To the Graduate Council:

I am submitting herewith a dissertation written by Eugene Leonard Schaefer entitled "The Metabolism, Sister Chromatid Exchanges and Macromolecular Binding of Benzo[a]pyrene by Mouse Hepatoma Cells in Culture." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biomedical Sciences.

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THE METABOLISM, SISTER CHROMATID EXCHANGES AND MACROMOLECULAR BINDING OF BENZO[a]PYRENE BY MOUSE HEPATOMA CELLS IN CULTURE

A Dissertation

Presented for the

Doctor of Philosophy

Degree

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DEDICATION

This dissertation is dedicated to Dr. John Vercellotti, who first introduced me to the wonders of biochemistry.

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ABSTRACT

A basic problem in cancer research is that individual humans and animals vary in susceptibility to initiation of cancer by carcinogens. Animals are used as test subjects in laboratory studies of cancer. However, animals are complex systems. Simpler systems such as animal cells in culture have been devised for the investigation of mechanisms of carcinogenesis. In the present research, the metabolism and some biological effects of the chemical carcinogen benzo[a]pyrene (B[a]P) were studied in a parent mouse hepatoma cell line, Hepa-1c1c7, and three of its variants, $BP^{r}c1$, MUL12, and $TAOc1BP^{r}c1$. The goal of this research was to see if biochemical and biological differences could be detected among subpopulations of a supposedly homogeneous population of cells. The study of metabolism was emphasized because it is thought that the metabolism of B[a]P is a prerequisite for other biological effects of B[a]P.

$BP^{r}c1$ metabolized B[a]P at a very low rate. Hepa-1c1c7 and MUL12 had high metabolic activity toward B[a]P. $TAOc1BP^{r}c1$ expressed intermediate metabolic activity which depended on culture conditions. Sister chromatid exchanges were induced by B[a]P in $TAOc1BP^{r}c1$ but not in $BP^{r}c1$. Metabolites of B[a]P were bound to nuclear RNA, DNA, and protein in Hepa-1c1c7, MUL12, and $TAOc1BP^{r}c1$, but not in $BP^{r}c1$. More B[a]P metabolites were bound to histones H3 and H2A in Hepa-1c1c7 and $TAOc1BP^{r}c1$ than in MUL12. Hepa-1c1c7 had the shortest population doubling time. MUL12 had the lowest saturation density, Hepa-1c1c7

and TAOclBP^{r}cl reached intermediate levels, and BP^{r}cl had the highest. MUL12 had the largest modal chromosome number, 100, compared with 58, 57, and 57 for Hepa-1c1c7, TAOclBP^{r}cl, and BP^{r}cl, respectively.

These results show that a cloned population of cells may be composed of subpopulations. The response of a cell population to B[a]P depends chiefly on the metabolic activities of these subpopulations.

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a	intercept of regression line
AHH	aryl hydrocarbon hydroxylase
anti	a stereoisomer with substituents on opposite sides of a plane
b	slope of regression line
BP or B[a]P	benzo[a]pyrene
BPDE	7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene
BrdU	5-bromodeoxyuridine
CHO	Chinese hamster ovary
Ci	Curie
deHase	dehydrogenase
d.f.	degrees of freedom
7,8-diol	7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene (Other diols are defined similarly.)
7,8-dihydrodiol	7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene (Other dihydrodiols are defined similarly; "diol" and "dihydrodiol" are used interchangeably, although "dihydrodiol" is the more correct term.)
DMSO	dimethylsulfoxide
dpm	disintegrations per minute
EDTA	ethylenediaminetetraacetic acid
EH	epoxide hydrolase
GSH	glutathione
GSH-T	glutathione-S-epoxide transferase

h	hours
HGPRT	hypoxanthine-guanine phosphoribosyl transferase
HPLC	high pressure liquid chromatography
IARC	International Agency for Research on Cancer
k	one thousand
μM	micromolar
MEM	minimal essential medium
mmol	millimole
MS	mean square
n	number of observations
nmol	nanomole
nM	nanomolar
N	cell number after incubation
N_o	initial cell number
ND	not detected
NE	nonenzymatic
ODS	octadecyl silane
3-OH	3-hydroxybenzo[a]pyrene (Other hydroxy derivatives are defined similarly.)
4,5-oxide	4,5-epoxy-4,5-dihydrobenzo[a]pyrene (Other oxides are defined similarly.)
P-450	a protein having a major absorption band at or near 450 nm which is able to metabolize one or more endogenous or exogenous substrates
P_1-450	a form of P-450 which has AHH activity
PAH	polycyclic aromatic hydrocarbon

PBS	phosphate-buffered saline
3-phenol	3-hydroxybenzo[a]pyrene (Other phenols are similarly defined.)
pmol	picomole
PMSF	phenylmethylsulfonyl fluoride
PPO	2,5-diphenyloxazole
RSB	reticulocyte swelling buffer
$s^2_{y \cdot x}$	variance of points about regression line of y on x
SCE	sister chromatid exchange
S.D.	standard deviation
SDS	sodium dodecyl sulfate
SE(b)	standard error of slope
$SE(T_D)$	standard error of population doubling time
ss	stereospecific
SS	sum of squares
ST	sulfotransferase
syn	a stereoisomer with substituents on the same side of a plane
TCPO	1,1,1-trichloropropene 2,3-oxide
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
T_D	population doubling time
UDPGA	uridine diphosphate glucuronic acid
UDPGA-T	UDP glucuronyl transferase
V.R.	variance ratio

CHAPTER I

INTRODUCTION

A. Experimental Rationale

Benzo[a]pyrene (B[a]P), a polycyclic aromatic hydrocarbon (PAH), was shown to be carcinogenic in laboratory animals (Cook et al., 1933), and has since been studied as a prototype carcinogen. Several hundred PAHs were synthesized and tested for carcinogenicity in mice (Bachmann et al., 1937; Badger et al., 1940; Badger et al., 1942; Barry et al., 1935; Cook, 1932, Cook et al., 1932). These compounds were found to vary widely in their carcinogenic potency. In an attempt to correlate this biological activity with chemical structure, Pullman and Pullman (1955) proposed that an area of high electron density, called the "K-region" (Figure I-1), was important in the carcinogenicity of these compounds. However, additional compounds were tested that were not carcinogenic as predicted by this theory. It became clear that the weakness of the theory was that it was based on the false premise that the parent PAHs themselves were biologically active (reviewed by Phillips, 1983). Later work has shown that PAHs are metabolized, and their biological activity is due to their metabolites.

B[a]P is lipophilic and relatively inactive metabolically (Selkirk et al., 1980). However, organisms have detoxification processes. During detoxification, B[a]P is transformed into an

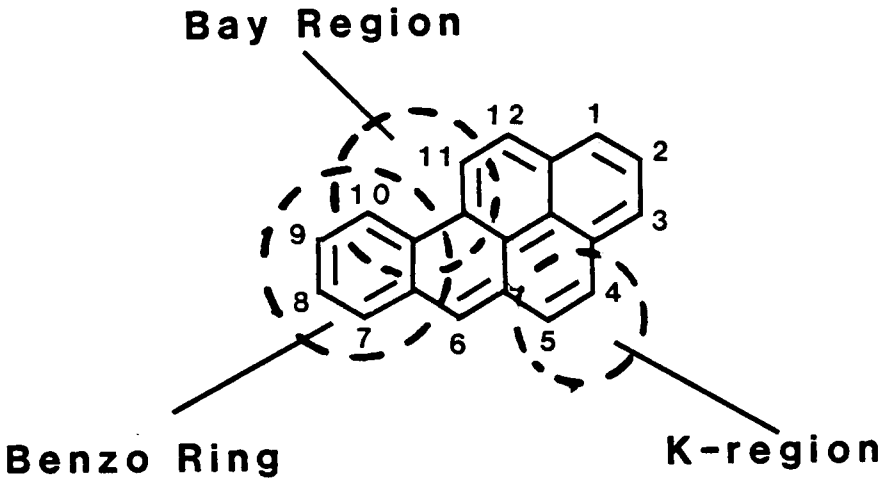


Figure I-1. Structure of Benzo[a]pyrene.

electrophilic reactive intermediate in preparation for further catabolism. While B[a]P is in this electrophilic state, it can react with cellular nucleophiles such as DNA, RNA, or protein. A reaction of this type may begin the process of malignant transformation (reviewed by Miller, 1978). However, the reactive intermediate could also be converted to a metabolically inactive polar structure for excretion from the body (Selkirk et al., 1980).

A large amount of information has been obtained regarding the metabolic conversions that are performed on B[a]P. However, the complete mechanism of malignant transformation by B[a]P remains obscure (Selkirk et al., 1980). It is possible that a mechanism could be elucidated by a kinetic approach. The availability of a reactive electrophile for reaction with a critical nucleophile such as DNA could depend on the relative rates of the reactions which produce the electrophile and those which detoxify the electrophile (Selkirk et al., 1980). The rates of the activating reactions may exceed the rates of the detoxifying reactions in an individual who is relatively susceptible to initiation of cancer by B[a]P, while the reverse may be true in a relatively resistant individual.

It is known which metabolites of B[a]P are carcinogenic and which are not (reviewed by Pelkonen and Nebert, 1982). However, it is not known how the carcinogenic metabolites actually cause malignant transformation. Studies designed to reveal the individual steps of this process are being conducted in mammalian cells in culture (reviewed by Heidelberger et al., 1983). However, Whitlock

et al. (1976) have shown that substantial heterogeneity in the metabolism of B[a]P can develop during the growth of cells in culture. Their data indicated the existence of subpopulations of cells in a supposedly homogeneous population.

The research described below was conducted to provide more insight into differences among subpopulations of cells isolated from a cell clone. The work consisted of studies of the metabolism of B[a]P, the induction of sister chromatid exchanges by B[a]P, and the binding of B[a]P metabolites to nuclear macromolecules in a parent line of mouse hepatoma cells and three of its variants.

B. Review of Literature on Benzo[a]pyrene

1. Carcinogenesis by B[a]P

The carcinogenicity of B[a]P depends on many factors, including route of administration, species, age, sex, nutritional state, dose, vehicle, schedule, etc. When B[a]P was administered orally to mice, rats, and hamsters, papillomas and carcinomas were observed in the esophagus (Gibel, 1964), forestomach (Hartwell, 1951), and intestine (Chu and Malmgren, 1965). In addition, leukemias (Rigdon and Neal, 1969) and lung adenomas (Wattenberg and Leong, 1970) were observed in mice, and mammary tumors were observed in rats (Huggins and Yang, 1962).

When B[a]P was applied to the skin, papillomas, carcinomas, and melanomas were observed in mice (Wynder and Hoffman, 1959), rats (Nakano, 1937), hamsters (Shubik et al., 1960), guinea pigs (Oberling

et al., 1937), and rabbits (Schürch and Winterstein, 1935).

Papillomas and carcinomas of the trachea, bronchi, and lungs were induced in rats (Ryazanov et al., 1961), hamsters (Mohr, 1971), ducks (Rigdon and Neal, 1965), and monkeys (Crocker et al., 1970) which received B[a]P by the inhalation, intratracheal, or intrabronchial routes.

Sarcomas or unspecified tumors were observed when B[a]P was administered subcutaneously or intramuscularly to mice (Bryan and Shimkin, 1943), rats (Oberling et al., 1939), hamsters (Halberstaedter, 1939), guinea pigs (Haagensen and Krehbiel, 1936), newts (Seilern-Aspang and Kratochwil, 1962), tree shrews (Noyes, 1968), or cotton-top marmosets (Noyes, 1969). When these routes were used to give B[a]P to newborn mice, however, hepatomas and lung adenomas were seen (Pietra et al., 1961).

Intraperitoneal administration of B[a]P to mice resulted in abdominal fibrosarcomas (Payne, 1958), whereas rats receiving B[a]P by this route developed mammary and uterine carcinomas (Payne, 1958). Rats also developed mammary carcinomas when they received B[a]P intravenously (Pataki and Huggins, 1969).

Finally, when pregnant mice received B[a]P subcutaneously or intraperitoneally, their offspring developed lung adenomas and skin tumors (Bulay, 1970).

Studies have not been done to prove that B[a]P is carcinogenic to humans. Nevertheless, based on these animal studies, the National Toxicology Program has designated B[a]P as a substance "which may

reasonably be anticipated to be carcinogenic to humans" (Third Annual Report on Carcinogens, December 1982).

2. Metabolism

a. Introduction. The rate and extent of metabolism of B[a]P depends on the animal species (Selkirk et al., 1983a), organ (Selkirk et al., 1983b), or cell type (Jones et al., 1983) studied. Metabolic differences at each of these three levels would be due to differences in genetic composition or expression. Metabolism results can also be influenced by experimental variables, such as substrate concentration, incubation time, or growth stage of cells in culture.

The aims of studies of B[a]P metabolism have been to elucidate the formation of particular metabolites under various conditions, and then to relate the formation of these metabolites to biological events, such as carcinogenesis, mutagenesis, transformation of cells, macromolecular binding, and the induction of sister chromatic exchanges in cells. Some reaction pathways form metabolites which are more carcinogenic or mutagenic, while other pathways form metabolites which decrease these biological effects.

The metabolism of B[a]P is quite complex. The structure and numbering system for B[a]P are shown in Figure I-1. Most of the reactions which metabolize B[a]P are enzymatic, but some are non-enzymatic. The enzymes which catalyze the enzymatic reactions can be divided into two groups (Pelkonen and Nebert, 1982). Phase 1 enzymes catalyze oxidative, reductive, or hydrolytic reactions which

introduce functional groups. The phase 1 enzymes involved in B[a]P metabolism include one or more forms of P-450 monooxygenase (Nebert, 1979), one or more forms of epoxide hydrolase (EH) (Guenthner and Oesch, 1981) and a soluble dehydrogenase (deHase) (Booth and Sims, 1976; Thakker et al., 1978). Phase 2 enzymes catalyze conjugative reactions between the newly-formed functional groups and low-molecular-weight-endogenous compounds. These enzymes include gluthathione-S-epoxide transferase (GSH-T) (Nemoto, 1981), UDP-glucuronyl transferase (UDPGA-T) (Nemoto, 1981), and sulfotransferase (ST) (Nemoto, 1981).

b. Phase 1 reactions. The first step in the metabolism of B[a]P is usually the P-450-mediated epoxidation of the substrate to form an arene oxide at the 4,5-, 7,8-, or 9,10- position. An arene oxide might also be formed at the 1,2- or 2,3- position (Gelboin, 1980).

An arene oxide can undergo three types of phase 1 reactions (Pelkonen and Nebert, 1982). First, it can be stereospecifically hydrolyzed by EH to form a trans-dihydrodiol (Guenthner and Oesch, 1981). Second, it may non-enzymatically rearrange to form a phenol (Jerina and Daly, 1976). Third, it can be reduced back to the parent hydrocarbon (Booth et al., 1975).

Phenols can be substrates for a second P-450-catalyzed epoxidation (Gelboin, 1980; Pelkonen and Nebert, 1982). 3-Hydroxybenzo[a]pyrene can be oxidized to the 3,6-quinone (Wiebel, 1975), and 9-hydroxybenzo[a]pyrene can be oxidized to the 4,5-oxide (Pelkonen

et al., 1978), which is hydrated to form the 9-hydroxy-4,5-dihydrodiol. The 6-phenol can be oxidized to the 6-oxy free radical, and then to quinones (Lesko et al., 1975).

Dihydrodiols can also be substrates for a second P-450-catalyzed epoxidation (Gelboin, 1980; Pelkonen and Nebert, 1982). The P-450 system metabolizes B[a]P 4,5-dihydrodiol to several uncharacterized metabolites (IARC Monographs, Vol. 32, 1983). The 9,10-dihydrodiol is metabolized predominantly to the 1- or 3-phenol, and minor quantities of a 9,10-diol-7,8-epoxide are formed. In contrast, the 7,8-dihydrodiol is mainly hydroxylated to form the 7,8-diol-9,10-epoxides (BPDE), while only small amounts of phenol-diol are formed (IARC Monographs, Vol. 32, 1983).

Dihydrodiols can also be dehydrogenated to catechols by a soluble dehydrogenase (Booth and Sims, 1976; Thakker et al., 1978).

Diol epoxides can be hydrolyzed to tetraols (Yang et al., 1977a) or reduced to triols (Yang and Gelboin, 1976).

In addition to formation from the 3- and 6-phenol, quinones are also produced non-enzymatically from the parent hydrocarbon (Morgenstern et al., 1981). It is possible that quinones are reduced back to phenols via DT-diaphorase (Lind et al., 1978) or by the action of NADPH-cytochrome P-450(c) reductase (Capdevila et al., 1978; Shen et al., 1979).

c. Conjugation reactions. Dihydrodiols, phenols and quinones can be conjugated to glucuronides by UDPGA-T (Nemoto and Gelboin,

1976) and to sulfate esters by ST (Nemoto et al., 1977). Arene oxides may be conjugated to glutathione, either non-enzymatically or via GSH-T (Nemoto, 1981), and then to a mercapturic acid (Boyland and Chasseaud, 1969), which can be detected in urine.

d. Summary. The reactions described above produce a great variety of metabolites of B[a]P. However, other studies described below have shown that the 7,8-diol-9,10-epoxide is probably the most important metabolite involved in carcinogenesis and mutagenesis. Therefore, the three step pathway from B[a]P to the 7,8-oxide, the 7,8-dihydrodiol, and the 7,8-diol-9,10-epoxide can be considered a central pathway leading to carcinogenesis, and all the other reactions can be considered as detoxifying mechanisms.

3. The Ah Locus

There is a difference in the induction of aryl hydrocarbon hydroxylase (AHH) activity by aromatic hydrocarbons among various mouse strains (Nebert et al., 1981). This responsiveness to aromatic hydrocarbons was designated the "Ah locus" and was first characterized in the C57Bl/6 inbred mouse strain, designated B6 or Ah^b. The first nonresponsive inbred mouse strain characterized was DBA/2, designated D2 or Ah^d. The Ah^b/Ah^d heterozygote obtained from the cross between the strains C57Bl/6 and DBA/2 is as responsive as the Ah^b/Ah^b homozygote, indicating that Ah^b is inherited as a Mendelian autosomal dominant trait (Eisen et al., 1983). However, Robinson et al. (1974) discovered other types of inheritance, which depend

on the mouse strains used for the cross. For example, the absence of AHH induction appears to segregate in an autosomal dominant fashion in the C57BL/6N x AKR/N cross, while AHH induction is inherited additively in the C3H/HeN x DBA/2N cross.

AHH activity is most commonly induced by 3-methylcholanthrene (MC) (Poland and Kende, 1976). However, Poland and Glover (1974) compared the induction of AHH by MC with induction by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). They found that the dose of TCDD that evoked half-maximal induction of AHH (ED_{50}) was 30,000 times less than the ED_{50} of MC. Therefore, TCDD is 30,000 times as potent as MC in the induction of AHH. Poland and Glover (1974) also found that TCDD and MC induced AHH to the same maximal response, the AHH inductions by TCDD and MC were not additive, and the log dose-response curves of AHH induction by TCDD and MC were parallel. They concluded that TCDD and MC either share the same receptor or compete at some later step(s) in the induction of AHH (Poland and Glover, 1974).

Poland and Glover (1975) discovered that AHH activity in strains of mice that are nonresponsive to MC can be induced by a dose of TCDD 12 to 18 times larger than that needed to achieve the same level of AHH induction in mice that are responsive to MC. This indicates that AHH activity is regulated by the Ah locus, and that the allelic difference is in a regulatory gene rather than in a structural gene. The results of Poland and Glover (1975) also showed that MC-responsive and MC-nonresponsive strains of mice have the same

binding site for TCDD and MC. If MC-responsive and MC-nonresponsive mice had different binding sites for TCDD and MC, the two groups of mice should be equally sensitive to AHH induction by TCDD. However, MC-nonresponsive mice are less sensitive to TCDD than MC-responsive mice.

Subsequent work has shown that the Ah locus does not reflect merely a single-gene difference. Robinson et al. (1974) crossed two AHH-responsive mouse strains, C57Bl/6N and C3H/HeN. Five out of 49 mice of the F_2 generation had noninducible hydroxylase activity. (The results in the progeny were independent of the sex of the parents.) Since this phenotypic incidence was close to 1 in 16, they concluded that two genes are associated with AHH responsiveness, and that the loci of the two genes need not be linked. These workers also observed the incidence of AHH induction in offspring derived from three or four inbred mouse strains. They compared these data with the results expected from crosses between two strains. For one group of seven genetic crosses, the observed results were significantly different from the expected results. Robinson et al. (1974) concluded that there are at least three alleles at each of the two nonlinked Ah loci.

Further work indicated that the product of the regulatory gene is a cytosolic Ah receptor which is bound by an inducer, such as TCDD or a polycyclic aromatic hydrocarbon such as B[a]P (Nebert, 1979). All nonresponsive mouse strains examined so far have no detectable Ah receptor (Nebert et al., 1981), but this alone cannot

explain the differences in AHH responsiveness among mouse strains (Robinson et al., 1974). A small difference in B[a]P-initiated tumorigenesis was observed between C57Bl/6N mice (AHH responsive) and DBA/2 mice (nonresponsive), but a large difference was observed between C57Bl/6N and C3H (both responsive strains) (reviewed in Thorgeirsson and Nebert, 1977). This review then states: "These data clearly demonstrate that factors other than metabolism of carcinogens regulated by the Ah locus may be critical in explaining an increased susceptibility to polycyclic hydrocarbon-initiated tumors." These other factors may include the conjugation reactions discussed above, or differences in accessibility of macromolecules to binding by B[a]P metabolites, discussed below.

4. Carcinogenesis by Metabolites of B[a]P

Most chemical carcinogens are active only after they have been metabolized to ultimate carcinogens (reviewed by Miller, 1978). Conney (1982) and his coworkers have performed studies to determine which metabolites of B[a]P are carcinogenic. BP-7,8-oxide applied to mouse skin resulted in a 90 to 100% tumor incidence, whereas BP-9,10-oxide was inactive and BP-4,5-oxide was only slightly active. BP-7,8-dihydrodiol was about as tumorigenic as B[a]P, and both were more potent than BP-7,8-oxide. BP-4,5-dihydrodiol and BP-9,10-dihydrodiol had little or no tumorigenic activity. BP-7,8-diol-9,10-epoxide (syn) was inactive as a complete carcinogen on mouse skin. The anti isomer was only weakly tumorigenic on mouse skin,

but was highly tumorigenic when injected intraperitoneally into newborn mice. Tests of optical isomers showed that (+)-BP-7,8-oxide was more tumorigenic than the (-) isomer, (-)-BP-7,8-dihydrodiol was more active than the (+) isomer, and (+)-BP-7,8-diol-9,10-epoxide (anti) showed higher tumorigenicity than its (-) isomer. In each of these three cases, the more tumorigenic optical isomer was the one that was metabolically predominant in mouse skin (Conney, 1982).

Other metabolites of B[a]P have been found to be noncarcinogenic or only weakly carcinogenic (Pelkonen and Nebert, 1982).

5. Macromolecular Binding

a. Introduction. Metabolites of B[a]P bind to DNA, RNA, and protein (MacLeod et al., 1979a). The binding of B[a]P metabolites to DNA may be the critical event in the initiation of carcinogenesis by B[a]P, but it is also possible that binding of B[a]P metabolites to RNA or protein is involved.

b. Binding to DNA. Brookes and Lawley (1964) proposed that covalent binding of a PAH metabolite to DNA is responsible for the initiation of cancer. The K-region epoxide (at the 4,5-position shown in Figure I-1, page 2) was considered the most likely candidate for reaction with DNA in vivo (Grover et al., 1972), but Baird et al. (1975) established that binding of B[a]P to DNA in vivo did not involve the K-region epoxide.

Sims et al. (1974) proposed that the 7,8-dihydrodiol-9,10-epoxide of B[a]P is responsible for in vivo DNA binding. The diol

epoxide of B[a]P is covalently bound to DNA from organs or cells in culture to a greater extent than other B[a]P metabolites (reviewed by Pelkonen and Nebert, 1982).

King et al. (1976) showed that the binding of B[a]P to DNA of embryo cells in culture involved mainly anti-BPDE compared with the syn isomers, but adducts with syn-BPDE have been detected in vivo by Cerutti et al. (1978).

Meehan and Straub (1979) prepared the two optical isomers of anti-BPDE. The found a ten-fold greater extent of reaction of the (+) isomer than the (-) isomer with native DNA, but this difference was not found on reaction with single-stranded DNA.

The major adduct of BPDE to DNA has a covalent bond between the C-10 position of the hydrocarbon and the N-2 position of quanine (Weinstein et al., 1976). There is indirect evidence for the reaction of BPDE at the N-7 position of guanine (Osborne et al., 1978) and with phosphates of the DNA backbone (Koreeda et al., 1978). BPDE has sometimes been found bound to adenine and cytosine of synthetic homopolymers, RNA, and DNA in vitro (Jennette et al., 1977; Jeffrey et al., 1979). (The above has been reviewed by Rajalakshmi et al., 1982).

c. Binding to RNA. In general, the interaction of chemical carcinogens with RNA has not been as extensively investigated as that with DNA. However, the structure of the major adduct of a B[a]P metabolite with RNA is the same as that with DNA, i.e., a covalent

linkage between the C-10 of the hydrocarbon and the N-2 of guanine (Jeffrey et al., 1976).

d. Binding to proteins. The study of the binding of carcinogens to proteins is important because cancer may be caused by alteration in gene expression or differentiation, due to changes in protein or RNA. Evidence for this epigenetic theory includes the observations that DNA binding and carcinogenicity do not correlate perfectly, some mutagens are not carcinogens, and tumor phenotypes can sometimes be reversed (Weinstein, 1976).

Proteins which regulate gene expression or differentiation are more likely to be located in the nucleus than in the cytoplasm. Nuclear proteins are of two types: histones and nonhistones. The histones which make up the core of the nucleosome are H2A, H2B, H3, and H4. In intact cells, B[a]P metabolites bind more to core histones H2A and H3 than to histones H2B and H4 (Kootstra, 1982; MacLeod et al., 1980, 1981, 1983; Sculley and Zytkovicz, 1983; Selkirk et al., 1983). This preferential binding of B[a]P metabolites to histones H2A and H3 may be a consequence of the types and amounts of B[a]P metabolites produced in intact cells, and of the accessibility of the core histones to binding.

The binding of B[a]P metabolites to nonhistone proteins of high molecular weight is less well documented because of the difficulty in separating and identifying the larger proteins.

6. Sister Chromatid Exchanges

B[a]P has been shown to induce SCEs, but only in cell or animal systems that are capable of metabolic activation of this compound (Latt et al., 1982; Perry, 1980; Abe and Sasaki, 1982b). Such systems include intact animals (Bayer et al., 1981) and certain cultured cells which have retained the ability to metabolize B[a]P (Abe et al., 1983a). Cells which lack this metabolic ability can be cocultivated with cells which have the ability (a feeder layer). Alternatively, this metabolic ability can be provided by the addition of a microsomal preparation obtained from homogenized liver by differential centrifugation (the "S9" fraction remaining after a 9000g spin).

There have also been studies of SCE induction by metabolites of B[a]P. For example, Pal et al. (1978) found that in CHO cells the 7,8-dihydrodiol was a more potent inducer of SCEs than the 9,10-dihydrodiol, the 4,5-dihydrodiol, or the parent hydrocarbon B[a]P. Pal et al. (1980) observed that the anti-7,8-diol-9,10-epoxide was the most potent inducer of SCEs among the diol epoxides, oxides, and phenols studied. The 7,8-oxide and the 4,5-oxide were more active than their respective dihydrodiols. The 7,8-dihydrodiol was more active than the two phenols studied, but less active than the oxides or diol epoxides. The 4,5- and 9,10-dihydrodiol were less active than the two phenols. These results were consistent with the results of Hsu et al. (1979), who found that the anti-7,8-diol-9,10-epoxide caused 50-100% more SCE in Chinese hamster V79 cells than the syn

isomer, and Connell (1979), who observed more SCE induction in V79 cells by anti-BPDE than by syn-BPDE or BP-4,5-oxide. The conclusion from these four papers is that the anti-BPDE was the most potent inducer of SCE among the B[a]P metabolites studied.

There is a large range of sensitivities of cell lines to induction of SCE by B[a]P (Table I-1). From literature reports, the concentration of B[a]P required to increase the SCE frequency to twice the background level varied from 4×10^{-8} to 5×10^{-4} M in various cell lines.

7. Mutagenesis

The mutagenesis by B[a]P and its metabolites has been studied in bacteria and established mammalian cell lines. In most of the bacterial systems, various histidine-requiring auxotrophs of Salmonella typhimurium have been used, and mutagenicity is detected by their reversion to histidine prototrophs (Nagao and Sugimura, 1978). The most popular mammalian cells for mutagenesis studies have been V79 cells, but CHO, mouse lymphoma, Syrian golden hamster embryo and human skin fibroblast cells have also been used. The genetic markers used in these studies have mainly been 8-azaguanine resistance, thioguanine resistance, and ouabain sensitivity (reviewed by Gelboin, 1980). The most important result of all of these studies is that BP-(+)-anti-7,8-diol-9,10-epoxide is the most active mutagen among all B[a]P metabolites in V79 cells (Brookes et al., 1979; Conney, 1982; Gelboin, 1980; Wood et al., 1977).

Table I-1

Sensitivity of Some Cell Lines to Induction of Sister Chromatid Exchanges by Benzo[a]pyrene

Cell Line	Cell Type	Doubling Concentration[a] (M)	Reference
TAOclBP^{r}cl	Mouse Hepatoma	4×10^{-8}	This study
b,c	Cultured Mouse Embryos	1×10^{-7}	Galloway et al. (1980)
Don	Chinese Hamster Lung	1×10^{-6}	Baker et al. (1983)
B-13	Chinese Hamster Embryo	1×10^{-6}	Ikeuchi & Sasaki (1981)
b	Human Lymphocytes	5×10^{-6}	Craig-Holmes & Shaw (1977)
ARL 18	Rat Liver Epithelial	1×10^{-5}	Tong et al. (1981)
GHE	Golden Hamster Embryo	1×10^{-5}	Ikeuchi & Sasaki (1981)
CHO	Chinese Hamster Ovary	3×10^{-5}	Pal et al. (1978)
C-HC-4	Human Hepatoma	4×10^{-5}	Abe et al. (1983a,b)
C-HC-20	Human Hepatoma	4×10^{-5}	Abe et al. (1983a,b)
RL4	Rat Liver Epithelial	5×10^{-5}	Cunningham & Ringrose (1983)
Don-6	Chinese Hamster Lung	5×10^{-4}	Abe & Sasaki (1982a)

Table I-1 (continued)

Cell Line	Cell Type	Doubling Concentration[a] (M)	Reference
R1	Rat Esophageal Tumor	5×10^{-4}	Abe & Sasaki (1982a)

[a]Concentration of B[a]P required to increase SCE response to twice the background level.

[b]Primary cultures.

[c]Various mouse strains were studied.

Besides the 7,8-diol-9,10-epoxides of B[a]P, the 4,5-epoxide is also quite mutagenic in nonmetabolizing V79 cells (Gelboin, 1980). Among phenols and diols studied in a cell-mediated assay, the trans-7,8-diol was the most mutagenic, presumably because of its conversion to the 7,8-diol-9,10-epoxide, and the (-)-trans-7,8-diol was six times more active than the (+)-trans-7,8-diol. The anti-7,8-diol-9,10-epoxide was more than 100 times as mutagenic as other B[a]P metabolites tested in nonmetabolizing V79 cells (Gelboin, 1980).

8. Transformation

In vitro transformation of mammalian cells has been reviewed by Heidelberger et al. (1983) and by Pelkonen and Nebert (1982). Briefly, cells in culture are created with a carcinogen, allowed to proliferate, and observed for an altered morphological phenotype, e.g., the production of foci or colonies of piled-up, crisscrossed cells. Such tests have been done in three main types of systems: in cell strains (cells with a limited lifespan); in cell lines (cells with an unlimited lifespan); and in viral enhancement systems, in which a chemical enhances transformation by a virus, or a virus enhances transformation by a chemical (Heidelberger et al., 1983). The parent compound B[a]P has been shown to be active in all three types of systems.

Berwald and Sachs (1963) reported transformation by B[a]P in secondary cultures from golden hamster embryos. Since then, studies of cell transformation by metabolites of B[a]P have also been

performed. For example, the transformation of mouse fibroblasts by the three dihydrodiols of B[a]P showed the 7,8-dihydrodiol to be more active than the 4,5- or the 9,10-dihydrodiol (Marquardt et al., 1976). The 4,5-oxide and the 6-phenol were shown to be inactive.

Marquardt et al. (1977) compared four diol epoxides of B[a]P in the same system. The anti isomer of the 7,8-diol-9,10-epoxide was quite active, the syn isomer of this compound was only weakly active, and both the anti and the syn isomers of the 9,10-diol-7,8-epoxide were devoid of transforming activity. The overall results from these two studies showed the anti-7,8-diol-9,10-epoxide to be the most active species among the B[a]P metabolites tested in transforming cells.

Barrett and Ts'o (1978a) showed that in vitro transformation by B[a]P has a multistage progressive nature. They exposed Syrian hamster embryo cells to B[a]P and allowed the cells to proliferate in culture while observing them for signs of transformation. Morphological alterations occurred within one week after carcinogen treatment, while enhanced fibrinolytic activity was expressed only after two to three weeks post treatment, but these two were not necessarily correlated. The ability to grow in soft agar and the ability to induce tumors were delayed until 32-75 population doublings (6-15 weeks) after carcinogen exposure. These altered characteristics were found to be acquired independently of each other. The development of the anchorage independent growth phenotype (the ability to grow in soft agar) was shown to be induced, rather than selected, by the carcinogen treatment (Barrett and Ts'o, 1978a).

Earlier studies in the laboratory of Heidelberger (reviewed by Heidelberger, 1978) had shown that 3-methylcholanthrene was able to transform single C3H mouse prostate cells in individual dishes with a high frequency. Mondal and Heidelberger (1970) concluded that the carcinogen was therefore not selecting for preexisting transformed cells. Furthermore, Mondal et al. (1970) demonstrated that individual transformed clones expressed tumor-specific transplantation antigens (TSTAs) that were noncrossreacting. Embleton and Heidelberger (1972) showed that the TSTAs arose during the process of transformation.

Possible mechanisms for in vitro transformations include somatic mutation (Barrett, 1981), genetic transpositions (Cairns, 1981; Weinstein, 1982), alteration of gene expression (Fahmy and Fahmy, 1980), aberrant chromosomal segregation (Kinsella et al., 1978), disruption of the cytoskeleton (Brinkley, 1982), or alteration of the binding of a growth factor to its receptor (Kamata and Feramisco, 1984).

C. History of Cell Lines Used in the Present Study

A permanent cell line, designated Hepa, was isolated in 1970 from a hepatoma, BW 7756, in mouse strain C57L/J (Bernhard et al., 1973). A clonal line, Hepa-1, and ten subclones were derived from Hepa in 1973 (Bernhard et al., 1973). Hepa-1 had high AHH activity and was very sensitive to the toxicity of B[a]P.

Hankinson (1979) isolated a clone, Hepa-1c1, from the cell line Hepa-1. He inoculated Hepa-1c1 cells into a microtest multiwell dish (Falcon), and identified wells containing single cells by microscopy. He isolated clones growing in selective medium (containing B[a]P, 4 μg/ml) by using the siliconized metal cylinder technique (Puck et al., 1956). One of these clones was designated Hepa-1c1c7. Miller and Whitlock (1981) incubated the cell line Hepa-1c1c7 with B[a]P, passed the cell suspension through a Becton-Dickenson cell sorter, and isolated new variants by detecting differences in the amount of B[a]P fluorescence. Highly fluorescent cells had lost the ability to metabolize B[a]P. Miller et al. (1983) screened many variants and compared the AHH activities of several of these variants with that of the parent cell line, Hepa-1c1c7. They found that the variants could be grouped into two classes: one with no detectable basal or inducible AHH activity (e.g., BP^rc1), and one with detectable but low levels of basal and inducible AHH activity (e.g., TAOc1BP^rc1). The low level of inducible AHH activity detected in TAOc1BP^rc1 cells is due to a slow rate of induction of AHH (Miller and Whitlock, 1982a).

Further work showed that variants of both classes have TCDD receptor defects. Using TCDD as an inducer, Miller et al. (1983) first suggested that members of the first class of variants (later designated Class II) do not translocate an inducer-receptor complex from the cytosol to the nucleus. They also suggested that members of the second class (later designated Class I) have cytosolic

receptors which are decreased either in number or binding ability. Cell fusion experiments indicated that the variants have recessive phenotypes and that the two lesions are in different genes (Miller et al., 1983). Whitlock and Galeazzi (1984) showed that the receptors of Hepa-1c1c7 cells are located primarily in the nucleus rather than in the cytosol or on the cell surface. Whitlock and Galeazzi (1984) also interpreted their data as implying that each receptor has two binding sites: one site for binding to TCDD (or other inducer, such as B[a]P), and another site for binding to the nucleus (presumably to chromatin). These two binding sites may be located on different proteins. Thus each receptor may be composed of two polypeptides. A Class I variant (e.g., TAOc1BP^{r}c1) might contain a lesion in the TCDD-binding domain of the receptor. A Class II variant (e.g., BP^{r}c1) might contain a lesion in the receptor's chromatin-binding domain.

Variant BP^{r}c3 contains no detectable TCDD-inducible P_1-450 mRNA. (Results were not reported for BP^{r}c1, but this variant is in the same class as BP^{r}c3.) TAOc1BP^{r}c1 contains a maximally induced amount of P_1-450 mRNA which is approximately ten-fold less than in Hepa-1c1c7 cells (Israel and Whitlock, 1983). (P_1-450 is the subclass of P-450 associated with AHH activity.) Furthermore, the rate of synthesis of P_1-450 mRNA was increased twenty-fold by TCDD in Hepa-1c1c7 cells, but TCDD had no effect on P_1-450 mRNA synthesis in BP^{r}c1 cells (Israel and Whitlock, 1984).

Hepa-1c1c7 makes P_1-450 mRNA and P_1-450 protein, and thus metabolizes B[a]P. However, Hepa-1c1c7 is resistant to B[a]P-induced

toxicity. This resistance may be due to conjugation of cytotoxic metabolites such as phenols.

High activity variants of Hepa-1c1c7 have been isolated (Jones et al., 1984). The DNA control element upstream from the 5' end of the cytochrome P_1-450 gene in these high activity variants contains at least three functional domains: a promoter, an inhibitory domain which may bind a repressor protein, and a TCDD-responsive domain which binds the TCDD-receptor complex (Jones et al., 1985). If this scheme is correct, the transcription of the P_1-450 gene is under both positive and negative control in these mouse hepatoma cells.

The parent cell line Hepa-1c1c7, the Class I variant TAOc1BP^{r}c1, and the Class II variant BP^{r}c1, all described above, and an additional variant, MUL12, whose characteristics have not been reported, were gifts of Dr. James P. Whitlock, Jr., of Stanford University, and were used in the present study.

CHAPTER II

PRELIMINARY STUDIES

A. Introduction

The population doubling times, modal chromosome numbers, and chromosome distributions of the parent cell line, Hepa-1c1c7, and three of its variants, $BP^{r}c1$, MUL12, and $TAOc1BP^{r}c1$, were measured. This information was used in deciding which of the cell subpopulations would be studied in the sister chromatid exchange experiment. In addition, morphologies and saturation densities were studied during the characterization of the differences among these four subpopulations of cells.

B. Morphology

The parent and three variants exhibited a spindle shaped, fibroblastic morphology upon initial plating. However, differences among the parent and three variants became apparent as the cells reached confluence. The parent cell line Hepa-1c1c7 and the variant $BP^{r}c1$ assumed a uniform cobblestone appearance, resembling epithelial cells, but $BP^{r}c1$ grew in patches. Variant $TAOc1BP^{r}c1$ retained a spindle shaped appearance and showed the greatest tendency to assume a crisscrossed, piled-up growth pattern. Variant MUL12 was more refractile than the other three, and if allowed to grow to high cell density and then trypsinized, the cells tended

to come off the plate in sheets, rather than as individual cells.

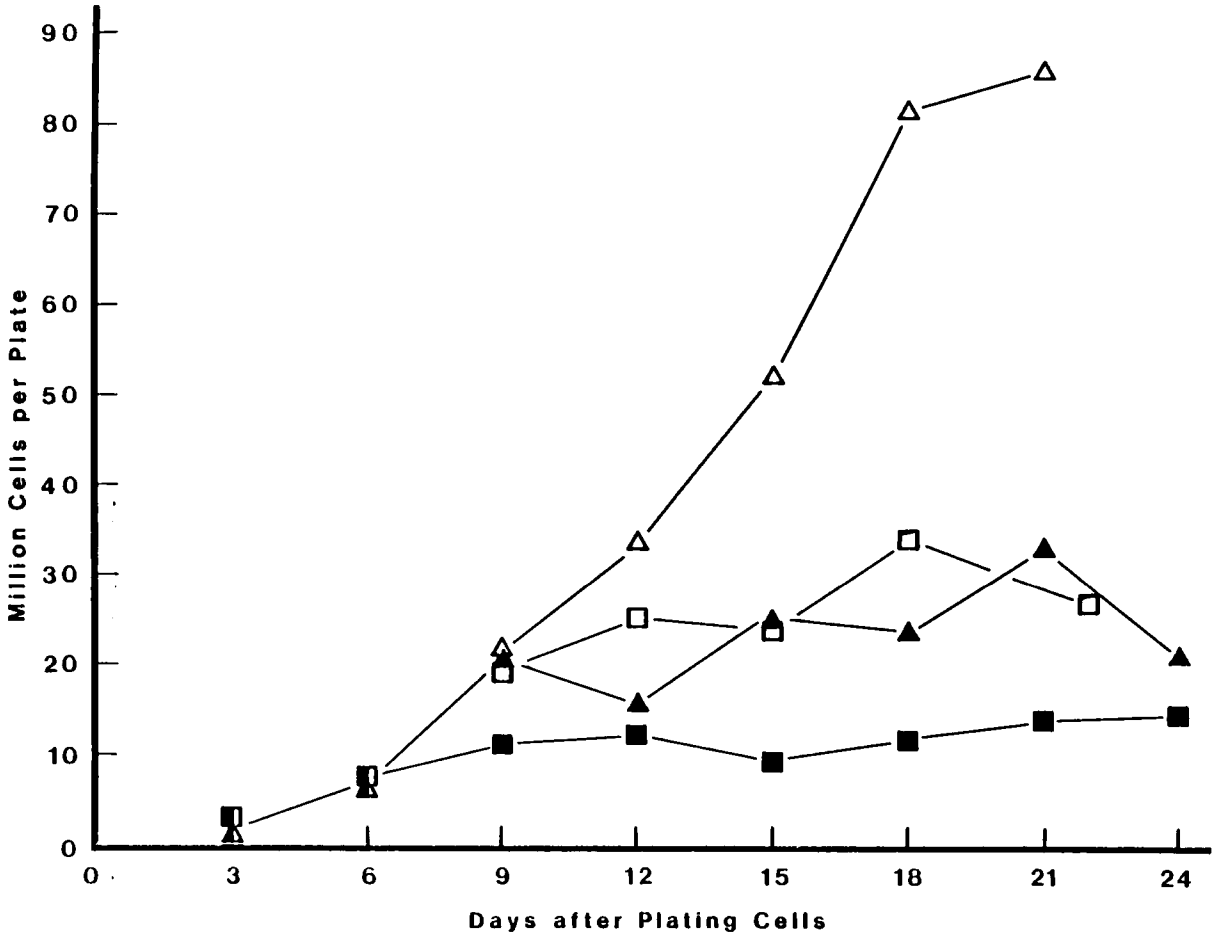
C. Saturation Density

The parent and three variants used in these studies had been derived from a mouse hepatoma, and are assumed to be transformed. One of the characteristics of transformed cells is that they are not contact inhibited (Holley, 1975). An experiment was performed to determine the saturation densities of the parent and three variants.

The cells were plated and the medium was changed every 3 days for up to 24 days. Cell counts were performed on the days of medium change. As shown in Figure II-1, MUL12 cells grew to the lowest saturation density of about 1.4×10^7 cells per 100 mm dish. The saturation densities of Hepa-1c1c7 and TAOc1BP^{r}c1 varied between 1.6 and 3.4×10^7 cells per dish. This variation may have been due to alternate proliferation and detachment of the cells from the dish. The variant BP^{r}c1 achieved a maximum number of about 8.6×10^7 cells per dish.

D. Population Doubling Times

An experiment was performed to measure the population doubling times of the parent and three variants. The cells were plated, allowed to proliferate, subcultured, and allowed to proliferate again. This process was repeated several times. Cell counts were performed and population doubling times were calculated as discussed in the Appendix.



△, $BP^r cl$; ▲, $TAOclBP^r cl$; □, Hepa-1clc7; ■, MUL12.

Figure II-1. Saturation densities of the parent cell line and three of its variants.

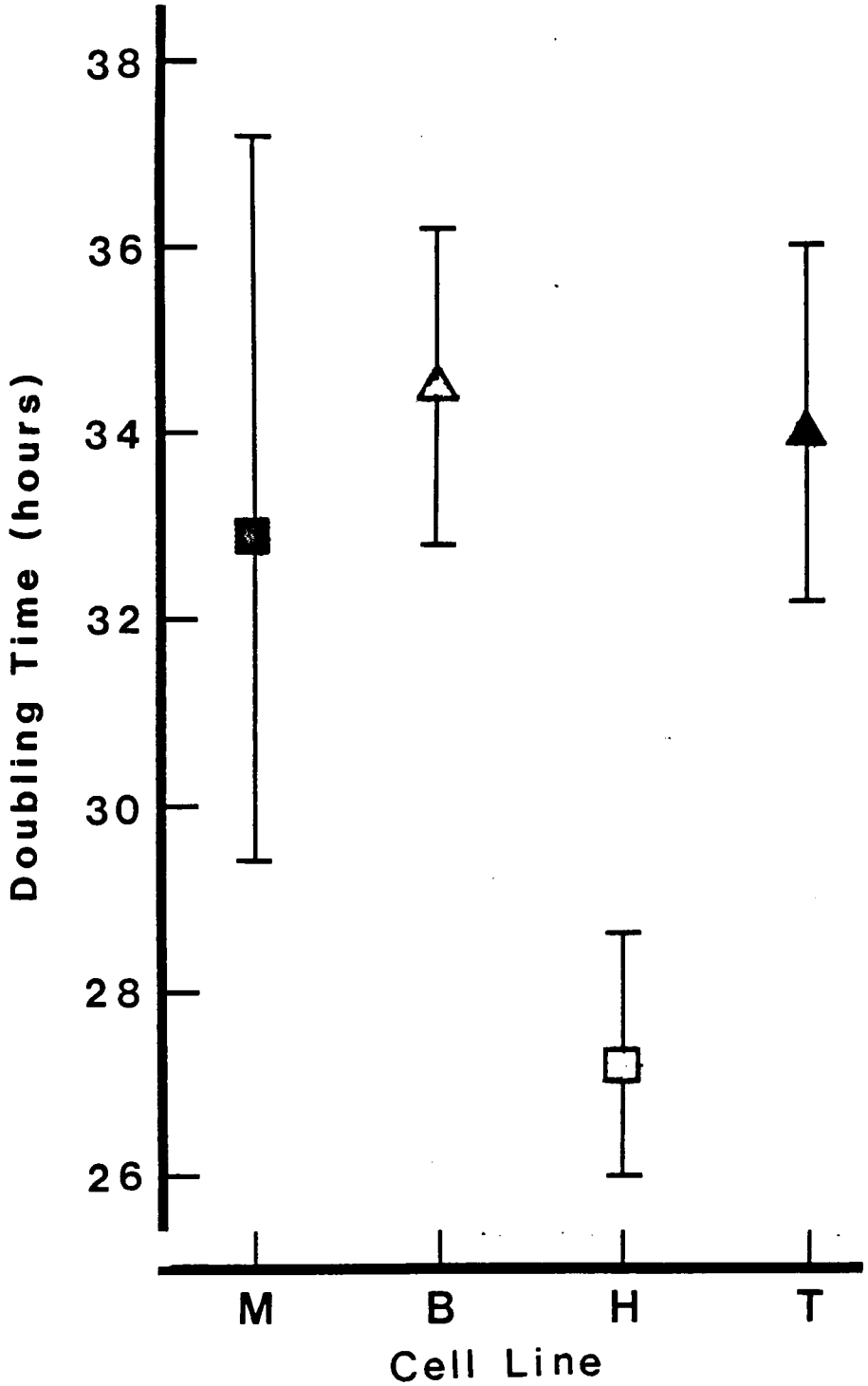
The population doubling times and error bars of the parent and three variants are presented in Figure II-2. Hepa-1c1c7 had a population doubling time shorter than that of the three variants. The slow growth of MUL12 was due to a longer lag time, since the population doubling time of MUL12 was comparable to that of BP^rc1 and TAOc1BP^rc1.

E. Modal Chromosome Numbers and Chromosome Distributions

In preparation for the sister chromatid exchange study, cells were harvested from the plates, swollen in 0.075 M KCl, fixed in methanol:acetic acid (3:1), spread on a slide, and stained with Giemsa. Chromosome counts were performed on 100 metaphases each from the parent and three variants. Chromosome distributions are presented in Figure II-3. The modal chromosome numbers obtained were:

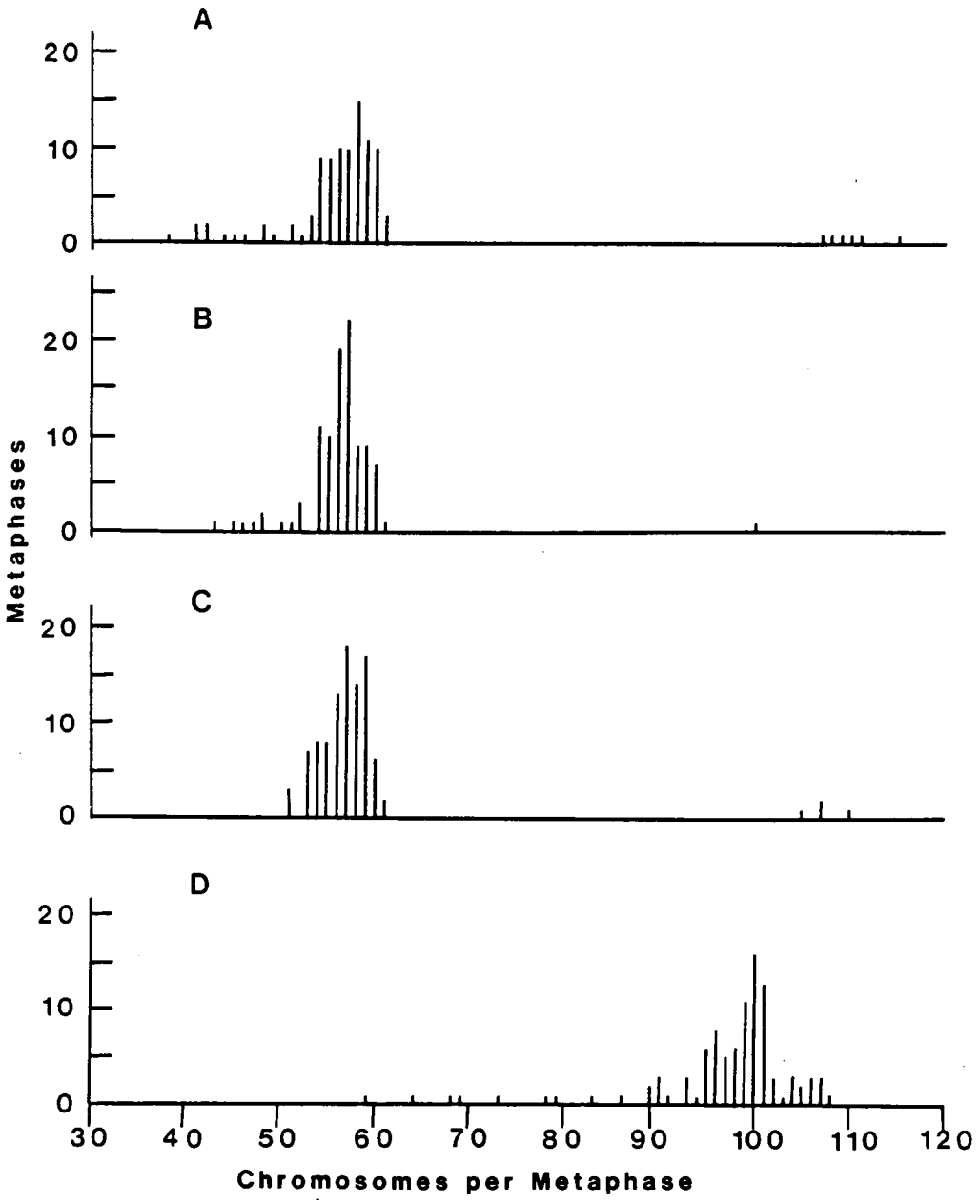
BP^rc1	57
TAOc1BP^rc1	57
Hepa-1c1c7	58
MUL12	100

Normal diploid mouse cells have 40 chromosomes each, so BP^rc1, TAOc1BP^rc1, and Hepa-1c1c7 are hypotetraploid, while MUL12 is hypertetraploid.



M, MUL12; B, BP^{r}c1; H, Hepa-1c1c7; T, TAOc1BP^{r}c1.

Figure II-2. Population doubling times of the parent cell line and three of its variants.



A, Hepa-1c1c7; B, TAOc1BP^{r}c1; C, BP^{r}c1; D, MUL12.

Figure II-3. Chromosome distributions of the parent cell line and three of its variants.

CHAPTER III

METABOLISM OF B[a]P BY CELLS DERIVED FROM A MOUSE HEPATOMA

A. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are common contaminants of the environment. Some of these compounds, including benzo[a]pyrene (B[a]P), have been shown to cause cancer in laboratory animals after metabolic activation to reactive electrophilic species that alkylate cellular macromolecules (Miller, 1978). This activation is performed by various enzyme systems known as the monooxygenases which contain one or more forms of cytochrome P-450 (Gelboin, 1980; Nebert, 1979).

These biochemical conversions have been studied extensively in established cell lines, in microsomes, and in tissue explants. These studies have shown differences in B[a]P metabolism by different species (Selkirk et al., 1983a), cell types (Jones et al., 1983), and organs (Selkirk et al., 1983b). It is often incorrectly assumed that a given cell line is composed of a population of cells which is homogeneous with respect to the ability to metabolize a foreign compound. However, this might not always be true (Selkirk et al., 1982; Whitlock et al., 1976). Therefore, efforts are routinely made to increase the homogeneity of a cell population by subcloning.

In the metabolism experiments to be described in this chapter, we show that a parent mouse hepatoma cell line (Hepa-1c1c7) and

three variants (MUL12, BP^rcl, and TAOclBP^{r}cl), which had been isolated from Hepa-1c1c7 by a fluorescence activated cell sorter, have different capacities to metabolize B[a]P.

B. Materials and Methods

1. Chemicals

Tritiated B[a]P (Amersham, ⩾ 25 Ci/mmol) was diluted with unlabeled B[a]P (Aldrich, gold label) to a specific activity of 3.15 Ci/mmol, dissolved in dimethylsulfoxide at 0.9 mM and stored at -20°C. The hydrocarbon preparation was more than 95% radiochemically pure as judged by HPLC. Bacterial beta-glucuronidase was obtained from Sigma Chemical Co.

2. Cell Culture

The cells were grown in 100 mm plastic tissue culture dishes (Falcon or Corning) in 10 ml of Dulbecco's MEM (high glucose) supplemented with 10% Fetal Bovine Serum, penicillin (100 units/ml), streptomycin (100 µg/ml), Fungizone (0.25 µg/ml) and L-glutamine (2 mM), all from Gibco, at 37°C in an atmosphere of 5% CO_2 in air. Trypsin (0.5 g/L)-EDTA (0.2 g/L) (Gibco) was used to detach cells from dishes for subculture or cell counting. In the majority of experiments, the cells were grown to confluence, but lower cell densities were sometimes used. The concentration of [^{3}H]-B[a]P applied to the cells was most often 4 µM, but lower concentrations down to 25 nM were also used. The final concentration of dimethylsulfoxide, 0.44%, had no measurable cytotoxicity at 24 h.

After incubation periods from 0.5 to 48 h, the medium was removed by aspiration, the cells were gently washed with phosphate-buffered saline (5 ml per plate), and the wash and medium were combined and frozen at -20°C for subsequent analysis. The cells were scraped from the plates with a rubber policeman, the plates were washed with 10 ml phosphate-buffered saline, and the wash and cells were combined and treated as above. Cell counts were performed by the use of hemacytometer or on a Coulter Counter Model ZF (Coulter Electronics, Inc.).

3. Analysis of Medium

Samples of medium were thawed and twice extracted with 2.5 vol ethyl acetate. After extraction the volumes of the aqueous and ethyl acetate phases were measured and aliquots taken from each for liquid scintillation counting.

The ethyl acetate was dried over anhydrous magnesium sulfate, evaporated under vacuum, and the residue dissolved in methanol for analysis by high-pressure liquid chromatography. Metabolite separation was performed with a Spectra Physics 3500B high-pressure liquid chromatograph fitted with a DuPont one meter ODS-Permaphase column. The mobile phase consisted of a solution of methanol in water. Samples were eluted from the column by increasing the percentage of methanol in the mobile phase from 10% to 70% over a period of 40 min (Selkirk et al., 1974). Flow rate was 0.6 ml/min and oven temperature was 50°C. The chromatography was monitored by a DuPont 836 multiwavelength fluorescence and UV spectrophotometer. [^{14}C]-

metabolite standards were coinjected with each sample for identification of metabolites. Fractions (0.2 ml) were added to Aquasol (New England Nuclear) and radioactivity was measured by the use of a Searle Mark III Scintillation Counter. The radioactivity data were converted to mass and plotted by a Tektronix 4051 Graphics Terminal.

4. Analysis of Cells

The harvested cells were thawed and extracted with ethyl acetate, the volumes of the ethyl acetate and aqueous phases measured, and aliquots taken and counted, as described above for the samples of medium.

5. Beta-Glucuronidase Treatment

After extraction of the medium with ethyl acetate, the aqueous phase was washed two more times with solvent, and treated with bacterial beta-glucuronidase in order to release metabolites which had been conjugated as glucuronides. After digestion, the aqueous phase was extracted with ethyl acetate and the metabolite analysis was performed as described above for the medium.

C. Results

1. Distribution of Radioactivity

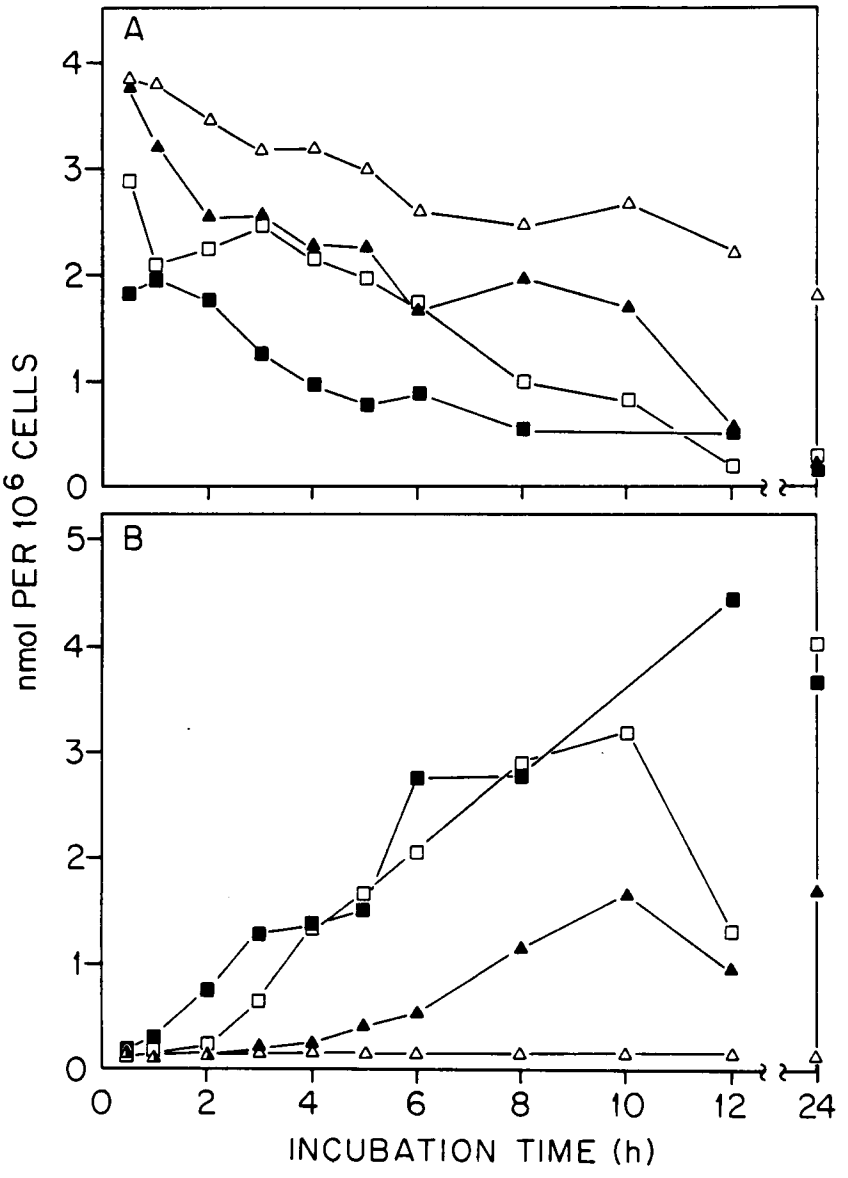
Between 65% and 93% of the radioactivity recovered from the plates was in the medium, with the rest of the radioactivity remaining in the cells. The distribution of radioactivity from the medium

and cells into the ethyl acetate and aqueous phases is shown in Figure III-1. The ethyl acetate-soluble radioactivity in the medium from the parent and three variants decreased with increasing incubation time. The water-soluble radioactivity in the medium correspondingly increased with time for the parent, Hepa-1c1c7, and two of the variants, MUL12 and TAOc1BP^{r}c1, indicating that the parent and these two variants were capable of converting B[a]P to water-soluble conjugates. Time course experiments (Figure III-1A) show that MUL12 converted B[a]P to ethyl acetate-soluble metabolites the most quickly, followed by Hepa-1c1c7 and TAOc1Bp^{r}c1. Furthermore, as shown in Figure III-1B, at incubation times of six h or less, TAOc1BP^{r}c1 resembled BP^{r}c1 with a low level of formation of water-soluble conjugates, but after longer incubation times it achieved levels comparable to those of MUL12 and Hepa-1c1c7.

In contrast, the third variant, BP^{r}c1, did not show an increase in water-soluble radioactivity in the medium. However, this variant showed a slight increase of ethyl acetate-soluble radioactivity in the cells (Figure III-1C).

2. Total Accumulation of Ethyl Acetate-Soluble Metabolites in Medium

The ethyl acetate extract of the medium was analyzed by HPLC. The total accumulation of ethyl acetate-soluble metabolites in the medium as a function of incubation time is presented in Figure III-2. With increasing incubation time, Hepa-1c1c7, TAOc1BP^{r}c1, and MUL12 showed an increasing accumulation, while BP^{r}c1 did not.



Cells and medium were extracted separately as described in "Materials and Methods." A, ethyl acetate fraction of medium; B, aqueous fraction of medium; C, ethyl acetate fraction of cells; D, aqueous fraction of cells. The concentration of B[a]P at the beginning of the experiment was 4 μM, and cell densities ranged from 5.8 to 19.5 x 10^6 cells per 100 mm dish. △, BP^rcl; ▲, $TAOclBP^rcl$; □, Hepa-1c1c7; ■, MUL12.

Figure III-1. Distribution of radioactivity in phases after extraction of cells and medium.

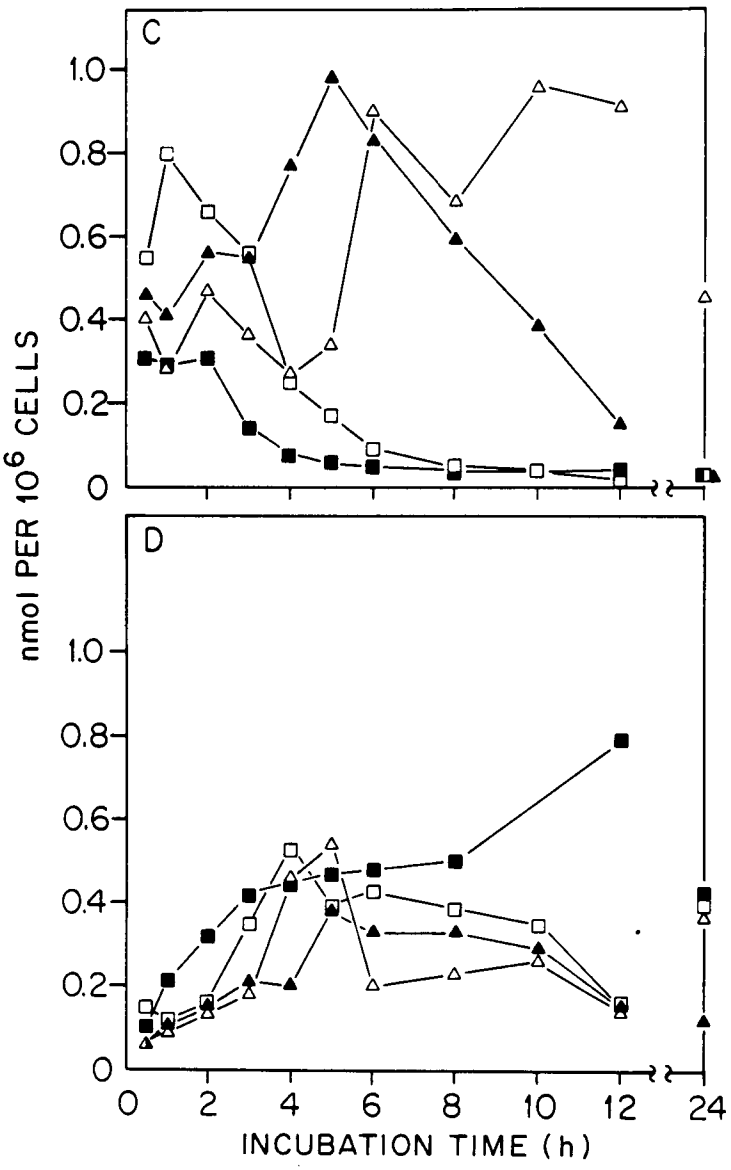
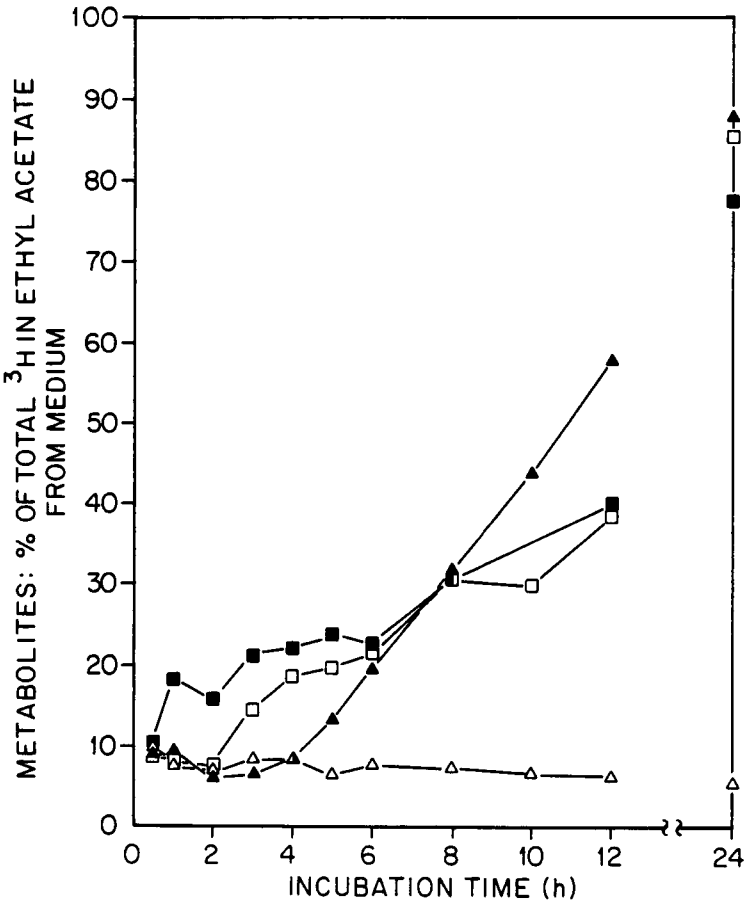


Figure III-1. (Continued)



Ethyl acetate-soluble metabolites of B[a]P were separated by HPLC, fractions were collected and counted by scintillation and the data were converted to pmol and plotted, as described in "Materials and Methods." The amount of radioactivity eluting as metabolites was determined as the percent of total ^{3}H present in the ethyl acetate phase after extraction of the medium. The concentration of B[a]P at the beginning of the experiment was 4 μM, and cell densities ranged from 5.8 to 19.5 x 10^6 cells per 100 mm dish. △, BP^{r}cl; ▲, TAOclBP^{r}cl; □, Hepa-1clc7; ■, MUL12.

Figure III-2. Accumulation of total ethyl acetate-soluble metabolites in medium.

The dependence of this accumulation on cell density is shown in Table III-1. At low cell number, BP^{r}cl and TAOclBP^{r}cl were similar in this accumulation, but at high cell number, TAOclBP^{r}cl showed a higher accumulation of ethyl acetate-soluble metabolites than BP^{r}cl, resembling Hepa-1c1c7 and MUL12.

3. Accumulation of Individual Ethyl Acetate-Soluble Metabolites in Medium

HPLC analysis showed that, of the ethyl acetate-soluble metabolites in the medium, the 9,10-diol and the 7,8-diol had been accumulated to the greatest extent. As shown in Figure III-3A, the 9,10-diol was accumulated most quickly in MUL12 cells, followed by Hepa-1c1c7 and TAOclBP^{r}cl. The 9,10-diol had been eliminated from the medium from MUL12 and Hepa-1c1c7 cells by 8 h, but this metabolite was still at an elevated level in the medium from TAOclBP^{r}cl cells at 12 h. TAOclBP^{r}cl cells accumulated more 9,10-diol than Hepa-1c1c7 or MUL12 cells.

The cells accumulated less 7,8-diol than 9,10-diol (compare Figure III-3B with Figure III-3A). MUL12 accumulated very little 7,8-diol, compared with Hepa-1c1c7 and TAOclBP^{r}cl. The amount of 7,8-diol in the medium reached a peak at 5 h for Hepa-1c1c7 cells, compared with 10 h for TAOclBP^{r}cl cells.

A cell density effect on the accumulation of individual ethyl acetate-soluble metabolites is shown in Table III-2. At high cell density, cell lines Hepa-1c1c7, MUL12, and TAOclBP^{r}cl accumulated more diols than phenols after 6 h of incubation with 4 μM B[a]P,

Table III-1

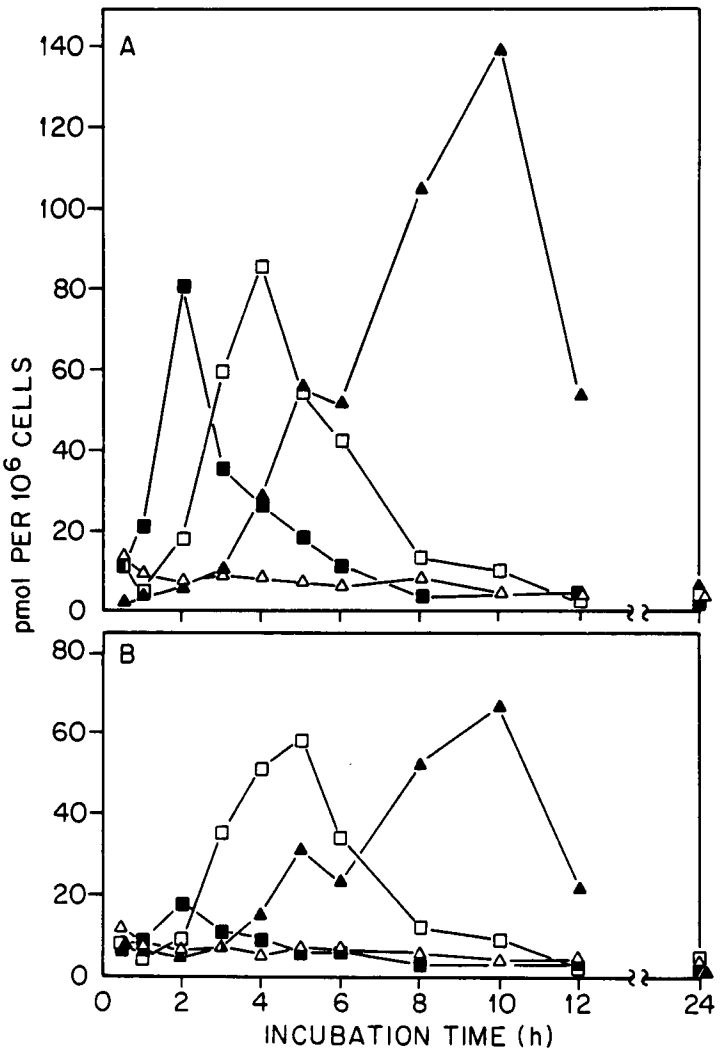
Effect of Cell Density on the Accumulation of Total Ethyl Acetate-Soluble Metabolites in Medium[a]

Cell Line	Cell Density[b]	
	Low	High
BP^rc1	9.52[c]	7.62
Hepa-1c1c7	20.22	21.39
MUL12	26.50	22.56
TAOc1BP^rc1	10.39	19.49

[a]An aliquot of the ethyl acetate fraction of medium was analyzed by HPLC and scintillation counting. The percentage of radioactivity eluting as metabolites was determined and normalized to cell number. The concentration of B[a]P at the beginning of the experiment was 4 μM, and the incubation time was 6 h.

[b]"High cell density" refers to apparent confluence, which ranged from 6.5 to 9.0 x 10^6 cells per 100 mm dish, and "low cell density" refers to 1.1 to 1.5 x 10^6 cells per 100 mm dish.

[c]Table entries are percent of total ethyl acetate-soluble ^3H.



Ethyl acetate-soluble metabolites of B[a]P were separated by HPLC, fractions were collected and counted by scintillation and the data were converted to pmol and plotted, as described in "Materials and Methods." A, accumulation of 9,10-diol; B, accumulation of 7,8-diol. The concentration of B[a]P at the beginning of the experiment was 4 μM, and cell densities ranged from 5.8 to 19.5 x 10^6 cells per 100 mm dish. △, BP^{r}c1; ▲, TAOc1BP^{r}c1; □, Hepa-1c1c7; ■, MUL12.

Figure III-3. Accumulation of diols in ethyl acetate phase after extraction of medium.

Table III-2

Accumulation of Individual Ethyl Acetate-Soluble Metabolites in Medium from High or Low Density Cells[a]

	BP^{r}c1		Hepa-1c1c7		MUL12		TAOc1BP^{r}c1	
Cell Density[b] / Metabolite	High	Low	High	Low	High	Low	High	Low
9,10-diol	6[c]	33	42	120	11	207	51	58
4,5-diol	9	41	14	102	6	110	20	39
7,8-diol	6	69	34	263	6	292	23	70
Quinones	42	520	48	497	29	583	47	428
9-phenol	9	149	13	437	4	257	7	134
3-phenol	16	468	21	415	7	258	14	204

[a]An aliquot of the ethyl acetate fraction of medium was analyzed by HPLC and scintillation counting. The percentage of radioactivity eluting as individual metabolites was determined and normalized to cell number. The concentration of B[a]P at the beginning of the experiment was 4 μM and the incubation time was 6 h.

[b]"High cell density" refers to apparent confluence, which ranged from 6.5 to 9.0 x 10^6 cells per 100 mm dish, and "low cell density" refers to 1.1 to 1.5 x 10^6 cells per 100 mm dish.

[c]Table entries are pmol per 10^6 cells.

but at low cell density, more phenols were accumulated than diols. Variant BP^rcl accumulated an appreciable amount of phenols when a lower number of cells was incubated with 4 μM B[a]P.

It is also seen in Table III-2 that at high cell density, the distribution of metabolites from TAOclBP^{r}cl resembles that from Hepa-1c1c7, but at low cell density, the distribution of metabolites from TAOclBP^{r}cl looks more like that from BP^rcl.

4. Release of Radioactivity by Beta-Glucuronidase

When the aqueous phase from the medium which had contained 4 μM [^{3}H]B[a]P was treated with beta-glucuronidase, radioactivity was released which eluted at the polar end of the chromatogram, and in the quinone region, but only low levels of phenols were released. In contrast, when the aqueous phase from 25 nM B[a]P-containing medium was treated with beta-glucuronidase, more phenols were released than quinones (Table III-3). The variant BP^rcl conjugated very small amounts of quinones and phenols to glucuronides, compared with Hepa-1c1c7, MUL12, and TAOclBP^{r}cl. These results are consistent with the lack of accumulation of water-soluble radioactivity in the medium from BP^rcl cells, shown in Figure III-1B, page 37.

D. Discussion

Our results indicate that subpopulations of cells separated from a parent clone can have markedly different metabolic abilities. The parent, Hepa-1c1c7, and one of the variants, MUL12, are similar

Table III-3

Release of B[a]P Metabolites from Glucuronide Conjugates by Beta-Glucuronidase

Metabolite	B[a]P Concentration			
	4 μM		25 nM	
	Quinones	Phenols	Quinones	Phenols
At 12 hours				
Hepa-1c1c7	176.9[b]	60.1	1.74	2.38
MUL12	--[c]	--	2.37	2.97
TAOc1BP^{r}c1	92.6	16.0	1.85	2.42
BP^{r}c1	--	--	0.03	0.04
At 24 hours				
Hepa-1c1c7	193.9	35.8	2.40	4.29
MUL12	324.7	63.3	4.18	4.05
TAOc1BP^{r}c1	70.4	ND[d]	1.11	1.91
BP^{r}c1	--	--	0.02	0.07

[a]The aqueous phase from the ethyl acetate extraction of medium was washed two more times with 2.5 vol ethyl acetate, then digested with bacterial beta-glucuronidase. After digestion, the aqueous phase was again extracted twice with 2.5 vol ethyl acetate. The ethyl acetate phase was evaporated, the residue was taken up in methanol and analyzed by HPLC.

[b]Values are pmol of ethyl acetate-soluble metabolites present after beta-glucuronidase treatment, normalized per 10^6 cells. Cell densities ranged from 2.1 to 23.1 x 10^6 cells per 100 mm dish.

[c]A dash (--) indicates "not done."

[d]Not detected.

in overall activity, with high ability to metabolize B[a]P, both in terms of accumulation of ethyl acetate-soluble metabolites and water-soluble conjugates. The variant BP^{r}c1 has low B[a]P metabolizing activity at low cell density, and TAOc1BP^{r}c1 has intermediate activity, depending on cell density, incubation time, and type of metabolism.

Analysis of individual metabolites of B[a]P is important because some metabolites are more carcinogenic than others. The 7,8-dihydrodiol-9,10-epoxide of B[a]P (BPDE) is the metabolite that is mainly responsible for binding to DNA (Sims et al., 1974). The metabolic conversion of B[a]P to BPDE occurs in three steps via the 7,8-oxide and the 7,8-diol (Deutsch et al., 1979; Levin et al., 1980; Thakker et al., 1977; Yang et al., 1977). Thus, it was of interest to determine the levels of 7,8-diol produced by the parent cell line and its three variants.

In addition, more recent studies have indicated that the 9,10-diol of B[a]P may be converted to a metabolite (presumably the 9,10-diol-7,8-epoxide) that covalently binds to a nuclear protein(s) having electrophoretic mobility similar to that of histone H1 (MacLeod et al., 1980, 1981). Our results indicate that MUL12 and Hepa-1c1c7 are more active in the further metabolism of 9,10-diol than is TAOc1BP^{r}c1. We speculate that the conversion of the 9,10-diol to the 9,10-diol-7,8-epoxide may be slower in TAOc1BP^{r}c1 than in MUL12 or Hepa-1c1c7.

There is a substrate concentration effect on the conversion of phenols and quinones to glucuronide conjugates. As shown in

Table III-3, at high substrate concentration, a larger amount of quinones than phenols was released from glucuronide conjugates by beta-glucuronidase. At low substrate concentration, a larger amount of phenols than quinones was released. A possible reason for this substrate concentration effect is that at the high substrate concentration there is a greater opportunity for phenols to be oxidized to quinones, whereas at the low substrate concentration the phenols are conjugated to glucuronides too rapidly for much oxidation to occur.

We conclude that Hepa-1c1c7, MUL12, and TAOc1BP^{r}c1 have inducible and basal AHH activity, but that AHH is more slowly induced in TAOc1BP^{r}c1 than in Hepa-1c1c7 or MUL12. Thus our rank order of inducible AHH activity for Hepa-1c1c7, TAOc1BP^{r}c1, and BP^{r}c1 agrees with that published by others (Miller et al., 1983). In addition, our work provides information on the individual metabolites of B[a]P produced in these cells, and the response of metabolic reactions in these cells to changes in cell density and substrate concentration.

Whitlock and Galeazzi (1984) have suggested that BP^{r}c1 lacks AHH activity because this variant has an inducer-receptor complex which does not bind to chromatin, whereas TAOc1BP^{r}c1 has low levels of AHH activity because this variant has receptors which do not bind to inducer. Our observation of a lack of AHH activity in BP^{r}c1 at high cell density agrees with their results, but the observation of some production of phenols by this variant at low cell density and the dependence of AHH activity in TAOc1BP^{r}c1 on cell density

are departures from the results of these workers. Possible reasons for these effects will be discussed in Chapter VI.

The shift in ratio of diols to phenols as a function of cell density which we have observed in this study also agrees with another report (Baird et al., 1981) in which confluent hamster or rat embryo cells metabolized B[a]P mainly to dihydrodiols, whereas low density cells showed an increased accumulation of free phenols.

In conclusion, this study has shown that subcultures of cells, isolated from a parent cell line by flow cytometry, are not biochemically identical. In particular, the parent cell line and its three variants show differences in the rate and extent of overall metabolism of benzo[a]pyrene, and in the production of individual metabolites. These differences will most likely influence the levels of carcinogenic metabolites formed and subsequent biological consequences in the cells. These results suggest that subpopulations of cells in a mass culture may differ in their metabolic activation capability and therefore their susceptibility to transformation to a neoplastic state by a chemical carcinogen such as benzo[a]pyrene.

CHAPTER IV

SISTER CHROMATID EXCHANGES INDUCED BY B[a]P IN CELLS DERIVED FROM A MOUSE HEPATOMA

A. Introduction

The metabolic studies described in Chapter III had shown that the parent Hepa-1c1c7 and the variants MUL12 and TAOc1BP^{r}c1 were more active in metabolism of B[a]P than the variant BP^{r}c1. In an extension of these studies, the relationship between the metabolic capabilities of two of these variants and the induction of sister chromatid exchanges by B[a]P was investigated. B[a]P is able to induce SCEs only in systems that are capable of metabolizing this compound (Abe and Sasaki, 1982b; Latt et al., 1982; Perry, 1980). TAOc1BP^{r}c1 and BP^{r}c1 were similar in their population doubling times and modal chromosome numbers, as discussed in Chapter II, but were different in their ability to metabolize B[a]P, as discussed in Chapter III. Therefore it was decided that this pair would be most suitable for a study of the induction of sister chromatid exchanges by B[a]P.

The experimental protocol used for this SCE study was:

1. to use intact cells without an exogenous activation system;
2. to use B[a]P itself, not its metabolites;
3. to correlate SCE responses with metabolism results.

This experimental protocol was used to ensure that SCEs would be induced only if B[a]P was metabolized. This study was conducted to see if a difference in SCE induction could be detected between such closely related cell variants.

B. Materials and Methods

1. Chemicals and Cell Culture

These were as described in Chapter III.

2. Chemical Exposure

Cells were seeded at 5 x 10^5 cells per plate and incubated for 26 h. The medium was then removed by aspiration, each plate was rinsed with 5 ml phosphate-buffered saline, and fresh medium was added. Plates for SCE analysis received unlabeled B[a]P at concentrations of 0, 40, 200, or 1000 nM for 6 h. Plates for metabolism analysis received B[a]P at the same concentrations but labeled with ^3H at a specific activity of 3.15 Ci/mmol.

3. SCE Analysis

After incubation with B[a]P, the medium was removed, the plates were rinsed with phosphate-buffered saline, fresh medium containing 5 μM BrdU was added, and incubation was continued for 40 or 45 h. Two hours before cell harvest, colchicine was added to the medium at a final concentration of 4 μM. At 40 or 45 h, cells were removed from the plates by scraping with a rubber tipped glass rod. They were swollen in 10 ml of 0.075 M KCl for 20 min at 37°C, then

fixed in a 3:1 mixture of methanol and acetic acid. After a wash in fixative, slides were prepared, coded and later stained for sister chromatid analysis according to the method of Goto et al. (1978), as follows:

After aging at room temperature for two or three days, the slides were immersed in Hoechst 33258 (5 μg/ml distilled water) for 30 min in a jar protected from light. The slides were rinsed with water three times, then dipped in McIlvaine's buffer (pH 8.0, made by mixing 35.36 ml of 0.2 M Na_2HPO_4 and 1.0 ml of 0.1 M citric acid). A long cover slip was set on the wet slide, excess buffer was blotted off, and the cover slip was sealed to the slide with rubber cement. The sealed slides were irradiated under BLB light (peak wavelength of 355 nm, 97% of emission in the range 300-400 nm) at 58°C for 10 to 30 min while being protected from drafts. The slides were removed from the heating tray, the rubber cement and cover slip were removed, and the slides were rinsed in distilled water. The slides were stained for 15 to 25 min in 4-6% Giemsa made up in phosphate buffer, pH 6.8. (Staining conditions were varied slightly to give the best differentiation of SCEs for each cell preparation.) After being rinsed and air dried for at least 30 min, each slide was mounted in a drop of Euparal and a coverslip, and the Euparal was allowed to harden overnight before analysis.

The coded and stained slides were scanned for differentially stained, properly spread intact metaphase cells containing 55 to 59 chromosomes (mode $+/-^2$ chromosomes). The chromosome number and SCE

frequency per metaphase were scored from 50 cells per group and statistical analyses were performed as described under Results.

C. Results

1. SCE Analysis

Pictures of representative metaphases are shown in Figure IV-1. A summary of the SCE frequencies induced by 40, 200, and 1000 nM B[a]P is presented in Table IV-1. A clear dose response was observed for TAOclBP^{r}cl cells, but not for BP^{r}cl cells.

A factorial two-way analysis of variance (Daniel, 1978) showed a dose effect (Factor B in Table IV-2), an effect between cell variants (Factor A in Table IV-2), and an interaction between effects (AB in Table IV-2), which indicated that results differed for the two variants. The variance ratio (V.R. in Table IV-2) exceeded the critical value of F (footnote c of Table IV-2) for Factor A, Factor B, and their interaction AB. Therefore the two effects and their interaction were each significant at the $p < .001$ level (Table IV-2).

Frequency distributions of SCEs per cell are shown in Figure IV-2. At a B[a]P concentration of 1000 nM, variant TAOclBP^{r}cl has a bimodal distribution of SCEs per cell.

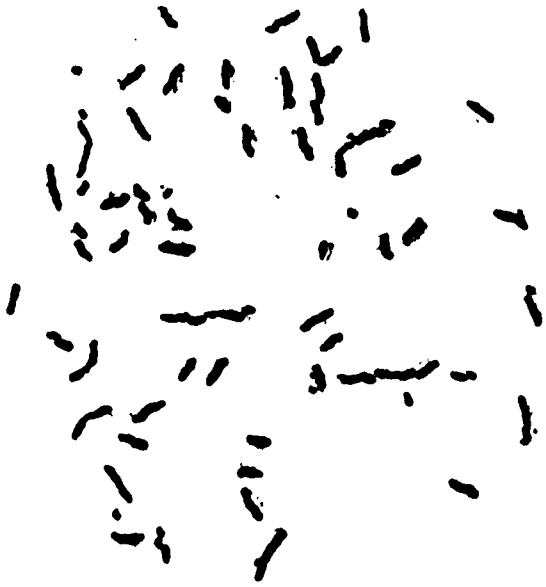
2. Metabolism of Benzo[a]pyrene

The studies described in Chapter III show that at high cell density variant TAOclBP^{r}cl was more active than variant BP^{r}cl in

Figure IV-1. Sister chromatid exchanges in BP^rcl and TAOclBP^{r}cl.

Sister chromatid exchanges were determined as explained in text. A, BP^rcl cells; B, TAOclBP^{r}cl cells, no B[a]P; C, TAOclBP^{r}cl cells, 40 μM B[a]P; D, TAOclBP^{r}cl cells, 200 nM B[a]P; E, TAOclBP^{r}cl cells, 1000 nM B[a]P.

A



B

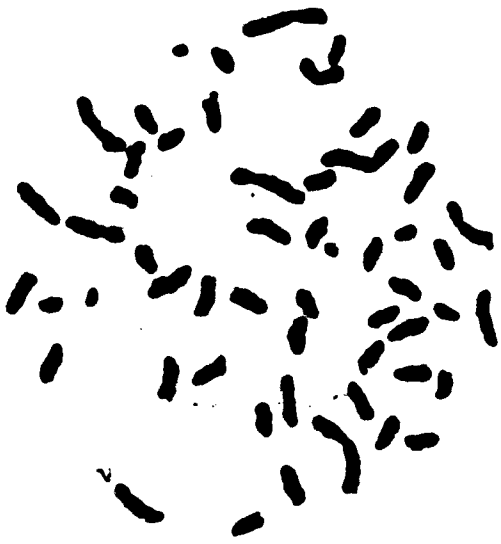


Figure IV-1.

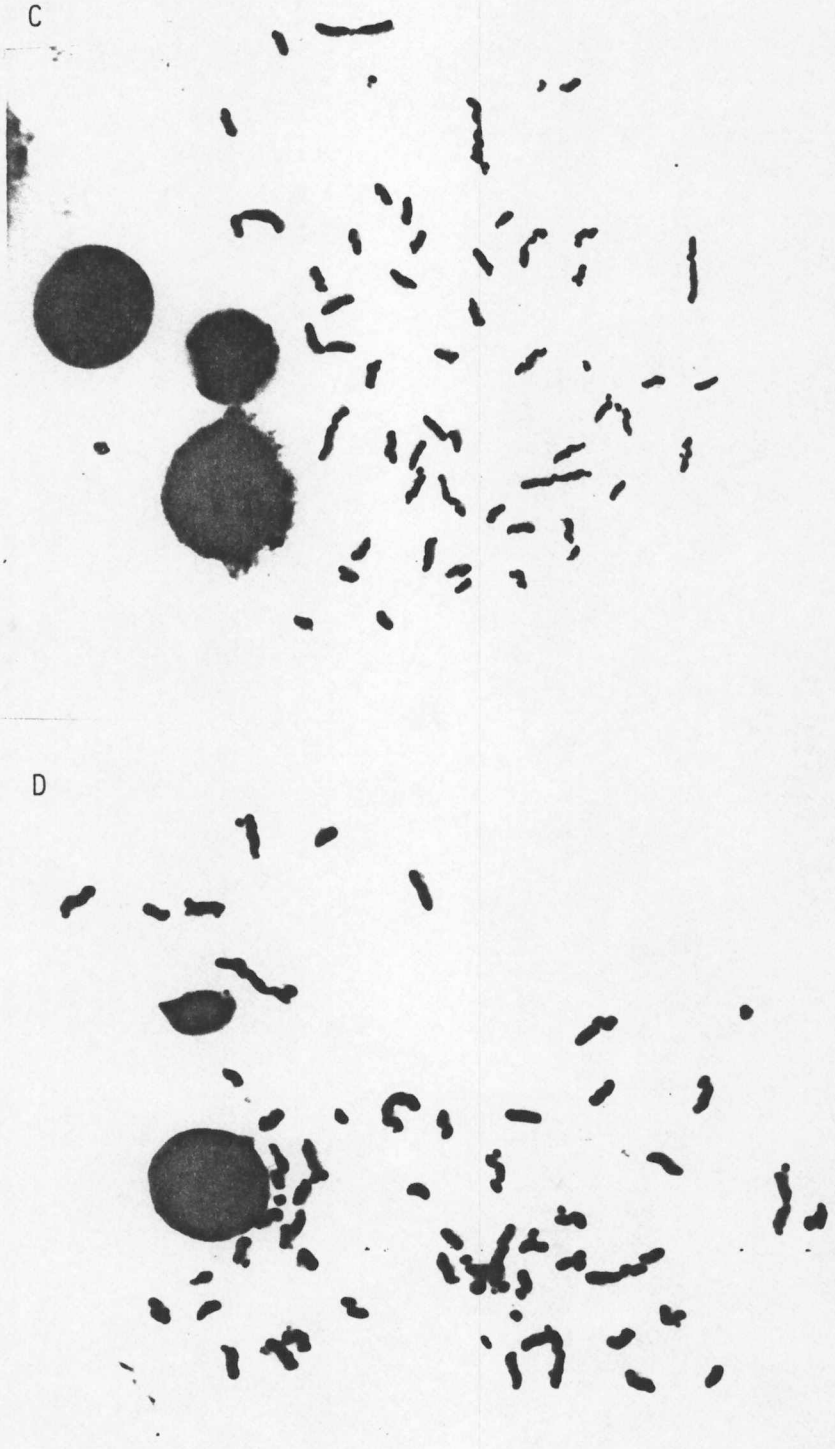


Figure IV-1. (Continued)

E

Figure IV-1. (Continued)

Table IV-1

Incidence of SCEs in BP^rcl and $TAOclBP^rcl$ Cells Exposed to Three Concentrations of Benzo[a]pyrene

Concentration of Benzo[a]pyrene (nM)	SCEs per Cell[a] (Mean ± S.D.)		SCEs per Chromosome[a] (Mean ± S.D.)	
	BP^rcl	$TAOclBP^rcl$	BP^rcl	$TAOclBP^rcl$
0	25.2 ± 8.0	22.9 ± 9.0	0.44 ± 0.14	0.40 ± 0.16
40	24.5 ± 10.4	44.2 ± 18.5	0.43 ± 0.18	0.78[b] ± 0.33
200	27.5 ± 10.3	60.4 ± 28.3	0.48 ± 0.18	1.06[c] ± 0.49
1000	27.6 ± 10.6	69.9 ± 32.0	0.48 ± 0.18	1.24[d] ± 0.56

[a]The SCE scores were obtained on 50 metaphases per group.

[b]Significantly greater than 0.40 for $TAOclBP^rcl$ and 0.43 for BP^rcl (t test, $p<.005$ for each).

[c]Significantly greater than 0.78 for $TAOclBP^rcl$ and 0.48 for BP^rcl (t test, $p<.005$ for each).

[d]Significantly greater than 1.06 for $TAOclBP^rcl$ and 0.48 for BP^rcl (t test, $p<.005$ for each).

Table IV-2

Analysis of Variance for SCE Dose Experiment[a]

Source[b]	SS	d.f.	MS	V.R.[c]
A	172,931	1	172,931	170.68
B	112,385	3	37,462	36.97
AB	87,369	3	29,123	28.74
Treatments	376,685	7		
Residual	397,178	392	1,013	
Total	769,863	399		

[a]SS = sum of squares.
d.f. = degrees of freedom.
MS = mean squares = SS/d.f.
V.R. = variance ratio = (mean square of Factor A or Factor B or Interaction AB) divided by mean square of residual.

[b]Factor A = cell variant.
Factor B = B[a]P concentration.
AB = interaction between Factor A and Factor B.
Treatments = A + B + AB.
Total = Treatments + Residual.

[c]For alpha = 0.001, critical values of F are:
For Factor A, $F_{1,392} = 10.83$.
For Factor B, $F_{3,392} = 5.42$.
For Interaction AB, $F_{3,392} = 5.42$.
In each case, V.R. > F.
Therefore, $p_A < .001$, $p_B < .001$, and $p_{AB} < .001$.

Figure IV-2. Frequency distributions of SCEs per cell.

Sister chromatid exchanges were determined as explained in the text. The numbers of SCEs per cell were grouped by intervals of 25, and the number of cells was determined for each interval. The symbols refer to different concentrations of B[a]P.

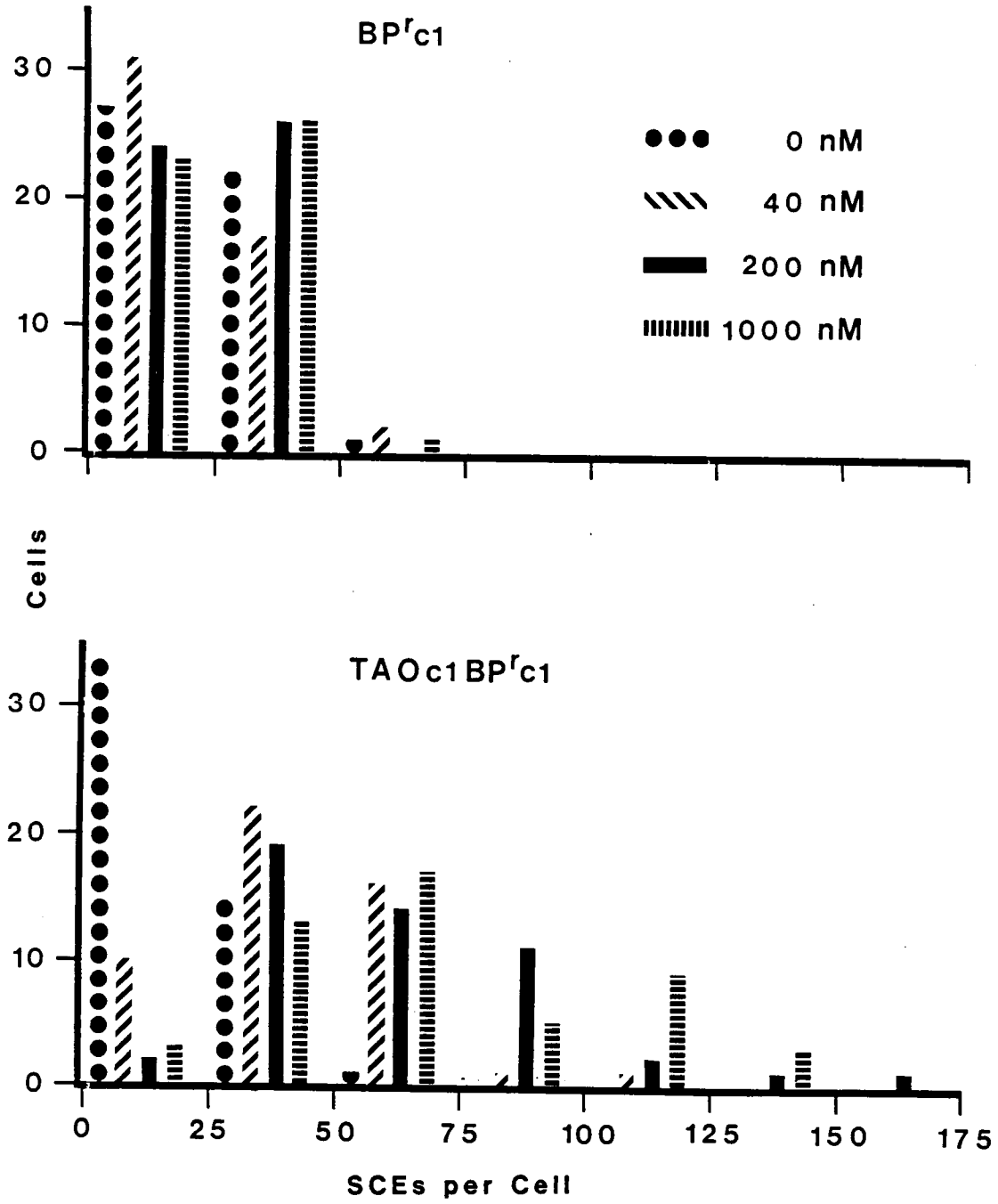

BP^r c1
30
20
10
0
0 nM
40 nM
200 nM
1000 nM
Cells
TAOc1BP^r c1
30
20
10
0
0
25
50
75
100
125
150
175
SCEs per Cell

Figure IV-2.

metabolism of B[a]P. However during preliminary experiments in preparation for the sister chromatid exchange experiments, it was discovered that conditions which had been suitable for the metabolic studies were not suitable for SCE studies. First, the metabolism studies had been done at confluence. However, confluent cultures were unsuitable for obtaining mitotic cells for cytogenetic analyses. Therefore the cell density was lowered. Second, the lower cell density required lower concentrations of B[a]P, because 4 μM B[a]P induced too many SCEs to be conveniently counted, and seemed to delay cell cycle progression from G_1 into S. Thirdly, a relatively short incubation time was chosen for the SCE studies.

Under the experimental conditions of the SCE studies, variant BP^rcl was as active as variant $TAOclBP^rcl$ in the overall production of ethyl acetate-soluble metabolites of B[a]P (Table IV-3). However, the two variants did not produce the various metabolites to the same extent. For example, BP^rcl did not produce a detectable amount of BP-7,8-dihydrodiol, whereas $TAOclBP^rcl$ produced this metabolite in amounts which increased as the substrate B[a]P concentration was increased (Table IV-3). Similarly, $TAOclBP^rcl$ produced BP-9,10-dihydrodiol in amounts which increased as the substrate B[a]P concentration was increased, but BP^rcl did not produce this metabolite.

D. Discussion

In these studies, BP^rcl and $TAOclBP^rcl$, subpopulations of a single parent cell line, showed a highly significant difference in

Table IV-3

Metabolism of Benzo[a]pyrene by BP^{r}cl and TAOclBP^{r}cl Cells

Concentration of Benzo[a]pyrene (nM)	Total Ethyl Acetate-Soluble Metabolites (pmol Per Plate)		BP-7,8-Dihydrodiol (pmol Per Plate)	
	BP^{r}cl	TAOclBP^{r}cl	BP^{r}cl	TAOclBP^{r}cl
40	63	73	ND[a]	2.7
200	283	330	ND	14.7
1000	1500	1184	ND	41.5

[a]Not detected.

the induction of SCEs by B[a]P. This difference in biological response was related to the biochemical differences between the two variants in their ability to metabolize B[a]P. The 7,8-diol and the 9,10-diol were produced in TAOclBP^{r}cl but not in BP^{r}cl. Sims et al. (1974) showed that BP-7,8-diol is converted to BP-7,8-diol-9,10-epoxide. Pal et al. (1978) showed that the 7,8-diol is the best inducer of SCEs among the diols tested, which included the 9,10-diol. Pal et al. (1980) showed that the 7,8-diol-9,10-epoxide is the species mainly responsible for the induction of SCEs by B[a]P. These reports support the conclusion that the production of a specific metabolite, BP-7,8-diol, is more relevant to the differential induction of SCEs in TAOclBP^{r}cl and BP^{r}cl than overall metabolism.

The bimodal distribution of SCEs per cell in TAOclBP^{r}cl could indicate that TAOclBP^{r}cl is actually composed of two (or more) subpopulations of cells, which exhibit different SCE responses to higher doses of B[a]P. This hypothesis could be tested by cloning single cells of this variant, then observing SCE responses in the progeny (Dr. Julian Preston, Biology Division, Oak Ridge National Laboratory, personal communication). Perhaps this would also explain the dependence of metabolic activity of this variant on cell culture conditions: maybe one subpopulation of TAOclBP^{r}cl has enzymes which are more inducible than those of the other.

Sister chromatid exchange analysis has been used extensively to assess the genotoxicity of chemical mutagens and carcinogens,

including B[a]P (Perry and Evans, 1975; Latt, 1981). Since many chemical mutagens and carcinogens require metabolic activation before they can exert a genotoxic effect (Miller, 1970), efforts have been made to develop in vitro systems of target cells with metabolic capability. This study utilized two variants of a mouse hepatoma cell line for determining the relationship of B[a]P metabolism to the induction of SCE. The availability of cell variants which differ in a given biological response to a chemical may allow investigators to study the genetic mechanisms and metabolic reactions responsible for the biological response.

CHAPTER V

BINDING OF B[a]P METABOLITES TO NUCLEAR MACROMOLECULES IN CELLS DERIVED FROM A MOUSE HEPATOMA

A. Introduction

B[a]P exerts its effects on cells in three steps. In the first step, B[a]P is metabolized to several activated electrophilic species. In the second step, these electrophiles bind to cellular nucleophiles, especially the three classes of macromolecules: DNA, RNA, and protein. In the third step, biological effects occur. These biological effects include sister chromatid exchanges, chromosomal aberrations, mutations, morphologic transformation, and neoplastic transformation. Therefore, as part of the characterization of these mouse hepatoma cells, the binding of B[a]P metabolites to nuclear DNA, RNA, and protein was studied and related to the results of the metabolism and sister chromatid exchange studies described in Chapter III and IV, respectively.

B. Materials and Methods

1. Chemicals

Reagents for cell culture and labeling have been described in Chapter III. Phenylmethylsulfonyl fluoride (PMSF), sodium deoxycholate, Triton X-100, and Tris base were obtained from Sigma Chemical Company. Acrylamide, bis-acrylamide, bromophenol blue, beta-

mercaptoethanol, glycine, ammonium persulfate, and Coomassie Blue R were obtained from BioRad Laboratories. Sodium dodecyl sulfate (SDS) was purchased from BDH Chemicals. Glycerol was obtained from the Preiser Company. Fluorescamine was obtained from the Roche Diagnostics Division of Hoffman-LaRoche, Inc. 2,5-Diphenyloxazole (PPO) came from Aldrich Chemical Co. Guanidine hydrochloride was obtained from Schwarz/Mann. Density grade cesium sulfate was purchased from Atomergic Chemetals.

2. Cell Culture and Labeling

Tertiary hamster embryo cells and the mouse hepatoma cells were cultured as described in Chapter III, labeled with [^{3}H]B[a]P at a concentration of 4 μM and a specific activity of 6 Ci/mmol for 12 or 24 h, and then prepared as described below.

3. Preparation of Nuclei

Cells were harvested by treatment for 15 min at 37°C with 0.05% trypsin, 0.02% EDTA (Gibco), and washed in RSB (reticulocyte swelling buffer) [10 mM NaCl, 10 mM Tris (pH 7.0), 1.5 mM $MgCl_2$]; PMSF (0.1 M in isopropanol) was added to this and all subsequent solutions to a concentration of 0.5 mM. Cells were suspended in RSB and lysed by Potter-Elvehjem homogenization after the addition of Triton-X 100 and sodium deoxycholate to 0.5% each. Nuclei were pelleted by low-speed centrifugation, and the homogenization and centrifugation steps were repeated to remove any cytoplasmic remnants from the nuclei (MacLeod et al., 1979).

4. Separation of Nuclear Macromolecules by Isopycnic Banding

The nuclear pellet from the second homogenization was lysed by dissolution in 6 M guanidine hydrochloride, 10 mM EDTA (pH 7.0), and its viscosity was reduced by sonication. Unbound B[a]P and its metabolites were removed by extracting the lysate three times with three volumes of ethyl acetate at room temperature. Residual ethyl acetate was evaporated with a stream of nitrogen. Aliquots of the nuclear preparation (2.0 ml) were layered over 2.8 ml of a solution containing 2.2 M cesium sulfate, 10 mM EDTA (pH 7.0), and 9% (v/v) dimethylsulfoxide in polyallomer tubes, and centrifuged for 40 +/- 2 h in a Spinco SW 50.1 rotor at 35,000 rpm (147,000 gmax) at 20°C. Tubes were pierced near the bottom and fractions of six drops each were collected and assayed for nucleic acid, protein, and radioactivity, as described below.

5. Analytical Methods

The amount of nucleic acid was determined by diluting 60- to 120-µl aliquots with 2.0 ml water and recording the absorbance at 260 nm. It was assumed that an absorbance of 1.0 corresponded to 40 µg/ml RNA or 50 µg/ml DNA. The diluted aliquots were mixed with 15 ml Aquasol and radioactivity was determined in a Searle Mark III liquid scintillation counter. Protein was determined by reaction with fluorescamine (Udenfriend et al., 1972). These raw data were plotted by a Tektronix Model 4662 Interactive Digital Plotter driven by a Tektronix 4051 graphics terminal. The specific activities of

B[a]P metabolites bound to nucleic acid and protein were calculated by the Tektronix 4051.

Analysis of total nuclear proteins was carried out using sodium dodecyl sulfate (SDS)--15% polyacrylamide slab gel electrophoresis (Kootstra et al., 1982; Laemmli, 1970). Fractions from the cesium sulfate gradient which contained protein but not nucleic acid were pooled, dialyzed, lyophilized, and dissolved in protein resuspension buffer, which was 100 mM NaCl, 1.0 mM EDTA, 1.0 mM PMSF, 1.0 mM dithiothreitol, and 1.0% SDS. Aliquots were removed for fluorescamine assay and scintillation counting. Two volumes of cold methanol were added, the proteins were precipitated overnight at 4°C, and the tubes were centrifuged at 2000 rpm for 30 min at 4°C. The methanol was decanted and residual methanol was blown off under nitrogen. The pellets were dissolved to a concentration of 3 µg/ml in SDS-sample application buffer, which contained 11.25% glycerol, 5% beta-mercaptoethanol, 2.25% SDS, 0.07 M Tris-HCl (pH 6.8), and 0.004% bromophenol blue. Samples, usually 15 µl/slot but sometimes up to 60 µl/slot, were subjected to electrophoresis at constant voltage (140 V) for ~4 h until the tracking dye, bromophenol blue, had reached the bottom of the gel. The proteins were visualized by staining the gels for at least 2 h in 25% acetic acid, 10% isopropanol, and 0.2% Coomassie Brilliant Blue R250. The gels were rinsed in 10% acetic acid, destained for several days in 10% acetic acid (sometimes containing 25% methanol), scanned with a Zeineh tungsten densitometer (Biomed Instruments, Inc.), and

photographed. For fluorography, the gels were treated with 2,5-diphenyloxazole in dimethylsulfoxide (Bonner and Laskey, 1974; Laskey and Mills, 1975). Kodak X-ray films (SB-5) were exposed to the gels for several months at -55°C, then developed and scanned densitometrically to visualize the position of the protein-bound radioactive carcinogen.

C. Results

1. Binding to Classes of Nuclear Macromolecule

Cells incubated with [^{3}H]B[a]P were lysed and centrifuged through cesium sulfate gradients to separate nuclear RNA, DNA, and protein adducts.

Examples of the profiles of nuclear macromolecules in cesium sulfate gradients are presented in Figure V-1. The binding of B[a]P metabolites to all three classes of nuclear macromolecule by BP^{r}c1 cells is very small (Figure V-1A). In contrast, Hepa-1c1c7, MUL12, TAOc1BP^{r}c1, and hamster embryo cells bind B[a]P metabolites to nuclear RNA, DNA, and protein.

Specific activities were calculated to obtain a more quantitative measure of B[a]P metabolite binding to each class of nuclear macromolecule by each type of cell. Average values are presented in Table V-1.

Factorial two-way analyses of variance (Daniel, 1978) support the contention that there are differences among the parent and three bariants in the binding of B[a]P metabolites to nuclear macromolecules.

Figure V-1. Cesium sulfate gradients.

A, BP^{r}c1; B, Hepa-1c1c7; C, MUL12; D, TAOc1BP^{r}c1.

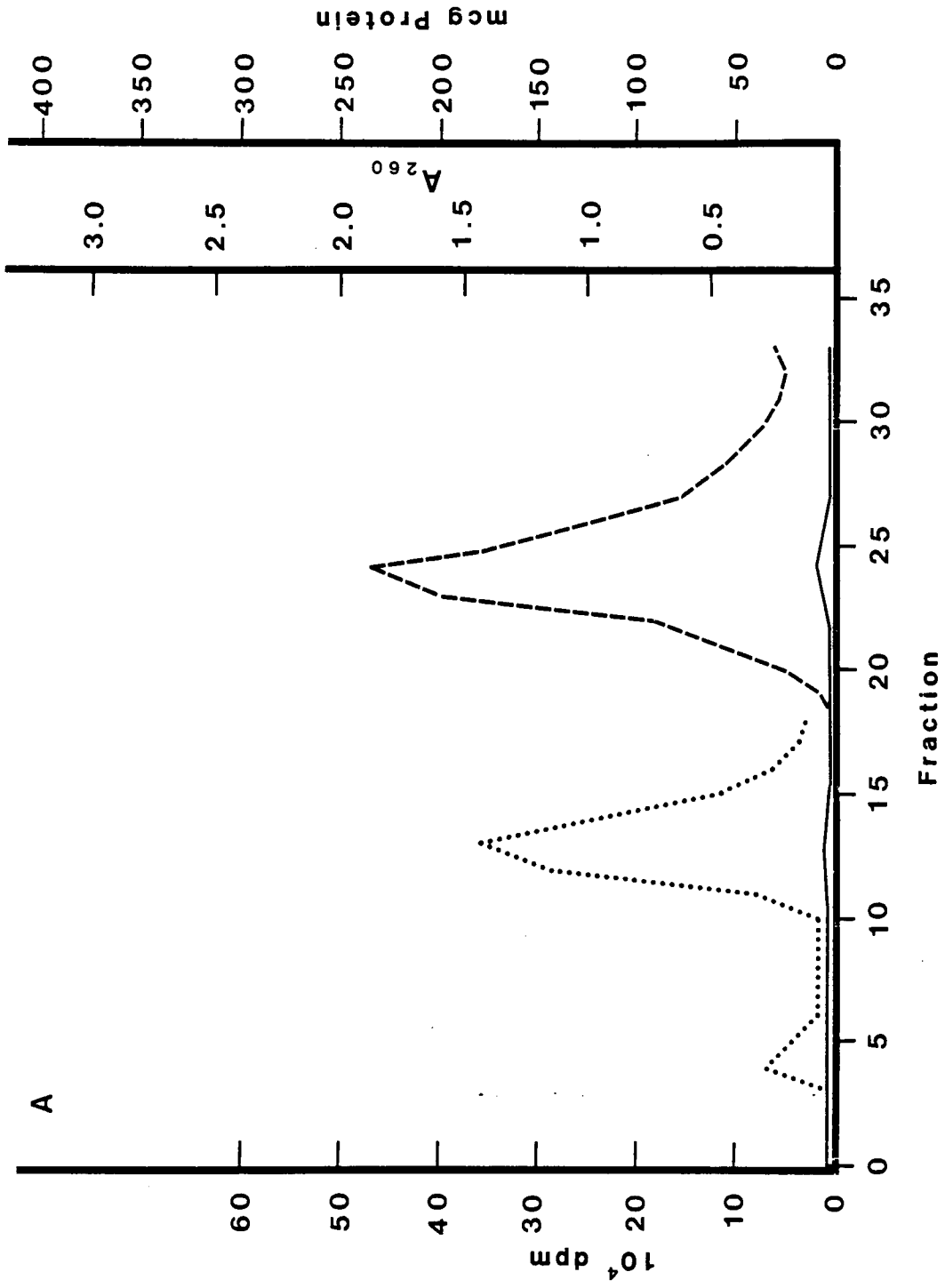

A
10^4 dpm
0
10
20
30
40
50
60
0
5
10
15
20
25
30
35
Fraction
A_{260}
0.5
1.0
1.5
2.0
2.5
3.0
mcg Protein
0
50
100
150
200
250
300
350
400

Figure V-1.

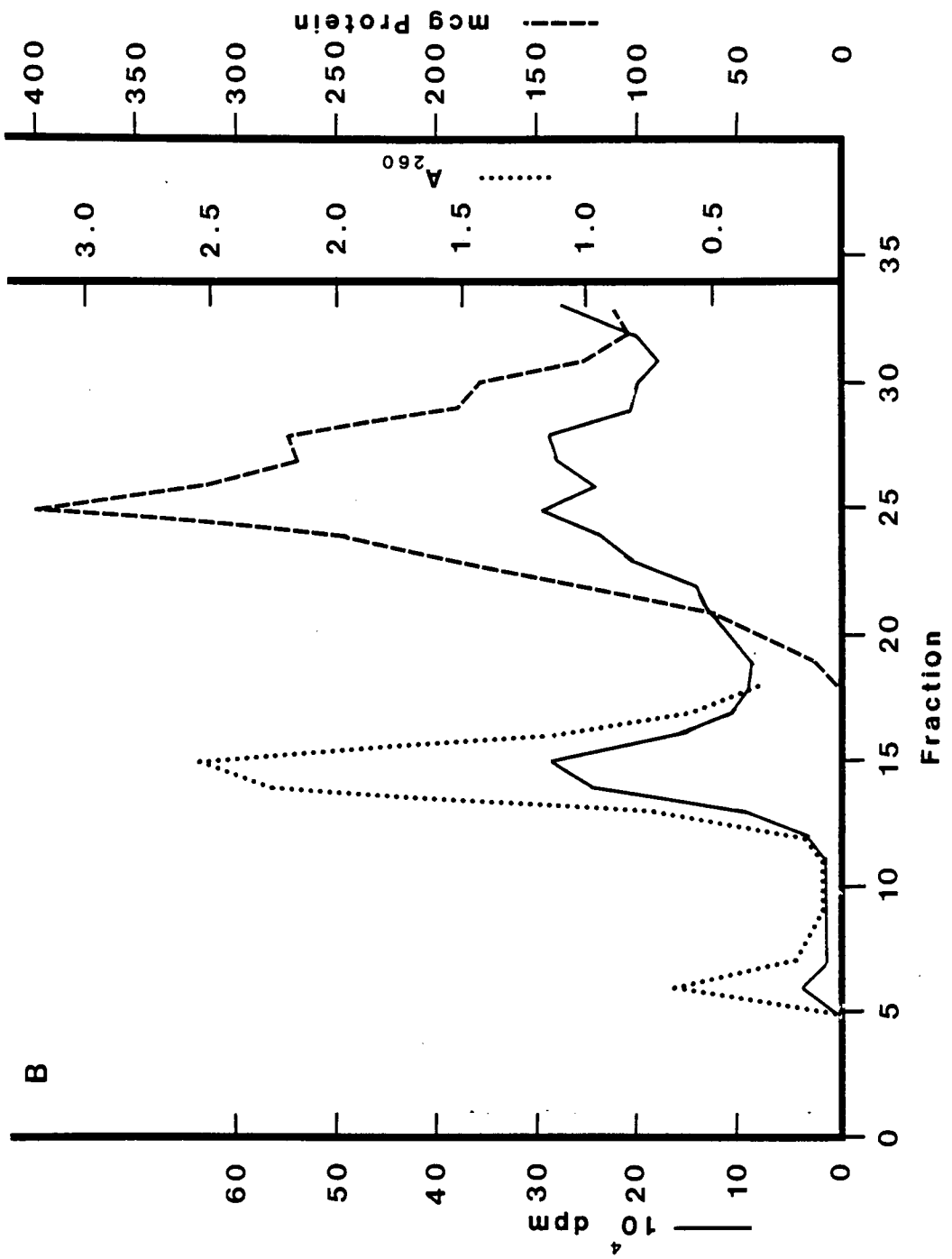


Figure V-1. (Continued)

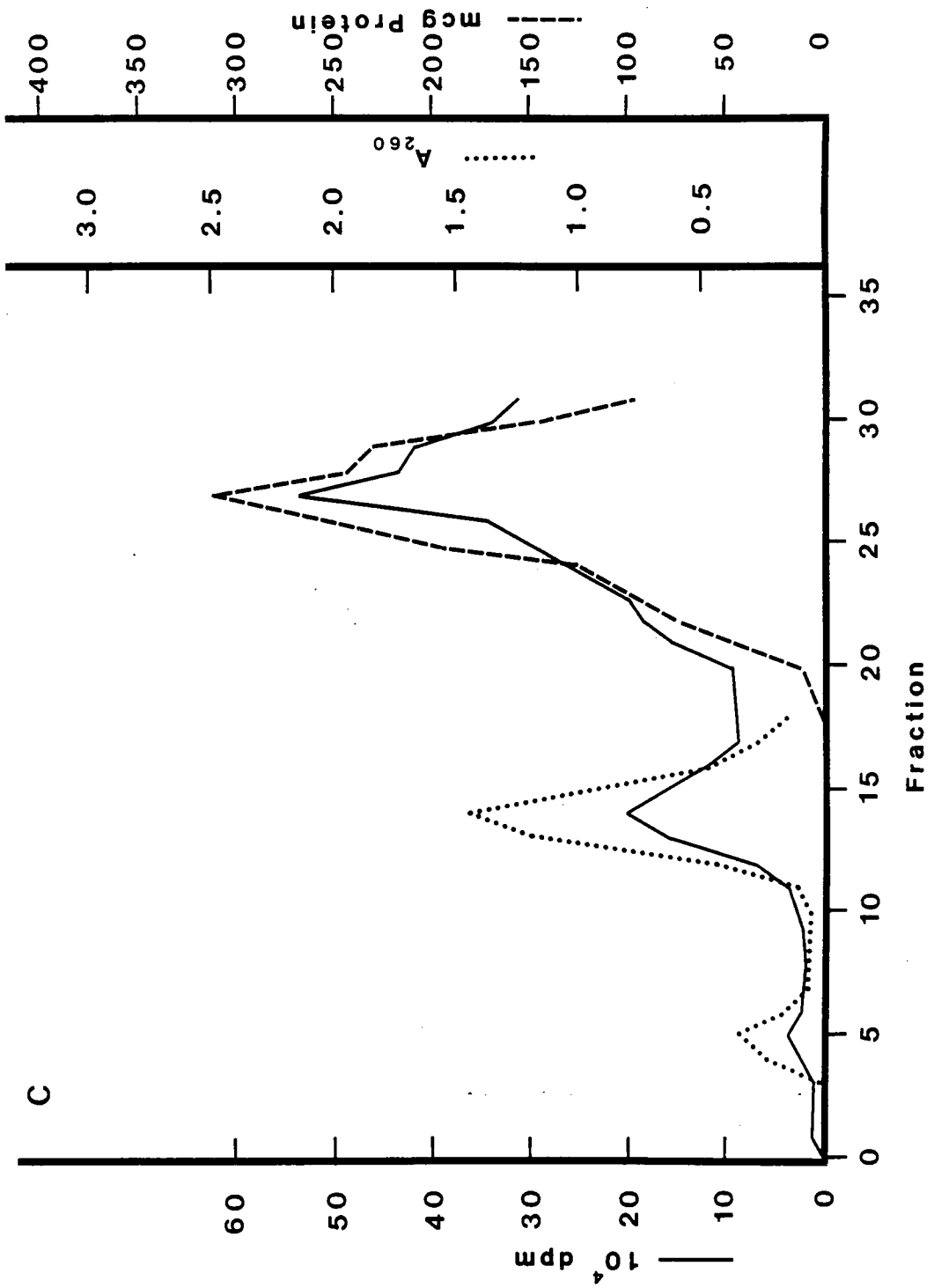


Figure V-1. (Continued)

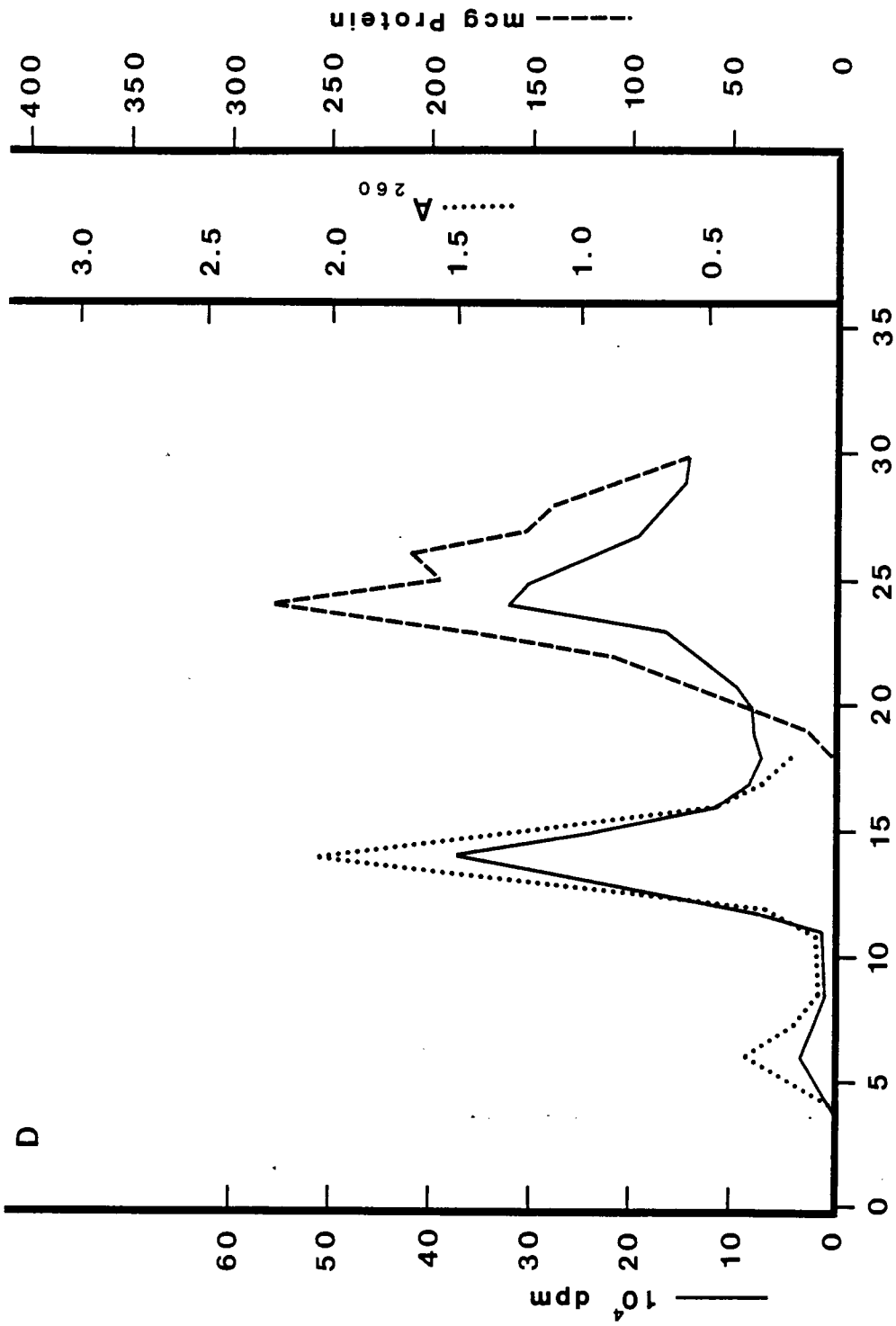


Figure V-1. (Continued)

Table V-1

Specific Activities of B[a]P Metabolites Bound to Nuclear Macromolecules: Average Values

Macro-Molecule	Incubation Time (h)	Cell Line			
		BP^{r}c1	Hepa-1c1c7	MUL12	TAOc1BP^{r}c1
RNA	12	10[a]	66	82	50
	24	8	52	74	84
DNA	12	5	102	154	82
	24	5	108	138	158
Protein	12	6	116	194	74
	24	6	98	170	116

[a]Table entries are pmol B[a]P per mg nucleic acid or protein.

The Newman-Keuls Multiple-Range Test (Bruning and Kintz, 1977) was performed to find which of these differences are statistically significant. Binding of B[a]P metabolites to nuclear macromolecules is less in $BP^{r}c1$ than in the parent or other two variants. MUL12 binds more B[a]P metabolites to nuclear protein than Hepa-1c1c7 or $TAOc1BP^{r}c1$ (for alpha = .05).

Differences in binding levels at 12 and 24 h were assessed by twelve individual two-sided t tests at alpha = .05 (four cell types x three classes of nuclear macromolecule). Variant $TAOc1BP^{r}c1$ bound more B[a]P metabolites to nuclear RNA and to nuclear DNA at 24 h than at 12 h. Other differences were not statistically significant.

2. Binding to Nuclear Proteins

The nuclear protein pools from the cesium sulfate gradients were analyzed by SDS-polyacrylamide gel electrophoresis. Photographs of representative stained gels are shown in Figure V-2. Densitometric scans were made of the gels to get semiquantitative estimates of the relative amounts of nuclear proteins in various molecular weight classes. Binding of $[^{3}H]$-B[a]P to nuclear proteins was estimated by fluorography followed by densitometric scans of X-ray films (Figure V-3).

Binding of B[a]P metabolites to nuclear proteins was not detected in variant $BP^{r}c1$ by fluorography. The parent Hepa-1c1c7 and the variants MUL12 and $TAOc1BP^{r}c1$ had detectable amounts of B[a]P

Figure V-2. Electrophoresis of nuclear proteins.

Nuclear proteins were separated by SDS-polyacrylamide gel electrophoresis as explained in the text. Migration was from top to bottom. Numbers across the top refer to lanes. Numbers down the side are molecular weights in kilodaltons, as determined by standards. Symbols below the molecular weight standards identify the core histones. In part A, 45 µg of protein were loaded per lane. In part B, different amounts of protein were loaded per lane.

A: Lanes 1 and 8, molecular weight standards; lane 2, Hepa-1c1c7, 12 h; lane 3, Hepa-1c1c7, 24 h; lane 4, TAOc1BP^{r}c1, 12 h; lane 5, TAOc1BP^{r}c1, 24 h; lane 6, MUL12, 12h; lane 7, MUL12, 24 h.

B: Lanes 1 and 8, molecular weight standards; lane 2, BP^{r}c1, 12 h, µg protein; lane 3, BP^{r}c1, 12 h, 90 µg protein; lane 4, BP^{r}c1, 12 h, 135 µg protein; lane 5, BP^{r}c1, 24 h, 45 µg protein; lane 6, BP^{r}c1, 24 h, 90 µg protein; lane 7, BP^{r}c1, 24 h, 135 µg protein.

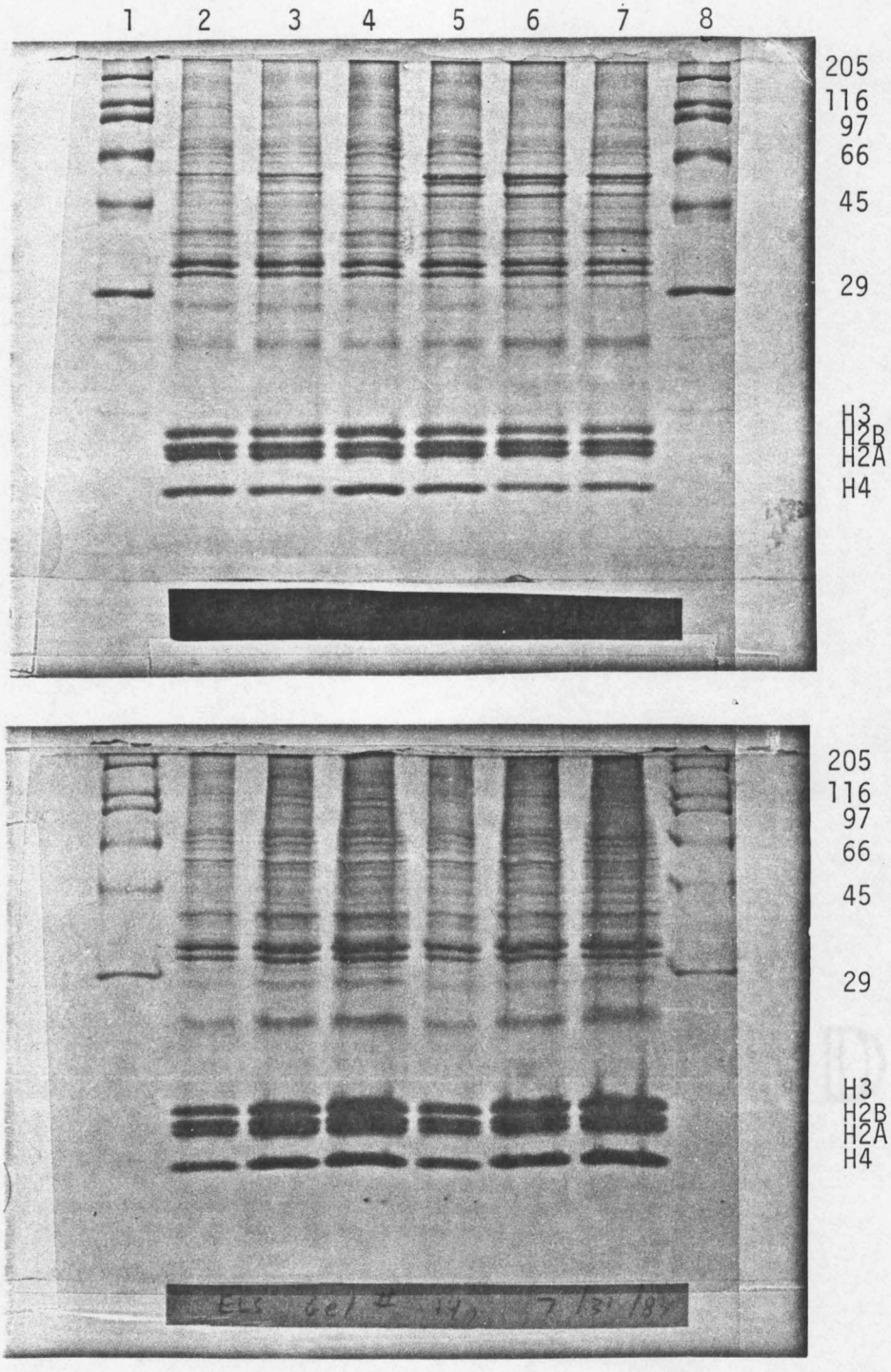

1
2
3
4
5
6
7
8
205
116
97
66
45
29
H3
H2B
H2A
H4
205
116
97
66
45
29
H3
H2B
H2A
H4

Figure V-2.

Figure V-3. Densitometric scans of X-ray films.

After fluorography, the X-ray films were scanned densitometrically, as explained in the text. Positions of histones H3 and H2A are indicated. Histone H2B migrates between H2A and H3, while H4 migrates ahead of H2A, as shown in Figure V-2.

A, Hepa-1c1c7; B, MUL12; C, TAOc1BP^{r}c1.

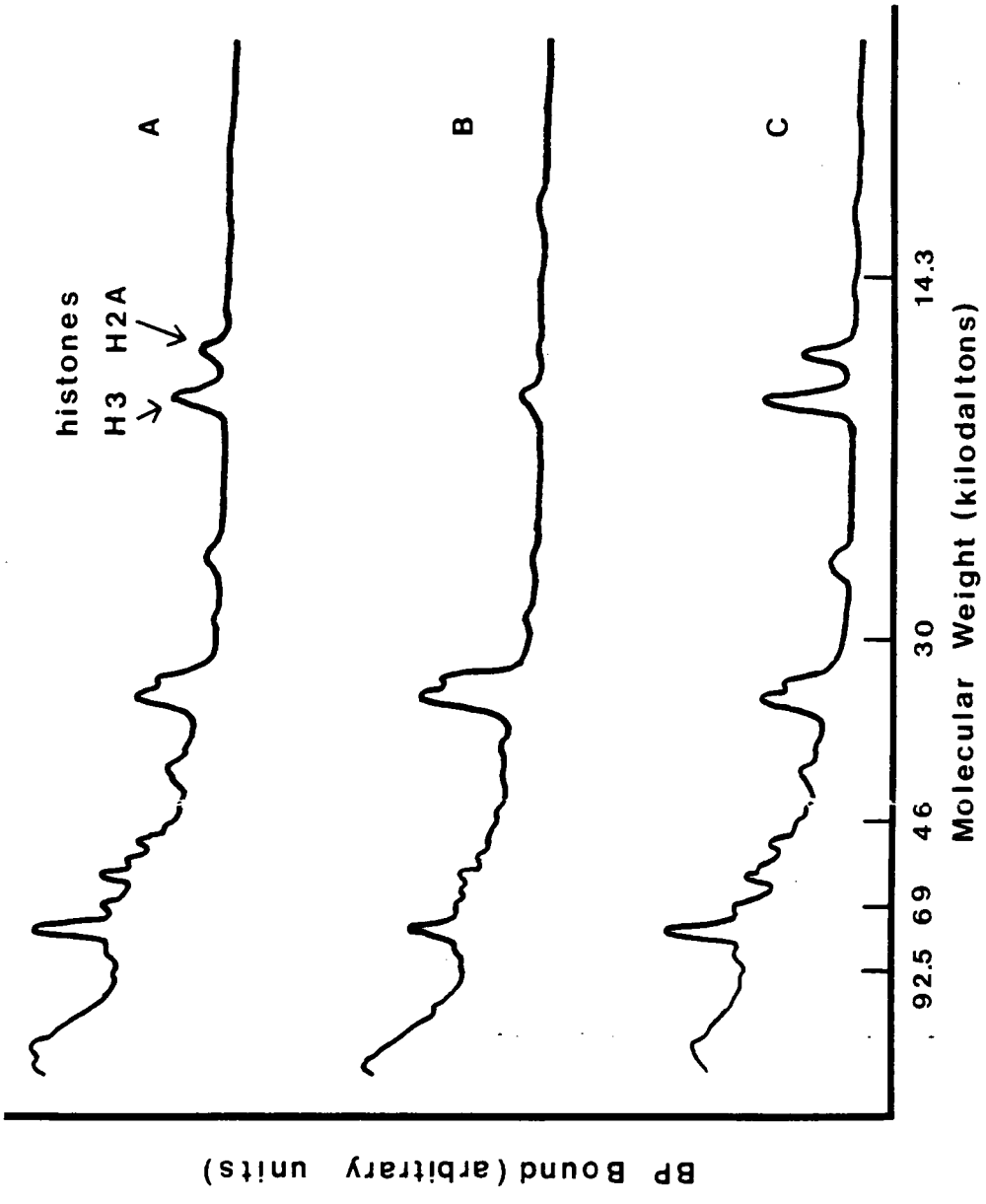

histones
H3
H2A
A
B
C
92.5
69
46
30
14.3
Molecular Weight (kilodaltons)
BP Bound (arbitrary units)

Figure V-3.

metabolites bound to nuclear proteins. B[a]P metabolites bound to core histones H3 and H2A in Hepa-1c1c7, MUL12, and TAOc1BP^{r}c1, but not to core histones H2B or H4. MUL12 cells bound less B[a]P metabolites to histones H3 and H2A than Hepa-1c1c7 or TAOc1BP^{r}c1 cells. Most of the binding by MUL12 was to nuclear proteins of molecular weights around 31k and 75k, whereas Hepa-1c1c7 and TAOc1BP^{r}c1 showed more complex nuclear protein binding patterns.

D. Discussion

The results of the macromolecular binding studies are consistent with the results of the metabolism and sister chromatid exchange studies. Variant BP^{r}c1, which exhibited little or no ability to metabolize B[a]P at high cell density, and which had poor induction of SCEs by B[a]P, also showed little binding of B[a]P metabolites to RNA, DNA, or protein in high density cells. In contrast, Hepa-1c1c7, MUL12, and TAOc1BP^{r}c1 did bind B[a]P metabolites to all three classes of nuclear macromolecule.

Histones H3 and H2A are the major targets of BP-7,8-diol in hamster embryo cells (MacLeod et al., 1980). Therefore, the different nuclear protein binding profile exhibited by MUL12 compared with Hepa-1c1c7 and TAOc1BP^{r}c1 may be due to the lower production of 7,8-diol by MUL12 compared with Hepa-1c1c7 and TAOc1BP^{r}c1 (discussed in Chapter III). This hypothesis could be tested by incubating [^{3}H]-BP-7,8-diol with Hepa-1c1c7, MUL12, and TAOc1BP^{r}c1, and seeing if the binding profile of MUL12 more closely resembles that of

Hepa-1c1c7 and TAOc1BP^{r}c1 than when [^{3}H]-B[a]P itself was used.

The differential binding pattern of core histones shown in this study was the same as that reported for hamster embryo cells (MacLeod et al., 1981). This binding pattern was also seen in mouse embryo cells, NIH-3T3 mouse fibroblasts, and T47D human mammary epithelial cells (MacLeod et al., 1983).

CHAPTER VI

SUMMARY AND CONCLUSIONS

A. Introduction

Mass cultures of cells have been routinely used for mutagenesis and transformation studies. The present work has shown that a cloned population of cultured cells may be composed of subpopulations of cells which differ genetically, biochemically, morphologically, and physiologically. Most of the effort in this work was devoted to the study of the metabolism of B[a]P by a clone of mouse hepatoma cells and three of its subclones. This approach was taken to test the hypothesis that the conversion of B[a]P to reactive metabolites is a prerequisite for other biological effects of B[a]P. Four mouse hepatoma cell subclones differed in metabolism of B[a]P, induction of sister chromatid exchanges by B[a]P, macromolecular binding by B[a]P metabolites, morphology, saturation density, population doubling times, modal chromosome numbers, and chromosome distributions.

B. Lower Activity of $BP^{r}c1$

Variant $BP^{r}c1$ has little or no ability to metabolize B[a]P, relative to Hepa-1c1c7, MUL12, and TAOc1$BP^{r}c1$. This reduced metabolic ability in $BP^{r}c1$ was reflected in all three major endpoints assayed: metabolism, sister chromatid exchange, and macromolecular binding. Whitlock and Galeazzi (1984) have shown that

the TCDD-receptor complex binds more weakly to the nucleus in BP^{r}cl cells than in Hepa-1c1c7 cells. Consequently, BP^{r}cl cells lack TCDD-inducible cytochrome P_1-450 mRNA (Israel and Whitlock, 1983) or AHH activity (Miller et al., 1983). B[a]P is 96% as effective as TCDD in displacing [^{3}H]TCDD from the TCDD receptor (Bigelow and Nebert, 1982). Therefore the receptor for B[a]P is most likely the same receptor as that for TCDD. The reduced ability of a B[a]P-receptor complex to bind to the nucleus in BP^{r}cl cells could account for the inability of B[a]P to induce AHH activity in these cells, and thus the reduced ability of these cells to metabolize B[a]P.

C. Slower Induction of AHH in TAOclBP^{r}cl

At incubation times of six hours or less, TAOclBP^{r}cl has low metabolic activity toward B[a]P, like BP^{r}cl, but at longer incubation times TAOclBP^{r}cl has high activity, like Hepa-1c1c7. This indicates that AHH activity is more slowly induced in TAOclBP^{r}cl than in Hepa-1c1c7. This slower induction of AHH in TAOclBP^{r}cl cells may be due to the reduced number of Ah receptors and/or the reduced affinity of these receptors for binding to inducer (TCDD or B[a]P) (Miller et al., 1983).

Even though AHH activity is more slowly induced in TAOclBP^{r}cl than in Hepa-1c1c7, the TAOclBP^{r}cl cells still have enough metabolic capability for B[a]P to induce SCEs in these cells after 6 h of incubation. Furthermore, by 12 h the TAOclBP^{r}cl cells have produced enough reactive metabolites to show a nuclear macromolecular binding profile similar to that of Hepa-1c1c7 cells.

D. Dihydrodiols

Differences were observed in the accumulations of BP-7,8- and 9,10-diol by Hepa-1c1c7, MUL12, and TAOc1BP^{r}c1. These differences could be due to the existence of P-450 enzymes having different abilities to hydroxylate B[a]P at different regions on the molecule.

Phenobarbital-induced rat microsomes produced elevated levels of BP-4,5-diol and 3-phenol relative to control, while hydrocarbon-induced microsomes increased the production of all identified metabolites (Rasmussen and Wang, 1974).

Wiebel et al. (1974) compared the production of B[a]P metabolites by different forms of purified cytochrome P-450 from phenobarbital-induced rabbit liver. P-450LM2 showed about a ten-fold greater hydroxylation of B[a]P at the 3-position compared with the 9-position, whereas P-450LM4 showed only a two-fold ratio of hydroxylation at these two positions.

In a more recent report, hepatic microsomes from control and phenobarbital-treated rats metabolized the 1,2-dihydrodiol of benz[a]anthracene to 1,2-diol-3,4-epoxides (i.e., the second hydroxylation occurred in the same ring as the first), but microsomes from 3-methylcholanthrene-treated rats produced primary bis-dihydrodiols from the 1,2-dihydrodiol (i.e., the second hydroxylation occurred in a ring different from the first) (Vyas et al., 1983).

Perhaps one of the best examples of different regioselectivities of B[a]P metabolizing enzymes was a comparison of seven

forms of cytochrome P-450 purified from rat liver microsomes (Wilson et al., 1984). Two forms (P-450's c and d) were purified from 3-methylcholanthrene-induced microsomes, four forms (P-450's a, b, e, and h) were from phenobarbital-induced microsomes, and one form (P-450 PCN) was from pregnenolone-16alpha-carbonitrile-induced microsomes. P-450's c and d produced similar metabolite distributions after incubation with B[a]P, except that P-450d produced less 7,8-diol compared with P-450c. P-450a produced largely BP-3-phenol and 9,10-diol. P-450e showed an almost absolute preference for formation of BP-4,5-diol, while P-450's b, h, and PCN produced both the 4,5-diol and the 3-phenol. This last comparison is interesting because P-450's b and e are immunologically identical and differ by only 13 of 491 amino acids.

Thus, there is a precedent for the idea that different subpopulations of cells, such as the parent and three variants of this study, may possess different forms of P-450. These different forms could arise from mutations in the P-450 structural genes, or in the regulatory genes coding for the Ah receptor. Alternatively, there could be other changes in induction pathways. These different enzymes could produce different types and amounts of B[a]P metabolites in different subpopulations of cells. These differences in production of metabolites could lead to differences in biological effects.

E. Cell Density Effects

In the present work, there was a small accumulation of phenols by BP^rcl cells at low cell density. In contrast, Miller et al. (1983) observed no AHH activity in BP^rcl cells, as measured by the fluorescence assay. Perhaps the weak metabolic activity of BP^rcl cells observed in the present work is due to a constitutively controlled enzyme which is active at low cell density but inactive at high cell density. Induced AHH activity was markedly reduced in confluent cultures of A31-714 cells (a subclone of mouse BALB/3T3 cells) compared to exponential phase cultures (Kakunaga, 1978). Similarly, a study of the induction of AHH in mouse liver cells showed that AHH activity peaked as the cells began entering the S phase of the cell cycle, indicating that the inducibility of AHH is greater when cells are actively proliferating (Becker and Bartholomew, 1979).

Whitlock and Gelboin (1974) have reported that culture medium and fetal calf serum, which is added to culture medium, both contain inducers of AHH activity. These unknown compounds might induce an enzyme(s) that is not P_1-450 but has some ability to metabolize B[a]P.

B[a]P could also be oxidized to quinones nonenzymatically, and these quinones could then be reduced to phenols by DT-diaphorase (Lind et al., 1978) or NADPH-cytochrome P-450(c) reductase (Capdevila et al., 1978).

The activity of TAOclBP^{r}cl depends on cell density, resembling that of BP^{r}cl at low cell density but resembling the activity of Hepa-1c1c7 at high cell density. This is contrary to what would be expected if AHH activity is turned off at high cell density. As discussed in Chapter I, AHH activity is regulated by the Ah locus. However, several other inducible enzyme activities are known not to be associated with the Ah locus. These activities include NADPH-cytochrome c reductase, NADPH-cytochrome P-450 reductase, epoxide hydrolase, and glutathione transferase (Eisen et al., 1983). Perhaps the dependence of B[a]P metabolism in TAOclBP^{r}cl on cell density is due to a change in induction of one or more of these enzymes rather than AHH.

A third cell density effect was the shift from production of diols to phenols. This effect could be due to epoxide hydrolase. If the activity of this enzyme were lower at lower cell density, fewer epoxide molecules would be hydrolyzed to dihydrodiols, and thus more epoxide molecules would be available for rearrangement to phenols. Another possibility is that fewer epoxides were conjugated to glutathione, either because of decreased activity of glutathione transferase, or decreased availability of the glutathione cosubstrate. Either of these causes would leave an increased number of epoxide molecules that could rearrange to phenols. A third possibility is that at low cell density there is greater conjugation of diols, e.g., to glucuronides or to sulfates. These conjugates would reduce the amount of diols observed in the ethyl acetate phase, thus increasing the phenol to diol ratio.

F. Substrate Concentration Effect

At higher substrate concentration, there was a higher accumulation of quinones than phenols, in contrast to the results at lower substrate concentration. This was true for both free and glucuronide-conjugated metabolites. A possible reason is that at high substrate concentration there was more oxidation of phenols to quinones, while at low substrate concentration most of the phenols were conjugated to glucuronides before being oxidized. This difference might be due to different reaction rates for oxidation and conjugation. Oxidation of phenols to quinones can occur non-enzymatically, but conjugation reactions are enzyme-mediated. It is possible that the conjugating enzyme UDP-glucuronyl transferase is saturated at the higher substrate concentration but not at the lower.

G. Sister Chromatid Exchanges

The major conclusion from this portion of the work was that the induction of sister chromatid exchanges was correlated with metabolic activities. This was probably due to the greater production of BP-7,8-dihydrodiol in TAOclBP^{r}cl than in BP^{r}cl. This conclusion is consistent with the report of Pal et al. (1978) that BP-7,8-diol is the best inducer of SCEs among the diols tested, and with the report of Pal et al. (1980) that the 7,8-diol-9,10-epoxide is the species mainly responsible for the induction of SCEs by B[a]P. The new aspect of the present study is that such a statistically

significant difference in SCE induction could be produced in such closely related cell variants.

H. Binding of B[a]P Metabolites to Nuclear Macromolecules

The nuclear protein binding profile of MUL12 was different from that of Hepa-1c1c7 and TAOc1BP^{r}c1. This was most likely due to the lower production of BP-7,8-diol by MUL12 compared with Hepa-1c1c7 and TAOc1BP^{r}c1. This lower production of BP-7,8-diol would probably lead to a lower amount of binding of B[a]P metabolites to histones H3 and H2A in MUL12 compared with Hepa-1c1c7 and TAOc1BP^{r}c1. This conclusion is consistent with the report of MacLeod et al. (1980) that histones H3 and H2A are the major targets of BP-7,8-diol.

I. Conclusions

The overall conclusion from this work was that a cloned population of cultured cells may be composed of genetically and biochemically distinct subpopulations.

These studies were performed on cells that had been derived from a mouse hepatoma. An extension of these studies could be to separate a population of normal cells into subpopulations with the fluorescence-activated cell sorter, and then study metabolism and the biological effects of that metabolism in these subpopulations. In designing such a further study, one would need to consider the source of the cells, the level of their differentiation, the

structure of the organ and tissue from which the cells are obtained, and the viability of the cells in primary culture.

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APPENDIX

CALCULATION OF POPULATION DOUBLING TIMES

A. Introduction

The purpose of this Appendix is to show the equations, data, and calculations that were used to obtain the population doubling times that were discussed in Chapter II. Section B of this Appendix is divided into two subsections. In subsection 1, it is shown how cell numbers were plotted as a function of time between subcultures. A straight line was fit to these data points by linear regression, and the slope of this line was obtained. From this slope, the population doubling time was calculated. Subsection 2 explains how the standard error of the slope was used to obtain a measure of the standard error of the population doubling time. This last value was used to construct the error bars in Figure II-2 of Chapter II, on page 30, which provide evidence for the shorter population doubling time of Hepa-1c1c7 compared with the three variants.

B. Equations

1. Slope and Population Doubling Time

According to Paul (1975), the kinetics of cell growth can be described by the equation

$$N = N_o 2^{t/T_D} ,$$

where

N_o = initial cell number,

N = cell number after incubation,

t = incubation time, and

T_D = population doubling time.

Taking logarithms,

$$\log N = \log N_o + \frac{t}{T_D} \log 2.$$

This equation has the form of a straight line,

$$y = a + bx \quad ,$$

where

$y = \log N$,

$a = \log N_o$ = intercept,

$x = t$, and

$$b = \frac{\log 2}{T_D} = \frac{0.301}{T_D} = \text{slope}.$$

Therefore

$$T_D = \frac{0.301}{b}$$

and the population doubling time is inversely proportional to the slope of the plot of the logarithm of cell number vs incubation time.

The slope of a straight line can be obtained by least squares linear regression, using the equation from Daniel (1978):

$$b = \frac{n\Sigma xy - (\Sigma x)(\Sigma y)}{n\Sigma x^2 - (\Sigma x)^2}$$

where x, y, and b are as defined above, and

n = number of observations.

To obtain data for calculating the slope b and subsequently the population doubling time T_D, the cells were plated, allowed to proliferate, counted daily, and subcultured before confluence was reached. This process was repeated several times. Cell counts were plotted on a logarithmic axis vs incubation time on a linear axis, and the results were also tabulated, as shown in Table A-1. The slopes, b, of the regression equations for the parent and three variants are presented in Table A-2, first row, and the population doubling times, T_D, are in Table A-2, second row.

2. Standard Error of the Slope and Standard Error of the Population Doubling Time

The standard error of the slope of the regression line was calculated by the method given by Colton (1974) as follows. First, the sums of squares and cross-products about the mean were calculated:

$$\Sigma(x - \bar{x})^2 = \Sigma x^2 - \frac{(\Sigma x)^2}{n}$$

$$\Sigma(y - \bar{y})^2 = \Sigma y^2 - \frac{(\Sigma y)^2}{n}$$

$$\Sigma(x - \bar{x})\,(y - \bar{y}) = \Sigma xy - \frac{(\Sigma x)(\Sigma y)}{n} \quad ;$$

second, the variance of the points about the regression line was calculated:

$$s^2_{y \cdot x} = \frac{1}{n-2} \left\{ (y - \bar{y})^2 - \frac{[\Sigma(x - \bar{x})(y - \bar{y})]^2}{\Sigma(x - \bar{x})^2} \right\} \quad ;$$

third, the standard error of the slope was calculated:

Table A-1

Data for Calculation of Population Doubling Times

Incubation Time (h)	Cell Number (10^6 Cells/Plate)				Log_{10} Cell Number			
	MUL12	BP^rc1	Hepa-1c1c7	TAOc1BP^rc1	MUL12	BP^rc1	Hepa-1c1c7	TAOc1BP^rc1
19.5		1.25		1.96		6.10		6.29
19.7	0.77	1.49	1.87	2.17	5.89	6.17	6.27	6.34
20.2		1.45	1.99	1.82		6.16	6.30	6.26
20.9		1.42	2.32	1.81		6.15	6.37	6.26
21.2	1.06				6.03			
22.9	0.86	1.39	1.78	2.05	5.93	6.14	6.25	6.31
24.0			1.93				6.29	
39.2	0.96	2.22	3.12	2.78	5.98	6.35	6.49	6.44
44.8			2.96				6.47	
46.0	1.12	2.22	3.75	3.66	6.05	6.35	6.57	6.56
46.8		2.35	3.56	2.98		6.37	6.55	6.47
47.5	1.66				6.22			
65.3		4.27	6.72	4.70		6.63	6.83	6.67
68.2	1.61	4.24	7.83	6.83	6.21	6.63	6.89	6.83
68.2	2.73				6.44			
69.0		3.80	8.19	4.64		6.58	6.91	6.67
71.2				4.92				6.69
71.3			5.65	4.38			6.75	6.64
71.8	1.98	4.09	7.82	7.07	6.30	6.61	6.89	6.85
74.5	3.12	3.12	6.57	5.19	6.49	6.49	6.82	6.72

Table A-1 (continued)

Incubation Time (h)	Cell Number (10^6 Cells/Plate)				Log_{10} Cell Number			
	MUL12	BP^rcl	Hepa-1c1c7	TAOc1BP^rcl	MUL12	BP^rcl	Hepa-1c1c7	TAOc1BP^rcl
85.8	2.77	5.47	10.94	9.29	6.44	6.74	7.04	6.97
88.7			12.13	8.24			7.08	6.92
90.5			11.90	8.08			7.08	6.91
90.5	5.03	5.44	11.02	7.02	6.70	6.74	7.04	6.85
94.4	3.48	6.17	9.77	9.46	6.54	6.79	6.99	6.98
95.3		6.48		8.10		6.81		6.91

Table A-2

Derived Parameters for Calculation of Population Doubling Times and Error Bars

Parameter[a]	MUL12	$BP^r c1$	Hepa-1c1c7	$TAOc1BP^r c1$
b	9.16×10^{-3}	8.74×10^{-3}	11.05×10^{-3}	8.86×10^{-3}
T_D	32.9	34.5	27.2	34.0
SE(b)	1.07×10^{-3}	0.43×10^{-3}	0.52×10^{-3}	0.50×10^{-3}
b + SE(b)	10.23×10^{-3}	9.16×10^{-3}	11.58×10^{-3}	9.36×10^{-3}
b - SE(b)	8.09×10^{-3}	8.31×10^{-3}	10.53×10^{-3}	8.36×10^{-3}
$\frac{0.301}{b + SE(b)}$	29.4	32.8	26.0	32.2
$\frac{0.301}{b - SE(b)}$	37.2	36.2	28.6	36.0

[a] b = slope of regression line.
T_D = population doubling time (in hours).
SE(b) = standard error of slope.

$$SE(b) = \sqrt{\frac{s^2_{y \cdot x}}{\Sigma(x - \bar{x})^2}}$$

The values of the standard error of the slope, SE(b), for the four cell lines are given in Table A-2, third row.

The standard error of the slope, SE(b), was then used to calculate a measure of the standard error of the population doubling time. The values b + SE(b) and b - SE(b) were calculated and are presented in Table A-2, fourth and fifth rows. These values were used to replace b in the expression $\frac{0.301}{b}$ to give

$$\frac{0.301}{b + SE(b)} \quad \text{and} \quad \frac{0.301}{b - SE(b)} ,$$

shown in Table A-2, sixth and seventh rows. Since $T_D = \frac{0.301}{b}$, the values

$$T_D - SE(T_D) = \frac{0.301}{b + SE(b)} \quad \text{and} \quad T_D + SE(T_D) = \frac{0.301}{b - SE(b)}$$

are measures of the standard error of the population doubling time, T_D. These numbers are plotted as the ends of the error bars in Figure II-2 of Chapter II, page 30. The lengths of the upper and lower error bars are not equal because T_D is proportional to the reciprocal of the slope of the regression line.

VITA

Eugene Leonard Schaefer was born in Sioux Falls, South Dakota on April 20, 1943. He attended elementary schools in and around that city and was graduated from Cathedral High School in June 1961. The following September he entered Marquette University, and in June 1966 he received a Bachelor of Science degree in Mathematics. In the fall of 1966 he accepted a teaching assistantship at Purdue University and began study toward a Master of Science degree in Chemistry. This degree was awarded in August 1968.

After a three-year tour of duty with the United States Army, he accepted a position in pharmaceutical quality control at The Upjohn Company in Kalamazoo, Michigan. He entered the Graduate School of The University of Tennessee, Knoxville, in September 1980. He received the Doctor of Philosophy degree with a major in Biomedical Sciences in August 1985.

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