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Estimation of Exposure to Two Potent Heterocyclic Aromatic Amines in Various Human Populations and Their Role in Colorectal Cancer

by

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ESTIMATION OF EXPOSURE TO TWO POTENT HETEROCYCLIC AROMATIC AMINES IN VARIOUS HUMAN POPULATIONS AND THEIR ROLE IN COLORECTAL CANCER

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Submitted to the Department of Toxicology on May 30, 1997 in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Toxicology

Abstract

The overall goal of this research project was to estimate exposure to food-derived heterocyclic aromatic amines (HAAs), PhIP and MeIQx, in various human populations and determine their role in colorectal cancer. Human exposure to PhIP and MeIQx was estimated by measuring the amounts of total HAAs (unmetabolized HAAs combined with acid-labile metabolites) present in urine samples. Purification and quantitative analysis of urine specimens was performed by immunoaffinity chromatography combined with ESI-LC/MS-MS. A population-based study of 130 male residents (44 blacks, 43 asians and 43 whites) of Los Angeles was performed in order to determine whether differences in ethnicity, dietary habits and smoking status have an effect on HAA disposition and metabolism. Non-whites excreted higher levels of urinary PhIP when compared to whites. The urinary excretion of PhIP was not correlated with the intake frequencies of beef, pork, chicken, bacon, fish, sausage or lard. However, a marginal association was observed between bacon consumption and the urinary excretion of total PhIP combined with MeIQx. A Chi-square test of association did not reveal a significant association between the urinary excretion of total MeIQx and PhIP. Neither smoking status nor N-acetylation activity had an impact on PhIP metabolism and disposition.

The effects of CYPIA2 and NAT2 activity on HAA disposition and metabolism were studied in a controlled food study. Subjects (n= 66) were phenotyped for CYPIA2/NAT2 activity after the consumption pan-fried beef containing undetectable levels of HAAs for seven days followed by seven days of fried beef containing high levels of HAAs (i.e., 9 ng PhIP/g_{-meat} and 32.8 ng PhIP/g_{-meat}). Subjects excreted approximately 9% and 4% of the ingested dose as total MeIQx and PhIP, respectively. Rapid CYPIA2 activity correlated with a weak but significant decrease in the urinary excretion of total MeIQx, but not PhIP. NAT2 activity did not affect HAA disposition nor metabolism. There was a marginal correlation between HAA intake and the excreted dose. The association between the urinary excretion of PhIP and MeIQx was also marginal.

Male residents of Shanghai, China participated in a nested case control study consisting of 59 colo-rectal cases and 240 controls. The main objective of this study was to determine whether individuals diagnosed with colorectal cancer excrete higher levels of food-derived HAAs when compared to matched controls. There were no differences in the levels of urinary excretion of PhIP nor MeIQx in a comparison between colo-rectal cases and their respective matched controls.

Dedication

to my grandparents.

On this day, I am quite fortunate to have two living grandmothers: Ms. Paula Staton (Nana), maternal grandmother; and Ms. Birdie Jenkins (Grandmommie), paternal grandmother. Unfortunately, my grandfather (Romulus E. Staton) is now deceased and could not witness the completion of my doctorate. If he were alive, I know he would be very proud of me.

During my matriculation at MIT, my grandparents would often ask "when are you going to graduate?" Since I completed my undergraduate studies at Spelman college within four years, they had a difficult time understanding why it took me so long to earn a doctorate. My college years only enabled me to scratch the surface of the research world. I graduated with a strong desire to study the role diet plays in cancer prevention or causation. This desire was further inspired by my grandparents who have suffered from prostrate cancer, breast cancer and most recently lung cancer. From my college days, I argued that lifestyle and dietary habits were the culprits. It took me 7 years to learn the necessary skills to establish a relationship between diet and health risks. Now I can finally say my matriculation at the Institute will conclude on the 6th of June 1997. I guess good things happen in sevens.

I would like to give a special dedication to my maternal grandmother. Everyone calls her Nana. Nana, born in 1927, was a native of Weldon, North Carolina. At the age of 16 she left her hometown, wedded two years later and bore 5 children. When her youngest child was at the tender age of 2, she became a single parent. Raising a family in the heart of Harlem in the late 50's was no easy task to say the least. Despite the statistical findings with regards to single mothers, poverty and total dependence upon public assistance was not part of her life goal. Determined to provide food, shelter and clothing for her children, she worked many hard and long nights as a short order cook. She had a special knack for stretching the dollar beyond its limits. When the dollar could no longer stretch she would simply "make do". During the day she instilled self pride, organization, self-discipline, and self motivation in her children. These strong principles enabled her children to obtain college degree's. My grandmother's teachings have also affected her grandchildren. Amongst her 14 grandchildren, ranging in ages' 7-30, we have brought home 8 high school diplomas, 3 bachelor's degrees and 2 master's degrees. Currently, three of her grandson's are enrolled in college. In conclusion, I come from a generation of well-educated family members. We are not afraid to work hard and long. Since we are over-achievers, we need reminders to quit. As a family we truly believe "a mind is a terrible thing to waste."

Acknowledgments

There are many players that have helped me through this long and arduous journey that I need to thank.

Spiritual Guidance:

First, I would like to thank God for giving me faith, self motivation and determination.

My Family:

I appreciate my family for instilling in me a thirst for knowledge and a standard of excellence. I have to credit my mother for my organizational skills, tremendous ability to perform numerous task simultaneously, and a home to rest and relax. My mother has been very supportive of me emotionally, even at the oddest hours. I cannot thank her enough for just listening. Many thanks go to my family for all the get-togethers (i.e., family reunions, Thanksgiving, Christmas, Kwanzaa, birthdays and baby showers). I attribute my extreme focus to my energetic and fun-loving daughter, Nayla Christene. I can't forget my deepest gratitude toward Michael for being there throughout all my emotional ups and downs. Michael has been extremely helpful by taking care of Nayla and maintaining our home away from home. This has been a tough academic year for both Michael and I, but we both completed our degrees at MIT. I would also like to thank my immediate family, for providing me with emergency funds and handling crisis situations well, especially when I could not. I totally appreciate the Vega Family, for taking care of Nayla and treating her like a granddaughter.

BGSA:

I would like to thank the BGSA family and the MIT community for often providing much needed breaks. My community service at MIT would not have been possible without the constant support of co-advisors of BGSA (i.e., Ike Colbert, Margaret Tyler, Blair Staton and Linda Hughes).

Administrators:

I have continuously received support from various administrators at MIT. Margaret Tyler always made time for a little chit chat (her way of getting the update on my progress). Clarence Williams encouraged me to pursue my dreams despite the many obstacles. Ayida Mthembu often invited me over for cooking sessions or an active-listening session. Ayida was constantly reminded me to take advantage of the available resources at MIT. Lynn Roberson always had a sensible, practical, up beat and positive attitude. Several administrators (Ayida Mthembu, Margaret Tyler and Mary Rowe) have

been extremely helpful in providing additional funds/resources. The emergency funds were used for extra food and child care services.

Faculty:

I probably would not have even learned about MIT if it were not for Professor William Thilly. Professor Thilly actively recruits minority students and commits himself toward bridging the gap between HBCU's and the Ivy League Tower. I clearly remember the day he came to the Atlanta University Center and encouraged me to apply to MIT. Moreover, Thilly has always believed in my ability to perform and constantly pushed me to do better. I fully appreciate his guidance in statistical analysis.

I would like to thank my advisor, Professor Tannenbaum for teaching me that you should not be in the field of Science because someone forces you to do so. One should study Science because of a natural desire to learn and expand. Steve played an important role in helping me to focus, something a graduate student must do in order to complete the marathon. Over the years I have learned to appreciate Steve's style. He encourages independent research and makes time to check on his students' progress. Although he can be fiery at times, he manages to remain calm when our experiments take a bad turn. He's also the advisor of the 90's. I can't thank him enough for allowing me to bring my daughter to the lab for the first six months of her life. His setting up a nursery in the lab saved me $3000 in child care services. His flexibility and great suggestions enabled me to juggle motherhood and school. Sometimes you need someone like Steve to say "just focus on one task at a time."

I must also thank my Thesis Committee (i.e., Professor Thilly, Professor Dedon, Professor Wogan and Professor Tannenbaum) for being supportive of my academic growth and sharing their constructive ideas concerning my research project. The common denominator amongst all Toxicology professors is their firmness and willingness to give praise for adequate progress.

Coworkers:

A special thanks go to my coworkers. Sara Stillwell has always been my soft whisper of confidence. Pete Wishnok has been an inspirational teacher and encouraged me to troubleshoot before asking (him) for help. Everything I know about analytical chemistry I have acquired from the mass spec duo (Sara and Pete). The final acid hydrolysis and urine extractions would not have been possible without the diligent help of Joe Glogowski. Olga is well known for her friendly and firm reminders of deadlines. Despite the sign in her office, she's the mom away from home. She keeps everyone on their toes. And life would not be the same without the little goodies (for example bunny slippers and night gowns) for Nayla. I can't think of many people who are as thoughtful as Olga. I would not have been able to complete my last experiments if it were not for Olga and Tim. They both helped to take care of Nayla while I worked feverishly on the Finnigan. And what will the Tannenbaum group be without "Timmy Ray". When he's

not filling out super rush orders or performing administrative tasks he's correcting our computer/printer glitches. I personally think the Tannenbaum group will be somewhat helpless without him. I can not thank Tim enough for giving me (free) ram for my personal computer which acquired the insufficient memory syndrome. When my stress zone was at its peak, I could always count on Jennifer to play with Nayla. Thank you Jen, Sara and Eric for editing my thesis. Don Brunson, a former graduate student, always brightened my day with his cheerfulness, humor, gourmet meals and chocolate treats. I think we should call Teresa Wright, "Don Part II". This woman can bake all kinds of pastries. I especially like her yummy homemade bread. Teresa has also tolerated my asking her all sorts of questions. I have often chuckled over Jacquin and Samar's humor (Jacquin constantly picks on Samar). Samar is my buddy. We've had many down to earth conversations about men (what else?). Since Samar works so hard and late at night, I often had someone to keep me company in the lab. Thanks Samar for the company. Can Ozbal participated in one of my preliminary food studies. I hope he at least enjoyed the burgers. What would we do without Finnigan time-sharing? Thanks Per, Vicky and Shawn for tolerating my extensive use of the Finnigan. Lastly, I would like to thank the whole Tannenbaum group for their patience. I will truly miss this family.

Close Friends:

Many thanks go to Shurronne for allowing me to get away from the stress by cooking, sewing, brainstorming together or playing with Nayla. I cannot forget to acknowledge Kara, my homegirl and my best friend for 9 years, for allowing me to regroup through our girl talk and taking on Nayla as if she was her own. I cannot forget to mention Alan and Kenneth for the quaint get-togethers, the many laughs (I do not know who is funnier). A special thanks goes to Alan, Ken, Kara and Angie for watching Nayla on weekends. Many thanks go to Damon Phillips, my Morehouse "brother", for being my first friend at MIT, confident, and buddy. I cherish Gavin Dutchin's words of wisdom "You cannot help anyone until you have helped yourself." Thanks Gavin for helping me get through the first two years at MIT.

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AαC; 2-amino-9H-pyrido[2,3-b]indole

AF-2; 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide

AfB_1; Aflatoxin B_1

aprt; adenine phosphori bosye transferase

B[a]P; benzo[a]pyrene

CHO; Chinese hamster ovary

Cre-P-1; 4-amino-1,6-dimethyl-2-methylamino-IH,6H-pyrrolo[3,4-f]benzimidazole-5,7-dione

CYPIA2; cytochrome P-450 IA2

DMIP; 2-amino-n,n-dimethylfuro[2,3(or 3,2)-e]imidazo[4,5-b]pyridine

EICI-LC-MS/MS; electron impact chemical ionization liquid chromatography mass spectrometry/mass spectrometry

GC/MS; gas chromatography mass spectrometry

Glu-P-1; 2-amino-6-methyldipyrido[1,2-a:3',2'-d]imidazole

Glu-P-2; 2-aminodipyrido[1,2-a:3',2'-d]imidazole

GSH; glutathione

HAAs; heterocyclic aromatic amines

HPLC; high pressure liquid chromatography

hprt; hypo xanthine phosphoribosye transferase

IQ; 2-amino-3-methylimidazo[4,5-f]quinoline

IQx; 2-amino-3-methylimidazo[4,5-f]quinoxaline

Lys-P-1; 3,4-cyclopentenopyrido[3,2-a]carbazole

MTD; maximum tolerated dose

MeAαC; 2-amino-3-methyl-9H-pyrido[2,3-b]indole

MeIFP; 2-amino-(1 or 3),6-dimethylfurol[2.3(or 3.2)-e]imidazo[4,5-b]pyridine

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

5-O-Glu MeIQx; 2 amino-4(or 5)-B-D-glucuronopy-ranosyloxy

5-O-Glucuronide MeIQx; 2-amino-5-(β-1-glucosiduronyloxy)-3,8-dimethylimidazo [4,5-f]quinoxaline

5-O-SO_3^- MeIQx; 2-amino-3,8-dimethylimidazo[4,5-f]quinoxalin-5-yl sulfate

8-CH_2OH MeIQx- 2-amino-8-hydroxymethyl-2-methylimidazo[4,5-f]quinoxaline

8-methyl-O-Glucuronide MeIQx;

8-methyl-OSO_3^- MeIQx; 2-amino-8-hydoxymethyl-3-methylimidazo [4,5-f] quinoxalin-5-yl sulfate

NA; nuclear aberration

N^2-Acetyl MeIQx; *N*-acetyl-2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline

N-(acetoxy)-MeIQx; 2-(acetoxy)-2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline

N^2-Glucuronide MeIQx; *2*-(β-1-glucosidurony)-2-amino-3,8-dimethylimidazo [4,5-f]quinoxaline

N^3-Glucuronide MeIQx;

N^2 -SO_3^- MeIQx ; *N*-(3,8-dimethylimidazo[4,5-f]quinoxalin-2yl) sulfamate

N(OH) MeIQx; 2-hydroxy-2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline

N(OH) N^2 Glucuronide MeIQx; N^2-(β-glucosiduronyl)-N-hydroxy-2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline

N(OH) N1 Glucuronide MeIQx;

$ng/g_{-creatinine}$; nanogram per gram of creatinine

ng/g_{-meat}; nanogram per gram of cooked meat

ng/ml_{-urine}; nanogram per gram of urine

N-(acetoxy)-PhIP; 2-acetoxy PhIP

NAT; N-acetyltransferase

NAT2; polymorphic acetyl transferase

N^2-Glucuronide PhIP;

N^3-Glucuronide PhIP;

N-(OH)-PhIP; 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-b]pyridine

N-(OH)-PhIP N^2-Glucuronide; 2-(N-ß-D)glucuronopyranosyl(hydroxamino)-methyl-6-phenylimidazo[4,5-b]pyridine

N-(OH)-PhIP N^3-Glucuronide;3-(N-B-1-glucosiduronyl)-2-hydroxy-amino-1-methyl-6 phenylimidazo[4,5-b] phyridine

OAT; O-acetyltransferase

Orn-P-1; 4-amino-6-methyl-1H-2,5,10,10b-tetraazafluoranthene

PBS; phosphate-buffered solution

Phe-P-1; 2-amino-5-phenylpyridine

4'-(OH)-PhIP; 4-(2-amino)-1-methyl-6-(-4-hydroxyphenyl)imidazo [4,5-b]pyridine

4'-O-Glucuronide PhIP-; 2-amino-4'-(b-1-glucosiduronyloxy)-1-methyl-6-phenylimidazo [4,5-b] phyridine

PhIP; 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine

4'-PhIP-sulfate; 4-(-2-amino-1-methylimidazo[4,5-b]pyrid-6-yl)phenyl sulfate

ppb; parts per billion

SCEs; sister chromatid exchanges

TD_{50}; dose required to produce cancer in 50% of treated animals in a lifetime

TMIP; 2-amino-n,n,n-trimethylimidazopyridine

Trp-P-1; 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole

Trp-P-2; 3-amino-1-methyl-5H-pyrido[4,3-b]indole

UDGT; uridine diphosphate glucuronyl transferase

UDS; unscheduled DNA synthesis

I. Introduction

Over a lifetime, humans consume over ten tons of food. In addition to providing nutritional sustenance and protection from diseases, our diet exposes us to numerous organic and inorganic substances which contribute to the etiology of human cancer. In fact, in their "Avoidable Risk" report, Doll and Peto (1981) suggested that diet contributed 35% toward all cancers of the United States. Moreover, epidemiological data and migration studies have shown that an over-consumption of fat and muscle-meat relate to an increased risk of colorectal cancer (Potter et al., 1990), the second leading cause of cancer death within the United States (Boring et al., 1994). Moreover, individuals who have preferences towards meats which have been browned or charred via broiling, barbecuing or pan frying are at higher risk for colorectal cancer than those who use cooking methods which require low to moderate temperatures (Gerhardsson de Verdier et al., 1991). Results of epidemiological studies have suggested that biologically active material formed during the cooking process may play a role in tumorigenesis.

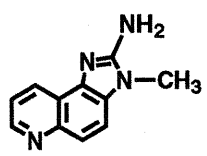
In 1939, Widmark initiated investigations on the occurrence of mutagenic-carcinogenic substances found in cooked muscle-meat. In a short term study it was found that rodents developed tumors of the mammary gland when their backs were continuously painted with organic solvent extracts of broiled muscle-meat. Sugimura and Nagao (1977) found that the charred surfaces of broiled beef steak exerted mutagenic activity toward *Salmonella typhimurium* TA98 with metabolic activation. A total of 5 g of charred surface from 190 g of beefsteak contained mutagenic activity that was 100,000 fold higher than the mutagenicity of its benzo(a)pyrene content. As a result of these and other studies, analytical methods were developed to isolate and identify the mutagenic substances present in cooked foods and pyrolysates of amino acids and proteins. High pressure liquid chromatography (HPLC) was used to purify solvent extracts of cooked muscle-meat. Fractions containing mutagenic activity were detected by the

Ames/*Salmonella* test. Structural analysis was made feasible through proton nuclear magnetic resonance and mass spectral data. The predominant compounds detected belong to a group of chemicals classified as heterocyclic aromatic amines (HAAs) (See Figure 1).

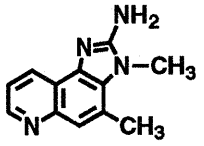
HAAs have been classified as either IQ-type compounds in which the amino group does not undergo deamination by treatment with 2 mM sodium nitrite; or non-IQ compounds, in which the amino group is converted to a hydroxyl group with 2mM sodium nitrite treatment (Wakabayashi et al., 1992). The amino group of IQ-type compounds is converted to a nitro group in the presence of 50 mM sodium nitrite and the resulting compound is as mutagenic as the parent compound in the absence of metabolic activation. In addition, IQ-type compounds have a 2-aminoimidazole moiety as a common structure and include the following compounds: aminoimidazoquinolines (i.e., IQ and MeIQ); aminoimidazoquinoxalines (i.e., IQ_x, MeIQx, MeIQx, 4,8-DiMeIQx); and aminoimidazopyridines (i.e., TMIP and PhIP). The aminoimidazole group is derived from creatine in muscle-meat and fish, and other parts of the IQ-type compounds are derived from amino acids and possibly sugars. Non-IQ compounds which contain a 2-aminopyridine moiety as a common structure have been subcategorized to include amino-γ-carbolines (i.e., Glu-P-1 and Glu-P-2) and amino-α-carbolines (i.e., AαC and MeAαC). In addition to the IQ-type and non-IQ compounds, two oxygen containing HAAs were isolated: 4-amino-1,6-dimethyl-2-methylamino-IH,6H-pyrrolo[3,4-f]benzimidazole-5,7-dione (Cre-P-1), isolated from creatine pyrolysate (Nukaya et al., 1991) and 2-amino-(1 or 3),6-dimethylfurol[2.3(or 3.2)-e]imidazo[4,5-b]pyridine (MeIFP), isolated from a creatine-supplemented fried-meat product (Knize et al., 1990).

The aforementioned HAAs have been tested for their biological effects, particularly their mutagenic and carcinogenic potency. Eight out of the 19 known HAAs have specific mutagenic potencies which are 10 to 10,000 fold stronger than other typical

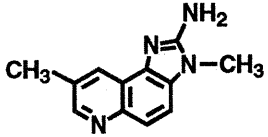
Figure 1. Structures of Heterocyclic Aromatic Amines



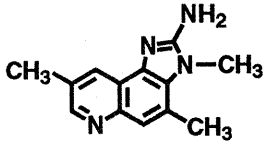
IQ



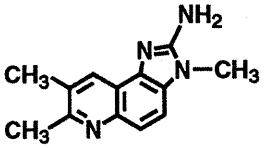
MeIQ



$MeIQ_x$



$4,8\text{-DiMeIQ}_x$



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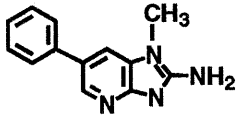
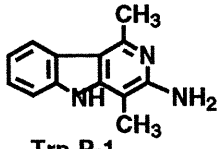
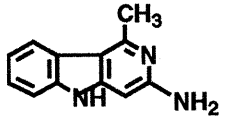


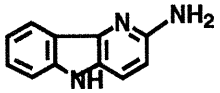
PhIP



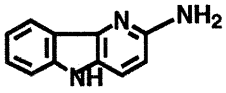
Trp-P-1



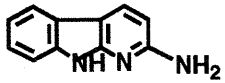
Trp-P-2



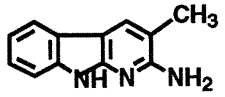
Glu-P-1



Glu-P-2



AαC



MeAαC

food-borne carcinogens, such as benzo[a]pyrene (B[a]P), 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide (AF-2), and aflatoxin B_1 (AfB_1) (see Table 1). Although the genotoxic potency of IQ-type HAAs (i.e., IQ and MeIQx) is higher than PhIP in bacterial cell lines, in mammalian cell lines PhIP is more mutagenic than IQ-type HAAs (Thompson et al., 1987). The reduced metabolic activation of PhIP relative to IQ-type HAAs in bacterial cell lines is due to the compound specificity of esterifying enzymes (i.e., O-acetyltransferase (OAT) and sulfotransferase) (Kato et al., 1986; Oda et al., 1995).

In order to exert their mutagenic potency, HAAs must first undergo conversion to metabolically active species, such as the N-hydroxylamine, N-acetoxy, and N-sulfoxy derivatives. The functional groups of these derivatives serve as good leaving groups resulting in the formation of electrophiles which can react with DNA and induce genetic alterations. In addition to inducing mutations in bacterial and mammalian cell lines, HAAs have been shown to induce: mutations, sister chromatid exchanges, unscheduled DNA synthesis, chromosomal aberrations, nuclear aberrations and DNA repair synthesis in mammalian cell lines and/or rodents *in vivo* (Aeschbacher and Turesky, 1991).

Long-term animal experiments with rodents fed a diet containing HAAs have been performed. Some of the major target sites for tumorigenesis in mice include the liver, forestomach, lung, blood vessels, hematopoietic system, and lymphoid tissue (Eisenbrand and Tang, 1993). In F344 rats, tumors developed in the liver, intestine, skin, oral cavity, mammary gland, zymbal gland, and clitoral gland (Eisenbrand and Tang, 1993). Unlike most HAAs, PhIP is not a hepatocarcinogen. Instead, PhIP induced tumors of the colon in male rats, mammary gland in female rats (Ito et al., 1991) and lymphomas in mice of both sexes (Esumi et al., 1989). Table 2 reveals the carcinogenicity of various HAAs in rodents.

Methods have been developed for measuring the levels of heterocyclic amines in cooked foods, cigarette smoke condensate, beer, wine and human urine specimens

Table 1. Mutagenicities of Heterocyclic Aromatic Amines and typical carcinogens in Salmonella typhimurium TA98 and TA100.

	Revertants/ug	
Compound	**TA98**	**TA100**
IQ	**433,000**	**7,000**
MeIQ	**661,000**	**30,000**
IQx	**75,000**	**1,500**
MeIQx	**145,000**	**14,000**
4,8-DiMeIQx	**183,000**	**8,000**
7,8-DiMeIQx	**163,000**	**9,900**
PhIP	**1,800**	**120**
Trp-P-1	**39,000**	**1,700**
Trp-P-2	**104,200**	**1,800**
Glu-P-1	**49,000**	**3,200**
Glu-P-2	**1,900**	**1,200**
AαC	**300**	**20**
MeAαC	**200**	**120**
Aflatoxin B_1	**6,000**	**28,000**
Af-2	**6,500**	**42,000**
B(a)P	**320**	**660**

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Table 2. Carcinogenicities of Heterocyclic Aromatic Amines in Rodents

Chemical	Species	%Concentration in diet	Target organs	Reference
MeIQx	Rats	0.06	Liver, Zymbal gland, skin, clitoral gland	Kato et al., 1988
	Mice	0.06	Liver, lung, hematopoietic system	Ohgaki et al., 1984a
IQ	Rats	0.03	Liver, sm. and lg. intestine, Zymbal gland, clitoral gland	Takayama et al., 1984a
	Mice	0.03	Liver, forestomach, lung	Ohgaki et al., 1984a
MeIQ	Rats	0.04	lg. intestine, Zymbal gland, skin, oral cavity, mammary gland	Kato et al., 1989
	Mice	0.04 and 0.1	Liver, forestomach	Ohgaki et al., 1986
PhIP	Rats	0.04	Large Intestine, mammary gland	Ito et al., 1989
	Mice	0.04	hematopoietic system	Esumi et al, 1989
Trp-P-1	Rats	0.02 and 0.15	Liver	Takayama et al., 1985
	Mice	0.02	Liver	Matsukura et al., 1981
Trp-P-2	Rats	0.02	Liver	Hosaka et al., 1981
	Mice	0.02	Liver	Matsukura et al., 1981
Glu-P-1	Rats	0.05	Liver, sm and lg intestine, Zymbal gland, clitoral gland	Takayama et al., 1984b
	Mice	0.05	Liver, blood vessels	Ohgaki et al., 1984b
Glu-P-2	Rats	0.05	Liver, sm and lg intestine, Zymbal gland, clitoral gland	Takayama et al., 1984b
	Mice	0.05	Liver, blood vessels	Ohgaki et al., 1984b
MeAαC	Mice	0.08	Liver, blood vessels	Ohgaki et al., 1984b
AαC	Mice	0.08	Liver, blood vessels	Ohgaki et al., 1984b

(Wakabayashi et al., 1992; Manabe et al., 1991; Manabe et al., 1993; Murray et al., 1989; Ushiyama et al., 1991; Lynch et al., 1992; Ji et al., 1994; Stillwell et al., 1994). It is generally assumed that exposure to HAAs via cigarette smoke, wine or beer is considerably less than through consumption of cooked muscle-meat. Analytical studies reveal that meats prepared under various cooking conditions (i.e., frying, grilling and broiling) contain HAAs ranging from 0.1-69.2 ppb (Wakabayashi et al., 1993). Based on dietary frequency questionnaires, the estimated human exposure to HAAs is about 0.4-16 μg per day per capita (Wakabayashi et al., 1993). A major portion of the daily intake of HAAs is represented by PhIP and MeIQx. Daily intakes of PhIP and MeIQx were estimated to be 0.1-13.8 μg and 0.2-2.6 μg per person, respectively (Wakabayashi et al., 1993). Our laboratory has detected the presence of MeIQx in the urine specimens collected from 133 healthy male residents of Los Angeles consuming normal diets (Ji et al., 1994). In addition to MeIQx, PhIP has been detected in the urine of Japanese consuming a Japanese-Western diet (Ushiyama et al., 1991). These studies indicate that humans are continuously exposed to HAAs in foods.

Due to their mutagenic-carcinogenic potency and their relatively high abundance in commonly consumed foods, PhIP and MeIQx may play a role in tumongenesis in humans. The overall objectives of this thesis are to determine: (i) the factors which may influence HAA metabolism or disposition; (ii) whether a relationship exists between the dietary intake of HAAS and the excreted dose; (iii) whether the urinary excretion of MeIQx can be used as an index of urinary PhIP; and (iv) whether colorectal cancer cases excrete higher levels of food-derived HAAS in their urine when compared to their matched controls.

II. Literature Review

1. Heterocyclic Aromatic Amines

1.1 Pyrolysates of Amino Acids and Proteins

In 1977, Sugimura and Nagao observed mutagenic activity in the smoke condensates of the charred parts of broiled or grilled beef and fish. In order to determine its origin, pyrolysates of pure proteins, DNA, RNA, starch and vegetable oil were tested for their mutagenicity. Only proteins produced considerable mutagenicity similar to that found in the cooked muscle-meat samples (Nagao et al., 1977). Consequently, pyrolysis of single amino acids and proteins led to the isolation of a series of mutagenic HAAs. Trp-P-1(2), Phe-P-1, Glu-P-1(2), Orn-P-1 and Cre-P-1 were isolated from the smoke condensates of tyrptophan, glutamic acid, ornithine and creatine, respectively (Sugimura et al., 1977b; Yamamoto et al., 1978; Yokota et al., 1981; Nukaya et al., 1991). Two α-carbolines (i.e., AαC and MeAαC) were isolated from the smoke condensate of the soybean globulin pyrolysate (Yoshida et al., 1981). Several of these mutagenic pyrolysates, namely Trp-P-1, Trp-P-1, Glu-P-1, Glu-P-2, AαC, MeAαC and Phe-P-1, have also been isolated from grilled or broiled beef, chicken, and fish (Wakabayashi et al., 1992). Table 3 reveals the amounts of HAAs detected in cooked muscle-meat.

1.2 Formation of Heterocyclic Aromatic Amines During the Cooking Process

The pyrolysate studies contributed to the isolation of a new class of food-borne mutagens from muscle-derived meats prepared under normal household cooking conditions. In the early eighties, Kasai and co-workers (1980a; 1980b; 1980c; 1981) identified mutagenic 2-aminoimidazoarenes, consisting of imidazoquinolines (IQ and MeIQ) and imidazoquinoxalines (MeIQx), in the charred surfaces of broiled sardines and fried beef, respectively, cooked at high temperatures ranging from 100 to 300 °C. In the late eighties,

Table 3. Amounts of Heterocyclic Aromatic Amines in Cooked Foods

	Amounts (ng/g)								
Sample	**IQ**	**MeIQ**	**MeIQ$_x$**	**DiMeIQ$_x$**	**PhIP**	**Trp-P-1**	**Trp-P-2**	**AαC**	**MeAαC**
Broiled beef	**0.19**		**2.11**		**15.7**	**0.21**	**0.25**	**1.2**	
Fried ground beef			**0.64**	**0.12**	**0.56**	**0.19**	**0.21**		
Broiled chicken			**2.33**	**0.81**	**38.1**	**0.12**	**0.18**	**0.21**	
Fried chicken[a]			**2**	**2**	**37**				
Grilled chicken[a, c]			**2**[a]	**1**[a]	**140**[a]			**180**[c]	**15**[c]
Fried cod fish[b]	**0.16**	**0.03**	**6.44**	**0.1**	**69**				
Fried bacon[d, e]	**10.5**[d]	**1.7**[d]	**0.9-18**[e]	**nd-0.6**[e]	**nd-53**[e]				
Bacon fat[e]			**1.4-27**	**nd**[*]**-2.4**	**nd-17**				
Falusausage[f] **pan residue**			 **0.2**	**0.07** **0.1**	**0.1** **0.4**				

Wakaybayashi et al., 1993; [a] Sinha et al., 1995a; [b] Zhang et al., 1988; [c] Matsumoto et al., 1981; [d] Johansson et al., 1994; [e] Gross et al., 1993; [f] Skog et al., 1995. [*] nd = not detected

imidazopyridines (PhIP and TMIP), 4,8-DiMeIQx and 7,8-DiMeIQx were detected in fried beef samples (Felton et al., 1986; Turesky et al., 1988). The two most abundant HAAs isolated from cooked muscle-meat (broiled beef, fried ground beef, broiled chicken, broiled mutton and fried cod) and food-grade beef extract are PhIP (0.56-69.2 ng/g) and MeIQx (0.8-12 ng/g) (see Table 1) (Wakabayashi et al., 1992). Gross and coworkers (1993) reported the occurrence of HAAs in pan residues and drippings derived from grilling or pan frying muscle-meat. For example, samples of bacon fat contained 1.4-27 ng/g MeIQx, 2.4 ng/g 4,8-DiMeIQx, 2.4 ng/g PhIP, and 0.1 ng/g AαC.

Several investigators suggest that estimating human consumption of dietary HAAs based on levels found in cooked muscle-meat samples may result in an underestimation, since these compounds have also been detected in pan drippings and residues used in the preparation of gravies or vegetable/rice dishes. Although the specific mutagens were not identified, Overik and coworkers (1987) revealed that gravies prepared from pan residues from fried beef contained high levels of mutagenic activity. Gross and coworkers (1993) showed that pan scrapings from bacon and grilled beef and fish contained 2 to 200-fold higher concentrations of MeIQx, 4,8-DiMeIQx and PhIP than those found in the cooked meats. HAAs were also detected in the pan residues of fried meats (i.e., pork chops, pork bellies, bacon minute steak, sirloin steak, ground beef, and meat balls) most commonly consumed by residents of Stockholm, Sweden (Gerhardsson de Verdier et al., 1991). In a 100g sample of meat, the amounts of MeIQx, DiMeIQx, PhIP and IQ detected in the pan residues corresponded to 0.02-2.3 μg, 0.01-0.4 μg and 0.03-8.2 μg, respectively. These levels represented 3.6%-91% (49.4% ± 26.3; mean ± STD) of the levels of individual HAAs detected in fried meat combined with pan residues.

1.3 Other Sources of HAAs

Several investigators have developed sensitive techniques which enabled the detection of HAAs in cigarette smoke, beer, wine and coffee beans. The HAAs isolated from cigarette smoke condensate include the following non-IQ HAAs: AαC (25-260 ng/cig), Me AαC (2-37 ng/cig), IQ (0.26 ng/cig), Glu-P-1 (0.37-0.89 ng/cig), Glu-P-2 (0.25-0.88ng/cig), Trp-P-1 (0.29-0.48 ng/cig), Trp-P-2 (0.82-1.1 ng/cig) and PhIP (11-23 ng/cig) (Manabe et al., 1991). The presence of HAAs in cigarette smoke suggests that these compounds can be formed through the combustion process. Support for this was based on the distribution of PhIP, AαC, MeAαC, Trp-P-1 and Trp-P-2 in various components of the environment such as airborne particles, rain water, cigarette smoke-polluted indoor air and diesel exhaust particles (Manabe et al., 1989; ibid. 1990; ibid. 1990; ibid. 1991). The concentrations of PhIP detected in beer and wine were 14.1ng/l and 30.4 ng/l, respectively (Manabe et al., 1993). The MeIQ content in roasted coffee beans was 0.016 ng/g for hot air-roasted, 0.032 ng/g for charcoal-roasted and 0.15 ng/g for high temperature roasted coffee beans (Kikugawa et al., 1989). The daily exposure to PhIP in an individual that smokes 20 cigarettes (0.32 μg) and drinks a half liter of beer (0.007 μg) or wine (0.015 μg) are significantly lower than the daily intakes through consumption of cooked muscle-meat (0.1-13.8 μg).

A few investigators have detected HAAs in foods other than muscle-meat. Gross and coworkers (1986) found 0.1 ng/g of IQ in fried eggs. Matsumoto and coworkers (1981) identified 47 ng/g of AαC and 5.4 ng/g of MeAαC in grilled onions. In the oil of charred egg yolk, a commercially available food in Japan, IQ (1.1 ng/g), Glu-P-1 (4.8 ng/g) and MeIQ were present. Gerhardsson de Verdier and coworkers (1991) detected several HAAs in the low ppb in pan fried falusausage, a commonly consumed sausage in Sweden, containing organ meat and modified food starch.

1.4 Mechanisms in HAA Formation

Several investigators proposed the involvement of Maillard reactions in the formation of IQ-type HAAs in cooked muscle-meat samples (Spingarn and Garvie, 1979; Shibamoto et al., 1981; Wei et al., 1981; Powrie et al., 1982; Jagerstad et al., 1983a; Aeschbacher, 1986). This reaction is a non-enzymatic browning which is necessary for the development of great flavor and texture during heat treatment of foodstuff. The precursors necessary for HAA formation include the following: reducing sugars (i.e., glucose, fructose or ribose), amino acids (i.e., glycine, phenylalanine, alanine or lysine) and creatin(in)e (Jagerstad et al., 1983b; Negishi et al., 1984; Jagerstad et al., 1984; Nyhammar et al., 1986). These precursors naturally occur in muscle-derived meat. The Maillard reaction involves an initial condensation between a reducing sugar and an amino acid resulting in the formation of strecker degradation products, such as pyrazines or pyridines and aldehydes (Jagerstad et al., 1983a). These products undergo further condensation to give rise to the quinoline or quinoxaline part of IQ-type HAAs. When subjected to reaction temperatures above 100 °C, creatine spontaneously undergoes dehydration and cyclization to form creatinine which in turn reacts with an aldehyde to form the 2-aminoimidazo group of IQ-type HAAs. The suggested route of formation of imidazoquinolines (quinoxalines) needs further substantiation since these compounds have also been produced by pyrolyzing a mixture of creatin(in)e and a specific amino acid without a reducing sugar (Yoshida et al., 1984; Knize et al., 1988; Overvik et al., 1989; Felton et al., 1990). Although the suggested route of mutagen formation was intended for IQ-type HAAs, it may also lead to the formation of imidazopyridines (i.e., PhIP). This is supported by the findings of Taylor et al., (see Aeschbacher and Turesky. 1993) and Felton (1990) who unequivocally demonstrated the incorporation of stable isotopic precursors (i.e., creatine and phenylalanine, respectively) into PhIP.

1.4.1 Pyrolysate Model Systems

Investigators in Japan and Sweden adopted a pyrolysis model system in order to generate data in support of the postulated route for HAA formation. In the model,

creatin(in)e, a specific amino acid (i.e., glycine, phenylalanine, alanine or threonine) and a reducing sugar (glucose, fructose or ribose) were boiled under reflux in a molar ratio of 1:1:0.5 in a solvent mixture of diethylene glycol-water with a reaction temperature of 128 °C for two hr. The addition of diethylene glycol ensured a reaction temperature above 100 °C and kept the reactants in solution. This system led to the isolation and identification of the following HAAs: MeIQx (Jagerstad et al., 1984; ibid. 1986; Grivas et al., 1986; Muramatsu and Matsushima 1985), 7,8-DiMeIQx (Negishi et al., 1984); 4,8-DiMeIQx (Jagerstad et al., 1986a; ibid. 1986b; Muramatsu and Matsushima 1985), MeIQ (Grivas et al., 1985), IQ (Grivas et al., 1986), 7,8-DiMeIQx (Negishi et al., 1984; Skog et al., 1990) and PhIP (Felton et al., 1987; ibid. 1989; ibid. 1990; Overvik et al., 1989; Shioya et al., 1987; Jagerstad 1990). These mutagenic substances can also be produced by dry heating the reactants using oven baking conditions at moderate cooking temperatures (150-250 °C).

1.5 Factors Effecting HAA Formation in Meats

Numerous studies have investigated the effects of time, temperature, style of preparation, type of food, meat content and fat/oils on the mutagenic activity present in various cooked foods, especially muscle-derived meats. In a comparison of several cooking styles, Bjeldanes and coworkers (1982a) revealed that broiling, grilling and frying produce higher mutagenic activity than baking, roasting, stewing or microwaving. This variation in mutagenic activity is due to the differences in heating temperature and meat surface temperature. For example, stewing and boiling require cooking temperatures near 100 °C resulting in low mutagenic activity. Oven baking and roasting, characterized by moderate temperatures (i.e., 180 °C), produce intermediate levels of mutagenic activity. Frying, grilling and broiling which require high cooking temperatures (i.e., 250 °C to 325 °C) exhibit the highest levels of mutagenicity. These direct high-temperature cooking processes enable the meat surface temperatures to get well over 100 °C (i.e., 200 °C for frying and 120 °C for broiling), resulting in appreciable levels of mutagen production. When compared to temperature, the effect of cooking time on mutagen formation are less

dramatic. In fact, mutagen formation occurs most rapidly during the first 5-6 min of frying beef (Bjeldanes et al., 1982a). The subsequent decline in mutagen formation in cooked beef after the first 6 min may be attributed to the removal of HAAs along with the fat and juice from the meat surface.

Moreover, foods containing high levels of protein and creatine (i.e., beef, chicken, pork and fish) exert higher mutagenicity toward *Salmonella typhimurium* when compared to high protein-low creatine food sources (i.e., cheese, tofu, eggs, legumes and organ meats) (Bjeldanes et al., 1982b). Thus, it was proposed that in addition to a high protein content, creatine derived from muscle-meat is a crucial factor involved in the formation of bacterial mutagens. This finding was substantiated by the studies of Becher and coworkers (1988) who demonstrated the formation of mutagenic HAAs (i.e., TMIP, DMIP and IQx) in fried Norwegian meat product supplemented with creatine.

Bjeldanes and coworkers (1982b) also showed that fish containing more pink or red color (i.e., red snapper and salmon) was more susceptible to mutagen formation than white meat (i.e., cod, sole or haddock). However, in the case of chicken, white meat forms more mutagens than dark meat under the same cooking conditions. These differences in mutagen formation in dark versus white meat may be due to the differences in the fat content or meat surface. In partial support of this hypotheses, Spingarn and coworkers (1981) showed that the mutagenicity in fried beef patties increased in a linear fashion when the added fat content was increased over a range of 5-30%. Thus, the low mutagenic activity in white-meat fish is a consequence of its relatively low fat content when compared to pink- or red-meat. The higher mutagenic activity detected in white meat of chicken when compared to dark meat may be attributed to its relatively flat surface which may optimize thermic transfer to the meat surface.

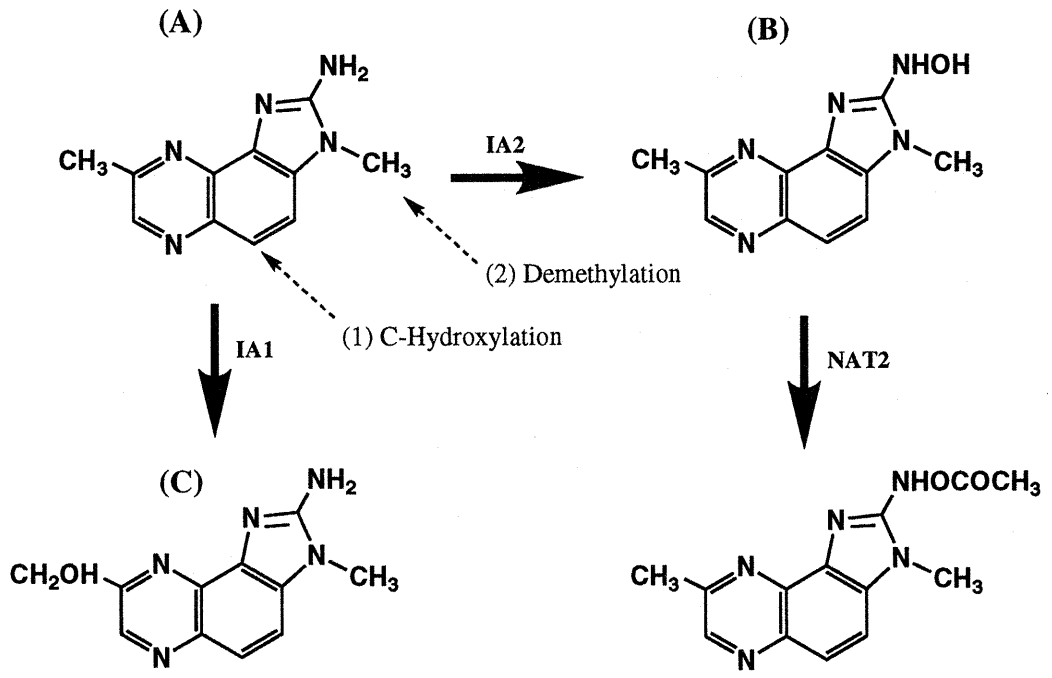
Nilsson and coworkers (1983) observed significant increases in mutagenicity in pork samples when fried at 250 °C with coconut oil, lard, butter and margarine. Frying fat may increase mutagenicity during the cooking process by: (1) increasing the temperature at

the surface of the meat; (2) enhancing the production of Maillard Reaction products, such as aldehydes and pyrazines; or (3) undergoing thermic oxidation resulting in the formation of free radicals which may in turn catalyze condensation reactions involved in HAA formation. Maillard reaction products may also be increased by the presence of small amounts of sugar in milk-based products, such as butter and margarine. Jagerstad and coworkers (ref) have shown that the mutagenicity of fried muscle-meat increases with increases in glucose content of the muscle-meat.

1.6 Metabolism of PhIP and MeIQx

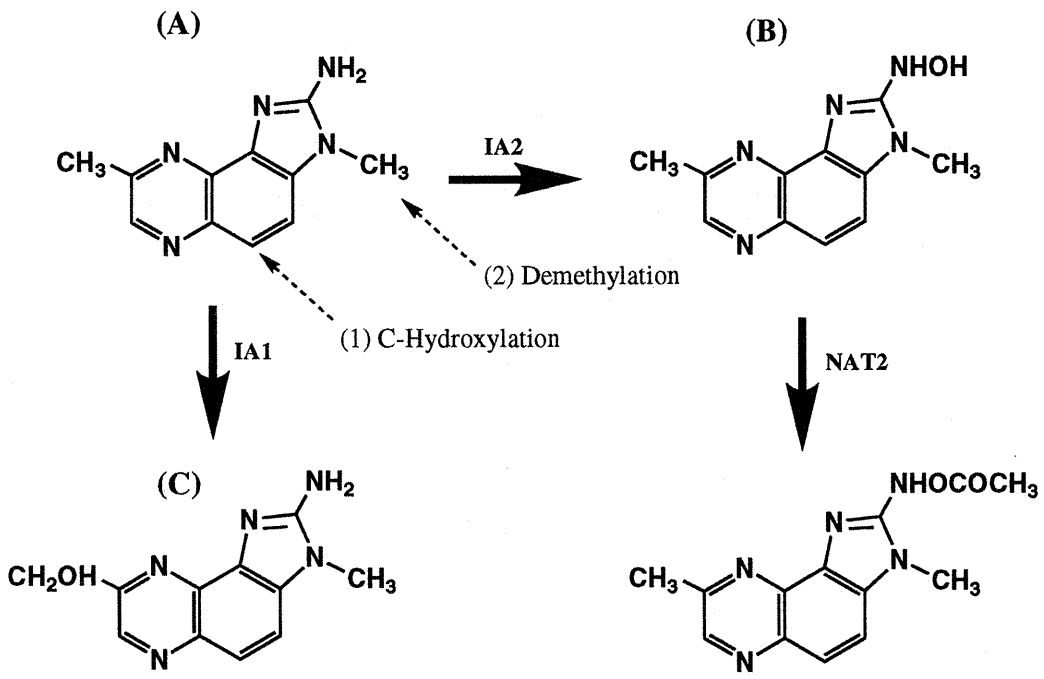
Heterocyclic amines require metabolic activation to their ultimate mutagenic/carcinogenic species. The metabolism of PhIP and MeIQx have been studied extensively *in vitro* (Yamazoe et al., 1988; Wallin et al., 1989; Mc Manus et al., 1989; Buonarati et al., 1990; Turtletaub et al., 1990; Turesky et al., 1990, ibid. 1994; Alexander et al., 1991) and *in vivo* (Gooderham et al., 1987; Turesky et al., 1988; Sjodin et al., 1989; Turtletaub et al., 1990; Watkins et al., 1991). Figures 2 and 3 reveal the proposed metabolic pathway of PhIP and MeIQx. In the presence of hepatic cytochrome P-450 IA2 (CYPIA2), both HAAs are bioactivated via N-oxidation, resulting in the formation of a N-hydroxylarylamine (Wallin et al., 1990; Rich et al., 1992), the principal metabolite leading to mutations in *Salmonella* and DNA damage in mammalian cells (Holme et al., 1989). Unlike other aromatic amines, *N*-acetylation is not an effective competing reaction for the N-oxidation of PhIP nor of MeIQx (Minchin et al., 1992; Turesky et al., 1991a; Hayatsu et al., 1987; Wild et al, 1995). However, the N-hydroxyarylamine may undergo

Figure 2. Suggested Routes of PhIP Metabolism in Rodents and Humans



Metabolites A, B and C are further metabolized to the following metabolites: (A) N-Glucuronide PhIP; (B) N(OH) N-Glucuronide PhIP; and (C) 4'-OSO_3^--PhIP Sulfate and 4'-O-Glucuronide PhIP, respectively.

Figure 3. Suggested Routes of MeIQx Metabolism in Rodents and Humans



Metabolites A, B and C are further metabolized to the following metabolites: (A) N-Glucuronide MeIQx and N-Sulfamate MeIQx; (B) N(OH) N-Glucuronide MeIQx; and (C) 8-methyl-meIQx Sulmate and 8-methyl-O-Glucuronide MeIQx, respectively

O-acetylation by hepatic or colon-derived polymorphic acetyltransferase (NAT2) to form *N*-acetoxy esters (Minchin et al., 1992; Turesky et al., 1991a). After formation in the liver, the *N*-hydroxylarylamines and *N*-acetoxy esters of PhIP and MeIQx are transported to extrahepatic tissue via the blood stream (Kadlubar et al., 1994). These proximate carcinogens may then be converted to ultimate reactive species, presumably a nitrenium ion which binds to macromolecules such as DNA (Kadlubar, 1985; Turesky et al, 1991a; Frandsen et al., 1992). ^{32}P-postlabeling experiments with rats revealed high levels of PhIP-DNA adducts in the lung>heart>pancreas>colon (Takayama et al., 1989). MeIQx-DNA.adducts were primarily detected in the liver, followed by the large intestine, stomach, spleen, small intestine and kidney of mice (Yamashita et al., 1988) and the liver, kidney, heart and colon of rats (Davis et al., 1993). *In vivo* and *in vitro* studies reveal that the N-hydroxylated and N-acetoxy derivatives of PhIP and MeIQx form major adducts with deoxyguanosine (dG). A major PhIP-DNA adduct was characterized as N-(deoxyguanosin-8-yl)-2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (Frandsen et al., 1992). The two major MeIQx DNA adducts have been classified as 5-(deoxyguanosin-N^2-yl)-2-amino-MeIQx (dG-N^2-MeIQx) and N-(deoxyguanosin-8-yl)-MeIQx (dG-C8-MeIQx) (Turesky et al., 1992).

The major pathways of detoxification of PhIP and MeIQx in rodents involve C-hydroxylation, resulting in the formation of 4'(OH) PhIP, 5(OH)-MeIQx and C-8-methyl(OH)-MeIQx. These metabolites may undergo further deactivation via conjugation to their respective sulfate and glucuronide conjugates (Hayatsu et al., 1987; Alexander et al., 1989). A minor pathway in rats involves glutathione of N-oxidized forms of PhIP and MeIQx conjugation (Alexander et al., 1991; Turesky et al., 1990). The *N*-(OH)-PhIP-N^2-glucuronide (Conjugate I) is the major urinary metabolite in dogs (Kadlubar et al., 1994); whereas *N*-OH-PhIP-N^3-glucuronide (Conjugate II) is the major urinary metabolite in rats (Alexander et al., 1991; Kaderlik et al., 1994). The N-glucuronides of PhIP, MeIQx and their respective N-hydroxyarylamines are formed by the uridine 5'-diphosphoglucuronic acid (UDP)-dependent *N*-glucuronidation by liver microsomal UDP-

glucuronosyltransferase UDDGT (Kaderlik et al., 1994). Both of the *N*-glucuronide conjugates of N(OH)PhIP are stable under acidic treatment (0.1N HCL 37 °C for 1 hr); however, only Conjugate II is hydrolyzed back to the *N*-hydroxylamine in the presence of E. coli derived β-glucuronidase (Turesky et al., 1990; Kaderlik et al., 1994). Conjugates I and II are believed to be formed from the N-glucuronidation of hydroxylamine/oxime tautomers of N(OH) PhIP and their proportion is species specific (Turesky et al., 1990, Styczynski et al., 1993). Kaderlik and coworkers (1994) showed that UDPGT activity in rat, dog and human resulted in the formation of conjugate I and II in a ratio of 1:15, 2.5:1.0 and 1.3:1.0, respectively.

Alternatively, MeIQx and PhIP can undergo direct conjugation leading to the formation of the following detoxified metabolites: N^2-glucuronide PhIP, N^3-glucuronide PhIP, N^2-glucuronide MeIQx and N^2-SO_3^- MeIQx. All of these metabolites can be hydrolyzed back to their respective parent amine under acid hydrolytic conditions (1N HCl 70°C for 4-6 hrs) (Stillwell et al., 1994). Unlike the N^2-glucuronide PhIP, N^3-glucuronide PhIP is susceptible to β-glucuronidase. It is has been speculated that the *N*-glucuronides of PhIP are formed as a result of N-glucuronidation of an amine/imine tautomer of PhIP (Styczynski et al., 1993). A number of the above detoxified metabolites have been detected in the urine and bile of various species (Kadlubar et al., 1994; Turesky et al., 1988a, ibid. 1991a; ibid. 1991b; Sjodin et al., 1989; Buonarati et al., 1992; Snyderwine et al., 1993; Alexander et al., 1991; Lynch et al., 1992).

1.6.1 Factors Affecting Bioavailability, Metabolism and Disposition

Factors which may have an impact on the rate of metabolic activation and disposition of HAAs include CYPIA2 phenotype, inducers or inhibitors of CYPIA2 activity, fiber intake, dietary microconstituents and microorganisms. In terms of CYPIA2 activity, Stillwell and coworkers (1997, accepted manuscript) revealed that higher CYPIA2 activity was weakly correlated (slope = -0.2; p(0.02); y-int = 2.07; and R^2 =

0.21) with lower levels of excretion of urinary of total MeIQx (unmetabolized plus acid-labile metabolites). However, this observation was not seen with urinary PhIP (slope = -0.06; p(0.55); y-int = 1.89; R^2 = 0.25). Degawa and coworkers (1989) demonstrated an increase in CYPIA2 activity in rat liver microsomes after treatment with various food related HAAs, such as Trp-P-1, Trp-P-2, Glu P-1, Glu P-2, AαC, MeAαC, IQ and MeIQx. Further investigation led by Kleman and coworkers (1990), revealed that PhIP induced CYPIA2 activity in the lung of rats; whereas, MeIQx was a weak inducer of CYPIA2 activity in various organs, such as the liver, kidneys and lungs. The ability of these food mutagens to induce CYPIA2 is species and gender dependent. In a controlled metabolic food study, Sinha and coworkers (1995b) found an inverse relationship between individuals with high CYPIA2 activity and urinary excretion of unmetabolized MeIQx. These studies suggests that HAAs detected in foods may influence their own rate of activation by inducing the enzyme (CYPIA2) responsible for converting them into their active forms.

Due to the presence of PhIP and other potential inducers of CYPIA2 activity in cigarette smoke, a few investigators have studied the effect of cigarette smoking on levels of urinary excretion of unchanged and total urinary MeIQx or PhIP; however, no association was observed (Lynch et al., 1992; Ji et al., 1994; Ushiyama et al., 1991).

The bioavailability of HAAs in the body is influenced by their absorption to dietary fibers. *In vitro* studies have shown that Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2 (Kada et al., 1984) and MeIQ and MeIQx (Sjoedin et al., 1985) are absorbed by the carboxylic groups, such as alginates (Nishiyama et al., 1990) which are found in vegetable fibers and bran. The binding capacity of fibers is greatly affected by the content of lignin. Substantial binding of IQ, MeIQ, and MeIQx was observed in sorghum fibers (Takeuchi et al, 1988; Vikse et al., 1992). Microorganisms such as *Leuconostoc paramesenteroides* (lactic acid bacteria), *Lactobacillus acidophilus* and *Bifidobacterium bifidum* (bacteria often found in yogurt), and *Eschericihia coli* and *Klebsiella pneumonia* (microorganisms of the intestinal flora) showed a high affinity toward various HAAs, including MeIQx.

A number of foods or dietary microconstituents have been recognized for their antimutagenic effect on HAAs. For example, polyphenolic substances found in vegetable and fruit dialysates reduce the mutagenicity of Trp-P-2 (Shinora et al., 1988). Flavonoides (i.e., quercetin, morin and myricetin) which occur in vegetables have a dose-dependent inhibitory effect on the mutagenicity of certain HAAs (i.e., MeIQ, MeIQx,Trp-P-1, Trp-P-2, Glu-P-1, and Glu-P-2) (Alldrick et al., 1986, 1989; Ogawa et al, 1987). Vegetables also inhibit HAA mutagenicity by directly binding to the HAA (Okuda et al, 1984). Lemon, black tea, green tea (Jain et al, 1987) and aqueous extracts of herbs (Natake et al., 1989) reduce the mutagenicity of Trp-P-2 toward *Salmonella typhimurium*. Antimutagenic activity was also demonstrated in proteins found in Brassica vegetables (i.e., cabbage and broccoli) (Morita et al., 1982). Cruciferous vegetables contain dithiothiones, which induce glutathione S-transferase known to catalyze the detoxification of *N*-acetoxy PhIP (Lin et al., 1994). In contrast to the above results, Ji and coworkers (1994) did not observe any relationship between vegetable consumption (salad greens, tomatoes, or green vegetables, yellow/orange vegetables, white vegetables or other vegetables) and HAA disposition.

2. Biological Activity

2.1 Mutagenicity in *Salmonella typhimurium*

Heterocyclic aromatic amines have been tested for their mutagenicity in bacterial and mammalian cell lines. Based on the results of the Ames mutation assay, *Salmonella typhimurium* TA98 (a frameshift sensitive strain) is more sensitive to the mutagenic activity of these compounds than the TA100 strain (a base-pair substitution tester strain) with metabolic activation (Sugimura and Wakabayashi, 1990). PhIP, Glu-P-2, AαC and MeAαC have much lower mutagenicities than IQ, MeIQ, MeIQx and certain amino acid pyrolysates (Sugimura and Wakabayashi, 1990). In addition to the tester strain used, the enzyme system used influences the mutagenicity of these compounds. The specific mutagenicities of HAAs in *Salmonella typhimurium* TA98 were ranked in descending order based on the

type of metabolic activation system used: (i) activation with hamster-liver S-9 resulted in MeIQ > IQ > 4,8-DiMeIQx >7,8-DiMeIQx > MeIQx > Trp-P-2 > IQx > Glu-P-1 > Trp-P-1 > Glu-P-2 > PhIP > AαC > MeAαC (Sugimura and Wakabayashi, 1990); (ii) activation with Rhesus monkey liver S-9 resulted in MeIQ > 7,8-DiMeIQx > IQ > 4,8 DiMeIQx >Trp-P-2 > Trp-P-1 > MeIQx > Glu-P-1 > MeAαC > AαC > Glu-P-2 (Ishida et al., 1987); (iii) activation with Rhesus monkey liver microsomes resulted in IQ > Glu-P-1 >Trp-P-2 (Ohta et al., 1989); and (iv) activation with human liver microsomes resulted in IQ> Trp-P-2 > Glu-P-1 (Ohta et al., 1989). Table 2 reveals the specific mutagenicity of HAAs and typical food-borne mutagens in *Salmonella typhimurium* TA98 and TA100.

2.2 DNA Damage in Mammalian Cells *In vitro* and *In vivo*

2.2.1 Gene Mutations

Compared to their specific mutagenicities in bacterial cell lines, HAAs have a much lower capacity to induce genetic mutation within mammalian cells using different selective markers (Thompson et al., 1983; ibid. 1987; Holme et al., 1987; Wild et al., 1985; Felton et al., 1988). In contrast to IQ, MeIQ and MeIQx, Trp-P-2 is significantly mutagenic in Chinese hamster ovary (CHO)-AA8 cells (hprt and aprt loci) supplemented with hamster liver S9 microsomal fraction for metabolic activation (Thompson et al., 1983; ibid. 1987). When Trp-P-2 and IQ were compared for their mutagenic efficiency (calculated from the ratio of the number of mutations/loci: number adducts/loci) in CHO-UV5 cells supplemented with hamster S9 microsomal fraction, IQ induced a higher number of mutations and adducts within the hprt and aprt loci. With the exception of PhIP and Trp-P-2, HAAs (i.e., MeIQ, IQ and MeIQx) induced very weak or negative hprt mutations in CHO-UV5 cells supplemented with hamster S9 mix for metabolic activation (Takayama et al., 1983; Holme et al., 1987; Thompson et al., 1983). Trp-P-2 is also a strong mutagen in a forward mutation assay using Chinese hamster lung cells in culture in the presence of a metabolic activation system using diphtheria toxin resistance as a selective marker. The specific mutagenicities of Trp-P-1>MeIQ>IQ>MeIQx>Glu-P-1>Glu-P-2 were 5-700 fold lower than Trp-P-2 (Takayama et al., 1983). Unlike the other compounds, Trp-P-2 did not require metabolic activation in order to express its mutagenic potency.

The aforementioned *in vitro* studies suggest that protein pyrolysates, in particular Trp-P-2, can readily induce point mutations in repair-proficient or deficient mammalian cell lines; whereas IQ-type compounds produce negative or weak responses. In support of this observation, Wild et al., 1985 showed that IQ does not induce point mutations (coat pigmentation) in the melanocytes of C57BL female mice offspring; however, Trp-P-2 (4.2 mg/kg) and Glu-P-1 (18 mg/kg) induce significant increases in the number of recessive spots when compared to the control group (Jensen et al., 1983).

Several hypotheses were developed to explain the reduced potency of aminoazoaarenes in mammalian cells. Since Trp-P-2, but not IQ, induced ouabain resistant mutants (with enhanced sensitivity to detect missense mutations), Takayama and coworkers (1983) suggested that the negative or weak effect of HAAs may be attributed to the inability of the cells to detect frameshift mutations. This hypothesis was refuted since thioguanine and diphtheria toxin resistance (Nakayasu et al., 1983; Holme et al., 1987) are appropriate markers for frameshift mutations (Gupta et al., 1980). Thompson and coworkers (1983) suggested that IQ was possibly a weak mutagen in CHO cells because the active form of IQ which was produced in the culture medium was not effective in damaging the nuclear DNA of the CHO cells. This phenomenon can be explained by: (i) poor uptake of activated IQ metabolites into the CHO cells; (ii) inactivation of the mutagens in the cytoplasm of the CHO cells, due to their instability, metabolic detoxification or reaction with cytoplasmic macromolecules; (iii) failure of the mutagen to react with the DNA within chromatin upon reaching the nucleus; (iv) or efficiency in repairing DNA damage (Thompson et al., 1983).

In order to examine the stability of mutagenic HAAs metabolically activated in culture in the presence of PCB-treated hepatocytes, activated medium (without hepatocytes) was incubated for various time periods at 37 °C before co-incubating with *Salmonella typhimurium* TA98 (Holme et al., 1987, ibid. 1989). HAA mutagenicity as a function of period of exposure to PCB-hepatocytes reached a maximum between 30-60 min. In addition the half-lives of PhIP, MeIQ and IQ in the hepatocyte activation system was estimated to be

< 18 hrs, 45 min and 45 min, respectively. Thus, mutagenic HAAs were fairly stable under physiological conditions. The reaction of activated HAAs was also unlikely since *in vivo* and *in vitro* studies revealed low binding affinity with protein macromolecules (i.e., albumin and hemoglobin). The genotoxic potency of IQ may, however, be inhibited by the presence of intact DNA repair systems in mammalian cell lines. This was demonstrated by Thompson and coworkers (1985) who showed that IQ induced weak but significant responses in repair-deficient CHO cells; whereas weak activity was observed in repair-proficient cells.

In addition to the presence of intact DNA repair systems, the mutagenic potential of HAAs may be affected by the capacity of cells to convert these compounds into active species. As a result, the higher mutagenic activity of HAAs against *Salmonella typhimurium* when compared to mammalian cell lines may be attributed to their higher levels of *O*-acetyltransferase activity (Kato et al., 1986). However, the capacity of bacterial cells to metabolically activate HAAs may be limited by compound specificity. This is best exemplified by the high mutagenicities of IQ-type HAAs toward *Salmonella typhimurium* when compared to PhIP. Unlike PhIP, bacterial derived *O*-acetyltransferase activity is highly specific toward IQ-type HAAs. The potency of PhIP toward *Salmonella typhimurium*, however, may be enhanced when human *O*-acetyltransferase activity is expressed, rather than bacterial activating enzymes (Oda et al., 1989). Moreover, the metabolic activation of HAAs is also species specific, as exemplified by Holme and coworkers who showed that hamster hepatocytes have a greater capacity to activate HAAs when compared to rat hepatocytes.

2.2.2 Chromosomal Aberrations

Based on the literature, IQ-type compounds are ineffective at inducing structural chromosomal aberrations (CA) in mammalian cells relative to TrpP-2. For example, Aeschbacher (1989) demonstrated that even at extremely high doses (0.4-100 μl/ml), IQ and MeIQx do not cause significant levels of CA in human lymphocytes with intact repair

systems. However, IQ induced CA in DNA repair deficient CHO-UV5 cells in the presence of metabolic activation (hamster-liver S9 fraction). At concentrations 10-fold lower than IQ, Trp-P-2 produced dose-dependent increases in CA in CHO-AA8 cells (repair proficient cells). A repair deficient strain was approximately 2-fold more sensitive to the mutagenic effects of Trp-P-2 than normal CHO cells. Despite the increased sensitivity of Syrian hamster embryo fibroblasts to the mutagenic potency of chemicals, IQ was classified as a weak mutagen due to its dose-dependent formation of low levels of chromosomal anomalies (micronuclei). These *in vitro* studies revealed that the genotoxic potential of Trp-P-2 was greater than IQ-type compounds (Trp-P-2>IQ), which was opposite to their potency in *Salmonella*, but similar to results obtained from animal carcinogenicity studies.

The relative potency of Trp-P-2 and PhIP *in vitro* was confirmed by *in vivo* studies. Dose-dependent CA were observed in the bone marrow of mice treated with 5-12 mg\kg Trp-P-2 (Minkler et al., 1984), but not IQ (Minkler et al., 1984; Wild et al., 1985). The detection of low levels of CA *in vitro* (Tucker et al., 1989) and negative responses in CHO-UV5 cells suggest that PhIP is a weak mutagen in mammalian cell lines. However, the mutagenic potency of PhIP was enhanced by using a cell line expressing the mouse cytochrome P_3-450 cDNA gene which encodes metabolic enzymes necessary for the conversion of PhIP to the genotoxic N-hydroxy metabolite. This confirms that the relative mutagenicity of these compounds is species-dependent.

2.2.3 Nuclear Aberrations

The formation of colon nuclear aberrations was proposed as a selective marker of colon tumorigenesis (Wargovich et al., 1983). *In vivo* studies using rodents revealed that HAAs, namely MeIQ and IQ (Bird et al, 1984 and Dolora et al., 1986), generated higher levels of nuclear aberrations (NA) in colonic crypts than Trp-P-2, Trp-P-1 and Glu-P-1. Despite the correlation between NA and high incidences of colon cancer, none of the aforementioned HAAs have been shown to induce tumors in the intestinal tract of mice.

However, HAAs such as IQ, MeIQ, Glu-P-1 and Glu-P-2 induced tumors of the small and large intestines in rats (Sugimura et al., 1990).

2.2.4 Sister Chromatid Exchange

All HAAs studied thus far induced sister-chromatid exchanges (SCEs) in various mammalian cell lines. IQ-type HAAs gave weak but significant responses for SCEs in repair deficient CHO cells and normal human lymphocytes (Thompson et al., 1987; Aeschbacher et al. 1989). However, Trp-P-2 produced significant dose-dependent increases in SCEs in both repair deficient cells and normal mammalian cell systems (Aeschbacher et al., 1989; Minkler et al., 1984; Thompson et al., 1983; Couch et al., 1987). Positive responses were also observed in repair deficient hamster cells treated with PhIP. Despite the relatively high production of SCEs in response to HAAs, some experiments suggest that there was no correlation between mutations and SCEs (Bradley et al., 1979). In contrast, Moralez-Ramirez (1988) reported a positive link between mutations and malignant transformations.

2.2.5 DNA Repair Synthesis

Although Trp-P-2 was the strongest inducer of gene mutations, chromosomal aberrations and SCEs, it was a weak inducer of unscheduled DNA synthesis (UDS) in rodent hepatocytes (Loury et al., 1983; Howes et al., 1986). However, other pyrolysis products, in particular Trp-P-1, showed significant levels of UDS measured as increased incorporation of 3H-thymidine into nuclear DNA (Loury et al., 1983). Moreover, dose-dependent increases of UDS were observed in hamster hepatocytes treated with IQ and MeIQ; however, weak responses were observed with MeIQx. (Howes et al., 1986). *In vitro* studies suggest that induction of UDS appears to be gender-species specific. For example, hamster hepatocytes were more sensitive to the DNA damaging effects of HAAs than rat hepatocytes (Howes et al., 1986). Moreover, higher levels of DNA damage were observed in cultures from female rats treated with Trp-P-1 than in cultures from male rats (Loury et al., 1983).

2.2.6 DNA Strand Breaks

Alkaline elution procedures have been used in several studies to measure the induction of DNA strand breaks in mammalian cells treated with HAAs. In the presence of metabolic activation systems, MeIQ and PhIP readily induced DNA strand breaks (Caderni et al., 1983; Dolora et al., 1985; Brunborg et al, 1988; Holme et al., 1989); whereas MeIQx caused a marginal effect (Dolora et al., 1985).

3. Carcinogenic Potency in Rodents and Non-human Primates

3.1 Species, Compound and Gender Differences in the Carcinogenicity of HAAs

Several heterocyclic aromatic amines (i.e., IQ, MeIQ, MeIQx, PhIP, Trp-P-1, Trp-P2, Glu-P-1, Glu-P-2, AαC, and MeAαC) were evaluated for their carcinogenic effect in rodents administered diets containing 0.02-0.08% of the mutagenic compound. All of the HAAs tested proved to be hepatocarcinogens, with the exception of PhIP. Instead, PhIP induced high incidences of mammary gland and large intestine carcinomas of rats (Ito et al., 1987) and lymphoid tissue of mice (Esumi et al., 1989). Tumors of the mammary gland were also induced in rats after treatment with MeIQ (Kato et al., 1989). In CDF_1 mice, hemangioendothelial sarcomas were induced by Glu-P-1, Glu-P-2, AαC and MeAαC (Ohgaki et al., 1984b). IQ and MeIQ induced tumors in the zymbal gland, large intestine and skin of rats (Takayama et al., 1984a; Kato et al., 1989) and forestomach of mice (Ohgaki et al., 1984a, Ohgaki et al., 1986). Tumors of the zymbal gland and skin were also demonstrated in rats fed a diet of MeIQx (Kato et al., 1988). High incidences of carcinomas were observed in the clitoral gland of rats (Kato et al., 1988; Takayama et al., 1984a) and lungs of mice after treatment with MeIQx and IQ (Ohgaki et al., 1984a; ibid. 1987b). In rats administered a diet of Glu-P-1 and Glu-P-2, carcinomas of the clitoral gland, small intestine and large intestine were detected (Takayama et al., 1984b). The carcinogenic potential of IQ has been evaluated in cynomolgus monkeys fed diets of 10 or 20 mg/kg/day, with 20 subjects in each dose group (Adamson et al., 1990). Three of the

monkeys developed hepatocarcinomas, with latency periods of 27-37 mos in one monkey receiving a dose of 10 mg IQ/kg and 2 animals given 20 mg/kg.

The carcinogenic potency of chemicals is normally expressed as the TD_{50} value, the daily dose that induces tumors in 50% of the animals tested when given throughout their lives (Gold et al., 1984). Based on the TD_{50} values (see Table 4), the listed mutagenic HAAs were more carcinogenic in rats (TD_{50} 0.1-5.7 mg/kg/day) than in mice (2.7-31.3 mg/kg/day). Moreover, rats were more susceptible to the hepatocarcinogenic effect of IQ than monkeys (Adamson et al., 1990; Takayama et al., 1984a). These species differences in the carcinogenic potency of HAAs may be attributed to varying abilities to metabolically activate the precarcinogenic compounds. This was substantiated by the findings of Davis and coworkers (1993) who studied the ability of rat, monkey and human microsomes to activate IQ, PhIP and MeIQx to metabolites mutagenic towards *Salmonella* TA98. Results of this investigation revealed that human microsomes expressed a two- and five-fold greater ability to convert PhIP to mutagenic products when compared to rats and monkeys, respectively.

In addition to target organ specificity, species-gender differences have an impact on tumor incidence in rodents. For example, higher incidences of liver tumors were observed in female mice fed MeIQx, IQ, Trp-P-1, Trp-P-2, Trp-P-2, Glu-P-1, AαC and MeAαC when compared to males. However, in rats, males were more susceptible to the hepatocarcinogenic effects of IQ, MeIQx, Glu-P-1, and Glu-P-2. Moreover, male rats were more prone to develop colon tumors when treated with IQ, PhIP, Glu-P-1, Glu-P-2 in comparison to females.

3.2 Carcinogenic Potency After Simultaneous Administration of HAAs

Takayama et al., 1987 have reported on the combination effect of five heterocyclic aromatic amines (i.e., Trp-P-1, Trp-P-2, Glu-P-2, AαC and IQ) in rats over a

Table 4. TD_{50}-Values of Heterocyclic Aromatic Amines in Mice and Rats

Compound	TD 50 (mg/kg/day)	
	Mouse	Rat
IQ	14.7	0.7 (0.33)[a]
MeIQ	8.4	0.1 (0.11)
MeIQx	11.0	0.7
Trp-P-1	8.8	0.1
Trp-P-2	2.7	(14)
Glu-P-1	2.7	0.8 (1.2)
Glu-P-2	4.9	5.7
AαC	15.8	
MeAαC	5.8	
PhIP	31.3	

Sugimura and Wakabayashi, 1990;

[a]Data in parenthesis from Takayama et al., 1989.

102 week period. The diets administered contained 1/5 of the TD-50 values for these compounds. As a result, high incidences of tumors were observed in the liver, colon, zymbal gland, skin and clitoral gland. The synergistic effect of these compounds on tumorigenesis suggests that humans may be at greater risk than rodents to the carcinogenic potency of HAAs, due to constant exposure to a variety of these food-derived compounds.

3.3 Dose-Response Data

Presently, only two animal bioassays have generated dose-response data using MeIQx. The incidences of liver tumors in mice fed a diet containing 0.06, 0.02, 0.006 and 0.002% MeIQx were 82%, 3%, 0% and 0%, respectively (Ohgaki et al., 1990). The results of this experiment suggest a possible threshold level in the carcinogenic potency of MeIQx in mice. However, in the aforementioned study, the level of DNA adducts formed in various organs in mice treated with 0.006% MeIQx was 1/10 of those given a 0.06% dose. Similarly, Yamashita and coworkers (1990), found that the levels of DNA adducts formed in rats after a 1 week administration of 0.00004%, 0.0004%, 0.004% and 0.04% of MeIQx in the diet were 0.04, 0.28, 3.34 and 39.0, adducts/10^7 nucleotides, respectively. This demonstrates that HAAs can form DNA adducts even at exposure levels which are lower than the doses used in animal carcinogenicity studies. This is supported by the findings of Kadlubar and coworkers (1994) who detected HAA-DNA adducts within humans who consume only trace amounts of these compounds. ^{32}P-postlabeling combined with GC/MS led to the isolation and identification of PhIP-DNA adducts from colon samples and IQ-DNA adducts from colon, pancreas and liver samples. The occurrence of HAA-DNA adducts within human organs may not only correspond to recent exposure to the carcinogens, but also represent an initiation event in chemical carcinogenesis.

4. Epidemiology of Colorectal Cancer

4.1 Effects of CYPIA2/NAT2 Activity on Colorectal Cancer risk

Several investigators have performed epidemiological studies in order to elucidate the factors involved in colorectal tumorigenesis in humans. Lang and coworkers (1994) performed a human case control study in order to determine whether the rate of N-oxidation or *O*-acetylation combined with HAA exposure are involved in the etiology of human colorectal cancer. The individual phenotypes for both CYPIA2 and NAT2 were determined by HPLC quantitative analysis of caffeine and four of its metabolites (i.e., 17X, 17U, AFMU and 1X) in the urine specimens of 205 controls and 75 patients with a history of colorectal cancer or non-familial polyps after the administration of 100 mg of caffeine. The control group consisted of persons ages 20-80; 63% were male, 37% were female, 88% were Caucasian, and 12% were African-American. The smoking rate among the controls was 36% for African-Americans and 23% for Caucasians. Cases ranged in age from 36 to 84 years; 56% were male, 44% were female, 83% were Caucasian, and 17% were African-American. Both rapid CYPIA2 (fast N-oxidation) and rapid NAT2 (rapid O-acetylation) were each slightly more prominent amongst cases when compared to controls (57% vs. 41% and 52% vs. 45%, respectively). Upon the examination of the combined rapid NAT2/CYPIA2 phenotypes, the cancer/polyp cases showed a 36% prevalence of having a combined rapid NAT2/CYPIA2 phenotype compared to 16% in the controls.

4.2 Age, Gender, Smoking Status, Ethnicity and Diet

In addition models were developed in order to determine whether age, gender, smoking status and ethnicity could serve as confounding factors. Although there were no differences in gender, ethnicity, and smoking status were observed between the healthy and polyps/cancer patients, ethnicity modulated the effect of cigarette smoking status on CYPIA2 activity. For example, Caucasian smokers had a mean N-oxidation rate that was two-fold higher than non-smoking Caucasians, while smoking and non-smoking African-Americans had levels similar to non-smoking Caucasians. These results combined with the finding that Caucasians and African-Americans smoke approximately one pack of cigarettes per day (17 vs. 21 cigarettes per day, respectively) suggest that CYPIA2 levels in

Caucasians are inducible, whereas CYPIA2 expression in African-Americans is not inducible by smoking. Although the effect of age on acetylator phenotype was significantly positive, there is little biological significance since the rate only increases by ca 0.01 units per year of age. Age, however, did not have a significant impact on CYPIA2 activity. This finding was confirmed by Corman and coworkers (1979) who suggested that age was a major risk factor involved in the etiology of colon cancer and polyps. Incidence and mortality rates for colorectal cancer start to increase dramatically after the fifth decade of life. This suggests that a certain length of exposure to initiators, such as HAAs, is required for the development of the genetic changes necessary to induce a polyp or a cancer (Gross et al., 1993). These results indicate that individuals with rapid-rapid NAT2/CYPIA2 phenotypes are more prone to colorectal cancer because they can metabolically activate HAAs more rapidly than controls. Analysis of the dietary questionnaire did not reveal any differences between cases and controls regarding type (i.e., beef, pork, chicken or fish) or quantity of muscle-meat consumed. In contrast, frequent use of cooking oil and a preference for well-done cooked muscle-meat were associated with increased risk for colorectal neoplasia. Nilsson and coworkers (1986) observed that an increase in temperature at the meat surface caused by adding frying fat during the broiling process resulted in increased mutagen formation in the crust and pan residue. The influence of temperature on mutagen formation was further studied by Sinha and coworkers (1995a) who showed that the amount of HAAs detected in cooked muscle-meat are increased by prolonged cooking at high temperatures. The correlation between HAA exposure and colon cancer or non-familial polyps was further supported by ^{32}P-postlabeling and GC/MS data (Kadlubar et al., 1994). The major PhIP-DNA adduct, N-(deoxyguanos-8-yl)-PhIP, was detected in 6 out of 40 surgical samples of human colon, but not in human liver (10 samples) nor pancreas (20 samples). Adducts derived from MeIQx were not detected in any of the samples.

4.3 Causative and Protective Affects of Diet

Several epidemiological studies have explored the possible linkages between muscle-meat consumption and colorectal cancer using case-control and prospective cohort methodologies. In addition to muscle-meat intake, various confounding factors were taken into consideration. For example, in residents of Northern Italy participating in a case-control study, La Vecchia and coworkers (1988) found an increase in relative risk for colon and rectal cancer associated with consumption of beef and veal. However, in this study, poultry consumption was inversely related to the risk of colorectal cancer. A similar association was observed between colon cancer and consumption of fish and chicken (Willett et al., 1990, Giovannucci et al., 1991 and Goldbohm et al. 1994). It is possible that individuals who select chicken and fish as their primary sources of meat may be protected by other health conscious practices: high consumption of vegetables, fruit and fiber and a low fat intake (La Vecchia et al., 1988, Willett et al., 1990; Peters et al., 1989). The negative association between colorectal cancer and vegetables, fruit and fiber is not universally accepted (Giovannucci et al., 1991). Willet (1990) and Giovannucci (1991) showed that men and women who consumed beef, pork or lamb daily had a relative risk of colon cancer that was 2.5 and 3.6 higher, respectively than those who consumed those meats less than once a month. Similarly, Goldbohm and coworkers (1994) detected an increase in relative risk of colon cancers with respect to consumption of processed meat, particularly sausages, in individuals participating in a cohort study in the Netherlands. Processed meats also contain nitrites which may result in the formation of nitrosamines which are known to cause intestinal tumors in rodents. In the same study, no risk was associated with consumption of total fresh muscle-meat, beef, pork, minced meat, chicken and fish.

There are several hypotheses which attempt to explain the relationship between high muscle-meat consumption and colorectal cancer. With few exceptions (Macquart-Moulin et al., 1986; Berta et al., 1985; Tuyns et al., 1987; Benito et al., 1991; Meyer et al., 1991), a link between dietary fat, particularly animal fat, and colon cancer has been supported by

numerous case control studies. However, the results of many of these studies may have been confounded by a high caloric intake within patients with colon cancer. Additional epidemiology studies using prospective cohort methodologies have reported positive (Bjelke et al., 1980; Gerhardsson de Verdier et al., 1988; Goldbohm et al., 1994), inverse (Stemmermann et al., 1984; Hirayama et al., 1986) and null associations with fat consumption (Garland et al., 1985; Phillips et al., 1983). Although these prospective studies are less prone to selection and recall bias, they are limited by the small number of cases, crude diet assessment and the lengthy time period between the collection of dietary data and diagnosis of colon cancer. After making adjustments for confounding factors and experimental bias, Willett (1990) and Gerharrdson de Verdier (1991) found that animal fat was positively correlated with colon cancer in women, but not in men. The speculated mechanism for tumorigenesis involves the following hypotheses: (i) dietary fat increases production of bile acids which can be converted into potential carcinogens by intestinal bacteria; or (ii) bile acids may act as tumor promoters by increasing the turnover of intestinal mucosal cells.

4.4 Meat and Heat Generated HAAs

High consumption of foods containing heat generated HAAs, which have been shown to cause intestinal tumors in rodents, may play a role in tumorigenesis in humans. Gerhardson de Verdier and coworkers (1991) observed an increased risk of colorectal cancer in individuals who preferred heavily browned meats and brown gravies. Surprisingly, the relative risk for colorectal cancer was reported to be higher in individuals who consumed meats which were stewed rather than meats which were heavily browned. Based on the findings of Sinha and coworkers (1995a), HAA formation is greater in meats which are browned via frying, grilling or broiling rather than boiled (stewed). This discrepancy can be explained if the meats were browned prior to stewing, a cooking technique which enhances flavor. Moreover, in white male residents of Los Angeles, elevated risk for tumors located in the ascending colon were associated with heavy

consumption of fried foods, fried bacon and barbecued or smoked foods (Peters et al., 1989). The only item associated with rectal cancer was deep fried food. Muscat and coworkers (1994) detected only a weak but positive association between colon cancer in men who consumed well done beef when compared to men who preferred meat rare or medium-rare; however no association was seen in women. Additionally, although no data was presented, Willett and coworkers (1990) did not find a positive relationship with the percentage of cooking time that muscle-meat was prepared. The proposed relationship between HAAs and colorectal cancer appears to be challenged by improper use of surrogates (i.e., cooking method and degree of doneness) of HAA exposure. Meat samples which are cooked well done may not necessarily have high levels of HAAs. Deleterious effects of HAAs may also be confounded by dietary intake of fat. However, adjustments for dietary fat intake may result in an underestimation of cancer risk, since fat (i.e., fat content of muscle-meat and cooking oil/fat) enhances the production of mutagenic HAAs.

The conflicting results regarding muscle-meat consumption and colorectal cancer may also be attributed to a number of sources: (i) surrogate respondents may not accurately report dietary habits of cancer patients; (ii) biased recall of diet in case control studies. This bias was eliminated in cohort studies since food intake data was collected before the diagnosis of colorectal cancer.; (iii) inadequate information on the amount of muscle-meat consumed; and (iv) differences in the average age of subjects between studies. Evidence suggests that younger individuals may be more susceptible to the etiological agents of tumorigenesis found in muscle-meat (Willet et al., 1990). Others argue that the development of colon cancer prior to 45 years of age may be attributed to a hereditary and/or predisposed medical condition (Peters et al., 1989).

5. Quantitative Analysis of HAAs in Human Urine Specimens

Ji and coworkers (1994) reported on the levels of urinary excretion of total MeIQx (unmetabolized MeIQx combined with acid-labile metabolites) amongst 133 male residents [44 blacks, 43 asians and 43 whites] of Los Angeles County. Quantitative

analysis revealed interracial differences in the urinary excretion of MeIQx. In the study, only 19% of the blacks had undetectable MeIQx vs. 63% of the whites. Moreover, 51% of the blacks and only 21% of the whites had levels above 2.5 $ng/g_{\text{-creatinine}}$ MeIQx. The asians had intermediate levels of urinary MeIQx. These results combined with frequency of consumption of selected food items suggest that the higher levels of urinary MeIQx observed in blacks may be attributed to their higher frequency of consumption of fried bacon when compared to their counterparts. Support for this is provided by Gross and coworkers (1993) who observed significant levels of HAAs (i.e., MeIQx, 4,8-DiMeIQx, and PhIP) in fried bacon and bacon fat drippings. Bacon fat drippings and residues are often used as a base for gravies, sauces and preparation of vegetable or rice dishes. Thus, cooking practices and dietary habits may have a strong impact on HAA exposure.

More recently, analytical methods have been developed to measure the levels of unmetabolized HAAs and their acid-labile metabolites excreted in human urine specimens after consumption of a diet high in HAAs. Murray and coworkers (1989) found that 1.8-4.9% of the ingested dose was excreted as unmetabolized MeIQx. Lynch and coworkers (1992) determined that the percentage of the oral dose of PhIP excreted as the parent amine was 0.6-2.5%. Stillwell and coworkers (1994) characterized acid-labile MeIQx metabolites as the N^2-sulfamate and N^2-glucuronide conjugates. Within a 12 hr. period after exposure, unmetabolized and total MeIQx (unmetabolized MeIQx plus acid-labile metabolites) excreted into the urine represented 0.5-4.7% and 1-14% of the ingested dose, respectively. Comparable results were obtained in a collaborative study involving the analysis of 66 urine specimens (Sara et al., 1997 and Sinha et al., 1995b). The levels of unmetabolized MeIQx and total MeIQx represented $1.3 \pm 0.6\%$ and $10.5 \pm 3.5\%$ of the ingested dose, while total PhIP represented $4.3 \pm 1.7\%$ of the ingested dose. Given the aforementioned literature values, urinary PhIP and MeIQx increased on average 4 and 10 fold as a result of acid hydrolysis, respectively. In addition, a marginal association ($R = 0.5$) between the ingested dose ($ng/g_{\text{-meat}}$) and the excreted dose (total PhIP and MeIQx) was observed.

6. Estimation of Human Risk from HAA Exposure

The carcinogenic potential of food-borne HAAs has been calculated using various models (Stavric et al., 1994). One model involves extrapolation from rodent carcinogenicity studies. Based on a TD_{50} values for PhIP (31 mg/kg/day) and MeIQx (11 mg/kg/day) in mice (20g), the exposure level over the 2-year life span would correspond to 23 g PhIP/kg/lifetime and 8 g MeIQx/kg/lifetime. Similar calculations using daily human exposure levels (13.8 μg PhIP/day and 2.6 μg MeIQx/day) would result in dietary consumption of 2.6 mg PhIP/kg and 0.5 MeIQx mg/kg over a life time (70 yrs). Thus, the estimated carcinogenic potency of these compounds would be approximately 1 in 1,000. Support for this risk assessment is provided by rodent genotoxicity studies for MeIQx. The formation of MeIQx-DNA adducts in mice, measured by accelerator mass spectrometry, was linear with doses ranging from 5 mg/kg to 500 ng/kg. This dose range was extremely relevant to estimated human daily intakes of MeIQx which range from 3 to 40 ng/kg/day. It is unclear whether there is a threshold for tumorigenicity, since tumors do not form at levels 1/3 of the TD_{50} value in mice (Ohgaki et al., 1990). Moreover, rodent bioassays may actually underestimate the carcinogenic potential of HAAs since humans are exposed to a mixture of HAAs in the diet. Multiple exposure may result in an attenuation of cancer risk due to a synergistic effect.

Bogen and coworkers (1994) developed a model for estimating cancer potencies of HAAs. This model takes into consideration that HAAs may induce tumors at multiple sites in humans, due to their multipotency in rodents. Additionally, in order to obtain dose equivalence between rats and humans, the carcinogenic potency [95% Upper Confidence Limit (UCL) cancer potency/mg/kg/day] of AαC (1.2/mg/kg/day), DiMeIQx (20/mg/kg/day), IQ (26/mg/kg/day), MeIQx (11/mg/kg/day and PhIP (3/mg/kg/day) were based on a surface area basis. Since no carcinogenicity data was available for DiMeIQx, its potency was calculated from a significant linear regression between log (highest cancer potency) vs. log(mutagenic potency) that used estimated obtained for one-tailed 95% UCL values on 10 other HAAs.

Most of the above animal carcinogenicity studies have been performed at doses close to the maximum tolerated dose (MTD), which exceed the daily levels consumed by humans by a factor ranging from 10^4 to 10^7. This suggests that no individual HAA can cause tumor formation in humans. However, the dose-dependent formation of DNA adducts in the liver of rats and mice administered doses of MeIQx equivalent to the low levels consumed by humans, suggests that HAAs can form DNA adducts in the human body, even when present at low levels relevant to human exposure. Additionally, rodent bioassays may actually underestimate the carcinogenic potential of HAAs since humans are exposed to a mixture of HAAs in the diet. Multiple exposure to these compounds may increase their overall carcinogenic potency due to their additive or synergistic effect on cancer incidence. In order to accurately estimate cancer risk associated with dietary consumption of HAAs, it is crucial to estimate human exposure.

Layton and coworkers (1995) estimated the incremental cancer risk associated with dietary consumption of HAAs by utilizing carcinogenic potencies (see Bogen et al., 1994), meat analysis studies and dietary questionnaires. Incremental cancer risk was calculated by multiplying carcinogenic potencies by estimated daily HAA intakes, generated from reported values of HAAs in cooked meat and meat intakes of 3,563 U.S. residents. The average daily intakes of the HAAs in descending order were PhIP>AαC>MeIQx>DiMeIQx > IQ. In contrast, their carcinogenic potencies were practically in reverse order: IQ >DiMeIQx>MeIQx>PhIP>AαC. The total upper-bound incremental risk associated with the food-borne HAAs was estimated as 1.1×10^{-4}. Due to their high abundance within most frequently consumed foods, PhIP and MeIQx contributed to the incremental cancer risk by 46% and 27%, respectively.

There were numerous sources of error or bias in Layton's risk assessment (Layton et al., 1995). Factors which could increase cancer risk are: (i) inclusion of other HAAs detected in food (33% increase); (ii) inclusion of fat drippings and pan residues which are often used in the preparation of gravies or rice/vegetable dishes as sources of exposure

(30% increase); (iii) correction of under-reporting of food intake (20% increase); and (iv) consideration of the synergistic effect of multiple HAAs in the diet (70% increase). Factors that would decrease risk include the following: (a) consideration of epidemiological studies which indicate that not all foods prepared well done contain high levels of HAAs (40% reduction); (b) use of mean cancer potency rather than the 95% upper confidence limit cancer potency/mg/kg/day (1.2-1.4 fold reduction) and (c) use of body surface area rather than body weight (6 fold reduction for rat data). Assuming that the factors which influence incremental risk act multiplicatively, the conservative upper and lower limits of incremental risk [i.e., HAA intake (ng/kg/d) * 95% UCL cancer potency] are $1x10^{-4}$ and $3.8x10^{-5}$, respectively.

III. Specific Aims

Due to their mutagenic-carcinogenic potency and relatively high abundance in foods most commonly consumed by westerners, it is hypothesized that PhIP and MeIQx may play a role in the incidence of colorectal cancer in humans. The specific aims of this study are to: (1) utilize analytical techniques to estimate the levels of urinary excretion of PhIP and MeIQx within various human populations; (2) determine whether HAAs detected in human urine samples is related to consumption of foods containing these compounds; (3) determine whether smoking status, CYPIA2/NAT2 activity effect HAA metabolism and disposition; (4) determine whether the urinary excretion of total MeIQx can serve as an index for total PhIP; and (5) determine whether the urinary excretion of unmetabolized plus acid-labile HAA metabolites can serve as an index for HAA exposure and risk.

IV. Urinary Excretion of 2-Amino-1-methyl-6-phenylimidazo[4,5-b]-pyridine in White, Black and Asian Men in Los Angeles County

1. Abstract

The primary goal of this research project was to determine whether differences in ethnicity, dietary habits, smoking status or NAT2 phenotype have an impact on HAA metabolism and disposition. Urine samples were collected from 130 healthy male residents (44 black, 43 asian and 43 white) of Los Angeles who consumed unrestricted western diets. The urinary excretion of PhIP was determined by measuring the amount of unmetabolized PhIP combined with acid-labile metabolites. Purification and quantitative analysis of urine specimens was performed by immunoaffinity chromatography combined with ESI-LC/MS-MS. Blacks and asians excreted higher levels of urinary PhIP when compared to whites. Although urinary excretion of PhIP was not associated with intake frequencies of cooked meat (i.e., beef, pork, chicken, fish, pork, sausage and bacon), a positive association was observed between bacon intakes and urinary PhIP levels combined with urinary MeIQx. A Chi-square test did not reveal an association between urinary excretion of MeIQx and PhIP [P(0.00); contingency coefficient = 0.38]. As a result, urinary MeIQx can not serve as an index of urinary PhIP. Smoking status did not affect PhIP disposition. Thus, potential inducers of CYPIA2 activity found in cigarette smoke may not modify PhIP metabolism.

2. Materials and Methods

2.1 Chemicals

PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine) and PhIP-d_3- were purchased from Toronto Research Chemicals (Ontario, Canada). Isotopic purity of the deuterated standards was determined by LC-MS/MS to be 98.7%. Stock solutions of deuterated PhIP was prepared in methanol and quantified with a Hewlett-Packard 8452A spectrophotometer (Hewlett-Packard Co., Palo Alto, CA) using extinction coefficient value of 18,133 M^{-1} cm^{-1} for PhIP at 316 nm (Friesen et al., 1994 - Stillwell 1996). Stock standard solutions were prepared as needed and were not stored for future use. Methanol and ethyl acetate were all Omnisolve grade solvents (EM Science, Gibbstown, NJ). High-purity water (double distilled) was used to prepare buffers and acid solutions. Monoclonal antibodies raised against PhIP was used in the preparation of immunoaffinity columns (Turesky et al., 1989). The antibodies were immobilized on CNBr-activated Sepharose 4B (Sigma Chem. Co., St. Louis, MO) at a concentration of 2-5 mg of protein/ml of gel. Blocked gel for precolumns was prepared by treatment of CNBr-activated Sepharose 4B with 0.2 M Tris-HCl, pH. 8.0.

2.2 Subject Profile

Detailed characteristics of subjects have been described by Yu et al., 1994. Subjects included 131 male subjects who were over the age of 35 years and were either black (n = 44) or asian (n = 43), or white (n = 43). Approximately half of the subjects were non-smokers (n = 69, 53%); the remaining (n = 62) were current cigarette smokers of varying intensity (Yu et al. 1994). Seventy-six of the participants were involved in a cross-sectional survey among Caucasian, African-American and Asian male residents of Los Angeles County, in which current dietary information was collected through a self-administered questionnaire (Yu et al., in review). The remaining subjects (n = 65) consisted of employees of the University of Southern California.

2.3 Determination of N-Acetylation Phenotype

Determination of acetylator phenotype has been described in more detail by Yu et al., 1994. On the day of the study, subjects were asked to refrain from chocolate and to limit their caffeine intake to no more than four cups of coffee (or 10 cans of cola). Each subject was asked to drink two packets of instant coffee (70 mg of caffeine) between 3 and 6 p.m. that same day. An overnight urine sample, ending with the first morning void, was collected in 1L plastic containers. All urine specimens were acidified (400 mg of ascorbic acid per 20 ml of urine) within 24 hr of collection and subsequently stored at -20 °C until analysis. High pressure liquid chromatography (HPLC) with photo-diode array detection and spectral validation was used to measure urinary excretion of three caffeine metabolites: 5-acetylamino-6-amino-3-methyluracil (AAMU), 1-methylxanthine (MX), and 1-methyluracil (MU). NAT2 phenotype was determined using the molar urinary ratio of AAMU to (AAMU + MX + MU). Subjects with values higher than 0.34 were classified as rapid acetylators, where as slow acetylators had lower values. For border-line cases, the AAMU/MX ratio was calculated; subjects with values in excess of 1.23 were designated as rapid acetylators, whereas slow acetylators had lower values.

2.4 Acid Hydrolysis and Extraction of Urinary PhIP

Isolation of unmetabolized and conjugated PhIP from human urine samples has been detailed previously (Stillwell et al., 1997). In brief, urine samples (10 ml) were placed in 50 ml glass-stoppered glass tubes, spiked with internal standard (ng of *d3*-PhIP), acidified with 6N HCl (2 ml) and incubated at 70 °C for 4 hrs. This protocol enables the liberation of PhIP from β-glucuronic acid (data not shown). After the incubation period, the samples were allowed to cool to room temperature and neutralized with 6N NaOH (2 ml). The samples were basified with sodium carbonate (0.67 g) and extracted twice with two volumes of ethyl acetate. The organic phase was placed in a -16 °C freezer for 30 min in order to freeze out residual aqueous phase, and then decanted into a clean glass centrifuge tube containing 2 ml of 0.1 N HCl. After vortexing the solutions, the acid layer was removed and a second extraction was performed with 1.0 ml of 0.1 N HCl. The acid

extracts were combined and placed into 7 ml vials and taken to dryness by vacuum centrifugal concentration (Savant Instruments, Inc., Farmingdale, NY). The dried samples were stored at -16 °C until further workup.

Creatinine levels in the 131 urine specimens were measured using standard procedures at the Clinical Laboratory of the Kenneth Norris, Jr. Cancer Hospital. As a means of adjusting for the variation in urine concentrations amongst individuals, the PhIP:creatinine ratio was used as a biomarker for PhIP exposure *in vivo*.

2.5 Purification by Immunoaffinity Chromatography

Dried samples were reconstituted in phosphate-buffered saline solution (PBS) (3 ml of 10 mM phosphate buffer, pH 7.4, 0.14 M NaCl), pH adjusted to 8.0 (0.2 to 0.4 ml of 0.1 N NaOH) and applied to small precolumns (1 ml of Tris-blocked Cyanide Bromide-activated Sepharose 4B) followed by monoclonal antibody columns (1.5 ml). The columns were washed with 15 ml of PBS solution, then the precolumns were removed and an additional 15 ml of PBS solution was applied to the antibody columns. The columns were then washed with an additional 30 ml of double distilled water. The PhIP fraction was eluted from the antibody columns by the addition of 3 ml of 1 N acetic acid. The vacuum dried acid fractions were basified with 1 N sodium carbonate (0.3 ml), extracted with ethyl acetate (2x1 ml) and chilled at -16 °C for 15 min. The ethyl acetate layers were dried by evaporation under nitrogen and then transferred with a small volume of ethyl acetate (2x100 µl) to 100 µl inserts positioned in screw-cap vials (DP-Target, Hewlett-Packard Co.). The samples were dried under nitrogen and stored in a -16 °C freezer until analysis via LC-ESI-MS/MS.

2.6 Quantitative Analysis by ESI/LC/MS/MS

Purified samples were analyzed by using a Hewlett-Packard HPLC 1090 series instrument coupled to a Finnigan TSQ 7000 triple-stage quadrupole mass spectrometer

(Finnigan-MAT, San Jose, CA) equipped with an electrospray ionization source. The HPLC and tandem mass spectrometer were interfaced via a Vydac narrow-bore C-18 reverse-phase column (150 x 2.1 mm, 5 μm particle size). The mobile phase, consisting of a solution of methanol:water (50:50) with 0.1% formic acid, was carried out at a flow rate of 200 μl/min. PhIP was analyzed in positive ion mode by using a selected reaction monitoring technique which monitors collision induced dissociation of a parent ion to its daughter ion. With the collision voltage and gas (argon) set at -32 volts and 3.2 mTorr, respectively, the protonated molecular ion was fragmented to its corresponding daughter ion, resulting in the loss of a methyl (or d_3-methyl) group ($[M+H]^+$ ---> m/z 210). The Finnigan data system monitored the positive ions at m/z 225, undeuterated PhIP (d_0 PhIP) and m/z 228, deuterated PhIP (d_3 PhIP). In order to ensure maximum sensitivity, instrumental parameters were optimized by adjusting the capillary and tube lens voltages. The heated capillary was set at 250 °C, while the nitrogen sheath gas was held at 80 psi and the auxiliary nitrogen set at 18 on the dial. Unextracted standards were analyzed under the same conditions and used to correct for background contribution of undeuterated PhIP present in the deuterated PhIP. The amount of PhIP in the specimens was determined by calculating the peak area ratio of 225/228. Percent recovery was determined by comparing the internal standard with unextracted standards.

2.7 Statistical Analysis

A Poisson approximation of PhIP exposure resulted in the generation of a frequency distribution curve that was beta distributed. As a result, the values of the PhIP:creatinine ratio were logarithmically transformed prior to applying formal statistical methods. The chi-square test was used to determine the relationship between urinary excretion of total PhIP and potential modifiers of HAA metabolism (i.e., cigarette smoking status and frequency intakes of cooked muscle meat and vegetables). All P-values cited were two-sided. The relative risk and 95% confidence interval was calculated in order to determine the association between the intake frequencies of selected food items and negative vs. low levels of urinary PhIP.

3. Results

The levels of urinary excretion of unmetabolized plus acid labile PhIP metabolites ranged from undetectable to 115 pg/ml_{-urine}. The limit of detection of PhIP in urine samples was approximately 4 pg/ml_{-urine}.

Detectable levels of urinary PhIP expressed in nanograms of PhIP per gram creatinine ($ng/g_{-creatinine}$) ranged from 30-1,425 $ng/g_{-creatinine}$ in blacks (n = 45), 32-661 $ng/g_{-creatinine}$ in asians (n = 42) and 38-147 $ng/g_{-creatinine}$ in whites (n = 43), as depicted in Figure 4. Table 5 reveals the geometric mean levels of urinary PhIP, which were lowest in whites (2.2 ng/g-creatinine) when compared to Blacks (3.2 ng/g-creatinine) and asians (3.1 ng/g-creatinine). In order to determine whether there were differences in the urinary excretion of PhIP amongst the three ethnic groups the levels of PhIP were divided into the following approximate tertiles: low (ND-6.5 $ng/g_{-creatinine}$), medium (6.5-7.5 $ng/g_{-creatinine}$) and high (> 7.5 $ng/g_{-creatinine}$). Approximately 54% of the non-whites (asians and blacks) had undetectable levels of PhIP in comparison to 77% of the whites (See Figure 5). In addition, approximately 29% of the non-whites but only 5% of the whites had levels of urinary PhIP in excess of 6.5$ng/g_{-creatinine}$ (See Figure 6). In the first tertile, approximately 18% of non-whites and whites excreted low levels (ND-6.5$ng/g_{-creatinine}$) of urinary PhIP. In terms of medium levels of urinary PhIP, the population size of non-whites was approximately two-fold higher in comparison to the whites. The differences between the non-whites and whites were more dramatic in the third

Figure 4. Urinary Excretion of Unmetabolized PhIP and Acid-Labile Conjugates Amongst Three Ethnic Groups (blacks, asians and whites).

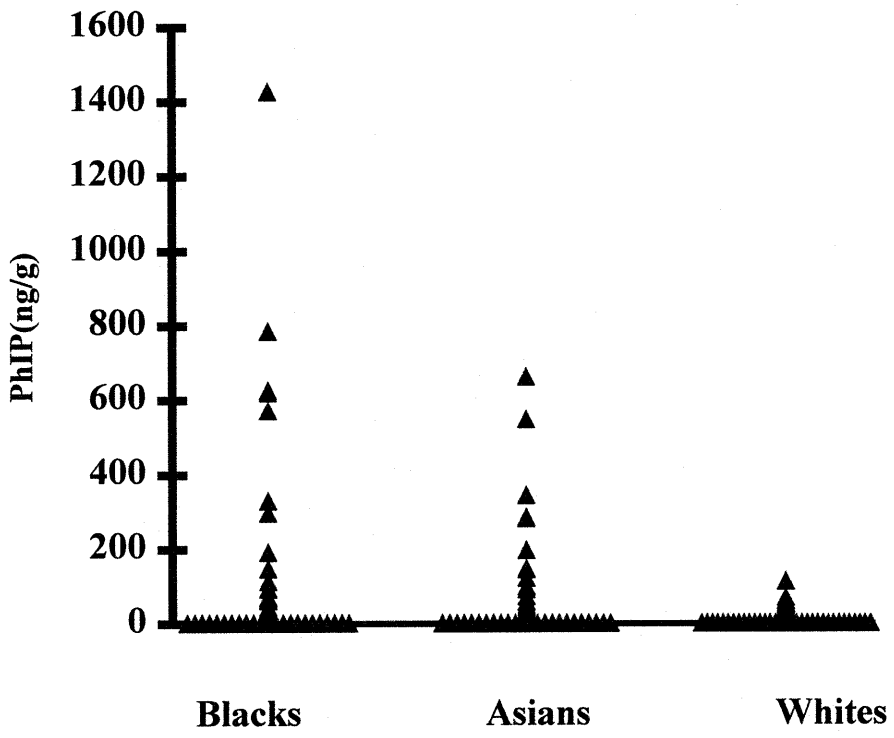
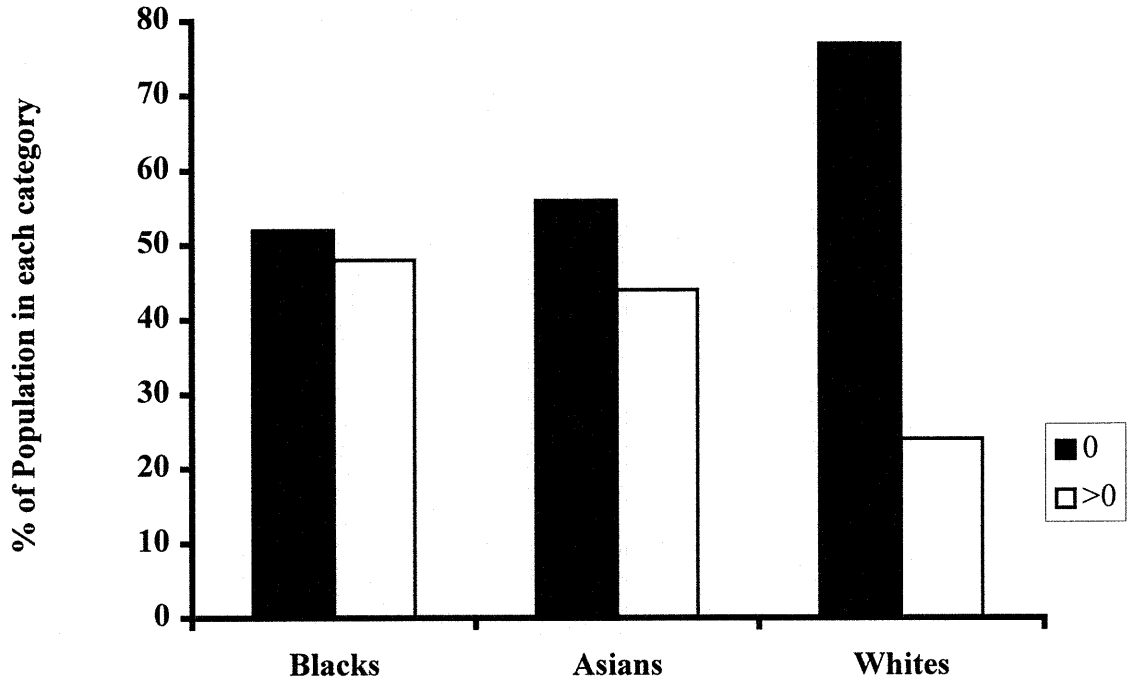


Table 5. Distribution of Urinary PhIP by Ethnicity

	PhIP[b]			
	Blacks	**Asian**	**Whites**	**Total**
ND	23 (52)[a]	24 (56)	33 (77)	80
ND-6.5	8 (18)	7 (16)	8 (19)	23
6.5-7.5	7 (16)	5 (12)	2 (5)	14
>7.5	6 (14)	7 (16)	0	13
Total	44	43	43	130
Geomean	3.21	3.09	2.20	3.15

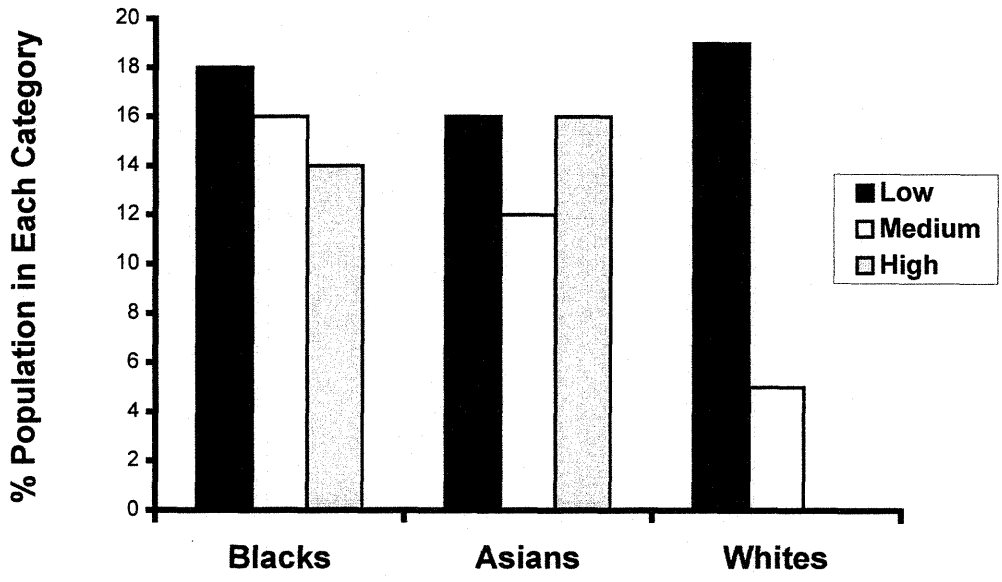
[a] Column Percentage. [b] Values are expressed in ng/$g_{creatinine}$
P (blacks vs. asians) = 0.92; P (blacks vs. whites) = 0.01;
P (asians vs. whites) = 0.02; P (All) = 0.07; all cited P-values are two-tailed.

Figure 5. Urinary Excretion of PhIP in Various Ethnic Groups



Urinary excretion of unmetabolized PhIP and acid-labile conjugates amongst three ethnic groups (blacks, asians and whites). The shaded and unshaded bars represent the percentage of the population with undetectable and detectable levels of urinary PhIP, respectively.

Figure 6. High, Medium and Low Levels of Urinary PhIP by Ethnicity



Urinary excretion of unmetabolized PhIP and acid-labile conjugates amongst three ethnic groups (blacks, asians and whites). The black, white and grey bars represent the percentage of the population with low (ND-6.5 ng/$g_{-creatinine}$), medium (6.5-7.5ng/$g_{-creatinine}$) and high (> 7.5 ng/$g_{-creatinine}$) levels of urinary PhIP, respectively. ND = not detected.

tertile: approximately 15% of the non-whites excreted high levels (>7.5ng/$g_{\text{-creatinine}}$) of urinary PhIP whereas none of the whites excreted high levels. Based on a two-tailed chi-square test, the distribution of urinary PhIP between asians and blacks was not statistically significant ($p = 0.92$). However, values for non-whites (blacks and asians) were significantly greater than whites ($p = 0.01$ and $p = 0.02$, respectively). Moreover, the observed differences amongst the three ethnic groups reflect a real difference in the total population, rather than chance variation at the 90% confidence level ($p = 0.07$). When urinary levels of PhIP and MeIQx were summed (see Table 6), the observed racial pattern was similar to the distribution of urinary PhIP amongst the three ethnic groups.

In order to determine whether the urinary excretion of MeIQx can be used as an index for urinary PhIP, a 3 x 3 contingency table was generated (see Table 7). A Chi-square test of association revealed that the distribution of MeIQx and PhIP were not correlated amongst the subjects at the 99% confidence level ($\chi^2 = 25.4$; $df = 4$; $p = 0.0$). Approximately 46% of total population displayed discordance between urinary PhIP and MeIQx. For example, 28% of 42 subjects had detectable levels of PhIP and undetectable levels of MeIQx. Additionally, 61% of 77 subjects had detectable levels of MeIQx and undetectable levels of PhIP. On the other hand, positive concordance was observed in 54% of the total population ($n = 127$) who either had undetectable (23%), moderate (9%) or high (10%) levels of both MeIQx and PhIP.

Urinary excretion of PhIP was not affected by cigarette smoking status ($\chi^2 = 0.01$; $df = 1$; $p = 0.94$ with Yates' correction) (See Figure 7). We confirmed previous findings which reveal that HAAs are not good substrates for *N*-acetylation (Wild et al., 1995; Minchin et al., 1992; Turesky et al., 1991a; Hayatsu et al., 1987) by demonstrating no difference in the distribution of urinary PhIP in rapid vs. slow *N*-acetylators ($\chi^2 = 0.54$; $df = 1$; $p = 0.46$ with Yates' correction). Thus, the validity of our statistical analysis was strengthened.

Table 6. Distribution of PhIP and MeIQx Combined by Ethnicity

	(PhIP + MeIQx)[b]			
(PhIP + MeIQx)	Blacks	Asians	Whites	Total
ND	6 (14)[a]	9 (21)	14 (33)	29
ND-6.0	12 (27)	13 (31)	15 (36)	40
6.0-7.0	13 (30)	8 (19)	11 (26)	32
>7.0	13 (30)	12 (28)	2 (5)	27
Total	44	42	42	128
Geomean	5.4	4.8	3.7	4.6

[a] Column Percentage.; [b] Values are expressed in $ng/g_{-creatinine}$

P(blacks vs. asians) = 0.61; P(asians vs. whites) = 0.03;
P(blacks vs. whites) = 0.008; P(All) = 0.04; all cited P-values are two-tailed.

Table 7. Contingency Table of Urinary PhIP vs. MeIQx

		MeIQx[b]		
PhIP	**ND**	**ND-6.5**	**> 6.5**	**Total**
ND	30 (71)[a]	41 (64)	6 (27)	77
ND-6.5	7 (17)	12 (19)	3 (14)	22
> 6.5	5 (12)	11 (17)	13 (59)	19
Total	42	64	22	128

[a] Column Percentage; [b] Values are expressed in ng/g$_{\text{-creatinine}}$;

X^2 =21; Df = 4; P(total)= 0.00 (99% confidence); Contingency Coefficient 0.38

Figure 7. A Comparison of Urinary PhIP in Smokers vs. Non-Smokers

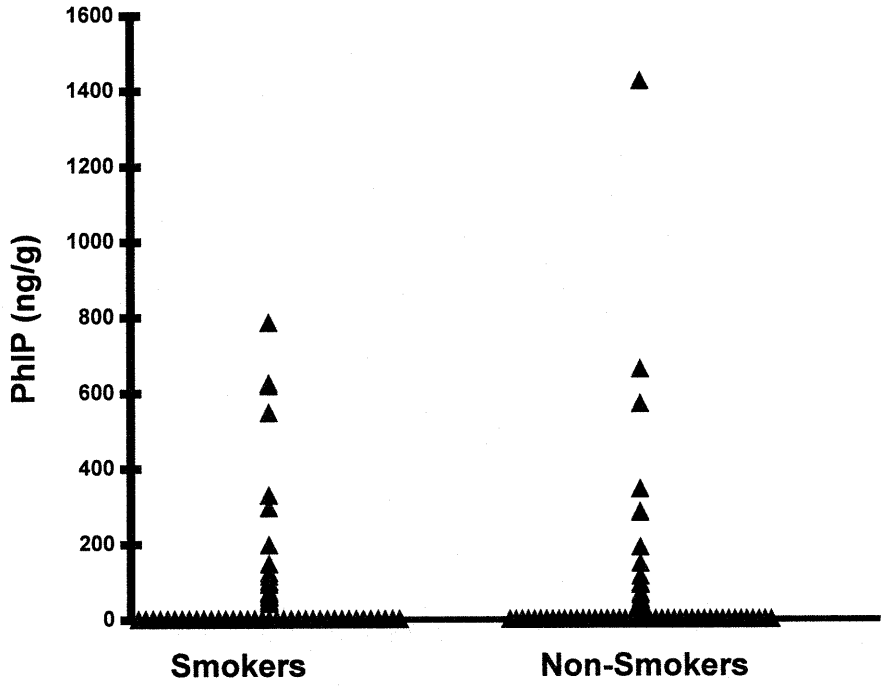


Table 8 compares the number (percentage) of subjects exhibiting low versus high levels and of urinary PhIP, and their corresponding intake frequencies of selected food items (i.e., beef, pork, chicken, fish, sausage and bacon) which have the potential of producing high levels of HAAs during the cooking process. There was no association between the meat intakes and urinary excretion of unmetabolized PhIP plus its acid-labile conjugates. However, a marginal association was observed between the frequency intakes of bacon and urinary excretion of PhIP combined with urinary MeIQx (See Table 9).

Table 8. Frequencies of Meat Intake According to Levels of Urinary PhIP Among Study Subjects[a]

Food	Neg	Pos	R(Prob) (95% CI)
Beef			
<wkly	20 (40)[b]	13 (50)	1.0
wkly	30 (60)[c]	13 (50)	0.7 (0.3,1.7)
Pork			
<wkly	37 (74)	17 (65)	1.0
wkly	13 (26)	9 (35)	1.5 (0.6,4.0)
Chicken			
<wkly	23 (46)	13 (50)	1.0
wkly	27 (54)	13 (50)	0.8 (0.3,2.2)
Fish			
<wkly	45 (88)	21 (81)	1.0
wkly	6 (12)	5 (19)	1.8 (0.5,6.2)
Sausage			
<wkly	32 (63)	20 (80)	1.0
wkly	19 (37)	5 (20)	0.4 (0.5,4.5)
Bacon			
<wkly	36 (73)	19 (76)	1.0
wkly	13 (27)	6 (24)	0.9 (0.3,2.7)

[a]Seventy-six of our subjects participated in survey in which current dietary information was collected through self-administered questionnaire. [b]Values represent percentage of subjects with less than once a week consumption of foods. [c]Values represent percentage of subjects with at least a week consumption of foods. Neg = undetected levels of PhIP; Pos = detectable levels of PhIP; R(Prob) = relative probability of excreting high levels of urinary PhIP due to high frequency intakes of meat; and (95% CI) = 95% confidence interval.

Table 9. Frequencies of Meat Intake According to Levels of Urinary PhIP and MeIQx Among Study Subjects[a]

Food	Low	High	R(Prob) (95% CI)
Beef			
<wkly	10 (30)[b]	21 (51)	1.0
wkly	23 (70)[c]	20 (49)	0.5 (0.2,1.3)
Pork			
<wkly	24 (73)	28 (68)	1.0
wkly	9 (27)	13 (32)	1.4 (0.5,3.8)
Chicken			
<wkly	12 (36)	22 (54)	1.0
wkly	21 (64)	19 (46)	0.5 (0.2,1.2)
Fish			
<wkly	24 (77)	31 (76)	1.0
wkly	7 (23)	10 (24)	1.2 (0.4,3.7)
Sausage			
<wkly	21 (64)	29 (71)	1.0
wkly	12 (36)	12 (29)	0.8 (0.3,2.2)
Bacon			
<wkly	26 (79)	27 (69)	1.0
wkly	7 (21)	12 (31)	2.1 (0.6,7.6)

[a]Seventy-six of our 131 study participated in survey in which current dietary information was collected through a self-administered questionnaire. [b]Values represent percentage of subjects with less than once a week consumption of foods. [c]Values represent percentage of subjects with at least a week consumption of foods. Low = ND-4.0 $ng/g_{\text{-creatinine}}$; High = > 4.0 $ng/g_{\text{-creatinine}}$; R(Prob) = relative probability of excreting high levels of urinary PhIP due to high frequency intakes of meat; and (95% CI) = 95% confidence interval.

4. Discussion

These reported values for PhIP can be compared to studies performed by two other investigators, although not directly. Our laboratory measured the amount of unmetabolized (free) PhIP plus its acid-labile conjugates and expressed it in terms of nanograms of PhIP per gram of creatinine; whereas other investigators measured unmetabolized PhIP and expressed it as nanograms of PhIP per 8-24 hr urine collection period. For example, Ushiyama and coworkers (1991) measured the amount of unmetabolized PhIP excreted in 24 hr urine samples collected from ten healthy Japanese men (natives of Tokyo, Japan) consuming Japanese/western diets and detected approximately 0.1-2 ng of PhIP. Japanese-Americans in our study excreted detectable levels of PhIP ranging from 32-661ng/$g_{-creatinine}$, equivalent to 4-115 pg/ml_{-urine}. In order to make a direct comparison our results must be multiplied by the volume of urine excreted within a 24 hr period (i.e., 1000 ml), resulting in approximately 4-115 ng of PhIP. Lastly, the values are adjusted downward by a factor of 4 in order to account for the findings that individuals excrete 1% and 4% of the ingested dose as unmetabolized PhIP and total PhIP (unmetabolized combined with acid-labile metabolites), respectively (Stillwell et al., 1997, accepted manuscript; Lynch et al., 1991). After adjustments, our values (approximately 1-30 ng of PhIP) are 0.5 to 300-fold higher than Ushiyama's. Assuming urinary excretion of free PhIP expressed in terms of the percentage of the ingested dose is a good marker of HAA exposure, 20% of the Japanese in our study may have consumed a recent meal containing 0.1-3 μg of PhIP. Although the Japanese in Ushiyama's study were exposed to dietary PhIP, the amounts did not exceed 0.2 μg of PhIP.

In another comparison, Lynch and co-workers (1992) found that 10 healthy male volunteers excreted approximately 38 ng of PhIP in their 8 hr urine samples (approximately 500 ml of urine) after the consumption of 4 hamburger patties (240g of cooked beef) containing 16 ng PhIP/g_{-meat}. After adjustments, the positive values (i.e., 4-115 pg/ml_{-urine})

within our three ethnic groups ranged from 0.5-14 ng in blacks and asians and 0.9-1.25 ng in whites. Although these values are 2-75 fold lower than those reported by Lynch and coworkers (1992), approximately 18% of our subjects, namely non-whites, may have consumed the equivalent of 1-2 hamburger patties. Alternatively, non-whites may have consumed the equivalent of 2-4 strips of bacon (approximately 56 g), containing 53 ng PhIP/g_{-meat} (Gross et al., 1993).

Prior to this investigation, our laboratory has reported on the urinary excretion of MeIQx amongst the same subjects as described earlier in this report (Ji et al., 1994). In comparison to whites, blacks and asians excreted higher levels of both PhIP and MeIQx. A Chi-square test did not reveal an association between the urinary distribution of PhIP and MeIQx across the three ethnic groups. This was further demonstrated by an extremely weak contingency coefficient of 38%.

The generation of a 2 x 2 contingency table of positive vs. negative HAA values resulted in the following observations: (1) 9% of the subjects only excreted PhIP, (2) 37% only excreted MeIQx, (3) 30% excreted both PhIP and MeIQx, (4) 23% did not excrete neither PhIP nor MeIQx and (5) 44% of the subjects excreted higher amounts of MeIQx in comparison to PhIP; whereas as 33% excreted relatively higher levels of PhIP.

The composition of urinary HAA metabolites is affected by their relative proportions in cooked meat samples. Style of preparation, cooking time and heating temperature have a great impact on the concentrations of HAAs in cooked muscle-meat (Bjeldanes et al., 1982a). For example, beef samples fried under low to moderate heating temperatures (150-190 °C) and under 5 min contain undetectable to low amounts of PhIP and MeIQx (See Lynch et al., 1995). In contrast, beef fried at high temperatures (230 °C) and over 5 min yield significantly higher levels of PhIP relative to MeIQx. Similar results are observed in grilled/broiled fish and chicken or well done bacon. However, concentrations of MeIQx exceed PhIP by 10 fold in fried bacon cooked just until done and bacon grease. Thus, subjects who excreted higher concentrations of MeIQx relative to PhIP

probably consumed a recent meal containing bacon. On the other hand, subjects that excreted higher levels of PhIP may have a preference for meats which are fried at high temperatures for prolonged periods or meats which have browned surfaces. This is consistent with the finding that non-whites excreted higher levels of dietary HAAs and consumed selected food items (i.e., bacon) more frequently than whites.

If HAAs are shown to play a role in the development of colorectal cancer in humans in epidemiological studies, then dietary consumption of foods containing high levels of HAAs may pose an increased risk. The high colorectal cancer incidence rates observed amongst Los Angeles males may be attributed to their high consumption of foods containing high levels of HAAs. For example, non-whites consumed meat more frequently and excreted higher levels of food related HAAs when compared to whites. The extremely low level of HAAs amongst whites, however, is not consistent with the above hypothesis. Perhaps other risk factors, such as rapid N-oxidation-rapid O-acetylation activity (Lang et al., 1994) or consumption of other fatty foods may contribute to the high risk for colorectal cancer amongst whites in this study. Alternatively, whites in this sub-population may represent a low exposure group due to preferences toward meats which are prepared under low to moderate cooking temperatures or via microwaving prior to cooking. Microwave pretreatment of meat enables the precursors necessary for HAA formation to leak out in the meat juices; thus, inhibiting their production during the cooking process. Alternatively, microwaving does not produce browned meat surfaces containing mutagenic HAAs due to its low heating temperature.

In order to estimate exposure to dietary HAAs, it is important to determine whether meat intakes are associated with urinary HAA levels. Our results reveal a marginal association between frequency of consumption of cooked muscle-meat, particularly bacon, and urinary excretion of PhIP combined with MeIQx. However, no association was observed between urinary excretion of total PhIP and frequency of consumption of meat (i.e., pork, chicken, sausage, bacon, fish and beef). These results emphasize the importance

of measuring exposure to multiple HAAs, especially since no individual HAA can be used as a surrogate of exposure of another HAA.

V. Urinary Excretion of 2-Amino-1-methyl-6-phenylimidazo[4,5-]pyridine (PhIP) and 2-amino-3,8-dimethylimidaza[4,5-f]quinoxaline in Colo-Rectal Cases and Matched Controls in Shanghai

1. Abstract

The primary goal of this research project was to determine whether individuals diagnosed with colorectal cancer excrete higher levels of food-derived HAAs when compared to matched controls. The levels of total PhIP and MeIQx (unmetabolized combined with acid-labile metabolites) were measured in the urine samples collected from 299 male residents (20 colon cases, 39 rectal cases and approximately 5 matched-controls per case) of Shanghai, a metropolitan area of the People's Republic of China. Purification and quantitative analysis of urine specimens was performed by immunoaffinity chromatography combined with ESI-LC/MS-MS. There were no differences in the geometric mean levels of HAAs between rectal and colon cancer cases and their respective matched controls. Moreover, a Chi-square test of association revealed no difference in the distribution of urinary HAAs in cases (rectal and colon cancer subjects) vs. controls .

2. Materials and Methods

2.1 Chemicals

PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine), MeIQx (2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline), PhIP-*d3*-methyl, and MeIQx-*d3*-methyl were purchased from Toronto Research Chemicals (Ontario, Canada). Isotopic purity of the deuterated standards was determined by LC-MS/MS to be 99.3% and 98.7%, respectively. Stock solutions of deuterated PhIP and MeIQx were prepared in methanol and quantified with a Hewlett-Packard 8452A spectrophotometer (Hewlett-Packard Co., Palo Alto, CA) using extinction coefficient values of 18,133 M^{-1} cm^{-1} for PhIP at 316 nm (Friesen et al., 1994 - Stillwell 1996) and 41,000 M^{-1} cm^{-1} for MeIQx at 274 nm (Turesky et al., 1988). Stock standard solutions were prepared as needed and were not stored for future use. Methanol and ethyl acetate were all Omnisolve grade solvents (EM Science, Gibbstown, NJ). High-purity water (double distilled) was used to prepare buffers and acid solutions. Monoclonal antibodies raised against MeIQx and PhIP were used in the preparation of immunoaffinity columns (Turesky et al., 1989). The antibodies were immobilized on CNBr-activated Sepharose 4B (Sigma Chem. Co., St. Louis, MO) at a concentration of 2-5 mg of protein/ml of gel. Blocked gel for precolumns was prepared by treatment of CNBr-activated Sepharose 4B with 0.2 M Tris-HCl, pH 8.0.

2.2 Subject Profile

Subjects included 299 male residents of Shanghai who were originally participants of a cohort study. Approximately 7% and 14% of the subjects were diagnosed with colon (n = 20) and rectal cancer (n = 39), respectively. Urine samples were collected prior to the diagnosis of cancer

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2.3 Acid Hydrolysis and Extraction of Urinary PhIP and MeIQx

Isolation of unmetabolized and conjugated HAAs from human urine samples has been detailed previously (Stillwell et al., 1997). In brief, urine samples (7.5 ml) were placed in 50 ml glass-stoppered glass tubes, spiked with internal standards (ng of d_3-PhIP and d_3-MeIQx), acidified with 6N HCl (1.5 ml) and incubated at 70 °C for 4 hrs. This protocol enabled the liberation of PhIP from its N^3-glucuronide conjugate and MeIQx from its exocyclic amine conjugates (N^2-sulfamate and N^2-glucuronide). After the incubation period, the samples were allowed to cool to room temperature and neutralized with 6N NaOH (1.5 ml). The samples were basified with sodium carbonate (0.5 g) and extracted twice with two volumes of ethyl acetate. The organic phase was placed in a -16 °C freezer for 30 min in order to freeze out residual aqueous phase, and then decanted into a clean glass centrifuge tube containing 2 ml of 0.1 N HCl. After vortexing the solutions, the acid layer was removed and a second extraction was performed with 1.0 ml of 0.1 N HCl. The acid extracts were combined and placed into 7 ml vials and taken to dryness by vacuum centrifugal concentration (Savant Instruments, Inc., Farmingdale, NY). The dried samples were stored at -16 °C until further workup.

2.4 Purification by Immunoaffinity Chromatography

Dried samples were reconstituted in phosphate-buffered saline solution (PBS) (3 ml of 10 mM phosphate buffer, pH 7.4, 0.14 M NaCl) and pH adjusted to 8.0 (0.2 to 0.4 ml of 0.1 N NaOH). The solutions were applied to small precolumns (1 ml of Tris-blocked Cyanide Bromide-activated Sepharose 4B) followed by PhIP and monoclonal antibody columns (1.5 ml) placed in series. In order to increase yield, the first eluant was passed through the precolumn and antibody columns twice. The columns were washed with 15 ml of PBS solution, then the precolumns were removed and an additional 15 ml of PBS solution was applied to the antibody columns. The antibody columns were then washed separately with an additional 30 ml of double distilled water. The PhIP fraction was eluted

from the antibody columns by the addition of 3 ml of 1 N acetic acid. The dried acid fractions were basified with 1N sodium carbonate (0.3 ml), extracted with ethyl acetate (2x1 ml) and chilled at -16 °C for 15 min. The ethyl acetate layers were dried by evaporation under nitrogen and then transferred with a small volume of ethyl acetate (2x100 µl) to 100 µl inserts positioned in screw-cap vials (DP-Target, Hewlett-Packard Co.). The samples were dried under nitrogen and stored in a -16 °C freezer until analysis via LC-ESI-MS/MS.

2.5 Quantitative Analysis by ESI/LC/MS/MS

Purified samples were analyzed by using a Hewlett-Packard HPLC 1090 series instrument coupled to a Finnigan TSQ 7000 triple-stage quadrupole mass spectrometer (Finnigan-MAT, San Jose, CA) equipped with an electrospray ionization source. The HPLC and tandem mass spectrometer were interfaced via a Vydac narrow-bore C-18 reverse-phase column (150 x 2.1 mm, 5 µm particle size). The mobile phase, consisting of a solution of methanol:water (50:50) with 0.1% formic acid, was carried out at a flow rate of 200 µl/min. PhIP and MeIQx were analyzed in positive ion mode by using a selected reaction monitoring technique which monitors collision induced dissociation of a parent ion to its daughter ion. With the collision voltage and gas (argon) set at -32 volts and 3.2 mTorr, respectively, the protonated molecular ion of PhIP ($[M+H]^+$) was fragmented to its corresponding daughter ion (m/z 210), resulting in the loss of a methyl (or d_3-methyl) group. For MeIQx analysis, the analogous transition of the protonated molecular ion ($[M+H]^+$ ----> m/z 199)was monitored. The corresponding parameters of the collision cell were set at -29 volts and 2.9 mTorr. The Finnigan data system monitored the positive ions at m/z 225, undeuterated PhIP (d_0 PhIP); m/z 228, deuterated PhIP (d_3 PhIP); m/z 214, undeuterated MeIQx (d_0-MeIQx); m/z 217, deuterated MeIQx (d_3-MeIQx). In order to ensure maximum sensitivity, instrumental parameters were optimized by adjusting the capillary and tube lens voltages. The heated capillary was set at 250 °C, while the nitrogen sheath gas was held at 80 psi and the auxiliary nitrogen set at 18 on the dial. Unextracted standards were analyzed under the same conditions and used to correct for background

contribution of the undeuterated standards present in their respective deuterated standards. The amount of PhIP and MeIQx in the specimens was determined by calculating the peak area ratio of 225/228 and 214/217, respectively. Percent recovery was determined by comparing the internal standard with unextracted standards.

2.6 Statistical Analysis

Values for the PhIP:creatinine ratio were logarithmically transformed in order to normalize the data. The chi-square test was used to determine whether colorectal cases excrete higher levels of urinary HAAs when compared to their respective matched controls. All P-values cited were two-sided.

3. Results

Detectable levels of urinary PhIP expressed in terms of nanograms of PhIP per gram creatinine ranged from 1-43 $ng/g_{\text{-creatinine}}$ in rectal cancer cases (n = 31) and 1-290$ng/g_{\text{-creatinine}}$ in the matched controls (n = 133). Comparable levels of urinary MeIQx were detected in these subjects. Rectal cancer cases (n = 39) excreted 1-33 ng of $MeIQx/g_{\text{-creatinine}}$ and matched controls (n = 141) excreted 1-5507 ng of $MeIQx/g_{\text{-creatinine}}$. Although the range for urinary MeIQx was fairly broad, only 2 percent of the rectal case population (n = 141) excreted more than 50 ng of $MeIQx/g_{\text{-creatinine}}$. For example, one subject excreted over 300 $ng\text{-}MeIQx/g_{\text{-creatinine}}$ and another excreted 5507 ng of $MeIQx/g_{\text{-creatinine}}$. A 2 x 3 contingency table was generated in order to compare HAA disposition between rectal cancer cases and matched controls. Values for the HAAs were categorized in the following manner: non-detectable, low (2.0-4.0 $ng/g_{\text{-creatinine}}$) and high (> 4.0 $ng/g_{\text{-creatinine}}$) levels. Due to the low number of cases, it was extremely difficult to categorize the values into approximate tertiles. As a result, a comparison between low and high levels of HAAs was performed. Approximately 56% and 62% of the total number of rectal cancer

cases and controls had undetectable levels of urinary MeIQx and PhIP, respectively. Moreover, low levels of urinary MeIQx and PhIP were observed in 19% of the subjects. An additional 20% of the rectal cases and controls excreted high levels of either PhIP or MeIQx. Tables 10-13 reveal the geometric mean levels of PhIP and MeIQx and their frequency of distribution between rectal cases vs. controls. There was no difference in the geometric mean values for either MeIQx or PhIP between rectal cases and controls. A Chi-square test did not reveal any differences in the urinary excretion of MeIQx [$\chi^2 = 0.39$; df = 2; p(0.82)] and PhIP [$\chi^2 = 0.39$; df =2; p(0.92) in rectal cases when compared to their matched controls. There were also no observed differences in geometric mean levels for urinary PhIP and MeIQx [p-values for PhIP (0.45) and for MeIQx (0.74)] in colon cases versus controls. However, all subjects excreted higher levels of urinary PhIP (geometric mean = 2.6) than MeIQx (geometric mean = 1.8). This difference was attributed to the large percentage of cases and controls which had undetectable levels of MeIQx (88% of 118 subjects) and PhIP (46% of 119 subjects). In addition, only 5% of the total population excreted greater than 3.5 ng of MeIQx/$g_{\text{-creatinine}}$; whereas 28% excreted more than 4.0 ng of PhIP/$g_{\text{-creatinine}}$.

4. Discussion

A majority of the Chinese of Shanghai excreted 1-50 ng/$g_{\text{-creatinine}}$ of either total urinary PhIP or MeIQx. In an earlier report, we observed that Chinese residents of Los Angeles excreted 30-100 ng/$g_{\text{-creatinine}}$ of the same HAAs. These differences may be attributed to the fact that Shanghai Chinese excreted 20 fold lower amounts of creatinine (mw = 113 g/mole) when compared to Los Angeles Chinese. For example, on average

Table 10. Distribution of Urinary PhIP in Colon Cases vs. Controls

PhIP[b]	Cases	Controls	RR (95% CI)
ND	8 (40)[a]	51 (52)	1.0
ND-4.0	5 (25)	26 (26)	0.8 (0.4,4.0)
> 4.0	7 (35)	22 (22)	2.0 (0.6,5.0)
Total	20	99	119
*Geomean	2.8	2.5	2.6

[a] Column Percentage. χ^2=1.57; df = 2; *p (0.45); *p (0.99); [b]Values are expressed in terms of $ng/g_{-creatinine}$; all p-values are two-tailed..

Table 11. Distribution of Urinary PhIP in Rectal Cancer Case vs. Controls

PhIP[b]	Cases	Controls	RR (95%CI)
ND	20 (65)[a]	80 (60)	1.0
2.0-4.0	5 (16)	28 (21)	0.7 (0.3,2.1)
> 4.0	6 (19)	25 (19)	1.0 (0.4,2.7)
Total	31	133	164
***Geomean**	2.2	2.3	2.3

[a] Column Percentage. χ^2=0.39; df = 2; * p (0.82); [b] Values are expressed in terms of ng/$g_{\text{-creatinine}}$; all p-values are two-tailed.

Table 12. Distribution of Urinary MeIQx in Colon Cancers Cases vs. Controls

MeIQx[b]	Cases	Controls	RR (95%CI)
ND	18 (90)[a]	82 (85)	1.0
ND-3.5	1 (5)	6 (6)	0.8 (0.4,17.7)
> 3.5	1 (5)	10 (8)	0.4 (0.1,3.8)
Total	20	98	118
***Geomean**	1.8	1.8	1.8

[a] Column Percentage. χ^2=0.6; df = 2; [*] p (0.74); [b] Values expressed in terms of ng/$g_{\text{-creatinine}}$; all p-values are two-tailed.

Table 13. Distribution of Urinary MeIQx in Rectal Cancer Cases vs.

MeIQx[b]	Cases	Controls	RR (95% CI)
ND	22 (56)[a]	79 (56)	1.0
ND-4.0	8 (21)	24 (17)	1.2 (1.4,8.46)
> 4.0	9 (23)	38 (27)	0.9 (1.0,5.6)
Total	39	141	180
Geomean	2.4	2.4	2.4

[a] Column Percentage. $\chi^2 = 0.39$; **df = 2; *p (0.82); [b]Values are expressed in terms of ng/g$_{\text{-creatinine}}$.**

Shanghai Chinese excreted 8.50 mg of creatinine/dL (0.07-35 mg/dL) and Los Angeles Chinese excreted 150 mg of creatinine/dL (19-401 mg/dL). According to the Merck Index (1968), the daily human output for creatinine is 25mg per kg of body weight. As a result, humans (70kg) should excrete approximately 17 mg of creatinine/dL. As a result, Los Angeles Chinese excreted approximately 10 fold higher levels of creatinine when compared to the literature values; whereas Shanghai Chinese excreted approximately 2 fold higher levels of creatinine.

Two subjects excreted extremely high levels of MeIQx when compared to the total Shanghai population (305 $ng/g_{-creatinine}$ and 5507 $ng/g_{-creatinine}$). These extreme values were attributed to the low levels (20-200 lower than the literature values) of creatinine excreted in the measured in the urine specimens.

Several studies suggest that high consumption of foods containing high levels of HAAs have higher risk for colorectal cancer. Our findings, however, are not in agreement with the aforementioned hypotheses. Recently, Sinha and coworkers (1994) reported an increase in CYPIA2 activity in 47 of 65 (72%) of the subjects after consuming pan-fried beef containing high levels of HAAs (i.e., 9.0 ng of $MeIQx/g_{-meat}$ and 32.8 ng of $PhIP/g_{-meat}$). Our laboratory (Stillwell et al., 1997, accepted manuscript) revealed a weak but statistically significant ($p = 0.02$) negative association between CYPIA2 activity and urinary excretion of total MeIQx (unmetabolized plus acid-labile metabolites); however, PhIP disposition and metabolism were not affected by CYPIA2 activity. These aforementioned studies prompted us to reconsider our original hypotheses. If colorectal cases consume higher levels of food-derived HAAs relative to controls, then they may also have higher rates for CYPIA2 activity. Induced CYPIA2 activity in colorectal cases would theoretically increase the metabolic activation of these compounds. As a result, detoxification pathways (i.e., direct N-glucuronidation and N-sulfation) may be less favored in colorectal cases versus controls. Unfortunately, the results of this study were not

consistent with this paradigm. Their were no differences in the geometric mean levels of urinary PhIP or MeIQx in colorectal case vs. controls.

Our laboratory has also observed a marginal but significant association between HAA intake expressed in terms of ng/g_{-meat} (Stillwell, et al., 1997, accepted manuscript) and urinary excretion of total PhIP or MeIQx (unmetabolized plus acid-labile metabolites). This suggest that urinary excretion of total HAAs may not be a good predictor of HAA exposure. Thus, we must take extreme precaution in using urinary metabolites as markers of HAA exposure and colorectal cancer risk.

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Urinary Excretion of Unmetabolized and Phase II Conjugates of PhIP and MeIQx in Humans: Relationship to Cytochrome P4501A2 and NAT2 Activity[1]

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[3]The abbreviations used are: HAA, heterocyclic aromatic amine; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; CYP1A2, cytochrome P4501A2; NAT2, N-acetyltransferase; PBS, phosphate-buffered saline; ESI, electrospray ionization; LC-MS/MS, liquid chromatography-tandem mass spectrometry.

ABSTRACT

Cooking meat, fish, or poultry at high temperature gives rise to heterocyclic aromatic amines (HAAs), which may be metabolically activated to mutagenic or carcinogenic intermediates. The enzymes cytochrome P4501A2 (CYP1A2) and *N*-acetyltransferase (NAT2) are principally implicated in such biotransformations. We have determined the relationship between the activity of these two enzymes and the urinary excretion of unmetabolized and phase II conjugates of the two HAAs MeIQx (2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline) and PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine) in individuals fed a uniform diet containing high-temperature cooked meat. The subjects in the study ate meat containing known amounts of MeIQx and PhIP and urine collections were made 0-12 and 12-24 hr postmeal. MeIQx and PhIP were measured in urine after acid treatment that quantitatively hydrolyzes the phase II conjugates to the respective parent amine. The extracts containing the HAAs were purified by immunoaffinity chromatography and analyzed by liquid chromatography employing electrospray ionization-tandem mass spectrometry. The MeIQx content in the 0-12 hr urine increased after acid hydrolysis by a factor of 3- to 21-fold. After acid treatment the total amount of MeIQx (unmetabolized plus the N^2-glucuronide and sulfamate metabolites) excreted in the 0-12 hr urine was (mean ± SD) 10.5 ± 3.5% of the dose, while the total amount of PhIP (unmetabolized plus acid-labile conjugate(s)) in the 0-12 hr period was (mean ± SD) 4.3 ± 1.7% of the dose. The total amount of PhIP in the 12-24 hr urine after acid treatment was (mean ± SD) 0.9 ± 0.4% of the dose. Linear regression analysis of the amounts of MeIQx and PhIP excreted in the 0-12 hr period expressed as percentage of the ingested dose, for all subjects, gave a low but significant correlation (r =

0.37, $P = 0.005$). Linear regression analyses showed that lower total MeIQx (unmetabolized plus the N^2-glucuronide and sulfamate metabolites) in urine was associated with higher CYP1A2 activity, while total PhIP (unmetabolized plus conjugated) in urine showed no association to CYP1A2 activity. These results indicate that in humans MeIQx metabolism and disposition are more strongly influenced by CYP1A2 activity than are those of PhIP. Linear regression analysis found no association between NAT2 activity and the levels (unmetabolized plus acid-labile conjugates) of MeIQx or PhIP excreted in urine.

INTRODUCTION

The heterocyclic aromatic amines (HAAs[3]) MeIQx (2-amino-3,8-dimethylimidazo[4,5-*f*]-quinoxaline) and PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine) illustrated in Fig. 1 are two of the principal HAAs which are present in cooked meats (1-3). MeIQx is responsible for a major portion of the mutagenic activity found in cooked beef (4), whereas PhIP exhibits moderate mutagenic activity but is often present in higher amounts in cooked meats compared to the other HAAs (5,6). MeIQx and PhIP, like other heterocyclic aromatic amines, require metabolic activation via *N*-oxidation to convert them to reactive species with genotoxic activity. The *N*-oxidation of heterocyclic aromatic amines is catalyzed primarily by hepatic cytochrome P4501A2 (7-9). Interindividual activity of CYP1A2 shows variability which is due in part to genetic polymorphisms and environmental factors (10). Dietary factors, including consumption of high-temperature cooked meats, were found to induce CYP1A2 activity in humans (11). The activity of NAT2, an enzyme involved in both the *N*-acetylation of aromatic amines and the *O*-acetylation of *N*-hydroxylamines, is regulated by genetic phenotype and is not modified by environmental factors (12). *N*-acetylation of aromatic amines represents a competing pathway for arylamine *N*-oxidation, whereas the *O*-acetylation of *N*-hydroxylamines is regarded as an activation process leading to more reactive intermediates.

The metabolic fate of MeIQx and PhIP in human subjects, and the factors affecting it, have been investigated in several studies. Murray *et al.* (13) found that the excretion of unmetabolized MeIQx in urine ranged between 1.8-4.9% of the oral dose. Subsequently we reported evidence for the elimination of acid-labile conjugates of MeIQx, *i.e.*, the N^2-

glucuronide and N^2-sulfamate derivatives (14). In the case of PhIP, Lynch and co-workers (15) reported that the excretion of unchanged PhIP in urine ranged between 0.6-2.3% of the oral dose. In an assessment of the intra- and interindividual variability in systemic exposure of MeIQx and PhIP in humans, they found that the urinary excretion of unmetabolized MeIQx and PhIP (expressed as the percentage of the ingested dose) remained relatively constant for an individual, but that intersubject variation was greater. In later work Boobis *et al.* (16) showed that the excretion of unchanged MeIQx and PhIP increased 14-fold and 4-fold, respectively, after human subjects were treated with furafylline, an inhibitor of CYP1A2. In a dietary study conducted with subjects consuming a controlled meat diet, Sinha *et al.* (17) found an inverse relationship between individuals with high CYP1A2 activity and excretion of urinary unconjugated MeIQx, while NAT2 activity showed no correlation with MeIQx excretion. These data indicate that in humans CYP1A2 activity has an effect on MeIQx metabolism.

At present, no information is available on the contribution of phase II conjugation to the metabolism of PhIP in humans nor has the relationship of CYP1A2 or NAT2 activity to the disposition of PhIP been described. The *N*3-glucuronide metabolite of PhIP was identified as the major detoxification product in studies with human liver microsomes *in vitro* and preliminary findings also indicated that this metabolite was excreted in human urine (18). Other studies with human liver microsomes have shown that PhIP is readily converted to the genotoxic product 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (19).

Recent advances in methodology in human metabolic phenotyping as well as the development of techniques for the analysis of arylamines in biological matrices have allowed

associations to be made on enzyme polymorphisms, metabolic pathways and exposure risks in humans (20). The excretion of urinary metabolites of MeIQx and PhIP could be used as an index of individual exposure to and metabolism of these compounds. In this investigation we quantify the extent of phase II conjugation reactions in the disposition and elimination of PhIP and MeIQx in humans consuming high-temperature cooked meat in a controlled dietary study using newly developed immunoaffinity purification and ESI-LC-MS/MS procedures. We also determined the relationship between the urinary excretion of unchanged plus metabolized MeIQx and PhIP within the individuals in the study. Moreover, we examined the role of CYP1A2 and NAT2 activity on the excretion levels of unmetabolized plus conjugated MeIQx and PhIP in urine.

MATERIALS AND METHODS

Chemicals. PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine), MeIQx (2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline), PhIP-d_3-methyl, and MeIQx-d_3-methyl were purchased from Toronto Research Chemicals (Ontario, Canada). Isotopic purity of the deuterated standards was determined by LC-MS to be 99.3% and 98.7%, respectively. Stock solutions of deuterated PhIP and MeIQx were prepared in methanol and quantified with a Hewlett-Packard 8452A spectrophotometer (Hewlett-Packard Co., Palo Alto, CA) using extinction coefficient values of 18,133 $M^{-1}cm^{-1}$ for PhIP at 316 nm (21) and 41,000 $M^{-1}cm^{-1}$ for MeIQx at 274 nm (22). Stock standard solutions were prepared daily when needed and not stored for usage at a later date. The sulfamate and N^2-glucuronide derivatives of [2-^{14}C]-MeIQx were kindly provided by Dr. R. J. Turesky, Nestlé Research Center, Lausanne, Switzerland (23). Omnisolve grade solvents obtained from EM Science (Gibbstown, NJ) and high-purity water (double-distilled) were used. Monoclonal antibodies raised against MeIQx and PhIP were used in the preparation of immunoaffinity columns (24). The antibodies were immobilized on CNBr-activated Sepharose 4B (Sigma Chem. Co., St. Louis, MO) at a concentration of 2-5 mg of protein/ml of gel. Blocked gel for precolumns was prepared by treatment of CNBr-activated Sepharose 4b with 0.2 M Tris-HCl, pH. 8.0.

Study Design. The subjects participating in the study and the protocol followed in the experimental design have been described in detail in a previous report (11). Briefly, the 66 subjects (33 males and 33 females) were recruited from the Beltsville, MD area. The enrollment criteria included being in good health, being a nonsmoker for at least 6 months, taking no medication other than an occasional analgesic, not consuming any atypical diet

(including vegetarian), and being able to consume caffeine.

Determination of CYP1A2 and NAT2 Phenotype. The subjects were phenotyped for CYP1A2 and NAT2 function by measurement of the urinary caffeine metabolites by the procedures described in detail elsewhere (10). In brief, caffeine and four of its metabolites were quantified by computerized high pressure liquid chromatography with photo-diode array detection and spectral validation. NAT2 was determined using the molar urinary ratio of 5-acetylamino-6-formylamino-3-methyluracil to 1-methylxanthine. CYP1A2 was calculated using the molar urinary ratio of 1,7-dimethylxanthine plus 1,7-dimethyluracil to 1,3,7-tri-methylxanthine.

Meat Preparation and Controlled Dietary Period. The meat preparation and dietary protocol have been described in detail elsewhere (11). In brief, the subjects ingested a controlled diet containing minced beef cooked at low temperature, with non-detectable amounts of MeIQx and PhIP for 7 days to minimize the exposure to the HAAs of all individuals. On the morning of day 8 the subjects were phenotyped for CYP1A2 and NAT2 activities. That evening the subjects ate a meal containing minced beef cooked at high temperature. The lean ground beef was made into quarter-pound patties and pan-fried on a griddle at 250 °C for 11 min per side. The subjects consumed meals containing differing amounts of meat based on their body weight (3.1 to 4.4 g meat/kg body weight). The well-done cooked meat in this phase of the study contained 9 ng/gm of MeIQx and 32.8 ng/gm of PhIP (11). The subjects collected all urine produced in the 0-12 and 12-24 hr postmeal period. The urine samples were frozen and stored at -30 °C until analysis. In a separate study the unconjugated MeIQx in the urine was measured by the GC-MS method described

previously (13, 15). The results in this analysis were reported earlier (17).

Hydrolysis of MeIQx and PhIP Conjugates and Urine Samples. One of the objectives in the present study was to determine the extent of the excretion of exocyclic amine (MeIQx) and ring-nitrogen (PhIP) conjugation products in human urine. Procedures were developed that employed acidic hydrolysis of urine in which the metabolites are quantitatively cleaved to liberate the parent HAA. In earlier studies it was shown that the amine conjugates of MeIQx (the sulfamate and N^2-glucuronide metabolites) are labile to 1 N HCl at 70 °C (14). In the present work optimal conditions for the hydrolysis of the urine samples were established by spiking control urine (10 ml) with radiolabeled (^{14}C) MeIQx-N^2-glucuronide and sulfamate standards. The solutions were made 1 N by the addition of 2 ml of 6 N HCL and incubated at 70 °C for various time periods. The samples were then neutralized, the pH adjusted to 9.5 by the addition of sodium carbonate, extracted with ethyl acetate (2 x 2 volume each), transferred to flasks and dried under vacuum. The recovery of MeIQx was measured by liquid scintillation counting. With each set of experiments control samples were analyzed in which no acid treatment was performed. Radiolabeled PhIP-*N*3-glucuronide was not available for establishing optimal hydrolysis conditions; however, a comparison of the recovery of PhIP was made on urine samples that were either (1) acid hydrolyzed at 70 °C for 4 hr or (2) enzymatically hydrolyzed (18) overnight at 37 °C with 1000 unit/ml of bacterial ß-glucuronidase, EC 3.2.1.31, Type X-A from *E. coli* (Sigma Chem. Co.).

Based on the above experiments urine samples in the study were hydrolyzed in 1 N HCl for 4 hr at 70 °C and extracted using the following conditions: 10 ml urine samples were transferred to 50 ml glass-stoppered glass tubes, spiked with a known amount of the internal

standards (typically 5 ng each of PhIP-d_3 and MeIQx-d_3), acidified by the addition of 2 ml of 6 N HCL and tightly stoppered. After heating for 4 h at 70 °C, the samples were cooled and neutralized by the addition of 2 ml of 6 N NaOH. Following the addition of 0.5 g Na_2CO_3 the samples were extracted twice with two volumes of ethyl acetate. The organic phase was placed in a -16 °C freezer for 30 min and then decanted into a glass centrifuge tube containing 1.5 ml of 0.1 N HCl. After extraction, the acid was transferred to a vial and a second extraction of the organic phase was performed with 1.0 ml of 0.1 N HCl. The acidic extracts were combined and placed into vials and dried by vacuum centrifugal evaporation (Savant Instruments, Inc., Farmingdale, NY).

Immunoaffinity Purification The dried samples were redissolved in 3 ml of PBS (10 mM phosphate buffer, pH 7.4, 0.14 M NaCl) and the pH was adjusted to 8.0 by the addition of 0.2-0.4 ml of 0.1 N NaOH. The solutions were passed through precolumns (1 ml of blocked gel) directly onto PhIP and MeIQx monoclonal antibody columns (1.5 ml) placed in series. The first eluant solution was collected and passed through the precolumn and antibody columns a second time. The precolumn and antibody columns were then washed with 15 ml of PBS, the precolumns were removed, and an additional 15 ml of PBS solution was applied individually to each PhIP and MeIQx antibody column. The respective antibody columns were washed with an additional 20 ml of distilled water. PhIP and MeIQx were individually eluted from the antibody columns with 3 ml of 1 N acetic acid. The acidic fractions were collected into separate vials and dried under vacuum. The dried samples were subsequently redissolved in 0.3 ml of 0.5 M Na_2CO_3 and extracted with ethyl acetate (2 x 0.5 ml). The organic layer was dried under a stream of nitrogen and subsequently transferred with a small

volume of ethyl acetate (2 x 90 μl) to microvolume glass inserts positioned in small screw-cap vials (DP-Target, Hewlett-Packard Co.). The samples were dried under a gentle flow of nitrogen and stored in a -16 °C freezer until analysis by LC-ESI-MS/MS.

For further reuse the monoclonal antibody columns were washed with additional 1 N acetic acid (20 ml), followed with distilled water (20 ml), then with sodium bicarbonate buffer (0.1M, pH 8.3) containing NaCl (0.5M), and finally with PBS solution (20 ml containing 2 mM sodium azide). The columns were then stored in PBS at 4 °C for later use.

LC-MS/MS Analysis. LC-MS/MS analyses of PhIP and MeIQx in the purified extracts were carried out using an on-line HPLC (Hewlett-Packard 1090) coupled to a Finnigan TSQ 7000 triple-stage quadrupole mass spectrometer (Finnigan-MAT, San Jose, CA) equipped with an electrospray ionization source. A Vydac narrow-bore C-18 reverse-phase column (150 x 2.1 mm, 5 μm particle size) was used at a flow rate of 200 μl/min. The mobile phase consisted of methanol:water (50:50) containing 0.1% formic acid. The heterocyclic aromatic amines were analyzed in positive ion mode by the technique of selected reaction monitoring in which the triple-stage quadrupole mass spectrometer was operated under collision-induced dissociation conditions to monitor the transition of a precursor ion to its corresponding product ion. For PhIP the transition of the protonated molecular ion $[M+H]^+$---> *m/z* 210 (loss of a methyl or d_3-methyl group) was monitored, while for MeIQx the analogous transition of the protonated molecular $[M+H]^+$---> *m/z* 199 was monitored. The instrumental parameters of the triple-stage quadrupole mass spectrometer were optimized for the analysis of the respective HAA by adjusting the capillary voltage and tube lens voltage for maximum sensitivity. For the analysis of PhIP the collision voltage was set at -32 volts, and a pressure

of 3.2 mtorr argon was maintained in the collision cell. For the analysis of MeIQx the collision voltage was set at -29 volts, and a pressure of 2.9 mtorr argon was maintained in the collision cell. The heated capillary was set at 240 °C, while the nitrogen sheath gas was held at 80 psi and the auxiliary nitrogen gas set at 18 on the dial. Alternate measurement of the deuterated internal standards and the unlabeled heterocyclic aromatic amine parent ions to the same product ions was performed by the Finnigan data system. The amounts of the HAA in the samples were determined by the ratios of the integrated areas of the ion chromatograms of the respective product ions. Deuterated standards (d_3) were analyzed under the same ESI-LC-MS/MS conditions to correct for background contribution of the amount of the unlabeled (d_0) MeIQx and PhIP present in the internal standards. The precision of the assay was quantified by measuring the coefficient of variation of quality-control samples analyzed with each set of five urine samples. All urine samples were coded and assayed blind.

Statistical Analysis. Comparison of the percent of the excreted dose of total PhIP and MeIQx for each individual in the 0-12 hr urine was by linear regression analysis using Statgraphics statistical software (Manugistics, Inc., Rockville, MD). To determine whether the amount of total PhIP and MeIQx excreted was related to interindividual variation in CYP1A2 and NAT2 levels, we performed a linear regression analysis of total MeIQx and PHIP *versus* levels of these enzymes. Since the largest determinant of MeIQx and PhIP excretion is the amount of HAA consumed, we evaluated the effect of these enzymes in a regression that controlled for the amount of meat ingested. A standard transformation ($\log_{10}$) from the Box-Cox family was used to increase the accuracy and efficiency of these statistical methods and to decrease the sensitivity of the analysis to individual points. Our regression equation for

estimating the effect of the enzyme levels on total MeIQx and PhIP excretion is:

$$Y_i = ß_0 + ß_1X_{1i} + ß_2X_{2i}$$

where Y_i is the $\log_{10}$ of individual i's total urinary MeIQx or PhIP excretion, X_{1i} and X_{2i} are individual i's meat consumption and $\log_{10}$ enzyme level (either CYP1A2 or NAT2), respectively.

RESULTS

Hydrolysis Conditions. The recoveries of ^{14}C-MeIQx were determined after urine samples were spiked with the radiolabled MeIQx-N^2-glucuronide or sulfamate standards and hydrolyzed in 1 N HCl at 70 °C for varying times. Urine samples that were not hydrolyzed had recoveries that were less that 0.8 % of the total amount added. With the addition of acid and an incubation time of 4 or 6 hr the recovery of MeIQx from the sulfamate metabolite was 72% of the total amount, while for the N^2-glucuronide conjugate the recovery was approximately 85% of the total. In the case of PhIP a comparison of recoveries from urine samples that were either acid hydrolyzed or enzymatically hydrolyzed showed similar results when analyzed for PhIP content, thus indicating that acid hydrolysis in 1 N HCL at 70 °C for 4 hr is sufficient for the hydrolysis of the expected *N*3-glucuronide metabolite in urine.

LC-MS/MS Characteristics. The LC-ESI mass spectra of MeIQx and PhIP were recorded in the positive ion mode. The two compounds showed abundant protonated molecular ions $[M+H]^+$ at *m/z* 214 and *m/z* 225, respectively. Under collision-induced dissociation conditions both HAAs readily lose a methyl group to yield the corresponding $[MH-15]^+$ fragment ions in high abundance; little other fragmentation was observed. Selective ion-reaction monitoring was utilized to analyze the aforementioned precursor and product ions of the HAAs and their respective stable-isotope labeled internal standards. The transition of the protonated molecular ion and internal standard at *m/z* 225 and 228 ---> *m/z* 210 was monitored for the analysis of PhIP, while for MeIQx the analogous transition of *m/z* 214 and 217---> *m/z* 199 was monitored. The precursor to product ion reactions involved in selective reaction monitoring provide great specificity of detection, while the use of immunoaffinity

column purification produces samples that are clean and amenable for direct analysis by LC-MS/MS. Good detection limits in the low picogram range were obtained for the standard compounds injected on column with no detectable background interference. The calibration curves for both MeIQx and PhIP showed good linearity over the range of 0.5 to 15 ng per sample.

Urine Analysis. The results of MeIQx and PhIP analysis in human urine using selected ion-reaction monitoring showed detection limits in the low (5-10) picogram per ml range for both MeIQx and PhIP. At these levels the signal-to-noise ratio was greater than 5 for each HAA. Overall recovery of PhIP and MeIQx from urine through the entire procedure is 25% or greater based on the analysis of unextracted deuterated internal standards. The precision of the urinary assay was determined by replicate analyses of MeIQx and PhIP from aliquots of a pooled urine collection. The quality-control samples were assayed as described above and analyzed over a four-month period. The percent coefficient of variation for the urinary analysis of MeIQx was 16, while the percent coefficient of variation for PhIP was 31. Illustrated in Fig. 2 and 3 are typical ion traces obtained from selected reaction monitoring of the precursor to product ions of MeIQx and PhIP and their respective deuterated internal standards isolated from human urine. These results characterize the high degree of selectivity and the lack of interference from matrix or endogenous components in these analyses.

Subject Characteristics and Urinary Excretion of MeIQx and PhIP. Table 1 summarizes the subject characteristics, the quantity of meat eaten, and other variables. The content of MeIQx and PhIP in the high temperature-cooked meat was 9.0 ng/gm and 32.8 ng/gm, respectively, and the quantity of meat eaten for each individual was 3.1-4.4 g meat/kg

body weight. The total amount of meat eaten by the subjects ranged from 180 to 328 gm with a median value of 248 gm.

Since MeIQx and PhIP were determined after acid treatment of urine specimens, the values of our measurements represent the sum of the free and conjugated forms of each compound. These values for the mean, median and range of the total amount (unchanged plus acid-labile conjugates) of MeIQx and PhIP excreted in 0-12 hr post-meal period are given in Table 2. The percentages of the ingested dose of MeIQx and PhIP eliminated in urine are shown in Table 2. The total amount of urinary MeIQx (unmetabolized plus N^2-glucuronide and sulfamate metabolites) excreted in the 0-12 hr period following meat consumption was 10.5 ± 3.5% (mean ± SD) of the ingested dose (Table 2). The amount of unmetabolized MeIQx in the urine of these subjects was found in the earlier report to be 1.3 ± 0.6% (mean ± SD) of the dose (17). Determination of the amount of MeIQx excreted in the 12-24 hr post-meal period was not conducted in this study inasmuch as a previous investigation showed that MeIQx is rapidly eliminated in urine and only minor quantities (0.2 to 1.3% of the dose) are excreted in this time period (14).

The total amount of urinary PhIP measured as unchanged plus acid-labile conjugate(s) in the 0-12 hr post-meal collection ranged from 1.9 to 9.8% of the dose with a mean ± SD of 4.3 ± 1.7% (Table 2). The total amount (unchanged plus conjugated) of PhIP eliminated in the urine of 16 subjects in the 12-24 hr post-meal period was also determined. The excreted amount in this period accounted for 0.9 ± 0.4% (mean ± SD) of the dose. A correlation value of $r = 0.37$ ($P = 0.005$) was found between the percent of the ingested dose of PhIP excreted in urine *versus* that of MeIQx excreted when compared for all the subjects (Fig. 4).

HAA Excretion versus CYP1A2 and NAT2 Phenotypes. CYP1A2 and NAT2 activities of the subjects were measured in the period prior to the consumption of high temperature-cooked meat (17). The median and range of these enzyme values are shown in Table 1. Tests for the association of CYP1A2 activity on total urinary MeIQx and PhIP excretion were conducted. Fig. 5a illustrates the effect of CYP1A2 on total (unmetabolized plus conjugated) urinary MeIQx excretion normalized to the median of meat consumed. The slope of the fitted line shows the average change in the $\log_{10}$ of total (unmetabolized plus conjugated) MeIQx per unit change in $\log_{10}$ CYP1A2 activity. The subjects with higher CYP1A2 activity have less total MeIQx in the urine than the subjects with lower enzyme activity. CYP1A2 activity showed a notable (P = 0.02) influence on total MeIQx excreted in urine. Tests for the association of CYP1A2 activity with other individual characteristics, such as age, gender, weight, and body mass index and tests for the importance of these features as main effects were all negative. The relationship between CYP1A2 activity and total (unmetabolized plus conjugated) urinary PhIP, adjusted for the amount of meat intake, is depicted in Fig. 5b. No correlation was found between CYP1A2 activity and PhIP excretion (P= 0.55). The intercept ($ß_0$), model R^2, slope of the fitted line and estimated standard error (SE), the level of significance (P value) for the meat intake ($ß_1$), and enzyme activity ($ß_2$) for both MeIQx and PhIP are given in Table 3.

Tests for the association of NAT2 activity on the total urinary excretion, adjusted for the amount of meat consumed, of MeIQx and PhIP were also conducted. No association was found between NAT2 activity and the total (unmetabolized plus conjugated) urinary excretion of MeIQx or PhIP in the subjects.

DISCUSSION

In this study we determined the contribution of phase II conjugation reactions to the metabolism and disposition of MeIQx and PhIP in individuals consuming a uniform diet under controlled dietary conditions. The data reported here provide evidence that in humans urinary phase II detoxification metabolites in the elimination of unoxidized MeIQx are comparatively higher than are those of unoxidized PhIP. The participants in this study excreted an average of 9-fold more MeIQx (expressed as percentage of the dose) in the form of acid-labile conjugates in urine than unmetabolized MeIQx. These results are in agreement with an earlier study showing that the N^2-glucuronide and sulfamate metabolites of MeIQx are important elimination products in humans (14). In the case of PhIP a previous investigation found that the urinary excretion of unchanged PhIP accounted for an average of 1.1% of the ingested dose (15). In the present study the excretion of PhIP (unmetabolized plus acid-labile conjugate(s)) in the 0-12 hr urine accounted for an average of 4.3% of the ingested dose, while another 0.9% of the dose was excreted in the 12-24 hr urine. Thus approximately 4-fold more PhIP in the form of acid-labile metabolites than unmetabolized PhIP is excreted in the combined 0-24 hr urine of the subjects. Several variables may influence the comparatively lower amount of PhIP excreted in urine compared to MeIQx. These are differential rates of phase II conjugation reactions which form the polar metabolites of PhIP for excretion and/or a preferential excretion of these metabolites of PhIP in feces.

A comparison of the amounts of MeIQx and PhIP in urine expressed as the percentage of the dose ingested, for all subjects, following consumption of uniform diet of well-done cooked beef showed a low correlation indicating that metabolic variation may be important in the

disposition and elimination of these HAAs. Differences were evident in the relationship between CYP1A2 activity and total (unmetabolized plus conjugated) urinary excretion of MeIQx and PhIP. The results reported here are in accordance with the earlier study and show that higher CYP1A2 activity is correlated with lower MeIQx excretion represented in terms of either unmetabolized MeIQx (17) or unmetabolized MeIQx plus the N^2-glucuronide and sulfamate metabolites. N^2-glucuronidation and sulfamation of MeIQx represent competing pathways for arylamine *N*-oxidation, thus an inverse relationship would be expected for the comparison of total MeIQx (unmetabolized plus amine conjugated) excreted *versus* CYP1A2 activity. The urinary excretion of total PhIP (unmetabolized plus conjugated) did not show an inverse relationship to the enzyme activity of CYP1A2. These results suggest that the metabolism of MeIQx in humans is more strongly influenced by CYP1A2 activity than is that of PhIP. These findings are in agreement with the previous *in vivo* investigation showing that the excretion of unchanged MeIQx in humans was increased by a greater degree after treatment with furafylline (an inhibitor of CYP1A2) than was PhIP (16). In contrast, experimental studies (9) with human liver microsomes have shown that the rate of *N*-oxidation of PhIP by CYP1A2 is comparatively high with respect to other HAAs. The lack of correlation between CYP1A2 activity and urinary PhIP excretion may involve other factors such as detoxification reactions of the *N*-acetoxy derivative of PhIP with glutathione. It was reported (25) that certain human hepatic glutathione *S*-transferases are active in detoxifying *N*-acetoxy-PhIP via a redox reaction to form PhIP and that this activity did not occur with the *N*-acetoxy derivatives of MeIQx or the related food mutagen IQ (2-amino-3-methylimidazo[4,5-*f*]quinoline). Other human cytochrome P-450s, including cytochrome P-

4501A1, may also be involved in the metabolism of PhIP (26, 27). Recent evidence has been reported that human cytochrome P-4501B1 expressed in yeast is involved in the metabolic activation of MeIQx and to a lesser extent PhIP (28).

Metabolism studies in nonhuman primates have demonstrated that PhIP is metabolized to several polar metabolites including the *N*3-glucuronide conjugate of N^2-hydroxy-PhIP (29). This metabolite was detected in small amounts in the urine, bile, and serum of treated animals and is believed to act as a transport form for the *N*-hydroxylarylamine to extrahepatic tissues. Our studies showed that in humans PhIP undergoes conjugation reactions to form an acid-labile metabolite(s) which is eliminated in urine. It remains to be determined whether the N^2-hydroxy-*N*3-glucuronide of PhIP is also a metabolic product and as such excreted in human urine.

No correlation between the enzyme activity of NAT2 and MeIQx or PhIP excretion was found. These findings are consistent with the results of an investigation showing that MeIQx and PhIP are not *N*-acetylated by human liver cytosol (9). Other investigations demonstrated that MeIQx was not *N*-acetylated by human NAT2 expressed in *COS-1* cells, while PhIP showed a markedly low rate of *N*-acetylation (30). Thus, NAT2 activity would not be expected to directly affect the systemic clearance of the HAAs.

The caffeine metabolic ratio varied from 2.1 to 28 among the participants of this study, and as reported earlier (11) this compares to an observed 40- to 60-fold range in hepatic CYP1A2 levels based on enzyme activity or protein content. The implications of this variation as a risk factor associated with development of certain human cancers are not known. However, in a case/control study Lang and co-workers (31) showed that individuals

at greatest risk to develop colorectal cancer or nonfamilial polyps are those who possess both the rapid NAT2 and rapid CYP1A2 phenotypes and are exposed to high dietary levels of HAAs. Human daily exposure to HAAs varies greatly and is dependent on several factors, including food preferences and cooking techniques (1-3, 5,6). Recent evidence in an epidemiological study has indicated that a dietary preference for fried meats, such as bacon, may play a role in the significant differences noted between the urinary excretion of unchanged plus conjugated MeIQx among men of different racial/ethnic background living in Los Angeles County (32).

In conclusion, the results in this investigation support the relevance of CYP1A2 activity to the metabolism and disposition of MeIQx in humans. The urinary excretion of PhIP showed no correlation to CYP1A2 activity, though significant associations between CYP1A2 activity and PhIP excretion may be missed because of the low proportion of the dose excreted in urine. The results presented here suggest that the excretion of urinary metabolites of MeIQx and PhIP may be used as an index of exposure to and metabolism of these compounds in humans. However, the interindividual variation in the proportion of the ingested dose excreted in urine for MeIQx (range, 3.2-22.7%) and PhIP (range, 1.9-9.8%) observed in the study group as well as possible variabilities in phase II conjugation reactions and elimination processes indicate that measurement of combined unmetabolized plus acid-labile conjugates of the HAAs in urine may not yield a direct comparison of the extent of human exposure of these compounds. Analysis of unmetabolized MeIQx in urine may provide a better estimate or biomarker for monitoring the degree of exposure to this heterocyclic aromatic amine in human studies (15). The excretion of unmetabolized PhIP may be too low to be useful as a

biomarker.

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Table 1. Characteristics of Study Subjects

	Median	(range)
Age (years)	37	(27-62)
Weight (kg)	70.8	(42.2-102.5)
BMI (kg/m^2)	23.9	(17.0-32.1)
Amount of meat eaten (gm)	248	(180-318)
NAT2 activity	0.6	(0.19-3.7)
CYP1A2 activity	8.8	2.1-28)

Table 2. Urinary Excretion of MeIQx and PhIP

	mean±SD	median	(range)
MeIQx[a] in urine (ng) 0-12hr	230±88	221	(72-644)
PhIP[a] in urine (ng) 0-12hr	351±160	322	(118-1012)
MeIQx[a] % of dose 0-12 hr	10.5±3.5	10.4	(3.2-22.7)
PhIP[a] % of dose 0-12 hr	4.3±1.7	4.0	(1.9-9.8)
PhIP[a] % of dose 12-24 hr	0.9±0.4	0.9	(0.5-1.9)

[a]Total of unmetabolized and acid-labile metabolites

Table 3. Variables examined in relationship to total (unmetabolized plus conjugated, $\log_{10}$ transformed) urinary MeIQx and PhIP

Independent variables	β-coefficient (SE)	*P* value	Intercept	R^2
MeIQx				
Multiple linear regression			2.07	0.21
Amount of meat consumed	0.002 (0.0006)	0.003		
Log_{10} CYP1A2	-0.20 (0.08)	0.02		
PhIP				
Multiple linear regression			1.89	0.25
Amount of meat consumed	0.003 (0.0006)	0.001		
Log_{10} CYP1A2	-0.06 (0.1)	0.55		

FIGURE LEGENDS

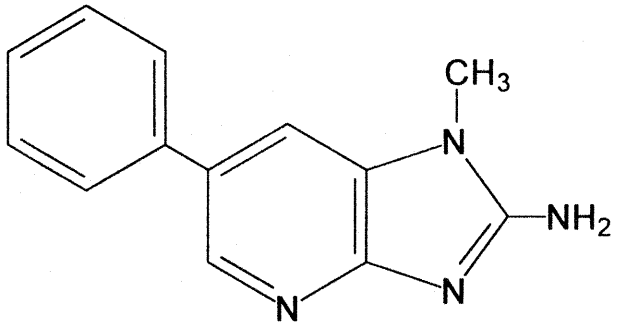
Figure 1. Chemical structures of PhIP and MeIQx.

Figure 2. ESI-LC-MS/MS analysis of MeIQx in human urine. The selected reaction-ion traces are of MeIQx (upper trace) and the d_3-internal standard (lower trace). The concentration of MeIQx in the sample was 410 pg/ml.

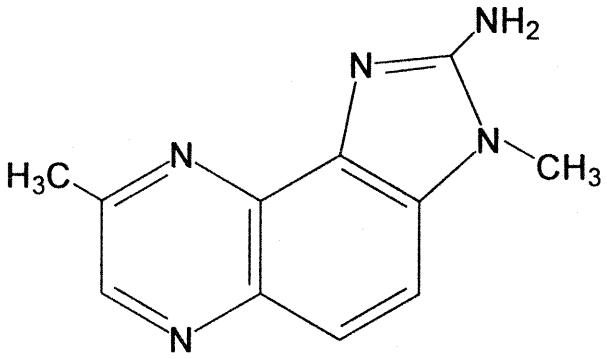
Figure 3. ESI-LC-MS/MS analysis of PhIP in human urine. The selected reaction-ion traces are of PhIP (upper trace) and the d_3-internal standard (lower trace). The concentration of PhIP in the sample was 460 pg/ml.

Figure 4. Correlation between the percent of the dose of MeIQx (unmetabolized plus conjugated) and PhIP (unmetabolized plus conjugated) excreted in 12-hr urine samples for all subjects.

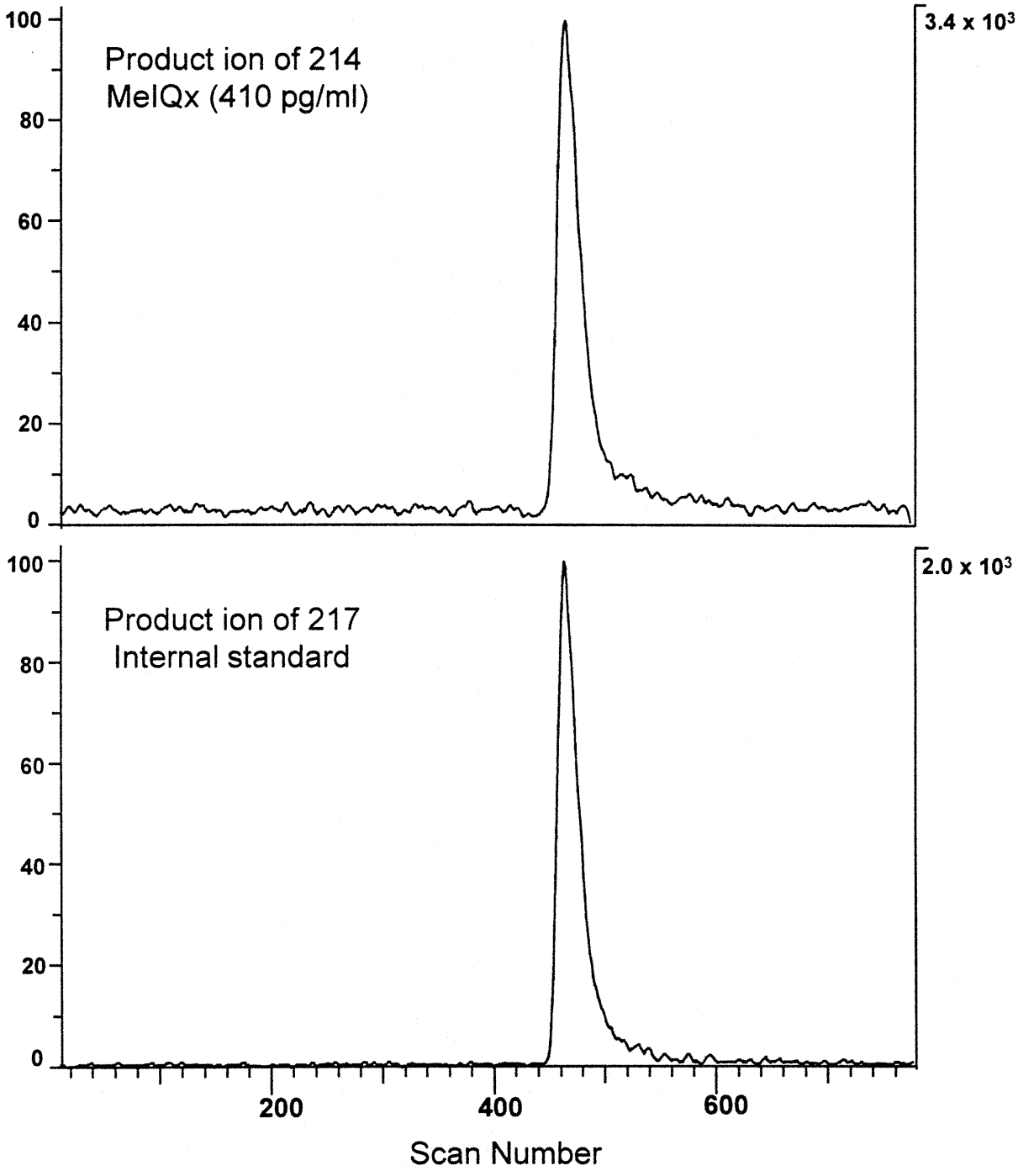
Figures 5a and 5b. Relationship of the total amount in ng (unmetabolized plus conjugated, $\log_{10}$ transformed data) of MeIQx (Fig. 5a) and PhIP (Fig. 5b) in the 12-hr urine samples to CYP1A2 (P4501A2) activity ($\log_{10}$ transformed), adjusted for the amount of meat eaten. The distance of the plotted points (Y_i) from the line demonstrates how closely an individual's observed total MeIQx (Fig. 4a) and PhIP (Fig. 4b) approximates their predicted level based on their CYP1A2 activity ($Y_i = ß_0 + ß_1 \times 248 + ß_2 X_{2i} + e_i$, where $ß_0$, $ß_1$, and $ß_2$ are the estimates of the coefficient in the linear regression model, 248 is the median amount of grams of meat consumed, X_{2i} is an individual i's log CYP1A2, and e_i is the deviation of the individual i's total MeIQx or PhIP from the predicted MeIQx or PhIP based on their actual meat intake and enzyme level and the estimated regression coefficient).

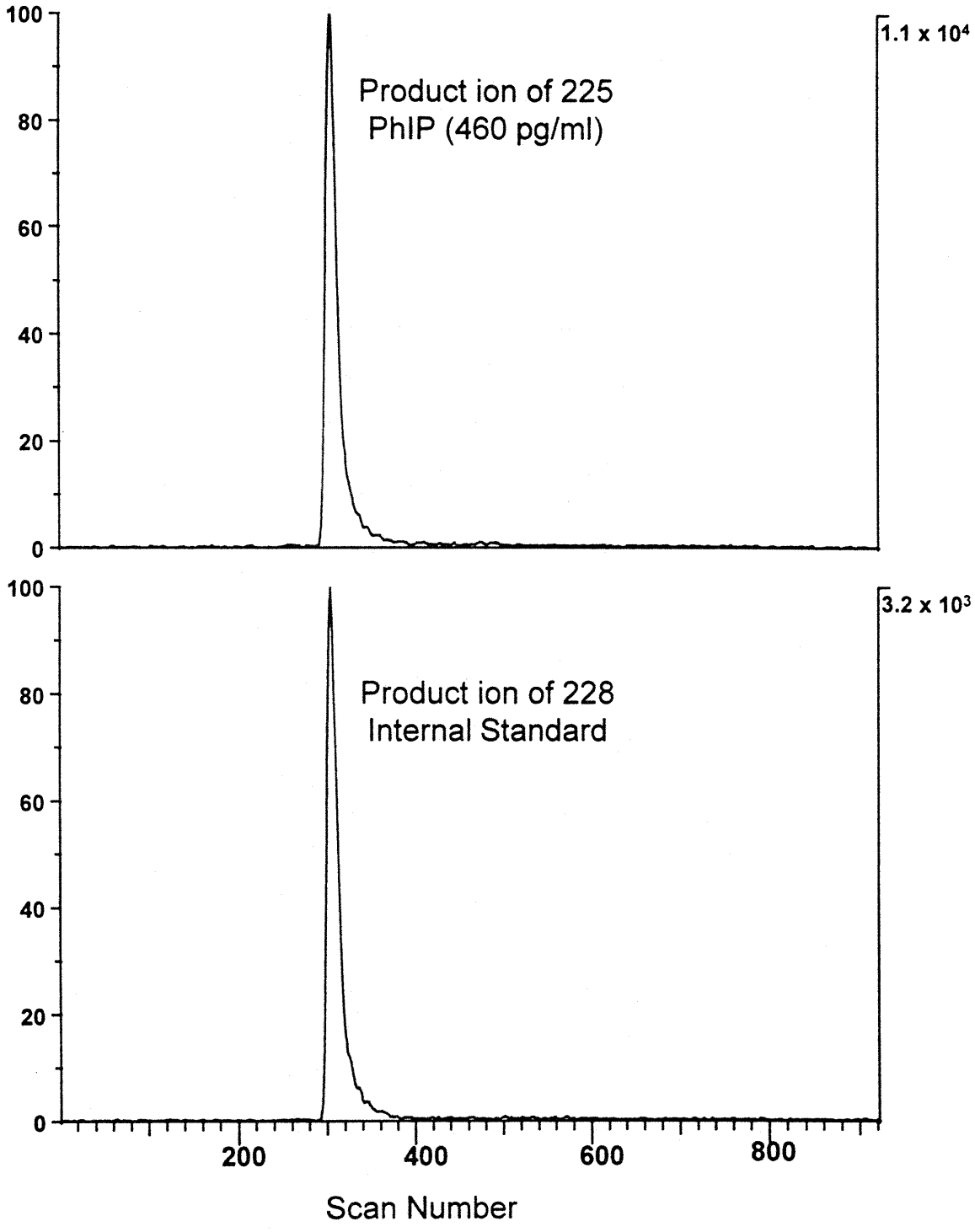


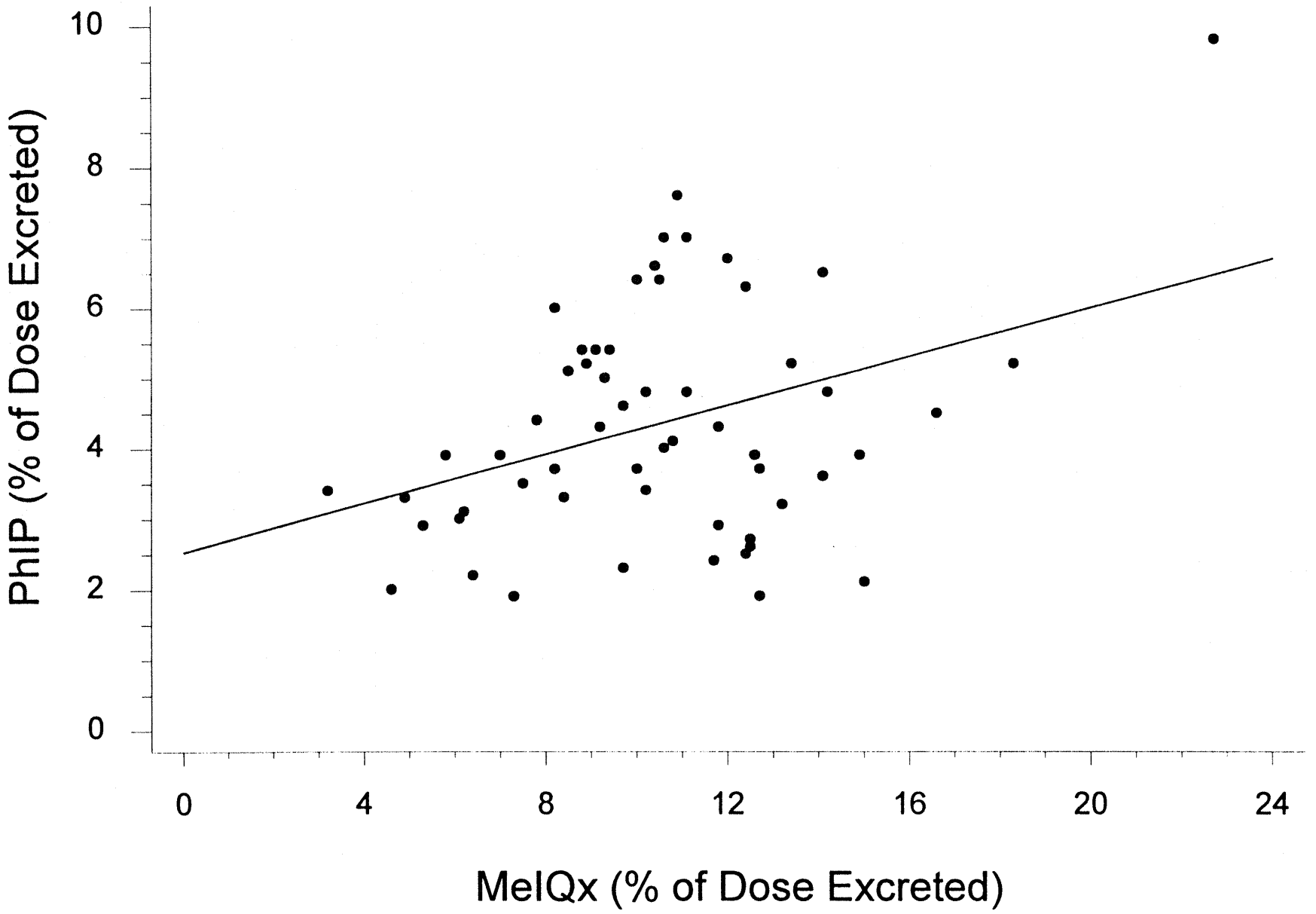
PhIP

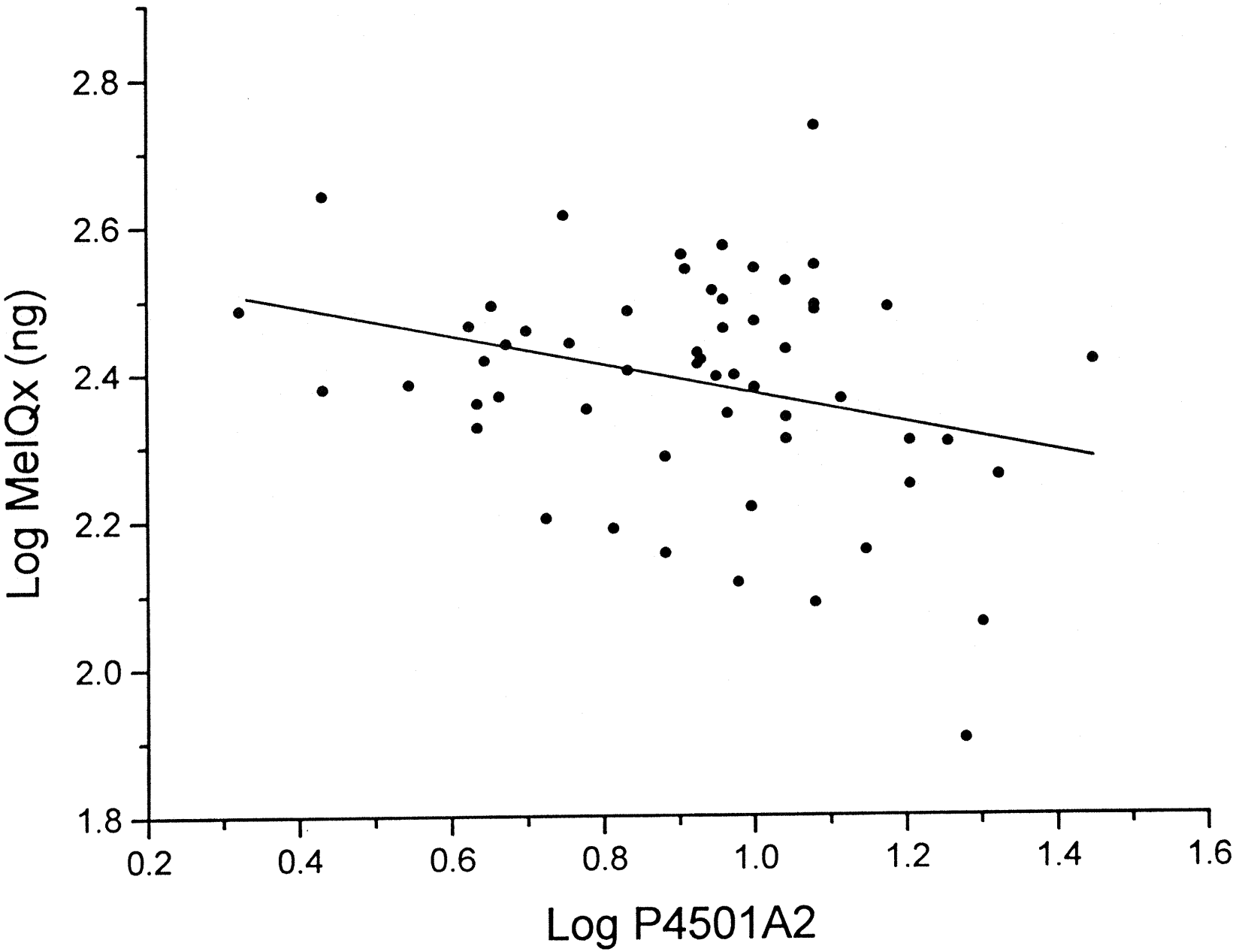


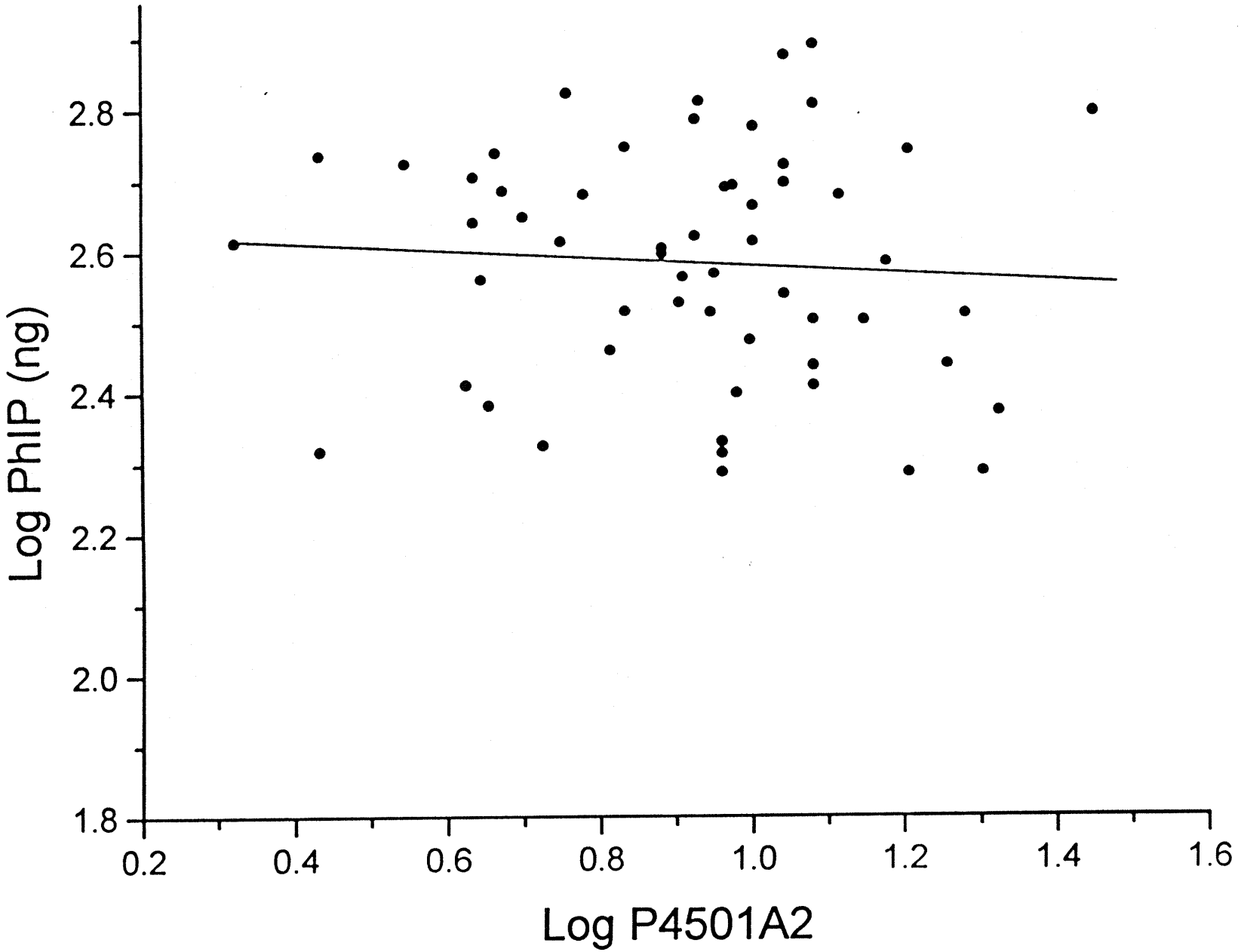
MeIQx


Product ion of 214
MeIQx (410 pg/ml)
3.4 x 10³
Product ion of 217
Internal standard
2.0 x 10³
100
80
60
40
20
0
200
400
600
Scan Number


100
80
60
40
20
0
1.1 x 10⁴
Product ion of 225
PhIP (460 pg/ml)
100
80
60
40
20
0
3.2 x 10³
Product ion of 228
Internal Standard
200
400
600
800
Scan Number


PhIP (% of Dose Excreted)
10
8
6
4
2
0
0
4
8
12
16
20
24
MeIQx (% of Dose Excreted)


Log MeIQx (ng)
2.8
2.6
2.4
2.2
2.0
1.8
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
Log P4501A2


Log PhIP (ng)
2.8
2.6
2.4
2.2
2.0
1.8
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
Log P4501A2