Heat-processed fats : analysis of genotoxicity

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HEAT-PROCESSED FATS: ANALYSIS OF GENOTOXICITY

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Rijksuniversiteit Limburg te Maastricht,
op gezag van de Rector Magnificus, Prof. Dr. F.I.M. Bonke,
volgens het besluit van het College van Dekanen,
in het openbaar te verdedigen op vrijdag, 22 juni 1990 om 16.00 uur

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Gerrigje Johanna Hageman

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Abbreviations

BHA	butylated hydroxyanisole
BHT	butylated hydroxytoluene
BrdU	5-bromodeoxyuridine
CO	coconut oil
DCM	dichloromethane
DMSO	dimethylsulphoxide
DPTG	dimeric and polymeric triglycerides
HGPRT	hypoxanthine guanine phosphoribosyl transferase
HPLC	high-performance liquid chromatography
ID	internal diameter
i.p.	intraperitoneally
LAHPO	linoleic acid hydroperoxides
LI	labeling index
LI _C	labeling index of control lymphocyte cultures
Me	methanol
MDA	malondialdehyde
min	minute(s)
NPF	non-polar fraction
PHA	phytohaemagglutinin
PBS	phosphate-buffered saline
PE	petroleum ether
PF	polar fraction
P/S	ratio of polyunsaturated fatty acids and saturated fatty acids
PO	polyunsaturated fatty acid-rich frying oil
PUFA	polyunsaturated fatty acids
rev	revertants
RP	reversed phase
SCE	sister chromatid exchange
SD	standard deviation
SEM	standard error of mean
TBA-RS	thiobarbituric acid-reactive substances
6-TG	6-thioguanine
TG ^r	6-thioguanine-resistant
THF	tetrahydrofuran
Tpot	potential doubling time
UV	ultra violet
Vf	variant frequency

Chapter 1

Heat-processing of food: Possible role of heat-processed lipids in colon tumorigenesis

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1. Heat-processing of food

The use and control of fire by early Human, some 14 million years ago, has had a tremendous impact on diet and availability of foods (Eaton et al., 1985; 1988). Heat processing of food, involving roasting, boiling, baking and frying, has been used ever since as a means of food preservation, and for improvement of taste and digestibility. Mild heat treatment of food has beneficial effects, since it prevents decomposition due to autolysis by endogenous lytic enzymes or putrefactive bacteria and fungi. Oxidizing enzymes in foods of animal and vegetable origin, which catalyze reactions leading to off-tastes, as well as flavors and colors, are also denatured by heat treatment. An additional benefit is the inactivation of antinutrients, such

as protease inhibitors which are present in many legumes, cereals and other vegetables (Gurr, 1988, Mauron, 1989). Heat processing of food, however may also cause loss of essential nutrients, in particular of water-soluble vitamins, which tend to be less stable than fat-soluble vitamins. Vitamin losses are highly determined by other physical factors as well, including pH, water activity and availability of oxygen (Quattrucci, 1988).

In industrial countries, between 1850 and 1950, a steadily increasing proportion of food has been prepared in factories, and after 1950, food industry has expanded rapidly (Mauron, 1989; Passmore and Eastwood, 1986). At present, industrially processed foods are estimated to provide approximately 75% of total daily energy consumed in The Netherlands and other developed countries (Gurr, 1988; Keuning and Beek, 1985). Concurrently with increasing consumption of processed foods in industrial countries, per capita consumption of dietary lipids has also increased. In The Netherlands, approximately 40% of daily energy intake is supplied by dietary fat (Anonymous, 1988b), while in less developed countries average per capita consumption of dietary fat is generally much lower (Rose et al., 1986). With most of our food being processed, constituents of food are likely to be heat-processed, and consequently may be subject to heat degradation.

Heat degradation products of dietary constituents receive increasing attention from the perspective of health risk assessment, since some of the compounds involved have been found to be mutagenic and carcinogenic (Larsen and Paulsen, 1987). Biological effects of heat degradation products of food have been extensively reviewed (Anonymous, 1983, 1985a; 1986; Frankel, 1984; Fretheim, 1983; Furihata and Matsushima, 1986; Gurr, 1988; Jägerstad et al., 1986; Larsen and Paulsen, 1987; O'Brien and Morrissey, 1989; Phillips, 1983; Powrie et al., 1986; Quattrucci, 1988; Smith and Johnson, 1989; Sugimura et al., 1986; Sugimura, 1985; Weisburger et al., 1986). In the following part, first the current hypotheses regarding the mechanisms of chemical carcinogenesis will be presented in a brief overview, as well as various test systems applied in genotoxicity and carcinogenicity evaluation of a chemical. Next, toxicological aspects of the most important classes of heat degradation products will be reviewed briefly. Since increased consumption of heat processed lipids is evident in Western societies, special attention will be paid to heat-degradation and oxidation products of lipids. This will be followed by a discussion dealing with evaluation of human health risk of some of the recently discovered heterocyclic amines, which are presently intensively evaluated, and which may serve as an illustrative case for other mutagens and carcinogens formed during processing of food. Finally, a possible role of lipid oxidation products in colon carcinogenesis will be postulated.

2. Toxicological aspects of heat degradation products

2.1. Carcinogenesis and genotoxicity

The process of carcinogenesis is presently considered to be a complex multiple-step event (Weinberg, 1989). In experimental models, such as in mouse skin carcinogenesis, at least three steps have been distinguished: initiation, promotion and progression. The process of tumor-initiation has been considered to involve an irreversible, heritable change in the DNA of a cell (Pitot, 1990). This alteration in the DNA may be a gene mutation, or structural and numerical chromosomal changes, and possibly also translocations, gene amplifications, or other gene or DNA rearrangements (Arlett and Cole, 1988; Barrett et al., 1988; Farber, 1987). It should be emphasized that the interaction of a chemical with DNA alone is not sufficient to initiate carcinogenesis; both *in vivo* and *in vitro* it has been observed that a round of cell proliferation is required as well (Farber, 1987; Pitot, 1990). During the promotional phase clonal expansion of initiated cells takes place. The principal characteristic of the stage of promotion in several model systems of multistage tumorigenesis has been reported to be its reversibility (Pitot, 1990). Agents which cause or enhance cellular proliferation are therefore considered to possess tumor-promoting potential. The stage of tumor progression has been defined as that stage of tumorigenesis exhibiting measurable and/or morphologically identifiable (karyotypic) changes in the structure of the cell genome (Pitot, 1990). Such changes are directly related to the increased growth rate, invasiveness, metastatic capability, and biochemical changes in the neoplastic cell. The stage of progression is considered to be irreversible owing to alterations in the cell genome which have been observed to occur during tumor-progression in various multistage tumorigenesis models (Pitot, 1990).

Proto-oncogenes are genes that regulate and control cell growth and proliferation, and have been recognized as molecular targets of special relevance for the process of carcinogenesis (Farber, 1987; Stowers et al., 1987; Weinberg, 1989). Neoplastic development is thought to be the result, at least in part, of abnormal activation of these proto-oncogenes, which were originally discovered as the transduced genes of acute transforming retroviruses (Land et al., 1983). The conversion of proto-oncogenes to activated oncogenes may occur via mechanisms including point mutations, gene amplification, chromosomal rearrangements, and promotor insertion (Farber, 1987). In addition to the activation of proto-oncogenes, the inactivation of tumor suppressor genes which are involved in negative regulation of oncogenes, may also be a necessary but distinct step in neoplastic development (Sager, 1986; Stowers et al., 1987; Weinberg, 1989). The activation of proto-oncogenes by genetic alterations results in changed levels of expression of the normal protein product, or in expression of an abnormal protein, consequently leading to abnormal cellular growth

and/or differentiation.

In different human and rodent tumors specific chromosome deletions and rearrangements as well as point mutations in specific oncogenes have been demonstrated (Cerutti, 1988; Fox, 1988; Weinstein, 1989). Although at present it is not clear whether genetic alterations observed in tumor cells are causative in initiation or progression or both (Fox, 1988; Weinstein, 1989), and although non-genotoxic carcinogens appear to act through mechanisms not involving genetic alterations, genetic change is part of many theoretical bases of the cause and nature of malignancy (Farber, 1987; Fox, 1988; Pitot, 1990). Therefore, detection of genetic alterations in short-term *in vivo* and *in vitro* assays is considered to indicate possible carcinogenic potential of a particular agent. Since at present the detection of changes at specific sites of the DNA (oncogenes or tumor suppressor genes) requires demanding procedures and is not yet available for routine testing, short-term genotoxicity assays developed during the past two decades which are based on measurement of "surrogate" end points, may still be useful for identification of possible genotoxic carcinogens, as well as for elucidation of their mode of action (Williams, 1989).

2.2. Toxicological risk assessment of carcinogens

Since carcinogens are widely distributed in the human environment and may not always be eliminated or banned, toxicological assessment of risk to man posed by possible carcinogens will be necessary to make a proper judgement with respect to the carcinogenic threat of each compound (Kroes, 1987). This may specifically apply to the dietary compounds which are produced during processing and preparation of food, and some of which have been found to possess carcinogenic properties in laboratory animals (Sugimura et al., 1986). Although human data provide the only direct evidence that a chemical produces cancer in man, it is obvious that for most chemicals such data will be not or only limited available. Moreover, epidemiologic evaluation of carcinogenic risk alone is unreliable due to the difficulties of estimating exposure levels, long latency periods, and to the presence of confounding factors (Doll and Peto, 1981). Consequently, negative epidemiologic results cannot ensure complete absence of carcinogenic risk from a compound or process. Additional data are therefore obtained from various experimental studies, which include (i) long-term bioassays with animals measuring the incidence of neoplasms; (ii) short-term *in vitro* and *in vivo* tests, designed to detect early effects of neoplasia, genetic alterations or cell transformation; and (iii) evaluation of biotransformation, pharmacokinetic properties and elucidation of the mechanism of chemical carcinogenesis. Over the past two decades various mutagenicity and carcinogenicity test systems with different biological end points have been developed (Table 1.1).

Table 1.1. Test systems in mutagenesis and carcinogenesis.

End point	Organism/cell	Test system	Target
1. Gene mutations	prokaryotic microbes	<i>Escherichia coli</i>	DNA strand
- point mutation	(bacteria)	<i>Salmonella typhimurium</i>	
within locus	eukaryotic microbes	<i>Saccharomyces cerevisiae</i>	
- multilocus deletions	(yeasts/fungi)	<i>Neurospora crassa</i>	
		<i>Aspergillus nidulans</i>	
	insects	<i>Drosophila melanogaster</i>	
	mammalian cells	germ cells	
		somatic cells	
		cell lines	
		human fibroblasts	
		human lymphocytes	
2. Chromosomal mutations	bacteria	<i>Bacillus subtilis</i>	Gene
- numerical		<i>E. coli</i>	
- structural aberrations	yeasts/fungi	<i>S. cerevisiae</i>	
- translocations		<i>A. nidulans</i>	
- DNA damage/repair	mammalian cells <i>in vitro</i>	various cells and cell lines	
		human lymphocytes	
	<i>in vivo</i> systems	bone marrow cells	
	(mouse, hamster, rat)	lymphocytes	
		tissues	
3. Cell biology	mammalian cells <i>in vitro</i>	various cells	Chromosome
- morphology		cell lines	
- transformation		human fibroblasts	
4. Organ neoplasia	<i>in vivo</i> systems	skin, lungs, liver,	Cell
- initiation-promotion-progression		bladder, mammary gland, gastro-intestinal tract	
			Organ

Modified from De la Iglesia et al., 1980.

Short-term *in vitro* and *in vivo* genotoxicity assays generally are designed to detect DNA damage or alterations, and these tests are associated primarily with the initiation phase of carcinogenesis (Williams, 1989). These tests do not detect agents that cause cancer by non-genotoxic mechanisms, and do not evaluate pharmacokinetic, metabolic and DNA-repair factors that influence species and tissue-specific susceptibilities. Although these tests have initially been considered to be an alternative for *in vivo* carcinogenicity assays, their predictive value has been found to be disappointingly low, ranging from 60 to 80% (Douglas et al., 1988; Tennant et al., 1987; Williams, 1989; Zeiger, 1987). The carcinogenicity of a compound in animals is established when administration in adequately designed and conducted experiments results in an increase in the incidence of one or more types of malignant neoplasms in treated animal as compared to untreated animals maintained under identical conditions except for exposure to the test compound (Anonymous, 1984). However, various tumor end points may be expected depending on route of administration, and species, strain and sex of the animals. In addition to increased tumor incidence, occurrence of tumors at sites not or rarely seen in control animals, and decreased latency periods may also be relevant end points. However, at present it is not clear whether these various patterns of tumor development are of different significance with respect to human risk (Anonymous, 1984). Therefore, it has been advised that animal studies, whenever possible, should be supplemented with studies of mechanisms, metabolism and pharmacodynamics, to provide additional information for risk evaluation (Anonymous, 1984; 1988a). Since none of the above presented single methods is able to produce adequate data, it is evident that results of various tests should be integrated for a reliable estimate of a chemical's risk to man.

2.3. Polycyclic aromatic hydrocarbons

Pyrolysis products of organic matter, belonging to the class of polycyclic aromatic hydrocarbons (PAHs) have been studied for a long time. Benzo(a)pyrene and other PAHs were recognized as potent carcinogens as early as 55 years ago (Phillips, 1983). Production of PAHs occurs at temperatures higher than 400 °C; at lower temperatures minimal amounts of PAHs are formed. Grilling or roasting of meat and fish on an open fire leads to PAHs contamination in several ways. PAHs may be "endogenously" formed from food constituents when the surface of the food is exposed to high temperatures, and they may also be derived from the combustion fuel. Highest contamination with PAHs is derived from melted fat which drips from the meat on the heat source; PAHs are produced and subsequently spread into the atmosphere and deposited on the surface of the meat (Fretheim, 1983). Levels of PAHs in heat-processed food may be as high as 200 µg/kg, depending on grilling or smoking conditions (Larsen and Paulsen, 1987). PAHs are present not only in broiled and smoked fish and meat and in

roasted coffee, but levels in the ppb range have also been found in fresh meats, seafood, vegetables, oils, grains, fruits, whiskey etc. (Anonymous, 1983). It can be assumed that in the latter cases the PAHs contamination derives primarily from air and water pollution or from food or beverage containers.

PAHs are promutagens and procarcinogens; upon oxidation by the cytochrome P₄₅₀ system ultimate mutagens and carcinogens are produced. The active metabolite of benzo(a)pyrene, used as model PAH in many studies, has been demonstrated to be the diol-epoxide (Anonymous, 1983). Many PAHs are genotoxic after microsomal metabolism in various *in vitro* short-term assays, both prokaryotic and eukaryotic (Anonymous, 1983; Phillips, 1983). More than 20 PAHs have been identified in food, of which more than 10 have been shown to be carcinogenic to animals; oral carcinogenicity data however, have been considered inadequate (Anonymous, 1973; 1983; Larsen and Paulsen, 1987).

2.4. Caramelization products

When mono- and disaccharides are heat-treated, they undergo dehydration first, which is followed by complex reactions not yet understood, eventually leading to many brown-colored intermediates and end products. This process is known as caramelization. Caramelization products of carbohydrates, treated in presence of ammonia, alkalis, sulphites and acids are widely used in food as colour additives (Quattrucci, 1988). In recent years, several doubts have arisen on their safety. In model browning systems, containing sugars and ammonia, mutagenic activity to *Salmonella typhimurium* has been observed to develop, which appeared to be related to the production of pyrazines. Subsequently, isolated pyrazines have been demonstrated to be mutagenic (Springarn and Garvie, 1979). A diversity of caramelization compounds with varying genotoxic activities in various short-term assays including mammalian cells, has been identified (Nishi et al., 1989; O'Brien and Morrissey, 1989; Powrie, 1986). These include direct-acting as well as promutagenic compounds. Animal studies have shown dose-related growth retardation, caecal enlargement and haematological changes (decrease in white blood cell count) after long-term administration of caramels produced with addition of ammonia (Quattrucci, 1988). No carcinogenicity data are available. In contrast, antimutagenic properties of caramelized sucrose have been reported as well (Chan et al, 1982; Powrie, 1986).

2.5. Products of Maillard reaction and amino acid pyrolyzates

The formation of brown pigments and flavor compounds from reducing sugars, heated in the presence of amines is called the Maillard reaction, which is dependent on temperature and water content of food (Jägerstad et al., 1986). In dry food systems, the reaction may take place at temperatures below 100 °C, and the rate increases rapidly at temperatures above 100 °C (O'Brien and Morrissey, 1989). Numerous Maillard reaction products can be isolated from either model browning systems or heated foods (Jägerstad et al., 1986, O'Brien and Morrissey, 1989). Maillard reactions have been implicated in the formation of imidazoquinolines and imidazoquinoxalines, heterocyclic amines which were first isolated from fried and boiled proteinaceous foods (Sugimura et al., 1977; Jägerstad et al., 1986). These compounds were found to be potent mutagens in the Salmonella/microsome assay after hepatic microsomal metabolism (Sugimura et al., 1986).

Other pyrolyzates of amino acids have been isolated from beef extracts and heated proteins and include indoles, imidazoles, carbolines, carbazoles and pyridines (Dolara and Bianchini, 1988; Furihata and Matsushima, 1986; Sugimura et al., 1986). Most of these bacterial mutagens have been found to be carcinogenic to rodents (Table 1.2). Their isolation, as well as their mutagenic and carcinogenic properties have been extensively reviewed (Anonymous 1986; Furihata and Matsushima, 1986; Sugimura et al., 1986; O'Brien and Morrissey, 1989). Most recently isolated and identified mutagen from fried ground beef is 2-amino-1-methyl-6-phenylimidazo[4,5-f]pyridine (PhIP; Felton et al., 1984, 1986). Heterocyclic amines show genotoxic effects in various short-term assays applying mammalian cells; potencies vary depending on the test system used and compound tested (Anonymous, 1986; Dolara et al., 1985; Furihata and Matsushima, 1986; Wild et al., 1985).

2-Amino-3,8-dimethylimidazo-[4,5-f]quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo-[4,5-f]quinoxaline (4,8-DiMeIQx) and PhIP have been found to be the major mutagens in fried beef, comprising approximately 75% of the total mutagenic activity, whereas 2-amino-3-methylimidazo-[4,5-f]quinoline (IQ) has only been found in trace amounts (Larsen and Paulsen, 1987). Concentrations of MeIQx in fried beef and beef extracts are reported to reach levels of 300 µg/kg (Jägerstad et al, 1986). Few data are available on concentrations of heterocyclic amines in heat processed meat products, due to complex analytical methods required (Hatch and Felton, 1986).

Table 1.2. Mutagenicity and carcinogenicity of pyrolysis products of amino acids and proteins in cooked foods.

Compound	Mutagenicity to Salmonella tester strain		Carcinogenicity to rodents
	TA98+S9 (rev/ μ g)	TA100+S9 (rev/ μ g)	
1. 2-Amino-9H-pyrido - [2,3-b]-indole (A-alpha-C)	300	20	+ (800 mg/kg in diet)
2. 2-Amino-3-methyl-9H-pyrido [2,3-b]-indole (MeA-alpha-C)	200	120	+ (800 mg/kg in diet)
3. 2-Amino-6-methyl-dipyrido- [1,2-a:3',2'-d]-imidazole (Glu-P-1)	49000	3200	+ (500mg/kg in diet)
4. 2-Aminodipyrido-[1,2-a:3',2'-d]- imidazole (Glu-P-2)	1900	1200	+ (500 mg/kg in diet)
5. 3,4-Cyclopenteno-pyrido- [3,2-a]-carbazole(Cys-P-1)	98	no data	no data available
6. 2-Amino-3-methylimidazo- [4,5-f]quinoline (IQ)	33000	7000	+ (300 mg/kg in diet)
7. 2-Amino-3,4-dimethylimidazo- [4,5-f]quinoline (MeIQ)	611000	30000	+ (100 and 400 mg/kg in diet)
8. 2-Amino-3,8-dimethylimidazo- [4,5-f]quinoxaline (MeIQx)	145000	14000	+ (300 mg/kg in diet)
9. 2-Amino-3,7,8-trimethylimidazo- [4,5-f]quinoxaline(7,8-DiMeIQx)	163000	9900	no data available
10. 2-Amino-3,4,8-trimethylimidazo- [4,5-f]quinoxaline (4,8-DiMeIQx)	183000	8000	no data available
11. 3-Amino-1,4-dimethyl-5H-pyrido- [4,3,6]-indole (Trp-P-1)	39000	1700	+ (2 mg/kg in diet)
12. 3-Amino-1-methyl-5H-pyrido- [4,3,6]-indole (Trp-P-2)	104000	1800	+ (1-2 mg/kg in diet)

After: Sugimura et al., 1986; Furihata and Matsushima, 1986; Felton et al., 1984. Mutagenicity is expressed as number of his⁺ revertant colonies induced per μ g of compound. + carcinogenic in rodents, the dose included in the diet is shown between parentheses.

Analysis of meals containing heated products and composed of regular products consumed in The Netherlands, has yielded estimates of 15-20 µg/kg for IQ, DiMeIQx, MeIQx and PhIP (Alink et al, 1988). Mutagen formation in fried ground beef appears to be temperature dependent (Pariza et al., 1979); increased mutagenicity has been observed in beef fried at temperatures ranging from 191 to 210 °C, whereas beef fried at 143 °C had low levels of mutagenicity. Increasing the fat content of fried ground beef has been shown to enhance mutagen formation, which has been attributed to a more efficient heat transfer (Springarn et al., 1981; Knize et al., 1985; Nilsson et al., 1986). However, interactions of amino acids with lipids may have produced mutagenic compounds as well, since heated reaction mixtures of lipids and amino acids have been reported to induce increased mutagenic activity (Vithayathil et al., 1983; Yoshida and Okamoto 1980). In contrast, antimutagenic activity of browning reaction products of L-lysine and D-fructose have also been demonstrated (Chan et al, 1982).

2.6. Lipid oxidation products

Heat degradation of dietary lipids may involve oxidation as well as chemical processes which do not necessarily involve oxygen (Frankel, 1984;1987). Oxidative deterioration of lipids is enhanced at high temperatures, but may occur at ambient or even freezer temperatures. The process of lipid oxidation or autoxidation of polyunsaturated fatty acids (PUFA) involves free radical mechanisms (Frankel, 1984;1987; Halliwell and Gutteridge, 1984). Lipid peroxidation proceeds in three stages: initiation, propagation and termination. Initiation takes place by loss of a hydrogen radical in presence of trace metals, light or heat. The resulting lipid free radicals ($L\cdot$) react with oxygen to form peroxy radicals ($LOO\cdot$). In the propagation process, $LOO\cdot$ react with more LH to form lipid hydroperoxides ($LOOH$), which are the primary products of autoxidation (Figure 1.1). Termination of lipid oxidation may follow several ways. Two lipid carbon radicals or peroxyradicals may combine and form dimeric and polymeric products. Alternatively, a peroxy radical can form a cyclic peroxide, which as a free radical itself, can undergo cyclization to a cyclic endoperoxide, which on heating in the presence of oxygen can split into a number of end-products characteristic of lipid oxidation, including aldehydes, ketones, alcohols, hydrocarbons, esters, furans and lactones. Lipid hydroperoxides can react again with oxygen to form secondary products, which may be volatile, or condense into dimers and polymers, which can also break down and produce volatile materials (Frankel, 1984; 1987).

Peroxidation of lipids in food occurs easily, because the reaction is catalyzed by components of the foods. Important non-enzymic catalysts are

iron and copper (Frankel, 1987; Gurr, 1988). Fresh plant and animal products contain the enzyme lipoxygenase which catalyzes the formation of hydroperoxides of PUFA (Halliwell and Gutteridge, 1985). Antioxidants such as naturally occurring alpha-tocopherol, beta-carotene, ascorbic acid, as well as the synthetic compounds butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) retard the process of lipid oxidation by scavenging free radicals (Frankel, 1984; 1989; Gurr, 1988). Cholesterol and related sterols are also subject to autooxidation during heating (Bascoul et al., 1986; Gurr, 1988). The lipid oxidation products malondialdehyde (MDA) or MDA-like substances have been detected in various foods, including oils, lipid containing vegetable products, and meat and fish (Shamberger et al., 1977; Siu and Draper, 1978); concentrations in heat-processed meats reached levels as high as 30-40 mg/kg. Heat-treated triglycerides may form considerable amounts of lipid oxidation products including cyclic monomers, peroxides, hydroperoxides, di- and polymeric triglycerides, as well as low molecular weight degradation products (Gurr, 1988; Sebedio et al., 1987; Stevenson, 1984; Thompson and Aust, 1983), which may involve 30 to 40% of the triglycerides. Oxidized lipids and aldehydes including MDA, are highly reactive compounds and are reported to bind covalently to proteins, amino acids and sugars (Esterbauer, 1982; Gurr 1988; Gutteridge, 1982; Halliwell and Gutteridge, 1984; Piche et al., 1988). MDA as well as other carbonyls have been observed to catalyze nitrosation reactions in the presence of free nitrite, which may result in the formation of potentially carcinogenic nitrosamines (Hartman, 1983; Kurechi et al., 1980).

Figure 1.1. Process of lipid oxidation.

The lipid oxidation products MDA, hydroperoxides of linoleic acid, various aldehydes and carbonyls, as well as oxidized sterols are mutagenic in the *Salmonella* mutagenicity assay (Basu and Marnett, 1983; MacGregor et al., 1985; Smith et al., 1986; Yamaguchi and Yamashita, 1979; 1980). Various lipid oxidation products, including oxidized sterols, hydroxy- and hydroperoxyeicosatetraenoic acids, alkenals, MDA and hydroperoxides of linoleic acid have been shown to be genotoxic to mammalian cells (Brambilla et al., 1986; Cajelli et al., 1987; Nakayama et al., 1986; Kelsey and Pienta, 1979; Vaca et al., 1988; Weitberg and Corvese, 1989). Acute or short-term oral administration of oxidized oils or isolated fractions containing oxidation products, to laboratory animals has been reported to induce adverse biological effects (Alexander, 1981; Gurr, 1988). These include cellular damage and increased liver and kidney weights, cellular damage to epidymides and testes, increased lipid oxidation in hepatic and renal tissue, as well as teratogenic effects (Alexander 1981; Alexander et al., 1987; Cutler and Schneider, 1973; Izaki et al., 1984; Kanazawa et al., 1986). Consumption of cooked, stored meat, containing MDA or MDA-like substances has been shown to increase urine excretion of these lipid oxidation products in humans (Jacobsen et al., 1983). Urinary excretion of MDA is considered an indicator of dietary intake of lipid oxidation products as well as of endogenously occurring lipid oxidation (Draper et al., 1984). Chronic oral administration of MDA at doses ranging from 0.1 to 10 µg/g bodyweight, has been associated with increased neoplastic and hyperplastic changes in the liver of mice (Bird et al., 1982; Siu et al., 1983). In another study however, no increased tumor incidence was seen after chronic administration of MDA with drinking water containing concentrations of MDA up to 0.5% (Apaja, 1980). MDA is the only lipid oxidation product on which carcinogenicity data are available. In contrast, antimutagenic and antitumorigenic oxidative derivatives of linoleic acid, which contain a conjugated double-bound system and are referred to as CLA, have also been isolated from fried ground beef (Ha et al., 1987; Pariza, 1988).

3. Heat degradation products and human health risk

At present, a small number of mutagens produced during heat-processing of food has been evaluated for carcinogenicity in animal experiments. Several heterocyclic amines as well as PAHs and MDA have been shown to be carcinogenic in animals and may therefore pose a serious hazard to human health. Although PAHs are widely distributed in the human environment, limited data are available on carcinogenicity after oral administration (Anonymous, 1973; 1983). Tobacco smoke and urban air are considered to be the main sources of PAH-production (Anonymous, 1983). Since epidemiologic evidence has indicated that intake of food containing traces of PAHs does not contribute appreciably to human cancer risk

(Anonymous, 1983), toxicological risk assessment of consumption of PAHs will not be discussed further. In addition, toxicological data available on caramelization products are too limited to allow a meaningful discussion of their toxicological risk. Since most data are available on the heterocyclic amines IQ, MeIQ and MeIQx, which have been found in heat-processed meat and fish, in the following part the current status of risk evaluation of these compounds will be discussed. This part is meant to illustrate the special issues encountered and problems to be solved in risk estimation of constituents which are commonly present in heat-processed foods. This will be followed by a discussion of the possible risks posed by oxidation and heat degradation products of lipids.

3.1. Current status of toxicological risk assessment of amino acid pyrolyzates

Carcinogenicity in rodents of the most potent bacterial mutagens, the amino acid pyrolyzates IQ and MeIQx, which are present in fried beef, has been observed at a single dose of 0.03% in diet for a single compound (Sugimura et al., 1986). Target organs have been reported to include liver, Zymbal gland, colon and small intestines (Anonymous, 1986; Kato et al., 1989). In contrast, chronic dietary administration of mutagen-containing charred fish meat at a level of 25% in diet, has not been reported to increase tumor incidence in rats (Fuji et al., 1987). However, in this latter study doses of heterocyclic amines consumed have not been determined. Recently, Alink et al. (1989) have reported increased incidences of epithelial tumors in male, but not in female rats fed a balanced human diet containing thermally processed foods, which resulted in an intake of mutagenic heterocyclic amines of 0.5-0.7 $\mu\text{g/kg}$ body weight/day. No increased tumor incidences have been observed in this study at sites reported to be specific for carcinogenic heterocyclic amines. These data suggest that risk assessment of intake of carcinogenic heterocyclic amines with human diet of doses which are 5000-fold lower than TD_{50} for rats (Alink et al., 1989; Felton et al., 1986), is hindered by the complexity of the human diet (Weisburger et al., 1986), of which carcinogenicity inhibiting as well as enhancing effects are not sufficiently known at present.

In short-term *in vitro* assays using mammalian cells, MeIQx and IQ have been observed to induce only weak positive effects (Dolara et al. 1985; Howes et al., 1986; Hatch and Felton, 1986; Nakayasu et al., 1983). High doses of IQ have been shown to be negative in inducing *in vivo* gene mutations or chromosome aberrations, and induced a borderline effect on frequencies of sister chromatid exchanges (SCE) (Minkler et al., 1984; Tucker et al., 1989; Wild et al., 1985). More recently, it has been shown that liver-microsomal metabolites of IQ and MeIQx are weak inducers of SCE in human lymphocytes at realistic exposure levels of 0.1 to 0.8 $\mu\text{g/ml}$ (Aeschbacher and Ruch, 1989). In the same study, chromosome aberrations have not been detected in human lymphocytes at 100-fold higher concentrations. IQ has been observed to be not capable either of increasing

numbers of micronuclei or mutation frequencies at the hypoxanthine guanine phosphoribosyl transferase (*hgp**rt*) locus in human lymphocytes, using human-liver microsomal activation at realistic as well as high exposure levels of 1 to 10 mM (McManus et al., 1988). The striking paradox between genotoxic potency in bacterial and mammalian assays may be due to differences in activities of acyltransferases, which have been shown to metabolize the mutagenic microsomal N-hydroxy derivatives of IQ and MeIQx, resulting in a 10- to 30-fold increase in mutagenic potency and DNA-binding capacity (Negishi et al, 1989; Snyderwine et al, 1988). In addition, specific DNA repair capacities of mammalian cells may make them less susceptible to genotoxic effects of MeIQx and IQ, and it has been postulated that these cells may be more relevant than prokaryotic organisms for genotoxic risk evaluation of dietary components (Aeschbacher and Ruch, 1989).

Few epidemiologic studies are available, which have evaluated health effects of consumption of heat-processed foods (Archer, 1989). In a prospective Japanese cohort study, Kuratsone et al. (1986) have observed increased mortality of cancer and increased stomach cancer mortality with increased consumption of broiled fish, whereas in a group of Japanese Seventh-day Adventists stomach cancer mortality has been found to be significantly lower. Seventh-day Adventists are known to abstain from broiled and fried meats and fish, and not to use alcoholic drinks. Colon cancer mortality of has been reported to be lower in Seventh-Day Adventists than in populations consuming mixed western diets (Phillips et al., 1980). Furthermore, in a case-control study high consumption of processed lunchmeat and pan-fried foods has been associated with increased colon cancer risk (Young and Wolf, 1988). Another case-control study however, has not shown increased risk for colon cancer in persons with more frequent use of broiled and fried meats five years before diagnosis (Lyon and Mahoney, 1988). These data are too limited to draw any conclusions, but it should be noted that the positive associations observed between meat or animal protein intake and colon cancer mortality (Armstrong and Doll, 1975; Rose et al., 1986; Willet, 1989) may be additional evidence for a role of heat-processed meat in human cancer, since most meats are heat-treated before consumption. In addition, increased faecal mutagenicity as well as the presence of metabolites of heterocyclic amines has been observed in 3 human volunteers after consumption of fried beef, indicating the colon may be a target for genotoxic effects of heterocyclic amines (Hayatsu et al., 1985).

The possible role of heterocyclic amines in colon cancer receives increasing attention (Bird and Bruce, 1984; Tudek et al., 1989). In animal carcinogenicity studies, increased tumor incidence has been observed in the colon of rats after chronic dietary administration 0.03% of IQ and MeIQ (Takayama et al., 1984; Kato et al., 1989; Sugimura, 1985). These heterocyclic amines have also been tested *in vivo* in animal models determining nuclear aberrations, SCE's or aberrant crypt foci in mucosal

tissue of the colon after a single oral dose (Bird and Bruce, 1984; Couch et al., 1987; Tudek et al., 1989; Wargovich et al., 1983). Using these models, it has been observed that IQ and MeIQ are inducers of nuclear aberrations in murine colon, with effective doses ranging from 10 to 200 mg/kg (Bird and Bruce, 1984). MeIQ has been shown to be a weak inducer of SCE's in colonic tissue of mice at doses ranging from 50 to 200 mg/kg (Couch et al., 1987). At even higher doses of 200 to 400 mg/kg, IQ and MeIQ have been shown to be potent inducers of aberrant foci in the colon of rats, but not in mice (Tudek et al., 1989). In addition, dietary modulation of genotoxic effects of IQ has also been demonstrated using this model. Feeding of 20% corn oil has been shown to increase nuclear aberrations in colon of mice induced by IQ, whereas cellulose was without effect and 20% olive oil reduced the nuclear damaging effect of IQ (Dolara et al., 1986). These data appear to support a role for specific heterocyclic amines in tumorigenesis of the colon, although the effective doses are extremely high and exceed realistic exposure levels at least a 1000-fold.

It will be clear from the above cited studies, that reliable risk estimation of mutagenic heterocyclic amines is not possible at present. Limited data are available, which do not allow for quantitative risk estimation. Mutagenic heterocyclic amines have been found in trace amounts (ppb level) in heat-processed food, while carcinogenicity in rodents, and genotoxicity *in vitro* in human cells, and *in vivo* in rodents has been observed at much higher than realistic exposure levels. Furthermore, fried foods have been found to contain mixtures of mutagens, whereas at present data are available of single compounds only. Therefore, *in vivo* and *in vitro* studies, which apply realistic doses, representative mixtures of mutagens and also involve modulating dietary factors, are needed to provide necessary additional information with respect to human carcinogenic risk evaluation.

3.2. Heat-processed fats and colon cancer risk

Epidemiologic and experimental data support an association between high dietary fat intake and cancer of breast, large bowel and prostate, although not all data are consistent with the association (Byers, 1988; Hill, 1987; Pariza, 1988; Reddy, 1987; Rogers and Longnecker, 1988; Willet, 1989). In contrast, per capita fat consumption has been found to be inversely related to stomach cancer mortality (Armstrong and Doll, 1975), whereas no other tumor sites have been reported to be associated with high fat intake. Some epidemiologic studies have evaluated specific effects of saturated and polyunsaturated fatty acids intake on cancer mortality or colon tumor incidence. Usually fat has been divided into two categories, animal or vegetable. These categories however, do not fully represent saturated and unsaturated fats, since some animal fats (e.g. chicken, pork and fish) may contain considerable amounts of polyunsaturated fatty acids (PUFA), whereas some vegetable fats (e.g. coconut oil) may contain minimal amounts of PUFA. Data obtained with these studies yield conflicting results,

with some pointing to a positive relation between animal or saturated fat consumption and colon cancer incidence, and others finding no association (Byers, 1988; Rogers and Longnecker, 1988; Willett, 1989). In contrast, some report an inverse relation between saturated fat intake and colon cancer incidence, whereas others have found an inverse relation between vegetable fat intake and colon cancer incidence (Stemmerman et al., 1984; Willett, 1989). One Dutch correlational study has shown a positive association between colon cancer mortality and per capita consumption of total fat, saturated fat, monounsaturated fat and polyunsaturated fat 10 years previously (Anonymous, 1985b).

Various explanations may be imaginable for these conflicting results, including difficulties in estimation of dietary intake as well as confounding effects of other dietary and environmental factors. High intake of meat with animal fats may explain the positive relation between saturated or animal fat intake and colon cancer, whereas intake of fiber and the antioxidants and presumable anticarcinogens alpha-tocopherol and beta-carotene (Ames, 1983; Hayatsu, 1988) may be associated with consumption of vegetable fats, which may result in an inverse relationship between vegetable fat intake and colon cancer. However, another confounding factor may be heat-processing of dietary lipids which has not yet been evaluated in epidemiologic studies. A specific effect of heat-processed lipids may be indicated by results of a case-control study recently reported by Peters et al. (1989). In this study high consumption of deep-fried foods has been observed to be associated with increased colon cancer risk. Since the authors do not report adjustment for total fat intake, this finding does not give a definitive clue for the involvement of heat-processed lipids in colon tumorigenesis and will have to be confirmed in other studies.

Although the secondary oxidation product MDA has been shown to possess carcinogenic properties in mice (Bird et al., 1982; Siu et al., 1983), no data are available on analysis of carcinogenicity of other oxidized lipids. Oxidized lipids however, may possess tumor-initiating properties since some have been found to exert genotoxic effects in the *Salmonella* microsome assay as well as in mammalian cells. MDA, hydroperoxides of linoleic and linolenic acid, and various aldehydic products and carbonyls have been reported to be mutagenic to *Salmonella typhimurium* (Basu and Marnett, 1983; Mukai and Goldstein, 1976; Marnett et al., 1985; Yamaguchi and Yamashita, 1979; 1980). Methyl linoleate hydroperoxide has been shown to induce single strand breaks in DNA of human fibroblasts at doses ranging from 50 to 100 μM (Nakayama et al., 1986), and the same effects have been reported for hydroperoxides and hydroxides of 6,8,11,14-eicosatetraenoic acid in human lymphocytes at doses ranging from 5 to 20 μM (Weitberg and Corvese, 1989). Several aldehydic products, including 4-hydroxynonenal have been shown to induce DNA single strand and double strand breaks, and increase SCE frequencies in Chinese hamster ovary cells (Brambilla et al., 1986). In addition, 4-hydroxynonenal has induced a dose-related increase in mutations at the *hgp* locus in V79 Chinese hamster cells at dose ranges from 10 to 50 μM (Cajelli et al., 1987). These data indicate that

oxidation products of fatty acids other than MDA may also be involved in tumor-initiation. Therefore, determination of possible tumor-initiating activity of heat degradation and oxidation products of lipids in experimental studies appears to be of relevance for evaluation of carcinogenic risk.

Whereas effects of heat-processed lipids have not been evaluated in experimental carcinogenicity studies, the role of unheated dietary fat in colon carcinogenesis has been comprehensively investigated in two-stage animal models. Using this initiation-promotion model, high intake of unheated dietary fat has been shown to enhance chemically induced colon tumorigenesis, but the enhancing effect appears to be dependent on the type of fat as well (Reddy, 1987). In carefully controlled studies, high intake of dietary corn oil, beef-fat, safflower oil, and lard has been shown to enhance colon tumorigenesis, whereas diets high in olive oil, coconut oil, and fish oil are without enhancing effects (Carroll, 1987). The enhancing effect of high-fat diets with *ad libitum* feeding as has been applied in earlier studies, has been related to high energy intake; increased bodyweights in the high-fat groups appeared to explain increased tumor incidence (Pariza, 1988; Rogers and Longnecker, 1988). It has also been demonstrated that linoleic acid is essential for optimal tumor development, as it is for normal growth and development (Pariza, 1988; Sakaguchi et al., 1986).

High levels of dietary fats are postulated to exert tumor enhancing effects via increased bile excretion and high levels of faecal fatty acids, which have been shown to be irritating and toxic to the colonic epithelial cells (Bird, 1986). This may increase cancer risk by affecting promotion and possibly also initiation of colon carcinogenesis (Bruce, 1987; Nair, 1988; Pariza, 1988). High fat diets may also influence composition and metabolic activity of colonic microflora, either directly or indirectly via enhanced bile excretion (Goldin, 1986; Nair, 1988), thereby causing altered metabolism of possible carcinogenic or carcinogenicity enhancing compounds, such as neutral steroids. Suzuki et al. (1986) have isolated the sterols cholest-4-en-3-one and 5- α -cholestan-3-one from human faecal extracts, which have been observed to be active compounds in the colonic nuclear aberration assay. These oxidation products of cholesterol may either be produced by bacterial transformations in the large bowel or may be derived from oxidized sterols in food (Bruce, 1987; Suzuki et al., 1986). Furthermore, oxidation products of linoleic and arachidonic acid have been reported to stimulate DNA synthesis and cell proliferation, and to induce ornithine decarboxylase (ODC) activity in rat colon after intrarectal administration (Bull et al., 1984, 1988), which effects are considered to be markers of tumor promotion (Hicks, 1983; Weinstein, 1988). Effects of oral administration of heat-processed or oxidized lipids on cell proliferation, tumor initiation or promotion in the colon however, have not yet been evaluated. These biologically active lipid oxidation products can be derived from dietary fats, and therefore, investigation of tumor-enhancing effects of lipid oxidation products present in the diet appears to be indicated.

Since the colon may be considered a possible target organ for possible carcinogenic lipid oxidation products present in heat-processed fats, the presence of these compounds in the colonic lumen will have to be demonstrated. Metabolic studies in rats, performed with ^{14}C -labeled linoleate hydroperoxides, low molecular weight secondary products and fractions rich in polymeric degradation products, have shown that radioactivity is excreted in faeces, being 45 to 53% for the polymer-containing fraction, and 13 to 40% for linoleate hydroperoxides, while low molecular weight compounds have been reported to be almost completely absorbed and metabolized, with only 3% excreted in faeces (Kanazawa et al., 1985; Oarada et al., 1986; Bergan and Draper, 1971). Thermally oxidized fats, or isolated fractions containing oxidation products, have been reported to induce microsomal enzyme activities in the liver as well as in the colon after short term dietary administration (Andia and Street, 1975; Perciballi and Pintauro, 1985). These data indicate that orally administered lipid oxidation products or their metabolites may actually reach the colon, either via the luminal contents or by the bloodstream.

The data presented above appear to provide some evidence for an involvement of thermal oxidation and degradation products of dietary lipids in colon tumorigenesis. However, no data are available which indicate that tumor-initiating or promoting effects may be exerted in the colon after consumption of heat-processed lipids. Therefore, determination of genotoxic and other biological effects of heat-processed dietary lipids appears to be indicated, with special attention to possible effects exerted in the colon.

3.3. Deep-frying fat as a source of oxidized lipids and mutagens

Heat-processed dietary lipids are found in many food products, but dietary lipids which are almost certainly subject to heat-treatment are considered to be deep-frying fats. Heated deep-frying oils are probably the most extreme example of heat-processed lipids, since these may be kept at high temperatures (160-200 °C) for long periods of time, with oxygen introduced into the oil during frying of foods, which are conditions favouring oxidative deterioration of the frying oil (Chang et al., 1978; Stevenson et al., 1984). Deep-fat frying is a common cooking practice in The Netherlands, and is applied for preparation of potatoe chips, French fries, various meat products, nuts etc., during home cooking as well as industrial food processing. Based on consumption data, recently collected in The Netherlands in a dietary record survey with 5898 participants representative of the Dutch population, mean daily intake of lipids from deep-fat fried foods is estimated to be at least 6.4 g per person (Anonymous, 1988b). Assuming that 20% of the fat of deep-fried foods is derived from the frying oil, mean daily intake of heat-processed fat is approximately 1.2 g per person. This is likely to be a minimum estimation of consumption of all possible heat-processed lipids, since it does only include deep-frying fat and does not take into account consumption of heat-processed fats from pan-fried meats,

confectioneries etc. In addition, mean data of the total population of 5898 persons, aged 0 to 75 years, have been used and categories may exist that consume considerable higher amounts of deep-frying fat.

Several authors have investigated mutagenicity of heated deep-frying fats, or deep-fat fried foods, applying the Salmonella/microsome assay. No mutagenicity has been detected in dimethyl sulphoxide (DMSO)-extracts of heated oil (Van Gastel et al., 1984), whereas mutagenicity of DMSO-extracts of fresh peanut oils has been reported to decrease after heating in another study, which has been suggested to be due to the presence of aflatoxins in the oils (Fong et al., 1980). Taylor et al. (1982; 1983) have shown mutagenic activity in the basic and acidic methylene chloride extracts of fried meat products, but not of potatoes and other starchy foods. Mutagenicity of basic methylene chloride extracts of deep-fat fried meat-products, which are considered to contain amino acid pyrolyzates, has been reported by these authors to be considerably lower as compared to pan-fried meats, indicating deep-fat frying is not a major source of dietary mutagenic amino acid pyrolyzates. Furthermore, these authors have reported great difficulties with testing of oxidized lipid fractions, due to insolubility of the lipid fractions in the test media and growth inhibition of the bacteria. Only one study has made an attempt to investigate mutagenicity of oxidized lipid fractions (Scheutwinkel-Reich et al., 1980), but data obtained in this study may be regarded as inconclusive due to toxic effects to the bacteria of oxidized lipid fractions. Since individual lipid oxidation products have been reported to possess genotoxic activity in the Salmonella/microsome assay (MacGregor et al., 1985; Yamaguchi and Yamashita, 1979; 1980), as well as in mammalian cells (Brambilla et al., 1986; Cajelli et al., 1987; Nakayama et al., 1986; Weitberg and Corvese, 1989), involvement of lipid oxidation products in mutagenicity of the diet cannot be excluded.

Assessment of the role of heat-processed fats in colon tumorigenesis will require analysis of tumor-initiating as well as tumor-enhancing properties of heat-processed dietary lipids. Since deep-frying oils represent a class of dietary lipids which may be heat-processed for long periods, which may produce biologically active lipid oxidation products, and which are consumed with deep-fat fried foods, deep-frying fats and deep-fried foods appear to be of primary interest with respect to health risk assessment of heat-processed lipids. Therefore, genotoxic and other biological effects associated with carcinogenic risk will have to be determined to provide data for a qualitative and quantitative evaluation of the contribution of heated deep-frying fats and deep-fried foods to the carcinogenic risk posed by the diet.

4. Aim of the thesis

Heat-processed lipids have been postulated to be possibly involved in tumorigenesis of the colon, since they may contain biologically active and

genotoxic lipid oxidation products. As discussed previously, specific lipid oxidation products have been shown to possess genotoxic potential, in bacteria as well as in mammalian cells, indicating possible tumor-initiating properties. However, the relation between mutagenicity of the diet and presence of lipid oxidation products in heat-processed dietary lipids such as deep-frying oils, is not known. In addition to the generally applied Ames mutagenicity assay, use of eukaryotic and more specifically human cells in genotoxicity testing of dietary compounds is indicated especially for purposes of human genotoxic risk evaluation (Anonymous, 1988a). Detection of genetic damage in human cells, such as peripheral blood lymphocytes, will enable evaluation of genotoxic risk in cells which are likely to be exposed *in vivo* as well. Therefore, analysis of genotoxic activity of heated deep-frying fats in the Salmonella microsome assay, as well as in human lymphocytes, in relation to the presence of lipid oxidation products is the first aim of this thesis. For *in vitro* mutagenicity testing in human lymphocytes, a modified procedure for the detection of 6-thioguanine-resistant lymphocytes has been developed, which is described in Chapter 2. Analysis of genotoxic potential of heated deep-frying fats, using the Salmonella/microsome assay as well as by measurement of mutagenicity and sister chromatid exchange (SCE) frequencies in human lymphocytes, in relation to the presence of lipid oxidation products is presented in Chapters 3, 4 and 5.

In this thesis it is also hypothesized that heat-processed lipids may be involved in colon tumorigenesis. In order to test this possibility, biological fate is assessed of dietary mutagens associated with heat-processing of lipids, by means of comparing intake with excretion in urine and faeces. Increased faecal mutagenicity has been shown to be associated with high-fat and low-fiber consumption, and is considered a risk factor for colon cancer (Schiffman, 1988). Therefore, effects on faecal mutagenicity of consumption of mutagens related to heat-processing of dietary fat appears to be of special relevance with respect to evaluation of a possible role in colon carcinogenesis. This requires good control of consumption of other diet-related mutagens, which may not always be possible in a human diet study. For this reason, evaluation of faecal and urine mutagenicity after consumption of fat-related dietary mutagens has been carried out in both humans and rats, and is presented in Chapters 6, 7 and 8.

Furthermore, biological effects of short-term consumption of heat-processed deep-frying oils in rats will be examined in relation to lipid oxidation products, and to the occurrence of mutagens in the oils. Toxic effects, as well as effects on cell proliferation in the gastro-intestinal tract, which are considered to be a marker of tumor-enhancing effects, will be analyzed and are reported in Chapter 8. Finally, in the general discussion presented in Chapter 9, the data obtained in various studies will be used to evaluate the possible role of heat-processed fats in colon carcinogenesis.

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Chapter 2

Detection of 6-thioguanine-resistant human peripheral blood lymphocytes using 5-bromodeoxyuridine labeling in combination with immunocytochemical staining: Application for *in vivo* and *in vitro* HGPRT-assay¹

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Abstract

Resistance to 6-thioguanine (TG) is considered to originate from single gene mutations at the hypoxanthine guanine phosphoribosyltransferase (*hgpert*) locus. Screening of TG-resistant (TG^r) human peripheral lymphocytes may thus provide a means for determination of genetic damage occurring in a population exposed to genotoxic chemicals or radiation. The currently applied clonal assays however, pose substantial technical problems, and for application of the short-term autoradiography assays special facilities are required. Therefore a modified procedure was developed for the detection of TG^r human peripheral blood lymphocytes, using 5-bromodeoxyuridine (BrdU)-labeling in combination with immunocytochemical staining and whole blood culturing. The procedure was evaluated for its applicability *in vivo* using blood obtained from two healthy donors. After correction for the effects of spontaneously cycling lymphocytes (phenocopies) by means of cold storage of the blood for 24 h at 4 °C, variant frequencies (Vf's) of $2.7 \cdot 10^{-5}$ (donor I) and $3.4 \cdot 10^{-6}$ (donor II) were found, which were in the normal range reported in the literature.

Determination of induction of TG^r T-lymphocytes may provide a test system for *in vitro* screening of potential mutagenic compounds, using human cells that are readily available, easily cultured and allow human genotoxic risk estimation without problems of interspecies extrapolation due to differences in DNA repair capacities. Its applicability was validated using the chemical mutagens ethyl methane sulphonate (EMS), N-ethyl-N-nitrosurea (ENU) and mitomycin C (MMC), which were tested in 2 separate assays performed within a two-week period using blood from the same donor. Dose-response effects were observed for ENU and MMC, although at considerable inter-assay variability.

It is concluded that the presented modified procedure for determination of TG^r T-lymphocytes provides a simple, quick and widely applicable method for measurement of primary effects of genotoxic agents *in vivo* in man. In combination with other short-term genotoxicity assays, the *in vitro* HGPRT-assay using human lymphocytes may be useful for the assessment the genotoxic potential of xenobiotics.

¹ based on a manuscript submitted for publication

Introduction

Mutational lesions in mammalian DNA in both *in vitro* and *in vivo* assays are usually determined to assess genotoxic properties of chemical and physical agents. Extrapolation of data obtained with non-human cells to human genotoxic risk however, may pose problems which are related to interspecies differences in cellular metabolism and DNA repair capacities (Fox, 1988; Fox and Roberts, 1987). Use of human cells is therefore preferred, for instance mitogen-stimulated lymphocytes (Kilby et al., 1984). Resistance to 6-thioguanine (6-TG) of human peripheral blood lymphocytes has been demonstrated to arise from somatic gene mutations at the hypoxanthine guanine phosphoribosyltransferase (*hgpert*) locus, and is used as a parameter in human mutagenicity monitoring (Albertini et al., 1985; Dempsey and Morley, 1983; Turner et al., 1985).

Generally, two assays have been developed to detect TG-resistant (TG^r) lymphocytes, a short-term autoradiography assay and a clonal assay (Albertini et al., 1987; Morley et al., 1982). The latter method appears to be preferential for monitoring of mutational lesions derived *in vivo* and for elucidation of mutational mechanisms *in vitro*, since it allows for verification of HGPRT deficiency, selection of mutant colonies and determination of *hgpert* structural gene alterations at a molecular level (Albertini, 1985; Nicklas et al., 1987). However, the clonal assay requires long culturing periods and appears to pose substantial technical problems, resulting in poor cloning efficiency (Morley et al., 1985). The autoradiography assay may be used as an indication of elevated mutant frequencies, but since it does not allow verification of the nature of mutant cells or elucidation of mutational lesions. The autoradiography assay however, is less laborious as compared to the clonal assay, and better applicable for initial screening of large groups (Albertini et al., 1987).

The use of bromodeoxyuridine (BrdU) which has been demonstrated to present a feasible alternative for tritiated thymidine, has made the *hgpert* somatic mutation assay available to any cytogenetic laboratory (Ostrosky-Wegman et al., 1987). However, serious problems were encountered in our laboratory when BrdU-incorporation was used in combination with the fluorescence-plus-Giemsa method, as described by these authors. These are caused mainly by poor discrimination between nuclei which actually had incorporated BrdU and nuclei of non-proliferating cells. Therefore, the feasibility of an immunocytochemical staining method using mouse monoclonal antibodies against BrdU (Grazner, 1982), in combination with a peroxidase-conjugated secondary antibody, was tested. In addition, the HGPRT-assay was modified with respect to culturing of T-lymphocytes; instead of isolation of lymphocytes by gradient centrifugation prior to culturing (Albertini et al., 1985; Morley et al., 1985), whole blood was

cultured corresponding to methods used in standard cytogenetic assays, which reduced the amount of blood required and resulted in a simple and quick procedure.

For *in vitro* mutagenicity testing human lymphocytes may provide an adequate tool, since these have been successfully applied for *in vitro* determination of chromosomal damage, and are readily available and easily cultured. The applicability of the modified procedure as an *in vitro* assay for detection of genotoxic properties of chemical mutagens to human DNA, was therefore also evaluated.

Materials and methods

Determination of in vivo variant frequency (Vf)

In all experiments, blood samples (10 ml) were collected from healthy, non-smoking volunteers, 1 male with a limited smoking history (donor I) aged 34, and 1 female (donor II), aged 29. Blood was drawn by venipuncture in heparinized tubes. Blood samples were kept at room temperature for 3 to 4 h, since we found that short-term storage of blood samples at room temperature yielded higher labeling indices as compared to culturing of fresh blood samples. Since separation of lymphocytes prior to culturing resulted in loss of large numbers of lymphocytes during harvesting of the cultures, whole blood culturing was applied to obtain sufficient numbers of cells and reduce the amount of blood required. Determination of *in vivo* derived TG^r lymphocytes may be confounded by the presence of phenocopies resulting in 10-fold higher frequencies, and was demonstrated to be eliminated by cryopreservation or cold storage (Albertini and Sylwester, 1984). In separate sets of experiments therefore, the effects of cold storage on observed Vf's, as well as the Vf of spontaneously cycling lymphocytes were determined. Effects of prolonged incubation with BrdU in order to increase the number of positive cells to obtain a more accurate assessment of the Vf, were also evaluated.

The definitive protocol for determination of the *in vivo* mutant T-lymphocytes was as follows: Whole blood was diluted (1:10, v/v) in RPMI 1640 medium (Gibco Europe, The Netherlands) without supplements, and kept at 4 °C for at least 24 h in order to eliminate the effects of phenocopies, caused by BrdU incorporation in cells which are spontaneously cycling and not sensitive to 6-TG at the particular moment of labeling (Albertini and Sylwester, 1984). After cold storage, cells were collected by centrifugation and resuspended at a density equal to 0.4 ml whole blood per 5 ml medium, in RPMI 1640 medium supplemented with 100 µg/ml streptomycin, 100 U/ml penicillin, 5 mM L-glutamine, 50 U/ml heparin and 10% foetal calf serum (FCS) (all obtained from Gibco Europe).

Two cultures were set up to determine the control labeling index (LI_c) and 4 to 8 cultures were used for TG-resistant (TG^r) cell selection. To all 5 ml cultures, 0.2 ml phytohaem-

agglutinin (PHA; Gibco Europe) was added; to the TG^f selection cultures 0.1 ml of a $1 \cdot 10^{-2}$ M TG stock in RPMI 1640 was added at a final concentration of $2 \cdot 10^{-4}$ M, and to control cultures 0.1 ml of RPMI 1640 adjusted to a pH equal to that of the TG stock. Cultures were incubated at 37 °C for 24 h, after which 0.05 ml of a filter-sterilized $2.5 \cdot 10^{-3}$ M BrdU solution was added to a final concentration of $2.5 \cdot 10^{-5}$ M. Cultures were incubated in presence of BrdU at 37 °C for an additional 16 h period.

After a total of 40 h of incubation, cells were harvested by centrifugation and pellets resuspended in 9 ml hypotonic 75 mM KCl solution of 37 °C, and incubated at 37 °C for 20 min. After centrifugation, pellets were resuspended by dropwise addition of fixative, methanol:acetic acid (3 : 1 v/v), first to a volume of 2 ml. To increase density of nuclei in TG cultures, at this point 2 to 4 TG cultures were combined and the final volume was completed to 9 ml with fixative. The control cultures were fixed by dropwise addition of 9 ml fixative and not combined. After centrifugation, nuclei were rinsed twice with 9 ml fixative and finally resuspended in 0.1 to 0.2 ml.

Nuclei were transferred to clean microscope slides which were pre-rinsed with ethanol; 2 drops of 10 µl of the control cultures were pipetted on a slide. The complete suspension of combined TG cultures was transferred on one slide in 25 µl portions, meanwhile determining the total volume of the TG culture. Thus, 2 slides were obtained from 2 to 4 originally set up 5 ml TG test cultures, thereby maximizing the number of nuclei per TG slide. The number of nuclei on TG slides was counted in triplicate in 5 µl of a 1:50 dilution in fixative.

Immunocytochemical staining using monoclonal anti-BrdU

Identification of nuclei that have incorporated BrdU, was achieved by immunochemical staining using mouse monoclonal antibodies against BrdU (Grazner, 1982), which were a gift from Dr. Bert Schutte (Department of Internal Medicine, University of Limburg, Maastricht, The Netherlands). The staining procedure was as follows: DNA was denatured by incubation of the slides in 4 N HCl for 25 min at room temperature. The slides were rinsed twice with 0.1 N Borax buffer of pH 8.5, followed by phosphate buffered saline (PBS) of pH 7.4. After rinsing, 250 µl of a solution of mouse monoclonal anti-BrdU antibodies in PBS containing 1% bovine serum albumin (PBS/BSA), were applied to the slides, which were carefully covered with a cover slide, ensuring the anti-BrdU layer was distributed over the whole area. The slides were incubated at 37 °C in a humid atmosphere for 60 min. Cover slides were carefully removed, after which two rinsing steps with PBS followed. Next, 250 µl of peroxidase-conjugated rabbit-anti-mouse immunoglobulin antibody (RAMPO, F114, Dakopatts, Denmark) solution in PBS/BSA were applied to the slides, which were covered with a cover slide and incubated, protected from light at room temperature for 60 min. After careful removal of the cover slides, 2 rinsing steps with PBS followed. Finally, the slides were incubated in 0.05 M Tris-KCl buffer of pH 7.6 containing 0.04% (w/v) 3,3'-diaminobenzidine (Fluka Chemie, Switzerland) and 0.01% (w/v) H₂O₂ at room temperature in the dark for 10 min. Slides were rinsed with distilled water and control slides were immediately counterstained by immersion in 3% Giemsa stain (BDH Chemicals, England) in 0.1 M phosphate buffer of pH 6.8 for 3 min. In the first series of experiments, anti-BrdU control slides containing nuclei of PHA-stimulated cells incubated without BrdU, were also stained in order to determine non-specific anti-BrdU

binding. Since non-specific anti-BrdU binding was not observed, these slides were included in later experiments only when new batches of anti-BrdU were used.

After staining, nuclei of non-proliferating cells appeared blue and BrdU-containing nuclei brown, and were clearly visible using normal light microscopy (400x). The slides of TG cultures were not immediately counterstained, but first encoded and screened for brown BrdU-containing nuclei. The number of brown nuclei per slide was determined by two independent observers. Next, the TG slides were also counterstained and the number of brown nuclei per slide was confirmed by re-evaluation of the nuclei earlier identified as positive. The LI_C was determined in duplicate by scoring 1000 nuclei per slide and was calculated as:

$$LI_C = \frac{\text{number of brown nuclei per 1000 nuclei scored}}{1000}$$

The labeling index of the TG culture (LI_t) was determined as:

$$LI_t = \frac{\text{number of brown nuclei on all TG slides}}{\text{total number of nuclei on all TG slides}}$$

The variant frequency (Vf) of TG^f nuclei (variants) was calculated as: $Vf = LI_t / LI_C$.

Chemical induction of TG^f T- lymphocytes in vitro

The applicability of the modified HGPRT-assay using whole blood culturing and BrdU incorporation for *in vitro* screening of chemical mutagens was evaluated by determination of the induction of TG^f T-lymphocytes by ethyl methanesulphonate (EMS), N-ethyl-N-nitrosourea (ENU) and mitomycin C (MMC) (all mutagens obtained from Sigma Chemical Co., St Louis, Mo.). These mutagens were chosen for their known capability to induce TG^f T-lymphocytes *in vitro* in the clonal HGPRT-assay (Vijayalaxmi and Evans, 1984). Heparinized blood samples (10 ml) were obtained by venipuncture from 2 healthy donors described earlier (donor I and donor II). Incubations with the chemical mutagens were set up using blood from one donor for each mutagen. Thus, EMS and MMC were tested with blood from donor II, while ENU was evaluated using blood of donor I. EMS and ENU were dissolved and diluted in distilled water and used immediately after preparation. For MMC, which is relatively stable in medium (Vijayalaxmi and Evans, 1984), RPMI 1640 without supplements was used for dilution. To a sterile 10 ml tube containing 5 ml of RPMI 1640 medium without supplements, 0.4 ml of whole blood and 0.1 ml of mutagen solution were added. Cultures were incubated at 37 °C; incubation times for EMS and MMC were 4 h and for ENU 30 min, these incubation times were chosen since the biological half-lives were reported to be 5.3 h, many hours and 0.15 h, respectively for EMS, MMC and ENU (Vijayalaxmi and Evans, 1984). After mutagen treatment, the cells were rinsed with pre-warmed PBS of 37 °C and resuspended in 5 ml RPMI 1640 medium containing 0.2 ml PHA. Per dose, a total of 6 cultures was set up; to 4 cultures 0.1 ml of a $1 \cdot 10^{-3}$ M TG stock and to 2 cultures 0.1 ml of TG control medium was added. Cultures were incubated for 24 h at 37 °C, after which BrdU was added to a final concentration of $2.5 \cdot 10^{-5}$ M. After an additional incubation of 16 h in presence of BrdU, cells were harvested, stained and evaluated as described previously for the *in vivo* determination of TG^f T-lymphocytes. In order to determine inter-assay variability, incubations with chemical mutagens were carried out in two separate experiments with blood obtained from the same donor, which

were performed within a 2 week period.

Statistical analyses

Statistical evaluation of differences between Vf's was performed as described by Sylwester and Albertini (1985). Vf's of different *in vivo* determinations and of different doses of chemical mutagens within one experiment, were compared by ratio using 95% or 99% binomial confidence interval tables (Sylwester and Albertini, 1985). Dose-response relations of chemical mutagens were evaluated using linear regression applied to non-toxic dose ranges.

Results

The effects of varying culturing conditions on the TGF α T-lymphocyte frequency are presented in Table 2.1. Cold storage at 4 °C for at least 24 h leads to a 2- to 6-fold decrease of the frequency of TGF α T-lymphocytes, as is evident from comparison of Vf's obtained in experiments 2 and 3 with those of experiment 1. Without prior cold storage of the blood, the spontaneously cycling lymphocyte fraction contributed significantly to the Vf of donor II, possessing a Vf of approximately $1 \cdot 10^{-3}$ (Table 2.1). In experiment 1 (Table 2.1) a considerably higher Vf was found for donor I compared to donor II. After correction for spontaneously cycling lymphocytes, using cold storage in experiment 3, Vf of donor I still showed a value which was approximately one order magnitude higher than the Vf of donor II. Incubation of the cultures with BrdU for 40 h in combination with increasing culturing time from 40 to 64 h, yielded Vf's which did not differ significantly from those observed for incubation with BrdU for 16 h and a total culturing time of 40 h. This was indicated by Vf's obtained in experiments 2.a and 2.d, 1.b and 2.d, as well as those in 2.b and 3.b (Table 2.1).

In two separate experiments, induction of TGF α T-lymphocytes by known chemical mutagens was determined, which showed greatly varying Vf's between the experiments (Table 2.2). Standard deviations were found for non-toxic doses ranging from 30 to 50 % of the observed mean. All mutagens were tested up to their cytotoxic limit, which is indicated by decreasing LI_C at high doses (Table 2.2). Significantly increased Vf's were observed for ENU in experiment 2 at doses of $1 \cdot 10^{-3}$ M ($\alpha=0.05$) and $5.0 \cdot 10^{-3}$ M ($\alpha=0.01$) (Table 2.2). Dose-response curves of EMS, ENU and MMC are shown in Figure 2.1. Within non-toxic dose ranges, dose-response curves obtained for ENU and MMC indicated linearity ($p<0.09$ and $p<0.06$, for ENU and MMC respectively).

Table 2.1. Variant frequencies (Vf's) in human peripheral blood T-lymphocytes with varying culture conditions.

Exp.	Donor	cold storage	PHA	Culturing time (h)	BrdU incubation time (h)	LI _c (mean±sd)	# variants	LI _t (•10 ⁻⁶)	Vf (•10 ⁻⁶)
1. a.	I	-	+	40	16	0.283 ±0.021	26	44.8	158.3 b
b.	II	-	+	40	16	0.192 ±0.035	1	1.8	9.2
2. a.	II	-	+	40	16	0.115 ±0.018	5	2.3	19.8 ac
b.	II	+	+	40	16	0.171 ±0.008	2	0.6	3.4 ad
c.	II	-	-	40	16	0.001 ±0.001	2	1.3	1066.7 cde
d.	II	-	+	64	40	0.534 ±0.012	7	5.5	10.3 e
3. a.	I	+	+	64	40	0.534 ±0.018	14	14.4	26.7 b
b.	II	+	+	64	40	0.503 ±0.004	2	2.0	4.0

a Identical superscripts indicate significant difference between Vf's at alpha=0.05.**b,c,d,e** Identical superscripts indicate significant difference between Vf's at alpha=0.01.

Table 2.2. Induction of *TGF* lymphocytes by ethyl methane sulphonate (EMS), *N*-ethyl-*N*-nitrosourea (ENU) and mitomycin C (MMC).

Test chemical	dose	exp.	L _C	# variants	L _t ($\cdot 10^{-6}$)	Vt ¹ ($\cdot 10^{-6}$)
EMS (10^{-3} M)	0.0	1	0.261	1	6.5	25.0
		2	0.133	5	5.7	42.7
	1.0	1	0.219	3	17.6	80.9
		2	0.159	1	6.5	40.8
	5.0	1	0.200	3	15.1	75.0
		2	0.214	1	8.5	40.0
ENU (10^{-3} M)	0.0	1	0.098	1	20.0	200.0
		2	0.045	3	2.5	55.6
	0.0	1	0.146	106	150.4	1030.1
		2	0.198	74	80.6	407.1 <i>ab</i>
	0.1	1	0.103	65	110.7	1074.8
		2	0.199	143	92.0	462.3
	1.0	1	0.088	72	98.0	1113.6
		2	0.193	162	131.3	680.3 <i>a</i>

Table 2.2. - continued

Test chemical	dose	exp.	L_C	# variants	L_t ($\cdot 10^{-6}$)	V_f^1 ($\cdot 10^{-6}$)
EMS (10^{-3} M)	5.0	2	0.181	82	264.5	1464.0 ^b
	10.0	1	0.034	9	33.3	979.4
MMC (10^{-6} M)	0.0	1	0.218	1	3.3	14.9
		2	0.192	1	1.8	9.3
	0.6	1	0.200	1	3.0	15.1
		2	0.227	1	2.2	9.8
	6.0	1	0.193	1	6.4	33.2
		2	0.200	3	3.6	18.1
	60.0	1	0.002	2	18.2	9091.0
		2	0.074	1	3.6	47.6

¹ V_f s are presented of 2 separate experiments. ^a Identical superscripts indicate significant difference between V_f s at $\alpha=0.05$; ^b Identical superscripts indicate significant difference between V_f s at $\alpha=0.01$.

Discussion

Effects of modifications in culturing conditions

The modified procedure for detection of TGr mutants in human T-lymphocytes using whole blood culturing and BrdU-labeling in combination with immunocytochemical staining, appears to provide a simple and generally applicable assay for the evaluation of genotoxic damage after *in vivo* and *in vitro* exposure to potential mutagens. The use of BrdU-labeling provides a reliable alternative for the autoradiography assay, as has first been demonstrated by Ostrosky-Wegman et al. (1987). When monoclonal anti-BrdU and peroxidase-conjugated secondary antibodies are used for detection of nuclei which have incorporated BrdU, a better contrast is observed between TGr and non-proliferating cells.

Small numbers of mutant cells have been reported to lead to large variations in the estimation of Vf's (Albertini and Sylwester, 1984). In order to reduce this variability, conditions of the modified procedure presented in this paper may easily be improved by setting up more 5 ml TG cultures, or by incubating larger cultures, for example 2 TG test cultures of 40 ml. Another way of increasing the number of mutant cells per culture is elongation of the incubation time with BrdU to 40 h, thus covering more than one cell cycle of stimulated T-lymphocytes, and enabling TGr T-lymphocytes to amplify and incorporate BrdU (Bender et al., 1988; deFazio et al., 1988). Previously, Morley et al. (1982), showed that elongation of the total culturing time to 64 is feasible. As is shown in experiment 2 (Table 2.1), prolonged incubation in presence of 2.5×10^{-5} M BrdU does not appear to result in increased Vf's, although BrdU itself is considered a chemical mutagen to mammalian cells, and has been found to cause pseudomutational effects in a lymphoblast cell line (Liber et al., 1985).

Cold storage at 4 °C for 24 h prior to culturing significantly reduces Vf's compared to Vf's obtained without cold treatment, and appears to eliminate the effects of spontaneously cycling phenocopies, resulting in Vf's that are well within the range of 10^{-6} to 10^{-5} reported for autoradiography assays (Albertini et al., 1987; 1988; Morley et al., 1982). Since Vf's obtained in the short-term autoradiography assay and the clonal assay have been reported to be similar (Albertini et al., 1988), and the modified short-term assay presented in this paper is simple and applicable at large scale, it may be useful for initial screening of large groups. The use of immunofluorescence staining and flow cytometry for enumeration of BrdU-labeled TGr T-lymphocytes may eventually provide a more sophisticated and less laborious technique (deFazio and Tattersall, 1985; Matsson et al., 1985).

Figure 2.1. Dose-response curves obtained for *in vitro* exposure of human peripheral T-lymphocytes to ethyl methane sulphonate (EMS; **A**), N-ethyl-N-nitrosourea (ENU; **B**) and mitomycin C (MMC; **C**). Data shown are the mean $\pm$ SEM based on results of 2 separate experiments. * significantly increased Vfi at $\alpha=0.01$.

Applicability for determination of in vivo derived variant T-lymphocytes

The autoradiography assay has been useful in determining the induction of TGR T-lymphocytes after chemotherapy treatment in cancer patients (Albertini et al., 1985; Lange and Prantner, 1982) and after cyclophosphamide infusion in multiple sclerosis patients (Ammenheuser et al., 1986). At present however, it is not clear whether detection of increased TGR T-lymphocytes *in vivo* is possible only shortly after exposure to chemical mutagens, since an *in vivo* selection against mutants appears to exist resulting in a decline of increased frequencies several months after exposure (Ammenheuser et al., 1986). However, TGR T-lymphocytes may also accumulate or even undergo clonal amplification upon *in vivo* stimulation, thereby leading to increased Vf's not induced by separate mutational events (Albertini et al., 1987; Lange and Prantner, 1982; Nicklas et al., 1987). When increased Vf's are observed, evaluation of the nature of mutations as well as determination of the origin of mutant T-lymphocytes will be necessary.

Since long-term exposure of nurses and pharmacists to cytostatic drugs has been reported to result in marginal increases of mutant frequencies as compared to the effects observed in treated patients (Chrysostomou et al., 1984), the applicability and sensitivity of determination of mutational lesions at the *hgp* locus in T-lymphocytes as a screening method for the assessment of genetic damage remains to be established. The observed absence of pronounced effects of exposure to genotoxic agents on Vf's may also be due to presence of confounding factors. Screening of exposed populations will therefore require strict control of possible confounding variables, among which are age (Amnéus et al., 1982; Morley et al., 1982; Trainor et al., 1984; Vijayalaxmi and Evans, 1984), smoking habits and history (Albertini et al., 1986), and possibly alcohol consumption and use of oral contraceptives, since these factors have been reported to influence the detection of sister chromatid exchanges (SCE) as well (Das, 1988). It seems therefore relevant to validate the applicability of the HGPRT-assay by determination of frequency of TGR T-lymphocytes in a population of heavy smokers with strict control of possible confounding variables.

Determination of variant T-lymphocytes after in vitro exposure to chemical mutagens

Chemical induction of TGR T-lymphocytes *in vitro* allows for evaluation of genotoxic effects of chemicals in human cells which are widely available and easily cultured. In addition, human lymphocytes allow determination of genetic damage to human DNA, and possess enzyme activities and DNA repair potentials which differ from other mammalian cells generally used in mutagenicity testing, as is illustrated by the lack of proficient O⁶-alkylguanine repair in Chinese hamster CHO fibroblast cell lines (Fox and Roberts, 1987).

Normal lymphocytes have also been reported to be genetically more stable than malignant cells used in short-term assays (Seshadri et al., 1987). Furthermore, the use of whole blood cultures appears to provide experimental conditions which more closely resemble the *in vivo* situation. Recently, comparison of results of whole blood culturing in the *in vitro* human lymphocyte micronucleus assay to results obtained for purified lymphocytes has shown that the presence of erythrocytes may play an important role in metabolic activation or detoxification of xenobiotics (Migliore et al., 1989).

Vf's observed in separate experiments in the present study, as well as standard errors of 20-50% reported by Vijayalaxmi and Evans (1984) for the *in vitro* induction of TGF T-lymphocytes by EMS, ENU and MMC using the clonal assay, indicate that inter-assay variability between Vf's may be substantial. This variability is probably due to inter-individual variability between different blood donors, as well as to differences in sensitivity between different classes of lymphocytes, and is considered to reflect biological variability as it occurs within a population. For *in vitro* testing of chemical compounds this may pose problems with respect to reproducibility of results when lymphocytes of different donors are used, and statistical evaluation of results is therefore limited to comparison within one assay. Dose-response curves obtained for ENU in separate assays in the present study also indicate variability in time course for lymphocytes obtained from one donor, which may be due to differences in sensitivity of lymphocytes to cytotoxic effects of ENU as is indicated by the decreased LI_C at a dose of 0.1 mM in experiment 1 which has not been observed in experiment 2 (Table 2.2). Furthermore, the induction of anti-mutagenic repair systems after mitogenic stimulation of T-lymphocytes by PHA, may also influence the detection of mutagenic lesions, and may also vary between different assays. (Sanderson and Morley, 1986; Waldstein et al., 1982).

In spite of the observed variability, use the HGPRT-assay with human lymphocytes for the assessment of mutagenicity of chemical compounds *in vitro*, does have advantages such as relatively simple culturing procedures, short duration of the assay, as well as testing of chemicals under conditions which closely resemble the *in vivo* situation. Further modifications of the standardized protocol as applied in the present study for the *in vitro* HGPRT-assay using human lymphocytes, such as variation of mutagen exposure and mutant expression times, may increase its sensitivity, and remain to be determined. In combination with analysis of other genotoxic effects, including induction of SCE's and micronuclei, determination of mutation frequencies at the *hgp* locus may prove useful in estimation of human genotoxic risk posed by xenobiotic compounds.

Summarizing, it is concluded that whole blood culturing in combination

with BrdU-labeling provides a simple and quick procedure for determination of TG^r T-lymphocytes after *in vitro* exposure to potential mutagens; further studies will be needed however, to determine effects of varying culture conditions, including exposure, mutant expression and labeling times. Furthermore, the modified procedure provides a simple procedure for initial large scale screening of mutational lesions in DNA of high-risk human populations.

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Chapter 3

Assessment of mutagenic activity of repeatedly used deep-frying fats

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Abstract

Mutagenic activity of repeatedly used deep frying fats was evaluated in relation to chemical characteristics. Deep frying fat samples were collected from local restaurants and snack bars after sensoric indication of abuse. A total of 20 deep frying fat samples and 2 unused control fat samples was tested. Fat samples were fractionated into non-polar and polar compounds by column chromatography. Amounts of polar compounds obtained ranged from 2% (by weight) for unused fat to 44% for used deep frying fat. Levels of di- and polymeric triglycerides (DPTG) were determined using gel permeation chromatography. DPTG concentrations of 13 used deep frying fat samples exceeded the threshold level of 10% above which fats are rejected for use. In addition thiobarbituric acid-reactive substances (TBA-RS) were measured. Amounts of TBA-RS were just above detection levels for most fat samples. Five used fat samples however contained relatively high concentrations of TBA-RS, ranging from 82 to 177 nmol malondialdehyde/g.

Non-polar and polar fractions were screened for mutagenic activity using the Ames mutagenicity assay. Mutagenic activity was found predominantly in polar fractions at doses higher than 1 mg/plate in strains TA97, TA100 and TA104, variously with and without metabolic activation. Highest number of mutagenic samples was detected by strain TA97, which appeared to be most sensitive. Some samples exhibited toxic effects. Chromatography blanks, consisting of solvents processed according to the same procedures as used for fat samples, were not mutagenic. Mutagenic activity was also detected in polar material obtained from unused frying fat. Non-polar fractions of unused frying fats showed no mutagenicity.

A frying experiment carried out under laboratory conditions indicated that during repeated and prolonged use of deep frying fat mutagenic polar substances were formed. Fat samples taken after 20 and 40 hours of frying contained increasing amounts of polar compounds. Mutagenic activity was highest after 20 hours of frying but was slightly decreased after 40 hours of frying. At this stage however mutagens also appeared in the non-polar fraction.

Mutagenic activity of polar fractions of used deep frying fats in strain TA97 was positively correlated with levels of TBA-RS, which may indicate the involvement of lipid oxidation products in mutagenicity of used deep frying fats. No significant correlations were found with other chemical characteristics. In the process of deep fat frying numerous degradation products are formed, which may include mutagenic heterocyclic amines and other pyrolyzates. The specific nature of mutagens formed during deep fat frying remains unclear.

Introduction

Heating of foods with high protein concentrations has been found to cause formation of mutagenic and carcinogenic substances (Sugimura et al., 1986). These substances are believed to explain in part the typical pattern of cancer prevalence that occurs in countries where intake of protein and fat is high. Fat is generally thought to act as tumor promotor. However, heating of fats, in particular those rich in polyunsaturated fatty acids, will generate numerous oxidative degradation products that may be potentially genotoxic (Alexander, 1980; Stevenson et al., 1984).

In the process of frying fats and oils are repeatedly heated at high temperatures up to 180 °C for prolonged periods of time. Under these conditions volatile and non-volatile oxidation products are formed (Stevenson et al., 1984) that may be absorbed by the food and subsequently eaten. Feeding experiments with laboratory animals have shown that thermally oxidized oils exert toxic effects *in vivo*. Consumption of heated oils or isolated fractions may cause growth retardation, increased liver and kidney weights, and cellular damage to liver, thymus, epididymides and testes (Andia and Street, 1975; Billek, 1976; Alexander, 1981; Alexander et al., 1987).

Evaluation of the genotoxic potential of heated deep frying fats *in vitro* using the Ames assay yielded negative or inconclusive results (Scheutwinkel-Reich et al., 1981; Taylor et al., 1982; Van Gastel et al., 1984). Fong et al. (1980) however, did report increased mutagenic activity of heated peanut oils as compared to uncooked oils, which could not be attributed to the presence of aflatoxins. Various isolated lipid oxidation products like malondialdehyde, some alkenals, carbonyls and hydroperoxides of linoleic and linolenic acid have also been found to be mutagenic (Mukai and Goldstein, 1976; Yamaguchi and Yamashita, 1979; 1980; Marnett et al., 1984; MacGregor et al., 1985). No studies evaluating the carcinogenic potential of heated fats are available.

Health risks of food preparation by deep frying are currently discussed primarily because reliable cut off levels based on chemical analysis seem unsatisfactory. In this study therefore, mutagenic activity of repeatedly used deep frying fats sampled at the retail level was evaluated in relation to chemical analyses used for quality control. In addition a frying experiment was carried out in the laboratory, in which the formation of mutagenic substances was followed during prolonged and repeated use.

Material and methods

Chemicals

Analytical grade, stabilized (with 250 ppm butylated hydroxytoluene) tetrahydrofuran used for HPLC-analysis of di- and polymeric triglycerides; analytical grade petroleum ether, boiling range 40-60 °C; methanol; acetic acid and silicagel 60, particle size 0.063-0.20 mm, used for fractionating fat samples; trichloroacetic acid; malonaldehyde bis-(diethylacetal); hydrochloric acid and 2-thiobarbituric acid, all of the highest chemical grade available, were purchased from Merck, Darmstadt, FRG. HPLC-grade diethyl ether and non-stabilized tetrahydrofuran used for dissolving fat fractions in mutagenicity testing were obtained from Fisons, Loughborough, England. All other chemicals and media used in the Ames mutagenicity assay were of the highest chemical grade commercially available.

Sampling

Deep frying fat samples were collected by officers of the Maastricht Office for Food Quality Control from local restaurants and snack bars after sensoric indication of abuse. Samples were taken according to internationally standardized procedures (ISO, 1983) and stored refrigerated until further testing.

Di- and polymeric triglycerides

At the Office for Food Quality Control di- and polymeric triglycerides (DPTG) were determined as part of routine quality assessment (ISO, 1980). Amounts of DPTG were analyzed using gel-permeation HPLC. HPLC-equipment consisted of a RI detector and a Styrogel 500 A column, 300 mm x 7.8 mm i.d. Degassed, stabilized tetrahydrofuran (THF) was applied as mobile phase at a flow rate of 1 ml/min. Samples of 200 µl of fat, liquified by heating at 50°C, or oil were mixed with 3.0 ml THF and 15 µl of this mixture was injected. Amounts of DPTG are calculated as follows:

$$\% \text{ DPTG} = \frac{\text{Sum of peak area's of DPTG}}{\text{Total sum of peak area's}} \times 100.$$

Fractionation of fat samples

Out of 60 samples originally collected 20 samples were randomly chosen, fractionated into non-polar and polar fractions, analyzed for thiobarbituric acid-reactive substances and screened for mutagenicity. Fractionation of samples into polar and non-polar components by column chromatography was modified from Billek et al. (1978), which procedure is proposed as an international standard (ISO, 1986). Briefly, one gram of fat was dissolved in 10 ml of a mixture of petroleum ether (boiling point 40-60 °C) and diethyl ether, 87:13 v/v (solvent 1). The fat solution and a 10 ml rinsing were carefully introduced onto a silica gel column (45 cm x 2.1 cm i.d.), filled with 25 gram deactivated silicagel. The non-polar fraction was eluted using 150 ml of solvent 1. The eluate was collected in a flask and designated the non-polar fraction. Subsequently the polar fraction was eluted with 150 ml diethyl ether acidified with 1% v/v acetic acid (solvent 2) and collected in a separate flask. Finally, very polar, yellow colored material was eluted with a mixture of methanol and water (75:25, v/v) acidified with 1% (v/v) acetic acid (solvent 3). Solvents were distilled off by vacuum evaporation at a maximum temperature of 60 °C. After evaporating most of the solvent, residues were transferred into

pre-weighed screw top vessels. Remaining solvent was evaporated under a stream of nitrogen at room temperature. Solvent 3 was evaporated in a vacuum oven at 50 °C. After complete evaporation of the solvents vessels were weighed and the content calculated as percentage of total fat weight. Fractionated samples were kept under nitrogen and stored under refrigeration.

Thiobarbituric acid-reactive substances

As an index of lipid peroxidation thiobarbituric acid-reactive substances (TBA-RS) were determined. Approximately one gram of fat was extracted with 4.0 ml of a reaction mixture consisting of 15% (w/v) trichloroacetic acid and 0.375% (w/v) TBA in 0.25 N HCl to which 0.02% butylated hydroxytoluene was added to prevent further fatty acid oxidation during the assay. The mixture was incubated in a shaking waterbad at 80 °C during 40 minutes. After centrifugation the absorbance of the aqueous phase was measured at 532 nm as well as at 600 nm to correct for non-specific background absorption. TBA-RS are calculated as nmol malondialdehyde (MDA) per gram of fat, using a calibration curve of malondialdehyde bis-(diethylacetal) dissolved in one gram of unused frying fat that contained no measurable TBA-RS.

Mutagenicity testing

Fractionated deep frying fat samples were dissolved in DMSO or THF. Some fractions required gentle heating (maximum temperature 50 °C) to facilitate dissolving. THF proved to be a more suitable solvent than DMSO; both non-polar and polar fractions were better dissolved in THF and suspended in the Ames incubation medium in the presence of THF. Doses were tested in duplicate or triplicate, total volumes of the solvents were 100 µl for DMSO and 25 µl for THF. The Salmonella/microsome assay was performed according to Maron and Ames (1983) using the preincubation procedure; in a medium consisting of 0.5 ml of S9 mix, that contained 50 µl of rat liver S9 fraction from Arochlor treated rats, or 0.5 ml of 0.1 M phosphate buffer (pH 7.4), the test dose and 0.1 ml of an overnight grown culture were incubated during 20 minutes at 37 °C, after which suspensions were plated. After initial prescreening strains TA97, TA100 and TA104 were selected for mutagenicity testing. For practical reasons however, strain TA104 was withdrawn from the set of tester strains since strain TA100 appeared equally sensitive and yielded lower plate counts on account of the smaller number of spontaneous revertants. Plates were counted manually with visual discrimination between fat particles and revertant colonies. Toxicity was evaluated by observing bacterial growth of the background lawn. Solvent controls (DMSO and THF) and positive controls were included in each assay. Chromatography blanks consisting of solvents processed following the same procedures as used for fat samples were also screened for mutagenicity. In addition, two different brands of unused frying fat obtained from local supermarkets were chemically analyzed and included in the mutagenicity assay.

Statistical analysis

Statistical analysis of the data was performed using the method of least significant difference (LSD) as described by MacGregor et al. (1985). The least significant difference between test and control data was calculated from the variance between sets of replicate control (solvent) values, summed over each set of strain and S9 conditions as follows:

$$LSD = t \sqrt{2 MS},$$

where t is the t -statistic value at a given confidence level with the degrees of freedom equal to the number of control data sets, and MS is the overall variance of individual replicate determinations from each individual experiment summed over all experiments. LSD values obtained in this study are at a confidence level of $p < 0.01$ and $p < 0.001$ respectively: for THF as solvent with TA97-S9 126 and 220; TA97+S9 142 and 235; TA100-S9 82 and 154; TA100+S9 76 and 142; TA104-S9 696 and 1329; TA104+S9 655 and 1255; for DMSO as solvent, with TA97-S9 132 and 211; TA97+S9 243 and 389; TA104-S9 588 and 975 and TA104+S9 592 and 980.

Frying experiment

A controlled frying experiment was performed under laboratory conditions. A batch of 2 kg of unused commercial brand frying fat was heated at 180 ± 5 °C during 10 hours per day on 4 consecutive days. Each day three portions of food: 100 g of meat croquettes, 2 egg roles (150 g) and 100 g of sliced potatoes (French fries) were fried according to instructions provided by manufacturers. The fat was allowed to cool overnight and was not replenished. Fat samples were collected once a day after 2 and 4 days of frying. TBA-RS were determined and samples were fractionated into polar and non-polar components, which were screened for mutagenic activity.

Results

Chemical analyses

Amounts of di- and polymeric triglycerides, polar compounds obtained with solvent 2, and TBA-RS of all samples tested are listed in Table 3.1. Of the twenty used deep frying fat samples chosen for mutagenicity testing thirteen showed concentrations of DPTG exceeding 10% (by weight) which is the threshold level above which fats are rejected for use. Amounts of polar compounds ranged from 3 to 44% (by weight) for used frying fats. Mean recovery after fractionating the samples into two fractions according to Billek et al. (1978) was $92 \pm 1\%$ (SEM). After eluting the remaining very polar compounds applying solvent 3 mean recovery increased to $102 \pm 2\%$ (SEM). Polar material obtained with solvent 2 and levels of DPTG were positively correlated ($r=0.86$, $p<0.01$). Concentrations of TBA-RS are just above the detection level, 1.0 nmol/g, in most frying fats with the exception of five fat samples that contained high levels ranging from 82 to 177 nmol MDA per gram of fat (Table 3.1). The presence of TBA-RS in frying fat samples was not associated with amounts of DPTG or polar compounds.

Mutagenicity testing

Dose-response curves describing mutagenic activity were determined by testing non-polar and polar fractions of six used frying fat samples at doses

Table 3.1. Amount of di- and polymeric triglycerides (DPTG), polar compounds and TBA-reactive substances (TBA-RS) of used deep frying fats

Sample no.	DPTG (% by weight)	Polar compounds (% by weight)	TBA-RS nmoles MDA/g of fat
1	32	44	157
2	10	25	10
13	28	41	6
14	12	32	6
17	<1	3	161
18	31	40	82
19	7	24	4
21	10	27	10
22	13	33	4
25	8	14	<1
30	12	34	177
35	11	34	18
43	11	18	103
44	32	38	ND *
45	8	26	44
49	22	35	8
50	2	7	4
55	25	39	6
56	22	35	6
58	3	11	8
<i>Unused frying fat</i>			
A	ND	2	4
B	ND	6	14
20 hours of frying (B)	ND	28	ND
40 hours of frying (B)	ND	40	14

* ND, not determined

Figure 3.1. Mutagenic activity of polar fractions of used deep frying fat samples 1 (●), 17 (○), 18 (■), 21 (□), 50 (▲) and 55 (△) in (A) strain TA97-S9; (B) strain TA97+S9; (C) strain TA104-S9; (D) strain TA104+S9. Samples 1, 55 and 18 were dissolved in DMSO, samples 17, 21 and 50 in THF. For DMSO as solvent mean numbers of spontaneous revertants of 134, 197, 504 and 476, and for THF of 152, 223, 476, and 551 for strain TA97-S9, TA97+S9, TA104-S9 and TA104+S9 respectively have been subtracted.* significantly different from control value, $p < 0.01$; ** significantly different from control value, $p < 0.001$.

ranging from 0.1 to 10 mg per plate. Mutagenic activity in strains TA97 and TA104 was found mainly in the second (polar) fraction at doses higher than 1 mg/plate (Figures 3.1 and 3.2). None of the very polar fractions that were eluted from the column using solvent 3 showed mutagenic activity (results not shown). In the absence of S9 mix polar fractions of three fat samples showed toxic effects at doses higher than 0.1 mg/plate (Figure 3.1.A). Addition of S9 mix to the polar fractions of two toxic samples (18 and 50) resulted in reduced toxicity and an increase in the number of revertants. In the presence of S9 mix however, mutagenic activity of the polar fractions of samples 55 and 17 showed a tendency to decrease in strain TA97. In strain TA104 addition of S9 mix to polar fractions increased the number of revertants in all but one sample (Figure 3.1.C, D). Two fat samples with mutagenic polar fractions (samples 1 and 17) showed also mutagenic activity in their non-polar fractions (Figure 3.2).

Since mutagenicity was found predominantly in fractions eluted with solvents 1 and 2 at doses ranging from 1 to 10 mg/plate, subsequent testing was performed with these only, applying doses of 5 and 10 mg/plate. Table 3.2 shows mutagenicity results of polar fractions obtained with sixteen used and two unused frying fat samples. From this table it is apparent that all samples showed mutagenic activity in strain TA97 without S9 mix. The addition of a metabolic system enhanced mutagenicity in some fractions but produced inhibiting effects in others. Table 3.3 shows mutagenicity results of non-polar fractions. Non-polar fractions of eight used frying fat samples exhibited mutagenic activity in strain TA97; the numbers of revertants induced by positive samples were far below those of concomitant polar fractions. Addition of S9 mix generally reduced mutagenicity of the non-polar fractions. Chromatography blanks showed no mutagenic activity, but polar fractions isolated from the unused frying fats induced increased numbers of revertants in all three tester strains without S9 mix. In strain TA97 the number of revertants induced by the polar fraction of unused fat B reached statistical significance at a dose of 5 mg/plate (Table 3.2). Non-polar fractions of unused frying fats showed no mutagenic activity (Table 3.3). The number of significantly mutagenic fat samples detected was higher in strain TA97 than in strains TA100 or TA104, thus indicating that strain TA97 was most sensitive.

Mutagenic activity in relation to oxidative indices

Mutagenic activity of polar fractions of twenty samples to strain TA97 was calculated as number of revertants per mg in order to obtain a mutagenicity index. This was achieved by linear regression of dose-response curves, excluding toxic fractions. Calculated coefficients of correlation are shown in Table 3.4. Mutagenic activity of polar fractions was not significantly associated with levels of DPTG or polar components. Significant positive correlations were observed between mutagenicity and levels of TBA-RS for

strain TA97. No significant associations were found between chemical indices and mutagenicity in strain TA100.

Frying experiment

The batch of frying fat (brand B) used for deep fat frying under controlled conditions was found to contain 6% (by weight) polar compounds. During frying this level increased to 28% and 40% after 20 and 40 hours of frying respectively (Table 3.1). No significantly increased formation of TBA-RS was observed after 40 hours of frying. Figure 3.3 shows mutagenicity results of the polar and non-polar fractions of fat samples taken during the frying experiment. Values are expressed as number of revertants per mg, and were obtained by linear regression. Mutagenic activity of the polar fraction obtained with solvent 2 was increased after 20 hours of frying, but declined after 40 hours of frying when compared to 20 hours. The non-polar fraction contained mutagenic substances after 40 hours of frying. Mutagenicity of the polar fraction was reduced in the presence of S9 mix.

Figure 3.2. Mutagenic activity of non-polar fractions of used deep frying fat samples 1 (●), 17 (○), 18 (■), 21 (□), 50 (▲) and 55 (△) in (A) strain TA97-S9; (B) strain TA97+S9; (C) strain TA104-S9; (D) strain TA104+S9. For further information see legend of Figure 3.1.

Table 3.2. Mutagenicity of polar fractions obtained from commercially used deep-frying fats

Sample no.	Dose (mg/plate)	Number of his ⁺ revertants in strain:			
		TA97 -S9	TA97 +S9	TA100 -S9	TA100 +S9
1.	5	341 <i>b</i>	250 <i>b</i>	21	0
	10	248 <i>b</i>	322 <i>b</i>	9	11
2.	5	102	33	(0)	NT *
	10	30 <i>a</i>	133	(0)	NT
13.	5	85	2	18	67
	10	248 <i>b</i>	132	154 <i>b</i>	82 <i>a</i>
14.	5	149 <i>a</i>	162 <i>a</i>	70	32
	10	92 <i>a</i>	208 <i>a</i>	152 <i>a</i>	58
19.	5	80	95	249 <i>b</i>	149 <i>b</i>
	10	335 <i>b</i>	346 <i>b</i>	236 <i>b</i>	215 <i>b</i>
22.	5	508 <i>b</i>	517 <i>b</i>	349 <i>b</i>	122 <i>a</i>
	10	480 <i>b</i>	284 <i>b</i>	370 <i>b</i>	136 <i>a</i>
25.	5	184 <i>a</i>	80	163 <i>b</i>	21
	10	250 <i>b</i>	132	134 <i>a</i>	0
30.	5	587 <i>b</i>	447 <i>b</i>	317 <i>b</i>	200 <i>b</i>
	10	411 <i>b</i>	1041 <i>b</i>	475 <i>b</i>	500 <i>b</i>
35.	5	156 <i>a</i>	204 <i>a</i>	83 <i>a</i>	139 <i>a</i>
	10	754 <i>b</i>	490 <i>b</i>	23	112 <i>a</i>
43.	5	346 <i>b</i>	182 <i>a</i>	146 <i>a</i>	33
	10	380 <i>b</i>	299 <i>b</i>	173 <i>b</i>	303 <i>b</i>
44.	5	176 <i>a</i>	170 <i>a</i>	81 <i>a</i>	185 <i>b</i>
	10	239 <i>b</i>	223 <i>a</i>	239 <i>b</i>	103 <i>a</i>

Table 3.2. - continued

Sample no.	Dose (mg/plate)	Number of his ⁺ revertants in strain:			
		TA97-S9	TA97+S9	TA100-S9	TA100+S9
45	5	95	192 ^a	115 ^a	34
	10	245 ^b	310 ^b	207 ^b	61
49.	5	327 ^b	220 ^a	99 ^a	39
	10	445 ^b	416 ^b	141 ^b	94 ^a
55.	5	257 ^b	142 ^a	92 ^a	3
	10	473 ^b	185 ^a	245 ^b	43
56.	5	276 ^b	98	98 ^a	35
	10	720 ^b	280 ^b	94 ^a	21
58.	5	193 ^a	60	68	0
	10	(68)	79	159 ^b	22
<i>Unused fat :</i>					
A	0.1	0	0	NT	NT
	1	108	0	NT	NT
B	5	232 ^b	222 ^a	NT	93 ^a

The number of spontaneous revertants (solvent controls) of 152, 223, 93 and 109 for TA97-S9, TA97+S9, TA100-S9 and TA100+S9 respectively has been subtracted. Values are given in parentheses when toxic effects are observed. All fractions were dissolved in THF with the exception of sample 55. * NT, Not tested ; ^a significantly different from control values, $p < 0.01$; ^b significantly different from control values, $p < 0.001$.

Table 3.3. Mutagenicity of non-polar fractions obtained from commercially used deep-frying fats

Sample no.	Dose (mg/plate)	Number of his ⁺ revertants in strain:			
		TA97 -S9	TA97 +S9	TA100 -S9	TA100 +S9
2.	5	63	12	6	0
	10	(0)	62	16	8
13.	5	101	53	22	7
	10	135 ^a	115	12	107 ^a
14.	5	54	0	31	8
	10	68	77	16	40
19.	5	24	25	13	35
	10	38	77	28	60
22.	5	172 ^a	36	11	0
	10	104	40	54	20
25.	5	104	67	38	1
	10	(49)	(0)	37	23
30.	5	117	29	37	39
	10	205 ^a	22	34	42
35.	5	56	0	0	7
	10	134 ^a	47	12	0
43.	5	149 ^a	166 ^a	21	77 ^a
	10	120	86	99 ^a	140 ^a
44.	5	316 ^b	211 ^a	25	19
	10	133 ^a	76	5	6

Table 3.3. - continued

Sample no.	Dose (mg/plate)	Number of his ⁺ revertants in strain:			
		TA97 -S9	TA97 +S9	TA100 -S9	TA100 +S9
45.	5	100	50	37	0
	10	68	79	14	14
49.	5	191 ^a	83	20	17
	10	287 ^b	149 ^a	105 ^a	24
56.	5	211 ^a	110	52	78 ^a
	10	149 ^a	296 ^b	121 ^a	20
58.	5	53	46	14	13
	10	42	45	12	21
<i>Unused fat</i>					
A	5	4	0	NT	NT
	10	0	0	NT	NT
B	10	55	28	7	0

The number of spontaneous revertants per plate have been subtracted. All fractions were dissolved in THF. For numbers of spontaneous revertants and an explanation of the used symbols, see legend of Table 3.2.

Discussion

This paper presents the first evidence that mutagens are present in repeatedly used deep frying fats. Mutagenic activity of used deep frying fats appears to be detected most sensitively by strain TA97. This new tester strain, that is reverted by frameshift mutations at the G.C. base pairs, has previously been found to be very suitable for the detection of mutagenic pyrolyzates (Kuroda et al., 1985). Application of this strain may account for the discrepancy with other reports on mutagenicity of used deep frying fat fractions (Scheutwinkel-Reich et al., 1981; Taylor et al., 1982b; Van Gastel et al., 1984). Mutagenic activity of the frying fat samples cannot be explained

by the presence of trace amounts of histidine in the frying fat. Increased reversion frequencies found for several samples in strain TA97 were not observed for the same samples using strain TA100. Also addition of S9 mix to non-toxic fractions often reduced the number of revertants which would not be observed if traces of histidine were present in the frying fat.

Deep frying fat fractions are weakly mutagenic, since significant mutagenic activity is found at high doses ranging from 1 to 10 mg. However, when numbers of revertants are calculated per gram of fat, mutagenic activity is ranging from $1 \cdot 10^3$ to $3 \cdot 10^4$ revertants per gram, approximating values that have been reported for fried beef fractions (Springarn et al., 1981; Taylor et al., 1982; 1983; Nilsson et al., 1986). In polar material isolated from unused frying fats mutagenicity is also detected, suggesting that during manufacturing or storage prior to use mutagenic substances are formed. Unused frying fats do not contain more than 2 to 6% polar compounds which implies that mutagenic activity per gram of unused frying fats is not exceeding 10^3 revertants.

Results from our own frying experiment showed decreased mutagenicity in the polar fraction after 40 hours of frying as compared to 20 hours. This effect might be explained by the fact that mutagenic products formed during earlier stages of frying may be diluted or deactivated by other polar compounds. Also degradation of mutagenic substances during prolonged heating may have caused the observed decline in mutagenicity. Based on sensoric evaluation of the fried foods after 40 hours of use, the fat would have been found not suitable for consumption and discarded or replenished. Mutagenic activity of repeatedly used frying fat is likely to be caused by various substances, since addition of S9 mix to different samples resulted in both increase and inhibition of mutagenicity. It has previously been shown that fat enhances mutagen formation during frying of meat (Springarn et al., 1981; Nilsson et al., 1986). This enhancing effect is considered to be the result of a more efficient heat transfer, but a more specific role of fat in mutagen formation cannot be excluded.

The positive correlation observed between mutagenicity in strain TA97 and TBA-RS may indicate the involvement of lipid oxidation products in mutagen formation during deep fat frying. Various oxidation products of fatty acids, including malondialdehyde, alkenals, carbonyls and hydroperoxides have already been found to be mutagenic (Mukai and Goldstein, 1976; Yamaguchi and Yamashita, 1979; 1980; Marnett et al., 1984; MacGregor et al., 1985). However, levels of TBA-RS in frying fats as shown in this study are generally low, which previously has been reported by Shamberger et al. (1977). Furthermore, since the TBA assay is non-specific, additional analysis elucidating the nature of mutagens formed during deep fat frying is necessary. Mutagenic heterocyclic amines may also be present in small

Figure 3.3. Mutagen formation during deep fat frying, (A) Mutagenic activity in strain TA97-S9; (B) Mutagenic activity in strain TA97+S9.

Table 3.4. Correlations between mutagenicity of polar fractions and levels of di- and polymeric triglycerides (DPTG), polar compounds (PC) and thiobarbituric acid-reacting substances (TBA-RS) of used frying fat samples.

Parameter	Coefficient of correlation with mutagenicity in strain:			
	TA97-S9	TA97+S9	TA100-S9	TA100+S9
DPTG	-0.27 (n=17)	-0.35 (n=19)	-0.44 (n=15)	-0.34 (n=15)
PC	-0.43 (n=17)	-0.36 (n=19)	-0.23 (n=15)	-0.26 (n=15)
TBA-RS	0.66 ^a (n=16)	0.58 ^a (n=18)	-0.02 (n=14)	0.25 (n=14)

In parentheses the number of samples is given, toxic fractions have been excluded; ^a significant correlation, $p < 0.01$.

quantities in the frying fat. Formation of these heterocyclic amines occurs during heating of mixtures of sugars, amino acids and creatinine at moderately high temperatures (Sugimura et al., 1986). Heterocyclic amines and other pyrolyzates may be formed in the foods during deep fat frying and small amounts possibly migrate into the frying fat.

During deep fat frying the fat is absorbed by the fried food; a portion of French fried potatoes can absorb 5-10% (by weight) of frying fat (Stevenson et al., 1984). Thus, consumption of one portion of French fried potatoes prepared in mutagenic frying fat may imply the intake of several grams of mutagenic polar compounds. Although repeatedly used deep frying fats are not highly mutagenic, chronic or large scale consumption may be of concern regarding possible health effects. Finally, mutagenicity of used deep frying fat samples appears not to be related to chemical indices currently used for quality assessment, emphasizing the need for a re-evaluation of applied chemical analyses with respect to biological effects.

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Chapter 4

Linoleic acid hydroperoxide concentration in relation to mutagenicity of repeatedly used deep-frying fats

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Abstract

A high-performance liquid chromatography procedure was developed to measure linoleic acid hydroperoxides (LAHPO) in 19 used deep-frying fat samples that were screened for mutagenicity. The detection limit of the method was found to be 10 nmol LAHPO/g of fat. In 8 samples, LAHPO were not detected, levels in the other samples ranged from 17 to 267 nmol/g of fat. LAHPO were not detectable in unused hydrogenated frying fat samples.

Concentrations of LAHPO correlated positively with mutagenicity to *Salmonella* tester strains TA97 and TA100, in presence of S9 mix only; coefficients of correlation were respectively $r=0.48$ ($p<0.05$) and $r=0.24$ (n.s.). Without metabolic activation no significant associations were observed.

These results suggest that metabolites or secondary autoxidation products of linoleic acid generated in presence of liver S9 mix may contribute to mutagenicity of some of the used deep-frying fat samples.

Introduction

Thermal and oxidative deterioration of fats and oils during deep-fat frying is of major concern with regard to possible adverse health effects, as well as the quality of foods prepared. When fed to laboratory animals, heated oils and fats have been reported to cause growth retardation, increase relative liver and kidney weights, induce detoxifying enzyme activities and lipid peroxidation in the liver, and produce cellular damage to liver, kidneys and epididymides (1, 2, 3, 4). Heating of fats and oils causes degradation of triglycerides, which may involve hydrolysis into free fatty acids as well as free radical mediated oxidation reactions of polyunsaturated fatty acids. Ultimately, di- and polymeric triglycerides as well as low molecular weight decomposition products are formed, both volatile and non-volatile (5). Isolated lipid oxidation products, autoxidation products of methyl linoleate and methyl linolenate, hydroperoxides of methyl linoleate, malondialdehyde and various other aldehydes and carbonyls have been found mutagenic in the Salmonella/microsome assay (6, 7, 8, 9, 10).

Recently, we have also observed mutagenic activity in repeatedly used deep-frying fats, sampled from local snack-bars and restaurants (11). In our study, highest mutagenic activity has been found in the polar fraction of these fats, separated by means of silicagel column chromatography, which indicates that polar oxidation and degradation products are involved. The contribution of lipid oxidation products to mutagenicity of these used fats has also been implicated by the positive correlation between mutagenicity of the polar fraction to strains TA97 and TA100 and levels of thiobarbituric acid-reactive substances (TBA-RS) of the fats (11). The thiobarbituric acid assay, however, is of limited value since it not only detects malondialdehyde but also other saturated aldehydes (12), as well as derivatives of amino acids, carbohydrates and nucleic acids that have been damaged by free radicals (13).

Since linoleic acid hydroperoxides (LAHPO) have been reported to exert mutagenic activity in the Salmonella/microsome assay using tester strains TA97 and TA100 (6), the objective of the present study was to evaluate further the contribution of lipid oxidation products to mutagenicity of used deep-frying fats by quantifying the concentrations of LAHPO and relating these to mutagenicity of the fats. Therefore, we developed a high-performance liquid chromatography (HPLC) assay measuring the primary oxidation products LAHPO, which has been modified from the method described by Teng and Smith (14)

Experimental

Reagents

Linoleic acid and soybean lipoxygenase, type V (EC 1.13.11.12), were obtained from Sigma, St. Louis (MO, USA). Sodium borate and analytical grade glacial acetic acid were purchased from Merck, Darmstadt, FRG. Chromatography solvents, all of the highest purity available were obtained from Fisons, Loughborough, England.

Preparation of LAHPO

Hydroperoxides of linoleic acid were enzymatically prepared using linoleic acid as substrate, and purified by thin layer chromatography as described by Teng and Smith (14). Hydroperoxides were visualized using N,N-dimethyl-p-phenylene-diamine spray and recovered from simultaneously developed chromatoplates by scraping off the correspondent area's. LAHPO were extracted from the silicagel using diethyl ether. After evaporating the diethyl ether under a stream of nitrogen, LAHPO were dissolved in hexane and the concentration was determined spectrophotometrically at 234 nm using a molar extinction coefficient of $2.7 \cdot 10^4$ l/mol.cm (15).

Determination of LAHPO in deep-frying fat samples

Twenty deep-frying fat samples, that had been screened for mutagenic activity, were stored under refrigeration until the moment of analysis. Unused control samples were stored and processed under the same conditions. One sample was withdrawn from the experiment because the amount of material left after mutagenicity testing was not sufficient for further analyses. Deep-frying fat samples of 50 mg were dissolved in 0.5 ml hexane, after which the mixture was extracted 3 times with 0.5 ml acetonitrile/water (1:1, v/v). The combined aqueous extracts were subjected to HPLC analysis. Aliquots of 20 μ l were separated on a Chromsep LiChrosorb RP18 column, 200x3.0 ID with guard column (Chrompack, Middelburg, The Netherlands) applying a quaternary eluent composed of tetrahydrofuran/acetonitrile-/water/acetic acid (45:100:100:1, v/v/v/v) at a flow rate of 0.2 ml/min. Peaks were detected by an UV-detector (Spectroflow 783 UV, Kratos Analytical) set at 234 nm. Positive identification of LAHPO was based on retention time and spiking of fat samples with a known amount of purified LAHPO. A calibration curve was obtained using enzymatically prepared and purified LAHPO, which were added to 50 mg of unused deep-frying fat that contained no detectable LAHPO.

Mutagenicity testing of deep-frying fat samples

Sixty deep-frying fat samples were obtained from local snack-bars and restaurants. Twenty were selected based on presence of di- and polymeric triglycerides, 10 samples had concentrations exceeding 10% (by weight). Samples were separated into two major fractions by means of silicagel column chromatography (11); a non-polar petroleum ether fraction, which has been reported to consist of non-polar degradation products of fatty acids and unaltered triglycerides, and a polar acidified diethyl ether fraction, which has been found to contain polar oxidation and degradation products (16). Non-polar and polar fractions were screened for mutagenicity using *Salmonella typhimurium* tester strains TA97 (20 samples) and TA100 (14 samples) in absence and presence of liver S9 mix obtained from Aroclor

induced rats. Samples were dissolved in tetrahydrofuran and tested at doses ranging from 0.1 to 10 mg/plate using a liquid preincubation of 20 min at 37 °C (17). Unused fat samples as well as solvent controls were also included. A more detailed description of the protocols used for mutagenicity testing has been given elsewhere (11). The mutagenic activity of the non-polar and polar fractions was estimated by extrapolation of the linear, non-toxic portion of dose-response curves applying linear regression. Mutagenicity per g of frying fat was calculated by summation of mutagenic activity of non-polar and polar fractions.

Results and discussion

Chromatograms of enzymatically obtained and purified LAHPO and of LAHPO extracted from a deep-frying fat sample are given in Figure 4.1. Isomers of LAHPO, *cis-trans* or *trans-trans* stereo isomers of 9-hydroperoxy-10,12-octadecadienoate and 13-hydroperoxy-10,12-octadecadieno-ate, elute in three peaks, of which the exact conformation is not known. Confirmation of the hydroperoxide identity has been obtained by analysis of aliquots of the mixture of linoleic acid and lipoxygenase after 0, 10, 20 and 30 minutes of incubation; heights of the three peaks have been found to increase as the reaction proceeded. The total amount of LAHPO is calculated from the chromatograms by summation of the three peak heights.

Figure 4.2 shows the calibration curve of LAHPO in deep-frying fat. Detection limit of the method is 10 nmol/g of fat. Purified LAHPO dissolved in hexane are detectable at concentrations of 0.1 nmol/ml. Recovery of added LAHPO is determined for 4 concentrations of the calibration curve. Mean recovery ($\pm$ SD) is $94\pm 6\%$. Reproducibility of the method, as determined by triplicate extraction and analysis of 39.5 nmol LAHPO added to 1 g of deep-frying fat, yields an intra-assay coefficient of variation of 3%, and an inter-assay coefficient of variation of 6%.

Levels of LAHPO measured in repeatedly used deep-frying fat samples range from below the detection level to 267 nmol/g (Table 4.1). LAHPO are below the detection level in two different brands of unused hydrogenated deep-frying fat (data not given). Seventeen out of 19 repeatedly used deep-frying fat samples tested are mutagenic; mean mutagenicity in strain TA97 without S9 mix is 18128 revertants/g (range: 943 - 86065 revertants/g). In presence of S9 mix 12 samples show mutagenic activity; mean mutagenicity is 16415 revertants/g (range: 5875 - 35671 revertants/g). Twelve out of 14 samples tested with strain TA100 show mutagenic activity without S9 mix; mean mutagenicity is 8319 revertants/g (range: 1760 - 12210 revertants/g). In presence of S9 mix 7 samples are mutagenic; mean mutagenicity is 10558 revertants/g (range: 3740 - 17010 revertants/g).

Figure 4.1. High-performance liquid chromatograms of hydroperoxides of linoleic acid (peaks 1, 2 and 3); (A) Enzymatically prepared and purified hydroperoxides of linoleic acid; (B) Hydroperoxides of linoleic acid extracted from a repeatedly used deep-frying fat sample.

Figure 4.2. Calibration curve of enzymatically prepared and purified LAHPO added to an unused deep-frying fat sample and extracted with acetonitrile:water (1:1 v/v). The detection level is 10 nmol/g of fat. Results are expressed as mean $\pm$ SD.

Table 4.1. Linoleic acid hydroperoxide (LAHPO) concentrations of 19 used deep-frying fat samples (mean $\pm$ SD).

Sample	LAHPO (nmoles/g)
1	65 $\pm$ 1
2	78 $\pm$ 4
13	< 10
14	200 $\pm$ 5
17	< 10
18	170 $\pm$ 12
19	< 10
21	20 $\pm$ 8
22	17 $\pm$ 1
25	< 10
30	257 $\pm$ 10
35	160 $\pm$ 16
43	107 $\pm$ 3
45	267 $\pm$ 2
49	49 $\pm$ 1
50	< 10
55	< 10
56	< 10
58	< 10

Since LAHPO have been found to be present in non-polar as well as in polar fat fractions separated by silicagel column chromatography, mutagenic activity calculated per g of frying fat has been compared with levels of LAHPO analyzed in unfractionated fat samples. LAHPO concentrations of repeatedly used deep-frying fat samples correlate positively with mutagenicity to *Salmonella typhimurium* tester strain TA97 in presence of S9 mix ($r=0.48$, $p<0.05$, $n=19$). In absence of S9 mix, no statistically significant association is observed ($r=0.02$). Correlations between LAHPO levels and mutagenicity to strain TA100 show the same tendency: without S9 mix mutagenicity of the fats to strain TA100 is not associated with LAHPO

levels ($r=0.07$, $n=14$), while in presence of S9 mix a weak, but statistically insignificant positive correlation has been found ($r=0.24$). In addition, LAHPO concentrations and mutagenic activity to both strains TA97 and TA100, added together reflecting the overall mutagenic potency to induce frameshift as well as base pair mutations, revealed a similar relationship. In absence of S9 mix total mutagenic potency and LAHPO concentrations showed no correlation ($r=0.05$, $n=14$), whereas in presence of S9 mix a positive, but statistically insignificant correlation was found ($r=0.48$, $n=14$).

Fatty acid hydroperoxides are the first isolatable oxidation products of polyunsaturated fatty acids, which now are reported to be present in used deep-frying fat samples. Although most brands of deep-frying fats consist of hydrogenated oils and thus contain low amounts of polyunsaturated fatty acids, during deep-fat frying of various foods polyunsaturated fatty acids migrate from the food into the fat, thereby increasing the P/S ratio (18). During heating of the frying fat, hydroperoxides of linoleic acid and other polyunsaturated fatty acids may be formed and remain in the frying fat in detectable amounts after cooling down. This has actually been demonstrated in a separate experiment in our laboratory, in which increased levels of LAHPO have been observed after repeated use in a frying oil containing 60% linoleic acid. LAHPO are not detected in unused frying oil of this particular type, while after 10, 20 and 30 h of frying LAHPO have been found to be present in a concentration of 17, 55 and 62 nmol/g, respectively. In this experiment it was also found that LAHPO in used deep-frying fats are stable for at least 2 weeks while stored under refrigeration. Stored fat samples analyzed within 3 days after frying and again after 2 weeks showed no systematically increased or decreased concentrations of LAHPO.

Whereas in general concentrations of TBA-RS (19) or the conjugated diene-fraction (20) are determined as an indication of lipid oxidation of fats or foods, identification of lipid oxidation products in relation to mutagenic activity requires more specific assays. In addition to determination of LAHPO, assays detecting unsaturated aldehydes and carbonyls appear to be of greater relevance (7). These oxidation products have been reported to possess mutagenic activity in *Salmonella* tester strain TA104 (7), and have also been detected in lipid oxidation studies *in vitro* using rat liver microsome preparations (21).

The positive correlation observed in the present study between LAHPO levels and mutagenic activity with S9 mix is in accordance with mutagenicity data obtained by other authors (6, 9), who report mutagenic activity of hydroperoxides of methyl linoleate and oxidized linoleic acid solutions with high peroxide values in presence of S9 mix. The positive correlation found between mutagenicity of repeatedly used deep-frying fat samples in presence of S9 mix and LAHPO concentrations may be explained by three

different mechanistic routes. Firstly, autoxidation of LAHPO and concomitant oxidation of other polyunsaturated fatty acids, is very likely to occur during incubation in the *Salmonella* mutagenicity assay due to the presence of trace amounts of iron or other transition metals (22), especially with S9 mix. It is not clear however, to what extent autoxidation may take place in S9 mix, since S9 homogenate also possesses free radical scavenging activities. Secondly, mutagenic metabolites of LAHPO may be derived as a result of degradation by cytochrome P₄₅₀ activities of LAHPO. Thirdly, oxidation of polyunsaturated fatty acids, present in fat samples or in microsomal membranes, by cyclooxygenase or lipoxygenase resulting in mutagenic intermediates, may be enhanced in presence of LAHPO, which specifically has been demonstrated by Marshall et al. (23) for the oxidation of arachidonic acid by cyclooxygenase in plasma.

Although used deep-frying fats have been observed to exert potent direct-acting mutagenic activity, in some of these samples mutagenicity in presence of S9 mix appears to be induced by metabolites or secondary autoxidation products of LAHPO as well. Future studies therefore, will be concentrated on isolation and identification of direct-acting mutagenic compounds as well as possible mutagenic metabolites or secondary oxidation products of LAHPO in used deep-frying fats.

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Chapter 5

Comparison of mutagenicity of repeatedly used deep-frying fats in the *Salmonella*/microsome assay with genotoxicity to human lymphocytes *in vitro*

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Abstract

Production of mutagenic compounds in repeatedly used deep-frying fats was evaluated for different types of frying fat and frying of various food products. In two polyunsaturated fatty acid (PUFA)-rich oils out of the three different fats tested in a first series of experiments and used for preparation of freshly sliced potatoes, extensive oxidative deterioration and foaming occurred after 30 h of use. These oils did not show significant direct-acting mutagenicity to strain TA97; one heated PUFA-rich oil increased numbers of revertants to strain TA97 in presence of S9 mix. In a second series of experiments, four different brands of frying oil, specifically developed for deep-fat frying, were used for preparation of various foods, including meat and starchy products. After 30 h of use, increased mutagenicity to strain TA97 without metabolic activation was observed for all fats, which appeared not to be related to the amount of various polar oxidation products formed.

Three heated fats which showed highest mutagenicity in the *Salmonella*/microsome assay, were selected for further genotoxicity testing, which involved analysis sister chromatid exchange (SCE) frequencies and mutations at the hypoxanthine guanine phosphoribosyl transferase (*hprt*) locus in human peripheral lymphocytes, in order to assess genotoxic risk of exposure to repeatedly used deep-frying fats. A dose-dependent, significant increase in SCE frequencies was observed after exposure of lymphocytes to the PUFA-rich frying oil, used for preparation of various foods. Other fats did not show dose-dependent increases in SCE frequencies. Significantly increased mutations at the *hprt* locus were observed after exposure of human lymphocytes to frying oil used for preparation of potatoes, but upon retesting this could not be confirmed, which might have been due to degradation of instable compounds during storage of the fat. Other heated fats did not significantly increase mutations at the HGPRT locus of lymphocytes. Two heated fats were observed to enhance cell proliferation of PHA-stimulated T-lymphocytes. No associations were observed between stimulation of cell proliferation, mutagenicity in the *Salmonella*/microsome assay, genotoxicity to human lymphocytes, and oxidative indices of repeatedly used deep-frying fats as determined by concentrations of di- and polymeric triglycerides, linoleic acid hydroperoxides and thiobarbituric-acid reactive substances.

The diversity of effects observed in the present study indicates that genotoxicity as well as other biological effects of repeatedly used deep-frying fats may be caused by various compounds not known at present, and may also be modulated by other factors, such as the presence of natural antioxidants. More information on these subjects is needed before a reliable estimation can be made of health risks posed by consumption of deep-fat fried foods.

Introduction

In a previous study performed at our laboratory, several repeatedly used deep-frying fats, sampled at local snack-bars and restaurants after sensory indication of abuse, were found to contain compounds that were mutagenic to *Salmonella typhimurium* tester strains TA97, TA100 or TA104 (Hageman et al., 1988). Mutagenicity was observed mainly in the polar fraction (PF) of the fat, isolated by silicagel column chromatography, which has been reported to consist mainly of polar oxidation and degradation products of lipids (Billek et al., 1978). In the same study, frying of various foods under controlled conditions, applying a temperature of 180 °C with repeated use of the fat and overnight cooling, was also demonstrated to result in increased mutagenicity of the polar fraction of the fat. Furthermore, mutagenic activity of repeatedly used deep-frying fats was found to be correlated with levels of thiobarbituric acid-reactive substances (TBA-RS), as well as with concentrations of linoleic acid hydroperoxides (LAHPO) of the fats, which suggests that lipid peroxidation products might be involved in mutagen formation during deep-fat frying (Hageman et al., 1988; 1989a).

Oxidative and thermal deterioration of fats during heating has been reported to be influenced by numerous factors, including source and fatty acid composition of the fat, presence of antioxidants, both naturally present and added during processing, contamination with trace amounts of transition metals, presence of trace amounts of oxidized lipids (peroxides), heating temperatures applied, nature and amounts of foods prepared, frying procedures followed, i.e. continuously heating versus intermittent heating, etc. (Stevenson et al., 1984; Chang et al., 1978; Pokorny et al., 1975). These factors may also be involved in mutagen formation, but only a few have been studied in this sense. Taylor et al. (1983) have shown that thermal abuse of frying oil, by heating at temperatures exceeding 200 °C or by over-cooking of foods, resulted in increased mutagenicity of the foods. The same authors have also reported that the nature of foods fried may influence mutagenicity of the fat and of deep-fat fried foods, since significant mutagenicity has been observed after frying of fish fillets with prolonged use of the oil, but not after frying of potatoes or onion rings.

Since we demonstrated significant mutagenic activity in lipid fractions isolated from commercially used deep-frying fat samples, as well as in commercially fried potatoes (Hageman et al., 1988; 1990), the first aim of the present study was to evaluate mutagen formation during deep-fat frying in relation to various deep-frying conditions. Factors of primary interest were nature of the frying fat, especially polyunsaturated fatty acid (PUFA)-content, and the nature of foods prepared. In a first series of experiments, effect of PUFA-content was investigated by measuring mutagen formation after frying of freshly sliced potatoes using standardized frying procedures, in 3 brands

of commercially obtained fats with varying levels of PUFA. Mutagenicity of the fat was assessed in the Salmonella/microsome assay using strain TA97, which was previously found to be most sensitive in detecting mutagenic activity of used deep-frying fats (Hageman et al., 1988). In a second series, mutagen formation during frying of various foods, including meat products, was determined. Four commercial brands of fat which are specifically developed for deep-fat frying, were evaluated.

Mutagenicity of compounds, as assessed by the Salmonella/microsome assay, is considered an indication of possible carcinogenic risk. However, genotoxicity to human DNA, may be a more valuable tool in assessing carcinogenic risk to man. Therefore, the second aim of this study was to assess genotoxicity of used deep-frying fats in short-term assays, using human peripheral T-lymphocytes. For this purpose, those fats were selected for further analysis, which showed increased mutagenic activity in the Salmonella/microsome assay after repeated use. Genotoxicity in human lymphocytes after *in vitro* exposure was determined measuring sister chromatid exchange (SCE) frequencies and single gene mutations at the hypoxanthine guanine phosphoribosyltransferase (*hprt*) locus, using a modified procedure developed at our laboratory (Hageman et al., 1989b).

Lipid oxidation products of linoleic and arachidonic acid were reported to exert genotoxic effects in various short-term assays using mammalian cells (Brambilla et al., 1986; Nakayama et al., 1986; Cajelli et al., 1987; Vaca et al., 1988; Weitberg et al., 1989). Furthermore, we previously found that mutagenicity of used deep-frying fats in the Salmonella/microsome assay was associated with presence of lipid oxidation products (Hageman et al., 1988; 1989a). Therefore, concentrations of di- and polymeric triglycerides (DPTG), TBA-RS and LAHPO were determined in the fats selected for further genotoxicity testing with human lymphocytes, in order to evaluate the possible involvement of these lipid oxidation products.

Materials and methods

Frying procedures

Mutagen formation during frying was followed in two different experiments, using 6 different brands of frying fat, which were obtained from local supermarkets. Batches of 3 to 10 kg were purchased and stored at 4 °C until use, which was within one week. The following types of frying fat were evaluated: I. Frying oil, consisting of vegetable oils (> 60% polyunsaturated fatty acids; PUFA), anti-foaming agent E900 (dimethyl polysiloxane) added; II. partly hydrogenated vegetable oils, anti-foaming agent added; III sunflower seed oil; IV. fractionated beef tallow (stearine fraction removed); V. coconut oil; VI hydrogenated vegetable and animal fats, anti-foaming agent added (all specifications as declared by

manufacturers).

In the first series of frying experiments, fats I (batch A) to III were heated in a home-style temperature-controlled fryer at 180 °C, for 10 h per day during 3 consecutive days. Every 2 h, alternating, 250 g portions of freshly peeled potatoes, or portions of 2 h earlier pre-fried potatoes were fried for 4 min. Actual frying temperatures ranged from 160 to 190 °C. After 30 h of frying excessive foaming occurred, which did not allow prolonged use of the fats. Unused fat, as well as heated fat samples were stored at 4 °C until analysis. In a second series of experiments fats I (new batch; B), and IV to VI were heated in a similar way as the initially heated fats, but instead of frying of potatoes, various foods were prepared according to manufacturers' instructions, including 300 g of freshly sliced potatoes, 300 g of meat croquettes, 300 g of hamburgers, 300 g of sausages and 250 g of pre-fried potatoes. For comparison with results of the first experiment, heating of the fat was limited to 30 h.

Salmonella mutagenicity testing

Mutagenicity of the fat samples in the *Salmonella*/microsome assay was determined as described previously (Hageman et al., 1988). For initial screening, mutagenic activity of fats heated for 30 h was compared to the unheated control samples, using strain TA97, which was found to be most sensitive in detecting mutagenic activity of heated frying fats (Hageman et al., 1988). Briefly, fat samples were separated into non-polar (NPF) and polar fractions (PF), using silicagel column chromatography. NPF and PF were dissolved in tetrahydrofuran (THF), which was distilled before use. Mutagenicity of NPF and PF was evaluated applying doses of 0.1, 1.0, 5.0 and 10.0 mg per plate, using *Salmonella typhimurium* tester strain TA97 for initial screening. Additional screening of mutagenicity in strain TA100 was applied only to fats selected for further analysis of genotoxicity. Lipid fractions were incubated both with and without liver S9 mix (50 µl of homogenate/plate) obtained from Aroclor induced rats. Chromatography and solvent controls as well as positive controls were included as described previously (Hageman et al., 1988). Statistical analysis of mutagenicity data was performed using the method of least significant difference (LSD; MacGregor et al., 1985). Doses of NPF or PF inducing numbers of revertants that exceeded the correspondent solvent control by LSD values at a confidence level of $p < 0.05$, were considered mutagenic. LSD values obtained in this study were at a confidence level of $p < 0.05$ for THF as solvent with TA97-S9, 120; TA97+S9, 105; TA100-S9, 146; TA100+S9, 114. Mean numbers of revertants observed for THF (solvent control) were for TA97-S9, 176; TA97+S9, 209; TA100-S9, 98; TA100+S9, 104. Mutagenic activity of the NPF and PF of frying fat was assessed applying linear regression to non-toxic portions of dose response curves of NPF and PF separately. Multiplication of the mutagenic activity per mg of NPF or PF, by the amount of the respective fraction present per g of fat, yielded mutagenic activity per g of fat.

Chemical analyses

Several indices of oxidative deterioration were determined in fats selected for further genotoxicity testing, including measurement of DPTG by means of gel-permeation high performance liquid chromatography (HPLC), analysis of TBA-RS using a colorimetric assay, and HPLC analysis of LAHPO, all performed as described previously (Hageman et al., 1988;1989a).

SCE frequency analysis

Induction of SCE in human T-lymphocytes *in vitro* after exposure to used deep-frying fats was evaluated according to the procedure described previously (Kleinjans et al., 1989). Briefly, heparinized blood samples were obtained from a healthy female donor, who was not under medication including use of oral contraceptives, and who had no history of smoking. An aliquot of 0.4 ml of whole blood was added to 4.5 ml RPMI 1640 complete medium. Cell proliferation was stimulated by addition of 0.2 ml of phytohemagglutinin (PHA). After a 24 h-incubation period at 37 °C, 5-bromodeoxyuridine (BrdU) was added to the lymphocyte culture to a final concentration of 58 µM. After 48 h of incubation, suspensions of frying fats were added to the cultures. Fats were suspended in sterile culture tubes with screw caps, in RPMI 1640 medium without supplements, under sonification at 50 °C for 30 min. Dilutions of the emulsions were made immediately after sonification, and 0.5 ml volumes were added to the cultures. Fat samples were tested at doses of 0.1, 1.0 and 10.0 mg/ml. After 72 h, metaphases were arrested by addition of colcemid (final concentration 0.2 µg/ml) and subsequent incubation for 2 h. Nuclei were collected using a hypotonic KCl solution (75 mM) and fixed in methanol:acetic acid (3:1, v/v), after which slides were prepared. All test doses were evaluated in duplicate; a solvent control (RPMI treated by sonification) and a positive control (mitomycin C, $3 \cdot 10^{-8}$ M) were also included. Since the mutagenic potency in the Salmonella/microsome assay of direct-acting compounds present in repeatedly used deep-frying fat samples was observed to be higher as compared to mutagenicity in presence of liver S9 mix (Hageman et al., 1988; 1990), genotoxic activity in human lymphocytes was not evaluated in presence of rat liver S9 mix.

Slides were stained using the fluorescence-plus-Giemsa method according to Perry and Wolff (1974). Slides were coded, after which 20 metaphases per cell culture were analyzed; a total of 40 metaphases was evaluated per test dose. All metaphases were analyzed by a single person; 10% of the slides was evaluated by a different observer, which revealed no systematic errors in scoring of SCE frequencies. SCE frequencies of test doses were compared to controls, statistical significance of differences was evaluated using Students' t-test for unpaired values. Linear trend of dose-response curves was assessed applying regression analysis.

In vitro HGPRT assay with human lymphocytes

Induction of TG^f T-lymphocytes by heated fats *in vitro* (HGPRT assay) was determined using a procedure developed at our laboratory (Hageman et al., 1989b). Since a considerable inter-assay variation was found in the HGPRT assay, fats were tested in 2 separate assays within a 1-week period. Briefly, heparinized blood samples were obtained from the same donor providing the blood for the SCE assay. Suspensions of the fats were prepared as described for the SCE assay. In a pilot study initially performed (experiments 1 and 2), fat I A, both fresh and heated, was evaluated at a concentration of 1 mg/ml, which is well within the range of triglyceride levels found in human blood (Anonymous, 1981). In a second series of tests (experiments 3 and 4), heated fat I A was evaluated at a dose range of 0.01 to 1.0 mg/ml. Fats I B and V, tested in experiments 5 and 6, which were performed within 1 month after the frying experiments, were evaluated at a dose range of 0.1 to 10.0 mg/ml. Solvent control cultures, consisting of RPMI medium which was ultrasonically treated, were also included in

each assay. Cultures consisting of 0.4 ml heparinized blood, 4.5 ml RPMI medium without supplements and 0.5 ml of fat suspension were incubated at 37 °C for 4 h, which was the maximum incubation period used in the standardized protocols (Hageman et al., 1989b). After mutagen treatment, cells were washed with pre-warmed PBS of 37 °C and resuspended in 5.0 ml RPMI complete medium to which 0.2 ml PHA and 0.1 ml of a 10 mM TG solution in RPMI (TG culture), or 0.1 ml of TG control RPMI solution (control culture) were added. Per test dose, a total of 6 cultures was set up; 2 cultures for measurement of the labeling index (LI_C), and 4 cultures for the enumeration of TG^+ T-lymphocytes. Cultures were incubated at 37 °C for 24 h, after which BrdU was added to a final concentration of 10 μ M. After an additional incubation period of 16 h, cells were harvested, nuclei prepared, fixed in methanol:acetic acid (3:1, v/v), and transferred to clean microscope slides. Two drops of 10 μ l of the control cultures were pipetted onto one slide for determination of the LI_C . During fixation, 2 TG cultures were combined in order to maximize number of nuclei per slide. In a 1:50 diluted suspension the number of nuclei present in the combined culture was determined in triplicate. The complete combined TG culture was transferred onto one slide in 25 μ l portions, meanwhile determining the volume. *tert*-Butylhydroperoxide (tBOOH), representing the class of mutagenic hydroperoxides and regarded as a possible positive control, was screened for its potential to induce TG^+ T-lymphocytes *in vitro*. tBOOH was dissolved in sterile distilled demineralized water shortly before use and tested up to its toxic limit, at doses ranging from $1 \cdot 10^{-6}$ to $1 \cdot 10^{-3}$ M.

TG and control slides were stained using mouse monoclonal antibodies against BrdU in combination with peroxidase-conjugated secondary antibodies. In short, DNA was denatured using 4 N HCl, after which slides were incubated at 37 °C for 1 h with mouse monoclonal anti-BrdU antibodies (clone IIBV), which were a gift from Dr. Bert Schutte, Department of Internal Medicine, University of Limburg, Maastricht, The Netherlands. Next, BrdU-containing nuclei were labeled by means of incubation with peroxidase-conjugated rabbit anti-mouse IgG antibodies (RAMPO; F114, Dakopatts, Denmark). BrdU-containing nuclei were stained brown by immersing the slides in 0.05 M Tris/HCl buffer of pH 7.6, containing 0.04% (w/v) 3,3'-diaminobenzidine (Fluka Chemie, Switzerland) and 0.01% (v/v) H_2O_2 , protected from light at room temperature. Non-proliferating nuclei of control slides were counterstained using Giemsa stain. TG slides were not immediately counterstained, but coded first, after which all TG^+ nuclei were enumerated using normal light microscopy (400x). LI_C was determined by evaluation of at least 1000 nuclei per control culture, a total of at least 2000 nuclei was evaluated per test dose.

Variant frequency (Vf) of TG^+ lymphocytes per test dose was calculated as:

$$Vf = \frac{\text{number of BrdU-containing nuclei}}{\text{number of evaluable nuclei}},$$

the number of evaluable nuclei being derived from the total number of nuclei present on TG slides, multiplied by the LI_C . Differences between Vf's of control and test doses within one experiment were statistically evaluated using 95% binomial confidence intervals of the ratio of Vf's, as described by Sylwester and Albertini (1985).

Results

In the first series of frying experiments, use of fats I and III had to be terminated after 30 h of heating due to excessive foaming during frying of sliced potatoes. Therefore, heating time of 30 h was chosen for comparison of mutagenic activity of all used fats to the unheated control samples, as shown in Table 5.1. The amounts of polar material found after 30 h of use appear to be highest in fats I A and III, indicating a relation between oxidative deterioration and practical cut-off points for use. Frying of freshly sliced potatoes appeared to enhance oxidative deterioration of the frying oils, especially if these contained high levels of polyunsaturated fatty acids (PUFA).

Polar fractions of used fats I B, IV, V and VI exhibited significant mutagenicity to strain TA97 without S9 mix, polar fractions of unused fats II and VI were also found to be mutagenic. Furthermore, it was observed that mutagenic activity found in unused fat II was decreased after 30 h of use. Mutagenicity of used fats I B, IV, VI and of unused fats II and VI appeared to be reduced in presence of S9 mix. Fat I A, both used and unused, contained compounds that exhibited toxic effects to the bacteria. Upon testing of this oil in presence of S9 mix, mutagenicity was detected in the polar fraction of the heated oil which appeared to be highest of all samples tested. Results presented in Table 5.1, indicated that mutagenicity of repeatedly used deep-frying fats was not related to the amount of polar oxidation products present. With the exception of results of fat I A, which might be confounded by the presence of toxic compounds, frying of various foods appeared to enhance mutagen formation as compared to frying of freshly sliced potatoes, indicated by the larger proportion of samples showing increased mutagenicity after repeated use for preparation of various foods.

Since the purpose of this study was to compare mutagenicity of repeatedly used deep-frying fats in the Salmonella/microsome assay with genotoxicity in human lymphocytes, fats I B and V were selected for further analyses. These fats fulfilled the selection criteria, since unused fats I B and V were not mutagenic, and showed highest mutagenicity after repeated use, whereas mutagenic activity appeared to be reduced in presence of S9 mix, which was observed in most of the mutagenic fats studied previously (Hageman et al., 1988: 1990). Therefore, these fats were considered to be representative of the group of mutagen-containing repeatedly used deep-frying fats. Fat I A was included in genotoxicity testing in human lymphocytes for comparison with fat I B, as well as for its apparent incompatibility with the Salmonella/microsome assay.

Table 5.1. Amounts of polar material (polar fraction; PF), and mutagenicity of unheated and heated deep-frying fats to *Salmonella* tester strain TA97.

Fat	Heating time(h)	PF (% by weight)	mutagenic activity in strain TA97	
			without S9 mix (rev/g of fat)	with S9 mix (rev/g of fat)
IA	0	3.1	tox ¹	tox
	30	30.0	tox	4067
II	0	3.5	1371	nm ²
	30	16.6	614	nm
III	0	2.8	nm	nm
	30	25.3	nm	nm
IB	0	3.5	nm	nm
	30	14.9	3200	2549
IV	0	<1	nm	nm
	30	17.9	1754	1074
V	0	5.2	nm	nm
	30	10.8	3775	nm
VI	0	2.0	1018	776
	30	18.0	1728	1368

Fats I A to III were used exclusively for frying of potatoes, while in fats I B to VI various foods were prepared. Mutagenicity was determined separately in non-polar (NPF) and polar (PF) fractions of the fats. Mutagenic activity per g of fat was obtained applying linear regression to non-toxic portions of dose-response curves of NPF and PF exhibiting significant mutagenicity. Contribution of the fractions to mutagenicity per g of fat was estimated by multiplying mutagenic activity per mg of fraction with the total amount of NPF or PF present per g of fat.

¹: toxic to the tester strain

²: not mutagenic

Mutagenic activity of unused and used fats I B and V was also studied using strain TA100. Unused fats and heated fat V did not show significant mutagenicity in strain TA100, neither in absence nor in presence of S9 mix. Polar fractions of used fat I B exhibited significant mutagenicity to strain TA100, either with and without metabolic activation. Numbers of revertants induced were 1851 and 1315, in absence and presence of metabolic activation respectively. Since fat I A was found to be toxic to the bacteria and was considered to yield unreliable results in the Ames mutagenicity assay, this fat was not further tested using strain TA100.

Results of chemical analyses for determination of the extent of oxidative deterioration of the fats selected for further testing are presented in Table 5.2. Highest concentrations of DPTG, TBA-RS and LAHPO after were observed in heated fat I A, whereas lowest levels were observed in heated fat V. Concentrations of TBA-RS in fats I B and V were just above the detection limit of 1 nmol/g. Results of chemical analyses indicated that frying of freshly sliced potatoes under the conditions used in our laboratory resulted in higher oxidative deterioration than frying of various foods.

SCE frequencies induced in human lymphocytes after *in vitro* exposure to frying fats are presented in Table 5.3. Elevated ($p < 0.05$) SCE frequencies were found in fat I A at a doses of 0.1 mg/ml and in fat V at a dose of 1.0 mg/ml, but failed to show dose-dependent increases at higher concentrations. Heated fat I B significantly increased SCE frequencies at a dose of 10.0 mg/ml ($p < 0.01$). A linear dose-dependent increase can be observed over the doses ranging from 0.1-10.0 mg/ml, inducing an increase in SCE frequencies of 1.0, 0.4 and 1.6, respectively ($p < 0.001$). Unheated fats did not significantly increase SCE frequencies at doses of 10.0 mg/ml. Mitomycin C ($3 \cdot 10^{-8}$ M), which was included as the positive control produced 15.2 ± 4.6 SCE/cell (mean $\pm$ SD).

Results of the HGPRT assay using human lymphocytes *in vitro* are presented in Table 5.4. Significant increase in frequency of TGR^r T-lymphocytes was observed for heated fat I A, tested at a dose of 1.0 mg/ml, whereas unheated fat appeared without activity (experiment 1 and 2). However, when analyzed in a separate series of experiments (3 and 4), performed 4 weeks later, no significantly increased frequencies were observed at doses ranging from 0.01 to 1.0 mg/ml. Heated fats I B and V, did not significantly increase Vf when tested at doses ranging from 0.1 to 10.0 mg/ml. Heated fats I A and V, and unheated fat I B appeared to stimulate cell proliferation of PHA-activated T-lymphocytes, whereas heated fat I B decreased LI_C at high doses, indicating cytotoxic activity (Figure 5.1). Due to severe oxidative damage to erythrocytes in whole blood cultures, highest evaluable dose of tBOOH appeared to be 100 μ M. tBOOH did not significant-

Table 5.2. Concentrations of di- and polymeric triglycerides (DPTG), thiobarbituric acid-reactive substances (TBA-RS) and linoleic acid hydroperoxides (LAHPO) measured in unheated and heated deep-frying fats.

Fat	Heating time (h)	DPTG (% by weight)	TBA-RS (nmol MDA/g)	LAHPO (nmol/g)
IA,	0	<1 ¹	1.5 ± 0.1	<2
	30	26.8 ± 1.0	16.5 ± 0.4	62 ± 4
IB,	0	<1	<1	<2
	30	6.4 ± 0.2	2.1 ± 0.3	4 ± 2
V,	0	<1	<1	<2
	30	<1	3.5 ± 0.3	<2

Fat IA was used exclusively for frying of potatoes, while in fats IB and V various foods were prepared. Results are presented as mean ± SD.

¹: not detectable; detection limits of the methods are 1% (by weight) for HPLC determination of DPTG; 1 nmol/g for colorimetric measurement of TBA-RS; and 2 nmol/g for HPLC determination of LAHPO.

ly increase Vf's at evaluable doses. At this dose however, tBOOH was found to enhance cell proliferation (Table 5.4).

Genotoxicity of heated fats as assessed in the Salmonella/microsome assay, as well as by induction of SCE frequencies and single gene mutations in human lymphocytes appeared not to be related to parameters of oxidative deterioration. No associations appeared to exist between induction of SCE frequencies, mutations at the HGPRT locus, and mutagenicity in the Salmonella/microsome assay either.

Discussion

Results obtained in the present study indicated that formation of products mutagenic in the Salmonella/microsome assay may be dependent on a number of variables, of which only a few could be elucidated. Frying of freshly sliced potatoes was observed to enhance formation of polar oxidation products especially in fats I A and III which were PUFA-rich, but failed to increase mutagenicity in strain TA97. Toxic effects exerted by fat I A to the bacteria prohibited reliable mutagenicity testing in the Salmonella-/microsome assay. Comparison of oxidative indices of fats I A and I B indicated that frying of potatoes was more deleterious with respect to oxidative degradation as well as autoxidation of PUFA. Enhanced formation of DPTG during frying of potatoe slices as compared to veal cutlets, was previously reported by Pokorny et al. (1975), who suggested that thiols, sul-

Table 5.3. Frequencies of sister chromatid exchanges (SCE) induced in vitro by repeatedly used deep-frying fats.

Test compound		dose (mg/ml)	SCE/cell	SD
1.	RPMI medium	8.0	2.2	
	fat I A, unheated	10.0	8.9	3.0
	fat I A, heated	0.1	9.3 ^a	3.1
		1.0	8.2	2.1
		10.0	8.7	2.3
2.	RPMI medium		7.2	2.8
	fat I B, unheated	10.0	7.7	3.8
	fat I B, heated	0.1	7.3	2.2
		1.0	7.6	2.7
		10.0	8.8 ^b	2.3
	fat V, unheated	10.0	7.3	2.7
		0.1	7.8	2.7
		1.0	8.5 ^a	2.5
		10.0	8.0	3.2

Fats were heated for 30 h; fat I A was used exclusively for frying of potatoes, while in fat I B and V various foods were prepared. Significant differences as compared to concurrent controls are indicated by superscripts: ^a $p < 0.05$; ^b $p < 0.01$.

Table 5.4. Variant frequency (Vf) of TGF T-lymphocytes after in vitro exposure to repeatedly used deep-frying fats.

Exp. Test compound	dose (mg/ml)	L ₁ C	# evaluable cells ($\times 10^5$)	# positive cells	Vf ($\times 10^{-6}$)
1. RPMI medium					
fat I A, unheated	1.0	0.181 $\pm$ 0.013	0.75	3	39.9
fat I A, heated	1.0	0.181 $\pm$ 0.014	0.94	3	32.1
		0.288 $\pm$ 0.057	0.96	816	8523.1 b
2. RPMI medium					
fat I A, unheated	1.0	0.178 $\pm$ 0.009	1.17	2	17.2
fat I A, heated	1.0	0.197 $\pm$ 0.013	0.51	2	38.8
		0.241 $\pm$ 0.021	0.81	184	2271.6 b
3. RPMI medium					
fat I A, heated	0.01	0.110 $\pm$ 0.013	1.28	0	<7.8
	0.10	0.130 $\pm$ 0.029	1.21	1	8.3
	1.00	0.142 $\pm$ 0.010	0.92	0	<10.9
		0.188 $\pm$ 0.012 a	0.54	2	37.0
4. RPMI medium					
fat I A, heated	0.01	0.138 $\pm$ 0.006	1.67	1	6.0
	0.10	0.195 $\pm$ 0.026 a	1.14	1	8.8
	1.00	0.213 $\pm$ 0.013 a	1.01	8	79.4
		0.265 $\pm$ 0.022 a	0.47	1	21.3
3. RPMI medium					
fat I B, unheated	10.0	0.203 $\pm$ 0.005	1.75	1	5.7
fat I B, heated	0.1	0.283 $\pm$ 0.021 a	0.60	0	<16.7
		0.187 $\pm$ 0.017	0.55	1	18.2

Table 5.4. - continued

fat I B, heated	1.0	0.122 ± 0.025 ^a	0.49	1	20.4
	10.0	0.067 ± 0.002 ^b	0.15	0	<66.7
fat V, unheated	10.0	0.235 ± 0.048	1.68	0	<6.0
fat V, heated	0.1	0.298 ± 0.015 ^a	1.84	2	10.9
	1.0	0.300 ± 0.010 ^a	2.38	1	4.2
	10.0	0.293 ± 0.035	0.63	3	47.6
6. RPMI medium					
fat I B, unheated	10.0	0.168 ± 0.013	1.32	5	37.5
fat I B, heated	0.1	0.217 ± 0.008 ^a	0.24	1	27.0
	1.0	0.205 ± 0.011	0.87	2	22.9
	10.0	0.219 ± 0.007 ^a	0.24	1	41.1
	10.0	0.099 ± 0.016 ^a	0.03	1	333.3
fat V, unheated	10.0	0.157 ± 0.008	0.71	5	70.4
fat V, heated	0.1	0.211 ± 0.004	1.03	2	19.4
	1.0	0.207 ± 0.019 ^a	1.15	4	34.8
	10.0	0.288 ± 0.028 ^a	0.62	1	16.1
7. RPMI medium					
tBOOH (100 µM)					
		0.046 ± 0.002	0.33	1	30.2
		0.061 ± 0.001 ^a	0.30	3	99.6

Fats were heated for 30 h; fat I A was used exclusively for frying of potatoes, while in fat I B and fat V various foods were prepared. Fat samples were tested twice in separate experiments as is indicated by the number of the experiment. Values of L/C are expressed as mean ± SD. Significant differences as compared to concurrent controls are indicated by superscripts: ^a $p < 0.05$; ^b $p < 0.01$.

phides or amines present in proteinaceous foods either inhibit autoxidation by free-radical scavenging activities or by binding of oligomers or their precursors.

Frying of various foods including proteinaceous meat products however, did increase mutagenic activity to strain TA97 of all repeatedly used fats evaluated. Previously Taylor et al. (1983) reported that frying of fish fillets, with repeated use of the fat, did result in mutagen formation in the fat, as well as in foods prepared after prolonged use of the fat, whereas after frying of potatoes or onion rings no increased mutagenicity was observed. Results of previous studies performed at our laboratory showed that the degree of mutagenicity was considerably higher in fat used for preparation of various foods (Hageman et al., 1988), than in a different batch of the same type of fat used for frying of potatoes (Hageman et al., 1990). This finding, in combination with results obtained in the present study, suggests that frying of various foods in the same fat enhances mutagen formation in the fat. Numbers of revertants induced in strain TA97 by the repeatedly used fats in the present study were in the lower range of those found in a previous study in which commercially, and presumably abused fat samples were found to induce $1 \cdot 10^3$ to $1 \cdot 10^4$ rev/g of fat (Hageman et al., 1988). It is therefore concluded that deep-fat frying of foods, by applying recommended frying practices, i.e. with frying temperatures not exceeding 180 °C, and limited heating times, is not likely to cause significant mutagenicity of the fat, independent of the nature of the fat.

Since mutagen formation was observed not to be consistently related to oxidative deterioration, PUFA concentration of the fats, or occurrence of mutagenic compounds present in unused fats, it was suggested that mutagen formation during deep-fat frying may be modulated by other factors including presence of antioxidants, both naturally occurring and synthetic (Pongracz, 1988; Stevenson et al., 1984). Evidence for an inhibiting effect of antioxidants was recently obtained at our laboratory. Polar fraction of a fat, which was commercially used for preparation of various foods, increased numbers of revertants in strain TA97 without S9 mix to 4 times the number of spontaneous revertants at a dose of 10 mg/plate. A dose-dependent decrease in mutagenicity was observed after addition of 10, 20 and 50 µg of beta-carotene per plate. Almost complete inhibition of mutagenicity was observed after addition of the highest dose. Other antioxidants evaluated, including alpha-tocopherol, ascorbic acid, glutathione, and butylated hydroxyanisole also exhibited reducing activity, but were less effective than beta-carotene on a molar base.

In addition, anti-mutagenic compounds might also have been produced during heating. Antioxidative as well as antimutagenic activities were

Figure 5.1. *In vitro* exposure to heated fats of PHA-stimulated human T-lymphocytes; effects on cell proliferation as determined by the LI_C of control cultures of the HGPRT assay. In A, LI_C 's are shown of fat I A, of unheated samples tested in experiments 1 (■) and 2 (●), and of heated samples in experiments 3 (□) and 4 (○); in B, LI_C 's are shown of fat I B, of unheated samples in experiments 5 (■) and 6 (●), and of heated samples in experiments 5 (□) and 6 (○); in C, LI_C 's are shown of fat V, of heated samples in experiments 5 (■) and 6 (●), and of heated samples in experiments 5 (□) and 6 (○). * significantly different from the RPMI control at $p < 0.05$; ** significantly different from the RPMI control at $p < 0.01$.

reported for oleic, linoleic acid and saturated fatty acids (Hayatsu et al., 1981; 1988; Negishi et al., 1984; Diplock et al., 1988; Anonymous, 1989), which are likely to occur in frying fats due to hydrolysis of the triglycerides during heating (Stevenson et al., 1984). Moreover, certain oxidative derivatives of linoleic acid, containing a conjugated double-bond system (CLA), have been isolated from fried ground beef, and were demonstrated to exert anti-mutagenic as well as anti-carcinogenic activities (Ha et al., 1987). Therefore, mutagenicity testing of non-fractionated fat samples seems to be of greater relevance than testing of isolated fractions, since this would allow determination of a net overall effect. Regrettably, mutagenicity testing of unfractionated fat samples appeared practically not feasible, since the required doses of 50 to 100 mg do not dissolve in 25 μ l THF, which is the maximum non-toxic volume of this solvent. Subsequently, suspending of the fats in sterile tubes in a sterile phosphate-buffered saline (PBS) solution, at 50 °C under sonification was applied. However, poor solution of the fats in top agar solution, resulting in poor discrimination between fat particles and revertant colonies at high doses did not allow reliable evaluation of mutagenic activity of unfractionated fat samples in the *Salmonella*/microsome assay.

Since mutagenicity results obtained with the *Salmonella*/microsome assay are considered insufficient evidence for possible carcinogenic risk, genotoxic effects of heated fats were determined using human lymphocytes. Determination of genotoxic damage in human lymphocytes was recently applied in order to assess carcinogenic risk of amino acid pyrolysates, which were isolated from broiled meats and found to be extremely potent bacterial mutagens as well as carcinogens in rodents (Sugimura, 1985; Kato et al., 1989), but of which carcinogenic potency in man is still unclear at present. The potent bacterial mutagens 2-amino-3-methylimidazo[4,5-*f*]quinoxaline (IQ) and 2-amino-3,8-dimethylimidazo-[4,5-*f*]quinoxaline (MeIQx) were reported to be considerably less active inducers of sister chromatid exchanges (SCE) or chromosome aberrations in human lymphocytes (Aeschbacher and Ruch, 1989), and IQ at realistic exposure levels did not cause detectable increases in mutations at the HGPRT locus or increases in micronuclei in human lymphocytes (McManus et al., 1988). Thus it was implicated that human cells with intact DNA repair and enzyme systems may be more relevant for evaluation of human genotoxic risk.

Genotoxicity testing of heated fats in the present study involved determination of the potency of repeatedly used fat samples to induce SCE, and single gene mutations at the HGPRT locus. SCE frequencies were slightly increased in lymphocytes exposed to heated fat samples, but the results obtained for the different fats appeared not consistent. A dose-related increase in SCE frequencies was observed for fat I B, however, the maximum increase measured was 1.6 SCE/cell, indicating weak potency of

this fat. However, effects were observed at concentrations which were close to possible realistic exposure levels.

Determination of the capacity of heated fats to induce single gene mutations in the *hprt* locus of human lymphocytes yielded variable and inconsistent results. Initially, significantly increased Vf's were observed for fat I A in 2 separate experiments performed within one week. This first series of experiments was performed 2 weeks after frying, but when the same fat sample was tested approximately 1 month later, no significantly increased Vf was found. The initially observed increases cannot be explained by instable autoxidation products of linoleic acid, which may be subject to degradation during storage of the fat. Subsequent analyses of LAHPO in this fat I A performed in the second and fourth week after frying, did not indicate significant changes in LAHPO concentration. Heated fats I B and V did not significantly increase mutations at the HGPRT locus in human lymphocytes under the conditions tested.

Heated fats I A and V, as well as the unheated fat I B, induced increased labeling indices in PHA-stimulated T-lymphocytes, indicating enhancing effects on cell proliferation. Since proliferation and activity of T-lymphocytes *in vitro* have been reported to be stimulated in the presence of free fatty acids (Spieker-Polet et al., 1981; Cuthbert et al., 1986), enhanced proliferation observed in our study might be ascribed to the presence of free fatty acids. However, autoxidation or degradation products of fatty acids could also have contributed to the observed stimulation cell proliferation, since after intra-rectal administration in rats, these products were demonstrated to enhance cell proliferation of the colonic mucosa (Bull et al. 1984; 1988). Furthermore, the increased LI_C observed for tBOOH in our study suggests that oxidative species may be involved in enhancement of cell proliferation. Effects on cell proliferation in the gastro intestinal tract after oral administration of autoxidized lipids have not been studied yet, but may be relevant since stimulation of cell proliferation is considered to facilitate tumorigenesis (Hicks, 1983; Weitberg, 1989).

In the present study, no associations appeared to exist between the observed biological effects and concentrations of DPTG, TBA-RS or LAHPO of the fats. It is therefore concluded that not only oxidation products of PUFA are involved in genotoxicity of heated frying fats. Reaction products of oxidized fats with proteins or carbohydrates, which are abundant in most deep-fat fried foods, likely contribute to genotoxicity of repeatedly used deep-frying fats as well, since preparation of various foods was observed to produce significant mutagenicity of repeatedly used deep-frying fats in the Salmonella/microsome assay. In addition, results obtained in the present study, suggest that repeatedly used deep-frying fats showing mutagenic activity in the Salmonella/microsome assay, may also exert genotoxic effects

to human lymphocytes *in vitro*. Furthermore, heated fats appeared to stimulate cell proliferation of PHA-stimulated T-lymphocytes. The nature of these biologically active compounds, however remains to be established.

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Chapter 6

Mutagenicity of deep-frying fat, and evaluation of urine mutagenicity in man after consumption of fried potatoes

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Abstract

Mutagen formation during deep-fat frying was evaluated using standard frying conditions. Portions of pre-fried, sliced potatoes obtained at the retail level, were fried in a commercial brand of hydrogenated vegetable frying fat which was used repeatedly and for a prolonged period of time. Concentrations of polar oxidation and degradation products, and of di- and polymeric triglycerides (DPTG) were found to increase in the frying fat as well as in fried potatoes with prolonged use of the fat. Thiobarbituric acid-reactive substances (TBA-RS) were not detectable, neither in the frying fat nor in fried potatoes.

Polar fractions of repeatedly used frying fat significantly increased the number of revertants in strain TA97 without S9 mix. In presence of S9 mix mutagenic activity was reduced. As a consequence of ongoing formation of polar degradation and oxidation products, mutagenicity of the fat increased after repeated use. Polar fractions of lipids extracted from commercially obtained pre-fried potatoes, as well as from fried potatoes marginally increased the number of revertants in strain TA97 without S9 mix. Mutagenicity of the lipid fractions of fried potatoes was not related to heating time of the fat. Methanol (Me)-extracts of fat-free residues of fried potatoes significantly increased numbers of revertants in strain TA97 after metabolic activation, indicating that a different class of mutagens had been isolated. Mutagenicity of Me-extracts was not increased after prolonged use and repeated use of the fat either.

Urine samples of 6 healthy, non-smoking volunteers collected during 24 hours after consumption of portions of fried potatoes prepared in repeatedly used frying fat showed no increased mutagenicity as compared to control samples. Since the exact identity of mutagens formed during deep-fat frying, as well as their metabolic fate in man is unclear at present, evaluation of possible adverse biological effects associated with consumption of fried foods will require strictly controlled metabolic studies.

Introduction

Repeatedly used deep-frying fat sampled at the retail level has been found to exert mutagenic activity in the *Salmonella*/microsome assay in a previous study performed in our laboratory (Hageman et al., 1988). Mutagenic activity was present mainly in the polar fraction of deep-frying fats, which has been reported to contain oxidation and heat degradation products of fatty acids (Billek et al., 1978). We also demonstrated that mutagenic activity of the fat increased after prolonged and repeated frying of various foods, under standard frying conditions according to manufacturers' instructions. Mutagenicity of the used deep-frying fats was positively correlated with thiobarbituric acid-reactive substances (TBA-RS) present in the fats, indicating that lipid peroxidation products might be involved in mutagenicity (Hageman et al., 1988).

In feeding experiments with laboratory animals growth retardation, increased liver and kidney weights and cellular damage in various organs have been observed after administration of oxidized fats and oils (Andia and Street, 1975; Billek, 1976; Alexander et al., 1987). In rats, consumption of thermally oxidized oils induced peroxidative deterioration of tissue lipids, as was shown by increased levels of TBA-RS in hepatic tissue, which correlated positively with concentrations of polar degradation products and the peroxidative value of the ingested fats (Izaki et al., 1984).

Previous evaluation of mutagenicity of deep-fat fried foods by Taylor et al. (1982) revealed weak mutagenic activity of basic methylene chloride extracts of fried foods. Mutagenic activity of deep-fat fried foods fried under laboratory conditions was detected only after severe abuse of the frying fat (Taylor et al, 1983). Since we found significant mutagenic activity in polar fraction of commercially used frying fat samples, the present study was undertaken to evaluate mutagenicity of various lipid fractions of deep-fat fried potatoes. In addition an attempt was made to assess endogenous exposure of humans to mutagenic and peroxidative products after consumption of fried potatoes prepared in repeatedly used deep-frying fat. Urines were screened for mutagenicity and analyzed for TBA-RS, which in animal studies have been found to be indicative of the extent of lipid oxidation in the diet and in tissues (Draper et al., 1984).

Materials and methods

Chemicals

Analytical-grade, stabilized (with 250 ppm butylated hydroxytoluene) tetrahydrofuran (THF) used for high-performance liquid chromatography (HPLC) analysis of di- and polymeric triglycerides (DPTG); acetic acid and silicagel 60, particle size 0.063-0.20 mm, used for fractionating fat samples; XAD-2 resin; analytical grade acetone; trichloroacetic acid;

malondialdehyde bis-(diethylacetal); hydrochloric acid and 2-thiobarbituric acid, all of the highest chemical grade available, were purchased from Merck, Darmstadt (F.R.G). HPLC-grade petroleum ether, boiling range 40-60 °C; methanol, diethyl ether, and non-stabilized THF used for dissolving fat fractions in mutagenicity testing were obtained from Fisons, Loughborough (Great Britain). All other chemicals and media used in the Ames assay were of the highest chemical grade available.

Frying experiment

Mutagen formation during deep-fat frying was studied in a frying experiment under standard conditions that closely resemble usual frying practices. A batch of 2 kg of hydrogenated vegetable frying fat, obtained from a local supermarket, was used for preparation of fried potatoes. The fat was heated in a home-style fryer at a temperature of 180 ± 5 °C during 8 hours each day on 5 consecutive days, allowed to cool overnight and not replenished. Fat samples were taken after 0, 8, 28 and 40 h of use.

Five 1 kg packages of frozen, pre-fried sliced potatoes, also commercially obtained, were divided in 200 g portions and stored at -20 °C until the moment of preparation. Portions of frozen potatoes were fried every 4 h, commencing after 4 h of heating of the fat. Frying time was 5 min, actual frying temperatures ranged from 160 to 182 °C.

Urine collection

Six healthy, non-smoking male volunteers, aged 22 to 42 years, were asked to collect 24-h urine samples before and after consumption of 200 g portions of fried potatoes. The subjects were asked not to consume other fried or grilled foods and not to drink alcoholic beverages while collecting urine samples. A 24-h control sample and 24-h urine samples collected immediately after consumption of fried potatoes on day 1, 3 and 5 of the experiment, prepared in fat that had been used for 8, 24 or 40 h respectively, were screened for mutagenicity. The samples were stored under refrigeration and processed within a day, or frozen at -20 °C.

Aliquots of 200 ml of urine were concentrated on XAD-2 resin columns, the columns were eluted with 100 ml of distilled water to remove traces of histidine and extracted with 10 ml of analytical-grade acetone as described by Yamasaki and Ames (1977). Acetone extracts were evaporated under a stream of nitrogen and stored under refrigeration until mutagenicity testing.

Chemical analysis

Portions of fried potatoes were freeze-dried, after which 10 g of dry material was homogenized with 10 g of anhydrous sodium sulphate. Lipids were isolated by overnight Soxhlet extraction with petroleum ether (PE). A second overnight extraction was performed using methanol (Me). PE and Me-extracts were concentrated on a rotary evaporator after which solvent residues were evaporated under a stream of nitrogen at room temperature.

DPTG in the frying fat and PE-extracts of fried potatoes were determined by gel-permeation HPLC (Hageman et al., 1988). In short, 200 µl of fat, liquified by heating at temperatures not exceeding 50 °C, was mixed with 3.0 ml of stabilized THF, after which 15 µl

of this mixture was injected on a Styrogel 500 A column, 300 mm x 7.8 mm ID. Mobile phase consisted of degassed, stabilized THF at a flow rate of 1.0 ml/min; peaks were monitored using a refractive index (RI)-detector. Concentrations of DPTG were calculated as percentage of total peak area.

Fractionation of deep-frying fat samples and PE-extracts into non-polar and polar compounds by column chromatography was carried out as described previously (Hageman et al., 1988). Briefly, 1 g of fat was dissolved in 10 ml of a mixture of petroleum ether: diethyl ether (87:13, v/v; solvent 1). The mixture was carefully applied to a silicagel column, after which the non-polar fraction was eluted with 150 ml of solvent 1. The polar fraction was obtained by elution with 150 ml of diethyl ether acidified with 1% acetic acid (solvent 2). Solvents were distilled off using a rotary evaporator.

TBA-RS of deep-frying fat samples and PE-extracts of fried potatoes were determined as described earlier (Hageman et al., 1988). To assess whether consumption of repeatedly used frying fats increases whole body lipid peroxidation, urine TBA-RS were also determined before and after consumption of fried potatoes as follows: in a screw top tube 2.0 ml of urine was mixed with 2.0 ml of a 10% trichloroacetic acid solution and 0.5 ml of a 1% TBA suspension in distilled water. Samples were incubated in a waterbath at 100 °C during 20 min, cooled and centrifuged, after which absorption was measured at 532 nm and at 600 nm. The difference between the absorption is a measure for the concentration of TBA-RS. Detection limit of the method is 0.1 nmol of malondialdehyde (MDA)/ml of urine.

Mutagenicity testing

Mutagenicity testing of deep-frying fat fractions, lipid fractions and methanol extracts of fried potatoes, and urine extracts was performed according to standard protocols (Maron and Ames, 1983) applying the liquid preincubation procedure during 20 min at 37 °C. Since strain TA97 appeared to be most sensitive in detecting mutagenic activity of used deep-frying fat, only this strain was used for mutagenicity testing of fat fractions as well as urine extracts. Liver S9 fraction, with a protein concentration of 34 mg/ml, was prepared from Aroclor 1254 treated rats, and added in a concentration of 50 µl per plate.

Fractionated fat samples were dissolved in analytical grade, unstabilized THF and tested at doses ranging from 0.1 to 10.0 mg/plate; the volume of the dose was 25 µl. Me-extracts of fried potatoes were suspended under sonification in distilled water, since Me-extracts appeared to be better dissolved in water than in dimethyl sulphoxide (DMSO). Me-extracts were tested at doses ranging from 0.1 to 10.0 mg/plate.

Urine concentrates were dissolved in DMSO and equivalents of 5.0, 10.0, 12.5 and 25.0 ml were tested both in absence and presence of liver S9 mix. Mutagenicity of 24-h urine samples was calculated using linear regression of dose-response curves. Extractions of fried potatoes and separation of fats into non-polar and polar fractions as well as mutagenicity testing were repeated at least once, including unheated unused control samples. Solvent controls, chromatography and extraction solvents, processed according to the protocols used for sample preparation, were also screened for mutagenic activity. All doses were tested

in duplicate or triplicate with and without metabolic activation. Estimation of mutagenic activity per g of frying fat or fried potatoes samples and urinary mutagen excretion per 24 h was achieved by linear regression of dose-response curves.

Statistical analyses

Statistical analysis of mutagenicity data of fats, extracts of fried potatoes and urines was performed using the method of least significant difference (LSD) (MacGregor et al., 1985). LSD values obtained in this study are at confidence levels of $p < 0.05$ and $p < 0.01$, respectively: for THF as solvent (lipid fractions) TA97-S9 256 and 388, TA97+S9 186 and 292; for distilled water as solvent (Me-extracts) TA97-S9 120 and 189, TA97+S9 142 and 215; for DMSO as solvent (urine extracts) TA97-S9 112 and 175, TA97+S9 92 and 144. Differences in urine mutagenicity between control and experimental samples were statistically analyzed using Student's t-test for paired values.

Results

During deep-fat frying and with prolonged use of the fat, the polar fraction representing oxidation and thermal degradation products of fatty acids, increased linearly with increasing heating time (Figure 6.1). The polar fraction isolated from PE-extracts of fried potatoes showed a similar tendency, but the polar fraction comprised a smaller percentage of the lipids as compared to the corresponding frying fat samples (Figure 6.1). PE-extracts of pre-fried frozen potatoes contained approximately 8% (by weight) of polar material. After frying in relatively fresh frying fat used for up to 24 h, polar fraction of fried potatoes was found to be decreased when compared to the pre-fried potatoes. DPTG present in the frying fat as well as in PE-extracts of fried potatoes samples are listed in Table 6.1. DPTG were detected in both deep-frying fat samples and in PE-extracts of fried potatoes after prolonged use of the fat. Neither the deep-frying fat nor the PE-extracts of fried potatoes contained detectable amounts of TBA-RS (data not shown).

Polar fractions of the frying fat samples taken after 8, 28 and 40 h of frying significantly ($p < 0.05$) increased the number of revertants at doses higher than 1 mg/plate. Non-polar fractions did not significantly increase the number of revertants and as a consequence, mutagenic activity estimated per g of fat was caused mainly by compounds present in the polar fractions (Table 6.2). Mutagenicity of the frying fat was increased after prolonged heating. In presence of S9 mix, mutagenicity of the polar fractions was reduced.

Figure 6.1. Formation of polar degradation products (PF) in deep-frying fat (▲) and in lipids extracted from fried potatoes (△) as function of heating time. Results are expressed as mean $\pm$ SD.

Figure 6.2. Mutagenicity to strain TA97-S9 mix (■) and to strain TA97+S9 mix (▨) of 24-h urine samples collected before and after consumption of fried potatoes prepared in repeatedly used frying fat. Results are expressed as mean $\pm$ SD of 6 subjects.

Table 6.1. Occurrence of di- and polymeric triglycerides (DPTG) in deep-frying fat and fried potatoes.

Sample	Heating time of the frying fat (h)	DPTG (% by weight)
<i>Frying fat</i>		
Control	0	< 1
	8	< 1
	28	2.5 ± 0.3
	40	3.3 ± 0.3
<i>Fried potatoes</i>		
Control	0	NA*
	4	< 1
	8	NA
	16	NA
	24	2.4 ± 0.2
	32	2.4 ± 0.3
	40	NA

* NA, not analyzed

Polar fractions of pre-fried sliced potatoes insignificantly increased numbers of revertants, with the exception of the fried potatoes sample taken after 8 h of use. For this sample at a dose of 5 mg/plate a significant ($p < 0.05$) increase was observed. Mutagenicity of polar fractions isolated from fried potatoes was also reduced in the presence of S9 mix. Mutagenicity of polar fractions of fried potatoes varied considerably and appeared not to be related to the heating time of the fat used for preparation of the potatoes. The non-polar fractions of PE-extracts of fried potatoes did not significantly increase numbers of revertants.

Me-extracts of fried potatoes showed no significant mutagenic activity without S9 mix, but significantly increased numbers of revertants were observed in 3 samples in the presence of S9 mix. At doses of 10 mg/plate mutagenic activity was observed in Me-extracts of potatoes fried after 8 ($p < 0.05$), 24 ($p < 0.01$) and 32 ($p < 0.01$) h of use. Me-extracts of potatoes prepared after 40 h of use showed weak toxic effects. Mutagenic activity of Me-extracts was not clearly increased after prolonged and repeated use of the fat either.

Table 6.2. Estimated mutagenicity of frying fat and contribution to mutagenicity of fried potatoes of petroleum ether (PE)-extracts (polar and non-polar fractions) and methanol (Me)-extracts.

		Mutagenicity (rev/g) of			
		Fat/PE-extracts		Me-extracts	
Sample	Heating time of the frying fat (h)	TA97 - S9	TA97 + S9	TA97 - S9	TA97 + S9
<i>Frying fat</i>					
Control	0	672	588		
	8	2160	324		
	28	2392	920		
	40	3451	2032		
<i>Fried potatoes</i>					
Control	pre-fried	192	72	200	395
	4	464	72	242	224
	8	1078	134	242	263
	16	168	252	133	115
	24	735	390	354	348
	32	195	0	183	648
	40	352	128	151	244

Mutagenic activity per g of fried potatoes was estimated by extrapolating dose-response curves of non-polar and polar fractions, and of Me-extracts (Table 6.2). Without S9 mix, a five-fold increase in mutagenicity was observed in fried potatoes prepared in fat used for 8 h. After repeated heating of the fat mutagenicity of the fried potatoes varied considerably, without reaching the peak level observed at the end of the first day. Mutagenic activity of the lipid fraction of fried potatoes was reduced in the presence of S9 mix. Me-extracts of fried potatoes induced significant mutagenic activity in presence of S9 mix only, however no relation was found with heating time of the fat.

To determine whether substances naturally occurring in potatoes could be responsible for the observed mutagenic activity of PE and Me-extracts of fried potatoes, 500 g of freshly peeled potatoes have been freeze-dried, extracted and tested according to protocols used for fried potatoes. No significantly increased numbers of revertants have been observed for either PE-extracts or Me-extracts, representing 50 g of fresh potatoes. In addition, portions of freshly peeled and sliced potatoes have been fried in fresh non-mutagenic deep-frying fat, and in fat that had been used for 100 h. Lipids extracted from these fried potatoes samples have been observed to contain respectively 4.2 ± 0.1 and $32.2 \pm 2.4\%$ (by weight) of polar components. Mutagenic activity of the fried potatoes to strain TA97 without S9 mix significantly increased from 0 in the control sample to 736 revertants/g of fried potatoes in the 100 h sample.

Urine mutagenicity of six volunteers after consumption of 200 g of fried potatoes prepared in repeatedly used frying fat was not significantly different from control levels, neither tested in absence nor in presence of liver S9 mix. At high doses representing 25 ml of urine, most samples were toxic, inducing a decreased response. After linear regression was applied to lower doses only, urine mutagenicity varied from 0 to 8 revertants per ml of urine. Extrapolated to 24-h volumes, urine mutagenicity ranged from 0 to 10^4 revertants in control as well as in experimental samples. Urine mutagenicity varied both intra- and interindividually and appeared not to be related to heating time of the fat used for preparation of the fried potatoes consumed (Figure 6.2). TBA-RS were detected in all urine samples, with concentrations ranging from 0.1 to 2.5 nmoles of MDA/ml. TBA-RS of urines following consumption of fried potatoes however, did not differ from control levels (data not shown).

Discussion

Prolonged heating and repeated use of deep-frying fat under standard conditions as employed in this study increases mutagenicity of polar fat fraction. Mutagenic activity of the frying fat which was used for preparation of fried potatoes only, appears to be moderate, reaching levels of 2,500 to 3,500 revertants per g of fat in strain TA97 without S9 mix. In our previous study, mutagenic activity of frying fat sampled at local restaurants and tested according to the same protocols, has been found to range from 1,000 to 30,000 revertants per g of fat (Hageman et al., 1988).

The mutagenicity results reported here differ from those described by Taylor et al. (1983), who did observe very low or even anti-mutagenic activity in lipid fractions of fat used for frying potatoes. This may be caused by differences in frying procedures, as well as fractionation and mutagenicity screening of fat samples. Taylor et al. (1983) fried 15 portions of potatoes per day as opposed to a maximum of 8 per day in our experiment. Thermal

abuse of the fat as a consequence of small amounts of food prepared, however, appeared to be minimal in our experiment since temperatures were found not to exceed 182 °C. Mutagenic activity of the polar fat fractions in our experiment was detected most sensitively by *Salmonella* tester strain TA97 without S9 mix, and applying the liquid preincubation procedure (Maron and Ames, 1983). Taylor et al. (1983) screened mutagenicity of lipid fractions using strain TA98 with S9 mix, probably detecting different classes of mutagens. These differences in fractionating and testing procedures together with endogenous properties of the fats used for frying, very likely explain the remarkable differences between mutagenicity results as reported by Taylor et al. (1983) and those observed in the present study.

Mutagenic activity of the polar fraction of PE-extracts of fried potatoes does not increase with prolonged heating time in a similar mode as the polar fraction of the frying fat. Mutagenic activity of polar fractions of fried potatoes varies from levels equal to those found for pre-fried potatoes to 2 to 4 times increased mutagenicity. This variation in mutagenic activity may reflect different mutagenic, or possibly antimutagenic properties of fried potatoes as compared to frying fat. A second extraction with methanol does only slightly improve isolation of possibly remaining direct-acting mutagens from the fried potatoes, since all methanol extracts have been found to marginally increase numbers of revertants in absence of S9 mix. Mutagenicity of PE and Me-extracts added together as an estimation of total mutagenic activity of fried potatoes, was not related to heating time of the fat. Extraction with methanol may have isolated a different class of mutagenic compounds, as is suggested by their metabolic activation.

The nature of mutagens formed during deep-fat frying remains to be established. Additional analysis of DPTG in non-polar and polar deep-frying fat fractions has shown that DPTG are present in the polar fraction and that non-polar fractions contain no detectable amounts of DPTG. Amounts of DPTG in the polar fraction of the fat have been found to increase from less than 1% to 30% (by weight) after 40 h of frying. Polymerized oxidation and degradation products of fatty acids, however, are not likely to be responsible for the observed mutagenic activity since mutagenicity of the polar fraction of the frying fat does not increase with increasing levels of DPTG. In our previous study, mutagenicity of deep-frying fat samples has not been found to correlate with DPTG either (Hageman et al., 1988).

Compared to meat products, potatoes contain low amounts of protein and no creatinin, and since the frying fat in the present study has been used for deep-frying of potatoes exclusively, substantial formation of amino-acid pyrolysates seems very unlikely. Furthermore, mutagenicity of amino-acid pyrolysates has been reported to require metabolic activation (Sugimura et al., 1986), whereas mutagens formed during deep-fat frying of potatoes and present in the polar fractions are direct-acting and deactivated by rat liver S9 mix. Mutagenicity of Me-extracts of fat-free residues however, is enhanced after hepatic metabolism and may be due to the presence of more polar

pyrolysis products. The absence of TBA-RS in the frying fat as well as in lipids extracted from fried potatoes appears to reject a possible contribution to mutagenic activity of MDA or other lipid oxidation products in this frying experiment.

Evaluation of endogenous exposure in humans of mutagens formed during deep-fat frying has been attempted by screening urine mutagenicity. Weak mutagenicity was found not only in urine samples collected after consumption of fried potatoes, but also in samples collected when subjects were consuming their habitual diet with the restriction of alcoholic drinks and fried or grilled foods. Since increased urine mutagenicity has also been observed by Sasson et al. (1985) in urine of subjects on a vegetarian diet, it is concluded that detection of marginally increased urine mutagenicity requires strictly controlled conditions with standardized diets and occupational and physical activity.

The absence of increased urine mutagenicity after consumption of fried potatoes may also indicate metabolic deactivation prior to excretion or lack of absorption by the digestive tract. Both possibilities are supported by data from feeding studies with laboratory animals (Oarada et al., 1986; Kanazawa et al., 1985). In these studies, rats fed (U-¹⁴C)-labeled secondary oxidation products of linoleic acid metabolized low molecular weight product but poorly absorbed polymeric degradation products which remained in the lumen. Consequently, screening of mutagenic activity and chemical analysis of faeces after consumption of oxidized deep-frying fat may elucidate metabolic fate of mutagenic polar compounds of the fat.

Epidemiological studies have shown a positive correlation among countries between fat consumption and cancer prevalence (Carroll and Khor, 1975). In industrialized countries fats contribute significantly to dietary fat intake. Based on household consumption expenditure statistics in the Netherlands (CBS, 1987), mean consumption of deep-fat fried food is estimated to contribute at least 6% to the daily fat intake. Assuming a mean level of 5% of polar fraction of the frying fat, daily intake per person of polar compounds will exceed 100 mg. Since processed dietary fat may possess tumor initiating as well as tumor promoting activity, the fate and exact nature of mutagens consumed with various deep-fat fried foods prepared in repeatedly used deep-frying fat, is of great interest with respect to possible health consequences and will be further evaluated.

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Chapter 7

Evaluation of faecal and urine mutagen excretion in man: effects of consumption of mutagenic, repeatedly used deep-frying oil

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Abstract

Effects on mutagenicity of urine and faeces of consumption of fried potatoes prepared in repeatedly used mutagenic coconut oil, were determined in 14 healthy volunteers. Using a cross-over design, volunteers consumed daily a 250-g portion of fried potatoes during a 5-day period; during a 5-day control period, volunteers consumed daily a portion of mashed potatoes to which unused frying oil was added. The lipid fractions of fried potatoes showed significant mutagenic activity to *Salmonella* tester strain TA97 in absence of S9 mix, estimated to amount to $1.5 \cdot 10^5$ rev/portion. Lipid fractions of fried potatoes were not found to contain detectable amounts of lipid oxidation products. Mutagenic activity was found in lipid fractions of mashed potatoes as well, which was estimated to amount to $2.6 \cdot 10^4$ rev/portion. During the experiment all foods were supplied by the investigators and food consumption was recorded daily. Urine and faeces were collected on days 4 and 5 of each period. In addition, faeces was sampled on the first day of the experiment, in order to determine faecal mutagenicity of the participants during consumption of their free-choice diets.

Mutagenicity of dichloromethane (DCM)- or acetone-concentrates of urine to strains TA97 and TA100 without S9 mix was not found to be increased after daily consumption of a portion of fried potatoes as compared to consumption of mashed potatoes. Neither was consumption of fried potatoes observed to increase mutagenic activity of DCM-extracts of faeces to these strains as compared to mashed potatoes. No effects of consumption of fried potatoes were observed on plasma clinical biochemical parameters of blood samples taken on the 5th day of each period, nor on urinary excretion of lipid oxidation products.

DCM-extracts of faeces samples collected during the experimental periods however, showed significantly increased mutagenicity to strain TA100 as compared to samples collected after free-choice diets of the volunteers. Mutagenicity of faecal extracts to strain TA100 was not associated with dietary intake of macro-nutrients, including fat, P/S ratio of lipids and fiber, but did show a significant positive correlation with faecal pH ($r=0.45$, $p<0.02$). Since faecal mutagen excretion was not found to be related to the pattern of mutagenicity of deep-fried potatoes, results of the present study do not indicate a contribution of consumption of mutagenic deep-frying oils to faecal mutagenicity. Faecal mutagen excretion or production appeared to be related to the diet, but the dietary factors affecting mutagenicity of faeces remain to be determined. Results obtained in the present study indicate that consumption of deep-fried potatoes prepared in mutagenic oil, containing low levels of lipid oxidation products, does not appear to pose a serious risk to human health.

Introduction

Dietary factors have been suggested to be strongly related to human cancer incidence (Doll and Peto, 1981). Epidemiologic data have indicated that prevalence of colorectal cancer is increased in developed countries as compared to many developing countries, and immigrants from low-incidence countries have been observed to assume cancer risk of their new homelands (Armstrong and Doll, 1975; Correa and Haenszel, 1978; Rose et al., 1986). Most, but not all epidemiologic studies have shown that high consumption of dietary fat and meat is associated with an increased risk for colon cancer (Byers, 1988; Hill, 1987; Willet, 1989). Recently it has been found in epidemiologic studies that high intake of fried meats (Young et al., 1988), as well as of fat-rich deep-fried foods (Peters et al., 1989) is associated with increased risk for colon cancer, which suggests that these food products contain compounds which may be causally related to colon tumorigenesis.

Dietary constituents are considered to act on the colonic mucosa largely via faeces, which may contain tumor-initiating, -promoting as well as -inhibiting factors (Bruce, 1987). Tumor-initiating compounds are generally considered to be genotoxic, and application of the Salmonella mutagenicity assay provided a relatively fast and simple method for determination and identification of possible carcinogens in the faecal stream. Persons at high risk for developing colon cancer, as well as populations consuming high-fat, low-fiber diets, have been reported to show high faecal mutagenicity, while vegetarians and populations consuming high-fiber, low-fat diets tend to have low levels of faecal mutagenicity (Ehrig et al., 1979; Kuhnlein et al., 1981; Mower et al., 1982).

fecapentaenes, very potent direct-acting bacterial mutagens, have been successfully isolated and identified from human faeces (Gupta et al., 1979). In addition, the existence of several not yet identified mutagens has been demonstrated, since faecal extracts have been observed to possess non-fecapentaene mutagenicity to Salmonella tester strains (Schiffmann et al., 1989), and have also been reported to contain pre-mutagenic compounds which require microsomal metabolism to become active (Reddy et al., 1984; Schiffman, 1986). Diet intervention studies have led to the identification of some determinants of faecal mutagenicity. Consumption of fried meat, which has been found to contain mutagenic amino acid pyrolyzates (Furihata and Matsushima, 1986), has been observed to increase faecal mutagenicity (Kuhnlein et al., 1983; Hayatsu et al., 1985b). In a pilot study, the addition of extra fat to a typical Western diet has been reported not to result in increased faecal mutagenicity (Vennit et al., 1986). In addition, supplementation of diets with fiber, vitamin C and vitamin E has been demonstrated to reduce faecal mutagenicity (Dion et al., 1982; Reddy et al., 1987; Vennit et al.,

1986). In another study however, supplementation of vitamin C or vitamin E has not been observed to affect fecapentaene production (Wilkins et al., 1981), thus indicating other compounds also contribute to faecal mutagen production. Effects of consumption of mutagen containing foods, other than fried meats, on faecal mutagen excretion have not yet been described.

In a previous study performed in our laboratory, repeatedly used, commercial deep-frying fat samples were found to exhibit mutagenicity to *Salmonella typhimurium* tester strains TA97, TA100 and TA104 (Hageman et al., 1988). Mutagenicity of most of the fat samples was observed to be reduced by microsomal metabolism. Mutagenic activity of these fat samples also showed a positive association with lipid oxidation products of polyunsaturated fatty acids (PUFA) determined in the fats (Hageman et al., 1988; 1989). Subsequent studies revealed that fats, which do not contain lipid oxidation products, also exhibit mutagenic activity, indicating that other mutagens, possibly also oxidation products, are formed during frying as well (Hageman et al., 1990; results shown in Chapter 5). It was also observed that consumption of potatoes prepared in repeatedly used, mutagenic deep-frying fat, did not increase urine mutagen excretion in 6 healthy volunteers (Hageman et al., 1990). It was therefore concluded that mutagens consumed with fried potatoes are either inactivated *in vivo*, or that mutagens may not be excreted in urine, but in faeces.

The present study was undertaken to determine primarily whether consumption of potatoes prepared in repeatedly used, mutagenic deep-frying fat affects faecal mutagenicity. For this purpose, 14 healthy volunteers were asked to consume daily, during a period of 5 days, a portion of fried potatoes, prepared in mutagenic deep-frying fat; during a 5-day control period, the same subjects consumed daily a portion of mashed potatoes, to which an equal amount of unheated frying fat was added. A cross-over design was used with equal food consumption during both 5-day periods. Diet was controlled with respect to consumption of known mutagen-containing foods, since increased mutagen excretion was repeatedly reported in urine as well as in faeces after consumption of fried meats and fish (Baker et al., 1982; Dolara et al., 1984; Hayatsu et al., 1985a, 1985b; Ohyama et al., 1987; Sousa et al., 1985). Therefore, all foods consumed by the participants of the study, were supplied by the investigators. Food consumption was recorded daily. Fried meats and meat extracts were excluded from the diet to prevent interference of genotoxic amino acid pyrolyzates (Alink et al., 1988; Furihata and Matsushima, 1986) with mutagenicity of compounds present in lipid fractions of deep-fat fried potatoes. For the same reason fried potatoes, and not meat snacks, were chosen to serve as a carrier of mutagens formed during deep-fat frying.

Urine and faeces samples were collected on the fourth and fifth day of

each diet period. Samples were tested for mutagenicity to strain TA97 in order to determine biological fate of direct-acting mutagens present in lipid fractions of fried potatoes. Mutagenicity to strain TA100 was analyzed to detect effects of mutagens acting by base-pair substitution, which were also found to be present in heated deep-frying fats (Hageman et al., 1988). Urinary pH was determined, to investigate a possible relationship with mutagenicity. In addition, the influence of the change from free-choice to experimental diets on faecal mutagenicity was studied. For this purpose, faeces samples were collected on the first day of the experiment as well. Blood was collected at the end of each diet period and plasma clinical biochemical parameters were measured to determine possible adverse effects of daily consumption of repeatedly used deep-frying fat on hepatic and renal tissues (Billek et al., 1979, Kanazawa et al., 1986). Excretion of thiobarbituric acid-reactive substances (TBA-RS) was measured as an index of whole body lipid oxidation, which was reported to be related with endogenous lipid oxidation, as well as with dietary lipid oxidation (Draper et al., 1984). Elevated faecal pH and increased mutagenicity are considered to be indicators for increased colon cancer risk; therefore, faecal pH was measured and related to mutagenicity (Bruce, 1987).

Materials and methods

Frying procedure

A batch of 10 kg of coconut oil was commercially obtained. According to the manufacturer (personal communication) the fat did not contain additives. Seven kg of the oil was used for frying, and was heated at 180 °C for 10 h per day during 3 consecutive days. Various fried foods were prepared, including meat products, breaded products and freshly sliced potatoes. After 30 h of heating, the polar fraction of the fat was found to be mutagenic, and at this point, 25 kg of freshly sliced potatoes were pre-fried in portions of 500 g during 4 min at 180 °C, using the mutagenic oil. After the complete batch was pre-fried, potatoes were fried again for 3 min. Actual frying temperatures ranged from 160 to 180 °C. Fried potatoes were mixed into a homogeneous batch, and in portions of 2 kg frozen at -20 °C until consumption, which was within 3 weeks.

Diet intervention study

Participants (7 males and 7 females) of the diet intervention study were selected from 25 volunteers using the following criteria: good health, aged between 18 and 30, non-smoker, no use of medication other than oral contraceptives, no abdominal complaints, regular stools (at least once per day), no extreme dietary habits, moderate alcohol consumption (maximally 2 glasses per day). Before entering into the study, volunteers were medically examined and a blood sample was collected for measurement of standard biochemical and hematological plasma parameters. A dietary history was obtained using a standard questionnaire. Energy and nutrient intake on free choice diet was calculated using the Dutch computerized food

composition table (Hautvast, 1975). The data obtained from the food questionnaire were used for composition of diets during the intervention period. The test protocol was approved by the Medical Ethical Commission of the University of Limburg, and all volunteers signed an informed consent.

During 2 periods of 5 days (monday till friday) of 2 consecutive weeks participants consumed a diet consisting of regular food products, with exclusion of fried or baked meat products, nuts, cacao products, meat extracts, and other heat processed foods rich in fat (confectioneries, cake, etc.), as well as alcohol. Participants received one hot meal daily at the institute between 12 and 14 h, which consisted of meat prepared in a microwave-oven without the use of browning plates, cooked as well as raw vegetables, fruit and a dairy product for desert. One period of 5 days participants consumed 250 g of fried potatoes with their hot meal (Period I). Fried potatoes were thawed at room temperature and heated in a stove for 15 min at 150 °C prior to consumption. During the other period (Period II); 250 g of mashed potatoes were consumed to which an amount of unused frying fat was added after cooking of the potatoes, equal to that present in fried potatoes. Mashed potatoes were prepared fresh daily and heated in a microwave-oven prior to consumption. Daily, participants received foods for breakfast and evening meals (whole wheat bread, margarine, preserves, cheese, low-fat milk and fresh fruits, as well as snack foods (low-fat biscuits, raisin bread, etc). Coffee, tea and soft-drink consumption were free, but subjects were asked to maintain their habits at a constant level during the study period. Food consumption during the experimental period was recorded daily. Menu's were equal during both periods of 5 days, and if possible, same batches of food products were used. A cross-over design was applied, with 7 participants (3 males, 4 females) starting with consumption of fried potatoes and 7 (4 males, 3 females) with mashed potatoes. During the weekend in between both periods, subjects were allowed to consume their free-choice diet.

faeces samples were collected in polypropylene bags on the first day of the experiment, as well as on days 4 and 5 of each period, and stored in dry-ice boxes at the homes of the participants. Samples were transferred to the laboratory and stored at -20 °C until analysis. Urine samples were collected on days 4 and 5; collection started immediately after the hot meal and was divided in 2 periods of 12 h. Urine samples were stored under refrigeration and brought to the laboratory on the following day, where the samples were stored at -20 °C until analysis. On the fifth day of each period, blood was collected by venipuncture in heparinized tubes. Plasma was obtained by centrifugation at 2000 g for 10 min, and stored at -20 °C until analysis.

Chemical analyses

Concentrations of TBA-RS were determined colorimetrically, and amounts of di- and polymeric triglycerides (DPTG) by gel permeation high performance liquid chromatography (HPLC), and were performed as described previously (Hageman et al., 1988). TBA-RS and DPTG were measured in frying oil and PE-extracts of fried and mashed potatoes. Unused frying oil did not contain detectable amounts of TBA-RS and DPTG. Frying oil used for preparation of fried potatoes was found to contain 3.5 nmoles of MDA/g. Urinary excretion of TBA-RS was measured as described previously (Hageman et al., 1990). Faecal pH was

determined potentiometrically in 0.5 g wet faeces which were homogenized with 2.0 ml of saline solution (0.9% NaCl, w/v) using a turrax mixer.

Determination of plasma clinical biochemical parameters

Clinical biochemistry of plasma consisted of determination of total protein, ureum (blood urea nitrogen, BUN), creatinin (Cr), lactate dehydrogenase (LDH; E.C. 1.1.27), alkaline phosphatase (AP; E.C. 3.1.3.1), creatine phosphokinase (CP; E.C. 2.7.3.2), glutamic oxaloacetic transaminase (GOT; E.C. 2.6.1.1) and glutamic pyruvic transaminase (GPT; E.C. 2.6.1.2), which were analyzed routinely using a centrifuge analyzer (type Cobas Bio, Hoffmann LaRoche)

Mutagenicity testing

Mutagenicity of the oil was evaluated after the fat was fractionated into a non-polar and a polar fraction using silicagel column chromatography, as described previously (Hageman et al., 1988). Unused coconut oil did not exhibit mutagenicity to *Salmonella* strain TA97, while after 30 h of use the polar fraction of the oil showed significant mutagenic activity, inducing approximately $4 \cdot 10^3$ revertants/g of fat. Before mutagenicity testing, fried potatoes as well as mashed potatoes containing unheated coconut oil, were lyophilized. Lipids were extracted from 20 g of dried product with 200 ml of petroleum ether (PE; boiling range 40-60 °C) by overnight Soxhlet extraction. Most of the PE was removed by vacuum evaporation, extracts were completely dried under a stream of nitrogen. Fat-free residues of fried and mashed potatoes were subsequently extracted with 200 ml of methanol (Me) to obtain more polar components which were previously shown to exhibit mutagenic activity as well (Hageman et al., 1990). PE extracts were separated into non-polar and polar fractions using silicagel column chromatography (Hageman et al., 1988), and tested for mutagenic activity using the liquid pre-incubation procedure as described previously (Hageman et al., 1990). In brief, non-polar and polar fractions were dissolved in tetrahydrofuran (THF), Me-extracts in methanol and tested in triplicate at doses of 0.1, 1.0, 5.0, 10.0 mg per plate, using *Salmonella* tester strains TA97 and TA100 with and without addition of liver S9 mix (50 µl of homogenate/plate), obtained from Aroclor induced rats. Aliquots of 25 µl of the THF solution, and 100 µl of the methanol solutions were applied to the plates. Negative controls, consisting of chromatography and extraction solvents processed according to the procedures used for lipid fractions were also included in mutagenicity testing. Mutagenicity testing of lipid fractions was repeated at least once in a separate assay.

Urine samples were thawed and vacuum filtered through a Whatman no. 4 filter. An aliquot representing 15% of the total 12-h volume was concentrated on XAD-2 resin as described previously (Hageman et al., 1990). In a previous study, acetone extracts of urine were not found to contain mutagenic activity after consumption of fried potatoes prepared in mutagenic frying fat. Since urine might possibly contain very polar mutagens derived from polar oxidation products of frying fat, extraction with dichloromethane (DCM) prior to acetone was applied to separate polar from non-polar compounds. DCM and acetone extracts were dried under a stream of nitrogen, stored at 4 °C, and analyzed for mutagenicity within a week. Prior to mutagenicity testing, urine extracts were dissolved in 0.6 ml dimethyl sulphoxide (DMSO). Aliquots of 100 µl (representing 2.5% of the total volume), and 100 µl of 1:3 diluted extracts

(0.8% of the total volume) were tested in duplicate for mutagenicity using strains TA97 and TA100. Negative controls consisting of 10 ml of DCM or acetone which were passed through XAD-2 resin, evaporated and dissolved in DMSO, were also included in mutagenicity testing. Urine mutagenicity was not evaluated in the presence of S9 mix, since mutagenicity of repeatedly used deep-frying fats as well as of deep-fried potatoes was not found to be enhanced in presence of liver S9 mix as compared to results obtained in absence of S9 mix (Hageman et al., 1988; 1990).

Faeces samples were thawed and homogenized in polypropylene bags using a stomacher lab blender (Seward Laboratory, London, Great-Britain). Homogenized samples were lyophilized, after which 1 g of dry faeces was extracted twice with 10 ml DCM under vigorous shaking for 30 min at room temperature. In a pilot study, it was observed that DCM was more efficient than diethyl ether, acetone, or methanol in extracting mutagenic substances from dry faeces. DCM-extracts were combined, filtered through a Whatman no. 4 filter and dried under a stream of nitrogen. Dry extracts were stored at -20 °C, and tested for mutagenicity within 2 days. Prior to mutagenicity analysis faecal extracts, representing 1.5 g-equivalent of dry faeces, were dissolved in 1 ml methanol; aliquots of 100 µl were tested per plate using strain TA97 and TA100 using the standard plate assay (Maron and Ames, 1983). Since consumption of mutagenic deep-frying fat was considered to influence faecal excretion of direct-acting mutagens, as well as possible production of pre-mutagenic compounds in faeces for instance by the intestinal microflora, mutagenicity of faecal extracts was evaluated in absence as well as in presence of liver S9 mix (50 µl/plate). Negative controls consisting of DCM which was evaporated and subsequently dissolved in methanol, as well as positive controls were also included in mutagenicity testing. Urine or faecal extracts of all samples obtained from a particular participant in both periods, were tested in the same assay.

Statistical analyses

Significance of mutagenicity results of lipid fractions and of Me-extracts was determined by the method of least significant differences (LSD) as described previously (Hageman et al., 1988). LSD values for THF as solvent (solvent control of non-polar and polar lipid fractions) were at a significance level of $p < 0.05$ for strain TA97-S9 mix, 120; for strain TA97+S9 mix, 105; for strain TA100-S9 mix, 146; and for strain TA100+S9 mix, 114. For methanol as solvent LSD values ($p < 0.05$) were for strain TA97-S9 mix, 106; for strain TA97+S9 mix, 142; for strain TA100-S9 mix, 91; and for strain TA100+S9 mix 94. Mutagenicity per mg of polar or non-polar fraction was estimated applying linear regression to non-toxic portions of dose-response curves, showing significant mutagenicity at one or more dose levels. LSD values for urine extracts were for DMSO as a solvent at a significance level of $p < 0.05$ in strain TA97-S9, 114; and in strain TA100-S9, 57. LSD values of faecal extracts at significance levels of $p < 0.05$ and $p < 0.01$ respectively, were for strain TA97-S9 mix, 56 and 99; for strain TA97+S9 mix, 54 and 96; for strain TA100-S9 mix, 42 and 74; and for strain TA100+S9 mix, 60 and 106.

Differences between the 2 periods in food consumption, urinary and faecal mutagen excretion, excretion of TBA-RS in urine, faecal pH, and plasma clinical biochemical parameters were statistically analyzed using Students' t-test for paired values. Differences between sexes and between the 2 groups of the cross-over design, were evaluated using Students' t-test for

unpaired values. Statistical significance of differences in frequencies of mutagenic faeces samples between diet periods was analyzed using Fisher's exact test. Associations between dietary intakes and mutagenicity of urine and faeces, as well as between different parameters measured, were evaluated by Pearson's coefficient of correlation.

Results

Results of nutrient intake of the participants during their free-choice diets, and during both experimental periods are shown in Table 7.1. Intake of energy, macro-nutrients and fiber was not found to differ between both experimental periods. In addition, energy intake, as well as consumption of protein, carbohydrates, fat and dietary fibre were not found to be significantly changed during the experimental periods as compared to the free-choice diet (Table 7.3). The P/S ratio of triglycerides consumed during the experimental period however, was significantly lowered ($p < 0.01$, $n = 14$) as compared to the P/S ratio of fats consumed during free-choice diets. Since only three participants reported regular alcohol consumption, differences in alcohol consumption between free-choice and experimental diets were obvious for these three only (Table 7.1).

Amounts of oxidation and degradation products of lipids in fried and mashed potatoes, are shown in Table 7.2. Fried potatoes were found to contain increased levels of polar oxidation products as well as Me-extractable compounds. DPTG and TBA-RS were not detectable in PE-extracts of fried and mashed potatoes. Results of mutagenicity testing of lipid fractions and Me-extracts are listed in Table 7.3. Mutagenicity was observed to be highest for fried potatoes to strain TA97 without S9 mix, although mashed potatoes were also found to possess mutagenic activity to strain TA97 without S9 mix. Significantly increased numbers of revertants in TA97 and TA100 in presence of S9 mix, were detected for polar lipid fractions and Me-extracts obtained from fried as well as from mashed potatoes, but mutagenicity per g of food product was considerably lower in mashed potatoes as compared to fried potatoes due to small amounts of these fractions present in mashed potatoes. Mutagenicity to strain TA97 of polar lipid fractions and Me-extracts was found to be reduced in presence of S9 mix. No mutagenicity was detected in lipid fractions and Me-extracts using strain TA100 without S9 mix, which for Me-extracts was found to be due to toxic effects to the bacteria. Mutagen intake due to consumption of fried potatoes, as determined with strain TA97 without S9 mix, was estimated to be equivalent to $1.5 \cdot 10^5$ rev/day, whereas mutagen intake with mashed potatoes approximately amounted to $2.6 \cdot 10^4$ rev/day.

Table 7.1. Energy and nutrient intake of participants on their free-choice diets and during the experimental periods in which either a portion of fried potatoes (Period I) or mashed potatoes (Period II) was consumed daily.

Daily intake	Free-choice diet		Period I		Period II	
	males	females	males	females	males	females
Energy (MJ)	16.1 ±4.8	8.9 ±2.0	13.6 ±2.1	8.6 ±1.3	13.3 ±1.6	8.7 ±1.5
Protein (g)	117 ±25	69 ±12	121 ±23	73 ±10	134 ±38	80 ±13
Carbohydrates (g)	428 ±117	266 ±62	409 ±80	280 ±46	401 ±85	335 ±54
Lipids (g)	179 ±74	83 ±25	122 ±29	68 ±11	117 ±19	73 ±14
P/S of triglycerides	0.50 ±0.23	0.52 ±0.22	0.33 ±0.09	0.35 ±0.07	0.35 ±0.09	0.31 ^a ±0.05
Dietary fibre (g)	43 ±8	31 ±7	39 ±6	26 ±3	37 ±5	26 ±3
Alcohol (g)	2 ±4	1 ±3	0	0	0	0

Results are presented as mean±SD of each group of 7 males or 7 females. ^a significantly different from free-choice diet, $p < 0.05$.

Consumption of fried potatoes by 14 human volunteers did not affect excretion of TBA-RS in urine, nor urinary and faecal pH as compared to consumption of mashed potatoes containing unheated frying oil (Table 7.4). Urinary excretion of TBA-RS were not significantly changed during the first 12 h after the hot meals as compared to urine samples collected 12-24 h after the hot meals (Table 7.4); no effect of consumption of heated vs. unheated oil was observed in either. The pH of faeces samples collected

immediately after the free-choice diets was not found to be significantly different from the pH of faeces samples collected during both experimental periods. Daily consumption during 5 days of fried potatoes prepared in repeatedly used deep-frying oil was not found to cause adverse effects as judged by results of plasma clinical biochemical parameters, which did not differ from results obtained for plasma of blood samples collected after daily consumption of mashed potatoes containing unheated frying oil (Table 7.5). Plasma clinical biochemical parameters measured during both periods, were found to be in the normal range reported in the literature (Anonymous, 1981).

Table 7.2. Results of chemical analyses of petroleum ether (PE)-extracts and methanol (Me)-extracts of fried and mashed potatoes.

Parameter	Mashed potatoes	Fried potatoes
dry matter (% by weight)	29.3	58.0
lipids (% by weight)	10.1	14.2
polar fraction (% by weight)	0.5	1.7
Me-extractable compounds (% by weight)	1.2	3.3
DPTG in PE-extracts (% by weight)	<1 ¹	<1
TBA-RS in PE-extracts (nmoles of MDA/g)	<1	<1

¹ Detection limit for analysis of di- and polymeric triglycerides (DPTG) is 1% (by weight), and for determination of thiobarbituric acid-reactive substances (TBA-RS) 1.0 nmol malondialdehyde (MDA)/g of fat.

Neither DCM- nor acetone-concentrates of individual urine samples collected during the first 12 h after the hot meal, significantly increased numbers of revertants in strains TA97 and TA100. Acetone concentrates slightly, but statistically not significantly, increased numbers of revertants in both tester strains, whereas DCM-concentrates induced numbers of revertants equal to the solvent control. Urine samples of four participants collected 12 to 24 h after the meal were analyzed, but since these were not found to induce increased numbers of revertants either, further analysis of urine samples collected 12 to 24 h after the hot meal was omitted. Mean mutagenicity of acetone extracts of urine samples collected during the first 12 h after the hot meal did not differ between the two diet periods (Figure 7.1), indicating that increased intake of mutagens with fried potatoes did not result in increased urine mutagenicity.

DCM-extracts of individual faeces samples of some, but not all participants, collected during the experimental periods were found to exhibit significant mutagenicity to strains TA97 and TA100. Mutagenicity results of DCM-extracts of faeces however, showed considerable inter- as well as intra-individual variation; intra-individual variations were found to range from reduced numbers of revertants to levels reaching 3 times the number of spontaneous revertants for 2 consecutive samples. In addition significantly reduced ($p < 0.05$) numbers of revertants were found for faecal extracts tested using strain TA97 in presence of S9 mix, which could not be ascribed to toxic effects since the background lawn did not show growth inhibition. Therefore, mean mutagenicity results of samples obtained on day 4 and 5 of each experimental period was determined and used for statistical analyses of the data. Incidences of mutagenic faeces samples are shown in Figure 7.2. Frequencies of faeces samples showing significant mutagenicity to strains TA97 and TA100 as compared to the solvent controls ($p < 0.05$) were found to be highest during period I when daily a portion of fried potatoes was consumed, but did not differ significantly from frequencies of mutagenic samples observed during free-choice diet and period II.

Mutagenicity of DCM-extracts of faeces to strains TA97 and TA100, tested with and without S9 mix, was not found to be increased during period I as compared to period II (Figure 7.3). However, significant differences were observed between mutagenicity of faecal extracts of samples reflecting excretion on free-choice diet and mean mutagenicity samples collected during Period I. Mutagenicity of the latter samples was significantly increased in strain TA100, tested both with and without S9 mix ($p < 0.05$). In contrast, mutagenicity of faecal extracts collected during period I showed significantly reduced mutagenicity in strain TA97 in presence of S9 mix ($p < 0.05$) as compared to the sample collected immediately after the free-choice diet. Results indicated that increased mutagen consumption with fried potatoes as determined by strain TA97, did not lead to increased faecal

Table 7.3. Mutagenicity to *Salmonella typhimurium* tester strains TA97 and TA100 of lipid fractions and methanol (Me)-extracts, obtained from mashed and fried potatoes.

	Mutagenicity to strain:			
	TA97-S9	TA97+S9	TA100-S9	TA100+S9
<i>Mashed potatoes:</i>				
Polar lipid fraction (rev/g of product)	28	112	nm ¹	nm
Non-polar lipid fraction (rev/g of product)	nm	nm	nm	nm
Me-extract (rev/g of product)	74	nm	tox ²	tox
Total mutagenicity (rev/g of product)	102	112	nm	nm
<i>Fried potatoes:</i>				
Polar lipid fraction (rev/g of product)	160	180	nm	92
Non-polar lipid fraction (rev/g of product)	nm	nm	nm	nm
Me-extract (rev/g of product)	422	nm	tox	tox
Total mutagenicity (rev/g of product)	582	180	nm	92

Mutagenicity of isolated fractions was estimated by determination of mutagenic activity per mg of polar and non-polar fractions and Me-extracts, applying linear regression to dose response curves. Contribution of the respective fractions to mutagenicity per g of product, was calculated by multiplication of mutagenicity per mg of fraction, with the total amount of this fraction present in one g of product.

¹ not mutagenic.

² toxic to the tester strain.

mutagenicity, but instead reduced faecal mutagenicity as compared to results obtained for samples representing excretion during free-choice diets. In addition, results of mutagen excretion as determined with strain TA100, indicated that the change of diet does affect the excretion of mutagens, which are not related to consumption of deep-fried potatoes.

Mutagenicity of urine and faeces samples, as well as results of other parameters tested were not found to differ between both groups formed in the cross-over design, and neither between the sexes. Use of oral contraceptives was not found to influence mutagenicity results, nor other parameters measured. A significant positive correlation was observed between mutagenicity of faecal extracts tested using strain TA100 with S9 mix, and pH of faeces samples ($r=0.45$, $p<0.02$). Weak, but statistically insignificant positive correlations were also observed between faecal pH and mutagenicity of faecal DCM-extracts to strains TA97-S9 ($r=0.33$) and TA100-S9 ($r=0.34$). A significant positive correlation was found between TA97 mutagenicity of faecal DCM-extracts and faecal mutagenicity to strain TA100 in absence of S9 mix ($r=0.79$, $p<0.001$), which was found within separate periods as well as when all faecal samples were included. Mutagenicity of urine extracts to strain TA97 was not found to be associated with mutagenicity to strain TA100. Mutagenicity of faecal extracts, as well as of urine extracts, was not found to be associated with any other relevant parameter measured, including dietary intake of fat, type of fat (P/S ratio) and fiber, indicating other dietary factors which were not determined in the present study may determine faecal mutagen excretion.

Figure 7.1. Mean mutagenicity of acetone extracts (0.8% of the total volume) of urine samples collected during the first 12 h after consumption of fried potatoes (Period I) or mashed potatoes (Period II). Results shown are the mean $\pm$ SD of mutagenicity results of 14 volunteers obtained for strains TA97 (▨) and TA100 (■).

Table 7.4. Excretion of thiobarbituric acid-reactive substances (TBA-RS) in urine, and pH of urine and faeces samples collected after daily consumption of fried potatoes, prepared in repeatedly used coconut oil (Period I), and consumption of mashed potatoes containing unused coconut oil (Period II).

Parameter	Free-choice diet	Period I	Period II
Urinary TBA-RS excretion 1-12 h after the hot meal (μ moles of MDA)	n.a. ¹	2.77 ± 0.85	2.63 ± 1.13
Urinary TBA-RS excretion 12-24 h after the hot meal (μ moles of MDA)	n.a.	2.08 ± 0.74	2.04 ± 0.64
Urinary pH	n.a.	7.03 ± 0.50	6.74 ± 0.49
Faecal pH	7.04 ± 0.59	7.14 ± 0.34	7.15 ± 0.23

¹ not analyzed.

Results are expressed as mean $\pm$ SD of all participants. Mean urinary excretion of TBA-RS, and mean urinary and faecal pH during periods I and II were determined per person using samples of days 4 and 5. Group means were calculated using mean values of each person.

Discussion

In the present study effects of consumption of mutagen containing deep-frying fats on urinary and faecal mutagen excretion were determined in human volunteers, to assess endogenous exposure and biological fate of mutagens produced during deep-fat frying. For this purpose, freshly sliced potatoes were prepared in repeatedly used coconut oil showing significant mutagenicity to *Salmonella* tester strain TA97, and 250 g-portion were consumed daily by 14 healthy volunteers during 5 consecutive days. Results obtained were compared to effects observed after daily consumption of unheated coconut oil, which was mixed through mashed potatoes. Intake of mutagenic compounds showing activity to strain TA97 without S9 mix, was found to be approximately 5 times higher during the period in which fried potatoes were consumed as compared to consumption of mashed potatoes. In contrast to a previous study (Hageman et al., 1990), in which Me-extracts of fried potatoes exhibited increased mutagenicity in presence of S9 mix, in

Figure 7.2. Frequencies of faeces samples showing significant mutagenicity as compared to solvent controls ($p < 0.05$) of 14 volunteers consuming their free-choice diets, and the experimental diets containing fried potatoes (Period I), or mashed potatoes (Period II). In (A), results are shown for strain TA97-S9 (■) and TA97+S9 (▨); in (B), frequencies of mutagenic faeces samples to strain TA100-S9 (▨), and TA100+S9 (■) are presented. Total number of samples tested was 14 for the free-choice diet period, and 28 for periods I and II.

Figure 7.3. Mean mutagenicity to strain TA97 (A) and TA100 (B) of DCM-extracts of faeces samples (150 mg-equivalent of dry faeces), representing mutagen excretion on free-choice diets, and during both experimental periods. Results shown are the mean $\pm$ SD of results of 14 volunteers obtained for strains TA97-S9 (■), TA97+S9 (▨), TA100-S9 (▩), and TA100+S9 (■). * significantly different from results obtained for samples representing the free-choice diet ($p<0.05$).

Table 7.5. Results of plasma clinical biochemical parameters of blood samples of 14 volunteers, taken after daily consumption during 5 days of fried potatoes prepared in repeatedly used frying oil (Period I), and mashed potatoes containing unheated frying oil (Period II).

Parameter	Period I	Period II
Glutamic oxaloacetic transaminase (GOT; U/l)	9.2 ±2.0	10.3 ±2.3
Glutamic pyruvic transaminase (GPT; U/l)	5.6 ±2.2	5.3 ±2.4
Creatine phosphokinase (CPK; U/l)	66 ±44	65 ±47
Alkaline phosphatase (AP; U/l)	83 ±19	85 ±22
Lactate dehydrogenase (LDH; U/l)	211 ±67	208 ±40
Plasma creatinin (Cr; µmol/l)	76 ±13	85 ±13
Plasma ureum (BUN; µmol/l)	5.1 ±1.4	5.2 ±0.9
Plasma protein (g/l)	66 ±2	65 ±3

Results are expressed as mean±SD of all participants (n=14).

the present study, Me-extracts were found to show direct-acting mutagenicity to strain TA97. This difference may be caused by the use of different types of fat in both studies, or by the nature of the fried potatoes. Compounds naturally present in potatoes may influence mutagenicity as well, since Me-extracts of mashed potatoes in this study also showed weak mutagenic

activity. PE-extracts of fried potatoes were not found to contain detectable amounts of DPTG or TBA-RS, thus excluding a significant contribution of lipid oxidation to mutagenicity of polar fractions of PE-extracts. Me-extracts were observed to contribute approximately 80% to mutagenicity of fried potatoes, indicating that this fraction may contain mutagenic compounds produced during deep-fat frying of potatoes. The nature of these compounds however, remains to be determined.

Daily consumption of heated coconut oil with fried potatoes during five days was not found to cause adverse effects as compared to daily consumption of unheated coconut oil with mashed potatoes, as evaluated by changes in plasma clinical biochemical parameters. This may be due to the fact that lipid fraction of fried potatoes did not contain detectable amounts of oxidation products of polyunsaturated fatty acids, which were reported to increase plasma activities of GOT and GPT, and cause cellular damage to liver and kidneys after oral administration to rats (Alexander, 1981; Billek, 1979; Kanazawa et al., 1986). In the present study, urinary excretion of TBA-RS of human volunteers was not found to differ either between both diet periods, indicating that endogenous exposure to lipid oxidation products was not increased after consumption of fried potatoes prepared in repeatedly heated coconut oil as compared to consumption of unheated coconut oil. This may also be considered to be due to the fact that concentrations of TBA-RS and DPTG in PE-extracts of potatoes in the present study were below detection limits. In a previous study, repeatedly used deep-frying fats were found to contain concentrations of TBA-RS up to 161 nmoles of MDA/g of fat and levels of DPTG as high as 32% (by weight; Hageman et al., 1988). Effects of daily consumption of fried foods prepared in heated fats containing extremely high levels of lipid oxidation products, on faecal and urine mutagenicity, on excretion of TBA-RS, as well as on tissues in liver and kidneys remain to be determined.

Urine mutagenicity was not found to be significantly increased after consumption of fried potatoes, which is in agreement with results found in our previous study (Hageman et al., 1990). Although the frequency of faeces samples showing significant mutagenicity was increased during the period in which fried potatoes were consumed as compared to the period in which mashed potatoes were consumed, no consistent changes in faecal mutagen excretion were found between the periods within individuals as evaluated with students' t-test for paired values. Mutagenicity to strain TA100 of faeces samples collected after consumption of fried potatoes did show increased mutagenicity as compared to samples representing free-choice diets. Since P/S ratio of fats consumed by the participants was significantly decreased during the experimental periods, a relation with increased faecal mutagenicity to strain TA100 was suggested. However, no significant correlations were found between P/S ratio of dietary lipids and faecal

mutagenicity, neither was the intake of other dietary compounds measured found to be associated with faecal mutagen excretion.

Faecal mutagenicity is considered an index of carcinogenic risk for the colon/rectum, since the high-risk Western-type of diet (low-fiber, high-fat) has been related to increased faecal mutagenicity (Ehrig et al., 1979; Kuhnlein et al., 1981; Mower et al., 1982). However the relation between presence of faecal mutagens, as well as mutagenicity-inhibiting factors detected in faecal extracts, to tumorigenesis in colon/rectum still remains to be established (Bruce, 1987; Hayatsu et al., 1981; Schiffman, 1988). In the present study, the change from free-choice to experimental diet marked the increase in faecal mutagenicity to strain TA100, but this increase appeared not to be related to the known high-risk dietary factors such as high-fat and low-fiber intake. Previously, Venitt et al. (1986) did not observe enhancing effects of increased intake of dietary fat either. This finding, as well as the results obtained in the present study suggest that other diet-related factors, which are not known at present, may be interacting. Since faecal pH in the present study was related to increased faecal mutagenicity, it is suggested that possible tumor-initiating factors may be produced at high faecal pH. The nature of faecal mutagens to strain TA100 remains to be determined. Since fecapentaenes were reported to possess direct-acting mutagenicity to strain TA100, and mutagenicity of fecapentaenes is reduced in presence of S9 mix (Gupta et al, 1983; Wilkins et al., 1981), the pattern of faecal mutagenicity obtained in the present study indicates that besides fecapentaenes other mutagens were present in faeces collected during the experimental periods.

Since daily consumption of mutagen containing deep-fat fried potatoes was not associated with increased urine or faecal mutagenicity in the present study, and mutagenicity of lipid fractions of fried potatoes was found to be reduced in presence of S9 mix, it appears likely that mutagens present in deep-fried potatoes are inactivated *in vivo*. A possible retention of mutagenic compounds within the tissues however, cannot be excluded. No acute toxic effects on liver or kidneys appeared to be exerted by daily consumption of approximately 35 g of heated fat either, since no changes were observed in serum clinical biochemical parameters either. It may be concluded from the present study that daily consumption for a short period of time of mutagenic deep-frying fat, containing low levels of lipid oxidation products, does not appear to pose serious health risks.

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Chapter 8

Effect of short-term feeding of mutagenic used deep-frying oils on cell proliferation in the gastro-intestinal tract and the excretion of mutagens in urine and faeces of rats ¹

Abstract

Effects of short-term feeding of mutagen containing, heated deep-frying oils on urinary and faecal mutagenicity, plasma clinical biochemical parameters and cell proliferation in the gastro-intestinal tract were determined in rats. Repeatedly used frying oils, i.e. a coconut oil (CO), containing <10% polyunsaturated fatty acids (PUFA) and a PUFA-rich frying oil (PO) were administered to groups of 7 rats at a level of 10% (by weight) in the diet for 4 weeks; control groups received an equal dose of unheated oils. Both heated oils showed direct-acting mutagenicity to *Salmonella* tester strain TA97; heated PO was also mutagenic to strain TA100. Unheated oils were not found to be mutagenic. Heated PO contained higher amounts of polar oxidation products, as well as polymerized triglycerides and hydroperoxides of linoleic acid, as compared to unheated oils and heated CO. Thiobarbituric acid-reactive substances (TBA-RS), indicating presence of lipid oxidation products, were detected in heated CO as well as in heated PO.

Rats receiving heated PO showed increased activities of plasma glutamic pyruvic transferase ($p < 0.05$) and alkaline phosphatase ($p < 0.01$) as compared to the control FO-group, which may indicate cellular injury in liver and kidneys. Urinary and faecal excretion of TBA-RS were increased in rats fed heated FO; excretion of TBA-RS was minimal as compared to intake, being 0.1% of the dose received with diet. Feeding of heated CO did not result in increased plasma enzyme activities or excretion of TBA-RS. Mutagenicity of urine and faeces to strain TA97 was not found to be increased in groups fed heated oils as compared to unheated oils. Faecal mutagenicity to strain TA100 was not observed to be increased after consumption of heated oils either. Urinary mutagen excretion detected with *Salmonella* strain TA100, was significantly increased in rats fed heated PO only; recovery in urine and faeces of the mutagenic load received with diet, was found to be 7%. Rats fed heated CO did not show increased mutagen excretion to strain TA100 as compared to the control group.

Labeling indices in tissues of oesophagus and forestomach were significantly increased in rats fed heated PO as compared to their control group, indicating a stimulation of cell proliferation. Tissues of glandular stomach and colon/rectum did not show significantly enhanced cell proliferation in the group receiving heated PO. Feeding of heated CO did not result in increased cell proliferation of gastro-intestinal tissues. No differences in cell kinetic parameters were observed between groups receiving unheated oils, indicating that the type of fat did not affect cell proliferation. Results obtained in this study indicate that consumption of oils containing oxidation products of linoleic acid does enhance cell proliferation predominantly in the upper gastro-intestinal tissues.

¹ based on a manuscript submitted for publication

Introduction

The relationship between dietary fat and intestinal cancer has been studied intensively in laboratory animals using initiation-promotion models. High intake of dietary fat has been found to promote chemically induced colon cancer in rats, but the type and source of the fat appears to be of importance as well, since fish oils containing long-chain polyunsaturated fatty acids (PUFA) of the linolenate type (n-3) have been observed to inhibit colonic tumorigenesis, while fatty acids of the linoleate type (n-6) have been reported to exert enhancing effects and are reported to be essential in tumor development (Carroll, 1987; Ip et al., 1985; Pariza, 1988; Reddy, 1987). Studies determining modulating effects of dietary fat and fiber on chemically induced tumors, showed enhanced cell proliferation of colonic mucosa in association with increased tumor incidence (Deschner et al., 1983; Jacobs and Lupton, 1986). Thus a mechanism was proposed for the promoting effect of high dietary fat on colon tumorigenesis, involving chronic irritation and stimulation of cell proliferation of the colonic mucosa, either directly or indirectly via excretion of bile acids (Bruce, 1987; Wargovich and Lointier, 1987).

Studies determining the effect of high fat diets on colonic cell proliferation in rats and mice however, have led to inconsistent results. Feeding of a diet containing 30% (by weight) corn oil for 10 or 15 weeks, was shown to increase colonic cell proliferation in mice as compared to feeding of 5% corn oil (Bird and Stamp, 1986), whereas feeding of diets with the same amount of corn oil or lard for 4 weeks was not found to significantly affect mitotic activity of the colon (Caderni et al., 1988; 1989; Jacobs, 1983). Furthermore, it has been demonstrated that the amount of starch, calcium and type of fiber in the diet modulate cell proliferation of the colon, which may explain the conflicting results obtained with high-fat diets containing different sources of starch and fiber (Caderni et al., 1988; 1989; Jacobs, 1988; Wargovich et al., 1984). In addition, stimulation of cell proliferation of the colonic mucosa was observed after intra-colonic installation of the fatty acids oleate, linoleate and arachidonate, as well as after rectal administration of hydroperoxy- and hydroxy-derivatives of linoleic acid (Bull et al., 1984; 1988; Craven and Rubertis, 1988), suggesting that oxidation products of unsaturated fatty acids may be involved. Effects of short-term oral administration of autoxidized fats on cell proliferation in the gastro-intestinal tract have not yet been determined. Neither have tumor-promoting or initiating effects of heat-processed lipids containing oxidized fatty acids been evaluated.

Feeding of thermally oxidized oils, or isolated oxidation products was found to cause adverse biological effects in laboratory animals, including growth retardation, teratogenicity, increased weights and tissue damage of

liver and kidneys, cellular damage to testes and epididymides, and increased peroxidation of membrane and tissue lipids (Alexander et al., 1981; 1987; Billek et al., 1979; Cutler and Schneider, 1973; Izaki et al., 1984; Kanazawa et al., 1985). Short-term feeding of thermally oxidized corn oil has been reported to induce drug-metabolizing enzyme systems in rat liver (Andia and Street, 1975). Furthermore, administration of fractions of oxidized corn oil by gastric intubation has been observed to induce benzo[a]pyrene hydroxylase activity in the colon, indicating the colon may be a target for biological effects of oxidation products of lipids (Perciballi and Pintauro, 1985).

In addition to these biological effects exerted *in vivo* by oxidized oils, we previously observed that repeatedly used deep-frying fats were mutagenic in the Salmonella/microsome assay (Hageman et al., 1988). Recently, we also found that *in vitro* exposure of lymphocytes to heated deep-frying oils enhanced cell proliferation (results presented in Chapter 5). Therefore, the present study was therefore undertaken to investigate the possible role of mutagenic oxidation and heat degradation products occurring in used deep-frying fats, in intestinal tumorigenesis. For this purpose, the effects of short-term feeding of heated deep-frying oils were determined on mutagen excretion in urine and faeces, and on cell proliferation in the gastro-intestinal tract of rats. Two types of heated oils were compared: a coconut oil (CO), containing less than 10% PUFA, and a PUFA-rich (>60%) frying oil (FO). These oils had been heated at 180 °C during 30 hours and used for preparation of fried foods, applying normal frying practices. These oils appeared not rejectable for human consumption based on chemical and sensory evaluation, but were found to contain varying amounts of different oxidative degradation products, and exhibited mutagenic activity in the Salmonella/microsome assay.

Heated as well as unheated oils were included at a concentration of 10% (by weight) in semi-purified diets which also contained 5% sunflower oil, and were fed to the rats for a period of 4 weeks. Urinary and faecal mutagen excretion and pH were determined. Thiobarbituric acid-reactive substances (TBA-RS) in urine and faeces were analyzed to assess biological fate of lipid oxidation products consumed as well as possible endogenous production (Draper et al., 1984). In addition, toxicity of heated oils was evaluated by analysis of plasma clinical biochemical parameters, and determination of relative liver and kidney weights. The effects on cell kinetic parameters were determined applying immunocytochemistry in combination with flow cytometry for the upper gastro-intestinal tissues and immunohistochemistry for the colon/rectum.

Experimental

Frying oils

Batches of 10 kg of CO (<10% PUFA) and PUFA-rich PO (>60% PUFA) were obtained from local supermarkets and used for frying of various food products, including starchy foods and meats, applying recommended frying practices. Amounts of 3.5 kg of the fats were heated at 180 °C for 10 h per day during 3 consecutive days, and were allowed to cool overnight; actual frying temperatures ranged from 155 to 181 °C. Unheated oils as well as heated oils were stored at 4 °C until analysis or use in the experimental diets. Oxidative deterioration of the oils was assessed by analysis of levels of di- and polymeric triglycerides (DPTG) using gel-permeation high performance liquid chromatography (HPLC), by determination of the amount of the polar oxidation and degradation products (polar fraction; PF) using silicagel chromatography, and by measurement of concentrations of thiobarbituric acid-reactive substances (TBA-RS) using a colorimetric assay, all performed as described previously (Hageman et al., 1988). Linoleic acid hydroperoxides (LAHPO) were determined by HPLC as described previously (Hageman et al., 1989a). Concentrations of oxidation products of unheated and heated oils are presented in Table 8.1.

Mutagenicity of the oil samples in the Ames mutagenicity assay was determined as described previously (Hageman et al., 1988). Briefly, fat samples were separated into non-polar (NPFs) and polar (PFs) fractions using silicagel column chromatography. NPFs and PFs were dissolved in tetrahydrofuran and mutagenic activity was determined testing doses of 0.1 to 10.0 mg per plate, using *Salmonella typhimurium* tester strains TA97 and TA100 with and without Aroclor induced rat liver S9 mix. Chromatography and solvent controls as well as positive controls were included in mutagenicity testing. NPFs of both unheated and heated oils did not show mutagenicity under the conditions used for mutagenicity testing, neither did PFs of unused oils. PFs of heated CO and PO exhibited mutagenic activity to strain TA97 when tested without S9 mix; mutagenicity was estimated to amount to 3775 and 3200 revertants per g of fat. In presence of S9 mix mutagenicity in strain TA97 was reduced; PF of heated CO was not mutagenic, while PF of heated PO did show mutagenic activity, amounting to 2549 revertants per g of fat. The PF of heated PO was also mutagenic to strain TA100, both in absence and presence of liver S9 mix, activities amounted to 1851 and 1315 revertants per g of fat, respectively. PF of unheated oils and heated CO did not exhibit mutagenic activity to strain TA100.

Feeding study with rats

Twenty eight male Wistar rats (Winkelmann, Borchern, FRG), weighing 82 ± 3 g (mean $\pm$ SD), were randomly divided into 4 groups of 7 animals. Rats were housed individually in metabolic cages in an air-conditioned environment at 22 °C, 50% humidity and a 12-h light/dark cycle. Powdered regular lab chow (Hope Farms, Woerden, The Netherlands) and tap water were allowed *ad libitum* for a 1-week acclimatization period. Experimental diets were made fresh weekly; fats were added to a fat-free basal diet (Hope Farms, Woerden, The Netherlands) at a concentration of 10% (by weight); 5% sunflower oil was included to prevent possible essential fatty acid deficiency. The composition of the purified diets is shown in Table 8.2. Fatty acid composition of the diets is presented in Table 8.3. Experimental diets

Table 8.1. Concentrations of polar fraction (PF), di- and polymeric triglycerides (DPTG), thiobarbituric acid-reactive substances (TBA-RS; expressed as nmoles of malondialdehyde (MDA) per g of fat) and linoleic acid hydroperoxides (LAHPO) of unheated and heated CO and PO.

Oil sample	PF (% by weight)	DPTG (% by weight)	TBA-RS (nmol MDA/g)	LAHPO (nmol/g)
CO, unheated	5.2	<1	<1	<2
CO, heated	10.8	<1	3.5	<2
PO, unheated	3.5	<1	<1	<2
PO, heated	14.9	6.4	2.1	4

Detection limits of the methods are 1% (by weight) for HPLC analysis of DPTG, 1.0 nmoles of MDA/g for colorimetric determination of TBA-RS, and 2 nmoles of LAHPO/g for HPLC determination of hydroperoxides of linoleic acid.

were fed for a period of 4 weeks, food consumption was recorded daily, body weights were measured 3 times per week. During the third week of the experimental period, urine and faeces were collected daily and stored at -20 °C until analysis.

After 4 weeks on the experimental diet, rats received i.p. 1 ml of 7.5 mg/ml 5-bromodeoxyuridine (BrdU; Serva, Heidelberg, FRG) dissolved in saline solution (0.9% NaCl), between 8 and 10 a.m., and were sacrificed 4 h later. The rats were killed under ether anaesthesia by exsanguination. Blood was collected in heparinized tubes and centrifuged 10 min at 2000 g, plasma was stored at -20 °C until analyses. Intestinal organs were dissected, opened lengthwise, cleaned from their contents, rinsed in saline solution, fixed in 70% ethanol, and stored at 4 °C in the dark. Liver and kidneys were removed, rinsed, blotted dry, and weighed.

Clinical biochemistry of plasma

Clinical biochemistry parameters were determined in rat plasma routinely using a centrifuge analyzer (type Cobas Bio, Hoffmann LaRoche), and comprised urea, creatinin, lactate dehydrogenase (LDH; E.C. 1.1.1.27), alkaline phosphatase (AP; E.C. 3.1.3.1), creatine phosphokinase (CPK; E.C. 2.7.3.2), glutamic oxaloacetic transaminase (GOT; E.C. 2.6.1.1) and glutamic pyruvic transaminase (GPT; E.C. 2.6.1.2).

Table 8.2. Composition of purified experimental diets

Ingredient	% in diet (by weight)
Acid casein	25.5
Corn starch	48.0
Cellulose	4.3
Sunflower oil	5.0
Frying fat ¹	10.0
Mineral mix ²	4.6
Standard vitamin mix ³	1.0
Standard trace element mix ³	1.0
dl-methionine	0.2
Choline Chloride 50%	0.4

Energy density of the diet was 15.75 kJ/g (3.75 kcal/g).

1. Added frying fat was either CO, unheated (control) or heated, or PO, unheated (control) or heated.
2. Mineral mix supplied 0.71% Ca, 0.55% P, 0.17% Mg, 0.12% Na, 0.59% K and 0.53% Cl to the diet.
3. Standard vitamin and trace element mixes supplied per kg of diet: 18,000 IU vitamin A (retinyl acetate); 2,000 IU cholecalciferol; 10 mg menadione sodium bisulfite; 6.25 mg dl-alpha-tocopherol acetate; 20 mg thiamine mononitrate; 12 mg riboflavin; 15 mg pyridoxine HCl; 40 mg niacin; 35 mg Ca dl-pantothenate; 50 µg cyanocobalamin; 300 µg biotin; 8 mg folic acid; 500 µg inositol; 115 mg Fe; 16.5 mg Cu; 62 mg Mn; 47 mg Zn; 180 µg Se; 0.50 Cr; 0.90 mg F; 0.38 mg I.

Analyses of urine and faeces

All urine and faeces samples collected during the third week of the experimental diet were pooled per rat prior to analysis. TBA-RS in urine and faeces were considered to reflect excretion of various lipid oxidation products (Gutteridge, 1982; Esterbauer and Zolner, 1989). TBA-RS in urine were determined as described previously (Hageman et al., 1989b). Urinary pH was determined by potentiometry. Faecal TBA-RS excretion was determined as follows: 1 g of faeces was homogenized with 5 ml 5% (w/v) trichloroacetic acid (TCA). Suspensions were centrifuged, after which 1 ml of TBA reagent (0.5% (w/v) TBA and 0.01% butylated hydroxytoluene in 2.5% (w/v) TCA solution) was added to 1 ml of supernatant. The reaction mixtures were heated at 100 °C for 1 h. After cooling, reaction mixtures were extracted with 2 ml n-butanol. Absorption of the butanol extracts was determined spectrophotometrically at 532 nm, and at 520 nm for correction of non-specific baseline absorption. A calibration curve was determined with concentrations of malondialdehyde-bis (diethylacetate) using the same procedure. Concentrations of TBA-RS were calculated as nmoles of malondialdehyde per g of faeces. Faecal pH was measured in supernatant of 1 g material which was homogenized 1:2 in saline solution.

Mutagenicity screening of urine and faeces

Acetone extracts of urine were prepared as follows: XAD-2 resin was washed as described by Yamasaki and Ames (1982). Volumes of 5-18 ml, representing 40% of the weekly excreted volume were concentrated on a column containing 1 ml of XAD-2 resin, which was subsequently eluted with 15 ml demineralized distilled water, followed by 10 ml acetone. Acetone extracts were dried under a stream of nitrogen, and residues were dissolved in 0.6 ml dimethylsulphoxide (DMSO) prior to mutagenicity testing. A solvent control prepared by concentrating and extracting 10 ml phosphate-buffered saline according to the protocol used for urine samples, was also included in mutagenicity testing. Mutagenicity of aliquots of 0.1 ml extracts dissolved in DMSO was determined in triplicate using *Salmonella* tester strains TA97 and TA100 as described previously (Hageman et al., 1989b). Since PF of frying oils was not activated in the presence of a liver metabolizing system, urine extracts were tested without S9 mix.

Table 8.3. Fatty acid composition of triglycerides of the experimental diets.

Fatty acid	SSO unheated	CO unheated	CO heated	PO unheated	PO heated
C10:0	-	2.6	2.4	-	-
C12:0	0.1	42.2	42.5	0.3	0.2
C14:0	0.2	19.7	19.7	0.2	0.2
C16:0	7.6	10.8	11.0	7.3	7.9
C16:1	0.2	0.1	0.1	0.2	0.3
C18:0	5.7	3.8	3.9	5.4	5.6
C18:1	21.3	10.0	10.2	20.6	21.5
C18:2	61.9	9.9	9.2	63.3	61.0
C18:3	0.2	0.1	0.1	0.1	0.1
C20:0	0.5	0.1	0.1	0.5	0.5
C20:1	0.3	-	-	-	-
C22:0	1.0	-	-	-	-
C24:0	0.1	-	-	-	-
unidentified	0.7	0.7	0.8	2.1	2.7

Values are expressed as percent of total triglycerides. Experimental diets contained 10% (by weight) unheated or heated coconut oil (CO) or PUFA-rich frying oil (PO) and 5% unheated sunflower seed oil (SSO). Fatty acid composition of triglycerides was determined by gas liquid chromatography (GLC) of the methyl esters of fatty acids. Triglycerides were hydrolyzed and fatty acids were methylated using a 14% (w/v) boron trifluoride (BF₃, Sigma, St. Louis, MO) solution in methanol. Methyl esters were analyzed by GLC as described by Rand et al. (25).

Faecal extracts for mutagenicity testing were prepared as follows: In a flask, 2 g of faeces and 2 g of anhydrous sodium sulphate were homogenized in 10 ml dichloromethane (DCM) using a turrax mixer for 30 s, under cooling with ice. Contents was transferred to a centrifuge tube, and after centrifugation and filtration of the supernatant through Whatman no. 4 filter paper, the residue was dried under a stream of nitrogen. Residues were dissolved in 0.8 ml methanol prior to mutagenicity testing. Solvent control was prepared by extraction of 2 g anhydrous sodium sulphate with 10 ml dichloromethane according to the protocol used for faecal extracts. Aliquots of 0.1 ml of faecal extracts in methanol were screened for mutagenicity in duplicate using strains TA97 and TA100, in absence as well as in presence of S9 mix in order to determine presence of possible pre-mutagens excreted or produced in faeces.

Preparation of tissues for determination of cell proliferation

Oesophagus, forestomach and glandular stomach tissues were processed for determination of the labeling index (LI) and potential doubling time (Tpot) by flow cytometry as described by Verhagen et al. (1988). Briefly, 4 samples per tissue per rat were randomly taken from the ethanol-fixed tissues and minced, after which single nuclei were released by pepsin digestion. Suspensions were filtered to remove cellular debris, and subsequently DNA was denatured by treatment with 2N HCl. Remaining nuclei were incubated with monoclonal anti-BrdU (clone IIB5; Schutte et al., 1987), followed by fluorescein-conjugated rabbit-anti-mouse IgG-FITC antibodies (F313; Dakopatts, Denmark). Total DNA was stained with propidium iodide (Calbiochem, Behring Diagnostics, San Diego, CA). Double stained nuclei were analyzed on a FACS IV cell sorter (Becton and Dickinson, CA). The LI was determined as the percentage of BrdU-positive cells. Tpot was calculated as described by Begg et al. (1985).

Since analysis by flow cytometry of colon/rectum was found to be not feasible, LI of this tissue was determined by immunohistochemistry, applying peroxidase-conjugated secondary antibody to detect BrdU-containing cells as described previously (Schutte et al., 1987; Verhagen et al., 1989). Briefly, 5 samples equally distributed over the full length of the colon/rectum were dissected and embedded in paraffin, after which 4 µm sections were cut and mounted to microscope slides. Before staining, slides were cleared of paraffin with xylene and treated with 0.3% H₂O₂ in methanol for 10 min to block endogenous peroxidases. Pepsin digestion and denaturation of DNA were essentially the same as described for flow cytometry. Instead of using rabbit-anti-mouse IgG-FITC antibodies, tissue sections were finally incubated with peroxidase-conjugated rabbit-anti-mouse IgG antibodies (F114, Dakopatts, Denmark) for 1 h at room temperature. The antibody binding sites were visualized by immersing the slides in 0.04% 3,3'-diaminobenzidine in 0.05 M Tris/HCl pH 7.6 containing 0.01% H₂O₂. Finally, sections were counterstained with haematoxylin. BrdU-positive cells appeared brown, while non-labeled cells were colored blue. Slides were coded and 20 full crypts per colon/rectum were evaluated by 2 independent observers. The total number of cells per crypt, as well as the number of BrdU-positive cells and the position of the uppermost labeled cell in the crypt column were recorded. LI was calculated as the percentage of positively stained cells, using data of both observers.

Statistical analyses

Differences between groups were evaluated using single-factor analysis of variance; in addition, multiple comparisons were made between groups using Dunnett's t-test. Comparisons were made between groups receiving unheated and heated oils: unused CO vs. heated CO and unused PO vs. heated PO, as well as between groups receiving unused oils: unheated CO vs. unheated PO, and between groups fed heated oils: heated CO vs. heated PO, to determine specific effects to the type of oil. Associations between various biological effects and characteristics of the oils consumed were analyzed using Spearman's rank correlation coefficient.

Results

General indices of toxicity

Feeding of deep-frying oils, heated for 30 h and used for preparation of various foods, at a level of 10% in the diet of rats did not result in growth retardation, since mean food consumption and weight gain were similar in all groups (Table 8.4). No pathologic conditions were indicated by relative liver and kidney weights, which were not found to differ between groups fed heated and unheated oils. Compared to the group fed unheated PO, significantly increased plasma activities of GPT ($p < 0.05$), and AP ($p < 0.01$) were observed in the group fed heated PO, possibly indicating adverse effects on cells in liver and kidneys. Plasma AP was also increased ($p < 0.01$) in rats consuming heated PO as compared to the group fed heated CO. Concentration of urea in plasma of the group consuming heated CO was significantly increased ($p < 0.05$) as compared to the group fed unheated CO. Plasma creatinin was significantly lower ($p < 0.05$) in the group fed unheated PO as compared to the group receiving unheated CO. Although some differences were noted, plasma clinical biochemical parameters were within the normal range reported in the literature (Benirschke et al., 1978). Generally, rats fed heated PO showed a higher plasma clinical biochemical parameters related to hepatic and renal cellular damage, than rats fed unheated oils and heated CO.

TBA-RS and pH in urine and faeces

Excretion of TBA-RS in urine was found to be significantly increased ($p < 0.01$) in the group consuming heated PO as compared to unheated PO (Table 8.5). No difference in urinary excretion of TBA-RS was observed between groups fed unheated and heated CO, nor between the 2 control groups. Rats receiving heated PO excreted significantly higher ($p < 0.05$) amounts of TBA-RS in urine as compared to rats consuming heated CO. No differences in faecal excretion of TBA-RS were found between the groups (Table 8.5). Urinary pH was found to be slightly, but not significantly elevated in groups fed heated fats in comparison to the control groups (Table 8.5).

Table 8.4. Food consumption, weight gain, and results of general toxicity testing after short-term consumption of diets containing 10% unheated or heated frying oils by rats.

Parameter	Diet group			
	CO control (n=7)	CO heated (n=7)	PO control (n=7)	PO heated (n=7)
Mean food consumption (g/day)	12.5 ±0.3	2.8 ±0.3	12.4 ±1.2	12.6 ±0.8
Mean weight gain (g)	168 ±12	168 ±10	175 ±21	171 ±16
Relative liver weight (% body weight)	3.46 ±0.22	3.53 ±0.16	3.46 ±0.17	3.46 ±0.21
Relative kidney weight (% body weight)	0.72 ±0.06	0.76 ±0.06	0.76 ±0.05	0.75 ±0.04
Glutamic pyruvic trans- aminase (U/l)	12.9 ±2.3	12.3 ±5.2	11.2 ±2.7	14.4 ^a ±2.8
Glutamic oxaloacetic transaminase (U/l)	39.9 ±6.6	36.3 ±9.4	40.0 ±8.9	43.5 ±6.7
Creatine phosphokinase (U/l)	188 ±65	117 ±70	211 ±111	205 ^c ±69
Alkaline phosphatase (U/l)	407 ±77	375 ±37	357 ±68	464 ^{bd} ±55
Lactate dehydrogenase (U/l)	601 ±214	340 ±270	719 ±330	565 ±224
Plasma urea (mmol/l)	3.4 ±0.3	4.0 ^a ±0.6	4.0 ±0.8	4.9 ±1.3
Plasma creatinin (μmol/l)	35.4 ±1.5	34.1 ±4.5	31.3 ^c ±4.8	36.1 ±3.9

Values are expressed as mean±SD. Significant differences between groups are indicated with superscripts: CO control vs. CO heated, and PO control vs. PO heated: ^a: $p < 0.05$; ^b: $p < 0.01$; CO control vs. PO control, and CO heated vs. PO heated: ^c: $p < 0.05$; ^d: $p < 0.01$.

Table 8.5. Excretion of thiobarbituric acid-reactive substances (TBA-RS) and pH in urine and faeces of rats fed diets containing 10% unheated or heated frying oils.

Parameter	Diet group			
	CO control (n=7)	CO heated (n=7)	PO control (n=7)	PO heated (n=7)
TBA-RS urine (nmol MDA/wk)	0.11 ±0.04	0.11 ±0.03	0.12 ±0.02	0.14 ^{bc} ±0.01
TBA-RS faeces (nmol MDA/wk)	1.35 ±0.13	1.33 ±0.44	1.44 ±0.21	1.52 ±0.19
pH urine	8.02 ±1.10	8.70 ±0.43	8.30 ±1.31	8.89 ±0.46
pH faeces	8.29 ±0.16	8.10 ±0.25	8.33 ±0.06	8.14 ^b ±0.13

Results are expressed as mean±SD. Significant differences between groups are indicated with superscripts, for an explanation of symbols used, see legend of Table 8.4.

Faecal pH was lower in groups receiving heated fats; a significantly lower ($p<0.05$) faecal pH was observed in the group fed heated PO as compared to unheated PO (Table 8.5).

Mutagenicity of urine and faeces

Mutagenic activity of acetone extracts of urine is shown in Figure 8.1. All urine extracts exhibited significant mutagenic activity to strain TA97 when compared to the solvent control, but excretion of mutagens did not differ between the experimental groups. All urine extracts were less mutagenic to strain TA100; significantly increased mutagenic activity was detected exclusively in urine extracts of rats fed heated PO (Figure 8.1). Excretion of mutagens detected by strain TA100 was significantly increased ($p<0.05$) in the group fed heated PO, as compared to the group receiving unheated PO. DCM-extracts of faeces exhibited no significantly increased mutagenic activity to strain TA97 as compared to the solvent control (Figure 8.2.A). Numbers of revertants appeared to be slightly, but not significantly, reduced in faecal incubations.

Comparison between groups showed significantly increased numbers of

revertants in strain TA97 without S9 mix for faecal extracts of rats fed heated PO as compared to their control group ($p < 0.05$), as well as compared to the group fed heated CO ($p < 0.01$). No differences were observed between mutagenicity of faecal extracts to strain TA97 in presence of S9 mix (Figure 8.2.A). Faecal extracts of all groups, did not significantly increase numbers of revertants in strain TA100, both in absence and presence of S9 mix, as compared to the solvent controls (Figure 8.2.B). Feeding of heated CO nor of heated PO resulted in increased faecal mutagenicity to strain TA100 as compared to the unheated oils. The number of revertants to strain TA100 induced by faecal extracts of rats fed heated PO was however, significantly increased as compared to faecal extracts of rats fed heated CO, both in absence and in presence of S9 mix ($p < 0.05$ and $p < 0.01$, respectively). Mutagenicity of faecal extracts to strain TA97 and TA100 appeared not to be influenced by the addition of the hepatic enzyme system (Figure 8.2). Data indicate that faecal mutagenicity was not affected by intake of mutagenic deep-frying oils. However, consumption of heated PO, which was the only oil showing mutagenic activity to strain TA100, was found to increase urinary mutagenicity to this strain.

Effects on cell proliferation

Labeling index (LI) and potential doubling time (Tpot) of oesophagus, forestomach and glandular stomach as measured by flow cytometry, and LI and number of cells per crypt of colon/rectum as determined by immunohistochemistry, are presented in Table 8.6. LI and Tpot of the tissues of the gastro-intestinal tract of rats fed heated CO did not differ significantly from those of the control group receiving unheated CO. Rats fed heated PO showed increased LI's and decreased Tpot's in the oesophagus ($p < 0.01$) and forestomach ($p < 0.05$) as compared to rats fed unheated PO. LI of glandular stomach and colon/rectum of the group fed heated PO were not statistically significantly increased. The number of cells/crypt was significantly decreased ($p < 0.05$) in rats fed heated PO as compared to their control group. No differences were observed between the groups with respect to the position of the uppermost labeled cells in colonic crypts. Comparison between the two groups fed heated oils, revealed significantly increased LI's and decreased Tpot's in the upper gastro-intestinal tissues of the group fed heated PO, which were significant for the oesophagus ($p < 0.05$) and glandular stomach ($p < 0.01$). No differences were observed between labeling indices of intestinal tissues of the groups fed unheated oils, indicating that the type of unheated oil had no effect on cell proliferation in the gastro-intestinal tract. Results indicated that consumption of heated PO stimulated cell proliferation in the upper gastro-intestinal tissues of rats.

Figure 8.1: Mutagenic activity of acetone extracts of urine to strain TA97 (▨) and TA100 (■). The equivalent of 6.5% of the weekly excreted volume was tested per plate, dissolved in 0.1 ml DMSO. Results are shown as mean±SD of groups fed unheated control CO (COC), unheated control PO (POC), heated CO (COH) and heated PO (POH); solvent control (control) is expressed as mean±SD of 3 plates. Significant differences between groups are indicated as follows: COH vs. COC, and POH vs. POC: * $p < 0.05$.

Origin of mutagen and TBA-RS excretion

In Table 8.7 weekly intake and excretion of mutagens and TBA-RS are compared. Mutagen intake, which varied considerably between groups, appeared not to explain mutagen excretion in urine and faeces as determined with strain TA97, since all groups excreted similar levels of mutagenicity. Data presented in Table 8.7 also show that mutagens to strain TA97, which were found to be induced during heating of the oils, were not recovered from urine and faeces after consumption. Mutagen excretion in urine and faeces determined with strain TA100 appeared to be associated with intake, since the group fed heated PO, exhibiting mutagenic activity in strain TA100, showed increased mutagen excretion in both urine and faeces. However, recovery of mutagenicity to strain TA100 was low, being 4% in urine and 3% in faeces. Excretion of TBA-RS in urine and faeces was not found to be related to dietary intake. TBA-RS were detected in urine and faeces of groups fed unheated CO and PO as well, indicating these may be derived from endogenous sources. Highest excretion of TBA-RS was observed in urine and faeces of the group fed heated PO. After correction for endogenous production of TBA-RS using values of groups fed unheated oils, effects of dietary TBA-RS on excretion appeared to be minimal, since only 0.1% of TBA-RS of heated PO was recovered from urine and faeces. Consumption of TBA-RS present in heated CO did not lead to increased excretion in urine or faeces, in spite of a higher dose as compared to heated PO.

Figure 8.2: Mutagenic activity of dichloromethane extracts of faeces: (A) to strain TA97 without S9 mix (□) and with S9 mix (▨); (B) to strain TA100 without S9 mix (▤) and with S9 mix (■). The equivalent of 0.22 g faeces was tested per plate. Significant differences between groups are indicated as follows: COH vs. COC, and POH vs. POC: * $p < 0.05$; POC vs. COC, and POH vs. COH: + $p < 0.05$, ++ $p < 0.01$. For an explanation of abbreviations used, see legend of Figure 8.1.

Table 8.6. Cell kinetic parameters in oesophagus, forestomach, glandular stomach and colon/rectum of rats fed diets containing 10% of unheated or heated frying oils for 4 weeks.

Tissue	Parameter	Diet group			
		CO control	CO heated	PO control	PO heated
Oesophagus	LI (%)	8.5	8.5	7.8	9.9 <i>bc</i>
		±1.4	±1.1	±1.3	±1.1
	Tpot (days)	2.8	2.7	2.8	2.1 <i>bc</i>
		±0.5	±0.4	±0.5	±0.1
Forestomach	LI (%)	9.1	10.0	7.8	9.8 <i>a</i>
		±2.3	±2.2	±1.3	±2.0
	Tpot (days)	2.8	2.4	2.8	2.3
		±0.9	±0.8	±0.6	±0.5
Glandular stomach	LI (%)	3.4	3.6	3.7	4.3
		±0.9	±0.7	±0.6	±0.8
	Tpot (days)	7.5	7.2	5.6	5.0 <i>d</i>
		±2.6	±1.5	±1.1	±0.8
Colon/rectum	LI (%)	9.9	9.1	9.8	10.8 <i>c</i>
		±2.5	±1.4	±1.4	±1.5
	Cells/crypt	67.5	66.4	68.7	64.8 <i>a</i>
		±3.9	±3.0	±4.2	±2.0
	Upper labeled cell in crypt (position)	13.7	14.2	14.1	13.4
		±1.3	±0.8	±1.5	±1.2

Results are expressed as mean±SD. LI and Tpot of oesophagus, forestomach and glandular stomach were measured by flow cytometry; LI and number of cells per crypt of colon/rectum were determined by immunohistochemistry. Significant differences are indicated with superscripts, for an explanation of symbols used, see legend of Table 8.4.

Table 8.7. Comparison of intake and excretion of mutagens and lipid oxidation products of rats fed diets with 10% unheated or heated oils.

Parameter	Diet group	Intake	Excretion	
		diet	urine	faeces
Mutagenicity to TA97-S9 ($\times 10^4$ rev/wk)	CO, unheated	ND [†]	0.37 ± 0.31	-0.19 ± 0.24
	CO, heated	3.39 ± 0.06	0.16 ± 0.15	-0.12 ± 0.06
	PO, unheated	ND	0.38 ± 0.63	-0.07 ± 0.06
	PO, heated	2.81 ± 0.19	0.40 ± 0.29	0.01 ± 0.07
Mutagenicity to TA100-S9 ($\times 10^4$ rev/wk)	CO, unheated	ND	0.00 ± 0.01	0.02 ± 0.04
	CO, heated	ND	0.02 ± 0.03	-0.04 ± 0.01
	PO, unheated	ND	0.00 ± 0.01	0.03 ± 0.14
	PO, heated	1.60 ± 0.08	0.06 ± 0.07	0.08 ± 0.12
TBA-RS (nmoles MDA/wk)	CO, unheated	ND	0.11 ± 0.04	1.35 ± 0.13
	CO, heated	31.6 ± 1.3	0.11 ± 0.03	1.33 ± 0.44
	PO, unheated	ND	0.12 ± 0.02	1.44 ± 0.21
	PO, heated	18.5 ± 1.2	0.14 ± 0.01	1.52 ± 0.19

Values are expressed as mean $\pm$ SD.

[†] ND, not detectable.

Lipid oxidation, mutagenicity and cell proliferation

Using data of individual rats, neither urinary pH, nor excretion of TBA-RS were found to be associated with mutagenicity of acetone extracts of urines. Neither was mutagenicity of faecal extracts observed to be correlated with faecal pH and excretion of TBA-RS. Individual intake of mutagens, TBA-RS, LAHPO nor DPTG was found to be associated with urinary and faecal mutagen and TBA-RS excretion, no significant correlations were found when data of the group fed heated PO, containing DPTG and LAHPO, were processed only. LI of the upper gastro-intestinal tissues was not found to be associated with mutagen and TBA-RS excretion in urine or faeces, nor with intake. No significant correlations were observed between LI and number of cells per crypt of the colon/rectum, and mutagenicity of faecal extracts or intake of mutagens, TBA-RS, LAHPO and DPTG, neither when data of the group fed heated PO were analyzed separately. LI and number of cells per crypt of colon/rectum were not found to be associated with faecal pH or TBA-RS concentration either.

Discussion

Short-term feeding of heated fats commonly used for frying of foods, did not appear to cause growth retardation, nor increase liver and kidney weights when administered to rats a level of 10% in the diet during 4 weeks. Slightly, but significantly increased plasma activities of GPT and AP, which were observed in the group fed heated PO as compared to the other experimental groups, however, suggested that short-term feeding of oxidized frying fats may affect cells in liver and kidneys. Lipid oxidation products are considered to cause the observed effects, since it has been reported that feeding rats with polar fraction of heated sunflower oil at a dose of 20% in the diet during 18 months caused growth retardation, elevated levels of GPT and GOT, and increased liver and kidney weights (Billek et al., 1979). A single dose of 1.2 g of secondary autooxidation products of linoleic acid to rats was also found to increase plasma GPT, liver weight and hepatic concentrations of lipid peroxides (TBA-RS) 12 to 24 h later (Kanazawa et al., 1985; 1986). The fact that the doses of oxidation products of linoleic acid administered in the present study, which were estimated by loss of linoleic acid in heated oils to be maximally 20 mg/day, were considerably lower, and the presence of alpha-tocopherol in the experimental diets, may explain the absence of an effect on liver and kidney weights and the marginal effects observed on plasma clinical biochemical parameters. TBA-RS of heated frying oils appeared not to be associated with adverse affects, since feeding of heated CO which contained slightly higher amounts of TBA-RS as compared to heated PO, did not show elevated levels of GPT and AP. Since LAHPO and DPTG were present in heated PO

but not in heated CO, it is suggested that lipid oxidation products derived from linoleic acid may specifically be associated with the observed effects on plasma clinical biochemical indices.

In the present study, feeding of heated PO was shown to enhance cell proliferation in tissues of oesophagus and forestomach, whereas heated CO did not affect cell proliferation of the gastro-intestinal tract. Stimulation of cell proliferation by heated PO appeared to be exerted predominantly in the upper gastro-intestinal tract; the decreased number of cells/crypt in the colon/rectum of rats fed heated PO was found to originate from a decrease of the non-proliferating compartment and may indicate an adaptive response to lytic effects caused by heated PO during the first week(s) of feeding of the experimental diet (Wargovich and Lointier, 1987). Enhancing effects exerted on cell proliferation by heated PO in tissues of oesophagus and forestomach, but not in glandular stomach and colon, indicated that these effects might have been caused by substances which were either highly reactive and not stable enough to reach the lower gastro-intestinal organs, or that were readily absorbed by mucosal cells of the oesophagus and forestomach. The nature of these substances is not clear at present, but may be related to oxidation and degradation products originating from linoleic acid, which was indicated by the presence of LAHPO in heated PO. The upper part of the gastro-intestinal tract may be considered as possible target tissues for effects observed after oral administration of LAHPO, since it has been reported that 12 to 24 h after administration of a single dose of ^{14}C -labeled methyl linoleate hydroperoxide by gastric intubation to rats, appreciable amounts of radioactivity were recovered in stomach, liver and urine, but recovery in the large intestine was only 3-5% (Bergan and Draper, 1971; Oarada et al., 1986). In contrast, ^{14}C labeled polymeric oxidation products were observed to be excreted largely in faeces (53%), although the stomach still contained approximately 1% of the orally administered labeled compounds 12 h after administration (Oarada et al., 1986). Since no significant stimulation of cell proliferation was observed in the colon of the group fed heated PO, it is suggested that DPTG present in heated PO may not be biologically active in the colon.

Hydroperoxides of linoleic acid or other oxidative degradation products and metabolites of polyunsaturated fatty acids were considered to be involved in stimulation of cell proliferation observed in the upper gastro-intestinal tissues of rats fed heated PO, since these have been reported to be capable of enhancing cell proliferation in intestinal tissues (Bull et al., 1984; 1988; Craven et al., 1987; Craven and Rubertis, 1988). Increased cell proliferation, DNA synthesis and induction of ornithine decarboxylase were reported in the colon after acute intrarectal administration of hydroperoxy and hydroxy-derivatives of unsaturated fatty acids dissolved in 1.0 ml saline solution at concentrations ranging from 5 to 10 mM (Bull et

al.,1984; 1988). In addition, it was demonstrated that intracolonicallly installed free arachidonate, linoleate and oleate dissolved in 2.0 ml saline at concentrations of 1-5 mM increased colonic DNA synthesis and mucosal ornithine decarboxylase activity (Craven et al.,1987; Craven and Rubertis,1988). In the present study, mean daily consumption of LAHPO was approximately 5 nmoles with the diet containing heated PO, and was found to be below the dose needed for *in situ* stimulation of cell proliferation in the colon. This may indicate a higher sensitivity of tissues of the forestomach and oesophagus for cell proliferation enhancing effects of lipid oxidation products. The absence of a correlation between individual consumption of LAHPO, TBA-RS and DPTG, and labeling indices, and the fact that short-term consumption of heated deep-frying oil does increase cell proliferation in the upper gastro-intestinal tissues, suggests that other oxidation or degradation products which were not determined in our study, may have been involved. These may also include free oleic and linoleic acids which may be produced by hydrolysis of triglycerides during heating of oil (Stevenson, 1984).

Stimulation of mucosal cell proliferation in the colon by high-fat diets is generally considered to be a secondary response to cell loss caused by nonspecific tissue injury by fatty acids or bile acids (Bruce, 1987; Wargovich and Lointier, 1987). Lipid oxidation products and oxidized fats however, were reported to damage tissues of liver and kidneys after oral administration (Izaki et al., 1983; Kanazawa et al., 1986; 1987), and therefore a similar mechanism may be considered to be involved in the enhancing effects exerted on oesophageal and forestomach tissues by heated PO in the present study. The mechanism by which unsaturated fatty acids stimulate cell proliferation in the intestine has also been postulated to involve activation of protein kinase C (Craven et al., 1988), a regulator of cell growth and many other functions (Anderson et al., 1985). Activation of protein kinase C may be mediated either through reactive oxygen species which has been reported for tumor promoters of the phorbol ester type (Craven et al., 1987; Weinstein, 1988), as well as by increased phosphoinositide breakdown, resulting in the release of diacylglycerols which directly activate protein kinase C (Rozengurt, 1984). Stimulation of cell proliferation may also be mediated through prostaglandin synthesis (Craven et al., 1983; Narisawa et al., 1987), and reactive oxygen species generated in cyclooxygenase or lipoxygenase pathways (DeRubertis et al., 1984; Craven et al., 1986). Since heated frying oils may contain precursors for several of the proposed stimulating mechanisms, and none of the oxidative parameters measured in diet as well as in urine and faeces, was found to be associated with stimulation of cell proliferation, no conclusion can be drawn with respect to the mechanisms by which heated PO enhanced cell proliferation in the present study. Neither mutagenicity of heated oils, nor mutagenicity of urine and faeces were found to be

associated with cell proliferation in gastro-intestinal tissues, indicating that compounds causing mutagenic activity of the oils were not involved in enhancement of cell proliferation.

Excretion of TBA-RS in urine and faeces was found to be minimal as compared to TBA-RS present in diet, being maximally 0.1% of the dose consumed. These data indicate that >99% of oxidation products were either retained in the tissues or inactivated by *in vivo*; these data do not suggest induction of *in vivo* lipid oxidation after short-term consumption of heated oxidized oils. It should be noted however, that levels of TBA-RS and LAHPO in heated oils in the present study were low in comparison with concentrations observed in a previous study in commercially used deep-frying oils, which contained levels of TBA-RS up to 161 nmoles MDA/g of fat (Hageman et al., 1988), and concentrations of LAHPO up to 267 nmoles/g of fat (Hageman et al., 1989a). Since the present data showed that consumption of low amounts of oxidation products significantly increased urinary concentrations of TBA-RS in combination with increased plasma activities of GPT and AP in rats fed heated PO as compared to rats fed unheated PO, chronic consumption of 5- to 10-fold higher doses of lipid oxidation products may be of concern with respect to oxidative damage to tissues.

Mutagenicity occurring in heated PO which was analyzed specifically by strain TA100, appeared to be detectable in urine and faeces after oral intake, and amounted to 7% of the ingested dose. This suggests that compounds present in heated frying oils and causing base-pair mutations in strain TA100, may not be totally inactivated after absorption. Consumption of frying oils containing TA97 mutagens did not increase excretion in urine and faeces, suggesting frame-shift mutagens may either be efficiently deactivated *in vivo*, or retained in the tissues. Although urine extracts exhibited significant mutagenicity to strain TA97, this was not related to mutagenicity of the oils consumed by the rats. Since no significant correlations were found between urine mutagenicity to either strain TA97 or TA100, and urinary pH or excretion of TBA-RS, the nature of these excreted mutagens remains to be determined.

Concluding, results of the present study indicate that heated deep-frying oils, containing autooxidation products, are capable of stimulating cell proliferation in the gastro-intestinal tract. The proliferation enhancing effects observed in this study however, do not appear to be extended to the colon/rectum, and are not directly pointing to an involvement of heat-processed lipids in colon tumorigenesis. Since data obtained in this study are limited to testing of a single dose of frying oils, and the nature of active compounds exerting an effect in the upper gastro-intestinal tissues is still unclear at present, determination of effects of thermally oxidized oils on

colon tumorigenesis therefore, requires further investigation. For a reliable risk estimation these studies will also have to include determination of interactions of heated oxidized lipids with other dietary factors, both possible initiators as well as inhibitors, that may be involved in the process of colon carcinogenesis (Bruce, 1987).

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Chapter 9

General discussion and concluding remarks

Heat-processing of food and colon cancer

High-meat and high-fat consumption have been recognized as dietary factors associated with increased cancer mortality and incidence of colonic and mammary tumors in populations of industrialized countries as compared to developing countries (Armstrong and Doll, 1975; Rose et al., 1986). Only a few dietary components however, have been isolated and identified which possess carcinogenic properties and may be causally related to tumorigenesis. Heat-processing of foods may be a factor of special importance since during heat-treatment degradation products are formed, which may possess toxic properties. With respect to intake of meat and meat products it appears of relevance that frying and broiling of proteinaceous foods has been demonstrated to result in formation of carcinogenic amino acid pyrolyzates (Sugimura et al., 1986), and at extremely high temperatures during broiling and roasting of meats and other foods, carcinogenic polycyclic aromatic hydrocarbons (PAHs) may be produced (Phillips, 1983). These compounds are considered to pose a serious risk to human health, but strong epidemiological or human toxicological evidence for a causal relation between high intake of heat-processed meat and tumor incidence is still lacking (Archer, 1988; Byers, 1988; Rogers and Longnecker, 1988; Willet, 1989). High intake of dietary fat has also been associated with increased cancer risk, especially of the colon/rectum, but epidemiologic studies have shown inconsistent results (Byers, 1988; Hill, 1987; Willet, 1989).

In this thesis it has been hypothesized in Chapter 1 that heat-processing of lipids may be one of the factors of relevance for the relation between dietary fat and cancer of the colon/rectum. It has been argued that thermal degradation and oxidation products of dietary lipids which possess mutagenic properties may be involved in tumorigenesis. With the exception of malondialdehyde (MDA), fatty acid oxidation and degradation products have not been tested for carcinogenicity. MDA, a secondary product of lipid oxidation has been reported to induce hepatic neoplasia in mice (Draper et al., 1982; Siu and Draper, 1983). Furthermore, experimental studies with animals have revealed a promoting effect on colon tumorigenesis of high intake of various dietary fats (Carroll, 1987; Reddy, 1986; Rogers and Longnecker, 1988), but tumor enhancing or initiating activities of heat degradation products of dietary lipids have not yet been studied. The aim of this thesis was therefore to investigate the possible involvement of heat-

Table 9.1. Overview of type and source of various fats used for repeatedly frying, treatment conditions and chemical parameters of oxidative deterioration.

Fat no.	type/source ¹	Treatment			Parameters of oxidative deterioration					
		additives ¹	heating time (h) ²	tempera- ture (°C) ³	foods prepared ⁴	PF5 (% by weight)	DPTG (% by weight)	TBA-RS (nmoles of MDA/g)	LAHPO (nmoles /g)	Ch.
1-20.	various	not known	not known; commercially used fat samples, sensory indication of abuse			3-44	<1-32	<1-177	<10-267	3,4
21.	partly hydroge- nated vegetable oils ^{a7}	polydime- thylsiloxane (E900)	0-40	180	various meat products, frozen pre-fried potatoes	6-40	NA ⁶	4-14	NA	3
22.	partly hydroge- nated vegetable oils ^a	E900	0-40	180	frozen, pre-fried potatoes	4-12	<1-3	<1-<1	NA	6
23.	partly hydroge nated vegetable oils ^b	E900, flavors and fragrances	0-100	180	frozen, pre-fried potatoes	4-35	NA	NA	NA	6
24.	vegetable oils (>60% polyunsaturated fatty acids; PUFA) ^c	E900	0-30	180	freshly sliced potatoes	3-30	<1-27	2-17	<2-62	5
25.	partly hydroge- nated vegetable oils ^b	E900, flavors and fragrances	0-30	180	freshly sliced potatoes	4-17	NA	NA	NA	5

Table 9.1.- continued

26.	sunflower seed oil	-	0-30	180	freshly sliced potatoes	3-25	NA	NA	5
27.	vegetable oils (>60% PUFA)	E900	0-30	180	various meat products, freshly sliced potatoes	4-15	<1-6	<1-2	5
28.	fractionated beef tallow	-	0-30	180	various meat products, freshly sliced potatoes	<1-18	NA	NA	5
29.	coconut oil	-	0-30	180	various meat products, freshly sliced potatoes	5-11	<1-<1	<1-4	5
30.	hydrogenated animal and vegetable oils	E900	0-30	180	various meat products, freshly sliced potatoes	2-18	NA	NA	5

1 Source and type of fat, as well as additives used are based on declarations of manufacturers.

2 Fats were heated 8-10 h/day and were allowed to cool overnight.

3 Fats were heated in temperature-controlled home-style fryers, maximum temperature measured was 190 °C.

4 Portion sizes of foods ranged from 100-250 g, portions of food were prepared every 2 h.

5 Ranges are presented for lowest and highest values observed in samples 1-20, and of unused and used fats for samples 21-30. The amount of polar fraction (PF) was determined by silicagel column chromatography (Chapter 3); the concentrations of di- and polymeric triglycerides (DPTG) by gel-permeation HPLC (Chapter 3), detection limit of the method was 1% (by weight); concentrations of thiobarbituric acid reactive-substances (TBA-RS) were analyzed colorimetrically, detection limit of the assay was 1 nmol of MDA/g (Chapter 3); concentrations of linoleic acid hydroperoxides (LAHPO) were measured by HPLC (Chapter 4), detection limit of the method was 10 nmol/g; in later assays detection limit was 2 nmol/g (Chapter 5).

6 NA, not analyzed

7 Identical superscripts indicate the same type of fat, in various experiments however, different batches were used.

Table 9.2. Biological effects of various repeatedly used deep frying fats, of which treatment procedures and chemical parameters of oxidative deterioration are shown in Table 9.1; the numbers of the fat samples correspond to the numbers shown in Table 9.1.

Mutagenicity to Salmonella tester strain TA97-S9 mix (rev/g of fat) ¹	Other <i>in vitro</i> activities	<i>In vivo</i> activities	Ch.
1-20. 0.3-10 ⁴	also mutagenic to TA97+S9 mix, several fat samples also mutagenic to Salmonella tester strains TA100 and TA104	NA ²	3, 4
21. 2·10 ³ -5·10 ⁴ (unused fat mutagenic)	also mutagenic +S9 mix, toxic to strain TA100-S9	NA	3
22. 0.6-3·10 ³ (unused fat mutagenic)	lipid fractions of fried potatoes prepared in oil mutagenic to strain TA97±S9 mix: 0.1-2·10 ³ rev/g	after consumption of fried potatoes by 6 human volunteers no effects on urinary mutagen excretion to strain TA97±S9	6
23. 0.3-10 ³	lipid fractions of fried potatoes prepared in oil used for 100 h, mutagenic to strain TA97-S9: 0.7·10 ³ rev/g	NA	6
24. toxic (+S9 mix: toxic-4·10 ³)	- increased frequencies of thioguanine-resistant (TG ^r) human T-lymphocytes after initial testing, could not be confirmed after retesting - enhanced cell proliferation of PHA-stimulated T-lymphocytes		5
25. 1·10 ³ -0.7·10 ³	NA	NA	5
26. 0-0	NA	NA	5
27. 0.3-10 ³	- mutagenic to strain TA100-S9: 2·10 ³ rev/g; to strain TA100+S9: 1·10 ³ rev/g	- enhanced cell proliferation in oesophagus and forestomach of rats after 4-week	5, 8

Table 9.2. - continued

28.	0.2•10 ³	- induced dose-dependent increase in SCE frequencies in human lymphocytes	feeding of heated fat (10% by weight, in a semipurified diet)	5
		- no effect on frequencies of TG ⁺ lymphocytes	- increased plasma glutamic pyruvic transaminase and alkaline phosphatase	
29.	0.4•10 ³	- cytotoxic to PHA-stimulated lymphocytes	- increased urinary excretion of TBA-RS in rats	5, 7, 8
		NA	- increased urinary excretion of mutagens to strain TA100, not to strain TA97; no increased faecal mutagen excretion in rats.	
30.	1.2•10 ³	NA	NA	5
		- not mutagenic to strain TA100	- no increased urinary and faecal mutagen excretion to strain TA97 and TA100 after consumption of fried potatoes by 14 volunteers, no other adverse effects were observed in human volunteers	
31.	0.6•10 ³	- lipid fractions of potatoes prepared in oil used for 30 h mutagenic to strain TA97-S9; 0.6•10 ³ rev/g.	- no effects on cell proliferation in the gastro-intestinal tract of rats after 4-week feeding of used fat (10% in diet)	5
		- no effect of SCE and TG ⁺ frequencies in human lymphocytes	- no effects on urinary and faecal mutagen and TBA-RS excretion in rats	
32.	1.2•10 ³	- enhanced cell proliferation in PHA-stimulated T-lymphocytes	- no effects on plasma enzyme activities in rats	5
		NA	NA	

¹ Mutagenicity of the fats was determined in non-polar and polar fractions separately; dose range tested was 0.1-10 mg/plate. Numbers of revertants/g were estimated by linear regression of dose response curves and multiplication of the obtained number of revertants/mg of fraction with the total amount of non-polar and polar fraction present per g of fat. Mutagenicity of lipid fractions isolated from fried potatoes was estimated similarly. ² NA, not analyzed.

processed lipids in tumorigenesis. For this purpose, (i) deep-frying fats were tested for genotoxicity *in vitro*, (ii) biological fate of mutagenic compounds associated with deep-fat frying was assessed (iii) in rats and (iv) in man, and (v) the effects of heat-processed fats on cellular growth in the rat gastro intestinal tract were determined. Data obtained were applied to evaluate a possible involvement of heat-processed lipids in colon tumorigenesis. In Tables 9.1 and 9.2 results of the experiments described in Chapters 3 to 8 are briefly summarized, and serve as a reference to this Chapter.

Mutagenicity testing of heated deep-frying fats in *Salmonella typhimurium*

In Chapters 3 to 6 genotoxicity of repeatedly used deep-frying fats to *Salmonella typhimurium*, as well as to human lymphocytes was analyzed. In Chapter 3, repeatedly used deep-frying fats, sampled from local snack-bars and restaurants by officers of the Maastricht Office for Food Quality Control after sensory indication of abuse and showing varying degrees of oxidative deterioration, were tested for mutagenicity in the *Salmonella*-/microsome assay. Results of this study showed that several repeatedly used deep-frying fats do possess mutagenic activity to strains TA97, TA100 or TA104. Mutagenic compounds appeared to be present predominantly in the polar fraction of these fats, which was isolated using silicagel chromatography and which was reported to contain polar oxidation and degradation products (Billek et al., 1978). Non-polar fractions of some used fat samples also exhibited mutagenic activity, but numbers of revertants induced by this latter fraction were lower as compared to the polar fraction. Mutagenicity of polar and non-polar fractions which did not show toxic effects to the bacteria, was reduced when tested in presence of liver S9 mix obtained from Aroclor induced rats, indicating mutagenic compounds might be deactivated by liver microsomal metabolism or by free-radical scavenging systems present in S9 homogenate. *Salmonella* tester strain TA97 appeared to be most sensitive in detecting mutagenicity of repeatedly used deep-frying fats; mutagenic activity of used fat samples to this strain in absence of S9 mix was estimated to range from $1 \cdot 10^3$ to $3 \cdot 10^4$ revertants per g. In this study, it was also demonstrated that mutagens found in these used deep-frying fat samples were likely to be produced during deep-fat frying and not during storage; in a frying experiment carried out in the laboratory, in which a commercially obtained deep-frying fat was used for regular preparation of various fried foods, and which involved mutagenicity testing of fat samples within one week, increased mutagenicity was observed in the polar fraction of this fat after repeated use.

Mutagenicity of deep-frying fats in relation to oxidative deterioration

Since mutagenic activity of heated deep-frying fats was found primarily in the lipid fraction containing oxidation products, it was suggested that lipid oxidation products might be involved in mutagenicity. Mutagenic activity of the used fat samples however, appeared not to be related to the extent of oxidative deterioration as determined by the amount of polar material or levels of di- and polymeric triglycerides (DPTG). Mutagenic activity of these fat samples to strain TA97, both detected in absence and in presence of S9 mix, did show a positive correlation with lipid oxidation products as determined by the colorimetric thiobarbituric acid assay. Since this assay non-specifically analyzes lipid hydroperoxides as well as several secondary oxidation products, such as MDA, alka-2,4-dienals and alk-2-enals (Kosugi and Kikugawa, 1989), the exact nature of lipid oxidation products involved remained unclear.

Hydroperoxides of linoleic and linolenic acid were reported to be mutagenic in the Salmonella/microsome assay (McGregor et al., 1985; Yamaguchi and Yamashita, 1979; 1980), and were therefore considered likely to contribute to mutagenicity of thermally oxidized fats. Consequently, the concentration of linoleic acid hydroperoxides (LAHPO) of repeatedly used deep-frying fat samples was determined using a high performance liquid chromatography (HPLC) method, which is described in Chapter 4. Mutagenic activity of the used deep-frying fat samples did show a weak positive correlation with concentrations of LAHPO, but this association was found for mutagenicity to strain TA97 in presence of S9 mix only. Concluding, these results indicate that secondary products of lipid oxidation may contribute to direct-acting mutagenicity of used deep-frying fat samples, whereas metabolites or non-enzymatic degradation products of LAHPO may be involved in mutagenicity detected in presence of liver S9 mix. The absence of a relation between mutagenicity of repeatedly used deep-frying fats and concentrations of DPTG and polar material, which are chemical parameters used for quality control of deep-frying fats (Billek et al, 1978; Stevenson et al., 1984), indicates that quality control as currently applied does not determine compounds that are associated with genotoxic risk, and may therefore be considered inadequate.

Mutagenicity of repeatedly used deep-frying fats in relation to the foods prepared

In Chapters 5 and 6, the influence of oxidative deterioration, lipid oxidation, type of frying fat and nature of the foods prepared on mutagen formation during deep-fat frying was investigated. Various types of frying fat, containing different concentrations of polyunsaturated fatty acids (PUFA), were used exclusively for frying of potatoes, or for preparation of various foods. Potatoes were initially used for frying, since these were considered to

present the least complex food matrix. In Chapter 6, it was demonstrated that prolonged and repeated use of the deep-frying fat for preparation of fried potatoes, resulted in increased mutagenicity of the fat to *Salmonella* strain TA97, which corresponded to increased concentrations of DPTG and of polar oxidation and degradation products. Levels of TBA-RS were below the detection level of 1.0 nmole of MDA/g, in the fat as well as in fried potatoes, indicating that mutagenicity of this used deep-frying fat was not related to autoxidation of PUFA, but that other compounds were involved. Mutagenicity of lipid fractions isolated from fried potatoes showed no clear tendency to increase with prolonged use of the deep-frying fat. In this frying experiment however, commercially obtained pre-fried, frozen potatoes were used, of which the polar lipid fraction was found to be mutagenic to *Salmonella* strain TA97. It was therefore suggested that either mutagens were produced during commercial preparation of pre-fried potatoes, or that naturally occurring components in potatoes contribute to mutagenicity of fried potatoes. Testing of mutagenicity of lipid fractions isolated from fresh, uncooked potatoes however, yielded negative results, thus providing no evidence for the latter possibility.

Mutagenic activity to *Salmonella* strain TA97 was also found in a fraction extracted with methanol from the fat-free residue of fried potatoes and mashed potatoes, but not from fresh uncooked potatoes (Chapters 6 and 7). Since fat-free residues of fried as well as of cooked potatoes, as described in Chapter 7, were found to contain appreciable amounts of methanol-extractable compounds, this extract is considered to contain very polar compounds produced during heating, which may be derived from lipids, carbohydrates or proteins, as well as from compounds naturally present in potatoes. Different frying experiments, in which various types of fat and potatoes were used, yielded varying mutagenic activity, showing the presence of pro-mutagenic compounds in methanol-extracts of fried potatoes in one experiment (Chapter 6), and direct-acting mutagenicity of methanol-extracts in another (Chapter 7). These data illustrate the complexity of assessment of mutagenic activity of deep-fat fried foods, since various factors which include the nature of the fats and the fried foods, as well as frying conditions, influence mutagen formation and presence of mutagens in fried foods. Furthermore, isolation of mutagens from complex matrices like fried foods using standard extraction procedures for the isolation of lipids, may not result in detection of reaction products of lipids with proteins or carbohydrates.

Results obtained in frying experiments described in Chapter 5, in which freshly sliced potatoes were prepared, indicated that lipid oxidation or oxidative deterioration of fats *per se* does not cause mutagenicity of the frying fat, since frying of freshly sliced potatoes caused no or only marginal mutagenicity of deep-frying fats to *Salmonella* strain TA97, of which some

showed severe oxidative deterioration and lipid oxidation after 30 h of use. Subsequently, both saturated and unsaturated fats were used for preparation of various products including proteinaceous foods, and heated for 30 h. In these experiments, no evidence was found either for a causal relation between the degree of oxidative deterioration or lipid oxidation of the fats and mutagenicity to strain TA97. Chemical as well as mutagenicity results obtained for different unused batches of the same type of fat which were tested in frying experiments described in Chapters 3, 5 and 6, suggest that the quality and source of the fat may also be factors causing poor reproducibility of mutagenicity results and parameters of oxidative deterioration; the presence of anti-oxidants, the nature of foods prepared, volume and water content of foods prepared, and quality of the equipment used may be additional factors causing variation (Chang et al., 1978; Stevenson et al., 1984).

Data obtained in Chapters 3 to 6 suggest that frying of various foods enhances mutagen formation during deep-fat frying as compared to frying of potatoes. This may be related to the high protein content of meat products as compared to potatoes, since basic dichloromethane fractions of commercially deep-fried meat products were previously reported to show mutagenic activity in the Salmonella/microsome assay, whereas in dichloromethane extracts of starchy foods no clear indication for mutagenicity was observed (Taylor et al., 1982; 1983). At present, it is not clear which constituents of foods or meat are involved in mutagen formation. Further studies applying heating of model systems consisting of various triglycerides, proteins or amino acids, various carbohydrates, catalyzing metal ions and other minor food constituents including vitamins and antioxidants, may elucidate which compounds enhance or inhibit mutagen formation during deep-fat frying.

Genotoxic effects of heated deep-frying fats to human lymphocytes in vitro

Repeatedly used deep-frying fats, which showed mutagenic activity to strain TA97, were tested for genotoxicity to human lymphocytes. End points determined were induction of sister chromatid exchanges (SCE) and single gene mutations at the hypoxanthine phosphoribosyl transferase (HGPRT) locus. A modified procedure was developed for the HGPRT assay with human lymphocytes, which is described in detail in Chapter 2. Results of genotoxicity testing of 3 different heated fats are presented in Chapter 5. *In vitro* exposure of human lymphocytes to heated deep-frying fats was observed to cause marginal effects. No dose-dependent induction of single gene mutations at the HGPRT-locus was found, although one heated fat significantly increased mutation frequencies at a single dose of 1.0 mg/ml when initially tested in two pilot experiments. Upon retesting one month later, no significantly increased mutation frequencies were observed over a

dose range of 0.01 to 1.0 mg/ml. Since this particular fat was found to contain appreciable amounts of LAHPO as compared to the other fats tested, it was suggested that mutagenicity to lymphocytes initially observed might have been due to instable lipid oxidation products, but this could not be confirmed by significant changes in the concentration of LAHPO after a two-week storage period.

In addition, it was observed that phytohaemagglutinin- (PHA)-stimulated lymphocyte cultures exposed to non-toxic doses of heated fats, showed increased labeling indices as compared to unheated fats, which indicated that heated fats contained compounds that stimulate cell proliferation. Although intra-colonically installed hydroxy- and hydroperoxy-derivatives of linoleic acid were reported to stimulate mucosal DNA synthesis, ornithine decarboxylase activity and cell proliferation in rats (Bull et al., 1984; 1988), increased labeling indices in lymphocytes *in vitro* appeared not to be associated with presence of lipid oxidation products as determined by concentrations of TBA-RS, DPTG and LAHPO in the heated fats, indicating that other compounds are likely to be involved. Since free fatty acids were reported to enhance proliferation of PHA-stimulated lymphocytes *in vitro* (Spieker-Polet et al., 1981), and may be present in heated fats due to hydrolysis of triglycerides, these possibly also contributed to the observed enhancement of cell proliferation in human lymphocytes as described in Chapter 5. Marginally, but dose-related increased SCE frequencies were observed in human lymphocytes after *in vitro* incubation with another heated fat at doses ranging from 0.1 to 10.0 mg/ml.

Genotoxicity of heated fats to human lymphocytes as assessed by *in vitro* induction of SCE's and mutations at the *hgp* locus, appeared to be marginal, and was not observed for all samples showing mutagenic activity in the Salmonella/microsome assay. Various oxidation products of PUFA were reported to possess genotoxic properties to mammalian cells (Cajelli et al., 1987; Nakayama et al., 1986; Weitberg and Corvese, 1989). Genotoxicity to human lymphocytes however, was not found to be associated with parameters of oxidative deterioration or concentrations of lipid oxidation products as determined by analysis of DPTG, TBA-RS and LAHPO of the heated fats. Since some heated fat samples did show weak genotoxic effects, which appeared not to be related to the presently applied parameters of oxidative deterioration of the fats, it is concluded that further identification of active compounds is required for a reliable assessment of genotoxic risk associated with consumption of heat-processed fats.

Effects of consumption of heated deep-frying fats on mutagen excretion in rats

In Chapter 8, various biological effects of short-term feeding of two types of mutagenic heated deep-frying fats to rats were determined. Semi-purified diets containing 10% unheated or heated PUFA-rich frying oil and coconut oil, containing <10% PUFA, were fed to groups of 7 rats for 4 weeks. Urine mutagenicity to *Salmonella* strains TA97 and TA100 was not significantly changed in rats fed heated coconut oil as compared to rats receiving unheated coconut oil. Consumption of PUFA-rich frying oil which showed mutagenicity to strains TA97 and TA100, did not show an effect on urinary mutagen excretion to strain TA97 in rats but was found to significantly increase urine mutagenicity to strain TA100, which indicated different biological fate of mutagens detected by strain TA100 as compared to strain TA97. Faecal mutagenicity to strains TA97 and TA100 was not found to be significantly increased in rats after consumption of heated oils as compared to unheated oils, but appeared to be decreased in the group fed heated coconut oil as compared to the group receiving unheated oil. Mutagenicity of heated PUFA-rich frying oil to strain TA100 appeared to be detectable in urine and faeces of rats, and amounted to 7% of the ingested dose. This suggests that compounds present in heated frying oils and causing base-pair mutations in strain TA100, may not be efficiently deactivated after absorption. Mutagenicity of frying oils to strain TA97 did not increase excretion in urine and faeces, suggesting frame-shift mutagens may either be efficiently deactivated *in vivo*, or are retained in the tissues.

In Chapter 8, excretion of TBA-RS in urine and faeces of rats was found to be minimal as compared to TBA-RS present in diet, being maximally 0.1% of the dose consumed. These data indicate that >99% of oxidation products were absorbed and metabolized or inactivated by *in vivo* acting antioxidative systems; these data do not suggest induction of *in vivo* lipid oxidation after short-term consumption of heated oxidized oils. In addition, plasma activities of glutamic pyruvic transaminase (GPT) and alkaline phosphatase (AP) were elevated in rats fed heated PUFA-rich frying oil as compared to the groups receiving unheated frying oil or heated coconut oil, which suggest that consumption of heated PUFA-rich frying oil may cause adverse effects to liver and kidneys. Heated PUFA-rich frying oil was found to contain detectable amounts of DPTG and LAHPO, whereas in heated coconut oil these lipid oxidation products were not detected. It should be noted however, that levels of TBA-RS and LAHPO in heated oils fed to the rats were low in comparison with concentrations reported in Chapters 3 and 4 for commercially used deep-frying oils. Since intake of high doses of lipid oxidation products was reported to cause tissue damage to liver and kidneys in rats (Alexander, 1981; Izaki et al., 1984; Kanazawa et al., 1985; 1986), and the present data showed that consumption of low amounts of oxidation products significantly increased urinary concentrations of TBA-RS in

combination with increased plasma activities of GPT and AP in rats fed heated PUFA-rich frying oil as compared to unheated oil, chronic consumption of 5- to 10-fold higher doses of lipid oxidation products may be of concern with respect to possible oxidative damage to tissues.

Although urinary excretion of TBA-RS was elevated in rats fed heated PUFA-rich frying oil, no significant correlation was found on an individual level between urine mutagenicity and concentration of TBA-RS. Since no significant correlations were found either between individual intake of DPTG, LAHPO or mutagenic compounds, and effects on plasma clinical biochemical parameters and faecal mutagen excretion, no evidence is found for a causal relation. The nature of compounds responsible for the observed effects in rats after consumption of PUFA-rich heated frying oil remains to be determined.

Mutagen excretion in urine and faeces of man after consumption of deep-fat fried potatoes

Consumption of deep-fat fried foods is considered likely to include intake of compounds which are produced during heat-processing of fats and foods and which are mutagenic to *Salmonella typhimurium*. Therefore, effects on mutagen excretion in urine and faeces of consumption of deep-fat fried potatoes were determined in human volunteers to investigate the biological fate of mutagens present in fried potatoes, and to assess endogenous exposure to mutagens associated with deep-fat frying. Using *Salmonella* strain TA97 as indicator of mutagens produced during deep-fat frying, results obtained in diet studies described in Chapters 6 and 7, showed that consumption of fried potatoes prepared in mutagenic deep-frying fats did not result in detectable increases in mutagen excretion in urine and faeces of human volunteers. These results were in agreement with the results observed for rats in Chapter 8, although the daily dose of heated coconut oil consumed by the rats ranged from 5 to 7 g/kg bodyweight, and was one order of magnitude higher as compared to the daily dose consumed by human volunteers with fried potatoes prepared in the same batch of oil (Chapter 7), which ranged from 0.40 to 0.45 g/kg bodyweight. Patterns found for human faecal mutagen excretion determined with strain TA97 corresponded to data obtained for faecal mutagenicity of the rats fed coconut oil observed in Chapter 8. These data implicate that in addition to mutagenic compounds, factors reducing faecal mutagenicity may also be present in heated coconut oil. Faecal mutagenicity to strain TA100 was not found to be increased in rats nor human volunteers consuming heated coconut oil, a striking difference however, was observed in the degree of mutagenicity. Twenty-five to 40% of faecal samples of human volunteers showed significant mutagenicity, whereas rat faecal samples did not show significantly increased mutagenicity to strain TA100. Obviously, this

difference may be explained by the greater complexity of human diets as compared to the semi-purified rat diet. In addition, in the study described in Chapter 7, no adverse effects were indicated by changes in plasma clinical biochemical parameters. Since in Chapters 3 to 7 mutagenic activity of heated fats and fried potatoes to strain TA97 has been observed to be decreased in presence of liver S9 mix, as well as in presence of antioxidants, it is suggested that after absorption and metabolism direct-acting mutagens present in heated fats and fried potatoes, are likely to be inactivated in vivo.

Furthermore, it was observed in Chapter 7 that human faecal mutagen excretion as determined with *Salmonella* strain TA100, was influenced by diet. During the two diet intervention periods, either fried potatoes prepared in mutagenic coconut oil or mashed potatoes containing unheated coconut oil were included in the diet for 5 days using a cross-over design, while consumption of other food products was kept equal. Mutagenicity to tester strain TA100, both in absence and in presence of rat liver S9 mix, of dichloromethane extracts of faeces collected during both intervention periods was found to be increased as compared to faeces representing free-choice diets of the volunteers. It is however, not clear which dietary changes were responsible for the observed increase in faecal mutagenicity during the experimental periods; comparison of food consumption of the volunteers during the experimental diets with habitual dietary intake revealed a significant reduction of total fat consumption and a decrease of the P/S ratio of dietary fats during the experimental periods. Individual faecal mutagen excretion however, did not correlate with these or other diet-related parameters measured. Faecal mutagenicity to strain TA97 as well as to strain TA100, did show a positive correlation with faecal pH, which implied that at higher pH faecal mutagen production may be enhanced, or inactivation inhibited. The nature of faecal mutagens detected by strain TA100 remains to be determined; fecapentaenes, bacterial products which have been isolated from human faeces and which are potent direct-acting mutagens to strain TA100 (Gupta et al., 1983; Schiffman, 1986), may be involved, but since dichloromethane extracts showed mutagenic activity to strain TA100 in absence and in presence of liver S9 mix, other mutagens are likely to be present in faeces as well.

Comparison of results obtained for human volunteers in Chapter 7 and results obtained for rats, showed that consumption of heated coconut oil did not cause adverse biological effects, neither in humans nor in rats, and although the daily dose received by rats was an order of magnitude higher as compared to human intake, no species differences appeared to exist with respect to the parameters measured. Since heated PUFA-rich frying oil did increase urine mutagenicity in rats, and possibly affected tissues of liver and kidneys, determination of biological effects in humans after consumption of

PUFA-rich frying oils seems indicated.

Effects of heated deep-frying fats on cell proliferation in the gastro intestinal tract of rats

In rats, effects of short-term feeding of mutagenic heated deep-frying fats on cell proliferation of the gastrointestinal tract were determined; results are also presented in Chapter 8. Since enhanced cell proliferation was observed in PHA-stimulated human T-lymphocytes exposed to heated deep-frying fats (Chapter 5), and intra-rectal administration of hydroperoxides and hydroxides of linoleic acid was reported to stimulate DNA synthesis and increase colonic cell proliferation, it was postulated that enhancement of colonic cell proliferation by oxidized polyunsaturated fatty acids might facilitate tumorigenesis and most likely indicated tumor promoting activity of oxidized fats (Bull et al., 1984; 1987). Results obtained in our study, showed that after short-term oral administration of mutagenic deep-frying oil containing lipid oxidation products, cell proliferation of tissues of the upper gastro-intestinal tract was stimulated as compared to feeding of unheated oil. In contrast, colonic cell proliferation, was not found to be significantly enhanced.

In combination with faecal mutagenicity data, these data indicated that compounds present in heated fats which stimulated cell proliferation in the upper gastro intestinal tract are not likely to reach the colon after oral administration. Consumption of heated coconut oil did not affect cell proliferation in the gastro intestinal tract of rats. Although results suggested that stimulation of cell proliferation by the heated PUFA-rich frying oil was related to the PUFA-content which resulted in formation of LAHPO and DPTG, no correlations were found between individual data of cell proliferation in the intestinal tissues in oesophagus and forestomach, and ingested or excreted mutagens and lipid oxidation products, thus not supporting a direct involvement of compounds determined by these parameters. Since differences between ingested doses of mutagenicity, DPTG and lipid oxidation products were marginal, determination of effects of heated frying fats containing higher doses of lipid oxidation and degradation products, will be required for a reliable assessment of the compounds and mechanisms involved.

The same batch of heated PUFA-rich frying oil (fat I B; Chapter 5) which was fed to the rats, was found to possess weak genotoxic properties, inducing a dose-dependent increase SCE frequencies in vitro in human lymphocytes when tested at concentrations ranging from 0.01-1.0% (w/v), which are 10-100 times lower than the concentration in the rat diets. This heated PUFA-rich frying oil also showed cytotoxic effects in human lymphocyte cultures in the HGPRT assay. The enhanced cell proliferation

observed in the upper gastro intestinal tissues of rats consuming this oil, may therefore be explained either by compensatory cell proliferation due to increased necrosis of epithelial mucosal cells or by effects on genetic regulation of cellular growth. Heated coconut oil (fat V; Chapter 5) was observed to enhance cell proliferation of PHA-stimulated lymphocytes, but did not show genotoxic effects to human lymphocytes. Estimation of in vivo genotoxic potential and health risk based on the data obtained in vitro experiments however, is not possible. Unknown factors, such as the nature of the active compounds, in vivo disposition and potency of these compounds at different dose-levels, and modulating effects of diet, which contains possible inhibitors of genotoxic and cellular injury such as α -tocopherol, calcium and fiber as well as possible enhancing components such as trace amounts of iron and copper (Caderni et al., 1988; 1989; Frankel, 1984; Hayatsu, 1988; Wargovich et al., 1984), will also have to be determined.

Heat-processed lipids and colon cancer

In various experiments described in this thesis, heated deep-frying fats were observed to exhibit genotoxic activity in *Salmonella typhimurium* as well as in human lymphocytes *in vitro*. Mutagenicity to *Salmonella* tester strains TA97 and TA100 of several heated commercially used deep-frying fats sampled after sensory indication of abuse, was found to reach levels which were an order of magnitude higher as compared to levels observed in fats tested in frying experiments in our laboratory, in which standard frying procedures and temperatures not exceeding 180 °C were applied. Since a causal relation could not be demonstrated between the presence of autoxidation products of PUFA and mutagenicity of the heated fats, the nature of mutagenic compounds remains to be established. Lipid fractions of fried potatoes prepared under recommended frying conditions were also found to be mutagenic in the *Salmonella*/microsome assay. Consumption of fried potatoes prepared in mutagenic deep-frying fats and showing mutagenic activity to *Salmonella* strain TA97, was not observed to increase urinary and faecal mutagen excretion in human volunteers. These data do not indicate a genotoxic risk for colonic mucosa of TA97-mutagens consumed with deep-fried potatoes.

In rats, a differential effect of mutagens present in heated deep-frying oils to *Salmonella* strains TA97 and TA100 was found. Mutagenicity to strain TA97 of heated deep-frying oils was not found to cause increased mutagen excretion in urine and faeces, whereas mutagenicity of a heated PUFA-rich frying oil to strain TA100 appeared to enhance urinary mutagen excretion to strain TA100. Faecal mutagen excretion to *Salmonella* strains TA97 and TA100 was not found to be determined by mutagenic activity present in the ingested heated oils. Consumption of the PUFA-rich heated deep-frying oil,

showing mutagenicity to strain TA100 and containing detectable amounts of LAHPO and DPTG, was found to enhance cell proliferation in upper gastro intestinal tissues of the rat. Since this oil was not found to affect colonic mucosal cell proliferation, results do not provide evidence for an involvement in colon tumorigenesis of biologically active compounds present in heated oils.

Finally, it is concluded that although heated deep-frying fats and fried potatoes have been found to possess genotoxic properties, the results presented in this thesis do not point to an involvement of these mutagens in colon tumorigenesis. In contrast, data indicate that active compounds may be retained in the upper gastro intestinal tissues, which may be possible target tissues for genotoxic or cellular damaging effects of heat-processed fats. Effects of consumption of heat-processed fats showing severe lipid oxidation, or exerting mutagenic activity equal to the "abused" fat samples from local snack-bars and restaurants, may be more pronounced and remain to be determined. Furthermore, in the frying experiments described in this thesis, mutagenicity to *Salmonella typhimurium* of fats used for preparation of various foods has been found to be moderate as compared to the initially tested "abused" commercial deep-frying fats. Results of these experiments therefore indicate that application of normal, recommended frying practices, i.e. frying at temperatures of maximally 180 °C and limited use of the fat, does not seem to result in high levels of mutagenicity.

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Summary

The typical Western diet, characterized by its high fat and meat consumption, has repeatedly been reported to be associated with increased colon cancer incidence. Although several plausible mechanisms have been postulated, various epidemiologic as well as experimental studies have yielded conflicting evidence for a causal relation between high fat intake and tumorigenesis in the colon. This inconsistency may be due to the fact that in general epidemiological studies do not discriminate between heat-processed and unheated dietary lipids. In the introduction of this thesis it has been hypothesized that heat-processing of lipids may give rise to the formation of oxidation and degradation products, which may possess tumor-initiating or -enhancing activity. Heat-processing of lipids has not yet been implicated in experimental studies investigating the role of dietary fat in colon tumorigenesis. The aim of this thesis was therefore to determine genotoxic and other adverse biologic effects of heat-processed fats, in order to investigate their possible role in colon tumorigenesis. For this purpose, used deep-frying fats which is heated for prolonged periods of time, subject to oxidative deterioration during preparation of food, have been tested for genotoxicity as well as for biological activity in the colon.

In first instance, repeatedly used deep-frying fat samples, which were collected from local snack-bars and restaurants by officers of the Maastricht Office for Food Quality Control after sensory indication of abuse, were analyzed for mutagenicity to *Salmonella typhimurium*, in order to obtain an indication of possible tumor-initiating activity. Seventeen out of twenty samples tested were found to possess mutagenic activity to tester strains TA97, TA100 or TA104. Highest mutagenicity was detected in the polar fraction of these used deep-frying fat samples, which was isolated using silicagel column chromatography and reported to contain oxidation and heat degradation products. Mutagenicity exerted by these polar fractions was detected most sensitively by strain TA97, and was generally reduced when tested in presence of rat liver S9 mix. Numbers of revertants induced in this strain by repeatedly used deep-frying fat samples were estimated to range from $1 \cdot 10^3$ to $3 \cdot 10^4$ per g of fat.

Mutagenic activity of these used deep-frying fat samples was found to correlate positively with concentrations of lipid oxidation products as determined by the colorimetric thiobarbituric acid assay, and with concentrations of linoleic acid hydroperoxides. The absence of an association between mutagenicity of repeatedly used deep-frying fats and levels of di- and polymeric triglycerides or polar material, which are chemical

parameters used in the quality control of deep-frying fats, indicated that quality control as currently applied does not determine compounds associated with genotoxic risk, and may therefore be considered inadequate. It has been shown that repeatedly used deep-frying fats do possess mutagenic activity in the Salmonella/microsome assay, which may be related to autoxidation of polyunsaturated fatty acids.

In a series of frying experiments, mutagen formation during deep-fat frying was investigated using various types of deep-frying fats. It was observed that frying of various foods, including proteinaceous products, appeared to enhance mutagen formation as compared to use of the fat for preparation of fried potatoes exclusively. In spite of substantial oxidative deterioration and lipid oxidation occurring in some used fats, it was found that after application of heating temperatures of maximally 180 °C and limiting heating time of fats to 30 hours, mutagenicity to strain TA97 of these fats amounted to $1\text{--}3 \times 10^3$ revertants per g of fat, which was moderate as compared to the previously tested "abused" samples. The results of these frying experiments did not support the existence of a causal relation between lipid oxidation or oxidative deterioration, and mutagenicity of heated deep-frying fats. It was therefore concluded that other compounds are also involved in mutagen formation during deep-fat frying. These remain to be determined but are most likely to be related to the nature of the foods prepared and to the frying procedures employed.

Subsequently, *in vitro* genotoxicity to human lymphocytes was tested of three heated deep-frying fats showing moderate mutagenic activity to Salmonella strain TA97, inducing $2\text{--}3 \times 10^3$ revertants per g of fat. Dose-related statistically significantly increased frequencies of sister chromatid exchanges were induced by one of the used deep-frying fats tested; the absolute increase observed however, was small indicating a relatively weak potency. Another fat increased mutation frequencies of 6-thioguanine-resistant T-lymphocytes in a first series of tests, but since this effect could not be duplicated after retesting, it was therefore suggested that instable compounds might be involved. Heated fats were found to increase cell proliferation of phytohaemagglutinin stimulated human T-lymphocytes. Neither concentrations of lipid oxidation products, nor oxidative deterioration of heated deep-frying fats appeared to be associated with genotoxicity and stimulation of cell growth of PHA-stimulated human lymphocytes. These data indicate that heated deep-frying fats may possess genotoxic properties to human lymphocytes. Since no evidence has been found for a causal role of autoxidation products of lipids, the nature of the active compounds remains to be established.

Consumption by human volunteers of fried potatoes prepared in deep-frying fats, showing mutagenicity to Salmonella strain TA97, was not found

to increase urinary nor faecal mutagen excretion to this strain, although lipid fractions isolated from deep-fat fried potatoes also showed mutagenic activity and the mutagenic load consumed with fried potatoes amounted approximately to $2\text{-}3 \cdot 10^5$ revertants. These data indicated that mutagenic compounds present in the heated deep-frying fats, are not likely to affect the colon. No other adverse effects were observed in human volunteers after daily consumption of a portion of fried potatoes prepared in mutagenic deep-frying fat.

In rats, consumption of heated mutagenic coconut oil or polyunsaturated fatty acid (PUFA)-rich deep-frying oil did not increase urinary and faecal mutagen excretion to *Salmonella* strain TA97 either. The heated PUFA-rich frying oil which showed mutagenic activity to strain TA100 however, was found to increase rat urinary mutagenicity to strain TA100. No effect was observed on rat faecal mutagen excretion to this strain. Urinary recovery of the dose consumed was $<1\%$, suggesting that after absorption mutagenic compounds were inactivated *in vivo*, or retained in the tissues. In addition, consumption of heated PUFA-rich frying oil increased urinary excretion of thiobarbituric acid-reactive substances (TBA-RS) in rats. Excretion in urine and faeces was found to be 4% and 3% of the ingested dose respectively, indicating most TBA-RS present in this heated PUFA-rich frying oil were bioavailable. Short-term feeding of this PUFA-rich frying oil to rats also increased plasma activities of glutamic pyruvic transaminase and alkaline phosphatase as compared to feeding of unheated frying oil or heated coconut oil, which may indicate adverse effects on liver and kidneys.

After short-term feeding of heated PUFA-rich frying oil cell proliferation in the upper gastro intestinal tissues of rats was slightly, but statistically significantly enhanced as compared to feeding of unheated oil or heated coconut oil, indicating that heated PUFA-rich frying oil contained compounds that stimulate cell growth *in vivo*. This cell proliferation enhancing activity is considered to indicate tumor-promoting effects, but this may also reflect initiating properties since increased cell proliferation has been reported to be an early effect in tumorigenesis. No enhanced cell proliferation was found in the colon of rats fed PUFA-rich heat-processed fat, implicating the enhancing effects were limited to the upper gastro-intestinal tract. The degrees of oxidative deterioration and mutagenicity of heated frying oils fed to the rats however, were moderate as compared to levels of lipid oxidation products found in commercially used deep-frying fat samples. Effects of consumption of high levels of specific oxidation products remain to be determined. Therefore, it can not be excluded that consumption of heated oils containing higher doses of lipid oxidation and mutagens may exert effects in the lower part of the gastro intestinal tract.

Finally, it is concluded that an involvement of heat-processed lipids in

colon tumorigenesis is not supported by the data presented in this thesis, since consumption of mutagenic deep-frying fats did not increase faecal mutagenicity in both rats and humans, and was not found to affect colonic cell proliferation in rats. Heated deep-frying fats however, have been found to show genotoxic properties to human lymphocytes, causing some concern with respect to human genotoxic risk. Elucidation of the nature of active compounds present in heated deep-frying fats, as well as determination of their concentration in deep-fat fried and other lipid-containing heat-processed foods is indicated before further testing of genotoxic, carcinogenic or tumor-enhancing activities is applied to evaluate health risk to man.

Samenvatting

Een hoge consumptie van vet en vleesprodukten, hetgeen kenmerkend is voor het "Westerse" voedingspatroon, is in epidemiologische onderzoeken herhaaldelijk in verband gebracht met een verhoogd risico voor colonkanker. Hoewel hiervoor verscheidene mechanistische verklaringen zijn gepostuleerd, hebben noch epidemiologische, noch experimentele onderzoeken consistente resultaten opgeleverd, die een causaal verband tussen een hoge vet inneming en het ontstaan van colonkanker ondersteunen. Deze tegenstrijdige onderzoeksresultaten kunnen onder meer worden toegeschreven aan het feit dat epidemiologische onderzoeken geen onderscheid maken tussen verhitte en onverhitte vetten in de voeding. In de inleiding van dit proefschrift is beschreven dat tijdens het verhitten van voedingsvetten oxydatie- en thermale degradatieprodukten ontstaan, die mogelijk tumor-initiërende of -bevorderende eigenschappen hebben. Experimentele onderzoeken naar de rol van voedingsvet in het ontstaan en ontwikkelen van colonkanker bestuderen hebben tot nu toe evenmin aandacht besteed aan een mogelijke rol voor componenten die aanwezig kunnen zijn in verhitte voedingsvetten. Het onderzoek beschreven in dit proefschrift had daarom tot doel de mogelijke rol van verhitte voedingsvetten in coloncarcinogenese te verkennen; hiertoe zijn verhitte voedingsvetten onderzocht op genotoxische en andere nadelige biologische effecten in het colon. Hierbij is gekozen voor herhaaldelijk gebruikte frituurvetten, omdat deze vetten langdurig verhit worden en zeer vatbaar zijn voor oxydatie tijdens het frituren van voedingsmiddelen.

In eerste instantie zijn herhaaldelijk gebruikte frituurvetmonsters getest op mutageniteit in de Salmonella mutageniteitstest om mogelijke tumor-initiërende activiteiten te bepalen. Deze frituurvetmonsters waren verzameld door de Maastrichtse Rijkskeuringsdienst van Waren op basis van sensorische indicatie van ondeugdelijkheid. Zeventien van de twintig geteste frituurvetmonsters vertoonden mutagene activiteit in Salmonella stammen TA97, TA100 of TA104. De hoogste mutagene activiteit werd aangetroffen in de polaire fraktie van het vet. Deze was geïsoleerd met behulp van silicagel kolomchromatografie en bevat voornamelijk polaire oxydatie- en degradatieprodukten. De mutagene activiteit van deze polaire frakties was het hoogst in Salmonella stam TA97, en was in het algemeen gereduceerd wanneer de frakties getest werden in aanwezigheid van rattelever S9 mix. Het aantal revertanten dat geïnduceerd werd door herhaaldelijk gebruikt frituurvet varieerde van $1 \cdot 10^3$ tot $3 \cdot 10^4$ per g vet.

De mutageniteit van deze frituurvetmonsters was positief geassocieerd met de colorimetrisch bepaalde gehalten aan lipid peroxydatieprodukten, en in mindere mate met de met behulp van HPLC gemeten concentratie linolzuur-hydroperoxyden. Er werden geen significante associaties gevonden tussen mutageniteit en gehalten aan di- en polymere triglyceriden of de hoeveelheid polair materiaal, hoewel dit chemische parameters zijn die worden gehanteerd bij de kwaliteitsbepaling van gebruikt frituurvet. Het ontbreken van een associatie tussen chemische kwaliteitsparameters en mutageniteit betekent dat deze parameters geen aanwijzing geven omtrent een eventueel genotoxisch risico, en impliceert dat verbetering van de kwaliteitscontrole gewenst is. Op grond van de gevonden resultaten wordt geconcludeerd dat herhaaldelijk gebruikte frituurvetten componenten kunnen bevatten die mutageen zijn voor *Salmonella typhimurium*. De mutageniteit blijkt geassocieerd met de aanwezigheid van auto-oxydatieprodukten van meervoudig onverzadigde vetzuren.

In een serie frituuronderzoeken is vervolgens de vorming van mutagene componenten tijdens het frituren onderzocht. De bereiding van verschillende produkten, waaronder eiwitrijke produkten bleek aanleiding te geven tot een hogere produktie van mutagenen dan het frituren van uitsluitend patat frites. Hoewel in sommige van deze herhaaldelijk gebruikte vetten aanzienlijke oxydatie optrad na verhitten bij een temperatuur van maximaal 180 °C gedurende 30 uur, bereikte de mutageniteit in stam TA97 waarden variërend van 1 tot $3 \cdot 10^3$ revertanten per gram vet, wat in vergelijking met de eerder geteste "ondeugdelijke" monsters een relatief geringe mutageniteit genoemd kan worden. De resultaten van deze serie frituurexperimenten leverden geen aanwijzing op voor een causaal verband tussen lipid peroxydatie of oxydatieve degradatie, en mutageniteit van verhitte frituurvetten. Hieruit wordt geconcludeerd dat andere componenten eveneens een rol spelen bij de produktie van mutagenen tijdens het frituren. De aard van deze verbindingen dient nog nader bepaald te worden, maar gesteld wordt dat de vorming van deze componenten onder meer afhankelijk is van de aard en kwaliteit van de frituurvetten en de daarin bereide voedingsmiddelen, en van de omstandigheden waaronder wordt gefrituurd.

Drie verhitte frituurvetten, waarvoor in de Ames-test (stam TA97) een mutagene aktiviteit gevonden was van 2 tot $3 \cdot 10^3$ revertanten per gram vet, werden met behulp van humane lymfocyten verder onderzocht op genotoxiciteit. Een dosis-afhankelijke statistisch significante toename in zuster chromatide uitwisselingen (sister chromatid exchanges, SCE's) werd geïnduceerd door één van de geteste gebruikte frituurvetten. De absolute toename was echter gering, wat duidt op een relatief lage genotoxische potentie. Een tweede vet veroorzaakte in een eerste serie van testen een verhoogde frequentie van 6-thioguanine-resistente T-lymfocyten. Echter, na

hertesten van hetzelfde vetmonster werd geen verhoogde frequentie van mutante T-lymfocyten waargenomen, hetgeen mogelijk wijst op de aanwezigheid van onstabiele mutagenen. Verhitte vetten bleken *in vitro* de celproliferatie van met phytohaemagglutinine-gestimuleerde humane T-lymfocyten te bevorderen. Dit effect was noch geassocieerd met de concentratie van lipid oxydatieproducten, noch met de mate van oxydatieve degradatie. Verhitte friuurvetten blijken dus componenten te kunnen bevatten die genotoxisch zijn voor humane lymfocyten. Er is echter geen duidelijke aanwijzing gevonden voor een causale relatie tussen genotoxiciteit en de aanwezigheid van specifieke oxydatieproducten van vetzuren.

Consumptie van patates frites gebakken in frituurvet dat mutagene activiteit in stam TA97 vertoonde, leidde niet tot verhoogde uitscheiding van mutagenen in urine en faeces van humane vrijwilligers, hoewel in de vetfrakties die waren geïsoleerd uit patates frites mutagene activiteit was gevonden en de inneming van mutagenen was berekend op 2 tot $3 \cdot 10^5$ revertanten. Dit impliceert dat de mutagene componenten in het verhitte frituurvet en de patat frites in het colon geen detecteerbare verhoogde mutagene activiteit veroorzaken. Evenmin werden andere nadelige gezondheidseffecten gevonden in humane vrijwilligers na dagelijkse consumptie van een portie in mutageen frituurvet bereide patates frites.

In ratten bleek dagelijkse consumptie van verhitte, mutagene cocosnoot olie, noch van verhit mutageen meervoudig onverzadigde vetzuren (MOV)-rijk frituurvet in een verhoogde uitscheiding van TA97-mutagenen in urine en faeces te resulteren. In de groep ratten die verhit MOV-rijk frituurvet consumeerde werd een verhoogde uitscheiding van TA100-mutagenen in de urine gevonden. De uitscheiding van TA100-mutagenen in de faeces was niet verhoogd in deze groep. Van de geconsumeerde dosis TA100-mutagenen werd in de urine minder dan 1% teruggevonden, wat erop wijst dat deze mutagene componenten na absorptie worden geïnactiveerd *in vivo*, of dat deze componenten achterblijven in de weefsels. De groep ratten die verhit MOV-rijk frituurvet consumeerde had eveneens een verhoogde urinaire uitscheiding van thiobarbituurzuur-reaktieve verbindingen (thiobarbituric acid-reactive substances; TBA-RS). De uitscheiding van TBA-RS in urine en faeces in deze groep was respectievelijk 4 en 3% van de ingenomen dosis, wat erop duidt dat de meeste TBA-RS biologisch beschikbaar zijn. In de groep ratten die verhit MOV-rijk frituurvet consumeerde werden een verhoogde activiteit van serum glutamic pyruvatic transaminase (SGPT) en alkalische fosfatase gevonden in vergelijking met de groepen die het onverhitte MOV-rijke frituurvet of de verhitte cocosnoot olie consumeerden, wat een aanwijzing is voor mogelijke celbeschadiging in lever en nieren.

Consumptie van verhit MOV-rijk frituurvet bleek de celproliferatie in slokdarm en voormag van ratten licht te verhogen in vergelijking met consumptie van onverhitte vetten of verhitte cocosnoot olie. Het verhitte MOV-rijke frituurvet bevatte blijkbaar verbindingen die *in vivo* de celproliferatie stimuleren. Een stimulatie van de celgroei wordt in het algemeen beschouwd als een tumorbevorderend effect, maar een verhoogde celproliferatie kan ook optreden als gevolg van bepaalde tumor-initiërende activiteiten. Er werd geen stimulatie van de celproliferatie in het colon gevonden na consumptie van verhit MOV-rijk frituurvet en het stimulerende effect op de celproliferatie beperkte zich tot het voorste deel van het maag-darm kanaal. De mate van oxydatieve degradatie en de mutageniteit van de verhitte frituurvetten die door de ratten werden geconsumeerd was relatief laag in vergelijking met gehalten aan lipid oxydatieproducten die waren gevonden in een aantal commerciële gebruikte "ondeugdelijke" frituurvetten. Niet bekend is of consumptie van verhitte vetten met hoge gehalten aan lipid oxydatieproducten wel leidt tot nadelige effecten in het colon; hiervoor is nader onderzoek vereist.

Tenslotte wordt op basis van de resultaten beschreven in dit proefschrift geconcludeerd dat voor verhitte vetten geen rol van betekenis in colon-carcinogenese lijkt weggelegd. Consumptie van mutageen frituurvet bleek noch in de mens, noch in de rat aanleiding te geven tot een verhoogde faecale mutageniteit, en bleek in ratten de celproliferatie in het colon niet te stimuleren. Verhitte frituurvetten vertoonden echter wel genotoxische activiteit in humane lymfocyten *in vitro*, wat een mogelijk genotoxisch risico voor de mens impliceert. Voor een evaluatie van het gezondheidsrisico voor de mens is daarom eerst opheldering vereist van de aard van deze actieve componenten, alsmede een bepaling van de gehalten hiervan in gefrituurde en andere vetrijke verhitte voedingsmiddelen. Vervolgens kan worden overgegaan tot een nadere analyse van specifieke genotoxische, carcinogene of tumorbevorderende eigenschappen van deze verbindingen.

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Curriculum vitae

Gerrigje Johanna (Geja) Hageman werd geboren op 10 juni 1960 te Zwollerkerspel. In 1978 behaalde zij het gymnasium- β diploma aan het Carolus Clusius College te Zwolle. Daarna begon zij met de studie Humane Voeding aan de Landbouwuniversiteit te Wageningen. In 1983 behaalde zij het kandidaatsdiploma. De doctoraalfase werd in 1985 afgesloten en bestond uit de vakken Voedingsleer (Prof. dr. M.B. Katan, Prof. dr. J.G.A.J. Hautvast), Levensmiddelenchemie (Dr. ir. A.G.J. Voragen, Prof. W. Pilnik) en een buitenlandse stage Klinische Voeding (Prof. dr. V.R. Young, Massachusetts Institute of Technology, Cambridge, MA, USA). Sedert 1985 is zij als wetenschappelijk assistent werkzaam aan de Rijksuniversiteit Limburg bij de vakgroep Humane Biologie/Biologische Gezondheidskunde i.o. (Prof. dr. F. ten Hoor, Dr. J.C.S. Kleinjans) waar het onderzoek zoals beschreven in dit proefschrift werd uitgevoerd. Vanaf 1990 is zij als universitair docent verbonden aan dezelfde vakgroep.

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