CHAPTER II

LITERATURE REVIEW

2.1 Antimutagenesis and Cancer Prevention

Cancer, a disease which today remains difficult to cure, was preventable (Weinstein, 1991; Wattenberg, 1992). Chemoprevention of cancer was a mean of cancer control in which the occurrence of this disease was prevented by administration of one or several chemical compounds (Wattenberg, 1985). More attention was devoted to investigating compounds in food with antimutagenic and / or anticarcinogenic potentials. These types of food components were classified as "chemopreventers" (Stavric, 1994). Chemopreventers were found in all categories of foods, fruits and vegetables being the main source as illustrated in Table 2.1. The amount of chemopreventer in different categories of foods varied considerably. The same type of food products, obtained from different regions, might sometimes contain different levels of a particular chemopreventer (Wattenberg, 1990).

Administration of certain vegetables and / or fruits or their constituents in the diet feed to animals could reduce chemically-induced tumor incidence (Wattenberg, 1992). Table 2.2 showed a list of some phytochemicals in fruits and vegetables that were able to inhibit carcinogenesis in experimental animal models (Huang *et al*, 1994).

Table 2.1 Categories of foods with the most prominent chemopreventers (Wattenberg, 1990)

Categories of foods	Chemopreventers
Fruit	Vitamins, flavonoids, polyphenolic acids, fiber, carotenes, monoterpenoids (*d*-limonene)
Vegetables	Vitamins, flavonoids, plant phenolics, chlorophyll, fiber, aliphatic sulfides, carotenes, aromatic isothiocyanates, dithiolthiones, phytic acid, calcium
Cereals	Fiber, α-tocopherol, phytic acid, selenium
Meats, fish, eggs, poultry	Conjugated isomers of linoleic acid, vitamins (A, E), selenites
Fat / Oil	Fatty acids, vitamin E, tocotrienols
Milk	Fermentation products, calcium , free fatty acids
Nuts, beans, grains	Polyphenolics, fiber, vitamin E, phytic acid, coumarins, proteins
Spices	Coumarins, curcumin, sesaminol
Tea	Plant phenolics, epigallocatechin
Coffee	Polyphenolic acids, diterpene, alcohol esters, melanoidins
Wine	Flavonoids
Water	Selenium

Table 2.2 List of some phytochemicals in fruits and vegetables with inhibitory effects on carcinogenesis in animal models (Huang *et al*, 1994)

Group	Phytochemicals	Source
Allylic compounds	Allylmercaptan	*Allium* sp. vegetables
	Allyl methyl disulfide	
	Allyl methyl trisulfide	
	Diallyl sulfide	
	Diallyl disulfide	
	Diallyl trisulfide	
Isothiocyanates	Benzyl isothiocyanate	Cruciferous vegetables
	Phenethyl isothiocyanate	
Indoles	Indole-3-cabinol	Cruciferous vegetable
	Indole-3-acetonitrile	
Monoterpenes	D-Limonene	Citrus fruit oils
	D-carvone	Caraway seed oil
Vitamins	Ascorbic acid	Fruits and vegetables
	α-Tocopherol	Vegetable oils
	Vitamin A	Vegetables
Carotenoids	β-Carotene	Orange-yellow vegetables
Chlorophyll	Chorophyll	Green vegetables
	Chlorophyllin	
Flavonoids	Quercetin	Vegetables and fruits
	Rutin	
	Tangeretin	Citrus fruits
	Nobiletin	
Cinnamic acids	Caffeic acid	Fruits, coffee bean, soybean
	Ferulic acid	Fruits and soybean
	Chlorogenic acid	Fruits, coffee bean, soybean

2.2 Anticarcinogenic and Antimutagenic Mechanisms

Many phytochemicals in fruits and vegetables were isolated and identified and were demonstrated to block different stages of the carcinogenic process in several animal models (Wattenberg, 1992; 1983; Boone *et al*, 1990). Chemicals that were able to prevent the formation of carcinogens from precursor substances or to prevented carcinogens from reaching with critical target DNA sites in the tissues were "blocking agents." Chemicals that acted by suppressing the expression of neoplasia in cell previously exposed to doses of a carcinogenic agent were "suppressing agents" (Wattenberg, 1992).

There were numerous examples of anitimutagenic / anticarcinogenic substances derived from edible plants. According to the terminology of Kada *et al* (1982), desmutagens were antimutagenic agents acting outside the cell by acting directing on mutagens or their precursors and inactivating them, while bio-antimutagens were antimutagenic agents acting inside the cell by interfering on the process of mutagenesis or repairing damaged DNA, thereby resulting in decreasing mutation. It was now clear that many antimutagens function as antioxidants; primary examples of antimutagens that might function in this manner include vitamins C and E. It was also clear that a number of antimutagens function as interceptors / desmutagens that intercepted, bond, or physically destroyed mutagens and carcinogens. These substances were extensively reviewed by Hartman and Shankel (1990). It was also evident that there are "extracellular" inhibitors that can be classified according to their mechanisms, and there are intracellular inhibitors that function only within the potentially damaged cells (table 2.3). The mechanisms by which antimutagens / anticarcinogens might act were summarized in an extensive review by DeFlora and Ramel (1988).

Table 2.3 Mechanisms by which dietary antimutagens could protect against mutation. (De Flora and Ramel, 1988)

Mechanism	Examples of dietary antimutagens
(1) Extracellular mechanisms	
(1.1) Inhibition of mutagen uptake	Dietary fibers, probiotic
(1.2) Inhibition of endogenous formation	
(1.2.1) Inhibition of nitrosation	Vitamins (ascorbic acid, α-tocopherol), sulphur compounds (cysteine, glutathione, *N*-acetyl cysteine), phenols (cinnamic acids, chlorogenic acid, butylated hydroxyanisole)
(1.2.2) Modification of the intestinal flora	Prebiotics, probiotics
(1.3) Complexation and / or deactivation	Dietary fibers, hemin, chlorophyllin
(1.4) Favouring absorption of protective agents	Vitamin D3 and analogues
(2) Cellular mechanisms	
(2.1) Blocking or competition	
(2.1.1) Scavenging of reactive oxygen species	Provitamins and vitamins (β-carotene, ascorbic acid, α-tocopherol), diterpenes (sarcophytol a), polyphenols including epigallocatechin gallate and various anthocyanins
(2.1.2) Protection of DNA nucleophilic sites	Ellagic acid, retinoids, polyamines
(2.2) Stimulation of trapping detoxification in non-target cells	*N*-Acetyl cysteine
(2.3) Modification of transmembrane transport	Short chain fatty acids (caproate, caprylate), acylglycosylsterols, dietary calcium
(2.4) Modulation of xenobiotic metabolizing enzymes	
(2.4.1) Inhibition of promutagen activation	Isothiocyanates, monocyclic monoterpenoids (limonene, methol, caveol), retinoids, flavonoids, wheat bran
(2.4.2) Induction of detoxification pathways	Polyphenols, indoles, diterpene esters, riboflavin 5' –phosphate, *S*-allyl-L-cysteine, allylic sulfides
(2.5) Modulation of DNA metabolism and repair	Cinnamaldehyde, vanillin, umbelliferone
(2.6) Enhancement of apoptosis	Retinoids, butyric acid, flavonoids
(2.7) Maintenance of genomic stability	Vitamins (folic acid, B12), minerals (selenium, zinc), polyphenols

2.3 Antimutagens in Plants

Recently, the use of antimutagens and anticarcinogens to prevent human cancer and genetic correlation diseases should be the most effective intervention. This suggestion led to an increase in studies of antimutagens. Many antimutagenic factors were found in plants (Vinitketkumnuen and Suaeyun, 1995). For example, some natural flavonoids were shown to inhibit the activity of cytochrome P450-dependent enzymes that metabolized drugs and carcinogens (Buening *et al*, 1981; Lasker, Huang and Conney, 1984). Moreover, it was significant that chlorophyll and chlorophyll-containing vegetables exhibit antimutagenic activities (Lai, Dabney and Shaw, 1978).

Natural product chemists isolated more than 100 chemical species from the plant kingdom that were animutagenic and anticarcinogenic (Wargovich, 1988). Various antimutagens were found in our daily food of plant origin. Edenharder, Leopold and Kries (1995) found antimutagenic activities in fruits and vegetables extracts against IQ and MeIQx. The edible part of fresh Chinese radish was extracted sequentially with hexane, chloroform and methanol. None of the three fractions exhibited any mutagenicity toward *S. typhimurium* strains TA98 and TA100 when tested either in the presence or absence of S-9 mix. Antimutagenic activities against aflatoxin B_1 induced in *Salmonella* spp. were detected in the n-hexane and chloroform extracts but not in the methanol extract of Chinese radish (Rojanapo and Tepsuwan, 1993). Significant inhibiting effects of vegetables, such as cauliflower, spinach and lettuce and some of their components, i.e. chlorophyll and ascorbic acid, on the mutagenicity of *in vivo* interaction of nitrite-aminopyrene model and nitrite-methylurea model which gave rise to dimethylnitrosamine (DMNA) and nitrosomethylurea (NMU) were reported (Barale *et al*,1983). Despite plants contained mutagens, they were antimutagenicity such as ginger juice contained 6-gingerol, a potent mutagen, and an antimutagenic compound against the 6-gingerol (Nakamura and Yamamoto, 1982).

Four Chinese medicinal plants with a long history of indigenous used as antitumor agents or as adjuncts (*Oldenlandia diffusa*, *Scutellaria barbata*, *Astragalus membranaceus*, and *Lingustrum lucidum*) were demonstrated to be antimutagenic activity in Ames assays with strain TA100 by reducing metabolic activation by P450

isoenzymes against aflatoxin B-1 (Wong, Lau and Teel, 1992) Organic solvent extracts of leaves of 4 common edible vegetable plants (*Bryophyllum pinnatum*, *Dialium guincense*, *Ocimum gratissimum*, and *Vernonia amygdalina*) had antimutagenic activity against ethyl methanesulfonate on *S. typhimurium* TA100 (Obaseiki-Ebor *et al*, 1993).

Some natural products inhibited only specific groups of mutagenic compounds depending on mechanism of mutagenesis. Pine cone extracts were found to inhibit the mutagenic action of the promutagens benz[a]pyrene, 2-aminoanthracene, 2-acetylaminofluorene etc., in Ames test but they failed against direct-acting mutagen. These findings suggested that the action was mediated by interference with P450 isoenzymes (Lee *et al*, 1993). Similar conclusions were reached in working with extracts of brown sea weeds, a part of the diet in Japan (*Laminaria japonica* and *Undaria pinnatifida*) (Okai, 1993).

Extracts of garlic (*Allium sativum*), an important part of the human diet for at least 3000 years, were found to be protective in mice against mitomycin C, cyclophosphamide and sodium arsenite (Das *et al*, 1993). Thai diet has been characterized by the intake of significant quantities of spices and yet there was no significant association between Thai food and increased incidence of gastrointestinal cancers. It was interesting in this context to note that the addition of nitrites to methanolic and aqueous extracts of several spices (caraway, coriander and black pepper) converted these materials from innocuous to mutagenic against Ames strain TA100. The hot water extracts themselves, without prior oxidation, were actually antimutagenic towards *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, dimethylnitrosamine and ICR-170. However, they were not antimutagenic towards 1-nitropyrene, *N*-ethyl-*N'*nitro-*N*-nitrosoguanidine, 2-aminoanthracene, 2-acetylaminofluorene, benzo[a]pyrene or IQ (Higashimoto *et al*, 1993).

A number of recent investigations dealt with plants which were studied well in other contexts or which were emerging from the previous category. Emphasis was placed on those which were used extensively as foods or condiments. For example extracts of chili, an important indigenous spice, were shown in Ames assays (TA98) to provide protection against nitroaromatics in the heavily polluted air of Mexico City. This

was attributed to the content of β-carotene and chlorophyll in presenting in the samples (Espinosa-Aguirre *et al*, 1993). Rosemary extracts, containing an active carnosic acid, were strongly antimutagenic in Ames assay (TA102) against hydrogen peroxide and *t*-butylhydroperoxide insults. This action was mostly antioxidation in nature (Minnumi, 1992).

Turmeric, *Curcuma longa*, a spice used extensively in Southern Asia. Some studies had shown that dietary curcumin and the glycoflavonoid hesperidin (both tumeric constituents) were orally protective to rats against oral carcinogenesis by 4-nitroquinoline 1-oxide (Tanaka *et al*, 1994). Other works confirmed these findings. It was shown that turmeric (and betel leaf extract) decreased mouse and rat mammary tumors caused by dimethylbenzanthracene (Bhide *et al*, 1994), decreased DNA adducts formed in rat livers by metabolites of benz[a]anthracene (Mukundan *et al*, 1993), inhibited DNA adducts formed in rat for stomach mucosa (Lahiri, Maru and Bhide,1993). The precise mechanisms were in determined but these agents would appear to inhibit cytochrome P450 mediated oxidation of the promutagen.

Pak Chee Lao (Dill, *Anethum graveolens* L; Umbelliferae): an annual plant. It grows to a height of 3 or 4 feet. Its flowers are yellow. The entire plant is sweet and aromatic, intermediate between anise and caraway. The herb, especially when fresh, has a much sweeter fragrance than dried fruits. Pak Chee Lao leaves are used as seasoning for soups, sauces, and particularly pickles; the seed is employed as a condiment (Guenther, 1950). There are some reports of its pharmacological effects, such as antimicrobial (Chaurasia and Jain, 1978; Delaquis *et al*; 2002), antihyperlipidaemic and antihyperchloresterolaemic (Yazdanparast and Alavi, 2001). As a folk remedy, Pak Chee Lao is considered for some gastrointestinal aliments such as flatulence, indigestion, stomachache and colic (Duke, 2001). Pak Chee Lao's fruit has an antispasmodic effect on the smooth muscles of the gastrointestinal tract (Fleming, 2000). Pak Chee Lao's water is believed to have a soothing effect on the digestive system and is given to babies to relieve hiccups and colic. Pak Chee Lao, especially its fruit, contains essential oil (3-4%) rich in *d*-carvone (main; 50-60%), and *trans*- and *cis*-dihydrocarveol, *trans*- and c*is*-carveol, limonene, *d*-dihydrocarveol, *l*-dihydrocarveol, α-

and γ-terpinene, α-phellandrene, β-terpineol, terpnene-4-ol *p*-cymene, thymol, carvacrol etc. were reported as other constituents (Niyazawa and Kameoka, 1974). However, only dillanoside has been published as a constituent of the water-soluble portion of this fruit (Kozawa *et al.*, 1976)

After investigating the genotoxicity and mutagenicity effect of Pak Chee Lao, the essential oils from Pak Chee Lao's seeds are found to be able to induce chromosome aberrations at low toxicity levels (less than 20% inhibition of mitotic activity). In Ames test, essential oils from Pak Chee Lao's herb and seeds are very toxic for *S. typhimurium* strains TA98 and TA100, but none induced mutations (Lazutka *et al.*, 2001).

Pak Ka Yang (Rice Paddy Herb, *Limnophila aromatica* Merr.; Scrophulariaceae): a synonym is *Limnophila gratissima* Blume. It is used as a spice and medicinal herb in Southeast Asia. This herb has a unique flavor. It is lemony, with a certain tickling quality. The essential oil in this plant exhibits significant anti-bacterial and anti-fungal activities (Kapil, Sinha and Sinha, 1983). The principle constituents of this oil are limonene, perillaldehyde, monoterpenoid ketone and *cis*-4-caranone. A flavone isolated from the aerial parts is identified as 7-desmethylartemetin (5, 7-dihydroxy-3, 6, 30, 40-tetramethoxyflavone), but later revised by Krishnan, Nair and Ramachandran (1999), who claimed that it was a mixture of nevadensin (5, 7-dihydroxy-6, 8, 40-trimethoxy-flavone) and salvigenin (5-hydroxy-6, 7, 40-trimethoxyflavone). They also reported that chlorogenic and caffeic acids as constituents of this species. Moreover, nevadensin exhibited inhibition activity against *Mycobacterium tuberculosis*, with equal 200 μg/ ml (Suksamran *et al.*, 2003).

Pak Gud (Paco, *Diplazium esculentum* (Retz.).; Athyriaceae): a fern-like plant, with curly leaves and small flowers, is used in many Thai dishes, such as eating with nam-prik, making spicy salad. There are some researches that antioxidant activity of Pak Gud was higher than alpha-tocopherol (Rahmat *et al.*, 2003). Nakahara *et al.* (2002) studied antimutagenicity of some edible Thai plants found that methanolic extracts of Pak Gud inhibit mutagenicity of Trp-P-1. Taungbodhitham (1995) found that Pak Gud was lack of thiamin and had antithiamin property and Srivastava *et al.* (1981) found

flavanone glycoside, namely f-riodietyol 5-*O*-Methyl ether 7-*O*-beta-D-xylosyigalactoside from *Diplazium esculentum* (whole plant).

Pak Krad Hua Wan (Paracress, *Spilanthes acmella* Murr.;Compositae): an annual herb. Its stems are branched with wing-like leaves which are simple, serrate and sessile. The flower shape is axillary, condense and round. Its seeds are yellowish brown. The plant has a local anesthetic property for gums and teeth and antiseptic. Pak Krad Hua Wan is an aromatic plant. The pungent flavour of Pak Krad Hua Wan is due to an unsaturated alkamide, spilanthol and other alkamides such as isobutylamides of hendeca-2E,7Z,9E-trienoic acid and hendeca-2E-en-8,10-diynoic acid (Ramsewak, Erickson and Nair, 1999).

Besides the alkamides, nonvolatile sesquiterpenoids have been found, e.g., polygodial and eudesmanolide II. From the flowers of Pak Krad Hua Wan, traces of an essential oil were isolated, whose main constituents were limonene, β-caryophyllene, Z-β-ocimene, γ-cadinen, thymol, germacrene D and myrcene. Prachayasittikul *et al* (2004) found stigmasterol and stigmasteryl-3-O-β-D-glucopyranoside together with a mixture of triterpenes, and long chain fatty esters in Pak Krad Hua Wan.

Pak Pai (Vietnamese Coriander, *Polygonum odoratum* Lour; Polygonaceae): It is noticed that the plants of genus *polygonum* provide many kinds of polyphenols, some of which showed interesting biological activities (Gong *et al.*, 2002). It has been traditionally used as an antidiabetic agent in Korea. Water and methanol extracts of Pak Pai alleviated insulin resistance with increased glucose use by skeletal muscles. Meanwhile, it contains low dialysable calcium (2-7%) with high level of oxalate (Kamchan *et al.*, 2004). The main constituents in Pak Pai are long-chain aldehydes e.g. decanal, dodecanal, decanol and sesquiterpenes e.g. α-humulene, β-caryophyllene. (Starkenmann *et al.*, 2006)

2.4 Nitrite and Gastric Cancer

Nitrate and nitrite have been used for centuries in curing and preserving meats and fish, and in manufacture of certain cheeses (Binkerd and Kolari, 1975). Being added to these foods, nitrite has at least three functions. Firstly, it contributes to the flavour; this may be due to the inhibition of development of rancid-off flavors (Cornforth, 1996). Secondary, it reacts with myoglobin to give mononitrosylhaemochrome, which gives the characteristic pink colour of cured meat. Thirdly, it inhibits the growth of food spoilage bacteria, most importantly, *Clostridium botulinum* (Cammack *et al*, 1991)

Nitrate and nitrite occur in the diet from numerous different sources (Knight *et al*, 1987). Vegetables are major sources of nitrate, which is converted to nitrite when such foods are stored at room temperature (Weisburger and Raineri, 1975). Many plants, such as leafy vegetables or certain roots, accumulate extremely high concentrations of nitrate under favourable conditions of soil and water (Phillips, 1968; Heisler *et al*, 1973). Since the 1970s there has been concern about a possible link between nitrite and cancer. There is no conclusive evidence that nitrite is directly carcinogenic (Cantor, 1997) but in high doses it has been implicated as a co-carcinogen (Schweinberg and Burkle, 1985).

Gastric cancer remains such a common neoplastic disease in many parts of the world (Kikuguwa and Nagao, 1990). Graham *et al* (1990) found that high ingestion of nitrate or nitrite in processed meats and fishes, heated fats and starch may be directly correlated with cancer risk. The correlation between nitrite ingestion and mortality from gastric cancer were reported (Fine *et al*, 1976).

It is proposed that nitrate involves in the formation of carcinogenic *N*-nitroso compounds via two district phases of gastric carcinogenesis. Firstly, after ingestion and absorption in the stomach, nitrate is secreted in the saliva in concentrated form. Oral bacteria can then produce nitrate to nitrite (Spiegelhader, Eisenbrand and Preussmann, 1976; Forman, 1989). In the second phase, nitrite is converted in the stomach to nitrous acid and reacts with certain substrates (amines, amides) to from carcinogenic *N*-nitroso compounds (Correa *et al*, 1983) which frequently demonstrated to be carcinogenic in animals (Choi *et al*, 1987; Forman, 1989).

2.5 Nitrite as a Converter for Direct mutagen

The majority of human cancer, 70-80%, is related to lifestyle (Doll and Peto, 1981). High fat, low fiber, consumption of well cooked meat, nitrite / nitrate, low vitamin C, alcohol and mycotoxins are all dietary factors which correlate most strongly with cancer. For example, a normal diet, well-cooked beef, chicken and pork, in New Zealand is a major source of mutagens (Norrish *et al*, 1999; Ferguson, 2002a; 2002b) .Epidemiologic studies showed the relationship between various sites including stomach, colon, prostate and breast and inappropriate diet consumption (WCRF/AICR, 1997). Several investigators (Marqauard *et al*, 1977; Llanes and Tannenbaum, 1982; Tomita *et al*, 1982; Wakabayashi *et al*, 1984) suggested that direct-acting mutagens / carcinogens formed from nitrite and the precursors of mutagen in the acid conditions of the stomach were possible candidates for the causation of human gastric cancer. For example, fava beans (*Vicia faba* L.) commonly eaten in Columbia where gastric cancer incidence is high (Correa *et al*, 1983) showed direct acting mutagenicity towards *Salmonella* strains after treated with nitrite in acid solutions (Llanes and Tannenbaum, 1982; Hoeven *et al*, 1984).

Many investigators examined the diets eaten mostly in Eastern European countries where the gastric cancer rate was high (Marquard, Rufino and Weisburger, 1977). Ohshima *et al* (1989) found that smoked foods, a frequent consumed food item, was associated with increased risk of gastric cancer; they were revealed to be direct acting genotoxicity after nitrosation *in vitro*. Münzner and Wever (1984) found that the products formed by the reaction of beef extract with nitrite were assayed in the *Salmonella* / microsome mutagenicity test on strains TA1538, TA98 and TA100. The products exhibited mutagenicity activity towards all tester strains with and without metabolic activation. Marquard, Rufino and Weisburger (1977) indicated that fish, beans and borscht showed the formation of one or more mutagenic on *Salmonella typhimurium* TA1535 after treated with 5000 ppm nitrite at pH 3.0. In addition, broiled chicken, pork, mutton, beef and sun-dried sardine were found to yield direct acting mutagenicity on TA98 and TA100 without metabolic activation (Yano *et al*, 1988) (Table 2.4)

Cooked meats, smoked food and charcoal-broiled foods are common foods which polycyclic aromatic hydrocarbon (PAH) were detected and quantified. The nitrite treated products of polycyclic aromatic hydrocarbon (PAH) extracts from smoked fish, skin of fresh water catfish, charcoal - broiled chicken wing, rice pork sausage, pork (medium fat) were mutagenic towards both strains TA98 and TA100 (Kangsadalampai Butryee and Manoonphol, 1996). New substances were not *N*-nitroso compound but might be nitro-polycyclic aromatic hydrocarbons (nitro-PAHs). In addition, Kangsadalampai and Peerawong (1997) found that commercial chicken extracts in the presence of nitrite showed their mutagenicity towards *Salmonella typhimurium* strains TA98 and TA100 in the absence of metabolic activation. Common dietary mutagens would include *N*-nitroso compounds in preserved meats, processed cheeses or beers, various fungal toxins including aflatoxins and fumonisins (Gelderblom *et al*, 2001) or cooked meat carcinogens (Layton *et al*, 1995).

Table 2.4 Mutagenicity of cooked foods after nitrite treatment (Yano *et al*, 1988)

Food	Revertants/gm original material	
	TA98	TA100
Chicken	33,300	12,800
Beef	22,600	7,400
Mutton	43,600	5,700
Pork	15,000	3,800
Sun-dried sardine	20,200	17,900

Japan also has a high incidence of gastric cancer which may relate to dietary habit. Japanese fish, soy sauce and Chinese cabbage, which are favorites of the Japanese showed direct acting mutagenicity on *Salmonella typhimurium* TA100 after nitrite treatment. Wakabayashi (1984) has made extensive studied on the appearance of direct-acting mutagenicity of various foodstuffs produced in Japan and Southeast Asia, such as The Philippines and Thailand, on nitrite treatment. After nitrite treatment, various kinds of pickled vegetable and sun-dried fishes produced in Japan showed direct-acting mutagenicity on *Salmonella typhimurium* TA100, inducing 1,900 - 18,000 revertants/gm. Palli (1996) indicated that salted/smoked and pickled/preserved foods

(rich in salt, nitrites and preformed nitroso compounds) were associated with an increased risk of gastric cancer. Salted pickled cabbage eaten by Korean three times a day contained high levels of total *N*-nitroso compounds (1,173 ug/ kg) after treatment with nitrite under simulated human stomach conditions. Thus, salted pickled cabbage may play an important role in the gastric cancer in Korea (Seel *et al*, 1994). Additionally, the extracts of raw and pickled vegetables and fruits, namely garlic, cabbage, shallot, mushroom, cucumber, ginger, Chinese mustard, bamboo shoot and mango were treated with nitrite in the absence of metabolic activation. All of them exhibited direct acting mutagenicity in *Salmonella* assay (Hankimhun, 1997).

Surprisingly, a dish composed mainly of carbohydrate was also turned to be direct mutagen. Hayatsu and Hayatsu (1989) treated an aqueous homogenate of boiled rice with nitrous acid at pH3 and found that mutagens were formed. The mutagen was active in *Salmonella typhimurium* TA98 and TA100 without metabolic activation.

Kangsadalampai and Butryee (1995) found that nitrite treated products of natural thai food colors from 5 plants, namely *Clitorea ternatea* Linn., *Hisbiscus sabdariffa* Linn., *Pandanus amaryllifolius* Roxb., caramellized coconut sugar and *Carthamus tinctorius* Linn., and of a synthetic color, Ponceau 4R, exhibited their mutagenicity on *Salmonella typhimurium* strains TA98 and TA100. Mutagenicity of the extracts of Thai medicinal plants after nitrite treatment was found on *Andrographis paniculata* Ness., *Carthamus tinctorius* Linn., *Cassia angustifolia* Vahl., *Cassia fistula* Linn., *Centella asiatica* Linn., *Curcuma domestica* Vahl., *Curcuma zedoaria* Rosc. (Kangsadalampai and Butryee, 1995). A food additive, namely sorbic acid could react with nitrite to yield mutagens (Namiki *et al,* 1981). Being treated with nitrite, pepper exhibited the strongest mutagenic activity in the Ames test, while nugmet, chili pepper and laurel also showed strong activities (Namiki *et al,* 1981). No mutagenicity was observed for spices alone. In addition, Shephard, Wakabayashi, and Nagao (1993) reported that aspartame (artificial sweetener) nitrosated for 10-30 minutes with 40 mM nitrite (pH3.5, 37°C) had mutagenic activity on *Salmonella typhimurium* strain TA100. Furthermore, the products formed by reacting with nitrite under gastric simulating condition (pH 3.0-3.5) of *Fornes japonica*, *Ganoderma applanatum*, *Acetobacter*

xylinum were mutagenic to both strains TA98 and TA100. While royal jelly was mutagenic to *S. typhimurum* strain TA98 (Katipagdeetham, 1996). Therefore, several nitrosable mutagen precursors in foods taken by people in high-risk areas might be the etiological factor of gastric cancer, investigation must be continued to elucidate whether nitrosable compounds are involved in the development of human cancer, particularly of the stomach.

2.6 1-Aminopyrene Nitrite Mutagenicity Model

1-Aminopyrene is a derivative of 1-nitropyrene in human gastrointestinal tract. Anaerobic bacteria metabolize 1-nitropyrene to 1-aminopyrene. 1-Nitropyrene is generally a product of incomplete combustion and is the predominant nitro-polycyclic aromatic hydrocarbons (nitro-PAHs) emitted in diesel engine exhaust, exhaust of kerosene heaters and petroleum gas burners and food products as a result of pyrolysis of fat in meat during barbecuing (Rosenkranz and Mermelstein, 1983; Handa *et al*, 1983; Tokiwa, Nakagawa and Horikawa, 1985; Kinouchi, Tsutsui and Ohnishi, 1986; Edenharder *et al*, 1993). The most primary route of potential human exposure to 1-nitropyrene is inhalation.

The metabolism of 1-nitropyrene was shown to involve both nitroreduction and oxidation as shown in Figure 2.1 (Howard, Beland and Cerniglia, 1983; Howard, Reed and Koop, 1988; Kataoka, Kinouchi and Ohnishi, 1991). Under aerobic conditions, cytochrome P450-mediated C-oxidation results in the formation of epoxides and/or phenols (Howard, Reed and Koop, 1988). Metabolite are presumably further metabolized via hydrolysis by epoxide hydrolases and / or by conjugation with glutathione, glucuronic acid, sulphate and then excreted as conjugates (Djuric, 1987; Kataoka, Kinouchi and Ohnishi, 1991).

Nitroreduction of nitro-PAHs *in vivo* occurs mainly by bacteria in the intestinal tract (El-Bayoumy *et al*, 1983; Moller *et al*, 1988; Ball *et al*, 1991). Nitroreduction in mammalian cells is catalysed by xanthine oxidase, aldehyde oxidase, or NADPH cytochrome P450 reductase. Complete nitroreduction of nitro-PAHs results, through a *N*-hydroxylarylamine intermediate, in the formation of the corresponding arylamine.

Arylamines can be reactivated through *N*-oxidation by a cytochrome P450-mediated reaction to form the *N*-hydroxylarylamine (Shimada *et al*, 1989). Although reactive arylnitrenium ion that can bind to DNA is formed when the hydroxyl group leaves the *N*-hydroxylarylamine, a more reactive species is formed through esterification of the *N*-hydroxylarylamine by acetyltransferases or sulfotransferases (Miller and Miller, 1981; Rosser *et al*, 1996)

1-Aminopyrene was known to be non-mutagenic when it was tested without metabolic activation (Kinouchi, Tsutsui and Ohnishi, 1986). Kato *et al* (1991) demonstrated that aminopyrene treated with nitrite at pH 3.0 and 37°C showed mutagenicity to *Samonella typhimurium* TA98 and TA100 without metabolic activation. The result agreed with the work of Kangsadalampai and Suharitamrong (1996) which stated that nitrite treated 1-aminopyrene exhibited stronger mutagenicity than the authentic aminopyrene towards *Salmonella typhimurium* both strains TA98 (frameshift mutation) and TA100 (base-pair substitution mutation), in the absence of metablic activation. The mutations appear to be due to the presence of nitroreductases (IARC, 1989) and *O*-acetyltransferase (Mesmelstein *et al*, 1981) which are the two activating systems presented in bacterial cells for nitrite treated aminopyrene (supposed to be 1-nitropyrene). Such enzymes metabolize 1-nitropyrene to be arylhydroxylamine, which is active to interact with DNA. Evidence had been shown that 1-nitropyrene induced tumors in experimental animals (Rosenkranz and Mermelstein, 1983; Busby, Penman and Crespi, 1994; El-Bayoumy *et al*, 1982). Thus, the mutagenicity of aminopyrene and nitrite in acid condition has been established as a model for antimutagenicity studies of some chemical concerning the phenomenon occurred during stomach digestion.

2.7 Conversion of Precursors in Beef Extract to Direct-Acting Mutagens (Beef Extract-Nitrite Mutagenicity Model)

Commonly eaten meat products prepared from beef, pork, mutton and chicken all show some level of mutagenic activity following cooking. Food preparation methods have a significant influence on the formation of the mutagenic activity, and many studies have been devoted to the mutagenic activity in fried/broiled food. Felton *et al* (1997) and

Sugimura (2000) found that common cooking procedures such as broiling, frying, barbecuing (flame-grilling), heat processing and pyrolysis of protein-rich foods induce the formation of potent mutagenic and carcinogenic compounds. Heterocyclic amines (HCAs) are formed during the high-temperature cooking of meat and fish, from amino acid, creatinine and polysaccharide pre-cursors (Layton *et al*, 1995). Although, their potencies vary, all of these compounds have shown to be mutagenic in the Ames *Salmonella* assay and carcinogenic in experimental animal studies (Layton *et al*, 1995; Sugimura, 1997; Nagao *et al*, 1994). The heterocyclic amine is a family of mutagenic / carcinogenic compounds produced during the heating of creatinine, amino acids and sugars.

Heterocyclic amines are classified into 2 groups by treatment with 2 mM sodium nitrite (Robbana -Barnet et *al*, 1996).

1. **Aminoimidazoazaarenes (AIAs) (IQ-type, polar)**

AIAs compounds have a 2-aminoimidazo group fused to a quinoline (IQ and MeIQ), a quinoxaline (MeiQx), or a pyridine (PhIP) ring. The amino group is not changed by treatment with 2 mM sodium nitrite but is converted to a nitro group with 50 mM sodium nitrite. Figure 2.1 shows the chemical structure of Aminoimidazoazaarenes AIAs (IQ-type, polar)

2. **Carboline (Non-IQ type , non-polar)**

Non-IQ type is produced by pyrolysis of amino acids and protein, being produced at higher temperature than IQ-type (Tsuda *et al* , 1985). The carboline and its analogues are the type non-IQ contrary to the AIAs. They include the aminopyridoindoles (Trp-P-1, Trp-P-2, AαC, MeAαC), the aminopyridoimidazoles (Glu-P-1, Glu-P-2, Lys-P-1, Orn-p-1) and an aminophenylpyridine (Phe-p-1). The amino group is ccnverted to a hydroxyl group with 2 mM sodium nitrite. Figure 2.2 shows the chemical structure of Carboline and its analogues.

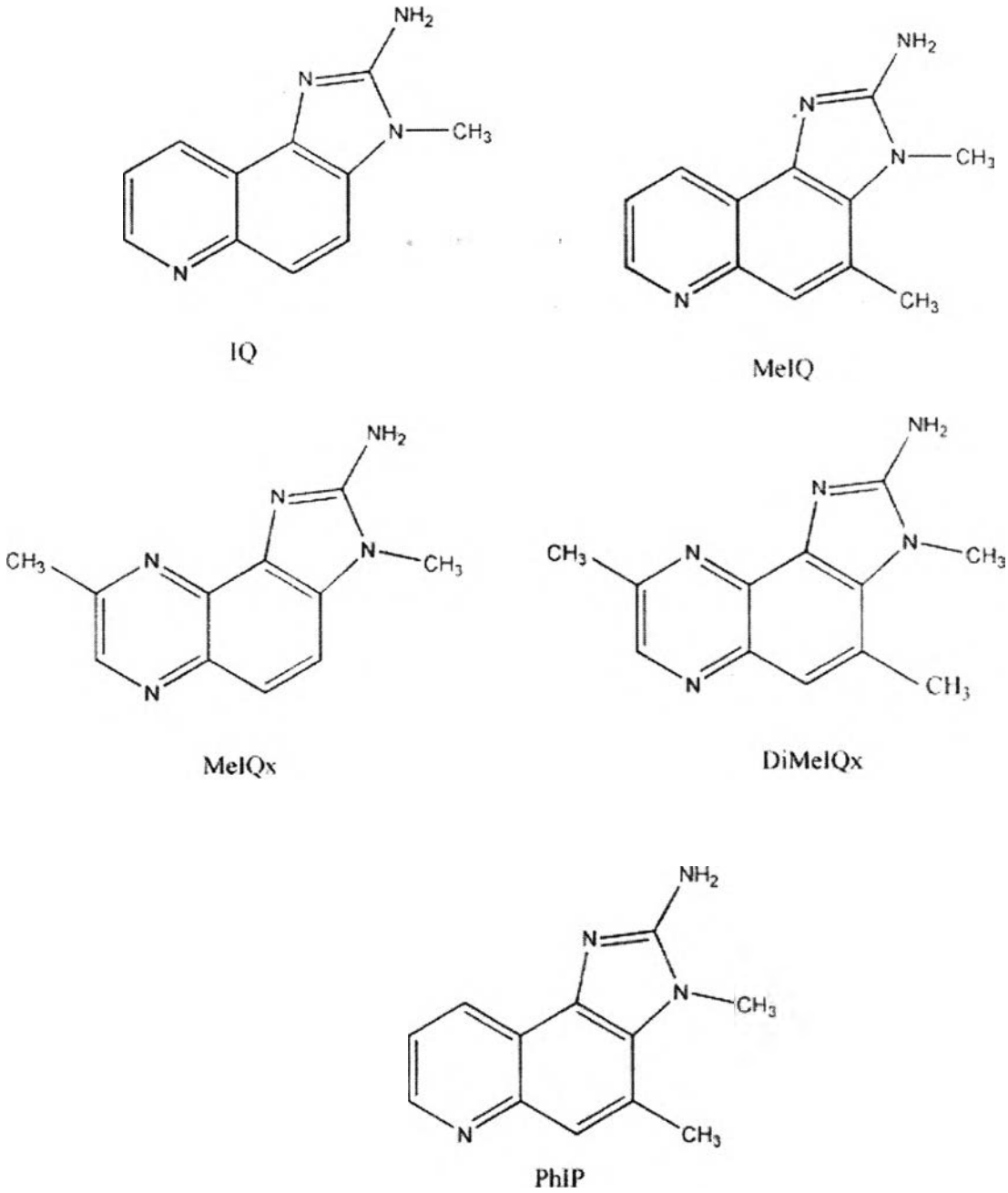

Figure 2.1 Chemical structure of Aminoimidazoazaarenes (IQ-type , polar) Quinolines (IQ= 2-amino-3-methylimidazo [4,5-*f*] quinoline ; MeIQ = 2-amino-3,4-dimethylimidazo[4,5-*f*] quinoline ; MeIQx = 2-amino-3,8-dimethylimidazo [4,5-*f*] quinoxaline ; DiMeIQx = 2-amino-3,4,8-trimethylimidazo [4,5-*f*] quinoxaline ; PhIP = 2-amino-1-methyl-6-phenylimid-azo [4,5-*b*] pyridine) (Herman *et al* , 1999)

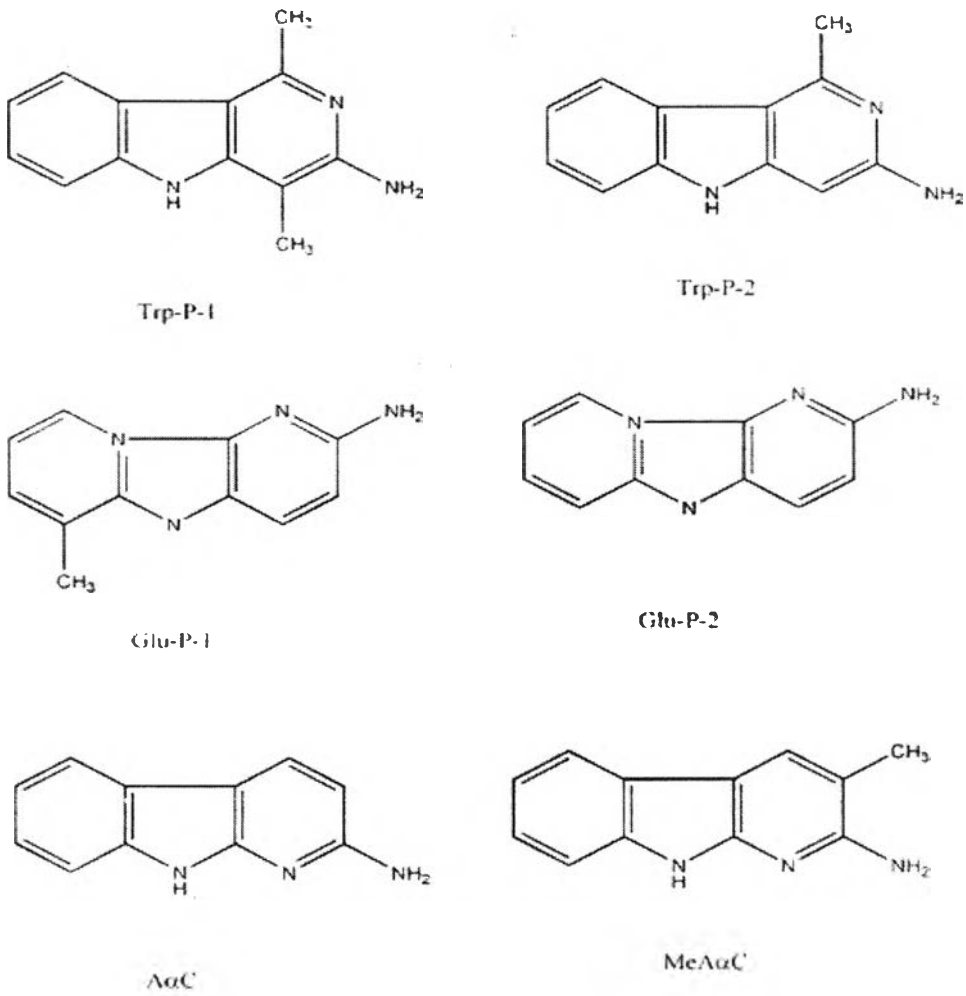

Figure 2.2 Chemical structure of Carboline and analogues (Non-IQ type , non-polar)Trp-P-1 = 3-3-Amino-1,4-dimethyl-5H-pyrido[4,3-*b*]indole ;

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

Glu-P-1 = 2-Amino-6-methyldipyrido[1,2*a*:3,2-*d*]imidazole ;

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

A**α**C = 2-Amino-9H-pyrido[2,3-*b*]indole ;

MeA**α**C = 2-Amino-3-methyl-9H-pyrido[2,3-*b*]indole (Sugimura , 2000).

The major subclass of heterocyclic amines found in human diet comprise aminoimidazoazaarenes (AIA), 2-amino-3-methyllimidazo[4,5-*f*]quinoline (IQ), 2-amino

3,4 dimethylimidazo[4,5*f*]quinoline (MeIQ), 2-amino 3,8 dimethylimidazole [4,5*f*] quinoxaline (MeIQx), 2-amino 3,4,8 trimethylimidazo [4,5-*f*] quinoxaline (DiMeIQx) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) (Schut and Snyderwine,1999).

The mutagens namely , 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) , 2-amino 3,4 dimethylimidazo[4,5*f*]quinoline (MeIQ) , 2-amino 3,8 dimethylimidazole [4,5*f*] quinoxaline (MeIQx) , 2-amino 3,4,8 trimethylimidazo [4,5-*f*] quinoxaline (DiMeIQx) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) were detected in food grade beef extract. The amounts of IQ type compounds were quantified to 70 ng/ gm while 2-amino-1-methyl-6-phenylimidazo [4, 5-*b*] pyridine (PhIP) was 3.6 ng/ gm. However, it should be keep in mind that beef extracts are always diluted before consumption (Skog, 1993).

All HCAs, IQ and non-IQ type , are mutagenic toward *Salmonella typhimurium*, as shown in Table 2.5 (Nagao , 2000). *Salmonella typhimurium* TA98, detecting frameshift-type mutagens, shows more susceptibility to HCAs than TA100, which detects base-pair change type mutagens. The specific mutagennicities of HCAs toward mammalian cell lines using the *Hprt* gene or the *Ef*-2 gene as a reporter were almost the same range among various HCAs as in *Salmonella typhimurium* (Terada *et al*, 1986; Thompson *et al*, 1987).

Table 2.5 Mutagenicity of HCAs in *Salmonella typhimurium* TA98 and TA100 with S9 mix (Nagao, 2000).

HCA	Revertants / μg	
	TA98	TA100
MeIQ	661,000	30,00
IQ	433,000	7,000
DiMeIQx	183,000	8,000
7,8-DiMeIQx	163,000	9,900
MeIQx	145,000	14,000
Trp-P-2	104,200	1,800
4-CH_2OH-8-MeIQx	99,000	3,000
IQx	75,400	1,500
Glu-P-1	49,000	3,200
Trp-P-1	39,000	1,700
Glu-P-2	1,900	1,200
PhIP	1,800	120
AαC	300	20
MeAαC	200	120
4'-hydroxy-PhIP	2	No data available

The major pathway of Aminoimidazoazaarenes (AIA) activation involves phase I hepatic cytochrome P450 (CYP1A2) mediated *N*-hydroxylation followed by phase II esterification of the *N*-hydroxylamines to reactive ester derivatives that are transient metabolites that react with nucleophilic sites in DNA. The ester moieties serve as leaving groups giving rise to putative electrophilic arylnitrenium ion intermediates (Figure 2.3) considered for many years to be involved in arylamine-DNA adduct formation. Although to a much lesser extent, arylnitrenium ions may also be generated directly from the *N*-hydroxylamine metabolite following the protonation of the *N*-hydroxylamino group. This reaction mechanism explains the DNA adduction of certain *N*-hydroxy-AIAs, such as *N*-hydroxy-IQ, in the absence of the esterification. The arylnitrenium ion is generally

considered to be the ultimate carcinogenic form of the Aminoimidazoaarenes (AIAs) responsible for the formation of AIA-DNA adducts. The reactivity of the arylnitrenium ions with particular nucleophilic sites on DNA give rise to specific DNA adducts, the major adduct being formed at the C8 position of guanine. Two AIAs, IQ and MeIQx, also from minor adducts at the N2 position of guanine (Snyderwine *et al*, 1988). A growing body of literature has reported on the mutation spectra induced by AIA-guanine adducts. Studies of animal tumors induce by AIAs have begun to relate AIA-DNA adduct induced mutagenic events with the mutations found in critical genes associated with oncogenesis (Sugimura, 1992; Schut and Snyderwine, 1999).

Several investigators have proposed that non-enzymatic browning (Maillard reaction) had an important role in the formation of mutagens, without specifying a reaction route (Powri, Wu, and Stich, 1982; Shibamoto, Nishimura and Mihara, 1981; Wei, Kitamura, and Shibamoto, 1981). The Maillard reaction takes place in foods through the reaction of carbonyl compounds (aldehydes and ketones), notably reducing sugar, such as glucose, fructose , *etc.* with compounds possessing free amino groups, such as amino acids, peptides and proteins. The reaction is of great importance for the development of flavors, texture, and brown pigments during heat treatment of foodstuffs, thereby contributing to the palatability of cooked foods.

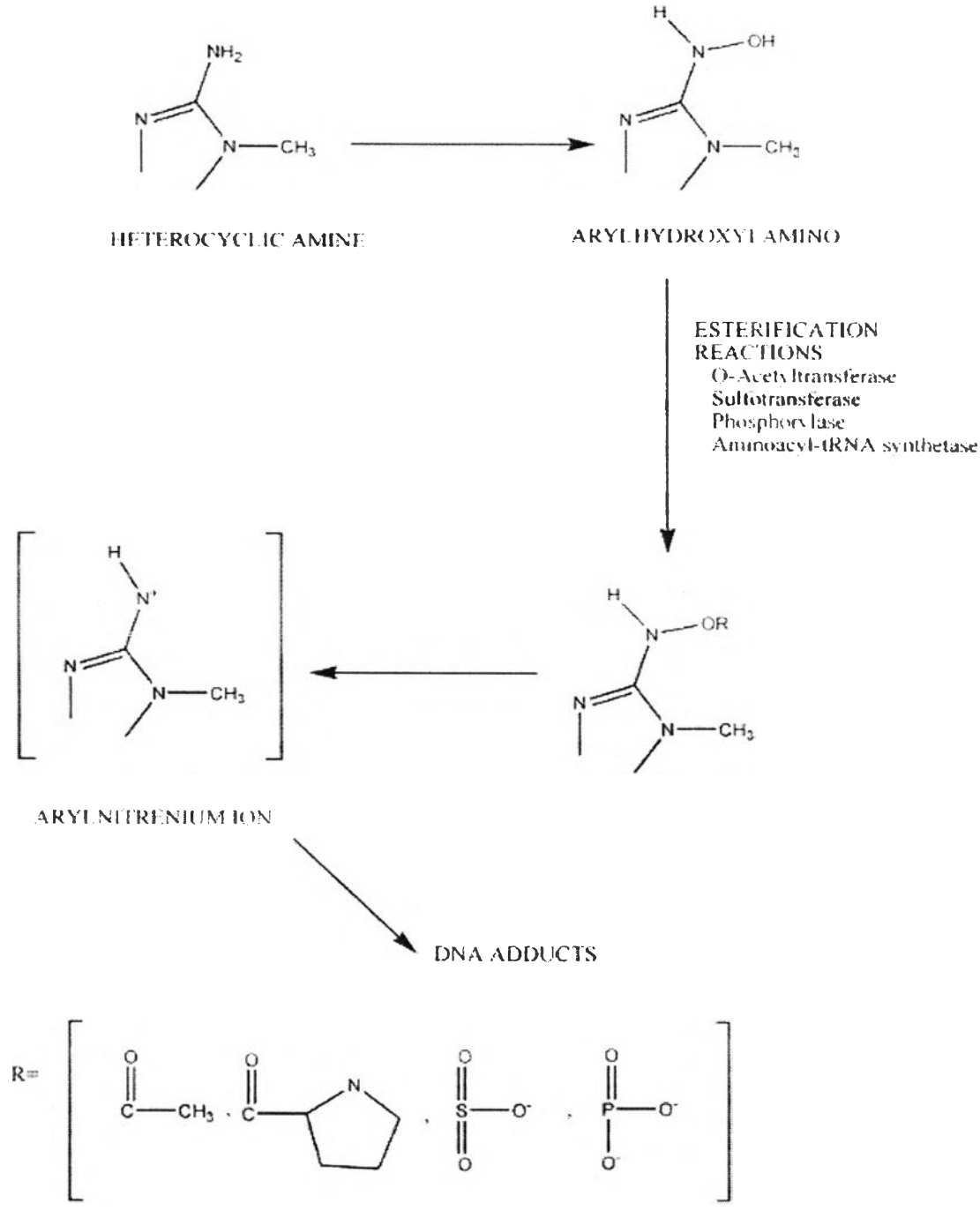

Figure 2.3 Bioactivation of heterocyclic amines. Pathway of Aminoimidazoazaarenes metabolic activation leading to DNA adduct formation (Herman *et al,* 1999)

Promutagens are formed in meat subjected to moderate cooking conditions and in commercially produced beef extract. Takahashi *et al* (1985) found 2-amino-3, 8-dimethylimidazo [4, 5-*f*] quinoxaline (MeiQx) in food–grade beef extract. The beef extracts requires metabolic activation for mutagenic expression (Munzner, 1986). The

products formed in the reaction between beef extract and nitrite under acid conditions showed direct-acting mutagenic response on the *Salmonella typhimurium* strains TA98, TA100 and TA1538 (Munzner and Wever , 1984).

IQ (2-amino-3-methylimidazo [4, 5-*f*]quinoline) was converted to 3-methyl-2-nitroimidazo[4,5-*f*]quinoline and expressed its mutagenicity towards *salmonella typhimurium* strains without activating system after 50 mM nitrite treatment at pH 3.0 (Sasagawa, Muramatsu and Mutsushima, 1988). Treatment of IQ with a much lower amount of nitrite (2 mM) produced no effect (Tsuda *et al*, 1985). The reaction of nitrite under mildly acidic conditions may have two aspects: one is the production of mutagens and the other is the destruction of mutagens (Tsuda *et al*, 1985). This result suggested that the non-enzymatic formation of direct-acting mutagens from indirect-acting mutagens such as IQ and MeIQ might be physiologically important, especially with regard to the etiology of human gastrointestinal tract tumors (Lin *et al*, 1992). IQ and MeIQ are very reactive to nitrite and the mutagenicity of nitrosated MeIQ and IQ is even higher than that of the parent compounds.

2.8 The *Salmonella* Mutagenic Assay (Ames test)

Bacterial mutagenicity assays, especially the Ames test (*Salmonella typhimurium his* reversion assay), have been used worldwide, in research laboratories. Their application is motivated by several goals; the identification of genotoxic hazards; the quantitation and regulation of health risks resulting from environment chemical exposures; and the elucidation of the biochemical mechanisms of mutagenesis. The potential of this method for used as a bioassay for the development of safe , useful chemicals raised many questions about the extent to which this kind of approach should be used in a program aimed at cancer prevention

The *Salmonella* histidine reverse mutation assay is based on the use of several selected histidine dependence (auxotrophy) to histidine independence (prototrophy) at an increased frequency in the presence of a mutagen. The test detects a wide variety of mutagens, including many that require an exogenous metabolic activation system. The test is used as a screen for mutagenic activity of pure compounds, complex mixtures,

and body fluids. At present the most commonly used *Salmonella* strains are TA1535, TA1537, TA1538, TA98 and TA100. The number and type of strains used depend upon the availability and type of sample, the focus of the study, and previous knowledge concerning the test material. In addition to having a mutation that impart other specific characteristics to the tester strain, one mutation (*rfa*) leads to a defective lipopolysaccharide coat ; another is a deletion of genes involved in the synthesis of the vitamin biotin (*bio*) and in the excision repair of DNA damage (*uvr* B). The *rfa* mutation increases the permeability of the strains to large molecules, thereby increasing the mutagenicity and/or toxic effects of these chemicals. The *uvr* B mutation leads to a reduced level of error-free repair of some types of DNA damage and thereby enhances the strains sensitivity to certain chemical and physical mutagens. Strain TA100 is derived from TA1535 by the introduction of the plasmid pKM101, which increases the sensitivity of mutagen detection by enhancing error-prone DNA repair. The presence of this plasmid makes TA100 respond to some frameshift mutagens as well as base-pair substitution mutagens, strain TA98 is derived from TA1538 by the introduction of plasmid pKM101. All tester strains should be maintained and stored according to published methods (Ames, Biek and Spudich, 1978; Maron and Ames, 1983). They should be analyzed on a frequent and rational basis for each characteristic that could affect the test. For example, strain identification could include the following: histidine and biotin requirement, UV sensitivity (presence of the *uvr* B deletion), crystal violet sensitivity (presence of the *rfa* mutation), ampicillin and /or tetracycline resistance (presence of the appropriate plasmid), spontaneous reversion frequency, and reversion characteristics to various positive controls.

Three of the most important his alleles found in the Ames tester strains (Hartman *et al*, 1986) are listed below, along with typical strains bearing the allele; the nature of the mutation in the target gene; and the most common pathway for its reversion:

- *hisD3052* ; TA1538 , TA98 : -1 frameshift ; ΔGpC frameshift in $(GC)_4$ run
- *hisG46* ; TA1535 , TA100 : missense ; base-substition at G:C base-pair
- *hisG428* ; TA102 , TA104 , TA2659 : ochre ; base-substitution at A:T base pair

Each Ames test strain evaluates mutagenic activity at a specific (reversion) target sequence. In the case of the frameshift allele *hisD3052* revertants bearing many different sequence changes (spanning a region of more than 50bp) can be recovered: of course, each such event restores the correct reading frame. Multiple classes of revertants of the base-substitution alleles can also recovered, including transitions, transversion, and some extragenenic suppressor mutations.

McCann and Ames (1977) discussed several aspects of the experimental basis for their current assessment of the value of the test as useful predictive tools:

1. The predictive value of the test as an indicator of carcinogenic potential, including both the strengths and weaknesses of the test at this stage in its development.

2. Current applications of the test method to problems that were not approachable using conventional animal test methods.

3. Some of the environmental chemicals that have already been pinpointed as potential carcinogens by the test and the current status of carcinogenicity tests of these chemicals in animals.

4. The evidence that the correlation between carcinogenicity and mutagenicity in the *Salmonella* test reflected more than a useful coincidence and fitted into a compelling collection of evidence supporting a central role for somatic mutation in the initiation of human cancer.

2.9 The mutagenicity Test (preincubation Method) Using *Salmonella typhimurium*

Some mutagen, such as, dimethylnitrosamine and diethylnitrosamine are poorly detected in the standard plate incorporation assay and should be tested using a modification of the standard procedure. The most widely used test modification is the preincubation assay first described by Yahagi (1975), in which carcinogenic azo dyes were found to be mutagenic. They incubated the mutagen and bacteria for 20-30min at 37°C and then added the top agar. The assay has been also used to detect the mutagenicity of 10 carcinogenic nitrosamines (Yahagi, Nagao, Matsushima, 1977) and several carcinogenic alkaloids (Yamanaka, 1979). The mutagenic activity of aflatoxin B1,

benzidine , benzo[a]pyrene and methylmethane sulfonate has been determined using both plate incorporation and preincubation procedures and in all cases the preincubation assay is of equal or greater sensitivity than the plate incorporation assay (Matsushima *et al* , 1980). The increased activity is attributed to the fact that the test compound and bacteria are incubated at higher concentration in the preincubation assay than in the standard plate incorporation test (Prival, King and Sheldon, 1979).

The preincubation modification can be used routinely or when inconclusive results are obtained in the standard plate incorporation assay. Nevertheless, many laboratories use it routinely because of the increased sensitivity towards some compounds. Its use in screening assays has been recommended by De Serres and Shelby (1979).