# QUANTITATIVE CYTOTOXICITY AND MUTAGENICITY STUDIES EMPLOYING MAMMALIAN CELLS IN CULTURE

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ABSTRACT

QUANTITATIVE CYTOTOXICITY AND MUTAGENICITY STUDIES
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This thesis describes the characterization of an in vitro mammalian assay for the detection and study of toxic and mutagenic chemicals. The postmitochondrial supernatant (S-15 fraction) prepared from rat liver homogenates is used as enzyme source and V79 Chinese hamster cells as targets for chemically induced damage. The assay advances the field of genetic toxicology because in addition to determining in a qualitative manner whether or not a chemical can be metabolically activated to a mutagen it may be used to elucidate quantitatively the relative importance of specific enzymes (ethoxyresorufin-O-deethylase, ethylmorphine-N-demethylase, epoxide hydrase) in the metabolic toxification/detoxification of that chemical.

To accomplish this objective the enzyme pattern contained in the S-15 fraction is modulated by pretreating experimental animals with enzyme inducers (phenobarbital, 3-methylcholanthrene, β-naphthoflavone or Aroclor 1254) and/or by adding enzyme inhibitors (α-naphthoflavone, metyrapone, cyclohexene oxide) in vitro. The enzymic activity in a given S-15 fraction is determined, and the ability of

the preparation to produce mutagenicity and/or cytotoxicity in the presence of the test chemical is assessed. Correlations are made between the activity of a particular enzyme or enzyme ratio in the S-15 fraction and the biological response produced by the test chemical. Two correlation coefficients are determined, one relating enzyme activity to cytotoxicity and the other relating enzyme activity to mutagenicity.

Benzo(a)pyrene (BP) was used to validate the assay. The results indicate that the rate of epoxide formation divided by the rate of epoxide destruction is an important determinant of the degree of BP-induced cytotoxicity and mutagenicity when it is metabolized in vitro. As this ratio increases BP becomes more cytotoxic and more mutagenic towards V79 cells. The relative importance of selected enzymes in metabolically activating dimethylnitrosamine was also determined using the assay. The genetic toxicology of the potent bacterial mutagen 2,4-dinitrofluorobenzene (DNFB) was assessed in the assay. The results of this study indicate that neither DNFB, nor its microsomal metabolites, are mutagenic. These observations emphasize the need for caution when using data derived from bacterial systems for use in human risk assessment and indicate that mammalian assays are needed to determine accurately the mutagenic potential of suspected carcinogens.

to Esther, Dorothy and Joan,

with love

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TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

LIST OF TABLES

LIST OF TABLES (continued)

LIST OF FIGURES

LIST OF FIGURES (continued)

LIST OF FIGURES (continued)

## LIST OF ABBREVIATIONS

| | |
|---|---|
| AHH | aryl hydrocarbon hydroxylase |
| ANF | α-naphthoflavone |
| BP | benzo(a)pyrene |
| BPDE | benzo(a)pyrene 7,8-diol, 9,10-epoxide |
| BPE | benzo(a)pyrene 4,5-epoxide |
| BNF | β-naphthoflavone |
| CCHO | cyclohexene oxide |
| DMN | dimethylnitrosamine |
| DNFB | 2,4-dinitrofluorobenzene |
| EH | epoxide hydrase |
| EMND | ethylmorphine-N-demethylase |
| EROD | ethoxyresorufin-O-deethylase |
| HGPRT | hypoxanthine-guanine phosphoribosyltransferase |
| MC | 3-methylcholanthrene |
| MET | metyrapone |
| MNNG | N-methyl-N-nitroso-N'-nitro-guanidine |
| PB | phenobarbital |
| PBS | phosphate buffered saline |
| PCB | polychlorinated biphenyl |
| S-15 | 15,000 x g supernatant of a rat liver homogenate |
| TCPO | 1,2-epoxy-3,3,3-trichloropropane |

# INTRODUCTION

## 1. The Need for Short-Term Tests and Their Applications

Methodologies must be developed which will facilitate the detection and study of chemicals in our environment which are carcinogens. At the present time the primary method for identifying human carcinogens is the rodent lifetime exposure bioassay. Unfortunately, the expense, in terms of both time and money of this assay limits its utility as a screening assay for the multitude of chemicals to which humans are exposed (for review see Ames, 1979). Therefore, many initial studies will be conducted utilizing so called "short-term tests." As their name implies, short-term tests deliver results on the biological activity of chemicals within only a few days or weeks. These assays have the additional advantage of being relatively inexpensive. However, while short-term tests may give a fast and cost-efficient estimate of the carcinogenic potential of a given chemical there are serious problems in the extrapolation of results to yield a human risk assessment. Short-term tests do not cover all aspects of tumor formation in animals and humans and therefore these tests will never completely supplant tumor induction tests in mammals. Nevertheless, short-term tests have been, and will continue to be, useful in determining if a given chemical is potentially carcinogenic to humans.

The current theoretical framework for the study of chemical carcinogenesis, first put forward thirty-four years ago (Berenblum and Shubik, 1947), divides the complex process into two major stages, initiation and promotion. The theory is supported by retrospective epidemiological studies which indicate that human carcinogenesis is a multistage process (Dorvlo *et al.*, 1980). Initiation requires only a single exposure to a carcinogen, is heritable, and irreversible. Promotion is a multistage process (Mufson *et al.*, 1979; Slaga *et al.*, 1980a,b) where, in the presence of agents termed tumor promoters, an initiated cell is allowed to express itself phenotypically and form a tumor. If short-term tests are to be effectively utilized in the field of chemical carcinogenesis, it seems desirable that methods be developed which are capable of discriminating between complete carcinogens and tumor promoters. Based on present scientific insights this has proved feasible because tumor promoters do not appear to damage DNA, whereas complete carcinogens, with few exceptions, cause DNA damage.

Recently a short-term *in vitro* method has been developed to detect and study tumor promoters (Yotti *et al.*, 1979) which is based on the observation that tumor promoters inhibit metabolic cooperation in V79 Chinese hamster cells. The development of short-term tests aimed at the identification and study of complete carcinogens has received a great deal of attention in recent years. A variety of cell types, including bacteria, fungi, insects, plants and cultured mammalian cells have been employed. The available test methods can be divided into

three broad categories: those that detect mutations, those that detect gross chromosomal effects and those that measure DNA repair. This dissertation deals with the characterization of a short-term test based on the observation that carcinogens are capable of acting as mutagens (McCann et al., 1975; McCann and Ames, 1976). In some cases the degree of mutagenic response in vitro has been shown to correlate with the carcinogenic potency of the chemical in vivo (Huberman and Sachs, 1976), while in other cases the mutagenic and carcinogenic potencies do not appear to correlate (Schut and Thorgeirsson, 1978; Bartsch et al., 1980; Wislocki et al., 1980; Brambilla et al., 1981). There are several probable reasons which could explain why in vitro responses do not always accurately predict in vivo responses. The two broad classes of inaccurate results in mutagenicity assays are false negative and false positive responses. A false negative test outcome could be due to: a) the carcinogenic mechanism may be unsuitable for detection in the short-term test being employed, e.g., the chemical in question is a tumor promoter, b) the test chemical may require metabolism into another form before it is active, and the conversion does not occur in the particular short-term test employed. This problem may be partially overcome by utilizing a battery of short-term tests. A false positive result in a short-term test may result from: a) inadequate carcinogenicity studies, i.e., the study was performed in the wrong species, at the wrong dose or via an inappropriate route of administration, b) pharmacologic factors are not taken into account in short-term tests. The absorption and

distribution pattern *in vivo* may profoundly affect the biological response to a particular chemical.

In the past mutagenicity assays have been used primarily as a qualitative prescreen to establish priorities for long-term carcinogenicity experiments. However, assays of the sort described in this dissertation are capable of testing a chemical's cytotoxic as well as mutagenic potential. This means that there are several other potential applications of this test in toxicological studies besides carcinogen screening. These include: 1) Quickly assessing the toxicological activity of a large number of compounds (e.g., structure-activity studies). This would be particularly useful when the test chemical is available in limited quantities because *in vitro* assays require much less chemical than do *in vivo* studies. 2) Metabolism Studies. This assay may be utilized to efficiently study the following questions: A) Is a chemical a direct acting toxicant or does it require metabolic activation prior to exerting toxic effects? B) If a chemical requires metabolic activation, then what is the active metabolite? C) Why do many toxic chemicals exhibit species and/or organ specificity? The ability of a particular organ to metabolize a potential toxicant to an active form may contribute to organ specificity. The metabolic potential of various organs could be analyzed quickly and inexpensively in a short-term test. The results from this type of analysis must be interpreted with caution because correlations between *in vitro* and *in vivo* drug metabolism are not always perfect (Pelkonen *et al.*, 1980). 3) Epidemiological Studies. One may analyze fecal, urine or blood samples for the presence of toxicants

utilizing this test. Such an analysis would be very useful in cases of suspected occupational or environmental exposure to toxic chemicals.

## 2. Test System Selection

The assay has been designed to complement other previously existing short-term mutagenicity tests and is intended to be useful in the construction of a battery of short-term tests so that chemicals which may pose a risk to man can be more fully evaluated.

Most chemical carcinogens must be metabolically converted into an electrophilic form in order to be active (for reviews see Miller, 1978; Miller and Miller, 1979). Cells in culture usually possess, at most, a limited capacity to metabolize foreign compounds (Huberman and Sachs, 1974, Meijer et al., 1980). Therefore, in vitro assays designed to study putative carcinogens/mutagens often utilize a mammalian enzyme source in order to incorporate metabolism into the test system. There are presently two widely used mammalian activation systems employed in short-term tests. These are either metabolically competent feeder cells or tissue subcellular fractions, which are primarily derived from liver. Several assays which utilize mammalian feeder cells have been described. For example, assay systems developed by Huberman and collaborators (Huberman and Sachs, 1974, 1976; Huberman et al., 1976, 1979), Langenbach et al. (1978), Newbold et al. (1977) and Gould (1980) utilize lethally irradiated hamster embryo cells, rat liver cells, BHK21 cells and rat mammary gland cells, respectively, as a source of xenobiotic metabolizing enzymes. These

systems have the advantage of producing metabolic profiles which approximate in vivo profiles (Selkirk, 1977; Glatt et al., 1981) and thus may prove particularly useful in the study of chemically induced, organ-specific carcinogenesis. For example, rat mammary gland cells appear unable to metabolically activate aflatoxin $B_1$, a potent liver carcinogen, whereas they can activate dimethylbenzanthracene, a mammary carcinogen (Gould, 1980). However, the cell in which the procarcinogen is initially metabolized may not necessarily be the cell which ultimately will suffer detrimental biological effects from that compound. That is, a procarcinogen could be metabolized to a proximate carcinogen in one organ and subsequently be transported to a second organ where tumor initiation may result. In a situation like this the use of feeder cells derived from the organ in which the tumor appears as a source of metabolic potential may yield a misleading result. An additional problem with cell mediated activation systems is the fact that the parent compound must be transported into the feeder cell and the activated metabolite(s) must be transported out to the target cell. The serum concentration in the culture medium may influence the amount of parent compound transported into the feeder cells (Coulomb et al., 1981), and the high degree of chemical reactivity of the active metabolites indicates that much of what is produced will bind to nucleophilic sites in the feeder cells (Newbold et al., 1977) thus eliminating their opportunity to produce biological effects in the target cells. Cell-mediated systems are also less useful than subcellular fractions for studies on the importance of alterations

in enzyme pattern and/or concentration in regard to the generation of mutagenic and/or cytotoxic metabolites from xenobiotics. For example, subcellular fractions have an obvious advantage over feeder cells in the evaluation of the relative importance of cytosolic, microsomal and nuclear enzymes in the process of metabolic activation. Therefore, I chose to utilize the hepatic postmitochondrial supernatant as an enzyme source in these studies.

Short-term tests utilizing mammalian subcellular fractions for activation and bacteria as target cells for chemically induced damage have been developed (Ames et al., 1973, 1975). However, there is a need for mutagenesis assays utilizing mammalian target cells. Some compounds may be metabolically activated to mutagens by enzymes found in bacterial, but not mammalian cells (Blumer et al., 1980, Speck et al., 1981). The mutagenicity of some compounds is dependent upon bacterial repair processes (Ivanovic and Weinstein, 1980; McCoy et al., 1981), which may differ from those found in mammalian cells. In addition, reports in the literature utilizing bacteria as target cells do not routinely quantify the toxicity of a given xenobiotic, precluding one's ability to express mutation frequency on a per-survivor basis. This information could be useful in comparing the potency of various mutagens (Peterson et al., 1979). The simultaneous measurement of cytotoxicity and mutagenicity may also aid in differentiating lesions which lead to mutagenesis from those responsible for cytotoxicity (Bradley et al., 1980). I chose to utilize V79 Chinese hamster cells as targets for chemically-induced damage and to measure cytotoxicity and mutation frequency in parallel.

Two loci which are commonly monitored for the induction of mutations in mammalian cells are the $Na^+,K^+$-ATPase locus (Baker et al., 1974; Chang et al., 1978) and the hypoxanthine-guanine phosphoribosyltransferase (HGPRT) locus (Chu and Malling, 1968; Krahn and Heidelberger, 1977; O'Neill et al., 1977a,b; Kuroki et al., 1977; Fox and Hodgkiss, 1981). Mutagenicity at the HGPRT locus is assessed by measuring resistance to purine analogs, 6-thioguanine or 8-azaguanine. Resistance to these compounds requires either a mutation which results in an abnormally functioning or non-functional HGPRT enzyme, or absence of HGPRT, possibly due to an epigenetic alteration of gene expression. Mutagenicity at the $Na^+,K^+$-ATPase locus is monitored by measuring resistance to ouabain, an inhibitor of the enzyme. In view of the fact that a functional $Na^+,K^+$-ATPase is essential for cell survival ouabain resistance cannot be caused by events which result in the enzyme being absent or non-functional. Therefore, ouabain resistance is probably due to a mutation involving a base pair substitution in the portion of the $Na^+,K^+$-ATPase gene that codes for the ouabain binding site. There are several potential problems associated with the assessment of mutagenicity at the HGPRT locus. These include the phenomenon of metabolic cooperation which makes the assay very dependent upon cell density (Burk et al., 1968; Van Zeeland et al., 1972; Fox, 1975), the presence in serum of hypoxanthine (Peterson et al., 1976), as well as enzymes capable of degrading purine analogs (Van Zeeland and Simons, 1975), a long expression period which may necessitate subculturing prior to mutant selection, high background (Arlett

et al., 1975), and finally the possibility that some mammalian cells which are resistant to the toxic effects of 8-azaguanine may arise as a result of non-mutational events, e.g., a stable shift in phenotypic expression (Harris, 1971; Sharp et al., 1973). For these reasons I chose to monitor the $Na^+,K^+$-ATPase locus. The advantage of this assay system seems to be the short expression time, the absence of metabolic cooperation and high inducibility by mutagens. I recognize that the assessment of mutagenicity using ouabain resistance will fail to detect certain classes of mutagenic agents such as X-rays (Chang et al., 1978) and gamma-rays (Arlett et al., 1975) which can be efficiently detected by monitoring the HGPRT locus.

In summary, I have chosen to utilize an in vitro mammalian assay system which employs the postmitochondrial supernatant prepared from a rat liver homogenate as an enzyme source and V79 Chinese hamster cells as the target for chemically-induced damage. Cytotoxicity is determined by measuring colony forming ability and mutagenicity is assessed at the locus coding for the membrane bound $Na^+,K^+$-ATPase by measuring development of resistance to ouabain.

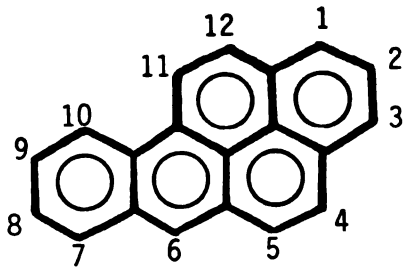
I have used this assay system to study quantitatively the metabolic activation of three chemical carcinogens which belong to three different classes of carcinogenic agents. The chemicals I chose to study are: a) Benzo(a)pyrene, a polycyclic aromatic hydrocarbon, b) dimethylnitrosamine, a nitrosamine, c) 2,4-dinitrofluorobenzene, an aromatic amine.

3. Benzo(a)pyrene (BP)

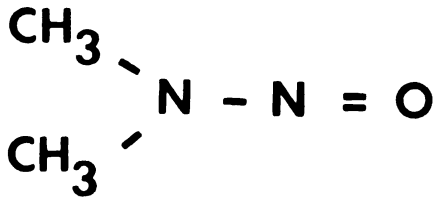
BP (Figure 1) is a common environmental pollutant (approximately 1320 tons emitted into the United States atmosphere in 1972, National Academy of Sciences, 1972), which requires metabolic activation and produces hepatocellular carcinomas in male Sprague-Dawley rats (Kitagawa et al., 1980). The different forms of cytochrome P-450 exhibit positional and stereospecificity in the metabolism of BP (Deutsch et al., 1978; Wang, 1981). Therefore, the pattern of metabolites produced during the microsomal metabolism of BP may be altered by pretreating the experimental animals with either PB or MC (Holder et al., 1974; Yang et al., 1975). Pretreatment with MC increases the production of the 4,5-diol, the 7,8-diol and the 9,10-diol, whereas PB pretreatment increases the production of only the 4,5-diol (Rasmussen and Wang, 1974).

Two theories have been proposed to relate the carcinogenic action of BP to the manner in which it is metabolized. The BP molecule contains two regions, the bay and the K, which are readily metabolized by mixed function oxidases. The theories relate the carcinogenic action of BP to metabolism in these regions; the K region theory predicting that the critical reactions leading to the ultimate reactive species occur in the K region, while the bay region theory predicts that these reactions will occur in the bay region. The K region theory was proposed (Pullman and Pullman, 1955) based upon the electronic characteristics of the BP molecule. According to this theory the 4,5-epoxide of BP (BPE) is considered to be the most likely ultimate carcinogen. However, the DNA adducts formed in cultured mammalian

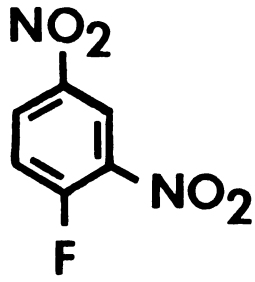
Figure 1. Structures of benzo(a)pyrene, dimethylnitrosamine, and 2,4-dinitrofluorobenzene.




**BENZO (a) PYRENE**




**DIMETHYLNITROSAMINE**




**2,4 - DINITROFLUOROBENZENE**

Figure 1

cells exposed to BP were found to be different from those formed when BPE is reacted with DNA in vitro (Sims et al., 1974; Baird et al., 1975). This work suggested that bay region diol-epoxides (BP 7,8-diol-9,10-epoxide) might be the ultimate carcinogenic species of BP (Sims et al., 1974). According to the bay region theory, in order for BP to become mutagenic and carcinogenic it must be initially acted upon at the 7,8 position, presumably by cytochrome P-448, to form the 7,8-oxide, followed by hydration catalyzed by epoxide hydrase to form the 7,8-dihydrodiol (Wood et al., 1976a; Neidle et al., 1981). In the absence of epoxide hydrase the 7,8-oxide may non-enzymatically rearrange to a relatively non-reactive phenol. This scheme is supported by the observation that the addition of epoxide hydrase to a purified microsomal system decreases the production of phenols and increases the production of dihydrodiols (Holder et al., 1974), indicating that dihydrodiols and phenols share arene oxides as a common precursor. The 7,8-dihydrodiol may be further epoxidated, again presumably by cytochrome P-448 (King et al., 1976) to the highly reactive diol epoxides, 7,8-dihydrodiol-9,10-epoxide (BPDE). There is recent evidence to suggest that prostaglandin synthetase may also catalyze this reaction (Sivarajah et al., 1981). The BPDE's exist as a pair of diastereomers due to the fact that the 7-hydroxyl group may be either cis or trans to the 9,10-epoxide. BPDE I is the diastereomer in which the 7-hydroxyl group is trans to the 9,10 epoxide, and BPDE II is the diastereomer in which the 7-hydroxyl group is cis to the 9,10 epoxide. BPDE I appears to be the more biologically important diastereomer due to the following considerations:

1) When BP is metabolized in vitro by rat liver microsomes a single enantiomer, (-) r-7, t-8-dihydroxy-7,8-dihydrobenzo(a)pyrene, is produced. This enantiomer is further metabolized predominantly to the diol-epoxide, r-7, t-8-dihydroxy-t-9, 10-oxy-7, 8, 9, 10-tetrahydrobenzo(a)pyrene (BPDE I) (Yang et al., 1976).

2) BPDE I is more mutagenic to mammalian cells than is BPDE II (Huberman et al., 1976; Wood et al., 1977).

3) BPDE I is more active than BPDE II in transforming hamster embryo cells in vitro (Mager et al., 1977).

4) BPDE I is more carcinogenic than BPDE II (Slaga et al., 1979).

5) When human or bovine bronchial explants are exposed to BP the major DNA adduct results from BPDE I (Jeffrey et al., 1977).

6) When 10T½ mouse embryo fibroblasts metabolize BP the major DNA adduct results from BPDE I (Brown et al., 1979).

When BPDE I interacts with mammalian DNA several nucleic acid adducts are produced, some of which may cause distortion of the double helix (Hogan et al., 1981). It is presently not known which of these nucleic acid adducts is(are) most critical with respect to the mutagenic and/or carcinogenic process. BPDE I produces multiple guanine, adenine and cytidine adducts but does not appear to bind covalently to thymidine (Jennette et al., 1977). The major adduct results from the covalent linkage of the N-2 of guanine to the 10-position of BPDE I (Jeffrey et al., 1976; Feldman et al., 1980; Osborne et al., 1981). BPDE I also interacts with guanine at the O-6 and N-7 positions to a minor extent (Osborne et al., 1981). The deoxyadenosine adducts result

from the addition of the N-6 amino group of adenine to the 10-position of BPDE I (Jeffrey et al., 1979), and the cytidine adducts result from the covalent interaction of the N-4 cytidine with the 10-position of the BPDE (Jennette et al., 1977).

The BPDEs have been reported to be relatively poor substrates for epoxide hydrase (Wood et al., 1976b) but if acted upon prior to their interaction with critical cellular nucleophilic sites they can be detoxified to the non-reactive metabolite, 7,8,9,10-tetrahydrotetrol. Therefore, it is clear that the role of epoxide hydrase in the metabolism of BP can be either toxification or detoxification depending on the metabolite which it acts upon. Furthermore, it appears probable that the ratio of P-448 activity to epoxide hydrase activity could determine the concentration of the reactive diol epoxide at any given time.

4. Dimethylnitrosamine (DMN)

DMN (Figure 1) is a hepatocarcinogenic nitrosamine (Craddock, 1971; Uchida and Hirono, 1979) which requires metabolic activation in order to acquire mutagenic properties (Malling, 1971; Umeda and Saito, 1975; Kuroki et al., 1977; Chin and Bosmann, 1980), although at high concentrations DMN is cytotoxic in the absence of metabolic activation due to its protein denaturing ability, which may result in cell membrane destruction (Argus and Arcos, 1978).

The metabolic activation of DMN is catalyzed by mixed function oxidases collectively termed DMN demethylase (Czygan et al., 1973).

DMN is dealkylated by DMN demethylase to a monoalkyl derivative which is spontaneously converted to the corresponding monoalkyl diazonium ion. This compound decomposes to yield a carbonium ion, which alkylates nucleophilic sites in DNA and RNA (Lijinsky et al., 1968). The DNA alkylation products are primarily $O^6$-methylguanine, $N^7$-methylguanine and $N^3$-methyladenine (Abanobi et al., 1980).

The DMN demethylase activity in an in vitro metabolic activation system correlates, in some cases, with DMN-induced mutagenicity (Frantz and Malling, 1975; Hutton et al., 1979b; Yoshikawa et al., 1980), while in other instances DMN demethylase activity does not correlate with DMN-induced mutagenesis (Hutton et al., 1979a). There is a correlation between the ability of a particular tissue to metabolize DMN in vitro and that tissue's susceptibility to DMN-induced carcinogenesis (Bartsch et al., 1975). Isolated microsomes from mouse livers exhibit only about half the DMN demethylase activity present in the crude homogenate (Lake et al., 1974; Lake et al., 1976; Anderson and Angel, 1980). The enhancing effect of the soluble fraction may be due to the presence of additional cofactors for the microsomal enzymes because the hepatic soluble fraction is unable to metabolize DMN in the absence of microsomes (Lake et al., 1975, 1976). Hepatic DMN demethylase activity (Czygan et al., 1973; Anderson and Angel, 1980) as well as DMN's enzymic activation to a mutagen (Czygan et al., 1973) are inducible by Aroclor 1254.

Recent evidence suggests that DMN demethylase activity may involve multiple enzymes (Lake et al., 1974; Kroeger-Koepke and Michejda, 1979;

Haag and Sipes, 980), e.g. both P-450 and P-448 (Guttenplan et al., 1976). The role that these mixed function oxidases and other enzymes play in the metabolic activation of DMN is obscure and requires further study.

5. 2,4-Dinitrofluorobenzene (DNFB)

DNFB (Figure 1), an aromatic amine used in the identification of N-terminal amino acids of polypeptides (Sanger, 1945) has been reported to be a potent, direct acting mutagen in bacteria (Hope, 1979; Jagannath et al., 1980; Summer and Goggelmann, 1980) and yeast (Fahrig, 1979). However, in vivo studies employing mice (Bock et al., 1969) have indicated that DNFB lacks initiating properties. To investigate this apparent discrepancy I chose to determine if DNFB is a direct acting mutagen in mammalian cells or if it can be metabolically activated to a form mutagenic towards mammalian cells.

6. Overall Objective

The objective of this research was to establish and characterize a mammalian short-term test which will be useful in conducting meaningful mechanistic studies regarding the metabolic toxification/detoxification of xenobiotics, particularly carcinogens.

It has become apparent in recent years that most carcinogens are electrophilic in their ultimate form(s). The majority of carcinogens exist as procarcinogens which must be metabolically converted into an electrophilic form. The target cells commonly used in short-term tests possess, at most, a limited capacity to metabolize foreign

compounds. Therefore, *in vitro* assays designed to study carcinogens must utilize a mammalian enzyme source in order to incorporate metabolism into the test. The concentration and pattern of enzymes responsible for the metabolic toxification/detoxification of xenobiotics in short-term tests is usually modulated by pretreating the mammals with selected compounds. To interpret properly the results obtained from these tests it is crucial that the effects of alterations in both the absolute and relative amounts of enzymes capable of metabolizing the putative toxicant on the outcome of the test be thoroughly examined and understood.

# MATERIALS AND METHODS

## 1. Materials

Calf serum, antibiotics and culture medium were purchased from Gibco (Grand Island, NY). Ouabain, NADPH, NADP, NADH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, 3-methylcholanthrene, benzo(a)pyrene, trypsin, 2,4-dinitrofluorobenzene, dimethylnitrosamine, metyrapone, α-naphthoflavone, quinine sulfate, bovine serum albumin and semicarbazide-HCl were obtained from Sigma Chemical Co. (St. Louis, MO). Cyclohexene oxide and β-naphthoflavone were from Aldrich Chemical Co. (Milwaukee, WI). Phenobarbital was from Mallinckrodt (St. Louis, MO), ethylmorphine (Dionin) from McKesson and Robbins (Lansing, MI), 7-ethoxyresorufin from Pierce Chemical Co. (Rockford, IL), resorufin from Eastman Organic Chemicals (Rochester, NY) and Giemsa stain (improved R66 solution) from Bio/medical Specialties (Santa Monica, CA). Aroclor 1254 brand of PCBs (Monsanto Chemical Co., St. Louis, MO) and [$^{3}$H]styrene oxide (Amersham, Arlington Heights, IL) were gifts from Dr. J.B. Hook, Michigan State University, and 3-OH benzo(a)pyrene (IIT Research Institute, Chicago, IL) was a gift from Dr. R.A. Roth, Michigan State University.

## 2. Cell Line and Culture Conditions

The cells used in these experiments are V79 Chinese hamster cells which grow in monolayer (obtained as a gift from Drs. J.E. Trosko and

C.C. Chang, Michigan State University). This cell line was derived from the lung of a normal, male Chinese hamster (Ford and Yerganian, 1958). The cells are routinely cultured at 37°C in humid air containing 5% $CO_2$, in modified Eagle's minimal essential medium (Gibco formula 78-5470, with Earle's salts, 1.5 X essential amino acids, 1 X glutamine, 2 X non-essential amino acids, 1.5 X vitamins; without glucose, sodium bicarbonate, and phenol red) supplemented with 20% (v/v) calf serum, penicillin (100 units/ml), streptomycin (100 µg/ml), fungizone (0.25 µg/ml), sodium pyruvate (110 µg/ml), glucose (1 mg/ml), sodium chloride (0.83 mg/ml) and sodium bicarbonate (1 mg/ml).

3. Preparation of Hepatic Postmitochondrial Supernatant (S-15 Fraction)

Male, Sprague-Dawley rats (Spartan Research Farms, Haslett, MI) weighing 180-240 g were maintained on a 12 hr light cycle (7 p.m.-7 a.m.), allowed free access to food (Wayne Lab-Blox, Allied Mills, Chicago, IL) and water, and they were acclimated to our animal quarters for at least 3 days prior to use.

Groups of animals were pretreated with i.p. injections of 3MC (80 mg/kg body weight in peanut oil, 24 hr prior to sacrifice), BNF (80 mg/kg body weight in peanut oil, 24 hr prior to sacrifice), Aroclor 1254 brand of PCBs (500 mg/kg body weight in peanut oil, 96 hr prior to sacrifice) or phenobarbital (80 mg/kg body weight in isotonic NaCl solution, 96, 72, 48 and 24 hr prior to sacrifice). Controls received vehicle only.

The hepatic postmitochondrial supernatant is prepared (Kuroki et al., 1977) as aseptically as possible, at 0-4°C, just prior to use.

Animals are killed by a sharp blow to the back of the head followed by cervical dislocation. The liver is removed, washed with 0.9% saline, and homogenized with a Potter-Elvehjem type homogenizer in 3 volumes of sucrose-HEPES buffer (0.25 M sucrose containing 2 mM $MgCl_2$ and 20 mM N-2-hydroxyethylpiperazine-N'-2 ethanesulfonic acid, pH 7.4). The homogenate then undergoes two successive centrifugations (Sorvall RC 2-B refrigerated centrifuge); 9000 x g for 10 minutes followed by 15,000 x g for 20 minutes. The postmitochondrial supernatant obtained is referred to as the S-15 fraction.

4. Cytotoxicity and Mutagenicity Assays

The assays are performed in the following manner (Figure 2): Stock cultures of V79 cells in the mid-logarithmic phase of growth are trypsinized (0.01% crystalline trypsin dissolved in phosphate buffered saline, pH 7.8, 37°C; 10 minutes) and the cell number determined using a hemocytometer. The cells are diluted to the desired concentration and seeded into 10 ml of warm (25°C) growth medium in 100 mm tissue culture dishes. The number of cells seeded per dish is 200-2000 for cytotoxicity assessment and $1x10^5$ to $5x10^5$ for the determination of mutation frequency. The actual number is dependent upon the anticipated toxicity of a particular treatment. The same stock population of V79 cells is used for parallel determinations of cytotoxicity and mutation frequency.

The cells are allowed 6 hours to attach to the culture dishes. The growth medium is then removed and the treatment medium is added. The treatment medium consists of the following components, added in

Figure 2. Protocol for the determination of cytotoxicity and mutation frequency in V79 Chinese hamster cells. Cytotoxicity is determined by measuring colony forming ability and expressed as cloning efficiency. Mutagenicity is determined by monitoring the locus coding for the membrane bound $Na^+,K^+$-ATPase (E.C. 3.6.1.3) by measuring the development of resistance to ouabain, an inhibitor of the enzyme.

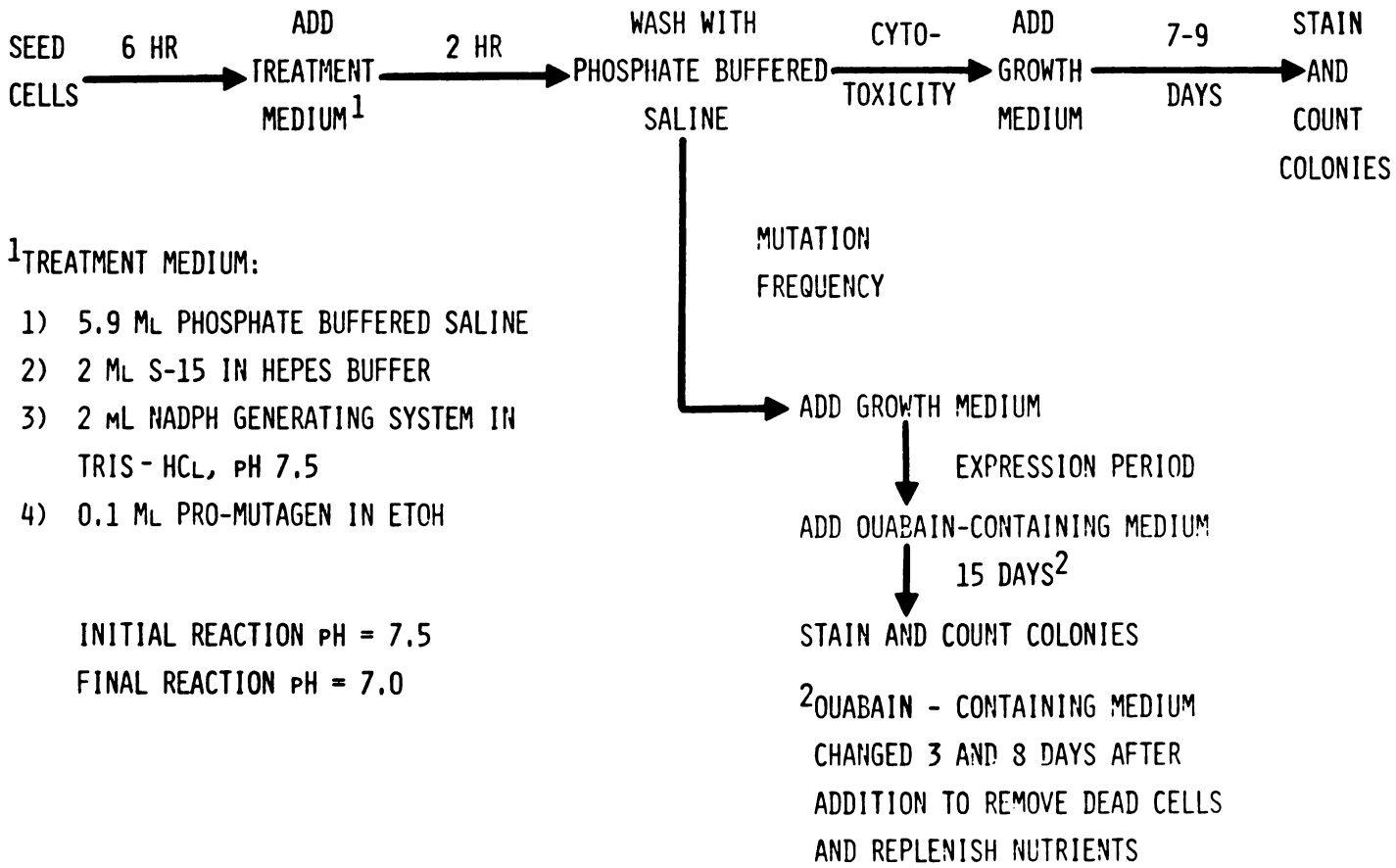

SEED CELLS
6 HR
ADD TREATMENT MEDIUM[1]
2 HR
WASH WITH PHOSPHATE BUFFERED SALINE
CYTO-TOXICITY
ADD GROWTH MEDIUM
7-9 DAYS
STAIN AND COUNT COLONIES
MUTATION FREQUENCY
ADD GROWTH MEDIUM
EXPRESSION PERIOD
ADD OUABAIN-CONTAINING MEDIUM
15 DAYS[2]
STAIN AND COUNT COLONIES
[1]TREATMENT MEDIUM:
1) 5.9 ML PHOSPHATE BUFFERED SALINE
2) 2 ML S-15 IN HEPES BUFFER
3) 2 mL NADPH GENERATING SYSTEM IN TRIS - HCL, pH 7.5
4) 0.1 ML PRO-MUTAGEN IN ETOH
INITIAL REACTION pH = 7.5
FINAL REACTION pH = 7.0
[2]OUABAIN - CONTAINING MEDIUM CHANGED 3 AND 8 DAYS AFTER ADDITION TO REMOVE DEAD CELLS AND REPLENISH NUTRIENTS

Figure 2

order: 1) 5.9 ml phosphate buffered saline (PBS) (8 gm NaCl, 0.2 gm KCl, 1.15 gm $Na_2HPO_4$, 0.2 gm $KH_2PO_4$ per liter glass distilled water, pH 7.3). 2) 2.0 ml of the cofactor mixture; comprised of the following (final concentration in the reaction mixture) dissolved in 66 mM Tris-HCl, pH 7.5; NADPH, 0.1 mM; NADP, 0.3 mM; NADH, 0.4 mM; $MgCl_2$, 3.0 mM; glucose-6-phosphate, 4.2 mM; and glucose-6-phosphate dehydrogenase, 1 unit/ml). 3) 2.0 ml of the S-15 fraction, diluted with sucrose-HEPES buffer to give the desired final protein concentration in the treatment medium. 4) 0.1 ml of the test compound, dissolved in ethanol (BP), dimethyl sulfoxide (DNFB) or glass distilled water (DMN). When enzyme inhibitors are employed the volume of PBS is reduced to 5.8 ml and 0.1 ml of the inhibitor dissolved in the appropriate solvent (metyrapone: 66 mM Tris-HCl, pH 7.5; cyclohexene oxide, α-naphthoflavone: dimethylsulfoxide) is added. Controls lack S-15 and cofactors or the test compound or all three of these components.

The pH of the treatment medium is 7.5 when the test compound is added. After the tissue culture dishes have been placed in the incubator (5% $CO_2$ in air) the pH becomes 7.0 and remains at that value for the duration of treatment.

Following a 2 hour incubation the treatment medium is removed, the tissue culture dishes are washed once with 10 ml of phosphate buffered saline and 10 ml of growth medium are added. The PBS and growth medium are warmed to room temperature prior to addition to avoid cold-stressing the cells. The cells to be used in the assessment of cytotoxicity are allowed to grow for 7-9 days, at which time the colonies that form are stained (25 ml Giemsa stain, 30 ml absolute

methanol, 945 ml glass distilled water) and counted. Cytotoxicity is expressed as cloning efficiency and is calculated for each treatment as follows:

$$\frac{\text{number of cells which form colonies}}{\text{number of cells seeded}} \times 100$$

The control cloning efficiency is normalized to 100% and the cloning efficiencies of the various treatments are expressed as a percentage of the control cloning efficiency. The cells to be used for the determination of mutation frequency are allowed an expression period equal to the time it takes for them to divide four times (16 cell stage, 48-80 hours after removal of the treatment medium, depending upon treatment toxicity). This is monitored with an inverted microscope. At this time growth medium is removed and selective medium is added. The selective medium consists of growth medium containing ouabain at the desired concentration (0.5 or 1.0 mM). The selective medium is changed 3 and 8 days after addition to remove dead cells and replenish nutrients. After 12-15 days of growth in the selective medium, the colonies which have formed are stained and counted. Mutation frequency is expressed on a per-survivor basis. The corresponding cytotoxicity plates are used to calculate cloning efficiency as follows:

$$\frac{\text{number of cells which form colonies}}{\text{number of cells seeded}} \times 100$$

This cloning efficiency is multiplied by the number of cells seeded on the mutation plates to give the number of viable cells. The number of

mutants recovered is divided by the number of viable cells to yield the mutation frequency.

5. Enzyme Assays

A. Aryl Hydrocarbon Hydroxylase (AHH)

Aryl hydrocarbon hydroxylase was assayed as follows (Nebert and Gelboin, 1968; Nebert and Gielen, 1972; Oesch, 1976): The 1 ml incubation mixture consists of 0.90 ml Tris-HCl, pH 7.5 containing (final concentration in the reaction mixture), NADH (0.4 mM), NADP (0.3 mM), NADPH (0.1 mM), glucose-6-phosphate (4.2 mM), and glucose-6-phosphate dehydrogenase (1 unit/ml), 0.050 ml 60 mM $MgCl_2$, 0.025 ml of the S-15 fraction and 0.025 ml of 10 mM benzo(a)pyrene dissolved in acetone. The reaction is initiated with benzo(a)pyrene, incubated in a 37°C water bath and terminated by the addition of 1 ml of ice-cold acetone. Blanks are treated in an identical fashion except the acetone is added prior to the benzo(a)pyrene. The metabolites are extracted from the reaction mixture with 6 ml of petroleum ether (bp 30-60°C). The metabolites from 3 ml of petroleum ether are then extracted into 3 ml of 1 M NaOH and the fluorescence in the NaOH layer is measured at 522 nm in a Perkin-Elmer model 204A fluorescence spectrophotometer using an excitation wavelength of 396 nm. Quinine sulfate is used to calibrate the instrument. Fluorescent units are expressed as 3-OH benzo(a)pyrene equivalents, using the purified compound as a standard.

B. Ethoxyresorufin-O-Deethylase (EROD)

Ethoxyresorufin-O-deethylase activity was assayed by a modification of the procedure previously described (Burke and Mayer, 1974;

Pohl and Fouts, 1980). Incubation mixtures consist of 0.78 ml 66 mM Tris-HCl, pH 7.4 containing (final concentration in the reaction mixture): NADPH (0.1 mM), NADP (0.3 mM), NADH (0.4 mM), $MgCl_2$ (3.0 mM), glucose-6-phosphate (4.2 mM), glucose-6-phosphate dehydrogenase (1 unit/ml), bovine serum albumin (1.6 mg/ml), 0.02 ml ethoxyresorufin (125 μM) dissolved in dimethyl sulfoxide, and 0.20 ml of S-15 fraction containing the desired protein concentration. When enzyme inhibitors are employed the volume of Tris-HCl in the reaction mixture is decreased to 0.68 ml and 0.1 ml of the inhibitor or its solvent are added. The reaction is initiated by addition of the S-15 fraction and terminated with 1 ml of ice-cold acetone. Blanks are treated identically except the acetone is added prior to the S-15 fraction. Two ml of Tris-HCl, pH 7.4 (66 mM) are added and fluorescence is measured at 585 nm in a Perkin-Elmer model 204A fluorescence spectrophotometer using an excitation wavelength of 550 nm. The amount of resorufin produced is quantified by comparison to a standard curve. The resorufin obtained from the manufacturer was recrystallized by dissolving in absolute ethanol, filtering (0.22 μm Millipore filter) and evaporating to dryness. The purified resorufin was redissolved in absolute ethanol for construction of the standard curve.

C. Ethylmorphine-N-demethylase (EMND)

Ethylmorphine-N-demethylase activity was assayed as previously described (Lu et al., 1972) with several modifications. The incubation mixture consists of 3 ml of 0.1 M dibasic potassium phosphate, pH 7.3, which contains (final concentration in the incubation mixture):

semicarbazide HCl (3.3 mM), ethylmorphine (6.7 mM), NADPH (1 mM), $MgCl_2$ (3.3 mM), glucose-6-phosphate (5 mM), glucose-6-phosphate dehydrogenase (0.5 units/ml) and the desired concentration of S-15 protein. When enzyme inhibitors are employed the volume of dibasic potassium phosphate is reduced to 2.97 ml and 0.03 ml of the inhibitor or its solvent are added. The reaction is initiated with the S-15 fraction and terminated with 0.3 ml perchloric acid (70%). Blanks are treated identically except the perchloric acid is added prior to the S-15 fraction. The reaction mixture is centrifuged (680 x g for 5 minutes) to pellet the protein. The amount of formaldehyde formed was determined as previously described (Nash, 1953; Cochin and Axelrod, 1959). One ml of double strength Nash reagent (75 gm ammonium acetate, 1 ml acetylacetone, dissolved in 249 ml glass distilled water) is added to 2.5 ml of the supernatant. This mixture is incubated for 30 min in a 60°C water bath. The absorbance at 415 nm is determined with a Gilford model 300-N microsample spectrophotometer and the amount of formaldehyde produced is determined by comparison to a standard curve constructed by carrying known amounts of formaldehyde through the incubation and assay procedures.

D. Epoxide Hydrase (EH, E.C. 3.3.2.3)

Epoxide hydrase activity is assayed as previously described (Oesch et al., 1971). The incubation mixture consists of 0.115 ml glass distilled water, 0.050 ml 0.5 M Tris, pH 9.0, 0.010 ml acetonitrile containing [$^3$H]styrene oxide (6.18 mM, 2.86 μCi/μmole) and 0.025 ml of the S-15 fraction. When enzyme inhibitors are employed the

volume of glass distilled water is reduced to 0.095 ml and 0.020 ml of the inhibitor or its solvent are added. The reaction is initiated with the S-15 fraction and terminated with 3.5 ml of petroleum ether (b.p. 30-60°C). Blanks are treated in an identical fashion except petroleum ether is added prior to the S-15 fraction. The samples are mixed well, centrifuged (680 x g for 5 min) and placed at -15°C to freeze the aqueous layer and thereby enhance the separation of the aqueous and organic layers. The petroleum ether is aspirated off and 3.5 ml of fresh petroleum ether are added. This differential extraction is repeated two more times to ensure removal of unreacted substrate. The product (styrene dihydrodiol) is extracted from the aqueous layer into 1 ml of ethylacetate. An aliquot (0.4 ml) of the ethylacetate is added to 10 ml of scintillation fluor (Patterson and Greene, 1965) and counted.

6. Protein Assay

The protein content of the S-15 fraction was measured by the Biuret method (Gornall et al., 1949), using a Gilford model 300-N microsample spectrophotometer. Bovine serum albumin was employed as the standard.

# RESULTS

## 1. Enzyme Activity in the S-15 Fraction

### A. Aryl Hydrocarbon Hydroxylase (AHH)

The assay is based on the enzymatic conversion of BP to phenols, a reaction believed to be catalyzed by cytochrome P-448. The phenols are quantified in a fluorescence spectrophotometer. Fluorescent units are converted into 3-OH BP equivalents (Figure 3). The time course of the AHH assay is illustrated in Figure 4, and indicates that the reaction velocity is linear for at least 10 minutes. The reaction velocity is linear at S-15 protein concentrations up to and including the concentrations utilized in Figure 4 (data not shown). When AHH activity in the S-15 fraction is assayed the reaction is terminated after 10 minutes and the amount of S-15 protein in the reaction mixture was: Aroclor 1254, MC, BNF pretreated; 0.20 mg; PB, non-induced; 0.50 mg. The results are expressed as pmoles 3-OH BP/minute/mg S-15 protein (Table 1).

### B. Ethoxyresorufin-O-Deethylase (EROD)

The assay is based on the enzymatic deethylation of ethoxyresorufin, a reaction believed to be catalyzed by cytochrome P-448. The product is resorufin, which is quantified in a fluorescence spectrophotometer and compared to a standard curve (Figure 5) constructedby

Figure 3. Standard curve for the aryl hydrocarbon hydroxylase assay. Each point represents the mean of three replicates.

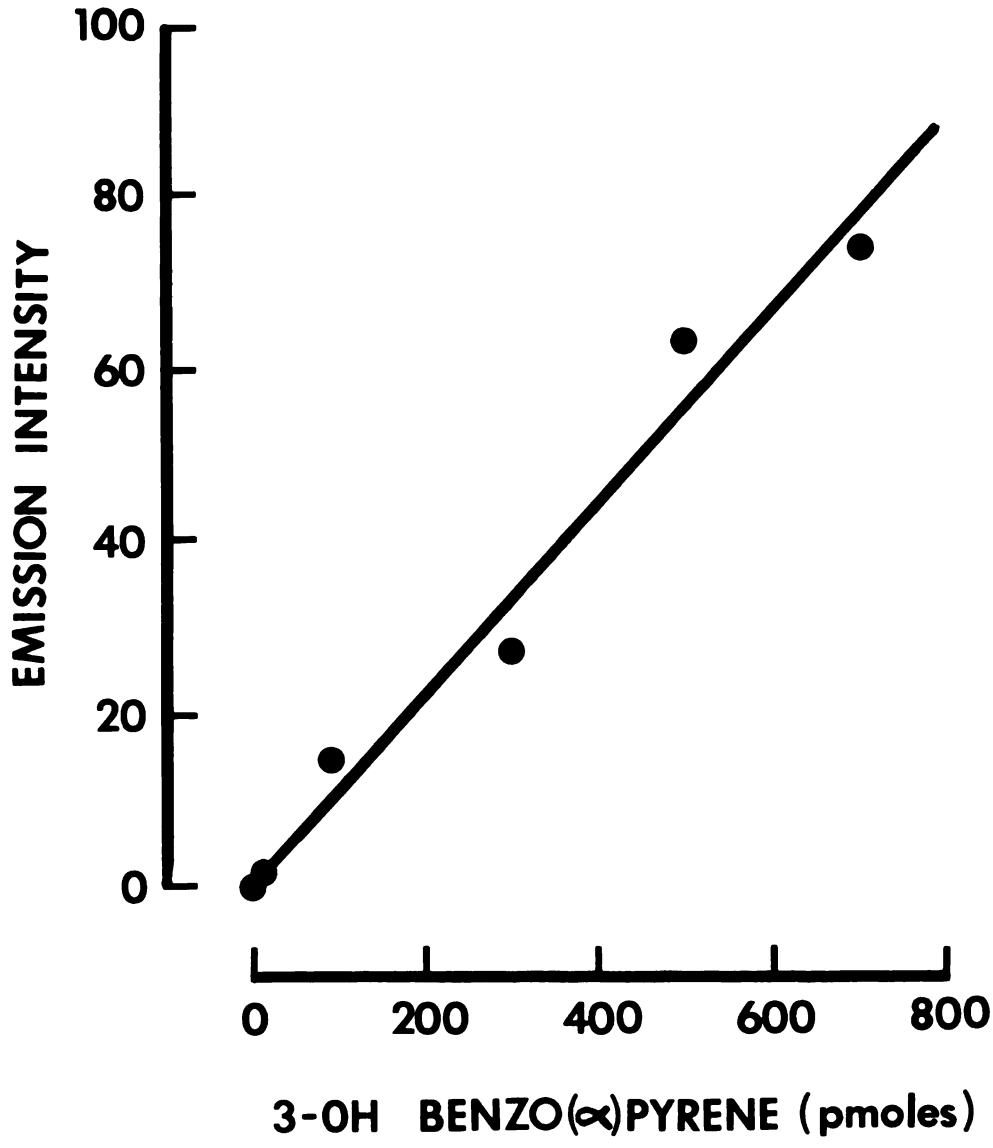

100
80
60
40
20
0
EMISSION INTENSITY
0
200
400
600
800
3-OH BENZO(∝)PYRENE (pmoles)

Figure 3

Figure 4. Time course of the aryl hydrocarbon hydroxylase assay. ●—●, 0.2 mg Aroclor 1254 pretreated S-15 protein; ■—■ , 0.5 mg non-induced S-15 protein. Each point represents the mean ± S.E. of three replicates.

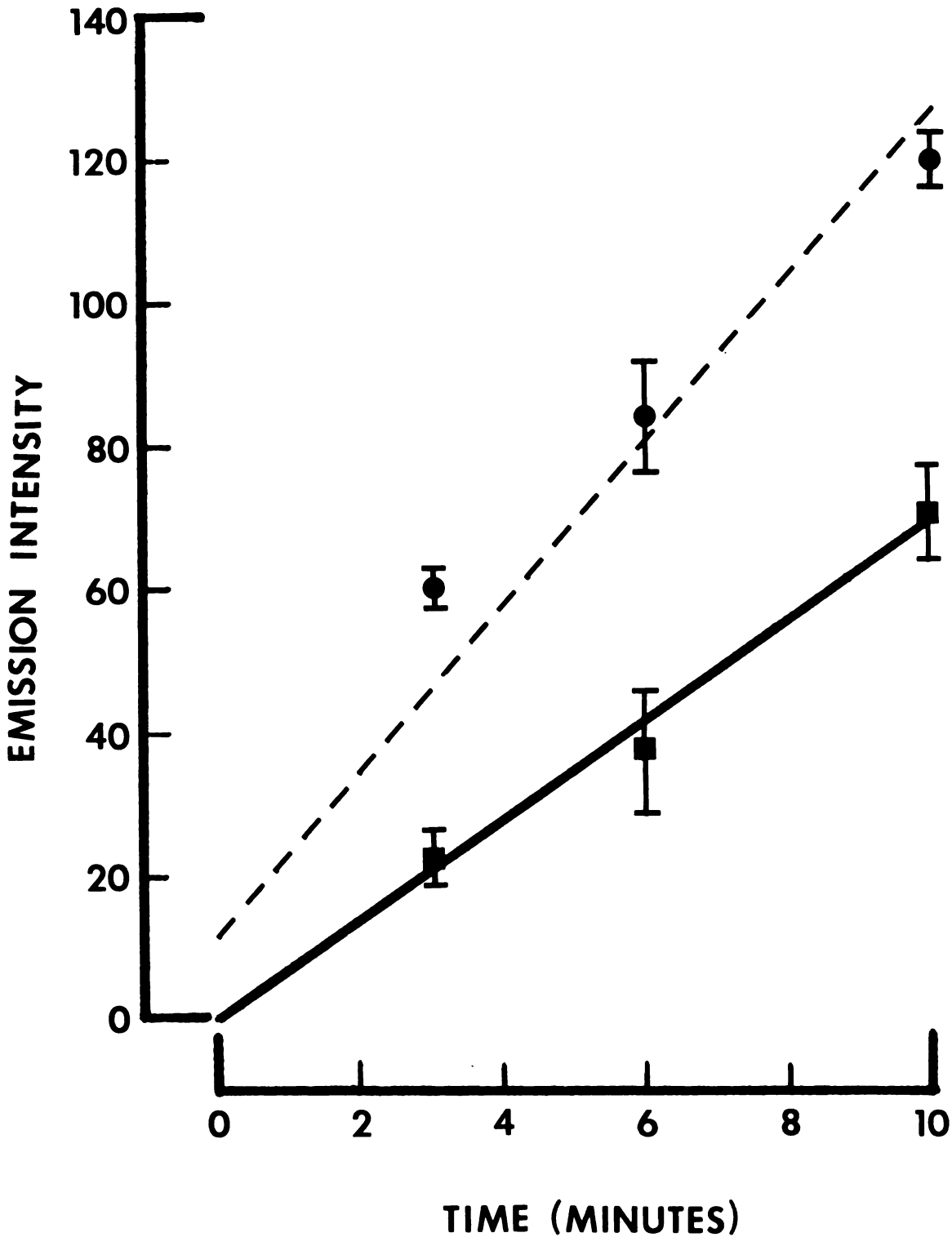

140
120
100
80
60
40
20
0
EMISSION INTENSITY
0
2
4
6
8
10
TIME (MINUTES)

Figure 4

TABLE 1

Aryl Hydrocarbon Hydroxylase Activity
in the S-15 Fraction

| Pretreatment | Enzyme Activity[a] |
|---|---|
| Control[b] | 132±45[c] |
| 3-Methylcholanthrene[d] | 420±41 |
| β-Naphthoflavone[e] | 426±23 |
| Aroclor 1254[f] | 457±18 |
| Phenobarbital[g] | 169±19 |

[a]pmoles 3-OH benzo(a)pyrene/minute/mg S-15 protein.

[b]Peanut oil or 0.9% saline.

[c]Mean ± S.E. of the values obtained from four animals.

[d]80 mg/kg, i.p., 24 hr prior to sacrifice.

[e]80 mg/kg, i.p., 24 hr prior to sacrifice.

[f]500 mg/kg, i.p., 96 hr prior to sacrifice.

[g]80 mg/kg, i.p., 96, 72, 48 and 24 hr prior to sacrifice.

Figure 5. Standard curve for the ethoxyresorufin-O-deethylase assay. Each point represents the mean of three replicates.

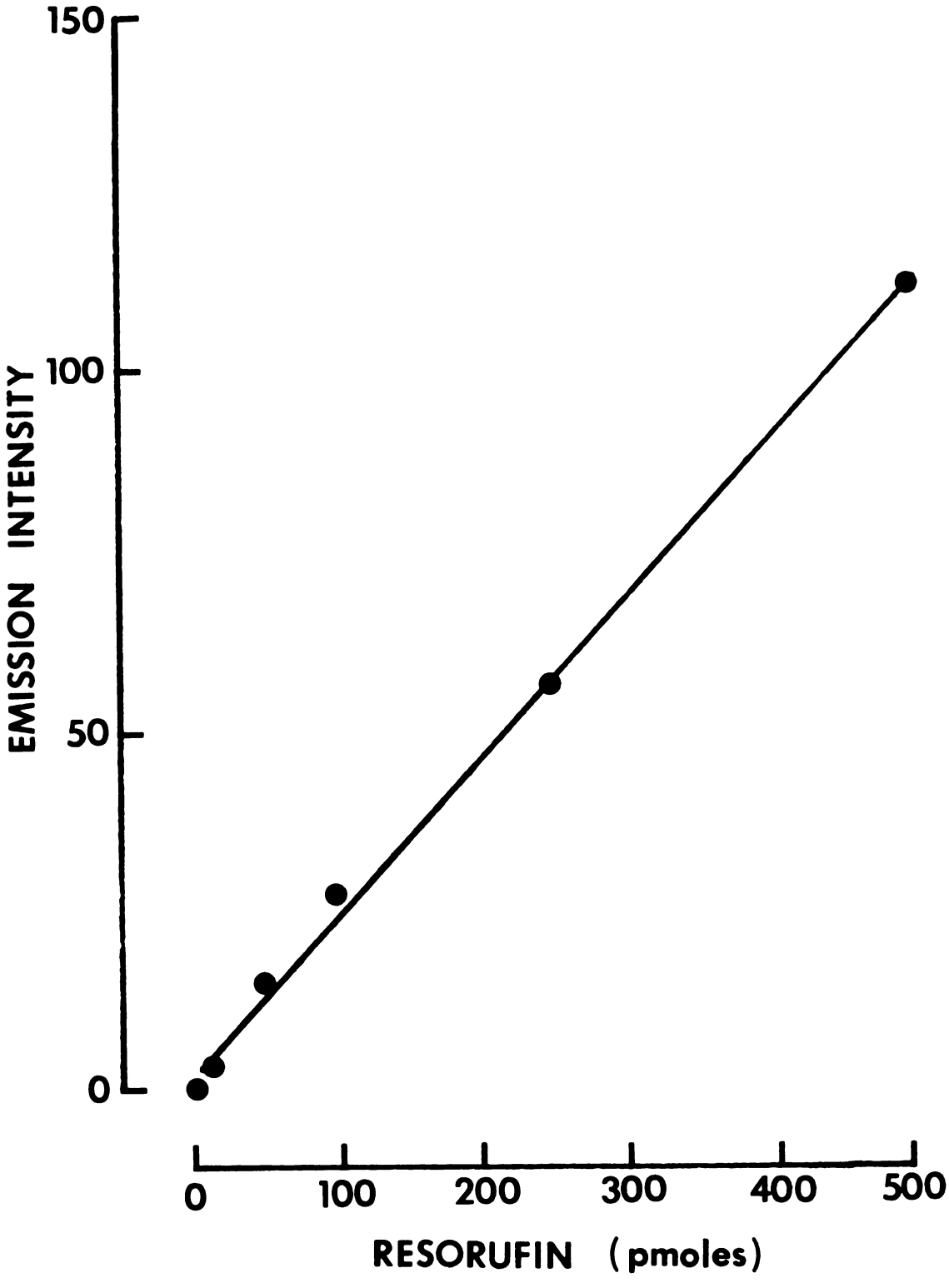

150
100
50
0
EMISSION INTENSITY
0
100
200
300
400
500
RESORUFIN (pmoles)

Figure 5

carrying known amounts of resorufin through the assay. In accordance with previous observations (Pohl and Fouts, 1980), the addition of 1.6 mg of bovine serum albumin per ml of reaction mixture increases apparent enzyme activity (2.5x) in this assay. Therefore, all EROD assays reported in this thesis contain bovine serum albumin (1.6 mg/ml). The time course of the reaction is illustrated in Figure 6, and indicates that the reaction velocity is linear for at least 6 minutes at appropriate concentrations of S-15 protein. Therefore, when the ethoxyresorufin-O-deethylase activity in the S-15 fraction is assessed the reaction is terminated after 5 minutes, and the following amounts of S-15 protein are employed: Aroclor 1254 pretreated, 0.035 mg; MC, BNF, 0.070 mg; PB, non-treated, 0.150 mg. The results are expressed as pmoles resorufin/minute/mg S-15 protein (Table 2).

C. Ethylmorphine-N-Demethylase (EMND)

This assay is based on the enzymatic N-demethylation of ethylmorphine, a reaction believed to be catalyzed by cytochrome P-450. The product is formaldehyde, and the reaction velocity is quantified by comparison to a standard curve constructed by carrying known amounts of formaldehyde through the assay (Figure 7). The time course of the reaction is illustrated in Figure 8, and indicates that the reaction velocity is linear for at least fifteen minutes at appropriate concentrations of S-15 protein. When ethylmorphine-N-demethylase activity in the S-15 fraction is assessed, the reaction is terminated after 10 minutes and the following amounts of S-15 protein are used: Aroclor 1254, PB, pretreated, 10 mg; MC, BNF, or non-treated, 20 mg. The results

Figure 6. Time course of the ethoxyresorufin-O-deethylase assay. ■—■, 0.12 mg 3-methylcholanthrene pretreated S-15 protein; ●—● , 0.06 mg 3-methylcholanthrene pretreated S-15 protein; ■ — ■, 0.24 mg non-induced S-15 protein; ● - ●, 0.12 mg non-induced S-15 protein. Each point represents the mean of three replicates.

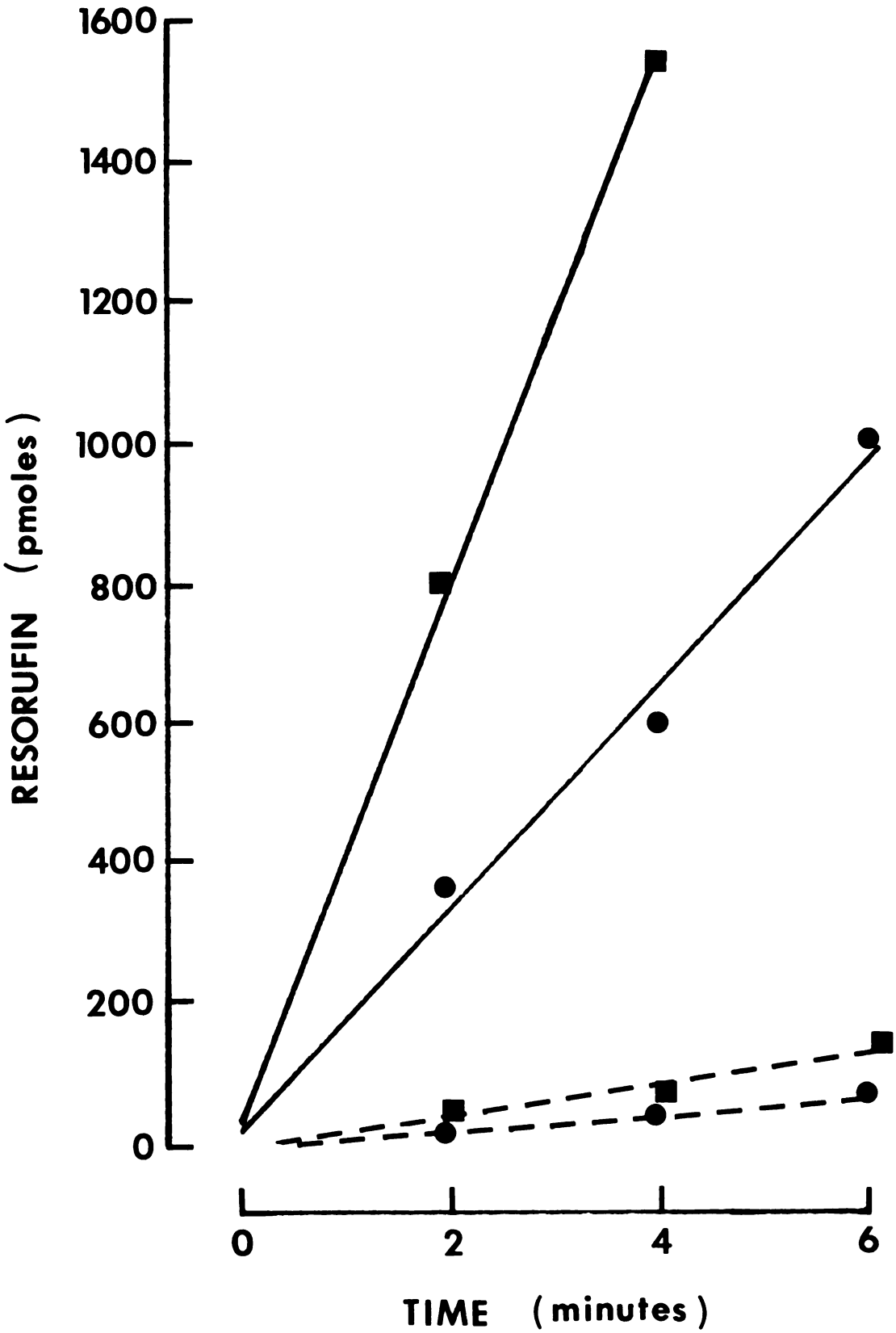

1600
1400
1200
1000
800
600
400
200
0
RESORUFIN (pmoles)
0
2
4
6
TIME (minutes)

Figure 6

TABLE 2

Ethoxyresorufin-O-Deethylase Activity in the S-15 Fraction

| Pretreatment | Enzyme Activity[a] |
|---|---|
| Control[b] | 124± 28[c] |
| 3-Methylcholanthrene[d] | 2089±405 |
| β-Naphthoflavone[e] | 2166±336 |
| Aroclor 1254[f] | 4611±338 |
| Phenobarbital[g] | 462±144 |

[a]pmoles resorufin/minute/mg S-15 protein.

[b]Peanut oil or 0.9% saline.

[c]Mean ± S.E. of the values obtained from three animals.

[d]80 mg/kg, i.p., 24 hr prior to sacrifice.

[e]80 mg/kg, i.p., 24 hr prior to sacrifice.

[f]500 mg/kg, i.p., 96 hr prior to sacrifice.

[g]80 mg/kg, i.p., 96, 72, 48 and 24 hr prior to sacrifice.

Figure 7. Standard curve for the ethylmorphine-N-demethylase assay. Each point represents the mean of three replicates.

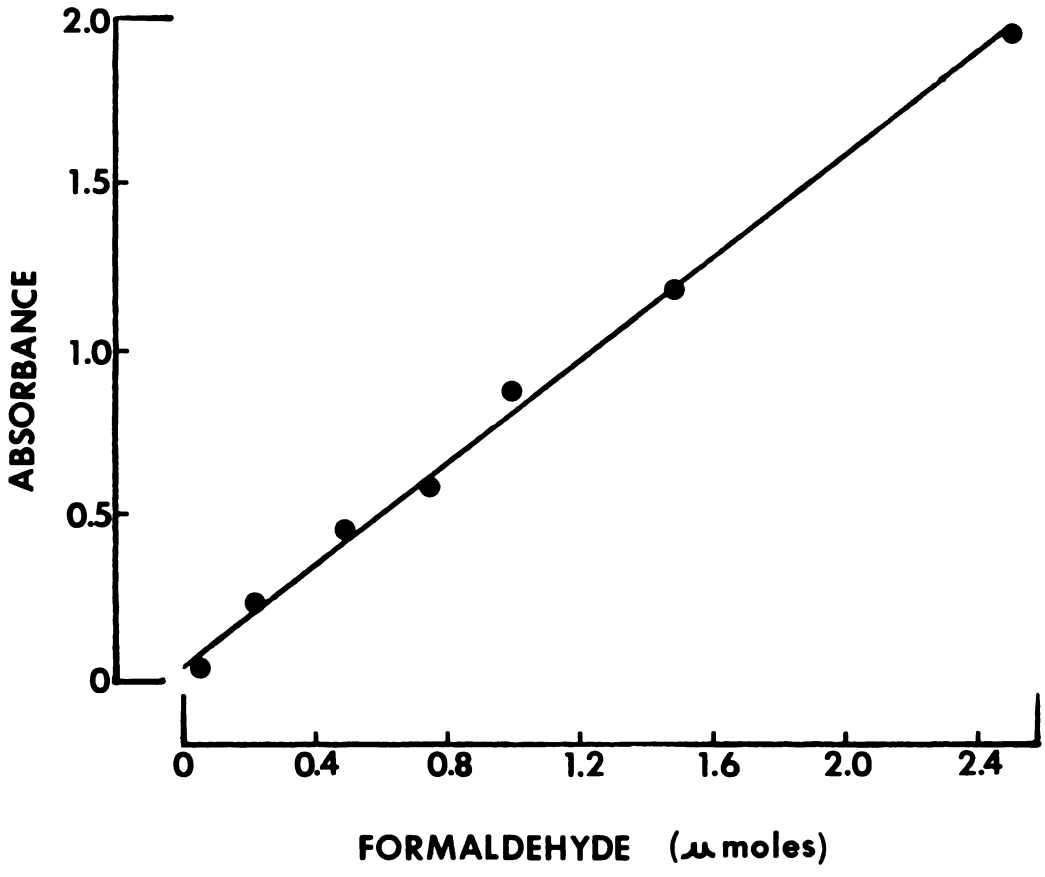

2.0
1.5
1.0
0.5
0
ABSORBANCE
0
0.4
0.8
1.2
1.6
2.0
2.4
FORMALDEHYDE (μmoles)

Figure 7

Figure 8. Time course of the ethylmorphine-N-demethylase assay. □—□, 10.7 mg Aroclor 1254 pretreated S-15 protein; ■—■, 4.4 mg Aroclor 1254 pretreated S-15 protein; ○—○, 8.6 mg non-induced S-15 protein; ●—● 3.6 mg non-induced S-15 protein. Each point represents the mean ± S.E. of three replicates.

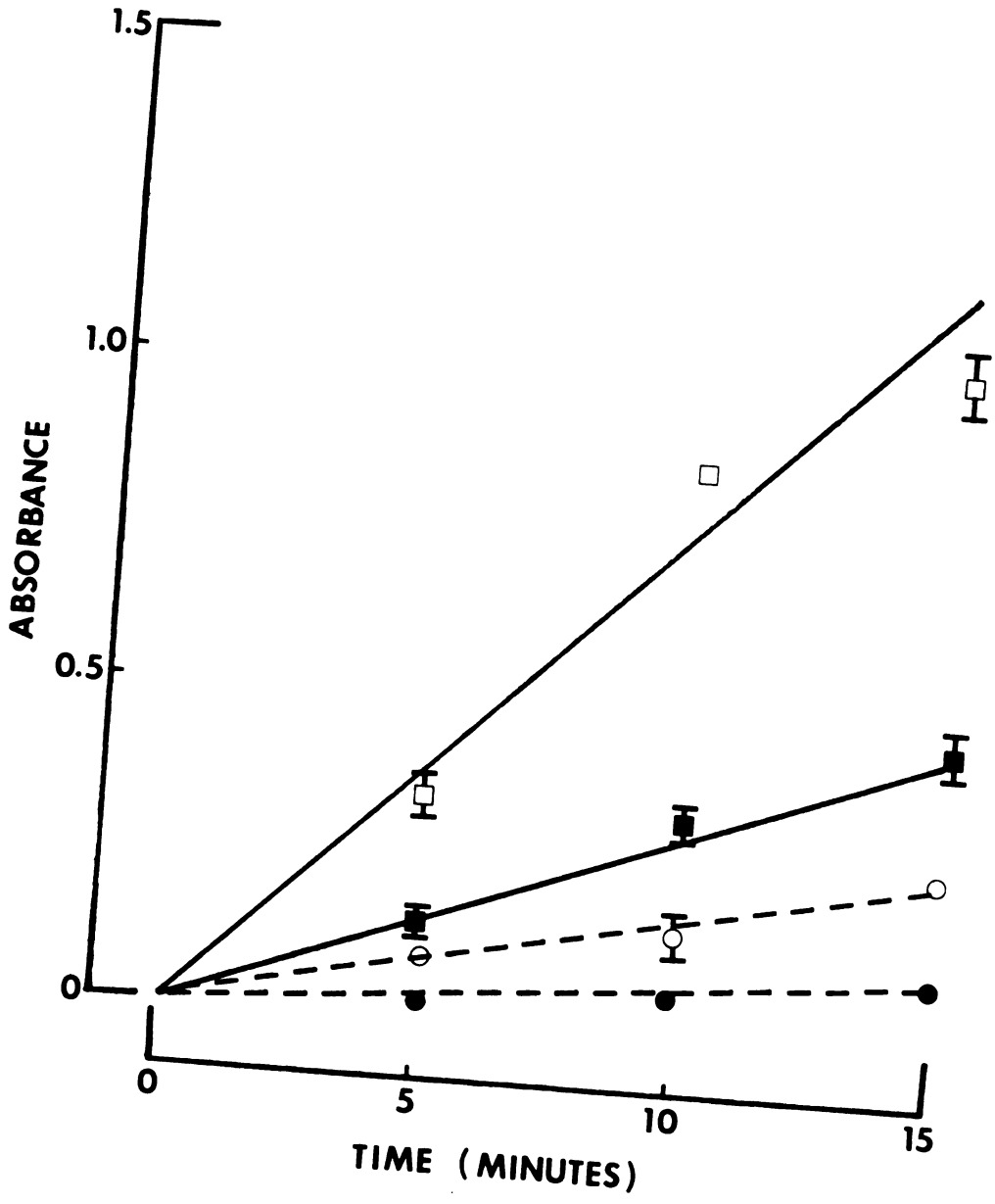



Figure 8

are expressed as nmoles formaldehyde produced per minute per mg S-15 protein (Table 3).

D. Epoxide Hydrase (EH)

The assay is based on the conversion of [$^3$H]styrene oxide to [$^3$H]styrene dihydrodiol by epoxide hydrase. Differential extraction of the incubation mixture is employed to separate substrate from product and the reaction velocity is determined by assaying the product ([$^3$H]styrene dihydrodiol) in a scintillation counter. The data in Figure 9 illustrate the time course of the reaction. At appropriate concentrations of S-15 protein the reaction velocity is linear for at least 9 minutes. Therefore, when assessing the epoxide hydrase activity in the S-15 fraction the reaction is terminated after 6 minutes and the following amounts of S-15 protein are employed: PB, Aroclor 1254 pretreated, 0.25 mg; MC, BNF or non-treated, 0.50 mg. The results are expressed as nmoles styrene dihydrodiol produced per minute per mg S-15 protein (Table 4).

2. The Effects of α-Naphthoflavone (ANF), Metyrapone (MET), and Cyclohexene Oxide (CCHO) on Enzyme Activity in the S-15 Fraction

A. Ethoxyresorufin-O-Deethylase (EROD) Activity

ANF, an inhibitor of cytochrome P-448, inhibits EROD activity in a concentration-dependent fashion when added to S-15 fraction derived from animals pretreated with MC or Aroclor 1254 (Table 5). Conversely, ANF does not inhibit EROD activity in the S-15 fraction derived from a non-induced animal (Table 5).

TABLE 3

Ethylmorphine-N-Demethylase Activity in the S-15 Fraction

| Pretreatment | Enzyme Activity[a] |
|---|---|
| Control[b] | 1.28±0.12[c] |
| 3-Methylcholanthrene[d] | 0.59±0.07 |
| β-Naphthoflavone[e] | 0.56±0.03 |
| Aroclor 1254[f] | 3.29±0.27 |
| Phenobarbital[g] | 4.15±0.04 |

[a] nmoles formaldehyde/minute/mg S-15 protein.

[b] Peanut oil or 0.9% saline.

[c] Mean ± S.E. of the values obtained from three animals.

[d] 80 mg/kg, i.p., 24 hr prior to sacrifice.

[e] 80 mg/kg, i.p., 24 hr prior to sacrifice.

[f] 500 mg/kg, i.p., 96 hr prior to sacrifice.

[g] 80 mg/kg, i.p., 96, 72, 48 and 24 hr prior to sacrifice.

Figure 9. Time course of the epoxide hydrase assay. ●—●, 0.4 mg Aroclor 1254 pretreated S-15 protein; □—□, 0.2 mg Aroclor 1254 pretreated S-15 protein; ■—■, 0.4 mg non-induced S-15 protein; ○—○, 0.2 mg non-induced S-15 protein. Each point represents the mean of three replicates.

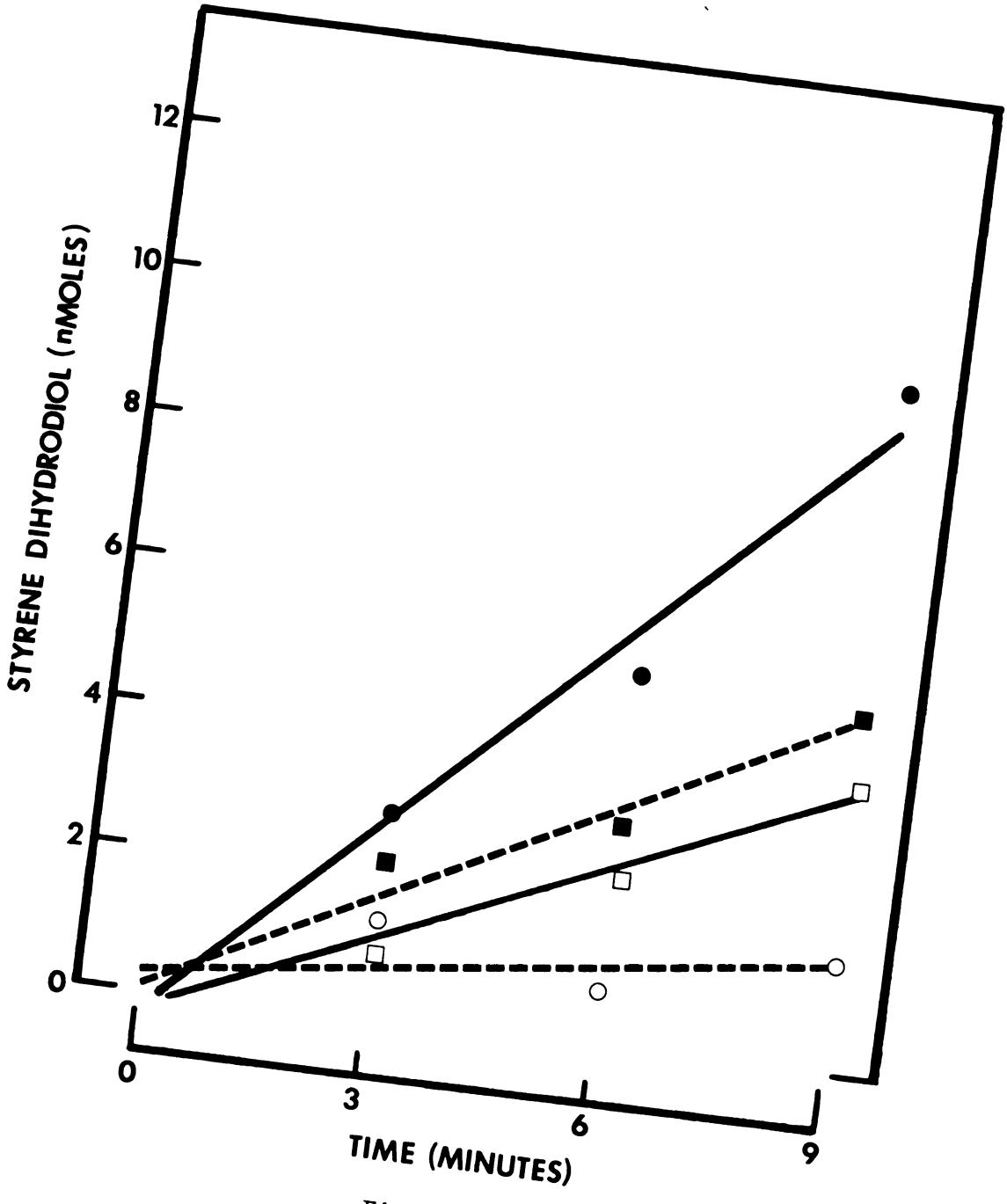



Figure 9

TABLE 4

Epoxide Hydrase Activity in the S-15 Fraction

| Pretreatment | Enzyme Activity[a] |
|---|---|
| Control[b] | 0.93±0.10[c] |
| 3-Methylcholanthrene[d] | 0.72±0.04 |
| β-Naphthoflavone[e] | 0.85±0.09 |
| Aroclor 1254[f] | 2.12±0.41 |
| Phenobarbital[g] | 1.85±0.12 |

[a] nmoles styrene dihydrodiol/minute/mg S-15 protein.

[b] Peanut oil or 0.9% saline.

[c] Mean ± S.E. of the values obtained from three animals.

[d] 80 mg/kg, i.p., 24 hr prior to sacrifice.

[e] 80 mg/kg, i.p., 24 hr prior to sacrifice.

[f] 500 mg/kg, i.p., 96 hr prior to sacrifice.

[g] 80 mg/kg, i.p., 96, 72, 48 and 24 hr prior to sacrifice.

TABLE 5

The Effect of α-Naphthoflavone on Ethoxyresorufin-O-Deethylase Activity in the S-15 Fraction

| α-Naphthoflavone | Enzyme Activity[a] | | |
|---|---|---|---|
| | Pretreatment | | |
| | 3-Methylcholanthrene[b] | Aroclor 1254[c] | Non-Induced[d] |
| 0[e] | 100.0±13.1[f] | 100.0±2.3 | 100.0± 3.6 |
| $10^{-7}$M | 34.0± 4.0 | 37.7±3.2 | 103.1± 3.6 |
| $10^{-6}$M | 7.9± 0.9 | 13.4±0.9 | 98.7± 7.1 |
| $10^{-5}$M | 5.0± 2.3 | 2.4±0.7 | 85.7±12.5 |

[a]Expressed as a percentage of solvent control, See Table 2.

[b]80 mg/kg, i.p., 24 hr prior to sacrifice.

[c]500 mg/kg, i.p., 96 hr prior to sacrifice.

[d]No pretreatment.

[e]Concentration in the reaction mixture.

[f]Mean ± S.E. of three replicates.

CCHO, an inhibitor of epoxide hydrase, and MET, an inhibitor of cytochrome P-450, were tested for their effects on EROD activity. MET inhibited EROD activity in a dose-dependent fashion (Table 6). This indicates that MET inhibits cytochrome P-448 and/or the deethylation of ethoxyresorufin may be catalyzed by cytochrome P-450. CCHO does not significantly affect EROD activity (Table 6).

B. Ethylmorphine-N-Demethylase (EMND) Activity

MET is an inhibitor of cytochrome P-450 and, as illustrated in Table 7, it inhibits EMND in a concentration dependent fashion. CCHO or ANF do not significantly affect EMND activity in the S-15 fraction (Table 8).

C. Epoxide Hydrase (EH)

CCHO inhibits EH activity in the S-15 fraction in a dose-dependent manner (Table 9). ANF has no effect on EH activity, whereas MET, at a concentration of $10^{-3}$M, stimulates EH activity (Table 10).

3. Characterization of the V79/Ouabain Resistance Mutagenicity Assay

When a culture of V79 Chinese hamster cells were first received they were routinely grown in modified Eagle's minimal essential medium supplemented with 5% (v/v) fetal calf serum and had a doubling time of 12 hours. In view of the high cost and diminishing availability of fetal calf serum, I selected out a cell line capable of growth in medium containing 20% (v/v) calf serum and employed this line in all experiments (Figure 10).

In order to develop this assay system, it was necessary to verify that the V79 cells in my possession were capable of detecting cytotoxic

TABLE 6

The Effect of Cyclohexene Oxide or Metyrapone on Ethoxyresorufin-O-Deethylase Activity in the S-15 Fraction[a]

| Compound | Enzyme Activity[b] |
|---|---|
| Cyclohexene Oxide | |
| 0[c] | 100.0±2.5[d] |
| $10^{-4}$M | 101.1±5.1 |
| $10^{-3}$M | 123.6±4.5 |
| $5x10^{-3}$M | 112.9±1.2 |
| Metyrapone | |
| 0 | 100.0±6.0 |
| $10^{-5}$M | 78.4±2.9 |
| $10^{-4}$M | 62.4±3.8 |
| $10^{-3}$M | 21.9±0.1 |

[a]S-15 fraction derived from animals pretreated with Aroclor 1254, 500 mg/kg, i.p., 96 hr prior to sacrifice.

[b]Expressed as a percentage of solvent control, see Table 2.

[c]Concentration in the reaction mixture.

[d]Mean ± S.E. of three replicates.

TABLE 7

The Effect of Metyrapone on Ethylmorphine-N-Demethylase Activity in the S-15 Fraction

| Metyrapone | Enzyme Activity[a] | | |
|---|---|---|---|
| | Pretreatment | | |
| | Phenobarbital[b] | Aroclor 1254[c] | Non-Induced[d] |
| 0[e] | 100.0±11.0[f] | 100.0±11.6 | 100.0±12.5 |
| $10^{-5}$M | 67.8± 4.7 | 45.8± 8.2 | 72.2±11.0 |
| $10^{-4}$M | 41.9± 8.7 | 17.3± 1.9 | 61.6± 3.4 |
| $10^{-3}$M | 10.5± 2.7 | 5.6± 1.7 | 13.7± 0.8 |

[a]Expressed as a percentage of solvent control, See Table 3.

[b]80 mg/kg, i.p., 96, 72, 48 and 24 hr prior to sacrifice.

[c]500 mg/kg, i.p., 96 hr prior to sacrifice.

[d]No pretreatment.

[e]Concentration in the reaction mixture.

[f]Mean ± S.E. of three replicates.

TABLE 8

The Effect of Cyclohexene Oxide or α-Naphthoflavone on Ethylmorphine-N-Demethylase Activity in the S-15 Fraction[a]

| Compound | Enzyme Activity[b] |
|---|---|
| Cyclohexene Oxide | |
| 0[c] | 100.0± 6.7[d] |
| $10^{-4}$M | 97.3± 3.5 |
| $10^{-3}$M | 91.1± 6.9 |
| $5x10^{-3}$M | 95.6± 3.9 |
| α-Naphthoflavone | |
| 0 | 100.0± 6.7 |
| $10^{-7}$M | 98.3±10.3 |
| $10^{-6}$M | 85.3± 7.2 |
| $10^{-5}$M | 103.0± 5.4 |

[a]S-15 fraction derived from animals pretreated with Aroclor 1254, 500 mg/kg, i.p., 96 hr prior to sacrifice.

[b]Expressed as a percentage of solvent control, see Table 3.

[c]Concentration in the reaction mixture.

[d]Mean ± S.E. of three replicates.

TABLE 9

The Effect of Cyclohexene Oxide on Epoxide Hydrase Activity in the S-15 Fraction

| Cyclohexene Oxide | Enzyme Activity[a] | |
|---|---|---|
| | Pretreatment | |
| | Aroclor 1254[b] | 3-Methylcholanthrene[c] |
| 0[d] | 100.0±3.2[e] | 100.0±11.9 |
| $10^{-4}$M | 73.4±5.0 | 84.9± 4.0 |
| $10^{-3}$M | 29.9±2.5 | 52.0± 3.6 |
| $5x10^{-3}$M | 20.7±2.4 | 22.9± 0.6 |

[a]Expressed as a percentage of solvent control, see Table 4.

[b]500 mg/kg, i.p., 96 hr prior to sacrifice.

[c]80 mg/kg, i.p., 24 hr prior to sacrifice.

[d]Concentration in the reaction mixture.

[e]Mean ± S.E. of three replicates.

TABLE 10

The Effect of Metyrapone or α-Naphthoflavone on Epoxide Hydrase Activity in the S-15 Fraction[a]

| Compound | Enzyme Activity[b] |
|---|---|
| Metyrapone | |
| 0[c] | 100.0± 2.1[d] |
| $10^{-5}$M | 81.0± 6.8 |
| $10^{-4}$M | 111.0± 3.4 |
| $10^{-3}$M | 142.0±11.8 |
| α-Naphthoflavone | |
| 0 | 100.0± 1.2 |
| $10^{-7}$M | 100.0± 2.5 |
| $10^{-6}$M | 101.2± 2.3 |
| $10^{-5}$M | 102.7± 5.8 |

[a]S-15 fraction derived from animals pretreated with Aroclor 1254, 500 mg/kg, i.p., 96 hr prior to sacrifice.

[b]Expressed as a percentage of solvent control, see Table 4.

[c]Concentration in the reaction mixture.

[d]Mean ± S.E. of three replicates.

Figure 10. Growth curves for V79 Chinese hamster cells. $1x10^5$ cells were seeded into 75 $cm^2$ flasks containing medium supplemented with the indicated concentration of calf serum (CS) or fetal calf serum (FCS). After various periods of time the cell number was determined. Each point represents the mean ± S.E. of 8 determinations.

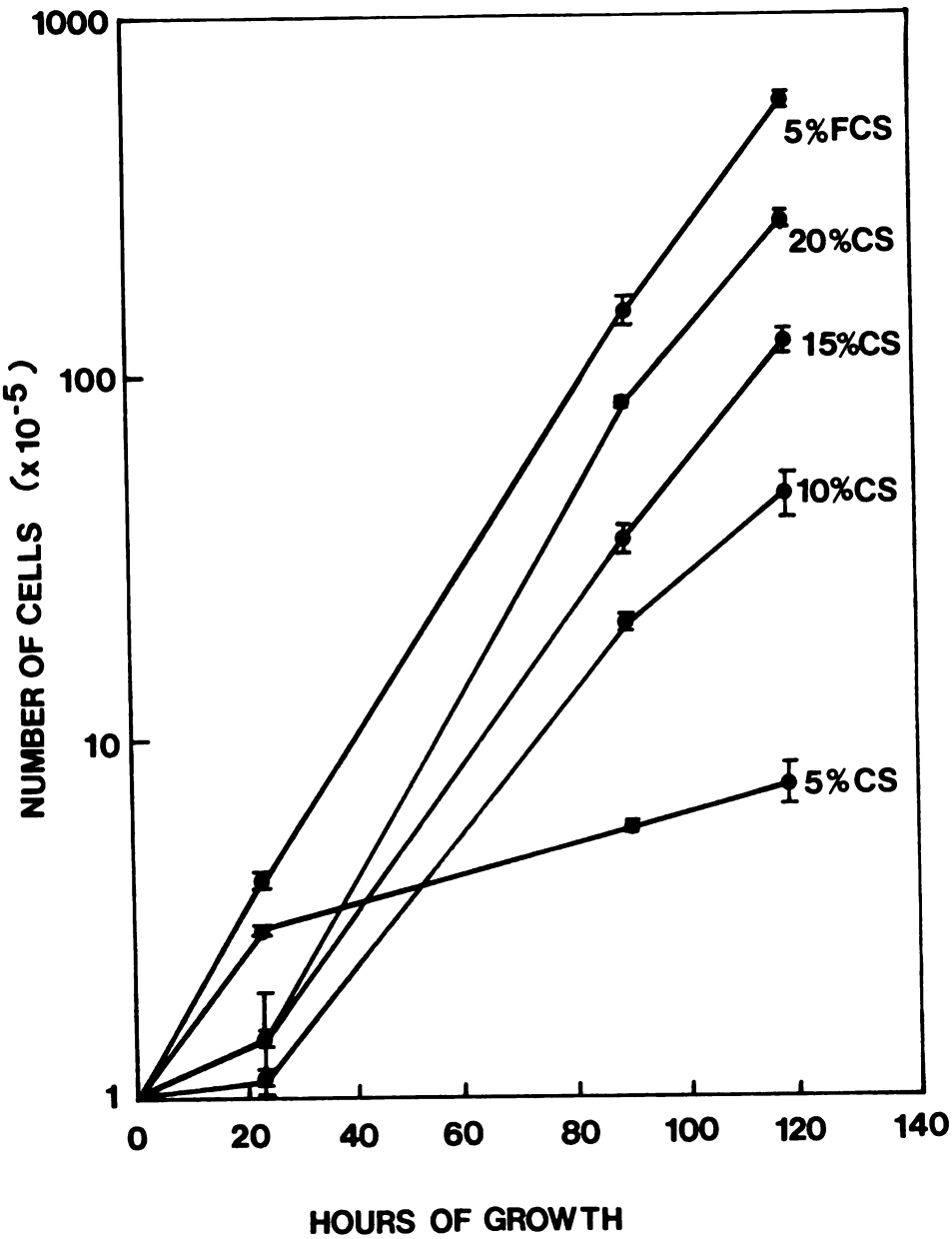

1000
100
10
1
NUMBER OF CELLS (x10-5)
0
20
40
60
80
100
120
140
HOURS OF GROWTH
5%FCS
20%CS
15%CS
10%CS
5%CS

Figure 10

agents in a dose-dependent fashion. MNNG is a direct acting alkylating agent which is toxic to mammalian cells (Peterson et al., 1979), and, MNNG produced cytotoxicity in our V79 cells in a concentration-dependent manner in the absence of the S-15 fraction (Figure 11). MNNG is also a direct acting mutagen in mammalian cells and it induces ouabain resistant mutants in our V79 cells (Table 11). The spontaneous as well as the induced mutation frequency is markedly influenced by the ouabain concentration employed in the selective medium (Table 11).

The S-15 fraction from a MC-pretreated animal along with the NADPH generating system is non-toxic to V79 cells (Table 12). The S-15 fractions prepared from control animals or from animals pretreated with PB, BNF or Aroclor 1254 are similarly non-toxic (data not shown).

4. Benzo(a)pyrene (BP)

Data on the cytotoxicity of BP in the absence of the S-15 fraction are presented in Table 13. The data in Figure 12 illustrate the cytotoxicity produced when a non-toxic concentration of BP (20 μM, Table 13) is titrated with non-toxic concentrations of the S-15 fraction (Table 12). When the S-15 is prepared from an animal pretreated with MC cytotoxicity occurs, but when the S-15 fraction is prepared from an untreated animal there is no cytotoxicity. The cytotoxicity curve is biphasic when MC pretreated S-15 is incorporated. When a portion of the S-15 fraction derived from a MC pretreated animal is inactivated by boiling (10 minutes) and various amounts are added to the reaction mixture along with 1.4 mg/ml active MC pretreated S-15 it can be seen that the BP is detoxified.

Figure 11. Cytotoxicity of N-methyl-N-nitroso-$N^1$-nitro-guanidine (MNNG) in the absence of the S-15 fraction. Cloning efficiency is expressed as a percentage of control. Values are the mean ± S.E. of 3 replicates.

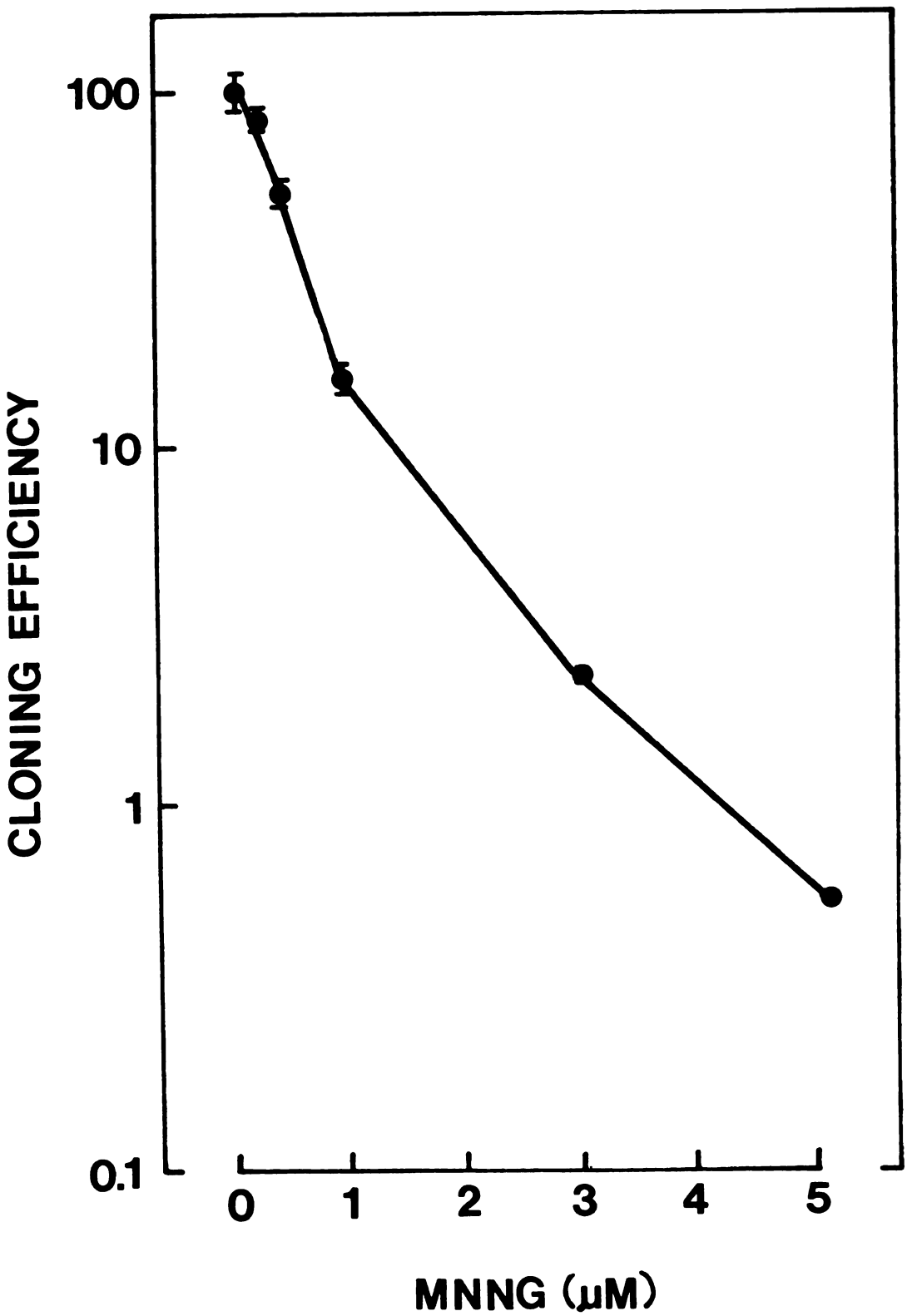



Figure 11

TABLE 11

The Influence of Ouabain Concentration on Mutation Frequency

| Treatment | Cloning Efficiency[a] | Mutation Frequency[b] | |
|---|---|---|---|
| | | Ouabain Concentration | |
| | | 0.5 mM | 1.0 mM |
| Control | 100[c] | 155[d] (26) | 1.3 (2) |
| 3 μM MNNG | 9 | 399 (139) | 152 (53) |

[a]Expressed as a percentage of solvent control.

[b]Mutants/$10^6$ viable cells.

[c]Mean of 3 replicates.

[d]Number of mutants recovered.

TABLE 12

Cytotoxicity of the S-15 Fraction and NADPH Generating System

| mg S-15 Protein[a] / ml Reaction Mixture | Cloning Efficiency[b] |
|---|---|
| 0 | 100.0±2.4[c] |
| 2.0 | 106.9±7.4 |
| 2.4 | 107.5±3.4 |
| 3.2 | 99.2±4.4 |
| 4.0 | 106.0±0.8 |
| 6.2 | 98.7±1.7 |

[a]Derived from animals pretreated with 3-methylcholanthrene, 80 mg/kg, i.p., 24 hr prior to sacrifice.

[b]Expressed as a percentage of control.

[c]Mean ± S.E. of 3 replicates.

TABLE 13

The Cytotoxicity of Benzo(a)pyrene in the Absence of the S-15 Fraction

| Benzo(a)pyrene (μM) | Cloning Efficiency[a] |
|---|---|
| 0 | 100±2.7[b] |
| 5 | 101±5.1 |
| 20 | 99.1±6.2 |
| 50 | 95.6±1.7 |
| 100 | 83.6±3.1 |

[a]Expressed as a percentage of control.

[b]Mean ± S.E. of 3 replicates.

Figure 12. Cytotoxicity of 20 μM benzo(a)pyrene in the presence of the S-15 fraction derived from non-treated (■—■) or 3-methylcholanthrene (●—●) pretreated animals. A portion of the 3-methylcholanthrene pretreated S-15 fraction was inactivated by boiling for ten minutes prior to use, and various amounts were added to the reaction mixture along with 1.4 mg/ml active 3-methylcholanthrene pretreated S-15 fraction (●—●). Cloning efficiency is expressed as a percentage of control. Values represent the mean ± S.E. of 3 replicates.

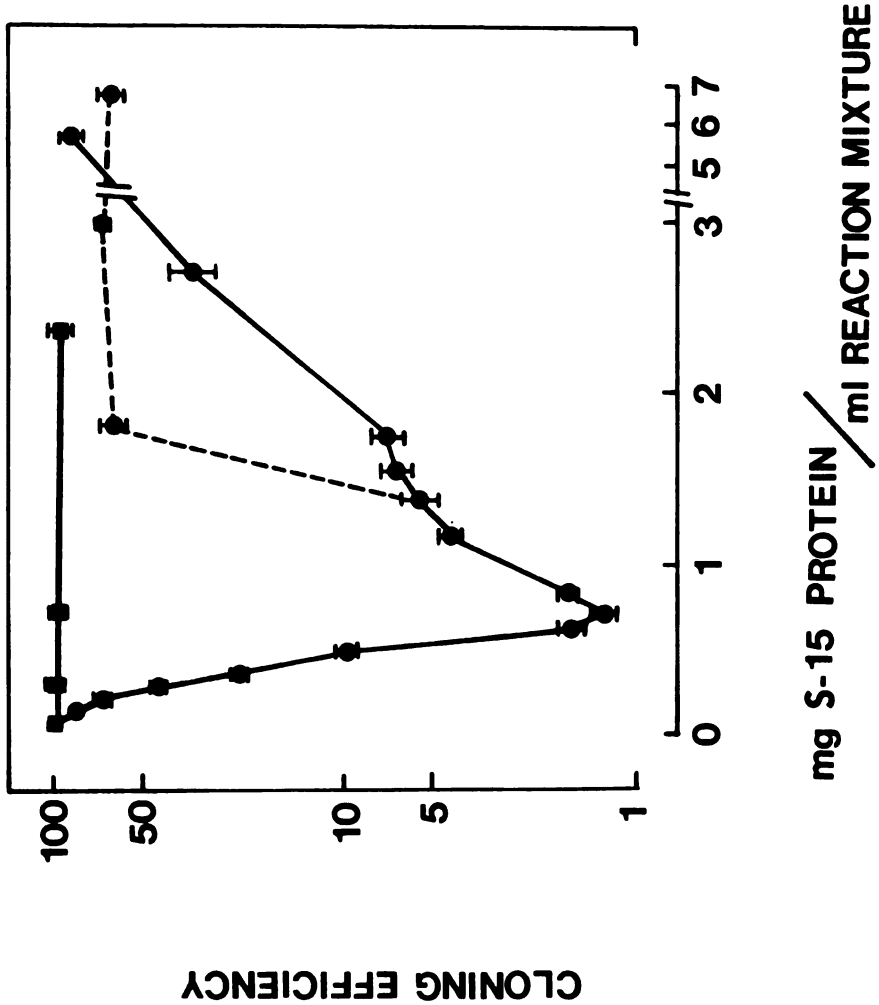

CLONING EFFICIENCY
100
50
10
5
1
0
1
2
3
5
6
7
mg S-15 PROTEIN / ml REACTION MIXTURE

Figure 12

It is apparent from Figure 12 that for S-15 derived from a MC pretreated animal the observed cytotoxicity is dependent upon the concentration of the S-15 fraction in the reaction mixture. To determine whether this dependence on S-15 concentration would be apparent when the animals were pretreated with other types of enzyme inducers I chose to test the P-450 type inducer PB (Conney, 1967), a P-448 type inducer other than MC (BNF) (Boobis et al., 1977) and a mixed type inducer (Aroclor 1254) (Alvares et al., 1973; Parkinson et al., 1980). The results from this study are depicted in Table 14 and indicate that in all three cases, cytotoxicity is dependent upon the concentration of S-15 protein in the reaction mixture. The results presented in Table 15 illustrate that the mutation frequency as well as the cytotoxicity, is dependent upon the concentration of S-15 protein in the reaction mixture when the S-15 is derived from a MC pretreated animal.

MC and BNF are P-448 type inducers (Boobis et al., 1977) and at the dose that I routinely employ both increase AHH activity to a similar degree (300% of control, Table 1). The ability of S-15 fractions prepared from animals pretreated with these compounds was compared regarding their ability to produce mutagenic and/or cytotoxic metabolites from BP. The results are illustrated in Figure 13 and indicate that at equivalent levels of cytotoxicity these S-15 fractions produced differing degrees of mutagenicity. BNF pretreatment produced a greater degree of mutagenicity per degree of cytotoxicity than did MC pretreatment.

TABLE 14

Influence of the S-15 Concentration on Benzo(a)pyrene-Induced Cytotoxicity

| S-15 Source | Cloning Efficiency[a] | |
|---|---|---|
| | 10 μM Benzo(a)pyrene | 20 μM Benzo(a)pyrene |
| Phenobarbital[b] | | |
| 1.1[c] | 83.3±6.5[d] | <1 |
| 2.4 | 91.2±4.8 | 34.0±3.4 |
| β-Naphthoflavone[e] | | |
| 0.9 | <1 | <1 |
| 2.3 | 68.7±5.4 | 7.5±0.5 |
| Aroclor 1254[f] | | |
| 1.1 | 53.1±3.7 | <1 |
| 2.4 | 101.8±5.7 | 19.3±3.0 |

[a]Expressed as a percentage of control.

[b]80 mg/kg, i.p., 96, 72, 48 and 24 hr prior to sacrifice.

[c]mg S-15 protein/ml reaction mixture.

[d]Mean ± S.E. of 3 replicates.

[e]80 mg/kg, i.p., 24 hr prior to sacrifice.

[f]500 mg/kg, i.p., 96 hr prior to sacrifice.

TABLE 15

Effect of the S-15 Concentration on Benzo(a)pyrene-Induced Cytotoxicity and Mutation Frequency

| mg S-15 Protein[a] / ml Reaction Mixture | Benzo(a)pyrene Concentration | | | | | |
|---|---|---|---|---|---|---|
| | 2.5 μM | | 5.0 μM | | 7.5 μM | |
| | Cloning Efficiency[b] | Mutation Frequency | Cloning Efficiency | Mutation Frequency | Cloning Efficiency | Mutation Frequency |
| 1.1 mg | 97.8±5.5[c] | 0.2[d] (2)[e] | 30.0±2.7 | 2.6 (21) | 3.2±0.2 | 29.5 (41) |
| 2.2 mg | 102.2±1.7 | 0.3 (3) | 91.1±2.0 | 0.6 (11) | 45.1±2.3 | 2.1 (31) |

[a]Derived from animals pretreated with 3-methylcholanthrene, 80 mg/kg, i.p., 24 hr prior to sacrifice.

[b]Expressed as a percentage of control.

[c]Mean ± S.E. of 3 replicates.

[d]Mutants/$10^5$ viable cells; selection in 1 mM ouabain.

[e]Number of mutants recovered.

Figure 13. The relationship between cytotoxicity and mutagenicity when benzo(a)pyrene is incubated in the presence of the S-15 fractions derived from rats pretreated with 3-methylcholanthrene (MC) or β-naphthoflavone (BNF). Cloning efficiency is expressed as a percentage of control. Values represent the mean of 3 replicates. Mutation frequency was determined in 0.5 mM ouabain.

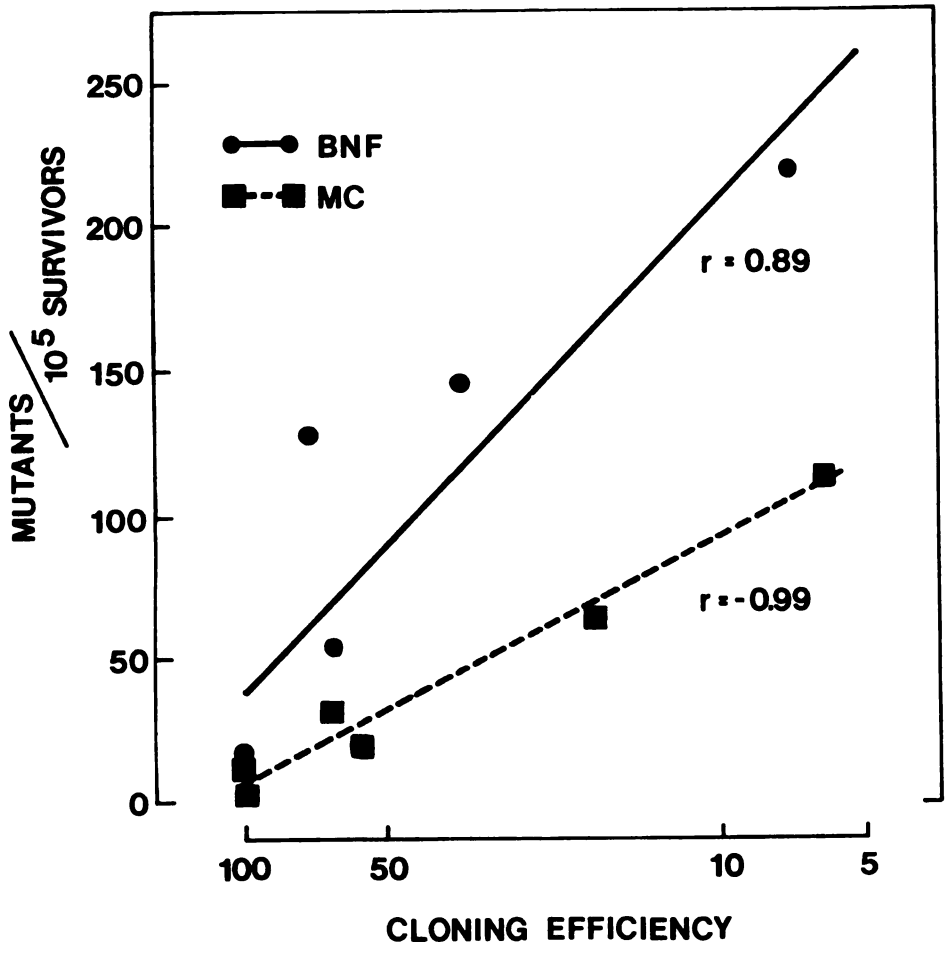

250
200
150
100
50
0
MUTANTS / $10^5$ SURVIVORS
BNF
MC
r = 0.89
r = -0.99
100
50
10
5
CLONING EFFICIENCY

Figure 13

In order to perform mechanistic studies on the metabolic activation of BP it is necessary to alter the pattern and relative activity of enzymes contained in the S-15 fraction. I chose to accomplish this by: 1) pretreating the experimental animals with Aroclor 1254, PB or MC because these compounds are known to alter hepatic enzyme patterns and 2) use the in vitro inhibitors CCHO, MET and ANF to inhibit specific enzymes in the S-15 fractions prepared from the pretreated animals. By combining these two approaches it is possible to generate a number of different enzymic patterns in the S-15 fraction. The ability of these various S-15 fractions to generate mutagenicity and/or cytotoxicity in the presence of BP was compared with the enzyme pattern contained in these fractions. This approach could allow one to elucidate the relative importance of various enzymes and/or enzyme patterns in the metabolic activation of BP.

MET, CCHO and ANF in concentrations which significantly inhibit enzyme activity in the S-15 fraction (Tables 5, 7, 9) were not cytotoxic (Table 16) or mutagenic (data not shown) to V79 cells either in the presence or absence of the S-15 fraction.

The concentration of S-15 protein in the reaction mixture markedly influences the cytotoxicity and mutagenicity of BP (Tables 14, 15, Figure 12). Therefore, in all studies employing enzyme inhibitors the concentration of S-15 protein was the same (1.0±0.1 mg protein/ml reaction mixture). The BP concentration (7.5 μM) was identical in all assays. The data in Table 17 illustrate that ANF decreases the cytotoxicity and mutagenicity of BP in the presence of the S-15 fraction

TABLE 16

The Cytotoxicity of Metyrapone, Cyclohexene Oxide and α-Naphthoflavone

| Compound | Cloning Efficiency[a] | |
|---|---|---|
| | Without S-15 Fraction | With S-15 Fraction[b] |
| Metyrapone | | |
| 0[c] | 100.0±2.3[d] | 100.0±5.5 |
| $10^{-5}$M | 86.6±7.6 | 98.4±4.1 |
| $10^{-4}$M | 101.7±6.3 | 81.5±2.7 |
| $10^{-3}$M | 97.2±4.7 | 102.2±2.3 |
| Cyclohexene Oxide | | |
| 0 | 100.0±9.9 | 100.0±5.1 |
| $10^{-4}$M | 86.5±7.1 | 103.0±5.2 |
| $10^{-3}$M | 102.4±8.5 | 100.8±4.9 |
| $5x10^{-3}$M | 78.4±4.6 | 94.4±6.3 |
| α-Naphthoflavone | | |
| 0 | 100.0±9.9 | 100.0±5.1 |
| $10^{-7}$M | 103.0±6.9 | 106.9±5.7 |
| $10^{-6}$M | 104.1±2.3 | 106.0±3.6 |
| $10^{-5}$M | 83.6±4.3 | 115.1±5.8 |

[a]Expressed as a percentage of control (no inhibitor).

[b]S-15 fraction derived from rats pretreated with Aroclor 1254, 500 mg/kg, i.p., 96 hr prior to sacrifice; 2.2 mg S-15 protein/ml reaction mixture.

[c]Final concentration in the reaction mixture.

[d]Mean ± S.E. of 3-9 replicates.

TABLE 17

The Cytotoxicity and Mutagenicity of Benzo(a)pyrene in the Presence of Various Concentrations of α-Naphthoflavone

| α-Naphthoflavone Concentration | S-15 Source | | | |
|---|---|---|---|---|
| | 3-Methylcholanthrene[a] | | Aroclor 1254[b] | |
| | Cloning Efficiency[c] | Mutation Frequency[d] | Cloning Efficiency | Mutation Frequency |
| 0 | 11.8±1.2[e] | 3.7[f] | 73.9±1.6 | 0.7[h] |
| $10^{-7}$M | 38.6±3.1 | 2.0[f] | 80.6±1.1 | 0.3[h] |
| $10^{-6}$M | 38.6±5.1 | 1.3[g] | 70.0±5.3 | 0.9[h] |
| $10^{-5}$M | 117.7±3.1 | 0.1[g] | 60.5±1.8 | 0.9[h] |

[a] 80 mg/kg, i.p., 24 hr prior to sacrifice.

[b] 500 mg/kg, i.p., 96 hr prior to sacrifice.

[c] Expressed as a percentage of control (no BP).

[d] Expressed as mutants/$10^5$ survivors.

[e] Mean ± S.E. of 3 replicates. [f] $36 \times 10^5$ cells assayed.

[g] $24 \times 10^5$ cells assayed. [h] $12 \times 10^5$ cells assayed.

derived from a MC pretreated animal, but does not affect either the cytotoxicity or mutagenicity of BP in the presence of the Aroclor 1254 induced S-15 fraction. CCHO increases the cytotoxicity and mutagenicity of BP in the presence of MC or Aroclor 1254 pretreated S-15 fractions (Table 18). At a concentration of $10^{-3}$M MET increases the cytotoxicity and mutagenicity of BP in the presence of the S-15 fraction derived from an Aroclor 1254 pretreated animal but does not affect BP toxicity or mutagenicity when present at lower concentrations (Table 19).

The effect of specific enzymes and/or specific enzyme patterns on the metabolic activation of BP was elucidated in the following manner:

A) the activity of EH, EROD and EMND in S-15 fractions derived from animals pretreated with selected enzyme modulators is known (Tables 2, 3, 4).

B) the inhibitory efficacy of various concentrations of MET, CCHO and ANF on these enzyme activities is known (Tables 5 to 10).

C) knowing (A) and (B) the activity of a given enzyme in a given S-15 fraction in the presence of a given inhibitor concentration may be calculated and expressed as amount of product/minute/mg S-15 protein.

D) the cytotoxicity and mutagenicity of BP in the presence of a particular S-15 fraction combined with a particular inhibitor is known (Tables 17, 18, 19).

E) graphs relating the cytotoxicity or mutagenicity of BP to the activity of a particular enzyme (or the ratio of two

TABLE 18

The Cytotoxicity and Mutagenicity of Benzo(a)pyrene in the Presence of Various Concentrations of Cyclohexene Oxide

| | S-15 Source | | | |
|---|---|---|---|---|
| | 3-Methylcholanthrene[a] | | Aroclor 1254[b] | |
| Cyclohexene Oxide Concentration | Cloning Efficiency[c] | Mutation Frequency[d] | Cloning Efficiency | Mutation Frequency |
| 0 | 26.7±2.2[e] | 2.2[f] | 48.5±8.5 | 0.5[g] |
| $10^{-4}$M | 8.5±2.8 | 11.7[f] | 30.1±2.8 | 1.3[g] |
| $10^{-3}$M | <1 | N.D.[h] | 9.3±4.2 | 2.5[g] |
| $5x10^{-3}$M | <1 | N.D. | 1.4±1.0 | 5.6[g] |

[a] 80 mg/kg, i.p., 24 hr prior to sacrifice.

[b] 500 mg/kg, i.p., 96 hr prior to sacrifice.

[c] Expressed as a percentage of control (no BP).

[d] Expressed as mutants/$10^5$ survivors.

[e] Mean ± S.E. of 3 replicates. [f] $18x10^5$ cells assayed.

[g] $15x10^5$ cells assayed. [h] not determined.

TABLE 19

The Cytotoxicity and Mutagenicity of Benzo(a)pyrene in the Presence of Various Concentrations of Metyrapone[a]

| Metyrapone Concentration | Cloning Efficiency[b] | Mutation Frequency[c] |
|---|---|---|
| 0 | 53.9±6.2[d] | 0.5[e] |
| $10^{-5}$M | 52.1±4.9 | 0.8[e] |
| $10^{-4}$M | 58.1±8.3 | 0.3[e] |
| $10^{-3}$M | 26.7±2.8 | 1.9[e] |

[a]S-15 fraction derived from rats which had been pretreated with Aroclor 1254, 500 mg/kg, i.p., 96 hr prior to sacrifice.

[b]Expressed as a percentage of control (no BP).

[c]Expressed as mutants/$10^5$ survivors.

[d]Mean ± S.E. of 3 replicates.

[e]$15x10^5$ cells assayed.

enzyme activities) were constructed by plotting the biological response to BP on the abscissa and enzyme activity in the S-15 fraction on the ordinate.

The results of this analysis are illustrated in Figures 14 to 19. EROD activity (Figure 14), EMND activity (Figure 15) or the ratio of EROD activity to EMND activity (Figure 16) in the S-15 fraction does not correlate with the induction of cytotoxicity or mutagenicity in the presence of BP. Conversely, EH activity (Figure 17) as well as the ratio between EMND activity and EH activity (Figure 18) do correlate with the induction of cytotoxicity and mutagenicity in the presence of BP. As the EH activity in the S-15 fraction increases, BP becomes less cytotoxic and less mutagenic and as the EMND/EH ratio increases, BP becomes more cytotoxic and more mutagenic. The correlations are statistically significant ($p<0.01$) (EH-cytotoxicity, 59% of total variability due to regression; EH-mutagenicity, 42% of total variability due to regression; EMND/EH-cytotoxicity, 62% of total variability due to regression; EMND/EH-mutagenicity, 46% of total variability due to regression). The ratio of EROD activity to EH activity in the S-15 fraction (Figure 19) correlates best with the production of toxic and mutagenic metabolites from BP ($p<0.01$, cytotoxicity - 79% of total variability due to regression; mutagenicity - 61% of total variability due to regression). As the ratio of EROD to EH activity in a given S-15 fraction increases, the cytotoxicity and mutagenicity of BP also increases indicating that the P-448/EH ratio in the S-15 fraction is important in determining the quantity of mutagenic and/or cytotoxic metabolites produced from BP.

Figure 14. The relationship between ethoxyresorufin-O-deethylase (EROD) activity in the S-15 fraction and the biological response of V79 cells to benzo(a)pyrene. EROD activity is expressed as pmoles resorufin/minute/mg S-15 protein. Cloning efficiency (●) is expressed as a percentage of control. Values represent the mean of three replicates. Mutation frequency (□) is expressed as mutants/$10^5$ viable cells and was determined in 1.0 mM ouabain.

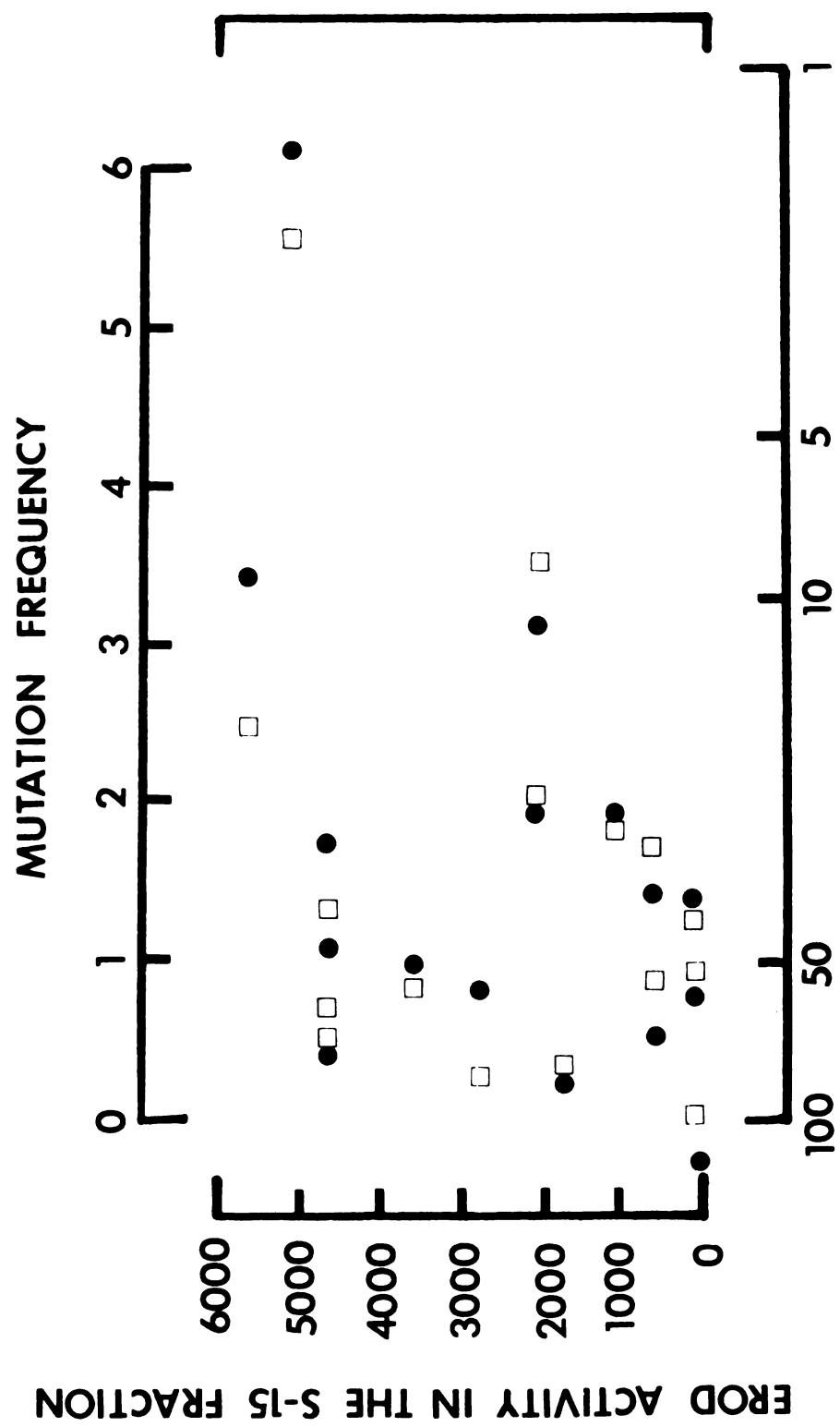

EROD ACTIVITY IN THE S-15 FRACTION
6000
5000
4000
3000
2000
1000
0
MUTATION FREQUENCY
0
1
2
3
4
5
6
CLONING EFFICIENCY
100
50
10
5
1

Figure 14

Figure 15. The relationship between ethylmorphine-N-demethylase (EMND) activity in the S-15 fraction and the biological response of V79 cells to benzo(a)pyrene. EMND activity is expressed as nmoles of formaldehyde/minute/mg S-15 protein. Cloning efficiency (●) is expressed as a percentage of control. Values represent the mean of three replicates. Mutation frequency (□) is expressed as mutants/$10^5$ viable cells and was determined in 1.0 mM ouabain.

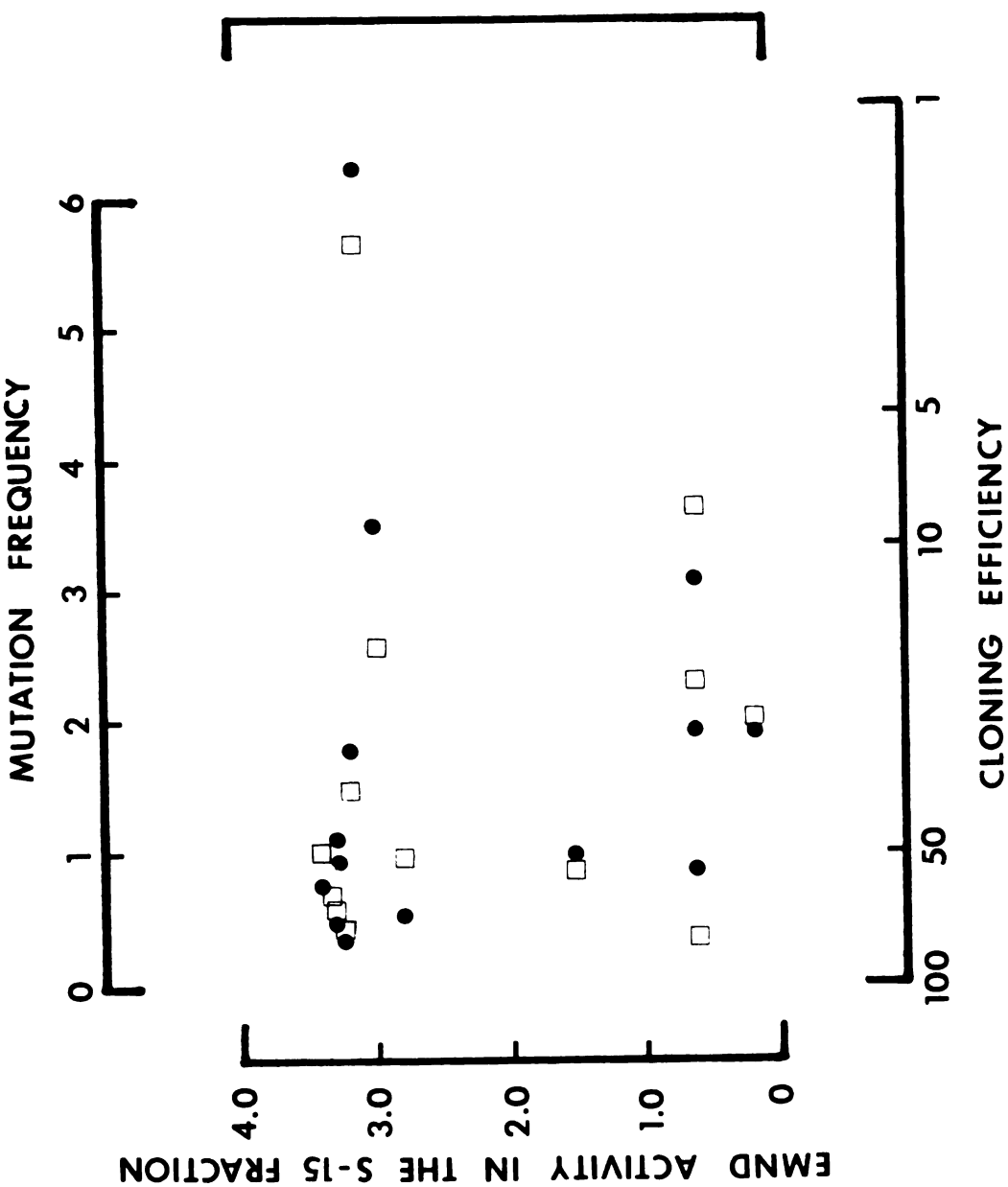

MUTATION FREQUENCY
0
1
2
3
4
5
6
CLONING EFFICIENCY
100
50
10
5
1
EMND ACTIVITY IN THE S-15 FRACTION
4.0
3.0
2.0
1.0
0

Figure 15

Figure 16. The relationship between the ratio of ethoxyresorufin-O-deethylase (EROD) to ethylmorphine-N-demethylase (EMND) activity in the S-15 fraction and the biological response of V79 cells to benzo(a)pyrene. EROD activity was expressed as pmoles resorufin/minute/mg S-15 protein; and EMND activity was expressed as nmoles formaldehyde/minute/mg S-15 protein. Cloning efficiency (●) is expressed as a percentage of control. Values represent the mean of three replicates. Mutation frequency (□) is expressed as mutants/$10^5$ viable cells and was determined in 1.0 mM ouabain.

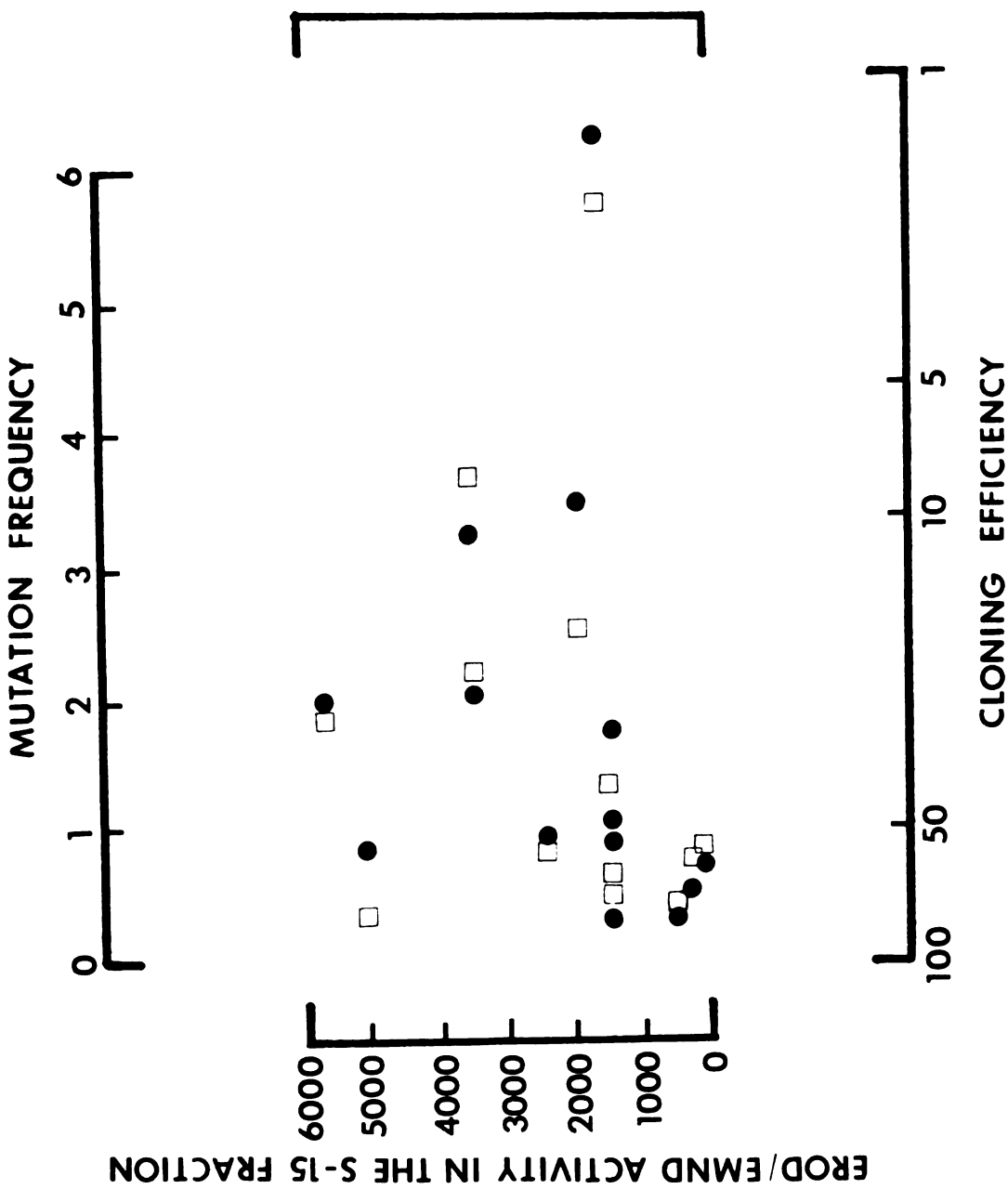

MUTATION FREQUENCY
0
1
2
3
4
5
6
CLONING EFFICIENCY
1
5
10
50
100
EROD/EMND ACTIVITY IN THE S-15 FRACTION
0
1000
2000
3000
4000
5000
6000

Figure 16

Figure 17. The relationship between epoxide hydrase (EH) activity in the S-15 fraction and the biological response of V79 cells to benzo(a)pyrene. EH activity is expressed as nmoles styrene dihydrodiol/minute/mg S-15 protein. Cloning efficiency (●—●, r = 0.77) is expressed as a percentage of control. Values represent the mean of three replicates. Mutation frequency (□—□, r = -0.65) is expressed as mutants/$10^5$ viable cells and was determined in 1.0 mM ouabain.

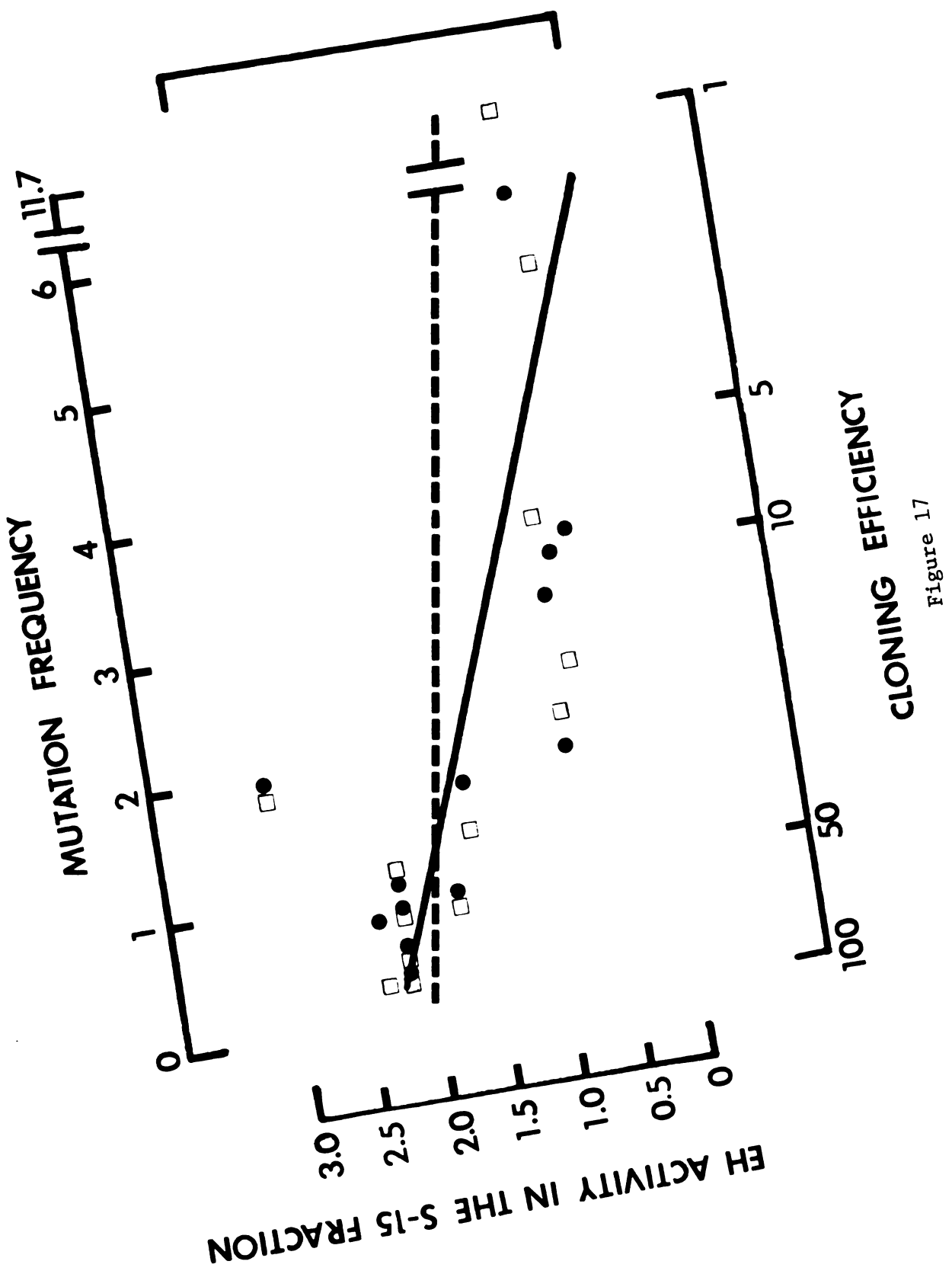

MUTATION FREQUENCY
11.7
0
1
2
3
4
5
6
CLONING EFFICIENCY
1
5
10
50
100
EH ACTIVITY IN THE S-15 FRACTION
3.0
2.5
2.0
1.5
1.0
0.5
0

Figure 17

Figure 18. The relationship between the ratio of ethylmorphine-N-demethylase (EMND) to epoxide hydrase (EH) activity in the S-15 fraction and the biological response of V79 cells to benzo(a)-pyrene. EMND activity was expressed as nmoles formaldehyde/minute/mg S-15 protein; and EH activity was expressed as nmoles styrene dihydrodiol/minute/mg S-15 protein. Cloning efficiency (●—●, r = -0.79) is expressed as a percentage of control. Values represent the mean of three replicates. Mutation frequency (□—□, r = 0.68) is expressed as mutants/$10^5$ viable cells and was determined in 1.0 mM ouabain.

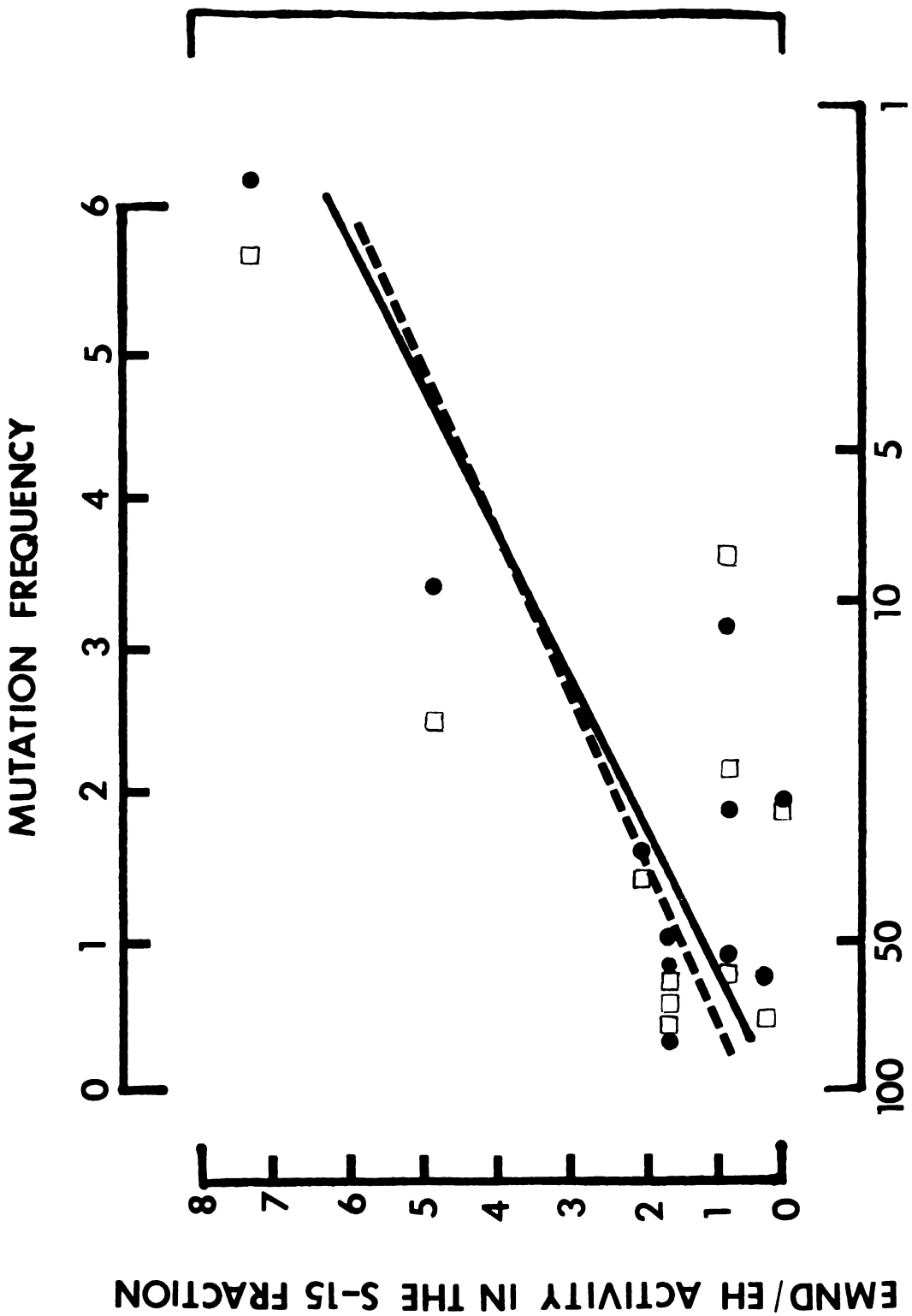

MUTATION FREQUENCY
0
1
2
3
4
5
6
CLONING EFFICIENCY
100
50
10
5
1
EMND/EH ACTIVITY IN THE S-15 FRACTION
0
1
2
3
4
5
6
7
8

Figure 18

Figure 19. The relationship between the ratio of ethoxyresorufin-O-deethylase (EROD) to epoxide hydrase (EH) activity in the S-15 fraction and the biological response of V79 cells to benzo(a)pyrene. EROD activity was expressed as pmoles resorufin/minute/mg S-15 protein; and EH activity was expressed as nmoles styrene dihydrodiol/minute/mg S-15 protein. Cloning efficiency (●—●, r = -0.89) is expressed as a percentage of control. Values represent the mean of three replicates. Mutation frequency (□—□, r = 0.78) is expressed as mutants/ $10^5$ viable cells and was determined in 1.0 mM ouabain.

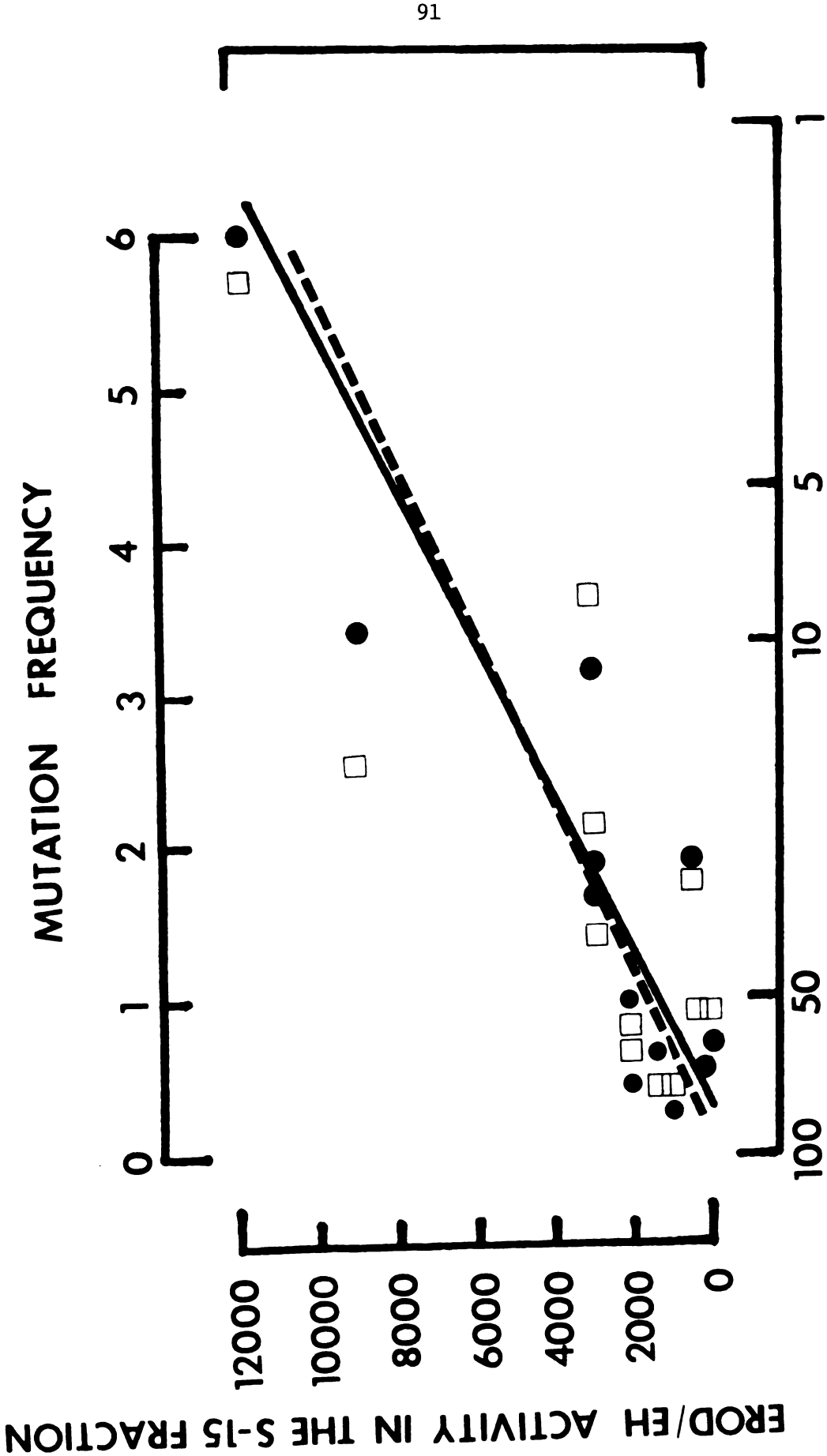

EROD/EH ACTIVITY IN THE S-15 FRACTION
12000
10000
8000
6000
4000
2000
0
MUTATION FREQUENCY
0
1
2
3
4
5
6
CLONING EFFICIENCY
100
50
10
5
1

Figure 19

5. Dimethylnitrosamine (DMN)

The cytotoxicity of DMN in the absence of the S-15 fraction is illustrated in Table 20. The cytotoxicity produced when 100 mM DMN is titrated with various concentrations of S-15 protein is depicted in Figure 20. It is apparent that the observed cytotoxicity is biphasic in the presence of all five S-15 fractions.

To determine if DMN-induced cytotoxicity and mutagenicity were related it is necessary to use a variety of treatments such that various numbers of cells survive each treatment and to measure the mutation frequency of each treatment in parallel with the cytotoxicity assessment. Toward that end, I chose to assay the cytotoxicity and mutagenicity of three different concentrations of DMN (10, 50, and 100 mM) in the presence of two different concentrations of S-15 protein (1.3 mg/ml and 3.5 mg/ml). This protocol was performed using S-15 fractions prepared from untreated rats as well as from rats pretreated with four different enzyme inducers (PB, Aroclor 1254, BNF, MC). After combining all of these data and plotting them on one graph (Figure 21), I conclude that the log of cloning efficiency is linearly related to mutation frequency ($p<0.01$) when DMN is metabolically activated by the S-15 fraction.

DMN is metabolically activated by (a) mixed function oxidase(s) termed DMN demethylase. I wished to determine: A) whether DMN demethylase activity was correlated with EROD activity (i.e., P-448), EMND activity (i.e., P-450), or the ratio of EROD to EMND activity and B) whether DMN demethylase activity could be inhibited by MET and/or ANF.

TABLE 20

The Cytotoxicity of Dimethylnitrosamine in the Absence of the S-15 Fraction

| Dimethylnitrosamine (mM) | Cloning Efficiency[a] |
|---|---|
| 0 | 100±2.9[b] |
| 10 | 96.8±6.3 |
| 50 | 101.4±2.4 |
| 100 | 87.3±3.1 |

[a]Expressed as a percentage of control.

[b]Mean ± S.E. of 12 replicates.

Figure 20. Cytotoxicity of 100 mM dimethylnitrosamine in the presence of the S-15 fraction. S-15 fractions were derived from animals pretreated with phenobarbital (○—○), Aroclor 1254 (□—□), β-naphthoflavone (●—●), 3-methylcholanthrene (■—■) or from animals which had not been pretreated (**). Cloning efficiency is expressed as a percentage of control. Values represent the mean of three replicates.

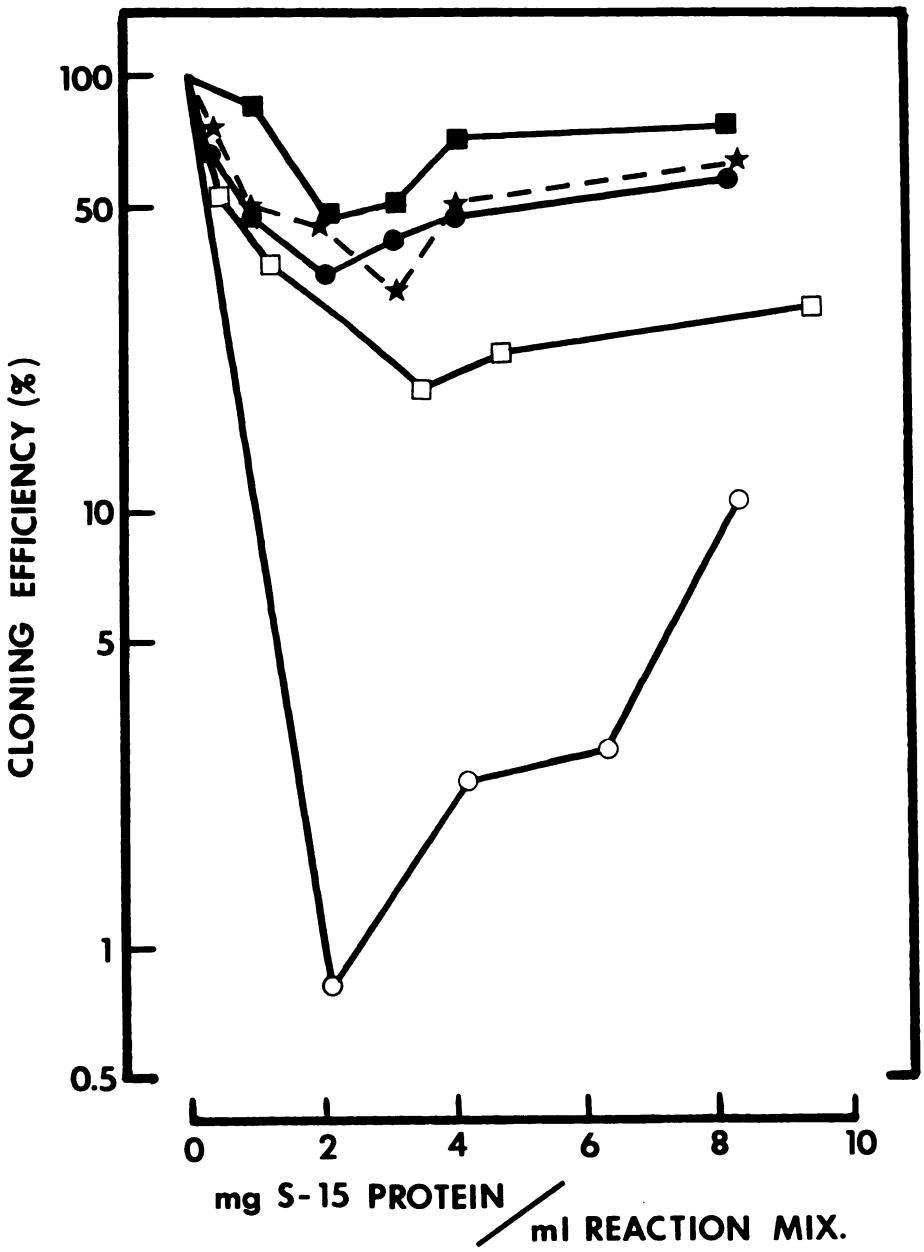

100
50
10
5
1
0.5
CLONING EFFICIENCY (%)
0
2
4
6
8
10
mg S-15 PROTEIN / ml REACTION MIX.

Figure 20

Figure 21. The relationship between cytotoxicity and mutagenicity when dimethylnitrosamine is incubated in the presence of the S-15 fraction. Cloning efficiency is expressed as a percentage of control. Values represent the mean of three replicates. Mutation frequency was determined in 1.0 mM ouabain (r = 0.96).

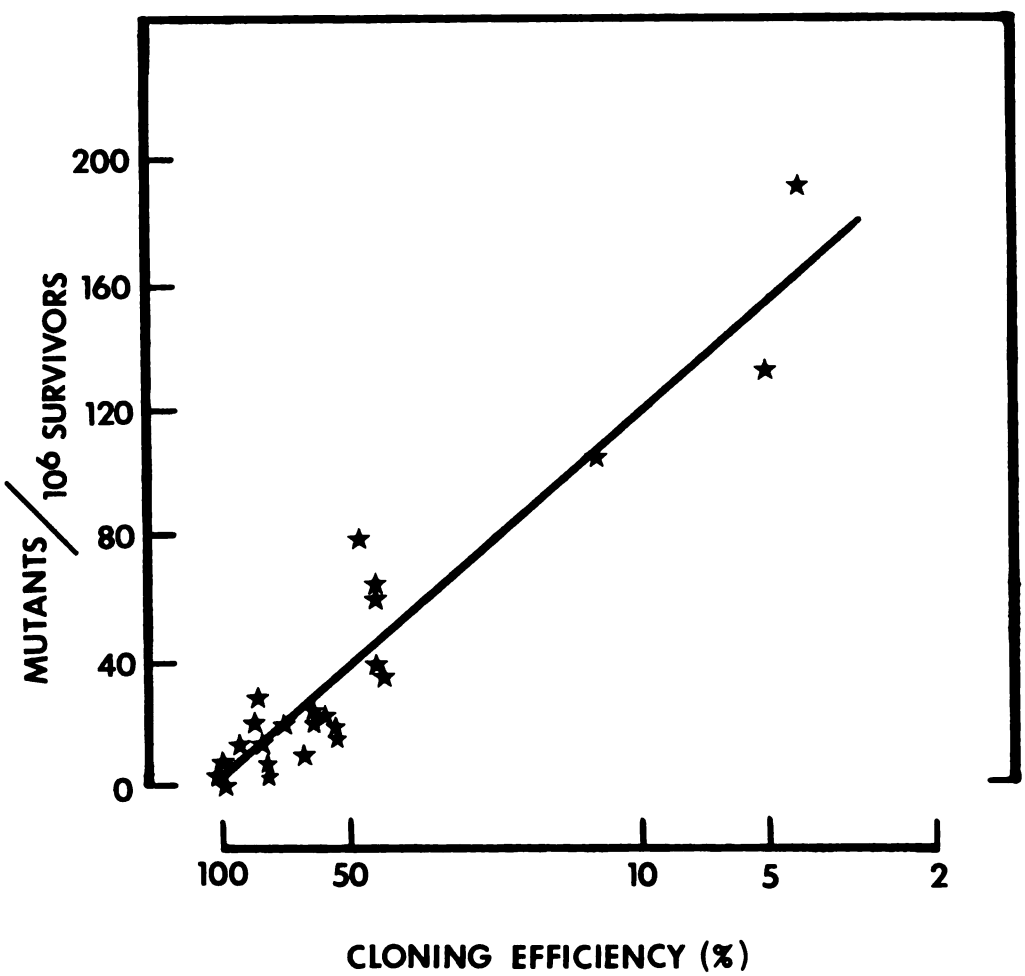

200
160
120
80
40
0
MUTANTS / $10^6$ SURVIVORS
100
50
10
5
2
CLONING EFFICIENCY (%)

Figure 21

The experimental approach for this series of experiments was identical with that previously described for studies on the metabolic activation of BP (see BP results section). Since the concentration of S-15 protein in the reaction mixture markedly influences the cytotoxic response to DMN (Figure 20), all samples in this series of experiments contained equal amounts of S-15 protein (2.4±0.1 mg/ml reaction mixture), while the DMN concentration was always 100 mM.

MET (Table 21) does not appear to affect DMN induced cytotoxicity or mutagenicity in a consistent manner in the presence of the S-15 fractions derived from PB or non-treated animals. However, when the S-15 fraction is derived from an Aroclor 1254 pretreated animal, MET decreases DMN induced cytotoxicity and mutagenicity in a dose-dependent fashion. In the presence of an Aroclor 1254 pretreated S-15 fraction ANF decreases DMN induced cytotoxicity, but only at $10^{-5}$M, the highest concentration tested (Table 22). Due to the extremely low cloning efficiency in the presence of $10^{-6}$M ANF (Table 22) the mutation frequency determination at this concentration of ANF is probably not very accurate.

In view of the fact that the activities of EROD and EMND vary substantially in S-15 fractions derived from animals pretreated in various manners (Tables 2 and 3) and that MET and ANF affect these enzymes in a rather complex fashion (Tables 5, 6, 7, 8) it is difficult to draw definitive conclusions from Tables 21 and 22. The analysis which is required involves calculating the activity of a given enzyme in a given S-15 preparation including, in most cases, an inhibitor and then plotting

TABLE 21

The Cytotoxicity and Mutagenicity of Dimethylnitrosamine in the Presence of Various Concentrations of Metyrapone

| Metyrapone Concentration | S-15 Source | | | | | |
|---|---|---|---|---|---|---|
| | Phenobarbital[a] | | Non-Induced[b] | | Aroclor 1254[c] | |
| | Cloning Efficiency[d] | Mutation Frequency[e] | Cloning Efficiency | Mutation Frequency | Cloning Efficiency | Mutation Frequency |
| 0 | 1.5±0.3[f] | 15[g] | 27.6±3.4 | 9[h] | 1.5±0.2 | 34[g] |
| $10^{-5}$M | 2.8±0.5* | 3[i] | 44.0±4.0* | 3[h] | 2.5±0.3 | 11[i] |
| $10^{-4}$M | 2.1±0.3 | 6[j] | 27.3±4.4 | 5[h] | 10.3±0.8** | 3[j] |
| $10^{-3}$M | 0.8±0.3* | 16[j] | 16.4±2.9* | 7[h] | 21.8±1.1** | 4[j] |

*Significantly different from each other ($p<0.05$). **Significantly different from all others in series ($p<0.05$).

[a]80 mg/kg, i.p., 96, 72, 48 and 24 hr prior to sacrifice. [b]Rats were not pretreated.

[c]500 mg/kg, i.p., 96 hr prior to sacrifice. [d]Expressed as a percentage of control (no BP).

[e]Expressed as mutants/$10^5$ survivors. [f]Mean ± S.E. of 3 replicates. [g]$36\times10^5$ cells assayed.

[h]$6\times10^5$ cells assayed. [i]$24\times10^5$ cells assayed. [j]$12\times10^5$ cells assayed.

TABLE 22

The Cytotoxicity and Mutagenicity of Dimethylnitrosamine in the Presence of Various Concentrations of α-Naphthoflavone[a]

| α-Naphthoflavone Concentration | Cloning Efficiency[b] | Mutation Frequency[c] |
|---|---|---|
| 0 | 4.0±1.3[d] | 4[e] |
| $10^{-6}$M | 1.7±0.6 | 17[e] |
| $10^{-5}$M | 24.2±1.3 | 4[e] |

[a] S-15 fraction derived from rats pretreated with Aroclor 1254, 500 mg/kg, i.p., 96 hr prior to sacrifice.

[b] Expressed as a percentage of control (no BP).

[c] Expressed as mutants/$10^5$ survivors.

[d] Mean ± S.E. of 3 replicates.

[e] $15x10^5$ cells assayed.

the calculated activity against the toxicity and mutagenicity of DMN in the presence of the S-15 fraction (see BP results section for detailed description of procedure). One can then look for correlations between the activity of a particular enzyme and the biological response to DMN. When such graphs are made (Figures 22, 23, 24) and statistically analyzed DMN induced toxicity or mutagenicity do not significantly correlate with EROD activity, EMND activity or the ratio of EROD to EMND activity in the S-15 fraction.

6. 2,4-Dinitrofluorobenzene (DNFB)

A. Cytotoxicity Studies

The data in Figure 25 illustrate that DNFB is cytotoxic to V79 cells in a dose-dependent fashion in the absence of the S-15 fraction. DNFB is more cytotoxic in protein free PBS compared to growth medium containing 5% (v/v) fetal calf serum. Presumably the serum protein (1.8 mg/ml medium) and amino acids in the growth medium produced a protective effect by covalently binding DNFB (Sanger, 1945), thus reducing the amount available to interact with the V79 target cells.

The addition of S-15 fraction derived from an untreated animal to the buffer had a pronounced protective effect on the cytotoxicity of DNFB (Figure 25). The protein concentration was 1.7 mg/ml reaction mixture, slightly less than the protein concentration of the growth medium, indicating that non-specific protein binding is not solely responsible for the detoxification of DNFB in the presence of the S-15 fraction.

Figure 22. The relationship between ethoxyresorufin-O-deethylase (EROD) activity in the S-15 fraction and the biological response of V79 cells to dimethylnitrosamine. EROD activity is expressed as pmoles resorufin/minute/mg S-15 protein. Cloning efficiency (●) is expressed as a percentage of control. Values represent the mean of three replicates. Mutation frequency (□) is expressed as mutants/$10^5$ viable cells and was determined in 1.0 mM ouabain.

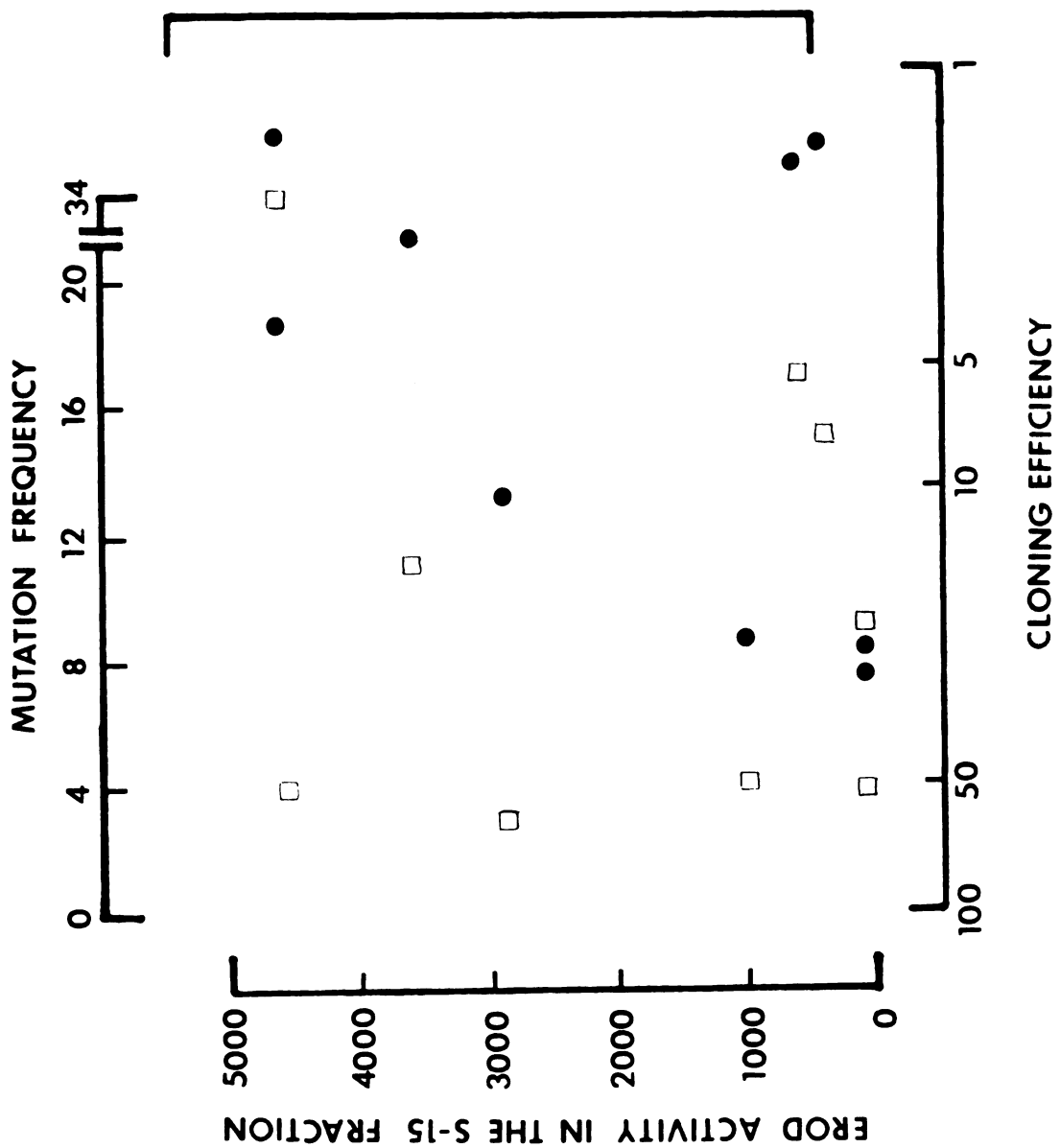

MUTATION FREQUENCY
0
4
8
12
16
20
34
EROD ACTIVITY IN THE S-15 FRACTION
5000
4000
3000
2000
1000
0
CLONING EFFICIENCY
100
50
10
5
1

Figure 22

Figure 23. The relationship between ethylmorphine-N-demethylase (EMND) activity in the S-15 fraction and the biological response of V79 cells to dimethylnitrosamine. EMND activity is expressed as nmoles formaldehyde/minute/mg S-15 protein. Cloning efficiency (●) is expressed as a percentage of control. Values represent the mean of three replicates. Mutation frequency (□) is expressed as mutants/$10^5$ viable cells and was determined in 1.0 mM ouabain.

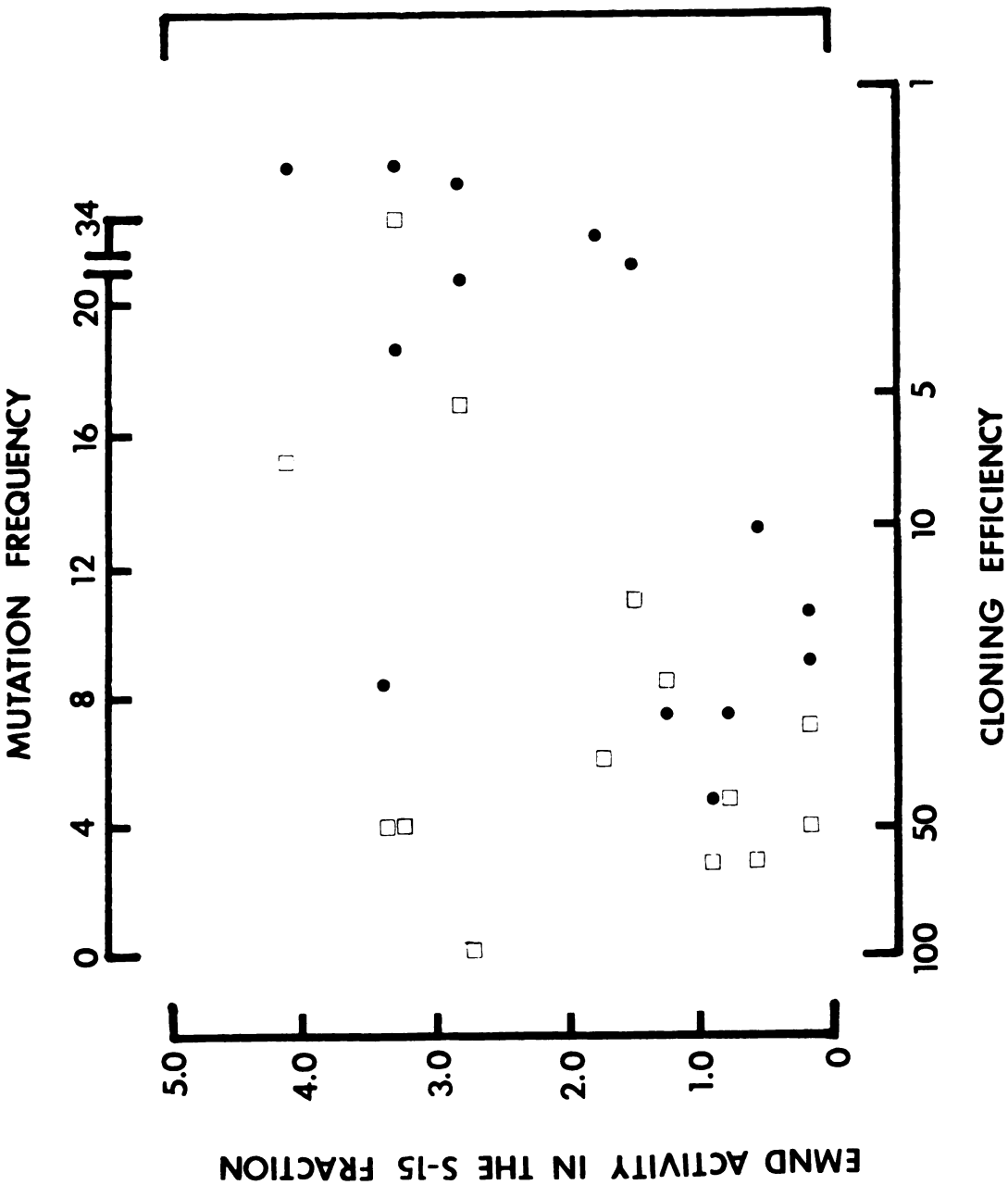

MUTATION FREQUENCY
0
4
8
12
16
20
34
CLONING EFFICIENCY
1
5
10
50
100
EMND ACTIVITY IN THE S-15 FRACTION
5.0
4.0
3.0
2.0
1.0
0

Figure 23

Figure 24. The relationship between the ratio of ethoxyresorufin-O-deethylase (EROD) to ethylmorphine-N-demethylase (EMND) activity in the S-15 fraction and the biological response of V79 cells to dimethylnitrosamine. EROD activity was expressed as pmoles resorufin/minute/mg S-15 protein; and EMND activity was expressed as nmoles formaldehyde/minute/mg S-15 protein. Cloning efficiency (●) is expressed as a percentage of control. Values represent the mean of three replicates. Mutation frequency (□) is expressed as mutants/$10^5$ viable cells and was determined in 1.0 mM ouabain.

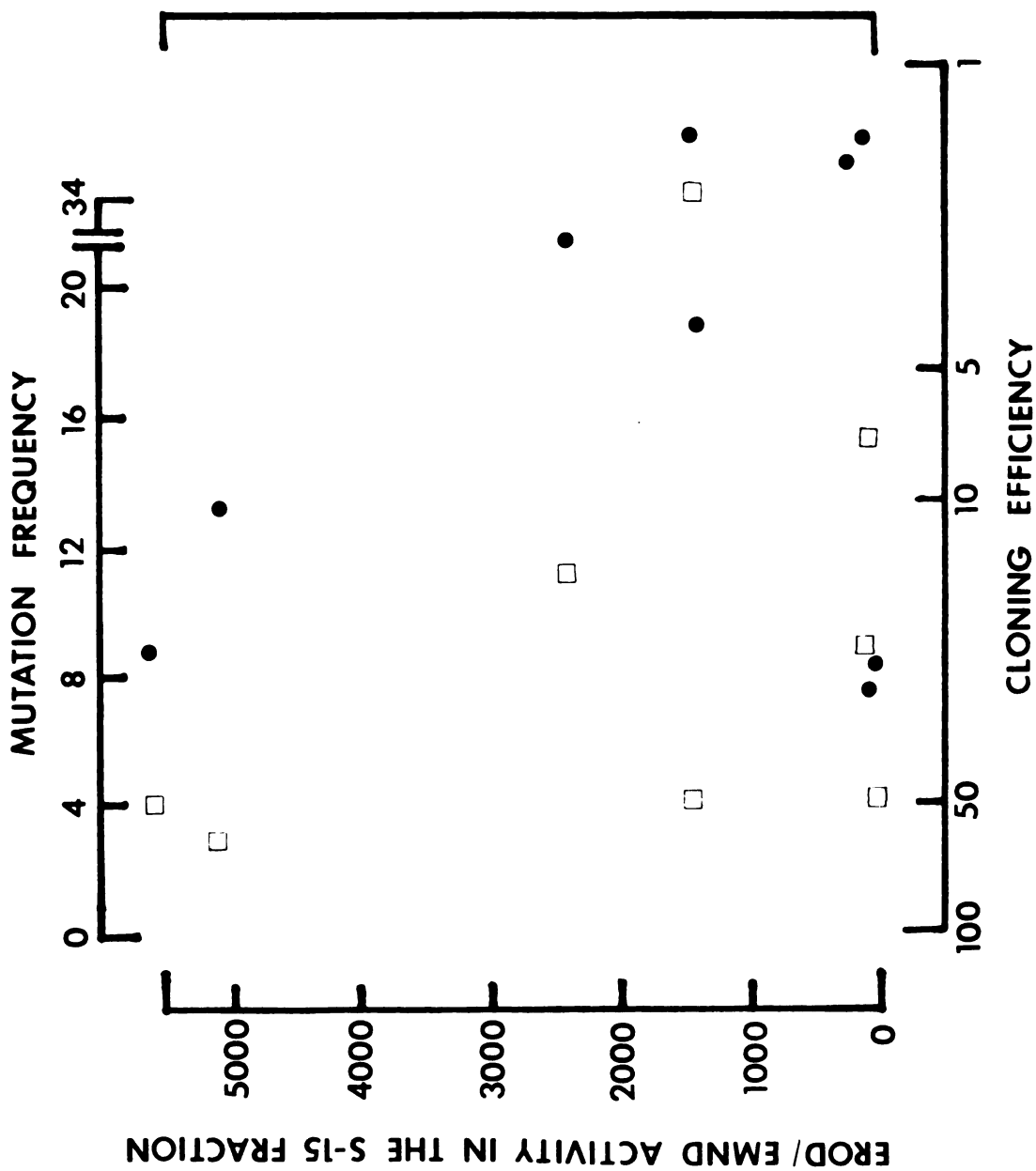
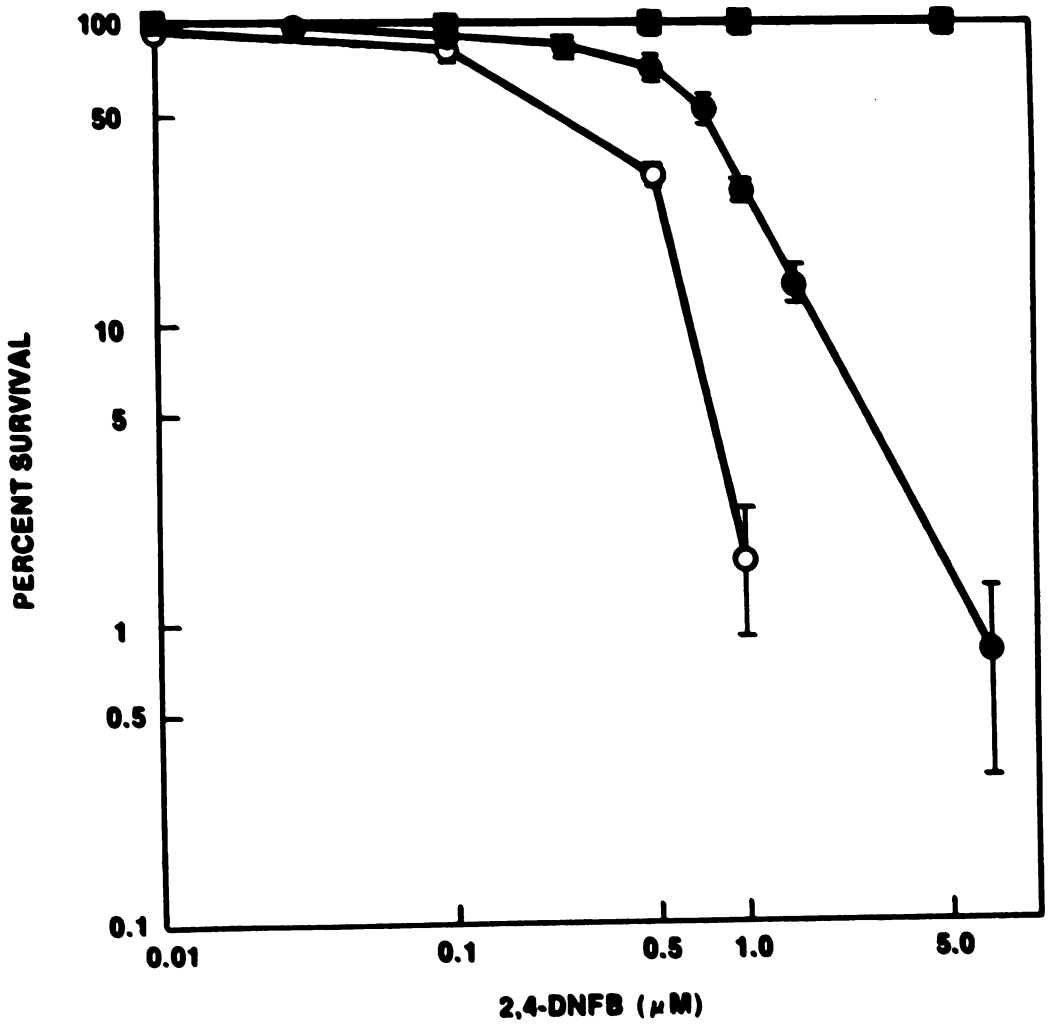

EROD/ EMND ACTIVITY IN THE S-15 FRACTION
0
1000
2000
3000
4000
5000
MUTATION FREQUENCY
0
4
8
12
16
20
34
CLONING EFFICIENCY
1
5
10
50
100

Figure 24

Figure 25. The cytotoxicity of 2,4-dinitrofluorobenzene (2,4-DNFB). (●), treatment in complete medium; (○), treatment in phosphate-buffered saline; (■), treatment in phosphate-buffered saline in the presence of the S-15 fraction from a non-induced animals. Each point represents the mean ± S.E. of three replicates.


100
50
10
5
1
0.5
0.1
PERCENT SURVIVAL
0.01
0.1
0.5
1.0
5.0
2,4-DNFB (μM)

To determine whether or not the enzymes responsible for the detoxification could be induced I pretreated animals with PB or MC and compared the cytotoxicity of DNFB in the resulting S-15 fractions with that derived from untreated animals. As illustrated in Table 23 these pretreatments did not appear to increase the ability of the S-15 fraction to detoxify DNFB, suggesting that the enzymes which are induced by these pretreatments are either not involved in the metabolic detoxification of DNFB or are not rate limiting in this instance. I was unable to test DNFB concentrations greater than 500 μM due to problems in the solubility of the compound.

B. Mutagenicity Studies

The experiments designed to determine whether DNFB is a direct acting mutagen in V79 cells were conducted in growth medium. The results are shown in Table 24. At doses resulting in 11.5 and 35.5 percent survival DNFB did not significantly change the mutation frequency at the ouabain locus, whereas UV irradiation used as a positive control did elevate the mutation frequency.

In order to determine whether DNFB requires metabolic activation in order to become mutagenic experiments were performed utilizing MC induced, PB induced and non-induced S-15 fractions. The results indicate that DNFB was not metabolically activated to a mutagen by these S-15 fractions (Figure 26). Note that the positive control BP in the presence of S-15 prepared from MC pretreated rats did increase the mutation frequency in a dose-dependent fashion.

TABLE 23

Effect of Pretreatment with Enzyme Modulators on the Detoxification of 2,4-DNFB by the S-15 Fraction

| Enzyme Source[a] | Cloning Efficiency[b] |
|---|---|
| Untreated[c] | |
| 50.0 μM[d] | 101.6±12.6[e] |
| 500.0 μM | 63.6± 4.1 |
| 3-Methylcholanthrene[f] | |
| 50.0 μM | 97.1±10.4 |
| 500.0 μM | 68.1± 1.0 |
| Phenobarbital[g] | |
| 50.0 μM | 101.3± 9.1 |
| 500.0 μM | 56.9± 5.8 |

[a] S-15 fraction prepared as described in Materials and Methods.

[b] Expressed as a percentage of control (no 2,4-DNFB).

[c] Animals were not pretreated.

[d] Concentration of 2,4-DNFB in the reaction mixture.

[e] Mean ± S.E. of 3 replicates.

[f] 80 mg/kg, i.p., 24 hr prior to sacrifice.

[g] 80 mg/kg, i.p., 96, 72, 48 and 24 hr prior to sacrifice.

TABLE 24

Mutagenicity of 2,4-DNFB in the Absence of the S-15 Fraction

| Treatment | Cloning Efficiency[a] | Mutation Frequency[b] |
|---|---|---|
| Control | 100.0[c] | 0.9 (1)[d] |
| 1.0 μM 2,4-DNFB | 35.5 | 1.4 (1) |
| 2.5 μM 2,4-DNFB | 11.5 | 2.3 (1) |
| UV light ($18J/M^2$) | 9.0 | 184.3 (68) |

[a]Expressed as % of control (no 2,4-DNFB).

[b]Mutants per $10^6$ viable cells.

[c]Mean of three replicates.

[d]Number of mutants recovered.

Figure 26. The mutagenicity of 2,4-dinitrofluorobenzene (2,4-DNFB) and benzo(a)pyrene (B(a)P). (●), 2,4-DNFB with 3-methylcholanthrene induced S-15; (■), 2,4-DNFB with phenobarbital induced S-15; (○), 2,4-DNFB with non-induced S-15; (□), benzo(a)pyrene with 3-methylcholanthrene induced S-15 as a positive control. Each point represents the mean ± S.E. of three replicates.

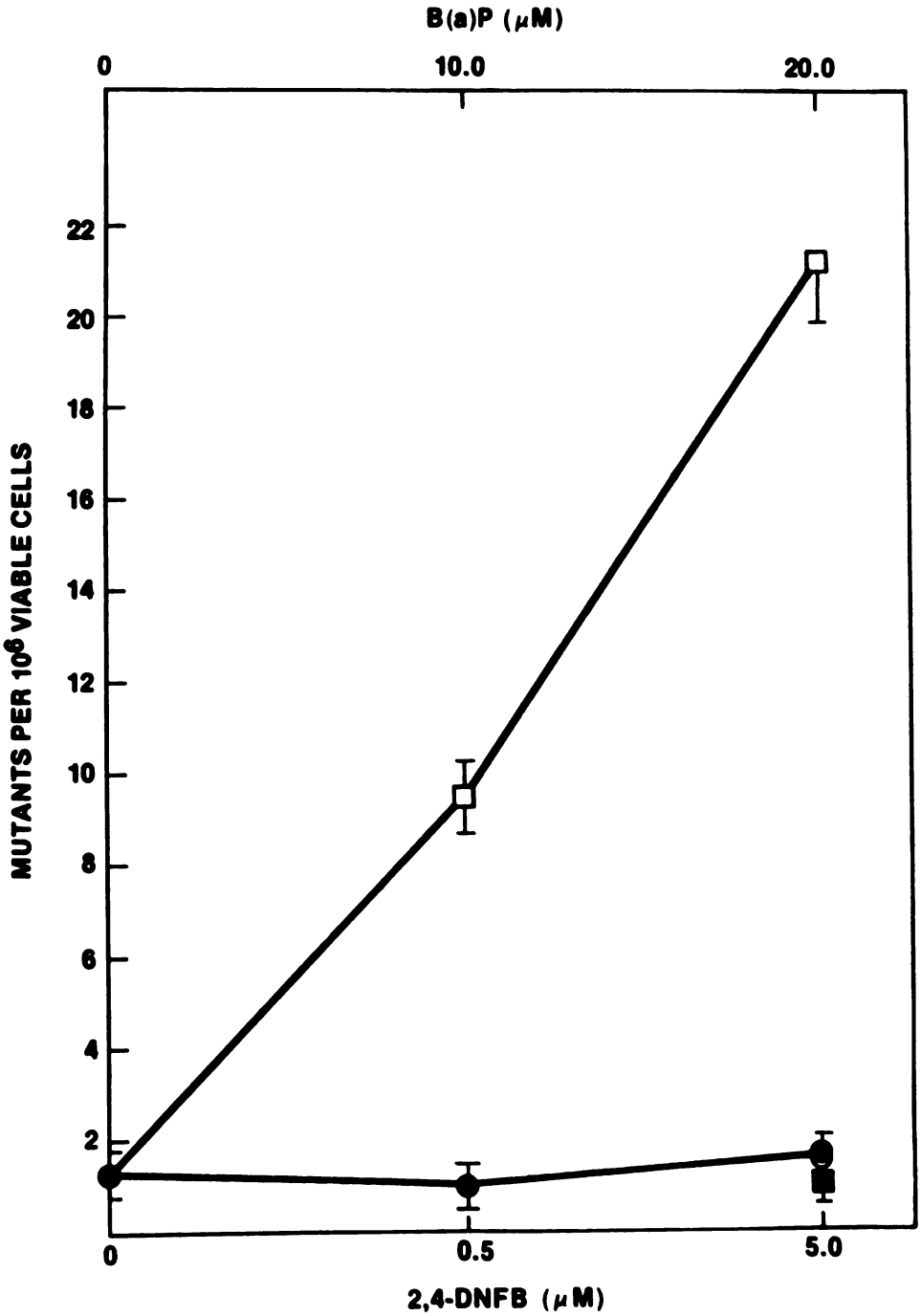

B(a)P (μM)
0
10.0
20.0
22
20
18
16
14
12
10
8
6
4
2
MUTANTS PER 10^6 VIABLE CELLS
0
0.5
5.0
2,4-DNFB (μM)

Figure 26

# DISCUSSION

## 1. Preliminary Characterization of the V79/Ouabain Resistance Assay

In order to develop this assay system it was necessary to verify that the V79 cells in my possession were capable of detecting cytotoxic agents in a sensitive, dose-dependent fashion. MNNG is a direct acting alkylating agent which induces dose-dependent cytotoxicity in my V79 cells at μM concentrations (Figure 11).

The inclusion of the hepatic S-15 fraction allows a quantitation of cytotoxicity and mutation frequency for many of those compounds which become active only after being metabolized to reactive species. The S-15 fraction is always prepared just prior to use because mixed function oxidase activity may deteriorate when subcellular fractions are frozen (-80°C) and stored (Yoshikawa et al., 1980; Dent et al., 1981). At the time the S-15 fraction is being added to the reaction mixture an aliquot is taken for the determination of AHH activity (Table 1) and protein content. AHH activity is assessed to be certain that the animals have been pretreated properly and that the microsomal mixed function oxidase activity has not deteriorated during preparation of the S-15 fraction.

I have chosen to assess the cytotoxic and mutagenic action of the test compounds following a two hour incubation period. It is probable

that at earlier time points (e.g., 5 minutes) the results would be different from those obtained after a two hour incubation due to pharmacokinetic differences between various enzymes. Since the vast majority of xenobiotics must be metabolized in vivo prior to excretion it is appropriate to allow all of the xenobiotic introduced into the test system to be metabolized prior to stopping the reaction. When employing a two hour incubation it appears as if all of the xenobiotic is metabolized because if one increases the concentration of test compound a greater degree of cytotoxicity and mutagenicity is observed.

Exposure of cells to xenobiotics is conducted in PBS to eliminate interaction of the test compound or its metabolites with constituents of the growth medium (e.g. serum proteins). Damage to DNA results in an inhibition of replication. The degree of inhibition is usually positively correlated with the amount of damage initially produced (Painter, 1978). In the assessment of mutagenicity at the $Na^+,K^+$-ATPase locus by measuring resistance to ouabain, it is desirable to add the ouabain-containing medium to the cells after allowing them to undergo an equal number of DNA replications regardless of treatment toxicity. This is important because the cells must replicate their DNA in order to convert a DNA adduct into a mutation. To accomplish this I monitor the cells with an inverted microscope and add ouabain-containing medium after the cells have divided four times (16 cell stage). The spontaneous mutation frequency is $<0.05$ ouabain-resistant mutants per $10^5$ viable cells ($29.3 \times 10^5$ viable cells assayed, zero mutants recovered).

I have investigated the ability of hepatic S-15 fractions to generate mutagenic and/or cytotoxic metabolites from BP, DMN and DNFB

in vitro. In an attempt to identify enzyme patterns which are optimal in this regard the enzyme pattern contained in the S-15 fraction is modulated by pretreating the animals with enzyme inducers. Some of the chemicals employed to influence hepatic enzyme activity such as PB (Peraino et al., 1971), MC (Miller et al., 1958) and PCBs (Makiura et al., 1974) have a protective effect on chemically-induced hepatocarcinogenesis. There are several probable reasons for this: A) fewer reactive metabolites may be produced from a precarcinogen due to qualitative changes in the manner in which it is metabolized (Miller et al., 1958). B) the reactive metabolites may be conjugated and excreted at a faster rate (Degen and Neumann, 1981; Chipman et al., 1981). C) the reactive metabolites may be detoxified at a faster rate (Leonard et al., 1981). The protective effect might not be reflected in the results obtained from in vitro mutagenicity assays using hepatic subcellular fractions to activate chemical carcinogens (Stout and Becker, 1979). It must be recognized that we are testing for the potential to metabolize a putative mutagen/carcinogen to a reactive species which may or may not be identical with the net effect in an intact organ. That is, in an in vitro assay we are dealing with only portions of the pharmacodynamic picture present in an intact, functioning organ, and this fact must be kept in mind when interpreting results from in vitro assays. However, by characterizing the metabolic parameters which are operational in in vitro assays and understanding how they may influence the results obtained it should be possible to conduct meaningful mechanistic studies on the potential for metabolic toxification/detoxification of xenobiotics. These studies represent a step in this direction.

2. Benzo(a)pyrene (BP)

Concentrations of BP which are non-toxic in the absence of metabolism have been defined (Table 13). A non-toxic concentration of BP (20 μM) was selected, various concentrations of S-15 protein were added, and the production of toxic metabolites was assessed by measuring cytotoxicity in V79 cells (Figure 12). The shape of the toxicity curve is dependent upon the source of the S-15 fraction. When the S-15 fraction is prepared from control animals I do not observe toxicity in the V79 cells. This could be due either to the inability of this preparation to generate toxic metabolites from BP, or alternatively it could be that this S-15 fraction has the ability to detoxify those toxic metabolites which are generated prior to their interaction with the V79 target cells. When the S-15 fraction is prepared from animals which have been pretreated with MC, a biphasic toxicity curve is produced. At low concetrations of S-15 protein (<0.8 mg S-15 protein/ml reaction mixture) an increase in S-15 protein concentration produces an increase in cytotoxicity, whereas at higher concentrations of S-15 protein (>0.8 mg S-15 protein/ml reaction mixture) an increase in S-15 protein concentration results in a decrease in observed cytotoxicity. There are two probable explanations for this biphasic effect: 1) the excess protein may be acting as a nucleophilic trap for the activated metabolites of BP, preventing them from interacting with V79 cells (Malaveille et al., 1979; Guenthner et al., 1979; Bartsch et al., 1980), or 2) the pattern of enzymatic activity present in the reaction mixture may shift from a pattern favoring the production of toxic metabolites from BP to a pattern of enzyme activity which is less favorable for the production

of toxic metabolites as the S-15 protein concentration is raised (Kuroki et al., 1979; Machanoff et al., 1981).

In an attempt to differentiate between these two alternatives I have taken a fixed concentration of active S-15 protein and added to it various concentrations of inactive S-15 protein (Figure 12). The S-15 fraction was inactivated by heating to 100°C for 10 minutes. The results indicate that protein binding is an important detoxification mechanism, but at high concentrations of postmitochondrial supernatant it appears as though a shift in the pattern of metabolites produced occurs. This conclusion is based on the observation that the active S-15 does not produce detectable toxicity when present in high concentrations, whereas the inactivated S-15 is unable to completely abolish the cytotoxic response to activated BP. To further investigate the interrelationship of S-15 protein concentration and cytotoxicity, S-15 fractions have been prepared from rats which have been pretreated with compounds known to modulate hepatic enzyme activity. The ability of such preparations to influence BP-induced cytotoxicity at several protein concentrations was assessed. I prepared the S-15 fraction from animals pretreated with a P-450 type inducer (PB) (Conney, 1967), a P-448 type inducer other than MC (BNF) (Boobis et al., 1977) and a mixed type inducer (Aroclor 1254) (Alvares et al., 1973; Parkinson et al., 1980). In all three instances I observed BP concentration dependent cytotoxicity which was markedly influenced by the concentration of S-15 protein employed (Table 14). In accordance with previous results (Kuroki et al., 1979; Malaveille et al., 1979) I have observed that the mutagenicity of BP is dependent upon the concentration of

postmitochondrial supernatant protein when the homogenate is derived from an animal which has been pretreated with MC (Table 15).

The degree of mutagenicity and cytotoxicity generated from BP by metabolism depends on the relative levels of individual BP metabolites. This is due to the fact that the degree of cytotoxicity and mutagenicity induced in V79 cells by individual BP metabolites varies substantially (Huberman et al., 1976). The levels of the individual metabolites which are generated from BP depends on the pattern of enzymatic activity present in the metabolic activation system. Therefore, I wished to investigate the role of specific enzymes and enzyme ratios in regard to the metabolic activation of BP to cytotoxic and mutagenic entities.

High inducibility of AHH in humans has been associated with the development of lung cancer (Kellermann et al., 1973; Emery et al., 1978), which is believed to be initiated primarily by polycyclic aromatic hydrocarbons present in cigarette smoke. AHH activity in the postmitochondrial supernatant prepared from rat liver has been reported to correlate with the ability of this fraction to metabolically activate BP to compounds which bind to calf thymus DNA in vitro (Raineri et al., 1981). However, AHH activity did not correlate with the amount of BP bound to DNA in an intact cell system (Gozukara et al., 1981). In order to study the effect of AHH induction on the metabolism of BP, I have induced hepatic AHH activity to equivalent levels (approximately 300% of control, Table 1) using two different inducing agents (MC and BNF) and assessed the effect of this induction on cytotoxicity and mutation frequency in the presence of BP (Figure 13). Pretreatment

with MC or BNF leads to the appearance of a new hepatic microsomal protein band (presumably P-448) at the same molecular weight (55,000) in AHH responsive mice (Wang, 1981). I found that at any given level of cytotoxicity these two pretreatments produced differing mutation frequencies. These results indicate that the cytotoxic metabolites of BP may not be identical with the mutagenic metabolites. This conclusion is supported by the observation that when hamster or rat embryo cells were used as feeder cells to metabolically activate BP to compounds which are mutagenic and/or cytotoxic to V79 Chinese hamster cells, these two biological responses were not a constant ratio when the two cell types were compared (Baird et al., 1981). At equal toxicity levels hamster embryo cells produced 3 times as many mutations in V79 cells as did rat embryo cells.

The comparison between MC and BNF (Figure 13) also indicates that AHH activity is not the sole determinant for the generation of mutagenic metabolites from BP, confirming previous results using hepatic subcellular fractions from mice (Hutton et al., 1979a), hamsters (Hutton et al., 1979b), and guinea pigs (Baker et al., 1980). These data may be explained by the observation (Gurtoo et al., 1980) that AHH activity as measured by the standard fluorometric assay (Nebert and Gelboin, 1968) does not correlate with the production of benzo(a)pyrene-7,8-dihydrodiol, the proposed proximate mutagenic form of BP (Huberman et al., 1976).

I have investigated the effect of AHH activity on the generation of toxic metabolites from BP by comparing the ability of the S-15

fractions prepared from control and MC pretreated animals to metabolize BP to toxic metabolites (Figure 12). Since the AHH activity/mg S-15 protein in MC pretreated S-15 is approximately 3 times that of control S-15 (Table 1) one may make the AHH activity/ml reaction mixture equal for these two preparations by incorporating 3 times as much non-treated S-15 protein into the reaction mixture as compared to MC S-15 protein. The extra protein in the non-treated S-15 preparation would be expected to act as a nucleophilic trap for the activated, electrophilic BP metabolites responsible for cytotoxicity (Figure 12). Nevertheless, S-15 preparations derived from animals pretreated with MC, BNF, Aroclor 1254 or PB induce a significant degree of cytotoxicity at this concentration of S-15 protein (Figure 12, Table 14). However, the S-15 fraction prepared from control animals is still ineffective in metabolizing BP to compounds cytotoxic to V79 cells, indicating that the AHH activity contained in the reaction mixture is not the sole determinant for the induction of V79 cytotoxicity in the presence of BP. There are two probable reasons for this: A) the AHH assay does not detect the metabolism of BP to non-phenolic products (Yang et al., 1975) such as diolepoxides, which are capable of killing V79 cells (Huberman et al., 1976). B) AHH activity in rat hepatic microsomes appears to be composed of two or more enzymes (Wiebel et al., 1971). The relative amounts of these enzymes in hepatic subcellular fractions vary depending on the type of inducer used (Wiebel and Gelboin, 1975). Therefore, even though "AHH activity" is equalized in two subcellular fractions, the enzymes which comprise "AHH" are probably not identical in the two preparations.

To examine the role of other enzymes in the metabolic activation of BP I chose to employ ANF, an inhibitor of cytochrome P-448, MET, an inhibitor of cytochrome P-450, and CCHO, an inhibitor of epoxide hydrase. The rationale for this approach was that, by inhibiting specific enzymes in the S-15 fraction to various degrees and concurrently measuring the ability of these fractions to metabolically activate BP, it might be possible to elucidate the relative importance of specific enzymes and/or enzyme patterns.

ANF is a synthetic flavonoid which acts as an _in vitro_ inhibitor of cytochrome P-448 (Wiebel and Gelboin, 1975). When employed _in vitro_ ANF inhibits primary (Selkirk _et al._, 1974) as well as secondary (Capdevila _et al._, 1975; King _et al._, 1976) oxidations of BP by microsomal enzymes. In the presence of MC induced mouse liver microsomes and DNA ANF decreases the concentration of all DNA-BP adducts with the exception of the adduct formed from BP 4,5-oxide (Boobis _et al._, 1979). This indicates that the metabolism of BP to the 4,5-oxide is mediated by an ANF insensitive form of cytochrome P-450.

The data in Table 5 illustrate that ANF inhibits EROD activity in a concentration dependent manner in S-15 fractions derived from animals pretreated with MC or Aroclor 1254, but does not significantly affect EROD activity in non-induced S-15 fractions. This result confirms and extends the observation that MC inducible AHH activity is inhibited by ANF, whereas AHH activity in a control animal is not inhibited by ANF (Wiebel _et al._, 1971). A possible reason for this specificity is that ANF may require metabolic activation (Nesnow and Bergman, 1981), probably at the number six position, before it is capable of inhibiting

AHH activity. This possibility is supported by the observation that hepatic microsomes from MC pretreated Sprague-Dawley rats metabolize ANF to the 5,6-epoxide, which inhibits AHH activity (Coombs et al., 1981). Uninduced microsomes may be unable to perform this oxidation. Further support for this hypothesis comes from the observation that, in order for ANF to have an inhibitory effect on MC induced microsomal drug oxidations, the number six position on the ANF molecule must be either unsubstituted or be substituted with an oxidizable moiety (Nesnow, 1979).

ANF inhibits BP-induced cytotoxicity and mutagenicity in the presence of a MC pretreated S-15 fraction, but does not alter BP-induced cytotoxicity or mutagenicity in the presence of the Aroclor 1254 pretreated S-15 fraction (Table 17). This result is possibly due to the fact that EROD activity in an Aroclor 1254 pretreated animal is substantially higher than in a MC pretreated animal (Table 2). Therefore, the EROD activity in the Aroclor preparation, even though significantly inhibited by ANF, may not have become rate limiting. Another possibility for the differential effect of ANF on BP activation is the fact that EH activity in an Aroclor 1254 pretreated animal is substantially higher than in a MC pretreated animal (Table 4).

MET is an inhibitor of cytochrome P-450 which has been reported to have little effect on the BP metabolite-nucleoside profile produced by MC induced mouse liver microsomes (Boobis et al., 1979). MET inhibits EMND activity (Table 7) and EROD activity (Table 6) in a concentration dependent manner and, in accordance with previous results

(Vaz et al., 1981), stimulates EH activity (Table 10). MET has no effect on the cytotoxicity and mutagenicity of BP when present at low concentrations, but at the highest concentration used ($10^{-3}$M) MET increased the toxicity and mutagenicity of BP (Table 19).

EH is a ubiquitous cellular enzyme found on nuclear as well as microsomal membranes, in mitochondria and in the cytosol (Gill and Hammock, 1981). I chose to examine the role of EH in the metabolic activation of BP because the majority of BP metabolites either are arene oxides or have arene oxides as intermediates. Selective inhibition of EH by TCPO increases the mutagenicity of BP (Oesch, 1976; Rasmussen and Wang, 1974). However, these results must be interpreted with caution because TCPO itself is a direct acting mutagen (Guest and Dent, 1980). Strong evidence for a role of EH in the metabolic toxification/detoxification of BP comes from the observation that the addition of purified EH decreases the mutagenicity of BP in the Ames assay (Oesch, 1976) and reduces the binding of $^{3}$H(-)t-7,8-diol metabolites to DNA in either purified reconstituted mixed function oxidase systems or intact cells (Gozukara et al., 1981). CCHO is an inhibitor of EH and has been reported to inhibit the formation of BP dihydrodiols in the presence of rat liver microsomes (Fahl et al., 1977). CCHO markedly potentiates the observed cytotoxicity and mutagenicity of BP in the presence of either a MC or Aroclor 1254 pretreated S-15 fraction (Table 18). This result provides circumstantial evidence that the toxic and mutagenic metabolites of BP are epoxides.

In view of the fact that the activities of EROD, EH and EMND in the S-15 fraction vary substantially depending on the type of inducer used (Tables 2, 3, 4) and that the activities of these enzymes are altered in a rather complex fashion by inhibitors (Tables 5, 6, 7, 8, 9, 10), it is difficult to draw definitive conclusions about the metabolic activation of BP from the results of experiments in which a single S-15 fraction is combined with various concentrations of a single inhibitor. Metabolism of BP involves several enzymes acting both sequentially and in concert. The interrelationships between toxification and detoxification pathways is crucial in determining the levels of reactive intermediates available to interact with cellular macromolecules. Therefore, in the study of BP metabolism patterns of enzyme activity should be considered in addition to studies on single enzymes.

The required analysis involves pooling the data from multiple experiments using several different S-15 sources and several different inhibitors. One may then look for correlations between a particular enzyme or enzyme ratio in the S-15 fraction and the ability of the fraction to metabolically activate BP. This type of analysis indicates that EH is the only single enzyme to correlate significantly ($p<0.01$) with BP induced cytotoxicity and mutagenicity (Figure 17). As the EH activity in the S-15 fraction increases the degree of cytotoxicity and mutagenicity which results when BP is metabolically activated by the S-15 fraction decreases. BPDE I is the most toxic and mutagenic metabolite of BP but BPE is also relatively cytotoxic and mutagenic (Huberman et al., 1976). Although the BPDEs are relatively poor substrates for EH (Wood et al., 1976b), they are detoxified to BP

7,8,9,10-tetrols by EH at some low rate. This is supported by the observation that in a purified, reconstituted mixed function oxidase system the addition of purified EH reduces the binding of $^{3}H(-)t$-7,8-diol metabolites to DNA (Gozukara et al., 1981). Presumably, as EH activity increases the rate of BPDE detoxification also increases, resulting in less V79 toxicity and mutagenicity. In addition, BPE may be detoxified by EH (Woood et al., 1976a), presumably to the 4,5-diol. Therefore, although the relative roles of the BPDEs and BPE in inducing cytotoxicity and mutagenicity in this assay has not been determined, both of these reactive intermediates would be expected to be detoxified by EH.

When I look at enzyme ratios I find two significant correlations ($p<0.01$), EMND/EH (Figure 18) and EROD/EH (Figure 19). This suggests that an important determinant of BP induced toxicity and mutagenicity is the rate of epoxide formation divided by the rate of epoxide destruction. As the rate of epoxide formation increases and/or the rate of epoxide destruction decreases BP becomes more cytotoxic and more mutagenic. These data support the role of epoxides in mediating the cytotoxic and mutagenic actions of BP.

EMND/EH does not correlate as strongly with BP induced cytotoxicity and mutagenicity as does EROD/EH Since EMND activity measures a PB inducible form of P-450 (Table 3) and EROD activity measures a MC inducible form of P-450 (Table 2) this result confirms and extends previous findings (Wood et al., 1976a) that MC inducible mixed function oxidases are better able to activate BP to a mutagen than are PB inducible mixed function oxidases.

3. Dimethylnitrosamine (DMN)

The cytotoxicity curve which results when 100 mM DMN is titrated with various amounts of S-15 protein has a biphasic shape, regardless of whether the S-15 fraction is derived from an untreated rat or from one which has been pretreated with MC, BNF, Aroclor 1254 or PB (Figure 20). Maximal toxicity is seen between 2.1 and 3.5 mg S-15 protein/ml reaction mixture, depending on the pretreatment used. The S-15 fraction which has the highest EMND activity (PB, Table 3) produces the greatest degree of cytotoxicity from DMN at all concentrations of S-15 protein. When the concentration of S-15 protein is 3.5 mg/ml or greater, it is apparent that an increase in the amount of S-15 protein produces a decrease in observed cytotoxicity. In view of the currently accepted hypothesis that the metabolic activation of DMN to a cytotoxic entity is due to a single mixed function oxidase catalyzed reaction, one would anticipate that the greater the amount of mixed function oxidase (i.e. S-15 fraction) in the reaction mixture, the greater the rate of DMN activation and the greater the amount of DMN induced cytotoxicity. Since DMN is less cytotoxic in the presence of large amounts of S-15 protein when compared to intermediate amounts, the excess protein is presumably acting as a nucleophilic trap for the DMN metabolite responsible for cytotoxicity.

The data depicted in Figure 21 illustrate that cloning efficiency is linearly related to mutation frequency when DMN is metabolically activated by the S-15 fraction. This indicates either A) the cytotoxic metabolite of DMN is identical with the mutagenic metabolite or B) the cytotoxic and mutagenic metabolites of DMN are not identical, but are

produced from DMN by microsomal enzymes in a constant ratio. A high pressure liquid chromatography analysis of the metabolite profile in the reaction mixture would be useful in distinguishing between these two possibilities.

The metabolic activation of DMN is currently believed to be catalyzed by mixed function oxidase(s) termed DMN demethylase (Czygan et al., 1973). It is unclear whether DMN demethylase is a P-450 type enzyme, a P-448 type enzyme or is comprised of several mixed function oxidases, including both P-450 and P-448. Therefore, I wished to determine if DMN demethylase activity could be correlated with EROD activity, a reaction catalyzed by P-448, with EMND activity, a reaction catalyzed by P-450, or with the ratio of EROD activity to EMND activity, which reflects the P-448/P-450 ratio. This study was facilitated through the use of the in vitro enzyme inhibitors MET and ANF. ANF inhibits primarily P-448 (Tables 5, 8), whereas MET inhibits primarily P-450 but also has a significant inhibitory effect on P-448 (Tables 6, 7). The results of this study (Figures 22, 23, 24) indicate that the cytotoxicity and mutagenicity of DMN neither correlate with EROD activity or with EMND activity nor with the ratio of EROD to EMND activity. There are several probable explanations for these results: A) The mixed function oxidases whose activities are measured by the EROD and EMND assays and/or inhibited by ANF and MET are not the enzymes which metabolically activate DMN to toxic and mutagenic forms. For example, pretreatment of rats with isopropanol, acetone or ethanol increases the hepatic microsomal demethylation of DMN, but

does not significantly increase EMND activity (Maling et al., 1975). B) The mixed function oxidases which are inhibited by MET or ANF are responsible for the metabolic activation of DMN, but these enzymes, even when substantially inhibited by MET or ANF, are present in such high concentrations that they do not become rate limiting. C) The metabolic activation of DMN may be catalyzed by any one of several mixed function oxidases. When one or more of these enzymes are inhibited by MET or ANF, other forms of cytochrome P-450 are able to compensate with little or no change in the rate of DMN activation.

4. 2,4-Dinitrofluorobenzene (DNFB)

DNFB has been shown to be a potent and presumably direct acting mutagen in several species of bacteria (Hope, 1979; Summer and Goggelmann, 1980; Jagannath et al., 1980; Warren et al., 1981). However, in vivo studies performed on mouse skin have indicated that DNFB is not a complete carcinogen, but rather acts as a tumor promoter (Bock et al., 1969). To investigate this apparent discrepancy I chose to determine if DNFB is mutagenic in V79 cells.

The results indicate that DNFB is extremely cytotoxic (Figure 25), but not detectably mutagenic (Table 24) in V79 Chinese hamster cells in the absence of the S-15 fraction. The addition of the S-15 fraction markedly reduces the cytotoxicity produced by DNFB (Figure 25). DNFB is not metabolically activated to a mutagen by the S-15 fraction as assessed by measuring ouabain resistance (Figure 26).

In additional studies employing V79 Chinese hamster cells, DNFB either in the presence or absence of the S-15 fraction was not mutagenic at the HGPRT locus as assessed by resistance to 6-thioguanine, did not cause unscheduled DNA synthesis, a measure of DNA excision repair, nor did it increase the frequency of sister chromatid exchanges, which is another indicator of DNA damage (Warren et al., 1981). Taken together these results suggest that neither 2,4-DNFB or its microsomal metabolites damage DNA in V79 cells. Therefore, there are qualitative differences between bacteria and V79 cells in terms of the mutagenic response to DNFB.

Two probable explanations may be proposed for the discordance between bacterial and mammalian cells. Since DNFB binds covalently to amino acids and proteins (Sanger, 1945) thus presumably leading to cytotoxicity, the relatively greater number of intracellular barriers to the DNA in mammalian cells (i.e., nuclear membrane, histones, etc.) as compared to bacteria may not allow DNA binding while maintaining viability. The survival data of DNFB treated cells indicate that proteins do provide a protective effect from cytotoxicity (Figure 25). Second and perhaps more likely is the possible existence of bacterial enzymes, which are lacking in mammals, that are capable of metabolizing DNFB to a mutagenic form. This possibility is supported by the existence of bacterial nitroreductases (Blumer et al., 1980) essential for niridazole mutagenesis which are not found in mammalian liver homogenates. If this possibility were true, then one could speculate that DNFB may be a complete carcinogen in mammals when ingested orally and subsequently metabolized by intestinal bacteria, a situation

previously described for other compounds (Batzinger et al., 1978). These data emphasize the need for caution when using data derived from bacterial systems for use in human risk assessment.

5. Significance

Most reports in the genetic toxicology literature dealing with short-term mutagenicity assays have addressed the qualitative question: Can a particular chemical be metabolically activated to a mutagen? The in vitro mammalian assay system developed and characterized in this thesis has advanced the field of genetic toxicology because in addition to answering the qualitative question posed above it may be used to elucidate the relative importance of specific enzymes and/or enzyme ratios in the metabolic toxification/detoxification of a chemical.

To accomplish these objectives the enzyme pattern contained in the hepatic S-15 fraction is modulated in two manners: 1) the experimental animals are pretreated with compounds known to alter hepatic enzyme activities and 2) selected enzymes in the S-15 fractions prepared from these animals are inhibited to various degrees in vitro. The enzymic activity and pattern in a given S-15 fraction is determined, and the ability of the preparation to produce mutagenicity and/or cytotoxicity in the presence of a particular xenobiotic is assessed.

Prior to conducting the mutagenicity and cytotoxicity assays aimed at elucidating the importance of specific enzymes in the metabolic toxification/detoxification of a chemical three initial procedures must be performed: 1) the cytotoxicity of various concentrations

of the test chemical in the absence of metabolism must be determined, 2) the cytotoxic response to fixed concentration of the test chemical in the presence of various concentrations of S-15 fraction must be determined. The concentration of test chemicals employed in this procedure should be the highest concentration which is non-toxic in the absence of metabolism. The procedure should be performed under four conditions: A) with the S-15 fraction derived from animals pretreated with a P-450 type inducer (e.g., PB); B) with the S-15 fraction derived from animals pretreated with a P-448 type inducer (e.g., MC); C) with the S-15 fraction derived from animals pretreated with a mixed type inducer (e.g., Aroclor 1254); and D) with the S-15 fraction from animals which have not been pretreated. This initial experiment is very important because the degree of observed cytotoxicity is biphasic and dependent on the type of pretreatment employed. At lower concentrations of S-15 protein an increase in the amount of S-15 fraction increases the degree of cytotoxic response, while at higher concentrations of S-15 protein an increase in the amount of S-15 fraction decreases the degree of cytotoxic response. The concentration of S-15 fraction which induces maximum cytotoxicity should be selected for use in the metabolic activation studies. The third of the orienting experiments is performed after a concentration of S-15 fraction has been selected and is aimed at assessing the effect of lowering the concentration of test chemical on cytotoxicity. The S-15 fraction which is most active in metabolizing the test chemical to a cytotoxic form should be employed in this procedure. The concentration of test chemical chosen for use in the metabolic activation studies should be

the concentration which produces a cloning efficiency of approximately 5%. This will allow an accurate assessment of cytotoxicity and mutation frequency.

Once selected the concentration of test chemical and the amount of S-15 fraction in the reaction mixture must be maintained at a constant level in all cytotoxicity and mutagenicity assays. Correlations are made relating the activity of a particular enzyme or enzyme ratio in the S-15 fraction to the cytotoxicity or mutagenicity of the test chemical in the presence of the S-15 fraction. This is done graphically by plotting enzyme activity on the ordinate and biological response on the abscissa. Two correlation coefficients (r) are determined, one relating enzyme activity to cytotoxicity and the other relating enzyme activity to mutagenicity. The significance of r is estimated from the expression:

$$t = \frac{r^2(N-2)}{1-r2}$$ (N is the number of samples)

The null hypothesis tested in this way is that r estimated from the sample represents a true correlation coefficient of zero. If the apparent correlation is real ($p<0.05$), the best fitting linear regression line is calculated and the strength of the correlation ($r^2$, fraction of total variability due to regression) is determined.

When BP is utilized as the test compound the degree of cytotoxicity and mutagenicity observed in V79 cells was neither correlated with EROD or EMND activity, nor with the ratio of EROD activity to EMND activity in the S-15 fraction. On the other hand, there were significant correlations between the degree of BP activation, as

assessed by V79 cytotoxicity and mutagenicity, and EH activity, the ratio between EMND activity and EH activity, as well as the ratio of EROD activity to EH activity. The latter two findings indicate that the rate of epoxide formation divided by the rate of epoxide destruction is an important determinant of the degree of BP induced cytotoxicity and mutagenicity when it is metabolized in vitro. As these ratios increase BP becomes more cytotoxic and more mutagenic towards V79 cells. In light of the fact that EH activity in the S-15 fraction correlates with the degree of cytotoxicity and mutagenicity produced by BP whereas EROD activity and EMND activity do not, the rate of epoxide destruction is more important than the rate of epoxide formation in determining the biological response to BP in this in vitro system. As EH activity in the S-15 fraction increases the degree of cytotoxicity and mutagenicity observed as a result of metabolism decreases. These results verify that the experimental approach described in this thesis will allow one to elucidate the relative importance of various enzymes and/or enzyme patterns in the metabolic toxification/detoxification of a potentially genotoxic chemical.

The results reported in this thesis also shed light on a discrepancy in the genetic toxicology literature regarding the carcinogenic potential of DNFB. Although DNFB is a potent bacterial mutagen, it does not initiate tumors when applied to mouse skin but rather acts as a tumor promoter at this site. According to the widely accepted two stage theory of chemical carcinogenesis initiators would be expected to be mutagenic whereas tumor promoters would not be expected to be mutagens. Therefore, the observation that DNFB acts as a tumor

promoter rather than as a tumor initiator *in vivo* contrasts with observations that DNFB is a potent bacterial mutagen. For this reason I chose to determine if DNFB was mutagenic towards V79 cells. The results of this study indicate that neither DNFB, nor its microsomal metabolites, are mutagenic. The most probable explanation for the discrepancy between bacteria and mammalian cells is that bacteria contain an enzyme that is not found in mammalian cells and can metabolize DNFB to a mutagenic form. These observations emphasize the need for caution when using data derived from bacterial systems for use in human risk assessment and indicate the need for mammalian assays in assessing the mutagenic potential of suspected carcinogens.

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