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EFFECTS OF THE COMUTAGENS, HARMAN AND NORHARMAN, ON THE INTERACTION OF A TRYPTOPHAN PYROLYSIS PRODUCT, 3-AMINO-1-METHYL-5H-PYRIDO(4,3-b)INDOLE WITH DNA

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SUMMARY

Harman and norharman, known as comutagens of many chemicals, were tested for their effect on the binding to DNA of 3-amino-1-methy-5H-pyrido(4,3-b)indole, (Trp-P-2), a potent mutagen found with harman and norharman in the pyrolysate of tryptophan(1). We demonstrated that the alteration of the DNA helix by intercalation of these comutagens to DNA does not affect the affinity of this potent mutagen for DNA. Covalent binding, however, was inhibited by the comutagens.

INTRODUCTION

Pyrolysis of the amino acid, D,L-tryptophan yielded a large amount of comutagens, harman and norharman and two potent mutagenic principles, 3-amino-1-methyl-5H-pyrido(4,3-b)indole, Trp-P-2 and 3-amino-1,4-dimethyl-5H-pyrido(4,3-b)indole, Trp-P-1(1,2) (Fig. 1). The same pyrolysis products were also found in charred meat(3), naturally-occurring foods and in cigarette tar(4).

There has been a dispute of the "comutagenic" action of harman or norharman in various laboratories. During the isolation of Trp-P-2 and Trp-P-1, Sugimura et al.(2) found that the total mutagenic activity was decreased markedly after harman and norharman were removed from the charred tryptophan mixture. They later claimed norharman or harman enhanced the mutagenic activities of Trp-P-2, Trp-P-1, benzo(a)pyrene, 4-dimethylaminoazobenzene, N-2-fluroenylacetamide and 4-dimethyl-aminostilbene(5,6,7). In contrary to their finding, Levitt et al.(8) found that both harman and norharman inhibit

Abbreviations: Trp-P-2, 3-amino-1-methyl-5H-pyrido(4,3-b)indole; Trp-P-1, 3-amino-1,4-dimethyl-5H-pyrido(4,3-b)indole; BP, Benzo(a)pyrene; S-9, the supernatant of mouse liver homogenate after 9,000 x g spin; MC, 3-methylcholanthrene.

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Figure 1. Structure of Trp-P-1 and Trp-P-2.

benzo(a)pyrene (BP) mutagenicity, BP metabolism and the covalent binding of all BP metabolites to DNA in vitro. Chang et al.(9) demonstrated that harman and norharman inhibit rather than enhance mutagenesis and harman decreases the capacity to repair DNA damage introduced by UV light or X ray. Sugimura and coworkers(10) later showed that the effect of norharman on the mutagenicity of BP was dependent on the amount of S-9 in the pre-incubation mixtures and the strains of Salmonella typhimurium. The enhancement effect was observed with an excess of rat-liver microsomal enzymes(S-9) and the inhibitory effect was observed with small amounts of the microsomal enzymes.(10) Similiar results with Trp-P-1 and Trp-P-2 were observed(15) by these workers.

This report is to re-investigate the possible comutagenic mechanisms, in particular, the effect of the comutagens on the interaction of Trp-P-2 with DNA in the presence or absence of microsomal enzymes.

MATERIALS AND METHOD

Trp-P-2 was synthesized by the method as described(11). Tritiation was performed by Moraveck Biochemicals (City of Industry, California). Harman and norharman were purchased from Aldrich Chemical Company(Milwaukee,Wisconsin).

Reversible binding: Equilibrium dialysis was performed as described(12). In brief, a fixed amount of DNA(calf thymus, Sigma) was placed in a dialysis bag, and the concentration of the free drug was estimated by the change in radioactivity before and after dialysis for 24 hours in the dark. Specific activity of [^{3}H]Trp-P-2 was 6 mC_i/mmole.

Fluorescence quenching was performed with a Turner fluorometer(model 430). Absorbance was measured with a Cary 15 spectrophotometer.

Measurements of reversible binding were carried out in a buffer which contained 0.1M NaCl, 20mM Tris-HCl(pH8.1), 1mM EDTA(pH8.1) and 3.5%(v/v)DMSO. Temperature was kept constant at 25°C.

Covalent binding: Male Fischer rats were sacrificed three days after injection i.p. with 25mg 3-Methylcholanthrene per Kg body weight per day. Washed microsome was obtained by two differential centrifugation runs at 9,000 rpm and 39,000 rpm. The pellets were homogenized in a medium containing 0.25M sucrose, 0.05M Tris-HCl(pH7.5) and stored in liquid nitrogen. The incubation mixture contained 45mM Tris-HCl(pH7.5),3mM $MgCl_2$, 5mM glucose-6-PO_4,0.8mM NADP,0.4U/ml glu-6-PO_4 dehydrogenase, 8 A_{260}/ml calf thymus DNA, 1mg/ml of

washed microsome prepared as above, and 5%(v/v) of [^{3}H] Trp-P-2 (6.34 mCi/mmole) and comutagens of the indicated amounts in methanol.

The reaction was terminated after 2 hr incubation at 37^{o}C in a shaker by centrifugation for 60 min at 39,000 rpm in a rotor 40. DNA was purified by phenol-$CHCl_3$-isoamyl alcohol extraction, followed by alcohol precipitation twice. The precipitate was dissolved in 0.05M Tris-HCl(pH7.5) and extracted with benzene, diethyl ether, ethyl acetate, and ethylene chloride.

RESULTS AND DISCUSSION

1. Effects on binding to DNA in the absence of microsomal proteins:

It was reported that quenching of fluorescence was observed when DNA was added to the solution of norharman, indicating that it interacted with norharman(13), which was also shown to unwind the DNA helix with an unwinding angle of 17 $\pm$ 3^{o} per intercalation(13). In Fig. 2, we demonstrated that fluorescence of Trp-P-2 was also quenched by DNA added to the solution. Therefore, this promutagen, which is not mutagenic by itself without microsomal activation, also interacts with calf thymus DNA in the absence of microsomal enzymes. Similar results were also observed with Trp-P-1 and harman(data not shown).

Since there is a comutagenic action of norharman on the proximate mutagen, N-acetoxy-AAF, which does not require S-9 mix for activation in the Ames test, Sugimura and coworkers(7) proposed that the comutagenic effect was not solely dependent on the alteration of the metabolic activation and that the comutagens might interact with DNA, thereby altering the conformation of the polynucleotide and increasing the affinity of DNA to the mutagens. This is analogous to the finding of Krugh and Young(14) who demonstrated that daunorubicin and adriamycin, which intercalate but do not covalently bind to DNA, facilitate the binding of actinomycin D to poly(dA-dT)·poly(dA-dT).

To re-examine this hypothesis, we have used [^{3}H] Trp-P-2 in equilibrium dialysis experiments similar to what Krugh *et al.*(14) have shown with actinomycin D binding to poly(dA-dT)·poly(dA-dT) in the presence of daunorubicin. Fig. 3 shows that the comutagens, harman and norharman, do not affect the affinity of Trp-P-2 to DNA by equilibrium dialysis. However, a slight decrease in affinity does occur at extremely high concentration(1mM) of the comutagen, harman. Therefore, we have shown that the comutagens, which are β-carbolines(5)

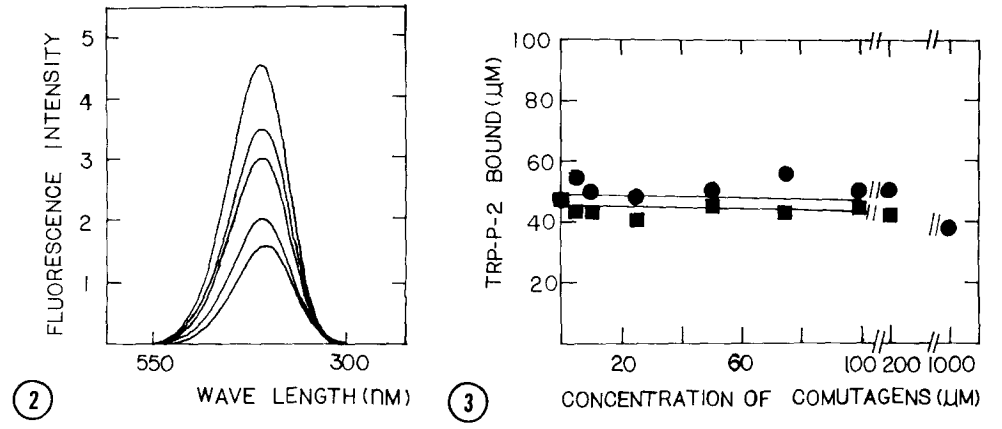

Figure 2. Fluorescence quenching of Trp-P-2 in the presence of calf thymus DNA. The concentrations of nucleotide corresponding to the decreasing fluorescence intensity were: 0, 151, 303, 757, and 1515μM. The concentration of Trp-P-2 was kept at 100μM. Excitation was at 350nm. Emission spectra was scanned from 550nm to 300nm.

Figure 3. Effect of harman or norharman on the reversible binding of Trp-P-2 to calf thymus DNA, as studied by equilibrium dialysis. ● , harman was added. ■ , norharman was added. Total concentration of Trp-P-2 was kept at 100μM. Total concentration of nucleotide was kept at 313μM.

and have structure similar to those of AAF and Trp-P-2, unlike the situation of daunorubicin mediated binding of actinomycin D to polynucleotide, do not increase the affinity of Trp-P-2 to DNA. We thus favor the hypothesis proposed by Chang et al.(9) that the intercalation of the comutagens with DNA alters the process of DNA repair and decreases the capacity of the bacteria to fix mutation rather than enhances the affinity of DNA to mutagens as proposed by Sugimura and coworkers(13,7).

2. Effects on binding to DNA in the presence of microsomal proteins:

Covalent binding study (Table 1) revealed that the comutagens inhibit rather than enhance the binding of Trp-P-2 to DNA in the presence of MC induced microsomes. This is in agreement with the results of BP binding to DNA after metabolic activation, as reported earlier(8). Mutagenesis of Trp-P-2 was also inhibited by the comutagens(unpublished results). It indicates that the inhibition action is largely enzymatic in contrast to non-covalent binding. Whether this is due to an alteration of the microsomal enzymes is not known.

Table 1. Effect of harman or norharman on the irreversible binding of the metabolized Trp-P-2 to DNA in vitro in the presence of MC induced microsomes.

Compounds	Binding ratio μmol metabolized Trp-P-2 bound /mol nucleotide	control value of binding ratio
Trp-P-2(500μM)	261	255
Trp-P-2(1mM)	305.4	300
Trp-P-2(500μM) plus:		
Norharman(50μM)	124	118
(100μM)	123	117
(500μM)	121	115
Harman (1mM)	102	96
Control: Boiled microsomes no NADPH		
Trp-P-2(500μM)	6	0
Trp-P-2(1000μM)	5.4	0

Input calf thymus DNA was 0.4 mg/ml. Binding ratios were estimated by the amount of recovery of radioactivity of tritiated Trp-P-2 bound to DNA.

However, it is probable that the comutagens utilize the same enzymes as those for the activation of Trp-P-2. Harman and norharman are both metabolized by mouse liver microsomes in the presence of NADPH(8). Therefore, the addition of the comutagens decreases the concentration of the microsomal enzymes available for activation of Trp-P-2.

Sugimura and coworkers(10) observed that the comutagenic inhibition of mutagenesis at a low concentration (5μl) of S-9 mix and an enhancement at a higher concentration (70μl) of S-9. At the higher concentration(70μl) of S-9, however, the mutagenicity of BP in the absence of norharman, was much lower than that at the lower concentration (5μl) of S-9 mix(10). The presence of norharman, however, did not increase the mutagenicity of BP at the higher concentration(70μl) of S-9 mix any larger than that at the lower concentration (5μl) of S-9 mix when the comutagen was not added(10). The "increase" in

mutagenicity leveled off at higher concentrations of the comutagen and remained to have the same value equal to that of the lower concentration(5μl) of S-9 mix when norharman was not present(10).

The bell-shaped curves of mutagenicity of BP and of Trp-P-2(10,15) vs the concentrations of S-9 mix in their studies in the absence of comutagens can be explained as follows: The microsomal proteins themselves also provide subtrate sites for binding of BP or Trp-P-2. At higher concentrations of microsomes, the excessive amount of microsomes might provide a significant amount of binding sites for metabolized BP or Trp-P-2. Binding to DNA of these metabolites, therefore, was decreased and so was the mutagenicity.

Harman and norharman, which are intercalative drugs, definitely bind to microsomal RNA. Addition of these intercalative drugs would decrease the number of the binding sites on the microsomes available to BP or Trp-P-2 in the presence of an excessive amount of the microsomal mix. In addition, the comutagens might utilize the same enzymes(s); the concentration of the enzyme(s) would then be relatively decreased. In any case, the effect of the addition of norharman to the incubation mixture containing an excessive amount of S-9 mix(70μl) would be changing the incubation condition to that of the optimum similar to the condition at the lower concentration(5μl) of S-9 mix in the absence of norharman(10). Therefore, the mutagenicity was increased to the original optimum(10) presumably due to a concomitant increase in mutagens binding to DNA.

If the above explanation proves to be true, the so-called "enhancement" effect of the comutagens on mutagenesis under an unfavorable enzymatic condition would be an artifact of the assay method. The inhibitory effect under the optimal enzymatic condition would be the real effect of norharman on mutagenesis of BP or Trp-P-2. Therefore, the validity of the Ames test in this regard only stands if the variables, in particular, the amount of S-9 and the input concentration of the test mutagens or chemicals, are adjusted to the condition such that the enzymatic reaction is at its optimum before the addition of the "comutagens".

Positive findings in animal studies, however, are required to show whether these chemicals are co-carcinogens in vivo or to prove that these tryptophan pyrolysis chemicals, which were found to exist in the smoke condensate and the charred surface of meats(16), are instrumental in the etiology of tumorigenesis.

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