Metabolic Activation of 1-Methyl-3-amino-5*H*-pyrido[4,3-*b*]indole and Several Structurally Related Mutagens†

John M. Pezzuto,‡ Patrick D. Moore,§ and Sidney M. Hecht*

ABSTRACT: It has recently been reported that the pyrolysis of tryptophan results in the production of 1-methyl-3-amino-5*H*-pyrido[4,3-*b*]indole (**1**) and 1,3-dimethyl-3-amino-5*H*-pyrido[4,3-*b*]indole (**2**), which are potent frame-shift mutagens. The covalent binding of these compounds to deoxyribonucleic acid (DNA) in the presence of rat liver microsomes and reduced nicotinamide adenine dinucleotide phosphate is demonstrated. In addition to **1** and **2**, we have synthesized the N^3-acetyl (**3**), N^3-ethyl (**4**), N^3,N^3-diethyl (**5**), 3-hydroxy (**6**), 7-methyl (**7**), and 8-methyl (**8**) derivatives of **1** for utilization in a forward-mutation assay employing *Salmonella typhimurium* strain TM677. The mutagenic activity of the compounds was found to follow the order **1** > **2** > **4** ≥ **5** ≥ **7** > **8** > **3** > **6**. Metabolic activation was necessary to convert the compounds to active mutagens, and the required activity was found to be localized in the liver microsomal fraction of 3-methylcholanthrene-induced rats. The reduced mutagenic activity of **3** and the inactivity of **6**, carbazole, norharman, and harman suggest oxidation of the aminopyridine portion of **1** in its activation. The reduced activity of **7** and **8**, and the remaining activity of **4** and **5**, may be due to factors such as less efficient physicochemical interaction with the bacterial DNA, although additional metabolic sites of activation of compound **1** are also possible. Microsomes isolated from phenobarbital-pretreated rats were much less effective for the activation of **1** or **3**, whereas 2-aminofluorene (a compound having mutagenic potential similar to that of **3**) or 2-acetylaminofluorene (AAF) was activated equally as well with either preparation of microsomes. Additionally, benzoflavone and norharman were shown to inhibit the mutagenic response obtained with **1**, in contrast to AAF which demonstrated higher mutagenic activity in the presence of these compounds. Since AAF is known to require N-oxidation for the expression of mutagenicity, the data suggest alternate sites of metabolic activation and detoxification for **1** and AAF which can be differentiated. Structural dissimilarities may result in highly efficacious metabolic activation of **1** to mutagenic species.

It has long been known that incomplete combustion of carbon-containing materials results in the formation of mutagens and carcinogens of great structural variety (Schmeltz & Hoffman, 1976). While many of these compounds do not react covalently with DNA[1] or other macromolecules, upon ingestion by humans or other mammals they may be metabolized to active species that can so react; some of these macromolecular transformations are believed to result in tumorigenesis (Miller & Miller, 1974; Heidelberger, 1975). A characteristic property of the activated species, analogous to that of common alkylating agents, is their sucseptibility to nucleophilic attack (Miller, 1970; Miller & Miller, 1974; Heidelberger, 1975).

Recently, 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (**1**) and 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (**2**) have been identified as potent mutagens and have been reported to arise during the pyrolysis of D,L-tryptophan, an essential amino acid (Sugimura et al., 1977a). These mutagens, which are related structurally to the aminofluorenes, are also produced by cooking proteinaceous foods over a direct flame (Sugimura et al., 1977b). As such, these γ-carbolines represent a structurally interesting class of compounds which may play a significant role in carcinogenesis.

The mechanism of action of these compounds is currently under investigation. It has been shown that metabolic activation is required for the expression of mutagenic activity (Sugimura et al., 1977a,b; Pezzuto et al., 1980) and for covalent reaction with DNA (Hashimoto et al., 1979; Pezzuto et al., 1980). Admixture with certain β-carbolines, such as norharman (**12**), may affect their mutagenic response (Nagao et al., 1977, 1978; Levitt et al., 1977). By the use of a number of structural analogues of compound **1**, we have shown previously that the extents of physicochemical interaction of such compounds with DNA (in the absence of metabolic activation) were correlated with their activities in a bacterial mutagenesis assay (Pezzuto et al., 1980). Thus, association with DNA was suggested to play a role in the expression of mutagenesis mediated by compound **1**.

Compound **1** can be metabolized efficiently to mutagenic compounds by components of the microsomal fraction of rat liver derived from MC-treated animals. Presently, we focus on the nature of the metabolically activated species derived from **1** that are responsible for mutagenic activity and the relationship of the activation process to that believed to be operative for the structurally related aminofluorenes. The possibility that the mutagenic pyrido[4,3-*b*]indoles are converted to more than one type of activated species is discussed.

Experimental Procedures

Materials. Norharman, AF, carbazole, trichloropropylene oxide, and 7,8-benzoflavone were purchased from Aldrich Chemical Co. AAF and MC were obtained from Sigma

† From the Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139. *Received April 30, 1980.* This work was supported in part by a research grant from Merck Sharp & Dohme.

‡ National Institutes of Health Postdoctoral Trainee, 1977–1978. National Institutes of Health Postdoctoral Fellow, 1978–1980. Present address: Department of Pharmacognosy and Pharmacology, University of Illinois at the Medical Center, Chicago, IL 60612.

§ American Cancer Society Postdoctoral Fellow, 1977–1978.

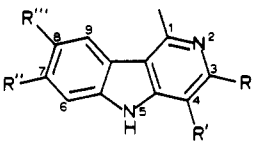
* Alfred P. Sloan Fellow, 1975–1979; National Institutes of Health Career Development Awardee, 1975–1980; John Simon Guggenheim Fellow, 1977–1978. Address correspondence to this author at Department of Chemistry, University of Virginia, Charlottesville, VA 22901.

[1] Abbreviations used: MC, 3-methylcholanthrene; PB, phenobarbital, MC or PB microsomes, microsomes isolated from rats pretreated with 3-methylcholanthrene or phenobarbital, respectively; AF, 2-aminofluorene; AAF, 2-acetamidofluorene; DPEA, (2,4-dichloro-6-phenylphenoxy)ethylamine; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; DNA, deoxyribonucleic acid; $NADP^+$, oxidized nicotinamide adenine dinucleotide phosphate; TLC, thin-layer chromatography; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

0006-2960/81/0420-0298$01.00/0

Chemical Co., as were $NADP^+$, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, 8-azaguanine, and calf thymus DNA. [^{3}H]Methyl iodide and L-[*methyl*-$^3H_3$]-methionine were from New England Nuclear Corp.; compound **1** was generally tritiated by Moravek Biochemicals. *N*-OH-AAF was obtained from the National Cancer Institute Chemical Repository with the assistance of Dr. A. R. Patel. DPEA was provided by Eli Lilly and Co. through the courtesy of Dr. Robert E. McMahon. PAPS was biosynthesized and purified by the method of Irving et al. (1971), using the enzyme preparation described by Robbins (1962). Sulfotransferase was partially purified from the 105000*g* supernatant of a rat liver homogenate by precipitation between 30 and 55% ammonium sulfate saturation (Wu & Straub, 1976). The resulting sulfotransferase and PAPS were shown to mediate the transfer of sulfate to *p*-nitrophenol (Robbins, 1963).

Synthesis of Compounds **1–8**. Compound **1** was prepared



1 R=NH_2, R'=R''=R'''=H

2 R=NH_2, R'=CH_3, R''=R'''=H

3 R=$NHCOCH_3$, R'=R''=R'''=H

4 R=NHC_2H_5, R'=R''=R'''=H

5 R=$N(C_2H_5)_2$, R'=R''=R'''=H

6 R=OH, R'=R''=R'''=H

7 R=NH_2, R'=R'''=H, R''=CH_3

8 R=NH_2, R'=R''=H, R'''=CH_3

from ethyl 2-cyanomethylindole-1-carboxylate as described (Takeda et al., 1977) by removal of the N-1 substituent to afford 2-cyanomethylindole and subsequent Lewis acid ($AlCl_3$) catalyzed cyclization in the presence of acetonitrile. Compounds **2–8** were synthesized as follows and the purity of each of the products was established by ^{1}H NMR, TLC, and where possible, melting point.

3-Amino-1,4-dimethyl-5H-pyrido[4,3-b]indole (**2**). The preparation of compound **2** has been reported (Akimoto et al., 1977) but was prepared for this study by a new procedure starting from ethyl 2-cyanomethylindole-1-carboxylate. To a flame-dried flask was added 100 μL (0.2 mmol) of 2 M *n*-butyllithium, and the flask was flushed with N_2 and immersed in a dry ice–acetone bath. Fifteen microliters (0.2 mmol) of dry diisopropylamine was added dropwise, and the combined solution was stirred for 10 min at −78 °C. Ethyl 2-cyanomethylindole-1-carboxylate (45.6 mg; 0.2 mmol) in tetrahydrofuran was added, and stirring was continued for an additional 10 min, after which 7 μL (0.2 mmol) of methyl iodide was added at −78 °C. The reaction mixture was stirred and allowed to warm to 25 °C over a period of 1 h. The reaction mixture was poured into 10 mL of water, and the combined solution was extracted with three 10-mL portions of methylene chloride. The combined organic extract was dried (Na_2SO_4) and concentrated to afford a crude product that was purified by preparative TLC (silica gel, development with CH_2Cl_2) to afford colorless crystals of ethyl 2-(1-cyano)ethylindole-1-carboxylate: yield 24 mg (50%); mp 112–114 °C; NMR [$CDCl_3$, $(CH_3)_4Si$] δ 1.52 (3 H, t, J = 7 Hz), 1.75 (3 H, d, J = 7 Hz), 4.62 (2 H, q, J = 7 Hz), 4.85 (1 H, q, J = 7 Hz), 6.88 (1 H, br s), 7.2–7.8 (3 H, m), and 8.1–8.4 (1 H, m). A radiolabeled sample of 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (7.1 Ci/mol) was prepared by the use of [^{3}H]methyl iodide in this step.

Ethyl 2-(1-cyano)ethylindole-1-carboxylate (24 mg; 0.1 mmol) was dissolved in 1.5 mL of absolute ethanol and stirred under N_2 for 1 h with 26 mg (0.1 mmol) of anhydrous potassium carbonate. The reaction mixture was filtered and concentrated to afford a crude product that was purified by preparative TLC (silica gel, development with CH_2Cl_2) to afford 2-(1-cyano)ethylindole as colorless needles: yield 13.5 mg (80%); mp 90–92 °C; NMR [$CDCl_3$, $(CH_3)_4Si$] δ 1.61 (3 H, d, J = 7 Hz), 3.90 (1 H, q, J = 7 Hz), 6.44 (1 H, br s), 7.1–7.4 (3 H, m), 7.5–7.7 (1 H, m), and 8.31 (1 H, br s).

2-(1-Cyano)ethylindole (150 mg; 0.9 mmole) was dissolved in 3 mL of acetonitrile and treated with a total of 1.5 g of aluminum chloride in portions. The resulting solution was heated at reflux for 14 h under N_2. The cooled solution was diluted cautiously with 50 mL of distilled water, and the aqueous solution was extracted with three portions of ether. The acidic aqueous layer was treated with solid sodium carbonate, and the resulting gelatinous mixture was extracted with four 50-mL portions of methylene chloride. The organic layer was filtered, dried, and concentrated under diminished pressure. Purification by preparative TLC (silica gel, development with 9:1 CH_2Cl_2–CH_3OH) afforded 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (**2**): yield 8 mg (~5%); $\lambda_{max}^{CH_3OH}$ (acid) 263 nm, $\lambda_{max}^{CH_3OH}$ (neutral) 264 nm ($\epsilon \approx 4 \times 10^4$); mass spectrum, *m/e* 211 (M^+), 194, 193, 184, 183, 169, 168, and 167 (Akimoto et al., 1977; Kosuge et al., 1978).

3-Acetamido-1-methyl-5H-pyrido[4,3-b]indole (**3**). 3-Amino-1-methyl-5*H*-pyrido[4,3-*b*]indole acetate salt (290 mg; 1.13 mmol) was treated with 3 mL of dry pyridine and 3 mL of acetic anhydride. The suspension was stirred at room temperature for 4 h and then diluted with 50 mL of ethyl acetate. The organic phase was washed with 3 volumes of saturated $NaHCO_3$ slution and 2 volumes of water. The ethyl acetate was then dried and concentrated under diminished pressure. The residue was partitioned between 1 M NaOH and 4:1 CH_2Cl–C_2H_5OH and the organic layer was washed with water, dried, and concentrated to afford 200 mg (74%) of 3-acetamido-1-methyl-5*H*-pyrido[4,3-*b*]indole (**3**) as a white solid: $\lambda_{max}^{CH_3OH}$ (10% CH_3COOH) 267 nm ($\epsilon \approx 7.3 \times 10^4$); NMR [$CDCl_3$ + CH_3OH, $(CH_3)_4Si$] δ 2.3 (3 H, s), 2.9 (3 H, s), 7.3–7.6 (3 H, m), and 8.0–8.2 (2 H, m); mass spectrum, *m/e* 239, 224, 197, 180, 127, and 43.

3-Ethylamino-1-methyl-5H-pyrido[4,3-b]indole (**4**). 3-Acetamido-1-methyl-5*H*-pyrido[4,3-*b*]indole (35 mg; 0.14 mmol) was added to 15 mL of dry tetrahydrofuran, and the reaction was initiated by the addition of excess lithium aluminum hydride. The reaction mixture was heated to 50–55 °C (under N_2) for 30 min and then at reflux for an additional 10 min. The cooled mixture was added in portions to a mixture of 35 mL of ethyl acetate and 10 mL of water. After the addition was complete, 10 mL of saturated Na_2CO_3 was added, and the organic layer was separated and retained. The aqueous phase was further extracted with two 35-mL portions of 4:1 ethyl acetate–ethanol. The combined organic extract was dried (Na_2SO_4) and concentrated under diminished pressure to give 25 mg of a solid residue. Purification by preparative TLC (silica gel, development with 4:1 CH_2Cl_2–CH_3OH) afforded 3-ethylamino-1-methyl-5*H*-pyrido[4,3-*b*]indole (**4**) as a clear glass: yield 8.8 mg (27%); $\lambda_{max}^{CH_3OH}$ (10% CH_3COOH) 270 nm ($\epsilon \approx 6.6 \times 10^4$); NMR [$CDCl_3$ + CH_3OH-d_4, $(CH_3)_4Si$] δ 1.3 (3 H, t, J = 7.0 Hz), 2.2 (3 H, s), 4.6 (4 H, q), 6.22 (1 H, s), 7.1–7.6 (3 H, m), and 7.7–8.0 (1 H, m); mass spectrum, *m/e* 225, 210, 149, 71, and 57.

3-(N,N-Diethylamino)-1-methyl-5H-pyrido[4,3-b]indole (**5**). 3-Ethylamino-1-methyl-5*H*-pyrido[4,3-*b*]indole (90 mg; 0.40 mmol) was treated with 3 mL of pyridine and 1.5 mL of acetic anhydride, and the reaction mixture was stirred for 5 h at 25 °C. The reaction mixture was further treated with several volumes of ethanol and stirred for an additional 30 min. After addition to 100 mL of ethyl acetate, the mixture was washed with two portions each of saturated sodium carbonate and water. Concentration of the dried organic phase gave 95 mg of crude product, presumably 3-(*N*-acetyl-*N*-ethylamino)-1-methyl-5*H*-pyrido[4,3-*b*]indole.

This material was dissolved directly in 40 mL of tetrahydrofuran and heated at reflux for 20 min in the presence of excess lithium aluminum hydride. The cooled reaction mixture was added in portions to a mixture of 70 mL of ethyl acetate and 20 mL of water. After the addition was complete, 10 mL of saturated sodium carbonate solution was added, and the mixture was shaken. The organic layer was washed with an additional 10-mL portion of saturated Na_2CO_3 and with two portions of water. The dried organic layer was concentrated to afford a colored solid residue which was purified by preparative TLC (silica gel, development with 85:15 methylene chloride–methanol) with a yield of 20 mg (20%) of 3-(*N*,*N*-dimethylamino)-1-methyl-5*H*-pyrido[4,3-*b*]indole (**5**): $\lambda_{max}^{CH_3OH}$ (10% CH_3COOH) 270 nm ($\epsilon \approx 7.9 \times 10^4$); NMR [$CDCl_3$ + CH_3OH-d_4, $(CH_3)_4Si$] δ 1.4 (6 H, m), 2.85 (3 H, s), 4.5–4.8 (4 H, m), 6.25 (1 H, s) 7.2–7.6 (3 H, m), and 7.8–7.95 (1 H, m).

3-Hydroxy-1-methyl-5H-pyrido[4,3-b]indole (**6**). 3-Amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (50 mg of the acetate salt; 0.19 mmol) was dissolved in 4 mL of 6 M sulfuric acid. The solution was cooled to 0 °C in an ice–salt bath and treated over a period of 10 min with 15.2 mg (0.22 mmol) of sodium nitrite dissolved in 0.5 mL of water. After being stirred at low temperature for an additional 1 h, the dark red reaction mixture was added dropwise to 150 mL of boiling 2 M sulfuric acid over a period of 20 min. The resulting yellow solution was neutralized to pH 6.5–7.0 with solid sodium hydroxide, and the neutral solution was extracted with three portions of 9:1 ethyl acetate–ethanol. The combined organic layer was dried (Na_2SO_4) and concentrated under diminished pressure to afford a crude product that was triturated with CH_2Cl_2–CH_3OH and further purified by preparative TLC (silica gel, development with 9:1 CH_2Cl_2–CH_3OH) to afford 3-hydroxy-1-methyl-5*H*-pyrido[4,3-*b*]indole (**6**) as a white solid: yield 28 mg (73%); $\lambda_{max}^{CH_3OH}$ (acid) 262 nm ($\epsilon \approx 3.1 \times 10^4$) and 282 nm ($\epsilon \approx 1.1 \times 10^4$); NMR [$Me_2SO$-$d_6$, $(CH_3)_4Si$] δ 2.7 (3 H, s), 5.85 (1 H, br s), 7.0–7.4 (3 H, m), and 7.6–7.9 (1 H, m); mass spectrum, *m/e* 198 (M^+), 170, 169, 154, 149, 127, and 115.

The syntheses of 3-amino-1,7-dimethyl-5*H*-pyrido[4,3-*b*]-indole (**7**) and 3-amino-1,8-dimethyl-5*H*-pyrido[4,3-*b*]indole (**8**) were carried out in analogy with the synthesis of compound **1** (Takeda et al., 1977), starting from ethyl 2-cyanomethyl-6-methylindole-1-carboxylate and ethyl 2-cyanomethyl-5-methylindole-1-carboxylate (Ikeda et al., 1976), respectively.

3-Amino-1,7-dimethyl-5H-pyrido[4,3-b]indole (**7**). Ethyl 2-cyanomethyl-6-methylindole-1-carboxylate (230 mg; 0.95 mmol) and potassium carbonate (230 mg; 1.66 mmol) were added to 14 mL of absolute ethanol, and the reaction mixture was stirred at 25 °C under N_2 for 2 h. The reaction mixture was filtered, and the filtrate was concentrated to dryness under diminished pressure and purified by chromatography on silica gel by elution with methylene chloride. The solid product was recrystallized from chloroform–petroleum ether, affording 2-cyanomethyl-6-methylindole: yield 132 mg (82%); mp 124–5 °C; NMR [$CDCl_3$ + $(CH_3)_2CO$-d_6, $(CH_3)_4Si$] δ 2.42 (3 H, s), 3.87 (2 H, s), 6.39 (1 H, br s), and 6.9–7.5 (4 H, m).

2-Cyanomethyl-6-methylindole (105 mg; 0.62 mmol) was dissolved in 2 mL of acetonitrile and treated with 1.0 g of aluminum chloride in portions. The resulting solution was cooled, diluted with 75 mL of ethyl ether, and treated carefully with 20 mL of water. The aqueous phase was extracted with two additional portions of ether and was then made basic by addition of sodium carbonate. The aqueous phase was then extracted with several equal volumes of methylene chloride and the combined organic phase was dried and concentrated to dryness under diminished pressure. The residue was purified by preparative TLC (silica gel, development with 7:3 CH_2Cl_2–CH_3OH) to give 22 mg (17%) of 3-amino-1,7-dimethyl-5*H*-pyrido[4,3-*b*]indole as a white solid having mobility on TLC and fluorescence similar to compound **1**: $\lambda_{max}^{CH_3OH}$ (10% CH_3COOH) 270 nm ($\epsilon \approx 9.8 \times 10^4$); NMR [$CDCl_3$ + CH_3OH-d_4, $(CH_3)_4Si$] δ 2.5 (3 H, s), 2.81 (3 H, s), 6.38 (1 H, s), 7.00 (1 H, d, J = 4.0 Hz), 7.16 (1 H, br s), and 7.80 (1 H, d, J = 4.0 Hz); mass spectrum, *m/e* 212 (M^+ + H), 211 (M^+), 184, 176, and 149.

3-Amino-1,8-dimethyl-5H-pyrido[4,3-b]indole (**8**). The conversion of 2-cyanomethyl-5-methylindole-1-carboxylate (700 mg; 2.9 mmol) to 2-cyanomethyl-5-methylindole was carried out with potassium carbonate in ethanol as described above for 2-cyanomethyl-6-methylindole-1-carboxylate. The product was obtained as yellow crystals from methylene chloride–petroleum ether following chromatography on silica gel: yield 189 mg (39%); mp 138–9 °C; NMR [$CDCl_3$ + CH_3OH-d_4, $(CH_3)_4Si$] δ 2.42 (3 H, s), 3.8 (2 H, s), 6.4 (1 H, br s), and 6.9–7.4 (3 H, m); mass spectrum, *m/e* 170 (M^+) 155, 143, 130, and 115.

2-Cyanomethyl-5-methylindole (140 mg; 0.82 mmol) was dissolved in acetonitrile and treated with $AlCl_3$ as described above for the isomeric 2-cyanomethyl-6-methylindole. Purification of the product by preparative TLC gave 21 mg (12%) of 3-amino-1,8-dimethyl-5*H*-pyrido[4,3-*b*]indole (**8**) having mobility on TLC and fluorescence similar to compound **1**: $\lambda_{max}^{CH_3OH}$ 265 nm ($\epsilon \approx 6.0 \times 10^4$) and 270 nm (sh); NMR [$CDCl_3$ + CH_3OH-d_4, $(CH_3)_4Si$] δ 2.45 (3 H, s), 2.58 (3 H, s), 6.34 (1 H, s), 7.12 (2 H, m), and 7.60 (1 H, m); mass spectrum, *m/e* 212 (M^+ + H), 211 (M^+), 184, 176, and 149.

Treatment of Animals and Isolation of Subcellular Fractions. Male Fischer rats (~125 g, Charles Breeding Farms) were given a daily intraperitoneal injection of MC (25 mg/kg, in corn oil) or PB (75 mg/kg, in H_2O) for four consecutive days. The following day the rats were sacrificed by decapitation, and the livers were excised immediately. All subsequent procedures were performed at 0–4 °C. The livers of each group of animals were pooled, rinsed with 0.14 M NaCl, and homogenized in 3 volumes (w/v) of 0.05 M Tris-HCl buffer, pH 7.5, containing 0.15 M KCl. The homogenate was filtered through cheesecloth and centrifuged at 800*g* for 10 min. The pellet was discarded and the supernatant was centrifuged at 9000*g* for 20 min. Several small aliquots of the 9000*g* supernatant were stored in liquid nitrogen, and the remainder was centrifuged at 105000*g* for 1 h to yield the cytoplasmic and microsomal fractions. The pellets were resuspended in 0.05 M Tris-HCl buffer, pH 7.5, containing 0.15 M KCl and centrifuged for an additional 1 h at 105000*g*. The cytosol was also recentrifuged. The microsomes were finally suspended in 0.25 M sucrose. Several small aliquots of each preparation were stored in liquid nitrogen. Protein was determined by the method of Lowry et al. (1951) and the cytochrome P-450

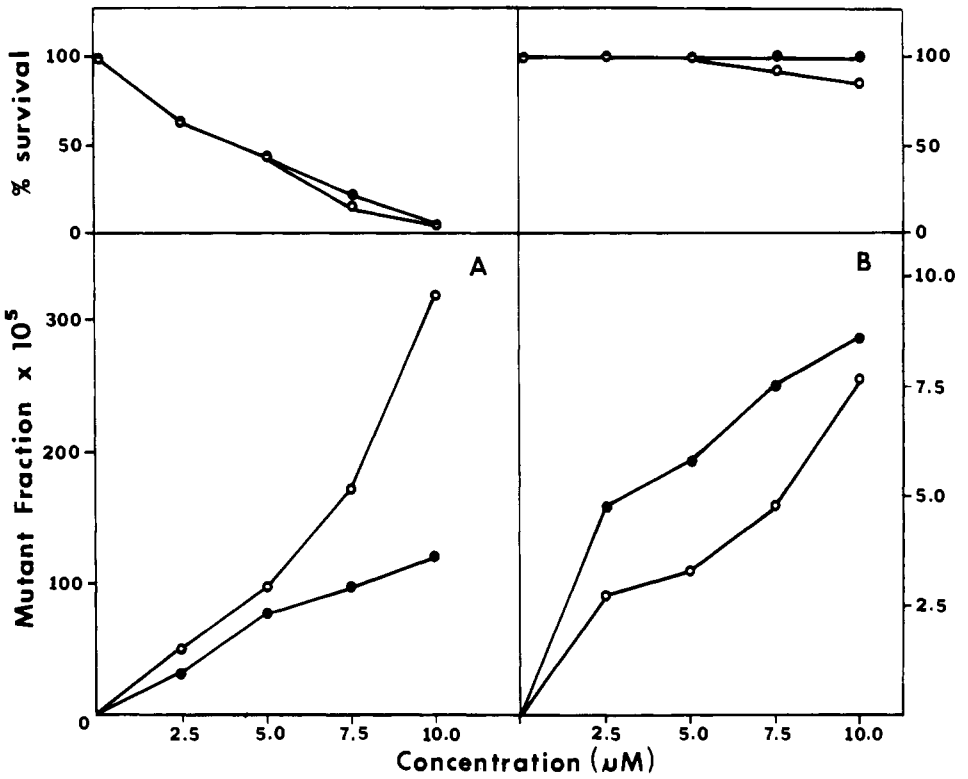


FIGURE 1: Concentration-dependent mutagenesis and cytotoxicity of **1** (○) and **2** (●) with 0.5 mg of MC microsomes (panel A) or 0.5 mg of PB microsomes (panel B) for metabolic activation. The results were corrected for the spontaneous rate of mutation obtained in the absence of **1** or **2**. No mutagenesis (mutant fraction $< 5 \times 10^{-5}$) was obtained in the absence of added microsomes.

content was measured by the method of Omura & Sato (1964).

Bacterial Mutation Assay. The assay utilized was that developed by Thilly and his co-workers, which quantitates the forward mutation of *Salmonella typhimurium* (strain TM 677, carrying the "R-factor" plasmid pkM 101) resulting in 8-azaguanine resistance (Skopek et al., 1978a,b). Duplicate incubation mixtures containing $\sim 1.5 \times 10^7$ bacteria, 1.0 mg of glucose 6-phosphate, 0.4 unit of glucose-6-phosphate dehydrogenase, 1.0 mg of $NADP^+$, 0.67 mg of $MgCl_2$, and the metabolic activating system (filtered through 0.3 μm nitrocellulose filters) were prepared in minimal Eagle's medium. The compounds to be tested were added in 10 μL of dimethyl sulfoxide (final volume, 1.0 mL), and the mixture was incubated at 37 °C for 1.5 h. Following the addition of 4.0 mL of phosphate-buffered saline and centrifugation, the bacteria were resuspended in this buffer, diluted into top agar in the presence or absence of 8-azaguanine, and plated in triplicate. After incubation at 37 °C for 36–40 h, the plates were scored and the averaged data were expressed as the mutant fraction, i.e., the number of 8-azaguanine-resistant colonies divided by the number of 8-azaguanine-sensitive colonies. The average mutant fraction obtained with no added mutagen was $(3.8 \pm 0.79) \times 10^{-5}$ ($N = 23$).

Covalent Binding of Compounds **1** *and* **2** *to DNA.* The reaction mixture contained 45 mM Tris-HCl buffer, pH 7.5, 3 mM $MgCl_2$, 5 mM glucose 6-phosphate, 0.8 mM $NADP^+$, 0.8 unit of glucose-6-phosphate dehydrogenase, 40 A_{260} units of calf thymus DNA, and MC microsomes corresponding to 2 mg of protein. After a 5-min incubation period at 37 °C, the reaction was initiated by the addition of ^{3}H-labeled **1** or ^{3}H-labeled **2** in a small volume of methanol (final reaction volume, 2.0 mL), and incubation was continued for 60 min. The mixture was then centrifuged at 4 °C to sediment the microsomes, and 0.16 mL of 4.0 M NaCl and 0.4 mL of 5% aqueous sodium dodecyl sulfate were added. Following extraction with an equal volume of 50:50:1 phenol–chloroform–isoamyl alcohol, the DNA was recovered from the aqueous phase by precipitation with 3 volumes of cold ethanol. The DNA was then rinsed with organic solvents and dissolved in 3 mL of 0.05 M Tris-HCl, pH 7.5, for the determination of radioactivity and absorption at 260 nm (Essigmann et al., 1977). Reprecipitation of the DNA from aqueous solution with ethanol did not alter the measured specific activity.

Adduct Formation with Methionine. The procedure employed was similar to that of DeBaun et al. (1970). A reaction mixture containing 0.03 M Tris-HCl buffer, pH 7.0, 9 mM $MgCl_2$, 15 mM [^{3}H]methionine (sp act. 67 mCi/mol), 0.8 mM $NADP^+$, 5 mM glucose 6-phosphate, 1.0 unit of glucose-6-phosphate dehydrogenase, 0.3 mM PAPS, and the 9000*g* supernatant isolated from the liver of MC-treated rats (2 mg of protein) was incubated at 37 °C for 5 min. Alternatively, PB microsomes and partially purified sulfotransferase (each 2 mg of protein) were used in the assay. The reaction was initiated by the addition of 200 nmol of the test compound in 20 mL of dimethyl sulfoxide (final volume, 1.0 mL), and the incubation was continued for 2 h. Following a 45-min incubation at 90 °C, the incubation mixture was cooled and treated with 0.2 mL each of ethanol and 11 M KOH. After standing at 25 °C for 15 min, the reaction mixture was extracted with 4 mL of 30% benzene in hexane, and the organic phase was used for scintillation counting.

Results

Compounds **1** and **2** were found to be potent mutagens requiring metabolic activation. As shown in Figure 1, the enzymatic activity needed for this activation was localized in the microsomal fraction of rat liver; pretreatment of the rats with MC, an inducer of aryl hydrocarbon hydroxylase [Conney, 1967; Kuntzmann, 1969; Lu & Levin (1974) and references cited therein], greatly enhanced the mutagenic response. Interestingly, utilization of microsomes isolated following

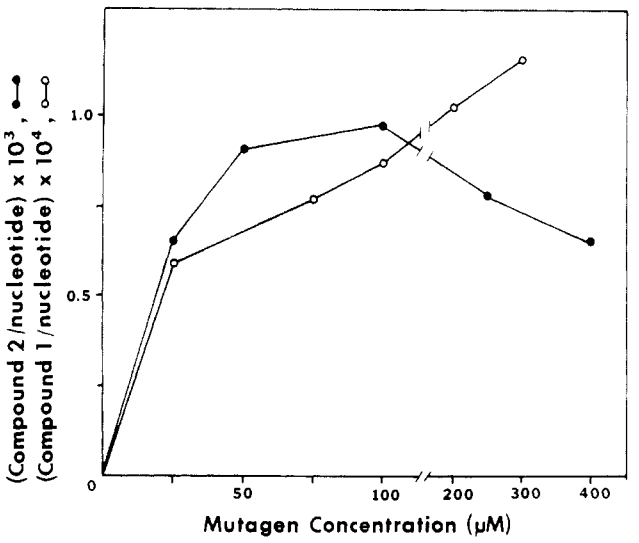


FIGURE 2: Concentration-dependent covalent binding of metabolically activated 4-methyl-$^3H_3$-labeled **2** (●, 7.1 Ci/mol) or generally labeled ^3H-labeled **1** (○, 39 Ci/mol) to calf thymus DNA.

treatment with PB, a more general inducer of microsomal enzymes [Conney, 1967; Kuntzmann, 1969; Lu & Levin (1974) and references cited therein], gave much less mutagenesis. Consistent with previous reports (Sugimura et al., 1977a; Pezzuto et al., 1980), mutagenesis mediated by compound **1** was greater than that obtained with compound **2**, although the degree of bacterial toxicity was similar for both of the compounds.

The covalent binding of **1** and **2** to calf thymus DNA in the presence of MC microsomes and a NADPH-generating system is illustrated in Figure 2. Compound **2** bound to DNA to the extent of ~1 molecule per 1000 nucleotides, whereas compound **1** was 8-fold less efficient.[2] The extent of covalent binding of the two compounds after microsomal activation was thus in the same order as that found for physicochemical interaction of the unactivated species with calf thymus DNA, although not in the same order as the results of the mutagenesis assays (Pezzuto et al., 1980). The concentration profiles for covalent DNA binding of **1** and **2** also differed significantly, an observation for which several explanations (e.g., microsomal inactivation by high concentrations of **2**) are possible.

In an effort to charcterize the mode(s) of activation of the 3-amino-5*H*-pyrido[4,3-*b*]indoles, a series of structural analogues of **1** was prepared chemically and tested for bacterial mutagenicity. As summarized in Table I, introduction of a methyl substituent at the 4, 7, or 8 positions of **1** or ethylation of the exocyclic amino group reduced the mutagenic response. Acetylation or oxidative deamination of the exocyclic amino group virtually abolished mutagenic activity at low concentrations of the test compounds. The general order of potency was **1** > **2** > **4** ≥ **5** ≥ **7** > **8** > **3** > **6**. As indicated in the table, formal methylation of the 8 position substantially reduced mutagenicity, whereas methylation of the 7 position or mono- or di-N-ethylation had much smaller effects.

While analogues **3** and **6** were virtually without mutagenic activity when tested at concentrations up to 10 μM, the former was much more mutagenic when tested at higher concentrations, a property also noted for 2-aminofluorene (Tables I and II). As found for the parent compound in the 3-amino-5*H*-pyrido[4,3-*b*]indole series (**1**), only MC microsomes elicited a mutagenic response from **3** even at the higher concentrations of test compound. Also compared in Table II are the mutagenic activities of AF (**9**) and AAF (**10**). Unlike **3** (and the other 3-amino-5*H*-pyrido[4,3-*b*]indoles so tested), **9** and **10** were activated by both MC and PB microsomes, with a slight preference for the latter at lower concentrations of the test compounds. Upon activation by MC microsomes, the activities of **3** and **9** as mutagens were similar; as in the 3-amino-5*H*-pyrido[4,3-*b*]indole series, acetylation of the exocyclic amino group (corresponding to the formal change **9** → **10**) diminished mutagenic potential.

For further characterization of the nature of the activation process, the ability of various enzymatic systems to activate compounds **1**, **3**, **10**, and **11** was also measured. As shown in Table III, the partially activated species *N*-hydroxy-AAF (**11**) was not mutagenic at concentrations up to 100 μM in the absence of any activating system. MC microsomes were totally incapable of activating **11**, whereas PB microsomes converted the test compound to an active mutagen. Interestingly, the cytoplasmic fraction (from PB-induced rats) elicited a mutagenic response from **11** greater than that obtained with either microsome preparation, although the cytosol was unable to

Table I: Concentration-Dependent Mutagenicity of Compound 1 and Several Structural Analogues[a]

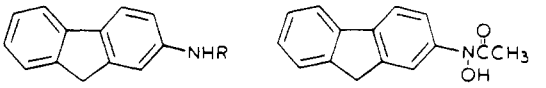
compd	concn (μM) 2.5	5.0	7.5	10.0
1	56.9	102.7	176.9	323.9
2	34.2	78.6	98.0	121.4
3	4.4	5.4	6.2	8.1
4	34.7	69.0	88.8	100.0
5	16.8	62.5	83.3	
6[b]	3.5	4.7	2.9	3.8
7	14.2	46.5	86.2	108.0
8	9.4	21.5	27.8	38.6
9	3.6	4.3	4.1	3.7

[a] MC microsomes corresponding to 0.5 mg of protein were used as the activating system. The values correspond to mutant fraction × 10^5. [b] A mutant fraction of 7.2 × 10^{-5} was obtained when this compound was tested at 100 μM concentration.

Table II: Mutagenic Activation of Compounds 3, AF (9), and AAF (10)[a]

compd	concn (μM)	mutant fraction × 10^5 MC microsomes	PB microsomes
none		3.6	7.6
3	50	35.3	6.2
	100	52.9	7.1
	150	102	5.7
9	50	35.8	64.9
	100	74.2	88.9
	150	92.2	72.1
10	50	7.5	30.3
	100	18.0	31.4
	150	20.3	31.0

[a] Each incubation contained microsomes corresponding to 0.5 mg of protein.



9 R = H

10 R = $\overset{O}{\overset{\|}{C}}CH_3$

11

[2] In comparison, aflatoxin B_1 has been reported (Essigmann et al., 1977) to bind to calf thymus DNA to the extent of ~1 molecule per 25 nucleotides, while benzo[*a*]pyrene bound to calf thymus DNA to the extent of about one per 50 000 nucleotides (Gelboin, 1969).

Table III: Mutation Resulting from Activation by Various Enzymatic Systems

compd	concn (μM)	activating system	mutant fraction × 10^5
11	50	none	3.3
11	100	none	6.4
11	5.0	MC microsomes[a]	5.2
11	2.5	PB microsomes[a]	50.6
11	5.0	PB microsomes[a]	98.6
11	2.5	cytosol[b]	138.6
11	5.0	cytosol[b]	216.7
10	100	cytosol[b]	7.0
3	100	cytosol[b]	4.5
1	50	cytosol[b]	10.1

[a] 0.5 mg of protein/incubation. [b] 2.0 mg of protein/incubation.

Table IV: Effect of DPEA on the Mutagenicity of N-Ethylated Derivatives 4 and 5[a]

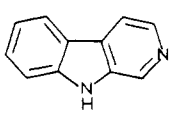
compd	DPEA (μM)	mutant fraction × 10^5
none	0	4.0
	100	6.7
4	0	97.7
	100	93.2
5	0	66.7
	100	83.3

[a] Each incubation contained MC microsomes corresponding to 0.5 mg of protein and, where indicated, 5 μM 4 or 5.

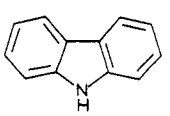
activate compounds **1**, **3**, or **10**.

The greatly reduced mutagenic potentials of compounds **3** and **6** (relative to **1**) suggested that a similar diminution in activity might be anticipated upon N-alkylation of **1**. As noted above, however, the *N*-ethyl (**4**) and *N*,*N*-diethyl (**5**) derivatives were found to be strongly mutagenic upon microsomal activation (Table I). Since a possible molecular basis for this observation could involve N-dealkylation, a well-known microsomal function [Kuntzmann, 1969; Lu & Levin (1974) and references cited therein] that would convert these derivatives to the parent mutagen, the mutagenicities of **4** and **5** were also measured in the presence of DPEA, a known inhibitor of certain O- and N-dealkylations (McMahon et al., 1969; Parli et al., 1973). As shown in Table IV, 100 μM DPEA failed to diminish the activity of compounds **4** and **5** in the bacterial mutagenesis assay. On the assumption that N-deethylation of **4** and **5** could not have occurred in the presence of DPEA,[3] the logical conclusion to be derived from this experiment is that activation of **4** and **5** occurs at some site other than the exocyclic amino group or that alkylation at the amino group is insensitive to the presence of the *N*-alkyl substituents.

Illustrated in Table V are the effects of two additional compounds on the mutagenicity of **1**. Benzoflavone, an inhibitor of aryl hydrocarbon hydroxylase (Wiebel et al., 1971), diminished the apparent mutagenicity of compound **1** in a concentration-dependent fashion. Interestingly, the mutagenicity of **10** was increased in the presence of benzoflavone (data not shown). The same pattern was also obtained upon addition of norharman (**12**) to the incubations; the mutagen-



12



13

[3] It should be noted that while DPEA is generally considered to be an inhibitor of dealkylations, the documented examples actually involve inhibition of demethylation.

Table V: Effect of Benzoflavone and Norharman on the Apparent Mutagenicity of 1[a]

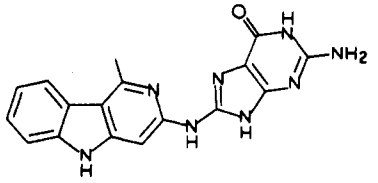
inhibitor	concn	mutant fraction × 10^5
none		102.7
benzoflavone	50 μM	58.8
	100 μM	34.9
norharman	50 μg	83.9
	100 μg	52.8
	150 μg	39.3

[a] Each incubation contained MC microsomes corresponding to 0.5 mg of protein and 5 μM compound 1.

icity of **1** was thereby decreased, as shown in the table, while the mutant fraction of **10** was significantly enhanced (from 34.4 × 10^{-5} to 159.1 × 10^{-5} in the presence of 100 μg of norharman).

Discussion

The likelihood that DNA serves as a critical target molecule for molecular species capable of inducing mutagenesis and carcinogenesis has prompted the study of the structures of the adducts that such species form with DNA [see, e.g., Irving (1973)]. While experimental evidence has sometimes permitted formation of a single type of adduct to be suggested as the relevant event leading to mutagenesis or carcinogenesis [e.g., for dimethylnitrosamine (Nicoll et al., 1975) and benzo[*a*]pyrene (Sims et al., 1974; Daudel et al., 1975)], more typically, the relationship between the structures of individual DNA adducts and their biological relevance is not well understood. In the present case, for example, Hashimoto et al. (1979) have isolated three fluorescent adducts formed by incubation of calf thymus DNA with **1** in the presence of a microsomal activating system and have identified guanine adduct **14** as one of those formed. However, at present no



14

evidence has been presented to suggest that formation of **14**, or either of the two other DNA adducts, represents (part of) the mechanism by which compound **1** exerts its mutagenicity.

In this report, we investigate a more general approach for studying the structural features of the mutagenic derivative(s) of compound **1** formed by metabolic activation. This approach involved a comparison of the mutagenicity of **1** with those of several structurally related compounds, in the presence of two types of microsomal preparations and certain characterized inhibitors of microsomal activation. The pyrido[4,3-*b*]indoles bear an obvious structural relationship to the aminofluorenes (e.g., **9** and **10**) and a more subtle one to benzo[*a*]pyrene and related species; therefore, the possible activation of **1** in analogy with these carcinogens was considered in detail.

The known P-450-mediated oxidations of polycyclic aromatic hydrocarbons are thought to involve initial formation of arene oxides. Although single epoxidations can yield species active as mutagens [e.g., aflatoxin 2,3-oxide (Essigmann et al., 1977)], diol–epoxide formation has also been demonstrated convincingly (Sims et al., 1974; Jennette et al., 1977; Moore et al., 1977; Yang et al., 1977). Since alkyl substituents have been shown to suppress arene oxide formation at their point

of attachment to aromatic rings (Jerina & Daly, 1974; Harvey & Dunne, 1978), compounds **7** and **8** were prepared to test the possible activation of mutagenic pyrido[4,3-*b*]indoles on the benzenoid ring. As shown in Table I, the bacterial mutagenicities of **7** and **8** were found to be considerably less than that of **1**, with **7** having the greater activity of the two. On the assumption that the presence of the 7- and 8-methyl substituents does not simply have a more general effect on metabolism, or on cell wall transport of **7** and **8** or physicochemical interaction of these species with DNA [cf. Pezzuto et al. (1980)], it may be concluded that the diminished activity of these compounds in comparison with **1** reflects a lesser conversion to an activated species such as an arene oxide.[4]

Comparison of the activation of **1** and its analogues with that of three aminofluorene derivatives was also instructive. As shown in Table I, when tested in the presence of MC microsomes 2-aminofluorene (**9**) was much less active as a bacterial mutagen than compound **1**. More nearly comparable in activity with **9** (and also with its *N*-acetyl derivative **10**) was the N-acetylated analogue of compound **1**, 3-acetamido-1-methyl-5*H*-pyrido[4,3-*b*]indole (**3**). It should be noted, however, that unlike compounds **9** and **10** the pyrido[4,3-*b*]indoles tested could be activated only by MC microsomes (Table II), indicating that at least the activating enzymes (if not the activation processes or activated species themselves) must differ for the two structural series of compounds.[5] Consistent with this putative difference in activation were the observations that *N*-hydroxy-AAF [**11**, which may be converted to an ultimate carcinogen by transformation to its respective sulfate ester (Miller, 1970; Miller & Miller, 1974; Heidelberger, 1975)] could be activated efficiently by a cytoplasmic fraction from PB- or MC-induced rats (Table III) and that the same cytoplasmic fraction enhanced the mutagenicity of **10** but not of **1** when these compounds were incubated in the presence of MC microsomes.[6] Further, while *N*-hydroxy-AAF was found to react with [^{3}H]methionine in the presence of microsomal preparations and added PAPS, little reaction was observed between methionine and compound **1** under the same conditions (data not shown).

While the mutagenic pyrido[4,3-*b*]indoles may not be activated in the same fashion as the structurally related 2-aminofluorenes, it seems clear that the exocyclic group participates in the expression of mutagenicity of compound **1** and its analogues since the deaminated derivative **6** is nonmutagenic and the respective N-acetylated species (**3**) was found to have greatly diminished mutagenic potential.[7] It might also have been anticipated that N-alkylated derivatives of **1** would also be much less active as mutagens than the parent compound. Interestingly, this was found not to be true; the N^3-ethyl and N^3,N^3-diethyl derivatives (**4** and **5**, respectively) demonstrated substantial mutagenic activity. Conceivably, the activity observed for **4** and **5** may simply reflect their initial conversion to **1** via dealkylation [as has been established for *N*,*N*-dimethylaminoazobenzene (Heidelberger, 1975)], although it has not been possible to verify this scheme by the use of DPEA (Table IV).

Although it seems likely that N-oxidation represents a major pathway for the activation of **1**, the results obtained with compounds **4** and **5** and especially those for **7** and **8** suggest the possibility that the pyrido[4,3-*b*]indoles may be metabolized in more than one fashion and that attempts to define individual pathways by the elaboration of analogues ostensibly blocked in single pathways simply results in greater utilization of an alternate activation scheme. Consistent with this possibility was the observation that benzoflavone, a known inhibitor of aryl hydrocarbon hydroxylase (Wiebel et al., 1971), diminished the apparent mutagenicity of compound **1**. As might have been expected, the mutagenicity of AAF (**10**), whose activation has only been shown to involve N-oxidation, increased in potency as a mutagen upon admixture of benzoflavone, presumably through the suppression of metabolic pathways leading to nonmutagenic products.

It has been shown previously that for several structurally related pyrido[4,3-*b*]indoles there is a correlation between the bacterial mutagenicities of the individual species and the extent of binding of the unactivated spcies to DNA. One exception to this correlation was compound **2**, whose noncovalent and covalent binding to DNA was somewhat greater than that of **1** but which was found to be somewhat less mutagenic than **1**. The reason(s) for this is not entirely clear, but the observation could conceivably be due to the formation of a (minor) DNA adduct by **1** that has a disproportionately large effect in the expression of mutagenicity [see, e.g., Kriek (1974) and Westra et al. (1976)].

Acknowledgments

We are grateful to Professor William G. Thilly, in whose laboratory bacterial mutation assays were performed, and Professor Gerald N. Wogan, in whose laboratory DNA binding experiments were performed. We also thank Dr. Robert E. McMahon, Eli Lilly and Company, for supplying DPEA and Dr. A. R. Patel, National Cancer Institute Chemical Repository, for *N*-OH-AAF. Certain of the synthetic procedures described herein were optimized by Dr. Yushi Luh.

References

Akimoto, H., Kawai, A., Nomura, H., Nagao, M., Kawachi, T., & Sugimura, T. (1977) *Chem. Lett.*, 1061.
Conney, A. H. (1967) *Pharmacol. Rev. 19*, 317.
Daudel, P., Duquesne, M., Vigney, P., Grover, P. L., & Sims, P. (1975) *FEBS Lett. 57*, 250.
DeBaun, J. R., Miller, E. C., & Miller, J. A. (1970) *Cancer Res. 30*, 577.
Essigmann, J. M., Croy, R. G., Nadzan, A. M., Busby, W. F., Reinhold, V. N., Büchi, G., & Wogan, G. N. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1870.
Gelboin, H. V. (1969) *Cancer Res. 29*, 1272.
Harvey, R. G., & Dunne, F. B. (1978) *Nature (London) 273*, 566.
Hashimoto, Y., Shudo, K., & Okamoto, T. (1979) *Chem. Pharm. Bull. 27*, 1058.
Heidelberger, C. (1975) *Annu. Rev. Biochem. 44*, 79.
Ikeda, M., Matsugashita, S., & Tamura, Y. (1976) *J. Chem. Soc., Perkin Trans. 1*, 2587.
Irving, C. C. (1973) *Methods Cancer Res. 7*, 189–244.

[4] Attempts to determine the mutagenicity of **1** in the presence of trichloropropylene oxide, a known inhibitor of epoxide hydrase (Oesch et al., 1971), were unsuccessful since the inhibitor itself was found to be mutagenic under the experimental conditions employed.

[5] The opposite effects of benzoflavone and norharman on the mutagenicities of **1** and **10**, respectively, are also consistent with the suggestion that the activation of the two series of compounds differ.

[6] The addition of 2.0 mg of cytosol to an incubation mixture containing MC microsomes enhanced the mutant fraction obtained with 50 μM compound **10** (29.5×10^{-5} vs. 15.0×10^{-5}) but failed to enhance the mutagenicity observed for 5 μM **1** in the absence of cytosol (mutant fraction 113.5×10^{-5}).

[7] Further support derives from the inactivity as mutagens of the structurally related compounds harman, norharman (**12**), and carbazole (**13**) (data not shown).

Irving, C. C., Janss, D. H., & Russell, L. T. (1971) *Cancer Res. 31*, 387.

Jennette, K. W., Jeffrey, A. M., Blobstein, S. H., Beland, F. A., Harvey, R. G., & Weinstein, I. B. (1977) *Biochemistry 16*, 932.

Jerina, D. M., & Daly, J. W. (1974) *Science 185*, 573.

Kosuge, T., Tsuji, K., Wakabayashi, K., Okamoto, T., Shudo, K., Iitaka, Y., Itai, A., Sugimura, T., Kawachi, T., Nagao, M., Yahagi, T., & Seino, Y. (1978) *Chem. Pharm. Bull. 26*, 611.

Kriek, E. (1974) *Biochim. Biophys. Acta 355*, 177.

Kuntzmann, R. (1969) *Annu. Rev. Pharmacol. 9*, 21.

Levitt, R. C., Legraverend, C., Nebert, D. W., & Pelkonen, O. (1977) *Biochem. Biophys. Res. Commun. 79*, 1167.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.

Lu, A. Y. H., & Levin, W. (1974) *Biochim. Biophys. Acta 344*, 205.

McMahon, R. E., Mills, J., Culp, H. W., Gibson, W. R., Miller, W. M., & Marshall, F. J. (1969) *J. Med. Chem. 12*, 207.

Miller, E. C., & Miller, J. A. (1974) in *The Molecular Biology of Cancer* (Busch, H., Ed.) pp 377–402, Academic Press, New York.

Miller, J. A. (1970) *Cancer Res. 30*, 559.

Moore, P. D., Koreeda, M., Wislocki, P. G., Levin, W., Conney, A. H., Yagi, H., & Jerina, D. M. (1977) *ACS Symp. Ser. No. 44*, 127–154.

Nagao, M., Yahagi, T., Kawachi, T., Sugimura, T., Kosuge, T., Tsuji, K., Wakabayashi, K., Mizusaki, S., & Matsumoto, T. (1977) *Proc. Jpn. Acad. 53*, 95.

Nagao, M., Yahagi, T., & Sugimura, T. (1978) *Biochem. Biophys. Res. Commun. 83*, 373.

Nicoll, J. W., Swann, P. F., & Pegg, A. E. (1975) *Nature (London) 254*, 261.

Oesch, F., Jerina, D. M., & Daly, J. (1971) *Biochemistry 10*, 4858.

Omura, T., & Sato, R. (1964) *J. Biol. Chem. 239*, 2370.

Parli, C. J., Lee, N. W., & McMahon, R. E. (1973) *Drug Metab. Dispos. 1*, 628.

Pezzuto, J. M., Lau, P. P., Luh, Y., Moore, P. D., Wogan, G. N., & Hecht, S. M. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1427.

Robbins, P. W. (1962) *Methods Enzymol. 5*, 964.

Robbins, P. W. (1963) *Methods Enzymol. 6*, 766.

Schmeltz, I., & Hoffman, D. (1976) *Carcinogenesis–Compr. Surv. 1*, 225–239.

Sims, P., Grover, P. L., Swaisland, A., Pal, K., & Hewer, A. (1974) *Nature (London) 252*, 326.

Skopek, T. R., Liber, H. L., Krolewski, J. J., & Thilly, W. G. (1978a) *Proc. Natl. Acad. Sci. U.S.A. 75*, 410.

Skopek, T. R., Liber, H. L., Kaden, D. A., & Thilly, W. G. (1978b) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4465.

Sugimura, T., Kawachi, T., Nagao, M., Yahagi, T., Seino, Y., Okamoto, T., Shudo, K., Kosuge, T., Tsuji, K., Wakabayashi, K., Iitaka, Y., & Itai, A. (1977a) *Proc. Jpn. Acad. 53*, 58.

Sugimura, T., Nagao, M., Kawachi, T., Honda, M., Yahagi, T., Seino, Y., Matsushima, T., Shirai, A., Sawamura, M., Sato, S., Matsumoto, H. & Matsukura, N. (1977b) *Cold Spring Harbor Conf. Cell Proliferation 4*, 1561–1577.

Takeda, K., Ohta, T., Shudo, K., Okamoto, T., Tsuji, K., & Kosuge, T. (1977) *Chem. Pharm. Bull. 25*, 2145.

Westra, J. G., Kriek, E., & Hittenhauser, H. (1976) *Chem.-Biol. Interact. 15*, 149.

Wiebel, F. J., Leutz, J. C., Diamond, L., & Gelboin, H. V. (1971) *Arch. Biochem. Biophys. 144*, 78.

Wu, S.-C., & Straub, K. D. (1976) *J. Biol. Chem. 251*, 6529.

Yang, S. K., McCourt, D. W., Leutz, J. C., & Gelboin, H. V. (1977) *Science 196*, 1199.