

Urinary Mutagenesis and Fried Red Meat Intake: Influence of Cooking Temperature, Phenotype, and Genotype of Metabolizing Enzymes in a Controlled Feeding Study

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Meat cooked at high temperatures contains potential carcinogenic compounds, such as heterocyclic amines (HCAs) and polycyclic aromatic hydrocarbons (PAHs). Samples from a 2-week controlled feeding study were used to examine the relationship between the intake of mutagenicity from meat fried at different temperatures and the levels of mutagenicity subsequently detected in urine, as well as the influence of the genotype of drug metabolizing enzymes on urinary mutagenicity. Sixty subjects consumed ground beef patties fried at low temperature (100°C) for 1 week, followed by ground beef patties fried at high temperature (250°C) the second week. Mutagenicity in the meat was assayed in *Salmonella typhimurium* TA98 (+S9), and urinary mutagenicity was determined using *Salmonella* YG1024 (+S9). Genotypes for *NAT1*, *NAT2*, *GSTM1*, and *UGT1A1* were analyzed using blood samples from the subjects. Meat fried at 100°C was not mutagenic, whereas meat fried at 250°C was mutagenic (1023 rev/g). Unhydrolyzed and hydrolyzed urine samples were 22× and 131× more mutagenic, respectively, when subjects consumed red meat fried at 250°C compared with red meat fried at 100°C. We found that hydrolyzed urine was ~8× more mutagenic than unhydrolyzed urine, likely due to the deconjugation of mutagens from glucuronide. The intake of meat cooked at high temperature correlated with the muta-

genicity of unhydrolyzed urine ($r = 0.32$, $P = 0.01$) and hydrolyzed urine ($r = 0.34$, $P = 0.008$). Mutagenicity in unhydrolyzed urine was not influenced by *NAT1*, *NAT2*, or *GSTM1* genotypes. However, a *UGT1A1**28 polymorphism that reduced *UGT1A1* expression and conjugation modified the effect of intake of meat cooked at high temperature on mutagenicity of unhydrolyzed urine (P for interaction = 0.04). These mutagenicity data were also compared with previously determined levels of HCAs (measured as MeIQx, DiMeIQx, and PhIP) and polycyclic aromatic hydrocarbons (PAHs) in the meat, levels of HCAs in the urine, and CYP1A2 and NAT2 phenotypes. The levels of mutagenicity in the meat fried at low and high temperatures correlated with levels of HCAs, but not levels of PAHs, in the meat. Also, levels of mutagenicity in unhydrolyzed urine correlated with levels of MeIQx in unhydrolyzed urine ($r = 0.36$; $P = 0.01$), and the levels of mutagenicity of hydrolyzed urine correlated with levels of MeIQx ($r = 0.34$; $P = 0.01$) and PhIP ($r = 0.43$; $P = 0.001$) of hydrolyzed urine. Mutagenicity in unhydrolyzed urine was not influenced by either the CYP1A2 or NAT2 phenotype. The data from this study indicate that urinary mutagenicity correlates with mutagenic exposure from cooked meat and can potentially be used as a marker in etiological studies on cancer. *Environ. Mol. Mutagen.* 43:53–74, 2004. Published 2004 Wiley-Liss, Inc.†

Key words: fried red meat; urinary mutagenicity; heterocyclic amines; genotype; phenotype

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INTRODUCTION

During the quarter century since Nagao et al. [1977] discovered that the charred surface of broiled fish or beef was mutagenic, numerous studies have confirmed and extended these initial observations to many other types of foods and cooking methods [Sugimura et al., 1988; Doolittle et al., 1989; Knize et al., 1994; Felton et al., 2002]. The mutagenic activity of cooked meat has been shown to increase with different types of cooking methods [Dolara et al., 1979; Knize et al., 1985; Laser-Reutersward et al., 1987a,b; Doolittle et al., 1989], and increased cooking temperature has been shown to increase the formation of heterocyclic amines (HCAs) in meats and amino acids [Gross and Gruter, 1992; Knize et al., 1994; Sinha et al., 1998a,b].

HCAs are a family of mutagens/carcinogens formed during the cooking of meats that are pyrolysis products of aromatic amino acids [Sugimura et al., 1988]. Although various mutagens, such as polycyclic aromatic hydrocarbons (PAHs), may be formed during the cooking of meat by grilling [Knize et al., 1999], we have shown that frying meat at low temperature (100°C) versus high temperature (250°C) can produce meat with virtually identical low PAH levels; in contrast, HCA levels are undetectable in the ground beef fried at low temperature but high in the meat fried as patties at high temperature [Sinha et al., 1994]. Chemical and mutagenicity analyses of cooked meat have suggested that HCAs are the primary chemical class responsible for the observed mutagenic activity of cooked meat [Sugimura et al., 1988; Felton et al., 2002].

Five years after the discovery that cooked meat was mutagenic, Baker et al. [1982] demonstrated that consumption of cooked meat resulted in mutagenic urine in humans. This observation has been confirmed and extended by others [Dolara et al., 1984; Hayatsu et al., 1985; Sousa et al., 1985; Baker et al., 1986; Ohshima et al., 1987a,b; Doolittle et al., 1989; Hayatsu and Hayatsu, 1993; DeMarini et al., 1997; Gabbani et al., 1998; Johansson et al., 1998; Murray et al., 2001; Pavanello et al., 2002]. With the exception of one study involving 32 subjects [Gabbani et al., 1998] and another involving 50 [Pavanello et al., 2002], these were small studies involving 3–21 subjects that examined variables such as the type of meat or cooking method as well as the kinetics of urinary mutagenicity. One study examined the levels of HCAs in the meat consumed and in the resulting urine [Murray et al., 2001]; however, no study has examined collectively the effect of high versus low cooking temperatures on the concentrations of HCAs and PAHs in the meat and the resulting urinary levels of HCAs and mutagenicity.

Four studies have examined the influence of either *N*-acetyltransferase (*NAT2*), glutathione *S*-transferase M1 (*GSTM1*), or cytochrome P4501A2 (*CYP1A2*) genotypes or *NAT2* and *CYP1A2* phenotypes on urinary mutagenicity after consumption of cooked meat [DeMarini et al., 1997;

Gabbani et al., 1998; Murray et al., 2001; Pavanello et al., 2002]. Although these studies have suggested a role for *NAT2* and *CYP1A2* activities, a study with larger numbers of subjects in a controlled feeding study would be helpful to confirm a modifying effect for these two metabolic enzymes on urinary mutagenicity.

Previously, we conducted a controlled feeding study involving 66 subjects over a 2-week period who ingested meat fried at low temperature for the first week and meat fried at high temperature during the second week. From this study, we found that the PAH levels of the meats were low and similar in the first and second week of the feeding study, whereas the HCA levels were undetectable in low-temperature fried meat consumed in the first week but high in high-temperature fried meat consumed in the second week [Sinha et al., 1994]. In addition, we also showed that *NAT2* activity remained unchanged throughout the study, whereas *CYP1A2* activity increased in 72% of the subjects after they consumed meat cooked at high temperature. Thus, consumption of such meat induces *CYP1A2* activity in humans.

We further reported from this feeding study on the levels of 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) in unhydrolyzed urine [Sinha et al., 1995] and the levels of MeIQx and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in acid-hydrolyzed urine [Stillwell et al., 1997] from the subjects who consumed meat fried at high temperatures during the second week. The levels of MeIQx metabolites and *NAT2* and *CYP1A2* activities in these subjects were also determined [Sinha et al., 1994, 1995; Stillwell et al., 1997, 1999]. These analyses showed that in humans, *CYP1A2* metabolizes MeIQx more than it does PhIP and that *NAT2* does not metabolize either HCA.

We present the remaining data from this feeding study, which address some of the outstanding issues in the literature regarding (1) the role of PAHs versus HCAs from cooked meat in urinary mutagenicity, (2) the relationship of cooking temperature alone to urinary mutagenicity, and (3) the influence of various metabolic polymorphisms on urinary mutagenicity. We report the intake of mutagenicity from meat fried at low versus high temperatures as well as the measured mutagenicity of hydrolyzed and unhydrolyzed urine samples collected at various times during the 2-week study. We also report on the genotypes of subjects for *NAT1* and *NAT2* (four alleles for each), *GSTM1*, and the number of polymorphic TA repeats in the TATA box motif in *UGT1A1**A and examine the modifying effect of these genotypes on urinary mutagenicity.

Based on the data presented in this report, along with data we have published previously from this feeding study, we examined the association of urinary mutagenicity with (1) the intake of mutagenicity from meat fried at low and high temperatures, (2) the HCA and PAH levels of the meats, (3) the levels of HCAs in unhydrolyzed and hydrolyzed urine, and (4) the phenotypic variants of *CYP1A2* and *NAT2* and genotypic variants of *NAT1*, *NAT2*, *UGT1A1*, and *GSTM1*.

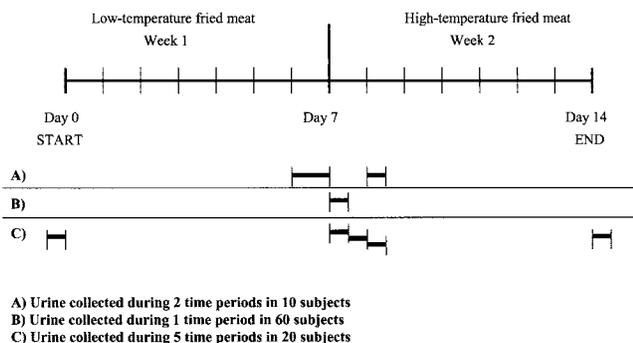


Fig. 1. Study design indicating periods of urine collections used to analyze urinary mutagenicity.

MATERIALS AND METHODS

Study Population and Design

The study design has been described previously [Sinha et al., 1994, 1995]. Briefly, 66 healthy, nonsmoking volunteers (33 men and 33 women) were included in the study, which was conducted at the Human Nutrition Research Center in Beltsville, MD, in 1992. Subjects ate a controlled diet throughout the 14 days of the study. During the first week, the evening meal contained pan-fried, ground-beef patties cooked at low temperature (100°C for 20 min and baked at 90°C for 20 min to reduce moisture), whereas during the consecutive second week, the ground beef was pan-fried as patties at 250°C for 22 min. The amount of meat consumed was adjusted for body weight (3.1 to 4.4 g of meat/kg body weight/day).

Multiple urine samples were collected throughout the 2 weeks of the study. These urine samples were collected for periods of 0–12 hr, 12–24 hr, or 0–24 hr after consumption of fried red meat. Urinary mutagenicity was measured in the following urine samples (Fig. 1): In a pilot phase (A), we first measured urinary mutagenicity in 10 subjects in urine collected during the following two different time periods: the first 24 hr after consumption of meat fried at low temperature on the last day of the first week; and the first 12 hr after consumption of meat fried at high temperature on the second day of the second week. Thereafter (B), we measured urinary mutagenicity from 0–12 hr after consumption of the first meal containing meat fried at high temperature in all subjects. Because of the amount of urine available when this study was conducted, urinary mutagenicity was measured in only 60 of the 66 subjects. To investigate within- and between-individual variability (C), we analyzed urinary mutagenicity in urine collected from 20 subjects during the following 5 time intervals: (1) 0–12 hr before initiation of the study, (2) 0–12 hr after consumption of the first meal containing meat fried at high temperature, (3) 12–24 hr after consumption of the first meal containing meat fried at high temperature, (4) 0–12 hr after consumption of the second meal containing meat fried at high temperature, and (5) 0–12 hr after consumption of the 6th meal containing meat fried at high temperature.

The study was approved by the Institutional Review Boards of the National Cancer Institute, Georgetown University, the Human Studies Review Committee of the U.S. Department of Agriculture, and the Human Subjects Research Review Official of the U.S. Environmental Protection Agency. Informed consent was obtained from all subjects.

Mutagenicity of Cooked Meat and Urine

The mutagenic activity of the cooked meat extracts was determined in *Salmonella* strain TA98 (*hisD3052*, *rfa*, Δ *uvrB*, pKM101) + S9 produced from Aroclor-induced rats (2 mg of S9 protein/plate) in the standard plate-incorporation assay as described [Knize et al., 1995]. Briefly, 25 g of

cooked beef was homogenized in 75 ml of 1-N NaOH, and 20 g of the homogenate was mixed with 20 g of diatomaceous earth material. The mixture was placed in a column, and the organics were extracted into coupled cation exchange Bond-Elute brand (Varian, Harbor City, CA) propylsulfonic acid (PRS) cartridges with 40 ml of a dichloromethane/toluene solution (95:5, v/v). The organics were then eluted from the cartridges with 2 ml of MeOH-NH₄OH (9:1), evaporated to dryness, and dissolved in 400 μ l of dimethylsulfoxide (DMSO) to make a concentrate containing 0.0125 g-equivalent/ μ l. The extract was tested in duplicate plates at 5, 10, 25, 50, and 100 μ l/plate, which represented a range of 0.06–1.25 g-eq/plate. The mutagenic potencies of the meat samples were determined by calculating the slope (rev/g-eq) of the linear portion of the dose-response curves. The positive control, 2-aminoanthracene, gave 800–1,200 rev/ μ g; the negative control, DMSO, gave 30–45 rev/plate. The laboratory was blinded to the type of meat. In addition, several blinded quality controls of beef patties fried at either low or high temperatures in the same way as the meat consumed in this study were included in the analysis (Appendix A).

Urinary mutagenicity was determined in blinded samples as described previously [DeMarini et al., 1997]. Briefly, urine (60 ml) was passed through C18 resin, and the organics were eluted by methanol. A portion of this extract was then solvent-exchanged into DMSO at 150 \times for bioassay and represented unhydrolyzed (free) urinary mutagenicity. The remaining portion of the extract was dissolved in water and hydrolyzed at 70°C for 6 hr in 6-N HCl at a 1:6 ratio of HCl to urine extract. The hydrolysate was neutralized with 6 N NaOH and Na₂CO₃, and the organics were extracted by C18/methanol and solvent-exchanged as described above [DeMarini et al., 1997]. This represented hydrolyzed urinary mutagenicity (i.e., a combination of free and previously conjugated urinary mutagenicity).

Because of the amount of urine available for mutagenicity evaluation, the extracts were evaluated once in single plates/dose at 0.5, 1, 3, 7, and 14 ml-equivalents/plate in the standard *Salmonella* mutagenicity plate-incorporation assay in the presence of Aroclor-induced rat liver S9 (Moltox, Boone, NC) (2 mg of S9 protein/plate). We used strain YG1024, which is derived from the frameshift strain TA98 and contains acetyltransferase activity, making it especially sensitive to aromatic amines and HCAs [Watanabe et al., 1990]. Mutagenic potencies, expressed as revertants (rev) per ml-eq, were calculated from the slope of the linear portion of the dose-response curves and multiplied by the number of ml of urine collected during a 12-hr period to give the number of rev/12 hr. Controls consisted of DMSO (100 μ l/plate), C18 resin blanks prepared by passing 60 ml of glass-distilled deionized water instead of urine through the columns (15 ml-eq/plate), and 2-aminoanthracene at 0.5 μ g/plate. The typical range for the results with the positive control were 1,600–2,200 rev/plate.

Genotype Analysis

Genotypes were analyzed using DNA extracted from blood cells (buffy coat). For *NAT2* genotyping we measured the single nucleotide polymorphisms that identify the 4 most common *NAT2* slow acetylator alleles: *NAT2*5* (T341C, C481T), *NAT2*6* (G590A), *NAT2*7*(G857A), and *NAT2*14* (G191A) as described previously [Bell et al., 1993a]. *NAT1* genotypes were determined for the following alleles: *NAT1*3* (C1095A), *NAT1*4* (WT), *NAT1*10* (T1088A, C1095A), *NAT1*11* (9-bp deletion between 1065–1090) as described previously [Bell et al., 1995]. Individuals with at least one *NAT1*10* allele were considered to have an “at-risk” genotype [Bell et al., 1995; Hein et al., 2000]. *GSTM1* was analyzed as described [Bell et al., 1993b]. The number of polymorphic TA repeats in the TATA box motif in the UDP-glucuronosyltransferase 1A1 promoter (*UGT1A1*28*) was determined as described by Monaghan et al. [1996].

Statistical Analysis

Paired t-tests were applied to test for differences between estimates measured at different time points in the same individuals, such as urinary

TABLE I. Intake and Excretion of Mutagenicity Among Subjects Consuming Red Meat Fried at Low or High Temperature

Variable	Low-temperature median (range)	High-temperature median (range)
Mutagenic intake from meat (rev/day, n = 60) ^a	Not detectable	253,700 (169,800–335,500) ^b
Urinary mutagenicity (rev/12 hr) ^c		
Unhydrolyzed (n = 10)	4,537 (0–10,606) ^d	101,181 (67,018–228,691) ^{b,e}
Hydrolyzed (n = 10)	5,966 (0–24,328) ^d	779,964 (219,848–1,284,696) ^{b,e}
Unhydrolyzed (n = 60)	Not determined	51,527 (11,378–122,205) ^e
Hydrolyzed (n = 60)	Not determined	399,582 (90,743–1,083,813) ^e

^aMeasured in TA98 + S9.

^bSignificant difference ($P < 0.001$) between values associated with meat cooked at low versus high temperature based on paired t-test.

^cMeasured in YG1024 + S9.

^dCalculated by dividing the number of rev/24-hr urine obtained on the last day of consumption of meat cooked at low temperature by 2, to permit comparison to the 12-hr urinary mutagenicity data obtained after consumption of the second meal containing meat fried at high temperature.

^eFrom urine collected 0–12 hr after second consumption of meat fried at high temperature.

mutagenicity or meat intake. Correlation coefficients were estimated as Spearman coefficients. Generalized linear models were applied to evaluate the impact on between-individual variation of urinary mutagenicity for the following variables: sex, age, and previously determined intake of meat cooked at high temperature (g/day), MeIQx (ng/day), CYP1A2 and NAT2 phenotypes (log-transformed and measured at two different times: on the last day of intake of meat cooked at low temperature and on the last day of intake of meat cooked at high temperature; Sinha et al., 1994), and all the genotypes determined here (*GSTM1*, *UGT1A1**28, *NAT1*, and *NAT2*). Two-way and three-way interactions of intake of meat cooked at high temperature, NAT2 and CYP1A2 phenotypes, as well as two-way interactions of intake of meat cooked at high temperature with the genotypes were investigated. Effects of phenotypes and genotypes of metabolizing enzymes were assessed only for unhydrolyzed urine because we assumed that hydrolysis would deconjugate the mutagens and cancel out the effects of metabolism.

To investigate within- and between-individual variability of urinary mutagenicity, we applied the mixed-effect model to repeated measures of urinary mutagenicity (dependent variable) for the 20 subjects for whom urinary mutagenicity was analyzed at different time intervals as fixed effects, and the study subjects were analyzed as random effects. We included all 5 time intervals as described earlier or included only the three time intervals collected within the first 12 hr after consumption of meat cooked at high temperature.

RESULTS

To examine the effect of consumption of meat cooked at low versus high temperature on urinary mutagenicity, we conducted a pilot study involving 10 of the subjects (Table I, Appendixes A and B). Although the meat fried at low temperatures was not mutagenic (Appendix A), the median urinary mutagenicity of unhydrolyzed urine from subjects who consumed meals composed of this meat was 4,537 rev/12 hr; a slightly higher value (1.3×) was obtained with acid-hydrolyzed urine (5,966 rev/12 hr) (Table I, Appendixes A and B). Urinary mutagenicity is an integrated measure of mutagenic activity from all sources; thus, factors other than meat, including the relative sensitivities of tester strains TA98 and YG1024, may influence these values.

In contrast, the fried red meat cooked at high temperature was highly mutagenic (1023 rev/g), resulting in a median intake of mutagenicity of 253,700 rev/day in TA98 during

the second week of the study (Table I). Compared with the subjects who consumed meat cooked at low temperatures, the median urinary mutagenicity in YG1024 was much higher, with 101,181 rev/12 hr for unhydrolyzed urine and 779,964 rev/12 hr for acid-hydrolyzed urine (Table I). Thus, with cooking temperature as the primary variable, consumption of meat fried at high temperature resulted in urinary mutagenicity that was 22× or 131× greater relative to that resulting from consumption of meat fried at low temperature for unhydrolyzed or hydrolyzed urine, respectively. During the first week of the study, subjects ate slightly more red meat (cooked at low temperature) than they did during the second week (cooked at high temperature) (see Table IV) due to higher loss of moisture in meat cooked at high temperatures.

We investigated the kinetics of urinary mutagenicity after consumption of meat cooked at high temperature by examining 20 subjects over 5 time intervals (Fig. 2, Appendix C). The highest mutagenicity levels were observed in urine sampled within any of the first 12-hr periods after intake of meat fried at high temperature (day 7, 8, and 14) relative to the levels of urine collected during the 12–24-hr period after intake of the meat (day 7.5; $P < 0.007$) or under free-living conditions (day 0; $P < 0.007$). A marginally significant increase in mutagenicity for hydrolyzed urine was observed over the three time intervals involving collections made within the first 12 hr after consumption of meat cooked at high temperature ($P = 0.05$). Using a mixed-effect model of repeated measures, between-individual variability explained only a small portion of the variance of urinary mutagenicity compared with the within-individual variability. Including either all five time intervals or only the three involving collections of the first 12 hr after consumption of meat fried at high temperature, between-individual variation did not exceed 6% for the mutagenicity of either hydrolyzed or unhydrolyzed urine.

Considering all 60 subjects, urine collected 0–12 hr after first consumption of meat fried at high temperature resulted in urinary mutagenicity values averaging 51,527 rev/12 hr

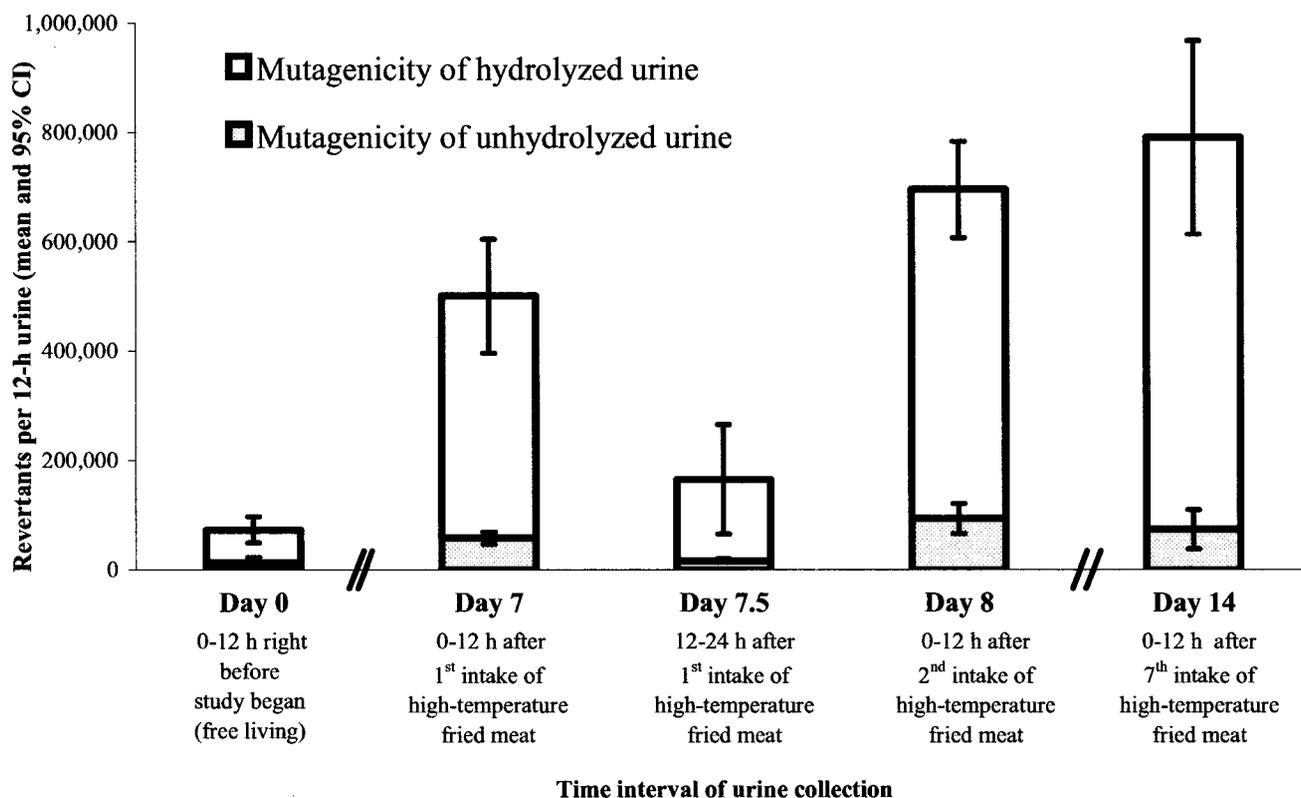


Fig. 2. Mean mutagenicity of unhydrolyzed and hydrolyzed urine measured over five different time intervals in 20 subjects.

for unhydrolyzed and 399,582 rev/12 hr for hydrolyzed urine (Table I). The individual values for all 60 subjects are given in Figure 3, which were calculated from the dose-response data in Appendix D. Thus, acid hydrolysis enhanced urinary mutagenicity resulting from consumption of meat cooked at high temperature by $\sim 8\times$ relative to unhydrolyzed urine.

We found that neither *GSTM1* polymorphism nor any variants of *NAT1* and *NAT2* had any modulating effect on unhydrolyzed urinary mutagenicity associated with fried-meat consumption (Table II). The one gene whose alleles influenced urinary mutagenicity in this study was *UGT1A1*. A TA-repeat polymorphism in the TATA box of the *UGT1A1* promoter (*UGT1A1**28) affects transcription and *UGT1A1* enzyme activity levels. Alleles containing 5, 6, 7, or 8 TA repeats have been reported, but only alleles with 6 or 7 repeats were observed in this population. We found no significant direct effect of *UGT1A1**28 (Table II); however, *UGT1A1**28 significantly modified the effect of meat intake on mutagenicity in unhydrolyzed urine ($P = 0.04$) (Table III). Intake of meat cooked at high temperature and unhydrolyzed urinary mutagenicity were significantly correlated ($r = 0.52$, $P = 0.003$) only in subjects who had one (6/7) or two (7/7) low-activity *UGT1A1* alleles. In contrast, no significant correlation was found between meat intake and urinary mutagenicity for subjects who were wild-type (6/6),

based on mutagenicity of unhydrolyzed urine ($r = 0.15$, $P = 0.45$).

DISCUSSION

Intake of HCAs, PAHs, and Mutagenicity of Fried Meat

Previously, we measured three HCAs (MeIQx, DiMeIQx, and PhIP) in the red meat fried at low and high temperatures that was consumed in this study [Sinha et al., 1994]. None of the three HCAs was detectable in meat fried at low temperature, but all 3 were present at high levels in the meat fried at high temperature. These results are consistent with other studies [Gross and Gruter, 1992; Knize et al., 1994; Sinha et al., 1998a,b] and confirm that high temperatures are required to produce detectable levels of HCAs in cooked meat. The median intake of HCAs from fried meat was 2.2, 0.5, and 8.1 $\mu\text{g}/\text{day}$ for MeIQx, DiMeIQx, and PhIP, respectively. These amounts are in the range consumed by people who eat very well done grilled meat or very crisp bacon [Sinha et al., 1998a,b, 1999, 2001].

In contrast to the increased levels of HCAs in the meat fried at high temperature, the PAH levels, measured previously [Sinha et al., 1994], were similar in the meat fried at low versus high temperature (10.7 and 10.1 ng/g, respec-

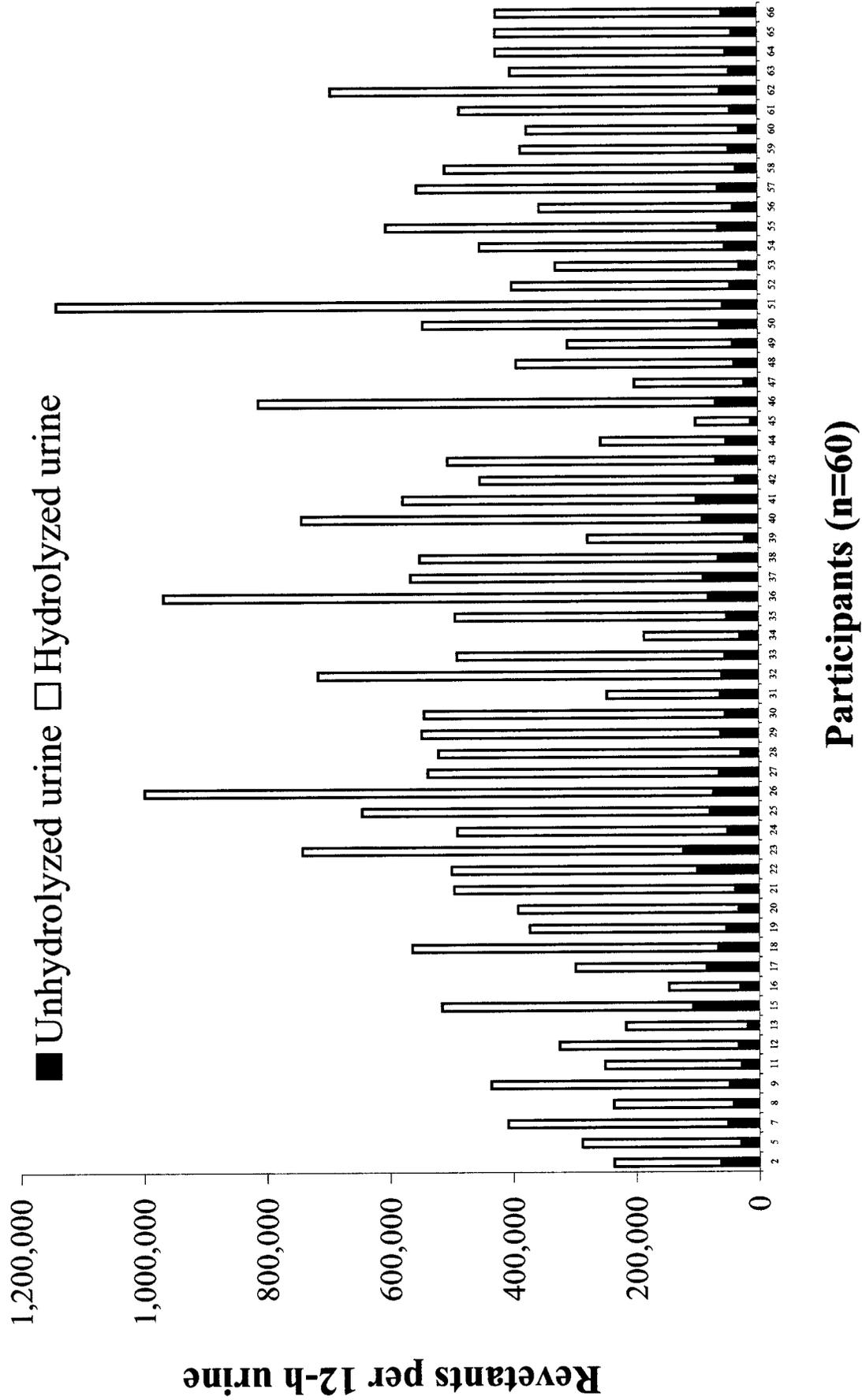


Fig. 3. Mutagenicity of unhydrolyzed and hydrolyzed urine collected 0–12 hr after first consumption of red meat fried at high temperature in 60 subjects.

TABLE II. Distributions of Genotypes of Metabolizing Enzymes and Their Effect Estimates on Mutagenicity of Unhydrolyzed Urine

Genotypes	n	Estimate	95% CI	P-value
<i>NAT2</i> slow/ <i>NAT2</i> rapid	30/30	-6840	-17843-4163	0.23
<i>NAT1</i> *10/ <i>NAT1</i> not- <i>NAT1</i> *10	26/34	10155	-809-21119	0.07
<i>UGT1A1</i> 6/6/ <i>UGT1A1</i> 6/7 or 7/7	29/30	10206	-1097-21509	0.08
<i>GSTM1</i> (1)/ <i>GSTM1</i> (3)	24/34	3202	-7925-14329	0.57

CI, confidence interval.

TABLE III. Association Between Unhydrolyzed Urinary Mutagenicity and Consumption of High-Temperature Cooked Meat Stratified by *UGT1A1* Genotype

<i>UGT1A1</i> genotype (no. of TA repeats)	Increase in urinary mutagenicity per 10 g of meat intake/day		
	Point estimate	95% CI	P-value ^a
6/6	747	-1166-2660	0.450
6/7 or 7/7	4062	1623-6511	0.003

CI, confidence interval.

^aP for interaction between *UGT1A1* and meat intake is 0.04.

tively). This resulted in a median intake of PAHs from fried meat of 2.9 µg/day in the first week and a lower PAH intake of 2.5 g/day in the second week (high temperature cooked meat). The concentrations of PAHs/g of the non-meat portion of the diet were 9.1 ng/g during the first week and 9.0 ng/g during the second week [Sinha et al., 1994]. Thus, unlike the intake of HCAs, which went from undetectable to high levels from the first to the second week of the study, the intake of PAHs was low in both weeks of the study.

When fried at low temperature, the red meat had no detectable mutagenic activity (Table I). In contrast, the fried red meat cooked at high temperature was highly mutagenic, resulting in a median intake of 253,700 rev/day during the second week of the study (Table I). These results are consistent with previous studies showing that the mutagenicity of meat increases with increasing cooking temperature [Dolara et al., 1979; Knize et al., 1985; Laser-Reutersward et al., 1987a,b; Doolittle et al., 1989]. Because we found similar and low levels of PAHs in meat fried at low or high temperatures, we assume that the mutagenicity in the meat cooked at high temperatures was caused by the high level of HCAs in that meat. Sugimura et al. [1988] and Felton et al. [2002] also concluded that HCAs are the major mutagens in high-temperature cooked meat.

Urinary Mutagenicity and Consumption of Meat Cooked at Low and High Temperatures

With cooking temperature as the primary variable, we showed that consumption of meat fried at high temperature resulted in urinary mutagenicity that was 22× or 131× greater relative to that resulting from consumption of meat fried at low temperature for unhydrolyzed or hydrolyzed

urine, respectively. Thus, our study provides for the first time a direct comparison between urinary mutagenicity and the effect of cooking temperature alone, as opposed to cooking temperature and cooking method. The results of our study are similar to those of Doolittle et al. [1989], who found that urinary mutagenicity was higher (10–50×) in subjects who consumed meats cooked at an elevated temperature, but using different methods for the different temperatures.

The kinetics of urinary mutagenicity observed in our study are consistent with those reported in other studies, which have shown that urinary mutagenicity peaks 2–6 hr after consumption of cooked meat and declines considerably after 12–16 hr [Dolara et al., 1984; Sousa et al., 1985; Hayatsu et al., 1985; Baker et al., 1986; Ohyama et al., 1987a,b]. The significant increase in mutagenicity of hydrolyzed urine over the 7-day period might suggest an accumulation of mutagens or an enhanced production of mutagenic metabolites. This increase is consistent with our observation that CYP1A2 activity, which can activate HCAs to mutagens, increased in 72% of the subjects of this study after consuming meat cooked at high temperature for 7 days ($P < 0.0002$) [Sinha et al., 1994].

Urinary Mutagenicity and Excretion of HCAs in Unhydrolyzed and Hydrolyzed Urine

The acid hydrolysis of urine used in our study was based on that of Stillwell et al. [1994] who showed an enhanced recovery of MeIQx from human urine from subjects who had consumed cooked meat. We found that this method also enhanced urinary mutagenicity from such subjects, with greater levels of mutagenicity (~8×) being obtained from hydrolyzed relative to unhydrolyzed urine (Table I and Fig. 3). Based on the observations of Stillwell et al. [1994, 1997, 1999] and the results reported here and elsewhere [DeMarini et al., 1997], it is likely that the mutagenicity of unhydrolyzed urine reflects excretion of unmetabolized mutagens, whereas the mutagenicity of hydrolyzed urine reflects the excretion of both metabolized and unmetabolized mutagens.

Correlating intake and urinary excretion levels of HCAs [Sinha et al., 1994, 1995; Stillwell et al., 1997, 1999] with urinary mutagenicity levels showed that urinary mutagenic-

TABLE IV. Correlations Between HCA Intake or Excretion and Urinary Mutagenicity

HCA intake and excretion	n	Spearman <i>r</i> (<i>P</i> -value) for urinary mutagenicity	
		Unhydrolyzed urine	Hydrolyzed urine
HCA intake from meat ^a	60	0.32 (0.01)	0.34 (0.01)
MeIQx in unhydrolyzed urine ^b	60	0.36 (0.01)	0.24 (0.1)
MeIQx in hydrolyzed urine ^c	52	0.26 (0.1)	0.34 (0.01)
PhIP in hydrolyzed urine ^c	52	0.26 (0.1)	0.43 (0.001)

HCA, heterocyclic amine; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine.

^aData from Sinha et al. [1994].

^bData from Sinha et al. [1995].

^cData from Stillwell et al. [1997].

ity was significantly and positively correlated with both the intake of HCAs ($r = 0.32$ or 0.34) and the excreted levels of HCAs in the urine (Table IV). Interestingly, the strongest correlations were between the levels of HCAs in unhydrolyzed urine and mutagenicity in unhydrolyzed urine ($r = 0.36$) and between the levels of HCAs in acid-hydrolyzed urine and mutagenicity in acid-hydrolyzed urine ($r = 0.34$ or 0.43). In the only other study in which the levels of HCAs and mutagenicity were measured in urine from subjects who had consumed cooked meat, no correlation was found between the levels of HCAs in the urine and the mutagenicity of the urine [Murray et al., 2001]. The reasons for this discrepancy are unclear.

The $\sim 8\times$ increase in mutagenicity of hydrolyzed relative to unhydrolyzed urine reported here (Table I) is consistent with our previous data from this same study population showing a 3–21 \times increase of MeIQx in hydrolyzed relative to unhydrolyzed urine [Sinha et al., 1995; Stillwell et al., 1997]. In this study population, only a small fraction of MeIQx and PhIP intake was detected as unmetabolized MeIQx and PhIP in the urine (2–5% and <1–2%, respectively), whereas 20–60% of MeIQx intake was detected in metabolized form, leaving some metabolites unidentified [Sinha et al., 1995; Stillwell et al., 1997, 1999; Turesky et al., 1998]. Thus, an $\sim 8\times$ increase in urinary mutagenicity after acid hydrolysis is within the expected range.

The mutagenicity of the hydrolyzed and unhydrolyzed urine from the subjects fed meat cooked at low temperature was similar to the levels found in our earlier study [DeMarini et al., 1997]. In contrast, the urinary mutagenicity from subjects who consumed meat cooked at high temperature was 10- and 40-fold greater than those from our earlier study for unhydrolyzed and hydrolyzed urine, respectively. This difference was most likely due to the higher cooking temperature used in the present study, which also resulted in a 4 \times greater intake of MeIQx and PhIP per day in the present study compared with our earlier study.

Influence of Genotype and Phenotype

Several studies indicate that certain genotypes and phenotypes can influence urinary mutagenicity. Two studies have shown that the induction of CYP1A2 activity can influence urinary mutagenicity [Murray et al., 2001; Pavanello et al., 2002]. Whereas two studies found no influence of the *GSTM1* genotype on urinary mutagenicity [DeMarini et al., 1997; Gabbani et al., 1998]. One study indicated that the rapid *NAT2* genotype increased urinary mutagenicity [DeMarini et al., 1997], whereas others showed that this genotype [Gabbani et al., 1998] or phenotype [Pavanello et al., 2002] decreased urinary mutagenicity.

In the present study, we also found no influence of *GSTM1* on unhydrolyzed urinary mutagenicity (Table II), consistent with other studies [DeMarini et al., 1997; Gabbani et al., 1998]. Based on the low PAH levels and the substrate-specific effects of *GSTM1* on PAH but not on HCAs, it is not surprising that we found no effect of *GSTM1* on urinary mutagenicity. The CYP1A2 phenotype [Sinha et al., 1994] did not influence the mutagenicity of unhydrolyzed urine (Table V). In contrast, we have shown previously in this study that CYP1A2 activity is induced in the subjects during the second week of the study [Sinha et al., 1994] and that this activity influences the metabolism of MeIQx [Sinha et al., 1995; Stillwell et al., 1997, 1999]. This is consistent with the finding by others that CYP1A2 activity modulates urinary mutagenicity after consumption of cooked-meat [Murray et al., 2001; Pavanello et al., 2002]. The reason for the discrepancy between our results here and those of Murray et al. [2001] and Pavanello et al. [2002] are unclear. However, substrate-specific differences for CYP1A2 activity, as we have shown previously in this study for MeIQx and PhIP [Sinha et al., 1995; Stillwell et al., 1997], might not be captured with urinary mutagenicity as an integrated measure of mutagen exposure.

As noted above, studies on *NAT2* genotypes have given conflicting results; here we found no effect of genotypic variants of *NAT2* on unhydrolyzed urinary mutagenicity (Table II). No study examined the effect of *NAT2* phenotype on unhydrolyzed urinary mutagenicity so far; in the present study, we found no effect of *NAT2* phenotype determined previously [Sinha et al., 1994] on unhydrolyzed urinary mutagenicity (Table V). A lack of effect of *NAT2* variants as well as *NAT2* activity is consistent with our previous observations in the same study in which we found that *NAT2* activity remained the same throughout both weeks of the study [Sinha et al., 1994] and showed no relation with urinary levels of unconjugated MeIQx [Sinha et al., 1995]. In addition to *NAT2* phenotype and variants of *NAT2* genotypes, we also found that genotypic variants of *NAT1* had no influence on urinary mutagenicity (Table II).

The homozygous 7/7 genotype (*UGT1A1**28/*28) has been associated with lower glucuronidation activity for various substrates [Iyer et al., 1999] and the mild bilirubinemia

TABLE V. Distributions of Phenotypes and Genotypes of Metabolizing Enzymes and Their Effect Estimates on Mutagenicity of Unhydrolyzed Urine

Phenotype	Median	Range	Estimate	95% CI	P-value
At end of low-temperature period ^a					
NAT2	0.97	0.19–3.70	6056	–6745–18857	0.36
CYP1A2	8.90	2.10–28.0	–1962	–13796–9872	0.75
At end of high-temperature period ^a					
NAT2	0.84	0.16–3.80	6146	–6670–18962	0.35
CYP1A2	13.0	2.50–34.0	6665	–6265–19595	0.32

CI, confidence interval.

^aEnzyme data from Sinha et al. [1994].

described as Gilbert's syndrome [Monaghan et al., 1996; Bosma et al., 1995]. Malfatti and Felton [2001] have shown that UGT1A1 is especially active in conjugating N²-hydroxy-PhIP to glucuronide. Thus, differences in the expression level of this enzyme might be expected to influence the ability of UGT1A1 to conjugate HCAs. We found no significant direct effect of *UGT1A1*28* (Table II); however, *UGT1A1*28* significantly modified the effect of meat intake on mutagenicity in unhydrolyzed urine ($P = 0.04$) (Table III). The intake of meat cooked at high temperature and unhydrolyzed urinary mutagenicity were significantly correlated ($r = 0.52$, $P = 0.003$) only in subjects who had one (6/7) or two (7/7) low-activity *UGT1A1* alleles. In contrast, no significant correlation was found between meat intake and urinary mutagenicity for subjects who were wild-type (6/6), based on mutagenicity of unhydrolyzed urine ($r = 0.15$, $P = 0.45$). Although the effect of meat intake on unhydrolyzed urinary mutagenicity differed substantially by *UGT1A1*28*, it is important to confirm this finding in further studies, in particular because we investigated direct and indirect effects of several phenotypes and genotypes of metabolizing enzymes, increasing the chance of false-positive findings. Because of the limited study size, we were not able to investigate small effects of the tested polymorphic genes or interactions between genetic polymorphisms.

SUMMARY AND CONCLUSIONS

This large, controlled feeding study has demonstrated that consumption of meat fried at low temperature, which was not mutagenic, had no detectable levels of HCAs and low levels of PAHs, correlated with weakly mutagenic urine. In contrast, consumption of meat fried at high temperature, which was highly mutagenic, had high levels of HCAs and low levels of PAHs, produced urine that was 22–131× more mutagenic than that produced after consumption of meat fried at low temperature. The urinary mutagenicity paralleled the levels of HCAs but not the levels of PAHs in the meat. Thus, urinary mutagenicity is strongly correlated with HCA intake from meat, indicating that HCAs are a likely cause of urinary mutagenicity in this study. As we noted in the Materials and Methods, strain

YG1024 is especially sensitive to heterocyclic amines. As other studies have shown, urinary mutagenicity has a short half-life, and much of it is eliminated in the first 12 hr after consuming high-temperature fried meat.

The mutagenicity of unhydrolyzed urine correlated with the level of MeIQx in unhydrolyzed urine, and the mutagenicity of hydrolyzed urine correlated with the levels of MeIQx and PhIP in hydrolyzed urine. This suggests that acid-hydrolysis liberates previously conjugated mutagenic activity. The importance of conjugation was highlighted by our finding that the mutagenicity of unhydrolyzed urine correlated with consumption of meat cooked at high temperature among subjects who had reduced conjugation ability due to a polymorphism in *UGT1A1*28*, but not in subjects lacking this polymorphism. This study shows that urinary mutagenicity correlates with mutagenic exposure from fried meat and can potentially be used as a marker in etiological studies on cancer. In support of this, we have shown recently in a case-control study that urinary mutagenicity is associated with an increased risk for colorectal adenoma [Peters et al., 2003].

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APPENDIX A. Mutagenicity of Meats Fried at Low and High Temperature

Meats	Revertants per g of meat ^a
Meats consumed in feeding study	
Meat cooked at low temperatures (week 1)	0
Meat cooked at high temperatures (week 2) (two separate analyses)	1381
	665
Average	1023
Quality controls ^b	
Meat cooked at low temperatures (nine separate analyses)	0
	0
	0
	0
	0
	0
	0
	0
	0
	0
Average	0
Meat cooked at high temperatures (13 separate analyses)	1975
	192
	1209
	568
	1815
	883
	1516
	918
	1191
	1115
	572
	659
Average	1051

^aDetermined in *Salmonella* strain TA98.

^bQuality controls were prepared in the same way as the meats used in the feeding study.

APPENDIX B. Mutagenicity of Urine Collected After Consumption of Meat Fried at Low and High Temperature*

I. Mutagenicity of Urine Collected 0–24 hr after Consumption of Meat Fried at Low Temperature on the Last Day of the First Week (n = 10)

Dose (ml-eq/plate)	Subjects					
	12		16		23	
	U	H	U	H	U	H
0	84 ^a	74 ^a	84 ^a	74 ^a	84 ^a	74 ^a
0.5	68	87 ^a	88	89 ^a	78	96 ^a
1	68	101 ^a	81	99 ^a	75	91 ^a
3	85	93 ^a	124 ^a	129 ^a	90 ^a	113 ^a
7	102 ^a	183 ^a	180 ^a	129	87 ^a	116 ^a
14	125 ^a	207 ^a	185	100	111 ^a	139 ^a
revertants/ml-eq	2.9	9.8	13.7	17.5	1.8	3.8
r squared	0.99	0.89	0.99	0.98	0.81	0.84

Dose (ml-eq/plate)	Subjects							
	26		27		35		42	
	U	H	U	H	U	H	U	H
0	84 ^a	74 ^a	84	74	84 ^a	74 ^a	84	74
0.5	87 ^a	110 ^a	90	76	101 ^a	85 ^a	87	85
1	93 ^a	135 ^a	93	89	92 ^a	88 ^a	100	78
3	122 ^a	229 ^a	88	102	109 ^a	86 ^a	96	112
7	202 ^a	365 ^a	38	1	114 ^a	125 ^a	102	112
14	326 ^a	505	0	6	157 ^a	146 ^a	NES	NES
revertants/ml-eq	17.7	40.6			4.6	5.0		
r squared	0.99	0.99			0.93	0.94		

Dose (ml-eq/plate)	Subjects					
	51		59		Subject 19	
	U	H	U	H	U	H
0	84 ^a	74 ^a	84 ^a	74 ^a	84 ^a	74 ^a
0.5	103 ^a	92 ^a	104	96 ^a	73	76 ^a
1	104 ^a	98 ^a	86	89 ^a	89 ^a	91 ^a
3	93 ^a	99 ^a	98	110 ^a	92 ^a	99 ^a
7	130 ^a	110 ^a	123 ^a	121 ^a	111 ^a	161 ^a
14	125	172 ^a	150 ^a	170 ^a	137 ^a	181
revertants/ml-eq	4.9	6	4.7	6.1	4.1	12.2
r squared	0.67	0.92	0.99	0.95	0.99	0.97

II. Mutagenicity of Urine collected 0–12 hr After Consumption of Meat Fried at High Temperature on the Second Day of the Second Week (n = 10)

Unhydrolyzed Urine

Dose (ml-eq/plate)	Subjects									
	12 U	16 U	19 U	23 U	26 U	27 U	35 U	42 U	51 U	59 U
0	56 ^a									
0.5	96 ^a	165 ^a	124 ^a	156 ^a	139 ^a	162 ^a	130 ^a	163 ^a	117 ^a	114 ^a
1	138 ^a	242 ^a	165 ^a	222 ^a	212 ^a	174 ^a	165 ^a	210 ^a	129 ^a	124 ^a
3	260 ^a	478 ^a	346 ^a	496 ^a	487 ^a	388 ^a	351 ^a	456 ^a	222 ^a	255 ^a
revertants/ml-eq	66.9	134.9	94	143	142	104.4	95.1	128	50.9	63.3
r squared	0.99	0.98	0.99	0.99	0.99	0.97	0.99	0.99	0.95	0.98

APPENDIX B. Continued

Hydrolyzed Urine								
Dose (ml-eq/plate)	Subjects							
	12 H	16 H	19 H	23 H	26 H	27 H	35 H	42 H
0	56 ^a							
0.05	93 ^a	145 ^a	103 ^a	79 ^a	122 ^a	131 ^a	104 ^a	110 ^a
0.1	139 ^a	206 ^a	142 ^a	147 ^a	149 ^a	201 ^a	122 ^a	152 ^a
0.3	249 ^a	540 ^a	327 ^a	296 ^a	329 ^a	389 ^a	271 ^a	296 ^a
0.7	507 ^a	927	565	582 ^a	623 ^a	709 ^a	526 ^a	598 ^a
1.0	629	1092	671	689	768	842	682	718
revertants/ml-eq	634	1606	902.2	756.3	797.7	908.1	666.9	760.2
r squared	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99

Dose (ml-eq/plate)	Subjects	
	51 H	59 H
0	73 ^a	73 ^a
0.5	318 ^a	196 ^a
1	413 ^a	291 ^a
3	852 ^a	635 ^a
7	1263	1157
revertants/ml-eq	243.9	183.1
r squared	0.97	0.99

U, unhydrolyzed urine; H, hydrolyzed urine; NES, not enough sample.

*Urinary mutagenicity determined using Salmonella YG1024 + 2X S9.

^aValues used to calculate the slope (revertants/ml-eq).

APPENDIX C. Mutagenicity of Urine Collected During Five Time Intervals (n = 20)*

I. Urine Collected 0–12 hr Before Initiation of the Study (n = 20)										
Dose (ml-eq/plate)	Subjects									
	2		8		13		17		20	
	U	H	U	H	U	H	U	H	U ^b	H
0	46 ^a	59 ^a	46 ^a	46 ^a	46 ^a	46 ^a				
0.25	63 ^a	61 ^a	43 ^a	64 ^a	63 ^a	52 ^a	53 ^a	103 ^a	55 ^a	97 ^a
0.5	64 ^a	57 ^a	43 ^a	74 ^a	57 ^a	116 ^a	49 ^a	124 ^a	62 ^a	189 ^a
1	75 ^a	53	57 ^a	42	51	115	66 ^a	172 ^a	72 ^a	369 ^a
3	56	41	84 ^a	33	3	0	75 ^a	62	124 ^a	479
7	79	39	110	39	0	—	103 ^a	31	123	237
14	104	32	137	8	0	—	104	17	137	91
revertants/ml-eq	26.1	22.0	14.1	56.0	22.0	114.0	7.8	119.0	25.5	331.1
r squared	0.86	0.50	0.95	0.97	0.41	0.70	0.95	0.95	0.99	0.99

Dose (ml-eq/plate)	Subjects									
	21		23		25		28		31	
	U ^c	H	U	H	U	H	U	H	U	H
0	46 ^a	46 ^a	46 ^a	46 ^a	46 ^a	46 ^a	46 ^a	46 ^a	46 ^a	46 ^a
0.25	60 ^a	61 ^a	52 ^a	49 ^a	62 ^a	44 ^a	70 ^a	56 ^a	73 ^a	56 ^a
0.5	64 ^a	53 ^a	44 ^a	53 ^a	63 ^a	65 ^a	51 ^a	60 ^a	56 ^a	62 ^a
1	53 ^a	99 ^a	48 ^a	75 ^a	60 ^a	91 ^a	55 ^a	74 ^a	61 ^a	97 ^a
3	73 ^a	309 ^a	60 ^a	122 ^a	69 ^a	151 ^a	64 ^a	126 ^a	57 ^a	170 ^a
7	86	598	103 ^a	102	89 ^a	110	84 ^a	125	76 ^a	112
14	96	632	155 ^a	62	139 ^a	78	104 ^a	114	81 ^a	51
revertants/ml-eq	3.2	91.1	8.0	26.2	5.9	36.3	3.7	26.2	1.8	41.8
r squared	0.83	0.97	0.99	0.99	0.97	0.97	0.86	0.99	0.54	0.99

Dose (ml-eq/plate)	Subjects									
	36		41		42		43		44	
	U	H	U	H	U	H	U	H	U	H
0	46 ^a	46 ^a	46 ^a	46 ^a	46 ^a	46 ^a	46 ^a	46 ^a	46 ^a	46 ^a
0.25	51 ^a	61 ^a	50 ^a	69 ^a	69 ^a	62 ^a	54 ^a	73 ^a	43 ^a	85 ^a
0.5	49 ^a	89 ^a	43 ^a	64 ^a	60 ^a	72 ^a	62 ^a	133 ^a	51 ^a	77 ^a
1	48 ^a	110	64 ^a	102 ^a	72 ^a	104 ^a	43 ^a	242 ^a	39 ^a	100 ^a
3	61 ^a	27	103 ^a	226 ^a	73 ^a	142	65 ^a	693	50 ^a	154 ^a
7	15	0	186 ^a	298	85 ^a	98	105 ^a	799	67 ^a	80
14	0	0	376 ^a	255	81	49	185 ^a	343	78 ^a	55
revertants/ml-eq	4.5	86.0	23.5	59.9	3.9	57.1	9.6	202.3	2.6	31.5
r squared	0.84	0.97	0.99	0.99	0.62	0.99	0.97	0.99	0.89	0.92

Dose (ml-eq/plate)	Subjects									
	51		54		58		62		65	
	U	H	U	H	U	H	U	H	U	H
0	46 ^a	46 ^a	46 ^a	46 ^a						
0.25	44 ^a	44 ^a	57	85 ^a	48 ^a	53 ^a	52 ^a	60 ^a	56 ^a	49 ^a
0.5	52 ^a	40 ^a	49	109 ^a	65 ^a	42 ^a	43 ^a	56 ^a	54 ^a	67 ^a
1	45 ^a	67 ^a	43	139	48	72 ^a	51 ^a	74 ^a	64 ^a	90 ^a
3	50 ^a	137 ^a	54	37	44	221 ^a	73 ^a	115 ^a	85 ^a	117
7	48 ^a	175	19	0	44	470 ^a	62	157	115 ^a	32
14	65 ^a	160	0	0	75	579	101	104	146	1
revertants/ml-eq	1.2	33.1	0	126.0	38.0	63.1	9.1	22.2	9.4	46.6
r squared	0.73	0.96	—	0.98	0.83	0.99	0.87	0.98	0.98	0.97

APPENDIX C. Continued

II. Urine Collected 0–12 hr After Consumption of the First Meal Containing Meat Fried at High Temperature (n = 20)

Dose (ml-eq/plate)	Subjects					
	2		8		13	
	U	H	U	H	U	H
0	67 ^a	127 ^a	66 ^a	100 ^a	66 ^a	100 ^a
1	172 ^a	358 ^a	103 ^a	608 ^a	116 ^a	516 ^a
3	331 ^a	804 ^a	209 ^a	957 ^a	153 ^a	949 ^a
7	642 ^a	1259	463 ^a	1065	50	609
14	987	1775	691	861	1	0
revertants/ml-eq	81	225	58	270	28	274
r squared	0.99	0.99	0.99	0.91	0.93	0.97

Dose (ml-eq/plate)	Subjects									
	17		20		21		23		25	
	U	H	U	H	U	H	U	H	U	H
0	58 ^a	58 ^a	75 ^a	75 ^a	75 ^a	77 ^a	75 ^a	77 ^a	75 ^a	77 ^a
0.5	—	248 ^a	—	516 ^a	—	449 ^a	—	266 ^a	—	520 ^a
1	146 ^a	428 ^a	139 ^a	746 ^a	116 ^a	812 ^a	123 ^a	479 ^a	183 ^a	853 ^a
3	417 ^a	1006 ^a	274 ^a	1174	251 ^a	1236	247 ^a	962	407 ^a	1009
7	513	1054	505 ^a	933	316	1302	633 ^a	1194	834 ^a	839
revertants/ml-eq	122	311	61	671	60	735	81	402	109	776
r squared	0.99	0.99	0.99	0.97	0.99	0.99	0.98	0.99	0.99	0.99

Dose (ml-eq/plate)	Subjects									
	28		31		36		41		42	
	U	H	U	H	U	H	U	H	U	H
0	75 ^a	75 ^a	75 ^a	75 ^a	57 ^a					
0.5	—	364 ^a	—	138 ^a	—	600 ^a	—	361 ^a	—	313 ^a
1	85 ^a	610 ^a	109 ^a	206 ^a	153 ^a	963 ^a	140 ^a	611 ^a	123 ^a	563 ^a
3	163 ^a	1236	172 ^a	396	338 ^a	1178	475 ^a	961	230 ^a	1031
7	502	1485	387 ^a	557	641 ^a	1223	849 ^a	1180	370 ^a	1013
revertants/ml-eq	31	535	45	131	83	906	116	554	44	506
r squared	0.95	0.99	0.98	0.99	0.99	0.99	0.98	0.99	0.98	0.99

Dose (ml-eq/plate)	Subjects									
	43		44		51		54		58	
	U	H	U	H	U	H	U	H	U	H
0	57 ^a	57 ^a	57 ^a	57 ^a	56 ^a					
0.5	—	297 ^a	—	162 ^a	—	404 ^a	—	219 ^a	—	196 ^a
1	130 ^a	528 ^a	91 ^a	281 ^a	97 ^a	671 ^a	115 ^a	366 ^a	72 ^a	274 ^a
3	267 ^a	1121	202 ^a	588 ^a	115 ^a	1017	172 ^a	801	101 ^a	492
7	568 ^a	1313	359 ^a	1067	220 ^a	1017	350 ^a	989	166 ^a	500
revertants/ml-eq	73	471	44	174	22	615	41	310	16	218
r squared	0.99	0.99	0.99	0.99	0.97	0.99	0.99	0.99	0.99	0.97

Dose (ml-eq/plate)	Subjects			
	62		65	
	U	H	U	H
0	56 ^a	56 ^a	56 ^a	56 ^a
0.5	—	762 ^a	—	265 ^a
1	163 ^a	1214 ^a	84 ^a	437 ^a
3	387 ^a	1648	163 ^a	951
7	547	1672	335 ^a	1139
revertants/ml-eq	111	1158	40	381
r squared	0.99	0.98	0.99	0.99

Appendix C. Continued

III. Urine Collected 12–24 hr After Consumption of the First Meal Containing Meat Fried at High Temperature (n = 20)

Dose (ml-eq/plate)	Subjects									
	2		8		13		17		20	
	U	H	U	H	U	H	U	H	U	H
0	46 ^a	46 ^a	46 ^a	46 ^a	46 ^a	46 ^a	46 ^a	46 ^a	46 ^a	46 ^a
0.25	78 ^a	114 ^a	62 ^a	96 ^a	78 ^a	60 ^a	57 ^a	84 ^a	64 ^a	42 ^a
0.5	88 ^a	150 ^a	68 ^a	147 ^a	64 ^a	77 ^a	68 ^a	137 ^a	60 ^a	78 ^a
1	85 ^a	235 ^a	68 ^a	99	67 ^a	147	97 ^a	176	77 ^a	128 ^a
3	175 ^a	295	128 ^a	54	63	173	190 ^a	182	85 ^a	201 ^a
7	395 ^a	170	225 ^a	43	13	0	346	130	103 ^a	188
14	564	73	379 ^a	20	0	0	540	166	183 ^a	159
revertants/ml-eq	47.9	183.0	23.6	202.0	0	62.0	48.3	182.0	8.6	53.5
r squared	0.99	0.99	0.99	0.99		0.99	0.99	0.99	0.96	0.94

Dose (ml-eq/plate)	Subjects									
	21		23		25		28		31	
	U	H	U	H	U	H	U	H	U	H
0	46 ^a									
0.25	54 ^a	130 ^a	50 ^a	52 ^a	52 ^a	100 ^a	64 ^a	91 ^a	54 ^a	51 ^a
0.5	63 ^a	152 ^a	62 ^a	72 ^a	79 ^a	169 ^a	68 ^a	130 ^a	66 ^a	72 ^a
1	66 ^a	172	66 ^a	102 ^a	90 ^a	197	100 ^a	85	76 ^a	101 ^a
3	165 ^a	104	108 ^a	219 ^a	155 ^a	268	219 ^a	56	80 ^a	172
7	282	54	136 ^a	223	297 ^a	295	342	54	149 ^a	185
14	385	74	250 ^a	177	404	381	546	60	217 ^a	133
revertants/ml-eq	40.0	212.0	14.0	58.9	35.2	246.0	57.7	168.0	11.9	57.8
r squared	0.97	0.9	0.99	0.99	0.99	0.99	0.99	0.99	0.98	0.97

Dose (ml-eq/plate)	Subjects									
	36		41		42		43		44	
	U	H	U	H	U	H	U	H	U	H
0	46 ^a	46 ^a	46 ^a	46 ^a	46 ^a	46 ^a	46 ^a	46 ^a	46 ^a	46 ^a
0.25	54 ^a	123 ^a	55 ^a	84 ^a	68 ^a	105 ^a	51 ^a	126 ^a	52 ^a	130 ^a
0.5	62 ^a	186 ^a	60 ^a	74 ^a	66 ^a	135 ^a	50 ^a	188 ^a	50 ^a	202 ^a
1	57	223	75 ^a	149 ^a	76 ^a	181	65 ^a	309	68 ^a	381
3	114	43	105 ^a	232 ^a	102 ^a	158	100 ^a	485	140 ^a	329
7	21	8	165 ^a	125	201 ^a	67	147	121	209	51
14	1	0	263	130	267	50	185	42	330	33
revertants/ml-eq	32.0	280.0	16.5	59.9	20.5	178.0	18.3	284.0	32.4	312.0
r squared	0.99	0.99	0.99	0.94	0.98	0.97	0.99	0.99	0.98	0.99

Dose (ml-eq/plate)	Subjects									
	51		54		58		62		65	
	U	H	U	H	U	H	U	H	U	H
0	46 ^a	46 ^a	46 ^a	46 ^a	46 ^a	46 ^a	46 ^a	46 ^a	46 ^a	46 ^a
0.25	54 ^a	45 ^a	54 ^a	72 ^a	60 ^a	50 ^a	64 ^a	226 ^a	60 ^a	101 ^a
0.5	43 ^a	73 ^a	72 ^a	116 ^a	49 ^a	53 ^a	56 ^a	658 ^a	37 ^a	158 ^a
1	63 ^a	76 ^a	55	185 ^a	52 ^a	92 ^a	91 ^a	1609 ^a	61 ^a	159
3	79 ^a	166 ^a	122	260	89 ^a	166 ^a	150 ^a	2569	97 ^a	25
7	111 ^a	221	51	0	117 ^a	250	267 ^a	2622	147 ^a	0
14	164 ^a	136	0	0	171 ^a	230	437	2190	5	0
revertants/ml-eq	8.4	40.7	52.0	142.1	8.9	41.8	31.2	1615.9	14.8	224.0
r squared	0.98	0.98	0.95	0.99	0.97	0.98	0.99	0.98	0.95	0.99

APPENDIX C. *Continued*

IV. Urine Collected 0–12 hr After Consumption of the Second Meal Containing Meat Fried at High Temperature (n = 20)

Dose (ml-eq/plate)	Subjects										
	2		8		13		17		20		
	U	H	U	H	U	H	U	H	U	H	
0	55 ^a	55 ^a	55 ^a	55 ^a	55 ^a	59 ^a	55 ^a				
0.25	65 ^a	309 ^a	76 ^a	188 ^a	88 ^a	392 ^a	81 ^a	116 ^a	101 ^a	205 ^a	
0.5	90 ^a	562 ^a	100 ^a	413 ^a	90 ^a	640 ^a	141 ^a	256 ^a	151 ^a	429 ^a	
1	172 ^a	879	158 ^a	599	88	812	170 ^a	412 ^a	237 ^a	665	
3	442 ^a	670	295	654	4	3	384 ^a	946	598	727	
7	718	329	707	231	0	—	540	1248	1120	270	
revertants/ml-eq	133.9	1014.0	103.9	716.0	70.0	1162.0	107.5	369.3	182.4	748.0	
r squared	0.99	0.99	0.99	0.98	0.79	0.99	0.99	0.98	0.99	0.99	

Dose (ml-eq/plate)	Subjects										
	21		23		25		28		31		
	U	H	U	H	U	H	U	H	U	H	
0	55 ^a	55 ^a									
0.25	81 ^a	117 ^a	86 ^a	123 ^a	99 ^a	197 ^a	75 ^a	159 ^a	101 ^a	187 ^a	
0.5	153 ^a	284 ^a	101 ^a	258 ^a	124 ^a	268 ^a	104 ^a	230 ^a	160 ^a	370 ^a	
1	196 ^a	404 ^a	177 ^a	454 ^a	221 ^a	515 ^a	112	356	245	668	
3	537 ^a	757	516 ^a	606	363	678	261	681	377	829	
7	955	393	933	488	865	476	537	544	37	478	
14	—	—	—	103	—	—	—	—	—	—	
revertants/ml-eq	160.8	363.9	156.7	410.3	163.5	448.8	98.0	350.0	210.0	630.0	
r squared	0.99	0.96	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	

Dose (ml-eq/plate)	Subjects										
	36		41		42		43		44		
	U	H	U	H	U	H	U	H	U	H	
0	55 ^a	55 ^a									
0.25	67 ^a	242 ^a	85 ^a	199 ^a	74 ^a	207 ^a	56 ^a	171 ^a	63 ^a	254 ^a	
0.5	98 ^a	447 ^a	105 ^a	408 ^a	105 ^a	388 ^a	75 ^a	332 ^a	92 ^a	440 ^a	
1	129 ^a	646	145 ^a	682	147 ^a	678	76 ^a	525	114 ^a	752	
3	232 ^a	655	263 ^a	764	343 ^a	713	114 ^a	1200	138	981	
7	503 ^a	37	562 ^a	340	554 ^a	267	205 ^a	1648	372	—	
14	—	—	—	—	1013	—	—	—	—	—	
revertants/ml-eq	63.1	784.0	70.6	706.0	72.2	666.0	21.1	554.0	62.2	770.0	
r squared	0.99	0.99	0.99	0.99	0.98	0.99	0.99	0.99	0.96	0.99	

Dose (ml-eq/plate)	Subjects										
	51		54		58		62		65		
	U	H	U	H	U	H	U	H	U	H	
0	55 ^a	55 ^a									
0.25	72 ^a	125 ^a	74 ^a	209 ^a	61 ^a	92 ^a	67 ^a	345 ^a	100 ^a	194 ^a	
0.5	92 ^a	221 ^a	85 ^a	358 ^a	57 ^a	127 ^a	112 ^a	697 ^a	76 ^a	416 ^a	
1	91 ^a	373 ^a	91 ^a	614	93 ^a	235 ^a	163 ^a	1198	101 ^a	710 ^a	
3	174 ^a	957 ^a	142 ^a	814	163 ^a	439	275	2193	181 ^a	1018	
7	321 ^a	950	218 ^a	—	224	673	430	—	287 ^a	—	
14	573	527	—	—	—	—	—	—	—	—	
revertants/ml-eq	37.1	300.2	22.1	606.0	37.6	180.7	113.5	1284	31.7	667.3	
r squared	0.99	0.99	0.98	0.99	0.98	0.99	0.97	0.99	0.97	0.99	

APPENDIX C. *Continued*

V. Urine Collected 0–12 hr After Consumption of the Sixth Meal Containing Meat Fried at High Temperature (n = 20)

Dose (ml-eq/plate)	Subjects									
	2		8		13		17		20	
	U	H	U	H	U	H	U	H	U	H
0	55 ^a	59 ^a	55 ^a	55 ^a	55 ^a	55 ^a				
0.25	56 ^a	255 ^a	77 ^a	321 ^a	68 ^a	399 ^a	75 ^a	323 ^a	91 ^a	587 ^a
0.5	69 ^a	300 ^a	81 ^a	493 ^a	88 ^a	644 ^a	65 ^a	584 ^a	81 ^a	992 ^a
1	86 ^a	601 ^a	114 ^a	649	73 ^a	—	105 ^a	977	139 ^a	2129 ^a
3	141 ^a	1043	262 ^a	403	141 ^a	—	200 ^a	923	231 ^a	1738
7	263 ^a	—	570 ^a	—	268 ^a	—	365 ^a	—	518 ^a	—
revertants/ml-eq	29.9	521.0	73.8	876.0	29.6	1170.0	44.6	1058.0	64.4	2057.9
r squared	0.99	0.97	0.99	0.98	0.98	0.99	0.99	0.99	0.99	0.99

Dose (ml-eq/plate)	Subjects									
	21		23		25		28		31	
	U	H	U	H	U	H	U	H	U	H
0	55 ^a									
0.25	64 ^a	85 ^a	67 ^a	308 ^a	64 ^a	416 ^a	67 ^a	256 ^a	76 ^a	148 ^a
0.5	80 ^a	87 ^a	99 ^a	521 ^a	100 ^a	575 ^a	74 ^a	396 ^a	79 ^a	235 ^a
1	105 ^a	148 ^a	110 ^a	914	108 ^a	662	109 ^a	561	102 ^a	408
3	230 ^a	476 ^a	302 ^a	815	268 ^a	257	225 ^a	321	181 ^a	708
7	480 ^a	—	468	—	425	176	353	—	259	—
revertants/ml-eq	61.3	143.5	82.9	932.0	71.2	1040.0	57.6	682.0	40.5	360.0
r squared	0.99	0.98	0.98	0.99	0.99	0.95	0.99	0.99	0.99	0.99

Dose (ml-eq/plate)	Subjects									
	36		41		42		43		44	
	U	H	U	H	U	H	U	H	U	H
0	55 ^a									
0.25	72 ^a	315 ^a	88 ^a	382 ^a	64 ^a	233 ^a	85 ^a	99 ^a	72 ^a	406 ^a
0.5	103 ^a	393 ^a	128 ^a	569 ^a	80 ^a	390 ^a	73 ^a	100 ^a	109 ^a	656 ^a
1	115 ^a	389	189	793	128 ^a	595	69 ^a	173 ^a	142 ^a	949
3	251 ^a	141	395	347	62	951	102 ^a	—	295 ^a	1179
7	476	—	403	—	65	—	150 ^a	—	621 ^a	774
revertants/ml-eq	64.0	676.0	146.0	1028.0	74.9	670.0	12.0	111.4	80.2	1202.0
r squared	0.99	0.91	0.99	0.98	0.97	0.99	0.92	0.95	0.99	0.99

Dose (ml-eq/plate)	Subjects									
	51		54		58		62		65	
	U	H	U	H	U	H	U	H	U	H
0		55 ^a	55 ^a	55 ^a	55 ^a	55 ^a	55 ^a	55 ^a	55 ^a	
0.25		172 ^a	79 ^a	245 ^a	44 ^a	109 ^a	173 ^a	249 ^a	85 ^a	
0.5		283 ^a	109 ^a	368 ^a	52 ^a	140 ^a	283 ^a	392 ^a	92 ^a	
1		479	141 ^a	389	77	259 ^a	438	583	124 ^a	
3		853	381 ^a	331	55	661 ^a	759	963	244 ^a	
7		—	456	—	61	—	791	—	441	
revertants/ml-eq	see	456.0	108.9	626.0	26.1	202.8	456.0	674.0	60.9	see
r squared	below	0.99	0.99	0.98	0.62	0.99	0.99	0.99	0.99	below

APPENDIX C. *Continued*

Dose (ml-eq/plate)	51 U	Dose (ml-eq/plate)	65 H
0	55 ^a	0	59 ^a
0.25	62 ^a	0.05	136 ^a
0.5	81 ^a	0.1	161 ^a
1	81 ^a	0.25	422 ^a
3	190 ^a	0.5	582
5.3	258 ^a	1	961
revertants/ml-eq	39.7	revertants/ml-eq	1445.7
r squared	0.98	r squared	0.98

U, unhydrolyzed urine; H, hydrolyzed urine.

*Urinary mutagenicity determined using Salmonella YG1024 + 2X S9.

^aValues used to calculate the slope (revertants/ml-eq).

^bTop dose = 13.

^cTop dose = 8.5.

APPENDIX D. Mutagenicity of Urine Collected 0–12 hr After Consumption of the First Meal Containing Meat Fried at High Temperature in All Participants (n = 60)*

Dose (ml-eq/plate)	Subjects			
	2		5	
	U	H	U	H
0	67 ^a	127 ^a	66 ^a	100 ^a
1	172 ^a	358 ^a	89 ^a	617 ^a
3	331 ^a	804 ^a	169 ^a	1009 ^a
7	642 ^a	1259	297 ^a	945
14	987	1775	638	568
revertants/ml-eq	81	225	34	288
r squared	0.99	0.99	0.99	0.93

Dose (ml-eq/plate)	Subjects					
	7		8		9	
	U	H	U	H	U	H
0	66 ^a	100 ^a	66 ^a	100 ^a	66 ^a	100 ^a
1	101 ^a	328 ^a	103 ^a	608 ^a	103 ^a	532 ^a
3	169 ^a	685 ^a	209 ^a	957 ^a	200 ^a	1120 ^a
7	261 ^a	1073	463 ^a	1065	350 ^a	1633
14	523	1273	691	861	545	1853
revertants/ml-eq	28	193	58	270	41	333
r squared	0.99	0.99	0.99	0.91	0.99	0.99

Dose (ml-eq/plate)	Subjects					
	11		12		13	
	U	H	U	H	U	H
0	66 ^a	100 ^a	66 ^a	100 ^a	66 ^a	100 ^a
1	102 ^a	380 ^a	97 ^a	380 ^a	116 ^a	516 ^a
3	162 ^a	856 ^a	154 ^a	811 ^a	153 ^a	949 ^a
7	295 ^a	1059	246 ^a	1229	50	609
14	508	1105	496	1587	1	0
revertants/ml-eq	32	250	25	234	28	274
r squared	0.99	0.99	0.99	0.99	0.93	0.97

Dose (ml-eq/plate)	Subjects									
	15		16		17		18		19	
	U	H	U	H	U	H	U	H	U	H
0	58 ^a	58 ^a	58 ^a	58 ^a	58 ^a	58 ^a	75 ^a	77 ^a	75 ^a	77 ^a
0.5	—	231 ^a	—	233 ^a	—	248 ^a	—	277 ^a	—	245 ^a
1	115 ^a	358 ^a	135 ^a	538 ^a	146 ^a	428 ^a	116 ^a	546 ^a	135 ^a	478 ^a
3	278 ^a	932 ^a	323 ^a	1096 ^a	417 ^a	1006 ^a	221 ^a	1086	323 ^a	1031
7	535	1131	626	1468	513	1054	511 ^a	1296	534	1119
revertants/ml-eq	75	288	89	343	122	311	63	469	84	401
r squared	0.99	0.99	0.99	0.98	0.99	0.99	0.99	0.99	0.99	0.99

Dose (ml-eq/plate)	Subjects									
	20		21		22		23		24	
	U	H	U	H	U	H	U	H	U	H
0	75 ^a	75 ^a	75 ^a	77 ^a	58 ^a	58 ^a	75 ^a	77 ^a	75 ^a	77 ^a
0.5	—	516 ^a	—	449 ^a	—	242 ^a	—	266 ^a	—	391 ^a
1	139 ^a	746 ^a	116 ^a	812 ^a	160 ^a	427 ^a	123 ^a	479 ^a	137 ^a	666 ^a
3	274 ^a	1174	251 ^a	1236	339 ^a	826	247 ^a	962	278 ^a	1085
7	505 ^a	933	316	1302	709	1333	633 ^a	1194	544 ^a	778
revertants/ml-eq	61	671	60	735	93	369	81	402	67	589
r squared	0.99	0.97	0.99	0.99	0.99	0.99	0.98	0.99	0.99	0.99

APPENDIX D. *Continued*

Dose (ml-eq/plate)	Subjects										
	50		51		52		53		54		
	U	H	U	H	U	H	U	H	U	H	
0	56 ^a	56 ^a									
0.5	—	313 ^a	—	404 ^a	—	265 ^a	—	451 ^a	—	219 ^a	—
1	128 ^a	514 ^a	97 ^a	671 ^a	111 ^a	395 ^a	116 ^a	763	115 ^a	366 ^a	—
3	220 ^a	873	115 ^a	1017	196 ^a	866	308 ^a	1154	172 ^a	801	—
7	476 ^a	940	220 ^a	1017	357 ^a	1199	533 ^a	986	350 ^a	989	—
revertants/ml-eq	59	458	22	615	42	339	69	707	41	310	—
r squared	0.99	0.99	0.97	0.99	0.99	0.98	0.99	0.99	0.99	0.99	0.99

Dose (ml-eq/plate)	Subjects										
	55		56		57		58		59		
	U	H	U	H	U	H	U	H	U	H	
0	56 ^a	56 ^a	56 ^a	56 ^a	75 ^a	75 ^a	56 ^a				
0.5	—	384 ^a	—	333 ^a	—	456 ^a	—	196 ^a	—	188 ^a	—
1	136 ^a	697 ^a	123 ^a	588 ^a	130 ^a	634 ^a	72 ^a	274 ^a	88 ^a	347 ^a	—
3	280 ^a	1228	223 ^a	1129	292 ^a	1004	101 ^a	492	145 ^a	842 ^a	—
7	587 ^a	1217	524	1200	499	916	166 ^a	500	302	992	—
revertants/ml-eq	76	641	55	532	74	559	16	218	30	261	—
r squared	0.99	0.99	0.99	0.99	0.99	0.96	0.99	0.97	0.99	0.99	0.99

Dose (ml-eq/plate)	Subjects										
	60		61		62		63		64		
	U	H	U	H	U	H	U	H	U	H	
0	56 ^a	56 ^a	56 ^a	56 ^a	56 ^a	56 ^a					
0.5	—	352 ^a	—	313 ^a	—	762 ^a	—	249 ^a	—	246 ^a	—
1	122 ^a	574 ^a	93 ^a	501 ^a	163 ^a	1214 ^a	127 ^a	455 ^a	152 ^a	461 ^a	—
3	206 ^a	843	161 ^a	851	387 ^a	1648	200 ^a	891	229 ^a	934	—
7	370 ^a	819	370 ^a	996	547	1672	420 ^a	1215	335	830	—
revertants/ml-eq	44	518	45	445	111	1158	51	399	55	405	—
r squared	0.99	0.99	0.99	0.99	0.99	0.98	0.99	0.99	0.94	0.99	0.99

Dose (ml-eq/plate)	Subjects			
	65		66	
	U	H	U	H
0	56 ^a	56 ^a	75 ^a	75 ^a
0.5	—	265 ^a	—	263 ^a
1	84 ^a	437 ^a	125 ^a	436 ^a
3	163 ^a	951	242 ^a	908
7	335 ^a	1139	424	1045
revertants/ml-eq	40	381	56	361
r squared	0.99	0.99	0.99	0.99

U, unhydrolyzed urine; H, hydrolyzed urine.

*Urinary mutagenicity determined using Salmonella YG1024 + 2X S9.

^aValues used to calculate the slope (revertants/ml-eq).