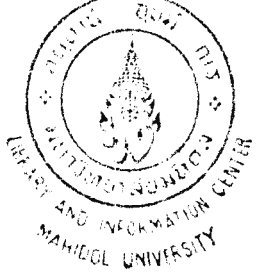

MUTAGENICITY OF 4-HEXYLRESORCINOL AND ITS MODIFICATION EFFECTS ON OTHER MUTAGENS IN TWO SHORT TERM ASSAYS

SUPHAPHAN SOIPHET

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Shrimp blackspot is an objectionable surface discoloration caused by enzymatic polyphenol oxidase formation of the precursors of insoluble polymeric pigments. It remains active during refrigeration, ice storage, and postfreeze thawing. A problem in virtually all commercial shrimp species, blackspot has a negative impact upon the commercial value and consumer acceptance of the shrimp product. Therefore, it has to be examined for quality and safety before exporting. Previous studies demonstrate that 4-hexylresorcinol presents no risk of toxicity at the levels proposed for treatment of shrimp, and the use of 4-hexylresorcinol as a processing aid to prevent melanosis in shrimp is GRAS. The main objective of this study was to determine the antimutagenicity of 4-hexylresorcinol. The methods used were two separate tests namely SMART and Ames. The former test was conducted to determine the modifying effects on both *in vivo* induction of mutation and mitotic recombination in somatic cells of *Drosophila melanogaster* (SMART assay). The second test was conducted to determine the modifying effects on the mutagen formed during aminopyrene-nitrite reaction mixture and on the final product of 4 h incubation of aminopyrene-nitrite reaction mixture using Ames assay with *Salmonella typhimurium* strains TA98 and TA100 in the absence of metabolic activation. The results showed that 4-hexylresorcinol was not mutagenic in both SMART and Ames tests. Co-administration of 4-hexylresorcinol with urethane to the larvae reduced the frequency of induced wing spots of the flies compared with the group fed urethane. 4-hexylresorcinol pretreatment did not change the frequency of mutant spots. However, the reduction of wing spot formation in 4-hexylresorcinol pretreatment group was less than that obtained from the study on simultaneous feeding of urethane with 4-hexylresorcinol. *In vitro* working on the Ames test, it was found that 4-hexylresorcinol was antimutagenic towards the product of AP-nitrite reaction at the higher doses and could modurate the formation of the mutagenic product. It is, thus, concluded that this compound is neither a direct nor indirect mutagen. Antimutagenicity effect of 4-hexylresorcinol may be due to the induction of glutathione-S-transferase activity or the increasing amount of glutathione in phase II detoxifying system as well as inhibition of the catalytic activities of cytochrome P-450 system of phase I. The lowest dose of 4-hexylresorcinol increased number of revertants of the final reaction product when it was added along with AP and nitrite. The increase may due to the stimulation on mutagen formation during acid incubation via the mechanism of C-nitroso formation. Whereas, 4-hexylresorcinol may act as a nitrite scavenger at the higher doses.

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สุภาพรรณ สร้อยเพ็ชร : การทดสอบฤทธิ์ก่อกลายพันธุ์ของสาร 4-HEXYLRESORCINOL และผลการเปลี่ยนแปลงของสารนี้ต่อสารก่อกลายพันธุ์ต่างๆ โดยวิธี SHORT TERM TEST 2 แบบ(MUTAGENICITY OF 4-HEXYLRESOLCINOL AND ITS MODIFICATION EFFECTS ON OTHER MUTAGENS IN TWO SHORT TERM ASSAYS). คณะกรรมการควบคุมการสอบวิทยานิพนธ์: แก้ว กังสดาลอำไพ, Ph.D., อาณาดี นิติธรรมยง, Ph.D., ครุณี บุรีภักดี ลอว์สัน, Ph.D., 132 หน้า. ISBN 974-663-699-5

จุดดำที่เปลือกกุ้งเป็นจุดที่เกิดจากการเปลี่ยนแปลงสีผิวของกุ้ง โดยมีสาเหตุจากเอ็นไซมโพลีฟีนอลออกซิเดสเป็นตัวตั้งต้นของการเกิดเม็ดสี โดยจะทำปฏิกิริยากับสิ่งที่ยังเหลืออยู่ระหว่างการแช่เย็น, การเก็บแช่ในน้ำแข็ง และหลังจากการถูกทำให้ละลาย ซึ่งก่อให้เกิดปัญหาต่อการค้ากุ้ง คือจุดดำมีผลกระทบด้านลบต่อราคาและการยอมรับผลิตภัณฑ์ของผู้บริโภค จึงต้องมีการควบคุมคุณภาพและความปลอดภัยก่อนการส่งออก จากการศึกษาที่ผ่านมาพบว่าสาร 4-HEXYLRESORCINOL (4-HR) ไม่ได้แสดงความเป็นพิษในปริมาณสารที่ใส่ในกุ้ง และปริมาณที่เติมลงในขบวนการผลิตเพื่อป้องกันการเกิดจุดดำในกุ้ง โดยปริมาณที่เติมเป็นที่ยอมรับว่าไม่เป็นอันตราย (GRAS) งานวิจัยนี้ต้องการศึกษาถึงฤทธิ์ด้านการก่อกลายพันธุ์ของสาร 4-HR ซึ่งทำการทดลอง 2วิธี คือSMART และ Ames test ในการทดลองแบบแรกต้องการศึกษาถึงผลการเปลี่ยนแปลงการเหนี่ยวนำการก่อกลายพันธุ์และการรีคอมบิเนชันในโซมาติกเซลล์ในแมลงหวี่ *Drosophila melanogaster* (วิธี SMART) ซึ่งจะทำการทดลองโดยให้ได้รับสารพร้อมกันและโดยการให้สาร4-HRก่อนในช่วงแรก (PRETREATMENT) รวมถึงการทดลองแบบที่สองต้องการศึกษาว่าสารนี้จะมีผลต่อการเปลี่ยนแปลงต่อฤทธิ์ก่อกลายพันธุ์ที่เกิดจากการทำปฏิกิริยาของสารอะมิโนพัยรีนกับเกลือไซเดียมไนไตรทโดยตรง และต่อผลิตภัณฑ์ขั้นสุดท้ายที่ได้จากอะมิโนพัยรีนที่ทำปฏิกิริยากับไนไตรทไปแล้ว 4 ชั่วโมง โดยใช้ *Salmonella typhimurium* สายพันธุ์ TA98 และ TA100 ในวิธี Ames test ที่ไม่มีระบบกระตุ้นสารพิษ ผลการทดลองสรุปได้ว่า สาร 4-HR ไม่แสดงฤทธิ์ก่อกลายพันธุ์ในการทดลองทั้งในวิธี SMART และ Ames test การทดสอบโดยให้สารนี้ร่วมกับสาร URETHANE ในช่วงที่เป็นหนอน พบว่าลดความถี่ของขนที่ผิดปกติ (MUTANT SPOTS) ในแมลงหวี่เมื่อเปรียบเทียบกับกลุ่มที่ได้รับเฉพาะสารURETHANE และการทดสอบให้ได้รับสาร4-HR PRETREATMENT พบว่าสารนี้ไม่ได้ไปเพิ่มความถี่ของขนที่ผิดปกติ แต่อย่างไรก็ตามการลดลงการเกิดขนที่ผิดปกติจากการให้สาร 4-HR PRETREATMENT พบว่าลดได้น้อยกว่าในการทดลองที่ให้ได้รับสารพร้อมกันของ URETHANE กับสาร 4-HR ส่วนผลการทดลองโดยวิธี Ames test พบว่าที่ความเข้มข้นสูงขึ้น(10μg/plate) สาร4-HRมีฤทธิ์ต้านการก่อกลายพันธุ์ของผลิตภัณฑ์ขั้นสุดท้ายของอะมิโนพัยรีนที่ทำปฏิกิริยากับไนไตรท และยังพบว่ามีผลลดการเกิดการก่อกลายพันธุ์ของสารก่อกลายพันธุ์ชนิดตรงด้วยดังนั้นจึงสรุปได้ว่าสาร4-HR ไม่ได้มีฤทธิ์ก่อกลายพันธุ์ทั้งชนิดตรงและชนิดอ้อม ส่วนฤทธิ์ต้านการก่อกลายพันธุ์ของสารนี้อาจเกิดเนื่องจากการกระตุ้นเอ็นไซมกลูทาไทโอน-*S*-ทรานส์เฟอเรส หรือโดยการเพิ่มปริมาณกลูทาไทโอนที่ระบบการทำลายสารพิษในวัฏภาคที่2 และไปยับยั้งการทำงานของระบบไซโตโครมพี-450ในวัฏภาคที่1 และพบว่าที่ความเข้มข้นต่ำสุดของสาร4-HRจะเพิ่มฤทธิ์ก่อกลายพันธุ์ของแบคทีเรียของการเกิดปฏิกิริยาของสารอะมิโนพัยรีนกับเกลือไนไตรท ซึ่งการเพิ่มนี้อาจเนื่องจากการกระตุ้นการเกิดสารก่อกลายพันธุ์ที่อยู่ในสภาวะที่เป็นกรดเลียนแบบกระเพาะอาหาร โดยผ่านกระบวนการเกิด C-nitroso ขณะเดียวกันสาร 4-HEXYLRESORCINOL อาจที่จะทำปฏิกิริยาโดยการจับไนไตรทไว้ที่ความเข้มข้นสูงขึ้น

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LIST OF ABBREVIATIONS

AP	Aminopyrene
°C	Degree celsius
µg	Microgram
µl	Microlitre
mg	Milligram
g	Gram
No.	Number
h	Hour
mg	Miligram
M	Molar
min	Minute
ml	Mililitre
S.typhimurium	*Salmonella typhimurium*
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
GSH	Glutathione
GST	Glutathione-*S*-transferase
Trp-p-2	3-Amino-1-methyl-5*H*-pyridol [4,3-*b*] indole

CHAPTER I

INTRODUCTION

Stomach cancer, while declining in most populations in recent years, still remains the most common cancer in both sexes on a worldwide basis (1-4). Most researchers suspect that certain components of diet will ultimately be identified as the major causal factors in this cancer (2,3,5-8). The source of hazard can be divided into four categories. Firstly, certain food additives may have harmful effect (9). Secondly, foodstuffs may be contaminated either by environmental pollutants or by microbial toxins (10). Thirdly, the processing of food (e.g. cooking, broiling, smoking, pickling, etc.) may produce carcinogenic compounds (11). Finally, certain natural constituents of foods are known to possess mutagenic and/or carcinogenic potential (12).

A variety of food additives are imposed on mankind daily, intentionally, of which many may represent carcinogenic hazards (13). From a regulatory standpoint, each of the food additive must provide some useful and acceptable function or attribute to justify its usage. Generally, improving keeping quality, enhancing nutritional value, functional property provision and improvement, processing facilitation, and enhancing consumer acceptance are considered acceptable functions for food additives (14). Only carefully selected chemicals can prevent contamination and spoilage of food that has to be produced in great quantities, stored, and transported. Chemicals are also used for flavoring and appearance (15). Some, such as nutrients, vitamins and minerals, are of

obvious benefit to man, whereas others may be quite hazardous (13). For instance, nitrite is an important food additive which has been used as meat preservative to prevent botulism; it also adds color to certain meats, especially bacon and sausages. Nitrite salts can react with other compounds to form potent carcinogens called nitrosamines (15).

In later years the recognition of chemical, which function as antimutagens and anticarcinogens by inhibitory action at different level of mutagenesis and tumor induction has lead to a rapidly expanding research field-chemoprevention of mutation and cancer. There is a wide array of chemicals, which have been shown to function as antimutagens and anticarcinogens, they may occurring as natural components in our food, and others made synthetically (16). Several antioxidants are widely used as preservative for both industrial products and foodstuffs. For example, vitamin E is an important intracellular antioxidant and theoretically could inhibit carcinogenesis though decreased lipid peroxidation and/or increased detoxication of carcinogenic compounds (17-20), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and propylgallate (PG), which are synthetic phenolic antioxidants currently approved for used in dried cereals, cooking oils, canned goods, and various animal feed. They have shown a wide spectrum of anticarcinogenicity (13,14,16,21).

Like other developing countries, Thailand has been increasingly exposed to chemicals. The demand for increasing the productivity in agriculture and the expansion of industry are major causes of rapid increase in the use of chemicals that they have result to positive economic of country (22). 4-Hexylresorcinol is a food additive and is an effective inhibitor of shrimp melanosis or "blackspot" in all species tested. Shrimp

blackspot is an objectionable surface discoloration caused by enzymatic formation of the precursors of insoluble polymeric pigments. The enzyme which catalyzes this reaction, polyphenol oxidase, remains active during refrigeration, ice storage, and postfreeze thawing. A problem in virtually all commercial shrimp species, blackspot has a negative impact upon the commercial value and consumer acceptance of the shrimp product (23). At present, frozen seafood products are very significant exporting goods for Thailand. Frozen shrimp is one of the most valued products, 40-60% of income from frozen seafood are generated from it. However, there are competitors in the global market. The problem is the quality of product to meet the customer satisfaction and compete with other countries. To reduce all problems of rejection from the consumer countries, the quality of product needs to be improved. Therefore, it has to be examined on quality and safety before exporting (24). The studies, along with the aforementioned data, demonstrate that 4-hexylresorcinol presents no risk of toxicity at the levels proposed for treatment of shrimp, and the use of 4-hexylresorcinol as a processing aid to prevent melanosis in shrimp is GRAS (25). However, it is of great interest to investigate whether co-administration of 4-hexylresorcinol along with genotoxins could also lead to *in vivo* modifying effects and determine the antimutagenicity of 4-hexylresorcinol, nitrosated product as well as effects on mutagens occurring during stomach digestion using 4 h aminopyrene-nitrite reaction as a model and their effect on the mutagenicity of aminopyrene-nitrite product were investigated by *in vitro* assay.

CHAPTER II

LITERATURE REVIEW

2.1 Antimutagenesis and Cancer Prevention

It has been estimated that about one third of all human cancer may relate to the diet. Although it would theoretically be possible to avoid exposure to all dietary carcinogens, it is becoming increasingly clear that these are many and varied, and that complete avoidance would be difficult (26). In later years the recognition of chemicals, which function as antimutagens and anticarcinogens (anti-M/C) by inhibitory action at different levels of mutagenesis and tumor induction has lead to a rapidly expanding research field-chemoprevention of mutations and cancer (16). Chemoprevention of cancer is a mean of cancer control in which the occurrence of this disease is prevented by administration of one or several chemical compounds (27). There is a wide array of chemicals, which have been shown to function as anti-M/C, many of them occurring as natural components in our food, and others made synthetically (16,18). Some are known to have important roles in the preservation of human health, whereas other such as fiber or flavonoids, which were considered for a long time to be "inert" components of foods, have not received much attention with regard to their subtle effects on human health. Compounds such as vitamins A,B,C or E, already known to possess distinctive physiological and pharmacological activities, until recently received only scant attention for additional roles as chemopreventers (CP) (18). Morse and Stoner suggested that a CP agent should have 5 qualities, which

they distinguished as: (i) little or no untoward side effects; (ii) high efficacy; (iii) capability of oral administration; (iv) a known mechanism of action and (v) low cost. Many antimutagens may fulfil these criteria (34). The most thoroughly investigated CP in foods are fibre, polyphenolic compounds, flavonoids (quercetin, ellagic acid, chlorogenic acid), conjugated isomers of linoleic acid, *d*-limonene, epigallocatechin gallate, soybean proteins, isoflavones, vitamins (A, B, C, E), tocopherols, calcium, selenium, chlorophylline, aliphatic sulfides, catechin, tetrahydrocurcumin, sesaminol, glutathione, coumarins, uric acid, indoles, thiocyanates, and protease inhibitors. Over 25 different classes of chemicals have been found to possess anti-M/C capacities. Chemopreventers are found in all categories of foods, fruits and vegetables being the main source as illustrated in Table1. The amount of CP in different categories of food can, however, vary considerably. Even the same type of food products, obtained from different regions, may sometimes contain different levels of a particular CP (18). Wattenberg therefore suggested that " the composition of the diet could be an important factor in determining the response of individuals to carcinogenic agents to which they were exposed" (28). Many micro nonnutrients in the diet were isolated and identified and were demonstrated to block different stages of the carcinogenic process in several animal models. Chemicals that are able to prevent the formation of carcinogens from precursor substances or to prevent carcinogens from reaching with critical target DNA sites in the tissues are "blocking agents". Chemicals that act by suppressing the expressing of neoplasia in cell previously exposed to doses of a carcinogenic agent, are "suppressing agents" (27,29,30).

There are numerous examples of antimutagenic/anticarcinogenic substances derived from nonnutrients. According to the terminology of Kada *et al.*, (31),

desmutagens are antimutagenic agents acting outside the cell by acting directly on mutagens or their precursors and inactivating them while bio-antimutagens are

Table 1. Categories of foods with the most prominent chemopreventer[a]

Type of food	Chemopreventers
Fruits	Vitamins, flavonoids, polyphenolic acids, fibre, carotenes, monoterpenoids (*d*-limonene)
Vegetables	Vitamins, flavonoids, plant phenolics, chlorophyll, fibre, aliphatic sulfides, carotenes, aromatic isothiocyanates, dithiolthiones, phytic acid, calcium
Cereals	Fibre, α-tocopherol, phytic acid, selenium
Meats, fish, egg, 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, fibre, vitamin E, phytic acid, coumarins, proteins
Spices	Coumarins, curcumin, sesaminol
Tea	Plant phenolics, epigallocatechin
Coffee	Polyphenolics acids, diterpene alcohol esters, melanoidins
Wine	Flavonoids
Water	Selenium

[a] From Wattenberg (28).

antimutagenic agents acting inside the cell by interfering on the process of mutagenesis or repairing of damaged DNA, thereby resulting in decreasing mutation.It is now clear that many antimutagens function as antioxidants; primary examples of antimutagens that may function in this manner include vitamins C and E. It is also clear that a number of antimutagens function as interceptors/desmutagens that intercept, bind, or physically destroy mutagens and carcinogens. These substances were extensively reviewed by Hartman and Shankel (32). It is 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. The mechanisms by which antimutagens/anticarcinogens may act were summarized in an extensive review by DeFlora and Ramel (33).

2.2 Anticarcinogenic and Antimutagenesis Mechanism

The anti-M/C mechanisms of CP appear to be complex and, in some cases, established. The beneficial activity of CP depends on many unrelated factors and conditions. This effect can be the result of a single event or the simultaneous action of several factors acting in concert. In a very arbitrary way, the mechanisms of action of the CP can be separated into two main categories and several subgroups according to the site or ambient at which they exert their influence, as illustrated in Table 2. Any of these actions, either alone or in combination, will reduce the hazardous activity of the mutagens and/or carcinogens species in the body.

Even if the inhibitory agents or steps are identified, the molecular mechanism underlying the inhibition is often not explained. This should not come as a surprise, since the science of chemoprevention is a relatively new area of research.

Although the mechanisms appear to be very heterogeneous, the antioxidative characteristics of CP seem to play the most significant part in their protective activity (18).

Table 2. Mechanisms of action of chemopreventers [a]

EXTRACELLULARLY
During the preparation of foods, by:
• reducing (inhibition) the formation of M/C
Effects in the intestine, by:
• formation of non-M/C complexs
• reducing bioavilability
• diluting with dietary fibres
• increasing adsorption on other food components
• accelerating intestinal transit
• protecting the mucosal barrier
• modifying intestinal microbial flora
• inhibiting the penetration of cells by M/C
INTRACELLULARLY
At cellular level, by:
• enhancing the activities of enzymes involved in detoxification of M/C
• inhibiting the activities of enzymes involved in formation of M/C metabolites
• trapping of electrophiles
• scavenging reactive oxygen species
• inhibiting metabolic activation
• protecting nucleophilic activation
• iinhibiting the detrimental effect of pro-carcinogens on DNA

M/C = mutagens and/or carcinogen

[a] From Stavric (18).

2.3 Antioxidants as Chemopreventers of Free Radicals

The role of vitamins and polyphenols, the naturally occurring antioxidants acting as CP, has been discussed above. This section reviews other types of food antioxidants involved in scavenging free radicals.

Lipid peroxidation can produce free radicals. Their formation may result in destabilization and disintegration of cell membranes, leading to liver injury, aging and greater susceptibility to cancer (34). Free radicals are highly reactive chemical species in the constant process of combining with other molecules or structures within the cells, including DNA. Cells possess a number of enzymatic systems that can repair the damage, with the aim of protecting themselves against damage caused by free radical. In addition to the enzymatic systems in the cell, antioxidants in foods have very important roles in preventing the formation of free radicals by way of non-enzymatic protective processes. Any agent, therefore, that scavenges free radicals directly, irrespective of mechanisms, or that interferes with the generation of events mediated by free radicals, presumably inhibits the neoplastic processes (35,36). It appears that dietary antioxidants are the most significant external factors to have a substantial role as CPs (37).

Selenium intake (in the form of selenite, selenate, selenomethionine, ect.) was reported as having a possible protective effect against cancer. The chemopreventive action of selenium is probably attributable to its antioxidatant properties and its involvement with the enzyme glutathione peroxidase (20,38). Several studies found that microgram quantities of dietary selenium can effectively reduce skin, colon, liver and mammary cancer in animals (39). However, some prospective epidemiological studies could not confirm an association between serum selenium concentration and

the prospective epidemiological studies could not confirm an association between serum selenium concentration and the prospective risk of cancer (40).

Chlorophylline (the sodium and copper salt of chlorophyll) possesses the ability to inhibit the mutagenic activity of a variety of complex environmental mixtures, including food mutagens formed during the frying of meat (41). It appears that chlorophylline, as a natural antioxidant, either acts as a scavenger of free radicals or interacts with the active part of mutagenic compounds. The antimutagenic activity of certain vegetable extracts has been correlated with their chlorophylline content (42). Chlorophylline was also active as an anticarcinogenic agent in rainbow trout challenged with aflatoxin B_1, 2-amino-3-methylimidazo[4,5-*f*]-quinoline(IQ) or 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]-indole (Trp-P-2) and also antimutagenic in the Ames Salmonella test against the same xenobiotics (18).

Considerable efforts are being directed at identifying new antioxidative (preferably heat-stable) factors in foods and spices. As a result, β-diketones from eucalyptus leaf, sesaminol from sesame seeds, tetrahydrocurcumin derived from curcumin (in curry cooking) and others were found to be food antioxidants of a novel lignin type (18,34). Curcumin a potent antioxidant and the active principle of turmeric, is consumed daily in considerable amounts in India and other Asian countries, and because of its antimutagenic effects against environmental mutagens [tobacco extracts, B[*a*]P, bidi (a type of Indian cigarette, generally smoked by those on low incomes) and cigarette smoke condensate], may afford protection against some environmental and dietary M/C in India (43).

Glutathione is present in food and is one of the major antioxidants and antimutagens in the soluble fraction of cells. The glutathione transferases (some of

which have peroxidase activity) are major defenses against oxidative and alkylating carcinogens. The concentration of glutathione may be influenced by dietary sulfur amino acids. *N*-acetylcysteine, a source of cysteine, raises glutathione concentrations and reduces the oxidative cardiotoxicity of adriamycin and the skin reaction to radiation. Glutathione concentrations are raised even more efficiently by L-2-oxothiazolidine-4-carboxylate, which is an effective antagonist of acetaminophen is thought to be toxic through radical and quinone oxidizing metabolites. Dietary glutathione may be an effective anticarcinogen against aflatoxin (20).

Previous studies shown that unprocessed palm oil had antitumorigenic effects. It appears that the non-saponifiable matter of palm oil, which is very rich in tocotrienols and possesses antioxidative capacity, is the reason for the chemopreventive properties of palm oil (44). In addition to the already mentioned beneficial effect of vitamin C in prevention of the formation of nitrosamines, other food-borne antioxidants such as vitamin C, tocopherols, polyphenols and sulfur-containing compounds, also inhibit the formation of nitrosamines (45).

2.4 Inhibition of Carcinogenesis by Specific Nonnutrient Constituents of the Diet

Foods contain major and minor constituents. The major ones are protein, fat, carbohydrate, and fiber. The minor ones are nutrients such as vitamins and a large number of nonnutrients, *i.e.*, compounds with no known nutritional value.

The mechanisms of action of many of the minor nonnutrient inhibitors of carcinogenesis are poorly understood, making it difficult to organize them into a precise pattern. One organizational framework is classification of inhibitors by the

time in carcinogenesis when they are effective. In this framework, inhibitors can be divided into three categories. The first consists of compounds that prevent the formation of carcinogens from precursor substances. The second contains compounds that inhibit carcinogenesis by preventing carcinogenic agents from reaching or reacting with critical target sites in the tissues. These inhibitors are called "blocking agents", which describes their mechanism of action (they exert a barrier function). Inhibitors of the third category act subsequent to exposures to carcinogenic agents. These are termed "suppressing agents" because they act by suppressing the expression of neoplasia in cells previously exposed to doses of carcinogens that otherwise would cause cancer (46).

2.4.1 Blocking Agents

Blocking agents prevent carcinogens from reaching or reacting with critical target sites by several mechanisms. One mechanism is inhibiting of reactions requiring metabolic activation. A second entails inducing of activities of enzyme systems that detoxify carcinogens. A third is the capacity to act by scavenging the reactive from of carcinogens. Some blocking agents prevent the action of tumor promoters by preventing the promoter from reaching or reacting with its cellular target or by blocking subsequent cellular events required for tumor promotion occur (29). A list of nonnutrient blocking agents is shown in Table 3.

2.4.1.1 Blocking Agents That Act by Inhibiting Carcinogen Activation.

Several groups of nonnutrient compounds prevent carcinogenesis by inhibiting carcinogen activation reactions. Almost all carcinogens occurring in food require metabolic activation. Examples of such carcinogens are include aflatoxin, nitrosamines, polycyclic aromatic hydrocarbons, hydrazines, and heterocyclic amines.

Table 3. Some nonnutrient blocking agents in food[a]

Organosulfides	Flavone	Coumarins
Conjugated dienoic linoleic acids	Phenol	Glucarates
Aromatic isothiocyanates	Tannin	Nucleophiles
Indoles	Ellagic acid	β-Carotene
Dithilethiones	Curcumin	18-β-Glycyrrhetinic acid

[a] From Wattenberg (29)

Thus, blocking agents inhibiting activation of these and other carcinogens may play a role in preventing neoplasia resulting from this type of exposure. Benzyl isothiocyanate is one such naturally occurring blocking agent. Benzyl isothiocyanate, found in cruciferous vegetables, is formed by the hydrolysis of the glucosinolate glucotropaeolin. The glucosinolate is the storage from in plant material. When plants are processed or damaged, the glucosinolate undergoes hydrolysis with resultant formation of the free aromatic isothiocyanate (29). Early experiments showed that benzyl isothiocyanate inhibited DMBA-induced mammary tumor formation in rats when given by p.o. intubation 2 hours before the carcinogen (47).

Organosulfur compounds found in *Allium* species, including garlic, onions leeks and shallots (29) are a second group of naturally occurring compounds that can inhibit carcinogenesis by preventing carcinogen activation in one series of experiments, they inhibited *N*-nitrosodiethylamine-induced carcinogenesis of the forestomach and, to alesser extent, the lungs in female A/J mice (48). The most potent of the naturally

occurring organosulfur compounds was diallyl disulfide. In other studies Wargovich *et al.* (49) showed that diallyl sulfide inhibits *N*-nitrosomethylbenzylamine-induced esophageal cancer in rats when the organosulfur compound was administered p.o. 3 h before nitrosamine. In related work, Brady *et al* (50) demonstrated that diallyl sulfide inhibits microsomal metabolism of nitrosamines by rats when administered 3 h before the rats are killed.

2.4.1.2 Blocking Agents That Act by Increasing Carcinogen Detoxification.

Blocking agents can prevent the occurrence of neoplasia by increasing the detoxification of carcinogens. Two general categories of blocking agents acting by this mechanism were identified. They are designated type A and type B inhibitors (27). Type A inhibitors induce an increase in activity of phase 2 enzymes, which perform conjugation and other reactions that detoxify many carcinogenic agents. Prominent among these is the marked increase in glutathione *S*-transferase activity. UDP-glucuronosyltransferase, epoxide hydrolase, and NAD(P)H-quinone reductase activities are also enhanced. Increases in tissue glutathione levels are found (27). The most extensively studied type A inhibitor is the phenolic antioxidant, BHA. It might be anticipated that compounds inducing a coordinated detoxification response would inhibit a wide range of chemical carcinogens. This has proved to be the case for BHA. BHA inhibits carcinogenesis resulting from adminstration of BP, benzo(a)pyrene 7,8-dihydrodiol, DMBA, uracil mustard, urethan (30). Type B inhibitors enhance both phase 1 and phase 2 enzyme include the cytochrome P-450 systems and are very complex (27).

Because type A inhibitors are effective, there have been a continuing effort to identify compounds in this category of chemopreventive agents. Previous work

focused on the organosulfur compounds found in *Allium* species. In initial studies, the capacity of AMT to induce increased glutathione *S*-transferase activity in rodent tissue was investigated. AMT induces increased activity of this enzyme system when given 4 and 2 days before assay. With this administration schedule, AMT was also found to inhibit BP-induced neoplasia of the forestomach of female A/J mice. Experimental protocols with the test compound being given 4 and 2 days before carcinogen challenge are commonly used in studying the inhibitory effects of agents that act by inducing increased activity of detoxification systems (29).

2.4.1.3 Blocking Agents Effective against Tumor Promoters.

A number of naturally occurring nonnutrient inhibitors of tumor promotion have been found. A property for identifying inhibitors of this type is their capacity to prevent an increased ornithine decarboxylase activity after exposure to the promoter. This assay has been particularly effective in mouse epidermis. However, in other instances, biological response such as inhibition of increase cellular proliferation or tumor formation in response to promoter administration has been the means by which the inhibitor was identified (29).

2.4.2 Suppressing Agent

Suppressing agents prevent the evolution of the neoplastic process in cells previously exposed to doses of carcinogenic agents that otherwise would cause cancer (27). Table 4 lists minor nonnutrient constituents of the diet that can act as suppressing agents in animal models. The number of suppressing agents identified is considerably smaller than the number of blocking agents. The mechanisms by which blocking agents act are relatively simple, although the systems themselves can be very complex. The mechanism of action for suppressing agents is not well defined, as might be

expected because the events that they counteract are those basic to cancer, also a poorly understood process. Suppressing agents act during the earliest stages of the neoplastic process, when the number of pathological alterations is considerably smaller than at later stages (29). As more information becomes available concerning the multiple events leading to malignancy, targeting of suppressing agents to counteract defined early changes may be feasible. The vast amount of data indicates that alterations in genetic material occur during carcinogenesis. Correcting genetic alterations during neoplastic changes in solid tissue is difficult. Suppressing agents can modulate consequences of the genetic changes but are not likely to correct the genetic defects themselves. Accordingly, it would be anticipated that suppressing agents may have reversible effects. Consequently, it is likely that continuous administration of suppressing agents will be necessary to sustain inhibition of carcinogenesis. Retinoids, the most extensively investigated of the suppressing agents, demonstrate this characteristic both *in vitro* and *in vivo*. Suppression is maintained as long as the agent is present; on removal, the neoplastic process recurs (29).

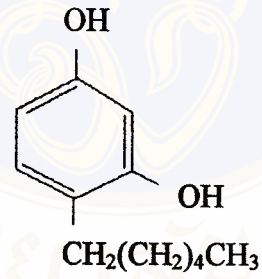
Suppressing agents have been found in individual dietary constituents. Studies with cruciferous vegetables and with orange oil demonstrated suppressing effects (30). In the initial studies with cruciferous vegetable, dehydrated powders prepared from Chieftain Savoy cabbage inhibited DMBA-induced mammary-tumor formation. In previous investigations, it was found that frozen-thawed segments of cabbage leaf placed directly in cages produced a comparable reduction of neoplasia in the animal (29).

Table 4. Some nonnutrient suppressing agents in food[a]

Protease inhibition
Inhibitors of the arachidonic acid cascade
Terpenes
Aromatic isothiocyanates
Inositol hexaphosphate
Nerolidol

[a] From wattenberg (29)

2.5 4-Hexylresorcinol



4-HEXYLRESORCINOL

CAS No. 136-77-6

$C_{12}H_{18}O_2$ Molecular weight 194.3

Synonyms: 4-hexyl-1,3-benzenediol; 4-hexyl-1,3-dihydroxybenzene

Figure 1. 4-Hexylresorcinol

4-Hexylresorcinol ($C_{12}H_{18}O_2$) is a dihydroxybenzene with a hexyl group in the 4 position and hydroxyl groups on positions 1 and 3 of the aromatic ring (25). 4-

hexylresorcinol (4-HR) is a white, microcrystalline solid. It forms needle-shaped crystals with a melting point of 67.5°-69°C . Its boiling point is 333°-335°C . The chemical has a pungent odor and a sharp astringent taste. The chemical is soluble in ether, chloroform, acetone, alcohol, and vegetable oils; it is slightly soluble in petroleum ether and is soluble in water at 1 part to 2,000 (51).

2.5.1 Historical and Proposed uses

4-HR, a phenolic compound, has a long history of use. The most widespread current use is as a topical antiseptic in throat lozenges, soaps and handwashes, and skin/wound cleaners. 4-HR was widely used for many years as an extremely effective anthelmintic, prior to recent development of more selective anthelmintics (25). Currently, 4-HR is being proposed for use as a processing aid for prevention of melanosis (black spot) in shrimp. At present, prevention of black spot in shrimp involves application of sulfites which have been prior sanctioned as a dip to control the incidence of melanosis. The problems associated with allergic reactions to sulfites, as well as other health risks associated with its use, are well known.

Shrimp melanosis, commonly known as black spot, is an objectionable surface discoloration caused by enzymatic formation of the precursors of insoluble polymeric pigments (25). The enzyme which catalyzes this reaction, polyphenol oxidase, remains active during refrigeration. Frozen storage will stop melanosis, yet accentuates black spot development when the product is thawed. Black spot is a problem in virtually all commercial shrimp species. The presence of black spot has a substantial negative impact upon the commercial value and consumer acceptance of the shrimp product, and has a direct bearing on the rating given to shrimp under current federal regulations (25). As noted, sulfiting agents were introduced in the 1950s to inhibit black spot

formation (52). Both ice boats and freezer boats employ sulfites to retard melanosis during onboard storage and subsequent transport and handling.

4-HR. is a functional sulfite alternative as it is a specific inhibitor of polyphenol oxidase, as compared to sulfite alternative as it is a specific inhibitor of polyphenol oxidase, as compared to sulfites which react chemically with black spot precursors. The functionality of 4-HR as a shrimp black spot inhibitor has been demonstrated both in laboratory tests with previously frozen shrimp and in field trials with fresh shrimp (53). The development of melanosis was evaluated by visual inspection by a trained panel and scored according to a melanosis rating system (25). Under this system, a score of 4 or greater (on a scale of 10), corresponding to a Grade B or below rating under National Marine Fisheries Service regulations (50 CFR, Part 265, Subpart A), indicates a degree of melanosis development which decreases product value.

Several 4-HR concentrations were tested and compared to the traditional 1.25% bisulfite treatment and to a seawater control (53). A concentration of 0.005% (50 ppm) kept melanosis development below the target limit (melanosis score of 4) for a storage period of 12 days,

The actual treatment of shrimp on a boat is performed by dipping 50-60 lbs. of shrimp into 25-30 gallons of solution. The results of testing indicate that approximately 500-600 lbs. Of shrimp could be treated with a single 4-HR solution (54).

In summary, a concentration of 0.005% (50 ppm) 4-HR has proven functional in the inhibition of shrimp black spot formation and corresponds to the lowest level of 4-HR which provides the intended technical effect of keeping melanosis development below the target limit (melanosis score of 4) for a storage period of 12 days. The

treatment has no negative impact upon product quality as measured by taste, texture, color, and overall appearance. The 4-HR treatment procedures require no changes in the traditional processing of shrimp except for the substitution of 4-HR for bisulfite in the dip tank.

2.5.2 Safety Data

2.5.2.1 Human Toxicity

The use of 4-HR in over-the-counter (OTC) drug products for more than 40 years has produced no evidence of systemic toxicity (55,56). The available human data indicate that 4-HR acts as a local irritant only at very high concentrations. Although human data on 4-HR are limited, there is no evidence that 4-h produces hypresensitivity reactions in humans (25).

2.5.2.2 Animal Toxicity on the Acute and Subacute

LD_{50} values for 4-HR by oral, intraperitoneal, and subcutaneous routes of administration have been reported for various laboratory of 4-HR in numerous animal species provide a sufficient basis for characterization of the acute/subacute effects of the chemical (55,57-63).

Table 5. LD_{50} studies of 4-Hexylresorcinol[a]

Test species	Route of administration	Test article vehicle	LD_{50} (mg/kg)
Rat	Oral	____	550
Mouse	Subcutaneous	5% solution of olive oil	750-1000
	Intraperitonueal	1% water emulsion	50
	Intraperitonueal	5% solution of olive oil	200
Guinea pig	Oral	____	470

[a]From Frankos (25)

2.5.2.3 Animal Toxicity on the Subchronic

In a study conducted in 1988 by the US National Toxicology Program (NTP) (64), F344/N rats and B6C3F$_1$ mice of both sexes were administered 0, 31.3, 62.5, 125, 250, or 500 mg 4-HR/kg body weight by gavage in corn oil, 5 days per week over a 16-day period (total of 12 doses). Body weight gain was significantly decreased relative to controls for only male rats given 250 or 500 mg/kg. The only clinical signs observed following treatment were hyperexcitability in male and female rats that received 500 mg/kg of 4-HR. Based on the results on these studies, doses of 0, 62.5, 125, 500, and 1000 mg/kg were selected for subsequent 13-week studies in both rats and mice.

In this subsequent 13-week study conducted by NTP, 4-HR was administered to F344/N rats and B6C3F1 mice of both sexes, by gavage in corn oil, at dose levels of 0, 62.5, 125, 250, 500, or 1000 mg/kg/day, 5 days per week for 13 weeks. In this study, all rats and male mice in the 1000 mg/kg/day dose group as well as 9 out of 10 female mice, died before the end of the study. Final mean body weight gains were significantly lower relative to controls for male and female rats following treatment with doses of 250 and 500 mg/kg. In addition, male rats administered 125 mg/kg exhibited a significant decrease in mean body weight gain. In mice, a dose of 250 or 500 mg/kg produced lower (but not statistically significant) mean body weights in males while mean body weights for female were not affected at any dose level. Clinical signs of toxicity observed in rats included nasal discharge, ocular irritation, alopecia, diarrhea, and cachexia, No overt signs of toxicity were observed in mice during the course of the study. No compound-related gross or microscopic pathologic effects were observed in rats of either sex. However, mice did exhibit a dose-related

increase in the incidence of mild to moderate nephropathy. A no-observed-effect level (NOEL) of 31.3 mg/kg/day was identified in this study for both rats and mice. Based on the results of these studies, doses of 0, 62.5, and 125 mg/kg 4-HR were selected for subsequent 2-year studies in the both rats and mice.

2.5.2.4 Animal Toxicity on the Chronic/Carcinogenicity

The potential chronic toxicity and carcinogenicity of 4-HR also were evaluated in a study conducted by the NTP (64). In this study, 4-HR in corn oil was administered by gavage to F344/N rats and $B6C3F_1$ mice of both sexes, at dose levels of 0, 62.5, or 125 mg/kg/day for 2 years. No significant differences in survival between treatment groups were noted for either rats or mice. Decreased body weight gain was observed in high dose male rats only and in male and female mice of both dosage levels during the last 16 weeks of the study only. There were no significant treatment-related carcinogenic effects in either male or female rats, or in female mice, for up to 2 years, However, a significant increase in the incidence of pheochromocytoma (control, 1/50; low dose, 2/50; and high dose, 5/49) and focal hyperplasia of the adrenal medulla (5/50, 16/50, and 10/49) was detected in male mice. The incidence of Harderian gland neoplasms was also slightly increased in male mice (0/50, 4/50, and 3/50). However, it was noted that the incidence of Harderian gland tumors in control mice was unusually low in this study. Nonneoplastic lesions observed following long-term treatment of mice with 4-HR included nephropathy and osteosclerosis. The renal lesion was described as varying from mild focal atrophy of tubules in the outer cortex to severe atrophy with dilatation of the tubular lumens and Bowman's space, tubular cysts, tubular regeneration, and lymphoplasmocytic in flammatory infiltrates. The incidence and severity of nephropathy increased in a dose-related manner in both male and

female mice. In male mice, the increase was marginal (control, 39/50; low dose, 47/50), while in female mice the increase was much more pronounced relative to controls (7/50, 40/49, and 47/50). The bone lesions detected in mice were described as focal or multifocal excesses of cancellous bone containing immature connective tissue and relatively few hematopoietic cells. The increased incidence if osteosclerosis occurred in both males (5/50, 5/50, and 15/50) and females (21/50, 25/49, and 40/50). In contrast to mice, the incidence of nonneoplastic lesions induced in rats was not significantly different from that of controls. It was concluded by the NTP Board of Scientific Counselors' Technical Reports Review Subcommittee and the Panel of Experts that the findings of pheochromocytoma and focal hyperplasia in the adrenal medulla, and the slight increase in the incidence of Harderian gland neoplasms in male mice provided equivocal evidence of carcinogenic activity. The finding of equivocal evidence of carcinogenic activity in male mice based on the findings at these two sites rested on borderline statistically significant positive trends, at best (Life Table Test or Incidental Tumor Test). Since there was no effect of 4-HR on survival in male mice, a more appropriate statistical analysis would be pairwise comparison which showed no increased tumor incidence. Therefore, significant questions arise with regard to the strength of evidence supporting the conclusion that there was equivocal evidence of carcinogenic activity for 4-HR based upon the adrenal medullary lesions and the proliferative lesions in the Harderian glands.

An evaluation of the NTP Carcinogenesis Studies on 4-HR (ENVIRON CORP.) and an independent pathology review (PATHCO, Inc.) was conducted to assess the weight of evidence for the equivocal carcinogenic activity 0f 4-HR. All adrenal and Harderian gland slides from the NTP 2-year gavage study of 4-HR were examined and

independent diagnoses were recorded. In addition, the individual animal data reports, tables, summary tables, Pathology Working Group (PWG) Report, NTP Technical Report Series No. 330, and quality assessment report were all examined. Data obtained in the independent pathology review indicated that bilateral proliferative lesions were found primarily in the low dose male mice and that the only malignant pheochromocytoma occurred in a low dose animal. Cytological differences and compression were generally not found for the pheochromocytomas in this study and thus size became the distinguishing factor for differentiating pheochromocytoma from focal medullary hyperplasia. Five pheochromocytomas were reported by NTP in the high dose males. This falls within the historical control range of 0 –10%. In the opinion of the reviewing pathologist, one and possibly two of the five pheochromocytomas were actually focal medullary hyperplasia as illustrated in Table 6. In addition, undiagnosed adrenal medullary hyperplasia was identified in the adrenal glands of four control mice. Based upon the aforementioned findings, it was concluded that the adrenal medullary pheochromocytomas appeared unlikely to be related to 4-HR administration.

Regarding the incidence of Harderian gland tumors in male mice, the reviewing pathologist considered all eight reported tumor to be adenomas. Additionally, the incidence of Harderian gland neoplasms was not dose-related and was within the historical control range for corn oil gavage studies (0–10%). Based up on the aforementioned findings, it was concluded that the weight of evidence suggested that the Harderian gland tumors were incidental findings. In summary, a recent independent pathology review indicated that the weight of evidence dose not support

the conclusion that there was equivocal evidence of carcinogenic activity of 4-HR in male mice but rather that 4-HR lacked carcinogenic activity in both rats and mice.

The only other information on the carcinogenic potential of 4-HR is from a study conducted by Boyland *et al.* that provided no scientifically supportable evidence of a carcinogenic response to 4-HR (65).

Table 6.Incidence of adrenal medullary hyperplasia[a]

	Treatment group		
	Control	Low dose	High dose
NTP PWG	5/50 (10%)	16/50 (32%)	10/50 (20%)
PATHCO	9/50 (18%)	16/50 (32%)	10/50 (20%)

[a]From Frankos (25)

2.5.2.5 Animal toxicity on the Reproductive and Development

There is no evidence of an association between 4-HR exposure and teratogenicity in humans (66) despite extensive use by human at exposure levels well above those expected from consumption of 4-HR-treated shrimp.

2.5.2.6 In Vitro Toxicity on the Mutagenicity

In vitro studies involving 4-HR are not indicative of mutagenic activity (64,67,68). Cortinas de Nava *et al* (67) found no increase in the number of revertant colonies following incubation of strains TA98, TA100, TA1535, TA1537, or TA1538 in the standard plate incorporation technique of Ames *et al.* with or without metabolic activation from PCB-induced male Sprague Dawley rat liver S9, with up to 30 μg 4-HR. When tested in a preincubation protocol with doses up to 100 μg/plate, 4-HR was not mutagenic in *S. typhimurium* strains TA98, TA100, TA1535, or TA1537 in the

presence or absence of metabolic activation from Aroclor 1254-induced male Sprague Dawley rat or Syrian hamster liver S9 (64). 4-HR demonstrated some mutagenic activity in cultured mammalian cells in NTP studies. It was mutagenic in the mouse lymphoma L5178Y/TK$^{+/-}$ assay in the presence of Aroclor 1254-induced F344 rat liver S9 at concentrations of 5-30 μg/ml; no response was observed in the absence of exogenous metabolic activation. Exposure to 4-HR at doses up to 50 μg/ml did not produce chromosomal aberrations in cultured Chinese hamster ovary (CHO) cells with or without Aroclor 1254-induced male Sprague Dawley rat liver S9. However, in sister chromatid exchange (SCE) assays with CHO cells, the compound produced a positive response at doses of 18 and 20 μg/ml in the absence of S9; on increase in the frequency of SCEs was observed in the presence of S9 activation. Furthermore, in order to evaluate the sensitivity and specificity of *in vitro* mutagenicity tests for predicting potential carcinogenic activity, chemicals tested for carcinogenicity by the NTP were classified according to chemical structure, mutagenicity to salmonella, and the level of carcinogenicity (69). The authors concluded, based upon their analysis, that 4-HR did not possess a chemical structure with the potential to interact with DNA.

2.5.2.7 Allergenicity

Few cases of sensitization to 4-HR were reported in the literature, despite widespread use as an OTC oral health drug product (70). In addition, animal studies indicate a lack of allergenic response to 4-HR as it did not induce delayed contact sensitivity in the guinea pig maximization test (71). There is no evidence from extensive clinical use of 4-HR that it produces hypersensitivity reactions in human.

2.5.3 ADI for 4-Hexylresorcinol

An extensive database, consisting primarily of animal studies, is available for derivation of an acceptable daily intake for 4-HR. The toxicologic and carcinogenic studies conducted by the NTP on this compound are considered the most suitable for determining a no-observed-effect level (64).

In the 2-year studies, mice were found to be the most sensitive species and, therefore, they are the most suitable for determining a NOEL. The NOEL for male mice, with regard to osteosclerosis and nephropathy, was 62.5 mg/kg/day; however, these studies did not demonstrate a no-observed-effect level for nephropathy in female mice, as the incidence of nephropathy was increased at all dose levels relative to controls. Extrapolation of the incidence of nephropathy induced in female mice at the lowest dose tested of 62.5 mg/kg/day (82%) to the control level (14%) yields an estimate of 10.7 mg/kg/day for the no-observed-effect level.

Derivation of the final ADI requires not only identification of a NOEL but also the selection of an appropriate safety factor as determined by qualified scientists. The FDA specified that when adequate chronic toxicity data are available a safety factor of 100 is appropriate. Application of this safety factor to the estimate NOEL (10.7 mg/kg/day) identified in chronic toxicity studies conducted by the NTP yields an acceptable daily intake for 4-HR of 0.11 mg/kg/day.

2.5.4 Residual Levels in Shrimp

Residual levels of 4-HR were evaluated in shrimp following experimental dip-treatment procedures (72). Residual data developed for fresh shrimp (heads-on and headless) are most representative of actual use conditions and potential consumption

of 4-HR and, therefore, are uses in estimates of the average and 90th percentile daily intake of 4-HR as a result of its antimelanosis use on shrimp.

The average residual 4-HR levels (mean ± standard deviation) measured on fresh heads-on shrimp were 0.4±0.09 ppm and 0.7±0.13 ppm for 1- and 15- min dip times, respectively. Likewise, the average residual 4-HR levels measured on fresh headless shrimp were 0.9±0.27 ppm and 2±0.41 ppm for 1- and 15-min dip times, respectively.

Based upon estimates of the U.S. shrimp supply in 1989, the most accurate estimate of 4-HR residuals will take into account headless and heads-on shrimp making up 38% and 62% of total shrimp production, respectively, prior to treatment.

2.5.5 Estimated Daily Intake of 4-Hexylresorcinol

The average residual level of 4-HR in shrimp compiled in field trials employing 4-HR under proposed use condition is approximately 0.4 and 0.9 ppm for fresh heads-on and headless shrimp, respectively. To provide a reliable estimate of 4-HR intake from consumption of shrimp, consumption data were compiled and reviewed.

The following consumption data are based upon Market Research Corporation of America Information Service menu census data (73). The shrimp consumption data reported include eaters only and was compiled over a 14-day survey period. The average and 90th percentile shrimp consumption data compiled by MRCA indicated portion sizes of 2.8 and 7.7 g per day, respectively.

The average and 90th percentile 4-HR intakes for a 70-kg individual employing MRCA consumption data for shrimp are calculated based on residual concentration of 4-HR in shrimp. Application of appropriate residual level data results in the following estimates of daily 4-HR intake.

Average Intake

Heads-on shrimp: (2.8 g/day x 0.4 μg/g x 0.62) ÷ 70 kg = 0.010 μg/kg/day

Headless shrimp: (2.8 g/day x 0.9 μg/g x 0.38) ÷ 70 kg = 0.014 μg/kg/day

Total 0.024 μg/kg/day

Heads-on shrimp: (7.7 g/day x 0.4 μg/g x 0.62) ÷ 70 kg = 0.027 μg/kg/day

Headless shrimp: (7.7 g/day x 0.9 μg/g x 0.38) ÷ 70 kg = 0.038 μg/kg/day

Total 0.065 μg/kg/day

In contrast, average daily exposure to 4-HR from OTC drug use can be estimated for specific exposure scenarios. 4-HR is the active ingredient in Sucrets Sore Throat Lozenges and Listerine Antiseptic Throat Lozenges at concentrations of 2.4 and 4.0 mg/lozenge (25). Assuming that an individual has four colds per year, each lasting 7 days, and that six lozenges are taken per day, the average daily exposure to 4-HR is estimated to be 6 mg/kg/year (0.0164 mg/kg/day) for individuals taking the regular strength lozenge (2.4 mg/lozenge) and 10 mg/kg/day (0.0274mg/kg/day) for individuals taking the maximum strength lozenge (4.0 mg/lozenge)-far greater that the estimated exposure from 4-HR use as an antimelanosis agent on shrimp (0.024 μ g/kg/day; see discussion above).

2.6 Dietary Nitrosating Compounds: Nitrate and Nitrite in Foods.

Nitrate in the environment originates from many sources, for example, as a product of nitrogen fixation by microorganisms and plants, as a decomposition product of plant and sewage wastes, from inorganic fertilizers used in agriculture, and in foodstuffs as a preservative in meat and fish. Nitrite is available either as a natural

constituent after reduction of nitrate or as food additive in meats, fish, and cheese. Sodium and potassium nitrate and nitrite are used to preserve meat products for the purpose of retaining color, improving flavor, and inhibiting growth and toxin formation by *Clostridium botulinum* (74). The toxicological significance of nitrates lies in its easy conversion to nitrite by nitrifying bacteria that may present in foodstuffs, the saliva and in the GI tract (75).

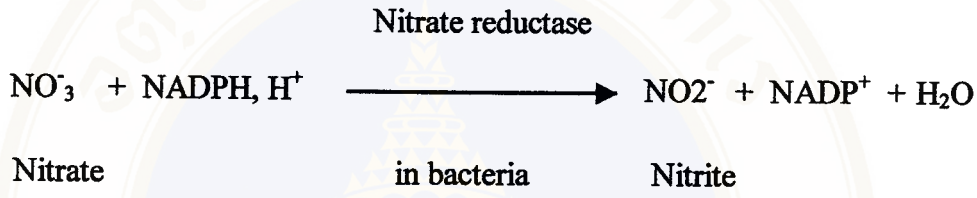


Figure 2. The conversion of nitrate to nitrite by nitrifying bacteria.

2.7 Mutagenicity and Carcinogenicity of Nitrite

High doses of sodium nitrite induced reversed mutation in *Salmonella typhimurium* and caused chromosomal aberrations in culture Chinese hamster fibroblast cells (76). Sodium nitrate did not cause an increase in single strand breaks in cultured mouse cells but there was a dose-related increase in gene mutations and chromosome aberrations at high concentrations, possibly due to deamination of bases (77). Chromosome aberrations were significantly increased by nitrate in culture hamster cells (78) and increase in 6-TG mutants were seen in V 79 hamster cells *in vitro* (79).

Exposure of newborn hamster cells *in vitro* to sodium nitrite at concentrations of 2100 or 4200 mg NO_2 / l for 24 h resulted in aneuploidy, chromosomal aberrations and cell transformations (80). It was also shown that the embrionic cell cultured of the

fetus obtained from pregnant Syrian hamsters given sodium nitrate at doses of 125, 250, or 500 mg/kg body weight by gavage on days 11 and 12 of pregnancy showed a dose-dependent increase in micronuclei and in 8 azaquanine and ouabain-resistant mutants. Cell transformation was also observed *in vitro* and implication of transformed cells led to tumor development (81).

Negative results were found in a host-mediated assay against *Salmonella typhimurium* at a dose level of 150 mg sodium nitrite/kg body weight (82) and in a mouse micronucleus test. However, administration of sodium nitrite in drinking water at a concentration of 1250 mg/l to non-pregnant or to pregnant fats (on days 5-18 gestation) induced chromosomal aberrations in the bone marrow of the adult (both pregnant and non-pregnant) and in the embryonic liver. The ratio of number of metaphases with aberrations in treated and control animals was higher for embryonic liver compared to adult bone marrow. The higher incidence might result from the higher numbers of mitotic cells in embryonic tissues (83). However, Wistar rats of both sexes were given sodium nitrite in distilled water at concentration of 0 to 3000 mg/l on 5 days/week for more than 100 weeks. Papilomas of the forestomach were seen in 8/45 treated animals (the group receiving sodium nitrite 3 g/l drinking water; total dose, 63/kg) compared with 2/91 controls (84).

Males Wistar rats given sodium nitrite in diet at concentrations of 0, 800, or 1600 mg/kg body weight for 90 weeks 1 out of 8 rats in the lower dose group developed a benign liver tumor and 5 out of 8 rats in the higher dose group developed liver tumor, three classified as benign and two as malignant. The tumors were derived from parenchymal or hemangioendothelial cells. However, the nitrite-containing diets were found to contain NDMA (N-nitrosodimethylamine) and NPYR (N-nitrosopyrollidine)

and the authors concluded that these preformed nitrosamines were probably the principle cause of the tumors (85).

2.8 Aminopyrene-Nitrite Model for Inhibition Study of 4-Hexylresorcinol

1-Aminopyrene is a natural compound produced in human feces or in anaerobic incubation of 1-nitropyrene with fecal bacterial (86-88). It was known to be mutagenic after metabolic activation to strain TA98 and TA100 but less mutagenic without activation. The mutagenicity of this compound with S-9 was decreased on treatment with nitrite whereas the activity was increased without S-9 (89). The numbers of His^+ revertant colonies on TA98 without S-9 mix were 870 colonies/0.022 μmole. The results indicated that this compound was transformed into potential direct action mutagens on treatment with nitrite. Kato *et al.* (90) also demonstrated that mutagenicity of 1-aminopyrene could be potentiated by nitrite. In their experiment, 1-aminopyrene (0.2 mmole) interacted with sodium nitrite (0.8 mmole) in 5 ml acetonitrile-water (1:1 v/v), the mixture was adjusted to pH 3 and incubated at 37 °C for 4 h. Then 0.8 mole ammonium sulfamate was added. The mixture was extracted with 5 ml ethyl acetate. The mixture was analyzed with HPLC and found to contain 1-nitropyrene and unidentified nitro-compounds. The mutagenicity of 1-aminopyrene is one-tenth that of its nitro-introduced product, 1-nitropyrene, in the Ames test (91). However, in the living system, 1-nitropyrene is converted back to 1-aminopyrene by microsomal enzyme (87).

2.9 The *Salmonella* Mutagenicity Test (Ames test)

The most generally used and validated bacterial reverse mutation test was devised by B.N. Ames and his colleagues (92). The Ames test was first validated in a study of 300 chemicals, most of which were known carcinogen (89,93). It was subsequently validated in study by the Imperial Chemical Industries (94), the National Cancer Center Research Institute in Tokyo (95) and International Agency for research on Cancer (96). Nearly 90% of the carcinogens tested were mutagenic in these studies. However, Ames and McCann estimated the correlation to be about 83%. All the validations showed that the test fails to detect a few classes of carcinogens such as polychlorinated pesticides (97-99).

Prior to the initial development of the *Salmonella*/microsome assay, there were several studies that employed bacterial systems to detect mutagenic (100). However, one of the problems with these earlier appearance was the use of screening techniques that did not employ bacterial strains designed to detect a broad range of mutagenic mechanism. Therefore, Ames *et al.* (101) developed a set of *Salmonella typhimurium* strains that were permeable to a wide range of chemicals and also were partially deficient in DNA repair.

2.9.1 The *Salmonella* Tester Strains

The reverse mutation system of *Salmonella typhimurium* uses that genetically well-defined histidine requiring mutants developed by Ames and his colleagues (102). The *Salmonella* histidine reverse mutation is based on the use of several *Salmonella typhimurium* strains that reversed from histidine dependence (auxotroph) to histidine independence (prototroph). Ames *et al.* collected and characterized a large number of *Salmonella typhimurium* strains containing mutations in different gene of the histidine

operon (Table7). Later, they developed the newly *Salmonella* tester strains to make them more effective in detecting mutagens that were not previously detected with the original strains (102). The newly standard tester strains contain other mutations that greatly increase their ability to detect mutagens such as :

- rfa mutation which causes partial loss of the lipopolysaccharide barriers that coat the surface of the bacteria and increases permeability of the cell wall to large molecules that do not penetrate the normal cell wall (91)

- *uvr* B mutation is a deletion of a gene coding for the DNA excision repair system, resulting in greatly increased sensitivity in detecting many mutagens (91,101).

- R-factor plasmid (pKM 101) This plasmid exhibits a greatly enhanced response to mutagen and also gives clear positive response to chemical described as weak, borderline or nonmutagens with the original set of tester strains (89,103). Furthermore, Macphee implied that pKM 101 contains gene products associated with errorprone repair which may be responsible for the enhance sensitivity seen in these strains (104,105).

Genotypes of the *Salmonella typhimurium* strains used for mutagenesis testing are shown in Table7. The standard tester strains.; TA97, TA98, TA100 and TA102 contain the R-factor parent strains (106,107).

Tester strains in brackets are recommended for general mutagenesis testing. All strains were originally derived from *Salmonella typhimurium* LT2. Wild-type genes are indicated by a +. The deletion (Δ) through *uvr* B also includes the nitrate reductase (*chl*) and biotin (*bio*) genes. The Δ*gal* strains and the rfa/uvr B strains have a single deletion through *gal chl bio uvr* B. The *rfa* repair - strains have a mutation in *gal E*.R = pKM 101. The tester strain TA1536, included in the original tester set (91), and all

other strains containing the histidine mutation his *C207* were discontinued due to the lack of specificity on few mutagens reversion and tester strains TA97 replaces TA1537 and TA2637. Genotypes of these discontinued strains and of other derivatives of his C3076 were listed by Ames *et al.*

Table 7.Genotypes of the *Salmonella typhimurium* strains used for mutagenesis testing

Histidine mutation				LPS	Repair	R-factor
his D6610			his G428			
His 01242	his D3052	His G46	(PAQ 1)			
TA90	TA1538	TA1535	-	*Rfa*	Δ*uvr* B	-R
(TA97)	(TA98)	(TA100)	-	*Rfa*	Δ*uvr* B	+R
-	(TA1978)	TA1975	-	*Rfa*	+	-R
TA110	TA94	TA92	-	+	+	+R
-	TA1534	TA1950	-	+	Δ*uvr* B	-R
-	-	TA2410	-	+	Δ*uvr* B	+R
TA89	TA1964	TA1530	-	Δgal	Δ*uvr* B	-R
-	TA2641	TA2631	-	Δgal	Δ*uvr* B	+R
-	-	-	(TA102)	*Rfa*	+	+R

Therefore these standard tester strains are recommended for mutagenesis testing. TA98 was derived from TA1538 by which plasmid pKM 101 was introduced. It can detect mutagens that cause frameshift mutation due to its DNA sequence (-CGCGCGCG-), which can be reverted to histidine independence by a variety of mutagens affected addition or deletion of the base pairs (108). TA100 containing R-factor plasmid derivative of TA1535 can detect mutagens that cause base-pair substitutions. The others *Salmonella* strains related to these 4 strains containing

different characteristics in terms of DNA-repair capacity, cell permeability and presence of plasmid pKM101 are also available and have been described (106,109).

However, some mutagens affect only one strain of frameshift mutation strain (TA1538 or TA98) or base-pair substitution strains (TA1535 or TA100), thus imparting a degree of mutagen specificity to the assay. But, many or even most mutagens can affect both types of strains at the different effective dose for each strain. Mutagen specificity, therefore, is frequently associated with quantitative rather than qualitative response (110).

2.9.2 Method used for Detecting Mutagens

There are three methods that have been used for testing mutagenicity of chemicals. These methods are spot test, plate incorporation test and preincubation method.

2.9.2.1 Spot test.

The spot test is the simplest way to test compounds for mutagenicity and is useful for the initial rapid screening of large numbers of compounds. This test is primarily a qualitative test and has distinct limitations. It can be used only for testing chemicals that are diffusable in the agar. It is be confirmed by demonstrating a dose-response relationship using the standard plate incorporation test.

2.9.2.2 Plate incorporation test.

The test is the standard method that has been used for the mutagenicity of chemicals. The test consists of combining the test compound and the bacterial tester strain in soft agar which is poured onto a minimal agar plate, After incubation at 37 °C for 48 h, revertant colonies are counted (91,102,111). For initial screening chemicals were tested in concentrations over a three-log dose range. A positive or questionable

result should be confirmed by demonstrating a dose response relationship using a narrower range of concentrations. In a modification of the plate incorporation procedure, a preincubation step precedes addition of the top agar. This modification is better for some compounds and appears to be at least as good for other compounds tested (91,102).

2.9.2.3 Preincubation method

The significant finding of preincubation assay was of equal or greater sensitivity than the standard plate incorporation assay, when the mutagenic activity of aflatoxin B_1, benzodine, benzo(a)pyrene and methyl methanesulfonate have been determined by both plate incorporation and preincubation procedure. Some mutagens, such as dimethyl and diethylnitrosamine are poorly detected in the standard plate incorporation assay a modification of standard procedures. The fact that the test compounds and bacteria were incubated 20-30 minutes at 37 °C at the higher concentrations before adding into top agar provided more sensitivity of mutagenic detection. The preincubation assay was first described by Yahagi *et al.* in which carcinogenic azo dyes were found to be mutagenic (112,113).

The preincubation modification can be used routinely or when inconclusive results are obtained in the standard plate incorporation assay. This assay required an extra step and therefore involves more work than the standard test but many laboratories has been used to detect mutagenicity of 10 carcinogenic nitrosamines (114) and several carcinogenic alkaloids (115). It was used in screening assays has also been recommended (116).

2.9.2.4 Positive control (diagnostic mutagens)

In each experiment, positive mutagenesis controls were used for diagnostic mutagens to confirm the reversion properties and specificity of each strain. The characteristic reversion patterns of the standard stains to some diagnostic mutagens are described. (117).

2.9.3 Evaluation Criteria for Ames Assay

The Ames assay used to evaluate the mutagenicity of test article is semiquantitative. The criteria used to determine positive effects was inherently subjective and based primarily on a historical database. The positive results in the present study were based on following criteria according to the Yahagi's recommendation. If the solvent control value was within the normal range a test article that produced a positive dose-response relationship over two concentration, with the highest increase was not less than twice the solvent control was considered mutagenic (113).

The criteria used to determine positive effects are inherently subjective and based primarily on the information shown in Table7. Most data sets should be evaluated using the following criteria (118).

a. Strains TA1535, TA1537, and TA1538. If the solvent control value is within the typical range for the laboratory, a test article that produces a positive dose response over three concentrations, with the highest increase equal to three times the solvent control value, is considered mutagenic.

b. Strains TA98 and TA100. If the solvent control value is within the normal range for the laboratory, a test article that produces a positive dose response over three concentration, with the highest increase equal to twice the solvent control value, is

considered mutagenic. Occasionally a doubling is not necessary for TA 100 if a clear dose-related pattern is observed over several concentrations.

c. Pattern. Because TA1535 and TA100 are derived from the same parental strain (G46), and because TA1538 and TA98 are derived from the same parental strain (D3052), to some extent there is a built-in redundancy in the microbial assay. In general, the two strains of a set respond to the same mutagen, and such a pattern is sought. Generally, if a strain responds to a mutagen in non-activation tests, it should do so in activation tests.

d. Reproducibility. If a test article produces a response in a single test that cannot be reproduced in additional runs, the initial positive test data lose significance.

The preceding criteria are not absolute and other extenuation factors may enter into a final evaluation decision. However, these criteria can be applied to the majority of situations and are presented to aid those individuals not familiar with this procedure. As the data base is increased, the criteria for evaluation can be more firmly established. It must be emphasized that modifications of the procedure involving preincubation conditions is necessary for evaluation of specific chemicals or classes of chemicals.

2.9.4 Antimutagenicity Test Using Ames Test

Considerable evidence indicated a strong association between the carcinogenicity and mutagenicity of chemicals (93,119). Since the antimutagenic activity has also been correlated with anticarcinogenic activity of various compounds, it should be possible to use mutagen-testing procedure or Ames test for screening various compounds for antimutagenic and hence for potential anticarcinogenic effects (120-123).

2.9.5 Criteria of Antimutagenic Activity

The antimutagenic effects expressed as inhibition (%):

Percentage of inhibition = (a-b)/(a-c)×100

where a = number of histidine revertants induced by the positive mutagen (product of AP-nitrite reaction); b = number of histidine revertants induced by the positive mutagen in the presence of sample; c = number of revertants of the negative control (spontaneous reversion).

Interpretation of data : compounds were classified as positive antimutagen based on the % inhibition of the mutagenicity of tested mutagen. Data were qualitatively ranked according the following scheme:

% inhibition	Ranking for antimutagenicity
>60 %	strongly active
60-40 %	Active
40-20 %	weakly active
20-0 %	not active

Positive antimutagens should be dose-responsive or positive dose-response relationship (124).

2.10 Somatic Mutation and Recombination Test (SMART)

The somatic mutation and recombination test (SMART) of *Drosophila* is a rapid and sensitive test which detects mosaic spots produced as cellular clones on the wings during the fly's development as a consequence of several genetic mechanisms: point mutation, deletion, mitotic recombination and chromosome non-disjunction.

This eukaryote offers the advantages of having a short generation time, needing very inexpensive culture media and allowing the breeding of large numbers of animals using simple facilities. In addition, it is well established that Drosophila possesses a versatile system for the metabolism of xenobiotics (125,126). The SMART provides a suitable substitute or at least a complementary *in vivo* method to mammalian *in vivo* investigation. *Drosophila* has detoxification-activating systems in many respects closely resembling the corresponding systems in mammals, which makes it possible to extrapolate data to mammals (125-127). The use of SMART assays is based on the treatment of larvae, and besides the number of mutated spots appearing in the adult flies, indicating the frequency of genetic events, the size of the spots indicates the time of action during embryogenesis. The SMART is also able to detect mutagenicity of unstable mutagens (128), pesticides (129), food colorants (130) and co-administration of some agents with mutagens (131-134).

2.10.1 Principle of the Somatic Assays:

Drosophila melanogaster as a dipteran insect develops through successive developmental stages of different duration. *Drosophila* undergoes complete metamorphosis:(duration 1 day at the optimal culture temperature of 25°C), 1st larval instar (L1, 1day), 2nd larval instar (L2, 1day), 3rd larval instar (L3, 2days), metamorphosis in pupal stage (prepupa 4 h, pupa 4.5 days) and adult stage (imago, up to 40 days) (135,136). During embryogenesis primarily larval tissues (cuticle, gut, fat body, nervous system, *etc.*) are formed, and during the larval period these tissues enlarge and finally form the body of a large L3 larva ready for pupation. The adult structures (wings, legs, eyes, etc.) are formed in the pupal stage from the so-called imaginal discs. These develop during larval life as proliferating entities derived

from groups of cells set apart during embryogenesis. Such discs grow during the larval period by cell proliferation. The cells of the imaginal wing disc derived from a sample of about 50 nuclei of the primitive egg syncytium, which happen to migrate to a given region of egg cortex. After these nuclei have been surrounded by cytoplasm and membranes, the corresponding cells become developmentally segregated from the neighboring ectodermal cells. They do not divide during embryonic development but can already be detected histologically, grouped in a discrete wing imaginal disc, in the newly hatched larva. Proliferative growth starts in the first instar and continues throughout the larval period. Cell proliferation is logarithmic in all the presumptive adult cuticular cells, although the number of cells per clone deviates from 2^n. On average, cells divide every 8.5 h and growth is complete after 9-10 cell divisions. After pupation, mitoses are still detectable, but somatic crossing over initiated at this time results in single marked cells. Twenty-four hours later, mitosis ceases altogether and visible cell differentiation begins. During differentiation about 50,000 cells give rise to single identifiable cuticular processes organized in the typical adult pattern.

During disc growth in the larval stage if a wing imaginal disc cell is genetically altered into a mutant form, a group of mutant cells will result from clonal expansion during disc growth. After pupation, in the course of metamorphosis of the imaginal disc into an adult wing the mutant phenotype will become expressed. The mutant clone will be recognizable as a group of phenotypically altered wing blade structure (called a "spot"), e.g. showing multiple hairs instead of the single hair formed by each wild-type wing cell.

2.10.2 Genetic Basis of the Effects Detected

The assay consists of exposing to a mutagen populations of cells that are destined to multiply in relatively fixed configurations so that an induced mutation in one of the exposed cells will give rise to a detectable clone. To ensure the clone is identifiable on the surface of the adult fly, one chooses genetic markers that are expressed autonomously in wing cells. The exposed cells of the larval wing imaginal discs are trans-heterozygous for two recessive markers located on the left arm of chromosome 3. Multiple wing hairs (*mwh*) is at position 0.0 cM and flare (flr^3) at 39.0 cM, while the centromere is located at 47.7 cM (137,138). The appearance of multiple wing hairs is a homozygous viable recessive mutation producing multiple trichomes per cell instead of the normally unique trichome. The second marker, flare (flr^3), are a zygotic recessive lethal but homozygous cells in the wing imaginal disc survive and lead to wing blade cells with short, thickened, and misshapen trichromes. In stock cultures, the flr^3 alleles have to be balanced over inverted chromosomes such as TM1 or TM3 because it is a recessive zygotic lethal. In all the experimental series analyzed, the occurrence of the various types of spots was as follows: most frequent were single spots expressing the *mwh* phenotype, less frequent twin spots with both a *mwh* and a flr^3 sub-clone, and quite rare single spots with the flr^3 phenotype.

There exist several mechanisms that lead to genetically marked clones (see Figure 3). An important possibility is a mitotic recombination event between two non-sister chromatids. Twin spots are expected if recombination occurs between flr^3 and the centromere (139). A recombination event between *mwh* and flr^3 may result in a *mwh* single spot. If both types of recombination events (one between flr^3 and the

centromere and a second between *mwh* and *flr*^{3}) take place within the same cell, a *flr*^{3} single spot may result.

A "twin spot" is an indicative of a recombinational event. Nondisjunctional or other loses of the chromosomes carrying the wild type allele represents another mechanism that may lead to single spots. Mitotic recombination in the chromosome section between the centromere (spindle fiber attachment site) and the marker *flr*^{3} leads to two daughter cells, one homozygous for *mwh*, the other homozygous for *flr*^{3}. Clonal expansion to these two cells will be recognizable on the wing blade from the two multicellular adjacent clones, one exhibiting the *mwh* phenotype (multiple hairs), the other the *flr*^{3} phenotype (misshape hairs).

On the other hand, the origin of "single spots", showing either the *mwh* or the *flr*^{3} phenotype, cannot be clearly determined. Multiple wing hairs single spots may result from a recombinational event occurring in the chromosome segment between the two marker genes. But also a gene mutation or deletion of the *mwh*$^+$ gene will result in a *mwh* single spot. A *flr*^{3} single spot may either result from a gene mutation or a deletion of the *flr*^{3} gene, or from a rare double recombination with one recombinational event to the left, and the other event to the right of the *flr*^{3} locus (140).

2.10.3 Approach of SMART

Three crosses of flies carrying the marker *mwh* and *flr*^{3} on the left arm of chromosome 3 were generally set up:

1. Standard cross: *flr*^{3} */In (3LR) TM3, ri p*^{p} *sep bx*^{34c} *e*^{s} Ser virgin females mated to *mwh* males. This is the reciprocal cross of the standard cross used previously (141, 142).

2. High bioactivation (HB) cross: *ORR; flr^3/TM3* females crossed with *ORR; mwh* males. This is the reciprocal cross of the one described by Frolich and Wurgler (143). A number of promutagens showed increased genotoxicity when the HB cross was used, compared to standard cross (143-145). However, the HB cross presents a number of difficulties and disadvantages (143,146).These are: (1) The presence of an irregular whorling in the pattern of wing hairs making spot classification difficult, especially for inexperienced scorers, (2) an undesirably high variation in results from repeated experiments, (3) the low egg production of the females used and the delay in development of the larvae of HB cross.

3. Improved HB cross: *ORR, flr^3 /TM3* females crossed with *mwh* males (147). The main advantage of the improved HB cross is to combine the high bioactivation capacity with the ease of scoring the wings using the same criteria as for the standard cross. The hybrid larvae of the improved HB cross show P450-dependent activation capacity equal to or even slightly higher than those of the original HB cross. In addition, the IHB cross is more sensitive than the standard cross in evaluating the genotoxicity of promutagens (147).

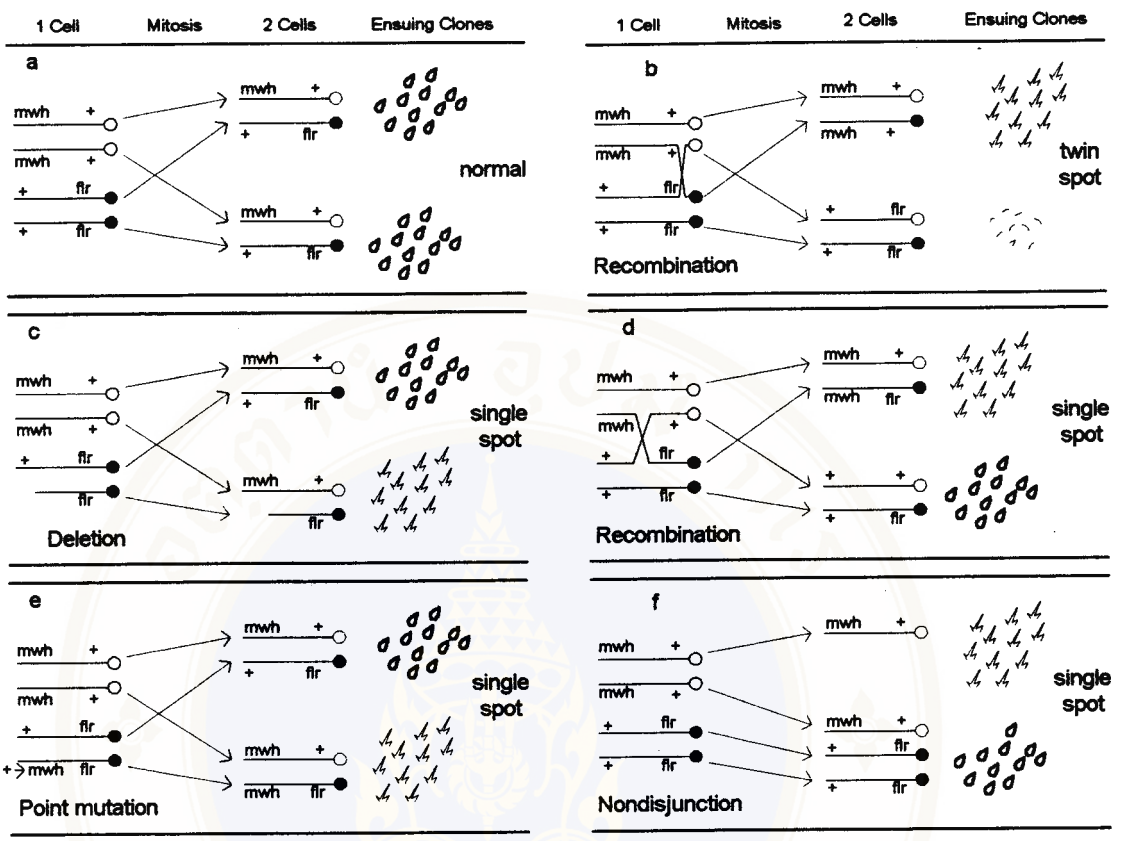


Figure 3.Genetic schemes illustrating various ways of spot formation in the somatic mutation and recombination test with the wing cell markers multiple wing hairs (*mwh*) and flare (flr^3). Twin spots are obtained by recombination proximal to the flr^3 marker (b), while more more distal recombination produces *mwh* single spots only (d). Deficiencies (c), point mutations (e) and nondisjunction events (f) give rise to *mwh* single spots, or in analogous ways to flr^3 single spots (not illustrated) (128).

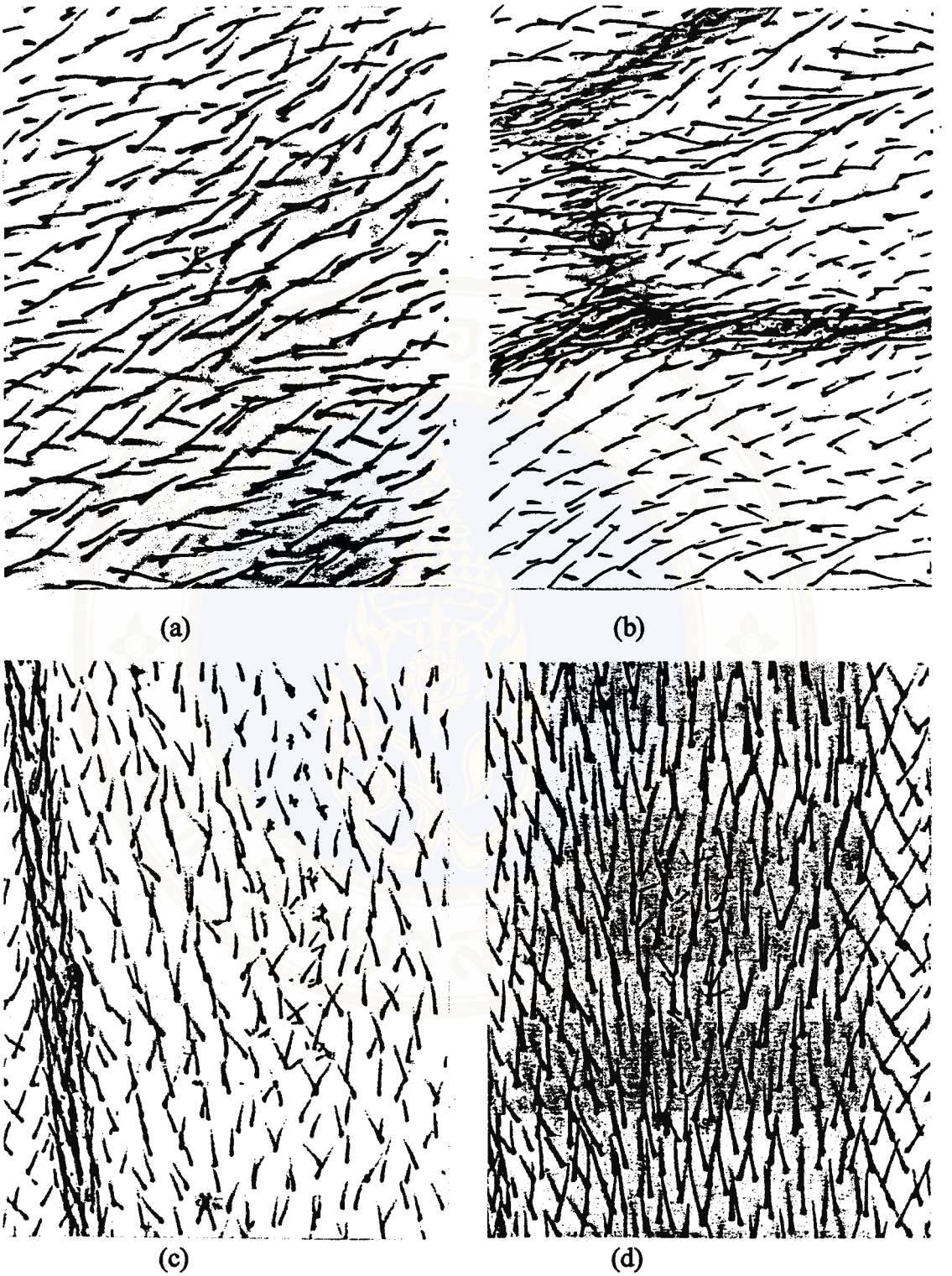


Figure 4. Marker mutations of wing surface to show clone of cuticle secreted by cells homozygous for multiple wing hairs, a) small single spots, b) flare on wing vein, c) twin spots, d) large single spots.

2.10.4 Standard Mutagens for Mutagenicity of SMART

Urethane is generally used as positive standard toxicants in evaluation genotoxicity of the unknown compounds in SMART (134). These chemical required metabolic activation to express their mutagenic activity (145).

2.10.4.1 Urethane (ethyl carbamate)

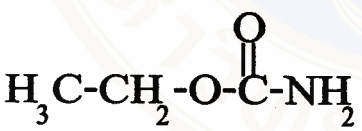
Urethane ($NH_2COOCH_2CH_3$), also known as ethyl carbamate, is the ethyl ester of carbamic acid (NH_2COOH). Urethane may occur as a colorless, odorless crystal or a white, granular powder. It is slightly soluble in olive oil and soluble in water, ethane, ether, glycerol, chloroform, and ethyl ether. In the 1940s, urethane was used as a hypnotic in man at doses of 1 g/person/day and as an anaesthetic for laboratory animals. In 1943, it was discovered that urethane had a carcinogenic effect in animals. It is regarded as an initiator, but also as a cocarcinogen and specifically as a promoter of radiation-induced cancer (148). Since 1948 it has been known that urethane is mutagenic in *Drosophila melanogaster*. Today, humans are exposed to urethane in their food and mainly alcoholic beverages.

2.10.4.2 Metabolic Activation and Detoxification of Urethane

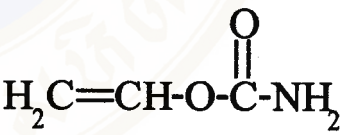
Urethane was found to induced point mutation, gene conversion, intrachromosomal recombination, chromosomal aberrations and sister chromatid exchanges in yeast, plant systems and mammalian cells (149). Urethane exerts its carcinogenic effect following bioactivation to vinyl carbamate and then to vinyl carbamate epoxide which forms RNA and DNA adducts and initiates tumorigenesis (150,151). The activation of urethane is important in exerting its carcinogenic effect. The two step oxidation of urethane to the active vinyl carbamate epoxide is catalyzed primarily by cytochrome P-450 subtype 2E1 (152). The metabolism of urethane is

shown in Figure 6. Urethane is metabolized by two different pathways. The major pathway, accounts in rodents for over 90%, is the hydrolysis of urethane by microsomal esterase and amidases to ethanol, ammonia and carbon dioxide (153,154) This major pathway is probably one for detoxification.

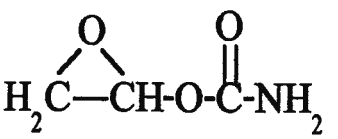
The minor pathway involves the oxidation of urethane via cytochrome P-450 subtype 2E1 (CYP2E1) to 2-hydroxyethyl carbamate, to *N*-hydroxyethyl carbamate and to vinyl carbamate, which is in turn converted by epoxidation to the putative ultimate carcinogen vinyl carbamate epoxide (152,155,156). Vinyl Carbamate epoxide is a major strong ultimate reactive electrophilic, mutagenic and carcinogenic metabolite of urethane and vinyl carbamate in mouse (154). Generation of the electrophilic vinyl carbamate epoxide leads to the formation of RNA and DNA adducts and the initiation of tumorigenesis (151).



(a)



(b)



(c)

Figure 5. (a) Urethane (ethyl carbamate); (b) Vinyl carbamate; (c) Vinyl carbamate epoxide.

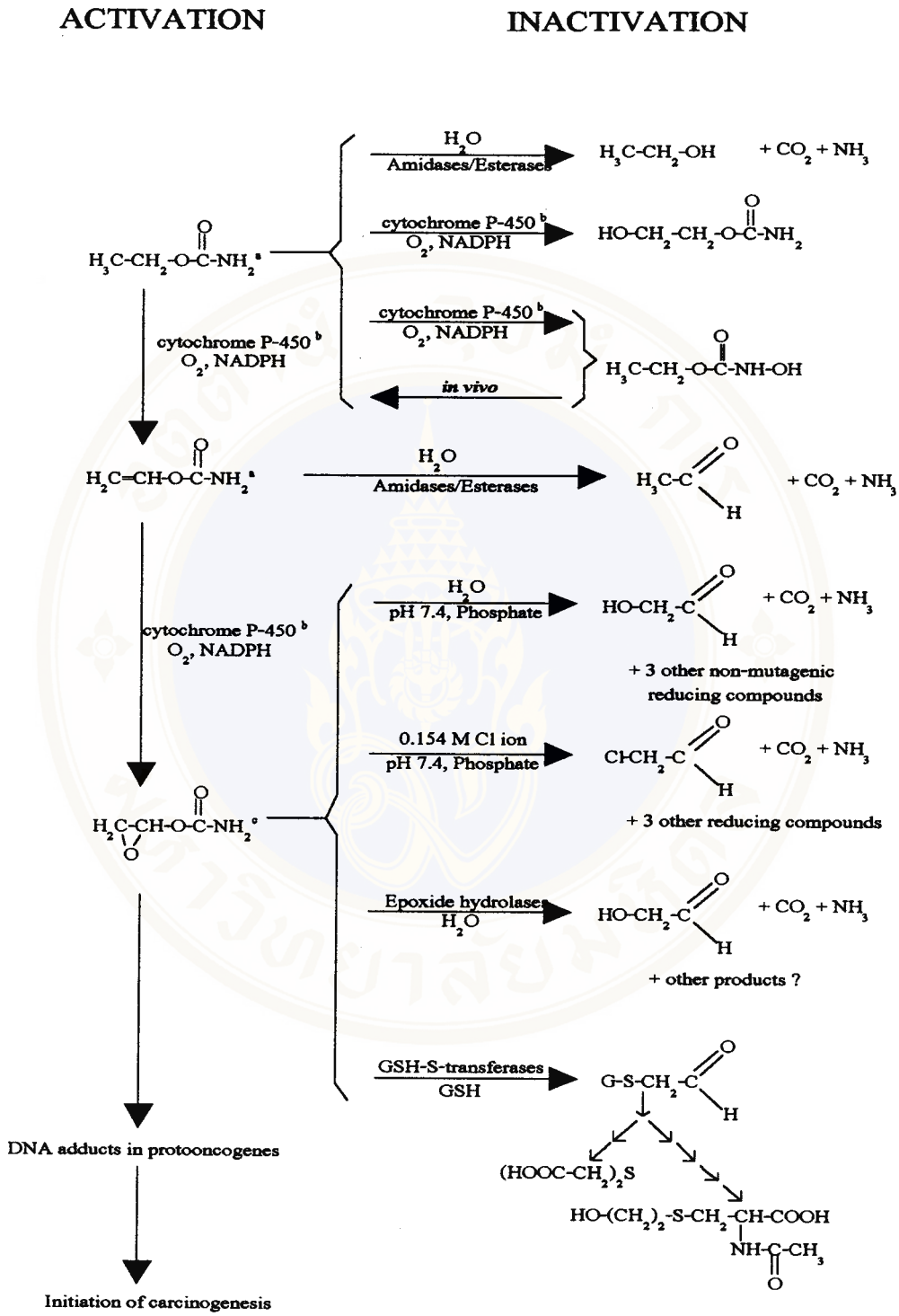


Figure 6. Known and probable activation and inactivation pathways of metabolism of urethane (ethyl carbamate), vinyl carbamate and vinyl carbamate epoxide. (**a**) Mouse liver microsomes + ethyl carbamate or vinyl carbamate + adenosine → 1,N^6-ethenoadenosine.(**b**) Human liver microsomal cytochrome P450 IIE1.(**c**) Vinyl carbamate epoxide + adenosine → 1,N^6-ethenoadenosine. GSH = glutathione (154).

2.10.4.3 Mutagenicity of of Urethane

Many studies were published concerning the mutagenicity of urethane in a wide range of organisms (157,158). In tests with eukaryotic cells, positive and negative findings were about equal in frequency. It seems that positive results are obtained only under conditions of appropriate metabolic activation. Urethane was genotoxic in the somatic mutation and recombination test in *Drosophila melanogaster* (number and shape of wing hairs after treatment of larvae), in a standard strain and in a strain in which genetic control of cytochrome P-450-dependent enzyme systems were altered (constitutively increased P-450 enzyme activities) (145,159). The effects were dose-dependent and the modified strain was more sensitive to urethane by about one order of magnitude than the standard strain. This further suggests that the P-450 enzyme system is involved in the activation of urethane. More than 10 publications are available with quantitative data on DNA-adduct formation by urethane. Several authors proposed a metabolic pathway which leads to the formation of vinyl carbamate and, after epoxidation, to DNA and RNA adduct (151,155,160). Since 7-(2-oxoethyl)-guanine is also formed by vinyl chloride (161). In bacterial test systems the results were mainly negative. An explanation for this may be that in the standard Salmonella/microsome assay, using rat-liver S-9, there was insufficient oxidation of urethane to vinyl carbamate (the first step in the metabolic activation) to give positive results. The fact that vinyl carbamate gave positive results in the Ames test (162) strongly supports this hypothesis. In addition, there is a vast literature on urethane carcinogenicity (153,163-165) and urethane is a pluripotent carcinogen with respect to tumor induction in different species, organs, and the stages of development of the animals (166).

2.10.4.4 Modification the Mutagenicity of Urethane

A number of compounds have been found to be good inducers of cytochrome P-450 subtype 2E1 (CYP2E1) including ethanol which can increase the metabolism of urethane or decrease it depending upon the condition of exposure (167,168). Acute administration of high doses of ethanol may postpone the metabolism of urethane, possibly by blocking metabolizing enzymes, including the group of cytochrome P450 (169,170). Chronic administration of ethanol, in contrast to the acute situation, may lead to induction of metabolizing enzymes systems such as P-450 (171) and thus modulated the carcinogenicity of urethane. Mirvish (153) reported that degradation of urethane was inhibited up to 90 % by blocking esterase activity, which indicated that ethanol may be formed in near equimolar amounts to the administered urethane dose. It remains to be shown whether the ethanol thus formed can modulated the further metabolism of urethane. Kurata *et al.* (172) demonstrated that acetone was a very potent, acute inhibitor of the *in vivo* metabolism of urethane when metabolically derived from 2-propanol. Conversely, pretreatment using acetone for 24 and 48 hours before urethane administration accelerated the clearance of urethane, indicating that enzyme metabolizing urethane was induced by acetone. Urethane is also metabolized by esterase to yield ethanol, carbondioxide and ammonia. Carlson (173) shown that the cytochrome P-450 inducers, phenobarbital and β-naphtholflavone, and esterase inhibitor, paraoxon, were without effect (to the conversion of (carbonyl-^{14}C) urethane to $^{14}CO_2$) while the CYP2E1 inhibitor, diethyldithiocarbamate, decreased metabolism to about 3% of control. Ethanol administered acutely inhibited urethane metabolism. Pyridine, shown previously to enhance this metabolism in microsomal preparations, greatly inhibited it *in vivo*.

Kemper *et al.* (174) investigated the role of glutathione in protection against vinyl carbamate epoxide-mediated adduct formation, and the involvement of glutathione-S-transferase in detoxification of vinyl carbamate epoxide. They reported that glutathione inhibited formation of ethenoadenosine, a measure of vinyl carbamate epoxide toxicity, in a concentration-dependent manner at concentration ranging from 1 to 8 mM. This effect was significantly enhanced by addition of rat liver glutathione-S-transferase. In addition, pretreatment of mice with 1% dietary butylated hydroxyanisole (BHA) caused parallel increases in cytosolic glutathione-S-transferase activity and cytosolic enhancement of detoxification of vinyl carbamate epoxide by glutathione. The major conclusions of the study were (1) vinyl carbamate epoxide could be detoxified by spontaneous conjugation with glutathione, (2) conjugation of vinyl carbamate epoxide with glutathione could be catalyzed by glutathione-S-transferase, (3) pretreatment with BHA protected against binding of active urethane metabolites *in vitro* and *in vivo*, and (4) the protective effect of BHA against urethane metabolite was mediated by increases in glutathione-S-transferase activity and glutathione concentration. De flora *et al.* (175) reported that N-acetylcysteine (NAC), a precursor of intracellular glutathione, induced a significantly increase in oxidizing glutathione reductase activity in rat liver preparations and counteracted the mutagenicity of direct acting compound (such as epichlorohydrin, hydrogen peroxide), as a result of its reducing and scavenging properties. At high concentrations, NAC completely inhibited the mutagenicity of procarcinogens such as cyclophosphamide, cigarette smoke condensate and aflatoxin B_1 by binding their electrophilic metabolites. In contrast, decreasing NAC concentrations stimulated their metabolic activation, especially when liver preparations from enzyme-induced rat were used. In addition,

when administrated with the diet, NAC markedly inhibited the induction of lung tumors in mice by urethane (175,176). Abraham *et al.* (177) investigated the change in glutathione-S-transferase activity in relation to the observed in vivo antigenotoxicity of fresh vegetables, spices, tea and coffee. This experiment showed that treatment with urethane alone resulted in inhibition of glutathione-S-transferase activity. In addition, only coffee could moderately increase glutathione-S-transferase activity, while extracts of vegetables, spices and tea did not exert any significant effect on glutathione-S-transferase level. However, combination of urethane with extracts of vegetables, spices and coffee attenuated the inhibitory effects observed with urethane alone in the mouse bone marrow micronucleus test. They concluded that there was no indication of a firm association between the observed protection against genotoxicity and glutathione-S-transferase activity (177).

A dose-dependent increase in the genotoxic activity of urethane was observed in SMART (145). The frequency of induction of mutations in the modification strain with increased P-450 enzyme activities was increased by about one order of magnitude compared with the standard strain. The frequencies of spots per wing in high bioactivation cross were higher than those of standard cross (144). This might result from the constitutive expression of the enzymes required for the transformation of urethane into ultimate genotoxic metabolites.

STATEMENT OF THESIS

Shrimp is a high commercial value export product due to the strong international market demand. Meanwhile, it is subjected to some oxidative processes seriously which affect the visual quality of the product. Melanosis of the shrimp carapace is a natural phenomenon caused by enzymic reactions that start as soon as they are drawn from the water and come into contact with atmospheric oxygen. 4-Hexylresorcinol (4-HR) is a synthetic phenolic compound containing antioxidative property. It shows a marked ability to inhibit or slow down melanosis in shrimp. Several workers reported that some phenolic compounds exhibited antimutagenic activity in a wide range of assays both *in vitro* and *in vivo*. 4-HR may have a possible role as an antimutagen. It is, therefore, of interest to elucidate the modulating effect of 4-HR on the mutagenicity of some mutagens using short term tests.

EXPERIMENT OBJECTIVES

General Objective

To determine the antimutagenicity of 4-hexylresorcinol (4-HR).

Special objectives

1. *In vivo* study using somatic mutation and recombination test

1.1 To determine the modulating effect of 4-HR simultaneously administered on urethane induced wing spot formation of *Drosophila melanogaster*.

1.2 To determine whether 4-HR pretreatment to parent flies could counteract the effect of urethane in wing spot mutation in the progeny flies.

2. *In vitro* study using Ames test

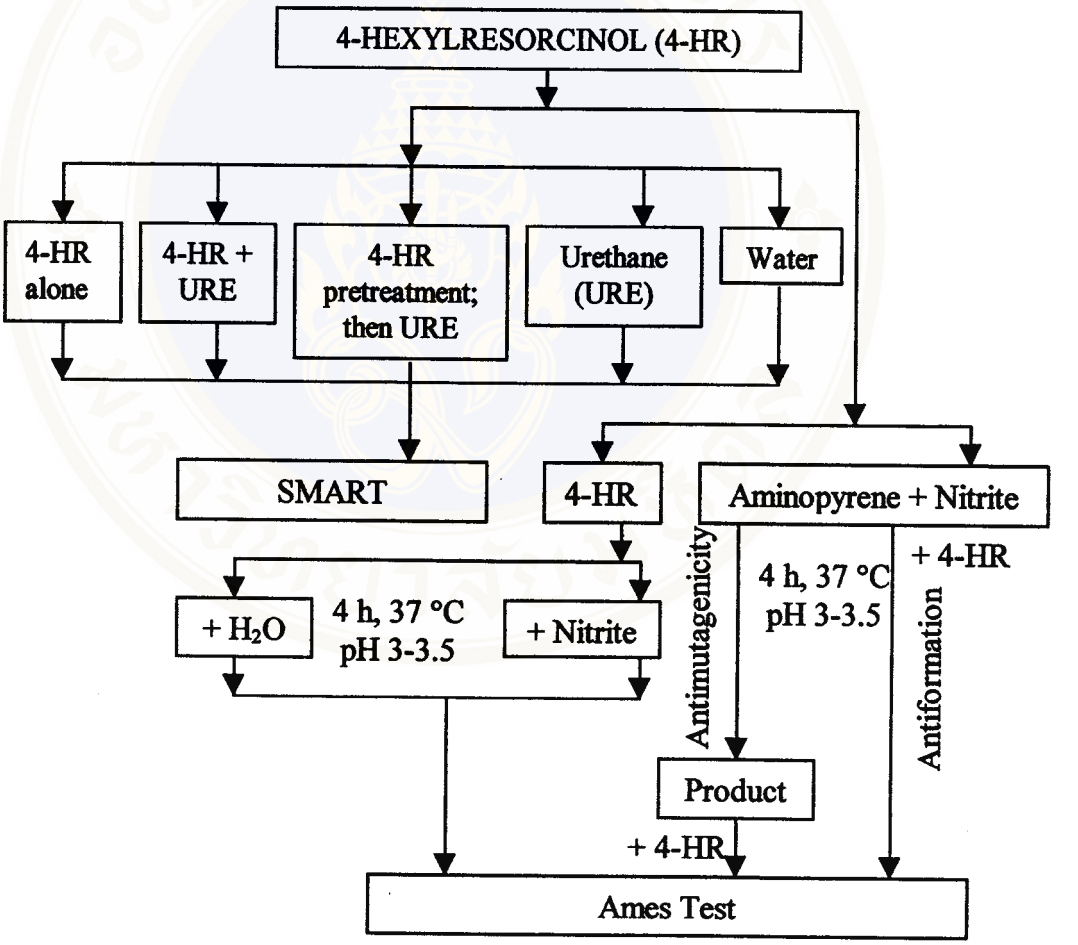
2.1 To determine the modifying effect of 4-HR incorporated into aminopyrene-nitrite mixture at the beginning of the reaction on the formation of mutagenic products.

2.2 To determine the effect of 4-HR on the mutagenicity of aminopyrene-nitrite reaction product after a 4 h incubation.

CHAPTER III

MATERIALS AND METHODS

3.1 Experimental design



3.2 SMART Test

3.2.1 Chemicals and samples

Urethane (URE) and 4-hexylresorcinol (4-HR) were purchased from Sigma Chemical (St. Louis, MO, USA). Glycerol was bought from Farmitalia Carlo Erba S.p.A. Arabic powder was purchased from BDH chemical Ltd., (Poole, England). Chloral hydrate was supplied by Srichand United Dispensary Co., Ltd., (Thailand). *Drosophila* medium was modified from the formular of Robert (178). Sugar (0.20 g), agar (0.028 g), yeast (0.10 g) and corn flour (0.25 g) were blended and boiled in a 15 ml test tube containing 2 ml water or test solution until sticky. Propionic acid (0.01 ml) was added as a preservative. Then, it was left for 10 min and covered with a cotton plug. Other chemicals were of laboratory grade.

3.2.2 Mutagenicity Assay

The mutagenesis assay was carried out according to the method described by Graf *et al.* (128,179). Virgin females of the DDT-resistant Oregon R (R) strain *Drosophila melanogaster* were allowed to mate with males of *mwh/mwh* strain to produced trans heterozygous (*mwh+/+flr*^{3}) larvae of improved high bioactivation cross (IHB). The three-day old larvae (72 h) were collected, washed with water and transferred (with the help of a fine artist's brush) to glass tubes with medium containing 4-HR or distilled water as negative control. Each 4-HR solution was tested for its mutagenicity by mixing 4-HR solution with *Drosophila* medium. Final concentrations of 4-HR were 50, 500 and 5000 ppm. Both control and treated larvae were allowed to feed on the culture medium at 25±1°C for 48 h until pupation. After metamorphosis, the survival flies were collected from the tubes between days 10-12 after egg laying and stored in 70%

ethanol. Only the insect bearing the marker trans-heterozygous (*mwh+/+flr*^{3}) indicated with round wings were collected as suggested by Graf and van Schaik (147) and their wings were mounted on microscope slides (described below); thus they were examined for the mutant spots.

3.2.3 Antimutagenicity Assay

Urethane (4500 ppm) was used as a positive control by mixing it with the fly medium. The final concentrations of 4-HR in the fly medium were 50, 500 and 5000 ppm. Three-day old larvae were transferred to test tube containing *Drosophila* medium and test compound (Figure 7). For each treatment, the test concentration of urethane (given either alone or in combination with 4-HR solution) was selected as suggested by Graf and Wurgler (179), Graf *et al.* (180) and Abraham (131). Experiment was proceeded as described in 3.2.2.

3.2.4 Effect of 4-HR Pretreatment to Parent flies on Urethane Mutagenicity

Virgin females of the DDT-resistant Oregon R (R) strain were allowed to mate with males of *mwh/mwh* strain. They were transferred to the medium containing 4-HR (50, 500 or 5000 ppm) and allowed to lay eggs. Larvae were fed on 4-HR for 3 days; then they were transferred to test tubes containing *Drosophila* medium and urethane (4500 ppm) (Figure 8). Both control and 4-HR treated larvae were treated with urethane at 25$\pm$1°C for 48 h until pupation. Experiment was proceeded as described in 3.2.2.

3.2.5 Wing Preparation and Microscopic Analysis.

The flies in 70% ethanol were washed with distilled water and placed in a drop of Faure's solution (30 g gum arabic, 20 ml glycerol, 50 g chloral hydrate and 50 ml

distilled water) as suggested by Graf *et al.* (128). Wings were separated from the body; with a fine paintbrush they were lined up on a clean slide. They were allowed to spread out. A droplet of Faure's solution was dropped on the slide and a cover slip was put on. A permanent preparation was obtained by sealing the cover slip with nail polish. Next, the wings were analyzed under a compound microscope at 400 x magnification The position of the spots was noted according to the sector of the wing (see Figure 9). Different types of spots namely, single spots showing either the multiple wing hairs *(mwh)* or the flare (*flr*^{3}) phenotype, and twin spots showing adjacent *mwh* and *flr*^{3} areas were recorded separately. The size of each spot was determined by counting the number of wing cells (hairs) exhibiting the mutant phenotype. The spots were counted as two spots if they were separated by three or more wide-type cell rows. Multiple wing hairs (*mwh*) were classified when a wing cell contained three or more hairs instead of one hair per cell as in wide-type (see Figure 10). Flare wing hair exhibited a quite variable expression, ranging from pointed, shortened and thickened hairs to amorphic, sometimes balloon-like extrusions of melanolic chitinous material (see Figure 10).

3.2.6 Data Evaluation and Statistical Analysis

3.2.6.1 Mutagenicity test

The wing spots data were evaluated by the statistical procedure described by Frei and Wurgler (181). Briefly, induction frequencies of wing spots of color treatment groups were compared with those of negative control group (water). The spots were grouped accordingly to the following 3 types: (1) small single spots of 1 or 2 cells in size (2) large single spots of 3 or more cells, and (3) twin spots. The estimation of spot frequencies and confidence limits of the estimated mutation frequency were performed

with significance level of $\alpha = \beta = 0.05$. A multiple-decision procedure was used to decide whether a result was positive, weakly positive, inconclusive or negative according to Frei and Wurgler (181). Statistical considerations and calculation step by step are shown in Appendix.

3.2.6.2 Antimutagenicity test

Antimutagenicity of 4-HR was estimated as suggested by Negishi *et al.* (132). Relative spot induction frequencies of co-administration of each concentration of 4-HR and a standard mutagen were compared with those of a standard mutagen group. Relative spot induction frequencies were calculated from the number of induced spot per wing subtracted by the value of the control group. For example, in Table 10, 100% of relative spot induction frequency of urethane of small single spots corresponds to 15.04-0.24 =14.8 (number of induced spot per wing), and 59 % relative spot induction frequency of co-administration of 4-HR (50 ppm) with urethane (4500 ppm) corresponds to 9.01-0.24 = 8.77.

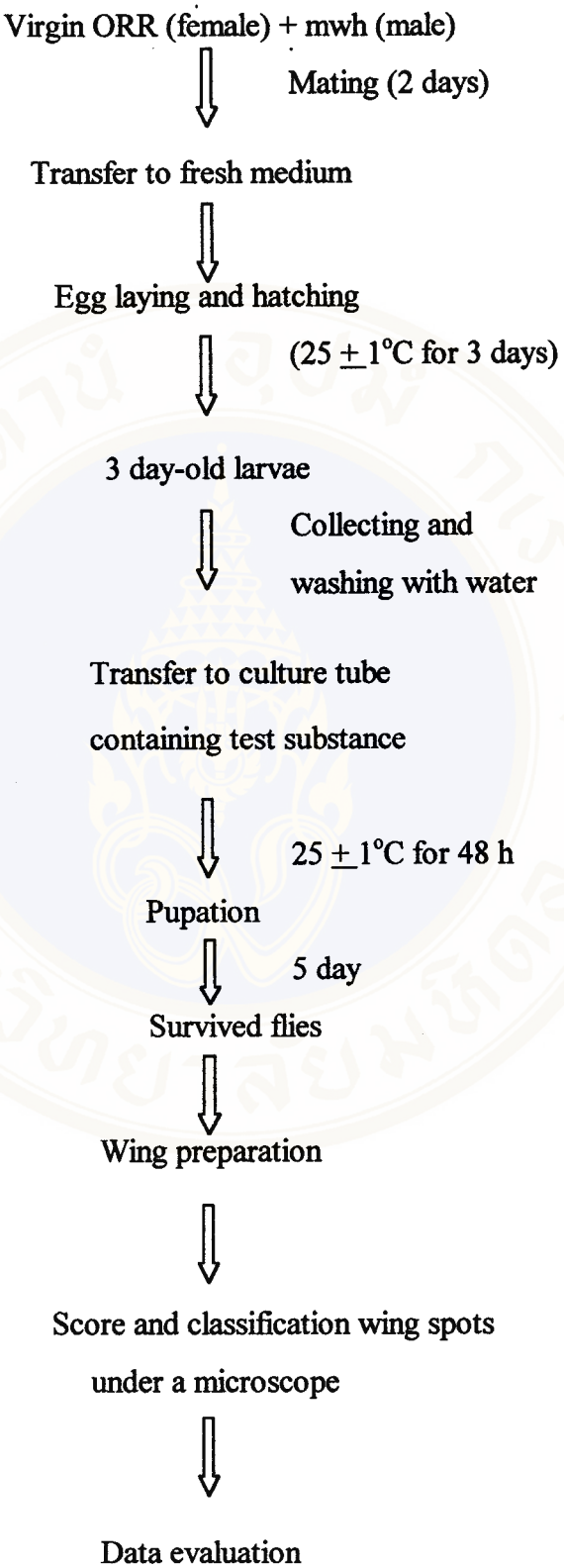


Figure 7. Mutagenicity testing and antimutagenicity testing

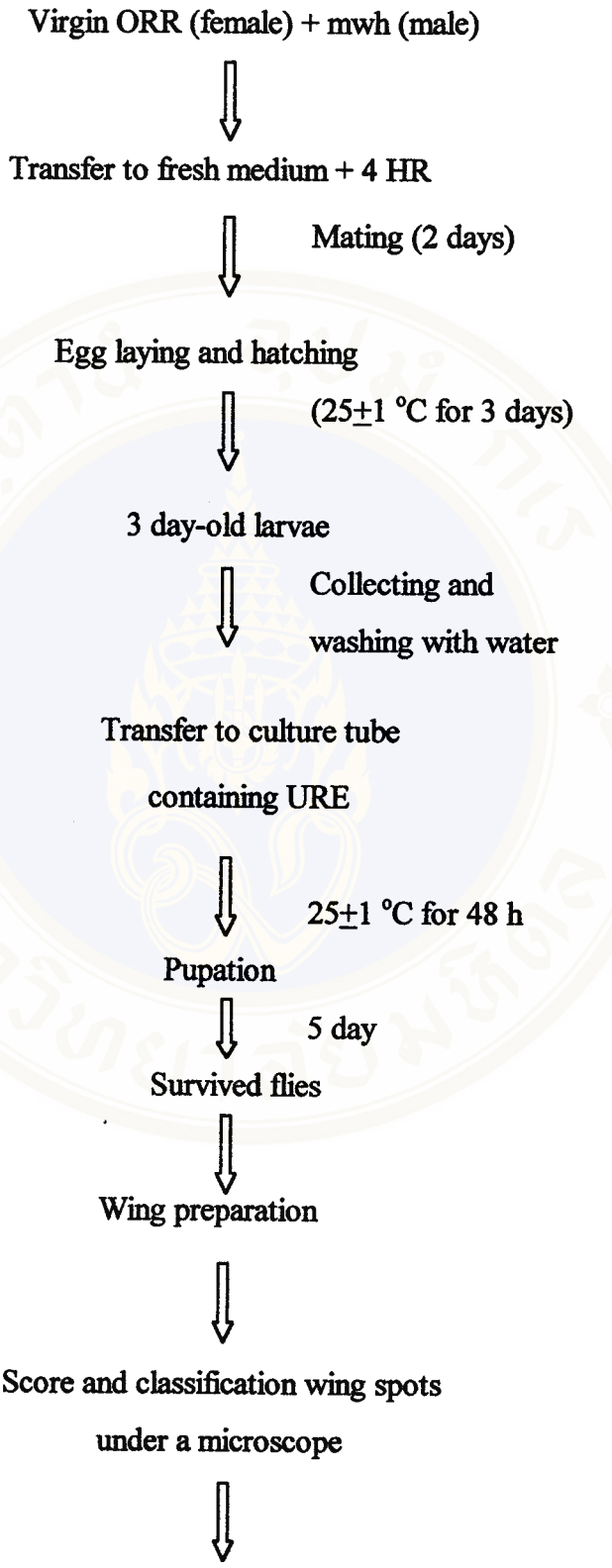


Figure 8. Metabolic activation study of 4-hexylresorcinol on mutagenicity of urethane in *Drosophila melanogaster*.

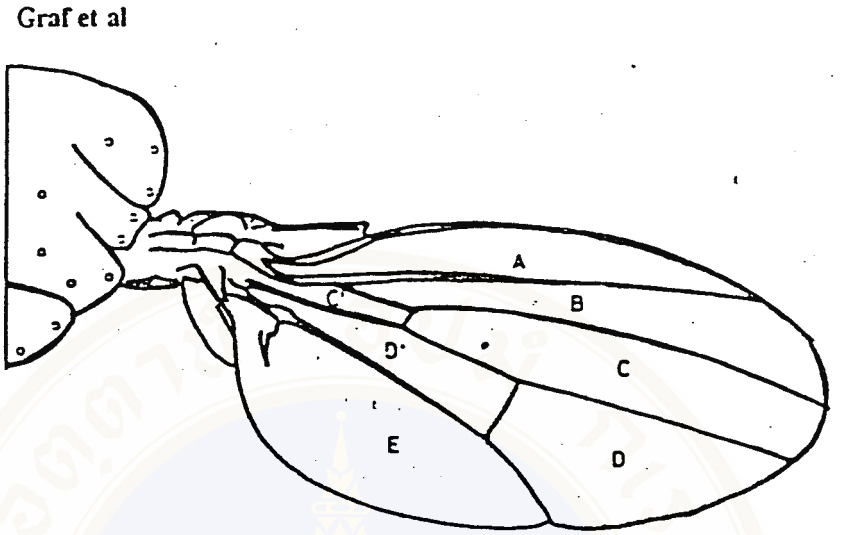


Figure 9. Normal half mesothorax showing the regions A-E of the wing surface scored for spots according to Graf *et al.* (128).

Figure 10. Trichomes on the wing blade, a) normal, b) deviate trichomes not counted as *mwh or flr*^{3}, c) configurations indicative of *mwh*, d) typical manifestation of *flr*^{3} (128).

3.3 AMES Test

3.3.1 Chemicals

1-Aminopyrene (Aldrich, St.Louis, U.S.A.), 4-hexylresorcinol (4-HR), d-biotin, ammonium sulfamate ($NH_2SO_3NH_4$), were purchased from Sigma Chemical Company (St. Louis, Missouri, U.S.A.). L-Histidine monohydrochloride, sodium chloride (NaCl), hydrochloric acid (HCl), magnesium sulfate heptahydrate ($MgSO_4.7H_2O$), citric acid monohydrate GR ($C_6H_8O_7.H_2O$), potassium chloride (KCl) and di-sodium hydrogenphosphate (Na_2HPO_4) were supplied by E. Merck (Darmstadt, Germany). D (+)-Glucose monohydrate, crystal violet indicator, di-sodium ammonium hydrogen phosphate tetrahydrate GR ($NaNH_4HPO_4.4H_2O$) were bought from Fluka AG (Buch, Switzerland). Bacto agar was purchased from Difco Laboratory (Detroit, Michigan, U.S.A.). Oxoid nutrient broth No.2 was supplied by Oxoid Ltd., (Basingstoke, Hants, England). Sodium di-hydrogen phosphate ($NaH_2PO_4.2H_2O$) was furnished by May & Baker Ltd., (Degenham, England). Ampicillin sodium was furnished by Verco pharmaceutical Ltd. (Bangkok, Thailand). Sodium hydroxide (NaOH), di-potassium hydrogen phosphate anhydrous (K_2HPO_4), and sodium nitrite ($NaNO_2$) were purchased from BDH chemicals Ltd., (Poole, England).

3.3.2 The Bacterial Tester Strain

Salmonella typhimurium tester strains used in this study were histidine-dependent strains (His-) TA98 and TA100 which were capable of detecting frameshift mutation and base-pair substitution, respectively, (Both strain were kindly provided by Dr.

Wannee Kusamran, National Cancer Institute, Ministry of Public Health Thailand). The tester strains were manipulated as suggested by Maron and Ames (117). Overnight cultures of bacteria inoculated from frozen stock culture in oxoid nutrient broth No. 2 at 37 °C were used for mutagenesis assay see in Appendix. Cultures were kept in refrigerator until use and were used within 24 h.

3.3.3 Nutrient Agar

3.3.3.1 Preparation of minimal agar plate

Minimal agar containing 1.5% Bacto-Difco agar was autoclaved and then it was mixed with 2% sterile glucose and Vogel-Bonner medium E (See in Appendix). About 30 ml of molten agar was poured on to the sterile petri dish. It was left until solidified and was stored at 37 °C in the incubator.

3.3.3.2 Preparation of top agar

Top agar containing 0.6% Bacto-Difco agar and 0.5% Sodium chloride was autoclaved and was stored at 45 °C. Before use, 10% of a sterile solution of 0.5 mM. histidine and biotin was added to the molten top agar and then it was maintained at 45 °C in the water bath

3.3.4 Sample preparations

4-HR solution was prepared to be the concentrations ranged from 0.625 mg/ml to 5 mg/ml in dimethylsulfoxide (DMSO). The combined solutions were vortex-mixed. 4-HR solution for testing was freshly prepared before each experiment.

3.3.5 1-Aminopyrene-Nitrite Model (AP-Nitrite model)

Aminopyrene treated with nitrite in acid solution was used as a standard mutagen because it has been shown to express direct-acting mutagenicity (182-186). Briefly explain, appropriate volume (10 µl for testing on *Salmonella typhimurium* TA98 and 40 µl for TA100) of aminopyrene (0.0375 mg/ml) in a tube fitted with a plastic stopper was mixed with 0.2N hydrochloric acid (sufficient to acidify the reaction mixture to pH 3.0-3.5) and 0.25 ml of 2M sodium nitrite. The volume was adjusted to be 1,000 µl giving the final concentration of nitrite was 500 mM. The reaction tube was shaken at 37 °C for 4 h and then it was stopped by placing the tube in an ice bath. In order to decompose the residual nitrite, 0.25 ml of 2M ammonium sulfamate was added to the reaction mixture; then it was allowed to stand for 10 min in an ice bath. The mixture (100 µl) was mixed with 0.5 ml phosphate buffer (pH 7.4), 100 µl of fresh overnight culture of tester strain and incubated at 37 °C in a shaking water bath for 20 min. After incubation, 2 ml of molten soft agar (45 °C) was added. It was mixed well and poured onto a minimal glucose agar plate. The plate was rotated to achieve uniform colony distribution and incubated at 37 °C in the dark for 48 h. After the incubation period, His^+ revertants colonies were counted.

3.3.6 Mutagenicity Study of 4-Hexylresorcinol

The concentrations of 4-HR were 0.625 mg/ml, 1.25 mg/ml, 2.5 mg/ml and 5 mg/ml. A specified concentration of 4-HR solution (0.1 ml) was added to the test tube containing 0.65 ml 0.0005 N HCl and 0.25 ml distilled water. The reaction tube was incubated at 37°C for 4 h. The reaction was stopped by placing the tube in an ice bath for 1 min; then, 0.25 ml 2 M ammonium sulfamate was added to the reaction mixture.

The reaction tube was immersed in an ice bath for 10 min and 0.5 ml of 0.2M phosphate buffer (pH 7.4) was added afterward. The overnight bacterial culture (0.1 ml) shaken at 37 °C for 16 h was added into the reaction tube. The reaction mixture was mixed gently and incubated in a shaking water bath at 37 °C for 20 min before 2 ml of molten top agar contain 0.05 mM each of L-histidine and D-biotin were added, mixed gently and poured onto a minimal glucose agar plate. The histidine revertant colonies were counted after incubation at 37 °C for 48 h. Each concentration was assayed in duplicated and done twice.

Positive and negative controls were included in each test. The negative control was DMSO. The positive control was prepared as describe in 3.3.5. Killing effect of each compound on tester strains was determined by the growth inhibition of the background lawn under a light microscope.

3.3.7 Mutagenicity of Nitrite Treated 4-Hexylresorcinol

To determine the mutagenicity of nitrite treated 4-HR were prepared to be 0.625 mg/ml, 1.25 mg/ml, 2.5mg/ml and 5mg/ml. Sample solution (0.1 ml) was added to the test tube containing 0.65 ml 0.2 N HCl and 0.25 ml 2 M sodium nitrite to obtain the final volume of 1,000 µl (Figure 11). The stoppered tube were incubated at 37°C for 4 h. Then, it was immersed in an ice bath for 1 min and 0.25 ml 2 M ammonium sulfamate was added to the reaction mixture and the reaction tube was left in an ice bath for another 10 min before mutagenicity assay.

3.3.8 Effect of 4-Hexylresorcinol on the Formation of Mutagen of Aminopyrene-Nitrite Mixture.

The experiment was also planned to find out whether 4-HR could interfere with the formation of direct mutagen in gastric liked pH. As shown in Figure 12, 0.1 ml of various concentrations of 4-HR solution were introduced to the tubes containing 10 µl (when TA98 was used) or 40 µl (when TA100 was used) of aminopyrene (0.0375 mg/ml), 0.25 ml 2 M sodium nitrite, 0.2 N hydrochloric acid (sufficient to acidify the reaction mixture to pH 3.0-3.5) and the final volume was 1,000 µl. All tubes were proceeded as described in 3.3.7 in order to evaluate the mutagenicity.

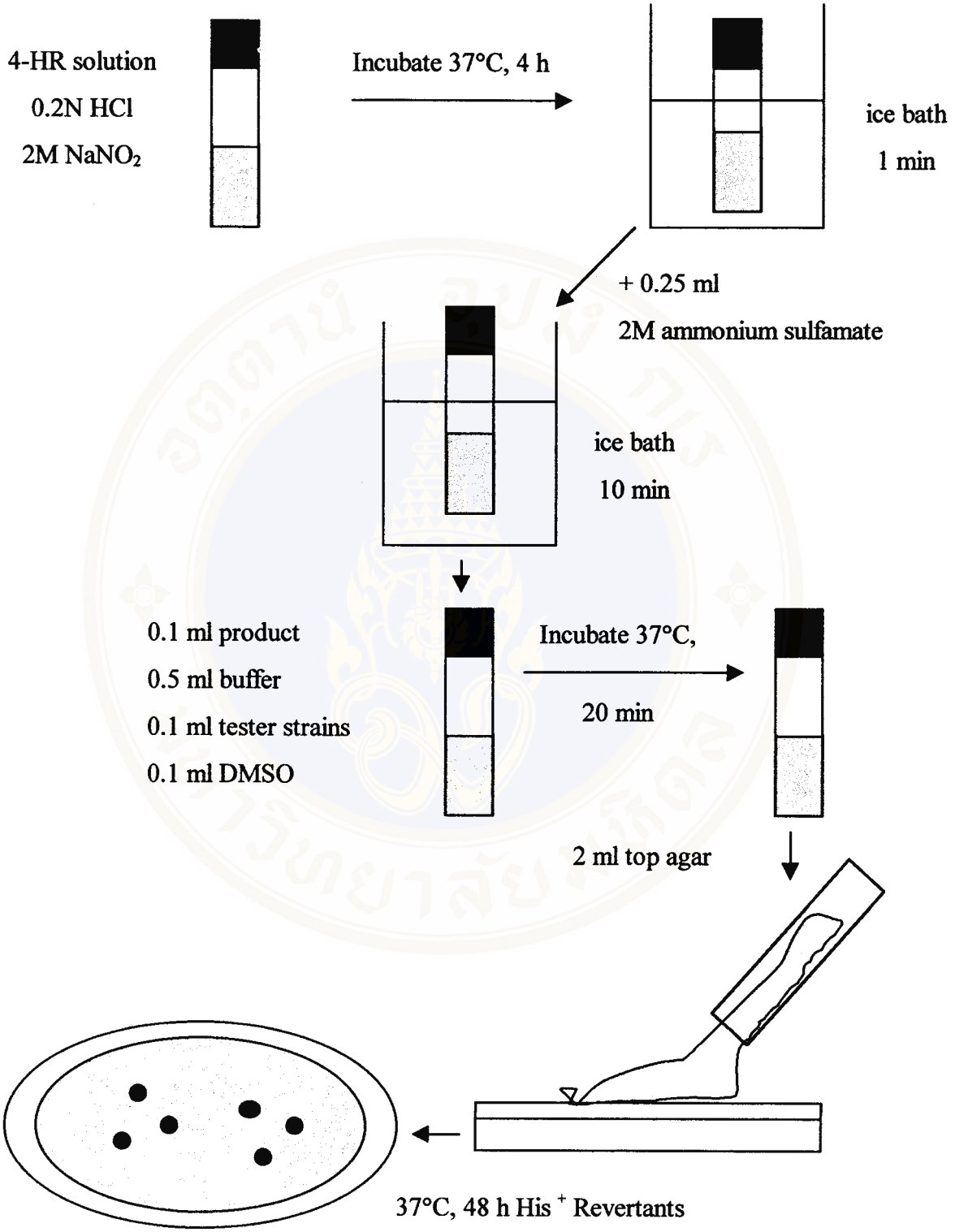


Figure 11. Direct mutagenicity evaluation of 4-hexylresorcinol using the Ames *Salmonella* mutagenicity test (pre-incubation modification).

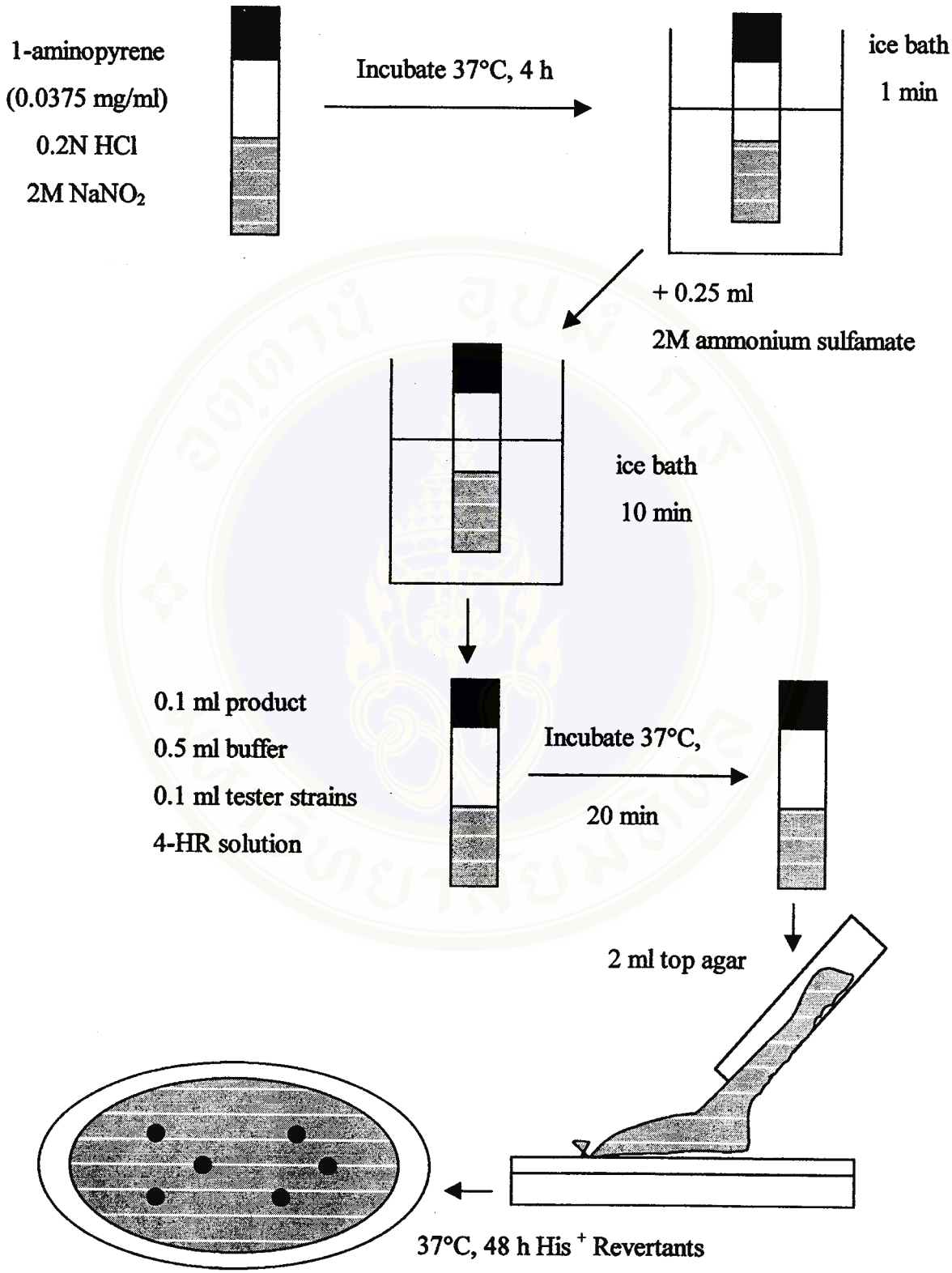


Figure 12. Steps in studying the effect of 4-hexylresorcinol on the mutagenicity of 4 hours incubation product of aminopyrene-nitrite model.

3.3.9 Effect of 4-Hexylresorcinol on the mutagenicity of 4 h Aminopyrene-Nitrite Product

4-HR solution (0.1 ml) was transfered into a sterile plastic stoppered tube containing 0.1 ml of AP-nitrite mixture (prepared as described in 3.3.5), 0.5 ml of 0.2M sodium phosphate buffer, pH 7.4 and 0.1 ml of overnight bacterial culture (Figure 13). The reaction tube was mixed gently and incubated in a shaking water bath at 37 °C for 20 minutes. Then 2 ml. of molten top agar containing 0.05 mM each of L-histidine and D-biotin were added, mixed gently and poured onto a minimal glucose agar plate. The His^+ revertant colonies were scored after incubation at 37 °C for 48 h. Percent inhibition of the mutagenicity was calculated as suggested by Calomme *et al.* (124):

$$\% \text{ inhibition} = 100 - \frac{\text{number of revertants with test compound}}{\text{number of revertants without test compound}} \times 100$$

Inhibition more than 60%, 60-40, 40-20% and 20-0% were classified as strongly active, active, weakly active and no effect, respectively. Whereas, increase of mutagenicity 0-20%, 20-40%, 40-60% and more than 60% were classified as no effect, weak enhancement, enhancement, strong enhancement, respectively.

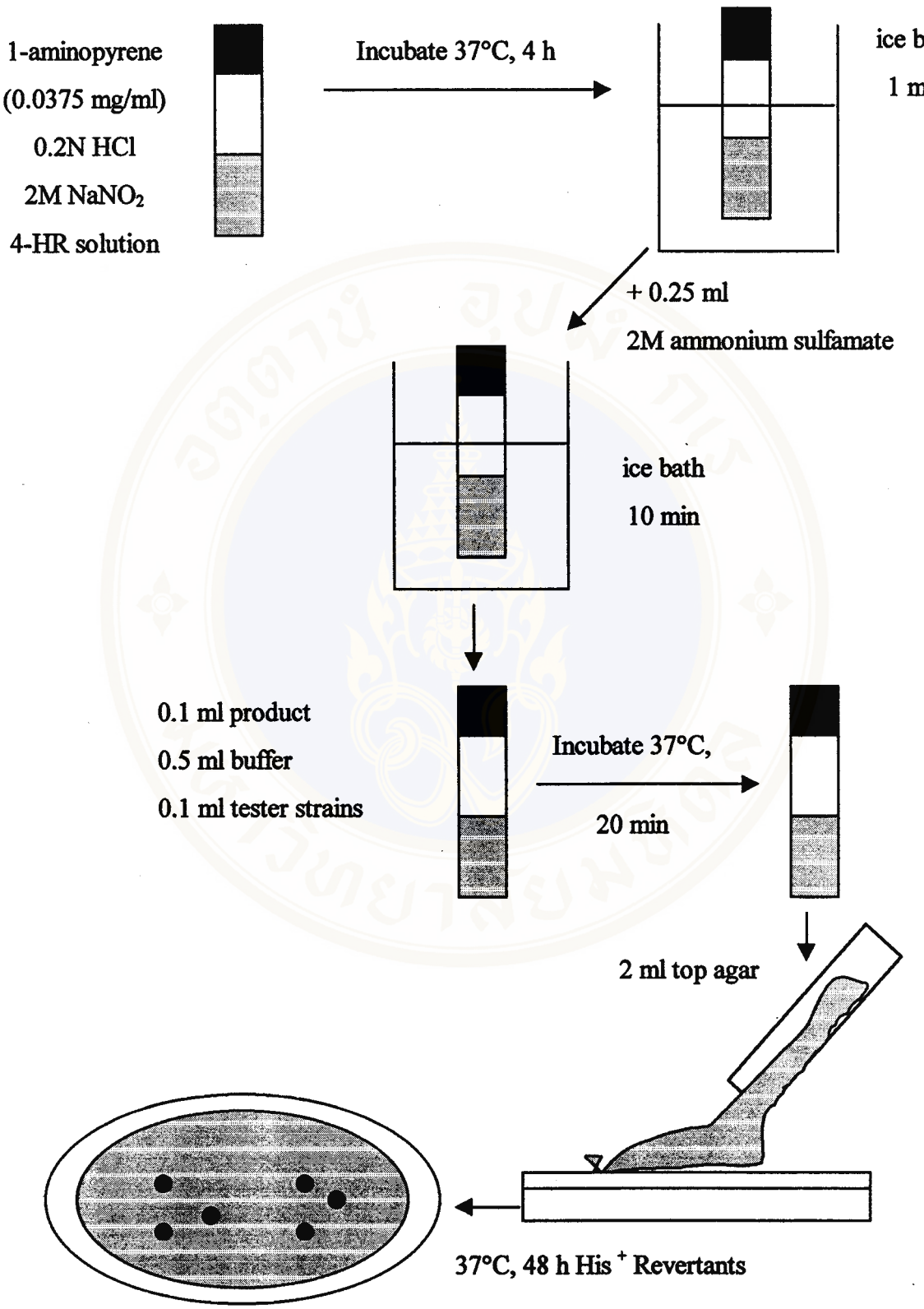


Figure 13. Steps in studying the effect of 4-hexylresorcinol on the 4 hours incubation aminopyrene-nitrite reaction.

CHAPTER IV

RESULTS

The first main aim of this study was to determine whether 4-hexylresorcinol (4-HR) affects the *in vivo* mutation and mitotic recombination of somatic cells by chemical mutagens. The somatic mutation and recombination test was employed. In addition, it is well established that *Drosophila* possesses a versatile system for the metabolic of xenobiotics. *Drosophila* has detoxification-activating systems in many respects closely resembling the corresponding systems in mammals, which makes it possible to extrapolate data to mammals. All experiments were chronic feeding studies (48 h). The spontaneous clone induction frequency expressed as total mutant spots per wing was 0.30. Urethane (URE) (4500 ppm) used as positive standard mutagens expressed the total mutant spots per wing of 19.75. These values were statistically significant according to the method described in Chapter III. In order to determine whether the *in vitro* expression mutagenicity and inhibitory effect on aminopyrene treated with nitrite during incubation in acid pH condition toward *Salmonella typhimurium* strains TA98 and TA100 in the absence of metabolic activation. Because nitrite is used as a preservative in many foods and it can convert some food constituents to be mutagen during the condition similar to gastric digestion.

4.1 Survival of Larvae and Adult Flies

Table 8 shows the number of larvae and adult flies survived from the treatment with 4-HR alone or the combination of 4-HR and urethane. It is shown that percent survival of flies of negative control is 80%.

Effect of 4-HR on the number of fly survival varied. Percents of fly survival are between 60% and 83% whereas, percents of fly survival of larvae treated with the combination of urethane (4500 ppm) and 4-HR (50 ppm to 5000 ppm) are between 48% and 60%.

Table 8. Number of survival trans-heterozygous (*mwh+/+flr*^{3}) adult flies from 200 larvae introduced to the medium containing (water) or 4-HR for 3 days; then they were fed on medium containing urethane

Control	4-HR	Number of survival	Percent of survival	Number of round wings[a]
Water	-	161	80	129
Water	50 ppm	167	83	186
Water	500 ppm	162	81	165
Water	5000 ppm	121	60	92
4500 ppm URE[b]	-	108	54	81
4500 ppm URE	50 ppm	117	58	123
Pretreatment	500 ppm	97	48	81
	5000 ppm	120	60	82

[a] = Some wings were damage during slide preparation.

[b] = larva were fed on urethane until they became adult flies.

Table 9 shows the number of larvae and adult flies survived from the treatment of 4-HR alone or combination of 4-HR and urethane. Percents of fly survival of flies treated with 4-HR (50 ppm to 5000 ppm) to combination treated with urethane (4500 ppm) are between 11-55%, whereas, percents of fly survival of positive control was 54%. This value was similar to those obtained with 50 ppm and 500 ppm 4-HR

Table 9. Number of the survival trans-heterozygous (*mwh+/+flr*^{3}) adult flies of 4-hexylresorcinal pretreated group before administration of urethane

Control	Concentration of 4-HR pretreatment	No. of larvae [a]	No. of fly survival	Percent of fly survival	No. of round wings[c]
Water	0	ND	161	-	129
	50 ppm	ND	74	-	84
	500 ppm	ND	86	-	104
	5000 ppm	ND	42	-	50
4500 ppm	0	200	108	54	81
URE	50 ppm	200	111	55	98
	500 ppm	200	111	55	86
	5000 ppm	200	22 [b]	11	2

ND = not determined

[a] = obtained from 5 pairs of parent flies

[b] = toxicity indicating by smaller size (about 0.5 of normal size) and some pupa did not transform to be adult

[C] = Some wings were damage during slide preparation.

4.2 Mutagenicity of 4-Hexylresorcinol and Effects of 4-Hexylresorcinol on Wing Spot Induction of Urethane

The data presented in Table10 show the frequencies of different categories of wing spots induced by three concentrations (50, 500 and 5000 ppm) of 4-HR. It is shown that 4-HR did not increase the frequency of mutant spots ($p<0.05$). Frequencies of total spots induced by 4-HR are within the range of 0.17 to 0.24 with no toxic effect while spontaneous frequency is 0.30.

The reduction of wing spots induced by urethane was observed when 4-HR to the larvae was simultaneously administered with urethane. 4-HR (50, 500 and 5000 ppm) reduced the number of small single spots, large single spots, twin spots and total spots compared with those of urethane treated group. The relative induction frequency of total spots formation was 55%, 52% and 16%, respectively. It was thus indicated that increasing concentrations of 4-HR decreased the incidence of small single spots, large single spots, twin spots and total spots of the relative induction frequencies.

4.3 Effects of 4-Hexylresorcinol Pretreatment on Wing Spot Induction of Urethane

Table 11 shows the frequencies of different categories of wing spots induced by 4-HR pretreatment (50, 500 and 5000 ppm) on Drosophila activating system. It is shown that 4-HR pretreatment (50 and 500 ppm) did not increase the frequency of mutant spots ($p<0.05$). Frequencies of total spots induced by 4-HR were within the range of 0.19 to 0.26 while spontaneous frequency was 0.30. It is noted that the highest

concentration of 4-HR (5000 ppm) was toxic to the larvae resulting in only two flies were available for analysis.

Urethane induced both small single spots and large single spots. Small numbers of twin spots were also induced. The administration of 4-HR pretreatment (50 or 500 ppm) to the larvae before the administration of urethane resulted in a remarkable reduce of frequency of mutant spots. The relative induction frequencies of small single spots formation were different in the groups that prefed with 50 or 500 ppm 4-HR. For total spots, modification on frequencies of mutant hair clones was not demonstrated in flies prefed with 50 ppm 4-HR (the relative induction frequency was 93%). The higher concentration of 4-HR (500 ppm) reduced the relative induction frequencies of wing spots to be 58% of urethane treated group. The highest concentration of 4-HR (5000 ppm) was very toxic to the larvae since smaller size of larvae and adult flies were obtained for mutant spot diagnoses as well as many pupae did not transform to be adult flies. However, the reductions of wing spot formation was less than those of the study on simultaneous feeding of URE with 4-HR.

4.4 Mutagenicity of 4-Hexylresorcinol Before and After Nitrite Treatment

4-HR was not mutagenic on both strains TA98 and TA100. Furthermore, no mutagenicity toward both TA98 and TA100 was observed after being treated with nitrite (Table 12). It is indicated that 4-HR was not toxic to Salmonella tester strains at concentrations up to 20 μg/plate. However the concentration of 40 μg/plate was toxic and expressed as partial killing effect on both strains.

4.5 Effect on 4-Hexylresorcinol on the Mutagenicity of Aminopyrene-Nitrite Product

4-HR inhibited the mutagenicity of the reaction product of mixture of nitrite and 1-aminopyrene in a dose-dependent manner on both strains TA98 and TA100 (Table 13). The mutagenicity of the model at the lowest testing amount (5 μg/plate) of 4-HR was inhibited (26%) only on TA98 and was increased to exhibit a partial killing effect with the highest testing amount (40 μg /plate). Maximum percent inhibition of 4-HR (at 20 μg/plate) were 35% on TA98 and 40% on TA100

Table 10. Inhibition of 4-hexylresorcinol on Wing Spot induced by 4500 ppm Urethane in trans-heterozygous (*mwh+/+flr*^{3}) adult flies

URE (ppm)	4-HR (ppm)	No. of wings	Small single spots			Large single spots			Twin spots			Total spots		
			No. found	Spots/ wing[a] m=2	% Rel.[b] frequency	No. found	Spots/ wing[a] m=5	% Rel.[b] frequency	No. found	Spots/ wing[a] m=5	% Rel.[b] frequency	No. found	Spots/ wing[a] m=2	% Rel.[b] frequency
4500	0	81	1218	15.04+	100	334	4.12+	100	48	0.59+	100	1600	19.75+	100
4500	50	123	1108	9.01+	59	200	1.63+	39	43	0.35+	58	1351	10.98+	55
4500	500	81	746	9.21+	61	93	1.15+	27	11	0.14+	21	850	10.49+	52
4500	5000	82	237	2.89+	18	29	0.35+	7	6	0.07+	9	272	3.32+	16
0	50	186	39	0.21-	0	5	0.03-	0	0	0-	0	44	0.24 -	0
0	500	165	32	0.19-	0	3	0.02-	0	1	0.01-	0	36	0.22-	0
0	5000	92	16	0.17-	0	0	0-	0	0	0-	0	16	0.17-	0
Water	0	129	31	0.24	0	6	0.05	0	2	0.02	0	39	0.30	0

[a] = Statistical diagnoses using estimation of spot frequencies and confidence limits according to Frei and Wurgler (1988) for comparison with distilled water : + = positive: - = negative; I = inconclusive; m = multiplication factor. Probability levels: $\alpha = \beta = 0.05$. One-sided statistical tests.

[b] = % Relative frequency = Calculated from the number-per-wing values by subtracting the background value. For example, 100% for small single spot corresponds to 15.04 - 0.24 = 14.8 (number per wing), and 59% corresponds to 9.01 – 0.24 = 8.77 (number per wing).

Table 11. Inhibition of 4-hexylresorcinol pretreatment on Wing Spot induced by 4500 ppm Urethane in trans-heterozygous (*mwh+/+flr*^{3}) adult flies

4-HR Pretreatment (ppm)	URE (ppm)	No. of wings	Small single spots			Large single spots			Twin spots			Total spots		
			No. found	Spots/ wing[a] m=2	% Rel.[b] frequency	No. found	Spots/ wing[a] m=5	% Rel.[b] frequency	No. found	Spots/ wing[a] m=5	% Rel.[b] frequency	No. found	Spots/ wing[a] m=2	% Rel.[b] frequency
0	4500	81	1218	15.04+	100	334	4.12+	100	48	0.59+	100	1600	19.75+	100
50	4500	98	1541	15.72+	105	237	2.42+	58	18	0.18+	28	1796	18.33+	93
500	4500	86	879	10.22+	67	117	1.36+	32	2	0.02+	0	998	11.60+	58
5000	4500	2[c]	37	-	-	24	-	-	8	-	-	69	-	-
50	0	84	19	0.23-	0	3	0.04-	0	0	0 I	0	22	0.26-	0
500	0	104	19	0.18-	0	1	0.01-	0	0	0-	0	20	0.19-	0
5000	0	50	7	0.14-	0	0	0-	0	1	0.02 I	0	8	0.16-	0
Water	0	129	31	0.24	0	6	0.05	0	2	0.02	0	39	0.30	0

[a] = Statistical diagnoses using estimation of spot frequencies and confidence limits according to Frei and Wurgler (1988) for comparison with distilled water : + = positive: - = negative; I = inconclusive; m = multiplication factor. Probability levels: $\alpha = \beta = 0.05$. One-sided statistical tests.

[b] = % relative frequency = Calculated from the number-per-wing values by subtracting the background value. For example, 100% for small single spot corresponds to 15.04 - 0.24 = 14.8 (number per wing), and 59% corresponds to 9.01 – 0.24 = 8.77 (number per wing).

[c] = Toxic dose indicating by smaller size of the adult treated flies than that of control (about 0.5 of normal size) and many pupae did not transform to be adult flies.

4.6 Effect of 4-Hexylresorcinol on the Mutagen Formation of Aminopyrene-Nitrite Mixture

It is shown in Table 14 that after 4 h incubation of the test material composing of aminopyrene and sodium nitrite mixture, its mutagenicity was reduced with respect to the increasing dose of 4-HR simultaneously added to the reaction except the lowest testing dose (5 μg/plate) of 4-HR increased number of revertants of the final reaction product. Using the doses ranging from 10 to 20 μg/plate of 4-HR, the mutagenicity on both *S. typhimurium* strains was inhibited by 24 – 42% (TA98) and 24 – 72% (TA100). The highest testing doses (40 μg /plate) of 4-HR showed its partial killing effect.

Table 12. Mutagenicity of 4-hexylresorcinol treated with nitrite in acid condition pH 3.0-3.5 on *S. typhimurium* TA98 and TA100. Data are expressed as mean and standard deviation of four plates from two experiments.

Sample	Amount (μg/plate)	No. of His^+ revertants/plate			
		TA98		TA100	
		without nitrite	with nitrite	without nitrite	with nitrite
4-HR	0	28±1	28±1	132±15	132±15
	5	35±7	36±1	135±12	133±19
	10	32±5	34±5	141±9	138±15
	20	31±3	20±5	136±8	146± 4
	40	PK	PK	PK	PK
Positive control[a]	10 (μl/plate)	-	1458± 44	-	-
	40 (μl/plate)	-	-	-	1473±36

[a] = Product of aminopyrene (0.0375 mg/ml) mixed with 2M sodium nitrite incubated at 37 °C (pH 3.0-3.5) for 4 h

PK = Partial killing effect

Table 13. Effect of 4-hexylresorcinol on the mutagenicity of aminopyrene-nitrite product on *S.* typhimurium TA98 and TA100. Data are presented as means and standard deviations of histidine revertants per plate and percentage of inhibition.

Sample	Amount (µg/plate)	TA98		TA100	
		No. of His^+ revertants/plate	(%) Inhibition	No. of His^+ revertants/plate	(%) Inhibition
4-HR	0	1458±44	-	1473±36	-
	5	1086±29	26	1493±45	0
	10	1030± 48	30	1358±28	9
	20	961±42	35	933±28	40
	40	PK	-	PK	-
	Spontaneous	28±1	-	132±15	-

% of inhibition = [(a – b)/(a-c)] x 100

when a is number of revertant per plate of the product after 4-h incubation of AP-nitrite model, b is number of revertant per plate of the product after 4-h incabation of AP-nitrite model in the presence of 4-HR, c is the spontaneous reversion

PK = Partial killing effect

Table 14. Effect of 4-hexylresorcinol on formation of mutagen occurring during aminopyrene-nitrite reaction on *S.* typhimurium TA98 and TA100. Data are presented means and standard deviations of histidine revertants per plate and percentage of inhibition.

Sample	Amount (μg/plate)	TA98		TA100	
		No. of His^+ revertants/plate	(%) Inhibition	No. of His^+ revertants/plate	(%) Inhibition
4-HR	0	861±34	-	960± 48	-
	5	467±36	0	1090±24	0
	10	660±36	24	763± 47	24
	20	513±37	42	368±19	72
	40	PK	-	PK	-
	Spontaneous	22±1	-	103±5	-

% of inhibition = [(a – b)/(a-c)] x 100

when a is number of revertant per plate of the product after 4-h incubation of AP-nitrite model, b is number of revertant per plate of the product after 4-h incabation of AP-nitrite model in the presence of 4-HR, c is the spontaneous reversion

PK = Partial killing effect

CHAPTER V

DISCUSSION

5.1 Mutagenicity of 4-Hexylresorcinol

Results of the present study demonstrated that 4-hexylresorcinol (4-HR) was not genotoxic toward *S.typhimurium* strains TA 98 and TA 100 in the absence of metabolic activation (*in vitro* study) as well as on the somatic cells of *Drosophila* (*in vivo* study). It is, thus, indicated that this compound is neither direct nor indirect mutagen. Mutagenesis study using Ames test also indicated that the interaction between 4-HR and nitrite did not produce any mutagenic product. This result ensures that the consumption of such compound as a carry-over antioxidant in the processed shrimp or others should be safe when it was digested in the stomach simultaneouly with the presence of nitrite salts. Its bactericidal effect to *Salmonella* tester strains should not post any health hazard since it showed when the highest dose was tested on plate. Previous data reported by Cortinas de Nava *et al.* (67) showed no increase in number of revertant colonies following incubation of strains TA 98, TA 100, TA1535, TA1537 or TA 1538 in the standard plate incorporation technique of Ames *et al.* with or without metabolic activation from PCB-induced male Sprague Dawley rat liver S9, with up to 30 μg 4-HR. National Toxicology Program (NTP) (64) reported that in most assays it exhibited little mutagenic activity. Forward mutations were detected at

the TK locus of cultured mouse lymphoma cells treated with 4-HR in the presence of metabolic activation; reverse mutations were not induced at the histidine locus of frameshift or base-pair substitution strains of Salmonella typhimurium in either the presence or absence of metabolic activation. Further, the chemical did not induce chromosomal aberrations in cultured Chinese hamster ovary (CHO) cells in either the presence or absence of metabolic activation. Treatment of CHO cells in vitro with 4-HR did produce an increase in sister chromatid exchange (SCEs) in one trial in the absence of metabolic activation at two doses, but the responses were weak.

5.2 Antimutagenicity of 4-Hexylresorcinol

In vivo mutagenic activity on *Drosophilla melanogaster* of this study demonstrated that 4-HR reduced the frequency of induced wing spots in larvae treated with urethane (URE). Vinyl carbomate epoxide, believed to be the metabolite of URE and ultimately responsible for its mutagenic effects (154), was detoxified via conjugation with glutathione (GSH); the conjugation was catalyzed by glutathione-S-transferase (GST) (174). It is proposed that the antimutagenicity effect of 4-HR may due to the inducing of glutathione-S-transferase activity or increasing amount of glutathione of phase II detoxifying system as well as inhibition the catalytic activities of cytochrome P-450 system of phase I. Abraham *et al.* (177) demonstrated an inhibition of GST activity 4 h after treatment with URE which rendered wing spot induction while the incorporation of 4-HR in this experiment should diminish the inhibition since the frequency of spot induction of URE was reduced. The mode of action of 4-HR on GSH activity on antimutagenic effect may be justified by comparing to the studies of

other antioxidants. The depletion of hepatic GSH by treatment with buthionine sulphoximine and diethyl meleate which increased the genotoxic effects of AFB_1 was abolished by the synthetic antioxidant ethoxyquin indicating by the decrease of AFB_1-DNA adduct formation in rat liver and the increase of GST activity towards epoxidated AFB_1 (187).

Since Brennan and Schiestl (188) showed that free radical species were produced in *Saccharomyces cerevisiae* following exposure to urethane, 4-HR might reduce the genotoxicity of URE by its free radical scavenging activity. Unfortunately, there is no information about the free radical scavenging activity of 4-HR. However, it is well known that phenolic antioxidant such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) exhibited antimutagenic activity. The studies on the mechanism of inhibitory effect of BHA and BHT which have a common chemical structure and activity related to 4-HR, demonstrated its free radical scavenging activity in many experiments. An example was shown in the experiment that insects fed on the diet supplemented with BHT had decreased rate of lipid peroxidation (measured by thiobarbituric acid test) with respect to the controls suggesting that the antioxidant BHT, which scavenged free radicals, prolonged the life span of *Drosophila* (189). In addition, Harman (190, 191) showed that BHT and other antioxidants increased the average survival time of mice and rats. The antioxidants administered at high levels for a long time appeared to delay certain aging processes and reduce so-called spontaneous tumor incidence under certain conditions. This was ascribed to the possible trapping of free radicals. In another study by Williams *et al.* (192), BHT at 100 ppm fed together with 2-acetylaminofluorene (AAF) at a low concentration of 50 ppm inhibited the

induction of liver altered foci and reduced the incidence of liver carcinomas by wk 76. Thus, the effective chemoprotective concentrations of BHT extend below 1000 ppm to 100 ppm. Anticarcinogenic activity at such low concentrations suggested to be due to free radical trapping activity.

The result of 4-HR pretreatment on the larvae before exposing to URE also suggested that this compound enhance glutathione-S-transferase resulting the decrease the active form of urethane for the induction of wing spot. Previous data reported by Kemper *et al.* (174) showed that pretreatment of mice with 1% dietary butylated hydroxyanisole (BHA) caused parallel increased in cytosolic GST activity and cytosolic enhancement of detoxification of vinyl carbamate epoxide by GSH. The enhancement of GST activity by BHA pretreatment postulated to play a role in the anticarcinogenic action of BHA (193). Sciuto and Moran (194) showed that dietary pretreatment with 0.75% and 1.5% BHA significantly enhanced lung tissue GSH, 1.8-fold and 5.8-fold, respectively, compared with phosgene-exposed control diet. In addition, Saprin *et al.* (195) found that pretreatment of rat with antioxidant BHT owing to its effect of animal protection against intoxication with diethylnitrosamine (DENA) increased the liver activity of enzymes of stage II metabolism of xenobiotics: GST. However, the stimulation might not be last longer because the reduction of wing spot formation was less than that of the study on simultaneous feeding of URE with 4-HR.

In the *in vitro* working on the Ames test, 4-HR was also found to be antimutagenic towards the product of aminopyrene-nitrite reaction at the higher doses (10 μg/plate). Reddy *et al.* (196) showed that the addition of phenolic compounds namely, BHT (100-250 μg/plate) or BHA (25-500 μg/plate) to the Ames test could

inhibit 3,2'-dimethyl-4-aminobiphenyl (DMAB)-induced mutagenicity in Salmonella strains TA98 and TA100. It, thus, supports the result obtained in the SMART.

5.3 Modification on Mutagenicity

The product aminopyrene-nitrite reaction, 1-nitropyrene, is a mutagen occurring during gastric-liked digestion as suggested by Kato *et al* (90). The present investigation shows that 4-HR could modify the formation of the mutagenic product. It is demonstrated that 4-HR (5 μg/plate) increased number of revertants of the final reaction product when it was added along with aminopyrene (AP) and nitrite. The increase may due to the stimulation on mutagen formation during acid incubation via the mechanism of *C*-nitroso formation. Generally some phenolic compounds form *C*-nitroso derivatives after reaction with nitrite and these intermediates further react with the nitrosating agent such as p-nitrosophenol and 2,4-dinitrosoresorcinol (197). When increasing concentrations of phenol (up to 100 mmol/l) or *p*-nitrosophenol (up to 10 mmol/l) were added to the reaction mixture, increases in the yield of N-nitrosoproline (NPRO) were observed; the efficiency of catalysis varied widely between the two compounds, being 18 times higher for *p*-nitrosophenol when compared on an equimolar (10 mmol/l) basis. The amount of NPRO formed increased linearly up to the highest concentration of *p*-nitrosophenol tested, and the straight line passed through the value for the uncatalysed reaction at zero concentration of *p*-nitrosophenol; this indicates a first-order kinetics of the catalysed reaction with respect to *p*-nitrosophenol, as demonstrated before for the nitrosation of diethylamine (197).

Resorcinol, a parent compound of 4-HR, increased the formation of nitrosoproline to be five folds of the control with a non-linear response (197). Resorcinol at the dose up to 5 mmole/l increased the nitrosation rate and with the higher dose the nitrosation rate was drop to zero at a dose of 30 mmole/l. This information is similar to that of the present experiment that only the low dose (5 μ g/plate) increased the mutagenicity of AP-nitrite product closely to 2 folds of the positive control plate (strain TA98) and then the mutagenicity of the other two doses was decreased. Pignatelli et al. (197) also worked on the experimental rat and they found that nitrosation of proline in the present of resorcinol was increased approximate 19 folds compared to the result of control animals. On the other hand, concentration of this phenolic compound higher than 5 μg/plate decreased the formation of mutagenic AP-nitrite product

4-HR may act as a nitrite scavenger at the higher doses (10 μg/plate). Stich *et al.* (198) demonstrated the capacity of phenolics to trapped nitrosating species, which results in a reduced nitrosation of amines and amides *in vitro* and *in vivo*, exerted a protective effect. However the interaction between phenolic compounds may result new mutagenic species or new desmutagenic species. Kanazawa *et al.* (199) identified the derivatives produced by incubating BHA with sodium nitrite at pH 2.0 or pH 5.0. Eight derivatives were detected. Their mutagenicity and desmutagenicity were assayed using *Salmonella typhimurium* strains. Although 2-tert.-*butyl*-p-*quinone* (BQ) and 3,3′-*di*-tert.-*butyl-biphenyldiquinone*-(2,5,2′,5′) (BBDQ) were the mutagens of base-substitution type, BHDQ was the potent desmutagen against a mutagenicity of *Trp-P-2*. This may explain why 4-HR could, somehow at one dose, activate the direct

mutagenicity in Ames test but decreased the activity in *in vivo* assay containing metabolic detoxification namely SMART.

The present investigation shows that 4-HR inhibited the mutagenesis induced by URE in SMART. This information may confrim the decreased incidences of three tumor types related to 4-HR administration in rats. In the NTP report (64), the inhibition of mononuclear cell leukemia by 4-HR was dose related in both male and female rats. Such negative trends were statistically significant. The incidences of thyroid gland C-cell neoplasms in male rats occurred with a marginal negative trend. The incidences of pancreatic islet cell adenomas, fibromas of mammary gland, and endometrial stromal polyps were also reduced. Incidence of hepatocellular neoplasms in mice were reduced in both low and high dose groups, and incidences of hemangiomas or hemangiosarcomas were reduced in high dose of 4-HR in male and female mice.

CHAPTER VI

CONCLUSION

4-Hexylresorcinol (4-HR) is not mutagenic to both in the Ames *Salmonella* mutagenicity assay in the absence of activating system and in the wing spot somatic mutation and recombination test. It, as well, showed no mutagenicity toward both TA98 and TA100 after being treated with nitrite. It is suggested that 4-HR is a mutagenic inhibitor of the product of aminopyrene-nitrite model. In addition, co-administration administration of 4-HR to larvae reduced the frequency of total spots induced by urethane. All concentrations of 4-HR decreased the frequencies of wing spots induced by urethane. Since the mutagenic effect of urethane depends on the cytochrome P-450 enzymatic activation and glutathione detoxification; thus, the antimutagenicity effect of 4-HR may involve the modification on detoxifying systems. 4-HR may inhibit the catalytic activities of cytochrome P-450 and induce of glutathione-*S*-transferase activity or glutathione. Unfortunately, there is no information abount the effect of 4-HR to both enzymes and glutathione. Further studies to determine the effect of 4-HR on cytochrome P-450 system, glutathione and glutathione-*S*-transferase are suggested.

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APPENDIX

SMART TEST

Preparation of Standard Culture Medium

Ingredient

1.	Corn flour	125	g
2.	Sugar	100	g
3.	Yeast	50	g
4.	Agar	14	g
5.	Proprionic acid	5	ml
6.	Water	1000	ml

Steps of preparations of standard medium for *Drosophila melanogaster* stocks.

1. Boil and blend sugar, agar, yeast and corn flour in 1000 ml water until sticky.
2. Add propionic acid.
3. Fill each 125 ml-erlenmeyer flask with 50 ml of the medium.
4. Close off the flask with a plug (made of gauze and cotton cover with aluminum foil).
5. Sterile the flasks in an autoclave microbial contamination that can harm the flies.

Statistical Consideration (181)

In experiments designed to assess the mutagenicity of a chemical, most often a treatment series were compared with a control series. One might like to decide whether the compound used in the treatment should be considered as mutagenic or non-mutagenic. The formulation of 2 alternative hypotheses allowed one to distinguish among the possibilities of a positive, inconclusive, or negative result of an experiment (200).

In the null hypothesis one assumes that there was no difference in the mutation frequency between control and treated series. Rejection of the null hypothesis indicated that the treatment resulted in a statistically increased mutation frequency. The alternative hypothesis postulated a priory that the treatment results in an increased mutation frequency compared to the spontaneous frequency.

The alternative hypothesis was rejected if the mutation frequency was significantly lower than the postulated increased frequency. Rejection indicates that the treatment did not produce the increase requires to consider the treatment as mutagenic. If neither of the 2 hypotheses was rejected, the results were considered inconclusive as one could not accept at the same time the 2 mutually exclusive hypotheses. In the practical application of the decision procedure, one defines a specific alternative hypothesis requiring the mutation frequency in the treated series be *m* times that in the control series and used together with the null hypothesis. It might happen in this case that both hypotheses had to be rejected. This should mean that the treatment was weakly mutagenic, but led to a mutation frequency which was significantly lower than *m* times the control frequency.

Testing against the null hypothesis (H_O) at the level α and against the alternative a hypothesis (H_A) at the level β led to the error probabilities for each of the possible diagnoses: positive, weakly but positive, negative, or inconclusive. The following four decisions were possible; 1) accept both hypotheses; these can not be true simultaneously, so no conclusions can be drawn--inconclusive result; 2) accept the first hypothesis and reject the second hypothesis--negative result; 3) reject the first hypothesis and accept the second hypothesis--positive result; 4) reject both hypotheses --weak effect.

Calculation step by step

Estimation of spot frequencies and confidence limits of m_e

Particularly in the case that both hypotheses, H_O as well as H_A, had to be rejected, one might be interested in knowing the confidence interval of m_e, i.e., of the estimated multiple by which the mutation frequency in the experimental series was larger than the spontaneous frequency. The estimated value was

$$m_e = \frac{(n_t / n)\, N_c}{(n_c / n)\, N_t}$$

where N_c and N_t represented the respective sample sizes in control and treatment series, n_c and n_t the respective numbers of mutations found, and n the total of mutations in both series together. Exact lower and upper confidence limits p_l and p_u for the proportion n_c/n on one hand, as well as q_l and q_u for the proportion n_t/n on the other hand, may be determined according to Sachs (201, 202). He gave an easy method to calculate these values using an F-distribution table. To determined q_l and p_0 one-sidedly at the level α , and q_u and p_l also one-sidedly at the level β. In this way and in

agreement with the foregoing section, a confidence limit $m_l > 1$ led to rejection of H_o, while a confidence limit $m_u < m$ led to rejection of H_A.

In the first step, F-distribution according to Sachs (201, 202) were used to determine the value $F_{\nu 1, \nu 2}$ at the level $\alpha = 0.05$, where the degrees of freedom ($\nu 1$, $\nu 2$) were given by the equations

$$\nu 1 = 2 (n - nt + 1) \text{ and } \nu 2 = 2n_t$$

In the second step, the F-value so obtained was used to calculate the lower confidence limit (q_l) for the proportion of spots in the experimental series

$$q_l = n_t / [n_t + (n-n_t + 1 F_{\nu 1,\nu 2}]$$

This gave a lower confidence limit for the frequency of spots per wing in the control which was equal to

$$f_{t,l} = q_l n/N_t$$

This was the following complementarity, namely that the lower confidence limit for the number of spots in the experimental series ($q_l n$) plus the upper confidence limit for the number of spots in the experiment ($p_u n$) was equal to the total number of spots (n) found in experimental and control series together, i.e.,

$$P_u n = (1-q_l) n$$

This gave an upper limit for the frequency of spots per wing for the control which is

$$f_{c,u} = q_u n/N_c$$

The lower confidence limit m_l of the multiple m_e was determined as the ratio between the lower confidence limit for the frequency in the treated series and the upper confidence limit for the frequency in the control, i.e.,

$$m_l = \frac{f_{t,l}}{f_{c,u}} = \frac{q_l\, n/N_t}{p_u n/N_c}$$

Only in the case that ml, the lower confidence limit of me, was larger than 1.0 would reject H_0. Since this was not the case, H_0 remain accepted.

In the same way, the lower confidence limit of the spot frequency may be determined in the control $f_{c,1}$ which will give $f_{t,u}$, the upper confidence limit of the spot frequency in the experimental series. This is also done one-sidedly, at the level β = 0.05. The inverse ratio of these values will provide the upper 5% confidence limit m_u for the multiple m_e.

Again, the F-distrivution according to Sachs (202) was used and detemined the value $F_{v1, v2}$ at the level $\beta = 0.05$, where the degrees of freedom $(_{v1, v2})$ were this time given by the equations

$$v1 = 2(n-n_c + 1) \text{ and } v2 = 2\, n_c$$

The F-value so obtained was used to calculate the lower confidence limit (p1) for the proportion of spots in the control

$$P_l = n_c / [n_c + (n-n_c + 1)\, F_{v1, v2}]$$

This gave a lower confidence limit for the frequency of spots per wing in the control which equal to

$$f_{c,1} = p_l n/N_c$$

Again, There was complementarity, in that the lower confidence limit for the number of spots in the control (p1n) plus the upper confidence limit for the number of spots in the experiment ($q_u n$) was equal to the total number of spots (n), so that

$$q_u n = (1-p_l)n$$

This gave an upper limit for the frequency of spots per wing for this series which is

$$f_{t,u} = q_u n/N_t$$

The upper confidence limit m_u of the multiple m_e can be determined as the ratio between the upper confidence limit for the frequency in the treated series and the lower confidence limit for the frequency in the control, i.e.,

$$m_u = \frac{f_{t,u}}{f_{c,l}} = \frac{q_u n/N_t}{p_l n/N_c}$$

H_A was rejected if m_u, the upper confidence limit of m_e, was less than m (m=2 for the total of all spots and for the small single spots, and m=5 for the large single spots as well as for the twin spots). Substitution of m_e by m_l or m_u in the above formulas provided the respective exact upper and lower confidence limits for the frequencies estimated.

AMES TEST

MANIPULATION OF THE TESTING STRAINS

1.Preparation of Stock Solution and Media

1.1 Vogel-Bonner medium E stock salt solution (VB salt)

Ingredient	1 liter	2 liter
Distilled H_2O	670 ml	1,340 ml
Magnesium sulfate ($MgSO_4.7H_2O$)	10 g	20 g
Citric acid monohydrate	100 g	200 g

Potassium phosphate, dibasic (anhydrous) (K_2HPO_4)	500 g	1,000 g
Sodium ammonium phosphate ($NaNH_4HPO_4.4H_2O$)	175 g	350 g

Add salts in the order indicated to water and allowed each salt to dissolve completely before adding the next. Filter the solutions and then autoclave at 121 °C for 20 min.

1.2 Minimal glucose agar plate

Ingredient	300 ml	350 ml
Bacto agar	4.5 g	5.25 g
Distilled H_2O	280 ml	330 ml
VB salts	6 ml	7 ml
40% glucose	15	17.5 ml

Add agar to distilled water in a glass bottle. Autoclave at 121°C for 20 min. When the solution has cooled slightly, add sterile VB salts and sterile 40% glucose. Mix and pour 30 ml into each sterile petri plate. Minimal glucose agar plates were kept in incubator at 37°C before using.

1.3 Oxoid nutrient broth No.2

Dissolve 2.5 g of nutrient broth No.2 in 100 ml distilled H_2O. Transfer 12 ml of nutrient broth for each flask (covered with sterile gauze). Autoclave at 121°C for 20 min.

1.4 Top agar

Ingredient	200 ml	300 ml
Bacto agar	1.2 g	1.8 g
Sodium chloride (NaCl)	1.0 g	1.5 g
Distilled H2O	200 ml	300 ml

Dissolve ingredients in water. Store in a glass bottle. Autoclave for 20 min at 121°C and then add 20 ml and 30 ml of 0.5 mM histidine HCl-0.5 mM biotin for 200 ml and 300 ml of top agar respectively.

1.5 0.1 M L-histidine HCl stock

Ingredient	100 ml
L-histidine HCL	2.096 g
Distilled H_2O	100 ml

Dissolve 2.096 g of L-histidine HCL (MW 209.63) in 100 ml distilled water. Autoclave at 121°C for 20 min.

1.6 1mM L-histidine HCL stock

Ingredient	100 ml
0.1 M L-histidine HCL	1 ml
Distilled H_2O	99 ml

Dilute 1 ml of 0.1 M L-histidine HCl in 99 ml of distilled water. Autoclave at 121°C for 20 min.

1.7 1mM Biotin stock

Ingredient	100 ml
Biotin	24.43 mg
Distilled H_2O	100 ml

Dissolve biotin (MW 244.3) in distilled water. Warm it until dissolve completely. Autoclave at 121°C for 20 min.

1.8 0.5 mM L-histidine HCl-0.5 mM biotin

Ingredient	200 ml
1 mM L-histidine HCl	100 ml
1 mM biotin	100 ml

Mix and autoclave at 121°C for 20 min.

1.9 $NaPO_4$-KCl buffer

Ingredient	330 ml
0.5 M $NaPO_4$ pH 7.4	100 ml
1 M KCl	16.5 ml
Distilled H_2O	213.5 ml

Mix and autoclave at 121°C for 20 min.

1.10 1.0 M KCl

Ingredient	1,000 ml
Potassium chloride	74.56 g
Distilled H_2O	1,000 ml

Mix and autoclave at 121°C for 20 min.

1.11 8 mg/ml Ampicillin solution

Ingredient	10 ml
Ampicillin (sodium)	800 mg
0.02 N NaOH	10 ml

Dissolved and store at 0°C.

1.12 0.1% Crystal violet

Ingredient	10 ml
Distilled H_2O	10 ml
Crystal violet	10 mg

Store at 0°C in glass bottle with screw cap.

2. Procedure for Reisolation and Growing Culture.

Tester strains, TA98 and TA100 are grown in Oxoid nutrient broth No.2 and incubated overnight in a 37°C shaking water bath. The growth period should not exceed 16 hours. These cultures are reisolation by streaking on minimal glucose agar plated which the surface were spread with 0.1 ml of 8 mg/ml ampicillin, 0.3 ml of 0.1 M histidine HCl and 0.1 ml of 1mM biotin. These plates are incubated at 37°C for 48 hours. After incubation, the 5 single colonies per strain TA98 and TA100 are picked up and growth in Oxoid nutrient broth No.2 overnight at 37°C in shaking water bath. Each culture is confirmed genotypes of the strains and kept the cultures as the source of bacteria for mutagenicity testing. For each 1.0 ml of culture, add 0.09 ml of spectrophotometric grade DMSO. Combine the culture and DMSO in a sterile tube and distribute 400µl of the culture aseptically into sterile cryotube (Nunc). The tubes should be filled nearly full and then transfer to a -80°C freezer.

3. Confirming Genotype of Tester Strains

The broth cultures of TA98 and TA100 are used to confirm genotypes in the following ways.

3.1 Histidine requirement

The his^- character of the strains is confirmed by demonstrating the histidine requirement for growth on the minimal glucose agar plates enriched with histidine and biotin.

Procedure:

plate a	no histidine and biotin
plate b	0.1 ml of 1mM biotin
plate c	0.3 ml of 0.1 M His-HCl
plate d	0.3 ml of 0.1 M His-HCl + 0.1 ml of 1 mM biotin

Four minimal glucose agar plates are required for each tester strains. Each of the plates is applied on the surface with 0.1 ml of 1 mM biotin, 0.3 ml of 0.1 M His-HCl, 0.3 ml of 0.1 M His-HCl plus 0.1 ml of 1 mM biotin and no application (plate b, c, d, a respectively). Made a single streak of each strain across these plates. Five strains could be tested on the same plate. Incubated at 37°C for 48 h. The growing of bacteria on histidine plus biotin plate is the result of histidine requirement.

3.2 R Factor

The R-factor strains (TA97, TA98, TA100 and TA102) should be tested routinely for the presence of the ampicillin resistance factor because the pladmid is somewhat unstable and can be lost from the bacteria.

Procedure: For each tester strain, add 0.3 ml of fresh overnight culture to a tube containing 0.1 ml of 0.1 M histidine-HCl followed by adding 2.0 ml of molten top agar containing 0.5 mM histidine-HCl and 0.5 mM biotin. Mixed and poured on a minimal agar plate. Rotated the plate to distribute the mixtures and allowed several minutes for agar to become firm. R-factor and rfa mutation (see the next section) are performed in the same plate by dividing the plate into 2 areas, one for R-factor and the other for rfa mutation. For R-factor, commercial ampicillin disc or filter paper disc containing 8 mg/ml ampicillin is applied on the surface of the agar by using sterile forceps. The disc is pressed lightly to embed in the overlay. The plates are incubated at 37°C for 24 hours. The absence of the clear zones of inhibition around the disc indicates resistance to ampicillin.

3.3 rfa mutation

Strains having the deep rough (rfa) character should be tested for crystal violet sensivity.

Procedure: Pipetted 0.1% solution of crystal violet to the sterile filter paper disc (1/4 inch) and transferred the disc to plates, seed with bacteria (the procedure is similar to R-factor). Incubated at 37°C for 48 h. The clear zone appeared around the disc indicated the presence of the rfa mutation the permitted crystal violet to enter and kill bacteria.

4. Spontaneous Reversion

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. The revertant colonies are clearly visible in a uniform background lawn of auxotrophic bacteria. Each tester strain reverts spontaneously at a frequency that is characteristic of the strain. Nevertheless, there is variability in the number of spontaneous revertants from one experiment to another and from one plate to another, and it is advisable to include at least 2-3 spontaneous mutation control plates for each strain in a mutagenicity assay.

Procedure: 0.1 ml of sterile water is added to capped culture tube. Add 0.5 ml of $NaPO_4$-KCl buffer pH 7.4, 0.1 ml of fresh overnight culture of TA98 or TA100, followed by 2.0 ml of molten top agar. Mixed and then poured on minimal glucose agar plate. Rotated plates and left it to become hardens. Incubated at 37°C for 48 h and the his^+ revertants colonies were counted.

5. The Response to Standard Carcinogen

Standard mutagens or positive mutagens are used routinely in mutagenicity experiments to confirm the reversion property and specificity of each strain. The standard mutagen, which used in this experiment, is nitrosoaminopyrene. Tester strain which highly response to positive mutagens be collected.

Procedure: 1.157 mg of aminopyrene (MW 217.27) was dissolved in 0.75 ml of 0.2 N HCl, 0.01 ml of the supernatant was pipetted to capped tube. Add 0.74 ml of 0.2 N HCl, 0.25 ml of 2 M $NaNO_2$. The final concentration of aminopyrene was 6.1706 x

10^{-4} M and the final concentration of nitrite was 0.5 M. Mixed and shaken in water bath at 37°C for 4 hours. Placed the tube in an ice bath and added 0.25 ml of 2 M $NH_2SO_3NH_4$. Standed for 10 min. Pipetted 50 µl and 100 µl of the mixture to each capped culture tube for test the stock culture TA98 and TA100. Then evaluated their mutagenicity as described in spontaneous reversion. The characteristic of the stock culture for TA98 and TA100 as the source of bacteria for mutagenicity is:

a) contained R-factor (pKM 101) and rfa mutation.

b) His+ requirement.

c) Low spontaneous reversion.

d) Highly response to standard carcinogen.

After the characteristic of the culture was tested, the mutagenicity test was started.

BIOGRAPHY

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