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Metabolic Aspects of Pyrolysis Mutagens in Food

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The first step in metabolic activation of mutagenic and carcinogenic heterocyclic amines has been elucidated to be *N*-hydroxylation by cytochrome P-448. *N*-Hydroxyamino compounds are further activated to form *N-O*-acyl derivatives that readily react with DNA. The adducts between the metabolites of Trp-P-2 and Glu-P-1 and DNA were shown to have a C^8-guanylamino structure. In the case of Glu-P-1, modification of guanine in GC clusters occurred preferentially. Glutathione transferases and myeloperoxidase were shown to inactivate some heterocyclic amines or their active metabolites. Hemin and fatty acids bind to and inactivate them. Fibers and other factors from vegetables also work to inactivate heterocyclic amines. Nitrite at low pH also degraded some heterocyclic amines, but those with an imidazole moiety were resistant. Glu-P-1 induced intestinal tumors in a high incidence when fed orally to rats. When ^{14}C-Glu-P-1 was administered by gavage into rats about 50% and 35% were excreted into feces and urine, respectively, within 24 hr. When the bile was collected, around 60% of radioactivity was excreted into it within 24 hr. In the bile, *N*-acetyl-Glu-P-1 was identified as one of the metabolites of Glu-P-1. It showed a mutagenic activity of about one fourth that of Glu-P-1 with S9 mix. Some radioactivity was also detected in the blood. At 24 hr after administration, most of the radioactivity was found to be bound to erythrocyte β-globins and serum proteins including albumin.

Metabolic Activation of Pyrolysis Mutagens

Most of these studies have been done with Trp-P-2 and Glu-P-1 by using S9 or microsomal fraction of rat liver or a reconstituted system including cytochrome P-450 by the groups of Kato and Shudo (*1–6*). The first step in activation is *N*-hydroxylation, and for this cytochrome P-448 induced by polychlorinated biphenyls or 3-methylcholanthrene was found by Ishii et al. (*1,2*) to be most effective. This was confirmed also by Watanabe et al. (*7*) using antibodies against P-450.

Hashimoto et al. found that the *N*-hydroxyamino derivative of Trp-P-2 reacted with DNA to some extent but its *N-O*-acyl derivative reacted more with DNA (*5*). In the case of Glu-P-1 also, the *N-O*-acyl derivative is regarded as an ultimate form which reacts with DNA (*4*).† The structures of the adducts of Trp-P-2 and Glu-P-1 have been elucidated to be C^8-guanylamino derivatives by Hashimoto et al. (*4,5*). They showed that this modification of guanine by Glu-P-1 occurred preferentially in GC clusters in the fragment of pBR322 plasmid DNA (*8*). This observation is interesting because the hot spot for reverse mutation in *Salmonella typhimurium* TA 98 is also GC clusters as was shown by Ames et al. (*9*). The *N*-hydroxyamino derivative of 2-amino-3-methylimidazo [4,5-*f*] quinoline (IQ) was also isolated by Okamoto et al. (*10*) and Yamazoe et al. (*11*). *N*-Hydroxylation as a step of activation was also suggested for 2-amino-9*H*-pyrido [2, 3-*b*] indole (AαC) by Niwa et al. (*12*).

For the further activation of *N*-hydroxy-Trp-P-2, the involvement of seryl or propyl tRNA synthetase has been proposed by Yamazoe et al. (*13,14*) as in the case of 4-hydroxy-aminoquinoline 1-oxide (*15*). Acetyl-CoA-dependent enzyme has also been shown by Shinohara et al. (*16*) to activate *N*-hydroxy-Trp-P-2 and *N*-hydroxy-Glu-P-1 to bind to DNA. The involvement of sulfotransferase in the activation of various heterocyclic amines was tested by Nagao et al. (*17*) by the addition of pentachlorophenol, an inhibitor of this enzyme, to the system of mutation assay. The mutagenicity of Glu-P-1 and IQ was markedly inhibited but that of Trp-P-2 was not. These results suggested that the ultimate forms of Glu-P-1 and IQ are sulfate esters of their *N*-hydroxy derivatives, but for the further activation of *N*-hydroxy-Trp-P-2, other mechanisms such as the involvement acetyl-transferases or prolyl-tRNA synthetase are responsible. The pathway of metabolic activation of heterocyclic amines as represented by Trp-P-2 is schematically illustrated in Figure 1.

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†Abbreviations used are listed in Table 1.

Table 1. Abbreviations used.

Abbreviation	Compound
Trp-P-2	3-Amino-1-methyl-5*H*-pyrido[4,3-*b*]indole
Glu-P-1	2-Amino-6-methyldipyrido[1,2-a:3′,2′-*d*]imidazole
N-Acetyl-Glu-P-1	2-*N*-Acetylamino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole
IQ	2-Amino-3-methylimidazo[4,5-*f*]quinoline
AαC	2-Amino-9*H*-pyrido[2,3-*b*]indole or 2-amino-α-carboline
Trp-P-1	3-Amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole
Glu-P-2	2-Aminodipyrido[1,2-*a*:3′,2′-*d*]imidazole
MeAαC	2-Amino-3-methyl-9*H*-pyrido[2,3-*b*]indole or 2-amino-3-methyl-α-carboline
Phe-P-1	2-Amino-5-phenylpyridine
MeIQ	2-Amino-3,4-dimethylimidazo[4,5-*f*]quinoline
MeIQx	2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline
4,8-DiMeIQx	2-Amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline
7,8-DiMeIQx	2-Amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline

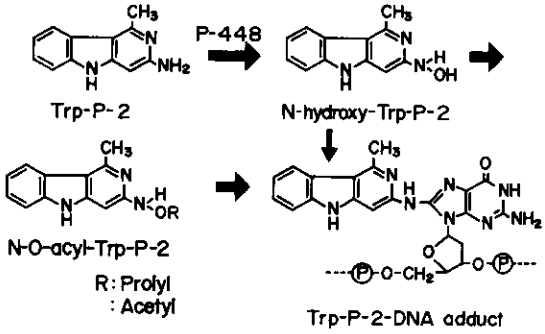

FIGURE 1. Pathway of metabolic activation of Trp-P-2.

Inactivation of Pyrolysis Mutagens

The mode of metabolic inactivation of heterocyclic amines is not fully elucidated. Glutathione transferases have been found by Saito et al. (*18*) to catalyze the conjugation of glutathione with *N*-hydroxy-Trp-P-2 to form some detoxicated products. On the other hand, there are some reports on the factors which inhibit the activity of heterocyclic amines. Hemin and other pyrrole pigments (*19*) and fatty acids such as oleic acid or linoleic acid (*20*) were shown by Hayatsu and his colleagues to inhibit the mutagenicity of heterocyclic amines by binding to them. Lipid peroxides were shown by Saito et al. (*21*) to convert an active metabolite of Trp-P-2 to nonreactive forms. Yamada et al. (*22*) found that myeloperoxidase degraded Trp-P-1, Trp-P-2, Glu-P-1, and AαC with loss of their mutagenicity in the presence of hydrogen peroxide.

Other than the above-mentioned endogenous factors there are some exogenous factors reported to inactive heterocyclic amines. They are lactoperoxidase (*22*) and peroxidases of plant origin (*22,23*). Vegetable fibers work to absorb heterocyclic amines and inhibit their mutagenicity (*24,25*). An irreversible inactivation of Trp-P-1 and Trp-P-2 by a factor with a molecular weight higher than 300,000 isolated from burdock was reported by Morita et al. (*26*). Nitrite under acidic conditions was found by Tsuda et al. (*27,28*) to inactivate some heterocyclic amines including Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2, AαC, MeAαC and Phe-P-1 (non-IQ type) through deamination. However, IQ, MeIQ, MeIQx, 4,8-DiMeIQx and 7,8-DiMeIQx (IQ type) are resistant to this treatment. This resistance is due to protonation under acidic conditions of the amino moiety of the IQ class of mutagens.

Inhibition of mutagenicity of Trp-P-1, Trp-P-2, Glu-P-1, and Glu-P-2 by retinol was reported by Busk et al. (*29*). Retinol in this case was interpreted to inhibit the metabolic activation of these heterocyclic amines. Cobaltous chloride was shown by Mochizuki and Kada (*30*) to inhibit the mutation of *S. typhimurium* TA 98 and TA 1538 by Trp-P-1. In this case cobaltous chloride did not seem to work directly on Trp-P-1 or on its metabolic activation but to inhibit the mutagenicity by improving the fidelity in DNA synthesis in the bacteria.

Metabolism of Glu-P-1 in the Rat

Glu-P-1, when fed to rats induced tumors in the liver, small and large intestines and Zymbal gland (*31*). The incidence of intestinal tumors was relatively high. Thus we were interested in the distribution and excretion of ^{14}C-Glu-P-1 which was administered orally to rats and its metabolites in the bile. [Imidazole-^{14}C]-Glu-P-1 was introduced into the stomach of male F344 rats by a gastric tube at 0.3 mCi/20.8 mg/kg body weight. At various times after administration various organs, urine, feces and blood were taken and their radioactivities were assayed. The radioactivity per unit wet weight of organ was highest in the liver through the observation period up to 48 hr, followed by the kidney. By 48 hr the radioactivity in these two organs had decreased to one fourth of the level at 5 hr. Five hours after administration, radioactivity comparable to that in the kidney was found in the mucosa of the small and large intestines. However, in these tissues, the activity remaining at 24 hr and 48 hr was very low. Cold ethanol-precipitable radioactivities in the liver homogenate were about one half that of the total activity at 5 hr and 75% at 48 hr. In the kidney they were 22% and 50% at 5 hr and 48 hr, respectively. In other organs some cold ethanol-precipitable radioactivity also remained, but actual counts were very low compared to those in the liver and kidney. The excretion of radioactivity into urine increased gradually up to 24 hr, when 35% of the administered amount had been excreted. Relatively high radioactivities bound to macromolecules in the liver

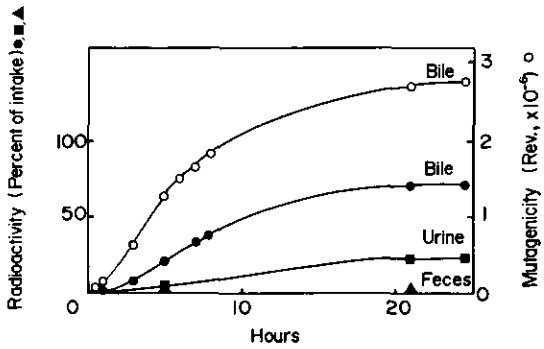

FIGURE 2. Excretions of radioactivity and mutagenicity after administration of ^{14}C-Glu-P-1 by gavage into a rat. Mutagenicity was assayed by *S. typhimurium* TA 98 with S9 mix.

throughout the observation period may be related to the high incidence of liver tumor induction by this heterocyclic amine. However, this was not the case with the kidney. Moreover, in the small and large intestines, which are also targets of Glu-P-1 carcinogenicity, the radioactivity bound to macromolecules was very low. In this study, the binding of radioactivity to DNA in various organs was not measured. To determine the correlation between the distribution of Glu-P-1 radioactivity and target organs for carcinogenicity, the modifications of DNA in different organs by Glu-P-1 should be studied.

For further studies on the mechanisms of induction of intestinal tumors by Glu-P-1 in the rat, the excretion of Glu-P-1 and its metabolites in the bile was investigated. The bile duct was cannulated, and the bile was collected after the intragastric administration of ^{14}C-Glu-P-1. As shown in Figure 2, under these conditions, the excretion of radioactivity into the bile gradually increased with time, reaching a plateau of about 60% of the administered amount at 24 hr. The excretion into the urine was about 20% at this time, and in the feces the radioactivity was almost undetectable. The mutagenic activity detectable in the bile with *S. typhimurium* TA 98 with S9 mix also increased with time, but that without S9 was only one thousandth of that with S9 mix. The bile collected for 24 hr was extracted with acetonitrile and applied to an HPLC column (LiChroprep RP-8) and eluted with acetonitrile/water (40/60, *v/v*). At least four peaks of radioactivity, named I to IV in order of elution, were separated. Significant mutagenicity with S9 mix was found only in peak IV. Peak IV was further subjected to another HPLC with an ODS column and elution with acetonitrile/water (30/70, *v/v*). Two major peaks of radioactivity were observed and those of the mutagenicity and OD_{254} coincided with these radioactivity peaks. Based on the elution position and mass and ^{1}H-NMR spectra, the material eluted as a later peak (Peak IV-2) under this condition was identified as unchanged Glu-P-1. The material at the earlier peak (peak IV-1) showed *m/z* of 240 on the mass spectrum, suggesting that it might be an N-acetyl derivative of Glu-P-1. Figure 3 shows that the ^{1}H-NMR spectrum of this material coincided with that

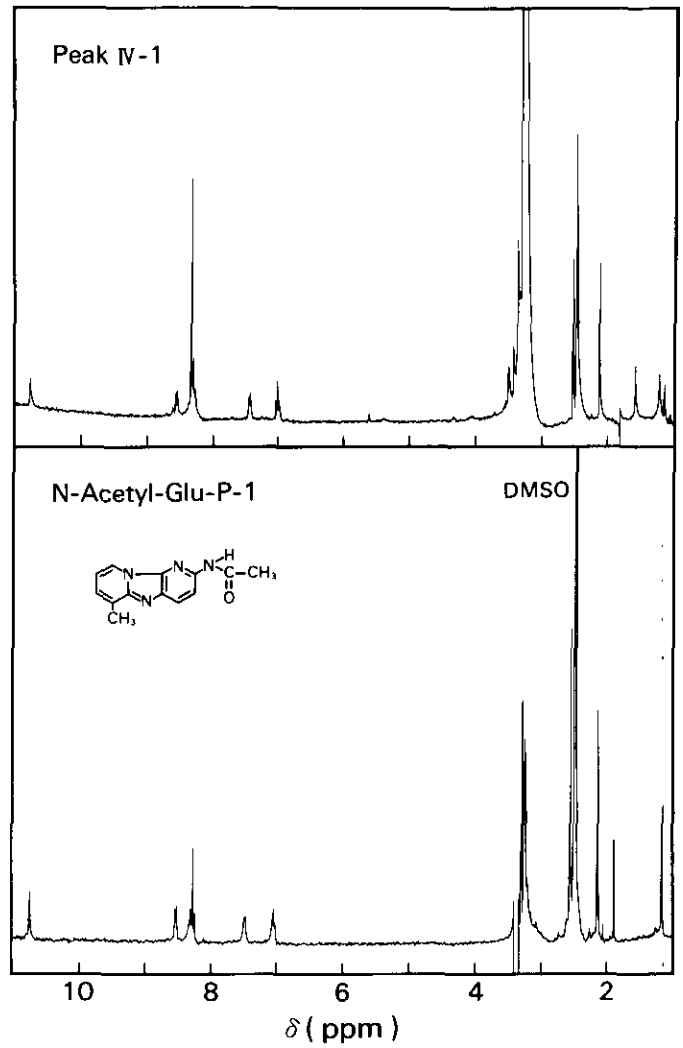

FIGURE 3. ^{1}H-NMR spectra of peak IV-1 and chemically synthesized *N*-acetyl-Glu-P-1.

of *N*-acetyl-Glu-P-1 which was synthesized chemically. The amounts of unchanged Glu-P-1 and *N*-acetyl-Glu-P-1 in 24-hr-bile were in both cases 3 to 7% of that which was administered to the rat. The mutagenic activities of chemically synthesized *N*-acetyl-Glu-P-1 were 12,800 and 740 revertants/μg toward *S. typhimurium* TA 98 and TA 100 with S9 mix, respectively. These specific activities were about one fourth those of Glu-P-1. No mutagenicity was detected without S9 mix.

Much lower mutagenicity of *N*-acetyl derivatives than the parent compounds was also observed with Trp-P-2 and 2-aminofluorene (*6*). However, 2-acetylaminofluorene, as 2-aminofluorene, is a well known carcinogen which is also activated by *N*-hydroxylation and *N-O*-acylation (*32*). Thus *N*-acetyl-Glu-P-1 excreted in the bile may also work as a carcinogen through metabolic activation similar to that for 2-acetylaminofluorene. It is not known if *N*-acetyl-Glu-P-1 and Glu-P-1 are activated in the intestinal mucosal cells and act as intestinal carcinogens. Their active metabolites formed in the liver and transported through the bile or blood may also

work at the intestinal mucosa. However, a high rate of excretion of Glu-P-1 metabolites into the bile suggests the importance of the metabolites in the bile to the carcinogenicity of Glu-P-1 in the intestine.

Binding of Metabolites of Glu-P-1 to Proteins in Erythrocytes and Plasma

At 24 hr after a single administration of ^{14}C-Glu-P-1 to rats, radioactivity which correspond to about 1% of the total administered was detectable in the blood, and this decreased with time. The blood was separated into blood cells and plasma and the radioactivity precipitated with cold ethanol or trichloroacetic acid was counted. The radioactivity in the fraction of plasma proteins reached a maximum around 24 hr and decreased to a nondetectable level 6 days later. In the blood cell fraction also, the maximum level, which was almost the same as that in the plasma protein fraction when normalized to that per unit volume of blood, was found at 24 hr. However, the decrease in the activity was gradual, and even 26 days later about half of the maximum level remained. The decay curve of the radioactivity in the blood cell fraction coincided with the lifetime erythrocytes (*33*).

From the 24-hr blood, the plasma was obtained and separated by gel filtration on Sephacryl S-300. Most of the radioactivity was found to be eluted with serum albumin. Some radioactivity was found also in fractions of α_2-macroglobulin and immunoglobulins.

Erythrocytes from the same blood sample were collected and washed. Most of the radioactivity was recovered in the supernatant of the hemolysate. From this supernatant, hemoglobin was crystallized (*34*), with the recovery of 85% of both protein and radioactivity. This hemoglobin was solubilized, and globin was separated from heme (*35*). The radioactivity was mostly recovered in the globin fraction. Globin was separated into its subunits by CM-cellulose column chromatography (*36*), and it was found that most of radioactivity was bound to the β chains. Distribution of radioactivity showed 21%, 47%, and 13% for $^{I}\beta$, $^{II}\beta$, and $^{III}\beta$ chains, respectively and only 6% and 5% for $^{I}\alpha$ and $^{II}\alpha$ chains, respectively. Under the conditions employed, the binding of Glu-P-1 metabolite to β chains was one molecule per 300 to 650 molecules. The nature of binding of Glu-P-1 metabolite to β-globin chains and albumin is not clear at present.

Binding of Glu-P-1 metabolite to hemoglobin and serum proteins may occur during the passage of the blood through the liver. Binding to serum proteins, such as albumin, secreted from the liver may also take place inside hepatocytes where Glu-P-1 is activated. A preferential binding of Glu-P-1 metabolite to β-globins may be due to the presence of reactive amino acid residue(s) in the β chains in positions highly accessible to Glu-P-1 metabolite. Such a preferential binding to β-globin has also been observed with simetryn sulfoxide, an *in vivo* metabolite of a herbicide simetryn, which modifies the cysteine-125 residue of the rat β-globin chain (*37*). Adduct formation with hemoglobin has also been reported for various mutagens and carcinogens (*38–42*), and the use of some of these adducts for the dosimetry of exposure of humans to chemicals in the occupational environment has been proposed (*39,42*).

Blood samples are easy to obtain and large amounts of hemoglobin and albumin are available from a small amount of blood. Thus, if a highly sensitive method to measure the adducts of heterocyclic amines to these proteins is developed like that for carcinogen–DNA adducts (*43*), it may serve as a good tool for the dosimetry of actual exposure of humans to the mutagenic and carcinogenic compounds in cooked foods.

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