 were seeded onto embryonic chick skin (organ culture) to assay for cellular neoplasia (130).
The chick embryonic skin organ culture technique was modified in the following manner to optimize the sensitivity and frequency of success for a rapid assay of cellular neoplasia for chemical carcinogen treated cells.

Eggs were incubated for 9 to 10 days in a humidified egg incubator (Humidaire Incubator Co., New Madison, Ohio). The embryos were removed from the egg and the skin separated from the dorsal part of the embryo and placed on previously prepared agar dishes. The agar base contained 10 parts of 1% agar (Bacto-agar) in Earle's balanced salt solution without NaHCO₃, 4 parts of FBS and 4 parts of chick embryo extract, prepared from ten day old chicken embryos by high speed mincing. Organ culture grids (stainless steel, 60 mesh size, Falcon Plastics, Oxnard, CA) were placed in 35mm. diameter plastic dishes and the enriched agar base was poured over the grids to cover them to a depth of 1 to 2mm. A 6 to 8 mm. diameter section of skin was layered on the medium over the wire grid, with the dermis side up. A sterile glass ring 2mm. in thickness and 8mm. in diameter was placed over the skin. Then, 10⁵ cells from treated and untreated cultures were suspended in 0.025 ml of complete medium (supplemented with 20% FBS) and seeded into the rings. Seven 35mm. closed agar dishes were set in a 150 mm. diameter Falcon petri dish. One 60mm. open petri plate was placed in each 150mm. plate in order to insure a humidified atmosphere. The 150 mm. petri dish was then covered and placed in a 4% CO₂ humidified atmosphere at 37°C. Two days after seeding, the cultures were fed with 0.025ml of complete medium supplemented with 20% FBS. Four days after seeding, the skins
were removed and fixed in Bouin's solution. The fixed skins were cut in half and embedded in paraffin with the cut edge towards the face of the block. Five micron transverse sections were made, stained with hematoxalin and eosin, and examined under the microscope.

RESULTS

Chemical carcinogen induced neoplastic transformation of normal human cells in culture was carried out by the procedure of Milo and DiPaolo (119). Rapidly growing low passage cells (PDL 4) seeded into amino acid deficient medium for 24 hours and blocked at the G1/S interphase (synchronized) were treated with a growth promoter (insulin) to release them from synchrony and 10 hours later, in the S phase of the cell cycle, treated with TDMA. Normal human fibroblast cells were treated with BPDE-I by a modification of this procedure (63), due to the reactive nature of this active metabolite of BP and its much shorter half life (30 minutes). Randomly proliferating low passage cells (PDL 4) blocked in the G1/S interphase for 24 hours, were released from synchrony as described above and 12 hours later (2 hours into S phase) treated with the carcinogen in serum free medium. Three hours after carcinogen treatment, the cells were refed with complete growth medium. The TDMA or BPDE-I treated cells were passaged in selection medium for 15 PDL's and then serially passaged into soft agar.

Cells transformed by both TDMA and BPDE-I grew to spherical colonies 10 to 12 days following seeding of single cell suspension in soft agar. The colonies derived from TDMA treated cells grew more rapidly and to a larger size compared to the colonies derived from
BPDE-I treated cells. Also, the frequency of colony formation in soft agar, of TDMA and BPDE treated cells, was 84 and 26 per $10^5$ cells.

Three weeks after seeding on soft agar, the colonies were removed and the cells were passaged in the selection medium. These transformed cell populations exhibiting anchorage independence were checked for cellular neoplasia on chick embryonic skin. Both the TDMA and BPDE-I treated cells exhibited invasiveness on the chick skin. Histopathology of the invaded chick skin was interpreted as fibrosarcoma.

DISCUSSION

Various parameters have been used to determine the ability of carcinogen-treated, altered cells to induce tumor formation in immunologically suppressed or deficient host animals (athymic nude mice), the ultimate criterion of neoplastic transformation.

Transformation can cause alterations in cell-cell, cell-substrate and cell-medium interactions leading to changes in cell arrangement, growth pattern and cell density. This morphological transformation leads to loss of contact inhibition (38), reduction in serum requirement (66), alterations in adhesive properties (135) and cyclic AMP content (147), changes in surface protease activity (174) and enhanced agglutinability with plant lectins like concanavalin A or wheat germ agglutinin (24,69). The transformed cells in culture also lack size and shape uniformity of nuclei and have an increased nucleus-to-cytoplasm volume ratio (149). The degree to which each individual parameter of morphological transformation is expressed depends on the histological origin of the cell. In fibroblasts, loss of contact inhibition results
in an increase in saturation density and the transformed cells grow in "pile-up" multi-layers (119).

Normal fibroblast or epithelial cells in tissue culture require attachment to a substrate as a necessary prerequisite for growth and cannot proliferate while suspended in regular growth medium containing agar, agarose or methylcellulose. On the other hand, cells transformed by chemical carcinogens exhibit anchorage independence and can proliferate in soft-agar suspension to form spherical colonies (boluses).\(^1\)

Neoplastic transformation by chemical carcinogens also leads to the expression in the transformed cells of neo-antigens (tumor specific transplantation antigens) not present on the normal cells (67). The transformed cells in culture exhibit an increased life span (171), a faster growth rate characterized by a decreased cell generation time (9) and characteristic changes in rate of uptake of glucose (61) and amino acids (47). There are also various changes in the plasma membranes of transformed and tumor cells, which include incomplete glycosyl extension of glycolipids (56), changes in membrane microviscosity (8), loss of a high molecular weight, extracellular glycoprotein (LETS glycoprotein)(68), and changes in fucose containing membrane glycoproteins (178).

Although Milo and DiPaolo have reported neoplastic transformation of LP synchronized human skin fibroblast cells in culture with various chemical carcinogens (119), Kakunaga has reported transformation of randomly proliferating normal human skin cells by 4-NQO and MNNG (82). Randomly proliferating normal cells have an efficient and "error-free" DNA repair system (63) and over 90% of the damage from chemical carcinogens is repaired within 4 to 10 hours (117). Thus, chemical carcinogen induced neoplastic transformation of randomly proliferating low passage cells has not been observed in our laboratory.

Various studies have implicated the bay region diol epoxides of PNH's like BP (2,3,6,45,58,59) and 7,12-dimethylbenzanthracene (71,124), as the ultimate carcinogenic forms of these hydrocarbons. Inbasekaran and co-workers have recently demonstrated that 1,2-dihydro DMB and 1,2,3,4-tetrahydro DMB, compounds in which the bay region is partially or completely reduced, exhibit mutagenic activity in the Ames assay, in the absence or presence of a microsomal activation system (70). In the present study also, TDDBA was as effective as BPDE-I (the "ultimate carcinogenic" form of BP), in inducing transformation in human skin fibroblast cells. On the other hand, Milo et al have not observed induction of neoplastic transformation in these cells by the parent compound 7,12-DMBA (unpublished data).

Other studies have also demonstrated that introduction of methyl or fluoro groups into the ring of a PNH, which is potentially capable of metabolic activation to a bay region diol-epoxide, may not necessarily abolish carcinogenic activity. Thus, 7-methyl BP, 4-methyl DMB and
4-fluoro DMBA have all been shown to be sarcomogenic on subcutaneous injection in rats (60). Shear has also reported induction of skin carcinoma in mice by 7-methyl BP (156). These studies thus suggest that metabolic activation of PNH's to diol-epoxides may not be a crucial step in induction of a carcinogenic event in these cells. It is also possible that metabolic activation by the MFO's may occur at alternate molecular regions when reaction at preferred sites is blocked. This may result in the production of multiple activated metabolites, the predominant one being determined by which molecular regions are accessible for activation.

In the present study, when HNF cells were treated with BP, a major portion of the PNH bound to the cytoplasmic protein complex was subsequently transported to the nucleus as the parent BP (Section D, Chapters 1 and 2). Also, HPLC analysis of the BP metabolites covalently bound to DNA indicated a low level of BP metabolism taking place in these cells, possibly at the chromatin level (Section D, Chapter 3). A major portion of the PNH was bound to the DNA as unmetabolized BP. It is thus possible that in the human skin fibroblast cells, one electron oxidation of BP to a radical cation intermediate, followed by interaction with cellular macromolecules may be an important mechanism of activation leading to carcinogenesis.
BIBLIOGRAPHY


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80. Kakunaga, T., Requirement for Cell Replication in the Fixation and 
Expression of the Transformed State in Mouse Cells Treated with 

81. Kakunaga, T., The Role of Cell Division in the Malignant Trans­
formation of Mouse Cells Treated with 3-Methylcholanthrene. Cancer 

82. Kakunaga, T., Neoplastic Transformation of Human Diploid Fibroblast 
Cells by Chemical Carcinogens. Proc. Natl. Acad. Sci. (U.S.A.), 75, 

83. Kelner, A., Effect of Visible Light on the Recovery of Streptomyces 

of Carcinogen-Binding Protein from Livers of Rats Given 4- 

J., 126, 3p, 1972.

86. Kinoshita, N., and Gelboin, H.V., Aryl Hydrocarbon Hydroxylase in 
7,12-Dimethylbenz(a)anthracene Skin Tumorigenesis: On the 
Mechanism of 7,8-Benzoflavone Inhibition of Tumorigenesis. Proc. 

87. Kinoshita, N., and Gelboin, H.V., β-Glucuronidase Catalyzed 
Hydrolysis of Benzo(a)pyrene-3-Glucuronide and Binding to DNA. 

88. Kirkland, D.J., Armstrong, C., and Harris, R.J.C., Spontaneous and 
Chemically Induced Transformation of Rat Embryo Cell Cultures. 

89. Koreeda, M., Moore, P.D., Yagi, H., Yen, J.C., and Jerina, D.M., 
Alkylation of Polyguanylic Acid at the 2-Amino group and Phosphate 
by the Potent Mutant (±)-7α,8β-Dihydroxy-9β,10α-Epox-7,8,9,10- 

90. Korenman, S.G., Radioligand Binding Assay of Specific Estrogens 

91. Kotin, P., and Falk, H.L., Organic Peroxides, Hydrogen Peroxides, 

92. Kruth, H.S., Avigan, J., Gamble, W., and Vaughan, M., Effect of 
Cell Density on Binding and Uptake of Low Density Lipoprotein by 


97. Lesko, S., Caspary, W., Lorentzen, R., and Ts'o, P.O.P., Enzymatic Formation of 6-Oxobenzo(a)pyrene Radical in Rat Liver Homogenates from Carcinogenic Benzo(a)pyrene. Biochemistry, 14, 3978, 1975.


