Biochemical Pharmacology Vol 35 No 13, pp 2129–2134, 1986
Printed in Great Britain

0006-2952/86 $3 00 + 0 00
Pergamon Journals Ltd

BINDING OF NITROPYRENES AND BENZO[a]PYRENE TO MOUSE LUNG DEOXYRIBONUCLEIC ACID AFTER PRETREATMENT WITH INDUCING AGENTS

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(*Received* 18 *March* 1985, *accepted* 29 *November* 1985)

Abstract—In assessing the biological effects of exposure to a complex chemical mixture, it is important to determine how the behavior of one compound may be influenced by the presence of other compounds in the mixture In this study the effect of pre-exposure to an organic extract of diesel exhaust or to selected compounds in diesel exhaust on the binding of diesel exhaust compounds to DNA was determined The amount of radiolabel covalently bound to mouse lung DNA following intratracheal administration of radiolabeled benzo[a]pyrene (BaP), 1-nitropyrene, 1,3,6-trinitropyrene, or a mixture of dinitropyrene was determined following pretreatment with benzo[a]pyrene, 1-nitropyrene, and diesel exhaust extract Male CD-1 mice, 15–18 weeks of age, received 10 mg/kg of putative inducing agents by intratracheal instillation and, after 24 hr, 0 03 to 1 2 mg/kg radiolabeled putative DNA binding agents Lung DNA was extracted, and covalent binding was quantitated by liquid scintillation spectroscopy 1-Nitropyrene was a potent lung DNA binding agent in the absence of inducing agents [Covalent Binding Index (CBI) = 970] and was extremely potent after benzo[a]pyrene pretreatment (CBI = 21,540, comparable to the CBI for aflatoxin B_1) Similar results were obtained for DNA binding of dinitropyrene and trinitropyrene with and without BaP pretreatment DNA binding of BaP was lower (CBI = 40) and less inducible (BaP-pretreatment CBI = 230) Pretreatment with diesel extract caused an elevation in the binding of benzo[a]pyrene but little or no elevation in the binding of the nitropyrenes Pretreatment with 1-nitropyrene did not increase significantly DNA binding of any of the agents tested These results indicate that nitropyrenes bind readily to lung DNA and this binding may be increased in the presence of respirable mixtures, especially those containing inducing agents such as BaP

Airborne toxicants often occur in combinations with one another and with various carriers, and their biological effects often depend on their interactions with one another *in vivo* One of the more important types of interaction is the induction by one xenobiotic of an enzyme responsible for the metabolism of another Induction of the mixed-function oxidases has been studied thoroughly [1], and other metabolic enzymes are also inducible [2] In assessing the biological effects of exposure to a complex mixture, it is important to determine the extent to which the metabolism of one component of the mixture can be facilitated or inhibited by prior or concurrent exposure to another component

The covalent binding of a genotoxic compound to DNA in a target tissue frequently provides a reliable measure of the effective dose of the compound [3, 4]. Most genotoxicants exert their effects through binding to DNA, although there are exceptions [5], and there is a high degree of correlation within chemical classes between covalent binding to DNA and genotoxicity [4, 6, 7] Thus, the alteration of DNA binding due to preadministration of an inducing agent may be a measure of the potency of the inducer in increasing the effective dose to DNA derived from a fixed administered dose. By measuring the covalent binding of a compound to DNA in the presence and absence of inducers we can assess an important part of the biological activity of the compound

Benzo[a]pyrene (BaP§, CAS Reg No 50–32–8) is a carcinogen, and its binding to DNA has been measured in a variety of systems [8–10] It is a known inducer of aryl hydrocarbon hydroxylase (AHH) activity [11]. It is found in many environmental mixtures, including diesel exhaust [12] 1-Nitropyrene (5522–43–0) is a mammary carcinogen in rats [13], and it is a potent DNA binding agent [8] It is present at fairly high levels in diesel exhaust [14] 1,3-, 1,6-, and 1,8-Dinitropyrene (75321–20–9, 42397–64–8, and 42397–65–9, respectively) are mutagenic in bacteria [15] and mammalian cells [16], and 1,3- and 1,8-dinitropyrene induce injection-site tumors in rats [17] The three isomers are present in roughly equal quantities in diesel exhaust [14]. 1,3,6-Trinitropyrene (75321–19–6) is suspected to be present in diesel exhaust [18] and a mutagen [15, 16], but little else is known of its biological effects Diesel exhaust

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§ Abbreviations AHH, aryl hydrocarbon hydroxylase, BaP, benzo[a]pyrene, CBI, Covalent Binding Index, HPLC, high-performance liquid chromatography, ITRI, Inhalation Toxicology Research Institute, and R_f, relative migration in a TLC experiment

particles have been shown to induce metabolism of 1-nitropyrene in rat nose and isolated perfused rat lung [19] and of benzo[*a*]pyrene in cultured lung epithelial cells [20] Inhaled diesel particles may induce AHH activity in some organs [21]; however, only a modest or no increase at all is seen in the lung after diesel inhalation exposure [21–23].

In previous work in this laboratory Mitchell [8] showed that benzo[*a*]pyrene, nitropyrene, and 2-aminoanthracene or their metabolites bind covalently to mouse lung DNA and that their initial binding and persistence are influenced by mixed-function oxidase inducers. The binding of 1-nitropyrene to lung DNA was strongly influenced by agents known to induce mixed-function oxidase activity; in Mitchell's study, prior administration of benzo[*a*]pyrene resulted in a 160-fold increase in nitropyrene binding.

To obtain information on the interaction of compounds in a complex mixture on the covalent binding of the compounds to DNA, we have measured the amount of radiolabel covalently bound to DNA following administration of radiolabeled benzo[*a*]-pyrene, 1-nitropyrene, 1,3,6-trinitropyrene, and a mixture of dinitropyrenes to mouse lung DNA (and/or the metabolic products of these compounds) The putative DNA binding agents were administered after pretreatment with an instilled vehicle or with 10 mg/kg of instilled benzo[*a*]pyrene, 1-nitropyrene, or a dichloromethane extract of diesel exhaust particles

MATERIALS AND METHODS

Purchased radiochemicals [7,10-^{14}C]Benzo[*a*]-pyrene (98% radiochemical purity, 45.6 and 60.7 mCi/mmole, depending on the batch) was purchased from Amersham (Arlington Heights, IL) and used without further purification The 45.6 mCi/mmole material was mixed with unlabeled BaP to produce a material with a specific activity of 26 2 mCi/mmole 1-Nitro-[4,5,9,10-^{14}C]pyrene (98% radiochemical purity; 43 mCi/mmole) was custom synthesized for ITRI by Amersham and used without further purification Identity was verified by direct exposure probe/mass spectrometry, no dinitropyrene was detected in the sample by this method Purity was assessed by high-performance liquid chromatography (HPLC) under the conditions used for purifying dinitropyrenes (see below); the radiochemical purity was >95%

Synthesized radiochemicals A mixture of 1,3-, 1,6-, and 1,8-dinitropyrene was synthesized by nitration of a mixture of ^{14}C-labeled 1-nitropyrene with an overall specific activity of 12.2 mCi/mmole using the method of Li and Dutcher [16] Nitropyrene and nitric acid (1 10 mole ratio) were dissolved in 5 1 dichloromethane–acetic anhydride and heated for 2 hr at 40° Excess acid was neutralized with 20 ml of 0 2 M sodium carbonate and the aqueous material discarded The organic-soluble material was enriched in dinitropyrene by silica gel thin-layer chromatography in 100% dichloromethane, a yellow-orange band near R_f = 0 6 was collected This product was purified by HPLC as described below A similar procedure was used to synthesize 1,3,6-trinitropyrene, but with 43 mCi/mmole 1-nitropyrene as starting material and a 1 13 nitropyrene nitric acid ratio, 3 hr of heat, and no TLC enrichment.

The synthetic products were purified by HPLC using a modification of the method of Rosenkranz *et al.* [24] An analytical (4.6 mm × 25 cm) Zorbax CN column (Dupont, Wilmington, DE) was attached to an SP8100 programmable HPLC (spectra-Physics, San Jose, CA) and used in a normal phase gradient (0%–16% 2-propranol in hexane over 24 min, flow rate 2 ml/min, ultraviolet detection at 254 nm) The identity and purity of the products were verified by mass spectrometry comparisons of the HPLC fractions with standards previously synthesized [16] The samples were introduced into the mass spectrometer on the direct exposure probe as the sensitivity was about 10-fold greater than the solid probe. The molecular ions of mono-, di-, and trinitropyrenes were differentiated by the presence or absence of ions at m/z 247, 292, and 337. For additional verification, the (M-46) ions were also considered, for the m/z 201, 246, and 291 are distinctive for each type of nitro derivative

Diesel exhaust extract. Particles were collected on a 20 in × 20 in high volume sample filter from the exhaust of an 8-cylinder Oldsmobile diesel engine mounted. The filter sample was collected over a 45-min period while the engine was running on two Federal Test Procedure cycles [25] Organic-soluble compounds were extracted from the particles by sonication for 2 hr in dichloromethane [26] Extractables were filtered through Millipore ultrafilters (Bedford, MA), flash-evaporated to dryness, and reconstituted in dichloromethane

Other unlabeled compounds 1-Nitropyrene (98% pure) was synthesized by nitration of pyrene with nitric acid [27] and analyzed by HPLC for purity It contained 1% dinitropyrene Benzo[*a*]pyrene (98% pure) was obtained from Eastman (Rochester, NY)

Suspensions for intratracheal instillation An 0.2% solution of gelatin (Bacto-Gelatin, Difco, Detroit) in 0 9% saline was used as a vehicle for the putative inducing and binding agents and as an instilling material for control animals. Putative inducing agents (benzo[*a*]pyrene, 1-nitropyrene, and diesel extract) were suspended by ultrasonication in 0 2% gelatin/saline at a concentration of 10 mg/ml Radiolabeled putative DNA binding agents (benzo-[*a*]pyrene, 1-nitropyrene, dinitropyrenes, and 1,3,6,-trinitropyrene) were dissolved in a minimum volume of acetone (0 1 to 0.3 ml) and suspended in 0 2% gelatin/saline at concentrations ranging from 0 03 to 1 1 mg/ml or 4 to 300 μCi/ml. The acetone was removed by bubbling air through the suspension prior to instillation

Animals used Specific pathogen free male CD-1 mice, 16 to 19 weeks of age and weighing 22–41 g, were used in these experiments The mice were raised in a barrier-maintained colony up to at least 8 weeks of age and then transferred to adult housing in plastic shoebox cages with hardwood chip bedding They were house 1–9 to a cage and fed Wayne Lab-blox (Allied Mills, Chicago) and water *ad lib* Three to five mice were assigned to each experimental group

Intratracheal instillations and sacrifices Mice were instilled with either the gelatin–saline vehicle or one of the inducing agents between 8:30 a.m and noon. Twenty-four hours later each animal was instilled with a putative DNA binding agent. The instillation technique was a modification [8] of the technique of Penã and Cabrera [28] The volume of instilled material in every case was 1 μl/g body weight, so each animal received 220 to 410 μg of a putative inducing agent and 0 63 to 41 μg or 0.08 to 12 μCi of a labeled compound. Each mouse was anesthetized with halothane before instillations Animals were killed by carbon dioxide asphyxiation 4, 28, and 172 hr after instillation of the DNA binding agents.

Isolation and analysis of lung DNA The technique for DNA isolation and quantitation of covalent binding has been described [8]. DNA content was measured as absorbance at 260 nm, and covalent binding of radiolabeled substrates was measured by liquid scintillation spectroscopy on a Packard 3255 spectrometer. DNA isolated by this procedure had 260 nm/280 nm ratios of → 1.98. DNA binding of metabolites of the labeled compounds was measured by the Covalent Binding Index (CBI) [7], defined as

$$\text{CBI} = \frac{(\mu\text{moles bound/mole nucleotides})}{(\text{mmoles administered/kg body weight})}$$

The results are expressed as the mean CBI ± S.E.M. for three to five mice The statistical significance of differences among CBI values was assessed with Student's *t*-test.

RESULTS

Purity of the synthesized materials. The mixture of dinitropyrenes was 99% radiochemically pure. 1,3,6-Trinitropyrene was 95% radiochemically pure The dinitropyrene mixture contained roughly equal quantities of the three isomers as estimated by the peak areas of the three HPLC peaks.

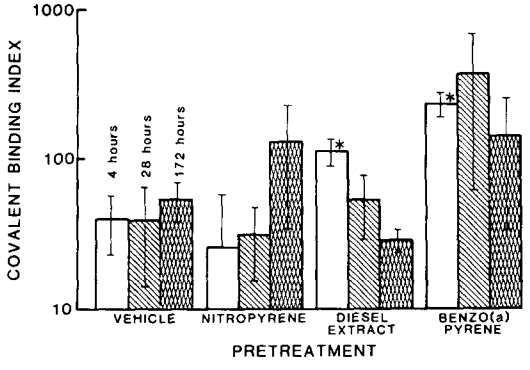

Fig 1 Covalent Binding Indices (CBI) for binding of metabolites of [^{14}C]benzo[*a*]pyrene to mouse lung DNA after pretreatment with 1 ml/kg of vehicle of 10 mg/ml 1-nitropyrene, diesel extract, or benzo[*a*]pyrene Twenty-four hours after pretreatment, mice were instilled with 1 ml/kg body weight of a 1 0 mg/ml suspension of 22 mCi/mmole benzo[*a*]pyrene in 0 2% gelatin/saline, so the instilled quantity was 1 mg/kg Sacrifice times in hours are given in the key Bars and error bars indicate mean ± S E M for three to five mice Asterisks indicate values significantly different from the vehicle 4-hr value ($P < 0.05$) The statistical significance of differences among CBI values was assessed with Student's *t*-test

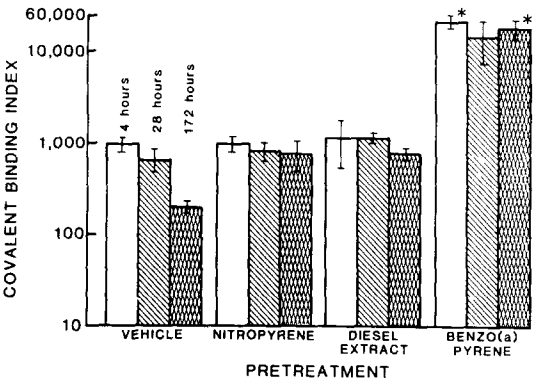

Fig 2 CBI of 1-nitro[^{14}C]pyrene to mouse lung DNA Mice were instilled with 1 ml/kg of an 0 8 mg/ml suspension of 43 mCi/mmole 1-nitro[^{14}C]pyrene in 0 2% gelatin/saline, the instilled quantity was 0 8 mg/kg Pretreatment conditions were as in Fig 1 Asterisks indicate values significantly different from the vehicle 4-hr and 172-hr values ($P < 0.05$)

Filter samples obtained from two separate runs of the Oldsmobile diesel engine yielded 4 g of diesel particle of which 18% (by weight) was dichloromethane-extractable.

Uninduced and induced DNA binding Covalent binding indices for benzo[*a*]pyrene and the three nitropyrenes are given in Figs. 1–4 at the three sacrifice times, with and without pretreatment with the putative inducers The covalent binding for each experimental group was compared with the value for vehicle-treated animals killed at the same sacrifice time and receiving the same putative DNA binding agent. As shown, BaP, 1-nitropyrene, dinitropyrenes, trinitropyrene, and/or their metabolites were bound to DNA. Pretreatment with benzo[*a*]pyrene significantly increased the binding for all the agents except trinitropyrene, and diesel extract increased the binding of benzo[*a*]pyrene at 4 hr Diesel extract

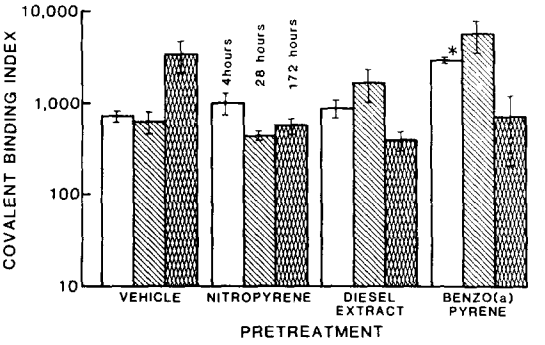

Fig 3 CBI for a mixture of dinitro[^{14}C]pyrenes to mouse lung DNA Mice were instilled with 1 ml/kg of an 0 8 mg/ml suspension of a 12 2 mCi/mmole mixture of 1,3-, 1,6-, and 1,8-dinitro[^{14}C]pyrene in 0 2% gelatin/saline, the instilled quantity was 0 8 mg/kg Pretreatment conditions were as in Fig 1 The asterisk indicates a value significantly different from the vehicle 4-hr value ($P < 0.05$)

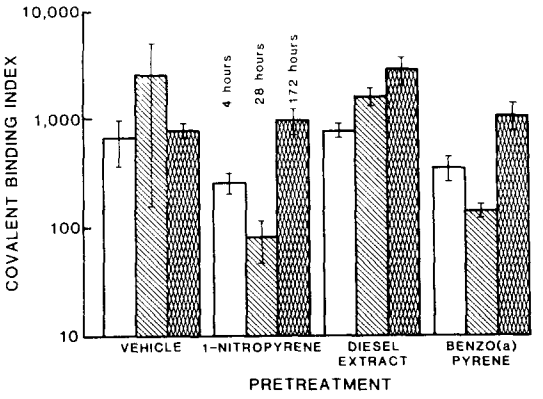

Fig 4 CBI for binding of 1,3,6-trinitro[^{14}C]pyrene to mouse lung DNA Mice were instilled with 1 ml/kg of 0 7, 0 07, or 0 03 mg/ml of a 43 mCi/mmole suspension of 1,3,6-trinitro[^{14}C]pyrene in 0 2% gelatin/saline, the instilled quantity was 0 7, 0 07, or 0 03 mg/kg Pretreatment conditions as in Fig 1

had insignificant effects on binding of other agents and of BaP at other times, and pretreatment with 1-nitropyrene did not alter significantly DNA binding of any of the agents

DISCUSSION

Uninduced DNA binding of BaP and nitropyrenes The covalent binding index found for benzo[*a*]pyrene in lung at 4 hr in this study (40 ± 17) is similar to that found previously [8]. The amount of uninduced 1-nitropyrene binding found in this study (CBI = 970 ± 180) is higher than that found by Mitchell [8] (CBI = 80). Dinitropyrene was a potent DNA binding agent (CBI = 720 ± 100) and trinitropyrene appeared to be similarly potent (CBI = 660 ± 210)

It is unclear why the uninduced 1-nitropyrene binding was higher in this study than in that of Mitchell [8] The mice used in this study were older—16–19 weeks versus 8–10 weeks in the previous study—and there are examples of age effects on constitutive levels of metabolic enzymes that could result in higher binding in more mature animals [29] However, these effects are not pronounced within a narrow range of age in adult animals A more likely explanation for the difference would be an unidentified change in the environment in which the animals were housed (chow, bedding, inhaled microorganisms, etc.), such changes have been implicated as effective inducers of mixed-function oxidases [30, 31] and could increase the constitutive levels of enzymes involved in 1-nitropyrene metabolism

Potency of benzo[a]*pyrene as an inducer* Figures 1–3 show that benzo[*a*]pyrene pretreatment enhanced the binding of metabolites of benzo[*a*]pyrene, 1-nitropyrene, and dinitropyrene to lung DNA at 4 hr, 4 and 172 hr, and 4 hr after pretreatment respectively The largest inductive effect, expressed as a ratio of induced to uninduced binding, occurred with 1-nitropyrene, for which the ratio was 22·1. This is smaller than the 160:1 ratio found by Mitchell [8] even though the induced CBI is higher in the present study (2,1540 vs 12,510) It is, nonetheless, a large increase and implies that the metabolic pathways leading to production of DNA adducts have been enhanced by the pretreatment

The magnitude of the BaP-induced CBI values for mono- and dinitropyrenes (21,540 and 2,920 respectively) is noteworthy It is in the same range reported by Lutz [7] for aflatoxin B_1 (3,000–31,000, depending on species, organ, and conditions) Strictly speaking, Lutz's definition of CBI applies to systemic binding distant from the site of administration (e g binding in liver after an intraperitoneal injection), whereas our values are obtained in an organ (lung) adjacent to the site of administration (trachea) Thus, our values are not entirely comparable to those given in Lutz's tables Nonetheless, the large CBI values for induced binding of nitropyrenes in respiratory tissue indicate their potential for causing DNA damage when present in inhaled mixtures The potency of a compound as a DNA binding agent does not correlate perfectly with carcinogenicity [32] or even with mutagenicity [7], so the large CBI values do not necessarily imply a high degree of carcinogenicity

Potency of diesel exhaust extract as an inducer Diesel exhaust has not been shown to be a potent inducer of mixed-function oxidase activity in the lung [21–23] A modest increase in mixed-function oxidase activity was seen in one study [21] On the other hand, increased enzyme activity has been observed in the lung after intratracheal administration of diesel particle extract with relative high doses (6 mg/kg body wt) [22] These results are in agreement with the data in the present study which indicate that the inductive effect of organic extracts of diesel exhaust is weak as measured by DNA binding There was a significant increase in DNA binding at 4 hr after diesel exhaust pretreatment only for benzo[*a*]pyrene, increases were absent or insignificant for the nitropyrenes It is of course conceivable that aryl hydrocarbon hydroxylase or some other activity is being induced to a considerable degree, but in a way that does not lead to increases in DNA binding for the agents studied

Potency of nitropyrene as an inducer The results show that nitropyrene pretreatment did not affect DNA binding of any of the binding agents Either the receptor(s) which binds inducers like benzo[*a*]pyrene fails to bind nitropyrene, or nitropyrene binds but does not activate an enzyme system, or it activates enzymes but the activations do not lead to DNA binding for any of the compounds under study The first suggestion is supported by the recent finding [33] that 1-nitropyrene does not compete for the 2,3,7,8-tetrachlorodibenzo-*p*-dioxin AHH receptor in rats

Retention of DNA adducts The retention time of any DNA lesion is important in assessing the significance of the damage We found a high degree of persistence of the covalent binding of metabolites of benzo[*a*]pyrene and the nitropyrenes to DNA At 172 hr the uninduced and induced binding of benzo[*a*]pyrene and nitropyrenes was about 75% of the 4-hr value BaP-induced DNA binding of 1-nitropyrene was more persistent than the uninduced binding during the first week, the 172-hr value was 85% of the 4-hr value after BaP induction, whereas

the uninduced 172-hr value was only 20% of the 4-hr value The mechanism for this increase in persistence is not obvious; it may be that different metabolites and therefore different DNA adducts form in induced lung as compared to uninduced lung, and that the adducts in the induced lung are less subject to repair On the other hand, the increase in persistence may arise from an alteration of a secondary metabolic pathway, e g inhibition of an enzyme responsible for conjugating electrophilic metabolites or inhibition of a DNA repair system. There is apparently a delicate balance between activation and detoxification pathways on the fate and persistence of potential mutagens and carcinogens. It is known that differential induction of cytochrome P-450 enzymes occurs after pretreatment with various inducers Aroclor-1254 is known to induce both cytochrome P-450 and P-448 enzymes, whereas BaP primarily induces P-448 enzymes [34]. The induced enzymes may have different substrate specificities [31] In addition, the levels of other enzymes, such as epoxide hydratase and UDP glucuronosyltransferase, may be affected by pretreatment with chemicals and thus have an overall effect on the forms and persistence of metabolites bound to DNA [35].

The persistence of DNA binding of BaP and nitropyrenes suggests that processes other than actual repair are responsible for the clearance of adducts. Most of the DNA damage cause by agents such as 4-nitroquinoline oxide is repaired within 48 hr of exposure [36], and enzymatic DNA repair processes tend to act on a time scale measured in hours or days rather than weeks [37] DNA lesions produced by BaP and nitropyrenes may actually persist until the affected cell is removed from the organ and could, in principle, give rise to somatic mutations at any point between exposure and cell turnover.

Mechanism of induction of DNA binding. The mechanism of BaP and diesel exhaust induced binding of BaP and nitropyrenes to DNA is not understood completely One explanation of the induction of binding is that it derives from induction of an enzyme system responsible for converting BaP and nitropyrenes to electrophilic intermediates capable of binding to DNA. BaP is known to be an inducer of AHH and inhaled diesel particles have a moderate effect on the induction of AHH activity [21] AHH activity is required for the conversion of benzo[*a*]pyrene to an intermediate capable of binding to DNA It is clearly established that polycyclic aromatic hydrocarbons such as benzo[*a*]pyrene are metabolized by the respiratory tract to metabolites that are bound to DNA and are responsible for the initiation of respiratory tract tumors However, little information exists on the metabolism and binding of nitroaromatics in these tissues It has been shown that rabbit lung explants and homogenates are capable of metabolizing and binding 1-nitropyrene to products that bind DNA [38]. In addition, lung tissue is capable of both oxidative and reductive metabolism which produce mutagenic metabolites of 1-nitropyrene [39] El-Bayoumy *et al* [40] have shown that 1-nitropyrene induces lung tumors in A/J mice and suggests that the 4,5-dihydro-4,5-epoxy-1-nitropyrene is a potential intermediate in the activation process We have shown in this study that nitroaromatics are metabolized and bound to mouse lung, *in vivo*, and that the binding is increased significantly following enzyme induction. This study has also shown that the binding is persistent for several days after administration Mechanisms involving AHH activation of nitroarenes have been postulated [8], the activation probably involves nitroreduction [41, 42]. 1-Nitropyrene is nitroreduced by an inducible cytochrome P-448 in mammalian cells in a reducing atmosphere [43] Induction of other enzymes may be an alternate or additional explanation for the increase in binding due to the pretreatments Induction of cytochrome P-448 remains the most plausible explanation, however, since that induction is itself well-documented and would lead directly to the results observed

It is not known which respiratory tract tissues and cells are at greater risk to injury from inhaled substances It has been reported that monooxygenases are induced in both Clara cells and Type II cells after β-naphthoflavone treatment [44] Additional studies show that rabbit alveolar macrophages can metabolize 1-NP to products that are bound to DNA [38]. Studies on DNA damage in rabbit lung Clara and Type II cells suggest that Clara cells may be a target because they are primary sites of cytochrome P-450-dependent monooxygenases and have a low capacity for DNA repair and a capacity for proliferation.

Conclusions. We found that metabolites of benzo[*a*]pyrene and nitropyrenes bound to mouse lung DNA and that this binding was increased by pretreatment with benzo[*a*]pyrene, increased in some instances by diesel extract, and unaffected by nitropyrene pretreatment. The binding was quite persistent and was made even more so in some cases by the action of the inducers As evidence accumulates that the nitroarenes are carcinogens [13, 17], it is becoming increasingly clear that the capacity of these compounds to bind to DNA has biological significance

Acknowledgements—This research was supported under U S Department of Energy Contract DE-AC04-76EV01013 We would like to thank Mary Stuever for performing the intratracheal instillations, Jack Lackey for collecting the diesel samples, Dr J D Sun for supplying unlabeled 1-nitropyrene, and Drs P H Ayres, J A Bond, R F Henderson and R K Wolff for suggestions and review of this manuscript

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