Mutagenic Activity of Heterocyclic Amines in Cooked Foods

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Mutagenic heterocyclic amines are generated in foods when they are cooked at temperatures over 150°C. These compounds are present from 0.1 to 50 ppb, depending on the food and cooking conditions. These heterocyclic amines are not only present in cooked red meat, fish, and chicken, but are also present at lower levels in baked and fried foods derived from grain. Mutagenicity of fried beef hamburgers cooked at 230°C is 800 ± 37 TA98 revertants per gram cooked weight. We measured 2-amino-3,8-dimethylimidazo[4,5-f]quinoline (MelOx), 2-amino-3,4,8-trimethylimi- dazo[4,5-f]quinoline (IQ), and 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) formation at this temperature and found 3.0 ± 2.0, 1.0 ± 0.18, and 0.06 ± 0.03 ng/g, respectively. 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhiP) was found at a higher concentration of 9.6 ng/g. In our laboratory we have shown these heterocyclic amines are capable of producing both reverse and forward mutations in Salmonella bacteria and forward mutations in Chinese hamster ovary cells (CHO). We have also been able to show a statistically significant increase in mutations in the pancreas of the "mutamouse" following PhiP exposure. The pancreas also shows relatively high DNA binding compared to other organs in the mouse. The number and type of mutations depend on the repair capacity of the cells for both Salmonella and CHO. In Salmonella the mutations are primarily 2-base deletions when the cells lack uvrB repair, but mutations are more complex (larger deletions and insertions) but lower in frequency when repair is functional. Efforts are now under way to assess specific sequence changes in the aprt gene in mutant CHO cells. — Environ Health Perspect 102(Suppl 6):201–204 (1994)

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Introduction

The etiology of human cancer has prompted the search for compounds in common foods in our diet that may act as tumor initiators by producing somatic cell mutations. Analyses of pyrolyzed amino acids and proteins and of cooked high-protein foods have led to the discovery of several classes of highly mutagenic heterocyclic amine compounds (1,2). Our laboratory has been concerned with the mechanisms of formation, the chemical identity, and the spectrum of genotoxicity of these mutagenic agents for more than 10 years (3,4) and this review will highlight some of our important results as well as some recent findings.

Analysis of Salmonella mutagens in major sources of cooked protein in the American diet (based on United States Department of Agriculture and United States Department of Health, Education and Welfare surveys) showed significant mutagen content in beef, eggs, pork, ham, and bacon, and a somewhat lesser amount in chicken and fish that has been fried or broiled (3). Tofu, beans, cheese, and some fish, when cooked under similar conditions, produced low or negligible mutagenic activity (6). Specific mutagen (in the case of these foods, heterocyclic amine) isolation has been improved dramatically from our original aqueous extraction method (pH 2 homogenization/extraction) followed by absorption-elution of mutagens on XAD-2 resin (7) by the methods of Gross (8) and of Murray (9). Although the original method allowed for the discovery of TMIP, IQx, DMIP, and IFP (4), the method of Gross, for example, allows for good quantification from small samples, and 1- to 2-day turnaround for results.

Mutagen Occurrence and Analysis

The quicker analysis (8) allows for direct comparison of the mass amount of each mutagen and its calculated mutagenicity (based on the standard specific activity of the compound) to the actual mutagenic activity of the food, based on the Ames test. This is a check on the ability of the analytic methods and the Ames/Salmonella test to predict, from a very complicated chemical matrix, the mutagen content of cooked food. Mutagen production in beef has been examined at different temperatures. Although total mutagenic activity increases dramatically with increasing temperature, chromatographic analysis shows that the relative amounts of the mutagenic peaks are generally similar (10), and that mutagen mass also increases proportionately (Table 1). An exception is the compound 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhiP). This mutagen is produced at greater concentrations at higher temperatures relative to the other heterocyclic amines.

Recently, we have assessed the mutagenicity of a number of foods derived from grain products. Commercially available

| Table 1. Mutagen content (expressed as ng/g) of fried beef patties cooked at various times and temperatures. |
|---------------------------------------------------------------|
| Mutagen          | Cooking temperature of grill | |
|                  | 150°C | 190°C | 230°C |
| IQ                |       |       |       |
| 4 min             | <0.1² | 0.1   | 0.15  |
| 6 min             | 0.1   | 0.45  | 0.25  |
| 10 min            | 1.5   | 0.85  | 1.8   |
| MelOx             |       |       |       |
| 4 min             | <0.1  | 0.25  | 0.4   |
| 6 min             | 0.15  | 1.3   | 1.1   |
| 10 min            | 2.7   | 5.1   | 8.0   |
| PhiP              |       |       |       |
| 4 min             | <0.1  | 0.15  | 1.4   |
| 6 min             | 0.25  | 1.9   | 4.1   |
| 10 min            | 0.9   | 6.0   | 21.5  |

²Method of Gross (8) ²Below the statistically significant detection level of 0.1 ng/g.
breadsticks, for example, have less than 5 revertants per gram in *Salmonella* strain TA98 (based on a 5 point dose-response curve), but when cooked at twice the manufacturer’s recommended time they have more than 40 revertants per gram (20% of the activity of a hamburger fried 6 min per side at 210°C) (Table 2). We have also seen up to 30 revertants per gram in commercially available meat substitutes when they are fried as patties. Both types of foods are high in gluten (wheat protein), and gluten has been found to be quite mutagenic when heated (11). The mutagenicity is primarily S9 dependent and greatest in *Salmonella* strain TA98. The reason for the mutagenicity in the absence of creatine/creatine (the guanidyl donor to heterocyclic amines in muscle meat) is under investigation in our laboratory. Preliminary data suggest that the mutagens may be heterocyclic amines, but they appear distinct in structure from the 18 previously described mutagens derived from cooked muscle meat products.

### Table 2. Mutagenic activity of heated grain products.

| Product          | Time and temperature as directed | Twice time directed |
|------------------|----------------------------------|---------------------|
| White bread      | 2°                              | 5                   |
| Pumpernickel bread | 6                               | 28                  |
| Breadstixs       | 6                               | 40                  |
| Crescent roll    | 1                               | 4                   |
| Pizza crust      | 3                               | 8                   |
| Graham cracker   | 5°                              | –                   |

*Derived from 4 point dose-response curves (2 plates each point). Standard Ames/Salmonella plate incorporation assay. All samples prepared by the method of Gross (8). *As purchased.

### Table 3. Role of DNA repair for heterocyclic amine response in the Ames/Salmonella assay (hisD gene).

| Strain | Large deletions | 2 base slippage (−CG) | Large deletions | 2 base slippage (−CG) |
|--------|-----------------|-----------------------|-----------------|-----------------------|
| TA1538* | 0               | 100                   | 37              | 63                    |
| TA98*   | 0               | 100                   | 30              | 70                    |
| TA1978c | 42              | 58                    | 100             | 0                     |

*uvrB repair deficient. †uvrB repair deficient, pkM101 plasma (error prone repair). ‡uvrB competent. † Two of these lesions were complex deletions and insertions.

Modelling of Mutagen-Forming Reactions

To identify the precursors and to elucidate the reaction conditions that yield mutagens in foods, we first used as a model reaction mixture a supernatant fraction prepared by centrifugation of beef steak homogenized in water. Compounds (less than 500 daltons) in the supernatant are the major precursors of *Salmonella* mutagenic activity in beef muscle. Incorporation of the dried supernatant fraction into meat shows that it contains the precursors for mutagen formation because it increases the mutagenic activity of fried ground beef. Boiling the beef supernatant results in the formation of 2-amino-3-methylimidazo[4,5-f]quinaline (MeIQ) (12). Aqueous pressure heating or oven baking the beef supernatant greatly elevates TA1538 activity, giving 45,000 revertants/g of beef; aqueous pressure heating at 300°C gave 64,000 revertants/g of beef; dry heating at 300°C gave 190,000 revertants/g of beef (12). Once the soluble supernatant precursors are concentrated to a freeze-dried powder, aqueous conditions are not essential for heterocyclic amine mutagen formation at ordinary cooking temperatures. Our findings with beef supernatants indicate that water is actually an inhibitor of IQ-type mutagen formation. While frying ground beef patties, water serves as a vehicle that transports precursors to the hot, drying contact surfaces. Importantly, brief microwave irradiation, which releases both water and the rate-limiting beef precursors, shows promise as a simple means to lower the mutagenic activity of subsequently fried patties (13,14).

Cretine was shown to be a precursor for mutagen production in beef and chicken. A 10-fold increase in cretine over the endogenous level prior to frying gave a 2- to 3-fold increase in total mutagenic activity. Chromatographic analysis of the mutagens produced showed a general increase of all of the mutagens types, suggesting that cretine is responsible for the imidazoline ring that is common to all of the beef mutagens identified to date (15). The most mass-abundant beef mutagen, PhIP, has been efficiently modeled from the simple dry heating of creatine and phenylalanine. Modeling experiments using isotopically labeled creatine or phenylalanine heated together at 200°C indicated that the atoms within each precursor molecule are present in PhIP as expected from the proposed route of formation (14). Dry heating the meat components creatine, glutamic acid, and glucose enabled us to identify a diaminomethylimidazobenzopyranone mutagen (mw = 243) as a result that shows the usefulness of model reactions for mutagen identification (16). In addition, the presence of TMIP has been reported in a dried mixture of threonine and creatine (along with other mutagens), and may be present in a high enough concentration to be identified in a reaction mixture.

**Genetic Toxicology**

**Microbial Mutagenesis**

De Meester has evaluated a number of food mutagens and analogs for microbial genotoxicity (17). All the heterocyclic amines, with the exception of the PhIP isomers and some carbolines, are extremely potent mutagens, active below 1 ng/plate in Ames/Salmonella tester strain TA1538 in the presence of induced liver S9 or microsomal preparations from rat, mouse, or hamster. They are also potent mutagens in strains TA98 and TA97, moderately active in TA1537, weakly active in TA100, and virtually inactive in TA1535 and TA102 (14). PhIP and its 3-methyl isomer are 100 to 10,000 times less potent than the imidazoquinoline and imidazoxinoline mutagens. But in comparison to the carcinogen benz[a]pyrene (B[a]P), PhIP is still 10 times more potent. All of the heterocyclic amines tested show frameshift mutagenic activity, but in some cases cause base substitution mutations at 100- to 1000-fold higher concentrations. In addition, they are 100-fold less active in the *uvrB* repair-proficient strain TA1978 (18).

**DNA Sequence Changes Induced by Heterocyclic Amines**

Analysis of the sequence changes in *Salmonella* after exposure to IQ, MeIQ, and PhIP shows that all induced mutations in TA1538 and TA98 are caused by a single -CG- deletion in a run of -CG-s. Levine et al. (19) suggest that a C8dG adduct can cause a 2-base slippage in the positive strand in the 8-base run of alternating -CG-s in the hisD gene. When revertants are examined in the repair-competent TA1978 strain, nearly 50% of the sequence changes are large deletions and insertions (Table 3). One explanation is the *uvrB* repair system, which is functional in this strain and can remove the lesions that normally lead to the -CG- deletion. A 100-
fold higher concentration of mutagen will give equivalent mutagenicity to that seen in the repair-deficient strain. PAHs like B[a]P behave much differently from the heterocyclic amines in this system, causing many more large deletions, even when the worB repair system is not functional (20).

**Mixing Microbial and Mammalian Metabolism**

Mutagenicity of IQ and PhIP have been shown to be quite high in studies using *Salmonella* TA1538 and S9 preparations from Chinese hamster ovary (CHO) cells expressing only P4501A2 derived from mice (P3 form) (21). This observation suggests that enzymes are needed from both types of cells to make the mutagenic intermediates, because neither cell is capable of carrying out the metabolism (especially of IQ) very well alone. Moreover, with *Salmonella* strains deficient for N-acetylation, a loss of mutagenicity for IQ and MelQx, but not for PhIP, suggests differences in activation for PhIP and other heterocyclic amines (22, Josephy, personal communication).

**In Vitro Mammalian Toxicology**

Trp-P-2 and PhIP showed cytotoxic, mutagenic (hppt and aprt loci), and chromosome damage (SCEs and aberrations) at doses as low as 1 μg/ml in CHO cells treated in the presence of liver S9 from PCB-induced hamsters (23). An excision-repair-deficient cell line (UV5) was more than twice as sensitive as the parental line (AA8) for both mutagens. Dose-related responses for IQ mutagenesis and SCE formation occurred in UV5 cells up to 30 μg/ml, but chromosomal aberrations were not significantly increased. No such responses occurred in the repair proficient cell line AA8 up to 300 μg/ml. Data for MelQx indicate that cytotoxicity occurs at approximately 150 μg/ml and above 300 μg/ml for MelQx. Thus, the cytotoxicity and genotoxicity of these representative thermic mutagens in CHO cells are inversely proportional to their responses in *Salmonella*, where MelQ and MelQx are extremely potent (23).

In order to evaluate these response differences with respect to DNA binding, both *Salmonella* and CHO cells were treated with radiolabeled Trp-P-2 and IQ, and DNA was purified for radioiodinated DNA adducts. Approximately 2 adducts per million bases were generated with 5 mM Trp-P-2 in both *Salmonella* and CHO cells; the induced mutation frequencies were found to be about equal at 25 to 30 per million surviving cells. Five millimolar IQ resulted in the same adduct level in *Salmonella* as Trp-P-2, but induced mutations were about 5 per million cells (one-fifth of that induced by Trp-P-2). The IQ concentration required to yield the Trp-P-2 level of two adducts per million bases was 132 mM (25 times greater). Under these conditions, the induced mutation frequency was 15 mutations per million surviving cells, 3-fold above the frequency found with *Salmonella*. From these results we conclude that, at the concentrations needed to give approximately similar numbers of DNA adducts, the two agents have similar mutagenic efficiencies (mutations per adduct) in both assay systems (24). Thus, the mutation frequency is directly proportional to the number of adducts formed. This appears to be related to the number of active intermediates formed, but could also be related to repair of specific DNA adducts.

These experiments do not explain the unexpectedly low mammalian genetic toxicity of IQ, but suggest CHO cells have different IQ metabolism that results in a weaker biologic response compared to *Salmonella*. Recent studies suggest CHO cells may lack the metabolic pathway N/O-acetyltransferase (C. King, personal communication) that functions in *Salmonella* strains TA98 and TA1538. In fact, the requirement for acetylation may explain the low biologic activity of the quinoline and quinoxaline compounds in these cells.

**In Vivo Mammalian Toxicology**

C57BL/6 mice pretreated with Aroclor 1254 showed LD₅₀ values of 15 mg/kg for Trp-P-2 and 150 mg/kg for IQ. Trp-P-2 was considerably more toxic than IQ in vivo, a result consistent with observations in vitro with CHO cells. Dose-dependent responses were obtained for SCEs in mouse bone marrow with Trp-P-2 at 1.2 to 20 mg/kg, and with IQ at 20 to 160 mg/kg. In both cases there was a plateau response at high doses. Chromosomal aberrations (chromosome and chromatid exchanges and deletions) in mouse bone marrow were dose-dependent with Trp-P-2 from 4 to 20 mg/kg; the response with IQ was negligible (23). Again, Trp-P-2 appears to be more potent in mammalian systems. Both PhIP and MelQx induced SCEs in vivo, but only aberrations were significantly increased with PhIP in peripheral blood lymphocytes (25,26). These data suggest reactive electrophiles are present extrahepatically and are able to react with DNA in peripheral tissues. This conclusion is supported by studies in mice and rats after long-term feeding bioassays with PhIP (27,28), as more nonhepatic tumors are seen in lymphocytic, colon, and mammary gland tissues. These conclusions are also supported by 32P-postlabeling experiments from several laboratories including our own. DNA binding is pronounced in the liver following exposure to IQ, MelQx, and DiMeIQx, but PhIP shows higher binding in nonhepatic tissues (2,26).

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