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METABOLIC ACTIVATION OF A MUTAGEN, 2-AMINO-6-METHYLDIPYRIDO-[1,2-a:3',2'-d]IMIDAZOLE. IDENTIFICATION OF 2-HYDROXYAMINO-6-METHYLDIPYRIDO[1,2-a:3',2'-d]IMIDAZOLE AND ITS REACTION WITH DNA

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SUMMARY:

A potent mutagen, 2-amino-6-methyldipyrido[1,2-a:3',2'-d]-imidazole(Glu-P-1), isolated from pyrolysates of L-glutamic acid and casein, was metabolically activated and bound to DNA. An activated form was identified as 2-hydroxyamino-6-methyldipyrido-[1,2-a:3',2'-d]imidazole(N-OH-Glu-P-1). Synthetic N-OH-Glu-P-1 reacted with DNA only after O-acetylation to give a modified DNA, which on hydrolysis gave 2-(C^8-guanyl)amino-6-methyldipyrido-[1,2-a:3',2'-d]imidazole(gua-Glu-P-1). The same adduct was isolated from DNA modified with Glu-P-1 by microsomes in vitro, as reported earlier.

INTRODUCTION:

2-Amino-6-methyldipyrido[1,2-a:3',2'-d]imidazole(Glu-P-1) is a potent mutagen isolated from pyrolysates of L-glutamic acid[1] and casein.[2] It shows mutagenic activity after microsomal activation, and binds to DNA only in the presence of microsomal protein.[3] The major modified base was identified as 2-(C^8-guanyl)amino-6-methyldipyrido[1,2-a:3',2'-d]imidazole (gua-Glu-P-1).[3] This suggests that the activated form leading to the modified base is probably 2-hydroxyamino-6-methyldipyrido-[1,2-a:3',2'-d]imidazole(N-OH-Glu-P-1), because N-OH-Glu-P-1 can be an electrophile and probably can attack the nucleophilic 8-position of guanine in DNA. The results in this paper indicate that N-OH-Glu-P-1 is a metabolically activated form of Glu-P-1

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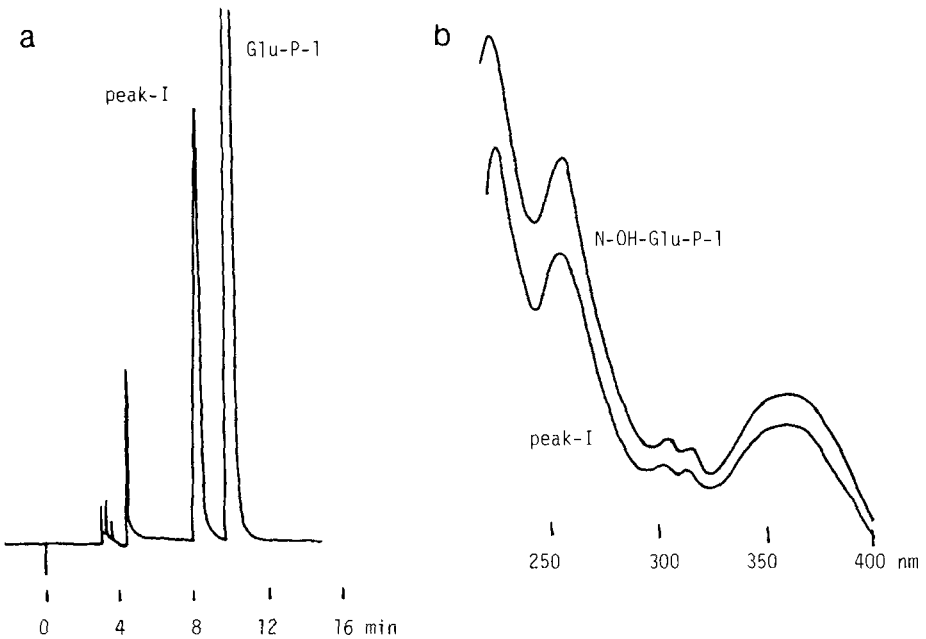
and that the major modification of DNA by N-OH-Glu-P-1 is identical to that by Glu-P-1 mediated by rat liver microsomes in vitro. A possible mechanism of enzymatic binding of Glu-P-1 is discussed.

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(100-150g) treated with polychlorinated biphenyls as described previously.[4)]

The microsomal metabolites of Glu-P-1 were obtained by in vitro incubation of a solution of Glu-P-1(HBr-salt, 0.3mg/ml) with rat liver microsomes(5mg protein/ml) and NADPH(1mg/ml) at 37°C for 10 min. For identification of N-OH-Glu-P-1, the incubation mixture was extracted with ethyl acetate and analysed by high performance liquid chromatography(HPLC, Fig. 1a). The stopped flow ultra-violet(UV) spectrum was obtained with a Shimadzu SPD-1 System, and compared with that of authentic material under the same conditions(Fig. 1b).

N-OH-Glu-P-1 was prepared from 2-nitro-6-methyldipyrido-[1,2-a:3',2'-d]imidazole, which was obtained by oxidation of Glu-P-1. The reagent used for oxidation contained 30%H_2O_2, CF_3COOH, $(CF_3CO)_2O$, CH_2Cl_2, and $Mo(CO)_6$. The reaction was performed at 0°C for 15 min. The mixture was extracted with CH_2Cl_2 after basification, and the product was separated by silica gel column chromatography, and recrystalized from ethyl acetate with a yield of about 80%. The structure was deduced by mass spectroscopy(M^+228) and NMR, and from the IR- and UV-(max. at 290 and 380 nm) spectra. Anal. Calcd. for $C_{11}H_8N_4O_2$: C, 57.86; H, 3.51; N, 24.56. Found: C, 57.82; H, 3.49; N, 24.45. Reduction of 2-nitro-6-methyldipyrido[1,2-a:3',2'-d]imidazole was performed by treatment with aluminum amalgamate in tetrahydrofuran(THF) at 0°C for 5 min. The mixture was evaporated after filtration, and the residue was washed with CH_2Cl_2, and recrystalized from n-C_6H_{14}, CH_2Cl_2, and CH_3OH. The yield was approximately 40%. The structure of the compound was deduced by mass spectroscopy (M^+214) and NMR, and from the UV-spectrum(max. at 253, 300, 312, and 364 nm). Anal. Calcd. for $C_{11}H_{10}N_4O$: C, 61.68; H, 4.71;



Column: Zorbax SIL 4.6ø x 250 mm
Solvent: CH_2Cl_2-CH_3CN-CH_3OH (75:20:5)
Flow rate: 1.0 ml/min
Detection: Absorbance at 254 nm

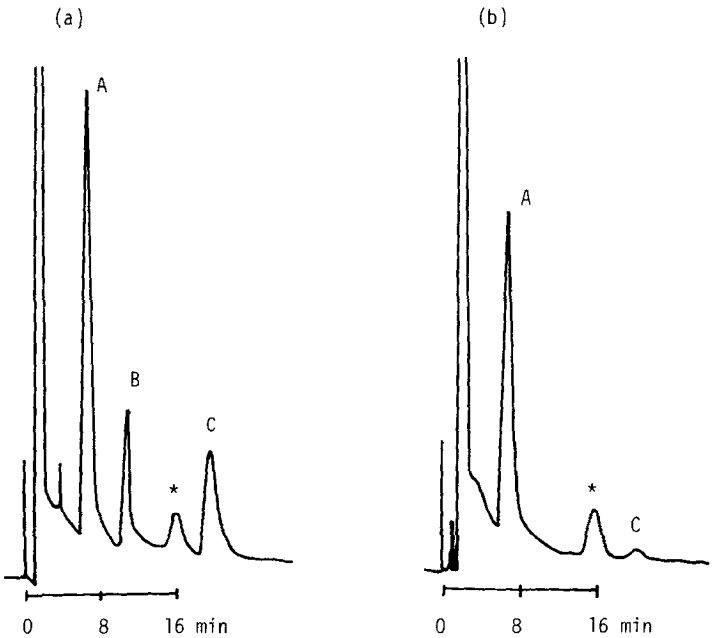
Fig. 1 (a) HPLC of microsomal metabolites of Glu-P-1; (b) UV spectra of peak-I and N-OH-Glu-P-1.

N, 26.16. Found: C, 61.96; H, 4.73; N, 25.99. Reaction of the compound with nitrosobenzene in the presence of acetic acid gave the azoxy derivative in good yield.

O-Acetylation of N-OH-Glu-P-1 was performed in THF at 0°C by bubbling with ketene gas. After most of the Glu-P-1 had disappeared, the excess ketene in solution was quenched with water. The solution was added directly to an aqueous solution of DNA without further work-up. Modified DNA was precipitated by addition of cold ethanol, and subjected to gel filtration chromatography(Sephadex G-25).

RESULTS:

HPLC analysis of the mixture of Glu-P-1 metabolites indicated that the major metabolite of Glu-P-1 is N-OH-Glu-P-1 (Fig. 1). That is, peak-I was chromatographically identical

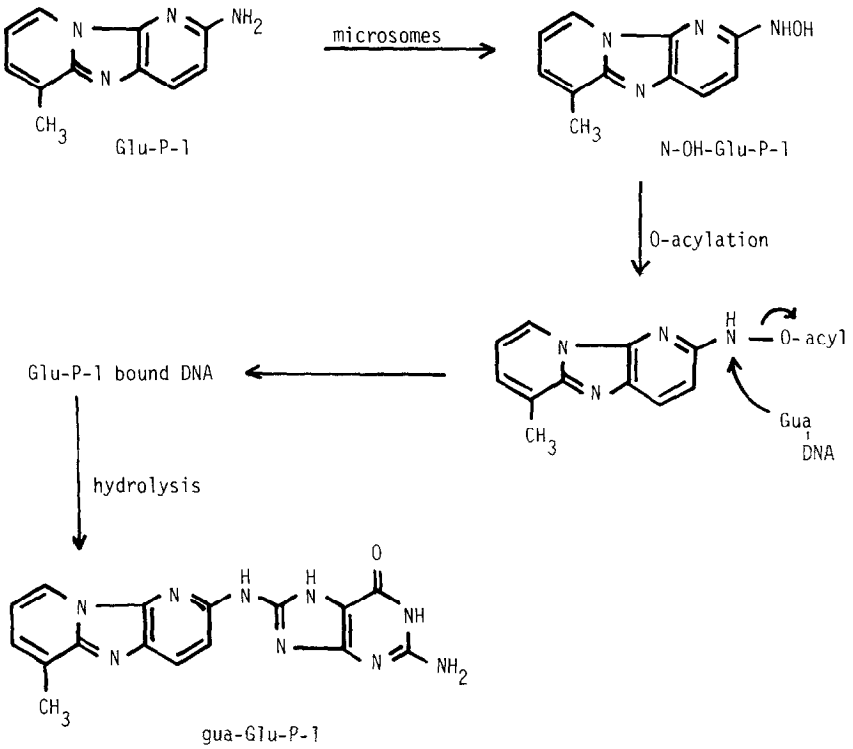


Peak-*: Glu-P-1
Column: Plygosil $_5C_{18}$, 4.6ø x 150 mm
Solvent: H_2O-CH_3OH-conc.NH_4OH (59:40:1)
Flow rate: 1.0 ml/min
Detection: Absorbance at 254 nm

Fig. 2 HPLC of bases modified by Glu-P-1 in vitro (a) and by N-OH-Glu-P-1 after O-acetylation (b).

with that of authentic N-OH-Glu-P-1. The UV-spectrum of peak-I was also identical with that of N-OH-Glu-P-1(Fig. 1b). Under the conditions of incubation used, about 20% of the Glu-P-1 was metabolized to N-OH-Glu-P-1.

No significant binding of N-OH-Glu-P-1 to DNA was observed on incubation under neutral or slightly acidic conditions. However, after its O-acetylation with ketene, N-OH-Glu-P-1 bound to DNA. More than 1 mmoles/mol P(fluorometric estimation) of N-OH-Glu-P-1 bound to DNA, which is about 40 times the microsomes-mediated binding of Glu-P-1 to DNA. When modified DNA was hydrolised enzymatically or with CF_3COOH, gua-Glu-P-1 was obtained as the major product. Gua-Glu-P-1 was identified by comparing



Scheme 1

its retention times on HPLC in several systems, and its fluorescence, excitation, and UV-spectra under acidic, neutral, and basic conditions with those of an authentic specimen synthesized as described previously.[3)]

Fig. 2 shows the chromatographic patterns of bases modified by Glu-P-1 in vitro (a), and bases modified by N-OH-Glu-P-1 after its O-acetylation (b). The yield of gua-Glu-P-1, calculated from the amount of guanotides, was approximately 2% (UV spectroscopic measurement of peak-A(Fig. 2b)). Peak-C is the other common product, whose UV-spectrum is very similar to that of Glu-P-1. (No conjugative substitution on the Glu-P-1 skeleton seems to occur.)

DISCUSSION:

The present results suggest a possible mechanism of binding of Glu-P-1 to DNA(Scheme 1). N-OH-Glu-P-1 is the major

metabolite of Glu-P-1, and binds to DNA after further activation. Consequently, N-OH-Glu-P-1 must be a proximate form of Glu-P-1 for reaction with DNA in vitro or in vivo. Though there is no evidence for biological O-acetylation of N-OH-Glu-P-1, O-esterification should be required for binding of N-OH-Glu-P-1 to DNA. Recent studies have demonstrated that some carcinogenic arylhydroxylamines react with nucleic acids after enzymatic phosphorylation or sulfonation in the presence of ATP or PAPS.[5] In the case of 4-hydroxyaminoquinoline-N-oxide, the activation of the hydroxylamino group involves a characteristic amino acylation.[6] The finding that O-acetylation is required for the binding is consistent with the postulated heterolytic cleavage of the N-O bond after O-acylation, O-phosphorylation, or O-sulfonation and attack by the guanine moiety in DNA shown in Scheme 1.

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