THE MUTAGENICITY AND METABOLISM OF 3-NITROPERYLENE

THE MUTAGENICITY, METABOLISM AND MACROMOLECULE BINDING OF THE NITRATED POLYCYCLIC AROMATIC HYDROCARBON 3-NITROPERYLENE

By

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Abstract

In recent years the nitrated polycyclic aromatic hydrocarbons (nitroPAH's) have been recognized as powerful mutagens in the Ames *Salmonella* test. Most nitroPAH's are direct-acting mutagens in the Ames test *i.e.* they induce mutation in the absence of S9, and appear to be activated through nitroreduction by bacterial enzymes. Others, however, such as 3-nitroperylene, are indirect-acting mutagens and show maximum activity only when S9 is present.

Studies using the Ames test have indicated that the cytochrome P-450-dependent mixed function oxidase system of S9 is responsible for the activation of 3-nitroperylene to mutagenic species. However, the pattern of P-450 isozymes involved in this process appears to be different from that involved in the conversion of most PAH's, such as the standard indirect-acting mutagen benzo(a)pyrene (B(a)P), to proximate mutagens. 6-NitroB(a)P, in contrast, behaves in an analogous manner to its parent hydrocarbon. Using appropriate *Salmonella* mutants, the activation of 3-nitroperylene was found to require bacterial involvement, although the nature of the bacterial contribution has yet to be determined. Studies with other mutants have indicated that nitroreduction, at least as a primary activation step, does not appear to be important.

Incubation of 3-nitroperylene with high concentrations of S9 led to the formation of a number of metabolites, of which phenolic derivatives were prominent. In addition, S9-derived microsomes were able to catalyse the conversion of 3-nitroperylene to species which

were able to bind to protein and DNA. Under the conditions employed in these binding studies, 3-nitroperylene appears to be acting like a simple PAH, and such experiments with very high concentrations of liver protein may be unrepresentative of the processes responsible for the mutagenesis of the compound.

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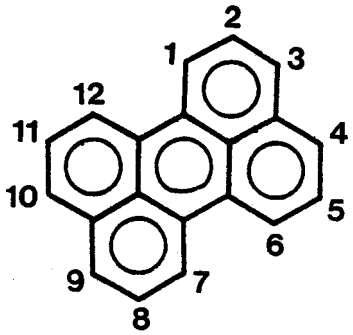

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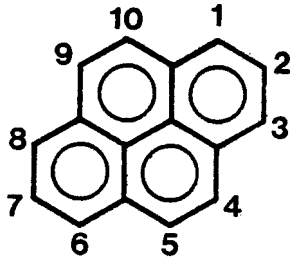

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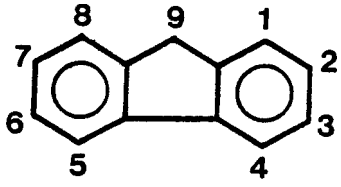

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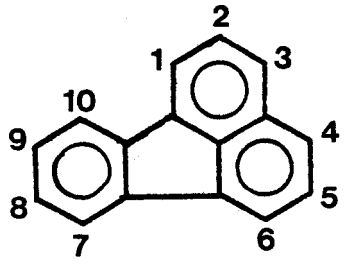

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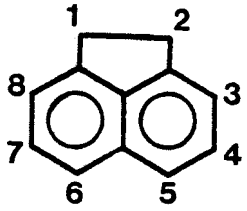

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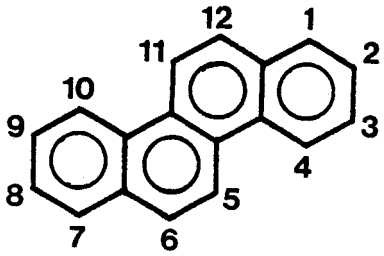

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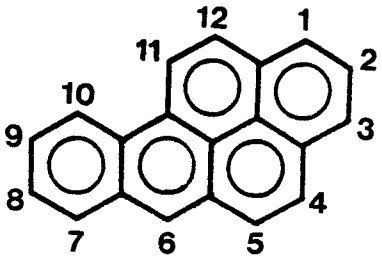


benzo(a)pyrene

1. LITERATURE SURVEY

1. Literature Survey

1.1. Introduction

Although significant advances in the understanding of the mechanisms of chemical carcinogenesis have been made only in recent times, observations indicating a link between environmental substances and cancer development date back several centuries. The earliest of these observations appear to have been made by two London physicians, John Hill and Percival Pott, in 1761 and 1775 respectively. Hill noted a correlation between nasal cancer and the excessive use of tobacco snuff (Redmond, 1971) while Pott recognized an unusually high incidence of scrotal cancer amongst men who had been chimney sweeps during childhood (Pott, 1775). Over the ensuing years, many more such associations were found, but it was not until 1933 that Cook, Hewett and Hieger succeeded in isolating and identifying a pure carcinogenic compound from an environmental sample (coal tar). The carcinogen they isolated was the polycyclic aromatic hydrocarbon (PAH) benzo(a)pyrene (B(a)P). Today the PAH's, of which B(a)P is the prototype, are recognized as one of the most widely distributed and significant classes of chemical carcinogens in the environment. Over the last 10-15 years the mechanisms by which they bring about the initiation of tumour development have been extensively studied and are relatively well understood (Conney, 1982 for review).

While many simple PAH's are known to possess mutagenic and tumorigenic properties, it was realized some time ago that the carcinogenicity of several types of environmental samples cannot be accounted for solely on the basis of their content of known

carcinogenic PAH's (Epstein et. al., 1966; Freeman et. al., 1971). In recent years it has become apparent that substituted PAH's, and in particular nitrated PAH's, could account for a significant portion of this "excess" carcinogenicity.

The observations of Pitts et. al. (1978) in the late 1970's led to the realization that nitro PAH's could be an important class of environmental mutagens. They found that under simulated atmospheric conditions of part per million levels of nitrogen dioxide and trace amounts of nitric acid, both B(a)P and perylene could be nitrated to products which were potent baterial mutagens (see also Pitts, 1979; Pitts et. al., 1979). Soon afterwards, Lofroth et. al. (1980) noted an unusually high level of mutagenicity in certain batches of photocopier toners, and subsequent studies led to the identification of the culprits as a series of nitrated pyrenes (Lofroth et. al., 1980; Rosenkranz et. al., 1980; Sanders, 1980). These compounds also proved to be potent bacterial mutagens (Mermelstein et. al., 1981).

NitroPAH's appear to be widely distributed in the environment and have been detected in diesel engine emissions (Clark et. al., 1982; Lewtas et. al., 1982b; Pitts et. al., 1982b; Pederson and Siak, 1981b; Salmeen et. al., 1982; Schuetzle et. al., 1982), gasoline engine emissions (Wang et. al., 1978), carbon blacks (Rosenkranz et. al., 1980; Sanders, 1980), cigarette smoke condensates (McCoy and Rosenkranz, 1982), wood stove emissions (Lewtas et. al., 1982a), urban atmospheres (Pitts et. al., 1982a; Talcott and Harger, 1981; Wang et. al., 1980), typewriter ribbons (Moller et. al., 1983) and fly ash (Li et. al., 1982; Wei et. al., 1982). Diesel engines are a particularly

rich source of nitroPAH's and, in view of the projected increase in the use of these engines (Rosenkranz, 1982), it is not surprising that diesel emissions have been extensively studied.

Modern analytical techniques have allowed the detection of a large number of nitroPAH's in environmental samples, however, the most widely distributed and abundant appears to be 1-nitropyrene. The dinitropyrenes, particularly 1,8- and 1,6-dinitropyrene, are present in much smaller quantities but may be more important biologically than 1-nitropyrene, as they are amongst the most potent bacterial mutagens known (Mermelstein et. al., 1981). In some environmental samples, for example diesel exhaust, the nitrated pyrenes are sufficiently strong mutagens to account for most of the "excess" mutagenicity which cannot be accounted for by simple PAH's. However, the role of other nitroPAH's and the effects of mixtures of PAH's and their derivatives remains to be evaluated.

Since the realization that nitroPAH's were a potentially significant class of environmental mutagens, considerable effort has been directed towards investigating their genotoxic effects in both bacterial and mammalian systems, and to identifying the mechanisms by which nitroPAH's exert their effects. A recent review (Rosenkranz and Mermelstein, 1983) provides an extensive coverage of the literature in this area so in the following pages I will present only some of the salient features of the published literature and assess the current state of knowledge in the field. Studies in bacteria have indicated that nitrated derivatives of 4 and 5 ring PAH's are the most potent mutagens in this class of chemicals and emphasis will be placed on

these in the following discussion. However, there is a considerable literature on the nitrated derivatives of 2 and 3 ring PAH's and this will be drawn upon to illustrate certain points.

1.2. Mutagenic and Genotoxic Effects of NitroPAH's

1.2.1. Bacterial Mutagenicity

Of the large number of procedures used in the short term testing of potential carcinogens, the most popular use bacteria as the tester organism, and mutation, usually a one locus, as the end point. The most frequently used bacterial mutagenicity test is the Ames Salmonella test, a reverse mutation assay in which the frequency of reversion of various mutant, histidine requiring strains of S. typhimurium to prototrophy (i.e. histidine independence) is measured (Ames et. al., 1975; Maron and Ames, 1983). The majority of the mutation data pertaining to the nitroPAH's has been obtained using this assay. Some data has been gathered from other bacterial systems and this will be covered later.

The mutagenicity of a variety of nitroPAH's in the Ames test has been summarized in several recent publications (Ho et. al., 1981; Lofroth, 1982; Rosenkranz and Mermelstein, 1983; Tokiwa et. al., 1981b) and some of the important features of this data are presented below.

The majority of the nitroPAH's are direct acting mutagens in the Ames test i.e. they exhibit maximum mutagenicity in the absence of an exogenous activating system. Direct acting mutagens comprise those chemicals which are capable of intercalating into the bacterial DNA, those which are so reactive that they are able to react directly with

the DNA to form covalent adducts, and those compounds which are activated to proximate and ultimate mutagens through metabolism by bacterial enzymes. Examples of this latter class, and the best known examples of direct acting nitroPAH's, are the nitropyrenes. Other nitroPAH's, in contrast, are indirect acting mutagens in the Ames test. 3-Nitroperylene and 6-nitro B(a)P are good examples. These compounds show maximum mutagenicity in the presence of an exogenous activating system, usually a liver postmitochondrial supernatant fraction (or S9) from rats pretreated with the polychlorinated biphenyl mixture Aroclor 1254. The fact that a compound is indirect acting does not preclude the involvement of bacterial enzymes in its activation, even though at least some part of the metabolism must be carried out by the added mammalian enzymes. There are yet other nitroPAH's which show both direct and indirect activity in Salmonella. Examples are 5-nitroacenaphthene (McCoy et. al., 1983) and 1- and 3-nitro B(a)P (Pitts et. al., 1982b). In the absence of S9 these compounds are activated solely by bacterial enzymes, while in the presence of S9, activation by bacterial enzymes, by S9 enzymes, or by a combination of the two are all possible pathways.

Studies with the nitroPAH's possessing three or more fused rings indicate that these compounds induce mutation predominantly of the frameshift type i.e. they are most active in the Salmonella tester strain TA98 (Rosenkranz and Mermelstein, 1983 for review). NitroPAH's generally are poor mutagens in the other standard tester strains, including strains that respond well to substances which induce base-pair substitution type mutations (TA100, TA1535), and strain TA

1537, which responds well to intercalating agents.

The mutagenicity of the nitroPAH's shows a marked dependence on their structure and, although structure-activity relationships have not yet been well defined, some trends may be noted. Mutagenic activity increases as we proceed from bicylic to tetracyclic systems but decreases considerably when the pentacyclic nitroPAH's are reached. Thus the tetracyclic nitropyrenes and nitrofluoranthenes are the most mutagenic nitroPAH's (Mermelstein et. al., 1981; Löfroth, 1982). Whether a compound is direct or indirect acting also depends in part on molecular size. For example, the nitropyrenes are all direct acting while most of the pentacyclic nitro compounds studied are indirect acting. To a certain extent, the degree of nitration also effects the mutagenicity of a compound. Thus in the nitropyrene series, the three dinitro isomers tested are potently mutagenic, whereas mono-, tri- and tetranitropyrenes are substantially less so (Mermelstein et. al., 1981).

Since many of the parent hydrocarbons of the nitroPAH's, for example pyrene and perylene, are essentially non-mutagenic in the Ames test, whereas their nitrated derivatives are potent mutagens, interest has centred on the nitro group as the likely site of activation. In view of this, it is of value to examine the mutagenicity of nitroPAH's in Salmonella strains deficient in nitroreductase enzymes. Several nitroreductaseless strains are available. Strains TA98NR and TA100NR (Rosenkranz and Speck, 1975, 1976), which were selected for resistance to niridazole (1-(5-nitro-2-thiazoyl)-2-imidazolidinone) have a biochemically demonstrable deficiency in an enzyme which reduces the

nitro group of nitrofurans and nitroimidazoles. This enzyme has been termed "classical" nitroreductase. A variety of nitroPAH's have been tested in these strains with varying results (Rosenkranz and Mermelstein, 1983 for summary). Some compounds such as 1- and 2-nitropyrene and 1,3-dinitropyrene, as well as several nitrated fluoranthenes and benzo(e)pyrenes, show considerably decreased activity in NR strains, which strongly suggests that reduction of their nitro groups(s) by the "classical" nitroreductase is involved at at least one point in their activation. The mutagenicity of some compounds, however, such as 1,6- and 1,8-dinitropyrene, is not diminished in the NR strains. This has led to the suggestions that there may be other nitroreductase enzymes in Salmonella which are responsible for activating the dinitropyrenes (Mermelstein et. al., 1981, Rosenkranz et. al., 1981). Strains of TA98 resistant to 1,8-dinitropyrene have been isolated ($TA98DNP_6$ and $TA98NR/1,8\text{-}DNP_6$) (McCoy et. al., 1981), but the lesion in these strains does not appear to be a nitroreductase deficiency (McCoy et. al., 1982). Its exact nature is unknown and is currently under investigation in several laboratories.

There is some evidence that under certain conditions direct acting nitroPAH's can become indirect acting mutagens. 1-Nitropyrene demonstrates very low mutagenicity in strain TA98NR, but it can be activated if low concentrations of S9 are added (Pederson and Siak, 1981b). This activation may be due to mammalian nitroreductase activity, but it is more likely that it involves oxidative metabolism by microsomal mixed function oxidase (MFO) enzymes in the S9 (see section 1.3.2). This suggests that 1-nitropyrene can be activated by

two different pathways - one utilizing bacterial enzymes and one involving mammalian enzymes. It is probable that other nitroPAH's, which show both direct and indirect acting behaviour, can be activated by two separate pathways.

The mutagenicity of nitroPAH's has also been investigated in several other bacterial systems. Some studies have been carried out in E. coli strain WP2 uvr A, but generally nitro PAH's show little, if any, mutagenicity in this system. For example, of a number of nitrated pyrenes tested using this assay, only 1,3,6-trinitropyrene demonstrated any activity (Mermelstein et. al., 1981). Since the E. coli assay responds primarily to mutagens causing base-pair substitutions (Green and Muriel, 1976), and the results in the Ames test indicate that most nitroPAH's induce frameshift type mutations, these results are not surprising. There is also some data on the ability of nitroPAH's to induce forward mutation in Salmonella. For example, 2-nitrofluorene will induce mutation to arabinose resistance (Pueyo, 1978) and 1,8-dinitropyrene can induce mutation to methyltryptophan resistance (Mermelstein et. al., 1981) but few other nitroPAH's have been studied for these activities.

1.2.2. Mutation in Mammalian Cells

The exceptional mutagenicity of many nitroPAH's in bacteria has prompted the investigation of the effects of these agents on mammalian cells in culture. Mutation in mammalian cell systems may be more relevant than microbial assays in assessing the risk of human exposure to nitroPAH's. The few studies which have been undertaken in this area

have been almost exclusively restricted to the nitropyrenes.

Cole et. al. (1982) found that 1,8-dinitropyrene was mutagenic in mouse lymphoma L5178Y cells when assayed for induced resistance at 4 loci (6-thioguanine, methotrexate, ouabain, and 1-β-D-arabinofuranosyl cytosine) although prolonged exposures were required. Plating efficiency studies indicated that the mutagen was not toxic in this cell line. However, in cultured hamster lung fibroblasts (CHL cells) 1,8-dinitropyrene was both highly toxic and mutagenic when diphtheria toxin resistance was used as a marker (Nakayasu et. al., 1982). In the same assay, 1,3- and 1,6-dinitropyrene and 1,3,6-trinitropyrene were also toxic and mutagenic, whereas 1-nitropyrene and 1,3,6,8-tetranitropyrene were without activity (Nakayasu et. al., 1982). The addition of S9 had no effect on the response to 1-nitropyrene but decreased both the toxic and mutagenic effects of 1,6-dinitropyrene. The results from this assay on the nitropyrene series generally parallel those results seen in Salmonella in the Ames test except that 1,6-dinitropyrene and not 1,8-dinitropyrene is the most potent mutagen in CHL cells.

The inactivity of 1-nitropyrene in inducing mutation in cultured CHL cells has been confirmed in CHO and L5178Y cells (Ball et. al., 1982), as well as in normal and XP human fibroblasts (Arlett, 1982). However, in contrast to the results with CHL cells, the addition of S9 substantially increases 1-nitropyrene induced mutation in L5178Y cells (Ball et. al., 1982). This result is not unlike its behaviour in Salmonella where, under appropriate conditions using a nitroreductase deficient bacterial strain, S9 can also activate

1-nitropyrene to mutagenic species (Pederson and Siak, 1981b).

There is a general paucity of knowledge of the mutagenic effects of nitroPAH's on cultured mammalian cells. Most compounds studied do appear to be mutagenic, although the levels of mutagenicity are not spectacularly high as they are in bacteria. Since the direct-acting nitroPAH's appear to depend in nitroreduction for the expression of their mutagenicity, it is likely that at least some cultured mammalian cells contain nitroreductase activity. However, the poor response to 1-nitropyrene by each of the cell lines studied suggests that the "classical" nitroreductase of bacteria, or an analogous enzyme, is either not present, or is only present at very low levels in these cells. There also exists the possibility that some other form of activation, probably ring oxidation, is important, particularly for 1-nitropyrene. Again, however, the MFO enzymes show only very low activities in cultured mammalian cells and in many cell lines are entirely lacking (Wood et. al., 1980; Bartsch et. al., 1982).

1.2.3. Other Effects in Mammalian Cells and Carcinogenicity in Animals

Chemicals prone to causing mutations are usually responsible for a variety of other effects on a cell's genetic material. The nitroPAH's are no exception and there is a growing body of literature on their genotoxic properties in cultured mammalian cells. Nitrated pyrenes, for example, are known to bring about a substantial increase in SCE's over control cultures in CHO cells (Nachtmann and Wolff, 1982), induce unscheduled DNA synthesis in several cell types (Ball et.

al., 1982; Campbell et. al., 1981; Kawachi, 1982) and induce substantial numbers of chromatid and chromosomal abberations in a rat epithelial cell line (Danford et. al., 1982). In some cases S9 enhanced these effects but in others it was inhibitory. Perhaps a more significant finding is that a number of nitroPAH's including 1-nitropyrene, 1,8-dinitropyrene, 6-nitrochrysene and 6-nitroB(a)P are able to induce transformation of Syrian hamster embryo cells (Di Paolo et. al., 1982). 1-Nitropyrene and 6-nitroB(a)P will also transform normal human skin fibroblasts (Howard et. al., 1983a) but nitrated pyrenes are not able to transform Balb/c-3T3 cells (Tu et. al., 1982), again indicating that not all cultured mammalian cells possess the necessary enzymes to convert these compounds to genotoxic forms.

Despite the limited amount of data available on the mutagenic and genotoxic effects of nitroPAH's on cultured mammalian cells, it is obvious that they do generally demonstrate high levels of activity and that, at least for the series of nitrated pyrenes, the results roughly correlate with those obtained in the Ames test. However, a variety of cell types have been used in the studies noted above and different cells do not necessarily give the same response to the same compound, making generalization of the results difficult. In addition, the role of exogenous activation has yet to be well defined. More valid interpretations of the effects of nitroPAH's on mammalian cells will be possible once the data base has been expanded and the nature of the xenobiotic metabolizing enzymes of cultured cells is better understood.

The most useful studies in terms of assessing carcinogenic risk to humans of any chemical are carcinogenicity studies in animals.

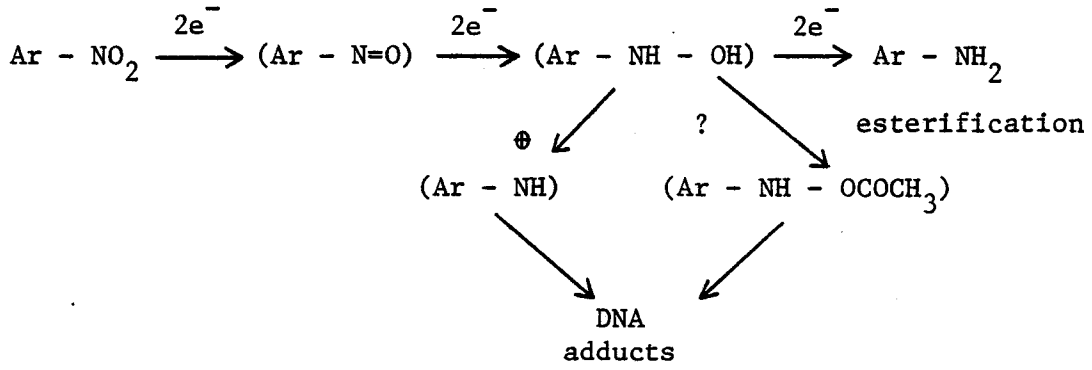
Since the highly mutagenic nature of nitroPAH's has only been recognized in recent years, few studies on the carcinogenicity of these compounds have yet appeared. Oghaki et. al. (1982) have reported the development of sarcomas at the site of subcutaneous injection of 1-nitropyrene and 3-nitrofluoranthene in Fisher rats, but it is likely that the 1-nitropyrene used was contaminated with dinitropyrenes which may have been responsible for the tumours observed. Using chromatographically pure 1-nitropyrene, El-Bayoumy et. al. (1982) found that the compound did not lead to tumour development in mouse skin, even after 13 weeks of promoter treatment, whereas 6-nitrochrysene was a tumour initiator. At the present time, it appears that some of the nitroPAH's are tumorigenic, but they do not have the potency that their spectacular bacterial mutagenicity may have suggested.

1.3. Metabolism of the NitroPAH's

1.3.1. Bacterial Metabolism

As noted above, most of the nitroPAH's are direct acting mutagens in the Ames test, and, since they do not appear to be intercalating agents, their mutagenicity is probably an expression of their activation by bacterial enzymes. Since unsubstituted PAH's are not direct acting mutagens in the Ames test, activation of nitroPAH's via ring oxidation is unlikely, whereas activation through reduction of the nitro moiety by bacterial nitroreductases has been shown to be responsible for the mutagenicity of several types of nitroaromatic and nitroheterocyclic compounds, and appears to be the route by which many nitro PAH's are metabolically activated.

The pathway for the reduction of the nitro group and its possible connections to DNA adduct formation are shown below:



The six electron reduction to the amino PAH must occur via the nitroso and hydroxylamino intermediates, although not necessarily by three two electron steps as indicated above. (The nitroaromatic anion radical and hydroxylamine radical, one- and three-electron reduction products respectively, are also possible intermediates.) The hydroxylamine is the putative proximate mutagen and evidence for this will be discussed shortly. It may decompose to a reactive aryl nitrenium ion, which could react with a variety of cellular nucleophiles, including DNA, or it could undergo esterification before binding to macromolecules.

Direct evidence for bacterial nitroreduction of two of the nitrated pyrenes has been obtained. When 1-nitropyrene was incubated with S. typhimurium several metabolites could be identified in the extracellular medium (Messier et. al., 1981). There was a time-dependent increase in the amounts of both 1-aminopyrene and N-acetyl-1-aminopyrene detected, indicating that reduction had occurred. Six minor products were also observed but were not identified. The reduction paralleled the binding of radioactivity to

DNA when tritium labelled 1-nitropyrene was used as the substrate, implicating an intermediate in the reduction pathway as a likely proximate mutagen. The involvement of bacterial nitroreductases in the metabolism was demonstrated by a decrease in both 1-aminopyrene production and DNA binding when nitroreductase deficient bacteria were used. 1,8-Dinitropyrene can also be reductively metabolized by bacteria to yield 1-amino-8-nitropyrene as the major product, and 1,8-diaminopyrene as a minor product (Andrews et. al., 1982). Both of these products exhibit mutagenicities at least two orders of magnitude lower than 1,8-dinitropyrene, suggesting that, if mutagenicity depends on nitroreduction, it is reduction of the first nitro group which is important. There is some evidence, however, that the mutagenicity of 1,8-dinitropyrene is not solely dependent on nitroreduction, if at all. Under certain conditions, using either whole bacteria or cell free extracts, two quite polar products, in addition to aminonitro- and diaminopyrene, are seen in metabolism experiments with 1,8-dinitropyrene (Bryant et. al., personal communication). These products, which are as yet unidentified, are also seen when bacteria deficient in the "classical" nitroreductase are used, even though nitroreduction in these cells is substantially reduced. In addition, the products are not formed when extracts of TA98DNP$_6$, a S. typhimurium strain selected for resistance to 1,8-dinitropyrene, are used, even though this strain still appears to be quite capable of reducing 1,8-dinitropyrene and remains fully sensitive to the mutagenic action of 1-nitropyrene. A more complete understanding of the mechanism of 1,8-dinitropyrene induced mutation will require the characterization of

the unknown metabolites and a better knowledge of the enzyme deficiency in 1,8-dinitropyrene resistant bacteria.

Nitroreduction is the major pathway of 1-nitropyrene activation in bacteria and it is likely that most other direct acting nitroPAH's are activated similarly. There are several lines of evidence which implicate N-hydroxyaminopyrene as the intermediate species from which the ultimate mutagen is derived.

Firstly, the mutagenic potency of 1-aminopyrene is substantially less than that of 1-nitropyrene (Tokiwa et. al., 1981a), indicating that the proximate mutagen arises during the reduction pathway. In addition, the intermediate 1-nitrosopyrene has been synthesized and has proved to be substantially more mutagenic than 1-nitropyrene, which is to be expected as it is necessarily an intermediate between the nitro compound and the hydroxylamine. It is interesting that the nitroso derivative has not been identified in metabolism experiments, even though it appears to be very stable (Elkhouri and McCarry, personal communication).

Secondly, studies on the activation of a variety of aromatic nitro and amino-compounds have indicated that arylhydroxylamines are notoriously oxygen sensitive (Weisburger and Weisburger, 1973). The presence of oxygen sensitive intermediates in the reductive activation of 1-nitropyrene has been inferred from the substantially higher mutagenicities of both the nitro compound and its nitroso derivative under low oxygen tensions (Löfroth, 1982).

More direct evidence for the involvement of the hydroxylamine comes from Pelroy and Gandolfi (1980) who found that, in the presence

of a mixed function amine oxidase, 1-aminopyrene was a potent mutagen in the Ames test. The enzyme they used has been shown to metabolize a number of aromatic amines to their N-hydroxylated products (Ziegler and Mitchell, 1972). Amine oxidase activity in S9 is probably responsible for the strong indirect acting mutagenicity of 1-aminopyrene (Ho et. al., 1981; Tokiwa et. al., 1981a).

Although the circumstantial evidence presented above strongly implicates the hydroxylamine as the proximate mutagen, unequivocal evidence has come from the identification of a 1-nitropyrene/DNA adduct. Howard et. al. (1983b) have shown that N-(deoxyguanosin-8-yl)-1-aminopyrene is the major adduct seen both in vivo in Salmonella DNA and in vitro in calf thymus DNA when xanthine oxidase/hypoxanthine is used to reduce the 1-nitropyrene, thus confirming that N-hydroxyaminopyrene is the source of the DNA binding species.

Although the mutagenicity of 1-nitropyrene is expressed via a derivative of its hydroxlamine, presumably the nitrenium ion, there has been some suggestion that esterification of hydroxylamines may be important in the mutagenicity of some other nitroPAH's, particularly 1,8-dinitropyrene (McCoy et. al., 1982). This claim is based on the observation that in the 1,8-dinitropyrene resistant Salmonella strain $TA98DNP_6$, the mutagenicity of hydroxylamines such as N-hydroxy-2-aminofluorene and N-hydroxy-2-acetylaminofluorene is significantly reduced, whereas they are highly mutagenic in wild type bacteria. The acetylated derivatives of both of these compounds are ultimate mutagens and esterification is known to be involved in the activation of a number of other aromatic amines to ultimate mutagens

(Kriek and Westra, 1979). Nevertheless, until the bacterial metabolites of 1,8-dinitropyrene have been identified, and more is known about the enzymes responsible for this metabolism, it is difficult to assess the role of esterification in its activation. The work with 1-nitropyrene has certainly indicated that S. typhimurium can acetylate a reduction product of that nitro compound (Messier et. al., 1981) and it is possible that similar reactions occur with 1,8-dinitropyrene.

1.3.2. Metabolism by Mammalian Enzymes

The majority of PAH's are indirect acting mutagens in the Ames test and are activated to proximate and ultimate mutagens by the microsomal mixed function oxidase (MFO) system of rat liver S9. Substituted PAH's, including nitro PAH's, appear to be susceptible to similar types of metabolism. For most PAH's a wide spectrum of products is obtained upon incubation with S9 under aerobic conditions (for example more than 20 B(a)P S9 metabolites have been found), although only a small number of these contribute to the mutagenicity of the compound. The products are predominantly phenols, quinones, hydroquinones and dihydrodiols, the latter being derived from the enzymic hydrolysis of epoxides. The major ultimate mutagens resulting from B(a)P metabolism by S9, for example, are the 7,8-dihydrodiol-9,10-epoxide and the 9-hydroxy-4,5-epoxide (Conney, 1982).

One of the nitroPAH's whose S9 metabolism has been relatively well studied is 6-nitroB(a)P. Incubation of this compound with rat

liver microsomes leads to the formation of 1- and 3-hydroxy-6-nitroB(a)P, 6-nitroB(a)P-1,9- and 3,9-hydroquinones and B(a)P-3,6-quinone (Fu et. al., 1981, 1982). Dihydroxy derivatives have also been isolated following the exposure of hamster embryo fibroblasts to 6-nitroB(a)P (Tong et. al., 1982). All these products are indicative of MFO metabolism. A mixture of 1- and 3-hydroxy-6-nitro B(a)P has proved considerably more mutagenic in the Ames assay than the parent nitro compound (Fu et. al., 1981, 1982; Pitts et. al., 1982b) but activation by S9 enzymes is necessary for the expression of this mutagenicity. The nature of the metabolites detected, the low mutagenicity of 6-nitroB(a)P in an oxygen-poor atmosphere (Löfroth, 1982) and the full sensitivity of the nitroreductase deficient Salmonella strain TA98NR to mutation by both 6-nitroB(a)P and 1- and 3-hydroxynitro B(a)P (Pitts et. al., 1982b), suggests that this latter activation step is also oxidative. On the basis of current evidence it appears that the metabolism of 6-nitroB(a)P to mutagenic species may be similar to that of its parent PAH, although nitroreduction by an enzyme other than the "classical" nitroreductase cannot be completely ruled out. Dihydrodiol metabolites, which are of great importance in the mutagenicity and carcinogenicity of B(a)P, have not been detected in the case of 6-nitroB(a)P. However, dihydrodiols have been found following incubation of both 6-nitrochrysene and 1-nitropyrene (see below) with S9 (El-Bayoumy et. al., 1982; El-Bayoumy and Hecht, 1983).

Oxidative metabolism of nitro PAH's is not restricted to the indirect acting mutagens and oxidation products resulting from the

incubation of 1-nitropyrene with S9 have been reported by several groups. 4,5-Dihydro-4,5-dihydroxy-1-nitropyrene is a major S9 metabolite (Wang and Burlingame, 1983; El-Bayoumy et. al., 1982; El-Bayoumy and Hecht, 1983) of 1-nitropyrene and it appears to be derived from 1-nitropyrene-4,5-epoxide which has also been detected (Wang and Burlingame, 1983). A number of phenolic metabolites are also produced (El-Bayoumy et. al., 1982; El-Bayoumy, 1982; Ball et. al., 1982). It is not known at the present time whether these oxidative metabolites of 1-nitropyrene are themselves mutagenic or, if not, whether they can be made so by subsequent nitroreduction. However, the presence of an epoxide as a metabolite suggests that oxidation alone may be sufficient to activate 1-nitropyrene to mutagenic species.

A nitroPAH for which a two stage activation of nitroreduction following ring oxidation has been strongly implicated is 5-nitroacenaphthene. A number of metabolites of this compound have been detected following incubation with S9, but the results of mutation assays indicate that 1-hydroxy-5-nitroacenaphthene and 1-oxo-5-nitroacenaphthene are the proximate mutagens (El-Bayoumy and Hecht, 1982). Both of these compounds are more mutagenic than their parent compound in the absence of S9, pointing to possible further metabolism by bacterial nitroreduction in the expression of the mutagenicity. The detection of several amino derivatives of 5-nitroacenaphthene and its metabolites shows that S9 enzymes certainly do have the capacity to reduce the nitro group, although the exact role of such activity in the mutagenicity is uncertain. The possibility that 5-nitroacenaphthene can be activated entirely by oxidative

metabolism cannot be discounted, since oxidative processes do predominate during its metabolism by S9, and both the parent compound and its major metabolites are strong indirect acting mutagens (El-Bayoumy and Hecht, 1982).

There is abundant evidence that mammalian nitroreductases present in S9 are able to reduce the nitro group of nitroPAH's. For example, several workers have identified 1-aminopyrene as the major product when 1-nitropyrene is incubated with S9 under anaerobic conditions (Tokiwa et. al., 1981a; Ball et. al., 1982; El-Bayoumy and Hecht, 1983). N-Acetylaminopyrene has also been identified as a minor product (Ball et. al., 1982) as it was in bacterial metabolism studies (Messier et. al., 1981). 6-Nitrochrysene is also converted to its amino derivative by S9 under conditions of low oxygen tension, whereas under aerobic conditions only a dihydrodiol is observed (El-Bayoumy et. al., 1982). Under the normal aerobic conditions employed in the Ames assay it is unlikely that S9 nitroreductase activity has any substantial effect on the mutagenicity of nitroPAH's. However, there is a possibility that mammalian nitroreductases may be involved in the S9 mediated activation of 1-nitropyrene to mutagenic species in the nitroreductase deficient Salmonella strain TA98NR (Pederson and Siak, 1981b).

1.4. Enzymes Involved in the Metabolism of NitroPAH's

1.4.1. Nitroreductases

The preceding discussion on the mutagenicity and metabolism of nitroPAH's has indicated that a major route of activation of these

compounds (at least in bacteria) is reduction of the nitro function by endogenous nitroreductases. A wide range of nitro compounds, including the nitrofurans, nitroimidazoles and nitroaryls, are activated by nitroreduction and are direct acting mutagens in the various indicator organisms used in short term assays (see Callen, 1982 for review). In addition, the toxic and carcinogenic effects of many aromatic nitro compounds *in vivo* appear to be related to their metabolic activation by nitroreduction to hydroxylamines and their corresponding hydroxamic acids (Poirier and Weisburger, 1974; Weisburger and Weisberger, 1973; Kriek and Westra, 1979).

Although nitroreductase activities have been studied most thoroughly in bacteria, particularly *S. typhimurium* and *E. coli*, they are also known to occur in other microorganisms such as fungi (Ong, 1978) and yeast (Shahin, 1975; Seibert *et. al.*, 1979). Of particular significance in terms of assessing the hazards associated with human exposure to nitro compounds is the finding that intestinal microflora also contain nitroreductase activity (Sheline, 1973; Kinoughi *et. al.*, 1982). In fact, a recent study has indicated that many types of human intestinal bacteria are able to reduce 1-nitropyrene to 1-aminopyrene (Kinoughi *et. al.*, 1982). Unfortunately, there has been little attempt to isolate and characterize the various microbial nitroreductases and only those in *E. coli* and *S. typhimurium* have been studied in any detail. Even so characterization of these enzymes remains on a relatively crude level.

Since *S. typhimurium* is the bacterium most frequently used in mutation assays, it is of some interest to examine its nitroreductases.

Studies with Salmonella mutants have suggested that the bacterium possesses several distinct nitroreductase activities (McCoy et. al., 1981; Rosenkranz et. al., 1982) (although of course unequivocal proof of this requires that the enzymes be isolated and separated chromatographically). For example, mutants resistant to niridazole but not 4-nitroquinoline-1-oxide and vice versa have been isolated (Rosenkranz and Speck, 1975; McCoy et. al., 1981). Both compounds are known to require nitroreduction for the expression of their mutagenicity, suggesting that at least two independent nitroreductases exist in Salmonella.

Nitroreductase enzymes have been partially characterized in S. typhimurium strains TA100 and TA98. In TA100 two oxygen-insensitive nitrofuran reductases have been found (Tatsumi et. al., 1982). One requires NADPH as a cofactor while the other works equally well with either NADPH or NADH. The NADPH-dependent enzyme is absent in nitrofuran low resistance mutants and the NAD(P)H activity can be removed by a second step of selection for high resistance to nitrofurazone. The NAD(P)H activity was shown to be a flavoenzyme which requires FMN. The situation in TA98 is a little more complex (Bryant et. al., personal communication). In the wild type strain, two nitrofurazone-reductase peaks are found following DEAE cellulose chromatography of cell extracts, although one of these peaks accounts for about 98% of the activity. Both components can use either NADPH or NADH as cofactors. In a mutant selected for resistance to niridazole (TA98NR) the small peak remains unchanged but the NADPH activity of the large peak has totally disappeared and its NADH activity is reduced to

less than 10% of its wild type value. This residual activity indicates that there are at least two components in the main wild type peak. These results were obtained with a nitrofuran as the substrate but the same pattern is seen when 1-nitropyrene is used as a substrate (Bryant et. al., personal communication). In this case the reduction to 1-aminopyrene may be followed by a sensitive fluorimetric assay (Lu et. al., 1983). The major nitrofuran reductase peak is also able to reduce 1,8-dinitropyrene to 1,8-diaminopyrene. Again, the fluorescence of the product is used as the assay for reduction. It is not known at the present time whether the small nitrofuran reductase peak is also able to catalyze 1,8-dinitropyrene reduction.

The nitroreductases of E. coli have been studied in somewhat more detail. Both oxygen-sensitive (Type II) and oxygen-insensitive (Type I) nitroreductases have been identified in the bacterium when nitrofurans are used as substrates (McCalla et. al., 1970; Peterson et. al., 1979). The Type I reductases have been best studied and several enzymes of this type have been partially characterized (Bryant et. al., 1981, Tatsumi et. al., 1981). At least two Type II activities are also present (McCalla et. al., 1975, 1978) but the enzymes have not yet been characterized. Some preliminary results in our laboratory indicate that extracts of several E. coli strains are able to reduce 1-nitropyrene to 1-aminopyrene and 1,8-dinitropyrene to 1-amino-8-nitropyrene and then to 1,8-diaminopyrene under either aerobic or anaerobic conditions (Gouin et. al., personal communication).

The presence of oxygen-sensitive nitroreductases in S9 with the

ability to reduce nitroPAH's was pointed out earlier. The major nitroreductase activity in rat liver extracts is due to the microsomal NADPH-cytochrome P450(c) reductase (Gillette et. al., 1968; Wang et. al., 1975). This nitroreductase is extremely sensitive to oxygen and appears to be mechanistically similar to the bacterial type II activity (Mason and Holtzman, 1975; Sealy, 1978). Other prominant nitroreductase activities in rat liver are due to the soluble enzymes xanthine oxidase (Morita et. al., 1971; Wang et. al., 1974) and DT-diaphorase (Kato et. al., 1970). Under anaerobic conditions xanthine oxidase is able to catalyze the reduction of 1-nitropyrene to 1-aminopyrene and to species which are capable of binding to calf thymus DNA (Howard and Beland, 1982) and causing cell transformation (Howard et. al., 1983a). The same enzyme is able to reduce 1,8-dinitropyrene (Bryant et. al., personal communication) to 1-amino-8-nitropyrene and 1,8-diaminopyrene. The xanthine oxidase catalyzed reduction of both of these nitro PAH's is oxygen sensitive, again indicating a similarity to the bacterial type II activity.

The reduction of nitro compounds in vitro by mammalian enzymes is highly sensitive to oxygen but the isolation of amines as urinary metabolites from animals treated with nitroaromatic compounds (Johnson and Cornish, 1978) suggests that nitroreduction can occur under normal oxygen tensions. Some recent findings have shown that both the native form of rat-liver xanthine oxidase, xanthine dehydrogenase, and NADPH-cytochrome P450(c) reductase can reduce nitrofurans aerobically (Kutcher and McCalla, 1983; Holtzman et. al., 1981). These observations have important implications in considering the toxic and

carcinogenic effects of nitro compounds in animals and humans.

1.4.2. Enzymes of Oxidative Metabolism

The enzyme system in S9 responsible for the activation of the majority of indirect acting mutagens is the cytochrome P-450 mixed function oxidase (MFO) system which is located in the smooth endoplasmic reticulum of whole cells and the microsomal fraction of S9. The term mixed function oxidase or monooxygenase refers to the fact that in P-450 catalysed reactions one atom of molecular oxygen is reduced to water while the other is incorporated into the substrate. The MFO system consists of three components - the hemoprotein cytochrome P-450, the flavo protein NADPH-cytochrome P-450 reductase, and a lipid component, mainly phosphatidyl choline. Of these three components, cytochrome P-450 is undoubtedly the most important in microsomal xenobiotic metabolism because of its vital role in oxygen activation and substrate binding. For normal function, oxygen, and a source of reducing equivalents in the form of NADPH, are required. NADH alone will not usually replace NADPH but may, in the presence of NADPH, have a sparing or even synergistic effect. Electrons are transferred from NADPH to the cytochrome by NADPH-cytochrome P-450 reductase. Cytochrome b_5, the other cytochrome characteristic of the endoplasmic reticulum, is not essential for MFO activity but in some reactions it can have a stimulatory effect. The mechanism of MFO catalyzed xenobiotic metabolism is the subject of numerous publications (Miwa et. al., 1978; Noshiro and Omura, 1978; Schenkman and Gibson, 1981 for example).

The MFO system shows a broad substrate specificity. Common endogenous substrates are the steroid hormones, bile acids, prostaglandins and biogenic amines. In addition, a large number of xenobiotics including PAH's, halogenated hydrocarbons, nitrosamines, azo compounds, aromatic amines, and nitroaromatics are substrates for the system (Conney, 1967, 1982). A large number of reactions such as aromatic hydroxylation and epoxidation, aliphatic hydroxylation, numerous dealkylations, N-oxidation and oxidative deamination are catalyzed by MFO enzymes (see Hodgson and Dauterman, 1980 for review). The epoxidation and hydroxylation of PAH's are amongst the best known MFO reactions (Conney, 1982 for review).

Even before purification of cytochrome P-450 had been achieved it was apparent from indirect evidence that multiple monooxygenases exist in the liver (Axelrod, 1956; Conney et. al., 1959; Weisberger et. al., 1957). The resolution and reconstitution of MFO system in the late 1960's (Lu and Coon, 1968; Lu et. al., 1969) paved the way for more detailed studies on the various forms of P-450. Five different cytochromes (P-450's a,b,c,d and e) have been purified to apparent homogeneity from rat liver microsomes (Ryan et. al., 1979, 1980, 1982) and more variants probably exist. For example, 11 P-450's have been found in rabbit liver microsomes, although they have yet to be purified and characterized (Nebert and Negishi, 1982). The five purified rat liver cytochrome P-450's have been partially characterized and show substantially different amino- and carboxyl-terminal amino acid sequences (Botelho et. al., 1979, 1982) suggesting that they are separate gene products. Indeed recent studies have shown that specific

mRNA's code for specific cytochrome P-450's in a cell-free protein synthesizing system (Bhat and Padmanaban, 1979; Colbert et. al., 1979) and work has commenced on the cloning of P-450 genes (Bresnick et. al., 1977; Fujii-Kuriyama et. al., 1982; Negishi et. al., 1981).

It has been known for over 30 years that the hepatic drug metabolizing system is inducible i.e. the metabolism of most substrates of the MFO system can be enhanced by pretreating the animals with a variety of compounds including 3-methylcholanthrene (MC), phenobarbital (PB), β-naphthoflavone (NF), Aroclor 1254 (AR), isosafrole or pregnenolone 16α-carbonitrile (see Conney, 1982 for review). Induction by these compounds is a reflection of the induction of various forms of P-450 by a mechanism which appears similar to the mechanism of steroid hormone action. For example, MC has been shown to bind to a hepatic cytosolic receptor (Poland et. al., 1976; Heintz et. al., 1981) to form a inducer-receptor complex which moves into the nucleus and stimulates the production of mRNA (Tukey et. al., 1982). The mRNA produced following inducer treatment has been shown to lead to the synthesis of P-450 in a cell-free protein synthesizing system (Bhat and Padmanaban, 1979; Colbert et. al., 1979).

The individual forms of P-450 are induced by different compounds to varying extents (Thomas et. al., 1979, 1981). Pretreatment of rats with MC causes a 50-fold increase in the concentration of P-450c in liver microsomes, a 4-fold increase in P-450a, and no increase in P-450b. Pretreatment with PB will cause a 50-fold increase in P-450b, a 3-fold increase in P-450a and no change in the amount of P-450c. Pretreatment with AR will cause a 45 to

60-fold increase in both P-450b and P-450c and a 5-fold increase in P-450a. Cytochrome P-450d is the major form of P-450 induced by isosafrole (Ryan et. al., 1980) and P-450e is found in small amounts following both PB and AR treatment (Ryan et. al., 1982). The ability of AR to increase greatly the levels of several P-450s probably explains why S9 from AR treated rats is so effective in metabolizing a diverse group of chemicals to ultimate mutagens in the Ames test.

Each of the various forms of cytochrome P-450 has its own characteristic range of substrate specificity, but these often overlap (Lu and West, 1980), suggesting that the rates of detoxification and metabolic activation of foreign chemicals will depend on the relative amounts of the various P-450s that are present in a tissue. The differing abilities of various forms of P-450 to metabolize various chemicals (Hara et. al., 1981, 1983; Gozukara et. al., 1982; Hutton et. al., 1979) and to activate them to mutagens in the Ames test (Hutton et. al., 1979; Kawajiri et. al., 1983, 1982; Robertson, 1983) has been well documented. For example, cytochrome P-450c (isolated from MC treated rats) is one of the most active enzymes which converts B(a)P (and indeed most PAH's) to mutagenic species, whereas other forms of the cytochrome are rather inactive (Wood et. al., 1976), a result consistent with metabolism studies (Gozukara et. al., 1982; Wood et. al., 1976). In addition to differences in overall metabolic rates, various forms of P-450 also exhibit positional selectivity and stereoselectivity in the oxidation of PAH's (Deutsch et. al., 1978; Thakker et. al., 1977). For example, when B(a)P is used as a substrate, the ratio of 3-hydroxy B(a)P to 9-hydroxy B(a)P formed

ranges from 1 to 20 depending on the forms of P-450 catalysing the reaction. Since each form of P-450 shows a particular stereoselectivity towards its substrate and different enantiomers of PAH metabolites have different mutagenic and carcinogenic activities (Conney, 1982 for review), the ratio of P-450 forms could be an important factor in determining the susceptibility of different tissues, species and strains to the hazardous effects of PAH's.

The role of various forms of P-450 in the metabolism and activation of indirect-acting nitro PAH's to mutagens is largely unknown as essentially all the mutagenicity data reported has been obtained using AR induced S9. One study, however, has reported on the metabolism of 6-nitroB(a)P using microsomes from PB treated, MC treated and control rats (Fu et. al., 1981). HPLC analysis of the metabolites revealed that PB microsomes were 1.5 times more active han control microsomes in metabolizing 6-nitroB(a)P, whereas MC microsomes were four times more effective than control microsomes. In each of the three cases the same metabolite pattern was observed. These results suggest that cytochrome P-450c is the most effective form of P-450 in the metabolism of 6-nitroB(a)P, a result consistent with the apparent similarity between the metabolism of this nitro compound and its parent PAH, B(a)P, which is also preferentially metabolized by P-450c.

1.5. Basis and Objectives of the Current Study

Although the potent mutagenicity of 3-nitroperylene was recognized when the study of the biological effects of nitroPAH's was in its infancy (Pitts et. al., 1978), there has been little published

on the compound since that time. Several groups have presented data on the mutagenicity of 3-nitroperylene in the Ames test (Pitts et. al., 1978; Löfroth, 1982; Ho et. al., 1981) and all agree that the compound is a very strong indirect acting mutagen, even though the reversion rates reported by the various groups differ considerably. The study of Löfroth (1982) was somewhat more extensive than the others and showed that S. typhimurium strain TA98 was the most sensitive to 3-nitroperylene, indicating that the compound induces frameshift type mutations. In addition, its activation by S9 enzymes was oxygen dependent. A crude nitration mixture of perylene has been shown to induce unscheduled DNA synthesis (UDS) in cultured HeLa cells and to show both direct and indirect activity in the Ames test (Campbell et. al., 1981).

While 3-nitroperylene is highly mutagenic in the Ames test, its parent hydrocarbon, perylene, is considered a non-mutagen in that assay, suggesting that the nitro group plays an important role in the mutagenesis of the compound. It could exert its effects in two basic ways. Firstly, the nitro group itself could be the site of metabolic activation by nitroreduction, as is the case with 1-nitropyrene. However, this would seem unlikely in view of the requirement for S9 to provide activation and the oxygen dependence of its activation. The second, and more likely, possibility is that the nitro group alters the electron distribution of the perylene molecule in such a way that its becomes a more attractive substrate for the microsomal MFO enzymes. There also exists the possibility that both oxidative MFO metabolism and nitroreduction are both involved in the activation of the compound.

The purpose of the present study was to investigate the basis of the mutagenicity of 3-nitroperylene and to ascertain the role played by the nitro function in this activity. Two approaches have been taken. Firstly, in view of the limited data on the mutagenicity of 3-nitroperylene in the literature, the mutagenic activity of the compound has been examined more thoroughly using the Ames assay, and compared with that of other mutagens (6-nitroB(a)P and B(a)P) whose mechanism of action is better understood. Secondly, the metabolism of 3-nitroperylene by rat liver S9 has been investigated in order to gain more direct insight into the mode of activation of the compound. As an adjunct to these studies, the ability of 3-nitroperylene metabolites to bind to biological macromolecules, protein and DNA, has also been investigated.

2. MATERIALS AND METHODS

2. Materials and Methods

2.1. Mutagens

B(a)P and perylene were obtained from Aldrich Chemical Co. (Montreal, P.Q.), and 1,8-dinitronaphthalene from Sigma Chemical Co. (St. Louis, MO.).

All other mutagens were synthesized and purified as described below. I am grateful to Dr. B.E. McCarry and Ms. M. Lew of the Chemistry Department, McMaster University, for performing the syntheses. Ms. Lew also assisted in the purification of some batches of 3-nitroperylene.

Nitrated perylene and B(a)P mixtures were prepared by treating the appropriate PAH with fuming nitric acid in acetic anhydride. 3-Nitroperylene, two dinitroperylenes, and 6-nitroB(a)P were purified by preparative reverse phase HPLC on a Whatman Partisil M9 10/50 ODS-2 column with pure methanol as the mobile phase (flow rate 4 ml/min). In some cases 3-nitroperylene was purified by preparative normal phase HPLC on an Altex Ultrasphere Cyano column (25 cm x 10 mm; 5 μm) using a mobile phase of 96% hexane/5% isopropanol (flow rate 4 ml/min). Insufficient quantities of the two dinitroperylenes were obtained for the assignment of structures by NMR spectroscopy, so the isomers were arbitrarily designated dinitroperylene (I) and dinitroperylene (II). Under the preparative HPLC conditions described above dinitroperylene (I) had a k' (capacity factor) of 2.1 and dinitroperylene (II) had a k' of 3.3. (NB $k' = (Vr-Vm)/Vm$ where Vr is the elution volume of the compound being considered and Vm is the void volume.)

The visible spectrum of 3-nitroperylene showed a single, broad

absorption peak at 465 nm. At this wavelength the molar extinction coefficient was calculated to be 1.559×10^7 cm^2 mol^{-1}, and all subsequent 3-nitroperylene concentrations were determined using this value.

3-Aminoperylene was prepared by hydrogenation of 3-nitroperylene over Adams catalyst in methanol. The amine was purified immediately before use by reverse phase HPLC on an Altex Ultrasphere ODS analytical column (4.6 mm x 25 cm; 5 µm) using a mobile phase of 100% methanol.

A mixture of bromoperylenes was prepared by treating perylene with bromine in carbon tetrachloride and the individual components of the mixture were purified by preparative reverse phase HPLC on a Whatman Partisil M9 10/50 ODS-2 column using an isocratic mobile phase of 30% methylene chloride/70% acetonitrile (flow rate 4 ml/min).

The [^{3}H]3-nitroperylene required for metabolism and macromolecule binding experiments was prepared as follows. 3-Bromoperylene was synthesized and purified as described above then labelled by catalytic exchange with tritium gas by Amersham Corporation (U.K.) to give 3,4,9,10-[^{3}H]perylene. TLC indicated that the sample was essentially free of bromoperylene. It was nitrated as described above and [^{3}H]3-nitroperylene was purified by reverse phase HPLC on a Hibar LiChrosorb 5 µm analytical column using an isocratic mobile phase of 80% acetonitrile/20% water. The final product had a radiochemical purity of 99% by HPLC and had a specific activity of 17.6 Ci/mmol.

The identities of all compounds were confirmed by mass spectrometry and purities were assessed by reverse phase HPLC. With

the exception of 3-aminoperylene, the purity of all compounds was greater than 99%. 3-Aminoperylene proved quite unstable, and the sample used in the Ames assay was only about 70% pure at the time the test was being conducted.

2.2. Other Chemicals

The other chemicals used were obtained from the following sources: 3-methylcholanthrene, α- and β- naphthoflavone, 2-methyl-1,2-di-3-pyridyl-1-propanone (metyrapone), uridine 5'-diphosphoglucuronic acid (UDPGA), bis(2-hydroxyethyl)-iminotris(hydroxymethyl)methane (bis-tris), cysteine, reduced glutathione (GSH), and calf thymus DNA from Sigma Chemical Company, St. Louis, MO.; nicotinamide adenine dinucleotide (NAD^+), nicotinamide adenine dinucleotide phosphate ($NADP^+$), glucose-6-phosphate dehydrogenase, and glucose-6-phosphate from Boehringer Mannhein, Canada, Dorval, P.Q.; acrylamide, N,N'-methylene bis acrylamide, ammonium persulfate, and N,N,N',N'-tetramethylethylenediamine from Bio-Rad Laboratories, Richmond, CA; Zn dust from McArthur Chemical Co., Montreal, P.Q.; sodium phenobarbital from Paul Maney Laboratories, Toronto, Ont.; and Aroclor 1254 from Monsanto Chemical Co., St. Louis, MO. 2-Diethylaminoethyl- 2,2-diphenylvalerate hydrochloride (SKF-525A) was a generous gift from Smith, Kline and French Canada Ltd., Mississauga, Ont.

All other chemicals and solvents were reagent grade or better.

2.3. Bacterial Strains

Salmonella typhimurium strains TA98, TA100, TA1535, TA 1537 and TA 1538 were obtained from Dr. B.N. Ames, University of California, Berkeley, CA.

Strains TA98NR, TA98DNP$_6$, and TA98NR/1,8-DNP$_6$ were obtained from Dr. H.S. Rosenkranz, Case Western Reserve University, Cleveland, OH.

Strain TA98MCF$_{50}$, which is resistant to the mutagenic effects of furacin, was isolated in our laboratory by Mrs. A. Bromke.

The genotypes of the various tester strains were confirmed by standard procedures (Maron and Ames, 1983). The presence of the deep rough (rfa) mutation in all strains was shown by their sensitivity to crystal violet, and the presence of the pKM101 plasmid in certain strains was confirmed by their resistance to ampicillin.

2.4. Liver S9 and Microsomes

All liver S9 and microsomal extracts were prepared from male Sprague-Dawley rats (Charles River, Montreal, P.Q.) weighing approximately 200 g. Prior to sacrifice, the animals were treated with inducers of the liver MFO system by intraperitoneal (i.p.) injection according to the following schedules: Aroclor 1254 (AR). A single injection of 500 mg/kg in corn oil five days before sacrifice; Sodium phenobarbital (PB). 80 mg/kg in water on three successive days. Sacrifice 24 hours following the last injection; 3-Methylcholanthrene (MC). 25 mg/kg in corn oil on three successive days. Sacrifice 24 hours after the third injection; β-Naphthoflavone (NF). 40 mg/kg in

corn oil on three successive days. Sacrifice 24 hours after the final injection. Control or uninduced (UN), S9's were prepared from animals killed five days following a single i.p. injection of 0.5 ml of corn oil.

Liver S9's were prepared in 0.15 M KCl according to the method of Ames et. al. (1975), frozen in liquid nitrogen, and stored at -70°C until required.

Microsomes for metabolism and macromolecule binding experiments were obtained by centrifuging freshly prepared S9 at 105,000 g for one hour at 4°C in an IEC Model B-60 ultracentrifuge using a type A-321 rotor. The supernatant (S105) was decanted and its volume measured. The microsomes were resuspended by homogenization using a Dounce homogenizer in a volume of 0.02 M tris-HCl (pH 7.4) (containing 0.15M KCl) equal to that of the S105. They were frozen in liquid nitrogen and stored at -70°C until required.

For mutation assays in which fractionated S9 was required, a measured volume of freshly thawed S9 was centrifuged at 105,000 g as described above. The supernatant was decanted and its volume adjusted to the initial volume by the addition of 0.15M KCl. The microsomes were resuspended in 0.15M KCl as described above. In some cases, the microsomes were resuspended in S105 to give reconstituted S9. Microsomes, S105 and reconstituted S9 were kept on ice until required and were used within one hour of preparation.

2.5. Mutation Assays

The Ames Salmonella plate incorporation test was conducted

according to standard procedures (Ames et. al., 1975; Maron and Ames, 1983). Tester strain cultures were grown overnight in either Penassay Broth (Difco) or Oxoid Nutrient Broth No. 2 to a density of 2×10^9 cells/ml ($A_{600} \approx 1.3$). On each test plate 2×10^8 cells were used. Test chemicals were always added in 50 µl of DMSO.

Most assay plates were counted following 48 hours incubation at 37°C. However, the revertant colonies from tester strains TA98 DNP_6 and TA98NR/1,8-DNP_6 tended to grow quite slowly on minimal glucose agar plates, and assays employing these strains were read after 72 hrs. Revertant colonies were counted on a Biotran II automated colony counter (New Brunswick Scientific).

The S9 mix employed in most assays was as described by Ames et. al. (1975). However, in experiments where S9 was fractionated into microsomal and soluble components, all mixes were supplemented with glucose-6-phosphate dehydrogenase (3 IU/plate). When NAD^+ was used in place of $NADP^+$ it was at the same concentration as the latter. S9 concentrations are reported as the percentage of S9 in the S9 mix. For example, a concentration of 5% indicates that 5% of the S9 mix used is S9.

When microsomal enzyme inhibitors (α- and β-naphthoflavone, SKF525-A, metyrapone) were used they were added in 50 µl of DMSO to give the desired concentration. GSH, cyteine, and UDPGA were all added in 50 µl of water when required.

In some experiments the preincubation modification of the Ames test was employed (Yahagi et. al., 1977; Maron and Ames, 1983). To a tube containing 50 µl of the appropriate chemical, 0.5 ml of S9 mix and

0.1 ml of an overnight culture of the bacterial tester strain were added. This mixture was preincubated at 37°C for 20 min, then mixed with 2 ml of top agar and plated on minimal glucose agar plates. The plates were incubated for 48 hours at 37°C before revertant colonies were scored.

In one series of assays the S9 mix was replaced by the zinc dust/ammonium chloride reducing system described by Karpinsky et. al. (1982). Standard plate incorporation assay conditions were employed except that 50 µl of a suspension of zinc dust in water, and 50 µl of an ammonium chloride solution, were added to the top agar before plating. The reducing system was used at two concentrations (2.28 mg/ml Zn dust/0.57 mg/ml NH_4Cl and 10 mg/ml Zn dust/2.5 mg NH_4Cl) but in each case the molar ratio of Zn to NH_4Cl remained the same at 13 to 4.

In all assays individual treatments were carried out in triplicate and all experiments were done at least twice and usually 3 to 5 times. The experiment with 3-aminoperylene was only carried out once in view of the instability of the compound.

2.6. Metabolism of 3-Nitroperylene by S9 and Microsomes

For metabolism experiments with S9, each ml of the incubation mixture contained the following: 125 nmol of 3-nitroperylene in 50 µl of DMSO; 8 µmol $MgCl_2$; 1.65 µmol KCl; 5 µmol glucose-6-phosphate; 4 µmol $NADP^+$; 100 µmol potassium phosphate buffer (pH 7.4) and 500 µl of S9. When tritiated 3-nitroperylene was required incubations contained 125 nmol of the labelled compound (specific activity 364

mCi/mmol) in 100 µl of DMSO.

All components, with the exception of 3-nitroperylene, were mixed and preincubated at 37°C for 2 min. The mutagen was then added and the incubation continued for the appropriate time at 37°C. Incubations were for 2 hours duration unless otherwise stated. Reactions were terminated by the addition of one volume of ice cold acetone and the mixtures were extracted twice with two volumes of ethyl acetate. The combined extracts were dried over anhydrous $MgSO_4$, filtered, and stored at -20°C until required. Storage as an ethyl acetate solution appeared to be the most effective way of preventing decomposition of the metabolites. Immediately before HPLC analysis samples were taken to dryness under reduced pressure at 40°C using a rotary evaporator and the residue was dissolved in 200 µl of tetrahydrofuran (THF).

Metabolite mixtures were analysed by reverse phase HPLC on an Altex Ultrasphere ODS column (4.6 mm x 25 cm; 5 µm) using a linear gradient of 60% methanol/40% water to 100% methanol over 50 minutes (flow rate 1 ml/min). A Beckman Model 332 gradient liquid chromatograph system was used for all HPLC. The column eluate was routinely monitored by absorption at 254 nm.

Incubations containing [^{3}H]3-nitroperylene had a total volume of 2 ml and were treated as described above. 50 µl of the final THF solution was subjected to HPLC. Fractions were collected at 0.5 min intervals and the radioactivity in each fraction was determined by liquid scintillation counting in a Beckman LS 780 instrument. Counting efficiency was measured by the H-number method.

When metabolites were required for UV-visible spectral analysis preparative scale reactions (5 or 10 ml volume) were carried out. Metabolites were extracted as described above and the final residue was dissolved in 200 µl of THF. 100 µl was subjected to HPLC. The appropriate peaks were collected and their UV-visible spectra determined using a Beckman Model 25 spectrophotometer. Difference spectra of ethyl acetate extracts were measured on the same instrument, where the reference cuvette contained an ethyl acetate solution of 3-nitroperylene with an absorbance at 465 nm equal to the 465 nm absorbance of the extract.

A mixture of the microsomal metabolites of 3-nitroperylene was obtained from incubations designed to examine the protein binding of the metabolites by PAGE (see section 2.8 for incubation conditions). The combined ethyl acetate extracts from such an experiment were dried over anhydrous $MgSO_4$ and filtered. The solvent was removed under reduced pressure at 40°C using a rotary evaporator and the residue was dissolved in 200 µl of THF. 25 µl was analysed by HPLC and the radioactivity profile determined as described above.

2.7. Covalent Binding to Protein

Each incubation contained the following: 125 nmol [^{3}H]3-nitroperylene (specific activity 182 mCi/mmol) in 75 µl DMSO; 8 µmol $MgCl_2$; 5 µmol glucose-6-phosphate; 3 IU glucose-6-phosphate dehydrogenase; 4 µmol $NADP^+$; 100 µmol sodium phosphate buffer (pH 7.4) and 1 mg of microsomal protein in a total volume of 1 ml.

All components except 3-nitroperylene were mixed and

preincubated at 37°C for 2 minutes before the nitroperylene was added. Following subsequent incubation for the appropriate time, the reaction was terminated by the addition of 1 ml of ice-cold acetone. Most of the unmetabolized nitroperylene and non-covalently bound metabolites were removed by extracting the mixture 3 times with 2 ml of ethyl acetate. Macromolecules were precipitated by the addition of 2 ml of ice-cold 95% ethanol. The precipitate was removed by centrifugation at 2000 g for 10 min at 4°C. The supernatant was decanted and retained for scintillation counting. The label recovered in this fraction was used as a measure of the water soluble metabolites of 3-nitroperylene.

The protein pellet was washed twice with 2 ml of diethyl ether: 95% ethanol (2:1) then dissolved in 2 ml of 0.2 M sodium phosphate buffer (pH 6.8) (containing 1% SDS and 0.1% β-mercaptoethanol) and placed in a boiling water bath for 2 minutes. A 0.5 ml sample was applied to a small (0.7 x 7.5 cm) Sephadex G-25 column and eluted with 0.2M sodium phosphate buffer (pH 6.8) (containing 1% SDS). Fractions were collected at 1 min intervals and the radioactivity profile determined by counting a 50 µl sample of each fraction. Preliminary experiments showed one main peak of radioactivity which eluted at the void volume along with the majority of the protein. The peak fractions were pooled and assayed for both radioactivity and protein content. Protein concentrations were determined by the method of Lowry et. al. (1951) using BSA as a standard. Results were expressed as nmol bound per mg of protein.

Several other procedures were employed to separate non-covalently bound label from covalently bound material. Extensive

washing with organic solvents proved rather ineffective. Prolonged dialysis was as effective as the Sephadex G-25 chromatography procedure described above, but the latter method was more convenient and was adopted as the standard procedure.

All incubations were carried out in duplicate and experiments were conducted at least twice.

2.8. Polyacrylamide Gel Electrophoresis

For the detection of metabolites of 3-nitroperylene covalently bound to protein by polyacrylamide gel electrophoresis (PAGE), the incubation conditions were modified slightly to achieve greater incorporation of radioactivity into the protein. The concentrations of each component, with the exception of 3-nitroperylene, were as described in the previous section. The total incubation volume was 2 ml. [^{3}H]3-Nitroperylene (specific activity 728 mCi/mmol) was added in 200 µl of DMSO to give a final concentration of 62.5 nmol/ml.

The mixture was incubated for 2 hours at 37^{o}C then the reaction was terminated by the addition of 2 ml of ice cold acetone. Non-covalently bound label was removed by extracting the sample 3 times with 4 ml of ethyl acetate, and protein was precipitated by the addition of 8 ml of ice-cold acetone. Precipitated protein was collected by centrifugation at 2000g for 10 minutes at 4^{o}C, then washed with 4 ml of diethyl ether:95% ethanol (2:1). The final pellet was dissolved in 2 ml of 0.01 M tris-HCl (pH 6.8) (containing 1% SDS and 0.1% β-mercaptoethanol) and placed in a boiling water bath for 2 minutes prior to electrophoresis.

SDS-PAGE was carried out using 7.5% tube gels (5.5 mm x 8.5 cm) according to the method of Laemmli (1970). Portions of the labelled protein sample prepared above were mixed with 0.4 volumes of glycerol and 5 µl of 0.1% bromophenol blue before application to gels.

Following electrophoresis, gels were stained for 8 hours in 0.8% (w/v) amido black in methanol:acetic acid:water (45:10:45), destained, then photographed. To analyse the gels for radioactivity they were frozen at -20°C then sliced into 1 mm segments using a battery of razor blades. Each segment was digested in 0.5 ml of 15% (v/v) hydrogen peroxide at 60°C overnight and the digests were analysed by scintillation counting using Formula 947 cocktail (New England Nuclear).

2.9. Covalent Binding to DNA

Each incubation contained the following: 80 nmol [^{3}H]-nitroperylene (specific activity 570 mCi/mmol) in 100 µl of DMSO; 8 µmol $MgCl_2$; 5 µmol glucose-6-phosphate; 3 IU glucose-6-phosphate dehydrogenase; 4 µmol NADP+; 50 µmol tris-HCl (pH 7.5); 1 mg microsomal protein and 0.5 mg of calf thymus DNA.

All components with the exception of 3-nitroperylene were mixed and preincubated at 37°C for 2 minutes before the substrate was added. The incubations were continued for the appropriate time at 37°C then the mixture was extracted 4 times with 2 ml of ethyl acetate to remove most of the non-covalently bound label. The aqueous phase was then extracted once with 1.5 ml of buffer (50 mM tris-HCl, pH 7.5) saturated phenol:isoamyl alcohol:chloroform (24:1:25) and 3 times with 1 ml of

the same phenol solution. In each case the phases were separated by low speed centrifugation in a Sorvall Superspeed benchtop centrifuge. The volume of the final aqueous phase was adjusted to 1 ml with 50 mM tris-HCl (pH 7.5) and nucleic acids were precipitated overnight at -20°C following the addition of 3 ml of ice-cold 95% ethanol. The precipitated nucleic acid was collected by centrifugation at 10,000g for 15 min at 4°C and dissolved in 1 ml of 5 mM bis-tris buffer (pH 7.1) (containing 0.1% EDTA). The DNA solution was extracted with 2 ml of ethyl acetate to ensure that no further non-covalently bound label remained. A sample of the extract was subjected to scintillation counting and in no case was a level of radioactivity above background found. The ionic strength of the remaining aqueous phase was increased by the addition of 25 µl of 0.4M $MgCl_2$ and 100 µl of 3.0 M sodium acetate (previously adjusted to pH 5.0 with acetic acid) and nucleic acids were again precipitated overnight at -20°C following the addition of 3 ml of 95% ethanol. The precipitated nucleic acid was collected by centrifugation and dissolved in 1 ml of bis-tris buffer as described above. The DNA concentration was determined spectrophotometrically at 260 nm using a Beckman Model 25 Spectrophotometer and the radioactivity in each sample was determined by scintillation counting. An A_{260} of 1.0 is assumed to represent a DNA concentration of 50 µg/ml. Results are presented as adducts per 10^6 nucleotides.

Duplicate incubations were carried out at each time point and the experiment was repeated on 2 occasions.

3. THE MUTAGENICITY OF 3-NITROPERYLENE AND RELATED COMPOUNDS

3. The Mutagenicity of 3-Nitroperylene and Related Compounds

3.1. Results

3.1.1. Comparative Mutagenicity of Perylene and B(a)P Derivatives

The mutagenicity of perylene, B(a)P and some of their derivatives has been investigated using the Ames test. Some comparative data are presented in Table 1. These data were obtained using S. typhimurium strain TA98, the strain most sensitive to the majority of the compounds being tested (see section 3.1.5.).

Perylene itself was a very poor mutagen in this test, whereas two of its nitrated derivatives, 3-nitroperylene and dinitroperylene (II), were potent mutagens. The mononitro compound was almost exclusively an indirect acting mutagen, since little mutagenicity was seen in the absence of S9, while dinitroperylene (II) was predominantly a direct acting mutagen. The latter, however, also retained substantial activity in the presence of S9. The other nitrated perylene studied, dinitroperylene (I), was only a weak indirect acting mutagen. 3-Aminoperylene proved a moderately strong mutagen in the presence of S9, although it is conceivable that this activity reflects the mutagenicity of impurities (since the preparation was only 70% pure), rather than that of 3-aminoperylene itself. The two bromoperylenes tested were non-mutagenic.

When compared to the highly mutagenic nitroperylenes, B(a)P was a relatively weak indirect acting mutagen. However, nitration of the hydrocarbon, to give 6-nitroB(a)P, brought about a 3-4 fold increase in mutagenic potency.

Further investigation of the mutagenicity of 3-nitroperylene

TABLE 1. The Comparative Mutagenicity of Perylene and B(a)P Derivatives in Salmonella typhimurium Strain TA98.

Compound[a]	Mutagenic Potency (Revertants/nmol)[b,c]	
	+ S9 [d]	- S9
Perylene	7 ± 3	e
3-Nitroperylene	1313 ± 27	17 ± 3
3-Aminoperylene	241 ± 31	32 ± 9
3-Bromoperylene	e	e
Dinitroperylene (I)	45 ± 4	16 ± 2
Dinitroperylene (II)	243 ± 6	1089 ± 63
Dibromoperylene	e	e
B(a)P	65 ± 6	e
6-NitroB(a)P	204 ± 6	5 ± 2

a. For 3-nitroperylene, 3-aminoperylene and the two dinitroperylenes results were obtained using 1 nmol of test compound per plate. The value for 6-nitroB(a)P was calculated from assays using 5 nmol/plate. Results for all other compounds were calculated from reversion frequencies obtained using 10 nmol/plate.

b. Means ± standard errors from triplicate plates.

c. Spontaneous reversion rates have been subtracted from all values. (+ S9, 40 revertants/plate; - S9, 25 revertants/plate.)

d. S9 from AR pretreated rats was used in each case. Perylene and its derivatives were tested using 2% S9, the optimum concentration for 3-nitroperylene. B(a)P and 6-nitroB(a)P were tested using 5% S9, their optimum concentration.

e. Value less than 5 revertants/plate above spontaneous reversion rate.

revealed that the S9 enzymes responsible for its activation were inducible, i.e. S9 prepared from control rats not treated with microsomal enzyme inducers provided some activation but was far less effective than S9 from AR, PB, MC or NF treated animals (Fig. 1). The activation was also dependent on NADPH or NADH as a source of reducing equivalents (Fig. 1; Table 2). When S9 was used to provide activation, NADPH was more effective than NADH, but when microsomes alone were used, NADH was almost as effective as NADPH (Table 2). Fractionation of S9 into microsomal and soluble protein components indicated that the enzymes involved in the activation of 3-nitroperylene reside almost entirely in the microsomes (Table 2), although a small amount of glucose-6-phosphate dehydrogenase, a cytosolic enzyme, had to be added to regenerate reduced pyridine nucleotide cofactors (data not shown).

The dose-response curve for the mutagenicity of 3-nitroperylene with AR induced S9 shows a slight deviation from linearity at low mutagen concentrations (Fig. 1). This deviation was investigated more thoroughly using concentrations of nitroperylene of less than 1 nmol/plate and was found to be both significant and reproducible (data not shown). Similar studies with S9 from PB and MC induced rats failed to show a non-linear response.

3.1.2. Differential Induction of S9 Enzymes and its Effect on the Mutagenicity of Selected Compounds

The use of S9's from rats pretreated with various inducers of the microsomal MFO system provides information on the various forms of cytochrome P-450 involved in the activation of compounds to proximate

FIGURE 1. The effects of S9's from rats treated with microsomal enzyme inducers on the mutagenicity of 3-nitroperylene in S. typhimurium strain TA98.

Dose response curves are shown for the mutagenicity of 3-nitroperylene in the absence of S9 (✕—✕) and in the presence of S9 from control rats (▲—▲), or rats treated with the MFO enzyme inducers PB (●—●), MC (△—△), NF (□—□) and AR (○—○). The curve for AR S9 in the absence of $NADP^+$ is also shown (■—■).

All S9's were used at 5%, which was their optimum concentration for 3-nitroperylene activation. (Determined in separate experiments).

Spontaneous reversion rates have been subtracted from all values (+ S9, 40 revertants/plate; - S9, 25 revertants/plate).

Points represent the means ± standard errors from triplicate plates. Standard errors have been omitted from the lower three curves.

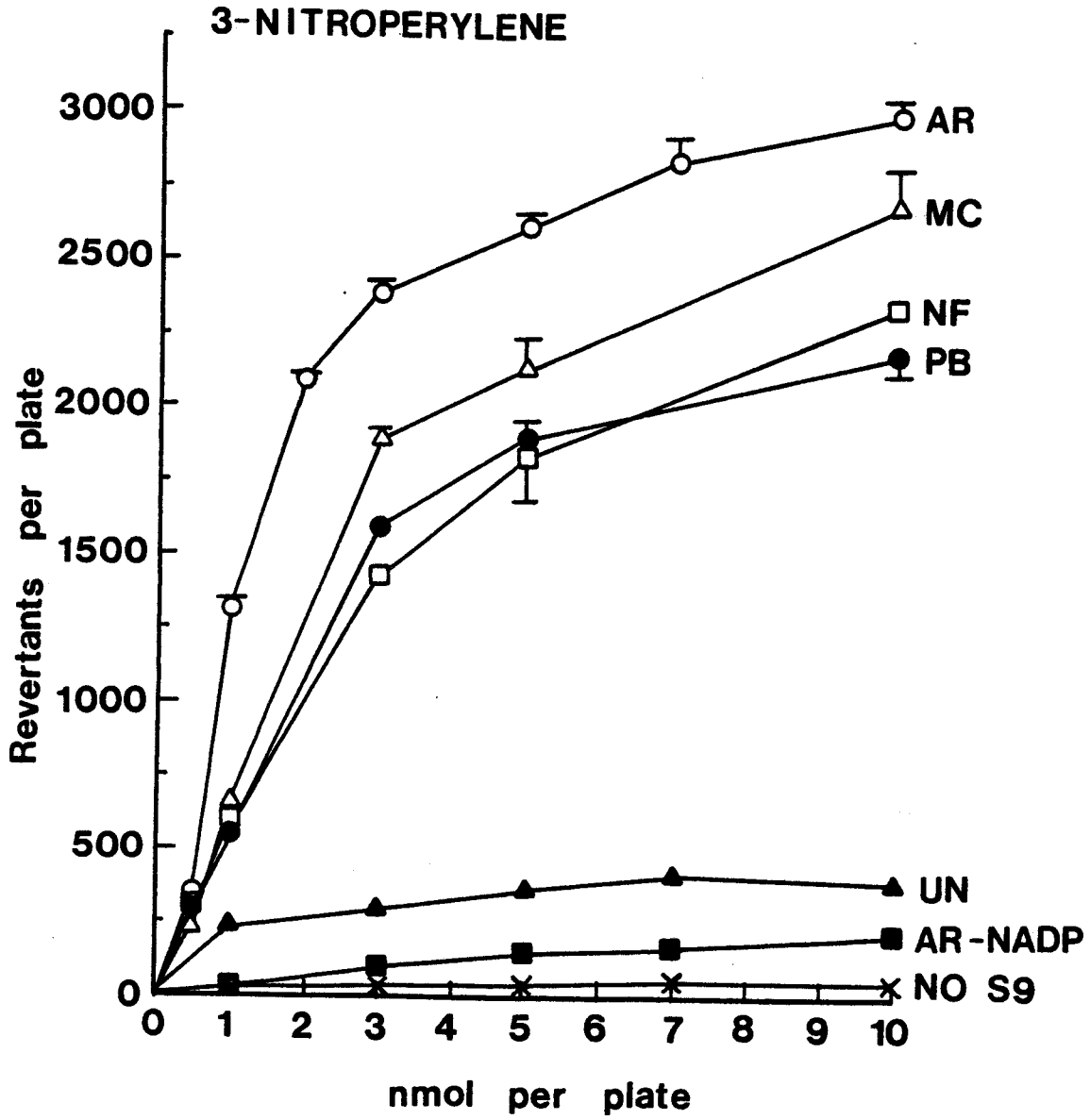

3-NITROPERYLENE
Revertants per plate
3000
2500
2000
1500
1000
500
0
0 1 2 3 4 5 6 7 8 9 10
nmol per plate
AR
MC
NF
PB
UN
AR-NADP
NO S9

TABLE 2. Distribution of 3-Nitroperylene Activating Ability in Aroclor Induced Rat Liver S9 and Its Cofactor Dependency.

S9 Fraction[a]	Revertants per nmol of 3-nitroperylene[b,c,d]	
	+ $NADP^+$	+ NAD^+
Whole S9	1425 ± 31	877 ± 56
Microsomes	1288 ± 39	1027 ± 53
S105	66 ± 6	44 ± 3
Reconstituted S9	1333 ± 87	1005 ± 125

a. All preparations were supplemented with glucose-6-phosphate dehydrogenase (3 IU/assay).

b. In S. typhimurium strain TA98 using 2% S9.

c. Means ± standard errors from triplicate plates.

d. The spontaneous reversion rate (40 revertants/plate) was subtracted from each valve.

mutagens (Conney, 1982). S9's were prepared from rats pretreated with PB, MC and NF in addition to AR. B(a)P was used as a standard indirect-acting mutagen in the Ames test and, as expected, was strongly activated by MC and NF S9 but showed little activity when PB S9 was used (Fig. 2b). When 3-methylcholanthrene was used as a mutagen the opposite result was observed, and only PB S9 provided strong activation (data not shown). These results are in accordance with mutagenicity data reported in the literature (Ames et. al., 1975) and reflect accurately the forms of P-450 known to be involved in the activation of these two mutagens (Conney, 1982), indicating that the pretreatments employed have been successful in inducing one or other of the two major forms of P-450 rather specifically.

The pattern of activation of 6-nitroB(a)P by PB, MC, and NF S9's was similar to that of its parent hydrocarbon (Fig. 2a) suggesting that the same major form of P-450 which is involved in B(a)P activation is also involved in 6-nitroB(a)P activation. The AR induced S9 used in these experiments was able to activate 6-nitroB(a)P more strongly than either MC or NF S9's while, surprisingly, it was less effective in activating B(a)P than MC and NF S9's, but still much more effective than PB S9. The result with B(a)P appears to reflect differences in the concentration of each S9 required for optimum activation, and in fact, additional experiments showed that other AR S9 preparations were able to activate B(a)P just as well, or even more effectively, than MC and NF S9's (data not shown). The particularly strong activation of 6-nitroB(a)P by AR S9 may also reflect differences in optimum S9 concentrations, but the possibility that additional forms of P-450,

FIGURE 2. The effects of S9's from rats treated with microsomal enzyme inducers on the mutagenicity of B(a)P and 6-nitroB(a)P in S. typhimurium strain TA98.

Symbols are explained in Figure 1.

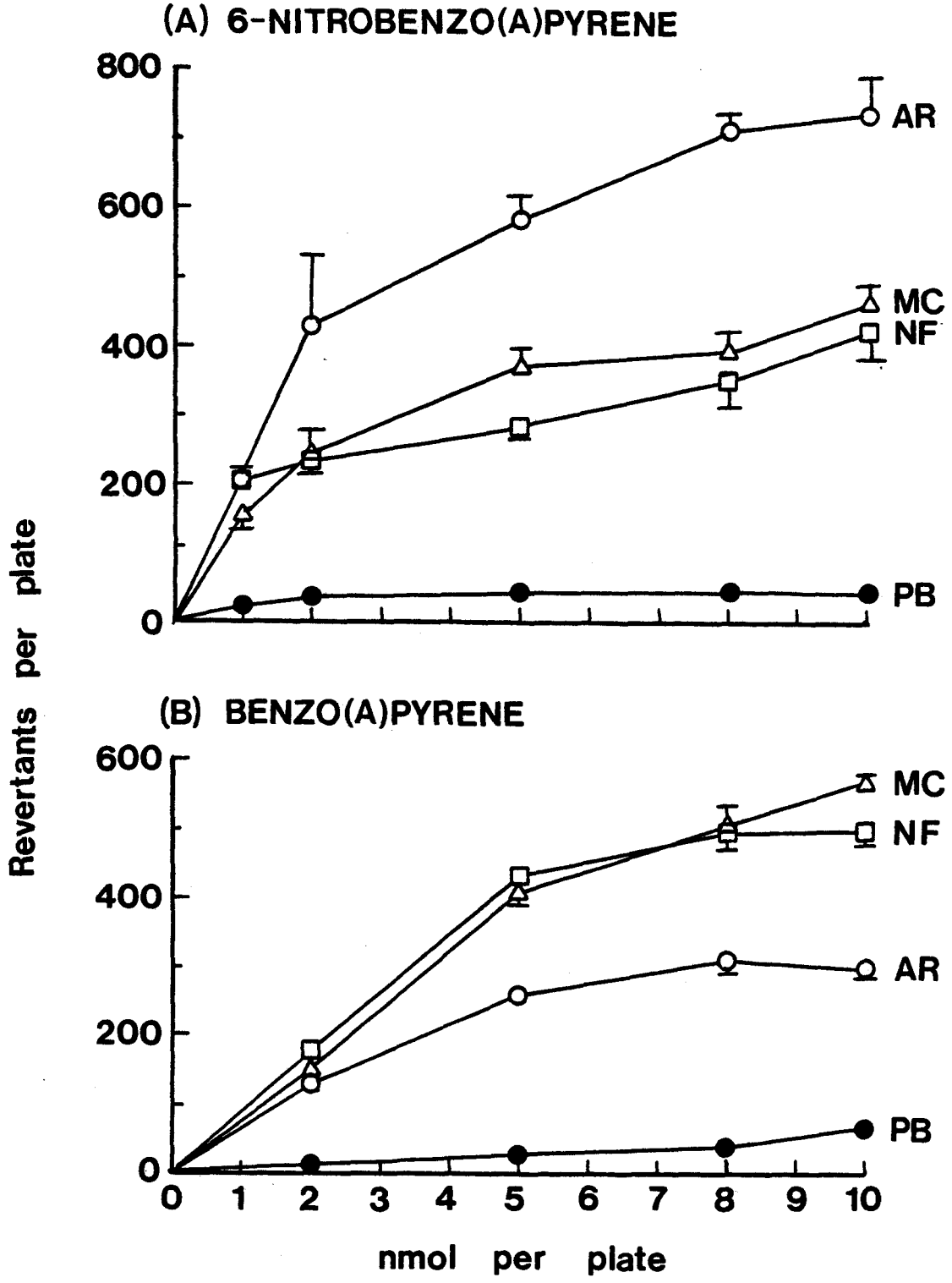
All S9's were used at a concentration of 5%.

The spontaneous reversion rate (40 revertants/plate) was subtracted from each value.

Points represent means ± standard errors from tripliate plates.

(A) 6-NitroB(a)P

(B) B(a)P


(A) 6-NITROBENZO(A)PYRENE
800
600
400
200
0
AR
MC
NF
PB
(B) BENZO(A)PYRENE
600
400
200
0
MC
NF
AR
PB
0 1 2 3 4 5 6 7 8 9 10
nmol per plate
Revertants per plate

induced by AR but not by MC or NF, are also involved in its activation cannot be discounted.

In contrast to both B(a)P and 6-nitroB(a)P, 3-nitroperylene was activated strongly and almost equally well by each of the induced S9's used (Fig. 1) implicating both major forms of P-450 in its activation.

3.1.3. The Effects of Microsomal Enzyme Inhibitors and Metabolite Conjugating Reagents

To complement the studies with S9's prepared from rats pretreated with various MFO inducers, the mutagenicities of 3-nitroperylene, 6-nitro B(a)P and B(a)P were examined in the presence of various MFO enzyme inhibitors (Fig. 3). In support of the earlier data, the mutagenicities of both B(a)P and 6-nitroB(a)P were substantially decreased in the presence of α- and β-naphthoflavone, both specific inhibitors of the major MC inducible form of cytochrome P-450 (P-450c), while metyrapone, an inhibitor of the major PB inducible form of P-450 (P-450b), slightly increased the mutagenicity of both compounds. The mutagenicity of 3-nitroperylene was also increased by metyrapone, but to a somewhat greater extent than that of the other two compounds. However, a clearly different pattern was observed when the effects of the naphthoflavones on 3-nitroperylene activation were investigated. The mutagenic activity of this compound was slightly inhibited by α-naphthoflavone, whereas β-naphthoflavone brought about a significant increase in the activity, a response opposite to that shown with B(a)P and 6-nitroB(a)P. This pattern of inhibition and activation for 3-nitroperylene does not conform with

FIGURE 3. The effects of microsomal enzyme inhibitors on the mutagenicity of 3-nitroperylene, B(a)P and 6-nitroB(a)P.

The effects of increasing concentrations of four MFO enzyme inhibitors on the mutagenicities of 3-nitroperylene (●—●), B(a)P (■—■) and 6-nitroB(a)P (▲—▲) were studied in S. typhimurium strain TA98. The amounts of each mutagen used were 0.5, 10 and 5 nmol/plate respectively.

For 3-nitroperylene 1% AR S9 was used. For B(a)P and 6-nitroB(a)P the S9 concentration was 5%.

All results were obtained using the liquid preincubation modification of the Ames assay and are reported as percentages of control assays which contained 50 µl of DMSO in place of 50 µl of the appropriate inhibitor.

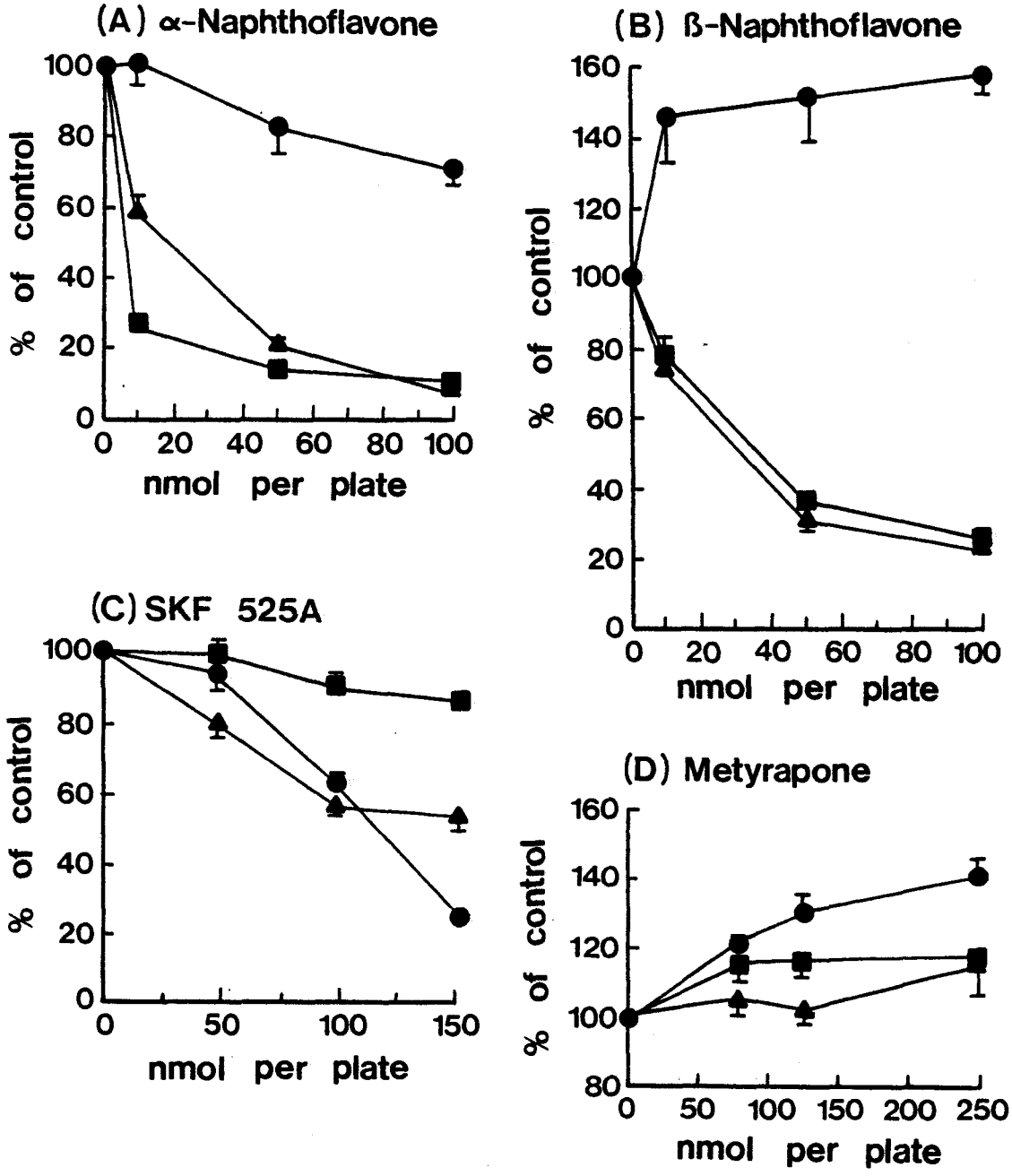
Means ± standard errors from triplicate plates are presented.

(A) α-naphthoflavone

(B) β-naphthoflavone

(C) SKF 525-A

(D) methyrapone


(A) α-Naphthoflavone
% of control
nmol per plate
(B) ß-Naphthoflavone
% of control
nmol per plate
(C) SKF 525A
% of control
nmol per plate
(D) Metyrapone
% of control
nmol per plate

that expected from the results with different S9's reported above.

In contrast to the results with the other inhibitors, SKF-525A was able to decrease the mutagenicities of both nitro compounds quite substantially, whereas the activation of B(a)P was only slightly depressed.

Reduced glutathione, cysteine and UDPGA are all compounds which are able to conjugate with many of the products of MFO reactions. Each of these decreased the mutagenicity of 3-nitroperylene, 6-nitroB(a)P and B(a)P (Fig. 4). UDPGA was particularly effective (Fig. 4c). 6-Nitro B(a)P showed an interesting response to GSH (Fig. 4a), with inhibition of mutagenicity being seen at the highest concentration used, but enhancement at lower concentrations.

3.1.4. Optimum S9 Concentration for 3-Nitroperylene Mutagenicity

When AR S9 was used to activate 3-nitroperylene to mutagenic species, strong activation was achieved only over a narrow range of S9 concentrations which were somewhat lower than the concentrations recommended for general screening procedures in the Ames assay. For example, Figure 5 provides a comparison of the S9 optima of 3-nitroperylene and B(a)P in both the standard plate incorporation assay and the liquid preincubation modification of the assay. The latter increases the sensitivity of the Ames assay for many mutagens, including 3-nitroperylene as Figure 5 indicates. In each form of the assay, the mutagenicity of B(a)P is maximal at an S9 concentration which is several fold higher than the optimum S9 concentration for 3-nitroperylene. In addition, the range of S9 concentrations over

FIGURE 4. The effects of conjugating reagents on the mutagenicity of 3-nitroperylene, B(a)P and 6-nitroB(a)P.

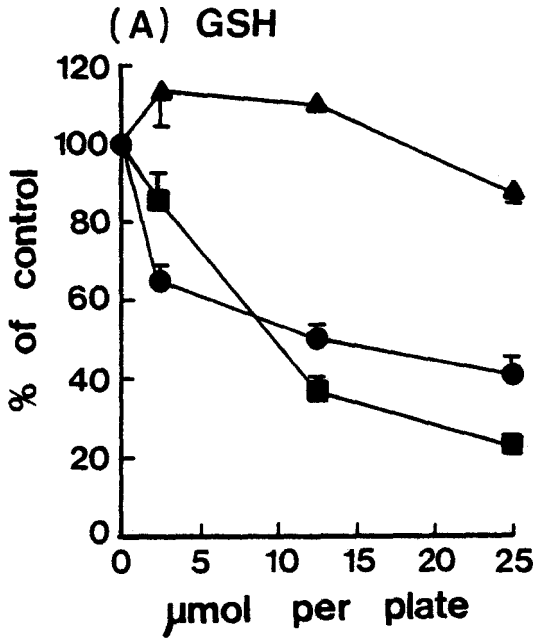
The effects of increasing concentrations of three conjugating reagents on the mutagenicity of 3-nitroperylene (●—●), B(a)P (■—■), and 6-nitroB(a)P (▲—▲) were studied in S. typhimurium strain TA98. The amounts of each of these mutagens used in each assay were 1, 10 and 5 nmol respectively. Assays with 3-nitroperylene used 1% S9 and those with B(a)P and 6-nitroB(a)P used 5% S9.

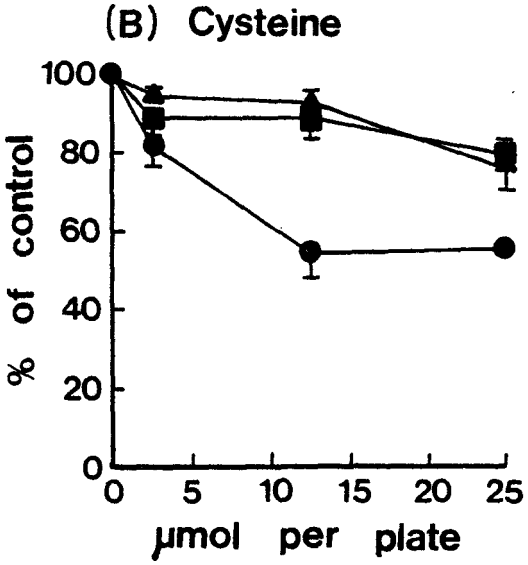
All results were obtained using the standard plate incorporation assay, and are reported as a percentage of control assays which contained 50 µl of water in place of 50 µl of the appropriate conjugating reagent. Means ± standard errors of triplicate plates are presented.

(A) reduced glutathione

(B) cysteine

(C) UDPGA


(A) GSH
% of control
120
100
80
60
40
20
0
0 5 10 15 20 25
μmol per plate


(B) Cysteine
% of control
100
80
60
40
20
0
0 5 10 15 20 25
μmol per plate

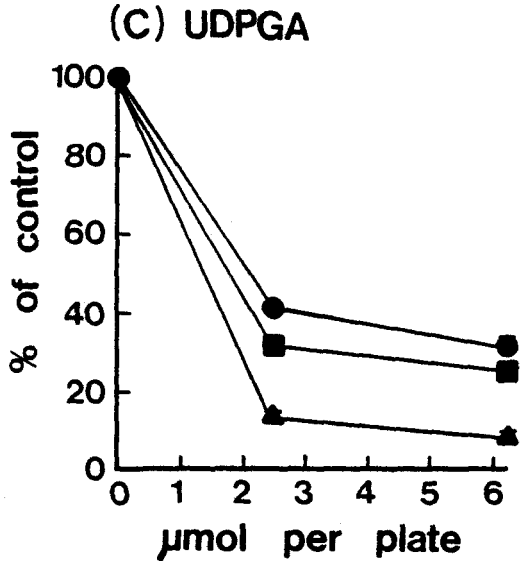

(C) UDPGA
% of control
100
80
60
40
20
0
0 1 2 3 4 5 6
μmol per plate

FIGURE 5. S9 optima of 3-nitroperylene and B(a)P in both the plate incorporation and preincubation forms of the Ames assay.

Solid lines indicate experiments with 3-nitroperylene. In the plate incorporation assay 1 nmol of mutagen was used per test plate, while in the liquid preincubation assay 0.5 nmol/plate was used.

Broken lines indicate experiments with B(a)P. 10 nmol/plate was used in each form of the Ames assay.

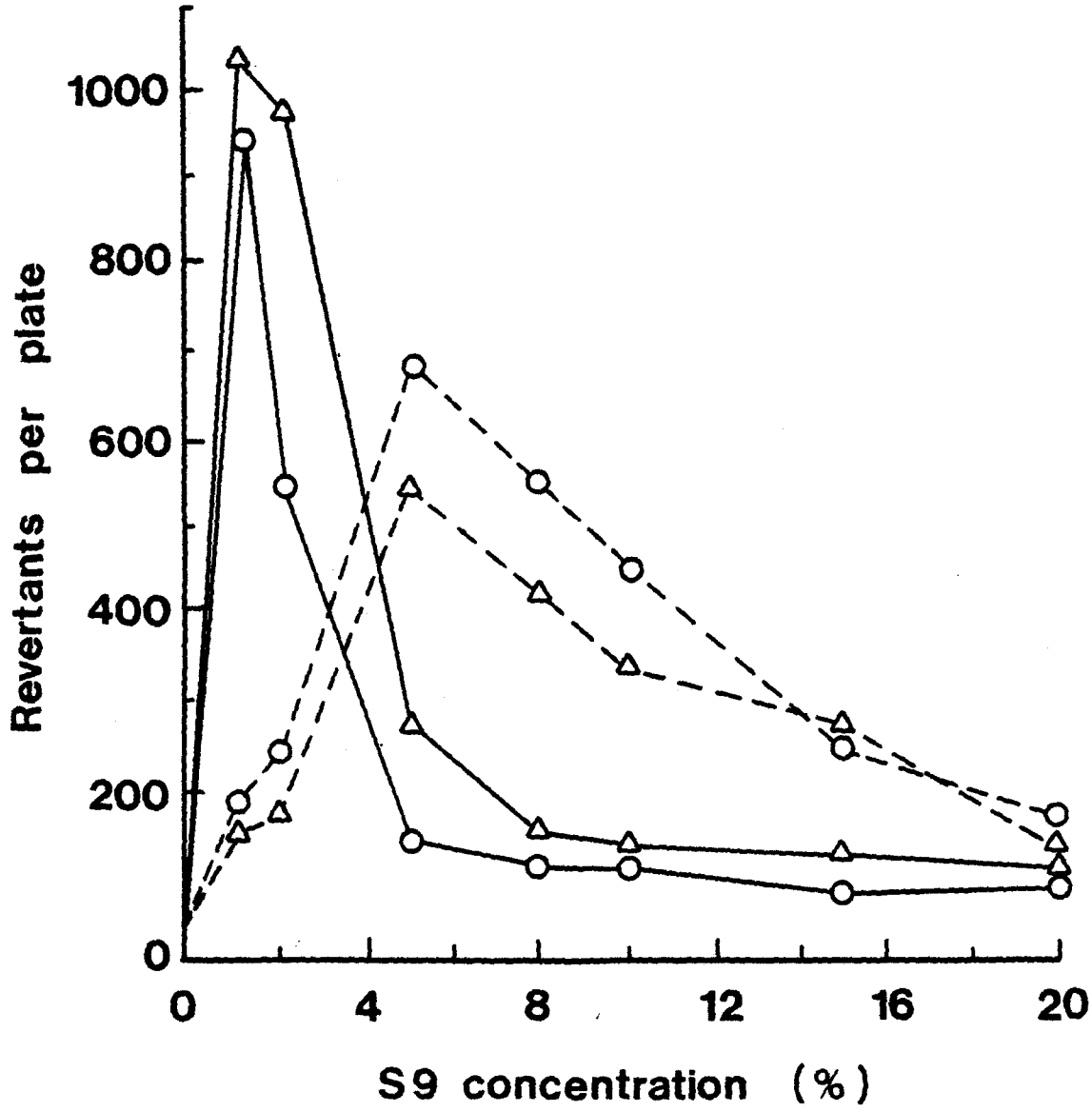
S. typhimurium strain TA98 was used for all assays.

Spontaneous reversion rates have not been subtracted.

Means of triplicate plates are presented. For the sake of clarity, standard errors have been omitted but in no case were they greater than 10% of the mean.

Plate incorporation (△–△).

Liquid preincubation (O–O).


Revertants per plate
1000
800
600
400
200
0
0
4
8
12
16
20
S9 concentration (%)

which reasonable activation of B(a)P occurred was much broader than the range of S9 concentrations which would activate 3-nitroperylene strongly.

When MC, NF, and PB S9's were used to activate 3-nitroperylene, all were effective over a wider range of S9 concentrations than AR S9 (Fig. 6). In contrast to these results with various induced S9's, the mutagenicity seen with uninduced S9 increased steadily with increasing S9 concentration rather than showing a peak of activity.

In view of the finding that strong activation of 3-nitroperylene occured only over a narrow range of AR S9 concentrations, each S9 preparation was screened to find its optimal concentration, and subsequent experiments with 3-nitroperylene were carried out at that concentration. All AR S9's provided maximum activation at concentrations from 1-2%, with the exception of one batch which showed an optimum between 2 and 5%.

3.1.5. Mutagenicity of 3-Nitroperylene and Related Compounds in Various Strains of S. typhimurium

The mutagenic activity of 3-nitroperylene has been determined in a number of S. typhimurium strains (Fig. 7). Strain TA98 was the most sensitive to the mutagen, and has been used routinely in the studies reported in this chapter. There was also substantial activity in TA100, but this strain has a very high spontaneous mutation rate and, in terms of the percentage increase over the frequency of spontaneous reversion, TA98 is many-fold more sensitive. There was some activity in TA1538, the strain from which TA98 was derived by

FIGURE 6. S9 optima of 3-nitroperylene using S9's prepared from control and PB, MC, NF or AR pretreated rats in S. typhimurium strain TA98.

All results were obtained using the plate incorporation assay at a mutagen concentration of 1 nmol/plate.

Spontaneous reversion rates have not been subtracted.

Values represent the means of triplicate plates. Standard errors have been omitted for the sake of clarity, but in no case were they greater than 10% of the mean indicated.

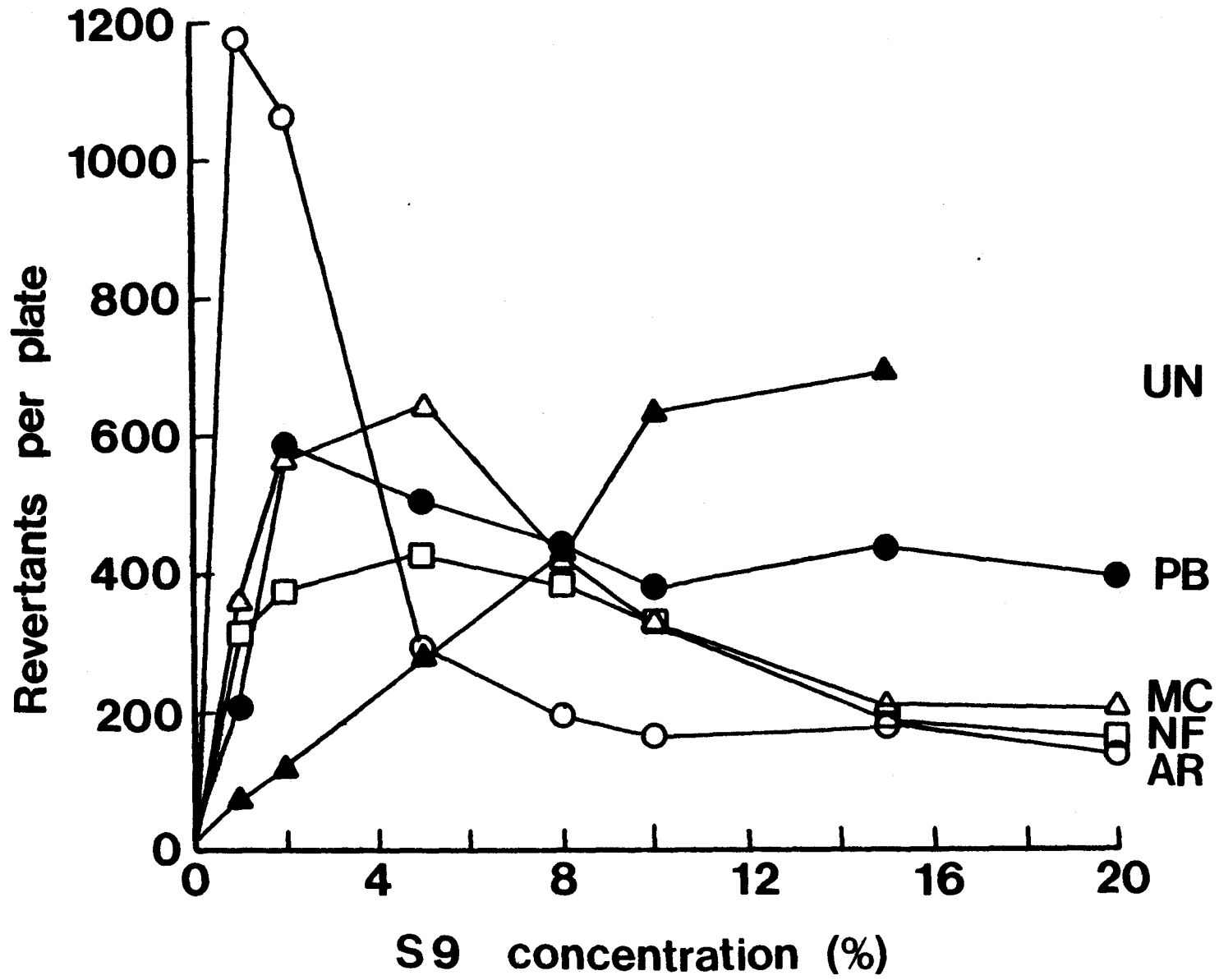

1200
1000
800
600
400
200
0
Revertants per plate
0
4
8
12
16
20
S9 concentration (%)
UN
PB
MC
NF
AR

FIGURE 7. The relative mutagenicity of 3-nitroperylene in various strains of Salmonella typhimurium.

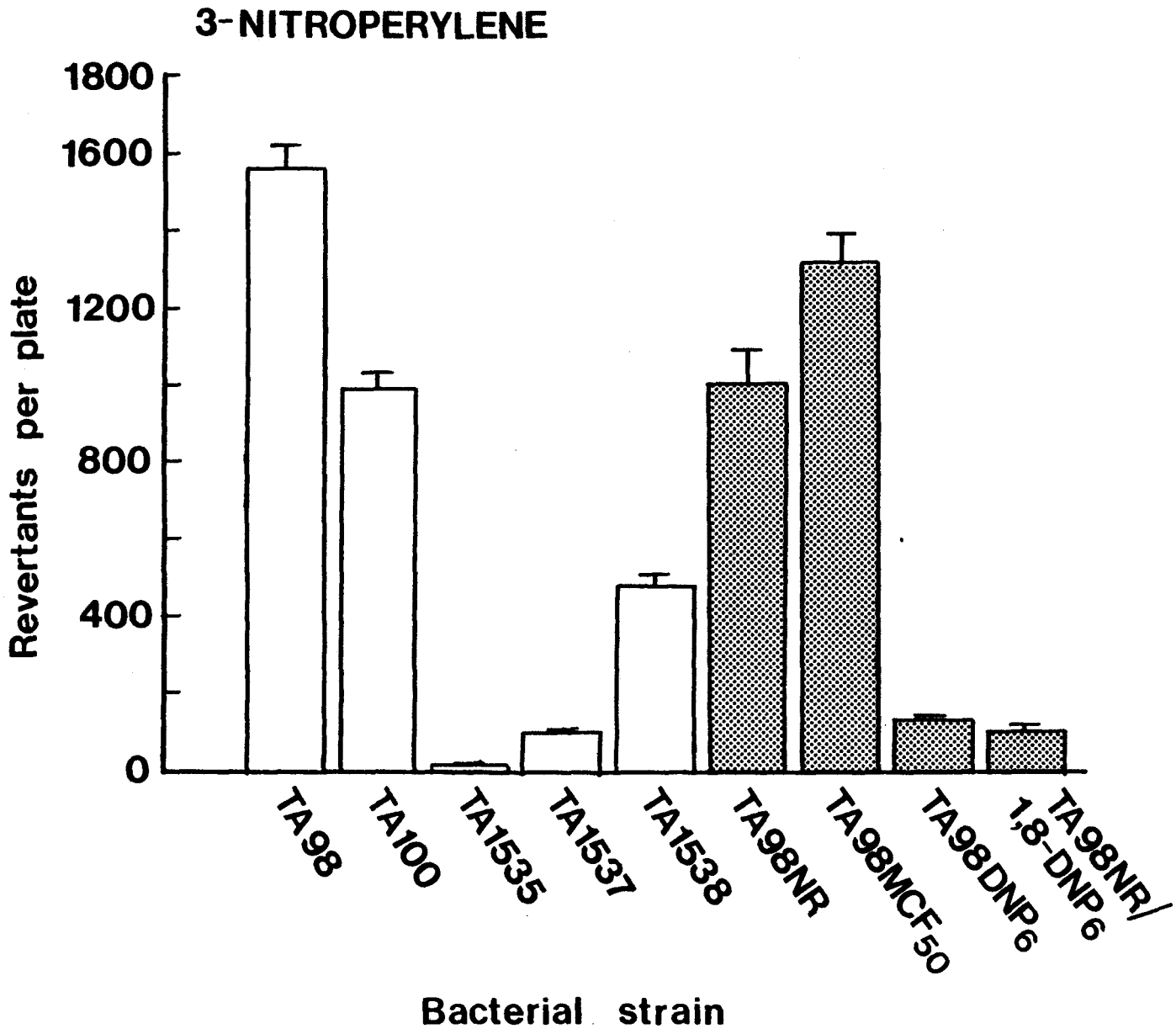
1 nmol of mutagen was used in each assay.

All experiments were carried out using 1% AR S9.

Spontaneous reversion rates were subtracted from each mean. The numbers of spontaneous his^+ revertants for each strain were as follows: TA98, 29; TA100, 214; TA1535, 32; TA1537, 10; TA1538, 27; TA98NR, 21; TA98 MCF_{50}, 40; TA98 DNP_6, 30; TA98NR/1,8-DNP_6, 12.

Shaded columns indicate nitroreductase-deficient strains.

Results are presented as means ± standard errors from triplicate plates.


3-NITROPERYLENE
1800
1600
1200
800
400
0
Revertants per plate
TA98
TA100
TA1535
TA1537
TA1538
TA98NR
TA98MCF50
TA98DNP6
TA98NR/
1,8-DNP6
Bacterial strain

introduction of the pKM101 plasmid, but only very low activity in TA1535 and TA1537.

Since reduction of the nitro group appears to be involved in the metabolic activation of at least some of the nitroPAH's, it was of interest to examine the mutagenicity of 3-nitroperylene in nitroreductase-deficient strains of Salmonella (Fig. 5). Strains TA98NR and $TA98MCF_{50}$, which have a known nitroreductase deficiency, were only slightly less sensitive to 3-nitroperylene than TA98 suggesting that the enzyme missing in these strains is not critically involved in the activation of the mutagen. However, TA98 DNP_6 and the double mutant TA98NR/1,8-DNP_6 were very resistant to mutation by 3-nitroperylene. The nature of the enzyme deficiency caused by the DNP_6 mutation in these strains is not well understood.

Some comparative data on the mutagenicities of B(a)P, 6-nitro B(a)P, and dinitroperylene (II) in the nitroreductase deficient Salmonella strains is presented in Figures 8 and 9. Dinitroperylene (II) showed essentially the same mutation pattern as 3-nitroperylene, both in the presence and absence of S9 (Fig. 9), whereas both B(a)P and 6-nitro B(a)P retained most of their mutagenic activity in each of the strains used with the exception of the double mutant (Fig. 8). For both of these mutagens the activity of TA98NR/1,8-DNP_6 was only about 50% of that seen with TA98. This probably reflects the slow growth of this strain rather than an effect of its enzyme deficiencies. For the nitroperylenes studied the activity in the double mutant was less than 10% of its value in TA98, indicating that, for these compounds, effects other than slow growth are responsible for the low mutagenicity in this

FIGURE 8. The relative mutagenicities of 6-nitroB(a)P and B(a)P in various strains of Salmonella typhimurium.

5 nmol of 6-nitroB(a)P or 10 nmol of B(a)P was used in each assay.

All experiments were conducted in the presence of 5% AR · S9.

Spontaneous reversion rates were subtracted in each case (see caption to Figure 7).

Results are presented as means ± standard errors from triplicate plates.

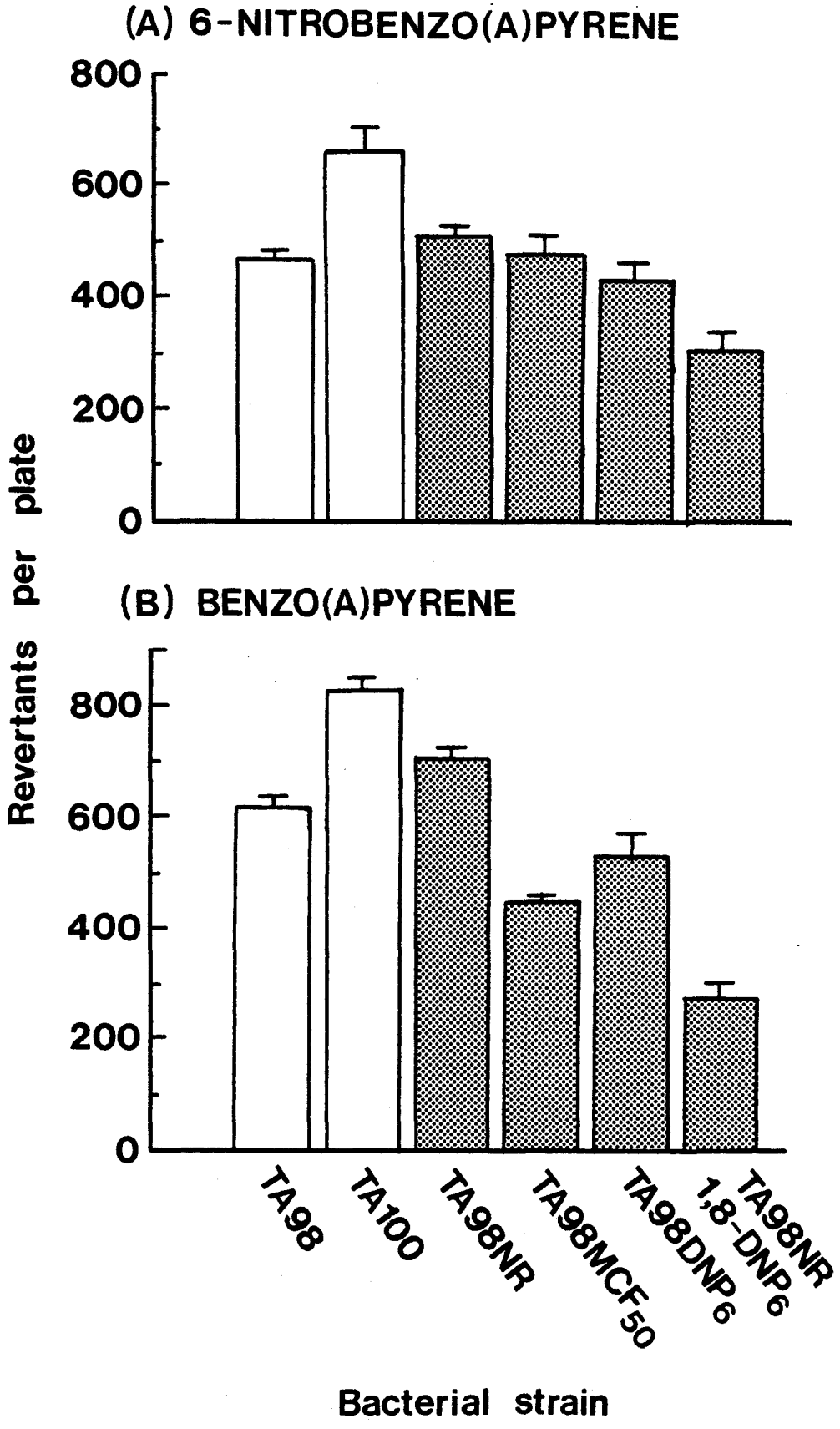

(A) 6-NITROBENZO(A)PYRENE
800
600
400
200
0
(B) BENZO(A)PYRENE
800
600
400
200
0
Revertants per plate
TA98
TA100
TA98NR
TA98MCF50
TA98DNP6
1,8-DNP6
TA98NR
Bacterial strain

FIGURE 9. The relative mutagenicity of dinitroperylene(II) in various strains of Salmonella typhimurium.

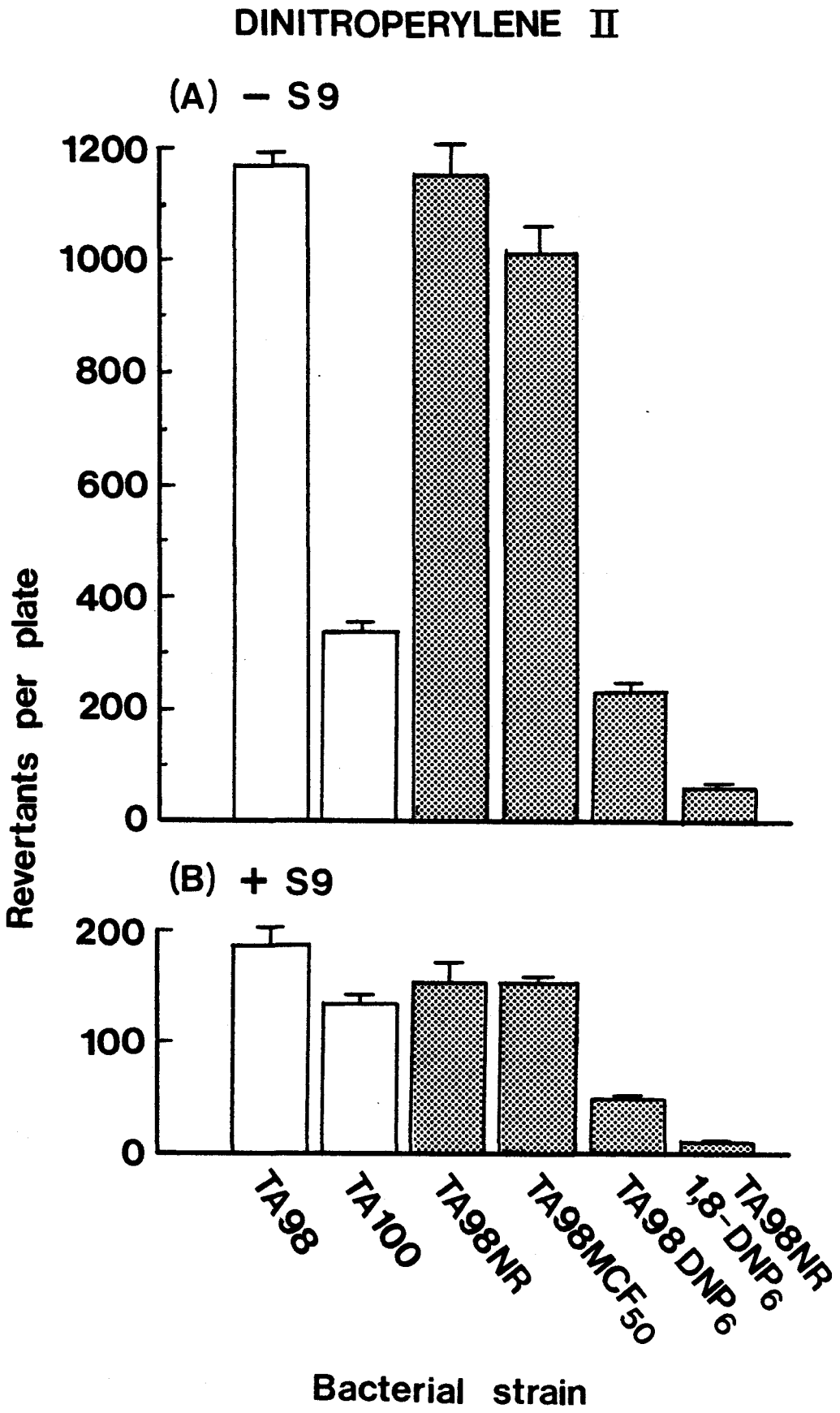
1 nmol of mutagen was used in each assay.

Experiments were carried out in the absence (A) or presence (B) of AR S9. When required S9 was used at 1%.

Spontaneous reversion rates were subtracted from each mean. The numbers of spontaneous his^+ revertants in the presence of S9 were detailed in the caption to Figure 7. The values in the absence of S9 were as follows: TA98, 19; TA100, 190; TA98NR, 15; TA98 MCF_{50}, 44; TA98 DNP_6, 22; TA98 NR/1,8-DNP_6, 22.

Shaded columns indicate nitroreductase deficient strains.

Results are presented as means ± standard errors from triplicate plates.


DINITROPERYLENE II
(A) − S9
1200
1000
800
600
400
200
0
Revertants per plate
(B) + S9
200
100
0
TA98
TA100
TA98NR
TA98MCF50
TA98DNP6
1,8-DNP6
TA98NR
Bacterial strain

strain. The low activity in strain TA98 DNP_6 supports this conclusion.

3.1.6. In situ Reduction of 3-Nitroperylene and 6-NitroB(a)P

In order to help assess the role of nitroreduction in the mutagenicity of 3-nitroperylene and 6-nitroB(a)P, these agents were tested in the Ames assay using a chemical reducing system (Zn/NH_4Cl) in place of S9 (Table 3). Slight but significant activation of both compounds was achieved indicating that, at least under some circumstances, they can be activated reductively. 1,8-Dinitronaphthalene was used as a positive control in this system and, as expected, was strongly activated.

TABLE 3. The Mutagenicity of 3-Nitroperylene, 6-NitroB(a)P and 1,8-Dinitronaphthalene Using *in situ* Reduction to Provide Activation in *S. typhimurium* strain TA98.

Mutagen	Amount per plate(nmol)	Number of his^+ revertants per plate[a,b]		
		No activation system	Zn/NH_4Cl(I)[c]	Zn/NH_4Cl(II)[d]
None	-	0	0	2 ± 1
3-nitroperylene	1	28 ± 3	37 ± 9	68 ± 10
	3	52 ± 13	48 ± 6	110 ± 8
6-nitroB(a)P	3	10 ± 1	8 ± 3	28 ± 5
	12	21 ± 3	27 ± 11	46 ± 2
1,8-dinitronaphthalene	115	8 ± 3	44 ± 4	198 ± 13
	230	22 ± 8	117 ± 7	346 ± 19

a. Spontaneous reversion rate (25 revertants / plate) subtracted from each value.

b. Means ± standard errors from triplicate plates.

c. 114 µg Zn dust/28.5 µg NH_4Cl per plate.

d. 500 µg Zn dust/125 µg NH_4Cl per plate.

3.2. Discussion

In view of the limited data on the mutagenicity of 3-nitroperylene (and indeed indirect acting nitro PAH's in general) available in the literature, the mutagenic activity of the compound has been extensively studied using the Ames assay. The aim of these experiments was to gain greater insight into the mechanisms by which 3-nitroperylene exerts its mutagenic effects, and in particular to assess the role of the nitro group in the mutagenicity.

Initial experiments confirmed the results of previous groups that 3-nitroperylene was a powerful indirect acting mutagen (Pitts et. al., 1981; Löfroth, 1982; Ho et. al., 1981). It was found to induce approximately 1500 His^+ revertants per nanomole of compound in strain TA98, a value in excellent agreement with that found by Löfroth (1982). Löfroth (1982) also reported that the dose-response curve for the mutagenicity of 3-nitroperylene was non-linear. In the present study this was confirmed at mutagen concentrations of less than 1 nmol/plate. The factors which determine the activity of various mutagens in the Ames test are complex, particularly when S9 is used to provide metabolic activation, but the observed departure from linearity probably reflects changes in the ratio of mutagen to MFO enzymes. With several PAH's, the pattern of metabolites produced by liver microsomal enzymes is known to be influenced by the substrate concentration (Levin and Conney, 1967; Holder et. al., 1975), and, in the case of 3-nitroperylene, those metabolites predominating at high S9 to substrate ratios may be less effective mutagens than those produced when the ratio is lower.

Examination of the mutagenicity of several other perylene derivatives revealed that two dinitroperylenes were also mutagenic in strain TA98. One of these, dinitroperylene (I), was a rather weak indirect acting mutagen while the other, dinitroperylene (II), was a potent direct acting mutagen. The latter also showed substantial activity in the presence of S9. This could reflect true indirect-acting behaviour or may merely be due to remaining S9-independent activity. Nevertheless, the lower mutagenicity of this dinitro isomer in the presence of S9 indicates that mammalian enzymes are able to metabolize the compound, but largely to inactive species. The fact that at least one of the nitrated perylenes is a direct acting mutagen explains why Campbell et. al. (1981) found that a crude nitration mixture of perylene demonstrated strong activity in the Ames test both in the presence and absence of S9. In the series of nitrated pyrenes, each of the mono-, di-, tri- and tetranitro derivatives is a direct acting mutagen (Mermelstein et. al., 1981) and, from the limited data available on the mutagenicity of other series of nitroPAH's, it appears that only one type of behaviour, usually direct acting, is shown within a series (Rosenkranz and Mermelstein, 1983 for summary). Thus the switch from indirect to direct activity by the addition of a second nitro group to 3-nitroperylene is a very unusual feature of this group of compounds, and could prove useful in defining the steric and electronic considerations which distinguish between direct and indirect acting nitroPAH's. However, before this can be achieved it will be necessary to determine the structures of the dinitroperylenes used in this study.

The majority of indirect acting mutagens in the Ames test are metabolized by the microsomal MFO system of S9 to species which are able to bind covalently to cellular macromolecules. Since 3-nitroperylene does require S9 to express its mutagenicity, and since its activation is oxygen-dependent (Löfroth, 1982), it too appears to be activated by the MFO system. The present study has provided several lines of evidence to support this conclusion. Firstly, the enzymes that are responsible for the activation are entirely localized in the microsomal fraction of S9. Secondly, the activation is inducible by inducers of MFO enzymes. Thirdly, inhibitors of MFO enzymes have substantial effects on the mutagenicity of 3-nitroperylene (see below), and, finally, the activation is strongly dependent on the presence of reduced pyridine nucleotide cofactors, which are essential to the function of P-450 dependent enzymes. This latter cofactor dependency was unusual in that NAD^+ seemed to be almost as effective as $NADP^+$ in facilitating for 3-nitroperylene mutagenesis. Most cytochrome P-450 dependent reactions have a strong requirement for $NADP^+$, but NAD^+ is not usually necessary (Schenkman and Gibson, 1981). However, in the presence of $NADP^+$, NAD^+ can have synergistic effect (Correia and Mannering, 1973) which is mediated by cytochrome b_5 (Sasame et. al., 1973). This stimulation is dependent on both the isozyme(s) of P-450 involved in a reaction and the substrate (Guengerich, 1979). Recent work has shown that NAD^+ alone can sustain P-450 dependent oxidations through an interaction with NADPH-cytochrome P-450(c) reductase, although at slower rates than the corresponding $NADP^+$-supported reactions (Noshiro and Omura, 1978). Thus, the surprising

effectiveness of NAD^+ in supporting 3-nitroperylene activation could reflect either a direct involvement of the cofactor in donating electrons to cytochrome P-450 via the reductase, or an indirect, synergistic effect in concert with residual amounts of $NADP^+$ in the S9 and microsome preparations.

The two major forms of cytochrome P-450 in rat liver S9, P-450's b and c, are induced by pretreating the rats with PB and MC (or NF) respectively. When the mutagenicity of 3-nitroperylene was examined using S9's from rats pretreated with PB, MC or NF, all preparations were able to activate the mutagen strongly and equally well. These results suggest that each of the two major forms of P-450 may be involved in the activation of 3-nitroperylene to mutagenic species. B(a)P, on the other hand, was activated strongly by MC and NF S9's, but poorly by PB S9. This latter result is in accord with well established findings (Conney, 1982 for review) and is true for many simple and substituted PAH's. Some PAH's, however, such as 3-methylcholanthrene, are activated more strongly by PB S9 (Ames et. al., 1975), and yet others, for example some methylbenz(a)anthracenes, are activated to mutagens equally well by both MC and PB S9's (Glatt et. al., 1981), like 3-nitroperylene. Other studies using purified MFO enzymes (Wood et. al., 1976; Robertson et. al., 1983), or antibodies to various P-450 isozymes (Kawajiri et. al., 1980, 1983) have demonstrated substantial variability in the patterns of activation of different compounds to mutagens in the Ames test by individual P-450 isozymes. Some compounds are activated well only by a single isozyme, whereas several isozymes are equally effective for others. Where several forms

of P-450 are able to activate a given compound, they often show remarkable regioselectivity and stereoselectivity. For example, B(a)P can be activated by several forms of P-450, albeit most strongly by the major MC induced form, and each isozyme provides a distinctive pattern of metabolites and isomers (Wiebel et. al., 1975; Conney, 1982).

If 3-nitroperylene is a substrate for several forms of P-450 then it is likely that the pattern of metabolites formed by each isozyme would be different. Indeed different metabolite patterns were seen when 3-nitroperylene was incubated with high concentrations of either PB or MC S9 (see Chapter 4). Thus, it is probable that a number of MFO products of this nitroPAH are proximate or ultimate mutagens in Salmonella. This would not be surprising, as the oxidative metabolism of many PAH's is known to give rise to a number of ultimate mutagens with a spectrum of potencies (Conney, 1982). The mutagenicity of a compound depends on the quantitative relationships between these active species, which in turn is a reflection of the forms of P-450 present in the activating system.

The observation that PB, MC and NF S9's all activate 3-nitroperylene equally well, whereas most PAH's and PAH derivatives are preferentially activated by MC and NF S9's, could also be explained by the presence of another form of P-450 which is induced by both PB and MC type inducers. Indeed, P-450a is induced following treatment of rats with both PB and MC (Ryan et. al., 1979) but there is only a 3-5 fold increase in the concentration of the isozyme, which alone would be insufficient to account for the large increase in mutagenicity associated with induction. In addition, the catalytic properties of

P-450a are very limited. It will preferentially hydroxylate testosterone in the 7 position but has low catalytic activity for the metabolism of a number of other compounds, including B(a)P (Ryan et. al., 1979). However, there is evidence that MC treatment of rats will not only induce aryl hydrocarbon hydroxylase (AHH) activity, but will also lower the K_m for the reaction (Razzouk et. al., 1978), presumably due to a change in the cytochrome-phospholipid interaction within the endoplasmic reticulum membranes. If a substantial change in the K_m of P-450a catalysed enzyme activities was also associated with the induction of this isoenzyme, then the mutagenicity of 3-nitroperylene could reflect the activity of a minor P-450 species.

In addition to studies using S9's from animals pretreated with various MFO inducers, the mutagenicity of 3-nitroperylene was examined in the presence of several MFO inhibitors. The pattern of inhibition seen when B(a)P was used as a mutagen was similar to that recorded in the literature (Yoshikawa et. al., 1981; Razzouk et. al., 1978) and, in agreement with the S9 studies presented above, implicated P-450c as the principal form of P-450 involved in the metabolic activation of the PAH. 6-NitroB(a)P showed similar behaviour to its parent compound, but 3-nitroperylene behaved somewhat differently. The differences were most apparent when α- and β-naphthoflavone were used as inhibitors. The effects of the various inhibitors are discussed in more detail below.

The synthetic flavones α- and β-naphthoflavone have long been recognized as MFO inhibitors (Diamond and Gelboin, 1969; Viswanathan and Alworth, 1981) with an apparent specificity for the major

MC-induced form of P-450 (Wiebel et. al., 1971; Wolf et. al., 1979). α-Naphthoflavone has proved the more effective of the two inhibitors (Diamond et. al., 1972). Although β-naphthoflavone has been found to inhibit some monooxygenase activities (Wiebel et. al., 1971; Diamond et. al., 1972) it is best known as an inducer of MFO enzymes (Haugen and Coon, 1976).

In the present study both naphthoflavones strongly inhibited B(a)P mutagenicity, as expected from their known effects on AHH activity (Gougon et. al., 1972). The mutagenicity of 3-nitroperylene was only slightly inhibited by α-naphthoflavone. This result is compatible with the data discussed earlier which implicated both major forms of P-450 in the activation of the mutagen. Surprisingly, however, the mutagenicity of 3-nitroperylene was substantially increased in the presence of β-naphthoflavone. This result is not without precedent as a number of both naturally occurring and synthetic flavones (including α- and β-naphthoflavone) have been shown to stimulate AHH activity in several tissues of various species, and to enhance B(a)P induced skin tumorigenesis in mice (Wiebel, 1980; Conney, 1982 for reviews). The stimulation of B(a)P hydroxylation has been seen in uninduced rat liver microsomes but not in microsomes from PB or MC treated rats (Wiebel et. al., 1971). However, the metabolism of N,N-dimethyl-4-aminoazobenzene is stimulated in both induced and uninduced rat liver microsomes by α-naphthoflavone (Levine and Lu, 1982). Thus the effects of naphthoflavones on MFO enzymes are complex and depend on the source of the microsomes, the nature of any enzyme induction, and the substrate being studied.

Few studies have been carried out regarding the effects of naphthoflavones on the mutagenesis of compounds in the Ames test, but those which have been undertaken show that α-naphthoflavone alters the mutagenicity in a fashion predictable from its effects on the different forms of P-450 (Felton and Nebert, 1975), as the results with B(a)P in the current project confirm. There appears to be only one report of α-naphthoflavone stimulating the mutagenicity of a compound (aflatoxin B_1) in the Ames assay (Buening et. al., 1978), but in this case human liver microsomes were used to provide activation and they are particularly susceptible to the stimulatory effects of this flavone (Kapitulnik et. al., 1977). The experiments with β-naphthoflavone reported in the current study appear to be the first examples of the use of the compound as an "inhibitor" in the Ames test.

The mechanism by which the naphthoflavones modify the effects of MFO enzymes is still a matter of controversy. Kinetic data suggest that α-naphthoflavone inhibits AHH activity competitively at low concentrations but at higher concentrations inhibits the hydroxylation by a more complex mechanism (Wiebel and Gelboin, 1975). Both α- and β-naphthoflavone inhibit 7-ethoxycoumarin deethylase activity non-competitively, although "pure" noncompetitive inhibition was not observed in either case (Viswanathan and Alworth, 1981). The hydroxylation of B(a)P by purified P-450 isozymes from rabbit liver is inhibited or stimulated by α-naphthoflavone depending on the isozyme under study (Huang et. al., 1981a). More detailed studies on the mechanism of the activating effects of α-naphthoflavone suggested that the stimulation was at least in part due to an enhancement of the

interaction between cytochrome P-450 and NADPH-cytochrome P-450(c)reductase, which facilitates the flow of electrons to the cytochrome (Huang et. al., 1981b). The enhancing effects of β-naphthoflavone on the mutagenic activity of 3-nitroperylene may also reflect a strong association between the flavone, the reductase, and P-450 which facilitates electron transport in a analogous manner to that proposed for α-naphthoflavone.

SKF 525-A is one of the most widely used inhibitors of MFO enzyme activities (Anders, 1971). It appears to exert its inhibitory effects by forming stable complexes with cytochrome P-450 (Franklin, 1976) and preferentially inhibits those MFO reactions associated with PB induction (Goujon et. al., 1972; Levine and Lu, 1982; Gontovnick and Bellward, 1982). However, it does also inhibit reactions involving P-450's associated with MC induction, but to a lesser extent (Gontovnick and Bellward, 1982; Yoshikawa et. al., 1982). In the present study, the mutagenicity of B(a)P was only slightly depressed by SKF 525-A. This result is in agreement with the known inhibitory effects of the compound, and supports the few studies which have been undertaken on the effects of the inhibitor in mutation assays (Razzouk, 1978; Yoshikawa et. al., 1982). SKF 525-A caused a far greater inhibition of 3-nitroperylene mutagenesis. This is not unexpected since the compound will bind to both major forms of P-450 (Franklin, 1976), and data presented above have suggested that both of these isozymes may be involved in the activation of 3-nitroperylene to mutagenic species. An interesting response to SKF 525-A was shown by 6-nitroB(a)P. The majority of the mutation data gathered in this study

suggest a very strong similarity in the activation of both 6-nitroB(a)P and its parent hydrocarbon to mutagenic species. However, SKF 525-A proved to be a surprisingly strong inhibitor of 6-nitroB(a)P mutagenesis. This may reflect slight differences in the P-450 isozymes involved in the activation of B(a)P and 6-nitroB(a)P, although the differential induction studies presented earlier do not support this, or could point to differing affinities of the MFO enzyme complex for the two mutagens. It is possible that the nitro group decreases the binding strength of the nitroPAH.

The final inhibitor used in this series of experiments was metyrapone. Like SKF 525-A, it preferentially binds to forms of P-450 not induced by MC treatment, and thus preferentially inhibits MFO activities associated with these isozymes (Kahl et. al., 1976; Goujon et. al., 1972; Guenthner et. al., 1980). In the present study metyrapone enhanced, rather than inhibited, the activation of each of the three mutagens under study. The stimulatory effect of metyrapone on B(a)P mutagenesis has been reported in the literature (Yoshikawa et. a., 1982) and its enhancing effects on some other MFO activities have also been noted (Anders, 1971). However, an extensive study by Goujon et. al. (1972) indicated that metyrapone inhibited AHH activity. The stimulation of some MFO reactions by metyrapone is thought to be a consequence of the formation of inactive complexes between the inhibitor and those forms of P-450 not induced by MC, which would decrease the proportion of substrate being drawn off by these isozymes for other reactions. All of those reactions which are enhanced by metyrapone are preferentially catalysed by MC induced enzymes (Anders,

1971). This would explain the increase in the mutagenic activity of B(a)P and, by analogy, 6-nitroB(a)P, but fails to account for the behaviour of 3-nitroperylene which is activated equally well by either PB or MC S9's. In fact the enhancement of 3-nitroperylene mutagenicity is substantially greater than that of the other two compounds. The mechanism by which metyrapone binds to cytochrome P-450 is not well understood (Kahl et. al., 1976), but it is possible that the stimulation it provides in some cases may be related to an enhancement of electron flow between NADPH-cytochrome P-450(c) reductase and P-450 as has been suggested for the naphthoflavones (Huang et. al., 1981b).

Although the experiments with various MFO inhibitors do support the earlier data which suggests that the P-450 system is involved in the activation of 3-nitroperylene, they fail to give clear cut results as to the P-450 isozymes involved in the activation. The strange effects of β-naphthoflavone and metyrapone may, in fact, indicate that neither of the major forms of P-450 is involved in the mutagenicity, and that it indeed reflects a minor isozyme, as was suggested earlier. The results with B(a)P clearly implicate the major MC-induced P-450 in the activation of that mutagen, and its nitrated derivative behaves in a very similar fashion as previous results have also shown.

The S9 mediated mutagenicity of 3-nitroperylene, 6-nitroB(a)P and B(a)P was found to be altered substantially by the addition of conjugating reagents such as reduced glutathione (GSH), cysteine and uridine 5'-diphosphoglucuronic acid (UDPGA). The cellular nucleophile, GSH is known to conjugate with the electrophilic metabolites of many xenobiotic compounds via both enzymic (glutathione-S-transferases) and

non-enzymic mechanisms (Jakoby, 1978). Cysteine, like GSH, also contains a free sulfhydryl group, but is a far less efficient electrophile scavenger. Conjugates appear to be formed solely through non-enzymic processes. Many phenols, amines and thiols will form conjugates with UDPGA through reactions which are catalysed by a family of UDP glucuronyltransferases (Bartsch et. al., 1982). These UDPGA conjugations, as well as those of GSH and cysteine, render xenobiotics more water soluble, and hence increase their rate of elimination from an organism. Thus, conjugating reactions are important in decreasing the toxicity of many foreign chemicals, but there are some instances in which conjugation can increase the toxicity and mutagenicity of various compounds (Caldwell, 1979).

The effects of GSH on the mutagenicity of B(a)P and aflatoxin B_1 have been relatively well studied (Malaveille et. al., 1981; Nemoto et. al., 1978) and provide some useful insights for the explanation of the experimental results presented in this chapter. Depending on the assay conditions (substrate, S9 and GSH concentrations; plate incorporation vs liquid preincubation) GSH can increase, decrease or have no effect on the mutagenicity of these compounds. The decrease is presumed to reflect inactivation of reactive epoxide intermediates by conjugation reactions with GSH. Enzymic conjugation is the major pathway of inactivation, but, since epoxides are quite reactive electrophiles, the non-enzymic pathway could also be important. The GSH-mediated decrease in the mutagenicities of both B(a)P and 3-nitroperylene in the current experiments is in accord with this type of behaviour. The effect of GSH on the 3-nitroperylene activity

strongly suggests that the mutagen is metabolized to a reactive electrophile, as would be predicted from the electrophile theory of carcinogenesis (Miller and Miller, 1981).

When 6-nitroB(a)P was studied in the Ames test in the presence of GSH, a decrease in the reversion rate was seen only when a very high GSH concentration was used. At concentrations below about 20 mM, enhancement of mutagenicity was actually seen. As indicated above (Malaveille et. al., 1981), similar results have been obtained with B(a)P and aflatoxin B_1 under certain conditions. The enhancement is thought to be the result of selective conjugation of simple oxides with GSH. For example, MFO enzymes metabolize B(a)P to a variety of epoxide intermediates, not all of which are important in the mutagenic action of that compound. If those epoxides irrelevant to the mutagenesis preferentially acted as substrates for glutathione-S-transferases, then the remaining intermediates could be further metabolized by microsomal enzymes to ultimate mutagens in the absence of competitive inhibition by the irrelevant epoxides. It is conceivable that a similar mechanism could explain the effects of GSH on 6-nitroB(a)P mutagenesis, although epoxides have not yet been detected as metabolites of the compound (Fu et. al., 1982).

Both cysteine and UDPGA were able to inhibit the mutagenic activity of each of the three mutagens studied. Cysteine was a far less effective inhibitor than GSH, as expected. Its effects on B(a)P and 6-nitroB(a)P were similar, but it was only weakly inhibitory. The mutagenicity of 3-nitroperylene was inhibited more strongly, suggesting that particularly reactive electrophiles may be formed during the

metabolic activation of this compound. UDPGA proved to be quite a potent inhibitor of the activity of all three compounds, but, like GSH, other studies have shown that its effects depend on the assay conditions, and in some instances it can even stimulate mutagenesis (Malaveille et. al., 1981). The results with B(a)P can be simply explained, since hydroxyl groups are prime targets for the formation of glucoside conjugates, and the major ultimate mutagens derived from B(a)P are dihydrodiol epoxides and phenol epoxides (Conney, 1982; King et. al., 1976). Since 3-nitroperylene also appears to be metabolized oxidatively by MFO enzymes, it is likely that the ultimate mutagen(s) derived from it also contain hydroxyl groups. The effects of UDPGA support this conclusion. Similar arguments apply to 6-nitroB(a)P.

One of the most interesting results from the studies on the mutagenicity of 3-nitroperylene was that strong activation was only achieved over a narrow range of low AR S9 concentrations. The response to increasing S9 concentration was biphasic. In the absence of S9, mutagenicity was very low, but as small amounts of the liver preparation were added, the number of revertants induced increased dramatically. A peak of activity was reached at quite low S9 concentrations (1-2% for AR S9), but as the amount of S9 was increased beyond this optimum level, mutagenicity declined appreciably. This biphasic response was also seen with MC, NF and, to a lesser extent, PB S9's but in each case the peak of optimum S9 concentration was much broader than that seen with AR S9, and occurred at higher S9 concentrations. In strong contrast to the results with induced S9's, the mutagenicity of 3-nitroperylene increased linearly in response to

increasing concentrations of UN S9. This latter behaviour is probably simply a response to increases in the levels of the MFO enzymes responsible for the activation of the mutagen, whereas the response to the other S9's reflects a more complex set of factors since monooxygenase levels are already very high.

Although there have been few studies on the factors which determine the amount of S9 needed for optimal mutagenesis in either the Ames test or other mutation assays, it is evident that the structure (lipophilic properties) and concentration of the test compound, as well as the type of MFO inducer with which the rats have been treated, are all important (Ames et. al., 1975; Malaveille et. al., 1979; Kuroki et. al., 1979, Nagao et. al., 1978). Lipophilicity is one of the most critical factors in determining the optimum S9 concentration. Studies have shown that lipophilic compounds, such as B(a)P and aflatoxin B_1, are strong mutagens at relatively low S9 concentrations, whereas polar compounds, for example the nitrosamines, require S9 concentrations at least 7 times higher than those required for B(a)P to show maximum mutagenicity (Malaveille et. al., 1979). When the octanol/water partition coefficients of a series of nitrosamines was correlated with their ability to induce mutations is mammalian cells, it was found that the more polar compounds required larger amounts of S9 to induce comparable levels of mutation (Kuroki et. al., 1979). The mutagenicity of 3-nitroperylene is maximal at very low S9 concentrations and this is, no doubt, in part due to its highly lipophilic nature. However, since the S9 optimum for the compound varies several fold amongst the various S9's used, there are almost certainly other factors operating

in determining the optimum value.

Some further clues as to why a specific concentration of S9 is required to obtain optimum mutagenesis for many compounds have been provided by the experiments of Malaveille et. al. (1979), in which they separated S9 into microsomal and cytosolic fractions. They found that if the concentration of cytosolic protein was kept constant while the concentration of microsomal protein was varied, the mutagenicity of B(a)P continued to increase as the amount of microsomal protein was increased. However, if the ratio of microsomal to cystolic protein was kept constant as the amount of S9 was increased, then a well defined optimum S9 concentration was observed. The response in the latter case was very similar to that shown by both B(a)P and 3-nitroperylene in the present experiments. Thus, the mutagenicity appears to be related to the trapping of mutagenic metabolites, either by non-enzymic reaction with nucleophiles in the S9 cytosolic fraction, or through enzymic conjugation with gluthathione. As the amount of S9 is increased over the amount required for optimal mutagenicity, mutagenic metabolites are inactivated by reaction with cytosolic nucleophiles at a rate that increases more rapidly than that of their formation.

Glatt and Oesch (1977) provided electron microscopic evidence that the deep rough character (loss of the outer lipopolysaccharide barrier) of the Salmonella mutants used in the Ames test causes some of the microsomes in the S9 preparation to stick directly to the bacteria. This result has important implications in the assessment of the mutagenicity of a compound, since it would obviate the need for at least some of the short-lived electrophilic metabolites to pass through

the nucleophilic environment of the S9 cytosol. Indeed it has been suggested that proximate contact between microsomes and bacteria or mammalian cells is essential for the mutagenesis of some compounds (Kuroki and Drevon, 1978). However, the results obtained by Malaveille et. al. (1979) indicated that, at least under the assay conditions used, the electrophilic metabolites of B(a)P are required to diffuse through the assay medium. In support of this obsevation, they failed to demonstrate microsomes associated with the membranes of S. typhimurium strains TA98 and TA100 despite the earlier reports to the contrary.

Although the concentrations of soluble factors in S9 can have a large bearing on the response of a mutagen to various S9 concentrations, relationships between microsomal enzymes are also important. In particular, the relationship between P-450 dependent enzymes and the enzyme epoxide hydrase (EH) has been found to be very important in modulating the mutagenicity of those compounds which are activated by metabolism to reactive electrophilic epoxides (Oesch and Glatt, 1976; Oesch, 1978). EH has a dual role in the metabolism of PAH's, such as B(a)P, to mutagenic species. It is essential for the formation of some ultimate mutagens, the dihydrodiol epoxides, but at the same time is able to inactivate simple epoxides quite effectively (Oesch and Glatt, 1976). Some simple epoxides are also ultimate mutagens. The addition of pure EH to mutation assays involving B(a)P will bring about a substantial decrease in the mutagenicity of the compound (Oesch, 1978), and, conversly, EH inhibitors will increase the reversion rate (Oesch and Glatt, 1976). Thus, the levels of reactive

intermediates will depend on a balance between activation by the P-450 system and inactivation by EH. Doolittle et. al. (1983) have examined the S9 mediated metabolic activation of B(a)P to species which are mutagenic in V79 Chinese hamster cells, and have correlated the results with P-450 dependent enzyme activity and EH levels. They showed that there was a strong correlation between the ratio of the two activities and the mutagenicity and toxicity of B(a)P. As the relative proportion of EH increased, the mutagenicity of B(a)P decreased, a result in accordance with the known effects of EH on the metabolism of the mutagen.

The factors determining the optimum S9 concentration for the activation of a compound to mutagenic species are complex, and some of the more important considerations have been discussed above. Another interesting observation is that for many compounds, there is not a linear relationship between the rate of metabolism and the S9 concentration (Parry et. al., 1976). This again appears to reflect the lipid solubility of the substrate. Such a non-linear relationship further complicates the interpretation of results in the Ames assay, since the actual concentration of mutagenic metabolites present during the exponential growth phase of the bacteria (which occurs after plating in the presence of biotin and traces of histidine) may influence the mutagenicity of the test compound (Malaveille et. al., 1977). Nevertheless, the major factors influencing the S9 optima of most mutagens, including 3-nitroperylene, appear to be the lipophilicity and concentration of the test substance, the concentration of non-critical cytosolic nucleophiles, and the relative

levels of the various enzymes responsible for activating and deactivating the compound and its metabolites.

The mutagenicity of 3-nitroperylene and some related compounds was examined in several different Salmonella typhimurium tester strains using the Ames test. Of the strains used, TA98 was by far the most sensitive to the mutagenic effects of 3-nitroperylene, which induced a level of reversion to histidine independence some 40 times higher than the spontaneous reversion rate. TA98 has proved to be the most sensitive Salmonella strain to mutation by the majority of nitroPAH's studied (Rosenkranz and Mermelstein, 1983 for summary). It is most sensitive to compounds which cause frameshift type mutations, thus placing 3-nitroperylene and many other nitroPAH's in this category.

Strain TA100 responds primarily to mutagens causing base-pair substitutions and is also reasonably sensitive to 3-nitroperylene. However, this strain has a very high spontaneous mutation rate, and when this is taken into consideration, the apparent sensitivity to 3-nitroperylene is substantially decreased, as the number of revertants induced is only about 5 times higher than the spontaneous rate. The other standard tester strains used in these studies were far less sensitive to 3-nitroperylene. Many chemicals which induce mutation by an intercalation mechanism preferentially elicit a strong response in TA1537. The very low sensitivity of this strain to 3-nitroperylene indicates that the mutagen is not an intercalating agent, and, in view of its strong activity in TA98, it probably induces mutation by forming covalent adducts with DNA.

Löfroth (1982) has reported on the mutagenicity of

3-nitroperylene in strains TA98, TA100, TA1537 and TA1538. The rates of reversion he obtained in each strain were in good agreement with those found in the current series of experiments.

Strains TA98 and TA100 were derived from TA1538 and TA1535 respectively by the addition of the pKM101 plasmid (Ames et. al., 1975) which appears to code for error-prone DNA repair enzymes (Goze and Devoret, 1979; McCann et. al., 1975). These plasmid-containing strains are much more sensitive to many chemicals, including PAH's and most nitroPAH's, such as 3-nitroperylene (McCann et. al., 1975; Rosenkranz and Mermelstein, 1983). This suggests that 3-nitroperylene reacts with the bacterial DNA to form adducts which are recognized by the error-prone enzymes. A comparison of the sensitivities of strains TA1538 and TA1535 to 3-nitroperylene probably provides a more valid measure of the frameshift versus base-pair substitution activity of the compound, since both strains have low spontaneous reversion rates (Maron and Ames, 1983). The ability of the mutagen to induce frameshift type mutations (TA1538) was much greater than its ability to cause base-pair substitutions (TA1535), in confirmation of the results with TA98 and TA100.

Nitroreduction is known to be involved in the activation of at least some nitroPAH's to mutagenic species, and these compounds often show substantially reduced activity in Salmonella strains deficient in nitroreductase enzymes (Rosenkranz and Mermelstein, 1983). Since the addition of a nitro group to the perylene molecule brings about such a large increase in the mutagenicity of the compound, it was of some interest to examine the mutagenicity of 3-nitroperylene in the Ames

test using some nitroreductase-deficient tester strains.

Strains TA98NR and $TA98MCF_{50}$ were selected for resistance to niridazole and nitrofurazone respectively, both mutagens which are known to be activated by nitroreduction (Speck et. al., 1981; McCalla et. al., 1975). TA98NR is deficient in a single enzyme (Bryant et. al., personal communication), which is referred to as "classical" nitroreductase, and $TA98MCF_{50}$ appears to contain a similar, if not identical, lesion. Although these strains are resistant to the mutagenic effects of 1-nitropyrene and several other nitroPAH's (Mermelstein et. al., 1981; Löfroth, 1982), they still remain quite sensitive to mutagenesis by 3-nitroperylene. This result indicates that the "classical" nitroreductases do not play a significant role in the activation of this compound to mutagenic species.

In contrast to the results with "classical" nitroreductase-deficient bacteria, strains which had been selected for resistance to 1,8-dinitropyrene (TA98 DNP_6 and TA98NR/1,8-DNP_6) were very resistant to the mutagenic action of 3-nitroperylene. As noted in the introduction, the nature of the enzyme deficiency in these strains is still a matter of controversy, although they do not appear to lack nitroreductase activity (McCoy et. al., 1982), making interpretation of the results difficult. Nevertheless, the resistance of these strains to 3-nitroperylene strongly implicates the involvement of bacterial enzymes in the activation of the mutagen. Thus both S9 enzymes and an endogenous bacterial enzyme appear to be required.

The mutagenicities of B(a)P, 6-nitroB(a)P and dinitroperylene (II) were also examined using a battery of nitroreductase-deficient

mutants. Each of the strains, with the exception of TA98NR/1,8-DNP_6, remained fully sensitive to the mutagenic effects of both B(a)P and 6-nitroB(a)P. The weaker response of the double mutant to these compounds probably reflects the slow growth of the strain rather than any enzyme deficiencies. The behaviour of B(a)P predictable from its known activation pathway, and from the data presented earlier, which show a close parallel between the mutagenic effects of B(a)P and 6-nitroB(a)P, the results with the nitro derivative are also not unexpected. The data indicate that nitroreduction , at least by the "classical" nitroreductase, is not involved in the activation of 6-nitroB(a)P, even though it has been suggested by some workers as a possible metabolic step subsequent to oxidative metabolism by S9 enzymes (Fu et. al., 1982). Dinitroperylene (II) showed a very similar pattern to 3-nitroperylene, with low activity in the dinitropyrene resistant mutants but full activity in the other two strains. Again a full interpretation of this result must await the identification of the enzyme deficiency in TA98DNP_6 and its associated strain.

Since "classical" nitroreductase-deficient strains remain sensitive to 3-nitroperylene, and in view of the demonstration that the mutagenicity of the compound is very low in an oxygen poor environment (Löfroth, 1982), nitroreduction would not appear to figure significantly in the activation of the mutagen. It is possible that nitroreductases in the S9 play a role in the activation of 3-nitroperylene, but there are two main lines of evidence against this. Firstly, mammalian nitroreductases only show low levels of activity under aerobic conditions (Holtzman et. al., 1981; Kutcher and McCalla,

1983), whereas mutagenesis is maximal under these conditions. Secondly, the most likely nitroreductase which could be involved in 3-nitroperylene reduction is the microsome associated NADPH-cytochrome P-450(c) reductase. This enzyme is inducible by PB and Aroclor 1254, but not by MC (Alvares and Kappas, 1977), which would preclude it from taking a major part in the activation, since all three S9's stimulate 3-nitroperylene mutagenesis equally well. There is also the possibility that bacterial nitroreduction could occur, but only in conjunction with an initial oxidative activation step or steps. Although, there does appear to be some involvement of bacterial enzymes in the activation of 3-nitroperylene, the nature of this involvement has yet to be evaluated. If nitroreduction does occur following oxidation of MFO enzymes, the "classical" nitroreductase is probably not involved.

To investigate further the possibility that reductive metabolites of 3-nitroperylene and 6-nitroB(a)P could be mutagenic in the Ames test, a modification of the test was employed which allowed chemical reduction of the compounds in the presence of bacteria (Karpinsky et. al., 1982). In this system slight, but significant, increases in the numbers of revertants induced by both mutagens were observed, although the degree of activation was far less than that required to account for the S9 mediated mutagenicity of either compound. Nevertheless, this is evidence that the reduction products of both 3-nitroperylene and 6-nitroB(a)P are mutagenic, albeit weakly under certain circumstances. There are some difficulties in attempting to correlate the results in this assay with those obtained under

standard test conditions (e.g. what proportion of the mutagen does the Zn/NH_4Cl system actually reduce), but the tests do appear to confirm that simple reduction of the nitro group is not involved, at least initially, in the activation of these nitroPAH's.

4. THE METABOLISM AND MACROMOLECULE BINDING OF 3-NITROPERYLENE

4. The Metabolism and Macromolecule Binding of 3-Nitroperylene

4.1. Results

4.1.1. S9 Metabolism of 3-Nitroperylene

Since 3-nitroperylene is a potent indirect acting mutagen in the Ames test, at least some step(s) in the metabolic activation of the compound must involve S9 enzymes. Thus a series of experiments was initiated to isolate and identify the S9 metabolites of the mutagen.

In preliminary experiments, 3-nitroperylene was incubated with low concentrations of S9, similar to those used in the Ames assay (1-5%), and the ethyl acetate extractable material was analysed by HPLC. Although a time-dependent decrease in the amount of 3-nitroperylene remaining in the extract was observed, no metabolic products of the compound were apparent on the HPLC profile when UV detection (A_{254}) was used (results not shown). However, as the S9 concentration was increased beyond 10%, several products with UV absorbance were detected. The amount of metabolism increased with increasing S9 concentration and 50% S9 was routinely used in metabolism studies.

HPLC analysis of the products of a typical metabolism experiment with AR S9 revealed the presence of several metabolites (Fig. 10a and b) (shaded peaks in Fig. 10a). The metabolite pattern seen with UV detection (Fig. 10a) was very similar to the radiochromatogram pattern (Fig. 10b), but the former has the disadvantage of numerous peaks of S9 origin, which appear on the UV trace, often masking true metabolites. This is an unavoidable consequence of using very high S9 concentrations. No sizeable fluorescent peaks were detected in any of the samples studied. Several

FIGURE 10. HPLC analysis of the S9 and microsomal metabolites of 3-nitroperylene and the dependence of their formation on $NADP^+$.

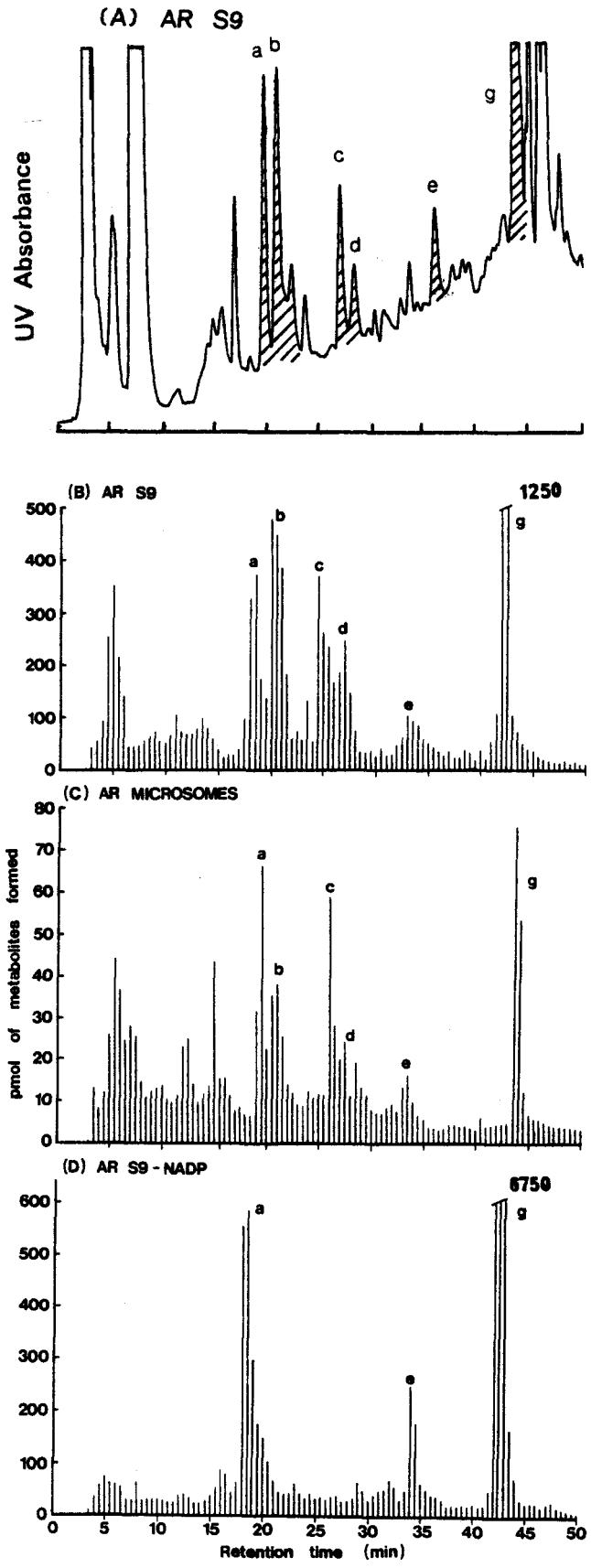
(A) AR S9. Detection by UV absorbance at 254 nm (0.06 absorbance units full scale).
Metabolites formed after 1 hour of incubation at 37°C.
3-Nitroperylene (peak g) and its metabolites (a-e) are indicated by shading. Other peaks are S9-associated.

(B) AR S9. Radiochromatogram.
Metabolites formed after 2 hours incubation at 37°C.

(C) AR microsomes. Radiochromatogram.
Metabolites formed after 1 hour of incubation at 37°C.

(D) AR S9-$NADP^+$. Radiochromatogram.
Metabolites formed after 2 hours incubation at 37°C.

Figures beside peak g indicate the maximum height of the peak (pmol of 3-nitroperylene remaining). N.B. Retention times for the individual metabolites tended to vary somewhat between chromatographic runs, and the assignment of metabolite peaks is based on a careful examination of both radiochromatograms and UV traces.


(A) AR S9
UV Absorbance
a
b
c
d
e
g
(B) AR S9
1250
(C) AR MICROSOMES
pmol of metabolites formed
(D) AR S9 - NADP
6750
Retention time (min)

prominent metabolites elute between 19 and 36 minutes and these have been lettered a-e for ease of reference. Other products are present, however, and there is a considerable amount of early-eluting radioactivity, particularly at the void volume, which suggests a number of more polar metabolites. No metabolites were seen in zero time controls and metabolite yield was low unless incubations were well aerated. Unmetabolized 3-nitroperylene constituted the largest peak, eluting at 43-44 minutes (peak g). Some initial studies on the kinetics of formation of the various metabolites showed a time dependent increase in the amounts of products b,c, and d, whereas the greatest amounts of products a and e appeared within 5 minutes of the commencement of incubation, then showed a subsequent time-dependent disappearance. In fact, after 5 minutes, a and e were the dominant metabolites (data not shown), suggesting that they are primary derivatives of 3-nitroperylene.

The pattern of metabolites seen when microsomes supplemented with glucose-6-phosphate dehydrogenase were used in metabolism experiments was essentially the same as that seen with whole S9 (Fig. 10c), although in the particular experiment shown in Fig. 10c, product a was surprisingly prominent. An interesting pattern was seen when 3-nitroperylene was incubated with AR S9 in the absence of added NADP. Most of the metabolites appeared to be absent or were present only in very small quantities, as would be expected, whereas, in contrast, both products a and e were present in very large amounts (Fig. 10d). This is consistent with the suggesting above that they are primary metabolic products.

FIGURE 11. HPLC analysis of the metabolites formed following incubation of 3-nitroperylene with PB, MC, NF or UN S9.

The results of each experiment are presented as radiochromatograms.

Incubations in each case were carried out for 2 hours at 37°C.

(A) PB S9

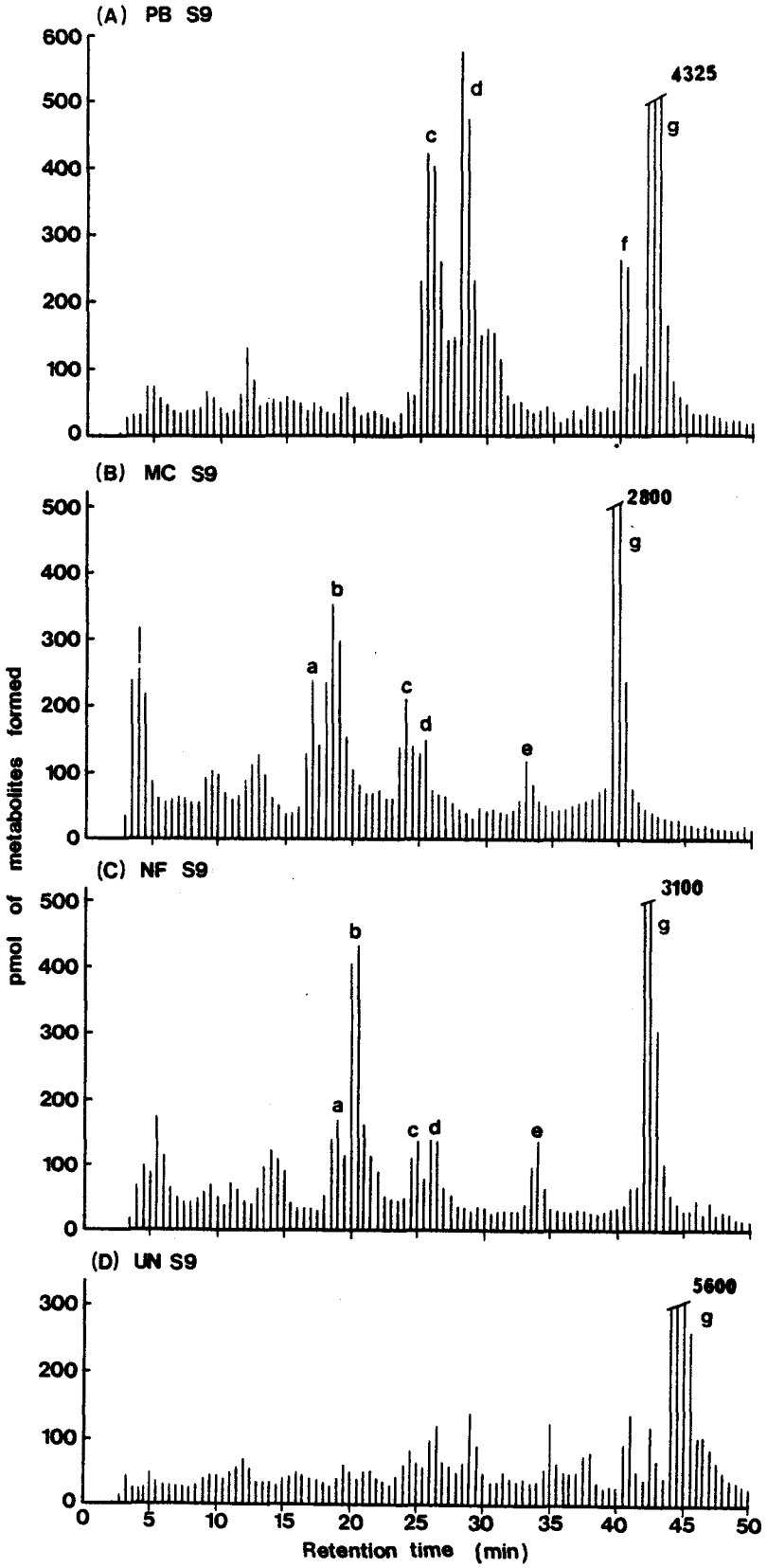
(B) MC S9

(C) NF S9

(D) UN S9

Figures beside peak g indicate the maximum height of the
peak (pmol of 3-nitroperylene remaining)

N.B. See note to Figure 10.


(A) PB S9
4325
(B) MC S9
2800
(C) NF S9
3100
(D) UN S9
5600
pmol of metabolites formed
Retention time (min)

S9 preparations from rats pretreated with PB, MC and NF, as well as UN S9, were also tested for their ability to metabolize 3-nitroperylene. Control (UN) S9 was very ineffective in metabolizing the compound (Fig. 11d), illustrating that the enzymes responsible for the metabolism are inducible, a result consistent with earlier mutation studies. Both MC and NF S9's produced metabolite patterns similar to that of AR S9, although in each case the extent of metabolism was substantially less than that seen with the latter S9 (Figs. 11b and c). PB S9 gave a considerably different pattern (Fig. 11a). The polar metabolites, including a and b and the void volume products, and product e were almost entirely absent, whereas derivatives c and d were present in very large quantities. Of interest was the presence of an additional peak eluting at about 40 min (peak f). This peak was not seen when AR S9 was used. The large difference between the metabolite patterns of PB and MC S9's is interesting in view of the equal facility with which they activate 3-nitroperylene to mutagenic species in the Ames assay.

4.1.2. The Nature of 3-Nitroperylene Metabolites

Attempts to identify some of the metabolites of 3-nitroperylene by mass spectrometry (MS) were without success. Products b, c and d were all examined, but in each case they proved very labile and merely charred on the MS probe upon heating. No ions from thermal decomposition were observed. Reaction of the compound with bis-trimethylsilyltrifuoroacetamide in the hope of preparing trimethylsilyl derivatives failed to stabilize them sufficiently for

acceptable spectra to be obtained. GC-MS analyses of mixtures of metabolites, both derivatized and underivatized, were also unsuccessful.

Some clues as to the identity of some of the metabolites were obtained from UV-visible spectroscopy studies. The ethyl acetate extract of zero time incubations in 3-nitroperylene metabolism experiments showed the strong yellow colour of the parent compound. However, the extract obtained after two hours incubation at 37^{o}C was bright pink, indicating the presence of new compounds which absorb strongly in the visible region at different wavelengths than the parent nitroPAH. Figure 12a shows a UV-visible spectrum of the ethyl acetate extract from a metabolism experiment using AR S9. The trace is presented as a difference spectrum (with 3-nitroperylene in the reference cuvette) because 3-nitroperylene, with its high extinction coefficient, dominated the standard spectrum of the metabolite mixture. The difference spectrum showed a single peak at 530 nm. Treatment of the sample with base produced a substantial red shift of approximately 150 nm which was reversed upon neutralization with acid. Acid treatment alone had no effect. The presence of a reversible base-induced red shift suggests that phenolic compounds are prominent components of the metabolite mixture. However, the inability of sodium hydroxide to shift the entire 530 nm peak to higher wavelengths indicates the presence of other types of compounds in the extract, as expected.

In order to obtain some more information on the identities of individual metabolites, peaks a,b, and d were collected and their

FIGURE 12. UV-visible spectra of a mixture of the S9 metabolites of 3-nitroperylene and of several purified metabolites.

(A) Difference spectrum of an ethyl acetate extract of 3-nitroperylene metabolites. 3-Nitroperylene itself is in the reference cuvette.

(i) ———— ethyl acetate extract at neutral pH

(ii) ——— (i) + 25 µl of 6 M NaOH (pH ≃ 13)

(B) Spectra of metabolite b at various pH's.

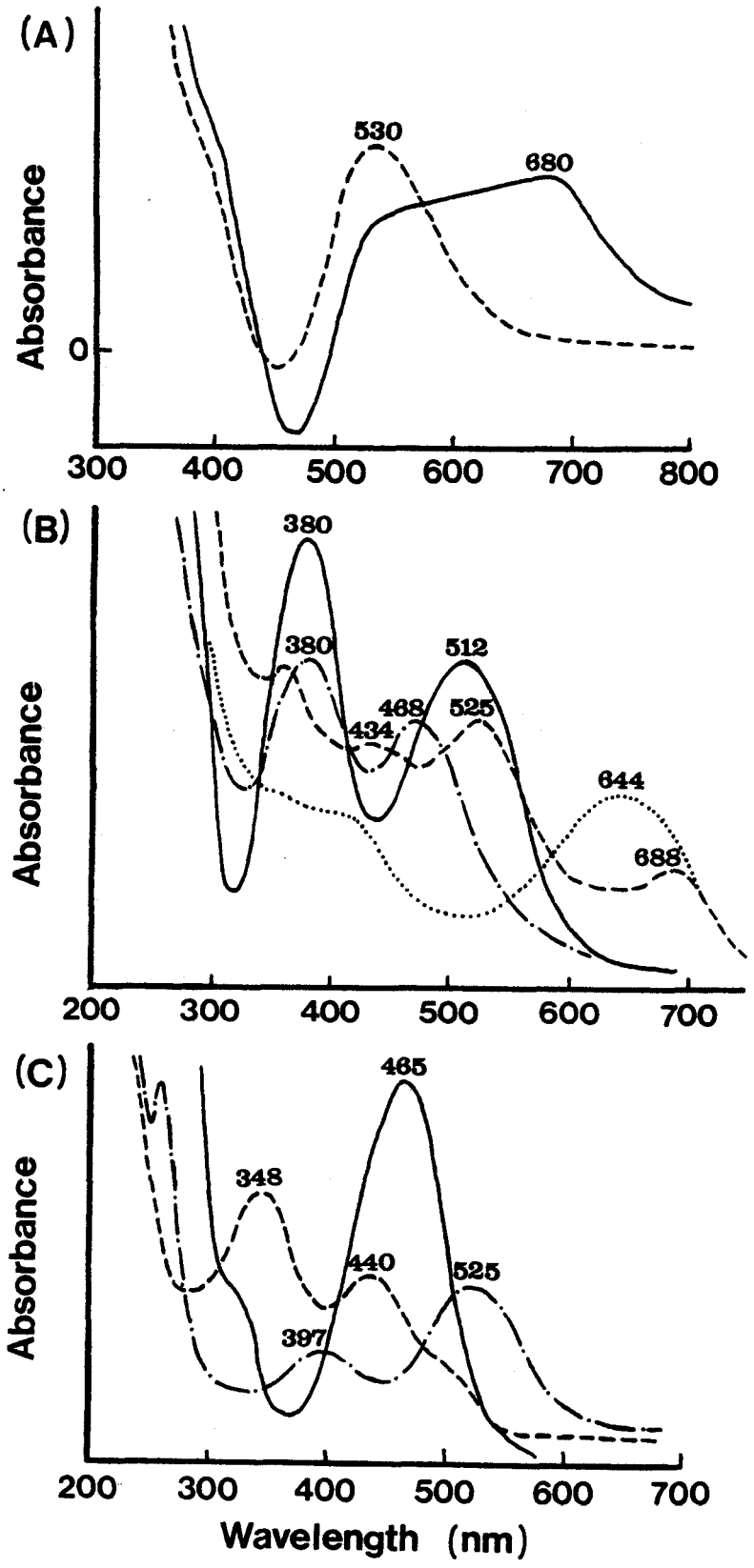
(i) ——— b at neutral pH

(ii) ————(i) + 50 µl of 1 M NaOH (pH ≃ 13)

(iii) —·—(ii) + 100 µl of 1 M HCl (pH ≃ 1)

(iv) •••••• (iii) + 150 µl of 1M NaOH (pH ≃ 13)

(C) Spectra of 3-nitroperylene (———) and metabolite c (— — —) and d (—·—) at neutral pH.


(A)
Absorbance
0
530
680
300
400
500
600
700
800
(B)
Absorbance
380
380
512
468
525
434
644
688
200
300
400
500
600
700
(C)
Absorbance
465
348
440
525
397
200
300
400
500
600
700
Wavelength (nm)

UV-visible spectra taken. Each of these three compounds gave a spectrum with two absorption maxima as indicated in Figures 12b and c. For comparison, the spectrum of 3-nitroperylene, which shows a single strong absorption maximum, is shown in Figure 12c. Sufficient quantities of product b were obtained to enable the effects of the addition of acid and base on its spectrum to be determined (Fig. 12b). When the pH was raised, a red shift was observed and subsequent neutralization restored the initial profile. When the neutralized sample was treated with more acid, one of the two absorption peaks (λ max 512 nm) was shifted to a shorter wavelength (λ max 468 nm) whereas the other (λ max 380 nm) remained unchanged. Upon subsequent treatment with base a large red shift was observed. These data are again consistent with a phenolic type metabolite. From the relatively short retention time of peak b the metabolite appears to be quite polar and may contain more than one hydroxyl group. The spectral data are consistent with a hydroquinone. A dihydrodiol is less likely since such a compound would not be expected to show a reversible red shift.

4.1.3. Covalent Binding of 3-Nitroperylene Metabolites to Protein

Since the majority of chemical mutagens and carcinogens bring about their genetic effects by binding covalently to cellular macromolecules, the ability of 3-nitroperylene metabolites to bind to microsomal protein and calf thymus DNA was examined.

Earlier studies showed that glucose-6-phosphate dehydrogenase-supplemented microsomes were alone sufficient to activate 3-nitroperylene to mutagenic species in the Ames test, and were able to

metabolise the compound in a similar manner to whole S9. In the protein binding studies presented below, microsomes alone were used (rather than whole S9) in order to decrease the protein concentration and hence the number of nucleophilic sites available for attack by reactive electrophiles. This should increase the specific activity of labelling. Only about 25% of S9 protein is microsome associated.

After tritiated 3-nitroperylene had been incubated with microsomes at 37°C for 60 minutes, 90-95% of the radioactivity could be removed by extraction with ethyl acetate. This consists of unmetabolized 3-nitroperylene, as well as ethyl acetate-soluble metabolites. If the protein was precipitated from the remaining aqueous phase, approximately 2% of the total radioactivity was associated with the precipitate, and the other 3-5% of the label remained as water soluble metabolites. About half of the precipitable counts were non-specifically associated with protein and could be removed by appropriate treatment of the sample, e.g. Sephadex G-25 chromatography or extensive dialysis. Only some 1% of the initial label appeared to be strongly (and presumably covalently) associated with protein.

When tritiated 3-nitroperylene was incubated with AR microsomes there was a time-dependent increase in both the amount of label associated with microsomal protein, and the amount of water soluble radioactivity (Figure 13). Under the conditions employed, binding reached a maximum of 1.5 - 2.0 nmol/mg protein after 30 to 60 minutes. Polyacrylamide gel electrophoresis of the labelled protein, followed by analysis of the gel for radioactivity, revealed that a substantial

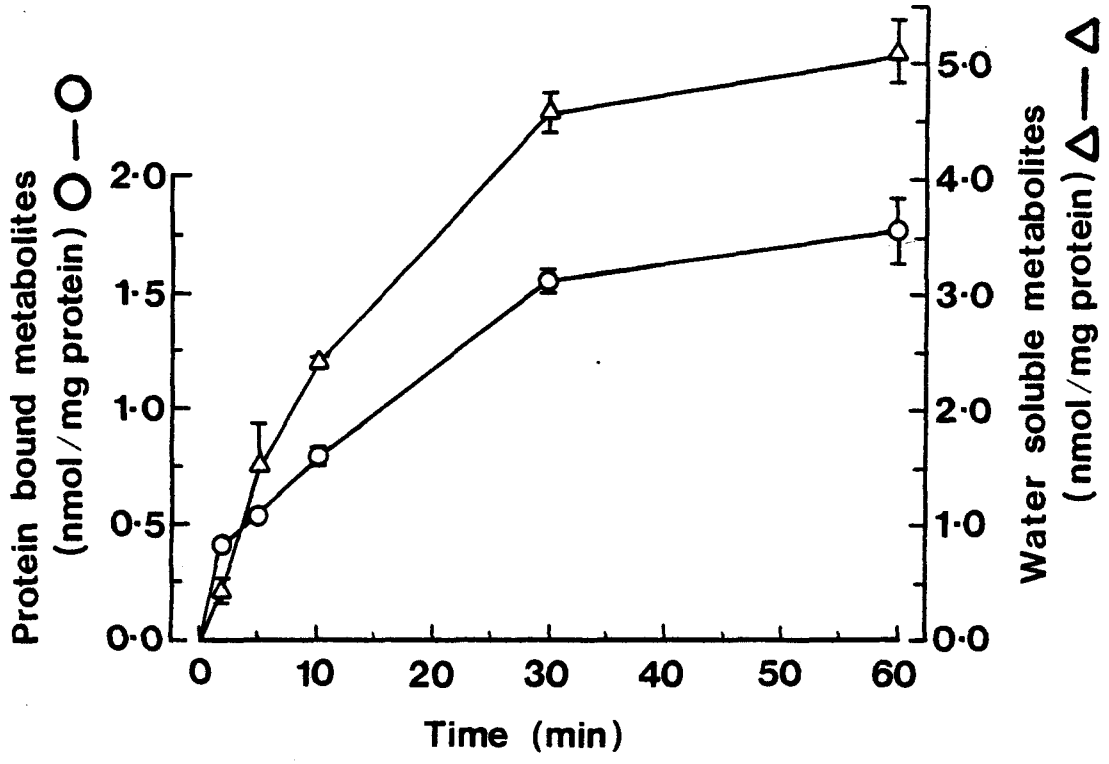
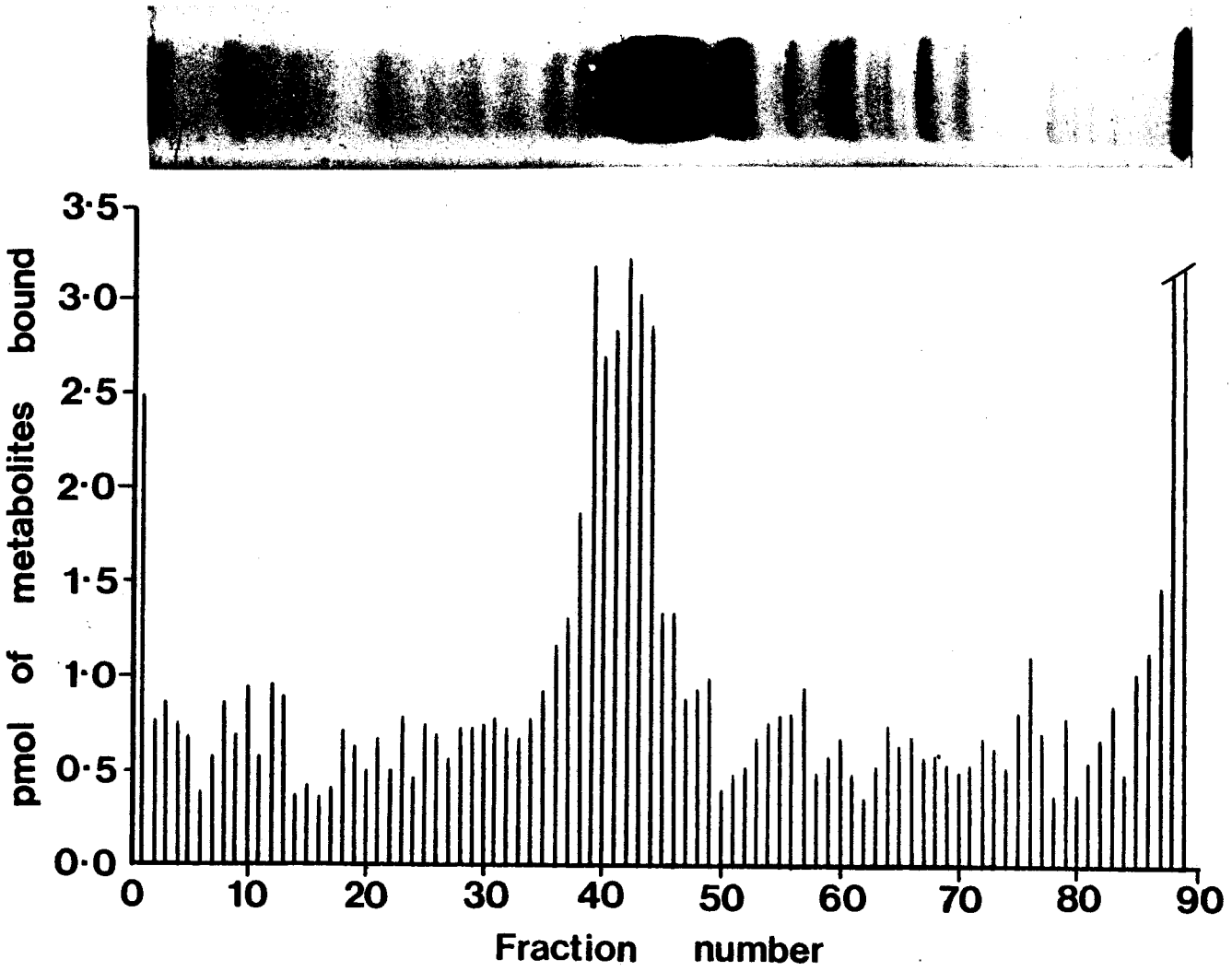


FIGURE 13. Time course of the microsome-mediated metabolism of (^{3}H) 3-nitroperylene to water soluble derivatives and to species which are able to bind covalently to microsomal protein.

Zero time values have been subtracted at each time point. Points represent means ± standard errors of duplicate determinations at each time point.

FIGURE 14. Electrophoretic analysis of the protein bound products resulting from the microsome-mediated binding of [^{3}H]3-nitroperylene to microsomal macromolecules.


pmol of metabolites bound
3·5
3·0
2·5
2·0
1·5
1·0
0·5
0·0
0
10
20
30
40
50
60
70
80
90
Fraction number

amount of label was associated with the major protein bands, confirming that covalent binding had indeed occurred (Figure 14). In similar experiments standard incubations were supplemented with 1 mg/ml of β-lactoglobulin and, following treatment with [^{3}H] 3-nitroperylene for the appropriate time at 37°C, the protein was extracted and electrophoresed. In confirmation of the results presented above, a substantial amount of radioactivity was associated with the β-lactoglobulin band (data not shown).

Earlier results using the Ames assay showed that strong activation of 3-nitroperylene occurred only over a narrow range of S9 concentrations, which were somewhat lower than those conventionally employed in the Ames assay. In view of this finding, an experiment was conducted to examine the effect of a range of microsomal enzyme concentrations on the covalent binding of 3-nitroperylene metabolites to protein (Figure 15). The total protein concentration was kept constant using BSA as the amount of microsomal protein was increased from 0.05 mg/ml to 2 mg/ml. Binding was found to increase with increasing microsome concentration rather than showing a peak of activity at low concentrations.

The ability of microsomes from control rats and rats treated with various MFO inducers to metabolize 3-nitroperylene to species which would bind to protein was also investigated (Figure 16). AR, MC, and NF microsomes all gave comparable levels of binding which were substantially higher than that seen with UN microsomes. However, PB microsomes generated only a small amount of binding which was only marginally above the level seen with control microsomes. These results

parallel the results of metabolism studies which showed that AR, MC and NF S9's all gave similar metabolite patterns, whereas PB S9 showed considerably different behaviour. Figure 16 also demonstrates that the covalent binding of label to protein was dependent on $NADP^+$, as would be expected from the foregoing mutation and metabolism data.

4.1.4. Covalent Binding of 3-Nitroperylene Metabolites to DNA

Of considerable interest in terms of defining a molecular basis for the mutagenicity of a particular compound is whether or not it can be activated to species which will bind covalently to DNA. When tritiated 3-nitroperylene was incubated with microsomes in the presence of calf thymus DNA there was a time dependent increase in the association of label with the nucleic acid (Figure 17). The binding was nearly linear over the first 30 minutes but thereafter levelled off rapidly. In accord with earlier results, the binding was dependent on NADP.

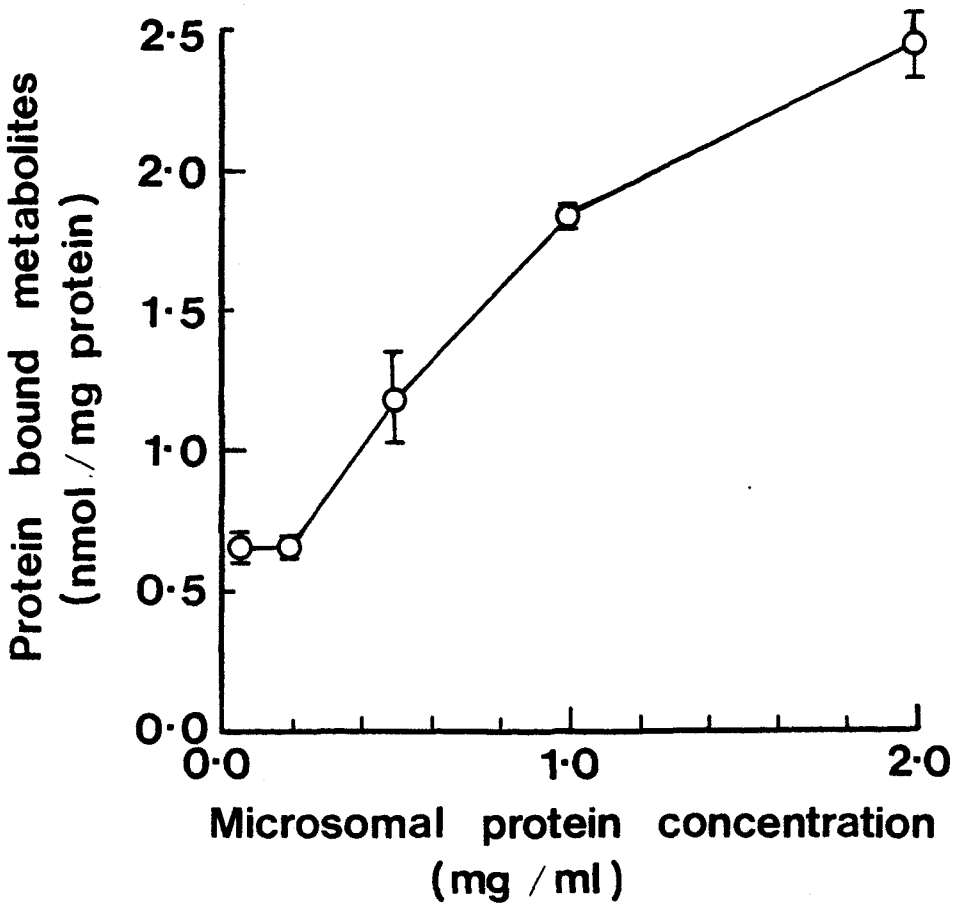


FIGURE 15. The influence of microsomal enzyme concentration on the covalent binding of (^{3}H) 3-nitroperylene metabolites to protein.

The total protein concentration was kept constant at 2 mg/ml by the addition of BSA while the microsomal protein concentration was varied from 0.05 mg/ml to 2.0 mg/ml.

Points represent means ± standard errors from duplicate determinations at each microsomal protein concentration.

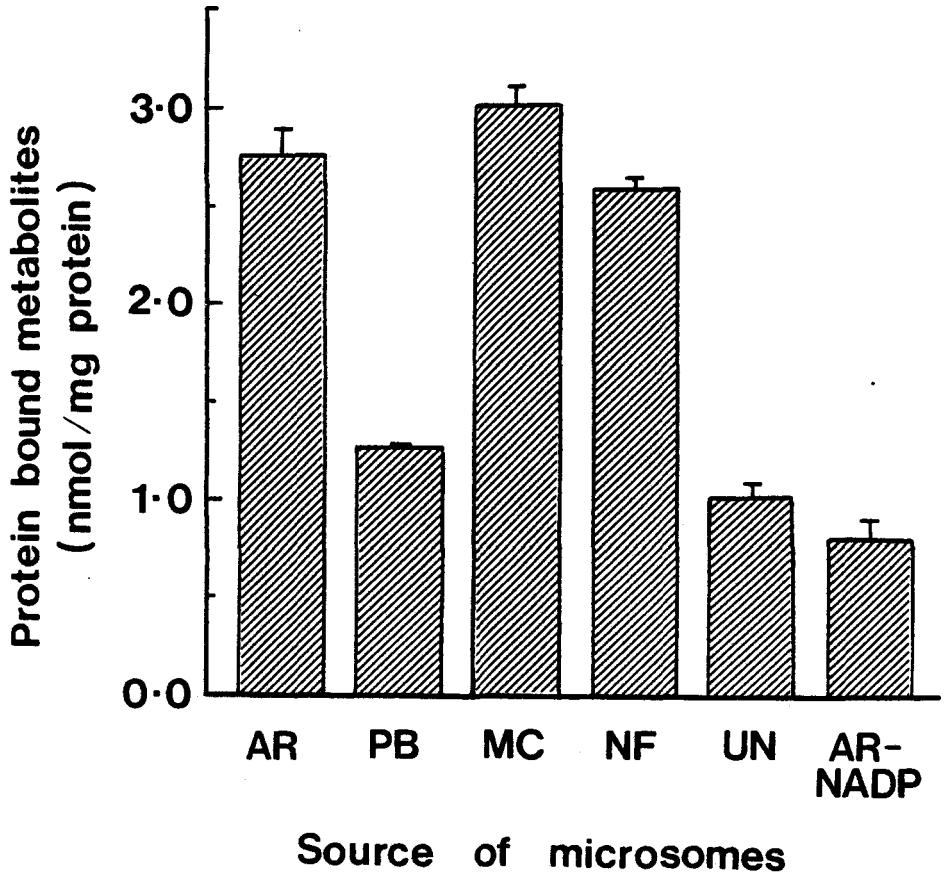


FIGURE 16. The relative abilities of microsomes prepared from rats pretreated with MFO inducers to activate (^{3}H) 3-nitroperylene to species which are able to bind to microsomal protein and the $NADP^{+}$ dependence of this activation.

Control levels of binding have not been subtracted. Columns represent means ± standard errors from duplicate experiments.

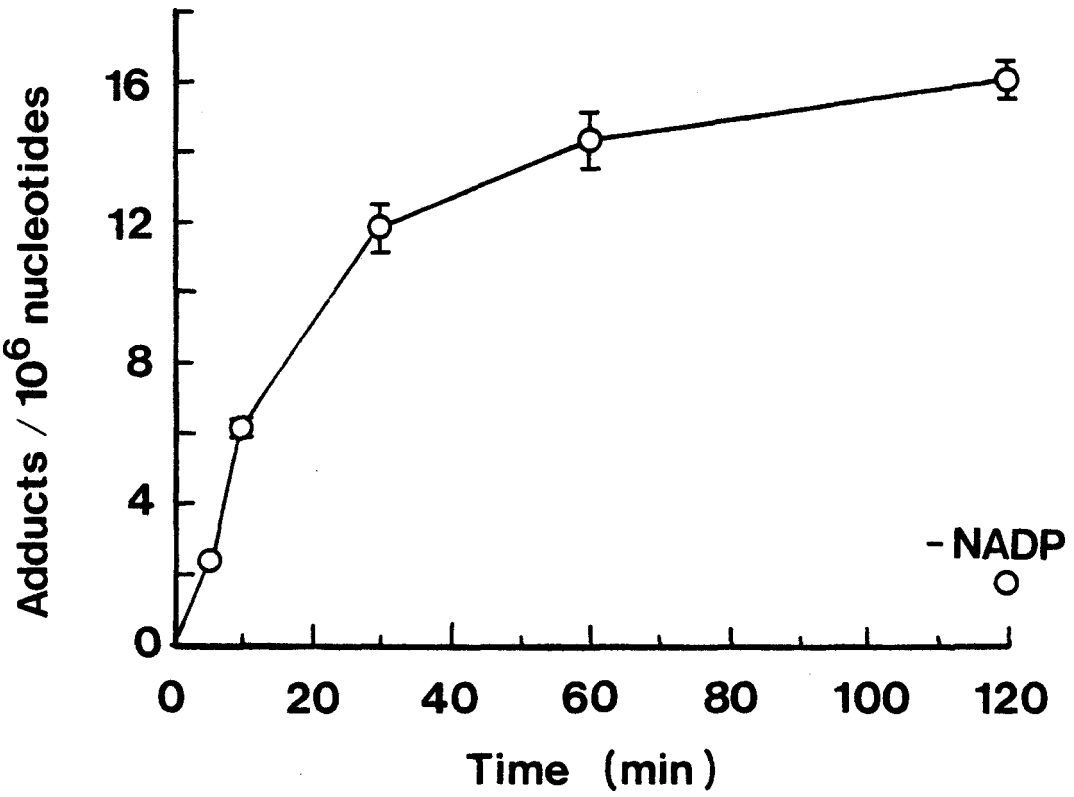


FIGURE 17. Time course of the microsome-mediated binding of (^{3}H) 3-nitroperylene metabolites to calf thymus DNA and the dependence of this binding on $NADP^+$.

Zero time values have been subtracted at each time point. Points represent means ± standard errors of duplicate experiments.

4.2. Discussion

Studies using the Ames test have shown that the mutagenicity of 3-nitroperylene is dependent upon metabolic activation by S9 enzymes. Thus, in order to obtain further information on the mechanism of activation, the metabolism of the mutagen by rat liver S9 was studied. When 3-nitroperylene was incubated with S9 prepared from AR treated rats it was metabolised to a number of ethyl acetate-extractable products as evidenced by HPLC analysis. The pattern of metabolites seen with microsomes was very similar to that seen with whole S9, but in each case quite high concentrations (> 10%) of the liver preparation were required before UV-absorbing products could be detected. Nevertheless, the time dependent disappearance of 3-nitroperylene from the incubation medium when concentrations of S9 as low as 2% were used indicated that metabolism was indeed occurring at these lower concentrations. It would be very useful to carry out experiments with [^{3}H]3-nitroperylene at these lower concentrations. The microsomal localization of the enzymes involved, the increase in the metabolism seen upon enzyme induction, its strong requirement for $NADP^{+}$, and the need to keep the system well oxygenated, all implicate the P-450 dependent monooxygenase activities of S9 in the metabolism of 3-nitroperylene. This supports the mutation data which suggested that the MFO system was involved in the activation of 3-nitroperylene to mutagenic species.

Attempts to identify 3-nitroperylene metabolites by standard procedures (MS, GC-MS) were without success as the compounds proved to be quite labile. However, UV-visible spectroscopy indicated that

phenolic products constituted a prominent proportion of the ethyl acetate extractable-material. In addition, one of the purified metabolites showed spectral behaviour indicative of a phenol, and, in view of its comparatively high polarity, it was tentatively identified as a hydroquinone. A metabolite of this type is not unlikely since aromatic hydroxylation is one of the major reactions catalysed by MFO enzymes (Hodgson and Dauterman, 1980). In addition, the hydroxylated metabolites of many PAH's are proximate mutagens in the Ames test, and may be further activated to reactive electrophilic ultimate mutagens (Conney, 1982).

The S9 metabolism of several nitroPAH's has been investigated and in each case phenolic compounds have been the predominant products (El-Bayoumy and Hecht, 1982, 1983; Fu et. al., 1982). As would be expected from the results with simple PAH's, however, a variety of other metabolites have been found including quinones, dihydrodiols and epoxides (El-Bayoumy and Hecht, 1982, 1983; El-Bayoumy et. al., 1982; Fu et. al., 1982; Wang and Burlingame, 1983). The finding that some nitroPAH's may be metabolized to epoxides by mammalian enzymes has important implications in interpreting the S9 mediated mutagenicity of these compounds. The phenolic metabolites of both 1-nitropyrene and 6-nitroB(a)P are substantially more mutagenic than their parent nitro compounds, but only in the presence of S9, indicating that they must undergo at least one further activation step. 9-HydroxyB(a)P is a prominant proximate mutagen resulting from the S9 mediated activation of B(a)P, and it is subsequently metabolized to 9-hydroxyB(a)P-4,5-oxide which is an ultimate mutagen (King et. al.,

1976). The mutagenicity of the hydroxyl derivatives of nitroPAH's is probably realized via a similar mechanism.

There have been several reports that, under anaerobic conditions, the incubation of nitroPAH's with S9 can lead to the formation of the corresponding amines (El-Bayoumy and Hecht, 1982, 1983; El-Bayoumy et. al., 1982; Wang and Burlingame, 1983). These reactions appear to be catalyzed by oxygen-sensitive mammalian nitroreductases. Under aerobic conditions the amines are either not formed, or are only formed in limited quantities, and oxidative metabolism by monooxygenases predominates. In experiments with 3-nitroperylene, no peak corresponding to 3-aminoperylene was apparent, supporting the results from mutation experiments which suggested that nitroreduction, at least under the conditions employed in the Ames assay, was unlikely to be involved in the mutagenicity of the compound.

When 3-nitroperylene was incubated with S9's prepared from rats which had been pretreated with various inducers of the MFO system, some interesting metabolite patterns were seen. With MC and NF S9's the profile of metabolites was very similar to that seen with AR S9, although the yield of products was much lower. A substantially different pattern was seen with PB S9. The total amount of metabolites produced was of the same order as that seen with MC and NF S9's, but the relative proportions of the various products were considerably different. Most of the radioactivity eluted in the region around metabolites c and d, while very little in the way of polar material was seen. In addition, metabolite e was missing and a new peak, eluting very close to the 3-nitroperylene peak, was present. The mutation data

presented in the previous chapter demonstrated much similarity in the ability of PB, MC and NF S9's to activate 3-nitroperylene to mutagenic species in bacteria, whereas the metabolite profiles indicate a dissimilarity in the metabolic capabilities of the various S9 preparations. There are several possible explanations for these apparently anomalous findings.

Firstly, the polar products seen with MC and NF S9's, but not with PB S9, may not contribute significantly to the mutagenicity of the parent nitro compound, and the proximate (or ultimate) mutagens could elute in the region where material formed by each of the different S9's was observed, i.e. around peaks c and d. However, if c or d themselves were the active species it would also be difficult to explain the mutagenicity data, because both are produced in much larger quantities with PB S9 than with MC or NF S9. Similarly, with this agument, the particularly effective activation provided by AR S9 is difficult to explain, as the quantities of compounds c and d produced when AR S9 is used in metabolism experiments are still less than the amounts produced when PB S9 is used. In addition, the major proximate and ultimate mutagens of many PAH's (for example the dihydrodiols of B(a)P) are relatively polar compounds, and elute fairly early under the usual HPLC conditions employed in the analysis of PAH metabolites (Selkirk, 1978). With the HPLC protocol used in the present series of experiments, dihydrodiol-type compounds should elute well before peaks c and d, although there is little evidence at the present time that this type of derivative is involved in the mutagenicity of 3-nitroperylene.

The second, and perhaps least likely, possibility is that the

equivalent levels of mutagenicity seen with each S9 are merely fortuitous and represent the cummulative effects of a spectrum of metabolites with varying mutagenic potencies. To assess this proposal effectively it would be necessary to determine the relative mutagenicities of individual metabolites.

A more likely explanation is that the profile of metabolites observed in these experiments with a very high S9 concentration (50%) is unrepresentative of that produced under the incubation conditions used in the Ames assay, where optimal mutagenicity is seen with very low levels of S9 (1-2% for AR S9; 2-5% for the other S9's). Mutagenicity is much lower at higher S9 concentrations. In support of this suggestion is the earlier observation (Chapter 3) that the optimum concentration of S9 in liquid preincubation mutation assays with 3-nitroperylene was the same as that in the plate incorporation assay. In fact, when liquid preincubation was used a substantially higher level of reversion was obtained. Additional evidence in favour of this proposal is the observation that both MC and NF S9's are effectively able to metabolize 3-nitroperylene to species which will bind to microsomal protein, whereas PB S9 is rather ineffective in promoting binding. These results will be discussed in more detail below. Despite several lines of evidence in support of the third suggestion, caution must be exercised in attempts to draw comparisons between results obtained in different systems and with different concentrations of both substrate and enzymes. The factors regulating the outcome can become rather involved as was pointed out in the discussion of the previous chapter (section 3.2.).

Differences in the patterns of 3-nitroperylene metabolites seen with either PB or MC S9 are not surprising in the light of results obtained with numerous simple and derivatized PAH's. Individual S9's vary substantially in the proportions of their constituent P-450 isozymes depending on the nature of the induction procedure used. In addition, different P-450's have their own range of substrate specificities and usually show significant regio- and stereoselectivity (Wiebel et. al., 1975; Wood et. al., 1976; Gozukara et. al., 1982; Hara et. al., 1983). Most PAH's are preferentially metabolized by MC S9 in reactions catalyzed by P-450c dependent MFO activities (Conney, 1982). For example, the conversion of B(a)P to metabolites by purified cytochromes from MC treated rats is about 20-fold greater than that seen with P450's from PB treated animals (Gozukara et. al., 1982). The greater effectiveness of MC and NF S9's than PB S9 in converting 3-nitroperylene to a variety of products suggests that the compound is susceptible to similar types of metabolism as simple PAH's, although the surprising ability of PB S9 to lead to the formation of some metabolites in quite high yield may be related to the mutagenicity of the compound and cannot be overlooked. The only other nitroPAH whose metabolism has been studied using PB and MC S9's is 6-nitroB(a)P (Fu et. al., 1982). The results were similar to those seen with B(a)P, as MC S9 was far more effective than PB S9 in converting the nitro compound to a variety of oxidative products. The metabolite pattern was similar with both S9's.

Most chemical mutagens and carcinogens exert their effects by binding covalently to critical cellular macromolecules such as DNA, RNA

and protein. Reactive electrophilic intermediates generated through metabolic activation of a compound may react with a variety of nucleophilic sites on both protein and nucleic acids. In proteins, the sulfur atons of cysteine and methionine, as well as sites on histidine and tyrosine are prominant targets, while in nucleic acids the N^7 and C-8 positions and the exocylic amino group of guanine are frequently the sites of covalent adduct formation (Holbrook, 1980). Although the modification of DNA appears to be the major change associated with the carcinogenesis of various chemicals (Brookes and Lawley, 1964; Phillips et. al., 1979), binding to protein is much greater in quantitative terms (Kuroki and Heidelberger, 1971).

As expected of a potent mutagen, 3-nitroperylene was activated by microsomal enzymes to species which were able to bind tightly, and presumably covalently, to both DNA and protein. The levels of protein binding found were comparable to those reported in the literature for PAH's of similar size (Tunek et. al., 1979), and the binding to both DNA and protein was dependent on $NADP^+$. In addition, the amount of label associated with protein was much higher when microsomes from rats which had been treated with MFO inducers were used, than it was when control microsomes were employed to provide activation. These characteristics of the binding, like the earlier mutation and metabolism data, strongly implicate the MFO system in the activation of 3-nitroperylene to electrophilic, macromolecule-binding species.

When the ability of microsomes to promote the binding of 3-nitroperylene metabolites to protein was examined using preparations from rats which had been pretreated with various inducers of MFO

enzymes, some interesting results were obtained. Whereas PB, MC and NF S9's were equally effective in activating the compound to mutagenic species in the Ames test, MC and NF microsomes were far more effective than PB microsomes in catalysing the conversion of 3-nitroperylene to protein-binding metabolites. This result, however, is consistent with metabolism experiments in which PB and MC (or NF) S9's gave rise to quite different profiles of metabolic products, and again suggests that under relatively high enzyme concentrations (1 mg of microsomal per ml of reaction mixture corresponds to 10-15% S9), 3-nitroperylene appears to be preferentially metabolized by the major MC-inducible form of P-450. Studies on the ability of the metabolites of several aromatic hydrocarbons (including B(a)P and MC) to bind to microsomal proteins have also indicated preferential activation by MC induced enzymes (Tunek et. al., 1979). The result with MC as a mutagen is notable in relation to the behaviour shown by 3-nitroperylene, because PB S9, and not MC S9, preferentially activates MC in the Ames test (Ames et. al., 1975), again indicating that the activities observed in metabolism or macromolecule binding studies do not necessarily reflect accurately the mechanisms involved in the expression of the mutagenicity of a compound.

In order to gain some more information as to how well the conditions of the protein binding experiments reflect those of mutation assays, the binding of radioactivity to protein was examined over a range of microsome concentrations (corresponding to S9 concentrations from 0.5% to 20%). In contrast to the mutation studies where the optimum S9 concentration occurred at quite low amounts of liver

protein, protein binding increased steadily as the microsome concentration was increased. The explanation of these apparently disparate findings probably lies in the use of microsomes in the protein binding studies but whole S9 in mutation assays. It was noted in the previous chapter that in the Ames test, the biphasic behaviour seen in the response of B(a)P to increasing S9 concentrations is critically dependent on increasing quantities of soluble S9 components (i.e. the cytosolic fraction) (Malaveille et. al., 1979). If the microsome concentration is increased in the presence of a constant amount of S9 cytosol, a simple dose-response relationship is seen in the mutagenic activity of B(a)P. A similar explanation could be invoked to explain the protein binding data. The amount of protein in each assay was kept constant using BSA while the amount of microsomes was increased, so the total concentration of nucleophilic groups on protein remained relatively constant. In addition, the capacity of microsomal preparations to form water soluble conjugates is limited since the concentrations of both the required low molecular weight cofactors and some of the prominant conjugate-forming enzymes are quite low (e.g. glutathione-S-transferases) (Wood et. al., 1980). Conjugation reactions are probably very important determinants in the biphasic response shown by both B(a)P and 3-nitroperylene in the Ames assay. Even in the absence of the soluble protein fraction of S9 there was a time-dependent increase in the amount of water soluble radioactivity when tritiated 3-nitroperylene was incubated with microsomes. This label presumably represents metabolite conjugates since UDP glucuronyltransferases, which are microsomal enzymes, are

still present, and there are probably sufficient residual quantities of UDPGA for some conjugates to be formed. Small amounts of other conjugated products are also likely. The formation of water soluble products following the incubation of a number of compounds with microsomes has been reported (Tunek et. al., 1978, 1980).

Several studies have shown that when various labelled compounds are incubated with microsomes, and the protein is subsequently analysed by polyacrylamide gel electrophoresis, radioactivity is found in a small number of protein bands, rather than being distributed non-specifically in all protein species (Tunek et. al., 1979; Kaderbhai et. al., 1981). Similar specific binding has been found in the nuclear proteins of mammalian cultured cells (Macleod et. al., 1980, 1981; Zytkovicz, 1981). The specificity of binding in microsomal proteins depends, firstly, on the concentrations of suitable nucleophilic sites within the individual proteins, secondly, on the accessibility of the proteins within the microsomal membranes and, thirdly, on the proximity of the target polypeptides to the sites of metabolism of the chemical i.e. to the MFO enzymes. For most compounds studied, binding has been maximal in proteins with molecular weights between 50 and 55K. With 3-nitroperylene, binding was also very heavy in this region, but the detection system used was not sensitive enough to determine with certainty whether other bands were specifically labelled. For several small aromatic compounds, such as benzene and phenol, highly specific binding to a protein with a molecular weight of 72K is observed (Tunek et. al., 1979).

The heavy concentration of labelling in proteins with molecular

weights of 50-55K does not appear to be due solely to the large quantity of protein in this region. This is the region in which the P-450 cytochromes and epoxide hydrase migrate, and it is conceivable, if not probable, that the proximity of these proteins to the activated species of various chemicals accounts for much of the binding in the 50-55K polypeptides. In addition, the major band labelled by benzene and phenol metabolites migrates close to the area in which NADPH-cytochrome P-450(c) reductase (another component of the MFO system) should be found (Kaderbhai et. al., 1981). Excellent support for this proposal comes from experiments with purified MFO components. When radioactively labelled B(a)P or polychlorinated biphenyls are incubated with a reconstituted monooxygenase system, covalent association with individual MFO proteins has been found (Gozukara et. al., 1981; Shimada et. al., 1981).

The covalent binding of the metabolites of many compounds to DNA has long been recognized as an important step in mutagenesis and carcinogenesis (Grover, 1979). The microsome-mediated binding of PAH metabolites, particularly those of B(a)P, to DNA has been extensively studied over the past 15 years (Grover and Sims, 1968; Gelboin, 1969; Thompson et. al., 1976; Conney, 1982) and the mechanisms of adduct formation are quite well understood (Phillips and Sims, 1979). The only nitro PAH whose DNA binding has been relatively well studied is 1-nitropyrene (Howard and Beland, 1982; Howard et. al., 1983; Messier et. al., 1981), but binding of the metabolites of 1,8-dinitropyrene (Andrews et. al., 1982) and 6-nitroB(a)P (Martin et. al., 1982) to DNA has also been reported. The binding of 1-nitropyrene metabolites to

calf thymus DNA using a pure enzyme (xanthine oxidase) to provide metabolic activation was much higher than that reported for 3-nitroperylene in the present experiments using a microsomal preparation to activate the mutagen. This is expected in view of the far greater number of potential target nucleophiles available when microsomes are used. Analysis of the major deoxyribonucleoside adduct formed from 1-nitropyrene activated by xanthine oxidase in vitro indicated an attachment at the C-8 of guanine. The same adduct was also formed in Salmonella in the absence of S9. This behaviour is characteristic of that shown by mutagenic species derived from the oxidative metabolism of aromatic amines and amides (such as 2-acetylaminofluorene) (Kriek and Westra, 1979), demonstrating the importance of the nitro group in the mutagenesis of both these compounds and 1-nitropyrene. The possibility that 1-nitropyrene can be activated to additional DNA-binding species in the presence of S9 via ring oxidation by MFO enzymes has yet to be investigated. However, the nature of the oxidative products of this compound which have been identified following its incubation with S9 (Wang and Burlingame, 1983) suggests that such adducts could be formed. 3-Nitroperylene, on the other hand, appears to be activated primarily via an oxidative pathway, and DNA adduct formation, at least using the in vitro conditions of the current study, probably involves the exocyclic amino group at C-2 of guanine, which is the typical site attacked by the epoxide ultimate mutagens of PAH's. However, in view of the suggestion from mutation studies that at least one bacterial enzyme is involved in the activation of the compound, the adduct formed in Salmonella, which is

responsible for the mutagenesis, could be quite different.

5. CONCLUSIONS AND PROSPECTS

5. Conclusions and Prospects

Like most indirect acting mutagens, 3-nitroperylene is activated to proximate, and perhaps ultimate, mutagenic species by the cytochrome P-450 MFO system of rat liver microsomes. Most PAH's are preferentially metabolized to mutagens by P-450c-dependent activities, and indeed this type of behaviour is also shared by at least one indirect-acting nitroPAH, 6-nitroB(a)P. However, the isozymes involved in the activation of 3-nitroperylene have yet to be identified with certainty. Under a variety of circumstances in the Ames test, using both microsomal enzyme inhibitors, and S9's prepared from animals which had been pretreated with MFO enzyme inducers, substantial differences between the behaviour of 3-nitroperylene, and 6-nitroB(a)P and its parent hydrocarbon, were noted. If both major forms of P-450 (P-450's b and c) are involved in the activation of 3-nitroperylene (as the induction data suggests) then the inhibitor experiments are difficult to explain, and a fairly complex activation mechanism would need to be invoked. The involvement of a minor isozyme of P-450, though, which is induced by each of the pretreatments used in this study, could account for all of the observations.

Any consideration of P-450 mediated activation must take into account the important finding that there is some bacterial involvement in the expression of the mutagenicity of 3-nitroperylene. The nature of this involvement is unknown at present, and an explanation must await the determination of the enzyme deficiency in the resistant Salmonella strain (TA98 DNP_6). Nitroreduction may be involved, but since some oxidative activation step(s) are necessary also, any attempt

to demonstrate nitroreduction by, say, conducting mutation assays under anaerobic conditions, would, of course, result in failure. Experiments using the appropriate Salmonella strains, however, indicate that the "classical" nitroreductase either does not play a significant role in 3-nitroperylene activation, or is involved but is not rate limiting.

Attempts to identify the microsomal metabolites of 3-nitroperylene met with little success, although phenolic products appeared to be present. Nevertheless, sufficient data was gathered both in these studies, and in studies on the in vitro binding of 3-nitroperylene metabolites to protein and DNA, to suggest that the processes occurring in these experiments were unrepresentative of those operating in mutation assays. High concentrations of S9 were required in vitro to lead to reasonable quantities of metabolites being formed, and the results indicated preferential metabolism by P-450c. Similar results were provided by the macromolecule binding experiments. This behaviour suggests that under the conditions employed in these studies, 3-nitroperylene was being activated like a simple PAH such as B(a)P. (It is interesting to note in this regard that under very high S9 concentrations ($\simeq$ 40%) perylene itself is mutagenic in the Ames test (Pitts et. al., 1979)).

There are a number of approaches which could be taken to gain further insight into the mechanism of activation of 3-nitroperylene. The most direct is to attempt to determine the structures of the S9 metabolites of the compound using concentrations of liver protein similar to those used in the Ames assay. From the experience in the present project it should be relatively easy to obtain sufficient

quantities of metabolites for analysis, using S9 concentrations below 5%, if large scale incubations are carried out, but refinements in the analytical procedures will be necessary as 3-nitroperylene derivatives have proved to be quite labile. A very useful approach would be to examine more closely the forms of P-450 involved in the activation. This could be achieved in several ways. The most thorough is to purify individual P-450 isozymes and to use a reconstituted MFO system to activate the compound in mutation studies. Perhaps an equally effective, yet less involved procedure is to use monospecific antibodies against individual isozymes to dissect the activating system. A third approach is to use very specific inducers (individual PCB or PBB isomers) of the various P-450's to prepare S9's with a very narrow range of activating capabilities. There is a growing body of literature on each of these three methods as they can be used to help determine what is actually happening in the mutation assay, thus complementing metabolism and macromolecule binding studies in vitro. The bacterial step(s) involved in the mutagenicity of 3-nitroperylene will become clearer once the enzyme deficiency in resistant bacterial strains has been identified. Work has commenced on this problem in our laboratory.

Although the nitroPAH's are spectacular bacterial mutagens, their effects on mammalian systems have yet to be well defined. Most of the direct acting nitroPAH's are largely inactivated in the presence of S9, so there are some doubts as to their genotoxic potency in the aerobic in vivo environment. For example, 1-nitropyrene is a strong bacterial mutagen, but current data indicates that it is

non-carcinogenic in mouse skin. Activation to mutagenic forms by gut microflora following ingestion may pose a more significant hazard. Since the indirect-acting nitroPAH's are activated under aerobic conditions, they have the potential for causing more widespread genotoxic damage in the body, and are perhaps worthy of a more significant research effort. 3-Nitroperylene has proved an interesting mutagen in bacteria with an unusual activation pathway. The elucidation of this mechanism, and those of other nitrated perylenes, should provide considerable insight into the factors determining the mutagenicity of many nitroPAH's.

6. REFERENCES

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