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ACTIVATION OF A MUTAGEN, 3-AMINO-1-METHYL-5H-PYRIDO[4,3-b]INDOLE. IDENTIFICATION OF 3-HYDROXYAMINO-1-METHYL-5H-PYRIDO[4,3-b]INDOLE AND ITS REACTION WITH DNA.

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SUMMARY:

A potent mutagen, 3-amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-P-2), isolated from a tryptophan pyrolysate, was activated metabolically by rat liver microsomes and bound to DNA. An active metabolite formed by rat liver microsomes was identified as 3-hydroxyamino-1-methyl-5H-pyrido[4,3-b]indole (N-OH-Trp-P-2). Synthetic N-OH-Trp-P-2 reacted with DNA efficiently after O-acetylation or to a lesser extent under acidic conditions(pH 5.5), but did not react appreciably under neutral conditions. Acid hydrolysis of DNA modified by O-acetylated N-OH-Trp-P-2 (N-OAc-Trp-P-2) gave 3-(8-guanyl)amino-1-methyl-5H-pyrido[4,3-b]indole (Gua-Trp-P-2), which is the main modified base of DNA formed by Trp-P-2 in the presence of microsomes. The glycoside bond of the modified base was found to be cleaved by heating at 100° for 1 hr at pH 7.0. In this way, the modified base was liberated from DNA modified by N-OAc-Trp-P-2 in good yield. N-OAc-Trp-P-2 bound to guanyl cytidine more effectively than to guanylic acid, suggesting that covalent binding with guanyl moiety of DNA involves intercalation of the ultimate mutagen into a base pair.

INTRODUCTION:

The potent mutagen 3-amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-P-2), isolated from a tryptophan pyrolysate[1] and proved to

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ABBREVIATIONS:
Trp-P-2: 3-amino-1-methyl-5H-pyrido[4,3-b]indole,
Glu-P-1: 2-amino-6-methyldipyrido[1,2-a:3',2'-d]imidazole

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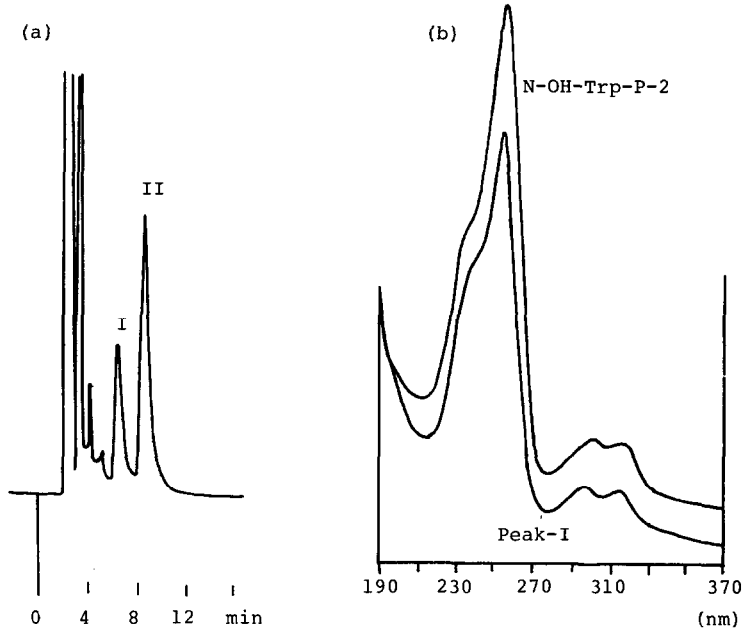
be carcinogenic,[2] shows mutagenic activity on Salmonella typhymurium TA 98 only after metabolic activation and binds to DNA in the presence of microsomal protein.[3] The major base obtained with Trp-P-2 was identified as 3-(8-guanyl)amino-1-methyl-5H-pyrido[4,3-b]indole (Gua-Trp-P-2).[4] The structure of this base suggests that the activated form that reacts with DNA is 3-hydroxyamino-1-methyl-5H-pyrido[4,3-b]indole (N-OH-Trp-P-2). This paper reports the identification of this metabolite, its reaction with DNA, and structure of the modified base.

MATERIALS AND METHODS:

Calf thymus DNA and NADPH were purchased from Sigma and Oriental Company, respectively. Rat liver microsomes were prepared from male Wistar rats treated with polychlorobiphenyls as described previously.[3]

Incubation: Microsomal metabolites of Trp-P-2 were obtained by in vitro incubation of a solution of Trp-P-2 (acetic acid salt,[5] 0.2 mg/ml) with rat liver microsomes (8 mg protein/ml) and NADPH (1.5 mg/ml) at 37° for 10 min. After incubation, an equal volume of cold acetone was added and the mixture was centrifuged. The supernatant was analyzed by high performance liquid chromatography (HPLC. Fig. 1a) with a UV detector, Shimadzu SPD-1. The stopped flow UV spectrum was obtained with the detector. Fluorescence and excitation spectra were obtained using a Hitachi MPF-4 fluorescence spectrometer.

Synthesis: N-OH-Trp-P-2 was prepared from 3-nitro-1-methyl-5H-pyrido[4,3-b]indole, which was synthesized by oxidation of Trp-P-2. The oxidation was performed in 30% H_2O_2 in the presence of Na_2WO_4 at room temperature for 6 hr. The reaction mixture was extracted with ethyl acetate, and crude product was chromatographed on a silica gel column, to give 3-nitro-1-methyl-5H-pyrido[4,3-b]indole, which was recrystalized from ethyl acetate, mp. 286-290°. The structure assigned was consistent with the IR, UV(nm, in MeOH: 235, 252, 280(sh), and 330), and mass spectrum, and with the result of elemental analysis. To a solution of the nitro compound in tetrahydrofuran(THF) was added aluminum



Column: Polygosil $_5C_{18}$, 4,6ø x 150 mm

Solvent: CH_3CN(40%)-0.02 M KH_2PO_4(60%)

Flow rate: 0.7 ml/min

Detection: Absorbance at 254 nm

Peak-II: Trp-P-2

Fig. 1 HPLC of microsomal metabolites of Trp-P-2 (a) and the UV spectra of the major metabolite of Trp-P-2 and authentic N-OH-Trp-P-2 (b).

amalgamate, and the mixture was stirred at 0° for 5 min. After filtration, the mixture was evaporated in vacuo, and the residue was washed with CH_2Cl_2 to give crude N-OH-Trp-P-2 (about 80% purity, 40-50% yield). Repeated reprecipitation from MeOH-CH_2Cl_2 and increasing amounts of n-C_6H_{14} gave chromatographically pure N-OH-Trp-P-2, dp. 176-178°. The yield of the pure product was 15%. UV(nm(ε), in MeOH: 204(3.0 x 10^4), 218(sh), 237(3.6 x 10^4), 256(4.7 x 10^4), 288(0.7 x 10^4), and 310(0.6 x 10^4)). The compound could not be recrystalized because it was heat-labile, but an azoxy derivative was prepared in good yield by reaction with nitrosobenzene and was correctly analyzed, confirming the structure.

Modification of nucleic acid: A solution of calf thymus DNA (2 mg

in 1 ml of 0.05 M citrate buffer, pH 5.5) was treated with a solution of N-OH-Trp-P-2 (3 mg in 1 ml THF) at 37° for 4 hr. Modified DNA was recovered by addition of cold ethanol and centrifugation, and was subjected to gel filtration chromatography (Sephadex G-25). The amount of binding was estimated from the fluorescence of the DNA obtained. Modification by 3-(N-acetoxy)-amino-1-methyl-5H-pyrido[4,3-b]indole (N-OAc-Trp-P-2) was performed at pH 7.5. N-OAc-Trp-P-2 was prepared in THF (1 ml) from N-OH-Trp-P-2 (3 mg) by treatment with an equimolar amount of acetic anhydride at 0° for 30-50 sec. The THF solution was used for modification of calf thymus DNA (2 mg in 1 ml 0.05 M phosphate buffer, pH 7.5). The reaction time was less than 10 min. Reactions of guanylic acid and guanyl cytidine with N-OAc-Trp-P-2 were performed similarly.

Modified DNA and nucleotides were hydrolyzed with 60% trifluoroacetic acid at 100° for 1 hr. The mixture was evaporated and the residue was dissolved in dimethylsulphoxide (DMSO) and analyzed by HPLC. Standard guanine bound Trp-P-2 (Gua-Trp-P-2) was prepared as reported previously.[4)]

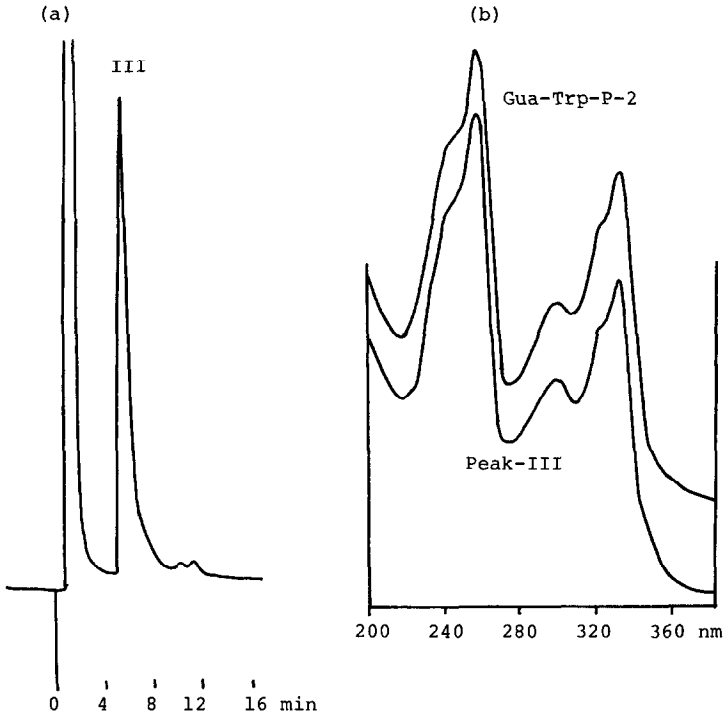
Gua-Trp-P-2 was liberated from modified DNA by heating an aqueous solution at 100° for 1 hr. The mixture was then cooled, mixed with cold ethanol and centrifuged. The supernatant, which contained the liberated base, was dried and the residue was dissolved in DMSO for analysis by HPLC.

RESULTS AND DISCUSSION:

The mixture of Trp-P-2 metabolites formed by rat liver microsomes was analyzed by HPLC (Fig. 1). The major metabolite (Peak-I) was identical with synthetic N-OH-Trp-P-2, judging by its chromatographic behavior and UV (Fig. 1b), fluorescence, and excitation spectra. The yield of this metabolite was fairly good (about 30%) when the work-up process was rapid. A longer incubation time did not increase the yield. This demonstration of N-OH-Trp-P-2 confirmed a previous suggestion of its formation from the base structure of the DNA modified by Trp-P-2.[4)] A recent paper claimed that this metabolite has also been found in microsomal metabolites of Trp-P-2.[6)]

Nonenzymatic binding of the metabolite to nucleic acid was examined. Under neutral conditions, the binding of N-OH-Trp-P-2 to DNA was negligible. However, under slightly acidic conditions (pH 5.5), N-OH-Trp-P-2 bound with DNA (0.3 mmoles of mutagen/mol P). After O-acetylation, the binding of the mutagen was very efficient and rapid under neutral conditions, modifying more than 1.6 mmoles/mol P (0.76% of the total guanine residues), which is about 30 times the microsome-mediated binding of Trp-P-2 to DNA.[3] This suggests that O-esterification may be involved in in vitro or in vivo modification of DNA, though the free hydroxylamine itself must also contribute to the modification to some extent. These results are in contrast with those on 2-hydroxyamino-6-methyldipyrido[1,2-a:3',2'-d]imidazole (N-OH-Glu-P-1), an active metabolite of 2-amino-6-methyldipyrido[1,2-a:3',2'-d]imidazole (Glu-P-1),[7] whose major binding occurs through O-esterification, DNA modification by free N-OH-Glu-P-1 being negligible.[7] This may reflect a difference in the heterolytic reactivities of the N-O bonds of N-OH-Trp-P-2 and N-OH-Glu-P-1, the former being more reactive.

When DNA modified by N-OH-Trp-P-2 was hydrolyzed with acid, Gua-Trp-P-2 was obtained as the major modified base (Fig. 2a, Peak-III), and its structure was confirmed by comparison of its chromatographic behavior and UV spectrum with those of authentic Gua-Trp-P-2 (Fig. 2b). During the present study, the modified base, Gua-Trp-P-2, was found to be liberated in good yield from DNA in neutral solution simply by heating at 100° for 1 hr. The same was found for the liberation of the major base modified by Glu-P-1, guanine bound Glu-P-1, from modified DNA. Thus the glycoside bonds of DNA modified by Trp-P-2 and Glu-P-1 are very weak.



Column: Polygosil $_5C_{18}$, 4.6ø x 150 mm

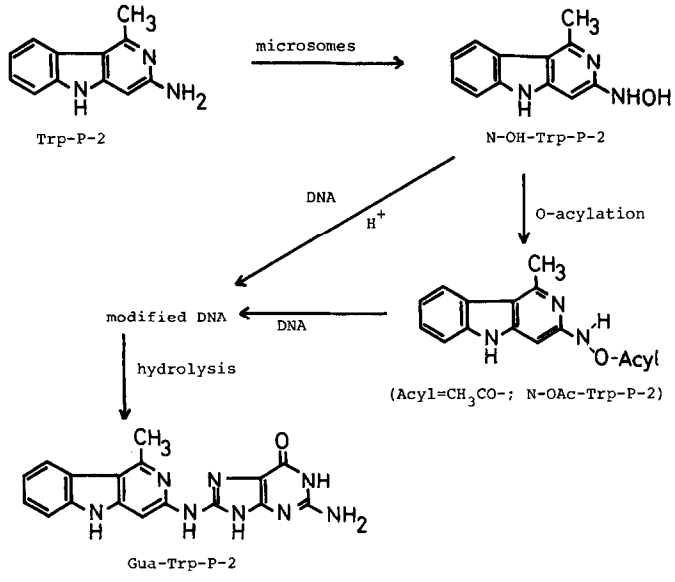
Solvent: CH_3OH(45%)-H_2)(54%)-NH_4OHaq.(1%)

Flow rate: 1.0 ml/min

Detection: Absorbance at 254 nm

Fig. 2 HPLC of bases obtained from DNA modified by N-OAc-Trp-P-2 (a) and UV spectra of the major modified base and authentic Gua-Trp-P-2 (b).

N-OAc-Trp-P-2 reacted with guanyl cytidine, and on hydrolysis the product gave Gua-Trp-P-2 in 1.7% yield. On the other hand, under similar conditions, guanylic acid was modified less than 0.3%. This suggests that intercalation of the activated mutagen into a complementary nucleotide base pair is important in the covalent reaction. We also found that N-OAc-Glu-P-1 reacts very efficiently with guanyl cytidine but poorly (about 1/100 less) with guanylic acid.



Scheme 1

It is concluded from this work that the potent mutagen Trp-P-2 is metabolized to N-OH-Trp-P-2, which reacts with DNA unchanged or after O-esterification. The activated mutagen modifies position 8 of guanine in the same manner as in the microsome mediated modification (Scheme 1). It modifies guanyl cytidine more readily than guanylic acid, suggesting that intercalation before reaction is important. The glycoside bond of the modified base is very weak. The present results are similar to those on Glu-P-1.[7, 8]

REFERENCES:

1) (a) Sugimura,T., Kawachi,T., Nagao,M., Yahagi,T., Seino,Y., Okamoto,T., Shudo,K., Kosuge,T., Tsuji,K., Wakabayashi,K., Iitaka,Y., and Itai,A. (1977) Proc. Japan Acad., 53, 58-61. (b) Kosuge,T., Tsuji,K., Wakabayashi,K., Okamoto,T., Shudo,K., Iitaka,Y., Itai,A., Sugimura,T., Kawachi,T., Nagao,M., Yahagi, T., and Seino,Y. (1978) Chem. Pharm. Bull., 26, 611-619.

2) Takayama,S., Hirakawa,T., and Sugimura,T. (1978) Proc. Japan Acad., 54, 418-421.

3) Hashimoto,Y., Takeda,K., Shudo,K., Okamoto,T., Sugimura,T., and Kosuge,T. (1978) Chem.-Biol. Interactions, 23, 137-140.
4) Hashimoto,Y., Shudo,K., and Okamoto,T. (1979) Chem. Pharm. Bull., 27, 1058-1060.
5) Takeda,K., Ohta,T., Shudo,K., Okamoto,T., Tsuji,K., and Kosuge, T. (1977) Chem. Pharm. Bull., 25, 2145-2146.
6) Yamazoe,Y., Ishii,K., Kamataki,T., Kato,R., and Sugimura,T. (1980) Chem.-Biol. Interactions, 30, 125-138.
7) Hashimoto,Y., Shudo,K., and Okamoto,T. (1979) Chem. Pharm. Bull., 27, 2532-2534.
8) Hashimoto,Y., Shudo,K., and Okamoto,T. (1980) Biochem. Biophys. Research Commun., 92, 971-976.