Roskilde
University

Food derived carcinogenic amnoimidazoazaarenes
bioactivation and DNA adduct formation

Frandsen, Henrik

Publication date:
1996

Citation for published version (APA):
Frandsen, H. (1996). *Food derived carcinogenic amnoimidazoazaarenes: bioactivation and DNA adduct formation*. National Food Agency of Denmark.

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain.
- You may freely distribute the URL identifying the publication in the public portal.

Take down policy
If you believe that this document breaches copyright please contact rucforsk@kb.dk providing details, and we will remove access to the work immediately and investigate your claim.

Download date: 14. Mar. 2025

FOOD DERIVED CARCINOGENIC AMINOIMIDAZOAZAARENES.

Bioactivation and DNA adduct formation

Ph.D. Thesis

Henrik Frandsen

Institute of Toxicology
Division of Biochemical and Molecular Toxicology
Laboratory of Biochemical and Genetic Toxicology
National Food Agency of Denmark

and

Institute for Life Sciences and Chemistry
Roskilde University Center
Denmark

DATA SHEET

Title: Food derived carcinogenic aminoimidazoazaarenes.
Subtitle: Bioactivation and DNA adduct formation.
Author: Henrik Frandsen.
Publisher: National Food Agency of Denmark.
Affiliation: Institute of Toxicology, Division of Biochemical and Molecular Toxicology, Laboratory of Biochemical and Genetic Toxicology
Address: Mørkhøj Bygade 19, DK-2860 Søborg, Denmark.
Telephone: +4539696600
Fax: +4539660100

Abstract: Carcinogenic aminoimidazoazaarenes are formed during cooking of meat and fish. Important factors for the formation of these compounds are meat type, cooking temperature and time. The compounds are genotoxic in bacterial and mammalian cells. In animal feeding studies the compounds tested so far were found to be multiple organ carcinogens.
The aminoimidazoazaarenes are metabolically activated by hydroxylation of the exocyclic aminogroup to the *N*-hydroxyamino derivative. The resultant proximate mutagens often need further activation by phase II transferases for formation of reactive species that form adducts with DNA. Adducts with 2-deoxyguanosine have been characterized for a number of aminoimidazoazaarenes. Adducts with DNA have also been found in animals after exposure to these compounds.
In vivo major metabolic detoxification pathways are ring hydroxylation followed by conjugation and conjugation of the exocyclic amino group. Estimations of human cancer risk have indicated that ingestion of food containing aminoimidazoazaarenes are of importance.

Key words: Aminoimidazoazaarene, Metabolism, Activation, DNA adducts.

Quotation: Henrik Frandsen (1996): Food derived carcinogenic aminoimidazoazaarenes. Bioactivation and DNA adduct formation. Ph.D. Thesis. National Food Agency of Denmark, DK-2860 Søborg, Denmark.

ISBN: 87-601-6161-2

Printing: Quickly Tryk A/S

SUMMARY

Several carcinogenic heterocyclic aromatic amines are formed during cooking of meat and fish. The important factors for the formation of these compounds are the type of meat being cooked, cooking temperature and duration of cooking. Under standard household cooking conditions, the aminoimidazoazaarenes (AIAs) which are condensation products of amino acids and creatinine, seem to be the most important compounds, due to the amounts formed and their possible biological effects.

The AIAs are genotoxic in a number of test systems such as those using *Salmonella typhimuriun* and mammalian cells. In rodent feeding studies IQ, 4-MeIQ, 8-MeIQx and PhIP were found to be multiple organ carcinogens.

These procarcinogens require metabolic activation to exert their genotoxic effects. Activation has been shown to occur via hydroxylation, catalysed by CYP1A enzymes, of the exocyclic amino group to the N^2-OH derivatives. These derivatives are proximate mutagens that to some extent bind covalently to DNA and other macromolecules. Further activation of the N^2-OH group through esterification by e.g. acetyltransferase or sulfotransferase results in formation of highly reactive species that form adducts with DNA and 2'-deoxyguanosine, but not with 2'-deoxyadenosine, 2'-deoxycytidine or thymidine. Adducts from 2'-deoxyguanosine and IQ, 4-MeIQ, 8-MeIQx, 4,8-DiMeIQx and PhIP have been characterized.

In rodents *in vivo*, major metabolic pathways, that result in detoxification products, are hydroxylation of the ring system followed by conjugation with sulphate or glucuronic acid. Also, conjugates of the exocyclic amino group with sulphate or glucuronic acid are major metabolites.

^{32}P-Postlabeling analyses of DNA from rodents given AIA compounds orally as well as studies using radiolabelled compounds showed that adducts with 2'-deoxyguanosine are also formed *in vivo*. Adduct levels seem to increase linearly with dose.

Risk estimates, based on extrapolations from results of long term animal cancer studies, have suggested that ingestion of AIA compound are important in human cancer etiology.

CONTENTS

SUMMARY 6
SUMMARY IN DANISH (SAMMENDRAG) 7
LIST OF PAPERS 8
ABBREVIATIONS 10
INTRODUCTION
    Chemical carcinogenesis 12
DIET AND CANCER 13
HETEROCYCLIC AROMATIC AMINES IN FOOD
    Formation and occurrence 14
MUTAGENICITY AND CARCINOGENICITY 18
METABOLISM 21
    The quinolines 21
    The quinoxalines 24
    The pyridine 27
DNA ADDUCT FORMATION 29
MECHANISM OF ADDUCT FORMATION 33
DNA ADDUCT FORMATION *IN VIVO* 35
ESTIMATES OF HUMAN INTAKE AND CANCER RISK 37
CONCLUSION 38
REFERENCES 41
PAPER I
PAPER II
PAPER III
PAPER IV

From the Department of Chemistry, Swedish University of Agricultural Sciences, Uppsala, Sweden:
Dr. Spiros Grivas
Dr. Rolf Andersson

From Nestec Ltd, Research Centre, Lausanne, Switzerland:
Dr. Robert J. Turesky

From Medical Research Council, Carshalton, Surrey, UK:
Dr. Peter Farmer

I also wish to thank my tutors: Dr. John C. Larsen, Institute of Toxicology, Danish National Food Agency and Dr. Ole Andersen, Institute for Life Sciences and Chemistry, Roskilde University Center, Denmark for valuable comments to the manuscript.

I wish to thank Vivian Jørgensen for performing the Ames test and Bo Lund Jensen for assistance with graphical work.
Especially, I wish to thank Joan E. Gluver for supplying me with skilful technical assistance throughout the studies.

Thanks also to Dr. James Felton, Lawrence Livermore Natl. Laboratory, Livermore, CA and Dr. Errol Zeiger, Cellular and Genetic Toxicology Branch, NIEHS, North Carolina, for providing PhIP for the present studies.

Copenhagen, January 1996

Henrik Frandsen

PREFACE

The present study was initiated to provide knowledge about the mechanism of activation and DNA binding of the food derived carcinogens PhIP and 4,8-DiMeIQx. PhiP and 4,8-DiMeIQx were chosen as representatives of the AIAs that are formed at high and medium frying temperatures, respectively.
Due to the presence of these potentially deleterious compounds in the normal human diet, there is a need for developing biomarkers of exposure and effects. Research on the mechanism of activation and adduct formation of several AIAs and pyrolysate mutagens have been conducted by a number of research groups throughout the world. PhIP, in particular, has attracted attention, which has resulted in a strong competitive environment but also in the establishment of many collaborative studies. The results presented here form part of the contribution to these collaborative international efforts.

In paper I, the microsomal metabolism of PhIP and identification of the genotoxic metabolite was published.

Paper II describes the formation of DNA adducts of PhIP and the structural characterization of the adduct.

In paper III, the microsomal metabolism of 4,8-DiMeIQx and identification of the genotoxic metabolites was published.

Paper IV describes the formation of DNA adducts of 4,8-DiMeIQx and the structural characterization of the adduct.

The studies on PhIP and 4,8-DiMeIQx are planned to continue. The future aims are to develop methods for measuring adducts in samples from humans exposed to AIAs by ingestion of meat prepared under normal household conditions. The development of such biomarkers will be useful in the estimation of individual human cancer risk associated with AIA ingestion.

I wish to thank all of the collaborators for a fruitful corporation.
From the Institute of Toxicology, Danish National Food Agency:
Dr. Preben A. Nielsen
Dr. Eva S. Rasmussen
Dr. Lars O. Dragsted
Dr. John C. Larsen

SAMMENDRAG

Ved stegning af kød og fisk dannes adskillige karcinogene heterocykliske aromatiske aminer. De vigtigste faktorer for dannelse af disse stoffer er kød typen, stegetemperaturen og varigheden. Ved almindelige stegebetingelser er aminoimidazoazaarenerne (AIA), som er kondensations produkter af aminosyrer og kreatinin, de vigtigste forbindelser. Både på grund af mængden de dannes i og den mulige biologiske effekt.

AIA forbindelserne er genotoksiske i mange test systemer som *Salmonella typhimurium* og mammale celler. I langtids studier i gnavere er IQ, 4-MeIQ, 8-MeIQx og PhIP vist at medføre kræft i flere organer.

Disse prokarcinogener kræver metabolisk aktivering for at udvise genotoksisk effekt. Aktiveringen er vist at ske ved CYP1A katalyseret hydroxylering af den exocykliske aminogruppe til et N^2-OH derivat. Disse derivater bindes i nogen grad kovalent til DNA og andre makromolekyler. En yderligere aktivering af N^2-OH gruppen kan ske ved esterifikation katalyseret af acetyltransferase eller sulfotransferase, hvorved der dannes meget reaktive derivater, som danner addukter med DNA og 2'-deoxyguanosin, men ikke med 2'-deoxyadenosin, 2'-deoxycytidin eller thymidin. Addukter mellem 2'-deoxyguanosin og IQ, 4-MeIQ, 8-MeIQx, 4,8-DiMeIQx og PhIP er blevet karakteriseret strukturelt.

I gnavere *in vivo* er det vist, at de vigtigste metaboliserings veje som fører til dannelse af detoksifiserings produkter, er hydroxylering af ringsystemet efterfulgt af konjugering med sulfat eller glucuronsyre. Konjugering af den exocykliske amino gruppe med sulfat eller glucuronsyre er også vigtige metaboliseringsveje.

^{32}P-Postlabeling analyse af DNA fra gnavere, som er doseret oralt med AIA, såvel som undersøgelser hvor der anvendt radioaktivt mærkede stoffer har vist at addukter med 2'-deoxyguanosin også dannes *in vivo*. Addukt niveauet stiger lineært med dosis størrelsen. Risiko extrapolering baseret på langtids studier i dyr indikerer at indtagelse af AIA forbindelser er af betydning for udvikling af kræft i mennesker.

LIST OF PAPERS

This thesis is based on the following papers. In the text they will be referred to by Roman numerals and author names.

I Frandsen, H., Rasmussen, E.S., Nielsen, P.A., Farmer, P., Dragsted, L. and Larsen, J.C. (1991). Metabolic formation, synthesis and genotoxicity of the *N*-hydroxy derivative of the food mutagen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP). *Mutagenesis*, **6**, 93-98.

II Frandsen, H., Grivas, S., Andersson, R., Dragsted, L. and Larsen, J.C. (1992) Reaction of the N^2-acetoxy derivative of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) with 2'-deoxyguanosine and DNA. Synthesis and identification of N^2-(2'-deoxyguanosin-8-yl)-PhIP. *Carcinogenesis*, **13**, 629-635.

III Frandsen, H., Nielsen, P.A., Grivas, S. and Larsen, J.C. (1994a) Microsomal metabolism of the food mutagen 2-amino-3,4,8-trimethyl-3*H*-imidazo[4,5-*f*]quinoxaline to mutagenic metabolites. *Mutagenesis*, **9**, 59-65.

IV Frandsen, H., Grivas, S., Turesky, R.J., Andersson, R., Dragsted, L.O. and Larsen, J.C. (1994b) Formation of DNA adducts by the food mutagen 2-amino-3,4,8-trimethyl-3*H*-imidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx) *in vitro* and *in vivo*. Identification of a N^2-(2'-deoxyguanosin-8-yl)-4,8-DiMeIQx adduct. *Carcinogenesis*, **15**, 2553-2558.

Papers relating to aminoimidazoazaarene research not included in this thesis.

Stevnsner, T., Frandsen, H. and Autrup, H. (1995) Repair of DNA lesions induced by ultraviolet irradiation and aromatic amines in normal and repair-deficient human lymphoblastoid cell lines. *Carcinogenesis*, **16**, 2855-2858.

Dragsted, L.O., Grivas, S., Frandsen, H. and Larsen, J.C. (1995) Antibodies to the food

mutagens, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine and 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline: useful for immunoassay and immunoaffinity chromatography of biological samples. *Carcinogenesis*, **16**, 2795-2806.

Dragsted, L.O., Frandsen, H., Reistad, R., Alexander, J. and Larsen, J.C. (1995) DNA-binding and disposition of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in the rat. *Carcinogenesis*, **16**, 2785-2793.

Reistad R., Frandsen H., Grivas S. and Alexander J. (1994) *In vitro* formation and degradation of 2-amino-1-methyl-6-phenylimidazo(4,5-*b*)pyridine (PhIP) protein adducts. *Carcinogenesis*, **15**, 2547-2552.

Dragsted L. O., Alexander J., Wallin H., Frandsen H., and Vang O. (1993) Bioactivation of 2-amino-1-methyl-6-phenylimidazo(4,5-*b*)pyridine by liver microsomes from three different rat strains. *Pharmacology and Toxicology*, **72**, 388-393.

Larsen J.C., Dragsted L.O., Frandsen, H., Kristiansen E., Rasmussen E.S., Nielsen P.A. and Knudsen I. (1990) Carcinogenicity of mutagens from cooked meats. In: Pariza M.W., Aeschbacher H., Felton J.S. and Sato S.(eds.), Mutagens and carcinogens in the diet, Progress in Clinical and Biological Research, Wiley-Liss Inc., New York, 347, 89-108.

ABBREVIATIONS

AαC	2-amino-9*H*-pyrido[2,3-*b*]indole
acetyl-CoA	acetyl coenzyme A
AIA	aminoimidazoazaarene
ATP	adenosine-5'-triphosphate
CYP	cytochrome P-450
DiMeIQx or	
4,8-DiMeIQx	2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline
4,7-DiMeIQx	2-amino-3,4,7-trimethylimidazo[4,5-*f*]quinoxaline
5,7-DiMeIQx	2-amino-3,5,7-trimethylimidazo[4,5-*f*]quinoxaline
5,8-DiMeIQx	2-amino-3,5,8-trimethylimidazo[4,5-*f*]quinoxaline
dG	deoxyguanosine
dG-C8-IQ	N^2-(deoxyguanosin-8-yl)-IQ
dG-N^2-IQ	5-(deoxyguanosin-N^2-yl)-IQ
dG-C8-MeIQx	N^2-(deoxyguanosin-8-yl)-8-MeIQx
dG-N^2-MeIQx	5-(deoxyguanosin-N^2-yl)-8-MeIQx
dG-C8-DiMeIQx	N^2-(deoxyguanosin-8-yl)-4,8-DiMeIQx
dG-C8-PhIP	N^2-(deoxyguanosin-8-yl)-PhIP
DNA	deoxyribonucleic acid
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
IQ	2-amino-3-methylimidazo[4,5-*f*]quinoline
IQx	2-amino-3-methylimidazo[4,5-*f*]quinoxaline
MeAαC	2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole
MeIQ or	
4-MeIQ	2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline
5-MeIQ	2-amino-3,5-dimethylimidazo[4,5-*f*]quinoline
4-MeIQx	2-amino-3,4-dimethylimidazo[4,5-*f*]quinoxaline
7-MeIQx	2-amino-3,7-dimethylimidazo[4,5-*f*]quinoxaline
MeIQx or	
8-MeIQx	2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline

NAT1	N-acetyltransferase type 1
NAT2	N-acetyltransferase type 2
2-nitro-PhIP	1-methyl-2-nitro-6-phenylimidazo[4,5-*b*]pyridine
N^2-OH-IQ	2-hydroxyamino-3-methylimidazo[4,5-*f*]quinoline
N^2-OH-MeIQx	2-hydroxyamino-3,8-dimethylimidazo[4,5-*f*]quinoxaline
N^2-OH-PhIP	2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine
5-OH-IQ	2-amino-5-hydroxy-3-methylimidazo[4,5-*f*]quinoline
4'-OH-PhIP	2-amino-1-methyl-6-(4-hydroxy-phenyl)imidazo[4,5-*b*]pyridine
7-oxo-MeIQx	2-amino-3,6-dihydro-3,8-dimethyl[4,5-*f*]quinoxaline-7-one
PAPS	3'-phosphoadenosine-5'-phosphosulphate
PCB	aroclor 1254
pdGp-C8-MeIQ	N^2-(deoxyguanosin-8-yl)4-MeIQ-3',5'-diphosphate
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine
RNA	ribonucleic acid
Trp-P-1	3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole
Trp-P-2	3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole

INTRODUCTION

Chemical Carcinogenesis

During the past two centuries it has been recognized that humans occupationally exposed to mixtures of chemical compounds, in particular tars from combustion, were at high risk of developing cancer. In the beginning of the 20th century the development of cancer in experimental animals was reported following repeated application of coal tar onto rabbit skin (reviewed by Miller, 1994 and Pitot, 1990). After the introduction of animal models in experimental carcinogenesis Kennaway and Heiger in 1930 identified the first pure chemicals, dibenz[*a,f*]anthracene and dibenz[*a,h*]anthracene, that could induce cancer (Kennaway and Heiger,1930).
Since then, several chemicals including polycyclic aromatic hydrocarbons, aromatic amines, *N*-nitroso-compounds, alkylating agents and several naturally occurring compounds have been shown to be carcinogens. These different chemical classes of carcinogens do not seem to share any structural or chemical similarities. However, in 1961 the Millers recognized that a metabolite of N-acetylaminofluorene was a more potent carcinogen than the parent compound. This indicated that chemical carcinogens were activated to proximate carcinogens by enzymatic processes taking place in the cell (Miller and Miller, 1961). Indications that metabolism was involved in the activation of carcinogens was also obtained from observations that carcinogens were covalently bound to macromolecules in animal tissue. In 1964, Brooks and Lawley discovered, by use of a radioactively labelled carcinogen, that DNA was the target molecule for reactive metabolites and they found a correlation between carcinogenic potency and DNA binding (Brookes and Lawley, 1964). Many of the procarcinogens are lipophilic substances which are subject to modifications by phase I and/or phase II enzymes in order to make them more hydrophillic to facilitate excretion via urine or faeces. For example, alkyl groups, aromatic rings and amino groups may be hydroxylated; nitro groups and azo groups may be reduced; phenolic groups, alcohols and amino groups may be conjugated with glucuronic acid, acetate, phosphate or sulphate.
These enzymatic processes usually result in formation of detoxified, water soluble metabolites which are readily excreted. However, some of these enzymatic modifications may result in metabolites that are more potent carcinogens than the parent compound.

Often more than one enzymatic activation reaction is necessary for the formation of ultimate carcinogenic metabolites, although, some carcinogens exist in their ultimate form (e.g. alkylating reagent) and do not need metabolic activation (Miller and Miller, 1966; Dipple, 1988).

The ultimate carcinogenic metabolites of the various classes of chemical carcinogens have one common feature. They are reactive electrophiles, that can react with cellular macromolecules. Especially, the reaction of an electrophilic metabolite with nucleophilic positions on the DNA bases resulting in the formation of DNA adducts seems to be a critical step in the development of cancer.

It is now realised that the development of cancer is a multistage process where several steps are involved. The process has been divided into four stages: initiation, promotion, conversion and progression, which each are thought to contain several different events (reviewed by Harris, 1991). The first stage, initiation, involves exposure to a carcinogen, formation of DNA adducts that result in a mutation leading to an initiated cell. During the second stage, promotion, the initiated cell starts to proliferate due to decreased responsiveness to regulatory growth factors. In the third stage, conversion, growing initiated cells are susceptible to genetic damage by chemical carcinogens resulting in conversion to a malignant cell. In the fourth stage, progression, genetic changes in the cells result in increased growth rate, the ability to invasive growth and the ability to metastasize.

Diet and cancer

Dietary constituents are now considered important factors both in the causation and in the prevention of important diseases, including cancer (Willett, 1994, Rogers et al., 1993). The diet has been estimated to account for 10% of cancers in some organs and up to 90% of cancers in other organs. A mean estimate of 35% has been suggested for the dietary importance on the cancer rate in the U.S. population (Doll and Peto, 1981). An association between intake of animal fat and cancers in the breast, pancreas and colon has been reported. However, in some epidemiological studies colon cancer seems to correlate stronger with intake of red meat and with preference for heavily browned meat surface. (Giovannucci, 1994; Gerhardson de Verdier, 1991, Willett et al., 1990)

Suggested mechanisms for these associations include: (1) Increased tumour incidence due to excessive calorie intake. A lower tumour incidence is observed in rodents on a calorie-restricted diet compared to controls. (2) Proliferative effects of bile acids on the colonic mucosa. (3) Increased iron absorption due to meat ingestion. Iron contributes to the production of reactive oxygen species that can damage cellular macromolecules e.g. DNA. (4) Formation of carcinogenic chemicals such as *N*-nitrosamines, heterocyclic aromatic amines and polycyclic aromatic hydrocarbons during cooking (reviewed by Ames et al., 1995).

The formation of carcinogenic substances in fried meat was for the first time reported in 1939 by Widmark, who had found that ethanol or petroleum ether extracts of horse meat, heat treated at a temperature of 275 °C resulted in the formation of mammary tumours, when painted on female mice in the occipital region (Widmark, 1939). In the 1970ies Sugimura and coworkers used the newly developed *Salmonella* mutagenicity assay (Ames et al., 1973) to look for mutagenic substances in grilled or broiled fish and meat (Sugimura, 1992). This led to the discovery of a series of mutagenic compounds identified as heterocyclic aromatic amines.

Heterocyclic aromatic amines in food.

Formation and occurrence.

Since it was first discovered that the charred surface of fish and meat contained mutagenic activity corresponding to that of 130-450 µg benzo[a]pyrene/ 100 g of meat (Nagao et al., 1977), a large number of mutagenic compounds has been isolated from heat processed proteins and foods and structurally characterized. The first class of mutagens that was structurally characterized was the pyrolysate mutagens which are formed during treatment of amino acids or proteins at very high temperatures. Examples of structures of the pyrolysate mutagens are shown in Figure 1. Later a second, more abundant class of mutagens, the aminoimidazoazaarenes (AIAs), was structurally characterized. The AIAs are formed during ordinary cooking conditions and are characterized by one or two heterocyclic rings fused to an aminoimidazo ring (Felton, 1986a). Figure 2 shows the structures of IQ (Kasia et al., 1980a); 4-MeIQ, (Kasia et al., 1980b); 8-MeIQx, (Kasia et

al., 1981); 4,8-DiMeIQx, (Felton et al., 1984) and PhIP (Felton et al., 1986a). The AIAs

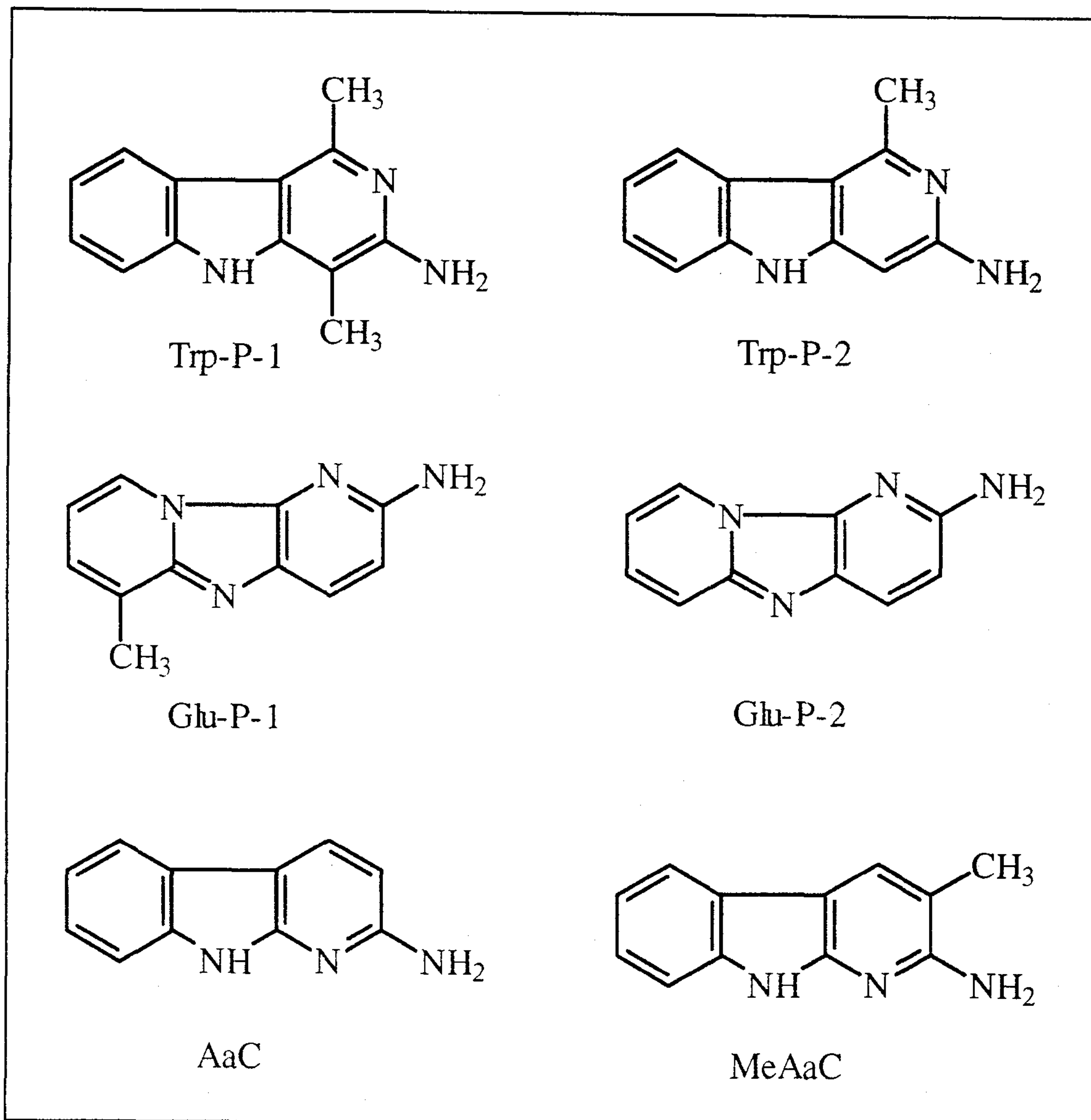

Fig. 1 Structures of selected pyrolysate mutagens

are found in higher concentrations compared to the pyrolysate mutagens when cooking is carried out under normal household conditions. Therefore, the following text will mainly concentrate on the AIAs.

The AIAs that until now have been characterized are subdivided into three structurally different groups; the quinolines, the quinoxalines and the pyridines (Figure 2). AIAs have been detected in a series of cooked foods including fish, beef, chicken, lamb and pork. The

formation of mutagenic substances does not seem to differ between the various sorts of meat, but seems to be qualitatively and quantitatively comparable under similar cooking conditions. Cooking time and cooking temperature are the most important factors that determine the amount of mutagens formed (Skog, 1993).

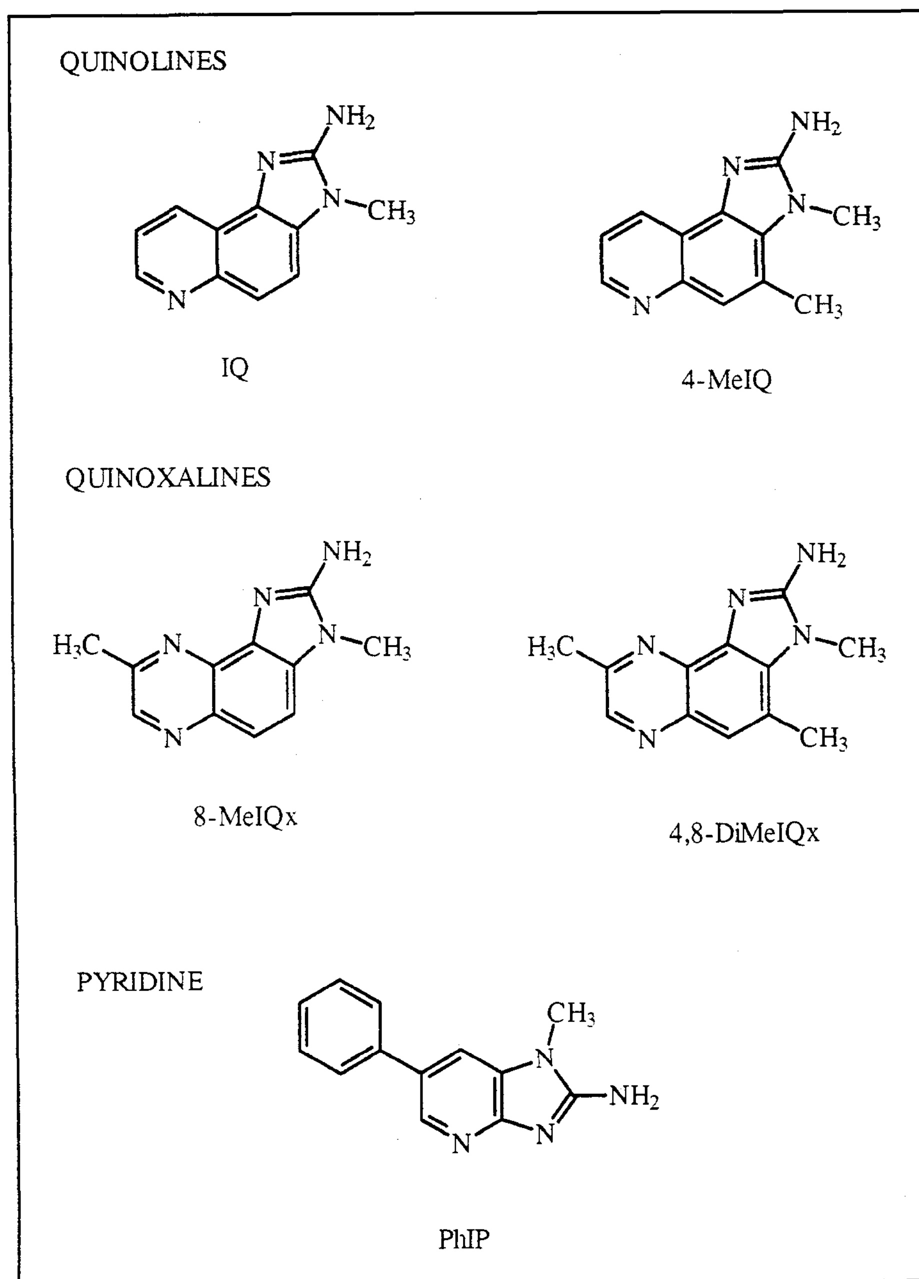

Fig. 2 Structures of selected aminomidazoazaarene mutagens.

During 10 min an almost linear increase was observed in the amount of mutagenicity

formed in beef patties fried at 150, 190 and 230 °C, respectively (Knize, 1994). Several investigation have shown that the mutagenicity in fried meat increases with increasing frying temperature (reviewed by Skog, 1993). For each 50 °C rise in frying temperature a doubling of the mutagenic activity has been detected in pork (Nielsen et al., 1988). Increasing frying temperature also resulted in a different distribution among the different AIAs formed. At low frying temperatures, up to 200 °C, mainly the more polar AIAs, IQ and MeIQx are formed. At temperatures above 200 °C at shift towards more apolar AIAs, especially PhIP takes place (Skog,1995, Felton et al., 1986b).

Table I. Concentrations of MeIQx, DiMeIQx and PhIP in meat samples and pan residues calculated as ng/g cooked product.

Sample/ Cooking temperature (°C)	MeIQx		DiMeIQx		PhIP	
	meat	residue	meat	residue	meat	residue
Pork chop/						
150	ND	ND	ND	ND	ND	0.02
175	0.2	0.8	0.04	0.2	0.02	0.8
200	0.2	1.9	0.05	0.3	0.02	2.4
225	2.6	1.8	1.1	0.5	4.8	3.8
Bacon/						
150	ND	ND	0.2	ND	0.3	0.06
175	0.1	ND	0.2	ND	0.2	0.1
200	0.7	ND	0.3	ND	0.6	0.8
225	23.7	0.9	1.4	ND	4.5	0.3
Minute steak/						
150	ND	0.1	ND	0.1	0.02	0.2
175	0.2	1.1	0.06	ND	0.03	1.0
200	0.6	2.9	0.1	0.4	0.3	0.9
225	6.2	23.3	2.7	4.1	12.7	82.4
Sirloin steak/						
150	0.02	0.07	0.02	ND	0.06	0.1
175	0.2	0.8	0.2	ND	0.02	0.7
200	0.7	3.3	0.3	ND	1.2	6.3
225	1.6	2.6	0.6	ND	1.8	5.3
Ground beef/						
150	ND	0.06	ND	0.02	0.01	0.08
175	0.2	1.1	0.02	0.2	0.04	0.5
200	1.2	5.8	0.4	1.1	0.5	7.2
225	2.2	3.3	0.8	0.7	1.1	11.2

ND, not detected.
Data from Skog et al., 1995.

AIAs are found not only in the surface of the fried meat, often the pan residue contains as high or higher levels. Examples of the content of MeIQx, DiMeIQx and PhIP in different meat samples and residues fried at different temperatures are shown in Table I. The important precursors for the formation of AIAs in foods are creatinine or creatine, amino acids and carbohydrate, all compounds that are abundant in meat. Formation of AIAs have been modelled by heating mixtures of creatinine and an amino acid or creatinine, an amino acid and a carbohydrate; IQ has been formed from creatinine/proline (Yoshida, 1984), 8-MeIQx from creatinine/glycine/glucose, (Jagerstadt et al., 1984); 4,8-DiMeIQx from creatinine/alanine/fructose (Grivas et al., 1985) and PhIP from creatinine/phenylalanine/glucose (Taylor et al., 1987).
Microwave treatment of meat prior to cooking reduced the mutagenicity of cooked meat. Microwave treatment for 1 min leaves only 38% of the mutagenicity compared to controls, 2 min leaves 10% and 3 min leaves 6%. It was suggested that the juice that leaves the meat during the microwave treatment contains the precursors for the mutagen formation (Felton et al., 1992).
AIA mutagens and pyrolysate mutagens have also been identified in non meat sources, although in lower concentrations. MeIQ has been found in roasted coffee beans; AαC, MeAαC, Glu-P-1, Glu-P-2, Trp-P-1, Trp-P-2 have been found in cigarette smoke condensates, airborne particles or diesel exhaust (Kikugawa et al., 1989; Manabe et al., 1990; Kanai et al., 1990; Manabe et al., 1989; Manabe et al., 1991). PhIP related DNA adducts has been detected in urine from smokers of black tobacco. (Peluso et al., 1991). PhIP has also been detected in beer and wine (Manabe et al., 1993)

Mutagenicity and Carcinogenicity

The AIAs are promutagens and require the presence of a metabolic activation system to show mutagenic activity in the Ames *Salmonella* assay. Two strains of *Salmonella* have usually been used in the detection of mutagenic activity, TA98 and TA1538. Table II lists the mutagenic potency of some of the AIAs found in cooked food together with some structurally related compounds which have been produced in the search of structural proof of the AIAs found in food. It can be seen that some of the AIAs are very potent bacterial

mutagens, others are quite weak and that small structural changes have large effects on the mutagenic potency (Felton and Knize, 1990).

Studies on quantitative structure activity relationship has resolved some of the structural elements that are of importance for the mutagenic potency. In the IQ and IQx type of mutagens higher mutagenic potency as compared to e.g. PhIP is related to the presence of Ring 3 with double bonds in conjugation with Ring 2 (Figure 3) and with the number and position of nitrogen atoms in Ring 3, e.g. 4-MeIQx is more mutagenic than 4-MeIQ (Hatch et al. 1991, Vikse et al., 1993a)(For comparison see Figure 2).

Table II. Mutagenicity of AIA compounds in *Salmonella typhimurium* strain TA98 and TA1538 expressed as revertants/µg.

Compound	TA98	TA1538
IQ	433.000	200.000
4-MeIQ	660.000	750.000
5-MeIQ	142.000	NT
4-MeIQx	NT	875.000
7-MeIQx	233.000	528.000
8-MeIQx	110.000	93.300
4,8-DiMeIQx	206.000	320.000
4,7-DiMeIQx	351.000	38.700
5,8-DiMeIQx	74.000	3.100
5,7-DiMeIQx	243.000	NT
PhIP	2000	NT

NT, not tested
Data from: Felton and Knize. 1990 and Felton et al., 1988.

Also the presence and position of methyl groups has a strong effect on the mutagenic potency. A methyl group in position 4 of IQx results in a higher mutagenic potency than a methyl in position 5 and the addition of a methyl group in position 7 of 4-MeIQx results in a higher mutagenic potency than addition of a methyl group in position 8 (Vikse et al., 1993b)(Consult Fig. 2).

The proposed ultimate mutagenic metabolite of the AIAs is a nitrenium ion generated after O-esterification of the proximate mutagenic N^2-hydroxylated metabolite (Figure 3). The presence of a long conjugated system of double bonds in the AIAs, that is able to delocalize the charge on the nitrenium ion will stabilize the ion and increase the probability that it will reach DNA before reacting with other cellular nucleophiles (Dipple et al., 1968). Recently, molecular orbital calculations on nitrenium ion stabilities of a series

of AIAs and pyrolysate mutagens have shown good correlation between mutagenic potency and nitrenium ion stability (Ford and Griffin, 1992).

In the Ames *Salmonella* assay the metabolic activation to the proximate mutagenic metabolite takes place outside the bacteria, and it has been proposed that the methyl groups in position 3 and 4 will provide a hydrophobic region that facilitates membrane transport. Also, methyl groups in certain positions will result in a different metabolic pattern by e.g. blocking a detoxification reaction and thereby increasing the mutagenic potency.

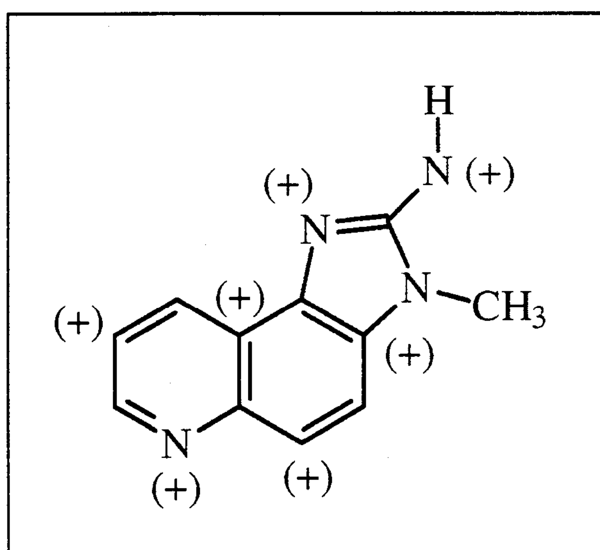

Fig. 3 Pattern of resonance delocalization of the positive charge of a nitrenium ion of IQ through the π electron system, Hatch et al., 1991.

The AIAs have been tested for genotoxicity in a large number of mammalian systems both *in vitro* and *in vivo* with both positive and negative results, possibly as a result of use of different protocols, different cell types, different metabolic activation system or none, and different doses (reviewed by Munro et al.,1993). It does, however, seem that the potency of the AIAs is reversed as compared to the potency in the *Salmonella* assay, with PhIP being more potent in inducing e.g. sister chromatid exchanges than MeIQ and MeIQx (Thompson et al., 1987).

Studies on carcinogenicity after oral treatment have been conducted with IQ, 4-MeIQ, 8-MeIQx and PhIP in mice and rats. All were found to be carcinogenic in various organs in rodents. A summary of selected oral carcinogenicity studies is shown in Table III.

Carcinogenicity studies in monkeys with oral dosing of IQ, MeIQx and PhIP are still ongoing. However, already after one-seventh of the expected life span of the monkeys IQ which was the first compound tested, turned out to be a liver carcinogen (Adamson et al., 1994; Table III).

Table III. Summary of carcinogenicity studies with AIA compounds.

AIA	Animal Species	Dose mg/kg/d	Number of animals with tumours	Most sensitive tissue
IQ	CDF_1 mouse	M 35.5 F 31	M 27/39 F 27/36	lung liver
	F344 rat	M 12 F 8.6	M 36/40 F 27/40	Zymbal gland zymbal gland
	SD rat	F 69.3	14/32	mammary gland
	monkey	M/F 10 M/F 20	11/20 19/20	liver liver
MeIQ	CDF_1 mouse	35	M 12/15 F 16/22	forestomach forestomach
	F344 rat	M 16 F 10.9	M 19/20 F 17/20	Zymbal gland Zymbal gland
MeIQx	CDF_1 mouse	M 77 F 70	M 15/37 F 32/35	liver liver
	F344 rat	M 19.4 F 13.1	M 20/20 12/19	liver clitoral gland
PhIP	CDF_1 mouse	M 86.7 F 53.3	M 11/35 F 26/38	lymphoma lymphoma
	F344 rat	20	M 16/29 F 14/30	colon mammary gland

Data from Munro et al., 1993 and Adamson et al., 1994.

Metabolism

The Quinolines: 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) and 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ)

In vitro studies have indicated that IQ and MeIQ are activated to mutagenic metabolites by microsomes or S9 from various species including mouse, rat, rabbit, monkey and man (Yamazoe et al., 1983, Aune et al., 1986, Alldrick et al., 1985, Turesky et al., 1991a, Davies et al., 1993). Microsomes from monkeys, however, had a low capacity for metabolic activation of IQ. IQ and MeIQ induces unscheduled DNA synthesis in hepatocytes from PCB treated rats, and sister chromatid exchanges in V79 cells when co-cultured with hepatocytes (Brunborg et al., 1988) The structure of the proximate mutagenic metabolite of IQ has been determined as the 2-hydroxyamino derivative, N^2-OH-IQ (Yamazoe et al., 1983). By transfection of cells with murine or human P450 isozymes, it has been shown that the activation of IQ and MeIQ to the mutagenic metabolite is catalyzed by cytochrome P450 1A2 (CYP1A2). CYP1A1 only showed very low capacity to activate IQ and MeIQ (Snyderwine and Battula, 1989, McManus et al., 1990). Prostaglandin H synthase was also able to activate IQ by oxidation of the exocyclic amino group to a nitro group (Morrison et al., 1993, Wild and Degen, 1987).

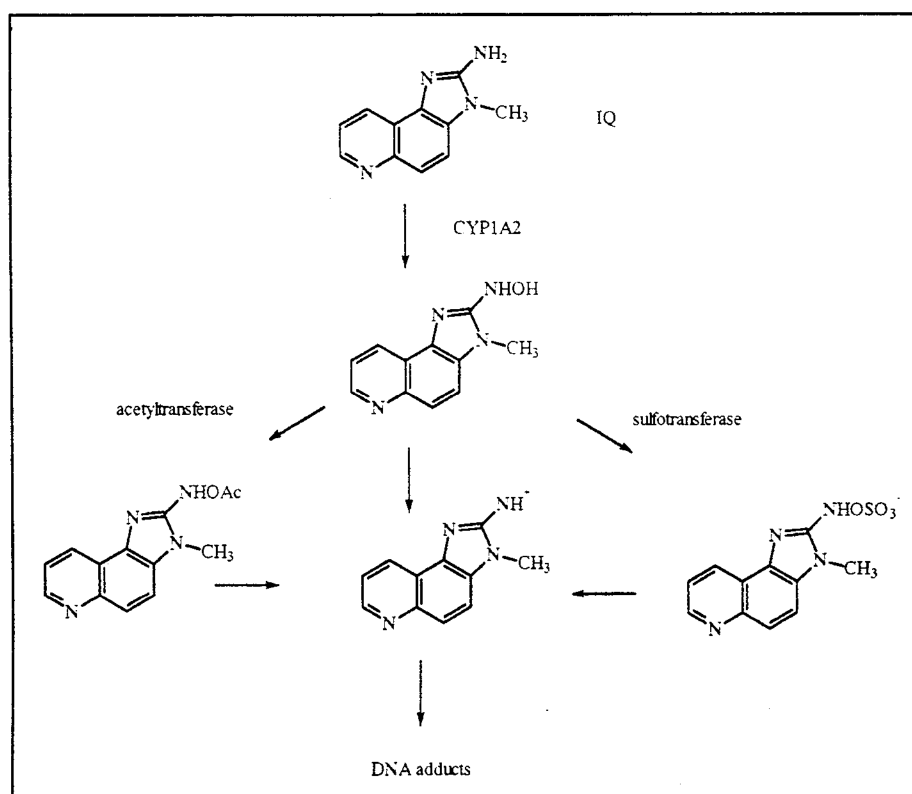

Fig. 4 Pathways for metabolic activation of IQ. Snyderwine et al., 1992.

N^2-OH-IQ needs further activation by esterification of the hydroxylamino group to exert its genotoxic activity, Figure 4. Incubation of N^2-OH-IQ with rat hepatic cytosol and pentachlorophenol, an inhibitor of both O-acetyltransferase and sulphotransferase, resulted in reduced mutagenicity in *Salmonella* TA98. Incubation with 2,6-dichloro-4-nitrophenol, a more specific inhibitor of sulphotransferase, did not result in reduced mutagenicity. This indicates that N^2-OH-IQ is a substrate for rat acetyltransferase but not for sulphotransferase (Snyderwine et al., 1988a). Activation of N^2-OH-IQ with cytosol from various organs from rats and monkeys has shown that there is a large interspecies and interorgan variation in the ability to generate DNA binding metabolites by acetyl-CoA, PAPS, L-Proline and ATP- dependant activating enzymes (Davis et al., 1993). Studies with human transferases, expressed in *Salmonella* bacteria or by use of human liver cytosol, have shown that N^2-OH-IQ not is a substrate for human sulphotransferase, a poor substrate for acetyltransferase isozyme NAT1, but is a substrate for human acetyltransferase isozyme NAT2 (Turesky et al., 1991a, Chou et al., 1995, Wild et al., 1995).

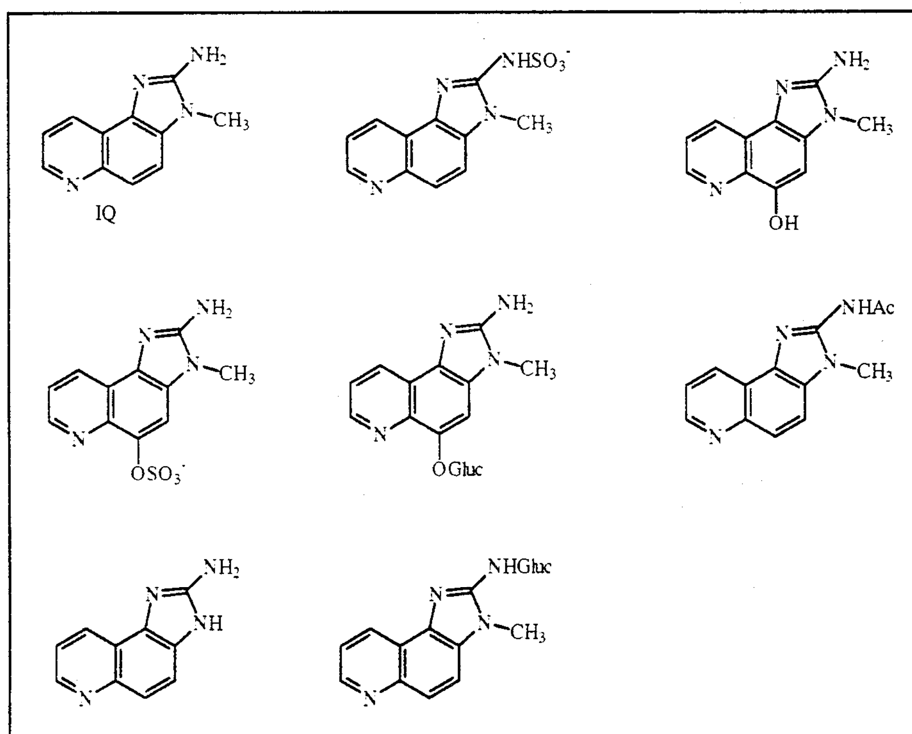

Fig. 5 Structures of metabolites of IQ identified in rodents.
Gluc = Glucuronic acid.

In vivo studies in rats have shown that IQ and MeIQ are readily absorbed and excreted. Within 24 hours after dosing with radiolabelled compounds more than 90% of the radioactivity had left the animals, 45-65% via the urine and 37-49% via the faeces (Sjodin et al., 1984, Inamasu et al., 1989). The major water soluble metabolites of IQ were identified as the N^2-sulfamate and the O-sulphate and glucuronide conjugates of a 5-hydroxylated metabolite. Minor metabolites were 5-OH-IQ, N^2-acetylated IQ, IQ-*N*-glucuronide and demethylated IQ (Inamasu et al., 1989, Luks et al., 1989, Størmer et al., 1987, Alexander et al., 1989, Turesky et al., 1986), Figure 5.
The water soluble metabolites of MeIQ were primarily the O-sulphate and glucuronide conjugates of the 5-hydroxylated metabolite. The N^2-sulfamate of MeIQ was much less abundant than the N^2-sulfamate of IQ. A minor nonpolar metabolite of MeIQ was identified as the N^2-acetyl derivative (Alexander et al., 1989a).

The Quinoxalines: 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) and 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (DiMeIQx).

In vitro studies have shown that microsomes from rats, humans and monkeys can metabolically activate MeIQx to mutagenic metabolites. Microsomes from monkeys showed the lowest capacity to activate MeIQx (Davis et al., 1993a). The proximate mutagenic metabolite from activation with rat hepatocytes and with human microsomes was isolated and identified as the N^2-hydroxylated derivative (Turesky et al., 1990, Rich et al., 1992, Turesky et al., 1991a). Human CYP1A2 was estimated to account for at least 66% to more than 90% of the phase I metabolism of MeIQx through N^2-hydroxylation (McManus et al., 1990, Rich et al., 1992). Murine CYP1A2 has also been shown to activate MeIQx, whereas CYP1A1 showed no activity (Snyderwine et al., 1989).
The phase II activation of N^2-OH-MeIQx was studied by use of cytosol from various organs from rats and monkeys and a large variation was observed in the ability of acetyltransferase, sulphotransferase, aminoacyl-tRNA synthetase and phosphatase to activate N^2-OH-MeIQX, depending on species and organ (Davis et al., 1993b). Studies with human transferases expressed in *Salmonella* or by use of human liver cytosol showed that N^2-OH-MeIQx not is a substrate for human sulphotransferase. Human acetyltransferase isozyme NAT1 has a low capacity to activate N^2-OH-MeIQx, whereas human

acetyltransferase isozyme NAT2 has a higher capacity for activation (Turesky et al., 1991a, Chou et al., 1995, Wild et al., 1995).

The metabolism of MeIQx was studied in hepatocytes from rats and ten metabolites were characterized. In hepatocytes from uninduced rats N^2-sulfamate formation is a major metabolic pathway, whereas ringhydroxylation followed by sulphate or glucuronide conjugation are major metabolites in rats induced with Aroclor 1254, ß-naphtoflavone or isosafrole. Formation of the mutagenic N^2-hydroxylated metabolite as well as its *N*-glucuronide was also detected. The formation of a 2-nitro derivative is indicated by the presence of a conjugate where the 2nd nitrogen atom of MeIQx was displaced by the sulphur atom of glutathione (Turesky et al., 1990, Wallin et al., 1989).

Liver microsomes from PCB induced rats metabolized 4,8-DiMeIQx to two major and three minor metabolites. One major and one minor metabolite were identified as the N^2-hydroxy and the 2-nitro derivatives of DiMeIQx, respectively. Both were mutagenic to *Salmonella typhimurium* TA98 without metabolic activation system. The other major and two minor metabolites were identified as the 8-hydroxymethyl derivative of DiMeIQx and its N^2-hydroxy and 2-nitro derivatives. The last two metabolites were mutagenic to *Salmonella typhimurium* TA98 without metabolic activation system (III, Frandsen et al., 1994a), Figure 6. Murine CYP1A2 is the predominant isoform responsible for the activation of DiMeIQx to mutagenic species, whereas CYP1A1 shows only little activation (Snyderwine et al., 1989).

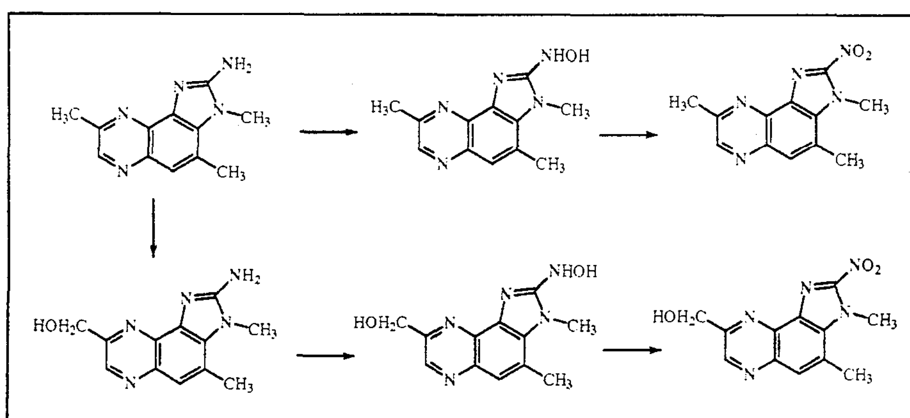

Fig. 6 Structures of metabolites of 4,8-DiMeIQx formed by hepatic microsomes from PCB induced rats. III, Frandsen et al., 1994a.

In vivo studies in rats dosed with ^{14}C labelled MeIQx showed that 36-41% of the dose is excreted with the urine during the first 24 hours, and 1-3% during the next 24 hours period. The remaining radioactivity is excreted with the faeces with less than 1% retained in the tissues after 72 hours (Sjodin et al., 1989). Several metabolites from urine, bile and faeces from rats have been isolated and characterized as: N^2-sulphamates of MeIQx, an 8-hydroxymethyl derivative together with its N^2-sulfamate and 4 (or 5) sulphate conjugate (structure uncertain), a N^2-glucuronide conjugate, sulphate and glucuronide conjugates of a 4 (or 5) hydroxylated metabolite, and N^2-acetylated and N-demethylated metabolites (Sjodin et al., 1989, Turesky et al., 1988, Hayatsu et al., 1987). Both the dose and use of enzyme inducers influenced the metabolism of MeIQx, with a higher percentage being N^2-hydroxylated after PCB induction (Turesky et al., 1991b). The metabolic profile in monkeys is quite similar to the profile in rats except for the presence of two additional metabolites, a N^1-glucuronide and 7-oxo-MeIQx. The latter was ascribed as an enteric bacterial metabolite of MeIQx (Snyderwine et al., 1995), Figure 7.

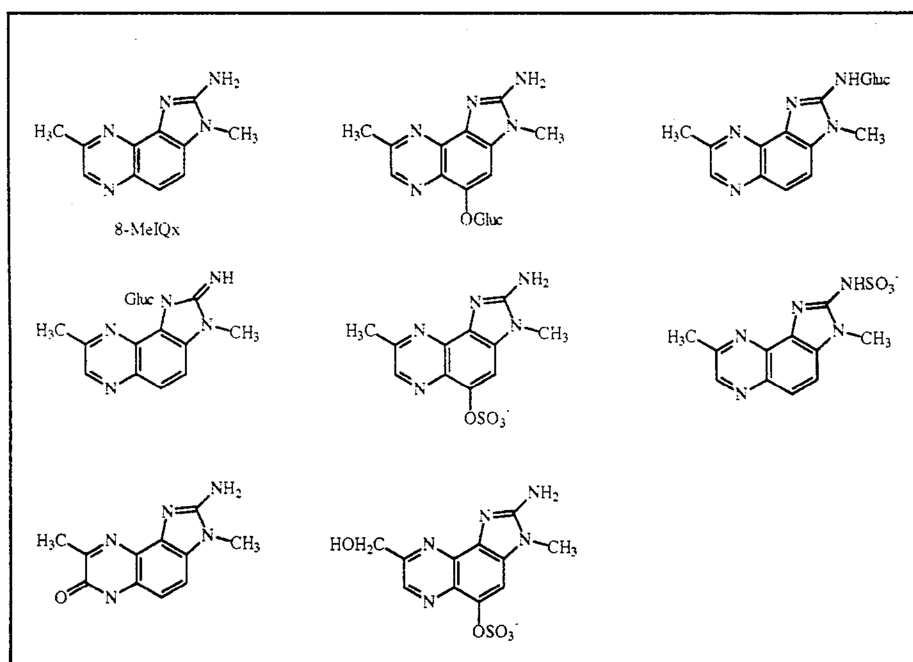

Fig. 7 Structures of MeIQx metabolites characterized in monkeys. Gluc = Glucuronic acid. Snyderwine et al., 1995.

An *in vivo* study in humans with and without the use of the CYP1A2 inhibitor furafylline

showed that CYP1A2, in humans, accounts for 91% of the metabolism of MeIQx, most likely via N^2-hydroxylation (Boobis et al., 1994).
An *in vivo* study of the metabolism of DiMeIQx in conventional and germ free rats indicated that the intestinal flora does not influence metabolism. Major metabolites were identified as 8-hydroxymethyl-DiMeIQx and its N^2-acetylated derivative (Knize et al., 1989).

The Pyridine: 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP).

Several studies have shown that PhIP is activated to a proximately mutagenic metabolite by rat hepatocytes and liver microsomes from rabbit, rat, mouse (Holme et al., 1989, McManus et al., 1989, Wallin et al., 1990, I, Frandsen et al., 1991, Buonarati et al., 1990a). The activated metabolite was identified as the N^2-hydroxylated metabolite N^2-OH-PhIP. The other major microsomal metabolite of PhIP was identified as the 4'-hydroxylated product (4'-OH-PhIP), which is a detoxification product with thousand times lower mutagenic potency than PhIP (Kurosaka et al., 1992), Figure 8. N^2-OH-PhIP has also been shown to induce sister chromatid exchanges in CHO cells and DNA strand breaks in V79 cells (I, Frandsen et al., 1991, Holme et al.,1989). Rat and mouse CYP1A2 N^2-hydroxylated PhIP at a high rate compared to other isozymes, and CYP1A1 detoxificated PhIP at a comparatively high rate (Wallin et al., 1990, McManus et al., 1990, Bounarati et al., 1991). Also human liver microsomes have a high capacity for CYP1A2 dependent activation of PhIP to N^2-OH-PhiP, however the capacity of human microsomes to detoxify PhIP to 4'-OH-PhIP is extremely low compared to rodents. The ratio of N^2-hydroxylation to 4'-hydroxylation was estimated to 97:1, 3,3:1, 1,7:1 in man, rat and mouse respectively (Davis et al., 1993a, Zhao et al., 1994, Lin et al., 1995).

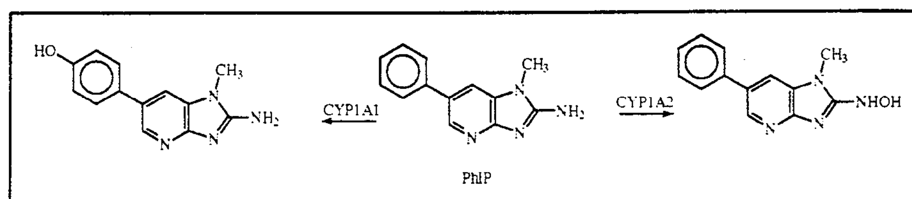

Fig. 8 Structures of microsomal metabolites of PhIP. Wallin et al., 1990.

N^2-OH-PhIP did not bind covalently to DNA, but addition of rat liver cytosol and PAPS, and to a much lesser extent acetyl-CoA, resulted in DNA adduct formation (Bounarati et al., 1990b). Human hepatic sulphotransferase activated N^2-OH-PhIP to DNA binding metabolites by a factor of 3.3 to 12.9 more efficiently than rat liver cytosol (Ozawa et al., 1994). In one study with *Salmonella typhimurium* expressing human acetyltransferases it was concluded that neither NAT2 nor NAT1 could acetylate N^2-OH-PhIP to an ultimate mutagenic metabolite (Wild et al., 1995). In other studies, however, using human microsomes and DNA binding as detection, NAT1 and to a larger extent NAT2 together with sulphotransferase and phosphotransferase were found to activate N^2-OH-PhIP to a DNA binding metabolite (Turesky et al., 1991a, Chou et al., 1995, Lin et al., 1995).

The metabolism of PhIP *in vivo* has been studied in mice, rats and monkeys. Following oral administration to mice of ^{14}C labelled PhIP, 31% of the radioactivity was recovered in the urine and 30% in the faeces after 24 hours. HPLC analysis of the urine demonstrated the presence of 11 metabolites (Turteltaub et al., 1989). In a study using doses equivalent to human dietary intakes, almost 100% of the dose was excreted during 96 hours, 90% in urine and 10% in faeces. Only 0.01 - 0.04% of the dose could be detected in the tissues 48 to 96 after exposure (Turteltaub et al., 1992). In the rat faeces is the major route of excretion, accounting for 78% of the dose after 24 hours, 51% was identified as unmetabolized PhIP (Watkins et al., 1991a). Several of the urinary and faecal metabolites have been isolated and characterized. 4'-PhIP sulphate and an *N*-glucuronide of N^2-OH-PhIP are the major urinary metabolites (Alexander et al., 1889b, Alexander et al., 1991, Watkins et al., 1991b, Bounarati et al., 1992), Figure 9. One minor metabolite was identified as a conjugate of glutathione, where the sulphur atom of glutathione is attached directly to the imidazole ring in position 2. The presence of this conjugate indicates that PhIP has been oxidized to 2-nitro-PhIP, as 2-nitro-PhIP has the ability to react with the thiol groups of glutathione and rat serum albumin (Alexander et al., 1991, Reistad et al., 1994). A study in monkeys demonstrated the presence of 4 metabolites in urine, PhIP-4'-glucuronide, PhIP-4'-sulphate, 4'-hydroxy-PhIP and an *N*-glucuronide of N^2-OH-PhIP. In faeces 4'-hydroxy-PhIP and PhIP were found. PhIP-4'-sulphate was the major urinary metabolite accounting for 64-72% of the dose (Snyderwine et al., 1993a).
An recent *in vivo* study in humans with and without the use of the CYP1A2 inhibitor

furafylline showed that CYP1A2 accounts for 70% of the metabolism of PhIP in humans, most likely via N^2-hydroxylation (Boobis et al., 1994).

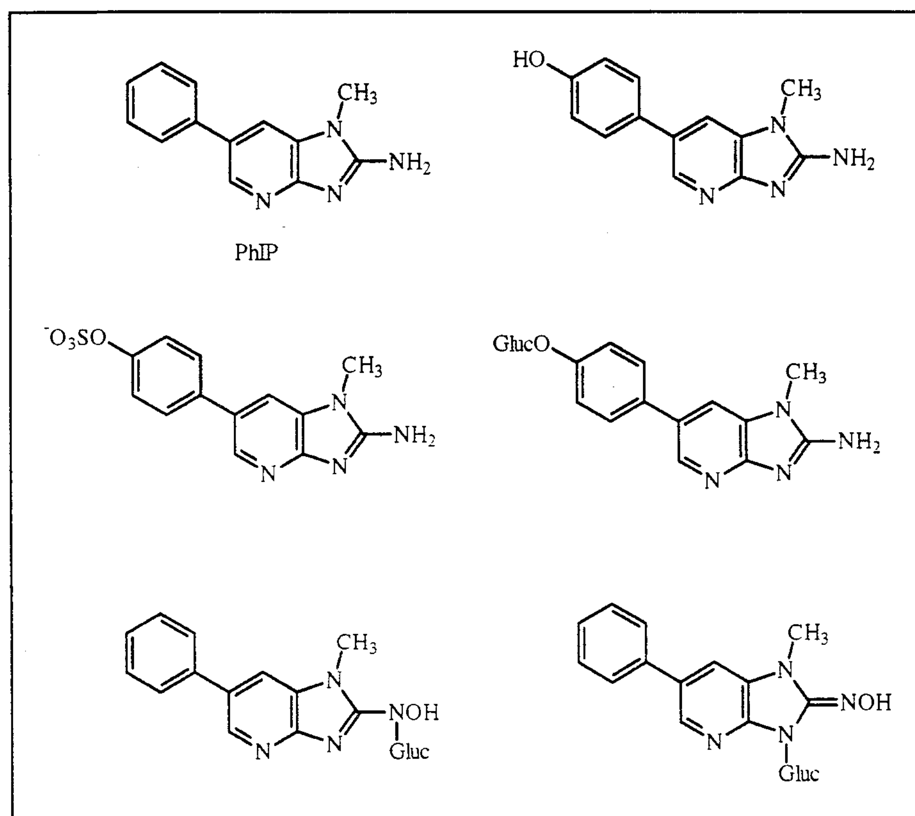

Fig. 9 Structures of metabolites of PhIP identified in rat urine. Gluc = Glucuronic acid. Kaderlik et al., 1994.

DNA adduct formation

Adduct formation of N^2-hydroxylated metabolites of AIAs with deoxynucleosides and DNA *in vitro* has been studied for a number of compounds including IQ, MeIQ, MeIQx, DiMeIQx and PhIP.

N^2-OH-IQ to a small extent binds covalently to calf thymus DNA at neutral pH. By reducing pH to 5 a minor increase in binding is observed. Addition of excess acetic anhydride to the reaction mixture, resulting in formation of the putative N^2-acetoxy derivative of IQ, dramatically increases DNA binding. N^2-acetoxy-IQ also reacts with

deoxyguanosine forming one major and one minor adduct. No reactions with deoxycytidine, deoxyadenosine or thymidine were observed. The major adduct was structurally characterized as N^2-(deoxyguanosin-8-yl)-IQ (dG-C8-IQ) (Snyderwine et al., 1988b, Turesky et al., 1992) and the minor adduct as 5-(deoxyguanosin-N^2-yl)-IQ (dG-N^2-IQ) (Turesky et al., 1992). The level of the dG-C8 adduct was approximately 8-10 times higher than the level of dG-N^2 adduct. HPLC analysis of DNA enzymatically digested after reaction with ^{14}C-N^2-acetoxy-IQ showed than both adducts had been formed. dG-C8-IQ accounted for 70% of bound radioactivity and dG-N^2-IQ accounted for 4% of the bound radioactivity (Turesky et al.,1992). ^{1}H-NMR spectroscopic analysis of the adducts revealed that the preferred conformation around the glycosidic bond was the anti form of the dG-N^2-IQ adduct and the syn form of the dG-C8-IQ adduct. It was suggested that this conformational difference may influence the persistence of the two adducts, as the dG-C8-IQ adduct is expected to induce greater distortion of the DNA structure than the dG-N^2-IQ adduct (Turesky et al., 1992), Figure 10.

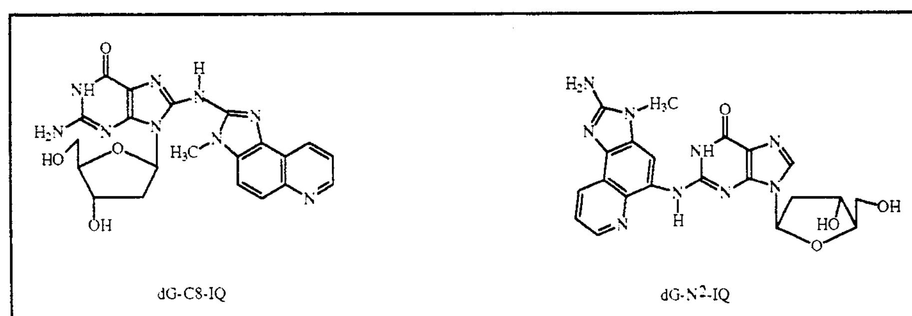

Fig. 10 Structures of DNA adducts of IQ. Turesky et al., 1992.

Adduct formation of 4-MeIQ was studied by use of the ^{32}P-postlabeling assay. N^2-OH-MeIQ was reacted with the four 2'-deoxynucleoside-3'-monophosphates in the presence of acetic anhydride and postlabeling analysis showed that only the guanine nucleotide had reacted forming one adduct. The adduct was structurally characterized as N^2-(deoxyguanosin-8-yl)-MeIQ-3',5'-diphosphate (pdGp-C8-MeIQ). Postlabeling analysis of DNA from livers of mice fed MeIQ, showed a single spot co-eluting with pdG-C8-MeIQ demonstrating that MeIQ also binds to C-8 of guanine *in vivo* (Tada et al., 1994).

Like IQ, 8-MeIQx produced one major and one minor adduct with deoxyguanosine when N^2-OH-MeIQx was reacted with deoxynucleosides or DNA in the presence of acetic anhydride. The major adduct which was formed in 8-10 times higher amounts than the minor was characterized as N^2-(deoxyguanosin-8-yl)-MeIQx (dG-C8-MeIQx). The minor adduct was characterized as 5-(deoxyguanosin-N^2-yl)-MeIQx (dG-N^2-MeIQx). ^{1}H-NMR studies revealed that dG-C8-MeIQx has acquired a syn conformation around the glycosidic bond, whereas dG-N^2-MeIQx is in the anti conformation (Turesky et al., 1992), Figure 11. In a ^{32}P-postlabeling study dG-C8-MeIQx was also detected as the major adduct formed when 3'-deoxyguanosine monophosphate reacted with N^2-OH-MeIQx in the presence of acetic anhydride. In liver DNA from rats given MeIQx intragastrically, dG-C8-MeIQx, was also the major adduct formed, together with some minor unidentified adducts (Ochiai et al., 1993).

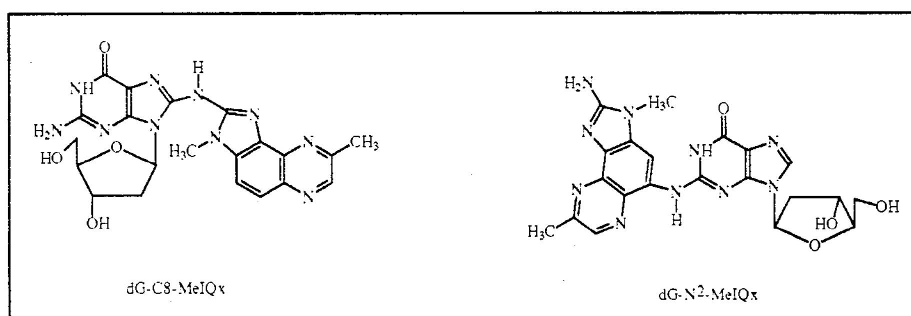

Fig. 11 Structures of DNA adducts of MeIQx. Turesky et al., 1992.

Reaction of the putative N^2-acetoxy derivative of 4,8-DiMeIQx, formed by acetylation of N^2-OH-DiMeIQx, with deoxynucleosides resulted in the formation of only one adduct. The structure was characterized as N^2-(deoxyguanosin-8-yl)-4,8-DiMeIQx (dG-C8-DiMeIQx) and NMR studies showed that the adduct has a syn conformation around the glucosidic bond, Figure 12. Absence of formation of a minor adduct of 4,8-DiMeIQx, although, structurally similar to MeIQx was ascribed to the presence of the 4-methyl group, which will sterically hinder attack of C-5 of 4,8-DiMeIQx on guanine-N^2 . N^2-acetoxy-4,8-DiMeIQx also formed dG-C8-DiMeIQx as the major adduct with calf thymus DNA, together with some minor adducts. The minor adducts had UV spectra similar to dG-C8-4,8-DiMeIQx and could be hydrolysed to dG-C8-4,8-DiMeIQx by nuclease P1, indicating

that the minor adducts was incompletely digested oligomers. *In vivo*, dG-C8-DiMeIQx was also the major adduct formed in liver DNA from rats dosed with 4,8-DiMeIQx (IV, Frandsen et al., 1994b).

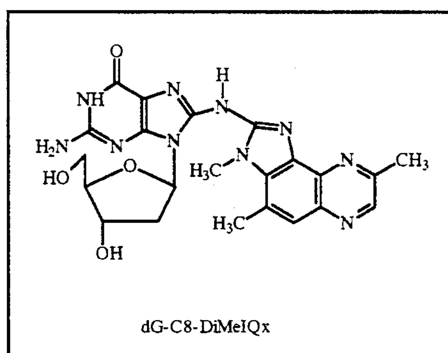

Fig. 12 Structure of the DNA adduct of 4,8-DiMeIQx. IV, Frandsen et al., 1994b.

N^2-OH-PhIP, in contrast to N^2-hydroxylated metabolites of other AIA, does not react to any extent with DNA or nucleosides. Acetylation with acetic anhydride to form the N^2-acetoxy derivative increases the reactivity. However, this compound is sufficiently stable to allow isolation and characterization (II, Frandsen et al., 1992). N^2-acetoxy-PhIP reacts with deoxyguanosine and its 3'-phosphate, but not with other deoxynucleosides, forming one adduct that has been identified as N^2-(deoxyguanosin-8-yl)-PhIP (dG-C8-PhIP) and its 3'-phosphate (II, Frandsen et al., 1992, Lin et al., 1992, Nagaoka et al., 1992), Figure 13.

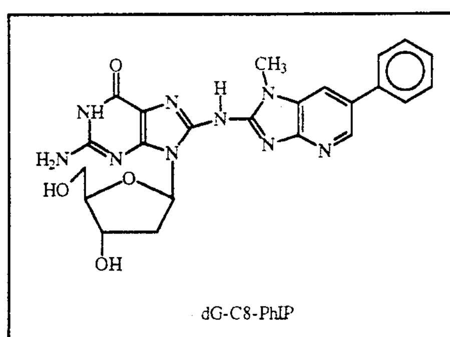

Fig. 13 Structure of the DNA adduct of PhIP. II, Frandsen et al., 1992.

N^2-acetoxy-PhIP also reacts with DNA forming an adduct that by HPLC following

enzymatic hydrolysis was identified as dG-C8-PhIP (II, Frandsen et al., 1992). ^{32}P-postlabeling analysis of DNA modified *in vitro* also showed dG-C8-PhIP as the major adduct, together with some minor adducts (Lin et al., 1992). Analysis of DNA from rats given PhIP orally revealed the presence of dG-C8-PhIP as the major adduct formed *in vivo* (II, Frandsen et al., 1992, Lin et al., 1992).

Mechanism of adduct formation

The electrophilic metabolites of the AIAs generally formed major adducts at the C-8 position of guanine, only minor adducts of a few of the AIAs are found at the N^2 position of guanine. The C-8 of guanine is only weekly nucleophilic and other alkylating or aralkylating agents tend to react with position N-7, O^6 or N^2 of guanine. It has been suggested that the formation of C-8 adducts of aromatic amines is a rearrangement product of an initially formed adduct with the more nucleophilic N-7 position of guanine. Evidence for this theory has been obtained by reacting N^2-acetoxy-2-aminofluorene with deoxyguanosine, methylated in position C-8 to prevent rearrangement.

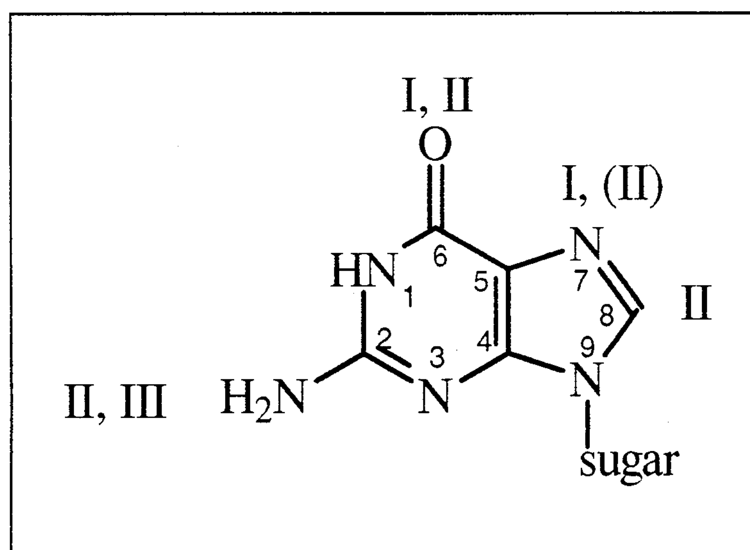

Fig. 14 Sites of substitution of 2'-deoxyguanosine. I: Sites modified by alkylating agents. II: Sites modified by arylaminating agents. III: Sites modified by aralkylating agents. Dipple, 1995.

The adduct obtained from this reaction was shown to have a structure where the N-7 position of guanine was attached to the exocyclic amino group of 2-aminofluorene (Humphreys et al., 1992). The evidence for initial adduct formation at the N-7 position of guanine, a common position for reaction with electrophilic alkylating agents, led to an attempt to make a general mechanistic explanation of the selectivity of adduct formation by alkylating, aralkylating and arylaminating ultimate electrophilic metabolites (Dipple, 1995).
Alkylating agents tend to react at the O^6 and N-7 positions of guanine, arylaminating agent form major adducts at position C-8 of guanine (rearranged from N-7) and minor adducts at position N^2 and aralkylating agents react almost exclusively at the N^2 position of guanine, Figure 14.
The theory is based on model studies of the benzylation of guanosine, and the difference in product distribution was ascribed to differences in the reaction character, S_N1 or S_N2, and ability of the developing ion to delocalize charge, Figure 15 (Moschel et al., 1979, Moschel et al., 1980, Moschel et al., 1986)). The theory suggests that reaction through a S_N2 mechanism results in formation of a N-7 guanine adduct, reaction through a S_N1 mechanism will result in O^6 adduct formation if the charge on the ionized intermediate is localized and in N^2 adduct formation if the charge is delocalized.

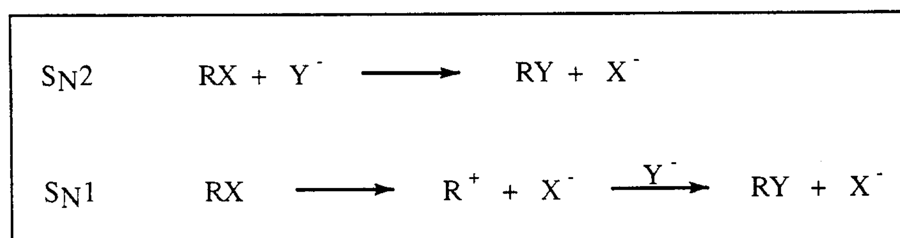

Fig. 15.

However, the evidence for the character of the reactions is limited. The theory has recently been questioned as theoretical basis was provided that some of the carbonium ions which in the above theory are assumed to react by an S_N1 mechanism can not possibly do so, because they are too unstable to exist (Loechler 1994).
The selectivity in adduct formation on guanine may instead be hypothesized by use of the concept of hard and soft nucleophiles/electrophiles, according to which hard electrophiles

tend to react with hard nucleophiles and soft electrophiles tend to react with soft nucleophiles (Carey and Sundberg, 1990). Other factors that have to be taken into consideration are that the free energy of the products has to be lower than the free energy of the reactants and that steric hindrance may prevent formation of certain adducts.

Using the hard-soft/nucleophile-electrophile concept, alkylating agents of the RCH_2X type, which are soft electrophiles, will react with soft nucleophiles, O^6 and N-7 of guanine. Also, nitreniumions derived from arylaminating agents are soft electrophiles and tend to react at positions O^6 and N-7 of guanine (rearranging to a C-8 adduct). Some arylaminating agents also to a lesser extent react with N^2 of guanine, which is borderline between hardness and softness. Reaction at the N^2 position affords that the electrophile is able to delocalize charge into the aromatic ring system so reaction takes place via a harder carbon electrophile.

Carbonium ions derived from aralkylating agents are borderline between hard and soft electrophiles and will react with N^2 of guanine, Figure 14.

DNA adduct formation *in vivo*

Numerous studies have been conducted to study DNA adduct formation of heterocyclic aromatic amines *in vivo*.

IQ-DNA adducts have been detected by ^{32}P-postlabeling analysis in liver, heart, kidney, colon, stomach and bladder in rats after oral administration of IQ (Yamashita et al., 1988, Overvik et al., 1991, Snyderwine et al., 1988c). Several adducts were detected in each organ, including a dG-C8-IQ adduct. Total adduct levels were highest in liver followed by kidney, colon, stomach and bladder (Snyderwine et al., 1988c). Studies using multiple dosing (5 or 50 mg/kg/day) for up to 15 days have shown that accumulation of adducts in several organs occurred in a dose and time dependant manner (Schut et al., 1994). Also, ^{32}P-postlabeling analysis of liver DNA from monkeys dosed with IQ revealed that dG-C8-IQ is the major adduct formed in monkeys (Snyderwine et al., 1993b).

Several DNA adducts of 4-MeIQ have been detected in the liver of CDF_1 female mice fed 0.04% MeIQ in the diet. However, addition of nuclease P1 and phosphodiesterase digestion

to the ^{32}P-postlabeling procedure reduced the number of adducts to a single spot, identified as the dG-C8-MeIQ adduct (Tada et al., 1994).

DNA-adduct formation was studied in mice given a single oral dose of ^{14}C labelled 8-MeIQx. Adduct levels were higher after 6 hours than after 24 hours, with the liver having the highest adduct level followed by large intestine, stomach, spleen, small intestine, kidney and lung (Alldrick and Lutz, 1989). By use of accelerator mass spectrometry, it was shown that the adduct level in mouse liver was linearly related to the dose down to an exposure as low as 500 ng/kg bw, the lowest dose administered (Turteltaub et al., 1990). ^{32}P-Postlabeling analysis of DNA from various organs of rats treated with 20 mg/kg bw/day, 5 days per week for two weeks, showed highest adduct level in the liver followed by kidney, heart and colon (Davis et al., 1993a). A study of the dose-response relationship in adduct formation in liver DNA of rats receiving 0.04, 0.4, 4, 40 and 400 ppm in the diet for one week revealed a linear response in adduct formation. Continued dosing at the highest level for 61 weeks resulted in induction of hepatocellular carcinomas (Yamashita et al., 1990). The structure of the major adduct formed in the rat *in vivo* was identical to the major adduct formed *in vitro*, dG-C8-MeIQx (Ochiai et al., 1993). Also in monkeys dG-C8-MeIQx was the major adduct found in liver DNA following treatment with MeIQx. The adduct level, however, was much lower than the adduct level found in rats treated at a comparable dose level (Snyderwine et al., 1993b, Davis et al., 1993a).

Several DNA adducts of 4,8-DiMeIQx were detected by ^{32}P-postlabeling analysis in rat liver DNA following oral administration of 4,8-DiMeIQx (Yamashita et al., 1988). By comparison with a synthetic standard the major adduct formed *in vivo*, accounting for 60-70% of the bound carcinogen, was identified as dG-C8-DiMeIQx (IV, Frandsen et al., 1994b).

DNA adduct formation of PhIP has been studied in various organs of mice, rats, monkeys and humans.
^{32}P-Postlabeling analysis of DNA from mice given a single oral dose of PhIP at 0, 1, 10, and 20 mg/kg bw showed highest adduct level in pancreas followed by thymus, heart and liver. The adduct level increased linearly with dose (Turteltaub et al., 1993). Also in rats

given a single oral dose of 0, 0.5, 5 and 50 mg/kg bw a dose related increase in adduct level was observed. The highest adduct levels were found in the large intestine followed by white blood cells, stomach, small intestine, kidney, liver and lung (Schut and Herzog, 1992). Studies in monkeys receiving either a single or multiple oral doses of 20 mg/kg bw showed that, upon multiple dosing, DNA adducts tend to accumulate in certain organs. Adduct levels in liver, pancreas, kidney, small intestine and colon increased 1.5 to 2.4 fold after multiple dosing for 10 days, adduct level in brain increased 5 fold, heart 10 fold and aorta 31 fold (Snyderwine et al., 1994).

The structure of the major adduct found in rat liver DNA after a single oral dose of PhIP was shown by chromatographic comparison to be identical to the adduct formed *in vitro*, dG-C8-PhIP (II, Frandsen et al., 1992, Lin et al., 1992).

In a pilot study with human DNA samples, using both GC/MS and ^{32}P-postlabeling analysis, evidence for PhIP-DNA adducts was found in two of six colon samples. PhIP-DNA adducts were not detected in samples from pancreas or urinary bladder (Friesen et al., 1994).

^{32}P-Postlabeling analyses of DNA adducted with AIA compounds have resulted in considerable interlaboratory variation, with up to six adduct spots being detected for each compound. However, inclusion of nuclease P1 digestion in the postlabeling analysis was recently shown to reduce the number of adduct spots to one or two, in accordance with the adducts characterized after *in vitro* formation. The additional adduct spots usually detected in ^{32}P-postlabeling analysis are suggested to be adducted oligonucleosides that are resistant to hydrolysis by micrococcal nuclease or spleen phosphodiesterase (Pfau et al., 1994, Fukutome et al., 1994).

Estimates of human intake and cancer risk

The concentration of heterocyclic aromatic amines (HAA) in cooked meat and fish is in the ppb range and large variations in concentration depending on meat type, frying temperature and frying time have been observed. This together with interindividual consumption habits will markedly influence the daily exposure of individual humans to heterocyclic aromatic amines.

An average daily intake has been estimated to 0.4 - 16 µg for the Japanese population (Wakabayashi et al., 1992). For the German population the average daily intake was estimated to 0.8 - 8.4 µg based on an annual consumption of 56 kg of meat and 5 kg of fish (Eisenbrand and Tang, 1993). Based on an annual consumption of 56 kg of meat and 9 kg of fish the intake was calculated to be 0 - 16.5 µg per day in the Danish population (Dragsted, 1994). By combining the concentrations of heterocyclic aromatic amines found in foods cooked under normal household conditions with a dietary survey of the U.S. population, daily intakes of PhIP, MeIQx, DiMeIQx and IQ have been estimated to 16.64, 2.61, 0.81 and 0.28 µg respectively (Layton et al., 1995).
Cancer risks associated with intake of HAAs have been estimated by combining the above intake estimates with the TD_{50} from animal tumour studies, mostly in rats, assuming a linear dose-response relationship. The assumption of linear dose-response relationship has been supported by linearity in DNA binding over a dose range covering 5 orders of magnitude down to a single dose of 500 ng/kg of MeIQx (Felton et al., 1992). The published cancer risk estimates associated with HAA ingestion vary from 1 in 1000 (Felton et al., 1992) to approximately 1 in 10,000 (Gaylor and Kadlubar, 1991, Layton et al., 1995). A total lifetime cancer risk interval of 0.2 - 2300 per million individuals has been estimated for the danish population (Dragsted, 1994). In a risk assessment covering five HAA's: PhIP, MeIQx, DiMeIQx, IQ and AαC, ingestion of PhIP accounted for almost 50% of the cancer risk (Layton et al., 1995).

Conclusion

Several heterocyclic aromatic amines of the AIA type are formed during cooking of meat and are found in amounts ranging from "not detected" to more than 80 µg/kg. The most important factors for the formation of the these compounds, both on the meat surface and in the pan residue, are the type of meat being cooked, cooking temperature and duration of cooking.
The most important of the AIA compounds, both due to the amount formed and due to possible biological effects, seem to be IQ, 4-MeIQ, 8-MeIQx, 4,8-DiMeIQx and PhIP. These AIA compounds are genotoxic in a number of test systems including bacterial and

mammalian systems.

Metabolic activation is required for these compounds to exert their mutagenic effect and hydroxylation of the exocyclic amino group has been shown to generate the proximate mutagenic metabolite. Further activation by transferases such as acetyltransferase, sulphotransferase, phosphotransferase or aminoacyltransferase to generate DNA binding metabolites are usually required.

IQ, MeIQ, MeIQx and PhIP were found to be multiple organ carcinogens in oral feeding studies in rodents. Also in monkeys IQ was found to be a liver carcinogen. In most of these studies, however, adequate data to provide a dose response relationship were not obtained (Munro et al., 1993). Furthermore, many of the experiments were terminated before the expected life span of the animals (Gold et al., 1994). Risk extrapolation to low dose human exposure, which is a factor of 100,000 less than the doses used in animal experiments, therefore is associated with great uncertainty.

Based on animal carcinogenicity data and estimated exposures the human lifetime cancer risk from ingestion of AIA compounds has recently been estimated within the range of 1 - 38 per 100,000 (Layton et al., 1995). In animals, however, metabolic transformation to non-genotoxic metabolites seems to be the major metabolic pathway, whereas in humans CYP1A2 mediated transformation to genotoxic metabolites is the major metabolic pathway. Assessment of human cancer risk based on animal bioassays may therefore result in an underestimation.

Large interindividual variations are found among humans in the activity of enzymes involved in activation and detoxification of chemical carcinogens. This results in differences in DNA adduct formation between individuals. Also rates of repair of DNA damage caused by chemical carcinogens vary among individuals (Harris, 1989). Large interspecies differences in metabolism are found, e.g. the ratio of phase I activation (N^2-hydroxylation) of PhIP to phase I detoxification (4'-hydroxylation) has been estimated to 97:1 in man, whereas in the rat, which is the species often used in cancer bioassay studies, the ratio has been estimated to 3.3:1. Also large interspecies variations in phase II activation are observed, the degree of activation depending on the substrate, tissue and species (Lin et al., 1995; Davis et al., 1993b).

Future developments of molecular biomarkers reflecting the exposure or the biological response to dietary exposures to AIA compounds, e.g. DNA adducts in urine, may provide

the means of identifying the most sensitive individuals. Also information obtained from studies on molecular biomarkers in humans and in animals from cancer bioassay studies can provide data for more accurate estimations of human cancer risk (Shuker and Farmer, 1992, Strickland and Groopman, 1995).

The results from own investigations have elucidated the structures of genotoxic metabolites and DNA adducts of PhIP and 4,8-DiMeIQx. These results provide a good basis for future development of molecular biomarkers based on measurement of DNA adducts, that can improve risk assessment of human exposure to these chemicals.

References

Adamsom, R.H., Takayama, S., Sugimura, T. and Thorgeirsson, P. (1994) Induction of hepatocellular carcinoma in nonhuman primates by the food mutagen 2-amino-3-methylimidazo[4,5-*b*]quinoline. *Environmental Health Perspectives*, **102**,190-193.

Alexander, J., Holme, J.A., Wallin, H. and Becher, G. (1989a) Characterization of metabolites of the food mutagens 2-amino-3-methylimidazo[4,5-*b*]quinoline and 2-amino-3,4-dimethylimidazo[4,5-*b*]quinoline formed after incubation with isolated rat liver cells. Chem. Biol. Interactions, **72**, 125-142.

Alexander, J., Wallin, H., Holme, J.A. and Becher, G. (1989b) 4-(2-amino-1-methylimidazo[4,5-*b*]pyrid-6-yl)phenyl sulfate-a major metabolite of the food mutagen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in the rat. *Carcinogenesis*, **10**, 1543-1547.

Alexander, J., Wallin, H., Rossland, J.O., Solberg, K.E., Holme, J.A., Becher, G., Andersson, R. and Grivas, S. (1991) Formation of a glutathione conjugate and a semistable transportable glucuronide conjugate of N^2-oxidized species of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in rat liver. *Carcinogenesis*, **12**, 2239-2245.

Alldrick, A.J. and Rowland, I.R. (1985) Activation of the food mutagens IQ and MeIQ by hepatic S9 fractions derived from various species. *Mutation Research*, **144**, 59-62.

Alldrick, A.J. and Lutz, W.K. (1989) Covalent binding of [2-^{14}C]2-amino3,8-diimethylimidazo[4,5-*f*]quinoxaline (MeIQx) to mouse DNA *in vivo*. *Carcinogenesis*, **10**, 1419-1423.

Ames, B.N., Durston, W.E., Yamasaki, E. and Lee, F.D. (1973) Carcinogens are mutagens: A simple test system combining liver homogenates for activation and bacteria for detection. *Proc. Nat. Acad. Sci. USA*, **70**, 2281-2285.

Ames, B.N., Gold, L.S. and Willett, W.C. (1995) The causes and prevention of cancer. *Proc. Natl. Acad. Sci. USA*, **92**, 5258-5265.

Aune, T. and Aune, K.T. (1986) Mutagenic activation of IQ and Me-IQ by liver and lung microsomes from rabbit and mouse, and with isolated lung cells from the rabbit. *Carcinogenesis*, **7**, 273-276.

Boobis, A.R., Lynch, A.M., Murray, S., de la Torre, R., Solans, A., Farre', M., Segura, J., Gooderham, N.J. and Davies, D.S. (1994) CYP1A2-catalyzed conversion of dietary heterocyclic amines to their proximate

carcinogens is their major route of metabolism in humans. *Cancer Research*, **54**, 89-94.

Brunborg, G., Holme, J.A., Alexander, J., Becher, G. and Hongslo, J.K. (1988) Genotoxic activity of the N-acetylated metabolites of the food mutagens 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) and 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ). Mutagenesis, 3, 303-309.

Brookes, P. and Lawley, P.D. (1964) Evidence for the binding of polynuclear aromatic hydrocarbons to nucleic acids of mouse skin: Relation between carcinogenic power of hydrocarbons and their binding to deoxyribonucleic acid. *Nature*, **4934**, 781-784.

Buonarati, M.H. and Felton, J.S. (1990a). Activation of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) to mutagenic metabolites. *Carcinogenesis*, **11**, 1133-1138.

Buonarati, M.H., Turteltaub, K.W., Shen, N.H. and Felton, J.S. (1990b) Role of sulfation and acetylation in the activation of 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine to intermediates which bind DNA. *Mutation Research*, **245**, 185-190.

Buonarati, M.H., Tucker, J.D., Minkler, J.L., Wu, R.W., Thompson, L.H. and Felton, J.S. (1991) Metabolic activation and cytogenetic effects of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in chinese hamster ovary cells expressing murine cytochrome p450 1A2. *Mutagenesis*, **6**, 253-259.

Bounarati, M.H., Roper, M., Morris, C.J., Happe, J.A., Knize, M.G. and Felton, J.S. (1992) Metabolism of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in mice. *Carcinogenesis*, **13**, 621-627.

Carey, F.A. and Sundberg, R.J. (1990) Advanced organic chemistry. Plenum Press, New York, pp. 287-288.

Chou, H.C., Lang, N.P. and Kadlubar, F.F. (1995) Metabolic activation of N-hydroxy arylamines and N-hydroxy heterocyclic amines by human sulfotransferase(s). *Cancer Research*, **55**, 525-529.

Davis, C.D., Schut, A.J., Adamson, R.H., Thorgeirsson, U.P., Thorgeirsson, S.S. and Snyderwine E.G. (1993a) Mutagenic activation of IQ, PhIP and MeIQx by hepatic microsomes from rats, monkey and man: low mutagenic activation of MeIQx in cynomolgus monkeys in vitro reflects low DNA adduct levels in vivo. *Carcinogenesis*, **14**, 61-65.

Davis, C.D., Schut, H.A. and Snyderwine, E.G. (1993b) Enzymatic Phase II activation of the *N*-hydroxylamines of IQ, MeIQx and PhIP by various organs of monkey and rats. *Carcinigenesis*, **14**, 2091-2096.

Dipple, A., Lawley, P.D. and Brookes, P. (1968) Theory of tumor initiation by chemical carcinigens: Dependence of activity on structure of ultimate carcinogen. *Europ. J. Cancer*, **4**, 493-506.

Dipple, A. (1988) Reactive metabolites of carcinogens and their interaction with DNA. In Politzer, P. and Martin Jr., F.J. (eds) *Chemical Carcinogens*, Elsevier, Amsterdam, p. 32-62.

Dipple, A. (1995) DNA adducts of chemical carcinogens. *Carcinogenesis*, **16**, 437-441.

Doll, R. and Peto, R. (1981) The causes of cancer: quatitative estimates of avoidable risks of cancer in the United States today. *J. Natl. Cancer Inst.*, **66**, 1192-1308.

Dragsted, L.O. (1994) Disposition, bioactivation and DNA-binding of food mutagens: The development of a strategy for immunoassay biomarkers. *National food Agency of Denmark.*

Eisenbrand, G. and Tang, W. (1993) Food-borne heterocyclic amines. Chemistry, formation, occurence and biological activities. A litterature review. *Toxicology*, **84**, 1-82.

Felton, J.S., Knize, M.G., Wood, C., Wuebbles, B.J., Healy, S.K., Stuermer, D.H., Bjeldanes, L.F., Kimble, B.J., and Hatch, F.T.1984) Isolation and characterization of new mutagens from fried ground beef. *Carcinogenesis*, **5**, 95-102.

Felton, J.S., Knize, M.G., Shen, N.H., Lewis, P.R.,Adresen, B.D., Happe, J. and Hatch, F.T. (1986a) The isolation and identification of a new mutagen in fried ground beef: 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP). *Carcinogenesis*, **7**, 1081-1086.

Felton, J.S., Knize, M.G., Shen, N.H., Adresen, B.D., Bjeldanes, L.F. and Hatch, F.T. (1986b) Identification of the mutagens in cooked beef. *Environmental Health Perspectives*, **67**, 17-24.

Felton, J.S., Knize, Shen, N.H., Wu, R. and Becher, G. (1988) Mutagenic heterocyclic imidazoamines in cooked foods. In: King, C.M., Romano, L.J. and Schuetzle, D.(eds), *Carcinogenic and mutagenic responses to aromatic amines and nitrosamines*. Elsevier Science Publishing Co. p.73-75.

Felton, J.S. and Knize, M.G. (1990) Heterocyclic-amine mutagens/carcinogens in foods. In: In Cooper, C.S. and Grover, P.L. (eds.) *Chemical carcinogenesis and mutagenesis*, Springer-Verlag, Berlin, p. 471-502.

Felton, J.S., Knize, M.G., Roper, M., Fultz, E., Shen, N.H., and Turteltaub, K.W. (1992) Chemical analysis, prevention and low-level dosimetry of heterocyclic amines from cooked food. *Cancer Res.*, **52**, 2103s-2107s.

Ford, G.P. and Griffin, G.R. (1992) Relative stabilities of nitrenium ions derived from heterocyclic amine food carcinigens: Relationship to mutagenicity. *Chem-Biol. Interactions*, **81**, 19-33.

Frandsen, H., Rasmussen, E.S., Nielsen, P.A., Farmer, P., Dragsted, L. and Larsen, J.C. (1991). Metabolic formation, synthesis and genotoxicity of the N-hydroxy derivative of the food mutagen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP). *Mutagenesis*, **6**, 93-98.

Frandsen, H., Grivas, S., Andersson, R., Dragsted, L. and Larsen, J.C. (1992) Reaction of the N^2-acetoxy derivative of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) with 2'-deoxyguanosine and DNA. Synthesis and identification of N^2-(2'-deoxyguanosin-8-yl)-PhIP. *Carcinogenesis*, **13**, 629-635.

Frandsen, H., Nielsen, P.A., Grivas, S. and Larsen, J.C. (1994a) Microsomal metabolism of the food mutagen 2-amino-3,4,8-trimethyl-3*H*-imidazo[4,5-*f*]quinoxaline to mutagenic metabolites. *Mutagenesis*, **9**, 59-65.

Frandsen, H., Grivas, S., Turesky, R.J., Andersson, R., Dragsted, L.O. and Larsen, J.C. (1994b) Formation of DNA adducts by the food mutagen 2-amino-3,4,8-trimethyl-3*H*-imidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx) *in vitro* and *in vivo*. Identification of a N^2-(2'-deoxyguanosin-8-yl)-4,8-DiMeIQx adduct. *Carcinogenesis*, **15**, 2553-2558.

Friesen, M.D., Kalderik, K., Lin, D., Garren, L., Bartsch, H., Lang, N.P. and Kadlubar, F.F. (1994) Analysis of DNA adducts of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine in rat and human tissues by alkaline hydrolysis and gas chromatography/electron capture mass spectrometry: validation by comparison with ^{32}P-postlabeling. *Chem. Res. Toxicol.*, **7**, 733-739.

Fukutome, K., Ochiai, M., Wakabayashi, K., Watanabe, S., Sugimura, T. and Nagao, M. (1994) Detection of guanine-C8-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine adduct as a single spot on thin-layer chromatography by modification of the ^{32}P-postlabeling method. *Jpn. J. Cancer Res.*, **85**, 113-117.

Gaylor, D.W. and Kadlubar, F.F. (1991) Quantitative cancer risk assessments of heterocyclic amines in cooked foods. In: H. Hayastu, (ed), *Mutagens in food. Dtection and prevention*, Boca Raton, Fl: CRC Press, pp 229-236.

Gerhardsson de Verdier, M., Hagman, U., Peters, R.K., Steineck, G. and Overvik, E. (1991) Meat, cooking methods and colorectal cancer: A case-referent study in Stockholm. *Int. J. Cancer*, **49**, 520-525.

Giovannucci, E., Rimm, E.B., Stampfer, M.J. Colditz, G.A., Ascherio, A., and Willett W.C. (1994) Intake of fat, meat, and fiber in relation to risk of colon cancer in men. *Cancer Research*, **54**, 2390-2397.

Gold. L.S.. Slone. T.H.. Manley. N.B. and Ames. B.N. (1994) Heterocyclic amines formed by ccoking food: comparison of bioassay results with other chemicals in the carcinogenic potency database. *Cancer Letters*, **83**, 21-29.

Grivas. S., Nyhammer. T., Olsson, K. and Jagerstadt. M. (1985) Formation of a new mutagenic DiMeIQx compound in a model system by heating creatinine, alanine and fructose. *Mutation Research*, **151**, 177-183.

Harris. C.C. (1989) Interindividual variation among humans in carcinogen metabolism. DNA adduct formation and DNA repair. *Carcinogenesis*, **10**, 1563-1566.

Harris. C.C. (1991) Chemical and physical carcinogenesis: Advances and perspectives for the 1990s, *Cancer Research* (supl). **51**, 5023s-5044s.

Hatch, F.T., Knize. M.G. and Felton. J.S. (1991) Quantitative structure-activity relationships of heterocyclic amine mutagens formed during the cooking of food. *Environmental and Molecular Mutagenesis*. **17**, 4-19.

Humphreys. W.G.. Kadlubar. F.F. and Guengerich. F.P. (1992) Mechanism of C^8 alkylation og guanine residues by activated arylamines: Evidence for initial adduct formation at the N^7 position. *Proc. Natl. Acad. Sci. USA*. **89**. 8278-8282.

Holme. J.A.. Wallin. H.. Brunborg. G.. Søderlund. E.J.. Hongslo. J.K. and Alexander J. (1989) Genotoxicity of the food mutagen 2-amino-1-methyl-6-phenylimidazo(4.5-*b*)pyridine (PhIP): formation of 2-hydroxyamino-PhIP. a direct acting genotoxic metabolite. *Carcinogenesis*. **10**. 1389-1396.

Hyayatsu. H.. Kasai. H.. Shigeyuki. Y.. Miyazawa. T.. Yamaizumi, Z.. Sato. S.. Nishimura. S.. Arimoto, S.. Hyatsu. T. and Ohara. Y. (1987) Mutagenic metabolites in urine and feces of rats fed with 2-amino-3,8-dimethylimiazo[4.5-*f*]quinoxaline. a carcinogenic mutagen present in cooked meat. *Cancer Research*, **47**, 791-794.

Inamasu. T.. Luks. H., Vavrek, T., and Weisburger, J.H. (1989) Metabolism of 2-amino-3-methylimidazo[4,5-*f*]quinoline in the male rat. Fd. Chem. Toxic., **27**, 369-376.

Jagerstadt. M., Olsson. K., Grivas. S. Negishi. C., Wakabayashi, K.. Tsuda. M.. Sato. S., and Sugimura, T. (1984) Formation of 2-amino-3.8-dimethylimidazo[4.5-*f*]quinoxaline in a model system by heating creatinine, glycine and glucose. *Mutation Research*. **126**. 239-244.

Kanai. Y.. Wada. O. and Manabe. S. (1990) Detection of carcinogenic glutamic acid pyrolysis products in

cigarette smoke condensate. *Carcinogenesis*, **11**, 1001-1003.

Kasia, H., Nishimura, S., Wakabayshi, K., Nagao, M. and Sugimura, T. (1980a) Chemical synthesis of 2-amino-3-methyl-3H-imidazo[4,5-*f*]quinoline (IQ),a potent mutagen isolated from broiled fish. *Proc. jpn Acad.*, **58**, 382-384.

Kasia, H., Yamaizumi, Z., Wakabayshi, K., Nagao, M., Sugimura, T., Yokoyama, S., Miyazawa, T. and Nishimura, S. (1980b). Structure and chemical synthesis of ME-IQ, a potent mutagen isolated from broiled fish. *Chem. Lett.*, **11**, 1391-1394.

Kasia, H., Shiomi, T., Sugimura, T. and Nishimura, S. (1981) Synthesis of 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoline (Me-IQx), a potent mutagen isolated from fried beef. *Chem. Lett.*, 675-678.

Kennaway, E.L. and Heiger, I. (1930) Carcinogenic substances and their flourescence spectra. *The British Medical Journal*, 1044-146.

Kikugawa, K., Kato, T. and Takahashi, S. (1989) Possible presence of 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline and other heterocyclic amine-like mutagens in roasted coffee beans. *J. Agric. Food Chem.*, **37**, 881-886.

Knize, M.G., Dolbare, F.A., Carroll, K.L., Moore, D.H. and Felton, J.S. (1994) Effect of cooking time and temperature on the heterocyclic amine content of fried beef patties. *Fd. Chem. Toxic.*, **7**, 595-603.

Knize, M.G., Övervik, E., Midtvedt, T., Turteltaub, K.W., Happe, J.A., Gustafsson, J. and Felton, J.S. (1989) The metabolism of 4,8-DiMeIQx in conventionel and germ-free rats. *Carcinogenesis*, **10**, 1479-1484.

Kurosaka, R., Wakabayashi, K., Ushiyama, H., Nukaya, H., Arakawa, N., Sugimura, T. and Nagao, M. (1992) Detection of 2-amino-1-methyl-6-(4-hydroxyphenyl)imidazo[4,5-*b*]pyridine in broiled beef. *Jpn. J. Cancer Res.*, **83**, 919-922.

Layton, D.W, Bogen, K.T., Knize, M.G., Hatch, F.T., Johnson, V.M. and Felton, J.S. (1995) Cancer risk of heterocyclic amines in cooked foods: An analysis and implications for research. *Carcinogenesis*, **16**, 39-52.

Lin, D., Kalderik, K.R., Turesky, R.J., Miller, D.W., Lay, J.O. and Kadlubar, F.F. (1992) Identification of *N*-(deoxyguanosin-8-yl)-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine as the major adduct formed by the food-borne carcinogen, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine, with DNA. *Chem. Res. Toxicol.*, **5**, 691-697.

Lin, D., Lang, N.P. and Kadlubar, F.F. (1995) Species differences in the biotransformation of the food-borne carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine by hepatic microsomes and cytosols from humans, rats and mice. *Drug Metabolism and Disposition*, **23**, 518-524.

Loechler, E.L. (1994) A violation of the Swain-Scott principle, and *not* S_N1 versus S_N2 reaction mechanisms, explains why carcinogenic alkylating agents can form different proportions of adducts at oxygen versus nitrogen in DNA. *Chem. Res. Toxicol.*, **7**, 277-280.

Luks, H.J., Spratt, T.E., Vavrek, M-T., Roland, S.F. and Weisburger, J.H. (1989) Identification of sulfate and glucuronic acid conjugates of the 5-hydroxy derivative as major metabolites of 2-amino-3-methylimidazo[4,5-*f*]quinoline in rats. *Cancer Research*, **49**, 4407-4411.

Manabe, S., Uchino, E. and Wada, O. (1989) Carcinogenic tryptophan pyrolysis products in airborne particles and rain water. *Mutation Research*, **226**, 215-221.

Manabe, S., Wada, O. and Kanai, Y. (1990) Simultaneous determination of amino-α-carbolines and amino-τ-carbolines in cigarette smoke condensate by high-performance liquid chromatography. *J- Chromatogr.*, **529**, 125-133.

Manabe, S., Izumikawa, S., Asakuno, K., Wada, O. and Kanai, Y. (1991) Detection of carcinogenic amino-α-carbolines and amino-τ-carbolines in diesel exhaust particles. *Environ. Pollut.*, **70**, 255-265.

Manabe, S., Suzuki, H., Wada, O. and Ueki, A. (1993) Detection of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in beer and wine. *Carcinogenesis*, **14**, 899-901.

McManus, M.E. Burgess, W., Veronese, M.E. Huggett, A., Quattrochi, L.C. and Tukey, R.H. (1990) Metabolism of 2-acetylaminoflourene and Benzo(a)pyrene and activation of food-derived heterocyclic amine mutagens by human cytochromes P-450. *Cancer Research*, **50**, 3367-3376.

McManus, M.E., Felton, J.S., Knize, M.G., Burgess, W.M., Roberts-Thomson, S., Pond, S.M., Stupans, I. and Veronese, M.E. (1989) Activation of the food-derived mutagen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine by rabbit and human liver microsomes and purified forms of cytochrome P-450. *Carcinogenesis*, **10**, 357-363.

Miller, C.M., Miller, J.A. and Hartmann, H.A. (1961) *N*-hydroxy-2-acetylaminofluorene: A metabolite of 2-acetylaminofluorene with increased carcinogenic activity in the rat. *Cancer Research*, **21**, 815-824.

Miller, C.M. and Miller, J.A. (1966) Mechanism of chemical carcinogenesis: Nature of proximate carcinogens and interactions with macromolecules. *Pharmacological Reviews*, **18**, 805-838.

Miller, J.A. (1994) Brief history of chemical carcinogenesis. *Cancer Letters*, **83**, 9-14.

Moschel, R.C., Hudgins, W.R. and Dipple, A. (1979) Selectivity in nucleoside alkylation and aralkylation in relation to chemical carcinogenesis. *J. Org. Chem.*, **44**, 3324-3328.

Moschel, R.C., Hudgins, W.R. and Dipple, A. (1980) Aralkylation of guanosine by the carcinogen *N*-nitroso-*N*-benzylurea. *J. Org. Chem.*, **45**, 533-535.

Moschel, R.C., Hudgins, W.R. and Dipple, A. (1986) Reactivity effects on site selectivity in nucleoside aralkylation: A model for the factors influencing the sites of carcinogen-nucleic acid interactions. *J. Org. Chem.*, **51**, 4180-4185.

Munro, I.C., Kennepohl, E., Erickson, R.E., Portoghese, P.S., Wagner, B.M., Easterday, O.D. and Manley, C.H. (1993) Safety assessment of ingested heterocyclic amines: Initial report. *Regulatory toxicology and pharmacology*, **17**, S1-S109.

Morrison, L.D., Eling, T.E. and Josephy, P.D. (1993) Prostaglandin H synthase-dependant formantion of the direct-acting mutagen 2-nitro-3-methylimidazo[4,5-*f*]quinoline (nitro-IQ) from IQ. *Mutation Research*, **302**, 45-52.

Nagao, M., Honda, M., Seino, Y., Yagahi, T. and Sugimura, T. (1977) Mutagenicities of smoke condensates and the charred surface of fish and meat. *Cancer Lett.*, **2**, 221-226.

Nagaoka, H., Wakabayashi, K., Kim, S., Kim, I., Tanaka, Y., Ochiai, M., Tada, A., Nukaya, H., Sugimura, T. and Nagao, M. (1992) Adduct formation at C-8 of guanine on *in vitro* reaction of the ultimate form of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine with 2'-deoxyguanosine and its phosphate esters. *Jpn. J. Cancer Res.*, **83**, 1025-1029.

Nielsen, P.A., Vahl, M. and Gry, J. (1988) HPLC profiles of mutagens in lean ground pork fried at different temperatures. *Z. Lebensm. Unters. Forsch.*, **187**, 451-456.

Ochiai, M., Nagaoka, H., Wakabayashi, K., Tanaka, Y., Kim, S. Tada, A., Nukaya, H., Sugimura, T. and Nagao, M. (1993) Identification of N^2-(deoxyguanosin-8-yl)-2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline 3',5'-diphosphate, a major adduct, detected by nuclease P1 modification of the ^{32}P-postlabelling method, in

the liver of rats fed MeIQx. *Carcinogenesis*, **14**, 2165-2170.

Ozawa, S., Chou, H., Kadlubar, F.F., Nagata, K., Yamazoe, Y. and Kato, R. (1994) Activation of 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine by cDNA-expressed human and rat arylsulfotransferases. *Jpn. J. Cancer Res.*, **85**, 1220-1228.

Overvik, E., Ochiai, M.m Hirose, M., Sugimura, T. and Nagao, M. (1991) The formation of heart DNA adducts in F344 rats following dietary administration of heterocyclic amines. *Mutation Research*, **256**, 37-43.

Peluso, M., Castegnaro, M., Malaveille, C., Friesen, M., Garren, L., Hautefeuille, A., Vineis, P., Kadlubar, F. and Bartsch, H. (1991) ^{32}Postlabelling analysis of urinary mutagens from smokers of black tobacco implicates 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) as a major DNA-damaging agent. *Carcinogenesis*, **12**, 713-717.

Pfau, W., Brockstedt, U., Sohren, K. and Marquardt, H. (1994) ^{32}P-Post-labeling analysis of DNA adducts formed by food-derived heterocyclic amines: evidence for incomplete hydrolysis and a procedure for adduct pattern simplification. *Carcinogenesis*, **15**, 877-882.

Pitot, H.C. (1990) Mechanism of chemical carcinogenesis: Theoretical and experimental basis. In Cooper, C.S. and Grover, P.L. (eds.) *Chemical carcinogenesis and mutagenesis*, Springer-Verlag, Berlin, p. 3-29.

Reistad, R., Frandsen, H., Grivas, S. and Alexander, J. (1994) *In vitro* formation and degradation of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) protein adducts. *Carcinogenesis*, **15**, 2547-2552.

Rich, K.J., Murray, B.P., Lewis, I., Rendell, N.B., Davies, D.S., Gooderham, N.J. and Boonies, A.R. (1992) *N*-hydroxy-MeIQx is the major microsomal oxidation product of the dietary carcinogen MeIQx with human liver. *Carcinogenesis*, **13**, 2221-2226.

Rogers, A.E., Zeisel, S.H. and Groopman, J. (1993) Diet and carcinogenesis. *Carcinogenesis*, **14**, 2205-2217.

Schut, H.A.J. and Herzog, C.R. (1992) Formation of DNA adducts of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in male Fischer-344 rats. *Cancer Letters*, **67**, 117-124.

Schut, H.A.J., Herzog, C.R. and Cummings, D.A. (1994) Accumulation of DNA adducts of 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) in tissues and white blood cells of the Fischer-344 rat after multiple oral dosing. *Carcinogenesis*, **15**, 1467-1470.

Shuker, D.E.G. and Farmer P.B. (1992) Relevance of urinary DNA adducts as markers of carcinogen exposure. *Chem. Res. Toxicol.*, **5**, 450-460.

Sjodin, P., and Jagerstadt, M. (1984) A balance study of 14C-labelled 3H-imidazo[4,5-f]quinolin-2-amines (IQ and MeIQ) in rats. *Fd. Chem. Toxic.*, **22**, 207-210.

Sjodin, P., Wallin, H., Alexander, J. and Jagerstadt, M. (1989) Disposition and metabolism of the food mutagen 2-amino-3,8-dimethylimiazo[4,5-*f*]quinoxaline (MeIQx) in rats. *Carcinogenesis*, **10**, 1269-1275.

Skog, K. (1993) Cooking procedures and food mutagens: A litterature review. *Fd. Chem. Toxic.*, **9**, 655-675.

Skog, K., Steineck, G., Augustsson, K. and Jagerstadt, M. (1995) Effect of cooking temperature on the formation of heterocyclic amines in fried meat products and pan residues. *Carcinogenesis*, **16**, 861-867.

Snyderwine, E.G., Wirth, P.J., Roller, P.P., Adamson, R.H., Sato, S. and Thorgeirsson, S.S. (1988a) Mutagenecity and in vitro covalent DNA binding of 2-hydroxyamino-3-methylimiazo[4,5-*f*]quinoline. *Carcinogenesis*, **9**, 411-418.

Snyderwine, E.G., Roller, P.P., Adamson, R.H., Sato, S. and Thorgeirsson, S.S. (1988b) Reaction of *N*-hydroxylamine and *N*-acetoxy derivatives of 2-amino-3-methylimidazolo[4,5-*f*]quinoline with DNA. Synthesis and identification of *N*-(deoxyguanosin-8-yl)-IQ. *Carcinogenesis*, **9**, 1061-1065.

Snyderwine, E.G., Yamashita, K., Adamson, R.H., Sato, S., Nagao, M., Sugimura, T., and Thorgeirsson, S.S. (1988c) Use of the ^{32}P-postlabeling method to detect DNA adducts of 2-amino-3-methylimidazolo[4,5-*f*]quinoline (IQ) in monkeys fed IQ: identification of the *N*-(deoxyguanosin-8-yl)-IQ adduct. *Carcinogenesis*, **9**, 1739-1743.

Snyderwine, E.G. and Battula N. (1989) Selective mutagenic activation by cytochrome P3-450 of carcinogenic arylaminrs found in foods. *J. Natl. Cancer Institute*, **81**, 223-227.

Snyderwine, E.G., Schut, H.A., Adamson, R.H., Thorgeirsson, U.P. and Thorgeirsson, S.S. (1992) Metabolic activation and genotoxicity of heterocyclic arylamines. *Cancer Research*, **52**, 2099s-2102s.

Snyderwine, E.G., Bounarati, M.H., Felton, J.S. and Turteltaub, K.W. (1993a) Metabolism of the food-derived mutagen/carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in nonhuman primates. *Carcinogenesis*, **14**, 2517-2522.

Snyderwine, E.G., Davis, C.D., Nouso, K., Roller, P.P., and Schut, A.J. (1993b) ^{32}P-Postlabeling analysis of IQ, MeIQx and PhIP adducts formed *in vitro* in DNA and polynucleotides and found *in vivo* in hepatic DNA from IQ-, MeIQx- and PhIP-treated monkeys. *Cacinogenesis*, **14**, 1389-1395.

Snyderwine, E.G., Schut, H.A.J., Sugimura, T., Nagao, M. and Adamson, R.H. (1994) DNA adduct levels of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in tissues of cynomolgus monkeys after single or multiple dosing. *Carcinogenesis*, **15**, 2757-2761.

Snyderwine, E.G., Welti, D.H., Davis, C.D., Fay, L.B. and Turesky, R.J. (1995) Metabolism of the food-derived carcinogen 2-amino-3,8-dimethylimiazo[4,5-*f*]quinoxaline (MeIQx) in nonhuman primates. *Carcinogenesis*, **16**, 1377-1384.

Strickland, P.T. and Groopman, J.D. (1995) Biomarkers for assessing environmental exposure to carcinogens in the diet. *AM. J. Clin. Nutr.*, **61** (suppl), 710s-720s.

Størmer, F.C., Alexander, J. and Becher, G. (1987) Fluorometric detection of 2-amino-3-methylimiazo[4,5-*f*]quinoline, 2-amino-3,4-dimethylimiazo[4,5-*f*]quinoline and their N-acetylated metabolites excreted by the rat. *Carcinogenesis*, **8**, 1277-1280.

Sugimura, T. (1992) Multistep carcinogenesis: A 1992 perspective. *Science*, **258**, 603-607.

Tada, A., Ochiai, M., Wakabayashi, K., Nukaya, H., Sugimura, T. and Nagao, M. (1994) Identification of *N*-(deoxyguanosin-8-yl)-2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (dG-C8-MeIQ) as a major adduct formed by MeIQ with nucleotides *in vitro* and with DNA *in vivo*. *Carcinogenesis*, **15**, 1275-1278.

Taylor, R.T., Fultz, E., Knize, M. and Felton, J.S. (1987) Formation of the fried ground beef mutagen 2-amino-3-methylimiazo[4,5-*f*]quinoline (IQ) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) from L-phenylalanine (Phe) + creatine (Cre) (or creatinine). *Environ. Mutagenesis*, **9**, Suppl. 8, 106.

Thompson, L.H., Tucker, J.D., Stewart, S.A., Christensen, M.L, Salazar, E.P., Carrano, A.V. and Felton, J.S. (1987). Genotoxicity of compounds from cooked beef in repair-deficient CHO cells versus Salmonella mutagenicity. *Mutagenesis*, **2**, 483-487.

Turesky, R.J., Skipper, P.L., Tannenbaum, S.R., Coles, B. and Ketterer, B. (1986) Sulfamate formation is a major route for detoxification of 2-amino-3-methylimiazo[4,5-*f*]quinoline in the rat. *Carcinogenesis*, **7**, 1483-1485.

Turesky, R.J., Ueaschbacher, H.U., Wurzner, H.P., Skipper, P.L. and Tannenbaum, S.R. (1988) Major routes of metabolism of the food-borne carcinogen 2-amino-3,8-dimethylimiazo[4,5-*f*]quinoxaline in the rat. *Carcinogenesis*, **9**, 1043-1048.

Turesky, R.J., Bracco-Hammer, I., Markovic, J., Richli, U., Kappeler, A., and Welti, D.H. (1990) The contribution of N-oxidation to the metabolism of the food-borne carcinogen 2-amino-3,8-dimethylimiazo[4,5-*f*]quinoxaline in rat hepatocytes. *Chem. Res. Toxicol.*, **3**, 524-535.

Turesky, R.J., Lang, N.P., Butler, M.A., Teitel, C.H. and Kadlubar, F.F. (1991a) Metabolic activation of carcinogenic heterocyclic aromatic amines by human liver and colon. *Carcinogenesis*, **12**, 1839-1845.

Turesky, R.J., Markovic, J., Bracco-Hammer, I. and Fay, L.B. (1991b) The effect of dose and cytochrome P450 induction on the metabolism and disposition of the food-borne carcinogen 2-amino-3,8-dimethylimiazo[4,5-*f*]quinoxaline (MeIQx) in the rat. *Carcinogenesis*, **12**, 1847-1855.

Turesky, R.J., Rossi, S.C., Welti, D.H., Lay, J.O. and Kadlubar, F.F. (1992) Characterization of DNA adducts formed *in vitro* by reaction of *N*-hydroxy-2-amino-3-methylimidazo[4,5-*f*]quinoline and *N*-hydroxy-2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline at the C-8 and N^2 atoms of guanine. *Chem. Res. Toxicol.*, **5**, 479-490.

Turteltaub, K.W., Knize, M.G., Heley, S.K., Tucker, J.D. and Felton, J.S. (1989) The metabolic disposition of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine in the induced mouse. *Fd. Chem. Toxic.*, **27**, 667-673.

Turteltaub, K.W., Felton, J.S., Gledhill, B.L., Vogel, J.S., Southon, J.R., Caffe, M.W., Finkel, R.C., Nelson, D.E., Proctor, I.D. and Davis, J.C. (1990) Accelerator mass spectrometry in biomedical dosimetry: Realtionship between low-level exposure and cocalent binding of heterocyclic amine carcinogens to DNA. *Proc. Natl. Acad. Sci. USA*, **87**, 5288-5292.

Turteltaub, K.W. Vogel, J.S., Frantz, C.E. and Shen, N. (1992) Fate and distribution of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine at a human dietary equivalent dose. *Cancer Research*, **52**, 4682-4687.

Turteltaub, K.W. Vogel, J.S., Frantz, C.E., Bounarati, M.H. and Felton, J.S. (1993) Low-level biological dosimetry of heterocyclic amine carcinogens isolated from cooked food. *Environmental Health Perspectives*, **99**, 183-186.

Vikse, R., Klungsøyr, L. and Grivas, S. (1993a) Mutagenic activity of three synthetic isomers of the food carcinogen 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) in the Ames test. *Mutation Research*, **319**, 273-278.

Vikse, R., Knapstad, A., Klungsøyr, L. and Grivas, S. (1993b) Mutagenic activity of the methyl and phenyl derivatives of the food mutagen 2-amino-3-methylimidazo[4,5-*f*]quinoxaline (IQx) in the Ames test. *Mutation Research*, **298**, 207-214.

Wakabayashi, K., Nagao, M., Esumi, H. and Sugimura, T. (1992) Food derived mutagens and carcinogens. *Cancer Research*, **52**, 2092s-2098s.

Wallin, H., Holme, J.A., Becher, G. and Alexander, J. (1989) Metabolism of the food carcinogen 2-amino-3,8-dimethylimiazo[4,5-*f*]quinoxaline. *Carcinogenesis*, **10**, 1277-1283.

Wallin, H., Mikaelsen, A., Guengrich, F.P., Ingelman-Sundberg, M., Solberg, K.E., Rossland, J. and Alexander, J. (1990). Differential rates of metabolic activation and detoxification of the food mutagen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine by different cytochrome P450 enzymes. *Carcinogenesis*, **11**, 489-492.

Watkins, B.E., Esumi, H., Wakabayashi, K., Nagao, M. and Sugimura, T. (1991a) Fate and distribution of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in rats. *Carcinogenesis*, **12**, 1073-1078.

Watkins, B.E., Suzuki, M., Wallin, H., Wakabayashi, K., Alexander, J., Vanderlaan, M., Sugimura, T.and Esumi, H. (1991b) The effect of dose and enzyme inducers on the metabolism of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in rats. *Carcinogenesis*, **12**, 2291-2295.

Widmark, E.M.P (1939) Presence of cancer-producing substances in roasted food. *Nature*, **143**, 984.

Wild, D. and Degen, G.H. (1987) Prostaglandin H synthase-dependant mutagenic activation of heterocyclic aromatic amines of the IQ-type. *Carcinogenesis*, **8**, 541-545.

Wild, D., Feser, W., Michel, S., Lord, H.L. and Josephy, P.D. (1995) Metabolic activation of heterocyclic aromatic amines catalyzed by human arylamine N-acetyltransferase isozymes (NAT1 and NAT2) expressed in *Salmonella typhimurium*. *Carcinogenesis*, **16**, 643-648.

Willett, W.C., Stamper, M.J., Colditz, G.A., Rosner, B.A. and Speizer, F.E. (1990) Relation of meat, fat, and fiber intake to the risk of colon cancer in a prospective study among women. *The new england journal of medicine*, **323**, 1664-1672.

Willett, W.C. (1994) Diet and Health: What should we eat? *Science*, **264**, 532-537.

Yamazoe, Y., Shimada, M., Kamataki, T: and Kato, R. (1983) Microsomal activation of 2-amino-3-methyl-imidazo[4,5-*f*]quinoline, a pyrolysate of sardine and beef extracts, to a mutagenic intermediate. *Cancer Research*, **43**, 5768-5774.

Yamashita, K., Umemoto, A., Grivas, S., Kato, S., Sato, S. and Sugimura, T. (1988) Heterocyclic amine-DNA adducts analyzed by ^{32}P-postlabeling method. *Nucleic Acids Research*, Symposium Series No **19**, 111-114.

Yamashita, K., Adachi, M., Kato, S., Nakagama, H., Ochiai, M., Wakabayashi, K., Sato, S. Nagao, M. and Sugimura, T. (1990) DNA adducts formed by 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline in rat liver: Dose-Response on chronic administration. *Jpn. J. Cancer Res.*, **81**, 470-476.

Yoshida, D., Saito, Y., Mizusaki, S. (1984) Isolation of 2-amino-3-methyl-imidazo[4,5-*f*]quinoline as mutagen fromthe heated product of a mixture of creatinine and proline. *Agric. Biol. Chem.*, **48**, 241-243.

Zhao, K., Murray, S., Davies, D.S., Boobis, A.R. and Gooderham, N.J. (1994) Metabolism of the food derived mutagen and carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) by human liver microsomes. *Carcinogenesis*, **15**, 1285-1288.

PAPER I

Mutagenesis vol.6 no.1 pp.93–98, 1990

Metabolic formation, synthesis and genotoxicity of the *N*-hydroxy derivative of the food mutagen 2-amino-1-methyl-6-phenylimidazo (4,5-*b*) pyridine (PhIP)

Henrik Frandsen, Eva S.Rasmussen, Preben A.Nielsen, Peter Farmer[1], Lars Dragsted and John C.Larsen

Institute of Toxicology, National Food Agency, Mørkhøj Bygade 19, DK-2860 Søborg, Denmark and [1]The Medical Research Council, Carshalton, Surrey, UK

Hepatic microsomes from rats pretreated with PCB were found to metabolize the food mutagen 2-amino-1-methyl-6-phenylimidazo(4,5-*b*)pyridine (PhIP) to two major metabolites, one of which was identified as the *N*-hydroxy derivative, 2-hydroxy-amino-1-methyl-6-phenylimidazo(4,5-*b*)pyridine (*N*-OH-PhIP). This identification was based on mass spectral (MS), UV and HPLC data by comparison with *N*-OH-PhIP prepared by chemical synthesis, as well as the specific activity of the compound in the Ames Salmonella test. Synthetic *N*-OH-PhIP was prepared by catalytic reduction of the nitro derivative of PhIP, which was synthesized from PhIP by diazotization and reaction with sodium nitrite. *N*-OH-PhIP was mutagenic to *Salmonella typhimurium* TA98 without metabolic activation and had a specific mutagenic activity of 2700 revertants/nmol. *N*-OH-PhIP thus seems to be a proximate mutagenic metabolite of PhIP. Other direct acting mutagens were not detected in the microsomal incubation mixture after HPLC separation. *N*-OH-PhIP also induced sister chromatid exchange (SCE) in Chinese hamster ovary cells (CHO cells) without metabolic activation. The specific activity of *N*-OH-PhIP in this assay was ~ 3 times higher than the activity of PhIP with microsomal activation.

Introduction

During cooking of meat several hetero-aromatic amine mutagens, imidazoazaarenes (AIAs), are formed (Felton *et al.*, 1986a,b; Nielsen *et al.*, 1988). When frying at high temperatures, PhIP on a weight basis accounts for ~80% of the mass mutagenic material produced and ~20% of the mutagenic activity as determined in the Ames Salmonella/mammalian microsome assay. Although the specific activity of PhIP in this assay is less than several of the other AIAs, recent studies have shown that PhIP, in contrast to the other AIAs is a potent mutagen/clastogen in mammalian cells inducing SCE and chromosomal aberrations in repair deficient CHO cells (Thomson *et al.*, 1987). PhIP was also found to be a potent inducer of SCE in V79 cells co-cultured with Aroclor-pretreated hepatocytes (Holme *et al.*, 1989).

Several studies on the metabolism of aromatic amines, including AIAs, show that these substances can be metabolically activated by microsomal oxidation of the exocyclic amino group, and that resultant *N*-hydroxy compounds are direct acting mutagens in the Ames Salmonella test (Kadlubar and Beland, 1985; Yamazoe *et al.*, 1988; Synderwine *et al.*, 1987; Holme *et al.*, 1989).

We have studied the metabolic activation of PhIP in microsomes from rats pretreated with polychlorinated biphenyls (PCB) and isolated a substance which was identified as 2-hydroxyamino-1-methyl-6-phenylimidazo(4,5-*b*)pyridine (*N*-OH-PhIP). The identification was based on comparison with chemically synthesized *N*-OH-PhIP, including mass spectral (MS), UV and HPLC data as well as the specific mutagenic activity in the Ames test. Moreover the NMR spectrum of synthetic *N*-OH-PhIP as well as its specific mutagenicity in the SCE assay are presented.

Materials and methods

Chemicals and apparatus

PhIP was kindly donated by Drs James Felton and Mark Knize, Lawrence Livermore National Laboratory, USA. The PhIP was judged > 99% pure by HPLC (316 nm). 2-Amino-4′-hydroxy-1-methyl-6-phenylimidazo(4,5-*b*)pyridine (4′-hydroxy-PhIP) was kindly donated by Dr Jan Alexander, National Institute of Public Health, Oslo, Norway. Isocitric dehydrogenase (from porcine heart), nicotinamide-adeninedinucleotide-phosphate, Trisodium-DL-isocitrate and Tris (Trizma Base) were obtained from Sigma, St Louis, MO, USA. PCB (Aroclor 1254) was obtained from Monsanto Industrial Chemical Co., St Louis, MO, USA. HPLC grade methanol was obtained from Rathburn, Walkerburn, UK. C-18 Sep-Pak columns were obtained from Waters, Milford, MA, USA. All other chemicals were obtained from Merck, Darmstadt, FRG, and were of analytical purity, except palladium on carbon and hydrazine hydrate which were of reagent grade. The Salmonella tester strain TA98 was kindly provided by Professor Bruce N.Ames, University of California, Berkeley, CA, USA. CHO cells (CHO Kl, no. 03-403-83) were from Flow, Rickmansworth, UK. UV spectra were recorded on a Shimatzu UV-160 double beam spectrophotometer. Mass spectra of the electron impact (EI) mode were recorded on a VG 70-250J at an electron energy of 70 eV. Positive fast atom bombardment (FAB) mass spectra were recorded at 8 kV using xenon as neutral beam and glycerol as matrix. HPLC was performed on a Hewlett-Packard model 1084 B liquid chromatograph. For analytical puroses a 250 × 4.6 mm Lichrosorb RP-18, 5 μm column (Merck, Darmstadt, FRG), flow 1 ml/min was used. For preparative purposes a 300 × 7.6 mm Lichrosorb RP-18, 5 μm column, flow 2.8 ml/min was used. Solvents: A, 50 mM ammonium acetate; B, methanol. For both analytical and preparative purposes the following solvent programme was used: 10% B for 5 min followed by a linear gradient to 60% B at 25 min, isocratic from 25 to 35 min and a linear gradient to 100% B at 40 min. Plasma spray liquid chromatography (LC) MS was performed on a VG Trio-3 coupled with a Waters 600 multisolvent delivery system. The solvents were 20 mM ammonium acetate and 20 mM ammonium acetate in 90% acetonitrile. The same column, flow and solvent programming as in analytical HPLC were used. NMR spectra were obtained in the solvents specified with a Varian VXR-400 FT NMR spectrometer. The oxygen electrode was from pHOX Systems Ltd, Ivel Road, Shefford, UK.

Synthesis of 2-nitro-1-methyl-6-phenylimidazo(4,5-b)pyridine (NO_2-PhIP)

PhIP (5.2 mg) was dissolved in a mixture of 0.5 ml phosphoric acid (85%) and 0.2 ml sulphuric acid. The solution was cooled on ice-water and four 5 μl portions of 2 M sodium nitrite were added during a 2 min period under vigorous stirring. The solution was stirred at 0°C for 50 min. The diazotization mixture was added dropwise to an ice-water cooled solution of 1.4 g sodium nitrite in 15 ml of water. The pH of the reaction mixture was kept at 3.5 by addition of first sulphuric acid and then 2 M sodium hydroxide. The mixture was heated to room temperature and after stirring for 25 min extracted twice with 25 ml of ethylacetate. The organic phase was washed with water, dried with magnesium sulphate and evaporated to dryness. The oily residue was dissolved in 0.5 ml of methanol and NO_2-PhIP was obtained as yellow crystals after addition of 1 ml of water and cooling. Yield 2.6 mg (44%). The NO_2-PhIP was judged > 95% pure by HPLC (254 nm). Mass spectrum (EI) m/z 254 (25%), M^+ and 225 (100%). UV absorption maximum in methanol was at 350 nm. The molar extinction coefficient was estimated to 11200 M^{-1} cm^{-1}.

Synthesis of N-OH-PhIP

A reaction tube containing 3 mg of 10% Pd/C was evacuated for 5 min and filled with argon. After addition of 2 ml of methanol the suspension was purged with oxygen free argon for 5 min. The suspension was cooled on ice-water and 10 μl of hydrazine hydrate (80%) was added under stirring. Stirring was continued for 30 min at 0°C before a solution of 2.6 mg of NO_2-PhIP in 2 ml of argon-purged

© Oxford University Press

methanol was added. After 10 min stirring at 0°C, 8 ml of cold, argon-purged methanol was added, and after centrifugation the supernatant was isolated and evaporated to dryness. The oily residue was dissolved in 0.3 ml of methanol and crystals of *N*-OH-PhIP were obtained after addition of 0.7 ml of water and cooling. Yield 1.4 mg (57%). The *N*-OH-PhIP was judged 90% pure by HPLC (316 nm). The mass spectrum (FAB) contained peaks at 241 $(M+H)^+$ and 225. UV absorption maximum in methanol was at 310 nm. The molar extinction coefficient was estimated to 15500 M^{-1} cm^{-1}.

Synthesis of 2-(phenylazoxy)-1-methyl-6-phenylimidazo(4,5-b) pyridine (phenylazoxy derivative of PhIP)

To a solution of 200 μg *N*-OH-PhIP in 0.5 ml argon-purged dimethylformamide was added 1 mg nitrosobenzene and 20 μl acetic acid. After 18 h at room temperature, the solution was evaporated to dryness and dissolved in 0.5 ml acetonitrile and analysed by LC MS.

Preparation of microsomes

Adult male Wistar rats (age 7–8 weeks, weight ~200 g), delivered from Møllegård Breeding Center Ltd., Lille Skensved, Denmark, were used to prepare PCB-induced hepatic microsomes. PCB (Aroclor 1254, 500 mg/kg, dissolved in corn oil) was injected i.p. 5 days before sacrifice and microsomes were prepared essentially as described by Lake (1987). The microsomes were stored in liquid nitrogen until use. The microsomes were characterized by measuring the concentration of protein (Sedmark and Grossberg, 1977), of P450 (Lake, 1987): 3.1 nmol/mg protein and of biphenyl hydroxylase activity (Yamazoe *et al.*, 1981): 2-hydroxy-biphenyl; 3.8 nmole formed/mg protein min, 4-hydroxy-biphenyl: 4.5 nmol formed/mg protein min.

Microsomal activation of PhIP

The incubation mixture consisted of: 1 mg/ml microsomal protein, 1 unit/ml DL-isocitrate-dehydrogenase, 0.5 mM $NADP^+$, 10 mM sodium-DL-isocitrate and 5 mM magnesium chloride in 50 mM Tris–HCl, 0.15 M KCl pH 7.4. The mixture was preincubated for 2 min at 37°C before addition of 10 μg PhIP (dissolved in 5 μl of methanol) per ml incubation mixture. The mixture was incubated at 37°C. In order to investigate the time course of formation of PhIP metabolites, aliquots of 0.5 ml were taken at fixed time points. After immediate addition of 1 ml of cold methanol and centrifugation, the supernatants were analysed by HPLC. For the isolation of microsomal metabolites the incubation time was 30 min, whereafter the incubation mixture was applied on a methanol and water washed C-18, Sep-Pak column through a 0.45 μm filter. After washing with water the metabolites were eluted with methanol, evaporated to dryness, extracted in a small volume of 50% methanol and separated by preparative HPLC. All solvents were purged with argon prior to use.

Mutation asssay

The mutation assays were carried out by the standard plate incorporation method as described by Maron and Ames (1983) with or without S9 mix (1 mg protein/plate) prepared from Aroclor 1254-induced male Wistar rat liver. In some experiments the metabolic activation system was substituted with 1 mM dithiothreitol in order to prevent oxidation of the test substance. To 2 ml of molten top agar at 45°C 0.1 ml of S9 mix, 0.1 ml of water or 0.1 ml 20 mM dithiothreitol was added. After 2 min the test compound, dissolved in 0.1 ml of methanol, and 0.1 ml of a 7 h nutrient broth (Oxoid) culture of the test bacteria (1.0 × 10^9 cells/ml) *Salmonella typhimurium* TA98 were added. After a gentle mixing the top agar was poured onto 20 ml minimal glucose plates. The number of His^+ revertant colonies on each plate was counted after incubation for 48 h at 37°C in the dark. The intended dose levels of *N*-OH-PhIP tested with addition of dithiothreitol were 0.02, 0.05, 0.16 and 0.50 nmol/plate. The exact concentrations were determined by HPLC analysis. Each dose level was tested in duplicates and the assays were repeated with new batches of both microsomally formed *N*-OH-PhIP and synthetic *N*-OH-PhIP. The results presented are the mean of these two independent experiments. The level of spontaneous revertants was not increased by the addition of dithiothreitol to the assay mixture. 2-Nitrofluorene and 2-aminoanthracene was used as positive control mutagens and solvent (methanol, 0.1 ml) as negative. 2-Nitrofluorene was tested without S9 mix at a concentration of 0.1 μg plate giving 176 ± 76 revertants/plate and 2-aminoanthracene was tested at a concentration of 0.1 μg/plate with S9 mix giving 114 ± 32 revertants plate. The specific mutagenic activity (revertants/nmol) was calculated by linear regression analysis.

SCE assay

KI CHO cells (2 × 10^5) were seeded in 25 cm^2 flasks with 10 ml growth medium [McCoys with HEPES buffer (Gibco) supplemented with 15% fetal bovine serum (Gibco)]. Penicillin and streptomycin sulphate (Gibco) both at 500 IU/ml of medium were used for all cultures. SCE assays were performed according to Dean and Danford (1984). Cells were incubated with the test substances for 2 h using 4.5 ml of medium without serum and 100 μl test compound or solvent control (100 μl methanol). *N*-OH-PhIP was tested with the addition of 0.5 mM dithiothreitol to the media. PhIP was tested without the addition of dithiotreitol and with 0.5 ml microsome mixture, containing 3 mg/ml of protein, 8 mM $MgSO_4$, 16 mM DL-isocitrate, 0.8 mM $NADP^+$ and 0.4 U/ml isocitrate dehydrogenase in 50 mM Tris–HCl, pH 7.6. Separate cultures for analysis of mitotic index were harvested by trypsination after 20 h incubation and stained in 3% Giemsa solution. Mitomycin C (0.1 μg/ml medium inducing 4.0 SCE/chromosome) and benzo[*a*]pyrene (20 μg/ml medium inducing 1.54 SCE/chromosome) were used as positive controls.

The oxygen concentrations in control incubation mixtures with or without microsomal activation system or with addition of 0.5 mM dithiothreitol was measured with an oxygen electrode. For SCE 2 × 25 metaphases were evaluated per concentration of PhIP and *N*-OH-PhIP. For mitotic index 2 × 1000 cells were evaluated per concentration. Two separate experiments were performed on each compound. For statistical evaluation the SCE values were transformed to $\log_{10}(x+1)$ and subjected to one-way analysis of variance (ANOVA) to compare each treatment group to solvent control. Randomized complete block design ANOVA analysis was used to evaluate differences between replica cultures and between experiments. Regression analysis were used to test dose–response curves for linearity.

Results

Nitro- and hydroxyamino derivatives of PhIP were synthesized from PhIP (Figure 1) by modifications of the procedures used by Yamazoe *et al.* (1988) for the synthesis of the corresponding derivatives of 2-amino-3,8-dimethylimidazo(4,5-*f*)quinoxaline ($MeIQ_x$). Based on HPLC areas at 254 nm the nitro-derivative (NO_2-PhIP) was obtained in ~80% yield. The structure of NO_2-PhIP was verified by MS (Figure 2) and NMR (Figure 3). The EI mass spectrum showed the expected molecular ion at *m/z* 254 and ions at 225, M^+-NCH_3 and 209, M^+-O-NCH_3. The ^{1}H-NMR spectrum of NO_2-PhIP in d_6-dimethylsulfoxide (DMSO) showed a singlet at δ 4.2 (N-CH_3), a triplet at δ 7.5 (H-4′), a triplet at δ 7.6 (H-3′ and H-5′), a doublet at δ 7.9 (H-2′ and H-6′), a singlet at δ 8.7 (H-7) and a singlet at δ 9.0 (H-5).

In the crude reaction mixture a by-product was detected in 20%

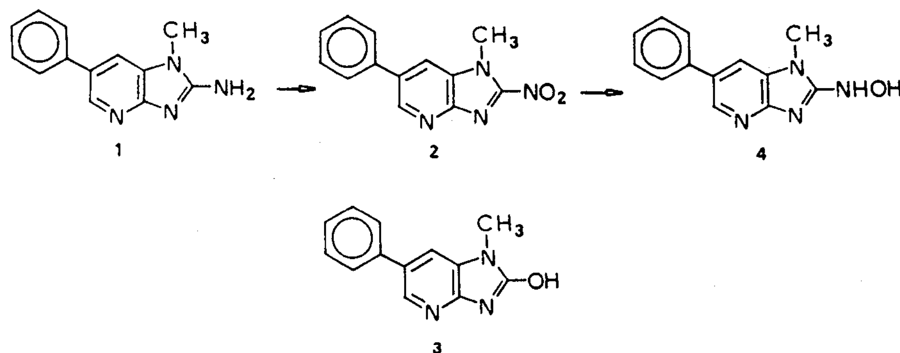

Fig. 1. The two-step synthesis of N-OH-PhIP (**4**) from PhIP (**1**), showing the intermediate NO_2-PhIP (**2**) and the main side reaction product with an molecular ion at *m/z* = 225 putatively identified 2-OH-PhIP (**3**).

yield on HPLC. The by-product was purified by preparative HPLC and assumed to be 2-hydroxy-1-methyl-6-phenylimidazo (4,5-*b*) pyridine (OH-PhIP) (MS, EI $m/z=225$, M^+, not shown) formed by attack on water on the intermediate diazoniumion or/and by hydrolysis of NO_2-PhIP. No_2-PhIP was unstable and was, especially in slightly acidic solution, converted to OH-PhIP (Figure 1).

The NO_2-PhIP was reduced to *N*-OH-PhIP (Figure 1) as described in Materials and methods. This compound was very sensitive to oxygen especially when dissolved in organic solvents. A methanolic solution slowly turned red, possibly due to formation of the azoxy-derivative of PhIP.

The positive FAB mass spectrum of *N*-OH-PhIP showed the expected molecular ion at 241, $(M+H)^+$ and an ion at 225 (not

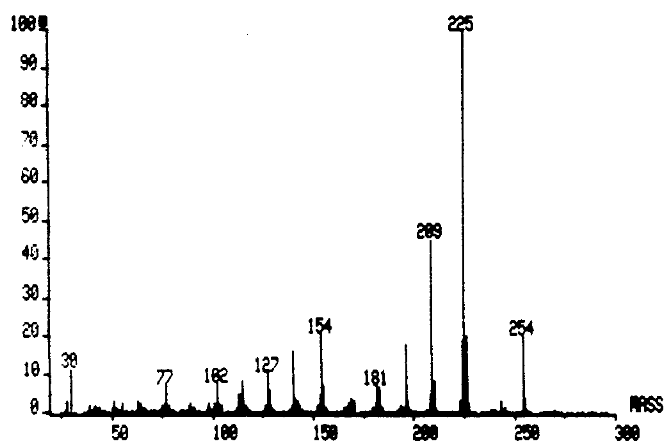

Fig. 2. EI mass spectrum of NO_2-PhIP, 70 eV ionization, solid probe inlet mode, showing the molecular ion at $m/z = 254$ and daughter ions at $m/z = 225$, M^+-NCH_3 and at $m/z = 209$, M^+-O-NCH_3.

shown). The ^{1}H-NMR spectrum (Figure 4) showed a singlet at δ 3.7 (N-CH_3), a doublet δ 7.5 (H-4'), a triplet at δ 7.6 (H-3' and H-5'), a doublet at δ 7.7 (H-2' and H-6'), and two doublets at δ 8.3 and 8.4 (H-7 and H-5). NH and OH protons are not seen due to exchange with deuterium.

Further confirmation that the *N*-OH-PhIP had been correctly synthesized was achieved through the synthesis of the phenylazoxy derivative (Hashimoto *et al.*, 1982). *N*-OH-PhIP reacted slowly with nitrosobenzene in acetic DMF. The reaction was followed on HPLC and as the *N*-OH-PhIP peak diminished a new peak arose. This product was purified by HPLC but a mass spectrum containing the molecular ion could not be obtained neither by EI nor FAB MS. However analysis of the crude reaction mixture by plasma spray LC MS showed the expected molecular ion at 330, $(M+H)^+$ for the phenylazoxy derivative and an ion at 314, $(M+H)^+$-O (Figure 5).

Two major metabolites were observed when PhIP was incubated with hepatic microsomes from rats pretreated with PCB, see Figure 6. As seen from Figure 7, the time course of the formation of the two metabolites was almost linear for 30 min when ~80% of the added PhIP had been consumed. The two metabolites were purified by preparative HPLC. One of the metabolites (peak b) was identified as *N*-OH-PhIP, as it gave the same mass and UV spectra as synthetic *N*-OH-PhIP and coeluted with this in HPLC. The fraction containing the *N*-OH-PhIP peak was the only fraction showing reactivity in the assay for *N*-hydroxylated amines by Belanger *et al.* (1981) and it was mutagenic in the Ames tester strain TA98 without metabolic activation system. Mutagenic activity without metabolic activation system was not detected when the remainder HPLC fractions were pooled and subjected to analysis. The other metabolite (peak a) was characterised by its MS spectrum showing 241, $(M+H)^+$, but no 225 peak as *N*-OH-PhIP. On HPLC it coeluted with a reference sample of 4'-hydroxy-PhIP kindly donated

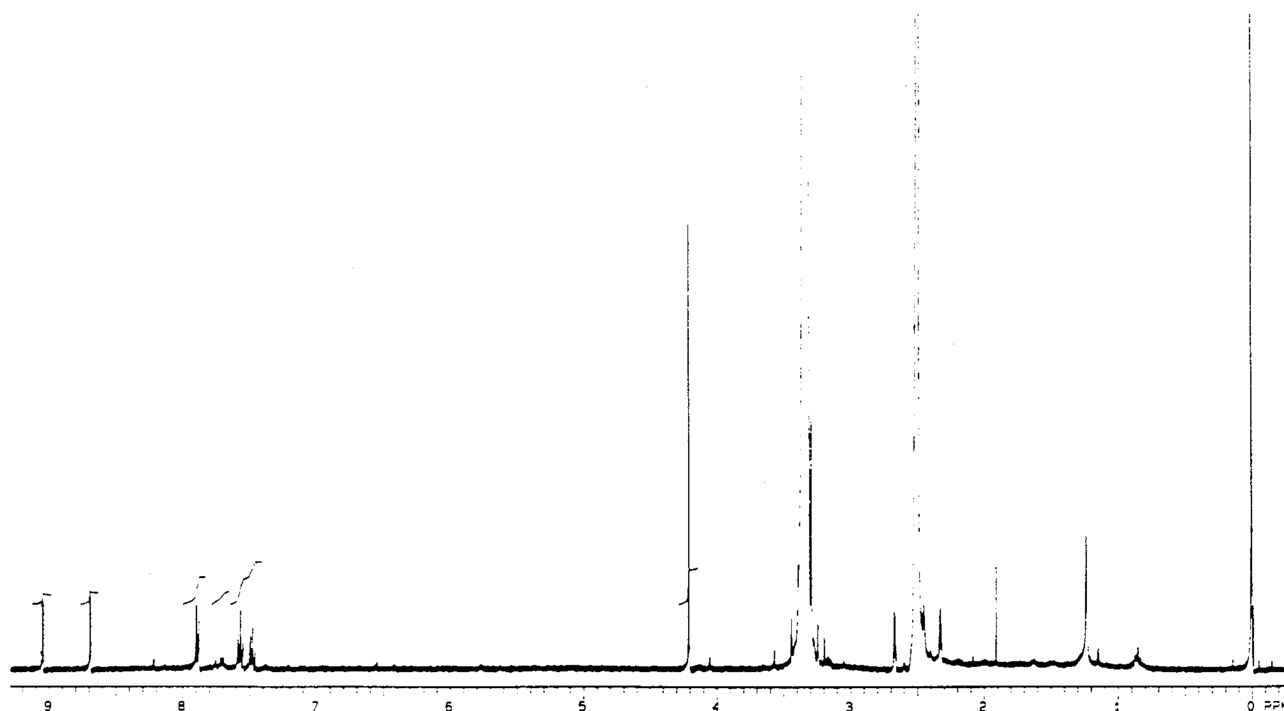

Fig. 3. The 400 MHz ^{1}H-NMR spectrum of NO_2-PhIP, dissolved in d_6-DMSO. Signals at δ 1.2, 1.9, 2.5 and 3.3 originate from the solvent. Proton assignment: singlet at δ 4.2 (N-CH_3), triplet at δ 7.5 (H-4'), triplet at δ 7.6 (H-3' and H-5'), doublet at δ 7.9 (H-2' and H-6'), singlet at δ 8.7 (H-7) and singlet at δ 9.0 (H-5).

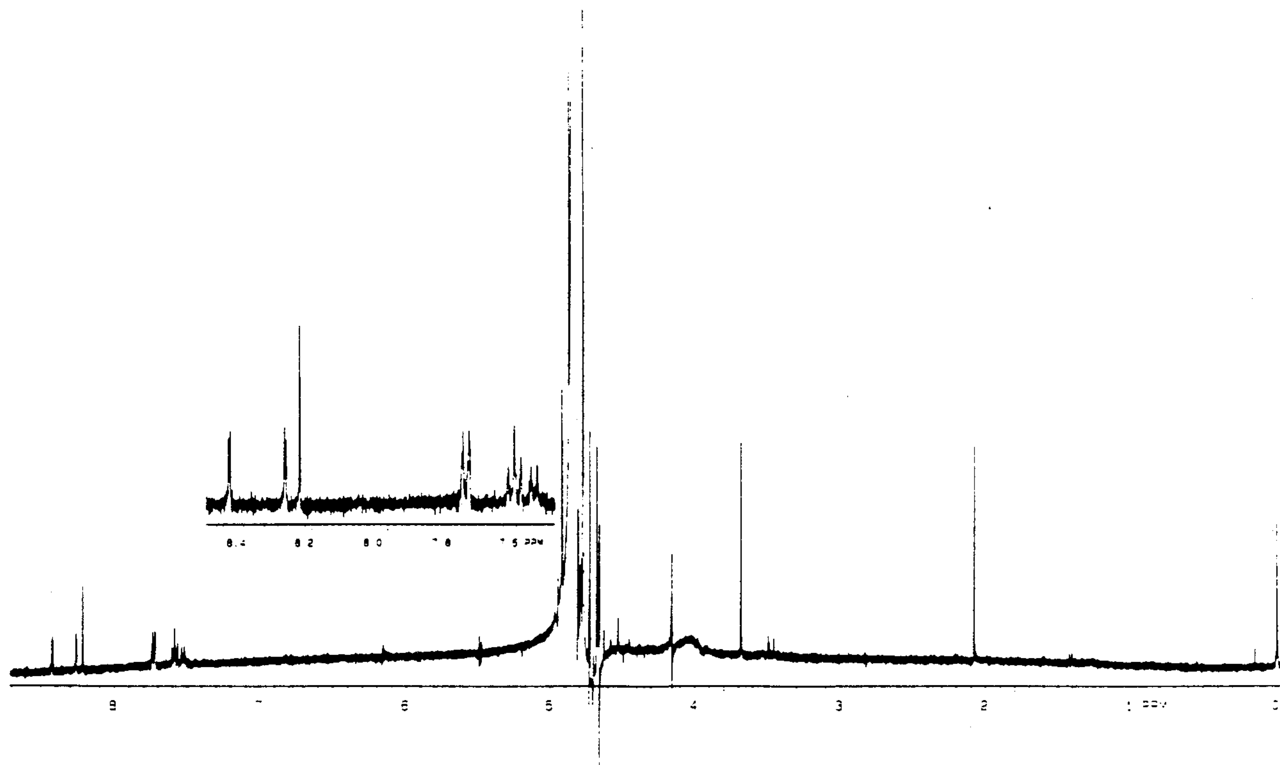

Fig. 4. 400 MHz ^{1}H-NMR spectrum of *N*-OH-PhIP, dissolved in D_2O made acidic to pH 4 with DCl. Signals at δ 2.1, 4.8 and 8.2 originate from the solvent. Inset: the aromatic region enlarged. Proton assignment: singlet at δ 3.7 (N-CH_3), doublet δ 7.5 (H-4'), triplet at δ 7.6 (H-3' and H-5'), doublet at δ 7.7 (H-2' and H-6'), and two doublets at δ 8.3 and 8.4 (H-7 and H-5). NH and OH protons are not seen due to exchange with deuterium.

by Alexander *et al.* (1989), indicating the identity of this compound.

N-OH-PhIP purified from either microsomal incubations or prepared by chemical synthesis was tested separately for mutagenic activity in the Ames tester strain TA98 without metabolic activation, and almost linear increases in the formation of mutant colonies with dose were observed. Two different preparations of synthesized and metabolically formed *N*-OH-PhIP were tested in duplicates and the specific mutagenic activities were calculated by linear regression analysis and found to be 2796 ± 237 (mean ± SD, r = 0.96) revertants induced per nanomol of the metabolically formed *N*-OH-PhIP and 2638 ± 158 (mean ± SD, r = 0.98) revertants induced per nanomole of synthetic *N*-OH-PhIP.

These results were obtained with the addition of 1 mM dithiothreitol prior to the addition of the test compound. If dithiothreitol was omitted from the incubation mixture *N*-OH-PhIP induced only 180 ± 52 (mean ± SD) revertants/nmol. If dithiothreitol was substituted with a metabolic activation system the specific mutagenic activity was 1970 ± 100 (mean ± SD) revertants/nmol.

The results of the SCE assays are presented in Figure 8. No significant differences between replica cultures and between experiments were found for PhIP with metabolic activation. The same apply to *N*-OH-PhIP without activation. For both substances the lowest concentration tested caused a significant increase in SCE (*N*-OH-PhIP: 0.51 nmol/ml, $P < 0.01$; PhIP: 2.67 nmol/ml, $P < 0.01$). Significant dose dependent increases in SCE (linear trend, $P < 0.01$) were found in all experiments. *N*-OH-PhIP and PhIP induced 0.062 and 0.022 SCE/chromosome/nmol added to the media respectively. At the

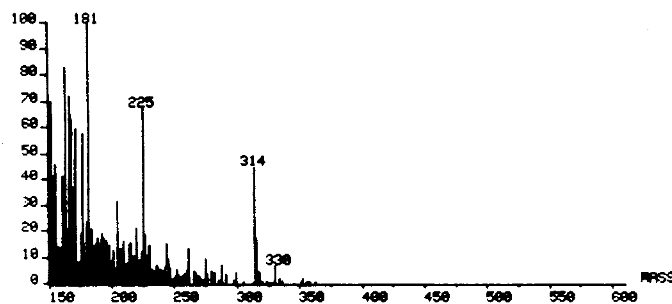

Fig. 5. Plasma spray LC MS spectrum of 2-(phenylazoxy)-1-methyl-6-phenylimiazo(4,5-*b*)pyridine, showing the molecular ion at m/z = 330 $(M+H)^+$ and a daughter ion at m/z = 314 $(M+H)^+$-O.

highest doses tested *N*-OH-PhIP and PhIP induced 13 and 19% mitotic inhibition, respectively. The positive controls benzo[*a*]pyrene with metabolic activation and Mitomycin C without also significantly increasing the number of SCE.

The oxygen concentrations in the SCE assay incubations mixtures was measured to 8.3 mg/ml without metabolic activation system, to 6.1 mg/ml with metabolic activation system and to 3.3 mg/ml with the addition of 0.5 mM dithiothreitol.

Discussion

Several of the heteroaromatic amines formed during frying of meat have been shown to be biotransformed into direct acting bacterial mutagens by hydroxylation of the exocyclic aminogroup (Kadlubar and Beland, 1985; Snyderwine *et al.*, 1987; Yamazoe

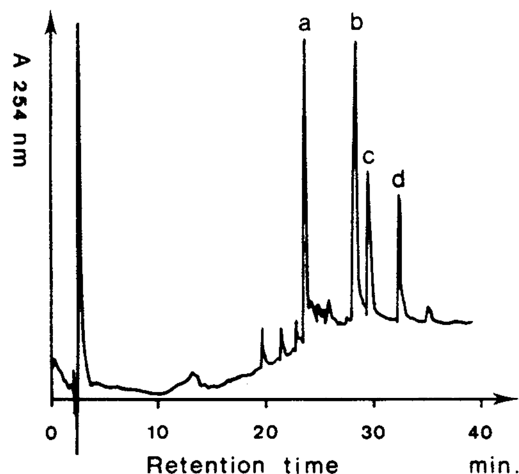

Fig. 6. HPLC analysis of microsomal PhIP metabolites after 30 min incubation. Peak **a** is identified as 4'-hydroxy-PhIP. Peak **b** is *N*-OH-PhIP. Peak **c** is PhIP. Peak d was also present in a control incubation not containing PhIP.

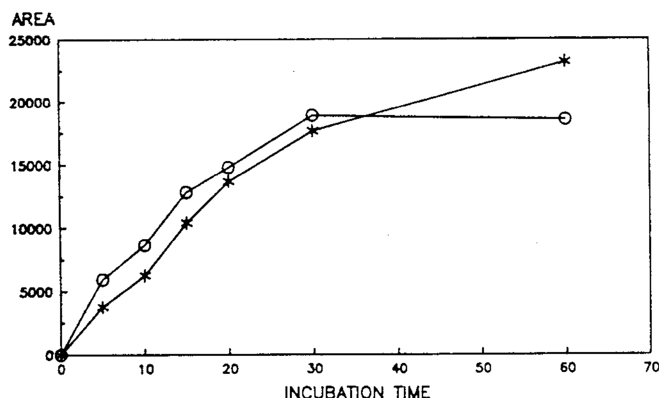

Fig. 7. Time course of formation of the two major microsomal PhIP metabolites. Each point represents the peak area, to the various incubations times (minutes), measured at 254 nm in HPLC analysis. ○: N-OH-PhIP; *: 4'-hydroxy-PhIP.

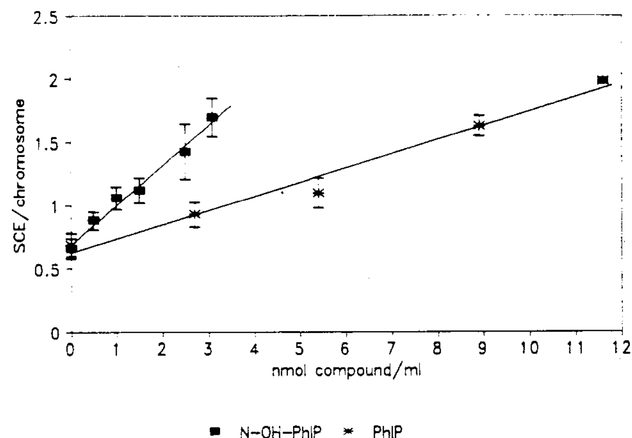

Fig. 8. SCE induced in CHO cells by *N*-OH-PhIP without metabolic activation and induced by PhIP with microsomal activation system. Values presented are means ± SD of duplicates of two separate experiments.

et al., 1988; Holme *et al.*, 1989; Wallin *et al.*, 1990). Holme *et al.* (1989) and Turteltaub *et al.*. (1990) have recently reported that PhIP is transformed by hepatocytes from PCB-treated rats or by microsomes from 3-methylcholanthrene treated mice to the directly mutagenic *N*-hydroxy derivative. Furthermore PhIP induced SCE in V79 cells when co-cultured with hepatocytes from PCB treated rats. The *N*-hydroxy derivative was tentatively identified by its reactivity in a colorimetric assay for *N*-hydroxylated amines and by co-elution in HPLC and identity of UV spectra with *N*-OH-PhIP obtained by enzymatic reduction of NO_2-PhIP (Holme *et al.*, 1989) or by co-elution in HPLC with synthetic *N*-OH-PhIP (Turteltaub *et al.*, 1990).

The present study describes a detailed method for synthesis of the nitro and *N*-hydroxy derivatives of PhIP and provides final evidence that PhIP, in accordance with the previously published results by Wallin *et al.* (1990) and Turteltaub *et al.* (1990), is metabolized by PCB-induced hepatic microsomes to *N*-OH-PhIP, and that this compound is mutagenic in *S.typhimurium* TA98 without metabolic activation. The structure is confirmed by comparing MS, UV and HPLC data on the *N*-OH-PhIP purified from microsomal incubations with data on the synthetic *N*-OH-PhIP. Furthermore we have characterized the synthetic *N*-OH-PhIP by NMR spectroscopy and by synthesis of the phenylazoxy derivative. The HPLC fraction containing the microsomally formed *N*-OH-PhIP shows, as the only fraction, reactivity in the assay for *N*-hydroxylated amines, in analogy with the results obtained with synthetic *N*-OH-PhIP. This further confirms the structure and we are also able to show that the specific mutagenic activities in *S.typhimurium* of microsomally formed and synthetic *N*-OH-PhIP are identical. The synthetic *N*-OH-PhIP was found to be directly genotoxic to CHO cells. In this assay *N*-OH-PhIP induced SCE with a specific activity three times higher than PhIP, the latter with metabolic activation. This result clearly indicates that *N*-OH-PhIP is a proximate mutagenic metabolite in eukaryotic cells as well as in bacteria.

As *N*-OH-PhIP is readily oxidized, we found it necessary to add an antioxidant to the test mixtures for genotoxic activity, in order to obtain reproducible results. If the antioxidant was omitted from the incubation mixture, large variations were observed in the specific mutagenic/genotoxic activities obtained from different experiments. *N*-OH-PhIP has a four times higher specific mutagenic activity in the Ames Salmonella assay than PhIP, after addition of a metabolic activation system. This is in agreement with that PhIP is only partially metabolized to *N*-OH-PhIP. When the metabolic activation system is substituted with 1 mM dithiothreitol the specific mutagenic activity of *N*-OH-PhIP is six times higher than the specific mutagenic activity of PhIP after metabolic activation. The lower oxygen content in assay mixtures containing dithiothreitol than in assay mixtures containing a microsomal activation system can, by improving the stability of *N*-OH-PhIP, account for this higher mutagenic activity of *N*-OH-PhIP. Addition of antioxidants has previously been shown to be beneficial in the testing of readily oxidized compounds (Yamazoe *et al.*, 1983). Addition of dithiothreitol to a solution of *N*-OH-PhIP did not change the HPLC retention time of *N*-OH-PhIP and dithiothreitol did not induce SCE in CHO cells or His^+ revertants in the Ames test. Thus dithiothreitol does not seem to react with *N*-OH-PhIP but only to exert a protective action toward oxidation.

Mutagens/carcinogens exert their action through reactions with DNA. In a preliminary experiment, we were not able to detect any reaction between DNA and *N*-OH-PhIP, when incubated for several days at room temperature under oxygen free conditions. Although *N*-hydroxylation seems to be a necessary metabolic step in the mutagenicity of PhIP, further activation is apparently

needed for reaction with DNA. Studies have shown that aryl hydroxylamines are very weak mutagens in an acetyl-CoA-dependent O-acetyltransferase deficient strain of *S.typhimurium* (TA98 1,8-DNP_6) (Saito *et al.*, 1983). Other studies indicate that sulphonylation or acylation of 2-hydroxyamino-3-methylimidazo(4,5-*f*)quinoline (*N*-hydroxy-IQ) by bacterial *O*-acyltransferase or sulfotransferase enhance the reactivity of *N*-hydroxy-IQ towards DNA 5- to 30-fold (Snyderwine *et al.*, 1988). Therefore, *N*-OH-PhIP may need further activation by acetylation or sulfonylation of the *N*-hydroxy group, improving the leaving group capability of the hydroxy group and facilitating the formation of a reactive nitrene or nitrenium ion. Such reactive intermediates have been generated by photolysis of the arylazide of 2-amino-3-methylimidazo(4,5-*f*)quinoline (IQ) and was shown to react with DNA (Wild *et al.*, 1989).

Acknowledgements

The authors wish to thank Rolf Andersson and Kjell Olsson, Swedish University of Agricultural Sciences for performing ¹H-NMR analysis, Arne Büchert for EI mass spectrometric measurements, J.Lamb, The Medical Research Council, Carlshalton, Surrey, UK, for assistance with FAB mass spectrometry, and Joan Gluver, Bo Lund Jensen, Jonna Madsen and Vivian Jørgensen for excellent technical assistance. This work was in part supported by grant no. 75 from 'Nordiska Kontaktorganet för Jordbruksforskning'.

References

Alexander,J., Wallin,H., Holme,J.A. and Becher,G. (1989) 4-(2-amino-1-methylimidazo(4,5-*b*)pyrid-6-yl)phenyl sulfate—a major metabolite of the food mutagen 2-amino-1-methyl-6-phenylimidazo(4,5-*b*)pyridine (PhIP) in the rat. *Carcinogenesis*, **10**, 1543–1547.

Belanger,P.M., Belanger,O.G. and Blouin,M. (1981) Colorimetric determination of *N*-hydroxylated metabolites in microsomal studies. *Anal. Biochem.*, **118**, 47–52.

Dean,B.J. and Danford,N. (1984) Assays for the detection of chemically-induced chromosome damage in cultured mammalian cells. In Venitt,S. and Parry,J.M. (eds), *Mutagenicity testing—a practical approach*. IRL Press, Oxford.

Felton,J.S., Knize,M.G., Shen,N.H., Andresen,B.D., Bjeldanes,L.F. and Hatch,F.T. (1986a) Identification of the mutagens in cooked beef. *Environ. Health Perspect.*, **67**, 17–24.

Felton,J.S,, Knize,M.G., Shen,N.H., Lewis,P.R., Andresen,B.D., Happe,J. and Hatch,F.T. (1986b) The isolation and identification of a new mutagen from fried ground beef: 2-amino-1-methyl-6-phenylimidazo(4,5-*b*)pyridine (PhIP). *Carcinogenesis.*, **7**, 1081–1086.

Hashimoto,Y., Shudo,K. and Okamoto,T. (1982) Modification of DNA with potent mutacarcinogenic 2-amino-6-methylpyrido(1,2-*a*:3′,2′-*d*)imidazole isolated from a glutamic acid pyrolysate: structure of the modified nucleic acid base and initial chemical event caused by the mutagen. *J. Am. Chem. Soc.*, **104**, 7636–7640.

Holme,J.A., Wallin,H., Brunborg,G., Søderlund,E.J., Hongslo,J.K. and Alexander J. (1989) Genotoxicity of the food mutagen 2-amino-1-methyl-6-phenylimidazo(4,5-*b*)pyridine (PhIP): formation of 2-hydroxyamino-PhIP, a direct acting genotoxic metabolite. *Carcinogenesis*, **10**, 1389–1396.

Kadlubar,F.F. and Beland,F.A. (1985) Chemical properties of ultimate carcinogenic metabolites of arylamines and arylamides. In Harvey,R.G. (ed.), *Polycyclic hydrocarbons and carcinogenesis. ACS Symposium Series*. American Chemical Society, Washington, DC, Vol. 283, pp. 341–370.

Lake,B.G. (1987) Preparation and characterisation of microsomal fractions for studies on xenobiotic metabolism. In Snell,K. and Mullock,B. (eds), *Biochemical Toxicology: A Practical Approach*. IRL Press, Oxford, pp. 183–215.

Maron,D. and Ames,B.N. (1983) Revised methods for the *Salmonella* mutagenicity test. *Mutat. Res.*, **113**, 173–215.

Nielsen,P.A., Vahl,M. and Gry,J. (1988) HPLC profiles of mutagens in lean ground pork fried at different temperatures. *Z; Lebensm. Unters. Forsch.*, **187**, 451–456.

Saito,K., Yamazoe,Y., Kamataki,T. and Kato,R. (1983) Mechanism of activation of proximate mutagens in Ames tester strains: The acetyl-CoA dependent enzyme in *Salmonella typhimurium* TA 98 deficient in TA 98/1,8-DNP_6 catalyzes DNA-binding as the cause of mutagenicity. *Biochem. Biophys. Res. Commun.*, **116**, 141–147.

Sedmark,J.J. and Grossberg,S.E. (1977) A rapid, sensitive and versatile assay for protein using coomassie brilliant blue G250. *Anal. Biochem.*, **79**, 544–552.

Snyderwine,E.G., Roller,P.P., Wirth,P.J., Adamson,R.H., Sato,S. and Thorgeirsson,S.S. (1987) Synthesis, purification and mutagenicity of 2-hydroxyamino-3-methylimidazolo(4,5-*f*)quinoline. *Carcinogenesis*, **7**, 1017–1020.

Snyderwine,E.G., Wirth,P.J., Roller,P.P., Adamson,R.H., Sato,S. and Thorgeirsson,S.S. (1988) Mutagenicity and *in vitro* covalent DNA binding of 2-hydroxyamino-3-methylimidazolo(4,5-*f*)quinoline. *Carcinogenesis*, **9**, 411–418.

Thomson,L.H., Tucker,J.D., Stewart,S.A., Christenson,M.L., Salazer,E.P., Carrano,A.V. and Felton,J.S. (1987) Genotoxicity of compounds from cooked beef in repair-deficient CHO cells versus *Salmonella* mutagenicity. *Mutagenesis*, **2**, 483–487.

Turteltaub,K.W., Knize,M.G., Buonarati,M.H., McManus,M.E., Veronese,M.E., Mazrimas,J.A. and Felton,J.S. (1990) Metabolism of 2-amino-1-methyl-6-phenyl(4,5-*b*)pyridine(PhIP) by liver microsomes and isolated rabbit cytochrome P450-isozymes. *Carcinogenesis*, **11**, 941–946.

Wallin,H., Mikalsen,A., Guengrich,F.P., Ingelman-Sundberg,M., Solberg,K.E., Rossland,J. and Alexander,J. (1990) Differential rates of metabolic activation and detoxification of the food mutagen 2-amino-1-methyl-6-phenylimidazo(4,5-*b*) pyridine by different cytochrome P450 enzymes. *Carcinogenesis*, **11**, 489–492.

Wild,D., Dirr,A., Fasshauer,I. and Henschler,D. (1989) Photolysis of arylazides and generation of highly electrophilic DNA-binding and mutagenic intermediates. *Carcinogenesis*, **10**, 335–341.

Yamazoe,Y., Kamataki,T. and Kato,R. (1981) A sensitive method for determination of biphenyl hydroxylase activities using high-performance liquid chromatography. *Anal. Biochem.*, **111**, 126–130.

Yamazoe,Y., Shimada,M., Kamataki,T. and Kato,R. (1983) Microsomal activation of 2-amino-3-methylimidazo(4,5-*f*)quinoline, a pyrolysate of sardine and beef extracts, to a mutagenic intermediate. *Cancer Res.*, **43**, 5768–5774.

Yamazoe,Y., Medhat,A., Shunichi,M., Toyama,S. and Kato,R. (1988) Metabolic activation of a protein pyrolysate promutagen 2-amino-3,8-dimethylimidazo (4,5-*f*)quinoxaline by rat liver microsomes and purified cytochrome P-450. *Carcinogenesis*, **9**, 105–109.

Received on August 15, 1990; accepted on October 8, 1990

PAPER II

Carcinogenesis vol.13 no.4 pp.629–635, 1992

Reaction of the N^2-acetoxy derivative of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) with 2′-deoxyguanosine and DNA. Synthesis and identification of N^2-(2′-deoxyguanosin-8-yl)–PhIP

Henrik Frandsen, Spiros Grivas[1], Rolf Andersson[1], Lars Dragsted and John C.Larsen

Institute of Toxicology, National Food Agency, Mørkhøj Bygade 19, DK-2860 Søborg, Denmark and [1]Department of Chemistry, Swedish University of Agricultural Sciences, PO Box 7015, S-750 07 Uppsala, Sweden

The direct acting mutagenic N^2-hydroxylated metabolite of the food mutagen 2-amino-1-methyl-6-phenylimidazo-[4,5-*b*]pyridine (PhIP) does not react with DNA. Upon acetylation of the N^2-hydroxy-PhIP with acetic anhydride two products could be detected. Mass spectrometric analysis showed that both products were monoacetyl derivatives of N^2-hydroxy-PhIP. One of the products did not show any reactivity towards DNA and is probably the *N*-acetyl derivative of N^2-hydroxy-PhIP. The other product which is most likely to be N^2-acetoxy-PhIP reacted with DNA and 2′-deoxyguanosine but not with 2′-deoxycytidine, 2′-deoxyadenosine or 2′-deoxythymidine. The PhIP-2′-deoxyguanosine adduct was purified and characterized by mass spectral, ^{1}H and [^{13}C]NMR analysis, showing that PhIP like the other cooked food mutagen 2-amino-3-methylimidazo[4,5-*f*]quinoline, had reacted with C-8 of guanine forming N^2-(2′-deoxyguanosin-8-yl)–PhIP. HPLC analysis of enzymatically hydrolyzed calf thymus DNA which had been reacted with N^2-acetoxy-PhIP showed one adduct which was chromatographically and spectroscopically identical to N^2-(2′-deoxyguanosin-8-yl)-PhIP. HPLC separation followed by liquid scintillation counting of hydrolyzed liver DNA from a rat dosed with [^{3}H]PhIP showed that radioactivity coeluted with the hydrolysis product of the synthetic PhIP–2-deoxyguanosine adduct, indicating that PhIP *in vivo* also forms an N^2-(2′-deoxyguanosin-8-yl)–PhIP adduct.

Introduction

The heterocyclic aromatic amine 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP*) is an indirect mutagenic compound formed during frying of meat (1–3). PhIP, which constitutes ~80% of the mass of mutagenic material in beef fried at high temperatures, has been shown to induce sister chromatid exchanges (SCEs) in mammalian cells (4–6). PhIP has also been shown to induce lymphomas in mice (7), to induce intestinal adenocarcinomas in rats (8), to produce DNA adducts in various organs in rats (9) and has recently been found to induce aberrant crypt foci in the large intestine of rats (10).

*__Abbreviations:__ PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; SCEs, sister chromatid exchanges; N^2-OH- PhIP, 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; N^2-OAc-PhIP, 2-acetoxyamino-PhIP; N^2-(2′-dG-8-yl)–PhIP, N^2-(2′-deoxyguanosin-8-yl)-PhIP; PCB, polychlorinated biphenyl; FAB, fast atom bombardment; BES, *N,N*-bis(2-hydroxyethyl)-2-aminoethane sulfonic acid; DMF, dimethylformanide; DMSO, dimethylsulfoxide; N^2-Ac-N^2-OH-PhIP, *N,N*-acetylhydroxyamino-PhIP; 2-OH-PhIP, 2-hydroxy-1-methyl-6-phenylimidazo-[4,5-*b*]pyridine; DEPT, distortionless enhancement by polarization transfer; N^2-OH-IQ, 2-hydroxyamino-3-methylimidazo[4,5-*f*]quinoline; Glu-P-1, 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole; Trp-P-2, 3-amino-1-methyl-5*H*-pyrido-[4,3-*b*]indole.

© Oxford University Press

PhIP is metabolically activated by hepatocytes or hepatic microsomes, from rat, mouse and rabbit, to the proximate mutagenic N^2-hydroxy derivative (5,6,11–13), but further activation is apparently needed for the formation of an ultimate mutagenic metabolite, which is reactive towards DNA (6,12). Previous studies have shown that 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (N^2-OH-PhIP) can bind covalently to DNA after further activation with cytosolic *O*-acetyltransferase or *O*-sulfotransferase or acetic anhydride (14,15). While PhIP, by [^{32}P]postlabeling, has been shown to form adducts with DNA *in vivo* and *in vitro*, the DNA adducts have not previously been characterized. In this study N^2-(2′-deoxyguanosin-8-yl)–PhIP [N^2-(2′-dG-8-yl)–PhIP] was synthesized and was shown by HPLC, combined with diode array detection or liquid scintillation counting, to be the major adduct formed, *in vitro* upon reaction between DNA in 2-acetyoxyamino-PhIP (N^2-OAc-PhIP) and *in vivo* in the rat after dosing with [^{3}H]PhIP.

Materials and methods

Chemicals and instrumentation

PhIP was kindly donated by Dr Errol Zieger, Cellular and Genetic Toxicology Branch, NIEHS, North Carolina. Non-specifically labeled [^{3}H]PhIP was made from PhIP by Amersham, UK. The exact position of tritium in the [^{3}H]PhIP is not known. The [^{3}H]PhIP was adsorbed on a SEP-PAK and washed with 0.1 M HCl, to remove exchangeable tritium, and eluted with acetonitrile followed by purification on HPLC. The sp. act. was 21.1 Ci/mol and the radiochemical purity was >95%. N^2-OH-PhIP was synthesized as previously described (6). Calf thymus DNA, 2′-deoxyadenosine, 2′-dexoycytidine, 2′-deoxyguanosine, 2′-deoxythymidine and nuclease P1 (EC3.1.30.2) from *Pencillium citrinum* were obtained from Sigma, St Louis, MO. Alkaline phosphatase (*Escherichia coli* C75) was obtained from Amersham, UK. Polychlorinated biphenyl (PCB) (Aroclor 1254) was obtained from Monsanto Industrial Chemical Co., St Louis, MO. C_{18} SEP-PAK columns were obtained from Waters, Milford, MA. HPLC grade acetonitrile was obtained from Rathburn, Walkerburn, UK. Silica gel 60 (70–230 mesh), silica TLC plates and all other chemicals were obtained from Merck, Darmstadt, Germany.

Evaporations of solvents under reduced pressure were accomplished with a Büchi rotary evaporator. HPLC analyses were performed with either a Hewlett Packard model 1084 B liquid chromatograph or a Hewlett Packard 1090 M liquid chromatograph equipped with a photodiode array detector. Nucleosil HPLC columns were obtained from HPLC technology, Macclesfield Cheshire, UK. Spherisorb HPLC columns were obtained from Phase Separation, Deeside Ind. Est., Queensferry, Clwyd, UK. Positive fast atom bombardment (FAB) mass spectra were recorded at 8 kV using Xenon as neutral beam and glycerol or glycerol/4-toluenesulfonic acid as matrix. Positive ion thermospray LC-MS was performed on a Kratos Profile mass spectrometer equipped with a thermospray interphase. The solvent was 50% 0.1 M ammonium acetate pH 4.5 in acetonitrile, flow 1 ml/min, source temperature 73°C. NMR spectra were recorded with a Varian VXR-400 FT spectrometer with DMSO-d_6 as solvent. Radioactivity was measured with a Packard Tri-Carb 460 CD liquid scintillation counter with external standardization.

Acetylation of N^2-OH-PhIP

660 μg of N^2-OH-PhIP (2.75 μmol) was dissolved in 0.5 ml argon purged 50% dimethylformamide in water and cooled to 0°C. Three times 3 μl of acetic anhydride was added during 10 min with stirring. After a further 10 min stirring at 0°C, HPLC analysis of the reaction mixture showed that two major products had been formed (Figure 1). Both products were purified by HPLC on a Nucleosil C_{18}, 5 μm semi-preparative column (250 × 10 mm) at room temperature with a flow rate of 2.0 ml/min. The eluents were 20 mM triethylamine/HCl pH 4.5 in I: water and II: ethanol. Elution programming: 20% II by 3 min, linear gradient to 50% II by 8 min, isocratic 50% II from 8 to 13 min and a linear gradient to 100% II by 20 min. The fractions containing the two products were collected under argon

and evaporated to dryness without heating on a rotary evaporator and analyzed by positive FAB mass spectrometry. One major acetylation product, peak B, tentatively identified as N^2-OAc-PhIP, (Figure 1) was obtained when 5 µl acetic acid was added prior to the addition of acetic anhydride. This methodology was used in the rest of this work.

Reaction of N^2-OAc-PhIP with 2'-deoxynucleosides and purification of N^2-(2'-dG-8-yl)–PhIP

2'-Deoxynucleosides: 2'-deoxyadenosine, 2'-deoxycytidine, 2'-deoxyguanosine and 2'-deoxythymidine were dissolved at a concentration of 1 mg/ml in 0.1 M *N-N*-bis(2-hydroxyethyl)-2-aminoethane sulfonic acid (BES)/NaOH pH 7.4. Two ml of each solution and a control not containing 2'-deoxynucleoside were placed in test tubes and heated to 37°C. One hundred µl of the crude acetylation mixture were added dropwise, during 2 min, to each of the vigorously stirred test tubes. After stirring for a further 15 min the reaction mixtures were analyzed by HPLC.

Since only reaction between N^2-OAc-PhIP and 2'-deoxyguanosine gave a product which was not present in the control incubation, this reaction was scaled up by a factor of 5.

The crude product from acetylation of 660 µg of N^2-OH-PhIP was added, during 10 min, to a well-stirred solution of 10 mg 2'-deoxyguanosine in 10 ml 0.1 M BES/NaOH pH 7.4 at 37°C. In the crude reaction product it was found that ~50% of the N^2-OH-PhIP had reacted with 2'-deoxyguanosine. After a further 10 min of stirring the precipitate was collected, washed with water and dried. The residue was taken up in 5 ml 20% DMF in chloroform and applied on a 1.6 × 9 cm column of silica gel equilibrated with DMF:chloroform, 1:4 v/v. After washing with this solvent system the adduct was eluted with DMF:chloroform, 1:1 v/v and evaporated to dryness. The yield of the adduct from N^2-OH-PhIP was 31%. The yield of adduct was calculated on the basis of experiments where [^{3}H]-N^2-OH-PhIP was used.

The synthesis was repeated several times until enough material for mass spectral and NMR analyses had been collected.

Binding of N^2-OAc-PhIP to DNA

The crude product from acetylation of 500 µg of N^2-OH-PhIP was added, during 10 min, to a well-stirred 37°C solution of 4 mg calf thymus DNA in 4 ml 0.1 M BES/NaOH pH 7.4. After a further 10 min of stirring, the DNA was precipitated by addition of 2 vol ice-cold ethanol, collected, dried and redissolved in 4 ml of water. The DNA solution was extracted twice with 4 ml of dichloromethane, to remove non-covalently bound reaction products and the DNA was precipitated by addition of 100 µl 3 M sodium acetate and 2 vol ice-cold ethanol.

Enzymatic hydrolysis of PhIP–DNA

Calf thymus DNA that had been reacted with N^2-OAc-PhIP was digested enzymatically and analyzed by a procedure modified after Palmgren *et al.* (16). PhIP–DNA was dissolved in a reagent glass at a concentration of 0.25 mg/ml in 30 mM sodium acetate, 1 mM $ZnSO_4$ pH 5.3 and 54 U nuclease P1 and 10 U of alkaline phosphatase were added per ml solution. After incubation at 70°C for 2 h, 54 U of nuclease and 10 U of alkaline phosphatase were added and incubation at 70°C was continued for 2 further hours. The digest was analyzed by HPLC and the eluate was monitored at 260, 280, 316 and 360 nm. Fractions (1 min) of the eluate from HPLC analysis of digested [^{3}H]PhIP–DNA was collected and analyzed by liquid scintillation counting. The amount of PhIP bound to DNA was estimated to be 12 nmol/mg DNA, indicating that one out of 50 guanine residues had formed adducts. The yield of adducts from N^2-OH-PhIP was estimated to be 2.5%.

Modification of DNA in vivo

One adult male Wistar rat (age 8 weeks, weight 250 g), delivered from Møllegård Breeding Center Ltd, Lille Skensved, Denmark, was injected i.p. with 200 mg PCB/kg (Aroclor 1254, dissolved in corn oil). After 5 days the rat was starved overnight and dosed by gavage with [^{3}H]PhIP (1 mg/kg, 21.1 Ci/mol, dissolved in 70% ethanol). The animal was killed after 2 h and the liver removed. The liver was homogenized and the DNA was isolated by the phenol/chloroform extraction procedure previously described (17). The 39 mg DNA obtained was dissolved in 15 ml of 0.1 M hydrochloric acid and hydrolyzed by heating to 100°C for 1 h. The DNA hydrolysate was loaded onto a C_{18} SEP-PAK, which had been washed with acetonitrile and water. After washing with 3 vol of water, the SEP-PAK was eluted with 3 ml of 10% ammonium formate pH 3.5 in acetonitrile. The eluate was evaporated to dryness and the residue was redissolved in 250 µl of 80% acetonitrile and separated by HPLC from which one min fractions were collected and analyzed by liquid scintillation counting. Synthetic N^2-(2'-dG-8-yl)–PhIP and calf thymus DNA reacted with N^2-OAc-PhIP were likewise hydrolyzed in 0.1 M hydrochloric acid for comparison with the rat liver DNA. The hydrolysis product from [^{3}H]PhIP–DNA was purified on a SEP-PAK. Liquid scintillation analysis showed that only a background level of radioactivity was present in the water wash. 90% of the applied radioactivity was recovered in the acetonitrile eluate. The eluate was analyzed by HPLC and TLC (1% acetic acid, 5% methanol in chloroform).

Kinetics

N^2-acetoxy-PhIP (15 µmoles) was added to 1 ml of a 0.15 mM solution of 2'-deoxyguanosine in 0.1 M BES/NaOH pH 7.4 held at 0°C and a control not containing 2'-deoxyguanosine. The reaction mixtures were stored at 0°C and analyzed by HPLC every 30 min. The residual concentration of N^2-acetoxy-PhIP was determined.

Results

Acetylation of N^2-OH-PhIP with acetic anhydride showed two products when analyzed by HPLC (Figure 1). Product A seemed to be quite stable relative to N^2-OH-PhIP, while product B was

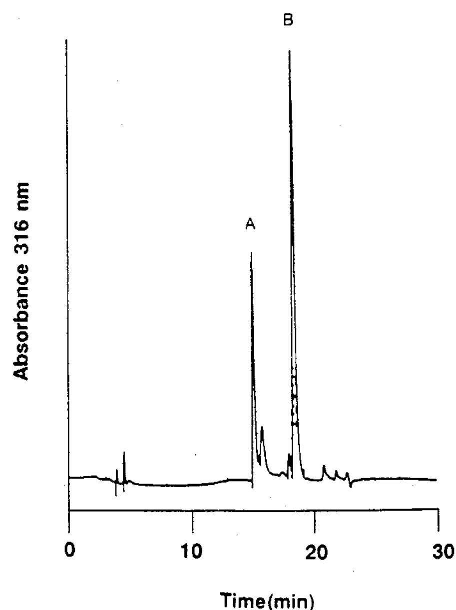

Fig. 1. Chromatogram of the crude product from acetylation of N^2-OH-PhIP with acetic anhydride, analyzed on a Nucleosil C_{18}, 5 µm 250 × 4.6 mm. Flow rate 0.8 ml/min. Solvents: I, 20 mM triethylamine/HCl pH 4.5 in water; II, 20 mM triethylamine/HCl pH 4.5 in ethanol. Elution programming: isocratic 20% II by 3 min, linear gradient to 50% II by 8 min, isocratic 50% II by 13 min and a linear gradient to 100% II by 20 min. **Peak B** tentatively identified as N^2-OAc-PhIP, while **peak A** is tentatively identified as N^2-Ac-N^2-OH-PhIP.

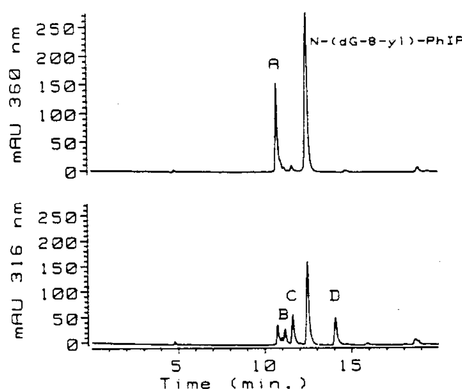

Fig. 2. Chromatograms, monitored at 316 and 360 nm, of the crude reaction mixture of acetylated N^2-OH-PhIP and 2'-deoxyguanosine, analyzed on a Nucleosil C_{18}, 5 µm, 25 cm × 4.6 mm, Flow rate 0.8 ml/min. Solvents: I, 50 mM ammonium formate pH 3.5; II, acetonitrile. Elution programming: isocratic 20% II by 3 min, a linear gradient to 50% II by 8 min, isocratic 50% II by 13 min and a linear gradient to 100% II by 20 min. **Peak A** is an unidentified rearrangement product of N^2-OAc-PhIP. **Peak B** is the putative N^2-Ac-N^2-OH-PhIP. **Peak C** is N^2-OH-PhIP and **peak D** is 2-OH-PhIP, a minor impurity in the N^2-OH-PhIP used. N^2-(2'-dG-8-yl)–PhIP is the adduct between PhIP and 2'-deoxyguanosine.

quite unstable and rapidly decomposed when stored at room temperature at neutral pH. The stability of product B was improved at acidic pH. Both products were purified by HPLC and analyzed by positive FAB mass spectrometry (spectra not shown). Both peaks A and B showed a molecular ion, $(M+H)^+$, at *m/z*, 283 and a daughter ion at 241 (loss of ketene). The mass spectra shows that both products are monoacetyl derivatives of N^2-OH-PhIP. The chemical reactivities of the two compounds, relative to N^2-OH-PhIP, indicate that A, which does not react with DNA or 2′-deoxynucleosides, is *N,N* -acetylhydroxyamino-PhIP (N^2-Ac-N^2-OH-PhIP) and that peak B, which does react with both DNA and 2′-deoxyguanosine, is N^2-OAc-PhIP (18). The relative yields of the two products could be varied by changing the reaction conditions. Product A was exclusively observed when pyridine was added to the reaction mixture, while addition of acetic acid resulted in formation of product B, tentatively identified as N^2-OAc-PhIP, as the major product.

Reaction of N^2-OAc-PhIP with 2′-deoxynucleosides. Synthesis of N^2-dG-8-yl) − PhIP

N^2-OAc-PhIP reacted with 2′-deoxyguanosine in neutral solution, whereas addition of N^2-OAc-PhIP to solutions of 2′-deoxyadenosine, 2′-deoxycytidine, and 2′-deoxythymidine only resulted in products which were also present in a control incubation not containing 2′-deoxynucleoside. Figure 2 shows a chromatogram of the crude product from reaction of N^2-OAc-PhIP and 2′-deoxyguanosine. Compound B eluting at 11.5 min is the putative N^2-Ac-N^2-OH-PhIP and compound D eluting at 14 min is 2-OH-PhIP, which is a minor impurity originating from the synthesis of the N^2-OH-PhIP. An unidentified compound (peak, A), with high absorption at 360 nm, eluted at ~11 min. The area of this peak was much larger when 2′-deoxyguanosine was not present in the reaction mixture, and this compound might be a decomposition product of N^2-OAc-PhIP. The adduct, which eluted at 12.5 min, precipitated quantitatively out of the solution and was further purified by silica gel chromatography, which removed a red colored impurity, that did not elute from the HPLC column. The UV spectrum of the adduct shows maxima at 364.5, 270.5 and 238.5 nm (Figure 3). The positive ion thermospray mass spectrum of the purified product showed a molecular ion at 490 $(M+H)^+$ (Figure 4), which is consistent with an adduct between PhIP and 2′-deoxyguanosine. As shown in Figure 5, its [^{1}H]NMR spectrum in DMSO-d_6 revealed the presence of all sugar protons, the two pyridine protons (H-5 and H-7), the five benzene protons (H-2′- H-6′), the 2″-amino group of the base and of the *N*-Me group of PhIP. However, the characteristic C-8 proton of guanine was not present. This observation is a strong indication that the nitrogen of the 2-amino group of PhIP was attached to the C-8 of 2′-deoxyguanosine. That was confirmed through [^{13}C]NMR spectroscopy where all 21 carbon atoms could be observed, and those with a single H bound to them were enhanced in the distortionless enhancement by polarization transfer (DEPT) experiment (see Table I). Again, the absence of a [^{13}C]signal from guanines C-9 when the DEPT technique was employed confirms that PhIP is bound to this carbon atom. These results from the [^{1}H]- and [^{13}C]NMR spectroscopy taken together with the thermospray mass spectrum corroborate that the structure of the adduct is N^2-(2′-dG-8-yl)−PhIP. The reaction scheme is depicted in Figure 6.

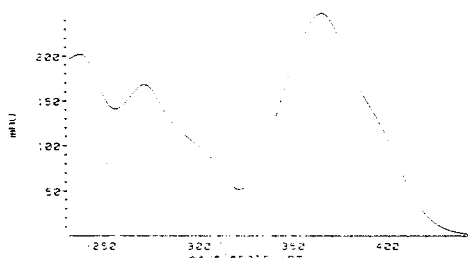

Fig. 3. UV spectrum of N^2-(2′-dG-8-yl)−PhIP obtained with a photodiode array detector at the elution conditions from the HPLC column (see Figure 2).

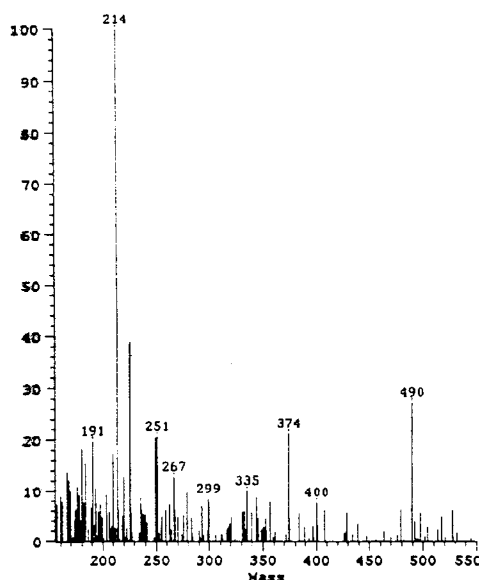

Fig. 4. Positive ion thermospray mass spectrum of N^2-(2′-dG-8-yl)−PhIP showing the molecular ion $(M+H)^+$ at m/z = 490 and a duaghter ion at m/z = 374, loss of deoxyribose. Solvent: 50% 0.1 M ammonium acetate pH 4.5 in acetonitrile, flow 1 ml/min, source temperature 73°C.

Table I. [^{13}C]NMR chemical shifts of N^2-(2′-dG-8-yl)−PhIP and PhIP

N^2-(2′-dG-8-yl)−PhIP	PhIP	Assignment
154.29	157.98	C-3a
152.89	156.29	C-2
152.74		C-6″
149.74		C-2″
148.95		C-4″
146.11		C-8″
139.48	139.29	C-5
137.98	139.03	C-1′
129.93	127.87	C-7a
128.97*	128.86	C-3′,5′
127.34*	126.56	C-4′
126.79*	126.43	C-2′,6′
125.88	126.22	C-6
113.42*	111.63	C-7
108.66		C-5″
87.37*		C-4‴
82.05*		C-1‴
71.32*		C-3‴
62.34		C-5‴
38.87		C-2‴
28.11	28.40	*N*-Me

[^{13}C]NMR chemical shifts of N^2-(2′-dG-8-yl)−PhIP at 20°C, referenced to the solvent [δ (DMSO-d_6) 39.50]. Assignments for PhIP are based on two-dimensional experiments and for N^2-(2′-dG-8-yl)−PhIP on comparisons to the chemical shifts of 2′-deoxyguanosine in DMSO-d_6 and to those of PhIP. Values marked with an asterisk are those peaks enhanced in the DEPT experiment.

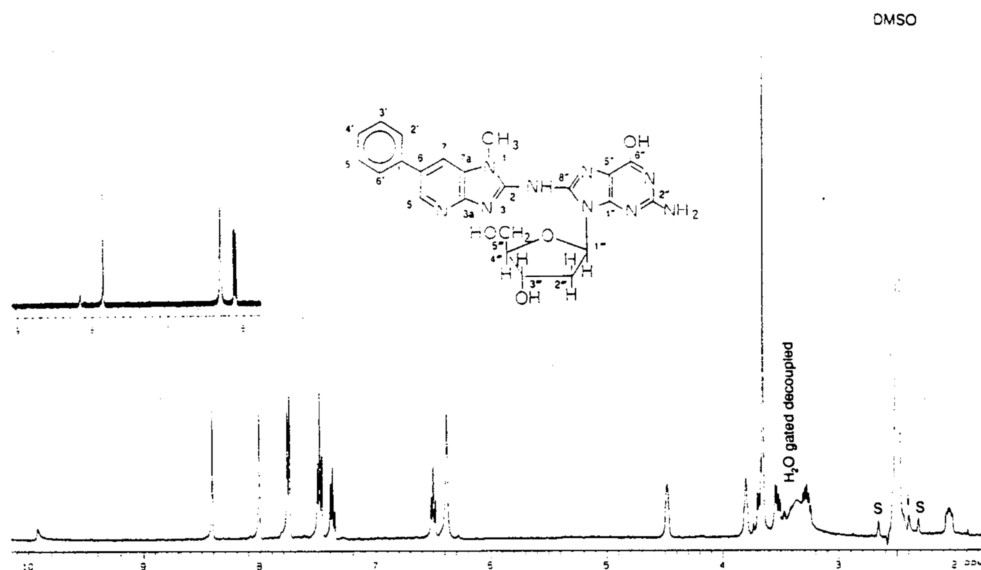

Fig. 5. 400 MHz ^{1}H-NMR spectrum of N^2(2-dG-8-yl)−PhIP at 20°C. The chemical shifts are referenced to the solvent [δ (DMSO-d_6)2.49]: δ = 9.9 (brs, 6″-OH), 8.41 (d, H-5), 8.01 (d, H-7), 7.76 (d, H-2′ and H-6′), 7.49 (t, H-3′ and H-5′), 7.38 (t, H-4′), 6.50 (t, H-1‴), 6.38 (brs, 2″-NH_2), 4.48 (m, H-3‴), 3.80 (m, H-4‴), ca. 3.7 (partly hidden, H-5‴b), 3.65 (s, N-CH_3), 3.53 (dd, H-5‴a), 3.27 (dt, H-2‴b), 2.05 (ddd, H-2‴a). $J_{5,7}$ = 2.0 Hz, $J_{2',3'}$ = 7.9 Hz, $J_{3',4'}$ = 7.9 Hz, $J_{1''',2'''a} = J_{1''',2'''b}$ = 7.4 Hz, $J_{2'''a,2'''b}$ = 13.7 Hz, $J_{2'''a,3'''}$ = 2.7 Hz, $J_{2'''b,3'''}$ = ca. 6 Hz, $J_{3''',4'''}$ = ca. 3 Hz, $J_{4''',5'''a} = J_{4''',5'''b}$ = 5.6 Hz and $J_{5'''a,5'''b}$ = 11.7 Hz. Inset: The aromatic region of a 400 MHz ^{1}H-NMR spectrum of 2′-deoxyguanosine showing H-8″ as a sharp singlet at δ = 7.85. i: impurity. S: sattelites (^{13}C).

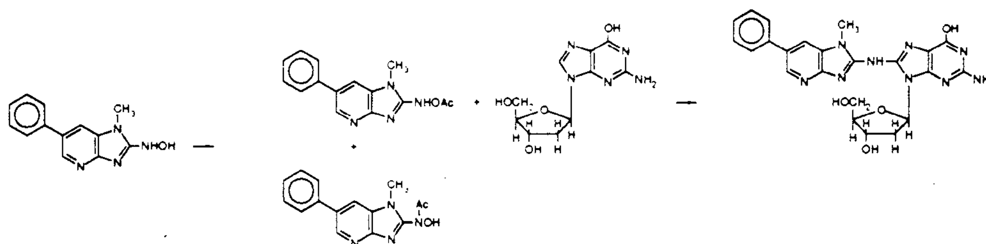

Fig. 6. Reaction scheme.

Reaction of N^2-OAc-PhIP with calf thymus DNA

N^2-OAc-PhIP reacted with calf thymus DNA giving a covalently bound PhIP residue, which could not be removed by repeated precipitations, solvent extractions or dialysis against 20% ethanol in water. The PhIP−DNA adduct was hydrolyzed enzymatically with nuclease P1 and analyzed by HPLC. Figure 7 shows the chromatograms monitored at 260 and 360 nm of the hydrolyzed PhIP−DNA adduct. Peaks A−D are the four nucleosides: 2′-deoxycytidine, 2′-deoxyguanosine, 2′-deoxythymidine and 2′-deoxyadenosine respectively. Peak E coeluted with synthetic N^2-(2′-dG-8-yl)−PhIP and had an identical UV spectrum. Any other possible adducts between PhIP and nucleosides were not detected to any appreciable extent, when the HPLC was monitored at 260 nm with enhanced sensitivity. HPLC analysis of an enzyme digest of [^{3}H]PhIP−DNA followed by liquid scintillation counting of collected fractions showed that 50% of the applied radioactivity coeluted with N^2-(2′-dG-8-yl)−PhIP. The remaining 50% of the applied radioactivity did not elute from the HPLC column. The small bump eluting at ~19.5 min may represent incompletely hydrolyzed DNA fragments bound to PhIP, as this bump was much larger and the adduct peak much smaller when hydrolysis was carried out at lower temperature (37°C). The high temperature (70°C), which is the temperature optimum of nuclease P1 (19), seems to be necessary for efficient hydrolysis of this DNA, with a high degree of modification. HPLC analysis of a solution of N^2-(2′-dG-8-yl)−PhIP incubated at 70°C showed that the adduct was stable under the hydrolysis conditions. Attempts to hydrolyze the PhIP−DNA and liberate the adducts with DNase 1, phosphodiesterase and alkaline phosphatase (37°C) were not successful.

Modification of DNA in vivo

Liver DNA from a rat dosed with [^{3}H]PhIP was isolated and hydrolyzed in dilute hydrochloric acid. The hydrolysate was separated by HPLC and fractions were collected and analyzed by liquid scintillation counting. N^2-(2′-dG-8-yl)−PhIP and the PhIP−DNA adduct, made *in vitro*, were hydrolyzed similarly for comparison. The results are shown in Figure 8. Panel 1 shows the HPLC profile of the acid hydrolysate of the N^2-(2′-dG-8-yl)−PhIP adduct. The hydrolyzed adduct eluted at 12 min. Panels 2 and 3 show the HPLC profile of the acid hydrolyzed PhIP−DNA adduct formed *in vitro*, monitored at 360 and 254 nm respectively. A product eluting at the same retention time as in panel 1 and showing an identical UV spectrum confirms the identity of the adduct formed from N^2-OAc-PhIP and DNA to be N^2-(2′-dG-8-yl)−PhIP. The small peak eluting just before the hydrolyzed adduct peak is also present, but to a lesser extent in panel 1. This peak grew larger when acid hydrolysis was carried out for a prolonged time. This early eluting peak may thus represent a further degradation product of the adduct. Panel 3 also shows that N^2-(2′-dG-8-yl)−PhIP is the major adduct formed in DNA. This is further confirmed in panel 4 showing

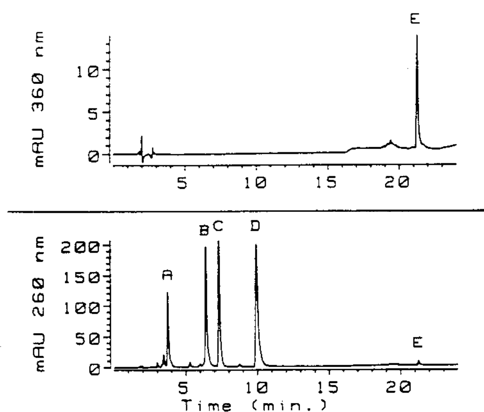

Fig. 7. Chromatograms of the enzymatically hydrolyzed PhIP−DNA adduct analyzed on a Spherisorb C_{18}, 3 μm, 150 × 4.6 mm. Flow rate: 0.8 ml/min. Solvents: I, 50 mM ammonium acetate pH 4.5. II: acetonitrile. Elution programming: isocratic 2% II by 1 min, linear gradient to 10% II by 13 min, linear gradient to 40% II by 17 min, isocratic 40% II by 20 min and a linear gradient to 80% II by 25 min. **Peaks A−D** are 2'-deoxycytidine, 2'-deoxyguanosine, 2'-deoxythymidine and 2'-deoxyadenosine respectively. **Peak E** is N^2-(2'-dG-8-yl)−PhIP.

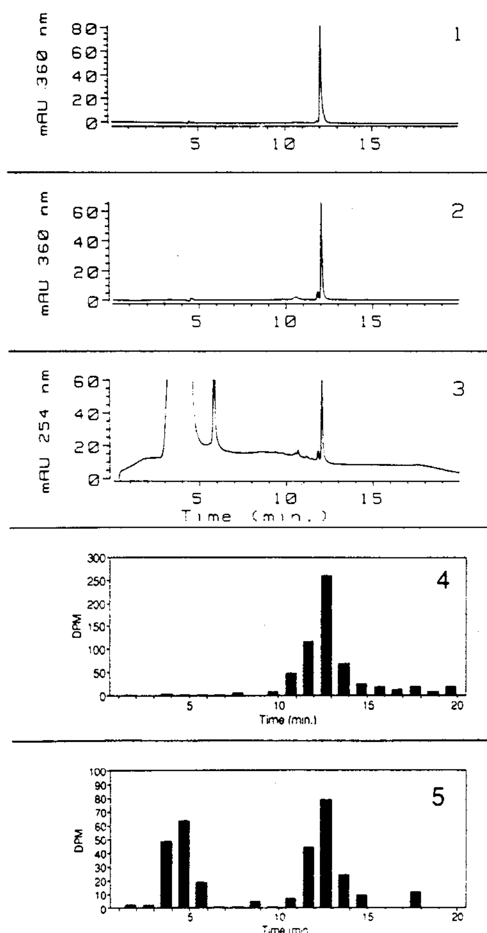

Fig. 8. HPLC profiles of N^2-(2'-dG-8-yl)]PhIP and the PhIP−DNA adduct subjected to acid hydrolysis. **Panel 1**: hydrolysate of N^2-(2'-dG-8-yl)−PhIP. **Panels 2** and **3**: hydrolysate of the *in vitro* formed PhIP−DNA adduct monitored at 360 and 254 nm. Peaks at 4 and 6 min in panel 3 originate from the calf thymus DNA. **Panel 4**: hydrolysate of [^{3}H]PhIP−DNA formed *in vitro*. Fractions were collected at 1 min intervals and radioactivity was measured by liquid scintillation counting. **Panel 5**: hydrolysate of liver DNA from a rat given [^{3}H]PhIP p.o. Fractions were collected by 1 min intervals and radioactivity was measured by liquid scintillation counting.

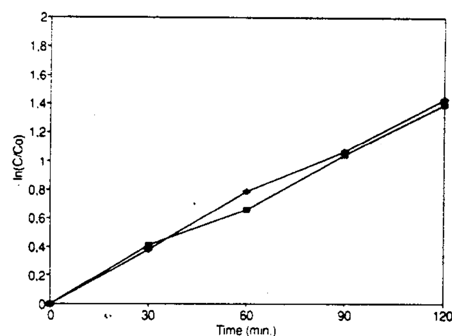

Fig. 9. Decay of N^2-acetoxy-PhIP at 0°C versus time. +; 2'-deoxyguanosine present in the incubation mixture. ■; 2'-deoxyguanosine not present in the incubation mixture.

the results from the liquid scintillation counting on fractions from the HPLC separation of the SEP-PAK eluate of hydrolyzed [^{3}H]PhIP−DNA. 60% of the applied radioactivity coeluted with the hydrolysis product of N^2-(2'-dG-8-yl)−PhIP. The remaining 40% of the radioactivity did not elute from the HPLC column. TLC analysis of the SEP-PAK eluate followed by liquid scintillation counting of collected spots showed that the radioactivity that did not elute from the HPLC column coeluted with a product which was also formed when N^2-acetoxy-PhIP was added to a buffer not containing 2'-deoxyguanosine. This byproduct from the reaction is probably azo-PhIP, formed by dimerization of two nitrenes. This byproduct seems to intercalate strongly into the DNA. Panel 5 shows the results from the liquid scintillation counting on fractions from the HPLC separation of hydrolyzed liver DNA from a rat dosed with [^{3}H]PhIP. The radioactivity eluting at 12 min indicates that PhIP, *in vivo*, also binds covalently to C-8 of guanine.

Kinetics

Results from the investigations of the reaction kinetics of 0°C, where the reaction between N^2-OAc-PhIP and 2'-deoxyguanosine was sufficiently slow to be followed by HPLC is shown in Figure 9. The decay of N^2-OAc-PhIP showed a linear relation of $-\ln/C/C_0)$ versus time, regardless of whether 2'-deoxyguanosine was present or not. As no solvolysis product (N^2-OH-PhIP) was detected, this linear relation indicates that the rate was first order with respect to N^2-OAc-PhIP and zero order with respect to 2'-deoxyguanosine.

Discussion

The proximate mutagenic N^2-hydroxylated metabolite of the food mutagen PhIP (N^2-OH-PhIP) did not bind covalently to DNA or 2'-deoxyguanosine, neither at neutral pH nor at slightly acidic pH. Acidic pH usually increases the binding of arylhydroxylamines e.g. 2-hydroxyamino-3-methylimidazo[4,5-*f*]quinoline (N^2-OH-IQ) to DNA, since protonation of the hydroxyl group facilitates the formation of a reactive nitrene. The fact that acidic pH does not increase the degree of binding of N^2-OH-PhIP to DNA, indicates that the heterolytic cleavage of the N−O bond proceeds less readily in N^2-OH-PhIP than in N^2-OH-IQ.

Covalent binding of *N*-hydroxylated amines to DNA is generally considered to proceed via activation by cytosolic acyl- or sulfotransferases and the subsequent formation of reactive nitrenes or nitrenium ions (20). Formation of the presumed *N*-acetoxy derivatives of 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) (21), 2-amino-6-methyldipyrido[1,2-*a*:3',2'-*d*]imidazole (Glu-P-1) (22) and 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2) (23), formed by reaction with acetic anhydride or ketene, have previously been shown to lead to reaction with DNA

and 2′-deoxyguanosine. After acetylation of N^2-OH-PhIP to N^2-OAc-PhIP, which enhances the heterolytic cleavage of the N–O bond, N^2-OH-PhIP reacted with both DNA and 2′-deoxyguanosine in high yields. Isolation of *N*-acetoxy-arylamines are usually precluded by their chemical instability (18), but the relative stability of the N–O bond in N^2-OAc-PhIP made possible its isolation and mass spectrometric characterization of this still rather reactive compound.

The highest yields of adduct were obtained when N^2-OAc-PhIP was added slowly to a 37°C solution of DNA or 2′-deoxyguanosine. The slow addition of N^2-OAc-PhIP combined with its fast rate of reaction keeps the ratio between nucleophilic DNA or nucleoside and electrophile high, whereby formation of side reaction products from the putative nitrene intermediate is kept low. One of these side reaction products is probably azo-PhIP, formed by dimerization of two nitrenes, which is a common reaction of arylnitrenes (24).

Analysis of the hydrolysis product of DNA, modified by N^2-OAc-PhIP *in vitro*, indicated that the N^2-OH-PhIP had mainly bound to the guanine of DNA, and since the nucleoside–PhIP adduct, on HPLC, coeluted with and had an identical UV spectrum as synthetic N^2-(2′-dG-yl)–PhIP, the major adduct formed *in vitro* thus appeared to be N^2-(2′-dG-8-yl)–PhIP.

PhIP also bound to liver DNA *in vivo*, upon dosing a rat with [^{3}H]PhIP. Since part of the eluted radioactivity which had bound covalently to DNA, upon acid hydrolysis coeluted with the hydrolysis product of synthetic N^2-(2′-dG-8-yl)–PhIP and PhIP–DNA made *in vitro*, N^2-(2′-dG-8-yl)–PhIP also appeared to be formed *in vivo*. It cannot be excluded that other nucleoside–PhIP adducts are formed *in vivo*.

Binding of the exocyclic amino group to the C-8 position of guanine seems to be the most common site of reaction of activated heterocyclic aromatic amines with DNA. N^2-OH-IQ, N^2-OH-Glu-P-1 and N^3-OH-Trp-P-2 have formerly been shown to bind to DNA in a similar way, after activation with acetic anhydride or ketene.

The yield of adduct from N^2-OH-PhIP was 2.5% when N^2-OAc-PhIP was reacted with DNA and 50% when N^2-OAc-PhIP was reacted with 2′-deoxyguanosine. This suggests that intercalation of N^2-OAc-PhIP into the DNA strand is not an important step in the binding of this *N*-acetoxy derivative to DNA. This is in contrast to findings with N^2-OH-IQ and N^2-OH-Glu-P-1, where intercalation was suggested to be of importance in the DNA binding of these substances (21,22). In these studies N^2-OAc-IQ was generated *in situ*, by addition of acetic anhydride to a mixture of DNA and N^2-OH-IQ (21), and N^2-OAc-Glu-P-1 was reacted with DNA at low temperature (22). The relatively low yield of adduct obtained when N^2-OAc-PhIP was reacted with DNA compared to that with 2′-deoxyguanosine indicates that the DNA structures reduces binding of N^2-OAc-PhIP to guanine, either by steric hindrance or by reduction of the nucleophilicity of guanine, due to hydrogen bonding.

The fast decomposition of N^2-OAc-PhIP at physiological conditions, 37°C and pH 7.4, prevented kinetic investigations of the reaction between N^2-OAc-PhIP and 2′-deoxyguanosine. Investigations of the kinetics at 0°C, where the reaction between N^2-OAc-PhIP and 2′-deoxyguanosine was sufficiently slow to be followed by HPLC, indicated that the rate was first order with respect to N^2-OAc-PhIP and zero order with respect to 2′-deoxyguanosine. This is in accordance with an SN1 mechanism, where heterolytic fission of the N–O bond in N^2-OAc-PhIP leads to the formation of a reactive nitrene or nitrenium ion.

From the results of this study it can be concluded that the proximate mutagenic N^2-hydroxylated metabolite of PhIP can be activated by acetylation and that the resulting N^2-OAc-PhIP reacts effectively with 2′-deoxyguanosine and DNA, resulting in the formation of N^2-(2′-dG-8-yl)–PhIP.

[^{32}P]Postlabeling analyses of DNA from various organs from rats dosed with PhIP, have recently been shown to result in high adduct levels in lung, pancreas and heart, but lower levels in the liver (9). The liver is usually the primary target organ for DNA-binding of heterocyclic amines with an exocyclic amino group. It can be speculated that the relatively higher stability of N^2-OH-PhIP, as compared to *N*-hydroxy derivatives of other heterocyclic aromatic amines, may explain this different mode of action, as PhIP upon phase I activation to N^2-OH-PhIP is sufficiently stable to allow its distribution throughout the whole organism. Adduct levels in the various organs may thus depend on their activity of phase II enzymes, and their DNA repair capacity in the various organs.

Acknowledgements

The authors wish to thank Peter Farmer, The Medical Research Council, Carshalton, Surrey, UK, for FAB mass spectrometric measurements, Kratos analytical, Manchester, UK, for thermospray mass spectrometric measurements, Joan Gluver and Bettina Lyngsøe Hansen for excellent technical assistance and Bo Lund Jensen for graphical work. This work was in part supported by grant no. 75 from Nordiska Kontaktorganet för Jordbruksforskning.

References

1. Övervik,E. and Gustafsson,J.-Å. (1990) Cooked-food mutagens: current knowledge of formation and biological significance. *Mutagenesis*, **5**, 437–446.
2. Jägerstadt,M., Skog,K., Grivas,S. and Olsson,K. (1991) Formation of heterocylic amines using model systems. *Mutat. Res.*, **259**, 219–233.
3. Felton,J.S. and Knize,M.G. (1991) Occurrence, identification, and bacterial mutagenicity of heterocyclic amines in cooked food. *Mutat. Res.*, **259**, 205–217.
4. Thompson,L.H., Tucker,J.D., Stewart,S.A., Christensen,M.L., Salazar,E.P., Carrano,A.V. and Felton,J.S. (1987) Genotoxicity of compounds from cooked beef in repair-deficient CHO cells versus *Salmonella* mutagenicity. *Mutagenesis*, **2**, 483–487.
5. Holme,J.A., Wallin,H., Brunborg,G., Søderlund,E.J., Hongslo,J.K. and Alexander,J. (1989) Genotoxicity of the food mutagen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP): formation of 2-hydroxamino-PhIP, a direct acting genotoxic metabolite. *Carcinogenesis*, **10**, 1389–1396.
6. Frandsen,H., Rasmussen,E.S., Nielsen,P.A., Farmer,P., Dragsted,L. and Larsen,J.C. (1991) Metabolic formation, synthesis and genotoxicity of the N-hydroxy derivative of the food mutagen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP). *Mutagenesis*, **6**, 93–98.
7. Esumi,H., Ohgaki,H., Kohzen,E., Takayama,S. and Sugimura,T. (1989) Induction of lymphoma in CDF_1 mice by the food mutagen, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine. *Jpn. J. Cancer Res.*, **80**, 1176–1178.
8. Ochiai,M., Kumiko,O., Wakabayashi,K., Sugimura,T., Nagase,S., Esumi,H. and Nagao,M. (1991) Induction of intestinal adenocarcinomas by 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine in Nagase analbuminemic rats. *Jpn. J. Cancer Res.*, **82**, 363–366.
9. Takayama,K., Yamashita,K., Wakabayashi,K., Sugimura,T. and Nagao,M. (1989) DNA modification by 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]-pyridine in rats. *Jpn. J. Cancer Res.*, **80**, 1145–1148.
10. Takahashi,S., Ogawa,K., Oshima,H., Esumi,H., Ito,N. and Sugimura,T. (1991) Induction of aberrant crypt foci in the large intestine of F344 rats by oral administration of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine. *Jpn J. Cancer Res.*, **82**, 135–137.
11. Wallin,H., Mikaelsen,A., Guengrich,F.P., Ingelman-Sundberg,M., Solberg,K.E., Rossland,J. and Alexander,J. (1990) Differential rates of metabolic activation and detoxification of the food mutagen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine by different cytochrome P450 enzymes. *Carcinogenesis*, **11**, 489–492.
12. Buonarati,M.H. and Felton,J.S. (1990) Activation of 2-amino-1-methyl-

6-phenylimidazo[4,5-*b*]pyridine (PhIP) to mutagenic metabolites. *Carcinogenesis*, **11**, 1133–1138.
13. Turteltaub,K.W., Knize,M.G., Buonarati,M.H., McManus,M.E., Veronese,M.E., Mazrimas,J.A. and Felton,J.S. (1990) Metabolism of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) by liver microsomes and isolated rabbit cytochrome P450 isozymes. *Carcinogenesis*, **11**, 941–946.
14. Buonarati,M.H., Turteltaub,K.W., Shen,N.H. and Felton,J.S. (1990) Role of sulfation and acetylation in the activation of 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine to intermediates which bind DNA. *Mutat. Res.*, **245**, 185–190.
15. Peluso,M., Castegnaro,M., Malaveille,C., Friesen,M., Garren,L., Hautefeuille,A., Vineis,P., Kadlubar,F. and Bartsch,H. (1991) ^{32}Postlabelling analysis of urinary mutagens from smokers of black tobacco implicates 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) as a major DNA-damaging agent. *Carcinogenesis*, **12**, 713–717.
16. Palmgren,G., Mattson,O. and Okkels,F.T. (1990) Employment of hydrolytic enzymes in the study of the level of DNA methylation. *Biochim. Biophys. Acta*, **1049**, 293–297.
17. Gupta,R.C. (1984) Nonrandom binding of the carcinogen *N*-hydroxy-2-acetylaminofluorene to repetitive sequences of rat liver DNA *in vivo*. *Proc. Natl. Acad. Sci. USA*, **81**, 6943–6947.
18. Lutgerink,J.T., Stavenuiter,J.F.C., Zomer,G., Hamzink,M., van Dijk,P., Westra,J.G. and Kriek,E. (1989) Synthesis of heterocyclic N-acetyoxy-arylamines and their reactivity with DNA. *Carcinogenesis*, **10**, 1957–1960.
19. Fujimoto,M., Kuninka,A. and Yoshino,H. (1974) Identity of phosphodiesterase and phosphomonoesterase activities with nuclease P_1 (a nuclease from *Penicillium citrinum*). *Agr. Biol. Chem.*, **38**, 785–790.
20. Kadlubar,F.F. and Beland,F.A. (1985) Chemical properties of ultimate carcinogenic metabolites of arylamines and arylamides. In Harvey,R.G. (ed.), *Polycyclic Hydrocarbons and Carcinogenesis. ACS Symposium Series*. American Chemical Society, Washington, DC, Vol. 283, pp. 341–370.
21. Snyderwine,E.G., Roller,P.P., Adamson,R.H., Sato,S. and Thorgeirsson,S. (1988) Reaction of *N*-hydroxylamine and *N*-acetoxy derivatives of 2-amino-3-methylmidazo[4,5-*f*]quinoline with DNA. Synthesis and identification of *N*-(deoxyguanosin-8-yl)–IQ. *Carcinogenesis*, **9**, 1061–1065.
22. Hashimoto,Y., Shudo,K. and Okamato,T. (1982) Modification of DNA with potent mutacarcinogenic 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole isolated from a glutamic acid pyrolysate: structure of the modified nucleic acid base and initial chemical event caused by the mutagen. *J. Am. Chem. Soc.*, **104**, 7636–7640.
23. Hashimoto,Y., Shudo,K. and Okamoto,T. (1980) Activation of a mutagen, 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole. Identification of 3-hydroxyamino-1-methyl-5*H*-pyrido[4,5-*b*]indole and its reaction with DNA. *Biochem. Biophys. Res. Comm.*, **96**, 355–362.
24. March,J. (1985) *Advanced Organic Chemistry*. John Wiley & Sons, NY, p. 177.

Received on September 16, 1991; revised on December 19, 1991; accepted on January 6, 1992.

PAPER III

Mutagenesis vol.9 no.1 pp.59–65, 1994

Microsomal metabolism of the food mutagen 2-amino-3,4,8-trimethyl-3*H*-imidazo[4,5-*f*]-quinoxaline to mutagenic metabolites

Henrik Frandsen, Preben A.Nielsen, Spiros Grivas[1] and John C.Larsen

Institute of Toxicology, National Food Agency, Mørkhøj Bygade 19, DK-2860 Søborg, Denmark and [1]Department of Chemistry, Swedish University of Agricultural Sciences, PO Box 7016, Uppsala, Sweden

Heterocyclic aromatic amines are formed in the crust of meat during ordinary cooking. These aromatic amines are potent bacterial mutagens and also potent rodent carcinogens. 2-Amino-3,4,8-trimethyl-3*H*-imidazo[4,5-*f*]quinoxaline (DiMeIQx) is one of the more abundant heterocyclic aromatic amines, accounting for ~20% of the mutagenic material found in cooked meat. DiMeIQx is metabolically activated, by hepatic microsomes from PCB treated rats, to two major and three minor metabolites. One major and one minor metabolite were identified as 2-hydroxyamino-3,4,8-trimethyl-3*H*-imidazo[4,5-*f*]quinoxaline and 3,4,8-trimethyl-2-nitro-3*H*-imidazo[4,5-*f*]quinoxaline, respectively, confirmed by comparison of HPLC retention times, and UV and mass spectra of synthetic standards. Both metabolites were mutagenic in *Salmonella typhimurium* TA98 without metabolic activation. The other major metabolite was identified as 2-amino-8-hydroxymethyl-3,4-dimethyl-3*H*-imidazo[4,5-*f*]-quinoxaline by mass and NMR spectral analysis. The two remaining minor metabolites were identified as the 2-hydroxy-amino- and 2-nitro- derivatives of 2-amino-8-hydroxy-methyl-3,4-dimethyl-3*H*-imidazo[4,5-*f*]quinoxaline by UV and mass spectral analysis. Both of these metabolites were mutagenic in *S.typhimurium* TA98 without metabolic activation.

Introduction

During frying of meat several mutagenic heteroaromatic amines of the aminoimidazoazaarene (AIA) type are formed in the meat crust. Several of these AIA compounds have been shown to be carcinogenic in rats and mice when administered in the diet, producing tumors in a variety of organs (Wakabayashi *et al.*, 1992).

A recent epidemiological investigation has indicated an increased risk of colorectal cancer associated with high meat intake and in particular with preference for browning of the meat surface (Gerhardsson de Verdier *et al.*, 1991). As AIA compounds are present in the meat surface they may be involved in the etiology of human cancer.

2-Amino-3,4,8-trimethyl-3*H*-imidazo[4,5-*f*]quinoxaline (DiMeIQx) is a potent bacterial mutagen which has been identified in beef extracts (Takahashi *et al.*, 1985), in smoked dried bonito (Kikugawa *et al.*, 1986), in fried ground beef (Knize *et al.*, 1987), in fried Norwegian meat products (Becher *et al.*, 1988), in fried beef (Murray *et al.*, 1988), in fried pork (Nielsen *et al.*, 1988) and in cooked beef products (Turesky *et al.*, 1988). The amounts of DiMeIQx found in fried meat range from 0.5 to 1.2 μg/kg and DiMeIQx accounts for ~20% of the total mutagenic activity found in beef fried at 300°C (Felton *et al.*, 1986). In a model system DiMeIQx was also formed by heating creatinine, amino acids and hexoses (Grivas *et al.*, 1985; Skog *et al.*, 1992).

Aromatic amines must undergo biotransformation to reactive electrophiles before they can interact with cellular macromolecules. Studies on the metabolism of aromatic amines with a structure similar to DiMeIQx show that these substances are metabolically activated by microsomal oxidation of the exocyclic amino group, and that the resultant N^2-hydroxy compounds are direct acting mutagens in the Ames Salmonella test (Yamazoe *et al.*, 1983, 1988; Snyderwine *et al.*, 1987; Holme *et al.*, 1989; Turteltaub *et al.*, 1990; Buonarati and Felton, 1990; Frandsen *et al.*, 1991).

The *in vivo* metabolism of DiMeIQx has previously been studied in conventional and germ-free rats. In this study only indirect mutagenic metabolites were structurally characterized (Knize *et al.*, 1989). In order to further clarify the mutagenic activation of DiMeIQx we have studied the *in vitro* metabolism and identified five metabolites four of which were found to be mutagenic in the Salmonella strain TA98.

Materials and methods

Chemicals

DiMeIQx and [2-^{14}C]DiMeIQx were synthesized as previously described (Grivas, 1985). DiMeIQx was determined to have >95% purity by HPLC with detection at 260 nm. [2-^{14}C]DiMeIQx was determined to have >92% radiochemical purity by HPLC with radioactivity detection. Isocitric dehydrogenase (from porcine heart), nicotinamide-adeninedinucleotide-phosphate, trisodium-DL-isocitrate and 3-(*N*-morpholino)propanesulfonic acid (MOPS) were obtained from Sigma (St Louis, MO), PCB (Aroclor 1254) was obtained from Monsanto (St Louis, MO). HPLC grade acetonitrile was obtained from Rathburn (Walkerburn, UK). All other chemicals were obtained from Merck (Darmstadt, FRG) and were of analytical purity, except palladium on carbon (Pd/C) and hydrazine hydrate which were of reagent grade. The Salmonella tester strain TA98 and TA98NR were kindly provided by Professor Bruce N.Ames (University of California, Berkeley, CA) and Professor Herbert S.Rosenkranz (University of Pittsburg, PA), respectively.

Synthesis of 3,4,8-trimethyl-2-nitro-3H-imidazo[4,5-f]quinoxaline (NO_2-DiMeIQx)

DiMeIQx, 11 mg, was suspended in 1 ml of 10% tetrafluoroboric acid, cooled on ice-water and 30 μl of 2 M sodium nitrite was added dropwise under stirring. The solution was stirred for 20 min at 0°C. The diazotization mixture was added dropwise to an ice-water cooled solution of 2.3 g sodium nitrite in 15 ml of water. The pH of the reaction mixture was maintained at 4.5 ± 0.1 by addition of first tetrafluoroboric acid and then 2 M sodium hydroxide. The mixture was allowed to warm to room temperature and, after stirring for 25 min, the mixture was neutralized with 2 M sodium hydroxide and extracted twice with 25 ml of ethyl acetate. The organic phase was washed with water and the water phase was extracted with ethyl acetate. The combined organic phase was dried with magnesium sulfate and evaporated to dryness. The product was purified by silica gel chromatography (Kieselgel 60, 1.6 × 9 cm, 2% methanol in ethyl acetate) and evaporated to dryness. Yield 10 mg (80%). The purity was shown to be >98% by HPLC (323 nm).

Synthesis of 2-hydroxyamino-3,4,8-trimethyl-3H-imidazo[4,5-f]quinoxaline (N^2-OH-DiMeIQx)

A reaction tube containing 20 mg of 10% Pd/C was evacuated for 5 min and filled with argon. After addition of 3 ml of tetrahydrofuran (THF) the suspension was purged with argon for 2 min. The suspension was cooled on ice-water and

© Oxford University Press

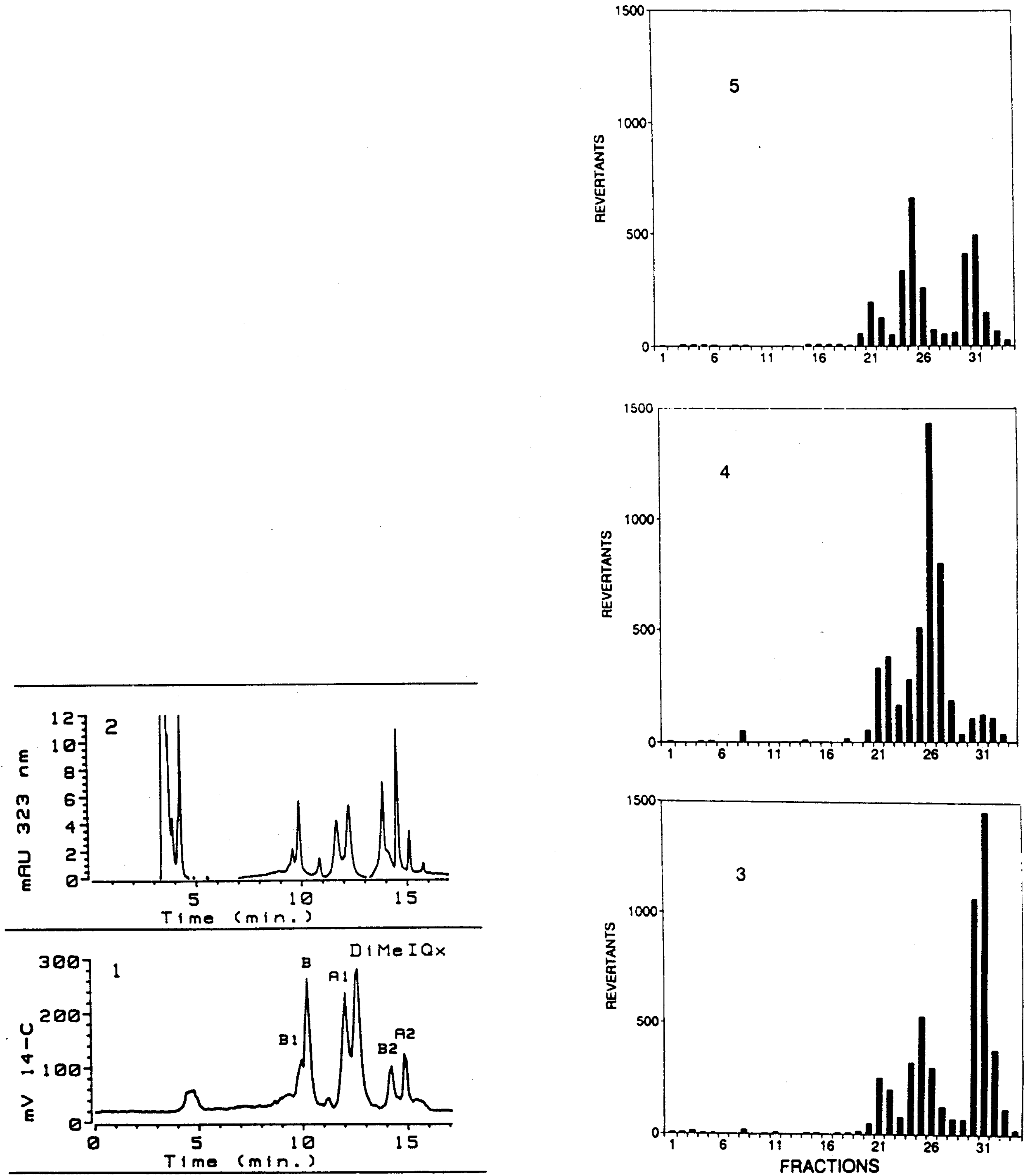

Fig. 1. [2-^{14}C]DiMeIQx and DiMeIQx were incubated for 40 minutes with hepatic microsomes from Wistar rats pretreated with PCB followed by HPLC analysis. **Panel 1 and 2**: HPLC profiles of metabolites of [2-^{14}C]DiMeIQx obtained with both an on-line radioactivity detector and a UV detector showing that five metabolites could be detected. **Panel 3–5** Metabolites of DiMeIQx were separated on HPLC, and 0.5 min fractions were collected and tested for mutagenic activity in TA98 without metabolic activation (**panel 3**), TA98 with S9 (**panel 4**) and in the nitroreductase deficient strain TA98NR without metabolic activation (**panel 5**). The experiment was conducted twice with identical results. There was a time delay of ~0.5 min to both radioactivity detector and fraction collector.

10 μl of hydrazine hydrate (80%) was added followed by addition of a solution of 2.6 mg of NO_2-DiMeIQx in 3 ml of argon purged THF. After 30 min of stirring at 0°C the suspension was centrifuged, the supernatant isolated, evaporated to dryness under vacuum and stored at −20°C until use.

Synthesis of 3,4,8-trimethyl-2-phenylazoxy-3H-imidazo[4,5-f]quinoxaline (phenylazoxy derivative of DiMeIQx)

Nitrosobenzene, 1 mg, dissolved in 0.1 ml dimethylformamide (DMF), was added to a solution of 200 μg N^2-OH-DiMeIQx in 0.5 ml argon purged DMF. After

standing for 1 h at room temperature, the solution was analyzed by HPLC/thermospray mass spectrometry.

Preparation of microsomes

Adult male Wistar rats (age 7–8 weeks, weight ~200 g), delivered from Møllegård Breeding Center (Lille Skensved, Denmark), were used to prepare PCB induced hepatic microsomes. PCB (500 mg/kg, dissolved in corn oil) was injected i.p. 5 days before sacrifice and microsomes were prepared essentially as described by Lake (1987). The microsomes were stored in liquid nitrogen until use. The protein content was determined by the method of Sedmark and Grossberg (1977). The concentration of P450 (Lake, 1987) was 2.3 nmol P450/mg protein. The biphenyl hydroxylase activities (Yamazoe *et al.*, 1981) were 3.0 nmol 2-hydroxy-biphenyl formed/(mg protein min) and 4.4 nmol 4-hydroxy-biphenyl formed/(mg protein min).

In vitro metabolism

The incubation mixture consisted of: 1 mg microsomal protein/ml, 1 unit DL-isocitrate-dehydrogenase/ml, 0.5 mM $NADP^+$, 10 mM sodium-DL-isocitrate and 5 mM magnesium chloride in 50 mM MOPS, 0.15 mM KCl, pH 7.4. The mixture was preincubated for 2 min at 37°C before addition of 10 μg test substance (dissolved in 5 μl of DMF)/ml incubation mixture. The mixture was incubated at 37°C. The reaction was terminated by the addition of two volumes of ice cold argon purged ethanol. After centrifugation the supernatant was isolated and analyzed by HPLC.

Analytical

HPLC analysis was performed on a Hewlett Packard model 1090 B liquid chromatograph equipped with a photo-diode array detector. Metabolites were separated on a Nucleosil C_8, 5 μm, 250 × 4.6 mm column obtained from HPLC Technology (Macclesfield Cheshire, UK). Flow rate 0.8 ml/min. Solvents: A, 50 mM ammonium formate, pH 3.5; B, acetonitrile. Solvent programming: isocratic 10% B by 1 min, a linear gradient to 25% B by 5 min, isocratic 25% B by 8 min, a linear gradient to 100% B by 13 min, isocratic 100% B by 15 min and a linear gradient to 10% B by 17 min.

In experiments with ^{14}C-labelled substrate the effluent from the column was monitored by both a photo-diode array detector and an on-line radioactivity detector (Radiomatic, Flo-one beta, Tampa, FL) with Packhard Pico aqua scintillation liquid (Mereda, CT). The flow rate of the scintillation liquid was four times the flowrate of the effluent.

In experiments with unlabelled substrate the HPLC fractions were collected in argon filled test tubes at 0.5 min intervals and analyzed for mutagenic activity.

Positive ion thermospray mass spectra were obtained at a resolution of 600 on a Profile mass spectrometer (Kratos, Manchester, UK). The effluent from the HPLC was introduced into the thermospray interphase at a flow rate of 0.8 ml/min. Both vaporizer and source temperature were 170°C. The same solvent programming as above was used; however, in order to avoid excessive fragmentation the ammonium formate was substituted by 0.5% formic acid.

NMR spectra were obtained with a Varian VXR-400 spectrometer with DMSO-d_6 as solvent.

Mutation assay

The Salmonella mutation assays were carried out by the standard plate incorporation method as described by Maron and Ames (1983). HPLC fractions were collected in argon filled test tubes at 0.5 min intervals and 0.1 ml aliquots were tested for mutagenicity in TA98 with and without S9 mix (1 mg protein/plate) and in TA98NR without S9 mix. S9 mix was prepared from the liver of Aroclor 1254 induced male Wistar rats. In assays not containing a metabolic activation system 1 mM dithiothreitol was added in order to prevent oxidation of the test substances (Yamazoe *et al.*, 1983; Frandsen *et al.*, 1991). To 2 ml of molten top agar at 45°C, 0.5 ml of S9 mix or 0.1 ml 20 mM dithiothreitol was added. After 2 min, 0.1 ml of the HPLC fractions and 0.1 ml of a 7 h nutrient broth (Oxoid) culture of the test bacteria (1–2 × 10^9 cells/ml) *Salmonella typhimurium* TA98 or TA98NR were added. After a gentle mixing the top agar was poured onto 20 ml minimal glucose plates. The number of His^+ revertant colonies on each plate was counted after incubation for 48 h at 37°C in the dark. 2-Nitrofluorene, 1,8-dinitropyrene and 2-aminoanthracene were used as positive control mutagens and solvent as negative. 2-Nitrofluorene was tested without S9 mix at a concentration of 0.1 μg/plate giving 99 ± 37 revertants/plate in TA98 and 42 ± 15 revertants/plate in TA98NR. 1,8-Dinitropyrene was tested in TA98NR without S9 at a concentration of 2 ng/plate giving 3184 ± 490 revertants/plate. 2-Aminoanthracene was tested in TA98 at a concentration of 0.1 μg/plate with S9 mix giving 181 ± 57 revertants/plate.

Results

Incubation of DiMeIQx with hepatic microsomes from Wistar rats pretreated with PCB produced five metabolites. Figure 1 (panels 1 and 2) shows the radiochromatogram and the UV profile, respectively, after 40 min of incubation with [2-^{14}C]DiMeIQx. After 20 min of incubation only two major metabolites (A1 and B) could be detected but longer incubation times resulted in increasing amounts of metabolites A2, B1 and B2.

To assess the mutagenic activity of the metabolites, fractions were collected at 0.5 min intervals and tested for mutagenicity in *S.typhimurium* TA98 both with and without the addition of

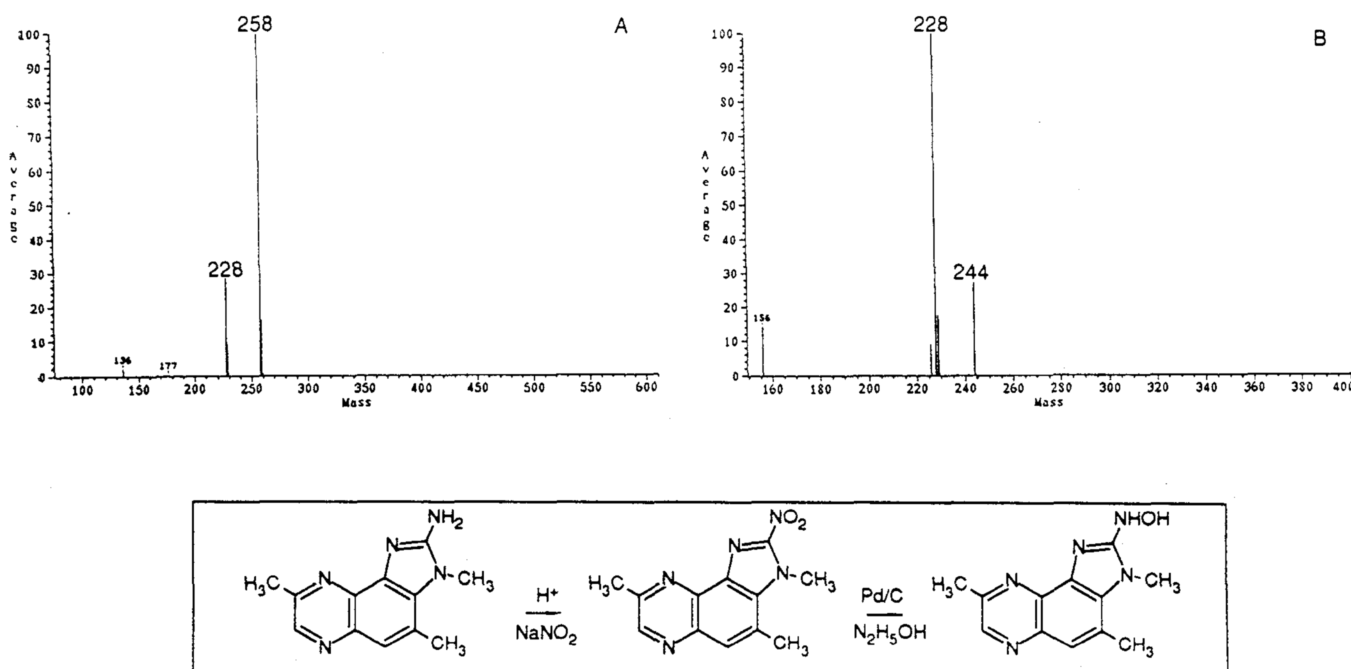

Fig. 2. Reaction scheme showing the syntheses of NO_2-DiMeIQx and N^2-OH-DiMeIQx derivatives. Both were characterized by thermospray mass spectrometry showing a molecular ion $(M+H)^+$ at m/z 258 of NO_2-DiMeIQx (**A**) and a molecular ion $(M+H)^+$ at m/z 244 with a daughter ion 228 of N^2-OH-DiMeIQx (**B**).

metabolic activation system (S9) and in the nitroreductase deficient strain TA98NR without addition of S9. The mutagenic activity of the HPLC fractions without S9 is shown in Figure 1 (panel 3). Fractions 21, 22, 24–26 and 30–32 all contained mutagenic activity. Addition of S9 resulted in increased mutagenicity in fractions 21–23 and in a highly increased mutagenicity in fractions 26–27, originating from the parent compound. The mutagenicity in fraction 25 was still present at the same level as without added activation system, whereas mutagenicity in fractions 30–31 had almost disappeared (Figure 1, panel 4). In the nitroreductase deficient strain TA98NR without S9 activation (Figure 1, panel 5) the mutagenicity of fractions 21, 22 and 24–26 was at the same level as in TA98. However, the mutagenicity in fractions 30–31 was reduced by >50% as compared with TA98.

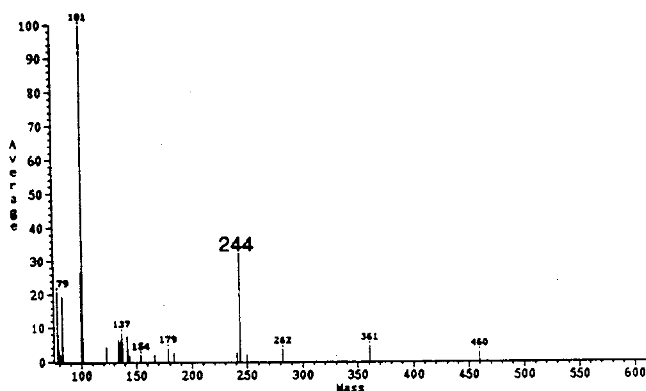

Fig. 3. Thermospray mass spectrum of metabolite B showing a molecular ion $(M+H)^+$ at m/z 244, indicating that B is a OH-derivative of DiMeIQx.

Identification of the microsomal metabolites

2-Nitro and N^2-hydroxyamino derivatives of DiMeIQx were synthesized by the two step procedure shown in Figure 2, and the compounds were characterized by UV spectroscopy and thermospray mass spectrometry. The positive ion thermospray mass spectrum of NO_2-DiMeIQx) showed a molecular ion $(M+H)^+$ at m/z 258 (Figure 2A) and the mass spectrum of N^2-OH-DiMeIQx showed a molecular ion $(M+H)^+$ at m/z 244 (Figure 2B), which confirms the structure of the two compounds. Further evidence that the N^2-OH-DiMeIQx had been formed was obtained by synthesis of the phenylazoxy derivative which by HPLC/thermospray mass spectrometric analysis showed the correct molecular ion $(M+H)^+$ at m/z 333 (not shown) (Hashimoto *et al.*, 1982). The identity of metabolites A1 and A2 was determined as N^2-OH-DiMeIQx and NO_2-DiMeIQx, respectively, by comparison of retention times in HPLC, UV spectra and mass spectra with synthetic standards.

Metabolite B was purified by preparative HPLC, and characterized by thermospray mass spectrometry and ^{1}H-NMR spectroscopy. The mass spectrum showed a molecular ion $(M+H)^+$ at m/z 244, which the mass spectrum of N^2-OH-DiMeIQx also did, but no daughter ion at m/z 228 (Figure 3). This indicates that metabolite B is hydroxylated in a different position of the molecule than N^2-OH-DiMeIQx. As shown in Figure 4 the ^{1}H-NMR spectrum revealed the presence of all protons. The assignments are based on comparisons with literature values of the chemical shifts of DiMeIQx (Grivas, 1985) and on irradiation experiments. Irradiation at the 4-methyl group at 2.8 p.p.m. resulted in an increased signal height of the 5-H proton due to elimination of long range coupling between the 4-methyl group and the 5-H proton. Similarly, irradiation at the CH_2 group at 4.76 p.p.m. resulted in an increased signal height of the 7-H proton. This indicates that the 8-methyl group is

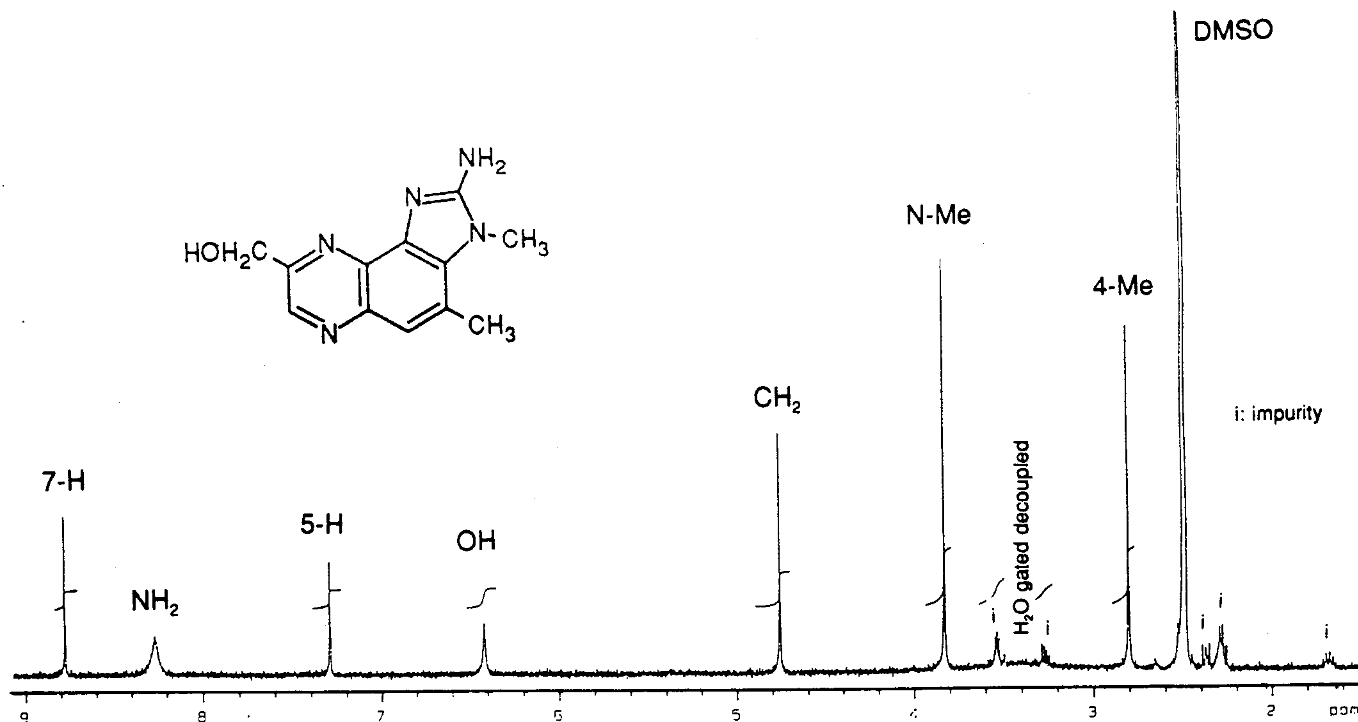

Fig. 4. The 400 MHz ^{1}H-NMR spectrum of metabolite B at 20°C. The chemical shifts are referenced to the solvent [δ (DMSO-d_6) = 2.49]; δ = 8.78 (s, 7-H), 8.27 (br s, 2-NH_2), 7.29 (s, 5-H), 6.42 (br s, 8-OH), 4.76 (s, 8-CH_2), 3.83 (s, N-Me), 2.80 (s, 4-Me).

oxidized to a hydroxymethyl group. The results from NMR experiments together with the mass spectrum corroborate that metabolite B is 2-amino-8-hydroxymethyl-3,4-dimethyl-3*H*-imidazo[4,5-*f*]quinoxaline (8-OH-DiMeIQx). The NMR and mass spectroscopy data on 8-OH-DiMeIQx are in accordance with data published by Knize *et al.* (1989) on a similar compound isolated from urine of rats dosed with DiMeIQx.

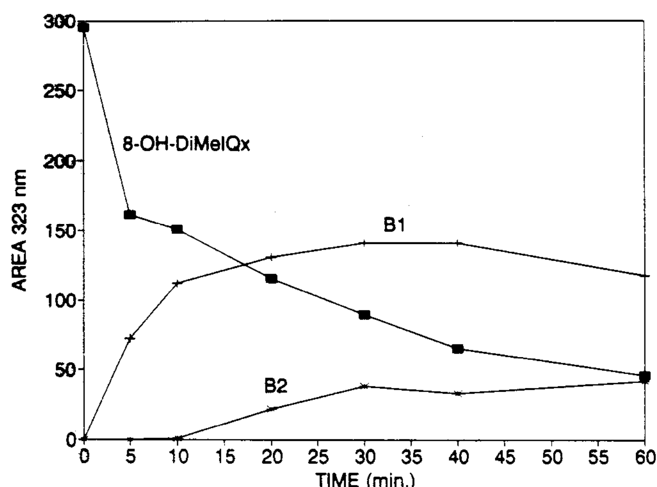

Fig. 5. Time course of the biotransformation of 8-OH-DiMeIQx (metabolite B) to metabolites B1 and B2.

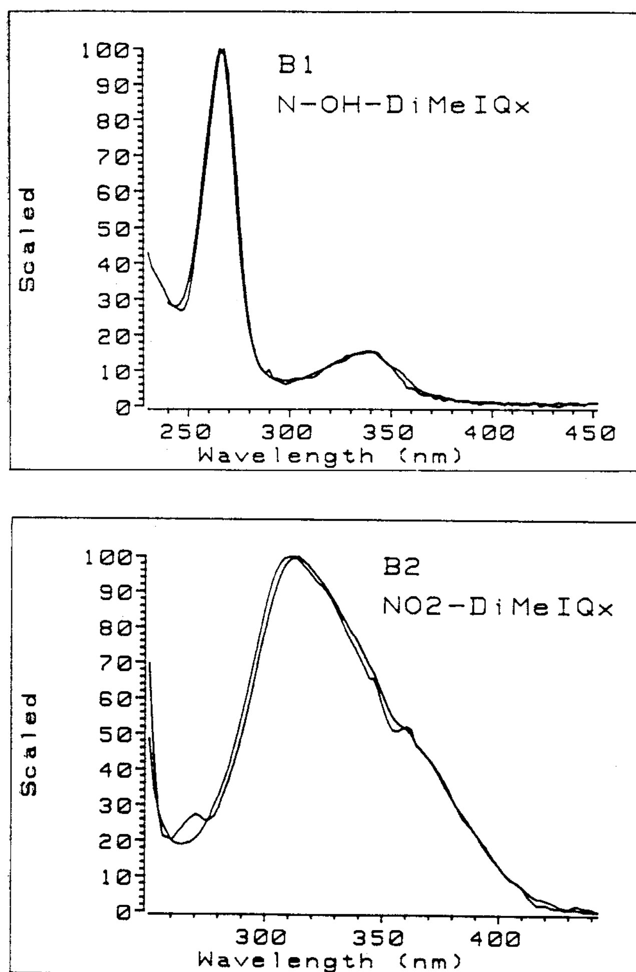

Fig. 6. Upper panel: UV spectra obtained with a diode array detector at the elution conditions from the HPLC column of metabolite B1 and N^2-OH-DiMeIQx. **Lower panel**: UV spectra obtained with a diode array detector at the elution conditions from the HPLC column of metabolite B2 and NO_2-DiMeIQx.

Incubation of purified metabolite B, 8-OH-DiMeIQx, with hepatic microsomes from PCB treated rats resulted in a transformation of 8-OH-DiMeIQx to metabolites B1 and B2. The time course of this transformation is shown in Figure 5. The initial disappearance of 8-OH-DiMeIQx is followed by a concomitant rise in the content of metabolite B1 in the incubation mixture. After 20 min, the levels of metabolite B1 began to plateau, whereas the amount of metabolite B2 increased. This time course indicates that 8-OH-DiMeIQx is biotransformed to metabolite B1, which is further transformed to B2.

A comparison of the UV spectra of metabolites B1 and B2 with the UV spectra of N^2-OH-DiMeIQx and NO_2-DiMeIQx is shown in Figure 6. The spectral similarity between metabolite B1 and N^2-OH-DiMeIQx and between metabolite B2 and NO_2-DiMeIQx together with the time course of their formation indicates that metabolite B1 in the N^2-OH derivative of 8-OH-DiMeIQx, 2-hydroxyamino-8-hydroxymethyl-3,4-dimethyl-3*H*-imidazo[4,5-*f*]quinoxaline, and that metabolite B2 is the 2-nitro derivative of 8-OH-DiMeIQx, 8-hydroxymethyl-3,4-dimethyl-2-nitro-3*H*-imidazo[4,5-*f*]quinoxaline. Further evidence for the proposed structures of metabolites B1 and B2 was obtained by analyses of the metabolites by HPLC/thermospray mass spectrometry.

Figure 7 (panel A) shows the mass spectrum of metabolite B1 having a molecular ion $(M+H)^+$ at *m/z* 260. This is in

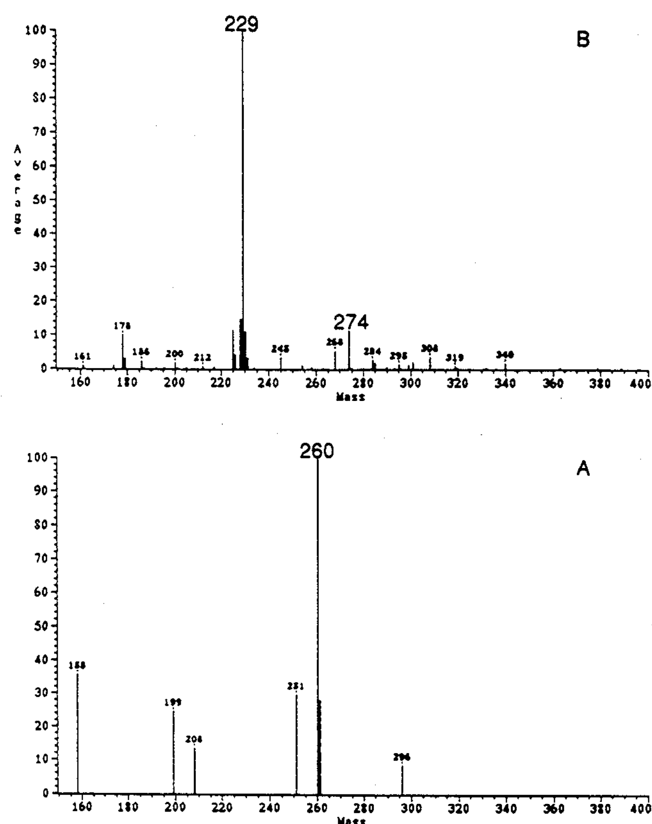

Fig. 7. HPLC thermospray mass spectra of metabolites B1 and B2. The mass spectrum of B1 showed a molecular ion $(M+H)^+$ at *m/z* 260 (**A**) and the mass spectrum of metabolite B2 (**B**) showed a molecular ion $(M+H)^+$ at *m/z* 274.

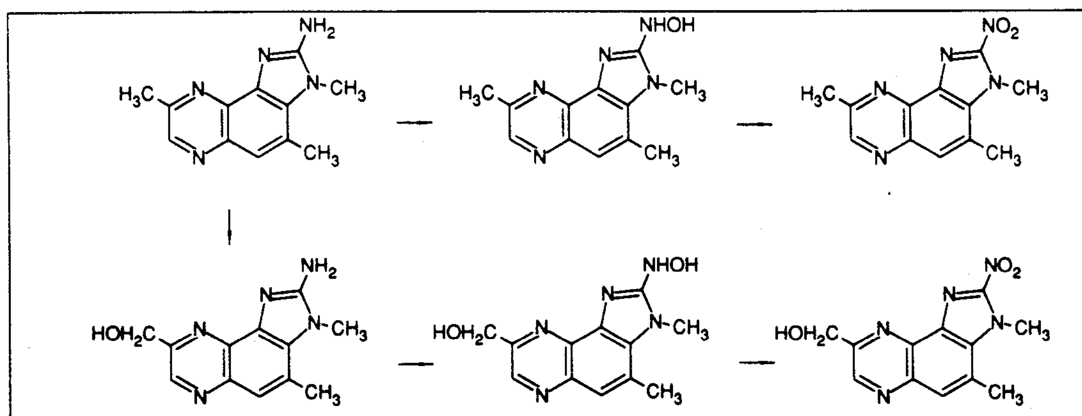

Fig. 8. Reaction scheme showing the proposed microsomal metabolism of DiMeIQx.

accordance with B1 being 2-hydroxyamino-8-hydroxymethyl-3,4-dimethyl-3*H*-imidazo[4,5-*f*]quinoxaline. The mass spectrum of metabolite B2 is shown in Figure 7 (panel B). The presence of a small molecular ion $(M+H)^+$ at *m/z* 274 is in accordance with B2 being 8-hydroxymethyl-3,4-dimethyl-2-nitro-3*H*-imidazo[4,5-*f*]quinoxaline.

The proposed reaction scheme for the microsomal metabolism of DiMeIQx is depicted in Figure 8. Oxidation of the exocyclic amino group results in the formation of two direct acting mutagenic metabolites (N^2-OH-DiMeIQx and NO_2-DiMeIQx). Hydroxylation of the methyl group in position 8 results in the formation of an indirect mutagenic metabolite (8-OH-DiMeIQx), which is further biotransformed to the two direct acting mutagenic metabolites N^2-OH-8-OH-DiMeIQx and NO_2-8-OH-DiMeIQx.

Discussion

It is well established that heterocyclic aromatic amines of the AIA type are metabolically activated to proximate mutagenic metabolites by hydroxylation of the exocyclic amino group (Yamazoe *et al.*, 1983, 1988; Snyderwine *et al.*, 1987; Holme *et al.*, 1989; Buonarati and Felton, 1990; Turteltaub *et al.*, 1990; Frandsen *et al.*, 1991). Incubation of DiMeIQx with hepatic microsomes from Aroclor induced rats for 20 min resulted in two major metabolites, N^2-OH-DiMeIQx and 8-OH-DiMeIQx, whereas longer incubation times resulted in three additional metabolites. It was thus shown that DiMeIQx was metabolized to a mutagenic N^2-hydroxy derivative and that this metabolite can be further oxidized to a mutagenic nitro derivative. This further oxidation is an enzymatically catalyzed reaction, as incubation of N^2-OH-DiMeIQx with microsomes resulted in the formation of the two nitro derivatives, whereas incubation with heat inactivated microsomes did not result in formation of the nitro derivatives (results not shown). This secondary reaction has not usually been observed for AIA type compounds. However, it has recently been reported that IQ can be biotransformed to a direct-acting mutagenic nitro derivative by prostaglandin-H-synthase (Morrison, 1993).

Microsomal hydroxylation of DiMeIQx at the 8-methyl group resulted in an indirect mutagenic metabolite with a specific mutagenic activity comparable with the specific mutagenic activity of the parent compound (results not shown). This is in accordance with observations published by Knize *et al.* (1989). 2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), a compound of similar structure to DiMeIQx, also retains its mutagenicity after 8-hydroxylation (Hayatsu *et al.*, 1987). We have also shown that the indirect mutagen 8-OH-DiMeIQx can be oxidized by microsomes to direct-acting mutagenic N^2-hydroxy and nitro derivatives. The N^2-hydroxy derivatives retained their mutagenic activity in the Salmonella strain TA98 with addition of S9 and in the TA98NR strain. However, the nitro derivatives almost completely lost their mutagenic activity with addition of S9 and lost ~50% of their mutagenic activity in the nitro reductase deficient strain TA98NR. A reduction in the mutagenic activity of nitro compounds with the addition of a metabolic activation system is a well known phenomenon (Rosenkranz and Mermelstein, 1983). As can be seen from the radiochromatograms in Figure 1 (panel 1), the two nitro derivatives of DiMeIQx (A2 and B2) were produced in smaller amounts than the corresponding N^2-hydroxy derivatives. However, the mutagenic activity without addition of S9 was much higher in the fractions containing the nitro derivatives than the fractions containing the N^2-hydroxy derivatives. The N^2-hydroxy derivatives are very reactive compounds, whereas the nitro-derivatives are quite stable. Decomposition of part of the N^2-hydroxy derivative during fraction collection and pippeting may explain the lower specific mutagenic potency of the N^2-hydroxy derivatives compared with the nitro derivatives.

Knize *et al.* (1989) isolated a putative 4-OH-DiMeIQx from rat urine and feces as a minor metabolite compared with 8-OH-DiMeIQx and N^2-acetyl-8-OH-DiMeIQx. In this *in vitro* experiment we did not observe any 4-OH-DiMeIQx. This may be due to the use of a different rat strain than Knize *et al.*, Wistar versus Sprague-Dawley, or the use of different inducers, Aroclor versus β-naphtoflavone or none. Furthermore we have investigated the microsomal metabolism, therefore hepatic enzymes located in the cytosol, which may affect the *in vivo* metabolism, were not considered.

In conclusion DiMeIQx is metabolically transformed to several metabolites that still retain mutagenic activity. This seems to be in contrast to most other AIAs where the predominant mutagenic metabolite found has been the N^2-hydroxy derivative of the parent compound.

N^2-OH-DiMeIQx is a very unstable compound and preliminary studies have shown that it reacts with 2′-deoxyguanosine to a small extent. However, acetylation of N^2-OH-DiMeIQx results in an extremely reactive product which gives adducts with 2′-deoxyguanosine in higher yields. Structural elucidation of this adduct is in progress.

Acknowledgements

The authors wish to thank Rolf Andersson (Swedish University of Agricultural Sciences) for recording the NMR spectra, and Joan Gluver, Bo Lund Jensen and Vivian Jørgensen for skilful technical assistance. This work was in part supported by The Danish Council for Agricultural Research as part of a Nordic collaborative project under the Scandinavian Contact Agency for Agricultural Research.

References

Becher,G., Knize,M.G., Nes,I.F. and Felton,J.S. (1988) Isolation and identification of mutagens from a fried Norwegian meat product. *Carcinogenesis*, **9**, 247–253.

Buonarati,M.H. and Felton,J.S. (1990) Activation of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) to mutagenic metabolite. *Carcinogenesis*, **11**, 1133–1138.

Felton,J.S., Knize,M.G., Shen,N.H., Andresen,B.D., Bjeldanes,L.F. and Hatch,F.T. (1986) Identification of the mutagens in cooked beef. *Environ. Health Perspect.*, **67**, 17–24.

Frandsen,H., Rasmussen,E.S., Nielsen,P.A., Farmer,P., Dragsted,L. and Larsen,J.C. (1991) Metabolic formation, synthesis and genotoxicity of the *N*-hydroxy derivative of the food mutagen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP). *Mutagenesis*, **6**, 93–98.

Gerdhardsson de Verdier,M., Hagman,U., Peters,R.K., Steineck,G. and Övervik,E. (1991) Meat, cooking methods and colorectal cancer: a case-referent study in Stockholm. *Int. J. Cancer*, **49**, 520–525.

Grivas,S. (1985) A convenient synthesis of the potent mutagen 3,4,8-trimethyl-3*H*-imidazo-[4,5-*f*]quinoxalin-2-amine. *Acta Chem. Scand.*, **B39**, 213–217.

Grivas,S., Nyhammar,T., Olsson,K. and Jägerstad,M. (1985) Formation of a new mutagenic DiMeIQx compound in a model system by heating creatinine, alanine and fructose. *Mutat. Res.*, **151**, 177–183.

Hashimoto,Y., Shudo,K. and Okamoto,T. (1982) Modification of DNA with potent mutacarcinogenic 2-amino-6-methylpyrido(1,2-*a*:3′,2′-*d*)imidazole isolated from a glutamic acid pyrolysate: structure of the modified nucleic acid base and initial chemical event caused by the mutagen. *J. Am. Chem. Soc.*, **104**, 7636–7640.

Hayatsu,H., Kasai,H., Yokoyama,S., Miyazawa,T., Yamaizumi,Z., Sato,S., Nishimura,S., Arimoto,S., Hayatsu,T. and Ohara,Y. (1987) Mutagenic metabolites in urine and feces of rats fed with 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline, a carcinogenic mutagen present in cooked meat. *Cancer Res.*, **47**, 791–794.

Holme,J.A., Wallin,H., Brunborg,G., Søderlund,E.J., Hongslo,J.K. and Alexander,J. (1989) Genotoxicity of the food mutagen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP): Formation of 2-hydroxyamino-PhIP, a direct acting genotoxic metabolite. *Carcinogenesis*, **10**, 1389–1396.

Kikugawa,K., Kato,T. and Hayatsu,H. (1986) The presence of 2-amino-3,4,8-trimethyl-3H-imidazo[4,5-*f*]quinoxaline in smoked dry bonito (Katsuobushi). *Jpn. J. Cancer Res.*, **77**, 99–102.

Knize,M.G., Happe,J.A., Healy,S.K. and Felton,J.S. (1987) Identification of mutagenic quinoxaline isomers from fried ground beef. *Mutat. Res.*, **178**, 25–32.

Knize,M.G., Övervik,E., Midtvedt,T., Turteltaub,K.W., Happe,J.A., Gustafsson,J. and Felton,J.S. (1989) The metabolism of 4,8-DiMeIQx in conventional and germ-free rats. *Carcinogenesis*, **10**, 1479–1484.

Lake,B.G. (1987) Preparation and characterisation of microsomal fractions for studies on xenobiotic metabolism. In Snell,K. and Mullock,B. (eds), *Biochemical Toxicology*, IRL Press, Oxford, pp. 183–215.

Maron,D. and Ames,B.N. (1983) Revised methods for the *Salmonella* mutagenicity test. *Mutat. Res.*, **113**, 173–215.

Morrison,L.D., Eling,T.E. and Josephy,P.D. (1993) Prostaglandin H synthase-dependent formation of the direct-acting mutagen 2-nitro-3-methylimidazo[4,5-*f*]quinoline (nitro-IQ) from IQ. *Mutat. Res.*, **302**, 45–52.

Murray,S., Gooderham,N.J., Boobis,A.R. and Davies,D.S. (1988) An assay for 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline and 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline in fried beef using capillary column gas chromatography electron capture negative ion chemical ionisation mass spectrometry. *Biomed. Environ. Mass Spectrom.*, **16**, 221–224.

Nielsen,P.A., Vahl,M. and Gry,J. (1988) HPLC profiles of mutagens in lean ground pork fried at different temperatures. *Z. Lebensm. Unters. Forsch.*, **187**, 451–456.

Rosenkranz,H.S. and Mermelstein,R. (1983) Mutagenicity and genotoxicity of nitroarenes. All nitro-containing chemicals were not created equal. *Mutat. Res.*, **114**, 217–267.

Sedmark,J.J. and Grossberg,S.E. (1977) A rapid, sensitive and versatile assay for protein using coomassie brilliant blue G250. *Anal. Biochem.*, **79**, 544–552.

Skog,K., Knize,M.G., Felton,J.S. and Jagerstad,M. (1992) Formation of new heterocyclic amine mutagens by heating creatinine, alanine, threonine and glucose. *Mutat. Res.*, **268**, 191–197.

Snyderwine,E.G., Roller,P.P., Wirth,P.J., Adamson,R.H., Sato,S. and Thorgeirsson,S.S. (1987) Synthesis, purification and mutagenicity of 2-hydroxyamino-3-methylimidazole[4,5-*f*]quinoline. *Carcinogenesis*, **7**, 1017–1020.

Takahashi,M., Wakabayashi,K., Nagao,M., Yamaizumi,Z., Sato,S., Kinae,N., Tomita,I. and Sugimura,T. (1985) Identification and quantification of 2-amino-3,4,8-trimethyl-3H-imidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx) in beef extracts. *Carcinogenesis*, **6**, 1537–1539.

Turesky,R.J., Bur,H., Huynh-Ba,T., Aeschbacher,H.U. and Milon,H. (1988) Analysis of mutagenic heterocyclic amines in cooked beef products by high-performance liquid chromatography in combination with mass spectrometry. *Fd. Chem. Toxic.*, **26**, 501–509.

Turteltaub,K.W., Knize,M.G., Buonarati,M.H., McManus,M.E., Veronese,M.E., Mazrimas,J.A. and Felton,J.S. (1990) Metabolism of 2-amino-1-methyl-6-phenyl[4,5-*b*]pyridine (PhIP) by liver microsomes and isolated rabbit cytochrome P450 isozymes. *Carcinogenesis*, **11**, 941–946.

Wakabayashi,K., Nagao,M., Esumi,H. and Sugimura,T. (1992) Food-derived mutagens and carcinogens. *Cancer Res.*, **52**, (Suppl.), 2092s–2098s.

Yamazoe,Y., Kamataki,T. and Kato,R. (1981) A sensitive method for determination of biphenyl hydroxylase activities using high-performance liquid chromatography. *Anal. Biochem.*, **111**, 126–130.

Yamazoe,Y., Shimada,M., Kamataki,T. and Kato,R. (1983) Microsomal activation of 2-amino-3-methylimidazo[4,5-*f*]quinoline, a pyrolysate of sardine and beef extracts, to a mutagenic intermediate. *Cancer Res.*, **43**, 5768–5774.

Yamazoe,Y., Medhat,A., Shunichi,M., Toyama,S. and Kato,R. (1988) Metabolic activation of a protein pyrolysate promutagen 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline by rat liver microsomes and purified cytochrome P-450. *Carcinogenesis*, **9**, 105–109.

Received on July 20, 1993; accepted on October 6, 1993

PAPER IV

Carcinogenesis vol.15 no.11 pp.2553–2558, 1994

Formation of DNA adducts by the food mutagen 2-amino-3,4,8-trimethyl-3*H*-imidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx) *in vitro* and *in vivo*. Identification of a N^2-(2′-deoxyguanosin-8-yl)-4,8-DiMeIQx adduct

Henrik Frandsen, Spiros Grivas[1], Robert J.Turesky[2], Rolf Andersson[1], Lars O.Dragsted and John C.Larsen

Institute of Toxicology, National Food Agency, Mørkhøj Bygade 19, DK-2860 Søborg, Denmark, [1]Department of Chemistry, Swedish University of Agricultural Sciences, S-75007 Uppsala, Sweden and [2]Nestec Ltd, Research Centre, CH-1000 Lausanne 26, Switzerland

The covalent binding of the mutagenic N^2-hydroxy metabolite of the food mutagen 2-amino-3,4,8-trimethyl-3*H*-imidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx) to 2′-deoxynucleosides and DNA was investigated *in vitro* and *in vivo*. N^2-Hydroxy-4,8-DiMeIQx reacted to a small extent spontaneously with 2-deoxyguanosine. However, acetylation of N^2-hydroxy-4,8-DiMeIQx with acetic anhydride to form the N^2-acetoxy derivative prior to reaction with 2-deoxyguanosine resulted in much higher yield of adduct. N^2-Acetoxy-4,8-DiMeIQx did not form adducts with 2′-deoxyadenosine, 2′-deoxycytidine or 2′-deoxythymidine. The adduct formed between the N^2-OH metabolite of 4,8-DiMeIQx and 2-deoxyguanosine was analysed by mass spectrometry and NMR spectroscopy and the structure of the adduct was shown to be N^2-(2′-deoxyguanosin-8-yl)-4,8-DiMeIQx. N^2-Acetoxy-4,8-DiMeIQx reacted with calf thymus DNA and formed a covalently bound 4,8-DiMeIQx residue, which could not be removed by repeated precipitations or solvent extractions. The 4,8-DiMeIQx-DNA was hydrolysed enzymatically with nuclease P1/acid phosphatase and HPLC analysis showed that 70% of the bound mutagen was recovered as N^2-(2′-deoxyguanosin-8-yl)-4,8-DiMeIQx. An additional minor adduct accounting for ~15% of the bound mutagen showed UV spectral characteristics similar to N^2-(2′-deoxyguanosin-8-yl)-4,8-DiMeIQx and is probably an undigested oligomer. ^{32}P-Postlabelling analysis of calf thymus DNA modified with 4,8-DiMeIQx *in vitro* and liver DNA from rats dosed with 50 mg/kg 4,8-DiMeIQx showed a similar adduct pattern. In both samples N^2-(2′-deoxyguanosin-8-yl)-4,8-DiMeIQx accounted for 60–70% of the bound mutagen. Thus, these results show that 4,8-DiMeIQx similar to other heterocyclic amines form adducts with C-8 of guanine both *in vitro* and *in vivo* via its N^2-OH metabolite.

Introduction

Heterocyclic aromatic amines (HAAs*) formed in the meat crust during ordinary cooking are potent mutagens and carcinogens (1). Daily exposure to these HAAs through the diet may be associated with diet related cancers, e.g. colorectal cancer (2).

2-Amino-3,4,8-trimethyl-3*H*-imidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx) is a potent bacterial mutagen found in a variety of cooked food products at levels of 0.5–1.2 μg/kg. In fried meat it accounts for ~20% of the mutagenic activity (3–10).

Covalent binding of carcinogens to nucleic acids of DNA is recognized as an important step in the initiation of cancer, and carcinogen–DNA adducts are considered to be promising biomarkers for DNA damage caused by chemical carcinogens (11).

HAAs must undergo bioactivation through *N*-oxidation of the exocyclic amino group to reactive N^2-hydroxy derivatives. These derivatives can either react directly with cellular macromolecules such as DNA and proteins, or prior to reaction they can be further activated by formation of reactive derivatives, such as acetate or sulfate esters (12).

The HAAs 2-amino-6-methylpyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1) (13), 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2) (14), 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) (15), 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) (16–18) and 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) (19) have been reported to form adducts with 2′-deoxyguanosine (dG), by binding of the exocyclic amino group of the HAA to the C-8 of guanine. In addition to the major C-8 adducts, IQ and MeIQx have also been shown to form adducts with N^2 of guanine to a lesser extent (20). In the present investigation we have examined the adduct formation of 4,8-DiMeIQx, and identified N^2-(2′-deoxyguanosin-8-yl)-4,8-DiMeIQx (dG-C8-[4,8-DiMeIQx]) as the major adduct formed both *in vitro* upon treament of DNA with N^2-acetoxy-4,8-DiMeIQx and *in vivo* after dosing rats with 4,8-DiMeIQx.

Materials and methods

Chemicals

4,8-DiMeIQx and [2-^{14}C]4,8-DiMeIQx were synthesized as previously described (21,22). 4,8-DiMeIQx was determined to have >95% purity by HPLC with detection at 260 nm. The sp. act. of [2-^{14}C]4,8-DiMeIQx was 48.7 mCi/mmol and the radiochemical purity was determined to be >92% by HPLC with radioactivity detection. The [2-^{14}C]4,8-DiMeIQx was diluted with unlabelled 4,8-DiMeIQx to a sp. act. of 2.4 mCi/mmol prior to use. N^2-OH-4,8-DiMeIQx and [2-^{14}C]N^2-OH-4,8-DiMeIQx were synthesized as previously described (23). Calf thymus DNA, 2′-deoxyadenosine, 2′-deoxycytidine, 2′-deoxyguanosine, 2′-deoxythymidine, micrococal nuclease (N-3755), phosphodiesterase I (P-6877), alkaline phosphatase (P-4377) and nuclease P1 (N-8630) from penicillium citrinum were obtained from Sigma, St Louis, MO. Acid phosphatase (from potato) and DNase I were obtained from Boehringer, Mannheim, Germany. [γ-^{32}P]ATP (7000 Ci/mmol) was obtained from ICN Chemicals. T4 polynucleotide kinase were obtained from New England Biolabs. Soluene 350 was obtained from Packard, Meriden, CT. PEI-cellulose membranes were obtained from Macherey Nagel, Düren, Germany. HPLC-grade acetonitrile was obtained from Rathburn, Walkerburn, Scotland. All other chemicals were obtained from Merck, Darmstadt, Germany, and were of analytical purity.

Analytical

HPLC analysis was performed on a Hewlett-Packard model 1090 B liquid chromatograph, Waldbronn, Germany, equipped with a photodiode array detector. Nucleosil C_{18} 5 μm, 250×4 mm column was obtained from Macherey

*__Abbreviations:__ HAAs, heterocyclic aromatic amines; 4,8-DiMeIQx, 2-amino-3,4,8-trimethyl-3*H*-imidazo[4,5-*f*]quinoxaline; Glu-P-1, 2-amino-6-methylpyrido[1,2-*a*:3′,2′-*d*]imidazole; Trp-P-2, 3-amino-1-methyl-5*H*-pyrido-[4,3-*b*]-indole; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; dG, 2′-deoxyguanosine; DMF, dimethylformamide; BES, *N,N*-bis(2-hydroxyethyl)-2-aminoethane sulfonic acid; <RAL>, relative adduct level.

© Oxford University Press

Nagel. The flow rate was 0.75 ml/min. Solvents were: A, 50 mM ammonium formate pH 3.5; B, acetonitrile, with the following solvent programming: isocratic 10% B by 3 min, a linear gradient to 30% B by 15 min, a linear gradient to 90% B by 22 min and a linear gradient to 10% B by 25 min. UV spectra were obtained with the photodiode array detector at the elution conditions from the HPLC column.

Positive-ion electrospray mass spectra were obtained at a resolution of 600 on a Profile mass spectrometer, Kratos, Manchester, UK. Samples were dissolved in 0.2% formic acid in 80% acetonitrile, flow rate 10 μl/min.

NMR spectra were recorded on a Varian VXR-400 spectrometer with DMSO-d_6 as solvent. Scintillation counting was done on a Packard Tri-carb model 2500TR liquid scintillation analyser with Hionic Flour as scintillation cocktail using external standardization.

Acetylation of N^2-OH-4,8-DiMeIQx and reaction with 2′-deoxynucleosides

The N^2-acetoxy derivative of N^2-OH-4,8-DiMeIQx was prepared immediately prior to reaction with 2′-deoxynucleosides. N^2-OH-4,8-DiMeIQx (1 mg, 4.1 μmol) was dissolved in 1 ml 75% dimethylformamide (DMF) containing 5 μl acetic acid and cooled to −50 °C; 12 μl of acetic anhydride was added with stirring over 10 min. Acetic acid was included in order to favour *O*-acetylation and suppress formation of the N^2-acetyl derivative (16).

2′-Deoxynucleosides: 2′-deoxyadenosine, 2′-deoxycytidine, 2′-deoxyguanosine and 2′-deoxythymidine were dissolved at a concentration of 2 mg/ml in 0.1 M *N,N*-bis(2-hydroxyethyl)-2-aminoethane sulfonic acid (BES)/NaOH, pH 7.4. Two millilitres of each solution and a control not containing 2′-deoxynucleoside were placed in test tubes and cooled in ice/water. Two hundred millilitres of the crude acetylation mixture was added dropwise, over 5 min, to each of the vigorously stirred test tubes. After stirring for a further 5 min the reaction mixtures were allowed to reach room temperature and analysed by HPLC.

Since only reaction between acetylated N^2-OH-4,8-DiMeIQx and 2′-deoxyguanosine gave a product which was not present in the control incubation, this reaction was scaled up by a factor of 5. The synthesis was repeated twice.

The product precipitated and was collected by centrifugation, redissolved in 25% DMF and purified by HPLC as described above. The sample was injected onto the HPLC column several times and fractions were collected and evaporated to dryness. The overall yield was 494 μg, 1.0 μmol (12%), which was sufficient for structural characterization of the adduct.

DNA binding of [2−^{14}C]N^2-acetoxy-4,8-DiMeIQx

The product from acetylation of [2−^{14}C]N^2-OH-4,8-DiMeIQx (200 μg, 0.82 μmol) was added dropwise to a vigorously stirred solution of calf thymus DNA (1 mg/ml) in 20 ml of 0.1 M BES/NaOH, pH 7.4, at 0 °C. After incubation for 10 min the reaction mixture was allowed to reach room temperature and extracted twice with 40 ml of water-saturated butanol and once with 40 ml of ethyl acetate in order to remove unbound 4,8-DiMeIQx. The DNA was precipitated by addition of 1 ml of 3 M sodium acetate and 40 ml of cold ethanol. The DNA was washed with ethanol, dried and dissolved in 20 ml of 0.1 M BES/NaOH, pH 7.4. The precipitation of the DNA was repeated twice and the DNA was finally dissolved in water at a concentration of 2 mg/ml and an aliquot was used for measurement of bound radioactivity. The DNA was solubilized in Soluene 350 according to the manufacturer's instructions (Packard) and the bound radioactivity was measured after addition of 10 ml of Hionic-Flour. It was estimated that 5.6% of the N^2-OH-4,8-DiMeIQx had bound to the DNA, corresponding to 2.3 nmol of 4,8-DiMeIQx/mg DNA.

Nuclease P1 digestion of 4,8-DiMeIQx-DNA

Modified calf thymus DNA was diluted to 0.25 mg/ml with 30 mM sodium acetate, pH 5.3, containing 1.5 mM $ZnSO_4$, and denatured by heating on a boiling waterbath for 5 min followed by cooling in ice/water. The DNA was enzymatically hydrolysed to nucleosides by addition of 20 units of Nuclease P1 and 1 unit of acid phosphatase per ml of incubation mixture. After incubation at 37°C for 30 min followed by cooling in ice/water, 3 vol of cold ethanol was added and the precipitate was removed by centrifugation. The supernatant, which contained >92% of the radioactivity, was evaporated to dryness by rotary evaporation. The residue was dissolved in a small volume of 10% acetonitrile in 50 mM ammonium formate, pH 3.5, and analysed by HPLC. The eluate was collected at 1 min intervals and mixed with scintillation cocktail for measurement of radioactivity.

DNase/phosphodiesterase digestion of 4,8-DiMeIQx−DNA

Modified calf thymus DNA was digested to nucleosides by a procedure previously used for digestion of IQ−DNA and MeIQx−DNA (20). Modified DNA was dissolved at a concentration of 0.5 mg/ml in 5 mM Tris, 10 mM $MgCl_2$, pH 7.5. After addition of DNase I at a concentration of 0.2 mg/ml the mixture was incubated for 5 h at 37°C. Phosphodiesterase I (0.03 unit/ml) and alkaline phosphatase (0.4 unit/ml) were then added and incubation was continued at 37°C for 18 h.

The digest was either further processed as described above, or was subjected to an additional Nuclease P1 digestion.

Twenty-five microlitres of 2 M sodium acetate, pH 4.75, and 25 ml 80 mM $ZnSO_4$ were added per ml of incubation mixture. After addition of 20 units of nuclease P1 and 1 unit of acid phosphatase per ml of the mixture, incubation was continued for 2 h at 37°C. The digest was further processed as described above.

More than 90% of the bound radioactivity was recovered in the supernatant from both digestion procedures.

Modification of DNA in vivo

Two adult male Wistar rats (age 8 weeks, weight 250 g), delivered from Møllegård Breeding Center Ltd, Lille Skensved, Denmark, were dosed by gavage with 4,8-DiMeIQx (50 mg/kg, dissolved in 50% ethanol). The animals were killed after 72 h and the liver removed. The liver was homogenized and the DNA was isolated by the phenol/chloroform extraction procedure previously described (24).

^{32}P-Postlabelling of 4,8-DiMeIQx−DNA adducts formed in vitro and in vivo

DNA was digested to 3′-phosphodeoxynucleosides as follows: 3.75 μg of DNA was digested in 50 μl of 20 mM sodium succinate and 10 mM $CaCl_2$, pH 6.0, containing 3 units of micrococal nuclease and 0.3 units of spleen phosphodiesterase for 4 h at 37 °C. Samples were then lyophilized.

3′-Phospho-dG-C8-[4,8-DiMeIQx] was used as reference substance for ^{32}P-postlabelling analysis. This compound was synthesized in the same manner as dG-C8-[4,8-DiMeIQx] by substitution of dG with 3′-phospho-dG. The product eluted earlier than dG-C8-[4,8-DiMeIQx] on HPLC, but showed an identical UV spectrum. HPLC analysis following treatment of the product with acid phosphatase resulted in a product that co-eluted with and showed an UV spectrum identical with dG-C8-[4,8-DiMeIQx]. This indicates that the product is 3′-phospho-dG-C8-[4,8-DiMeIQx].

Samples were postlabeled under ATP limiting conditions as follows. The samples were resuspended in 20 ml of polynucleotide kinase buffer (30 mM Bicine, 10 mM $MgCl_2$, 2 mM spermidine and 10 mM DTT, pH 9.6, containing 55 pmol of [γ-^{32}P]ATP, followed by 1 μl buffer containing 10 units of T4 polynucleotide kinase and incubated for 1 h at 37°C.

Adducts were analysed by TLC (PEI−cellulose) developed by adapting previously published methods (25). Development in D1 was done overnight in 2.3 M NaH_2PO_4, pH 5.8. Development in D3 (bottom to top) was done in 3.0 M lithium formate, 8.5 M urea, pH 3.0. D4 (rotated 90°) was done with 0.7 M Na_3PO_4 containing 0.5 M Tris−HCl and 8.5 M urea, pH 8.0. D5 was done in the same direction as D4 with 1.7 M NaH_2PO_4, pH 5.8.

Kodak scientific imaging film (X-OMAT) and Du Pont Cronex intensification screens were used for autoradiography. DNA adducts were located on the plates by autoradiography by exposing the films at −80 °C for varying periods of time. Radioactive spots (adducts) were cut out and the radioactivity was quantified by Cerenkov counting. Relative adduct levels were calculated as described by Randerath *et al.* (26).

Results

Reaction of acetylated N^2-OH-4,8-DiMeIQx with 2′-deoxynucleosides

Room temperature incubation of N^2-OH-4,8-DiMeIQx with dG at neutral pH resulted in the formation of small amounts of adduct (not shown). However, acetylation of N^2-OH-4,8-DiMeIQx to the putative N^2-acetoxy derivative prior to reaction with dG gave a much higher yield of adduct. Addition of N^2-acetoxy-4,8-DiMeIQx to solutions containing 2′-deoxycytidine, 2′-deoxyadenosine or 2′-deoxythymidine only resulted in decomposition products which were also present in a control incubation not containing 2′-deoxynucleoside. Figure 1 shows the chromatogram of the crude product from reaction of N^2-acetoxy-4,8-DiMeIQx with dG. The only product, not seen in control incubations, eluted at 21.3 min, and the UV spectrum recorded at the elution conditions from the column showed maxima at 326 and 258 nm, and a valley at 282 nm (Figure 1, inset). The product, of which ~90% precipitated from solution, was further purified by HPLC and characterized by mass spectrometry and NMR spectroscopy. The positive ion electrospray mass spectrum of the purified product showed a molecular ion $[M + H]^+$ at 493.5 (Figure 2), which is in accordance with an adduct between 4,8-DiMeIQx and dG.

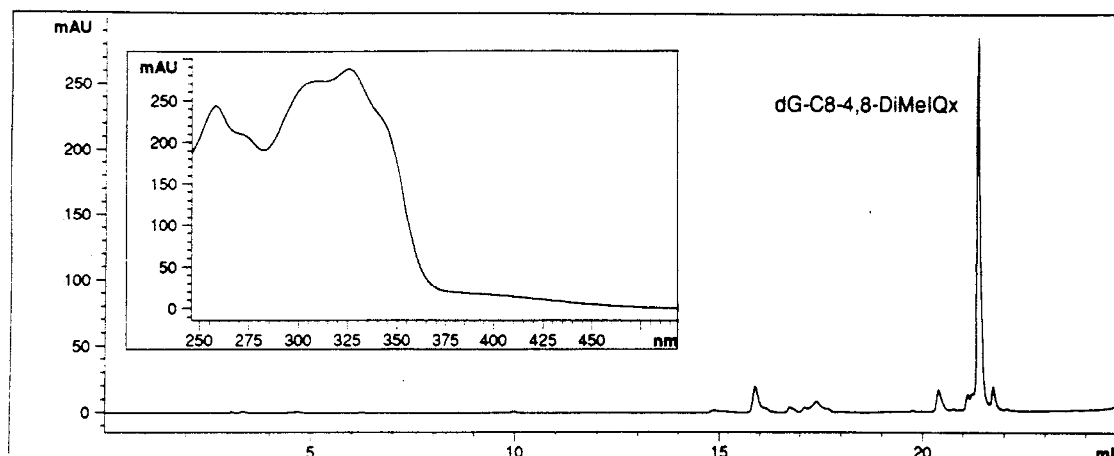

Fig. 1. Chromatogram monitored at 323 nm of the crude product from reaction of acetylated N^2-hydroxy-4,8-DiMeIQx with 2′-deoxyguanosine. **Inset**, UV spectrum at the elution conditions from the column of the adduct, dG-C8-[4,8-DiMeIQx], eluting to 21.4 min.

As shown in Table I, the ^{1}HNMR spectrum revealed the presence of all non-exchangeable protons: all sugar protons, the two aromatic protons of 4,8-DiMeIQx (H-5 and H-7) and the three methyl groups at N-3, C-4 and C-8 of 4,8-DiMeIQx. However, the characteristic signal of the C-8 proton of guanine at δ 7.85 p.p.m. was absent. This indicates that the exocyclic amino group of 4,8-DiMeIQx is attached to the C-8 of guanine. On the basis of the mass spectrometry and NMR analysis it was concluded that the structure of the adduct was N^2-(deoxyguanosin-8-yl)-4,8-DiMeIQx (dG-C8-[4,8-DiMeIQx]) (Figure 3).

The resonance for H-2′cb is somewhat downfield compared to what is observed for 2′-deoxyguanosine. Such a downfield shift is considered to be due to a deshielding effect of the nearby guanine N-3 atom and suggests that the adduct is in a *syn* conformation (31). A similar *syn* conformation of C-8 adducts has previously been observed for other heterocyclic amines (20).

The conformation of the glycosylic bond is in the *anti* conformation in unmodified DNA. A change to *syn* conformation in adducted DNA may have an influence on the structure of the DNA and on adduct persistence *in vivo*.

Reaction of N^2-acetoxy-4,8-DiMeIQx with calf thymus DNA

[2−^{14}C]N^2-acetoxy-4,8-DiMeIQx reacted with calf thymus DNA, giving a covalent bound 4,8-DiMeIQx residue, which could not be removed by repeated solvent extractions or precipitations. The adduct level was 2.3 nmol/mg DNA, corresponding to one out of 300 guanine residues forming adducts. Higher adduct levels could be obtained by using a higher ratio of N^2-acetoxy-4,8-DiMeIQx/DNA; however, this resulted in increasing difficulties in succesful enzymatic hydrolysis of the modified DNA. HPLC analysis of heat denaturated [2−^{14}C]4,8-DiMeIQx−DNA subjected to enzymatic hydrolysis with nuclease P1/acid phosphatase showed one major and a few minor peaks (Figure 4). The major peak, eluting at 21.3 min accounted for 70% of the total eluted radioactivity. This peak co-eluted with dG-C8-[4,8-DiMeIQx] and had an identical UV spectrum which corroborated its identity. The most important of the minor peaks, eluting at 20.4 min, accounted for 14% of the radioactivity and this peak also had a UV spectrum that was identical to the spectrum of dG-C8-[4,8-DiMeIQx] (Figure 5).

HPLC analysis of [2−^{14}C]4,8-DiMeIQx−DNA subjected to enzymatic hydrolysis with DNase, phosphodiesterase and alkaline phosphatase, showed one major and at least four minor peaks (Figure 6). The major peak, which accounted for

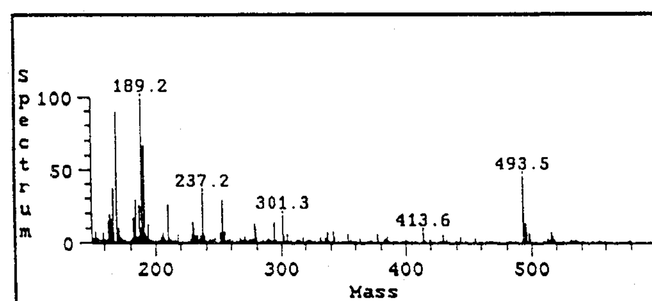

Fig. 2. Positive-ion electrospray mass spectrum of purified dG-C8-[4,8-DiMeIQx] showing the molecular ion [M + H]$^+$ at m/z 493.5.

Table I. ^{1}HNMR chemical shifts of N^2-(dG-8-yl)-4,8-DiMeIQx referenced to the solvent, DMSO-d_6 = 2.49 p.p.m.

Chemical shift (p.p.m.)	Multiplicity	Assignment
8.74	s	H-7
7.60	s	H-5
6.56	t	H-1′c
4.46	m	H-3′c
3.97	s	N-CH_3
3.82	m	H-4′c
3.68	dd	H-5′cb
3.54	dd	H-5′ca
3.25	m	H-2′cb
2.89	s	4-CH_3
2.76	s	8-CH_3
2.05	m	H-2′ca

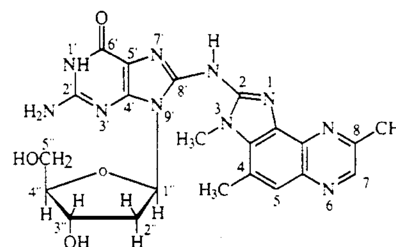

Fig. 3. Structure of dG-C8-[4,8-DiMeIQx].

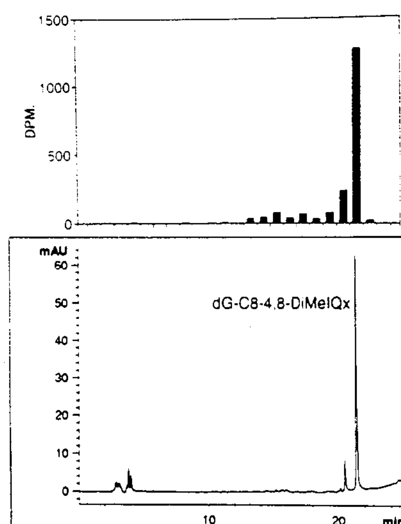

Fig. 4. HPLC profiles of calf thymus DNA modified with [2−^{14}C]4,8-DiMeIQx after heat denaturation and digestion with nuclease P1 and acid phosphatase. **Lower panel**: UV trace monitored at 323 nm. **Upper panel**: Fractions were collected at 1 min intervals and radioactivity measured by scintillation counting.

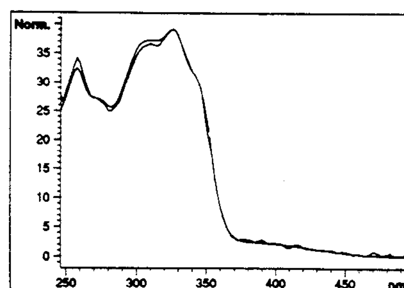

Fig. 5. Comparison of the UV spectrum of dG-C8-[4,8-DiMeIQx] eluting to 21.3 min and the UV spectrum of the minor peak eluting to 20.4 min.

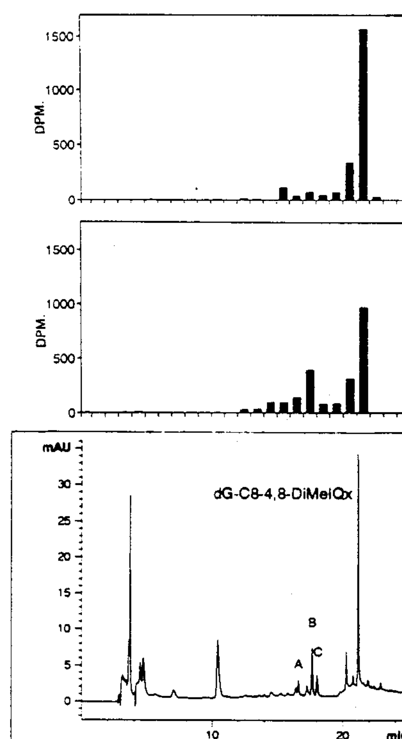

Fig. 6. HPLC profiles of calf thymus DNA modified with [2−^{14}C]4,8-DiMeIQx after digestion with DNase, phosphodiesterase and alkaline phosphatase. **Lower panel**: UV trace monitored at 323 nm. **Middle panel**: Fractions were collected at 1 min intervals and radioactivity measured by scintillation counting. **Upper panel**: After aditional digestion with nuclease P1 and acid phosphatase. Fractions were collected at 1 min intervals and radioactivity measured by scintillation counting.

44% of the eluted radioactivity, co-eluted with and showed a UV spectrum identical to dG-C8-[4,8-DiMeIQx]. The four minor peaks, A, B, C and the peak eluting at 20.4 min also seen in the Nuclease P1 digest, all showed UV spectra similar to dG-C8-[4,8-DiMeIQx]. HPLC analysis of the above hydrolysis product after an additional digestion with Nuclease P1 and acid phosphatase showed that peaks A, B and C had disappeared (not shown) and that the radioactivity associated with the dG-C8-[4,8-DiMeIQx] peak had increased to 70% (Figure 6).

This indicates that the minor peaks, eluting earlier than dG-C8-[4,8-DiMeIQx] are incompletely digested oligomers.

^{32}P-Postlabelling of 4,8-DiMeIQx−DNA formed in vitro and in vivo

Three adducts were detected by ^{32}P-postlabelling under adduct intensification conditions both in calf thymus DNA modified with N^2-acetoxy-4,8-DiMeIQx and *in vivo*. The major adduct co-migrated with synthetic 3′P-dG-C8-[4,8-DiMeIQx] (Figure 7) and the two minor adducts formed *in vitro* also co-migrated with those seen *in vivo*, Figure 7. Thus, all adducts appear to be derived from the N^2-hydroxy metabolite of 4,8-DiMeIQx. The relative adduct level (<RAL>) values under adduct itensification conditions from calf thymus DNA modified with 4,8-DiMeIQx at a level of 1.0 µg/mg DNA and from rat liver DNA from an animal dosed at 50 mg/kg and analysis 72 h post-exposure are shown in Table II.

Table II. Relative adduct level (<RAL>) values under adduct intensification conditions of (I) calf thymus DNA modified with N^2-acetoxy-4,8-DiMeIQx; (II) liver DNA from a rat dosed with 50 mg/kg of 4,8-DiMeIQx

	I	II
dG-C8	$10.6 \pm 0.8 \times 10^{-3}$	$3.54 \pm 2.42 \times 10^{-5}$
Adduct 2	$4.1 \pm 2.5 \times 10^{-3}$	$1.43 \pm 0.54 \times 10^{-5}$
Adduct 3	$0.8 \pm 0.4 \times 10^{-3}$	$1.30 \pm 1.30 \times 10^{-5}$

DNA adducts were measured 72 h post-exposure (average ± SD, $n = 3$).

Therefore, the dG-C8 adduct accounts for ~70% of adducts *in vitro* and ~60% of adducts *in vivo* under adduct intensification conditions. Note that adduct intensification conditions preferentially label adducts over non-modified nucleotides and provide an 'overestimation' of actual adduct levels (25). It is probable that these two other adducts may be incompletely digested oligomers, based upon the relative amount of dG-C8 in postlabelling versus adduct digested to the deoxynucleoside which was analysed by HPLC. The amount of dG-C8-[4,8-DiMeIQx] measured relative to other uncharacterized adducts or oligomers is comparable to what has been reported for other heterocyclic amines assayed by ^{32}P-postlabelling (18,27,28).

Discussion

4,8-DiMeIQx is, like other HAAs, biotransformed to a mutagenic metabolite by oxidation of the exocyclic amino group

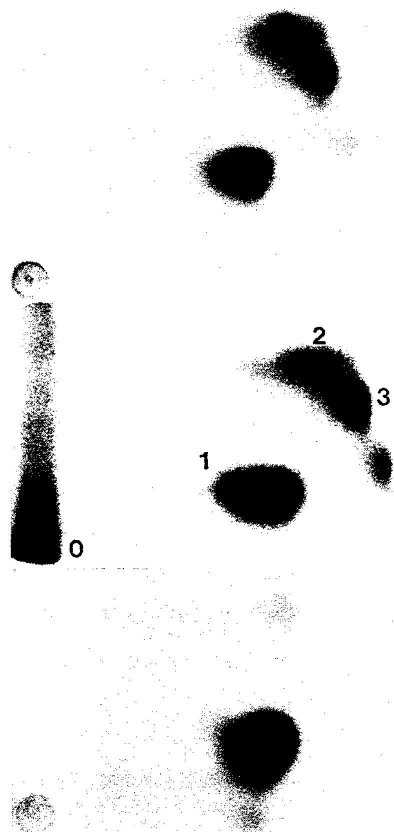

Fig. 7. ^{32}P-Postlabelling of calf thymus DNA modified with *N*-acetoxy-4,8-DiMeIQx (**upper**), rat liver DNA 72 h following exposure to 4,8-DiMeIQx (50 mg/kg) (**middle**) and 3′-phospho-dG-C8-[4,8-DiMeIQx] (**lower**). 0 = origin; adduct 1 = dG-C8-[4,8-DiMeIQx].

to the N^2-hydroxy derivative (22). Although N^2-OH-4,8-DiMeIQx at room temperature spontaneously reacts to a small extent with dG to form an adduct, acetylation of the N^2-OH-4,8-DiMeIQx to the putative N^2-acetoxy derivative prior to reaction with dG resulted in a much more reactive species, giving much higher yields of adducts. Formation of the presumed N^2-acetoxy derivative of Glu-P-1, Trp-P-2, MeIQx and PhIP by reaction with either acetic anhydride or ketene prior to reaction with dG have previously been shown to result in efficient adduct formation (13,14,16,18,29).

The synthesis of the N^2-acetoxy-4,8-DiMeIQx was performed at −50°C, because of the rapid decomposition of N^2-acetoxy-4,8-DiMeIQx at temperatures above −30°C. The reaction with deoxynucleosides and DNA was performed at 0°C. At this temperature the highest yield of adduct was obtained; at higher temperatures the N^2-acetoxy-4,8-DiMeIQx probably decomposed before it could efficiently react with the dG or DNA.

N^2-Acetoxy-4,8-DiMeIQx reacted with dG, but not with other deoxynucleosides, giving dG-C8-[4,8-DiMeIQx] in high yield. Similarly, other heterocyclic aromatic amines, IQ (15), Glu-P-1 (13), Trp-P-2 (14), PhIP (16–18) and MeIQx (19) have been reported to form adducts with dG, by binding of the exocyclic amino group of the aromatic amine to the C-8 of guanine. However, Turesky *et al.* (20) have recently reported that IQ and MeIQx, in addition to the major C-8 adduct, also form minor adducts where the C-5 of the aromatic amine is attached to the N^2 of guanine. These minor adducts showed UV spectra dissimilar to the UV spectra of the major C-8 adducts. Although 4,8-DiMeIQx, MeIQx and IQ are compounds of similar structure, we did not observe indications of a minor adduct in addition to the dG-C8-[4,8-DiMeIQx] adduct. 4,8-DiMeIQx, in contrast to IQ and MeIQx, has a methyl group in position 4 of the aromatic ring system. Steric hindrance by this methyl group probably prevents nucleophilic attack by the 2-amino group of guanine on the C-5 of 4,8-DiMeIQx.

N^2-Acetoxy-4,8-DiMeIQx also readily reacted with calf thymus DNA. HPLC analysis of the modified DNA after heat denaturation and enzymatic hydrolysis showed that 70% of the bound radioactivity co-eluted with dG-C8-[4,8-DiMeIQx]. However, one minor peak eluting earlier and accounting for 14% of the bound radioactivity was also present. This earlier eluting compound had a UV spectrum which was identical to the UV spectrum of dG-C8-[4,8-DiMeIQx]. Some additional minor peaks were too small for UV spectral analysis. However, digestion of the modified DNA with DNase, phosphodiesterase and alkaline phosphatase resulted in a hydrolysate where dG-C8-[4,8-DiMeIQx] only accounted for 44% of the radioactivity. In addition, four earlier eluting peaks with UV spectra similar to dG-C8-[4,8-DiMeIQx] could be identified. The radioactivity associated with three of these peaks was added to the dG-C8-[4,8-DiMeIQx] peak after further hydrolysis with nuclease P1 and acid phosphatase (Figure 6). This indicates that enzymatic hydrolysis is incomplete and results in the presence of oligomers. Furthermore, increasing the degree of modification of the DNA with this bulky adduct resulted in less efficient enzymatic hydrolysis.

^{32}P-Postlabelling analysis of modified calf thymus DNA and liver DNA from rats dosed with 4,8-DiMeIQx showed a similar adduct pattern. dG-C8-[4,8-DiMeIQx] accounted for 60–70% of the measured radioactivity, and two minor adducts, probably undigested oligomers, accounted for 15–30% and 5–20% of the radioactivity respectively. These results are comparable with the results from HPLC analysis of DNA modified *in vitro*. 4,8-DiMeIQx DNA adduct formation *in vivo* analysed by the ^{32}P-postlabelling technique have previously been described, resulting in three major and two minor adducts in liver DNA of rats feed 4,8-DiMeIQx (30). It has, however, recently been shown that the number of adduct spots found in the postlabelling assay of DNA adducted with HAAs are reduced when an additional nuclease P1 digestion is included in the assay (32). It is suggested that many of the additional adduct spots observed are oligomers. This is in accordance with our observation that additional nuclease digestion or nuclease digestion of heat-denatured DNA results in a reduced number of adduct peaks.

We have recently reported that 4,8-DiMeIQx is metabolized *in vitro* to the direct-acting mutagenic N^2-hydroxy-4,8-DiMeIQx and an indirect-acting mutagenic 8-hydroxymethyl derivative (8-CH_2OH-4,8-DiMeIQx). Moreover, prolonged incubation times also resulted in formation of a direct-acting mutagenic N^2-hydroxy derivative of the 8-CH_2OH-4,8-DiMeIQx (22). The adduct patterns, as measured by ^{32}P-postlabelling analysis, between DNA modified with N^2-acetoxy-4,8-DiMeIQx and DNA modified *in vivo* were similar. This indicates that activation of 8-CH_2OH-4,8-DiMeIQx to a DNA-reactive species does not take place *in vivo* to any appreciable extent, because additional adduct spots, originating from 8-CH_2OH-4,8-DiMeIQx, would have been expected to be present in ^{32}P-postlabelling analysis of DNA modified *in vivo* as compared to DNA modified with N^2-acetoxy-4,8-DiMeIQx.

In conclusion, metabolic activation of 4,8-DiMeIQx to the N^2-hydroxy derivative results in formation of DNA adducts *in vivo* as well as in *in vitro*. The N^2-hydroxy derivative either directly reacts with dG of DNA or reacts after esterification to a more reactive species, resulting in formation of dG-C8-[4,8-DiMeIQx] as the major adduct.

Acknowledgements

The authors wish to thank Joan Gluver for skilful technical assistance. This work was in part supported by The Danish Council for Agricultural Research as part of a Nordic collaborative project under the Scandinavian Contact Agency for Agricultural Research.

References

1. Wakabayashi,K., Nagao,M., Esumi,H. and Sugimura,T. (1992) Food-derived mutagens and carcinogens. *Cancer Res. (Suppl.)*, **52**, 2092s–2098s.
2. Gerdhardsson de Verdier,M., Hagman,U., Peters,R.K., Steineck,G. and Övervik,E. (1991) Meat, cooking methods and colorectal cancer: a case-referent study in Stockholm. *Int. J. Cancer*, **49**, 520–525.
3. Takahashi,M., Wakabayashi,K., Nagao,M., Yamaizumi,Z., Sato,S., Kinae,N., Tomita,I. and Sugimura,T. (1985) Identification and quantification of 2-amino-3,4,8-trimethyl-3*H*-imidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx) in beef extracts. *Carcinogenesis*, **6**, 1537–1539.
4. Kikugawa,K., Kato,T. and Hayatsu,H. (1986) The presence of 2-amino-3,4,8-trimethyl-3*H*-imidazo[4,5-*f*]quinoxaline in smoked dry bonito (Katsuobushi). *Jpn. J. Cancer Res. (Gann)*, **77**, 99–102.
5. Knize,M.G., Happe,J.A., Healy,S.K. and Felton,J.S. (1987) Identification of the mutagenic quinoxaline isomers from fried ground beef. *Mutat. Res.*, **178**, 25–32.
6. Becher,G., Knize,M.G., Nes,I.F. and Felton,J.S. (1988) Isolation and identification of mutagens from a fried Norwegian meat product. *Carcinogenesis*, **9**, 247–253.
7. Murray,S., Gooderham,N.J., Boobis,A.R. and Davies,D.S. (1988) An assay for 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline and 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline in fried beef using capillary column gas chromatography electron capture negative ion chemical ionisation mass spectrometry. *Biomed. Environ. Mass Spectrom.*, **16**, 221–224.
8. Nielsen,P.A., Vahl,M. and Gry,J. (1988) HPLC profiles of mutagens in lean ground pork fried at different temperatures. *Z. Lebensm. Unters. Forsch.*, **187**, 451–456.
9. Turesky,R.J., Bur,H., Huynh-Ba,T., Aeschbacher,H.U. and Milon,H. (1988) Analysis of mutagenic heterocyclic amines in cooked beef products by high-performance liquid chromatography in combination with mass spectrometry. *Food Chem. Toxicol.*, **26**, 501–509.
10. Felton,J.S., Knize,M.G., Shen,N.H., Andresen,B.D., Bjeldanes,L.F. and Hatch,F.T. (1986) Identification of the mutagens in cooked beef. *Environ. Health Perspect.*, **67**, 17–24.
11. Hemminki,K. (1993) DNA adducts, mutations and cancer. *Carcinogenesis*, **14**, 2007–2012.
12. Kadlubar,F.F. and Beland,F.A. (1985). Chemical properties of ultimate carcinogenic metabolites of arylamines and arylamides. In Harvey,R.G. (ed.), *Polycyclic Hydrocarbons and Carcinogenesis, ACS Symposium Series*. American Chemical Society, Washington, DC, Vol. 283, pp. 341–370.
13. Hashimoto,Y., Shudo,K. and Okamoto,T. (1982). Modification of DNA with potent mutacarcinogenic 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole isolated from a glutamic acid pyrolysate: structure of the modified nucleic acid base and initial chemical event caused by the mutagen. *J. Am. Chem. Soc.*, **104**, 7636–7640.
14. Hashimoto,Y., Shudo,K. and Okamoto,T. (1980) Activation of a mutagen, 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole. Identification of 3-hydroxyamino-1-methyl-5*H*-pyrido[4,3-*b*]indole and its reaction with DNA. *Biochem. Biophys. Res. Commun.*, **96**, 355–362.
15. Snyderwine,E.G., Roller,P.P., Adamson,R.H., Sato,S. and Thorgeirsson,S. (1988) Reaction of *N*-hydroxylamine and *N*-acetoxy derivatives of 2-amino-3-methylimidazo[4,5-*f*]quinoline with DNA. Synthesis and identification of *N*-(deoxyguanosin-8-yl)-IQ. *Carcinogenesis*, **9**, 1061–1065.
16. Frandsen,H., Grivas,S., Andersson,R., Dragsted,L. and Larsen,J.C. (1992) Reaction of the N^2-acetoxy derivative of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) with 2′-deoxyguanosine and DNA. Synthesis and identification of N^2-(2′-deoxyguanosin-8-yl)-PhIP. *Carcinogenesis*, **13**, 629–635.
17. Nagaoka,H., Wakabayashi,K., Kim,S., Kim,I., Tanaka,Y., Ochiai,M., Tada,A., Nukaya,H., Sugimura,T. and Nagao,M. (1992) Adduct formation at C-8 of guanine on *in vitro* reaction of the ultimate form of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine with 2′-deoxyguanosine and its phosphate esters. *Jpn. J. Cancer Res.*, **83**, 1025–1029.
18. Lin,D., Kalderik,K.R., Turesky,R.J., Miller,D.W., Lay,J.O. and Kadlubar,F.F. (1992) Identification of *N*-(deoxyguanosin-8-yl)-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine as the major adduct formed by the food-borne carcinogen, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine, with DNA. *Chem. Res. Toxicol.*, **5**, 691–697.
19. Ochiai,M., Nagaoka,H., Wakabayashi,K., Tanaka,Y., Kim,S., Tada,A., Nukaya,H., Sugimura,T. and Nagao,M. (1993) Identification of N^2-(deoxyguanosin-8-yl)-2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline 3′,5′-diphosphate, a major DNA adduct detected by nuclease P1 modification of the ^{32}P-postlabelling method, in the liver of rats fed MeIQx. *Carcinogenesis*, **14**, 2165–2170.
20. Turesky,R.J., Rossi,S.C., Welti,D.H., Lay,J.O. and Kadlubar,F.F. (1992) Characterization of DNA adducts formed *in vitro* by reaction of *N*-hydroxy-2-amino-3-methylimidazo[4,5-*f*]quinoline and *N*-hydroxy-2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline at the C-8 and N^2 atoms of guanine. *Chem. Res. Toxicol.*, **5**, 479–490.
21. Grivas,S. and Olsson K. (1985) An improved synthesis 3,8-dimethyl-3*H*-imidazo[4,5-*f*]quinoxalin-2-amine ('MeIQx') and its 2-^{14}C-labelled analogue. *Acta Chem. Scand.*, **B39**, 31–34.
22. Grivas,S., Tian,W., Ronne,E., Lindstrom,S. and Olsson,K. (1993) Synthesis of mutagenic methyl- and phenyl-substituted 2-amino-3*H*-imidazo[4,5-*f*]quinoxalines via 2,1,3-benzoselenadiazoles. *Acta Chem. Scand.*, **47**, 521–528.
23. Frandsen,H., Nielsen,P.A., Grivas,S. and Larsen,J.C. (1994) Microsomal metabolism of the food mutagen 2-amino-3,4,8-trimethyl-3*H*-imidazo[4,5-*f*]quinoxaline to mutagenic metabolites. *Mutagenesis*, **9**, 59–65.
24. Gupta,R.C. (1984) Nonrandom binding of the carcinogen *N*-hydroxy-2-acetylaminofluorene to repetitive sequences of rat liver DNA *in vivo*. *Proc. Natl. Acad. Sci. USA*, **81**, 6943–6947.
25. Nouso,K., Bohr,V.A., Schut,H.A.J. and Snyderwine,E.G. (1993) Quantitation of 2-amino-3-methylimidazo[4,5-*f*]quinoline and 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline DNA adducts in specific sequences using alkali or uvrABC exinuclease. *Mol. Carcinogenesis*, **7**, 126–134.
26. Randerath,E., Agrawall,H.P., Weaver,A., Bordelon,C.B. and Randerath,K. (1985) ^{32}P-Postlabelling analysis of DNA adducts persisting for up to 42 weeks in the skin, epidermis and dermis of mice treated topically with 7,12-dimethylbenz[*a*]anthracene. *Carcinogenesis*, **6**, 1117–1126.
27. Snyderwine,E.G., Yamashita,K., Adamsom,R.H., Sato,S., Nagao,M., Sugimura,T. and Thorgeirsson,S.S. (1988) Use of the ^{32}P-postlabeling method to detect DNA adducts of 2-amino-3-methylimidazolo[4,5-*f*]quinoline (IQ) in monkeys fed IQ: identification of the *N*-(deoxyguanosin-8-yl)-IQ adduct. *Carcinogenesis*, **9**, 1739–1743.
28. Fukutome,K., Ochiai,M., Wakabayashi,K., Watanabe,S., Sugimura,T. and Nagao,M. (1994) Detection of guanine-C8-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine adduct as a single spot on thin-layer chromatography by modification of the ^{32}P-postlabeling method. *Jpn. J. Cancer Res.*, **85**, 113–117.
29. Yamashita,K., Umemoto,A., Grivas,S., Kato,S. and Sugimura,T. (1988) *In vitro* reaction of hydroxyamino derivatives of MeIQx, Glu-P-1 and Trp-P-2 with DNA: ^{32}P-postlabelling analysis of DNA adducts formed *in vivo* by the parent amines and *in vitro* by their hydroxyamino derivatives. *Mutagenesis*, **3**, 515–520.
30. Yamashita,K., Umemoto,A., Grivas,S., Kato,S., Sato,S. and Sugimura,T. (1988) Heterocyclic amine-DNA adducts analyzed by ^{32}P-postlabelling method. *Nucleic Acids Res., Symp. Series*, no. 19, pp. 111–114.
31. Evans,F.E., Miller,D.W. and Beland,F.A. (1980) Sensitivity of the conformation of deoxyguanosine to binding at the C-8 position by *N*-acetylated and unacetylated 2-aminofluorene. *Carcinogenesis*, **1**, 955–959.
32. Pfau,W., Brocksted,U., Söhren,K. and Marquardt,H. (1994) ^{32}P-Postlabelling analysis of DNA adducts by food-derived heterocyclic amines: evidence for incomplete hydrolysis and a procedure for adduct pattern simplification. *Carcinogenesis*, **15**, 877–882.

Received on April 28, 1994; revised on July 26, 1994; accepted on August 1, 1994