Pathways for the Mutagenesis of 1-Nitropyrene and Dinitropyrenes in the Human Hepatoma Cell Line HepG2

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The mutagenicity, metabolism, DNA adduction and induction of unscheduled DNA synthesis (UDS) of 1-nitropyrene and 1,8-dinitropyrene were investigated in the human hepatoma cell line HepG2. Previous results had demonstrated that 1-nitropyrene was both mutagenic at the *hgprt* locus and induced UDS in these cells. In the present study, we find that the dinitropyrenes, although highly mutagenic in *Salmonella typhimurium*, are not mutagenic and do not induce UDS in the HepG2. Although the rate of 1,8-dinitropyrene nitroreduction was less than that of 1-nitropyrene nitroreduction, this did not explain the lack of mutagenicity and UDS induction by the dinitropyrenes. Therefore, it is proposed that the arylhydroxylamine *O*-esterificase is not expressed in these cells. Since cytochrome P450-mediated C-oxidation is the predominant metabolic pathway *in vivo*, we sought to determine if an increase in the ratio of cytochrome P450-mediated C-oxidation over nitroreduction would result in increased or decreased DNA adducts in the HepG2. The administration of 2.5 μM 3-methylcholanthrene to the HepG2 increased the ratio of C-oxidation/nitroreduction from 2.8 ± 1.9 to 50.4 ± 46.1. This was accompanied by a decrease in the C8-guanyl adduct of 1-nitropyrene (via nitroreduction) from 18.7 ± 7.0 to 4.8 ± 1.7 fmoles/μg DNA, without any further increase in other 1-nitropyrene DNA adducts. These results suggest that the cytochrome P450-mediated metabolism of 1-nitropyrene to epoxides, phenols, and dihydrodiols is not an activation pathway in the HepG2 cells, and may explain the weak carcinogenicity of 1-nitropyrene *in vivo*, where cytochrome P450-mediated C-oxidation predominates. — Environ Health Perspect 102(Suppl 6):195–200 (1994)

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Introduction

The method for assessing the human risk of a chemical is to use the *in vivo* and *in vitro* data on the mutagenicity and tumorigenicity of the chemical, and to determine the likelihood for human genotoxicity or adverse health effects. Risk assessment evaluations take into consideration *in vitro* data from both prokaryotic and eukaryotic cell studies, as well as *in vivo* toxicity studies from several species. It would be most advantageous to study the metabolism and genotoxicity of chemicals *in vitro* in human cells that metabolically resemble the cell of interest in humans. While studying the metabolism and genotoxicity of chemicals *in vitro* ignores the interaction of chemicals and metabolites between cells or organs (e.g. enterohepatic circulation), it may present a simulated environment in which to study the generation of reactive metabolites within a human cell.

The human hepatoma cell holds good promise as an *in vitro* candidate for studies on xenobiotic metabolism. The isolation of primary human hepatocytes is restricted by a lack of availability of tissue, and poses the additional problem of interindividual variation. Several hepatoma cell lines have been isolated, with the most promising being the HepG2 cell line. This cell line was derived from a primary hepatoblastoma isolated from an 11-year-old Argentinean male (*1*). These cells retain many characteristic enzyme pathways of hepatocytes (*1–15*), and have been cultured successfully for more than 100 passages.

The HepG2 has been shown by several groups to possess the enzymes necessary for the activation of many chemicals. For instance, Diamond and coworkers (*16*) demonstrated that when X-ray-irradiated HepG2 was cocultured for 48 hr with Chinese hamster V79 lung cells and 1 μg/ml 15,16-dihydro-11-methylcyclopenta[*a*]phenanthrene-17-one, 7.8 mutations per 10^5 clonable cells were noted in the V79 cells. Dearfield et al. (*17*) demonstrated that the HepG2 was capable of activating cyclophosphamide to induce sister chromatid exchanges (SCEs), and demonstrated that the content of cytochrome P450 is very low in the HepG2 cells (a phenomenon that has been reported by several laboratories). Other compounds that have been reported to be activated by the HepG2 include benzo[*a*]pyrene (*18,19*), 7,12-dimethylbenz[*a*]anthracene (*20,21*), aflatoxin B_1, (*22,23*), several *N*-nitroso compounds (*24*), benzidine (*23,25*), acetylbenzidine (*23*), 2-aminofluorene (*23*), 2-aminoanthracene (*23*), dibenz[*a,h*]anthracene, 7-methyldibenz[*a,h*]anthracene, 7,12-dimethyldibenz[*a,h*]anthracene, 3-methylcholanthrene, 3,6-dimethylcholanthrene, 1-methylbenzo[*e*]pyrene, 7,12-dimethylbenz[*a*]anthracene, and 1-,4-, or 10-fluoro-7,12-dimethylbenz[*a*]anthracene (*21*).

The metabolism of 1-nitropyrene has been shown to involve both cytochrome P450-mediated C-oxidation (*26–30*) and nitroreduction (*31–35*). The C-oxidation of 1-nitropyrene by cytochrome P450 can result in the formation of two arene K-

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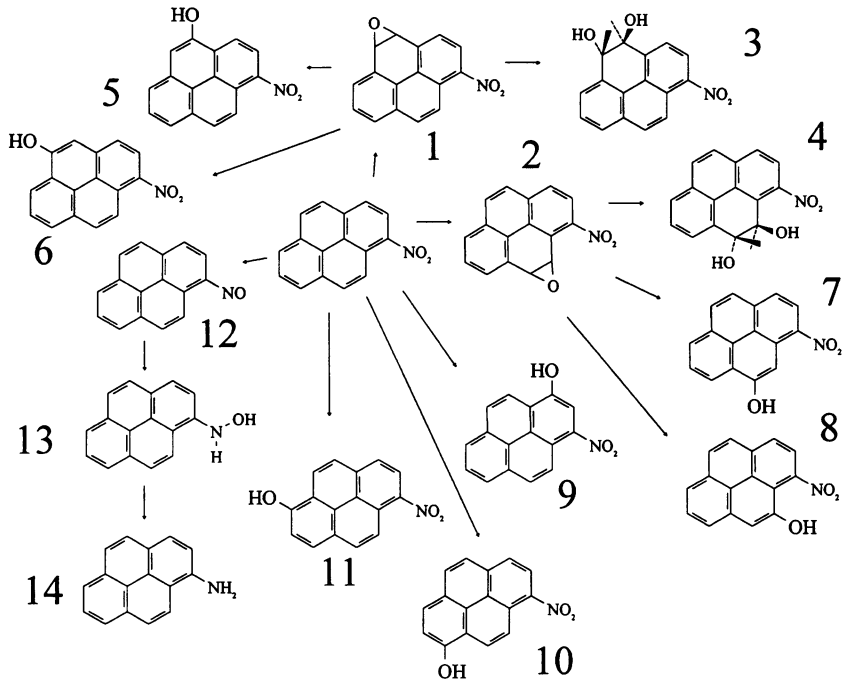

Figure 1. Scheme for the metabolism of 1-nitropyrene.

region oxides, 1-nitropyrene-4,5-oxide and 1-nitropyrene-9,10-oxide (Figure 1, structures 1,2). The K-region oxides are mutagenic in *Salmonella typhimurium* either with or without exogenous activating enzymes (*35,36*). The K-region oxides can be hydrolyzed by epoxide hydrolase to the corresponding K-region *trans*-dihydrodiols (structures 3,4), or can rearrange to form four K-region phenols (structures 5–8). Alternatively, cytochrome P450 can catalyze the direct formation of three phenols (structures 9–11). Each of these phenols has been reported to have differing mutagenicities in *S. typhimurium*, presumably through nitroreduction to hydroxylamine derivatives (*37,38*). Not shown in Figure 1 are the conjugation of these phenols to sulfate and glucuronide derivatives, or the hydrolysis of the epoxides via glutathione and glutathione transferases.

In *S. typhimurium*, 1-nitropyrene is mutagenic through nitroreduction to the corresponding nitroso (structure 12) then the hydroxylamino (structure 13) derivative, which has been shown to form a C8-guanyl adduct (*32*). This adduct also is responsible for the mutagenicity of 1-nitropyrene in CHO cells (*34*), and cultured human diploid fibroblasts (*39–40*).

We demonstrated earlier that HepG2 activates 1-nitropyrene to a mutagenic metabolite (*41*), inducing 76 mutants per 10^5 clonable cells at 4 µM 1-nitropyrene. However, the pathways responsible for this activation were not known. Moreover, both cytochrome P450-mediated C-oxidized metabolites and nitroreduced metabolites (1-aminopyrene) were detected (*41*). Therefore, we were unable to deduce the pathway or the DNA adduct responsible for the mutagenesis of 1-nitropyrene in HepG2.

The C-oxidative metabolism of 1-nitropyrene in different species is catalyzed by different cytochrome P450s [P450 2C3 in rabbit (*28*); P4503A4 in human (*30*); and P4502B1 and P4502C in rat (Howard, unpublished)]. Additionally, the nitroreduction of 1-nitropyrene is catalyzed by several enzymes in bacteria (*42,43*), and several enzymes, including NADPH-dependent cytochrome P450 reductase, *DT*-diaphorase, aldehyde oxidase, xanthine oxidase, and lipoyl dehydrogenase, in mammalian cells (*44–47*).

Therefore we sought to determine whether 1,8-dinitropyrene, like 1-nitropyrene, was mutagenic in HepG2 cells, and to describe the pathway involved in the metabolic activation of 1-nitropyrene and 1,8-dinitropyrene in HepG2.

Materials and Methods

Culture of HepG2 Cells

HepG2 cells were obtained from L. Diamond (Wistar Institute, Philadelphia, PA), and from the American Type Culture Collection (Rockville, MD). The cells were grown essentially as described in Eddy et al. (*41*) at 37°C in 5% CO_2 in humidified air on 100 mm tissue culture plates in Minimal Essential Medium (MEM; GIBCO) with 10% heat-inactivated (56°C, 30 min) fetal bovine serum (hi-FBS; ICN Biomedicals), and 10 units/ml penicillin and 10 g/ml streptamycin sulfate (GIBCO). The cells were subcultured (1:3) every 3 to 4 days using trypsin (GIBCO).

Mutagenesis and Unscheduled DNA Synthesis in HepG2 Cells

The mutagenesis of the chemicals by selection of mutations at the *hgprt* locus and the quantitation of induction of unscheduled DNA synthesis (UDS) were essentially as described in Eddy et al. (*41*).

Metabolism of [^{3}H]1-nitropyrene and [^{3}H]1,8-dinitropyrene

For the metabolism studies, HepG2 cells were plated at 1.5×10^6 cells/100 mm culture plates and incubated overnight with MEM + 10% hi-FBS. Immediately prior to adding the radiolabeled compounds, the medium was changed to MEM + 2% hi-FBS. Either 4 µM [4,5,9,10-^{3}H] 1-nitropyrene (1 Ci/mmole; radiolabel purity >98%; Chemsyn, Inc., Lenexa, KS) or 4 µM [4,5,9,10-^{3}H] 1,8-dinitropyrene (2.2 Ci/mmoles; radiolabel purity >99%; Chemsyn, Inc.) were added to the media and incubated for up to 24 hr. The metabolism of the compounds was terminated by decanting and cooling the media on ice, removal of the cells with trypsin, and extraction of the parent compounds and metabolites with chloroform:methanol (2:1) followed by chloroform. Additionally, cells and media were incubated at 37°C overnight in the presence of β-glucuronidase (Sigma) or arylsulfatase (Sigma), followed by extraction with chloroform.

The analysis of the metabolites of [^{3}H]1-nitropyrene and [^{3}H]1,8-dinitropyrene was essentially as described for 1-nitropyrene (*30*) using HPLC (Varian Instr., Walnut Creek, CA) and reverse-phase columns (Waters Assoc., Milford, MA). Nonradiolabeled standards were routinely coinjected to verify retention times of the metabolites. The conversion of the parent compound was quantified using a flow-through scintillation counter (Flo-One, Radiomatic Instruments). Representative chromatograms of [^{3}H]1-nitropyrene and [^{3}H]1,8-dinitropyrene metabolism are shown in Figure 2. Baseline separation of the major metabolites of both [^{3}H]1-

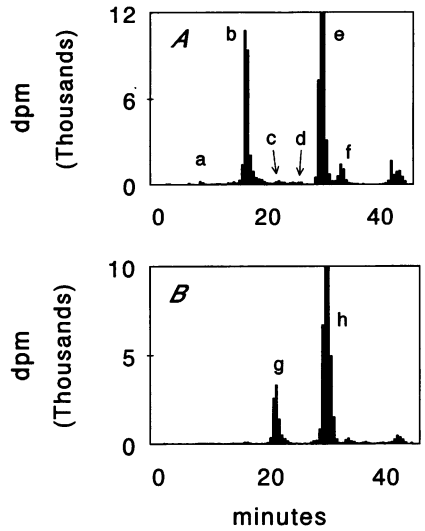

Figure 2. Representative chromatograms of the HPLC analysis of the HepG2 metabolism of [^{3}H]1-nitropyrene (*A*) and [^{3}H]1,8-dinitropyrene (*B*).The following metabolites or parent compounds were determined by cochromatography with non-radiolabeled compounds: (*a*) 1-nitropyrene-4,5-dihydrodiol; (*b*) 1-aminopyrene; (*c*) 1-nitropyren-6-ol and 1-nitropyren-8-ol; (*d*) 1-nitropyren-3-ol; (*e*) 1-nitropyrene; (*f*) 1-nitrosopyrene; *g*) 1-amino-8-nitropyrene; (*h*) 1,8-dinitropyrene.

nitropyrene and [^{3}H]1,8-dinitropyrene were achieved.

^{32}P-Postlabeling of DNA from HepG2

DNA adducts were assayed by ^{32}P-postlabeling on DNA by the *n*-butanol enrichment and contact transfer procedures as indicated in Smith et al. (*48*). The adducts were quantified by comparison to DNA standards that were modified to a known extent with 1-nitropyrene.

Results and Discussion

While 1-nitropyrene has been shown to be a weak carcinogen in rodent bioassays, 1,6- and 1,8-dinitropyrene have been shown to possess very high carcinogenic potential (*49–51*). This correlates with the mutagenicity of 1-nitropyrene and 1,6- and 1,8-dinitropyrene in *S. typhimurium*, where the dinitropyrenes are 1000- to 2000-fold more mutagenic in *S. typhimurium* strain TA98.

In Table 1 we show the results of the incubation of 1-nitropyrene and 1,8-dinitropyrene with HepG2, and the subsequent selection for mutations at the *hgprt* locus. The induction of 96.1 mutants per 10^5 clonable cells at 10 μM 1-nitropyrene is slightly lower than the values reported by Eddy et al. (*41*). The inclusion of 1,3-dinitropyrene in HepG2 resulted in an increase of mutations over the background, yet did not result in a dose-dependent increase in the mutation frequency. The inclusion of up to 17 μM 1,6- or 1,8-dinitropyrene did not result in a dose-dependent increase in mutations.

Table 1. Mutagenicity of 1-nitropyrene and dinitropyrenes at the *hgprt* locus in HepG2 cells.

Chemical[a]	μM	Mutations per 10^5 clonable cells
none		6.3
1-nitropyrene	10	96.1
MNNG	1.0	92.6
1,3-dinitropyrene	3.4	23.8
1,3-dinitropyrene	8.6	28.3
1,3-dinitropyrene	17	18.9
1,6-dinitropyrene	3.4	9.9
1,6-dinitropyrene	8.6	2.1
1,6-dinitropyrene	17	0.8
1,8-dinitropyrene	3.4	2.2
1,8-dinitropyrene	8.6	0
1,8-dinitropyrene	17	0

[a]*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. The compounds were added to HepG2 and mutagenicity at the *hgprt* gene was determined as described in the text using 6-thioguanine.

Another method for detecting genotoxic damage to the HepG2 cells is by monitoring the repair of DNA adducts (unscheduled DNA synthesis). The results of incubation of 1-nitropyrene and the dinitropyrenes and determination of the UDS is shown in Table 2. The presence of 4 μM 1-nitropyrene resulted in a 52% increase in UDS. As with the mutation results, there was not a dose-dependent increase in UDS with 1,3-dinitropyrene, although UDS was increased approximately 16% above the background values. Neither 1,6- or 1,8-dinitropyrene induced UDS above the background values. These results suggest that the highly mutagenic and carcinogenic 1,6- and 1,8-dinitropyrene are not metabolically activated in HepG2 cells, as evidenced by the lack of genetic damage.

Table 2. Induction of unscheduled DNA synthesis by 1-nitropyrene and dinitropyrenes in HepG2 cells.

Chemical	μM	Unscheduled DNA synthesis, dpm per μg DNA
DMSO-hydroxyurea		60,189
DMSO		2,879
1-nitropyrene	4	4,381
1,3-dinitropyrene	1.7	3,699
1,3-dinitropyrene	3.4	3,274
1,3-dinitropyrene	6.8	3,102
1,6-dinitropyrene	1.7	3,927
1,6-dinitropyrene	3.4	2,083
1,6-dinitropyrene	6.8	1,852
1,8-dinitropyrene	1.7	2,108
1,8-dinitropyrene	3.4	2,248
1,8-dinitropyrene	6.8	2,372

In order to determine if the lack of genotoxicity of 1,8-dinitropyrene resulted from a lack of metabolic activation, the nitroreduction of 1-nitropyrene and 1,8-dinitropyrene were contrasted. Both of these compounds have been shown to be metabolically activated by nitroreduction to arylhydroxylamines that bind to DNA. In prokaryotic cells, the metabolic activation of 1,6- and 1,8-dinitropyrene additionally requires the esterification of the arylhydroxylamine to an acyloxy ester via acyltransferase enzymes (*52,53*), while this step is not required for 1-nitropyrene or 1,3-dinitropyrene. Figure 3 shows the results of incubation of 4 μM [^{3}H]1-nitropyrene and [^{3}H]1,8-dinitropyrene with HepG2. The metabolism of both compounds to nitroreduced metabolites plateaued at approximately 6 to 12 hours. While the extent of metabolism of 1-nitropyrene was approximately 4-fold higher than of [^{3}H]1,8-dinitropyrene, the rates of metabolism between 4 and 6 hours were approximately the same. This diminished nitroreduction of 1,8-dinitropyrene cannot account for the lack of induction of mutations at the *hgprt* locus and induction of UDS by this compound. In *S. typhimurium* TA98, a 4-fold loss in nitroreduction for 1,8-dinitropyrene would still result in mutation rates approximately 250- to 500-fold higher than 1-nitropyrene. The only case where a reduction of 1,8-dinitropyrene nitroreduction would result in mutagenicities either equal to or less than [^{3}H]1-nitropyrene would be in strains missing the arylhydroaxylamine *O*-esterificase, such as TA98/1,8DNP$_6$ and TA100/Tn5-1012. Therefore, the lack of mutagenicity of 1,6- and 1,8-dinitropyrene

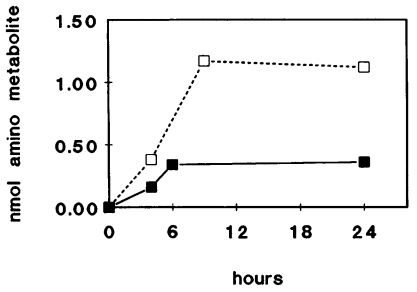

Figure 3. Nitroreduction of 4 μM [^{3}H]1-nitropyrene (□) and [^{3}H]1,8-dinitropyrene (■) in HepG2 cells.The metabolites were extracted and analyzed as described in "Materials and Methods." The results are from duplicate analyses from two experiments.

in HepG2 cannot only be attributed to the loss of nitroreduction, but additionally results from a lack of arylhydroxylamine *O*-esterificase activity.

One possible explanation for the weak tumorigenicity of 1-nitropyrene and high tumorigenicity of 1,6- and 1,8-dinitropyrene could be a lack of activation of 1-nitropyrene *in vivo* and a predominance of activation for 1,6- and 1,8-dinitropyrene *in vivo*. The metabolism of 1-nitropyrene *in vitro* with microsomes and *in vivo* is dominated by cytochrome P450-mediated C-oxidation to phenols or dihydrodiols. However, no cytochrome P450-mediated C-oxidation has been reported for 1,6- or 1,8-dinitropyrene, leaving nitroreduction as the sole pathway for metabolism and removal of the compound. This would obligate cells to metabolize the dinitropyrenes through a pathway that results in mutagenicity in cells *in vitro*.

A method for testing the hypothesis that nitroreduction of 1-nitropyrene is responsible for the mutagenesis of 1-nitropyrene in the HepG2, and that cytochrome P450-mediated C-oxidation is not an activation pathway, would be to vary the ratio of cytochrome P450-mediated C-oxidation to nitroreduction in HepG2, and then determine the effect on DNA adduction. In Table 3 we show the results of the administration of 2.5 μM 3-methylcholanthrene on the metabolism of 1-nitropyrene in HepG2 cells. The inclusion of 3-methylcholanthrene induced the cytochrome P450-mediated C-oxidation of 1-nitropyrene by 2.2-fold, and reduced the nitroreduction to 1-aminopyrene by 81%. There was a variability in the metabolism of 1-nitropyrene between the experiments, as indicated by the standard deviations. The nature of this variability is not understood, yet there was a consistency in the ratio of C-oxidation/nitroreduction between experiments. An increase in the formation of 1-nitropyren-6-ol and 1-nitropyren-8-ol over the formation of 1-nitropyren-3-ol (6+8/3-ols) is a hallmark of cytochromes P4501A (*28*). In the HepG2 cells, treatment with 3-methylcholanthrene resulted in an increase of the 6+8/3-ol ratio from 1.5 to 6.8, suggesting the induction of the cytochromes P4501A.

To determine the effect of the altered ratio of C-oxidation/nitroreduction on the DNA adduct formation, control and 3-methylcholanthrene-treated HepG2 cells were exposed to 10 μM 1-nitropyrene. The DNA was isolated, hydrolyzed, and analyzed for the presence of DNA adducts by the ^{32}P-postlabeling method (Figure 4). When *S. typhimurium* TA98 is exposed in suspension to 1-nitropyrene, the C8-guanyl adduct of 1-nitropyrene (dG-C8-AP), C8-guanyl adduct of either 1,6- or 1,8-dinitropyrene (dG-C8-ANP), and a polar adduct presumed to be the ring-opened product of dG-C8-AP ("a") are detected (Figure 4*A*). While no adducts appear in HepG2 in the absence of added compounds (Figure 4*B*), the inclusion of 10 μM 1-nitropyrene induced two adducts (Figure 4*C*). One of the adducts comigrated with dG-C8-AP and the other with "a" of panel A. Since we used 1-nitropyrenes of identical purity (~99.5%), the lack of DNA adducts in HepG2 from the dinitropyrenes that contaminate 1-nitropyrene, and the presence of these adducts in the *S. typhimurium* TA98, argue in favor of our conclusion that HepG2 lack the arylhydroxylamine *O*-esterificase necessary for the activation of the dinitropyrenes. Pretreatment of HepG2 cells with 3-methylcholanthrene and exposure to 1-nitropyrene resulted in the formation of less dG-C8-AP, and the formation of 3-

Table 3. The effect of pretreatment of HepG2 with 3-methylcholanthrene on metabolism of 10 μM 1-nitropyrene (pmole/24 hr).

Quantified 1-nitropyrene metabolites	Untreated HepG2	3-Methylcholanthrene-pretreated HepG2
1-Nitropyrene-4,5 and 9,10-dihydrodiols	15.6 ± 6.4	10.8 ± 10.1
1-Nitropyren-6- and 8-ol	74.5 ± 25.1	299.1 ± 121.4
1-Nitropyren-3-ol	72.6 ± 50.4	52.4 ± 36.5
Total C-oxidation[a]	163.8 ± 44.8	364.2 ± 152.1
1-Aminopyrene	76.8 ± 39.0	14.3 ± 18.3
(C-Oxidation/nitroreduction)	2.8 ± 1.9	50.4 ± 46.1
(6+8-ol/3-ol)[b]	1.5 ± 0.8	6.8 ± 2.5

[a]The total C-oxidation was calculated as the sum of dihydrodiol and phenol metabolites produced. [b]The ratio of the formation of 1-nitropyren-6-ol plus 1-nitropyren-8-ol divided by 1-nitropyren-3-ol formation. The results are the sum of five experiments and are presented as the mean ± SD.

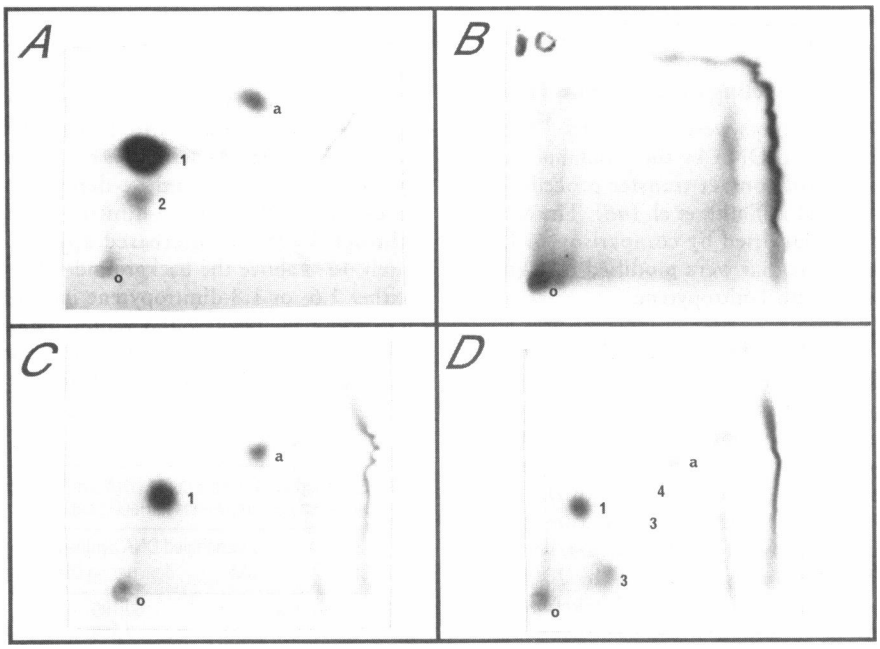

Figure 4. ^{32}P-Postlabeling of DNA isolated from (*A*) *Salmonella typhimurium* TA98 treated with 20 μM [^{3}H]1-nitropyrene in suspension; (*B*) HepG2 cells; (*C*) HepG2 cells exposed for 24 hr to 10 μM [^{3}H]1-nitropyrene; and (*D*) HepG2 cells preexposed for 24 hr to 2.5 μM 3-methylcholanthrene followed by exposure for 24 hr to 10 μM [^{3}H]1-nitropyrene. The adducts are identified as: (1), C8-guanyl adduct of 1-nitropyrene [dG-C8-AP; N-(2′-deoxyguanosin-8-yl)-1-aminopyrene]; (2), C8-guanyl adduct of 1,6-dinitropyrene or 1,8-dinitropyrene [N-(2′-deoxyguanosin-8-yl)-1-amino-(6 or 8)-nitropyrene]; (a), adduct of undetermined identity that has chromatographic characteristics consistent with the ring-opened product of dG-C8-AP; (3), uncharacterized DNA adducts from 3-methylcholanthrene; (4), location of DNA adduct resulting from the incubation of 1-nitropyrene-4,5-epoxide with calf thymus DNA; (o), origin.

methylcholanthrene DNA adducts (Figure 4*D*). There was a lack of formation of other DNA adducts that could be attributable to 1-nitropyrene K-region epoxides. The DNA from the HepG2 treated with 1-nitropyrene contained 18.7 ± 7.0 fmoles/μg DNA of dG-C8-AP, while the cells pretreated with 3-methylcholanthrene then treated with 1-nitropyrene had 4.8 ± 1.7 fmoles dG-C8-AP per μg DNA.

These results demonstrate that when the ratio of cytochrome P450-mediated C-oxidation of 1-nitropyrene is increased over the nitroreduction pathway, there is a decrease in the formation of dG-C8-AP DNA adducts, which arise from the nitroreduction pathway, and that this decrease in adducts is not associated with the formation of DNA adducts arising from cytochrome P450-mediated C-oxidation, e.g., the 4,5-epoxide DNA adduct. Nitroreduction is not a favored pathway *in vivo* in tissues where cytochrome P450 concentrations are significantly higher than in HepG2 cells. Additionally, the epoxides of 1-nitropyrene are quickly hydrolyzed by epoxide hydrolase in human liver tissue (*54*). These results, along with the weak tumorigenic response of rodents to 1-nitropyrene, indicate that 1-nitropyrene may not pose a significant tumorigenic risk to the human population.

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