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Bioactivation of the food mutagen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)

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BIOACTIVATION OF THE FOOD MUTAGEN 2-AMINO-1-METHYL-6-PHENYLIMIDAZO[4,5-*b*]PYRIDINE (PhIP)

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ABSTRACT

BIOACTIVATION OF THE FOOD MUTAGEN
2-AMINO-1-METHYL-6-PHENYLIMIDAZO[4,5-*b*]PYRIDINE (PhIP)

by Michael A. Malfatti

The bioactivation pathways of the cooked-food mutagen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) were investigated in *Salmonella* and rat hepatocytes. Dichloro-4-nitrophenol (DCNP), but not pentachlorophenol (PCP) (both inhibitors of acetyltransferase and sulfotransferase activity) produced a concentration-dependent decrease in mutagenic activity of N-hydroxy-PhIP in the Ames/*Salmonella* assay. Both PCP and DCNP produced a slight decrease (~10%) in covalent binding to proteins in hepatocytes derived from uninduced rats co-incubated with [^{3}H]-PhIP (100 μM), but this was not observed in Aroclor-induced hepatocytes. Metabolism of PhIP in Aroclor-induced hepatocytes was approximately eight-fold higher than in uninduced cells. PCP and DCNP decreased 4'-PhIP-sulfate by 80% and 40%, respectively and in uninduced cells by 90% and 65%, respectively. Alpha-naphthoflavone (ANF) produced a 90% decrease in PhIP metabolites in induced cells, but protein binding was reduced by only 18%. These studies suggest that additional pathways besides sulfation, acetylation, and perhaps cytochrome P450 may be involved in PhIP bioactivation.

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INTRODUCTION

Chemical Carcinogens in Food

Environmental and occupational exposure to chemicals has been well documented over the years as two important factors involved in the etiology of human cancers. For instance, occupational exposure to the aromatic amine 2-naphthylamine has been linked to a high incidence of bladder cancer in dye industry workers (*reviewed in* 1). Also, in the late eighteenth century, a high incidence of scrotal cancer in chimney sweeps was associated with chronic dermal exposure to soot and coal tars which contain carcinogenic polynuclear aromatic hydrocarbons (PAHs) (*reviewed in* 1). Because exposure to these chemical carcinogens occurred at high levels and over a long period of time, it was possible to link exposure with effect. In contrast to occupational exposure, chemical carcinogen exposure from environmental or dietary sources is often at very low levels and is difficult to relate to tumor induction. Nonetheless, humans are exposed to a large number of food-derived chemical carcinogens; therefore, the potential for an association with cancer may be significant.

There are many sources of genotoxic carcinogens present in the human diet. Naturally occurring mutagens include pyrrolizidine alkaloids which occur in many plant species, hydrazines found in mushrooms, alkylating agents found in spice oils, and nitrites which produce nitrosamines from the degradation products of proteins or other food components (2). Aflatoxin B_1 is one of the most widely studied of the naturally occurring chemical carcinogens. This mycotoxin is produced by various species of the mold *Aspergillus* and is a common contaminant of foods such as peanuts, corn, and some grains that are harvested and often stored under warm humid conditions which promote aflatoxin B_1 formation. Aflatoxin B_1 is a potent hepatotoxin producing liver tumors in several mammalian species after dietary intake at parts per billion (ppb) levels (2,3). Recent

studies have implicated Hepatitis B virus as a cooperative etologic agent for hepatic cancer with Aflatoxin B_1 exposure (*reviewed in* 2). Aflatoxin-mediated carcinogenesis is postulated to result from metabolism to an epoxide reactive intermediate capable of binding DNA. Historically, sources of mutagens/carcinogens in food have been derived from pesticides and artificially added chemicals such as food preservatives and coloring agents. For instance, 4-dimethylaminoazobenzene (butter yellow) was used to color butter in the 1930s, but was shown to induce liver and bladder cancer, and subsequently its use was discontinued (*reviewed in* 1). A more recently discovered source of food-derived mutagens/carcinogens are those produced during the cooking of food (4). Carcinogenic compounds derived from cooking food include pyrolysis products which are formed at temperatures of 300° to 600°C and low temperature (< 300°C) thermic mutagens produced in high protein-foods derived from muscle. One of the first observations of the carcinogenic potential of cooked foods was made in 1939 by Widmark who reported malignant tumors in the mammary glands of female mice chronically exposed to extracts of horse muscle cooked at a temperature of 275°C (5). It was not until about 15 years ago, however, when better analytical techniques were developed, that significant advances were made in the study of dietary thermic mutagens. These studies were a derivative of earlier reports on the mutagenicity of cigarette smoke condensate (6). The mutagenic potency of tobacco smoke correlated not with the amount of carcinogenic PAHs present, but with the protein content of tobacco leaves. These findings led to pyrolysis studies of proteins and amino acids, and then later on foods.

Data from studies by Nagao *et al.* indicated that condensed smoke from meat or fish broiled over an open gas or charcoal flame contained material that was mutagenic in the Ames/*Salmonella* assay (7). The mutagenic activity detected was much too high to be accounted for by the benzo[*a*]pyrene content in the smoke condensates. This finding

suggested that other mutagens besides PAHs may arise from the pyrolysis of tissue protein and amino acids. Later studies by Nagao *et al.* (8), revealed that smoke condensates obtained from the pyrolysis of lysozyme and histone produced a strong mutagenic response in the Ames/*Salmonella* assay, while smoke from starch and vegetable oil showed only slight mutagenic activity. Thus, only protein produced highly mutagenic products by the pyrolytic process. Based on these findings, it was suggested that charred parts of fish and beef broiled at high temperatures contain mutagenic compounds which arise from protein pyrolysis (8). Furthermore, results from the smoke condensate studies led to experiments on cooked food.

Mutagens distinguishable from benzo[*a*]pyrene and from amino acid and protein pyrolysis products were detected when ground beef was cooked under normal household cooking conditions (electrical hot plate at 200°C) or when beef stock was concentrated to a paste known commercially as beef extract (9). Lean ground beef was cooked on an electric cooking appliance at 200°C for 1.5 minutes, 3.0 minutes and 5.5 minutes, to consistencies of rare, medium, and well-done, respectively. Portions of the beef were acid extracted and assayed for mutagenic activity using the Ames/*Salmonella* test system. All of the samples yielded substantial mutagenic activity when compared to control, uncooked beef. The mutagenicity increased with cooking time, with the well-done samples being the most mutagenic. The concentrated beef extract had higher mutagenic activity than the cooked beef samples. At 0.3 grams dry weight per plate, the beef extract produced 1,572 revertants per plate, while at 5.0 grams dry weight per plate of the "well-done" beef produced only 954 revertants per plate. Chromatographic analysis of these samples indicated that the mutagens produced from the beef extract were indistinguishable from those produced when ground beef was cooked under "normal household conditions" and that the mutagens were chromatographically distinguishable from benzo[*a*]pyrene and from

pyrolysis products of amino acids and proteins (9). Since mutagenic activity had been found in beef cooked under "normal household conditions" it was essential that these mutagens be accounted for when assessing human carcinogenic risk of cooked foods. Knize *et al.* reported that the increase in mutagenicity in well-done beef was not due to a change in the types of mutagens formed, but rather to an increase in the quantities of the same mutagens produced at lower temperatures (10). High performance liquid chromatography (HPLC) profiles of mutagenic components of ground beef patties fried at 200, 250, and 300°C for 6 min/side were similar although the total mutagenic activity in *Salmonella* TA1538 was about four times as high in the beef fried at 300°C than in the sample fried at 200°C (10). Later studies with cooked meats have led to the isolation and characterization of many of these mutagenic compounds.

Formation of Amino-Imidazoazaarenes in Cooked Meat

Several amino-imidazoazaarene (AIA) compounds have been isolated from cooked food and shown to be mutagenic in the Ames/*Salmonella* assay. Many of these chemicals also were shown to be carcinogenic in rodents (11-16). Six mutagenic AIAs have been isolated from fried beef and their structures fully characterized (Fig. 1). All of these compounds contain either a quinoline, quinoxaline, or pyridine ring system, an imidazo group moiety, a methyl group on one of the imidazo ring nitrogens, and one or more aromatic rings fused to the imidazo ring (17). It is the common amino-imidazole ring portion of the molecule which is responsible for the mutagenic activity of these compounds (18). Resistance to deamination (due to the guanidyl constituent in the imadazole ring) following nitrite treatment under acidic conditions (19,20) differentiates these compounds from the high temperature amino acid pyrolysis products, which are nitrite sensitive (19). These nitrite resistant compounds isolated from fried beef include 2-amino-3-methylimidazo[4,5-

f]quinoline (IQ), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo[4,5-*f*]-quinoxaline (DiMeIQx), 2-amino-*N,N,N*-trimethylimidazopyridine (TMIP), and the most abundant AIA, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), which is also the most nonpolar of the six compounds (17). Because PhIP is the most mass abundant AIA mutagen found in cooked beef, PhIP may be the most important AIA mutagen involved in carcinogenic risk because of the similarity of carcinogenic potency of the whole class of AIAs.

Studies have shown that the formation of PhIP may result from the condensation of creatinine and/or creatine with phenylalanine (21). These very common food constituents were also found to be implicated in the formation of the carcinogenic aminoimidazo-quinoxaline and aminoimidazoquinoline compounds (22). A mixture of creatinine, L-phenylalanine, and D-glucose in diethylene glycol containing 14% water was heated to 128°C for 2 hours. The heated material was purified and analyzed by HPLC and the isolate co-eluted with a synthetic PhIP standard. Shioya *et al.* suggested that creatinine and/or creatine is required for the formation of the amino-imidazole moiety of PhIP, while the rest of PhIP could be derived from various reaction products of amino acids, particularly phenylalanine, and sugars (22).

Bacterial Mutagenicity of PhIP

Studies of the mutagenic potential of AIA compounds in bacteria using the Ames/*Salmonella* assay have indicated that the mutagenic potency of all AIAs tested range over five orders of magnitude, and are among some of the most potent mutagens ever tested (23). For optimal expression of mutagenic activity the frameshift sensitive strains, *Salmonella typhimurium* TA98 and TA1538, are often used. In order to express mutagenic

activity from PhIP, as well as from other AIAs, metabolic activation by a liver S9 fraction is required. When compared to other AIAs isolated from fried beef, PhIP was the least potent in the Ames/*Salmonella* assay. This could be due to differences in metabolism or adduct effects. However, PhIP is reported to account for about 75% by mass, of the AIA mutagens found in fried meats (18). The N-hydroxylated intermediate of PhIP, 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (N-Hydroxy-PhIP), was shown to be mutagenic to *Salmonella* TA98 without further metabolic activation by a liver S9 fraction (24). Further studies have shown that synthetic N-hydroxy-PhIP is directly mutagenic in both *Salmonella* strains TA98 and the acetyltransferase-deficient TA98/1,8-DNP_6 (25). Studies have indicated that acetylation and/or sulfation are the major metabolic pathways for the further bioactivation of N-hydroxy intermediates (26,27,28). Since TA98/1,8-DNP_6 is an acetyltransferase-deficient strain (29,30,31), and mutagenic activity from N-hydroxy-PhIP was detected in this strain, acetylated intermediates appear not to be of consequence in the expression of bacterial mutagenicity with N-hydroxy-PhIP. These results are contrary to studies with other AIA mutagens, such as IQ, MeIQ, and MeIQx, which show little or no mutagenic activity in TA98/1,8-DNP_6, with or without liver S9 activaton (29,32). These three compounds also exhibit activation by acetyl CoA-dependent bacterial enzymes (27,32-34).

To evaluate the difference in the metabolic activation of N-hydroxyarylamines in *Salmonella* and mammalian systems, the characteristics of bacterial cytosolic activation in comparison with rat hepatic cytosolic activation was examined using the mutagenic metabolites 2-hydroxyamino-3-methylimidazo[4,5-*f*]quinoline (N-hydroxy-IQ) and the glutamic acid pyrolysate 2-hydroxyamino-6-methyldipyrido-[1,2-a:3',2'-*d*]imidazole (N-hydroxy-Glu-p-1) (27). Cytosols prepared from *Salmonella* strains TA98 and TA98/1,8-DNP_6 containing the sulfate donor 3'-phosphoadenosine 5'-phosphosulfate (PAPS)

incubated with either N-hydroxy compound and exogenous DNA did not result in significant DNA binding, whereas both N-hydroxyarylamines were activated by the PAPS fortified hepatic cytosols. In addition, sulfotransferase activity was assayed in both bacterial strains using *p*-nitrophenol as the substrate and no measurable activity was detected. Since no activity was detected in the bacterial cytosols containing PAPS, these data suggested that sulfotransferases may not be important in the further activation of N-hydroxy IQ and N-hydroxy-Glu-p-1 in bacteria, but sulfotransferases may contribute to activation in rat hepatic cytosols. Also, cytosol from the acetyltransferase deficient bacteria TA98/1,8-DNP$_6$, was unable to activate N-hydroxy-IQ (27). Furthermore, this study indicated that the difference in substrate specificity of bacterial and hepatic acetyltransferases may, in part, account for the difference in metabolic activation in bacteria and mammals. This is contrary to the results from bacterial activation studies with PhIP. PhIP was shown to be active in TA98/1,8-DNP$_6$ suggesting acetyltransferases may not play a significant role in the mutagenic activation of PhIP in bacteria (25). What is not clear is the significance of sulfotransferases in the activation of PhIP in the bacterial system.

To further characterize the metabolic activation pathways in bacteria, enzyme inhibitors such as 2,6-dichloro-4-nitrophenol (DCNP) and pentachlorophenol (PCP) have been used. These compounds inhibit both sulfation and acetylation (33,35,36). DCNP is more selective for sulfation. To determine whether metabolism of N-hydroxy-IQ by bacterial *O*-acetyltransferase and sulfotransferase are important in mutagenic activation, Snyderwine *et al.* examined the effects of PCP and DCNP on the mutagenicity of N-hydroxy-IQ in the bacterial strain TA98NR which is nitroreductase deficient. The TA98NR strain was used because some of the oxidative products of N-Hydroxy-IQ have been shown to be nonmutagenic with TA98NR (34). PCP produced a concentration-dependent inhibition of mutagenicity up to 80% of controls, while DCNP inhibited mutagenic activity by only 50%

at the highest concentration of inhibitor tested that did not kill the bacteria. The decreased inhibition by DCNP compared to PCP is consistent with previous studies suggesting that acetylation is more important than sulfation in the bacterial activation of N-hydroxy-IQ. In another study using *Salmonella* strains TA98 and TA98/1,8-DNP_6, incubations with PhIP and PCP showed no appreciable change in mutagenic activity when compared to control incubations without PCP (37). This finding suggests that the bacterial mutagenic activity of PhIP may involve pathways other than acetylation and/or sulfation. To fully characterize the bacterial mutagenic activation pathways of PhIP, further studies are warranted.

Mammalian Mutagenicity and Carcinogenicity of PhIP

The carcinogenicity of PhIP has been evaluated in both mice and rats. PhIP produced lymphomas in the mesenteric lymph nodes, mediastinal lymph nodes, and spleen of CDF_1 mice exposed to 400 parts per million (ppm) dietary PhIP for 579 days (38). In F344 rats, PhIP produced high incidences of colon and mammary carcinomas when administered at a concentration of 400 ppm in the diet for 365 days (39). The lack of hepatic tumor formation in either species contrasts with findings of other heterocyclic amines such as IQ, MeIQ, and MeIQx. (12-16). These data suggest that the major metabolic activation pathways of PhIP may be different from those of other AIAs. Though PhIP was less active than other AIA compounds such as IQ, MeIQ, and MeIQx in the Ames/*Salmonella* assay, PhIP has been shown to be more potent in mammalian genotoxicity assays (40). Exposure to PhIP resulted in cell killing, hypoxanthine phosphoribosyltransferase (*hprt*) mutation induction, and sister chromatid exchange in Chinese hamster ovary cells where MeIQ and MeIQx gave very weak responses. PhIP, when compared to MeIQ, produced a much larger increase in DNA damage as measured by alkaline elution or by unscheduled DNA synthesis in Chinese hamster V79 cells co-incubated with Aroclor 1254 (PCB)-

induced hepatocytes (37). PhIP also caused an 8-fold increase in sister chromatid exchange in the V79 cells co-cultured with the Aroclor-induced hepatocyctes, whereas MeIQ caused an increase of less than 2-fold. The mechanisms behind the inverse relationship between the mammalian and Ames/*Salmonella* assays of PhIP and the other AIAs isolated from fried beef are currently unknown, but could be related to differences in metabolism.

Metabolism of PhIP

Metabolic activation of arylamines and AIAs to reactive intermediates is highly dependent on N-hydroxylation to the corresponding hydroxylamine by specific enzymes belonging to the cytochrome P450 system. The specific cytochrome P450 isozymes involved in the metabolism of AIAs to mutagenic intermediates were shown to be cytochrome P450 IA1 and IA2 (24,41), which are inducible by 3-methylcholanthrene (3-MC) and by PCB congeners present in Aroclor 1254. While most N-hydroxylated intermediates may be chemically reactive and capable of nonenzymaticly producing DNA or protein adducts, further metabolism to more proximate reactive intermediates may be required for genotoxic activity (42). Most AIAs studied have been shown to form electrophilic intermediates which are capable of causing DNA damage (43,44). Two major metabolites were formed from PhIP by liver microsomes derived from 3-MC induced mice. The metabolites were isolated by High Performance Liquid Chromatography (HPLC) and determined to be N-hydroxy-PhIP and 4'-hydroxy-PhIP. N-Hydroxy-PhIP was shown to be directly mutagenic to *Salmonella* TA98, while 4'-hydroxy-PhIP showed no mutagenic activity in TA98 with or without metabolic activation (24). In a separate study, hepatic microsomal preparations from 3-MC induced mice were 10-fold more active in producing N-hydroxy-PhIP than control microsomes (25). To further characterize the metabolic

pathways of PhIP, hepatocytes were isolated from Aroclor-induced rats (38). PhIP was metabolized to reactive genotoxic metabolites that caused DNA strand breaks and sister chromatid exchanges in Chinese Hamster V79 cells co-incubated with the hepatocytes. Hepatocytes prepared from uninduced rats were unable to activate PhIP to the same extent as the cells isolated from the induced rats. These results indicate that the mutagenic activation of PhIP is mediated by cytochrome P450 dependent pathways (Fig. 2).

While the mutagenic activation of PhIP was shown to be dependent upon cytochrome P450 activation to the N-hydroxylated intermediate, additional phase II metabolic pathways appear to be involved in the genotoxicity (26). N-Hydroxylated intermediates can be converted via acetylation, sulfation, and glucuronidation to highly reactive electrophilic species capable of covalently binding to nucleic acids and protein. The enzymes that catalyze these reactions are cytosolic acetyltransferases, sulfotransferases, and microsomal glucuronyltransferases, respectively. Although phase II conjugation reactions usually result in detoxification, studies on secondary metabolic pathways of AIAs indicate that both acetylation and sulfation may be activation pathways (25,26,34). Although glucuronides constitute a large portion of the metabolites of most N-arylhydroxylamines, and glucuronidation is quantitatively the most important phase II pathway in humans, it is not known whether this pathway plays a significant role in the production of carcinogenic effects (42). N-O-Glucuronides of N-arylhydroxyl-amines have not been directly detected in or isolated from biological systems, presumably because of their extreme instability. Since glucuronyltransferase activity is found in most tissues, the possibility that glucuronidation is an activation pathway of N-arylhydroxylamines must be considered. However, UDP-glucuronate-dependent conjugation of N-hydroxy-2-naphthylamine in the presence of DNA and rRNA resulted in less than 0.02% conversion of the hydroxylamine to the nucleic acid-bound adducts in rat hepatic microsomal preparations. Thus, if the O-

glucuronide was formed under these conditions, it may have undergone intramolecular rearrangement or reaction with nucleophiles other than those present on nucleic acids (42). Because of these and similar findings with other N-arylhydroxylamines, N-O-glucuronide conjugates of N-arylhydroxylamines are considered to be compounds that do not readily lead to the generation of reactive electrophiles capable of binding DNA. However, N-hydroxy-N-glucuronides have been implicated in the transport of potentially reactive compounds from the liver to target tissues, where deconjugation under acidic conditions to N-hydroxy metabolites occurs (42). This may be relevant to PhIP since most carcinomas that have been detected occurred in extrahepatic tissues.

To determine the role of sulfation and acetylation in the activation of PhIP, the N-hydroxylated intermediate of PhIP was incubated with mouse liver cytosolic fractions containing exogenous DNA, in the presence of the sulfate donor PAPS, or the cofactor for acetylation, acetyl Coenzyme A (26). DNA adducts were detected in the presence of N-hydroxy-PhIP in incubations with both PAPS and acetyl Coenzyme A. The adduct levels in the acetyl Coenzyme A preparations were significantly lower than those in the PAPS samples. Under the conditions of the experiment, DNA adducts were not detected in incubations lacking PAPS or acetyl Coenzyme A, indicating the importance of sulfation and acetylation dependent pathways in the mammalian genotoxic actions of PhIP (26). Also, slight increases in mutagenicity resulted from the addition of PAPS, but not acetyl Coenzyme A, to microsomal/cytosolic incubations containing PhIP, further suggesting that secondary metabolism to a sulfate conjugate may be relevant in the mutagenicity of PhIP (26). Further studies using inhibitors of sulfotransferase and acetyltransferase may reveal additional information on the mammalian metabolic pathways involved in the genotoxic activity of PhIP.

Experimental Rationale

In the present studies the characterization of the secondary metabolic activation pathways of PhIP in *Salmonella* as well as both primary and secondary activation pathways in mammalian cellular systems were investigated. The roles of acetylation and sulfation were assessed using the acetyltransferase and sulfotransferase inhibitors PCP and DCNP to modulate metabolism and genotoxicity. Previous studies with *Salmonella* have indicated that acetylation has little or no role in the activation of PhIP in the bacterial system (26). Therefore, these inhibitors were used to assess the role of sulfation in the mutagenicity of PhIP in the Ames/*Salmonella* assay. By inhibiting sulfotransferase activity with PCP or DCNP, secondary metabolic activation of N-hydroxy-PhIP by this pathway could be measured. The bacterial system, although much simpler than mammalian metabolic systems, could reveal mechanisms that are relevant to mammalian cells.

To assess effects of acetylation and sulfation in mammalian cells, isolated rat hepatocytes were pre-incubated with PCP or DCNP and the effects on PhIP metabolite formation, covalent binding to proteins, and DNA adduct formation were assessed. In addition, the P450 IA1 and IA2 isozyme inhibitor alpha-naphthoflavone (ANF) was pre-incubated with hepatocytes to assess whether modulation of P450 activity would affect these parameters. Modulation of PhIP metabolism using these inhibitors was studied in both cytochrome P450 induced and uninduced hepatocytes.

By characterizing the metabolic pathways involved in the activation of PhIP to reactive metabolites which could potentially form DNA and protein adducts, the mechanisms of toxicity and the potential of this compound to pose a human health risk can be better understood.

MATERIALS AND METHODS

Chemicals

PhIP was purchased from Toronto Research Chemicals, Inc. (Downsview, Ontario). [^{3}H]-PhIP was synthesized previously by catalytic tritiation of 2-amino-1-methyl-6-(2'-bromo)phenylimidazo[4,5-*b*]pyridine (2'-bromo-PhIP), according to methods similar to those used to synthesize unlabelled PhIP (45). [^{3}H]-PhIP was subsequently purified by HPLC. The HPLC system consisted of a Waters Associates M-6000A pump with a Nucleosil C_{18} column (0.4 x 25 cm). The desired separations were attained using an isocratic mobile phase consisting of 40% methanol/59.9% water/0.1% diethylamine, adjusted to pH 4 with acetic acid. Fractions were eluted at a flow rate of 1.0 ml/min, monitored at a wavelength of 280 nm, using a Waters Associates 440 detector, and collected at 0.5 minute intervals. Radioactivity was quantitated using a Beckman LS8100 scintillation counter. Fractions eluting at the retention time of PhIP and containing significant radioactivity were combined, evaporated under vacuum, and reconstituted in 200 µl of methanol. The sample was then repurified following the same procedure as above. The fractions containing PhIP were combined and evaporated. The sample was reconstituted in 1 ml of 1% acetic acid and further purified using solid-phase extraction with a C_{18} Prep Sep column (Fisher Scientific, Pittsburgh, PA). The column was washed with 5 ml of 1% acetic acid and then PhIP was eluted with 3 ml of 90% methanol in water and 0.1% triethylamine (HPLC grade, Fisher Scientific, Pittsburgh, PA). The eluate was evaporated under nitrogen, and reconstituted in 500 µl of methanol. The radiochemical purity was assessed and shown to be greater than 98%. N-Hydroxy-PhIP was synthesized from PhIP by converting the heterocyclic amine to a nitro derivative and then reducing it to the N-hydroxylated derivative as described by Turteltaub *et al.* (24). This metabolite was

then purified by HPLC. Pentachlorophenol (PCP) and alpha-naphthoflavone (ANF) were purchased from Sigma Chemical Company (St. Louis, MO). 2,6-Dichloro-4-nitrophenol (DCNP) was supplied by Aldrich Chemical Company, Inc. (Milwaukee WI). Aroclor 1254 was supplied by Analabs (Norwalk, CT). All other reagents were of reagent-grade quality or better. All solvents used for HPLC separation were of spectral grade quality.

Animals

Male Sprague-Dawley rats weighing 160-250 grams were obtained from Simonsen Farms (Gilroy, CA) and allowed to acclimate to their quarters for at least 10 days prior to use. All animals were housed on hardwood bedding in polystyrene cages and given food and water *ad libitum*. Animals were kept on a 12 hour light/dark cycle in a ventilated room maintained at 24°C. In experiments requiring hepatocytes isolated from induced animals, rats were treated with a single intra-peritoneal (i.p.) injection of Aroclor 1254, at 500 mg/kg in 1 ml/kg corn oil, five days prior to hepatocyte isolation.

Mutagenesis Assay

The mutagenesis assay used in these studies was the standard plate incorporation technique of Ames *et al.* (46), using the acetyltransferase-deficient *Salmonella* tester strain TA98/1,8-DNP$_6$ (47). Bacteria were grown overnight in Oxoid nutrient broth to a concentration of 1 x 10^9 cells/ml. Molten top agar (0.6% Difco agar with 0.5% NaCl) was maintained at 45°C. To the top agar a histidine-biotin solution was added to a final concentration of 10%, and 2 ml of this was placed into culture tubes. To the top agar was added in the following order: 0.1 ml of the *Salmonella* bacteria; 0, 2.5, 5, 10, 25, or 50 µg PCP or DCNP (dissolved in 25 µl of spectral grade methanol); and 1 nmol N-hydroxy-PhIP (in 10 µl methanol). Each sample was vortex mixed immediately after chemical

additions and poured onto gamma-irradiated minimal glucose agar plates. The plates were gently swirled while pouring the top agar to insure even distribution of the agar on the plate. The top agar was allowed to solidify, and plates were incubated at 37°C for 48 hours. After the 48 hour incubation time revertant numbers were counted with a Biotran III Automatic Colony Counter (New Brunswick Scientific Co., Edison, NJ).

Isolation of Hepatocytes

Buffers and Solutions: Hepatocytes were isolated using a modification of the collagenase perfusion technique of Moldeus *et al.*, (48), and Seglen, (49). Buffer *A*, consisted of a Ca^{2+} free HEPES supplemented Hank's Buffer [8.0 g NaCl; 0.4 g KCl; 0.12 g Na_2SO_4; 3.9 g HEPES (15 mM); 0.06 g KH_2PO_4; 2.19 g $NaHCO_3$; 0.10 g $NaHPO_4 \cdot 7H_2O$; 5.0 mg phenol red, pH 7.4, into 1 liter H_2O] containing 0.5 mM EGTA. The addition of the calcium chelator EGTA before perfusion with collagenase enhances liver dispersion (49). Buffer *B* consisted of a HEPES supplemented Hank's buffer containing 5 mM $CaCl_2$ and 110 units /ml collagenase (Sigma Chemical Company, St. Louis, MO). Since collagenase is a Ca^{2+} requiring enzyme, calcium must be re-introduced into the collagenase containing buffer (buffer *B*) for enhanced enzymatic activity and accelerated dispersion. The collagenase was added to buffer *B* just prior to liver perfusion. Buffer *C* was Waymouth's MB752/1 medium (Gibco, Scotland) containing 1% bovine serum albumin, 15 mM HEPES, and 10 mg/l of gentamycin (Sigma Chemical Company, St. Louis, MO). All buffers were brought to 37°C, oxygenated with 95% O_2/5% CO_2 and adjusted to pH 7.4 prior to use. Percoll solution consisted of Hank's buffer containing 36% percoll (Sigma Chemical Co., St. Louis, MO). This was prepared in a capped Erlenmeyer flask, oxygenated and put on ice until use.

Perfusion Apparatus: The perfusion apparatus consisted of two 250 ml beakers which served as buffer reservoirs for buffers *A* and *B*. These were kept in a water bath at 37°C. A tightly fitted mesh nylon screen was placed in the bottom of each beaker to act as a filter for the perfusate. From each beaker, a buffer line consisting of C-flex tubing (ID 4 mm) was fixed beneath the filter screen with the other ends connected to a three way stopcock. This allowed the switching from buffer *A* to buffer *B* during the perfusion with minimal interuption of flow. A Cole-Palmer peristaltic pump (model # 7553-20) was used for perfusion. A bubble trap was connected to the line immediately before connection to the liver. The bubble trap consisted of a 5 cc plastic syringe with the plunger removed and stoppered with a Vacutainer cap. A 3 inch 18 gauge stainless steel needle was pushed through the Vacutainer cap and served as the perfusate outflow. Also, a 1 inch 25 gauge needle was inserted into the cap with the opposite end connected to a stopcock. This allowed for pressure relief in the syringe. The perfusate entered the bubble trap from the bottom of the syringe, and as it filled with buffer, any bubbles in the buffer line were trapped in the syringe while the perfusate was allowed to flow through the 18 gauge needle out through Tygon tubing (3 mm ID) attached to a cannula. The cannula was an 18 gauge Angiocath (Becton-Dickinson, Rutherford, NJ) which consisted of a stainless steel needle with a Teflon sheath. The sheath was notched with a file 5 mm from the tip to secure the cannula in the portal vein with surgical thread. All tubing, except for the line attached to the cannula, was wrapped with foil coated insulated tape to minimize heat loss.

Surgical Procedure: The rat was anesthetized by placing it into a large capped glass jar containing gauze soaked with diethyl ether. A suitable screen was placed above the gauze to avoid dermal contact with the ether. After 2-3 minutes, when the animal was under anesthesia, it was removed from the jar and placed on its back and immobilized on the

surgical tray. A 50 ml plastic culture tube with ether-soaked gauze was placed over the nose of the rat to maintain anesthesia during surgery and adjusted to maintain proper anesthesia and air intake. The peritoneal cavity was opened by making a lateral incision in the lower abdominal region and carefully cutting up the sides of the animal to the plural cavity, taking care not to puncture it. The intestines were displaced to the right (animal's left) side of the abdominal cavity. The animal was injected with 500 units of sodium heparin into the posterior vena cava, proximal to the common iliac vein, to prevent blood coagulation during surgery. Gauze was held over the injection sight with light pressure to prevent significant blood loss. The heparin was allowed to circulate for 1-2 minutes, then the plural cavity was opened and further cuts up the side of the animal were made to facilitate easy manipulation of the liver. The portal vein was located and a loose ligature was tied around it in close towards the liver. An 18 gauge catheter with a notched Teflon sheath was carefully inserted into the portal vein just below the ligature. As the catheter was inserted, the needle was pulled back and removed. The remaining Teflon portion was gently pushed into the vein to where it enters the liver. Once the catheter was properly inserted, it was tied off with the ligature around the notch in the catheter. With successful cannulation of the vein the perfusion apparatus was attached and buffer *A* was immediately pumped through the liver, first slowly and then gradually increasing the rate to 30 ml/min. The posterior vena cava was cut below the liver, and the perfusate flow was increased to 40 ml/min. At this time, the liver started to clear of blood and blanching to a pale brown color was evident. Buffer *A* was allowed to perfuse through the liver for four minutes *in situ*. Successful perfusion revealed no blotching or red spots on the liver, which was a uniform light tan color. While perfusion with buffer *A* continued, the liver was gently removed from the carcass. Care was taken to handle the liver as little as possible, to avoid cutting the liver tissue, and to not dislodge the cannula. Once the liver was free from the body it

was gently transferred to a pre-warmed deep glass petri dish. After the 4 minute perfusion time with buffer *A*, the flow was reduced and switched to the collagenase-containing buffer *B*. The initial perfusate was removed from the petri dish by aspiration. Once the dish was filled with buffer *B* and the liver completely submerged, the buffer was recirculated at 40 ml/min. Total perfusion time with buffer *B* was 8-12 minutes. During perfusion with buffer *B*, the liver began to swell to about twice its original size and lose its integrity. After perfusion with buffer *B*, the cells were gently dispersed into the glass petri dish containing buffer *B* by cutting the capsula and allowing the cells to flow out into the petri dish. Gentle mincing with scissors helped to disperse the cells. The areas of the liver that were not completely perfused were cut away and removed, as well as any remaining connective tissue. The free cells and associated tissue were filtered through a fine mesh polyethylene screen into a small beaker on ice and rinsed with buffer *B*. The cell suspension was gently transferred to centrifuge tubes and centrifuged at 50 rpm for 1 minute. The supernatant was removed and the cells were resuspended in 20 ml of buffer *C* (Waymouth's media). To remove any nonviable cells from the cell suspension, 25 ml of chilled percoll solution was added to each of two Teflon FEP centrifuge tubes and each was layered with 10 ml of the cell suspension, and centrifuged for 20 minutes at 11,500 rpm (Sorvall SS-34 rotor). The supernatant, which contained nonviable cells, was removed and the pelleted cells were resuspended in 10 ml of buffer *C* and centrifuged at 50 rpm for 1 minute to remove residual percoll. After removal of the supernatant, the cells were resuspended in 20 ml of buffer *C*, and placed on ice. To assess cell viability and to determine cell count, 50 µl of cells were added to 150 µl of a 0.4% trypan blue solution and gently mixed. The cells were counted on a hemocytometer using light microscopy. Nonviable cells were stained blue since trypan blue enters cells with nonintact membranes. To determine the total cell count, the number of cells from the hemocytometer was multiplied by 4 (dilution factor)

and then by 10,000 to give the total number of cells/ml. The cells were then diluted to 2 x 10^6 cells/ml and viability was again assessed. Initial viability was always greater than 90%.

Incubation of [^{3}H]-PhIP and Enzyme Inhibitors with Hepatocytes

Hepatocytes were divided into 1-2 ml aliquots and placed into silanized 10 ml serum bottles which were on ice. Each vial received 5 µl of [^{3}H]-PhIP (25, 50, 100 µM, or DMSO vehicle) per 1 ml hepatocytes. The hepatocytes were incubated under an atmosphere of 95% O_2/5% CO_2 for 0, 30, 60, and 120 minutes in a shaking water bath at 37°C. For protein binding analysis, 1 ml aliquot of each sample was placed into a conical glass centrifuge tube and 2 volumes of ice-cold methanol were added to precipitate proteins. The samples were stored at -80°C until analysis. For DNA adduct analysis, the remaining 1 ml of each sample was placed in a microcentrifuge tube and centrifuged at 1000 rpm for 1 minute. The supernatant was removed and the pellet was washed with 1 ml of phosphate buffered saline (PBS). Each sample was centrifuged again at 1000 rpm for 1 minute and the supernatant was removed leaving a small layer of PBS over the pellet. The samples were stored at -20°C until analysis. In experiments assessing the effect of enzyme inhibitors on DNA adduct formation and protein binding, PCP (0.05 and 0.1 mM), DCNP (0.05 and 0.1 mM), and ANF (0.1 mM) at 2.5 µl per ml of cells were pre-incubated 15 minutes prior to addition of [^{3}H]-PhIP, and then incubated for 2 hours.

Protein Binding Studies

Samples were centrifuged at 2,000 rpm for 5 minutes (Sorvall SS-34 rotor) at 4°C. The aqueous methanol supernatant was removed and stored at -80°C until metabolite analysis by HPLC. Initially, protein binding was assessed by exhaustive extraction with methanol

and ethanol (data in Fig. 6A). Due to high levels of nonspecific binding the procedure was modified as follows: To the protein pellet, 3 ml of methanol were added and the samples were vortexed until the protein was well dispersed. The samples were then centrifuged at 2,000 rpm for 5 minutes at 4°C. The supernatant was discarded and the procedure was repeated until no more radioactivity could be detected in the washes. The protein pellet was then washed with a 1% acetic acid/90% methanol/9% water solution to further remove any unbound [^{3}H]-PhIP. This was followed by another methanol wash, two ethanol washes and then once again with methanol. The protein pellet was solublized in 1.5 ml 1 N NaOH at 60°C. Once the protein was completely dissolved, a 100 µl aliquot was taken from each sample and saved for protein quantification by the method of Lowry *et al.*, (50). One ml of the dissolved protein solution was placed into a 20 ml scintillation vial and 1 ml of H_2O was added. To neutralize the samples, approximately 0.25 ml of 3 N HCl was added. Each sample received 15-20 ml of Universol cocktail (ICN Biomedicals, Inc., Irvine, CA). The vials were placed in the dark for 24 hours before analysis by liquid scintillation counting. Each sample was counted for at least 10 minutes. Radioactivity was assessed using external-quench correction and standardized to protein content.

DNA Adduct Analysis

Stored cells were removed from the -20°C freezer and allowed to thaw. Each sample was resuspended in 5 ml ice cold PBS and centrifuged at 1000 rpm for 10 min at 4°C to wash away any unbound proteins. The PBS supernatant was removed and discarded. The cell pellet was resuspended in cold TE buffer (10 mM Tris/1 mM EDTA, pH 8) at 10^8 cells/ml. Ten volumes of freshly prepared lysis buffer were added to each sample [0.1% sodium dodecylsulfate (SDS), 10 mM Tris-HCl pH 7.9, 10 mM EDTA, 0.2 mg/ml proteinase K (Sigma Chemical Co., St. Louis MO)]. The samples were incubated for 6

hours at 37°C with gentle shaking. After the incubation period, the samples were gently extracted for ten minutes with an equal volume of 1 M Tris-saturated phenol (pH 8), then centrifuged for ten minutes at 10,000 rpm. The top aqueous layer containing the DNA was removed, taking care not to remove the protein layer sandwiched between the aqueous layer and the phenolic layer. The aqueous layer was extracted two separate times with an equal volume of PCI (phenol : chloroform : isoamyl alcohol, 24 : 24 : 1) until protein could no longer be detected in the top layer. Each sample was treated with 100 μg/ml RNAase A and T for 2 hours at 37°C to remove RNA. The samples were then extracted once with PCI and then once with CI (chloroform : isoamyl alcohol, 24 : 1). After the extractions, 0.1 volume 3 M sodium acetate (pH 5.2) was added, followed by two volumes of ice-cold 100% ethanol to precipitate the DNA. The DNA was spooled out with a fire-polished Pasteur pipette and washed with 70% ice-cold ethanol to remove any unbound material. The DNA was then dissolved in 1 ml of water and quantified using a Shimadzu UV2100 UV-Visible spectrophotometer at 260 nm. A 5 μg aliquot of DNA was evaporated under nitrogen to dryness and analyzed for adduct formation by ^{32}P-postlabeling analysis as described by Randerath *et al.* (51). Adduct formation from [^{3}H]-PhIP was also analyzed via scintillation counting.

Hepatocyte Metabolism of PhIP

The aqueous methanol supernatant from the protein binding analysis was evaporated under vacuum and reconstituted in 300 μl of 20% methanol. This solution was centrifuged at 2,000 rpm for 2 minutes and an aliquot of the sample was injected into a Rainin Rabbit HP HPLC system equipped with a Novapak C_{18} column (4 μm, 0.8 x 10 cm) and monitored with a Milton Roy detector at 315 nm. Metabolites were eluted isocratically at 1.5 ml/min using a solvent of 10% methanol/0.1% diethylamine, pH 6, for 5 minutes.

This was followed by a gradient to 33% methanol at 45 minutes, followed by a final gradient to 55% methanol/0.1% diethylamine at 60 minutes. The methanol concentration was maintained at 55% from 60 to 70 minutes. The column eluate was collected at 1 minute intervals and radioactivity content was quantified.

Statistical Analysis

All statistical comparisons were done using ANOVA and Dunnett's *t*-test or Student's *t*-test. Comparisons with p-values < 0.05 were considered statistically significant.

RESULTS

BACTERIA:

The Effect of PCP and DCNP on N-hydroxy-PhIP Mutagenicity in the Ames/Salmonella Assay

To determine the role of bacterial sulfotransferases in the mutagenic activation of N-hydroxy-PhIP, the effect of PCP and DCNP co-incubation on mutagenicity was examined. No evident inhibitory effect on revertant formation was observed with PCP (Fig. 3). At a concentration of 5 μg/plate, mutagenicity of N-hydroxy-PhIP was decreased by 17%. Increasing concentrations of PCP produced less inhibition and at 50 μg/plate, the mutagenicity was inhibited by only 2%. The decrease in mutagenicity, at any point tested, was not significantly different from control incubations without inhibitor. DCNP, a more specific inhibitor of sulfotransferase than of O-acetyltransferase (28), produced a concentration-dependent inhibition of N-hydroxy-PhIP mutagenicity (Fig. 3). Only 10% of the mutagenic activity remained at the highest concentration of DCNP (50 μg/plate) examined. Interestingly, co-incubation with DCNP produced a slight inhibitory effect at 2.5 μg/plate, but addition of 5 μg/plate resulted in revertant formation approaching levels similar to that of the controls. This finding was observed in two separate experiments. However, the decrease in mutagenicity at 2.5 μg DCNP was not statistically significant from the 0 and 5 μg concentrations (ANOVA, $p<0.05$). Background plates with PCP or DCNP (no N-hydroxy-PhIP) indicated the inhibitors were neither mutagenic nor toxic to the bacteria at the concentrations tested (Fig. 4).

HEPATOCYTES:

Viability of Hepatocytes

The trypan-blue-exclusion test was used to assess hepatocyte viability of cells incubated with PhIP, PCP or DCNP at various concentrations, over a 4 h time period. Initial viabilities of the incubations containing PhIP were greater than or equal to 90% at all concentrations tested (Table 1). After 4 h, the viability decreased to between 75-85%, depending on the concentration of PhIP. At 4 h, the viability of cells treated with the DMSO vehicle was 80%. Viability of cells exposed to 200 μM PhIP was 77%, whereas for cells exposed to 100 μM PhIP, viability was 87%. These data indicate that PhIP, at the concentrations tested, had little effect on cell viability.

Viability assessment of cell incubations with PCP and DCNP indicated that at 0.01 and 0.1 mM concentrations, viability was maintained at greater than 80% after 4 h (Fig. 5A and B). At 2 h cell viability was approximately 90% at both concentrations of PCP and at 0.1 mM DCNP. At 1.0 mM PCP or DCNP viability quickly declined over the 4 h period. The decline in viability at 2 h for DCNP treated cells at 0.1 mM was probably not accurate since viability increased at 4 h at this concentration. Since these initial studies suggested that PCP or DCNP treatment produced cytotoxicity in hepatocytes, concentrations of these inhibitors used in subsequent studies were set at 0.05 and 0.10 mM, while incubation times were set at 2 h. Based on the above data these conditions should produce no significant inhibitor-related effects on cytotoxicity.

Covalent Binding to Proteins

Covalent binding of [^{3}H]-PhIP to endogenous and exogenous proteins was assessed to evaluate the formation of reactive intermediates from PhIP metabolism. Hepatocytes were exposed to 3 different doses of [^{3}H]-PhIP over 2 h. Covalent binding was concentration-

dependent (Fig. 6A). At 25 μM PhIP after 2 h, there were10 pmol bound per mg protein, while at 100 μM PhIP there were 28 pmol of PhIP bound. Also, there was a somewhat linear increase with time in the amount of [^{3}H]-PhIP bound at all concentrations tested. At time zero, nonspecific binding by [^{3}H]-PhIP was detected at significant levels. Because the reaction was terminated immediately after the addition of substrate by precipitation of the proteins with ice-cold methanol, it is unlikely that this binding was the result of metabolism of [^{3}H]-PhIP. The nonspecific binding was subtracted from each data point at each concentration and replotted as indicated in Figure 6B.

The Effect of PCP and DCNP on the Covalent Binding of PhIP

The covalent binding of [^{3}H]-PhIP (100 μM) in hepatocytes pretreated with PCP or DCNP (0.05 and 0.1 mM) was assessed in both induced and uninduced cells to evaluate the effect of these enzyme inhibitors on the formation of reactive intermediates from PhIP. In uninduced vehicle-pretreated control hepatocytes, the level of protein binding was measured at 36 pmol/mg protein. PCP pretreatment decreased binding at 0.1 mM to 32 pmol/mg, but not at 0.05 mM. DCNP reduced protein adduct formation to levels averaging 25 and 30 pmol/mg protein at 0.05 and 0.1 mM, respectively (Fig. 7). In hepatocytes incubated with 25 μM PhIP and pretreated with PCP or DCNP, the same trend in the decrease of covalent binding by the inhibitors was evident (Fig. 8). The decline in covalent binding was not statistically significant based on ANOVA ($p<0.05$); however, the trend was consistent over the two concentrations of PhIP tested. In Aroclor-induced hepatocytes, protein adduct levels were detected at levels comparable to those observed in the uninduced cells. No decrease in binding due to PCP or DCNP pretreatment at either concentration was detected in the induced cells incubated with [^{3}H]-PhIP (Fig. 9).

The Effect of PCP and DCNP on DNA Adduct Formation from PhIP

No radioactivity was detected by scintillation counting of isolated nucleic acids derived from either uninduced or Aroclor-induced hepatocytes. DNA adducts were detected, however, by ^{32}P-postlabeling, but due to analysis time associated with this detection method, the effect of PCP or DCNP could not be assessed at this time.

The Effect of PCP and DCNP on the Metabolism of PhIP

To determine the effect of PCP and DCNP pretreatment (0.1 mM) on the metabolism of PhIP, 100 μM [^{3}H]-PhIP was incubated with rat hepatocytes for 2 h and metabolite formation was assessed by HPLC. Five metabolites were detected at significant levels in incubations derived from uninduced control hepatocytes. Metabolites eluting at 29, 43 and 54 minutes had retention times similar to those of PhIP metabolite standards and were determined to be 4'-PhIP-sulfate, 4'-hydroxy-PhIP, and N-hydroxy-PhIP-glucuronide, respectively (Fig. 10A). The metabolites that eluted at 18 and 48 minutes have not been identified and are referred to as unkown 1 and 2 (UK1 and UK2), respectively. Incubations from the uninduced hepatocytes containing 0.1 mM PCP had a significant decrease in the amount of 4'-PhIP-sulfate levels relative to controls (Fig. 10B). Analysis of samples with 0.1 DCNP indicated a decrease in 4'-PhIP-sulfate as well, but the decrease was not as great as in the PCP-pretreated samples (Fig. 10C). A decrease in 4'-PhIP-sulfate levels of approximately 90% and 65% in the PCP and DCNP pretreated samples, respectively, was observed (Fig. 11). All other metabolites were detected at levels comparable to those observed in control incubations.

Incubations from Aroclor-induced hepatocytes produced the same metabolites from PhIP as did the uninduced samples, but at levels approximately eight-fold higher (Fig. 12A). In induced hepatocytes containing 0.1 mM PCP, a decrease in 4'-PhIP-sulfate and

an increase in the 4'-hydroxy-PhIP metabolites compared to control incubations without PCP was noted (Fig 12B). DCNP pretreatment produced a decrease in 4'-PhIP-sulfate formation and an increase in 4'-hydroxy-PhIP levels as well, but not to the same extent as PCP (Fig 12C). The amount of PhIP converted to 4'-PhIP-sulfate in the PCP treated samples was decreased by 80% compared to control levels, while the 4'-hydroxy-PhIP was increased by 55% (Fig. 13). DCNP reduced the amount of 4'-PhIP-sulfate formed by 40% of control levels and 4'-hydroxy-PhIP was increased by 37%. All other metabolite levels were similar to those of controls.

The Effect of ANF on the Covalent Binding and Metabolism of PhIP

To determine the role of P450 activation, the effect of the P450 inhibitor ANF on covalent binding and metabolite formation of [^{3}H]-PhIP in Aroclor-induced and uninduced hepatocytes was assessed. In [^{3}H]-PhIP (100 μM) treated uninduced cells, pretreatment with ANF had no effect on protein binding compared to the control cells (Fig. 14). In induced cells, a decrease of 18% in the amount of PhIP bound per mg protein was observed. However, this was not statistically significant. When ANF was incubated with uninduced hepatocytes treated with 25 μM [^{3}H]-PhIP, protein binding was detected at levels of 14 pmol PhIP bound per mg protein, while the vehicle-pretreated control levels were lower at 4.9 pmol PhIP bound per mg protein (data not shown).

The effect of ANF on metabolite formation in uninduced hepatocytes treated with 100 μM [^{3}H]-PhIP was a decrease in 4'-hydroxy-PhIP levels by 53% relative to control cell incubations (Fig. 15A). All other metabolites detected remained relatively unchanged with ANF pretreatment. ANF produced a substantial decrease in PhIP metabolite formation in Aroclor-induced cells. 4'-PhIP-sulfate was decreased by 89%, while 4'-Hydroxy-PhIP

and N-hydroxy-PhIP-glucuronide levels decreased by 91% and 94%, respectively, compared to control cell levels (Fig. 15B).

The Effect of PCP, DCNP, and ANF on the Loss of PhIP due to Metabolism

To assess the effect of PCP, DCNP, and ANF on the inhibition of metabolism, the amount of unchanged PhIP present after 2 h incubations in both induced and uninduced hepatocytes was determined. In uninduced control cells, 80.7 nmol of PhIP was detected by HPLC. In cells pretreated with PCP, DCNP or ANF the amount of PhIP recovered was 74.4, 86.6, and 75.1 nmol, respectively (Table 2). The amount of PhIP detected in incubations of Aroclor-induced, control hepatocytes was 87.7 nmol PhIP. In PCP and DCNP pretreated cells the levels were approximately the same as controls at 89.3 and 87.4 nmol, respectively (Table 2). In ANF treated samples, however, the amount of PhIP recovered was 103 nmol. This was consistent with lower amounts of metabolites produced in the ANF-pretreated induced samples.

DISCUSSION

The effect of PCP and DCNP on the bacterial mutagenicity of N-hydroxy-PhIP was assessed to determine the role of bacterial sulfotransferases in the mutagenic activation of N-hydroxy-PhIP. DCNP, which is a much more selective inhibitor of mammalian sulfotransferases than is PCP (28,33), produced a concentration-dependent inhibition of mutagenicity. No significant inhibition of mutagenic activity from PCP was observed (Fig. 3). Since mutagenic activity from PhIP was observed in the acetyltransferase-deficient *Salmonella* strain TA98/1,8-DNP$_6$ (25), acetyltransferases appear not to be important in the mutagenic activation of N-hydroxy-PhIP in bacteria. This is in contrast to that observed for other heterocyclic amine mutagens such as IQ and MeIQ (28). At the lowest concentration of DCNP (2.5 μg/plate) a slight inhibitory effect on PhIP mutagenicity was seen, but at 5.0 μg/plate of DCNP, mutagenicity increased to levels approaching that of the control incubations with no inhibitor (Fig. 3). The unusual concentration-mutagenicity curve produced via the addition of DCNP to incubations with N-hydroxy-PhIP was also observed in incubations of DCNP with N-hydroxy-IQ (34). At higher concentrations of DCNP (10-50 μg/plate), mutagenic activity of PhIP was clearly inhibited. The decrease in mutagenicity due to DCNP observed in the current studies indicates that bacterial sulfotransferases may play an important role in the activation of N-hydroxy-PhIP. In contrast to what was observed for N-hydroxy-PhIP, both PCP and DCNP produced decreases in N-hydroxy-IQ mutagenicity in *Salmonella* (34). PCP was shown to have a greater inhibitory effect on mutagenicity than did DCNP for N-hydroxy-IQ, and perhaps this was due to greater inhibition of O-acetyltransferases by PCP. In the present study with N-hydroxy-PhIP, PCP did not appear to inhibit sulfotransferase activity in the TA98/1,8-DNP$_6$ strain, but in a previous study by Snyderwine *et al.*, PCP inhibited mutagenicity from N-hydroxy-IQ in TA98NR (34), a strain having acetyltransferase activity. The

differential effects of PCP and DCNP on N-hydroxy-IQ (34) versus N-hydroxy-PhIP activity could be consistent with a mechanism of PCP inhibition due to inhibition of acetyltransferases. It is unclear why DCNP, but not PCP inhibited bacterial mutagenicity, but the current data suggests that bacterial sulfotransferases may be significantly different from mammalian sulfotransferases at least with respect to inhibition by these classical enzyme inhibitors.

To evaluate the formation of reactive intermediates from PhIP metabolism in mammalian cells, [^{3}H]-PhIP was incubated with rat hepatocytes and covalent binding to proteins was assessed. Covalent binding was time and concentration-dependent, though significant nonspecific binding was detected as evidenced by high levels of radioactivity detected at time zero in the hepatocyte samples (Fig. 6A). A modification of the protein purification method by adding a 1% acetic acid/methanol wash step (see Methods) decreased some of the nonspecific bound substrate, but complete removal could not be attained. It is unlikely the nonspecific binding was the result of metabolism of [^{3}H]-PhIP since the reaction was terminated immediately after the addition of substrate by precipitation of the proteins with ice-cold methanol. Furthermore, addition of [^{3}H]-PhIP after protein precipitation with methanol also resulted in comparatively high levels of nonspecific binding (data not shown). Apparently, rather tight-binding of PhIP to hepatocytes or the exogenous bovine serum albumin occurred under the conditions of the experiment.

Unlike the bacterial system, the addition of PCP or DCNP to rat hepatocytes produced no statistically significant changes in protein binding by PhIP at both 25 and 100 μM PhIP in cells derived from uninduced rats. There was, however, an observable decrease in binding at both concentrations of [^{3}H]-PhIP by these inhibitors (Fig. 7 and 8) suggesting that inhibition may have occurred. No decrease in binding was evident at the lower PCP concentration (0.05 mM), while at 0.1 mM PCP, a slight decrease of approximately 10%

was observed. In the DCNP incubations, a decrease in binding was observed at both concentrations of DCNP tested. In contrast, pre-incubation of PCP or DCNP with Aroclor-induced hepatocytes had no effect on the covalent binding of [^{3}H]-PhIP (Fig. 9). The rather marginal effect of PCP or DCNP in hepatocytes derived from uninduced rats and the negative effect in the hepatocytes from Aroclor-induced animals suggests that other metabolic pathways besides sulfation and acetylation may be important in the activation of PhIP to reactive intermediates which covalently bind proteins. Possibly, N-hydroxy-PhIP formation may be sufficient for binding, as observed in microsomal assays (52). However, the finding that the levels of protein adducts in Aroclor-induced hepatocytes (which produced significantly more metabolites) were comparable to that in uninduced hepatocytes suggests that additional metabolites besides N-hydroxy-PhIP may be involved in protein binding.

The formation of DNA adducts in hepatocytes due to [^{3}H]-PhIP exposure was examined via scintillation counting and ^{32}P-postlabeling. No adducts were detected from scintillation counting presumably because of the lack of sensitivity of the detection method. However, adducts were detected using the ^{32}P-postlabeling technique (data not shown) indicating further studies for DNA adduct analysis in hepatocytes exposed to PhIP and these inhibitors are warranted.

Metabolic profiles of rat hepatocytes incubated with [^{3}H]-PhIP were qualitatively similar to urinary metabolite profiles of mice treated with [^{14}C]-PhIP (52). At least five metabolites were detected in control uninduced hepatocyte incubations (Fig 9A). The major metabolites formed were 4'-PhIP-sulfate and the unconjugated 4'-hydroxy-PhIP. This is consistent with previous studies by Alexander *et al.* using rat hepatocytes (53). N-Hydroxy-PhIP-glucuronide also was detected. Two additional metabolites were not identified. The addition of PCP to the cell incubations produced a decrease in the 4'-PhIP-

sulfate metabolite compared to the control cells (Fig. 10B). Addition of DCNP also produced a decrease in 4'-PhIP-sulfate, but not to the extent of the PCP-pretreated samples. The decrease in the levels of 4'-PhIP-sulfate suggested the sulfation pathway was inhibited by PCP or DCNP. However, no increase in 4'-hydroxy-PhIP or N-hydroxy-PhIP-glucuronide was observed with uninduced cells pre-incubated with PCP or DCNP (Fig. 11).

To determine the effect of P450 induction on PhIP metabolite formation, Aroclor-induced hepatocytes were incubated with [^{3}H]-PhIP. Metabolite formation was approximately eight times higher than in the uninduced cells (Fig 12A) which was fairly consistent with Aroclor-induced effects on microsomal metabolite formation (41). Pre-incubation with PCP or DCNP produced a decrease in 4'-PhIP-sulfate, and a concomitant increase in 4'-hydroxy-PhIP as might be expected. An increase in N-hydroxy-PhIP-glucuronide levels via DCNP or PCP pre-incubation (which might be expected from increased N-hydroxy-PhIP available for glucuronidation) was not observed, and this may suggest that either these inhibitors do not affect N,O-sulfation or acetylation, or that sulfation and/or acetylation are quantitatively minor pathways for N-hydroxy-PhIP metabolism in the hepatocyte system used in these studies. This would be consistent with the protein binding data showing no effects of the inhibitors on adduct formation.

To determine the role of cytochrome P450 in adduct formation of [^{3}H]-PhIP, the cytochrome P450 inhibitor ANF was added to both uninduced and Aroclor-induced hepatocytes. In the uninduced hepatocytes, a significant decrease in the formation of 4'-hydroxy-PhIP was observed, but all other metabolites were unchanged. In the Aroclor-induced cells pre-incubated with ANF, a decrease in the formation of all detectable metabolites by at least 90% of control incubations was observed. In fact, the levels of metabolites were similar to that produced by uninduced cells without inhibitor. This was

consistent with previous studies showing that ANF blocks the mutagenic activity of PhIP and the formation of PhIP metabolites in rat hepatocytes (37,53). Since only minor changes in metabolite profiles were observed in ANF-pretreated uninduced cells incubated with 100 μM [^{3}H]-PhIP, other P450 isozymes not inhibited by ANF (ANF inhibits P450 IA1 and IA2 which have been shown to metabolize PhIP) may be involved in the mutagenicity of PhIP in isolated hepatocytes. Other oxidative enzymes besides cytochrome P450 may also be involved in the mutagenicity of PhIP in this system. ANF produced no significant change in protein binding in uninduced hepatocytes treated with 100 μM [^{3}H]-PhIP, and only a slight, but not statistically significant, decrease in binding of approximately 18% in the induced cells. The much lower decline in covalent binding via ANF-pretreatment was not consistant with the large decrease (90%) in metabolite formation. Interestingly, when uninduced cells were co-incubated with 25 μM [^{3}H]-PhIP and 0.1 mM ANF, a 65% increase in covalent binding was observed. Additional studies are warranted for a better understanding of these results.

The effect of PCP, DCNP or ANF on PhIP levels due to conversion to metabolites was determined in both Aroclor-induced and uninduced hepatocytes. The addition of PCP, DCNP, or ANF to uninduced hepatocytes as well as PCP and DCNP in the induced hepatocytes produced no statistically significant changes in the amounts of PhIP detected after incubation when compared to controls. The addition of ANF to induced cells produced an increase in the amount of parent compound detected relative to control hepatocytes with no inhibitor. The fact that no significant differences in the amount of PhIP present after pre-incubation with PCP or DCNP was observed suggests that metabolism of PhIP by cytochrome P450 or some other oxidative pathway was not inhibited by PCP or DCNP. Also, the concentrations of PCP and DCNP used in these experiments (0.05 and 0.1 mM) did not cause a decrease in the amount of metabolite

produced due to cytotoxicity. The increase in PhIP levels due to pre-incubation with ANF in induced cells confirms previous studies suggesting that cytochrome P450 catalyzed oxidation reactions are important in the metabolism of PhIP in hepatocytes.

The mechanisms involved in the mutagenic/carcinogenic actions of PhIP are still not fully understood. In the present studies, attempts have been made to gain a better understanding of the metabolic processes involved in the biotransformation of PhIP to mutagenic metabolites. In the bacterial system, sulfotransferases appear to play an important role in mutagenic activation. In rat hepatocytes, due to the inconsistencies in protein binding and metabolite data, it is still unclear how much sulfotransferases and/or acetyltransferases contribute to the activation of PhIP. The present results also lead to some speculation that additional metabolic pathways may be involved in the activation of PhIP to genotoxic metabolites. Mutagenicity of PhIP has been clearly shown to involve cytochrome P450 and the present studies confirm a role of cytochrome P450 in the metabolism of PhIP in Aroclor-induced cells, but the effects of cytochrome P450 activation on covalent binding to protein are still unclear. Additional studies such as *in vivo* metabolism experiments using the specific enzyme inhibitors PCP, DCNP, and ANF, and additional DNA adduct studies are needed to fully characterize the mechanisms involved in the mutagenic and or carcinogenic actions of PhIP before human health risks can be properly assessed.

Table 1. Viability of hepatocytes incubated with PhIP over 4 hours (percent)*

PhIP (μM)	Time (min) 0	30	60	120	240
0	90	94	89	88	80
25	94	79	85	84	81
50	94	95	86	83	77
100	96	94	86	88	87
200	98	87	88	86	77

*Data are the means of two incubations

Table 2. Effect of PCP, DCNP, and ANF on loss of PhIP due to metabolism

PhIP (μM)	Pretreatment	nmol ^{3}H-PhIP recovered Control	PCP*	DCNP*	ANF*
100	None	80.7 (1.7)	74.4 (1.9)	86.6 (4.4)	75.1 (2.7)
100	Aroclor	87.7 (5.4)	89.3 (5.4)	87.4 (3.9)	102.9 (0.41)

*Concentration of inhibitors was 0.1 mM
Data are the means (± S.E.) of three incubations

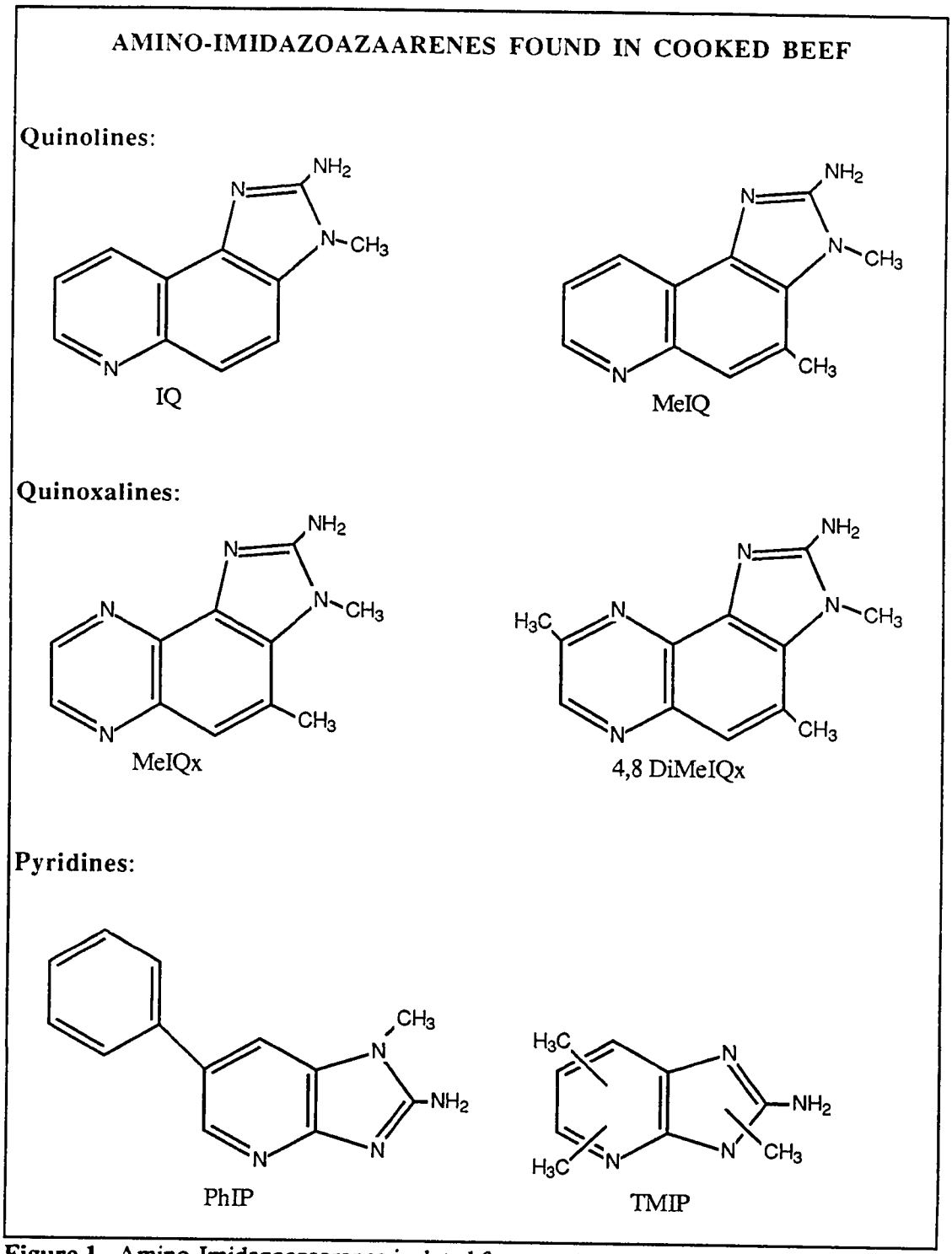

Figure 1. Amino-Imidazoazaarenes isolated from cooked beef.

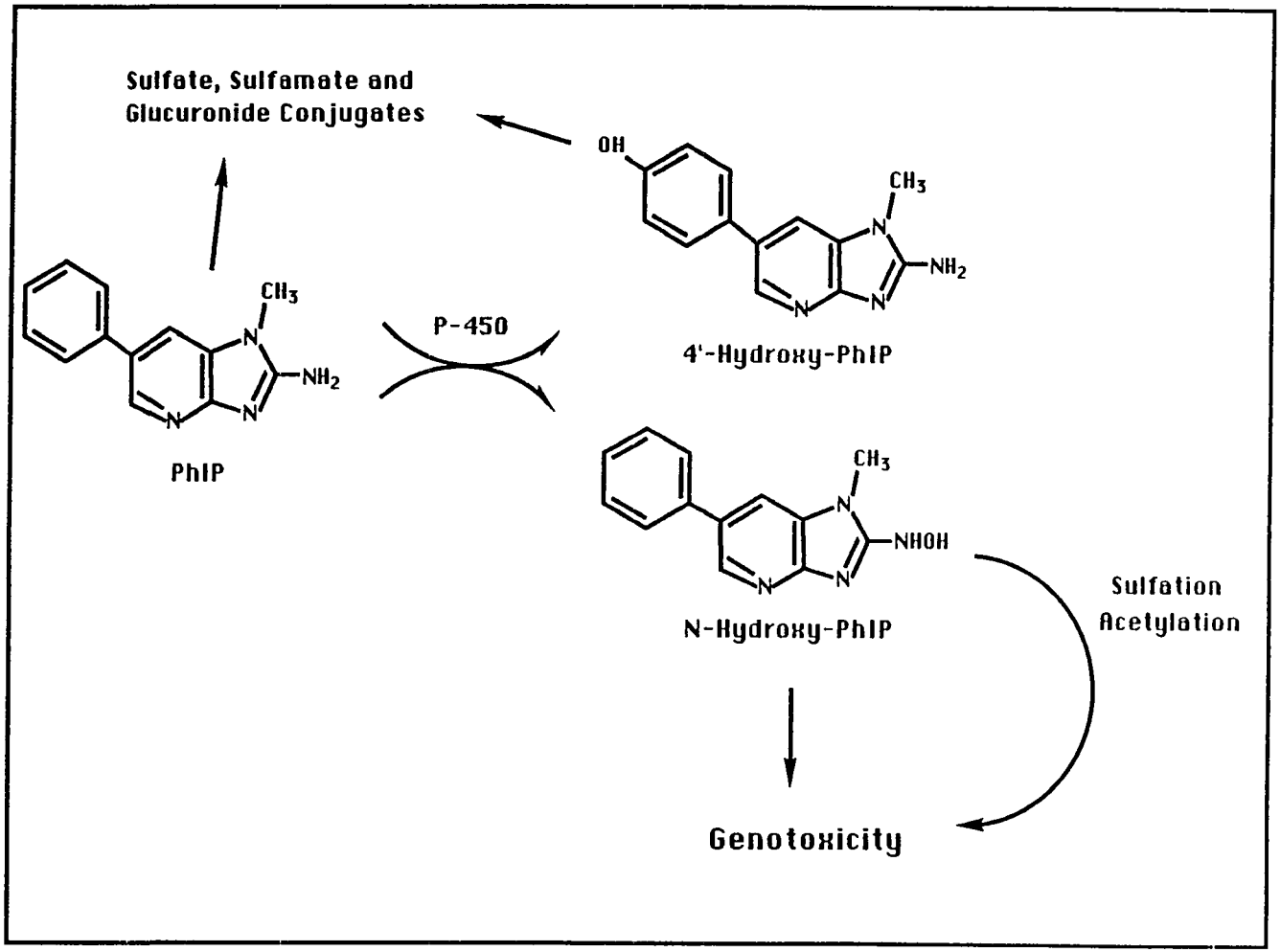

Figure 2. Proposed pathways for PhIP Metabolism. Used with permission, Buonarati and Felton, 1990.

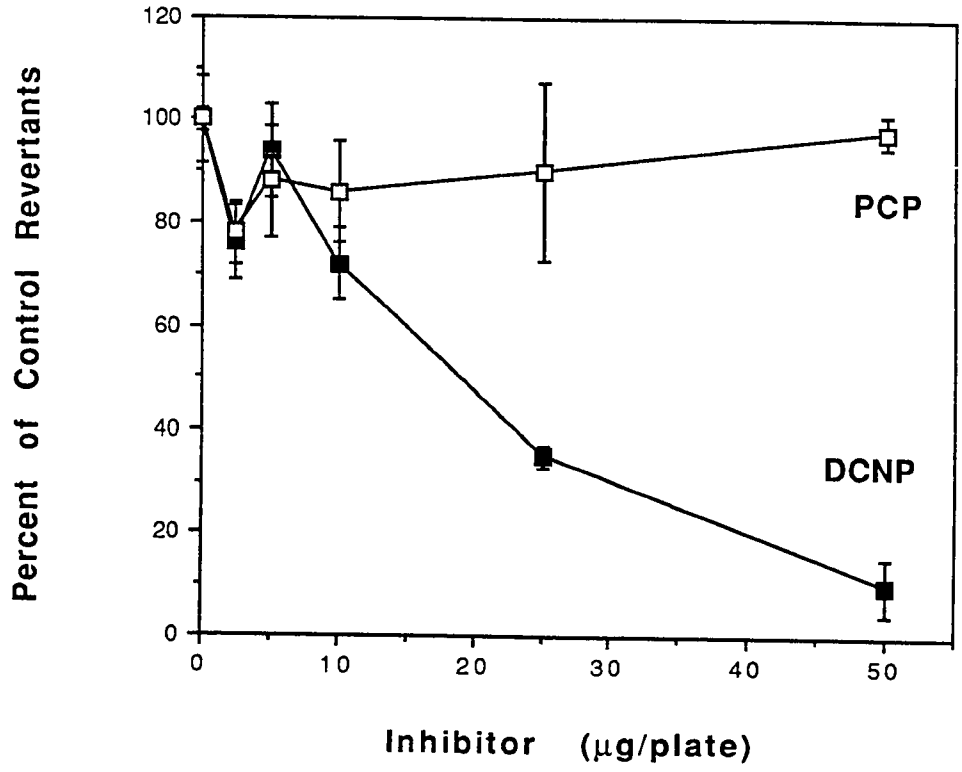

Figure 3. Effect of PCP and DCNP on the mutagenicity of N-hydroxy-PhIP in the Ames/*Salmonella* assay. Data are expressed as the percentage of control revertants (incubations of N-hydroxy-PhIP with no inhibitor) and are the means of two separate experiments on different days. Data are the means ± S.E. of five incubations.

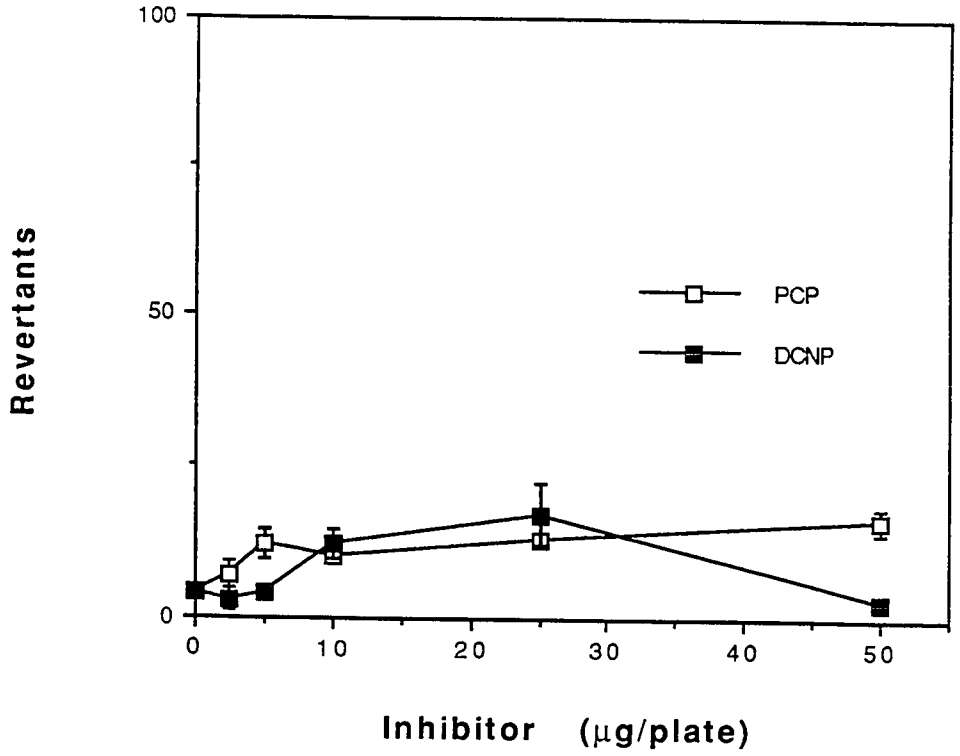

Figure 4. Background revertants from PCP and DCNP in the Ames/*Salmonella* assay. Incubations consisted of bacteria, PCP or DCNP. Data are the means ± S.E. of three incubations.

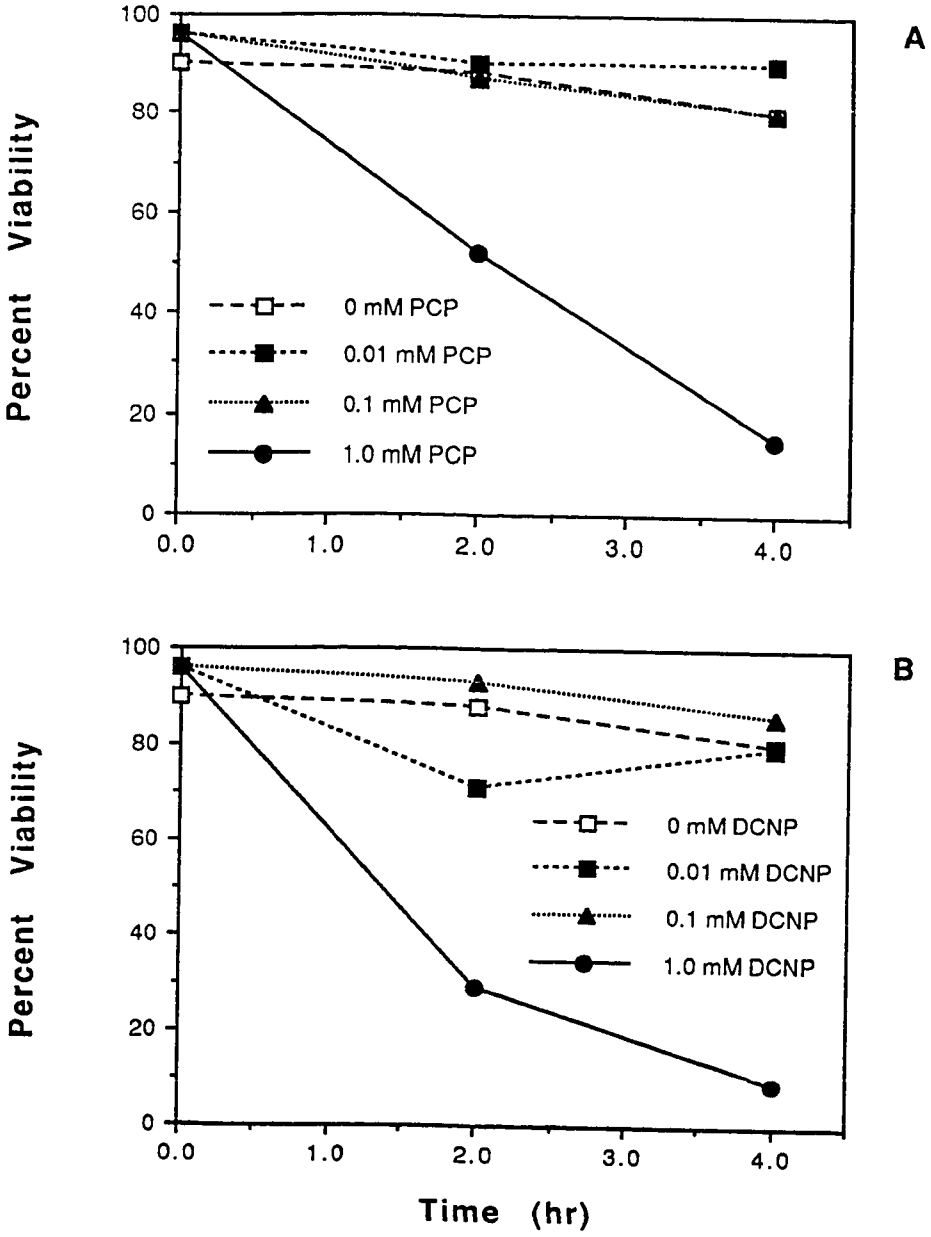

Figure 5. Percent viability of rat hepatocytes incubated with PCP (A) or DCNP (B) at 0, 0.01, 0.1, and 1.0 mM for 4 h. Data are the means of two incubations

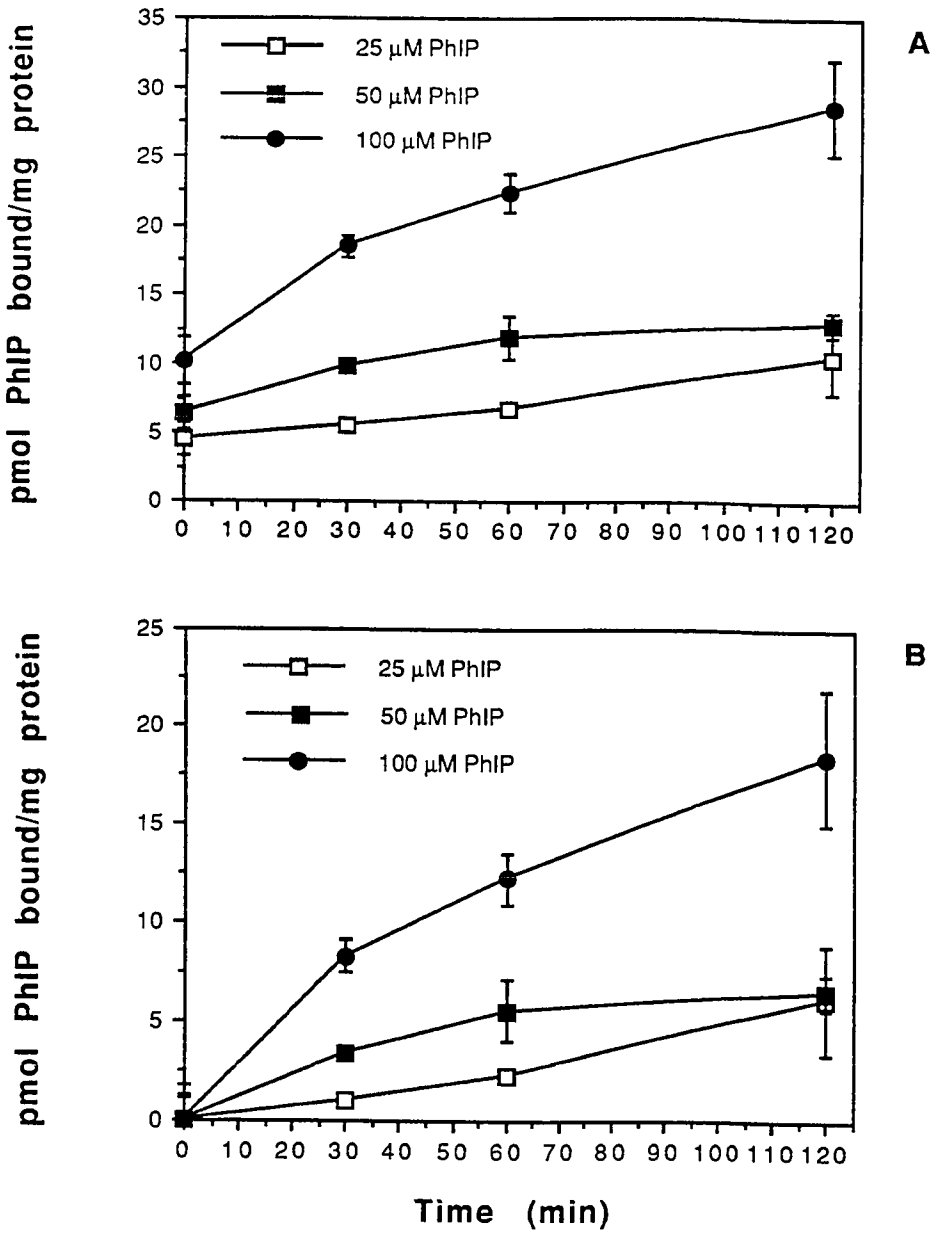

Figure 6. Covalent binding by [^{3}H]-PhIP in rat hepatocyte preparations at 25, 50, and 100 μM. Total binding (A), and binding minus nonspecific binding (B) are expressed as pmol PhIP bound per mg protein. Data are the means ± S.E. of three incubations.

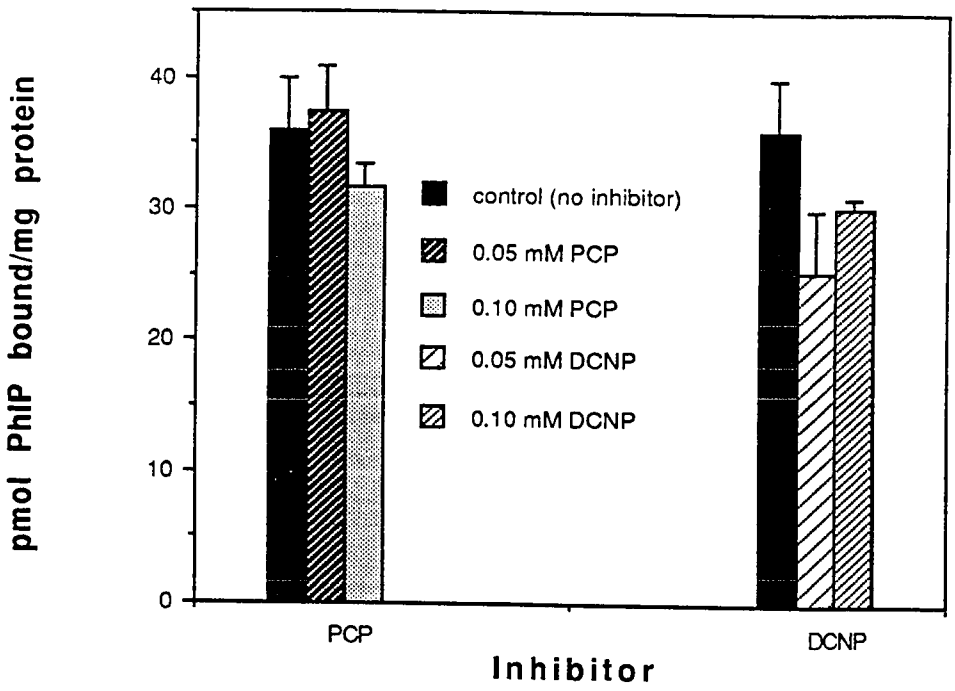

Figure 7. Effect of PCP and DCNP on covalent binding by 100 μM [^{3}H]-PhIP in uninduced rat hepatocyte preparations. Data are the means ± S.E. of three incubations.

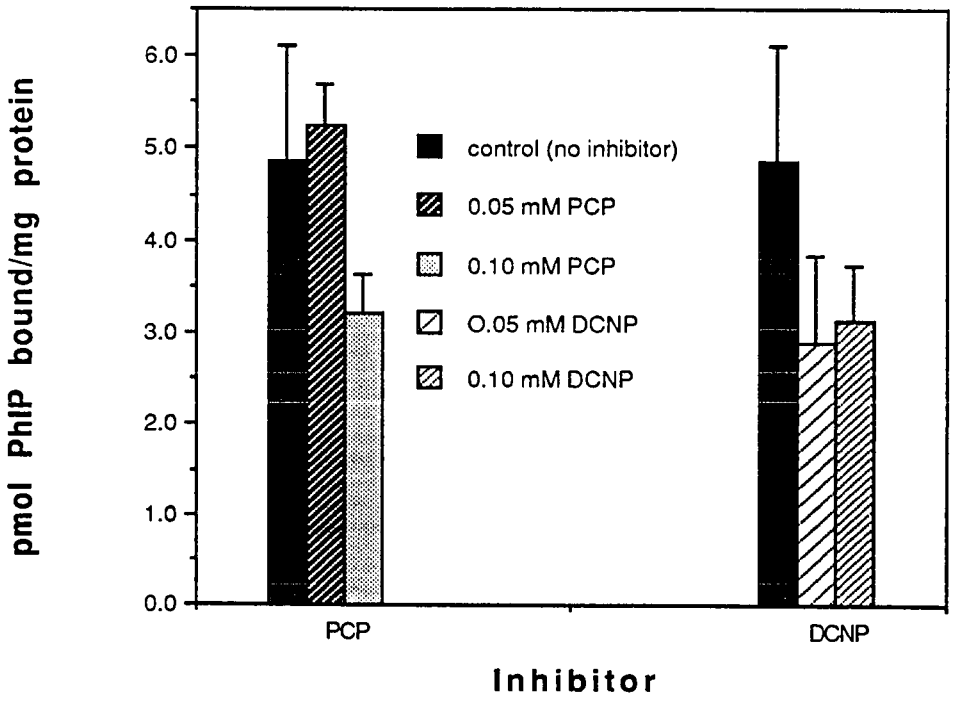

Figure 8. Effect of PCP and DCNP on covalent binding of 25 μM [^{3}H]-PhIP in uninduced rat hepatocyte preparations. Data are the means ± S.E. of three incubations.

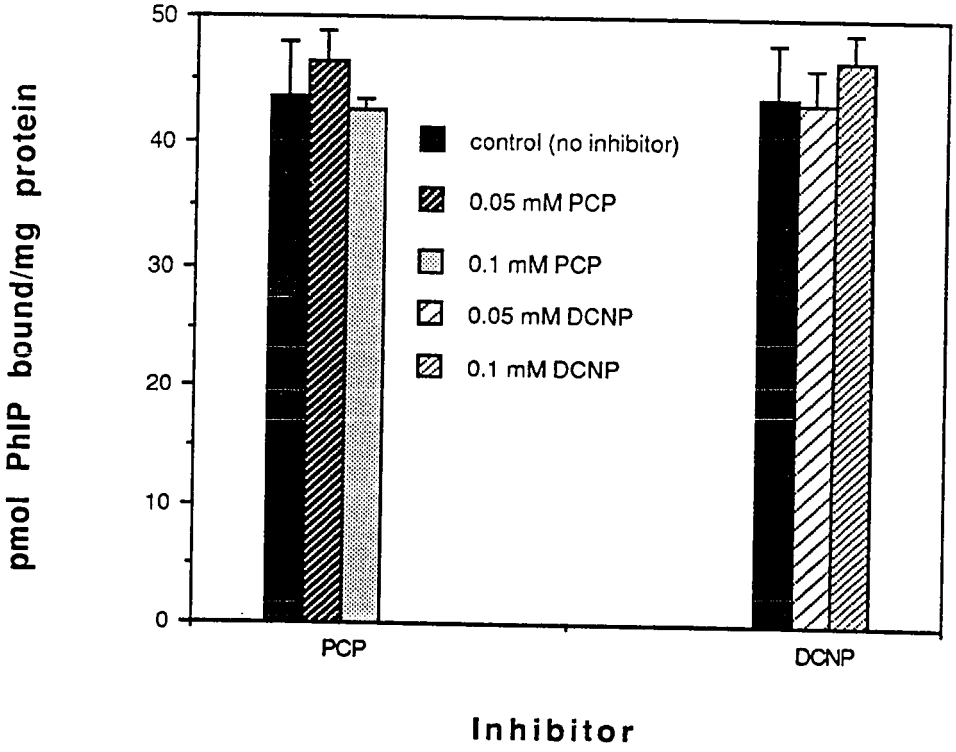

Figure 9. Effect of PCP and DCNP on covalent binding of 100 μM [^{3}H]-PhIP in Aroclor-induced rat hepatocyte preparations. Data are the means ± S.E. of three incubations.

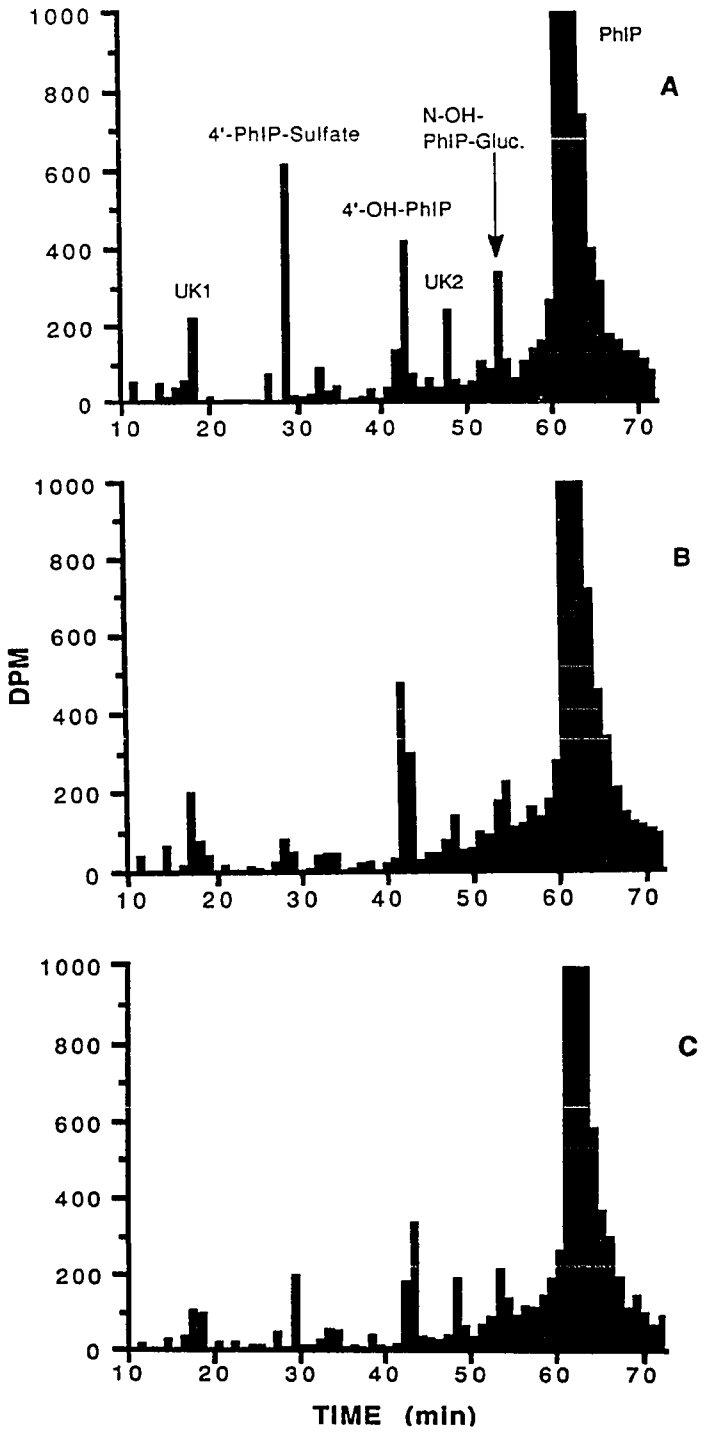

Figure 10. HPLC radioprofiles of hepatocyte preparations from uninduced rats at 100 μM [^{3}H]-PhIP pretreated with DMSO vehicle-control (A), 0.1 mM PCP (B) and 0.1 mM DCNP (C).

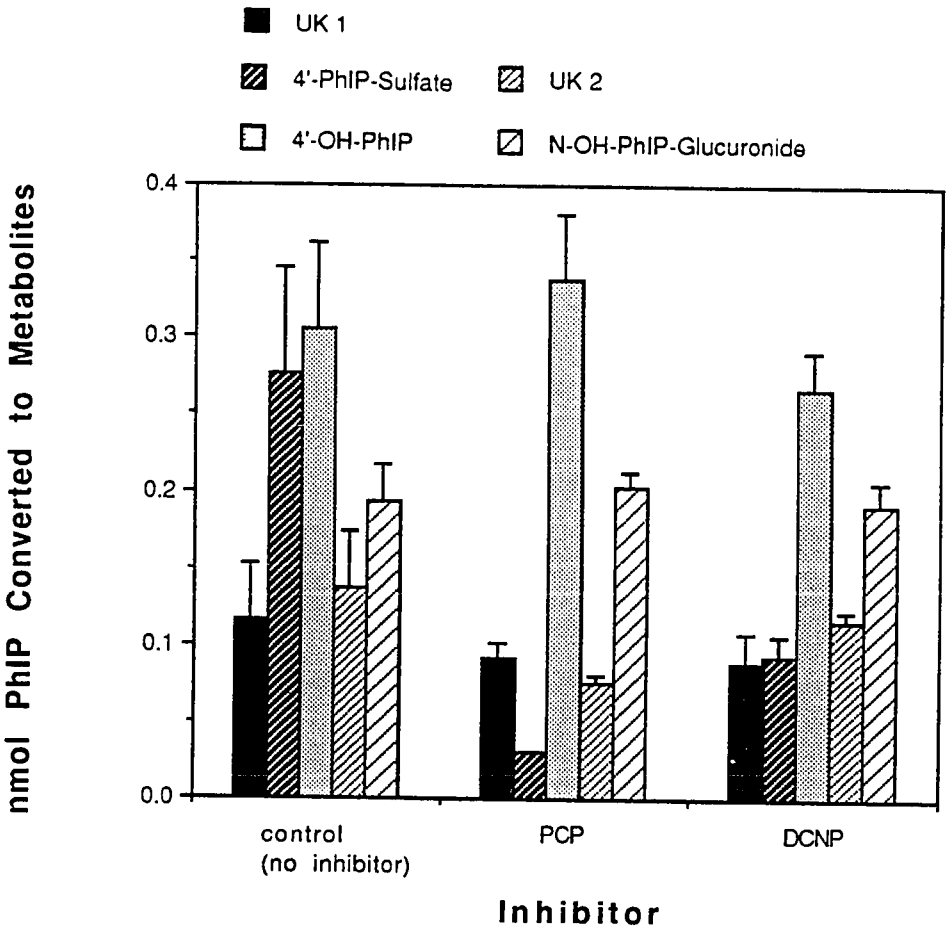

Figure 11. Effect of PCP and DCNP on the amount of [^{3}H]-PhIP (100 μM) converted to metabolites by uninduced rat hepatocyte preparations ($2x10^6$ cells) pretreated with 0.1 mM PCP or 0.1 mM DCNP after 2 h. Data are the means ± S.E. of three incubations.

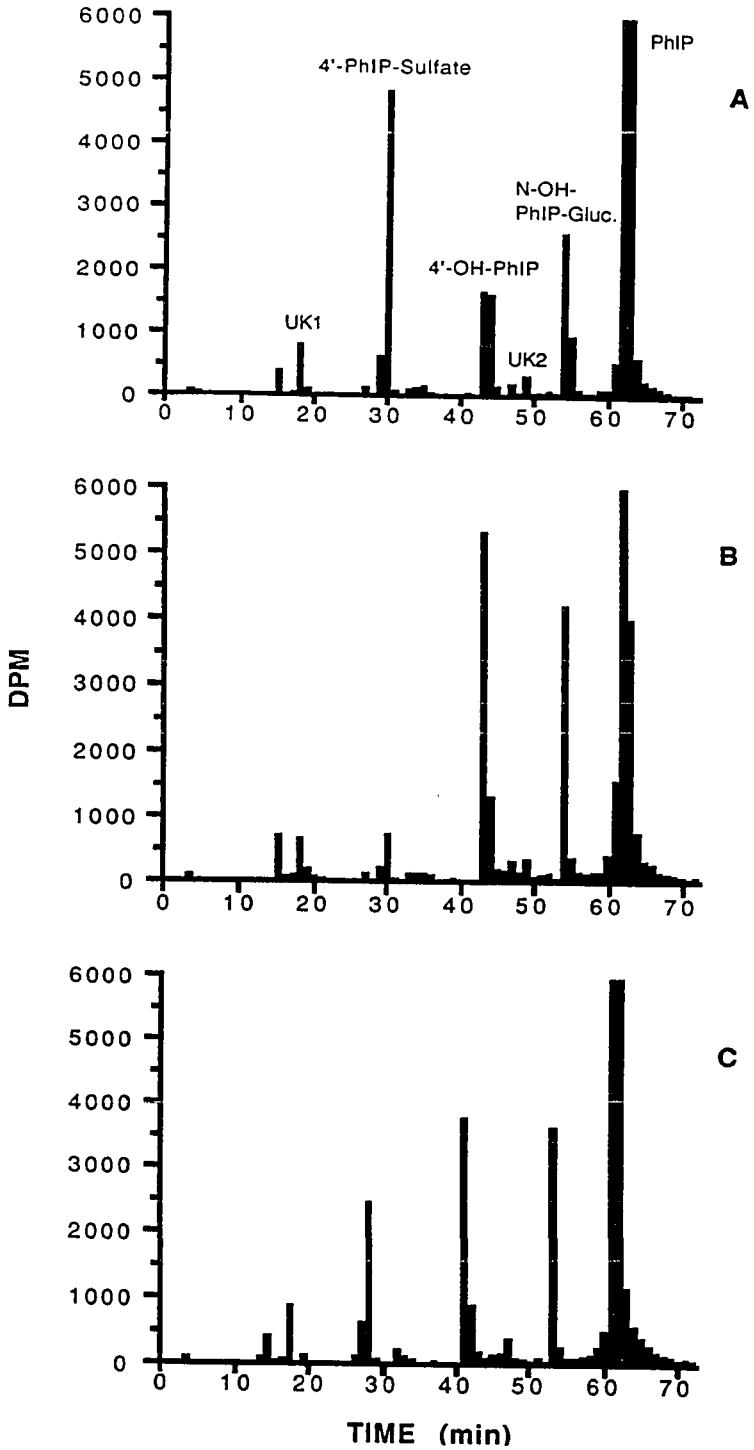

Figure 12. HPLC radioprofiles of hepatocyte preparations from Aroclor-induced rats at 100 μM [^{3}H]-PhIP pretreated with DMSO vehicle-control (A), 0.1 mM PCP (B) or 0.1 mM DCNP (C).

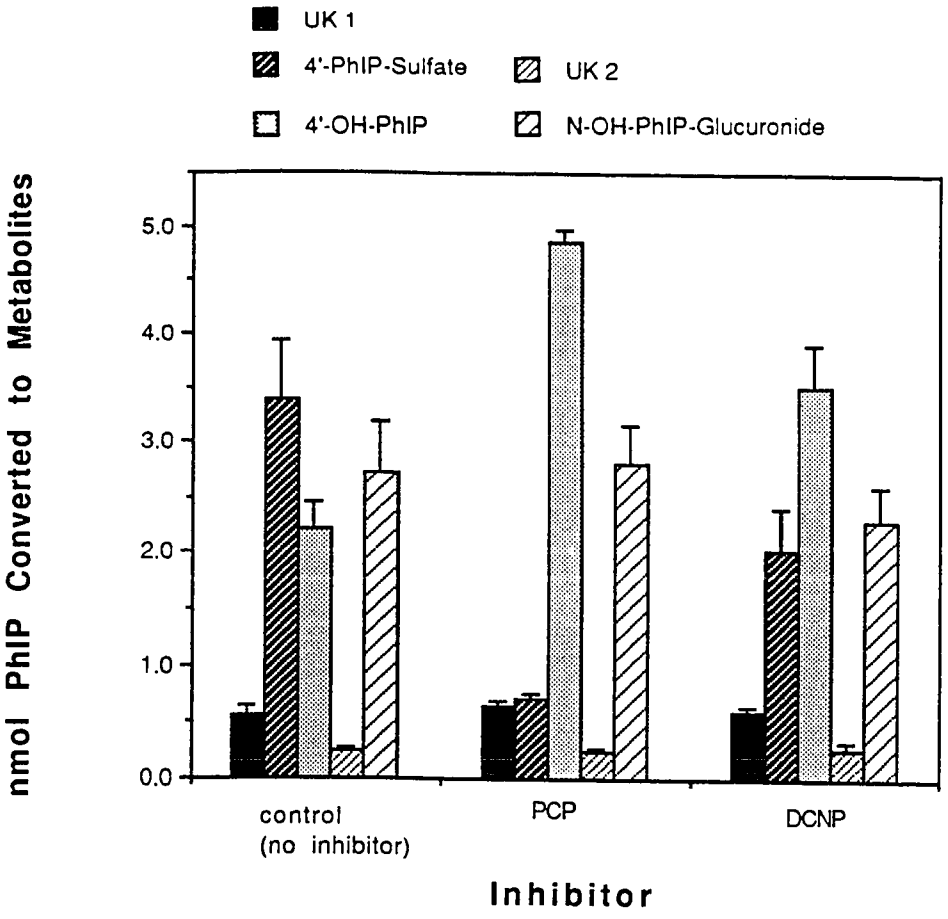

Figure 13. Effect of PCP and DCNP on the amount of [^{3}H]-PhIP (100 μM) converted to metabolites by Aroclor-induced rat hepatocyte preparations (2x10^6 cells) pretreated with 0.1 mM PCP or 0.1 mM DCNP after 2 h. Data are the means ± S.E. of three incubations.

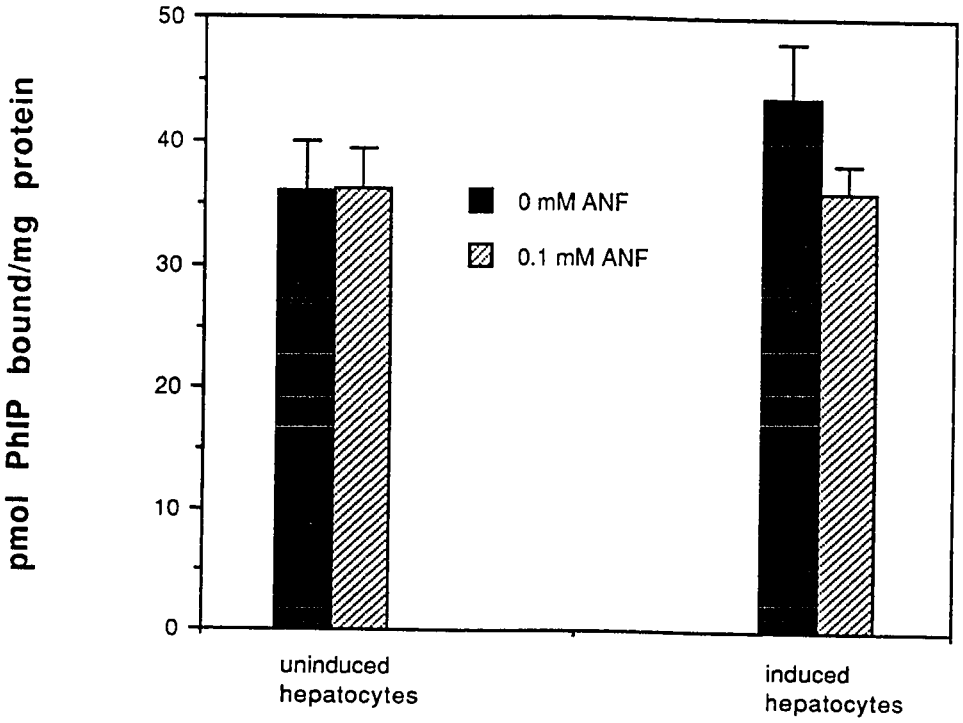

Figure 14. Effect of ANF pretreatment on covalent binding of 100 μM [^{3}H]-PhIP in Aroclor-induced and uninduced rat hepatocyte preparations. Data are the means ± S.E. of three incubations.

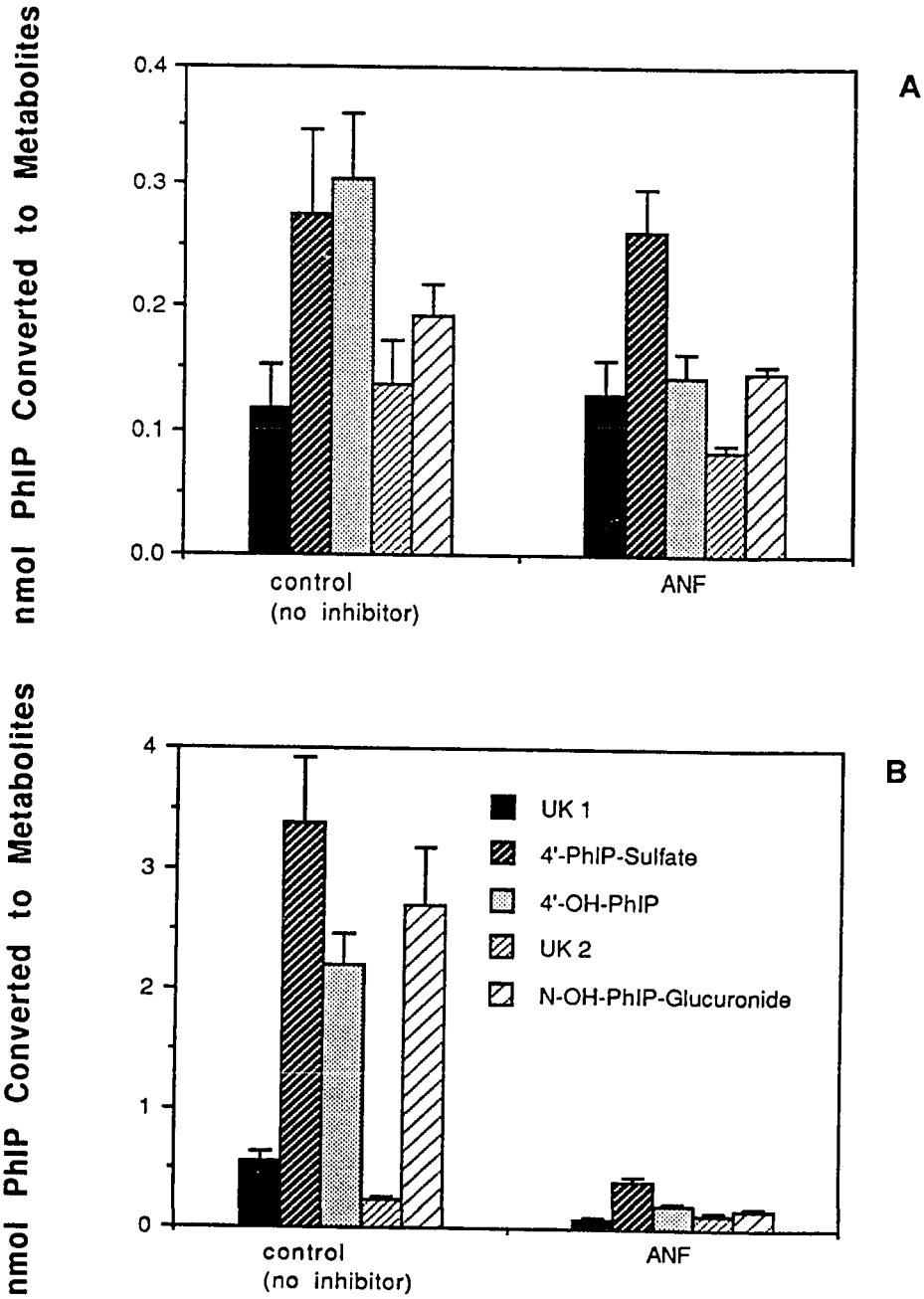

Figure 15. Effect of ANF (0.1 mM) pretreatment on the amount of [^{3}H]-PhIP (100 μM) converted to metabolites from uninduced (A) and Aroclor-induced (B) rat hepatocyte preparations ($2x10^6$ cells). Data are the means ± S.E. of three incubations.

REFERENCES

1. Casarett, L. and Doull, J. Toxicology: The Basic Science of Poisons. (Klaassen C., Amdur M., and Doull J. eds.) Macmillan Publishing Co. N.Y. N.Y., 1986, pg. 99.

2. Miller, E.C. and Miller, J. (1986) Carcinogens and mutagens that may occur in foods. *Cancer*, 58, 1795-1803.

3. Groopman, J.D., Cain, L.G., and Kensler, T.W. (1988) Aflatoxin exposure in human populations: measurements and relationship to cancer. *CRC Critical Reveiws in Toxicology*, 19, 113-145.

4. Sugimura, T. and Sata, S. (1983) Mutagens-Carcinogens in foods. *Cancer Research* (Suppl), 43, 2415s-2421s.

5. Widmark, E.M.P. (1939) Presence of cancer-producing substances in roasted food. *Nature*, 143, 984.

6. Hatch, F.T., Felton, J.S., and Knize, M.G. (1988) Mutagens formed in foods during cooking. ISI Atlas of Science: Pharmacology, 222-228.

7. Nagao, M., Honda, M., Seino, Y., Yahagi, T., and Sugimra, T. (1977) Mutagenicities of smoke condensates and the charred surface of fish and meats. *Cancer Letters*, 2, 221-226.

8. Nagao, M., Honda, M., Seino, Y., Yahagi, T., Kawachi, T., and Sugimura, T. (1977) Mutagenicities of protein pyrolysates. *Cancer Letters*, 2, 335-339.

9. Commoner, B., Vithayathil, A., Dolara, P., Nair, S., Madyastha, P., and Cuca, G. (1978) Formation of mutagens in beef and beef extract during cooking. *Science*, 201, 913-916.

10. Knize, M.G., Andresen, B.D., Healy, S.K., Shen, N.H., Lewis, P.R., Bjeldanes, L.F., Hatch, F.T., and Felton, J.S. (1985) Effects of temperature, patty thickness and fat content on the production of mutagens in fried ground beef. *Food and Chemical Toxicology*, 12, 1035-1040.

11. Ohgaki, H., Kusama, K., Matsukura, N., Morino, K., Hasegawa, H., Sato, S., Takayama, S., and Sugimura, T. (1984) Carcinogenicity in mice of a mutagenic compound 2-amino-1-methylimidazo[4,5-*f*]quinoline from broiled sardine, cooked beef and beef exrtract. *Carcinogenesis*, 5, 921-924.

12. Kato, T., Ohgaki, H., Hasegawa, H., Sato, S., Takayama, S., and Sugimura,T. (1988) Carcinogenicity in rats of a mutagenic compound 2-amino-3,8-dimethyl-imidazo[4-5-*f*]quinoxaline. *Carcinogenesis*, 9, 71-73.

13. Sugimura, T. (1985) Carcinogenicity of mutagenic heterocyclic amines formed during the cooking process. *Mutation Research*, 150, 33-41.

14. Ohgaki, H., Hasegawa, H., Kato, T., Suenaga, M., Ubukata, M., Sato, S., Takayama, S., and Sugimura, T. (1986) Carcinogenicity in mice and rats of heterocyclic amines in cooked foods. *Environmental Health Perspectives,* 67, 129-134.

15. Ohgaki, H., Hasegawa, H., Suenaga, M., Kato, T., Sato, S., Takayama, S., and Sugimura, T. (1986) Induction of hepatocellular carcinoma and highly metastatic squamous cell carcinomas in the forestomach of mice by feeding 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline. *Carcinogenesis,* 7, 1889-1893.

16. Ohgaki, H., Hasegawa, H., Suenaga, M., Sato, S., Takayama, S., and Sugimura, T. (1987) Carcinogenicity in mice of a mutagenic compound 2-amino-3,4-dimethyl-imidazo[4,5-*f*]quinoxaline (MeIQx) from cooked food. *Carcinogenesis,* 8, 665-668.

17. Felton, J.S., Knize, M.G., Shen, N.H., Andresen, B.D., Bjeldanes, L.F., and Hatch, F.T. (1986) Identification of the mutagens in cooked beef. *Environmental Health Perspectives*, 67, 17-24.

18. Felton, J.S., Knize, M.G., Shen, N.H., Lewis, P.R., Anderson, B.D., Happe, J., and Hatch, F.T. (1986) The isolation and identification of a new mutagen from fried ground beef: 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). *Carcinogenesis*, 7, 1081-1086.

19. Sugimura, T. (1982) Mutagens, carcinogens and tumor promotors in our daily food. *Cancer,* 49, 1970-1984.

20. Felton, J.S., Knize, M., Wood, C., Wuebbles, B., Healy, S. K., Stuermer, D.H., Bjeldanes, L.F., Kimble, B.J., and Hatch, F.T. (1984) Isolation and characterization of new mutagens from fried ground beef. *Carcinogenesis,* 5, 95-102.

21. Taylor, R.T., Fultz, E., Knize, M.G., and Felton, J.S. (1987) Formation of the fried ground beef mutagens 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) from L-phenylalanine (Phe) + creatinine (Cre) (or creatine). *Environmental Mutagenesis* , 9, (suppl. 8), 106.

22. Shioya, M., Wakabayashi, K., Sato, S., Nagao, M., and Sugimura, T. (1987) Formation of a mutagen, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in cooked beef, by heating a mixture containing creatinine, phenylalanine, and glucose. *Mutation Research,* 191, 133-138.

23. Felton, J.S., Knize, M.G, Shen, N.H., Wu, R., and Becher, G. Mutagenic heterocyclic imidazoamines in cooked foods. Carcinogenic and Mutagenic Responses to Aromatic Amines and Nitroarenes. (King C., Romano L., and Schuetzle D., eds.) Elsevier Science Publishing Co. Inc. N.Y. N.Y. 1988, 73-85.

24.Turteltaub, K.W., Knize, M.G., Buonarati, M.H., McManus, M.E., Veronese, M.E., Mazrimas, J.A., and Felton, J.S. (1990) Metabolism of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) by liver microsomes and isolated rabbit cytochrome P450 isozymes. *Carcinogenesis*, 11, 941-946.

25. Buonarati, M.H. and Felton, J.S. (1990) Activation of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) to mutagenic metabolites. *Carcinogenesis,* 11, 1133-1138.

26. Buonarati, M.H., Turteltaub, K.W., Shen, N.H., and Felton, J.S. (1990) Role of sulfation and acetylation in the activation of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine to intermediates which bind DNA. *Mutation Research,* 245, 185-190.

27. Yamazoe, Y., Abu-Zeid, M., Gong, D., Staiano, N., and Kato, R. (1989) Enzymatic acetylation and sulfation of N-hydroxyarylamines in bacteria and rat livers. *Carcinogenesis,* 10, 1675-1679.

28. Kato, R. (1986) Metabolic activation of mutagenic heterocyclic aromatic amines from protein pyrolysates. *CRC Critical Reveiws in Toxicology,* 16 no. 4, 307-348.

29. Nagao, M., Fujita, K., Wakabayashi, K., and Sugimura, T. (1983) Ultimate forms of mutagenic and carcinogenic heterocyclic amines produced by pyrolysis. *Biochemical and Biophysical Research Communications,* 114, 626-631.

30. Saito, K., Yamazoe, Y., Kamataki, T., and Kato, R. (1983) Mechanisms of activation of proximate mutagens in the Ames' tester strains: The acetyl-CoA dependent enzyme in *Salmonella typhimurium* TA98 deficient in the TA98/1,8-DNP6 catalyses DNA binding as the cause of mutagenicity. *Biochemical and Biophysical Research Communications,* 116, 141-147.

31. McCoy, E.C., Anders, M., and Rosenkranz, H.S. (1983) The bases of the insensitivity of *Salmonella typhimurium* strain TA98/1,8-DNP6 to the mutagenic action of nitroarenes. *Mutation Research,* 121,17-23.

32. Turteltaub, K.W., Watkins, B.E., Vanderlaan, M., and Felton, J.S. (1990) Role of metabolism on the DNA binding of MeIQx in mice and in bacteria. *Carcinogenesis,* 11, 43-49.

33. Saito, K., Shinohara, A., Kamataki, T., Kato, R. (1985) Metabolic activation of mutagenic N-hydroxylarylamines by O-acetylation in *Salmonella typhimurium* TA98. *Archives of Biochemistry and Biophysics,* 239, 286-295.

34. Snyderwine, E.G., Wirth, P.J., Roller, P.P., Adamson, R.H., Sato, S., and Thorgeirsson, S.S. (1988) Mutagenicity and *in vitro* covalent DNA binding of 2-hydroxyamino-3-methylimidazolo[4,5-*f*]quinoline. *Carcinogenesis,* 9, 411-418.

35. Mulder, G.J. and Scholtens, E. (1977) Phenol sulfotransferase and uridine diphosphate glucuronyltransferase from rat liver *in vivo* and *in vitro. Biochemical Journal,* 165, 553-559.

36. Shinohara, A., Saito, K., Yamazoe, Y., Kamataki, T., and Kato, R. (1986) Inhibition of acetyl coenzyme A dependent activation of N-hydroxy arylamines by phenolic compounds, pentachorophenol and 1-nitro-2-naphthol. *Chemico-Biological Interactions,* 60, 275-285.

37. Holme, J.A., Wallin, H., Brunborg, G., Soderlund, E.J., Hongslo, J.K., and Alexander, J. (1989) Genotoxicity of the food mutagen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP): formation of 2 hydroxamino-PhIP, a directly acting genotoxic metabolite. *Carcinogenesis*, 10, 1389-1396.

38. Esumi, H., Ohgaki, H., Kohzen ,E., Takayama, S., and Sugimura, T. (1989) Induction of lymphoma in CDF_1 mice by the food mutagen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine. *Jap. J. Cancer Res., Gann*, 80, 1176-1178.

39. Ito, N., Hasegawa, R., Sano, M., Tamano, S., Esumi, H., Takayama, S., and Sugimura, T. (1991) A new colon and mammary carcinogen in cooked food, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). *Carcinogenesis*, 12, 1503-1506.

40. Thompson, L.H., Tucker, J.D., Stewart, S.A., Christensen, M.L., Salazar, E.P., Carrano, A.V., and Felton J.S. (1987) Genotoxicity of compounds from cooked beef in repair-deficient CHO cells versus *Salmonella* mutagenicity. *Mutagenesis*, 2, 483-487.

41. Wallin, H., Mikaalsen, A., Guengerich, F.P., Ingelman-Sunberg, M., Solberg, K.E., Rossland, O.J., and Alexander, J. (1990) Differentail rates of metabolic activation and detoxification of the food mutagen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*] pyridine by different cytochrome P450 enzymes. *Carcinogenesis*, 11, 489-492.

42. Hanna, P.E. and Banks, B.R. Arylhydroxylamines and arylhydroxamic acids: Conjugation Reactions. Bioactivation of Foreign Compounds. (M.W. Anders ed.) Academic Press Inc. Orlando Florida , 1985, 375-402.

43. Thompson, L.M., Carrano, A.V., Salazar, E., Felton, J.S., and Hatch, F.T. (1983) Comparative genotoxic effects of the cooked-food-related mutagens Trp-P-2 and IQ in bacteria and cultured mammalian cells. *Mutation Research*, 117, 243-257.

44. Holme, J.A., Hongslo, J.K., Soderlund, E.J., Brunborg, G., Christensen, T., Alexander, J., and Dybing, E. (1987) Comparative genotoxic effects of IQ and MeIQ in*Salmonella typhimurium* and cultured mammalian cells. *Mutation Research*, 187, 181-190.

45. Knize, M.G., and Felton, J.S. (1986) The synthesis of the cooked-beef mutagen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine and its 3-methyl isomer. *Heterocycles*, 24, 1815-1819.

46. Ames, B.N., McCann, J., and Yamasaki, E. (1975) Method for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsomal mutagenicity test. *Mutation Research*, 31, 347-364.

47. McCoy, E.C., Rosenkranz, H.S., and Mermelstein, R. (1981) Evidence for the existance of a family of bacterial nitroreductases capable of activating nitrated polycyclics to mutagens. *Environmental Mutagenesis*, 3, 421-427.

48. Moldeus, P., Hogberg, J., and Orrenuis, S., Isolation and use of liver cells. Methods in Enzymology: Biomembranes (Fleischer, S. and Packer, L., eds.) vol. LII. Academic Press Inc. N.Y. N.Y. 1978, 66-71.

49. Seglen,P.O. Preparation of isolated rat liver cells. Methods in Cell Biology (Prescott, D.M., ed.) vol. XIII. Academic Press Inc. N.Y. N.Y. 1976, 29-83.

50. Lowry, O.H., Rosenbrough, N.J., Farr, A.L., and Randell, R.J. (1951) Protein measurement with folin phenol reagent. *Journal of Biological Chemistry*, 193, 265-275.

51. Randerath, K., Reddy, M.V., and Gupta, R.C. (1981) ^{32}P-labeling test for DNA damage. *Procedings of the National Acadamy of Science USA*, 78, 6126-6129.

52. Buonarati, M., Roper, M., Morris, C., Happe, J., Knize, M., and Felton, J. (1992) Metabolism of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in mice. *Carcinogenesis*, (in press).

53. Alexander, J., Wallin, H., Holme, J., and Becher, B. (1989) 4-(2-amino-1-methyl-imidazo[4,5-*b*]pyrid-6-yl)phenyl sulfate—a major metabolite of the food mutagen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in the rat. *Carcinogenesis*, 10, 1543-1547.

APPENDIX I

Assay Protocols

Ames/*Salmonella* Assay

Bacteria

Inoculate bacteria (*Salmonella* TA98/1,8-DNP_6) into 30 ml Oxoid nutrient broth. Incubate at 37°C with shaking for 16 hours and refrigerate at 4°C used that day.

Top Agar Tubes

Melt top agar and cool to about 60°C and add Histidine-biotin solution to a final concentration of 10% (10 ml His-bio. to 100 ml top agar). Dispense 2 ml of top agar into each tube and keep tubes in 45°C heating block.

Assay Procedure

To each agar tube add in the following order:

1. 0.1 ml bacteria (fresh overnight culture with approximately $1x10^9$ bacteria /ml)
2. Appropriate concentration of PCP or DCNP (dissolved in 25 µl methanol)
3. 1 nmol N-hydroxy-PhIP (in 10 µl methanol)

Vortex and add immediately to room temperature minimal glucose agar plates. Swirl plate, and incubate at 37°C for 48 hours.

Remove plates from incubator and score for revertant formation.

Lowry Protein Assay

Run all samples in duplicate.

Set up the following in large test tubes:

Standards

blank, 0.5 ml H_2O

25 µl of 1 mg/ml BSA + 0.475 ml H_2O

50 µl of 1 mg/ml BSA + 0.450 ml H_2O

75 µl of 1 mg/ml BSA + 0.425 ml H_2O

100 µl of 1 mg/ml BSA +0.400 ml H_2O

Samples

10 µl of protein sample + 0.490 ml H_2O

Procedure

1. Add 5 ml of Lowry reagent to each tube (100 ml of sol. A + 1 ml of sol. B + 1 ml of sol C, made fresh each time).
2. Mix contents and let sit at room temperature for 10 minutes.
3. Add 0.5 ml of Folin reagent to each tube (dilute Folin 1:1 with water just before use).
4. Vortex each sample vigorously and immediately after adding Folin reagent.
5. Let tubes sit at room temperature for 30 minutes.
6. Read absorbance at 660 nm.

Lowry reagent:

solution A- 30 grams anhydrous Na_2CO_3 + 4 grams NaOH into 1liter H_2O.

solution B- 2% $CuSO_4 \cdot 5H_2O$ (10 grams/500 ml H_2O).

solution C- 4% potassium tartrate (20 grams/500 ml H_2O).

Folin and Ciocalteu's Phenol reagent, 2 N.

APPENDIX II

Buffers and Solutions

Hank's Buffer-	8.0 g NaCl	0.06 g KH_2PO_4
	0.4 g KCl	2.19 g $NaHCO_3$
	0.12 g Na_2SO_4	0.10 g $Na_2HPO_4 \cdot 7H_2O$
	3.9 g HEPES (15 mM)	5.0 mg phenol red

Add 0.9 L of distilled H_2O, dissolve salts and adjust pH to 7.4. Bring up to 1 L with H_2O. Filter through EH type filter. Store in refrigerator at 4°C.

EGTA solution- (25 mM) 1.9 g EGTA into 200 ml H_2O.

$CaCl_2$ solution- (250 mM) 7.3 g $CaCl_2 \cdot 2H_2O$ into 200 ml H_2O.

Trypan Blue- (0.4%) 0.24 g into 60 ml saline.

Buffer A- To 200 ml Hank's buffer add 4.0 ml EGTA solution (final concentration of 0.5 mM EGTA). Bring to 37°C, oxygenate (95% O_2/5% CO_2) and adjust pH to 7.4.

Buffer B- To 200 ml Hank's Buffer add 4.2 ml $CaCl_2$ solution (final concentration of 5 mM $CaCl_2$). Bring to 37°C , oxygenate and adjust pH to 7.4. Add 110 units/ml collagenase just as a successful perfusion of the liver with buffer A is initiated.

Buffer C- To 100 ml Waymouth's MB752/1 medium* add 1 g bovine serum albumin (1%) and 0.39 g HEPES (15 mM)**. Oxygenate and adjust pH to 7.4. Place on ice.

* Sterile techinque should be used when pipetting media.

** After opening a new bottle of media, add 5 mg gentamycin/500 ml media as a precaution to inhibit bacterial growth.

Percoll Solution- (36% percoll in Hank's buffer). Add 18 ml percoll to 32 ml Hank's buffer. Prepare in capped Erlenmeyer flask. Oxygenate, shake and chill.

DNA Isolation Buffers:

TE buffer

10 mM Trizma base (Tris)

1 mM Ethylenediaminetetraacetic acid (EDTA)

Add 0.5 ml of a 2 M Tris stock solution and 0.2 ml of a 0.5 M EDTA stock solution to 99.3 ml sterile distilled H_2O for a final concentration of 10 mM Tris/1 mM EDTA.

Lysis buffer

0.1% sodium dodecylsulfate (SDS)	10 mM EDTA
10 mM Tris-HCl pH 7.9	0.2 mg/ml proteinase K

Combine 10 ml of a 100 mM Tris-HCl stock solution, 2 ml of a 0.5 M EDTA stock solution, and 1 ml of a 10% SDS solution to 87 ml sterile distilled H_2O. Add proteinase K immediately before use.

APPENDIX III

Sample Calculations

Protein Binding Quantification:

$$\frac{\text{sample DPM}}{\text{specific activity DPM/nmol}} = \text{nmol}$$

$$\frac{\text{nmol}}{\text{protein conc. (mg)}} = \frac{\text{nmol PhIP}}{\text{mg protein}}$$

$$\frac{178 \text{ DPM}}{6537 \text{DPM/nmol}} = 0.027 \text{ nmol}$$

$$\frac{0.027 \text{ nmol}}{4.19 \text{ mg}} = 0.00649 \text{ nmol}$$

$$= 0.00649 \times 1000$$

$$= 6.50 \text{ pmol PhIP/mg protein}$$

Metabolite Quantification:

$$(\text{DPM of HPLC fraction})\left(\frac{\text{sample volume}}{\text{sample volume inj. HPLC}}\right)\left(\frac{\text{total sample volume}}{\text{sample volume used}}\right) = \text{DPM}$$

$$\frac{\text{DPM}}{\text{specific activity DPM/nmol}} = \text{nmol}$$

$$(5502 \text{ DPM})\left(\frac{400 \text{ µl}}{100 \text{ µl}}\right)\left(\frac{3.0 \text{ ml}}{2.5 \text{ ml}}\right) = 26410 \text{ DPM}$$

$$\frac{26410 \text{ DPM}}{6478 \text{ DPM/nmol}} = 4.08 \text{ nmol PhIP converted to metabolite}$$