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FACTORS INFLUENCING MUTAGEN FORMATION
DURING FRYING OF GROUND BEEF

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A. M. Pearson
Major professor

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FACTORS INFLUENCING MUTAGEN FORMATION DURING FRYING OF GROUND BEEF

BY

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ABSTRACT

FACTORS INFLUENCING MUTAGEN FORMATION DURING FRYING OF GROUND BEEF

BY

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The effects that different antioxidants and other food additives have on mutagen formation during frying of ground beef were investigated. Results demonstrated that the basic fraction of fried ground beef is mutagenic toward Salmonella TA98 or TA100 + S-9. Meat mutagenicity was shown to be unrelated to fat content. Internal temperature studies indicated that: (1) Longer frying times did not necessarily increase the internal temperature of the meat; (2) Although the internal temperature did not increase significantly with frying time, mutagen formation was positively correlated with frying time; (3) Less mutagens were formed in thick patties than in thin patties; and (4) Formation of meat mutagens was shown to be time-temperature dependent.

In the Ames test, mutagenic potency was in the order of MeIQ > IQ > MeIQx, with specific activities toward TA98 + S-9 being 5644, 3836 and 642 revertants/μg, respectively. With TA100 + S-9, the respective numbers of revertants/μg were 540, 261 and 154. BHA and PG significantly inhibited the mutagenicity of IQ, MeIQ and MeIQx. BHT slightly inhibited the mutagenicity of MeIQx, had little effect on

the mutagenicity of IQ and MeIQ at low concentrations, but significantly increased their mutagenicity at high concentrations.

To evaluate the relationship between antioxidants and meat mutagen formation, antioxidants were added to raw beef patties before frying. All added antioxidants (BHA, PG and Tenox 4) decreased mutagenicity by inhibiting formation of IQ, MeIQ and 4,8-DiMeIQx, except for BHT, which enhanced mutagenicity and increased the amount of 4,8-DiMeIQx by 4-fold.

Different food additives (bisulfite, nitrite, polyphosphates, citrate, ascorbate, tocopherols and liquid smoke) were evaluated for their effects on mutagen formation in fried ground beef. All additives, except polyphosphates, were shown to inhibit the formation of IQ-like compounds. A possible mechanism for formation of IQ-like meat mutagens was proposed, which suggests that imidazoquinoline type meat mutagens (IQ and MeIQ) are formed from a reaction mixture containing alkyl-pyridine free radicals and creatinine. The imidazoquinoxaline type mutagens (MeIQx and 4,8-DiMeIQx) may be produced from reacting a mixture containing dialkyl-pyrazine free radicals and creatinine. Under mildly acidic conditions, the reaction would favor formation of MeIQx and 4,8-DiMeIQx.

To my dear parents

Mr. and Mrs. Pao-Shiang and Char-Shun Chen

and to my lovely wife Yeong-Tsuey

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INTRODUCTION

Cancer is a serious health problem in many countries
d is often one of the leading causes of mortality. In
stern countries, cancer is second only to cardiovascular
sease as the most frequent cause of death (Rauscher, 1975;
easey, 1985; Stalder, 1986).

As many as 70-90% of human cancers have been estimated
be associated with environmental causes (Higginson, 1969;
nder and Gori, 1977; Doll and Peto, 1981). Cancer causes
e often misunderstood and misconstrued as being primarily
e to ubiquitous chemicals derived from modern technology
d industrial development. It is true that a number of food
ditives, pesticides, insecticides and industrial chemicals
troduced commercially in the last 40 years have exhibited
rcinogenic properties in animal models (Roberts, 1984).
cording to Stich (1982), however, the main causes of human
ncer in the Western world do not stem from such chemical
ntaminants. It is, therefore, important to identify the
tual causes of cancer in order to develop an effective
sis for cancer prevention.

Epidemiological studies have shown that diet and life-
rle are closely related to human cancer (Higginson and
r, 1979; Doll and Peto, 1981). For instance, cancer of
stomach is much more common in Japan than in the U.S.,
reas, cancer of the large intestine, the breast and the

prostate are more common in U.S. (Haenszel et al., 1972). According to epidemiological studies by Haenszel et al. (1973), when Japanese emigrate to the U.S., these differences are lost within a generation or two. Since the Japanese immigrants and their children tend to marry within the group, the change in incidence must be caused by the changed environment rather than by genetic factors (Haenszel et al., 1973). Moreover, since the incidence of cancer may take more than one generation to reach levels typical of the U.S., some of the causative agents must be factors such as diet, which tend to persist as part of a cultural heritage, rather than factors such as air pollution that tend to be the same for everyone in a given place (Kolonel et al., 1980). Ikeda et al. (1983) reported that people who frequently eat charred fish have a higher incidence of gastric cancer.

Mutagens can be formed in muscle foods when subjected to various cooking and processing methods. For example, charcoal-broiled and grilled beef has been shown by Lijinsky and Shubik (1964) to contain benzo[a]pyrene (BaP). Pensabene et al. (1974) have demonstrated that carcinogenic nitrosamines can be formed in meat products when nitrite and secondary amines are heated together. It now appears that heating of most, if not all, muscle foods by a variety of cooking methods can also produce mutagens.

Miller (1985) classified the mutagens formed in processed muscle foods into two groups: (1) mutagens induced by high temperatures, and (2) those formed at moderate

temperatures. The high temperature-induced mutagens are likely to be produced during cooking of proteinaceous foods at temperatures in excess of 300°C (Sugimura et al., 1977). Most of these compounds are protein pyrolysates (Sugimura, 1986) and are 2-amino-pyridine-type mutagens (Furihata and Matsushima, 1986). The moderate temperature-induced mutagenic compounds are 2-amino-imidazole-type mutagens (Furihata and Matsushima, 1986), and contribute most of the mutagenicity found in cooked meat (Kasai et al., 1979). These mutagens are probably produced from creatinine, aldehydes, and Maillard reaction products (Furihata and Matsushima, 1986). However, the mechanisms involved in the formation of these compounds are still largely unknown.

The effect of antioxidants on mutagen production in cooked ground beef was first described by Wang et al. (1982), who showed that adding butylated hydroxyanisole (BHA) to meat before cooking successfully reduced its mutagenicity after frying. Later, Barnes et al. (1983) showed that BHA can inhibit 2-amino-3-methyl-imidazo[4,5-f]quinoline (IQ) formation by 40% during cooking of beef. Based on these results, the hypothesis is proposed that free radical reactions may be involved in the formation of mutagenic compounds. If this hypothesis is true, then any antioxidant that can scavenge free radicals should also inhibit the formation of mutagens produced in the cooking of meat and other muscle foods.

The present study was designed to test: (1) the effects of different antioxidants on mutagen formation

during frying of ground beef; (2) the influence of different types of Maillard reaction inhibitors on the mutagenic compounds formed during cooking of meat; (3) the effects of some other food additives on the mutagenicity of fried ground beef and the mutagenic compounds formed during frying of ground beef; and (4) to study the mechanism(s) by which antioxidants influence the Ames test for mutagenicity in cooked meat.

LITERATURE REVIEW

MUTATION AND MUTAGENICITY TESTS

MUTATION

Carcinogens are chemical, physical or biological agents to which exposure of animals or humans increases the probability of tumor induction (Epstein, 1974). The carcinogenic process consists of at least two main steps: initiation and promotion (Slaga, 1983). Initiation is a process related to mutagenesis, in which mutagens produce mutations in crucial genes in the cell nucleus. Promotion, the second step, is a process related to phenotypic expression (Sugimura, 1982a). It is generally believed that mutation is necessary for the initiation stage of carcinogenesis (Slaga et. al., 1978).

Mutation can be defined as any permanent alteration in the sequence of DNA bases, and may or may not have a detectable phenotypic effect (Stanier et al., 1986). The sequence of nucleotides within a gene can be altered by mutation in any of several ways, the most frequent of which are base-pair substitutions, frameshift mutations, and chromosome mutations (Thilly and Call, 1986).

Thilly and Call (1986) have defined base-pair mutations as replacement of one base or base pair by another, which is called a base-pair substitution. It can be further divided into two classes known as transition and transversion mutations. In a transition mutation, a purine is replaced

by another purine, and at the same time a pyrimidine is replaced by another pyrimidine: for instance, A•T -> G•C or G•C -> A•T. In a transversion mutation, a purine is replaced by a pyrimidine and a pyrimidine is replaced by a purine: for instance, A•T -> T•A or A•T -> C•G.

Frameshift mutations have been explained by Thilly and Call (1986) as any addition or deletion of one or more base pairs. An important example of this type occurs in the DNA sequences that encode polypeptides. Frameshift mutations are important because they have the ability to disrupt gene function. Because the genetic code consists of codons of three contiguous bases to each amino acid, the addition or deletion of any non-three-fold number of bases causes their ending frame to be out of order.

Weinberg (1983) has described chromosome mutations as those mutations affecting from tens to many thousands of base pairs. The gain or loss of whole chromosomes is termed aneuploidization. The term clastogenesis is used to designate the process of genetic change that appears as microscopically observable addition, deletion, or rearrangement of parts of the chromosomes in eukaryotic species (Thilly and Call, 1986).

IN VITRO MUTAGENICITY TESTS

There are many short-term tests that have been proposed to measure the mutagenic activity of different mutagens (IARC Monogr. Suppl. 2, 1983; Garattini et al., 1982; Williams et al., 1983; Ramel et al., 1986). These tests can

be classified into two main groups, either in vitro or in vivo tests.

I. THE AMES TEST

Ames (1971) developed a mammalian microsomal mutagenicity assay, which is currently one of the best known and most widely used in vitro test systems for detecting the mutagenic effects of chemicals. The tester organism is a strain of Salmonella typhimurium bearing a mutation (His-) that renders it unable to manufacture one of the enzymes required for the synthesis of histidine. As a result of the mutation the bacterium is unable to grow in a mineral nutrient medium unless it is supplemented with an external supply of histidine (Ames and McCann, 1976).

On very rare occasions a His- mutation undergoes reversion, i. e., a back mutation restores the normal DNA coding sequence for the needed enzyme, and thereby, produces an internal supply of histidine. The reversion can be scored because only the revertant bacteria form colonies on a medium that lacks histidine. Obviously the spontaneous rate of reversion, which is ordinarily very low, will be considerably enhanced if the His- deficient bacteria are exposed to a chemical that induces mutations. This is the theoretical basis of the Ames test.

Three important modifications were introduced into the original His- strain to make it a more sensitive and versatile tester bacterium: (1) Ames et al. (1973b) identified and isolated a mutation (rfa) which causes partial loss of

the lipopolysaccharide barrier that coats the surface of the bacteria. This increases its permeability to large molecules, such as benzo[a]pyrene, that do not normally penetrate the cell wall; (2) Ames et al. (1971) also isolated a second mutation (uvrB), which makes the strain more sensitive to DNA-damaging agents by eliminating its capacity for excision repair, and thus, leaves most of the primary lesions unhealed; and (3) several research groups (McCann et al., 1975; Walker and Dobson, 1979; Shanabruch and Walker, 1980) introduced into the bacterium the R factor plasmid, pKM101, a foreign genetic element that increases chemical and spontaneous mutagenesis by enhancing an error-prone DNA repair system, which is normally present in these organisms. By means of these three modifications, a strain was constructed in which a few molecules of a carcinogen are able to create DNA lesions, such that it is likely to engender a mutation (Ames et al., 1975). Some of the mutations will be such that the internal supply of histidine is restored. The genotypes of the various tester strains are listed in Table 1.

The tester strains are mutants that contain either a base-pair substitution or frameshift mutation as explained by Ames et al. (1975). For example, the mutation in base-pair substitution tester strains TA100 and TA1535 is in the HisG gene coding for the first enzyme involved in histidine biosynthesis (Ames, 1971). This mutation, which is determined by DNA sequence analysis, substitutes =글글글= (proline) for =글수글= (leucine) in the wild type organism. In contrast,

Table 1: Genotypes of the TA strains used for mutagenesis testing[a, b]

Histidine mutation				LPS[c]	Repair	R-factor
HisD6610 His01242 =TA88	HisD3052	HisG46	HisG428 (pAQ1)			
TA90	TA1538	TA1535	-	rfa	duvrB[d]	-R
TA97	TA98	TA100	TA104	rfa	duvrB	+R
-	TA1978	TA1975	-	rfa	+	-R
TA110	TA94	TA92	-	+	+	+R
-	TA1534	TA1950	-	+	duvrB	-R
-	-	TA2410	-	+	duvrB	+R
TA89	TA1964	TA1530	-	dgal[e]	duvrB	-R
-	TA2641	TA2631	-	dgal	duvrB	+R
-	-	-	TA102	rfa	+	+R

a) Adapted from Maron and Ames (1983) and Marnett et al. (1985)
b) All strains were originally derived from S. typhimurium LT2. Wild-type genes are indicated by a +.
c) LPS = Lipopolysaccharides; R = pKM101 plasmid
d) The deletion (d) through uvrB also includes the nitrate reductase (chl) and biotin (bio) genes.
e) The dgal strains and the rfa/uvrB strains have a single deletion through gal, chl, bio and uvrB. The rfa repair+ strains have mutated in galE.

the site of the histidine mutation in frameshift tester strains TA1538 and TA98 is by deletion of one C•G-pair (C•G(-1)) in the normal genome of the gene coding for histidinal dehydrogenase (Maron and Ames, 1983). With this aberration, the strains cannot survive on minimal media without histidine supplementation. TA104 and TA102 are two base-pair substitution tester strains containing the multi-copy plasmid, pAQ1, which carries a nonsense mutation (-TAA-) at the site of reversion that is present in single copy on the chromosome (His-G428 mutation) and a tetra-cycline resistance gene (Levin et al., 1982).

The real breakthrough, and the one that made the

Salmonella test truly effective, was mixing the tester bacteria with an extract of rat liver (S-9), and thereby, subjecting the tested chemical to the mammalian metabolic processes (Ames et al., 1973a).

II. MAMMALIAN CELL CULTURE MUTAGENICITY TESTS

There are a number of *in vitro* mammalian cell culture systems that have been applied in testing for the genotoxicity of meat mutagens. The results of these mutagenic tests are summarized in Table 2.

1. Diphtheria Toxin Resistance Assay

Diphtheria toxin, which is composed of polypeptides A and B, binds to the cell surface and then fragment A enters the cell. It then catalyses ADP-ribosylation of the elongation factor 2, which is required for protein synthesis. ADP-ribosylation occurs at a particular peptide called diphthamide in elongation factor 2, which is produced by a post translational modification of the histidine residue. ADP-ribosylated elongation factor 2 loses activity for peptide elongation during protein synthesis and eventually the mammalian cells die. Diphtheria toxin resistant mutants are classified into two categories, mutants of events involved in membrane binding of toxin and mutants lacking diphthamide. The latter are resistant to higher concentrations of the toxin than the former.

Table 2: Genotoxicity of Pyrolysis Products in Mammalian Cells in vitro[a]

Endpoint measured	Cells	Test compound	Exposure concentration		Exposure time	Results	Reference
Diptheria toxin resistance (DRGr)	Human embryonic diploid cells	Trp-P-1	0.3	ug/ml	4 hr	7.0 DRGr/10^5 survivors	Kuroda, 1980, 1981
		Trp-P-2	1.0	ug/ml	4 hr	2.8 DRGr/10^5 survivors	Kuroda and Asakura, 1981
		Glu-P-2	0.3-30	ug/ml	4 hr	0.9-2.7 DRGr/10^5 survivors	Kuroda, 1980
Diptheria toxin resistance (DTr)	Chinese hamster lung cells (CHL)	AaC	25-100	ug/ml	3 hr	180-500 DTr/2.5 x 10^5 survivors	Nakayasu et al., 1983
		Glu-P-1	250-750	ug/ml	3 hr	100-170 DTr/2.5 x 10^5 survivors	
		Glu-P-2	500-1500	ug/ml	3 hr	50-120 DTr/2.5 x 10^5 survivors	
		IQ	5-40	ug/ml	3 hr	75-120 DTr/2.5 x 10^5 survivors	
		MeIQ	10-50	ug/ml	3 hr	80-150 DTr/2.5 x 10^5 survivors	
		MeIQx	10-100	ug/ml	3 hr	80-150 DTr/2.5 x 10^5 survivors	
		Trp-P-1	7-20	ug/ml	3 hr	70-130 DTr/2.5 x 10^5 survivors	
		Trp-P-2	1-5	ug/ml	3 hr	50-260 DTr/2.5 x 10^5 survivors	
Ouabain resistance (Oubr)	Chinese hamster V79 cells	Lys-P-1	10-50	ug/ml	2 days	19.5-22.2 Oubr/10^6 survivors	Takayama et al., 1985
		Trp-P-2	1-5	ug/ml	2 days	1.9-13.1 Oubr/10^6 survivors	
Chromosomal aberration	PHA-stimulated human lymphocytes (HL)	Trp-P-1	0.2-0.5	ug/ml	48 hr	0.04-0.18 chromatid breaks/cell	Sasaki et al., 1980
		Trp-P-2	2-3	ug/ml	48 hr	0.03-0.08 chromatid breaks/cell	
	Chinese hamster cells (Don-6)	Trp-P-1	0.5-2.0	ug/ml	26-30 hr	0.07-1.17 chromatid breaks/cell	
		Trp-P-2	5-7.5	ug/ml	26-30 hr	0.15-0.35 chromatid breaks/cell	
	Chinese hamster embryonic cells (B-131)	Trp-P-1	0.25-2.0	ug/ml	25-27 hr	0.10-0.31 chromatid breaks/cell	
		Trp-P-2	2.5-10	ug/ml	26-27 hr	0.07-0.20 chromatid breaks/cell	
Sister Chromatid exchange	Human lymphoblastoid cells (MLS)	AaC	1-100	uM	2 hr	1.6-9.2 induced SCEs/cell	Tohda et al., 1980
		3AN	100-500	uM	2 hr	1.1-5.5 induced SCEs/cell	Tohda et al., 1983
		3AN	100-10000	uM	2 hr	3.5-5.5 induced SCEs/cell	
		Glu-P-1	1-50	uM	2 hr	1.4-9.0 induced SCEs/cell	Tohda et al., 1980
		Trp-P-1	1-50	uM	2 hr	2.8-11.0 induced SCEs/cell	
		Trp-P-2	0.1-10	uM	2 hr	5.3-14.7 induced SCEs/cell	
	Human embryonic fibroblasts (Hs 2149)	Trp-P-1	0.2-0.3	ug/ml	44 hr	6.7-7.9 induced SCEs/cell	Sasaki et al., 1980
	PHA-stimulated human lymphocytes (HL)	Trp-P-1	0.2-0.5	ug/ml	48 hr	23.2-46.0 induced SCEs/cell	
		Trp-P-2	1-3	ug/ml	48 hr	5.6-10.8 induced SCEs/cell	
	Chinese hamster cells (Don-6)	Trp-P-1	0.1-1	ug/ml	26-30 hr	3.3-11.3 induced SCEs/cell	
		Trp-P-2	0.1-7.5	ug/ml	26-30 hr	2.1-9.3 induced SCEs/cell	
	Chinese hamster embryonic cells (B-131)	Trp-P-1	0.25-1	ug/ml	25-27 hr	20.0-33.1 induced SCEs/cell	
		Trp-P-2	2.5-5	ug/ml	25-27 hr	3.8-4.1 induced SCEs/cell	
Morphological	Syrian Golden hamster embryo cells	Glu-P-1	10, 20	ug/ml	8 days	2 t.c.(b/230 or 811 survivors	Takayama et al., 1979
		Trp-P-1	0.1, 0.5	ug/ml	8 days	2 t.c./412 and 3 t.c./223 survivors	Takayama et al., 1977
		Trp-P-2	0.1, 0.5	ug/ml	8 days	3 t.c./505 and 7 t.c./459 survivors	
	Golden hamster embryo cells	Trp-P-2	0.5	ug/ml	10 days	30 t.c./2433 survivors	Borek and Ong, 1981
		Trp-P-2	X rad 50				
			rad + 0.5	ug/ml	10 days	68 t.c./4431 survivors	
		Trp-P-2	X ray 100				
			rad + 0.5	ug/ml	10 days	95 t.c./2220 survivors	

a). Adapted from Furihata and Matsushima, 1986. b). t.c. = transformed colonies.

2. HGPRT Test System

HGPRT (hypoxanthine, guanine phosphoribosyl transferase) is a distinctive enzyme that catalyzes the transfer of a ribose phosphate group from 5-phospho-α-D-ribose-1-pyrophosphate (PRPP) to either guanine or hypo-xanthine (Lehninger, 1975). HGPRT ordinarily converts hypoxanthine, guanine and xanthine to their respective nucleotides. 6-Mercaptopurine (MP) is a purine analogue, while 6-thioguanine (TG) and 8-azaguanine (AG) are pyrimidine analogues. These purine or pyrimidine analogues can be converted to toxic ribonucleotides by HGPRT. The proliferation of normal cells that have HGPRT is inhibited in medium containing any of these guanine analogues, but because HGPRT is dispensable under ordinary culture conditions, HGPRT-deficient mutant cells can proliferate to form resistant colonies (Demars and Held, 1972; Demars, 1974). The disadvantages of this test system include: (1) The test gives false positive results if the enzyme is barely turned off but no genetic damage has occurred; (2) When many cells detach during the mutagenic treatment, it is possible that the surviving fraction and the incidence of mutants differ in the attached and detached cell populations; and (3) The cell population density at the time of treatment may markedly affect cell survival. It has been reported that at greater population densities, the toxic effects of the mutagen may be greatly reduced. When HGPRT-deficient mutant cells in contact with wild-type cells become sensitive to guanine analogues, they do not proliferate to form scorable colonies, thus causing underestimation

of the mutant frequency (Jacobs and DeMars, 1984; Williams and Weisburger, 1986).

3. Ouabain Resistance Assay

Kuroki et al. (1980) have shown that ouabain can inhibit the Na/K-ATPase activity of the plasma membrane. Genetic alteration may affect the Na^+- and K^+-transport system of mammalian cells. Active Na^+- and K^+-transport is associated the Na^+/K^+, $-Mg^{++}$- activated ATPase of the plasma membrane, an enzyme that is specifically inhibited by the steroid compound ouabain (Kuroki et al., 1980). The growth of wild-type cells in culture is inhibited by ouabain, while the ouabain-resistant cells are mutants and differ from the wild type principally in the relative resistance of their plasma membrane Na/K-ATPase activity to ouabain.

According to Kuroki et al. (1977) and Kuroki and Drevan (1978), there are two main disadvantages of the ouabain system: (1) Like many other in vitro tests, it can only pick up the Na/K-ATPase mutation, and (2) The uptake of K^+ at 37°C is principally attributable to active transport and is inhibited by about 75% in the presence of 1mM ouabain. The residual uptake at 30°C in the presence of ouabain indicates incomplete inhibition of Na/K-ATPase at this concentration as well as a ouabain-insensitive component due to K^+ influx.

III. DNA DAMAGE OF CULTURED MAMMALIAN CELLS

Sister chromatid exchanges (SCE) and chromosome aberrations are two other methods that treat mammalian cells

with suspect chemicals. The SCE were first described by Taylor (1958) who found that if chromosomes were allowed to replicate once in the presence of tritiated thymidine, and then again in the absence of the isotope, autoradiographs showed that only one chromatid of each chromosome was labelled as a result of the semiconservative replication of DNA. Occasional symmetrical switches in the label between sister chromatids were observed, which Taylor (1958) called "sister chromatid exchanges". According to Perry and Thomson (1984), the SCE assay is based on the higher affinity for heavy atoms of some chromosome stains, like the fluorescent stain Hoechst 33258. By selective incorporation of a heavy atom (bromine) in DNA, the fluorescence is quenched and differential staining of the chromatids of one chromosome can be accomplished. The bromine required is added to the cultures as bromodeoxyuridine (BrdUrd). For evaluation of the SCE system in short-term screening tests, a wide range of compounds from different chemical classes (carcinogens and non-carcinogens) were tested (De Serres and Ashby, 1981; Latt et al., 1981, 1982; Abe and Sasaki, 1982).

The protocol details of chromosome aberration assays were reviewed by Dean et al. (1985). In these assays, the cell cultures are grown in media containing the suspect compound for a period of time. They are then transferred to fresh media for a period of time, after which they are fixed and examined for chromosome aberrations under the microscope.

Both the SCE system and the chromosome aberration system

are especially designed for detecting chromosome mutations (Thilly and Call, 1986).

IN VIVO MUTAGENICITY TESTS

There also are some in vivo mutagenicity tests that have been used for identifying mutagens formed during processing or cooking of meats. These in vivo tests are outlined in Table 3.

I. DROSOPHILA ASSAYS

Tests with Drosophila melanogaster represent an in vivo indicator system which permits the simultaneous and efficient testing of various types of genetic lesions from the molecular up to the chromosomal levels. Clark (1959) first suggested that Drosophila has the enzymes necessary for converting procarcinogens into genetically active metabolites. Special test protocols have been devised to detect aneuploidy (loss of a pair of chromosomes) resulting from nondisjunctional events. Description and evaluation criteria for these protocols are listed below:

(1) Wing spot mosaicism is a protocol measuring the wing spots from mutation, deletion, chromosome breakage, mitotic recombination or aneuploidy (Lindsley and Grell, 1968; Garcia-Bellido and Dapena, 1974).

(2) The white-zeste eye mosaic system, which was developed by Rasmuson et al. (1974), is based on the scoring of somatic mutations in an unstable white locus, leading to red sectors against a yellow eye background. A series of

Table 3: Genotoxicity of Pyrolysis Products in vivo(a

Endpoint measured	Species	Strain	Sex	Organ	Test compound	Exposure concentration	Exposure time	Results	References
Somatic eye-color mutation	Drosophila melanogaster		M	Eye	Trp-P-1	200, 400 ppm	24 hr	19 red spots/7574 flies, 22 red spots/5059 flies	Fujikawa et al., 1983
					Trp-P-2	400, 800 ppm	24 hr	11 red spots/6657 flies, 7 red spots/2304 flies	
Wing spot test	Drosophila melanogaster		M,F	Wing	AαC	400-1000 ppm	1 day	0.43-0.57 spot/wing	Yao et al.,1985
					Glu-P-1	100- 800 ppm	1 day	0.36-0.81 spot/wing	
					Glu-P-2	100- 800 ppm	1 day	0.50-0.67 spot/wing	
					IQ	100-1000 ppm	1 day	0.42-0.59 spot/wing	
					MeAαC	400 ppm	1 day	0.39 spot/wing	
					MeIQ	100 ppm	1 day	0.53 spot/wing	
					MeIQx	100- 200 ppm	1 day	0.44-0.51 spot/wing	
					Trp-P-1	200- 800 ppm	1 day	0.36-0.87 spot/wing	
					Trp-P-2	200- 800 ppm	1 day	0.55-0.89 spot/wing	
Skin color spot test	Mouse	C57Bl /6J Han	F	Fur	Trp-P-1	4.2 mg/kg BW ip on days 8,9,10 of pregnancy		8 recessive spots/317 offspring	Janson, 1983
					Glu-P-1	10 mg/kg BW ip on days 8,9,10 of pregnancy		12 recessive spots/293 offspring	
ATPase-deficient foci	Rat	Sprague-Dawley	M	Liver	Trp-P-1	10 mg/kg BW/day x 6, ip + 0.05% phenobarbital diet 16 weeks		7.2 ATPase-deficient foci/10 cm	Ishikawa et al., 1979
					Trp-P-2	10 mg/kg BW/day x 6, ip + 5 mg/kg BW x 2/day x 3, ip + partial hepatectomy + 0.05% phenobarbital diet 16 weeks		11.4 ATPase-deficient foci/10 cm	

a) Adapted from Furihata and Matsushima, 1986.

chemical mutagens, including both directacting agents and procarcinogens give positive results with this system (Nylander et al., 1979; Fahmy and Fahmy, 1980). This is also the system that Fujikawa et al. (1983) used to test the mutagenic activities of tryptophan pyrolysates. (3) The recessive lethal test of Fujikawa et al. (1983) has been used to test the mutagenicity of tryptophan pyrolysates. Test results have shown that it is less sensitive than the white-zeste mosaic system.

According to Vogel et al. (1985), the disadvantages of the Drosophila recessive lethal assay are two-fold: (1) It is a relatively tedious and time-consuming method in comparison to systems utilizing bacterial or lower eukaryotes; and (2) It has the possibility of strong elimination of cells carrying a mutation from the body during meiosis (divisions involved in the production of germ cells, 2n -> 1n).

The advantages of the Drosophila recessive lethal assay are three-fold: (1) The criterion used to determine the presence of a mutation is highly objective; (2) Lethals are much more frequent than other types of genetic lesions; and (3) A specific part of the Drosophila genome is involved by this multilocus test (Vogel and Ramel, 1980).

MUTAGENS IN MEAT

Sugimura et al. (1977) tested the mutagenicities of smoke condensate and the charred surface of broiled fish

and broiled steak using Salmonella typhimurium tester strain TA98, which is a test for frameshift mutations. The smoke condensate of broiled fish was found to be very mutagenic with metabolic activation. Its mutagenicity was about 10,000 times higher than the mutagenic activity that was derived from its benzo(a)pyrene (BaP) content. The dimethyl sulfoxide (DMSO) extract of the charred surface of both fish and steak was fairly mutagenic to TA98. The DMSO extract from the charred surface of one broiled fish had mutagenicity equivalent to 356 μg of BaP. A 5 g sample from the charred surface of beef steak had mutagenic activity equivalent to that of 855 μg of BaP. However, only 9 ng of BaP was found to be present in the smoke condensate from 100 g of broiled fish (Masuda et al., 1986).

Sugimura et al. (1977) compared the mutagenicities of smoke condensates obtained on heating calf thymus histone, chicken egg-white lysozyme, L-tryptophan, calf thymus DNA, yeast DNA, potato starch, and vegetable oil. Results showed that proteins having a high L-tryptophan content, and L-tryptophan itself, possessed strong mutagenicity.

Further studies on mutagenicity showed that pyrolysis products of proteins and certain amino acids contain strong mutagenic activity (Matsumoto et al., 1976, 1977; Nagao et al., 1977). Active mutagenic compounds were isolated from the pyrolysis products of various amino acids and their structures were characterized by Sugimura et al. (1977), Yamamoto et al. (1978), Wakabayashi et al. (1978) and Yokota et al. (1981). These compounds were designated as

Trp-P-1 (3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole) and Trp-P-2 (3-amino-1-methyl-5H-pyrido[4,3-b]indole), with both originating from tryptophan pyrolysates; Glu-P-1 (2 amino-6-methyldipyrido[1,2-α:3',2'-d]imi-dazole) and Glu-P-2 (2-amino-dipyrido[1,2-α:3',2'-d]imidazole), both of which were isolated from glutamic acid pyrolysates; Lys-P-1 (3,4-cyclopentenopyrido[3,2-α]carbazole), which came from a lysine pyrolysate; Orn-P-1 (4-amino-6-methyl-1H-2,5,10,10b-tetraazafluoranthene) from an ornithine pyrolysate; and Phe-P-1 (2-amino-5-phenylpyridine) from a phenylalanine pyrolysate. In addition, AαC (2-amino-α-carboline) and MeAαC (2-amino-3-methyl-α-carboline) were isolated as mutagens from a pyrolysate of soy-bean globulin by Yoshida et al. (1978) and shown to be mutagenic. Trp-P-1 and Trp-P-2 were stronger frameshift mutagens than aflatoxin B_1 toward Salmonella typhimurium mutant strain TA98 in the Ames test, and they also induced transformation in primary cultures of cryopreserved Syrian golden hamster embryo cells (Takayama et al., 1979).

Commoner et al. (1978a,b) determined that the increased mutagenic activity resulted from a S-9 dependent component in the nutrient broth used to grow the bacterial cells. Results led this research team to detect mutagenic activity both in commercial beef extract and in cooked ground beef (Vithayathil et al., 1978). The presence of mutagens in cooked beef was soon confirmed by others (Spingarn and Weisburger, 1979; Pariza et al., 1979a,b; Rappaport et al., 1979; Felton et al., 1981), who found that moderate

temperature cooking at 190-200°C, such as frying and broiling, induced high levels of mutagenic activity.

Kasai et al. (1979) first demonstrated that mutagenicity was present in the smoke from broiling or grilling fish, and the charred surfaces of fish and beefsteak. However, the mutagenic principles found in broiled sardines could not be attributed to the then-known mutagenic compounds, Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2, Lys-P-1, AαC or MeAαC. The authors then demonstrated that the mutagenic principle was present in the basic fraction of broiled sardines using reversed-phase column high-pressure liquid chromatography (Kasai et al., 1979).

Two potent mutagens formed in moderately heated foods, 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) and 2-amino-3,4-dimethylimidazo[4,5-f]quinoline (MeIQ), were then isolated from broiled fish by Kasai et al. (1980a). In addition, IQ was also isolated from beef extract and fried beef (Yamaizumi et al., 1980; Hargraves and Pariza, 1983; Turesky et al., 1983). The structure of IQ was elucidated based upon its ^{1}H-NMR spectrum, and low- and high-resolution mass spectra (Kasai et al., 1980b). Yokoyama et al. (1980) determined the crystalline and molecular structure of IQ. The formulas for IQ and MeIQ are given in Table 4.

The structure of MeIQ was determined by Kasai et al. (1980c) after comparing ^{1}H-NMR and high resolution mass spectral data for MeIQ with IQ. Later Kasai et al. (1981) also characterized a third potent mutagen, 2-amino-3,8-dimethylimidazo-[4,5-f]-quinoxaline (MeIQx) in fried beef

(Table 4).

There are two structurally different isomers of 2-aminotrimethylimidazo[4,5-f]quinoxaline (DiMeIQx) that have been isolated from a heated mixture of creatinine, amino acids and sugars. One of these was 2-amino-3,7,8-trimethylimidazo[4,5-f]quinoxaline (7,8-DiMeIQx), which was isolated from a heated mixture of creatinine, glycine and glucose (Negishi et al., 1984). The other isomer was 2-amino-3,4,8-trimethylimidazo[4,5-f]quinoxa-line (4,8-DiMeIQx), which was isolated from a heated mixture of creatinine, threonine and glucose (Negishi et al., 1985) or from a mixture of creatinine, alanine and fructose (Grivas et al., 1985).

Knize et al. (1985) detected six mutagenic peaks in meat fried at 200, 250 and 300°C after separation by HPLC. The two major peaks were identified as MeIQx and DiMeIQx. IQ was also detected as a minor component, but was present in samples fried at all three frying temperatures. The DiMeIQx isolated from fried-beef patties was later identified as the 4,8-DiMeIQx isomer (Knize et al., 1987). Kikugawa and Kato (1987) have recently shown MeIQx and 4,8-DiMeIQx to be the major mutagens in heated fish.

A new mutagenic compound, 2-amino-1-methyl-6-phenylimidazo-[4,5-b]pyridine (PhIP), was recently isolated from fried ground beef by Felton et al. (1986). This compound contains an aminoimidazole moiety like aminoimidazoquinoxaline and aminoimidazoquinoline compounds.

CHEMISTRY OF MUTAGENS FORMED ON COOKING MEAT

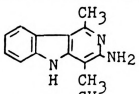
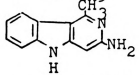
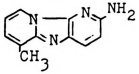
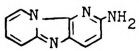
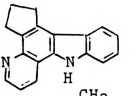
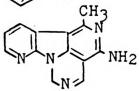
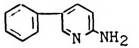
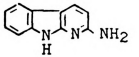
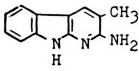
The chemical structures and formulas of the mutagens formed during cooking of meat are listed in Tables 4 and 5. Among these is Lys-P-1 which is the only heterocyclic imino compound. All other mutagens formed in cooking of meat are heterocyclic amines and include AαC, MeAαC, Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2, Phe-P-1, Orn-P-1, IQ, MeIQ, MeIQx, 4,8-DiMeIQx, 7,8-DiMeIQx, PhIP and TMIP.

Miller (1985) classified the mutagens formed in processed muscle foods into two groups: (1) mutagens induced by high temperatures, and (2) those formed at moderate temperatures.

The high temperature-induced mutagens are likely to be produced during cooking of proteinaceous foods at temperatures in excess of 300°C (Sugimura et al., 1977). Most of these compounds are protein pyrolysates (Sugimura, 1986). Except for Lys-P-1, all the high temperature-induced meat mutagens (AαC, MeAαC, Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2, Phe-P-1 and Orn-P-1) have a common 2-amino-pyridine structure. Felton et al. (1984a) showed that none of the high temperature-induced mutagens are present in detectable amounts in cooked ground beef based on analysis of total mutagenic profiles and recovery data.

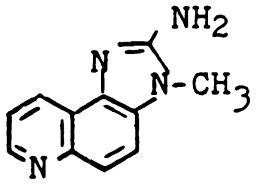
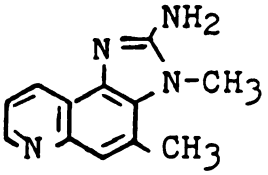
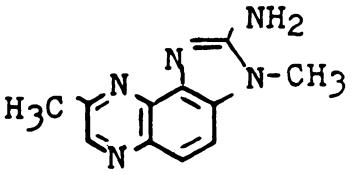
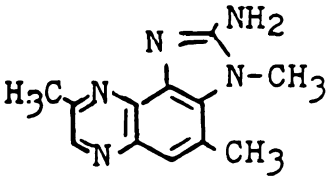
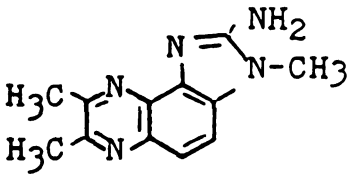
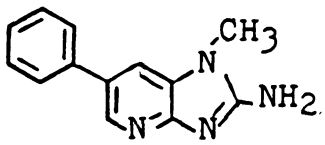
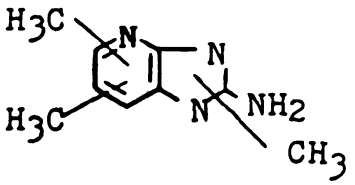
The moderate temperature-induced mutagens, so called IQ-like compounds, are 2-amino-imidazole-type mutagens (Furihata and Matsushima, 1986), and contribute most of the mutagenicity found in cooked meat (Kasai et al., 1979). Based on their chemical structures, Felton et al. (1986)

Table 4: High temperature-induced meat mutagens[a]

Chemical name	Abbreviation	Structure
3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole	Trp-P-1	
3-amino-1-methyl-5H-pyrido[4,3-b]indole	Trp-P-2	
2-amino-6-methyldipyrido-[1,2-a:3',2'-d]imidazole	Glu-P-1	
2-aminodipyrido[1,2-a:3',2'-d]imidazole	Glu-P-2	
3,4-cyclopenteno-pyrido[3,2-a]imidazole	Lys-P-1	
4-amino-6-methyl-1H-2,5,10,10b-tetraazafluoranthene	Orn-P-1	
2-amino-5-phenyl-pyridine	Phe-P-1	
2-amino-9H-pyrido-[2,3-b]indole	AαC	
2-amino-3-methyl-9H-pyrido[2,3-b]indole	MeAαC	

a) Adapted from Nagao et al. (1983) and Furihata and Matsushima (1986).

Table 5: Moderate temperature-induced meat mutagens(a

Chemical name	Abbreviation	Structure
2-amino-3-methylimidazo-[4,5-f]quinoline	IQ	
2-amino-3,4-dimethyl-imidazo[4,5-f]quinoline	MeIQ	
2-amino-3,8-dimethyl-imidazo[4,5-f]quinoxaline	MeIQx	
2-amino-3,4,8-trimethyl-imidazo[4,5-f]quinoxaline	4,8-DiMeIQx	
2-amino-3,7,8-trimethyl-imidazo[4,5-f]quinoxaline	7,8-DiMeIQx	
2-amino-1-methyl-6-phenyl-imidazo-[4,5-b]pyridine	PhIP	
2-amino-N,N,N-trimethyl-imidazopyridine	TMIP	

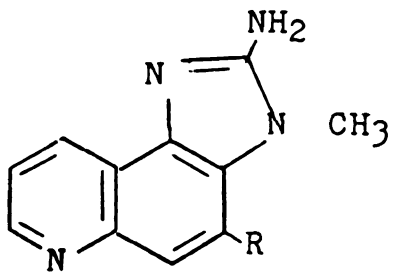
a) Adapted from Nagao et al. (1983), Furihata and Matsushima (1986) and Felton et al. (1986).

classified the IQ-like compounds as aminoimidazoazaarenes (AIAs). They further divided the moderate temperature-induced mutagens into three groups: imidazoquinolines, imidazoquinoxalines and imidazopyridines. The basic structures of these three groups are shown in Table 6. All of the IQ-like compounds possess an imidazo group with an amino moiety at the 2 position. They also have a methyl group on one of nitrogens in the imidazo ring and one or more aromatic rings are fused to the imidazo ring.

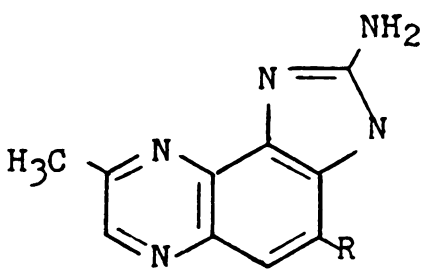
The high and moderate temperature-induced meat mutagens can be differentiated by their chemical resistance to nitrite. Tsuda et al. (1980, 1981) showed that moderate temperature-induced mutagens are resistant to deamination following nitrite treatment under acidic conditions. This is due to the "guanidine" structure in the imidazole ring (Sugimura, 1982b; Felton et al., 1984a). However, the mutagenicity of the high temperature amino acid pyrolysis products (Trp-P-1, Trp-P-2, AαC, MeAαC, Glu-P-1 and Glu-P-2) can be easily inactivated by adding dilute nitrite solution under weakly acidic conditions (Sugimura, 1982b). According to Nishioka et al. (1981), nitrate is present in saliva and foods and can be reduced to nitrite in the stomach, especially in subjects with intestinal metaplasia. Inactivation of high temperature-induced heterocyclic amines can occur at physiologically possible concentrations of nitrite (Nishioka et al., 1981).

Tsuda et al. (1981) demonstrated that all of these heterocyclic amines can be quickly degraded and lose their

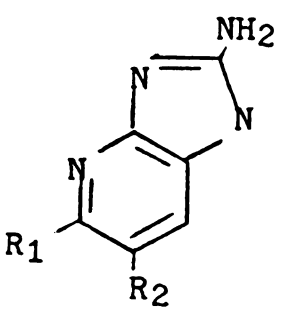
Table 6: Three basic structures of moderate temperature-induced meat mutagens (Felton et al., 1986).



Quinolines
R = H or CH_3



Quinoxalines
R = H or CH_3



Pyridines
R_1 = H or CH_3
R_2 = H, CH_3 or phenyl

mutagenic activity on treatment with hypochlorite, which is usually present in chlorinated tap water. These heterocyclic amines have a UV absorption peak at 250-260 nm, which disappears on treatment with hypochlorite. The half-life of 10 μM IQ in a solution containing 1.5 ppm of residual chlorine is less than 10 sec at room temperature, while the half-lives of Glu-P-1 and Trp-P-2 under the same conditions are 0.5-1 and 2-3 min, respectively. Tsuda et al. (1981) have identified the substance produced from Glu-P-1 by hypochlorite as an azo-dimer of Glu-P-1. Inactivation can be used to estimate the proportion of mutagenic heterocyclic amines in crude materials, because benzo[a]pyrene and other polyaromatic hydrocarbons are not inactivated by hypochlorite at such a low concentrations (Sugimura et al., 1982).

TOXICOLOGY OF IQ AND IQ-LIKE COMPOUNDS

Sugimura (1982c) demonstrated that IQ, MeIQ, and MeIQx are potent frameshift bacterial mutagens. The specific mutagenicities of these three compounds towards TA98 + S-9 were shown to be 433,000, 661,000 and 145,000 revertants/μg, respectively. The specific mutagenicities of IQ and MeIQ toward TA1538 + S-9 were 400,000 and 1,000,000 revertants/μg, respectively (Felton, 1987). According to Sugimura (1986), these IQ-like compounds are much more potent than Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2, and aflatoxin B_1 (AFB_1). Table 7 shows the comparative mutagenicities of these compounds.

Table 7: Specific mutagenic activities of the compounds isolated from pyrolysates and well known carcinogens(a

Revertants/μg			
S. typhimurium	TA98	S. typhimurium	TA100
MeIQ	661,000+	AF-2	42,000*
IQ	433,000+	MeIQ	30,000+
4,8-DiMeIQx	183,000+	Aflatoxin B1	28,000+
7,8-DiMeIQx	163,000+	4,8-DiMeIQx	11,200+
MeIQx	145,000+	4NQO	9,900*
Trp-P-2	104,200+	MeIQx	8,540+
Glu-P-1	49,000+	7,8-DiMeIQx	8,100+
Trp-P-1	39,000	IQ	7,000+
AF-2	6,500*	Glu-P-1	3,200+
Aflatoxin B1	6,000+	Trp-P-2	1,800+
Glu-P-2	1,900▪	Trp-P-1	1,700+
4NQO	970*	Glu-P-2	1,200▪
B(a)P	320+	MNNG	870*
AαC	300ϕ	B(a)P	660+
MeAαC	200ϕ	MeAαC	120ϕ
Lys-P-1	86ϕ	Lys-P-1	99ϕ
Phe-P-1	41ϕ	Phe-P-1	23ϕ
DEN	0.02ϕ	AαC	20ϕ
DMN	0.00ϕ	DMN	0.23ϕ
MNNG	0.00*	DEN	0.15ϕ

a) Adapted from Sugimura (1982c, 1986) and Knize et al. (1987).
* Without S-9 mix; + 10μl; ▪ 30μl; ϕ 150μl S-9/plate

Analysis of the DNA sequence changes responsible for the IQ-induced TA1538 reversion shows that deletion of a -GC- base pair results in a corrected reading frame and a viable revertant bacterial colony. The base-pair substitution strains, TA100, TA102, and TA104, were at least two orders of magnitude lower in their test responses than TA1538 (Felton, 1987).

Felton (1987) indicated that MeIQ is one of the most potent mutagens that has been tested in the Ames/Salmonella

bacterial mutagenesis assay to date. Alldrick et al. (1986) observed that MeIQ is a more potent bacterial mutagen when S-9 mixes from uninduced rats were used. Felton (1987) showed that PhIP, on the other hand, which is the most abundant mutagen by mass in fried ground beef, gave only 1,800 revertants/μg with TA1538 + S-9. This mutagenic response is much lower than that for IQ, MeIQ, and MeIQx.

A number of studies, however, indicates that the IQ-like compounds, while still biologically active, are not as potent in other genetic assays as with Salmonella. IQ was determined to be mutagenic in mammalian cells in vitro, but less so than Trp-P-2 (Thompson et al., 1983; Nakayasu et al., 1983). Nevertheless, Takayama and Tanaka (1983) found that IQ and MeIQ were not genotoxic in their in vitro Chinese hamster V97 cell mutagenicity assay. The authors suggested that the discrepancy may be due to differences in the genetic markers used for selection of mutation.

Radermacher et al. (1987) assessed the in vivo mutagenic potential of IQ with a rat granuloma pouch assay (Maier et al., 1978, 1980). The assay was performed with and without preinduction by Aroclor 1254. In the initial experiment, IQ was injected directly into the pouch of non-induced rats. The dose of IQ administered varied from 0.1 to 2.0 mg/pouch. There was a positive correlation between the mutation frequency and the administered dose of IQ. A 10-fold increase in mutation frequencies was obtained with the 2.0 mg/pouch dose of IQ with uninduced cell populations. In a second trial IQ was injected intraperitoneally and into

the pouch of rats that had been preinduced with Aroclor 1254. The Aroclor 1254 treatment produced no significant increase in mutation frequencies over that for the uninduced animals. The mutagenic effect of IQ in this study was about 10-fold weaker than that of benzo[a]pyrene or N-methyl-N'-nitro-n-nitrosoguanidine (MNNG).

Bird and Bruce (1984) determined that feeding of IQ and MeIQ to rats caused high levels of nuclear aberrations in colonic crypt cells. The compounds tested were tryptophan pyrolysates (Trp-P-1 and Trp-P-2), a glutamic acid pyrolysate (Glu-P-1), IQ and MeIQ. The number of nuclear aberrations (NA) per crypt was determined 24 hours after oral administration of these amines at various dose levels. Trp-P-1, Trp-P-2, and Glu-P-1 increased the incidence of NA approximately 2-fold to 3-fold above the back-ground levels (0.15 NA/crypt), even at near-lethal dose levels. However, IQ and MeIQ increased the incidence by 5-fold and by 10-fold when administered at non-lethal dose levels ranging from 200 to 800 mg/kg body weight and at 50 to 200 mg/kg body weight, respectively. The colon-specific toxicity of the heterocyclic amines at approximately 35% of their maximum tolerated dose levels was in the order of MeIQ > IQ > Trp-P-2 > Trp-P-1 ≥ Glu-P-1 according to Bird and Bruce (1984).

IQ has a planar skeletal structure, suggesting that it is intercalated between DNA bases, thus promoting the adduct-forming reactions between DNA and the active metabolite(s) of IQ (Yokoyama et. al., 1980). Cortesi and Dolara

(1983) showed that addition of IQ induced neoplastic transformations in Balb3T3 mouse embryo fibroblasts at concentrations of 1, 5 and 15 ng/ml.

Experiments have shown that IQ is moderately carcinogenic in mice, producing tumors in the liver, forestomach, and lungs when fed at 300 ppm (Ohgaki et al., 1984). In a similar rat study, tumors were observed in the zymbal gland of the ear duct, in the intestines and in the mammary gland (Takayama et al., 1984b). MeIQ is also carcinogenic to both male and female mice at a dose of 0.04% in the pelleted diet, inducing tumors in the forestomach and liver (Ohgaki et al., 1985).

Sugimura (1983) suggested that the discrepancy between the high mutagenic and moderate carcinogenic potency of IQ may be idiosyncratic to the Salmonella assay, since these organisms contain high concentration of guanines and cytosines at the mutation sites. IQ has a high affinity for guanine, and runs of this "hot spot" would make the strain particularly sensitive to this compound.

Ohgaki et al. (1987) gave MeIQx orally to both sexes of CDF_1 mice at a concentration of 0.06% in a diet for 84 weeks. Liver tumors were induced in 43% of the males and 91% of the females. The incidence of liver tumors in mice of both sexes was significantly higher in groups fed MeIQx than in the control group. The incidence of lung tumors in females and of lymphomas and leukemia in both sexes were also significantly higher than in their respective controls.

METABOLISM

The metabolic aspects of mutagens formed in cooked meat have been extensively reviewed by Sato et al. (1986). Most of the metabolic activation of mutagens formed by pyrolysis were carried out using Trp-P-2 and Glu-P-1 with S-9 or another concentrated microsomal fraction of rat liver (Ishii et al., 1981; Yamazoe et al., 1980; Wakata et al., 1985).

Hashimoto et al. (1980a,b) have indicated that after esterification, the metabolites of the pyrolyzed mutagens become more active. They may bind to DNA and cause strand scissions. Hashimoto et al. (1980b) found that a N-hydroxyamino derivative of Trp-P-2 reacted with DNA to some extent, but its N-O-acyl derivative reacted more strongly. In the case of Glu-P-1, the N-O-acyl derivative is regarded as the ultimate form that reacts with DNA (Hashimoto et al., 1980a). Chemically synthesized N-O-acetyl-Glu-P-1 can form 2-(C^8-guanyl)-amino-6-methyldipyrido[1,2-a:3',2'-d]-imidazole, which is a DNA base adduct (Hashimoto et al., 1980a,b). The structures of the adducts of Trp-P-2 and Glu-P-1 have been shown to be C^8-guanylamino derivatives by Hashimoto et al. (1980a,b).

The N-hydroxy derivative of 2-amino-3-methylimidazo-[4,5-f]-quinoline (IQ) was also isolated by Okamoto et al. (1981). Yamazoe et al. (1983) showed that the mechanism of mutagenic activation of IQ appears to be via N-hydroxylation of the exocyclic amino group by cytochrome P-450 monooxygenase(s) to form 2-hydroxyamino-3-methyl-imidazo-[4,5-f]-quinoline (N-hydroxy-IQ). Using the metabolic inhibitor,

ellipticine, Yamazoe et al. (1983) also demonstrated that cytochrome P-448-mediated N-hydroxylation was necessary for the formation of the active mutagen. Mutagenicity studies with TA98 showed that N-hydroxy-IQ is a direct mutagen with the specific activity of 2 x 10^4 revertants/nm (Snyderwine et al., 1987). The data confirm that N-hydroxy-IQ is a mutagenic metabolite of IQ and further implicates this hydroxylamine in the carcinogenicity of IQ-like compounds.

Loretz and Pariza (1984) using a hepatocyte assay observed that methimazole, a flavin monooxygenase inhibitor (Prough and Ziegler, 1977), failed to reduce macromolecular binding of IQ, which suggests that this enzyme is not involved in N-hydroxylation. Studies on the potential mechanisms of IQ detoxication have also been carried out by Alldrick et al. (1986) who observed that both acetyl-CoA and glutathione can reduce mutagenicity of IQ and MeIQ. Loretz and Pariza (1984) observed that addition of glutathione to the assay led to a reduction in the macromolecular binding of IQ, while Shinohara et al. (1984) demonstrated that IQ could be N-acetylated by enzymes in the cytosolic fraction of hepatic homogenates.

Alldrick et al. (1986) utilized a modified Ames assay with S-9 fractions derived from either corn oil (uninduced) or Aroclor 1254-treated Sprague-Dawley rats with different metabolism modifiers and inhibitors to study the metabolic conversion of IQ and MeIQ to direct bacterial mutagens. The activation of both compounds was inhibited by ellipticine, indicating a role for cytochrome P-448 and methimazole,

which suggests that flavin monooxygenases may also play a role in activation of IQ and MeIQ.

Alldrick et al. (1986) also demonstrated the importance of N-oxidation in the biotransformation of IQ and MeIQ. This was confirmed by the inhibitory effects of tryptamine and tyramine on the mutagenicity of IQ and MeIQ. Rice et al. (1976) have shown that both of these biogenic amines, which are common in several foods and beverages, can competitively inhibit amine oxidases.

IQ and MeIQ may share a similar affinity for the enzymes involved in their activation, but MeIQ appears to be more sensitive to the effects of acetyl-CoA (Alldrick et al., 1986). These results, therefore, imply that methylation at the 4-position increases the reactivity of the active MeIQ metabolite, and hence, its ability to exert a mutagenic effect.

For further activation of N-OH-Trp-P-2, the involvement of propyl tRNA synthetase has been proposed by Yamazoe et al. (1981, 1982). An acetyl-CoA-dependent enzyme has also been shown by Shinohara et al. (1985) to activate N-OH-Trp-P-2 and N-OH-Glu-P-1 and cause them to bind to DNA. Wakata et al. (1985) reported that N-OH-Trp-P-2 is a directacting mutagenic compound. They showed that N-OH-Trp-P-2 can induce lesions in DNA in two distinct ways: (1) by covalent binding, and (2) by strand cleavage. Results using N-OH-Trp-P-2 indicated that cleavage is caused not by N-OH-Trp-P-2 itself, but by agents formed during spontaneous degradation of this compound.

Since sulfhydryl reagents can inhibit degradation, results suggest that it is an oxidative process. DNA cleavage can also be inhibited by catalase, which indicates that the active oxygen species rather than compounds derived from Trp-P-2, are responsible for strand cleavage. It also has been shown that oxygen radicals can cause DNA single-strand cleavage (Totter, 1980).

Wakata et al. (1985) showed that the N-OH-Trp-P-2 binding to DNA is through N-OH-Trp-P-2 itself. This view is consistent with results reported by Mita et al. (1981), in which binding of N-OH-Trp-P-2 to DNA was demonstrated. Wakata et al. (1985) reported that cysteamine inhibited spontaneous degradation of N-OH-Trp-P-2 and enhanced covalent binding of N-OH-Trp-P-2 to DNA. This result is consistent with that of Negishi and Hayatsu (1979), who reported that addition of cysteamine enhances Trp-P-2 mutagenicity.

Nagao et al. (1983) tested the involvement of sulfotransferase in the activation of various heterocyclic amines. They added pentachlorophenol, an inhibitor of this enzyme, to the assay and showed that the mutagenicity of Glu-P-1 and IQ was markedly inhibited but Trp-P-2 was not affected. Results suggested that the activated metabolites of Glu-P-1 and IQ are sulfate esters of their N-hydroxy derivatives, but further activation of N-OH-Trp-P-2 must involve other mechanisms, such as acetyl-transferase.

Many researchers have indicated that ingested heterocyclic amines are metabolized and excreted into the bile, and probably are further converted to other active

substances by microbial enzymes in the gut (Kosuge et al., 1978; Sugimura, 1982a,b,c; Sugimura and Sato, 1983; Sjodin and Jagerstad, 1984; Sugimura, 1985; Sato et al., 1986). Bashir et al. (1987) incubated IQ with mixed human fecal microflora under anaerobic conditions. The major metabolite detected was 2-amino-3,6-dihydro-3-methyl-7H-imidazo[4,5-f]-quinoline-7-one (HO-IQ). Bashir et al. (1987) stated that they are currently measuring the mutagenicity of HO-IQ but results have not yet been reported.

To study the distribution and excretion of Glu-P-1, Sato et al. (1986) introduced [imidazole-^{14}C]-Glu-P-1 into the stomach of male F344 rats by gastric intubation at 0.3 mCi in water at 20.8 mg/kg body weight. At various times post-administration, samples of organs, urine, feces and blood were taken and their radioactivities were assayed. They found that total radioactivity per unit wet weight was highest in the liver during the first 48 hr, which was followed by the kidney. By 48 hr, the radioactivity in these two organs had decreased to one-fourth of their 5 hr values. Five hours after administration, total radioactivity comparable to that in the kidney was found in the mucosa of the small and large intestines. In the intestinal tissues, however, the activity remaining after 24 hr or 48 hr was very low. Cold ethanol-precipitable radioactivity in the liver homogenate was about one-half of total activity at 5 hr, and 75% of the total activity at 48 hr. In the kidney, activity was 22 and 50% at 5 and 48 hr, respectively. In other organs, some cold ethanol-precipitable radioactivity

also remained, but the actual counts were very low compared to those in the liver and kidney. Excretion of radioactivity into the urine increased gradually up to 24 hr, when 35% of the administered amount had been excreted. A relatively high amount of radioactivity was bound to the macromolecules in the liver throughout the observation period. However, this was not the case with the kidney where the amount bound by the macromolecules was minuscule. Moreover, in the small and large intestines, which may also be targets of Glu-P-1 carcinogenicity, the amount of radioactivity bound to the macromolecules was very low.

To study the mutagens after intragastric administration of ^{14}C-Glu-P-1, Sato et al. (1986) collected the bile for 24 hr. The collected bile was then extracted with acetonitrile and applied to an HPLC column (Li-Chroprep RP-8) and eluted with acetonitrile/water (40/60, v/v). At least four peaks of radioactivity, numbered from I to IV in order of elution, were separated. Significant mutagenicity with S-9 was found only in peak IV. Peak IV was subjected to further HPLC with an ODS column and eluted with acetonitrile/water (30/70, v/v). Two major peaks of radioactivity were observed, and their mutagenicity was shown to coincide with their radioactivity. Based on the elution position, mass and ^{1}H-NMR spectra, the material in the later eluting peak was identified as unchanged Glu-P-1. The material in the earlier peak had an m/s of 240, suggesting that it might be N-acetyl-Glu-P-1. The amount of unchanged Glu-P-1 and N-acetyl-Glu-P-1 in 24-hr-bile accounted for only 3 to 7% of the total

dose, respectively. The specific activities of N-acetyl-Glu-P-1 toward S. typhimurium TA98 and TA100 were about one-fourth those for Glu-P-1 with S-9. No mutagenicity was detected without S-9.

Sato et al. (1986) also found that radioactivity in the blood at 24 hr after administration of a single dose of ^{14}C-Glu-P-1 corresponded to about 1% of the total dosage, and decreased with time. The radioactivity was mostly recovered in the globin fraction of hemoglobin, especially the β-chains.

In a balance study, Sjodin and Jagerstad (1984) measured absorption and excretion of ^{14}C-labelled IQ and MeIQ in rats of both sexes. Excretion was rapid and within 24 hr more than 90% of the radioactivity had been excreted by the rats. After 72 hr the fecal excretion of both compounds had accounted for approximately 45-65%, and the corresponding excretion via the urine amounted to 37-49%. Only 1-2% of the residual activity was still present in the carcasses, and less than 0.2% was found in the expired air. In a separate 24-hr study, about 70% of the IQ and 80% of the MeIQ was found in the bile. The two compounds had different biliary excretion patterns, with IQ radioactivity being excreted in one major peak within 4-5 hr, while MeIQ radioactivity was excreted in several peaks spread over a longer period of time. Mutagenicity of the bile correlated closely with excretion of radioactivity.

Using humans, Kuhnlein et al. (1983) fed a non-meat diet for 7 days followed by a diet high in meat and refined

grains. The meat diet resulted in an increase in fecal mutagenic activity within two weeks as shown on testing with S. typhimurium TA100 and TA98. Also working with humans, Hayatsu et al. (1985a) demonstrated that feeding of fried ground beef (equivalent to 150g raw weight) resulted in greatly increased fecal mutagenicity for the next two days on testing with S. typhimurium TA98.

Baker et al. (1982) also reported mutagenic activity in urine samples obtained from individuals who had eaten either fried pork or fried bacon. Mutagenic activity was reported to persist as long as 24 hours after eating either of these products. Sousa et al. (1985) and Hayatsu et al. (1985b) also have reported that ingestion of fried ground beef also increases urinary mutagenicity toward TA98 + S-9.

After the ingestion of fried ground beef, mutagenic heterocyclic amines, possibly MeIQx and its metabolites, were recovered from human feces and urine (Hayatsu et al., 1985a,b). Trp-P-1 and Trp-P-2 were detected in the dialysis fluid of a patient with uremia (Manabe et al., 1987). These results indicate that mutagenes generated by frying of meat can be ingested, absorbed, and excreted by humans in biologically detectable quantities.

MECHANISMS OF IQ-LIKE COMPOUND FORMATION

MAILLARD REACTION AND IQ-LIKE COMPOUND FORMATION

Many investigators have tried to explain how the thermally induced mutagens are formed. Spingarn et al.

(1980) compared mutagens formed in the pan frying of beef with these formed during cooking of some high starch foods (baked biscuits, fried pancakes, fried potatoes, and toasted bread). All these foods showed mutagenicity in the presence of S-9. However, fried beef had at least 10-fold more mutagenic activity than the high starch foods.

Jagerstad et al. (1983) fried beef patties with either normal or low glucose levels. The outer meat crust from the low-glucose meat showed very low mutagenic activity compared to normal beef when frying conditions were the same. On spreading 1 ml. of a 5% D-glucose solution over the upper surface of the low-glucose patties just before frying, the brown color increased as well as the mutagenic activity. Jagerstad et al. (1983) noticed that the mutagenicity of the crusts from fried beef patties as well as the amount of brown color increased in parallel to increases in heating conditions. Holtz et al. (1985) also noticed a high correlation between mutagenicity and color development in baked meat loaves. These results suggest that the Maillard reaction may play an important role in the formation of mutagenic substances.

A number of investigators have demonstrated that mutagens can be generated in model Maillard reaction systems. Spingarn and Garvie (1979) found that refluxing of reducing sugars (especially rhamnose, xylose, glucose, and galactose) plus an ammonium salt produced strong mutagenic activity. These reactions were basecatalyzed and were inhibited by the antioxidant propyl gallate.

Jagerstad et al. (1983) added one of the Maillard reaction products (2,5-dimethylpyrazine or 2-methylpyridine) to a refluxing model system containing creatinine, d-glucose and one amino acid (either glycine or alanine) in diethylene glycol:water (6:1, v/v). Results indicated that addition of either of the Maillard reaction products enhanced mutagenicity by about 50%.

Shibamoto et al. (1981) showed that heating of maltol and ammonia at 100°C for 5 hr produced mutagens that were detectable by TA98 and S-9. These investigators determined that alkylpyridine derivatives were responsible for the mutagenic activity.

ROLE OF CREATINE AND CREATININE

In skeletal muscle of vertebrates and to a lesser extent in other tissues, creatine (α-methylguanidoacetate) is an important reservoir of high-energy phosphate groups. Phosphorylation of the terminal amino acid group on the creatine molecule results in formation of the high-energy compound phosphocreatine (Bessman and Carpenter, 1985). Creatine is lost from the metabolic pool *in vivo* by spontaneous cyclization of either phosphocreatine or creatine itself to yield creatinine (Zubay, 1983).

Mutagenicity was observed by Yoshida and Okamoto (1980a) on heating mixtures of creatine and glucose above 150°C for 1-2 hr, and could be detected by TA98 and S-9. No activity was observed without glucose being present in the reaction mixture.

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Yoshida and Okamoto (1980b) refluxed 0.05 mole of each of 17 different amino acids with creatine, adenine or 0.5 g of albumin in 100 ml of 0.5 M glucose for 8 hr. After refluxing, these solutions were extracted and subjected to the Ames test in the presence of S-9. Only the solution of creatine and two amino acids (arginine and lysine) were found to have significant mutagenicity towards TA98. Since creatine and glucose are common components of muscle, they may provide a significant contribution to the formation of mutagens during the heating of meat (Yoshida and Okamoto, 1980b).

Yoshida and Fukuhara (1982) observed mutagenic responses from mixtures of heated creatine with cystine, threonine, phenylalanine, methionine, tryptophan, valine, proline, or serine on heating at 200°C, and using TA98 and S-9 as the test system. Since free cystine, lysine and tryptophan are not normally present in meat (Nikuni and Kata, 1966), Yoshida and Fukuhara (1982) concluded that the formation of mutagens may occur by the reaction between creatine and other amino acids during the cooking of beef.

Raw beef contains creatine, which on heating is converted to creatinine according to Jagerstad et al. (1983), who then studied the effect of creatinine on mutagen formation in fried meat. Chemical analyses to determine creatine and creatinine, were performed on both low-glucose and normal beef before and after frying. During frying of beef, varying amounts of creatinine were produced from creatine in all samples. The mutagenic activity increased

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irectly with the formation of creatinine. On some beef atties, a 2% solution of creatine was spread over the upper urface just before frying, which also increased the utagenic activity.

MeIQx (Jagerstad et al., 1983, 1984) and 7,8-DiMeIQx Negishi et al., 1984) were detected on refluxing creatinine ith glucose and an amino acid (glycine or alanine) in a odel system. Similarly, IQ was generated from a heated ixture of proline and creatine (Yoshida et al., 1984), from heated mixture of creatinine, glycine and glucose (Barnes nd Weisburger, 1983) and from a heated mixture of creatiine, glycine and fructose (Grivas et al., 1986). Muramatsu nd Matsushima (1984) showed that formation of 4,8-DiMeIQx ccurred during heating of a mixture of creatinine, sugar glucose or ribose) and an amino acid (alanine or lysine).

Negishi et al (1984) showed that 4,8-DiMeIQx can also e formed on refluxing of creatinine, glucose, and hreonine. Shioya et al. (1987) showed that PhIP can be ormed by heating a mixture containing creatinine, phenyllanine and glucose in diethylene glycol-water solution for hour at 128°C. The yield of PhIP was 3.6 nmoles/millimole f creatine equivalents.

To identify the precursors that yield heterocyclic mine mutagens in cooked meat products, Taylor et al. (1986) repared a lean round steak H_2O-homogenate. The homogenate as centrifuged to yield a residue and homogenate that were abelled R_1 and S_1, respectively. On pan heating of S_1 for 0 min at 95 to 100°C and recentrifugation, a second residue

(R_2) and second supernatant (S_2) were obtained. Results of the Ames/Salmonella TA1538 mutagen assay with S-9 indicated that although S_2 comprised only 5% of the dry weight and only 10% of the water soluble protein in the original homogenate, the compounds in S_2 were responsible for all of the S-9 dependent mutagenic activity. The authors demonstrated that mutagenic activity can be generated by 3 different cooking conditions: (1) by prolonged boiling, (2) by pressure cooking at 200°C, and (3) by dry oven baking at 200 to 300°C. In addition, HPLC experiments showed that the compounds in S_2 are also the precursors for mutagen formation in the outer surfaces of 200°C griddle-fried ground beef.

In order to determine the precursors of mutagens in S_2, Taylor et al. (1986) added test compounds to S_1 before it was converted to S_2. They tested 20 separate amino acids or combinations of amino acids along with various non-amino acid nitrogenous compounds. They found that maximal S_2 mutagenic activity was obtained by adding 10 mM Trp or 2.5 mM creatine phosphate (CP), or synergistically by 10 mM Trp, 2.5 mM CP and 1.0 mM $FeSO_4$.

By HPLC, paper electrophoresis, and resistance of the active HPLC fractions to acid-nitrite inactivation, Taylor et al. (1986) demonstrated that boiled S_2 contained IQ, MeIQ and Trp-P-2. When S_2 was boiled with creatine phosphate, it doubled the IQ content and decreased Trp-P-2 production by one-half, produced a trace amount of MeIQ, and generated an unknown nitrite-resistant mutagen. On boiling S_2 that was

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repared from S1 with 10 mM Trp, 2.5 mM CP and 1.0 mM $FeSO_4$, he same four mutagens were produced. However, yields of oth IQ and Trp-P-2 were increased and large amounts of rp-P-1 were also generated. These results indicate that rp (or its degradation products) and creatine phosphate (or ts degradation products) are the precursors in beef for the ndole ring in Trp-P-type mutagens and the NH_2-imidazole ing in IQ-type mutagens, respectively. It seems that the minoimidazole moiety of aminoimidazoquinoxaline and minoimidazoquinoline compounds is derived from creatinine nd/or creatine, while the remainder of their structures is erived from amino acids and sugars.

It is noteworthy that creatine is present only in muscle oods of vertebrate origin (Lehninger, 1975). Mutagenic ests of a variety of heated foods have shown that meat roduces mutagens at levels an order of magnitude higher han plant foods (Sugimura et al. 1977), a fact that could e due to the lack of creatine in plants.

Miller (1986) reported that fried shell fish (shrimp and callop) does not contain any mutagenicity. This may be due o the fact that shell fish use arginine instead of creatine s the high energy reservoir in the muscle system (Zubay, 983).

OLE OF FAT IN MUTAGEN FORMATION

The role of the lipid fraction in the development of mutagenic activity of meat is still not very clear. Barnes et al. (1983) and Barnes and Weisburger (1983, 1984) claimed

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that fat is important in the formation of the mutagenic compounds. Barnes et al. (1983) developed a quantitative assay for IQ based on TLC and HPLC. Using this method, high fat (25% of total wet weight) and low fat (11%) beef patties, cooked 5 min/side, were found to contain 20.1 and 0.5 μg of IQ per kg of sample, respectively. Barnes and Weisburger (1983) reported that inclusion of beef lipids into a heated mixture of creatinine, glycine and glucose can increase the mutagenic activity three-fold. Barnes and Weisburger (1984) showed that adding either corn oil or beef fat (beef suet) can increase the mutagenic activity of fried ground beef. Both of these lipids doubled the amount of mutagens formed in fried meat when added to the samples at a concentration of 20% based on the wet weight of the ground beef. Barnes and Weisburger (1984) showed the addition of glycine and creatinine to ground beef prior to cooking enhances mutagen formation by approximately 50%. On supplementation of the system with glycine, creatinine and glycerol, mutagen formation increased by approximately 100%. These results indicate that lipid decomposition may contribute precursor(s) for mutagen formation and that glycerol may account for at least part of the mutagen-enhancing effect of fat.

Felton et al (1984b) and Knize et al. (1985) carefully examined the role of fat content on mutagen production, and specifically its effects on the amount of IQ produced. In a comparison of thick patties of lean beef (15% fat) and of high-fat beef (30% fat), the total revertants/g of sample

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were lower at the higher fat content. This could be explained by a decrease in the level of mutagen precursors in the lean by dilution with fat, thus decreasing the proportion of mutagenic compounds. In a separate trial, the amount of IQ in the thick high-fat patty (21,000 revertants/kg) was not significantly different from that of the thin low-fat patty (22,000 revertants/kg). The percentage of IQ present was estimated by purifying the IQ peak and determining the number of revertants. Equivalent IQ values for the two samples were confirmed by mass spectrometric analysis of IQ in the HPLC-purified material.

Knize et al. (1985) compared the effect of fat concentration on cooking at 180 and 240°C. At both temperatures, 8% fat produced the least mutagenic activity. However, at 15% fat there was an approximate doubling of activity, while 30% fat slightly reduced mutagenic activity in comparison to the 15% fat sample. Thus, increasing the fat content from 8 to 15% enhanced mutagenicity on cooking at both 180 and 240°C. On the other hand, increasing the fat content from 15 to 30% resulted in a slight reduction in overall mutagenic activity. These researchers suggested that increasing the lipid concentration from 8 to 15% enhanced the conduction of heat into the meat, but above 15% of additional lipid had no further effect. In other words, the increased mutagenic effect was not believed to be due to the fat *per se* but rather due to increased heat penetration associated with the increase in fat content between 8 and 15%.

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ROLE OF PYROLYSIS IN MUTAGEN FORMATION

Since cooking procedures range from mild heating to strong heating, the formation of mutagens during cooking may be due to both browning and pyrolysis. Yoshida and Okamoto (1982a) found that mutagenic activity could be detected for the pyrolysis products of the organic ammonium salts of malate, citrate, tartarate and oxalate on heating at 550°C for 1 min on using TA98 and S-9 as the test system.

Ohe (1982) tested 21 nitrogen-containing compounds for mutagenic activity after pyrolysis at 300-600°C for 3 min using TA100 and TA98 in the presence of S-9. Methylguanidine, agmatine, dihydrouracil, dimethylamine, diethylamine, trimethylamine, triethylamine, pyrrolidine, morpholine, sarcosine, piperazine, piperidine, spermine, and spermidine showed mutagenic activity, especially with TA98.

Spingarn et al. (1980) proposed that the mechanism for mutagen formation is by breaking down sugars or starches to form smaller, more reactive, unsaturated aldehydes and ketones. They suggested that amino acids or other amines may be degraded by heat and through formation of Amadori compounds may add ammonia to the carbonyls. These fragments can then combine, cyclize and dehydrate to yield heterocyclic structures as first demonstrated by Koehler (1969). The mutagens are probably produced from creatinine, aldehydes, and Maillard reaction products (Furihata and Matsushima, 1986).

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MUTAGEN FORMATION DURING COOKING AND FOOD PROCESSING

Krone and Iwaoka (1981) suggested that mutagens are probably formed not only on cooking of beef, but also during the heating of other foods. An extensive survey of mutagen formation during cooking of the major and secondary sources of protein in the US diet was carried out by Bjeldanes et al. (1982). They found that fried ground beef, beef steak, ham, pork chops and bacon, as well as baked and broiled chicken and broiled beef steak, exhibited significant amounts of mutagenicity. Other sources of protein, such as milk, cheese, tofu and organ meats, produced negligible mutagenicity upon cooking.

Herikstad (1984) screened some Norwegian food products for mutagenicity, including seafoods, bakery products and cheese. Only fried fish cakes and baked pudding were reported as being mutagenic.

Krone and Iwaoka (1981) reported that mutagens were formed during pan frying of salmon, sole, snapper and turbot fillets at 190°C, but not during broiling in an electric oven. Krone and Iwaoka (1984) also reported that some canned food products contain mutagenic substances. The results of mutagenic analysis of canned foods are listed in Table 8, and as shown beef and beef-containing products consistently displayed mutagenic activity. However, seafoods were more varied in their mutagenic responses. Pink salmon was the most mutagenic canned product tested, whereas, tuna (water pack) and sardines contained no detectable or very low levels of mutagens. Basic extracts

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of canned turkey, chicken, beef stew, ham, Vienna sausages and corned beef also exhibited a low mutagenic activity ratio (MAR) of less than 2.5, as was also true in raw salmon, beef, chicken and turkey (Krone and Iwaoka, 1984).

Table 8: Mutagenicity of some commercially canned meats and seafoods(a

	Mutagenic activity ratio(b,c	
Product	-S9(d	+S9(e
Pink salmon (Brand #1)	0.8	17.6
Beef broth	2.2	13.0
Pink salmon (Brand #2)	0.6	11.9
Red salmon	1.3	8.5
Beef stew (retort pouch)	0.9	7.4
Mackerel	1.2	7.2
Roast beef hash	0.4	6.0
Chili with beans	1.1	4.9
Roast beef	2.1	4.6
Tuna (oil pack)	1.8	3.8
Minced clams	1.9	3.8
Corned beef hash	1.2	3.0

a. Adapted from Krone and Iwaoka (1984).
b. Mutagenic activity ratio is calculated by dividing the number of revertant colonies on plates containing food extracts by the number of spontaneous revertants.
c. Mutagenic activity ratio using Salmonella typhimurium TA1538 with the basic organic extracts from 80 g of product.
d. Without metabolic activation.
e. With 80 µl S9 per plate.

Frying of meat and fish products usually results in formation of mutagens near the surface of the product in contact with the heating source (Dolara et al., 1979; Felton et al. 1981). Krone and Iwaoka (1987) attempted to determine if this was also the case in the canned salmon. They divided the contents of a 1-lb can into three portions, i.e., a cylindrical core from the center of the can (~5 cm

in diameter), an outer cylindrical shell (~1 cm in thickness), and the broth which was first drained from the can. Each portion was extracted and tested by the Ames assay. It was found that 76% of the mutagenicity was located in the outer shell, which comprised only 50% of the total can contents. On the other hand, the core (30% of total weight) and broth (20% of total weight) contained 17 and 7% of the total mutagenicity, respectively. If an outer shell of smaller thickness could have been obtained, the differences would probably be even more marked.

During the canning process, canned salmon is sometimes reprocessed or reconditioned, which leads to an increase in total heating time. Krone and Iwaoka (1987) have attempted to determine if there is more mutagen formation in the reprocessed products. They opened single processed canned salmon, and drained the fluid. The salmon was then repacked into new cans. Brine or water was added, the cans were resealed and the heat treatment was repeated. There was a two-fold increase in the mutagenicity of the flesh and broth. It appears that the mutagen forming reactions had not been completed during the initial heat treatment so that an increase in heating time increased mutagenicity.

Krone and Iwaoka (1987) compared three different canning processes: (1) 100°C for 139 min; (2) 116°C for 85 min; and (3) 121°C for 64 min. There was no significant difference in the amount of mutagens formed by these three processes. All three treatments resulted in low mutagenicity. The authors suggested that processing temperatures

below 135°C result in low mutagenicity, whereas, those above 135°C cause a sharp increase in mutagen formation.

In the canned salmon system, Krone and Iwaoka (1987) demonstrated that browning reaction inhibitors decreased the quantity of mutagens formed. Sodium bisulfite (a browning inhibitor), when added at 0.5% to canned salmon, completely eliminated mutagen formation. Dipping salmon steaks in a 0.5% $NaHSO_3$ solution and draining for 30 s. before packing into cans and heat processing also decreased mutagenicity. Nearly the same degree of reduction in mutagen formation was accomplished by adding 1% ascorbic acid. The products with added bisulfite exhibited a distinct sulfur odor, whereas, the ascorbic acid treatment did not alter the appearance or odor. Hence, it may be possible through relatively simple means, such as addition of ascorbic acid before processing, to minimize mutagen formation in canned meat and fish products.

Pariza et al. (1979a) found that canned chicken broth and beef broth exhibited moderately high levels of TA98 mutagenic activity, while crackers, corn flakes, rice cereal, and bread crust had low levels of mutagenic activity. Bread crumbs, toast (surface), and coconut cookies also had low levels of mutagenic activity.

Krone and Iwaoka (1981) found mutagenic activity in fish fried at 190°C on using TA1537, TA1538, and TA98. Levin et al. (1981) used a modification of the Salmonella assay with TA98 and found that many commercial food preparations had significant levels of mutagenic activity. These

included dehydrated products such as: beef broth, vegetable beef soup mix, seasoning, beef bouillon cubes, beef barley soup, and ox-tail soup. Other products showing activity were canned chicken broth and evaporated milk.

A number of reports have appeared in the literature concerning formation of mutagens in cooked pork and pork products. Gocke et al. (1982) found mutagenic activity in pan-fried sausages in seven test systems. Bjeldanes et al. (1982) found that many protein-rich foods normally consumed by Americans, including pork products, formed genotoxic components when cooked and tested by TA1538 and S-9. Miller and Buchanan (1983a,b) detected mutagens in both nitrite-free and nitrite-treated bacon. Overvik et al. (1984) observed similar mutagenic activity in pan-broiled pork.

Krone and Iwaoka (1987) examined one commercial product, which was packed in a retort pouch (beef stew), and found it to contain significantly higher levels of mutagenicity than a similar product in a standard metal can. This may be due to the fact that the maximum retort temperature at which pouch laminates are processed has been increased from 121 to 135°C (Krone and Iwaoka, 1987).

Nader et al. (1981) showed that broiled beef surfaces contained elevated mutagenic activity when tested with TA98 and S-9. On the other hand, microwave-heated beef held for up to three times the normal cooking period at 2440 MHz did not exhibit any genotoxicity. Similar observations were also reported by Commoner et al. (1978a,b) and Baker et al. (1982) in beef, and by Miller and Buchanan (1983b) in pork.

However, Taylor et al. (1982) observed little mutagenic activity in ground beef that was deep-fat fried for 3 min. They concluded that, in general, deep-fat fried foods possess low levels of mutagenic activity, and that severe frying conditions must be employed to obtain appreciable levels of activity. These results may be due to volatilization or the inability to extract the mutagens from the oil (Rappaport et al., 1979).

COOKING CONDITIONS AND MUTAGEN FORMATION

COOKING TIME AND TEMPERATURE

The effect of temperature on mutagen production in cooked ground beef was first described by Commoner et al. (1978b). A number of investigators have subsequently shown that mutagen production increases with the temperature of cooking (Spingarn and Weisburger, 1979; Hatch et al., 1982; Bjeldanes et al., 1983).

Temperature is the most important established determinant for mutagen formation in muscle foods. Cooking methods that employ higher heating temperatures generally induce greater mutagenic activity than low temperature methods (Miller and Buchanan, 1983b).

Pariza et al. (1979b) investigated the mutagenic activity of hamburgers fried at 143, 191 and 210°C. Mutagenic activity assayed with the Ames test was not detected in uncooked hamburger. In hamburgers fried at 143°C,

mutagenic activity remained low at all cooking times studies (4-20 min). In contrast, frying at 191°C or 210°C for up to 10 min resulted in the generation of considerably higher levels of mutagenic compounds.

Dolara et al. (1979) investigated the effects of temperature on the formation of mutagens on reflux boiling of beef. The results showed that at 100°C mutagenic activity increases approximately linearly with time over a 13 hr period. The rate of production of mutagenic activity at temperatures between 68 and 98°C conformed closely to the Arrhenius equation, yielding an activation energy of 23,738 calories per mole. Extrapolation from these data predicted that a sharp rise in the rate of mutagen formation would occur between 140 and 180°C. This was confirmed on cooking ground beef patties in various conventional electrically-heated appliances, operating at different cooking temperatures within the 140 to 180°C temperature range.

Dolara et al. (1979) in another trial indicated that ground beef patties cooked in different appliances showed dissimilar mutagenicity. The mutagenic activities were in the following descending order: electric hamburger cooker (5.5 min) > electric frying pan (3 min) > electric broiler (10 min) ≥ microwave oven equipped with a browning plate (10 min) > microwave oven using a paper plate (10 min). These results suggest that mutagens are not produced in beef cooked at 100°C for a 5 to 10 min period, but are produced when the same meat is cooked at surface temperatures in the range of 190 to 210°C for 3 to 6 min. Since the center

internal temperature of a hamburger cooked in this way never exceeds 100°C, this study suggests that mutagen formation is restricted to the outer surfaces where the temperature is the highest.

Spingarn and Weisburger (1979) compared mutagen formation on cooking by frying, broiling and boiling. High levels of mutagenic activity were formed rapidly on frying, but more slowly during broiling. Formation of mutagens in boiled beef stock required several days under reflux conditions and showed a strong concentration dependence.

Jagerstad et al. (1983) measured mutagenicity in the crust of beef patties containing 2.0% fat using TA98 + S-9. The surface temperature of the fry pan was maintained at 120, 180 or 250°C, and the frying time was varied between 1.5 and 9 min. When fried at either 120 or 180°C, there were no significant differences between mutagenicity at different frying times. However, frying at 180°C for 3 min, resulted in slightly higher mutagenic activity than frying at 120°C for the same time period. At 250°C the mutagenic activity in the crust increased directly with increasing frying times.

Holtz et al. (1985) compared the mutagenicity of meat loaves having the same fat and water content on baking at 39 to 97 min. With increasing baking times, the final surface temperature rose from 129 to 170°C. The mutagenicity increased in parallel up to a surface temperature of 150°C, but did not increase further on raising of the temperature.

Knize et al. (1985) showed that frying increased total

mutagenic activity with increasing cooking times. They compared the HPLC profiles of mutagenic compounds in the extract of ground beef patties fried at 200, 250 and 300°C for 6 min per side. The HPLC profiles of the mutagenic compounds were similar. However, the total mutagenic activity measured by TA1538 for an extract of meat fried at 300°C was roughly 4-fold higher than that fried at 200°C. In general, cooking temperature appears to greatly affect the quantities of mutagens produced, but does not appear to influence their HPLC profiles, i.e., the total number of mutagenic compounds formed.

Knize et al. (1985) pointed out that the thickness of the meat patties did not affect the total yield of mutagens except at longer cooking times. Furthermore, thickness of the meat patties did not influence the number of mutagenic components.

MODULATION OF ACTIVITY OF HEAT-INDUCED MUTAGENS

Man is seldom exposed to a single mutagen or carcinogen, but rather to complex mixtures of several chemical and/or physical agents. Mixtures may consist of one or more toxic compounds in combination with a variety of possible positive and negative modulators (Ames, 1983). The following agents may either increase or suppress the biological risks associated with the heat-induced mutagens: antioxidants, peroxidases in the presence of H_2O_2, plant extracts, fatty acids, the acidic fraction from meat extract, vitamins, β-thials, pyrrole pigments, human saliva, soy

protein concentrate, defatted glandless cotton-seed flour, xanthine derivatives, germanium and cobaltous chloride, liquid smoke and others. These compounds will each be discussed below.

1. Antioxidants

In a model system, Spingarn and Garvie (1979) found that refluxing of reducing sugars (especially rhamnose, xylose, glucose, and galactose) plus an ammonium salt produced strong mutagenic activity. These reactions were base-catalyzed and were inhibited by the antioxidant propyl gallate.

The effects of antioxidants on mutagen production in cooked ground beef was first described by Wang et al. (1982), who showed that adding butylated hydroxyanisole (BHA) to meat before cooking successfully reduced its mutagenicity after frying. Later, Barnes et al. (1983) showed that BHA can inhibit 2-amino-3-methyl-imidazo[4,5-f]-quinoline (IQ) formation by 40% during cooking of beef.

2. Peroxidases with H_2O_2

Yamada et al. (1979) showed that Trp-P-1, Trp-P-2, Glu-P-1 and AαC are broken down by peroxidases (myeloperoxidase, lactoperoxidase, and horseradish peroxidase) in the presence of H_2O_2. The degradation of the mutagenic compounds could befollowed by changes in their absorption spectra, by thin layer chromatography of the reaction products, and by a decrease in the mutagenicity of these compounds. The

degradation of Trp-P-1 and related compounds by myeloperoxidase was not stimulated by adding 0.3M NaCl and was not affected by β-carotene, which is an effective quencher of singlet molecular oxygen (Kearns, 1971).

3. Plant Extracts

Fresh extracts from vegetables and fruits, such as cabbage, broccoli, green pepper, eggplant, apple, burdock (Arctium Lappa L.), stone-leek (Allium fistulosum L.), ginger, mint leaf, and pineapple have been shown to inactivate the mutagenic principles in tryptophan pyrolysates (Morita et al., 1978). The factor in extracts of leaves of cabbage (Brassica oleracea) responsible for inactivation of Trp-P-1 and Trp-P-2 was identified as a peroxidase. Its molecular weight was 43,000 and it contained a sugar moiety (Inoue et al., 1981).

4. Fatty Acids

Hayatsu et al. (1981a) have shown that ether extracts of human feces can inhibit the mutagenicity of Trp-P-1, Glu-P-1 and AαC. The inhibitors in the feces extracts were identified as oleic and linoleic acid. Hayatsu et al. (1981b) suggested that oleic acid can interfere with Trp-P-2 mutagenesis at 2 stages: (1) by inhibiting the S-9-mediated activation process, and (2) by blocking the subsequent process of attaching the activated mutagen to DNA. Whether inhibition of activation results from blocking of the process at stage 1 or at stage 2 is still not clear.

However, Hayatsu et al. (1981b) indicated that oleic acid can inhibit mutagenesis of S-9-treated Trp-P-2 (the direct mutagen) and suggested that an interaction takes place between oleic acid and the mutagen.

5. The Acidic Fraction of Meat Extract

Hayatsu et al. (1981b) found that the mutagenicity present in the basic fraction of cooked ground beef was completely suppressed by addition of the acidic fraction obtained from cooked beef. Pariza et al.(1986) showed the modulator(s) in the acidic fraction of fried ground beef that was responsible for the antimutagenic effect is a nonpolar molecule or a class of nonpolar molecular molecules. The modulator(s) is/are insoluble in water, 1.2N HCl and 2.5N NaOH, but is/are soluble and stable in concentrated H_2SO_4. These solubility properties appear to eliminate several possible molecular structures, for example, proteins, peptides, charged or polar lipids, carbohydrate molecules with 5 carbons or less, phenol or polyhydroxy phenols, hydroxy acids, amino acids, amides, amines, acids or anhydrides. Pariza et al.(1986) showed that a modulator(s) from beef extract inhibited the mutagenicity of 7,12-dimethylbenz[a]anthracene (DMBA) on using TA98 and S9. They found that the modulator(s) inhibited the metabolism of DMBA by rat liver microsomes. Furthermore, liver microsomal preparations from untreated or phenobarbital treated rats were much more sensitive to inhibition by the modulator(s) than were chromosomes from 3-methyl-cholanthrene treated

rats. The formation of the DMBA-3,4-diol (a precursor to the ultimate diol epoxide) was substantially more sensitive than the other DMBA metabolites. These results suggest that the modulator(s) act(s) selectively on certain forms of cytochrome P-450 and notably on form(s) producing high levels of 3,4-diol. Hayatsu et al. (1981b) attributed the antimutagenic effect in the fried ground beef to the presence of oleic acid in the acidic fraction. They then indicated that the acidic fraction contained 3% by weight of palmitoleic acid, 7% palmitic acid, 4% stearic acid, 22% oleic acid and 3% linoleic acid. Pariza (1987) have isolated and identified a modulator in fried hamburger. The modulator, designated CLA (conjugated linoleic acid), is a derivative of linoleic acid. Pariza (1987) stated that CLA is an effective inhibitor of skin cancer in mice. Besides being found in fried hamburger, Pariza (1987) also found CLA in uncooked beef and some dairy product, especially cheese.

6. Vitamins

Busk et al. (1982) showed that the mutagenic activity of Trp-P-1, Trp-P-2, Glu-P-1 and Glu-P-2 can be inhibited by the addition of vitamin A (retinol) *in vitro*. The effect was interpreted as being due to inhibition of metabolic activation of their respective ultimate mutagenic forms. Retinol was shown to have no toxic effects on the survival of *Salmonella* cells. It also had little or no effect on direct acting mutagens or on the formation of NADPH in the test system. The results demonstrate the need for an

increased understanding of the interaction of dietary components on the mutagenic/carcinogenic risks from processed foods containing different dietary constituents.

7. β-Thiols

β-thiols, such as cysteamine, cysteine, and N-acetyl-cysteine, and the comutagens harmon and norharmon, have been shown to enhance genotoxicity (Sugimura et al., 1977; Negishi and Hayatsu, 1979; Deflora et al., 1984). Cysteine and its derivatives were found to increase the mutagenic activity of the tryptophan-pyrolysis products, Trp-P-1 and Trp-P-2, as assayed by the Salmonella-microsomal system. A several-fold increase in the number of revertant colonies was caused by addition of cysteine, cysteine ethyl ester and cysteamine at 10 mM concentrations in the reaction mixture containing the bacteria and the mutagens. Studies on the structural requirement for the enhancing effect suggest that both the thiol and the amino groups are necessary in order for the compound to exhibit the increased mutagenic effect. The cysteine derivatives did not affect the mutagenic activity of either benzo(a)pyrene or the beef-extract mutagen(s) (Negishi and Hayatsu, 1979).

8. Pyrrole Pigments

Pyrrole pigments, such as hemin, biliverdin, chlorophyllin, and protoporphyrin, have been shown to have a strong inhibitory effect towards the meat mutagens (Arimoto et al., 1980a,b.; Hayatsu et al., 1981b). Barnes and

Weisburger (1984) showed that Fe^{3+} or Fe^{2+} can be released through denaturation of heme proteins and can catalyze mutagen formation in processed meat. Addition of 10 ppm Fe^{3+} and Fe^{2+} to ground beef prior to cooking doubled the mutagenic activity, but higher concentrations of Fe^{2+} were less effective. On the other hand, increasing the concentration of Fe^{3+} from 10 to 60 ppm caused a further increase in mutagen formation. Taylor et al. (1986) found that synergistically adding 10 mM Trp + 2.5 mM CP + 1.0 mM $FeSO_4$ was the most effective means for enhancing the mutagenic activity of cooked meat homogenates.

9. Human Saliva

Nishioka et al. (1981) showed that the mutagenic activity of Trp-P-1 as measured by TA98 or TA100 with metabolic activation can be greatly inhibited by human saliva. They also demonstrated that the mutagenic activity of pyrolysates of beef, salmon and sodium glutamate were significantly decreased by adding human saliva to tester strain TA98 + S-9. However, adding human saliva to the polypeptone pyrolysate did not significantly effect mutagenicity. In a subsequent trial, it was observed that preincubation with saliva instead of S-9 did not activate the mutagenicity of Trp-P-1.

10. Soy Protein Concentrate

Wang et al. (1982) reported that they were successful in preventing the formation of mutagens in fried beef by

adding soy protein concentrate. However, volumetric or dilution effects seemed to be largely responsible for the reduction of mutagenicity by the soy product.

11. Defatted Glandless Cottonseed Flour (GCF)

Rhee et al. (1981), Ziprin et al. (1981) and Rhee (1986) have shown glandless cottonseed flour to be effective in retarding lipid oxidation in various soy and meat products. Rhee et al. (1987) have reported that 5% GCF (by weight) significantly reduced mutagen formation in fried beef patties. The magnitude of mutagen reduction tended to be much greater than the meat dilution effect from adding GCF.

12. Xanthine-Derivatives

Yamaguchi and Nakagawa (1983) reported that the xanthine-derivatives (theophylline, caffeine, and 3-isobutyl-1-methyl-xanthine) reduced the mutagenicity of Trp-P-2, 2-acetyl amino-fluorene (AAF) and B(a)P. The effect of these compounds may be due to their interference with the metabolism of these mutagens by S-9. As far as the relationship between the antimutagenic effect and the structural specificity of these derivatives were concerned, the imidazole rather than the uracil moiety in xanthine appeared to be responsible for the reduction in mutagenicity. The 1(N)-methyl-derivatives seemed to be more antimutagenic than the original xanthine compounds.

13. Germanium Oxide (GeO_2) and Cobaltous Chloride ($CoCl_2$)

Mochizuki and Kada (1982) and Kada et al. (1984) showed that germanium oxide (GeO_2) and cobaltous chloride ($CoCl_2$) were potent antimutagens on Trp-P-1-induced reverse mutations in Salmonella typhimurium TA98 and TA1538. The antimutagenic effects of $CoCl_2$ and GeO_2 appear to be exclusively related to cellular events. Thus, the authors suggested that these two compounds may interfere with the inducible error-prone DNA-repair system (SOS repair system).

14. Liquid Smoke

Liquid smoke is a concentrated acidic solution containing the natural phenols and carbonyls characteristically present in wood smoke (Toth and Potthast, 1983). It imparts the flavor, odor and color familiarly associated with smoked foods. Liquid smoke has long been known to have antioxidative properties (Kurko, 1963; Tilgner, 1967; Daun, 1969; Tilgner and Daun, 1970; Daun and Tilgner, 1977). Some of these compounds have structures similar to the known antioxidants (BHA, BHT and PG) according to Daun (1969, 1979). Chuyen (1986) reported that the neutral fraction of liquid smoke inhibits the mutagenicity of Trp-P-1, Trp-P-2, Glu-P-1 and IQ by 50, 80, 70 and 30%, respectively.

15. Others

Barnes et al. (1983) showed that when either Celite (10%, w/w) or casein (10%, w/w) were added to the ground meat, the IQ content in the final fried beef extract were

decreased by 48.7% and 69.5%, respectively. On the basis of this study, it was suggested that the physical properties and texture of the meat may influence mutagen formation.

MATERIALS AND METHODS

MATERIALS

Solvents and Chemicals

All solvents were either glass distilled reagent grade or HPLC grade. MeIQ and MeIQx were obtained as a gift from Dr. S. Grivas, at the Swedish University of Agricultural Science, Lund, Sweden. PhIP and 4,8-DiMeIQx were provided courtesy of Dr. James M. Felton of Lawrence Livermore National Laboratory, University of California, Livermore, CA. IQ was purchased from Wako Chemicals USA, Inc. (Dallas, TX). Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), n-propyl gallate (PG) and disodium dihydrogen pyrophosphate were purchased from Sigma Chemical Company (St.Louis, MO). Tertiary butylhydroquinone (TBHQ) and Tenox 4 (20% BHA and 20% BHT dissolved in corn oil) were purchased from Eastman Chemical Products, Inc. (Kingsport, TN). Sodium sulfate and sodium nitrite were purchased from J.T. Baker Chemical Company (Phillipsburg, NJ). Citric acid was purchased from MCB Manufacturing Chemists, Inc. (Cincinnati, OH). Ascorbic acid was purchased from Mallinckrodt Inc. (Paris, KY). Covitol (mixed tocopherol concentrate) was purchased from Henkel Corporation (Minneapolis, MN). Liquid smoke was manufactured by E.H. Wright Company (Brentwood, TN) and purchased at a local grocery store. Amberlite XAD-2 resin

was purchased from Aldrich Chemical Company (Milwaukee, WI).

Source of Meat

Beef muscle was obtained from either the Michigan State University Meat Laboratory or from a local meat packer (Vanalstine Packing Co., Inc., Okemos, MI). Portions of the semitendinosus muscle (eye of round) were excised from cull dairy cow carcasses at 24-48 hr postmortem. After all visible fat was trimmed from the muscle, it was ground twice through a meat grinder (Model 84181D, The Hobart Manufacturing Company, Troy, OH). The ground meat was weighed, wrapped, frozen and stored at -20°C until removed for cooking and/or analysis. Samples of meat were taken randomly before frozen for fat and moisture determination.

Beef fat from the kidney knob was removed and frozen at -20°C for one hour. The frozen beef fat then was chopped with the food processor attachment using the same Hobart grinder. The chopped beef fat was frozen and stored at -20°C until needed for the experiments described later.

EXPERIMENTAL

EVALUATION OF THE MUTAGENICITY OF FRIED GROUND BEEF

The purpose of this experiment was to determine the mutagenicity of pan fried ground beef with or without metabolic activation.

FRYING METHOD

A stainless steel, Teflon-coated electric fry pan was used for the frying procedure. The temperature control of the fry pan was set at 215°C. After thawing, the ground beef was made into 100g patties about 0.5 cm in thickness. Before frying, the fry pan was preheated for 5 min. In the present study, the patties were fried for 9 min per side.

EXTRACTION METHOD

The fried patties were extracted using modifications of the methods of Pariza et al. (1979) and of Felton et al. (1981) as shown in Figure 1. The fried ground beef patties were blended and extracted in a Waring blender at moderate speed with 1 volume of distilled water for 3 min. The homogenate was filtered through cheese cloth to remove the solid material. After filtration, the solid material was blended and extracted in the Waring blender at moderate speed with 1 volume of distilled water for 2 min and then was filtered through the same cheese cloth again. This procedure was repeated one more time. After combining all of the filtrates, the sample was acidified to pH 2 with HCl then saturated with NaCl (35 g NaCl/100 ml). The filtrate then was filtered through glass wool.

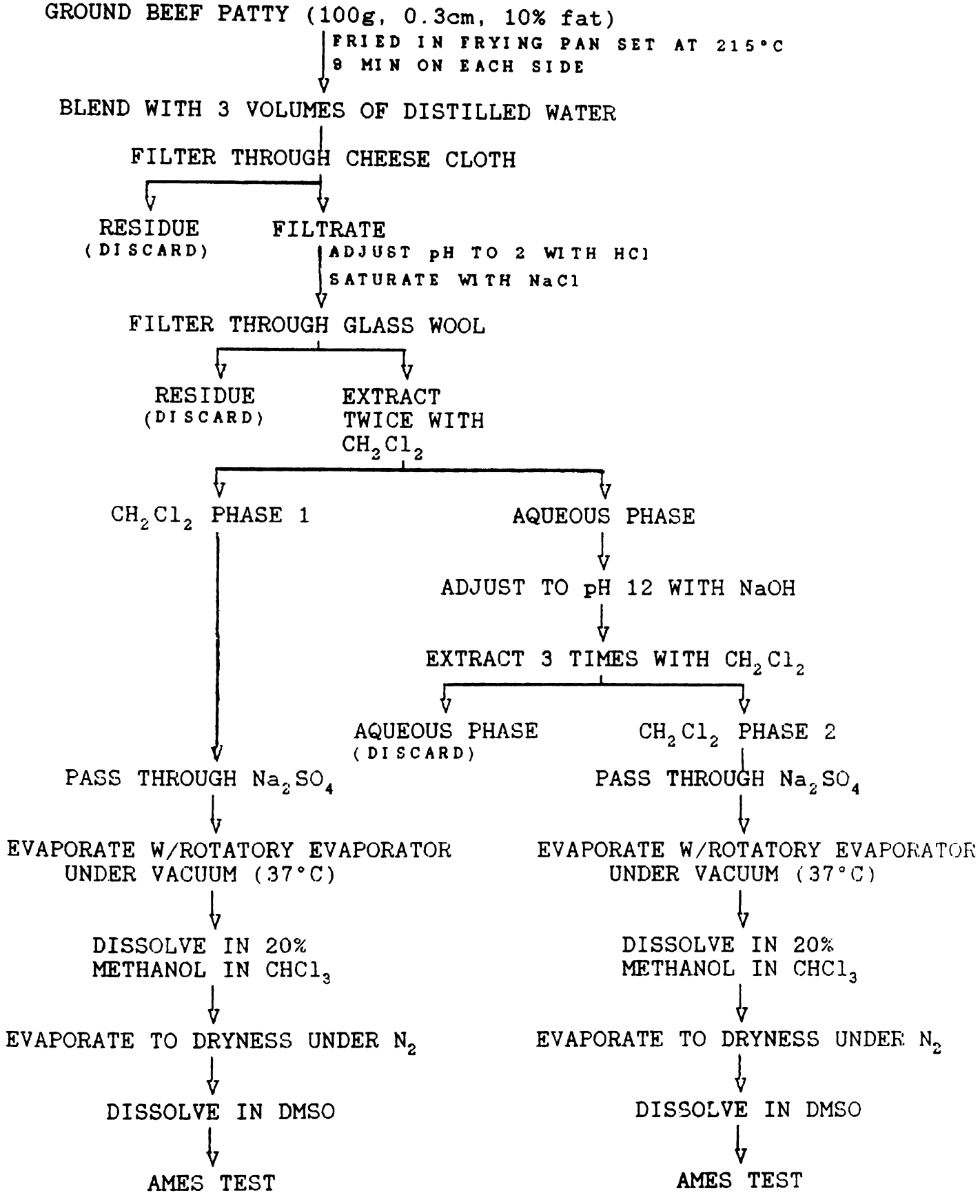


Figure 1. Extraction Method No 1.

LIQUID-LIQUID PARTITION

The acidic filtrates were transferred to 1000 ml separatory funnels. Then an equal volume of methylene chloride was added. The separatory funnels were shaken vigorously for 1 min. The layers were allowed to separate, and the lower methylene chloride layer, which contained the soluble acidic components, was drained into a 4000 ml flask. The methylene chloride extraction was repeated one more time and the methylene chloride extracts were combined. The upper aqueous layer was adjusted to pH 12 with saturated NaOH solution. The basic aqueous phase was extracted three times with methylene chloride to remove the basic components. The two methylene chloride extracts containing the acidic and basic components were passed through anhydrous sodium sulfate separately to remove excess water. These two extracts then evaporated to dryness in a rotatory evaporator (Buchi Rotary Evaporation System, Switzerland) using a water bath setting of 37°C. The residues were dissolved in 20% methanol in $CHCl_3$ (v/v) and quantitatively transferred to 2 dram vials using disposable glass pipets. The solutions in the vials were evaporated to dryness (N-Evap Evaporator, Model 106, Organomation Assoc., Worcester, MA) under a stream of nitrogen using a water bath setting of 40°C. Special care was taken to avoid over-heating of the dry extract. Then the residues were dissolved in dimethylsulfoxide (DMSO) and used for the Ames test.

AMES TEST

The Ames test was carried out as described by Ames et al. (1975) and Maron and Ames (1983). Salmonella typhimurium strains TA98, TA100, TA102, TA104, TA1535 and TA1538 were provided courtesy of Dr. Bruce N. Ames at University of California, Berkeley, CA.

Tester Strains

The tester strains were received on a piece of filter paper in saline solution in a sterilized plastic bag. The plastic bags were opened with sterilized scissors. The filter papers were aseptically transferred into 18 x 150 mm culture tubes with loose fitting caps containing 5 ml of Oxoid 25% nutrient broth No. 2 (Oxoid Ltd., Hants, England). The liquid tester strain cultures were grown in a 37°C gyrotory incubator (Model C-25, New Brunswick Scientific Co., Inc. Edison, NJ) set at approximately 210 rpm for 10 hr. The liquid culture of each tester strain was streaked on a nutrient agar plate, incubated at 37°C for 24 hr and stored at 5°C until the liquid culture was checked out and found to be satisfactory.

Confirming Genotypes of Tester Strains

The tester strains were confirmed: (a) immediately after receiving the culture, (b) when a new set of frozen permanent or lyophilized cultures was prepared, (c) when the number of spontaneous revertants per plate failed to fall in the normal range, or (d) when there was a loss of

sensitivity to standard mutagens.

Fresh broth cultures were used for these tests. All reagents, glassware, petri plates, inoculating sticks, and cotton swabs were sterile. The reagents and growth media are listed in Appendix A.

Histidine Requirement. The His- character of the tester strains was confirmed by demonstrating the histidine requirement for growth on selective agar plates. A wire loop was dipped into the culture, a single streak across the biotin control plate was made and then across the histidine/ biotin plate. Some 5 or 6 strains can be tested on each plate. The strains were identified by labelling each streak with a marking pen on the bottom of the petri plate. The histidine/biotin plates were incubated for 24 hr at 37°C and examined for growth. There should be no growth on the control plates.

rfa Mutation. Strains having the deep rough character (rfa) were tested for crystal violet sensitivity (Ames et al., 1973a,b). For this test, nutrient agar plates were seeded with cultures of the strains to be tested and a sterile filter paper disc containing crystal violet was placed on the surface of each seeded plate. For each tester strain, 0.1 ml of a fresh overnight culture was added to a tube containing 2 ml of molten top agar held at 45°C. It was not necessary to add histidine and biotin. The tubes were vortexed for 3 sec at low speed and poured on a nutrient agar plate. The plates were tilted and rotated to distribute the top agar evenly. Then it was placed on a

level surface and allowed several min to become firm. Afterwards, 10 µl of a 1 mg/ml solution of crystal violet were pipeted on the center of sterile filter paper discs (1/4 inch) and transferred to each of the seeded plates using sterile forceps. The disc was pressed lightly with the forceps to embed it slightly, taking care not to move it laterally. The plate was inverted and incubated at 37°C. After 12 hr incubation, a clear zone of inhibition (approximately 14 mm) appears around the disc indicating the presence of the rfa mutation, which permits large molecules such as crystal violet to enter and kill the bacteria. Wild-type strains or strains containing the gal deletion are not inhibited because the crystal violet cannot penetrate the cell.

uvrB Mutation. The uvrB mutation can be confirmed by demonstrating UV sensitivity in strains that contain this mutation (Ames et al., 1973b). With sterile swabs, the tester strain cultures were streaked across a nutrient agar plate in parallel stripes. Non-R-factor strains should be streaked on a separate plate. A piece of cardboard was placed over the uncovered plate so that half of each bacterial streak was covered. The plates were irradiated with a UV lamp at a distance of 33 cm. Non-R-factor strains (TA1535 and TA1538) were irradiated for 6 sec and R-factor strains (TA97, TA98 and TA100) were irradiated for 8 sec. A strain with the wild-type excision repair enzymes (e.g., TA102) should be tested on the same plate as a control for the UV dose. The irradiated plates were incubated at 37°C for 24 hr. Strains with the uvrB deletion will grow only on

the unirradiated side of the plate.

R-Factor. The R-factor strains (TA97, TA98, TA100 and TA102) were tested routinely for the presence of the ampicillin resistance factor because the plasmid is somewhat unstable and can be lost from the bacteria (McCann et al., 1975). To test for ampicillin resistance, the cultures were streaked across the surface of an ampicillin plate using the procedure described for confirming the histidine requirement. Several strains can be tested on the same plate. The non-R-factor strains (TA1535, TA1537 or TA1538) can be tested for ampicillin activity on the same plate as the control. After incubation for 24 hr at 37°C, there should be growth along the streaks for the R-factor strains and no growth along the control streak.

pAQ1 Plasmid. The pAQ1 strains (TA102 and TA104) should be tested for both ampicillin and tetracycline resistance on ampicillin/tetracycline plates. An R-factor strain is used as a control for tetracycline.

Spontaneous Reversion. The spontaneous reversion of the tester strains to histidine independence is measured routinely in mutagenicity experiments and is expressed as the number of spontaneous revertants per plate. Each tester strain reverts spontaneously at a frequency that is characteristic of the strain. Acceptable ranges of spontaneous reversion may be somewhat different in different laboratories, but they should be relatively consistent within a laboratory. The estimate of spontaneous reversion rates for different tester strains are listed in Table 9. In the

present study, at least 3 spontaneous mutation control plates were run for each strain in the mutagenicity assays.

Table 9: Estimates of spontaneous reversion rate ranges for different tester strains[a].

Strain	Revertant/Plate (No S-9 Fraction[b])	Type[c]
TA1535	10-35	BS
TA100	120-200	BS
TA1537	3-15	FS
TA97	90-180	FS
TA1538	15-35	FS
TA98	30-50	FS
TA102	240-320	FS

[a]. Adapted from Maron and Ames, 1983.
[b]. The number may be slightly different on plates containing the S-9 fraction.
[c]. BS - Base-pair substitution; FS-Frameshift mutation.

S-9 Fraction Induction

Sprague-Dawley male rats (Harlan Sprague-Dawley Inc., Indianapolis, IN) weighing approximately 200 g were used for S-9 fraction induction and preparation. Aroclor 1254 (poly-chlorinated biphenyls) was provided by Dr. S.J. Bursian of the Animal Science Department at Michigan State University. It was diluted in corn oil to a concentration of 200 mg/ml. The rats were injected intra peritoneally with the Aroclor 1254 solution at a rate of 80 mg/kg for 3 successive days. The rats were allowed drinking water ad libitum and Purina Laboratory Chow until 12 hr before sacrificing them when the food (but not the water) was removed. At day 4 (one day after the last injection) the rats were killed by cervical dislocation.

S-9 Fraction Preparation

In the procedure used for preparation of the liver S-9 fraction, all steps were carried out at 0-4°C using cold sterile solutions and glassware. The freshly excised livers were placed in pre-weighed beakers containing approximately 1 ml of chilled 0.15 M KCl per g of wet liver. Each liver weighed approximately 10-15 g. After weighing, the livers were washed several times in fresh chilled KCl to remove hemoglobin, which can inhibit the activity of the cytochrome P450 enzymes. The washed livers were transferred to a beaker containing 3 volumes of 0.15 M KCl (3 ml/g wet liver), minced with sterile scissors, and homogenized with a Polytron homogenizer (Virtis Research Equipment, Gardiner, NY). The homogenate was centrifuged in a Sorval Superspeed Centrifuge (Model RC-2-B, Sorvaly Scientific Equipment Co., Norwalk, CN) for 10 min at 9000xg, and the supernatant containing the S-9 fraction was decanted off. The freshly prepared S-9 fraction was distributed in 1-2 ml portions in small plastic tubes, frozen quickly in a bed of crushed dry ice, and stored immediately at -20°C.

Preparation of S-9 Mixtures

The frozen S-9 fractions were thawed, mixed with the other ingredients (H_2O, $MgCl_2$, NADP solution, phosphate buffer and glucose-6-phosphate solution) to yield the S-9 mixture. The components of the standard S-9 mixture are listed in Appendix A. The mixture was prefiltered through glass fiber membrane filter paper. To insure a clean S-9

mixture, the filtrates were then filtered through a 0.45 μm Nalgene disposable membrane filter unit. The S-9 mixture was prepared fresh for each mutagenicity assay. The activity of the freshly prepared S-9 fraction was tested by determining the mutagenicity of 2-aminofluorine (2AF) (20 μg/plate) toward TA98 with different concentrations of S-9 mixtures ranged from 2 to 10%. In the present study, either 8 or 10% of the S-9 mixture was shown to give the optimum response and was used in all subsequent experiments.

Storage of The Tester Strains

Frozen Permanent Copies. A fresh Oxoid nutrient broth culture was grown to give a density of 1-2 x 10^9 bacteria per ml. For each 1.0 ml of culture, 0.09 ml of spectrophotometric grade DMSO was added. The culture and DMSO were combined in a sterile tube, flask or bottle, according to the number of permanents to be prepared. The solution was swirled gently until the DMSO was dissolved and distributed throughout the cultures. Then the cultures were transferred aseptically into sterile 1.2 ml labeled cryotubes. The tubes were filled nearly full, allowing for expansion due to freezing. This eliminated the air space at the top and helped to minimize oxidative damage. The tubes were placed upright in a bed of crushed dry ice until the cultures were frozen solid and then were transferred to a -80°C freezer.

Master Plates. In addition to the frozen permanents, it was found to be convenient to have cultures of the tester strains on master plates, which could then be stored at 4°C.

These are minimal glucose-agar plates enriched with histidine and biotin. Ampicillin is added to plates used for the R-factor strains. For TA102, tetracycline was also added. Master plates were used routinely in this study as the source of bacteria for inoculating the overnight cultures of frequently used strains. The use of these plates for routine work avoided the problems that arise when the frozen permanents were opened frequently.

The bacteria were scraped from the surface of a frozen permanent or taken from another master plate and transferred to the surface of a nutrient agar plate followed by incubation for 48 hr at 37°C. With a sterile wire loop, a well-isolated colony was removed and resuspended in 0.3 ml or less of phosphate-buffered saline in a small culture tube. A sterile cotton swab was dipped into the bacterial suspension and the excess liquid was removed by pressing it against the inside of the tube. It was then used to make 4 or 5 parallel streaks across the surface of the appropriate agar plate. The plates were incubated for 12 hrs at 37°C to give the master plates. The spontaneous reversion frequency characteristic of the strains was tested whenever new master plates were prepared.

The Mutagenicity Test

The preincubation technique of Maron and Ames (1983) was used to assay for mutagenic activity. With a sterile wire loop, a well-isolated colony was removed and resuspended in a 18 x 150 mm culture tubes with loose fitting

caps containing 5 ml of Oxoid 5% nutrient broth No. 2. The liquid tester strain cultures were grown in a 37°C gyrotory incubator shaken at approximately 210 rpm for 10 hrs to give a density of 1-2 x 10^9 cells per ml. The tester strains were then kept in a 5°C cooler until needed for the assay (< 4 hrs). Exactly 0.5 ml of S-9 (or phosphate buffer, pH 7.4) was added to sterile 13 x 100 mm capped culture tubes held in an ice bath. Then 0.1 ml or less of the test solution dissolved in DMSO (extracted meat) was added followed by 0.1 ml of the bacterial culture. This order of addition of the test components is used to avoid placing the bacteria in direct contact with the undiluted mutagenic compounds and their solvents. The tubes were vortexed gently and incubated at 37°C for 20 min. After the incubation, 2 ml of molten top agar (45°C) was added to the mixture. After gently vortexing to mix, the mixture was poured onto a minimum glucose-agar plate. To achieve uniform distribution of the top agar on the surface of the plate, it was quickly tilted and rotated. Then the plate was placed on a level surface to harden. The mixing, pouring and distribution should take less than 20 sec. The plates were covered promptly with brown paper or other suitable material to avoid the effects of light on any photosensitive chemicals. Within an hour, the plates were inverted and placed in a 37°C incubator. After 48 hrs, the number of revertant colonies on the test plates and on the control plates was counted. The presence of the "background lawn" on all plates was confirmed. The absence of the "background lawn" was

considered as the toxic effect of the tested compound. Sodium azide (NaN_3) was used as the positive control for TA100. 2-Aminofluorine (2AF) was used as the positive control for TA98 and TA100 along with the S-9 fraction.

EVALUATION OF THE RELATIONSHIP BETWEEN FAT CONTENT AND MUTAGENICITY OF FRIED GROUND BEEF

Lean ground beef (1.8% fat) was thawed and divided into five groups. Each group was mixed with a different amount of chopped frozen beef fat in order to give a fat content 1.8, 5, 10, 15 and 20%. Three samples were taken from each group for fat and moisture analyses. Then, 500 g of sample was taken from each group and divided into five patties for frying, extraction and mutagenicity determination as described previously.

FAT AND MOISTURE ANALYSIS

Moisture Content

The A.O.A.C. (1975) procedure for determining moisture was used. Four grams of tissue were accurately weighed to four decimal places into a previously dried and tared aluminum dish (100°C for at least 1 hr). The sample plus the dish were then dried overnight for 18 to 24 hr in a air convection oven at 100°C. The dried sample was cooled in a desiccator and weighed to four decimal places. The loss in weight was calculated as percentage moisture. Three replicate determinations were run for each sample.

Fat Content

The fat content was determined using the Goldfisch extraction method of the A.O.A.C. (1975). The dried sample from the moisture analysis was utilized. The aluminum dish containing the dried meat sample was carefully folded into a porous thimble and clipped into a Goldfisch apparatus. The fat was extracted with anhydrous diethyl ether for approximately 3 hr into a previously dried and tared beaker. The extract was then dried for 1 hr at 100°C in an air convection oven, cooled in a desiccator and weighed as before. The percent fat was calculated as grams of fat extracted from each 100 g of tissue. Three replicates were run for each sample.

EVALUATION OF THE RELATIONSHIP BETWEEN ANTIOXIDANTS AND MUTAGENICITY OF FRIED GROUND BEEF

Ground beef was thawed and prepared to contain antioxidants at the following levels: (a) control - no added antioxidant and without corn oil; (b) corn oil - no antioxidant with 1 ml of corn oil per 500 g of meat; (c) propyl gallate (PG) added in 1 ml of corn oil to contain either 0.01 or 0.1% - based on the fat content of meat; (d) butylated hydroxyanisole (BHA) at the same levels as in treatment c; (e) butylated hydroxytoluene (BHT) at same levels as treatment c; and (f) Tenox 4 (20% BHA and 20% BHT dissolved in corn oil) added at 0.02 and 0.2% (w/w) based on the fat content.

The ground beef patties were fried for 9 min per side

in the same fry pan with the same frying method as used previously. After frying, the fried ground beef were extracted by the extraction method used previously. The DMSO extracts were subjected to Salmonella typhimurium tester strains TA98 and TA100. The effects of the different antioxidants on mutagenicity were then compared.

QUANTITATIVE ANALYSIS OF IQ-LIKE COMPOUNDS

The purpose of developing this method was to quantitatively determine the mutagens formed in pan fried ground beef. The ground beef patties were thawed and fried 9 min per side as described previously.

EXTRACTION METHOD

The fried patties were extracted using modifications of the methods of Bjeldanes et al. (1982), Hayatsu et al. (1983) and Felton et al. (1984a) as shown in Figure 2. The fried patties were blended and extracted in a Waring blender at moderate speed with 1 volume of 0.01N HCl for 3 min. The homogenate was filtered through cheese cloth to remove the solid material. After filtration, the solid material was blended and extracted in the Waring blender at moderate speed with 1 volume of 0.01N HCl for 2 min and then was filtered through the same cheese cloth again. This procedure was repeated one more time. After combining all of the

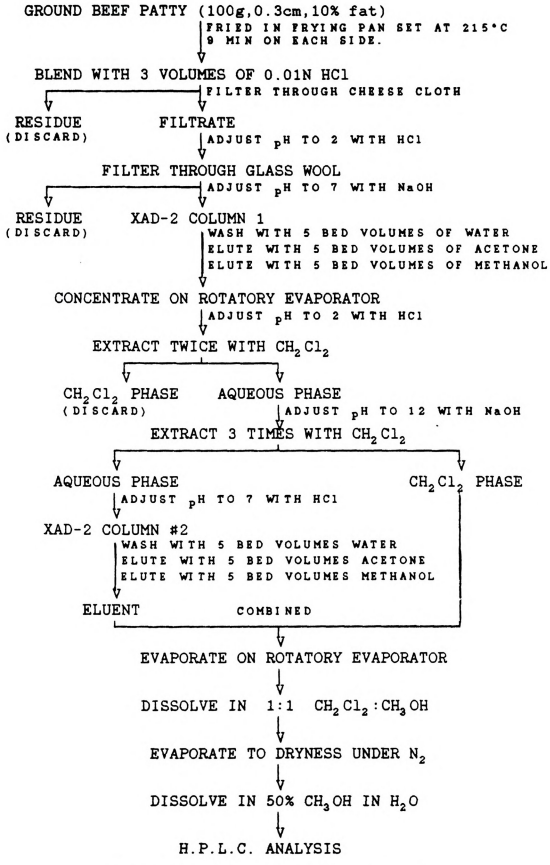


Figure 2: Extraction Method No 2.

filtrates, the sample was acidified to pH 2 with HCl. The filtrate was filtered through glass wool and then through Z13,261-6 filter paper (Aldrich). The clear decanted supernatant was collected and passed over an XAD-2 Amberlite column.

XAD-2 AMBERLITE COLUMNS

It is important to clean the resin before use. The resin was washed in sequence in a Soxhlet apparatus for 48 hr with methanol, benzene, acetonitrile, and again with methanol. It was then stored in methanol until removed for use.

Two XAD-2 Amberlite columns were utilized for each sample. One was 2.5cm x 45cm diameter (175 ml bed volume) and the other one was 1.5cm x 50cm diameter (70 ml bed volume).

After draining the excess methanol, the Amberlite XAD-2 resin was weighed into a 600 ml beaker. Distilled water was used to transfer the Amberlite polymeric adsorbent resin to the column. The column itself contained some water at the start of the operation and the resin was poured into the column as a water slurry. Occasionally excess water was drained through the bottom of the column. The liquid level was not permitted to fall below the resin level. Resin was continuously added in this manner until all the resin was transferred to the column. Then a 2.5 x 25 cm diameter empty column was connected to the upper end of the resin filled column (2.5 x 45 cm). The 1.5 x 50cm diameter resin

packed column was connected to a 250 ml column reservoir.

A silicone tube was attached to the bottom of both columns. Distilled water was introduced through the silicon tubes and the pressure was adjusted so the water flowed upward at a slow rate. The flow was increased until the bed of resin expanded approximately 100% and maintained until all air pockets were removed and all the particles were free and mobile. Extremely small particles flowed out of the top of the column. The flow was then stopped and the resin was permitted to settle by gravity. The liquid level was adjusted to one inch or more above the top of the resin bed. Then four bed volumes of distilled water were passed downward through the columns at a rate of 2 bed volumes/hr.

The clear decanted supernatant containing the meat extract was passed over the first XAD-2 Amberlite column (2.5cm x 45 cm diameter, 170 ml bed volume) at 0.025 bed volumes/min. The column was then rinsed with 5 bed volumes of distilled water at 0.025 bed volumes/min. The mutagenic activity, which was retained on the resin was eluted with 5 bed volumes of acetone at 0.05 bed volumes/min. Elution with 5 bed volumes of methanol at 0.05 bed volumes/min was then carried out to recover any residual mutagenic activity.

The eluents were combined and reduced in volume by rotatory evaporation, leaving water as the primary solvent. The concentrated eluent was diluted to 250 ml with distilled water.

LIQUID-LIQUID PARTITION

The eluent was adjusted to pH 2 with HCl. The acidic eluent was transferred to a 500 ml separatory funnel, then an equal volume of methylene chloride was added, and the separatory funnel and contents were mixed thoroughly by shaking for 1 min. The layers were allowed to separate, and the lower methylene chloride layer, which contained the soluble acidic components, was discarded. Occasionally, centrifugation or adding of saturated NaCl solution was necessary to break the emulsion. The methylene chloride extraction was repeated one more time.

The upper aqueous layer was adjusted to pH 12 with saturated NaOH solution. The basic aqueous phase was extracted three times with methylene chloride to remove the basic components. The methylene chloride extracts were collected and passed through anhydrous sodium sulfate to remove excess water. The upper basic aqueous phase was diluted to 500 ml with distilled water and adjusted to pH 7 with HCl. The diluted neutral aqueous phase was passed through the second XAD-2 column (1.5 cm x 50 cm), using the same washing and elution procedures.

The acetone and methanol eluents from the second XAD-2 column were combined with the methylene chloride extract from liquid-liquid partition. The mixture was evaporated in a rotatory evaporator using a water bath setting of 37°C until only the aqueous phase containing the mutagenic compounds remained. The remaining water was removed by twice adding absolute ethanol and evaporating the ethanol-

water azeotrope from the sample. The residue was dissolved in 50% methanol in CH_2Cl_2 (v/v) and quantitatively transferred to 2 dram vials using disposable glass pipets. The solutions in the vials were evaporated to dryness under a stream of nitrogen using a water bath setting of 40°C. Special care was taken to avoid overheating of the dry extract. Then the residues were dissolved in 50% methanol in water (v/v) and used for HPLC analysis and the Ames test.

HPLC

All HPLC analyses were carried out on a liquid chromatograph (Model 600, Water Associates, Inc., Milford, MA) equipped with a model 440 spectrophotometric absorbance detector. A Versapack C18 10μ 300 x 4.1 mm reverse phase HPLC column was used with a C18 reverse phase guard column (Alltech Associates, Inc., Deerfield, IL) and a silica presaturation column (Alltech Associates, Inc.). A mobile phase 1 M ammonium sulfate solution in methanol:ethanol: water (35:6:59, v/v/v) was used at flow rate 0.9 ml/min.

EVALUATION OF THE CARRY-THROUGH ACTIVITY OF BHA AND BHT

The fate of BHA and BHT, and their associated reaction products produced during frying and extraction, were followed in the present experiment. Ring labelled [^{14}C]BHA and [7-^{14}C]BHT were provided courtesy of Dr. Charles R. Warner of the Division of Chemistry and Physics, Food and

Drug Administration, Washington, D.C.

FATE OF RADIOLABELLED ANTIOXIDANTS

Preparation of Ground Beef with Radioactive Labelled Antioxidants

Based on the specific activity of ^{14}C-labelled BHA and BHT, both radioactive labelled antioxidants were quantitatively mixed separately with the corresponding unlabelled antioxidants and dissolved in tocopherol stripped corn oil (United States Biochemical Corporation, Cleveland, OH). The corn oil mixtures were compounded to contain either BHA or BHT at a concentration of 10 mg of antioxidant with 1μCi of activity per ml. Then 1 ml of the radioactive labelled mixture (either BHA or BHT) was introduced into 100 g of ground beef (10% fat). The labelled ground beef samples were fried and extracted as described previously. Samples were taken after each extraction step and the fate of the antioxidants was followed by measurement of radioactivity.

Determination of Radioactivity

The radioactivity in each sample was counted with a Beckman LS-3133P liquid scintillation spectrometer (Beckman Instruments, Inc., Fullterton, CA).

Liquid Sample. After diluting with aqueous counting scintillant (Amersham Corporation, Arlington Heights, IL), the amount of ^{14}C in the liquid samples was determined by scintillation counting.

Solid Sample. Exactly 1 g of solid sample was weighed

into capped scintillation vials and mixed with 1 ml of distilled water, after which 12 ml of NCS solubilizer (Amersham Corporation, Arlington Heights, IL) was added. The mixtures were swirled gently and digested at 50°C overnight. The completely dissolved sample mixtures were neutralized to pH 7 with glacial acetic acid (ca. 0.03 x the volume of NCS). After the mixtures were cooled, the scintillation fluid was added and counted as explained previously.

EVALUATION OF BHA AND BHT IN THE MEAT EXTRACT WITH GLC

About 0.5 g of accurately weighed meat extract with 1 ml 1N KOH in ethanol was transferred into a tube and kept at room temperature for 16 hr. After adding 2 ml distilled water and 1 ml isopropyl ether, the tube was centrifuged at 3,000 rpm for 3 minutes. The solvent layer was collected and transferred to another test tube. The isopropyl ether extraction step was repeated 3 more times. To remove trace amounts of water soluble materials, the isopropyl ether extracts were pooled and washed 3 more times with 1 ml distilled water. In the washing step, the aqueous layer was removed with a disposable pipet. Then 1 teaspoon of anhydrous sodium sulfate was added to remove trace amounts of water from the sample. The solution was transferred to a 2 dram vial, and evaporated to dryness (N-Evap Evaporator, Model 106, Organomation Assoc., Worcester, MA) under a stream of nitrogen. The sample was silylated by adding 100 µl pyridine and 50 µl of silylation reagent (BSTFA with 1%

TMCA obtained from Pierce Chemical Co., Rockford, IL). To complete the silylation reaction, the sample was kept at room temperature for 30 min.

A Hewlett-Packard Model 5890A Gas Chromatograph (Hewlett Packard Co., Avondale, Pa) equipped with a flame ionization detector and utilizing a 30 m silicone capillary column was used. The flow rate of the carrier gas (Helium) was set at 16.3 ml/min. A mixture of hydrogen (30 ml/min) and compressed air (300 ml/min) was used for the flame ionization detector. The oven temperature was programmed from 180°C to 260°C at a rate of 5°C per min. Temperatures of detectors and injection ports were maintained at 300°C and 200°C, respectively. BHA and BHT identifications were based on a comparison of GC retention times with those of the standard antioxidants.

EVALUATION OF THE MUTAGENICITY OF IQ, MeIQ AND MeIQx

IQ, MeIQ and MeIQx standards were carefully transferred and separately weighed into preweighed 2 dram vials to four decimal places. The weighed standards were dissolved quantitatively in DMSO. Through a series of dilution steps, each standard was diluted to give five different concentrations: 0.5, 5, 50, 500 and 5000 ng/µl. Each standard solution was then subjected to Salmonella typhimurium tester strains TA98 and TA100 to determine the most sensitive concentration range for each tester strain. Afterwards, 5 different concentrations of each standard were prepared and

subjected to the Ames test in the most sensitive concentration range. Standard curves for each compound tested for both tester strains was then prepared.

EVALUATION OF THE EFFECTS OF ANTIOXIDANTS ON MUTAGENICITY OF IQ, MeIQ AND MeIQx

The effects of antioxidants (BHA, BHT and PG) to the mutagenicity of IQ, MeIQ or MeIQx were evaluated in the present experiment. Based on the mutagenicity determined in the previous experiment, a concentration was chosen for each standard that was mutagenic to TA98 and TA100 + S-9. The concentration chosen was 20 μg/plate for IQ, 3.5 μg/plate for MeIQ and 300 μg/plate for MeIQx.

In the Ames test, both the S-9 fraction and the tested standards were added to the culture tubes as described previously. Different concentrations of BHA, BHT and PG (0.1, 0.2, 0.3, 0.4, 0.5, 5, 10, 20, 30, 40 and 50 μg/plate) were added to the tubes before the addition of the tester strains. Each concentration were tested in triplicate.

EVALUATION OF THE EFFECTS OF DIFFERENT FOOD ADDITIVES ON IQ-LIKE COMPOUND FORMATION IN FRIED GROUND BEEF

Ground beef was thawed and prepared to contain antioxidants at the following levels: (a) control - no added anti-oxidant; (b) propylgallate (PG) added in 1 ml of corn oil to contain 0.1% - based on the fat content of the meat;

(c) butylated hydroxyanisole (BHA) at the same levels as in treatment b; (d) butylated hydroxytoluene (BHT) at the same levels as in treatment b; (e) tertiary butylhydroquinone (TBHQ) added in 1 ml of corn oil to contain 0.01 or 0.1%-based on the fat content of meat; (f) covitol (mixed tocopherol concentrate) added in 1 ml of corn oil to contain 1 or 10% - based on the fat content of the meat; (g) ascorbic acid added in 1 ml of distilled water to contain 100 or 1000 ppm - based on the total meat weight; (h) citric acid added in 1 ml of distilled water to contain 10 or 100 ppm - based on the total meat weight; (i) sodium sulfate at the same levels added in treatment h; (j) sodium pyrophosphate at the same levels as in treatment h; (k) nitrite at the same levels as in treatment h; and (l) liquid smoke at the same levels as in treatment g.

The ground beef patties were fried 9 min per side using the frying method previously described. After frying, the fried ground beef was extracted as described previously. The extracts were subjected to HPLC and the Ames test in order to quantitatively determine the type and amount of IQ-like compounds formed in the presence of the different additives.

EVALUATION OF THE RELATIONSHIP BETWEEN THE INTERNAL TEMPERATURE AND MEAT MUTAGEN FORMATION

Frozen ground beef was thawed and made into 100 g patties about 1 cm in thickness. The meat patties were

fried at 0, 3, 6 and 9 mins per side. The temperature of 5 points in the center of the meat and 4 points on the fry pan surface were monitored throughout the cooking process with a potentiometer equipped with a multipoint strip chart recorder (Honeywell-Industrial Products Group, Philadelphia, PA). The tip of the thermocouple was attached to a wood stick embedded in a wooden block (Figure 3) so that the points were spaced equidistantly from each other. Temperature measurements were taken in the center of each ground beef patty and surface temperatures were made on the surface of the fry pan. In this way, the surface heating temperatures were taken along with the temperatures in the center of the patties.

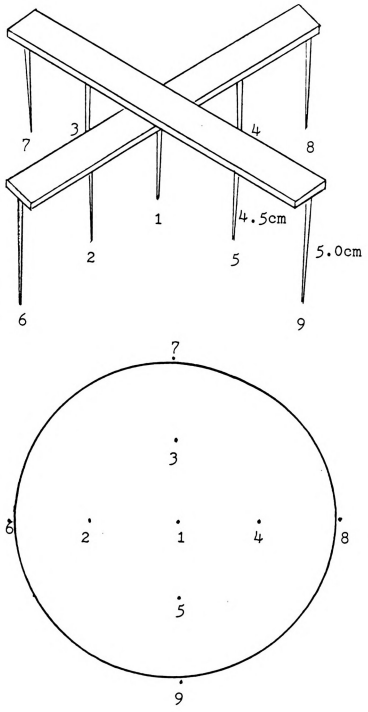


Figure 3. Diagram showing the points where the temperature was measured by thermocouples. Points 1-5 were used to measure the internal temperature; points 6-9 were utilized for measuring the pan surface temperature.

RESULT AND DISCUSSION

EVALUATION OF THE MUTAGENICITY OF FRIED GROUND BEEF

The mutagenicity produced during frying of ground beef was determined using the Ames test (Maron and Ames, 1983). Each of the fractions shown in Figure 1 were tested for mutagenicity using the frameshift tester strain TA98 and the base-pair substitution tester strain TA100.

No mutagenicity was detected in the acidic fraction (Figure 1) with either tester strain, however, potent mutagenicity was detected in the basic fraction. The standard curves for mutagenicity of the basic fraction toward TA98 and TA100 are shown in Figures 4 and 5. The data in Figure 4 show that there was a linear response of the number of revertants versus the amount of basic extract added to the plate with the microsomal fraction (S-9). The basic extract was not mutagenic when the S-9 fraction was absent. The specific activity of TA98 with S-9 toward the basic meat extract was 54.4 colonies per gram equivalent of ground beef.

The data in Figure 5 indicate that there was also a linear response of the number of revertants versus the amount of basic extract (Figure 1) added in the range of 0-36 g equivalent of meat. The specific activity of TA100 with S-9 toward the basic meat extract in this concentration range was 47.8 colonies per gram equivalent of ground beef.

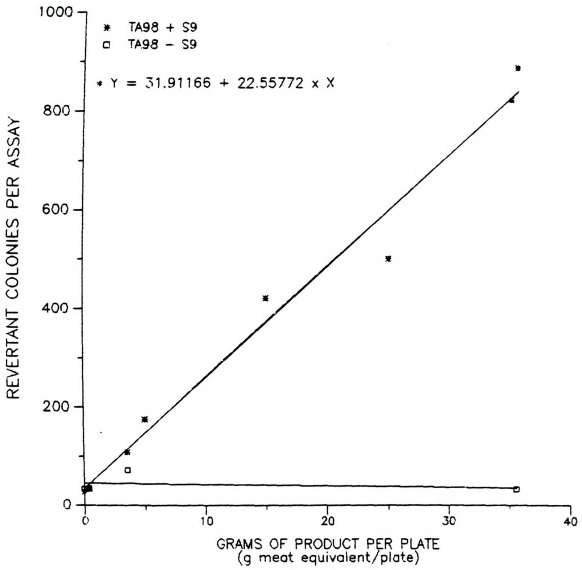


Figure 4. Mutagenicity of the basic fraction extract of ground beef fried at 9 min per side as assayed by TA98. The number of spontaneous revertants (30) has been subtracted.

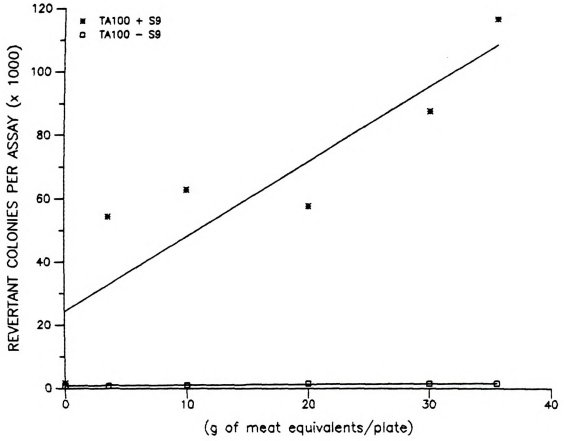


Figure 5. Mutagenicity of the basic fraction extract of ground beef fried at 9 min per side as assayed by TA100. The number of spontaneous revertants (122) has been subtracted.

However, if the dose was increased above 36 g meat equivalent/plate, the mutagenic response by TA100 decreased. This suggests that adding the basic extract at concentrations over 36 g meat equivalent/plate exhibited a toxicogenic affect. Apparently, the meat extract at the higher levels was toxic to the tester strain, and thus, decreased the mutagenic response. The cause of the decreased mutagenic response at the higher levels of the added basic extract was not determined in this study. There was no mutagenic response in the absence of the S-9 fraction.

This study demonstrated that fried ground beef is mutagenic in the Ames test, but S-9 is required for promotion of mutagenicity. Fractionation of the meat extract revealed that all of mutagenic activity was localized in the basic fraction.

EVALUATION OF THE RELATIONSHIP BETWEEN FAT CONTENT AND MUTAGENICITY OF FRIED GROUND BEEF

After determining the fat content the ground beef samples that were supposed to contain 1.8, 5, 10, 15 and 20% fat, they were found to actually contain 2, 4, 8, 12 and 18% fat, respectively. The meat was fried at either 6 or 9 mins per side. After extraction by the method listed in Figure 1, the basic fraction was tested for mutagenicity using tester strain TA98 + S-9.

The results are shown in Figure 6 and indicate that samples with fat concentrations ranging from 4 to 8% showed

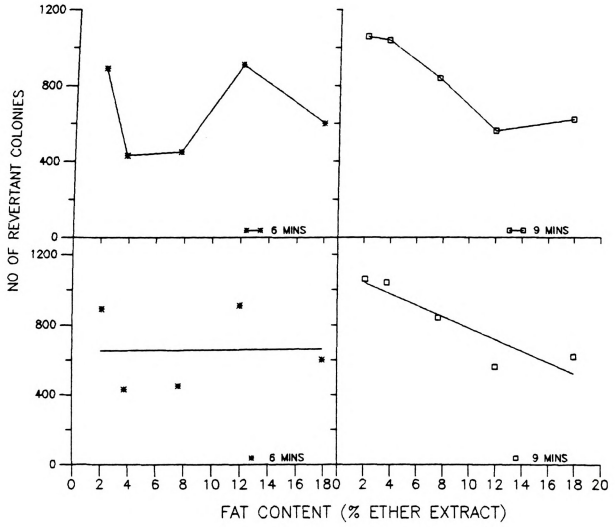


Figure 6. Relationship between the fat content of ground beef patties and mutagen formation after cooking for either 6 or 9 min per side. Each point represents 6 replicates. The concentration of the basic fraction added was 50g meat equivalents/plate. The lower two graphs present the regression lines of the data shown in the upper two graphs.

the least amount of mutagenicity. At 14% fat there was an approximate doubling of mutagenic activity, while the 18% fat sample had less mutagenic activity than that of the 14% fat sample. These results agree with Knize et al. (1985), who reported that increasing the fat content from 8 to 15% enhanced mutagenicity on cooking at either 180 or 240°C for 6 mins per side. However, it was found in the same study that increasing the fat content from 15 to 30% resulted in a slight reduction in overall mutagenic activity, which is similar to the results in the present study.

On frying the ground beef at 9 mins per side, the mutagenicity decreased directly with fat content (Figure 6). Although the meat fried at 9 mins per side showed less mutagenicity than that fried at 6 mins per side, the reason that there is less fluctuation in mutagenicity on frying at 9 mins per side is not clear. A possible explanation is that with longer frying times most of the fat was cooked out of the patties, which would probably concentrate the precursor(s) of mutagen formation and reduce the dilution effects from the fat. These data confirm the fact that fat is not the major contributor of precursor(s) for mutagen formation. This is in agreement with earlier studies by Felton et al. (1984b) and Knize et al. (1985), who demonstrated that fat content did not contribute precursor(s) for mutagen formation. However, the results are in contrast to studies by Barnes et al. (1983) and Barnes and Weisburger (1983, 1984), who reported that mutagenicity increased directly with fat content.

The regression line (Figure 6) indicates that the fat content of ground beef fried at 6 mins per side did not effect mutagenicity. No doubt the fluctuation in mutagenicity at different fat concentrations accounts for the low straight line relationship showing no effect of fat on mutagenic activity. However, at 9 mins per side, the fat concentration was negatively related to mutagenicity.

Results of this study show that meat mutagenicity is not directly related to fat content. Apparently, the mutagenic compounds are produced from heating the non-fatty components in the meat.

EVALUATION OF THE RELATIONSHIP BETWEEN ADDED ANTIOXIDANTS AND MUTAGENICITY OF FRIED GROUND BEEF

All of the antioxidants tested had an inhibitory effect on mutagenicity, except for BHT (Figure 7) which will be discussed later. Although the basic fraction from the meat fried with 0.1% BHA (group E) showed some mutagenicity on testing with TA100 (10.5 revertants/g equivalent of meat), at the 0.01% level of BHA mutagenicity was markedly inhibited in the fried meat. On testing with TA98, however, BHA inhibited mutagenicity at both added levels (0.01 and 0.1%). These results agree with those reported earlier by Wang et al. (1982) and Barnes et al. (1983), who reported that BHA inhibited mutagenicity during frying of ground beef.

Tenox 4 (a mixture of 20% BHA and 20% BHT in corn oil) inhibited mutagenicity of the fried beef patties at both

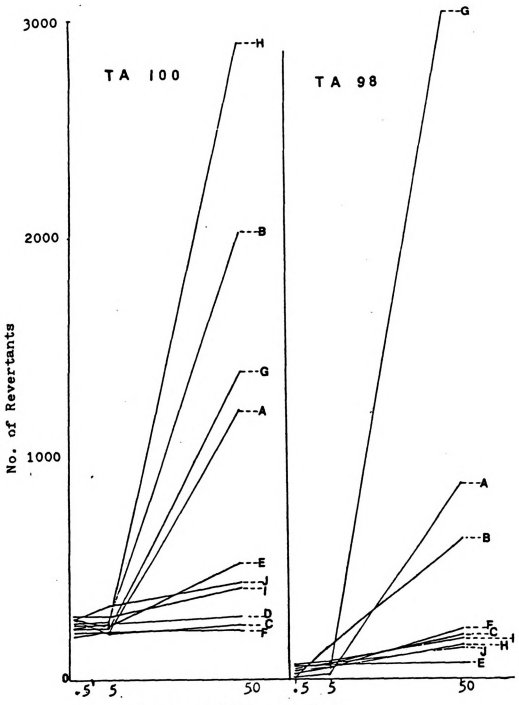


Figure 7. Effects of adding different antioxidants (AO) on the mutagenic activity of the basic fraction of beef patties (10% fat) toward TA98 and TA100 + S-9. Corn oil (CO) was used as the carrier for AO (1ml/500g). A-No AO nor CO added; B-No AO but CO added; C-50mg PG/500g added; D-5mg PG/500g added; E-50mg BHA/500g added; F-5mg BHA/500g added; G-50mg BHT/500g added; H-5mg BHT/500g added; I-250mg Tenox 4/500g added and J-20mg Tenox 4/500g added.

levels of addition (0.02 and 0.2%). This was true on testing with both TA98 and TA100. PG also inhibited mutagenicity with TA98 and TA100 at both levels of usage (0.01 and 0.1%).

The basic fraction obtained from beef containing added BHT had more mutagenicity with TA98 and TA100 at both antioxidant levels (0.1 and 0.01%) than control samples. As indicated earlier in this section, the reason that BHT tends to increase mutagenicity and the other antioxidants decrease the mutagenic activity in the basic fraction of the fried beef is not known. It could be associated with the degree of carry-through of the different antioxidants during extraction, which will be discussed later in this dissertation.

In summary, all antioxidants utilized in this study (BHA, PG and Tenox 4) inhibited formation of mutagens on frying of ground beef, except for BHT which enhanced mutagenicity.

EVALUATION OF THE MUTAGENICITY OF IQ, MeIQ AND MeIQx

The concentration effects of IQ, MeIQ and MeIQx toward TA98 and TA100 with added S-9 are shown in Figures 8, 9, 10 and 11. All three compounds were toxic to TA98 in the presence of S-9 at all levels above 100 µg/plate as is shown in Figure 8. Concentrations in the range of 0 to 0.5 µg/plate were tested and the results are presented in Figure 9. In this range the specific activity of MeIQ, IQ and MeIQx are

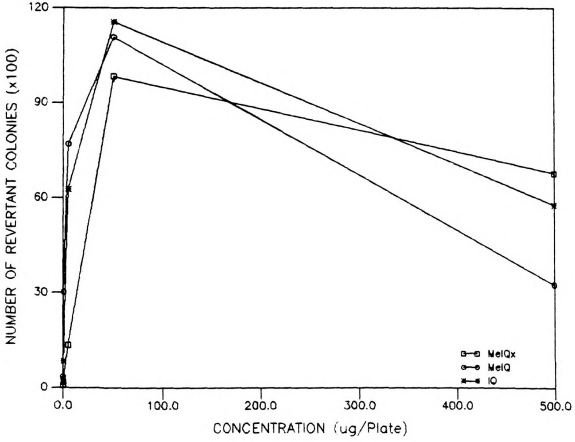


Figure 8. Concentration effects of IQ, MeIQ and MeIQx with TA98 + S-9. Spontaneous revertants have been subtracted. Concentration range was from 0 to 500 μg/plate.

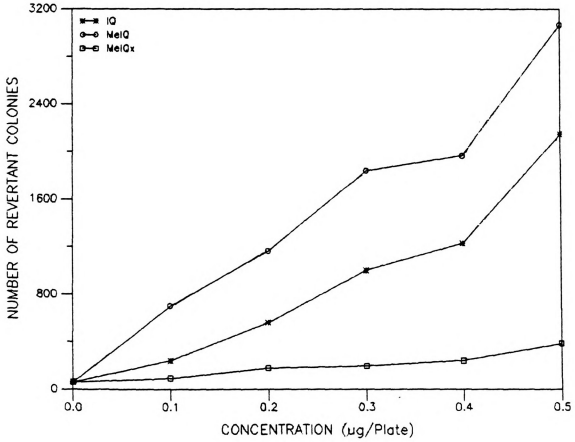


Figure 9. Concentration effects of IQ, MeIQ and MeIQx with TA98 + S-9. Spontaneous revertants have been subtracted. Concentration range was from 0 to 0.5 µg/plate.

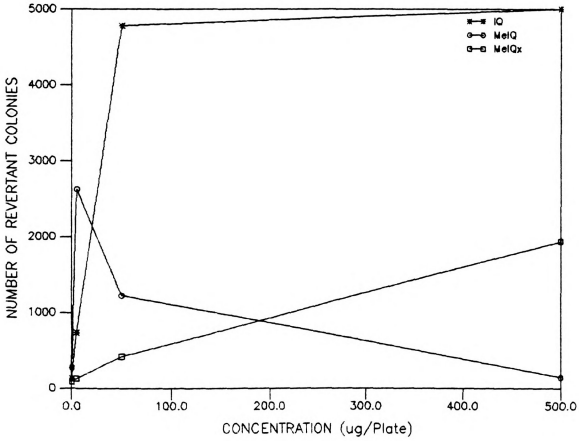


Figure 10. Concentration effects of IQ, MeIQ and MeIQx with TA100 + S-9. Spontaneous revertants have been subtracted. Concentration range was from 0 to 500 µg/plate.

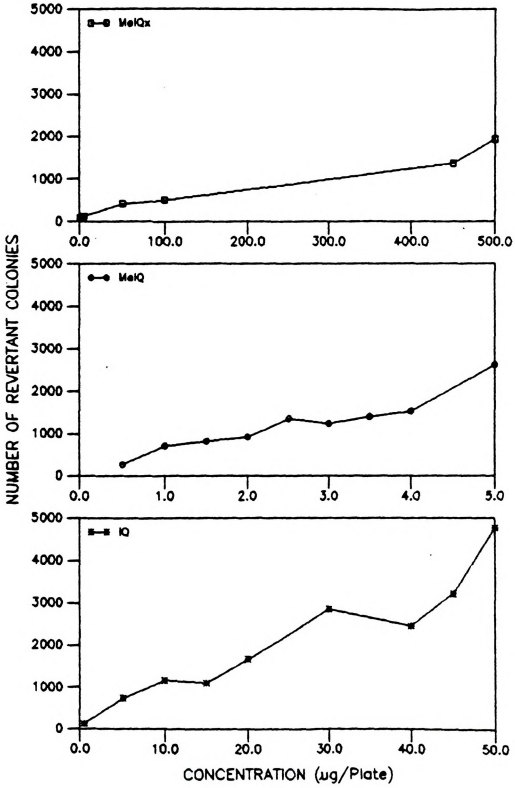


Figure 11. Concentration effects of IQ, MeIQ and MeIQx with TA100 + S-9. Spontaneous revertants have been subtracted.

5644, 3836 and 642 revertants/μg, respectively. MeIQ showed the most mutagenicity and was followed by IQ and MeIQx in that order. These results are in agreement with Sugimura (1982c, 1986) and Felton (1987), who have demonstrated that MeIQ is the most potent mutagen and is followed by IQ and MeIQx.

In the present study, on testing with TA100 + S-9, MeIQ was more toxic than IQ and MeIQx (Figure 10). The mutagenic test results for these compounds in the most sensitive concentration range are presented in Figure 11. In this range, the specific activities of MeIQ, IQ and MeIQx are 540, 261 and 154 revertants/μg, respectively. The mutagenicity of MeIQ when tested with TA100 + S-9 was higher than that for IQ and MeIQx.

Results of this study demonstrated that MeIQ, IQ and MeIQx were all mutagenic when tested with the Ames test with their potency being in the order listed above. This was true on testing with both TA98 and TA100, both of which required added S-9 for activation.

EVALUATION OF THE EFFECTS OF ANTIOXIDANTS ON MUTAGENICITY OF IQ, MeIQ AND MeIQx

Many antioxidants have been shown to inhibit carcinogenesis (Ames, 1983). For example, BHA (Wattenberg, 1972; McKee and Tometsko, 1979), BHT (Weisburger et al., 1977; McKee and Tometsko, 1979) and PG (Rosin and Stitch, 1978a,b) have been shown to reduce reversion induced by chemicals

requiring metabolic activation in the Ames test. The present study was carried out to evaluate the effects of these phenolic antioxidants (BHA, BHT and PG) on the mutagenicity of IQ, MeIQ and MeIQx using the Ames test.

Based on the mutagenicity determined in the previous experiment, the concentrations of each mutagen tested in this study was taken from the midpoint of the linear portion of the concentration response curve. The concentrations chosen were 20 μg/plate for IQ (Figures 12-14), 3.5 μg/plate for MeIQ (Figures 15-17) and 300 μg/plate for MeIQx (Figure 18). Different concentrations of each of the antioxidants (BHA, BHT and PG) were added to each of the mutagens (IQ, MeIQ and MeIQx) in order to determine their effects upon the mutagenic response.

On testing IQ with BHA, BHT or PG at concentrations ranging from 0 to 5000 μg/plate with TA100 + S-9, it was demonstrated that BHA and PG are both concentration responsive and inhibit mutagenicity (Figure 12). When the amount of BHA and PG added was below 500 μg/plate, these two antioxidants exhibited inhibitory effects on the mutagenicity of IQ, which were not due to toxicity as demonstrated by the presence of the background lawn. However, upon adding 500 μg/plate of either BHA or PG to the test solution (IQ + antioxidant + tester strain + S-9), the solutions became cloudy and interfered with identification of the background lawn. This made it difficult to determine if the decrease in growth was due to an antimutagenic effect of if it was associated with toxicity of the antioxidants. Therefore,

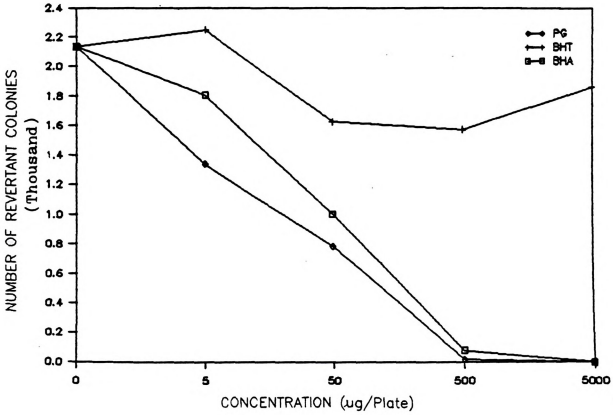


Figure 12. Effects of BHA, BHT and PG on the mutagenicity of IQ when tested with TA100 + S-9 at an IQ concentration of 20 ug/plate.

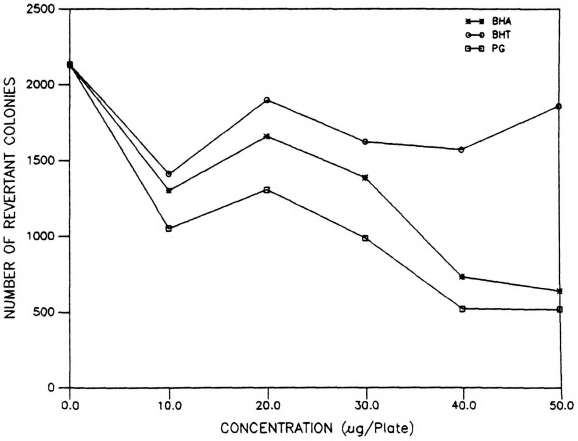


Figure 13. Effects of BHA, BHT and PG on the mutagenicity of IQ when tested with TA100 + S-9 at an IQ concentration of 20 µg/plate.

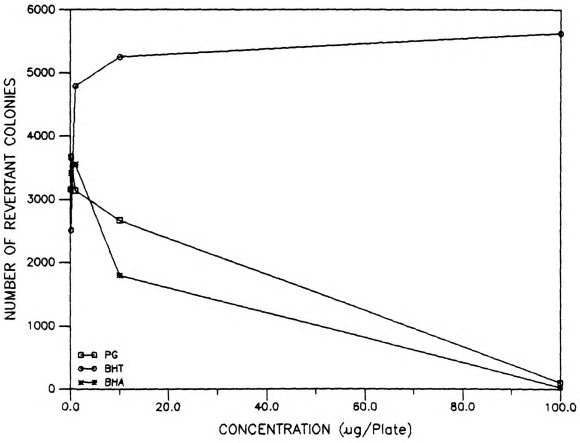


Figure 14. Effects of BHA, BHT and PG on the mutagenicity of IQ when tested with TA98 + S-9 at an IQ concentration of 20 ug/plate.

the three antioxidants were added at concentrations of 0 to 50 μg/plate, and the test was repeated with the results being shown in Figure 13. The inhibitory effects of BHA and PG towards the mutagenicity of IQ were confirmed by testing against TA100 + S-9. Results demonstrated that the two antioxidants had an antimutagenic effect against IQ. On the other hand, BHT had little effect on the mutagenicity of IQ on testing with TA100 + S-9 in both trials.

When tested with TA98 + S-9 (Figure 14), both BHA and PG inhibited the mutagenicity of IQ, which was shown to not be the result of any toxic effects from either additive. On the other hand, BHT significantly increased the mutagenicity of IQ (Figure 14).

When testing MeIQ with TA100 + S-9, BHA and PG had similar inhibitory effects as observed on testing with IQ (Figures 15). BHT decreased the mutagenicity of MeIQ at a dose of 5 μg/plate. On testing concentrations from 50 to 5000 μg/plate, however, BHT significantly increased the mutagenicity of MeIQ. To confirm the results, concentrations ranging from 0 to 50 μg/plate for BHA, BHT and PG were repeated (Figure 16). BHA and PG decreased the mutagenicity of MeIQ at all concentrations tested. On the other hand, BHT had little effect on the mutagenicity of MeIQ on testing with TA100 + S-9 in this trial. These results demonstrated that BHA and PG inhibit the mutagenicity of MeIQ toward TA100 + S-9. At low concentrations, BHT had little or no inhibitory effect on the mutagenicity of MeIQ when tested with TA100 + S-9. At higher concentrations

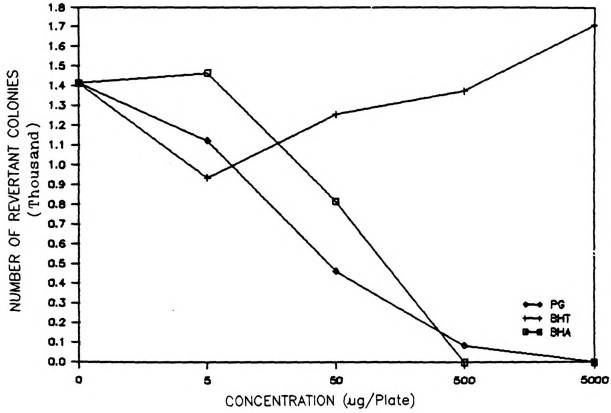


Figure 15. Effects of BHA, BHT and PG on the mutagenicity of MeIQ when tested with TA100 + S-9 at a MeIQ concentration of 3.5 ug/plate.

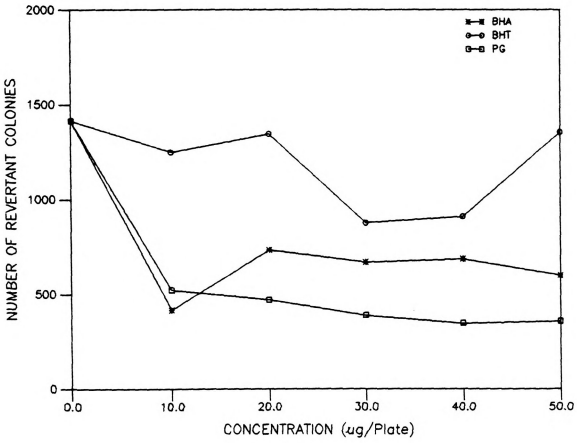


Figure 16. Effects of BHA, BHT and PG on the mutagenicity of MeIQ when tested with TA100 + S-9 at a MeIQ concentration of 3.5 ug/plate.

(above 50 μg/plate), however, BHT increased the mutagenicity of MeIQ.

When tested with TA98 + S-9 (Figure 17), a similar pattern was observed. BHA and PG were concentration responsive and inhibited the mutagenicity of MeIQ. On the other hand, BHT slightly decreased the mutagenicity of MeIQ at a concentration of 10 μg/plate, but at all higher concentrations it increased the mutagenicity of MeIQ in a concentration responsive manner.

On testing MeIQx with TA100 + S-9 (Figure 18), both BHA and PG inhibited the mutagenicity of MeIQx in a concentration responsive manner. BHT, however, had little or no inhibitory effect on MeIQx.

In summary, it was clearly demonstrated that BHA and PG significantly inhibited the mutagenicity of IQ, MeIQ and MeIQx. On the other hand, BHT had little effect on the mutagenicity of IQ and MeIQ at low concentrations, but significantly increased their mutagenicity at high concentrations. BHT slightly inhibited the mutagenicity of MeIQx at all concentrations tested. Unfortunately, large enough quantities of 4,8-DiMeIQx were not available for the mutagenicity tests.

QUANTITATIVE ANALYSIS OF IQ-LIKE COMPOUNDS

A number of mutagens (Tables 4 and 5) have been identified in cooked beef prepared under different conditions by using various analytical chemical methods. On using the

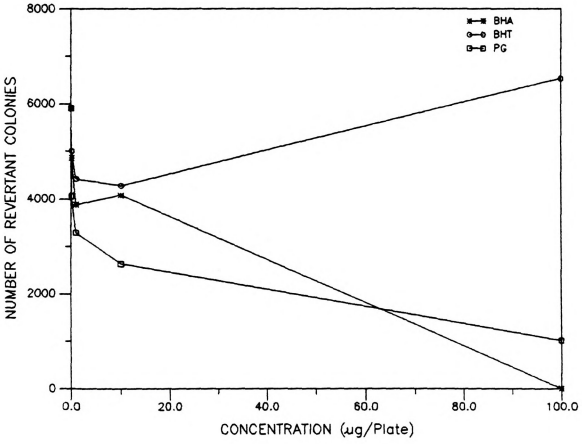


Figure 17. Effects of BHA, BHT and PG on the mutagenicity of MeIQ when tested with TA98 + S-9 at a MeIQ concentration of 3.5 µg/plate.

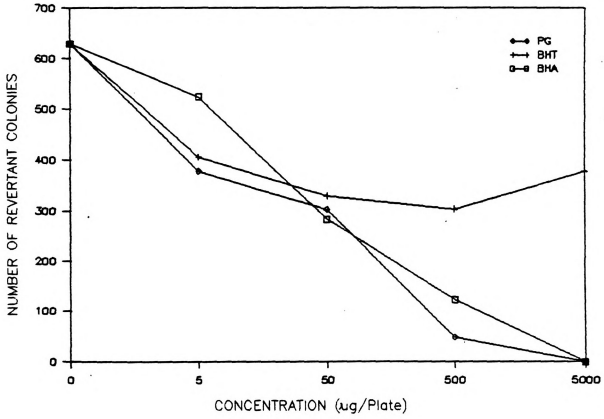


Figure 18. Effects of BHA, BHT and PG on the mutagenicity of MeIQx when tested with TA100 + S-9 at a MeIQx concentration of 300 ug/plate.

Ames test, it was demonstrated in the present investigation that adding of BHA, PG and Tenox 4 to ground beef before frying inhibited mutagenicity, however, BHT tended to increase mutagenicity. It is still not clear whether inhibition was caused by: (1) repression of meat mutagen formation, or (2) through carry-over of the antioxidants into the final meat extract, which could then inhibit the mutagenic activity of the compounds formed in frying ground beef. Thus, quantitative analysis of the meat mutagens in the fried ground beef was carried out to clarify the mechanisms involved in their formation.

Using a modification of the procedures utilized by Bjeldanes et al. (1982), Hayatsu et al. (1983) and Felton et al. (1984), the fried meat patties were extracted, and the extract was purified and subjected to HPLC analyses. Many peaks were present in the HPLC profile as shown by Figure 19. By using internal standards and retention times, the meat mutagens, IQ, MeIQx and 4,8-DiMeIQx, were identified in the HPLC profile. In the present study, the retention times for MeIQx, IQ and 4,8-DiMeIQx were found to be 12.2, 15.1 and 19.2 minutes, respectively. In no case did any of the known mutagen peaks co-chromatograph with MeIQ, which had a standard retention time of 23.31 minutes. Thus, we were unable to identify MeIQ in the HPLC profile of the extract from fried beef patties. These findings agree with those of Felton et al. (1984), who were unable to detect MeIQ in fried beef.

Tsuda et al. (1980, 1981) have shown that high

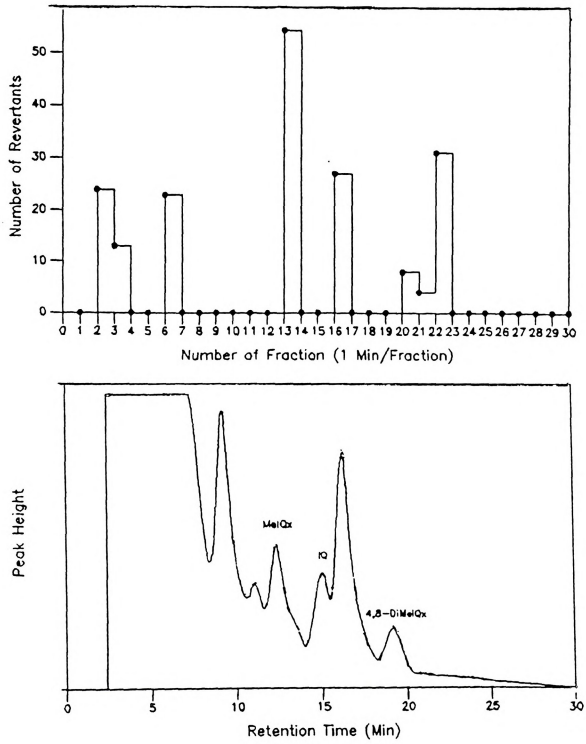


Figure 19. Plot showing the relationship between the Ames test (top) and the HPLC profile (bottom) of control meat sample fried at a temperature setting of 215 C. 100 ul out of each fraction (ca. 900 ul) was used in each test (TA98 + S-9). No antioxidant was added.

temperature-induced mutagens (Trp-P-1, Trp-P-2, AαC, MeAαC, Glu-P-1 and Glu-P-2) can be easily inactivated by adding dilute nitrite solution under weakly acidic conditions. However, the moderate temperature-induced mutagens (IQ-like compounds) are resistant to deamination following nitrite treatment under acidic conditions.

For further identification of the meat mutagens in the present study, the HPLC eluent was collected at a rate of one fraction per minute. The collected fractions were tested with TA98 + S-9 either with or without nitrite treatment. Results indicated that the fractions containing IQ, MeIQx and 4,8-DiMeIQx are mutagenic (Figure 19), but their mutagenicity was not effected by nitrite treatment. These results further confirm the identification of IQ, MeIQx and 4,8-DiMeIQx and agree with the results of Tsuda et al. (1980, 1981).

The relative amount of each identified mutagen was calculated by measuring the area under the HPLC peaks and compared with standard peaks of known concentrations. The concentration of each mutagen was calculated according to the following formula:

ng/g of meat equivalent = (A x B x C)/(D x E x F)

where:

A = Area under the mutagen peak in the sample;

B = Concentration of standard mutagen in ng/µl;

C = µl of mutagen standard injected;

D = Area under the standard mutagen peak;

E = Concentration of meat extract (g equivalent of

meat/μl); and

F = μl of meat extract injected.

Recovery studies using known amounts of IQ, MeIQ and MeIQx demonstrated that 67, 73 and 58% were recovered, respectively. The limited quantity of 4,8-DiMeIQx precluded recovery studies with this mutagens.

Results indicated that meat fried at 9 mins per side at a temperature setting of 215°C contained IQ, MeIQx and 4,8-DiMeIQx at concentrations of 1557, 5028 and 730 ng/g of meat equivalents, respectively. The MeIQx content was significantly higher than that of IQ and 4,8-DiMeIQx. MeIQx and 4,8-DiMeIQx have been reported to be the major mutagens produced in cooked fish (Kikugawa and Kato, 1987) and beef (Knize et al., 1987). Thus, results of the present investigation are in essential agreement with the earlier studies showing that MeIQx and 4,8-DiMeIQx are produced during frying of meat.

In summary, IQ, MeIQx and 4,8-DiMeIQx were identified in the HPLC profile of the basic fraction from ground beef fried for 9 min per side at a temperature setting of 215°C. However, neither MeIQ nor PhIP could be identified.

EVALUATION OF THE RELATIONSHIP BETWEEN THE INTERNAL TEMPERATURE AND MEAT MUTAGEN FORMATION

The relationship between the internal temperature of the meat patties and the surface temperature of the frying pan was monitored and is shown in Figure 20. In this test,

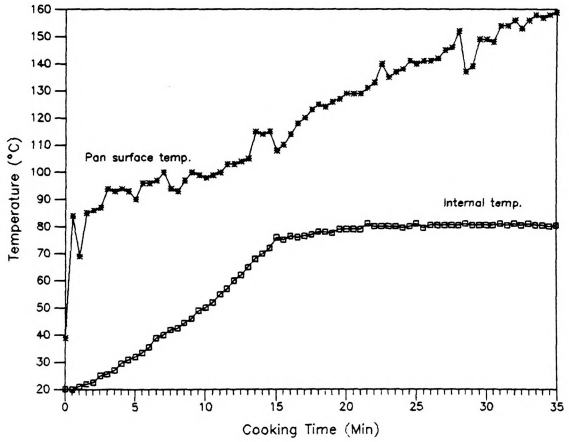


Figure 20. Pan surface temperature and internal temperature of meat fried for 35 min on one side. The temperature control for the fry pan was set at 215°C.

the beef patties were fried for 35 mins on one side only.

Results showed that even when the temperature setting of the fry pan was 215°C, the actual pan surface temperature was much lower than that. The initial pan surface temperature immediately after the beef patties were placed in the fry-pan was 42°C. The low initial temperature apparently occurred because the raw meat patties cooled down the surface temperature. As cooking proceeded, the pan surface temperature increased gradually, but did not reach over 160°C. Longer cooking times may be needed in order to reach the pan setting of 215°C. These results showed that cooking time and cooking temperature are interrelated and must both be considered when evaluating mutagenicity on cooking of meat. Increasing the cooking time will increase the temperature effect to which the meat is exposed. As the internal temperature of the meat patties reached a plateau after 15 mins, the internal temperature was about 75 - 80°C and remained in this temperature range until cooking was completed.

To determine the relationship between mutagen formation and the internal temperature of the beef patties, the pan surface temperature and the internal temperature of beef patties fried at 3, 6 and 9 mins per side were monitored. The results are shown in Figures 21, 22 and 23, respectively. After extraction, the concentrations of IQ, MeIQx and 4,8-DiMeIQx were measured and are presented in Table 10.

The internal temperature of all meat samples increased steadily during frying on the first side. However, on

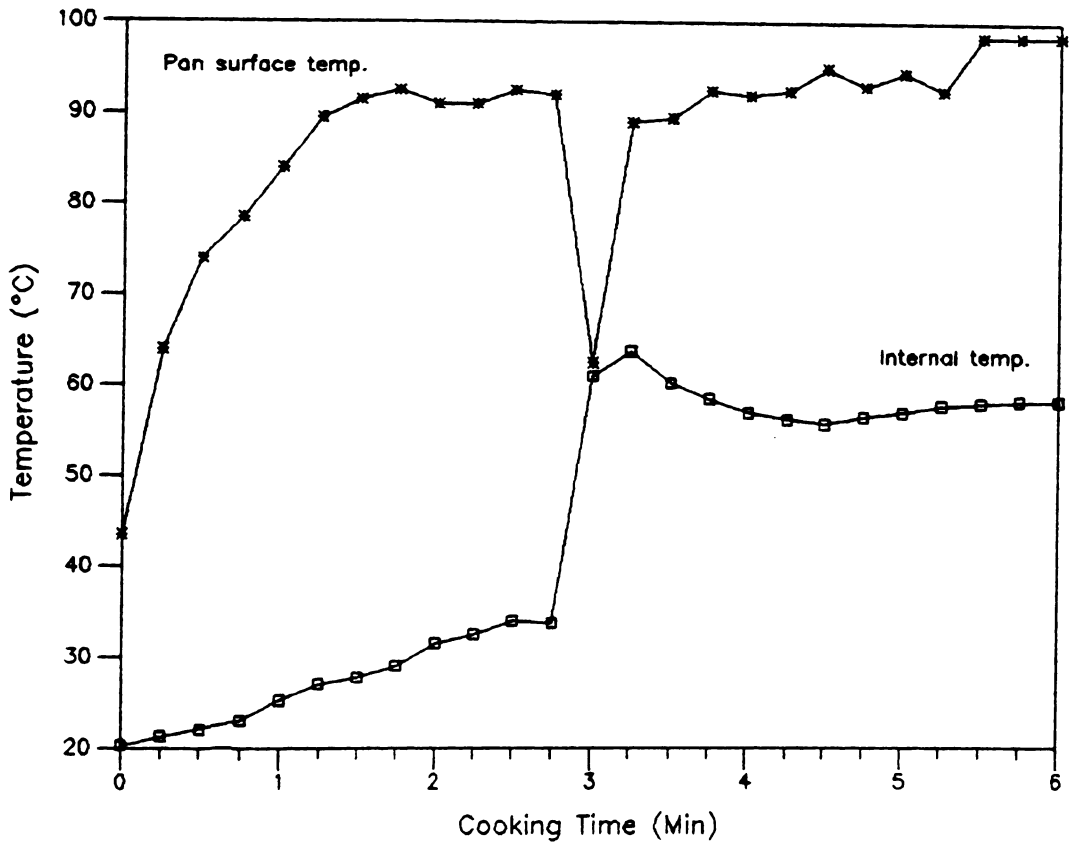


Figure 21. Pan surface temperature and internal temperature of meat fried for 3 min per side. The temperature control of the fry pan was set at 215°C.

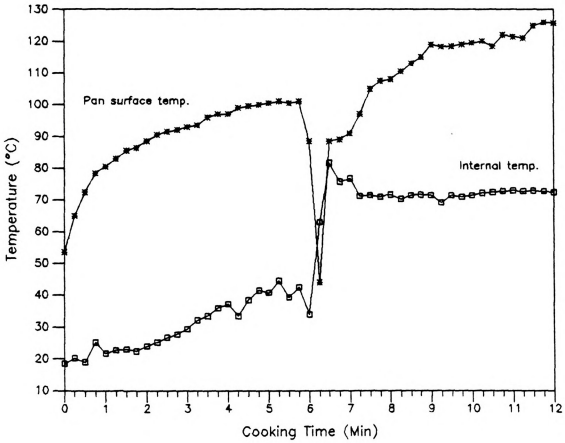


Figure 22. Pan surface temperature and internal temperature of meat fried for 6 min per side. The temperature control for the fry pan was set at 215°C.

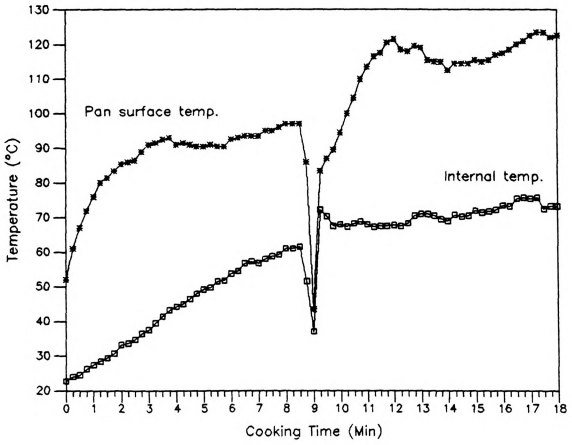


Figure 23. Pan surface temperature and internal temperature of meat fried for 9 min per side. The temperature control for the fry pan was set at 215°C.

turning the meat to the other side, the internal temperature suddenly increased from 34 to 61°C for 3 mins, from 42 to 73°C for 6 mins and from 61 to 72°C for 9 mins. Then the temperature tended to remain constant until frying on the second side was completed (Figures 21, 22 and 23). The final internal temperatures were 60°C for 3 mins, 74°C for 6 mins and 73°C for 9 mins per side. This phenomenon may be explained as being due to crust formation on the surface of the meat in contact with the fry-pan surface during frying on the first side. The moisture in the meat was evaporated from the other side of the patties and tended to keep the meat temperature low. Therefore, the internal temperature of the meat patties steadily increased during heating on the first side. On turning the meat to the other side, the fry-pan surface temperature was cooled down by the moist surface. In the meantime, the internal temperature of the meat patty increased markedly until the crust was formed and prevented moisture evaporation. Thereafter, the temperature tended to remain constant until frying was completed.

HPLC results showed that even though the internal temperature did not increase significantly with frying time, mutagen formation was positively correlated with frying time (Table 10). These results agree with Dolara et al. (1979) and Knize et al. (1985) who showed that the total mutagenic activity increased directly with cooking time.

Results of the present study showed that less mutagens were formed in the thicker patties (1 cm in thickness) than in the thinner patties (0.5 cm in thickness), but the HPLC

Table 10. Concentrations of IQ, MeIQx and 4,8-DiMeIQx in beef patties fried at 0, 3, 6 and 9 min per side[a,b].

SAMPLE TREATMENT	ng of Mutagens / g of Meat		
	IQ	MEIQX	4,8-DiMEIQx
Raw meat (0 min)	0	0	0
3 min	24±3	39±1	10±12
6 min	60±3	73±1	229±93
9 min	141±38	105±6	490±20

a) No MeIQ was detected in any of the samples.
b) The data show the means and the standard deviations (in parentheses) for two replicate samples.

profiles were qualitatively similar. This is in agreement with the results of Knize et al.(1985) who pointed out that the thickness of the meat patties was negatively related to the mutagenic activity of fried beef patties, especially with longer cooking times.

The present study indicated: (1) An increase in frying time did not necessarily increase the internal temperature of the meat patties. (2) Even though the internal temperature did not increase significantly with frying time, mutagen formation was positively correlated with frying time. (3) Less mutagens were formed in the thicker than in the thinner patties. (4) This study clearly demonstrates that formation of meat mutagens during cooking is a function of both time and temperature.

EFFECTS OF DIFFERENT ANTIOXIDANTS ON FORMATION OF IQ-LIKE COMPOUND IN FRIED GROUND BEEF

Table 11 shows the effects of adding different antioxidants (BHA, BHT, PG or TBHQ) upon formation of the meat mutagens. The ground beef patties used were 0.5 cm in thickness and contained 10% fat. They were fried at 9 mins per side at a pan setting of 215°C.

Table 11. The effects of BHA, BHT, PG and TBHQ on the formation of IQ, MeIQx and 4,8-DiMeIQx in ground beef[a,b,c].

Group		IQ	MeIQx	4,8-DiMeIQx	TOTAL
CONTROL	Mean	1558	5028	730	7316
	S.D.	180	2392	374	
BHA	Mean	313	2893	38	3244
	S.D.	102	1258	5	
BHT	Mean	221	2237	5902	8360
	S.D.	25	982	1298	
PG	Mean	225	1738	136	2099
	S.D.	23	578	49	
TBHQ	Mean	106	1332	321	1759
	S.D.	73	1053	31	

a) Ground beef was 0.3-0.5 cm in thickness and contained 10% fat. It was fried at a fry pan temperature setting 215°C at 9 min per side.
b) The data in are given as ng/g of meat equivalent.
c) The antioxidant was added at a concentration of 0.1% of the fat content of the meat.

As expected, no detectable amounts of IQ-like compounds were found in any of the raw meat samples either with or without the added antioxidants. However, measurable amounts of IQ, MeIQx and 4,8-DiMeIQx were detected in all the cooked meat samples, both in the controls and those containing added antioxidants.

The concentrations of IQ and MeIQx were highest in the control meat samples, which contained 1558 and 5028 ng/g (meat equivalents), respectively. Addition of BHA, BHT, PG and TBHQ to the meat before frying significantly lowered the IQ content ($P < 0.0005$) in comparison to that of the control. All of the antioxidant treated groups also contained significantly less MeIQx than the control ($P < 0.05$).

The concentrations of 4,8-DiMeIQx in the samples containing BHA ($P < 0.025$), PG ($P < 0.05$) and TBHQ ($P < 0.1$) were all significantly lower than that of the control. However, such was not the case for the BHT-treated group, which contained a significantly higher concentration of 4,8-DiMeIQx ($P < 0.005$) than the control group.

As shown in Figure 24, the total amount of meat mutagens (IQ + MeIQx + 4,8-DiMeIQx) was highest in the BHT-treated group (8360 ng/g of meat equivalent), which was higher than that of the control group (7316 ng/g of meat equivalent). These two treatments groups both exhibited significant mutagenicity when tested with the Ames test (Figure 7). The BHA, PG and TBHQ treated groups contained less total meat mutagens (3244, 2099 and 1759 ng/g of meat equivalents, respectively) than either the untreated control or the BHT treatment.

Results of the Ames test indicated that BHA, PG and Tenox 4 inhibit the mutagenicity of fried ground beef (Figure 7). Barnes et al. (1983) have shown that BHA inhibits IQ formation by 40% during frying of ground beef. Results of the present study demonstrated that BHA not only

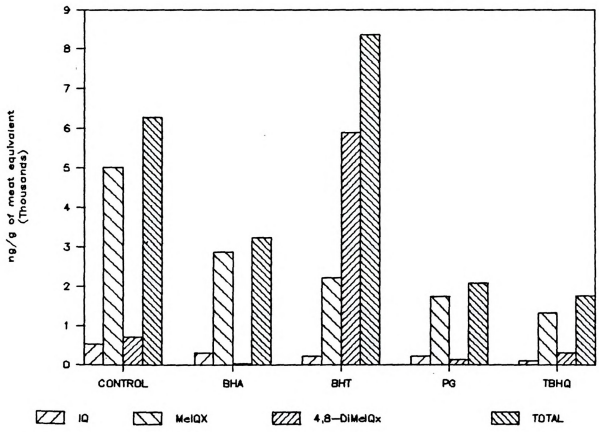


Figure 24. Effects of added BHA, BHT, PG and TBHQ on formation of IQ, MeIQx and 4,8-DiMeIQx in 0.5 cm thick ground beef (10% fat) fried at 9 min per side at a fry pan setting of 215°C.

inhibited IQ formation by about 80%, but also inhibited formation of MeIQx and 4,8-DiMeIQx by about 40 and 90%, respectively. PG was found to inhibit the formation of IQ, MeIQx and 4,8-DiMeIQx by some 85, 65 and 80%, respectively, whereas, TBHQ inhibited the formation of the same mutagens by approximately 90, 70 and 55%, respectively.

Figure 25 shows the effects of two different levels of TBHQ (0.02 and 0.1% of fat content) upon formation of mutagens in fried ground beef. All the meat mutagens detected (IQ, MeIQx 4,8-DiMeIQx) were inhibited by TBHQ, with the degree of inhibition being greatest at the highest concentration.

BHA, BHT, TBHQ and PG are free-radical scavenger type antioxidants. They interrupt the free-radical chain of oxidative reactions by contributing hydrogen from their phenolic hydroxyl groups and form stable free radicals, which do not initiate or propagate further oxidation of lipids (Sherwin, 1978).

Results of the present study clearly demonstrate that these antioxidants (BHA, PG and TBHQ) inhibit formation of the meat mutagens, except for BHT, which enhanced mutagenicity. TBHQ had the greatest inhibitory effect followed by PG and BHA. Results suggest that free radical reactions may be involved in formation of IQ and MeIQ as their formation was inhibited by all added antioxidants.

The reason that the BHT treated group had higher mutagenicity than all other treatments can be partially explained by the higher concentration of 4,8-DiMeIQx. According to

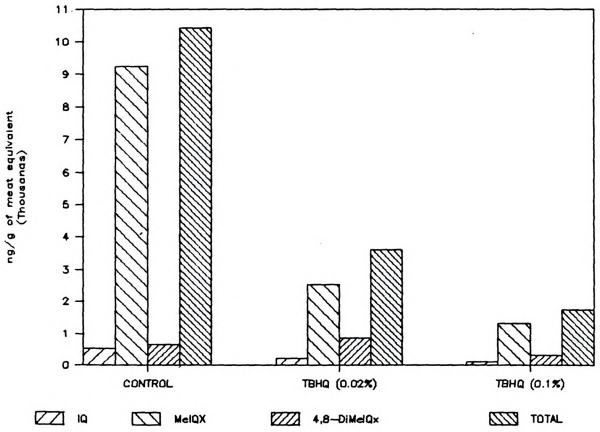


Figure 25. Relationship between levels of TBHQ and formation of IQ, MeIQx and 4,8-DiMeIQx in 0.3 cm thick ground beef (10% fat) fried at 9 min per side at a fry pan setting of 215°C.

Sugimura (1986) and Knize et al. (1987), 4,8-DiMeIQx is more mutagenic than MeIQx but less mutagenic than IQ towards TA98 and TA100 + S-9 (Table 7). Although the BHT treated group has less IQ and MeIQx than the control, it had more 4,8-DiMeIQx than the other samples (Table 11). On calculating mutagenicity based on reported Ames test results (Table 7), the BHT treated group would have 1,500,124 revertants/g of meat equivalent in comparison to 1,537,264 for the untreated control. It is, however, possible that some other mechanism(s) may be responsible for the increased mutagenicity of the BHT treated samples.

EFFECTS OF DIFFERENT FOOD ADDITIVES ON FORMATION OF IQ-LIKE COMPOUNDS IN FRIED GROUND BEEF

Not all antioxidant activity is due to the effects of free-radical termination. Sulfiting agents and ascorbic acid are reducing agents and function by transferring hydrogen atoms, thus serving as oxygen scavengers. They have been widely used in foods to control nonenzymatic browning as well as certain enzyme-catalyzed reactions (Taylor et al., 1986). Krone and Iwaoka (1987) reported that both sodium bisulfite (0.5% solution) and ascorbic acid (1% solution) inhibit the mutagenicity of canned salmon.

Table 12 shows that effects of adding different food additives (bisulfite, nitrite, pyrophosphate, citrate, ascorbic acid, vitamin E and liquid smoke) upon formation of the meat mutagens. In the present study, sodium bisulfite

Table 12. The effects of different food additives on the formation of IQ, MeIQx and 4,8-DiMeIQx in ground beef fried at 215°C, 9 min per side[a,b].

Treatment		IQ	MeIQx	4,8-DiMeIQx	TOTAL
		ng/g of meat equivalent			
CONTROL	Mean	1558	5028	730	7316
	S.D.	180	2392	374	
B.S.1	Mean	110	1272	17	1399
	S.D.	68	481	4	
B.S.2	Mean	212	2127	494	2833
	S.D.	21	424	204	
N.1	Mean	1534	2077	204	2435
	S.D.	44	579	97	
N.2	Mean	214	2734	141	3089
	S.D.	104	1359	115	
P.P. 1	Mean	436	2373	4958	7768
	S.D.	147	1243	1246	
P.P. 2	Mean	464	3627	370	4461
	S.D.	24	1191	73	
C.A.1	Mean	347	3760	121	4228
	S.D.	31	1162	29	
C.A.2	Mean	152	1695	709	2556
	S.D.	20	851	262	
AA 1	Mean	178	1452	657	2287
	S.D.	137	1320	74	
AA 2	Mean	345	2464	290	3099
	S.D.	45	585	229	
VE 1	Mean	206	2299	493	2998
	S.D.	16	179	55	
VE 2	Mean	282	2094	0	2376
	S.D.	130	742	0	
L.S. 1	Mean	177	2033	35	2245
	S.D.	63	97	7	
L.S. 2	Mean	183	2232	202	2617
	S.D.	45	934	76	

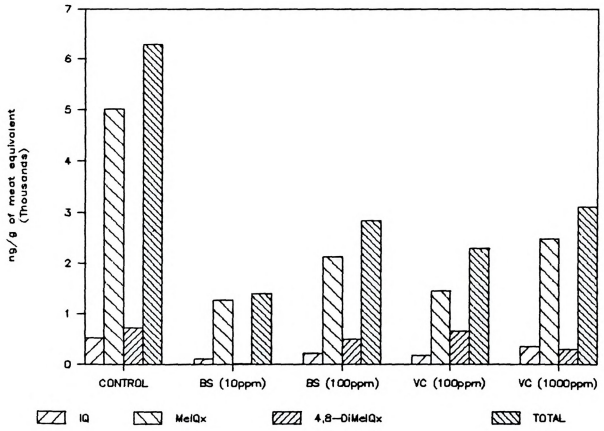
a) 10% fat ground beef.

b) B.S.1 = bisulfite, 10ppm; B.S.2 = bisulfite, 100ppm; N.1 = nitrite, 10ppm; N.2 = nitrite, 100ppm; P.P.1 = pyrophosphate, 10ppm; P.P.2 = pyrophosphate, 100ppm; C.A.1 = citric acid, 10ppm; C.A.2 = citric acid, 100ppm; AA.1 = ascorbic acid 100ppm; AA.2 = ascorbic acid, 1000ppm; VE.1 = vitamin E, 1% (fat); VE.2 = vitamin E, 10% (fat); L.S.1 = liquid smoke, 100ppm; L.S.2 = liquid smoke, 1000ppm.

(10 and 100 ppm) and ascorbic acid (100 and 1000 ppm) were added to raw ground beef before frying to determine if they inhibited formation of IQ-like compounds. Results (Figure 26) indicated that sodium bisulfite significantly inhibited formation of IQ and MeIQx at both concentrations ($P < 0.0005$). Bisulfite also inhibited the formation of 4,8-DiMeIQx at 10 ppm ($P < 0.025$) but had no statistically significant effect at 100 ppm ($P < 0.2$).

Figure 26 indicates that ascorbic acid inhibits formation of IQ, MeIQx and 4,8-DiMeIQx, but was not as effective as bisulfite. At 100 ppm, ascorbic acid inhibited IQ and MeIQx ($P < 0.025$ and $P < 0.01$, respectively) but had no statistically significant effect on formation of 4,8-DiMeIQx ($P < 0.25$). At 1000 ppm, ascorbic acid significantly inhibited formation of IQ and MeIQx ($P < 0.005$ and $P < 0.05$, respectively), but caused only a slight inhibition of 4,8-DiMeIQx ($P < 0.1$).

It is interesting to note that both sodium bisulfite and ascorbic acid were more effective in blocking mutagen formation at low levels than at high levels. On adding sodium bisulfite, the total meat mutagens detected were 1399 ng/g (meat equivalent) at 10 ppm but 2833 ng/g (meat equivalent) at 100 ppm. For ascorbic acid, the total amount of meat mutagens amounted to 2287 ng/g (meat equivalent) at 100 ppm and 3099 ng/g at 1000 ppm. The reason that the lower levels were more effective than higher levels is not clear. One possible explanation is that different pHs may effect the amount of mutagens formed. Taylor et al. (1986) have



Figrue 26. Effects of sodium bisulfite (BS) and ascorbic acid (AA) on formation of IQ, MeIQx and 4,8-DiMeIQx in fried ground beef.

shown that a boiled meat homogenate produces two mutagenic peaks as a function of pH, one at pH 4 and another at pH 9. Both sodium bisulfite and ascorbic acid are acidic in aqueous solution and theoretically a higher concentration would make the meat more acidic, and may increase the yield of total meat mutagens. Nitrite and liquid smoke also show a similar tendency in the present study, with higher concentrations producing more IQ-like compounds than lower concentrations. However, the effects of high concentrations of sodium bisulfite and ascorbic acid on mutagenicity were more pronounced than those of nitrite and liquid smoke.

Both citric acid and polyphosphates are chelating agents, which complex with prooxidant metal ions such as iron and copper thus, chelating agents can block the catalysis of lipid oxidation (Dziezak, 1986).

In the present study, two concentrations of sodium citrate (10 and 100 ppm) were used (Figure 27). At 10 ppm, sodium citrate significantly inhibited formation of IQ and 4,8-DiMeIQx ($P < 0.005$ and $P < 0.025$, respectively) but had little effect upon formation of MeIQx ($P < 0.2$). At 100 ppm, sodium citrate significantly inhibited both IQ and MeIQx ($P < 0.0005$ and $P < 0.005$, respectively), but there were no great difference ($P > 0.25$) in the concentration of 4,8-DiMeIQx in the control and the citrate treated samples.

It has been reported that short chain polyphosphates, for example pyrophosphate, are better heavy metal sequestrants than long chain polyphosphates (Steinhauer, 1983). In the present study, sodium pyrophosphate was tested at two

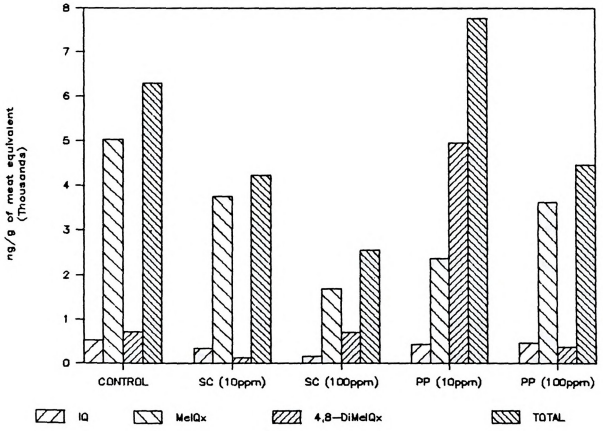


Figure 27. Influence of sodium citrate (SC) and sodium pyrophosphate (PP) on formation of IQ, MeIQx and 4,8-DiMeIQx in fried ground beef.

concentrations (10 and 100 ppm). Results in Figure 27 indicate that at 100 ppm, sodium pyrophosphate slightly inhibited formation of IQ, MeIQx and 4,8-DiMeIQx ($P < 0.1$). At 10 ppm, however, sodium pyrophosphate caused a small increase in formation of 4,8-DiMeIQx ($P < 0.1$), but it had no great effect on formation of IQ and MeIQx in comparison to the control.

On comparing the effects of reducing agents (Figure 26) with chelating agents (Figure 27), it is clear that reducing agents (bisulfite and ascorbic acid) are more effective in inhibiting meat mutagen formation than chelating agents (sodium citrate and sodium pyrophosphate).

Tocopherols are the best known natural antioxidants and exist ubiquitously in nature as a mixture of α-, β-, τ- and δ- homologs. Their antioxidant activity decreases from δ through α, while their vitamin E activity increases in the reverse order. In nature α-tocopherol is the most abundant and active source of vitamin E (Dugan, 1980). In the present study, a commercial tocopherol mixture (Cavitol) was used in two concentrations (1 and 10% of fat content). The results presented in Figure 28 indicate that 1% of mixed tocopherols significantly reduced IQ formation ($P < 0.0005$), but at 10% was less effective ($P < 0.1$). The tocopherols also inhibited formation of MeIQx at both concentrations tested ($P < 0.05$ for 1 % and $P < 0.01$ for 10%). Addition of tocopherols at a concentration of 1% slightly inhibited formation of 4,8-DiMeIQx ($P < 0.2$), but at 10% completely prevented its formation.

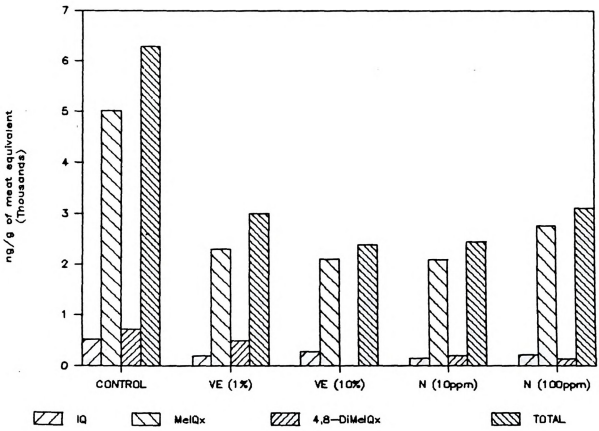


Figure 28. Effects of adding tocopherol mixture (VE) and nitrite (N) on formation of IQ, MeIQx and 4,8-DiMeIQx in fried ground beef.

In the present study, nitrite was tested at two concentrations (10 and 100 ppm). Results shown in Figure 28 demonstrated that at 10 ppm, nitrite significantly inhibited IQ, MeIQx and 4,8-DiMeIQx ($P < 0.0005$, $P < 0.005$ and $P < 0.05$, respectively). At 100 ppm, nitrite also significantly inhibited the formation of all IQ-like mutagens ($P < 0.005$ for IQ, $P < 0.025$ for MeIQx and 4,8-DiMeIQx). Thus, both concentrations of nitrite were about equally effective in blocking mutagen formation.

Liquid smoke is a concentrated acidic solution containing the natural phenols and carbonyls characteristically present in natural smoke. In the present study, commercial liquid smoke was tested at concentrations of 100 and 1000 ppm. Results presented in Figure 29 demonstrate that both concentrations of liquid smoke inhibited the formation of IQ, MeIQx and 4,8-DiMeIQx. At 100 ppm of added liquid smoke, formation of IQ ($P < 0.005$), MeIQx ($P < 0.005$) and 4,8-DiMeIQx ($P < 0.025$) was significantly inhibited. At 1000 ppm, liquid smoke inhibited formation of IQ ($P < 0.0005$), MeIQx ($P < 0.025$) and 4,8-DiMeIQx ($P < 0.05$).

Jagerstad et al. (1983) have indicated that addition of Maillard reaction products (2,5-dimethylpyrazine or 2-methyl-pyridine) enhance the mutagenicity in fried beef patties. Sodium bisulfite (a known Maillard reaction inhibitor) was shown in the present study to be a very effective inhibitor of meat mutagen formation. This suggests that the non-enzymatic browning reaction is involved in meat mutagen formation and can be inhibited by blocking the reaction.

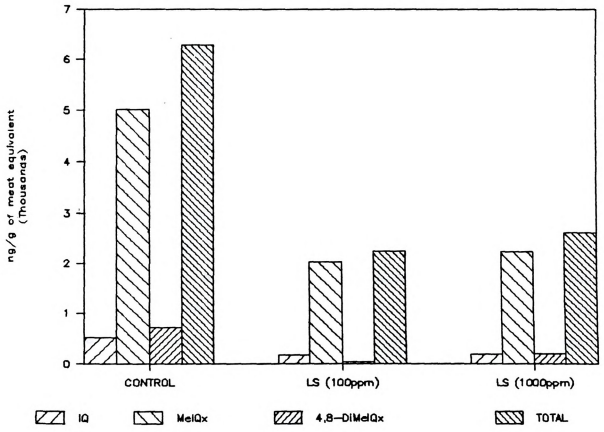


Figure 29. Influence of liquid smoke (LS) on formation of IQ, MeIQx and 4,8-DiMeIQx in fried ground beef.

In this study, all the food additives tested except pyrophosphates were shown to inhibit the formation of IQ-like compounds. All of these additives have been reported to inhibit lipid oxidation in one way or another and may be indirectly involved in the inhibition of free radicals. In an earlier phase of this study, it was concluded that mutagenic compounds are produced by heating the non-fatty components. This suggests that IQ-like meat mutagens may be produced through a mechanism similar to that involved in lipid oxidation and that Maillard reaction products may also be involved in their formation.

Namika and Hayashi (1983) demonstrated that free radicals, probably N,N'-disubstituted pyrazine cation radical products, are produced through sugar fragmentation at an early stage of the Maillard reaction. They have shown that either a mixture of N-N'-dialkylpyrazinium salt with compound(s) having a free amino group or a mixture of glycolaldehydes with an amino compound are highly active in free radical formation as well as in browning. The results of the present study suggest that antioxidants may stabilize the sugar fragment or react with the free radicals formed in the earlier stages of the browning reaction, and thus indirectly inhibit the formation of the meat mutagen precursors.

EVALUATION OF THE CARRY-THROUGH ACTIVITY OF BHA AND BHT

Buck (1985) defined "carry through" of an antioxidant as its ability to be added to a food component, survive processing, such as frying or baking, and impart stability to the finished food product. In the previous section of the present study, it was demonstrated that adding of BHA to ground beef before frying inhibited mutagenicity of the final extract. However, BHT tended to increase mutagenicity. It was also demonstrated that BHA and PG significantly inhibited the mutagenicity of IQ, MeIQ and MeIQx. On the other hand, BHT had little effect on the mutagenicity of IQ and MeIQ at low concentrations, but significantly increased their mutagenicity at high concentrations. Furthermore, BHT had little effect on the mutagenicity of MeIQx at all concentrations tested.

Based on these results, it is possible that some of the added antioxidants carry through frying and extraction of the ground beef and remain in the final meat extract, which could then either inhibit or stimulate the mutagenic activity of the compounds formed during frying. On the basis of this assumption, extracts from control samples of fried ground beef and samples containing added antioxidants (BHA or BHT) were subjected to gas liquid chromatography to ascertain if the antioxidants were carried through to the meat extract. The resulting chromatogram were extremely complex. However, none of the peaks in the chromatogram coincided with the retention times for BHA or BHT. Thus, as was explained previous, the fate of these antioxidants

during frying and extraction of ground beef were studied using [7-^{14}C]BHT and ring-labelled [^{14}C]BHA . The radioactivity of each fraction was monitored during extraction and results are shown in Table 13.

After frying, 90% of the BHA and 26% of the BHT radiolabels were still present in the original meat extract. During extraction, most of the radioactivity was located in the first methylene chloride extract that contained 58 and 24% of the original radioactivity for BHA and BHT, respectively. These fractions were then discarded as shown in FIgure 1. Only 0.4 and 0.5% of the total radioactivities of the added BHA and BHT, respectively, were found in the final DMSO extract that was used for the Ames test (Table 13). These results indicate that only minor amount of the original antioxidants and/or their derivatives were present in the final extract.

Because the final meat extract was concentrated, it is possible that the carry through of the antioxidats may be high enough to influence the Ames test. Therefore, the effects of the antioxidants and/or their derivatives in the final DMSO extract were tested for mutagenicity by the Ames test. Equivalent amounts of BHA or BHT that were present in the final meat extract
were added to the control meat extract and subjected to testing with TA98 + S-9. Results indicated that there was no significant difference in the mutagenic response for control samples and samples containing equivalent amounts of unlabelled BHA or BHT (Figure 30).

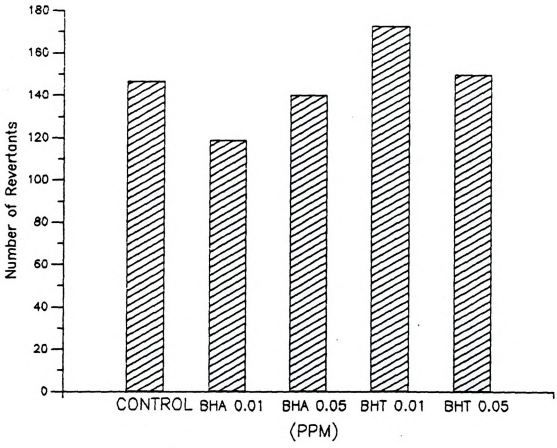


Figure 30. Comparison of Ames test results for control sample extract before or after adding BHA or BHT.

Table 13. [^{14}C]BHA and [7-^{14}C]BHT carry-over during extraction[a].

Fraction No.		BHA	BHT
		% of total radioactivity	
Fraction 1 (Raw beef patty)	Mean	100	100
	S.D.	0	0
Fraction 2 (H_2O filtrate after frying)	Mean	90	26
	S.D.	0.1	0.9
Fraction 3 (CH_2Cl_2 phase of acid extract)	Mean	58	24
	S.D.	4.4	0.7
Fraction 4 (Basic aqueous extract)	Mean	0.1	0
	S.D.	0.1	0
Fraction 5 (Final DMSO extract)	Mean	0.4	0.5
	S.D.	0.4	0.2

a) See figure 1 for extraction procedure for the various fractions.

Results demonstrated that the increased mutagenicity of the BHT treated sample was not due to any carry-over effect of the antioxidant to the final meat extract. Thus, the increased amount of 4,8-DiMeIQx formed during frying of ground beef containing added BHT appears to account for the mutagenic effect of BHT. It is still not clear why the BHT treated samples have a higher concentrations of 4,8-DiMeIQx than the controls. Therefore, further research is needed to clarify the mechanism(s) that result(s) in an increase in the concentration of this mutagen formed during frying after adding BHT.

MECHANISM(S) OF MUTAGEN FORMATION AND INHIBITION

Maillard reaction, one of nonenzymatic browning reactions, is among the most important reactions affecting food quality during storage and heat treatment (Kawamura, 1983). The mechanism proposed by Hodge (1953) for the early stages of the Maillard reaction, involving Amadori rearrangement as a key step, has been accepted over a quarter of a century. Namiki and Hayashi (1983) have proposed that the Maillard reaction involves sugar fragmentation and free radical formation. Results of the present study support the concept that free radicals may be involved in formation of the IQ-like meat mutagens.

A possible mechanism for the formation of the meat mutagens is shown in Figure 31. The basic concept of the proposed mechanism is that imidazoquinoline type meat mutagens (IQ and MeIQ) are formed from a reaction mixture containing alkylpyridine free radicals and creatinine. The imidazoquinoxaline type meat mutagens (MeIQx and 4,8-DiMeIQx) may be produced from reacting a mixture containing dialkylpyrazine free radicals and creatinine (Figure 31).

The mechanism would involve the formation of an isolated two-carbon fragment to produce a glyoxal diimine derivative. The initial two-carbon fragment could be a glycosylamino compound produced by a reverse-aldol-type reaction. However, it would be easily oxidized to produce a glyoxal monoimine derivative, and would subsequently give a glyoxal diimine derivative, which has been isolated from the reaction mixture by Namiki and Hayashi (1983). Although

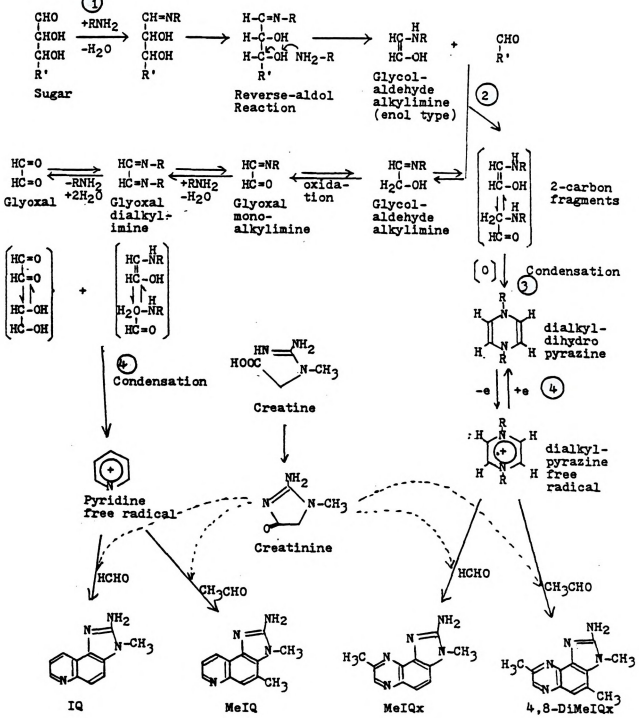


Figure 31. Suggested pathway for formation of IQ-like compounds

glycolaldehyde alkylimines were proposed as initial fragmen-tation products, they would be easily oxidized to glyoxal monoalkylimine, and may subsequently give rise to glyoxal dialkylimines.

Two different pathways for free radical product formation in the Maillard reaction are suggested in Figure 31. The first is bimolecular ring formation from the enaminol form of the glycolaldehyde alkylimine and is followed by oxidative formation of the free radical product. The second pathway suggested involves formation of N,N'-dialkylpyrazinium ions from glyoxal monoalkylimine followed by reduction to yield the free radical products. The respective intermediates in these pathways (Glycolaldehyde alkylimine and glyoxal monoalkylimine) may be formed by reaction of glycolaldehyde or glyoxal with amino compounds. According to Namiki and Hayashi (1983) the glycolaldehyde system reacts much faster and produces more free radicals than the glyoxal system, which gives only a weak ESR signal. Results indicate that the glycolaldehyde system is by far the predominant intermediate. Furthermore, the results help to explain formation of the imidazoquinoxaline meat mutagens (MeIQx and 4,8-DiMeIQx) and why they are the predominant mutagens formed in fried fish (Kikugawa and Kato, 1987) and beef (Knize et al., 1987). This pathway is also supported by the results of the present study which demonstrated that the concentration of imidazoquinoxaline type meat mutagens (MeIQx and 4,8-DiMeIQx) is present in fried ground beef at higher concentrations than the imidazoquinoline type meat

mutagens (IQ and MeIQ) (Tables 11 and 12).

Namiki and Hayashi (1983) have shown that under acidic conditions formation of glyoxal dialkylimine is readily reversed to yield the glyoxal monoalkylimine derivative. Subsequent reduction will provide glycolaldehyde monoalkylimine, which is the active precursor for free radical (dialkylpyrazine free radicals) formation. This pathway helps to explain how pH can influence the amount of mutagens formed in fried ground beef (Figure 31).

Namiki and Hayashi (1983) have shown that both a N,N'-dialkylpyrazinium salt and a mixture of glycolaldehyde with an amino compound are highly active in free radical formation. Results of the present study have shown that all the free radical scavenger type antioxidants (BHA, PG and TBHQ) can inhibit the formation of meat mutagens, except for BHT, which enhances mutagenicity. The present study suggests that antioxidants may stabilize the sugar fragment or react with the free radicals formed in the browning reaction, either alkylpyridine free radicals or dialkylpyrazine free radicals (Figure 31 step 4), and thus, indirectly inhibit the formation of the meat mutagen precursors.

Figure 32 shows a possible mechanism by which BHT may enhance mutagenicity. This scheme proposes that BHT may act as an alkylating agent and increase the formation of the precursors for 4,8-DiMeIQx. The methyl group, probably in the para-position, would be split off from BHT and react with some of the precursors and then become the methyl group attached to the 8-position of 4,8-DiMeIQx. The reason that

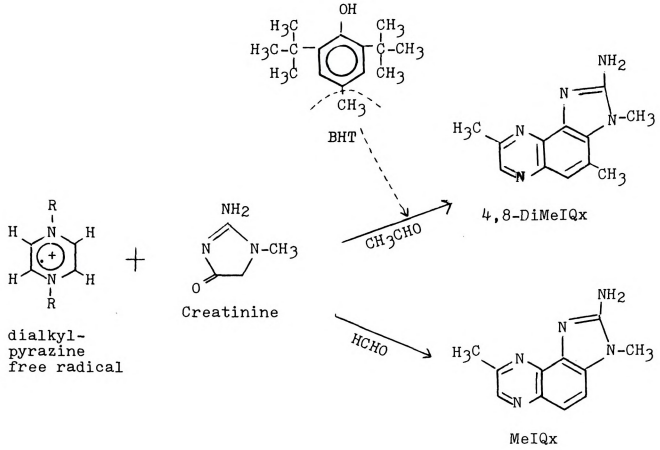
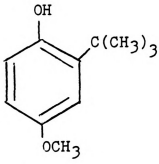


Figure 32. Possible mechanism by which BHT increase formation of 4,8-DiMeIQx.

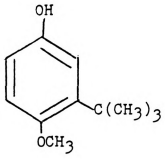
the methoxy group of BHA does not act like that from BHT is because after the methyl group leaves BHA, the molecule becomes a quinone-like compound (a very potent free radical scavenger). It then blocks step 4 in Figure 31 before it can react with the other meat mutagen precursors. This also explains how TBHQ and PG inhibit meat mutagen formation, since they have more than one hydroxy group attached to their aromatic ring structures (Figure 33).

Tocopherols are the best known natural antioxidants, and also work as free radical scavengers. Results of the present study have shown that tocopherols can inhibit meat mutagen formation, in fact, at high concentrations of tocopherol mixture (10% of fat content) 4,8-DiMeIQx formation is completely inhibited. Tocopherols may inhibit IQ-like compound formation in two ways: (1) It may block formation of free radicals (Figure 31 step 4); and (2) Some break-down product of tocopherols may react with some of the meat mutagen precursors and prevent formation of 4,8-DiMeIQx.

Sulfiting agents have long been known to serve as inhibitors of the browning reaction. Many mechanisms have been proposed (McWeeny, 1981; Whistler and Daniel, 1985) to explain how sulfiting agents inhibit the Maillard reaction. Sulfites readily react with a variety of food constituents, including reducing sugars, aldehydes, ketones and proteins, to form various sulfite combinations. Therefore, sodium bisulfite may react with other precursors of the meat mutagens and render them unavailable for meat mutagen formation.

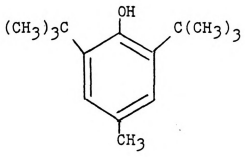


2-BHA



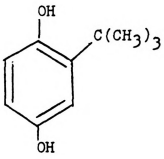
3-BHA

(Butylated hydroxyanisole)



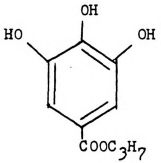
BHT

(Butylated hydroxytoluene)



TBHQ

(Tertiary butylhydroquinone)



PG

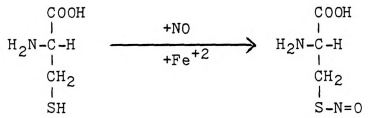
(Propyl gallate)

Figure33 . Chemical structure of BHA, BHT, TBHQ and PG.

Support for this viewpoint is found in the observation that the amount of browning on adding sulfite to the meat was reduced and could be see visually. Sulfiting agents had also been used as food additives for other purposes besides their ability to control the Maillard reaction, e.g., they have been used as a preservatives in wines, beverages and a variety of other products to control bacterial growth (Chichester and Tanner, 1968).

The mechanism by which nitrite prevents or inhibits the formation of the IQ-like compounds is still not clear. Kanner (1979) demonstrated that cysteine reacts with nitrite to form S-nitrosocysteine, which he demonstrated to be a strong antioxidant (Figure 34). Morgan et al. (1975) have shown that S-nitrosocysteine is formed in a model system containing cysteine, Fe^{2+}, and NO_2^- at a retort temperature of 121°C for 1 hour. These studies support the concept that S-nitrosocysteine may prevent oxidation, and thus inhibit meat mutagen formation.

Both citrate and polyphosphates are chelating agents, which complex with prooxidant metal ions such as iron and copper. Kato et al. (1981) have reported that copper, and iron enhance browning, with Fe^{3+} ions being more effective than Fe^{2+} ions. Taylor et al. (1986) have indicated that Fe^{2+} can increase meat mutagen formation in the model system. Results of the present study suggest that prooxidant metal ions may be involved in some of the reactions in the scheme shown in Figure 31. Kato et al. (1981) have suggested that metal ion catalysts may be involved in the



Cysteine S-Nitrosocysteine

Figure34. Reaction of cysteine with nitrite to produce S-nitrosocysteine (Morgan et al., 1975).

latter stages of the Maillard reaction, perhaps in those contributing to pigment formation.

The reason polyphosphates tend to increase the formation of 4,8-DiMeIQx can be explained by their effect on changing the pH of the meat prior to heating. Phosphate have been reported to increase the rate of browning due to their buffering capacity (Saunders and Jervis,1966). Polyphosphates are not reducing agents or free radical scavenger type antioxidants. Therefore, they do not have the ability to stabilize the free radicals formed, with the end result being an increase in formation of imidazoquinoxaline type meat mutagens. However, this does not explain why high concentrations of polyphosphates decrease meat mutagen formation. A possible explanation for the effects of polyphosphates in decreasing meat mutagen formation at high concentions may be related to the fact that they increase the water binding capacity of the meat (Bendall, 1954; Sherman, 1962). At high concentrations (100 ppm) of polyphosphates, the meat patties may contain more moisture and decrease the temperature to which the meat patties are exposed, thus decreasing mutagen formation, which is time-temperature dependent.

As discussed earlier, a boiled meat homogenate produces two mutagenic peaks as a function of pH, one at pH 4 and another at pH 9 (Taylor et al., 1986). The facts that more mutagenic compounds can produced at both low and high pHs suggests that more than one mechanism may be responsible for generating the precursors for the meat mutagens. Mauron

(1981) have reported that pyrazine can also be formed through the condensation of Strecker degradation products (Figure 35), which then can form the pyrazine free radical and then may result in the formation of the direct meat mutagen precursors.

In addition to the other precursors of meat mutagen formation, i.e., free radicals and creatinine, an aldehyde may be involved. The aldehyde could also be a Strecker degradation product (Figure 35), since Namiki and Hayashi (1983) have shown that an aldehyde can increase free radical formation.

Pariza (1987) has reported that presence of some anti-mutagenic compounds in fried ground beef. Results of the present study also indicate that some chemical compounds may decrease while others may increase formation of meat mutagens. It also has been reported that the physical properties of the meat patties may influence the amount of mutagens formed (Barnes et al., 1983). This indicates that there are many different factors which influence the formation of meat mutagens. The results presented in the present study could be due to a combination of many different factors. In order to more clearly understand the mechanism(s) involved in meat mutagen formation, much more research will be necessary.

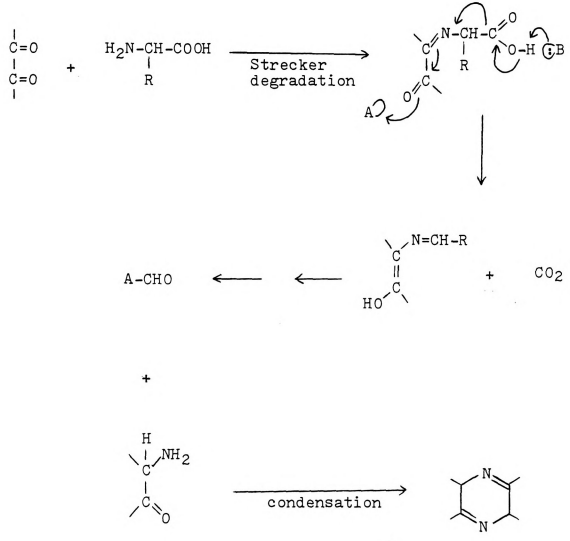


Figure 35. Production of pyrazines through Strecker degradation.

FUTURE RESEARCH SUGGESTIONS

Role of Pyrolysis in the Formation of IQ-Like Compounds

When a molecule is broken down by heat, the process is called pyrolysis. In general, pyrolysis results in fission of the weakest bond of a molecule and forms free radicals. Although the internal temperature achieved during the frying of the meat was not as high as expected (Figures 20-23), pyrolysis may still be involved in the formation of IQ-like compounds. Free radical scavenger type antioxidants (BHA, PG and TBHQ) may play a role in stabilizing the free radicals formed by pyrolysis.

Pyrolysis occurs in an environment lacking in oxygen. If oxygen is present, the free radicals easily react with oxygen and initiate peroxidation. In the presence of oxygen, however, some of the precursors of IQ-like compounds may be broken down and become unavailable to form the mutagenic IQ-like compounds.

Based on the assumption that the proposed mechanism shown in Figure 34 is due to pyrolysis in the absence of oxygen, if the atmosphere during frying is controlled, it should be possible to alter the amount of IQ-like compounds produced. Several experiments are proposed to test this assumption.

Experiment 1. Ground beef patties could be fried in a covered fry pan connected to a consistent flow controlled gas supply. Three different gas sources are suggested: (1) helium gas, (2) compressed air, and (3) nitrogen gas. The fried patties would then be extracted and analyzed with HPLC

as shown in Figure 2. The amounts of IQ-like compounds produced under each gaseous atmosphere would then be compared. On the assumption that the gaseous atmospheres would alter the concentration of the IQ-like compounds, the helium gas should yield the greatest amount of IQ-like compounds. The compressed air group should yield the least amount of IQ-like compounds and the nitrogen gas would produce an intermediate amount of IQ-like compounds.

Experiment 2. Ground beef patties would be fried under the same sources of gas as explained in experiment 1 using spin traps, e.g., nitron type spin traps, to stabilize the free radicals produced. The fried patties would be analyzed by electron-spin resonance (ESR) to measure the amounts of free radicals formed. The helium gas treated group should give the strongest ESR signal, while the compressed air should produce the lowest ESR signal. The nitrogen gas should produce intermediate ESR values.

The Relationship Between pH and IQ-Like Compound Formation

As discussed earlier, the pH may change the amount and type of IQ-like compounds. Thus, the relationship between pH and IQ-like compound formation needs further study. The pH could be altered by adding either acid (HCl) or base (NaOH) to the ground beef and measuring the amount of IQ-like compounds formed under these conditions.

Relationship Between Moisture Retention, Internal Temperature and Amount of IQ-Like Compounds

As discussed earlier, water binding agents (i.e., polyphosphates) may decrease the amount of IQ-like compounds through their effects on increasing the water binding capacity of the meat. Barnes et al. (1983) also have shown that adding of either Celite (10%, w/w) or casein (10%, w/w) to ground meat decreased IQ formation by 49% and 70%, respectively. In this case, further study may be required to determine the relationship between moisture retention, internal temperature and formation of IQ-like compounds. Different gums, e.g., locust bean gum, and different salt concentrations could be used as water binding agents in these studies.

Model System to Confirm the Mechanism Proposed

The mechanism proposed for IQ-like compound formation (Figure 31) may be confirmed by monitoring free radical formation with ESR and measuring the amount of IQ-like compounds formed in model systems using different intermediates. For example, mixing of N,N'-dialkyl-pyrazinium compounds, creatinine and a simple aldehyde (formaldehyde or acetaldehyde) should significantly influence the amounts of free radicals formed, which could be monitored with ESR. One could also quantitate the amounts of imidazoquinoxaline type mutagens (4,8-DiMeIQx or MeIQx) formed by the reaction mixture, while imidazo-quinoline type mutagens (IQ or MeIQ) would not be expected to be produced.

Effects of Other Antioxidants in the Formation of IQ-Like Compounds Formed During Frying

Antioxidants are widely distributed in the environment and are active ingredients in spices, oil seeds (Chipault, et al., 1952), vegetable extracts (Pratt and Watts, 1964), citrus waste, tree barks and in animal and plant proteins and their hydrolysates (Bishov and Henick, 1972, 1975). Many natural antioxidants are phenols and polyphenols, such as tocopherols, flavones, catechins, coumarins, and hydroxycinnamic acids (Bishov and Henick, 1976). They may act as free radical scavenger type antioxidants and terminate the free radical reactions involved. The effects of these natural antioxidants on the formation of IQ-like compounds is also an area which needs further study in order to more clearly understand the mechanism(s) involved in formation of IQ-like compounds.

Effects of Fe^{2+} and Fe^{3+} on Formation of IQ-Like Compounds During Frying of Ground Beef

It was demonstrated in the present study that metal sequestrant type antioxidants (sodium citrate and polyphosphates) can inhibit the formation of IQ-like compounds at certain concentrations. The inhibitory effect may due to metal sequestering. It has also been reported that $FeSO_4$ can increase formation of IQ-like compounds on mixing with creatine phosphate and tryptophan in a model system (Taylor et al.,1986). Barnes and Weisburger (1984) showed that Fe^{3+} or Fe^{2+} can be released through denaturation of heme

proteins and can catalyze mutagen formation in cooked meat. However, pyrrole pigments, such as hemin, biliverdin, chlorophyllin, and protoporphyrin, have been shown to have a strong inhibitory effect towards the meat mutagens (Arimoto et al., 1980a,b.; Hayatsu et al., 1981b). The role of Fe^{2+} and Fe^{3+} involved in the formation of IQ-like compounds is still not clear. By adding creatinine, glucose or other proposed precursors for the formation of IQ-like compounds to a model meat system from which all of the heme pigments and other water soluble components have been removed (Igene et al., 1979) may be a useful technique for these studies. Different concentrations of heme and non-heme iron, pyrrole pigments, or various metal sequestrants, e.g., ethylenediaminetetraacetic acid (EDTA), can be added to this model to study their respective roles in the formation of IQ-like compounds.

SUMMARY AND CONCLUSIONS

Results of this study demonstrated that fried ground beef is mutagenic in the Ames test, but that the S-9 fraction is required for promotion of mutagenicity. Fractionation of the meat extract revealed that all of mutagenic activity was localized in the basic fraction.

To study the relationship between internal temperature and meat mutagen formation, ground beef patties were fried for different time intervals. Internal temperature studies indicated that: (1) An increase in frying time did not necessarily increase the internal temperature of the meat patties; (2) Even though the internal temperature did not increase significantly with frying time, mutagen formation was positively correlated with frying time; (3) Less mutagens were formed in thick than in thin patties; and (4) This study clearly demonstrates that formation of meat mutagens during cooking is a function of both time and temperature.

To evaluate the relationship between fat content, cooking time and mutagenicity, fried ground beef samples with different amounts of fat were fried at different time intervals. Results demonstrated that meat mutagenicity is not directly related to the fat content of the patties. Apparently, the mutagenic compounds are produced from heating the non-fatty components in the meat.

On testing IQ, MeIQ and MeIQx in the Ames test, all

three compounds were mutagenic towards both TA98 and TA100 + S-9. Their potency was in the order of MeIQ > IQ > MeIQx, with their specific activities being 5644, 3836 and 642 revertants/μg, respectively, for TA98 + S-9. On testing with TA100 + S-9, the specific activities of MeIQ, IQ and MeIQx were 540, 261 and 154 revertants/μg, respectively.

BHA, BHT and PG were tested at different concentrations for their effects on the mutagenicity of IQ, MeIQ and MeIQx in the Ames test. BHA and PG significantly inhibited the mutagenicity of IQ, MeIQ and MeIQx. BHT slightly inhibited the mutagenicity of MeIQx at both low and high concentrations. On the other hand, BHT had little effect on the mutagenicity of IQ and MeIQ at low concentrations, but significantly increased their mutagenicity at high concentrations. Unfortunately, large enough quantities of 4,8-DiMeIQx were not available for mutagenicity tests.

To evaluate the relationship between antioxidants and meat mutagen formation in fried ground beef, antioxidants were added to raw beef patties before frying. All the antioxidants used (BHA, PG and Tenox 4) inhibited formation of mutagens on frying of ground beef, except for BHT, which enhanced mutagenicity.

IQ, MeIQx and 4,8-DiMeIQx were all identified in the HPLC profile of the basic fraction from ground beef fried for 9 min per side at a temperature setting of 215°C. Neither MeIQ nor PhIP could be identified in any of the samples. Quantitative determination of the meat mutagens formed in fried ground beef containing antioxidants

demonstrated that BHA, PG and TBHQ inhibited formation of all meat mutagens. TBHQ had the greatest inhibitory effect followed in order by PG and BHA. Although BHT also inhibited the formation of IQ and MeIQx, it greatly enhanced total mutagenicity by increasing the amount of 4,8-DiMeIQx by about 4-fold.

The carry through of radio-labelled BHA and BHT was followed during frying and extraction. Only minor amounts of the original antioxidants and/or their derivatives were present in the final extract. Equivalent amounts of BHA or BHT found in the final meat extract were added to the control meat extract and subjected to testing with TA98 + S-9. There was no significant difference in the mutagenic response for control samples and samples containing equivalent amounts of unlabelled BHA or BHT. Results demonstrated that the increased mutagenicity of the BHT treated sample was not due to any carry-over effect of the antioxidant to the final meat extract. Thus, the increased amount of 4,8-DiMeIQx formed during frying of ground beef containing added BHT appears to be responsible for the mutagenic effect of BHT.

In addition to the antioxidants tested. different food additives (bisulfite, nitrite, polyphosphates, citrate, ascorbic acid, tocopherol mixture and liquid smoke) were evaluated for their effects on mutagen formation in fried ground beef. All food additives tested, except polyphosphates, were shown to inhibit the formation of IQ-like compounds. All of these additives have been reported to

inhibit lipid oxidation in one way or another and may be indirectly involved in the inhibition of free radical formation. Since it was shown earlier in this study that mutagenic compounds are produced by heating of the non-fatty components, results suggest IQ-like meat mutagens may be produced through a mechanism similar to that involved in lipid oxidation. Evidence also indicated that nonenzymatic browning reaction products may be involved in mutagen formation.

A possible mechanism was proposed for formation of IQ-like meat mutagens. The basic concept of the proposed mechanism is that imidazoquinoline type meat mutagens (IQ and MeIQ) are formed from a reaction mixture containing alkylpyridine free radicals and creatinine. The imidazoquinoxaline type meat mutagens (MeIQx and 4,8-DiMeIQx) may be produced from reacting a mixture containing dialkylpyrazine free radicals and creatinine. Strecker degradation products may also involved in the formation of these mutagens. Under mild acidic conditions, the reaction would favor the formation of the MeIQx and 4,8-DiMeIQx. To more clearly understand the mechanism(s) involved in meat mutagen formation will require much more research.

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APPENDIX A

FORMULAS FOR STOCK SOLUTIONS AND MEDIA

1. Vogel-Bonner Medium E (50X VB salts)

Use: Minimal agar

Ingredients	Amount Per liter
Warm distilled water (45°C)	670 ml
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	10 g
Citric acid monohydrate	100 g
Potassium phosphate, dibasic (anhydrous) (K_2HPO_4)	500 g
Sodium ammonium phosphate ($NaHNH_4PO_4 \cdot 4H_2O$)	175 g

The salts were added in the order indicated to warm water in a 2-liter beaker or flask and placed on a magnetic stirring hot plate. Each salt was dissolved completely before adding the next. The final volume was adjusted to 1 liter. The final solution was transferred to 1-liter glass bottles. After loosely capping, the bottles were autoclaved at 121°C for 20 min. When the solutions were cool, the caps were tightened.

2. 0.5 mM Histidine/Biotin Solution

Use: Mutagenicity assay (add 10 ml to 100 ml of top agar)

Ingredients	Amount Per 250 ml
D-Biotin (F.W. 247.3)	30.9 mg
L-Histidine;HCl (F.W. 191.7)	24.0 mg
Distilled water	250 ml

The biotin was dissolved by heating to boiling, which can be done in a microwave oven. Sterilize by filtration through a 0.22-µm membrane filter or autoclave for 20 min at 121°C. Store in a glass bottle at 4°C.

3. Top Agar

Use: Mutagenicity assay

Ingredients	Amount Per liter
Agar	6 g
Sodium chloride (NaCl)	5 g
Distilled water	1000 ml

The agar may be dissolved in a steam bath or microwave-oven, or by autoclaving briefly. Mix thoroughly and transfer 100-ml aliquots to 250-ml glass bottles with screw caps. Autoclave for 20 min with loosened caps. Slowly exhaust. Cool the agar and tighten the caps. Before use, the cap was loosened and the agar was melted by placing the bottle in a steam bath or a microwave-oven. 10 ml of sterile solution of 0.5 mM L-histidine•HCl/0.5 mM biotin were added to the molten agar and mixed thoroughly by gentle swirling.

4. Salt Solution (1.65 M KCl + 0.4 M $MgCl_2$)
Use: S-9 mix for mutagenicity assay

Ingredients	Amount Per 500 ml
Potassium chloride (KCl)	61.5 g
Magnesium chloride ($MgCl_2$)	40.7 g
Distilled water	to final volume of 500 ml

Dissolve the ingredients in water. Autoclave for 20 min at 121°C. Store in glass bottles in the refrigerator or at room temperature.

5. 0.2 M Sodium Phosphate Buffer, pH 7.4

Use: S-9 mix for mutagenicity assay

Ingredients	Amount Per 500 ml
0.2 M sodium dihydrogen phosphate ($NaH_2PO_4 \cdot H_2O$) (13.8 g/500 ml)	60 ml*
0.2 M disodium hydrogen phosphate (Na_2HPO_4) (14.2 g/500 ml)	440 ml*

* These are approximate values. Test the pH. If it is too low, add more 0.2 M disodium hydrogen phosphate to bring the pH to 7.4. Sterilize by autoclaving for 20 min at 121°C.

6. 0.1 M NADP Solution (nicotine adenine dinucleotide phosphate)

Use: S-9 for mutagenicity assay

Ingredients	Amount Per 5ml
NADP (F.W. 765.4)	383 mg*
Sterile distilled water	5 ml

Add NADP to pre-weighed sterile glass tubes with screw caps without adding water. The tubes were wrapped with metal foil to protect against light and labeled with the correct weight. It is not necessary to weigh exactly 383 mg as long as the weight IS indicated on the label along with the calculated volume of water to give a 0.1 M solution. Place all the tubes of NADP in a jar with a tight fitting lid. Silica gel or other desiccant should be placed in the bottom of the jar. Store in a -20°C freezer. When needed for making S-9 mix, remove one tube from the jar, add the specified amount of water and mix by vortexing until the NADP has dissolved. Place the tube in an ice bath. We have not found it necessary to filter-sterilize NADP solutions prepared this way but it can be done, if necessary, using a 0.22-µm filter. Replace the left-over solution in the storage jar and return to the freezer for future use. Solutions of NADP stored in the freezer are stable for at least 6 months.

* This amount of NADP applies to a formula weight of 765.4. Check the corrected formula weight indicated for each lot of NADP.

7. 1 M Glucose-6-Phosphate

Use: S-9 mix for mutagenicity assay

Ingredients	Amount Per 10 ml
Glucose-6-phosphate (G-6-P)	2.82 g
Sterile distilled water	10 ml

Pre-weighed aliquots of glucose-6-phosphate are prepared as described for NADP and stored in desiccator jars in a freezer. Solutions of G-6-P can also be stored in the freezer and are stable for at least 6 months. If necessary, solutions may be filter-sterilized using a 0.22 µm filter.

8. S-9 Mix (rat liver microsomal enzymes + cofactors)*

Use: Mutagenicity assay

Ingredients	Amount Per 50 ml Standard S-9 mix	High S-9 mix
Rat liver S-9 (Aroclor-1254-induced)	2.0 ml (4%)	5.0 ml (10%)
$MgCl_2$-KCl salts	1.0 ml	1.0 ml
0.1 M glucose-6-phosphate	0.25ml	0.25ml
0.2 M phosphate buffer, pH 7.4	25.0 ml	25.0 ml
Sterile distilled water	19.75ml	16.75ml

* Liver from other mammalian species such as the hamster or mouse may be used. Other tissues may be used. The ingredients were added in the reverse order indicated above so that the S-9 fractions were added to a buffered solution. The solution must be prepared fresh and kept on ice. All ingredients were chilled. Any left over S-9 or S-9 mixture should be discarded. Never refreeze S-9.

9. Ampicillin Solution (8 mg/ml)

Use: Tests of ampicillin resistance
Master plates for R-factor strains

Ingredients	Amount Per 100 ml
Ampicillin trihydrate	0.8 g
Sodium hydroxide (0.02 N)	100 ml

It is not necessary to sterilize ampicillin solutions but they can be filtered through a 0.22-µm membrane filter. Store in glass bottle at 4°C.

10. Crystal Violet Solution (0.1%)

Use: Tests for crystal violet sensitivity (to confirm rfa mutation)

Ingredients	Amount Per 100 ml
Crystal violet	0.1 g
Distilled water	100 ml

The glass bottle with a screw cap was stored at 4°C. The bottle was wrapped with metal foil to protect against light.

11. Minimal Glucose Plates (bottom agar plates)

Use: Mutagenicity assay

Ingredients	Amount Per liter
Agar	15 g
Distilled water	930 ml
50X VB salts	20 ml
40% glucose	50 ml

15 g of agar was added to 930 ml of distilled water in a 2-liter flask. Autoclave for 20 min and slowly exhaust. When the solution has cooled slightly, 20 ml of sterile 50X VB salts and 50 ml of sterile 40% glucose were added. For mixing, a large magnetic stir bar was added to the flask before autoclaving. After all the ingredients have been added, the solution was stirred thoroughly. Pour 30 ml into each petri plate.

Note: The 50X VB salts and 40% glucose were autoclaved separately.

12. Histidine/Biotin Plates

Use: Master plates for non R-factor strains
Tests for histidine requirement

Ingredients	Amount Per liter
Agar	15 g
Distilled water	914 ml
50X VB salts	20 ml
40% glucose	50 ml
Sterile histidine•HCl•H_2O (2g per 400ml H_2O)	10 ml
Sterile 0.5 mM biotin	6 ml

The agar and water were autoclaved. The sterile 40% glucose, 50X VB salts, and histidine were added to the hot agar solution. The solution was allowed to cool slightly. Then the sterile biotin was added, mixed and the mixture was poured into the plates.

Note: A magnetic stir bar was added before autoclaving to facilitate mixing. The 50X VB salts, 40% glucose, and histidine solution were autoclaved separately.

13. Ampicillin Plates and Ampicillin/Tetracycline* Plates
Use: Master plates for strains carrying the plasmid pKM101 and pKM101 + pAQ1**

Ingredients	Amount Per liter	Plate concentration
Agar	15 g	1.5%
DIstilled water	910 ml	-
50X VB salts	20 ml	1X
40% glucose	50 ml	2.0%
Sterile histidine•HCl•H_2 O (2g per 400ml H_2O)	10 ml	260 µM
Sterile 0.5 mM biotin	6 ml	3 µM
Sterile ampicillin solution (8 mg/ml 0.02 N NaOH)	3.15ml	25 µg/ml
Sterile tetracycline solution* (8 mg/ml 0.02 N HCl)	0.25ml	2 µg/ml

Agar and water were autoclaved for 20 min. Sterile glucose, 50X VB salts, and histidine were added to the hot solution. The mixture was mixed and cooled to approximately 50°C. Then sterile biotin and ampicillin solutions were added aseptically.

* Tetracycline was added only for use with TA102 which is tetracycline-resistant. It is essential not to exceed or fall below this concentration.

The 50X VB salts and 40% glucose solutions were sterilized separately by autoclaving for 20 min. Histidine and biotin solutions were autoclaved or filter-sterilized.

Plates to be used for tests of tetracycline and/or ampicillin resistance can be stored for approx. 2 months at 4°C. After 2 months they should be tested for ampicillin/tetracycline activity with a non R-factor strain such as TA1535. Plates should be discarded if the non R-factor strain grows.

Master plates should use only plates prepared within a few days after the agar was poured.

** TA102 master plates should be discarded after 2 weeks.

14. Nutrient Agar Plates

Use: 1. Tests for genotypes
(a) crystal violet sensitivity (rfa)
(b) UV sensitivity (uvrB)
2. Tests for viability of bacteria

Ingredients	Amount Per liter
Oxoid nutrient broth No.2	25 g
Agar	15 g
Distilled water	1000 ml

Add the ingredients to a 2-liter flask containing a magnetic stir bar. Autoclave for 30 min and slowly exhaust. Mix and pour the plates.

* Difco Bacto Nutrient Broth (8g) and NaCl (5g) can be substituted for Oxoid nutrient broth No. 2.

APPENDIX B

Table of abbreviations

Abbreviation	Chemical Name
AαC	2-amino-α-carboline
AAF	2-acetyl amino-fluorene
AFB1	aflatoxin B1
AG	8-azaguanine
AIAs	aminoimidazoazaarenes
BaP	Benzo(a)pyrene
BHA	butylated hydroxyanisole
BHT	butylated hydroxytoluene
BrdUrd	Bromodeoxyuridine
CLA	conjugated linoleic acid
DEN	N,N-diethylnitrosamine
DMN	N,N-dimethylnitrosamine
DiMeIQx	2-aminotrimethylimidazo[4,5-f]quinoxaline
4,8-DimeIQx	2-amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline
7,8-DiMeIQx	2-amino-3,7,8-trimethylimidazo[4,5-f]quinoxaline
DMBA	7,12-dimethylbenz[a]anthracene
DMSO	Dimethyl sulfoxide
GCF	glandless cottonseed flour
Glu-P-1	2-amino-6-methyldipyrido[1,2-α:3',2'-d]imidazole
Glu-P-2	2-amino-dipyrido[1,2-α:3',2'-d]imidazole
HGPRT	hypoxanthine, guanine phosphoribosyl transferase
HO-IQ	2-amino-3,6-dihydro-3-methyl-7H-imidazo[4,5-f]-quinoline-7-one
IQ	2-amino-3-methylimidazo[4,5-f]quinoline
Lys-P-1	3,4-cyclopentenopyrido[3,2-α]carbazole
MAR	mutagenic activity ratio
MeAαC	2-amino-3-methyl-α-carboline
MeIQ	2-amino-3,4-dimethylimidazo[4,5-f]quinoline
MeIQx	2-amino-3,8-dimethylimidazo-[4,5-f]-quinoxaline
MNNG	N-methyl-N'-nitro-n-nitrosoguanidine
MP	6-mercaptopurine
NA	nuclear aberrations
4NQO	4-nitroquinoline-N-oxide
Orn-P-1	4-amino-6-methyl-1H-2,5,10,10b-tetraazafluoranthene
PG	n-propyl gallate
Phe-P-1	2-amino-5-phenylpyridine
PhIP	2-amino-1-methyl-6-phenyl-imidazo[4,5-b]pyridine
PRPP	5-phospho-α-D-ribose-1-pyrophosphate
SCE	Sister chromatid exchanges
TBHQ	butylhydroquinone
TG	6-thiogua-nine
Trp-P-1	3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole
Trp-P-2	3-amino-1-methyl-5H-pyrido[4,3-b]indole