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There is a correlation between the DNA affinity and mutagenicity of several 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indoles

(mechanism of mutagenesis/physicochemical and covalent interaction with DNA/metabolic activation/environmental carcinogenesis/chemical syntheses of mutagenic indoles)

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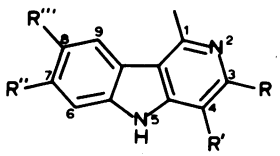
ABSTRACT **3-Amino-1-methyl-5*H*-pyrido[4,3-*b*]indole, previously reported to be a component of tryptophan pyrolysates, is an intensely mutagenic compound requiring microsomal activation for expression of mutagenicity. We have found that this species and several synthetic analogs interact noncovalently with calf thymus DNA, as judged by both fluorescence quenching and differential dialysis. Remarkably, this noncovalent interaction correlated with the mutagenic potential of the compounds in a bacterial mutagenesis assay. Therefore, it is suggested that the mechanism of mutagenesis involves metabolic activation followed by physicochemical interaction with DNA; for these compounds, the latter step may be limiting for the expression of mutagenicity.**

Carcinogenic chemicals produced by incomplete combustion processes represent a significant hazard to which we are exposed. The molecular mechanism(s) by which these chemicals initiate and promote the development of tumors has not been elucidated despite many years of intensive investigation, although it is now generally accepted that covalent reactions with critical cellular target molecules play an integral role in the process. Many carcinogens require metabolic activation prior to adduct formation (1, 2); due largely to the work of Miller and Miller (2, 3) it is known that the activated species are electrophilic in nature. It has become increasingly clear that carcinogenic chemicals can generally also behave as mutagens (4), supporting the concept of somatic mutation as the basis of carcinogenesis (4–6).

An active, and somewhat controversial, area of investigation has involved the correlation of the carcinogenic or mutagenic potential of several chemicals with their relative affinities for nucleic acids. For example, Brookes and Lawley (7) reported a good correlation relating carcinogenic potency and covalent binding to mouse skin DNA for a series of polycyclic aromatic hydrocarbons, but the observation was subsequently challenged by Goshman and Heidelberger (8), who found that noncarcinogenic dibenz[*a*,*c*]anthracene was bound to a greater extent than the carcinogen dibenz[*a*,*h*]anthracene. Analogous studies of the covalent reactions of other classes of chemicals with nucleic acids have been carried out (9–12), as have numerous studies of the noncovalent interactions of structurally related compounds with DNA (13), but no obvious correlations could be demonstrated among polycyclic aromatic hydrocarbons (14), aflatoxins (15), or acridine dyes (16). Nevertheless, physicochemical interaction has been considered a necessary if not sufficient step in mutagenesis (16). The critical series of events has been proposed as metabolism of the mutagen followed by intercalation into DNA and covalent reaction (11, 17, 18), the last of which is thought to be requisite for the expression of intense mutagenicity (18).

3-Amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (**1**) is a potent



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

mutagen structurally reminiscent of 2-aminofluorene and reported to be identical with a product resulting from pyrolysis of tryptophan (19). In the present study, compound **1**, and several synthetic analogs thereof, have been shown to be mutagenic toward *Salmonella typhimurium* only in the presence of a microsomal activating system. Remarkably, for these compounds there is a reasonable correlation between mutagenic activity and the extent of physicochemical interaction with calf thymus DNA. Discussed herein is the nature of this correlation and its possible implications for the mechanism of action of these species as mutagens.

MATERIALS AND METHODS

Synthesis of Test Substances. The syntheses of compounds **1** and **2** have been reported (20, 21). As will be described in detail elsewhere, compound **3** was prepared from **1** (acetic anhydride, pyridine, 25°C) as a white solid in high yield and then converted to 3-ethylamino-1-methyl-5*H*-pyrido[4,3-*b*]-indole (**4**) by treatment with lithium aluminum hydride in tetrahydrofuran; both were characterized by UV, NMR, and mass spectrometry. Analogous acetylation of **4** and reductive treatment of the formed acetate afforded 3-(*N*,*N*-diethylamino)-1-methyl-5*H*-pyrido[4,3-*b*]indole (**5**). Deamination of **1** was effected by diazotization (6 M H_2SO_4, $NaNO_2$, 0°C) and addition of the dark-colored reaction mixture to boiling 2 M H_2SO_4; extractive workup and subsequent purification of the product by chromatography on silica gel afforded **6** as a white solid in 70% yield. The syntheses of **7** and **8** were effected starting from 7- and 6-methyl quinolines, respectively, in analogy with the preparation of species **1** from quinoline (21, 22).

Treatment of Animals and Isolation of Microsomes. Male Fischer strain rats (100–125 g, Charles Breeding Farms, Wil-

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mington, MA) were housed in air-conditioned quarters with a 12-hr light–dark cycle and fed a semisynthetic diet (23) *ad libitum*. After four consecutive daily intraperitoneal injections of 3-methylcholanthrene (25 mg/kg of body weight, in corn oil), the rats were sacrificed by decapitation. The livers were excised rapidly and rinsed several times with ice-cold 0.14 M NaCl solution. Microsomes were isolated from the 10,000 × *g* supernatant as described (24) and stored in small portions at −90°C. Protein was determined by the method of Lowry *et al.* (25) and cytochrome *P*-448 content, by the method of Omura and Sato (26). Prior to use in the bacterial mutation assay, the microsomes were passed through nitrocellulose filters with pore diameters of 0.8 and 0.3 μm.

Bacterial Mutation Assay. The forward mutation assay described by Thilly and his coworkers (27, 28) was utilized. This assay, which is based on the development of 8-azaguanine resistance, employed *S. typhimurium* strain TM677 (carrying the R-factor plasmid pKM101) (28). Duplicate 1.0-ml incubation mixtures were prepared containing approximately 1.5×10^7 bacteria, 0.5 mg of microsomal protein, 1.0 mg of glucose 6-phosphate, 0.4 unit of glucose-6-phosphate dehydrogenase, 1.0 mg of $NADP^+$, and 0.67 mg of $MgCl_2$ in minimal Eagle's medium. The compound to be tested was added in 10 μl of dimethyl sulfoxide and the mixture was incubated at 37°C for 1.5 hr. The reaction was then quenched by the addition of 4.0 ml of phosphate-buffered saline followed by centrifugation. The bacteria were resuspended in phosphate-buffered saline and plated in triplicate in the presence and absence of 8-azaguanine. After incubation at 37°C for 36–40 hr, the plates were scored and the data were expressed as the mutant fraction—i.e., the average number of 8-azaguanine-resistant clones divided by the average number of 8-azaguanine-sensitive clones.

Interaction of Test Substances with DNA. *By fluorescence quenching.* Solutions containing calf thymus DNA and single test compounds were prepared in 20 mM Tris·HCl buffer, pH 8.1, containing 0.1 M NaCl, 1.0 mM EDTA, and 3.75% (vol/vol) dimethyl sulfoxide. The concentration of either the DNA or the test compound was varied, holding the other constant. Fluorescence spectra were taken on a Turner model 430 fluorimeter at ambient temperature. The excitation wavelength was 350 nm; the fluorescence emission was scanned from 550 to 300 nm, and the intensities corresponding to the emission maxima were used in the calculations. The addition of DNA to the solutions did not shift the emission maxima of the test compounds.

By equilibrium dialysis. A 0.5-ml solution of a single test compound and DNA in 20 mM Tris·HCl, pH 8.1, containing 0.1 M NaCl, 1.0 mM EDTA, and 3.75% dimethyl sulfoxide was sealed in a length of dialysis tubing and immersed in 2.5 ml of the same buffer containing the test compound at the same concentration, but no DNA. After the solutions were allowed to equilibrate at ambient temperature for 16 hr, the concentration of the test compound in the external medium was determined on a Cary 15 recording spectrophotometer (employing the λ_{max} for each compound) and compared with that obtained prior to dialysis.

Covalent Binding of Compounds 1 and 2 to DNA. Reaction mixtures (2.0 ml) containing 45 mM Tris·HCl buffer at pH 7.5, 3 mM $MgCl_2$, 5 mM glucose 6-phosphate, 0.8 unit of glucose-6-phosphate dehydrogenase, 2 mg of microsomal protein, 40 A_{260} units of calf thymus DNA, and various amounts of generally labeled [^{3}H]**1** (39 Ci/mol) or [4-*methyl*-^{3}H]**2** (7.1 Ci/mol) (1 Ci = 3.7×10^{10} becquerels), added in small volumes of methanol, were incubated at 37°C for 1 hr. The microsomes were removed by centrifugation, and 0.16 ml of 4.0 M NaCl and 0.4 ml of 5% aqueous sodium dodecyl sulfate were added to the supernatant. After extraction with an equal volume of phenol/chloroform/isoamyl alcohol (50:50:1, wt/vol/vol), the DNA was recovered from the aqueous phase by ethanol precipitation and rinsed with organic solvents (29). The amount of binding was determined by liquid scintillation counting.

Table 1. Mutagenic activities of 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (1) and several synthetic analogs

Compound	Mutant fraction × 10^5 (% survival)		
	2.5 μM	5.0 μM	7.5 μM
1	57 (63)	103 (44)	177 (17)
2	34 (65)	79 (45)	98 (23)
4	35 (70)	69 (40)	89 (18)
5	17 (41)	63 (8)	84 (2)
7	14 (45)	47 (18)	86 (12)
8	9.4 (32)	22 (23)	28 (29)
3	4.4 (100)	5.4 (100)	6.2 (100)
6	3.5 (100)	4.7 (100)	2.9 (100)

The assay was carried out as described in *Materials and Methods*. Control incubations containing no test compound were treated in similar fashion and gave an average mutant fraction of about 3.5×10^{-5}.

RESULTS

Consistent with previous reports (19, 30), compounds **1** and **2** were found to be potent mutagens (Table 1). The mutagenic activity obtained with strain TM677 was much greater than the activities associated with compounds such as benzo[*a*]pyrene or 2-acetamidofluorene and was comparable to the activity of aflatoxin B_1. Expression of mutagenicity was dependent on metabolic activation and the requisite activity was found to be localized in the microsomal fraction of rat liver. Pretreatment of the rats with 3-methylcholanthrene, an inducer of cytochrome *P*-448 (31), was necessary; phenobarbital was ineffective as an inducer of the required activating system.

Also recorded in Table 1 and in Fig. 1 are the mutagenic activities of several synthetic analogs of compound **1**. Although it is difficult to produce precise quantitative results employing mutagenesis assays (28), and comparisons among compounds **1**–**8** are additionally dependent on the concentration of test compound and complicated by varying levels of toxicity, the general pattern of mutagenic potencies can be represented as **1** > **2** > **4** ≥ **5** ≥ **7** > **8** > **3** > **6**. The *N*-acetylated derivative (**3**) exhibited significant mutagenicity when tested at higher concentrations (mutant fraction, 53×10^{-5}; 100 μM concentration), whereas compound **6** was nonmutagenic at all tested concentrations.

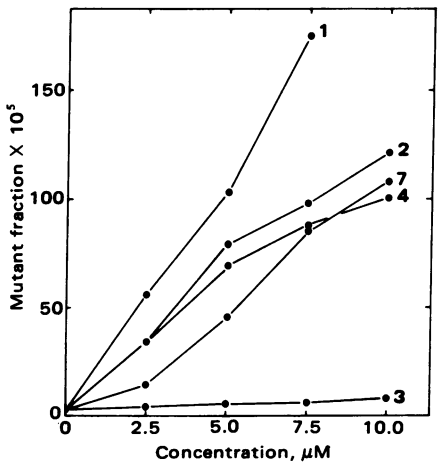


FIG. 1. Concentration-dependent mutagenicity of 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole and various analogs.

Table 2. Noncovalent binding to DNA of 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (1) and several synthetic analogs

Compound	Fluorescence quenching ($\Delta I/I$) 0.76 mM	1.52 mM	2.22 mM	Equilibrium dialysis ($\Delta A/A$) 0.55 mM
2	0.81	0.88	0.93	0.75
1	0.55	0.65	0.68	0.60
7	0.43	0.44	0.58	0.59
4	0.28	0.38	0.44	0.51
5	0.19	0.31	0.34	0.47
8	0.16	0.17	0.17	0.45
3	0.06	ND	ND	0.26
6	0	0	0	ND

The assays were carried out with each test compound at 100 μM and the DNA nucleotide concentration given in the column heading. For the fluorescence quenching experiments, a control experiment involving addition of heat-denaturated DNA (1.52 mM) to a 100 μM solution of 1 gave a value of 0.79 for $\Delta I/I$. ND, not determined.

The fluorescence of compounds **1–8** was measured in the presence and absence of DNA; fluorescence quenching was obtained with each species except **6**. The diminution of fluorescence provided a measure of the relative extents of interaction of the various compounds with DNA (Table 2); these are expressed in normalized form as $\Delta I/I$, in which I is the fluorescence intensity of the compound in the absence of DNA and ΔI is the diminution in intensity observed upon admixture of a known amount of DNA. As indicated in the table, these experiments were performed at a single (100 μM) concentration of test compound with various concentrations of DNA. The relative extents of interaction for the eight compounds were suggested by these experiments to be in the order **2** > **1** > **7** > **4** > **5** > **8** > **3** > **6**. The order was not a function of DNA concentration. The affinities of the test compounds for DNA were also determined by equilibrium dialysis (Table 2) and expressed as $\Delta A/A$, in which A = the absorbance of each compound prior to dialysis against DNA and ΔA = the change in absorbance after dialysis. As shown, the affinities were in the same qualitative order as determined by fluorescence quenching, although the two methods of assay did give some quantitative differences between analogs within the series.

In order to obtain a quantitative measure of the interaction with DNA, and additionally verify the order of affinities, association constants were measured for some of the analogs. Assuming the binding sites are noninteracting, an apparent association constant K_a can be expressed as

$$K_a = \frac{C_b}{\nu_m C_f N_T}, \qquad [1]$$

in which C_b and C_f are the concentrations of bound and free compound, respectively, N_T is the nucleotide concentration, and ν_m is the maximum value of the intrinsic binding ratio (C_b/N_T). The magnitude of K_a indicates the affinity for DNA and ν_m corresponds to the maximum number of binding sites; these values can be obtained either by rearrangement of Eq. 1 to afford a double reciprocal plot,

$$\frac{1}{C_b} = \frac{1}{K_a \nu_m N_T C_f} + \frac{1}{\nu_m N_T}, \qquad [2]$$

or by rearrangement to the Scatchard equation

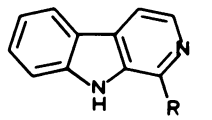
$$\frac{\nu}{C_f} = K_a(\nu_m - \nu). \qquad [3]$$

Values for C_b were obtained either by equilibrium dialysis or fluorescence quenching. According to the latter method,

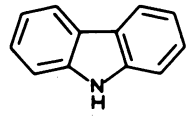
$$C_b = \Delta I \cdot \frac{C_T}{\Delta I_s}, \qquad [4]$$

in which C_T is the total concentration of the analog ($C_f + C_b$) and ΔI_s is the quenching at the DNA concentration at which all of the molecules are bound (32). ΔI_s is accessible by extrapolation of a ΔI vs $1/N_T$ plot.

As shown in Table 3, the values obtained for K_a were proportional to those for $\Delta I/I$ (and $\Delta A/A$) and were similar regardless of the method employed. Harman (**9**) and norharman (**10**) were chosen as structurally related compounds for which



9 R = CH_3
10 R = H



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values determined spectrophotometrically have been reported (30). The fluorescence quenching method gave a Scatchard plot identical with that reported when the ionic conditions of the report were reproduced.

DISCUSSION

The intense mutagenicity and cytotoxicity of certain 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indoles in bacterial and mammalian (33) assays and the implications of their possible occurrence as dietary constituents (19, 30) prompted us to investigate the mechanism(s) by which they exert their mutagenic effects. Although the compounds of interest bear an obvious structural relationship to those in the 2-aminofluorene series,

Table 3. Affinity of 3-amino-1-methyl-5*H*-pyrido-[4,3-*b*]indole (1) and several synthetic analogs for DNA

Compound	Fluorescence quenching $\Delta I/I$*	$K_a \times 10^{-4}$, M^{-1}	ν_m	Equilibrium dialysis $\Delta A/A$†	$K_a \times 10^{-4}$, M^{-1}	ν_m
1	0.55	3.6	0.26	0.44	4.9	0.39
2	0.81	4.0	0.26			
8	0.16	0.4	0.26			
Harman (**9**)	0.15	1.1	0.26	0.25	1.1	0.37
Norharman (**10**)	0.058	—‡	—‡	0.15	0.61	0.39

* Determined at 100 μM for each test compound and 757 μM DNA nucleotide concentration.

† Determined at 100 μM for each test compound and 379 μM DNA nucleotide concentration.

‡ The value for ΔI_s could not be determined accurately due to the minimal fluorescence quenching obtained upon admixture of DNA.

the much greater mutagenic activity of the indole derivatives suggested that the mechanism(s) by which they are activated metabolically and react with DNA might not parallel the pathways utilized by the aminofluorenes. It was hoped that some of the *C*- and *N*-alkylated derivatives of **1** would be fully as mutagenic as the parent compound, whereas others would be substantially less mutagenic, thus providing suggestive evidence regarding sites required for binding or activation (see, e.g., ref. 34). As shown in Table 1, all of the structural modifications reduced the mutagenicity of **1.** The most significant reductions in activity resulted from *N*-acylation and oxidative deamination of the exocyclic nitrogen atom, consistent with its possible functional role. It should be noted, however, that (mono- or di-) alkylation of this atom had less effect on activity than introduction of a methyl group at C-7 or C-8.

Also examined was the physicochemical interaction of each of the unactivated indole derivatives with calf thymus DNA, as judged by both fluorescence quenching and equilibrium dialysis. The latter method confirmed the more sensitive technique of fluorescence quenching as accurately reflecting the affinity of the derivatives for DNA; also consistent with these findings were the measured association constants for compounds **1, 2,** and **8.** For comparative purposes, we determined K_a values for the structurally related but nonmutagenic compounds harman (**9**) and norharman (**10**)[‡] and found these to be comparable to those determined previously by spectrophotometric procedures (37).

The affinity of 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole and its derivatives for DNA was similar to that noted previously for several other compounds (13, 15, 32, 37, 38), although the precise nature of the noncovalent interaction obtained with **1–8** is unclear. Unlike polycyclic aromatic hydrocarbons, the indole derivatives are quite water soluble, so that a solubilization effect (39) is not likely. Given the size of the heterocyclic ring system involved (40), intercalation between DNA bases would seem to be a reasonable basis for the observed affinity, especially because the structurally related species norharman (**10**) has been shown to unwind double-stranded covalently closed circular DNA and **9** and **10** gave well-defined isosbestic points when admixed with calf thymus DNA (37). It should be noted, however, that compound **1** was found to have an affinity even for denatured calf thymus DNA, as judged both by fluorescence quenching and equilibrium dialysis (data not shown), which seems inconsistent with association occurring exclusively via intercalation.[§]

The structural parameters involved in DNA interaction may also be considered. The unsubstituted, nonmutagenic, heterocycle carbazole (**11**) was found not to bind to DNA (data not shown), whereas its aza analog norharman (**10**) was bound rather strongly. Even stronger binding was observed for harman (**9**), the methylated analog of **10.** Among the pyrido[4,3-*b*]indole derivatives, which have an additional exocyclic amino group relative to **9** and **10,** compound **1** was bound to DNA even more strongly than harman, and its 4-methylated analog (**2**) had the highest affinity of all the compounds studied. Interestingly, methylated derivatives **7** and **8** had a lesser affinity for DNA than **1,** as did *N*-alkylated derivatives **4** and **5.** Consistent with the putative contribution of the exocyclic amino group, the *N*-acetylated (**3**) and deaminated (**6**) derivatives of **1** bound to DNA poorly, if at all.

In agreement with the results obtained in other laboratories (14–16), we found that DNA binding was a necessary but not sufficient condition for mutagenesis. Compounds **9** and **10,** for example, efficiently bind noncovalently to DNA but are nonmutagenic. However, among those species found to be mutagens, there was a general correlation between mutagenic potential and the degree of physicochemical interaction with DNA. This indicates the importance of DNA binding in the mechanism by which **1** and its analogs exert their mutagenicity, as has previously been suggested for other mutagens (11, 16–18). Moreover, the observed correlation is consistent with a scheme in which the compounds are activated metabolically, bound noncovalently to DNA (in a step that is limiting for expression of mutagenicity), and then bound covalently.[¶]

Compound **2** represents an obvious exception to the correlation between extent of DNA binding and mutagenicity, because it has a greater affinity for DNA than **1** but is less mutagenic (Tables 1–3). Although these measurements were obtained with the unactivated indole derivatives, we have found that compound **1** binds to DNA covalently to the extent of about one molecule per 10,000 nucleotides (see also ref. 42), whereas **2** was bound almost an order of magnitude more efficiently. The ostensibly diminished mutagenicity of **2** may reflect factors such as transport through the bacterial cell wall or differences in the assay conditions used for measurement of covalent and noncovalent DNA binding and mutagenicity. Alternatively, it is also possible that the 4-alkyl substituent affects qualitatively the nature of the covalent adducts formed with DNA[‖] and that, although the adducts derived from **2** are more numerous, they effect mutagenesis at the cellular level less efficiently than those derived from **1.** Consistent with this possibility was the observation that compound **2** was more cytotoxic than **1** when incubated with hamster embryo cells (33).

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‡ Although nonmutagenic, **9** and **10** have been shown to enhance the mutagenicity of some compounds under a given set of conditions (30) and to inhibit mutagenesis with **1** (unpublished results) and benzo[*a*]pyrene (35, 36).

§ Analogous behavior has been noted previously for harmaline (41).

¶ An important and unproven consequence of this proposed scheme is that the metabolically activated analogs of **1–8** would also have the same relative affinities for DNA. An alternative scheme, also consistent with the observed results, might involve binding of the compounds to the DNA prior to metabolic activation and covalent binding. Clearly, the existence of intranuclear activating enzymes would be necessary if this process were to obtain *in vivo* (43).

‖ This could be expressed either in the mode of activation of the indole derivatives or at the level of reaction of the activated species.

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